nature reviews clinical oncology

https://doi.org/10.1038/s41571-025-01016-y

Review article

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HER2 testing: evolution and update for a companion diagnostic assay

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Abstract

Human epidermal growth factor receptor 2 (HER2; encoded by *ERBB2*) testing has been a cornerstone of patient selection for HER2-targeted therapies, principally in breast cancer but also in several other solid tumours. Since the introduction of HercepTest as the original companion diagnostic for trastuzumab, HER2 assessment methods have evolved substantially, incorporating various testing modalities, from western blots, immunohistochemistry and fluorescence in situ hybridization, to early chromogenic quantitative methods and, probably in the future, fully quantitative methods. The advent of highly effective HER2-targeted antibody–drug conjugates with clinical activity at low levels of HER2 expression, such as trastuzumab deruxtecan, has necessitated the re-evaluation of HER2 testing, particularly for HER2-low tumours. In this Review, we provide an in-depth overview of the evolution of HER2 testing, the current clinical guidelines for HER2 testing across various solid tumours, challenges associated with current testing methodologies and the emerging potential of quantitative techniques. We discuss the importance of accurately defining HER2-low expression for therapeutic decision-making and how newer diagnostic approaches, such as quantitative immunofluorescence and RNA-based assays, might address the limitations of traditional immunohistoch emistry-based methods. As the use of HER2-targeted therapies continues to expand to a wider range of tumour types, ensuring the precision and accuracy of HER2 testing will be crucial for guiding treatment strategies and improving patient outcomes.

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Key points

- The advent of new antibody–drug conjugates (ADCs) capable of targeting breast cancers and other solid tumours with much lower levels of human epidermal growth factor receptor 2 (HER2) expression has created a need to rethink the current approach to companion diagnostics owing to the insufficient sensitivity of current assays.
- Accurate and precise identification of HER2-low cancers is crucial for drug selection in light of the emerging landscape of novel ADCs.
- New approaches, such as quantitative immunofluorescence and RNA-based assays, have the potential to provide more accurate and precise assessments of HER2 expression, especially in the range of HER2 expression typically seen in non-malignant breast ducts.
- Better assessment criteria are needed for HER2 testing in solid tumour types beyond breast cancer given that expression patterns and response to therapy vary by cancer type.
- We believe that the future of HER2 testing lies in standardized, quantitative assays, which will be essential for personalized treatment, particularly with multiple ADCs targeting different tumour types.

Introduction

Human epidermal growth factor receptor 2 (HER2) is a well-known biomarker and can be targeted using a range of therapeutic agents across an expanding number of solid tumour types[1]. Over the past decades, the way HER2 is assessed in tumour samples to assign clinical HER2 status has continued to evolve[2]. Now, over the past few years, with the closer examination of HER2-low as a category that is associated with clinical benefit from the HER2-targeted antibody–drug conjugate (ADC) trastuzumab deruxtecan (T-DXd) and the introduction of competing therapeutic agents targeting alternative biomarkers, re-evaluating methods of determining and assigning HER2 status has become more important than ever.

In this Review, we discuss the evolution of HER2 companion diagnostic assays from the identification of HER2 as a therapeutic target in the 1980s to the present day and describe the current guidelines for testing and scoring HER2 expression in a variety of solid tumour types. We further elaborate on the current challenges associated with HER2 testing and explore the various novel approaches to HER2 assessment that are being developed to address these issues. We also discuss the future of companion diagnostic assays and their important role in modern oncology.

History of HER2 assessment

The *Erbb22* gene (also known as *Neu*, HER2/*Neu* and HER2) was first identified in 1984 as a novel oncogene in rats[3]. Within the same year, monoclonal antibodies were used to identify the protein encoded by the *neu* oncogene as a ~18 kDa phosphoprotein originally called p185 but now better known as HER2 (ref. 4). These authors predicted that "p185 may prove useful as a potential target for directing monoclonal antibodies against tumours containing activated *neu* oncogenes"[4]. This idea was validated in 1986 when, in a follow-up study, this group demonstrated that monoclonal antibodies targeting HER2-overexpressing cancer cells cause these cells to revert to a non-malignant phenotype[4,5]. Through additional research involving cell lines and primary breast cancer tissues, HER2 was found to be overexpressed on malignant cells in 15–30% of breast cancers[6–9]. In 1987, such overexpression was found to predict an unfavourable prognosis, including associations with a higher risk of breast cancer recurrence and metastatic dissemination[7]. Realizing that HER2 had potential as a therapeutic target, a team of scientists began developing an antibody designed to bind to HER2 aiming to block the oncogenic activity of this protein[10]. In 1998, this research culminated in the introduction of trastuzumab — the first clinically available monoclonal antibody targeting an oncoprotein and the first of several HER2-targeted therapies[11]. With the introduction of trastuzumab, creating an accurate, reproducible and standardized method of assessing HER2 expression became crucial.

During the preclinical and early clinical development of trastuzumab, an immunohistochemistry (IHC) assay known as the clinical trial assay (CTA) was used to assess HER2 expression and determine patient eligibility. Although this assay proved to be an effective tool in these early studies, it was not well suited for clinical use owing to its time-consuming and technically challenging protocol, which required the use of two different mouse monoclonal anti-HER2 antibodies (4D5, a trastuzumab precursor that recognizes an extracellular epitope, and CB11, which recognizes an intracellular epitope) as well as four or more tissue samples per patient[9,12,13]. For trastuzumab to be clinically effective, a companion diagnostic with good performance that could be reproduced in any pathology laboratory was also necessary. To meet this need, a commercial IHC assay called HercepTest was developed[14]. Notable differences between HercepTest and the CTA include the use of a rabbit polyclonal antibody in the former as well as improvements in sensitivity, which are attributed to the inclusion of signal amplification systems. Despite these differences, the evaluation method for HercepTest remained unchanged, with samples scored as 0, 1+, 2+ or 3+ on the basis of the original CTA guidelines. HercepTest was compared to both the CTA and a fluorescence in situ hybridization (FISH) assay using binary categories to define HER2 status as positive (2+ or 3+ on IHC or a *ERBB2*-to-*CEN17* ratio of ≥2.0 on FISH) or negative (0 or 1+ on IHC, *ERBB2*-to-*CEN17* ratio <2.0 on FISH). HercepTest demonstrated a concordance of 79% with the CTA and 86% with FISH[13,15,16]. Although the assay had not been used to determine HER2 status in the clinical trials testing trastuzumab, HercepTest was nonetheless approved by the FDA alongside trastuzumab (in combination with chemotherapy as first-line therapy and as later-line monotherapy for metastatic HER2-positive breast cancer) as the companion diagnostic in September 1998 (ref. 14). Over the following years, HercepTest was used to assess HER2 overexpression in a number of clinical trials and the scoring criteria were adjusted, with HER2 positivity defined as an IHC score of 3+ and equivocal HER2 expression as an IHC score of 2+. Samples scored as equivocal on IHC could then be assessed for *ERBB2* amplification (defined as *ERBB2*-to-*CEN17* ratio ≥2.0 on FISH) to determine eligibility for trastuzumab, which established the practice of reflex FISH testing of IHC 2+ samples[17]. After HercepTest entered the market, other diagnostic companies developed their own HER2 assays. Ventana introduced two versions of a HER2 IHC assay: the first used the mouse monoclonal antibody CB11, and the subsequent version used the rabbit monoclonal antibody 4B5. Concordance rates between assays were 93.3% within a single-institution cohort and 84.7% within a multinational cohort[18]. Today, the PATHWAY 4B5 version is the most widely used HER2 companion diagnostic test[18].

Since the introduction of trastuzumab, IHC has been the primary method of evaluating HER2 expression. However, the accuracy of

HER2 IHC assays is limited by the wide dynamic range and heterogeneity of HER2 protein expression, various pre-analytical variables and the subjective nature of IHC scoring. HER2 expression can span over three orders of magnitude (or 'logs'), with individual cells expressing around 1,000 to >1,000,000 HER2 molecules[19]. By contrast, IHC assays typically have a dynamic range of only one or two logs at best[19]. HER2 expression in breast tissue is also not necessarily exclusive to cancer cells and can also be detected in non-malignant breast ductal cells. Furthermore, the heterogeneous pattern of HER2 expression within a tumour means that not every cancer cell in a HER2-positive tumour will express detectable HER2 (ref. 16). Consequently, a patient's HER2 score can vary based on the tissue area selected for analysis, whether the level of HER2 expression falls within the dynamic range of the assay and on the subjective interpretation of IHC staining by pathologists[20,21]. Pre-analytical variables can also introduce a substantial amount of inconsistency in IHC results. Factors such as the choice of antibody and assay, level of adherence to the recommended assay protocol, time to and duration of tissue fixation, type of fixative used, and storage conditions have all been identified as influencing the accuracy of HER2 testing[2,18,20]. The introduction of standardized HER2 scoring guidelines by the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) helped improve the consistency of IHC results but did not eliminate the inherent subjectivity arising from the need for a pathologist to visually score each sample[17,22]. Attempts to make scoring more objective by partially or fully automating this process have thus far included the development of an automated cell imaging system (ACIS) digital microscope and the creation of automated quantitative analysis; both of these methods use signal intensity to score HER2 expression. ACIS provided categorical scores corresponding to IHC 0, 1+, 2+ or 3+ on the basis of chromogenic assays, whereas automated quantitative analysis provided a continuous score from immunofluorescence assays on the basis of the intensity of the signal within the area of tumour cells[23–25] without standardization or association with traditional ordinal scores.

