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T-cell immune checkpoint inhibition plus hypomethylation for locally advanced HER2-negative breast cancer: a phase 2 neoadjuvant window trial of decitabine and pembrolizumab followed by standard neoadjuvant chemotherapy

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ABSTRACT

Background Higher levels of tumor-infiltrating lymphocytes (TILs) in breast cancers are associated with increased likelihood of pathologic complete response (pCR) to chemotherapy. DNA methyltransferase inhibitors (DNMTi) can augment immune responses to cancers, decreasing myeloid-derived suppressor cells (MDSCs) and increasing T lymphocyte responsiveness. We have shown that the DNMTi decitabine augments the effectiveness of immunotherapy using murine triple-negative breast cancer (TNBC) models. The primary objective was to determine whether DNMTi+immune checkpoint blockade would increase stromal TIL (sTIL) in primary breast cancers before neoadjuvant chemotherapy (NCT).

Methods In a phase 2 study (NCT02957968), patients with human epidermal growth factor receptor 2-negative breast cancer received window immunotherapy—decitabine (15 mg/m^2×4 doses over 5 days) followed by 2 doses of pembrolizumab (200 mg, 2 weeks apart)—before starting NCT. Biopsies before and after window immunotherapy quantified TILs and programmed death-ligand 1 (PD-L1) expression. Patients proceeded to NCT and tumor resection per standard of care. Mid-study, results of the KEYNOTE 522 trial led to patients with TNBC receiving additional pembrolizumab concurrently with standard NCT and in the adjuvant setting.

Results 46 patients (median age 54.5 years, range 28–72; 71.7% white, 28.3% black; 100% female) were treated. 21 patients had TNBC and received neither neoadjuvant pembrolizumab concurrently with NCT nor adjuvant pembrolizumab (Cohort A), 7 patients had TNBC and did receive concurrent and/or adjuvant pembrolizumab (Cohort A2), and 18 patients were estrogen receptor positive and/or progesterone receptor positive and received neither concurrent nor adjuvant pembrolizumab (Cohort B). Blood samples collected after decitabine administration before pembrolizumab showed a 59% decrease (p<0.01) in monocytic MDSCs compared with baseline. 38 patients had paired biopsies for sTIL and 37 for PD-L1 evaluation. Cohorts A/A2 experienced an sTIL increase of 6.1% (p<0.008); Cohort B experienced an sTIL increase of 8.3% (p=0.006). PD-L1 expression increased by 73.9% (p<0.01). 14 of 43 patients (32.6%)

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Evidence of immune responsiveness in breast cancer tumor microenvironments (TME) predict for better prognosis and higher likelihood of response to neoadjuvant chemotherapy (NCT), but little had been done to modify the TME prior to initiation of NCT. Moreover, myeloid-derived suppressor cells (MDSC), which can be depleted by treatment with DNA methyltransferase inhibitors (DNMTi), are known to suppress immune responses in murine cancer models and are associated with poor prognosis in women with breast cancer. Finally, gene methylation in T lymphocytes contributes to T cell exhaustion and resistance to immune checkpoint blockade (ICB).

WHAT THIS STUDY ADDS

⇒ This study demonstrates that treatment with DNMTi depleted systemic MDSC and, combined with ICB, significantly increased infiltration with stromal lymphocytes and expression of programmed death-ligand 1 in primary breast cancers.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This suggests that the addition of DNMTi to ICB is a safe and potentially useful strategy for breast cancer treatment.

who proceeded to resection achieved pCR (n=11 of 27 (40.1%) in Cohorts A/A2 and n=3 of 16 (18.8%) in Cohort B). The most frequently reported immune-related adverse events were adrenal insufficiency (AI) (n=6, 13.0%), maculopapular rash (n=3, 6.5%), and hypothyroidism (n=3, 6.5%). Five of the six AI instances were at least partially attributable to hypophysitis/pituitary dysfunction, and one remains uncertain.

Conclusions Treatment in the pre-neoadjuvant window with decitabine and pembrolizumab could sensitize breast cancers to standard NCT by recruitment of TILs to the tumor tissue. The treatment was well-tolerated.

Trial registration number NCT02957968.

INTRODUCTION

Although originally reserved for inoperable locally advanced breast cancer (LABC), primary systemic therapy, particularly neoadjuvant chemotherapy (NCT) has increasingly been used to treat patients with earlier stages of breast cancer, especially triple-negative breast cancer (TNBC) and human epidermal growth factor receptor 2 (HER2)-positive breast cancer.[1–5] Advantages to this approach include the potential for de-escalation of local and regional therapies, with increased chances for breast conservation and less need for radical axillary node surgery. Perhaps the most important benefit is guiding postsurgical adjuvant therapies based on pathologic response.[6 7] Until recently, single-modality immune therapies for breast cancer have had limited success, with the prominent exception of monoclonal antibodies targeting HER2-positive tumors; however, successful immune therapies for HER2-negative breast cancers have remained elusive.[8]

For over four decades, evidence has supported the significance of host immune responses to breast cancer.[9] Early studies revealed that lymphocyte infiltration in breast cancers indicated a better prognosis.[10 11] Modern techniques, like immunohistochemistry (IHC), further supported these findings, showing that CD8+T cell infiltration or tumor-infiltrating lymphocytes (TILs) in breast cancers are associated with increased likelihood of pathologic complete response (pCR) to chemotherapy and improved outcomes.[10–16] Gene signatures related to immune responses have been found to pinpoint patients less likely to face recurrences.[17] Lymphocytic infiltration and its corresponding gene expression profiles are also linked to positive responses to NCT, especially when platinum compounds are included for TNBC.[12 18 19] Recently, immune checkpoint blockade (ICB) treatments for breast cancer have shown promising results when combined with chemotherapy in metastatic, neoadjuvant or, less well established, prechemotherapy "window" periods.[2 20–25]

However, not all patients with breast cancer benefit from ICB therapy due to barriers including lack of non-self-antigens, immunoediting, low expression of major histocompatibility complex (MHC) antigens required for T cell recognition, induction/infiltration of myeloid-derived suppressor cells (MDSCs), secretion of immunosuppressive cytokines, vascular endothelial growth factor, induction/infiltration of suppressive Treg cells, tumor-associated suppressive macrophages, and stimulation of immune checkpoint molecules on T cells.[12 17–19 26–33] Research from our laboratory and others has demonstrated that DNA methyltransferase inhibitors (DNMTi) exert an array of effects that may increase the likelihood of responses to immune therapies by upregulating tumor antigen and MHC expression, decreasing numbers and activity of MDSC, and increasing responsiveness of T lymphocytes.[34–40] Moreover, inhibition or knockout of DNMT in T lymphocytes can increase their persistence and efficacy in vivo and increase the efficacy of ICB.[41]

In this trial, we recruited patients with locally advanced HER2-negative breast cancer to explore the efficacy of using decitabine, a DNMTi, followed by pembrolizumab, an anti-programmed cell death protein-1 monoclonal antibody, in the pre-neoadjuvant "window" period to increase lymphocyte infiltration into tumor and stroma, deplete MDSC, increase antigen presentation to T cells, and potentially sensitize breast cancers to subsequent standard NCT.

