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Efficacy of Pembrolizumab and Biomarker Analysis in Patients with WGS-Based Intermediate to High Tumor Mutational Load: Results from the Drug Rediscovery Protocol

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ABSTRACT

Purpose: To evaluate the efficacy of pembrolizumab across multiple cancer types harboring different levels of whole-genome sequencing–based tumor mutational load (TML; total of nonsynonymous mutations across the genome) in patients included in the Drug Rediscovery Protocol (NCT02925234).

Patients and Methods: Patients with solid, treatment-refractory, microsatellite-stable tumors were enrolled in cohort A: breast cancer cohort harboring a TML of 140 to 290, cohort B: tumor-agnostic cohort harboring a TML of 140 to 290, and cohort C: tumor-agnostic cohort harboring a TML >290. Patients received pembrolizumab 200 mg every 3 weeks. The primary endpoint was clinical benefit [CB; objective response or stable disease (SD) ≥16 weeks]. Pretreatment tumor biopsies were obtained for whole-genome sequencing and RNA sequencing.

Results: Seventy-two evaluable patients with 26 different histotypes were enrolled. The CB rate was 13% in cohort A [3/24 with partial response (PR)], 21% in cohort B (3/24 with SD; 2/24 with PR), and 42% in cohort C (4/24 with SD; 6/24 with PR). In cohort C, neoantigen burden estimates and expression of inflammation and innate immune biomarkers were significantly associated with CB. Similar associations were not identified in cohorts A and B. In cohort A, CB was significantly associated with mutations in the chromatin remodeling gene *PBRM1*, whereas in cohort B, CB was significantly associated with expression of MICA/MICB and butyrophilins. CB and clonal TML were not significantly associated.

Conclusions: Although pembrolizumab lacked activity in cohort A, cohorts B and C met the study's primary endpoint. Further research is warranted to refine the selection of patients with tumors harboring lower TMLs and may benefit from a focus on innate immunity.

See related commentary by Hsu and Yen, p. 3652

Introduction

Over the past decade, immune checkpoint blockade (ICB) has impacted anticancer treatment by demonstrating robust, durable responses (1). However, a substantial proportion of patients still do not achieve benefit, although toxicity could be severe, highlighting the urgent need to identify additional predictive biomarkers for adequate patient selection.

High tumor mutational burden (TMB) is increasingly recognized as a predictor of favorable response to ICB. The underlying assumption is that a high TMB leads to increased generation of neoantigens, subsequently enhancing the immunogenicity of tumors and thereby rendering them more sensitive to immunotherapy (2–4). Several studies have reported that high TMB is associated with higher response rates to ICB therapy across a wide variety of cancer types (5–8). For instance, the KEYNOTE-158 study, evaluating pembrolizumab, demonstrated an objective response rate of 29% in patients with tumors harboring high TMB [defined as ≥10 mutations per megabase (mut/Mb) as determined by the FoundationOne CDx assay] compared with an

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Translational Relevance

The need for biomarkers to predict immunotherapy efficacy remains urgent. Within the Drug Rediscovery Protocol (NCT02925234), we evaluated the efficacy of pembrolizumab (anti-PD1) in patients with tumors harboring an intermediate to high tumor mutational load (TML; total number of nonsynonymous mutations per genome). Patients were selected based on whole-genome sequencing–derived intermediate TML (140–290) or high TML (>290) and treated with pembrolizumab 200 mg every 3 weeks. We observed the highest clinical benefit rate (42%) in patients with tumors harboring high TML. Biomarker analysis provided insights that may improve precision immunotherapy, particularly in patients with tumors harboring lower TMLs. In these patients, only the expression of the innate immune biomarkers MICA/MICB and butyrophilins was significantly associated with clinical benefit. These findings may provide a rationale for future studies to focus on innate immune biomarkers to refine patient selection and develop novel treatment approaches.

objective response rate of 6% in the non–TMB high group (9). Based on these results, the FDA approved pembrolizumab for the treatment of patients with unresectable or metastatic TMB-high solid tumors, defined as ≥10 mut/Mb (as determined by an FDA-approved test), who progressed on prior treatment and for whom no satisfactory alternative treatment options are available (10).

Importantly, neoantigens are largely derived from nonsynonymous mutations that result in novel peptides. Synonymous mutations, in contrast, do not result in amino acid changes and are therefore less likely to be directly involved in tumor immunogenicity (4, 11, 12). Based on this assumption, we selected tumor mutational load (TML), defined as the total number of only nonsynonymous somatic mutations across the genome, rather than TMB, which generally also takes synonymous mutations into account, as a biomarker for patient selection.

The Drug Rediscovery Protocol (DRUP) is a clinical platform trial in which patients with advanced solid tumors who have exhausted all standard-of-care options are treated based on their tumor mutational profile with targeted therapies and immunotherapies outside the registered indications (13). DRUP aims to facilitate access to these drugs and to systematically collect efficacy and safety data. Furthermore, the DRUP structure creates the opportunity to explore determinants of (non)response by performing whole-genome sequencing (WGS) and RNA sequencing (RNA-seq) on fresh frozen pretreatment tumor biopsies. Details on trial design and results from other cohorts have been previously reported (13–19).

In this study, we report the results of three different TML-guided cohorts in DRUP: pembrolizumab (anti-PD1) in a metastatic breast cancer cohort harboring a TML of 140 to 290 nonsynonymous somatic mutations per genome (cohort A) and in two tumor-agnostic cohorts harboring either a TML of 140 to 290 nonsynonymous somatic mutations per genome (cohort B) or more than 290 nonsynonymous somatic mutations per genome (cohort C). Furthermore, we performed exploratory genomic and transcriptomic biomarker analyses to refine patient selection.

Patients and Methods

Study design

DRUP is an ongoing Dutch prospective, multicenter, non-randomized platform trial in which patients with advanced or metastatic solid tumors, multiple myeloma, or non–Hodgkin lymphoma, who have exhausted all standard-of-care options, are treated based on their tumor molecular profile with approved targeted therapies or immunotherapies outside the registered indication (13). Patients are enrolled in parallel cohorts, each defined by one tumor type, one molecular variant, and one study treatment. For selected biomarkers, such as TML, the protocol allows for tumor-agnostic cohorts.

DRUP was approved by the Medical Ethical Committee of the Netherlands Cancer Institute in Amsterdam and is conducted in accordance with Good Clinical Practice guidelines and the ethical principles of the Declaration of Helsinki for medical research. Written informed consent was obtained from all included patients. DRUP is registered with ClinicalTrials.gov, number NCT02925234.

Study population

Eligible patients were ≥18 years old with advanced, treatment-refractory, and immunotherapy-naïve, microsatellite-stable solid tumors. Patients were selected based on TML as determined by WGS during routine diagnostics prior to study enrollment. Patients with breast cancer harboring an intermediate TML of 140 to 290 nonsynonymous mutations across the genome were eligible for a tumor-specific breast cancer cohort (cohort A). In two tumor-agnostic cohorts, patients with all other cancer types harboring either an intermediate TML of 140 to 290 or a high TML of >290 nonsynonymous mutations across the genome were included (cohorts B and C, respectively). These thresholds were chosen in consultation with the drug sponsor before the tumor-agnostic FDA approval of pembrolizumab. Patients with prostate or head and neck squamous cell carcinomas harboring a TML of 140 to 290 were enrolled in additional tumor-specific cohorts that were still open for accrual at the time of data cutoff. Patients with gastrointestinal tumors harboring a TML of 140 to 290 were also enrolled in additional tumor-specific cohorts, but these were closed at the time of data cutoff due to a lack of clinical activity of pembrolizumab (13). Patients with melanoma, urothelial cell carcinoma, and non–small cell lung carcinoma were ineligible as at the time of enrollment, ICB was the standard-of-care therapy for these tumor types. Patients were accrued by 35 participating hospitals throughout the Netherlands.

Patients had measurable disease according to the Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST v1.1; ref. 20) or Response Assessment in Neuro-Oncology (RANO) criteria (21), an acceptable organ function, and an Eastern Cooperative Oncology Group performance status of 0 or 1. Patients were considered evaluable if response was radiologically or clinically evaluable and had received at least two treatment cycles. Nonevaluable patients were replaced and excluded from efficacy and biomarker analyses.

