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ORIGINAL REPORT

Tumor-Infiltrating Lymphocytes and Response to Neoadjuvant Chemotherapy With or Without Carboplatin in Human Epidermal Growth Factor Receptor 2–Positive and Triple-Negative Primary Breast Cancers

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Terms in blue are defined in the glossary, found at the end of this article and online at www.jco.org.

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ABSTRACT

Purpose
Modulation of immunologic interactions in cancer tissue is a promising therapeutic strategy. To investigate the immunogenicity of human epidermal growth factor receptor 2 (HER2) –positive and triple-negative (TN) breast cancers (BCs), we evaluated tumor-infiltrating lymphocytes (TILs) and immunologically relevant genes in the neoadjuvant GeparSixto trial.

Patients and Methods
GeparSixto investigated the effect of adding carboplatin (Cb) to an anthracycline-plus-taxane combination (PM) on pathologic complete response (pCR). A total of 580 tumors were evaluated before random assignment for stromal TILs and lymphocyte-predominant BC (LPBC). mRNA expression of immune-activating (*CXCL9*, *CCL5*, *CD8A*, *CD80*, *CXCL13*, *IGKC*, *CD21*) as well as immunosuppressive factors (*IDO1*, *PD-1*, *PD-L1*, *CTLA4*, *FOXP3*) was measured in 481 tumors.

Results
Increased levels of stromal TILs predicted pCR in univariable ($P < .001$) and multivariable analyses ($P < .001$). pCR rate was 59.9% in LPBC and 33.8% for non-LPBC ($P < .001$). pCR rates ≥ 75% were observed in patients with LPBC tumors treated with PMCb, with a significant test for interaction with therapy in the complete ($P = .002$) and HER2-positive ($P = .006$), but not the TNBC, cohorts. Hierarchic clustering of mRNA markers revealed three immune subtypes with different pCR rates ($P < .001$). All 12 immune mRNA markers were predictive for increased pCR. The highest odds ratios (ORs) were observed for *PD-L1* (OR, 1.57; 95% CI, 1.34 to 1.86; $P < .001$) and *CCL5* (OR, 1.41; 95% CI, 1.23 to 1.62; $P < .001$).

Conclusion
Immunologic factors were highly significant predictors of therapy response in the GeparSixto trial, particularly in patients treated with Cb. After further standardization, they could be included in histopathologic assessment of BC.

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INTRODUCTION

Treatment of human epidermal growth factor receptor 2 (HER2) –positive and triple-negative breast cancers (TNBCs) with neoadjuvant therapy leads to pathologic complete response (pCR) rates of 30% to 50%,[1,2] which have been linked to long-term benefit.[3,4] For TNBC, the use of platinum-based chemotherapy is currently under evaluation as a new chemotherapeutic option.[5,6] However, a relevant number of patients still do not respond to treatment, and therefore, new treatment options are needed.

Previous investigations have shown that immunologic parameters are relevant for response to neoadjuvant chemotherapy in BC[7-10] as well as for outcome after adjuvant therapy.[11-13] These immune signals are particularly strong in HER2-positive and TNBCs.[14,15] Comparably high response rates were reported in initial clinical trials evaluating inhibitors of immune checkpoints, such as anti–PD-L1,

anti–PD-1, or anti-CTLA4 antibodies.[16-18] Therefore, it would be interesting to evaluate expression levels and predictive capacity of immune markers in clinical cohorts as a basis for future combined immunotherapy approaches in BC. These combinations might be particularly promising, because it has been shown that many chemotherapeutic agents are immunogenic.[19]

In this study, we prospectively validated tumor-infiltrating lymphocytes (TILs) in 580 tumor samples of HER2-positive and TN breast carcinomas in the neoadjuvant GeparSixto trial (Fig 1A).[5] On the molecular level, mRNA expression levels of 12 immune genes were measured, including immune-activating (*CXCL9*, *CCL5*, *CD8A*, *CD80*, *CXCL13*, *IGKC*, *CD21*) and putative immunosuppressive factors (*IDO1*, *PD-1*, *PD-L1*, *CTLA4*, *FOXP3*). The rationale for selection of markers was to include T-cell markers, B-cell markers, chemokines, and immune checkpoint markers that are currently under evaluation as therapeutic targets. We prospectively evaluated the hypothesis that pCR rates would be higher in tumors with increased levels of TILs in the pretherapeutic core biopsy. Secondary aims were the evaluation of the immunologic infiltrate and immune mRNA markers in subgroups with or without carboplatin (Cb) for TN and HER2-positive BCs.

PATIENTS AND METHODS

Study Population

In GeparSixto (ClincalTrials.gov NCT01426880), patients with centrally confirmed HER2-positive or TNBC were treated for 18 weeks with paclitaxel 80 mg/m^2 once every week and nonpegylated liposomal doxorubicin 20 mg/m^2 once every week (PM). Patients were randomly assigned at a 1:1 ratio to receive simultaneously Cb (PMCb; area under curve, 1.5 [initially 2.0] once every week) or not (ie, PM only). Patients with TNBC received bevacizumab 15 mg/kg once every 2 weeks, and patients with HER2-positive disease received trastuzumab 6 mg/kg (loading dose 8 mg/kg) once every 3 weeks and lapatinib 750 mg daily simultaneously. Pretherapeutic formalin-fixed paraffin-embedded core biopsies were collected after written informed consent. Hormone receptor positivity was defined as $\geq$ 1% positive cells for estrogen (ER) and/or progesterone receptors (PRs). Ethical approval was obtained for all clinical centers and from the institutional review board of the Charité Berlin. We defined pCR as the absence of residual invasive or noninvasive tumor cells in breast and lymph nodes (ypT0 ypN0). This study is reported according to the REMARK (Reporting Recommendations for Tumor Marker Prognostic Studies) criteria.[20]

Statistical Analysis Plans

The investigations on TILs and mRNA markers were conducted as two separate translational research studies. The evaluation of TILs was part of the original study protocol of GeparSixto as a secondary end point. TILs were evaluated prospectively before random assignment, and the results were documented in a signed histopathologic report that was sent to the central study office for each patient. A prespecified statistical analysis plan for TIL evaluation was defined before statistical evaluation started.

The evaluation of mRNA markers was performed as a separate project using the existing tumor samples after the clinical study was completed. This separate project had a separate statistical analysis plan and study outline, which were also completely defined before the evaluation was started.

Prospective Histopathologic Evaluation of Inflammatory Infiltrates

TIL evaluation was performed on hematoxylin and eosin–stained sections in a routine diagnostic setting.[7] Each patient case was evaluated by two of six pathologists. We evaluated the percentage of stromal as well as intratumoral TILs separately. The predominant lymphocytic infiltrate was located in the stroma (Figs 1B to 1E). Intratumoral TILs had lower levels and were correlated with stromal TILs (Appendix Fig A1, online only). Therefore, in this analysis, we focused on stromal TILs as a continuous parameter. For some analyses, we used lymphocyte-predominant (LP) BC ($\geq$ 60% of either intratumoral or stromal TILs) as a predefined categorical parameter, based on a previous study.[8] To evaluate interobserver variance of TIL assessment, three independent pathologists (C.D., B.M.P., W.D.S.) evaluated 87 digital images of selected regions from 29 tumors. The results were compared with automated image analysis.[21,22]

Evaluation of mRNA Markers

Immunologically relevant genes were selected based on previous evaluations[7] and published data on checkpoint inhibitors.[16-18] Total RNA was extracted from 5-μm whole formalin-fixed paraffin-embedded sections with $\geq$ 30% tumor area, as defined in a previous study,[23] using a fully automated method (VERSANT; Siemens, Tarrytown, NY).[24] Genes were measured in triplicate by quantitative real-time polymerase chain reaction (RT-PCR) using the SuperScript III Platinum One-Step quantitative RT-PCR system with ROX (Invitrogen, Karlsruhe, Germany) in a ViiA 7 RT-PCR system (Applied Biosystems, Darmstadt, Germany). The thermal profile included 30 minutes at 50°C, 20.5 minutes at 8°C and 2 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 30 seconds at 60°C. Primer and probe sequences (Appendix Table A1, online only) were selected by empiric rules and Primer Express software (version 3.0; Applied Biosystems). No-template controls, a standardized reference RNA control (Clontech Laboratories, Saint-Germain-en-Laye, France), and a pooled RNA control from TNBC samples (for *CTLA4*, *FOXP3*, and *PD-1*) were measured in parallel. Cycle threshold (Ct) values were calculated using ViiA 7 software (version 1.2.1; Applied Biosystems). Relative expression levels of genes of interest were calculated as ΔCt values ($\Delta Ct = 20 - [Ct_{GOI} - Ct_{(mean\ of\ RPL37A,\ CALM2,\ OAZ1)}]$), where GOI indicates gene of interest. Trained laboratory personnel strictly blinded to clinical data performed all mRNA analyses after the end of the recruitment period.

Statistical Analyses

Associations between LPBC and pCR were investigated with χ^2 tests for categorical variables using SPSS software (version 21; SPSS, Chicago, IL) and with univariable logistic regression. Odds ratios (ORs) and 95% CIs with two-sided *P* values were used. A *P* value $\leq$ .05 was considered statistically significant; no adjustment for multiple comparisons was performed. For the logistic regression, the following clinical variables were used: ER negative/PR negative versus ER positive and/or PR positive; HER2 positive versus HER2 negative; grade 3 versus grade 1 or 2; cT1-2 versus cT3-4; cN0 versus cN+; PM versus PM plus Cb; and age $\geq$ 50 versus $<$ 50 years. Stromal TILs were also included in an additional exploratory multivariable analysis of mRNA markers. An interaction test was performed for some analyses.

Interobserver variance was measured using Cohen's kappa and the intraclass correlation coefficient (ICC).[25] The ICC was calculated using the mixed model and absolute agreement. The ICC for single measures is an index for reliability of single raters, whereas the ICC for average measures is an index for reliability of different raters averaged together (which is similar to diagnostic approach used in GeparSixto); we report both ICC values.

