

Phase II study of pembrolizumab plus nab-paclitaxel in Hormone Receptor–positive, HER2-negative metastatic breast cancer: clinical and molecular insights into response

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Abstract

Purpose

We investigate the addition of PD-1 blockade to chemotherapy in HR + MBC to assess efficacy, safety, and changes in the tumor microenvironment utilizing serial tumor biopsies.

Methods

Single-arm phase 2 clinical trial (NCT02752685) of 20 HR+/HER2- MBC patients received nab-paclitaxel (100mg/m² IV d1/8, q 3 weeks) and pembrolizumab (200mg IV d1, q 3 weeks, starting with cycle 2). Primary endpoint: best overall response rate (BORR) by RECIST v1.1; secondary endpoints: safety, progression free survival (PFS), duration of response (DOR), and overall survival (OS). Changes in PD-L1 score, stromal tumor-infiltrating lymphocytes (TIL), T-Cell Receptor (TCR) sequence, gene expression, and quantitative multiplex immunofluorescence (qMIF) were evaluated serially during treatment.

Results

The BORR for partial response (PR) was 26.3% (5/19). Median (m) PFS was 4.1 months (mo) (95% CI 2.2–12.5), mOS was 15.7 mo (95% CI 10.0- NA), and mDOR was 5.6 mo (3.9–52.7). 14 patients experienced one or more grade 3 or higher adverse events (AEs), the most common being anemia, fatigue, diarrhea, and neutropenia, and there were no grade 5 AEs. Longitudinal spatial qMIF and transcriptomic profiling indicated that tumor immune remodeling and proliferative programs shape treatment response, with adaptive immune activation alone being insufficient, supporting combination approaches targeting tumor cell-cycle control and immune competence. No predictive biomarkers emerged, however higher T-cell fraction at baseline was associated with OS ($p = 0.0009$).

Conclusions

Chemoimmunotherapy was efficacious with manageable toxicity profile. Importantly, responses were seen in patients previously treated with CDK 4/6 inhibitors. Further research is needed for predictive biomarkers in HR+/HER2- MBC to checkpoint blockade.

Introduction

Pembrolizumab, a programmed cell death protein 1 (PD-1) checkpoint inhibitor, is currently the only FDA-approved immunotherapy for breast cancer, with demonstrated survival benefit in both early-stage and metastatic triple-negative breast cancer (TNBC), a subtype characterized by higher immune infiltration and PD-ligand 1 (PD-L1) expression [1–3]. In contrast, hormone receptor-positive (HR+), human epidermal growth factor receptor (HER-2)-negative breast cancer, the most common subtype, has

demonstrated limited benefit from immune checkpoint blockade. Compared with TNBC, HR+/HER2- tumors are generally less immunogenic, with lower PD-L1 expression, fewer tumor-infiltrating lymphocytes (TILs), and lower tumor mutational burden (TMB) [4–6].

Nonetheless, recent evidence suggests that immunotherapy may hold promise for selected subsets of HR+/HER2- breast cancer. In early-stage disease, neoadjuvant studies have suggested higher response rates with the addition of checkpoint blockade to chemotherapy in tumors with more immunogenic features, including high genomic risk (e.g., MammaPrint) or increased TIL [7–10]. In the metastatic setting, however, results have been less encouraging. For example, a randomized phase II trial evaluating eribulin with or without pembrolizumab did not improve progression-free survival (PFS), overall response rate (ORR), or overall survival (OS) with the addition of pembrolizumab [11]. Moreover, neither PD-L1, TILs, nor TMB clearly identified patients most likely to benefit [11, 12]. These findings highlight both the limited activity of immunotherapy in unselected HR+/HER2- metastatic breast cancer (MBC) and the need for improved biologic selection strategies.

Although endocrine therapy and targeted agents, especially cyclin-dependent kinase 4/6 (CDK4/6) inhibitors, have substantially improved outcomes in endocrine-resistant HR+/HER2- MBC, patients progressing after these therapies often transition to sequential antibody-drug conjugates and single-agent chemotherapy, which provides limited durability of benefit. At the same time, HR+/HER2- MBC is biologically heterogeneous, and a subset of tumors may retain features consistent with immune responsiveness, including an inflamed tumor microenvironment, immune-related gene expression programs, increased T-cell clonality, elevated TMB, or, rarely, deficient DNA mismatch repair [13–16]. High TMB in breast cancer has been demonstrated as biomarker of response in some studies [17, 18].

Here, we evaluated pembrolizumab in combination with nab-paclitaxel in patients with HR+/HER2- MBC and incorporated serial tumor biopsies to characterize treatment-associated changes in the tumor microenvironment (TME). Nab-paclitaxel was selected because it can be administered without corticosteroid premedication and because of prior clinical experience combining taxanes with immune checkpoint inhibitors or other biologic therapies [19]. To distinguish early changes induced by chemotherapy from later changes associated with combined therapy, nab-paclitaxel was administered alone during Cycle 1 of treatment, followed by the addition of pembrolizumab from Cycle 2 onward. This was done to assess the effects of chemotherapy alone in the post-cycle 1 biopsy based on known early effects of cytotoxic therapy on biomarkers in TME and to test the delayed effects of immune checkpoint blockade in the combination treatment in post-cycle 2 biopsy, given the expected delayed effects of immunotherapy on TME [19, 20]. In addition, in the TONIC trial, this strategy of using a short chemotherapy induction prior to immune checkpoint blockade improved responses in metastatic TNBC as it enhanced sensitivity to the PD-1 blockade [21]. Our design enabled longitudinal assessment of PD-L1 expression, TIL density, gene expression, T-cell receptor (TCR) repertoire, and spatial TME organization. Given the modest activity of immunotherapy reported in this disease setting, our objective was not only to assess the clinical activity and safety of this regimen, but also to identify biologic

features associated with response, resistance, and survival that may inform future biomarker-enriched studies.

Methods

Study Design

Figure 1. A. Study schema (C=cycle). **B.** CONSORT diagram. From July 2016 to May 2019, 20 eligible patients with Hormone-Receptor-Positive breast cancer were enrolled in this cohort and followed through February 2020. All enrolled patients received at least one dose of study therapy and were included in the safety analysis; 19 patients were evaluable for response.

In this single-arm, open-label, multi-cohort Phase II study (NCT02752685, registration date 6/2016) we evaluated the safety/tolerability and clinical activity of the combination of nab-paclitaxel and pembrolizumab in patients with HER2-negative metastatic breast cancer (n = 70). There were three cohorts of patients: two TNBC cohorts comprising a total of 50 subjects and a hormone receptor (HR)-positive cohort comprising 20 subjects (Fig. 1A). There was an initial safety run-in with 12 subjects (5 from the HR+ cohort and 7 from the TNBC cohort; patients from both cohorts were eligible). Given that no unexpected toxicity was observed (as defined in the study protocol), enrollment continued to completion in both cohorts (50 TNBC, 20 HR-positive). The subjects from the run-in safety portion were included in the Phase II analysis. Tumor expression of PD-L1 was not required for study enrollment but was assessed as a potential predictive marker.

Here we report the results of the HR+ cohort. From July 2016 to May 2019, 20 eligible patients with HR+ breast cancer were enrolled into this cohort. Patients were followed for progression and survival through February 2020. No patients remain on study treatment. All 20 patients received therapy in accordance with the study protocol (Fig. 1B). Eligible patients in this cohort had HR-positive (estrogen receptor > 1% and/or progesterone receptor positive > 1%), HER2-negative (per American Society of Clinical Oncology/College of American Pathologists guidelines) MBC treated with 0–2 prior lines of cytotoxic chemotherapies and allowed prior lines of endocrine and/or targeted therapy. Participants with previously treated brain metastases were eligible if imaging confirmed stability (without evidence of progression by imaging for at least four weeks prior to the first dose of trial treatment and any neurologic symptoms had returned to baseline), had no evidence of new or enlarging brain metastases, and were not using steroids for at least 7 days prior to trial treatment. Participants could not have received prior therapy with an anti-PD-1, anti-PD-L1, or anti-PD-L2 agent or a taxane in the past 90 days prior to study enrollment. CT of the chest and abdomen/pelvis with IV contrast were used, and per investigator's discretion, PET/CT can be substituted. If IV contrast is contraindicated, a non-contrast CT of chest/abdomen/pelvis is acceptable. Bone scans could be used if clinically indicated. MRI brain or CT brain with IV contrast is required at baseline and as clinically indicated thereafter. The same imaging methods should be used throughout the trial. The gold standard for evaluating tumor response and best overall response rate (BORR) during the study was RECIST 1.1 criteria. A 7-day window was allowed

around post-baseline imaging scans (i.e. should be obtained every 9 weeks from the date of allocation (+/- 7 days)). Frequency of imaging studies was every 12 weeks (+/- 7 days) after 12 months on study treatment. Tumor assessment dates were fixed and should not be adjusted for dose interruptions, delays, or modifications.

