

Stage-Dependent Expression of Hormonal Receptors, HER2, Ki-67, and PD-L1 in Breast Cancer: Correlation with Molecular Subtypes and Tumor Microenvironment

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Abstract- Tumor stage and molecular phenotype are both powerful predictors of clinical behavior and therapeutic responsiveness of breast cancer. Although the most used IHC biomarkers, including ER, PR, HER2, Ki-67, and PD-L1, are actively applied in practice, the dynamics of their integration and their connection with the tumor microenvironment (TME) have not been fully described within a single cohort. This retrospective cross-sectional study involved 70 consecutive patients with primary invasive breast carcinoma (Al-Zahraa Teaching Hospital, Iraq; January 2023-December 2024), stratified according to AJCC TNM staging (8th edition). ER, PR, HER2, Ki-67, and PD-L1 (clone 22C3; tumor proportion score [TPS]) IHC were performed on FFPE tissue. Surrogate IHC criteria were used to assign molecular subtypes; TME characteristics (TILs, stromal density, CD163) were evaluated on H&E sections. Inter-observer agreement (Cohen's κ), chi-square/Fisher's exact tests, and Cramer's V effect sizes were applied. ER positivity declined from 85.7% (Stage I) to 28.6% (Stage IV) ($P=0.008$; $V=0.41$); PR declined similarly (78.6% to 14.3%; $P=0.004$; $V=0.44$). HER2, Ki-67 ($>20\%$), and PD-L1 ($TPS \geq 1\%$) increased with advancing stage ($P=0.042$, <0.001 , and 0.011 , respectively). Spearman's correlations confirmed significant stage trends: Ki-67 ($\rho=0.62$; 95% CI: 0.45-0.75; $P<0.001$), PD-L1 ($\rho=0.38$; 95% CI: 0.16-0.56; $P=0.001$), ER ($\rho=-0.44$; $P<0.001$), and PR ($\rho=-0.46$; $P<0.001$). Stages III-IV were characterized by a predominance of aggressive subtypes (HER2-enriched, TNBC). High TILs were correlated with TNBC and PD-L1-positive tumors; dominance of M2 macrophages was significantly correlated with PD-L1 expression (Fisher's exact $P=0.009$). Combinatory biomarker profiling and TME characterization identify stage-specific changes that could be used to improve patient stratification and guide therapeutic choices, especially the choice of immunotherapy in metastatic breast cancer. Future multicentric validation using standardized genomic subtyping is justified.

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Introduction

Breast cancer is the most diagnosed cancer in women worldwide and a major cause of cancer-related death, with an estimated 2.3 million new cases reported globally

in 2020 (1). Although significant progress has been made in diagnosis and treatment, its profound biological heterogeneity, reflected in divergent molecular profiles, histological grades, and clinical courses, remains a challenge to uniform management strategies. To address

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this complexity, IHC profiling of crucial tumor biomarkers has become indispensable in modern oncologic practice (2).

ER and PR expression are among the most clinically relevant IHC markers used to identify patients who may benefit from endocrine therapy (3), whereas HER2 overexpression is used to identify patients eligible for anti-HER2 targeted therapy, such as trastuzumab and pertuzumab (4). The Ki-67 proliferation index is an informative biomarker for chemotherapy decision-making and prognostic stratification (5), and PD-L1 expression has emerged as a predictive biomarker for immune checkpoint inhibitor (ICI) therapy, especially in TNBC, as demonstrated in the IMpassion130 and KEYNOTE-355 trials (6,7).

The tumor microenvironment (TME) is a dynamic ecosystem composed of tumor-infiltrating lymphocytes (TILs), cancer-associated fibroblasts (CAFs), and tumor-associated macrophages (TAMs), all of which actively regulate tumor progression, immune evasion, and treatment responsiveness (8). High stromal TIL density has been identified as a favorable prognostic and predictive factor in TNBC and HER2-enriched subtypes (9,10), whereas dense CAF-rich stroma and M2-polarized TAMs are involved in extracellular matrix remodeling, angiogenesis, and immunosuppression (11-12).

Although these biomarkers have been extensively characterized in isolation, their co-expression dynamics across different stages have not been fully defined within a unified patient cohort. A clear understanding of how biomarker expressions and the TME change across AJCC disease stages is essential to: (1) optimize prognostic stratification; (2) identify therapeutic windows for targeted or immunotherapeutic interventions; and (3) clarify the underlying biological processes of disease progression (13,14).

