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LETTER TO THE EDITOR

Predictive power of tertiary lymphoid structure signature for neoadjuvant chemotherapy response and immunotherapy benefit in HER2-negative breast cancer

Dear Editor,

Pembrolizumab, an immune checkpoint inhibitor (ICI), was recently approved for early-stage triple-negative breast cancer (eTNBC), and promising results have been reported in advanced hormone receptor-positive/human epidermal growth factor receptor 2-negative (HR+/HER2-) and HER2+ BCs [1]. Given the costs and potential toxicity, markers predictive of ICI benefits are needed. Currently, neither programmed death-ligand 1 (PD-L1) expression nor other tested markers (i.e., tumor mutational burden, microsatellite instability) effectively predict ICI benefit [2]. Tertiary lymphoid structures (TLS) are secondary lymphoid organ-like aggregates that reside in the tumor microenvironment and are associated with clinical response to ICIs in metastatic melanoma, renal cell carcinoma, and sarcoma [3, 4]. In BC, two studies reported a predictive value for pathological response to neoadjuvant chemotherapy (NACT) [5, 6], and no data exist regarding the predictive value of TLS for the response to or the benefit from ICIs. Due to the limitations of TLS analysis on pathologic slides, gene expression signatures associated with TLS enrichment have been generated, including Coppola's 12-chemokine signature, the most studied one [7]. We report the predictive value of this signature for pathological response to NACT in eBC and its predictive value for the benefit of adding pembrolizumab to NACT.

We analyzed two clinical cohorts with available tumor gene expression data (Supplementary Table S1). Cohort 1 included data from 1,203 patients treated with anthracycline-based NACT. Cohort 2 included prospective data from 248 HER2-negative patients enrolled in the I-SPY2 trial [8], treated with NACT alone (control arm: sequential weekly paclitaxel followed by doxorubicin/cyclophosphamide, $n = 179$) or NACT plus pembrolizumab (investigational arm: same regimen but with pembrolizumab added to paclitaxel, $n = 69$). The study design and methods are detailed in the Supplementary Methods. We applied the Coppola's TLS signature to each tumor sample, as well as two signatures associated with response to ICIs: the immunologic constant of rejection (ICR) classifier [9] and the Ayers' T-cell-inflamed signature (TIS) [10]. For each signature, the cut-off distinguishing "high" *vs.* "low" tumors was the median value of the score in the whole cohort. Pathological complete response (pCR) to neoadjuvant therapy was defined on the surgical specimen as the absence of invasive cancer in both the breast and lymph nodes (ypT0/Tis ypN0). In cohort 1, 281 patients (23%) achieved pCR, while in cohort 2, 30 (17%) achieved pCR in the control arm and 31 (45%) in the investigational arm (Supplementary Table S2).

First, we investigated the association of the TLS signature with obtaining pCR in cohort 1. The pCR rate was 34% (198/588) in the "TLS-high" tumors *vs.* 13% (83/615) in the "TLS-low" tumors ($P < 0.001$, Fisher's exact test, Supplementary Table S2). The odds ratio (OR) for pCR between the "TLS-high" and "TLS-low" tumors was 3.25 (95% CI = 2.44-4.34; $P < 0.001$, logit-link; Figure 1A). In univariate analysis (Figure 1A), grade 3, HER2+ and TN subtypes were associated with pCR, as well as other immune scores ($P < 0.001$ for ICR, and $P < 0.001$ for TIS). In multivariate analysis (Figure 1A), the TLS status remained associated with pCR ($P < 0.001$, logit-link), as well as grade (OR = 11.7, 95% CI = 2.83-48.6; $P < 0.001$, logit-link) and molecular subtype (OR = 3.85, 95% CI = 2.55-5.79; $P < 0.001$, logit-link). This result demonstrates the favorable independent predictive value of the TLS signature for pCR, as already reported in two smaller series of eBC treated with NACT [5, 6].

Then, we assessed whether the TLS signature could predict the pathological response to NACT plus

Abbreviations: BC, breast cancer; eBC, early-stage BC; CI, confidence interval; HR, hormone receptor; ICI, immune checkpoint inhibitor; ICR, immunologic constant of rejection; NACT, neoadjuvant chemotherapy; OR, odds ratio; pCR, pathological complete response; TILs, tumor-infiltrating lymphocytes; TIS, T-cell-inflamed signature; TLS, tertiary lymphoid structures; TN, triple-negative..

