Analyses of PD-L1 and Inflammatory Gene Expression Association with Efficacy of Nivolumab ± Ipilimumab in Gastric Cancer/Gastroesophageal Junction Cancer A C

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ABSTRACT

Purpose: In advanced gastric cancer/gastroesophageal junction cancer (GC/GEJC), there is a need to identify biomarkers of response to therapies, such as immune checkpoint inhibitors.

Patients and Methods: In *post hoc* exploratory analyses from CheckMate 032 (GC/GEJC cohort), we evaluated associations between nivolumab ± ipilimumab (NIVO ± IPI) efficacy and programmed death ligand 1 (PD-L1) expression, defined by tumor cells (% TC) or combined positive score (CPS; sum of PD-L1–staining TCs + immune cells, divided by total viable TCs, × 100) using the Dako PD-L1 IHC 28-8 pharmDx assay, or inflammatory gene expression.

Results: There was a trend toward increased efficacy (objective response and overall survival) when PD-L1 expression was determined by CPS compared with % TC at higher cutoffs of ≥5 and ≥10 in the pooled analysis of all treatment regimens. In this analysis, 19%

and 26% of patients with PD-L1–positive tumors at a CPS cutoff of ≥5 and ≥10, respectively, had an objective response compared with 8% and 9% of patients at the equivalent % TC cutoffs. Longer survival was demonstrated in patients with PD-L1–positive (defined by CPS cutoffs of ≥5 and ≥10) versus PD-L1–negative status. Similar results were observed in the NIVO 1 mg/kg + IPI 3 mg/kg subgroup. Multiple inflammatory gene signatures/transcripts, including a signature consisting of four genes (*CD274, CD8A, LAG3,* and *STAT1*), showed associations with response to NIVO ± IPI.

Conclusions: This study suggests a greater association of PD-L1 expression by CPS with NIVO ± IPI efficacy compared with % TC PD-L1 expression in patients with GC/GEJC. Inflammatory signatures were also associated with NIVO ± IPI response, warranting further investigation.

See related commentary by Moutafi and Rimm, p. 3812

Introduction

With over a million new cases in 2018, gastric cancer/gastroesophageal junction cancer (GC/GEJC) is currently the fifth most common malignancy and the third leading cause of cancer-related mortality worldwide (1). Most patients are diagnosed with advanced, unresectable disease, resulting in poor prognosis with a median survival time of approximately 4 months without treatment (2). Although systemic chemotherapy remains a standard-of-care first-line treatment for

advanced GC/GEJC (and in combination with trastuzumab in patients with HER2-positive tumors), most patients experience disease progression, at which point treatment options are further limited (2). Accordingly, there is an urgent need for both new therapies and biomarkers to identify patients who may respond best to emerging treatments such as immune checkpoint inhibitors (ICI).

Nivolumab (NIVO), a programmed death-1 (PD-1)–blocking antibody, has been investigated in GC/GEJC in the CheckMate 032, 577, 649, and ATTRACTION-2 and -4 studies (3–7). Based on the results of the ATTRACTION-2 study, NIVO is currently approved in Russia, Chile, China, Japan, Taiwan, Singapore, Switzerland, and South Korea for the treatment of patients with chemotherapy-refractory GC/GEJC regardless of PD-L1 status (8, 9). In the United States, the anti–PD-1 antibody pembrolizumab is approved for the treatment of patients with chemotherapy-refractory, PD-L1–positive GC/GEJC based on the results of the KEYNOTE-059 study (10), with PD-L1 expression by immunohistochemistry (IHC) defined as a combined positive score (CPS; number of PD-L1–staining tumor cells and immune cells divided by the total number of viable tumor cells, multiplied by 100) ≥1 (Dako PD-L1 IHC 22C3 pharmDx assay; Agilent Technologies Inc.; ref. 11).

Given the observed clinical benefit of ICIs, including NIVO ± IPI [a cytotoxic T lymphocyte antigen-4 (CTLA-4)–blocking antibody], in GC/GEJC (4, 7), there is currently an effort to systematically evaluate drivers of benefit in this tumor type. Biomarkers currently under investigation include PD-L1 expression by IHC and inflammatory gene expression signatures by gene expression profiling (GEP; refs. 12–14).

In patients with metastatic GC/GEJC in CheckMate 032, responses to NIVO ± IPI were observed regardless of tumor cell PD-L1 expression, although objective response rates (ORRs) seemed numerically higher in patients with PD-L1–positive tumors defined by ≥1% PD-L1 staining of tumor cell membranes (% TC) than in patients with

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Translational Relevance

Given the limited treatment options for patients with gastric cancer/gastroesophageal junction cancer (GC/GEJC), efforts are ongoing to identify patients more likely to respond to therapies such as immune checkpoint inhibitors. Combined assessment of programmed death ligand 1 (PD-L1) expression on both tumor and immune cells, as well as inflammatory gene expression signatures, may provide a more comprehensive representation of inflammation within the tumor microenvironment (TME). We explored associations of these biomarkers with clinical efficacy of nivolumab ± ipilimumab in patients with GC/GEJC. Our analyses identified a greater association of efficacy with PD-L1 expression defined by scoring both tumor and immune cells than by scoring tumor cells alone. In addition, inflammatory gene signatures were associated with response. These results highlight the importance of understanding mechanisms of inflammation in the TME and support use of PD-L1 assays and gene expression profiling as viable approaches to guide further research.

PD-L1–negative tumors (4). ORR also seemed higher in patients with PD-L1–positive tumors (defined by CPS ≥ 1) than with PD-L1–negative tumors in the study of pembrolizumab in advanced GC/GEJC (KEYNOTE-059; ref. 10). However, sample sizes were small, with 95% confidence intervals (CI) overlapping in these studies. In other tumor types, there has been further investigation into the utility of PD-L1 expression by various scoring methods, assays, and cutoffs. For example, in subgroup analyses of patients with advanced squamous non–small cell lung cancer (NSCLC; CheckMate 017) or nonsquamous NSCLC (CheckMate 057), higher levels of tumor cell PD-L1 expression (defined by increasing % TC cutoffs of <1%, ≥1%, ≥5%, ≥10%, and ≥50%) were associated with greater magnitude of overall survival (OS) benefit with NIVO in the second-line setting (15). Similar analyses in GC/GEJC have been scarce; this provides a rationale for investigating PD-L1 expression by % TC and CPS as potential predictive markers for clinical efficacy in patients with GC/GEJC who were treated with NIVO ± IPI in CheckMate 032.