RESULTS

Baseline Clinical Data

A total of 580 tumors (98.6%) were prospectively evaluated for TILs, including 266 HER2-positive (45.9%) and 314 TN tumors (54.1%; Appendix Table A2, online only). Eight patient cases were excluded from the evaluation, because the diagnostic biopsy was from a lymph node. Median patient age was 47 years. mRNA markers were measured in 481 samples (82.9%; Fig 1A). For 99 patients (17.1%), the mRNA markers could not be measured because of low tumor-cell content.

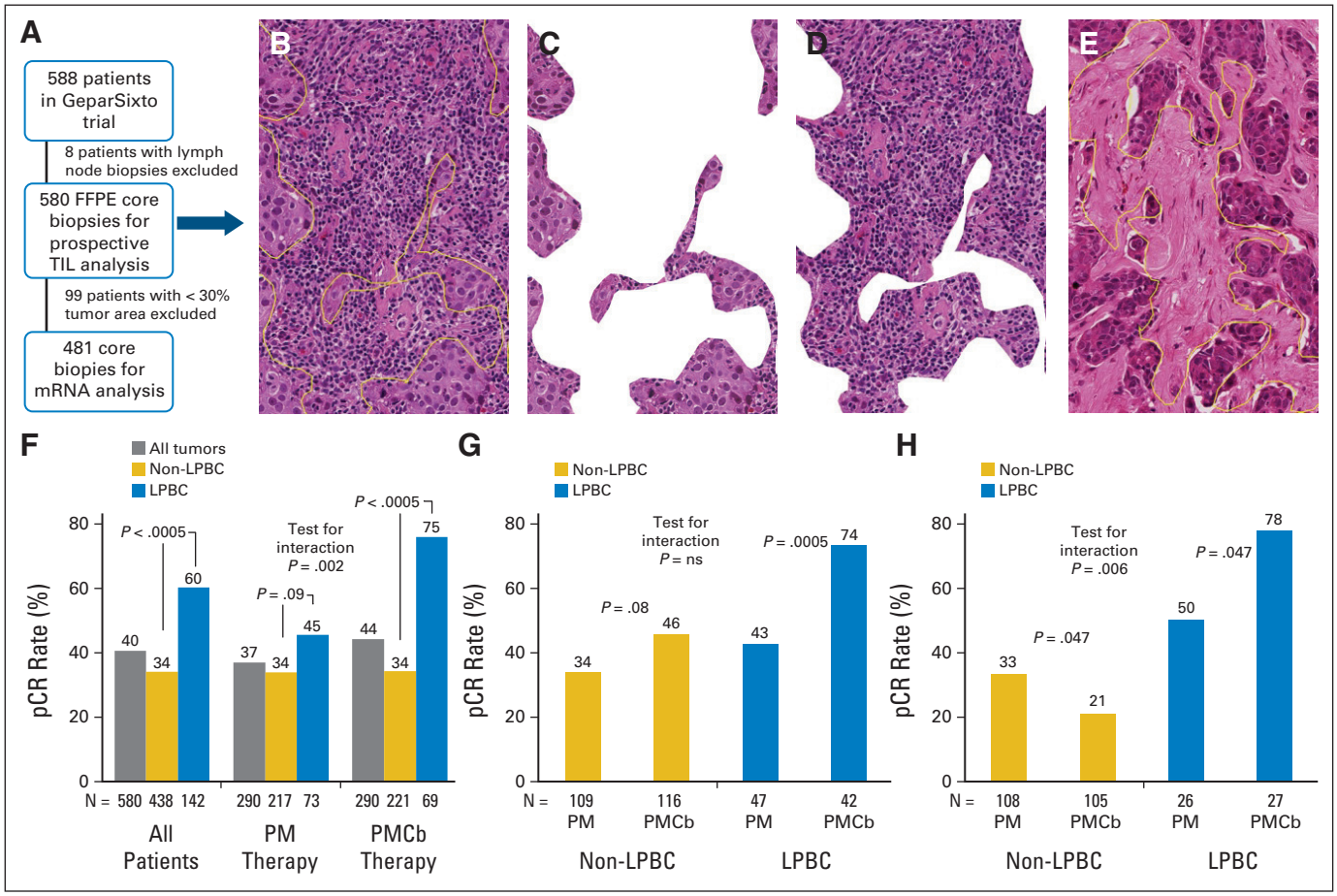

Fig 1. (A) CONSORT diagram of patients included in prospective tumor-infiltrating lymphocyte (TIL) and mRNA marker evaluations. (B) Breast carcinoma with predominant lymphocytic infiltrate (LPBC). TILs were evaluated separately in (C) epithelial and (D) stromal components. Stromal lymphocytes were usually denser and more straightforward for evaluation; therefore, only stromal lymphocytes are reported. (E) Breast carcinoma without lymphocytic infiltrate. (F to G) Increased pathologic complete response (pCR) rates in tumors with LPBC phenotype, compared with non-LPBC tumors, in (F) complete GeparSixto cohort, (G) Triple-negative breast cancer subgroup, and (H) human epidermal growth factor receptor 2 (HER2) –positive subgroup. Test for interaction between LPBC status and response to paclitaxel plus nonpegylated liposomal doxorubicin (PM) versus PM with carboplatin (PMCb) therapy were significant in (F) complete cohort ($P = .002$) and (G) HER2-positive subgroup ($P < .001$). FFPE, formalin fixed paraffin embedded; NS, nonsignificant.

pCR Rates in LPBC

Of the 580 patients, 142 (24.5%) had an LPBC phenotype (Figs 1B to 1D; Appendix Table A3, online only), which was predictive for increased response to neoadjuvant chemotherapy. In the complete cohort, LPBC tumors had a pCR rate of 59.9%, compared with 33.8% for non-LPBC tumors ($P < .001$; Fig 1F). The OR for prediction of pCR by LPBC was 2.92 (95% CI, 1.98 to 4.31; $P < .001$); this was in the same range as that for hormone receptor status (OR, 2.78; 95% CI, 1.84 to 4.20; $P < .001$; Table 1).

The percentage of stromal TILs (Fig 1D) was significantly linked to pCR, with an OR of 1.22 (95% CI, 1.14 to 1.31) per 10% increase in lymphocytes ($P < .001$; Table 1; Appendix Fig A1, online only). In a multivariable analysis adjusted for clinicopathologic parameters, LPBC was an independent predictor of pCR (OR, 2.66; 95% CI, 1.76 to 4.02; $P < .001$; Table 1). A similar independent prediction was observed for stromal TILs as a continuous parameter (OR, 1.20 per 10% increase; 95% CI, 1.11 to 1.29; $P < .001$; Table 1), as well as for intratumoral TILs (Appendix Table A4, online only).

Subgroup Analysis for HER2-Positive and TN Tumors and Therapy Arms

An LPBC phenotype was found in 53 (19.9%) of the 266 HER2-positive tumors and 89 (28.3%) of the 314 TN tumors (Appendix Table A3, online only). Stromal TILs as well as LPBC were significant for prediction of pCR in univariable and multivariable analyses in TN (Table 1; Fig 1G) and HER2-positive tumors (Table 1; Fig 1H; Appendix Table A5, online only).

In a separate analysis for the two therapy arms (PM *v* PM plus Cb; Fig 1F), pCR rates in patients with LPBC tumors were significantly higher with Cb therapy. In the LPBC subset, the addition of Cb increased the odds of pCR 3.71-fold, whereas in non-LPBCs, the increase was only 1.01-fold, leading to an interaction OR of 3.67 (Appendix Table A6, online only). In TN and HER2-positive tumors with PMCb therapy, high response rates of 74% and 78% were observed for LPBC tumors; the test for interaction was significant in the complete cohort ($P = .002$; Fig 1F) and in the HER2-positive ($P = .006$; Fig 1H), but not the TNBC, subgroup (Fig 1G; Appendix Tables A5 and A6, online only). Similarly, for stromal TILs as a continuous

Table 1. Univariable and Multivariable Analyses of Stromal TILs and LPBC for Prediction of pCR (ypT0ypN0)

