Unraveling triple-negative breast cancer tumor microenvironment heterogeneity: towards an optimized treatment approach

Yacine Bareche1* MSc, Laurence Buisseret1* MD PhD, Tina Gruosso2 PhD, Edwina Girard1 MD, David Venet1 PhD, Floriane Dupont1 MSc, Christine Desmedt1 PhD, Denis Larmsimont3 MD PhD, Morag Park2 PhD, Françoise Rothé1 PhD, John Stagg4 PhD & Christos Sotiriou1 MD PhD

1Breast Cancer Translational Research Laboratory J.-C. Heuson, Institut Jules Bordet, Université Libre de Bruxelles, Brussels, Belgium; 2Department of Oncology, McGill University, Montreal, Canada; 3Pathology Department, Institut Jules Bordet, Université Libre de Bruxelles, Brussels, Belgium; 4Centre de Recherche du Centre Hospitalier de l’Université de Montréal, Québec, Canada; *Equal contribution.

Corresponding author:

Prof. Christos Sotiriou
Breast Cancer Translational Research Laboratory, J.-C. Heuson
Department of Medical Oncology,
Institut Jules Bordet, Université Libre de Bruxelles,

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Rue Héger Bordet 1, 1000 Brussels, Belgium

Telephone: +32 2 541 34 28

Email: christos.sotiriou@bordet.be

Keywords: breast cancer, TNBC, tumor microenvironment, immune response, gene expression profiling
Abstract:

**Background:** Recent efforts of gene expression profiling analyses recognized at least 4 different triple-negative breast cancer (TNBC) molecular subtypes. However, little is known regarding their tumor microenvironment (TME) heterogeneity.

**Methods:** Here, we investigated TME heterogeneity within each TNBC molecular subtype including immune infiltrate localization and composition together with expression of targetable immune pathways using publicly available transcriptomic and genomic datasets from a large TNBC series totaling 1512 samples. Associations between molecular subtypes and specific features was assessed using logistic regression models. All statistical tests were two-sided.

**Results:** We demonstrated that each TNBC molecular subtype exhibits distinct TME profiles associated with specific immune, vascularization, stroma and metabolism biological processes together with specific immune composition and localization. The immunomodulatory subtype was associated with the highest expression of adaptive immune-related gene signatures and a fully inflamed spatial pattern appearing to be the optimal candidate for immune check-point inhibitors. In contrast, most mesenchymal stem-like and luminal androgen receptor (LAR) tumors showed an immunosuppressive phenotype as witnessed by high expression levels of stromal signatures. Basal-like, LAR and Mesenchymal subtypes exhibited an immune cold phenotype associated with stromal and metabolism TME signatures and enriched in margin restricted spatial pattern. Tumors with high chromosomal instability and copy number loss in
chromosome 5q and 15q regions including genomic loss of MHC-related genes showed reduced cytotoxic activity, as a plausible immune escape mechanism.

**Conclusion:** Our results demonstrate that each TNBC subtype is associated with specific TME profiles, setting the ground for a rationale tailoring of immunotherapy in TNBC patients.
TNBC, representing 15% to 20% of all breast cancers (BCs), has the worst outcome among BC subtypes and is known to be a heterogeneous disease, at the clinical, biological and genomic levels. Gene expression analyses have led to the identification of several molecular subtypes with distinct mutational profiles, genomic alterations and biological processes including basal-like (BL), immunomodulatory (IM), luminal-androgen receptor (LAR), mesenchymal (M) and mesenchymal stem-like (MSL)(1)–(4). TNBC treatment remains challenging since therapeutic options are essentially limited to chemotherapy (5).

Cancer development, progression and treatment resistance are known to be influenced by genetic and epigenetic alterations, as well as crosstalk between tumor cells and their microenvironment. The tumor microenvironment (TME) is composed of multiple cell types including fibroblasts, adipose and immune-inflammatory cells, as well as blood and lymphatic vascular networks(6). TNBC subtype is associated with the highest tumor-infiltrating lymphocytes (TILs) levels. High TILs were associated with better clinical outcome and response to neoadjuvant chemotherapy(7)–(9).

Several agents targeting TME immune components by inducing or enhancing anti-tumor immunity are under development(10). Cancer immunotherapies, in particular, immune checkpoint blockers (ICB), have changed treatment paradigm in a variety of neoplastic diseases(11). In BC, immunotherapy with ICB has demonstrated clinical activity and survival benefit, mostly in advanced TNBC and HER2+ subtype(12)–(14). However, benefit to ICB was only observed in a minority of BC patients highlighting
the need to better elucidate the mechanisms of treatment resistance allowing to identify patients who will benefit the most from immunotherapy.

**Methods**

Additional information on samples and methods used in this study is provided in Supplementary Methods.

