

Molecular landscape: a new framework for personalized medicine in Breast Cancer

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Abstract

Estrogen receptor (ER) signaling is an important factor in breast carcinogenesis. It drives different subtypes in breast cancer and treatment decisions. In most of the cases, if a patient is diagnosed with breast cancer and has immunohistochemistry (IHC) percentage of 1% for estrogen receptor, they will receive standard endocrine therapy. We show here that estrogen signaling is a continuous status rather than dichotomous. To further elucidate the effects of estrogen signaling and other molecular pathways, we propose a new framework for personalized medicine. First, we introduce a novel embedding technique based on RNA-seq and microarray data that integrates distinct cohorts, such as TCGA, METABRIC and SCANB. We show that the embedding, also called molecular landscape, captures the true biology of breast cancer, dividing patients based on ER status and the different molecular subtypes (PAM50). We show that the embedding is not dependent of tumor purity, age and other clinical variables and other pathways, such as G2M Checkpoint, Epithelial to Mesenchymal Transition (EMT) and PI3K AKT MTOR signaling drives the embedding. The embedding works in a way that the algorithm is able to project new samples together within the three cohorts, allowing to compare them to what is publicly available and avoiding the need of specific reference sets. We demonstrate how to use the molecular landscape to ask specific questions about patients in terms of estrogen signaling in the context of the POETIC trial and what could be possible reasons that explain the response to treatment. Lastly, we analyse specific parts of the molecular landscape to look for potential subgroups and alternative therapies, more specifically, we show that PI3K AKT MTOR signaling might be a potential target pathway to be considered in future treatments among specific subgroups on the embedding.

Introduction

Breast cancer is the most commonly diagnose tumor disease in the world (1). It is also a very heterogeneous disease that can be subdivided in different subtypes. Patients that are diagnosed

with breast cancer are classified based on the expression of the estrogen, progesterone receptors and HER2 membrane protein. The estrogen receptor positive (ER+) breast cancer subtype is the most frequent found in clinical practice (2). Tumor cells of this subtype contain the estrogen receptor whose main mechanism is the target of selective estrogen receptor modulator (SERM), selective estrogen receptor degrader (SERD) and aromatase inhibitors (AI). The degree of the expression of the protein can range from 1% to 100% according to ASCO guidelines (3). It has been shown that the patients in the low spectrum of ER positivity, from 1% to 10%, do not benefit as much as patients with 10% to 100% of ER positive cells in the tumor (4).

Transcriptomics and genomics have revolutionized how cancer research is done. The new related tools opened the option to better understand the molecular underpinnings of cancer biology. In breast cancer, microarray technologies and RNA-sequencing have been used to develop gene signatures that are able to assign a risk score, usually for node-negative, post-menopausal and ER+ BC patients. The risk score is based on survival data found in the literature and the higher the risk score of a patient, the higher the benefit of additional chemotherapy besides the first-line therapy, such as aromatase inhibitors. Several of these signatures are already available to be used in hospitals (5–9). Moreover, some of the risk scores are already recommended in international guidelines (10). These signatures though do not provide a possible explanation on why a patient should receive additional therapy. These signatures are composed of several submodules, they are in most of the cases related to estrogen, proliferation, HER2 and invasion. In some of the cases the risk score is more strongly associated with the estrogen module and in other cases to the proliferation module (11). There is still a lack of understanding of the patient's tumor molecular biology. There are challenges to overcome in the pathway analysis for patients individually and how to compare patients molecularly when considering complete transcriptomics data. There is no tool that allows to integrate patients in a continuous way. Usually integration is a one step procedure and cannot be updated. The usual tools are (12–14) and they do not provide batch effect removal for new samples. The only way is to re-run the procedure together with the new sample. The problem of doing this is usually you don't have enough samples to estimate batch effects across groups and therefore correct it.

We propose here a framework for personalized medicine and the understanding of the signaling pathways in a patient level. By developing a normalization and projection technique, we show that it is possible to integrate publicly available molecular datasets, such as TCGA, SCAN-B, METABRIC and microarray data of some patients from the POETIC trial (15–18), and that it is possible to compare patients in a neighborhood of the projected data, providing a context to compare patients and better understand the biology of the tumor. Moreover, we propose to use the different gene sets from the hallmarks collection of the Molecular Signature Database (MSigDB) (19,20) to understand the biology of the tumors in patient and neighborhood levels.

Methods

Cohorts

The breast cancer cohorts TCGA, SCANB, METABRIC and POETIC (15–18) were used to calculate the PCA embeddings and scores. The samples are of primary breast cancer samples from different molecular subtypes and age distribution. TCGA was download from Firebrowse, SCANB data was downloaded from GEO (accession code GSE96058) using the package GEOquery (21) in R along the clinical data from the <https://oncogenomics.bmc.lu.se/MutationExplorer/> website. METABRIC was downloaded directly from cBioPortal. POETIC data was downloaded from GEO (accession code GSE105777) using the R package GEOquery. More details about the download and preprocessing steps are described in <https://chronchi.github.io/transcriptomics>.

