











# Biomarker analysis from CheckMate 214: nivolumab plus ipilimumab versus sunitinib in renal cell carcinoma

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## ABSTRACT

**Background** The phase 3 CheckMate 214 trial demonstrated higher response rates and improved overall survival with nivolumab plus ipilimumab versus sunitinib in first-line therapy for advanced clear-cell renal cell carcinoma (RCC). An unmet need exists to identify patients with RCC who are most likely to benefit from treatment with nivolumab plus ipilimumab.

**Methods** In exploratory analyses, pretreatment levels of programmed death ligand 1 were assessed by immunohistochemistry. Genomic and transcriptomic biomarkers (including tumor mutational burden and gene expression signatures) were also investigated.

**Results** Biomarkers previously associated with benefit from immune checkpoint inhibitor-containing regimens in RCC were not predictive for survival in patients with RCC treated with nivolumab plus ipilimumab. Analysis of gene expression identified an association between an inflammatory response and progression-free survival with nivolumab plus ipilimumab.

**Conclusions** The exploratory analyses reveal relationships between molecular biomarkers and provide supportive data on how the inflammation status of the tumor microenvironment may be important for identifying predictive biomarkers of response and survival with combination immunotherapy in patients with RCC. Further validation may help to provide biomarker-driven precision treatment for patients with RCC.

## BACKGROUND

Nivolumab combined with ipilimumab or the tyrosine kinase inhibitor (TKI) cabozantinib are among several immune checkpoint inhibitor (ICI) regimens approved as first-line treatment for patients with advanced renal cell carcinoma (RCC).<sup>1–6</sup> After a 48-month minimum follow-up analysis of CheckMate 214, superior survival benefit was observed with nivolumab plus ipilimumab versus sunitinib, the only double-ICI combination therapy currently approved for patients with RCC.<sup>1,7</sup> Given these promising data, there still remains an unmet need to understand the underlying biology of RCC and identify

patients who are most likely to respond to treatment.<sup>8</sup>

Programmed death ligand 1 (PD-L1) expression has shown promise as a biomarker for response to anti-programmed death-1 (PD-1) therapy in a number of cancer types.<sup>9</sup> However, PD-L1 expression alone has been a suboptimal biomarker for patients with RCC and its predictive value is, to date, unclear. A number of studies have indicated that PD-L1 expression is negatively prognostic, but not predictive of response to treatment with ICIs alone or in combination with vascular endothelial growth factor (VEGF) inhibitors in patients with RCC.<sup>10–12</sup> In CheckMate 214, exploratory analysis showed that longer overall survival (OS) with nivolumab plus ipilimumab versus sunitinib was independent of PD-L1 expression on tumor cells (TCs).<sup>1</sup>

A number of DNA-based biomarkers have been associated with response to ICIs. High tumor mutational burden (TMB) and the number of small insertions and deletions (tumor indel burden; TIB) are associated with increased numbers of tumor neoantigens presented on major histocompatibility complexes, facilitating immune recognition of the tumor and the development of an antitumor response.<sup>13</sup> In addition, human leukocyte antigen (HLA) zygosity may affect the presentation of immunogenic antigens, potentially impacting response to ICIs.<sup>14</sup> In RCC, TMB, TIB, and HLA, zygosity have demonstrated limited value in predicting clinical benefit with ICI monotherapy or ICIs in combination with VEGF inhibitors.<sup>12 15 16</sup>

Mutational inactivation of individual genes, including *VHL*, *PBRM1*, *BAP1*, and *SETD2*, is frequent in clear-cell RCC.<sup>15–18</sup> In particular, results are conflicting regarding the association of *PBRM1* mutation with clinical benefit from TKIs. *PBRM1* mutation was associated



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with clinical benefit from ICI monotherapy following prior treatment with VEGF inhibitors,<sup>16,19</sup> but not in previously untreated patients, whether given as monotherapy or in combination with VEGF inhibitors.<sup>12,15</sup>

Gene expression profiling (GEP) can be used to classify disease types and characterize biological processes such as inflammation in the tumor microenvironment (TME).<sup>20,21</sup> An angiogenesis (Angio) gene expression signature was found to be predictive for benefit with sunitinib in the IMmotion150 and JAVELIN Renal 101 trials.<sup>12,15</sup> In patients with sarcomatoid RCC, low Angio signature scores, poor response to sunitinib, and improved responses to ICIs have been observed in some studies.<sup>22,23</sup>

Immune-cell (IC) infiltration has been reported to be associated with prognosis in patients with RCC.<sup>24</sup> The GEP-based microenvironment cell population (MCP)-counter method has been used to identify neutrophils and endothelial cells as having potential roles in immune suppression that may result in poorer clinical outcomes with nivolumab.<sup>25</sup> Improved clinical efficacy with ICIs alone or in combination with VEGF inhibitors has been associated with high scores from a T-effector function (Teff) gene expression signature.<sup>12,15,22</sup> High Teff and low Angio gene expression signature scores were associated with improved response rates with nivolumab,<sup>25,26</sup> and a combined high score from both Teff and Myeloid signatures was associated with improved progression-free survival (PFS) with atezolizumab plus VEGF inhibitor, but not with avelumab plus VEGF inhibitor.<sup>12,15</sup>

In summary, there exists extensive literature evaluating PD-L1 expression, genomic mutations, gene expression, and IC infiltration for associations with clinical benefit from ICI and other therapies in multiple tumor types, including RCC. However, associations between these factors and response to nivolumab plus ipilimumab have not yet been fully investigated in patients with RCC. This comprehensive exploratory post-hoc analysis of patients with RCC from the CheckMate 214 phase 3 trial with a 42-month follow-up period represents a thorough investigation of PD-L1, as well as genomic and transcriptomic biomarkers for response to nivolumab plus ipilimumab.

## METHODS

### Trial design and clinical assessments

The CheckMate 214 (NCT02231749) trial design and clinical assessments have been reported previously.<sup>1</sup> See online supplemental methods for details.

### Exploratory analysis of patient samples

Histological analysis and biomarker assessments were performed on archival formalin-fixed, paraffin-embedded (FFPE) primary and metastatic tumor samples and baseline blood samples from patients subsequently treated in CheckMate 214 (online supplemental table 1).

### Histology and immunohistochemistry

The presence of sarcomatoid features was determined by reviewing local pathology reports accompanying

preassessment tumor samples (n=843) and by independent central review (n=1009) of available hematoxylin and eosin-stained slides, as previously described.<sup>23</sup>

PD-L1 expression was assessed manually by a single pathologist at Covance (Princeton, New Jersey) using the Dako PD-L1 immunohistochemistry (IHC) 28–8 pharmDx assay (Agilent, Santa Clara, California). Tissue sections chosen for analysis were representative of the majority of the tumor. If present, sarcomatoid or rhabdoid areas were selected for analysis. Tissue processing and PD-L1 staining were performed following protocols optimized and approved for FFPE non-squamous non-small cell lung cancer samples.<sup>27</sup> PD-L1 expression was quantified as the percentage of PD-L1-expressing TCs (% TC) as described previously for RCC,<sup>1,28</sup> and patients were categorized as having tumors with PD-L1 expression <1% and ≥1% TCs. PD-L1 was also quantified on TCs and ICs using the combined positive score (CPS), defined as the number of PD-L1-expressing TCs, lymphocytes, and macrophages, divided by the total number of TCs, and multiplied by 100.<sup>29</sup> Patients were categorized as having tumors with CPS <1 or ≥1.

