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Review Article

Update on HER2 Testing in Breast Cancer: HER2-ultralow, HER2-low, HER2-null Clinical Categories and Challenges in HER2 Assessment

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Abstract

Background and objectives: The success of trastuzumab deruxtecan (T-DXd) has expanded the therapeutic relevance of human epidermal growth factor receptor 2 (HER2) expression beyond the traditional HER2-positive population. This has led to evolving concepts such as HER2-low, HER2-ultralow, and HER2-null. Accurately and reproducibly distinguishing HER2 immunohistochemistry (IHC) scores 0, 0+, and 1+ remains challenging because of intratumoral heterogeneity and interobserver variability. This review discusses the challenges in HER2 assessment.

Methods: The evolution of HER2 testing and recent guideline updates were reviewed. The implications of HER2-low and HER2-ultralow breast cancers for HER2 testing were discussed.

Results: Challenges in HER2 assessment include interobserver variability and intratumoral heterogeneity. Potential strategies for accurately and reproducibly distinguishing all HER2 IHC categories, especially HER2 IHC 0 from HER2 IHC 0+, are also discussed. Significant discordance has been reported in interpreting low-level HER2 expression. Revisiting HER2 testing guidelines, improving HER2 assessment, and developing sensitive, objective, and reproducible detection methods may reduce variability. No alternative method has been validated as a companion diagnostic for determining eligibility for T-DXd or other HER2-targeted antibody–drug conjugates. Consequently, HER2 immunohistochemistry, supplemented by reflex *in situ* hybridization testing for equivocal cases, remains the standard despite its limitations at the HER2-low threshold. Digital pathology, quantitative image analysis, artificial intelligence-assisted interpretation, molecular profiling, and other emerging technologies may provide more objective, sensitive, and reproducible approaches to HER2 assessment.

Conclusions: The evolving concepts of HER2-low, HER2-ultralow, and HER2-null emphasize the need to accurately and reproducibly distinguish HER2 IHC categories. Further research and technological advances are needed to better classify HER2 status.

Introduction

Human epidermal growth factor receptor 2 (HER2), a receptor tyrosine kinase, is encoded by the *ERBB2* gene located on chromosome 17q12. The human *ERBB2* (HER2/neu) gene was reported by King *et al.*[1] in 1985, representing a major milestone in cancer research. In 1987, Slamon *et al.*[2] published a landmark study in *Science* demonstrating that HER2/neu amplification occurred in approximately 30% of primary human breast cancers and was a significant predictor of both overall survival and time to relapse. This study established HER2 amplification as an important prognostic biomarker in breast cancer. These findings ultimately led to the development of the monoclonal antibody trastuzumab, which has proven highly effective in treating breast cancers with HER2 overexpression, resulting from either *ERBB2* gene amplification or increased HER2 protein expression. Furthermore, these advances prompted the development of standardized HER2 testing methods and a binary pathological

Keywords: HER2-ultralow; HER2-low; HER2-null; Interobserver variability; Intratumoral heterogeneity; HER2 testing; ASCO/CAP; Guidelines; Breast cancer.
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classification of HER2 status by the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) in 2007.

Trastuzumab deruxtecan (T-DXd), a next-generation antibody-drug conjugate, consists of trastuzumab linked to the potent topoisomerase I inhibitor deruxtecan. The phase 3 DESTINY-Breast04 trial demonstrated that T-DXd significantly improved progression-free survival and overall survival compared with physician's choice chemotherapy in patients with HER2-low metastatic breast cancer who had previously received one or two lines of chemotherapy.[3] More recently, the phase 3 DESTINY-Breast06 trial showed that T-DXd significantly improved progression-free survival compared with physician's choice treatment in patients with HER2-low or HER2-ultralow hormone receptor-positive metastatic breast cancer who had received one or more lines of endocrine-based therapy but had not yet undergone chemotherapy in the metastatic setting.[4]

The success of T-DXd has elevated HER2-targeted therapy to a new level by demonstrating meaningful clinical activity even in breast cancers with low levels of HER2 protein expression, thereby substantially broadening the population of patients who may benefit from HER2-directed therapies. These findings have reshaped the contemporary understanding of HER2 biology in breast cancer and have led to the introduction of new clinical concepts, including HER2-low, HER2-ultralow, and HER2-null breast cancer. At the same time, they have raised important questions regarding current pathology practice and HER2 testing methodologies and assessment. It remains unclear whether the traditional binary classification of HER2-positive versus HER2-negative disease is sufficient or whether current testing methods are adequately sensitive and reproducible to assess HER2 status.

In addition, variability in HER2 assessment, including interobserver variability, interlaboratory differences, and intratumoral heterogeneity, has been a persistent challenge since the introduction of HER2 testing. These issues are likely to remain important challenges in HER2 assessment.

The objective of this review is to provide a comprehensive and critical evaluation of HER2 testing in breast cancer. Specifically, it examines the recent evolution of HER2 testing and HER2 status assessment, reviews HER2-low and HER2-ultralow breast cancers, and discusses longstanding and emerging challenges related to these clinical categories, with particular emphasis on interobserver variability, intratumoral heterogeneity, and potential strategies to reduce variability. The review also addresses the limitations of current HER2 testing approaches and highlights emerging methodologies that may improve the accuracy and reproducibility of HER2 assessment and reduce variability.

Evolution of HER2 testing

In this review, we divide the evolution of HER2 testing into two major periods: before 2023 and after 2023. Because different terminology has been used to designate HER2 expression in the literature and clinical practice, we define HER2 protein test results as protein expression (at normal or low levels) or protein overexpression (i.e., equivalent to a HER2 immunohistochemistry (IHC) score of 3+); HER2 DNA test results as gene amplification, *HER2* copy number, or the *HER2/CEP17* ratio; and HER2 RNA test results as RNA expression.

Evolution of HER2 testing: Before 2023

Owing to the remarkable clinical success of trastuzumab, ASCO/CAP recommends HER2 testing for all newly diagnosed primary and metastatic breast cancers. To ensure appropriate patient selection for HER2-targeted therapies and to minimize false-positive and false-negative results, standardized methods and guidelines for determining HER2 status in breast cancer were first developed by ASCO/CAP in 2007 and subsequently updated in 2013,[5,6] 2018,[7] and 2023.[8]

Currently, standard HER2 testing methods and guidelines established by the 2018 ASCO/CAP recommendations are used to assess HER2 status. These include IHC to assess HER2 protein expression and *in situ* hybridization (ISH) techniques to evaluate *HER2* gene amplification. ISH methodologies include fluorescence *in situ* hybridization and chromogenic *in situ* hybridization, using single- or dual-probe assays to determine *HER2* gene copy number and/or the *HER2*/centromere enumeration probe for *CEP17* ratio.