METHODS

Setting and trial design

This study was a non-randomized, open-label, multicenter, phase 2 study of a short course of immunotherapy consisting of sequential decitabine followed by pembrolizumab administered prior to a standard NCT regimen (online supplemental table 1) for patients with locally advanced HER2-negative breast cancer. The full protocol document can be found in online supplemental file 1. The primary objective of the study was to determine whether initial administration of DNMTi+ICB would increase stromal TIL (sTIL) in primary breast cancers prior to NCT, as measured by the increase of tumor and stroma with infiltrating lymphocytes from baseline pretreatment biopsy to postimmunotherapy biopsy. The long-term objective, given the strong relationship between sTIL and pCR with NCT, was that increasing TIL prior to NCT might result in an increase in the likelihood of pCR. Programmed death-ligand 1 (PD-L1) expression can also predict pCR[42–44]; therefore, change in PD-L1 expression was examined as a secondary endpoint.

A safety lead-in phase was conducted in the first 11 patients enrolled during immunotherapy and receipt of dose-dense doxorubicin and cyclophosphamide (AC), to determine adequate safety based on assessment of immune-related adverse events (irAEs). Initially, only six patients were planned for the safety lead-in phase, receiving decitabine at 20 mg/m^2 for 5 days; however, one of the first five patients treated during the safety lead-in phase developed Guillain-Barré syndrome. Also, of the first five patients, two patients developed grade 3 neutropenia, and one patient developed grade 4 neutropenia, resulting in a delayed start to NCT. Based on the observed neutropenia, we reduced the total dose of decitabine to 15 mg/m^2 delivered on 4 days out of 5 (further explained under treatment plan). In view of these toxicities (and

subsequent dose reduction), we extended the safety lead-in by 5 additional patients to a total of 11 patients.

After enrollment, patients were stratified into two cohorts based on their hormone receptor (HR) status (Cohort A: estrogen receptor (ER) and progesterone receptor (PgR) negative; Cohort B: ER and/or PgR positive). After the report of the KEYNOTE-522 trial, which demonstrated a significant increase in pCR for patients with TNBC receiving concurrent pembrolizumab with NCT,[22] some patients with TNBC were allowed to receive pembrolizumab concurrently with their NCT and in the adjuvant setting according to treating physician and patient choice. Those patients were classified as Cohort A2. Cohorts A and A2 were evaluated collectively for the primary endpoint, since their treatments were the same at that time point, but were evaluated separately otherwise.

All cohorts were to receive the same window immunotherapy treatment schedules of decitabine and pembrolizumab, and all window immunotherapy was followed by a NCT regimen that was standard for treatment of primary breast cancer at that time (online supplemental table 1).

Study population

Recruitment occurred from March, 2017 to April, 2022. The last follow-up occurred in February, 2024. Participants had a confirmed diagnosis of invasive adenocarcinoma of the breast, as determined by a core needle biopsy. Only those with HER2-negative tumors were included, which was determined as per current American Society of Clinical Oncology/College of American Pathologists HER2 Guidelines, which includes results of IHC 0 or 1+ if IHC was performed, or a HER2/CEP17 ratio of <2.0 with an average HER2 copy number <4.0 signals/cell if fluorescence in situ hybridization or another in situ hybridization test was done.[45] Additionally, breast cancers were subgrouped based on HR-positive (≥10% staining by IHC for either ER or PgR) or HR-negative (<10% staining by IHC for both ER and PgR).

LABC was defined according to The American Joint Committee on Cancer's (AJCC) Staging Criteria; eligible tumors included T2 tumors with clinically positive regional lymph nodes (cN1 or cN2), HR-negative tumors sized 3–5 cm with clinically negative regional lymph nodes (cN0), any T3 tumors, and any T4 tumors, including inflammatory breast cancer. Tumor measurements and eligibility were determined by breast ultrasound, or MRI, but not mammography. Additionally, ipsilateral axillary lymph nodes were evaluated by MRI or ultrasound within 12 weeks prior to study registration. Nodal status was ascertained by imaging and fine needle aspiration or core needle biopsy—when warranted—and classified into positive or negative prior to study treatment and qualification. Breast imaging was completed within 12 weeks (ipsilateral) and 24 weeks (contralateral) prior to study registration.

Selected patients were ≥18 years and had an Eastern Cooperative Oncology Group performance status of 0–1. Their bone marrow function was adequate, as evident by an absolute neutrophil count $\geq 0.0015\times10^9$/L, platelet count $\geq 0.1\times10^9$/L, and hemoglobin ≥100.0 g/L at screening. Renal function was suitable, with serum creatinine levels ≤the lab's upper limit of normal or a creatinine clearance ≥60 mL/min. Hepatic function criteria included total bilirubin, aspartate transaminase, alanine transaminase, and alkaline phosphatase within specified limits. Appropriate cardiac function with a left ventricular ejection fraction (LVEF) of ≥50% was required, as assessed within 12 weeks before screening. Women who were not postmenopausal or had not undergone hysterectomy had a negative pregnancy test within 72 hours before treatment. All participants agreed to use appropriate contraception methods and signed the study consent form.

Participants were excluded if they had any previous breast cancer treatment, received a live vaccine within the last 30 days, or were treated with a monoclonal antibody or investigational agent within the past 4 weeks. Those with extensive metastatic disease, a history of previous ipsilateral invasive or in situ breast carcinoma were ineligible. However, patients with limited or oligometastatic disease who were considered suitable for possible locoregional surgery were considered eligible. Previous recipients of a solid organ or allogeneic stem cell transplant, or those who have had specific therapies for other cancers were excluded. Participants must not have had severe cardiac disease, significant nervous system disorders, or have used systemic steroids or immunosuppressive therapies within the last 7 days prior to joining the trial. A history of treatment with certain immunomodulatory agents, known allergies to study drugs, immunodeficiency diagnosis, active autoimmune disease, interstitial lung diseases, active tuberculosis, active hepatitis B or C, or pregnancy/breastfeeding were all excluded. Any diagnosis or treatment for another malignancy within the last 5 years, except complete resection of basal cell carcinoma or squamous cell carcinoma of the skin, any in situ malignancy, and low-risk prostate cancer after curative therapy, resulted in exclusion, as did any condition the investigator deemed risky or potentially affecting study adherence.

