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ORIGINAL ARTICLE

Analytical and clinical validation of PATHWAY HER2 (4B5) assay for assessment of HER2-low/HER2-ultralow status and eligibility for trastuzumab deruxtecan in DESTINY-Breast06

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Background: The DESTINY-Breast06 study demonstrated a statistically significant and clinically meaningful improvement in progression-free survival benefit with trastuzumab deruxtecan (T-DXd) versus chemotherapy treatment of physician's choice in patients with hormone receptor-positive metastatic breast cancer (mBC) whose tumors were scored as human epidermal growth factor receptor 2 (HER2)-low [immunohistochemistry (IHC) 1+, or IHC 2+/*in situ* hybridization (ISH)-negative] and who had received one or more lines of endocrine therapy and no previous chemotherapy in the metastatic setting. An exploratory analysis consistently showed a benefit for patients with HER2-low and HER2-ultralow (IHC 0 with membrane staining) expression.

Materials and methods: Analytical validation of the PATHWAY HER2 (4B5) assay (Roche HER2 4B5 assay; Roche Diagnostic Solutions) at the HER2-ultralow cut-off was carried out, including intermediate precision, inter-reader precision, and inter-laboratory reproducibility. Patients with tumors historically classified as HER2-negative (IHC 0, 1+, and 2+/ISH-negative) based on pre-existing local test results were eligible for screening for DESTINY-Breast06; tumor samples taken in the metastatic setting were assessed centrally to confirm eligibility regarding HER2 status. Additionally, central and pre-existing HER2 test results were compared, and the prevalence of IHC scores based on central test results was assessed.

Results: Intermediate precision met the pre-specified endpoints across all parameters tested; agreement was observed within and between readers and sites, demonstrating that the HER2-ultralow cut-off can be scored accurately and reproducibly using the Roche HER2 4B5 assay (intra- and inter-laboratory and inter-reader agreement $\geq$95%). A clinically meaningful progression-free survival (hazard ratios 0.43-0.78) and objective response rate (odds ratios 2.5-4.5) improvement for T-DXd over chemotherapy was consistently observed across IHC expression levels. For cases with a pre-existing HER2 IHC 0 result, central testing found 24% with HER2-low, 40% with IHC 0 with membrane staining, and 35% with IHC 0 absent membrane staining.

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Conclusions: For patients with HER2 IHC 0 mBC, rescoring and/or retesting with the Roche HER2 4B5 assay is encouraged to determine eligibility for T-DXd.
Key words: HER2-low, HER2-ultralow, trastuzumab deruxtecan, metastatic breast cancer, 4B5

INTRODUCTION

In breast cancer (BC), human epidermal growth factor receptor 2 (HER2)-targeted antibody treatments have improved outcomes for patients with tumors that express high levels of HER2 (i.e. HER2-positive BC tumors),[1,2] defined as immunohistochemistry (IHC) 3+ or IHC 2+ with gene amplification [*in situ* hybridization (ISH)-positive]. However, ∼70% of United States patients with BC have tumors that are hormone receptor-positive (HR-positive) but are considered to be HER2-negative (IHC 0, IHC 1+, IHC 2+/ISH-negative) by the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines.[3-5] Among this HR-positive, HER2-negative population, it is estimated that ∼60%-65% of patients may have tumors that are HER2-low (IHC 1+, IHC 2+/ISH-negative).[6,7] The remaining ∼35%-40% of the HR-positive, HER2-negative population comprises patients whose tumors are classified as HER2 IHC 0 according to the ASCO/CAP guidelines.[5-7] Within this category, a proportion of tumors nevertheless express some level of HER2, detectable by IHC assay and characterized by the observation of membrane staining in a small proportion of tumor cells.[5] Such tumors can be classed as HER2 IHC 0 with membrane staining (or 'HER2-ultralow'). It is estimated that ∼20%-25% of tumors with HR-positive, HER2-negative status per the ASCO/CAP guidelines are actually HER2-ultralow.[7,8] The remainder of HER2 IHC 0 tumors (per the ASCO/CAP guidelines) where no staining is observed can be termed HER2 IHC 0 absent membrane staining.

