Clinical Cancer Research

Interaction between Molecular Subtypes and Stromal Immune Infiltration before and after Treatment in Breast Cancer Patients Treated with Neoadjuvant Chemotherapy

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Abstract

Purpose: High levels of tumor-infiltrating lymphocytes (TIL) before neoadjuvant chemotherapy (NAC) are associated with higher pathologic complete response (pCR) rates and better survival in triple-negative breast cancer (TNBC) and *HER2*-positive breast cancer. We investigated the value of TIL levels by evaluating lymphocyte infiltration before and after NAC.

Experimental Design: We assessed stromal TIL levels in 716 pre- and posttreatment matched paired specimens, according to the guidelines of the International TIL Working Group.

Results: Pre-NAC TIL levels were higher in tumors for which pCR was achieved than in cases with residual disease (33.9% vs. 20.3%, $P = 0.001$). This was observed in luminal tumors and TNBCs, but not in *HER2*-positive breast cancers ($P_{\text{Interaction}} = 0.001$). The association between pre-NAC TIL levels and pCR was nonlinear in TNBCs ($P = 0.005$). Mean TIL levels decreased after chemotherapy completion (pre-NAC TILs: 24.1% vs. post-NAC TILs: 13.0%, $P < 0.001$). This decrease was strongly associated with high pCR rates, and the variation of TIL levels was strongly inversely correlated with pre-NAC TIL levels ($r = -0.80$, $P < 0.001$). Pre-NAC TILs and disease-free survival (DFS) were associated in a nonlinear manner ($P < 0.001$). High post-NAC TIL levels were associated with aggressive tumor characteristics and with impaired DFS in *HER2*-positive breast cancers (HR, 1.04; confidence interval, 1.02–1.06; $P = 0.001$), but not in luminal tumors or TNBCs ($P_{\text{Interaction}} = 0.04$).

Conclusions: The associations of pre- and post-NAC TIL levels with response to treatment and DFS differ between breast cancer subtypes. The characterization of immune subpopulations may improve our understanding of the complex interactions between pre- or post-NAC setting, breast cancer subtype, response to treatment, and prognosis.

Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer-related death in women. Neoadjuvant chemotherapy (NAC) is increasingly prescribed for patients with locally advanced breast cancer and provides opportunities for studying and monitoring the treatment sensitivity of tumors "*in vivo*." A pathologic complete response (pCR) after NAC is a surrogate marker of good prognosis in triple-negative breast cancer (TNBC) and *HER2*-positive breast cancer, and is now used in FDA trials as a means of accelerating the approval of new drugs.

The role of tumor-infiltrating lymphocytes (TIL) in breast cancer has been studied over the last decade. Many studies have reported associations between high TIL levels at diagnosis and a better response to NAC (1–3), and a better prognosis in both neoadjuvant and adjuvant chemotherapy settings (4–7), particularly for TNBC and *HER2*-positive breast cancer. In 2015, an international consortium provided guidelines for the standardized evaluation of TILs in clinical practice (8), and their assessment is encouraged in routine practice, although the results of such evaluations currently have no impact on therapeutic strategy in clinical practice.

The analysis of residual tumor burden after systemic neoadjuvant treatment is an underexplored area that may help us to understand the mechanisms of resistance to specific treatments in breast cancer. However, only a few studies have investigated the variation of TIL levels in response to NAC. Furthermore, studies of

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Translational Relevance

In breast cancer, the evaluation of tumor-infiltrating lymphocytes (TIL) is encouraged in routine practice. However, little is known on their variations between before and after neoadjuvant chemotherapy (NAC), and few data are available on their value after treatment. We investigated TIL levels before and after NAC in 716 paired biopsy and surgical specimens. Pre-NAC TILs levels were associated with pathologic complete response (pCR) in a nonlinear manner in triple-negative breast cancer and were not associated with pCR in *HER2*-positive breast cancer. TIL levels decrease after chemotherapy completion, and this decrease was strongly associated with pCR. High post-NAC TIL levels were associated with impaired survival in *HER2*-positive breast cancer but not in the other subtypes. TILs' subsetting would be critical to (i) further identify the different immune subpopulations in residual specimen and (ii) understand if their localization, their quantity, or their state of activation is associated with the nonlinear predictive impact and/or their different prognostic value before and after NAC among breast cancer subtypes.

the prognostic significance of postchemotherapy TILs have focused almost exclusively on TNBCs (9, 10).

The aim of this study was to report and compare the predictive and prognostic values of TIL levels (before and after NAC) as a function of breast cancer subtype, in a real-life cohort of 718 breast cancer patients treated with NAC.

Materials and Methods

Patients and treatments

We analyzed a cohort of 718 patients with nonmetastatic breast cancer treated with NAC with or without trastuzumab, followed by surgery, at the Institut Curie (Paris and Saint Cloud, France). The cohort and treatments have been described in detail elsewhere and are summarized in the Supplementary Material. This study was approved by the Institutional Review Board of Institut Curie and was conducted in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. By the law, no informed consent from the patient was required in this observational study.

Tumor samples

Breast cancer tumors were classified into subtypes [TNBC, *HER2*-positive, and luminal *HER2*-negative (referred to hereafter as "luminal")] on the basis of IHC and FISH (see the Supplementary Material). In accordance with the guidelines used in France (11), cases were considered estrogen receptor (ER)–positive or progesterone receptor (PR)–positive if at least 10% of the tumor cells expressed ER/PR, and endocrine therapy was prescribed when this threshold was exceeded.

Pathologic review

Pretreatment core needle biopsy specimens and the corresponding post-NAC surgical specimens were reviewed independently by two experts in breast diseases (M. Laé and D. De Croze).

