nature portfolio

Peer Review File

Spatial transcriptomics reveals substantial heterogeneity in triple negative breast cancer with potential clinical implications

Corresponding Author: Dr Christos Sotiriou

This manuscript has been previously reviewed at another journal. This document only contains reviewer comments, rebuttal and decision letters for versions considered at Nature Communications.

Parts of this Peer Review File have been redacted as indicated to remove third-party material.

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

Version 0:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
Wang and colleagues examine a series of 92 patients with triple-negative breast cancer by Spatial Transcriptomics. Samples were from fresh, frozen specimens. Bulk RNA sequencing was conducted with analysis via Salmon. Extensive histopathology was performed, and the integrated analysis with spatial transcriptomics is a strength of the paper. Over all the paper is well done, but could benefit from further discussion and clarification in several sections.
Major critiques:
There is a broader question of whether ecotypes are sub-categories of the molecular subtypes. Obviously, there will be imperfections and noise, but for example, ET5 is nearly equivalent to the LAR subtype. Can the authors address how we should think of these ecotypes in relation to the molecular subtypes? I am interpreting their data as perhaps sub-categories, accepting that there is some noise & misclassification driven by the unsupervised analysis. This is a more major topic of clarification.
A broad question is whether ST was needed to identify the subtypes. There isn't an extensive analytical analysis of the spatial orientation of ecotypes with respect to one another. I wondered if these events would be seen in single-cell multi-omics. Why or why not?
An example: "As illustrated in Fig. 1b and c, TNBC appears as a highly heterogeneous entity made up of a mosaic of variable morphological features". Is it possible to quantify this spatial arrangement so that we can make true conclusions of associations? It seems overly qualitative.
Another example of where the paper would be better served by looking analytically at spatial orientation is in this sentence: "These results indicate substantial intra-patient heterogeneity within each TNBC molecular subtype, with different contributions of tumor and stroma features associated with distinct molecular characteristics and clinical outcome."
Additional Critiques
The paper could better delineate the individual-level characteristics of the samples as they relate to the different features in the figures and tables (e.g., histopathological features, BRCA status, TILs, ecotypes, etc.). Specifically, it is recommended to have a single table that shows the 92 samples*3 slides, with columns for each characteristic. Right now, some of that detail is lost in the summary tables.
Graph 1D: It would be helpful to show the variability of % pixels by sample (such as through a boxplot or violin plot). This allows a better sense of the sample-to-sample variability, somewhat analogous to 1F.
It is a bit difficult to follow Table 1 vs. Supplemental Table 1. One would like to look at these by molecular subtype, but Table 1 aggregates.
Figure 6A & 6C don't fully agree and appear inconsistent. For example, all ecotypes show that at least some samples are of the LAR molecular subtype, but looking at 6A, LAR is only found in ET5. Could the authors clarify?

It would be interesting to see the authors discuss the benefits (or lack thereof) of greater resolution than the 100-micron spots. Does the deconvolution address it, and to what extent would better resolution help? Reading the methods, I sensed

that additional clarity on the experimental methods needs to be provided, rather than referring to Stahl et al. from 2016.

Reviewer #4

(Remarks to the Author)
In this manuscript, Wang and colleagues present an extensive body of work to study the spatial intra- and inter-tumoral heterogeneity of triple negative breast cancer. To do so, they have performed spatial transcriptomics on an impressive cohort of TNBC tumors. Their computational approach is rigorous and innovative and address some of the technological limits of spatial transcriptomics, while returning interesting results. As such, I believe this study is timely and will be of interest to the community.
The text itself is well organized, but is at times difficult to follow due to a systematic use of many abbreviations, which tends to hinder comprehension.
While the manuscript is overall sound, I have some concerns. I think addressing them would enhance the quality of the manuscript.

1) In several instances, the authors only rely on visual similarities, rather than on accurate, reliable statistical approaches. This is notably the case for the analyses presented in figures 3a and 3e. Figure 3e only relies on visual cues in a single sample. Figure For all these, the authors need to derive proper, cohort-wide statistical approaches to support their claims. Similarly, Figure 3b would also benefit from statistical tests to compare the results.

2) The association between annotations and deconvolution estimates are only scarcely validated in the current version. This is an important foundation of most of the results presented in the manuscript, and it needs to be assessed quantitatively and thoroughly.

3) Figure 3b presents results using xCell, which notably includes M1/M2 macrophages. The consensus among macrophages experts is now widely against this nomenclature. M1 and M2 are rather theoretical opposites of the activation spectrum of macrophages in vitro, and most tumor associated macrophages would exhibit both markers that used to be considered associated to M1 and M2. I would suggest the authors to refrain from referring to this nomenclature, or to perhaps use other deconvolution approaches that do not use them.

4) The authors show quite well the prognostic relevance of the megaclusters and ecotypes. How about their association with response to immunotherapy? The data is available, as they used it with regards to the TLS signature.

5) Evaluating the prognostic impact on a cohort that includes the discovery cohort (figure 5f) can be biased. This analysis should be carried in fully independent cohorts. The authors could consider validating results on an independent cohort such as TCGA?

6) The manuscript is at times very difficult to follow. This is due to several things:

a. There are far too many abbreviations in the text, and some paragraphs are basically unreadable unless the reader is intimately familiar with all of them.
b. The annotations of megaclusters and ecotypes are all listed in the text, but presenting a summary figure highlighting their main hallmarks would strongly help readers.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I appreciate the authors' considerable effort in addressing the concerns raised during the previous round of review. Their integration of spatial transcriptomics with histomorphological data to redefine TNBC subtypes is an innovative approach, and the manuscript is now appropriately aligned with the scope of Nature Communications. The authors have clearly presented their methodology and the clinical implications of their findings, especially regarding immune-enriched subtypes and actionable targets, which enhance the overall value of the study.

However, I still hold reservations regarding the use of the term "ecotype" to describe these TNBC subtypes. While the authors acknowledge that their use of this term does not strictly adhere to its biological definition, I believe that introducing such terminology in tumor stratification might introduce unnecessary confusion. A more conventional term could enhance clarity without detracting from the novelty of the findings. Nonetheless, with this adjustment, the manuscript would be a strong contribution to the field.