Treatment, assessment, and evaluation

Patients were treated with pembrolizumab 200 mg intravenously once every 3 weeks until disease progression or unmanageable toxicity. Radiological imaging for tumor response assessment, either CT or MRI, was performed at baseline and every 9 weeks

(three cycles) thereafter. Interruption of treatment could be considered for patients with ongoing benefit after 2 years of treatment. For these patients, the protocol allowed recommencement of treatment in case of radiological or clinical progression, provided that the patient still met all eligibility criteria.

Primary endpoints included clinical benefit (CB) and safety. CB was defined as an objective response (OR) or stable disease for at least 16 weeks, according to RECIST v1.1 or RANO criteria and measured at least two times. An OR was defined as a confirmed complete or partial response (confirmed when response was observed on subsequent tumor measurements) according to RECIST v.1.1 and RANO criteria. Safety was measured by the frequency of grade ≥3 treatment-related adverse events (AE) occurring up to 30 days after the last administration of the study drug. All AEs were graded according to the Common Terminology Criteria for Adverse Events version 4.03. Secondary endpoints included progression-free survival (PFS) and overall survival (OS). Biomarker analysis was an exploratory endpoint.

During accrual, safety and accrual data were regularly reviewed by an independent data monitoring committee who monitored and advised on trial conduct.

Biomarker analyses

Prior to the start of treatment, all patients underwent a tumor biopsy for biomarker analysis including WGS and RNA-seq. This procedure was mandatory, unless patients had received WGS on tumor tissue prior to study enrollment, provided that no anticancer therapy was administered between the collection of tissue for WGS and treatment initiation in DRUP, or if deemed unsafe. For patients that did not undergo a biopsy for WGS in the context of our trial, previously obtained WGS data from routine diagnostics were used for biomarker analyses, if available. For biomarker analyses, we applied the WGS pipeline of Hartwig Medical Foundation (https://github.com/hartwigmedical/pipeline5, version 3.4.2; detailed values per patient are reported in Supplementary Table S1). The results were assessed per cohort, and further subgroup analysis of patients was performed based on primary tumor type (breast vs. other tumor types).

WGS

WGS and preprocessing were performed on tumor biopsies and matched 10 mL blood sample for germline DNA. Sequencing was performed on the Illumina NovaSeq (2 × 151 bp reads) platform by the Hartwig Medical Foundation, as previously described (22–24). Samples with low tumor purity (<20%), with low DNA yield (<300 ng), or lacking sufficient informed consent for the present study were excluded from the analysis. Briefly, reads were aligned to the reference genome GRCH37 using Burrows–Wheeler Aligner (RRID: SCR_010910). Somatic single-nucleotide variants, small insertions, and small deletions were called using SAGE (https://github.com/hartwigmedical/hmftools/tree/master/sage). TMB, TML, structural variant load (SVL), clonality, ploidy, and purity were computed using publicly available bioinformatic tools GRIDSS, PURPLE, and LINX (https://github.com/hartwigmedical/pipeline5, version 3.4.2). TMB was defined as the total number of all nonsynonymous and synonymous mutations across the whole genome of the tumor per Mb, whereas TML was defined as the total number of nonsynonymous mutations across the whole genome. TML and TMB were determined by performing WGS on fresh frozen biopsy samples, which does not allow for a direct comparison with panel-based TMB scores on formalin-fixed, paraffin-embedded material. Somatic clonal TML (cTML) was determined with a subclonal likelihood threshold <0.05. The aneuploidy score was calculated as the total number of chromosome arms with arm-level copy number alterations in a sample (25). Neoantigen repertoires were computed based on WGS, and if available RNA-seq, through the NEO pipeline (26). Fusion-derived neoepitopes were characterized from in- and out-of-frame gene fusions in both RNA and DNA (26). Driver mutations were determined using PURPLE. Genetic variants were annotated using SnpEff v5.0e (RRID: SCR_005191) and filtered using SnpSift v5.0e (RRID: SCR_015624) selecting oncogenes and biallelic tumor suppressor genes, including splice site regions, with moderate and high impact (27, 28). Genetic variants were selected from pathways for antigen presentation (29), chromatin remodeling [the switch/sucrose nonfermentable (SWI/SNF) complex; ref. 30], insensitivity to IFNγ signaling (31), key oncogenes, or tumor suppressor genes (22).

RNA-seq

Total RNA was extracted using the QIAGEN QIAsymphony RNA kit. The KAPA RNA Hyper + RiboErase HMR method was used to prepare samples containing approximately 100 ng total RNA. RNA libraries were paired and sequenced on the Illumina NextSeq 550 platform (2 × 75 bp reads) or Illumina NovaSeq 6000 platform (2 × 150 bp reads). Adapters were trimmed using Trim Galore! (v0.6.10; (bioinformatics.babraham.ac.uk/projects/trim_galore/trim_galore) with the specified parameters: –quality 20, –length 25, and –paired and were subsequently aligned to the human reference genome GRCh38 (GENCODE v35, RRID: SCR_014966) utilizing STAR (v2.7.10b, RRID: SCR_004463) with default settings (32). Next, featureCounts (subread v2.0.6, RRID: SCR_012919) was used to generate count data from the aligned reads with reverse strandedness (33). Counts per million was $\log_2$-normalized, and genes with an absolute count below 10 were removed. To assess immune infiltration, deconvolution analysis was performed on markers proposed by Danaher and colleagues (34). The IFNγ signature gene set (35), tumor-infiltrating lymphocyte (TIL) signature (34), and other signatures were calculated as the sum of the $\log_2$ (RPM + 1) for each gene within the respective gene sets (Supplementary Table S2).

Statistical analysis

In DRUP, cohorts are monitored using a Simon-like two-stage "admissible" monitoring plan to identify cohorts with evidence of activity (36, 37). The cohorts were evaluated in a two-stage design. If no CB was observed in any of the first enrolled eight participants in the cohort, the cohorts were closed. When at least one of eight patients experienced CB according to the study definition, an additional 16 patients were included in the cohort. Four or fewer patients with CB would suggest a lack of activity, whereas five or more patients with CB suggested that further investigation of the drug in the tumor/variant was warranted. The null hypothesis and alternative hypothesis were defined as clinical benefit rate (CBR) of 10% versus ≥30%. This monitoring rule had 85% power to reject the null hypothesis of a CBR of 10% when the true CBR was 30%, with a one-sided α-error rate of 7.8%.

All statistical analyses were performed using R version 4.0.3. Patient characteristics, AEs, and tumor responses were summarized using descriptive statistics. Kaplan–Meier methods were used to estimate PFS (calculated from the start of treatment to progression or death from any cause and censoring patients alive without

progression) and OS (calculated from the first day of treatment administration to the date of death from any cause, censoring patients who were alive at the last follow-up). The duration of response (DoR) was calculated from the first response date until disease progression or death. Differences in CB between different patient groups were calculated using the Fisher exact test (categorical data) or the Mann–Whitney *U* test (continuous data). A Pearson correlation coefficient was computed to assess the relationship between TML and TMB. All tests were two-sided, and a *P* value less than or equal to 0.05 was considered statistically significant.

Data availability

The data described in this study are available for academic use upon request. WGS data can be obtained through the Netherlands Cancer Institute and Hartwig Medical Foundation. Procedures and requested forms can be found at https://www.hartwigmedicalfoundation.nl/en. An independent data access board will evaluate whether the intended use of the data is compatible with the consent given by the patients and whether there would be any applicable ethical or legal constraints. Clinical data can be obtained at a per-patient level by emailing the Institutional Review Board of the Netherlands Cancer Institute (IRB@nki.nl).

Results

Accrual

Between June 2017 and August 2021, 114 patient cases with tumors harboring TML ≥140 were submitted to the central study team for the evaluation of potential study participation in one of the three cohorts described in this analysis. All patients exhausted standard-of-care treatment options. Ninety-nine patients were approved by the central team for pembrolizumab treatment screening. After allocation, 18 patients dropped out, mainly because they no longer met the study selection criteria ($n = 10$). Eighty-one patients with 26 different tumor types were found eligible and started study treatment (Supplementary Table S1). Nine patients were not evaluable for the primary endpoint according to the protocol definition of evaluability, with rapid clinical deterioration as the most common reason ($n = 8$), and were excluded from the efficacy and biomarker analyses (Supplementary Fig. S1; Supplementary Table S1). The representativeness of study participants is reflected in Supplementary Table S3.

