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Reproducibility of Equivocal *HER2* In Situ Hybridization Groups 3 and 4, and Comparison With *HER2* mRNA Expression

The Thin Line Between Amplified and Nonamplified Breast Cancers

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• **Context.**—Breast carcinomas (BCs) with equivocal *HER2* (human epidermal growth factor receptor 2) immunohistochemistry are subjected to in situ hybridization (ISH) to assess *HER2* copy numbers. Infrequently, dual-probe ISH also provides equivocal results, designated as ISH groups 2, 3, or 4.

Objective.—To evaluate the reproducibility of *HER2* ISH groups 3 and 4, and to compare the integrated immunohistochemistry/ISH *HER2* result with *HER2* mRNA expression.

Design.—Dual-probe ISH slides of 50 BCs were re-evaluated by 2 independent observers. mRNA was extracted from microdissected tumor cells. Quantitative real-time polymerase chain reaction (RT-qPCR) was performed for quantitative evaluation of *HER2* mRNA, using the MammaTyper assay.

Results.—Reproducibility of ISH groups 1 (amplified; $n = 5$) and 5 (nonamplified; $n = 4$) was good with only 1 case differently classified. Many group 4 ($n = 28$) and

group 3 ($n = 13$) tumors were reclassified after recounting: of 41 patients, 9 and 18 might have been treated differently as based on assessment by observers 1 and 2, respectively. Concordance for MammaTyper *HER2* status was 56% ($n = 18/32$) for the original report; 59% ($n = 19/32$) for observer 1; 72% ($n = 23/32$) for observer 2; and 63% ($n = 20/32$) for the majority opinion.

Conclusions.—The reproducibility of equivocal ISH groups is limited. Although *HER2* ISH has long been considered as the gold standard to establish the *HER2* status in BC, the equivocal “gray zone” between amplified and nonamplified BCs is prone to interobserver variability. Potential solutions could comprise the involvement of at least 3 different observers for assessment of equivocal ISH cases, and/or evaluation of *HER2* mRNA as a more objective alternative method.

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Approximately 15% of invasive breast cancers (BCs) involve amplification of the human epidermal growth factor receptor 2 gene (*HER2*) and overexpression of the corresponding protein.^{1,2} A positive *HER2* status is associated with poor prognosis. The development of *HER2*-targeted therapies has proved effective in achieving excellent outcomes for this subset of patients with BC. Recently, novel therapies called antibody-drug conjugates have also proved effective in BC with low and ultralow *HER2* protein expression in the absence of *HER2* amplification. Therefore, determining *HER2* status is crucial for selecting the most effective personalized treatment.^{3,4}

The 2018 *HER2* testing guideline from the American Society of Clinical Oncology and the College of American Pathologists (ASCO/CAP) recommends using immunohistochemistry (IHC) as the primary method for assessing *HER2* status.⁵ When an equivocal IHC 2⁺ score is obtained, which occurs in about 15% to 20% of all invasive BCs, in situ hybridization (ISH) is required to further clarify the *HER2* status.^{1,5,6} Using dual-probe ISH testing, tumors with a mean *HER2* copy number of 4.0 or greater and a *HER2*:CEP17 ratio of 2.0 or greater are classified as *HER2*-positive (ISH group 1), while those with a mean *HER2* copy number less than 4.0 and a *HER2*:CEP17 ratio less than 2.0 are considered *HER2*-negative (ISH

group 5).⁵ Three ASCO/CAP ISH groups are regarded as equivocal and require additional workup.⁵ ISH group 2 tumors have a mean *HER2* copy number less than 4.0 and a *HER2*:CEP17 ratio of 2.0 or greater. ISH groups 3 and 4 each have a *HER2*:CEP17 ratio less than 2.0, with ISH group 3 tumors having a mean *HER2* copy number of 6.0 or greater and ISH group 4 tumors showing a mean *HER2* copy number of 4.0 or greater and less than 6.0.⁵ These equivocal ISH results require correlation with the *HER2* IHC. In case of a double equivocal result, that is, an equivocal ISH result in combination with an IHC 2⁺ score, a second observer blinded to the initial ISH result must evaluate at least 20 nuclei. If the equivocal ISH result is confirmed by the second observer, ISH groups 2 and 4 are considered as *HER2*-negative with a comment without further testing on the same sample.⁵ It is uncertain whether patients in this ISH group benefit from *HER2*-targeted therapy in the absence of protein overexpression.^{6–12} In contrast, ISH group 3 tumors with an IHC 2⁺ score are regarded as *HER2*-positive in case of confirmation of the ISH result. If the additional observer's review shifts the count into a different ISH category, the final category should be determined through adjudication following internal procedures.⁵ However, the 2018 ASCO/CAP guideline does not specify the workflow of such an internal procedure.⁵

High reproducibility in ISH quantification has been reported, with a study by Polonia et al¹³ showing 97% concordance ($k = 0.879$). However, this study included few cases from ISH groups 3 and 4, and interobserver agreement for these groups remains unclear. To address *HER2* ambiguity in ISH group 4, alternative assays like genomic profiling and quantitative real-time polymerase chain reaction (RT-qPCR) have been proposed. Studies comparing ISH and next-generation sequencing in ISH group 4 tumors have shown no detectable *HER2* amplification by next-generation sequencing, regardless of the ISH result.^{8,14} Additionally, discordant findings have been observed in mRNA quantification for ISH group 4, with studies showing varying relationships between mRNA levels, IHC scores, and ISH status.^{6,9,15}

OBJECTIVE

The objective of this study was to assess the interobserver reproducibility of ISH groups 3 and 4, using the originally reported score as well as scores determined by 2 additional observers who were blinded to the initial results. Additionally, we aimed to assess the *HER2* mRNA expression level by using the MammaTyper RT-qPCR kit. The integrated *HER2* IHC/ISH result, as determined by each observer, was compared to *HER2* mRNA expression level determined by MammaTyper.

