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Review

Evolving concepts in HER2 evaluation in breast cancer: Heterogeneity, HER2-low carcinomas and beyond

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ABSTRACT

The human epidermal growth factor receptor 2 (HER2) is a well-known negative prognostic factor in breast cancer and a target of the monoclonal antibody trastuzumab as well as of other anti-HER2 compounds. Pioneering works on HER2-positive breast cancer in the 90s' launched a new era in clinical research and oncology practice that has reshaped the natural history of this disease. In diagnostic pathology the HER2 status is routinely assessed by using a combination of immunohistochemistry (IHC, to evaluate HER2 protein expression levels) and *in situ* hybridization (ISH, to assess *HER2* gene status). For this purpose, international recommendations have been developed by a consensus of experts in the field, which have changed over the years according to new experimental and clinical data. In this review article we will document the changes that have contributed to a better evaluation of the HER2 status in clinical practice, furthermore we will discuss HER2 heterogeneity defined by IHC and ISH as well as by transcriptomic analysis and we will critically describe the complexity of HER2 equivocal results. Finally, we will introduce the clinical impact of *HER2* mutations and we will define the upcoming category of HER2-low breast cancer with respect to emerging clinical data on the efficacy of specific anti-HER2 agents in subgroups of breast carcinomas lacking the classical oncogene addition dictated by *HER2* amplification.

1. Introduction

The human epidermal growth factor receptor 2 (HER2) is an orphan tyrosine kinase receptor belonging to the Human Epidermal Receptor family. HER2 has no ligand but is a preferred dimerization partner of the other three receptors of the family [1]. Upon (homo/hetero-)dimerization among the receptors, downstream tyrosine kinase signaling cascades are activated thus triggering cell proliferation, migration, invasion, and survival [1].

HER2 is amplified leading to HER2 overexpression in about 15 % of breast carcinomas [2]. If on one side this mechanism confers a poor prognosis (due to the effect on cell proliferation, migration, invasion, and survival, all hallmarks of cancer), on the other side it offers the unique possibility to use a targeted treatment approach with the monoclonal antibody trastuzumab, which targets the extracellular domain of HER2 thus altering the normal tyrosine kinase signaling (Fig. 1). Such therapeutic option has radically changed the natural history of HER2-positive disease since the 90s'. Besides the monoclonal antibody trastuzumab, which is currently administered together with chemotherapy in the neoadjuvant, adjuvant and metastatic settings, other anti-HER2 therapeutic compounds have been developed over the years (Fig. 1). Pertuzumab, a humanized monoclonal antibody that binds to HER2 on a different domain than trastuzumab and prevents homo- and hetero-dimer formations, is routinely added to taxanes and trastuzumab as the preferred regimen in the first-line setting of advanced disease [3], and it is also administered in the neoadjuvant setting [4]. Other anti-HER2 compounds (tyrosine kinase inhibitors, such as lapatinib, or antibody-drug conjugates such as T-DM1) are used mainly in the metastatic setting or within the context of clinical studies. It is important to note that although several markers with prognostic value have been identified in HER2-positive disease over the past years, at present the best predictive factor for likeliness of response to anti-

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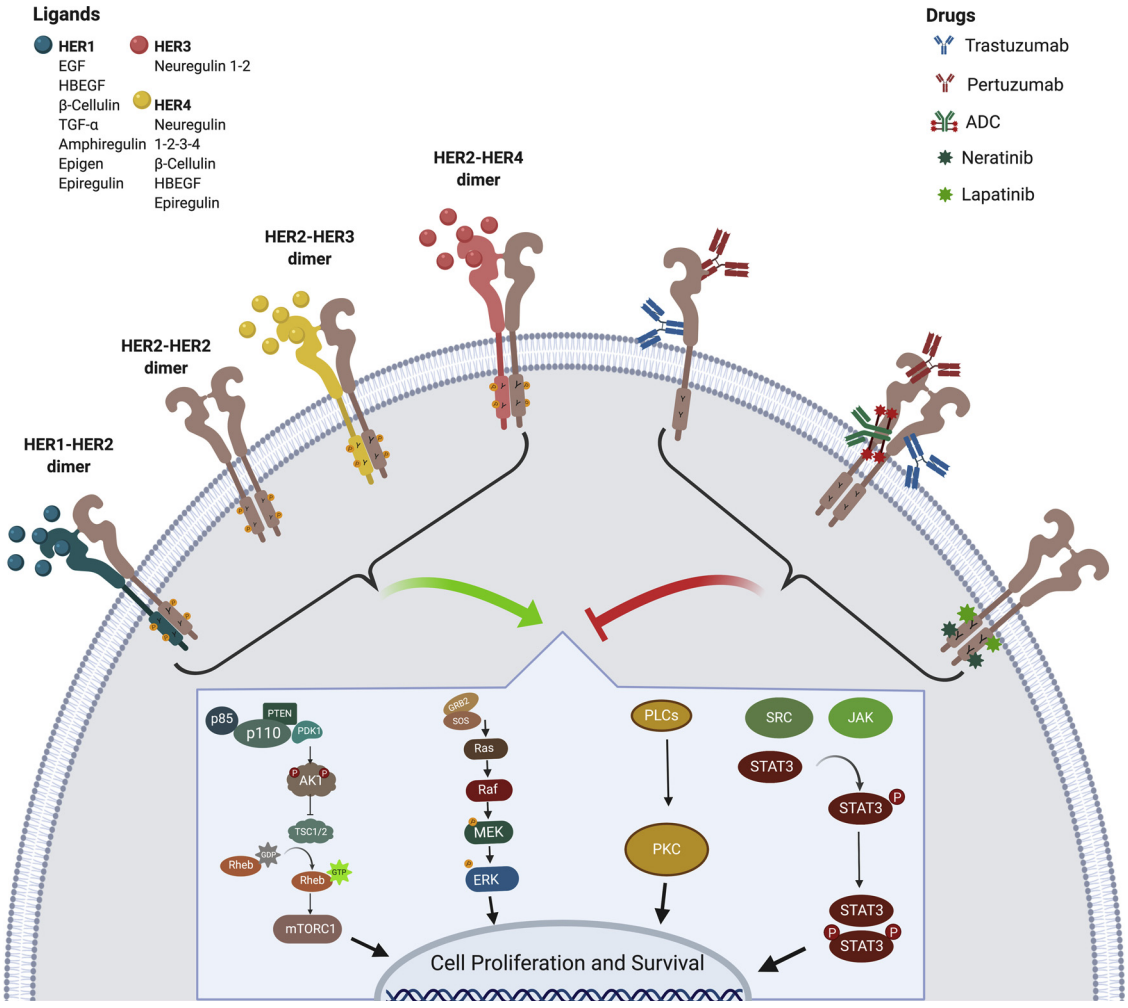

Fig. 1. HER2 downstream pathway activation, anti-HER2 targeted agents. HER2 is a ligand-orphan transmembrane tyrosine kinase receptor belonging to the Human Epidermal Receptor (HER) family. HER2 homo- or hetero-dimerization with one of the other three receptors (HER1 or EGFR, HER3 and HER4), triggers the activation of different signaling pathways that are associated with cell proliferation, invasion and survival of cancer cells [1,106]. Therapeutic approaches, based on the modulation of HER2 activation, include different targets of the protein. Monoclonal antibodies bind the extracellular domain of HER2, with a selective antigen preference for trastuzumab or pertuzumab, leading to a reduction of the signaling cascade [107]. Antibody-drug conjugates are designed to release the drug after internalization by the tumor cell, with the decrease of systemic side effects. The small molecules tyrosine kinase inhibitors (TKIs), such as lapatinib and neratinib, inhibit the catalytic activity of the HER2 receptor in a reversible or irreversible way, respectively, through the specificity for the ATP binding site of the kinase domain [108]. ADC: Antibody-Drug Conjugate. This image has been created with BioRender.

HER2 agents is represented by HER2 overexpression/amplification [5].

In diagnostic pathology the HER2 status is routinely assessed by using a combination of immunohistochemistry (IHC, to evaluate HER2 protein expression levels) and *in situ* hybridization (ISH, to assess *HER2* gene status), the only two techniques with acknowledged clinical validation [2,5,6]. For this purpose, international recommendations have been developed by a consensus of experts in the field. These recommendations have changed over the years according to new experimental and clinical data. In this review we will document the changes that have contributed to a better evaluation of the HER2 status in clinical practice; in addition we will discuss the different scenarios encompassing HER2 heterogeneity defined by IHC and ISH as well as by transcriptomic analysis, the complexity of HER2 equivocal results, *HER2* mutations and the upcoming category of HER2-low breast cancer.