Patient characteristics and clinical benefit

Cohort A: Breast cancer cohort harboring a TML of 140 to 290

Twenty-six female patients were enrolled in the breast cancer cohort (Supplementary Fig. S1). The median age was 57 years (range 30–73 years). Patients with hormone receptor (HR)–positive/HER2-negative ($n = 12$; 46%) and triple-negative tumors ($n = 9$; 35%) were most frequently included. Patients were heavily pretreated with a median of 7.5 prior lines of treatment. The median WGS-based TML in this cohort was 177 nonsynonymous mutations per genome (range 143–274). Baseline characteristics are presented in **Table 1**.

Twenty-four patients were evaluable for the primary endpoint (Supplementary Fig. S1). CB was observed in three patients [13%; 95% confidence interval (CI), 2.7%–32.4%], all of whom showed partial response. Among them, two patients had triple-negative breast cancer, and one patient was diagnosed with HR-negative/HER2-positive breast cancer. Detailed responses are presented in Supplementary Fig. S1.

At the time of data cutoff (January 27, 2023), the response in one patient was ongoing for more than 24 months, and other responses lasted 6 and 10 months (**Fig. 1**). After a minimal follow-up of 2.0 months, the median PFS and OS were 1.8 months (95% CI, 1.7–1.9 months) and 6.5 months (95% CI, 3.3–10.8 months), respectively (**Fig. 2A** and **B**).

Cohort B: Tumor-agnostic cohort harboring a TML of 140 to 290

Twenty-eight patients with 15 different tumor types were enrolled in the tumor-agnostic cohort for tumors harboring a TML between 140 and 290 (Supplementary Fig. S1). The median age was 59 years (range 34–80 years), and the majority of patients were female [$n = 19$, (68%)]. The median number of prior lines of systemic treatment was three. The median WGS-based TML in this cohort was 176 nonsynonymous mutations per genome (range 143–277). Baseline characteristics are presented in **Table 1**.

Twenty-four patients were evaluable for the primary endpoint (Supplementary Fig. S1). CB was observed in five patients (21%; 95% CI, 7.1%–42.2%), with an OR in two patients (8%; 95% CI, 1.0%–27.0%). Detailed responses are presented in Supplementary Fig. S2. The median DoR was 5.2 months (95% CI, 4.1 months–not reached). CB was observed across different histotypes, including vaginal cancer ($n = 2$), cancer of unknown primary ($n = 1$), cervix carcinoma ($n = 1$), and rhabdomyosarcoma ($n = 1$; Supplementary Table S1).

At the time of data cutoff, none of the patients were still on study. The median time on treatment was 1.5 months (95% CI, 1.2–3.5 months; **Fig. 1**). After a minimal follow-up of 11.0 months, the median PFS and OS were 2.0 months (95% CI, 1.8–3.0 months) and 9.1 months (95% CI, 4.7–13.5 months), respectively (**Fig. 2A** and **B**).

Cohort C: Tumor-agnostic cohort harboring a TML >290

Twenty-seven patients with 16 different tumor types were enrolled in the tumor-agnostic cohort for tumors harboring a TML of >290 (Supplementary Fig. S1). The median age was 64 years (range 45–85 years), and 15 patients (56%) were male. The median number of prior lines of systemic treatment was two. The median TML in this cohort was 348 nonsynonymous mutations per genome (range 299–2,915). Baseline characteristics are presented in **Table 1**.

Twenty-four patients were evaluable for the primary endpoint (Supplementary Fig. S1). CB was observed in 10 patients (42%; 95% CI, 22.1%–63.4%), with an OR in 6 patients (25%; 95% CI, 9.8%–46.7%). Detailed responses are presented in Supplementary Fig. S2. The median DoR was not reached. CB was observed across different histotypes, including skin cancer ($n = 3$), cancer of unknown primary ($n = 2$), esophagus cancer ($n = 2$), anaplastic oligodendroglioma ($n = 1$), breast cancer ($n = 1$), and penile cancer ($n = 1$; Supplementary Table S1). Interestingly, in this cohort, CB was more frequently observed in males than in females [9 of 12 (75%) vs. 1 of 15 (7%), $P = 0.0045$], potentially attributed to a higher occurrence of ultraviolet light– or human papillomavirus–associated histotypes among males.

At the time of data cutoff, two patients were still on study. The median time on treatment was 3.5 months (95% CI, 1.5–5.8 months; **Fig. 1**). After a minimal follow-up of 2.1 months, the median PFS and OS were 3.4 months (95% CI, 1.9–6.0 months) and 11.8 months (95% CI, 7.3–16.9 months), respectively (**Fig. 2A** and **B**).

Safety

Among the 81 treated patients, 19 (23%) experienced a total of 35 grade ≥3 AEs deemed at least possibly related to treatment. Grade 3 and 4 AEs were reported for 19 and 2 patients, respectively, and no grade 5

Table 1. Baseline characteristics of enrolled patients.

Characteristic	Enrolled patients, *n* (%)		Cohort A, *n* (%)		Cohort B, *n* (%)		Cohort C, *n* (%)	
Total patients enrolled	81		26		28		27	
Age (years) at submission								
Median (range)	61	(30–85)	57	(30–73)	59	(34–80)	64	(45–85)
Gender								
Female	57	70%	26	100%	19	68%	12	44%
Male	24	30%	—	0	9	32%	15	56%
ECOG performance status								
ECOG 0	26	32%	9	35%	8	29%	9	33%
ECOG 1	47	58%	16	62%	16	57%	15	56%
Not done	8	10%	1	4%	4	14%	3	11%
Primary tumor type								
Breast	32	40%	26	100%	—	—	6	22%
Small cell lung	7	9%	—	—	5	18%	2	7%
Cervix	6	7%	—	—	4	14%	2	7%
Skin	5	6%	—	—	2	7%	3	11%
Squamous cell carcinoma of (peri)genitals[a]	5	6%	—	—	4	14%	1	4%
Unknown primary	4	5%	—	—	2	7%	2	7%
Lower gastrointestinal tract[b]	4	5%	—	—	1	4%	3	11%
Upper gastrointestinal tract[c]	3	4%	—	—	-	—	3	11%
Sarcoma[d]	3	4%	—	—	2	7%	1	4%
Endometrium	2	2%	—	—	2	7%	—	—
Ovarian	2	2%	—	—	2	7%	—	—
Urachus	2	2%	—	—	2	7%	—	—
Anaplastic oligodendroglioma	1	1%	—	—	—	—	1	4%
Biliary tract	1	1%	—	—	1	4%	—	—
Bladder (small cell)	1	1%	—	—	—	—	1	4%
LCNEC	1	1%	—	—	1	4%	—	—
Neuroendocrine tumor	1	1%	—	—	—	—	1	4%
Prostate	1	1%	—	—	—	—	1	4%
Number of prior systemic therapy lines								
Median (range)	3	(0–15)	8	(5–15)	3	(0–6)	2	(0–9)
TML at entry								
Median (range)	226	(143–2,915)	177	(143–274)	176	(143–277)	348	(299–2,915)
HR and HER2 status (breast cancer)								
HR^+ and $HER2^-$	17	53%	12	46%	—	—	5	83%
Triple-negative	10	31%	9	35%	—	—	1	17%
$HER2^+$	5	16%	5	19%	—	—	0	—

Cohort A: breast cancer cohort harboring a TML of 140 to 290. Cohort B: tumor-agnostic cohort harboring a TML of 140 to 290. Cohort C: tumor-agnostic cohort harboring a TML >290.

Abbreviations: ECOG, Eastern Cooperative Oncology Group; LCNEC, large-cell neuroendocrine carcinoma of the lung.

[a]Anal, $n = 2$; vaginal, $n = 2$; and penile, $n = 1$.

[b]Colorectal, $n = 3$; small intestinal, $n = 1$.

[c]Esophageal, $n = 2$; gastroesophageal junction, $n = 1$.

[d]Angiosarcoma, $n = 1$; uterine leiomyosarcoma, $n = 1$; and rhabdomyosarcoma, $n = 1$.

AEs occurred (Supplementary Table S4). AEs led to treatment discontinuation in four (5%) patients. Of note, one case of immune-related grade 4 neutropenia occurred after the administration of six treatment cycles, which was resolved after the administration of granulocyte colony–stimulating factor and immunosuppressive therapy. Remarkably, despite treatment discontinuation, the patient experienced durable CB (>24 months) from treatment. Otherwise, the safety profile across cohorts was consistent with that reported previously for pembrolizumab in patients with advanced cancers, and no unexpected toxicities were observed.