### Assessment of genomic status

Germline and somatic genomic statuses were assessed using whole exome sequencing (WES), see online supplemental methods.

TIB, defined as the total number of indels that are commonly called by two variant callers, Strelka<sup>30</sup> and TNscope,<sup>31</sup> and TMB, defined as the total number of somatic missense mutations,<sup>32</sup> were assessed. HLA genotyping was performed on HLA loci A, B, and C, as previously described.<sup>14</sup> Patients were classified as heterozygous if all three loci were heterozygous and homozygous if one or more loci were homozygous.

### Mutation status

WES data were used to categorize patients as wild type (WT) or mutant (MUT) for all protein-coding genes, based on somatic variant calls of single-nucleotide variants and indels. See online supplemental methods for more details.

### Assessment of transcriptomic signatures

Whole transcriptome analysis was performed using RNA-sequencing (RNA-seq) (see online supplemental methods). In the 213 RNA-seq-evaluable samples from treated patients, scores were derived for three IMmotion150 signatures Angio, Teff, and Myeloid,<sup>15</sup> the JAVELIN Renal 101 signature (JAVELIN),<sup>12</sup> and tumor inflammation signature (TIS).<sup>21</sup> Signature scores were calculated as the median value of Z-scored expression for the constituent transcripts.

### Estimation of cell type abundance in the TME

The abundance of eight tumor-infiltrating IC populations (CD3 +T cells, CD8 +T cells, cytotoxic lymphocytes, natural killer cells, B lymphocytes, monocytic lineage cells, myeloid dendritic cells, and neutrophils) and two

stromal cell populations (endothelial cells and fibroblasts) was assessed within the TME using MCP-counter deconvolution. See online supplemental methods for details.

### Gene set enrichment analysis

A Cox proportional-hazards model was used to estimate the association of each gene's expression with PFS and OS as a continuous variable in RNA-seq-evaluable patients within each treatment arm.

Reverse signed Wald test Z scores from the Cox proportional-hazards models were ranked and then evaluated with the 'GSEA' algorithm (Bioconductor V.3.8)<sup>33</sup> on the HALLMARK gene sets from the Molecular Signatures Database.<sup>33,34</sup>

### Statistical analyses

All analyses were conducted using SAS (SAS Institute) and R V.3.6.1 unless otherwise stated. Patient characteristics and clinical outcomes were compared between the biomarker-evaluable population and the intent-to-treat population using frequency statistics and descriptive statistics. Survival analyses were conducted using the R survival (V.2.44–1.1) and survminer (<https://github.com/kassambara/survminer>) (V.0.4.6) packages. HRs and their 95% CIs for associations with PFS or OS in patient subgroups were assessed by Cox proportional-hazards models and were illustrated with forest plots and Kaplan-Meier plots. Descriptive p values were calculated from Wald tests unless otherwise stated and adjusted for multiple hypothesis testing using the Benjamini-Hochberg Procedure<sup>35</sup>; all comparisons were exploratory, and p values should be interpreted with caution. All comparisons for continuous variables use the two-sided Mann-Whitney test for two groups.

## RESULTS

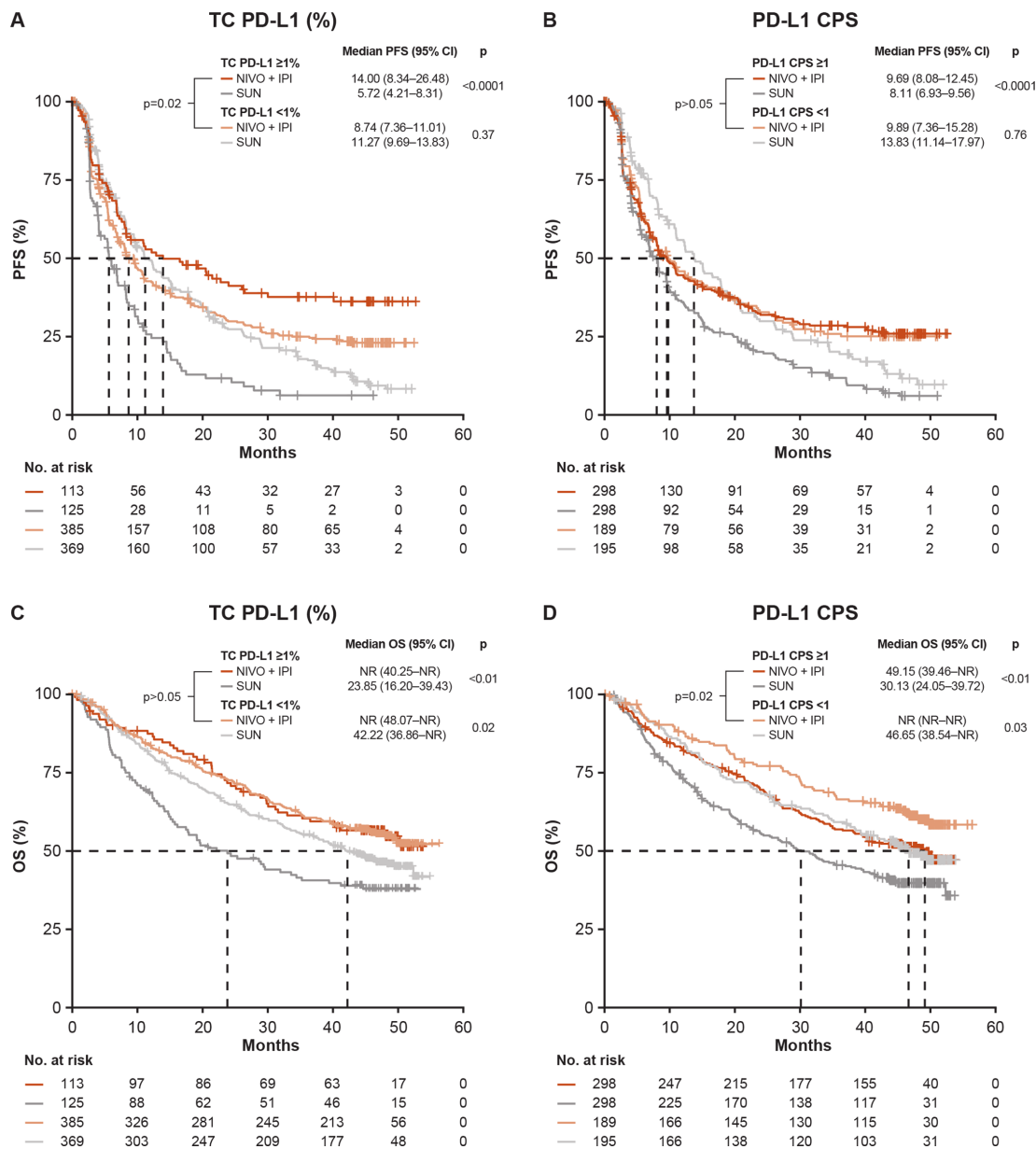
In this exploratory analysis of 1082 treated patients from CheckMate 214, 992 patients were evaluable for PD-L1 expression by IHC, 481 patients were evaluable by WES, and 213 patients were evaluable by RNA-seq (online supplemental tables 1 and 2). Baseline characteristics were similar between each category of biomarker-evaluable patients and the entire treated population (online supplemental table 2). PFS and OS were similar across biomarker-evaluable and treated populations with nivolumab plus ipilimumab and sunitinib (online supplemental figures 1 and 2). Sarcomatoid RCC was identified in 143 patients (13%): 5 with favorable risk and 138 with intermediate/poor risk.<sup>23</sup> Of note, the success rate of RNA analysis was low. Factors influencing the availability of RNA-seq data included the age of the tissue (the median sample age at which RNA-seq data passed quality control was 104 days, while 335 samples that failed had a median age of 219 days from sample procurement), the availability of tumor tissue for biomarker analyses allowed

by patient informed consent, the amount of tissue available, and tissue quality.