Reviewer #2

(Remarks to the Author)

The manuscript describes a medium-resolution spatial transcriptomic analysis of a cohort of TNBC samples. This is a revision of a previous submission. The present analysis assesses how distinct biological structures within spatially heterogeneous samples contribute to bulk RNA signatures. This is useful information and advances our understanding of how to interpret bulk RNA sequences. The manuscript also usefully subdivides TNBCs into subgroups associated with clinical outcome and/or response to treatment. The associations are validated using previously published clincial cohorts. The manuscript also provides some suggestions for improved therapeutic attacks. The data in the manuscript are publically available and so will enable following secondary analyses by the the extended compoutational community. I recommend publication of this revised manuscript.

Reviewer #3

(Remarks to the Author)
Overall, the reviewers have addressed my prior concerns. Upon reading the other reviewers responses, I felt that the responses to reviewer 1 would actually serve the paper & should be more directly included, rather than just as part of the dialogue with reviewer 1.

There is of course considerable different types of validation that could be done, and these are highlighted by the other reviewers. Given the steps required, I am ok with the other reviewer responses. My comments were generally adddressed earlier.

Reviewer #4

(Remarks to the Author)
The revised version of the manuscript is substantially improved, and the new manuscript reads more easily. Most of my comments have been adequately addressed.

My point 5 in the original review has however not been answered. Keeping the ST TNBC cohort in the analysis of now figure 7f presents poses a risk of bias. Even though no clinical information was used to establish MCs, this still cannot be considered to be an independent validation cohort. Using this cohort in both Fig 7e and 7f does not allow to see validation on a really independent cohort, and the authors should remove the ST TNBC bulk cohort from the analysis presented in Fig. 7f.

Regarding the impact of ecotypes on response to immunotherapy, it would be very valuable if the data the authors are waiting on accessing (rebuttal to my point 4) could be incorporated into the final manuscript.

Finally, a minor comment on the new figure 1. This figure strongly helps to follow the story presented here and I really appreciate the effort the authors have done. However, the current layout (mostly because of the arrow) of the figure could give the impression that there is an evolution or development of tumors from LAR to M to MSL to BL to IM instead of a spectrum of presentations that do not evolve from one state to another. Also, the legend of that figure shows different colors for CAFs and all CAFs drawn on the figure are the same color, which is likely a simple omission.

We thank the reviewers for the critical assessment of our manuscript and the valuable comments. We have addressed all the comments and performed revisions in the manuscript, the main figures, and the supplementary figures/tables/data according to the suggestions. Please find hereunder a point-by-point reply to the reviewers' comments, with the comment appearing in regular text and our reply following in bold text. The revised manuscript version (manuscript, main figures) has been uploaded in two forms, one clean and one where all the changes to the manuscript are marked with the red words/track changes.

[Editorial note: reports from previous journals have been redacted**]**

[redacted]

REVIEWERS <NATURE COMMUNICATIONS

Reviewer #3 (Remarks to the Author): with expertise in translational genomics, spatial transcriptomics, breast cancer

Wang and colleagues examine a series of 92 patients with triple-negative breast cancer by Spatial Transcriptomics. Samples were from fresh, frozen specimens. Bulk RNA sequencing was conducted with analysis via Salmon. Extensive histopathology was performed, and the integrated analysis with spatial transcriptomics is a strength of the paper. Overall the paper is well done, but could benefit from further discussion and clarification in several sections.

Major critiques:

There is a broader question of whether ecotypes are sub-categories of the molecular subtypes. Obviously, there will be imperfections and noise, but for example, ET5 is nearly equivalent to the LAR subtype. Can the authors address how we should think of these ecotypes in relation to the molecular subtypes? I am interpreting their data as perhaps sub-categories, accepting that there is some noise & misclassification driven by the unsupervised analysis. This is a more major topic of clarification.

Reply:
We thank Reviewer #3 for addressing this major question about the significance of the ecotypes in light of previously established bulk RNA-seq-derived molecular subtypes. Ecotypes are derived exclusively from spatial transcriptomics (ST) data, based on the identification of geographical information and the clustering of spatial genes regardless of the five molecular subtypes. This bioinformatic process is distinct from the bulk RNA-seq-derived TNBC molecular classification as defined by Bareche et *al*.
Defining ecotypes merely as subcategories of the five molecular subtypes is an oversimplification. Ecotypes provide higher granularity in understanding TNBC heterogeneity. For example, while the LAR subtype can be associated with a distinct ecotype (ET5), this is not the case for other molecular subtypes. At the spatial level, the combination of certain gene expression clusters leads to ecotypes with specific molecular features, which are associated with different clinical outcomes and potential different therapeutic vulnerabilities. To highlight this, we now added a new main figure (Figure 9).

Figure 9:

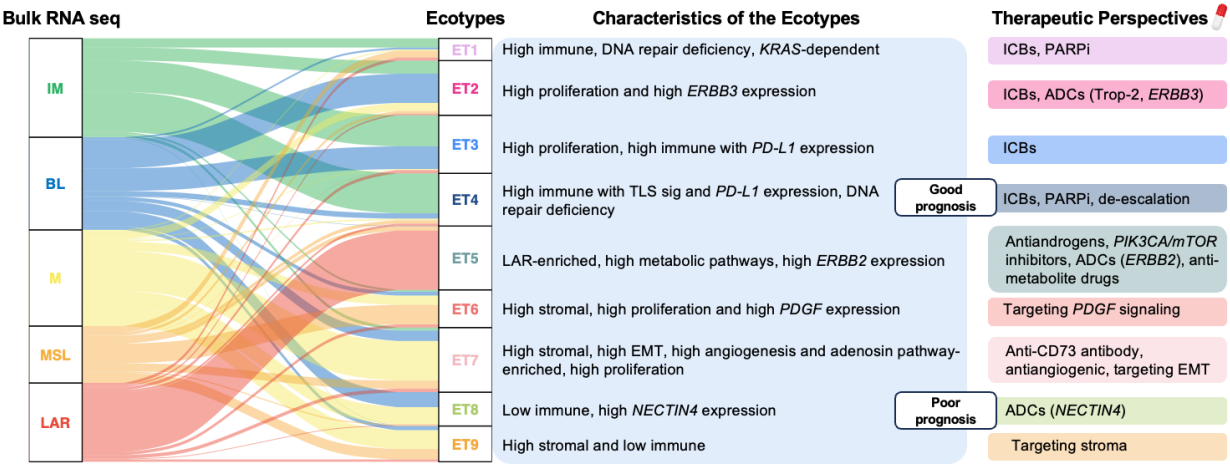


Fig. 9. Evolution of the molecular subtypes in TNBC from bulk RNA-seq analysis to ST-derived ecotypes.
The alluvial plot shows the distribution of the five pre-existing molecular subtypes into different ecotypes in the ST TNBC, METABRIC and SCAN-B cohorts. The molecular subtype was defined from the ST global pseudobulk and from METABRIC and SCAN-B bulk transcriptomes. The main characteristics of each ecotype are summarized, highlighting the potential for precision medicine in TNBC with specific therapeutic strategies for each ecotype.
Source data are provided as a Source Data file.
ADC: antibody-drug conjugate; BL: basal-like; EMT: epithelial-mesenchymal transition; ET: ecotype; ICB: immune checkpoint blockade; IM: immunomodulatory; LAR: luminal androgen receptor; M: mesenchymal; MSL: mesenchymal stem-like; PARPi: poly-ADP ribose polymerase inhibitor; TLS: tertiary lymphoid structures.