Formalin-fixed paraffin-embedded tumor tissue samples were studied. TILs, residual cancer burden (RCB) indices, and pre- and post-NAC cellularity were reviewed simultaneously, specifically for the purposes of this study, between January 2015 and March 2017. In accordance with the recommendations of the international TILs Working Group (8), we checked for presence of a mononuclear cell infiltrate in the stroma on hematoxylin and eosin–stained sections without additional staining, after excluding areas around ductal carcinomas *in situ*, and tumor zones with necrosis and artifacts. Infiltrates were scored on a continuous scale, as the mean percentage of the stromal area occupied by mononuclear cells. After NAC, we assessed TIL levels within the borders of the residual tumor bed, as defined by the RCB index (12). Nothing is known about the clinical, biological, and prognostic significance of TILs in the area of regression in cases of pathologic response, but the TILs' international working group recently called for their evaluation for research purposes. In cases of pCR, the scar area was measured on macroscopic examination. The scar appeared as a white area in the breast parenchyma corresponding to the tumor bed modified by NAC. It was characterized by the presence of histiocytes, lymphocytes, macrophages, fibrosis, and elastosis. The whole fibro-inflammatory scar was evaluated on hematoxylin and eosin sections (size in mm and stromal TIL level evaluation; Supplementary Fig. S1). We determined the RCB index, as described by Symmans and colleagues (12), with the web-based calculator freely available via the Internet (www.mdanderson.org/breastcancer_RCB). Invasive tumor cellularity before and after NAC was determined as the percentage of the tumor area occupied by invasive cancer.

Study endpoints

We defined pCR as the absence of invasive residual tumor from both the breast and axillary nodes (ypT0/is N0). Disease-free survival (DFS) was defined as the time from surgery to death, locoregional recurrence, or distant recurrence, and overall survival (OS) was defined as the time from surgery to death. For patients for whom none of these events were recorded, we censored data at the time of last known contact.

Quantitative data handling and statistical analysis

Pre- and post-NAC TIL levels were analyzed as continuous variables, after performing linearity tests (see the complementary statistical methods section of the Supplementary Material). RCB index was assessed as a continuous variable in both univariate and multivariate analyses. All analyses were performed on the whole population and after stratification by breast cancer subtype. TIL levels and qualitative variables in classes were compared by ANOVA, with *post hoc* Tukey analysis when required, or in Mann–Whitney *U* or Kruskal–Wallis tests, where indicated. Absolute and relative changes in TIL levels were calculated as the difference between pre- and post-NAC TIL levels and as these levels divided by pre-NAC TIL levels, respectively. Changes in mean values were investigated in paired *t* tests. The classical statistical methods used to analyze univariate and multivariate associations with pCR (logistic regression models) and survival (Cox proportional hazard models) are described in the complementary statistical methods section of the Supplementary Material.

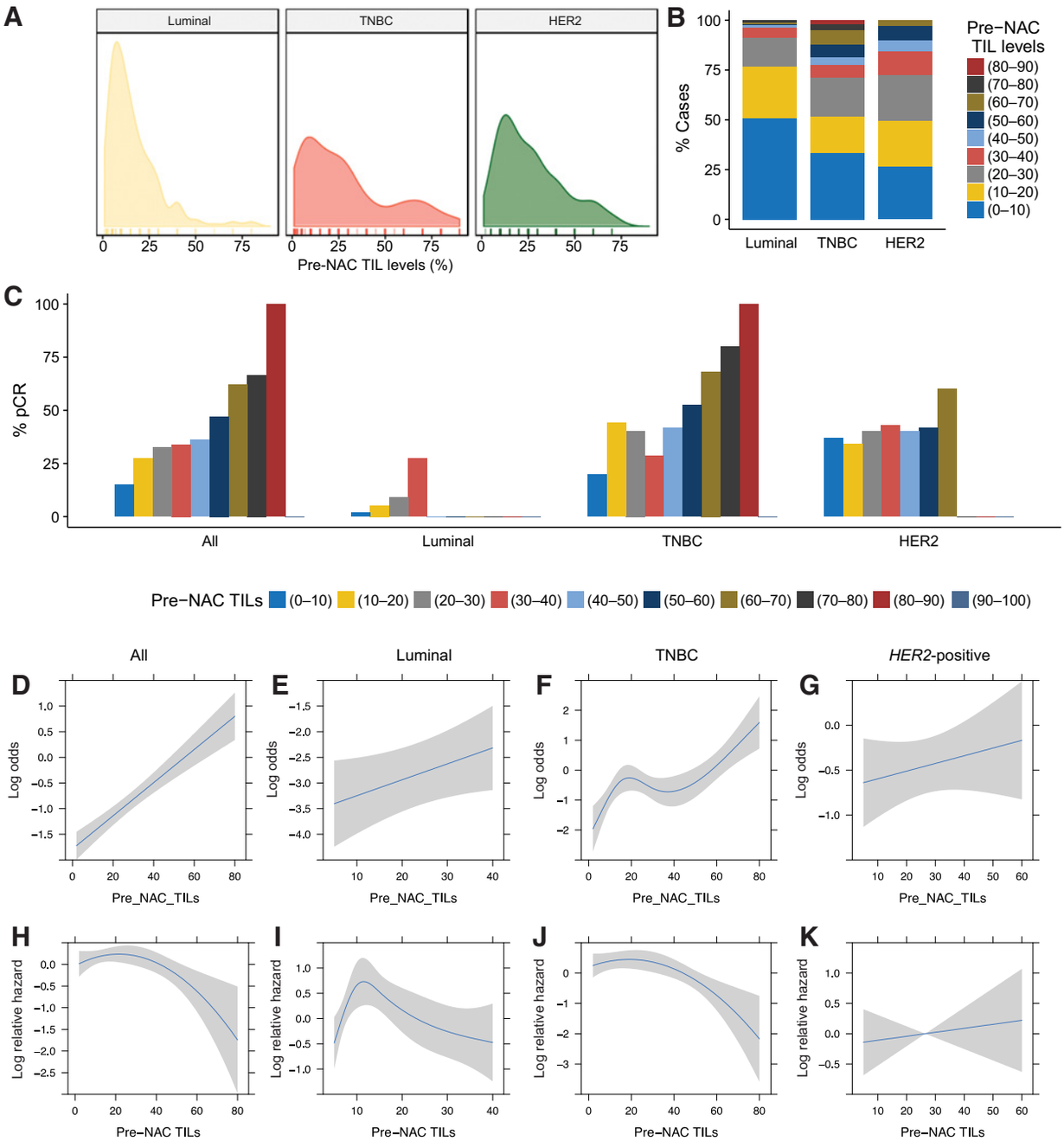


Figure 1.

