Immune cell composition and functional marker dynamics from multiplexed immunohistochemistry to predict response to neoadjuvant chemotherapy in the WSG-ADAPT-TN trial

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ABSTRACT

Background The association of early changes in the immune infiltrate during neoadjuvant chemotherapy (NACT) with pathological complete response (pCR) in triple-negative breast cancer (TNBC) remains unexplored.

Methods Multiplexed immunohistochemistry was performed in matched tumor biopsies obtained at baseline and after 3 weeks of NACT from 66 patients from the West German Study Group Adjuvant Dynamic Marker-Adjusted Personalized Therapy Trial Optimizing Risk Assessment and Therapy Response Prediction in Early Breast Cancer - Triple Negative Breast Cancer (WSG-ADAPT-TN) trial. Association between CD4, CD8, CD73, T cells, PD1-positive CD4 and CD8 cells, and PDL1 levels in stroma and/or tumor at baseline, week 3 and 3-week change with pCR was evaluated with univariable logistic regression.

Results Compared with no change in immune cell composition and functional markers, transition from 'cold' to 'hot' (below-median and above-median marker level at baseline, respectively) suggested higher pCR rates for PD1-positive CD4 (tumor: OR=1.55, 95% CI 0.45 to 5.42; stroma: OR=2.65, 95% CI 0.65 to 10.71) and PD1-positive CD8 infiltrates (tumor: OR=1.77, 95% CI 0.60 to 5.20; stroma: OR=1.25, 95% CI 0.41 to 3.84; tumor+stroma: OR=1.62, 95% CI 0.51 to 5.12). No pCR was observed after 'hot-to-cold' transition in PD1-positive CD8 cells. pCR rates appeared lower after hot-to-cold transitions in T cells (tumor: OR=0.26, 95% CI 0.03 to 2.34; stroma: OR=0.35, 95% CI 0.04 to 3.25; tumor+stroma: OR=0.00, 95% CI 0.00 to 1.04) and PD1-positive CD4 cells (tumor: OR=0.60, 95% CI 0.11 to 3.35; stroma: OR=0.22, 95% CI 0.03 to 1.92; tumor+stroma: OR=0.32, 95% CI 0.04 to 2.94). Higher pCR rates collated with 'altered' distribution (levels below-median and above-median in tumor and stroma, respectively) of T cell (OR=3.50, 95% CI 0.84 to 14.56) and PD1-positive CD4 cells (OR=4.50, 95% CI 1.01 to 20.14).

Conclusion Our exploratory findings indicate that comprehensive analysis of early immune infiltrate dynamics complements currently investigated predictive markers for pCR and may have a potential to improve guidance for individualized de-escalation/escalation strategies in TNBC.

BACKGROUND

Triple-negative early breast cancer (TNBC) is a heterogeneous disease associated with poor prognosis. However, pathological complete response (pCR) to neoadjuvant chemotherapy (NACT) is relatively frequent and associated with good long-term outcomes.[1–3] Consequently, NACT allows individualized therapy approaches for further escalation or de-escalation according to response at surgery (pCR status). However, consensus on the optimal NACT offering the best benefit/risk profile is currently lacking, partially due to lack of a direct comparison between several NACT agents and the biological heterogeneity observed in TNBC.[4] Several studies in TNBC demonstrated a number of genetic, molecular, and cell signatures associated with response to different types of therapy.[5 6] Elevated tumor-infiltrating lymphocytes (TILs) levels were associated with pCR and improved survival in most, but not all trials.[7–12] A recent analysis using multiplexed ion beam imaging revealed distinct patterns of tumor-immune landscape in TNBC characterized by lack of immune infiltrates

(frequently termed as 'cold'), immune cells in contact ('hot' or 'mixed') or spatially separated from tumor cells (termed 'altered', 'immune excluded' or 'compartmentalized'[13–15]). Interestingly, altered tumor organization translated to longer survival than that associated with hot tumors.[15] Capitalizing on the advances in deciphering the molecular interactions in tumor-immune microenvironment, the clinical research on immune checkpoint inhibitors is increasingly gaining traction in TNBC. For instance, high PDL1 expression was positively associated with pCR in response to NACT alone or combined with pembrolizumab, atezolizumab or durvalumab.[9 16–19] Yet, PDL1 status does not seem to be predictive of response to immunotherapy in early TNBC,[18 20] and available data offer limited insight into marker dynamics during NACT. Marker levels in residual tumors (non-pCR) and their value for prediction of long-term outcomes may not reliably reflect the situation in tumors achieving pCR. In this context, identification of reliable prognostic or predictive markers would allow development of patient-tailored therapeutic approaches that optimize outcome without overtreating patients.