The IHC scoring system evaluates the completeness and intensity of HER2 membrane staining, as well as the percentage of breast cancer cells demonstrating membranous reactivity, using a 10% tumor cell threshold as the principal criterion for score assignment. HER2 IHC results are categorized into four scores: 0, 1+, 2+, and 3+. An IHC score of 2+ is classified as equivocal and requires reflex ISH testing to determine *HER2* gene amplification status and establish a definitive HER2 classification.

HER2-positive breast cancers are defined as tumors demonstrating either IHC 3+ protein overexpression or IHC 2+ with *HER2* gene amplification by ISH (IHC 2+/ISH+). In contrast, HER2-negative breast cancers are defined as IHC 0, IHC 1+, or IHC 2+/ISH−.[7]

A summary of the HER2 IHC and ISH scoring criteria and associated testing algorithm from the 2018 guidelines is presented in Tables 1, 2, and 3.[7]

Evolution of HER2 testing: After 2023

The clinical success of T-DXd has made distinctions among IHC score 0 (without membranous staining), IHC score 0+ (with membranous staining), IHC score 1+, and IHC score 2+/ISH-negative breast cancers clinically relevant, and a more precise characterization of HER2 IHC staining patterns has become increasingly important in routine pathology practice. It has also solidified the clinical terminology of HER2-low, HER2-ultralow, and HER2-null. Because of the emergence of the new clinical categories of HER2-low and HER2-ultralow breast cancer and their impact on HER2 testing, we discuss these breast cancer subgroups in greater detail below.

HER2-low breast cancer

The term "HER2-low" breast cancer entered widespread clinical use between 2020 and 2022 and became firmly established after the phase 3 DESTINY-Breast04 results were presented at ASCO in June 2022. HER2-low breast cancer is clinically defined as breast cancer with a HER2 IHC score of 1+ or 2+ and negative ISH results.

This clinical category represents approximately 31%–67% of breast cancers historically classified as HER2-negative.[9-25]

Accumulating evidence suggests that HER2-low breast cancers exhibit distinct clinicopathologic characteristics compared with

Table 1. Summary of the HER2 IHC scoring criteria and associated testing algorithm (ASCO/CAP 2018)

Score	Staining pattern	Interpretation	Next step
0	No membrane staining observed or Incomplete, faint/barely perceptible membrane staining in ≤10% of tumor cells	Negative	No further testing
1+	Incomplete membrane staining, faint/barely perceptible in >10% of tumor cells	Negative	No further testing
2+	Weak to moderate complete membrane staining in >10% of tumor cells, or intense complete staining in ≤10% of tumor cells	Equivocal	Reflex to ISH testing: if ISH+ → HER2-positive; if ISH− → HER2-negative
3+	Intense complete membrane staining in >10% of tumor cells (readily appreciated at low-power objective)	Positive	No further testing; treat as HER2-positive disease

Less common HER2 staining patterns, such as moderate to intense but incomplete membrane staining, including the basolateral or lateral staining pattern frequently observed in invasive micropapillary cancer, are also classified as IHC 2+ (equivocal).[7] ASCO, American Society of Clinical Oncology; CAP, College of American Pathologists; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; ISH, *in situ* hybridization.

Table 2. *HER2* ISH classification, interpretation criteria, and associated testing algorithm for dual-probe assays (ASCO/CAP 2018)

ISH group	*HER2/CEP17* ratio	Average *HER2* Copy number/cell	Next step
Group 1 (Positive)	≥ 2.0	≥ 4.0	None needed
Group 2	≥ 2.0	< 4.0	Concurrent IHC required*
Group 3	< 2.0	≥ 6.0	Concurrent IHC required*
Group 4	< 2.0	≥ 4.0 and < 6.0	Concurrent IHC required*
Group 5 (Negative)	< 2.0	< 4.0	None needed

*For Groups 2–4, the final HER2 status depends on concurrent IHC: cases with IHC 3+ are classified as HER2-positive, while those with IHC 0 or 1+ are negative. When IHC is 2+, a second blinded reviewer recounts the ISH in the targeted area. The final result depends on the recount result.[7] ASCO, American Society of Clinical Oncology; CAP, College of American Pathologists; *CEP17*, centromere enumeration probe for chromosome 17; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; ISH, *in situ* hybridization.

Table 3. *HER2* ISH classification, interpretation criteria, and associated testing algorithm for single-probe assays (ASCO/CAP 2018)

Average *HER2* copy number per cell	ISH Interpretation	Next step
≥ 6.0 (positive)	Amplified	None needed
≥ 4.0 to < 6.0	Equivocal	Correlate with concurrent HER2 IHC and/or dual-probe ISH*
< 4.0 (negative)	Not amplified	None needed

*For a single-probe *HER2* ISH assay, the interpretation is based solely on the average *HER2* gene copy number per tumor cell, since no *CEP17* probe is included and a *HER2/CEP17* ratio cannot be calculated. Cases with an average *HER2* copy number of ≥ 4.0 to < 6.0 signals per cell require integration with concurrent HER2 IHC and/or dual-probe ISH findings to determine the final HER2 status. If IHC 3+ and/or dual-probe ISH group 1, classify as HER2-positive. If IHC 0, 1+ and/or dual-probe ISH group 5, classify as HER2-negative. If IHC 2+, perform dual-probe ISH and interpret the result based on dual-probe ISH result. If initial assessment of dual-probe ISH is suggestive of groups 2, 3, or 4, follow the algorithm described in Table 2. Dual-probe assays are generally preferred because they provide additional information regarding *chromosome 17* status and allow calculation of the *HER2/CEP17* ratio. Concomitant IHC review should be part of the interpretation of all single-probe ISH results.[7] ASCO, American Society of Clinical Oncology; CAP, College of American Pathologists; *CEP17*, centromere enumeration probe for chromosome 17; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; ISH, *in situ* hybridization.