Inflammatory gene expression signatures can be used to assess a complex, inflamed phenotype in the tumor microenvironment (TME), which may be indicative of pre-existing T-cell immunity that could be enhanced by checkpoint blockade (12, 16). Several signatures of inflammation within the TME have been associated with clinical response across multiple tumor types, consistent with the antitumor T-cell–promoting mechanisms of action of ICIs (12).

In the current *post hoc* exploratory analyses from CheckMate 032, we sought to assess associations between PD-L1 expression or multiple inflammatory gene signatures and efficacy outcomes in patients treated with NIVO ± IPI. This may help characterize key drivers of NIVO ± IPI efficacy in patients with metastatic GC/GEJC that potentially have diverse inflammatory characteristics.

Patients and Methods

Study design and patients

CheckMate 032 (NCT01928394) is a phase I/II, open-label, dose-escalation and expansion study of NIVO ± IPI in advanced or metastatic solid tumors, including GC, GEJC, and esophageal adenocarcinoma (EAC), defined here as the GC/GEJC cohort (4). Key eligibility criteria included one or more prior chemotherapy regimens, Eastern Cooperative Oncology Group performance status (ECOG PS) of ≤1, and no prior treatment with T-cell costimulation ICIs or antitumor vaccine therapy. Patients were assigned to receive either NIVO monotherapy at 3 mg/kg i.v. every 2 weeks (Q2W) or in combination with IPI for four cycles followed by NIVO monotherapy using one of two regimens: NIVO 1 mg/kg + IPI 3 mg/kg every 3 weeks (Q3W; NIVO1 + IPI3) or NIVO 3 mg/kg + IPI 1 mg/kg Q3W (NIVO3 + IPI1; Supplementary Fig. S1; ref. 4). In addition, there were three patients treated with NIVO 1 mg/kg + IPI 1 mg/kg (NIVO1 + IPI1) as part of the initial dose-escalation safety evaluation, followed by NIVO3 Q2W. The study protocol and all amendments were approved by local institutional review boards, and the protocol was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice Guidelines, as defined by the International Conference on Harmonisation of Technical Requirements for Pharmaceuticals for Human Use. All patients provided written informed consent before enrollment.

Efficacy assessments

Objective responses were assessed by investigators and by blinded independent central review (BICR). BICR-assessed response was used for analyses presented here. Best overall response (BOR) was evaluated as complete response (CR), partial response (PR), stable disease (SD), progressive disease (PD), or not evaluable (NE), according to RECIST v1.1. Tumor response was assessed using imaging every 6 weeks for 24 weeks, then every 12 weeks until disease progression or treatment discontinuation. ORR was calculated as the percentage of patients with CR or PR in each patient subgroup. OS was also assessed (4).

Biomarker assessments

PD-L1 expression

PD-L1 expression was assessed in baseline formalin-fixed, paraffin-embedded (FFPE) archival tumor samples or fresh biopsy specimens (obtained during screening period prior to treatment start). PD-L1 expression was determined at a central laboratory using a validated automated IHC assay (Dako PD-L1 IHC 28-8 pharmDx assay; Agilent Technologies Inc.) and reported as the percentage of PD-L1–positive tumor cells as defined by complete or partial membrane staining at any level (% TC).

PD-L1 expression according to CPS was determined retrospectively by Agilent-certified pathologists in a Clinical Laboratory Improvement Amendments–certified central laboratory for available patient samples by rescoring the PD-L1 IHC 28-8–stained slides using the CPS algorithm [defined as the number of tumor cells with PD-L1 cell membrane staining plus the number of immune cells (lymphocytes, macrophages) with cell membrane or intracellular PD-L1 staining, divided by the total number of viable tumor cells, and multiplied by 100; ref. 17]. A total of three certified pathologists assessed PD-L1 expression on 104 tumor samples using CPS. Of these samples, 32 were scored by one pathologist and verified and approved by another, with the remaining 72 only scored by one pathologist. There were no disagreements between the two pathologists for any of these 32 samples. Details on pathologist information can be found in Supplementary Table S1. Corresponding % TC and CPS OS and response data were collated (Supplementary Table S2) for the entire cohort.

Gene expression profiling

GEP of inflammatory gene expression signatures was performed retrospectively using RNA sequencing (RNA-seq) on a subset of baseline FFPE tumor samples. Nine inflammatory gene expression signatures or individual transcripts were evaluated. These included several published signatures (12, 16) and a 4-gene inflammatory signature

[CD274 (*PD-L1*), *CD8A*, *LAG3*, and *STAT1* (patent numbers WO2020/198672 and WO2020/198676); refs. 18–20]. The components of this inflammatory signature reflect key functions in immune modulation and prediction of cytotoxic response [*CD274* (*PD-L1*) and *LAG3*], cytotoxic T-cell tumor infiltration (*CD8A*), and IFNγ signaling (*STAT1*; refs. 21–24).

For RNA-seq, paired-end FASTQ files were analyzed using the Seven Bridges platform (Seven Bridges Genomics). FASTQ reads were mapped to the GRCh37 genome reference using Spliced Transcripts Alignment to a Reference (STAR) software (25). Gene expression was calculated using the RSEM package (26) v1.1.13 and Ensembl GRCh37 v75 gene annotation. An additional step of calculating gene quantile-normalized read counts was performed using a custom Practical Extraction and Report Language (Perl) script from the Cancer Genome Atlas mRNA-seq pipeline. Ensembl gene identifiers were translated to gene symbols, adding multiple Ensembl gene values to obtain a symbol-level value where appropriate. All gene-normalized expression data for samples were collated into a table (Supplementary Table S3) for the entire cohort (27–30).