In the present study, we employed multiplexed immune cell detection to explore the value of the inflammatory tumor microenvironment and its early dynamic changes during NACT for prediction of pCR. This research was conducted within the framework of the preplanned exploratory objectives of the West German Study Group Adjuvant Dynamic Marker-Adjusted Personalized Therapy Trial Optimizing Risk Assessment and Therapy Response Prediction in Early Breast Cancer - Triple Negative Breast Cancer (WSG-ADAPT-TN) subtrial in order to identify markers predicting pCR and thus enabling selection of candidates for future therapy de-escalation.[21]

METHODS
Patients
WSG-ADAPT-TN (NCT01815242) is a prospective, multicenter, controlled, non-blinded, randomized, investigator-initiated trial in women ≥18 years of age with histologically confirmed unilateral, primary invasive cT1c-cT4c or cN+ TNBC (centrally confirmed hormone receptor positivity in <1% of tumor nuclei and HER2 score of 0–1+ or 2+ with negative fluorescence in situ hybridization test). Histopathology subtype was assessed by local pathologists at the time of diagnosis. pCR (ypT0/is, ypN0), assessed after 12 weeks of NACT, was a primary endpoint of the study. Surgery was performed within 3 weeks after NACT. In case investigators opted for further NACT, prior histological confirmation of non-pCR by core needle biopsy was obligatory. Recommended poststudy therapy (neoadjuvant and/or adjuvant) followed national guidelines. Core biopsies used for the present analysis were performed at the time of diagnosis and after 3 weeks of NACT (online supplemental figure 1).

Molecular subtypes of TNBC
The PAM50 assay was used to classify TNBC into five intrinsic molecular subtypes (luminal A, luminal B, HER2-enriched, basal-like and normal-like) as described elsewhere.[22] The expression signatures of 50 genes included in PAM50 were compared with each of the five intrinsic molecular subtype centroids using Spearman's rho correlation levels as a distance measure (the subtype was assigned to the nearest centroid).

Assessment of Ki-67 levels
Ki-67 was assessed by central pathology using rabbit monoclonal anti-Ki-67 antibody 30–9 (Ventana Medical Systems, Tucson, Arizona, USA). Early Ki-67 response in the 3-week biopsy was defined as >30% decrease in Ki-67 versus baseline or <500 invasive tumor cells in the 3-week biopsy as previously described.[21]

Analysis of TILs levels
Stromal TILs were evaluated by a two-independent observer approach following the guidelines of the International TILs Working Group 2014[23] as previously described.[12] Briefly, the evaluation was based on H&E staining on digital whole slide image (WSI), and consensus was obtained by joint re-evaluation. In single cases, assessment of a third evaluator or additional information to visualize tumor composition (eg, CK5/14 and E-cadherin immunohistochemistry (IHC) staining) was considered. In case of heterogeneous spatial distribution, results were averaged. Brisk focal infiltrates ('hot spots'), obviously perivascular infiltrates, and tertiary lymphoid structures were excluded from the analysis. For statistical analysis, TILs levels, recorded as percentage, were expressed as a continuous variable.

Multiplexed IHC
Multiplexed IHC was performed on 3 μm-thick sections cut from the formalin-fixed paraffin-embedded tissue blocks from surplus material after central pathology review, which was available for 83 out of 385 study cases. These additional sections were stored at 4°C in the dark to minimize antigen aging over time until further processing. Inclusion criteria for the multiplex study were (1) availability of paired biopsies before and on treatment, (2) sufficient size of the biopsies on visual inspection, (3) representation of cases covering the entire study recruitment time, and (4) sufficient remaining representative tissue for quantitative analysis on microscopic inspection and comparison with the initial H&E staining, resulting in a series of 66 fully evaluable cases.

Assay optimization was performed following the manufacturer's instructions (OPAL Multiplex IHC Assay Development Guide; Akoya Bioscience, Menlo Park, California, USA). Slides were deparaffinized; initial antigen retrieval was performed by microwave cooking in Tris-buffered saline (TBS) at pH 9; and blocking of unspecific protein binding was performed using Protein Block Serum-free solution (Agilent/Dako, Hamburg,

Germany). Subsequent antigen retrieval and deactivation of the preceding staining step were performed according to the previous optimization by microwave cooking either in TBS at pH 9 or citrate buffer at pH 6. Consecutive IHC staining using the OPAL 7-plex fluorescence system was performed using the following primary antibodies: anti-CD4 (clone SP35, Zytomed Systems), anti-CD8 (clone C8/144B, Agilent/Dako), anti-PD1 (clone NAT105, Cell Marque), anti-CK7 (clone OV-TL12/30, Agilent/Dako), anti-CD73 (clone EPR6114, Abcam), anti-PDL1 (clone QR1; Quartett, Berlin, Germany), and a laboratory-developed assay[24 25] robustly detecting PDL1 that is not linked to a specific therapy and was validated in several QuIP ring studies (https://www.pdl1portal.eu/). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole. The following fluorophores in combination with the tyramide signal amplification system were used for detection of bound antibodies: Opal 520, Opal 540, Opal 570, Opal 620, Opal 650, or Opal 690. Fluoromount-G mounting medium (Thermo Fisher Scientific, Braunschweig, Germany) was applied to cover slides before imaging.

Multispectral image analysis and quantitative evaluation

WSI scanning was done at ×20 magnification using the Vectra Polaris instrument (Akoya Bioscience, online supplemental figure 2). Three-channel fluorescent WSIs were used to select regions of interest (ROIs) for subsequent targeted scanning of image stacks at ×40 magnification across the visible spectrum (420–720 nm) for multispectral imaging. The full available tumor area including the adjacent stromal area was included, excluding from analysis only ROIs containing obvious artifacts, pre-existing mammary gland tissue, and stromal areas with brisk perivascular inflammatory infiltrates outside of the tumor areas. Spectral libraries were generated using single-stained scans of tonsil tissue for each reagent and image deconvolution was performed with the inForm Advanced Image Analysis software (inForm V.2.4.8, Akoya). Training for the machine learning-based segmentation, tissue classification and cell phenotyping algorithms was performed using the inForm software on 20 images covering the full spectrum of expected variability regarding staining intensities and cell densities. Batch analysis and data export into text format comprised 2188 images representing all evaluable tissue areas in the data set. Visual quality control of all analyzed ROIs was performed by reviewing all composite images in the context of the quantitative output using Image Miner software V.2.7.0 (Definiens, Munich, Germany) (FF), and in-depth control of quantitative output in context of the corresponding H&E-stained sections was performed in 10% of randomly selected cases (FF and MC).