These insights cannot be inferred by simply "subcategorizing" the five molecular subtypes. In our study, we were able to identify two distinct immune-enriched subtypes among the IM TNBC subtype, namely ET3 and ET4. These subtypes exhibit different clinical outcomes that cannot be captured by routine biomarkers in the clinic, such as TILs. Additionally, we have identified ET8 as an immunosuppressed subtype with a particularly poor prognosis, enriched in NECTIN4, a potential target for antibody-drug conjugates.

A broad question is whether ST was needed to identify the subtypes. There isn't an extensive analytical analysis of the spatial orientation of ecotypes with respect to one another. I wondered if these events would be seen in single-cell multi-omics. Why or why not?

Reply:
We thank the reviewer for this valuable comment. The spatial orientation of the ecotypes is challenging to analyze because ecotypes are defined at the patient level. As aforementioned, ecotypes are established based on a local combination of tumor and stromal cells and the identification of spatial genes in the initial step. The methodology inherently relies on the spatial information from each TNBC sample. Since ecotypes are derived from ST data, it is difficult to predict if the same ecotypes could be observed at the single-cell multi-omics level, where spatial information is absent, and different cell subtypes are individualized.

An example: "As illustrated in Fig. 1b and c, TNBC appears as a highly heterogeneous entity made up of a mosaic of variable morphological features". Is it possible to quantify this spatial arrangement so that we can make true conclusions of associations? It seems overly qualitative.

Reply:

Fig. 1b (now Fig. 3a) is shown for illustrative purposes to highlight TNBC heterogeneity. Fig. 1c (now Fig. 3b) represents the prevalence of different histomorphological categories across the whole cohort. These figures do not provide the quantification of the morphological analysis, which is detailed in Fig. 1d (now Fig. 3c), Fig. 1f (now Fig. 3e) and Extended Data 1 (now Supplementary Fig. 1a-b). In these figures, we quantify the prevalence of histomorphological categories and metrics related to spatial organization into tumor and stroma patches in the context of TNBC molecular subtypes. The morphological features were also analyzed quantitatively within the molecular subtypes of the tumor and stroma compartments (now Supplementary Fig. 3a-b: annotations), as well as across the ecotypes (Supplementary Fig 16c-d, now Supplementary Fig. 19c-d).

Another example of where the paper would be better served by looking analytically at spatial orientation is in this sentence: "These results indicate substantial intra-patient heterogeneity within each TNBC molecular subtype, with different contributions of tumor and stroma features associated with distinct molecular characteristics and clinical outcome."

Reply:
The sentence highlighted by Reviewer #3 serves as the conclusion of the section where we analyzed tumor and stroma compartments separately at the gene expression level. In this section, we investigated the heterogeneity within each compartment. The analysis of the spatial interaction between these compartments is the focus of a subsequent section in the paper (see Section "Identification of fourteen spatial molecular patterns shared across TNBC patients").

Additional Critiques

The paper could better delineate the individual-level characteristics of the samples as they relate to the different features in the figures and tables (e.g., histopathological features, BRCA status, TILs, ecotypes, etc.). Specifically, it is recommended to have a single table that shows the 92 samples*3 slides, with columns for each characteristic. Right now, some of that detail is lost in the summary tables.

Reply:
We thank the Reviewer for the suggestion. We have now updated Supplementary Table 1 (now Supplementary Data 1), which originally summarized primarily the clinico-pathological data, including clinical outcomes, TILs, and TIME classification. We enhanced this table by adding information about molecular subtypes and ecotypes. More specific data, such as QC by array/slide, morphological annotations, TLS signatures, and regression/deconvolution-derived results, are provided in successive Supplementary Data files to maintain the flow and order of the manuscript. The details about the Supplementary Data are reported in the file "Description of Additional Supplementary Files."

Graph 1D: It would be helpful to show the variability of % pixels by sample (such as through a boxplot or violin plot). This allows a better sense of the sample-to-sample variability, somewhat analogous to 1F.

Reply:
We agree with the reviewer on this point. This information was indeed already present in the previous Extended Data 1a (now Supplementary Fig. 1a), which shows the distribution of morphological annotations as a percentage of pixels for a given annotation across the samples, analyzed within the five TNBC molecular subtypes. Each dot represents a sample.

It is a bit difficult to follow Table 1 vs. Supplemental Table 1. One would like to look at these by molecular subtype, but Table 1 aggregates.

Reply:
We understand the Reviewer's concern about the difficulty in distinguishing Table 1 from Supplementary Table 1 (now Supplementary Data 1). Table 1 provides a global view of the clinicopathological data across the molecular subtypes, highlighting the heterogeneity of our TNBC cohort. In contrast, Supplementary Table 1 (now Supplementary Data 1) offers a comprehensive overview of each TNBC sample, including all relevant information such as clinicopathological data, morphological results, and ST-derived results.

Figure 6A & 6C don't fully agree and appear inconsistent. For example, all ecotypes show that at least some samples are of the LAR molecular subtype, but looking at 6A, LAR is only found in ET5. Could the authors clarify?

Reply:
We apologize for the lack of clarity on this Figure. Figure 6A (now Fig. 8a) was computed using the study dataset (ST TNBC cohort). Figure 6C (now Fig. 8c) was computed using the ecotypes (ETs) derived from our deconvolution method applied to the bulk expression data from the combined study ST TNBC dataset (bulk), METABRIC, and SCAN-B. To clarify this, we have now modified the title of Figure 6C (now Fig. 8C).

