

Comprehensive Assessment of Immune Phenotype and Its Effects on Survival Outcomes in HER2-Low versus HER2-Zero Breast Cancer

Heidi Chwan Ko^{1,*}, RJ Seager^{2,*}, Sarabjot Pabla², Maria-Fernanda Senosain², Erik Van Roey², Shuang Gao², Kyle C Strickland^{1,3}, Rebecca Ann Previs^{1,4}, Michelle F Green¹, Maureen Cooper¹, Mary K Nesline¹, Stephanie B Hastings¹, Kobina Agyaful Amoah¹, Shengle Zhang², Jeffrey M Conroy², Taylor J Jensen¹, Marcia Eisenberg⁵, Brian Caveney⁵, Eric A Severson¹, Shakti Ramkissoon^{1,6}, Shipra Gandhi⁷

¹Labcorp Oncology, Durham, NC, USA; ²Labcorp Oncology, Buffalo, NY, USA; ³Department of Pathology, Duke University Medical Center, Duke Cancer Institute, Durham, NC, USA; ⁴Department of Obstetrics & Gynecology, Division of Gynecologic Oncology, Duke University Medical Center, Duke Cancer Institute, Durham, NC, USA; ⁵Labcorp, Burlington, NC, USA; ⁶Department of Pathology, Wake Forest Comprehensive Cancer Center, Wake Forest School of Medicine, Winston-Salem, NC, USA; ⁷Department of Hematology and Oncology, Roswell Park Comprehensive Cancer Center, Buffalo, NY, USA

*These authors contributed equally to this work

Correspondence: Heidi Chwan Ko, Email Heidi.ko@labcorp.com

Background: The understanding of molecular characteristics of HER2-low breast cancer is evolving since the establishment of trastuzumab deruxtecan. Here, we explore the differences in expression patterns of immune-related genes in the tumor immune microenvironment (TME) and survival between HER2-low and HER2-zero breast cancers.

Methods: Comprehensive genomic and immune profiling, including RNA-seq gene expression assessment of 395 immune genes, was performed on FFPE samples from 129 patients with advanced HER2-negative (immunohistochemistry (IHC) 0, 1+ or 2+ with negative *ERBB2* amplification by in-situ hybridization) breast cancer. Both estrogen receptor (ER) and HER2 statuses were obtained from available pathology reports. mRNA expressions of immune biomarkers, except for PD-L1 IHC and TMB, were derived from RNA-seq. Statistical comparisons were performed using the Kruskal–Wallis or Wilcoxon Rank-Sum test or the two-sample test for equality of proportions with continuity correction ($p \leq 0.05$ for significance). Survival differences were calculated using Kaplan–Meier analysis ($p \leq 0.05$ for significance).

Results: There were no significant differences in mRNA expressions of immune-related genes between HER2-low and HER2-zero breast cancers. However, HER2-low breast cancers were associated with a higher proportion of ER-positivity. When ER was analyzed along with HER2, we observed a significantly higher tumor immunogenic signature (TIGS) expression in HER2-zero/ER-negative tumors than in HER2-low/ER-positive tumors ($p = 0.0088$). Similarly, lower expression of PD-L1 and T cell immunoglobulin and ITIM domain (TIGIT) mRNA was observed in HER2-low/ER-positive tumors when compared to HER2-zero/ER-negative tumors ($p = 0.014$ and 0.012 , respectively). Patients with HER2-low tumors had a longer median OS than those with HER2-zero tumors (94 months vs 42 months, $p = 0.0044$).

Conclusion: Patients with HER2-low breast cancer have longer survivals yet display no differences in immune-related gene expression when compared to those with HER2-zero cancers. The differences in survival can be attributed to the higher rate of ER-positivity seen in HER2-low breast cancers, compared to HER2-zero tumors.

Keywords: HER2-low breast cancer, estrogen receptor, tumor immune microenvironment, immune checkpoint biomarkers, gene expression profiling, immune profiling

Introduction

Breast cancer is a biologically heterogeneous disease, harboring distinct molecular features that result in abnormal cellular proliferation and invasiveness of cancer cells. Accurate prognostication and therapeutic decisions rely on understanding

the intrinsic biological factors that differentiate breast cancer into four groups with unique gene expression profiles: luminal A, luminal B, HER2-enriched, and basal-like breast cancer.¹ For practical considerations, these four biological entities are abridged into three main clinical subtypes of breast cancer based on biomarkers reflecting cell surface receptor statuses: luminal-like (ER-positive and/or PR-positive and HER2-negative), HER2-positive (positive HER2 expression with any ER and PR expression), and triple-negative (ER-negative, PR-negative and HER2-negative).²

The luminal-like subtype, having ER and/or PR expression, accounts for about 75% of breast cancer diagnoses.^{1,3} Approximately 15% of patients with breast cancer overexpress the transmembrane glycoprotein HER2/*ERBB2* and are therefore defined as having HER2-positive breast cancer.² HER2-positivity is defined as having a HER2 immunohistochemistry (IHC) staining score of 3+ or 2+ when the gene amplification is confirmed in the latter by in-situ hybridization (ISH) of *ERBB2*/CEP17 ratio ≥ 2.0 and an average *ERBB2* gene copy number ≥ 4.0 .⁴ Historically, HER2-positivity (defined as IHC 3+ or 2+ with amplification by ISH) was considered as the only predictive biomarker for any anti-HER2 therapy.^{5,6} However, this treatment paradigm has shifted because tumors with low levels of HER2 expression (defined as those with IHC 1+ or 2+ with no amplification of the *ERBB2* gene) have shown improved responses to a HER2 targeting antibody drug conjugate (ADC) called trastuzumab deruxtecan (T-DXd).^{7,8} In the Phase III DESTINY-Breast04 trial, treatment with T-DXd in patients with HER2-low metastatic breast cancer resulted in improved outcomes compared to chemotherapy. The risk of disease progression was about 50% lower, and the risk of death was 36% lower with T-DXd relative to chemotherapy, regardless of hormonal receptor status.⁷ These results led to the U.S Food and Drug Administration (FDA) approval of T-DXd for the treatment of advanced HER2-low breast cancer.

Although HER2-low cases represent a large proportion (55%) of all breast cancers,⁴ the intrinsic biological characteristics of HER2-low breast cancer is not yet well-understood. In a study of 5,235 patients with HER2-negative breast cancer, clinical outcome differences between HER2-low and HER2-zero (IHC 0) breast cancers were mainly attributed to hormone receptor (HR) expression, and HER2 expression level did not independently affect prognosis when considering HR status.⁹ Tarantino et al also demonstrated that there were no significant differences in the genomic landscape between HER2-low and HER2-zero tumors, apart from the rate of *ERBB2* copy number alterations.¹⁰ Recently, a large-scale retrospective cohort study conducted using the National Cancer Database of over 1,100,000 patients diagnosed with breast cancer in the US found minimal prognostic differences between HER2-low and HER2-zero breast cancers.¹¹ Therefore, the study concluded that clinical outcomes in HER2-low breast cancer were attributed to HER2-low being a predictive biomarker for a HER2-targeted ADC rather than the intrinsic biological characteristics of HER2-low expression.

