Unusual Patterns of HER2 Expression in Breast Cancer: Insights and Perspectives

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Abstract
The biomarker human epidermal growth factor receptor-2 (HER2) has represented the best example of successful targeted therapy in breast cancer patients. Based on the concept of “oncogene addiction,” we have learnt how to identify patients likely benefitting from anti-HER2 agents. Since \textit{HER2} gene amplification leads to marked overexpression of the HER2 receptors on the cell membrane, immunohistochemistry with clinically validated antibodies and scoring system based on intensity and completeness of the membranous expression constitute the screening method to separate negative (score 0/1+) and positive (score 3+) carcinomas and to identify those tumours with complete yet only moderate HER2 expression (score 2+, equivocal carcinomas), which need to be investigated further in terms of gene status to confirm the presence of a loop of oncogene addiction. This process has demanded quality controls and led to recommendations by Scientific Societies, which pathologists routinely need to follow to guarantee reproducibility. In this review, we will span from the description of classical HER2 evaluation to the discussion of those scenarios in which HER2 expression is unusual and/or difficult to define. We will dissect HER2 heterogeneity, HER2 conversion from primary to relapsed/metastatic breast cancer, and we will introduce the new category of HER2-low breast carcinomas.

Introduction
The human epidermal growth factor receptor-2 (HER2) has represented the main character of the precision medicine era for breast cancer patients and holds a profound impact on tumour boards in the process of treatment decision-making that ascertains the need to tackle the disease with anti-HER2 agents. This biomarker has taught us the concept of “oncogene addiction,” i.e., the phenomenon of dependency of certain tumour cells on a single activated oncogenic protein or pathway to maintain their malignant properties, despite the likely accumulation of multiple gain- and loss-of-function mutations that contribute to tumorigenicity \cite{1}. As a proof of principle, oncogene-driven tumours in preclinical models have been shown to undergo regression (in association with proliferative arrest, apoptosis, and/or differentiation) following the acute inhibition of oncprotein function \cite{1}.
From this concept, we have learnt how to deal with the identification of patients benefitting from the addition of anti-HER2 agents. The impact of HER2 addiction in HER2-positive breast carcinomas is evident from the tremendous responses obtained over the course of a neoadjuvant therapy with anti-HER2 therapy associated with chemotherapy, with 60% of patients experiencing a pathological complete response (pCR) [2].

From a pathologist standpoint, this process has demanded quality controls on pre-analytical, analytical, and post-analytical factors and has led to specific recommendations by Scientific Societies, which pathologists routinely follow to adhere to standard assessment, thus guaranteeing reproducibility. More recently, we have witnessed exciting data stemming from novel antibody-drug conjugates (ADCs) that seem to have efficacy even in those patients affected by breast carcinomas that would be classically classified as HER2-negative but show a certain degree of HER2 protein on the tumour cell membrane. This scenario is likely to drive a paradigm shift in how we deal with HER2 evaluation, thus integrating the spectrum of “HER2-low breast carcinomas.” We are currently revisiting the importance of HER2 (over)expression assessment under a new perspective, where the expression of a given protein may represent an anchor to a novel therapeutic option. IHC is therefore currently under the spotlight and is playing an essential role beyond a screening method to investigate HER2 oncogene addiction.

In this review, we will span from description of classical HER2 evaluation to the discussion of those scenarios in which HER2 expression is unusual and/or difficult to define. Hence, we will deepen the description of HER2 heterogeneity, HER2 loss/conversion from primary to relapsed/metastatic breast carcinomas, and the new category of HER2-low breast carcinomas, from theory to practice.

**Evaluation of HER2 Expression “by the Guidelines”: Usual and Unusual Patterns**

HER2 status is assessed by combining IHC to assess the protein levels and in situ hybridization (ISH) to assess gene amplification. Although a frontline approach by ISH can be pursued, most pathology laboratories adopt a screening by IHC to assess the degree of HER2 (over)expression, followed by reflex ISH testing in equivocal cases. A scoring system has been devised, and it is based on the intensity and completeness of the membrane staining and on the percentage of positive cells. A four-tier scoring system is used (shown in Fig. 1) and acknowledged as follows:

- A score 0 (negative), where no staining can be observed or if it is incomplete and faint/barely perceptible in ≤10% of tumour cells (shown in Fig. 1).
- A score 1+ (negative) with an incomplete membrane staining that is faint/barely perceptible in >10% of tumour cells (shown in Fig. 1).
- A score 2+ representing equivocal cases that show a weak to moderate complete membrane staining in >10% of tumour cells (shown in Fig. 1).
- A score 3+ (positive) whenever there is a complete and intense circumferential membrane staining in >10% of tumour cells (shown in Fig. 1).

In all IHC score 2+ cases, a reflex test on the same sample using ISH or a new test on a new sample using IHC or ISH must be ordered. HER2 amplification is expected in about 25% of score 2+ cases that are reflexed to ISH [3].

The evaluation of membrane staining intensity is subjective to human-eye perception. Especially when it comes to distinguish between scores 1+ and 2+, the subjectivity of the interpretation of the scoring may lead to discordant results among pathologists and/or institutions. Given the central role of IHC HER2 evaluation in guiding therapy, some authors have developed the so-called magnification rule [4]. The latter uses the different magnifications of the objectives to perform a sort of triage across scores. A clear-cut intense staining perceived at low magnification (×2.5–×5) corresponds to a score 3+; stainings that can be appreciated at 10–20× would most likely fit into a score 2+, whereas whenever a 40× objective lens is needed to identify some staining, a score 1+ should be assigned. This rule may assist pathologists and may lead to an increased reproducibility in IHC scoring, especially with the perspective of introducing the new category of HER2-low breast carcinomas; see below. Some national guidelines have already implemented the rule in both breast [5, 6] and gastric cancer [7].

The ASCO/CAP guidelines acknowledge the presence of staining patterns that are not exactly fulfilling the definitions, yet they should be recognized and categorized accordingly (score 2+ is assigned) in order not to miss a possible HER2-amplified tumour [8]. The unusual patterns are mainly grouped into two categories, one featuring cases with IHC staining that is moderate to intense but incomplete (basolateral or lateral) and one representing carcinomas with a limited extent of bold HER2 over-expression.
One example for the first category is provided by micropapillary carcinomas that typically show the so-called U-shaped pattern of HER2 expression, featuring a basolateral lining of the cell membrane (shown in Fig. 2). The intensity of expression can be variable, typically moderate to intense; however due to the lack of completeness along the cell membrane, this basolateral pattern would not fulfill even the criteria of a score 2+. Nevertheless, it must be considered score 2+ and reflexed to ISH analysis given that these carcinomas harbour HER2 amplification in about 30% of cases [9, 10].