Figure 8c:

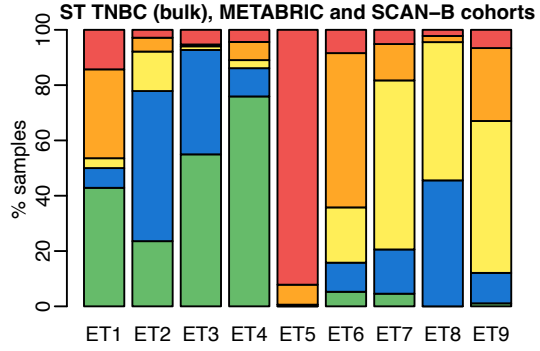


Fig. 8c: *Barplot showing the distribution of TNBC molecular subtypes across the ecotypes in the merged TNBC cohorts (ST TNBC bulk, METABRIC and SCAN-B).*

It would be interesting to see the authors discuss the benefits (or lack thereof) of greater resolution than the 100-micron spots. Does the deconvolution address it, and

to what extent would better resolution help? Reading the methods, I sensed that additional clarity on the experimental methods needs to be provided, rather than referring to Stahl et al. from 2016.

Reply:
Smaller ST spots, as provided by Visium technology, will offer higher resolution of spatial gene expression. In our 2K spots ST array, deconvolution helped us understand the composition of each ST spot by treating each spot as a mini-bulk RNA-seq sample. However, due to the size of each spot on the 2K array, the detailed spatial cell-cell layout is lacking. In this context, we believe that recent ST technologies would enable more detailed analyses. For example, with the 2K array, only a few spots (1-3) cover TLS. The analysis of TLS composition and the spatial distribution of different cell types within them would be enhanced by deploying recent technologies.
We now added this paragraph, as suggested by the Reviewer, to the Discussion section of the paper (raw 717 to 730): *"Our study faced several challenges and limitations. The resolution of spatial transcriptomics has dramatically increased, from our ST technology (2K 100 µm diameter spots) to the most recent Visium (4992 spots of 55 µm diameter) and Visium HD technology (2 x 2 µm barcoded squares), which approaches single-cell scale[66]. Our spots were analyzed as individual mini-RNA bulks containing up to 200 cells, which obscured the cellular and molecular heterogeneity within each spot. This limitation led to issues such as the presence of the BL subtype in the stroma compartment and the IM subtype in the tumor compartment, due to the discrimination limits of the regression tool. Additionally, deeper analysis of small structures like TLS is constrained, as only a few spots (1-3) cover one TLS. The analysis of TLS composition and the spatial distribution of different cell types within them would benefit from recent technologies. Another limitation is the validation of the clinical relevance of our results in external cohorts. We are eagerly awaiting access to data from clinical trials to assess the clinical utility of the ET and TLS signatures."*

For the experimental methods, we referred to the previously published papers of Lundeberg's team, as we applied the same methodology in our study in collaboration with his team. It is important to reference these prior publications. However, we have also detailed all the steps of the experimental method across different sections to ensure consistency with the previous publications:
-ST Arrays
-Tissue handling, staining and imaging
-Permeabilization and cDNA synthesis
-Tissue removal and cDNA release from the surface
-Library preparation and sequencing

Reviewer #4 (Remarks on code availability):

Code is available. README appears fine. Data links under restriction, pending publication.

Reply:
We thank the Reviewer for the comment about the code availability.

Reviewer #5 (Remarks to the Author): with expertise in TLS/cancer immunology, spatial transcriptomics, computational

In this manuscript, Wang and colleagues present an extensive body of work to study the spatial intra- and inter-tumoral heterogeneity of triple negative breast cancer. To do so, they have performed spatial transcriptomics on an impressive cohort of TNBC tumors. Their computational approach is rigorous and innovative and address some of the technological limits of spatial transcriptomics, while returning interesting results. As such, I believe this study is timely and will be of interest to the community.

The text itself is well organized, but is at times difficult to follow due to a systematic use of many abbreviations, which tends to hinder comprehension.

Reply:
We apologize for the lack of clarity. To enhance the comprehensibility of the paper and facilitate the use of abbreviations, we modified some details of the main figures and the supplementary figures to improve the consistency of the use of different terms (e.g., naming "tertiary lymphoid structures" or "TLS" instead of "Lymphoid nodule"). The modified figures are notified by the red color (e.g., Fig. 3a or 3b, Supplementary Fig. 2a-b).
Moreover, we have included a graphical abstract in the main figures (now Fig. 1) to provide an introduction to the paper. We aim for this figure to aid in understanding the molecular subtypes (LAR, M, MSL, IM, and BL), the immune microenvironment (TILs, spatial organization by TIME classification: FI, SR, MR, ID, and TLS), and the stromal fibroblasts (myCAF, iCAF). Additionally, we have reduced the use of unnecessary abbreviations. We hope that these efforts address the concerns raised by the Reviewer.

Figure 1:

[figure redacted]

Fig. 1: Overview of tumor heterogeneity in triple-negative breast cancer.
Previous studies using bulk RNA-seq analysis of TNBC patients have identified five molecular subtypes: luminal androgen receptor, mesenchymal, mesenchymal stem-like, basal-like, and immunomodulatory. These subtypes are associated with distinct tumor microenvironments, characterized by variations in the rate of tumor-infiltrating lymphocytes, spatial immune organizations (TIME classification), and the presence or absence of tertiary lymphoid structures and different cancer-associated fibroblasts.
BL: basal-like; CAF: cancer-associated fibroblast; detox-iCAF: detoxification pathway inflammatory cancer associated fibroblast S1; ecm-myCAF: extracellular matrix myofibroblastic cancer associated fibroblast S1; DC: dendritic cell; FI: full inflamed; iCAF: inflammatory cancer associated fibroblast S1; ID: immune desert; IFNγ-iCAF: interferon gamma signaling pathway cancer associated fibroblast S1; IL-iCAF: IL pathway inflammatory cancer associated fibroblast S1; IM: immunomodulatory; LAR: luminal androgen receptor; M: mesenchymal; MR: margin restricted; MSL: mesenchymal stem-like; myCAF: myofibroblastic cancer associated fibroblast; SR: stroma restricted; TGFβ-myCAF: TGFbeta signaling pathway myofibroblastic cancer associated fibroblast S1; TILs: tumor-infiltrating lymphocytes; TIME: Tumor Immune Micro-Environment; TLS: tertiary lymphoid structures; wound-myCAF: wound healing myofibroblastic cancer associated fibroblast S1.