2. HER2 evaluation in breast cancer by ASCO/CAP guidelines

In diagnostic practice every newly diagnosed breast carcinoma as well as any relapses or metastatic deposits are assessed for the HER2 status, which is assigned based on recommendations by an international group of experts in the field. The American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) have drafted the recommendations for HER2 testing since 2007 [7] by taking also care to discuss the optimal pre-analytical and analytical requirements for the performance and interpretation of HER2 testing using IHC and fluorescence ISH (FISH). The pre-analytical phase has a huge impact on the correct performance of both IHC and ISH and proper control of cold ischemia time is paramount in ensuring the preservation of antigens, DNA and RNA in formalin-fixed, paraffin-embedded (FFPE) tissues [2,8–10].

The initial clinical trials for trastuzumab used an IHC score 3+ or a score 2+ with a positive FISH test (defined by *HER2*/CEP17 ratio ≥2 in > 50 % neoplastic cells) as eligibility criteria. In these trials, an IHC score 3+ was defined as intense/strong complete circumferential membrane staining in > 10 % cells of neoplastic cells and 2+ was defined as weak to moderate complete circumferential membrane staining in > 10 % cells of neoplastic cells [11]. In the 2007 ASCO/CAP guidelines the threshold for positivity was elevated to > 30 % of neoplastic cells in IHC and to *HER2*/CEP17 ratio ≥2.2 by FISH in order to lower the number of false positive results, decreasing the administration of ineffective anti-HER2 therapy [7].

In 2009 the ASCO/CAP published a supplement to the 2007

guidelines regarding *HER2* heterogeneity in FISH assays. It defined *HER2* genetic heterogeneity as the presence of ≥5% to < 50 % of infiltrating tumor cells with a ratio ≥2.2 when using dual probes or ≥6 HER2 signals/cell using single probes [12]. They recommended two to four representative areas of the invasive tumor to be selected and assessed after scanning the entire slide to look for heterogeneity; clusters (> 20 cells) with *HER2* amplification by FISH should be separately assessed and reported in terms of *HER2*/CEP17 ratio and/or *HER2* signals/cell [12].

The 2013 ASCO/CAP guidelines reverted the positivity threshold to the original > 10 % cells of neoplastic cells in IHC and *HER2*/CEP17 ratio ≥2 in FISH in order to avoid false negative results [13]. These recommendations brought the attention to false negative results, which could deny a potentially life-saving treatment (anti-HER2 therapy) to breast cancer patients [13]. This position was taken based on the relatively low frequency of side effects this treatment may lead to [13]. In particular, these guidelines introduced the concept of ISH algorithm, which represents a two-step approach when scoring ISH results taking into account first the *HER2*/CEP17 ratio, followed by the analysis of *HER2* mean copy numbers when the *HER2*/CEP17 ratio is < 2. This algorithm helped avoid misclassification of *HER2* amplification in those cases harboring abnormal copy numbers of the centromeric region of chromosome 17 (CEP17). Indeed, we [14] and others [15–17] have demonstrated that breast carcinomas with such FISH patterns more frequently harbor true *HER2* amplification coupled with gain/amplification of the centromeric region of chromosome 17, rather than chromosome 17 (Chr17) polysomy.

Finally, the 2013 ASCO/CAP recommendations further addressed the issue of HER2 heterogeneity by adopting the same threshold of > 10 % used in IHC assessment, thus defining HER2 heterogeneity as the presence of a separate population of cells with a different *HER2* copy number and/or *HER2*/CEP17 ratio accounting for at least 10 % of the overall tumor cell population [13]. The guidelines recommended that in these cases a separate counting should be done in at least 20 of these cells [13].

In 2018 the ASCO/CAP panelists provided an update of these guidelines, which focuses on five specific clinical questions (Table 1) [6]. In this respect, the 2018 ASCO/CAP guidelines should be best considered as an integration of the 2013 edition, rather than a new version of recommendations. Neither cut-offs or definitions were amended, rather literature data were reviewed to assess the level of evidence to suggest a positive or negative result in borderline cases.

As first question to be answered the panelist focus on the correct definition of score 2+ breast carcinomas that would require a reflex ISH testing: a weak to moderate complete membrane staining in more than 10 % of the tumor cell population is required to classify breast carcinomas as score 2 + . Nevertheless, it is acknowledged that unusual patterns of HER2 expression can be encountered, featuring for instance strong but incomplete membrane staining (typical of micropapillary carcinomas) or strong and complete staining but only in a subpopulation of cells that account for less than 10 % of the entire tumor population. In all of these scenarios ISH has to be performed. In the latter scenario, which is suggestive of high intratumoral heterogeneity, reasonable common practice would include to test additional tumor blocks to assess the real degree of HER2 overexpression/amplification. These highly heterogeneous carcinomas are those that would likely benefit most from retesting at recurrence and/or metastatic progression since *HER2*-amplified clones may become dominant and therefore leading to the addition of anti-HER2 therapy [18].

A second clinical question relates to the mandatory recommendation that was given in 2013 regarding the repetition of the HER2 testing on surgical specimens whenever core needle biopsy specimens harbor negative HER2 results at the initial assessment. The 2018 update tempers this statement by suggesting that HER2 test may be repeated whenever encountering one of the following instances: i) amount of invasive tumor in the core biopsy specimen is small; ii) resection specimen contains high-grade carcinoma that is morphologically distinct from that in the core; iii) core biopsy result is equivocal for HER2 after testing by both ISH and IHC; iv) there is doubt about the handling of the core biopsy specimen (long ischemic time, short time in fixative, different fixative) or the test is suspected by the pathologist to be negative on the basis of testing error [6].

The remaining three clinical questions are related to ISH results, for which the recommendations refer to the 5-Group classification proposed by Press and co-workers in 2016 [19]. Group 1 (*HER2*/CEP17 ratio ≥2, *HER2* copy number > 4) and Group 5 (*HER2*/CEP17 ratio < 2, *HER2* copy number < 4) represent the two extremes of the spectrum of HER2 evaluation (presence and lack of *HER2* amplification, respectively) and the authors indicate that would comprise 95 % of all dual-probe ISH test results. The remaining 5% would be categorized as Groups 2–4 [6], featuring challenging scenarios of non-univocal interpretation that have been a matter of debate over the years and for which the 2018 ASCO/CAP update suggests to perform an additional work-up based on a combined reinterpretation of ISH and IHC assays to reach the most accurate HER2 status attribution (positive *versus* negative) (Table 1).

In particular, Group 2 identifies tumors in which ISH test results show *HER2*/CEP17 ratio ≥2 but mean *HER2* copy numbers < 4, a pattern that often reflects the presence of chromosome 17 monosomy. In the retrospective analysis of the initial clinical trials for trastuzumab, although the small sample size for this group made it impossible to statistically rule out a possible benefit from therapy, most patients did not seem to have improved outcomes with anti-HER2 treatment [6]. Hence, if the ISH pattern is confirmed during the additional work-up it is recommended to consider these cases as negative, unless tumor cells display a score 3+ by IHC [6].

In Group 3 we include cases harboring mean *HER2* copy number ≥6 and a *HER2*/CEP17 ratio < 2 due to increased CEP17 copy numbers, which were labeled as positive thanks to the adoption of the ASCO/CAP 2013 algorithm. These cases are very uncommon (0.4–3 %) and were not included in the initial trastuzumab clinical trials, therefore there is limited clinical evidence regarding the benefit from trastuzumab-containing chemotherapy. Nevertheless, based on the demonstration by several independent groups of occurrence of true *HER2* amplification in these cases [14–17], in particular when *HER2* copy numbers are high, the 2018 update supports the definition of HER2-positivity for these tumors, unless there is lack of HER2 overexpression (*i.e.* the specimen shows a score 1+ or 0 by IHC) [6].