Selection of highly variable genes

In order to perform the PCA, we sub selected 1000 common and highly variable genes in the TCGA and METABRIC cohorts. For each gene and in each cohort separately, the standard deviation of that gene was calculated. Then an average standard deviation was calculated using the formula below.

$$\text{average sd} = \sqrt{\frac{(sd_{TCGA}^2 + sd_{METABRIC}^2)}{k}},$$

where k corresponds to the number of cohorts, in this case $k = 2$.

qPCR-like normalization

Since samples are coming from different platforms, they need to be scaled in a way that they are comparable. For this we developed a new way to scale the data based on the ranking of the samples. Given a list of 44 stable genes across different cancers (22) and the 1000 genes selected previously, all genes were ranked from lowest to highest expression for each sample separately and the rankings were divided by the average ranking of the stable genes. Stable genes are considered the housekeeping genes.

PCA embedding

Using the normalized data, a total of 1000 random samples coming from TCGA and METABRIC were selected to perform the initial PCA. This is an unsupervised learning method, therefore there is no need to label the samples with respect to some category. The package PCATools (23) in R was used to perform the PCA and to obtain the loadings for downstream analysis. PCA was used with centering and scaling set to `FALSE`. The embedding for new individual samples is obtained by multiplying the loadings matrix with the normalized data for that sample. Since the normalization procedure is performed sample-wise, this step is independent of the number of samples. If there are missing genes in a sample, the normalization is performed and the missing genes are padded with 0.

Scoring strategies

For the 4 big cohorts, TCGA, SCANB, METABRIC and POETIC, GSVA (24) was applied along with the $SET_{ER/PR}$ signature (25) and the hallmark collection from the molecular signature database (19,26). Default parameters were used in the `gsva` function from the GSVA package.

Average neighborhood scores

In order to calculate the posterior distribution of the average scores in each neighborhood, a linear regression with only intercept was fit using `rstanarm` (27). The package `tidybayes` (28) was used to extract the draws and put them in a tidy format.

Survival analysis

Survival analysis was done using cox regression with the `survival` package from R. The variables used for adjustment were age, tumor stage and number of lymph nodes for TCGA and SCANB. For METABRIC the variables used were Age and the Nottingham Prognostic Index (NPI). Overall survival was performed for all three cohorts and recurrence free survival for METABRIC only. The causal model used here is that the confounders affect both the outcome and the pathway of interest. It could be that there are unmeasured confounders that are affecting the pathways and outcomes at the same time, leading to a biased result.

Code availability

The code used to generate all the analysis is available on https://github.com/chronchi/molecular_landscape. Descriptions for a docker image to reproduce the analysis are available on the github repository. An online version with a website containing all the analysis can be found on https://chronchi.github.io/molecular_landscape

Results

Estrogen receptor is a clinical continuous variable

Estrogen signaling is an indicative of how well a patient will respond to endocrine therapy. There are several ways to represent the estrogen signaling, one of them is to calculate scores derived from gene expression levels of bulk RNA-sequencing. We used three independent breast cancer molecular bulk RNA-seq datasets, TCGA, SCANB and METABRIC, (15–17) to calculate estrogen signaling scores. They are cohorts with over 1000 patients each that cover the whole molecular spectrum of primary breast cancer samples. The estrogen signatures HALLMARK_ESTROGEN_RESPONSE_EARLY and HALLMARK_ESTROGEN_RESPONSE_LATE were extracted from the molecular signature database (19) (MSigDB) and $SET_{ER/PR}$ from (25). The two signatures from MSigDB contain 200 genes and the later signature has 18 genes that are associated with estrogen and progesterone signaling. The individual scores for each patient sample are shown in Figure 1 (a) for each cohort stratified by estrogen receptor status. The scores capture the differences between estrogen receptor positive and negative breast cancer as expected. There is a wide range of values in the estrogen receptor positive (ER+) subgroup.

Cox regression was used to determine the hazard ratio of the estrogen signaling in overall survival (OS) for TCGA, SCANB and METABRIC and recurrence free survival (RFS) for METABRIC. Each survival analysis was done independently and adjusted for available clinical variables. Tumor size and number of lymph nodes were used for TCGA and SCANB cohorts. The Nottingham prognostic index (NPI) was used for METABRIC. Age was used in all cohorts as a clinical variable for adjustment. To better understand the effect of estrogen signaling in the clinics, only ER+ BC patients were used and when possible only those that received endocrine therapy. The selected sub population represents patients that could benefit from additional adjuvant therapies given unfavorable scores. Figure 1 (b) shows the forest plots for each cohort individually when calculating the hazard ratio for the $SET_{ER/PR}$ estrogen signaling signature. In all the three cases, the hazard ratio for $SET_{ER/PR}$ was below 1, with values ranging from 0.23 to 0.61. There is moderate variability for each hazard ratio. This shows the continuous aspect of estrogen receptor status. For both estrogen pathways of the hallmark collection of gene sets, hazard ratios were below 1 in all cases (Table S1), with a HR below 1 meaning that the higher the score the better the chances of survival for a patient.