Statistical methods

Associations between PD-L1 expression status (% TC and CPS) and objective response were assessed using descriptive statistics, ROC analyses, and logistic regression models. A Welch *t* test was used to evaluate association of CPS and % TC with objective response (CR/PR vs. SD/PD/NE). Nested logistic regression models were used to assess the relative contributions of CPS and % TC to the association with objective response probability. These models included CPS alone, % TC alone, or both CPS and % TC as predictor variables, with treatment arm and ECOG PS as covariates. The area under the ROC curve (AUC) for CPS was compared with that for % TC by stratified resampling, with bootstrapping 100,000 times using the pROC R package (31). Kaplan–Meier curves were used to illustrate association between OS and PD-L1 expression, classified using various thresholds.

Welch *t* tests were used to assess the association of gene expression signatures or individual transcripts with objective response. Linear models of signature scores or individual gene expression levels, including objective response, treatment arm, and ECOG PS, were also used to assess such associations. Analyses were performed using the R language for statistical computing (32). Limma was used for fitting linear models and performing associated hypothesis tests (33). Corrections for multiple testing were based on the FDR using the p.adjust R function (34, 35).

Results

Baseline characteristics: overall GC/GEJC cohort and biomarker-evaluable subgroups

A total of 160 patients were treated in the GC/GEJC cohort of the CheckMate 032 study. Three eligible patients who were treated with NIVO1 + IPI1 in the dose-escalation phase were also included in the GC/GEJC cohort analyses (Supplementary Fig. S1). Median follow-up was 23 months for the entire GC/GEJC cohort (*n* = 163). Median follow-up for each treatment group was 28, 24, and 22 months for the NIVO3, NIVO1 + IPI3, and NIVO3 + IPI1 subgroups, respectively (Supplementary Fig. S1), and 35 months for the NIVO1 + IPI1 subgroup. PD-L1 expression defined by % TC and by CPS was determined for 130 and 104 patient samples, respectively. Of 163 patients, 63 had tumor tissue samples available for GEP by RNA-seq, 40 of which passed quality control and were included in this analysis. Similar baseline characteristics and

Table 1. Baseline characteristics and clinical response in the CheckMate 032 GC/GEJC cohort and biomarker-evaluable subgroups.

Characteristic, *n* (%)	Overall population *N* = 163	Evaluated by % TC *n* = 130	Evaluated by CPS *n* = 104	GEP analysis *n* = 40
Treatment arm				
NIVO3	59 (36.2)	42 (32.3)	32 (30.8)	11 (27.5)
NIVO3 + IPI1	52 (31.9)	43 (33.1)	36 (34.6)	13 (32.5)
NIVO1 + IPI3	49 (30.1)	42 (32.3)	33 (31.7)	2 (5.0)
NIVO1 + IPI1[a]	3 (1.8)	3 (2.3)	3 (2.9)	14 (35.0)
Age, years				
<65	117 (71.8)	95 (73.1)	78 (75.0)	30 (75.0)
≥65	46 (28.2)	35 (26.9)	26 (25.0)	10 (25.0)
Sex				
Female	37 (22.7)	30 (23.1)	26 (25.0)	10 (25.0)
Male	126 (77.3)	100 (76.9)	78 (75.0)	30 (75.0)
ECOG PS				
0	75 (46.0)	56 (43.1)	45 (43.3)	12 (30.0)
1	88 (54.0)	74 (56.9)	59 (56.7)	28 (70.0)
Disease site				
Esophagus	26 (16.0)	21 (16.2)	14 (13.5)	5 (12.5)
Gastric	61 (37.4)	51 (39.2)	43 (41.3)	13 (32.5)
Gastroesophageal junction	76 (46.6)	58 (44.6)	47 (45.2)	22 (55.0)
Response, *n* (%)				
CR/PR	16 (10.0)	14 (11.0)	11 (10.6)	4 (10.0)
SD/PD/NE	147 (90.0)	116 (89.0)	93 (89.4)	36 (90.0)

[a]Three patients in the dose-escalation phase of NIVO1 + IPI1 were also included in the analysis if data were available.

clinical responses were observed in the overall GC/GEJC cohort of CheckMate 032 and in the PD-L1 expression and GEP biomarker-evaluable subgroups (**Table 1**).

Association of PD-L1 expression: all treatment regimens pooled

Analysis of data pooled across all treatment groups examined % TC and CPS stratified by BOR. Mean % TC in patients with CR/PR was similar to those in patients with SD/PD/NE (*P* = 0.45). In contrast, mean CPS was greater in patients with CR/PR than in those with SD/PD/NE (*P* = 0.0067; **Fig. 1A** and **B**). In addition, analysis of nested logistic regression models of objective response using treatment and ECOG PS as covariates showed that removal of CPS from a model that included both CPS and % TC significantly degraded model fit (*P* = 0.020), whereas the removal of % TC from this model did not (*P* = 0.98). This suggests that evidence for an association of PD-L1 expression with objective response probability was stronger for CPS than for % TC.

ROC curves were also used to assess the association of PD-L1 expression determined by CPS and by % TC with objective response. The estimated AUC for CPS was 76% (95% CI, 63–90; *n* = 104), compared with 60% (95% CI, 46–74; *n* = 130) for % TC (**Fig. 1C** and **D**). In the 104 patients with PD-L1 expression assessed by both CPS and % TC, the AUC for % TC was 63% (95% CI, 46–79; *n* = 104; Supplementary Fig. S2). A direct comparison of the AUCs for CPS and % TC in this population, by significance testing through stratified resampling, was consistent, albeit not statistically significant, with a stronger association for CPS (*P* = 0.071).