On the contrary, a pooled analysis of four prospective neoadjuvant clinical trials showed that patients with HER2-low tumors had a significantly longer survival but lower pathologic complete response (pCR) rates than HER2-zero tumors, suggesting that HER2-low breast cancer should be considered a different subtype from HER2-zero tumors, regardless of hormone receptor (HR) expression.¹² Similarly, our previous study also showed improved clinical outcomes of HER2-low de novo metastatic breast cancer compared to HER2-zero metastatic breast cancer, irrespective of HR expression.¹³ We further showed that this survival advantage was primarily observed among tumors treated with chemotherapy in the first line (HR 0.92 vs 0.99, p-interaction=0.04).¹³ Retrospective analysis of PAM50 data from 3,689 patients revealed that HER2-low tumors were significantly associated with HR status, and luminal genes were upregulated in HER2-low tumors.⁴ HER2-low was also more frequently observed in older, male patients and associated with more regional metastases compared to HER2-zero tumors.⁴ Current data, therefore, are conflicting and not well-understood to support HER2-low breast cancer as a separate clinical entity with unique biological and clinicopathological characteristics. A deeper understanding of the differences in the clinical and biological characteristics of HER2-low breast cancer will provide further insights into its prognosis and potentially discern unique targets for investigating novel therapeutic strategies beyond anti-HER2 ADCs.

Since anti-tumor immunity has been shown to play a key role in determining prognosis, it is crucial to understand the interplay between tumor and immune cells in the tumor immune microenvironment (TME). Triple-negative breast cancer (TNBC) is known to have a more active TME than HER2-positive and HR-positive breast cancers, harboring a higher proportion of tumor infiltrating lymphocytes (TILs) and higher median TMB when compared to other breast cancer subtypes.^{14,15} To our knowledge, there have been no studies that have reported on immune-related gene expression

signatures underlying the different prognosis between HER2-low and HER2-zero breast cancers. Herein, we aim to investigate the differences in the immune-related gene expression pattern of the TME as well as survival outcomes of HER2-low compared to HER2-zero cancers.

Methods

Patient Cohort

Ethics approval for this study was obtained from Roswell Park Comprehensive Cancer Center Institutional Review Board (Study# BDR 162622) and determined to be non-human subject research by the IRB due to conducting research using only unidentifiable or de-identified data. The study was conducted in accordance with the ethical standards of the Declaration of Helsinki. We analyzed comprehensive genomic and immune profiling (CGIP) data from a retrospective cohort of breast cancer FFPE tissue specimens that were submitted for CGIP testing as part of standard of care from 2017 to 2022.

HER2 and ER Status Determination

Both estrogen receptor (ER) and HER2 statuses were obtained from the available pathology reports. HER2-low was defined as immunohistochemistry (IHC) 1+ or IHC2+/in-situ hybridization negative, and HER2-zero was defined as IHC0, based on the American Society of Clinical Oncology/College of American Pathologists guidelines. ER positivity was defined as Allred score ≥ 3 or ER expression of $\geq 1\%$ on IHC.

Comprehensive Genomic and Immune Profiling

DNA and RNA were co-extracted from FFPE tumor tissue specimens and submitted for NGS-based CGIP (OmniSeq[®] INSIGHT, OmniSeq[®] Advance, and OmniSeq[®] Immune Report Card CGIP Tests, OmniSeq, Buffalo, NY, USA), performed in a laboratory accredited by the College of American Pathologists (CAP) and certified by the Clinical Laboratory Improvement Amendments (CLIA).¹⁶ DNA sequencing was performed using the TruSight[®] Oncology 500 (TSO500) assay (Illumina, San Diego, CA, USA), testing for small variants in 523 genes (single and multi-nucleotide substitutions, insertions, and deletions), copy number alterations in 59 genes (gains and losses), and TMB genomic signatures. RNA sequencing, performed using a targeted RNA sequencing assay (OncoPrint[™] Immune Response Research Assay, ThermoFisher, Waltham, MA, USA), assessed the expression of 395 immune-related genes, including lymphocyte activation gene-3 (LAG-3), T cell immunoglobulin and ITIM domain (TIGIT), PD-L1, and T cell immunoglobulin and mucin-domain containing-3 (TIM-3).

Immune Gene Expression Analyses

Absolute read counts for each gene transcript were generated using the Ion Torrent Suite Software plugin immuneResponseRNA (ThermoFisher, Waltham, MA), then converted to percentile gene expression ranks as previously described.¹⁷ From these gene expression rank values, three immune gene expression signatures previously shown to be associated with immune checkpoint inhibition therapy outcomes were calculated: tumor immunogenic score (TIGS),¹⁷ cellular proliferation (CP) score¹⁷ and cancer testis antigen burden (CTAB) score.¹⁸ TIGS was calculated by averaging the gene expression ranks of 161 immune-associated genes to assess the degree of intratumoral immune activity.¹⁷ CP was calculated by averaging the gene expression ranks of 10 genes associated with cell proliferation to gauge proliferative activity, both originating with tumor cells and immune cells.¹⁷ Finally, CTAB sums the expression of 17 CTA genes in order to assess both the overall expression and co-expression of CTAs, a class of genes known to be associated with the anti-tumor immune response.¹⁸

Immunohistochemical Studies

In addition to the assessment of PD-L1 by mRNA gene expression signatures as a part of CGIP, tumor cell surface expression of PD-L1 was measured by Dako[®] PD-L1 IHC 22C3 pharmDx (Agilent, Santa Clara, CA). Expression was scored by a board-certified anatomical pathologist according to published guidelines as combined proportion score

(CPS), which is the percentage of tumor cells and immune cells (including lymphocytes and macrophages) with positive linear membranous PD-L1 staining, divided by the number of all viable tumor cells.

Statistical Analysis

Statistical comparisons of variables between groups were calculated using the Kruskal–Wallis or Wilcoxon Rank-Sum test for continuous variables and the two-sample test for equality of proportions with continuity correction for categorical variables ($p \leq 0.05$ for significance). Overall survival was defined as the time in months from the date of diagnosis to the date of the last follow up appointment. Kaplan-Meier (KM) analyses were used for survival analysis. In all cases, p -values ≤ 0.05 were considered significant.

Results

Cohort Clinical and Tumor Characteristics

A total of 129 patients with canonically defined HER2-negative breast cancer (IHC 0, 1+ or 2+ with negative *ERBB2* amplification by ISH) were included in the study. Further classification revealed 32 patients with HER2-zero and 97 with HER2-low tumors, defined as either IHC 1+ or IHC 2+ without *ERBB2* amplification on FISH (Figure 1). The median age of diagnosis was 49.5 years (range 47.5–52.9 years); most patients in the cohort (79.1%) were under 50 years of age. In addition, HER2-low patients were slightly younger at diagnosis than HER2-zero patients (49.3 vs 49.5 years of age; $p=0.025$), with a higher proportion of HER2-zero patients older than 50 years of age (83.5% vs 65.6%; $p=0.044$). Most patients were White (108 patients, 83.7%), and of non-Hispanic descent (125 patients, 96.9%). A total of 78 cases were mammary adenocarcinoma, not otherwise specified (60.5%), 44 cases were invasive ductal carcinoma, not otherwise specified (34.1%), 3 cases were invasive lobular carcinoma (2.3%), 3 cases were metaplastic carcinoma (2.3%), and 1 had unknown histology (0.8%). A total of 74 patients (57.4%) had tumors positive for estrogen receptor (ER) expression (defined as Allred score ≥ 3 or ER expression of $\geq 1\%$ on IHC) (Figure 1 and Table 1). HER2-low tumors had a significantly higher proportion of ER-positivity than did HER2-zero tumors (63.9% vs 37.5%; $p=0.021$) (Table 1). Most patients had PD-L1 negative tumors (73.6%), defined by a combined positive score (CPS) <10 . Most tumors analyzed were from metastatic sites (75.2%).

