

Multicellular immune ecotypes within solid tumors predict real-world therapeutic benefits with immune checkpoint inhibitors

Corresponding Author: Dr Xuefeng Wang

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Wang et al. leverages real-world transcriptomic and clinical data from the ORIEN Avatar cohort to identify immune ecotypes that predict response to ICI. Using EcoTyper, tumors were classified into ten carcinoma ecotypes linked to better survival and lymphocyte-deficient ecotypes. A melanoma-specific risk model incorporating five ecotypes was developed and validated using external datasets, confirming its predictive accuracy. Additionally, three novel melanoma-specific ecotypes were discovered, further refining immune profiling. These findings establish immune ecotypes as clinically relevant biomarkers, offering a scalable framework for personalized immunotherapy and improved patient selection.

The dataset includes 1,610 ICI-treated patients, making it one of largest transcriptomic datasets used for ICI analysis. The lack of large-scale transcriptomic datasets in ICI research has been a long-standing challenge, making this dataset particularly valuable.

I have a few suggestions for the authors to consider before publication.

1. Biological Relevance of Ecotypes (CE1–CE10)

The manuscript effectively classifies tumors into ten carcinoma ecotypes, but providing additional insights into their biological significance would enhance interpretability. Currently, the functional implications of each ecotype are not extensively discussed. The authors should elaborate on the molecular and cellular composition of these ecotypes, highlighting their roles in shaping the tumor microenvironment TME. If possible, integrating insights from previous literature or pathway enrichment analyses could help explain why certain ecotypes correlate with favorable or poor prognosis in ICI-treated patients.

2. Differences Between PD-(L)1 and CTLA-4-Treated Patients

The study does not specify whether the identified immune ecotypes exhibit distinct responses to different ICI therapies, such as PD-(L)1 inhibitors versus CTLA-4 inhibitors. Since these inhibitors act through different immune-modulatory mechanisms, certain ecotypes may be more responsive to one class of therapy than the other. Conducting a subgroup analysis to examine whether specific ecotypes correlate more strongly with PD-(L)1 or CTLA-4 responses would provide valuable clinical insights. If direct comparisons are not possible due to sample size limitations, at least discussing the potential implications of ecotype-specific responses to different ICI therapies would be beneficial.

3. Confounding Factors in Survival Analysis

While the survival analysis was performed multiple times, it seems potential confounders were not explicitly stated or acknowledged. If adjustments for these confounders are not feasible due to data availability, a clear acknowledgment of their impact in the limitations section would strengthen the study's rigor.

Reviewer #2

(Remarks to the Author)

The authors present an analysis of a large cohort of immune checkpoint inhibitor (ICI) treated patients (from the ORIEN resource) for whom bulk gene expression is available. They apply the EcoTyper tool to identify and quantify immune cell

states in these samples and then related them to ICI response. They also do a deeper dive into melanoma (SKCM) that are specific for predicting response in that cancer. These findings were validated in additional cohorts.

1610 patients in ORIEN were treated with ICIs, comprising a mix of cancer types for which >160 samples were available in each. Pembrolizumab is the most common ICI though more SKCM patients received Ipilimumab than other cancer types.

The authors applied the EcoTyper approach across cancers and found that the majority of samples (typically >75%) could be assigned to ecotypes, they also show that the individual cell states that make up these ecotypes are generally validated in the ORIEN data. There are differences in the representation across different cancer types, as one would expect.

As the authors note, a large proportion of samples in each cancer type are assigned to CE6 from the original EcoTyper paper. That paper identified this as being the normal/adjacent normal collection of cell states, rather than being tumor. Can the authors comment on why so many of their samples are assigned to be 'normal'?

ORIEN does not have responder/non responder labels for patients and the authors therefore used overall survival (OS) as a metric for evaluating the clinical relevance of ecotypes. This seems reasonable, though it would of course be interesting to have seen PFS. But ultimately survival is the key outcome. They found that the pancancer CE9 from original EcoTyper paper is the most predictive of favorable response to ICIs, across tumor types. This is a nice validation of the original paper.