Treatment plan

The study treatment regimen was initiated with window immunotherapy for all participating patients. The first five patients received decitabine at 20 mg/m^2 as an intravenous infusion over 60 min daily for 5 days, but after three instances of neutropenia causing delays in NCT initiation, subsequent patients received decitabine at a dose of 15 mg/m^2 on 4 days out of 5 (ie, a total of four doses, with no more than 5 days elapsing from the first dose to the fourth). Patients were treated with two doses of pembrolizumab at 200 mg intravenous infusion over 30 min on Day 8 and Day 22.

NCT was started approximately 1 week after the second dose of pembrolizumab. Each cohort received a different regimen. Cohort A (TNBC) participants received doxorubicin at a dose of 60 mg/m^2 intravenous push over 15 min every 2 or 3 weeks, combined with cyclophosphamide

at 600 mg/m^2 intravenous infusion over 30–60 min (AC). If dose-dense AC was used, patients also received granulocyte-colony stimulating factor support. This treatment was administered for a total of four cycles. Subsequently, patients were transitioned to either paclitaxel at 80 mg/m^2 or nab-paclitaxel at 125 mg/m^2. These drugs were given as intravenous infusions over 60 and 30 min, respectively, once weekly for 12 doses. Carboplatin was administered concurrently with the taxane, either at area under the curve (AUC) 1.5 weekly or AUC 5 every 3 weeks for 12 weeks. After the 11 safety lead-in subjects, treating physicians could choose to reverse the sequence of chemotherapy regimens, giving the taxane-based regimen followed by AC.

Cohort A2 participants received pembrolizumab at 200 mg as an intravenous infusion over 30 min every 3 weeks throughout their NCT. They also received adjuvant pembrolizumab at 200 mg intravenous infusion every 3 weeks for up to eight additional cycles postsurgery.

Cohort B participants with HR+ cancers were to receive doxorubicin (60 mg/m^2 intravenous push over 15 min every 2 or 3 weeks) and cyclophosphamide (600 mg/m^2 intravenous infusion over 30–60 min) for a total of four cycles. This was followed by either paclitaxel at 80 mg/m^2 or nab-paclitaxel at 125 mg/m^2, administered as intravenous infusions over 60 and 30 min, respectively, once weekly for 12 doses.

Breast surgery was recommended for eligible patients. Three patients developed progressive disease and therefore did not have surgery, but all other patients underwent breast-conserving surgery or mastectomy, with or without reconstruction. This surgery was accompanied by axillary staging after recovery from the last cycle of NCT. Patients also underwent adjuvant radiotherapy per standard of care.

Evaluation of primary and secondary endpoints

The primary endpoint was to measure the shift in the percentage of tumor and stroma with infiltrating lymphocytes (intratumoral TIL (iTIL) and sTIL). Tumor samples were collected via core needle biopsy at baseline and after the completion of study treatment (decitabine and two doses of pembrolizumab), prior to the start of NCT. TILs were assessed by VCU/Massey pathologists, per the International TIL Working Group guidelines described by Salgado *et al*.[46] Additionally, the shift in the proportion of patients meeting criteria for lymphocyte-predominant breast cancer (LPBC) after treatment with decitabine and pembrolizumab was examined.

PD-L1 expression was evaluated in the same pre-window and post-window treatment tumor biopsy specimens by two methods. One used a proprietary H-score performed by Discovery Life Sciences (formerly known as QualTek) using the Merck 22C3 antibody on their in-house platform. Scoring was performed by a board-certified pathologist using the H-score system as described in Dolled-Filhart *et al*, which calculates a score based on intensity of tumor staining and the percentage of cells stained.[47] PD-L1 was also assessed by the combined positive score (CPS) performed by VCU/Massey pathologists, also using 22C3 antibody.[48]

Variations in MDSC levels in blood samples were determined before decitabine, after decitabine and after the second window dose of pembrolizumab. After collecting whole blood, PBMC were isolated using a density gradient solution. PBMCs were used for flow cytometry analysis of MDSC. After blocking Fc receptors with Human TruStain FcX (BioLegend) cells were stained with anti-CD11b, CD33, CD14, CD15 and HLA-DR antibodies. Antibodies were obtained from BioLegend and conjugated to the following fluorescent dyes: FITC- CD33 (#303304), PE-CD11b (#301306), PE/Cy7- HLA-DR (#307616), APC-CD15 (#301908) and APC/Fire750- CD14 (#367120). Acquisition of cell counts was performed using a BD FACSCanto system. BD FACSDiva Software was used for analysis of data and using the following combinations for identification of MDSCs: total MDSC: CD11b+CD33+HLA-DR−, monocytic MDSC: CD11b+CD33+ HLA-DR−CD14+CD15−, and granulocytic MDSC: CD11b+CD33+ HLA-DR− CD14− CD15+.

Clinical outcomes

For patients who had completed their NCT course (but had not yet undergone surgery), a clinical examination was conducted to document the presence or absence of clinical complete response (cCR). This determination of cCR was based on the resolution of all palpable disease identified at baseline and the absence of new lesions or other indicators of disease progression. The study also looked at the proportion of patients alive without disease progression or relapse a year postsurgery.

Following surgery, the local pathologist determined the pCR by examining the excised tissue (both breast and nodes). The definition of pCR in both the breast and axillary lymph nodes was based on the absence of any invasive tumor cells in the surgical breast specimen and nodes after the neoadjuvant therapy, regardless of ductal carcinoma in situ (DCIS) presence or absence (T0/Tis N0).

AEs were captured and analyzed using the guidelines set by the National Cancer Institute Common Terminology Criteria for Adverse Events V.5.0.

Research ethics

The study followed ethical guidelines for human research. This study is registered on ClinicalTrials.gov (NCT02957968). Before joining the study, participants were informed about its purpose and potential risks. They provided written consent to confirm their understanding and voluntary participation.

Statistical analyses

In the analysis of the primary endpoint, a one-sided paired t-test or Wilcoxon signed-rank test was used to evaluate the differences between baseline and post-treatment percentages of tumor and stromal areas that exhibited infiltrating lymphocytes. The paired t-test was

implemented distinctly for Cohorts A and B. The choice of the paired t-test and the Wilcoxon signed-rank test was dictated by the assessment of Gaussian assumptions of the response variable. Under Gaussian assumptions, we used the t-test; under violations of the Gaussian assumption, we used the Wilcoxon signed-rank test. The analysis of pre-window and post-window treatment values for Cohort A included data from patients in Cohort A2.

For the secondary endpoints, serious adverse events (SAEs) and unanticipated problems (UPs) were characterized for Cohorts A and B by determining their frequencies and percentages. Both the aggregate number and the proportion of patients experiencing an SAE or UP are presented.