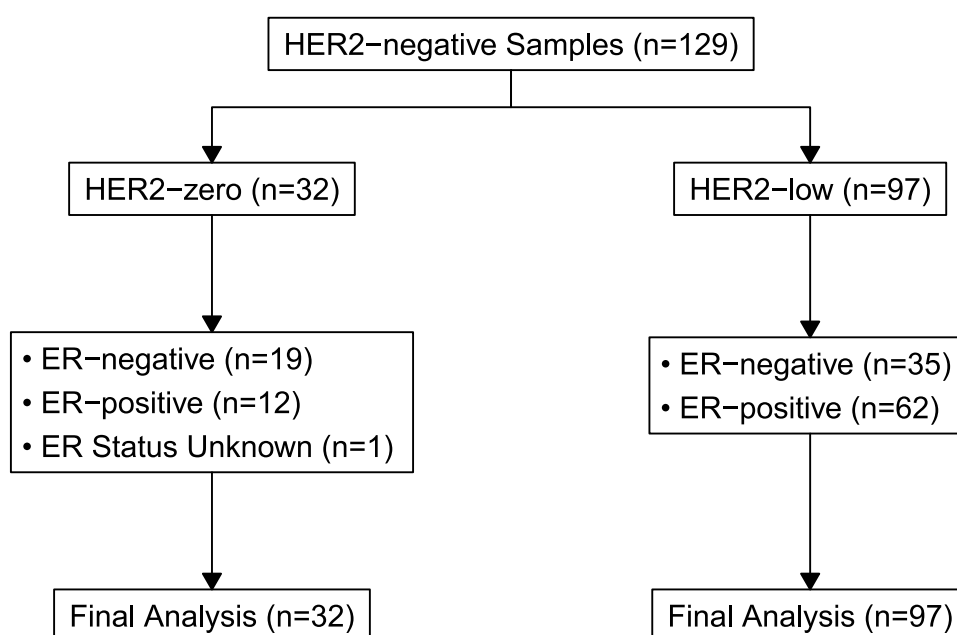


Figure 1 A consort diagram of cohort selection.

Table I Summary of Breast Cancer Patient and Tumor Characteristics by HER2 Expression Status

Variable	Group	All Patients (n=129)	HER2-zero (32, 24.8%)	HER2-low (97, 75.1%)	p (HER2-low vs HER2-zero)
Age	Median age in years (range)	49.5 (47.5–52.9)	49.5 (47.6–52.9)	49.3 (47.5–52.8)	0.025 (Wilcoxon Rank-Sum)
	Age < 50, n (%)	102 (79.1%)	21 (65.6%)	81 (83.5%)	0.044 (Fisher's Exact Test)
	Age ≥ 50, n (%)	27 (20.9%)	11 (34.4%)	16 (16.5%)	
Race	White	108 (83.7%)	25 (78.1%)	83 (85.6%)	0.16 (Fisher's Exact Test)
	African American	18 (14.0%)	5 (15.6%)	13 (13.4%)	
	Other	3 (2.3%)	2 (6.3%)	1 (1.0%)	
Ethnicity	Hispanic	3 (2.3%)	2 (6.3%)	1 (1.0%)	0.26 (Fisher's Exact Test)
	Non-Hispanic	125 (96.9%)	30 (93.8%)	95 (97.9%)	
	No Data	1 (0.8%)	0 (0.0%)	1 (1.0%)	
Tumor Histology	Mammary adenocarcinoma, NOS	78 (60.5%)	20 (62.5%)	58 (59.8%)	0.48 (Fisher's Exact Test)
	Invasive ductal carcinoma, NOS	44 (34.1%)	10 (31.2%)	34 (35.1%)	
	Invasive lobular carcinoma	3 (2.3%)	0 (0.0%)	3 (3.1%)	
	Metaplastic carcinoma	3 (2.3%)	1 (3.1%)	2 (2.1%)	
	Other	1 (0.8%)	1 (3.1%)	0 (0.0%)	
Estrogen receptor (ER) status	ER-Negative	54 (41.9%)	19 (59.4%)	35 (36.1%)	0.021 (Fisher's Exact Test)
	ER-Positive	74 (57.4%)	12 (37.5%)	62 (63.9%)	
	Missing Data	1 (0.8%)	1 (3.1%)	0 (0.0%)	
PD-L1 Score	CPS <10	95 (73.6%)	25 (78.1%)	70 (72.2%)	0.31 (Fisher's Exact Test)
	CPS ≥ 10	12 (9.3%)	5 (15.6%)	7 (7.2%)	
	No Data	22 (17.1%)	2 (6.3%)	20 (20.6%)	
Site of Specimen	Primary	30 (23.3%)	7 (21.9%)	23 (23.7%)	1 (Fisher's Exact Test)
	Metastatic	97 (75.2%)	25 (78.1%)	72 (74.2%)	
	Recurrent	2 (1.6%)	0 (0.0%)	2 (2.1%)	

Biomarkers of Tumor Inflammation Between HER2-Low and HER2-Zero

Three gene expression-derived biomarkers of tumor inflammation - tumor immunogenic signature (TIGS), cell proliferation (CP), and cancer testis antigen burden (CTAB) along with mutational burden (TMB) were compared between the HER2-low and HER2-zero breast cancers. There were no statistical differences in the expression of these biomarkers between HER2-low and HER2-zero tumors although there was a trend towards higher TIGS in HER2-zero when compared to HER2-low tumors (Figure 2A-D).

Biomarkers of Tumor Inflammation Between HER2-Low and HER2-Zero by ER Expression

Three gene expression-derived biomarkers of tumor inflammation such as TIGS, CP, and CTAB along with TMB were compared between the HER2-low and HER2-zero breast cancers, stratified by positive or negative ER expression. There were no differences in expression of TIGS, CP, CTAB, and TMB between HER2-low and HER2-zero among ER-positive or ER-negative cases (Figure 3A-D). However, a statistically higher TIGS expression was seen in HER2-zero/ER-negative tumors compared to HER2-low/ER-positive tumors (Figure 3A).

Comparison of Immune Checkpoint Gene mRNA Expression Between HER2-Low and HER2-Zero Breast Cancer

mRNA expressions of four immune checkpoint genes, PD-L1, TIM-3, LAG-3, and TIGIT, were compared between HER2-low and HER2-zero breast cancers. There were no statistical differences in mRNA expression levels of PD-L1,