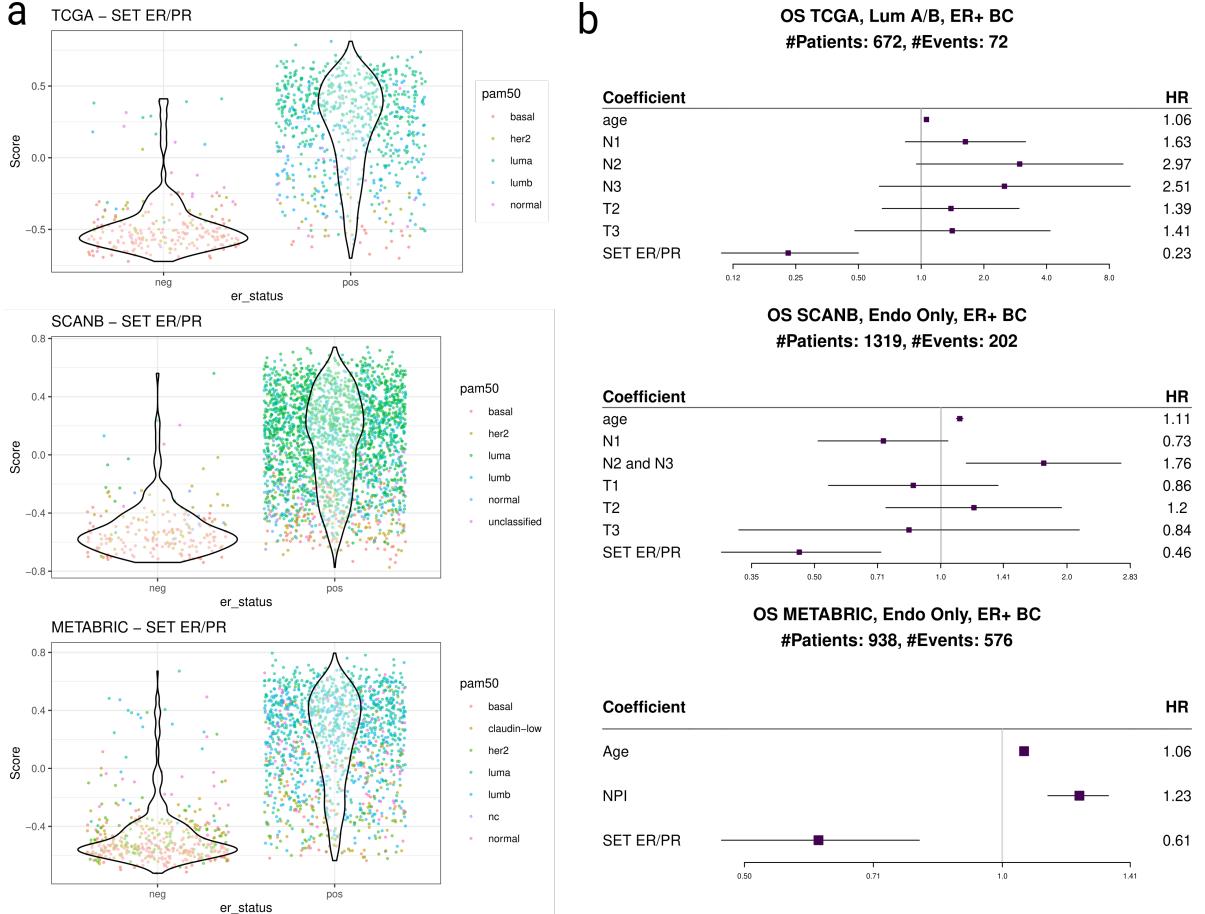


Figure 1: Scores and survival analysis results from TCGA, SCANB and METABRIC cohorts.

(a) GSVA scores for the SET ER/PR signature for each cohort. Each point corresponds to a patient sample and they are divided by estrogen receptor status. (b) Forest plot of the survival analysis for each cohort separately. NPI: Nottingham prognostic index. Ti: i-th stage of tumor. Ni: i lymph nodes with breast cancer cells.

Single sample integration preserves relevant breast cancer properties

Since each patient has a different ER signaling score, we assumed that patients should be treated individually, not just binned in two big subgroups as ER+ and ER-. Therefore, it is important to consider each patient individually. We developed a single sample batch effect removal method (see methods section) to integrate microarray and bulk RNA-seq and create a molecular landscape. The advantage of the method is that given a new sample, it can easily be integrated with all the other previous samples without any retraining.

The first step is to normalize the samples to the same scale. The average ranking of house-keeping genes distribution is similar among the two RNA-seq datasets, SCAN-B and TCGA and their mean values are higher than the mean of the distribution on METABRIC (Figure S1 (a)). The normalization preserves the distinctions of gene expression levels between ER+ and ER- breast cancer samples. Examples of genes where see such differences are TFF1 and ESR1 (Figure S1 (b)).

