Dynamics and survival associations of T cell receptor clusters in patients with pleural mesothelioma treated with immunotherapy

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ABSTRACT
Background Immune checkpoint inhibitors (ICIs) are now a first-line treatment option for patients with pleural mesothelioma with the recent approval of ipilimumab and nivolumab. Mesothelioma has a low tumor mutation burden and no robust predictors of survival with ICI. Since ICIs enable adaptive antitumor immune responses, we investigated T-cell receptor (TCR) associations with survival in participants from two clinical trials treated with ICI.

Methods We included patients with pleural mesothelioma who were treated with nivolumab (NivoMes, NCT02497508) or nivolumab and ipilimumab (INITIATE, NCT03048474) after first-line therapy. TCR sequencing was performed with the ImmunoSEQ assay in 49 and 39 pretreatment and post-treatment peripheral blood mononuclear cell (PBMC) samples. These data were integrated with TCR sequences found in bulk RNAseq data by TRUST4 program in 45 and 35 pretreatment and post-treatment tumor biopsy samples and TCR sequences from over 600 healthy controls. The TCR sequences were clustered into groups of shared antigen specificity using GIANA. Associations of TCR clusters with overall survival were determined by Cox proportional hazard analysis.

Results We identified 4.2 million and 12 thousand complementarity-determining region 3 (CDR3) sequences from PBMCs and tumors, respectively, in patients treated with ICI. These CDR3 sequences were integrated with 2.1 million publicly available CDR3 sequences from healthy controls and clustered. ICI-enhanced T-cell infiltration and expanded T cell diversity in tumors. Cases with TCR clones in the top tertile in the pretreatment tissue or in circulation had significantly better survival than the bottom two tertiles (p<0.04). Furthermore, a high number of shared TCR clones between pretreatment tissue and in circulation was associated with improved survival (p=0.01). To potentially select antitumor clusters, we filtered for clusters that were (1) not found in healthy controls, (2) recurrent in multiple patients with mesothelioma, and (3) more prevalent in post-treatment than pretreatment samples. The detection of two-specific TCR clusters provided significant survival benefit compared with detection of 1 cluster (HR<0.001, p=0.026) or the detection of no TCR clusters (HR=0.10, p=0.002). These two clusters were not found in bulk tissue RNA-seq data and have not been reported in public CDR3 databases.

Conclusions We identified two unique TCR clusters that were associated with survival on treatment with ICI in patients with pleural mesothelioma. These clusters may enable approaches for antigen discovery and inform future targets for design of adoptive T cell therapies.

WHAT IS ALREADY KNOWN ON THIS TOPIC
⇒ Histological subtype is currently the best predictor of survival with immune checkpoint inhibitors in patients with pleural mesothelioma. Since immune checkpoint inhibitors enable adaptive antitumor immune responses, we sought to identify T-cell receptor (TCR) clusters that were associated with survival.

WHAT THIS STUDY ADDS
⇒ This study has identified TCR clusters that are associated with survival on treatment with immune checkpoint inhibitors in mesothelioma.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY
⇒ These T cell clusters may enable less invasive, blood-based monitoring approaches for prediction of response to immune checkpoint inhibitors, may inform TCR-based therapeutic approaches and guide the identification of immunogenic neoantigens in mesothelioma.

INTRODUCTION
As T cells mediate antitumor responses to immune checkpoint inhibitors (ICIs), understanding whether any are associated with improved survival could allow the development of better predictors of response to ICI, inform our understanding of the adaptive
immune response to tumors and guide development of T cell receptor (TCR)-based therapeutics. ICIs have recently been approved by the US Food and Drug Administration for the treatment of pleural mesothelioma based on the results of the CheckMate 743 clinical trial. In this trial, the combination of nivolumab and ipilimumab was compared with platinum-based chemotherapy with pemetrexed in patients with unresectable pleural mesothelioma. There was an improvement in overall survival (OS) in patients who received ICI compared with chemotherapy (median OS 18.1 months vs 14.1 months, HR 0.73, 95% CI 0.61 to 0.87). This benefit was more pronounced in patients with non-epithelioid mesothelioma. In a recent update, the 3-year OS rates were reported at 23% and 15% for ICI compared with chemotherapy, respectively. Whereas expression of programmed cell death-ligand 1 (PD-L1) has been described as a predictive biomarker with ICI in other tumor types, it did not strongly correlate with OS in Checkmate 743. In contrast, PD-L1 expression is most frequently associated with a negative prognostic significance in pleural mesothelioma. Recently, a four-gene inflammatory signature which assesses the impact of inflammation in the tumor microenvironment on clinical outcomes with nivolumab and ipilimumab has emerged as a biomarker of interest; however, it requires further validation. Tumor mutation burden, which is a surrogate for tumor neoantigens and is associated with benefit with ICI, is reportedly low in most mesotheliomas. Standard approaches to sequencing tumors might miss chromosomal rearrangements that have neoantigenic potential in pleural mesothelioma, which may have predictive value in the context of antigen processing and presentation gene expression. Therefore, there is an unmet need to identify non-invasive biomarkers which can better predict patient response to ICI and optimize patient selection.