Finally, Group 4 breast carcinomas show equivocal *HER2* gene status (*HER2*/CEP17 ratio < 2.0 with mean *HER2* copy numbers ≥4 and < 6) and would be defined HER2 double-equivocal if showing also a score 2+ of HER2 expression by IHC. The number of such cases within a laboratory varies based on the patient population referred for ISH testing, but it seems to account for approximately 5% of cases (range: 1 %–16 %) [6]. Breast carcinomas harboring this ISH pattern were labeled as “equivocal” according to the 2013 recommendation and the use of anti-HER2 agents was left to the clinician’s choice [13]. There is no doubt that Group 4 cases have posed a challenge to oncologists and patients due to a perceived ambivalence about whether to recommend HER2-targeted therapy. It should also be noted that the absence of an unequivocally positive or negative test result has led to multiple testing of the same tissue sample as well as to the use of alternative Chr17 probe testing in the attempt to resolve the HER2 status. It is important to stress the concept that Chr17 in breast cancer is highly recurrently altered [14,20,21], therefore the use of alternative Chr17 probes mapping to regions different from the CEP17 may be misleading rather than of support in these scenario: as an example, by using probes mapping to the short Chr17 arm, which happens to harbor large chromosomal losses, the *HER2*/Chr17-probe ratio may increase and even lead to a positive result based on mere artefactual calculation. Furthermore, these alternative Chr17 probes are not analytically or clinically validated, *i.e.* they have not been used in clinical trials

Table 1
Summary of HER2 evaluation by ISH following the 2018 ASCO/CAP guidelines.

Assay	Method	Result	Additional work-up	Final HER2 assignment
Dual-probe ISH	**ISH algorithm**	**Group 1** *HER2*/CEP17 ratio ≥**2.0** and average *HER2* copy number ≥**4.0** signals/cell	/	***AMP/Positive***
		Group 2 *HER2*/CEP17 ratio ≥**2.0** and average *HER2* copy number < **4.0** signals/cell	Concurrent IHC score **0/1**+	NOT AMP/Negative with Comment
			Concurrent IHC score **2**+, recount ISH: preliminary result confirmed	NOT AMP/Negative with Comment
			Concurrent IHC score **2**+, recount ISH: other ISH result	Result should be adjudicated per internal procedures
			Concurrent IHC score **3**+	***AMP/Positive***
		Group 3 *HER2*/CEP17 ratio < **2.0** and average *HER2* copy number ≥**6.0** signals/cell	Concurrent IHC score **0/1**+	NOT AMP/Negative with Comment
			Concurrent IHC score **2**+, recount ISH: preliminary result confirmed	***AMP/Positive***
			Concurrent IHC score **2**+, recount ISH: other ISH result	Result should be adjudicated per internal procedures
			Concurrent IHC score **3**+	***AMP/Positive***
		Group 4 *HER2*/CEP17 ratio < **2.0** and average *HER2* copy number ≥**4.0** and < **6.0** signals/cell	Concurrent IHC score **0/1**+	NOT AMP/Negative with Comment
			Concurrent IHC score **2**+ **(HER2 double-equivocal)**, recount ISH: preliminary result confirmed	NOT AMP/Negative with Comment
			Concurrent IHC score **2**+ **(HER2 double-equivocal)**, recount ISH: other ISH result	Result should be adjudicated per internal procedures
			Concurrent IHC score **3**+	***AMP/Positive***
		Group 5 *HER2*/CEP17 ratio < **2.0** and average *HER2* copy number < **4.0** signals/cell	/	NOT AMP/Negative
Single-probe ISH	***HER2* copy number**	Average *HER2* copy number < **4.0** signals/cell	/	NOT AMP/Negative
		Average *HER2* copy number ≥**4.0** and < **6.0** signals/cell	Concurrent IHC score **0/1**+ and/or concurrent dual-probe ISH **Group 5**	NOT AMP/Negative
			Concurrent IHC score **2**+	Perform dual-probe ISH for final result
			Concurrent IHC score **3**+ and/or concurrent dual-probe ISH **Group 1**	***AMP/Positive***
		Average *HER2* copy number ≥**6.0** signals/cell	/	***AMP/Positive***

Legend: AMP: amplified; IHC: immunohistochemistry; ISH: *in situ* hybridization. Scenarios in which the final result is adjudicated as positive are highlighted in Bold and Italic.

showing efficacy of anti-HER2 therapy [6]. The Expert Panel of the 2018 ASCO/CAP update also strongly recommends against this as a routine testing strategy and suggest to rely on a re-evaluation of the IHC score if not performed and to a recount by a second observer of the ISH experiment [6]. If the double-equivocal result is confirmed after additional work-up, the carcinoma is preferentially labeled as negative, since there is uncertainty as whether patients may benefit from HER2-targeted therapy in the absence of protein overexpression (IHC score 3+) [6]. It should be noted that this is a recommendation conceived in spite of a gap of knowledge on the potential beneficial effect of a HER2-targeted treatment in patients with an average of ≥4.0 and < 6.0 *HER2* signals per cell and a *HER2*/CEP17 ratio of < 2.0. The NSABP B-47 trial has recently demonstrated that HER2-negative carcinomas (score 1+/2+ in IHC and ISH-negative) do not benefit from the addition of trastuzumab [22]. Regrettably, *HER2* equivocal carcinomas were not included in this study and data about treatment response for these carcinomas are therefore missing.

Not surprisingly, studies available so far on the impact of the 2018 guidelines indicate that application of the new guidelines leads to an increase in the negativity rate on HER2 testing. This observation stems from the reclassification of Group 2 and Group 4 cases that are now recommended to be scored as negative [23,24].

3. Intratumor and intertumor heterogeneity of HER2-positive breast carcinomas

Intratumor genetic heterogeneity has been well documented in various types of human cancer, including breast cancer [25]. It is well known to pathologists routinely assessing HER2 in diagnostic practice that HER2 overexpression and amplification can present a heterogeneous pattern (Fig. 2). Three distinct types of distribution of cells with heterogeneous *HER2* status have been described [26]: “clustered”

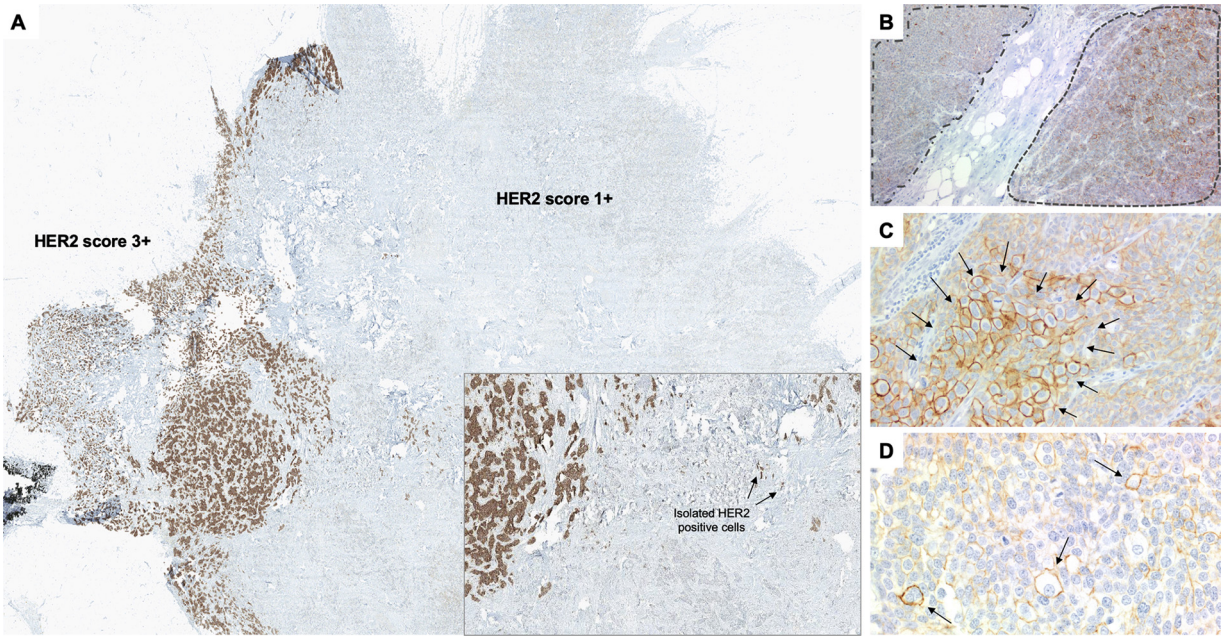

Fig. 2. Intratumor heterogeneity of HER2 expression. Representative micrographs of heterogeneous patterns of HER2 protein expression assessed by immunohistochemistry in breast carcinomas. (A) A sample showing two discrete tumor clones, one showing a HER2 expression scored a 3+ and one lacking HER2 overexpression with some interspersed isolated HER2-positive cells (inset). (B) A breast carcinoma with a variable degree of HER2 expression with some areas featuring a score 2+ (dashed lines) and other areas score 1+ (dashed lines with dots). (C) A sample with aggregated HER2 score 2+ cells (arrows) surrounded by HER2 score 1+ cells. (D) Scattered HER2 score 2+ cells (arrows) within a HER2 score 1+ tumor cell population. Micrographs were taken from samples routinely analyzed for HER2 assessment and for which an informed consent for research studies was also signed.

type, featuring the presence of two topographically distinct tumor clones of tumor cells, one harboring *HER2* amplification and the other with normal HER2 status; "mosaic" type, displaying either diffuse intermingling of cells with different HER2 statuses; "scattered type", with isolated *HER2*-amplified cells in a HER2-negative tumor cell population.