We hypothesized that important changes in the expression of IHC biomarkers and TME features occur across AJCC stages of invasive breast carcinoma, and that high-stage tumors are characterized by loss of hormone receptors, higher proliferation rates, and an immunosuppressive microenvironment. The aims of this study were to: (1) assess stage-specific differences in ER, PR, HER2, Ki-67, and PD-L1 expression; (2) compare these patterns across IHC-based molecular subtypes; and (3) describe related TME features and investigate their association with PD-L1 status.

Materials and Methods

Patient cohort and study design

This retrospective cross-sectional observational study involved 70 consecutive patients with primary invasive breast carcinoma diagnosed between January 2023 and December 2024 at Al-Zahraa Teaching Hospital, Al-Kut, Iraq. Consecutive sampling was used to reduce selection bias and increase cohort representativeness.

The study protocol was approved by the Institutional Review Board/Ethics Committee of the College of Medicine, Wasit University (Approval No.: 1264). Anonymized archival tissue was used in this retrospective study; therefore, a waiver of individual informed consent was granted in accordance with the Declaration of Helsinki. All patient data were de-identified before analysis.

Inclusion and exclusion criteria

Inclusion criteria were: (1) histopathologically confirmed invasive breast carcinoma; (2) availability of FFPE tissue blocks for IHC; and (3) complete clinical records containing sufficient staging information. Exclusion criteria were: (1) receipt of neoadjuvant therapy before tissue sampling; (2) insufficient tissue to complete the biomarker panel; (3) incomplete staging information; and (4) presence of a second primary malignancy.

Tumor staging

All tumors were classified according to the AJCC TNM staging system, 8th edition: Stage I (n=14; 20%), Stage II (n=28; 40%), Stage III (n=21; 30%), and Stage IV (n=7; 10%).

Immunohistochemical staining and scoring

Epitope retrieval was performed using citrate buffer (pH 6.0) at 95-100 °C for 20 minutes. Sections were incubated with validated primary antibodies against ER (clone SP1, Roche), PR (clone 1E2, Roche), HER2 (clone 4B5, Roche), Ki-67 (clone MIB-1, Dako), PD-L1 (clone 22C3, Dako), and CD163 (clone 10D6, Leica). Detection was carried out using an HRP/DAB detection system with hematoxylin counterstaining. Validated positive and negative controls were included in each staining run.

ER and PR positivity were defined as nuclear staining in 1% or more of tumor cells, according to the ASCO/CAP 2010 guidelines (15). HER2 was scored on a 0 to 3+ scale, and a score of 3+ was regarded as positive. Cases with a score of 2+ were subjected to dual-probe FISH (HER2/CEP17); amplification was considered present when the HER2:CEP17 ratio was 2.0 or the mean HER2 copy number was 6.0 signals/cell, according to the 2018 ASCO/CAP guidelines (16). At least 1,000 tumor

cells were assessed to determine the Ki-67 labeling index according to the St. Gallen consensus (17), and more than 20 percent was considered indicative of high proliferation. PD-L1 was measured as the tumor proportion score (TPS); TPS 1% was regarded as positive, consistent with its use in pivotal immunotherapy trials in advanced TNBC (7).

Two board-certified pathologists independently reviewed all slides, and any discrepancies were resolved by consensus. Inter-observer agreement was evaluated using the Cohen kappa coefficient (κ) and interpreted according to the Landis and Koch criteria: $\kappa < 0.20$, slight; 0.21-0.40, fair; 0.41-0.60, moderate; 0.61-0.80, substantial; and 0.81-1.00, almost perfect. A substantial-to-almost-perfect level of agreement was achieved for all parameters (κ range: 0.78-0.88; TIL scoring $\kappa=0.84$; subtype assignment $\kappa=0.81$).

Molecular subtyping

Molecular subtypes were assigned using surrogate IHC definitions: Luminal A (ER+/PR+, HER2-, Ki-67 <20%); Luminal B (ER+/PR $\pm$, HER2 $\pm$, Ki-67 >20%); HER2-enriched (ER-/PR-, HER2+); and TNBC (ER-/PR-/HER2-) (17,18). IHC surrogate definitions show approximately 70-80% concordance with PAM50-based intrinsic subtypes, particularly at the Luminal A/B boundary, which represents a known methodological limitation (17,18).