As a rule, whenever a moderate to intense basolateral or lateral pattern of HER2 expression is encountered the case should be reflexed to ISH, regardless of the histologic type. A basolateral/lateral pattern is indeed also detected in carcinomas with pervasive glandular features (shown in Fig. 3).

The second category of unusual HER2 expression described in the recommendations is represented by breast carcinomas showing a very limited proportion of tumour cells displaying an intense and circumferential HER2 overexpression. In other words, these breast carcinomas show an area with score 3+ features for intensity of the HER2 expression; however, they cannot be classified as score 3+ because of the very limited extent of the HER2-positive tumour cell population (<10%) (shown in Fig. 3, 4). In such instances, the case should be reflexed to ISH to ascertain the degree of HER2 gene amplification within the invasive tumour. It is advisable to test additional blocks if available and/or other samples (typically a lymph-node metastasis) to account for the effective degree of HER2 amplification within these highly heterogeneous carcinomas. If the HER2-amplified component is overall below 10% of the tumour population, the case is scored as HER2-negative. A practical approach would suggest annotating in the report the presence of this limited HER2-positive component to suggest a prompt accurate re-assessment of the HER2 status in case of relapse or metastatic disease over the natural history of the patient.

To the best of our knowledge, there are no specific studies addressing the impact of unusual HER2 expression patterns on the HER2 rates. Nevertheless, from a practical standpoint common sense would lead to hypothesize that whenever unusual patterns are observed a reflex test may be requested to rule out any possibilities.
of HER2 amplification, thus raising the number of ISH tests. Finally, we should mention that cytoplasmic granular staining can be occasionally encountered (for instance, in apocrine carcinomas), as well as nuclear staining (often in conjunction with cytoplasmic staining) and basal membrane pattern or pseudo-luminal staining (in welldifferentiated breast carcinomas): these types of staining are best considered artefactual and not associated with HER2 gene alterations.

The Challenge of Heterogeneous Patterns of HER2 Expression

Heterogeneity is present across breast carcinomas and expressed across several features. HER2 expression/amplification is no exception to this rule with a variable frequency spanning from 1% to 40% [11, 12]. It is well known that heterogeneity of HER2 expression/amplification features three main patterns: we can encounter either two topographically distinct tumour clones of tumour cells, one harbouring HER2 amplification, and the other with normal HER2 status (“clustered or clonal type”) or, more frequently, an intermingling of cells with different HER2 statuses that can be either diffuse or featuring only scattered isolated HER2-amplified cells in a HER2-negative tumour cell population (“mosaic type”).

When deepening the analysis of the relative frequency of HER2 heterogeneity in breast cancer, it should be acknowledged that the clustered type is reported to be much rarer compared to the mosaic type [13, 14]; nevertheless although many studies have reported prevalence of HER2 heterogeneity, few specify the granular data about type of heterogeneity observed (clustered vs. mosaic type). Data from our institutional series of unselected breast carcinomas (cases diagnosed in year 2021, reported according to the latest ASCO/CAP guidelines update [8]), which were reviewed with the specific purpose of assigning the type of HER2 heterogeneity, revealed 0.01% of clustered-type HER2 heterogeneity in an unselected cohort (i.e., all newly diagnosed breast carcinomas), 4% of all score 3+ and score 2+ cases subjected to fluorescence ISH (FISH), and 10% of all positive cases. Conversely, the mosaic-type heterogeneity was observed in 3% of the unselected cohort.

Fig. 2. Unusual pattern of HER2 positivity in micropapillary carcinomas. Micropapillary carcinomas have been described to show the so-called U-shaped pattern of HER2 expression, featuring a basolateral lining of the cell membrane with lack of HER2 expression on the luminal portion of the membrane, which is facing the stroma in micropapillary carcinomas due to the reverted polarity. The intensity of expression can be variable, typically moderate to intense. These cases must be considered score 2+ and reflexed to ISH analysis given that these carcinomas harbour HER2 amplification in about 30% of cases.
and in 15% of score 3+ and score 2+ subjected to FISH analysis and was restricted to score 2+ cases. These data are in line with reports from unselected [15] and HER2-positive cohorts [14].

HER2 heterogeneity has also been reported to be significantly more common in cases with HER2 equivocal status by ISH and/or IHC [13, 16–20], and some authors have shown that HER2 heterogeneity is an important cause of equivocal HER2 results in breast cancer by FISH and IHC [19, 21, 22]. We have demonstrated that whenever assessing HER2 status in breast carcinomas with a diffuse intermingling of HER2-amplified and nonamplified cells, the overall scoring leads to HER2 equivocal counts [23], the so-called Group 4 by ISH, as reported by Press et al. [24]. Of note, in these cases, cells with HER2 signals >6 typically harbour low levels of HER2 amplification [15, 18, 21, 23, 25].

The clustered type of HER2 heterogeneity is easier to be defined as the tumour cell populations are clear-cut and easily recognized on IHC and ISH grounds. This pattern also suggests the presence of two different tumour types into one lesion or a sort of “collision tumour.” Whether one tumour cell population may derive from the other is yet to be demonstrated. A genomic study exploiting gene copy number (CN) profiling and massively parallel sequencing separately analysed the HER2-negative and HER2-positive components of a small series of 12 HER2 heterogeneous breast carcinomas and identified potential driver genetic alterations restricted to the HER2-negative cells, thus suggesting that the HER2-negative compo-

Fig. 3. Usual and unusual HER2 patterns in breast carcinomas, as assessed by IHC. In these images, usual and unusual patterns of HER2 expression in breast cancer are summarized. On the top panel, cases with score 3+ intensity and complete circumferential staining. When this pattern is encountered in >10% of tumour cells, we can report the usual score 3+. On the other hand, whenever a breast carcinoma shows a very limited proportion (<10%) of tumour cells displaying an intense and circumferential HER2 overexpression, the tumour is scored 2+ and must be reflexed to ISH to ascertain the degree of HER2 gene amplification within the invasive tumour. In the middle panel examples of variable intensity of usual HER2 stainings compatible with a score 2+ (weak to moderate complete membrane staining in >10% of tumour cells). In the bottom panel a portfolio of unusual HER2 patterns scored as score 2+ (basolateral staining in micropapillary carcinomas and in carcinomas with glandular features and cases with slightly fragmented membrane staining).