Several studies have demonstrated the presence of *HER2* genetic heterogeneity at variable frequencies (1–34 %) in breast cancer [27–36], being significantly more common in cases with *HER2* equivocal status by ISH and/or IHC [27,30,33,34,37]. For instance, Allison et al. [27] and Chang et al. [29] found that > 90 % of FISH *HER2* equivocal cases presented *HER2* heterogeneity; Bukley et al. observed that the highest levels of heterogeneity were detected in HER2 border line cases as well as in HER2-negative cases with highest *HER2*/CEP17 ratios [18]. Some have suggested that HER2 heterogeneity is an important cause of equivocal HER2 results in breast cancer by FISH and IHC [18,36,37]. In this respect, our group has demonstrated that whenever assessing HER2 status in breast carcinomas with a diffuse intermingling of *HER2*-amplified and non-amplified cells leads to *HER2* equivocal counts [38]. These cases typically feature low levels of *HER2* amplification [38], as observed also by others [28,33,36,39].

Some studies have also associated increased frequency of chromosome 17 polysomy with HER2 heterogeneity [37,39,40]. As already mentioned, chromosome 17 polysomy is a rare event in breast cancer, which features more frequently gain or amplification of the chromosome 17 centromere (CEP17) [14–17]. Hence, the term CEP17 abnormal copy number should be used in this context (especially if assessed by FISH in interphase nuclei). Nevertheless, this phenomenon of increased CEP17 copy numbers independently observed in HER2 heterogeneous carcinomas may support the theory that HER2 heterogeneity is caused, at least in part, by chromosomal instability [40].

In terms of impact on patient outcome, Shafi et al. [37] found that *HER2* FISH non-amplified tumors with genetic heterogeneity were associated with larger size, higher histologic grade and frequency of lymph node metastasis, which are important negative prognostic factors in breast cancer. Several studies indicate that the presence of *HER2* heterogeneity is associated with worse patient outcomes, in terms of shorter disease free survival and overall survival [32,33,40]. When analyzing the response to treatment, patients with HER2 heterogeneity seem to be less responsive to anti-HER2 targeted therapy [30,41–43], as also demonstrated by lower achievement of pathologic complete response (pCR) following neoadjuvant treatment with trastuzumab-containing chemotherapy [35,38]. These results have been recently confirmed in an elegant study presented at ASCO 2019 by Metzeger Filho and co-workers, who designed a Phase II study in which evaluation of response of stage II and stage III centrally confirmed HER2-positive breast carcinomas to neoadjuvant T-DM1 and pertuzumab was performed. *HER2* heterogeneity in diagnostic core needle biopsy samples was defined either as i) *HER2* positivity by FISH in > 5% and < 50 % of tumor cells [12]; ii) an area of the tumor that was tested negative. *HER2* heterogeneity was found in 10 % of cases (n = 16/157). pCR was not achieved in any of the heterogeneous cases compared to 55 % of HER2-positive carcinomas without evidence of *HER2* heterogeneity. The association between *HER2* heterogeneity and pCR remained significant when adjusted by estrogen receptor (ER) status. These data suggest that HER2 heterogeneous breast carcinomas may represent a distinct subset of HER2-positive tumors for which different therapeutic approaches may be considered [44].

A genomic analysis based on gene copy number profiling and massively parallel sequencing of HER2-negative and HER2-positive components of 12 HER2 heterogeneous breast cancers identified potential driver genetic alterations restricted to the HER2-negative cells in each case. In *in vitro* models *BRF2* and *DSN1* overexpression/amplification and the *HER2* I767M mutation were shown to be alterations that compensate for the lack of *HER2* amplification in the HER2-negative components of HER2 heterogeneous breast carcinomas [45].

Another important aspect to consider is intertumor heterogeneity of HER2-positive disease. First, up to 45 % of cases may also express variable levels of hormone receptors; second, there is a variable composition of molecular subtypes; finally, different genetic alterations can be observed across tumors.

Although hormone receptor status seems not to determine the

overall genetic profile of *HER2*-amplified breast carcinomas, specific genetic aberrations may be characteristic of subgroups of HER2-positive breast cancers [46–48]. Hormone receptor status is also impacting, at least in part, on the distribution of intrinsic molecular subtypes of clinically defined (*i.e.* by IHC/FISH) HER2-positive carcinomas. The large majority is represented by HER2-enriched carcinomas, however a non-negligible number of luminal carcinomas (luminal B being more prevalent than luminal A) and basal-like carcinomas have been consistently reported [46,49,50].

Of note, HER2-positive breast cancers defined as "HER2-enriched" by transcriptomic analysis have high response rates to anti-HER2 therapy, thus identifying a category of super-responders to anti-HER2 compounds [50,51]. Prat and co-workers [52] have recently analyzed *HER2* mRNA levels and the HER2-enriched intrinsic subtype by PAM50 in HER2-positive disease treated with dual HER2-blockade without chemotherapy in five neoadjuvant clinical trials (SOLTI- PAMELA, TBCRC023, TBCRC006, PER-ELISA, EGF104090). The PAM50-defined HER2-enriched intrinsic subtype was significantly associated with high *HER2* mRNA levels and higher pCR rate compared to the rest [52]. These data suggest that this biomarker could help de-escalate chemotherapy in approximately 40 % of patients with HER2-positive breast cancer [52].

As a matter of fact, there is a wide interpatient variability across the different intrinsic subtypes in *HER2*-amplified patients. In a set of 66 *HER2*-amplified breast carcinomas analyzed by whole genome sequencing, *TP53* mutations were detected only in tumors characterized by a HER2-enriched PAM50-derived subtype, without any alterations in the *PI3KCA* gene. Conversely, breast carcinomas classified as luminal showed a high frequency of *PIK3CA* mutations and no *TP53* alterations [46]. Interestingly, in a cohort of HER2-positive carcinomas subjected to neoadjuvant anthracycline-taxane-based chemotherapy plus anti-HER2 treatment *PIK3CA* mutated carcinomas (21.4 % of the overall cohort) were less likely to achieve a pCR compared to *PIK3CA* wild-type tumors. The difference in terms of pCR rate between *PIK3CA* wild type and *PIK3CA* mutated tumors was more pronounced for hormone receptor positive carcinomas [53].

In a series of HER2-positive/ER-positive breast carcinomas showing a better prognosis and reduced benefit from trastuzumab compared with the HER2-positive/ER-negative carcinomas, Zhao and co-workers reported a reduced rate of *TP53* mutations and a lesser degree of HER2 expression at both mRNA and protein levels [54].

4. HER2 status by RNA-based methods

A high correlation between *HER2* gene status and *HER2* mRNA levels is well known, as documented by several studies [55–57]. This has allowed to consider RNA evaluation as a possible option for HER2 assessment. The aim is to understand whether RNA-based testing could be able to complement IHC and DNA FISH analysis, allowing a full resolution of the HER2 status. Several techniques have been applied for *HER2* mRNA analyses, including quantitative procedures and *in situ* detection methods, however they are not widely used in clinical practice even though the determination of biomarkers by RNA evaluation is becoming more feasible.

The possibility to perform *HER2* mRNA testing by real-time quantitative PCR (qPCR) has been extensively evaluated [57–59]. The opportunity to use qPCR to assess in a single reaction *HER2* DNA amplification and mRNA expression in microdissected breast carcinomas has been investigated and, despite the unavoidable degradation of nucleic acids that occurs in FFPE tissue samples, the detection of *HER2* mRNA was successful in 94 % of samples [57].