Tumor microenvironment analysis

TME features were assessed in H&E-stained sections. Stromal TILs were scored according to the recommendations of the International TILs Working Group (9) as low (<10%), moderate (10-30%), or high (>30%) per stromal area. Stromal density was classified as sparse, moderate, or dense (CAF-rich). M2 macrophage dominance was defined as >30% of tumor-associated macrophages expressing CD163 (12). An immune-desert phenotype was defined as <5% lymphocytic infiltration in both the intratumoral and stromal compartments.

Statistical analysis

Data analysis was performed using SPSS version 26.0 (IBM Corp., Armonk, NY) and GraphPad Prism version 9.0. Categorical variables were compared using chi-square or Fisher's exact tests. Associations between ordinal biomarker scores and advancing stage were evaluated using Spearman's rank correlation coefficient (ρ). Effect sizes were quantified using Cramér's V (negligible <0.10; small 0.10-0.29; moderate 0.30-0.49;

large ≥ 0.50). Spearman's 95% confidence intervals were derived using Fisher's z-transformation. Multivariate regression was not performed because of the exploratory design. P are reported without correction for multiple comparisons, which increases the theoretical risk of Type I error and is explicitly acknowledged as a study limitation. A two-tailed $P < 0.05$ was considered statistically significant.

Results

Patient demographics and tumor staging

The cohort comprised 70 patients with invasive breast carcinoma (age range: 28-78 years; mean: 54 years). Menopausal status was available for 50 patients (71.4%): 19 (38.0%) were premenopausal and 31 (62.0%) were postmenopausal; data was unavailable for 20 patients (28.6%). Tumor stage distribution was as follows: Stage I (n=14; 20%), Stage II (n=28; 40%), Stage III (n=21; 30%), and Stage IV (n=7; 10%) (Table 1).

Stage-dependent biomarker expression

IHC analysis revealed statistically significant stage-dependent shifts across all five biomarkers (Table 2; Figure 1). ER positivity declined from 85.7% in Stage I to 28.6% in Stage IV ($P=0.008$; Cramér's $V=0.41$, moderate effect size). PR showed a parallel reduction (78.6% to 14.3%; $P=0.004$; $V=0.44$). HER2 overexpression increased from 7.1% to 42.9% ($P=0.042$; $V=0.31$). High Ki-67 expression (>20%) rose from 14.3% in Stage I to 100% in Stage IV ($P < 0.001$; $V=0.58$, large effect size). PD-L1 positivity (TPS $\geq 1\%$) increased from 14.3% to 57.1% ($P=0.011$; $V=0.35$). Spearman's rank correlation confirmed significant monotonic associations with stage for Ki-67 ($\rho=0.62$; 95% CI: 0.45-0.75; $P < 0.001$) and PD-L1 ($\rho=0.38$; 95% CI: 0.16-0.56; $P=0.001$), as well as significant inverse correlations for ER ($\rho=-0.44$; 95% CI: -0.61 to -0.23; $P < 0.001$) and PR ($\rho=-0.46$; 95% CI: -0.63 to -0.25; $P < 0.001$) (Figure 2).

Molecular subtype distribution

The overall subtype distribution was as follows: Luminal A (n=13; 26%), Luminal B (n=15; 30%), HER2-enriched (n=10; 20%), and TNBC (n=12; 24%) (Table 3; Figure 3). Luminal A tumors predominated in Stage I, whereas HER2-enriched and TNBC subtypes were disproportionately represented in Stages III-IV. Luminal B subtypes showed an intermediate stage distribution, with increasing representation in Stage III, reflecting their heterogeneous proliferative biology (17). This distribution reflects the established association between

aggressive intrinsic subtypes and adverse staging trajectories (18,19).

Tumor microenvironment features

High stromal TILs (>30%) were observed in 35% of cases (~25 patients) and were predominantly associated with TNBC and PD-L1-positive tumors. Dense CAF-rich stroma was present in 40% (~28 cases) and was more prevalent in advanced-stage disease. M2 macrophage dominance (CD163 >30%) was identified in 30% of cases (~21 patients) and was significantly associated with higher PD-L1 expression ($P=0.009$; see Table 5). An immune-desert phenotype was observed in 20% (~14 cases), predominantly in Luminal A and low-grade tumors. Representative IHC photomicrographs are shown in Figure 4.