Associations between pre-NAC TIL levels, clinical and pathologic factors, and response to treatment. **A,** Distribution of pre-NAC TIL levels, by breast cancer subtype (kernel density plot). **B,** Barplot of the repartition of the percentage of tumors according to pre-NAC TIL levels binned by 10% increment by breast cancer subtype. The proportion of tumors with TILs $\geq$ 60% is 11% ($n = 80$; luminal: 2.3%, $n = 5$; *HER2*-positive: 9.7%, $n = 17$; TNBC: 18.2%, $n = 58$). **C,** Percentage of pCR rate by pre-NAC TIL levels in the global population and by breast cancer subtype (TILs were binned by increments of 10%, as previously described in ref. 6). The shape of the TNBCs bars enables a visual representation of the deviation to the linearity assumption. **D–G,** Graphical representation of the best statistical model retained for analyzing the association between pre-NAC TIL levels and pCR. *X*-axis represents the increasing value of pre-NAC TILs, and *y*-axis represents the increasing OR for pCR. **D,** Whole population, linear model. **E,** Luminal, linear model. **F,** TNBC: restricted cubic spline. **G,** *HER2*-positive: linear model. **H–K,** Graphical representation of the model best fitting the data for the association between pre-NAC TILs and DFS. *X*-axis represents the increasing value of pre-NAC TILs, and *y*-axis represents the increasing HR for DFS. **H,** Whole population, second-order fractional polynomial. **I,** Luminal, restricted cubic spline. **J,** TNBC, second-order fractional polynomial. **K,** *HER 2*-positive, linear model.

Table 1. Association between clinical and pathologic factors with pCR (univariate and multivariate analysis, whole population)

		Total population			Univariate		Multivariate	
Characteristics	**Class**	***N***	**pCR**	**%**	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
Pre-NAC parameters								
Age (years)	<45	286	76	26.6%	1			
	45–55	254	66	26%	0.97 (0.66–1.42)	0.877		
	>55	178	60	33.7%	1.4 (0.93–2.11)	0.101		
Menopausal status	Post	259	80	30.9%	1			
	Pre	452	119	26.3%	0.8 (0.57–1.12)	0.193		
BMI class	(19–25)	414	125	30.2%	1			
	<19	41	8	19.5%	0.56 (0.24–1.19)	0.156		
	(25–30)	166	41	24.7%	0.76 (0.5–1.14)	0.186		
	>30	96	27	28.1%	0.9 (0.55–1.47)	0.69		
Tumor size	T1–T2	529	155	29.3%	1			
	T3	189	47	24.9%	0.8 (0.54–1.16)	0.245		
Clinical nodal status	N0	282	83	29.4%	1			
	N1–N2–N3	435	119	27.4%	0.9 (0.65–1.26)	0.546		
ER status	Negative	397	163	41.1%	1			
	Positive	321	39	12.1%	0.2 (0.13–0.29)	<**0.001**		
PR status	Negative	474	183	38.6%	1			
	Positive	221	17	7.7%	0.13 (0.08–0.22)	<**0.001**		
HER2 status	Negative	543	134	24.7%	1			
	Positive	175	68	38.9%	1.94 (1.35–2.78)	<**0.001**		
BC subtype	Luminal	223	11	4.9%	1		1	
	TNBC	320	123	38.4%	12.03 (6.58–24.27)	<**0.001**	10.96 (5.64–24)	<**0.001**
	HER2	175	68	38.9%	12.25 (6.46–25.36)	<**0.001**	11.08 (5.52–24.8)	<**0.001**
Histology	NST	661	188	28.4%	1			
	Other	53	13	24.5%	0.82 (0.41–1.52)	0.543		
Grade	I–II	211	33	15.6%	1			
	III	491	164	33.4%	2.71 (1.8–4.16)	<**0.001**		
Ki67	<20	33	6	18.2%	1			
	≥20	146	53	36.3%	2.56 (1.05–7.23)	0.051		
NAC regimen	Anthra-tax	610	169	27.7%	1			
	Anthra	62	17	27.4%	0.99 (0.54–1.74)	0.962		
	Taxane-based	23	7	30.4%	1.14 (0.43–2.73)	0.774		
	Others	23	9	39.1%	1.68 (0.69–3.9)	0.236		
Mitotic index	<11	176	36	20.5%	1			
	11–22	202	53	26.2%	1.38 (0.86–2.25)	0.187		
	>22	319	110	34.5%	2.05 (1.34–3.19)	**0.001**		
Invasive tumor cellularity	≤60%	372	107	28.8%	1			
	>60%	344	95	27.6%	0.94 (0.68–1.31)	0.733		
DCIS component	Yes	605	174	28.8%	1			
	No	112	28	25%	0.83 (0.51–1.3)	0.417		
Pre-NAC TIL levels (10% increment)	0–10	266	40	15.0%	1			
	10–20	157	43	27.4%	2.13 (1.65–2.62)	**0.002**		
	20–30	135	44	32.6%	2.73 (2.24–3.22)	<**0.001**		
	30–40	53	18	34.0%	2.91 (2.25–3.57)	**0.002**		
	40–50	25	9	36.0%	3.18 (2.29–4.06)	**0.01**		
	50–60	34	16	47.1%	5.02 (4.27–5.77)	<**0.001**		
	60–70	29	18	62.1%	9.25 (8.42–10.07)	<**0.001**		
	70–80	12	8	66.7%	11.3 (10.05–12.55)	<**0.001**		
	80–90	6	6	100.0%				
	90–100	0	0					
Pre-NAC TILs	Linear				1.03 (1.02–1.04)	<**0.001**	1.03 (1.02–1.03)	<**0.001**

NOTE: OR for pCR and corresponding CI are calculated with a univariate logistic regression model. Pre-NAC TILs are considered as a continuous variable in the analyses. Due to the difficulty to translate a continuous variable into a pCR rate, we also reported pre-NAC TILs binned by 10% increment to enable comparison with further studies using other TIL threshold values. *P* values ≤0.05 are shown in bold.

Abbreviations: BC, breast cancer; BMI, body mass index (kg/m^2); CI, confidence interval; DCIS, ductal carcinoma *in situ*; NST, no specific type.