Given that qPCR is quantitative and objective, while IHC is semi-quantitative and observer-dependent, qPCR approaches have been proposed as alternative to IHC to reduce the variability of immunohistochemistry. Denkert and co-workers [60] found that *HER2* mRNA expression was higher and detectable with gradually increasing expression levels in ER-positive tumors, while distinct groups of *HER2*-positive and *HER2*-negative tumors were identified in ER-negative cases [60]. The continuum of *HER2* mRNA expression in ER-positive cases has called into question the selections of cut-off levels for HER2 assessment, highlighting the need of further evaluation of *HER2* mRNA expression in additional cohorts. Dabbs and co-workers [61] evaluated the concordance between laboratory HER2 results by FDA-approved IHC and FISH assays and *HER2* mRNA assessment by qPCR separately reported by the Oncotype DX test. Their results demonstrated that the false-negative rate for Oncotype DX qPCR for *HER2* was greater than 50 % [61]. The authors spoke a word of caution especially for those equivocal or negative qPCR results in unequivocally HER2-positive patients by using FDA-approved procedures. This is why mRNA-based techniques are not recognized as alternative methods to assess HER2 status in diagnostic practice, as stated also by the ASCO/CAP guidelines.

Many causes have been suggested to explain discordant results. Heterogeneity of HER2 expression/amplification is likely key in this respect, but cases with low *HER2* level amplification may contribute, at least in part, to these discordances: as a matter of fact thresholds in ISH analysis are used and unselected cases classified as HER2-positive by ISH may have a wide range of *HER2* amplification.

Droplet digital PCR (ddPCR) is another method for mRNA evaluation and is based on partitioning the PCR reaction mixture into thousands of droplets so that each droplet contains either 1 or 0 molecules of target RNA. It is capable of quantifying mRNA expression with high precision. From the advent of this technique, several studied have been proposed with the aim to assess *HER2* expression in breast carcinomas. Meehan and colleagues demonstrated that ddPCR can be applied in FFPE tissues to measure *HER2* mRNA [62]. When applying a cut-off of 490 mRNA *HER2* copies/μl a perfect (100 %) agreement was found in discriminating HER2-positive from HER2-negative cases. They included in their analysis also 6 HER2 equivocal cases based on the 2013 ASCO/CAP Guideline Recommendations and these cases were consistently classified as negative by ddPCR [62]. In another study ddPCR was applied for *HER2* mRNA determination in both breast and gastric cancer samples, with the final ambition to find a possible role for ddPCR in handling the two clinical challenges of equivocal cases and intratumor heterogeneity [63]. The ddPCR assay defined the IHC and FISH double-equivocal case as HER2-negative patients [63]. The authors acknowledged that the weakness of ddPCR is that high intratumor heterogeneity of clinical sample is likely to generate false negative results [63]. The loss of spatial information represents indeed one of the major disadvantages of *in vitro* mRNA-based assays [64].

To avoid the drawbacks related to standard molecular procedures, *in situ* mRNA detection methods have been proposed (Fig. 3). Wang et al. developed a novel *in situ* RNA analysis platform for FFPE tissues, the so called "RNAscope" [65], which has been reported superior to qPCR in cases with intratumor heterogeneity or equivocal/double-equivocal results by IHC and FISH [65]. The RNAscope *HER2* assay was performed in seven cases with heterogeneous IHC staining patterns but unequivocal FISH results (five amplified and two unamplified) and RNAscope classified them with 100 % accuracy when compared with FISH. In contrast, qPCR showed only 42.8 % agreement with FISH. In equivocal cases, RNAscope was able to classify those cases that were equivocal by FISH into *HER2*-positives and negatives, which agreed with IHC when the IHC results were unequivocal. The FISH/IHC double-equivocal cases were not different at the mRNA level from those that were FISH equivocal only and RNAscope classified 7 on 26 cases (27 %) as positive [66].

Our group has participated to the validation of a single-molecule RNA FISH protocol for the detection of RNA in FFPE tissue sections (FFPE-smFISH), which was applied to quantify the expression and the intratumor spatial heterogeneity of *HER2* in a series of breast carcinomas with different HER2 status [67]. In our series we were able to observe that one IHC 2+/DNA FISH-negative case had *HER2*

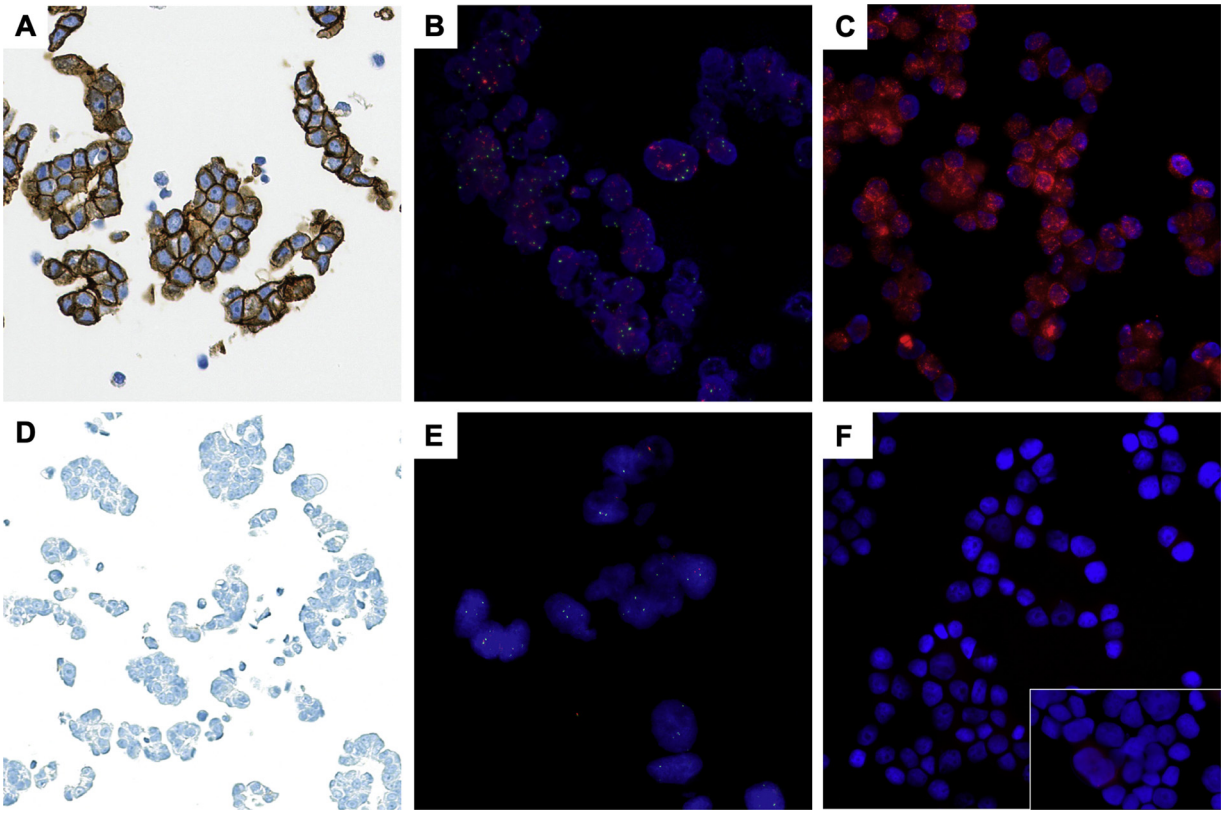

Fig. 3. Immunohistochemistry, DNA-FISH and RNA-FISH for HER2. Assessment of HER2 status in two human breast cancer cell lines. BT-474 cells are HER2-positive, showing a score 3+ protein expression (A), *HER2* amplification detected by DNA FISH (B; red: HER2, green: CEP17) and high levels of HER2 mRNA (C) assessed by HuluFISH probes (PixelBiotech GmbH, provided from MetaSystems s.r.l.). MCF7 cells are HER2 negative at protein level (D) and lack *HER2* amplification (E; red: HER2, green: CEP17): when assessed by mRNA FISH only rare red signals can be appreciated (F).

expression levels similar to tumors in the IHC 2+/DNA FISH-positive and IHC 3+ groups. Conversely, several tumors from patients in the IHC 2+/DNA FISH-positive group, who received targeted therapy in agreement with 2013 ASCO/CAP guidelines, showed *HER2* expression levels close to those in the IHC 2+/DNA FISH-negative group [67]. These data raise the question as whether this assessment may be of support in identifying patients more likely or less likely to respond to treatment, especially in those rare cases currently categorized as HER2-negative, in which *HER2* mRNA levels are similar to those of score 2+/*HER2*-amplified and score 3+ cases.