HER2-zero breast cancers. HER2-low breast cancer is more commonly observed in hormone receptor (HR)-positive breast cancers than in HR-negative disease.[9,15-19,21,23-26] Most studies have reported a lower Ki-67 proliferation index in HER2-low breast cancers compared with HER2-zero breast cancers,[9,13,18,21,27] and HER2-low breast cancers tend to be associated with a lower histologic grade.[25,27] At the molecular level, HER2-low breast cancers are predominantly classified as luminal subtypes, with reported distributions of luminal A (29.3%–65.5%), luminal B (28.8%–50.5%), HER2-enriched (1.1%–4.1%), and basal-like (4.6%–13.3%).[10,27,28] When HER2-low breast cancers are stratified by HR status, the HER2-zero/ estrogen receptor (ER)-positive subgroup contains significantly more grade 3 breast cancers than the HER2-low/ER-positive subgroup.[25] Moreover, HER2-low/HR-positive breast cancers demonstrate a higher proportion of the luminal A molecular subtype and lower Ki-67

expression than HER2-zero/HR-positive breast cancers.[9,10]

Compared with HER2-zero breast cancers, HER2-low cancers generally demonstrate lower response rates to neoadjuvant chemotherapy.[9,21,26,29-32]

Denkert *et al.*[9] analyzed 2,310 HER2-negative breast cancers from four neoadjuvant clinical trials, including 1,098 HER2-low and 1,212 HER2-zero breast cancers, and reported longer disease-free survival among patients with HER2-low disease. Several studies have also reported more favorable disease-free survival among patients with HER2-low/HR-positive breast cancer.[33-35]

Zhang *et al.*,[27] in a study of 281 consecutive breast cancers, found that HER2-low breast cancers were more frequently associated with clinical stage I–II disease at presentation.

HER2-low status is not consistently maintained during disease progression, with approximately 30%–40% of breast cancers changing HER2 category between primary and metastatic specimens or between sequential biopsies of the same lesion.[36,37] This observation highlights the importance of reassessing HER2 expression in recurrent or metastatic disease when treatment decisions may be affected.

HER2-ultralow breast cancer

The terms "HER2-ultralow" and "HER2-null" breast cancer were introduced by the DESTINY-Breast06 clinical trial in 2024. HER2-ultralow breast cancer is clinically defined as breast cancer with an IHC score of 0 and faint and incomplete membrane staining in ≤10% of breast cancer cells (reported as score 0+ in the current CAP protocol),[38] whereas HER2-null breast cancer is clinically defined as a tumor with IHC score 0 and complete absence of membrane staining (reported as score 0 in the current CAP protocol).[38]

HER2-ultralow breast cancer accounts for approximately 15%–36% of all breast cancers, although the reported incidence varies depending on the patient population, antibody platform, and scoring methodology employed.[39-44]

Several studies suggest that HER2-ultralow tumors are biologically and clinically more similar to HER2-low tumors than to HER2-null tumors.[39,41] Compared with HER2-null cancers, HER2-ultralow tumors are associated with significantly higher rates of ER and progesterone receptor (PR) positivity,[39,41,43,44] older age at diagnosis (mean age approximately 56–61 years versus 50–57 years for HER2-null tumors),[39-41] and lower histologic grade. HER2-ultralow tumors are also associated with a substantially lower prevalence of triple-negative breast cancer (TNBC) than HER2-null cancers.[39,43]

When stratified by ER status, Hu *et al.*[39] reported that, among ER-positive cancers, HER2-ultralow tumors were associated with older patient age (61.9 *vs.* 58.1 years, $P = 0.0458$), fewer grade 3 tumors (7.4% *vs.* 31.7%, $P < 0.0001$), and fewer PR-negative tumors (12.0% *vs.* 23.3%, $P = 0.0361$) compared with HER2-null tumors. However, no significant clinicopathologic differences were observed between HER2-ultralow/ER-positive and HER2-low/ER-positive cancers, suggesting clinicopathologic similarity between HER2-ultralow and HER2-low tumors.

Regarding histologic subtype, HER2-ultralow tumors are predominantly invasive ductal carcinomas, whereas HER2-null tumors appear to have a relatively higher frequency of invasive lobular carcinoma (ILC).[39,40] This finding appears contradictory to the observation that HER2-ultralow tumors are associated with a lower prevalence of TNBC. However, both TNBC enrichment and ILC enrichment can coexist because they involve different tumor subsets. Across large cohorts, HER2 protein expression is positively correlated with ER expression; the proportion of HER2-low tumors increases with ER positivity, whereas ER-negative/basal-like tumors cluster within the HER2-null category.[24] This may explain why HER2-null tumors are enriched for triple-negative, ER-negative, grade 3, and high Ki-67 expression. In the study by Hu *et al.*,[39] HER2-null tumors exhibited a much higher frequency of TNBC than HER2-ultralow tumors (42.6% *vs.* 16.2%; $P < 0.0001$). Importantly, once cases were stratified by ER status, the differences between HER2-null and HER2-ultralow tumors were no longer evident within the ER-negative subgroup, indicating that the observed differences may largely reflect differences in hormone receptor distribution rather than intrinsic HER2 biology.

In addition, the majority of TNBCs are invasive ductal carcinomas, with ILC accounting for only approximately 1%–3%.[45] Classic ILC is overwhelmingly ER-positive and HER2-negative but also exhibits an intrinsically low rate of *ERBB2* amplification and expression.[46] In a study of approximately 8,000 tumors, HER2-negative ILCs showed a significantly higher frequency of HER2-zero tumors (59.4% *vs.* 53.7%) and a lower frequency of HER2-low tumors than HER2-negative ductal carcinomas ($P < 0.001$).[47] Thus, ILC represents a notable exception to the general ER–HER2 correlation: despite high ER expression, its intrinsically low *ERBB2* expression shifts it disproportionately toward the HER2-null rather than the HER2-ultralow category.

The tumor immune microenvironment also appears to differ among these categories. Takada *et al.*[41] reported that HER2-null tumors exhibited a significantly higher density of tumor-infiltrating lymphocytes compared with HER2-ultralow tumors (50.0% *vs.* 36.8%, $P = 0.016$). In the same study, overall survival was significantly worse in HER2-null tumors than in non-HER2-null tumors ($P = 0.026$). HER2-ultralow tumors were reported to have an intermediate prognosis between HER2-low and HER2-null tumors.