Results

Associations between pre-NAC TILs, clinicopathologic patterns, response to treatment, and survival

Patient and tumor characteristics before NAC. In total, 718 patients were included in the cohort [luminal ($n = 223$), TNBC ($n = 320$), and *HER2*-positive ($n = 175$); Supplementary Table S1). Mean pre-NAC TIL level was 24.2% (luminal: 16.2%; TNBC: 28.5%; *HER2*-positive: 26.5%; $P < 0.001$), and the distribution of TILs differed between breast cancer subtypes (Fig. 1A and B).

Pre-NAC TILs and response to treatment. Pre-NAC TIL levels were significantly higher in tumors for which pCR was achieved than for tumors for which residual disease (RD) was detected, except in *HER2*-positive breast cancers (Supplementary Table S2, $P_{\text{Interaction}} = 0.001$). Pre-NAC TILs were

Table 2. Association with clinical and pathologic pre- and post-NAC parameters with DFS (whole population, univariate, and multivariate analysis)

				All				
				Univariate			Multivariate	
Characteristics	**Class**	***N***	**Event[a]**	**HR (95% CI)**	***P***	**P_{wald}[b]**	**HR (95% CI)**	***P***
Pre-NAC parameters								
Age (years)	<45	286	60	1		0.666		
	45–55	254	54	0.98 (0.68–1.41)				
	>55	178	31	0.83 (0.53–1.27)				
Menopausal status	Post	259	53	1		0.864		
	Pre	452	89	0.97 (0.69–1.36)				
BMI class	<25	455	84	1		0.143		
	≥25	262	61	1.28 (0.92–1.78)				
Tumor size	T1–T2	529	96	1		**0.004**		
	T3	189	49	1.66 (1.18–2.34)				
Clinical node status	N0	282	54	1		0.449		
	N1–N2–N3	435	91	1.14 (0.81–1.6)				
ER status	Negative	397	91	1		**0.009**		
	Positive	321	54	0.64 (0.46–0.9)				
PR status	Negative	473	106	1		**0.006**		
	Positive	222	32	0.57 (0.38–0.84)				
HER2 status	Negative	543	127	1				
	Positive	175	18	0.45 (0.28–0.74)		**0.002**		
BC subtype	Luminal	223	44	1		<**0.001**	1	-
	TNBC	320	83	1.64 (1.14–2.37)			2.45 (1.55–3.87)	<**0.001**
	HER2	175	18	0.61 (0.35–1.05)			1.05 (0.53–1.7)	0.95
Histology	NST	661	130	1		0.206		
	Other	53	14	1.43 (0.82–2.48)				
Grade	I–II	211	41	1		0.344		
	III	491	101	1.19 (0.83–1.71)				
Ki 67	<20%	33	7	1		0.292		
	≥20%	146	41	1.54 (0.69–3.43)				
Invasive tumor cellularity	≤60%	372	82	1		0.345		
	>60%	344	63	0.85 (0.61–1.19)				
Mitotic index	<11	176	27	1		0.061		
	11–22	202	43	1.47 (0.91–2.37)				
	>22	319	73	1.7 (1.1–2.65)				
Pre-NAC TILs	As FP[c]					<**0.001**		**0.01**
Post-NAC parameters								
pCR status	No pCR	516	131	1		<**0.001**		
	pCR	202	14	0.26 (0.15–0.46)				
RCB index	Continuous			1.63 (1.42–1.86)		<**0.001**	1.66 (1.4–1.95)	<**0.001**
Post-NAC TILs	Linear			1.01 (0.99–1.02)		0.325		
Mitotic index	<11	524	64	1		<**0.001**	1	-
	11–22	34	8	1.94 (0.93–4.04)	0.078		0.95 (0.43–2.1)	0.89
	>22	120	61	5.54 (3.9–7.88)	<**0.001**		2.92 (1.95–4.35)	<**0.001**
Invasive tumor cellularity	≤30%	456	59	1		<**0.001**		
	>30%	237	79	2.6 (1.85–3.64)	<**0.001**			
Size of nodal metastasis (mm)	≤2	135	45	1		**0.046**		
	3–5	73	14	0.51 (0.28–0.93)	**0.028**			
	>5	59	21	1.15 (0.68–1.93)	0.604			

Abbreviations: BC, breast cancer; BMI, body mass index (kg/m^2); DCIS, ductal carcinoma *in situ*; DFS, disease-free survival; FP, fractional polynomial; NAC, neaodjuvant chemotherapy; NST, no specific type.

