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Valentina Silvestri, Virginia Valentini, Agostino Bucalo, Virginia Porzio, Giorgia Scafetta, Bruna Cerbelli, Ines Zanna, Simonetta Bianchi, Maria Grazia Tibiletti, Ileana Carnevali, Daniele Calistri, Valentina Arcangeli, Valentina Zampiga, Elisa Gasparini, Laura Cortesi, Giuseppe Giannini, Carlo Capalbo, Stephen B. Fox, Giovanna Masala, Silvia Mezi, Giulia d'Amati & Laura Ottini

Transcriptomic Deconvolution Reveals Prognostic Immune Signatures and Immunotherapy-Responsive Subtypes in Male Breast Cancer

Valentina Silvestri[1], Virginia Valentini[1], Agostino Bucalo[1], Virginia Porzio[1], Giorgia Scafetta[2], Bruna Cerbelli[3], Ines Zanna[4], Simonetta Bianchi[5], Maria Grazia Tibiletti[6], Ileana Carnevali[7], Daniele Calistri[8], Valentina Arcangeli[8], Valentina Zampiga[8], Elisa Gasparini[9], Laura Cortesi[10], Giuseppe Giannini[1], Carlo Capalbo[11], Stephen B Fox[12], Giovanna Masala[13], Silvia Mezi[3], Giulia d'Amati[3], Laura Ottini[1]

Affiliations:

[1] Department of Molecular Medicine, Sapienza University of Rome, Rome, Italy

[2] Translational Oncology Research Unit, IRCCS Regina Elena National Cancer Institute, Rome, Italy

[3] Department of Radiological, Oncological and Pathological Sciences, Sapienza University of Rome

[4] Cancer Risk Factors and Lifestyle Epidemiology Unit, Institute for cancer research, prevention and clinical network (ISPRO), Florence, Italy

[5] Department of Health Sciences, Division of Pathological Anatomy, University of Florence, Florence, Italy

[6] Hereditary Cancer Research Center, University of Insubria, Varese, Italy

[7] SSD Laboratorio Specialistico Genetica Medica Citogenetica e Genetica Molecolare ASST Settelaghi Varese

[8]Istituto Romagnolo per lo Studio dei Tumori "Dino Amadori" IRCCS, Meldola

[9] Medical Oncology, AUSL- IRCCS Reggio Emilia, Reggio Emilia, Italy

[10] Medical Oncology, AUSL- IRCCS Reggio Emilia, Reggio Emilia, Italy; Department of Medical and Surgical Sciences, University of Modena and Reggio Emilia, 41124 Modena, Italy.

[11]Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, 87036 Rende; Oncology Unit, Annunziata Hospital Cosenza, 87100 Cosenza, Italy

[12] Department of Pathology, Peter MacCallum Cancer Centre, Melbourne, VIC, Australia;
Sir Peter MacCallum Department of Oncology and Collaborative Centre of Cancer Genomic Medicine, University of Melbourne, Melbourne, VIC, Australia.

[13] Clinical Epidemiology Unit, Institute for cancer research, prevention and clinical network (ISPRO), Florence,Italy

Corresponding Author: Laura Ottini[1], email address: laura.ottini@uniroma1.it

Abstract

Background: Male breast cancer (MBC) is a rare malignancy with distinct biological and clinical features compared to its female counterpart. Emerging evidence suggests that a subset of MBCs may exhibit an immunogenic tumor microenvironment; however, the lack of sex-specific data on immune biomarkers has limited the inclusion of male patients in immunotherapy trials.

Methods: We performed transcriptomic profiling of 123 MBCs, including 41 with germline pathogenic variants (PVs) in *BRCA2* (n=26), *BRCA1* (n=12), and *PALB2* (n=3). Immune characterization included *PD-1* and *PD-L1* expression, immune scores, and immune cell infiltration using deconvolution tools. The Tumor Immune Dysfunction and Exclusion (TIDE) algorithm was applied to predict potential response to immune checkpoint inhibitors.

Results: A distinct subset of MBCs showed high *PD-L1* expression and high immune scores. Immune deconvolution revealed that CD4+ memory resting T cells (24.5%), M2 macrophages (14.4%), and M0 macrophages (13.8%) were the most abundant infiltrating immune populations within the tumor microenvironment. Notably, 37.4% of tumors were predicted to respond to immunotherapy, primarily within the Luminal B subtype. These tumors demonstrated significantly higher PD-1/PD-L1 expression, higher immune scores, and enriched immune cell infiltration, compared to non-responders. Unsupervised clustering identified two transcriptionally distinct molecular subgroups. Cluster 1 was enriched for immune-related pathways and comprised the majority of predicted responders. Immune infiltration patterns varied significantly according to germline mutation status, intrinsic subtype, histological grade, androgen receptor expression, and Ki-67 proliferation index.

Conclusions: This study identifies a transcriptionally defined, immunogenic subset of MBCs with potential sensitivity to immune checkpoint inhibitors. These findings highlight the need for sex-specific immune profiling and provide a rationale for incorporating immunotherapy into precision treatment strategies for men with breast cancer.

Keywords: Male breast cancer; Transcriptomics; Molecular Biomarkers; Germline mutations; *BRCA1*/*BRCA2*; Molecular subtypes; PD-1/PD-L1; Immune cell infiltration; Immunotherapy; Precision oncology

Introduction

Male breast cancer (MBC) represents less than 1% of all cancers in men and less than 1% of all breast cancers (BCs), with its incidence increasing over the last 30 years.[1,2] At genetic level, hereditary germline *BRCA2*, *BRCA1* and *PALB2* pathogenic variants (PVs) play the main role in MBC susceptibility, accounting for about 13%, 2% and 1% of unselected MBCs, respectively.[2-4]

The rarity of MBC has precluded the development of ad hoc clinical trials and currently, clinical management and therapeutic options of MBC patients are informed almost entirely by female BC (FBC) research.[5] Notably, mortality after cancer diagnosis is higher among male patients with BC compared with their female counterparts, even after accounting for known prognostic factors, suggesting that such disparity may be due to factors yet to be identified.[6] Indeed, increasing evidence indicates that MBC may be different, with unique molecular features, suggesting sex-specific differences in terms of biological and clinical behavior.[7-9] Although most MBCs are estrogen receptor positive (ER+), thus treated with endocrine therapy, MBCs have been shown to display molecular features that may help in identifying subgroups of patients that may benefit from other targeted therapeutic approaches.[10,11] For example, there is a tremendous interest in using immunotherapy to treat BC, although the vast majority of breast tumors are considered immunologically quiescent.[12] The identification of BC patients who are more likely to respond to immunotherapy with immune checkpoint inhibitors (ICI), is currently based on immune-related biomarkers, including the abundance of tumor-infiltrating lymphocytes (TILs), tumor microenvironment composition and expression of PD-1 and PD-L1.[13]

In FBC, triple negative breast cancer (TNBC) is more primed to respond to immunotherapy given the presence of more TILs and higher PD-L1 expression relative to the other BC subtypes, and therefore, immuno-oncology represents a key area of promise for TNBC research.[14] On the other hand, the role of immune-related biomarkers is less clear in BC of luminal subtype, which represents the majority of MBCs.[15,16]

Considering the known sex-driven differences in immune responses as potential factors contributing to diverse outcome as well as response to therapy in male and female cancer patients, a further understanding of the MBC-specific immune landscape is essential for the clinical applicability of personalized immune therapeutic strategies.

To date, limited information is available regarding immune-related biomarkers in MBC, thus largely cutting out male patients from the possibility of receiving ICI treatment. Single-cell transcriptome analysis demonstrated that MBC had a significantly lower degree of immune infiltration than FBC, especially T and B cells.[17] However, it was recently shown that about 4% of MBCs displayed a TIL density >50% and about 7%, all of luminal subtype, a PD-L1 cell score >1%.[18] Importantly, in our previous study, immune response emerged as the most relevant process able to discriminate MBC subgroups at the transcriptional level. From unsupervised clustering approach, two distinct MBC subgroups emerged, named Cluster 1 and Cluster 2, with Cluster 1 enriched for *BRCA2*-associated cases, and displaying up-regulation of genes involved in immunity pathways and a possible worse overall survival compared with Cluster 2.[19] Overall, all these data pointed to the possibility that a subset of MBCs, not overlapping with responsive subsets identified in FBCs, such as TNBC, may harbor an immunogenic microenvironment and may potentially benefit from ICI therapy.

To further address this issue, this study aims to perform a comprehensive, transcriptomic-based profiling of the immune landscape of MBC, by characterizing immune-related biomarkers according to germline mutational status, molecular and pathology features, to identify MBC subtypes with possible clinical significance. This approach may provide insights into the determinants of immune responses in MBCs and, eventually, inform the identification of MBC patients that may benefit from personalized treatment.