The Institutional Review Board at the Grossman School of Medicine at New York University (NYUGSoM) approved the study protocol (**Supplement 1**), and written informed consent was obtained from all participants prior to study entry. The study was monitored by the Data Safety Monitoring Committee of the New York University Perlmutter Cancer Center.

Treatment and Study Procedures

For Cycle 1 (C1), patients received nab-paclitaxel monotherapy at 100mg/m² intravenous (IV) on Days 1 and 8 of a 21-day cycle, followed by pembrolizumab 200mg IV on Day 1 of a 21-day cycle, with nab-paclitaxel continued in Cycle 2 (C2) and subsequent cycles. Participants continued on trial until progression or intolerable side effects.

Statistical Methods

The primary objective of this clinical trial was to evaluate the safety and tolerability of the combination of nab-paclitaxel and pembrolizumab in patients with metastatic HR+/HER2-negative breast cancer. Secondary objectives were to estimate the best overall response rate (BORR) using RECISTv1.1. Additional exploratory objectives included evaluating PFS, OS, DOR (duration of response), and CBR (defined as complete response (CR) plus partial response (PR) plus stable disease (SD) for more than 6 months) using RECISTv1.1. Demographic and baseline characteristics, as well as safety results, were summarized descriptively.

The distribution of the maximum grade 3 or greater adverse event (AE) (CTCAE v5.0) possibly, probably, or definitely related to study treatment, experienced by each patient, is provided. In addition, immune-related adverse events were reported. Additional exploratory analyses addressed the relationships between PD-L1, TIL, and other biomarkers evaluated at baseline and post-cycles 1 and 2, and with ORR, PFS, and OS, with descriptive statistics and summaries of changes from baseline over time.

Estimates of BORR and other categorical outcomes are provided with an exact 95% Clopper-Pearson confidence intervals. Time-to-event outcomes are evaluated with Kaplan-Meier estimates over time and confidence intervals for median times to event.

Tumor Biopsy Collection

Serial formalin-fixed paraffin-embedded (FFPE) tumor biopsy samples were collected at three time points: pre-treatment (baseline), treatment post-cycle 1, and treatment post-cycle 2. Hematoxylin and

eosin (H&E) stained sections from each biopsy were reviewed by a board-certified breast pathologist. Tumor samples were evaluated for PD-L1 modified proportion score (MPS), TIL score based on H&E morphology, TMB, NanoString gene-expression profiling, TCR sequencing, and quantitative multiplex immunofluorescence (qmIF) (**Table S1**).

PD-L1 immunohistochemistry and modified proportion score (MPS)

PD-L1 protein expression was assessed on FFPE tumor sections by immunohistochemistry (IHC) using the Merck 22C3 antibody [22] according to the manufacturer's instructions. An adequate specimen was defined as having at least 50 viable neoplastic cells overall or at least 5 PD-L1-positive tumor cells; specimens not meeting these criteria were considered non-evaluable. PD-L1 staining was evaluated by a board-certified breast pathologist, who assigned a modified proportion score (MPS). At the time the study was designed, no FDA-approved companion diagnostic assay for pembrolizumab use in breast cancer was available, and no established cutoff for PD-L1 expression in HR+/HER2- breast cancer had been defined; therefore, MPS testing was used. Membranous staining of any intensity (weak, moderate, or strong) in tumor cells and associated mononuclear inflammatory cells was considered positive. The stromal interface was assessed qualitatively; when present, it appeared at low magnification (4×) as a lichenoid band of PD-L1-positive cells bordering tumor nests and, at higher magnification (20×), was composed predominantly of macrophages. Only PD-L1 staining within tumor nests and immediately apposed mononuclear inflammatory cells was included in the MPS.

TIL scoring

TIL were quantified on H&E sections by the same board-certified pathologist using a semi-quantitative 4-point scale. For each case, 3–5 representative high-power fields (HPFs; 20× objective) within tumor nests and the invasive margin were examined, and the average number of lymphocytes per HPF was estimated. Scores were assigned as follows: 0, < 1 TIL/HPF; 1, 1–10 TIL/HPF; 2, 11–20 TIL/HPF; and 3, > 20 TIL/HPF. This score reflects the relative abundance of TILs within the tumor and was used as a global histologic measure of immune infiltration.

Quantitative multiplex immunofluorescence (qmIF) staining and imaging

Multiplex immunofluorescence was performed on 5 µm FFPE sections [23, 24]. Hematoxylin and eosin (H&E) slides were reviewed by an expert pathologist to exclude samples lacking viable tumor regions. Adjacent FFPE sections were stained on a Leica Bond RX automated stainer (Leica Biosystems) using Opal reagents (Akoya Biosciences) according to the manufacturer's instructions with minor optimization. Following deparaffinization and rehydration, endogenous peroxidase activity was quenched, and heat-induced epitope retrieval was performed at 100°C using either Epitope Retrieval Solution 1 (ER1) or Epitope Retrieval Solution 2 (ER2), as appropriate for the target antigen. Two 6-plex

panels were used to interrogate T-cell and myeloid compartments: Panel 1: Cluster of Differentiation 3 (CD3), CD8, Forkhead box P3 (FoxP3), Human Leukocyte Antigen-DR isotype (HLA-DR), CD68, Pan-cytokeratin (PanCK). Panel 2: CD33, Colony Stimulating Factor 1 Receptor (CSF1R), CD163, HLA-DR, CD68, PanCK. Each panel was applied using iterative staining cycles consisting of primary antibody incubation, secondary horseradish peroxidase (HRP)-conjugated antibody, and tyramide signal amplification with Opal fluorophores, followed by heat-mediated stripping of antibodies between cycles. The fluorophore remained covalently bound to the tissue at each round, enabling multiplex detection. After completion of all cycles, slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and coverslipped. Whole-slide images were acquired on Leica AT2 or Akoya Vectra Polaris/Phenolmager HT systems with spectral unmixing. Image acquisition was performed using either a Leica AT2 whole-slide scanner (40x) or an Akoya Vectra Polaris/Phenolmager HT system (20x). Multispectral images were unmixed using PhenolmagerHT 2.0, Phenochart 2.0, and inForm 3.0 software to generate whole-slide qPTIFF files. All images were stored in NYUGSoM's OMERO Plus database (Glencoe Software).

qMIF analysis

Spectrally unmixed whole-slide qptiff files were analyzed in HALO (Indica Labs, v3.4+) [25]. HALO's deep-learning classifiers (Mininet) delineated tumor versus stroma compartment, nuclei segmentation tool was used for nuclei detection and segmentation, and HighPlex FL (v4.2.3) classifier was used for cell phenotyping and extraction of per-cell marker intensities. These classifiers were custom-trained on HALO by a learn-by-example approach. Positivity thresholds were optimized iteratively and confirmed by manual review of randomly selected regions on the image. Cell-level outputs, including coordinates, fluorescence intensities, and compartment annotations, were exported as csv files. Single-cell phenotyping and quantitative analyses were performed in R (v4.x) using custom scripts and cells were assigned a unique phenotype based on binary marker expression in a hierarchical rule-based classification scheme (**Table S5**). Relative abundance was calculated as the percentage of each phenotype. Cells labeled as "Other" were not covered by our panels and were excluded. Data visualization and statistical analyses were subsequently performed in GraphPad Prism (v9).