**Datasets**

Bioinformatic analyses were performed on four publicly available datasets assembled as cohorts A, B and C (Supplementary Figure 1 and Supplementary Table 1-2). Cohort A was composed of 555 TNBC samples from the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) (15) and The Cancer Genome Atlas Consortium (TCGA) (16) with available gene expression, somatic mutation profiles, segmented copy-number and overall survival (OS) data. Cohort B was composed of 360 TNBC samples from patients with Asian ancestry, retrieved from Jiang and colleagues (3), including gene expression, somatic mutation and whole-genome copy number data. The validation cohort C was composed of 597 TNBC samples with gene expression data retrieved from the Gene Expression Omnibus platform (GSE31519) together with relapse-free survival data (17).

**TNBC molecular subtyping**

Three different classifications were used to define TNBC molecular subtypes according to the recent publications by Bareche et al. (2), Jiang et al. (3) and Burstein et
**Tumor Micro-Environment gene signature analysis**

Ten gene signatures related to the TME were used for this analysis (Supplementary Table 3): two immune-related (reflecting “Cytolytic activity”\(^{(18)}\) and “Lymphocytes”\(^{(19)}\)), three vascularization-related (reflecting “Angiogenesis”\(^{(2)}\), “Hypoxia”\(^{(20)}\) and “Lymphangiogenesis”\(^{(21)}\)), three stroma-related (reflecting “Cancer Associated Fibroblast (CAFs)”\(^{(22)}\) and the “Stroma”\(^{(23)}\)) and three metabolism-related (reflecting “Glycolysis”\(^{(24)}\), “Lipid Metabolism”\(^{(25)}\) and a “Pentose Phosphate Pathway”\(^{(26)}\)). The “Cytolytic activity”, “Lymphocyte” and “Stroma” signature scores were calculated as a weighted sum of genes normalized expression, with gene-specific weights equal to +1 or -1 depending on the association’s direction with the gene expression phenotype. The other signatures scores were calculated as an unweighted mean of the genes normalized expression. Gene signature scores were then scaled so that the 2.5% and 97.5% quantiles equaled -1 and +1\(^{(27)}\).

**Tumor Immune Micro-Environment molecular subtype (TIME)**

The Tumor Immune Micro-Environment (TIME) molecular subtypes were assigned to each tumor sample, as described by Gruosso et al. \(^{(28)}\).

**Computational immune composition quantification**

Gene signatures allowing to identify 16 distinct immune cell types were obtained...
from Tamborero et al. (2018) (29). Gene Set Variation Analysis (GSVA) was performed to compute the 16 immune cell types scores, using the gsva (30) R package with default parameters. The 16 Gene Set Variation Analysis (GSVA) signature scores were then scaled so that the 2.5% and 97.5% quantiles equaled −1 and +1, respectively.

**Statistical Analysis**

Associations between TME gene signatures, GSVA immune cell types and immune gene targets, used as continuous variables, together with TNBC molecular and TIME subtypes, used as categorical variables, were evaluated using logistic regression model with P-values computed from parametric Mann Whitney U tests.

Spearman correlation analysis was performed between CIN scores and genes expression. Genes with spearman coefficient rho > 0.30 or < -0.30 and p-values corrected for multiple testing (FDR) < 0.05 were considered statistically significant for the gene ontology (GO) analysis. GO enrichment was performed using GO.db (version 3.5.0) and limma (version 3.34.9) R packages.

Associations between TME gene signatures and GSVA immune cell types with OS were evaluated using Cox proportional hazards regression univariable and multivariable models (coxph function, R package “genefu” (27), version 2.11.2) in cohort A and with relapse-free survival using Cox proportional hazards regression model in cohort C. Multivariable analyses were performed adjusting for the dataset (TCGA vs. METABRIC), nodal status (0 vs. 1), age (≤40 years vs. > 40 years), tumor size (≤2 cm vs. > 2 cm) and histological grade (I/II vs. III). Survival data from cohort B were not used, because of a
relatively low relapse rate (only 49/360 TNBC patients relapsed with a median follow-up of 3.8y). Cox proportional hazards assumptions of proportionality were checked and verified using the Schoenfeld residual test in cohorts A and B.

All statistical analyses were performed using R (version 3.4.4). P-values were corrected for multi-testing using the Benjamini–Hochberg false discovery rate (FDR). Statistical tests were considered statistically significant if FDR <0.05. All tests were two-sided and P values <0.05 were considered statistically significant.

Results

TNBC molecular subtypes association with distinct TME and clinical outcome

To evaluate whether TNBC molecular subtypes as defined by Bareche et al. (2) exhibit distinct TME patterns, we interrogated several specific gene expression signatures capturing different biological features or cellular components including immune response (18,19), vascularization (2,20,21), stroma compartment (22),(23) and metabolic processes (24)–(26) using cohorts A and B (Figure 1A; Supplementary Tables 3,4).