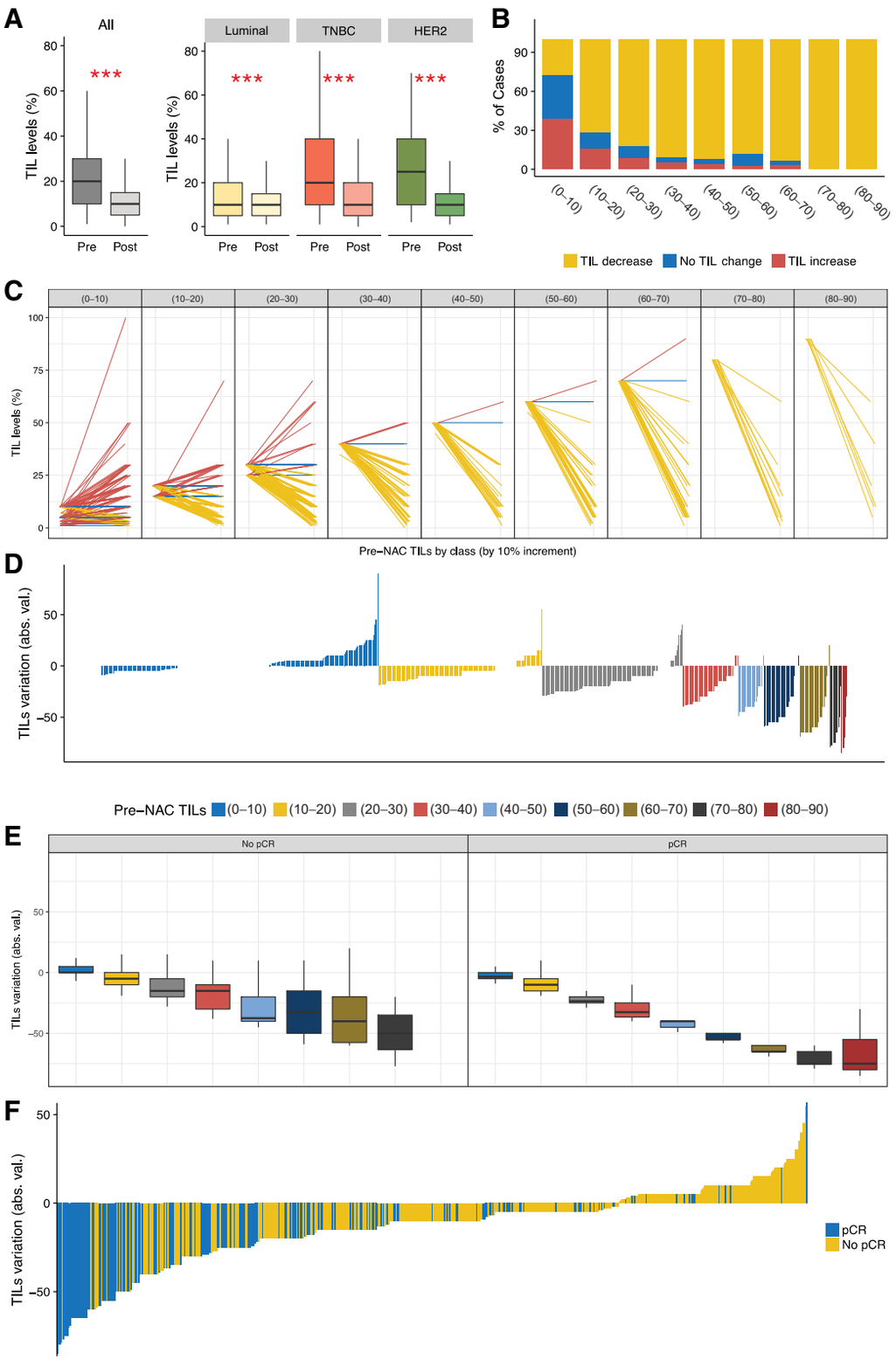
[a]An event includes either locoregional recurrence, distant recurrence, or death.

[b]P_{wald} is the *P* value for the Wald test, and *P* represents the test of a given class versus the reference class.

[c]Due to a significant deviation to the linearity assumption, pre-NAC TILs are considered as a continuous variable but are modelized with a fractional polynomial. Post-NAC TILs are considered as a continuous, linear variable.

significantly associated with pCR (all: OR, 1.03; confidence interval, 1.02–1.04; $P < 0.001$; Table 1]. However, after stratification by breast cancer subtype, this association was found to be significant only in TNBCs (luminal: OR, 1.03; CI, 1–1.06, $P = 0.058$; TNBC: OR, 1.03; CI, 1.02–1.04; $P < 0.001$; *HER2*-positive: OR, 1.01; CI, 0.99–1.03, $P = 0.341$; Supplementary Table S3). The association between TILs and pCR (Fig. 1C) was linear for all groups (Fig. 1D, E, and G) except TNBCs, for which it was best fitted by a cubic spline ($P = 0.006$; Fig. 1F). In univariate and multivariate analysis, pre-NAC TIL levels were significantly associated with pCR in the whole population and in the TNBC subtype.

Prognostic impact of pre-NAC TILs. Pre-NAC TIL levels were significantly associated with DFS in the whole population (HR, 0.988; CI, 0.979–0.998; $P = 0.017$; Table 2) and in the TNBC subgroup (HR, 0.982; CI, 0.971–0.993; $P = 0.002$), but not in the other subgroups (luminal: HR, 0.994; CI, 0.971–1.018; $P = 0.641$; *HER2*-positive: HR, 1.007; CI, 0.981–1.032; $P = 0.611$; Supplementary Table S4). Statistical tests revealed significant



deviations from the assumption of linearity in the whole population, and in the luminal and TNBC subgroups, consistent with a nonlinear prognostic effect of TILs. No such deviation from linearity was observed in the *HER2*-positive population (Fig. 1H–K). In addition, the interaction test between pre-NAC TILs and chemotherapy regimen on DFS was significant ($P_{interaction} = 0.05$), suggesting that the positive impact of TILs on DFS was different according to the NAC used (Anthra-taxanes: HR, 0.993; 95% CI, 0.983–1.003; $P = 0.18$; Others: HR, 0.968; 95% CI, 0.944–0.994; $P = 0.014$).

TIL variations before and after NAC

After chemotherapy, TIL levels decreased in 61.6% of tumors ($n = 441$), did not change in 17.7% ($n = 127$), and increased in 20.7% ($n = 148$). Mean TIL levels were higher before than after NAC (all: 24.1% vs. 13.0%, $P < 0.001$; luminal: 16.0% vs. 11.2 %; TNBC: 28.5% vs. 15.4 %; *HER2*-positive: 26.5% vs. 10.9 %, $P < 0.001$; Fig. 2A). These results were similar according to NAC regimen (Supplementary Fig. S2).

Mean TIL variation differed according to pCR status (pCR: −25.2 vs. no pCR: −5.6, $P < 0.001$). TIL levels were more likely to increase or remain stable after NAC if pre-NAC TIL levels were low than if they were high (Fig. 2B–D). PCR status was strongly associated with the magnitude of TIL level decrease (Fig. 2F); however, the variation of TIL level was strongly inversely correlated with pre-NAC TIL levels ($r = -0.80$, $P < 0.001$) regardless of pCR status (Fig. 2E). Overall, these findings suggest a strong inverse correlation between pre-NAC TIL levels and the variation of TIL levels, both of which are also strongly associated with pCR (Supplementary Fig. S3). This was true irrespective of breast cancer subtypes and NAC regimen (Supplementary Figs. S4 and S5).

Association between post-NAC TILs, clinicopathologic patterns, and survival

Association between post-NAC TILs and tumor characteristics. After NAC, mean TIL levels were 13%, and differences were observed between breast cancer subtypes (TNBC: 15.4%; luminal: 11.3%; *HER2*-positive: 10.9%, $P < 0.001$; Fig. 3A and B).