Translational analyses

Baseline biopsies

To explore potential predictors of response or resistance to refine patient selection, we performed genomic and transcriptomic analyses on fresh, frozen pretreatment tumor biopsies. WGS data were available for 66 of 72 evaluable patients (92%), obtained either in the context of the trial that were successfully sequenced ($n = 41$) or outside of the trial ($n = 25$; Supplementary Table S1). Successful RNA-seq was available for 54 patients (54/72, 75%) in total (Supplementary Table S1). Stratified by CB (yes or no), the cohorts were analyzed collectively, and the patients were further divided into breast cancer versus other tumor types.

Neoantigen burden estimates predicted clinical benefit in cohort C

To understand the relationship between TMB and TML, we first investigated the correlation between these two variables per cohort (Supplementary Fig. S3). For all cohorts combined, these variables

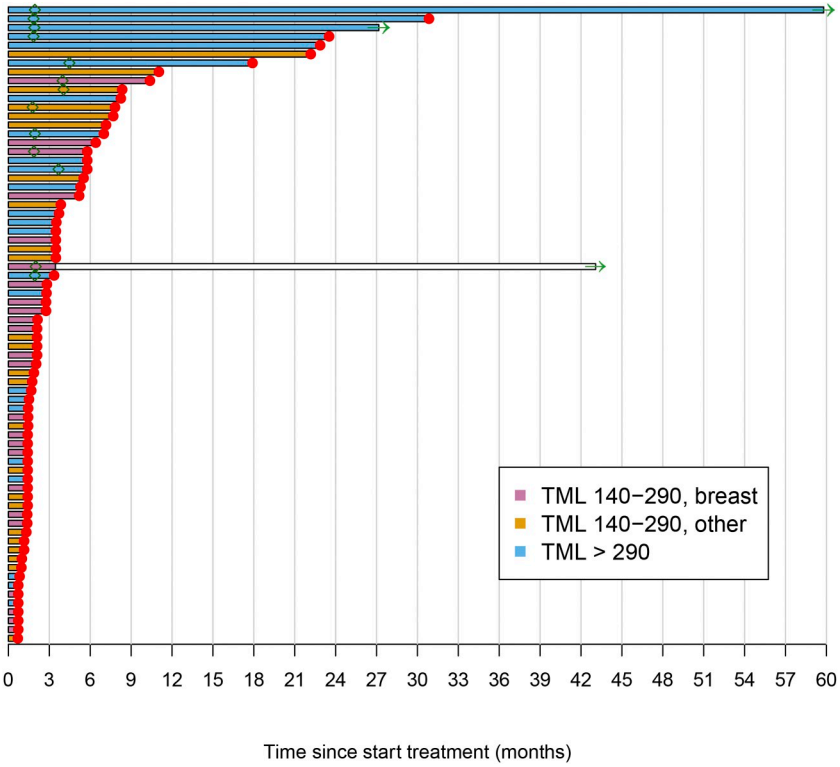

Figure 1.
Swimmer plot. Swimmer plot of the time on treatment (in months) for individual patients ($n = 72$). The diamond marks the moment of partial response, the red dot indicates the end of treatment, and the arrow indicates that the patient was still on study at the time of analysis. The white bar represents the time during which pembrolizumab was interrupted (which was optional per protocol after 2 years of treatment) for patients who still experienced CB and were thus still in follow-up. Patients included in the breast cancer cohort harboring a TML of 140–290 are indicated in pink. Patients included in the tumor-agnostic cohort harboring a TML of 140–290 are indicated in orange. Patients included in the tumor-agnostic cohort harboring a TML >290 are indicated in blue.

were highly correlated ($R = 0.97$, $P = 2.20 \times 10^{-16}$), which was also observed separately within each cohort (cohort A: $R = 0.94$, $P = 1.42 \times 10^{-11}$; cohort B: $R = 0.76$, $P = 4.82 \times 10^{-5}$; cohort C: $R = 0.97$, $P = 3.96 \times 10^{-13}$).

For some patients, variations were observed between initial TML at inclusion and TML of the biopsy used for biomarker analyses. The median TML of biopsies used for biomarker analyses was 177 (range 65–364) in cohort A, 175 (range 34–281) in cohort B, and

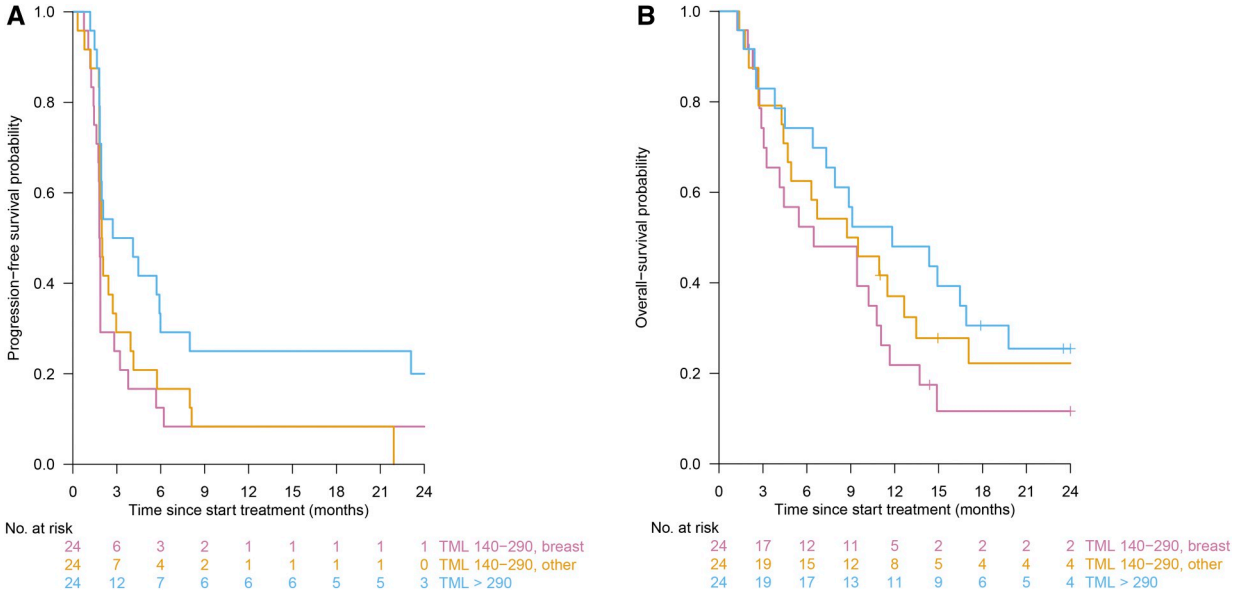

Figure 2.
PFS and OS curve including the number of patients at risk. Kaplan–Meier curve for (**A**) PFS and (**B**) OS including the number of patients at risk.