RNA isolation and NanoString gene-expression profiling

RNA was extracted from FFPE biopsy specimens corresponding to each study time point using the Qiagen RNeasy FFPE Kit according to the manufacturer's instructions. Gene expression was prospectively assessed using the NanoString nCounter Breast Cancer 360 Panel, including 770 genes [26]. Samples were processed according to the manufacturer's protocol, and raw RCC files were analyzed using the R package nSolver Analysis Software (v. 4) using predefined quality control metrics; samples failing quality control were excluded. Data were normalized using background subtraction, positive control normalization, and housekeeping gene normalization according to the manufacturer's recommended default parameters. Normalized expression data were analyzed using the nSolver Advanced Analysis module to generate gene-expression and pathway scores. Differential expression

analyses were performed using two-sided statistical tests on normalized baseline expression data to compare tumors from patients with PD versus those with PR and SD [27], and p-values were adjusted for multiple testing using the Benjamini–Hochberg method. Results were summarized by log2 fold change and p-value, and the top 20 ranked genes were displayed in the volcano plot. Genes with an absolute log2 fold change of > 1 and an adjusted p-value of < 0.05 were considered significant. Pathway enrichment analysis was performed using Gene Set Enrichment Analysis; pathways with FDR $q < 0.05$ were considered enriched.

Tumor Mutational Burden

TMB was available for 8 of the 20 patients in this cohort and was retrieved by chart review based on previously performed standard-of-care clinical assays such as the Memorial Sloan Kettering Impact or Foundation Medicine testing (see **Table S2**).

T-cell receptor variable beta chain sequencing

The CDR3 regions of human TCR variable beta chain (TCR β) and IgH chains were sequenced using Adaptive Immunosequencing (Adaptive Biotechnologies, Seattle, WA). Extracted genomic DNA was amplified in a bias-controlled multiplex PCR, followed by high-throughput sequencing. Sequences were collapsed and filtered to identify and quantify the absolute abundance of each unique CDR3 region for further analysis as previously described [28–30]. The fraction of T-cells was calculated by normalizing TCR β template count to the total number of cells sequenced on the assay using a panel of reference genes found in all nucleated cells.

Two quantitative components of TCR β diversity were compared across samples in this study. First, Simpson clonality was calculated on productive rearrangements. Second, sample richness was calculated as the number of unique productive rearrangements in a sample after computationally downsampling to a common number of T-cells to control for variation in sample depth or T-cell fraction [28–30].

Results

Patient Characteristics and Clinical Outcomes

Baseline characteristics are summarized in **Table 1**. The median age at enrollment was 56 years (range, 34–75). At study entry, 75% (15/20) of patients had ER expression $> 10\%$, and 25% (5/20) had PR expression $> 10\%$. 70% (14/20) had received prior hormone therapy in the metastatic setting. Five of the six patients who had not received prior hormone therapy had tumors that were ER and/or PR 1–10%. 55% (11/20) had prior exposure to CDK4/6 inhibitors. The median number of prior cytotoxic chemotherapy lines was 1 (range, 0–2) (**Table 1**).

Figure 2. Patient outcomes. (A) Kaplan–Meier survival curves showing progression-free survival (PFS) and overall survival (OS). Tick marks indicate censored observations. Median survival times and

corresponding 95% confidence intervals are shown. **(B)** Swimmer plot of individual patient outcomes. Each bar represents one patient, showing the duration of treatment and follow-up from enrollment to the last available assessment. Bar colors indicate best overall response: partial response (PR), stable disease (SD), progressive disease (PD), and not evaluable (NE). Symbols denote key clinical events: disease progression, death, and censoring. Time is shown in years from enrollment. Note that this figure represents all data collected on all patients; analysis was limited to follow up through February 2020.

Table 1. Patient demographic and treatment characteristics

Age in years	N = 20	
	median (range)	56 (34–75)
ECOG PS	0	3 (15%)
	1	17 (85%)
Ethnicity	Hispanic/Latino	2 (10.0%)
	Not Hispanic/Latino	18 (90.0%)
Race	White	15 (75.0%)
	Asian	3 (15.0%)
	Black	1 (5.0%)
	Other	1 (5.0%)
ER at Study Entry	Positive (> 10%)	15 (75.0%)
	Low (1–10%)	2 (10.0%)
	Negative (< 1%)	3 (15.0%)
PR at Study Entry	Positive (> 10%)	5 (25.0%)
	Low (1–10%)	7 (35.0%)
	Negative (< 1%)	8 (40.0%)
Lines of Chemotherapy in Metastatic Setting	median (range)	1(0–2)
	0	8 (40.0%)
	1	6 (30.0%)
	2	6 (30.0%)
Lines of Hormone Therapy in Metastatic Setting	median (range)	1 (0–3)
	0	6 (30.0%)
	1	7 (35.0%)
	2	3 (15.0%)
	3	4 (20.0%)
Prior CDK 4/6 inhibitors and duration of treatment	Yes	11 (55.0%)
	< 3 mo	3 (27.3%)
	3-6mo	1 (9.1%)
	6-12mo	4 (36.4%)

Age in years	N = 20	
	median (range)	56 (34–75)
	≥ 12mo	3 (27.3%)
	No	9 (45.0%)
Site of Metastatic Biopsy	Breast	5 (25%)
	Liver	8 (40%)
	Lung	1 (5%)
	Lymph Node	4 (20%)
	Soft Tissue	1 (5%)
	Bone	1 (5%)

The BORR was partial response (PR) in 5 of 19 patients (26.3%, 6.5–46.1%), stable disease (SD) in 7 of 19 (36.8%, 15.2–58.5%), and progressive disease (PD) in 7 of 19 (36.8%, 15.2–58.5%) (Table 2). Although there were 7 patients with SD, only 1 patient had SD for > 6 months. The clinical benefit rate (CBR) was 31.58% (6/19). One patient was not evaluable for response due to early death from complications of metastatic disease shortly after receiving Cycle 1, Day 1 treatment.

Among the fifteen patients with baseline ER expression of 76–100%, 5 achieved PR (33.3%), and 5 achieved SD (33.3%). Clinical and pathologic features of the 5 patients with PR are provided in **Table S3**. All of these patients had received at least one prior line of endocrine therapy in the metastatic setting.

Among the 11 patients who had previously received CDK4/6 inhibitors, 4 (36.4%) achieved PR, all of whom had received a CDK4/6 inhibitor for > 3 months, including 3 patients for > 6 months; 4 (36.4%) had SD, including 2 patients treated for < 3 months and 2 patients for ≥ 3 months; and 3 (27.3%) experienced PD, including 1 patient who progressed on CDK4/6 inhibitor within < 3 months. All patients had received palbociclib; one patient had also received ribociclib prior to study enrollment. Among the remaining 9 patients who did not receive prior CDK4/6 inhibitors, 1 (11.1%) achieved PR, 3 (33.3%) achieved SD, 4 (44.4%) experienced PD, and 1 (11.1%) was not evaluable.

Median PFS was 4.1 months (95% CI, 2.2–12.5), and median OS was 15.7 months (95% CI, 10.0–NA) (Fig. 2), and the median duration of response (DOR) was 5.6 months (95% CI, 3.9–52.7) (Table 2). PFS rates at 6 and 12 months were 35% (95% CI, 19.3–63.6%) and 25% (95% CI, 11.7–53.4%), respectively. OS rates were 75% (95% CI, 58.2–96.6%) at 6 months, 58.9% (95% CI, 40.6–85.5%) at 12 months, and 42.2% (95% CI, 24.7–72.0%) at 24 months (Table 2).

Treatment-related AE grade ≥ 3 and immune-related

adverse events (irAE), defined as AE felt to be secondary to pembrolizumab, are summarized in Table 3. The most common treatment-related AEs of any grade included anemia (50%), diarrhea, nausea, and liver function test (LFT) abnormalities (40% each). Fourteen patients (70%) experienced one of more grade 3 or higher AE, the most common being neutropenia. One patient had several grade 4 AE (pneumonitis, neutropenia, hyponatremia). No grade 5 AE was observed. IrAE included abnormal liver function tests, mucositis, hypothyroidism, hyponatremia, diarrhea, and

pneumonitis, with grade 3–4 IrAE toxicities seen in 5 patients. Notably, three of these patients from the PR group experienced grade 3 irAEs (**Table S3**).