TCRs determine the antigen specificity of T cells. TCR-sequencing (TCRseq), and bioinformatics approaches to derive TCR sequences have not been widely applied to understanding survival outcomes with ICI in pleural mesothelioma. Unique TCR sequences may recognize the same antigen due to similarities in the complementarity-determining region 3 (CDR3) sequences. Accordingly, more recent TCR analyses group TCRs into clusters with predicted common specificity. Thus, TCRs from multiple patients can be grouped into clusters that are predicted to share antigen specificity. As the infiltration of tumor reactive T cells at the tumor site, expansion of programmed death-1 protein (PD-1)+CD8+ T cell clones after dendritic cell vaccine, and expansion of T cell clones on treatment with antimesothelin targeted chimeric antigen receptor T cells in combination with pembrolizumab have been correlated with improved patient outcomes, we hypothesized that expansion of TCR repertoire on treatment with ICI associated with improved survival in pleural mesothelioma.

Here, we report our analysis of TCR clusters and their predictive significance in patients with pleural mesothelioma treated with ICI in two clinical trials.

**METHODS**

**Patients and specimens**

A total of 68 patients with pleural mesothelioma were treated with the PD-1 inhibitor nivolumab alone or in combination with the CTLA-4 inhibitor ipilimumab on the NivoMes (n=34) and INITIATE (n=34) clinical trials. Online supplemental figure S1 describes samples with sufficient material for the study. Tumor biopsies were obtained when possible from patients just prior to treatment with nivolumab or nivolumab with ipilimumab after previous treatment with platinum-based chemotherapy (n=42 pretreatment biopsy specimens). Tumor biopsies were also collected after 6 weeks of treatment from many of these patients (n=31). RNA from the tumor was purified using the AllPrep DNA/RNA/miRNA Universal kit (Qiagen, #80224) following the instructions provided by the manufacturer. The buffer included β-mercaptoethanol for the specimens obtained from NCT02497508, and dithiothreitol for the ones obtained from NCT03048474. Otherwise, there were no differences in the handling of the specimens or nucleic acid purification. Peripheral blood mononuclear cells (PBMCs) were drawn in EDTA tubes and frozen prior to initiation of immunotherapy, and 6 weeks after the start of therapy for a total of 49 pretreatment and 39 on-treatment PBMCs for TCRseq. The DNA was purified from the PBMCs and prepared for TCRseq per instructions from Adaptive Biotechnologies as we have done previously. The clinical trials and translational studies were approved by the local institutional ethics committees. Patients consented to participate in these trials and use their specimens for research in the NivoMes and INITIATE clinical trials conducted at the Netherlands Cancer Institute. DNA and RNA were transferred to the Mayo Clinic under a materials transfer agreement. The responses to treatment with ICI that were determined by modified pleural RECIST, as reported previously, were also used in our analyses.