Methods

Study population

A series of 123 MBC cases, collected within the Italian multicenter study on MBC, which comprises samples and data from more than 1.000 MBCs, were selected for the present study. Transcriptome data by RNA-sequencing were already available for 63 MBCs, as previously described in detail.[19] Here, we expanded the series by analyzing additional 60 MBCs based on the availability of formalin-fixed, paraffin-embedded (FFPE) tumor samples that could provide an adequate quantity and quality of RNA to carry out molecular analyses. Overall, the series of 123 MBCs was collected from 6 research centers (**Supplementary Table 1**). All MBC cases analyzed were of self-reported European (Caucasian) ethnicity.

All MBC cases were tested for germline *BRCA1* and *BRCA2* PVs, performed in the frame of genetic counseling programs at the center of origin.[4] The majority of cases negative for *BRCA1/2* PVs were retested using Next-Generation Sequencing for germline PVs in 50 cancer-related genes, allowing the identification of PVs in other BC susceptibility genes.[4] Overall, 41 MBCs with germline PVs in MBC risk genes (26 *BRCA2*, 12 *BRCA1* and 3 *PALB2*) and 82 MBCs without germline PVs were included in this study.

Pathology information for each MBC was obtained, including histology, grade, lymph node status, estrogen, progesterone and androgen receptors (ER/PR/AR), HER2 expression, Ki67 status.

Data on vital status, in terms of overall survival (OS) were available for 109 out of 123 recruited cases. All MBC patients analyzed in this study underwent surgery without neoadjuvant therapy administration. For each MBC case, informed consent was obtained. The study was approved by the Local Ethical Committee (Sapienza University of Rome, Prot. 669/17).

IHC analysis

A subset of 70 MBCs was analyzed by IHC analysis, to evaluate the correlation between molecular and pathology data. First, hematoxylin and eosin-stained FFPE tumor slides were evaluated for the presence and percentage of stromal TILs, according to the standardized method proposed by the International TILs Working Group in 2014.[20] TILs were quantified as a percentage of the stromal area of the tumor and expressed as a continuous parameter. Cases were stratified into high- and low-TILs according to the cut-off of 50%.[21] To assess and quantify T lymphocytes specifically, IHC was carried out using CD3 antibody (1: 100 Roche Diagnostics, Basilea, Switzerland). The number of CD3 positive lymphocytes was quantified and expressed as a percentage of the total number of TILs.

Scoring of PD-L1 expression by IHC testing was assessed in FFPE tumor slides using either the 22C3 clone (Agilent DAKO, Santa Clara, CA, US) or the SP263 clone with Ventana BenchMark Ultra platform (Ventana Medical Systems, Inc., AZ, U.S). Preparation and staining were done according to the manufacturer´s instructions. PD-L1 staining was evaluated according to the combined positive score (CPS), defined as the number of PD-L1–staining cells (tumor cells, lymphocytes, and macrophages) divided by the total number of viable tumor cells, multiplied by 100.[22]

RNA Isolation and Sequencing

RNA from breast tumors was extracted from microdissected FFPE sections using the MiReasy FFPE kit (Qiagen, Hilden, Germany) and subsequently processed as previously described.[19]
Briefly, libraries were prepared using the TruSeq RNA Access Library Prep kit (Illumina, San Diego, CA, USA) and sequenced in paired-end mode (2x75 bp) on an Illumina NextSeq platform.
A bioinformatic pipeline including FastQC for quality control, cutadapt to remove the adapter sequence (if present) and the very short reads (read length < 25 bp), STAR for alignment on reference homo sapiens hg19 (Ensembl version GRch37), and FeatureCounts for counting reads was used to analyze the data. Raw read counts and transcripts per million (TPM) counts were obtained for all samples analyzed.

Transcriptomic-based Analysis
Gene expression of the immune checkpoint markers PD-1 (*PDCD1* gene) and PD-L1 (*CD274* gene) were estimated as TPM. A score for the presence of immune infiltrate (Immune Score) was inferred by transcriptomic data using ESTIMATE (Estimation of STromal and Immune cells in MAlignant Tumor tissues using Expression data) tool.[23] Immune cell infiltration abundances and fractions were estimated based on transcriptomic data using CIBERSORTx and MCP-counter.[24,25] CIBERSORTx is a deconvolution-based approach which provides both the immune cell fractions relative to total immune cell content for each tumor, and an absolute proportion of 22 distinct infiltrating immune cell types within a tumor sample, allowing for both intra-sample comparisons between immune cell types and inter-sample comparisons of the same cell type.[24] MCP-counter tool is a marker-gene-based approach which allows the quantification of the absolute abundance of eight immune, two stromal cell populations and a cytotoxicity score, comparable among different samples.[25]
TIDE (Tumor Immune Dysfunction and Exclusion) module was used to predict patient response to immune checkpoint blockade.[26]
To validate our results, ESTIMATE, CIBERSORTx and TIDE analyses were also performed on two MBC series from available external datasets, including 46 MBCs from Severson et al. 2018 [27] (available on NCBI-GEO, accession n. GSE104730), and for 12 MBCs from The Cancer Genome Atlas (TCGA) dataset (available at https://www.cancer.gov/tcga).[28]
An unsupervised hierarchical clustering analysis was performed using pvclust [29] on the sample correlation matrix, constructed using the 2.000 most variable transcripts among the 123 MBC samples, to validate previously reported MBC subgroups with distinct gene expression patterns.[19] Differential expression analysis was performed using R package DESeq2 as previously described.[19] PAM50 subtype classification was performed using genefu package in R studio.[30]

Statistical analysis
Association between transcriptome-based profiles and IHC data, pathology features, molecular subtypes and germline mutational status were evaluated by using linear regression, Wilcoxon rank-sum test, Kruskall-wallis test and Chi-square test, as appropriate. A receiver operating characteristic (ROC) curve analysis, with evaluation of the area under the curve (AUC), was performed as an additional correlation metric. Survival time was calculated from the date of interview to the date of death from any cause or the last follow-up for alive patients, as previously described.[19,31] OS was estimated using the Kaplan–Meier method, and differences between groups of patients were assessed by the log-rank test and Cox regression models. For continuous

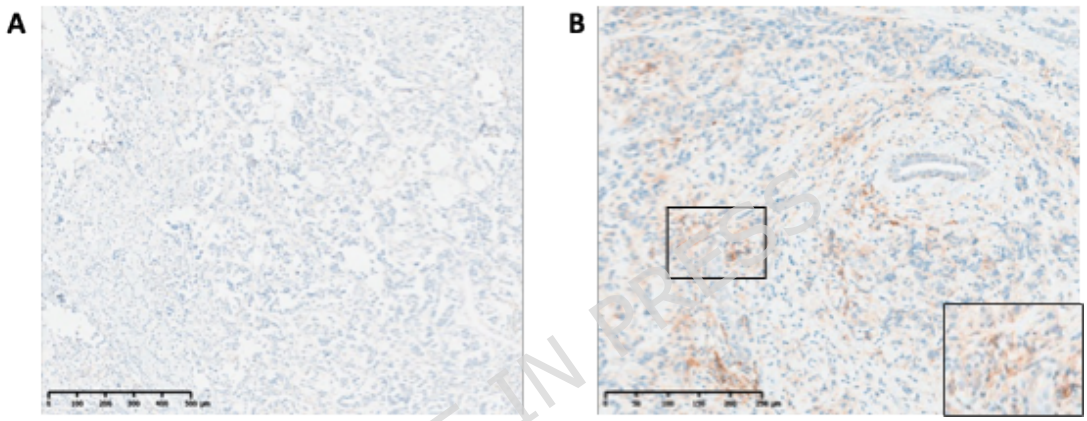
variables, the cut-off for dividing cases in two groups (high and low) in the survival analysis was based on median values in the whole MBC series. In all analyses, p-values $\leq$0.05 were considered statistically significant. All statistical analyses were performed with the R Studio software and Stata software version 18.

Results

Characterization of the immune landscape of MBCs

TIL density was estimated based on pathology evaluation. The range of TIL density was 1-70%, with a median value of 10%. The majority of MBCs (68.9%) had <50% TILs. T cells (CD3+) represented ≥ 80% of TILs in 96.4% of analyzed MBCs.
PD-L1 status was first estimated by IHC. Using a CPS >10 as the cut-off for PD-L1 positivity, in line with criteria established for FBC, positive PD-L1 IHC staining was observed in 2 out of 70 MBC cases analyzed (**Figure 1A-B**). When adopting a CPS >1 threshold for PD-L1 positivity, as previously proposed for MBC,[32] a total of 6 out of 70 cases (8.6%) were classified as PD-L1 positive by IHC.