The molecular basis of HER2-ultralow disease remains incompletely understood. In an analysis of 723 residual tumors from the Penelope-B trial, Denkert *et al.*[42] found no significant difference in HER2 mRNA expression between HER2-ultralow and HER2-null tumors ($P = 0.08$), whereas significant differences were observed between HER2-ultralow and HER2-low tumors ($P < 0.0001$) and between HER2-low and HER2-positive tumors ($P < 0.0001$). These findings suggest that HER2-ultralow breast cancer may not represent a distinct molecular subtype and may instead reflect the lower end of a continuous spectrum of HER2 expression.

Hu *et al.*[39] reported that HER2-ultralow breast cancers exhibited significantly lower *HER2* copy numbers and *HER2/CEP17* ratios than HER2-low breast cancers ($P < 0.0001$) but were indistinguishable from HER2-null breast cancers. Similar findings were observed when analyses were restricted to ER-positive breast cancers. Findings reported by Denkert and Hu may appear inconsistent with observations that the clinicopathologic features of HER2-ultralow breast cancers more closely resemble those of HER2-low than HER2-null tumors. However, they

describe different aspects of tumor biology. The apparent discrepancy may be explained once the distinction between clinicopathologic phenotype (largely driven by hormone receptor biology) and molecular substrate (*ERBB2* gene dosage) is made explicit. The clinicopathologic features driving the resemblance between HER2-ultralow and HER2-low tumors, such as ER/PR positivity, lower grade, lower Ki-67, older age, and lower prevalence of TNBC, are largely influenced by hormone receptor status rather than *HER2* gene biology. HER2 protein expression is positively correlated with ER expression; therefore, tumors with even faint membranous staining tend to be ER-positive, luminal, and lower grade, whereas completely unstained tumors are disproportionately ER-negative, triple-negative, and higher grade.[39,44] These clinicopathologic characteristics may reflect hormone receptor biology rather than directly indicate *ERBB2* activation. Therefore, HER2-ultralow tumors resemble HER2-low tumors from a clinicopathologic perspective, yet at the level of *ERBB2* gene copy number they occupy the same non-amplified, near-diploid range as HER2-null tumors. The minimal increase in detectable HER2 protein expression in ultralow tumors may not correspond to a meaningful increase in *ERBB2* gene dosage or transcript abundance. Because these represent different biological measurements, concordance between them should not necessarily be expected.

It is worth noting that Morrar *et al.*,[40] who analyzed 400 primary breast cancers diagnosed in 2024 using the PATHWAY anti-HER2/neu (4B5) assay, found no significant clinicopathologic differences among HER2-null, HER2-ultralow, and HER2-low groups.

In summary, most studies suggest that HER2-ultralow breast cancers are clinicopathologically closer to HER2-low breast cancers than to HER2-null breast cancers. Nevertheless, distinguishing between these clinical categories remains challenging because low-level HER2 protein expression and small differences in *HER2* gene copy number are particularly susceptible to pre-analytic, analytic, and interpretive variability. Additional molecular and clinical studies are needed to determine whether HER2-ultralow disease represents a biologically distinct entity or simply part of a continuum of HER2 expression within pathologically HER2-negative breast cancer.

ASCO/CAP current update

Based on the therapeutic efficacy of T-DXd and emerging studies on HER2-low and HER2-ultralow breast cancer, ASCO/CAP updated the HER2 testing guidelines in 2023. The updated guideline states that the creation of new pathological interpretive categories such as “HER2-low” and “HER2-ultralow” remains premature and is not recommended for determining HER2 status in pathology reports.[8] HER2 testing methods and reporting recommendations remain unchanged from the 2018 ASCO/CAP recommendations. Nevertheless, the current CAP Breast Biomarker Reporting Protocol distinguishes two negative categories at the lowest end of HER2 expression: score 0, with no membrane staining, and score 0+, with faint, incomplete membrane staining in ≤10% of tumor cells.[38] Figure 1 summarizes the clinically relevant HER2 testing algorithm and its associated therapeutic implications.[7,38]

Challenges in HER2 testing: HER2 assessment variability

Since the introduction of the ASCO/CAP HER2 testing guidelines in 2007, HER2 testing has faced numerous challenges, particularly variability in HER2 assessment. This review focuses on HER2 assessment variability and strategies to overcome it.

Variability in HER2 assessment has been recognized at both the protein expression and gene amplification levels. This variability may arise from interobserver differences, interlaboratory differences, specimen handling and processing, tumor-related factors (e.g., intratumoral heterogeneity), among other factors. With the emergence of the clinically relevant categories of HER2-low, HER2-ultralow, and HER2-null breast cancers, a new question has arisen: do these categories exacerbate existing challenges or help reduce them? Here, we discuss variability in two main areas: interobserver variability and intratumoral heterogeneity.

HER2 assessment variability: Interobserver variability

Three aspects of interobserver variability are examined: incidence, contributing factors, and strategies to improve concordance.

Incidence

The degree of interobserver agreement varies substantially across the HER2 IHC scoring spectrum. Evidence has shown that interobserver variability is greatest at the lower end of HER2 protein expression, particularly when distinguishing IHC scores 0 and 0+ combined from IHC score 1+. Overall percent agreement (OPA) for identifying IHC scores 0 and 0+ combined has been reported to be as low as 26%, whereas agreement for identifying HER2 IHC score 3+ breast cancers is substantially higher, approaching 85% OPA.[48]

Robbins *et al.*[49] evaluated 170 breast cancer biopsies independently scored by 18 breast pathologists from 15 institutions. Complete agreement was achieved in fewer than 1% of IHC score 1+ cases and in only 25% of combined IHC score 0 and 0+ cases (Fleiss $\kappa = 0.49$). Even when up to 10 pathologists were included in the analysis, the OPA for distinguishing combined IHC scores 0 and 0+ from scores 1+, 2+, and 3+ plateaued at only 59.4%, compared with 87.1% for distinguishing IHC score 3+ from the combined group of IHC scores 0 and 0+, 1+, and 2+ cases.

Baez-Navarro *et al.*[50] evaluated 105 HER2-nonamplified breast cancers scored by 16 pathologists using the 2018 ASCO/CAP criteria. Only 4.7% of cases achieved complete agreement among all observers. Reproducibility improved when categories were grouped; the highest concordance (74.3% complete agreement) was observed when comparing HER2 IHC score 0 without staining against a combined HER2-ultralow/HER2-low/HER2 IHC 2+ category.

