# Computational prediction of TNBC tumor subtypes from an integrative single cell atlas elucidates immune evasion and immunotherapy resistance mechanisms

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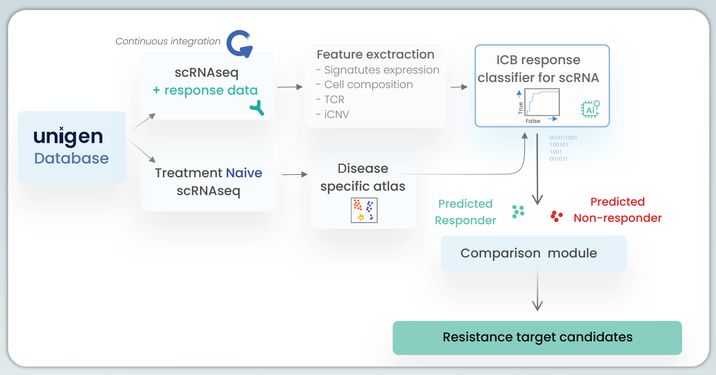
## Abstract

Though approved for neoadjuvant treatment of TNBC patients, anti-PD-1 therapy combined with chemotherapy often fails, indicating a need for improved treatments. In order to study resistance mechanisms, we developed an integrated TNBC single-cell atlas by combining 12 datasets, encompassing 250,000 cells from 72 individuals. Combining Leiden clustering with gene signatures and enrichment analysis, we identified diverse cell subtypes. Through principal component analysis (PCA) of tumor samples using 102 low-level cell subtype fractions, we identified four molecular phenotypes, identified for the first time in single cell TNBC data. These correspond to subtypes previously described in bulk-RNA-seq: 'Cold' (low immune infiltration), 'Dysfunctional' (stressed and naïve T-cells), 'Functional' (activated/exhausted T-cells), and 'LAR-like' (Luminal androgen receptor).

We utilized TCR expansion data (as response-proxy) and RECIST1.1 information from two datasets to explore the connection between anti-PD1 treatment responses and tumor phenotypes. We found that most TCR-expanded tumors belonged to the functional phenotype, whereas most non-expanded samples aligned with the cold and dysfunctional phenotypes. Consequently, we categorized treatment-naïve patients as either potential responders or non-responders to anti-PD-1 treatment based on their molecular phenotype. By examining both naïve and treated samples, we aimed to uncover mechanisms of resistance.

Our research identified specific subtypes of luminal cells, fibroblasts, and macrophages associated with immune exclusion and dysfunction resistance phenotypes. Through differential expression and receptor-ligand interaction analyses of these subtypes, we identified potential genes and pathways linked to immunotherapy resistance in TNBC. These insights may lead to the development of new drug targets or combination therapies for TNBC non-responders.

### Methodology




### The challenge:

There are many datasets of naïve patients but very few datasets with post PD1 treatment data, limiting the understanding of resistance and response mechanisms.

### Solution:

Stratify tumor samples with response data into responders / non-responders and project onto naïve samples to construct a cohort of predicted responders / non-responders.

## 1. TNBC single cell RNA-seq data integration

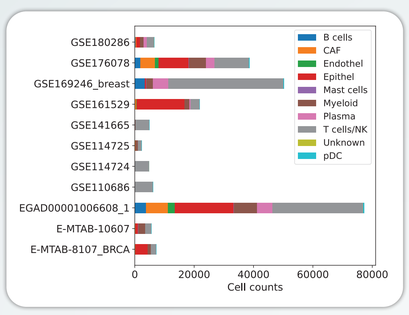
We started with 11 public TNBC single cell RNA-seq datasets, where EGAD00001006608 from Bassez et al. (2021) had TCR expansion data, a proxy for response, and 4 datasets had only immune cells.

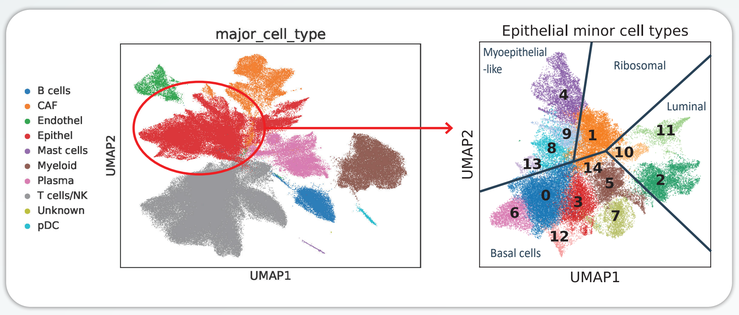
To limit heterogeneity, we excluded BRCA1-mutated and metastatic sites.

Using AI-driven label transfer, we later added a newly published dataset, GSE246613, with additional patients and clinical response data.