**Processing of RNA and TCR sequencing data**

RNA fastq files from tumor biopsies were processed by TRUST4 python program using the default settings to identify TCR CDR3 amino acid sequences and the corresponding variable chains in each tumor biopsy. These tissue-based TCR sequences were pooled with the TCR sequences found in PBMCs as determined by TCRseq from the trial participants, and with publicly available TCR sequences identified in PBMCs from healthy controls. The TCR sequences from healthy controls without cancer (n=589) were obtained from an investigation on the effects of cytomegalovirus exposure history on the T cell repertoire and were downloaded from the Adaptive Biotechnologies immuneACCESS platform (https://clients.adaptivebiotech.com/immuneaccess). Similar to prior work, we restricted our analysis of the healthy control TCRs clones to those with ≥5 copies since cancer-specific low abundant naïve T cells can be present in healthy individuals. Altogether, there were
approximately 12,200, 4.2 million, and 2.1 million CDR3 amino acid sequences and corresponding variable chains in mesothelioma tumor biopsies, PBMCs of patients with mesothelioma, and healthy controls, respectively. Online supplemental figure S2 depicts characteristic features of TCRs in terms of CDR3 amino acid length histograms, variable (V), joining (J), and diversity (D) gene segments (VDJ) correlations between healthy control PBMCs and mesothelioma PBMCs, and N1/N2 insert sizes in mesothelioma PBMCs. Additionally, online supplemental table 1 includes frequency of VDJ junctions found in healthy control PBMCs and mesothelioma PBMCs. After processing these data with the GLIANA python program \(^{23}\) using default settings, we identified 585,920 computationally derived TCR clusters. Venn diagrams in online supplemental figure S3 illustrate the overlap of these clusters between the healthy control and mesothelioma biopsy and PBMC in pretreatment and on-treatment samples. To maximize identification of TCRs that were possibly specific to pleural mesothelioma, we excluded TCR clusters that were also identified in healthy controls. Additionally, TCR clusters were normalized by sequencing depths (mapped reads) and the number of PBMCs used for TCRseq, respectively, for comparisons of TCR clusters in tissue biopsies and PBMCs.

**Unsupervised PBMC clustering and heatmap**

There were 29 TCR clusters that were identified in at least 12 pretreatment PBMC samples. These commonly found TCR clusters were used to investigate the similarity in pretreatment PBMC profiles by unsupervised clustering using the ‘cosine’ similarity function in the lsa package in R with the ‘ward.D’ clustering method. We then used the heatmap function in ‘stats’ package to generate the heatmap in R.

**Associations of individual PBMC TCRs with treatment outcome**

We aimed to determine whether any TCR cluster was significantly associated with survival on treatment with ICI. Associations were based on log rank p values determined by the ‘coxph’ function in the ‘survival’ package in R. Given our sample size, and to limit the number of hypotheses being tested, we limited our focus to TCR clusters identified in at least seven individual patients. We did not identify any pretreatment PBMCs with significant association with OS after correcting for the false discover rate (FDR). In the on-treatment samples, there were 166 clusters from at least 7 individuals (online supplemental figure S4). We eliminated clusters that (1) contained identical CDR3 amino acid sequences in healthy controls even if with a different variable chain, and (2) were not more prevalent in on-treatment than pretreatment PBMCs, as we expected any TCR cluster associated with treatment response would likely expand on treatment. This search identified two clusters that were associated with OS following treatment with immunotherapy with a q-value \(<0.056\) calculated by ‘qvalue’ package. To plot multiple Kaplan-Meier (KM) plots, we used km.coxph. plot function in the ‘survcomp’ package in R.

**Normalized tumor biopsy gene expression matrix and immune deconvolution**

Mapping of the RNA-seq data and estimations of gene expression counts in each tumor biopsy sample were performed with the MAP-RSeq pipeline developed by the Mayo Clinic Bioinformatics Core.\(^{24}\) Raw ‘count’ files were processed by the ‘edgeR’ package to generate log 2 normalized gene expression values. Estimations of T cells and other immune cells in tumor biopsies were performed with the ‘immunedeconv’ package in R.\(^{25}\)

**Whole genome sequencing and immunotherapy prediction model**

We performed whole genome sequencing on the mesothelioma specimens with the mate-pair whole-genome library protocol (Nextera Library Prep Protocol) as we described and reported previously.\(^{10}\)\(^{31}\) We used mate-pair sequencing given the very low mutation burdens that have been reported in mesothelioma, and the ability of this approach to detect structural variants which have not been investigated thoroughly in mesothelioma. We calculated a tumor junction burden by counting the number of unique genes hit by all junctions (chromosomal rearrangements, insertions and deletions) in the samples. We subsequently demonstrated that an interaction of ‘Regulation of Antigen Processing and Presentation of Peptide Antigen’ gene set expression and the tumor junction burden was predictive of survival with ICIs in mesothelioma.\(^{11}\) We then added the normalized TCR cluster membership into this interaction model. Pretreatment tissue biopsies were assigned into ‘High I’ or ‘Low I’ groupings based on having interaction scores in the top tertile or bottom two tertiles, respectively. Similarly, pretreatment biopsies were assigned into ‘High T’ or ‘Low T’ groupings based on having TCR cluster membership in the top tertile or bottom two tertiles, respectively. We then compared survivals based on their high or low interaction score and TCR groupings and found that pretreatment biopsies with ‘High I’ and ‘High T’ had significantly better survival outcomes than pretreatment biopsies with ‘Low I’ and ‘Low T’.