Figure 1. Exemplificative pictures of immunohistochemical expression of PD-L1 in MBC. (A) MBC characterized by the absence of PD-L1 expression; (B) MBC positive for the SP263 antibody.



In addition, gene expression of both PD-1 and PD-L1 were estimated as TPM from transcriptomic data. In our MBC series, PD-1 expression ranged from 0 to 14.9 TPM (median=0 TPM), whereas PD-L1 expression ranged from 0 to 30.5 TPM (median=1.7 TPM) (**Supplementary Figure 1A-B**). Immune score was inferred from transcriptomic data using ESTIMATE tool. In our MBC series, immune score values ranged from -1,218.5 to 2299.9 (median=48.2) (**Supplementary Figure 1C**).
Similar immune score value ranges and medians were observed in MBCs from the two external datasets used as validation series (**Supplementary Figure 2**).[27-28]
Correlations between PD-L1 IHC, PD-1 and PD-L1 gene expression and immune score emerged. MBCs showing a positive PD-L1 staining in IHC displayed a significantly higher PD-L1 gene expression compared with MBCs cases with negative PD-L1 IHC (p=0.0008) (**Figure 2A**). The association between PD-L1 IHC and gene expression was further confirmed by the ROC analysis (AUC=0.88, 95% CI 0.78-0.99) and linear regression model (p=0.01) (**Supplementary figure 3A-B**).
MBCs showing a positive PD-L1 staining in IHC displayed significantly higher ESTIMATE immune score values compared with MBCs cases with negative PD-L1 IHC (p=0.0013) (**Figure 2B**). Notably, ESTIMATE immune score values were also positively correlated with PD-1 (p<0.00001) and PD-L1 (p<0.00001) gene expression, and with the percentage of TILs (p=0.0005) (**Figure 3A-B-C**).

Figure 2. Box plots showing significantly different levels of PD-L1 TPM gene-expression (A) and ESTIMATE Immune Score (B) based on PD-L1 IHC status. 0 = negative; 1 = positive.

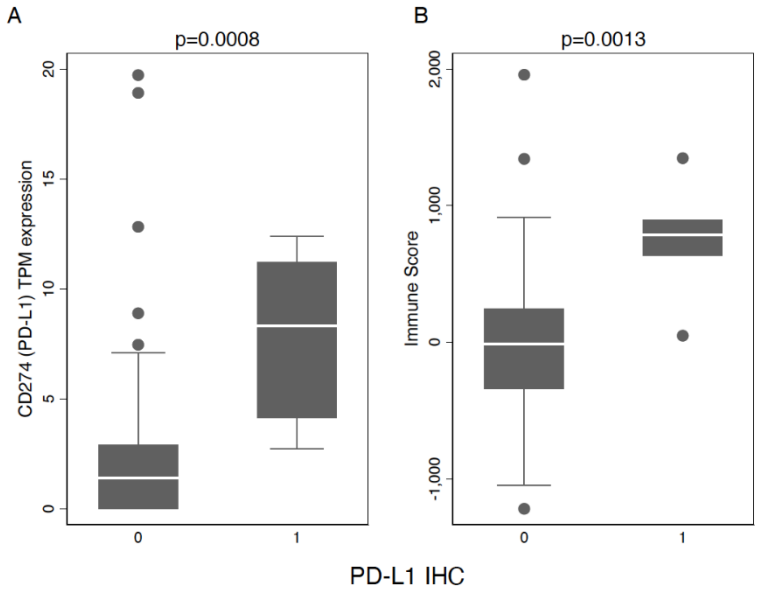
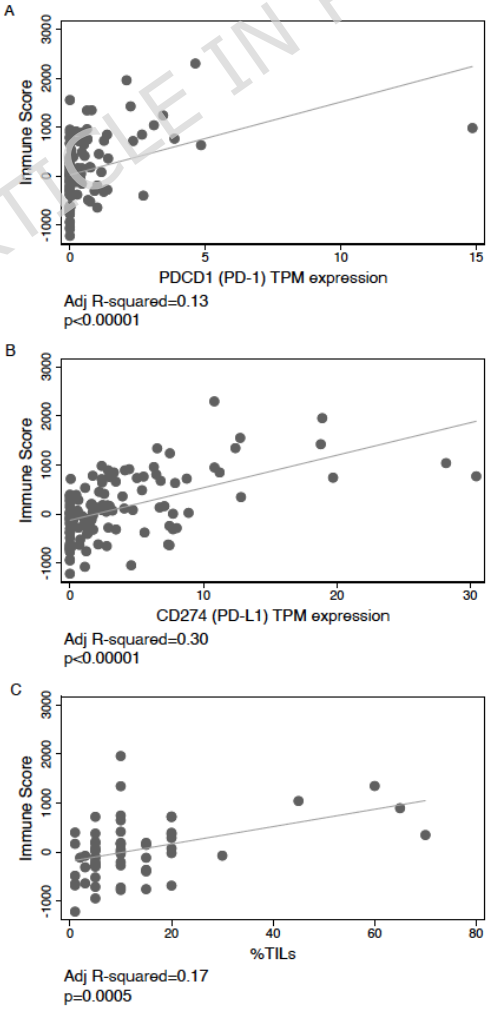


Figure 3. Scatter plots showing the significant linear correlation between ESTIMATE Immune Score and (A) PD-1 TPM gene-expression, (B) PD-L1 TPM gene-expression, and (C) percentage of TILs in IHC.



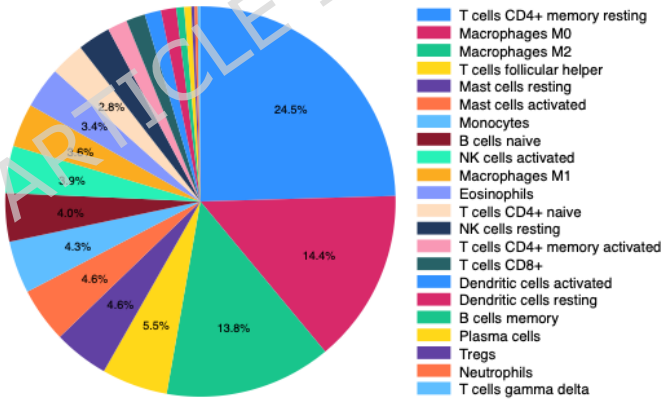
To estimate immune cell infiltration profiles based on transcriptomic data, CIBERSORTx and MCP-counter tools were used. Absolute immune cell quantification was consistent among cell types, including monocytes, macrophages, neutrophils, NK cells, T cells and B cells, estimated by both quantification tools (**Supplementary Figure 4**).

We first evaluated the reliability of transcriptomic-based quantification methods by comparing computational data with IHC. A positive correlation was observed between CIBERSORTx and IHC in evaluating overall TILs (Adjusted R2=0.38; p<0.0001), as well as CD3+ T cells (Adjusted R2=0.42; p<0.0001) (**Supplementary Figure 5A**). Similar results were obtained with MCP-counter tool (**Supplementary Figure 5B**).

CIBERSORTx relative abundance data showed that CD4+ memory resting T cells (24.5%), M2 macrophages (14.4%) and M0 macrophages (13.8%) represented the top three highest infiltrating fractions (**Figure 4**). Mast cells, both resting and activated, together represented about 9% of the entire immune infiltrate. Each of monocytes and naïve B cells represented about 4% of the immune infiltrate. Other cell types were more scarcely represented within the immune infiltrate (**Figure 4**).

Immune deconvolution analysis performed on the two MBC validation series from external datasets [27,28] confirmed, although with different percentages, that macrophages M0/M2 and CD4+ memory resting T cells are the top three highest infiltrating fractions, accounting for more than 50% of MBC immune infiltrate **(Supplementary Figure 6)**.

Figure 4. Relative fractions of 22 infiltrating immune cells estimated by CIBERSORTx in the whole MBC series analyzed.