Eren *et al.*[51] assessed 116 breast cancer cases and reported that overall interobserver agreement was highest for HER2 IHC score 3+ cases ($\kappa = 0.76$–0.78) but was substantially lower for HER2 IHC scores 1+ and 2+ ($\kappa = 0.59$–0.60).

Karakas *et al.*[52] studied 114 breast cancer specimens reviewed by six breast pathologists using both HercepTest and Ventana 4B5 assays. Concordance for distinguishing combined HER2 IHC scores 0 and 0+ from IHC score 1+ was higher than that reported

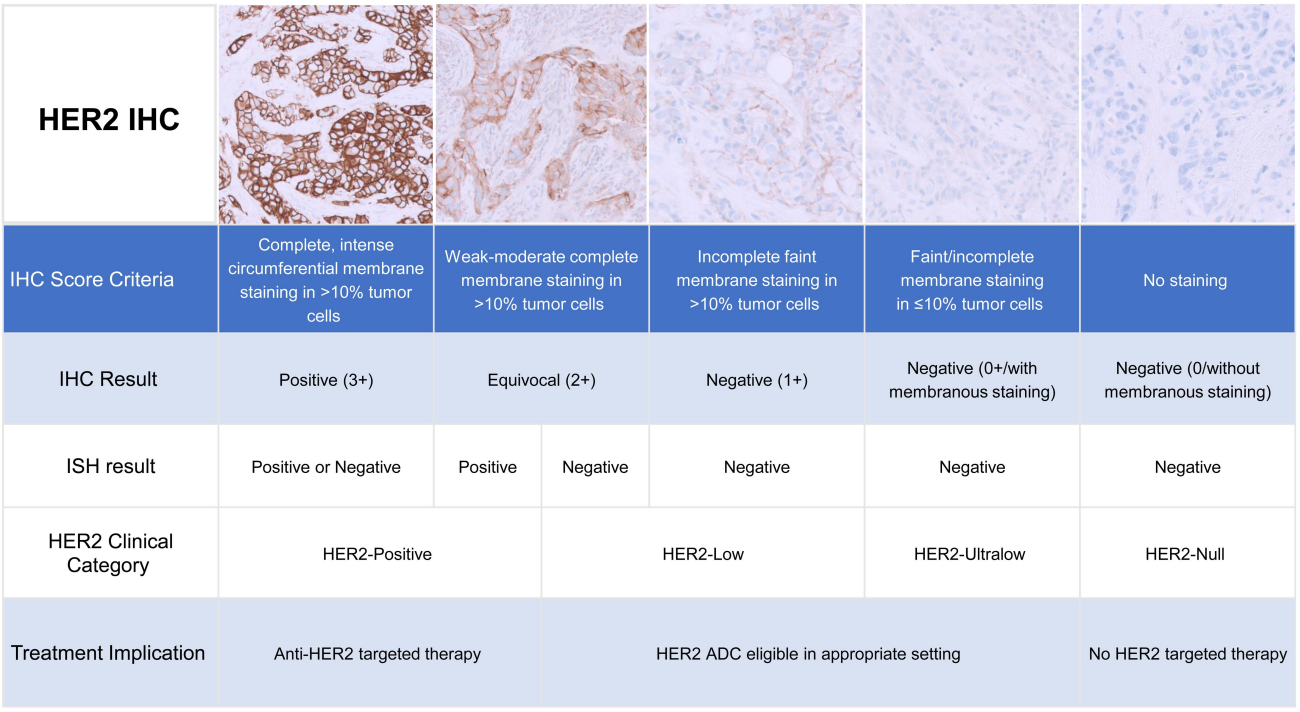


Fig. 1. Summary of the HER2 IHC scoring criteria, associated testing algorithm, and HER2 clinical categories and treatment implications.[7,38] The magnifications of the five representative HER2 IHC images (from left to right) are 5×, 20×, 40×, 40×, and 40×, respectively. ADC, antibody–drug conjugate; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; ISH, *in situ* hybridization.

in most previous studies, reaching 78.1% for HercepTest and 72.2% for Ventana 4B5. Nevertheless, complete interobserver agreement was achieved in only 44.7%–45.6% of cases.

Tseng *et al.*[53] evaluated 60 breast cancer cases scored by nine breast pathologists and demonstrated particularly poor reproducibility among HER2-low breast cancers. Among pathologist pairs, 50% exhibited κ values of 0 or lower, indicating no meaningful agreement, and only 14% achieved substantial agreement.

Contributing factors to interobserver variability

The factors contributing to interobserver variability are summarized as follows:

Subjective interpretation

HER2 IHC assessment relies on visual estimation of staining intensity, completeness, and the percentage of positive breast cancer cells. Consequently, interpretation is inherently subjective and susceptible to observer variation.

Recognition of staining patterns

Distinguishing true faint membrane staining from staining artifacts can be challenging. Tseng *et al.*[53] identified faint and heterogeneous membrane staining as the most common source of disagreement at the threshold between combined HER2 IHC scores 0 and 0+ and IHC score 1+. Cytoplasmic blush may be misinterpreted as weak membranous staining, while dislodged tumor cells and ductal carcinoma *in situ* may be inadvertently included in the assessment of invasive carcinoma.

Cases near the 10% threshold

Breast cancers with staining involving approximately 10% of tumor cells are inherently difficult to classify because small differences in interpretation can change the final HER2 score from combined 0 and 0+ to 1+ or from 1+ to 2+.[48]

Strategies to improve concordance among observers

Given the substantial interobserver variability associated with HER2 IHC interpretation, particularly at the lower end of the HER2 expression spectrum, several strategies have been proposed to improve scoring accuracy and reproducibility.

The 2023 ASCO/CAP HER2 testing guideline update emphasizes several best-practice recommendations for improving HER2 assessment, especially in distinguishing combined HER2 IHC scores 0 and 0+ from IHC score 1+ cases. These include the use of appropriate controls spanning a range of HER2 protein expression levels (including IHC score 1+ controls) and consideration of a second pathologist review for borderline cases. In addition, recognition of nonspecific staining artifacts, such as granular cytoplasmic staining, cytoplasmic blush, edge artifact, and dot-like staining, is important for improving concordance in HER2 assessment.[40]

The magnification rule has been suggested as a practical aid. It has been shown to reduce interobserver variability, particularly in distinguishing IHC score 0 from IHC score 1+ and IHC score 1+ from IHC score 2+ breast cancers.[48] HER2 IHC score 3+ staining should be readily appreciable at low-power magnification (2×–4×).[8,54] IHC score 2+ staining is clearly visible at intermediate

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magnification (10×–20×), whereas IHC score 1+ staining is discernible only at high-power magnification (40×). To improve discrimination among combined IHC scores 0 and 0+, and IHC score 1+, ASCO/CAP recommends initial screening at approximately 20× magnification, followed by confirmatory assessment at 40× magnification. If faint membranous staining is identified in more than 10% of invasive breast cancer cells, regardless of staining completeness, the tumor should be classified as IHC score 1+.