Treatment endpoint	Response	Response rate
BORR (95% CI)	CR	0
	PR	5 (26.3%, 6.5–46.1%)
	SD	7 (36.8%, 15.2–58.5%)
	PD	7 (36.8%, 15.2–58.5%)
	Not evaluable	1
	BORR (CR + PR)	5 (26.3%, 6.5–46.1%)
CBR	CR + PR+SD > 6 mo	6/19 (31.58%)
DoR (months)	median (range)	5.6 (3.9–52.7)
PFS (months)	median (95% CI)	4.1 (2.2–12.5)
at 6 months		0.35 (95%CI 0.19–0.64)
at 12 months		0.25 (95%CI 0.12–0.53)
OS (months)	median (95% CI)	15.7 (10.08-NA)
at 6 months		0.75 (95%CI 0.58–0.97)
at 12 months		0.59 (95%CI 0.41–0.86)
at 24 months		0.42 (95%CI 0.25–0.72)

Tumor Immune Correlates of Clinical Response

To explore tumor-based correlates of clinical response, we evaluated tumor PD-L1 expression and T-cell infiltration across all available serial tumor biopsies (Figs. 3, **Table S1A**). Longitudinal assessment of PD-L1 protein expression by the MPS assay at baseline, C1, and C2, showed increased PD-L1 levels at C1 with a further rise at C2 in several patients with PD and PR (Fig. 3A, **B**). Four of 5 PR patients were PD-L1 MPS-negative at baseline (MPS < 1); 1 patient had a baseline MPS score of 2 (**Table S3**).

Figure 3. Longitudinal dynamics of the tumor PD-1/PD-L1 axis at baseline (C0), cycle 1 (C1), and cycle 2 (C2). (A) Representative PD-L1 immunohistochemistry (IHC) images from formalin-fixed, paraffin-embedded tumor sections of HR+/HER2- metastatic breast cancer with low (MPS < 1) and high (MPS > 20) PD-L1. Boxed regions are shown at higher magnification. (B) Tumor PD-L1 MPS at baseline, cycle 1, and cycle 2. Each point represents an individual tumor sample, with lines connecting longitudinal samples from the same patient.

(C) Intratumoral gene expression scores for PDCD1 (PD-1), CD274 (PD-L1), and PDCD1LG2 (PD-L2), quantified using the NanoString platform at C0, C1, and C2. Each point represents an individual tumor sample, with lines indicating matched longitudinal samples when available. Colors denote best overall response (BOR): partial response (PR, blue), stable disease (SD, yellow), and progressive disease (PD, orange). Sample numbers were as follows: **C0**: PR, $n = 4$; SD, $n = 3$; PD, $n = 5$; **C1**: PR, $n = 3$; SD, $n = 2$; PD, $n = 3$; **C2**: PR, $n = 3$; SD, $n = 3$; PD, $n = 3$.

Legend: CR=complete response, PR=partial response, SD=stable disease, PD=progressive disease, ORR=overall response rate, CBR=clinical benefit rate, DoR=duration of response, PFS= progression free survival, OS= overall survival

NanoString expression profiling showed a similar pattern at the transcript level. Longitudinal analysis demonstrated a trend toward increased expression of both CD274 (PD-L1) and PDCD1LG2 (PD-L2) from baseline (C0) ($n = 13$) to on-treatment biopsies (C2) ($n = 8$), which may reflect PD-1-associated immune activation (Fig. 3C). Induction of PD-1-axis transcripts was observed in tumors from PR ($n = 3$) and SD ($n = 3$) patients, while tumors from patients with PD ($n = 5$) showed comparatively lower on-treatment expression of PD-L1 and PD-L2. Overall, on-treatment PD-1 axis induction may be consistent with adaptive immune mechanisms rather than a clear association with clinical benefit in this MBC cohort.

Table 3
Patients with Maximum Grade ≥ 3 Treatment-Related Adverse Events, Including Immune-Related AEs, Possibly/Probably/Definitely Attributed to nab-Paclitaxel and/or Pembrolizumab (Maximum CTCAE Grade)

Adverse Event	Grade 3	Grade 4	Immune-Related Adverse Events
Abnormal Liver Function Test	1	1	1
Allergic Reaction	1	0	0
Fatigue	2	0	0
Febrile Neutropenia	2	0	0
Gait Disturbance	1	0	0
Nausea/Vomiting	3	0	0
Diarrhea	2	0	2
Hematologic	4	1	0
Hyponatremia	3	1	1
Hypothyroidism	1	0	1
Infection	1	0	0
Mucositis	1	0	1
Pain	1	0	0
Pneumonitis	0	1	1
Vaginal Dryness	1	0	0

TILs were assessed by pathologic review (Fig. 4A) and T-Cell fraction was assessed by ImmunoSeq TCR sequencing (Fig. 4B). TCR analysis showed an increase in T-Cell fractions in baseline-to-on-treatment biopsies, especially in C2, across PR and SD patients (Fig. 4B). In contrast, little change was observed in tumors from patients with PD. Baseline tumor T-cell fractions overlapped across response groups. When dichotomized at the cohort median (0.119), was no association between elevated T-cell fractions and PFS (log-rank $p = 0.82$), however a significant association between T-cell fraction and improved OS (log-rank $p = 0.0087$) was observed (Fig. 4C-D). Together, these data suggest that while baseline immune infiltration alone is not predictive of response, higher baseline tumor T-cell fraction and on-treatment increases in T-cell fraction may reflect a biologically relevant immune contexture linked to longer-term clinical outcomes.

TCR analysis was performed on matched blood and tumor samples collected longitudinally (**Figure S1**). Peripheral blood TCR repertoire metrics, including Simpson clonality, remained largely stable over time, with no consistent differences across response groups (**Figure S1A, S1B**). This suggests that the treatment regimen used in the trial had neither immunosuppressive nor immunostimulatory effect on

systemic T cell immunity. In tumor samples, diversity metrics also showed no consistent longitudinal patterns, and interpretation was limited by low T-cell abundance in several specimens (**Figure S1C, S1D**).

Somatic mutations were analyzed in 9 patients, and TMB data were available for 8 (median, 5 mutations/Mb; range, < 1–24) (**Table S2**). Given the limited number of samples, these findings are descriptive. Two patients achieving PR demonstrated elevated TMB values (9 and 24 mutations/Mb) with one PR patient who did not have elevated TMB value (2 mutations/mb) compared to patients with SD (n = 3) (0, 2.0, and 6.1 mutations/Mb), and PD (n = 2) (< 1 and 4 mutations/Mb). Three of 9 tumors harbored PIK3CA mutations, including two in the PD group and one in the PR group. The latter patient also had multiple additional genomic alterations, including NOTCH1, and a TMB of 24.

TME and Transcriptional Programs Associated with Treatment Response

Figure 5. Spatial analysis of TME using quantitative multiplex immunofluorescence. (A) Representative multiplex images (left) from (a) Immune Panel, (b) H&E image, (c) nuclei segmentation, (d) tissue segmentation showing tumor bed (red) and stroma compartments (e) cell phenotyping and (f) digital image. (B) Log₂ fold changes in cell type abundance between post-Cycle 2 (C2) and baseline (C0) (total n = 12: PR, n = 4, SD, n = 2, PD, n = 6). Changes are calculated based on the percentage of each cell type relative to the total cells in each biopsy. Abbreviations: TAM, tumor-associated macrophage; aiTAM, activated inflammatory tumor-associated macrophage; piTAM, pro-inflammatory tumor-associated macrophage; CTL, cytotoxic T lymphocyte; Th, T-helper cell; Treg, regulatory T cell.

Treatment-associated TME remodeling was assessed using spatial qMIF analysis of serial tumor biopsies. The TME profiling workflow is shown in **Fig. 5A**. Within this limited cohort (n = 12; PR = 4, SD = 2, PD = 6), tumors from patients with PR showed increases in T-cell populations, including FOXP3⁺ Tregs, together with a decrease in myeloid subsets (Fig. 5B), suggesting a relative shift towards a more adaptive immune microenvironment. In SD subgroup (n = 2), both T-cell and myeloid populations increased, with the exception of cytotoxic T lymphocytes (CTLs) (CD3⁺CD8⁺), which decreased. In the PD subgroup, remodeling appeared heterogeneous, with an increase in CD68⁺ and pro-inflammatory or piTAMs (CD33⁺CD68⁺HLA-DR⁺) together with CTLs and Thelper (Th, CD3⁺CD8⁺FOXP3⁺) cells, while other immune populations declined (Fig. 5B). Representative longitudinal qMIF images from each PR and PD case showing different phenotypes presented in **Figure S3**. Overall, these findings may reflect differential TME immune remodeling during therapy across response groups, but they should be interpreted cautiously given the small sample size and subgroup heterogeneity.