Fig. 4. HER2 heterogeneity in core biopsy samples. Examples of HER2 evaluation on CNB samples. Three breast carcinomas: (a, b) invasive carcinoma of no special type; (c, d) invasive carcinoma of no special type; (e–h) invasive lobular carcinoma showing two distinct tumour cell populations with divergent HER2 status (clustered type of HER2 heterogeneity). The HER2-positive (score 3+) component accounts for 20% (a, b), 11% (c, d), and 40% (e–h), respectively.

(For figure see next page.)
nents are likely driven by genetic alterations not present in the HER2-positive component [26].

This scenario of clustered heterogeneity is considered by the guidelines, which suggest reporting separately these populations whenever the HER2-positive population is representing at least 10% of the whole lesion. An important aspect of these cases relates to treatment decision-making, as medical oncologists face the treatment of a tumour composed of distinct populations with likely differential response compared to pure HER2-positive and HER2-negative lesions. This impact is much evident in the neoadjuvant setting, where the HER2 status is assessed on core biopsy specimens, and therefore, representativeness of the lesions is of utmost importance to administer the right therapy (shown in Fig. 4). Pathologists and radiologists often liaise in this scenario to obtain the most representative tissue material. It is recommended to examine more than one core to adequately sample the tumoral lesion and to guarantee proper assignment of histologic type and phenotype of tumour cells.

Of note, if the patient subjected to chemotherapy in the neoadjuvant phase experiences pCR, the core biopsy specimens would represent the only material available related to the primary tumour for any further assessments. If HER2 heterogeneity is properly captured through an appropriate sampling of the lesion, the neoadjuvant setting offers the unique opportunity to assess the degree of responsiveness to anti-HER2 therapies. A recent phase II neoadjuvant clinical trial has provided data on the impact of HER2 heterogeneity on treatment response of early-stage heterogeneous HER2-positive breast carcinomas [27]. A careful examination of core biopsies was performed to identify topographically distinct tumour cell populations with different HER2 statuses (positive and negative). HER2 heterogeneity (defined as an area with HER2 amplification in >5% but <50% of tumour cells, or a HER2-negative area by FISH) was detected in 10% of evaluable cases (16/157). In this study, treatment was planned with the ADC trastuzumab emtansine (T-DM1) in combination with pertuzumab, so that chemotherapy was administered through the action of the anti-HER2 antibody. None of the HER2 heterogeneous tumours achieved a pCR compared to a 55% pCR rate observed in the non-heterogeneous subgroup (p value <0.0001, adjusted for hormone receptor [HR] status). These data demonstrate that the fraction of HER2 nonamplified cancer cells is a strong predictor of resistance to anti-HER2 therapies in HER2 heterogeneous breast carcinomas. Hence, HER2 heterogeneity should be considered in efforts to optimize treatment strategies.

The mosaic type of HER2 heterogeneity can feature scattered HER2-positive cells or a diffuse intermingling of groups of cells with a different HER2 expression and/or gene status. This pattern is more frequent and typically encountered in HER2 score 2+ carcinomas (shown in Fig. 5). At FISH analysis, a subgroup of these carcinomas may show an admixture of tumour cells with increased HER2 CNs. Typically, these cases would be classified as “HER2-double equivocal,” due to the equivocal result of both diagnostic tests. Of note, the IHC staining can be diffuse and moderate and ISH analysis typically highlights a variable proportion of tumour cells with increased HER2 CN between 6 and 10 or more [23]. In diagnostic practice cases as such can be quite challenging as one would not miss a patient benefitting from a potentially life-saving treatment such as anti-HER2 agents. We do not have solid data on the possible benefit of anti-HER2 therapies in such patients. We have performed a hypothesis-generating study.

![Fig. 5. Mosaic-type heterogeneity applied to HER2. An invasive breast carcinoma showing a score 2+ overexpression of HER2 with scattered cells showing intense and complete membranous staining (score 3+, a). Corresponding FISH images (b) show a diffuse intermingling of tumour cells with different HER2 statuses (red, HER2 gene signals; green, CEP17 signals). Arrow-headed lines point to nuclei harbouring >6 signals.](image-url)
in a cohort of patients treated with neoadjuvant trastuzumab-containing chemotherapy and observed that pCR rates were significantly lower in double-equivocal carcinomas with HER2 heterogeneity compared with HER2-positive (score 3+) carcinomas (10% vs. 60%, Fisher exact test, \( p = 0.009 \)) [23]. Three cases showed a near-pCR (minimal residual disease \( \text{RD}/\text{near-total effect}/<10\% \) of tumour remaining). When pCR and near-pCR categories were grouped, the difference in terms of response rate was not statistically significant between double-equivocal and score 3+ carcinomas (40% vs. 63.3%, \( p = 0.27 \)). Nevertheless, we cannot rule out that this was due to the beneficial effect of chemotherapy, as this rate was not significantly different from the rates accrued in a cohort of oestrogen receptor-positive/HER2-negative patients treated with chemotherapy only [23].

Larger studies comparing patients who received the same chemotherapy regimens \( \pm \) trastuzumab treatment are warranted to ascertain the real impact of anti-HER2 therapy in this specific subset of breast carcinomas. According to the latest ASCO/CAP update on HER2 testing recommendations, HER2 ISH equivocal carcinomas should be reported as “negative” unless the HER2 pattern of expression fulfils a score 3+. This recommendation relies on the fact that there is insufficient evidence at present as whether patients with an average of 4–6 HER2 signals per cell and a HER2/CEP17 ratio of \(<2.0 \) benefit from HER2 targeted therapy in the absence of protein overexpression. Hence, whenever IHC results are not score 3+ positive, it is recommended that the sample be considered HER2-negative and a comment should be added to contextualize the result.