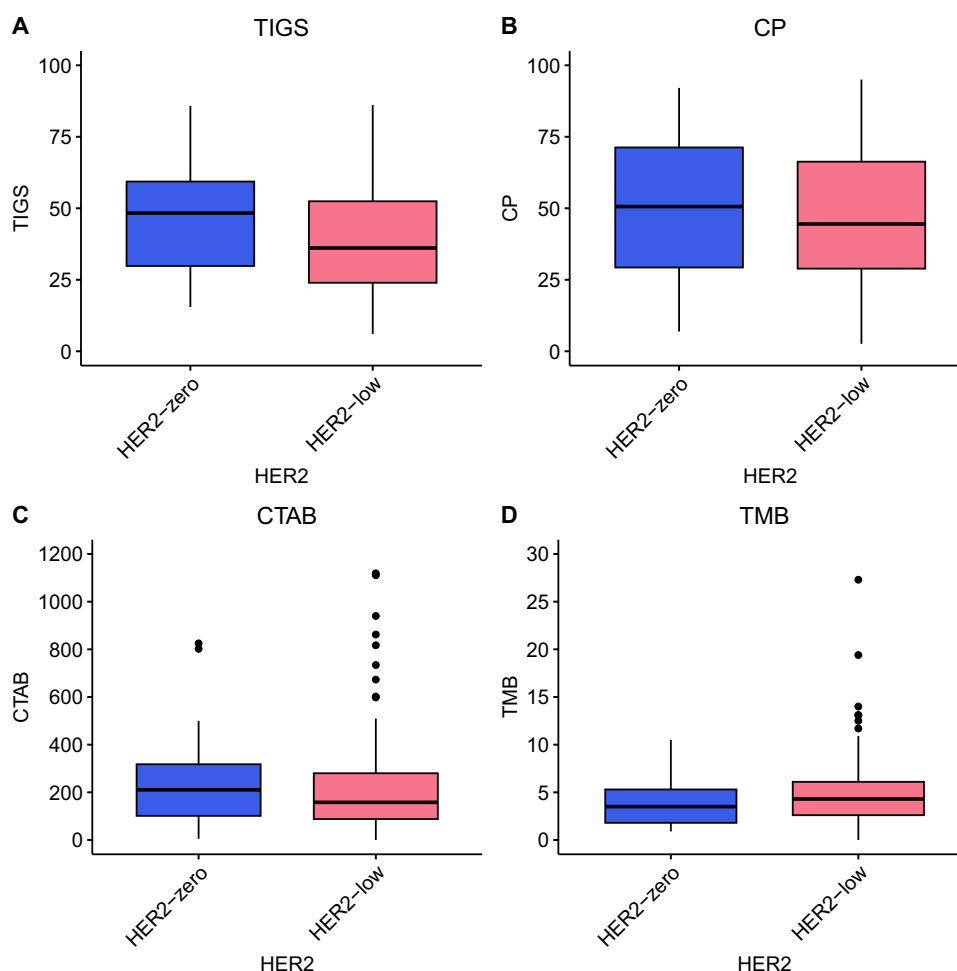


Figure 2 Distributions of TIGS (A), CP (B), CTAB (C), and TMB (D) within each HER2 group for all patients in the cohort.

TIM3, LAG-3, and TIGIT between HER2-low and HER2-zero tumors although there was a trend towards greater PD-L1 and TIGIT mRNA expressions in HER2-zero when compared to HER2-low tumors (Figure 4A-D).

Comparison of Immune Checkpoint Targets Between HER2-Low and HER2-Zero Breast Cancer by ER Expression

mRNA expressions of four immune checkpoint genes, PD-L1, TIM-3, LAG-3, and TIGIT were compared between the HER2-low and HER2-zero breast cancers, stratified by ER expression. There were no differences in mRNA expression of PD-L1, TIM-3, LAG-3 and TIGIT between HER2-low and HER2-zero among ER-positive or ER-negative cases (Figure 5A-D). However, a statistically higher level of PD-L1 ($p=0.014$) and TIGIT ($p=0.012$) mRNA expression was seen in HER2-zero/ER-negative tumors compared to HER2-low/ER-positive tumors (Figure 5B and C).

PD-L1 Expression by Immunohistochemistry (IHC) in HER2-Low versus HER2-Zero, Stratified by ER Expression

PD-L1 protein expression levels by combined positive score (CPS) were compared between HER2-low and HER2-zero groups, stratified by positive or negative ER expression. There were no differences in PD-L1 CPS protein expression cut off of 10 between HER2-low and HER2-zero among ER-positive or among ER-negative cases (Figure 6). While not statistically significant, there was a higher proportion of patients having PD-L1 CPS $\geq 10\%$ in HER2-zero/ER-negative group when compared to HER2-low/ER-positive ($p=0.061$, Figure 6).

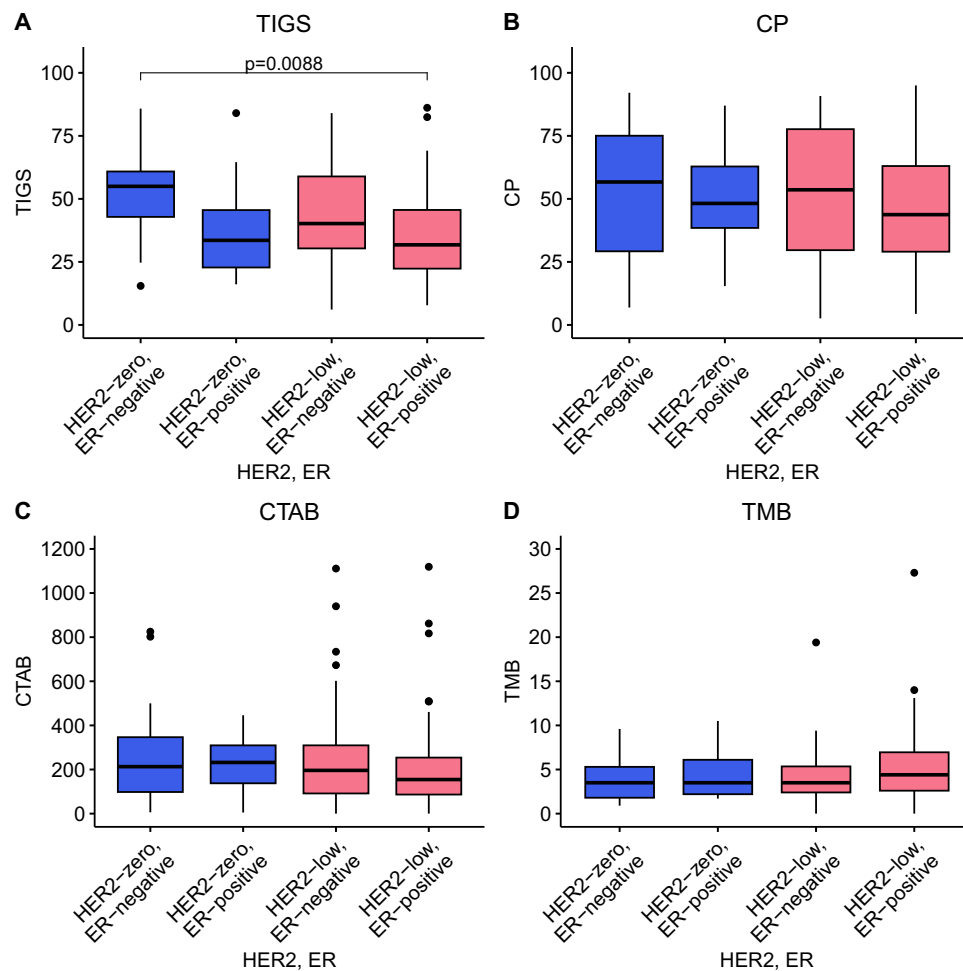


Figure 3 Distributions of TIGS (A), CP (B), CTAB (C), and TMB (D) among the four combined HER2-ER groups for all patients in the cohort.

Overall Survival (OS) Analysis of the Study Cohort by HER2 Expression

Follow up data were available for all 129 patients in the cohort. The median follow-up time was 59 months (range 2–367 months). During the follow-up period, 102 cases of death were observed, and 85 patients died of complications from breast cancer. There was a statistical difference in median OS between HER2-zero and HER2-low groups, where patients with HER2-low tumors had improved OS than those with HER2-zero tumors (median OS=94 months vs median OS=42 months; $p=0.0044$, [Figure 7](#)).