Using McNemar's test, a comparison was drawn between patients exhibiting LPBC pretreatment and post-treatment with decitabine and pembrolizumab. The observed frequency and percentage of patients achieving pCR were compiled, followed by the application of a one-sample binomial test. This was done to contrast the observed proportion against 0.28—the anticipated pCR rate within the studied population. All inferential tests were deemed significant at a threshold of 0.05.

Additionally, descriptive statistics were provided for both pCR in the breast and post-therapy lymph nodes and cCR rate. Peripheral blood MDSCs were presented using measures like the mean, median, SD, and range. To discern the appropriate model for the data distribution of cell counts, potential models including Gaussian, Poisson, and negative binomial were explored. Among these, the latter two were prioritized for count or rate data, especially in instances of skewed distribution. As an exploratory measure, regression analyses were conducted to identify if any baseline demographic or clinical attributes significantly influenced the cell counts.

Subset analyses were executed to account for potential variability observed in the actual therapeutic approaches administered during the standard neoadjuvant phase.

RESULTS

Patient population

A total of 46 patients (median age 54.5 years, range 28–72) were enrolled and treated (table 1, figure 1). The population was 71.7% white, 28.3% black, and 100% female. Four patients had metastatic disease at enrollment (n=2 in Cohort A, n=2 in Cohort B). Of the 46 total patients, 21 (45.7%) had TNBC and did not receive neoadjuvant pembrolizumab concurrently with NCT nor adjuvant pembrolizumab (Cohort A), 7 (15.2%) had TNBC and did receive concurrent and/or adjuvant pembrolizumab (Cohort A2), and 18 (39.1%) were ER+ and/or PR+ and received neither concurrent nor adjuvant pembrolizumab (Cohort B). There were no significant differences in baseline conditions between the cohorts.

MDSC changes following window treatments

Blood samples collected after decitabine administration, before the first pembrolizumab dose showed a 59% decrease (p=0.000002) in monocytic MDSCs compared with baseline (figure 2); changes in granulocytic MDSCs were not statistically significant. For blood samples collected after the two "window" doses of pembrolizumab, M-MDSC levels remained significantly below the baseline level (p=0.0006). Granulocytic MDSCs showed a significant increase after pembrolizumab (p=0.0273).

TIL evaluation

38 patients had paired biopsies adequate for sTIL and iTIL evaluations. As shown in figure 3A, sTIL had a mean increase from 22.8% to 29.7% (absolute increase 6.9%, p<0.001). For cohorts A/A2, sTIL increased from 27.0% to 33.0% (6.1% increase, p=0.008) and for cohort B, sTIL increased from 16.4% to 24.7% (8.3% increase, p=0.006). As shown in figure 3B, iTIL had a mean change of 2.0% (p<0.01). Cohorts A/A2 tumors showed an absolute iTIL increase of 2.2% (p=0.08); Cohort B tumors demonstrated an absolute iTIL increase of 1.7% (p=0.04). 47.4% of tumors demonstrated an increase in sTIL of ≥10% and 50% demonstrated an increase in sTIL of ≥5%. Two patients in Cohort A showed sTIL >50% after treatment. For iTIL, 15.8% of tumors increased by ≥10% and 29% increased by ≥5%. There were no LPBC tumors at baseline, and three tumors developed the characteristic feature of LPBC after window treatment (defined as >60% sTIL). We also assessed whether tumor characteristics were related to sTIL change. We saw no statistically significant differences in clinical T-stage, clinical M-stage, clinical N-stage, histology, nor tumor size in patients with a ≥10% change in sTIL compared with patients with a <10% change in sTIL (online supplemental table 2).

PD-L1 expression changes

PD-L1 expression in both tumor biopsies were scored for 39 patients for the H-score using MoAb 22C3 clone, which increased by 43% from a mean of 29.13 to 41.77 overall (p<0.0095). The increase for A/A2 was 51.1%, from 39.71 to 60.04 (p=0.0122); for cohort B, PD-L1 H-score increased from 12.20 to 12.53 (p=0.22). Using the standard CPS score (also using 22C3 antibody), assessed by our local pathologists, PD-L1 expression was assessed for 37 patients. Using this method, PD-L1 increased from a mean of 28.78 to 46.22 for Cohorts A/A2 (60.5%, p=0.0006) and from 13.07 to 29.07 for cohort B (122%, p=0.0037, figure 4). For the total of assessable tumors, the increase was from 22.84 to 39.73 (73.9%, p=0.000006).

Tumor response

As shown in table 2, 14 of the 43 patients (32.6%) who proceeded to resection achieved pCR (n=11 of 27 (40.7%) in Cohorts A/A2 and n=3 of 16 (18.8%) in Cohort B). 24 of 43 patients (55.8%) achieved cCR (n=16 of 27 (59.3%) in Cohorts A/A2 and n=8 of 16 (50%) in Cohort B). Three patients experienced disease progression during

Table 1 Demographics

MCC-15–11083 demographic table					
Characters	**Total n=46 n (%)**	**Cohort A n=21 n (%)**	**Cohort A2 n=7 n (%)**	**Cohort B n=18 n (%)**	**P value**
Age (year, median (range))	54.5 (28–72)	56 (35–72)	55 (28–65)	54 (28–70)	0.8172
Race					0.6427
Black or African American	13 (28.3)	6 (28.6)	1 (14.3)	6 (33.3)	
White	33 (71.7)	15 (71.4)	6 (85.7)	12 (66.7)	
Gender					
Female	46 (100)	21 (100)	7 (100)	18 (100)	
Menopausal status					0.9945
Premenopausal	20 (43.5)	9 (42.9)	3 (42.9)	8 (44.4)	
Postmenopausal	26 (56.5)	12 (57.1)	4 (57.1)	10 (55.6)	
Clinical T-stage					0.6913
T2	32 (69.6)	16 (76.2)	6 (85.7)	10 (55.6)	
T3	11 (23.9)	4 (19.1)	1 (14.3)	6 (33.3)	
T4	2 (4.3)	1 (4.8)	0 (0)	1 (5.6)	
T4b	1 (2.2)	0 (0)	0 (0)	1 (5.6)	
Clinical M-stage					0.6705
M0	42 (91.3)	19 (90.5)	7 (100)	16 (88.9)	
cM1	4 (8.7)	2 (9.5)	0 (0)	2 (11.1)	
Clinical N-stage					0.4247
cN0	14 (30.4)	9 (42.9)	2 (28.6)	3 (16.7)	
cN1	26 (56.5)	10 (47.6)	5 (71.4)	11 (61.1)	
cN2	2 (4.3)	0 (0)	0 (0)	2 (11.1)	
cN3	3 (6.5)	2 (9.5)	0 (0)	1 (5.6)	
cN3c	1 (2.2)	0 (0)	0 (0)	1 (5.6)	
Histology					0.2034
Invasive ductal	42 (91.3)	20 (95.2)	7 (100)	15 (83.3)	
Invasive lobular	3 (6.5)	0 (0)	0 (0)	3 (16.7)	
Other	1 (2.2)	1 (4.8)	0 (0)	0 (0)	
Tumor size (cm, mean)	5.4	6.0	4.7	5.0	0.5542