Statistical analysis

Clinical characteristics between patients included in this immune response assessment study and all other patients included in the WSG-ADAPT-TN trial were compared by independent two-sample t-tests, Wilcoxon rank-sum tests, χ^2 tests or Fisher's exact tests. Cellular marker levels at baseline and after week 3 were summarized by median values and 25th and 75th percentiles. Changes between the two time points were evaluated with the Wilcoxon signed-rank test.

Correlations between cellular marker levels and TILs levels (evaluated as a continuous variable) were calculated using Pearson's correlation coefficient (r). Associations between pCR and patient characteristics, as well as baseline cellular marker levels in stroma and tumor, were evaluated with univariable logistic regression. ORs were calculated per IQR, for example, ORs compared two patients with marker values at the 75th and 25th percentiles. Associations between pCR and changes in cellular marker levels between baseline and week 3, dichotomized as decrease versus increase, were evaluated with logistic regression adjusted for baseline marker levels. A trend test was based on the significance of the continuous marker level. Separate analyses were performed for measurements in stroma and tumor. Dynamics in T cells, PDL1 and PD1-positive CD4 and CD8 cells during 3 weeks of therapy were evaluated by grouping tumors into cold and hot (below-median and above-median marker levels at baseline, respectively), according to a recently proposed nomenclature.[14] However, these definitions are currently emerging and might change, depending on tumor type and therapy. For analyses of combined stroma and tumor measurements, tumors were categorized into cold, hot and altered (figure 1). Tumors were considered cold when marker levels in stroma and tumor were below the respective median value, hot when marker levels in tumor were above the median value, irrespective of levels in stroma, or altered when marker levels were below-median in tumor and above-median in stroma. We used univariable logistic regression to evaluate the association between pCR and transitions between the different states under therapy, that is, 'cold-to-hot' and 'hot-to-cold', from or to altered, and 'no change'. For all test results, two-sided p values were reported and interpreted as significant at 5%. P values were not corrected for multiple testing because of the exploratory nature of this study. To aid interpretation, ORs for pCR below 0.5 or above 2.0 were considered potentially clinically meaningful. Statistical analyses were performed using STATA SE V.6 software.

RESULTS
Patient characteristics
Between June 2013 and February 2015, 336 patients were randomized at 45 centers in the WSG-ADAPT-TN substudy (figure 2). pCR occurred in 36% of patients, that is, 28.4% of patients on
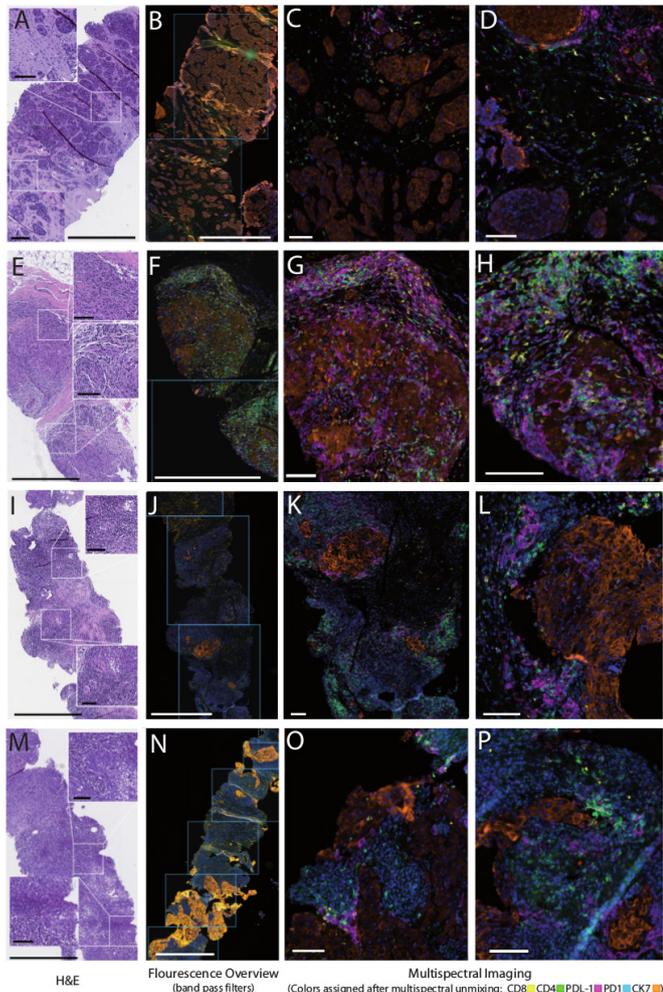
Figure 1 Representative examples for histopathological patterns of the TNBC inflammatory microenvironment and the immunological response. (A–D) Baseline biopsy and (E–H) corresponding paired biopsy under treatment from a patient with largely 'cold' tumor at diagnosis and brisk immunological response (cold to 'hot' transition). (I–L) Baseline biopsy and (M–P) corresponding paired biopsy under treatment from a patient with largely constant inflammatory pattern before and on treatment with heavily infiltrated stromal compartment but only a few lymphocytes in direct contact with tumor cells, referred to as 'immune excluded', 'compartmentalized' or 'altered' pattern. The left parts of each image set (A, E, I and M) depict H&E staining. (B, F, J, and N) Whole slide image fluorescence scans used to select regions of interest and right parts of each image set (C/D, G/H, K/L, and O/P) show representative examples of multiplexed immunohistochemistry displaying the five markers in the panel in pseudocolors: CK7 (orange), PDL1 (magenta), PD1 (cyan), CD4 (green) and CD8 (yellow) with blue nuclear stain (4′,6-diamidino-2-phenylindole). bars indicate 800 µm in the H&E and overview images and 100 µm in the inserts and the multiplex images. TNBC, triple-negative breast cancer.