When the cohort was subdivided by ER status in addition to HER2 status, the survival relationship between HER2-zero and HER2-low remained the same within each ER group. HER2-low had better survival than HER2-zero, but neither of these associations were statistically significant. When ER status was taken into consideration, among patients with HER2-low status only, those with ER-positive tumors had significantly longer median OS than those with ER-negative tumors (123 months vs 61 months, $p=2.5 \times 10^{-4}$) ([Figure S1](#)). Patients with HER2-low/ER-positive tumors also had significantly longer median OS than those with HER2-zero/ER-negative tumors (123 months vs 31 months, $p=2.3 \times 10^{-6}$) ([Figure S1](#)).

Discussion

The breakthrough approval of trastuzumab-deruxtecan (T-DXd) has led to a new clinically defined subset of HER2-low breast cancer that was once canonically known as HER2-negative.⁷ Despite the predictive nature of HER2-low for treatment with T-DXd, distinct biological and molecular characteristics of HER2-low breast cancer as a clinical subtype

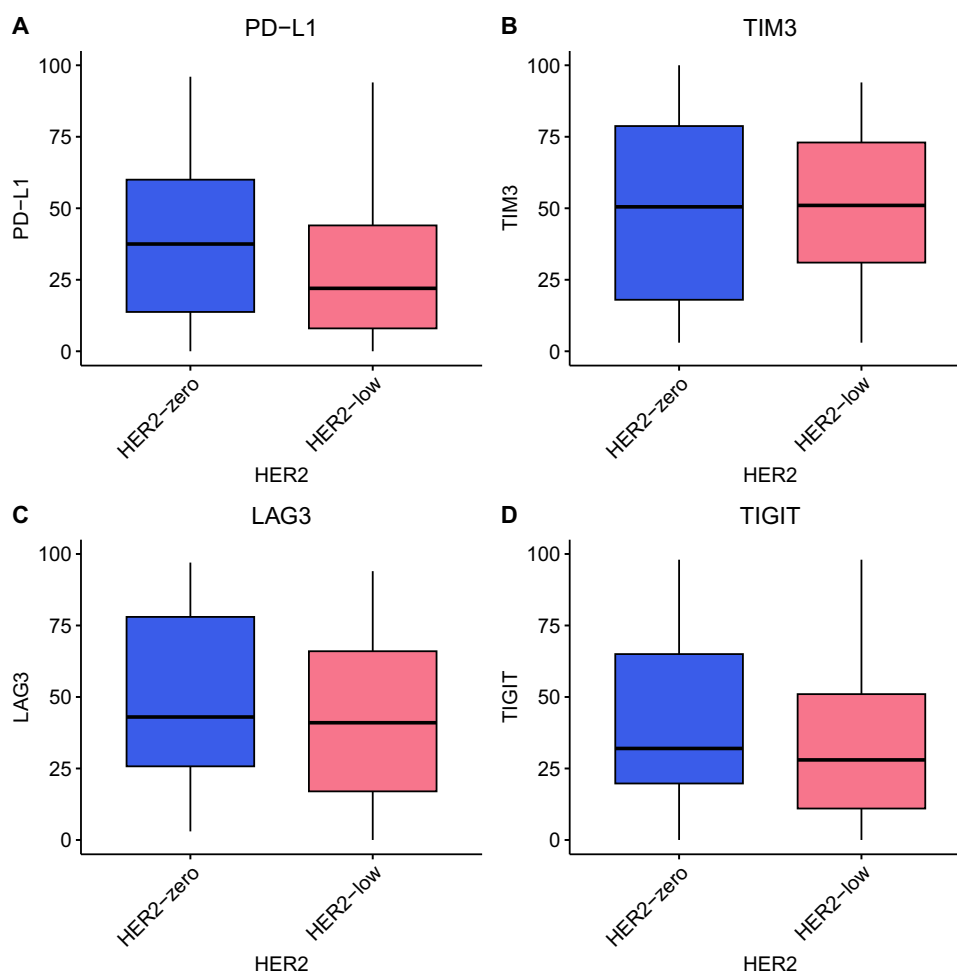


Figure 4 Distributions of PD-L1 (A), TIM-3 (B), LAG-3 (C), and TIGIT (D) gene expression ranks, assessed by RNA-seq, within each HER2 group for all patients in the cohort.

remain elusive.¹⁹ Historically, breast cancer has been considered a poorly immunogenic cancer, and immunotherapies were less prioritized in the treatment paradigm. There have now been a growing number of studies that have shown that triple-negative breast cancer (TNBC) is strongly immunogenic, harboring a higher proportion of tumor infiltrating lymphocytes (TILs) along with higher median TMB when compared to other breast cancer subtypes.¹⁵ Since HER2-low tumors account for about 55% of all breast cancers,^{4,20} around 40% of patients with TNBC have HER2-low tumors that may have a distinct immunogenic profile compared to canonically defined HER2-negative breast cancer.²¹ In this study, we aimed to analyze whether HER2-low breast cancers (IHC 1+ or 2+ with negative FISH) have different expression of immune biomarkers and survival compared to HER2-zero tumors.

Most breast cancer cases in our study were HER2-low (n=97, 75%). This finding shows an overrepresented number of patients with HER2-low breast cancers when compared to previous studies describing that over 55% of all breast cancer diagnoses are HER2-low tumors.^{4,20} It can be attributed to our study inclusion criteria of selected patients with HER2-negative breast cancers. When clinicopathological variables were compared between HER2-low and HER2-zero tumors, we observed a higher proportion of ER-positivity in HER2-low tumors compared to HER2-zero tumors. This finding is also consistent with previous studies that have shown that HER2 low expression has a positive correlation with ER expression due to bidirectional cross-talk between ER and HER2.^{22–25}

Although PD-L1, TMB, and MSI are the most established biomarkers for immunotherapy response, the complexity of the tumor immune microenvironment (TME) cannot be attributed to an individual biomarker, reflective of the host immune system or tumor cells. Further, neither TMB nor MSI signatures have demonstrated efficacy as predictive