Cohort A: TNBC, no concurrent pembrolizumab nor adjuvant pembrolizumab; Cohort A2: TNBC, concurrent and/or adjuvant pembrolizumab; Cohort B: ER+ or PR+, no concurrent nor adjuvant pembrolizumab.
ER, estrogen receptor; PR, progesterone receptor; TNBC, triple-negative breast cancer.

treatment and did not have surgery. In order to find out whether the response of PD-L1 or sTIL was a predictor for pCR, several logistic regressions were conducted. We found that these independent variables were not significant predictors for the outcome pCR. We also assessed whether there was a greater clinical benefit seen in patients with an sTIL change ≥10%. While not statistically significant, we saw a trend for a higher proportion of pCR in patients with a change in sTIL ≥10% compared with those with a change in sTIL <10% (p=0.0551, table 2). Additionally, we assessed whether there was a greater clinical benefit seen in patients with a PD-L1 CPS change ≥10% compared with those with a PD-L1 CPS change of <10%. We found no statistical significance (table 2).

Safety

Overall, the study treatment was well-tolerated (table 3). The most frequently reported treatment-related AEs of grade 3 or above were neutrophil count decreased (n=8, 17.4%), white blood cell count decrease (n=3, 6.5%), and maculopapular rash (n=3, 6.5%). All grade 3 or above neutropenias were attributed to decitabine and/or pembrolizumab and occurred before NCT. irAEs were also evaluated separately from general AEs. The most frequently reported irAEs were adrenal insufficiency (AI) (total n=6 (13.0%), n=1 ≥Grade 3, n=4 unresolved), maculopapular rash (total n=3 (6.5%), n=3 ≥Grade 3, n=0 unresolved), and hypothyroidism (total n=3 (6.5%), n=0

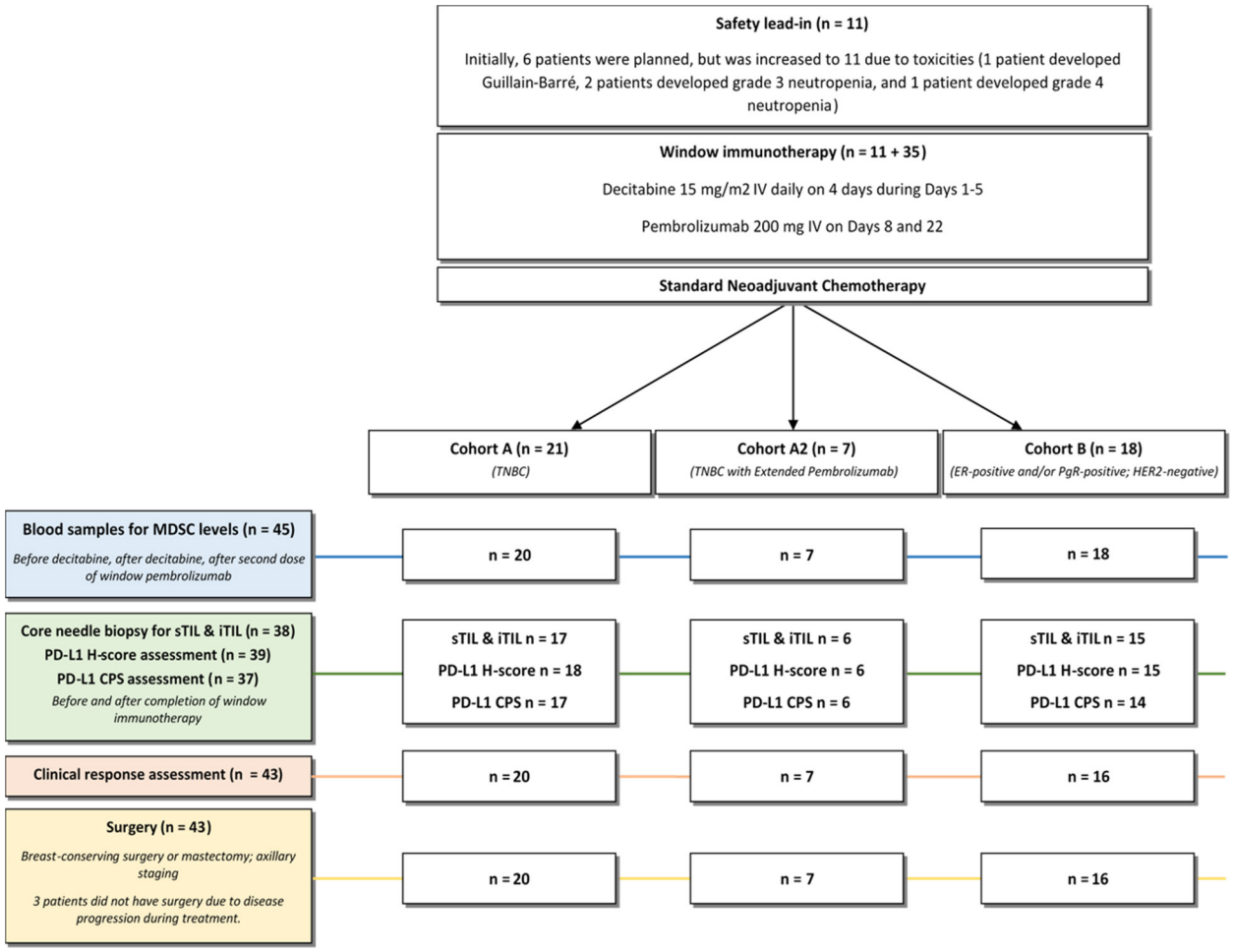

Figure 1 CONSORT diagram. CONSORT, Consolidated Standards of Reporting Trials; CPS, combined positive score; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; iTIL, intratumoral tumor-infiltrating lymphocyte; IV, intravenous; MDSC, myeloid-derived suppressor cell; PD-L1, programmed death-ligand 1; PgR, progesterone receptor; sTIL, stromal tumor-infiltrating lymphocyte; TNBC, triple-negative breast cancer.