Finally, specialized training and standardized scoring conventions may improve reproducibility. Farshid *et al.*[55] demonstrated that a group of nine breast pathologists who developed HER2-low-specific scoring criteria achieved excellent interobserver agreement, with a mean Cohen κ value of 0.81 for distinguishing HER2-low from non-HER2-low breast cancers. Importantly, this level of agreement was maintained across two independent validation sets. However, educational interventions alone may not be sufficient. Tseng *et al.*[53] found that a single training session did not significantly improve concordance among pathologists, suggesting that limitations inherent to current HER2 IHC assays contribute substantially to variability at low expression levels.

HER2 assessment variability: Intratumoral heterogeneity

HER2 intratumoral heterogeneity (ITH) comprises two major forms:

1) Genetic heterogeneity refers to the coexistence of *HER2*-amplified and nonamplified tumor cell populations within the same tumor.[56,57] HER2 genetic heterogeneity exists when more than 5% but fewer than 50% of infiltrating tumor cells demonstrate a *HER2/CEP17* ratio > 2.2 by dual-probe testing (or > 6 *HER2* signals per cell by single-probe testing).[58] The reported incidence of intratumoral *HER2* amplification heterogeneity ranges from approximately 5% to 30% in the literature.[58]

Based on the spatial distribution of heterogeneous tumor cell populations, HER2 genetic ITH has been classified into three morphologic patterns[59,60]: a) Clustered (regional) pattern: a population of *HER2*-amplified tumor cells bordered by a *HER2*-nonamplified tumor cell population. b) Mosaic (intermixed) pattern: diffuse intermingling of amplified and nonamplified tumor cells within the same tumor. c) Scattered pattern: isolated *HER2*-amplified tumor cells dispersed within a predominantly nonamplified tumor cell population.

HER2 genetic ITH is observed more frequently in breast cancers with HER2 IHC score 2+ protein expression than in breast cancers with HER2 IHC score 3+ protein overexpression.[61-66] It was also more frequent in cases with HER2 IHC 1+ or 2+ than IHC 3+ ($P < 0.001$).[63]

2) Non-genetic (phenotypic) heterogeneity is characterized by variable HER2 protein expression detected by IHC within the same tumor without corresponding differences in gene amplification status.[56,57] However, there is currently no consensus definition of non-genetic (phenotypic) heterogeneity. Examples of HER2 IHC heterogeneity are shown in Figure 2.

In their study, Zhang *et al.*[27] defined HER2 IHC heterogeneous staining patterns as geographic differences in HER2 staining within the same tumor. The clustered (regional) pattern was defined as segregated populations of HER2-stained and unstained tumor cells. The mosaic (intermixed) pattern was defined as a tumor displaying HER2-stained cells intermingled with unstained tumor cells. The scattered pattern was defined as a tumor demonstrating isolated HER2-stained cells within a background of unstained tumor cells. Both mosaic and clustered patterns were observed in HER2-low breast cancers.

Wu *et al.*[67] defined HER2 IHC heterogeneity as the presence of different staining intensities and incomplete HER2 membrane staining in > 5% but < 50% of tumor cells. They further classified HER2 IHC ITH into three major types: scattered, clustered, and mixed, based on the distribution and staining intensity of tumor cells. They reported that 28% of HER2-ultralow cases and 86% of HER2 IHC score 1+ cases exhibited ITH. Scattered-type heterogeneity, followed by clustered and mixed types, was most common in HER2-ultralow cases, whereas all three patterns, especially the mixed type, were observed in HER2 score 1+ cases.

Based on the total percentage of cells exhibiting 2+ and 3+ staining intensity, Perez *et al.*[68] subjectively categorized staining patterns as focal (10%–29%), heterogeneous (30%–79%), or homogeneous (≥80%) in tumors with IHC scores of 2+ or 3+. The study showed that focal and heterogeneous HER2 IHC staining patterns were infrequently observed in tumors with IHC score 3+ (2.8% and 11.8%, respectively), whereas a much higher proportion of IHC score 2+ tumors (48.1%) exhibited focal staining.

Using a combined *HER2* gene and HER2 protein assay, Hou *et al.*[69] classified 64 cases into five patterns: Type 1, all tumor cells with homogeneous *HER2* gene amplification and HER2 protein overexpression ("classic" HER2-positive cells); Type 2, all tumor cells with homogeneous *HER2* gene amplification but without HER2 protein overexpression ("nonclassic" HER2-positive cells); Type 3, a mixture of classic HER2-positive cells and HER2-negative cells (no *HER2* gene amplification and no HER2 protein overexpression); Type 4, a mixture of classic and non-classic HER2-positive cells; and Type 5, a mixture of classic HER2-positive cells, non-classic HER2-positive cells, and HER2-negative cells. Types 1 and 2 were defined as homogeneous HER2 status, whereas Types 3–5 were considered heterogeneous HER2 status. Nineteen of the 64 cases (30%) demonstrated HER2 ITH.

HER2 ITH presents a significant challenge to accurate assessment of HER2 status by both IHC and ISH because sampling different regions of the same tumor may yield different HER2 results. HER2 ITH can also lead to discordance between IHC and ISH findings.[59,66,70]

To minimize the impact of HER2 ITH on HER2 assessment, the 2018 ASCO/CAP guideline recommends the following approaches[7]: a) Careful evaluation of the entire tumor section, including assessment of multiple tumor blocks when available. b) Separate ISH testing of morphologically distinct areas showing different HER2 IHC staining patterns.

Limitations

This review is based primarily on data collected before 2025, and most previous studies of HER2-zero breast carcinomas combined HER2-null and HER2-ultralow cases into a single category. Consequently, only limited data are currently available for HER2-ultralow breast carcinoma.