MATERIALS AND METHODS

Patient Selection

Fifty archived BCs of patients who received a diagnosis between January 1, 2019, and July 31, 2024, at the Department of Pathology of the Cliniques universitaires Saint-Luc (Brussels, Belgium), with complete available IHC and ISH data, were selected. These BCs were initially reported as follows: 5 cases were ISH group 1; 13 cases were ISH group 3; 28 cases were ISH group 4; and 4 cases were ISH group 5. No equivocal ISH group 2 cases with residual tumor tissue were available in our center. Cases were selected by availability of residual tumor tissue for subsequent mRNA expression analysis. Clinicopathologic data were collected from the original report and included the following: sex, age at time of diagnosis,

specimen type (ie, biopsy or surgical specimen), tissue type (ie, primary BC versus metastasis), histologic type, grade, hormone receptor status including estrogen (ER) and progesterone (PR) receptors (as percentages), *HER2* status, Ki-67 index (as percentage), and treatment with neoadjuvant chemotherapy (NAC). One pathologist reviewed the archived hematoxylin-eosin-stained slides for assessment of tumor-infiltrating lymphocytes (TILs) as a percentage, according to the method proposed by the International Immuno-oncology Biomarkers Working Group.¹⁶

Immunohistochemistry

IHC for ER, PR, *HER2*, and Ki-67 was performed in the ISO15189-accredited Department of Pathology of the Cliniques universitaires Saint-Luc, as previously described in detail.¹⁷ *HER2* IHC was assessed as negative (scores 0/1⁺), equivocal (score 2⁺), or positive (score 3⁺) as per the ASCO/CAP 2018 guideline.⁵ ER and PR expression in BC cells was classified as negative (<1% nuclear immunoreactivity), weakly positive (1%–10% nuclear immunoreactivity), or positive (>10% immunoreactivity), based on the ASCO/CAP 2020 guideline.¹⁸ Ki-67 IHC scoring was categorized as 5% or less (low), 6% to 29% (intermediate), and 30% or more (high) according to the recommendations proposed by the International Ki67 in Breast Cancer Working Group.¹⁹ Our laboratory participates biannually in the Nordic Immunohistochemical Quality Control (NordiQC) for ER and *HER2* IHC, and annually for PR IHC. We participate in the external quality assessments organized by the Belgian public research institution Sciensano^{20,21}: annually for ER and PR IHC, and biannually for *HER2* IHC. We also participate in the NordiQC and Sciensano external quality assessments for Ki-67 IHC, which are organized on a less regular basis.

Dual-Probe *HER2* In Situ Hybridization

Silver in situ hybridization (SISH) was performed at the ISO15189-accredited Department of Pathology of the Cliniques universitaires Saint-Luc, using the INFORM *HER2* Dual ISH DNA Probe Cocktail Assay (Roche Diagnostics, Rotkreuz, Switzerland) on an automated slide stainer (BenchMark Ultra, Ventana Medical Systems, Arizona), according to the manufacturer's instructions, as previously described.¹⁷ Both the number of *HER2* (black signals) and CEP17 (red signals) copies were counted in the nuclei of 20 invasive tumor cells at ×630 magnification, using a brightfield microscope, according to the ASCO/CAP 2018 guideline.⁵ The mean *HER2* and CEP17 copy numbers and the *HER2*:CEP17 ratio were calculated. The originally reported copy numbers reflect the SISH assessment conducted by 2 independent observers, with the final reported values provided by the second observer. For this study, 2 additional independent observers, referred to as observer 1 and observer 2, evaluated the *HER2* and CEP17 signals, applying the aforementioned guideline. Both additional observers were trainees in pathology who had followed a course on *HER2* ISH evaluation, including a test set, whereas the observers involved in the original report were 3 experienced laboratory technologists. *HER2* dual-probe SISH in our laboratory is evaluated biannually by NordiQC, and evaluated annually by Sciensano.^{20,21}

RNA Extraction and mRNA Quantification

RNA extraction and qPCR were performed at the Department of Pathology of the Erasmus Medical Center (Rotterdam, the Netherlands). All available BC samples with a group 3 or group 4 ISH result were microdissected manually with a sterile scalpel under a stereomicroscope (Zeiss, Oberkochen, Germany) to ensure a reliable comparison with the *HER2* IHC and ISH result. Microdissected samples were stored into RNase/DNase-free tubes containing RNALater (Thermo Fisher). Before microdissection, the areas of invasive tumor were marked by a breast pathologist using the last 4-μm-thick hematoxylin-eosin-stained slide of 5 to 12 sequential slides (depending on the total tumor content in the tissue block). The remaining slides of formalin-fixed paraffin-embedded (FFPE) tissue

were dewaxed and rehydrated. RNA was isolated by using the RNeasy FFPE kit (Qiagen) according to manufacturer's instructions. MammaTyper RT-qPCR (Cerca Biotech, Berlin, Germany; distributed by Sysmex Europe SE, Norderstedt, Germany) was used for quantitative evaluation of mRNA levels of ER, PR, HER2, and Ki-67. Cutoff values of the MammaTyper assay according to the manufacturer's instructions were used as reference for interpretation of mRNA levels. These corresponded to the following groups based on $\Delta\Delta C_q$ cutoff values: (1) for ER: negative ($\leq 1\%$) ($< 36.8 \Delta\Delta C_q$), positive ($\geq 36.8 \Delta\Delta C_q$); (2) for PR: negative ($\leq 1\%$) ($< 34.9 \Delta\Delta C_q$), positive ($\geq 34.9 \Delta\Delta C_q$); (3) for HER2: 0/ultralow ($< 38.0 \Delta\Delta C_q$), low (≥ 38.0 – $40.1 \Delta\Delta C_q$), positive ($\geq 40.2 \Delta\Delta C_q$); (4) for Ki-67: low ($< 33.7 \Delta\Delta C_q$), intermediate (≥ 33.7 – $35.6 \Delta\Delta C_q$), high ($\geq 35.7 \Delta\Delta C_q$).

Statistical Analysis

For the evaluation of the clinicopathologic characteristics of cases obtained from the original report, descriptive statistics were performed for frequencies of categorical values. We calculated the mean, median, and range for continuous variables. ISH reproducibility was assessed by calculating the percentage of cases that shifted from one ISH group to another, based on the original report compared to the additional observers included for this study. ISH reproducibility was defined as concordant ISH groups, without the necessity of reproducing identical mean *HER2* and *CEP17* copy numbers as previously reported in the patient files, since the *HER2* status and the subsequent treatment depend on the ISH group, and not on the precise copy numbers. Percentage concordance was calculated. Sankey diagrams, made in SankeyMATIC.com, were used to depict the distribution of *HER2* ISH groups between the original report and the additional 2 observers; and between ER, PR, and Ki-67 IHC from the original report and MammaTyper mRNA levels. Analyses were performed in SPSS (IBM Corp; version 28.0.1, released 2021).