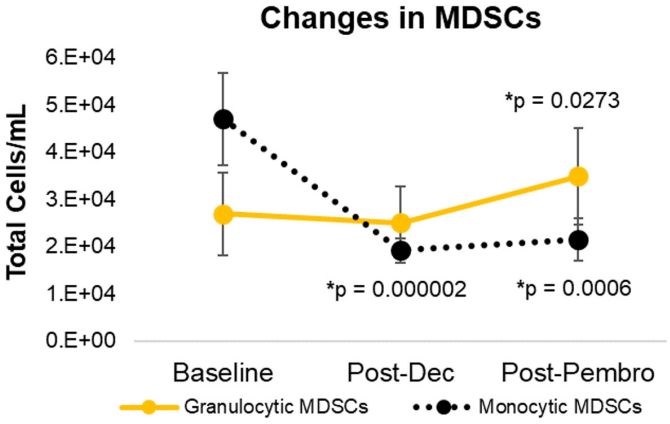

Figure 2 MDSC counts in peripheral blood at baseline, after decitabine and after two "window" doses of pembrolizumab, before initiation of NCT were determined using a BD FACSCanto system. BD FACSDiva Software was used for analysis of data following combinations for identification of MDSCs. Monocytic MDSC: CD11b+CD33+ HLA-DR– CD14+CD15 and granulocytic MDSC: CD11b+CD33+ HLA-DR– CD14– CD15+. Error bars represent SEMs. MDSC, myeloid-derived suppressor cell; NCT, neoadjuvant chemotherapy.

≥Grade 3, n=2 unresolved). Five of the six instances of AI were at least partially attributable to hypophysitis/pituitary dysfunction, and one remains uncertain (table 3). One patient in Cohort B developed Guillain-Barré syndrome while receiving standard NCT. She elected not to proceed with further chemotherapy, but her AE resolved while being treated with neoadjuvant endocrine therapy. This patient proceeded to curative-intent breast surgery. The AE profiles were not noticeably different between cohorts.

Postsurgical outcomes

The proportion of patients alive without disease progression or relapse a year after surgery was calculated for patients that did not have progression prior to surgery. Three patients were excluded from the progression-free survival (PFS) analysis as they did not have surgery because of disease progression. An additional three patients who experienced disease progression before undergoing surgery were excluded from the PFS analysis. 38 of the 40 remaining patients (95.0%) were alive without disease progression or relapse a year after surgery. The two patients with

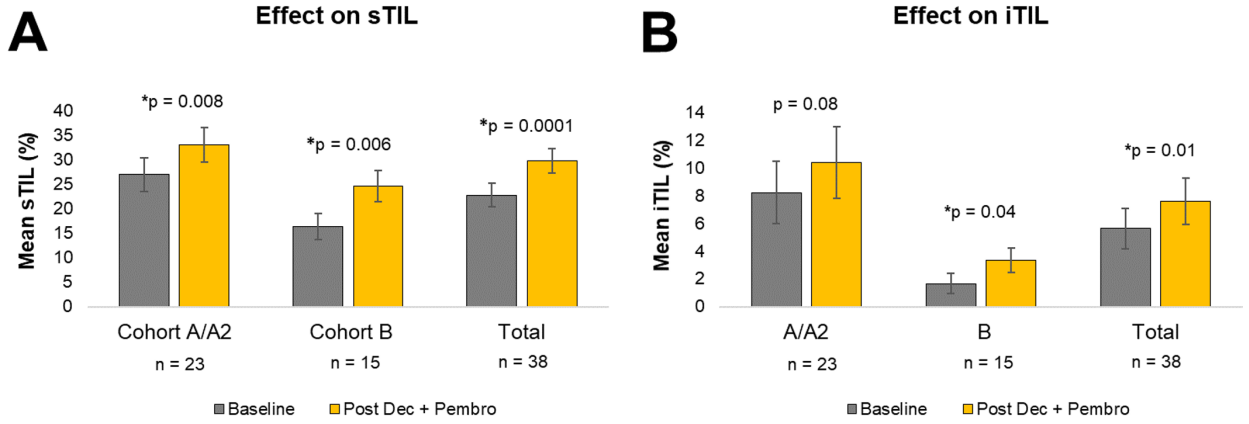

Figure 3 Effect of decitabine and pembrolizumab on stromal TIL (A) and intratumoral TIL (B) in breast tumor biopsies. For stromal TIL, the t-test was used for A/A2; the Wilcoxon signed-rank test for B and total. For intratumoral TIL, the t-test was used for B; the Wilcoxon signed-rank test for A/A2 and total. Error bars represent SEMs. Cohorts A and A2 are combined, since treatments at the time of the second biopsy were identical. iTIL, intratumoral tumor-infiltrating lymphocyte; sTIL, stromal tumor-infiltrating lymphocyte.

disease progression <1 year after surgery belonged to Cohort A.

DISCUSSION

This trial is one of only a few studies to examine the possibility that pre-chemotherapy "priming" of the immune response in the neoadjuvant setting for breast cancer might mobilize the immune system and thereby potentially increase the likelihood of a good response. And this is the only study to test the potential for DNMTi to augment the efficacy of immune therapy in the primary breast cancer setting. The GeparNuevo study included a subset of patients who received ICB prior to and concurrently with NCT, and it was this subset (limited by halting the "window" treatments midtrial) who seemed to benefit most from the addition of ICB.[25] However, other studies reported recently did not demonstrate an advantage to starting ICB treatment ahead of NCT versus concurrent ICB treatment alone.[49 50]

In this relatively small phase 2 trial, we did not observe pCR rates that were significantly higher than expected with NCT alone or with NCT+ICB. However, it should be noted that most of the patients with TNBC cancers and all of those with HR+ tumors did not receive ICB concurrently with NCT, which is now considered standard therapy for TNBC since the KEYNOTE-522 trial. Our pCR rate in the B cohort was 18.8% (table 2), which was similar to the KEYNOTE-756 rate of 24.3%, but KEYNOTE-756 gave pembrolizumab through chemotherapy.[51] It should also be noted that many of our patients had very advanced tumors, including a few with stage IV disease (which was allowed if there was intention for the patient to undergo surgery for the primary tumor).