Trastuzumab deruxtecan (T-DXd) is an antibody—drug conjugate composed of a humanized immunoglobulin G1 monoclonal antibody specifically targeting HER2, a tetrapeptide-based cleavable linker, and a potent topoisomerase I inhibitor payload.[9,10] T-DXd is approved in the United States (and elsewhere) for adult patients with unresectable or metastatic HER2-positive (IHC 3+, ISH+) BC who have received a prior anti-HER2-based regimen, and was recently approved in the United States and the European Union for patients with HR-positive, HER2-low (IHC 1+, IHC 2+/ISH-negative) or HER2-ultralow (IHC 0 with membrane staining) BC following disease progression after one or more endocrine therapies in the metastatic setting; the latter extends the previous approval of T-DXd for use after prior chemotherapy in the metastatic setting, or after disease recurring within 6 months of completing adjuvant chemotherapy, in patients with HER2-low BC.[11-14] The approvals by the United States Food and Drug Administration (FDA) and the European Union for use in patients with HER2-low and HER2-ultralow tumors were based on results from the DESTINY-Breast04 and DESTINY-Breast06 studies.[11-16] Results from DESTINY-Breast04 contrasted with previous reports that found trastuzumab-containing regimens offered no clinical benefit to patients with HER2-low tumors.[17,18] The ASCO/CAP guidelines were updated in 2023 to reaffirm previous guidelines (specifically, IHC 3+ is denoted as HER2-positive, IHC 2+ is equivocal, and IHC 1+ and IHC 0 are HER2-negative); none the less, best practice outlined in the guidelines aims to ensure that patients who may be eligible for treatment with T-DXd (i.e. those with HER2-low BC) are identified.[5]

DESTINY-Breast06 (NCT04494425) was a randomized, open-label, phase III study that enrolled 866 patients with HR-positive metastatic BC (mBC) characterized as HER2-low or HER2-ultralow. Eligible patients had received one or more lines of endocrine therapy, but no prior chemotherapy in the metastatic setting. Central testing of tumor samples was used to determine HER2 status and was carried out using the PATHWAY HER2 (4B5) assay (henceforth referred to as Roche HER2 4B5 assay; Roche Diagnostic Solutions, Tucson, AZ) (see Supplementary Materials, available at https://doi.org/10.1016/j.esmoop.2025.105310). T-DXd demonstrated a statistically significant and clinically meaningful improvement in progression-free survival (PFS) compared with chemotherapy treatment of physician's choice (TPC) in patients with HER2-low mBC (median PFS of 13.2 versus 8.1 months; hazard ratio 0.62, $P < 0.001$). The results were consistent in the intent-to-treat (ITT) population (median PFS of 13.2 versus 8.1 months; hazard ratio 0.64, $P < 0.001$), which comprised the HER2-low and HER2-ultralow groups.[16] On the basis of these results, T-DXd became the first HER2-directed therapy for patients with HR-positive, HER2-low, or HER2-ultralow mBC.[12]

In this article, we outline the analytical validation of the Roche HER2 4B5 assay at the HER2-ultralow cut-off and provide insights into central HER2 testing results as well as the efficacy of T-DXd by tumor characteristics in DESTINY-Breast06.

MATERIALS AND METHODS

HER2 IHC scoring method

IHC scoring was carried out according to the Roche HER2 4B5 assay product-specific scoring algorithm, which was adapted from the 2018 ASCO/CAP guidelines; the IHC 0 category was further divided into two subcategories: IHC 0 *absent* membrane staining and IHC 0 *with* membrane staining (HER2-ultralow).

The breast tissue section was examined for the intensity and completeness of cell membrane staining (Figure 1). The criteria for scoring were as follows:

- IHC 0 'absent' membrane staining: no membrane staining is observed
- IHC 0 'with' membrane staining (HER2-ultralow): any staining of the membrane in >0 and ≤10% of the cancer cells

- IHC 1+: faint, partial staining of the membrane in >10% of the cancer cells
- IHC 2+: weak-to-moderate complete staining of the membrane in >10% of the cancer cells
- IHC 3+: intense, complete staining of the membrane in >10% of cancer cells

Review at 40× magnification was recommended to discern the presence or absence of any staining such as faint, partial staining (i.e. to discriminate between scores of IHC 0 absent membrane staining, IHC 0 with membrane staining, and IHC 1+). Staining in BC cells using the Roche HER2 4B5 assay can be membranous and/or cytoplasmic. Cytoplasmic-only staining is not known to be clinically relevant[19] and thus was excluded from HER2 status determination. The guidelines for assessing HER2 IHC cut-offs are included in the instructions for use and interpretation guide for the Roche HER2 4B5 assay.

Pre-analytical and staining protocol and analytical validation studies

Pre-analytical conditions and the staining protocol are given in the Supplementary Material, available at https://doi.org/10.1016/j.esmoop.2025.105310. Methods for the analytical validation, including within-case and within-block heterogeneity, comparison of primary and metastatic matched samples, and precision and reproducibility studies are described in the Supplementary Materials, available at https://doi.org/10.1016/j.esmoop.2025.105310.