Assessment of *ERBB2* amplifications

While the IHC assays discussed previously enable semiquantitative assessments of HER2 expression, challenges relating to the reproducibility of IHC 2+ scores led to the development of alternative companion diagnostic methods such as determining the presence of *ERBB2* amplifications using FISH and quantification of *ERBB2* RNA using reverse transcriptase polymerase chain reaction (RT-PCR). Among these methods, FISH quickly gained acceptance as a method of distinguishing between samples harbouring *ERBB2* amplifications and those without when IHC results are inconclusive. Three *ERBB2* FISH assays — INFORM[26], PathVysion[27] and PharmDx Kit[28] — received FDA approval for the assessment of *ERBB2* amplifications in 1997, 1998 and 2005, respectively. In a 2007 study, agreement between PathVysion and PharmDX Kit was demonstrated to be 100%, while agreement between PathVysion and INFORM differed based on the threshold used for INFORM (76% with a threshold of 4+ signals per nucleus, 91% for 5+ signals and 98% for 6+ signals)[29]. FISH was officially included in the ASCO/CAP clinical scoring guidelines for HER2-equivocal (IHC 2+) specimens in 2007 (refs. 14,16).

In an attempt to address the platform-based limitations of FISH, specifically the need for fluorescence microscopy, chromogenic in situ hybridization (CISH) was introduced as an alternative method of visualizing *ERBB2* amplifications using a bright-field microscope. The first CISH assay for *ERBB2*, SPOT-LIGHT[30], received FDA approval in 2008, and more CISH assays were approved in the following years. Nonetheless, CISH has thus far not been broadly implemented in clinical pathology laboratories[14].

RT-PCR offers an alternative and substantially different method enabling quantitative assessments of *ERBB2* mRNA. Whereas FISH and CISH require visual assessments of tissue on a microscope slide, RT-PCR removes this source of subjectivity by providing a result indicating the relative amount of *ERBB2* mRNA present in each sample[31]. Despite this advantage, RT-PCR was seldom used clinically owing to the difficulties of performing RT-PCR relative to FISH[31] as well as the inability to exclude signals arising from non-malignant ducts and ductal carcinoma in situ, which also express varying levels of *ERBB2* mRNA. More recently, closed-system quantitative RT-PCR methods have been introduced[32]; however, these assays have not yet gained traction in clinical practice and are not included in the ASCO/CAP guidelines.

ASCO/CAP HER2 testing guidelines in breast cancer

Since 2001, the ASCO Tumour Marker Guidelines Panel has recommended routine HER2 testing for patients with newly diagnosed and metastatic breast cancer. Despite this recommendation, substantial discordance in testing outcomes has emerged, partly owing to variability in the testing methods. At that time, approximately 20% of HER2 testing results were found to be inaccurate, resulting in a risk of inappropriate disease classification and/or treatment decisions[17]. In response to the growing awareness of the variability in HER2 testing and its implications for patient outcomes, ASCO and CAP established a clinical practice expert panel with the goal of developing recommendations to standardize HER2 testing protocols and thus improve reliability. The ASCO/CAP guidelines for HER2 biomarker testing, originally published in 2007 and updated in 2013, 2018 and 2023, refined the evaluation criteria for HER2 IHC and in situ hybridization (ISH) testing in breast cancer.

The 2007 ASCO/CAP guidelines aimed to improve the accuracy of HER2 testing in invasive breast cancers, particularly in the adjuvant setting. The expert panel conducted a systematic review of the literature to develop their recommendations for an optimal HER2 testing protocol[17]. These guidelines formed the basis for the two-phase HER2 testing algorithm that is still in use today, with HER2 IHC serving as the primary screening method that reflexes to assessment of *ERBB2* amplifications for samples with equivocal protein expression (HER2 IHC 2+). The guidelines also provided recommended procedures for specimen handling, testing, exclusion criteria, assay interpretation and reporting practices to mitigate sources of variation in HER2 IHC testing. These guidelines also established a series of external quality assurance procedures, including biannual laboratory proficiency testing for reporting HER2 results, CAP accreditation requirements and pathologist competency assessments.

In 2013, the ASCO/CAP expert panel reconvened to amend the 2007 guidelines. The decision by the 2007 panel to increase the threshold for HER2 positivity on FISH (*ERBB2*-to-*CEP17* ratio from 2.0 to 2.2 or *ERBB2* copy number from 4 to 6 copies per cell) and for a HER2 IHC 3+ score (strong circumferential staining from >10% to >30% of cancer cells) raised concerns that initial trials testing adjuvant trastuzumab would have excluded many patients had the 2007 criteria been applied. A retrospective analysis of data from the North Central Cancer Treatment Group N9831 trial, which tested trastuzumab plus adjuvant chemotherapy, demonstrated that 0.78–1.1% of patients deemed to have HER2-positive breast cancer at the time of enrolment would not have qualified if the 2007 ASCO/CAP criteria had been used[33,34]. The 2013 Update Committee elected to lower

these thresholds for the 2013 ASCO/CAP guideline (that is, IHC 3+ was defined as strong circumferential staining on >10% of cancer cells and HER2 positivity by FISH was defined as a *ERBB2*-to-*CEP17* ratio of 2.0) and introduced the concept of ambiguous HER2 expression on ISH (IHC 2+ with a *ERBB2*-to-*CEP17* ratio of <2 and *ERBB2* copy number ≥4 and <6)[35]. Nonetheless, the pathology community had several concerns regarding the consequences of implementing this update. In a critical review of the 2013 ASCO/CAP update, several issues were highlighted, including the ambiguity of directions for IHC assessment of equivocal samples, inadequate information on rare forms of breast cancer harbouring strong HER2 expression without a circumferential distribution, the role of FISH-based assessments in the context of *ERBB2* heterogeneity, and the optimal next step in the workflow after receiving equivocal results from both IHC and FISH[36]. Many studies on the implications of the modified 2013 ASCO/CAP guidelines for HER2 testing in breast cancer were subsequently conducted, with several reporting an increase in the number of equivocal cases that did not contribute to increases in definitive HER2 assessment[37–42]. This guideline update might have instead resulted in delays in the definition of HER2 status for some patients[37] as well as false-positive interpretations of HER2 FISH-equivocal samples when investigated further using the recommended alternative control probes[42].

In 2018, ASCO/CAP provided another HER2 testing guideline update in an attempt to remedy a range of issues that arose in clinical practice[43]. One key change was the removal of the requirement to retest surgical excision specimens if the initial HER2 test result from a core needle biopsy sample obtained from the primary breast cancer was negative. The panel also redefined HER2 IHC 2+ equivocal specimens as those showing weak-to-moderate complete, as opposed to incomplete, membrane staining in >10% of cancer cells. The algorithm for interpreting dual-probe ISH results was also restructured to account for the approximately 5% of patients with equivocal *ERBB2* amplification results. The controversial 'double equivocal' category was replaced with new ISH categories[35].

In August 2022, the FDA approval of T-DXd for patients with metastatic HER2 IHC 1+ or 2+ and ISH-negative breast cancer, referred to as HER2-low, led to the publication of a short review of the ASCO/CAP guidelines, published in September 2023, following the DESTINY-Breast04 trial[44,45]. This update acknowledged the issues with interpreting 'low HER2' samples, yet no changes to the official guidelines were introduced owing to insufficient evidence. Instead, a series of best practice recommendations for handling such cases were provided. A timeline for the events related to the HER2 companion diagnostic in breast cancer and associated HER2 targeting drugs are shown in Fig. 1.

Although the ASCO/CAP HER2 testing guidelines are the most prevalent, guidelines provided by the European Society for Medical Oncology (ESMO)[46] and a panel of UK-based pathologists are also available[47]. Interestingly, both the ESMO and UK guidelines recognize the HER2-low category. The ESMO guidelines, phrased in a question-and-answer format, suggest challenges in scoring HER2 but do not provide a definition for each score class. These guidelines also suggest consideration of re-biopsy following a finding of HER2 IHC 0, to "open new therapeutic opportunities in a relevant proportion of patients"[46], thus showing recognition of the potential variability and/or inaccuracy of IHC-based HER2 scoring in differentiating between low and absent HER2 expression. The UK guidelines are more prescriptive and provide an algorithm for scoring HER2 that includes a definition of the HER2-low category as IHC 1+ or 2+ and ISH-negative. Specifically, these guidelines recommend that "the term HER2-low should be included as a descriptive term in pathology reports"[47], thereby going further than the guidance provided by ASCO/CAP for laboratories in the USA.

HER2 positivity and testing guidelines for other solid tumours

The success of HER2-targeted therapies in patients with breast cancer spurred interest in applying similar treatments to other HER2-expressing solid tumours. Most recently, findings from the DESTINY-PanTumor02, DESTINY-Lung01 and DESTINY-CRC02 trials led to the FDA accelerated approval of T-DXd as a tumour-agnostic therapy for patients with unresectable or metastatic HER2-positive (IHC 3+) solid tumours[48–50]. Collectively, the tissue-agnostic utility of HER2-targeted agents underscores the importance of HER2 testing in solid tumours.

Defining HER2 positivity across many different solid tumours as *ERBB2* amplification and/or overexpression of HER2 protein might seem like a simple task. However, in reality, the biology of *ERBB2* and HER2 alterations, the histopathology of HER2 expression, and the responsiveness to HER2-targeted therapies varies substantially across tumour types. These challenges necessitated the development of specific workflows for HER2 testing in other solid tumours beyond breast cancer, including oesophageal, gastro-oesophageal junction, gastric, colorectal, uterine and non-small-cell lung cancers. The clinical landscape of HER2-targeted therapies and the current pathology workflows for determining HER2 positivity are summarized in detail elsewhere[1,51].