The prevalence of PD-L1 positivity and ORR by PD-L1 expression was examined according to different cutoff values and scoring methods (**Table 2**); as expected, PD-L1 prevalence was higher when defined
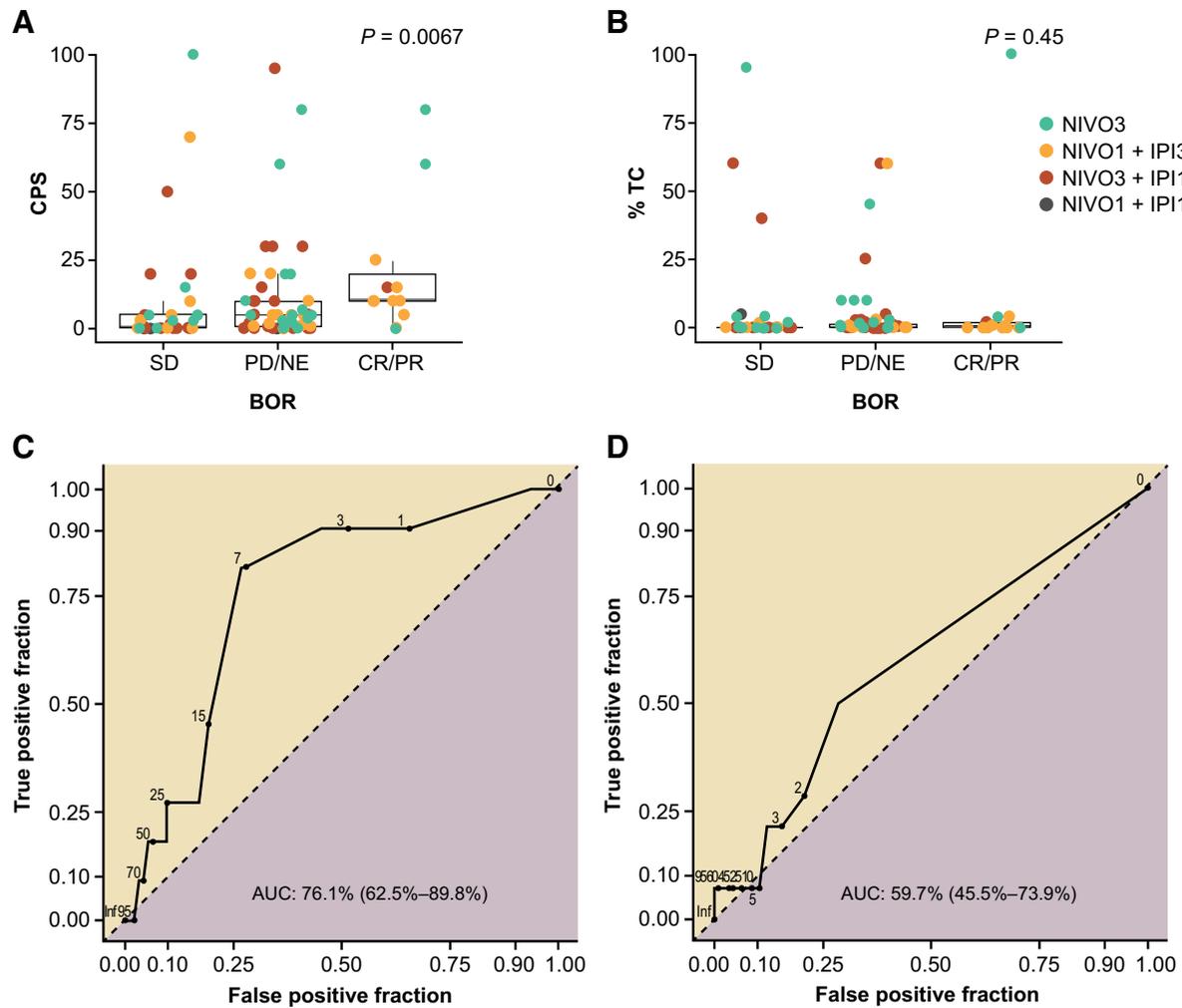
Figure 1.
Association of PD-L1 expression with BOR by (**A**) CPS and (**B**) % TC (all treatment regimens pooled). **C,** ROC analysis (CPS). **D,** ROC analysis (% TC). For boxplots, boxes extend from the first to third quartiles, the middle line shows the median, and the whiskers extend to the most extreme data point, which is no more than 1.5 times the IQR from the box. These hypothesis tests were not prespecified; therefore, *P* values (CR/PR vs. SD/PD/NE) are not intended to demonstrate statistical significance.

by CPS than by % TC alone across all cutoffs, due to the inclusion of immune cells in the CPS. At cutoff values of CPS ≥1 versus % TC ≥1%, the proportion of patients with PD-L1–positive tumors was 68% versus 31%, respectively. At cutoff values of CPS ≥5 and ≥10, PD-L1 prevalence was 50% and 33% versus 10% and 8% at cutoff values of % TC ≥5% and ≥10%, respectively. Nineteen percent and 26% of patients had an objective response at higher cutoff values (≥5 and ≥10) of CPS, respectively, compared with 8% and 9% of patients with an objective response at those same higher cutoffs of % TC, respectively. Odds ratios were also higher at higher cutoff values (≥5 and ≥10) of CPS compared with those same higher cutoffs of % TC, with overlapping CIs (**Table 2**).

Although this study was not designed to demonstrate statistical significance in OS, increasing PD-L1 expression defined by CPS did appear to be associated with improved OS (**Fig. 2A** and **E**). A similar pattern was not apparent for PD-L1 expression defined by % TC (**Fig. 2B** and **E**). The OS HRs for CPS cutoffs of ≥1, ≥5, and ≥10 were

1.13 (95% CI, 0.70–1.82), 0.69 (95% CI, 0.44–1.09), and 0.59 (95% CI, 0.36–0.98), respectively, relative to 0.86 (95% CI, 0.56–1.33), 1.06 (95% CI, 0.57–1.98), and 1.01 (95% CI, 0.51–2.01) for equivalent % TC cutoffs, respectively (**Fig. 2A**, **B**, and **E**).

Association of PD-L1 expression: NIVO 1 mg/kg + IPI 3 mg/kg

A similar analysis was performed comparing PD-L1 positivity and ORR in patients treated with NIVO1 + IPI3. Consistent with the pooled analysis, greater ORRs were seen with increasing PD-L1 expression defined by CPS, where 28%, 41%, and 55% of patients experienced an objective response at the cutoff values of ≥1, ≥5, and ≥10, respectively. In comparison, 10 patients had PD-L1 expression defined by a % TC cutoff of ≥1%, with four (40%) experiencing an objective response. Only one patient had PD-L1 expression defined by % TC cutoffs of ≥5% and ≥10% and did not experience an objective response. At PD-L1 levels <1% defined by % TC, 19% of patients experienced an objective response compared with no patients for

Table 2. Prevalence and response rate by % TC and CPS.