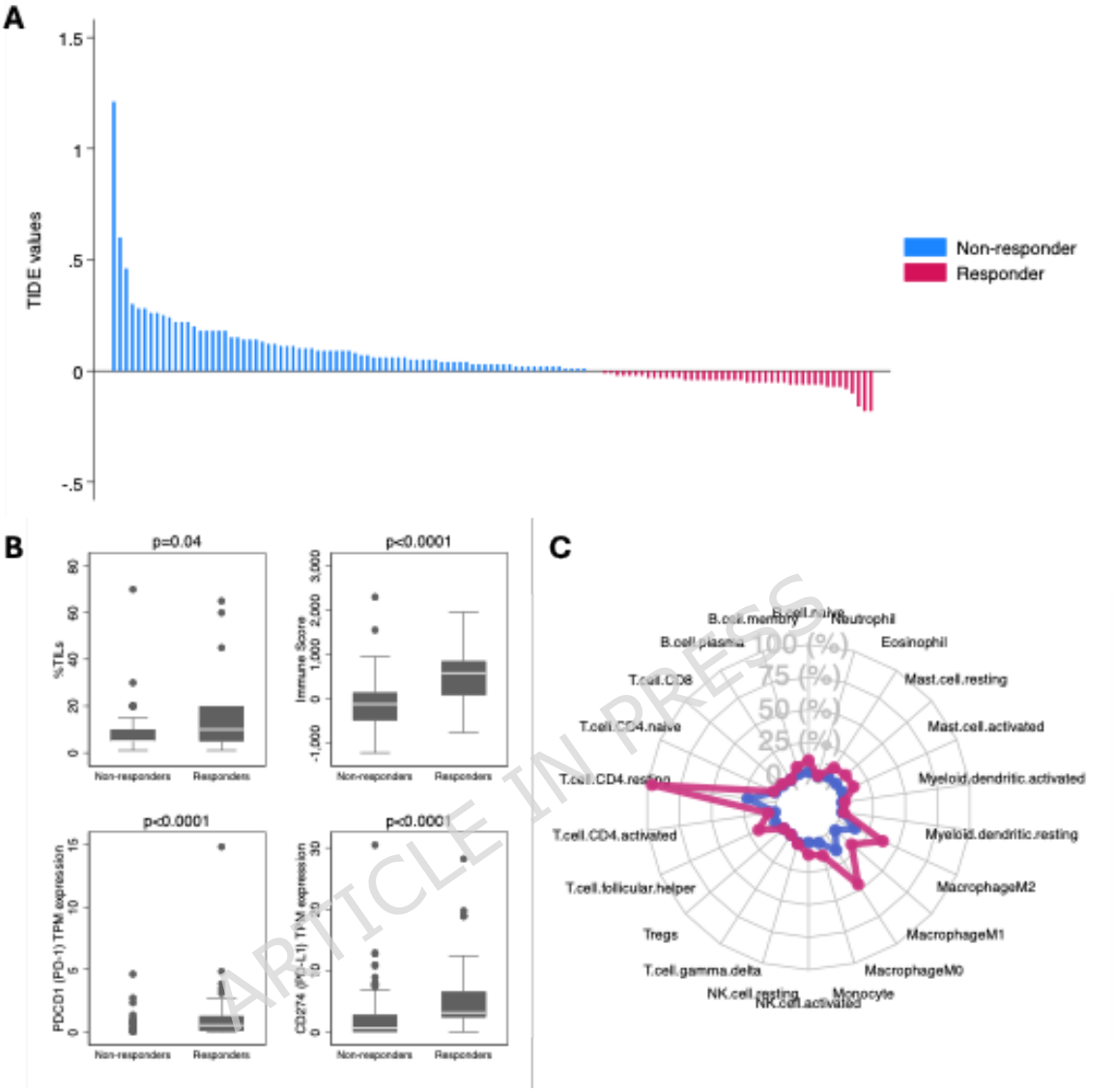
Prediction of response to immunotherapy based on gene signatures

To predict patient response to ICI-based immunotherapy, we applied the TIDE module. Overall, 77 out of 123 (62.6%) MBC cases were predicted as potential non-responders, while 46 out of 123 (37.4%) MBCs were predicted as potential responders (**Figure 5A**). TIDE module applied to the two MBC validation series from external datasets showed that 26 out of 46 (56.5%), MBCs from Severson et al.[27], and 3 out of 12 (25%) MBCs from TCGA [28] were predicted as potential responders **(Supplementary Figure 7)**.

Notably, responders showed significantly higher PD-1 (p<0.0001) and PD-L1 (p<0.0001) gene expression, higher ESTIMATE immune score values (p<0.0001) and a higher percentage of TILs (p=0.04) compared with non-responders (**Figure 5B**). Consistently, MBCs predicted as responders displayed higher abundance of all types of infiltrating immune cells compared with non-responders (**Figure 5C**). Notably, within

immune infiltrate, the fraction of M1 Macrophages and CD4+ memory resting T cells was higher in responders compared with non-responders (**Supplementary Figure 8A**).

Figure 5. (A) Classification of MBCs based on the prediction of immunotherapy response by TIDE tool; (B) Box plots showing significantly different levels of immune-related biomarkers based on responder status; (C) Abundances of infiltrating immune cell types based on responder status.

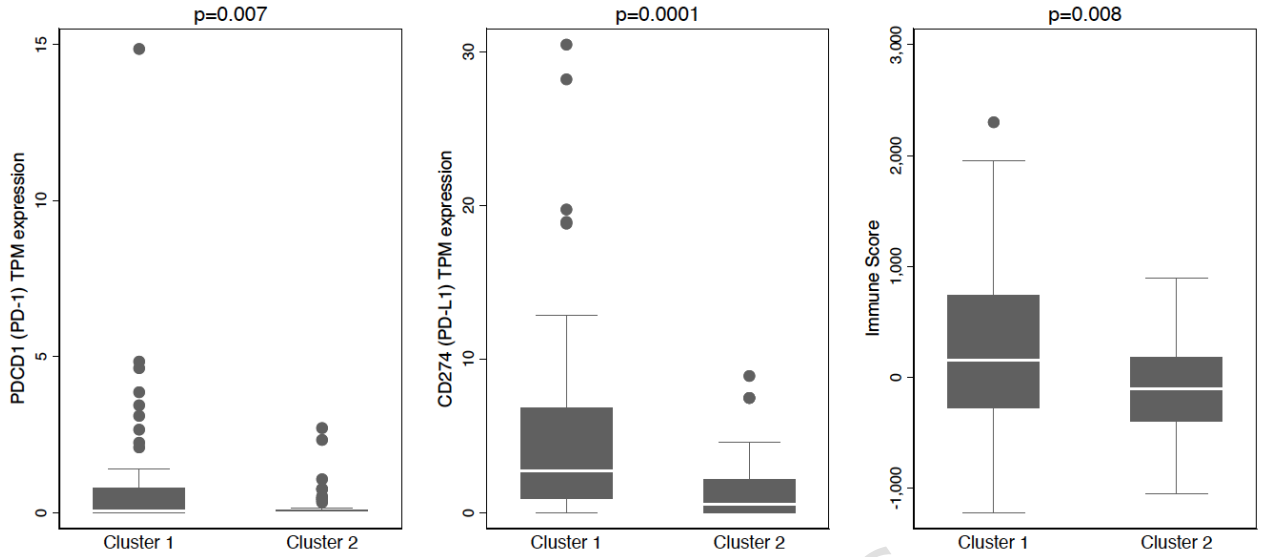


Associations of immune-related features with MBC molecular subgroups

To validate and further characterize the previously identified molecular subgroups of MBC based on transcriptomic data, an unsupervised clustering analysis was performed on the whole series of 123 MBC samples. This approach led to the identification of two distinct subgroups, designed as Cluster 1 (n=78) and Cluster 2 (n=45), consistent with findings from a previous, smaller dataset [19] (**Supplementary Figure 9A**). Differential gene expression analysis between the two clusters revealed that immune-related processes were the most relevant features distinguishing Cluster 1 and Cluster 2 at transcriptional level. In particular, 2338 differentially expressed genes between the two Clusters emerged, 1498 over-expressed in Cluster 1 (top hits include *IGKV3-20*, *FUT6*, and *IRX6* genes) and 840 over-expressed in Cluster 2 (top hits include NPY, PLEC, and *SNORD116-24* genes) (**Supplementary Figure 9B**). Enrichment analysis highlighted a significant up-regulation for genes mainly involved in immunity, including both innate and adaptive immune response, in Cluster 1 (**Supplementary Table 2**). Notably, Cluster 1 showed a higher PD-1 (p=0.007) and PD-L1 (p=0.0001)

gene expression, and a higher immune score (p=0.008), compared with Cluster 2 (**Figure 6**).

Figure 6. Box plots showing significantly different levels of immune-related biomarkers based on MBC Cluster classification.



Consistently, Cluster 1 exhibited a significantly higher absolute abundance of various infiltrating immune cell types, including T cells, NK cells, B cells, Monocytes, Macrophages, and Dendritic cells, compared with Cluster 2 (**Supplementary Figure 10**). Notably, among the immune infiltrates, Cluster 1 showed a greater proportion of M1 macrophages, and a lower proportion of activated mast cells was lower compared with Cluster 2 (**Supplementary Figure 8B**).
Furthermore, MBCs predicted to be potential responders to immunotherapy, as assessed by the TIDE module, were significantly enriched in Cluster 1 (82.6%) compared with Cluster 2 (17.4%) (p=0.001).