Interestingly, CE6 (the normal ecotype) is predictive of favorable response. In the original paper, CE6 was variably associated with OS in TCGA (which does not have ICI treated patients) and tended towards unfavorability in the ICI patients of that study. The data for ICI response were limited, which might explain this difference, and also there was a mix of therapies. Nonetheless do the authors have any possible explanation?

The authors then fit a multivariate model to the ICI SKCM data that selected 5 ecotypes (including pro-inflammatory CE9 and CE10) in a model with positive weights for C1, CE5, CE7 and negative for C9, CE10. This indicates that, as expected, C9 and CE10 are favorable whereas others are adverse. Interestingly, CE10 gets higher weighting than CE9 even though the latter is the original "ICI response" ecotype. This is a little surprising since CE10 is made up of actively cytotoxic CD8 and CD4 T cells whereas CE9 is made up of exhausted ones. The original paper found that CE10 was however excluded from the tumor mass in general, whereas CE9 occurred within it. Thus the spatial location of cells is informative as well as their state. It would also have been interesting to see the univariate association of ecotype fractions with ICI OS data.

In addition to previously derived ecotypes the authors apply EcoTyper in discovery mode to melanoma data and found 3 ecotypes. Their ecotype E3 showed particularly poor response to ICIs. Unfortunately there is no comparison to the pancancer ecotypes in terms of what the specific transcriptional differences are in the new ecotypes compared to the others. For example do new ecotypes have similar cell states to the prior ones; are there different associations of cell types/states in ecotypes compared to pancancer ecotypes etc.? For example one of the 'new' CD4 states (S04) contains CTLA4 suggesting it is similar to the CD4 state from original ecotype CE9.

The authors note several of the limitations including lack of analysis of combination therapies, intensity of treatment; however, these data are not generally available.

Overall the study is confirmatory of the applicability of cellular ecotypes in predicting response to immunotherapies. The methods and approach are solid and the results are described appropriately.

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)

The manuscript by Wang et al, utilised a large pan cancer cohort, ORIEN Avatar ICI, with pre-treatment RNAseq for n= 1610 ICB treated patients. Including patients with melanoma, H&N and lung cancer. The gene expression data then underwent immune phenotyping and clustering, ecotyping, using a previously published method EcoTyper. The study finds that patient biopsies that group into immunogenic ecotypes do better on immunotherapies than those with lymphoid depleted tumours. The team utilises these ecotypes within multivariate models to make response predictions and validate their findings in other cohorts. Overall the study's strength is the large pancancer cohort and application of subtyping developed in TCGA but this is also its weakness with intra cancer heterogeneity not addressed and limited integration of clinical data known to impact outcomes.

Major comments:

- 1) The cancer within this study are a genomically subclasses that have distinct response rates to ICB. Features such as HPV status (H&N) and sun exposure (cutaneous vs acral/mucosal) and smoking greatly affect the overall mutation burden of the tumours and this is broadly associated with ICB response.
 - Can the authors supply the breakdown of the HPV status, melanoma subtype and smoking status of the NSCLC patients in

the table 1.

2) Major clinical features are missing from table 1 that are known to impact ICB response.

- Can the authors please add commonly reported features such as LDH, ECOG status into table 1.

3) Multivariate clinical analysis is lacking. The authors do not include any critical features such as stage, treatment, age and cancer type in the multivariate models. It is therefore difficult to determine if major clinical difference are driving some of the results, although they are expected results.

- Can the authors include the major clinical features in the MV models.

4) The immunogenic subtype of tumours is not strongly consistent from primary to metastatic disease (Paired primary and metastatic lesions of patients with ipilimumab-treated melanoma: high variation in lymphocyte infiltration and HLA-ABC expression whereas tumor mutational load is similar and correlates with clinical outcome - PMC).

- Can the authors examine an confounding impact "tumour origin" might be having on the results as all cancer types have a mixture of primary and metastatic disease tested.

5) The annotations of the CEs is not well introduced and this makes interpretation of the data difficult.

- Can the authors please define the meaning of the CE subtypes either as they mentioned in the results or in the introduction.