All treatments pooled	Prevalence, n (%)		Number of responders [ORR % (95% CI)]		OR (95% CI)	
	% TC	CPS	% TC	CPS	% TC	CPS
	n = 130	n = 104	n = 130	n = 104	n = 130	n = 104
PD-L1 cutoff[a]						
<1	90 (69)	33 (32)	7 [8 (3–15)]	1 [3 (0–16)]		
≥1	40 (31)	71 (68)	7 [18 (7–33)]	10 [14 (7–24)]	2.50 (0.69–9.07)	5.19 (0.68–234.41)
≥5	13 (10)	52 (50)	1 [8 (0–36)]	10 [19 (10–33)]	0.67 (0.01–5.27)	11.91 (1.58–535.64)
≥10	11 (8)	34 (33)	1 [9 (0–41)]	9 [26 (13–44)]	0.82 (0.02–6.65)	11.91 (2.25–120.79)
NIVO1 + IPI3 arm	% TC	CPS	% TC	CPS	% TC	CPS
	n = 42	n = 33	n = 42	n = 33	n = 42	n = 33
PD-L1 cutoff[a]						
<1	32 (76)	8 (24)	6 [19 (7–36)]	0		
≥1	10 (24)	25 (76)	4 [40 (12–74)]	7 [28 (12–49)]	2.81 (0.44–17.07)	NR (0.49–NR)
≥5	1 (2[b])	17 (52)	0	7 [41 (18–67)]	0 (0–124.56)	NR (1.77–NR)
≥10	1 (2[b])	11 (33)	0	6 [55 (23–83)]	0 (0–124.56)	22.07 (2.05–1201.37)

Abbreviation: NR, not reached.
[a]For % TC, the cutoff is represented as a percentage. For CPS, the cutoff is represented as a score.
[b]Only one patient had % TC PD-L1 ≥5% and ≥10%.

CPS < 1 (**Table 2**). Also consistent with the pooled analysis, there was a higher prevalence of patients with PD-L1–positive tumors defined by CPS than those defined by the equivalent cutoffs for % TC (76%, 52%, and 33% vs. 24%, 2%, and 2% at cutoffs of ≥1, ≥5, and ≥10 for CPS and % TC, respectively; **Table 2**).

Similar to the pooled analysis, increasing PD-L1 expression defined by CPS appeared to be associated with improved OS in the NIVO1 + IPI3 subgroup (**Fig. 2C** and **F**), but not with PD-L1 expression defined by % TC (**Fig. 2D** and **F**). The OS HRs for CPS cutoffs of 1, 5, and 10 were 0.81 (95% CI, 0.31–2.14), 0.21 (95% CI, 0.08–0.58), and 0.16 (95% CI, 0.05–0.56), respectively, and HR for % TC at the cutoff of 1% was 0.49 (95% CI, 0.19–1.31; **Fig. 2C, D**, and **F**). As there was only one patient with % TC above the cutoffs of 5% and 10%, Kaplan–Meier curves were not plotted. These associations with OS in the NIVO1 + IPI3 subgroup were more pronounced than those for all pooled treatment regimens.

Associations between inflammatory gene expression signatures and clinical response

Nine inflammatory gene expression signatures or individual transcripts were analyzed for association with objective response. Despite a limited number of responders and a small sample size, seven of the nine signatures or individual transcripts had positive associations with objective response (defined as an FDR <0.1, as will be discussed later). Responders (CR/PR) tended to have increased signature scores/expression levels compared with non-responders (SD/PD/NE) as assessed by BICR [data are shown in **Fig. 3A** for four of these signatures or individual transcripts (CD8 signature, 4-gene inflammatory signature, 10-gene IFNγ signature, and PD-L1 transcript); data for five additional signatures or individual transcripts are shown in Supplementary Fig. S3A]. We also provided P values for each gene signature or individual transcript in Supplementary Table S4. In addition, linear modeling, with treatment arm and ECOG PS as covariates, suggested association with objective response (defined as an FDR <0.1) for seven of the signatures or transcripts. In particular, objective response was significantly associated (defined as an FDR <0.05) with the 4-gene inflammatory signature, with an FDR equal to 0.038 and an estimated effect size of 3.3 (95% CI, 0.75–9.31; **Table 3**).

ROC curves were also used to assess the predictive performance of the nine inflammatory signatures or transcripts (Supplementary Fig. S3B). Despite the small number of responding patients in the RNA-seq analysis subcohort (n = 4), discrimination of response with an AUC of 90% (95% CI, 78–100) was observed for the 4-gene inflammatory signature (**Fig. 3B**).

Discussion

The *post hoc* exploratory analyses of CheckMate 032 in patients with metastatic GC/GEJC presented here suggest that efficacy of NIVO ± IPI increases with higher PD-L1 levels as measured by CPS. In addition, seven of nine inflammatory signatures or individual transcripts tested showed evidence for association with objective response. These results support the initial utility of assessing PD-L1 expression and inflammatory signatures for predicting response in patients receiving ICI therapy and highlight the need for further investigation.

Previously, the potential association of PD-L1 expression status with clinical response and survival outcomes to NIVO ± IPI in metastatic GC/GEJC has been explored using PD-L1 expression defined by ≥1% TC, with results demonstrating clinical activity regardless of PD-L1 expression status (3, 4). However, in the KEY-NOTE-059 study, in which PD-L1 expression was assessed on tumor and immune cells with CPS, greater ORR and longer duration of response to pembrolizumab were observed in patients with PD-L1–positive tumors defined by CPS ≥1 compared with PD-L1–negative tumors, with CIs overlapping (10). In the current analysis, the association of PD-L1 with efficacy by both % TC and CPS was evaluated across different cutoffs. Our nested model analysis suggested that the association between objective response and CPS was stronger than that for % TC. In addition, increasing PD-L1 expression defined by CPS, but not by % TC, appeared to be associated with improved OS across all pooled treatment regimens and in the NIVO1 + IPI3 subgroup. These results suggest that PD-L1 defined by CPS may offer greater predictive utility as a biomarker for ICIs than by % TC alone in GC/GEJC.