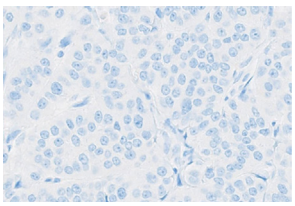
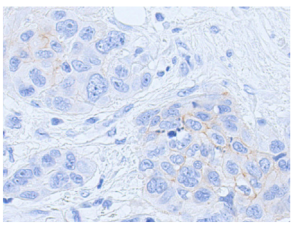
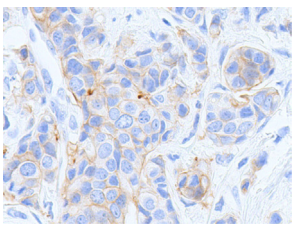
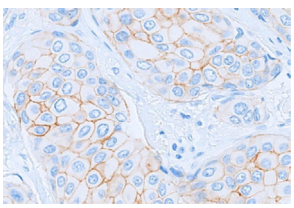
IHC staining pattern	HER2 (4B5) score	Recommended reporting status	Representative images
No membrane staining is observed[a]	IHC 0 *absent* membrane staining	HER2-null	
Any staining of the membrane in >0 and ≤10% of the cancer cells[a,b,c]	IHC 0 *with* membrane staining	HER2-ultralow expression	
Faint, partial staining of the membrane in >10% of the cancer cells[a]	IHC 1+	HER2-low expression	
Weak to moderate complete staining of the membrane in >10% of the cancer cells[d]	IHC 2+ *Reflex test: HER2 non-amplified*	HER2-low expression	
	IHC 2+ *Reflex test: HER2 amplified*[e]	HER2-positive/ overexpression	

Figure 1. HER2 IHC scoring patterns in BC and categorization used in DESTINY-Breast06 inclusion criteria. The IHC 3+ cut-off is not shown because patients whose tumors were scored as IHC 3+ were ineligible for DESTINY-Breast06.
ASCO/CAP, American Society of Clinical Oncology/College of American Pathologists; BC, breast cancer; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; ISH, *in situ* hybridization.
[a]Review at 40× magnification is recommended to discern the presence or absence of any staining such as faint, partial staining.
[b]Recommend re-reading by a second pathologist for cases with 'faint, partial staining of the membrane' and percentage tumor cells ≤5%.
[c]In the HER2-ultralow 'IHC 0 with membrane staining' category, partial membranous staining is usually faint but may exhibit stronger intensities, and such rare cases are scored as HER2-ultralow if they do not otherwise qualify for a higher score. Refer to the Interpretation Guide for case examples.
[d]Recommend reflex test to assess gene amplification per ASCO/CAP guidance.
[e]Patients whose tumors were IHC 2+/ISH+ were ineligible for DESTINY-Breast06.

HER2 testing in DESTINY-Breast06

The DESTINY-Breast06 study was conducted in accordance with the International Council for Harmonisation Good Clinical Practice guidelines, the Declaration of Helsinki, and local regulations on the conduct of clinical research. All patients provided written informed consent. The clinical methodology for DESTINY-Breast06 has been reported previously.[16]

Patients with tumors that had a pre-existing local (historical) classification as HER2-negative per the ASCO/CAP guidelines,[4] defined as IHC 2+/ISH-negative, IHC 1+, or IHC 0, were eligible for screening. Tumor samples were evaluated at a central laboratory to determine HER2 status; patients whose tumors were HER2-low or HER2-ultralow by central testing were considered for study enrollment. The analyses presented here include all patients with valid central HER2 results, those randomized to DESTINY-Breast06, and subgroups thereof.

Clinical sites were provided with a pathology manual that included guidance on pre-analytical and sample collection practices to maximize quality in alignment with manufacturer guidance. A sample collected at the time of metastatic disease or later was required for central testing. Samples were either formalin-fixed, paraffin-embedded (FFPE) tumor tissue blocks or tumor slides. Further details on sample collection are provided in the Supplementary Material, available at https://doi.org/10.1016/j.esmoop.2025.105310.

Central testing was conducted at Labcorp Geneva, Switzerland, Labcorp Los Angeles, CA, or Labcorp Shanghai, China, using the Roche HER2 4B5 assay (investigational for HER2-low and HER2-ultralow cut-offs when the DESTINY-Breast06 study started, but subsequently approved for HER2-low and HER2-ultralow) and scored as described in the preceding text.[20,21] The pathologists received specific HER2-low and HER2-ultralow scoring training for assessment of BC tissue specimens. All study readers carried out a competency examination and achieved acceptable levels of performance [≥85% positive percent agreement (PPA) and negative percent agreement (NPA) with consensus scores] before reading for the trial. Per the Roche HER2 4B5 assay instructions for use, ISH status was confirmed for all samples scored as IHC 2+ using an on-market assay according to the manufacturer's guidelines.