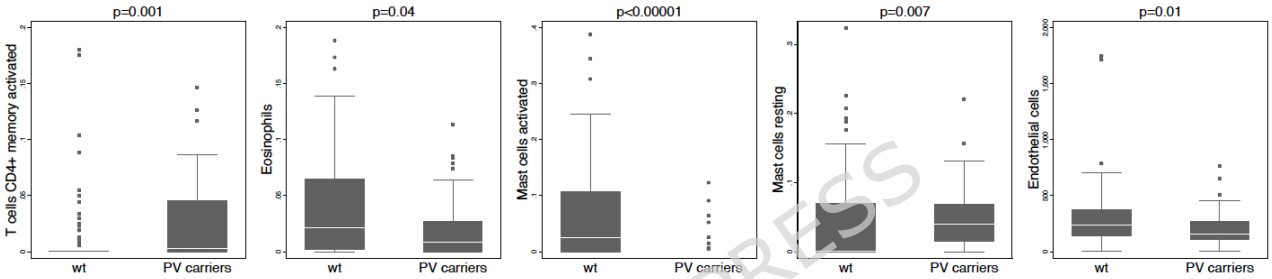
Associations of immune-related features with clinicopathological characteristics

To explore potential predictive and prognostic characteristics of immunogenicity in MBCs, we analyzed immune-related features in relation to germline PV status, pathological characteristics, and clinical outcomes.
Our series included 41 MBC cases with germline PVs, including 26 *BRCA2*, 12 *BRCA1* and 3 *PALB2*, and 82 cases without identified PVs. The main clinicopathological characteristics are detailed in **Supplementary Table 3**. The median age at diagnosis was 66 years (range 32-91). Most tumors were invasive ductal carcinomas (95.9%) of intermediate-high histologic grade (91.3%) with a high prevalence of hormone receptor positivity: ER (95.7%), PR (91.2%) and AR (92.8%). The majority were HER2 negative (87.6%) and exhibited high Ki67 expression (58.7%). Intrinsic molecular subtypes were assigned using transcriptome profiling based on the PAM50 gene signature. Luminal B was the most frequent subtype (47.1%), followed by Luminal A (30.1%), HER2-enriched (12.2%), Basal-like (6.5%) and Normal-like (4.1%). Overall survival analysis was performed on 109 MBCs with available follow-up data (mean follow-up: 8 years; range 0–20), during which 39 death events (35.8%) were recorded. No significant differences were observed in TILs percentages, PD-1 and PD-L1 expression, ESTIMATE Immune scores, TIDE-predicted immunotherapy responder

status or transcriptomic cluster classification between MBCs with and without germline PVs. Similar findings emerged when *BRCA1, BRCA2* and *PALB2* were analyzed individually.

However, differences were noted in the composition of the immune infiltrate. MBCs with germline PVs showed a significantly higher abundance of CD4+ memory activated T cells (p=0.001) and resting mast cells (p=0.007), and a lower abundance of eosinophils (p=0.04) and activated mast cells (p<0.00001), compared with MBCs without germline PVs (**Figure 7**). In addition, endothelial cell abundance was significantly lower in MBCs with germline (p=0.01) (**Figure 7**). When stratified by specific gene, variations in immune cell composition were observed among wild-type, *BRCA1*-, *BRCA2*- and *PALB2*-associated MBCs (**Supplementary Figure 8C**). Notably, the M2 Macrophages fraction was significantly lower in *BRCA1*-associated MBCs compared with the other subgroups (p=0.02).

Figure 7. Box plots showing differentially abundant infiltrating immune cell types between MBCs arising in wild-type (wt) patients and in PV carriers.



With regard to pathology features, no statistically significant differences were found in TILs percentages, PD-1 and PD-L1 expression, ESTIMATE Immune scores and TIDE-predicted responder status. However, MBCs with positive PD-L1 IHC staining were more likely to be ER-negative compared to PD-L1 negative cases (p=0.04). In terms of transcriptional clustering, Basal-like, HER2-enriched, and Luminal B subtypes were more frequently observed in Cluster 1, whereas Luminal A and Normal-like subtypes in Cluster 2 (p=0.02). Notably, all five ER-negative MBCs in the series were classified within Cluster 1 (p=0.09). Moreover, MBCs in Cluster 1 more frequently exhibited high Ki-67expression (p=0.02).

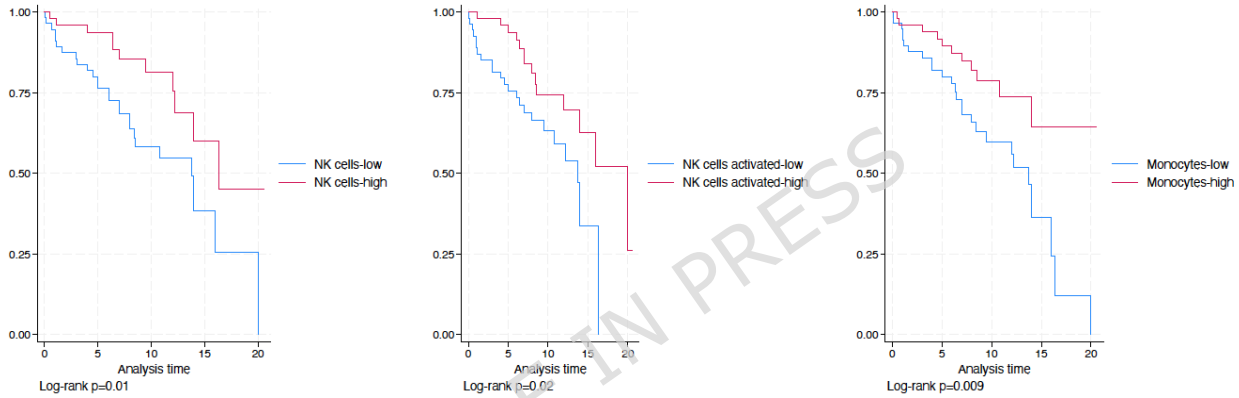
In MBCs with high histologic grade, the abundance of M1 macrophages was significantly higher compared to low histology grade MBCs (p=0.02). Similarly, high Ki-67 MBCs were characterized by higher abundance of M1 macrophages (p=0.01), endothelial cells (p=0.01), cancer associated fibroblast (CAFs) (p=0.02) and a higher cytotoxicity score (p=0.03), compared with Ki-67 low MBCs. In AR+ MBCs, there was a significantly higher abundance of eosinophils (p=0.01), endothelial cells (p=0.02), CAFs (p=0.04) compared with AR- MBCs. A higher abundance of eosinophils was also observed in HER2- MBCs compared with HER2+ MBCs (p=0.01) (**Supplementary Figure 11**).

Differences in immune cell composition were also observed across intrinsic molecular subtypes as defined by PAM50, with M0 and M1 macrophages, and activated and resting mast cells showing the most pronounced variation among molecular subtypes (**Supplementary Figure 8D, Supplementary Figure 12**).

No significant differences in OS were observed according to TILs percentages, PD-1 and PD-L1 expression, ESTIMATE Immune scores, TIDE predictions, or Cluster subtype classification. However, a higher abundance of NK cells, particularly activated NK cells, and monocytes was associated with a better survival (**Figure 8**).

In a multivariate Cox regression model including NK cells, activated NK cells and monocytes, as well as age at diagnosis, germline PV status and histologic grade as possible confounding factors, the abundance of NK cells and activated NK cells emerged as independent prognostic factors (**Supplementary Table 4**).
Cut-off for survival analysis were based on the median value of each cell abundance in the whole MBC series. Given that the abundance of NK cells is very low (median for NK cells: 3, range 0-174.5; median for activated NK cells: 0.038, range 0-0.18), a sensitivity analysis was performed with a different cut-off considering absence/presence of these cells (i.e., abundance values 0 *vs.* >0). Associations with survival according to this cut-off were confirmed (NK cells p=0.009; activated NK cells=0.047).

Figure 8. Log-Rank analysis of overall survival by NK cells, activated NK cells, and Monocytes levels. Cut-off based on the median values of each cell type abundance in the MBC series.