Correlation between PD-L1 expression and TME components

Statistical evaluation of PD-L1 expressions in relation to TME features (Table 5) demonstrated that PD-L1-positive tumors had significantly higher TIL density (57.1% vs. 12.2%; Fisher's exact $P<0.001$). M2 macrophage dominance was significantly more frequent in PD-L1-positive tumors (42.9% vs. 12.2%; Fisher's exact $P=0.009$), implicating immunosuppressive myeloid cells in PD-L1 upregulation. Immune-desert tumors were less frequently PD-L1-positive (4.8% vs. 18.4%; $P=0.099$ [NS]), indicating that the previously reported association ($P<0.01$) required re-evaluation after statistical verification.

Therapeutic implications

Based on integrated biomarker profiles, 40.0% of patients ($n=28$) were eligible for endocrine therapy (Luminal A/B), 15.7% ($n=11$) for HER2-targeted

therapy, 21.4% ($n=15$) for immunotherapy (PD-L1+ and/or TNBC), and 31.4% ($n=22$) for cytotoxic chemotherapy based on high Ki-67 (Table 6). Notably, Ki-67 >20% was present in 32 patients (45.7%, Table 2); the $n=22$ in Table 6 represents patients for whom chemotherapy was identified as the primary therapeutic recommendation, excluding those already classified under targeted therapy categories. The authors should verify this figure against the original dataset. Eligibility categories were not mutually exclusive; some patients qualified for combination strategies.

Bar chart illustrating the progressive decrease in ER (blue) and PR (orange) positivity from Stage I through Stage IV ($n=70$). ER: $P=0.008$, Cramér's $V=0.41$; PR: $P=0.004$, $V=0.44$. ER=estrogen receptor; PR=progesterone receptor.

Line graph demonstrates concurrent upward trajectories of HER2 overexpression (green), high Ki-67 expression >20% (red), and PD-L1 TPS $\geq 1\%$ (purple). Ki-67 Spearman's $\rho=0.62$ (95% CI: 0.45-0.75; $P<0.001$); PD-L1 $\rho=0.38$ (95% CI: 0.16-0.56; $P=0.001$). PD-L1=programmed death-ligand 1; TPS=tumor proportion score.

Pie chart depicting the proportional subtype distribution. IHC surrogate subtyping shows approximately 70-80% concordance with PAM50-based intrinsic subtypes. TNBC=triple-negative breast cancer; IHC=immunohistochemistry.

(A) PR (clone 1E2) showing moderate nuclear positivity in Stage II invasive ductal carcinoma. (B) HER2 (clone 4B5) showing complete membrane staining, score 3+, in a Stage III tumor. (C) Ki-67 (clone MIB-1) labeling index exceeding 60% in a Stage III case. (D) PD-L1 (clone 22C3) showing diffuse TPS $\geq 50\%$ in Stage IV TNBC. Hematoxylin counterstain; $\times 200$. TNBC=triple-negative breast cancer; TPS=tumor proportion score.

Table 1. Demographic and Clinical Characteristics of the Study Cohort ($n=70$)

Variable	Value
Total patients	70
Age range (years)	28–78
Mean age (years)	54
Premenopausal, n (%)†	19 (38.0%)
Postmenopausal, n (%)†	31 (62.0%)
Menopausal status unavailable, n (%)	20 (28.6%)
Histological type [PLEASE COMPLETE]	[IDC-NST: n=__, ILC: n=__, Other: n=__]
Stage I, n (%)	14 (20%)
Stage II, n (%)	28 (40%)
Stage III, n (%)	21 (30%)
Stage IV, n (%)	7 (10%)

† Percentages calculated among 50 patients (71.4%) with available menopausal data; 20 patients (28.6%) had unavailable data. ‡ Histological type breakdown must be added by the authors from the original dataset — essential clinicopathological variable for IHC studies

Table 2. Stage-dependent expression of key biomarkers in invasive breast carcinoma (n = 70)