Central and pre-existing HER2 test results were compared for patients screened for DESTINY-Breast06 where available. Pre-existing HER2 tests were conducted as part of routine clinical practice and did not form part of this study. Prevalences of IHC scores were determined using central test results.

Efficacy of T-DXd in tumor subgroups

PFS and objective response rate (ORR) were assessed by blinded independent central review (BICR) according to the Response Evaluation Criteria in Solid Tumours version 1.1 (RECIST 1.1). Subgroup analyses were carried out on all randomized patients (i.e. the ITT population). Hazard ratios and corresponding 95% confidence intervals (CIs) for PFS were estimated using an unstratified Cox proportional hazards model. Confirmed ORR was defined as the percentage of patients with a confirmed complete or partial response (according to RECIST 1.1). Odds ratios and associated 95% CI estimates were obtained using an unstratified logistic regression model. All subgroup analyses were exploratory; as such, results were not adjusted for multiplicity.

RESULTS

Analytical validation

Within-case and within-block heterogeneity studies were conducted to evaluate the variation that may exist between BC tissue blocks from the same patient and throughout BC tissue blocks. Out of 20 tissue blocks representing 10 patient cases (two blocks per patient) used to assess within-case heterogeneity, all 20 tissue blocks demonstrated concordant HER2-ultralow status within each case, resulting in an overall percent agreement (OPA) of 100%. Of the 15 FFPE cases used to characterize within-block heterogeneity, 14 cases showed consistent HER2-ultralow status between block sections. In the case that displayed heterogeneity, the change in clinical interpretation status between tissue sections was due to a reduction in viable tumor cells expressing HER2, as the block was sectioned to exhaustion.

The OPA for HER2-ultralow status between patient-matched primary and metastatic tumor specimens was 81.3% (26/32). Out of the six discordant cases for primary versus metastatic samples, two went from IHC 0 absent membrane staining to IHC 0 with membrane staining, two went from IHC 0 with membrane staining to IHC 0 absent membrane staining, and two went from IHC 1+ to IHC 0 absent membrane staining.

Across all intermediate precision parameters evaluated (between antibody lot, detection kit lot, instrument and day, within run), the PPAs and NPAs were 100% (Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2025.105310).

For intra-reader precision, the average positive agreement (APA) and average negative agreement (ANA) combined across readers was 97.7% (95% CI 95.9% to 99.3%) and 97.6% (95% CI 95.6% to 99.3%), respectively (Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2025.105310). For inter-reader precision, the APA and ANA combined across both reader pairs was 95.5% (95% CI 91.7% to 98.4%) and 95.2% (95% CI 91.1% to 98.2%).

The overall inter-laboratory reproducibility, measured across three sites with two pathologists per site, for HER2-ultralow status was 99.2% (95% CI 98.3% to 99.9%) with a PPA and NPA of 98.3% (95% CI 96.7% to 99.8%) and 100% (95% CI 99.1% to 100%), respectively (Table 1). The PPA and NPA agreement rates for within-site and within-reader reproducibility were all ≥98.3%. In addition, pairwise comparisons were made between sites, between readers, and between days for HER2-ultralow status. The between-site APA and ANA were 98.4% (95% CI 96.7% to 99.8%) and 98.4% (95% CI 96.9% to 99.8%), respectively. The

Table 1. Inter-laboratory reproducibility of the Roche HER2 4B5 assay on BC tissue with HER2-ultralow scoring on BenchMark ULTRA

Inter-laboratory reproducibility ($n = 28$)	Agreement			
	Type	*n/N*	%	95% CI
Overall	PPA	413/420	98.3	96.7-99.8
	NPA	420/420	100	99.1-100
Within-site	PPA	413/420	98.3	96.7-99.8
	NPA	420/420	100	99.1-100
Within-reader	PPA	413/420	98.3	96.7-99.8
	NPA	420/420	100	99.1-100
Between-site	APA	8124/8260	98.4	96.7-99.8
	ANA	8404/8540	98.4	96.9-99.8
Between-reader	APA	406/413	98.3	96.6-99.8
	ANA	420/427	98.4	96.8-99.8
Between-day	APA	1628/1652	98.5	97.2-99.8
	ANA	1684/1708	98.6	97.4-99.8

ANA, average negative agreement; APA, average positive agreement; BC, breast cancer; CI, confidence interval; HER2, human epidermal growth factor receptor 2; NPA, negative percent agreement; PPA, positive percent agreement.

between-reader APA and ANA were 98.3% (95% CI 96.6% to 99.8%) and 98.4% (95% CI 96.8% to 99.8%), respectively. The between-day APA and ANA were 98.5% (95% CI 97.2% to 99.8%) and 98.6% (95% CI 97.4% to 99.8%), respectively.