OS was improved with nivolumab plus ipilimumab compared with sunitinib, regardless of PD-L1 expression. The association between PD-L1 expression on TCs and survival was previously reported.<sup>36</sup> In this study of patients from CheckMate 214 with a minimum follow-up of 42 months, we expanded this evaluation to include association of combined PD-L1 expression on TCs and ICs by CPS (see Methods) with PFS and OS. In the nivolumab plus ipilimumab arm, 113 of 498 (22.7%) patients were PD-L1-positive by % TC ( $\geq 1\%$  TC); in the sunitinib arm, 125 of 494 (25.3%) patients were PD-L1-positive by % TC. When assessed by CPS, 298 of 487 (61.2%) and 298 of 493 (60.4%) patients were PD-L1-positive (CPS  $\geq 1$ ) in the nivolumab plus ipilimumab arm and sunitinib arm, respectively.

PD-L1 assessed by CPS did not provide a greater predictive power than PD-L1 on TCs for PFS with nivolumab plus ipilimumab. For patients with PD-L1 expression on  $\geq 1\%$  TCs, the median PFS was longer in the nivolumab plus ipilimumab arm than in the sunitinib arm (HR, 0.43 (95% CI, 0.31 to 0.60);  $p < 0.0001$ ). A similar observation was made when comparing PFS with nivolumab plus ipilimumab versus sunitinib in patients evaluated by PD-L1 CPS (HR, 0.68 (95% CI, 0.56 to 0.82);  $p < 0.0001$ ). For patients with PD-L1 expression on  $< 1\%$  TCs or PD-L1 CPS  $< 1$ , PFS was similar in the nivolumab plus ipilimumab and sunitinib arms ( $p > 0.05$ ). Median PFS with nivolumab plus ipilimumab was longer in patients with PD-L1 expression on  $\geq 1\%$  compared with  $< 1\%$  TCs (HR, 0.73 (95% CI, 0.55 to 0.95);  $p = 0.02$ ), while median PFS was similar in CPS  $\geq 1$  and in CPS  $< 1$  (HR, 1.01 (95% CI, 0.81 to 1.26)) (figure 1A,B). The significant associations observed between PD-L1 expression, assessed using either TC scoring or CPS, and PFS were maintained after adjustment for multiple hypothesis testing (online supplemental table 3).

PD-L1 assessed by CPS did not provide a greater predictive power than PD-L1 on TCs for OS with nivolumab plus ipilimumab. For patients with PD-L1 expression on  $\geq 1\%$  TCs, OS was longer in the nivolumab plus ipilimumab arm (median not reached) compared with OS in the sunitinib arm (HR, 0.57 (95% CI, 0.40 to 0.82);  $p < 0.01$ ). A similar observation was made for OS when PD-L1 was evaluated by CPS (HR, 0.72 (95% CI, 0.57 to 0.89);  $p < 0.01$ ). For patients with PD-L1 expression on  $< 1\%$  TC, a trend towards longer median OS was observed in the nivolumab plus ipilimumab arm compared with the sunitinib arm (HR, 0.79 (95% CI, 0.64 to 0.97);  $p = 0.02$ ). The same observation was made for OS when PD-L1 CPS  $< 1$  (HR, 0.71 (95% CI, 0.52 to 0.96);  $p = 0.03$ ). Significant associations between PD-L1 expression and OS were maintained after adjustment for multiple hypothesis testing (online supplemental table 3). Median OS with nivolumab plus ipilimumab was similar between patients with PD-L1 expression on  $< 1\%$  and  $\geq 1\%$  TCs (HR, 0.99 (95% CI, 0.72 to 1.36)), while median OS appeared



**Figure 1** PFS and OS by PD-L1 expression assessed by % TCs or CPS. (A) PFS with nivolumab plus ipilimumab vs sunitinib in patients with PD-L1 expression on <1% and  $\geq 1\%$  TCs. (B) PFS with nivolumab plus ipilimumab vs sunitinib in patients with PD-L1 CPS <1 and  $\geq 1$ . (C) OS with nivolumab plus ipilimumab vs sunitinib in patients with PD-L1 expression on <1% and  $\geq 1\%$  TCs. (D) OS with nivolumab plus ipilimumab vs sunitinib in patients with PD-L1 CPS <1 and  $\geq 1$ . CPS, combined positive score; IPI, ipilimumab; NIVO, nivolumab; NR, not reached; OS, overall survival; PD-L1, programmed death ligand 1; PFS, progression-free survival; SUN, sunitinib; TC, tumor cell.

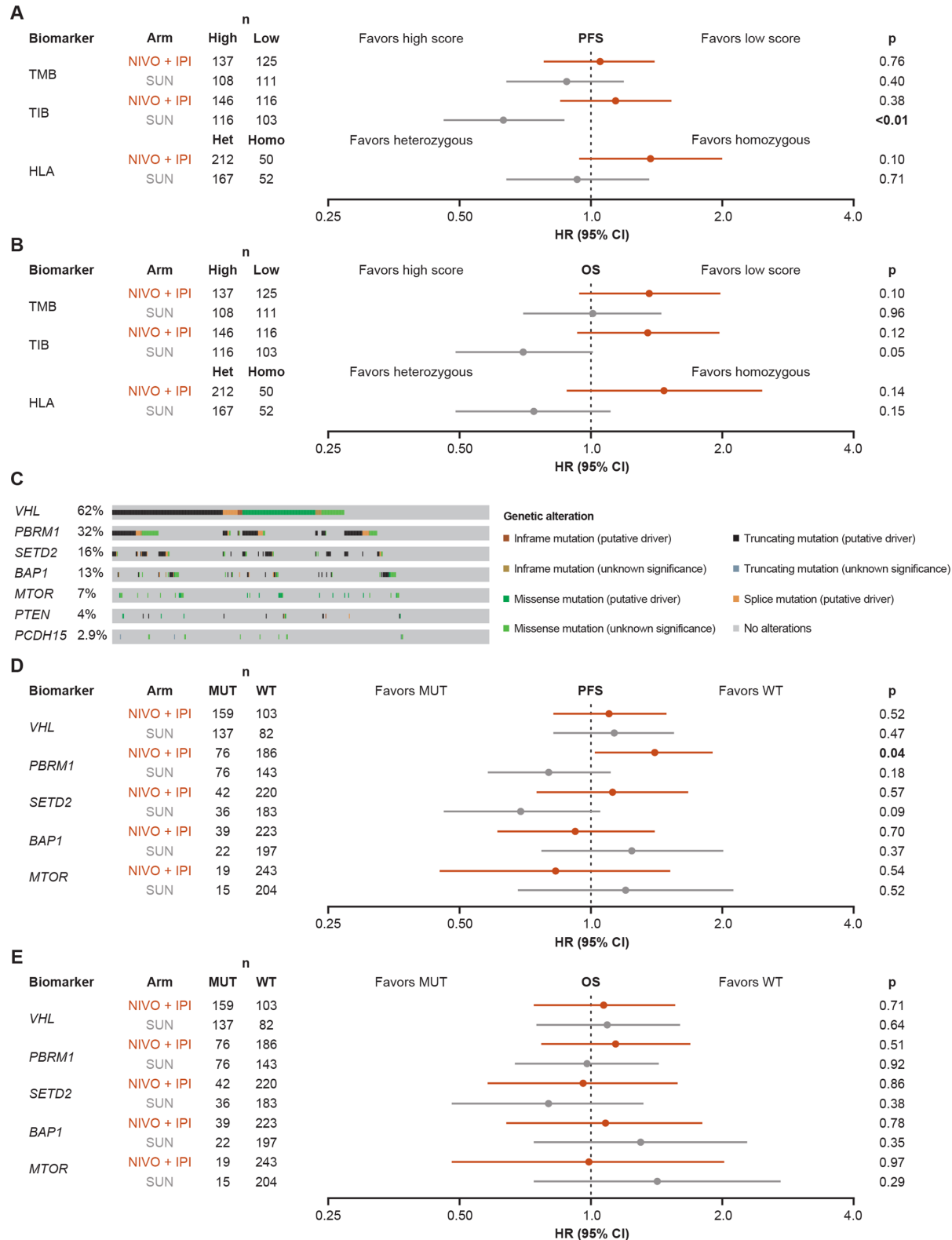
shorter in CPS  $\geq 1$  than in CPS <1 (HR, 1.40 (95% CI, 1.06 to 1.87)) (figure 1C,D).