Ethical Approval

This study was approved by the Ethics Committee of the Cliniques Universitaires Saint-Luc (file name: 2024/18MAR/137 – RETRO-HER2-P24). The Medical Research Involving Human Subjects Act does not apply to this work; consequently, there was no need for an informed consent. The study was performed in accordance with the Declaration of Helsinki.

RESULTS

Clinicopathologic Characteristics

One male and 49 female patients with BC were included, with a median age of 61 years (range, 22–91 years). Tissue samples were collected from various sources: 30 (60%) were obtained from surgical resections, while 20 (40%) came from core needle biopsies. Of these 50 samples, 45 (90%) were taken from primary tumors, 4 (8%) from lymph node metastases, and 4 (8%) from distant metastases (liver, meninges, ovary, and skin). The most common histologic type was no special type (NST) BC, accounting for 42 of 50 (84%), followed by invasive lobular carcinoma at 4 (8%), of which 2 were pleomorphic and 2 were classic-type invasive lobular carcinoma. The other tumor types comprised 2 micropapillary carcinomas (4%), 1 mixed NST/micropapillary carcinoma (2%), and 1 invasive papillary carcinoma (2%) (Table 1). Most tumors were hormone receptor-positive: 46 (92%) were ER-positive, and 37 (74%) were PR-positive. In terms of *HER2* IHC, 46 samples (92%) were scored as 2+, 3 (6%) were scored as 3+, and 1 (2%) was scored as 1+. For the ISH classification, 28 (56%) were categorized as group 4; 13 (26%) as group 3; 5 (10%) as group 1;

Table 1. Clinical and Pathologic Characteristics of 50 Patients With Breast Cancer		
Original Report Characteristics	No.	%
Sex		
Female	49	98
Male	1	2
Age, median (range), y	61	(22–91)
Type of tissue		
Resection	30	60
Biopsy	20	40
Origin of tissue		
Primary tumor	45	90
Lymph node	1	2
Distant metastasis	4	8
NAC		
Yes	11	22
No	34	68
Not applicable	5	10
Histologic type		
NST	43	86
Lobular	4	8
Other	3	6
Grade		
1	1	2
2	23	46
3	26	52
HER2 IHC score		
0	0	0
1+	1	2
2+	46	92
3+	3	6
HER2 ISH group		
1	5	10
2	0	0
3	13	26
4	28	56
5	4	8
Ki-67		
≤5% (low)	12	24
6%–29% (intermediate)	18	36
≥30% (high)	20	40
TILs		
0%–10% (low)	44	88
11%–49% (intermediate)	5	10
≥50% (high)	1	2

Abbreviations: *HER2*, human epidermal growth factor receptor 2; IHC, immunohistochemistry; ISH, in situ hybridization; NAC, neoadjuvant chemotherapy; NST, no special type; TILs, tumor-infiltrating lymphocytes.

and 4 (8%) as group 5. No ISH group 2 cases were available in our institution. The amplified and nonamplified BC samples were randomly added to obtain a heterogeneous study cohort, aiming to prevent bias of the observers involved in the interobserver variability study. Most tumors had an intermediate to high Ki-67 index ($n = 38$, 76%) and low level of TILs ($n = 44$, 88%) (Table 1).

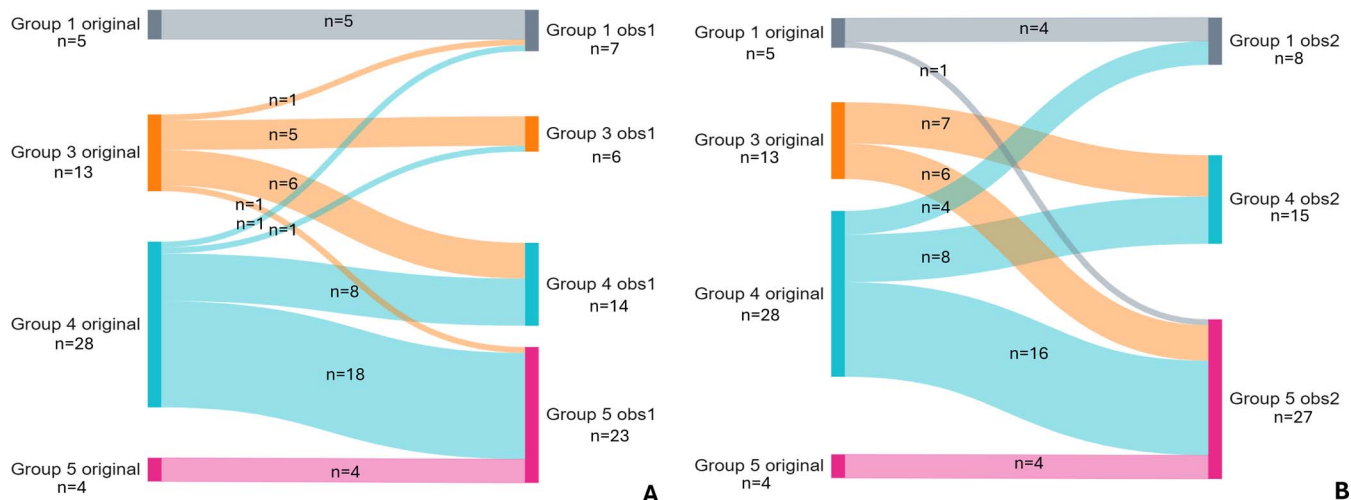


Figure 1. Comparison of in situ hybridization groups between the original report and observer 1 (A), and between the original report and observer 2 (B). Abbreviations: obs1, observer 1; obs2, observer 2.

ISH Interobserver Reproducibility

To assess the reproducibility of ISH, with an emphasis on the equivocal ISH groups 3 and 4, the ISH groups of the original report were compared to the additional observers included for this study. Figure 1, A, illustrates the comparison between the ISH groups from the original report and those as assessed by observer 1. The overall concordance was 22 of 50 (44%). Groups 1 and 5 remained the same. In the discordant cases, 8 of 13 cases originally classified as ISH group 3 were assigned to a different group by observer 1. Specifically, 1 case changed to group 1 (amplified), 6 cases changed to group 4, and 1 case changed to group 5 (nonamplified). Furthermore, 20 of 28 cases originally classified as ISH group 4 were assigned to a different group by observer 1. From this, 1 case changed to group 1 (amplified), 1 case changed to group 3, and 18 cases changed to group 5 (nonamplified).