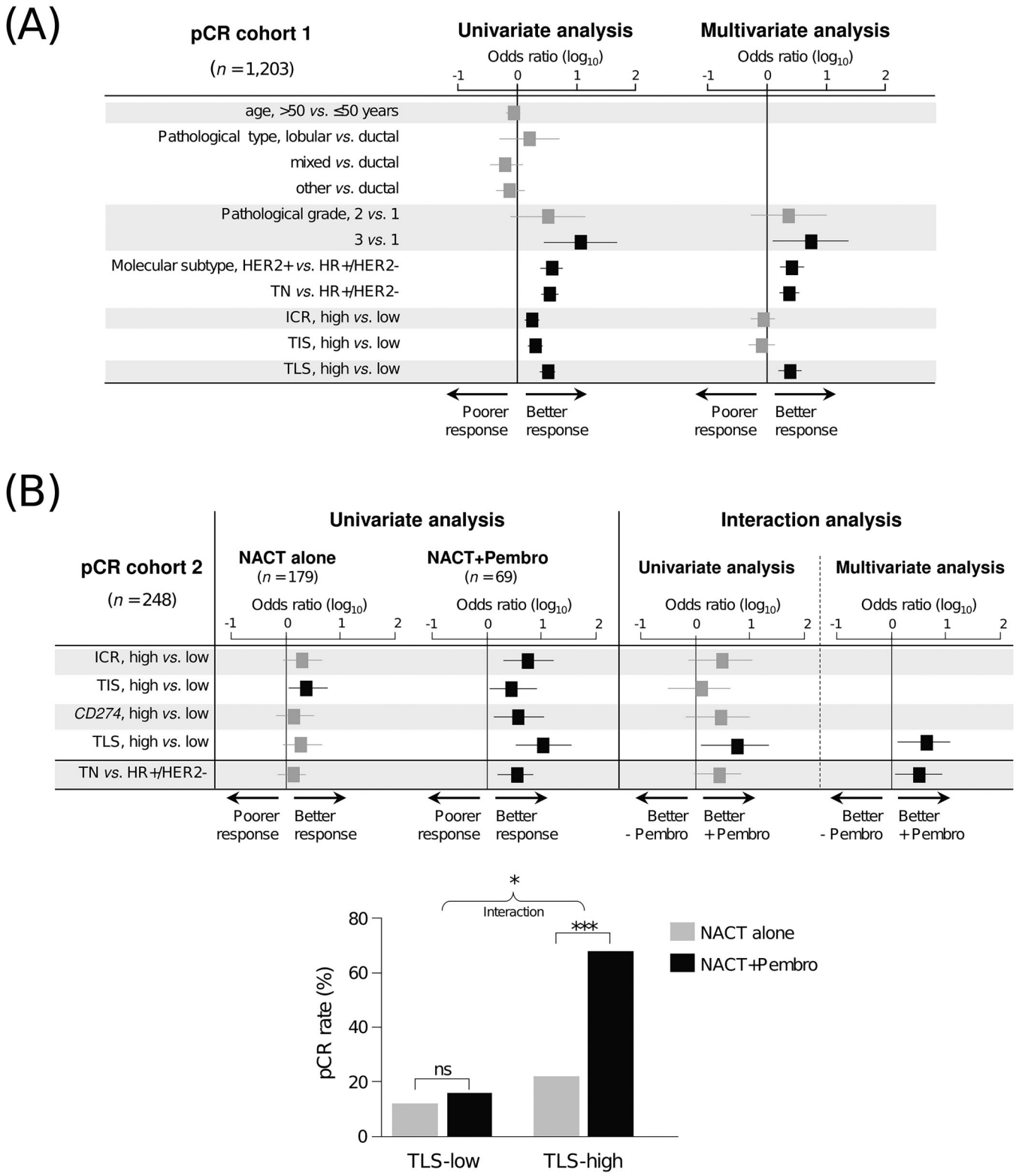

FIGURE 1 Correlations of TLS classes with pathological complete response to NACT alone and to NACT plus pembrolizumab, and with benefit, in terms of pathological response, of neoadjuvant pembrolizumab in HER2-early breast cancer. The TLS classes ("high" and "low") were defined using the median value of the TLS score in the whole cohort. **(A)** Cohort 1: uni- and multivariate analyses for pCR to NACT alone. The odds ratios were log10-transformed. The boxes and whisker plots are colored in black when significant and grey when not significant. **(B)** Cohort 2: *Top*, univariate analyses for pCR to NACT alone (control arm) and to NACT plus pembrolizumab (investigational arm), and uni- and multivariate interaction analyses. *Bottom*, Bar plots showing the pCR rates in "TLS-high" and in "TLS-low" tumors according to the therapeutic arm (NACT alone or NACT plus pembrolizumab). The odds ratios were log10-transformed. The boxes and whisker plots are colored in black when significant and in grey when not significant.