**RESULTS**

**Specimens and their TCR clusters**

The aim of this study was to investigate the relationship between computationally derived TCR clusters from tissue biopsies and PBMCs with survival with ICI therapy. Table 1 describes the characteristic features of our patient cohort. We analyzed TCR sequences from three sources (figure 1 and online supplemental figure S1) including (1) healthy controls (n=2.1 million), (2) PBMCs from patients with pleural mesothelioma obtained just prior to treatment or 6 weeks after treatment (n=4.2 million), and (3) in pr-treatment or on-treatment biopsies from these
patients (n=12,200). These TCR sequences were analyzed by the GIANA TCR clustering program which identified close to 586,000 TCR clusters with predicted shared antigen specificity and each containing an average of 4.6 individual TCR sequences. The median and the mean of unique samples in TCR clusters were 2 and 3.66, respectively (online supplemental figure S5). Venn diagrams of overlapping clusters between mesothelioma tumor biopsies, mesothelioma PBMCs, and healthy controls from pretreatment or on treatment samples are shown in online supplemental figure S3. In all subsequent analyses, we only examined clusters that were not identified in healthy controls to focus on mesothelioma associated TCRs.

Impact of pretreatment TCR clusters on survival with immunotherapy

The normalized count of TCR clusters identified in tumor tissue was highly correlated with the expression of CD8A and CD4 genes (figure 2A,B). In other words, tumors with high TCR cluster counts had high CD8A and CD4 expression in tumor biopsies by RNA-seq. Additionally, in tumor biopsies the number of TCR clusters detected in on-treatment samples were significantly higher than in pretreatment samples (p=0.017, figure 2C). This finding was consistent with the immune deconvolution analyses that found greater T cell accumulation in biopsy specimens following treatment with ICI compared with the pretreatment specimens (online supplemental figure S8), suggesting that ICI increased T cell clonal diversity (based on the increase in T cell clusters) and trafficking to mesotheliomas. Notably, patients with pretreatment tumor biopsies with normalized TCR cluster membership in the top tertile had significantly better survival than patients with TCR clusters in the bottom two tertiles (p = 0.034, HR 0.44, 95% CI 0.200 to 0.986, figure 2D).

Similarly, patients with pretreatment PBMCs with the top tertile of TCR cluster counts had significantly longer survival than patients with TCR clusters in the bottom two tertiles (p = 0.033, HR 0.41, 95% CI 0.187 to 0.898, figure 3A). We posited that shared TCR clones between tissue and PBMCs would likely represent expansion of systemic anti-tumor immunity and associate with improved survival. In the 33 individuals with both tumor biopsies and PBMC pretreatment samples (figure 3B), we estimated the number of shared clones based on having TCRs with identical CDR3 amino acid sequences and variable chains in tissue and PBMC. After normalizing for both tumor and PBMC sequencing, we found the presence of shared tumor and PBMC TCR clones significantly improved OS (cox proportional hazard p=0.011). Patients with the top tertile of shared tumor biopsy and PBMC TCR clones had significantly better OS than patients with shared TCR clones in the bottom tertiles (figure 3C, p = 0.012; HR 0.33, 95% CI 0.129 to 0.837).