nab-paclitaxel+gemcitabine and 44.5% of patients on nab-paclitaxel+carboplatin (p=0.004[21]). The 7-plex IHC and multispectral imaging were performed in a subset of sequential biopsies from 66 patients obtained at baseline and after 3 weeks of NACT. The median age

of this group was 49 years, and 54.5% (n=36/66) were premenopausal (table 1). Most tumors were of basal type according to PAM50 (86.4%, n=57). Most patients had cT2 (65.2%, n=43) and cN0 (77.3%, n=51) cancer. Forty (60.6%) and 26 (39.4%) patients received nab-paclitaxel+gemcitabine or nab-paclitaxel+carboplatin, respectively. pCR was achieved in 36.4% (n=24) of tumors, and early response rate (Ki-67 decrease or low cellularity) was 43.1% (n=28). Characteristics of the 66 patients with immune response assessment were not statistically different from the characteristics of the remaining 270 patients with the exception of a lower rate of early response (43.1%, n=28/65 vs 58.9%, n=116/197, p=0.026; table 1).

Marker expression levels

Compared with baseline levels, the absolute number of CD4, CD8 and total T cells significantly increased after 3 weeks of NACT in stroma (median change: 29.1, 20.1 and 49.8 cells/megapixel, respectively) and in tumor (median change: 10.3, 21.6 and 39.0 cells/megapixel, respectively; table 2). NACT also induced significant accumulation of PD1-positive CD8 cells in stroma (median change: 3.5 cells/megapixel) and in tumor (median change: 9.5 cells/megapixel). Furthermore, we observed a significant increase of CD73 cells in stroma (median change: 6.4 cells/megapixel) and PDL1-positive cells in tumor (median change: 32.3 cells/megapixel).

Correlation between TILs and immune markers

Correlation between the levels of TILs and immune markers either at baseline or their changes during the
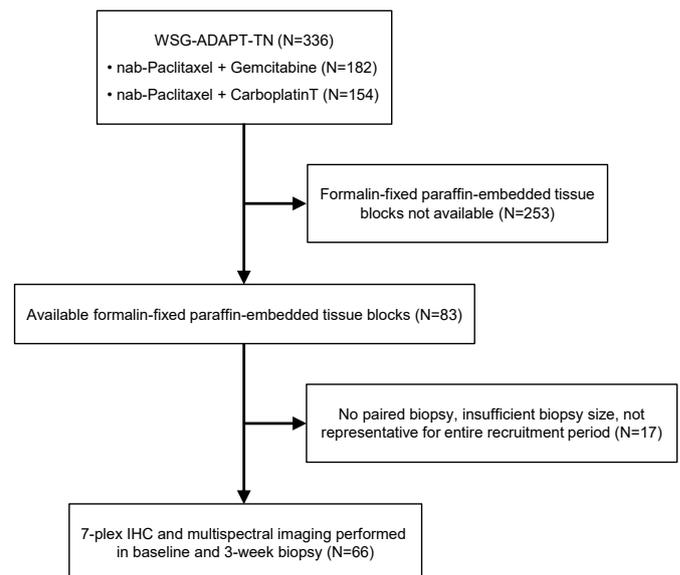
Figure 2 Consolidated Standards of Reporting Trials diagram. IHC, immunohistochemistry; WSG-ADAPT-TN, the West German Study Group Adjuvant Dynamic Marker-Adjusted Personalized Therapy Trial Optimizing Risk Assessment and Therapy Response Prediction in Early Breast Cancer - Triple Negative Breast Cancer trial.

Table 1 Patient characteristics for subjects with immune response assessed by 7-plex immunohistochemistry and multispectral imaging and the remaining subjects in the West German Study Group Adjuvant Dynamic Marker-Adjusted Personalized Therapy Trial Optimizing Risk Assessment and Therapy Response Prediction in Early Breast Cancer - Triple Negative Breast Cancer substudy