Characteristic	Univariable Analysis			Multivariable Analysis					
				Stromal TILs			LPBCs		
	OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*
Complete cohort (N = 580)									
Stromal TILs (per 10%)	1.22	1.14 to 1.31	< .001	1.20	1.11 to 1.29	NS		Not included	
LPBC (≥ 60% *v* < 60%)	2.92	1.98 to 4.31	< .001		Not included		2.66	1.76 to 4.02	< .001
HR status (negative *v* positive)	2.78	1.84 to 4.20	< .001	2.92	1.67 to 5.09	< .001	2.99	1.72 to 5.21	< .001
HER2 status (negative *v* positive)	1.54	1.10 to 2.16	.01	0.68	0.42 to 1.10	NS	0.68	0.42 to 1.09	NS
Tumor grade (3 *v* 1 to 2)	1.73	1.21 to 2.47	.003	1.30	0.87 to 1.93	NS	1.37	0.93 to 2.04	NS
Clinical T stage (cT1-2 *v* cT3-4)	1.34	0.82 to 2.18	NS	0.98	0.57 to 1.69	NS	1.03	0.60 to 1.76	NS
Clinical N stage (cN0 *v* cN+)	1.62	1.15 to 2.30	.006	1.81	1.24 to 2.66	.002	1.76	1.20 to 2.57	.004
Therapy (PMCb *v* PM)	1.35	0.97 to 1.89	.076	1.32	0.92 to 1.88	NS	1.36	0.95 to 1.94	NS
Age group (≥ 50 *v* < 50 years)	0.97	0.69 to 1.35	NS	0.97	0.68 to 1.40	NS	0.96	0.67 to 1.38	NS
TNBC cohort (n = 314)									
Stromal TILs (per 10%)	1.15	1.05 to 1.26	.004	1.17	1.06 to 1.30	NS		Not included	
LPBC (≥ 60% *v* < 60%)	2.01	1.22 to 1.31	.006		Not included		2.17	1.27 to 3.73	.005
Tumor grade (3 *v* 1 to 2)	1.69	0.996 to 2.86	.052	1.84	1.03 to 3.30	.04	1.85	1.03 to 3.32	.04
Clinical T stage (cT1-2 *v* cT3-4)	2.94	1.29 to 6.72	.01	1.76	0.73 to 4.29	NS	1.81	0.75 to 4.37	NS
Clinical nodal status (cN0 *v* cN+)	2.72	1.65 to 4.48	< .001	2.75	1.60 to 4.74	< .001	2.63	1.53 to 4.50	< .001
Therapy (PMCb *v* PM)	1.97	1.26 to 3.10	.003	2.04	1.25 to 3.31	NS	2.08	1.28 to 3.46	.003
Age group (≥ 50 *v* < 50 years)	0.77	0.49 to 1.22	NS	0.92	0.56 to 1.52	NS	0.89	0.54 to 1.46	NS
HER2-positive cohort (n = 266)									
Stromal TILs (per 10%)	1.30	1.17 to 1.45	< .001	1.28	1.14 to 1.44	NS		Not included	
LPBC (≥ 60% *v* < 60%)	4.78	2.53 to 9.05	< .001		Not included		4.19	2.11 to 8.31	< .001
HR status	3.33	1.97 to 5.65	< .001	2.74	1.56 to 4.80	< .001	2.77	1.57 to 4.87	< .001
Tumor grade (3 *v* 1 to 2)	1.52	0.91 to 2.54	NS	0.97	0.54 to 1.73	NS	1.10	0.62 to 1.95	NS
Clinical T stage (cT1-2 *v* cT3-4)	0.67	0.35 to 1.27	NS	0.62	0.30 to 1.27	NS	0.64	0.31 to 1.33	NS
Clinical nodal status (cN0 *v* cN+)	0.85	0.51 to 1.41	NS	1.12	0.63 to 1.97	NS	1.09	0.62 to 1.92	NS
Therapy (PMCb *v* PM)	0.84	0.51 to 1.39	NS	0.76	0.43 to 1.33	NS	0.79	0.45 to 1.38	NS
Age group (≥ 50 *v* < 50 years)	1.28	0.77 to 2.13	NS	1.04	0.60 to 1.83	NS	1.06	0.61 to 1.87	NS

Abbreviations: Cb, carboplatin; HER2, human epidermal growth factor 2; HR, hormone receptor; NS, nonsignificant; LPBC, lymphocyte-predominant breast cancer; OR, odds ratio; pCR, pathologic complete response; PM, paclitaxel plus nonpegylated liposomal doxorubicin; TIL, tumor-infiltrating lymphocyte; TNBC, triple-negative breast cancer.

parameter, the test for interaction with therapy was also positive in the complete cohort ($P = .006$) and the HER2-positive subgroup ($P = .007$; Table 2), but not in the TNBC subgroup (Appendix Tables A5 and A6, online only).

In an exploratory logistic regression using the alternative pCR definition (ypT0is ypN0), stromal lymphocytes as well as LPBCs were still highly significant (stromal lymphocytes: OR, 1.19; 95% CI, 1.11 to 1.28; $P < .001$; LPBCs: OR, 2.83; 95% CI, 1.89 to 4.23; $P < .001$). With the wider pCR definition, the pCR rate of LPBC tumors with Cb treatment was 79% (for TNBC) and 85% (for HER2-positive BC). Using ypT0is ypN0 as an end point, the test for interaction between therapy and LPBC was still significant in the complete cohort

Table 2. Analysis of Interaction of Stromal TILs and LPBCs With Chemotherapy With or Without Carboplatin

Treatment Group	No. of Patients	Stromal TILs				LPBCs			
		OR	95% CI	*P*	*P**	OR	95% CI	*P*	*P**
Complete cohort					.006				.002
PM	290	1.11	1.004 to 1.22	.048		1.63	0.95 to 2.79	NS	
PMCb	290	1.35	1.21 to 1.49	< .001		5.96	3.22 to 11.01	< .001	
TNBC subgroup					.27				.12
PM	156	1.09	0.96 to 1.25	NS		1.44	0.72 to 2.91	NS	
PMCb	158	1.22	1.06 to 1.39	.004		3.35	1.54 to 7.30	.002	
HER2-positive subgroup					.007				.006
PM	134	1.13	0.98 to 1.30	NS		2.00	0.84 to 4.76	NS	
PMCb	132	1.53	1.29 to 1.82	< .001		13.21	4.75 to 36.7	< .001	

Abbreviations: Cb, carboplatin; HER2, human epidermal growth factor 2; NS, nonsignificant; LPBC, lymphocyte-predominant breast cancer; OR, odds ratio; PM, paclitaxel plus nonpegylated liposomal doxorubicin; TIL, tumor-infiltrating lymphocyte; TNBC, triple-negative breast cancer.
*Test for interaction.

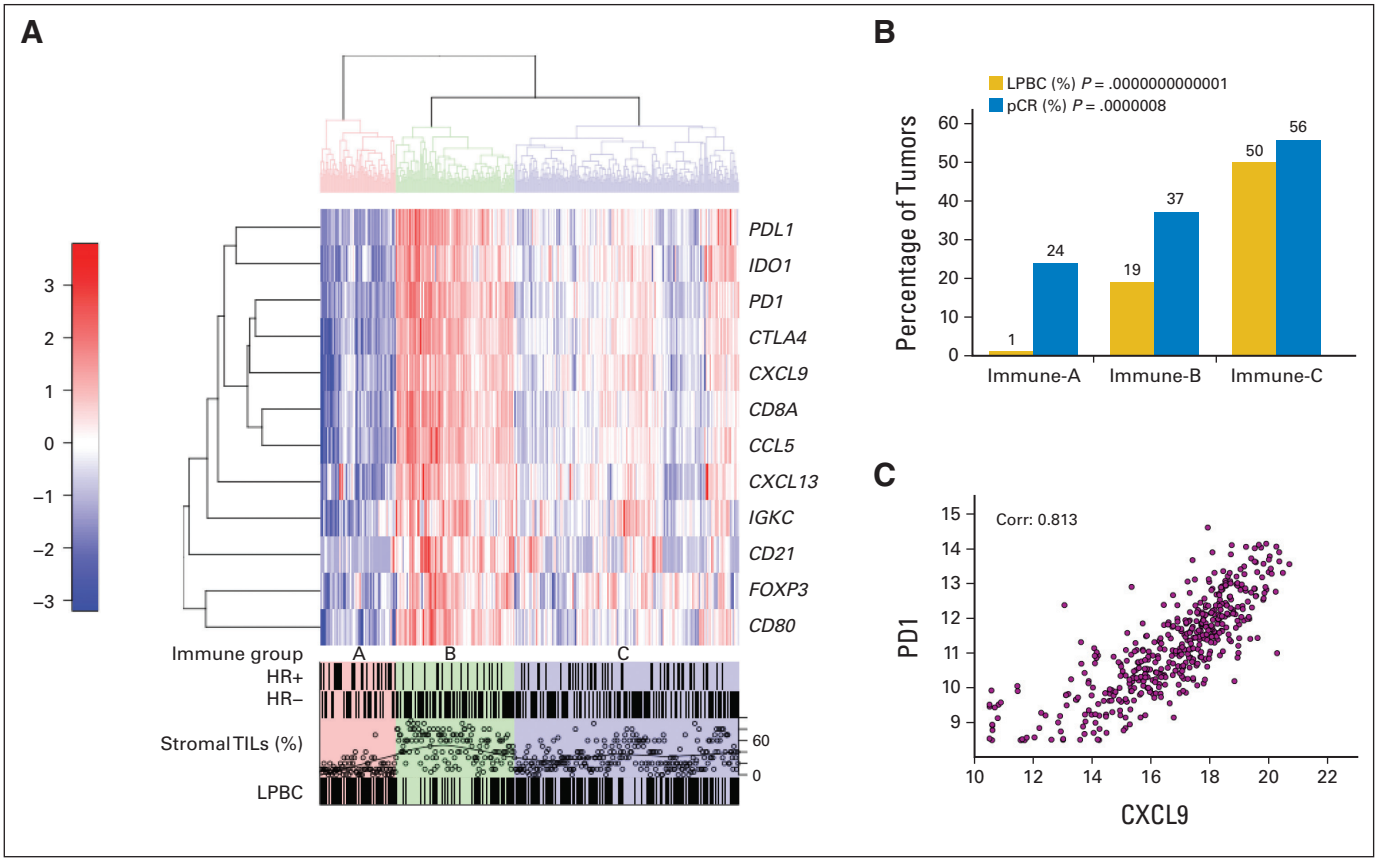

Fig 2. (A) Hierarchic clustering of 12 immunologically relevant genes in 481 tumors from GeparSixto showed three different immune groups of tumors with different expression of immunologic genes and different amounts of tumor-infiltrating lymphocytes (TILs). Immune group A tumors showed low expression of all immune genes; immune group C tumors showed high expression of immunologic genes; immune group B tumors showed intermediate gene expression levels. Corresponding levels of stromal TILs for each tumor as well as lymphocyte-predominant breast cancer (LPBC) status is shown in lower section of panel. (B) Evaluation of *CXCL9* and *PD-1* mRNA levels in 481 tumors from GeparSixto showed positive correlation (corr) between both markers (Pearson correlation coefficient, 0.813). (C) Three immune clusters were significantly different for percentage of LPBC tumors (χ^2 test for trend $P < .001$) and pathologic complete response (pCR) rate (χ^2 test for trend $P < .001$). HR, hormone receptor.

(interaction $P = .009$), but it was not significant in the TNBC or HER2-positive subgroup (data not shown).