By performing quantitative measurements at multiple, distinct spatial areas in the tumor, we were able to measure various aspects of intratumor transcriptional heterogeneity: we observed that tumors belonging to the same molecular subtype had different transcriptional intratumor heterogeneity profiles, suggesting that not only the type and average expression level, but also the spatial distribution of cells expressing a given biomarker might influence how a tumor evolves and responds to therapy [67].

Schilz and co-workers tried to link transcript, protein, and signaling networks in tissues with spatial resolution: they proposed an application of imaging mass cytometry to enable multiplexed detection of mRNA and proteins in tissues [68]. A good correlation was found for *HER2* mRNA and HER2 protein in a series of 70 breast carcinomas with different HER2 status. A significant upregulation of the HER2 mRNA was identified in 21 of the 26 samples with *HER2* amplification and in 16 of these cases *HER2* mRNA levels were more than 10-fold higher than levels in control tissues. In 19 of the 26 samples with *HER2* amplification a significant expression of HER2 protein was detected compared with control tissues. Three of these 19 HER2-amplified samples showed significant overexpression of the HER2 protein but their levels of *HER2* mRNA were lower than in most other amplified samples. Due to the complexity of the technique and the use of FFPE samples some of these differences may be related to RNA degradation, nevertheless it would be of interest to ascertain whether some of these mRNA-to-protein level ratio differences across patient samples could be due to patient-specific deregulation that might be reflected in clinical features [68].

5. HER2-low breast carcinomas: a new entity in the field

Pathologists have traditionally aimed at separating HER2-positive from HER2-negative breast carcinomas. This dichotomous diagnostic work-up, strengthened by the 2018 ASCO/CAP update, has been dictated by solid clinical data demonstrating that only tumors driven by HER2 oncogene addition benefit from the addition of trastuzumab to chemotherapy [22]. Hence, medical oncologists have applied a binary treatment decision making when considering HER2, based on the assumption that only HER2-positive patients defined by HER2 overexpression and amplification should be offered anti-HER2 agents.

In recent years new therapeutic compounds have been developed, in particular antibody drug conjugates (ADCs) that are designed to target and deliver chemotherapy inside cancer cells thus reducing systemic exposure to the cytotoxic agent (Fig. 4). The first in class ADC targeting HER2 is composed of trastuzumab and the cytotoxic agent emtansine (T-DM1) at a drug antibody ratio of 3.5. T-DM1 has showed superior efficacy and a favorable risk-benefit profile as compared with capecitabine plus lapatinib or treatment of the physician's choice in two phase 3 trials involving patients with HER2-positive advanced breast cancer who had previously received HER2-targeted therapy including trastuzumab and chemotherapy [69–71]. T-DM1 is currently approved for use in patients with HER2-positive metastatic breast cancer who previously received treatment with trastuzumab and a taxane [72]. More

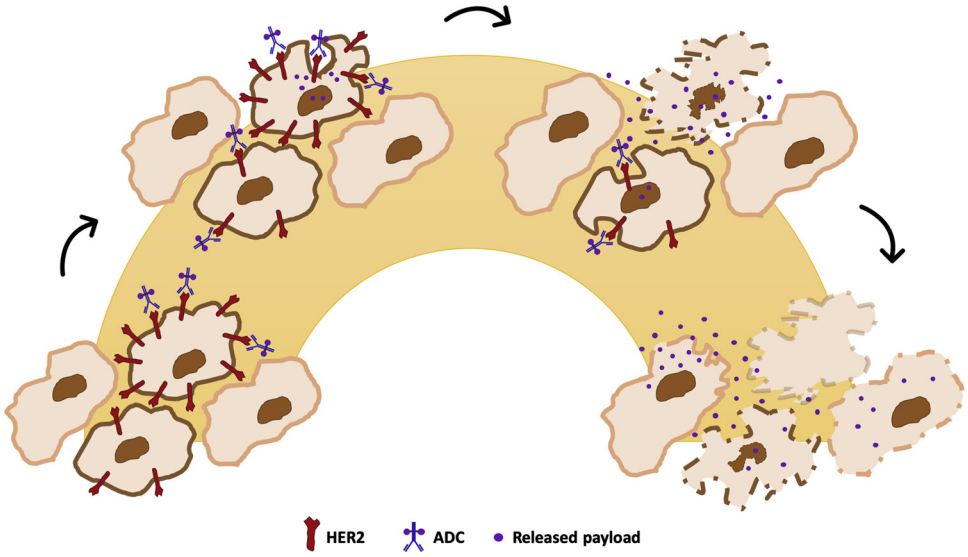

Fig. 4. Mechanism of action of antibody drug conjugates (ADCs). Antibody-drug conjugates (ADCs) are monoclonal antibodies covalently bound to cytotoxic agents and are designed to target and deliver chemotherapy inside cancer cells thus reducing systemic exposure to the cytotoxic agent. After ADC binding to cell surface antigens, the ADC-antigen complex is internalized and the released payload mediates the killing of antigen expressing cells. ADCs have been shown to be active also against surrounding cells: the payload released into the extracellular space can be taken up also by neighboring HER2-negative cells causing their death even without expressing the specific target (bystander killing or bystander effect).

recently, results from the Katherine study has shown a significant advantage for T-DM1 in patients with a residual disease following neoadjuvant trastuzumab-containing chemotherapy [73].

Other ADCs have been introduced, such as trastuzumab duocarmazine (SYD-985) and trastuzumab deruxtecan (DS-8201), which have demonstrated encouraging response rates not only in HER2-positive but also in the so called "HER2-low" breast cancer patients [74–77].

Trastuzumab duocarmazine (SYD-985) comprises the monoclonal antibody trastuzumab covalently bound to a linker drug containing duocarmycin, with a drug:antibody ratio of 2.8:1. The linker drug contains a cleavable linker and the prodrug seco-DUBA: following HER2-binding and internalization, the linker is cleaved in the lysosome by proteases that release the active toxin (DUBA), which alkylates DNA thus resulting in DNA damage in both dividing and non-dividing cells and ultimately cell death. Treatment with trastuzumab duocarmazine led to partial response in 28 % and 40 % of HER2-low ER-positive and ER-negative breast cancer patients, respectively [74].

Trastuzumab deruxtecan (DS-8201) is comprised of trastuzumab attached to a novel topoisomerase I inhibitor payload by a tetrapeptide-based linker. It shows a drug-to-antibody ratio higher than that of T-DM1 (7.8:1) and a potent bystander effect. The results of the phase 2 study in HER2-positive heavily pretreated metastatic breast cancer patients (median of 6 previous lines of therapy with T-DM1, trastuzumab, pertuzumab, and other anti-HER2 therapies) showed a response rate of 60.9 %, with 6% of patients achieving a complete response, and 54.9 % a partial response [78]. Interestingly, the phase 1 study of DS-8201 reported also data on pretreated HER2-low metastatic breast cancer patients demonstrating an overall response rate by independent central review of 37 % with a median duration of response of 10.4 months in 54 extensively pre-treated patients [75]. At present, a phase 3 study evaluating the safety and efficacy of DS-8201 in patients with HER2-low, unresectable and/or metastatic breast carcinomas previously treated with standard chemotherapy is ongoing (DESTINY-Breast04; ClinicalTrials.gov Identifier: NCT03734029).

Taken together these data may call into question the need of a paradigm shift in the definition of HER2 status in breast cancer, which in the next future could be based on a 3-tier system featuring i) HER2-positive, ii) HER2-negative, and iii) HER2-low breast carcinomas (Fig. 5). The latter category would represent an example of carcinomas potentially benefitting from a targeted therapy because of the presence of the target (which allows the drug to attach and exert the action) even in the absence of the addition of the tumor cells to the *HER2* oncogene (Fig. 5).

Two factors may be relevant in the mechanism of action of these drugs and may help define possible responsive carcinomas: on one side, the minimum quantity of HER2 receptors to be present on tumor cell to offer an anchor to the drug to work; on the other side, the degree of bystander effect the drugs can exert to spread their activity also to neighboring cells that could be even HER2-negative (Fig. 4).