Gastro-oesophageal adenocarcinomas

Following the landmark ToGA trial, in which patients with advanced-stage gastric, gastro-oesophageal junction or gastro-oesophageal adenocarcinomas (GEAs) harbouring *ERBB2* amplifications and/or HER2 overexpression received trastuzumab plus chemotherapy, HER2-targeted therapy has become central to systemic therapy for this subgroup. ToGA demonstrated a significant improvement in median overall survival (OS) for patients with GEAs deemed to be HER2 positive (either IHC 3+ or FISH positive) who received trastuzumab plus chemotherapy (n = 294) versus chemotherapy alone (n = 290): 13.8 months versus 11.1 months (P = 0.046)[52]. Post-hoc subgroup analysis by HER2 IHC and FISH status revealed that patients with IHC 2+ and FISH-positive or IHC 3+ tumours had further improvements in median OS when receiving the trastuzumab-containing regimen (n = 446; 16 months versus 11.8 months) compared to those with IHC 0 or 1+ and FISH-positive tumours (n = 131; 10 months versus 8.7 months). These results established trastuzumab plus chemotherapy as a standard treatment option for patients with HER2-positive (IHC 3+, or IHC 2+ and ISH-positive) GEAs, with FDA approval in 2010 (ref. 52). On the basis of more recent results from KEYNOTE-811, pembrolizumab can also be included in this regimen (with variations in the chemotherapy component) for patients with HER2-overexpressing and PD-L1-expressing (combined positive score ≥1) GEAs[53].

The incidence of HER2 positivity in patients with gastric cancer ranges from 12% to 23%, varying by histological subtype (greater in intestinal versus diffuse-type cancers) and tumour grade (greater in moderately differentiated cancers)[54–59]. For oesophageal and gastro-oesophageal junction cancers, reported incidences of HER2-positive disease vary more widely from 2% to 45%[60], with oesophageal adenocarcinomas more likely to be HER2-positive compared with

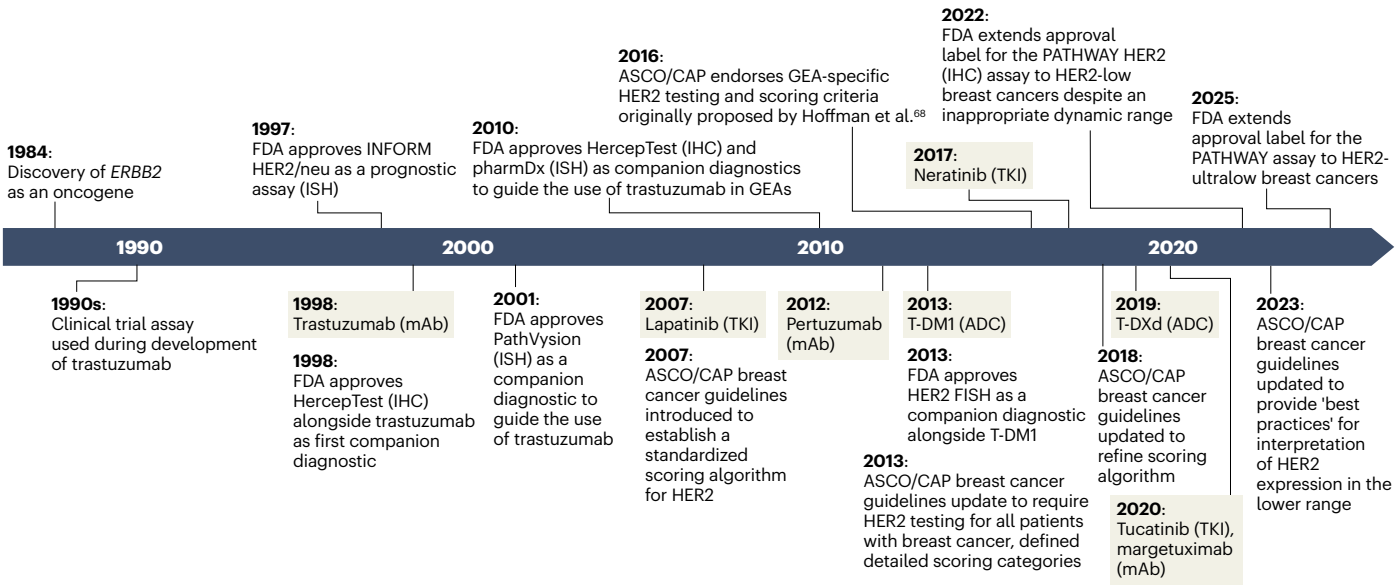

Fig. 1 | Timeline of developments in HER2 assessment. This timeline provides an overview of the major developments in human epidermal growth factor receptor 2 (HER2) testing in the context of the approvals of various HER2-targeted therapies over the same period. ADC, antibody–drug conjugate; ASCO, American Society of Clinical Oncology; CAP, College of American Pathologists; FISH, fluorescence in situ hybridization; GEA, gastro-oesophageal adenocarcinoma; IHC, immunohistochemistry; ISH, in situ hybridization; mAb, monoclonal antibody; T-DM1, trastuzumab emtansine; T-DXd, trastuzumab deruxtecan; TKI, tyrosine-kinase inhibitor.

squamous cell carcinomas[61–64]. Unlike breast cancers, in which full circumferential staining is included in the IHC scoring criteria, GEAs frequently have a U-shaped pattern of membrane staining, with expression restricted mostly to the basolateral cell membrane[65]. HER2 expression in these cancers is often also highly heterogeneous, with focal or patchy areas of overexpression often observed across the specimen[66]. This variability and differences in staining patterns necessitated the development of specific scoring systems to ensure consistent and accurate determination of HER2 status[66,67].

In 2016, ASCO, CAP and the American Society of Clinical Pathology officially endorsed the GEA-specific HER2 testing and scoring criteria originally established by Hofmann et al. in 2008 (refs. 65,68). These guidelines emphasize the importance of both IHC and ISH assays in HER2 evaluation, although the interpretation criteria differ substantially from those used in breast cancer owing to differences in the biology of HER2 expression in GEA. Mysteriously, the GEA scoring criteria for the IHC biomarker assay also differ between biopsy and resection specimens. For a positive result, biopsy samples must have staining of at least one tumour-cell cluster containing five or more cancer cells, whereas resection specimens require staining of at least 10% of tumour cells in the selected block. Investigations of the effects of specimen type on HER2 status found that the type of specimen (biopsy versus excision) has minimal effects on the extent of IHC–FISH concordance (n = 386 patients, 83.7% agreement), whereas the level of concordance between different specimens obtained from the same patient is lower (n = 130 patients, 75.4%), partly owing to intratumoural heterogeneity[69]. This study reinforces the view that current guidelines for determining HER2 positivity in patients with GEAs are reasonably concordant but could benefit from including the recommendation to test multiple specimens from the same patient or possibly the introduction of quantitative methods.

Colorectal cancer

Testing for *ERBB2* amplifications in patients with colorectal cancer (CRC) remains controversial owing to the absence of universally accepted criteria. Various guidelines, such as the HERACLES diagnostic criteria, the ASCO/CAP 2018 breast cancer guidelines and CAP/American Society of Clinical Pathology/ASCO GEA guidelines, are currently used. Overall, HER2 is rarely amplified and/or overexpressed in CRC (in approximately 3% of patients)[70,71], although this prevalence is higher in patients with *RAS*-wild-type and *BRAF*-wild-type tumours (5–14%)[72,73]. The HERACLES diagnostic criteria, which were specifically developed for CRC, were used to assess the effectiveness of dual HER2 blockade with a combination of trastuzumab and lapatinib in patients with HER2-positive CRC[72,73]. These criteria are endorsed by the current USA National Comprehensive Cancer Network (NCCN) guidelines for CRC across all second-line regimens indicated for patients with HER2-positive CRC[74], including trastuzumab plus pertuzumab, trastuzumab plus lapatinib, trastuzumab plus tucatinib, and T-DXd.

According to the HERACLES criteria[73], intense (3+) complete, basolateral or lateral membranous HER2 staining of ≥50% of tumour cells is considered IHC positive. However, if the staining intensity is 3+ in ≥10% but <50% of tumour cells, confirmatory FISH testing is required to verify the presence of *ERBB2* amplifications. Investigators compared the performance of HERACLES scoring of CRC specimens when applied to IHC images stained using the 4B5 assay or HercepTest, with silver ISH serving as the gold standard[73]. This study demonstrated that the 4B5 assay produces fewer false-negative results than HercepTest. The 4B5 assay also showed higher cellularity of intense IHC positivity (at a 50% cellularity cut-off) for amplified specimens compared to HercepTest, indicating that the 4B5 assay provides a more sensitive method of quantifying HER2 expression. Besides IHC and ISH, use of next-generation sequencing (NGS) for the detection of *ERBB2* amplifications is also

recommended in the NCCN guidelines on CRC, although this approach is not specified in the ESMO equivalent[75].