Analytic Validation of TIL Assessment

To obtain data on interobserver variance of TIL assessment, three pathologists evaluated a set of digital images of 87 regions of interest from 29 tumors. For LPBC versus non-LPBC, Cohen's kappa values for comparison of the three evaluators with one another were 0.90, 0.69, and 0.60. For stromal TILs, the ICC for the 29 patient cases and three pathologists was 0.92 (95% CI, 0.83 to 0.96; $P < .001$) for single measures and 0.97 (95% CI, 0.93 to 0.99; $P < .001$) for average measures. Measurement of stromal TILs by automated image analysis showed that the lymphocytic infiltrate varied between < 500 and $> 11{,}000$ lymphocytes per mm^2 of stromal tissue (Appendix Fig A2, online only). The differences between the three observers were particularly relevant in those tumors with intermediate TIL levels between 20% and 50% that also showed increased intratumoral heterogeneity between the three regions of interest.

Evaluation of Immunologic mRNA Markers in Tumor Samples From GeparSixto

On the basis of previous reports by our group and others, we selected 12 immunologically relevant mRNA markers for detailed evaluation in breast cancer tissue, including T-cell markers, B-cell markers, chemokines, and immune checkpoint parameters (*CXCL9*, *CCL5*, *CD8A*, *CD80*, *CXCL13*, *IGKC*, *CD21*, *IDO1*, *PD-1*, *PD-L1*, *CTLA4*, *FOXP3*).

Hierarchic clustering of mRNA expression revealed three different immune subtypes of tumors with different expression of immunologic genes and different amounts of TILs (Fig 2A). Immune group A tumors showed low expression of all immune genes, immune group C tumors had high immunologic gene expression levels, and immune group B tumors had intermediate gene expression levels. Similar patterns were observed in the HER2-positive and TN subgroups (Appendix Fig A3, online only). The distribution of the three groups in the different subtypes is summarized in Appendix Table A3 (online only). The three immune subtypes had largely different response rates to chemotherapy. The pCR rates of immune groups A, B, and C were 24%, 37.4%, and 56.2%, respectively (χ^2 test for trend $P < .001$; Fig 2B). The percentages of LPBC tumors in immune groups A, B, and C were 1.1%, 19.1%, and 50.4%, respectively (χ^2 test for trend $P < .001$; Fig 2B).

All immune markers had highly significant ($P < .001$) positive correlations with one another and with stromal TILs (Appendix Fig A4, online only). Interestingly, even those markers that were linked to immunosuppressive activity in tumor

Table 3. Univariable and Multivariable Analyses of Immunologic mRNA Markers for Response to Chemotherapy and Interaction With Therapy Groups

Marker	Univariable Analysis			Multivariable Analysis				
				Clinical Parameters			Clinical Parameters and Stromal TILs	PM Versus PMCb Therapy
	OR*	95% CI	*P*	OR*	95% CI	*P*	*P*	*P*†
Complete cohort (n = 481)								
Stromal TILs	1.26	1.16 to 1.36	< .001	1.24	1.14 to 1.35	< .001	—	.007
CCL5	1.41	1.23 to 1.62	< .001	1.39	1.20 to 1.61	< .001	.04	.002
CXCL9	1.25	1.14 to 1.38	< .001	1.21	1.09 to 1.34	.003	NS	NS
CXCL13	1.16	1.06 to 1.26	.001	1.14	1.04 to 1.25	.006	NS	NS
CD8A	1.29	1.13 to 1.48	< .001	1.28	1.11 to 1.48	.001	NS	.01
PD-1	1.43	1.24 to 1.66	< .001	1.41	1.20 to 1.65	< .001	NS	.02
PD-L1	1.57	1.34 to 1.86	< .001	1.53	1.29 to 1.82	< .001	.005	NS
CTLA4	1.38	1.19 to 1.60	< .001	1.35	1.16 to 1.58	< .001	NS	NS
FOXP3	1.23	1.003 to 1.50	.05	1.29	1.04 to 1.60	.02	NS	NS
IDO1	1.25	1.14 to 1.36	< .001	1.22	1.11 to 1.34	< .001	.05	.03
IGKC	1.15	1.06 to 1.24	< .001	1.14	1.05 to 1.23	.002	NS	NS
CD80	1.59	1.26 to 2.01	< .001	1.59	1.24 to 2.05	< .001	NS	NS
CD21	1.11	1.02 to 1.21	.01	1.07	0.98 to 1.18	NS	NS	NS
TNBC cohort (n = 255)								
Stromal TILs	1.16	1.04 to 1.28	.007	1.19	1.06 to 1.33	.004	—	NS
CCL5	1.30	1.07 to 1.56	.007	1.36	1.11 to 1.68	.004	NS	.02
CXCL9	1.17	1.02 to 1.33	.02	1.16	1.005 to 1.34	.04	NS	NS
CXCL13	1.18	1.04 to 1.35	.01	1.19	1.03 to 1.38	.02	NS	NS
CD8A	1.21	1.01 to 1.46	.04	1.24	1.01 to 1.52	.04	NS	.02
PD-1	1.27	1.05 to 1.53	.01	1.35	1.09 to 1.66	.005	NS	NS
PD-L1	1.44	1.18 to 1.77	< .001	1.45	1.16 to 1.82	.001	.04	NS
CTLA4	1.30	1.07 to 1.58	.009	1.37	1.10 to 1.71	.005	NS	NS
FOXP3	1.09	0.84 to 1.42	NS	1.23	0.92 to 1.65	NS	NS	NS
IDO1	1.18	1.05 to 1.32	.004	1.21	1.06 to 1.37	.004	NS	.05
IGKC	1.10	0.998 to 1.21	NS	1.11	0.998 to 1.24	NS	NS	NS
CD80	1.74	1.28 to 2.38	< .001	1.93	1.36 to 2.73	< .001	.005	NS
CD21	0.99	0.89 to 1.11	NS	0.98	0.87 to 1.12	NS	NS	NS
HER2-positive cohort (n = 226)								
Stromal TILs	1.37	1.22 to 1.55	< .001	1.37	1.20 to 1.57	< .001	—	.008
CCL5	1.52	1.24 to 1.87	< .001	1.46	1.17 to 1.81	.001	NS	NS
CXCL9	1.34	1.16 to 1.56	< .001	1.30	1.11 to 1.53	.001	NS	NS
CXCL13	1.12	0.99 to 1.26	NS	1.12	0.98 to 1.27	NS	NS	NS
CD8A	1.39	1.13 to 1.70	.002	1.34	1.08 to 1.66	.008	NS	NS
PD-1	1.67	1.31 to 2.12	< .001	1.58	1.22 to 2.05	.001	NS	NS
PD-L1	1.79	1.37 to 2.34	< .001	1.75	1.31 to 2.33	< .001	NS	NS
CTLA4	1.45	1.17 to 1.80	.001	1.40	1.11 to 1.76	.005	NS	.05
FOXP3	1.61	1.15 to 2.26	.005	1.53	1.08 to 2.16	.02	NS	NS
IDO1	1.31	1.14 to 1.50	< .001	1.29	1.11 to 1.49	.001	NS	NS
IGKC	1.21	1.07 to 1.37	.002	1.18	1.04 to 1.35	.01	NS	NS
CD80	1.38	0.96 to 1.97	NS	1.29	0.88 to 1.88	NS	NS	NS
CD21	1.25	1.10 to 1.43	.001	1.19	1.04 to 1.38	.02	NS	NS

Abbreviations: Cb, carboplatin; HER2, human epidermal growth factor 2; NS, nonsignificant; OR, odds ratio; PM, paclitaxel plus nonpegylated liposomal doxorubicin; TIL, tumor-infiltrating lymphocyte; TNBC, triple-negative breast cancer.
*For stromal TILs, OR is reported per 10%; for RNA markers, OR is reported per Δ cycle threshold.
†Test for interaction.

tissue (*PD-1*, *PD-L1*, *CTLA4*, *IDO1*) had a significant positive correlation with the other immune markers and with TILs (Fig 2C; Appendix Fig A4, online only).

Prediction of Response to Neoadjuvant Chemotherapy by Immunologic mRNA Expression

All 12 immune mRNA markers were significantly linked to increased pCR (Table 3; Fig 3A). The highest ORs were observed for *PD-L1* (OR, 1.57 per ΔCt; 95% CI, 1.34 to 1.86; $P < .001$) and *CCL5* (OR, 1.41 per ΔCt; 95% CI, 1.23 to 1.62; $P < .001$). The ORs were generally higher in HER2-positive compared with TN tumors (Figs 3B and 3C), which might be explained by an interaction of the immune system with the additional anti-HER2 therapy.

Eleven of the 12 mRNAs were also significant in multivariable analysis adjusted for clinicopathologic factors (Table 3). As shown in Figures 3A to C, even putative immunosuppressive markers such as *PD-1*, *PD-L1*, *CTLA4*, and *IDO1* had a positive correlation with chemotherapy response. The test for interaction with PM versus PMCb therapy was significant for *CCL5* ($P = .002$), *CD8A* ($P = .01$), *PD-1* ($P = .02$), and *IDO1* ($P = .03$; Table 3). Inclusion