The analyses described above were also conducted using % TC cut-offs of  $\geq 5\%$  and  $\geq 10\%$ , as well as CPS cut-offs of  $\geq 5$  and  $\geq 10$ , with no substantive difference in findings (data not shown).

#### Genomic features did not associate with survival in patients treated with nivolumab plus ipilimumab

To assess the association between mutation burden and survival, WES-evaluable samples from 262 and 219 patients in the nivolumab plus ipilimumab and the sunitinib arms, respectively, were categorized around the

median values for TMB (50 missense mutations) and TIB (six indels). For the nivolumab plus ipilimumab and the sunitinib arms, 137 and 108 patients were categorized as having high TMB ( $\geq$ median) while 125 and 111 had low TMB (<median), respectively. No association was identified between high versus low TMB levels and PFS or OS in either treatment arm (PFS: nivolumab plus ipilimumab HR, 1.05 (95% CI, 0.78 to 1.40),  $p=0.76$ ; sunitinib HR, 0.88 (95% CI, 0.64 to 1.19),  $p=0.40$ ; OS: nivolumab plus ipilimumab HR, 1.36 (95% CI, 0.94 to 1.98);  $p=0.10$  vs sunitinib HR, 1.01 (95% CI, 0.70 to 1.45);  $p=0.96$ ) (figure 2A,B). For the nivolumab plus



**Figure 2** Association of genomic biomarkers with survival. (A) Forest plot for PFS comparing high ( $\geq$ median) vs low TMB, high ( $\geq$ median) vs low TIB, and heterozygous vs homozygous HLA in the nivolumab plus ipilimumab and the sunitinib arms. (B) Forest plot for OS comparing high ( $\geq$ median) vs low TMB, high ( $\geq$ median) vs low TIB, and heterozygous vs homozygous HLA in the nivolumab plus ipilimumab and the sunitinib arms. (C) Representation of mutations found across both treatment arms in seven selected genes. (D) Forest plot of PFS by mutation status. (E) Forest plot of OS by mutation status. Forest plots show HRs and 95% CIs for given comparisons, and p values compare patient subgroups within each treatment arm. Het, heterozygous; HLA, human leukocyte antigen; Homo, homozygous; IPI, ipilimumab; MUT, mutant; NIVO, nivolumab; OS, overall survival; PFS, progression-free survival; SUN, sunitinib; TIB, tumor indel burden; TMB, tumor mutational burden; WES, whole-exome sequencing; WT, wild type.

ipilimumab and the sunitinib arms, 146 and 116 patients were categorized as having high TIB ( $\geq$ median) while 116 and 103 had low TIB ( $<$ median), respectively. TIB was not associated with either PFS or OS with nivolumab plus ipilimumab (PFS: HR, 1.14 (95% CI, 0.85 to 1.53);  $p=0.38$ ; OS: HR, 1.35 (95% CI, 0.93 to 1.97);  $p=0.12$ ). However, TIB was positively associated with both PFS and OS with sunitinib (PFS: HR, 0.63 (95% CI, 0.46 to 1.87);  $p<0.01$ ; OS: HR, 0.70 (95% CI, 0.49 to 1.01);  $p=0.05$ ) (figure 2A,B). The association between TIB and PFS with sunitinib was maintained after adjustment for multiple hypothesis testing (adjusted  $p=0.01$ ) and in multivariate analyses accounting for age, sex, International Metastatic RCC Database Consortium criteria, and presence of sarcomatoid features ( $p=0.04$ ); however, associations between TIB and OS were not maintained in multivariate analyses or after adjustment for multiple hypothesis testing (online supplemental table 4). Patients were also categorized on the basis of HLA zygosity. No differences were observed for PFS or OS with nivolumab plus ipilimumab in patients with homozygous ( $n=50$ ) or heterozygous ( $n=212$ ) HLA status (PFS: HR, 1.37 (95% CI, 0.94 to 2.00);  $p=0.10$ ; OS: HR, 1.47 (95% CI, 0.88 to 2.47);  $p=0.14$ ) (figure 2A,B).

#### Associations between mutation status and survival in patients treated with nivolumab plus ipilimumab

The association of individual gene mutation status with OS was also assessed in WES-evaluable patients ( $n=481$ ). In total, 382 genes that were mutated in  $\geq 10$  patients (prevalence  $>2\%$ ) were chosen for the association analysis. Online supplementary figure 3 shows the association between mutation status of those genes and OS in the nivolumab plus ipilimumab arm, based on Cox proportional-hazards models. Mutation of either *PTEN* or *PCDH15* was associated with unfavorable OS with nivolumab plus ipilimumab ( $p<0.05$ ). This association should be interpreted with caution, given that only 11 patients with *PTEN* mutations and 4 patients with *PCDH15* mutations were identified in WES-evaluable patients who received nivolumab plus ipilimumab (figure 2C), and no associations with mutation status were maintained after adjustment for multiple hypothesis testing (online supplemental table 5). A subset of five genes were selected for further analysis based on the findings of previous publications and known prevalence in RCC (*PBRM1*, *VHL*, *BAP1*, *SETD2*, and *MTOR*).<sup>12–15–17</sup> The frequency of mutations in these five selected genes was  $>5\%$  across all treated patients (figure 2C). Although an association was suggested for prolonged PFS, but not OS, with nivolumab plus ipilimumab in patients with *PBRM1*<sup>WT</sup> RCC ( $p=0.04$ ), the mutation status of none of these genes was significantly associated with PFS or OS in the nivolumab plus ipilimumab arm or the sunitinib arm after adjustment for multiple hypothesis testing (figure 2D,E; online supplemental table 5).