Although the measures discussed above have been implemented to improve diagnostic reproducibility and reduce variability in HER2 assessment, persistent challenges remain. The introduction of the new clinical categories of HER2-low, HER2-

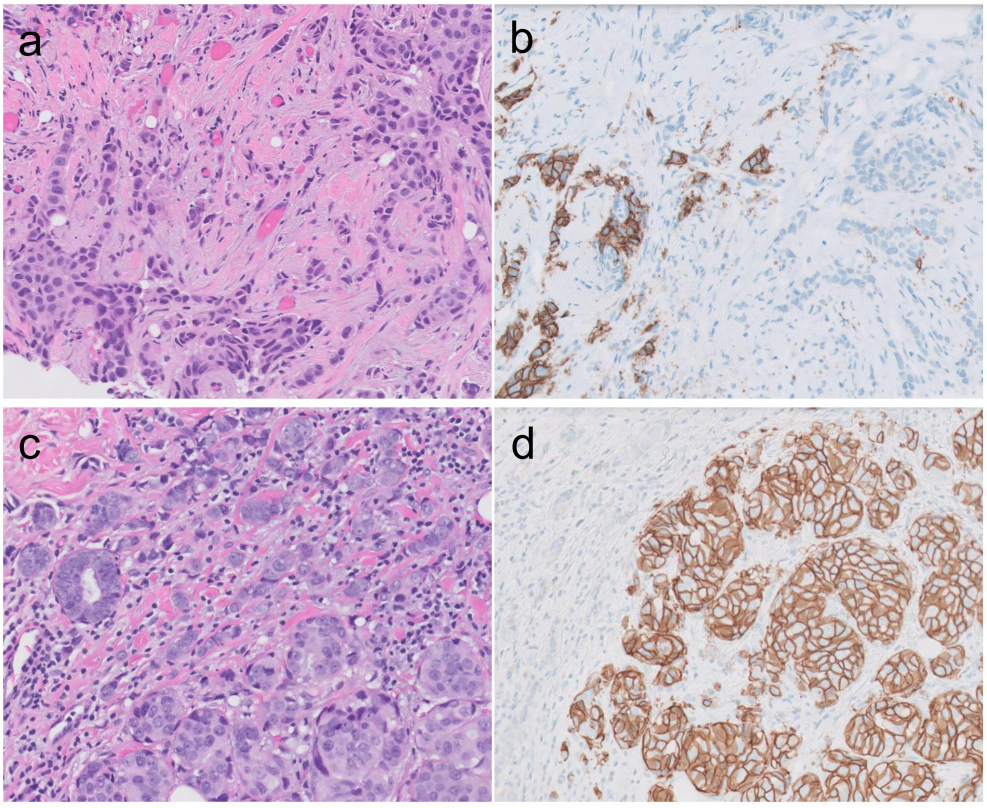


Fig. 2. Examples of intratumoral heterogeneity. (a, b) HER2 intratumoral heterogeneity with admixed HER2 IHC 3+ and HER2 IHC 0 breast cancer cell populations. (c, d) HER2 intratumoral heterogeneity with coexisting HER2 IHC 3+ and HER2 IHC 1+ breast cancer cell populations. Magnification: 20×. HER2 IHC antibody: VENTANA HER2 (4B5). HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry.

ultralow, and HER2-null has further complicated HER2 assessment. These categories require pathologists to accurately distinguish among HER2 IHC scores 0, 0+, and 1+.

Furthermore, other factors, such as differences in testing platforms, remain important contributors to discordance in HER2 assessment. Current testing methods are not sufficiently sensitive to objectively distinguish all HER2 IHC categories.

Future directions

The emergence of T-DXd and the recognition of HER2-low and HER2-ultralow breast cancer have fundamentally changed the clinical significance of HER2 testing. These developments raise important questions regarding whether current HER2 testing methods, reporting practices, and classification systems, originally developed to identify HER2 protein overexpression and gene amplification, can reliably identify HER2-low, HER2-ultralow, and HER2-null breast cancers. Based on current evidence, a major challenge is the poor reproducibility of HER2 IHC assessment at the lower end of the expression spectrum. With the success of the DESTINY-Breast06 clinical trial, accurately and reproducibly distinguishing HER2-null from HER2-ultralow tumors has become increasingly important. Several measures warrant consideration.

Classification system modifications

1) A proposed three-tier classification system of "HER2-null / HER2-ultralow+low / HER2-positive," which corresponds to HER2 IHC groups of 0/HER2 0+ plus HER2 1+ and HER2 2+ with negative ISH/HER2 2+ with positive ISH and HER2 3+, could be adopted. Jiang *et al.*[71] demonstrated that this scheme improved reproducibility compared with the HER2-null/HER2-ultralow/HER2-low/HER2-positive classification, with odds ratios of 1.472 versus 1.338, respectively ($P < 0.01$). 2) Baez-Navarro *et al.*[50] showed that the highest level of agreement was achieved when HER2 IHC 0+ (i.e., ultralow), IHC 1+, and IHC 2+ cases were clustered into a single group and compared with HER2 0 (i.e., null). Complete agreement among all pathologists was 74.3% (78/105), whereas agreement among 14 of 16 pathologists reached 80% (84/105). This study suggests that a modified version of the 2007 ASCO/CAP scoring criteria may be more reproducible than the 2018 criteria for distinguishing HER2-null from HER2-low breast cancers.

Development of new quality assurance programs

Current HER2 proficiency testing may not adequately evaluate laboratory performance in distinguishing HER2-null, HER2-ultralow, and HER2-low breast cancers. As discussed in the section on strategies to improve interobserver concordance, future

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quality assurance initiatives should include: 1) Standardization and validation of HER2 IHC 0, 0+, 1+, and 2+ reference materials. 2) External proficiency testing specifically targeting low-level HER2 protein expression. 3) Interlaboratory comparison programs focused on borderline HER2 cases. 4) Standardized educational and competency assessment programs for pathologists.

Development of novel HER2 assessment methods

Although studies have shown that HER2 protein expression exists along a broad continuum in breast carcinoma, the differences among HER2 IHC categories are often subtle, particularly at the lower end of the expression spectrum. Therefore, more sensitive, objective, and reproducible methods for HER2 assessment may ultimately be required. Several approaches have been proposed and are currently under investigation.

ISH

The recognition of HER2-low and HER2-ultralow breast cancer has raised important questions regarding the relationship between HER2 protein expression and *HER2* gene copy number. Studies have demonstrated statistically significant differences in *HER2* copy number among HER2-negative subgroups, although the degree of overlap between categories limits the clinical utility of ISH for discrimination.