Patient disposition in DESTINY-Breast06

From July 2020 to April 2023, a total of 2311 patients were screened or pre-screened, and 1999 of these had tumor samples submitted for central testing. Of these, a valid central result was obtained from 1856 patients. There were 1629 tumor samples, for which both a valid central and pre-existing result were available. Of the 1616 patients whose tumor samples were centrally confirmed as HER2-low or HER2-ultralow, 1349 were screened and 866 were randomized into DESTINY-Breast06 using interactive response technology (IRT) software. Among the 866 randomized patients, 713 (82.3%) had HER2-low tumors and 153 (17.7%) had HER2-ultralow tumors (Supplementary Figure S1, available at https://doi.org/10.1016/j.esmoop.2025.105310). Patients with centrally confirmed HER2-low or HER2-ultralow status who were not randomized were screen failed for one or more of the reasons outlined in the eligibility criteria of the clinical study.[16] One patient with HER2-low status was incorrectly randomized into the HER2-ultralow group. As a result, this patient's data were excluded from the efficacy analysis in the HER2-ultralow group. Consequently, there were 152 patients in the HER2-ultralow group (based on the final central test result) instead of 153 (as per IRT).

Tumor sample characteristics

From the 2311 patients screened, 462 (20.0%) tumor samples were obtained from breast or regional lymph nodes, while 1537 (66.5%) were obtained from other metastatic sites [for the remaining 312 (13.5%) patients, a sample was not submitted for central testing] (Supplementary Table S2, available at https://doi.org/10.1016/j.esmoop.2025.105310). Approximately three-quarters of samples screened [1759 (76.1%)] were obtained via biopsy; 240 (10.4%) were from excisions/resections. A range of commercial tests were used for pre-existing tests, including HercepTest™ (Agilent, Santa Clara, CA) [322 (13.9%)], Roche HER2 4B5 assay (Roche Diagnostics Solutions, Tucson, AZ) [543 (23.5%)], Bond Oracle HER2 IHC system (Leica Biosystems, Nussloch, Germany) [27 (1.2%)], and others [62 (2.7%)]; however, in >50% of samples, the identity of the pre-existing test was either missing or otherwise unknown. Samples were collected in the USA (6.3%), Europe (41.6%), Asia (excluding China) (17.7%), and China (10.5%), with the rest of the world accounting for 10.4% of screened samples [for the remaining 312 (13.5%) patients, a sample was not submitted for central testing]. Samples ranged in age from <6 months (41.4%), 6 months to 1 year (10.0%), 1 to 3 years (23.5%), and >3 years (11.6%) [for the remaining 312 (13.5%) patients, a sample was not submitted for central testing]. Tumor characteristics of the randomized population were broadly similar to that of the screened population (Supplementary Table S2, available at https://doi.org/10.1016/j.esmoop.2025.105310).

HER2 status prevalence

Among patients screened for DESTINY-Breast06 with a valid central test result, 402/1856 (22%) tumor samples were scored as HER2-ultralow and 1214/1856 (65%) were scored as HER2-low. Prevalence across HER2 IHC scores were largely similar regardless of tumor location, sample type (Table 2), testing region, or sample age (data not shown). No obvious differences in prevalence of HER2-low or HER2-ultralow were observed by metastatic sample location (Supplementary Table S3, available at https://doi.org/10.1016/j.esmoop.2025.105310).

Of the 349 samples with a pre-existing HER2 IHC 0 score, central HER2 testing found 85 (24%) IHC 1+, IHC 2+/ISH-negative (HER2-low), 140 (40%) IHC 0 with membrane staining (HER2-ultralow), and 123 (35%) IHC 0 absent membrane staining (Table 3). Of the 1270 samples with a pre-existing HER2 IHC 1+ or IHC 2+/ISH-negative score (i.e. those that could be categorized as HER2-low), 999 (79%) were classed as HER2-low, 196 (15%) as HER2-ultralow, and only 65 (5%) as IHC 0 absent membrane staining, by central test.