#### Gene expression signatures are related to tumor characteristics in pretreatment biopsies

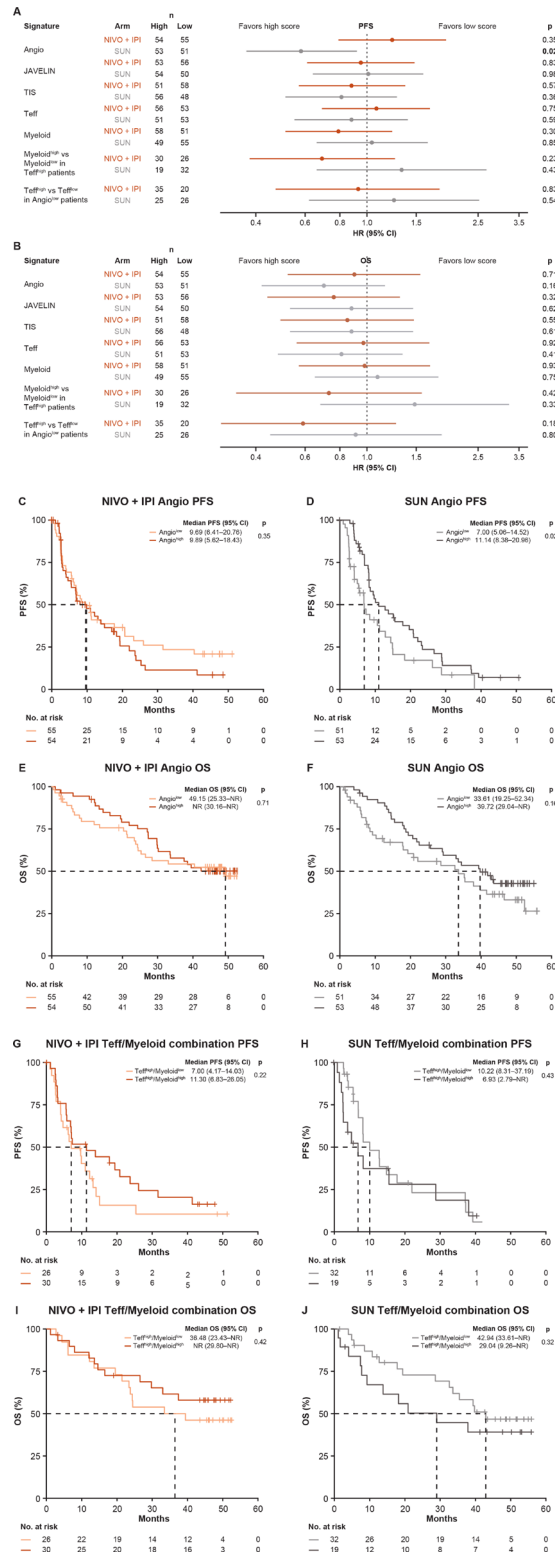
In total, 109 patients in the nivolumab plus ipilimumab arm and 104 patients in the sunitinib arm were RNA-seq-evaluable. RNA-seq data were analyzed to establish scores for published gene expression signatures, and MCP-counter was used to predict the abundance of cell populations in each sample.

Clustering of all 213 RNA-seq samples based on their signature score revealed overlap in sample classification by three gene expression signatures (JAVELIN Renal 101 Immuno signature (JAVELIN), TIS, and IMmotion150 Teff) despite minimal overlap in gene content (online supplemental figure 4). In addition, lower Angio signature scores were associated with sarcomatoid RCC samples ( $p=0.02$ ), while no significant relationship was observed between presence of sarcomatoid features and Myeloid ( $p=0.05$ ) or Teff ( $p=0.56$ ) signature scores (online supplemental figure 5).

#### Previously evaluated gene expression signatures were not associated with survival in patients treated with nivolumab plus ipilimumab

In our RNA-seq-evaluable population, we evaluated associations between published gene expression signatures and survival in each treatment arm. Patients were categorized as having high ( $\geq$ median) or low ( $<$ median) gene expression signature scores using the median score across both treatment arms. Higher Angio scores associated with longer PFS and a numerically higher (although not significant) OS in patients receiving sunitinib (median PFS in Angio<sup>high</sup>, 11.14 months (95% CI, 8.38 to 20.96) vs median PFS in Angio<sup>low</sup>, 7.0 months (95% CI, 5.06 to 14.52);  $p=0.02$ ; median OS in Angio<sup>high</sup>, 39.72 months (95% CI, 29.04 to NR) vs median OS in Angio<sup>low</sup>, 33.61 months (95% CI, 19.25 to 52.34);  $p=0.16$ ) (figure 3A–D). However, the association between Angio score and PFS with sunitinib was not maintained after adjustment for multiple hypothesis testing (online supplemental table 6). Angio score did not predict PFS or OS in patients treated with nivolumab plus ipilimumab (median PFS in Angio<sup>high</sup>, 9.89 months (95% CI, 5.62 to 18.43) vs median PFS in Angio<sup>low</sup>, 9.69 months (95% CI, 6.41 to 20.76);  $p=0.35$ ; median OS in Angio<sup>high</sup>, NR (95% CI, 30.16 to NR) vs median OS in Angio<sup>low</sup>, 49.15 months (95% CI, 25.33 to NR);  $p=0.71$ ) (figure 3A–F). Early separation of OS curves for nivolumab plus ipilimumab vs sunitinib was observed (figure 3E,F), but median OS could be unstable as data to establish 95% CIs have not been reached.

No association between JAVELIN, TIS, Teff, or Myeloid gene expression signature scores with PFS and OS was observed for patients treated with nivolumab plus ipilimumab or sunitinib in the RNA-seq-evaluable population (figure 3A,B). Similarly, no association with PFS or OS was seen in composite analysis of Myeloid<sup>high</sup> versus Myeloid<sup>low</sup> patients in the Teff<sup>high</sup> group, or in Teff<sup>high</sup> versus Teff<sup>low</sup> patients in the Angio<sup>low</sup> group. HRs improved for the association of Myeloid<sup>high</sup> versus Myeloid<sup>low</sup> in Teff<sup>high</sup>



**Figure 3** Association of gene expression signatures and molecular subtypes with survival. (A) Association of gene expression signatures with PFS comparing high ( $\geq$ median) vs low gene expression signature scores in the nivolumab plus ipilimumab and the sunitinib arms. P values compare patient subgroups within each treatment arm. (B) Association of gene expression signatures with OS comparing high vs low gene expression signature scores in the nivolumab plus ipilimumab and the sunitinib arms. P values compare patient subgroups within each treatment arm. PFS according to Angio signature score with (C) nivolumab plus ipilimumab and (D) sunitinib. OS according to Angio signature score with (E) nivolumab plus ipilimumab and (F) sunitinib. Association of Teff<sup>high</sup>/Myeloid<sup>low</sup> and Teff<sup>high</sup>/Myeloid<sup>high</sup> with PFS with (G) nivolumab plus ipilimumab and (H) sunitinib. Association of Teff<sup>high</sup>/Myeloid<sup>low</sup> and Teff<sup>high</sup>/Myeloid<sup>high</sup> with OS with (I) nivolumab plus ipilimumab and (J) sunitinib. Angio, angiogenesis; IPI, ipilimumab; MUT, mutant; NIVO, nivolumab; NR, not reached; OS, overall survival; PFS, progression-free survival; SUN, sunitinib; Teff, T-effector; TIS, tumor inflammation signature; WT, wild type.

with PFS and OS compared with HRs observed in the analysis of Myeloid or Teff signatures alone, and a trend for prolonged PFS and OS was observed in patients with high versus low Myeloid scores in Teff<sup>high</sup> patients treated with nivolumab plus ipilimumab but not with sunitinib (figure 3G–J).