As illustrated in Figure 1A, BL tumors were enriched with metabolism processes together with low levels of stroma and lymphangiogenesis signatures whereas IM subtype was predominantly associated with tumor immune response signatures. LAR and M subtypes showed similar TME patterns with high stroma and metabolism expression levels together with low immune signatures expression levels. Finally, MSL
was mainly associated with high levels of lymphangiogenesis and low levels of metabolism processes.

We then investigated whether different TME patterns were associated with distinct clinical outcome. As shown in Figure 1B, the two immune-related signatures were associated with a better OS whereas the CD10+/GPR77+ CAF signature showed an association with poor outcome. These associations being statistically significant both in the univariable and multivariable analyses (Figure 1B, Supplementary Table 5). Altogether, our results highlight that each TNBC molecular subtype presents specific TME patterns associated with distinct clinical outcome.

Spatial immune organization within TNBC molecular subtypes

Immune-related gene signatures were derived from bulk tumor gene expression, ignoring immune cells spatial distribution. To investigate the spatial heterogeneity of tumor immune infiltration within each of the TNBC molecular subtype, we first applied the recently reported Tumor Immune Micro-Environment (TIME) classification grouping tumors into 3 patterns according to CD8+ TIL spatial distribution, namely the Fully Inflamed (FI), Stroma Restricted (SR) and Margin Restricted (MR) subtypes to cohorts A and B (28). We then examined whether TNBC molecular subtypes present specific TIL localization according to TIME classification. As illustrated in Figure 2A-B, IM tumors showed the highest prevalence of FI patterns, which were absent in the M subtype. M, MSL and LAR molecular subtypes presented the highest levels of MR pattern whereas BL subtype was predominantly associated with the SR pattern (28).
To investigate whether this classification reliably reflects TIL localization as assessed by a pathologist, we reviewed 161 H&E slides available from the TCGA series and compared TIL localization with TIME molecular classification. Interestingly, we observed a statistically significant concordance of 67% with a Cohen’s κ coefficient value of 0.48 (P≤0.001) (Supplementary Figure 2A). Furthermore, the pathologist classification showed similar results as found using the TIME molecular classification (Supplementary Figure 2B-D), thus demonstrating that the TIME classification faithfully captures TIL spatial localization.

TIME classification was also associated with distinct expression of TME signatures (Figure 2C; Supplementary Table 6). FI tumors were positively and negatively associated with immune and stroma/metabolism-related signatures respectively, in contrast to MR tumors showing opposite TME features. Finally, SR tumors were mainly associated with metabolism-related signatures.

We next investigated whether TIME classification was associated with distinct clinic-pathological features and clinical outcome. FI tumors were statistically significantly enriched for medullary carcinoma and smaller tumors, SR tumors were associated with younger patients while MR tumors were enriched for low grade tumors (Supplementary Table 2). Furthermore, in line with Gruosso et al., TIME patterns were statistically significantly associated with 10-year OS (Log-rank test, p=0.048), patients with FI tumors exhibiting the best prognosis (Supplementary Figure 2E). These results highlight that each TNBC molecular subtype is associated with distinct immune
localization together with specific TME processes and clinico-pathological characteristics.

**Immune infiltrate composition of TNBC molecular subtypes**

To gain more insight into the relative composition of the immune infiltrate between the different TNBC molecular subtypes, we used a compendium of mRNA gene signatures capturing 16 immune cell types depicting innate and adaptive immune responses (29). IM subtype was mainly enriched with adaptive immune cells as compared to MSL mainly composed of innate immune cells (**Figure 2D; Supplementary Table 7**). LAR subtype was enriched with innate immune cells although to a lesser extent as compared to MSL. Of note, BL and M subtypes were characterized by poor adaptive and innate immune responses. Overall, our results show a statistically significant heterogeneity in the immune cell composition characterizing each TNBC molecular subtype.

**Association of tumor genomic and TME features with cytotoxic immune response**

We next aimed to determine the genomic and TME features associated with local cytotoxic immune activity (CYT), evaluated through the geometric mean expression of **GZMA** & **PFR1** genes (18), including chromosomal instability (CIN), intra-tumor heterogeneity (ITH), homologous recombination deficiency (HRD) scores, tumor mutational burden (TMB) and several TME signatures as previously described. High CYT was statistically significantly associated with high expression levels of
lymphangiogenesis signature whereas reduced CYT was correlated with high levels of CD10+/GPR77 CAF and high CIN as well as to a lesser extent with hypoxia, glycolysis, lipid metabolism as well as HRD and TMB (Figure 3A).