Baseline transcriptional profiling identified distinct gene expression patterns between tumors from patients with PD and those with PR + SD (**Figure S2A**). Tumors from patients with PD showed higher expression of genes including PARP1, SFRP1, SFRP4, E2F5, BIRC5, MYCN, and FOXC1, with the broader ranked gene list suggesting greater representation of proliferation/cell-cycle, DNA repair, and wingless-related integration site (WNT)-associated features consistent with an intrinsic phenotype associated with poor prognosis across breast cancer subtypes [31, 32] (**Figure S2B**). Baseline PD tumors also

exhibited elevated expression of the apoptosis inhibitor BIRC5 (survivin) (**Figure S2A**), supporting enhanced tumor cell survival signaling [33, 34]. In contrast, tumors from patients with PR or SD showed higher expression of INHBB, SMAD5, TGFB2, DLL1, LFNG, JAK2, and ALDH1A1, with additional transcripts in the larger dataset consistent with enhanced cytokine/growth factors, immune-related, vascular/stromal, and transforming growth factor beta (TGF- β) signaling, in line with prior reports in breast cancer [35–37] (**Figure S2A**). Expression of DLL1 and LFNG further suggested involvement of neurogenic locus notch homolog (NOTCH)-related signaling pathways.

Overall, integration of spatial qMIF and targeted transcriptional profiling suggests that intrinsic tumor biology may influence subsequent immune remodeling during therapy. These observations are broadly consistent with the TIL and TCR analyses, in which tumors from patients with PR or SD showed greater on-treatment increases in tumor-associated T-cell content, whereas tumors from patients with PD showed more limited immune enrichment.

Discussion

In this prospective phase II study, nab-paclitaxel plus pembrolizumab demonstrated an acceptable safety profile but only modest clinical activity in patients with HR+/HER2- MBC. Toxicities were consistent with the known safety profiles of the individual agents, and no unexpected toxicities (grade 5 events) have occurred. Although the regimen was feasible in this pretreated metastatic population, the antitumor activity observed in our cohort was limited overall and comparable to outcomes reported in prior studies of chemotherapy with or without immune checkpoint blockade in HR+/HER2- MBC [5, 7, 8, 9, 11].

Clinical outcomes in this study were modest, with 26.3% of patients achieving a PR and 36.8% experiencing SD. Median PFS and OS were 4.1 months and 15.7 months, respectively. These outcomes are comparable to those reported in other trials evaluating chemotherapy with or without ICIs in this disease context [5, 11], including the eribulin $\pm$ pembrolizumab study, which similarly failed to demonstrate a clear survival advantage with the addition of immunotherapy, with mPFS of 4.1 months vs. 4.2 months [11]. Given the single-arm design, we cannot distinguish the relative contributions of nab-paclitaxel, pembrolizumab, and patient selection to the observed outcomes.

Longitudinal IHC and transcriptomic analyses suggested increased PD-L1 protein expression and a trend toward increased *CD274* (PD-L1)/*PDCD1LG2* (PD-L2) transcripts during treatment, consistent with therapy-associated immune activation and the presence of a substantial fraction of activated myeloid cells, typically expressing elevated PD-L1 and PD-L2. Notably, induction of PD-1 axis transcripts was similar across BORR categories, even though tumors from patients with PD exhibited the lowest on-treatment expression of PD-L1 and PD-L2. In HR+/HER2 – MBC, the baseline PD-L1 expression demonstrated only modest and inconsistent predictive value for PD-1/PD-L1 blockade in contrast with TNBC [4, 5, 11, 12]. This finding is important because it suggests that on-treatment immune engagement alone does not necessarily indicate therapeutic susceptibility in HR+/HER2- MBC. Rather, PD-L1

dynamics and related immune activation markers appear to require interpretation in the context of broader tumor-intrinsic and microenvironmental features.

Our data support a model in which baseline biologic context may be more important than treatment-induced immune changes alone in determining outcome. Tumors from patients achieving PR or SD showed transcriptional programs consistent with preserved cytokine and immune signaling pathways, including *JAK2*, *IL11RA*, *SOCS2*, *INHBB*, *SMAD5*, and *TGFBR2*, together with NOTCH-associated genes such as *DLL1* and *LFNG*. This suggests preservation of immune and stromal signaling networks that may support adaptive immune remodeling during therapy [38] and TGF- β -associated remodeling as previously described [39]. In contrast, tumors from patients with PD were characterized by highly proliferative, therapy-resistant transcriptional profiles, including upregulation of *E2F5*, *BIRC5*, *MYCN*, *FOXC1*, *PARP1*, and *MK167*. This indicates enhanced cell-cycle progression, apoptosis resistance, DNA damage repair, and lineage plasticity- all associated with aggressive basal-like tumor features and endocrine-independent growth [15]. Upregulation of *SFRP1* and *SFRP4* further suggested dysregulated WNT-associated signaling linked to tumor cell migration and therapeutic resistance [40, 41]. Enhanced GABRP expression supports intra-tumoral features of stemness and invasive potential described in aggressive basal-like and triple-negative subtypes [42], while increased CHRNA5 is closely linked to secondary E2 signaling and a worse prognosis in breast cancer [43]. Collectively, these findings suggest that resistance to PD-1-based therapy in HR+/HER2 – MBC may reflect not only insufficient immune activation, but also dominant tumor-intrinsic programs that limit effective antitumor responses.

Although exploratory, several observations may help refine future patient selection strategies. First, a higher proportion of responders had prior exposure to CDK4/6 inhibitors. Preclinical work has shown that CDK4/6 inhibition can influence antigen presentation, T-cell activation, and suppressive immune populations [38]. While these findings raise the possibility that prior CDK4/6 inhibition may prime tumors for subsequent chemoimmunotherapy by modulating proliferative and immune programs [44], this requires confirmation in larger prospective studies. Second, two responding patients exhibited elevated TMB values (9 and 24 mutations/Mb), consistent with the tissue-agnostic approval of pembrolizumab for tumors with TMB $\geq$ 10 mutations/Mb [17, 18]. Although TMB alone did not discriminate outcomes in this small subset, our data suggest that mutational load may be most informative when considered together with a permissive immune contexture rather than as an isolated biomarker.

These findings should be interpreted in the context of metastatic HR+/HER2 – disease, in which prior endocrine therapy, CDK4/6 inhibition, TMB, and metastatic evolution may substantially influence the intratumoral state [5, 17, 18, 45]. In this framework, treatment-associated immune activation may be necessary but not sufficient; tumors dominated by proliferative and resistant transcriptional programs may remain clinically refractory despite evidence of immune modulation. Although exploratory due to the limited sample size, these findings underscore the need for integrated biomarkers that capture both tumor-intrinsic and immune-contextual determinants of response and survival and suggest that combination strategies targeting tumor cell-cycle control and immune competence may be necessary to improve outcomes in HR+/HER2 – MBC.

We also observed that a higher tumor T-cell fraction, measured by TCR sequencing, was significantly associated with prolonged OS, whereas conventional TIL assessment by hematoxylin and eosin was not. This may reflect the low overall abundance of TILs in HR+/HER2- MBC and suggests that more granular immune profiling may be needed in this subtype. In contrast to TNBC, where PD-L1 and TILs have established predictive and prognostic relevance [6], immune biomarkers in HR+/HER2- disease may need to capture not only the presence of immune cells but also their clonality, functionality, and spatial organization.

Spatial qMIF analysis provided additional context to the bulk TIL assessment by describing response-associated patterns of TME remodeling. In patients with PR, the treatment was associated with an increase in all intratumoral T-cell populations, along with decrease in myeloid subsets, suggesting that combination therapy may promote partial alleviation of myeloid-driven immunosuppression rather than simply increasing immune cell quantity [46, 47]. This is consistent with the modest on-treatment increases in TIL density observed by conventional assessment. In contrast, tumors from SD subgroup showed concurrent increase in both T-cell and myeloid compartments, coupled with a decline in CTLs. PD subgroup exhibited a heterogeneous but potentially maladaptive remodeling pattern, characterized by expansion of CD68⁺ and pro-inflammatory CD33⁺CD68⁺HLA-DR⁺ myeloid cells, whereas T cells varied [48, 49]. Despite evidence of immune cell recruitment, this spatial configuration supports ineffective or dysregulated immune engagement and may help explain why treatment-induced increases in TIL may not translate into clinical benefit in this group [50]. Overall, these findings suggest that treatment response may be associated not only with TIL abundance, but also with the composition of the TME.

This study has several important limitations. The cohort was small, the study was single-arm, and not all translational assays were available for all patients, limiting statistical power and preventing definitive conclusions regarding predictive biomarkers or the independent contribution of pembrolizumab. In addition, the metastatic setting introduces substantial biologic complexity, including prior endocrine therapy, prior CDK4/6 inhibition, and clonal evolution across disease sites, all of which may influence both tumor-intrinsic and immune states. Accordingly, our translational findings should be interpreted as exploratory.