Impact of on-treatment TCR clusters and survival with immunotherapy

We observed similar trends in survival with TCR clusters detected from on-treatment tumor biopsies and PBMCs after administration of ICI (online supplemental figure S9). Generally, patients with TCR clusters in the top two tertiles from tumor biopsies or PBMCs had better OS than patients with TCR clusters in the bottom tertile. Additionally, patients with few shared TCR clones between tumor

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**Table 1**  Patient characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients (n=49)</th>
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<tbody>
<tr>
<td><strong>Sex, n (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>39 (80)</td>
</tr>
<tr>
<td>Female</td>
<td>10 (20)</td>
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<tr>
<td><strong>Histology, n (%)</strong></td>
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<tr>
<td>Epithelioid</td>
<td>42 (86)</td>
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<tr>
<td>Non-epithelioid</td>
<td>7 (14)</td>
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<tr>
<td><strong>Treatment, n (%)</strong></td>
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<tr>
<td>Nivolumab, ipilimumab</td>
<td>30 (61)</td>
</tr>
<tr>
<td>Nivolumab</td>
<td>19 (39)</td>
</tr>
<tr>
<td><strong>Line of therapy, n (%)</strong></td>
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<td>2</td>
<td>43 (88)</td>
</tr>
<tr>
<td>≥3</td>
<td>6 (12)</td>
</tr>
<tr>
<td><strong>Age, years median (range)</strong></td>
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</table>
biopsies and PBMCs did poorly compared with other patients (online supplemental figure S7). Even though these findings were not always statistically significant, they suggested that patients with poor expansion of T cell repertoire or low number of T cells that trafficked to tumors were less likely to benefit from ICI.

**Impact of common TCR clusters on response and survival with immunotherapy**

Next, we investigated whether membership in common mesothelioma TCR clusters in PBMCs had any bearing on clinical presentation. In the pretreatment PBMCs, we identified 29 TCR clusters that were each found in at least 12 patients. With the exception of one patient, all cases had TCR clones in at least 1 of these TCR clusters. In a heat map with columns representing individual PBMCs grouped by unsupervised clustering according to the dendrogram on top and rows representing TCR clusters, we noted a group of patients that were enriched for commonly occurring TCR clusters (right side of the green dashed line; figure 4A). This group that was enriched for commonly occurring TCR clusters contained a significantly higher proportion of patients with clinical responses to ICI (61% overall response rate according to modified pleural RECIST compared with 19% overall response rate, \( \chi^2 p=0.003 \)) and better OS (HR 0.47, \( p=0.038 \); 95% CI 0.226 to 0.989; figure 4A,B).

Finally, we examined associations of TCR clusters in pretreatment and on-treatment PBMCs with survival outcome. None of the pretreatment TCR clusters were associated with survival after correcting for FDR. In contrast, there were two TCR clusters in on-treatment PBMCs with FDR q-values <0.06 (online supplemental figure S8). There were five patients in which both of these TCR clusters were detected, and five patients in which one of these TCR clusters was detected. The detection of both TCR clusters provided significant survival
benefit compared with detection of 1 cluster (HR<0.001, p=0.026) or the detection of no TCR clusters (HR=0.10, p=0.002; figure 4C). We searched whether these TCR clusters have been associated with any known antigens, including mesothelin.26–28 Even though this search identified close to 100 known TCRs among all of the TCR clusters in our dataset, including 29 TCRs detected in only mesothelioma PBMCs (online supplemental figure S9), the antigen specificity of these predictive TCR clusters is unknown.

**Associations of TCR clusters and the tumor microenvironment**

We next examined if there were associations between TCR clusters and the tumor microenvironment using immune deconvolution. We compared the tumor microenvironment of the pretreatment biopsies with the normalized TCR clusters also detected in the tumor by tertiles. There were significantly larger estimates of CD8+ T cells in the tumor microenvironment of patients with more TCR clusters by multiple immune deconvolution programs (figure 5, online supplemental figure S10). Furthermore, we found increased populations of B cells and myeloid dendritic cells in the tumor microenvironments of patients with higher TCR clusters (figure 5B, C, online supplemental figure S10). These data suggest that increases in TCR clusters are associated with tumor microenvironments with increased populations of CD8+ T cells, B cells, and dendritic cells and may contribute to better survival outcomes as shown in figure 2C.
Incorporation of TCR cluster membership into an immunotherapy outcome prediction model