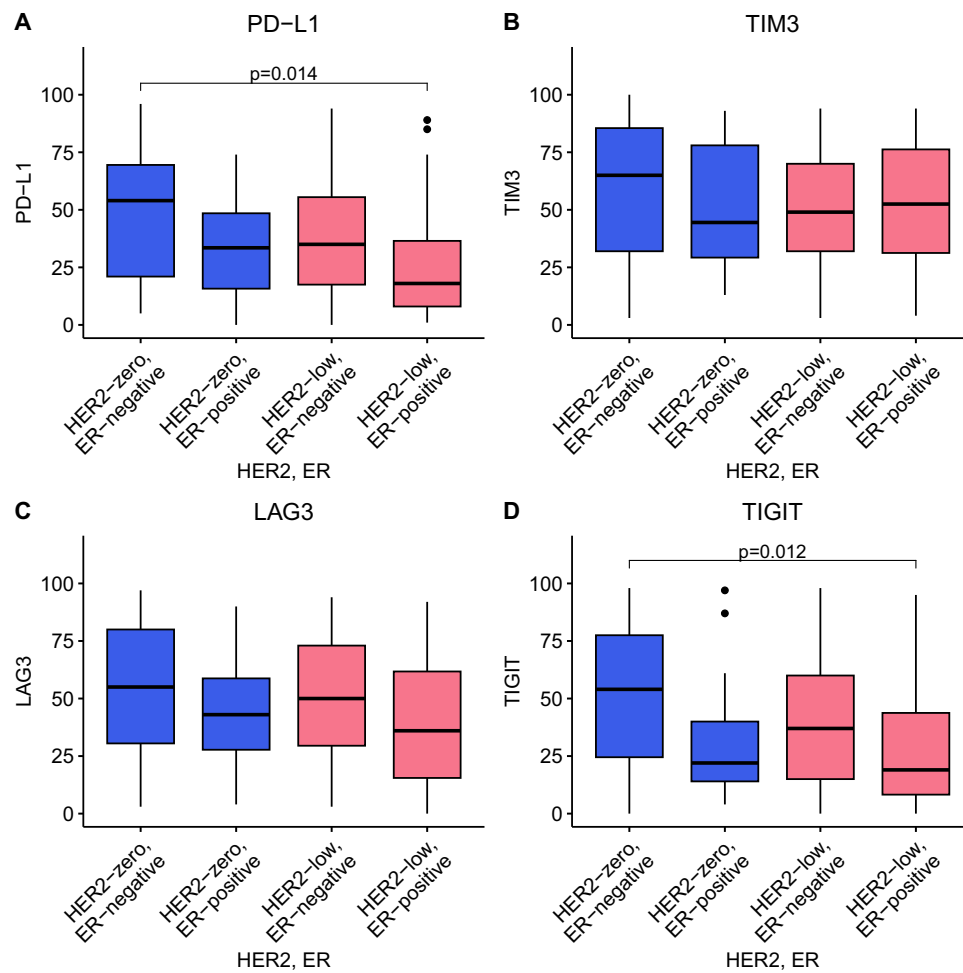


Figure 5 Distributions of PD-L1 (A), TIM-3 (B), LAG-3 (C), and TIGIT (D) gene expression ranks, assessed by RNA-seq, for the four combined HER2-ER groups for all patients in the cohort.

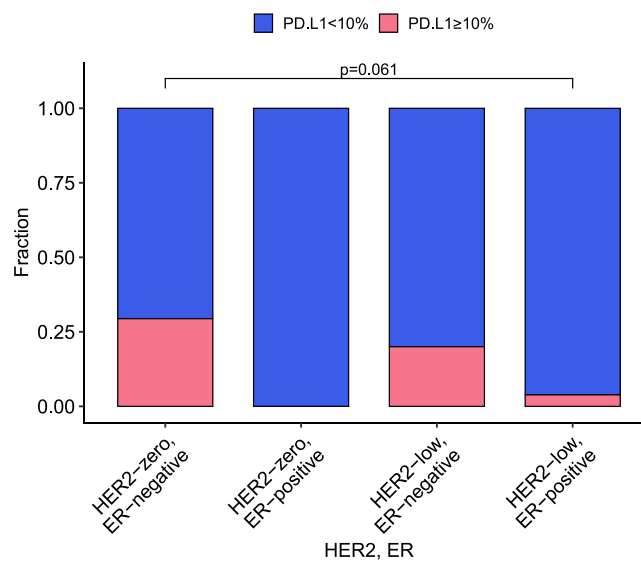


Figure 6 Bar plot comparison of PD-L1 IHC expression in HER2-low versus HER2-zero, stratified by ER expression.

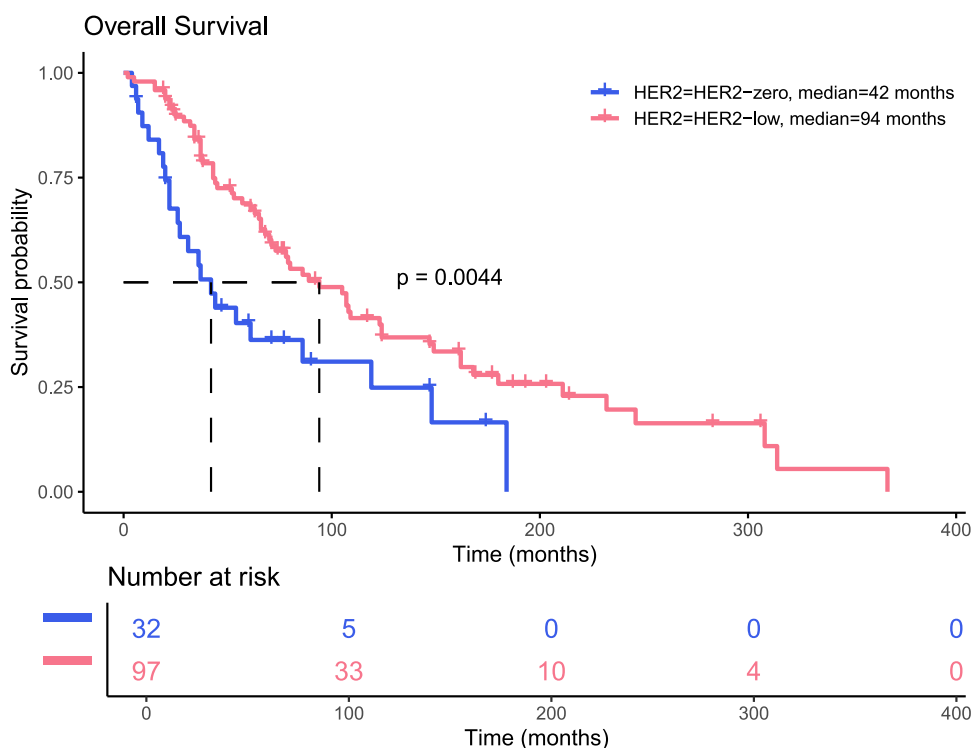


Figure 7 Kaplan-Meier survival analysis of HER2-low and HER2-zero groups for all patients in the cohort.

markers for immunotherapy in breast cancer, leaving an unmet need for additional predictive immune biomarkers. Our previous work has shown that gene expression-based multiplex immune biomarkers offer a more comprehensive insight into the state of the TME primed for immunotherapy response.¹⁷ Tumor immunogenic signature (TIGS), a signature combining the ranked expression of 161 genes associated with tumor inflammation response, was strongly correlated with tumor-infiltrating lymphocytes (TILs) in tumor samples and identified patients with higher response to immunotherapy in NSCLC.^{16,17} In addition, high TIGS score was more commonly observed in tumors with high TMB and PD-L1 protein expression and served as a predictive biomarker of response to immune checkpoint inhibitors alone or in combination with TMB and PD-L1.¹⁷

In this study, we found that there was a trend towards higher TIGS expression in HER2-zero compared to HER2-low tumors, although it was not statistically significant. Even when ER expression was adjusted for, there were no differences in immune phenotypes between HER2-low and HER2-zero breast cancers. However, when ER expression was different among HER2-low and HER2-zero tumors, HER2-zero/ER-negative tumors exhibited a significantly higher TIGS expression than HER2-low/ER-positive tumors. This observation highlights that the differences between HER2-low and HER2-zero tumors are likely driven by ER status. Previous studies have demonstrated that HER2-zero tumors harbor a more basal-like molecular profile with higher immunogenicity like TNBC in the absence of ER expression.^{25,26}

Another important aspect of the TME that plays a role in tumor development and metastasis is the immune escape phenomenon defined by promoting cytotoxic immune cell exhaustion via increased expression of immune checkpoint proteins. PD-1/PD-L1 and CTLA-4 are previously established biomarkers of this immune checkpoint escape mechanism.^{27,28} In the recent years, LAG-3, TIM-3, and TIGIT have emerged as the second wave of promising immune checkpoint targets.²⁸ Our study demonstrated that HER2-zero tumors had higher, though not statistically significant, levels of PD-L1 mRNA expression than HER2-low tumors. When adjusted for ER expression, there were no differences in biomarkers of immune escape mechanism between HER2-low and HER2-zero tumors. However, HER2-zero/ER-negative tumors had significantly higher PD-L1 and TIGIT mRNA expression than HER2-low/ER-positive tumors. This finding again suggests that the differences in immune escape phenotype between HER2-low and HER2-zero tumors are

likely driven by ER expression, and that in the absence of ER, HER2-zero breast cancers exhibit similar immunologically active phenotype as basal-like TNBC.