#### Cellular composition of the TME and clinical benefit with nivolumab plus ipilimumab

MCP-counter analysis<sup>37</sup> was performed on RNA-seq-evaluable samples to quantify the abundance of 10 different cell types in the TME. No associations were observed between these scores and survival, although among patients treated with sunitinib, potential trends were noted for prolonged PFS in patients with high endothelial cell abundance scores and for prolonged OS in patients with high B lineage cell abundance scores (figure 4A,B).

Unsupervised clustering based on the 10 cell abundance scores identified five distinct clusters of tumor samples (C1–C5), reflecting different compositions of ICs (eg, T cells, natural killer cells, and myeloid dendritic cells) and stromal/immune suppressing components (eg, neutrophils, endothelial cells, and fibroblasts) in the TME of each patient (cluster 1: immune low, cluster 2: stromal/immune suppression low, cluster 3: stromal/immune suppression high and immune low, cluster 4: stromal/immune suppression high and immune high, cluster 5: stromal/immune suppression low and immune high (figure 4C,D)). Only cluster 1 was completely separated from the rest of the clusters by principal component 1 (PC1) and PC2. Differences in PFS and OS with nivolumab plus ipilimumab versus sunitinib did not reach statistical significance when the 213 RNA-seq-evaluable patients were grouped into these five clusters of patients (online supplemental figure 6).

#### Biological processes associated with survival in patients treated with nivolumab plus ipilimumab

To further investigate biological processes that may drive prolonged survival with nivolumab plus ipilimumab, we used a Cox proportional-hazards model to evaluate the association between gene expression levels and PFS or OS in each treatment arm. Gene set enrichment analysis was then performed on these results to identify HALLMARK gene sets that were significantly enriched in the association analysis (false discovery rate <0.05). For several gene sets, higher expression of transcripts was associated with longer PFS in patients treated with nivolumab plus ipilimumab but with shorter PFS in patients treated with sunitinib (figure 5). These gene sets included Apoptosis, the Reactive Oxygen Species Pathway, IL-6/JAK/STAT3 Signaling, Allograft Rejection, and Inflammatory Response. Interestingly, expression of transcripts from the HALLMARK Adipogenesis and fatty acid metabolism gene sets were positively associated with OS in both nivolumab plus ipilimumab and sunitinib arms, suggesting a potential prognostic value of these gene sets in RCC. In contrast, expression of transcripts from gene

sets for E2F targets and MYC targets were negatively associated with OS in both treatment arms.

#### DISCUSSION

In CheckMate 214, nivolumab plus ipilimumab demonstrated superior clinical outcomes versus sunitinib in patients with advanced RCC.<sup>1,36</sup> An unmet need exists for biomarkers with the potential to identify patients with advanced RCC who are most likely to respond to available treatments.<sup>8</sup> The present study used pretreatment tumor samples in exploratory analysis of associations of histological, genomic, and transcriptomic biomarkers with survival outcomes.

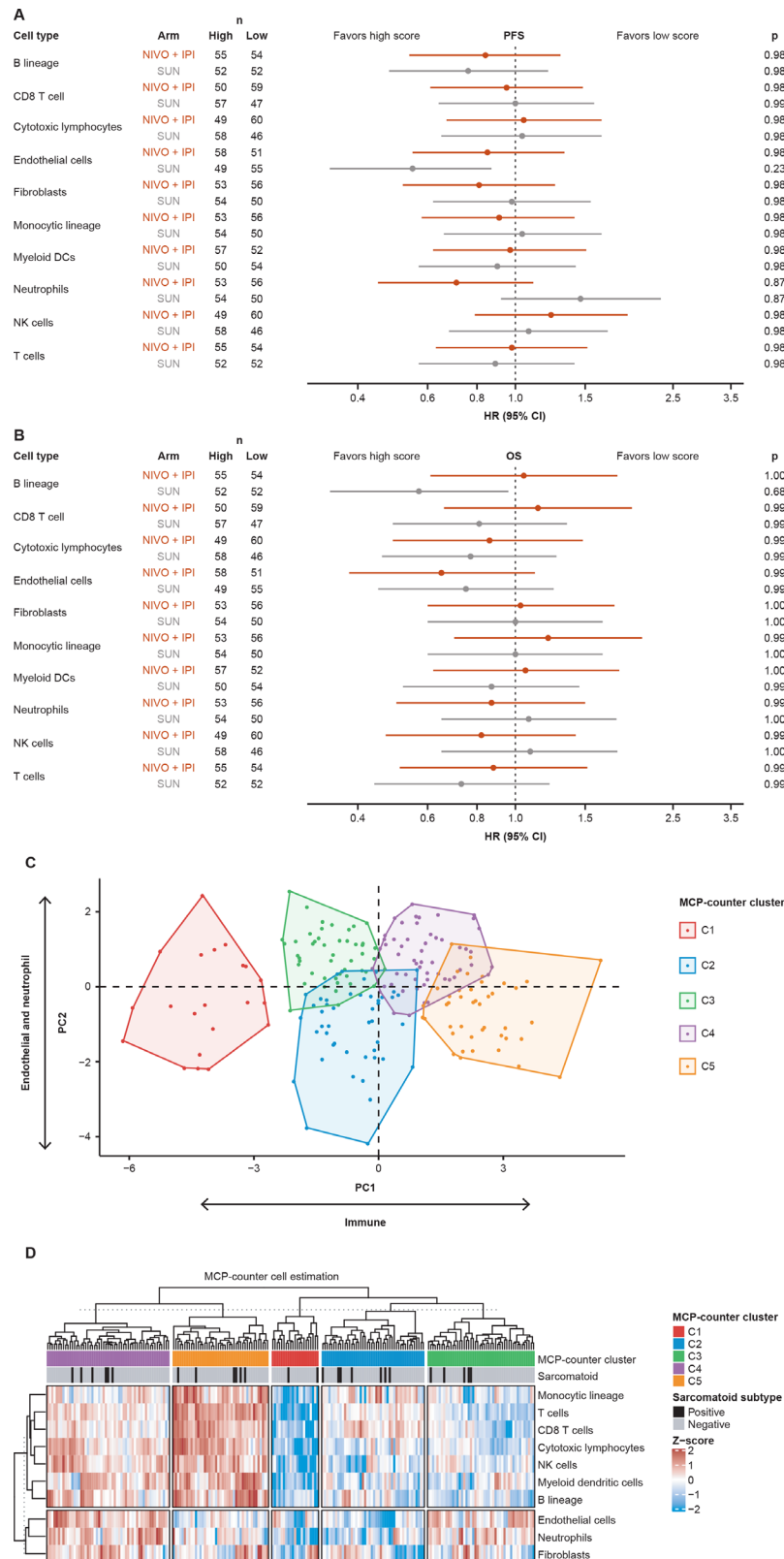
There is currently no evidence for the value of PD-L1 alone as a biomarker of response to treatment in RCC. Compared with sunitinib, improved OS in patients with RCC was observed with nivolumab plus ipilimumab regardless of PD-L1 expression level or scoring method used.