A similar need for investigating both tumor and immune cell PD-L1 expression has been observed in various other tumor types. In a study using lung and breast tumor samples, IHC revealed three distinct patterns of PD-L1 expression, as assessed by the Ventana PD-L1
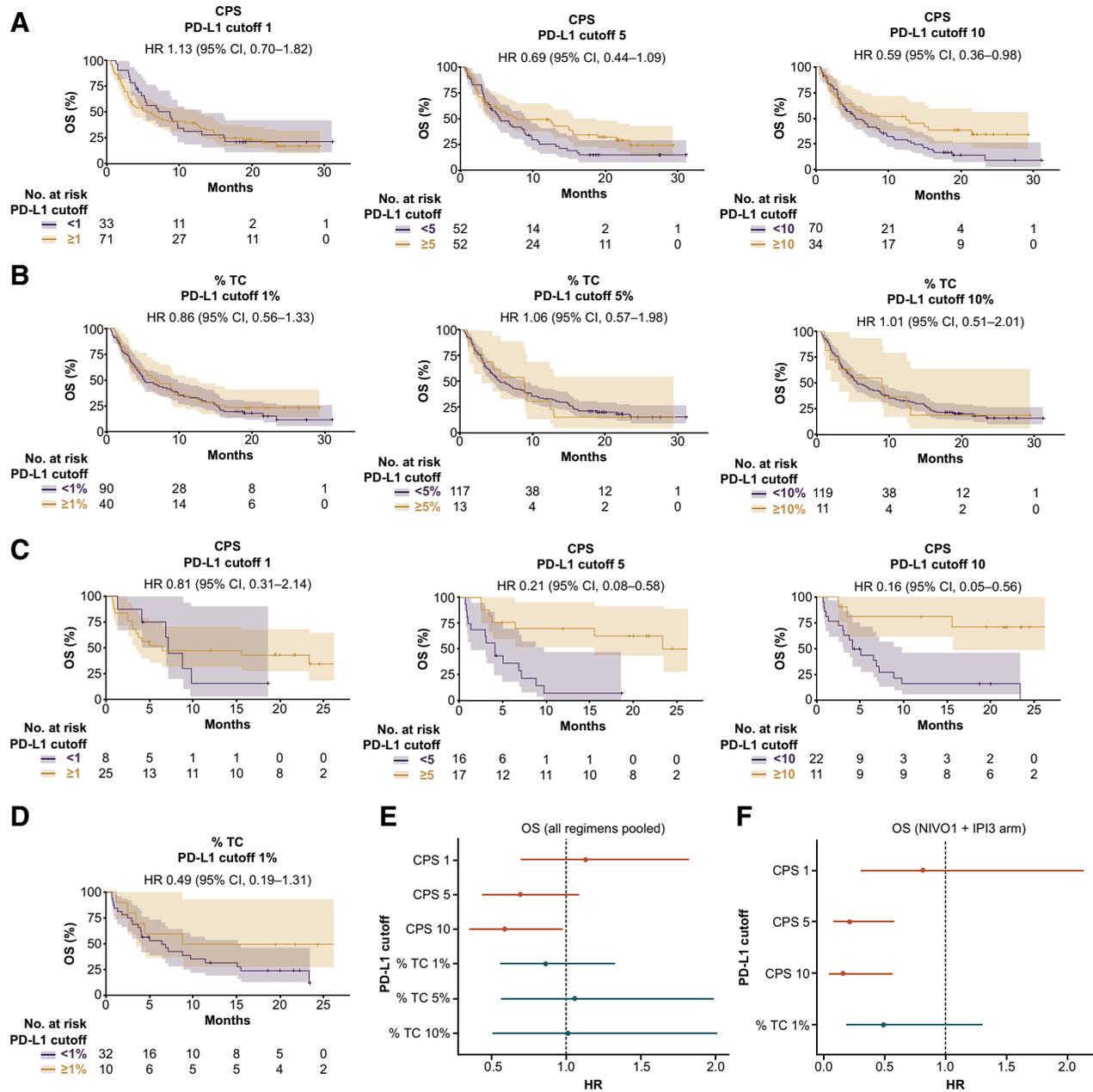
Figure 2.
Association of PD-L1 expression with OS by (**A**) CPS (all treatment regimens pooled), (**B**) % TC (all treatment regimens pooled), (**C**) CPS (NIVO1 + IPI3 arm), and (**D**) % TC (NIVO1 + IPI3 arm). Forest plots of HR estimates for patient subgroups defined by CPS and % TC cutoffs are shown in **E** for all treatment regimens pooled and in **F** for the NIVO1 + IPI3 arm. Gray and orange shaded areas represent survival curve 95% CIs for patients < or ≥ for a given % TC or CPS cutoff, respectively. % TC curves not shown for cutoffs 5% and 10% for the NIVO1 + IPI3 arm as there was only one patient whose % TC PD-L1 expression was ≥5% and ≥10%.

(SP142) assay (Ventana Medical Systems Inc.): mostly associated with epithelial tumor cells, in infiltrating immune cells only, or in both immune cells and tumor cells (36). In mouse models, studies using targeted genetic deletions of PD-L1 in host and tumor cell compartments indicated that PD-L1 expression in both compartments might play important but nonredundant roles in tumor-specific immuno-suppression (36). This prediction was confirmed clinically in studies of NSCLC in which patients with high levels of PD-L1 in tumor cells only