Despite these recommendations and the variations in HER2 expression patterns across tumour types, most pathology laboratories continue to apply HER2 testing guidelines designed for breast cancer to the analysis of GEA and CRC specimens[76]. This approach has been influenced by clinical trials, including the MyPathway trial[77] (testing trastuzumab plus pertuzumab) and the MOUNTAINEER trial[78] (testing trastuzumab plus tucatinib), in which the HER2 IHC criteria for breast cancer were used to select patients with HER2-overexpressing CRC. By contrast, the DESTINY-CRC01 trial testing T-DXd used the GEA guidelines for HER2 scoring[79]. These alternative scoring approaches have the advantage of permitting the use of both the 4B5 and HercepTest assays but might overcall HER2 positivity, where less stringent criteria, in which intense IHC staining of as few as 10% of cells can qualify as a positive result, are applied[71,73,76]. Conversely, with the emergence of more effective HER2-targeted agents with the potential to provide clinical benefit to patients with HER2-low cancers, this amalgamation of current scoring guidelines for CRC might underestimate the size of the population of patients who are likely to have a response. Overall, these discrepancies between recommended guidelines and real-world pathology practice highlight an ongoing debate as well as the need for a standardized approach to HER2 testing.

Uterine cancer

The approach to testing for HER2 overexpression in uterine cancers is similar to that used in CRC, with no established scoring system available. Historically, patients with HER2-overexpressing tumours had worse clinical outcomes[80–83], and case reports from the 2000s describe responses to trastuzumab in patients with uterine serous carcinoma (USC)[84–86]. Yet, responsiveness to trastuzumab was found to be inconsistent when investigated in early-phase trials[87,88] and estimates of the percentage of USCs harbouring HER2 overexpression have varied substantially (14–80% of patients)[82,83,85,89–94]. This situation is further complicated by the rarity of high-risk USC, resulting in small cohort sizes, variations in testing methodologies and scoring criteria, and the inclusion of tumours with mixed histologies[51]. In 2013, one of the largest studies of the extent of HER2 expression in USC at the time demonstrated that approximately 35% of USCs harbour HER2 overexpression and/or *ERBB2* amplifications on IHC and/or FISH[95]. This study determined HER2 positivity according to the 2007 ASCO/CAP breast cancer guidelines, in which IHC 3+ is restricted to samples with intense complete, basolateral or lateral membranous staining on at least 30% of tumour cells. Applying this more stringent scoring system increased the extent of concordance between HER2 IHC and FISH (from 75% to 81%) compared to the FDA HercepTest package insert criteria, which require >10% cancer cell staining. However, over half of the specimens had significant visual evidence of heterogeneous HER2 protein expression, similar to that seen in other solid tumour types outside of breast cancer. Nonetheless, the scoring system applied in this study went on to guide the patient enrolment criteria for subsequent trials testing HER2-targeted therapies in patients with high-risk uterine cancers.

In 2019, the NCCN endorsed a HER2-targeted triplet therapy combining trastuzumab with carboplatin and paclitaxel for HER2-positive USC and uterine carcinosarcomas. Although USC and carcinosarcomas account for ~10% of all endometrial carcinomas, these high-risk subtypes are responsible for up to 40% of endometrial cancer mortality[96]. This recommendation, as the primary therapy for stage III–IV disease and as a first-line regimen for recurrent disease, is based on data from a randomized phase II trial[97,98]. To date, this trial is the only one to demonstrate that the addition of trastuzumab to standard-of-care chemotherapy improves the outcomes of patients with HER2-positive uterine cancers[99].

The success of trastuzumab triplet therapy for patients with HER2-positive uterine cancers has been attributed to patient selection using a USC-specific HER2 scoring protocol[100,101]. Although not formally endorsed, these USC-specific criteria are included in the March 2023 biomarker testing template for analysis of gynaecological cancer specimens provided by CAP[102]. A multi-institutional study with data published in 2021 demonstrated a reasonable level of inter-operator concordance for scoring HER2-overexpressing USCs using this IHC scoring system, with overall levels of agreement ranging from 72.3% to 83.3% and K values between 0.6 and 0.65 for all categories, or two categories combined (IHC 0 or 1+ versus 2+ or 3+), respectively, across seven pathologists[99]. Despite this concordance, the standardization of HER2 testing in USCs remains in its early stages, with challenges arising from the complex and variable morphologies of this tumour subtype. For example, some samples have full circumferential HER2 staining similar to that seen in breast cancer, whereas others have basolateral HER2 staining patterns akin to those observed in gastric cancer (Fig. 2). This complexity is further compounded by the heterogenous nature of HER2 expression in USC, which is similar to that seen in other solid tumour types. Therefore, additional research is needed to gain a deeper understanding of the biology of HER2-positive USC and to optimize the assessments of HER2 specifically for this tumour subtype.

The combination of trastuzumab plus pertuzumab or trastuzumab plus carboplatin and paclitaxel is being tested in a phase II–III trial enrolling patients with HER2-positive advanced-stage USC or uterine carcinosarcoma, with HER2 status determined according to 2018 ASCO/CAP breast cancer HER2 testing guidelines, presumably with a HER2 IHC 3+ threshold of >10% cancer cell staining (NCT05256225). This trial, once mature data are available, might help determine the relevance of morphology and IHC intensity to clinical outcomes. In the meantime, the lack of specific standardized testing guidelines for HER2-positive USC and uterine carcinosarcoma might have deterred testing in many laboratories, as only 31.9% of laboratories reported performing HER2 testing for uterine cancers in a survey of 1,195 pathology laboratoriess included in the CAP HER2 IHC proficiency testing programme in 2021 (ref. 76). For those that do test uterine cancer specimens, few laboratories reported using uterine cancer-specific scoring criteria (16.0%) with most applying the 2018 ASCO/CAP guidelines for breast cancer (69.4%)[76].

Non-small-cell lung cancer

The clinical implications of HER2 alterations in patients with non-small-cell lung cancer (NSCLC) are complex. Mutations in *ERBB2* can be found in up to 4% of patients, with a higher prevalence in women, non-smokers and individuals of Asian ethnicity, and are often associated with brain metastases[103–107]. *ERBB2* amplifications are rare, occurring in about 3% of patients with newly diagnosed disease, although such amplifications can be detected in up to 13% of patients with *EGFR*-mutant NSCLC with disease progression on earlier-generation EGFR tyrosine-kinase inhibitors[103,108]. HER2 overexpression on IHC can be detected in up to 38% of NSCLCs[103] but has poor concordance with *ERBB2* amplifications. A meta-analysis of data from 1,217 patients found a concordance rate of only 11% for HER2 IHC scores of 2+ or 3+, compared to 99% for scores of 0 or 1+, indicating that current criteria for interpreting HER2 IHC or the IHC assays themselves are not suitable for predicting the presence of

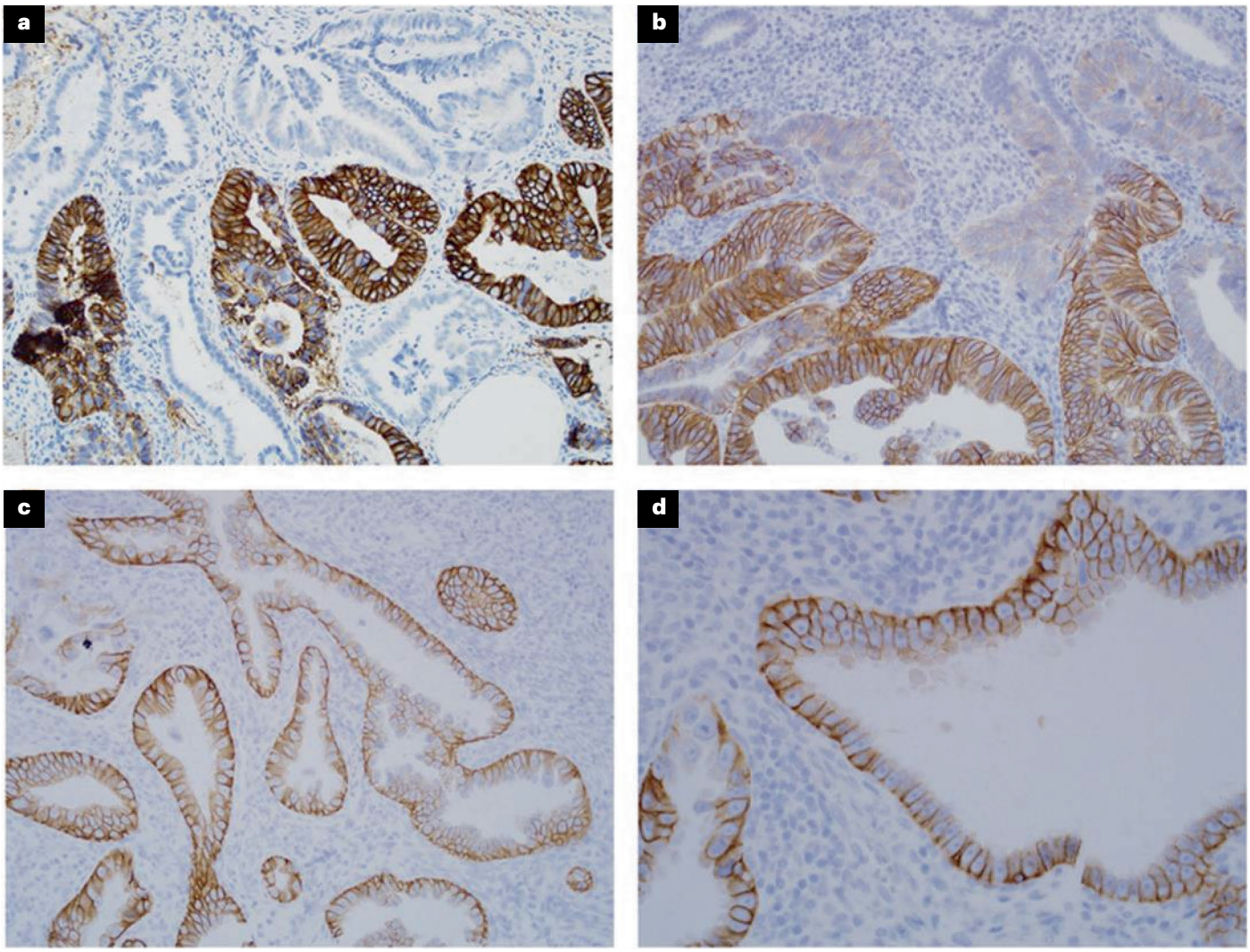

Fig. 2 | Visualization of HER2 expression using immunohistochemistry in tumour tissue samples from patients with uterine serous carcinoma. **a**,**b**, Heterogeneous human epidermal growth factor receptor 2 (HER2) expression (brown). **c**,**d**, Lack of apical HER2 immunostaining, resulting in a lateral or basolateral 'U-shaped' staining pattern. Reprinted from ref. 95, Springer Nature Limited.