Of note, higher CIN scores were observed in BL and M subtypes as well as in the SR tumors (Figure 3B). Gene ontology analysis using Spearman correlation test (|Spearman ρ|≥0.30) showed that CIN scores were positively correlated with genes involved in cell cycle processes and inversely correlated with genes involved in immune response. 73.2% (361/493) of these inversely correlated genes also showed higher copy number losses in tumors with high CIN scores (Figure 3C; Supplementary Table 8). Of interest, 33 of these genes were associated with reduced CYT levels including B2M and TMEM173 (STING) genes, both involved in MHC class I and II complex molecules, required for tumor antigen presentation, nearly all of them being located in chromosomes 5q and 15q (Figure 4A). We further showed that among the 23000 genes with copy number alterations, 84.1% of the genes associated with reduced CYT were located in chromosome 5q and 15q regions, independently from CIN status (Supplementary Table 9). Of note, CIN and chromosome 5q region remained statistically significantly associated with reduced CYT in a multivariable model suggesting that both CIN and 5q regions are independently associated with CYT (Supplementary Figure 3). Finally, we identified two regions on chromosome 5q (5q11.2-5q21.2 and 5q31.1-5q31.3) and two regions on chromosome 15q (15q14-15q21.1 and 15q22.31) statistically significantly associated with decreased CYT levels (Figure 5). Of great interest, similar findings were observed in cohort B demonstrating the reproducibility of
our results (Figure 3-5; Supplementary Figure 3 and Supplementary Tables 9,10).

Altogether, these data highlight the importance of CIN as well as chromosome 5q and 15q regions loss as potential immune escape mechanisms in TNBC tumors.

**Association of targetable immune markers expression with TNBC molecular subtypes**

We next evaluated the expression of key immune targets including co-inhibitory as well as co-stimulatory ligands and receptors, chemokines and enzymes in order to guide immune therapeutic strategies according to each TNBC and TIME subtype (Supplementary Table 11).

Our analyses showed that most of the evaluated immune targets were highly expressed in the IM and to a lesser extent to MSL subtype (Figure 6A; Supplementary Table 12) in contrast to the M, LAR and BL subtypes which showed low immune target expression. Notably, BL tumors were characterized by an immunosuppressed TME, including association with immunomodulatory proteins involved in negative immune regulation of T cells (B7-H4) and myeloid cells (CD47). GARP was the only immune target observed in the LAR subtype and was strongly enriched in MSL tumors.

Other molecules from immune negative regulation pathways were associated with MSL tumors, including CD39 and CD73 ectoenzymes, responsible for the generation of immunosuppressive adenosine in the TME (31). In contrast to MSL tumors, IM tumors were characterized by a more balanced pattern of immune targets with the association of immune stimulatory receptors such as 4-1BB, OX40, IL2-R and ligands such as ICOS and CD40L, as well as molecules involved in the dysfunction of pro-inflammatory status.
as inhibitory receptors (TIGIT, PD-1, CTLA4). Mesenchymal tumors did not appear immune reactive, with a negative association for most tested immune targets except for two immune inhibitory receptors, B7-H3 and B7-H4, also expressed by tumor cells and involved in the regulation of cancer initiation and progression (32).

Finally, we evaluated the expression of immune targets according to TIME subtypes (Figure 6B; Supplementary Table 13). Almost all immune targets were highly expressed in the FI subtype. MR tumors were enriched in B7-H3 and GARP expression while being negatively associated with most other targets, reflecting the immunosuppression of these tumors. Finally, SR tumors were characterized by a low expression of most evaluated immune targets. Overall, these analyses show that different immune targets are differentially expressed across TNBC and TIME subtypes allowing a rationale tailoring of immunotherapy in TNBC patients.

Validation of the TME heterogeneity within TNBC molecular subtypes

The robustness of our findings was further evaluated using an independent cohort of 497 TNBC samples with available clinical and transcriptomic data (cohort C). Similar TME patterns were found for each TNBC subtype with immune signatures being statistically significantly associated with better relapse-free survival (Figure 7A,B and Supplementary Tables 4,6). The same distribution of the TNBC molecular subtypes according to TIME classification was observed between all cohorts (Figure 7C). In line with our previous results, the TIME classification was also statistically significantly associated with distinct TME processes and relapse-free survival (Figures
7D,E; Supplementary Table 5). IM subtype was mainly enriched with adaptive immune cells compared to MSL which were mainly composed of innate immune cells (Figure 7F). Finally, IM and FI subtypes were positively associated with almost all evaluated immune targets, in contrast to MSL and MR subtypes which were more associated with a protumoral TME, overall validating our results (Supplementary Figure 4; Supplementary Table 12,13). Finally, TME heterogeneity was also explored in all 3 cohorts using two other TNBC molecular classifications reported by Burstein et al. (4) and Jiang et al. (3), showing similar TME profiles and clinical outcome associations (Supplementary Figures 5-6).