- Can the authors please add the cell proportions of each CE per the cancer type as a figure 1C? It is hard to compare cell composition from the heatmap colour legends alone and will aid in introducing the CEs for those not used to Ecotyper.

6) Overall survival is used as a clinical endpoint, although with modern treatments OS may not be the best outcome measure. Can the authors explain why progression free or recurrence free survival was not used?

7) The statistical analysis and modelling methods is difficult to follow. Please add a figure overview of the cohorts used and the modelling steps used in the study to aid in the interpretation.

Minor comments:

1) Figure 4C: Specific time metric OS PFS?

2) Figure 5A. what are the missing row names genes? Please add.

3) "Ecotype E3 was identified by the co-occurrence of CD8-S03 and EPI-S02, which was related to gene SOX10 (Figure 5B)." Please explain how Figure 5B shows this and test that the

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have solved my concerns in the last round. I only have a couple minor language and style comments:

The manuscript use both "immune-checkpoint inhibitor" and "immune checkpoint inhibitor"; choose one format and apply consistently.

Harmonize hyphenation for terms like pro-inflammatory or proinflammatory.

For "cell states" and "carcinoma ecotypes," occasionally abbreviations are introduced but later not used (e.g., "cell states (CSs)" vs. "cell states"); ensure consistent abbreviation use.

Reviewer #2

(Remarks to the Author)

I could not find Table 1 in the files provided.

Could the authors please increase the size of gene labels in Supp Figs 3 and 9. They are not readable. Also the ecotype labels in Supp Fig 4 are blurred (I realize this is often because of journal upload systems).

Otherwise the authors made significant efforts to address prior comments, and have done so clearly.

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)

The authors have addressed my concerns.

Open Access This Peer Review File is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.

The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>

Reviewer #1(Remarks to the Author):

Wang et al. leverages real-world transcriptomic and clinical data from the ORIEN Avatar cohort to identify immune ecotypes that predict response to ICI. Using EcoTyper, tumors were classified into ten carcinoma ecotypes linked to better survival and lymphocyte-deficient ecotypes. A melanoma-specific risk model incorporating five ecotypes was developed and validated using external datasets, confirming its predictive accuracy. Additionally, three novel melanoma-specific ecotypes were discovered, further refining immune profiling. These findings establish immune ecotypes as clinically relevant biomarkers, offering a scalable framework for personalized immunotherapy and improved patient selection.

The dataset includes 1,610 ICI-treated patients, making it one of largest transcriptomic datasets used for ICI analysis. The lack of large-scale transcriptomic datasets in ICI research has been a long-standing challenge, making this dataset particularly valuable. I have a few suggestions for the authors to consider before publication.

Response: We thank the reviewer for the positive evaluation of our manuscript and for providing detailed comments to improve the quality and rigor of the paper.

1. Biological Relevance of Ecotypes (CE1–CE10)

The manuscript effectively classifies tumors into ten carcinoma ecotypes, but providing additional insights into their biological significance would enhance interpretability. Currently, the functional implications of each ecotype are not extensively discussed. The authors should elaborate on the molecular and cellular composition of these ecotypes, highlighting their roles in shaping the tumor microenvironment TME. If possible, integrating insights from previous literature or pathway enrichment analyses could help explain why certain ecotypes correlate with favorable or poor prognosis in ICI-treated patients.

Response: We thank the reviewer for this comment. We agree that a deeper discussion of the biological significance of each carcinoma ecotype (CE1–CE10) enhances interpretability and translational relevance. In response, (1) we have added a new paragraph (the 3rd paragraph) in the revised **Introduction** section to provide biological context and insights from previous literature for each ecotype; (2) In the revised **Results**, we added new analyses (**Figure 1C** and **Supplementary Figure 2**) to further explore the relationships between ecotypes and underlying immune cell state compositions across cancer types in the real-world setting; and (3) in the revised **Discussion**, we elaborated on the functional interpretation and prognostic implications related to ICI-treated patients based on our findings.