ERBB2 amplifications[109]. An assessment of HER2 expression in NSCLC published in 2024 suggests that conventional IHC assays might have the wrong dynamic range, and that >60% of NSCLC tumours have levels of HER2 expression that are above the limits of detection of a more sensitive quantitative assay[110]. Independent of IHC, *ERBB2* exon 20 insertions have been shown to be crucial for the efficacy of T-DXd in patients with NSCLC, leading to the FDA approval of this agent on the basis of data from the DESTINY-Lung01/02 trials, which showed objective response rates (ORRs) of ~50% in patients with often heavily pretreated advanced-stage *ERBB2*-mutant disease[105]. Assessments of HER2 protein expression using IHC are currently not recommended in patients with NSCLC, although this situation might change depending on the results of trials testing T-DXd in patients with HER2-expressing NSCLC. Notably, a second publication from DESTINY-Lung01 describes a number of patients with HER2 IHC 2+, *ERBB2*-unamplified NSCLC who derived benefit from T-DXd[49]. Molecular testing, particularly NGS, is recommended in the ESMO, NCCN and ASCO NSCLC guidelines for precise diagnosis and treatment planning[104]. Given the small amounts of tumour tissue typically present in lung cancer biopsy samples, the possibility of future integration of nucleic acid (NGS or PCR) and/or protein testing remains uncertain.

HER2 testing in HER2-low cancers

The growing list of indications for HER2-targeted ADCs is expected to increase the demand for HER2 testing across many solid tumour types in the coming years. However, the lack of established scoring systems for HER2 overexpression and amplification outside of breast and gastric cancers poses a major challenge in providing reliable results that can support clinical decision-making regarding these new ADCs. In addition, cancers characterized by low levels of HER2 expression (HER2-low, defined as IHC 1+ or 2+ without *ERBB2* amplifications) have emerged as a clinically relevant entity largely owing to the success of

T-DXd, which might reflect 'bystander effects' of this agent[111–113]. T-DXd is of particular interest owing to the outstanding results of the phase III DESTINY-Breast04 trial, in which patients with HER2-low breast cancer receiving T-DXd had significantly improved median PFS (10.1 months versus 5.4 months; HR 0.51, 95% CI 0.40–0.64; P <0.001) and median OS (23.9 months versus 17.5 months; HR 0.64, 95% CI 0.48–0.86; P = 0.003)[45].

The thresholds for target expression and the extent of spatial heterogeneity required for clinical benefit from T-DXd remain largely undefined. This knowledge gap is underscored by results from the phase II DAISY trial of T-DXd, in which 29.7% of patients with HER2 IHC 0 metastatic breast cancer had an objective response[114]. Whether these responses reflect bystander effects of T-DXd or inaccuracies in reading or heterogeneity in IHC assessments remains unclear. When 31 specimens from patients in the IHC 0 subgroup were reread, some level of HER2 expression was detected in 15 samples, including 7 that were classified as IHC 1+. Nonetheless, responses were seen both in patients with samples that were reread as >0 (IHC 1+ and IHC 'ultralow') and in samples reread as IHC 0. Thus, accurate interpretation of these findings will require a new, adequately powered trial involving patients with IHC 0 disease, hopefully including some kind of standardization to define true IHC 0 status. However, observations from DAISY have generated considerable research interest in the potential of T-DXd for patients with HER2-ultralow cancers, potentially with IHC scores falling between 0 and 1+. This hypothesis was further examined in the DESTINY-Breast06 trial, which demonstrated prolonged median PFS durations for patients with both HER2-low (IHC 1+ or 2+ with a negative ISH result) and HER2-ultralow (classed as IHC 0 per the 2018 ASCO/CAP guidelines, with some HER2 staining on cancer cell membranes in <10% of cancer cells) metastatic breast cancer receiving T-DXd compared with chemotherapy (13.2 months versus 8.1 months; HR 0.64, 95% CI 0.54–0.76; P <0.001)[115,116]. These findings raise crucial questions about whether current FDA-approved HER2 assays are sufficiently sensitive to establish reliable thresholds for predicting a therapeutic response to T-DXd.

Existing HER2 IHC assay platforms and interpretation guidelines were designed to distinguish high levels of HER2 expression from low levels in a binary manner and are therefore not optimized for discriminating between low and absent HER2 expression[117]. Owing to the wide spectrum of HER2 expression in cancer — which spans at least 4 orders of magnitude based on measurements of the number of HER2 receptors per cell[118,119] — as well as the poor dynamic range of IHC assays involving diaminobenzidine-based detection of 1–1.5 orders of magnitude[19], providing accurate and reproducible assessments of HER2-low disease on IHC is often challenging. An evaluation of 2 years of CAP survey data from over 1,400 laboratories, each of which scored 80 breast cancers for HER2 expression, demonstrates that 19% of tissue cores produced highly discordant results (Fig. 3), with <70% agreement at the HER2 IHC 0 versus 1+ or 2+ cut-off[120]. The same study also assessed the level of concordance among 18 board-certified pathologists, most with >5 years of experience, who were asked to score the same set of scanned IHC slides, revealing a 75% discordance rate in scoring HER2 IHC 0 versus 1+ or 2+ disease[120,121]. Pathologists participating in these studies were not first informed that their performance at the IHC 0 versus 1+ cut-off was being tested. Several independent studies have also demonstrated poor inter-rater reliability for distinguishing between HER2 IHC 0 and

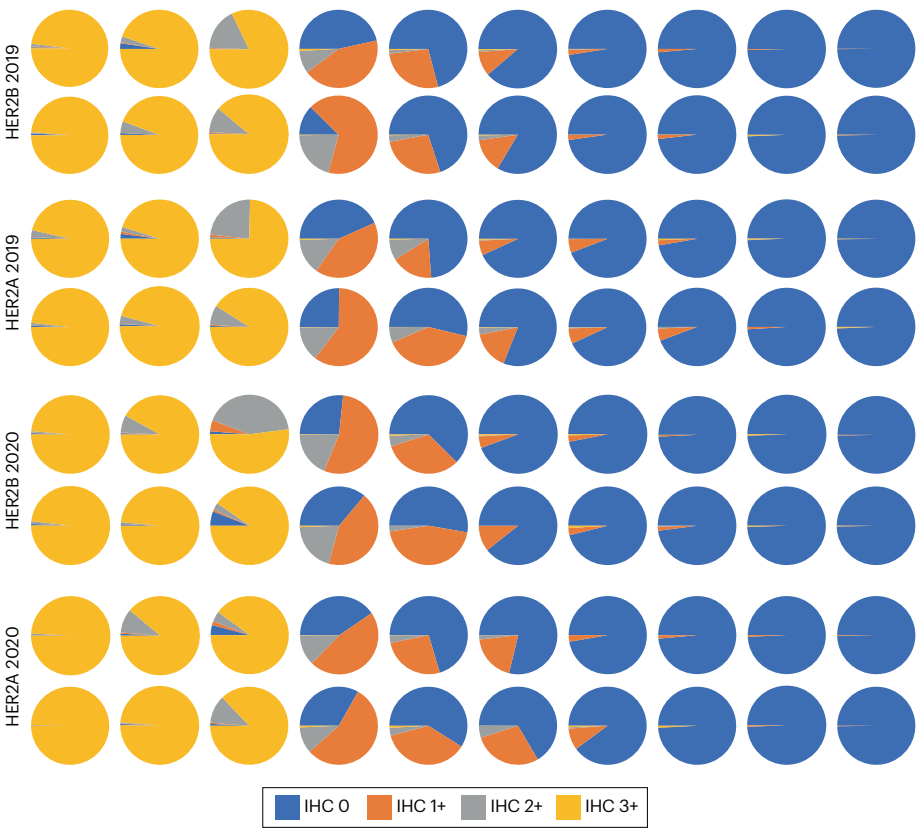

Fig. 3 | A pie chart matrix showing the scoring proportions of laboratory readings for each scoring category for HER2 IHC. Two years of data from the College of American Pathologists' immunohistochemistry (IHC) proficiency surveys for human epidermal growth factor receptor 2 (HER2) expression in breast cancer from 2019 and 2020. Participating laboratories (n = 1,391–1,452) each received two tissue microarrays comprising 10 HER2 cores twice per year (designated HER2A and HER2B). The laboratories then performed the HER2 assay using the standard method used in their clinical laboratory improvement amendments (CLIA) laboratories. These tissue microarrays were then scored and returned to the College of American Pathologists as part of their quality assessment programme. The total survey dataset covers the scores from 40 HER2 cores per laboratory over a 2-year period from ~1,400 laboratories. The extremes (IHC 3+ and IHC 0) generally have high levels of concordance amongst laboratories, yet intermediate HER2 expression (IHC 1+ and IHC 2+) is often highly discordant. This figure is constructed from data collected and published in Fernandez et al.[120] and Robbins et al.[121].