Discussion

TILs and immune signatures were previously reported to be associated with better survival and response to treatment in TNBC (7)–(9), however, it is not clear which immune cell types nor which spatial organization drive clinical outcome. This is the largest analysis, exploiting 1512 TNBC samples from 4 large and independent public datasets, demonstrating the extent of TME heterogeneity that characterizes each TNBC molecular subtype beyond the genomic and transcriptomic diversity (2,3).

We also explored for the first time the TIME classification integrating immune cells spatial localization (28). We showed that IM and FI subtypes are associated with high expression of most evaluated immune targets (e.g. immune checkpoint receptors) and adaptive immune-related cell populations suggesting that these “immune hot” tumors are the best potential candidates for immune checkpoint blockers (ICB).
contrast, most MSL and LAR tumors showed an immunosuppressive and pro-tumorigenic phenotype with high expression levels of stromal signatures, known to promote an immunosuppressed TME, suggesting that they are potential candidates for treatment targeting regulatory T cells or immunosuppressive pathways such as the adenosine pathway. The M and MR subtypes could be considered as “immune cold” tumors with low expression of different immune cell populations and downregulation of most immune targets.

Tumor metabolic reprogramming is a known cancer hallmark characterized by an adaptive mechanism promoting tumor development in a hostile TME. Our analysis revealed an activation of the metabolic pathways in the “immune cold” BL, LAR and M molecular subtypes as well as in the SR subtype, pointing out the tight interplay between tumor metabolism and TME. Targeting the metabolic pathways therefore appears as a promising anti-cancer strategy in these specific TNBC subtypes.

We are the first to demonstrate that TIME classification faithfully reflects TIL localization, as witnessed by a good 67% concordance rate between TIL localization assessed by pathologists and the gene expression-based TIME classification. Discordant cases could notably be explained by the use of H&E instead of CD8+ IHC-stained slides to assess the TIL localization, differences in tumor sampling used for H&E staining and RNA-sequencing and/or the exclusion by pathologists of highly infiltrated necrotic areas captured by gene expression analysis from bulk tumors. Indeed, about 25% of discordant cases showed an immune infiltration either in necrotic areas or in normal adjacent tissue. Our findings suggest that TIME classification should be evaluated to
discriminate responders from non-responders to immunotherapy, as a complementary biomarker to TIL assessment (14),(33).

Furthermore, our analysis provides novel evidence of potential mechanisms of resistance to ICB. We have notably shown that tumors with high CIN and chromosomal 5q and 15q regions loss, including *TMEM173* (5q31.2; STING) and *B2M* (15q21.1) genes leading to the downregulation of several MHC class-I/II genes, were independently associated with reduced immune cytotoxicity. Downregulation of MHC class-I molecules by tumor cells decreases tumor antigenicity and could be an immune escape mechanism to avoid recognition and tumor cell death by the immune system (10). In some cases, the absence of MHC class-I on tumor cells could explain the low tumor immune infiltration and the lack of response to ICB(34). Inducing MHC recovery to overcome the MHC loss using oncolytic viruses or demethylating agents, as well as NK cell therapy activated by the loss of MHC-I on tumors cells therefore appear as potential treatment option for these particular tumors (35)–(37). Chromosomal 5q region loss includes also *GZMA* encoding for proteins involved in cytotoxic activity of immune cells (5q11.2).

Although, our bulk sequencing analyses do not allow us to discriminate between tumor and immune cells expression, we considered that this loss doesn’t interfere with our findings as it is mainly expressed by immune cells (38),(39).

The recent results from the first phase III trial evaluating anti-PD-L1 therapy in combination with chemotherapy demonstrated a survival benefit in metastatic TNBC patients(13). Of note, the survival benefit was only observed in the PD-L1+ subgroup. In light of our results, the identification of novel predictive biomarkers could allow to
determine the most appropriate immunotherapeutic strategy in order to optimally enhance antitumor immune responses. Hence, combinatorial approaches, targeting diverse immune escape mechanisms may potentially improve the response rate and clinical benefit to ICB (Figure 8).

To conclude, despite being limited by its retrospective nature and restricted to bulk tumor sequencing data analysis, this study allowed us to gain more insight into the complex interactions between tumor cells and their microenvironment, in particular immune cells. However, prospective validation of our findings is warranted before their clinical implementation. New technologies such as single cell sequencing and spatial transcriptomics (40,41) may further allow to investigate the extent of tumor heterogeneity that characterizes TNBC at an unprecedented level.

**Funding**

YB and CS are supported by the Télévie and the Fonds National de la Recherche Scientifique (F.R.S.-FNRS). LB is supported by “Les Amis de l’Institut Bordet” foundation. DV is supported by the Walloon region under the WALInnov program. This study was supported by a grant from Breast Cancer Research Foundation (BCRF). No grant numbers applied.