The biplot in Figure 2 (a) with the third and fourth components from TCGA and METABRIC samples shows that the samples are well integrated. All samples, including those used for training and validation, are plotted. The third components corresponds to the separation between ER+ and ER- BC patients in both cohorts (Figure 2 (b)). A combination of the third and fourth components shows a good distinction among the PAM50 molecular subtypes (Figure 2 (c)). The fourth component mostly divides the luminal A and luminal B subtypes, whereas the normal-like subtype is spread across the third and fourth component. This also highlights the fact that one cannot interpret the PCA locations globally, rather when comparing samples one should consider only its neighborhood.

As pointed out before, ER status should be considered continuous and not dichotomous, Figure 2 (d) shows a gradient of the ER signaling score $SET_{ER/PR}$. The higher values are on the far right of the third component, going to negative values as one goes from right to left, i.e., moving from a more ER+ status to the ER-.

In order to check the robustness of the procedure, we redid all the pipeline 10 times with 10 random sets of patient samples from TCGA and METABRIC, simulating a cross validation process. The PCA embedding is invariant to rotation, translation and reflection (Figure 2 (e)). Another problem inherent to publicly available datasets, is the fact that there are missing genes. We try to understand the effect of missing genes in the embedding based on their loading values. Ideally if a low amount of genes with high loadings are missing, this should not affect very much the embedding. On the other hand, the more genes missing with high loadings, the more it will impact the embedding. We removed 200 genes in total with a varying proportion of top loading genes (ranging from 0 to 100% in a 5% step). The number of top loading genes missing from the dataset is key for the embedding (Figure 2 (f)). The higher the proportion the less precise the embedding is. Other clinical factors, such as tumor stage, node stage, age, NPI and tumor purity show no influence in the embedding (Figure S2), besides the fact that ER- BC patients usually have higher stage in general.