1+ or 2+ tumours[51,122–127]. This inconsistency has created a conundrum for pathologists[128,129], who are increasingly being asked to interpret 'zeros' and 'ones' despite mounting evidence that legacy IHC assays are not fit for this purpose[130].

This repurposing of traditional HER2 IHC assays, although approved by the FDA, has been likened to "weighing elephants when you are trying to accurately weigh mice"[128]. The absence of a sensitive analytical assay designed specifically for low levels of HER2 expression has resulted in some patients receiving T-DXd when no target is present, or worse, being denied treatment (incorrectly classified as having IHC 0 disease and therefore being deemed ineligible for T-DXd) when they might, in fact, have tumours expressing the target and could benefit from this highly effective therapy. The combination of inadequate assay sensitivity and considerable inter-rater variability in pathologist scoring for low HER2 expression provides an explanation as to why some patients with 'HER2-low' cancers might respond to T-DXd. This issue is even more pronounced in other solid tumour types in which HER2 testing guidelines are less well developed as pathologists now face the additional challenge of determining HER2-low status without clearly defined, clinically relevant criteria.

Unlike for patients with HER2 IHC 2+ disease, in whom *ERBB2* amplifications can be used to confirm HER2 positivity, no reflex test or evidence-based recommendations exist for HER2-low disease; only 'best practices' for assessing for this type of HER2-positive disease have been recommended[43,44]. The 2023 reaffirmation of the 2018 ASCO/CAP guidelines maintains the legacy approach to HER2 testing[44]. The best practices that pathologists are supposed to use to distinguish between IHC 0 and 1+ include[44] (1) examining HER2 IHC-stained slides using scoring criteria provided by standardized ASCO/CAP guidelines; (2) examining the HER2 IHC slide at a higher than usual magnification (40×) when attempting to discriminate IHC 0 from 1+ staining; (3) considering a second pathologist review when results are close to the IHC 0 versus 1+ interpretive threshold; (4) using controls with a range of protein expression (including IHC 1+) to ensure an appropriate limit of detection; and (5) paying careful attention to the pre-analytical conditions of breast cancer tissue samples obtained from both primary and metastatic lesions. A comprehensive study, which included 77 pathologists from 14 countries, was conducted to assess the accuracy of HER2-low diagnosis. Pathologists' performance was assessed, followed by training and re-assessment[131]. Small numerical improvements were seen in the overall level of inter-rater agreement for both HER2 IHC 0 and HER2-low (IHC 1+ or 2+), although training did not lead to any statistically significant improvements. Perhaps future studies will provide evidence that these approaches improve the reproducibility and accuracy of patient selection. However, the underlying design and dynamic range of the assays suggest that a new approach, assay and/or method of assessment might be required to best serve patients.

Despite the controversies surrounding HER2-low testing, early evidence suggests that HER2-low cancers beyond breast cancer could benefit from next-generation HER2-targeted ADCs such as T-DXd. The DESTINY-Gastric01 phase II trial tested this approach in exploratory cohorts with HER2-negative (IHC 2+/ISH-negative or IHC 1+) GEAs[132]. The HER2 IHC 2+/ISH-negative cohort ($n = 19$) had a confirmed ORR of 26.3%, with median PFS and OS durations of 4.4 months and 7.8 months, respectively[132]. Surprisingly, the ORR was 9.5% in the IHC 1+ cohort ($n = 21$), indicating that some patients with very low levels of HER2 expression can have a response to T-DXd[132]. The STATICE trial tested T-DXd in patients with HER2-high (IHC ≥2+; $n = 22$) and in those with HER2-low (IHC 1+; $n = 10$) advanced-stage or recurrent uterine carcinosarcoma[133]. ORRs of 54.5% and 70% were observed in the HER2-high and HER2-low groups, respectively[133], although the cohort sizes were small and these results should therefore be viewed with caution. In the phase II HERB trial, which tested T-DXd in 32 patients with unresectable and/or recurrent HER2-expressing biliary tract cancer, the ORR was 36.4%[134]. Some evidence of efficacy was also seen among the seven patients with HER2-low disease (HER2 IHC 0/ISH-positive, IHC 1+, or IHC 2+/ISH-negative), with an ORR of 12.5%, a median PFS of 4.2 months and a median OS of 8.9 months[134]. However, not all HER2-low cancers seem to respond to T-DXd. In the DESTINY-CRC01 trial[79], no objective responses were observed in the HER2-low subgroup, and patients with HER2 IHC 3+ tumours had considerably more favourable response rates than those with IHC 2+/ISH-positive previously treated metastatic CRC (ORR 57.5% versus 7.7%). Nonetheless, these results must be interpreted in light of the limitations of current HER2 assays, which are designed to detect HER2 overexpression or *ERBB2* amplification, as well as the inconsistent scoring criteria used across different tumour types and the effects of interlaboratory workflow variations.

Emerging approaches to HER2 testing and quantification

The clinical demand to select patients with HER2-low disease for treatment with T-DXd, coupled with the largely inadequate HER2 testing procedures for HER2-low cancers, underscores the urgent need for standardized protocols and potentially new, more sensitive methodologies designed specifically for HER2-low testing. Several emerging approaches and techniques that aim to address the limitations of the current assays and scoring systems are currently in development.

Standardizing HER2 IHC

HER2 IHC scoring, like that applied to any IHC-detected biomarker, hinges on two key factors: the chemical preparation of the slide and the pathologist's scoring or interpretation of the resulting visualization of expression. The discordances in scoring seen in previous studies often reflect the inability to separate these two variables. The invention and now widespread use of autostainers is an attempt to standardize the chemical preparation component. The reproducibility provided by mechanization and the proprietary, quality-controlled reagents are designed to minimize variance in the staining process compared to manual staining with 'home brew' reagents. While a general consensus exists that the introduction of autostainers has indeed improved consistency, variability persists in the intensity of chromogenic staining, which is often influenced by laboratories 'tweaking' protocols to optimize appearance based on laboratory directors' preferences.

This issue has been recognized in the context of HER2 detection since 2008 (ref. 135) and, after a few different approaches[136], the same group successfully standardized the performance of several key IHC companion diagnostic tests, including testing for HER2 expression. These investigators used cell-sized microbead calibrators, in which synthetic antigens are conjugated to microbeads in precise quantities[137]. These calibrators are attached to slides with a range of antigen concentrations, creating a standardized slide for each IHC test. The autostainer is then adjusted so that the limit of detection produces a visible brown chromogenic signal at the appropriate antigen concentration, while the next lowest concentration remains undetectable. These standardization slides can be run with each autostainer batch to ensure reproducible limit of detection results. This method has received FDA approval[138] and uses units of measures for antigens traceable to National Institute of Standards and Technology reference

material[139]. This approach holds great potential for optimizing the reproducibility of IHC tests for a wide range of antigens, although it has yet to be validated prospectively.

Calibration standards could potentially address the variability of chromogenic assays, although they cannot resolve the issue of image interpretation. Chromogenic assays continue to be read by pathologists, and this interpretation remains a major source of variability. Interpretation is not a trivial issue as has been shown in various studies in which multiple pathologists have assessed the same scanned images and reported high levels of discordance on the basis of interpretation alone[121]. To address this problem, expression signals must be assessed quantitatively or 'measured' rather than 'read'. Many attempts to quantify IHC signals have been made, most involving a chromogenic substrate. Immunofluorescent quantification has also been used extensively, albeit with attempts at standardization only reported in 2022 (ref. 140). Regardless of the method, signals must be objectively measured to address the issue of variable interpretation.

Quantitative analysis of HER2 IHC images

Quantitative image analysis (QIA) of IHC for HER2 and other biomarkers has been a staple of digital pathology research for years. Several algorithms incorporated into commercially available systems for HER2 IHC have advanced to FDA premarket approval, including PATHIAM[141], ScanScope XT System[142], VIAS[143], ARIOL[144], ACIS[145] and QCA[146]. In response, the CAP developed guidelines for the use of these QIA tools for HER2 IHC in 2018 (ref. 147). However, adoption of these tools has been extremely slow, partly owing to challenges in integrating digital pathology infrastructure and algorithms into clinical laboratories[148–153]. The most recent FDA approval of a QIA system for HER2 IHC was 15 years ago. All of these methods predated the emergence of the new challenge of assessment of low levels of HER2 expression.

A deep learning-based quantitative continuous scoring (QCS) method was developed to objectively quantify HER2 expression by measuring membranous optical density in IHC images[154]. Using samples from patients with breast cancer who received T-DXd as part of a phase I trial, QCS outperformed manual HER2 scoring in predicting responders, particularly when >98% of cells had membranous optical density above a specified threshold. The group also introduced a spatial proximity score that reflects the proximity of HER2-negative cells to HER2-positive cells, which might capture the extent of the bystander effect of T-DXd. This score was highly correlated with PFS, thus underscoring the importance of spatial heterogeneity in HER2 expression. By combining these objective metrics, QCS quantifies HER2 expression more granularly and potentially with less subjectivity compared to manual interpretation of IHC. QCS is a novel method and data on the development of this method were only published in 2024. Therefore, and despite great promise, this approach needs to be validated and might be challenging to introduce into the clinical laboratory improvement amendments (CLIA) laboratory setting.