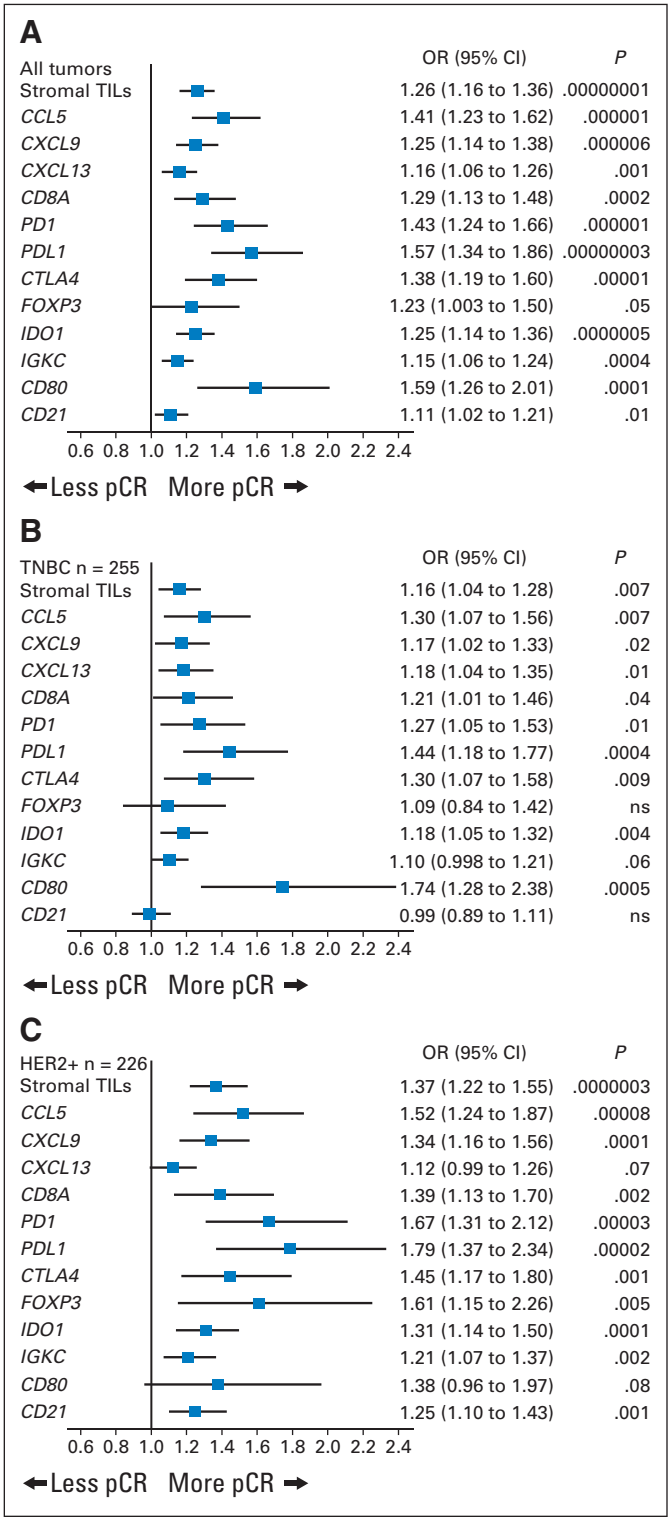

Fig 3. Univariable logistic regression for prediction of response to neoadjuvant chemotherapy, including stromal tumor-infiltrating lymphocytes (TILs) and 12 immunologically relevant genes measured by quantitative real-time polymerase chain reaction, for (A) complete cohort, (B) triple-negative breast cancer (TNBC) tumors, and (C) human epidermal growth factor receptor 2 (HER2) –positive tumors. All mRNA markers, including immunosuppressive markers *IDO1*, *PD-1*, *PD-L1*, *CTLA4*, and *FOXP3*, were correlated positively to response to neoadjuvant therapy. For stromal TILs, odds ratio (OR) is shown per 10% change. For mRNA markers, OR is shown per one Δ cycle threshold value, which approximately corresponds to doubling of mRNA levels. pCR, pathologic complete response.

of TILs and mRNA markers in a combined exploratory multivariable analysis demonstrated that pathologic as well as molecular parameters provided comparable information in many analyses (Table 3). However, some mRNA markers, such as *CCL5*, *PD-L1*, and *IDO1* in the complete cohort as well as *PD-L1* and *CD80* in TN tumors, were significant in multivariable analysis even if the stromal TILs were included. In TNBC, the markers *CCL5*, *CD8A*, and *IDO1* provided predictive information for Cb response (test for interaction $P = .02$ for *CCL5*; $P = .02$ for *CD8A*; $P = .05$ for *IDO1*; Table 3).

DISCUSSION

In this study, we performed a prospective validation of TILs in a large clinical trial. Two pathologists performed the analysis for each tumor at the time of random assignment in a setting that was comparable to routine diagnostic histopathology. Although a general positive role for TILs in chemotherapy response has been reported in many studies,[7-10,13] our study is the first to our knowledge to suggest that some types of chemotherapy, such as Cb, have a particularly strong interaction with the immune system. It has been shown that platinum chemotherapeutics have the ability to induce an immunogenic type of cell death,[26] which might explain the effects observed in our study. A recent study evaluating postneoadjuvant samples also described a role for immune cells in BC outcome.[27]

TILs in hematoxylin and eosin–stained sections are a basic parameter, considering the complexity of the immune system. Therefore, we further validated our results by investigation of mRNA expression of key modulators of immune reactions. All mRNA markers were significantly linked to pCR, and *CCL5*, *CD8A*, *CTLA4*, and *IDO1* had a positive test for interaction with PM-plus-Cb versus PM chemotherapy. In TNBC, where stromal TILs had no significant interaction with PM-plus-Cb versus PM therapy, *CCL5*, *IDO1*, and *CD8A* had a significant test for interaction. Additional validations in larger cohorts are needed to validate mRNA signatures for additional predictive information beyond TILs.

Our evaluation included promising therapeutic targets such as *CTLA4*, *PD-1*, and *PD-L1*, which are already in clinical evaluation.[16-18] There is an ongoing debate about the best biomarkers for these new immunomodulatory therapies. In our study, mRNA markers such as *PD-1*, *PD-L1*, *CTLA4*, and *FOXP3* showed a positive correlation with proimmune markers, stromal TILs, and treatment response. Immunosuppressive checkpoint markers were expressed in parallel with the proimmune markers, suggesting a feedback activation of immunosuppressive pathways as part of the immune reaction. Our results are concordant with a recent study by Schalper et al,[28] who showed that increased mRNA expression of *PD-L1* was positively correlated with increased TILs as well as improved survival. The positive correlation of immunosuppressive markers with improved outcome and improved therapy response has been described in other studies as well.[29-32]

The cluster analysis shows that there are distinctive immunologic subtypes of BC and that a considerable amount of those tumors show features of immunogenicity. Therefore, it might be interesting to include certain types of BC in clinical evaluations of immunomodulatory agents. Such approaches might be able to change the intratumoral immune patterns observed in our hierarchic clustering and increase response rates to chemotherapy.

There are some limitations to our study. We showed the interaction with Cb response for the specific comparison with the PM control arm in GeparSixto, and a validation in other clinical studies of Cb should be performed. The reduced pCR rate with PMCb in HER2-positive tumors with low TILs needs further validation, because this was not observed in the TNBC subcohort. We did not correct for multiple testing; however, the internal consistency of the results supports the conclusions regarding the relevance of immunologic interactions. It should be further noted that the TNBC and HER2-positive patient cohorts had somewhat uneven sample sizes and slightly different event rates, which translated into uneven power to detect marker-outcome associations for the same markers in the two distinct groups.

The analytic validation of TILs was not the main focus of this study and was just performed on a subset of samples. In this subset, the assessment of TILs by three observers had an ICC of 0.92 to 0.97. It is not clear at present if the methods for TIL evaluation would lead to similar results in a multicenter setting. However, even our single-center evaluation was performed by randomly assigned pathologists. Recently, a first guideline paper was published for further standardization of TIL evaluation.[33]

In summary, we prospectively validated the relevance of TILs and mRNA markers as response predictors in a large clinical trial. Interestingly, the effect size measured as OR was in a similar range for LPBC (OR, 2.92; 95% CI, 1.98 to 4.31; $P < .001$) and hormone receptor status (OR, 2.78; 95% CI, 1.84 to 4.20; $P < .001$). This suggests that after further international standardization, TILs could become an additional parameter for chemotherapy response prediction, with an importance similar to that of the established parameter of hormone receptor status.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Disclosures provided by the authors are available with this article at www.jco.org.

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REFERENCES

1. Gianni L, Pienkowski T, Im YH, et al: Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): A randomised multicentre, open-label, phase 2 trial. Lancet Oncol 13:25-32, 2012
2. Baselga J, Bradbury I, Eidtmann H, et al: Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): A randomised, open-label, multicentre, phase 3 trial. Lancet 379:633-640, 2012
3. Cortazar P, Zhang L, Untch M, et al: Pathological complete response and long-term clinical benefit in breast cancer: The CTNeoBC pooled analysis. Lancet 384:164-172, 2014
4. Untch M, Fasching PA, Konecny GE, et al: Pathologic complete response after neoadjuvant chemotherapy plus trastuzumab predicts favorable survival in human epidermal growth factor receptor 2–overexpressing breast cancer: Results from the TECHNO trial of the AGO and GBG study groups. J Clin Oncol 29:3351-3357, 2011
5. von Minckwitz G, Schneeweiss A, Loibl S, et al: Neoadjuvant carboplatin in patients with triple-negative and HER2-positive early breast cancer (GeparSixto; GBG 66): A randomised phase 2 trial. Lancet Oncol 15:747-756, 2014
6. Sikov WM, Berry DA, Perou CM, et al: Impact of the addition of carboplatin (Cb) and/or bevacizumab (B) to neoadjuvant weekly paclitaxel (P) followed by dose-dense AC on pathologic complete response (pCR) rates in triple-negative breast cancer (TNBC): CALGB 40603 (Alliance). Presented at the San Antonio Breast Cancer Symposium, December 10-14, 2013 (abstr S5-01)
7. Denkert C, Loibl S, Noske A, et al: Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. J Clin Oncol 28:105-113, 2010
8. Issa-Nummer Y, Darb-Esfahani S, Loibl S, et al: Prospective validation of immunological infiltrate for prediction of response to neoadjuvant chemotherapy in HER2-negative breast cancer: A substudy of the neoadjuvant GeparQuinto trial. PLoS One 8:e79775, 2013
9. West NR, Milne K, Truong PT, et al: Tumor-infiltrating lymphocytes predict response to anthracycline-based chemotherapy in estrogen receptor-negative breast cancer. Breast Cancer Res 13:R126, 2011
10. Yamaguchi R, Tanaka M, Yano A, et al: Tumor-infiltrating lymphocytes are important pathologic predictors for neoadjuvant chemotherapy in patients with breast cancer. Hum Pathol 43:1688-1694, 2012
11. Schmidt M, Hellwig B, Hammad S, et al: A comprehensive analysis of human gene expression profiles identifies stromal immunoglobulin κ C as a compatible prognostic marker in human solid tumors. Clin Cancer Res 18:2695-2703, 2012
12. Loi S, Sirtaine N, Piette F, et al: Prognostic and predictive value of tumor-infiltrating lymphocytes in a phase III randomized adjuvant breast cancer trial in node-positive breast cancer comparing the addition of docetaxel to doxorubicin with doxorubicin-based chemotherapy: BIG 02-98. J Clin Oncol 31:860-867, 2013
13. Gu-Trantien C, Loi S, Garaud S, et al: CD4+ follicular helper T cell infiltration predicts breast cancer survival. J Clin Invest 123:2873-2892, 2013
14. Ignatiadis M, Singhal SK, Desmedt C, et al: Gene modules and response to neoadjuvant chemotherapy in breast cancer subtypes: A pooled analysis. J Clin Oncol 30:1996-2004, 2012
15. Bianchini G, Gianni L: The immune system and response to HER2-targeted treatment in breast cancer. Lancet Oncol 15:e58-e68, 2014
16. Brahmer JR, Tykodi SS, Chow LQ, et al: Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med 366:2455-2465, 2012
17. Hodi FS, O'Day SJ, McDermott DF, et al: Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 363:711-723, 2010
18. Wolchok JD, Kluger H, Callahan MK, et al: Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med 369:122-133, 2013
19. Galluzzi L, Senovilla L, Zitvogel L, et al: The secret ally: Immunostimulation by anticancer drugs. Nat Rev Drug Discov 11:215-233, 2012
20. McShane LM, Altman DG, Sauerbrei W, et al: Reporting recommendations for tumor marker prognostic studies (REMARK). J Natl Cancer Inst 97:1180-1184, 2005
21. Wienert S, Heim D, Saeger K, et al: Detection and segmentation of cell nuclei in virtual microscopy images: A minimum-model approach. Sci Rep 2:503, 2012
22. Wienert S, Heim D, Kotani M, et al: CognitionMaster: An object-based image analysis framework. Diagn Pathol 8:34, 2013
23. Denkert C, Loibl S, Kronenwett R, et al: RNA-based determination of ESR1 and HER2 expression and response to neoadjuvant chemotherapy. Ann Oncol 24:632-639, 2013