**Notes**
The funding sources had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication. JS is a permanent member of the Scientific Advisory Board and holds stocks of Surface Oncology. The other authors declare no potential conflicts of interest.

**Author contributions:** Dr. Sotiriou, Dr. Buissneret and Y. Bareche had full access to all the data in the study and take the responsibility for the integrity of the data and the accuracy of the data analysis. Y. Bareche and Dr Buissneret contributed equally to this work. Drs Stagg and Sotiriou are joint senior authors.

*Study concept and design: Bareche, Buissneret, Gruosso, Stagg, Sotiriou.*

*Acquisition, analysis, or interpretation of data: All authors.*

*Drafting of the manuscript: Bareche, Buissneret, Stagg, Sotiriou.*

*Critical revision of the manuscript for important intellectual content: Bareche, Buissneret, Gruosso, Rothé, Stagg, Sotiriou.*

*Statistical analysis: Bareche, Venet*

*Obtained funding: Sotiriou*

*Administrative, technical, or material support: Bareche, Buissneret, Girard, Dupont, Desmedt, Rothé, Sotiriou*

*Study supervision: Bareche, Buissneret, Gruosso, Park, Stagg, Rothé, Sotiriou*

**Additional Contributions:** We are thankful to the TCGA Research Network (http://cancergenome.nih.gov/) and the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) for providing the data analyzed in this study. We
are also grateful to both Pr. Van Loo and Dr. Haase for sharing with us their TCGA ASCAT data.

TG’s present address is: Forbius, 750 Boul St-Laurent, Montréal, Quebec, Canada.

CD’s present address is: Laboratory for Translational Breast Cancer Research, Department of Oncology, KU Leuven, Herestraat 49, box 818, 3000 Leuven.

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**Figure 1.** Tumor Micro-Environment features associated with TNBC molecular subtypes and overall survival. (A) Associations between TME gene expression signatures and TNBC molecular subtypes. A logistic regression model was used to evaluate associations between each specific gene signature and each TNBC molecular subtype. P-values were obtained from parametric Mann Whitney U tests and corrected for multi-testing. Only statistically significant associations are shown (FDR≤ 0.05), with negative and positive associations being represented in red and green, respectively. The left half-circle and the right half-circle represent cohorts A and B respectively. (B) Associations between TME gene signatures and 10-year overall survival (OS), using univariable and multivariable Cox regression models, adjusted for the dataset (TCGA vs. METABRIC), patient age (≤40y vs. >40y), nodal status (Positive vs. Negative), tumor size (<2cm vs. ≥2cm) and histological grade (I/II vs. III). The x and y axis represent, hazard ratio (HR) and the -log10 (FDR), respectively. The horizontal bold dotted line represents the FDR threshold at 0.05 for statistically significant associations.

**Figure 2.** Characterization of the spatial immune landscape and immune composition in TNBC molecular subtypes.

Associations between TNBC molecular subtypes and TIME subtypes in cohorts A (A) and B (B). (C) Associations between TME gene expression signatures and TIME subtypes. Logistic regression model was used to evaluate the association between each feature
and each subtype. (D) Associations between 16 immune cell subsets scores with TNBC molecular subtypes. A logistic regression model was used to evaluate associations between each immune cell population and each tumor TNBC molecular subtype. P-values were obtained from parametric Mann Whitney U tests and corrected for multi-testing. Only statistically significant associations are shown (FDR≤ 0.05), with negative and positive associations being represented in red and green, respectively. The left half-circle and the right half-circle represent cohorts A and B respectively.

**Figure 3. Chromosomal instability association with immune escape.** (A) Associations between TME and tumor specific features with local cytotoxic immune activity in cohorts A and B. Spearman correlation was used to evaluate associations between each TME and tumor specific gene expression signature with the cytotoxic immune activity gene signature. The x and y axis represent, spearman ρ and the -log10(FDR), respectively. The horizontal bold dotted line represents the FDR threshold at 0.05 for statistically significant association. Chromosomal Instability (CIN) distribution within each (B) TNBC molecular and (C) TIME subtypes in cohorts A and B. Differences between each subtype and the rest of the cohort was assessed using two-sided Mann-Whitney U test. A black star was displayed when statistically significant CIN scores enrichment were observed within a specific TNBC and TIME subtype. (D) Gene ontology analyses of genes with mRNA expression significantly (FDR≤0.05) negatively (left, in red) and positively (right, in blue) correlated with CIN scores (spearman correlation) in cohorts A and B.
Figure 4. Loss of chromosome 5q and 15q regions is associated with reduced CYT levels. **(A)** Copy number aberration (CNA) status distribution according to the 3 different TNBC classifications of the 33 genes associated with high CNA loss and low CYT scores in CIN High (CIN≥0.38, 3rd tertile) and CIN Low (CIN≤0.08, 1st tertile) tumors in cohort A. **(B)** CNA status distribution of the 27 out of these 33 genes validated in cohort B associated with high CNA loss and low CYT scores in CIN High (CIN≥0.39, 3rd tertile) and CIN Low (CIN≤0.11, 1st tertile) tumors. Differences in CNA status between both CIN subgroups were assessed using a two-sided Fisher exact test. P-values were adjusted for multiple testing using the Benjamini-Hochberg procedure. Logistic regression model was used to evaluate the association between each gene CNA loss with CYT activity. P-values were obtained from parametric Mann Whitney U tests and corrected for multi-testing.