2. Differences Between PD-(L)1 and CTLA-4-Treated Patients

The study does not specify whether the identified immune ecotypes exhibit distinct responses to different ICI therapies, such as PD-(L)1 inhibitors versus CTLA-4 inhibitors. Since these inhibitors act through different immune-modulatory mechanisms, certain ecotypes may be more responsive to one class of therapy than the other. Conducting a subgroup analysis to examine whether specific ecotypes correlate more strongly with PD-(L)1 or CTLA-4 responses would provide valuable clinical insights. If direct comparisons are not possible due to sample size limitations, at least discussing the potential implications of ecotype-specific responses to different ICI therapies would be beneficial.

Response: As recommended, we conducted a stratified analysis by ICI class, as shown in **Supplementary Figure 5A**. In a real-world data like the ORIEN cohort, the number of patients receiving

anti-CTLA4 monotherapy is very limited. Therefore, we grouped patients receiving anti-CTLA4-based (either mono or combo) into a single group for analysis. Despite these limitations, we observed consistent ecotype-outcome associations across two subgroups.

3. Confounding Factors in Survival Analysis

While the survival analysis was performed multiple times, it seems potential confounders were not explicitly stated or acknowledged. If adjustments for these confounders are not feasible due to data availability, a clear acknowledgment of their impact in the limitations section would strengthen the study's rigor.

Response: We agree with the reviewer on the importance of accounting for potential confounding factors in the survival analysis. As suggested, we performed multivariable Cox regression models in the melanoma data (**Supplementary Figure 7A**), adjusting for key clinical variables available, including age, sex, and sample type (primary vs. metastatic). Notably, the EcoRIS signature remained significantly associated with both OS and rwPFS after adjustment. While the other covariates were not statistically significant (as expected), this supports as a sensitivity analysis of our univariable findings. We have also acknowledged the limitations from other confounders in the revised **Discussion** section.

Reviewer #2, (Remarks to the Author):

The authors present an analysis of a large cohort of immune checkpoint inhibitor (ICI) treated patients (from the ORIEN resource) for whom bulk gene expression is available. They apply the EcoTyper tool to identify and quantify immune cell states in these samples and then related them to ICI response. They also do a deeper dive into melanoma (SKCM) that are specific for predicting response in that cancer. These findings were validated in additional cohorts. 1610 patients in ORIEN were treated with ICIs, comprising a mix of cancer types for which >160 samples were available in each. Pembro is the most common ICI though more SKCM patients received Ipi than other cancer types.

The authors applied the EcoTyper approach across cancers and found that the majority of samples (typically >75%) could be assigned to ecotypes, they also show that the individual cell states that make up these ecotypes are generally validated in the ORIEN data. There are differences in the representation across different cancer types, as one would expect.

Response: We thank the reviewer for the nice summary and positive evaluation of our manuscript.

As the authors note, a large proportion of samples in each cancer types are assigned to CE6 from the original EcoTyper paper. That paper identified this as being the normal/adjacent normal collection of cell states, rather than being tumor. Can the authors comment on why so many of their samples are assigned to be 'normal' ?

Response: We appreciate the reviewer's observation. We believe this unexpected pattern arises from two main reasons. First, such pattern is most prominent at pan-cancer level and is largely driven by kidney cancer cases assigned to this ecotype (Supplementary Fig. 6), where the transcriptomic profiles of these tumors may resemble those of adjacent normal tissues. Second, in real-world setting, the classification may occasionally favor CE6 (TCGA-derived) when multiple ecotypes exhibit similar transcriptional features. We have incorporated this discussion into the revised manuscript to address this point.

ORIEN does not have responder/non responder labels for patients and the authors therefore used overall survival (OS) as a metric for evaluating the clinical relevance of ecotypes. This seems reasonable, though it would of course be interesting to have seen PFS. But ultimately survival is the key outcome. They found that the pancancer CE9 from original Ecotyper paper is the most predictive of favorable response to ICIs, across tumor types. This is a nice validation of the original paper.