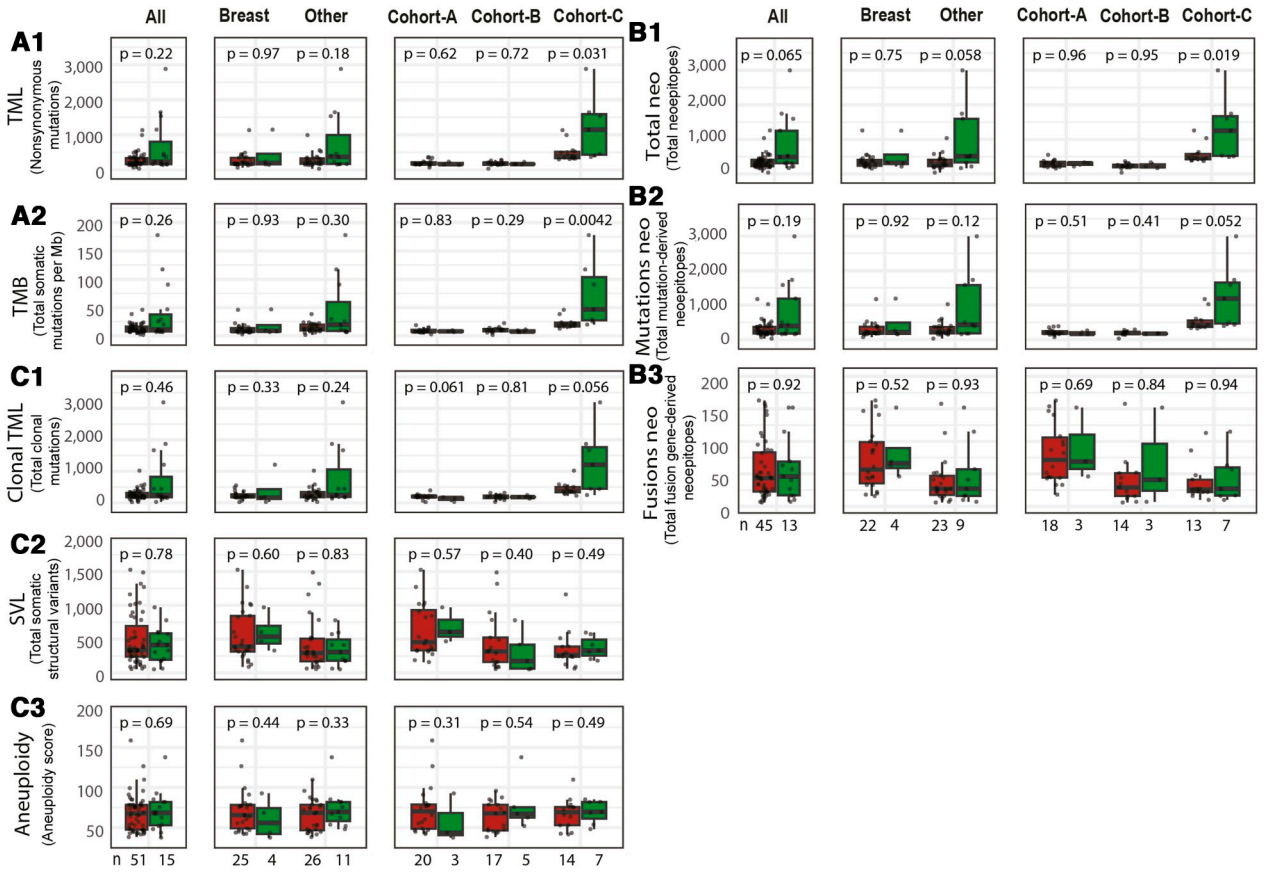

Figure 3.

Neoantigen burden estimates of clinical benefit to ICB. **A1,** Neoantigen burden estimates of patients ($n = 66$) experiencing CB (green) or no CB (red) according to coding mutations (TML). **A2,** All coding mut/Mb (TMB). Neoantigen peptide prediction of patients ($n = 52$) according to total neoantigens (**B1**), mutation-derived neoantigens (**B2**), and fusion-derived neoantigens (**B3**). **C1,** cTML, (**C2**) SVL, and (**C3**) aneuploidy in patient experiencing CB (green) or no CB (red; $n = 66$).

448 (302–2,880) in cohort C, showing wider spreads than at baseline (**Table 1**; Supplementary Table S1). For consistency, all biomarker analyses were performed according to the cohorts that were prespecified at the time of inclusion.

In cohort C (tumor-agnostic cohort with TML >290), we found both TML and TMB to be significantly associated with CB (TML: $P = 0.031$; TMB: $P = 0.0042$; **Fig. 3A1** and **A2**). In cohort A (breast cancer cohort with TML 140–290) and cohort B (tumor-agnostic cohort with TML 140–290), however, these associations were not observed (**Fig. 3A1** and **A2**).

Although neoantigen burden estimates reflect the extent of neoantigens produced by tumors, not all nonsynonymous mutations give rise to expressed neoantigens (4). Therefore, we hypothesized that a direct measurement of neoantigen load might yield more predictive power with regard to ICB effectiveness compared with TMB and TML. Hence, we determined the neoantigen peptide load using both WGS and RNA-seq. The total neoantigen load was significantly associated with CB in patients in cohort C ($P = 0.019$), in line with our observations for TML and TMB, and was near-significant for the non–breast cancer subgroup ($P = 0.058$) and all patients combined ($P = 0.065$; **Fig. 3B1**). Tumors with a high abundance of fusion-derived neoepitopes generate highly potent neoantigens and are hypothesized to respond to ICB even with low TML (38), but this effect was not observed in our study (**Fig. 3B2** and **B3**).

We next hypothesized that the impact of neoantigens would increase with higher clonality or exhibition of high SVL (refs. 39–41). cTML showed a near-significant trend with CB in cohort C ($P = 0.056$; **Fig. 3C1**). However, SVL did not show a significant association with CB for any of the groups (**Fig. 3C2**). We further compared aneuploidy scores between patients with and without CB, as previous research has shown that higher aneuploidy scores were associated with poor prognosis following ICB (42), but this association was not observed in our study (**Fig. 3C3**).

Mutations in SWI/SNF were associated with clinical benefit in cohort A

Mutations in T cell–mediated killing pathways have emerged as potential mechanisms leading to ICB resistance (1). To this end, we analyzed mutations in IFNγ signaling pathways, antigen presentation, specifically *HLA* genes, and carcinogenic pathways for chromatin remodeling, such as the SWI/SNF complex (Supplementary Fig. S4). The SWI/SNF complex plays a crucial role in chromatin remodeling and alterations that could cause changes in gene

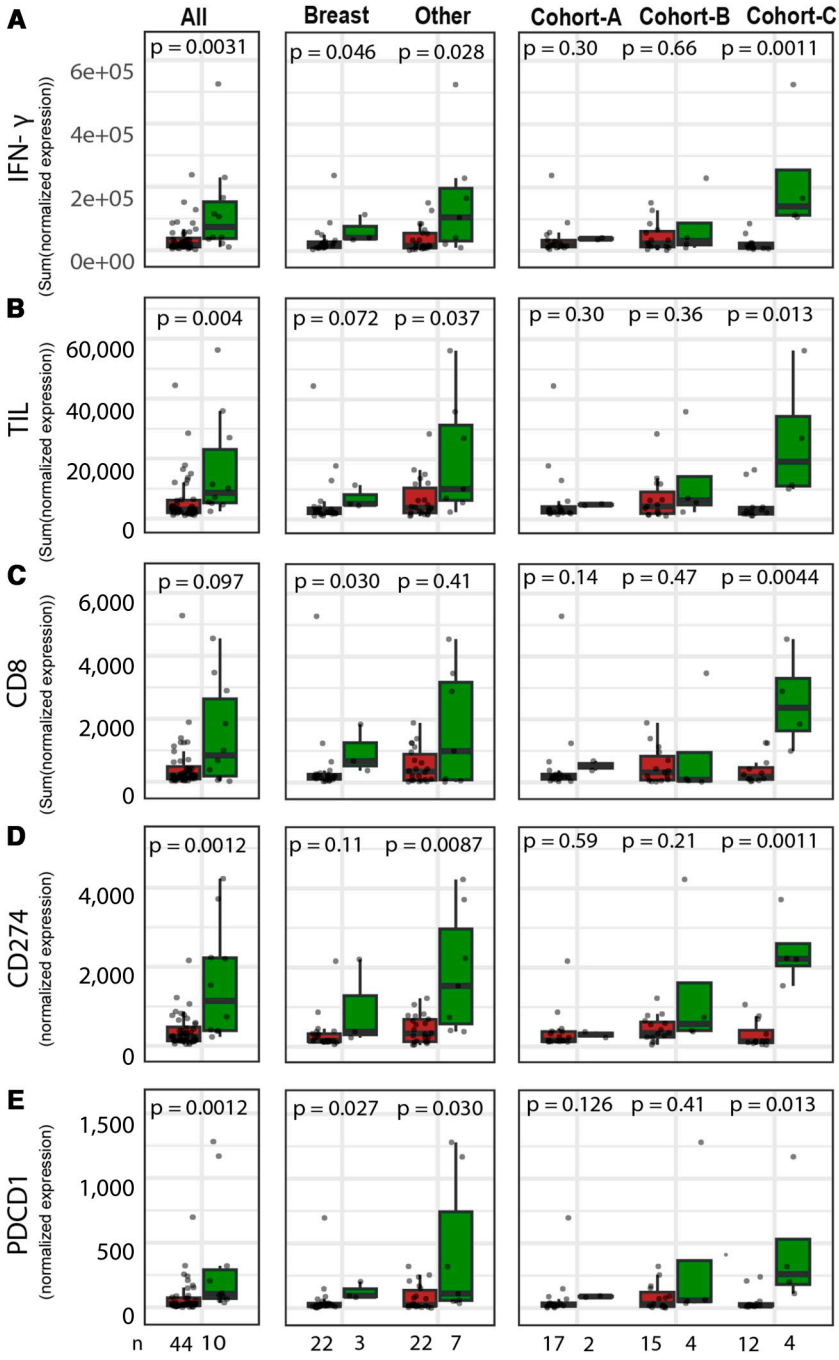

Figure 4.