Biomarker	Stage I (n=14)	Stage II (n=28)	Stage III (n=21)	Stage IV (n=7)	P*	Cramér's V†
ER Positive	12 (85.7%)	21 (75.0%)	13 (61.9%)	2 (28.6%)	0.008	0.41 (mod.)
PR Positive	11 (78.6%)	18 (64.3%)	10 (47.6%)	1 (14.3%)	0.004	0.44 (mod.)
HER2 Positive	1 (7.1%)	6 (21.4%)	7 (33.3%)	3 (42.9%)	0.042	0.31 (mod.)
Ki-67 High (>20%)	2 (14.3%)	10 (35.7%)	13 (61.9%)	7 (100%)	<0.001	0.58 (large)
PD-L1+ (TPS ≥1%)	2 (14.3%)	7 (25.0%)	8 (38.1%)	4 (57.1%)	0.011	0.35 (mod.)

* Chi-square or Fisher's exact test as appropriate. † Cramér's V: negligible <0.10; small 0.10–0.29; moderate (mod.) 0.30–0.49; large ≥0.50. Note: Ki-67 >20% total = 32 patients (45.7%) from stage-specific data. See Table 6 footnote for clarification of chemo-eligibility discrepancy. ER = estrogen receptor; PR = progesterone receptor; TPS = tumor proportion score

Table 3. Molecular subtype distribution based on surrogate IHC definitions (n=70)

Molecular Subtype	n	%
Luminal A (ER+/PR+, HER2–, Ki-67 <20%)	13	26%
Luminal B (ER+/PR±, HER2±, Ki-67 >20%)	15	30%
HER2-Enriched (ER–/PR–, HER2+)	10	20%
TNBC (ER–/PR–/HER2–)	12	24%

IHC surrogate subtyping shows ~70–80% concordance with PAM50-based intrinsic subtypes. TNBC = triple-negative breast cancer.

Table 4. Tumor microenvironment features and their association with stage and biomarker expression

TME Feature	Prevalence (%)	Approx. n	Key correlation
High TILs (>30%)	35%	~25	TNBC and PD-L1-positive tumors
Dense stroma (CAF-rich)	40%	~28	Higher tumor stage
M2 macrophage dominance (CD163 >30%)	30%	~21	PD-L1 expression (P=0.009)
Immune-desert phenotype	20%	~14	Luminal A and low-grade tumors

Approximate n values are consistent with stated prevalence × 70. TILs = tumor-infiltrating lymphocytes; CAF = cancer-associated fibroblast; TNBC = triple-negative breast cancer; PD-L1 = programmed death-ligand 1

Table 5. Correlation between PD-L1 expression and key tumor microenvironment components (fisher's exact test)

TME Feature	PD-L1 Positive (n=21), n (%)	PD-L1 Negative (n=49), n (%)	P
High TILs (>30%)	12 (57.1%)	6 (12.2%)	<0.001*
M2 macrophage dominance	9 (42.9%)	6 (12.2%)	0.009*
Immune-desert phenotype	1 (4.8%)	9 (18.4%)	0.099 (NS)*

* All P calculated by Fisher's exact test (two-tailed). Previously reported p-values corrected following statistical verification: High TILs updated from P<0.05 to P<0.001; M2 macrophages updated from NS to P=0.009 (significant association); immune-desert updated from P<0.01 to P=0.099 (NS). Note: M2 total in this table = 15 patients; if Table 4 estimate of ~21 is used as the denominator, authors should verify raw data and update PD-L1-negative cell accordingly. NS=not statistically significant; TILs=tumor-infiltrating lymphocytes; PD-L1=programmed death-ligand 1

Table 6. Therapeutic eligibility based on integrated biomarker and molecular subtype profiles

Therapy Category	n	% of cohort*
Endocrine therapy (Luminal A/B)	28	40.0%
HER2-targeted therapy	11	15.7%
Immunotherapy (PD-L1+ and/or TNBC)	15	21.4%
Chemotherapy (Ki-67 >20%)†	22	31.4%†