At the same time, the study has notable strengths. Prospective collection of baseline and serial on-treatment biopsies enabled integrated analysis of transcriptional, spatial, and immune repertoire changes within the same patients over time, a dataset that is uncommon in HR+/HER2- MBC. This longitudinal, multimodal approach provides insight beyond conventional clinical endpoints and helps generate biologically grounded hypotheses for future trials. Overall, our results support continued investigation of immunotherapy-based strategies in HR+/HER2- breast cancer, but favor biomarker-enriched approaches and rational combinations designed to address both immune competence and tumor-intrinsic resistance programs. These results do not support broad use of PD-1 blockade in unselected HR+/HER2- MBC, but they do suggest that a biologically defined subset may derive benefit and that future progress will likely depend on integrated biomarker strategies and rational combination approaches.

Conclusion

In this single-arm phase II cohort, nab-paclitaxel plus pembrolizumab showed an acceptable safety profile and modest clinical activity in HR+/HER2- MBC. Longitudinal translational analyses demonstrated treatment-associated immune modulation, including PD-1 axis engagement, but these changes alone did not distinguish clinical benefit. Instead, benefit appeared more likely in tumors with a more favorable baseline immune contexture and less dominant proliferative or resistance-associated transcriptional programs. Although exploratory, these findings suggest that PD-1 blockade may be relevant for a biologically selected subset of patients with HR+/HER2- MBC and support future biomarker-enriched studies integrating tumor-intrinsic and immune microenvironment features.

Declarations

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Competing Interests

No competing interests

Author Contributions

SA, JDG, MK contributed to the clinical study conception and design; **SA** obtained clinical trial funding and principal investigator/regulatory oversight; **NK, SA, MK, DM** contributed to the translational study design and funding; **NK, JDG, AL, AT, TH, FD, MK, MM, YN, RO, MH, CL, YP, DM, IV, MK, SA** contributed to data collection and/or analysis and interpretation; **FD** and **BB** performed biomarker analysis; **JG** and **TH** performed statistical analysis. **NK, JDG, IV, AT** have drafted the manuscript; all authors read, provided critical revisions, and approved the final manuscript.

Ethics Approval and Consent to Participate

This study's (NCT02752685) protocol was approved by The Institutional Review Board at the Grossman School of Medicine at New York University (NYUGSoM) and written informed consent was obtained from all participants prior to study entry. The study was monitored by the Data Safety Monitoring Committee of the New York University Perlmutter Cancer Center.

Data Availability

Data supporting the conclusions of this study are available from the corresponding author upon reasonable request; however, public access is restricted due to ethical considerations and patient privacy.

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Figures

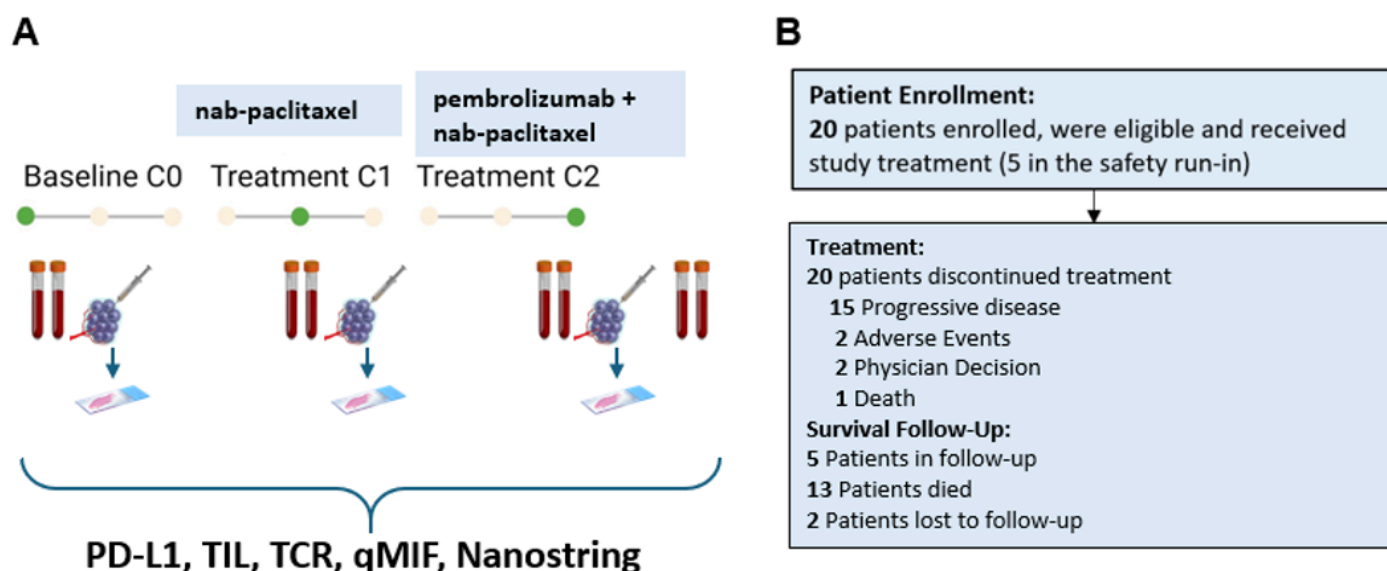


Figure 1

A. Study schema (C=cycle). **B.** CONSORT diagram. From July 2016 to May 2019, 20 eligible patients with Hormone-Receptor-Positive breast cancer were enrolled in this cohort and followed through February 2020. All enrolled patients received at least one dose of study therapy and were included in the safety analysis; 19 patients were evaluable for response.