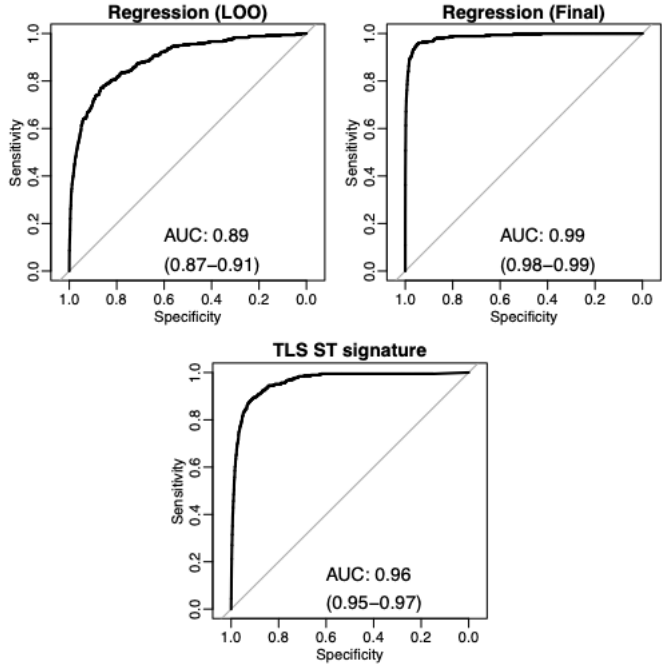
While the manuscript is overall sound, I have some concerns. I think addressing them would enhance the quality of the manuscript.

1) In several instances, the authors only rely on visual similarities, rather than on accurate, reliable statistical approaches. This is notably the case for the analyses presented in figures 3a and 3e. Figure 3e only relies on visual cues in a single sample. For all these figures, the authors need to derive proper, cohort-wide statistical approaches to support their claims. Similarly, Figure 3b would also benefit from statistical tests to compare the results.

Reply:
We wholeheartedly agree that proper statistical tests are crucial. Initially, we chose to present an illustrative case in our figures (as in Figure 3a) to elucidate

the methodology and showcase a typical result of the analysis. However, the same figures for other samples are available on the Zenodo repository.
The statistical approach for evaluating the 'similarities' between the TLS signature and TLS annotation was previously reported in Supplementary Fig 2a (now Supplementary Fig. 6d), where we computed the TLS signatures (both ours and Lundeberg's) on bulk RNA and pseudobulk RNA data. We assessed the accuracy using areas under the curve (AUCs) to predict the presence of TLS based on morphological annotations.
To further address the Reviewer's concerns regarding statistics, we have now added Supplementary Fig. 6c. This figure displays the AUCs for predicting the TLS category (considered present in a spot if the proportion is > 25%) using the regressor and our ST TLS signature. We also added this paragraph to the Results section of the paper (raw 370 to 374): *"The high accuracy of TLS prediction by our signature was quantitatively assessed by the area under the curve (AUC) (Supplementary Fig. 6c). Even when compared with other TLS signature, it demonstrated its high specificity for TLS detection (Supplementary Fig. 6d, Supplementary Data 16-17)."*

Supplementary Figure 6c:



Supplementary Fig. 6c: *AUCs for the prediction of the TLS, using the regressor in a leave-a-patient-out cross-validation (top left), the final regressor, computed using all data (top right) and the 30-gene TLS signature (bottom). Spots with TLS as at least 25% of their morphological annotation were binarized as TLS.*

We then enhanced the reporting of various statistical results in the figures and supplementary data.
For instance, all the results in Fig 3b (now Fig. 5b) are significant correcting for multiple testing (FDR<.05, as stated in the legend). More precisely, all the FDRs of Fig 3b are <.001 and should be stared as * (FDR < 0.001 and ≥ 0.0001) or **** (FDR < 0.0001). The updated figure is provided as below:**

Figure 5b

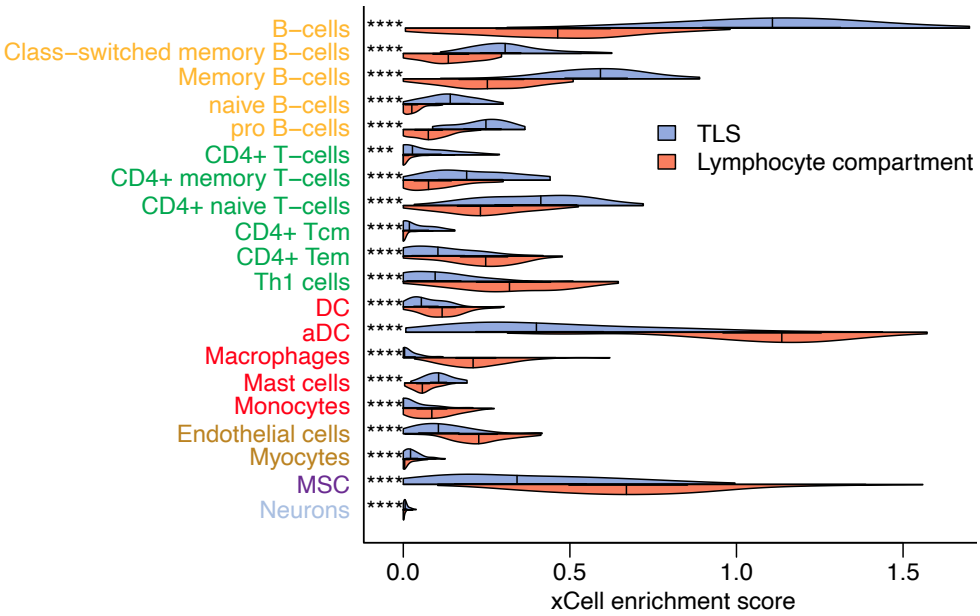


Figure 5b: *Violon plot showing the cell types assessed using xCell that are significantly enriched either in TLS or lymphocyte compartments. Median values are represented by vertical lines. Differences between both compartments were assessed using Wilcoxon rank sum tests. FDRs were obtained adjusting P values using Benjamini & Hochberg method (*FDR < 0.05 and ≥ 0.01; **FDR < 0.01 and ≥ 0.001; ***FDR < 0.001 and ≥ 0.0001; ****FDR < 0.0001).*

The same for Figures 5f and 5g (+ Supplementary Data 16-17):