Despite the promise of QIA as a method of quantifying HER2 IHC, clinical implementation might be challenging owing to variations in slide preparation and staining protocols, which might affect measurements of intensity and/or optical density. Therefore, standardization of tissue processing and digitization processes will also be essential for reproducibility and broader adoption in clinical practice. Nonetheless, QIA and new algorithms such as QCS could provide an important opportunity to optimize the accuracy of HER2 testing and potentially improve the selection of patients for HER2-targeted therapies.

Quantitative immunofluorescence

Quantitative immunofluorescence (QIF) enables objective spatial measurements of HER2 expression, thus overcoming the limitations of traditional IHC. QIF quantifies fluorescence intensity within the tumour compartment defined by a cancer cell-specific marker such as pan-cytokeratin, thereby removing the challenge of cell segmentation that limited many previous methods. The signal is converted into a quantitative measurement in molecular units (moles) per unit area, providing a continuous measure of protein expression analogous to measures of concentration in liquid specimens.

A high-sensitivity HER2 (HS-HER2) QIF assay tailored for low HER2 expression in breast cancer was developed using a cell-line array calibrated by protein mass spectrometry to establish a standard curve[140] (Fig. 4). This assay enables HER2 to be quantified in attomoles per square millimetre of tumour area. In the analysis of samples from 364 patients with breast cancer, 67% had HER2 expression above the limit of quantification but below the levels associated with overexpression. QIF revealed a broad range of HER2 expression in tumour samples that were traditionally classified as IHC 0, 1+ or 2+. Although response thresholds are not yet known, early attempts to define such thresholds indicate an increase in responsiveness to T-DXd (as time to next treatment) for every additional attomole per square millimetre of HER2 protein detected[155].

Outside of breast cancer, this assay has been tested in 741 patients with NSCLC[110], of whom 63% had detectable HER2 and 17% were above the limit of quantification, despite low rates of *ERBB2* alterations. This finding suggests that the HS-HER2 assay could enable the identification of additional patients for HER2-targeted ADC treatment beyond those with *ERBB2* amplifications or mutations. In addition, this study provides proof-of-concept that the HS-HER2 QIF assay can be readily translated to other cancers beyond breast cancer, potentially including gastric, colorectal, ovarian, endometrial, bladder and head-and-neck carcinomas, in which HER2 is an established or emerging therapeutic target.

The ability of QIF to multiplex targets for the sensitive quantification of ADC target proteins is further supported by the development of a standardized quantitative multiplexed protein assay for both HER2 and TROP2, a cell-surface glycoprotein that can be targeted using emerging ADCs such as sacituzumab govitecan[156]. Large-scale studies across multiple cancer types will be needed to better characterize low HER2 expression and thus fully harness the potential of QIF. Integrating QIF into clinical trials will be crucial to define predictive thresholds. With further development, this objective and quantitative approach could transform HER2 testing and patient selection for HER2-targeted therapies.

HER2-low RNA expression and molecular correlates

RNA expression analysis, including measurements of transcript abundance and RNA hybridization techniques, provides an alternative to protein-based HER2 testing. Research to determine if low levels of HER2 protein correlate sufficiently with RNA levels to inform clinical decision-making is currently ongoing. RT-PCR-based methods provide the most obvious method of assessing and quantifying *ERBB2* mRNA. Although such assays are not part of the routine diagnostic workflow for all breast cancers, they have the potential to assist in assessments of low levels of mRNA and, most likely, protein expression. Studies testing the Oncotype DX assay for this purpose have shown a significant difference in *ERBB2* mRNA levels between patients with an HER2 IHC score of 0 versus 1+, suggesting a correlation between HER2 protein and mRNA levels in the HER2-low range[157,158]. However, the Oncotype

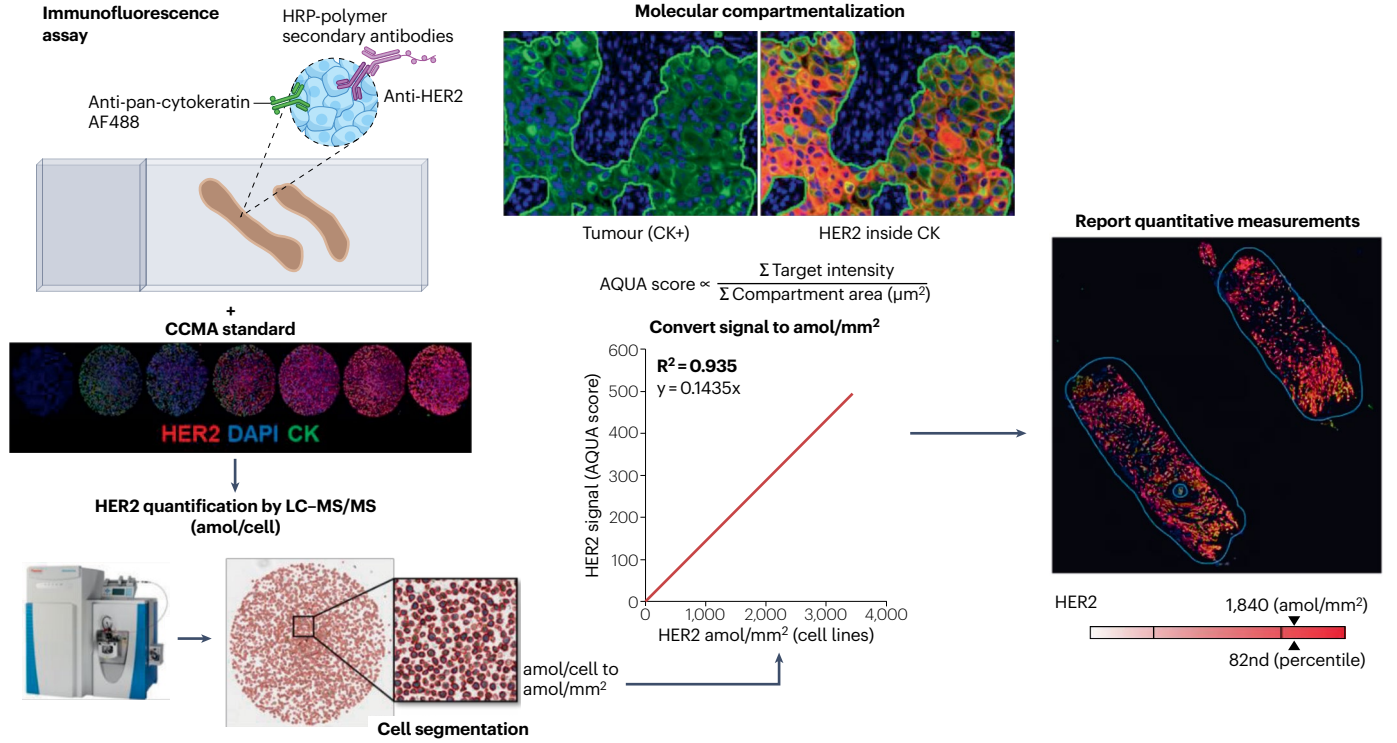

Fig. 4 | Overview of a novel high-sensitivity HER2 quantitative immunofluorescence assay. As originally described by Moutafi et al.[140] in 2022, breast cancer tissue is stained and human epidermal growth factor receptor 2 (HER2) signal within the pan-cytokeratin (CK) molecular compartment is quantified. Cell lines with a range of HER2 expression undergo HER2 quantification using liquid chromatography–tandem mass spectrometry (LC–MS/MS) to generate a cell-line microarray (CMA) standard, which is run in the same tray as the patient samples on the autostainer. Proteomics measurements are converted to attomoles (amol) per cell area using cell segmentation on digital images of the CMA standard. The standard curve is used to convert the HER2 signal into amol per square millimetre for each staining batch. This approach enables the high-sensitivity HER2 assay to provide quantitative measurements of HER2 expression in abundance units (amols) as well as percentile-ranked scores that are suitable comparisons with those for other targets of antibody–drug conjugates. AQUA, automated quantitative analysis; DAPI, 4′,6-diamidino-2-phenylindole.

DX assay has so far only been compared to protein measured categorically according to IHC score and has not yet been assessed against a continuous method of protein measurement, which could more precisely determine the relationship between *ERBB2* mRNA levels and HER2 protein expression in the low range.

The Xpert Breast Cancer STRAT4 assay is another RT-PCR assay that is also not widely used in clinical practice. This method measures *ERBB2* mRNA levels by comparison with the abundance of a control gene (*CYFIP1*)[159]. The STRAT4 assay returns continuous values for the measurement of *ERBB2* mRNA and uses a cut-off to classify the samples as positive or negative[159]. However, unlike QIF, this technique does not incorporate spatial information, which could potentially be an issue if heterogeneity needs to be assessed or if non-malignant ducts are included in the specimen. However, when this assay was used to assess 63 HER2-equivocal (IHC 0/1+ or IHC 2+) breast cancer specimens, *ERBB2* mRNA and HER2 protein (measured using QIF) were only moderately correlated ($r = 0.52$) with a concordance of 69.8% for positive versus negative classification[159]. The STRAT4 assay has also been tested in larger cohorts spanning the full dynamic range of HER2 expression, and *ERBB2* mRNA has demonstrated a robust correlation with HER2 protein scored using IHC[160,161].