A Possible Role for Computational Pathology in Assessing HER2 Patterns

Thanks to the availability of whole slide imaging, several efforts have been invested in automated image analysis algorithms that have also been applied to quantification of HER2 expression on IHC slides. The need stems from the fact that even when guidelines/recommendations are adopted challenging cases remain, especially with borderline HER2 evaluation across scores [28].

Studies comparing HER2 scoring between pathologists and computer-aided methods have revealed the latter to be as accurate and reproducible as pathologists [29]. Some have reported good agreement for score 3+ and for score 0/1+, whereas score 2+ proved to be the most problematic category [28]. Some tools have been shown to identify score 2+ cases to a lesser extent compared to human eye [30]. The implementation of computer-aided IHC scoring led to FDA approval of many of these algorithms [28, 31], and in 2019, the College of American Pathologists published recommendations to improve accuracy, precision, and reproducibility of HER2 interpretation in breast cancer when using quantitative image analysis (QIA) [29]. Readers should refer to these recommendations whenever in the process of implementing QIA-based systems for HER2 evaluation in diagnostic practice. The guidelines clearly state that validated procedures are needed before implementation, and that quality control/assurance is mandatory. Of note, performance, interpretation, and reporting of HER2 results using a QIA system should be supervised by pathologists with expertise in the field [29]. Hence, QIA can be used as an auxiliary tool for pathologists to achieve consistent interpretation, and the use of QIA should be acknowledged in the report [29]. Nevertheless, some have reported that these systems seem not to perform well with some type of samples, such as heterogeneously stained slides [32]; therefore, their reliability in helping assess unusual patterns is yet to be demonstrated.

The New Category of HER2-Low Carcinomas

Besides classic agents targeting HER2 (i.e., monoclonal antibodies trastuzumab and pertuzumab), novel anti-HER2 drugs have been developed during the years. ADCs, formed by a monoclonal antibody linked to a cytotoxic agent, are characterized by the capability to selectively deliver the chemotherapeutic compound inside cancer cells, reducing systemic side effects typically deriving from standard chemotherapy. The first ADC being tested was T-DM1 that showed an improvement in overall survival (OS) and a better toxicity profile when compared to capecitabine + lapatinib (EMILIA) or to a treatment of physician’s choice (TH3RESA) in HER2-positive metastatic breast cancer previously treated with anti-HER2 agents [12]. T-DM1 is currently approved in HER2-positive unresectable locally advanced or metastatic disease previously treated with a trastuzumab + taxane regimen and in the adjuvant setting of HER2-positive early breast cancer with residual-invasive disease in the breast and/or lymph nodes after neoadjuvant taxane-based and trastuzumab-based therapy [33, 34]. The more recently developed ADC trastuzumab deruxtecan (DS-8201) conjugates trastuzumab with the topoisomerase I inhibitor Ddx and has been tested in HER2-positive metastatic...
breast cancer previously treated with T-DM1 [35] showing an overall response rate (ORR) of 60.9% (6.0% complete response, 54.9% partial response) and leading to Food and Drug Administration (FDA) and European Medicines Agency (EMA) approval for HER2-positive metastatic breast cancer already treated with two or more anti-HER2 therapeutic regimens [36, 37]. The ongoing phase III DESTINY-Breast03 trial is comparing efficacy and safety of DS-8201 and T-DM1 in 524 HER2-positive metastatic breast cancer patients already treated with taxane + trastuzumab. Early results presented at the ESMO 2021 meeting showed an improvement in progression-free survival (PFS) of DS-8201 over T-DM1 and an ORR of 79.7% and 34.2%, respectively [38]. These data encouraged FDA to grant the breakthrough therapy designation for DS-8201 in HER2-positive unresectable or metastatic breast cancer patients already treated with at least one anti-HER2 therapeutic regimen [39]. This decision may soon lead to a change in the second line of therapy for HER2-positive metastatic breast cancer patients. Studies testing T-DM1 only included HER2-positive tumours, but retrospective analyses conducted by reviewing HER2 status identified HER2-low tumours and showed that no benefit can be derived from T-DM1 in this subtype [40]. On the other hand, DS-8201 showed antitumour activity in a cohort of 34 pretreated HER2-low metastatic breast cancers [41]. The ORR was 50%, increasing to 55.2% when only considering the HR-positive subgroup (29/34); interestingly, when excluding patients already treated with an anti-HER2-containing regimen, the ORR lowers to 46.2%.

At present, the DESTINY-Breast06 trial is actively recruiting patients with HER2-low disease. This study is designed to evaluate the efficacy, safety, and tolerability of DS-8201 compared with investigator’s choice chemotherapy in HER2-low, HR-positive breast cancer patients whose disease has progressed on endocrine therapy in the metastatic setting.

Trastuzumab duocarmazine (SYD985) links the monoclonal antibody to the alkylating agent duocarmycin. Its safety and efficacy are now being compared to a treatment of physician’s choice in the phase III TULIP clinical trial in 437 HER2-positive locally advanced or metastatic breast cancer patients already treated with at least two therapeutic regimens or with T-DM1. Preliminary results showed an improvement in PFS (7 months vs. 4.9 months), while no significant differences were found in terms of ORR [42]. SYD985 has also been tested in a phase I dose-escalation and dose-expansion study and showed a partial objective response in 28% of HR-positive/HER2-low and 40% of HR-negative/HER2-low metastatic breast carcinomas [43] indicating that SYD985 might represent, in future, another treatment option in the metastatic setting.

Taken together, these data suggest that patients with lesions showing low levels of HER2 might still benefit from new-generation ADCs, even if lacking HER2 amplification. Hence, it does not come as a surprise if experts in the field have developed an interest in the identification of a new category labelled as “HER2-low” breast carcinomas, i.e., tumours displaying some degree of expression of the HER2 protein not stemming from HER2 gene amplification (Fig. 6). These carcinomas would not be therefore “HER2-addicted,” but rather “HER2-equipped” or “HER2-scaffolded.” The scaffold offered by the degree of HER2 receptors expressed on the membrane seems to be enough to favour the delivery of a cytotoxic agent through anchorage of the anti-HER2 antibody to the HER2 receptors that here serve as simple vehicles.