In our study, patients with HER2-zero breast cancers had worse overall survival than patients with HER2-low breast cancers (42 months vs 94 months, $p=0.0044$). A recent meta-analysis of 42 studies, including 1919 identified records of patients with breast cancer, showed that HER2-low status was associated with significantly better disease-free and overall survival compared to HER2-zero tumors in both early and metastatic setting, regardless of hormone receptor status.²⁹ Our findings are consistent with this meta-analysis by Molinelli C et al²⁹ as patients with HER2-low tumors had significantly better survival compared to those with HER2-zero. When ER expression was taken into consideration, the differences in survival between HER2-low and HER2-zero breast cancers dissipated except in HER2-zero/ER-negative and HER2-low/ER-positive cases (median OS: 31 months, HER2-zero/ER-negative vs median OS: 123 months, HER2-low/ER-positive; $p=2.3 \times 10^{-6}$) (Figure S1). This observation suggests that the survival differences between HER2-low and HER2-zero breast cancers that we observed initially were driven by ER expression.

Overall, our study suggests that HER2-low breast cancer is associated with higher ER expression and better survival compared to HER2-zero tumors. Patients with HER2-low breast cancers harbored a higher rate of ER positivity and therefore resulted in better survival, compared to those with HER2-zero tumors. Similarly, the differences in immune profiles between the two groups were only evident when ER expression was different between HER2-low and HER2-zero tumors. The findings support the notion that HER2-zero tumors, in the ER negative state, behave similarly to basal-like tumors and display a more immune-enriched TME primed for immunotherapy response and have worse survival outcomes.^{26,29}

Strengths of this study include our characterization of the tumor-immune microenvironment (TME) of HER2-low and HER2-zero using the emerging biomarkers TIGS, CP, and CTAB, each of which quantifies a distinct aspect of the TME. These biomarkers each summarize multi-gene expression data collected for each tumor sample into a single value, allowing a succinct phenotypic comparison to be made between various groups of patients while accounting for a broad group of functionally associated genes.^{17,18} To our knowledge, this is the first study that provides a comprehensive immune signature profile encompassing the distinct elements of both tumor and immune-related drivers of the TME in HER2-low and HER2-zero breast cancers.

Our study has some limitations. First, the retrospective nature of the study leads to inevitable selection bias. Although manual abstraction was performed to gather the clinical data, patients with incomplete records or unavailable gene expression Results were excluded. Second, there may be inter-observer variability in scoring HER2 status^{30,31} and heterogeneity of HER2 can exist between tumors as there was a lack of study-specific central rescore for HER2 IHC.^{32,33} Third, our study is relatively small and lacks patient clinical data such as staging and treatment, which can impact the power of statistical analysis and results.

Conclusions

In summary, our study highlights that HER2-low breast cancers do not display distinctive immune gene expression patterns compared to HER2-zero cancers. However, HER2-low breast cancers have a higher rate of ER positivity that resulted in better survival, compared to HER2-zero tumors. Future studies with a larger sample size are warranted to characterize the immune microenvironment of HER2-low and HER2-zero tumors to effectively integrate and sequence immunotherapy with T-DXd.

Data Sharing Statement

The datasets generated and/or analyzed during the current study are not publicly available due to a non-provisional patent filing covering the Methods used to analyze such datasets but are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

Ethics approval for this study was obtained from Roswell Park Comprehensive Cancer Center Institutional Review Board (Study# BDR 162622) and determined to be non-human subject research by the IRB due to conducting research using only unidentifiable or de-identified data. The study was conducted in accordance with the ethical standards of the Declaration of Helsinki.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

Institutional funds.

Disclosure

HCK, RJS, SP, MFS, EVR, SG, KCS, RAP, MFG, MC, MKN, SBH, KAA, SZ, JMC, TJJ, ME, BC, EAS, and SR are employees of Labcorp Oncology, a business unit of Labcorp. SG is an employee at Roswell Park Comprehensive Cancer Center. ME and BJC are employees of Labcorp. RJS, EVR, SG, SP, and JMC are listed as authors on a pending patent US2022/049867 entitled Methods and Systems for Analyzing and Utilizing Cancer Testis Antigen Burden. JC and SP report a pending patent US20220136070A1 Methods and Systems for Characterizing Tumor Response to Immunotherapy Using an Immunogenic Profile pending to OmniSeq, Inc. SP reports patents US11427873B2 Methods and systems for assessing proliferative potential and resistance to immune checkpoint blockade and US11515008B2 Methods and systems of prioritizing treatments, vaccination, testing and/or activities while protecting the privacy of individuals issued to Omniseq. TJJ, EAS, MKN, KCS are shareholder of Labcorp. TJJ is a shareholder in Fortrea. The authors report no other conflicts of interest in this work.