The PAM50 assay offers an alternative RT-PCR-based method of measuring mRNA expression. This method uses sequence-specific probes labelled with unique barcodes to identify specific genes, which are then quantified using the nCounter platform. The PAM50 assay uses a 50-gene signature to classify each breast cancer sample as luminal A, luminal B, HER2-enriched or basal-like, each of which is associated with a distinct recurrence risk[162,163]. *ERBB2* is one of the 50 genes assessed by the PAM50 assay and is quantified as a continuous variable; therefore, this assay can be used to explore the relationship between *ERBB2* mRNA and HER2 protein expression in the HER2-low range. Interestingly, a study comparing *ERBB2* mRNA measured using the PAM50 assay to IHC scores in samples from patients with HER2-low or HER2-negative breast cancer found significantly higher levels of *ERBB2* mRNA in HER2 IHC 1+ samples than in IHC 0 samples within the hormone receptor-positive disease subgroup but no significant difference in *ERBB2* expression across samples that were IHC 2+, 1+ or 0 within the triple-negative breast cancer subgroup[123]. The role of the PAM50 assay in patients with HER2-low breast cancer continues to be explored, although this assay has not yet been compared with continuous methods of protein quantification and its utility in predicting responsiveness to HER2-targeted ADCs has thus far not been determined.

RNAscope is an ISH-based method of mRNA assessment that has also been used to investigate *ERBB2* expression in HER2-low breast cancer and can additionally provide spatial information. When *ERBB2* was measured using RNAscope and compared with HER2 expression

measured using QIF, RNA and protein levels were found to be moderately correlated when the entire range of HER2 expression was considered but poorly correlated in the low range[164]. When *ERBB2* RNAscope scores were compared with IHC scores, two studies found significantly higher RNAscope scores in tumour samples deemed IHC 3+ versus 2+ and in those deemed IHC 2+ versus 1+ but no significant difference in RNAscope scores between IHC 0 and 1+ samples were observed[164,165]. No standard method of quantification exists for RNAscope, although most studies use some form of dot counting to score the results, where each dot is a diaminobenzidine or fluorescent signal[164–166]. These dot-counting methods are probably subjective as the size of a dot must be defined and clusters of dots must be assigned a number based on the area they cover, with the number of mRNA molecules represented by each dot remaining uncertain. Whether or not the correlation between *ERBB2* mRNA and HER2 protein levels would change if RNAscope was quantified using a less subjective method has not yet been explored.

The HER2DX multimodal signature is another commercially available method that incorporates *ERBB2* measurements. This test, designed to stratify patients with *ERBB2*-amplified breast cancers, involves a supervised learning algorithm that combines analysis of tumour characteristics and gene signatures to calculate a prognostic risk score for each patient, classifying them into either low-risk or high-risk groups[163]. One of the four gene signatures incorporated into HER2DX is a HER2 signature, and RNA-sequencing data is used for the *ERBB2* mRNA component of this signature[163]. The level of concordance between the HER2DX *ERBB2* mRNA measurements and HER2 scores has been assessed in multiple studies, albeit always over the entire range of HER2 expression[163,167]. When *ERBB2* scores provided by HER2DX were compared between the HER2-positive (IHC 3+ or 2+/ISH-positive) and HER2-negative categories (IHC 0, 1+ or 2+/ISH-negative), *ERBB2* scores were significantly higher in the HER2-positive group[167]. When *ERBB2* scores from HER2DX were divided into low-score and high-score groups instead of being used as a continuous variable, 100% of patients in the HER2-negative group received a HER2DX score of *ERBB2*-low, while 83.6% of patients in the HER2-positive group were scored as *ERBB2*-high and 16.4% were scored as *ERBB2*-low[163]. Several trials testing HER2-targeted ADCs have begun to investigate the predictive value of HER2DX, and the data available thus far indicate a relationship between a higher HER2DX score and response to trastuzumab emtansine[168,169]. However, much of this research is ongoing and associations between HER2DX score and response to T-DXd have yet to be published. Performance of the HER2DX score has also not yet been compared with that of continuous protein measurements, such as QIF, or analysed in a prospective cohort to test performance in patients with HER2-low cancers.

Future directions

The role of IHC as a companion diagnostic test or method of assessment of drug targets has been controversial. Following the introduction of HER2 IHC assays[170], a precedent was set to allow pathologist judgement or reading to replace analytical assays such as the oestrogen receptor ligand-binding assay. Shortly thereafter, as discussed above, the limitations of IHC for HER2 assessment led to orthogonal assay development and, ultimately, to the use of FISH as an adjudicator for target expression as a companion diagnostic for trastuzumab[171]. IHC continues to be considered 'fit for purpose'[172] and remains the gold standard approach for virtually all clinical pathology laboratories; however, as technologies improve, this might no longer be the case. In laboratory medicine, analytical assays with well-defined limits and coefficients of variation have long been relied upon by clinical colleagues to assure accuracy and reproducibility in patient care. With the increased digitization of pathology, increased computational power and growing demands for more accurate information, new technologies seem likely to replace subjective assessments of IHC as companion diagnostic tests.

Among cancer biomarkers, HER2 might be the leader in the migration from subjective to objective testing of protein expression on histopathology slides. The demands for accurate measurements of HER2 in the low range discussed above, even with better assays, are unlikely to be entirely addressed by subjective assessments of chromogenic intensity. More accurate quantitative methods, either involving QIF, protein mass spectrometry or mRNA assessment, are primed to replace IHC. Although the accuracy of these tests might be less important in the metastatic setting, trials testing ADCs in the neoadjuvant setting, in which the administration of drugs to patients with early-stage disease arguably comes with higher stakes, are ongoing (NCT05113251 and NCT05710666). A drug with a 10% chance of clinically significant toxicities must be carefully considered for this patient population and will probably require more accurate assessments of the likelihood of response than would be required in the metastatic setting. This consideration as well as the availability of multiple HER2-targeted ADCs suggest that better identification of responders, beyond the current capacity of IHC, will be required.

The availability of multiple treatment options in the same clinical setting indicates a need for a new type of biomarker. Classically, three types of biomarkers exist: diagnostic, prognostic and predictive, each with established criteria[173] and standardized methods of evaluation[174]. The presence of two or more options for different ADCs with different targets in the same clinical setting presents a novel biomarker category that we propose could be referred to as a 'selective' biomarker. That is, the new biomarker would be multiplexed and enable accurate relative assessments of target expression for each ADC to enable the clinician to select the drug that best matches the tumour biology for each patient. The selective biomarker is likely to be of increased importance as more ADCs are approved with a wide range of targets in more and earlier clinical settings. Subjective methods are unlikely to be sufficiently precise to address the accuracy and specificity requirements of selective biomarkers. By contrast, RT-PCR-based assays, mass spectrometry and QIF are, by design, multiplexed and suitable for rigorous quantification. As selective biomarkers become more crucial for optimal patient care, this will be another force moving diagnostic target assessment away from IHC and towards quantitative methods.

Once new quantitative methods are introduced for selective biomarkers, this will probably bring new levels of accuracy and precision to the protein biomarker space. These assays will probably provide results in biochemical units (femtomoles or attomoles per unit area) and thus enable the determination of limits and cut-off values that are currently the standard approach for most other laboratory medicine results (such as blood glucose levels). Although absolute comparisons of molar levels of targets might not be biologically informative, the availability of quantitative results will enable clinicians to make decisions based on ranks or percentiles of target amounts within a population. Thus, a clinician could choose drug A over drug B when a tumour expresses target A at the 80th percentile compared with target B at the 10th percentile. Another major advantage of the adoption of quantitative multiplexed approaches is the ability to simultaneously assess both targets and resistance factors. Future ADC diagnostics might include measurements of the target but also of some trafficking or drug transport-related channel proteins associated with resistance

that are specifically associated with responsiveness to a given ADC and/or payload[175].

Conclusions

In summary, we believe HER2 is the 'canary in the coal mine' for the next generation of assays for therapeutic targets. The long track record of HER2 as a companion diagnostic provides data related to the various challenges and solutions associated with the IHC-based approach to target assessment. Furthermore, the successful use of trastuzumab as one of the earliest carriers of a range of toxic payloads in ADCs provides additional support for the role of HER2 as a good candidate for future assay development and optimization. Such assays will be essential to optimizing the level of benefit that patients can derive from current and future targeted therapies, especially ADCs.

Published online: 7 April 2025

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Acknowledgements
The work of the authors is supported by the Breast Cancer Research Foundation 23-138 (D.L.R.) and the National Institutes of Health P30CA016359 and F30CA287869 (C.J.R.).

Author contributions
All authors contributed equally to all aspects of the preparation of this manuscript.

Competing interests
D.L.R. has acted as an advisor of Agendia, Amgen, AstraZeneca, Bristol Myers Squibb, Cell Signalling Technology, Cepheid, Danaher, Daiichi Sankyo, Genoptix/Novartis, GSK, Halda Biotherapeutics, Incendia, Konica Minolta, Merck, NanoString, Nucleai, PAIGE.AI, Perkin Elmer, Regeneron, Roche, Sanofi, and Ventana and has received research funding from Amgen, Cepheid, Konica Minolta, Leica, NavigateBP and NextCure. The other authors declare no competing interests.

Additional information
Peer review information *Nature Reviews Clinical Oncology* thanks F. Penault-Llorca who co-reviewed with M. Kossai, D. Y. Oh who co-reviewed with J. Yoon, A. Prat and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.