At present, HER2-low breast carcinomas are being defined on immunohistochemical grounds by exploiting the current scoring system and include tumours reported as HER2 score 1+ or score 2+ with a negative ISH result. HER2-low tumours represent up to 55% of breast cancer, estimated to be HR-positive in the 65–83% of the cases and HR-negative in the remaining percentage. A study comparing HER2 status between primary versus relapsed lesions observed 15% of HER2-negative tumours converting to HER2-low and 14% of HER2-low to HER2-negative. HER2-low status was found in 34% and 38% of primary and relapsed tumours, respectively [44].

The reasons for the presence of HER2 protein expression without an underlying HER2 gene amplification have not been clarified yet, but an increased HER2 CNs may explain this phenomenon. In addition, the upregulation of HER2 induced by NF-kB pathway activation induced by chemotherapy and radiation therapy or by epigenetic changes has been associated with increased HER2 expression in the absence of HER2 gene amplification. Furthermore, the crosstalk between HER2 and oestrogen receptor pathways or modifications deriving from adaptation or resistance to treatments has been reported to upregulate HER2 protein levels [40].

In two retrospective analyses, Schettini et al. [45] and Agostinietto et al. [46] investigated the molecular and pathological characteristics and prognostic impact of HER2-low breast cancer. Schettini retrospectively collected clinicopathological and PAM50 gene expression data from 13 publicly available datasets deriving from different studies for a total of 3,689 HER2-negative breast
cancers with known IHC and HER2 amplification status. The purpose was to provide a first characterization of HER2-low breast cancer and compare the features of HER2-low and HER2 0 disease. HER2-low tumours were found to be associated with larger tumour size and more nodal involvement, being mostly HR-positive. Luminal A was the most frequent intrinsic subtype, followed by the luminal B. Within the HR-positive disease, HER2-low presented higher levels of HER2 mRNA compared to HER2 score 0 carcinomas. No differences in OS between HER2-low and HER2 score 0 carcinomas were observed.

To further unravel HER2-low features, Agostinetto performed a retrospective analysis of more homogeneous data from The Cancer Genome Atlas (TCGA) including 1,097 primary breast cancers with known HR and HER2 status. They confirmed previous findings by observing HER2-low tumours to be mostly HR-positive, Luminal A. HR-positive/HER2-low HER2 mRNA levels were higher compared to HR-negative/HER2-low tumours. No statistically significant differences were observed between HER2-low and non-HER2-low regarding progression-free interval, disease-free interval, and OS.

In a recent analysis, Denkert et al. [47] compared clinical and molecular features of HER2-low and HER2 0 breast cancer with a particular focus on response to therapy by exploiting the neoadjuvant setting. Besides confirming a higher prevalence of HER2-low disease among HR-positive tumours, they detected a lower pCR rate in HER2-low/HR-positive compared to HER2 0/HR-positive, but no significant difference was found among the HR-negative disease. On the other hand, longer disease-free survival (DFS) and OS were present among HER2-low/HR-negative tumours compared to HER2 score 0 but not among HR-positive breast cancer patients.

Based on the data available so far, it is still premature to draw definitive conclusions on whether HER2-low car-

**Fig. 6.** Spectrum of HER2 overexpression by IHC across breast carcinomas. A spectrum of distinct levels of HER2 expression in breast carcinomas as assessed by IHC is represented. ISH data are annotated in italics in close proximity to the pictures. The combination of the two data allows to show a category of breast carcinomas with clear-cut HER2 overexpression, in the form of either a score 3+ or a score 2+, both driven by HER2 gene amplification. These carcinomas are HER2-positive and HER2-addicted: they have the potential to be responsive to anti-HER2 agents that block the signal transduction pathway activated by HER2 overexpression. Whenever levels of HER2 compatible with a score 2+ without HER2 gene amplification or with a score 1+ are encountered in breast carcinomas, these have been correlated with response to new anti-HER2 compounds, i.e., ADCs, formed by a monoclonal antibody anti-HER2 linked to a cytotoxic agent. These carcinomas are defined “HER2-low” and are “HER2-equipped”: they have the potential to respond to these drugs because the scaffold offered by the degree of HER2 receptors expressed on the membrane seems to be enough to favour the delivery of a cytotoxic agent through anchorage of the anti-HER2 antibody to the HER2 receptors that here serve as simple vehicles.
cincmas may constitute a distinct subtype of breast carcinomas. At present, the “HER2-low” definition may represent an umbrella term including different types of breast carcinomas sharing different degrees of HER2 (over)expression. Nevertheless, as mentioned above the promising results deriving from the ADCs studies even in the HER2-low breast carcinomas have urged the need of a change in the clinical classification of HER2-expressing breast cancer. Pathologists have been used to applying a dichotomous separation of HER2 status, positive (score 3+ and score 2+ to be verified by IHS) versus negative (score 0 and 1+). Hence, the distinction between score 0 and 1+ has not been under the spotlight; however, this will have to be emphasized to reach the new categorization into (i) HER2-negative (score 0), (ii) HER2-low (score 1+ and score 2+ not amplified), and (iii) HER2-positive (score 2+ HER2 amplified and score 3+) carcinomas. Further data on the degree of response across HER2-low carcinomas may also help in the fine tune of the real needs from the pathological evaluation of these carcinomas.

It should be emphasized that, at least at present, IHC protocols for HER2 testing should not be changed or adapted to be able to detect HER2-low carcinomas. As discussed above, the definition relies on the current IHC scoring system based on traditional IHC staining protocols. The challenge lies in the reproducibility of the scoring [45, 48], which in turn may suffer from technical issues, including pre-analytical factors. Quality assurance programmes are of utmost importance in this context. Of note, some national expert groups such as the French GEFPICS group have already endorsed this new category in their guidelines [6] as well as in their quality assurance programmes (see, for instance, AFAQAP programme in France).

Education on optimal HER2 scoring to adequately identify score 1+ versus score 0 carcinoma may be beneficial as well. Nevertheless, it is worth mentioning that at the latest San Antonio Breast Cancer Symposium the phase II study “DAISY” reported 30% of response rates in score 0 breast carcinoma patients treated with DS-8201 [49]. Although data are still premature to draw any definitive conclusions, HER2-low and ultra-low breast cancers are now – even more than before – under the spotlight and the correct definition of the lowest limit of HER2-low expression is still under proper definition. As a matter of fact, we should remember that the IHC protocols that we routinely use with clinically validated antibodies have been devised to discriminate HER2-addicted versus HER2-not addicted carcinomas. Data analysis on tissue samples collected in clinical trials focused on the treatment of HER2-low breast cancer patients may help understand whether IHC is an adequate method to detect the best responders.