Figure 5. Specific 5q/15q region losses associated with reduced CYT. Associations between chromosome 5q **(A)** and 15q **(B)** specific region logR with cytotoxic activity using a linear regression model. Forestplots displaying hazard ratios (HR) and 95% confidence intervals (CI). Horizontal bars represent the 95% confidence intervals of HR. Variable with significant effect (p-values≤0.05) are shown in red. Grey boxes highlight common regions significantly associated in both cohorts.

Figure 6. Therapeutic immune targets according to TNBC molecular and TIME subtypes. Associations of 44 immune genes corresponding to immunomodulatory targets with TNBC molecular **(A)** and TIME **(B)** subtypes. A logistic regression model was
used to evaluate associations between each gene expression with each subtype. P-values were obtained from parametric Mann Whitney U tests and corrected for multi-testing. Only significant associations are shown (FDR≤ 0.05), with negative and positive associations being represented in red and green, respectively. The left half-circle and the right half-circle represent cohorts A and B respectively.

**Figure 7. Validation of Tumor Micro-Environment heterogeneity within TNBC molecular subtypes.** (A) Associations between TME gene expression signatures and TNBC molecular subtypes within cohort C. A logistic regression model was used to evaluate associations between each specific gene signature and each TNBC molecular subtype. P-values were obtained from parametric Mann Whitney U tests and corrected for multi-testing. Only significant associations are shown (FDR≤ 0.05), with negative and positive associations being represented in red and green, respectively. (B) Associations between TME gene signatures and relapse-free survival (RFS), using a cox regression model. The x and y axis represent, hazard ratio (HR) and the -log10 (FDR), respectively. The horizontal bold dotted line represents the FDR threshold at 0.05 for significant associations. (C) Associations between TNBC molecular subtypes and TIME subtypes in cohort C. (D) Associations between TME gene expression signatures with TIME subtypes in cohort C. A logistic regression model was used to evaluate associations between each specific gene signature and each TNBC molecular subtype. P-values were obtained from parametric Mann Whitney U tests and corrected for multi-testing. Only significant associations are shown (FDR≤ 0.05), with negative and positive associations being
represented in red and green, respectively. (E) Kaplan-Meier analysis of relapse-free survival (RFS) of cohort C stratified according to TIME subtypes (FI vs. SR vs. MR). (F) Associations between 16 immune cell populations scores with TNBC molecular subtypes in cohort C. A logistic regression model was used to evaluate associations between each immune cell population and each tumor subtype. P-values were obtained from parametric Mann Whitney U tests and corrected for multi-testing. Only significant associations are shown (FDR≤ 0.05), with negative and positive associations being represented in red and green respectively.

**Figure 8. Response to immunotherapy through targeting TNBC tumor micro-environment and genomic heterogeneity.** The inner pie chart represents the relative proportion of the TNBC molecular subtypes. The outer pie chart represents the relative proportion of the TIME subtypes within each TNBC molecular subtype. Observed TNBC subtype specific aberrations are listed in each quadrant with the corresponding rational therapeutic strategies presented in the dotted box.
Figure 1

A

Lymphocyte
Cytolytic Activity
Lymphangiogenesis
Inducing Angiogenesis
Hypoxia
Stroma
CD10+/GPR77+ CAF
Pentose Phosphate
Lipid Metabolism
Glycolysis

Effect Size
≥ 4
2
1.5
0.66
0.50
≤ 0.25

cohort A
cohort B

TME signature

B

Univariable

TME signature

Multivariable
Figure 2

A cohort A

| TIME subtype | Frequency (%) |
|--------------|---------------|
| FI | 103 |
| SR | 115 |
| MR | 69 |
| Total | 117 |

B cohort B

| TIME subtype | Frequency (%) |
|--------------|---------------|
| FI | 59 |
| SR | 48 |
| MR | 11 |
| Total | 65 |