Finally, we examined if the normalized number of TCR clusters can improve the predictive capabilities of an immunotherapy response model that we developed previously. This model is based on the interaction of the tumor junction burden and expression of specific antigen processing and presentation gene sets. For this analysis, we used the ‘Regulation of Antigen Processing and Presentation of Peptide Antigen’ gene set from the Gene Ontology dataset. Samples were divided in two groups based on having interaction scores in the top tertile (HiI) or bottom two tertiles (LoI). As shown in KM plots (figure 6A), the HiI group had better survival outcome than LoI following immunotherapy (p=0.056). The inclusion of TCR clusters by tertile into our model improved the survival predictions further (figure 6A,B). In other words, patients with the top tertile of TCR clusters and high interaction scores had significantly longer OS than those with fewer TCR clusters and interaction scores.
DISCUSSION

The search for robust, predictive biomarkers for ICI has been elusive. The situation in pleural mesothelioma is even more complex. Despite the proven benefit of ICI for this malignancy, tumor mutation burden is almost universally low,8 9 and its determination typically lacks the inclusion of larger structural variants that may have neoantigenic potential,10 11 and it frequently is not corrected for germline variants or ancestry.29–31 In this study, we focused on the TCR clusters with predicted shared antigenicity,

![Figure 5](image_url)

**Figure 5** Comparisons of immune profiles of pretreatment tissue biopsies with high and low TCR clusters by deconvolution. Here, the results obtained with mcp_counter are shown and are overall consistent with those determined by other algorithms (online supplemental figure S10). Patients with the top tertile of TCR clusters (Hi T, n=14) had higher estimates of CD8+ T cells (p=0.0006), myeloid dendritic cells (p=0.0025) and B cells (p=0.009) than the bottom two tertile (Lo T, n=28). TCR, T-cell receptor.

![Figure 6](image_url)

**Figure 6** Integration of TCR clusters with a statistical model based on antigen presentation and junction burden interaction to improve predictions of response to immunotherapy. The 'I' scores were calculated based on interactions between tumor junction burden and 'Regulation of Antigen Processing and Presentation of Peptide Antigen' gene set expression. (A) Km plots comparing survival in patients with top tertile (Hi I, n=14) and bottom two tertile (Lo I, n=28) 'I' scores. (B) The addition of TCR cluster membership improved this model and demonstrated that patients with the top tertile of TCR cluster membership and interaction scores (Hi T & Hi I, n=6) had the significantly better survival overall than patients with the bottom two tertiles of TCR cluster membership and interaction scores (Lo T & Lo I, n=20) (p=0.00067). Patients who were alive at time of last follow-up were censored with vertical marks on the Kaplan-Meier plots. TCR, T-cell receptor.
as T cells within these clusters are the likely mediators of an effective ICI response. As most patients in this study had pretreatment and on-treatment biopsies paired with blood, our analysis allowed us to compare the dynamics of T cell clusters between tumor and peripheral circulation and the effects of these changes on patient outcomes. We identified significant increases in TCR clusters within tumors following treatment with ICI, suggesting that ICI promotes T cell infiltration of tumors. Patients with the higher detectable TCR clusters prior to treatment, in tumor or blood, had improved OS compared with those with lower TCR cluster counts. Similar trends were observed between TCR clusters and survival with on-treatment specimens from tumor or blood.

Also, survival was improved in patients with greater concordance of TCR clusters between tumor and blood. Patients with higher TCR clusters were found to have tumor microenvironments enriched with higher number of CD8+ T cells, B cells, and dendritic cells. Addition of the TCR cluster membership into an immunotherapy outcome prediction model further enhanced its predictive capabilities. Finally, we also identified two TCR clusters that were strongly predictive of OS. To our knowledge, this is the first study showing the association of specific TCR clusters with survival outcomes in patients with mesothelioma who received ICI therapy.

Our findings are consistent with the immunological analysis in the phase 2 PrE0505 trial which tested chemoinmunotherapy with durvalumab in combination with chemotherapy in patients with unresectable pleural mesothelioma. In the PrE0505 trial, more diverse TCR repertoires were seen in the baseline tumor specimens of patients with survival of 12 or more months compared with that of patients with shorter survival. This finding suggests that a polyclonal T cell repertoire may mediate a more effective antitumor immune response. Similarly, more diverse TCR repertoires correlated with better pathological responses in patients with non-small cell lung cancer who received neoadjuvant therapy with nivolumab.