Figure 5f

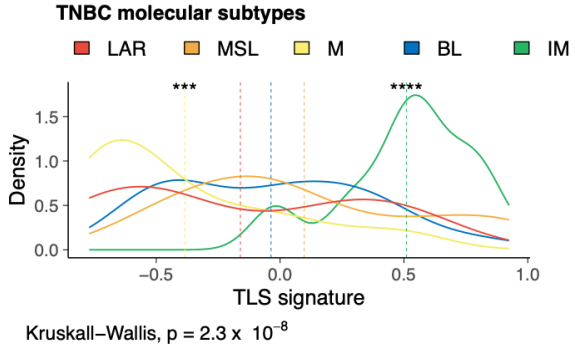
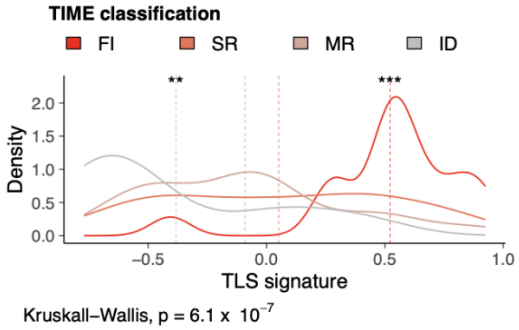


Figure 5g



Figures 5f-g: *Density plots showing the distributions of TLS signature expression across TNBC molecular subtypes **(f)** and TIME classification **(g)**. Dashed lines represent the mean of each subgroup. Statistical differences across subtypes or TIME classes were assessed using Kruskal-Wallis tests and Wilcoxon rank sum test (when comparing each class to all classes). For Wilcoxon tests, FDRs were obtained by adjusting P values using Benjamini & Hochberg method.*

Additionally, we enhanced all the dot plots (e.g., Fig.4c, 8e; Supplementary Fig. 3) by also displaying the dots with non-significant FDR (FDR ≥ 5%) in lighter colors to aid in the interpretation of the results.

Figure 4c:

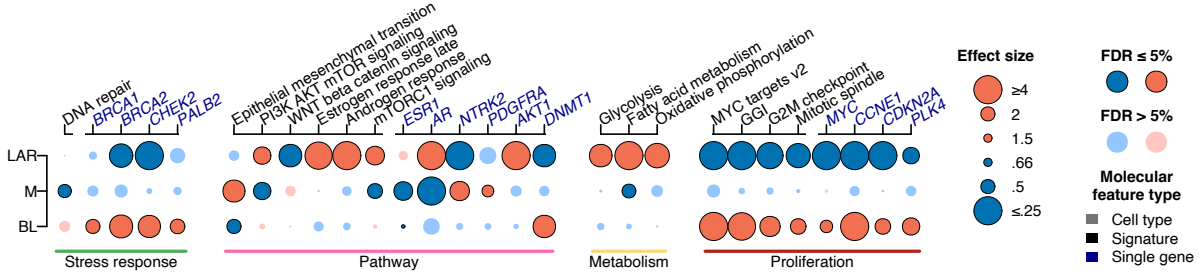
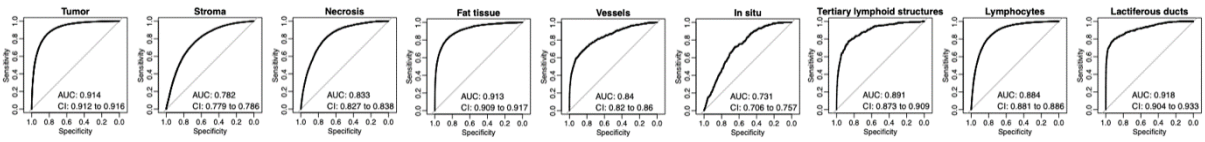


Figure 4c: *Molecular and cellular characterization of the tumor compartment from the different TNBC subtypes. Illustration of representative statistically significant molecular and cellular features associated to tumor compartment including specific single gene expression, gene signatures and xCell cell type enrichment analysis according to logistic regression model. Dots are bordered and dark-colored when FDRs < 0.05, compared to lighter-colored dots when FDRs ≥ 0.05. Negative and positive associations are represented in blue and red, respectively. The effects size and FDRs referred to source analyses are available in the Supplementary Figures 3a and 3b.*

2) The association between annotations and deconvolution estimates are only scarcely validated in the current version. This is an important foundation of most of the results presented in the manuscript, and it needs to be assessed quantitatively and thoroughly.

Reply:
We thank the Reviewer for addressing this important question about the association between annotation and deconvolution. We have reported the results of morphological annotation and regression by sample in Supplementary Data 3 and 6. Additionally, we statistically assessed this association in the previous Extended Data Fig 2 (now Supplementary Fig. 2a) by computing the AUCs for each histomorphological category using the regressor.

Supplementary Figure 2a:



Supplementary Fig. 2a*: Areas Under the Curve (AUCs) for the prediction of each histomorphological category (binarized as present in a spot if proportion > 25%) by the regressor. Confidence intervals (CI) for the AUC of each histomorphological category are reported.*

3) Figure 3b presents results using xCell, which notably includes M1/M2 macrophages. The consensus among macrophages experts is now widely against this nomenclature. M1 and M2 are rather theoretical opposites of the activation spectrum of macrophages in vitro, and most tumor associated macrophages would exhibit both markers that used to be considered associated to M1 and M2. I would suggest the authors to refrain from referring to this nomenclature, or to perhaps use other deconvolution approaches that do not use them.

Reply:
We agree with Reviewer's suggestion and have now deleted M1 and M2 macrophages from the Xcell deconvolution in all figures and supplementary

figures. We have recalculated the false discovery rates (FDRs) and updated the figures accordingly. The revised figures are now available as: Figure 4c-stroma compartment-, Figure 5b, Figure 7d, Supplementary Figure 3b, Supplementary Figure 5a, Supplementary Figure 13, Supplementary Figure 20a, Supplementary Figure 24-26. The revised Supplementary Tables and Data are available as: Supplementary Table 5, Supplementary Table 6, Supplementary Table 9, Supplementary Table 10, Supplementary Table 13, Supplementary Table 14, Supplementary Data 25.

4) The authors show quite well the prognostic relevance of the megaclusters and ecotypes. How about their association with response to immunotherapy? The data is available, as they used it with regards to the TLS signature.