24. Bohmann K, Hennig G, Rogel U, et al: RNA extraction from archival formalin-fixed paraffin-embedded tissue: A comparison of manual, semiautomated, and fully automated purification methods. Clin Chem 55:1719-1727, 2009

25. Polley MY, Leung SC, McShane LM, et al: An international Ki67 reproducibility study. J Natl Cancer Inst 105:1897-1906, 2013

26. Hato SV, Khong A, de Vries IJ, et al: Molecular pathways: The immunogenic effects of platinum-based chemotherapeutics. Clin Cancer Res 20: 2831-2837, 2014

27. Dieci MV, Criscitiello C, Goubar A, et al: Prognostic value of tumor-infiltrating lymphocytes on residual disease after primary chemotherapy for triple-negative breast cancer: A retrospective multicenter study. Ann Oncol 25:611-618, 2014

28. Schalper KA, Velcheti V, Carvajal D, et al: In situ tumor PD-L1 mRNA expression is associated with increased TILs and better outcome in breast carcinomas. Clin Cancer Res 20:2773-2782, 2014

29. West NR, Kost SE, Martin SD, et al: Tumour-infiltrating FOXP3(+) lymphocytes are associated with cytotoxic immune responses and good clinical outcome in oestrogen receptor-negative breast cancer. Br J Cancer 108:155-162, 2013

30. Salama P, Phillips M, Grieu F, et al: Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. J Clin Oncol 27:186-192, 2009

31. Jacquemier J, Bertucci F, Finetti P, et al: High expression of indoleamine 2,3-dioxygenase in the tumour is associated with medullary features and favourable outcome in basal-like breast carcinoma. Int J Cancer 130:96-104, 2012

32. Loi S, Michiels S, Salgado R, et al: Tumor infiltrating lymphocytes is prognostic and predictive for trastuzumab benefit in early breast cancer: Results from the FinHER trial. Ann Oncol 25:1544-1550, 2014

33. Salgado R, Denkert C, Demaria S, et al: Harmonization of the evaluation of tumor infiltrating lymphocytes (TILs) in breast cancer: Recommendations by an international TILs-working group 2014. Ann Oncol [epub ahead of print on September 11, 2014]

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GLOSSARY TERMS

CTLA4 (CD152): receptor on activated T cells that binds B7 molecules with a higher affinity than CD28, downregulating T-cell responses by inhibiting CD28 signaling.

immune checkpoint: immune inhibitory pathway that negatively modulates the duration and amplitude of immune responses. Examples include the CTLA-4:B7.1/B7.2 pathway, and the PD-1:PD-L1/PD-L2 pathway.

immunogenic: capable of inducing an immune response.

immunotherapy: a therapeutic approach that uses cellular and/or humoral elements of the immune system to fight a disease.

neoadjuvant therapy: the administration of chemotherapy prior to surgery. Induction chemotherapy is generally designed to decrease the size of the tumor prior to resection and to increase the rate of complete (R0) resections.

pathologic complete response: the absence of any residual tumor cells in a histologic evaluation of a tumor specimen.

PD-1: programmed cell death protein 1 (CD279), a receptor expressed on the surface of activated T, B, and NK cells that negatively regulates immune responses, including autoimmune and antitumor responses.

PD-L1: programmed cell death 1 ligand 1 (CD274; also known as B7-H1), the major binding partner (ligand) for the PD-1 inhibitory immune receptor. PD-L1 is expressed on the surface of activated antigen presenting cells, such as dendritic cells, and by many types of cancer cells. Its expression is induced by the inflammatory cytokine interferon.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Tumor-Infiltrating Lymphocytes and Response to Neoadjuvant Chemotherapy With or Without Carboplatin in Human Epidermal Growth Factor Receptor 2–Positive and Triple-Negative Primary Breast Cancers

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Appendix

Table A1. Genes of Interest/References Genes and Corresponding Primer/Probe Sequences

Sequence Identifier	Gene	Probe	Forward Primer	Reverse Primer
1	*CALM2*	TCGCGTCTCGGAAACCGGTAGC	GAGCGAGCTGAGTGGTTGTG	AGTCAGTTGGTCAGCCATGCT
2	*CCL5*	CTCTGCGCTCCTGCATCTGCCTC	CGCTGTCATCCTCATTGCTACT	TGTGGTGTCCGAGGAATATGG
3	*CD21* (*CR2*)	CCCTGGCGGTTTGCAGATCCC	GCCAATCGGATCACCAATG	ACCACAAAGGACAGGAGCAAGT
4	*CD80*	AGGCCAGCGCCAGAACCCAGA	CAGGGAGGTGACCCGAATTA	AAAGGGAAAGAGCACCAGAGTTAG
5	*CD8A*	CAAATGTCCCCGGCCTGTGGTC	CAGGGAACCGAAGACGTGTT	TAGACGTATCTCGCCGAAAGG
6	*CTLA4*	CCTGGGCATAGGCAACGGAACCC	TCATGTACCCACCGCCATACT	GGCACGGTTCTGGATCAATT
7	*CXCL13*	TGGTCAGCAGCCTCTCTCCAGTCCA	CGACATCTCTGCTTCTCATGCT	AGCTTGTGTAATAGACCTCCAGAACA
8	*CXCL9*	CCACTAACCGACTTGGCTGCTTCCTCTAG	AAAGGGAA CGGTGAAGTACTAAGC	AACTGGGCACCAATCATGCT
9	*FOXP3*	TGACAGTTTCCCACAAGCCAGGCTG	GCGTGGTTTTTCTTCTCGGTAT	TGGTGAAGTGGACTGACAGAAAAG
10	*IDO1*	CGCCTGTGTGAAAGCTCTGGTCTCC	GCCTGCGGGAAGCTTATG	GTACTTAGTCACGATTTGCAGATGGT
11	*IGKC*	AGCAGCCTGCAGCCTGAAGATTTTGC	GATCTGGGACAGAATTCACTCTCA	GCCGAACGTCCAAGGGTAA
12	*OAZ1*	TGCTTCCACAAGAACCGCGAGGA	CGAGCCGACCATGTCTTCAT	AAGCCCAAAAAGCTGAAGGTT
13	*PD-1* (*PDCD1*)	TGAGCCCCAGCAACCAGACGG	CAACACATCGGAGAGCTTCGT	GGAAGGCGGCCAGCTT
14	*PD-L1* (*CD274*)	CAGAAGTGCCCTTTGCCTCCACTCAA	CCCTAATTTGAGGGTCAGTTCCT	CTCAGTCATGCAGAAAACAAATTGA
15	*RPL37A*	TGGCTGGCGGTGCCTGGA	TGTGGTTCCTGCATGAAGACA	GTGACAGCGGAAGTGGTATTGTAC

Table A2. Clinicopathologic Data of GeparSixto Cohort

Characteristic	TIL Evaluation (N = 580)		mRNA Analysis (n = 481)	
	No.	%	No.	%
Age group, years				
< 50	338	58.3	283	58.8
≥ 50	242	41.7	198	41.2
Tumor type				
Ductal/other	570	98.3	475	98.7
Lobular	10	1.7	6	1.2
Tumor grade				
1 to 2	207	35.7	171	35.6
3	373	64.3	310	64.4
ER/PR status (central IHC)				
ER negative/PR negative	420	72.4	341	70.9
ER positive and/or PR positive	160	27.6	140	29.1
Receptor status combined (central IHC/SISH)				
HER2 negative and ER negative/PR negative (TNBC cohort)	314	54.1	255	53.0
HER2 positive (HER2-positive cohort)	266	45.9	226	47.0
HER2 positive and ER negative/PR negative	106	18.3	86	17.9
HER2 positive and ER positive and/or PR positive	160	27.6	140	29.1
Clinical tumor stage				
cT1-2	496	85.5	413	85.9
cT3-4	82	14.1	67	13.9
Missing	2	0.3	1	0.2
Clinical nodal status				
cN0	336	57.9	265	55.1
cN+	232	40.0	206	42.8
Missing	12	2.1	10	2.1
Type of chemotherapy				
PMCb	290	50.0	238	49.5
PM	290	50.0	243	50.5
Pathologic response (ypT0 ypN0)				
No pCR	347	59.8	287	59.7
pCR	233	40.2	194	40.3

Abbreviations: Cb, carboplatin; ER, estrogen receptor; HER2, human epidermal growth factor 2; IHC, immunohistochemistry; pCR, pathologic complete response; PM, paclitaxel plus nonpegylated liposomal doxorubicin; PR, progesterone receptor; SISH, silver in situ hybridization; TIL, tumor-infiltrating lymphocyte.