References

- Brigham & Women's Hospital & Harvard Medical School. Comprehensive molecular portraits of human breast tumours. *Nature*. 490(7418):61–70. doi:10.1038/nature11412
- Gennari A, André F, Barrios CH, et al. ESMO Clinical Practice Guideline for the diagnosis, staging and treatment of patients with metastatic breast cancer. *Ann Oncol*. 2021;32(12):1475–1495. doi:10.1016/j.annonc.2021.09.019
- Yersal O, Barutca S. Biological subtypes of breast cancer: prognostic and therapeutic implications. *World J Clin Oncol*. 2014;5(3):412–424. doi:10.5306/wjco.v5.i3.412
- Schettini F, Chic N, Braso-Maristany F, et al. Clinical, pathological, and PAM50 gene expression features of HER2-low breast cancer. *NPJ Breast Cancer*. 7(1):1. doi:10.1038/s41523-020-00208-2
- Saini KS, Azim HA Jr, Metzger-Filho O, et al. Beyond trastuzumab: new treatment options for HER2-positive breast cancer. *Breast*. 2011;20(Suppl 3):S20–7. doi:10.1016/S0960-9776(11)70289-2
- Capelan M, Pugliano L, De Azambuja E, et al. Pertuzumab: new hope for patients with HER2-positive breast cancer. *Ann Oncol*. 2013;24(2):273–282. doi:10.1093/annonc/mds328
- Modi S, Jacot W, Yamashita T, et al. Trastuzumab Deruxtecan in Previously Treated HER2-Low Advanced Breast Cancer. *N Engl J Med*. 387(1):9–20. doi:10.1056/NEJMoa2203690
- Roy AM, Kumarasamy VM, Dhakal A, O'Regan R, Gandhi S. A review of treatment options in HER2-low breast cancer and proposed treatment sequencing algorithm. *Cancer*. 2023;129(18):2773–2788. doi:10.1002/cnrc.34904
- Tarantino P, Jin Q, Tayob N, et al. Prognostic and Biologic Significance of ERBB2-Low Expression in Early-Stage Breast Cancer. *JAMA Oncol*. 8(8):1177–1183. doi:10.1001/jamaoncol.2022.2286
- Tarantino P, Gupta H, Hughes ME, et al. Comprehensive genomic characterization of HER2-low and HER2-0 breast cancer. *Nat Commun*. 14(1):7496. doi:10.1038/s41467-023-43324-w
- Peiffer DS, Zhao F, Chen N, et al. Clinicopathologic Characteristics and Prognosis of ERBB2-Low Breast Cancer Among Patients in the National Cancer Database. *JAMA Oncol*. 9(4):500–510. doi:10.1001/jamaoncol.2022.7476
- Denkert C, Seither F, Schneeweiss A, et al. Clinical and molecular characteristics of HER2-low-positive breast cancer: pooled analysis of individual patient data from four prospective, neoadjuvant clinical trials. *Lancet Oncol*. 2021;22(8):1151–1161. doi:10.1016/s1470-2045(21)00301-6
- Jiang C, Perimbeti S, Deng L, Shapiro CL, Gandhi S. Clinical outcomes of de novo metastatic HER2-low breast cancer: a National Cancer Database Analysis. *NPJ Breast Cancer*. 2022;8(1):135. doi:10.1038/s41523-022-00498-8
- Abdou Y, Goudarzi A, Yu JX, Upadhyaya S, Vincent B, Carey LA. Immunotherapy in triple negative breast cancer: beyond checkpoint inhibitors. *NPJ Breast Cancer*. 2022;8(1):121. doi:10.1038/s41523-022-00486-y
- Barroso-Sousa R, Jain E, Cohen O, et al. Prevalence and mutational determinants of high tumor mutation burden in breast cancer. *Ann Oncol*. 2020;31(3):387–394. doi:10.1016/j.annonc.2019.11.010
- Conroy JM, Pabla S, Glenn ST, et al. A scalable high-throughput targeted next-generation sequencing assay for comprehensive genomic profiling of solid tumors. *PLoS One*. 2021;16(12):e0260089. doi:10.1371/journal.pone.0260089
- Pabla S, Seager RJ, Van Roey E, et al. Integration of tumor inflammation, cell proliferation, and traditional biomarkers improves prediction of immunotherapy resistance and response. *Biomark Res*. 9(1):56. doi:10.1186/s40364-021-00308-6

18. Seager RJ, Senosain MF, Van Roey E, et al. Cancer testis antigen burden (CTAB): a novel biomarker of tumor-associated antigens in lung cancer. *J Transl Med.* 22(1):141. doi:10.1186/s12967-024-04918-0
19. Zhang H, Peng Y. Current Biological, Pathological and Clinical Landscape of HER2-Low Breast Cancer. *Cancers.* 2023;15(1):126. doi:10.3390/cancers15010126
20. Tarantino P, Hamilton E, Tolaney SM, et al. HER2-Low Breast Cancer: pathological and Clinical Landscape. *J Clin Oncol.* 38(17):1951–1962. doi:10.1200/JCO.19.02488
21. Nicolò E, Boscolo Bielo L, Curigliano G, Tarantino P. The HER2-low revolution in breast oncology: steps forward and emerging challenges. *Ther Adv Med Oncol.* 2023;15:17588359231152842. doi:10.1177/17588359231152842
22. Chen M, Chen W, Liu D, et al. Prognostic values of clinical and molecular features in HER2 low-breast cancer with hormonal receptor overexpression: features of HER2-low breast cancer. *Breast Cancer.* 2022;29(5):844–853. doi:10.1007/s12282-022-01364-y
23. Shirman Y, Lubovsky S, Shai A. HER2-Low Breast Cancer: current Landscape and Future Prospects. *Breast Cancer (Dove Med Press).* 2023;15:605–616. doi:10.2147/bett.S366122
24. Loi S, Dafni U, Karlis D, et al. Effects of Estrogen Receptor and Human Epidermal Growth Factor Receptor-2 Levels on the Efficacy of Trastuzumab: a Secondary Analysis of the HERA Trial. *JAMA Oncol.* 2(8):1040–1047. doi:10.1001/jamaoncol.2016.0339
25. Won HS, Ahn J, Kim Y, et al. Clinical significance of HER2-low expression in early breast cancer: a nationwide study from the Korean Breast Cancer Society. *Breast Cancer Res.* 2022;24(1):22. doi:10.1186/s13058-022-01519-x
26. van den Ende NS, Smid M, Timmermans A, et al. HER2-low breast cancer shows a lower immune response compared to HER2-negative cases. *Sci Rep.* 12(1):12974. doi:10.1038/s41598-022-16898-6
27. Shen H, Yang ES, Conry M, et al. Predictive biomarkers for immune checkpoint blockade and opportunities for combination therapies. *Genes Dis.* 2019;6(3):232–246. doi:10.1016/j.gendis.2019.06.006
28. Cai L, Li Y, Tan J, Xu L, Li Y. Targeting LAG-3, TIM-3, and TIGIT for cancer immunotherapy. *J Hematol Oncol.* 2023;16(1):101. doi:10.1186/s13045-023-01499-1
29. Molinelli C, Jacobs F, Agostinetti E, et al. Prognostic value of HER2-low status in breast cancer: a systematic review and meta-analysis. *ESMO Open.* 2023;8(4):101592. doi:10.1016/j.esmoop.2023.101592
30. Layfield LJ, Frazier S, Esebua M, Schmidt RL. Interobserver reproducibility for HER2/neu immunohistochemistry: a comparison of reproducibility for the HercepTest™ and the 4B5 antibody clone. *Pathol Res Pract.* 2016;212(3):190–195. doi:10.1016/j.prp.2015.11.016
31. Turashvili G, Leung S, Turbin D, et al. Inter-observer reproducibility of HER2 immunohistochemical assessment and concordance with fluorescent in situ hybridization (FISH): pathologist assessment compared to quantitative image analysis. *BMC Cancer.* 9(1):165. doi:10.1186/1471-2407-9-165.
32. Tarantino P, Gandini S, Nicolo E, et al. Evolution of low HER2 expression between early and advanced-stage breast cancer. *Eur J Cancer.* 2022;163:35–43. doi:10.1016/j.ejca.2021.12.022
33. Tarantino P, Curigliano G, Tolaney SM. Navigating the HER2-Low Paradigm in Breast Oncology: new Standards, Future Horizons. *Cancer Discov.* 2022;12(9):2026–2030. doi:10.1158/2159-8290.CD-22-0703

Breast Cancer: Targets and Therapy

Dovepress

Publish your work in this journal

Breast Cancer - Targets and Therapy is an international, peer-reviewed open access journal focusing on breast cancer research, identification of therapeutic targets and the optimal use of preventative and integrated treatment interventions to achieve improved outcomes, enhanced survival and quality of life for the cancer patient. The manuscript management system is completely online and includes a very quick and fair peer-review system, which is all easy to use. Visit <http://www.dovepress.com/testimonials.php> to read real quotes from published authors.

Submit your manuscript here: <https://www.dovepress.com/breast-cancer—targets-and-therapy-journal>