Conventional immunity biomarkers of clinical benefit to ICB. Immune marker gene set expression of patients ($n = 54$) according to (**A**) IFNγ, (**B**) TILs, (**C**) CD8 expression, (**D**) CD274 expression, and (**E**) PDCD1 expression in patients experiencing CB (green) or no CB (red).

expression and therefore overexpression of neoantigens potentially rendering the tumor susceptible to ICB (43). Interestingly, two genes from the SWI/SNF complex were found mutated in patients that experienced CB in the total patient cohort (*PBRM1*: $P = 0.0099$; *SMARCB1:* $P = 0.034$), of which *PBRM1* was specifically mutated in cohort A ($P = 0.012$; Supplementary Fig. S4). The presence of at least one mutated gene of the SWI/SNF complex attained statistical significance in patients within cohort A ($P = 0.011$), as well as within all cohorts combined ($P = 0.0055$). However, none of these mutations passed the multiple testing limit.

All general markers of immune infiltration were associated with clinical benefit in cohort C

We proceeded to compare expression profiles of individual genes and marker gene sets (2, 35, 44, 45) between patients with and without CB. All immune infiltration markers were significantly associated with CB in cohort C, including IFNγ signature expression ($P = 0.0011$), TIL score ($P = 0.013$), CD8 expression ($P = 0.0044$), CD274 (encoding PDL1; $P = 0.0011$), and PDCD1 (encoding PD1; $P = 0.013$; **Fig. 4A–E**). IFNγ signature expression and *PDCD1* gene expression were significantly associated with CB

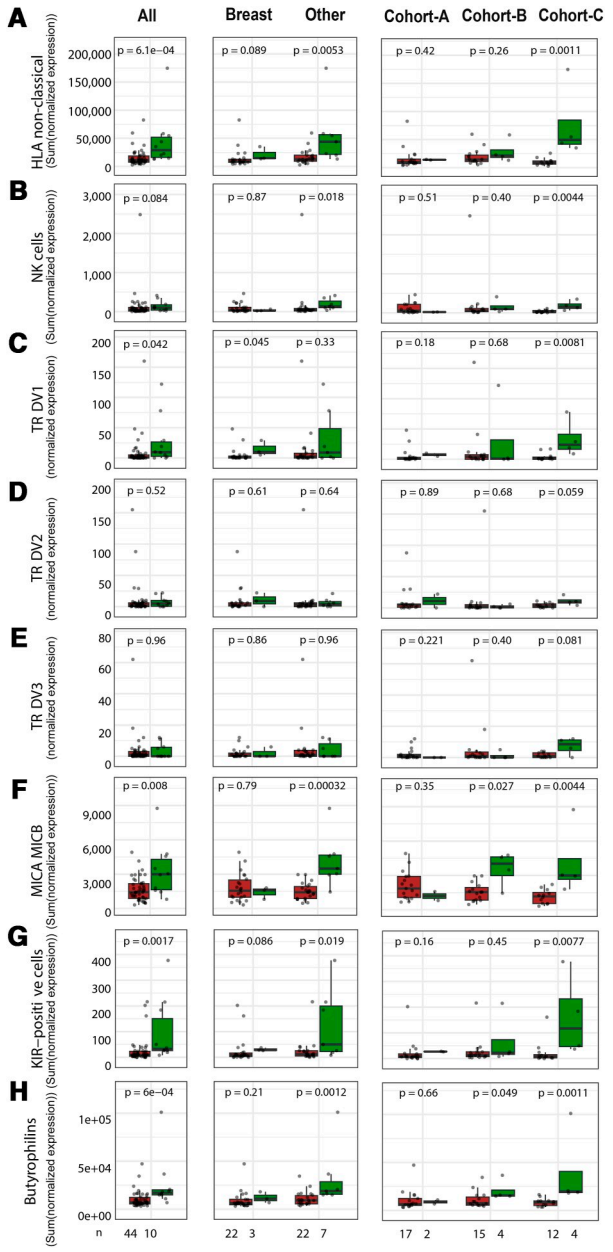

Figure 5.

Innate immunity biomarkers of clinical benefit to ICB. Innate immunity marker gene set expression of patients ($n = 54$) according to (**A**) nonclassical HLA, (**B**) NK cells, (**C–E**) TDRV1 to TDRV3 expression, (**F**) MICA and MICB expression, (**G**) KIR-positive cells, and (**H**) butyrophilins in patients experiencing CB (green) or no CB (red).

in both the breast cancer and other cancers subgroups (breast cancer: $P = 0.046$ and $P = 0.027$, respectively; other cancers: $P = 0.028$ and $P = 0.030$, respectively; **Fig. 4A** and **E**). Furthermore, in patients with breast cancer, CD8 expression ($P = 0.030$) was significantly associated with CB (**Fig. 4C**). However, in patients with non–breast cancer, the TIL score ($P = 0.037$) was also significantly associated with CB (**Fig. 4B**).

Expression of a variety of innate immune biomarkers was associated with clinical benefit in cohorts B and C

Previous studies in advanced microsatellite instable tumors have indicated that modulators of the innate immunity also play a role in ICB effectiveness (46, 47). Therefore, expression of innate immune biomarkers was compared between patients with and without CB across our cohorts (**Fig. 5A–H**). In cohort C, expression of the majority of these innate immune biomarkers was significantly associated with CB, including nonclassical human leukocyte antigens (HLA; $P = 0.0011$), NK cells ($P = 0.0044$), and TRVDV1 ($P = 0.0081$), encoding the variable regions of the γδ T-cell receptor (46), ligands MICA/MICB ($P = 0.0044$), killer-cell immunoglobulin-like receptors (KIR; $P = 0.0077$), and butyrophilins ($P = 0.0011$; **Fig. 5**). Interestingly, we found only the expression of MICA/MICB ($P = 0.027$) and butyrophilins ($P = 0.049$) to be significantly associated with CB in cohort B (**Fig. 5F** and **H**). In patients with breast cancer, expression of the marker TRVDV1 ($P = 0.045$) showed a significant association with CB (**Fig. 5C**), whereas in patients with non–breast cancer, expression of nonclassical HLA ($P = 0.0053$), NK cells ($P = 0.018$), MICA/MICB ($P < 0.001$), KIRs ($P = 0.019$), and butyrophilins ($P = 0.0012$) was significantly associated with CB (**Fig. 5A**, **5B**, and **5F–H**).

Discussion

The current notion is that antitumor immune responses induced by ICB are primarily driven by mutation-derived neoantigens. Therefore, high TMB has been proposed as a predictive biomarker for ICB efficacy. Previous studies have reported robust responses to ICB in tumors harboring high TMB including advanced microsatellite-instable tumors (9, 48), melanoma (49), and lung cancer (50, 51). However, an important subset of patients still does not benefit, highlighting the need to further refine patient selection. Therefore, we evaluated pembrolizumab efficacy in three different TML-guided cohorts: a breast cancer cohort harboring a TML 140 to 290 (cohort A), a tumor-agnostic cohort harboring a TML 140 to 290 (cohort B), and a tumor-agnostic cohort harboring a TML >290 (cohort C). In cohort A, the CBR was only 13% (3/24 patients), suggesting a lack of activity of pembrolizumab in this patient group according to our protocol definition. However, cohorts B and C were considered positive and met the study's primary endpoint, as the CBRs in cohorts B and C were more favorable, with 21% (5/24 patients) and 42% (10/24 patients), respectively. Although these outcomes seem to align with previous studies evaluating ICB efficacy in TMB-defined subgroups (5, 9, 52), the interpretation of mutation estimates is complex due to differences in gene panels, variations in number of genes assessed, and types of mutations considered (12, 53), limiting direct comparability between previous work and this study.

Interestingly, in some patients, we observed notable differences in the initial TML at entry and TML values derived from the biopsies used for biomarker analysis, even resulting in situations in which patients, in retrospect, no longer fulfilled the cohort inclusion criteria. As per the predefined categorization, these patients remained within their cohort for biomarker analysis, but these variations may have obscured signals of (non)response. Differences in TML likely arose from biological variations, such as spatial and temporal tumor heterogeneity, in patients who underwent a new tumor biopsy at baseline, and pipeline optimization for patients who did not undergo a new baseline biopsy. Tumor heterogeneity in mutational burden has been previously described (54) and should be taken into account in clinical decision-making and evaluation of response to ICB.