Table A3. Distribution of LPBC Tumors and Immune mRNA Groups in Complete Cohort and TNBC and HER2-Positive Subgroups

Group	TILs					mRNA Clustering						
	No. of Patients	Non-LPBC		LPBC		No. of Patients	Immune Group A (low)		Immune Group B (intermediate)		Immune Group C (high)	
		No.	%	No.	%		No.	%	No.	%	No.	%
All tumors	580	438	75.5	142	24.5	481	87	18.1	257	53.4	137	28.5
TNBCs	314	225	71.1	89	28.3	255	46	18.0	82	32.2	127	49.8
HER2 positive	266	213	80.1	53	19.9	226	52	23.0	138	61.1	36	15.9

Abbreviations: HER2, human epidermal growth factor 2; LPBC, lymphocyte-predominant breast cancer; TIL, tumor-infiltrating lymphocyte; TNBC, triple-negative breast cancer.

Table A4. Univariable and Multivariable Logistic Regression for Evaluation of Intratumoral TILs in Complete Cohort and TNBC and HER2-Positive Subgroups

	Univariable Analysis			Multivariable Analysis*		
Intratumoral TILs (per 10%)	OR	95% CI	*P*	OR	95% CI	*P*
Complete cohort	1.30	1.12 to 1.50	< .001	1.24	1.07 to 1.45	.006
TNBC subgroup	1.22	1.03 to 1.43	.02	1.28	1.07 to 1.54	.007
HER2-positive subgroup	1.47	1.05 to 2.08	.03	1.26	0.87 to 1.84	NS

Abbreviations: HER2, human epidermal growth factor 2; NS, nonsignificant; OR, odds ratio; TIL, tumor-infiltrating lymphocyte; TNBC, triple-negative breast cancer.
*Including clinical parameters shown in Table 1.

Table A5. Correlation Between LPBC Status and pCR Rate in Different Subgroups of GeparSixto

		pCR Rate (%)			
Subgroup	No. of Patients	All Patients	Non-LPBC	LPBC	*P**
Complete cohort					
Both arms	580	40.2	33.8	59.9	< .001
PM therapy	290	36.6	33.6	45.2	NS
PMCb therapy	290	43.8	33.9	75.4	< .001
TNBC subgroup					
Both arms	314	44.9	40.0	57.3	.006
PM therapy	156	36.5	33.9	42.6	NS
PMCb therapy	158	53.2	45.7	73.8	.002
HER2-positive subgroup					
Both arms	266	34.6	27.2	64.2	< .001
PM therapy	134	36.6	33.3	50.0	NS
PMCb therapy	132	32.6	21.0	77.8	< .001

Abbreviations: Cb, carboplatin; HER2, human epidermal growth factor 2; LPBC, lymphocyte-predominant breast cancer; NS, nonsignificant; pCR, pathologic complete response; PM, paclitaxel plus nonpegylated liposomal doxorubicin; TNBC, triple-negative breast cancer.
*Non-LPBC versus LPBC; two-sided Fisher's test.

Table A6. Interaction Between LPBC and Therapy in Complete Cohort and HER2-Positive and TNBC Subgroups

Treatment Group	OR for pCR	95% CI	*P*
Complete cohort (N = 580)			
Non-LPBC			
PM	1.00		
PMCb	1.01	0.68 to 1.51	NS
LPBC			
PM	1.00		
PMCb	3.71	1.81 to 7.59	< .001
Interaction term	3.67	1.62 to 8.29	.002
TNBC subgroup (n = 314)			
Non-LPBC			
PM	1.00		
PMCb	1.64	0.96 to 2.81	NS
LPBC			
PM	1.00		
PMCb	3.81	1.55 to 9.35	.004
Interaction term	2.32	0.82 to 6.63	NS
HER2-positive subgroup (n = 266)			
Non-LPBC			
PM	1.00		
PMCb	0.53	0.29 to 0.98	.04
LPBC			
PM	1.00		
PMCb	3.5	1.1 to 11.5	.04
Interaction term	6.60	1.73 to 25.2	.006

Abbreviations: Cb, carboplatin; HER2, human epidermal growth factor 2; LPBC, lymphocyte-predominant breast cancer; NS, nonsignificant; OR, odds ratio; pCR, pathologic complete response; PM, paclitaxel plus nonpegylated liposomal doxorubicin; TNBC, triple-negative breast cancer.

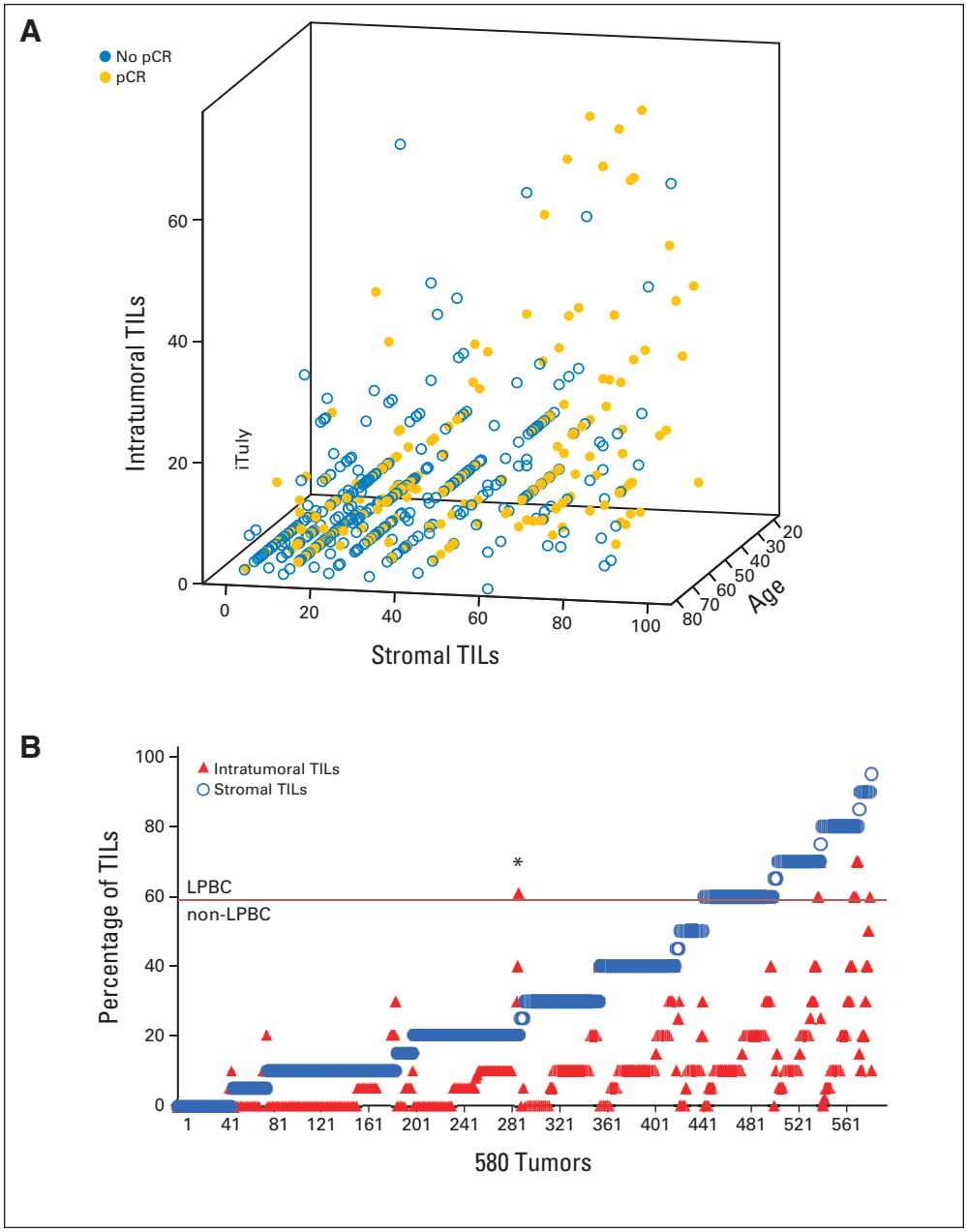

Fig A1. (A) Correlation of stromal and intratumoral tumor-infiltrating lymphocytes (TILs) in GeparSixto. Stromal TILs typically have higher level than intratumoral TILs. Filled circles indicate patient cases of pathologic complete response (pCR). Variable patient age was added for improved visualization to reduce overlay of spots. (B) Levels of intratumoral and stromal TILs. All 580 tumors from GeparSixto were sorted first by ascending stromal TILs (circles) and then by ascending intratumoral TILs (triangles). For each tumor, stromal and intratumoral TILs are shown. Figure indicates that stromal TILs have higher values in majority of tumors and reach higher values. (*) Lymphocyte-predominant breast cancer (LPBC) status is mainly driven by stromal TILs; there was only one LPBC tumor that had high intratumoral and low stromal TILs. All other LPBC tumors were defined solely on basis of increased stromal TILs.