Genomic analyses performed in this study showed that, in contrast to previous studies in other tumor types such as non-small cell lung cancer and melanoma,<sup>38–40</sup> genomic features such as TMB and TIB did not associate with response to ICIs in CheckMate 214. These findings are consistent with biomarker analyses carried out as part of the JAVELIN Renal 101 trial<sup>12</sup> and in CheckMate 010 and CheckMate 025<sup>16</sup> in patients with advanced RCC and may be due to the relatively low TMB and TIB levels, as well as their narrow distribution, in RCC samples compared with tumor types where the predictive value of TMB has been reported.<sup>41</sup> Other factors may play a more significant role in determining response to ICIs in RCC, or act synergistically with TMB and TIB.

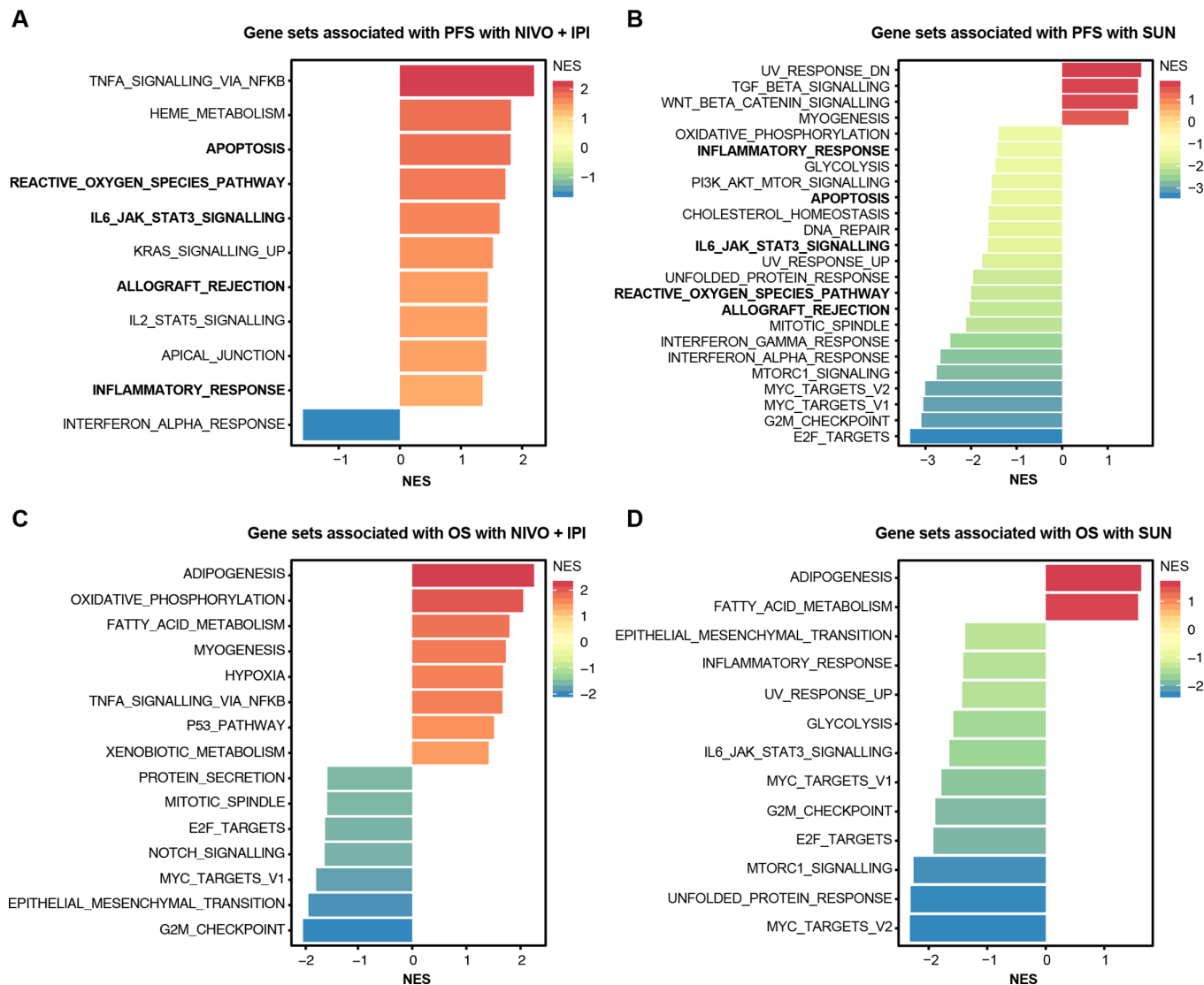
Among the 382 genes alterations assessed in this study for an association between mutation status and response to nivolumab plus ipilimumab, none were found to be associated with survival after adjustment for multiple hypothesis testing. Mutation frequencies for genes that are commonly mutated in RCC were consistent with other reports.<sup>12,15–17</sup> Similarly, our results are consistent with other studies that reported no association between *VHL*, *PBRM1*, *BAP1*, and *MTOR* mutation status and outcomes in patients with RCC treated with first-line PD-L1 inhibitor monotherapy or in combination with TKIs.<sup>12,15</sup> Our findings that are suggestive of prolonged PFS with nivolumab plus ipilimumab in patients with *PBRM1*<sup>WT</sup> RCC are in contrast to other reports of improved clinical benefit with PD-1 or PD-L1 inhibitor monotherapy or in combination with anti-cytotoxic T lymphocyte antigen-4 inhibitors in patients with *PBRM1*<sup>MUT</sup> RCC.<sup>16,19</sup> Differences in the reported associations between the mutation status of *PBRM1* and treatment efficacy may be influenced by processes within the TME such as T-cell infiltration or immunosuppression.

RNA-seq data from CheckMate 214 were analyzed to evaluate the association between previously published gene expression signatures and clinical benefit with nivolumab





**Figure 4** Analyses of cell populations in the TME estimated in RNA-seq data. (A) HRs for the association of high ( $\geq$ median) vs low cell abundance scores with PFS. P values compare patient subgroups within each treatment arm and were corrected for multiple hypothesis testing using Benjamini-Hochberg procedure.<sup>35</sup> (B) HRs for the association of cell abundance scores with OS. P values compare patient subgroups within each treatment arm. (C) RNA-seq-evaluable samples grouped into five clusters (C1–C5) by unsupervised clustering. (D) Heatmap showing abundance scores for the 10 cell types in each evaluable sample. Sample annotation tracks show the MCP-counter cluster assignment and sarcomatoid status. DC, dendritic cell; IPI, ipilimumab; MCP, microenvironment cell population; NIVO, nivolumab; NK, natural killer; OS, overall survival; PC, principal component; PFS, progression-free survival; SUN, sunitinib; TME, tumor microenvironment.