C

| Metabolism | Immune System |
|------------|---------------|
| Lymphocyte | Immune |
| Cytolytic Activity | Immune |
| Lymphangiogenesis | Vascularization |
| Inducing Angiogenesis | Vascularization |
| Hypoxia | Stroma |
| Stroma | Stroma |
| CD10+/GPR77+ CAF | Stroma |
| Pentose Phosphate | Metabolism |
| Lipid Metabolism | Metabolism |
| Glycolysis | Metabolism |

D

Effect Size

- ≥ 4
- 2
- 1.5
- 0.66
- 0.5
- ≤ 0.25

TME signature

- Immune
- Vascularization
- Stroma
- Metabolism

Immune System

- Adaptive Immunity
- Innate Immunity

CD56bright NK cells

CD56dim NK cells

Figure 2--FINAL

Downloaded from https://academic.oup.com/jnci/advance-article-abstract/doi/10.1093/jnci/djz208/5609116 by ULB user on 27 November 2019
Figure 3

A  

Cohort A

Cohort B

Association with local cytotoxic immune activity

- CIN
- HRD
- TMB
- ITH
- Lymphangiogenesis
- Inducing Angiogenesis
- Hypoxia
- Stromal
- CD10+/GPR77+ CAF
- Pentose Phosphate
- Lipid Metabolism
- Glycolysis

B  

CIN scores according to the TNBC molecular classification

C  

CIN scores according to the TIME classification

D  

Cohort A Top 10 Gene Ontology

Inversely correlated with CIN

Positively correlated with CIN

- Regulation of apoptotic cell clearance
- Immunological cell lineage
- Regulation of autophagosome maturation
- Oligosaccharide metabolic process
- Innate immune response
- Regulation of autophagosome maturation
- Mature B cell differentiation
- Regulation of autophagosome maturation
- Innate immune response
- TNF-alpha signaling pathway

Cohort B Top 10 Gene Ontology

Inversely correlated with CIN

Positively correlated with CIN

- Regulation of cellular stress
- Regulation of autophagosome maturation
- Oligosaccharide metabolic process
- Innate immune response
- Regulation of autophagosome maturation
- Mature B cell differentiation
- Regulation of autophagosome maturation
- Innate immune response
- TNF-alpha signaling pathway
- Cell cycle
Figure 7

A

B

cohort C

Effect Size

≤ 0.25

0.50

0.66

1.5

2

≥ 4

TME signature

Immune

Vascularization

Stroma

Metabolism

Cytolytic Activity

Lymphangiogenesis

Inducing Angiogenesis

Hypoxia

Stroma

CD10+/GPR77+ CAF

Pentose Phosphate

Lipid Metabolism

Glycolysis

C

cohort C

Effect Size

≤ 0.25

0.50

0.66

1.5

2

≥ 4

TME signature

Immune

Vascularization

Stroma

Metabolism

Cytolytic Activity

Lymphangiogenesis

Inducing Angiogenesis

Hypoxia

Stroma

CD10+/GPR77+ CAF

Pentose Phosphate

Lipid Metabolism

Glycolysis

D

cohort C

Effect Size

≤ 0.25

0.50

0.66

1.5

2

≥ 4

TME signature

Immune

Vascularization

Stroma

Metabolism

Cytolytic Activity

Lymphangiogenesis

Inducing Angiogenesis

Hypoxia

Stroma

CD10+/GPR77+ CAF

Pentose Phosphate

Lipid Metabolism

Glycolysis
Pro-tumoral Immune Infiltration

Stroma

Lymphangiogenesis

Retained RB1

CD39 / CD73

GARP / TGFβ

CSF1R

Immune Hot

Adaptive & Innate Immunity

Most Immunomodulatory target (including PD1, PDL1 & CTLA4)

Immune Cold

Hypoxia

Stroma

CAF

CIN high - 5q/15q region loss

B7-H4

IDO1

Oncolytic virotherapy

HDAC / DNMT inhibitors

NK therapy

B7-H4 & IDO inhibitors

Anti-metabolite

Innate Immunity

Metabolism

Stroma

Retained RB1

CDK4/6 inhibitors

Anti-metabolite

VEGF inhibitors

Oncolytic virotherapy

HDAC / DNMT inhibitors

NK therapy

B7-H4 inhibitors

Anti-metabolite

Immune Cold

Hypoxia

Stroma

CAF

CIN high - 5q/15q region loss

B7-H4

CDK4/6 inhibitors

VEGF inhibitors

HDAC / DNMT inhibitors

NK therapy

B7-H4 inhibitors

Anti-metabolite

Immune checkpoint blockers (ICB)

Pro-tumoral Immune Infiltration

Stroma

Lymphangiogenesis

Retained RB1

CD39 / CD73

GARP / TGFβ

CSF1R

TIME Classification:

- Fully Inflamed
- Stroma Restricted
- Margin Restricted
- Immune Cold

Figure 8