In a study using HER2-targeted liposomal doxorubicin as a model-targeted nanoparticle to quantitatively investigate the effect of HER2 expression levels on delivery of doxorubicin to the nucleus, a quantitatively greater nuclear doxorubicin delivery was observed with increasing HER2 expression. A threshold effect was seen at approximately 200,000 HER2 receptors/cell [79], which may also constitutes the threshold of HER2 expression that separates toxic from therapeutic actions of HER2-targeting drug conjugates.

Studies have shown that a range of 100,0000–500,000 HER2 receptor molecules are present on the membrane of score 1+ and 2+ breast carcinoma cells, compared to more than two millions on score 3+ cells [80]. Although there are no formal definitions of HER2-low breast carcinomas, at present it seems reasonable to identify as HER2-low breast carcinomas those displaying a score 1+ or a score 2+ of HER2 expression and lacking *HER2* amplification (Fig. 5). Based on this definition, up to 55 % of all breast carcinomas would be categorized as HER2-low. Of note, for this subtype there are no targeted treatments approved, and this stresses once more the impact agents effective in HER2-low breast cancer may have. At present, ER-positive breast cancer patients are treated with a combination of endocrine therapy +/- chemotherapy whereas ER-negative (at present defined as "triple negative") patients are treated with chemotherapy.

The category of HER2-low breast cancer clearly constitutes a spectrum of carcinomas with different degrees of HER2 expression, the highest end of which is represented by score 2+ carcinomas. Independent groups have demonstrated that these carcinomas have a poorer prognosis compared to HER2-negative breast carcinomas [81–83] and this holds true when cases are categorized in the different immunophenotypical subtypes (luminal-like and triple negative) [81]. Although HER2-low carcinomas can populate both luminal and non-luminal subtypes, they are typically ER-positive [81,84–86] and preferentially pertain to the luminal B molecular subgroup by gene expression analysis [38]. They tend to be of higher histologic grade and to harbor higher proliferation rates compared to ER-positive/HER2-negative disease [81,84–86]. One relevant point to discuss in this context relates to heterogeneity, as these carcinomas are those in which the highest prevalence of HER2 heterogeneity has been reported, more frequently featuring a pattern of diffuse intermingling of HER2-positive

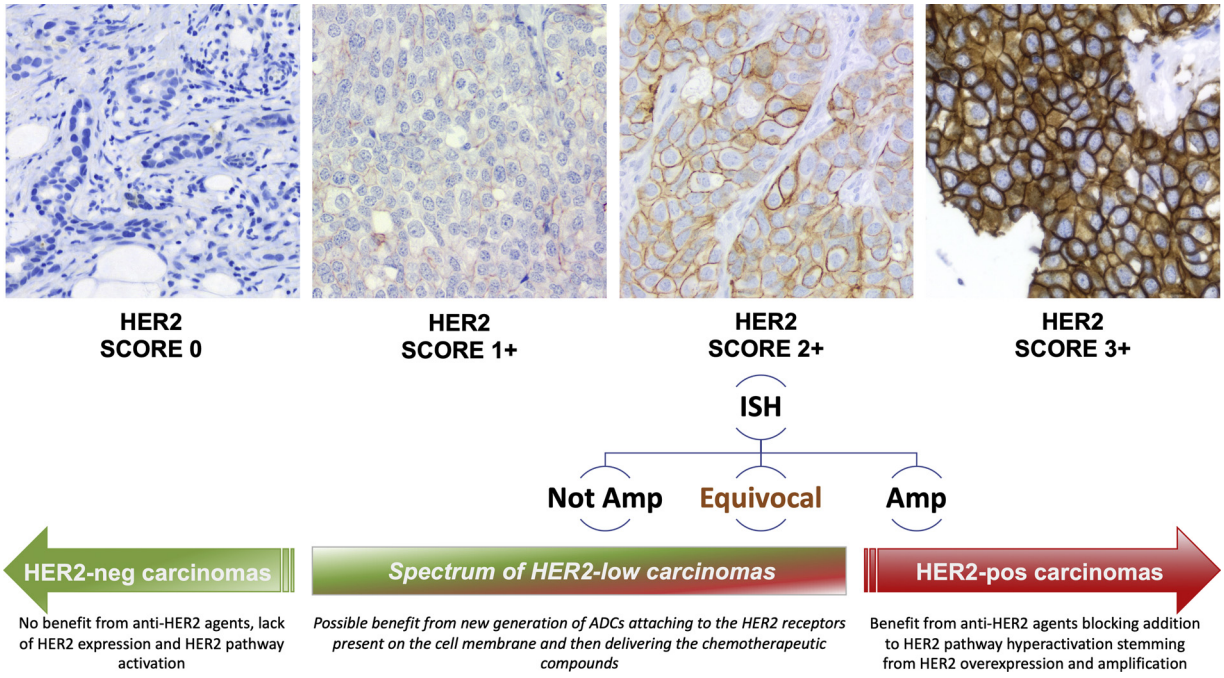

Fig. 5. A paradigm shift in the definition of HER2 status in breast cancer. The present dichotomous way of scoring HER2 (positive *versus* negative) may be revisited soon by introducing a 3-tier system, here exemplified, featuring i) HER2-positive (red arrow, including score 3+ and score 2+ with *HER2* amplification), ii) HER2-negative (score 0) and iii) HER2-low breast carcinomas (score 1+ and score 2+ without *HER2* amplification). The category of HER2-low breast cancer constitutes a spectrum of carcinomas with different degrees of HER2 expression: at present, as depicted in the Figure they are defined as carcinoma showing HER2 expression classified as score 1+ or score 2+ and lacking *HER2* amplification, however they may harbor *HER2* gain (former *HER2* equivocal category). The relevance of this classification is based on promising results on efficacy of some anti-HER2 agents also in the subpopulation of patients affected by breast carcinomas not HER2-addicted yet showing some degree of HER2 expression (HER2-low) [74,75].

and HER2-negative tumor cells [2]. We have recent data showing a higher response rate in score 2+ than score 1+ breast cancer patients [75], however whether or not the degree of response may vary according to presence and type of heterogeneity is yet to be elucidated. Nevertheless, one could speculate that mosaic-type heterogeneity may not be a relevant issue in this context thanks to the potent bystander effect that these drugs show (Fig. 4).

Data from ongoing trials may provide more information on the correlation between response to treatment and HER2 expression patterns as well as with possible other predictors of response. One may wonder that, on top of the presence of a certain degree of HER2 receptor expression on the cell membrane, which is key for the ADCs to exert its action, mRNA levels and degree of HER2 pathway activation may also impact in this context. This could be studied by assessing the correlation between HER2 protein and mRNA levels or by studying the assignment of the HER2-enriched subtype in the whole lesion. As an example, by studying HER2 double-equivocal carcinomas at the transcriptomic level, although we observed *HER2* mRNA levels more similar to those of score 0 and score 1+ carcinomas, a subset of these carcinomas could be classified as HER2-enriched [38].

Finally, HER2-low breast carcinomas may be heterogeneous in terms of tumor infiltrating lymphocytes (TILs) enrichment in the microenvironment, which could be relevant in terms of antibody dependent cellular cytotoxicity.

6. *HER2* mutations

Activation of the HER2 downstream pathway has always been thought by being exclusively driven by gene amplification, nevertheless studies based on massively parallel sequencing (next generation sequencing, NGS) have brought to the forefront that breast carcinomas can harbor *HER2* activating mutations. *HER2* somatic mutation are less prevalent than *HER2* amplification as they occur in about 2.7 % of breast cancer patients [87] and they more frequently occur in HER2-negative or HER2-low breast carcinomas [2,88]. Among breast carcinomas harboring *HER2* alterations, the co-occurrence of gene amplification and mutation has been identified in less than 1% of the specimens [89–91]. Targeted sequencing of three independent cohorts of unselected breast carcinomas reported in the TCGA dataset a *HER2* mutation rate comprised between 2.8 % and 4.6 % and surprisingly revealed also a 0.2%–1.4% prevalence of fusion between the *HER2* gene and different partners [92–95].

The large majority of *HER2* mutations described so far affect exons 19–20 coding for the tyrosine kinase domain (Fig. 6), whereas the remaining relate to the extracellular domain (exon 8 mainly, Fig. 6) [88,89]. In a large series of 5605 cases of advanced/metastatic breast cancer 90 % of *HER2* mutations occurred in the kinase domain and the remaining 10 % in the extracellular domain [89]. The most common mutations in this study were L755S, V777L and D769H/Y, which are missense mutations in the kinase domain of the protein [89]. Missense mutations (S310F and S310Y) were the most common alterations in the extracellular domain [89], affecting the furin-like cysteine-rich domain that is crucial for the receptor dimerization [96]. In this study *PIK3CA* was the most common (42 %), statistically significant, co-mutated gene in tumors harboring *HER2* mutation. They also found a concomitant *CDH1* mutation in 37 % of tumors with *HER2* mutation [89], which is not surprising given that studies correlating *HER2* somatic mutation with clinicopathological features reported an enrichment in the lobular histologic type [89,90,97] and some authors have reported an association with the solid variant of high grade invasive lobular carcinomas [98].