**HER2 Conversion and HER2 Loss**

The so-called receptor conversion, i.e., the discordance of HR or HER2 status between primary tumor and metastatic deposits over the clinical history of a given patient, is a relevant issue since it would require to reconsider a possible shift in treatment decision-making. On the one side, it is universally recommended to retest one or multiple metastatic deposits for biomarker evaluation to better target the disease under evolution. On the other side, it must be acknowledged that biomarker conversion in retesting does not necessarily lead to a prompt change of therapy based on the new profile. In general, medical oncologists consider the issue of heterogeneity (whenever multiple metastases are present) and the availability of therapeutic options. Whenever the conversion is toward a positivity in a biomarker, this would mean a therapeutic agent to be added to the treatment schedule, which holds a potent therapeutic impact compared to loss of expression of a given biomarker.

Different studies have investigated the phenomenon of HER2 conversion between primary and relapsed/metastatic diseases (Table 1), and early studies reported a wide range of discordance rate varying from 0% to 44%. To best understand the true extent of this phenomenon, meta-analyses have been conducted. Aurilio et al. [50] selected published data from 48 studies evaluating concordance in receptor expression and HER2 status between primary tumour and both local and distant metastases. HER2 discordance rates were heterogeneous (0–24%). The meta-analytic pooling assessed a discordance rate of 8%. When stratified according to site of relapse, the discordance rate was 10% and 6% for distant and locoregional metastases, respectively. Regarding the quality of HER2 conversion, 13% of patient changed from positive to negative and 5% from negative to positive.

In a more recent meta-analysis, Schrijver et al. [51] analysed data from 39 studies considering only distant metastases, focusing on the type of technique used for HER2 assessment and on metastasis location-specific differences. HER2 conversion was identified in a range between 0% and 34%, with a mean of 10.3%. Conversion from HER2-positive to HER2-negative disease was observed in 21.3% of cases, whereas in 9.5% of cases HER2 status...
Table 1. Summary of studies reporting HER2 status conversion over treatment

| Study                          | Setting                      | N     | Conversion, n | HER2 loss | HER2 gain | Outcome main result |
|-------------------------------|------------------------------|-------|---------------|-----------|-----------|---------------------|
| Aurilio et al. [50]           | Primary versus local and distant mts | 2,987 | 8             | 13% of the HER2-positive | 5% of the HER2-negative | –                   |
| Schrijver et al. [51]         | Primary versus distant mts    | 2,440 | 10.3          | 21.3% of the HER2-positive | 9.5% of the HER2-negative | –                   |
| Chen et al. [52]              | Primary versus mts            | 320   | 13.7          | 28.7% of the HER2-positive | 8.1% of the HER2-negative | MFS and OS not significant |
| ESME report [53]              | Primary versus mts            | 1,076 | 7.8           | 45.2% of the total cohort experiencing a conversion | 54.8% of the total cohort experiencing a conversion | PFS and OS not significant |
| Mittendorf et al. [54]        | Primary versus RD             | 25    | 32            | 32% of the cases with residual carcinomas | – | Significantly worse PFS for HER2 loss |
| Guarneri et al. [55]          | CT versus CT + anti-HER2      | 107   | –             | 40% of the cases with residual carcinoma treated with CT versus 14.7% of the cases with residual carcinoma treated with CT + anti-HER2 | – | Significantly worse PFS for HER2 loss |
| Niikura et al. [56]           | Primary versus RD             | 16,271 | 24.7 (trast) 18.2 (no trast) | 21.4% of the cases with residual carcinoma | 3.4% of the cases with residual carcinoma | – |
| Wang et al. [57]              | CT versus CT + anti-HER2      | 459   | –             | 19.8 (CT + trast) versus 9.4 (CT only) | – | Significantly worse PFS for HER2 loss |
| Ignatov et al. [59]           | Primary versus RD             | 205   | –             | 42% of the cases with residual carcinomas | – | Significantly worse PFS and trend for OS (not significant) for HER2 loss |
| Tural et al. [60]             | Primary versus RD             | 186   | 18            | 18% of the cases with residual carcinomas | – | Significantly worse PFS for HER2 loss |
| Branco et al. [58]            | Primary versus RD             | 108   | 13            | 13% of the cases with residual carcinomas | – | Significantly PFS and OS worse for HER2 loss |
| Katayama et al. [61]          | Primary versus RD             | 221   | –             | 22.3% of the cases with residual carcinomas | 15.9% of the cases with residual carcinomas | – |

MFS, metastasis-free survival; PFS, progression-free survival; OS, overall survival.
shifted from negative to positive. Total discordance rates accounted for 11.5%, 12.7%, and 9.8% when using FISH, IHC, and both, respectively. No statistically significant metastatic location-specific differences for pooled discordance were found.

To produce data as homogenous as possible, Chen et al. [52] studied 320 paired primary and metastatic breast carcinomas from a single institution, considering also the prognostic impact of HER2 status conversion. HER2 conversion was observed in 13.7% of cases, with positive to negative conversion occurring in 28.7% of cases and negative to positive conversion in 8.1% of cases. No statistically significant site-specific differences were found; however, the author reports a 14.3% discordance among different sites of relapse in patients with multi-organ metastasis. No significant differences were found in terms of OS.

The ESME report recently published results from a large cohort of patients and showed that at time of diagnosis of metastatic disease HER2 status discordance was detected in 7.8% of cases (loss of HER2 amplification in 45.2% of cases and HER2 gain in 54.8%), whereas after first progression the discordance rate was 10% (loss = 50.9% and gain = 49.1%). HER2 discordance was not significantly associated with OS [53].

Taken together, these data suggest that HER2 discordance between primary tumour and metastatic disease more frequently features “HER2 loss.” The underlying mechanisms have not been clarified yet, but plausible explanations are represented by tumour heterogeneity, and selective selection due to clonal evolution or therapeutic pressure. As mentioned above, therapies are not necessarily modified based on the profile identified in a biopsy of metastatic deposit; hence, insufficient data are available regarding the long-term effect of a possible therapy switch.