Response: We thank the reviewer for the thoughtful summary and positive evaluation. As noted, responder/non-responder labels are not available in the ORIEN dataset. However, in the revision, we have included real-world progression-free survival (rwPFS) as an additional clinical outcome metric to complement OS. The results shown in **Supplementary Figure 4**, which include pan-cancer and major cancer types, along with the additional EcoRIS analysis in **Supplementary Figure 7**, further support the prognostic relevance of the ecotypes. Furthermore, in this revision, we explored the association between ecotypes and binary treatment response as predicted by COMPASS, a newly developed foundation model for predicting immunotherapy response from transcriptomic data. The observed concordance in **Figure 4D** further supports the predictive value of the EcoRIS score.

Interestingly, CE6 (the normal ecotype) is predictive of favorable response. In the original paper, CE6 was variably associated with OS in TCGA (which does not have ICI treated patients) and tended towards unfavourability in the ICI patients of that study. The data for ICI response were limited, which might explain this difference, and also there was a mix of therapies. Nonetheless do the authors have any possible explanation?

Response: We thank the reviewer for this thoughtful question. First, we note that Figure 1A shows an overall pattern across pan-cancer types, which can be influenced by differences in survival across cancer types and sample composition. In our dataset, nearly half of the CE6-assigned samples come from kidney cancer, which generally shows better survival outcomes in our cohort (**Supplementary Figure 6**). Also, we found that in our data, CE6-assigned samples were enriched for primary tumors, which may partly explain the observed discrepancy. We have added this explanation to the revised Discussion section.

The authors then fit a multivariate model to the ICI SKCM data that selected 5 ecotypes (including pro-inflammatory CE9 and CE10) in a model with positive weights for CE1, CE5, CE7 and negative for C9, CE10. This indicates that, as expected, C9 and C10 are favorable whereas others are adverse. Interestingly, CE10 gets higher weighting than CE9 even though the latter is the original "ICI response" ecotype. This is a little surprising since CE10 is made up of actively cytotoxic CD8 and CD4 T cells whereas CE9 is made up of exhausted ones. The original paper found that CE10 was however excluded from the tumor mass in general, whereas CE9 occurred within it. Thus, the spatial location of cells is informative as well as their state. It would also have been interesting to see the univariate association of ecotype fractions with ICI OS data.

Response: We appreciate the reviewer's comments. As suggested, we have added the univariable associations between individual ecotype fractions and OS in the ICI-treated melanoma cohort to the Supplementary Materials (**Supplementary Figure 7B**). The results are consistent with the multivariable model and align with the patterns shown in Figure 3C. Regarding the stronger contribution of CE10 compared to CE9, we agree this is an interesting observation. One of our hypotheses is that CE10 may reflect more naïve or recovering cytotoxic immune responses, as suggested in a recent breast cancer study (PMID: 40069179), whereas CE9 represents more exhausted or dysfunctional immune states. This distinction may be particularly relevant in real-world settings, where a substantial proportion of patients have received prior treatment. There is also a prior study noted that CE10 is more enriched in lymph node, which may confound the signatures in metastatic tissue types such as in melanoma. We have expanded on this interpretation in the revised **Results** and **Discussion** section.

In addition to previously derived ecotypes the authors apply EcoTyper in discovery mode to melanoma data and found 3 ecotypes. Their ecotype E3 showed particularly poor response to ICIs. Unfortunately, there is no comparison to the pancancer ecotypes in terms of what the specific transcriptional differences are in the new ecotypes compared to the others. For example do new ecotypes have similar cell states to the prior ones; are there different associations of cell types/states in ecotypes compared to pancancer ecotypes etc. ? For example one of the 'new' CD4 states (S04) contains CTLA4 suggesting it is similar to the CD4 state from original ecotype CE9.

Response: As suggested, we have added **Supplementary Figures 8B & 8C** to further explore the transcriptional differences between the melanoma-specific ecotypes (E1–E3) identified in discovery mode and the relationship with previously defined pan-cancer ecotypes. We observed that the new ecotype E3, which is associated with poor ICI response, is characterized by a lower proportion of CE9 and CE10 (immunogenic ecotypes) and is instead enriched for CE7. In addition, as the reviewer pointed out, we found that E1, E2, and E3 are characterized by distinct CD4+ T cell states—S04, S02, and S01, respectively (**Supplementary Figure 9**), which also contribute to the observed prognostic patterns. We have expanded the discussion of these findings in the revised **Results** section.