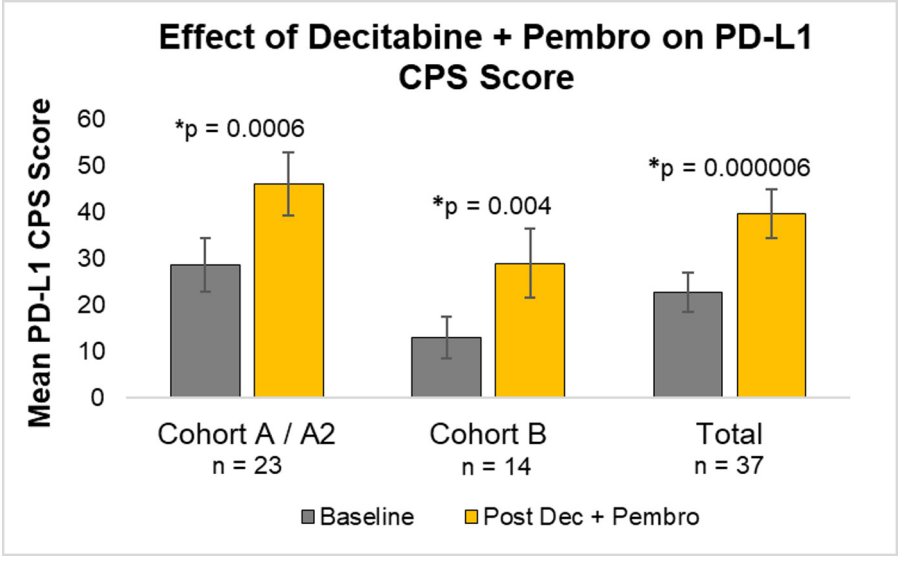

Figure 4 Effect of decitabine and pembrolizumab on PD-L1 CPS score. The t-test for A/A2; Wilcoxon signed-rank test for B and total. Error bars represent SEMs. CPS, combined positive score; PD-L1, programmed death-ligand 1.

Table 2 pCR and cCR by cohort and comparison of clinical outcome to change in sTIL and PD-L1

pCR	pCR by cohort (number of patients (%))				Comparison of pCR to change in sTIL			Comparison of pCR to change in PD-L1		
	Cohort A n=20	Cohort A2 n=7	Cohort B n=16	Total n=43	sTIL change <10% n=19	sTIL change ≥10% n=17	P value	PD-L1 CPS change<10% n=20	PD-L1 CPS change≥10% n=15	P value
No	10 (50)	6 (85.7)	13 (81.3)	29 (67.4)	17 (89.5)	10 (58.8)	0.0551	15 (75)	11 (73.3)	1
Yes	10 (50)	1 (14.3)	3 (18.8)	14 (32.6)	2 (10.5)	7 (41.2)		5 (25)	4 (26.7)	
cCR	**cCR by cohort (number of patients (%))**				**Comparison of cCR to change in sTIL**			**Comparison of cCR to change in PD-L1**		
	Cohort A n=20	Cohort A2 n=7	Cohort B n=16	Total n=43	sTIL change <10% n=19	sTIL change ≥10% n=17	P value	PD-L1 CPS change<10% n=20	PD-L1 CPS change≥10% n=15	P value
No	6 (30)	5 (71.4)	8 (50)	19 (44.2)	11 (57.9)	6 (35.3)	0.1751	9 (45)	7 (46.7)	0.922
Yes	14 (70)	2 (28.6)	8 (50)	24 (55.8)	8 (42.1)	11 (64.7)		11 (55)	8 (53.3)	

Cohort A: TNBC, no concurrent pembrolizumab nor adjuvant pembrolizumab; Cohort A2: TNBC, concurrent and/or adjuvant pembrolizumab; Cohort B: ER+ or PR+, no concurrent nor adjuvant pembrolizumab.
For comparison of clinical outcomes to change in sTIL, Fisher's exact test and χ^2 test were used due to the small sample size. 36 patients who had sTIL, pCR, and cCR data were included in this analysis (2 patients had sTIL but no pCR and cCR). For comparison of clinical outcome to PD-L1 CPS, Fisher's exact test, Cochran-Mantel-Haenszel test, and Wilcoxon test were used due to the small sample size and multiple levels. 35 patients who had PD-L1 CPS, pCR, and cCR data were included in this analysis (3 patients had PD-L1 CPS but no pCR and cCR).
cCR, clinical complete response; CPS, combined positive score; ER, estrogen receptor; pCR, pathologic complete response; PD-L1, programmed death-ligand 1; PR, progesterone receptor; sTIL, stromal tumor-infiltrating lymphocyte; TNBC, triple-negative breast cancer.

We did observe significant reductions in the level of peripheral blood M-MDSC and significant increases in sTIL. Whether these changes were substantial enough to have a potential for clinical significance is unknown, although prior studies have shown that each 10% increase in TIL can significantly impact prognosis and likelihood of pCR, and nearly half of our patients experienced ≥10% increase in sTIL after the window treatment.[10 52–54] Unfortunately, biopsied tissue was not adequate for paired TIL assessments in nearly 1/5 of the subjects.

Our lab and others have reported preclinical data showing that DNMTi, with or without HDAC inhibitors, can increase the efficacy of immune therapies in murine models of TNBC.[34–40] It has also been shown that inhibition or knockout of DNMT prevents T cell exhaustion and increases in vivo persistence of therapeutically active T cells and responsiveness to ICB in animal models.[41] Our published results with decitabine and guadecitabine (a dimer more stable in circulation than decitabine) demonstrated increased antitumor effects when combined with adoptive cellular therapies, and our unpublished data show that DNMTi also increases the efficacy of ICB, with or without low-dose cyclophosphamide. We have also found that DNMTi restored the effectiveness of ICB in a subline of the E0771 murine breast cancer that was selected for resistance to ICB (manuscript in preparation). We acknowledge that the changes we observed in the microenvironment cannot be attributed specifically to the combination of DNMTi and ICB, as either alone might have had this effect. Likewise, it is possible that an inflammatory response to needle biopsy might have had this effect. However, it should be noted that our first research biopsy was obtained after the patients had already undergone their initial diagnostic needle biopsies.

The relative contribution to the TIL and PD-L1 changes of decitabine versus the combination with ICB is uncertain here, but it is clear that decitabine significantly reduced the prevalence of M-MDSC in peripheral blood, which persisted after the two doses of pembrolizumab, approximately 3 weeks after the last decitabine dose. While sTIL was the primary endpoint, PD-L1 was also examined, since PD-L1 has also been shown to be predictive of pCR with NCT.[42–44] PD-L1 expression has been predictive of benefit of ICB in the metastatic setting, although it has not been found to correlate with the benefit of adding ICB to NCT for primary TNBC. However, the long-term goal of exploring the window treatment with DNMTi+ICB was to suggest an approach that might increase pCR to NCT. Food and Drug Administration approval of adding pembrolizumab to NCT for TNBC occurred in July, 2021, and most of the TNBC subjects (22 of 28 Cohort A/A2 patients) were enrolled in this trial prior to that approval; thus most of the patients with TNBC in this trial received NCT without ICB after the window of opportunity experimental therapy. A