Reply:
We thank the reviewer for this valuable comment. The presence of megaclusters and ecotypes is specific to TNBC and not applicable to other types of cancer. Unfortunately, publicly available external datasets containing TNBC patients who have undergone immunotherapy are scarce. The only available dataset with TNBC patients treated with immunotherapy is I-SPY2, which consists of only 35 TNBC cases that received immunotherapy. However, dividing these cases into 9 ecotypes would result in groups too small to yield meaningful insights.

Nevertheless, as previously mentioned, we are currently discussing for access to data from randomized clinical trials investigating immunotherapy for early TNBC, and we anticipate that we will soon be able to validate the clinical value of the ET and TLS signatures in this context.

5) Evaluating the prognostic impact on a cohort that includes the discovery cohort (figure 5f) can be biased. This analysis should be carried in fully independent cohorts. The authors could consider validating results on an independent cohort such as TCGA?

Reply:
We agree with the reviewer's point. The majority of the samples come from METABRIC and Scan-B, two independent cohorts. We included our ST TNBC cohort in the pooled analysis since no clinical outcome information was utilized to derive the ecotypes. We chose not to incorporate TCGA data due to the limited number of TNBC cases (approximately 80) compared to METABRIC and Scan-B and the poor quality of their survival data.

6) The manuscript is at times very difficult to follow. This is due to several things:

a. There are far too many abbreviations in the text, and some paragraphs are basically unreadable unless the reader is intimately familiar with all of them.

Reply:
We apologize for the lack of clarity. We now addressed these concerns in the responses provided to Reviewer #4.

b. The annotations of megaclusters and ecotypes are all listed in the text, but

presenting a summary figure highlighting their main hallmarks would strongly help readers.

Reply:
The characteristics of the fourteen megaclusters and ecotypes are reported in Supplementary Tables 13 and 14. Figures 7d and 8e show a selection of the most relevant molecular features of the megaclusters and ecotypes. To improve clarity, following the Reviewer's suggestion, we have now added a summary in Figure 9, which recapitulates the main characteristics of the ecotypes and their therapeutic perspectives.

Figure 9

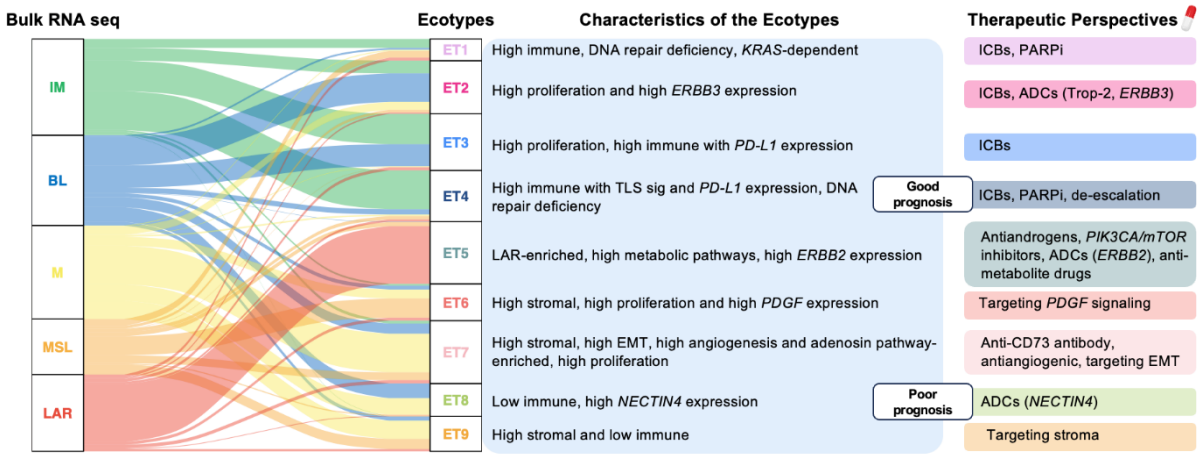


Fig. 9. *Evolution of the molecular subtypes in TNBC from bulk RNA-seq analysis to ST-derived ecotypes. The alluvial plot shows the distribution of the five pre-existing molecular subtypes into different ecotypes in the ST TNBC, METABRIC and SCAN-B cohorts. The molecular subtype was defined from the ST global pseudobulk and from METABRIC and SCAN-B bulk transcriptomes. The main characteristics of each ecotype are summarized, highlighting the potential for precision medicine in TNBC with specific therapeutic strategies for each ecotype. Source data are provided as a Source Data file.*

ADC: antibody-drug conjugate; BL: basal-like; EMT: epithelial-mesenchymal transition; ET: ecotype; ICB: immune checkpoint blockade; IM: immunomodulatory; LAR: luminal androgen receptor; M: mesenchymal; MSL: mesenchymal stem-like; PARPi: poly-ADP ribose polymerase inhibitor; TLS: tertiary lymphoid structures.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

I appreciate the authors' considerable effort in addressing the concerns raised during the previous round of review. Their integration of spatial transcriptomics with histomorphological data to redefine TNBC subtypes is an innovative approach, and the manuscript is now appropriately aligned with the scope of Nature Communications. The authors have clearly presented their methodology and the clinical implications of their findings, especially regarding immune-enriched subtypes and actionable targets, which enhance the overall value of the study.

However, I still hold reservations regarding the use of the term "ecotype" to describe these TNBC subtypes. While the authors acknowledge that their use of this term does not strictly adhere to its biological definition, I believe that introducing such terminology in tumor stratification might introduce unnecessary confusion. A more conventional term could enhance clarity without detracting from the novelty of the findings. Nonetheless, with this adjustment, the manuscript would be a strong contribution to the field.
We now changed the term "ecotype" to "spatial archetypes" (SA) as suggested by the reviewer in the manuscript. We modified the following figures and tables according to the new term: Figures 7a, 8a-h, 9, Supplementary Figures 19 to 33, Supplementary Tables 15 and 16, Supplementary Data 25 to 27.

Reviewer #2 (Remarks to the Author):

The manuscript describes a medium-resolution spatial transcriptomic analysis of a cohort of TNBC samples. This is a revision of a previous submission. The present analysis assesses how distinct biological structures within spatially heterogeneous samples contribute to bulk RNA signatures. This is useful information and advances our understanding of how to interpret bulk RNA sequences. The manuscript also usefully subdivides TNBCs into subgroups associated with clinical outcome and/or response to treatment. The associations are validated using previously published clincial cohorts. The manuscript also provides some suggestions for improved therapeutic attacks. The data in the manuscript are publically available and so will enable following secondary analyses by the the extended compoutational community. I recommend publication of this revised manuscript.
We thank the Reviewer #2 for the valuable comments.