Parameter	Total (N=336)	Immune response assessed (n=66)	Immune response not assessed (n=270)	P value
Age (years), median (IQR)	50 (43–61)	49 (42–58)	51 (43–61)	0.396
Age group (years), n (%)				
<40	59	12 (18.2)	47 (17.4)	
40–50	94	21 (31.8)	73 (27.0)	
50–60	90	18 (27.3)	72 (26.7)	
>60	92	14 (21.2)	78 (28.9)	
Missing	1	1 (1.5)	0 (0)	
Menopausal status, n (%)				
Premenopausal	163	36 (54.5)	127 (47.0)	0.187
Postmenopausal	157	25 (37.9)	132 (48.9)	
Missing	16	5 (7.6)	11 (4.1)	
Histological subtype, n (%)				
No special type, ductal	304	60 (90.9)	244 (90.4)	0.999
Invasive lobular	7	1 (1.5)	6 (2.2)	
Medullary	9	2 (3.0)	7 (2.6)	
Other	12	2 (3.0)	10 (3.7)	
Missing	4	1 (1.5)	3 (1.1)	
PAM50 subtype, n (%)				
Basal	250	57 (86.4)	193 (71.5)	0.487
HER2	19	2 (3.0)	17 (6.3)	
LumA	5	0 (0)	5 (1.9)	
Normal	26	6 (9.1)	20 (7.4)	
Missing	36	1 (1.5)	35 (13.0)	
Clinical tumor stage, n (%)				
1	125	20 (30.3)	105 (38.9)	0.353
2	188	43 (65.2)	145 (53.7)	
3	18	2 (3.0)	16 (5.9)	
4	5	1 (1.5)	4 (1.5)	
Clinical nodal status, n (%)				
0	248	51 (77.3)	197 (73.0)	0.854
1	77	13 (19.7)	64 (23.7)	
2–3	11	2 (3.0)	9 (3.3)	
Treatment, n (%)				
nab-paclitaxel+gemcitabine	182	40 (60.6)	142 (52.6)	0.242
nab-paclitaxel+carboplatin	154	26 (39.4)	128 (47.4)	
pCR, n (%)				
No	206	42 (63.6)	164 (60.7)	0.992
Yes	118	24 (36.4)	94 (34.8)	
Missing	12	0 (0)	12 (4.4)	
Ki-67, median (IQR)	72.5 (50–80)	75 (60–90)	70 (50–80)	0.152
Missing	12	0	12	

Continued

Table 1 Continued

Parameter	Total (N=336)	Immune response assessed (n=66)	Immune response not assessed (n=270)	P value
Early response, n (%)				
No	118	37 (56.9)	81 (41.1)	0.026*
Yes	144	28 (43.1)	116 (58.9)	
Missing	74	1 (1.5)	73 (27.0)	

*Among patients with evaluable pCR.
pCR, pathological complete response.

3 weeks of NACT was low to moderate (table 3). The highest correlation coefficients (Pearson's r) were noted for change in T-cell levels in stroma (0.52) and tumor (0.40), baseline PDL1 levels in stroma (0.47) and tumor (0.45), and for change in CD4 cell levels in stroma (0.46).

Association between cellular markers at baseline and pCR
Baseline levels for any of the cellular markers in tumor or stroma were not associated with pCR (online supplemental table 1). Increases in the baseline CD4 to CD8 ratio in stroma of an IQR appeared associated with about a twofold reduced chance of pCR, although the association was not statistically significant.

Association between early dynamic changes in the immune markers and pCR
A decrease in the PD1-positive CD4 count in tumor was significantly associated with lower pCR rates compared

with an increase (OR=0.25, 95% CI 0.08 to 0.80, p-trend=0.023; table 4). The pattern in the stroma was similar, though not statistically significant. Lower pCR rates after a marker decrease (compared with an increase) were also observed for CD8, T cells, PD1 in CD8, and PDL1, although trends were not statistically significant.

For T cells and PDL1 separately in stroma or tumor and both compartments combined, transitions from cold at baseline to hot at week 3 were associated with similar pCR rates as no change (figure 3). Increased ORs appeared to suggest higher pCR rates after cold-to-hot transitions in PD1-positive CD4 (in tumor and stroma separately) and PD1-positive CD8 cells (in tumor and in tumor and stroma combined). Based on PD1-positive CD8 cells, hot-to-cold transitions separately in stroma or tumor and combined were associated with significantly reduced pCR rates compared with no change. However, these results

Table 2 Change in marker levels between baseline and week 3

Marker	Baseline, median (IQR)	Week 3, median (IQR)	Change, median (IQR)	P value for change
Stroma (cells/megapixel)				
CD4	63.9 (29.4–119.9)	102.2 (50.8–199.2)	29.1 (–8.0 to 112.1)	<0.001
CD8	27.8 (17.6–42.0)	52.1 (32.7–80.3)	20.1 (0.0–42.1)	<0.001
CD4/CD8	2.1 (1.1–3.8)	2.3 (1.1–3.1)	0.0 (–1.3 to 0.8)	0.423
T cell	102.3 (50.0–167.8)	168.4 (86.2–270.9)	49.8 (5.3–153.9)	<0.001
CD73	39.9 (25.8–73.3)	51.2 (30.7–100.1)	6.4 (–9.7 to 35.3)	0.015
PD1 in CD4	5.8 (3.1–15.0)	6.1 (2.5–12.2)	–1.0 (–4.5 to 3.3)	0.265
PD1 in CD8	25.5 (11.5–43.4)	31.9 (20.5–50.9)	3.5 (–7.4 to 19.2)	0.019
PDL1	54.0 (15.8–146.4)	68.1 (37.5–149.9)	6.9 (–25.5 to 78.0)	0.279
Tumor (cells/megapixel)				
CD4	13.4 (3.9–29.3)	24.9 (11.8–72.8)	10.3 (–4.3 to 48.9)	0.003
CD8	19.0 (6.5–29.4)	35.2 (17.1–74.9)	21.6 (–1.4 to 43.8)	<0.001
CD4/CD8	0.8 (0.3–2.2)	0.8 (0.3–2.0)	0.0 (–0.8 to 0.4)	0.492
T cell	34.7 (13.6–88.5)	85.8 (27.9–143.5)	39.0 (–0.8 to 103.5)	<0.001
CD73	33.1 (18.4–80.0)	43.8 (18.4–74.5)	–1.3 (–25.7 to 22.9)	0.982
PD1 in CD4	11.0 (4.9–22.5)	12.7 (4.2–33.3)	1.8 (–5.6 to 10.9)	0.144
PD1 in CD8	15.6 (5.2–38.0)	29.7 (14.3–44.7)	9.5 (–5.1 to 26.5)	0.001
PDL1	35.0 (14.1–117.1)	72.0 (22.5–194.8)	32.3 (–6.6 to 112.7)	0.003