Discussion

Accumulating evidence comparing male and female BCs has revealed sex-related differences in immune-related characteristics. Notably, PD-1 expression on TILs is significantly less frequent in males, and MBCs display lower immunogenicity, with reduced T and B cell infiltration compared to FBCs.[17, 32, 33] Similar findings were also reported in murine models, where male mice showed lower levels of intratumoral leukocyte infiltration.[34]

Given these emerging sex-related immunological differences, we hypothesized that distinct molecular features, beyond those recognized in FBC, may define immunogenic subgroups in MBC. To our knowledge, this is the first study focusing on immune-related transcriptomic profiling in a well-characterized series of MBCs to enable sex-specific patient stratification for personalized immunotherapy.

Our findings support the hypothesis that while most MBCs are immunologically quiescent, a subset may harbor immunogenic features. Analysis of established immunotherapy biomarkers showed that a subset of MBCs was characterized by high TIL density and PD-L1 expression (via IHC), aligning with prior MBC studies.[18,32,35] Notably, transcriptomic-based immune scores strongly correlated with PD-1/PD-L1 gene expression and IHC assessments, indicating their reliability as indicators of an immunogenic microenvironment.

PD-L1 positivity thresholds by IHC have been validated in FBC but not in MBC; nevertheless, the same cutoffs are currently applied in clinical practice to both sexes, with CPS ≥10 being the most commonly used threshold for immune checkpoint inhibitor eligibility in BC, including for agents such as pembrolizumab.[22] Based on a previous study of PD-L1 expression in MBC [32], we additionally explored CPS ≥1 as a potentially more appropriate positivity threshold for male disease. Notably, in our cohort, PD-L1 IHC assessed using the CPS ≥1 cutoff showed a strong correlation with PD-L1 gene expression and transcriptome-based immune scores, providing biological support for the further investigation and prospective validation of sex-specific PD-L1 positivity thresholds in the breast cancer clinical setting.

Among immune populations, CD4+ memory resting T cells, M2 macrophages, and M0 macrophages were the most abundant, consistent with single-cell RNA-seq data from MBC.[17] Immune cell deconvolution correlated with IHC data for TILs and CD3+ T cells. Overall, these observations further strengthen the reliability of the *in silico* deconvolution tools used in this study.

Using the TIDE score, originally validated in melanoma and NSCLC, and subsequently applied to BC,[36-38] we predicted response to ICI. Approximately 37% of MBCs were classified as potential responders, characterized by elevated immune biomarkers and higher immune cell infiltration compared with non-responders. These findings suggest that MBC may exhibit features associated with immune checkpoint inhibitor responsiveness. However, in the absence of real-world immunotherapy response data for our cohort and given that the external validity of TIDE in MBC has not yet been established, the clinical relevance of these observations remains preliminary. Accordingly, our results should be regarded as hypothesis-generating and primarily support the need for future studies aimed at the prospective validation of immunotherapy response predictors, including TIDE, in MBC.

Although publicly available transcriptomic datasets for MBC are limited, we were able to externally replicate our findings on immune scores, immune deconvolution, and TIDE-based classification with a high degree of consistency in two independent MBC

cohorts, comprising a total of 58 additional cases [27,28]. Nevertheless, some discrepancies in immune cell infiltrate abundances and in the prevalence of TIDE-predicted responder status were observed across datasets, likely reflecting differences in the composition of the three MBC series analyzed. Notably, our cohort is enriched for *BRCA* PV carriers, whereas in the TCGA dataset only two of 12 MBCs harbored *BRCA* variants. In contrast, although Severson et al. [27] did not report *BRCA* status, their cohort was strongly enriched for the M1 molecular subtype as defined by Johansson et al. [10], which we and others have previously shown to resemble our Cluster 1 subtype and to be enriched for potentially immunogenic MBCs [19]. This may account for the higher proportion of predicted immune checkpoint inhibitor responders observed in that series.

To define male-specific immunogenic subgroups, we performed unsupervised clustering, which revealed two distinct transcriptomic clusters, consistent with our previous findings.[19] Immune response emerged as the most relevant process discriminating the two clusters at the transcriptional level. Cluster 1 was enriched for predicted ICI responders and showed significantly higher PD-1/PD-L1 expression, immune scores, and immune cell infiltration. This cluster mainly included basal-like, HER2-enriched, and Luminal B MBCs, encompassing all ER-negative tumors, and thus may represent the subgroup most likely to benefit from immunotherapy. Given that MBC is predominantly ER-positive,[39] transcriptomic profiling could expand treatment eligibility beyond traditional standard pathology-driven criteria.

Accumulating evidence on ICI response in FBCs has confirmed TNBC as the most responsive subtype. By contrast, the role of ICI in luminal BCs, which represent the majority of MBCs, remains unclear and has been considered of limited benefit.[14-16] Interestingly, early results from large phase III trials demonstrate that adding ICIs to standard neoadjuvant chemotherapy improves response rates in early-stage, high-risk luminal BC. The benefit was greater in tumors with PD-L1 positivity or high levels of immune cell infiltrates, reaching response rates comparable to TNBC, thereby suggesting that a subset of luminal FBCs may also be highly responsive to immune-based treatments.[40] Accordingly, the use of ICIs in combination with chemotherapy, as already approved for TNBC, could be extended to additional BC subtypes displaying immunogenic features. In line with this, our data on MBC support the possibility that luminal MBCs may harbor an immunogenic microenvironment and may thus potentially benefit from ICI therapy.

If further validated in future clinical trials, these findings may inform improved therapeutic strategies for luminal BC in both sexes.

BRCA1/2-associated cancers may develop immune-evasive microenvironments.[41] In FBC, *BRCA1/2*-deficient tumors have been linked to increased immune infiltration and higher expression of PD-1 and PD-L1.[42] In our study, no significant differences were found in TILs density, PD-1 and PD-L1 expression, immune scores or TIDE predictions between MBCs with or without PVs; however, the composition of the immune infiltrate differed. Specifically, CD4+ memory activated T cells and resting mast cells were enriched in PV carriers, while eosinophils, endothelial cells, and activated mast cells were more abundant in non-carriers, suggesting that germline mutation status could impact on MBC microenvironment, highlighting immune cell types that may be driven by different underlying molecular pathways.

CD4+ T cells, especially memory subtypes, are pivotal in anti-tumor immunity.[43,44] Their higher abundance in PV carriers suggests that DNA repair defects may drive neoantigen formation and subsequent immune activation. Conversely, the role of mast

cells remains controversial, as they can exhibit both pro- and anti-tumor effects.[45,46] An *in silico* analysis in FBC indicated a significantly higher infiltration of mast cells in luminal tumors, particularly Luminal A, compared with more aggressive HER2-positive and TNBC subtypes.[47] In MBC, mast cell abundance and activation state varied with germline PV status and molecular subtype, being more activated in Luminal A tumors and more resting in basal-like and Luminal B.

Eosinophils and endothelial cells were more prevalent in *BRCA* wild-type MBCs. Eosinophils may contribute to angiogenesis partially through the release of VEGF stored in their granules,[48] while endothelial cells play a key role in tumor vascular regulation.[49-51] Interestingly, *BRCA* genes have been implicated in the regulation of endothelial cell survival and inflammation, suggesting a potential mechanistic link between *BRCA* status and vascular features.[52,53] These findings underscore the need for further investigation into endothelial subpopulations and their relationship to *BRCA* mutational status in MBC.

A complex interplay among mast cells, eosinophils, and CD4+ T cells was observed in MBC. Specifically, the presence of eosinophils was associated with a reduced abundance of resting mast cells and CD4+ memory T cells, consistent with findings reported in female BC [54-56]. Eosinophils were less abundant in HER2+ MBCs consistent with the observation that MBC with *BRCA* PVs are enriched in HER2+ subtype.[57] A similar association between eosinophilic infiltration and HER2 status was recently reported in FBC.[58]

Distinct from female BC, where *BRCA1* is often associated with triple-negative tumors,[59] *BRCA1*-associated MBCs lack this phenotype. *BRCA2*-associated MBCs, however, appear more aggressive than their female counterparts.[8] We found that these tumors harbored higher levels of M2 macrophages, consistent with their pro-tumorigenic role and the aggressive behavior of *BRCA2*-driven MBC.[60] Meanwhile, M1 macrophages, typically displaying anti-tumor effects,[61] were enriched in high-grade, Ki-67-high, Luminal B tumors, suggesting an immunogenic profile reminiscent of TNBC in females.[62] These findings may indicate that sex-specific roles of *BRCA1* and *BRCA2* may influence immune phenotypes in *BRCA*-associated breast tumors, further underscoring the biological divergence between male and female BCs.