Figure 1, B, illustrates the comparison between the ISH groups from the original report and those assessed by observer 2. The overall concordance was 16 of 50 (32%). Group 5 remained the same among observers. In the discordant cases, 1 of 5 cases originally classified as ISH group 1 (amplified) was assigned to ISH group 5 (nonamplified) by observer 2. Moreover, 13 of 13 cases originally classified as ISH group 3 were changed to other groups by observer 2, including 7 cases to group 4 and 6 to group 5 (nonamplified). Furthermore, 20 of 28 cases originally classified as ISH group 4 were assigned to a different group by observer 2. From these, 4 cases changed to group 1 (amplified) and 16 cases changed to group 5 (nonamplified).

After integration of ISH and IHC data, the observed discordances between the original report and observer 1 resulted in 7 of 50 cases (14%) changing from a positive to a negative HER2 status, and 2 of 50 cases (4%) changing from a negative to a positive HER2 status. From the original report to observer 2, the data indicate that 14 of 50 cases (28%) changed from a positive to a negative HER2 status, and 4 of 50 cases (8%) changed from a negative to a positive HER2 status. In other words, of the 50 patients, 9 (18%) and 18 (36%) could have been treated differently, based on the counts of observer 1 and observer 2, respectively. Nine of 28 ISH group 3 and 4 cases (32%) fell into 3 different ISH groups, based on the initial report and the ISH recounts by

the 2 additional observers. Five of these were initially reported as ISH group 3, among which 4 patients with BC might not have received anti-HER2 treatment, based on the majority opinion. The remaining 4 BCs were ISH group 4 cases for which the treatment would not have been altered by the majority opinion (Table 2).

Based on the overall majority opinion in the hypothetical scenario of 3 observers per double equivocal BC, 7 of 41 patients with BC (17%) would have been reconsidered as HER2-negative, and 1 of 41 patients with BC (2%) would have been reconsidered as HER2-positive, resulting in a potential treatment change for 8 of 41 patients (20%) (Supplemental Table; see the [supplemental digital content](#) containing 1 table and 3 figures). The level of discordance was not influenced by the specimen type (ie, biopsy versus resection specimen). Figure 2 shows examples of invasive breast tumors originally reported as HER2 ISH group 3 (Figure 2, A through C), ISH group 4 (Figure 2, D through F), and reclassified as group 5 by the majority opinion (Figure 2, G through I).

Table 2. In Situ Hybridization (ISH) Groups for 9 Breast Cancers With Discordant Results Based on the Original Report and the Recounts by Observers 1 and 2, With HER2 Status Based on the Majority Opinion

Patient ID	ISH Original Report	ISH Observer 1	ISH Observer 2	HER2 Status Majority Opinion ^a
6	3	1	5	HER2 positive
28	3	4	5	<i>HER2 negative</i>
30	3	4	5	<i>HER2 negative</i>
35	3	4	5	<i>HER2 negative</i>
45	3	4	5	<i>HER2 negative</i>
16	4	5	1	HER2 negative
20	4	3	5	HER2 negative
29	4	5	1	HER2 negative
31	4	5	1	HER2 negative

Abbreviation: HER2, human epidermal growth factor receptor 2.

^a A modified HER2 status with potential clinical impact (ie, HER2-targeted therapy or not) is highlighted in italics.

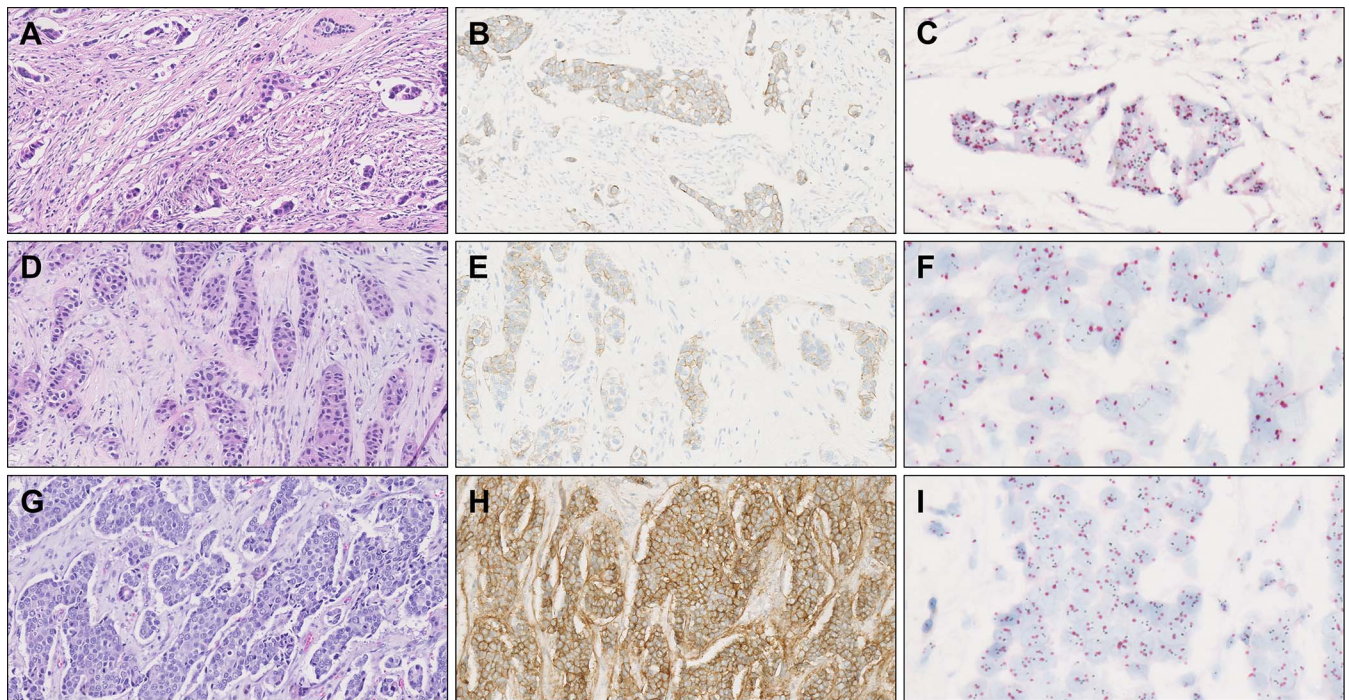


Figure 2. Photomicrographs of hematoxylin-eosin-stained slides (A, D, G), HER2 immunohistochemistry (B, E, H), and HER2 SISH (C, F, I) illustrating an invasive breast carcinoma of no special type originally reported as HER2 ISH group 3 (A through C), an invasive breast carcinoma of no special type originally reported as HER2 ISH group 4 (D through F), and an invasive breast carcinoma of no special type reclassified as group 5 on the basis of the majority opinion (G through I) (original magnification $\times 200$ [A, B, D, E, G, and H]; dual-probe SISH, original magnification $\times 800$ [C, F, and I; with red dots representing the CEP17 probe and black dots representing the HER2 signals]). Abbreviations: HER2, human epidermal growth factor receptor 2; ISH, in situ hybridization; SISH, silver in situ hybridization.