Table 3 Pearson's correlation coefficients between TILs and cellular markers

Correlation between TILs and	Stroma		Tumor	
	Baseline*	Change†	Baseline*	Change†
CD4	0.257	0.464	0.087	0.324
CD8	0.264	0.39	0.226	0.374
CD4/CD8	−0.06	0.003	−0.096	0.024
T cell	0.314	0.522	0.204	0.404
CD73	0.09	0.277	−0.194	−0.126
PD1 in CD4	−0.068	0.035	0.07	0.161
PD1 in CD8	0.066	0.193	0.177	0.291
PDL1	0.47	0.27	0.447	0.124

*Correlation between TILs and immune marker levels measured at baseline.
†Correlation between change in TIL levels and change in immune marker levels (changes between baseline and week 3 assessments).
TIL, tumor-infiltrating lymphocyte.

were based on the absence of pCRs in patients with tumors changing from hot to cold. Lower pCR rates in tumors after hot-to-cold transitions compared with no change were also observed for T cells and PD1-positive CD4 cells, but numbers were too small to reach statistical significance. In a combined analysis of changes in tumor and stroma compartments, TNBC with altered T-cell and PD1-positive CD4 cell levels had higher odds of pCR than those with no change (figure 3).

DISCUSSION
We addressed the role of immune cell infiltrates in TNBC with particular emphasis on early dynamic changes in immune markers that remain underexplored to date and on the spatial distribution of immune cells in tumor subcompartments. This study complements the investigation of early response to NACT and its association with pCR, which was one of the major exploratory objectives of the WSG-ADAPT-TN substudy, in addition to assessment of clinical response/resistance after one cycle of NACT. These objectives are clinically highly relevant given their potential to guide individualized de-escalation/escalation strategies.

We present a unique cohort as we evaluated dynamic changes in immune markers induced early during NACT (sequential assessments at baseline and after only one cycle of combination chemotherapy) and their association with pCR as opposite to prior approaches relying exclusively on post-treatment samples. We observed increases in the levels of CD4, CD8, PD1-positive CD8 and T cells and in both tumor and stroma, CD73 in stroma and PDL1 in tumor compartments. Thus far, investigations of immune marker levels in post-NACT residual tumor specimens demonstrated increased levels of stromal and intratumoral TILs, either referring to total TILs[23] or specifically to CD4+ or CD8+ lymphocytes[7 26 27]. Expression of stromal PD1 was shown to increase after NACT, whereas PDL1 on TILs and tumor cells did not change.[26] In the NeoTRIP study, one cycle of nab-pacli

taxel+carboplatin±atezolizumab increased TIL levels.[28] Interestingly, nab-paclitaxel+carboplatin reduced PDL1 expression on immune cells; however, addition of atezolizumab increased PDL1 expression on immune and tumor cells.[28] Previously, baseline TIL levels were found to correlate with PDL1 expression,[17] which is in line with our results. Lee and colleagues reported that PDL1 expression decreased in the residual tumor or was maintained at low levels in tumor bed or stroma in 35.3% and 13.3% of patients, respectively.[27] Furthermore, in the residual tumor tissue, the number of stromal CD4+/CD8+/FOXP3+ TILs increased or remained stable in 70% of patients. In our study, 3 weeks of NACT induced the same effect on stromal T-cell infiltration in a similar proportion of patients (71.2%), whereas more patients had corresponding changes in PDL1 expression in tumor (72.7%) and stroma (75.8%). Potentially, this variability in findings could result from differences in the population of investigated lymphocytes and the definitions used for low/high PDL1 expression. Alternatively, the timing of the biopsy could play a role as it was suggested that the levels of TILs and PDL1 decrease during the natural history of TNBC.[29] This could therefore explain the more pronounced changes after one NACT cycle observed in our study compared with the changes observed in residual tumors after two to six NACT cycles.[27]

Baseline immune cell infiltrates were not predictive of pCR in our exploratory substudy cohort. Lack of predictive power may be due to a relatively low number of samples available for multiplexed IHC and the accuracy of this approach involving the exact phenotyping (restricted to CD4+ and CD8+ lymphocytes) as opposed to the H&E-based approaches including all mononuclear cells (including natural killer cells, plasma cells, and others in addition to CD4+ and CD8+ lymphocytes). Additionally, a potential trend favoring slightly higher inclusion of cases with unfavorable outcome could be associated with more vital tumor and less signs of regression in the on-treatment biopsies in patients with delayed pathological response.