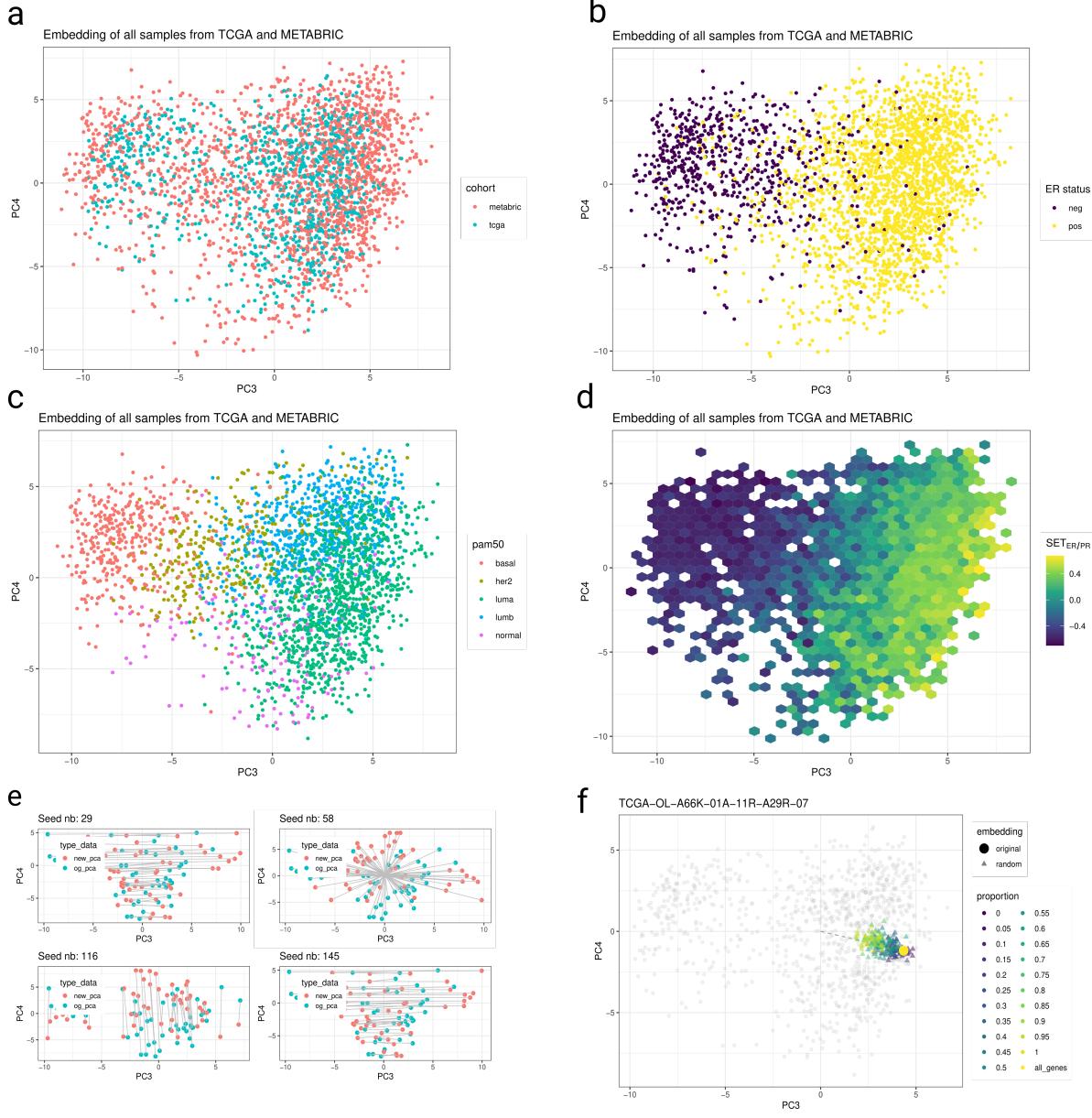


Figure 2: (a) Biplot using the third and fourth components on TCGA and METABRIC samples. Colored by cohort. (b) Same as a, colored by ER status. (c) Same as a, colored by PAM50 molecular subtype. (d) Hex grid calculated on the biplot of the fourth and third component. Each hex is colored based on its average value of the SET ER/PR signature. (e) Embedding of random samples given different training sets for PCA. Blue dots correspond to the original embedding of a sample and red dots correspond to the new embedding given the new training set. (f) Biplot of all possible embeddings of sample given a certain proportion of top loadings missing in the dataset.

Embedding generalizes to a validation cohort

So far only samples from TCGA and METABRIC were used in the training and projection. An external validation cohort is needed. SCANB was used as an external validation cohort. SCANB is well mixed with both METABRIC and TCGA samples (Figure 3 (a)). ER+ and ER- BC patients are well separated (Figure 3 (b)) and the procedure can also distinguish the molecular subtypes (Figure 3 (c)). As an RNA-seq cohort, it is expected that SCANB samples will be closer to TCGA than to METABRIC when removing batch effects, due to platform biases and initial scale of the genes. Biplot of PC1 and PC2 (Figure 3 (d)) shows that SCANB is closer to TCGA than to METABRIC. It is also in between the two cohorts. We also show that the embedding works for the SMC cohort (29) (supplementary figure 3; 3.14 from the book).

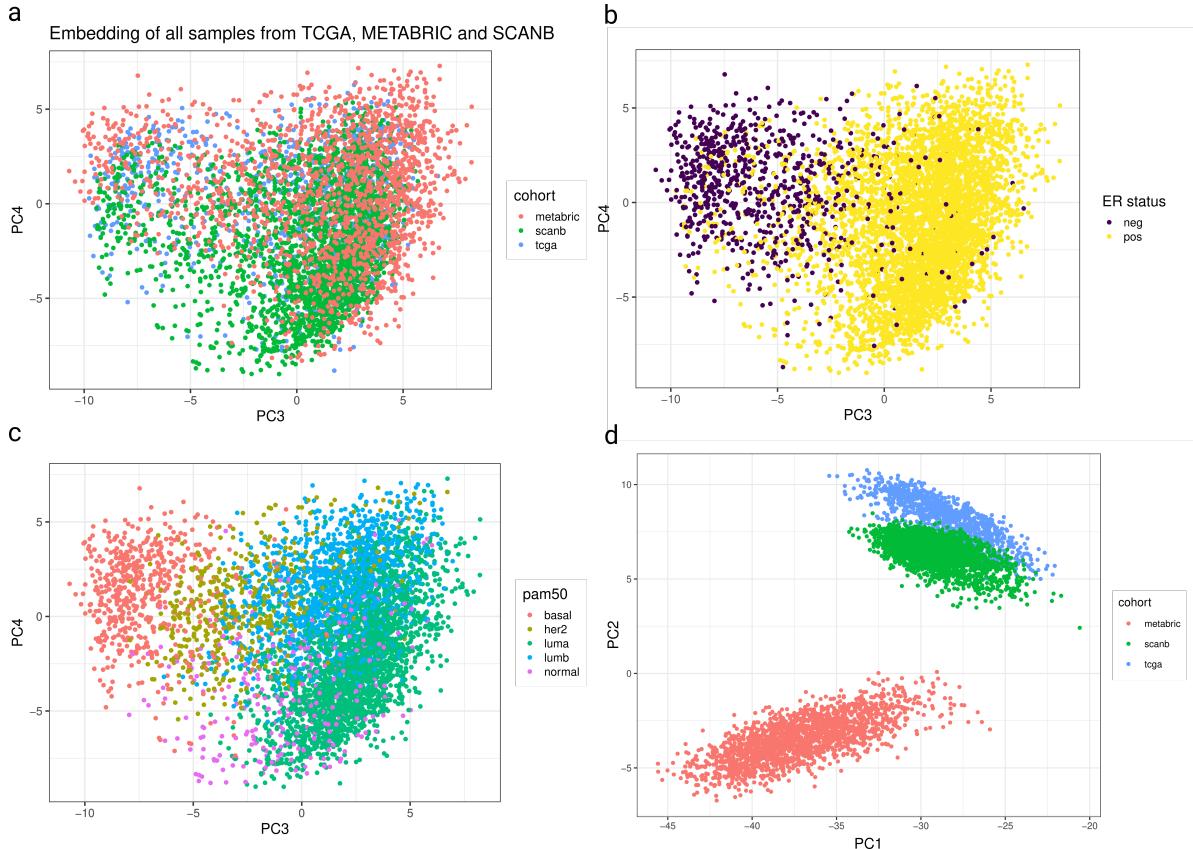


Figure 3: Validation of the molecular landscape with an external cohort (a) Biplot using the third and fourth components and now including all samples from the three cohorts: TCGA, METABRIC and SCANB. (b) Same as a, colored by ER status. (c) Same as a, colored by PAM50 molecular subtype. (d) Biplot using the first and second component of TCGA, METABRIC and SCANB.