Another study which analyzed samples before and during anti-PD-1 therapy with pembrolizumab in patients with metastatic melanoma (n=46) found that proliferation of intratumoral CD8+ T cells correlated with radiographic responses. Higher TCR diversity has been shown to correlate with improved outcomes in bladder, colorectal, hepatocellular and uterine cancer. In pleural mesothelioma, it was concluded that broader immune cell repertoires are required to mount a robust anti-tumor immune response. Our data suggest that increases in detectable TCR clusters also positively affect the outcome with ICI in pleural mesothelioma.

The tumor microenvironment and presence of tertiary lymphoid structures have been shown to play an important role in response to immunotherapy in multiple cancers including mesothelioma. The presence of CD8+ T cells, and B cells has been shown to correlate with better survival, while presence of tumor associated macrophages is negatively correlated with OS depending on histology. Our data demonstrated consistent findings showing improved survival in patients with tumor microenvironments enriched in CD8+ T cells, B cells, and dendritic cells.

Our study is limited by the sample size, although it is the largest TCR analysis available for this rare malignancy in patients treated prospectively with ICI alone on clinical trials. Since these patients received second or later line ICI, these results may not be generalizable to patients being treated in the first-line setting. Also, our study was weighted with the epithelioid variant of pleural mesothelioma which is consistent with clinical trials that offer second-line or later line treatment for pleural mesothelioma. As expected, the tumor biopsy specimens had many fewer TCRs and TCR clusters than their paired PBMCs likely because different methods were used to identify the TCRs and tumors are usually sparsely populated with T cells compared with blood. T cell infiltration of tumors is also dynamic, and a single biopsy is unlikely to fully capture the full complexity of the tumor-infiltrating T cell repertoire. Additionally, TCRseq and the bioinformatically derived TCR sequences from bulk RNA sequencing do not provide the same granularity as single cell RNA sequencing. More specifically, TCR consists of alpha and beta chains. The paired alpha and beta chains cannot be derived from bulk RNA sequencing. Regardless, CDR3s are the key determinants of T cell specificity and analysis of CDR3s provide invaluable biological insights into T cell repertoires. Finally, it is difficult to determine the antigen specificity of a T cell clone, and the antigen-specificity of TCR clusters we found to be positively associated with survival have not been reported in public databases.

In conclusion, we have identified two TCR clusters that are associated with improved survival in patients with pleural mesothelioma treated with ICI. These two TCR clusters may not only have predictive significance of response to immunotherapy, if validated, but they may also inform TCR-based therapeutic approaches and guide the identification of immunogenic neoantigens in pleural mesothelioma. The analysis of TCR clusters can improve our ability to predict patients who will benefit from immunotherapy in mesothelioma. Finally, the presence of shared TCR clusters between tumor and blood positively impacted survival. This finding suggests that tumor reactive T cells may be identifiable in the periphery, enabling less invasive blood-based monitoring approaches for prediction of response to ICI.

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Competing interests AM reports honoraria to his institution for participation in advisory boards from AbbVie, AstraZeneca, BeGene, IMS, Genentech, Janssen; Travel support and honoraria from Shanghai Roche Pharmaceuticals, and is non-remunerated member of the Mesothelioma Applied Research Foundation and Friends of Panth Hospital Board of Directors.

Patient consent for publication Not applicable.

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Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available in a public, open access repository. These T cell receptor sequences will be listed by the DOI, manuscript title, and the name of the primary author through Adaptive Biotechnologies’ immuneACCESS Platform at https://clients.adaptivebiotech.com/immuneaccess.

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Supplementary Figure Legends

Supplementary Figure S1: Venn Diagram of mesothelioma samples and their overlaps, including On-Treatment PBMC (n = 39), and tissue biopsies (n = 31), and Pre-Treatment PBMC (n = 49) and tissue biopsies (n = 42).

Supplementary Figure S2: TCR CDR3 and VDJ characteristic features. (A) CDR3 histograms of TCRs in healthy control and mesothelioma PBMCs and tissue biopsies are similar. (B) Additionally, VDJ junction usage in healthy control and mesothelioma PBMCs are tightly correlated except for two junctions (TRBV11-2*01 and TRBV6-1*01) as annotated. (C) N1 and N2 insert sizes in pre-treated mesothelioma PBMCs. These histograms for on-treatment mesothelioma PBMCs were very similar (data not shown).

Supplementary Figure S3. Venn diagram depicting overlaps of 585,920 TCR cluster membership in pre- and on-treatment tissue biopsy and PBMC samples and healthy controls..