Concordance Between IHC, ISH, and mRNA Expression

Thirty-two BC samples had enough FFPE material available and yielded sufficient mRNA after RNA extraction. All BC samples subjected to mRNA quantification belonged to ISH groups 3 and 4. mRNA levels were determined by using the MammaTyper kit. Based on this analysis, 23 tumors were classified as HER2-negative (1 tumor was considered as HER2-ultralow and 22 were HER2-low), and 9 tumors as HER2-positive. The concordance between MammaTyper HER2 groups and final HER2 status as determined by IHC and ISH from the original report was 56% ($n = 18/32$), between MammaTyper and observer 1 this concordance was 59% ($n = 19/32$), and between MammaTyper and observer 2 this concordance was 72% ($n = 23/32$).

According to the original report, all 13 tumors in ISH group 3 were classified as the luminal B HER2⁺ surrogate molecular subtype. In the case of the 28 ISH group 4 tumors, 18 were luminal A, 8 were luminal B HER2⁻, and 2 were triple-negative breast cancer. In other words, most of the equivocal ISH group 3 and 4 cases were hormone receptor–positive. Equivocal ISH results were a rare finding in hormone receptor–negative BC. Regarding hormone receptor status, the concordance between ER from the original report and MammaTyper was 30 of 32 (94%) (Supplemental Figure 1). The 2 discordant cases had ER-positive status by IHC but were classified as ER-negative by MammaTyper. These cases presented with 20% and 50% nuclear immunoreactivity, respectively. Furthermore, the concordance between PR from the original report and MammaTyper was 26 of 32 (81%) (Supplemental Figure 2). The 6 discordant cases had PR-positive status by IHC but were classified as PR-negative by MammaTyper: 3 of these cases had a low-positive PR IHC (1%–9%), and the other 3

had a respective PR rate of 20%, 60%, and 95%. Ki-67 had a concordance of 19 of 32 (59%) between IHC and MammaTyper (Supplemental Figure 3). From the 13 discordant cases, 2 had a low Ki-67 expression according to IHC but intermediate according to MammaTyper; 6 had an intermediate Ki-67 expression according to IHC but high according to MammaTyper; and 5 had a high Ki-67 expression according to IHC, but intermediate according to MammaTyper.

Ki-67 Expression and TILs

TIL levels were correlated with the Ki-67 expression assessed by IHC and by MammaTyper, to exclude a potential confounder and source of discordance between IHC and MammaTyper results, as lymphocytes can also express Ki-67. No significant association was found between TIL levels and Ki-67 expression as assessed by IHC ($P = .97$) or by MammaTyper ($P = .92$).

Follow-up Analysis

Follow-up data were available for 25 patients with primary BC whose tumor was subjected to RT-qPCR analysis with the MammaTyper assay. The average length of follow-up was 22 months for 23 patients without disease progression (range, 6–60 months). Two patients developed locoregional recurrence at 4 months and 10 months after their initial treatment. One of the patients had a tumor originally classified as IHC 2⁺ ISH group 3 (based on the original report of the primary tumor and majority opinion) and received anti-HER2 therapy. HER2 ISH could not be repeated on the bone metastasis owing to technical failure due to decalcification. The other patient with recurrence had a tumor classified as

IHC 2⁺ ISH group 4 (original report) and IHC2⁺ ISH group 5 (majority opinion) and did not receive anti-HER2 therapy (Supplemental Table). The skin metastases of this patient showed an equivocal 2⁺ IHC score in the absence of amplification (ISH group 5).

DISCUSSION

Invasive BCs with an equivocal HER2 IHC 2⁺ score are evaluated by additional ISH testing to investigate the presence of *HER2* gene amplification. Depending on the mean *HER2* gene copy number and the *HER2*:CEP17 ratio, tumors are classified into 5 different groups, with ISH group 1 tumors being amplified, ISH group 5 tumors being nonamplified, and ISH group 2, 3, and 4 tumors being designated as equivocal.⁵ The 2018 ASCO/CAP guideline on HER2 testing recommends to perform additional workup for such “double equivocal” tumors.⁵ As the selection of the precise tumor area wherein 20 nuclei are counted can differ among different observers, especially in resection specimens with large tumors, we questioned the degree of reproducibility of such “borderline” cases. Moreover, counting of *HER2* signals can differ among observers, as this might be influenced by the distance between the individual signals. We therefore retrieved a series of double equivocal BCs, diagnosed since the introduction of the recent version of the ASCO/CAP guideline,⁵ to re-count the archived SISH slides. Four and 5 BCs, previously reported as nonamplified and amplified, respectively, were added to the series to avoid bias of the involved observers and served as a control group for the reproducibility study. For 32 of 41 ISH group 3 and 4 cases (78%), there was sufficient residual tumor tissue to allow for mRNA expression analysis by RT-qPCR. Our findings show that interobserver agreement for amplified and nonamplified cases was substantial. There was 100% concordance with the originally reported result for the 4 nonamplified BCs for each observer. For the 5 amplified BCs, concordance ranged from 80% to 100%, with only 1 amplified case reclassified as nonamplified by a single observer. This misclassification was most likely due to incorrect selection of the tumor area when counting heterogeneously distributed *HER2* signals at ×630 magnification.