Molecular landscape is a tool to understand and explore patient heterogeneity

Since the molecular landscape relies in a single sample to obtain the embedding, we can add samples from any cohort. The POETIC trial (30) was a trial that evaluated the use of perioperative aromatase inhibitors in ER+, postmenopausal BC patients. Its primary endpoint was time to recurrence. The POETIC trial team sent for microarray hybridization matched samples from baseline (before treatment) and at surgery (after an average 14 days of treatment) (18). There are also untreated patients, used to control for sample processing artefacts. The patients have matched Ki67 percentage levels, which can be considered an indication of how well a patient responded to the endocrine therapy. Patients with more than 5% of baseline Ki67 and a reduction of 60% upon endocrine therapy are considered responders, otherwise they are called non responders.

The molecular landscape can shed light on the differences between responders and non responders. We embedded the POETIC trial samples using the procedure (Figure 4 (a) left). The samples are spread across the whole molecular landscape, showing that patients indeed have different molecular biological properties. Furthermore, the POETIC samples are embedded closer to the METABRIC samples (supplementary figure 4; 4.3 from the book). Given the available information, the patients that are ER+ and in the left part of the landscape (ER+ patients), are all non responders (Figure 4 (a) right). This highlights the importance to look more carefully to ER+ patients. We selected two patients, a responder and non responder that are close in the embedding (Figure 4 (b)) to highlight their molecular differences and see what is their context. Figure 4 (c) shows the average posterior distribution of the neighborhood for the responder patient. The responder patient has a ER signaling score higher than the average. On the other hand, the non responder has a smaller ER signaling score than the average (Figure 4 (d)) and also a higher androgen signaling score (Androgen response). Other pathways and their average posterior distributions are shown for both patients.

Generating hypothesis: subgroups and alternative treatments

One key aspect of drug discovery is finding the right groups for targeting with the proper drug. With the molecular landscape we can try to identify different subgroups, based on molecular pathway scores. There are several driving the distinction between samples in the embedding, such as G2M checkpoint, epithelial to mesenchymal transition, DNA repair and PI3K AKT MTOR signaling (Figure 5 (a)). The scores go from one direction to other across the two different principal components, indicating that they complement each other when capturing information. Not only G2M checkpoint is differentiating luminal A and B patients, but also EMT.

Understanding a pathway and its clinical relevance is key for drug development and subtyping. The overall and recurrence free survival calculated from patients that have ER+ BC, received only endocrine therapy (METABRIC: OS/RFS and SCANB: OS) and have $PC3 > 0$ show that G2M checkpoint has a hazard ratio higher than 1 (Figure 5 (b)) and a tight confidence