Table 4 Association between pCR and changes in cellular marker levels between baseline and week 3

Marker	Stroma			Tumor		
	pCR, n	Non- pCR, n	OR* (95% CI)	pCR, n	Non- pCR, n	OR* (95% CI)
CD4						
Increase	17	30	1.00	17	28	1.00
Decrease	7	12	1.24 (0.39 to 3.96)	7	14	1.27 (0.37 to 4.41)
P-trend			0.260			0.249
CD8						
Increase	22	28	1.00	21	28	1.00
Decrease	2	14	0.10 (0.01 to 0.66)	3	14	0.28 (0.07 to 1.16)
P-trend			0.522			0.198
CD4/CD8						
Increase	15	18	1.00	12	20	1.00
Decrease	9	24	0.69 (0.21 to 2.30)	12	22	0.79 (0.26 to 2.34)
P-trend			0.711			0.205
T cell						
Increase	20	31	1.00	21	28	1.00
Decrease	4	11	0.60 (0.16 to 2.19)	3	14	0.31 (0.07 to 1.41)
P-trend			0.331			0.184
CD73						
Increase	13	28	1.00	11	21	1.00
Decrease	11	14	1.64 (0.58 to 4.62)	13	21	1.32 (0.44 to 3.94)
P-trend			0.458			0.930
PD1 in CD4						
Increase	13	13	1.00	18	19	1.00
Decrease	11	29	0.36 (0.12 to 1.07)	6	23	0.25 (0.08 to 0.80)
P-trend			0.213			0.023
PD1 in CD8						
Increase	16	21	1.00	19	25	1.00
Decrease	8	21	0.53 (0.17 to 1.61)	5	17	0.45 (0.12 to 1.63)
P-trend			0.098			0.196
PDL1						
Increase	15	22	1.00	18	27	1.00
Decrease	9	20	0.50 (0.16 to 1.59)	6	15	0.53 (0.16 to 1.81)
p-trend			0.781			0.652

*OR adjusted for baseline marker value.
pCR, pathological complete response.

Available evidence on association of high TILs levels, immune checkpoint and T-cell differentiation markers prior to NACT is conflicting with studies indicating either a high predictive value,[9 26 28 30–32] no predictive value[33] or even a negative prognostic/predictive role.[34] Collectively, available data suggest that improved scoring approaches, concise immune cell phenotyping and increased numbers of patients in concert with harmonization of treatments are needed for consensus between different studies.[35 36]

Thus far, only a few studies investigated the association between patient outcomes and NACT-induced changes in immune markers (and mostly in postsurgery specimens). Low stromal PDL1 expression after NACT was associated with shorter disease-free survival,[27] while increased CD8+ TILs correlated with improved breast cancer-specific survival.[32] However, since these findings were obtained in residual tumor tissue, changes in immune markers may not reflect the dynamics occurring in tumors particularly sensitive to NACT, that is, in those who will achieve pCR. Therefore, results from surgical specimens could be biased towards 'no response' and therapy resistance or may have more necrosis/regression in responders.

Change	Patients, n	pCR, n	OR	95% CI
T cells				
Tumor				
No change	38	15	1.00	
Cold to hot	21	8	0.94	0.32-2.82
Hot to cold	7	1	0.26	0.03-2.34
Cold: T cells tumor <34.7 Hot: T cells tumor ≥34.7				
Stroma				
No change	41	15	1.00	
Cold to hot	19	8	1.26	0.42-3.83
Hot to cold	6	1	0.35	0.04-3.25
Cold: T cells stroma <102.3 Hot: T cells stroma ≥102.3				
Tumor and stroma				
No change	33	11	1.00	
Cold to hot	17	6	1.09	0.32-3.73
Hot to cold	5	0	0.00	0.00-1.04
Altered at baseline or week 3	11	7	3.50	0.84-14.56
Cold: T cells tumor <34.7 and T cells stroma <102.3 Hot: T cells tumor ≥34.7 and stroma ≥102.3 or <102.3 Altered: T cells tumor <34.7 and T cells stroma ≥102.3				

Change	Patients, n	pCR, n	OR	95% CI
PDL1				
Tumor				
No change	42	15	1.00	
Cold to hot	18	6	0.90	0.28-2.89
Hot to cold	6	3	1.80	0.32-10.05
Cold: PDL1 tumor<35 Hot: PDL1 tumor ≥35				
Stroma				
No change	41	16	1.00	
Cold to hot	16	6	0.94	0.28-3.08
Hot to cold	9	2	0.45	0.08-2.42
Cold: PDL1 stroma<54 Hot: PDL1 stroma ≥54				
Tumor and stroma				
No change	40	14	1.00	
Cold to hot	12	5	1.33	0.35-4.96
Hot to cold	6	3	1.86	0.33-10.45
Altered at baseline or week 3	8	2	0.62	0.11-3.48
Cold: PDL1 tumor <35 and PDL1 stroma <54 Hot: PDL1 tumor ≥35 and stroma ≥54 or <54 Altered: PDL1 tumor <35 and PDL1 stroma ≥54				

Change	Patients, n	pCR, n	OR	95% CI
PD1 in CD4				
Tumor				
No change	45	16	1.00	
Cold to hot	13	6	1.55	0.45-5.42
Hot to cold	8	2	0.60	0.11-3.35
Cold: PD1 in CD4 tumor <11 Hot: PD1 in CD4 tumor ≥11				
Stroma				
No change	47	17	1.00	
Cold to hot	10	6	2.65	0.65-10.71
Hot to cold	9	1	0.22	0.03-1.92
Cold: PD1 in CD4 stroma <5.8 Hot: PD1 in CD4 stroma ≥5.8				
Tumor and stroma				
No change	41	14	1.00	
Cold to hot	8	2	0.64	0.11-3.61
Hot to cold	7	1	0.32	0.04-2.94
Altered at baseline or week 3	10	7	4.50	1.01-20.14
Cold: PD1 in CD4 tumor <11 and stroma <5.8 Hot: PD1 in CD4 tumor ≥11 and stroma ≥5.8 or <5.8 Altered: PD1 in CD4 tumor <11 and stroma ≥5.8				