Post-NAC TIL levels differed significantly between tumors with and without pCR (no pCR/pCR: 14.7% vs. 8.8 %, $P < 0.001$; Fig. 3C; Supplementary Table S5) except in luminal breast cancers (TNBC: 18.6% vs. 10.3%, $P < 0.001$; *HER2*-positive: 14.0% vs. 6.2%, $P < 0.001$; luminal: 11.4% vs. 7.8%, $P = 0.27$). Post-NAC TIL levels were associated with aggressive tumor characteristics in the *HER2*-positive population, but not in luminal tumors and TNBCs (Fig. 3D–E). Significant interactions were observed for the association between post-NAC TILs, breast cancer subtype, and post-NAC mitotic index ($P_{Interaction}$: 0.037), invasive tumor cellularity ($P_{Interaction} < 0.001$), and RCB class ($P_{Interaction} = 0.05$, Fig. 3F).

Survival as a function of post-NAC TIL levels. Post-NAC TIL levels were not associated with DFS in the whole population (HR, 1.01; 95% CI, 0.099–1.02; $P = 0.325$; Table 2), but a significant interaction with breast cancer subtype was observed ($P_{Interaction} = 0.04$). Post-NAC TILs had no impact on prognosis in the luminal subgroup (HR, 0.996; CI, 0.964–1.029; $P = 0.79$) or TNBC subtypes (HR, 0.998; CI, 0.983–1.013; $P = 0.786$), but had a significant adverse impact in *HER2*-positive breast cancers (HR, 1.04; 95% CI, 1.016–1.064; $P = 0.001$; Supplementary Table S4). No significant deviation from the assumption of linearity was observed. In the population with RD, an adverse impact of post-NAC TILs was observed for patients with *HER2*-positive disease (HR, 1.029; CI, 1.002–1.057; $P = 0.034$), whereas a trend toward a protective effect of high post-NAC TIL levels was observed for TNBC (HR, 0.984; CI, 0.966–1.003; $P = 0.095$).

Multivariate survival analyses

After multivariate analysis, pre-NAC TIL levels, breast cancer subtype, RCB index, and post-NAC mitotic index were significantly associated with DFS (Table 2). In TNBCs, pre-NAC TIL levels were an independent predictor of better DFS (Supplementary Table S4), whereas post-NAC TIL levels were an independent predictor of impaired DFS in the *HER2*-positive subgroup. Neither pre-NAC nor post-NAC TIL levels were associated with DFS in the luminal subgroup.

TIL analyses by RCB class and trastuzumab use

Relationship between TIL levels and survival, by RCB class. Detailed analyses performed after stratification by RCB class are provided in Supplementary Tables S6 and S7. The association between post-NAC TILs and DFS was not significant in the RCB-0-I or RCB-II classes, whereas post-NAC TILs were associated with poor outcome in the RCB-III class (HR, 1.02; CI, 1.001–1.037; $P = 0.036$).

Survival analysis in the HER2-positive population, according to neoadjuvant trastuzumab use and ER status. We investigated survival as a function of neoadjuvant trastuzumab use ($n = 144$, 82.3%) or nonuse ($n = 31$, 17.7%) in the *HER2*-positive population (Supplementary Table S8). Pre-NAC TIL levels were not associated with DFS in any of the two groups, and post-NAC TIL levels were significantly associated with impaired DFS only

Figure 2.

TIL levels' variation before and after NAC. **A,** Bar plots of TIL levels before and after NAC in the whole population and in the various breast cancer subtypes. Bottom and top bars of the boxplots represent the first and third quartiles, respectively, the medium bar is the median, and whiskers extend to 1.5 times the interquartile range. **B,** Repartition (as percentages) of TIL variation classes, according to the pre-NAC TIL levels, binned by increments of 10%. TIL level variation is classified into three categories (TIL level decrease: yellow/no change: blue/increase: red). **C,** Variation of TIL levels according to the pre-NAC TIL levels binned by increments of 10%. Lines represent pre- and post-NAC paired TIL levels values of a given patient and are colored according to TIL variation category (TIL level decrease: yellow/no change: blue/increase: red). **D,** Waterfall plot representing the variation of TILs according to the pre-NAC TILs levels, binned by increments of 10%. Each bar represents the absolute TIL variation, that is, the difference between TIL levels after and before NAC and is colored according to the pre-NAC TIL levels. Within each pre-NAC TIL levels category, the change in TIL levels is ranked by increasing TIL level variation. **E,** Association between pre-NAC TIL levels by 10% increment and absolute difference in TIL levels before and after NAC, by pCR status (no pCR tumor, left panel; pCR tumor, right panel); each boxplot represents the median value and associated interquartile range. **F,** Waterfall plot representing the variation of TIL levels according to pCR status; each bar represents one sample, and samples are ranked by increasing order of TIL level change. Paired samples for which no change was observed have been removed from the graph.