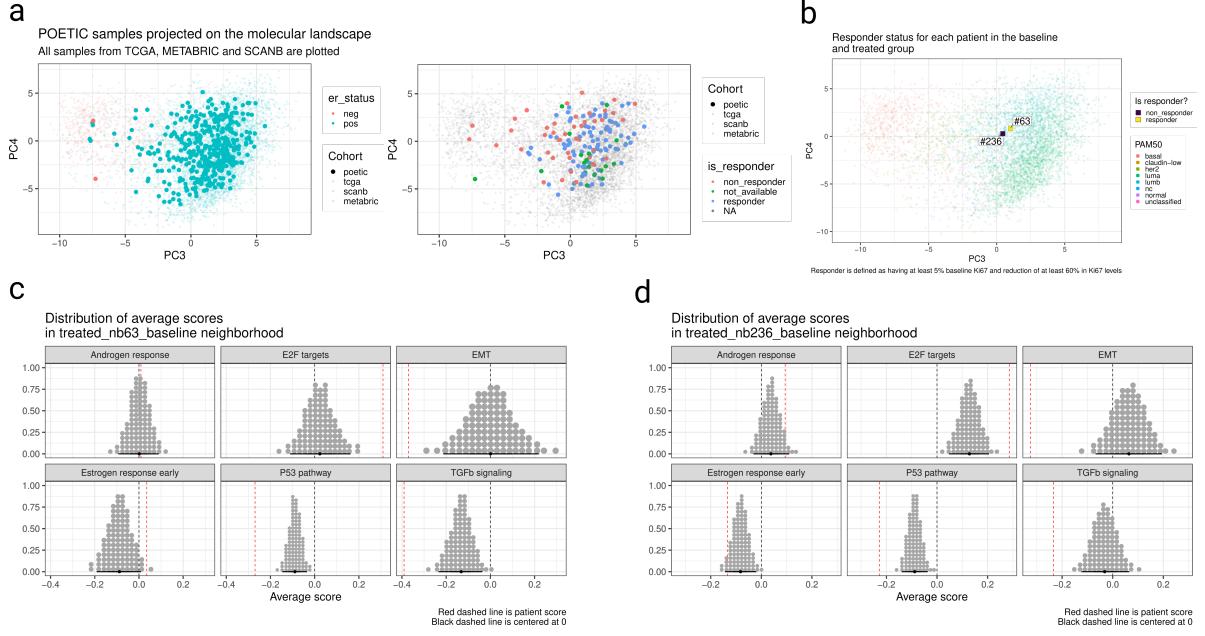


Figure 4: Embedding of the POETIC cohort into the molecular landscape and pathway analysis for patient samples. (a) Biplots of the POETIC samples (baseline and surgery) into the molecular landscape. Left plot is colored by ER status and right plot is colored by molecular subtype PAM50 when available. (b) Biplot highlighting two patients with similar embedding and different response status. (c) Posterior distributions of the average scores in the neighborhood of the responder patient. Red line corresponds to the patient score. (d) Posterior distributions of the average scores in the neighborhood of the **non**-responder patient. Red line corresponds to the patient score.

interval, which reflects the fact the higher the proliferation the worse the outcome, and reflects the subtyping differences between luminal A and B. The PI3K AKT MTOR signaling has a hazard ratio higher than 1 with a wider confidence interval, indicating a possible subgroup among all selected patients that could benefit from additional adjuvant therapy targeting this specific pathway.

Discussion

Personalized medicine is a key topic in medicine. The goal of better understanding the molecular underpinnings of the diseases leads to a better allocation of treatments and resources in the patient care. We have shown here a possible framework to deal with personalized medicine in breast cancer in general with a focus on ER+ BC patients.

In general, estrogen receptor status is considered either a dichotomous or trichotomous state (3). Breast cancers are classified either in ER+ or ER- based on their protein expression levels and IHC. For those tumors that are ER+, they can be subdivided into low ER positive ($1\% < \text{IHC}\% < 10\%$) and simply ER positive. Those that are into the low ER positive do not usually benefit as much on endocrine therapy. Here we show using cox regression and multiple big study cohorts (15–17) with both RNA-seq and microarray data, that ER status is more of a continuous rather than a categorical state. This can aid in treatment decision when evaluating the breast cancer. Several gene signatures already are being used, such as OncotypeDX, Mammaprint and Prosigna to assign chemotherapy for those patients with higher risk of recurrence (5–7). This score might be associated with the commercial signatures, as it has been shown that OncotypeDX's estrogen module is highly correlated with the signature itself (11).

Integrating molecular data stemming from different sources is a challenge. On one hand batch effect tools are usually able to remove the batch effects across the different sources of variability (12,13), on the other hand they are not single sample based, meaning each time a new sample comes the algorithm runs. It is also based on the fact one has enough data in the different datasets, otherwise it skews the possible integration towards one of the datasets. Here we show by using TCGA, METABRIC and SCANB that it is possible to integrate the samples from these cohorts in a single sample manner. The samples show good mixing when using test samples not seen during the training stage. The embeddings preserve key molecular features of breast cancer. PC3 is clearly driven by estrogen signaling where from right to left there is a gradient of ER positivity. On the other hand, PC4 is what makes a difference between the molecular subtypes luminal A and B, which in practice differ by proliferation status in terms of Ki67 levels (31).

Moreover, the embeddings in a validation set preserve the key features of breast cancer. Samples are projected by their different PAM50 molecular subtypes and there is a gradient of estrogen signaling pathway from ER+ BC towards ER- BC patients. The first two components are the batch effect removal components and SCANB is projected closer to TCGA, since