In a study of 1,309 consecutive HER2-negative invasive breast cancers diagnosed between 2018 and 2021, Hu *et al.*[25] found that HER2-low breast cancers exhibited significantly higher mean *HER2* copy numbers and *HER2/CEP17* ratios than HER2-zero breast cancers ($P < 0.0001$). Similarly, Polónia *et al.*[72] demonstrated that breast cancers classified as HER2 IHC 1+ had significantly higher average *HER2* copy numbers than HER2 IHC 0 breast cancers when evaluated by bright-field ISH.

In a cohort comprising 1,486 primary invasive breast cancers, Hu *et al.*[39] reported that HER2-ultralow breast cancers exhibited significantly lower *HER2* copy numbers and *HER2/CEP17* ratios than HER2-low breast cancers ($P < 0.0001$) but were indistinguishable from HER2-null breast cancers. Similar findings were observed when analyses were restricted to ER-positive breast cancers.

Several independent studies have reached similar conclusions. Peiffer *et al.*[73] used data from the National Cancer Database to evaluate whether *HER2* ISH parameters could distinguish HER2-low from HER2-zero breast cancers. An optimal *HER2/CEP17* ratio cutoff of > 1.09 yielded a sensitivity of 76.6% but a specificity of only 36.8% for identifying HER2-low disease. Likewise, a *HER2* copy number cutoff of > 2.01 achieved a sensitivity of 61.1% and a specificity of 58.2%. The authors concluded that ISH parameters demonstrate poor discriminatory performance for identifying HER2-low breast cancers and should not be used as a surrogate for IHC classification.

Chen *et al.*[74] explored the possibility of using ISH as an alternative approach for distinguishing HER2-low from HER2-zero breast cancers. In proficiency-testing specimens and institutional tissue microarrays, IHC 1+ breast cancers exhibited significantly higher HER2 signal counts than IHC 0 breast cancers (median 2.6 *vs.* 2.0 and 2.0 *vs.* 1.7, respectively). However, substantial overlap existed between the groups.

Luen *et al.*[75] analyzed 1,795 centrally reviewed HR-positive/HER2-negative early breast cancers from the BIG 1–98 and SOFT clinical trials. They reported that *HER2* copy number was significantly higher in HER2-low than in HER2-zero breast cancers; however, the absolute difference was small.

In summary, these studies suggest that *HER2* copy number and *HER2/CEP17* ratios tend to be higher in HER2-low cancers than in HER2-null or HER2-ultralow cancers, whereas the latter two categories may overlap substantially. Nevertheless, the absolute differences are modest, and substantial overlap exists among the groups. As a result, ISH alone cannot reliably distinguish HER2-low, HER2-ultralow, and HER2-null breast cancers. Consistent with these observations, the 2023 ASCO/CAP HER2 Testing Guideline Update emphasizes that IHC remains the primary method for classifying HER2-low and HER2-ultralow disease, whereas ISH should continue to be reserved for reflex testing of equivocal (IHC 2+) cases.

Other methodologies are currently under investigation

Quantitative reverse-transcription polymerase chain reaction for HER2 transcript quantification[76-79]; RNAscope RNA *in situ* hybridization, which enables highly sensitive spatial assessment of HER2 mRNA expression within tissue sections[80,81]; targeted mass spectrometry for direct quantitative measurement of HER2 protein levels[82]; quantitative immunofluorescence, which provides objective and continuous measurement of HER2 protein expression[83-85]; and digital image analysis and artificial intelligence-assisted scoring systems, which have shown promise in improving reproducibility and reducing observer-dependent variability through objective assessment.[86,87] These technologies may ultimately provide more precise assessment of HER2 protein or RNA expression, particularly among HER2-null, HER2-ultralow, and HER2-low breast cancers, where current IHC methods exhibit the greatest limitations. As HER2-directed therapies continue to expand beyond traditionally HER2-positive disease, future diagnostic approaches will likely move away from purely categorical scoring systems toward more quantitative assessments of HER2 expression at the protein and RNA levels.

Conclusions

The HER2 testing guidelines have undergone several revisions since their initial publication by ASCO/CAP in 2007 in response to accumulating evidence and recognized assessment variability. With the success of T-DXd in treating patients with low levels of HER2 protein expression and the emergence of new clinical categories such as HER2-low, HER2-ultralow, and HER2-null, the need to accurately and reproducibly distinguish all HER2 IHC categories has become increasingly important. However, studies have shown greater interobserver discordance among tumors with low levels of HER2 expression. Revisiting HER2 testing guidelines, implementing measures to reduce variability in HER2 assessment, and developing more sensitive, objective, and reproducible detection methods appear necessary to improve HER2 assessment.

The 2023 ASCO/CAP guideline update acknowledges that some HER2 IHC 0 cases, including both 0 and 0+ categories, may have low levels of HER2 protein expression detectable by more sensitive assays. The guideline also recognizes that current HER2 IHC assays were originally optimized to detect HER2 protein overexpression rather than to distinguish among low levels of HER2 expression. Therefore, additional studies and clinical trial

data are needed to determine whether newer, more sensitive, reproducible, quantitative, and objective methods can accurately quantify low-level HER2 protein expression and whether differences in HER2 expression are associated with differential clinical benefit from HER2-targeted therapies.

At present, no alternative method has been validated. Consequently, HER2 IHC remains the standard method for HER2 assessment despite its recognized limitations, particularly at the HER2-low and HER2-ultralow thresholds. Ultimately, advances in HER2 testing may lead to a more refined pathological classification of HER2 subgroups.

Finally, it is worth mentioning the ongoing DESTINY-Breast15 clinical trial evaluating T-DXd in patients with HER2-low or HER2 IHC 0 (including both 0 and 0+) unresectable or metastatic breast cancer. This study may help address an important question: whether T-DXd is effective in patients with HER2-null disease. If efficacy is demonstrated in HER2-null patients, the HER2 IHC threshold that currently determines eligibility for T-DXd could become less clinically relevant. Such findings could broaden the population of patients with HER2-negative metastatic breast cancer who may benefit from T-DXd. Under that scenario, a more refined and clinically meaningful HER2 scoring system would likely be required.

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Conflict of interest

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Author contributions

Conception and design (YH, ZL), drafting the manuscript (YH, GT, DJ, WZ, AP, ZL). All authors have approved the final version and publication of the manuscript.

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