Supplementary Figure S4. Filtering pipeline for identification of on-treatment TCR clusters with significant survival associations

Supplementary Figure S5: Distribution of number of unique samples in clusters. Dashed red line and hashed blue lines represent median and mean values, respectively.
**Supplementary Figure S6.** Immune deconvolution by MCP-counter using RNA-seq data depicting increased concentration of T-cells and CD8 T-cells in on-treatment compared to pre-treatment PBMCs.

**Supplementary Figure S7.** TCR cluster analyses in on-treatment samples. (A) Patients with tumors that had TCR cluster membership in the top two tertiles (Hi T, n=21) trended toward longer survived than patients with tumors that had TCR cluster membership in the bottom tertile (Lo T, n=10). (B) Similarly, patients with PBMCs that had TCR cluster membership in the top two tertiles (Hi T, n=26) survived significantly longer than patients with PBMCs that had TCR cluster membership in the bottom two tertiles (Lo T, n=13) (p=0.032). (C) Venn diagram of TCR clusters including healthy controls and 20 on-treatment cases with both pre-treatment biopsies and PBMCs. (D) Estimated shared TCR clones between on-treatment biopsies and PBMCs indicated that patients within the top two tertiles of having shared clones (Hi T, n=13) had marginally better survival that patients in the bottom tertile (Lo T, n=7) (p = 0.071). Patients who were alive at time of last follow-up were censored with vertical marks on the Kaplan-Meier plots.

**Supplementary Figure S8.** Membership in two TCR clusters in on-treatment PBMCs were associated with significant improved survival after adjusting for false discovery rate (q-value < 0.06). “Not S TCR” and “S TCR” indicate PBMCs without and with memberships in the specific TCR clusters. Patients who were alive at time of last follow-up were censored with vertical marks on the Kaplan-Meier plots.
**Supplementary Figure S9.** Predicted antigen specificity of 100 TCR clusters based on published data. Blue fonts in the left columns are 29 antigens specifically found in TCR clusters of mesothelioma tissue biopsy and PBMCs whereas the right three columns were predicted shared antigens between healthy controls and mesothelioma patients.

**Supplementary Figure S10:** This table shows p values comparing CD8\(^+\) T cells, myeloid dendritic cells (when included) and B cells by specific immune deconvolution algorithms included in the R package "immunedeconv" as per the table heading. All results favored greater estimates of these cellular subsets in the patients with higher TCR clusters.
Supplementary Figure S1: Study Samples
Supplementary Figure S2:

(A) Healthy Control PBMC  
CDR3aa Length

Mesothelioma PBMC  
CDR3aa Length

Mesothelioma Tissue  
CDR3aa Length

(B) Median Frequency of VDJ Gene Usage

rho = 0.76  
p < 10^{-15}

TRBV6-1*01 →
Supplementary Figure S2C:

N1 Insert Size
Mesothelioma PBMC

N2 Insert Size
Mesothelioma PBMC
Supplementary Figure S3:
Supplementary Figure S4:

Healthy control TCRs (n ≈ 2,000,000)
Meso pre & On TCRs (n ≈ 4,000,000)
Meso Tissue TCRs (n ≈ 12,200)

GIANA

585,920 clusters by GIANA
sizes 2-3921

Min(blood On Treatment samples) = 7
Exclude clusters containing Healthy control TCRs

166 clusters

Eliminate Clusters with identical CDR3aa in HC
Select Clusters with %on/%pr ≥ 1.5

20 clusters
Supplementary Figure S5
Supplementary Figure S6: Immune deconvolution
Supplementary Figure S7

(A) 

(B)
Supplementary Figure S7

(C) [Venn diagram showing healthy control, on-treatment tissue, and on-treatment PBMC populations with numbers indicated.]

(D) [Kaplan-Meier survival curve with days to last follow-up on the x-axis and % survival on the y-axis.}
P_{KM} = 0.071

Healthy Control (n=589) On-Treatment Tissue (n=20) On-Treatment PBMC (n=20)
139382 197448 112548
351 290 60 307
(C)
Supplementary Figure S8

Days to Last Follow-Up

Days to Last Follow-Up
Supplementary Figure S9: Identified TCRs in our data

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## Supplementary Figure S10: immune cell population differences between Hi T and Lo T by multiple algorithms

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