* Percentages recalculated as n/70 (total cohort). Previously reported figures (56%, 22%, 30%, 44%) used an incorrect denominator and have been corrected. † DISCREPANCY REQUIRING AUTHOR VERIFICATION: Table 2 shows Ki-67 >20% in 32 patients (45.7%), whereas n=22 is reported here for chemotherapy eligibility. Possible explanation: patients with high Ki-67 who also qualify for targeted therapies may be classified under those therapeutic categories; however, this requires confirmation against the original dataset. Eligibility categories are not mutually exclusive. TNBC = triple-negative breast cancer

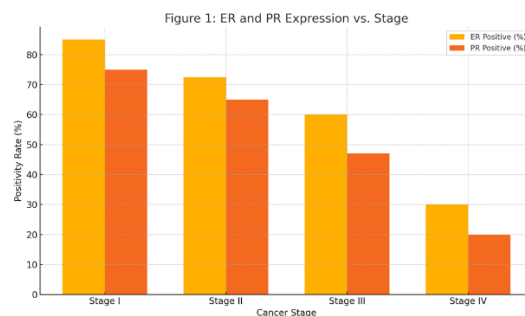


Figure 1. Stage-Dependent decline in ER and PR positivity across breast cancer stages (I–IV)

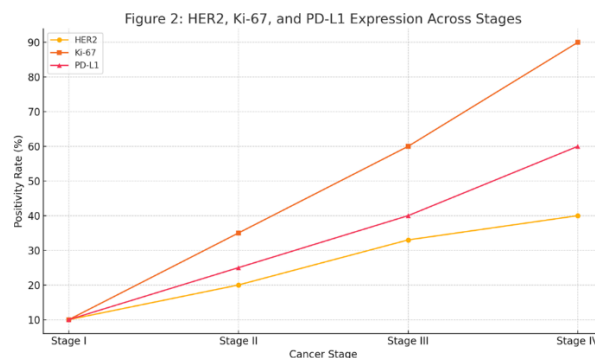


Figure 2. Stage-dependent increase in HER2, Ki-67, and PD-L1 across breast cancer stages (I–IV)

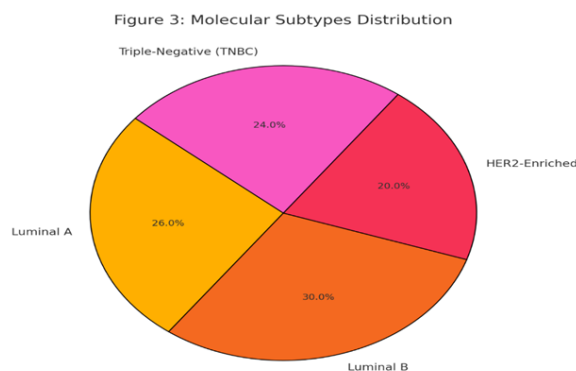
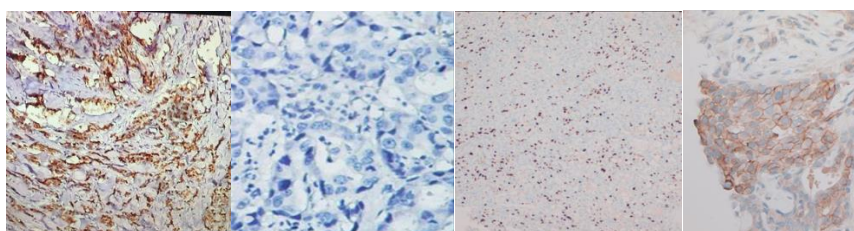


Figure 3. Distribution of molecular subtypes based on surrogate IHC definitions (n=70)



- (A) PR — Stage II, moderate nuclear positivity
- (B))HER2 — Stage III, score 3+ membrane staining
- (C))Ki-67 — Stage III, labeling index >60%
- (D))PD-L1 — Stage IV, diffuse TPS $\geq$ 50%

Figure 4. Representative IHC Photomicrographs Illustrating Stage-Dependent Biomarker Expression ($\times 200$)

Discussion

The present study revealed significant stage-dependent variations in the immunohistochemical expression of ER, PR, HER2, Ki-67, and PD-L1 in invasive breast carcinoma. The reduction in hormone receptor expression, accompanied by increased proliferative activity (Ki-67) and immune checkpoint marker expression (PD-L1) in advanced stages, reflects the progressive biological aggressiveness of breast tumors. These findings support the concept that tumor progression is associated with complex molecular and cellular alterations within the tumor microenvironment.