Wang et al. [91] studied a cohort of 1348 breast cancer patients and demonstrated that patients with *HER2* mutations had a significantly worse relapse free survival compared to those with wild-type tumors considering the entire cohort and also when analyzing patients with *HER2* non-amplified tumors only.

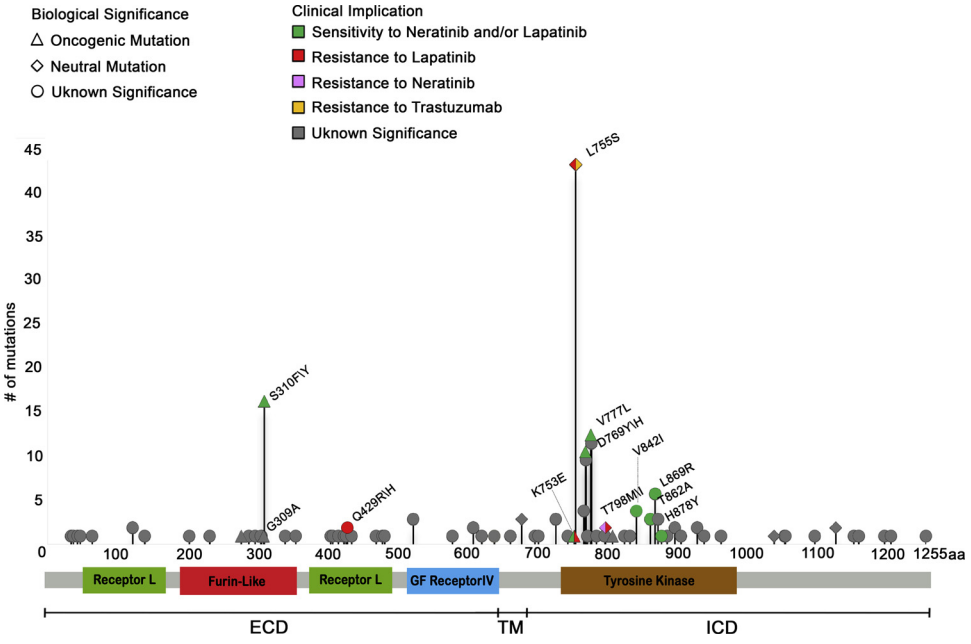

Fig. 6. Domain distribution, prevalence and significance of *HER2* somatic mutations. Lollipop plot representing the absolute frequency and the aminoacidic residues involved in the most common mutations reported for the *HER2* gene. The lollipop plot underlines the presence of rare hot-spot variants, belonging to exons 19-20 and encompassing the tyrosine kinase domain [88,89]. The biological significance for each mutation is represented by different shapes: mutation with oncogenic boost effect are pictured with triangles, whereas squares and circles represent variants of neutral or unknown significance [88,95,104]. Some of the reported mutations showed differential behaviors with respect to anti-HER2 treatments: in particular, green alterations describe mutations conferring sensitivity to the tyrosine kinase inhibitor neratinib and lapatinib [88]. The red, pink and orange filled shapes summarize the different mutations associated with resistance to lapatinib, neratinib and trastuzumab, respectively [88,102,104,105]. Most of the mutations are with unknown significance (grey). ECD: extracellular domain, TM transmembrane domain, IND: intracellular domain.

Bose and co-workers [88] used transformed cell lines to test the oncogenic capability of the most common *HER2* mutations. Seven of the 13 functionally characterized *HER2* mutations (G309A, D769H, D769Y, V777L, P780 in., V842I, and R896C) were activating mutations, as they were found to have greater tyrosine kinase activity and/or increased dimer formation capacity than the wild-type counterpart. The L755S *HER2* mutation did not promote oncogenic transformation in cell cultures but showed resistance to lapatinib. One mutation, del. 755-759 increased phosphorylation of HER2 heterodimerization partners, EGFR and HER3. When testing the sensitivity to various HER2 targeted-therapy, the irreversible HER2/EGFR tyrosine kinase inhibitor neratinib was capable of inhibiting the proliferation of cells bearing all mutations.

The relevance of detection of *HER2* mutations in breast cancer patients resides indeed in the identification of patients who can possibly be treated with specific tyrosine kinase inhibitors (TKIs), such as neratinib. Hyman and co-workers [99] have reported data from a large basket trial with neratinib observing the greatest degree of activity in patients with breast cancer with an objective response rate at week 8 of 32 %. Responses were observed in patients with missense mutations involving the extracellular and kinase domains, as well as insertions in the kinase domain [99].

HER2 mutation can also be a mechanism of resistance to anti-HER2 therapeutic compounds (Fig. 6). Xu et al. [100] showed that the gain of the *HER2* L755S mutation is an acquired mechanism of resistance to HER2 targeted-therapies and that dual HER1/2 irreversible kinase inhibitors can overcome the resistance, as observed in *in vitro* models of breast cancer cell lines harboring this mutation [100]. Moreover, the *HER2* K753E mutation gave resistance to reversible but not to irreversible HER2 tyrosine kinase inhibitors in three different breast cancer cell models [101]. Recently, Kong and co-workers demonstrated that three *HER2* mutations (Q429R, Q429H and T798M) established in MCF7 breast cancer cells strongly reduced the effect of the trastuzumab compared to wild-type MCF7 cells, through the dysregulation of the PI3K-AKT pathway [102]. Similarly, mutations in the *HER2* transmembrane domain occurring in the residue 659 and 660 have been associated with resistance to trastuzumab in other malignancies, such as lung adenocarcinomas, retaining the sensitivity to the afatinib [103]. Furthermore, Hanker and co-workers [104] in a series of metastatic breast cancer patients identified a novel *HER2* mutation (T798I) occurring in the "gatekeeper" residue within the kinase ATP-binding pocket mutation and inducing resistance to neratinib *in vivo*. In silico analysis predicted that isoleucine substitution at position 798 is able to confer resistance to neratinib by diminishing the size of its binding site. Cells harboring the *HER2* T798I mutation lacked transforming ability. This suggests that *HER2* T798I is not a driver mutation but is most probably acquired due to therapeutic pressure. The authors also demonstrated that *in vitro* afatinib was able to overcome *HER2* T798I-mediated drug resistance [104]. Finally, an unpredictable role of *HER2* mutations during the treatment of non-HER2-enriched breast cancer has been described by Nayar and co-workers [105], who analyzed a series of biopsies from metastatic sites of eight patients, who received fulvestrant or aromatase inhibitors and developed resistance. Each patient showed a *HER2* metastasis-private mutation, not detected in the primary breast cancer, suggesting a therapeutically evolutionary pressure at the base of the development of the *HER2* alteration [105]. *In vitro* analysis on breast cancer cells confirmed that mutations on both tyrosine kinase and transmembrane domain conferred resistance to both hormonal therapy and aromatase inhibition [105].

7. Conclusions

The assessment of HER2 is key to treatment decision making for breast cancer patients. At present, this is achieved by a combination of immunohistochemical and DNA *in situ* hybridization analyses. Nevertheless, it has become clear that HER2-positive carcinomas can be highly heterogeneous and this may hamper the correct identification of true responders to anti-HER2 agents. Transcriptomic analysis may be instrumental in the identification of "HER2-enriched" carcinomas that are highly sensitive to anti-HER2 agents regardless of the addition of chemotherapy, thus opening the possibility to de-escalate chemotherapy for a subgroup of HER2-positive breast cancer patients.

In addition, although rare, *HER2* mutations are emerging as important molecular alterations to be identified for instance in metastatic patients since *HER2* mutated tumors may be responsive to specific tyrosine kinase inhibitors, as showed by recent clinical studies.

Finally, the dichotomous definition of HER2-positive *versus* HER2-negative disease is currently experiencing a wave of changes by including the identification of the "HER2-low" category, for which new therapeutic compounds in the form of potent antibody drug conjugates

may be effective.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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