We proceeded with patients with complete TME composition of primary TNBC samples:

| | Total | TNBC atlas | GSE246613 |
|---|---|---|---|
| Responders | 21 | 4 | 17 |
| Non-responders | 25 | 7 | 18 |
| Naïve | 36 | 36 | |




**Main Tools:**

Unigen: in-house single cell data processing

Scanpy: single cell data processing and analysis

Harmony: dataset integration

CellTypist: major cell type annotation and label transfer

InferCNV: Labeling Malignant cells

### Cell type annotations

Within each major cell types the cells were manually annotated into mid and minor-level cell types based on Leiden clustering.

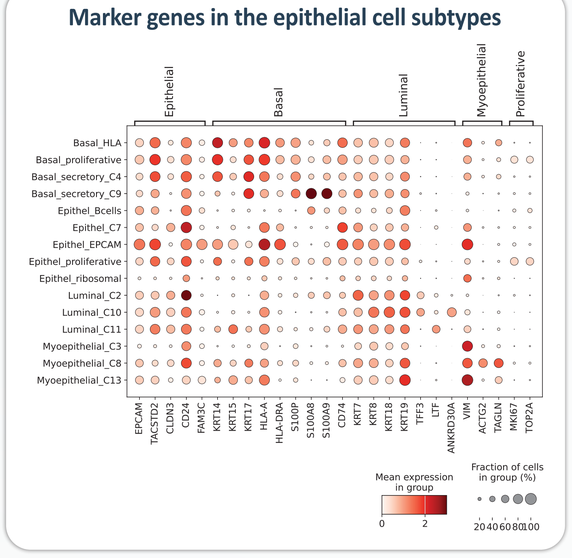
**A total of ~150K cells were initially annotated into:**

- 10 major cell types
- 42 mid cell types
- 106 minor cell types

**Tumor epithelial cells, for example, were classified into:**

- Mid: Basal, luminal, myoepithelial-like cells, …
- Minor: Basal secretory, Basal HLA, Luminal C2, Luminal C10, Luminal C11, …

### Marker genes in the epithelial cell subtypes

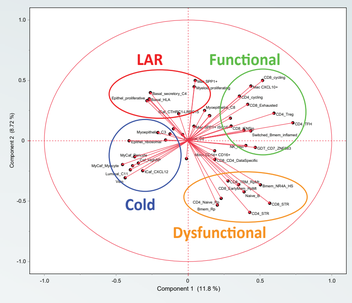


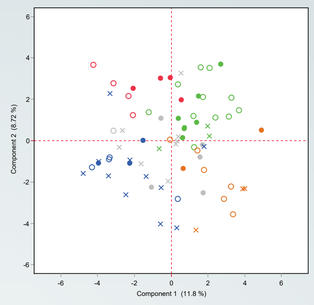

## 2. Stratification uncovers four distinct tumor phenotypes

The samples were projected onto a PCA space that is based on the fraction of minor cell types in each sample. This projection yielded a clear separation of expanded and non-expanded samples.
The samples were further divided into phenotypes based on the minor cell types that dominate the relevant eigen vectors:

- **Functional group (20):** enriched with activated and follicular T-cells and Inflammatory Macrophages
- **Dysfunctional group (11):** enriched with "naïve" and stressed T-cells and FOLR2+ MHC- macrophages
- **Cold group (18):** enriched with luminal cells, CAFs and no immune infiltrate
- **LAR-like group (8):** enriched with basal cells and SPP1+ Macrophages

The cold and dysfunctional subtypes seem to represent resistance mechanisms.