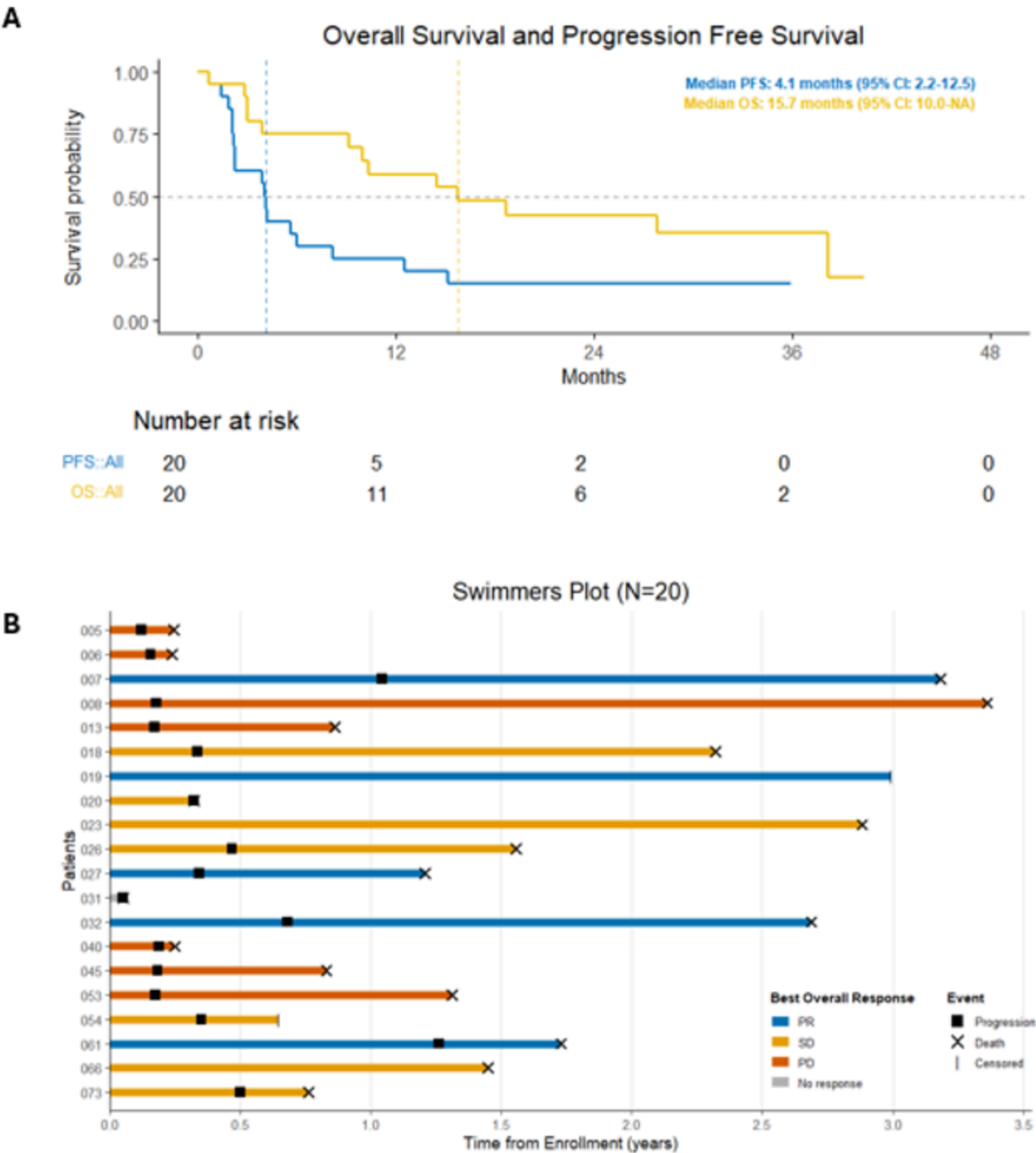


Figure 2

Patient outcomes. (A) Kaplan–Meier survival curves showing progression-free survival (PFS) and overall survival (OS). Tick marks indicate censored observations. Median survival times and corresponding 95% confidence intervals are shown. (B) Swimmer plot of individual patient outcomes. Each bar represents one patient, showing the duration of treatment and follow-up from enrollment to the last available assessment. Bar colors indicate best overall response: partial response (PR), stable disease (SD), progressive disease (PD), and not evaluable (NE). Symbols denote key clinical events: disease progression, death, and censoring. Time is shown in years from enrollment. Note that this figure represents all data collected on all patients; analysis was limited to follow up through February 2020.

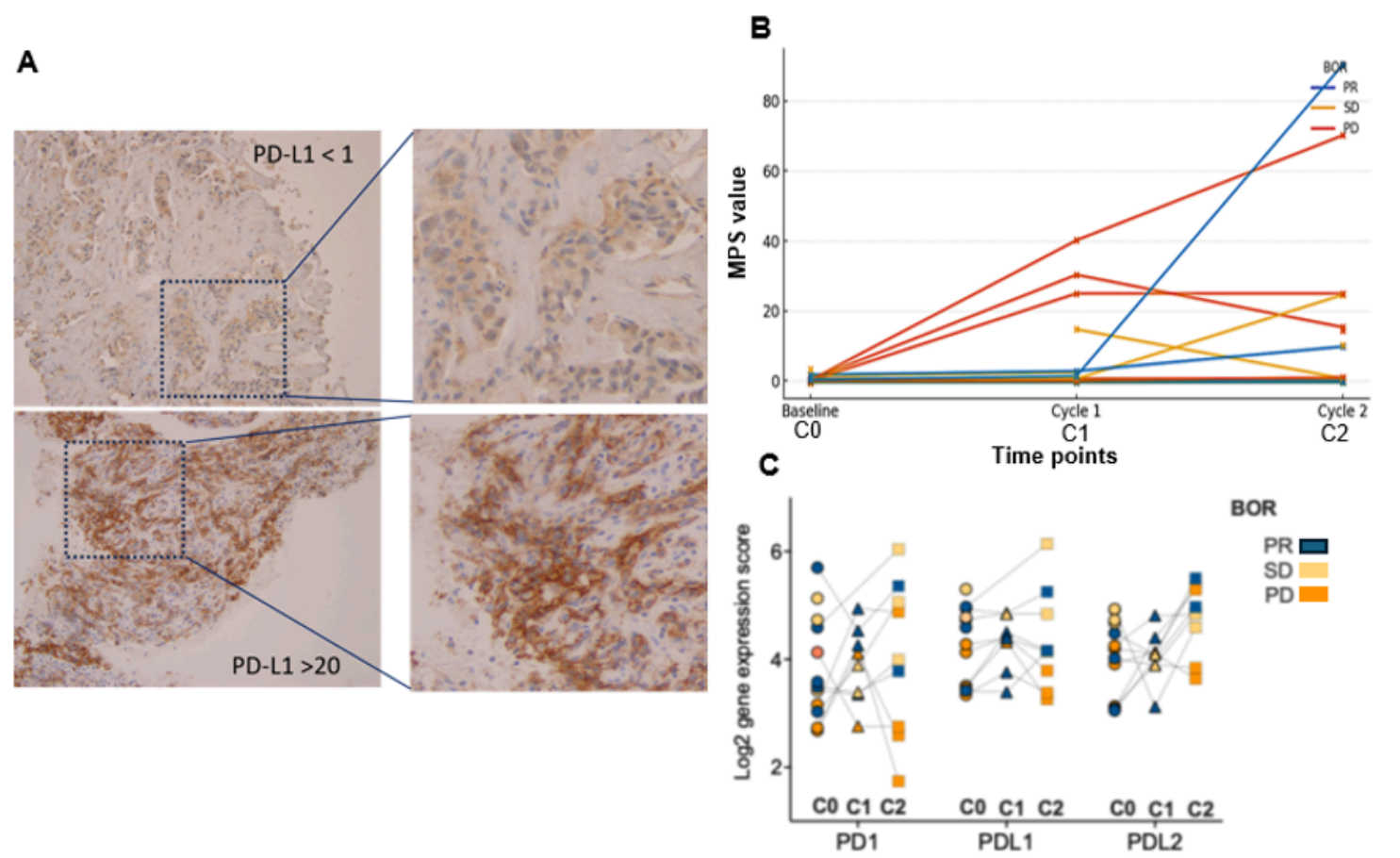


Figure 3

Longitudinal dynamics of the tumor PD-1/PD-L1 axis at baseline (C0), cycle 1 (C1), and cycle 2 (C2). (A)Representative PD-L1 immunohistochemistry (IHC) images from formalin-fixed, paraffin-embedded tumor sections of HR+/HER2- metastatic breast cancer with low (MPS <1) and high (MPS >20) PD-L1. Boxed regions are shown at higher magnification. (B) Tumor PD-L1 MPS at baseline, cycle 1, and cycle 2. Each point represents an individual tumor sample, with lines connecting longitudinal samples from the same patient. (C) Intratumoral gene expression scores for PDCD1 (PD-1), CD274 (PD-L1), and PDCD1LG2 (PD-L2), quantified using the NanoString platform at C0, C1, and C2. Each point represents an individual tumor sample, with lines indicating matched longitudinal samples when available. Colors denote best overall

response (BOR): partial response (PR, blue), stable disease (SD, yellow), and progressive disease (PD, orange). Sample numbers were as follows: **C0**: PR, $n = 4$; SD, $n = 3$; PD, $n = 5$; **C1**: PR, $n = 3$; SD, $n = 2$; PD, $n = 3$; **C2**: PR, $n = 3$; SD, $n = 3$; PD, $n = 3$.