HER2 discordance has also been studied between native primary tumours and RD following neoadjuvant therapy (Table 1). In one of the earliest studies, Mittendorf et al. [54] evaluated HER2 status in 25/142 patients who did not reach a pCR following neoadjuvant chemotherapy + trastuzumab and observed 32% of cases with HER2 loss on the RD. Moreover, loss of HER2 overexpression was associated with worse recurrence-free survival: the 3-year recurrence-free survival estimates were 87.5% for patients maintaining HER2 amplification and 50% for patients whose tumour showed HER2 loss. Guarneri et al. [55] enrolled 107 patients in two cohorts and administered neoadjuvant chemotherapy (cohort A) or neoadjuvant chemotherapy + an anti-HER2 agent (cohort B). Loss of HER2 expression was observed in 40% of patients with RD of cohort A and in 14.7% of patients of cohort B. This study highlights that HER2 loss can be experienced also following chemotherapy regimens only. Patients with HER2 loss tended to have a poorer DFS compared to patients who maintained HER2 positivity. Niikura et al. [56] reported similar findings with 21.4% of HER2-positive tumours converting to HER2-negative after treatment versus 3.4% of HER2-negative patients showing a HER2-positive residual tumour. Discordance was observed in 24.7% of patients who received trastuzumab and in 18.2% of who did not receive trastuzumab.

Other neoadjuvant studies have reported HER2 loss over the course of a neoadjuvant treatment (range: 18–42%), but with a higher percentage of HER2 loss in residual tumours of patients treated with trastuzumab-containing chemotherapy compared to chemotherapy only (Table 1) [57–60]. These studies have also reported a higher risk of relapse for patients experiencing HER2 loss [57–60].

Interestingly, Ignatov et al. [59] reported comparative data for trastuzumab versus the dual blockade trastuzumab + pertuzumab. Out of the 205 patients with HER2-overexpressing carcinomas that were studied, 42% showed HER2 loss over the course of the neoadjuvant treatment. When the combination of trastuzumab and pertuzumab was adopted, 63.2% of cases experienced a decrease in HER2 levels, whereas only 47.3% of cases had a HER2 loss when trastuzumab was employed. In terms of outcome, the 5-year DFS was 74.4% for HER2-concordant and 59.6% for the HER2-discordant cases, respectively. No influence on OS was observed.

Finally, Katayama et al. [61] also performed assessment of ISH data. Out of the HER2-positive carcinomas, a subgroup of 22.3% shifted to HER2-negative; in addition, 15.9% of the HER2-negative carcinomas became HER2-positive. Average HER2 gene and CEP17 CN on pre- and post-treatment samples were also evaluated for IHC score 2+ cases. Within the patients who changed from IHC2+/HER2-amplified to IHC2+/HER2-not-amplified, 65.2% maintained the same HER2 gene CN, while 34.8% showed a decreased HER2 gene CN. Among those that shifted from IHC2+/HER2-not-amplified to IHC2+/HER2-amplified, 76.9% maintained the same HER2 gene CN and 23.1% showed an increased HER2 CN. These results may highlight that variability was minimal in terms of HER2 gene alterations and shifts from negative to positive tests were most likely because of values close to thresholds of positivity.

Taken together, these data suggest that HER2 status may be influenced by neoadjuvant therapy. HER2 change
occurs more frequently as a loss, and it seems to be particularly influenced by the addition of anti-HER2 agents. It remains unclear if this phenomenon is the reflection of response/resistance to therapy or the expression of an underlying HER2 heterogeneity. Nevertheless, HER2 loss following neoadjuvant chemotherapy seems to represent a negative prognostic factor in terms of PFS, without affecting the OS.

Of note, HER2 re-assessment following neoadjuvant chemotherapy is a debated matter for HER2-positive patients; nevertheless, it seems to be rather informative and would add prognostic information. The KATHERINE clinical trial [62], which has compared T-DM1 and trastuzumab in the adjuvant setting of HER2-positive breast cancer patients with residual-invasive disease after neoadjuvant therapy containing taxanes + trastuzumab, has shown that the risk of recurrence or death was 50% lower in the T-DM1 cohort, thus leading to a practice change on how we tackle HER2-positive RD following neoadjuvant chemotherapy. Of note, the requirement for the trial was to have a positive HER2 assessment either in the pre-treatment core biopsy or in the RD. Given the known phenomenon of HER2 loss over the course of trastuzumab-containing chemotherapy, it would be interesting to understand whether or not patients with RD characterized by HER2 loss would still benefit more from T-DM1 over trastuzumab-containing chemotherapy.

**Pre-Analytical Phase and Impact on HER2 Expression Assessment in Different Types of Tissue Samples**

The sensitivity of the IHC method and accuracy of interpretation of HER2 expression assessment in breast cancer represent an old issue [63]. As a matter of fact, the protein detection on formalin-fixed, paraffin-embedded tissue samples by IHC is strongly influenced by pre-analytical variables, in particular by the fixation process [64, 65]. For instance, an uncontrolled formalin fixation timing and duration modulate immunoreactivity, with structural and macromolecular alterations caused by both under- and over-fixation [66, 67].

To maximize the standardization of the IHC procedure, the ASCO/CAP guidelines for HER2 evaluation recommend (i) an immediate incubation of tissue sample in the fixative, thus minimizing ischemic time (≤1 h), and (ii) a fixation time of 6–48 h. The time between tissue collection and tissue fixation must not exceed 60 min [68, 69], with a potential reduction of macro-degradation through a controlled temperature protocol (4°C) [70] or by using a vacuum storage up to 72 h [71]. The fixative of choice is represented by 10% neutral buffered formalin [69, 72].

In the context of HER2 testing, fixation issues have led to poor reproducibility of results [73]. To better understand the effect of fixation time, comparative studies were conducted to show the consequences of a reduced or an excessive formalin incubation time. Studies assessing under-fixation (reviewed in [74]) or over-fixation [75] alone, as well as comparative studies [76, 77], have shown a limited impact of formalin over tissues characterized by a “clear-cut” expression of the HER2 protein. Interestingly, a complete agreement in the interpretation for HER2-negative (score 0) and HER2-positive (score 3+) cases was reported, regardless of the time of fixation [76, 77]. On the other hand, HER2 detection was affected in breast carcinomas showing intermediate levels of expression (score 1+ and score 2+), thus suggesting a significant impact of the fixation time on the HER2-low category that may undergo important reclassifications in the context of poorly preserved tissues [75, 78–81].