pembrolizumab by analyzing I-SPY2 data [8] and, more specifically, whether it might predict benefit, in terms of response, of pembrolizumab addition (Figure 1B, Supplementary Table S2-S3). In the control arm (NACT), the pCR rate was 22% (18/81) in the "TLS-high" tumors *vs.* 12% (12/98) in the "TLS-low" tumors ($P = 0.107$, Fisher's exact test), with a 2.05 OR for pCR (95% CI = 0.92-4.55; $P = 0.079$, logit-link), consistently with our above-reported result. In the investigational arm (NACT+Pembrolizumab), the difference in pCR rates was higher, with a 68% pCR rate (26/38) in the "TLS-high" tumors *vs.* 16% (5/31) in the "TLS-low" tumors ($P < 0.001$, Fisher's exact test), and an 11.27 OR for pCR(95% CI = 3.47-36.53; $P < 0.001$, logit-link). The univariate interaction analysis between the TLS signature and the addition of pembrolizumab was significant ($P = 0.019$; Figure 1B). The addition of pembrolizumab to chemotherapy increased the pCR rate by >3-folds in the "TLS-high" tumors (68% *vs.* 22%, $P < 0.001$), whereas it had no statistically significant effect in the "TLS-low" tumors (16% *vs.* 12%, $P = 0.554$). Then, we compared these predictive values and interactions to those of other immune variables (CD274/PD-L1 expression, ICR, and TIS scores). Although most of them were associated with pCR to NACT plus pembrolizumab, none showed significant interaction with treatment, by contrast to the TLS signature. The molecular subtype (TN *vs.* HR+/HER2-) tended towards significance by univariate interaction analysis ($P = 0.066$), with a stronger benefit of pembrolizumab addition in TN tumors (Figure 1B). In multivariate analysis for interaction, both TLS signature and molecular subtypes were significant. Analysis per molecular subtype (Supplementary Figure S1) showed that the addition of pembrolizumab to NACT increased the pCR rate more strongly in the "TLS-high" tumors than in the "TLS-low" tumors, by respectively 2.3-folds (58% *vs.* 25%, $P = 0.035$) *versus* 0.5-folds (5% *vs.* 10%, $P = 0.673$) in the HR+/HER2- patients ($P = 0.087$ for interaction), and 4-folds (79% *vs.* 20%, $P < 0.001$) *versus* 2.4-folds (40% *vs.* 17%, $P = 0.189$) in the TN patients ($P = 0.151$ for interaction).

One limitation of our study was the use of a TLS expression signature rather than directly detecting TLS, but such an approach is more adapted to small biopsies. In the future, we will analyze, at both morphological and gene levels, the TLS in samples collected in the prospective PELICAN trial (NCT03515798). Another limitation may be related to the signature cut-off we applied. Thus, we repeated all analyses by applying another cut-off defined according to a glm logistic regression model as previously described [7] (Supplementary Methods). Here too, the TLSglm classification (Supplementary Tables S2-S4) displayed independent predictive value for pCR to NACT with better response in "TLSglm-high" tumors (Supplementary Figure S2A) and independent predictive value for benefit from pembrolizumab addition with higher benefit in "TLSglm-high" tumors (Supplementary Figure S2B). The analyses per molecular subtype also showed that the addition of pembrolizumab to NACT increased the pCR rate more strongly in the "TLSglm-high" tumors than in the "TLSglm-low" tumors, by respectively 2.3-folds (58% *vs.* 25%, $P = 0.035$) *versus* 0.5-folds (5% *vs.* 10%, $P = 0.673$) in the HR+/HER2- patients ($P = 0.087$ for interaction; Supplementary Figure S3A), and 4-folds (79% *vs.* 20%, $P < 0.001$) *versus* 2.4-folds (40% *vs.* 17%, $P = 0.189$) in the TN patients ($P = 0.151$ for interaction; Supplementary Figure S3B).

In conclusion, we confirm the independent predictive value of the TLS signature for pathological response to NACT and show its independent predictive value for the benefit, in terms of pathological response, of adding pembrolizumab to NACT. Despite the limitations indicated above, our results suggest the possibility to better tailor neoadjuvant chemotherapy and immune therapy in eBC, calling for validation of this TLS signature in larger series.

DECLARATIONS

AUTHOR CONTRIBUTIONS

Concept and design: François Bertucci; Acquisition, analysis, or interpretation of data: all authors. Drafting of the manuscript: François Bertucci; Critical revision of the manuscript for important intellectual content: all authors. Statistical analyses: Pascal Finetti, François Bertucci. Validation: all authors; Supervision: François Bertucci. The corresponding author (Pr François Bertucci) is responsible for all aspects of this work.

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The authors have nothing to report.

CONFLICT OF INTEREST STATEMENT

Alexandre de Nonneville declares the following competing interest: Daiichi Sankyo (lecture fees, congress invitation), Gilead (lecture fees, congress invitation), MSD (congress invitation), and Seagen (consulting fees). The other authors declare no Competing Financial or Non-Financial Interests.

Abbreviations: ns, not statistically significant, *, $P < 0.05$; ***, $P < 0.001$; NACT, neoadjuvant chemotherapy; pCR, pathological complete response; HR, hormone receptor; TN, triple negative; ICR, immunologic constant of rejection, TIS, T-cell-inflamed signature; TLS, tertiary lymphoid structures.

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CONSENT FOR PUBLICATION

Not applicable.

ETHICS IN APPROVAL AND CONSENT TO PARTICIPATE

Our *in-silico* study is based upon public data from published studies in which the informed patient's consent to participate and the ethics and institutional review board were already obtained by authors. The study was approved by our institutional review board according to good clinical practices and applicable laws and regulations.

DATA AVAILABILITY STATEMENT

All datasets analyzed during the current study are publicly available, and the respective sources are indicated in Supplementary Table S1.

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SUPPORTING INFORMATION

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