The authors note several of the limitations including lack of analysis of combination therapies, intensity of treatment; however, these data are not generally available. Overall the study is confirmatory of the applicability of cellular ecotypes in predicting response to immunotherapies. The methods and approach are solid and the results are described appropriately.

Response: We thank the reviewer again for the thorough reading and insightful comments.

Reviewer #3, ECR (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response: no action is needed.

Reviewer #4, (Remarks to the Author):

The manuscript by Wang et al, utilised a large pan cancer cohort, ORIEN Avatar ICI, with pre-treatment RNAseq for n= 1610 ICB treated patients. Including patients with melanoma, H&N and lung cancer. The gene expression data then underwent immune phenotyping and clustering, ecotyping, using a previously published method EcoTyper. The study finds that patient biopsies that group into immunogenic ecotypes do better on immunotherapies than those with lymphoid depleted tumours. The team utilises these ecotypes within multivariate models to make response predictions and validate their findings in other cohorts. Overall the

study's strength is the large pancancer cohort and application of subtyping developed in TCGA but this is also its weakness with intra cancer heterogeneity not addressed and limited integration of clinical data known to impact outcomes.

Response: We thank the reviewer for the thoughtful summary and careful review of our manuscript.

Major comments:

1) The cancer within this study are a genomically subclasses that have distinct response rates to ICB. Features such as HPV status (H&N) and sun exposure (cutaneous vs acral/mucosal) and smoking greatly affect the overall mutation burden of the tumours and this is broadly associated with ICB response.

- Can the authors supply the breakdown of the HPV status, melanoma subtype and smoking status of the NSCLC patients in the table 1

Response: We appreciate the reviewer's important comment regarding key clinical and genomic features that may influence ICB response. However, as is often the case with real-world datasets, HPV status was missing for a large proportion of patients. Therefore, we conducted a sensitivity analysis restricted to HPV-negative patients. While the sample size was limited, the observed trends remained consistent with our overall findings. For melanoma, all included cases were cutaneous melanoma, based on clinical annotation. Regarding NSCLC, we performed a sensitivity analysis restricted to patients with known smoking history. The three ecotype groups (CE1–2, CE3–8, and CE9–10) remained clearly separated in overall survival. These additional subgroup analyses are included in **Supplementary Figures 5B and 5C**.

2) Major clinical features are missing from table 1 that are known to impact ICB response.

- Can the authors please add commonly reported features such as LDH, ECOG status into table 1.

Response: As suggested, we have additional clinical features such as LDH and ECOG into **Table 1**.

3) Multivariate clinical analysis is lacking. The authors do not include any critical features such as stage, treatment, age and cancer type in the multivariate models. It is therefore difficult to determine if major clinical difference are driving some of the results, although they are expected results.

- Can the authors include the major clinical features in the MV models.

Response: We thank the reviewer for this important point. In our multivariable models, we included key clinical covariates available in our dataset, including age, sex, and tumor sample type (primary vs. metastatic), as shown in the forest plot (**Supplementary Figure 7A**). For the melanoma cohort in the immunotherapy-treated group, most patients were late-stage, and detailed staging information was either unavailable or not uniformly recorded in the real-world dataset. Treatment type was considered in our stratified analyses for anti-PD-(L)1 and CTLA-4 therapies, as presented in **Supplementary Figure 5A**.

4) The immunogenic subtype of tumours is not strongly consistent from primary to metastatic disease (Paired primary and metastatic lesions of patients with ipilimumab-treated melanoma: high variation in lymphocyte infiltration and HLA-ABC expression whereas tumor mutational load is similar and correlates with clinical outcome - PMC).

- Can the authors examine an confounding impact “tumour origin” might be having on the results as all cancer types have a mixture of primary and metastatic disease tested.