In contrast, the reproducibility of ISH groups 3 and 4 was limited. The combined concordance for ISH groups 3 and 4 was 13 of 41 tumors (32%) for observer 1, with 8 of 13 ISH group 3 tumors (62%) being reclassified and 20 of 28 ISH group 4 tumors (71%) being reclassified. The combined concordance for ISH groups 3 and 4 was 8 of 41 tumors (20%) for observer 2, with all ISH group 3 tumors being reclassified as ISH group 4 or 5 (ie, 0% concordance) and 16 of 28 ISH group 4 tumors (57%) being reclassified as nonamplified ISH group 5. In the case of an equivocal HER2 IHC 2⁺ score, a switch from group 4 to group 5 will not affect treatment, that is, these patients are considered to have HER2-negative BC, as there is insufficient evidence to support HER2-targeted treatment in ISH group 4 BCs.^{6,22} However, a switch from ISH group 3 to ISH groups 4 or 5 will have an impact on the patient's treatment, as IHC 2⁺ ISH group 3 tumors are considered as HER2-positive. Based on the counts of observer 1 and observer 2, in our series 9 patients and 18 patients, respectively, could have been treated differently. Our findings show that a large proportion of double equivocal HER2 BCs end up with a discordant ISH group result after evaluation by a second observer. None of the 13 initially reported ISH group 3 BCs was confirmed by both observers, and only

4 of 28 initially reported ISH group 4 cases were confirmed by both observers. Finally, HER2 status, and thus subsequent systemic treatment, could have been potentially different for 8 of 41 patients (20%) who were initially reported as having ISH group 3 or 4 tumors, if 3 observers would have been involved from the start. It seems therefore more prudent to involve a third observer, blinded to the previous results, to re-count at least 20 nuclei. In those BC cases flirting with the threshold between amplification and absence of amplification, the majority opinion could be used to establish the final decision regarding HER2 status. Of note, the observers who were involved in the ISH reassessment for this study had less experience with HER2 ISH evaluation than the observers involved in the original evaluation, which might have influenced our results.

Automated algorithms or so-called artificial intelligence (AI) could help in the automated scoring of HER2 IHC and ISH through imaging software. In the case of HER2 IHC, according to a meta-analysis by Wu et al,²³ the ability of AI to identify HER2 2⁺ was slightly inferior compared to the recognition of HER2 0/1⁺ and HER2 3⁺. This could be explained by the changes in guidelines affecting the definition of HER2 2⁺, and the greater heterogeneity in HER2 2⁺ than in HER2 3⁺. Concerning ISH, in a study by Xue et al,²⁴ the overall classification accuracy between the Aitrox model and manual counting was 85.33% (157 of 184). In group 5, the most common group, the consistency was as high as 95.90%. However, consistency was low in other groups owing to limited numbers of cases. Other models have shown similar reproducibility rates.^{25,26} For now, standardized AI algorithms that can be extrapolated to different hospital settings are still required to demonstrate robustness and applicability. Of note, even across different computational pathology models for HER2 IHC, substantial variability was observed for all IHC categories (ie, 3⁺, 2⁺, 1⁺, and 0). This discordance was most likely due to these models being trained by pathologists, who do not entirely agree among themselves.²⁷

It needs to be emphasized that large-scale prospective data are not available about treatment response to HER2-targeting therapies in the adjuvant setting in this particular patient population. Follow-up data on treatment response to HER2-targeting therapies in clinical trial setting are required to guide future treatment decisions for these patients.⁶ A retrospective analysis showed that ISH group 2 cases might benefit from HER2-targeted therapies in the neoadjuvant setting, with a complete response rate of 27%.²⁸ Moreover, multiple studies have shown that the mean *HER2* copy number strongly correlates with the chance of achieving a pathologic complete response after neoadjuvant chemotherapy in patients with HER2-positive BC, with lower *HER2* copy numbers and lower *HER2*:CEP17 ratios being associated with a higher chance of residual disease.^{17,29–31}

Several studies have assessed mRNA quantification in ISH group 4 BCs. In a study by Tong et al,⁹ similar mRNA expression levels were reported for ISH dual-equivocal IHC 2⁺ (ISH group 4 nonamplified, according to ASCO/CAP guideline 2018⁵) and ISH-negative IHC 2⁺ tumors. Gupta et al⁶ observed that mRNA levels were higher in ISH group 4 tumors with IHC 2⁺ than with IHC 0/1⁺, although protein quantification (determined by quantitative fluorescence) was similar to that observed in HER2-negative cases. However, Marchio et al¹⁵ found significantly different *HER2* mRNA levels between HER2-positive, HER2-negative, and IHC 2⁺ ISH

group 4 carcinomas, with greater overlap of *HER2* mRNA levels between IHC 2⁺ ISH group 4 and *HER2*-negative carcinomas. Given the interobserver discordance for equivocal ISH tumors demonstrated in our study, one may wonder whether ISH should be regarded as the gold standard in this ambiguous gray zone. RT-qPCR provides an objective single value for *HER2* mRNA expression levels. On the one hand, RT-qPCR could cause a false sense of security by relying too much on this single value and its associated positive or negative *HER2* status, determined by an arbitrary cut point. On the other hand, however, RT-qPCR might provide a more reliable view on the actual *HER2* status of the tumor, as it analyzes all tumor cells, and not just a subjective selection of 20 tumor nuclei. Interestingly, we observed a difference between the *HER2* status provided by the different observers and the mRNA-based *HER2* status, with observer 2 showing the highest percent concordance of 72%. This observation insinuates that mRNA expression could be used in selected IHC/ISH double equivocal cases to make a final decision.

Clinicians generally want to obtain a definitive *HER2* status as soon as possible. Future discussion about a potential clarification of the current version of the ASCO/CAP guideline should also consider how a timely report about the definitive *HER2* status can be achieved for double equivocal cases, and how those results should be communicated. It is likely that turnaround times will increase if additional evaluation steps are added, such as implementing more observers for ISH and/or RNA-based testing.

In the past years, a novel antibody-drug conjugate targeting *HER2*—trastuzumab deruxtecan—has shown extended progression-free and overall survival in patients with *HER2*-positive, but also *HER2*-low, BC.^{32–34} According to the European Society for Medical Oncology consensus and ASCO/CAP guideline of 2023, *HER2*-low BC is defined as BC with *HER2* IHC 1⁺ or 2⁺ scores without *HER2* amplification.^{35,36} No substantial molecular or pathologic differences have been demonstrated between *HER2*-low and *HER2*-0 BCs, after correcting for the expression of hormone receptors.^{37,38} For this reason, *HER2*-low BCs are not considered as a distinct molecular entity; rather they represent a term used for clinical-trial purposes.³⁶ In light of these findings, the final *HER2* status of BC with mean *HER2* copy numbers of 4.0 or greater and less than 6.0 becomes increasingly important, as patients with *HER2*-low ISH-negative BC could potentially benefit from treatment with trastuzumab deruxtecan, whereas patients with *HER2* IHC 2⁺ ISH-positive BC are still eligible for treatment with the “classic” anti-*HER2*-targeting therapies such as trastuzumab and pertuzumab.