Within our cohort, Ki-67, a proliferation marker used to distinguish Luminal A from Luminal B subtypes, alongside AR status, emerged as a key determinant of the immune microenvironment. Specifically, AR-positive, highly proliferative MBCs exhibited higher levels of endothelial cells and cancer-associated fibroblasts (CAFs) compared to AR-negative, Ki-67-low tumors. Both cell types can express Ki-67, contributing to a proliferative signaling environment that may influence angiogenesis and tumor invasion. [63,64] Furthermore, mesenchymal/stromal AR plays a well-established role in prostate cancer development, a disease sharing notable molecular parallels with MBC.[65] In particular, AR and androgen-dependent signaling are active in CAFs and exert significant immunosuppressive effects on prostate cancer cells.[66] In this context, AR activity is enhanced by IL-6/STAT3 signaling, contributing to immunosuppressive tumor progression.[67] In general, androgen signaling shapes male-specific immune landscapes, often dampening antitumor inflammatory responses and influencing cancer therapy outcomes.[68-69] This evidence, together with our results, may add to the hypothesis that sex-related differences observed in BC may be driven by androgen signaling.

In terms of prognostic immune biomarkers, our multivariate Cox analysis revealed that high abundance of NK cells was an independent favorable prognostic factor. NK cells,

essential for tumor immunosurveillance, have been linked to improved outcomes in various cancers, including BC, particularly TNBC. [70–72] Our study is the first to report this positive prognostic role in MBC, highlighting their potential clinical relevance.

Limitations of this study include its retrospective design and the absence of treatment data, which restricts the ability to draw definitive conclusions regarding therapeutic response. Additionally, immune cell infiltration was inferred through *in silico* deconvolution of bulk transcriptomic data, rather than through single-cell analysis. However, our findings showed strong concordance between transcriptomic data and IHC, as well as consistency with previous single-cell RNA-seq results,[17] supporting the reliability of transcriptome-based approaches for immune profiling in MBC. Given the rarity of MBC and the limited availability of large cohorts of fresh-frozen tissue samples, transcriptomic profiling of FFPE samples represents a feasible and informative alternative for dissecting biologically and clinically relevant immune features. Nonetheless, future studies should aim to evaluate cell-specific transcriptomic profiles and the spatial localization of immune cells within the MBC tumor microenvironment, in order to validate and expand upon these observational findings.

In conclusion, our data provide the first comprehensive evidence that a subset of MBCs may benefit from ICI-based immunotherapy, featuring immunogenic properties distinct from those in FBC. Immune profiling identified CD4+ memory T cells, M1/M2 macrophages, mast cells, and NK cells as potential predictive or prognostic biomarkers in MBC. These findings contribute to a deeper understanding of sex-specific immune features in BC, laying the groundwork for precision medicine approaches specifically tailored to male patients, rather than extrapolated from the female counterpart.

Abbreviations

AR	Androgen receptor
BC	Breast cancer
BRCA1	BReast CAncer gene 1
BRCA2	BReast CAncer gene 2
CPS	Combined positive score
ER	Estrogen receptor
ESTIMATE	Estimation of STromal and Immune cells in MAlignant Tumor tissues using Expression data
FBC	Female Breast Cancer
FFPE	Formalin-fixed paraffin-embedded
HER2	Human epidermal growth factor receptor 2
ICI	Immune checkpoint inhibitors
IHC	Immunohistochemistry
Ki-67	Antigen Ki-67
MBC	Male breast cancer
NSCLC	Non-small cell lung cancer
OS	Overall survival
PALB2	Partner and localizer of BRCA2
PAM50	Prediction analysis of microarray 50
PD-1	Programmed cell death 1
PD-L1	Programmed death-ligand 1
PR	Progesterone receptor
PV	Pathogenic variant
TIDE	Tumor Immune Dysfunction and Exclusion
TILs	Tumor infiltrating lymphocytes
TNBC	Triple negative breast cancer
TPM	Transcripts per million

Declarations

Ethics approval and consent to participate
For each participant, informed consent to participate in the study was obtained. The study was approved by the Local Ethical Committee (Sapienza University of Rome, Prot. 669/17).

Consent for publication
Not applicable

Availability of data and materials
The data generated and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests
The authors declare no competing interests.

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Authors' contributions
LO and VS conceptualized and designed the study. IZ, SB, MGT, IC, DC, VA, VZ, EG, LC, GG, CC, SBF, GM, SM, GdA acquired patient samples and data. VV, AB and VP performed molecular analyses. GS, BC, SB, GdA performed immunohistochemistry analyses. VS performed data analysis. VS and LO performed data interpretation drafted the manuscript. All authors critically revised the manuscript and approved the final version for submission.