Change	Patients, n	pCR, n	OR	95% CI
PD1 in CD8				
Tumor				
No change	36	13	1.00	
Cold to hot	22	11	1.77	0.60-5.20
Hot to cold	8	0	0.00	0.00-0.53
Cold: PD1 in CD8 tumor <15.6 Hot: PD1 in CD8 tumor ≥15.6				
Stroma				
No change	41	16	1.00	
Cold to hot	18	8	1.25	0.41-3.84
Hot to cold	7	0	0.00	0.00-0.54
Cold: PD1 in CD8 stroma <25.5 Hot: PD1 in CD8 stroma ≥25.5				
Tumor and stroma				
No change	34	13	1.00	
Cold to hot	18	9	1.62	0.51-5.12
Hot to cold	8	0	0.00	0.00-0.49
Altered at baseline or week 3	6	2	0.81	0.13-5.05
Cold: PD1 in CD8 tumor <15.6 and stroma <25.5 Hot: PD1 in CD8 tumor ≥15.6 and stroma ≥25.5 or <25.5 Altered: PD1 in CD8 tumor <15.6 and stroma ≥25.5				

Figure 3 Association between pCR and changes in marker levels in tumor and/or stroma for T cells, PDL1, PD1 in CD4 and PD1 in CD8 based on univariable logistic regression. pCR, pathological complete response.

Furthermore, biopsies and surgical specimens are differently processed thus introducing tissue fixation-related variability. Our strategy reduces these risks as 3-week biopsies capture the entire spectrum of responses to NACT and they are processed identically as pretherapy biopsies. This approach revealed that hot-to-cold change in PDL1 expression (stroma) and in T-cell number (either stroma or tumor) was associated with low pCR rate. Conversely, tumors with increases in stromal CD4+ and intratumoral CD8+ cells more frequently achieved pCR. Additionally, altered T-cell status (cold in tumor, hot in stroma) at baseline or at week 3 was predictive of pCR. In a recent analysis of the WSG-ADAPT-TN data, TIL levels in non-paired biopsies increased during 3 weeks of NACT and higher TIL numbers at either time point corresponded to higher pCR rates.[37] It is well established that CD8 T cells directly promote the apoptosis of cancer cells, whereas the role of CD4 TILs is twofold. CD4 helper T cells initiate and maintain antitumor functions of CD8 T cells, while CD4 regulatory T cells may suppress immune response. Therefore, association of increased CD4 and CD8 number with pCR indicates an ongoing active adaptive immune response in tumors responding well to NACT. We also found that increased number of PD1-positive CD8 and CD4 cells and altered distribution of PD1-positive CD4 cells were associated with pCR. Classically, PD1 expression marks an exhausted population of CD8+ TILs, and PD1 interaction with PDL1 on tumor cells leads to inhibition of proliferation, cytokine release and cytotoxic functions of TILs. However, we observed that pronounced increase in PD1 and decrease in PDL1 during NACT were associated with pCR. Although this finding appears to challenge the

current paradigm on the role of PD1–PDL1 interaction in immune escape mechanisms, a decrease in PDL1 early during NACT could also indicate an active elimination of tumor cells. Furthermore, induced PD1 expression on immune cells appears to prevent lymphovascular invasion.[38] Considering the favorable outcome in tumors with changes in CD8+ TILs and PDL1 in our study, early assessment of tumor response to NACT could potentially select patients for therapy de-escalation. Given that high pCR rates were obtained already after 12 weeks of NACT in the WSG-ADAPT-TN study,[21] patients with CD8+ TILs increase accompanied by PDL1 decrease could benefit from this shorter therapy. Conversely, high PDL1 levels could be used to identify candidates for therapy escalation with immunotherapy. Addition of pembrolizumab to NACT was recently shown to increase pCR rate in the KEYNOTE-522 and I-SPY two trials.[20 39] This observation appears to support the hypothesis that PDL1 blockade may sensitize cancer cells to chemotherapy.[40] Recently, atezolizumab+chemotherapy improved pCR rates over chemotherapy in the IMpassion031 study[41] but not in the NeoTRIP study.[42] However, in both studies, PDL1-positive disease was associated with higher odds for pCR than PDL1-negative disease. Yet, PDL1 status was not predictive for atezolizumab response. Interestingly, in unresectable, locally advanced or metastatic TNBC, PDL1 status is predictive of response to atezolizumab with first-line atezolizumab plus nab-paclitaxel being approved only for PDL1-positive TNBC based on the prolonged overall survival in the Impassion130 study.[43]

In conclusion, our multiplexed IHC approach provided important insights into the immune infiltrate dynamics during tumor response to NACT. These results might help to find immune markers to identify tumors most likely to respond to chemotherapy, thus potentially allowing for individualized de-escalation/escalation strategies in TNBC. Nevertheless, both TILs and multiplex immune markers need to be further investigated in prospective trials to confirm their value for prediction of tumor response to NACT and to investigate their function in response to immunotherapy.

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