Our study has a few limitations. First, the cohort size was limited, as ISH group 3 and 4 cases are infrequently encountered and our institution did not have any archived ISH group 2 cases with available residual tumor tissue. Second, owing to the short follow-up time, we were able to retrieve follow-up data of only 25 patients with primary BC. Therefore, to determine which assay (MammaTyper versus combined IHC/ISH) correlates strongest with progression-free survival, prospective validation in a large multicenter patient cohort is required, particularly for those patients with IHC 2⁺ BC and an *HER2* ISH finding bordering on the ISH threshold. Third, the level of experience of the observers, both in this study setting and in real life, might influence the obtained results. Fourth, our study used brightfield ISH instead of fluorescence ISH (FISH), and we therefore cannot extrapolate our results to FISH without additional validation.

Future studies should investigate whether laboratories applying dual-probe FISH for *HER2* observe a similar number of double equivocal breast cancers. We are currently exploring this via an internationally organized survey. Fifth, we were unable to investigate the impact of retesting on a subsequent specimen, as this is not routinely performed in our institution. One study on ISH group 2 cases demonstrated that 23% of ISH group 2 tumors convert from a *HER2*-negative to a *HER2*-positive status upon retesting, suggesting that tumor heterogeneity may substantially influence the *HER2* ISH results.³⁹

In conclusion, the reproducibility of the equivocal ISH groups 3 and 4 is poor. Although the 2018 ASCO/CAP guideline for *HER2* testing recommends adjudicating BC with double equivocal IHC and ISH results as per internal procedure,⁵ we show here that the number of observers involved in *HER2* testing likely influences the final *HER2* status, and thus, patient treatment. We therefore recommend to routinely involve a third observer in the case of “borderline” ISH BCs with discordant ISH results between 2 observers, and to rely on the majority opinion to establish a final diagnosis. Evaluation of *HER2* mRNA is a more objective method to determine *HER2* status and could be considered as an alternative solution for tumors with equivocal ISH.

References

1. Baez-Navarro X, van Bockstal MR, Andrinopoulou ER, van Deurzen CHM. *HER2*-low breast cancer: incidence, clinicopathologic features, and survival outcomes from real-world data of a large nationwide cohort. *Mod Pathol*. 2023; 36(4):100087. doi:10.1016/j.modpat.2022.100087
2. Martin V, Cappuzzo F, Mazzucchelli L, Frattini M. *HER2* in solid tumors: more than 10 years under the microscope; where are we now? *Future Oncol*. 2014;10(8):1469–1486. doi:10.2217/fon.14.19
3. Early Breast Cancer Trialists' Collaborative Group. Trastuzumab for early-stage, *HER2*-positive breast cancer: a meta-analysis of 13 864 women in seven randomised trials. *Lancet Oncol*. 2021;22(8):1139–1150. doi:10.1016/S1470-2045(21)00288-6
4. Plosker GL, Keam SJ. Trastuzumab: a review of its use in the management of *HER2*-positive metastatic and early-stage breast cancer. *Drugs*. 2006; 66(4):449–475. doi:10.2165/00003495-200666040-00005
5. Wolff AC, Hammond MEH, Allison KH, et al. Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Focused Update. *Arch Pathol Lab Med*. 2018;142(11):1364–1382.
6. Gupta S, Neumeister V, McGuire J, et al. Quantitative assessments and clinical outcomes in *HER2* equivocal 2018 ASCO/CAP ISH group 4 breast cancer. *NPJ Breast Cancer*. 2019;5:28. doi:10.1038/s41523-019-0122-x
7. Woo JW, Lee K, Chung YR, Jang MH, Ahn S, Park SY. The updated 2018 American Society of Clinical Oncology/College of American Pathologists guideline on human epidermal growth factor receptor 2 interpretation in breast cancer: comparison with previous guidelines and clinical significance of the proposed in situ hybridization groups. *Hum Pathol*. 2020;98:10–21. doi:10.1016/j.humpath.2020.01.003
8. Bai Q, Lv H, Bao L, et al. Invasive breast cancer with *HER2* $\geq$ 4.0 and $<$ 6.0: risk classification and molecular typing by a 21-gene expression assay and MammaPrint Plus BluePrint testing. *Breast Cancer*. 2023;15:563–575. doi:10.2147/BCTT.S420738
9. Tong Y, Chen X, Fei X, et al. Can breast cancer patients with *HER2* dual-equivocal tumours be managed as *HER2*-negative disease? *Eur J Cancer*. 2018; 89:9–18. doi:10.1016/j.ejca.2017.10.033
10. Press MF, Sauter G, Buysse M, et al. *HER2* gene amplification testing by fluorescent in situ hybridization (FISH): comparison of the ASCO-College of American Pathologists guidelines with FISH scores used for enrollment in breast cancer international research group clinical trials. *J Clin Oncol*. 2016; 34(29):3518–3528. doi:10.1200/JCO.2016.66.6693
11. Press MF, Villalobos I, Santiago A, et al. Assessing the new American Society of Clinical Oncology/College of American Pathologists guidelines for *HER2* testing by fluorescence in situ hybridization: experience of an academic consultation practice. *Arch Pathol Lab Med*. 2016;140(11):1250–1258. doi:10.5858/arpa.2016-0009-OA
12. Shah MV, Wiktor AE, Meyer RG, et al. Change in pattern of *HER2* fluorescent in situ hybridization (FISH) results in breast cancers submitted for FISH testing: experience of a reference laboratory using US Food and Drug Administration criteria and American Society of Clinical Oncology and College of American Pathologists guidelines. *J Clin Oncol*. 2016;34(29):3502–3510. doi:10.1200/JCO.2015.61.8983