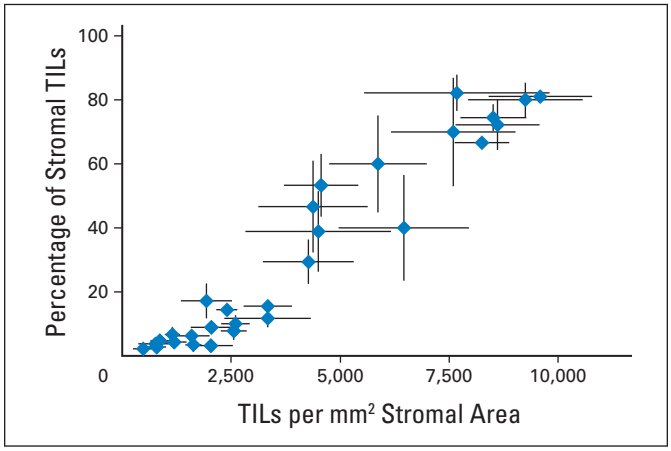

Fig A2. Validation of tumor-infiltrating lymphocytes (TILs) by automated image analysis and interobserver assessment; 29 tumors were selected from GeparSixto to cover complete range of TILs. From each tumor, three representative stromal regions of interest (ROIs) were marked on digital slide. These regions were evaluated by three pathologists and by automated image analysis; these pathologists assessed each ROI, and mean of three ROIs was used as value for tumor. Diagram shows on *y*-axis pathologist assessment as mean and standard deviation (SD) of three pathologists for each tumor. For image analysis, mean and SD of three ROIs are shown. Therefore, SDs on *y*-axis indicate interobserver variance among three pathologists; SDs on *x*-axis indicate intratumoral heterogeneity of TILs based on image analysis of three ROIs. Those tumors with increased interobserver variance often also had increased intratumoral heterogeneity; these tumors were more often found in intermediate TIL range.

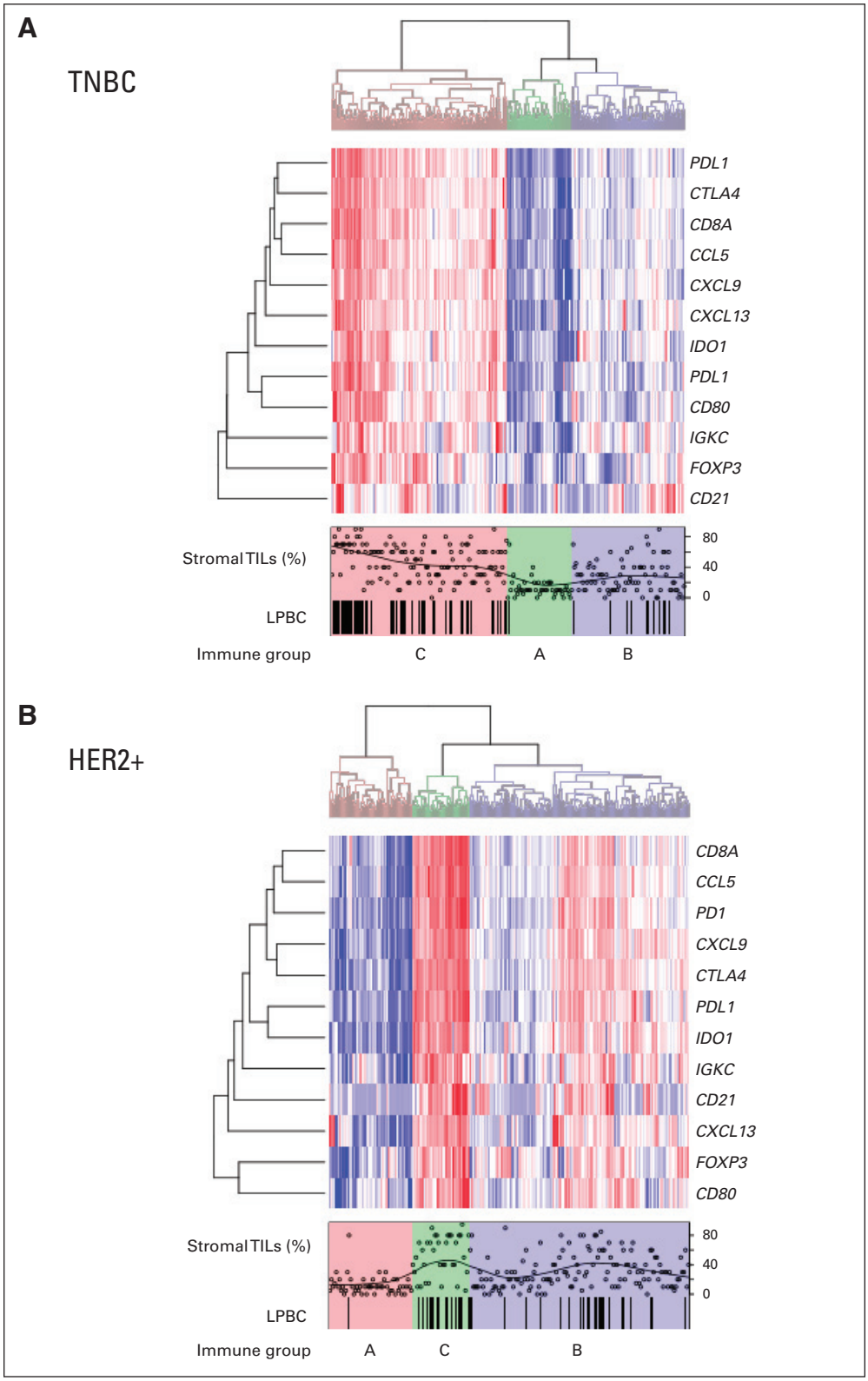

Fig A3. Hierarchic clustering of 12 immune genes in (A) triple-negative breast cancer (TNBC) and (B) human epidermal growth factor receptor 2 (HER2) –positive subgroups of GeparSixto. Corresponding levels of stromal tumor-infiltrating lymphocytes (TILs) and lymphocyte-predominant breast cancer (LPBC) status for each tumor are shown in lower panel sections.

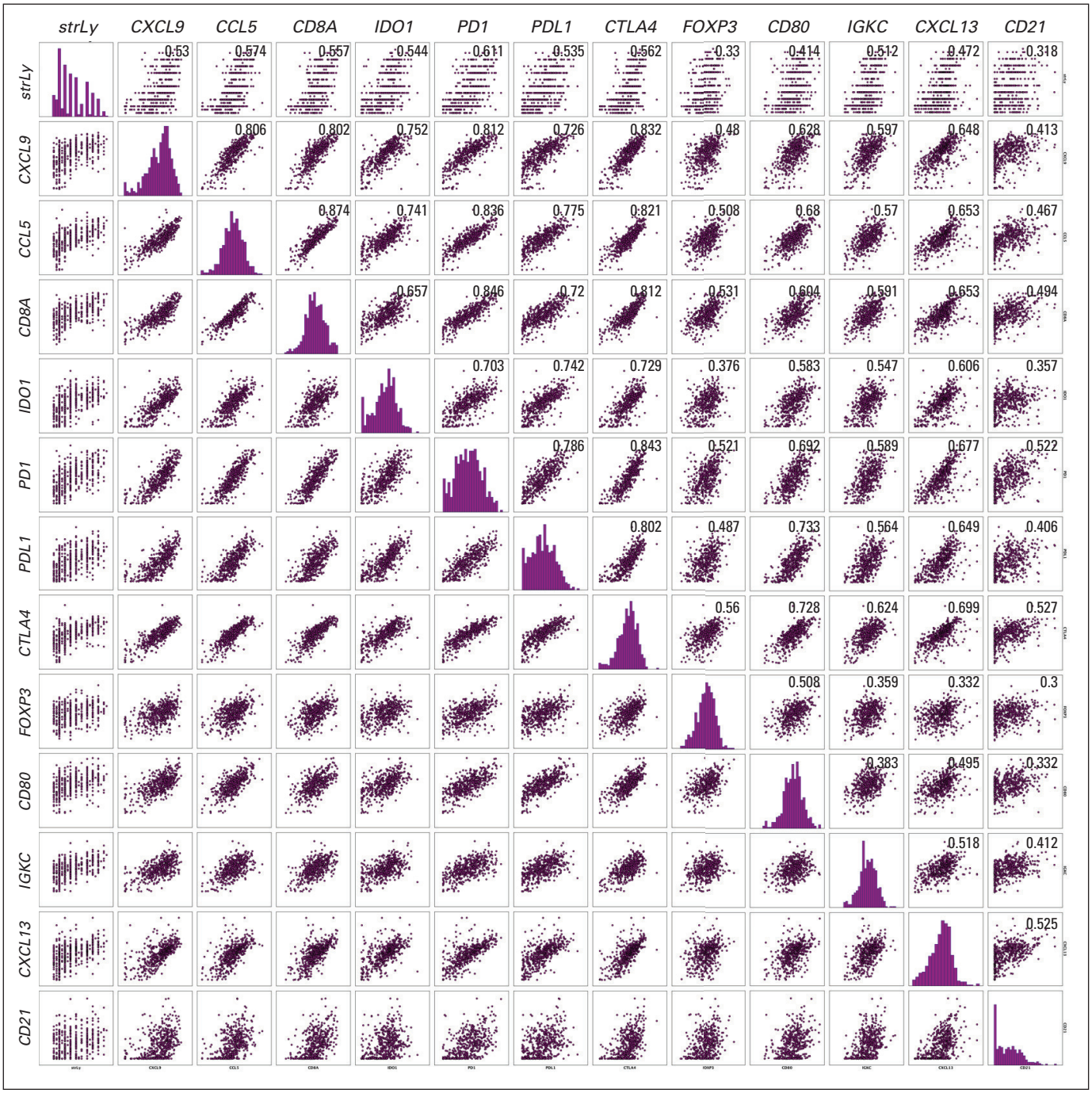

Fig A4. Correlation analysis of 12 immunologically relevant genes and stromal tumor-infiltrating lymphocytes (TILs). All genes showed positive correlation with one another and with stromal TILs; correlation coefficients are also shown. This positive correlation also included those genes with reported immunosuppressive function, such as *PD-1*, *PD-L1*, *IDO1*, and *CTLA4*.