Table 2. Central HER2 testing results in DESTINY-Breast06 by tumor sample characteristics

Variable, *n* (%)	IHC 0 absent membrane staining[a]	IHC 0 with membrane staining (HER2-ultralow)[b]	IHC 1+	IHC 2+/ISH−	IHC 2+/ISH+	IHC 3+	Total
Overall	225 (12)	402 (22)	829 (45)	385 (21)	11 (<1)	4 (<1)	1856
Sample type							
Biopsy	202 (12)	344 (21)	729 (45)	338 (21)	8 (<1)	4 (<1)	1625
Resection	23 (10)	58 (25)	100 (43)	47 (20)	3 (1)	0	231
Sample location							
Breast or regional lymph nodes	47 (11)	119 (27)	204 (46)	67 (15)	3 (<1)	1 (<1)	441
Metastatic	178 (13)	283 (20)	625 (44)	318 (22)	8 (<1)	3 (<1)	1415

HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; ISH, *in situ* hybridization.
[a]No membrane staining is observed.
[b]Faint, partial staining of the membrane in ≤10% of the cancer cells.

Efficacy of T-DXd in tumor subgroups

T-DXd consistently demonstrated a PFS benefit across sample types analyzed (i.e. breast/regional lymph node versus metastatic; biopsy versus resection, sample age) (Figure 2; Supplementary Figure S2, available at https://doi.org/10.1016/j.esmoop.2025.105310). A PFS benefit was also consistently shown for T-DXd versus TPC across IHC expression levels [IHC 0 with membrane staining (HER2-ultralow), 1+, or 2+/ISH-negative], albeit with numerical differences between hazard ratios (0.78, 0.73, and 0.43, respectively) (Figure 2). Confirmed ORR by BICR consistently showed odds ratios (2.5-4.5) that favored T-DXd versus TPC, across IHC scores (Supplementary Table S4, available at https://doi.org/10.1016/j.esmoop.2025.105310).

DISCUSSION

The approval of T-DXd for patients with HER2-low and HER2-ultralow mBC has highlighted the need for IHC scoring to go beyond the HER2-positive and HER2-negative categories as described in the ASCO/CAP guidelines.[5] The recent results from DESTINY-Breast06 showed the clinical relevance of the newly defined HER2-ultralow cut-off, thus highlighting the importance of accurate scoring at this cut-off.[16] Based on the data described here, the Roche HER2 4B5 assay was recently approved by the FDA for the selection of patients for T-DXd treatment. Currently, the Roche HER2 4B5 assay is the only FDA-approved HER2 IHC companion diagnostic assay for assessing HER2-ultralow expression.

The analytical validation presented here demonstrates that the HER2-ultralow cut-off in BC samples can be scored accurately and reproducibly using the Roche HER2 4B5 assay. The within-case and within-block concordance was very high, and a strong agreement within and between readers and sites was found. These results are consistent with those previously reported for the HER2-low cut-off.[22] In the majority of cases, results were consistent between a patient's primary and metastatic tumor samples, with just 6 out of 32 cases that were discordant. Some discordance is expected, however, owing to receptor conversion, which has been reported to occur in ∼8%-10% of patient-matched primary and metastatic samples.[23,24] Results from DESTINY-Breast04 and DESTINY-Breast06 demonstrated a PFS benefit for T-DXd versus TPC regardless of whether the samples used to confirm HER2 status were from primary or metastatic sites.[22] Overall, although digital pathology tools that make use of artificial intelligence will likely continue to evolve and help pathologists score HER2 cut-offs ever more accurately and reproducibly,[25,26] the consistent results demonstrated here with manual reading using the Roche HER2 4B5 assay provide reassurance regarding the current standard approach.

The results from central laboratory testing in the DESTINY-Breast06 study provide an assessment of the prevalence of patients with HER2-low (65%) or HER2-ultralow (22%) tumors in the HR-positive HER2-negative population. These proportions are in line with previous estimates[6-8] and, based on the results from DESTINY-Breast06, demonstrate that

Table 3. Pre-existing and central HER2 IHC test results in DESTINY-Breast06

HER2 status by central testing, *n*		HER2 status by pre-existing[a] result, *n*					
		IHC 0[b]	IHC 1+	IHC 2+/ISH−	IHC 2+/ISH+	IHC 3+	Total
IHC 0[b]	Absent membrane staining[c]	123	45	20	0	1	189
	With membrane staining[d] (HER2-ultralow)	140	146	50	2	1	339
IHC 1+		70	534	146	3	0	753
IHC 2+/ISH−		15	83	236	3	0	337
IHC 2+/ISH+		1	1	6	0	0	8
IHC 3+		0	1	2	0	0	3
Total		349	810	460	8	2	1629

HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; ISH, *in situ* hybridization.
[a]Pre-existing results were obtained at a local center and did not form part of this study.
[b]Per American Society of Clinical Oncology/College of American Pathologists 2018 guidelines.
[c]No membrane staining is observed.
[d]Faint, partial staining of the membrane in ≤10% of the cancer cells.