(cutoff 50%; ORR: 40%) or immune cells only (cutoff 10%; ORR: 22%) had durable responses to the anti–PD-L1 antibody atezolizumab (37). A similar study investigated the predictive value of tumor proportion score (defined as the % viable tumor cells showing partial or complete membrane staining divided by the total number of viable tumor cells present in the sample, and multiplied by 100) and CPS (as assessed by the Dako PD-L1 IHC 22C3 pharmDx assay) in patients with previously treated GC/GEJC. With a CPS cutoff of ≥1, the
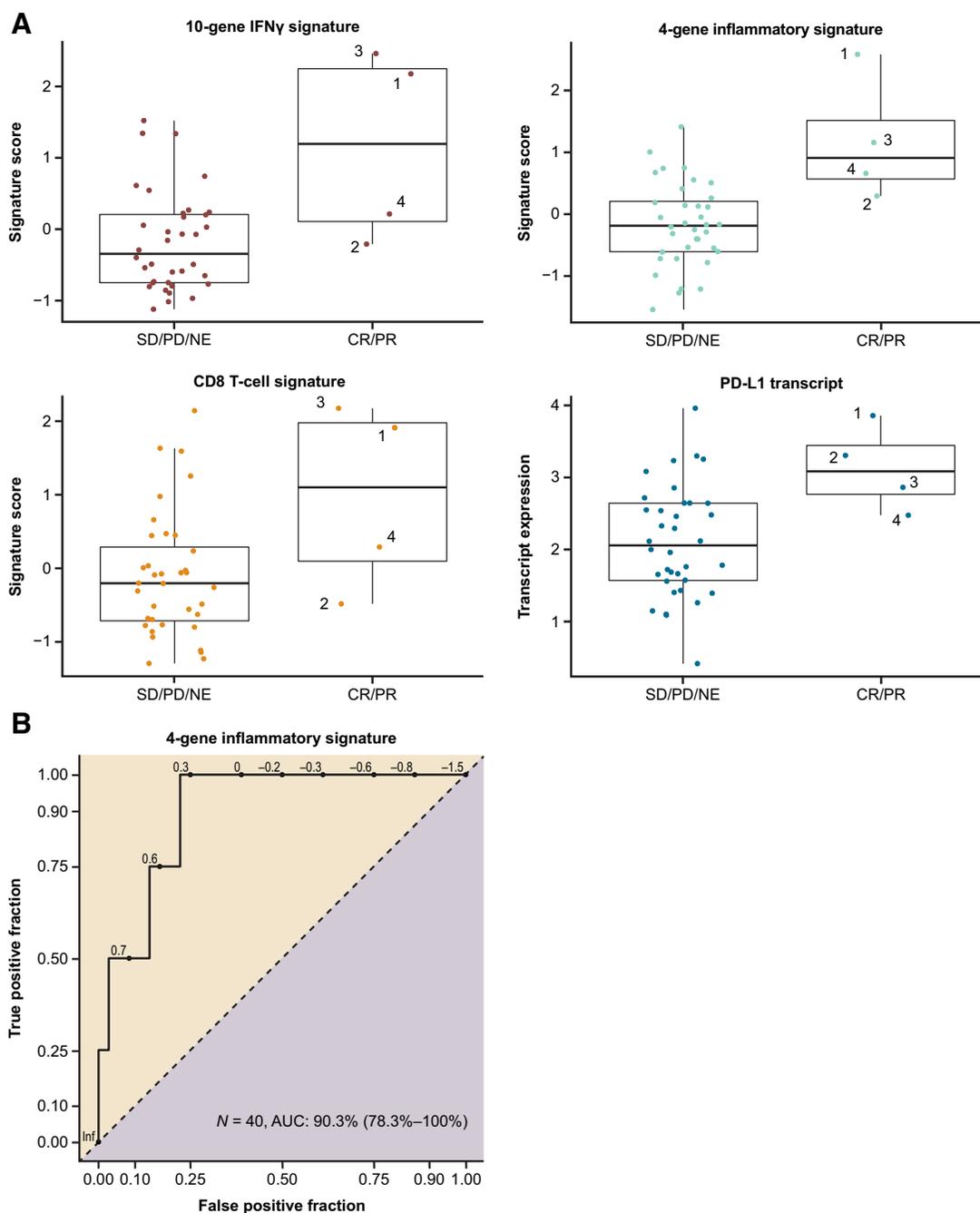
Figure 3.
A, Association of gene expression signatures or individual transcripts with response to NIVO ± IPI. Individual patients with clinical benefit are labeled 1 to 4. **B,** ROC analysis (4-gene inflammatory signature). For boxplots, boxes extend from the first to third quartiles, the middle line shows the median, and the whiskers extend to the most extreme data point, which is no more than 1.5 times the IQR from the box.

prevalence of PD-L1 expression was 57.6% with reasonable enrichment of responses to pembrolizumab (OR, 2.8), whereas with a tumor proportion score cutoff of ≥1, prevalence was 12.5% with minimal enrichment (OR, 1.4; ref. 38).

It has been hypothesized that analysis of CPS may be particularly important when the prevalence of tumor cell PD-L1 positivity is low, as observed in patients with GC/GEJC (3). In our study, as expected, a

greater number of patients had PD-L1–positive tumors based on CPS compared with % TC. Therefore, CPS may identify a greater number of patients more likely to respond to ICI therapy, driven largely by the inclusion of patients whose tumors possess PD-L1–positive immune cells in addition to those with only tumor cell positivity. In fact, in GC, a higher percentage of tumor-infiltrating immune cells express PD-L1 than tumor cells (39). This may provide a more comprehensive picture

Table 3. Gene expression signatures or individual transcripts and association with response: all treatment regimens pooled.

Gene signatures/transcripts	Genes included in signature	Estimate[a] (95% CI)	FDR[b]
4-gene inflammatory signature (16)	*CD274* (PD-L1), *CD8A, LAG3, STAT1*	3.3 (0.75–9.31)	0.038
LAG3 transcript	*LAG3*	2.3 (0.25–5.86)	0.081
CD8 T-cell signature (16)	*CD8A, CD8B*	1.7 (0.14–4.06)	0.081
10-gene IFNγ signature (12)	*CCR5, CXCL9, CXCL10, CXCL11, GZMA, HLA-DRA, IDO1, IFNG, PRF1, STAT1*	1.4 (0.08–3.34)	0.081
IFNγ signature	*IFNG*	0.96 (0.01–2.19)	0.086
PD-L1 transcript	*CD274*	1.8 (−0.08–4.6)	0.087
13-gene inflammatory signature (20)	*CCL2, CCL3, CCL4, CD8A, CXCL9, CXCL10, GZMK, HLA-DMA, HLA-DMB, HLA-DOA, HLA-DOB, ICOS, IRF1*	1.7 (−0.11–4.85)	0.087
T-cell signature (12)	*CD2, CD3D, CD3E*	1.2 (−0.47–3.31)	0.19
DC1 signature	*CLEC9A, FLT3, XCR1*	0.79 (−0.42–2.35)	0.20

[a]For *LAG3* and *CD274* (PD-L1) transcripts, estimated effect size is represented as the log2 change in transcript expression between responder (CR/PR) and nonresponder (SD/PD/NE) groups. For all signatures, estimated effect size is represented as the change in mean signature expression between CR/PR and SD/PD/NE groups in units of signature standard deviation. Transcripts comprising the gene signature were individually fit to a standard distribution across all samples, and the signature score was computed as a median of z-transformed signature transcript values within each sample. Logistical regression was used to estimate association of response and 95% CI.
[b]FDR derived from testing using nine prespecified signatures and transcripts. An FDR <0.1 suggests a positive association with response, whereas an FDR <0.05 suggests a significant association with response.

of the inflammatory TME and might be a better indicator of NIVO ± IPI efficacy in GC/GEJC than assessing expression in tumor cells only. The increased efficacy observed with PD-L1 positivity defined by CPS, compared with % TC, supports the relevance of assessing PD-L1 expression in both tumor and immune cells in GC/GEJC. Additional data from larger controlled prospective studies are needed to investigate clinically relevant cutoffs, predictive value, and clinical utility of CPS in GC/GEJC.