Response: We appreciate the reviewer’s important observation. As noted, tumor origin (primary vs. metastatic) can influence the tumor immune and transcriptomic profiles. In our analysis, we accounted for this by including tumor sample type as a covariate (primary vs. metastatic) in the multivariable Cox models (**Supplementary Figure 7A**). The EcoRIS score remained significantly associated with survival outcomes after adjustment. We agree that transcriptomic differences between primary and metastatic tumors could introduce variability; however, our dataset includes very few matched primary-metastatic pairs, limiting our ability to systematically assess this effect. Notably, the EcoRIS signature was developed using the melanoma cohort, in which ~2/3 samples are from metastatic lesions. Due to limited numbers of primary tumor samples, we do not currently have sufficient power to construct and validate a separate EcoRIS model. This limitation has been noted in the revised **Discussion** section.

5) The annotations of the CEs is not well introduced and this makes interpretation of the data difficult.

- Can the authors please define the meaning of the CE subtypes either as they mentioned in the results or in the introduction.

- Can the authors please add the cell proportions of each CE per the cancer type as a figure 1C? It is hard to compare cell composition from the heatmap colour legends alone and will aid in introducing the CEs for those not used to Ecotyper.

Response: We thank the reviewer for this helpful suggestion. To improve clarity for readers unfamiliar with Ecotyper, we have added a new paragraph (the third paragraph) in the revised **Introduction** section that provides biological context and summarizes key insights from prior literature for each ecotype. (Please also refer to the first comment to Reviewer #1). As suggested, we have also added **Figure 1C** to include cell-type proportions per CE for each cancer type, allowing easier interpretation beyond the heatmap. Additional supporting data have been included in **Supplementary Figure 2**. We hope these additions improve the interpretability of the ecotype signatures.

6) Overall survival is used as a clinical endpoint, although with modern treatments OS may not be the best outcome measure. Can the authors explain why progression free or recurrence free survival was not used?

Response: We appreciate the reviewer’s comment. In addition to OS, we included real-world progression-free survival (rwPFS) as a complementary clinical endpoint. The results based on rwPFS are consistent with the OS findings and are included in the revised manuscript (**Supplementary Figures 4 and 7**).

7) The statistical analysis and modelling methods is difficult to follow. Please add a figure overview of the cohorts used and the modelling steps used in the study to aid in the interpretation.

Response: We thank the reviewer for this helpful suggestion. To improve clarity, we have revised the Statistical Analysis section to more clearly describe the modeling workflow. An overview of the cohorts used in the study is provided in **Supplementary Fig 1**.

Minor comments:

1) Figure 4C: Specific time metric OS PFS?

Response: We have clarified that the time metric in Figure 4C is overall survival (OS).

2) Figure 5A. what are the missing row names genes? Please add.

Response: In Figure 5A, the row names correspond to cell types and states, as indicated by the color legend at the bottom of the figure—not individual gene names.

3) “Ecotype E3 was identified by the co-occurrence of CD8-S03 and EPI-S02, which was related to gene SOX10 (Figure 5B).” Please explain how Figure 5B shows this and test that the

Response: We apologize for the confusion. We have now revised the text. The cell state EPI-S02 is related to a set of genes including SOX10. The supporting cell state–level gene expression heatmap is now included in Supplementary Figure 9 to provide additional context.

Reviewer #1 (Remarks to the Author):

The authors have solved my concerns in the last round. I only have a couple minor language and style comments: The manuscript use both "immune-checkpoint inhibitor" and "immune checkpoint inhibitor"; choose one format and apply consistently. Harmonize hyphenation for terms like pro-inflammatory or proinflammatory. For "cell states" and "carcinoma ecotypes," occasionally abbreviations are introduced but later not used (e.g., "cell states (CSs)" vs. "cell states"); ensure consistent abbreviation use.

Response: We have standardized these terms throughout the revised manuscript.

Reviewer #2 (Remarks to the Author):

I could not find Table 1 in the files provided. Could the authors please increase the size of gene labels in Supp Figs 3 and 9. They are not readable. Also the ecotype labels in Supp Fig 4 are blurred (I realize this is often because of journal upload systems).

Otherwise the authors made significant efforts to address prior comments, and have done so clearly.

Response: Table 1 has been added by the editor. We have also improved the quality of supplementary figures in the revised version.