13. Polonia A, Caramelo A. HER2 in situ hybridization test in breast cancer: quantifying margins of error and genetic heterogeneity. *Mod Pathol*. 2021; 34(8):1478–1486. doi:10.1038/s41379-021-00813-x
14. Hoda RS, Bowman AS, Zehir A, et al. Next-generation assessment of human epidermal growth factor receptor 2 gene (ERBB2) amplification status in invasive breast carcinoma: a focus on Group 4 by use of the 2018 American Society of Clinical Oncology/College of American Pathologists HER2 testing guideline. *Histopathology*. 2021;78(4):498–507. doi:10.1111/his.14241
15. Marchio C, Dell'Orto P, Annaratone L, et al. The dilemma of HER2 double-equivocal breast carcinomas: genomic profiling and implications for treatment. *Am J Surg Pathol*. 2018;42(9):1190–1200. doi:10.1097/PAS.0000000000001100
16. Salgado R, Denkert C, Demaria S, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. *Ann Oncol*. 2015;26(2):259–271. doi:10.1093/annonc/mdl450
17. Van Bockstal MR, Dano H, Benhaddi N, et al. Predictive markers for pathological complete response (pCR) after neo-adjuvant chemotherapy in HER2-positive breast carcinoma. *Histol Histopathol*. 2024;39(2):153–164. doi:10.14670/HH-18-626
18. Allison KH, Hammond MEH, Dowsett M, et al. Estrogen and Progesterone Receptor Testing in Breast Cancer: ASCO/CAP Guideline Update. *J Clin Oncol*. 2020;38(12):1346–1366. doi:10.1200/JCO.19.02309
19. Nielsen TO, Leung SCY, Rimm DL, et al. Assessment of Ki67 in breast cancer: updated recommendations from the International Ki67 in Breast Cancer Working Group. *J Natl Cancer Inst*. 2021;113(7):808–819. doi:10.1093/jnci/djaa201
20. NordiQC. HER2 ISH assessment. <https://www.nordiqc.org/index.php>. Updated 2024. Accessed November 29, 2024.
21. Sciensano. <https://www.sciensano.be/en>. Updated 2024. Accessed November 29, 2024.
22. Hoda RS, Brogi E, Xu J, et al. Impact of the 2018 American Society of Clinical Oncology/College of American Pathologists HER2 Guideline Updates on HER2 Assessment in Breast Cancer With Equivocal HER2 Immunohistochemistry Results With Focus on Cases With HER2/CEP17 Ratio <2.0 and Average HER2 Copy Number $\geq$ 4.0 and <6.0. *Arch Pathol Lab Med*. 2020;144(5):597–601. doi:10.5858/arpa.2019-0307-OA
23. Wu S, Li X, Miao J, et al. Artificial intelligence for assisted HER2 immunohistochemistry evaluation of breast cancer: a systematic review and meta-analysis. *Pathol Res Pract*. 2024;260:155472. doi:10.1016/j.prp.2024.155472
24. Xue T, Chang H, Ren M, et al. Deep learning to automatically evaluate HER2 gene amplification status from fluorescence in situ hybridization images. *Sci Rep*. 2023;13(1):9746. doi:10.1038/s41598-023-36811-z
25. Jian Z, Song T, Zhang Z, et al. Deep learning method for detecting fluorescence spots in cancer diagnostics via fluorescence in situ hybridization. *Sci Rep*. 2024;14(1):27231. doi:10.1038/s41598-024-78571-4
26. Jian Z, Song T, Zhang Z, et al. An improved nested U-net network for fluorescence in situ hybridization cell image segmentation. *Sensors (Basel)*. 2024; 24(3):928. doi:10.3390/s24030928
27. McKelvey B, Torres-Saavedra PA, Li J, et al. Agreement across 10 artificial intelligence models in assessing HER2 in breast cancer whole slide images: findings from the Friends of Cancer Research Digital PATH Project. In: *San Antonio Breast Cancer Symposium*; 2024. Abstract P2-02-18.
28. Rakha EA, Miligy IM, Quinn CM, et al. Retrospective observational study of HER2 immunohistochemistry in borderline breast cancer patients undergoing neoadjuvant therapy, with an emphasis on Group 2 (HER2/CEP17 ratio $\geq$ 2.0, HER2 copy number <4.0 signals/cell) cases. *Br J Cancer*. 2021;124(11):1836–1842. doi:10.1038/s41416-021-01351-8
29. Zhao J, Krishnamurti U, Zhang C, et al. HER2 immunohistochemistry staining positivity is strongly predictive of tumor response to neoadjuvant chemotherapy in HER2 positive breast cancer. *Pathol Res Pract*. 2020;216(11):153155. doi:10.1016/j.prp.2020.153155
30. Antolin S, Garcia-Caballero L, Reboredo C, et al. Is there a correlation between HER2 gene amplification level and response to neoadjuvant treatment with trastuzumab and chemotherapy in HER2-positive breast cancer? *Virchows Arch*. 2021;479(4):853–857. doi:10.1007/s00428-021-03104-7
31. Choi JH, Jeon CW, Kim YO, Jung S. Pathological complete response to neoadjuvant trastuzumab and pertuzumab therapy is related to human epidermal growth factor receptor 2 (HER2) amplification level in HER2-amplified breast cancer. *Medicine (Baltimore)*. 2020;99(46):e23053. doi:10.1097/MD.00000000000023053
32. Modi S, Jacot W, Yamashita T, et al. Trastuzumab deruxtecan in previously treated HER2-low advanced breast cancer. *N Engl J Med*. 2022;387:9–20. doi:10.1056/NEJMoa2203690
33. Modi S, Saura C, Yamashita T, et al. Trastuzumab deruxtecan in previously treated HER2-positive breast cancer. *N Engl J Med*. 2020;382(7):610–621. doi:10.1056/NEJMoa1914510
34. Mosele F, Deluche E, Lusque A, et al. Trastuzumab deruxtecan in metastatic breast cancer with variable HER2 expression: the phase 2 DAISY trial. *Nat Med*. 2023;29(8):2110–2120. doi:10.1038/s41591-023-02478-2
35. Wolff AC, Somerfield MR, Dowsett M, et al. Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: ASCO-College of American Pathologists Guideline Update. *J Clin Oncol*. 2023;41(22):3867–3872. doi:10.1200/jco.22.02864
36. Tarantino P, Viale G, Press MF, et al. ESMO Expert Consensus Statements (ECS) on the definition, diagnosis, and management of HER2-low breast cancer. *Ann Oncol*. 2023;34(8):645–659. doi:10.1016/j.annonc.2023.05.008
37. Tarantino P, Gupta H, Hughes ME, et al. Comprehensive genomic characterization of HER2-low and HER2-0 breast cancer. *Nat Commun*. 2023; 14(1):7496. doi:10.1038/s41467-023-43324-w
38. Schettini F, Chic N, Braso-Maristany F, et al. Clinical, pathological, and PAM50 gene expression features of HER2-low breast cancer. *NPJ Breast Cancer*. 2021;7(1):1. doi:10.1038/s41523-020-00208-2
39. Wang M, Ding Q, Gu J, et al. Breast cancer with a HER2 FISH Group 2 result: should HER2 tests be repeated? *Clin Breast Cancer*. 2023;23(4):415–422. doi:10.1016/j.clbc.2023.02.006