Alongside the PD-L1 IHC results, analysis of gene expression signatures using RNA-seq provided a more expansive method for assessing inflammation within the TME, complementing information about potential involvement of the PD-1/PD-L1 pathway provided by CPS analysis. PD-L1 transcript expression from both tumor and immune cells showed a positive association with NIVO ± IPI efficacy. Associations have been reported between immune-related gene signatures and efficacy with anti–PD-1 inhibitor monotherapy in patients with GC (12, 40). In addition, seven of the nine gene expression signatures or individual transcripts evaluated in this exploratory study, including the 4-gene inflammatory signature (*CD274, CD8A, LAG3,* and *STAT1*; ref. 18), showed evidence for association with objective response, providing a snapshot of the baseline inflammatory milieu within the TME. Although the number of responding patients was small, results from an ROC analysis of the 4-gene inflammatory signature suggest its potential as a predictive biomarker for ICI therapy in GC/GEJC.

NIVO and IPI have distinct but complementary mechanisms of action. By blocking CTLA-4, IPI induces *de novo* antitumor T-cell responses in the lymph nodes (41, 42). Meanwhile, anti–PD-1 blockade with NIVO increases cytokine production and reactivates preexisting T-cell cytotoxicity in the TME (41–43). The 4-gene inflammatory signature may reflect this pre-existing antitumor immunity with tumor-infiltrating CD8$^+$ T cells (*CD8A* expression) that are in an active IFNγ-mediated antitumor environment (*STAT1* expression) but are in a nonresponsive or exhaustive state driven by PD-L1 (*CD274* expression) and LAG-3 (*LAG3* expression). TMEs such as these may be the most responsive to ICI therapy; indeed, similar trends have been observed in GEP studies of anti–PD-1-based immunotherapy for advanced melanoma and hepatocellular carcinoma (18, 19). Investi-

gation of other gene expression signatures that may reflect inflammatory responses in the TME is ongoing. For example, CD8-derived gene expression signatures have been shown to reflect T-cell inflammation in the TME or its stromal and parenchymal compartments in melanoma and squamous cell carcinoma of the head and neck (44, 45), which may be predictive of the degree of response to checkpoint blockade.

The exploratory analyses presented here have some limitations. First, CheckMate 032 is an open-label study with no standard-of-care comparator to treatment with NIVO ± IPI. Therefore, a prognostic versus predictive nature of the proposed biomarkers cannot be distinguished and requires further evaluation in future controlled studies. In addition, the identification of potential biomarkers of response was limited by the small sample sizes. It is important to note that the GEP cohort ($n = 40$) was a small subset of the overall study population ($N = 163$) because of the limited tissue available for RNA-seq analysis; however, baseline characteristics and response rate were comparable with the overall population. Due to the small number of patients with CR/PR within the GEP cohort ($n = 4$), data relating to the association between gene expression signatures and clinical efficacy should be considered exploratory and hypothesis-generating only.

Additional investigations in larger controlled studies, including those with combination regimens, are needed to evaluate the predictive value and clinical utility of PD-L1 status and inflammatory gene expression signatures as biomarkers of efficacy for ICI-based therapies in GC/GEJC. For example, the phase III CheckMate 649 (NCT02872116) trial evaluated the efficacy of NIVO + chemotherapy versus chemotherapy alone in patients with advanced GC/GEJC or EAC. NIVO + chemotherapy demonstrated significant survival benefit in patients with CPS ≥5, as well as those with CPS ≥1 and all randomized patients, with OS benefit enriched at CPS ≥5 (7).

To our knowledge, this report is the first comparison between % TC and CPS across different cutoffs and analysis of the 4-gene inflammatory signature, among other gene signatures or individual transcripts, with respect to clinical efficacy of ICIs in GC/GEJC. Our results demonstrate the value of evaluating both tumor and immune cell PD-L1 by CPS relative to % TC and at higher cutoff values (i.e., ≥5

or ≥10), which could potentially identify more responders to NIVO ± IPI therapy in advanced GC/GEJC. In addition, results from multiple inflammatory gene expression signatures reported here add validity to this approach, furthering our understanding of molecular drivers of response to ICIs in advanced GC/GEJC.

Authors' Contributions

M. Lei: Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing–original draft, project administration, writing–review and editing, data interpretation. **N.O. Siemers:** Conceptualization, data curation, software, formal analysis, validation, visualization, methodology, writing–review and editing. **D. Pandya:** Data curation, validation, methodology, writing–review and editing. **H. Chang:** Resources, data curation, formal analysis, supervision, validation, investigation, visualization, methodology, writing–review and editing. **T. Sanchez:** Conceptualization, writing–original draft, writing–review and editing, data interpretation. **C. Harbison:** Conceptualization, supervision, writing–review and editing. **P.M. Szabo:** Data curation, formal analysis, validation, investigation, visualization, methodology, writing–review and editing. **Y. Janjigian:** Conceptualization, resources, data curation, software, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing–original draft, project administration, writing– review and editing. **P.A. Ott:** Data curation, writing–review and editing. **P. Sharma:** Writing–review and editing. **J. Bendell:** Resources, investigation, writing–review and editing. **T.R.J. Evans:** Resources, investigation, methodology, writing–original draft, writing–review and editing. **F. de Braud:** Formal analysis, writing–review and editing. **I. Chau:** Data curation, writing–review and editing. **Z. Boyd:** Conceptualization, supervision, writing–review and editing.

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