To refine patient selection, we explored potential determinants of response or resistance at baseline. In cohort C, TML, TMB, total neoantigen load, expression of transcriptomic inflammation markers, and expression of a variety of innate immune biomarkers were significantly associated with CB. Interestingly, in cohort B, we found only the expression of MICA/MICB and butyrophilins to be significantly associated with CB, suggesting that these unconventional innate immune biomarkers might be more predictive for ICB efficacy here than other well-understood conventional biomarkers. In this regard, it is important to note that TML does not account for immunologic features of the tumor microenvironment. Arguably, TML could be a starting point for patient selection and be further improved with additional biomarkers that consider factors from the tumor microenvironment, among others.

In the era of precision oncology, tumor-agnostic approaches have emerged as a strategy to treat patients; however, they challenge the search for biomarkers by potentially diluting tumor type–specific signals. In that regard, breast cancer constituted the largest subgroup in this study and was selected for focused analyses. Although clinically relevant responses were observed, these were too few to consider this biomarker–treatment combination a suitable option for all patients, which is consistent with previous data showing ICB effectiveness only in small subsets of patients with metastatic breast cancer (52, 55). This might be explained by the relatively "cold" phenotype of breast tumors compared with that of other tumor types (56). Interestingly, pretreatment expression of inflammation markers, including IFNγ, CD8, and PDCD1, could significantly distinguish responders from nonresponders, indicative of a more inflamed phenotype in responders compared with nonresponders. Investigations are underway to explore methods to enhance the immunogenicity of these tumors (57), and our exploratory biomarker analyses may support these efforts. Additionally, within this subgroup, mutations in *PBRM1*, encoding a subunit of the SWI/SNF complex, were significantly associated with CB. Attributed to decreased resistance to T cell–mediated killing (58), similar findings have been previously reported across several other tumor types (30, 58, 59), but limited data exist with regard to its role in breast cancer (30), warranting future research.

Our study also has limitations, including the lack of both randomization and a control group and the heterogeneous study population. We included a total of 26 different tumor (sub)types with previous treatment lines that varied in nature and quantity, a wide range of TML levels, and variations in survival prognosis across tumor types, which may have impacted the interpretation of our results. Furthermore, although our cohort represents a unique dataset of clinical, genomic, and transcriptomic data, the cohorts are small for biomarker analysis, resulting in limited statistical power. Furthermore, it is uncertain whether the significant associations between immune markers and CB found in the breast cancer and non–breast cancer groups are driven by the immune marker itself, the addition of tumors with high TML, or the interaction between the immune marker and TML. Therefore, our findings should be interpreted with caution and regarded as hypothesis-generating. Nevertheless, in the context of a pan-cancer clinical platform trial, our study provides useful insights about the efficacy and safety of pembrolizumab in a wide variety of tumors with an intermediate to high TML. Moreover, especially for patients with lower TMLs, it provides a rationale for follow-up studies to focus on unconventional biomarkers and to apply these, potentially, in combination with TML.

In conclusion, our study demonstrated that pembrolizumab could be an effective treatment option for some pretreated patients with advanced microsatellite-stable tumors harboring an intermediate to high TML, but additional predictive biomarkers are still highly needed. Neoantigen burden estimates, expression of immune inflammation markers, and expression of a variety of innate immune biomarkers were mainly predictive of CB in patients with tumors harboring high TML. In tumors harboring lower TMLs, only pretreatment expression of unconventional innate immunity–associated biomarkers predicted CB. To improve clinical treatment outcomes in these patients, future studies may benefit from a focus on innate immune biomarkers in the context of patient selection and development of novel personalized treatment strategies.