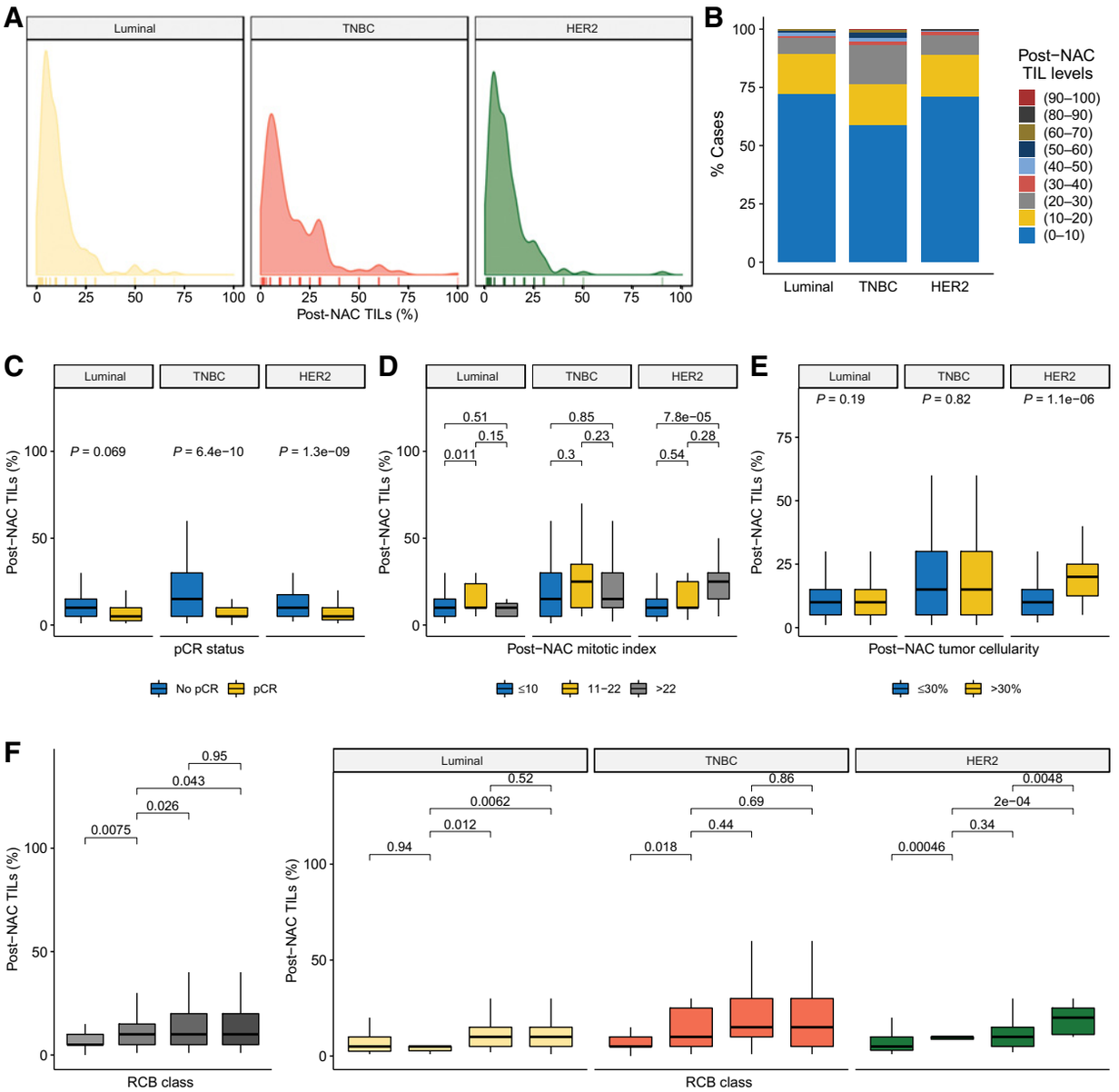


Figure 3.

Post-NAC TIL levels and their association with post-NAC pathologic factors. **A,** Distribution of post-NAC TIL levels by breast cancer subtype (kernel density plot). **B,** Barplot of the repartition of the percentage of tumors according to post-NAC TIL levels binned by 10% increment by breast cancer subtype. The proportion of tumors with TILs $\geq$ 60% is 2% ($n = 16$; luminal: 1%, $n = 3$; *HER2*-positive: 1%, $n = 1$; TNBC: 4%, $n = 12$). **C,** Post-NAC TIL levels by pCR status and by breast cancer subtype. **D,** Associations between post-NAC TIL levels and post-NAC mitotic index. **E,** Associations between post-NAC TIL levels and post-NAC cellularity. **F,** Associations between post-NAC TIL levels and RCB in the whole population, and after stratification by breast cancer subtype. Bottom and top bars of the boxplots represent the first and third quartiles, respectively, the medium bar is the median, and whiskers extend to 1.5 times the interquartile range. The results are considered statistically significant at a P value < 0.05 (*), <0.01 (**), or <0.001 (***).

in the population treated with neoadjuvant trastuzumab (HR, 1.038; CI, 1.011–1.065; $P = 0.005$).

We analyzed the *HER2*-positive population according to ER status (ER positive, $n = 98$; ER negative, $n = 77$; Supplementary Table S9). Tumors from the ER$^-$/HER2$^+$ subgroup were of higher grade, and TIL levels were higher before chemotherapy than in the ER$^+$/HER2$^+$ subgroup (30.6% vs. 23.2%, $P < 0.01$). After chemotherapy, there was no difference in the TIL levels.

Pre-NAC TIL levels were neither associated with pCR nor DFS in any of the ER-positive or ER-negative subgroups. Post-NAC TILs were associated with impaired DFS in ER-positive population (HR, 1.04; 95% CI, 1.02–1.07; $P < 0.01$) but not in the ER-negative population (HR, 1.04; 95% CI, 0.99–1.09; $P = 0.13$). This difference might be explained by a lack of statistical power ($P_{interaction\ with\ ER\ status}$ = NS).

Discussion

We report here detailed analyses of associations between baseline and posttreatment immune infiltration levels in a large cohort of paired pre- and post-NAC breast cancer samples. Our findings extend existing knowledge in this field in several ways.

First, our results confirm the widely reported association between pre-NAC TILs and pCR (1–3, 13–17), but we nevertheless observed (i) a nonlinear effect in TNBCs and (ii) a significant interaction with breast cancer subtype.

Nonlinear effects have been reported for the association of pCR and TIL levels in *HER2*-positive tumors (2, 18, 19), but linearity has never been investigated in detail for TNBCs in the neoadjuvant setting (no linearity test reported; refs. 1, 3, 9, 10, 15–17, 20–27). In addition, our data also revealed a nonlinear prognostic impact of TILs, differing by breast cancer and by NAC regimens.

Second, significant interactions with breast cancer subtype have been described only in the GeparSixto trial (3) so far. It is unclear why pre-NAC TILs were associated with pCR only in TNBC. Although the relationship we found here is almost constant in TNBC studies (1, 3, 17, 23), this effect seems less clear in *HER2*-positive breast cancer (2, 14, 20, 28). Several studies on *HER2*-positive breast cancer [NeoSPHERE (20), NeoALTTO (2), and GeparSepto (28)]—including ours—showed no association between pre-NAC TILs and pCR, whereas other did [GeparQuattro and GeparQuinto (14)]. Several hypotheses could explain such differences: (i) Differences in tumor biology; (ii) quantitative and qualitative differences in the immune infiltration and corresponding threshold values for defining high-TILs tumors (2, 18); (iii) the use, the type, and the interaction of TILs with anti–*HER2*-targeted therapies (2, 7, 29); (iv) the type and the sequences of NAC regimen, as interactions have been previously described between TILs, subtype, and chemotherapy regimen (3, 4, 5); and (v) and the difference in the percentages of ER-positive disease in the different *HER2*-positive breast cancer cohorts. Regarding luminal breast cancer, the number of patients whose tumor reached pCR was very low, and a lack of statistical power may partially explain why the association between pre-NAC TILs and pCR failed to reach statistical significance ($P = 0.058$).