Another matter of interest related to pre-analytical conditions is associated with the different types of specimens that may be subjected to HER2 testing. Both core needle biopsies (CNBs) and surgical resections represent adequate specimens for HER2 testing (full sections of the tissue blocks) in primary tumours. A high level of agreement has also been reported between both surgical resections and CNBs [82], in particular whenever multiple core biopsies are available for a given lesion [83]. The latter helps capture intratumoral heterogeneity, whose full picture is well obtained in samples from surgical specimens. Recently, Miglietta et al. [84] have reported higher rates of HER2-low cases when assessing HER2 expression on CNBs compared to surgical specimens, thus suggesting that the reliability of CNBs evaluation when including the HER2-low category needs to be deeper assessed. This observation needs to be further explored in independent studies.

The advantages of using core biopsies are mainly related to anticipation of the information about tumour phenotype before surgical intervention and the optimal tissue fixation obtained thanks to the small size of CNB samples [85, 86]. Recent studies have shown that reduced fixation times (even a few hours) do not compromise the quality and concordance of the HER2 test [87, 88], thus confirming the indication of the original ASCO/CAP guidelines which indicate 6–8 h as the optimal fixation time for biopsies, with reduction to 1 h in case of extreme need [72].
Biopsies are also the typical sample available for metastatic lesions, whose re-assessment in terms of biomarkers is mandatory in breast cancer. Heterogeneity across metastatic site can be encountered; hence, a change in treatment schedule is typically embraced when a conversion from a HER2-negative to a HER2-positive result is observed on a metastatic site. In some instances, limited tumour material may be sampled with biopsies, thus further complicating the conduction of a reliable evaluation of the real biomarker profile of the lesion. Active interaction with medical oncologists is advised in this context.

Cytological specimens may also represent a tissue source in these scenarios; however, careful management of the fixation method is needed. Although alcohol fixation has been reported to be very effective in preserving the molecular components of cytologic specimens [89, 90], the ASCO/CAP guidelines for the evaluation of HER2 status recommend formalin fixation for cytological samples [8, 69, 72].

When discussing different types of tissue specimens and metastatic lesions, bone tissue management merits a separate chapter. Bone is the first metastatic site for breast cancer [91], thus representing an important sampling site for re-evaluation of HER2 status. Decalcification procedures aim to remove calcium phosphate, thus allowing sectioning, with several protocols available [92]. The detrimental effect of decalcification mainly affects the quality of nucleic acids [93], while methods such as IHC seem not to be significantly affected [94]. Studies focusing on HER2 assessment have shown that decalcification does reduce the success rate of the IHC [95, 96] resulting in the lack of a decalcification protocol recognized as the “gold standard” for the downstream IHC application [97]. The use of an EDTA-based method should be considered in view of the possible downstream FISH evaluation for HER2 gene status, since better results have been shown with this protocol [98].

In the context of pre-analytical factors, it should be emphasized that ASCO/CAP guidelines [69] highlight the importance of both internal and external quality control assessments for laboratories conducting HER2 assays. External quality control assessment relies on the participation and completion of external proficiency tests and onsite inspections; moreover, the recommendations provide examples of international quality assurance programmes. On the other hand, internal quality assessment programmes are not well defined, besides the recommendation to use FDA-approved assays.

A final remark to exhaustively cover this section is dedicated to alternative tissue samples as a source for HER2 testing. When dealing with histological samples, research studies often exploit the advantage of tissue microarray (TMA). This is a technique that has also been explored to some extent for routine practice [99]. We reported, for instance, in the context of breast cancer biomarker assessment (hormonal receptors, HER2, and Ki67) a 95% agreement between TMA and full sections; however, four cores per lesion were sampled to allow proper representativeness of the tumoral lesion. Intratumoral heterogeneity greatly limits the implementation of TMA in routine diagnostics, and TMA use is not recommended in diagnostic practice.

Conclusions

HER2 expression assessment by IHC plays a central role in breast cancer diagnostic pathology and has been used to identify breast carcinomas addicted to HER2 gene amplification, which leads to a massive overexpression of the HER2 protein on the cell membrane. We know that HER2 overexpression can be present in variable degrees and shapes, even featuring some unusual patterns that do not exactly fulfill the categories according to the historical scoring system proposed by ASCO/CAP. The ASCO/CAP recommendations acknowledge such patterns and speak a word of caution on how to address these unusual scenarios. HER2 heterogeneity can create difficulties in the final definition of the HER2 status of individual patients and needs to be discussed with medical oncologists. Another chance of active discussion with medical oncologists is encountered whenever the HER2 status changes over the course of the clinical history of the patient. Loss of HER2 expression seems to be more frequent than acquisition of HER2 positivity. HER2 loss has been described also following neoadjuvant chemotherapy and seems to hold prognostic information, thus highlighting the informativeness of re-assessing biomarkers on carcinomas not reaching pCR.

Finally, at present we are revisiting the importance of HER2 (over)expression assessment under a new perspective, where the expression of a given protein may represent an anchor to a novel therapeutic option, as it has been shown for the subgroup of “HER2-low” carcinomas. IHC is therefore again under the spotlight. Although simple and widespread across pathology laboratories, IHC is not free from pre-analytical issues and pathologists must be aware of the critical steps that may hamper immunohistochemical performance of HER2 assays and their evaluation. This seems to be particularly important for
the lower end of the HER2 expression spectrum; hence, it may have an impact on the identification of the so-called HER2-low carcinomas.

Conflict of Interest Statement

Caterina Marchiò has received personal consultancy fees from Roche, Bayer, AstraZeneca, Daiichi Sankyo, and Novartis. Caterina Marchiò serves as an Associate Editor for Pathobiology. Dora Grassini, Eliano Cascardi, Ivana Sarotto, Laura Annaratone, Anna Sapino, and Enrico Berrino have no conflicts of interest to declare.

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Author Contributions

Dora Grassini, Enrico Berrino, and Eliano Cascardi performed literature search and contributed to writing and to illustrations. Caterina Marchiò has drafted the review structure and wrote the first draft of the manuscript, which was revised and integrated by Laura Annaratone, Anna Sapino, and Ivana Sarotto.
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