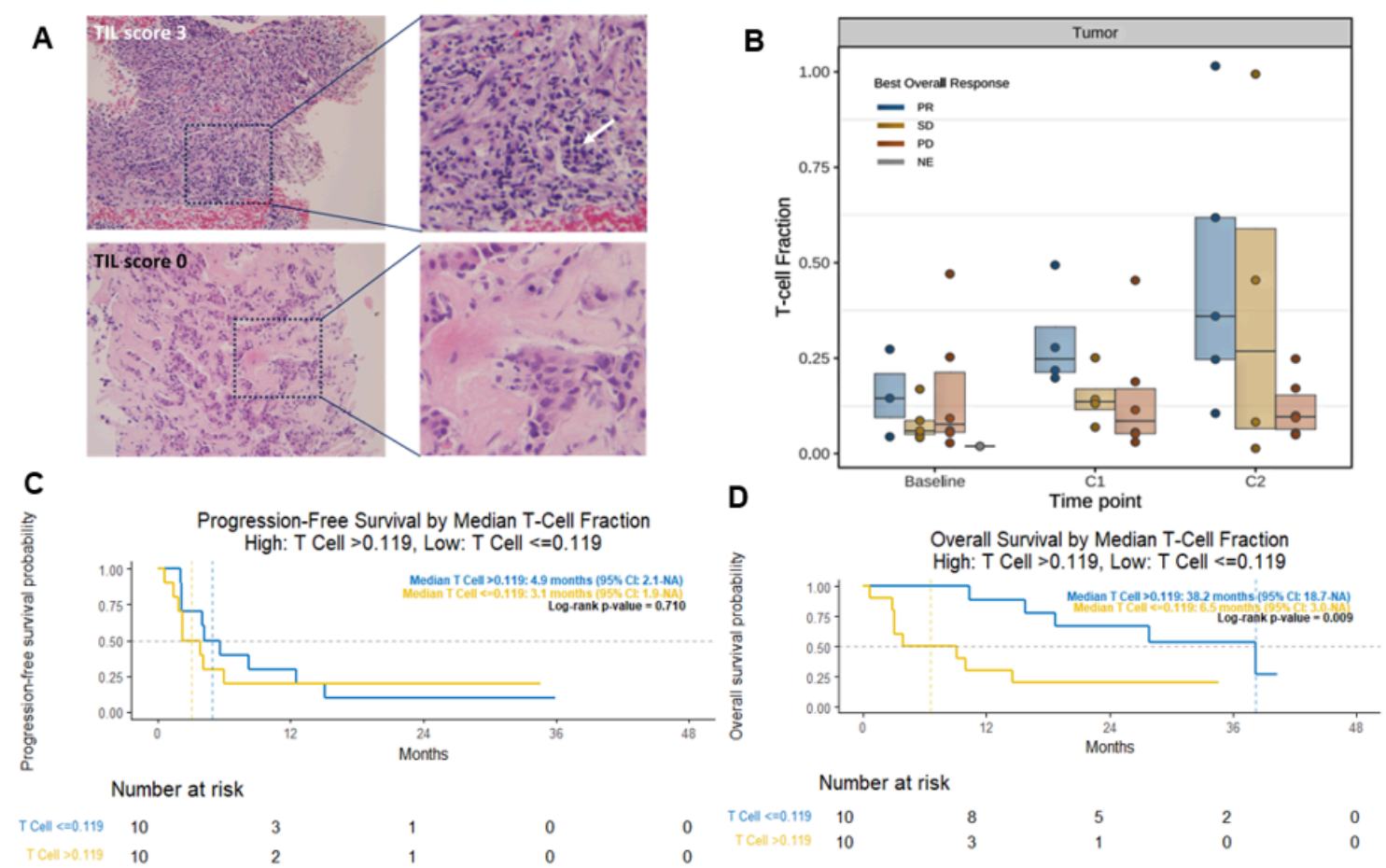


Figure 4

Assessment of T-cell fraction in baseline and on-treatment tissue samples, H&E and ImmunoSeq data. **(A)** Representative H&E images of HR+ MBC with high and low TIL scores, arrow - TILs. **(B)** ImmunoSeq data: T cell fraction was comparable by response group (sample sizes by time point = PR [3,4,5]; SD [5,4,4]; PD [6,6,6]). Increases over time were observed in patients with partial response (PR) and a subset of those with stable disease (SD). Sample sizes were limited, precluding statistical comparisons across groups. **(C)** PFS stratified by baseline tumor T-cell fraction, dichotomized at the cohort median (0.119). Patients with high T-cell fraction (>0.119) and low T-cell fraction (≤0.119) demonstrated similar PFS outcomes, with median PFS of 4.9 months (95% CI, 2.1–NA) and 3.1 months (95% CI, 1.9–NA), respectively (log-rank $p = 0.71$). **(D)** Overall survival (OS) stratified by baseline tumor T-cell fraction, dichotomized at the cohort median (0.119). Patients with high T-cell fraction (>0.119) had significantly improved OS compared to those with low T-cell fraction (≤0.119), with median OS of 38.2 months (95% CI, 18.7–NA) versus 6.5 months (95% CI, 3.0–NA), respectively (log-rank $p = 0.0009$).

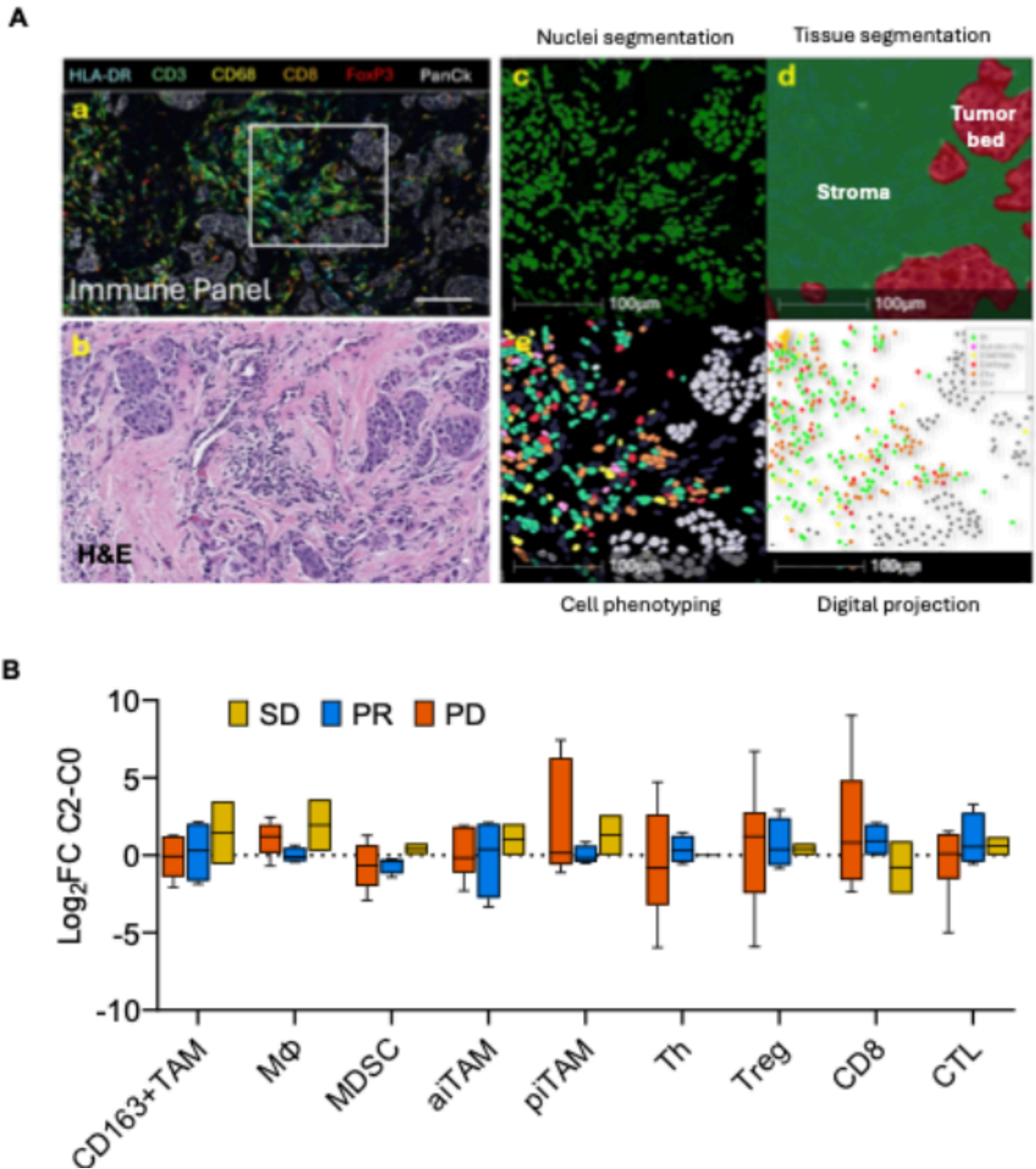


Figure 5

Spatial analysis of TME using quantitative multiplex immunofluorescence. (A) Representative multiplex images (left) from (a) Immune Panel, (b) H&E image, (c) nuclei segmentation, (d) tissue segmentation showing tumor bed (red) and stroma compartments (e) cell phenotyping and (f) digital image. (B) Log₂fold changes in cell type abundance between post-Cycle 2 (C2) and baseline (C0) (total n = 12: PR, n = 4, SD, n = 2, PD, n = 6). Changes are calculated based on the percentage of each cell type relative to the total cells in each biopsy. Abbreviations: TAM, tumor-associated macrophage; aiTAM, activated

inflammatory tumor-associated macrophage; piTAM, pro-inflammatory tumor-associated macrophage; CTL, cytotoxic T lymphocyte; Th, T-helper cell; Treg, regulatory T cell.

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