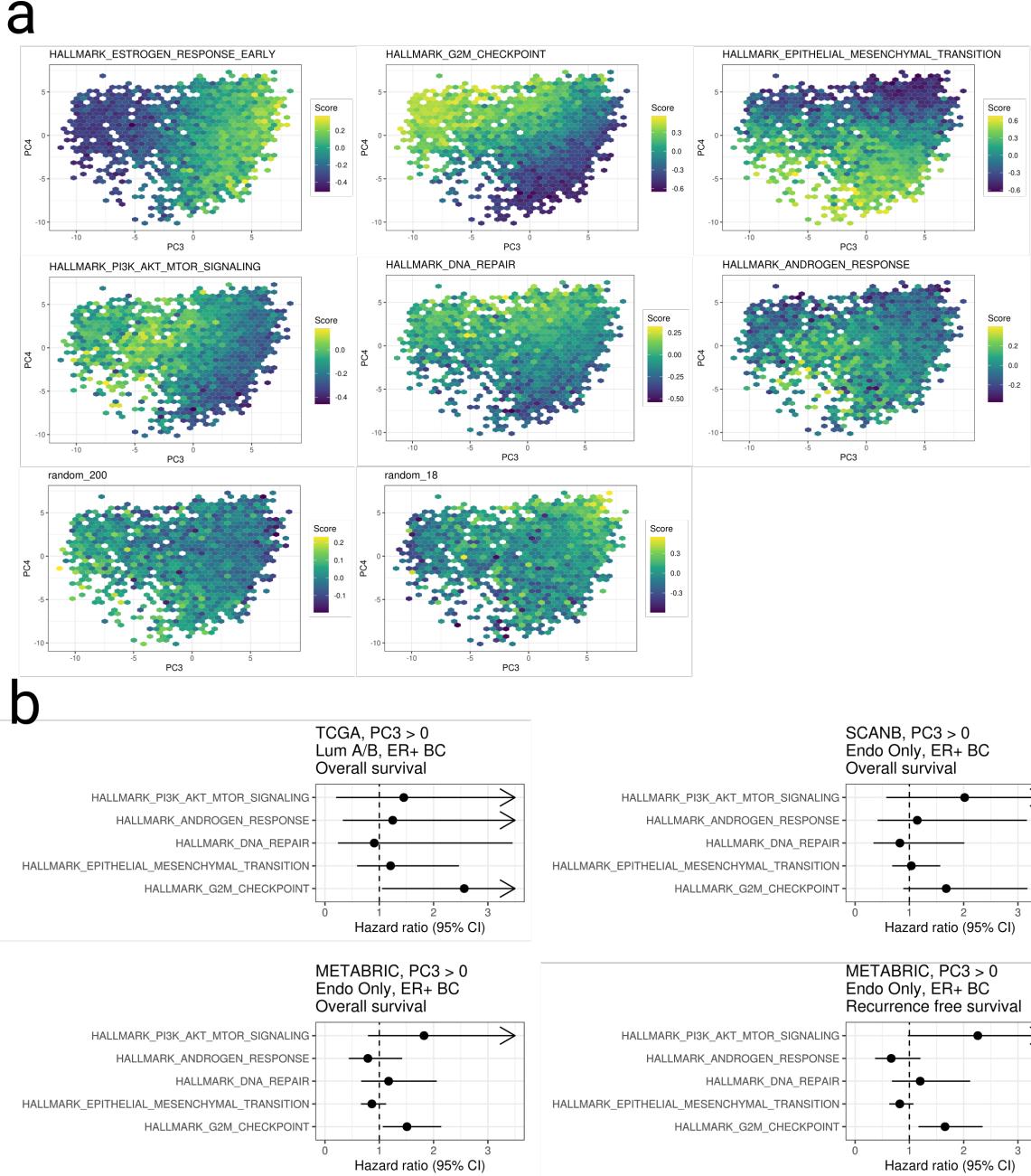


Figure 5: Average scores in hex regions using all three cohorts together and survival analysis in each cohort individually. (a) Biplots of all samples from TCGA, SCANB and METABRIC that were grouped in small hex regions. Colors are depicted as the average score value in each hex region. Selected pathways are shown. (b) Survival analysis results obtained for each pathway individually. The pathways were adjusted as described in the methods section. CI: confidence interval.

both datasets are RNA-seq. On the other hand, part of the POETIC trial was sequenced using microarray dataset (18,30), and we show that it is projected closer to METABRIC as expected according to the first two components. We also show that the embedding is independent of the training set and that when changing the training set the embedding is only rotated or translated, thus not affecting the global structure of the projection.

Sometimes when dealing with publicly available datasets, not all of the genes are available due to ethical (16), pre-processing or technological reasons. Therefore we showed how robust the projection is to missing genes with high loadings in the projection. If less than 20% of the genes we can recover almost surely the position of the embedding if all the genes are available. The more genes that are missing, the closer the projection will be to the origin, i.e., the (0,0) coordinate in the plane.

To show the clinical validity of the projection, we used a subset of patients from the POETIC trial with microarray and clinical information (18). The samples are projected as expected and surprisingly the samples that are considered to be non responders upon 2 weeks of aromatase inhibition are projected among the ER- BC patients. When looking further upon two different patients that have similar embedding but different response to endocrine therapy, we see that the responder has a higher value of estrogen signaling than the average. On the other hand, the non responder patient has a smaller estrogen signaling score than the average, which suggests a possible explanation for the difference in response. Moreover, estrogen and androgen receptor signaling have been show to be tightly linked (32) and these two patients have different androgen signaling scores. The non responder has a higher score than the average compared to the responder, whose score is just the same as the average.

Complementing the personalized approach, the molecular landscape can be used as a starting point to understand breast cancer molecular subgroups in a more intuitive way using pathways. Using survival analysis we show that some of the pathways that are important for the embedding can be used for subgrouping and hypothesis generation.

Some weaknesses of the proposed method is that we rely on GSVA scores, which can be used and compared across different cohorts since they have a representation of all molecular subtypes. A possible way to circumvent this problem is by using singscore or stingscore (22,33), a single sample pathway scorer. There is still a cost barrier for using RNA-seq dataset in the clinical setting, but efforts are being made across the industry to reduce the sequencing costs and make it more widespread.

In conclusion we provide some evidence that ER status should be considered a continuous marker rather than a categorical one. We also extend this notion to a framework for personalized medicine, where each patient is embedded in some context that can be used for the interpretation of its molecular underpinnings. In this paper we only discussed some pathways, but when analysing patient data, several pathways can be considered and should be taken into account when deciding tumor treatment.

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