Table 3 Related grade 3 or higher toxicities

Related grade 3 or higher toxicities		Cohort A n=21 n (%) E	Cohort A2 n=7 n (%) E	Cohort B n=18 n (%) E	Total n=46 n (%) E
Toxicity category	Toxicity				
Blood and lymphatic system disorders	Febrile neutropenia	0 (0.0) 0	1 (14.3) 1	1 (5.6) 1	2 (4.3) 2
Endocrine disorders	Adrenal insufficiency	0 (0.0) 0	1 (14.3) 1	0 (0.0) 0	1 (2.2) 1
Endocrine disorders	Endocrine disorders—other	0 (0.0) 0	1 (14.3) 1	0 (0.0) 0	1 (2.2) 1
Gastrointestinal disorders	Gastrointestinal disorders—other	0 (0.0) 0	1 (14.3) 1	0 (0.0) 0	1 (2.2) 1
Gastrointestinal disorders	Nausea	0 (0.0) 0	1 (14.3) 3	0 (0.0) 0	1 (2.2) 3
Gastrointestinal disorders	Vomiting	0 (0.0) 0	1 (14.3) 3	0 (0.0) 0	1 (2.2) 3
Investigations	Aspartate aminotransferase increased	0 (0.0) 0	0 (0.0) 0	1 (5.6) 1	1 (2.2) 1
Investigations	Neutrophil count decreased	6 (28.6) 6	0 (0.0) 0	2 (11.1) 2	8 (17.4) 8
Investigations	Platelet count decreased	0 (0.0) 0	2 (28.6) 2	0 (0.0) 0	2 (4.3) 2
Investigations	White blood cell count decreased	1 (4.8) 1	1 (14.3) 1	1 (5.6) 1	3 (6.5) 3
Metabolism and nutrition disorders	Hyponatremia	1 (4.8) 2	1 (14.3) 1	0 (0.0) 0	2 (4.3) 3
Musculoskeletal and connective tissue disorders	Muscle weakness lower limb	0 (0.0) 0	0 (0.0) 0	1 (5.6) 1	1 (2.2) 1
Musculoskeletal and connective tissue disorders	Pain in extremity	0 (0.0) 0	0 (0.0) 0	1 (5.6) 1	1 (2.2) 1
Nervous system disorders	Guillain-Barre syndrome	0 (0.0) 0	0 (0.0) 0	1 (5.6) 1	1 (2.2) 1
Skin and subcutaneous tissue disorders	Rash maculopapular	1 (4.8) 1	1 (14.3) 1	1 (5.6) 1	3 (6.5) 3
Vascular disorders	Hypotension	0 (0.0) 0	1 (14.3) 3	0 (0.0) 0	1 (2.2) 3
Vascular disorders	Vascular disorders—other	0 (0.0) 0	1 (14.3) 1	0 (0.0) 0	1 (2.2) 1
Vascular disorders	Vasculitis	1 (4.8) 1	0 (0.0) 0	0 (0.0) 0	1 (2.2) 1

number of studies have also suggested additional benefits of DNMTi, including sensitization to subsequent chemotherapy in resistant breast cancer cells by the depletion of DNMT proteins.[55 56] An ongoing trial is testing an orally administered DNMTi combined with ICB and chemotherapy for patients with advanced TNBC.[57]

When postsurgical outcomes were calculated for patients who underwent surgery but did not experience progression prior to surgery, we found that 2 out of 18 patients from Cohort A developed disease progression <1 year after surgery (11.1%). This finding was similar to the KEYNOTE-522 finding[22]—the percentage of patients that developed disease progression in the pembrolizumab-chemotherapy group after a median follow-up of 15.5 months was 11.8%.

It has been observed that the use of pembrolizumab in patients with breast cancer can lead to AI.[22 58] The reasons for this are not clear, and in many cases, it may be secondary to hypophysitis. Here, we found that six patients developed AI (online supplemental table 3). Two of the six patients were taking steroids for nausea/vomiting when AI developed. Five of the six instances of AI could be attributed to low adrenocorticotropic hormone (ACTH) levels, indicating pituitary dysfunction/hypophysitis and two also had evidence of primary adrenal unresponsiveness. The protocol did not specify how AI was to be evaluated. It is conceivable that the use of decitabine in this study affected the incidence of AI/hypophysitis. Future trials evaluating pembrolizumab, as well as its routine clinical use, in breast cancer should include diagnostic steps (specifically measuring ACTH levels) to distinguish primary versus secondary AI when it arises.

In the future, we plan additional transcriptomic assessments of the tumor biopsies collected from this trial to

assess messenger RNA expression for immune-related genes, including markers of T cell states and MHC Class I and II expression and levels of DNMT proteins. Future trials could assess the possible benefits of chemotherapy-free options for patients with advanced breast cancer by novel combination of DNMTi and ICB agents.

Study limitations

This study was fairly small, with a total of 46 patients, and patients with TNBC were divided into two cohorts based on whether they received pembrolizumab concurrent with chemotherapy and a separate cohort with HR+ tumors who received a different chemotherapy regimen. Unfortunately, adequate paired biopsy specimens for TIL and PD-L1 analysis were not obtained for all of the enrolled and treated patients. Moreover, because of the timing of the biopsies, it is difficult to determine whether the changes in TIL and PD-L1 in the paired biopsies that were adequate were attributable to the DNMTi treatment, the ICB treatment or the combination. In addition, the eligibility allowed for a broad spectrum of stages, even including stage IV patients who were initially considered to be candidates for surgery after NCT. This makes it difficult to compare pCR rates to larger published studies in the literature.

CONCLUSIONS

The combination of decitabine and pembrolizumab was well-tolerated once the dosage of decitabine was reduced to avoid chemotherapy delays because of neutropenia. Otherwise, AEs and irAEs were comparable to those seen with ICB alone. No unexpected or increased frequency of irAEs was observed. We saw statistically significant decreases in peripheral blood M-MDSC after decitabine, which persisted for about a month at the end of the window treatments. Most importantly, the window treatments resulted in significant increases in sTIL and iTIL as well as PD-L1 expression compared with the pretreatment tumor biopsies. Further trials combining DNMTi with ICB for patient with breast cancer, either as neoadjuvant treatment or for advanced metastatic disease can be justified on the basis of these results.

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Competing interests HDB owns stock in Pfizer, Abbvie and Viatris—all unrelated to this manuscript. HDB received payments from Merck for consulting and advisory boards in 2018-2020. PD acts as a site principal investigator for a Merck clinical trial unrelated to the present manuscript. KPM is on the advisory board for Mammotome and Kubtec, and the speakers bureau for Endomag, LLC—all unrelated to the present manuscript. All other authors declare that they have no conflicts of interest relevant to the content of this manuscript.

Patient consent for publication Not applicable.

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