Authors' Disclosures

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Authors' Contributions

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Note

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References

1. Sharma P, Hu-Lieskovan S, Wargo JA, Ribas A. Primary, adaptive, and acquired resistance to cancer immunotherapy. Cell 2017;168:707–23.
2. McGrail DJ, Pilie PG, Rashid NU, Voorwerk L, Slagter M, Kok M, et al. High tumor mutation burden fails to predict immune checkpoint blockade response across all cancer types. Ann Oncol 2021;32:661–72.
3. Cristescu R, Mogg R, Ayers M, Albright A, Murphy E, Yearley J, et al. Pan-tumor genomic biomarkers for PD-1 checkpoint blockade-based immunotherapy. Science 2018;362:eaar3593.
4. Chan TA, Yarchoan M, Jaffee E, Swanton C, Quezada SA, Stenzinger A, et al. Development of tumor mutation burden as an immunotherapy biomarker: utility for the oncology clinic. Ann Oncol 2019;30:44–56.
5. Goodman AM, Sokol ES, Frampton GM, Lippman SM, Kurzrock R. Microsatellite-stable tumors with high mutational burden benefit from immunotherapy. Cancer Immunol Res 2019;7:1570–3.
6. Johnson DB, Frampton GM, Rioth MJ, Yusko E, Xu Y, Guo X, et al. Targeted next generation sequencing identifies markers of response to PD-1 blockade. Cancer Immunol Res 2016;4:959–67.
7. Samstein RM, Lee CH, Shoushtari AN, Hellmann MD, Shen R, Janjigian YY, et al. Tumor mutational load predicts survival after immunotherapy across multiple cancer types. Nat Genet 2019;51:202–6.
8. Yarchoan M, Hopkins A, Jaffee EM. Tumor mutational burden and response rate to PD-1 inhibition. N Engl J Med 2017;377:2500–1.
9. Marabelle A, Le DT, Ascierto PA, Di Giacomo AM, De Jesus-Acosta A, Delord JP, et al. Efficacy of pembrolizumab in patients with noncolorectal high microsatellite instability/mismatch repair-deficient cancer: results from the phase II KEYNOTE-158 study. J Clin Oncol 2020;38:1–10.
10. Marcus L, Fashoyin-Aje LA, Donoghue M, Yuan M, Rodriguez L, Gallagher PS, et al. FDA approval summary: pembrolizumab for the treatment of tumor mutational burden-high solid tumors. Clin Cancer Res 2021;27:4685–9.
11. Schumacher TN, Schreiber RD. Neoantigens in cancer immunotherapy. Science 2015;348:69–74.
12. Mankor JM, Paats MS, Groenendijk FH, Roepman P, Dinjens WNM, Dubbink HJ, et al. Impact of panel design and cut-off on tumour mutational burden assessment in metastatic solid tumour samples. Br J Cancer 2020;122:953–6.
13. van der Velden DL, Hoes LR, van der Wijngaart H, van Berge Henegouwen JM, van Werkhoven E, Roepman P, et al. The drug rediscovery protocol facilitates the expanded use of existing anticancer drugs. Nature 2019;574:127–31.
14. Spiekman IAC, Geurts BS, Zeverijn LJ, de Wit GF, van der Noort V, Roepman P, et al. Efficacy and safety of panitumumab in patients with RAF/RAS-wild-type glioblastoma: results from the Drug Rediscovery Protocol. Oncologist 2023;oyad320.
15. Geurts BS, Battaglia TW, van Berge Henegouwen JM, Zeverijn LJ, de Wit GF, Hoes LR, et al. Efficacy, safety and biomarker analysis of durvalumab in patients with mismatch-repair deficient or microsatellite instability-high solid tumours. BMC Cancer 2023;23:205.
16. van der Wijngaart H, Hoes LR, van Berge Henegouwen JM, van der Velden DL, Zeverijn LJ, Roepman P, et al. Patients with biallelic BRCA1/2 inactivation respond to olaparib treatment across histologic tumor types. Clin Cancer Res 2021;27:6106–14.
17. van Berge Henegouwen JM, Jebbink M, Hoes LR, van der Wijngaart H, Zeverijn LJ, van der Velden DL, et al. Trastuzumab and pertuzumab combination therapy for advanced pre-treated HER2 exon 20-mutated non-small cell lung cancer. Eur J Cancer 2022;171:114–23.
18. Zeverijn LJ, Looze EJ, Thavaneswaran S, van Berge Henegouwen JM, Simes RJ, Hoes LR, et al. Limited clinical activity of palbociclib and ribociclib monotherapy in advanced cancers with cyclin D-CDK4/6 pathway alterations in the Dutch DRUP and Australian MoST trials. Int J Cancer 2023;153:1413–22.
19. Hoes LR, van Berge Henegouwen JM, van der Wijngaart H, Zeverijn LJ, van der Velden DL, van de Haar J, et al. Patients with rare cancers in the drug rediscovery protocol (DRUP) benefit from genomics-guided treatment. Clin Cancer Res 2022;28:1402–11.
20. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer 2009;45:228–47.
21. Wen PY, Macdonald DR, Reardon DA, Cloughesy TF, Sorensen AG, Galanis E, et al. Updated response assessment criteria for high-grade gliomas: response assessment in neuro-oncology working group. J Clin Oncol 2010;28:1963–72.
22. Priestley P, Baber J, Lolkema MP, Steeghs N, de Bruijn E, Shale C, et al. Pan-cancer whole-genome analyses of metastatic solid tumours. Nature 2019;575:210–6.
23. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, et al. Signatures of mutational processes in human cancer. Nature 2013;500:415–21.
24. Roepman P, de Bruijn E, van Lieshout S, Schoenmaker L, Boelens MC, Dubbink HJ, et al. Clinical validation of whole genome sequencing for cancer diagnostics. J Mol Diagn 2021;23:816–33.
25. Taylor AM, Shih J, Ha G, Gao GF, Zhang X, Berger AC, et al. Genomic and functional approaches to understanding cancer aneuploidy. Cancer Cell 2018;33:676–89.e3.
26. Martinez-Jimenez F, Priestley P, Shale C, Baber J, Rozemuller E, Cuppen E. Genetic immune escape landscape in primary and metastatic cancer. Nat Genet 2023;55:820–31.
27. Cingolani P, Platts A, Wang LL, Coon M, Nguyen T, Wang L, et al. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w1118; iso-2; iso-3. Fly (Austin) 2012;6:80–92.
28. Cingolani P, Patel VM, Coon M, Nguyen T, Land SJ, Ruden DM, et al. Using *Drosophila melanogaster* as a model for genotoxic chemical mutational studies with a new program, SnpSift. Front Genet 2012;3:35.
29. Zaretsky JM, Garcia-Diaz A, Shin DS, Escuin-Ordinas H, Hugo W, Hu-Lieskovan S, et al. Mutations associated with acquired resistance to PD-1 blockade in melanoma. N Engl J Med 2016;375:819–29.
30. Yang Q, Shen R, Xu H, Shi X, Xu L, Zhang L, et al. Comprehensive analyses of *PBRM1* in multiple cancer types and its association with clinical response to immunotherapy and immune infiltrates. Ann Transl Med 2021;9:465.
31. Darnell JE Jr, Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 1994;264:1415–21.
32. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 2013;29:15–21.
33. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 2014;30:923–30.
34. Danaher P, Warren S, Dennis L, D'Amico L, White A, Disis ML, et al. Gene expression markers of tumor infiltrating leukocytes. J Immunother Cancer 2017;5:18.
35. Ayers M, Lunceford J, Nebozhyn M, Murphy E, Loboda A, Kaufman DR, et al. IFN-gamma-related mRNA profile predicts clinical response to PD-1 blockade. J Clin Invest 2017;127:2930–40.
36. Simon R. Optimal two-stage designs for phase II clinical trials. Control Clin Trials 1989;10:1–10.
37. Jung SH, Lee T, Kim K, George SL. Admissible two-stage designs for phase II cancer clinical trials. Stat Med 2004;23:561–9.
38. Velaga R, Koo KM, Mainwaring PN. Harnessing gene fusion-derived neoantigens for "cold" breast and prostate tumor immunotherapy. Immunotherapy 2022;14:1165–79.
39. Trinh A, Polyak K. Tumor neoantigens: when too much of a Good thing is bad. Cancer Cell 2019;36:466–7.
40. Craig DJ, Bailey MM, Noe OB, Williams KK, Stanbery L, Hamouda DM, et al. Subclonal landscape of cancer drives resistance to immune therapy. Cancer Treat Res Commun 2022;30:100507.
41. McGranahan N, Furness AJ, Rosenthal R, Ramskov S, Lyngaa R, Saini SK, et al. Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade. Science 2016;351:1463–9.
42. Spurr LF, Weichselbaum RR, Pitroda SP. Tumor aneuploidy predicts survival following immunotherapy across multiple cancers. Nat Genet 2022;54:1782–5.
43. Miao D, Margolis CA, Gao W, Voss MH, Li W, Martini DJ, et al. Genomic correlates of response to immune checkpoint therapies in clear cell renal cell carcinoma. Science 2018;359:801–6.
44. Gong J, Chehrazi-Raffle A, Reddi S, Salgia R. Development of PD-1 and PD-L1 inhibitors as a form of cancer immunotherapy: a comprehensive review of registration trials and future considerations. J Immunother Cancer 2018;6:8.
45. Luen SJ, Savas P, Fox SB, Salgado R, Loi S. Tumour-infiltrating lymphocytes and the emerging role of immunotherapy in breast cancer. Pathology 2017;49:141–55.

46. de Vries NL, van de Haar J, Veninga V, Chalabi M, Ijsselsteijn ME, van der Ploeg M, et al. γδ T cells are effectors of immunotherapy in cancers with HLA class I defects. Nature 2023;613:743–50.
47. Geurts B, Zeverijn LJ, Battaglia TW, van de Haar J, van Berge Henegouwen J, Hoes LR, et al. Efficacy and predictors of response of nivolumab in treatment-refractory MSI solid tumors: results of a tumor-agnostic DRUP cohort. J Clin Oncol 2023;41:2590.
48. Overman MJ, McDermott R, Leach JL, Lonardi S, Lenz HJ, Morse MA, et al. Nivolumab in patients with metastatic DNA mismatch repair-deficient or microsatellite instability-high colorectal cancer (CheckMate 142): an open-label, multicentre, phase 2 study. Lancet Oncol 2017;18:1182–91.
49. Snyder A, Wolchok JD, Chan TA. Genetic basis for clinical response to CTLA-4 blockade. N Engl J Med 2015;372:783.
50. Alborelli I, Leonards K, Rothschild SI, Leuenberger LP, Savic Prince S, Mertz KD, et al. Tumor mutational burden assessed by targeted NGS predicts clinical benefit from immune checkpoint inhibitors in non-small cell lung cancer. J Pathol 2020;250:19–29.
51. Huang D, Zhang F, Tao H, Zhang S, Ma J, Wang J, et al. Tumor mutation burden as a potential biomarker for PD-1/PD-L1 inhibition in advanced non-small cell lung cancer. Target Oncol 2020;15:93–100.
52. Alva AS, Mangat PK, Garrett-Mayer E, Halabi S, Hansra D, Calfa CJ, et al. Pembrolizumab in patients with metastatic breast cancer with high tumor mutational burden: results from the targeted agent and profiling utilization registry (TAPUR) study. J Clin Oncol 2021;39:2443–51.
53. Passaro A, Stenzinger A, Peters S. Tumor mutational burden as a pan-cancer biomarker for immunotherapy: the limits and potential for convergence. Cancer Cell 2020;38:624–5.
54. Kazdal D, Endris V, Allgauer M, Kriegsmann M, Leichsenring J, Volckmar AL, et al. Spatial and temporal heterogeneity of panel-based tumor mutational burden in pulmonary adenocarcinoma: separating biology from technical artifacts. J Thorac Oncol 2019;14:1935–47.
55. Emens LA. Breast cancer immunotherapy: facts and hopes. Clin Cancer Res 2018;24:511–20.
56. Vonderheide RH, Domchek SM, Clark AS. Immunotherapy for breast cancer: what are we missing? Clin Cancer Res 2017;23:2640–6.
57. Pellegrino B, Tommasi C, Cursio OE, Musolino A, Migliori E, De Silva P, et al. A review of immune checkpoint blockade in breast cancer. Semin Oncol 2021;48:208–25.
58. Dai J, Cui Y, Liang X, Xu J, Li J, Chen Y, et al. PBRM1 mutation as a predictive biomarker for immunotherapy in multiple cancers. Front Genet 2022;13:1066347.
59. Jiang T, Chen X, Su C, Ren S, Zhou C. Pan-cancer analysis of ARID1A alterations as biomarkers for immunotherapy outcomes. J Cancer 2020;11:776–80.