Our results are in line with a recently published pooled analysis from German Breast Group (13) analyzing the relationships between TIL levels in baseline samples and oncologic outcomes in a large cohort of 3,771 patients receiving NAC. Denkert and colleagues (13) found that higher TIL levels were associated with a DFS benefit in *HER2*-positive and TNBC tumors, but with a poor OS in luminal *HER2*-negative breast cancers. These results underline the complexity of the relevance of TILs to each specific breast cancer subtype. We believe that both (i) TILs' subsetting and (ii) analyses of previous data taking into account chemotherapy regimen, type of targeted therapy, and breast cancer subtypes may help understanding these noticeable discrepancies.

Third, we demonstrated a decrease in mean TIL levels when comparing levels before and after chemotherapy. Only a few cohorts (9, 10, 15, 21, 24–27, 30–32) have reported pathologic TIL evaluations on paired matched samples, and all these previous cohorts were small (Supplementary Table S10). Two studies assessing lymphocyte density by computational pathology on large cohorts of patients from neoadjuvant trials [NeotAnGo (25) and ARTemis (27)] found that both pre-NAC immune infiltration and a decrease in immune infiltration were associated with pCR, and another study found that larger decreases in CD3 levels after treatment were associated with better DFS and OS (15). Due to the strong association between pre-NAC TILs, TILs changes, pCR status, post-NAC TILs, and DFS, their respective part regarding the association with prognostic remains unknown. Notably, TILs' changes might be an interesting parameter, as it was both strongly associated with pCR and DFS. As these data on TILs' variation are unprecedented on a large cohort of breast cancer patients in the literature, it calls for further validation of this endpoint on independent cohorts.

Fourth, regarding the immune infiltration after treatment, there was almost no post-NAC LPBCs (TILs $\geq$ 60%), highlighting the need for a revision of TIL level cutoff points after NAC. Post-NAC TIL levels were higher in tumors with RD than in areas of scarring in tumors displaying pCR. TIL levels have never been reported from pCR specimens, but recent guidelines (33) have suggested that these levels could be evaluated for research purposes. We found that TIL levels were extremely low in most, but not all, tumors scars. Our findings might suggest that, once the immune cells have eradicated the tumor, they would move into the periphery similar to responses to infection or other anomalies eliciting an immune response. Research on post-NAC TILs in pCR specimen could be of interest notably to analyze their association with the rare subgroup of patients experiencing relapse after their tumor reached pCR.

In cases of RD, TILs were associated with aggressive post-NAC patterns only in the *HER2*-positive subgroup. It remains unclear whether this difference reflects inherent differences between the three breast cancer subtypes, the use of neoadjuvant trastuzumab, or differences in the immune infiltration in RD. Two hypotheses can be drawn. On the one hand, TILs in specimen with RD could be active but may not have had sufficient time to completely eradicate the tumor. Our data do not support this hypothesis, because we found no correlation at all between time from biopsy to surgery and post-NAC TIL levels (Supplementary Fig. S6). On the other hand, the immune response may not recognize the tumor cells and post-NAC TILs could be unable to exert their antitumor function, possibly due to a surrounding immunosuppressive milieu.

Finally, our results suggest that pre- and post-NAC TIL levels may have different impacts on outcome. In TNBC, high pre-NAC TIL levels were an independent predictor of good prognosis, whereas in *HER2*-positive breast cancer, high post-NAC TIL levels were an independent predictor of poor outcome. We are currently characterizing the immune subpopulations, immune checkpoint, and immune checkpoint ligand expression in residual tumor specimens, in the hope that this will shed further light on the mechanisms underlying the observed differences in the prognostic impact of post NAC-TILs in the three breast cancer subtypes. Analyses of spatial and temporal dynamics, particularly to determine whether TIL location (intratumoral vs. stromal) has a differential effect on outcome, will also be of interest.

The strengths of this study include the large sample size and the availability of paired matched pre- and post-NAC samples for 716 patients. In addition, the patients and samples were derived from an institutional cancer center cohort, and therefore reflect real-life conditions more faithfully than analyses of results for randomized trials including only highly selected patients. The limitations of this study include the lack of data

on TILs during NAC (i.e., on-treatment biopsies), which would have (i) provided insights into the mechanisms underlying immune response to chemotherapy and (ii) confirmed if pre-NAC TIL levels go straightforward to the levels observed after NAC, or if it is preceded by an initial increase. On-treatment data from the I-SPY trial suggest that the immune genes expression decreases as soon as 1 to 4 days after NAC beginning (34). In addition, the study was performed at a single center, making external validation necessary. Large integrative and collaborative analyses may make it possible to decipher the role of immune infiltration in breast cancer in more detail, particularly in cases of RD after NAC. We therefore provide our original data as an open-access resource for the medical and scientific community, for pooling with existing datasets (Supplementary Table S11).

Our results have several implications. First, they suggest that future studies should include interaction and linearity tests, to help determining and validating TIL thresholds values relevant to each breast cancer subtype, both in the pre- and in the post-NAC setting. Second, due to the multiplicity of interactions (breast cancer subtypes, NAC regimen, benefit from targeted therapy, RCB score), efforts should be paid in routinely score TILs both in the pre and the post-NAC setting, and share data within collaborative projects, as such complex associations may only be deciphered with a very large amount of patients and samples. Finally, the adverse outcome associated with high TIL levels after the completion of NAC in some subgroups (*HER2*-positive patients; RCB-III tumors) highlights the urgent need for second-line trials in the post-NAC setting. Immunotherapies may theoretically be of interest for the treatment of tumors with an immune infiltrate associated with a poor prognosis.

Disclosure of Potential Conflicts of Interest

F. Reyal is an advisory board member/unpaid consultant for Agendia. No potential conflicts of interest were disclosed by the other authors.

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