Reviewer #3 (Remarks to the Author):

Overall, the reviewers have addressed my prior concerns. Upon reading the other reviewers responses, I felt that the responses to reviewer 1 would actually serve the paper & should be more directly included, rather than just as part of the dialogue with reviewer 1.

According to Reviewer #3's suggestion, we integrated the response to Reviewer #1 in the "Introduction" section 'row 88 to row 92') and in the "Discussion" section ('row 519 to 528', row '537 to 540', row '570 to 576').

There is of course considerable different types of validation that could be done, and these are highlighted by the other reviewers. Given the steps required, I am ok with the other reviewer responses. My comments were generally addressed earlier.

Reviewer #3 (Remarks on code availability):

The data is restricted pending publication, and thus it couldn't be fully reproduced. I have a concern that the data on zenodo is not in a primary format, noting I do see various Rdat files which are typically downstream.

On Zenodo, the raw UMI count matrices (output from the ST-pipeline) are available. These matrices provide the UMI count for each gene in every spot, which can be used for downstream analyses and to verify the reproducibility of our results. Along with the spot positions and images—also available—we believe these represent the key raw data most users will utilize. Cy3 images are available for researchers wishing to check spot positioning. Pixel-level slide annotations are provided both as images and as R objects. Additionally, raw RDS files for each sample, containing the image, spot positions, and UMI counts for each gene in each spot, are available for seamless use within R.

Reviewer #4 (Remarks to the Author):

The revised version of the manuscript is substantially improved, and the new manuscript reads more easily. Most of my comments have been adequately addressed.

My point 5 in the original review has however not been answered. Keeping the ST TNBC cohort in the analysis of now figure 7f presents poses a risk of bias. Even though no clinical information was used to establish MCs, this still cannot be considered to be an independent validation cohort. Using this cohort in both Fig 7e and 7f does not allow to see validation on a really independent cohort, and the authors should remove the ST TNBC bulk cohort from the analysis presented in Fig. 7f.

We follow the request of the Reviewer #3 and we removed ST cohort for the analysis. Without ST TNBC cohort, FDRs for MC 5 and 9 are not significant and we adapted the manuscript ('Row 398 to 403'): "Interestingly, the association between all MCs and clinical outcomes showed a consistent trend in a large cohort combining the METABRIC and SCAN-B datasets (Fig. 7f, Supplementary Fig. 18). Additionally, in this extensive dataset, survival analyses identified an additional MC, specifically MC 9, which showed a trend toward being associated with better outcome and is characterized by an organized immune response (Fig. 7f)."

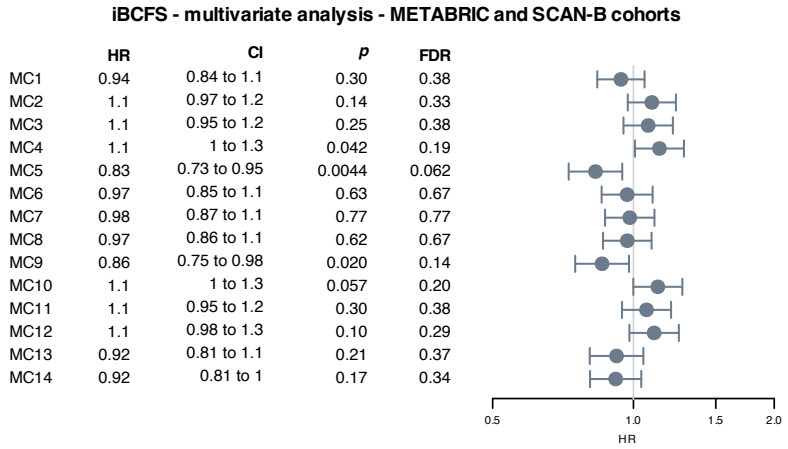
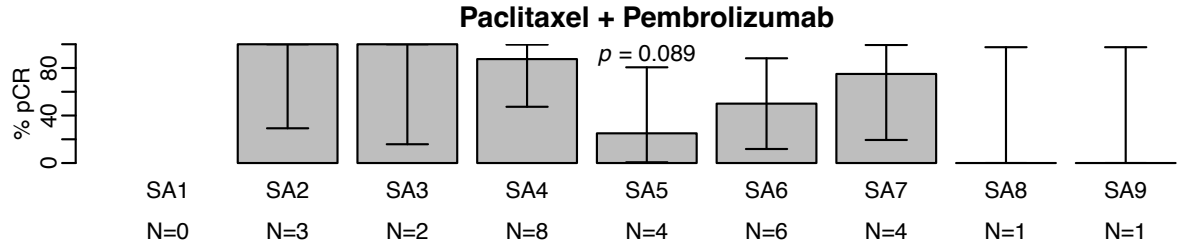


Fig. 7f Association of the 14 megaclusters with iBCFS in the combined METABRIC and SCAN-B cohorts (N = 1007), using deconvolution of ST spots and RNA bulk expression, respectively. Analyses were adjusted for age, tumor size, and nodal status. Two-sided P values were derived from likelihood ratio tests on nested models, with significant FDRs (< 0.05) shown in blue. Circles represent hazard ratios (HR), and error bars indicate the 95% confidence interval (CI).

Regarding the impact of ecotypes on response to immunotherapy, it would be very valuable if the data the authors are waiting on accessing (rebuttal to my point 4) could be incorporated into the final manuscript.

The boxplot below illustrates the percentage of pathological complete response in TNBC patients treated with immune checkpoint blockade (pembrolizumab) in the I-SPY2 trial. Due to the low number of patients per spatial archetype (SA) (previously named "ecotypes"), a robust interpretation of the predictive value of SA for immunotherapy response is not possible. We hope to gain access to data from larger randomized clinical trials investigating immunotherapy in early TNBC to validate our findings.



Finally, a minor comment on the new figure 1. This figure strongly helps to follow the story presented here and I really appreciate the effort the authors have done. However, the current layout (mostly because of the arrow) of the figure could give the impression that there is an evolution or development of tumors from LAR to M to MSL to BL to IM instead of a spectrum of presentations that do not evolve from one state to another. Also, the legend of that figure shows different colors for CAFs and all CAFs drawn on the figure are the same color, which is likely a simple omission.

We modified the Figure 1 as following:

[figure redacted]

We hope that these changes met the Reviewer #4's expectations.