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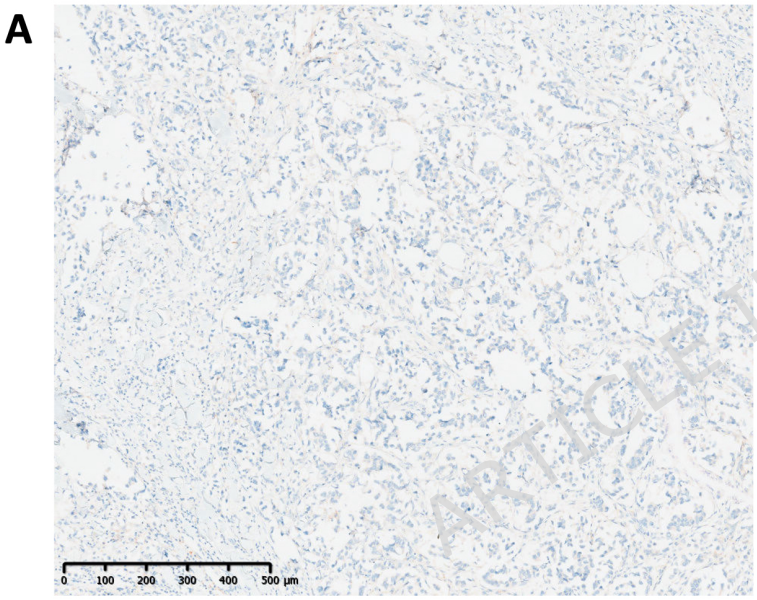
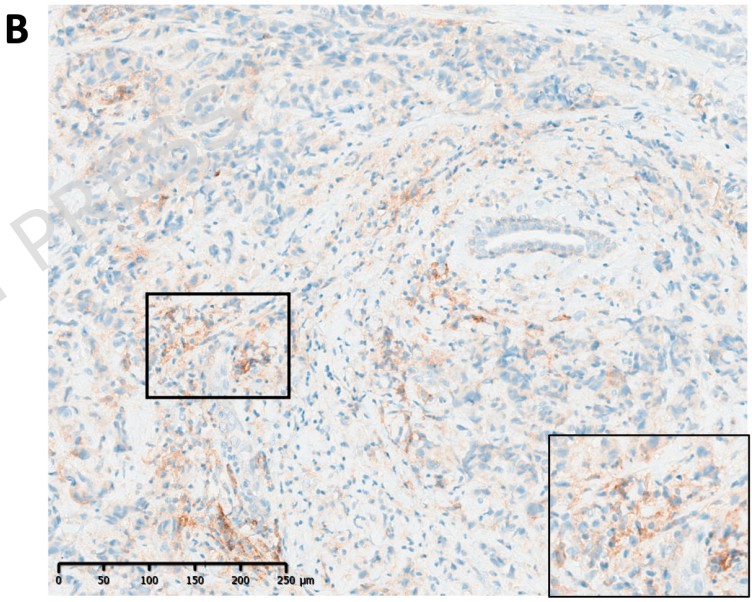
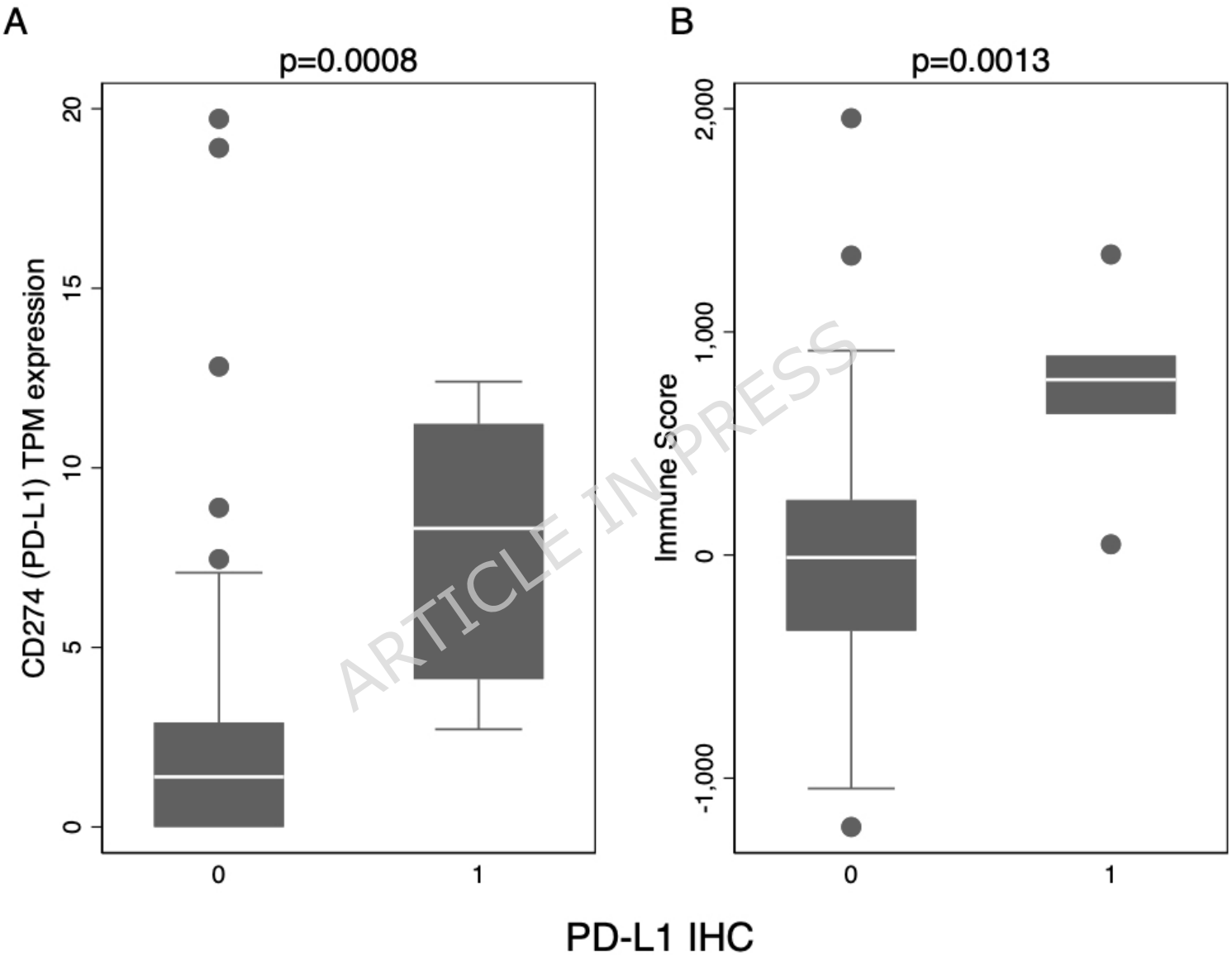
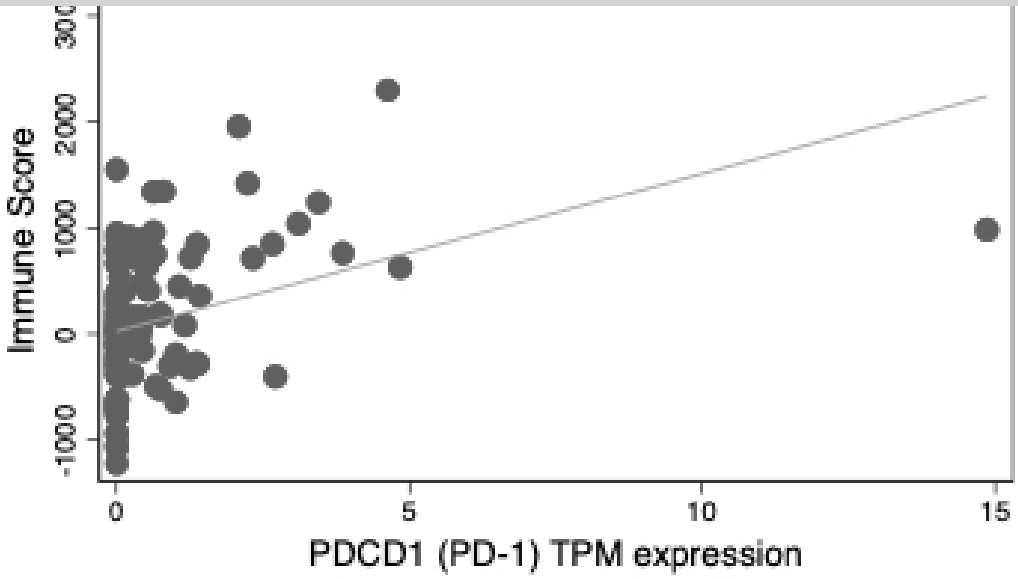


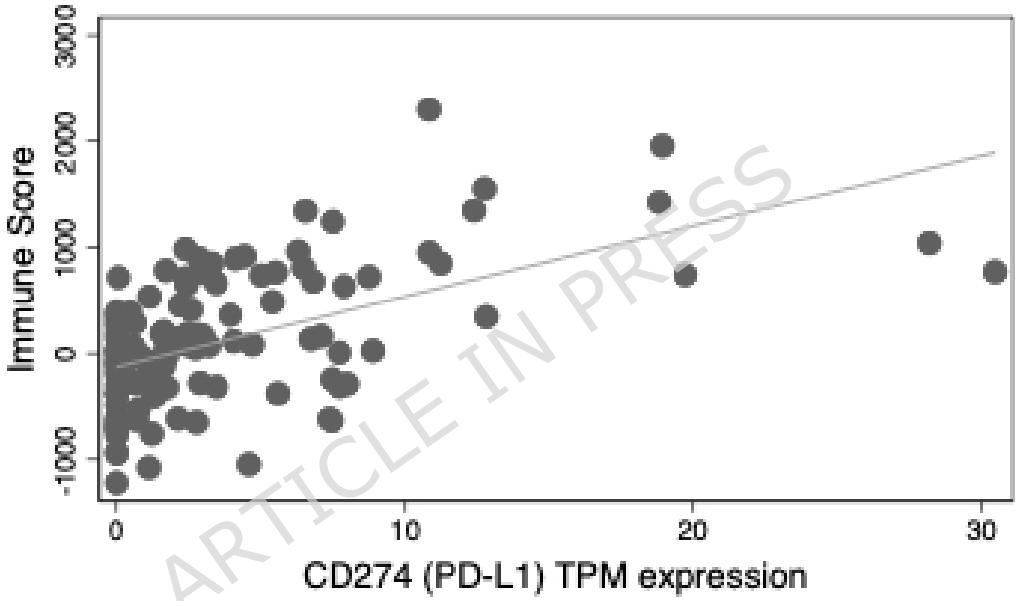






Adj R-squared=0.13
p<0.00001

B



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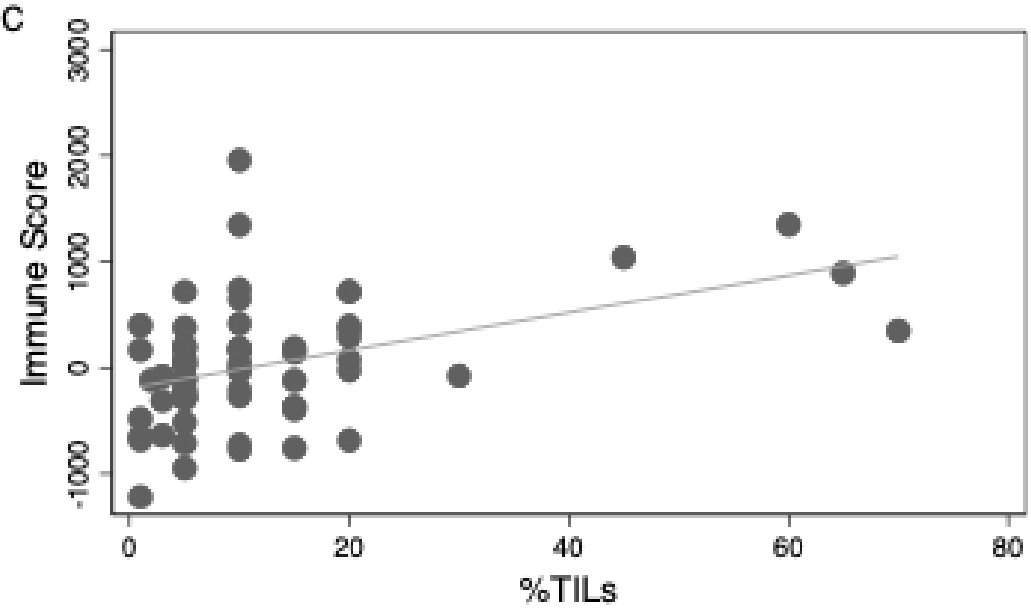
p<0.00001

C



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p=0.0005