**Figure 5** HALLMARK gene sets with significant enrichment (FDR < 0.05) in the transcripts associated with PFS or OS by a Cox proportional-hazards analysis. Gene sets enriched in transcripts associated with longer PFS in the (A) nivolumab plus ipilimumab or (B) sunitinib arms. Bold typeset indicates gene sets with opposing association in the two arms (ie, transcripts associated with longer PFS in patients treated with nivolumab plus ipilimumab but shorter PFS in those treated with sunitinib). Gene sets enriched in transcripts associated with longer OS in the (C) nivolumab plus ipilimumab and (D) sunitinib arms. FDR, false discovery rate; IPI, ipilimumab; NES, normalized enrichment score; NIVO, nivolumab; OS, overall survival; PFS, progression-free survival; SUN, sunitinib.

plus ipilimumab versus sunitinib. Higher levels of the Angio signature were associated with improved response to sunitinib in agreement with a previous report,<sup>15</sup> but are not predictive of PFS and OS with nivolumab plus ipilimumab. In our analysis, Angio scores were lower in sarcomatoid-positive than in sarcomatoid-negative tumors, suggesting that Angio is not the main driver of sarcomatoid RCC, or that Angio may be driven by other factors beyond the Angio signature. A recent report indicates that *MYC*-regulated transcriptional programs may drive the aggressive nature and poor prognosis associated with sarcomatoid RCC.<sup>42</sup>

Gene expression signatures of tumor inflammation previously reported to be associated with response to anti-PD-L1 therapy<sup>15 26</sup> did not independently predict

PFS or OS with nivolumab plus ipilimumab when assessed in pretreatment samples from CheckMate 214. The use of combined gene expression signatures to categorize patients with RCC has previously been shown to be predictive of response to anti-PD-L1 therapy alone and in combination with VEGF inhibitors.<sup>12 15 25</sup> Our data also suggest that combining multiple gene expression signatures may improve the ability of gene expression signatures to predict response to treatment with nivolumab plus ipilimumab, and combination signatures such as Angio<sup>low</sup>/Teff<sup>high</sup> and Teff<sup>high</sup>/Myeloid<sup>high</sup> may provide further information about biological factors that are important for response. Further studies are needed to optimize the most appropriate gene expression signatures for predicting response to this combination treatment

in patients with RCC. Combinations of nivolumab plus ipilimumab have previously demonstrated synergistic antitumor activity.<sup>43,44</sup> Evidence suggests that nivolumab plus ipilimumab combination therapy may enhance T-cell infiltration and bypass a need for pre-existing Teff cells in the TME prior to therapy compared with nivolumab monotherapy.<sup>44</sup> This may explain the differential association between gene expression signatures representing T-cell inflammation and survival in patients treated with a single ICI (alone or in combination with VEGF inhibitors) and in patients treated with nivolumab plus ipilimumab in this study.<sup>12,15</sup> This is further supported by previous transcriptomic analyses from CheckMate 009 that identified ipilimumab-mediated transcripts as baseline predictors of response to nivolumab monotherapy in RCC, suggesting mechanistically how ipilimumab may act in synergy with nivolumab and lead to improved efficacy over ICI monotherapy, regardless of baseline inflammation status.<sup>26</sup>

The potential immune suppression caused by neutrophils, endothelial cells, and fibroblasts, and their negative impact on clinical outcomes in patients treated with nivolumab monotherapy, were recently reported.<sup>25</sup> In the present study, no significant associations between individual cell types and survival were identified with nivolumab plus ipilimumab. Patients with higher levels of ICs and/or lower levels of stromal/immune-suppressive cells could be hypothesized to show improved response to nivolumab plus ipilimumab. Although no significant association with PFS or OS was observed for any of the five patient subgroups derived by unsupervised clustering and characterized by levels of IC and stromal/immune suppression components, these results are suggestive, and further investigation in larger sample numbers may demonstrate the enrichment of clinical benefit with nivolumab plus ipilimumab in some patient subgroups. As previously reported, however, it is possible that combination therapy with nivolumab plus ipilimumab may bypass the effect of immune-suppressive cell types in the TME.<sup>45</sup>

In the 42-month follow-up of CheckMate 214, PFS curves for the two treatment arms notably diverged at 24 months, with over 30% of patients receiving nivolumab plus ipilimumab achieving long-term PFS.<sup>36</sup> In the current study, analysis of gene expression in pretreatment samples suggested that higher baseline expression of particular biological processes may be prognostic for survival in patients with RCC. For example, expression of transcripts corresponding to MYC and E2F targets were negatively associated with OS in both treatment arms, supporting the association of E2F-driven and MYC-driven gene expression with poor prognosis in patients with RCC.<sup>46–48</sup> While the association of the HALLMARK fatty acid metabolism gene set with response to sunitinib is in accordance with previously reported data,<sup>49</sup> the current study shows that enrichment of the same gene set is also associated with prolonged survival with nivolumab plus ipilimumab, supporting the hypothesis that expression of

genes involved in fatty acid metabolism is a prognostic biomarker for RCC.<sup>50</sup> This is in contrast to the negative correlation between the HALLMARK fatty acid metabolism gene set and response to nivolumab monotherapy in CheckMate 009, where the majority of patients had received prior therapy with VEGF inhibitors.<sup>26</sup> The observed difference may be due to the first-line setting of Checkmate 214 or to expansion of the pool of responsive patients resulting from combination therapy. Other biological processes may be specifically predictive for response and survival with nivolumab plus ipilimumab. For example, the association of the HALLMARK Inflammatory Response gene set with PFS in patients treated with nivolumab plus ipilimumab may help to dissect the role of baseline IC infiltration in durable responses to ICI therapy.<sup>51</sup> Expression of transcripts related to IL-6/JAK/STAT3 signaling, which has been shown to be involved in the induction of PD-1 and/or PD-L1 expression,<sup>52,53</sup> was also associated with long PFS in patients treated with nivolumab plus ipilimumab. Further investigation and clinical validation of the gene sets associated with longer PFS in patients treated with nivolumab plus ipilimumab may provide additional clues towards identification of prognostic or predictive biomarkers of response to this therapy.

The FFPE samples evaluated in the present study may have originated from primary or metastatic sites; therefore, caution should be used in interpreting the results due to the potential for tumor heterogeneity at the pathological and molecular level. Furthermore, this retrospective study may have been limited by the availability of quality samples for DNA and RNA analysis. While our transcriptomic analyses showed potential associations between gene expression signatures and outcomes to treatment, no distinct profile identifying patients more likely to respond to nivolumab plus ipilimumab was identified. Further studies are warranted to evaluate transcriptomic biomarkers of response to nivolumab plus ipilimumab in patients with RCC.

The results reported here offer insights into the biology of RCC, reveal relationships between molecular biomarkers, and provide supportive data for identifying predictive biomarkers that have either positive or negative associations with response and survival in patients with RCC treated with immunotherapy. Further validation of these exploratory analyses will be conducted in ongoing studies to identify biomarkers predictive of response to ICIs in patients with RCC, with the ultimate aim to provide biomarker-driven precision treatment for patients with RCC.

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**Patient consent for publication** Not applicable.

**Ethics approval** This trial was approved by the institutional review board or ethics committee at each site and was conducted according to Good Clinical Practice guidelines, defined by the International Conference on Harmonisation. All the

patients provided written informed consent that was based on the Declaration of Helsinki principles. Participants gave informed consent to participate in the study before taking part.

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**Data availability statement** Data are available in a public, open access repository. Data in support of the findings of this study, including anonymized raw FASTQ files of WES and RNA-seq data from 87, and 39 patients, respectively, who granted informed consent to share such data, are made available at the European Genome-Phenome Archive (EGA) under accession number EGAS00001005501 and EGAD00001007938. More information on Bristol Myers Squibb's data sharing policy can be found here: <https://www.bms.com/researchers-and-partners/clinical-trials-and-research/disclosure-commitment.html>.

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