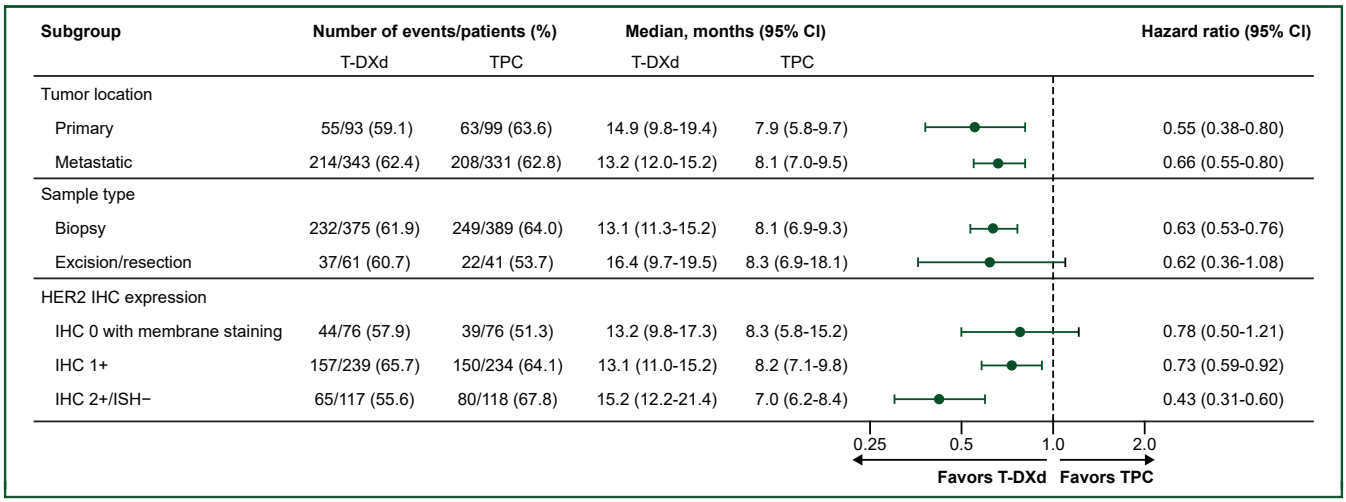


Figure 2. PFS by BICR by tumor characteristic subgroup, specimen collection type, and IHC expression subgroups.
BICR, blinded independent central review; CI, confidence interval; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; ISH, *in situ* hybridization; PFS, progression-free survival; TPC, chemotherapy treatment of physician's choice; T-DXd, trastuzumab deruxtecan.

many patients with a historical HER2-negative status could benefit from treatment with T-DXd.

Most samples that had a pre-existing IHC 0 score were found to have detectable and potentially actionable levels of HER2 (either HER2-low or HER2-ultralow), which suggests that reassessment of historically scored HER2 IHC 0 cases can be used to determine patients who may benefit from T-DXd. On the other hand, only a small proportion (5%) of samples that were HER2-low by local (historical) test showed no detectable level of HER2 (i.e. HER2 IHC 0 absent membrane staining) by central test, while 79% and 15% were classed as HER2-low and HER2-ultralow, respectively. Overall, therefore, the vast majority (∼94%) of samples that were HER2-low by local (historical) test were found centrally to have detectable and potentially actionable levels of HER2. There are several factors that need to be considered when interpreting our findings. The first is the status of the HER2-low and HER2-ultralow cut-offs in international guidelines. It is unsurprising that samples with a pre-existing HER2 IHC 0 score were found to be HER2-ultralow by central testing, given that the 2018 ASCO/CAP guidelines include both IHC 0 absent membrane staining and IHC 0 with membrane staining (HER2-ultralow) within the definition of HER2 IHC 0.[4] The European Society of Medical Oncology HER2-low Expert Consensus Statement does differentiate HER2-ultralow and HER2 IHC 0 absent membrane staining (referred to as HER2-null) as subcategories of HER2 IHC 0,[27] but it was published in 2023, so would not have been available to local pathologists during the time of the DESTINY-Breast06 study. By contrast, the HER2-low cut-off (including IHC 1+ and IHC 2+/ISH-negative) is differentiated from HER2 IHC 0 in the 2018 ASCO/CAP guidelines.[4] None the less, it is likely that the HER2-low cut-off had no clinical relevance at the time when pre-existing HER2 scores were collected; pathologists would therefore have been unlikely to have received training on these cut-offs and, consequently, awareness of these cut-offs is likely to have been low. In addition to issues of awareness, differences may have arisen owing to different assays or samples being used for the pre-existing test versus that used for central testing.[28,29] Therefore, agreement between central and pre-existing tests cannot be adequately assessed using these data.