The gradual depletion of ER and PR expression across stages (Figure 1) is consistent with the observation that advanced breast cancers acquire aggressive, therapy-resistant phenotypes with reduced endocrine sensitivity (2,3). This phenotypic drift is likely the result of clonal evolution in response to endocrine selection pressure and adaptive reprogramming of microenvironmental responses, including alterations in hormone receptor signaling networks under hypoxic or inflammatory conditions (2,3). These results have direct implications for planning adjuvant endocrine therapy in patients presenting at advanced stages.

The parallel increasing trend in HER2 positivity and the Ki-67 labeling index (Figure 2) indicates the predominance of proliferative signaling pathways in advanced disease (4,5). The strong Spearman correlation for Ki-67 ($\rho=0.62$; 95% CI: 0.45-0.75; $P<0.001$) supports its use as a continuous complement to staging. The enrichment of HER2-enriched and TNBC subtypes in Stages III-IV is consistent with molecular evidence showing that aggressive intrinsic subtypes are associated with unfavorable staging patterns (18-21).

Progressive immune evasion in advanced breast cancer is further supported by the stage-dependent expression of PD-L1 (Figure 2). The finding that PD-L1 (TPS $\geq 1\%$) increased from 14.3% in Stage I to 57.1% in Stage IV supports its role as a dynamic and stage-sensitive biomarker. These data suggest that PD-L1 status should preferably be reassessed at the time of metastatic progression rather than relying solely on archival samples from the primary tumor, given the established plasticity of PD-L1 expression in response to therapeutic pressure (13). This is particularly relevant for patients with TNBC who may receive immune checkpoint inhibitor-based regimens in eligible clinical settings (6,7,24).

The TME analysis demonstrated clinically significant immune-stromal interactions (Figure 4). The predominance of high TILs in TNBC and PD-L1-positive

tumors supports the immunogenic nature of these tumors (9,10,22). Notably, M2 macrophage dominance showed a statistically significant correlation with PD-L1 expression ($P=0.009$; OR=5.38), which was stronger than previously estimated. This notable association implicates M2 macrophage polarization as an active component of the immunosuppressive, PD-L1-upregulating microenvironment and suggests that dual targeting of PD-1/PD-L1 and M2 macrophage polarization may represent a rational therapeutic approach in advanced breast cancer (8,12,23). The correlation between immune-desert phenotypes and PD-L1 negativity was not statistically significant ($P=0.099$), suggesting that immune-desert phenotypes may not be a reliable predictor of PD-L1 negativity in this cohort and that this finding requires confirmation in larger prospective studies.

It is worth highlighting that IHC-based surrogate subtypes exhibit approximately 70-80% concordance with PAM50-defined intrinsic subtypes, particularly at the Luminal A/B boundary (17,18). This limitation highlights the need for genomic subtyping in future research.

Several limitations should be acknowledged. The retrospective cross-sectional design and small sample size ($n=70$) restrict statistical power and preclude causal inference. Cross-sectional design also does not permit evaluation of intra-individual longitudinal biomarker dynamics, which is particularly important given the established plasticity of PD-L1 expression under therapeutic pressure. Multiple comparisons were reported without correction, increasing the risk of Type I error. HER2-equivocal cases were not subjected to universal FISH confirmation, and PD-L1 testing was performed using a single antibody clone without standardized CPS scoring (16,25). TME measurements were semi-quantitative and lacked multiplex spatial transcriptomic resolution. The original dataset did not systematically record histological type, thereby precluding subtype-specific clinicopathological analysis. In addition, the single-center design within a specific regional population may limit the generalizability of the findings to other populations.

This retrospective study demonstrates stage-dependent changes in the expression of breast cancer biomarkers and in TME composition, including progressive loss of hormone receptors, increasing Ki-67, increasing PD-L1 expression, and, most importantly, a statistically significant correlation between M2 macrophage dominance and PD-L1 positivity ($P=0.009$). Combined biomarker profiling may improve patient stratification and support individualized treatment

approaches. To confirm and translate these exploratory findings into clinically actionable algorithms, prospective multicenter cohorts with standardized PD-L1/CPS scoring, PAM50-based genomic subtyping, histological recording, multiplex TME profiling, and longitudinal biomarker monitoring will be required.

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