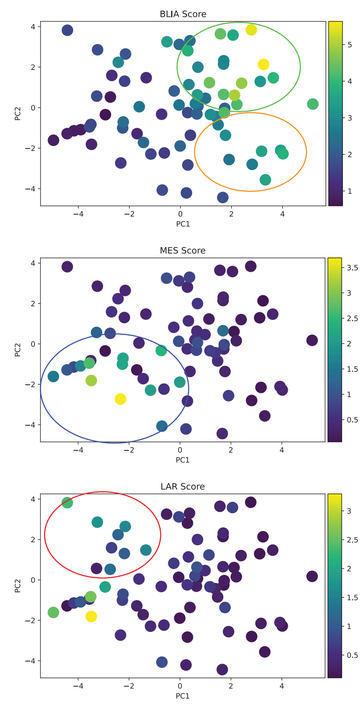



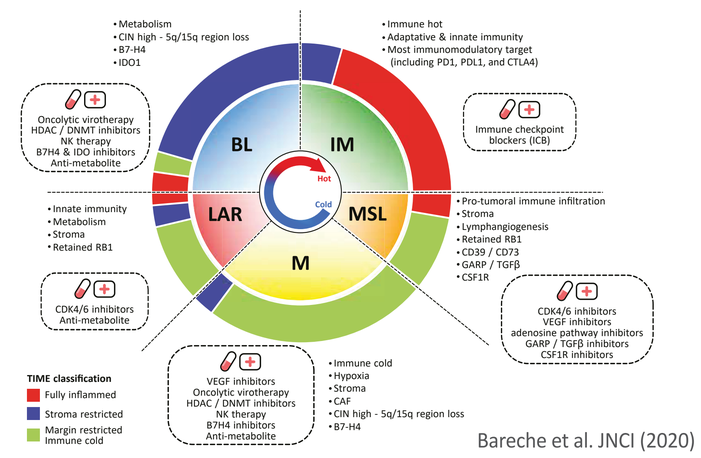

**Tumor phenotype:**
**Blue:** Cold
**Green:** Functional
**Orange:** Dysfunctional
**Red:** LAR
**Grey:** Other

**Expansion status:**
- Responders (expanded TCR)
- × Non-responders (no-expansion)
- ○ naïve (NA)

### Similar phenotypes were identified as TNBC subtypes observed in large bulk cohorts

Tumor samples in the same PCA space as a above colored by bulk signature scores as listed below



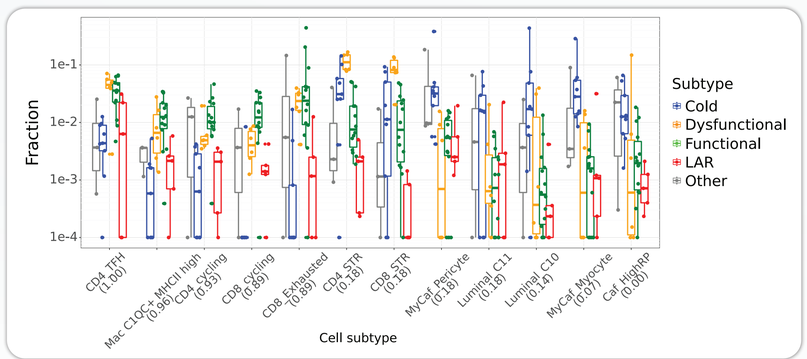




Bareche et al. JNCI (2020)

| SC phenotype | Bulk subtype | Signature |
|---|---|---|
| Functional immune | Immunomodulatory (IM) | Basal-like immune-activated (BLIA) |
| Dysfunctional immune | Basal-like (BL) | Basal-like immune-suppressed (BLIS) |
| Cold | Mesenchymal (M), Mes. stem-like (MSL) | MES - Mesenchymal |
| LAR | LAR | LAR |

## 3. Top minor cell types distinguishing expanded (E) from non-expanded (NE) samples

The fraction of different cell types can be used as a classifier score for distinguishing responders (expanded TCR - E) from non-responders (no-expansion - NE). The area under the ROC curve (AUC) quantifies how each minor cell type is more (less) prevalent in E (NE) samples.

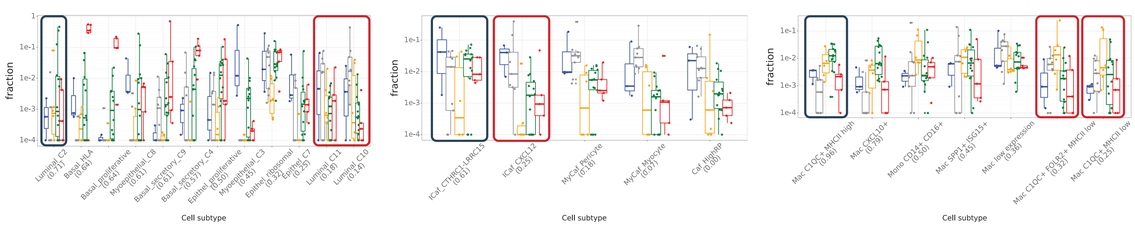



### Diverse cell types highlight multiple evading mechanisms

**Tumor (Epithelial) cells:**
Luminal C2 vs. C10 and C11

**Stromal cells (CAFs):**
iCAFs_CXCL12 vs. iCAFs_CTHRC1_LRRC15

**Macrophage (TAM) Comparisons:**
Macs_C1Q_MHChigh vs. C1Q_MHClow
Macs_C1Q_MHChigh vs. C1Q_FOLR2_MHClow



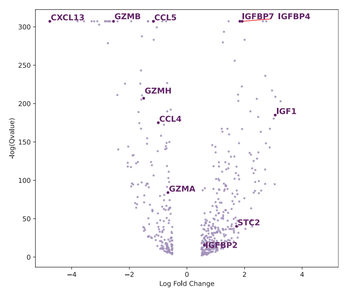

## 4. Differential expression analyses of cell subtypes highlight response and resistance mechanisms

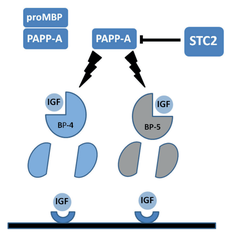
We performed diverse differential expression analyses to highlight genes that are overexpressed in non-responding tumors:

- Mid-cell types in different response-associated phenotypes, e.g., all luminal cells in cold vs. the rest of the samples
- Related minor cell types associated with different phenotypes, e.g., luminal C10 vs. C2
- Pseudo-bulk at the sample level and mid cell type level in different response-associated phenotypes

While the over-expressed genes in the Functional phenotype (left hand side) represent immune activation mechanisms such as chemokine and granzymes, in the Cold phenotype (right hand side) we observed several over-expressed pathways such IGF/IGFBPs.

### Example: Volcano plot of secreted proteins in the Cold vs. functional phenotypes



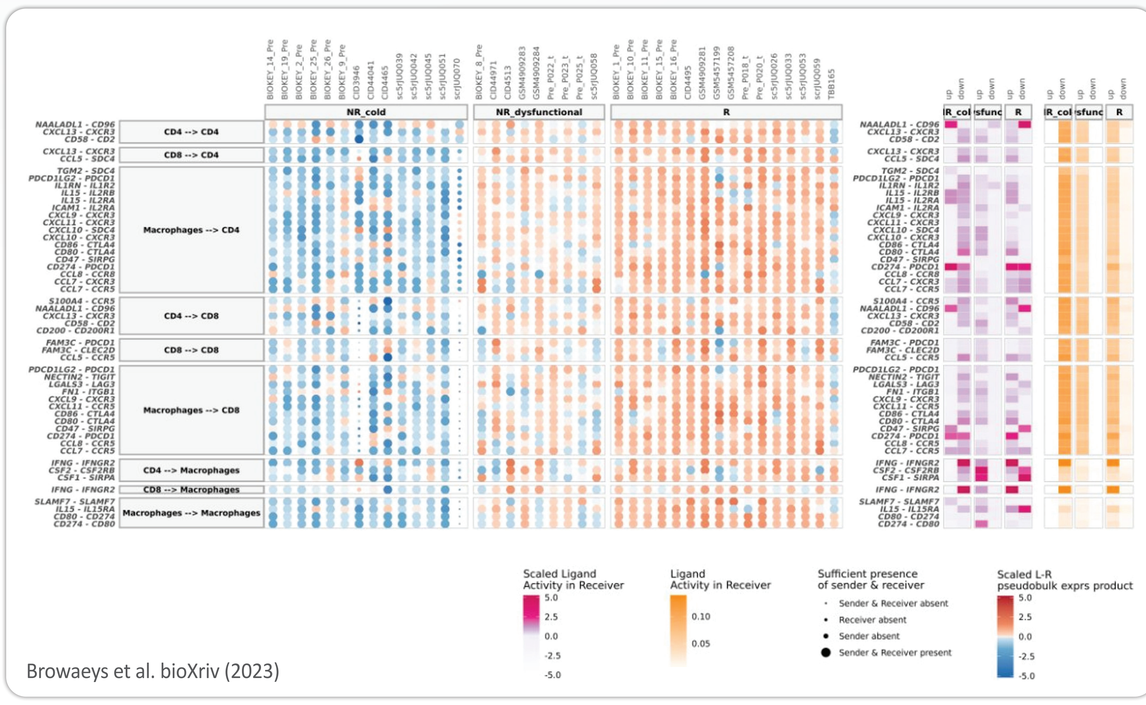




Beattie et al. F. Endocrinol. (2018)

## 5. Differential cell-cell communications between the phenotypes

MultiNicheNet was implemented to highlightligand-receptor interactions that are modified in the various tumor phenotypes

For example: PDL1 (CD274) / PD1 in functional immune (R) in Macrophage – CD8 and Macrophages – CD4 T-cells




Browaeys et al. bioXriv (2023)

## Summary and conclusions

We developed a comprehensive TNBC single-cell atlas by integrating machine learning and AI methodologies to support and refine analytical elements. Through principal component analysis of tumor samples using low-level cell subtype fractions, we identified four molecular phenotypes, identified for the first time in single cell TNBC data, which stratified patients into potential responders or resistant to anti-PD1. Furthermore, our analysis identified two distinct types of resistant tumors distinguished by the composition of specific cells subtypes, suggesting different approaches for combination therapy. Through differential expression and receptor-ligand interaction analyses of these subtypes, we identified potential genes and pathways linked to immunotherapy response and resistance in TNBC.