The prevalence of IHC scores as determined centrally was consistent regardless of sample location, including by metastatic organ site, and no differences were observed by geographical region. Similarly, and in agreement with previous findings,[30] the prevalence of IHC scores was consistent between biopsy and resection samples. While the prevalence of IHC scores appeared generally consistent regardless of sample age, multiple confounding factors, such as storage conditions, prevent these data being meaningfully interpreted. As such, further data are required to more specifically understand the potential impact of sample age on HER2-low and HER2-ultralow scores.

The baseline tumor sample characteristics were balanced between the TPC and T-DXd arms.[16] A PFS benefit for T-DXd versus TPC was observed regardless of tumor sample characteristics, consistent with the results of DESTINY-Breast04.[22] This study confirms these findings for the HER2-low cut-off while additionally demonstrating that tumor samples obtained either through biopsy or resection can be used to identify tumors with HER2-ultralow expression.

Results from DESTINY-Breast06 showed a statistically significant and clinically meaningful PFS benefit versus TPC in patients with HR-positive, HER2-low mBC after one or more endocrine-based therapies, with similar results in the ITT population.[16] While this study was not powered to detect differences between subgroups, numerical differences in hazard ratios were observed across IHC levels, with a numerically greater benefit for T-DXd over TPC observed for the IHC 2+/ISH-negative group than for the IHC 1+ or IHC 0 with membrane staining groups. It should be noted that the sample size for the IHC 0 with membrane staining group was small, which limits the interpretation of this finding. Notably, a difference between IHC 1+ and IHC 2+/ISH-negative was not seen in DESTINY-Breast04, in which

hazard ratios were similar between these subgroups.[15] Furthermore, in DESTINY-Breast06, the same numerical trend was not observed for ORR across IHC levels. Overall, a clinical benefit was consistently observed across all HER2 IHC categories, and additional data would be required to explore any potential trend in more detail.

On the other hand, there are limited data on the effectiveness of T-DXd in patients with tumors scored as IHC 0 absent membrane staining. The DAISY trial included a small population ($n = 37$) of patients with a HER2 IHC 0 score from routine care. Upon review of 31/37 of the tumor samples by two pathologists to determine whether there was a detectable level of HER2 expression, 8 were scored as IHC 0 with membrane staining and 7 as IHC 1+.[31] In an exploratory analysis, a confirmed objective response was observed in 6/15 [40% (95% CI 16.3% to 67.7%)] patients found by the pathologist review to have detectable HER2 expression (i.e. IHC 0 with membrane staining or IHC 1+), and in 4/16 [25% (95% CI 7.3% to 52.4%)] of those without detectable HER2 expression (i.e. IHC 0 absent membrane staining).[31] Further data regarding T-DXd efficacy in patients with tumors scored as IHC 0 will be generated in the ongoing DESTINY-Breast15 study.[32]

In conclusion, identification of HER2-low and HER2-ultralow expression levels is clinically important and could be achieved with high accuracy and reproducibility using the Roche HER2 4B5 assay (and ISH when applicable). Patients with HR-positive mBC determined to be HER2-low or HER2-ultralow derived clinical benefit from T-DXd, irrespective of sample type or location used to determine HER2 status. As expected, some patients who had tumors with a pre-existing HER2 IHC 0 score were scored as HER2-low or HER2-ultralow by central testing, suggesting that patients with HR-positive HER2 IHC 0 mBC should be reassessed to determine whether they may be eligible for treatment with T-DXd. As the HER2-ultralow cut-off is not yet fully part of standard clinical practice, increased awareness of low HER2 expression levels is desirable, which may be achieved through education and training for pathologists.

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DATA SHARING

Data underlying the findings described in this manuscript may be obtained in accordance with AstraZeneca's data-sharing policy, described at https://www.astrazenecaclinicaltrials.com/our-transparency-commitments/. Data for studies directly listed on Vivli can be requested through Vivli at www.vivli.org. Data for studies not listed on Vivli could be requested through Vivli at https://vivli.org/members/enquiries-about-studies-not-listed-on-the-vivli-platform/. The AstraZeneca Vivli member page is also available, outlining further details: https://vivli.org/ourmember/astrazeneca/.

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