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Biomarkers for HER2-positive metastatic breast cancer: Beyond hormone receptors

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ABSTRACT

The overexpression of human epidermal growth factor receptor-2 (HER2) results in a biologically and clinically aggressive breast cancer (BC) subtype. Since the introduction of anti-HER2 targeted agents, survival rates of patients with HER2-positive metastatic BC have dramatically improved. Currently, although the treatment decision process in metastatic BC is primarily based on HER2 and hormone-receptor (HR) status, a rapidly growing body of data suggests that several other sources of biological heterogeneity may characterize HER2-positive metastatic BC. Moreover, pivotal clinical trials of new anti-HER2 antibody-drug conjugates showed encouraging results in HER2-low metastatic BC, thus leading to the possibility, in the near future, to expand the pool of patients suitable for HER2-targeted treatments. The present review summarizes and puts in perspective available evidence on biomarkers that hold the greatest promise to become potentially useful tools for optimizing HER2-positive metastatic BC patients' prognostic stratification and treatment in the next future. These biomarkers include HER2 levels and heterogeneity, HER3, intrinsic molecular subtypes by PAM50 analysis, DNA mutations, and immune-related factors. Molecular discordance between primary and metastatic tumors is also discussed.

Introduction

Human epidermal growth factor receptor-2 (HER2) is overexpressed in 15–20% of breast cancer (BC) cases, resulting in an aggressive clinical behaviour [1]. The introduction of trastuzumab has contributed to revert the poor prognosis of HER2-positive metastatic BC patients [2,3]. In Europe, at present, the pool of approved drugs for the treatment of HER2-positive metastatic BC includes trastuzumab, lapatinib, pertuzumab and T-DM1. Thanks to these therapeutic advances the overall survival of HER2-positive metastatic BC patients now exceeds 50 months from the diagnosis of advanced disease, with data from the real-world setting matching the results of clinical trials [4,5].

The current therapeutic algorithms can be further personalized according to hormone receptors (HR) co-expression. The only treatment specifically approved for HR−/HER2+ disease is the combination of lapatinib and trastuzumab [6]. More options are available for the subset of HR+/HER2+ patients. Combinations of endocrine therapy with single agent anti-HER2 drug represent an option for selected HR+/HER2+ patients according to the results of randomized trials showing benefit from the addition of trastuzumab or lapatinib to an aromatase inhibitor [7-9]. More recently, two randomized studies evaluated the role of dual blockade combined with endocrine therapy for HR+/HER2+. The PERTAIN trial [10] randomized 129 patients to first line trastuzumab and pertuzumab or trastuzumab, in combination with an aromatase inhibitor. Patients could receive induction chemotherapy with taxane according to physician's decision. The study demonstrated a significant progression-free survival (PFS) benefit for the dual blockade arm in the overall cohort (median 18.89 vs 15.80 months, HR 0.65 95%CI 0.48–0.89, p = 0.007) and in the cohort of patients who did not receive induction chemotherapy (median 21.72 vs 12.45 months, HR 0.55 95%CI 0.34–0.88, p = 0.0111). The ALTE-RNATIVE trial [11] randomly assigned patients in the ≥2 line setting to receive: trastuzumab + lapatinib + aromatase inhibitor or trastuzumab + aromatase inhibitor or lapatinib + aromatase inhibitor. The dual blockade arm showed a significant improvement in PFS as compared to the trastuzumab arm (median 11 vs 5.7 months, HR = 0.62, p = 0.0064).

Although for therapeutic decisions, at present, we dichotomize HER2-positive BC in HR+ and HR−, there are many other sources of biologic heterogeneity including: gene expression, DNA mutations and the immune microenvironment (Fig. 1). None of these new potential biomarkers is ready for clinical application, however research in the
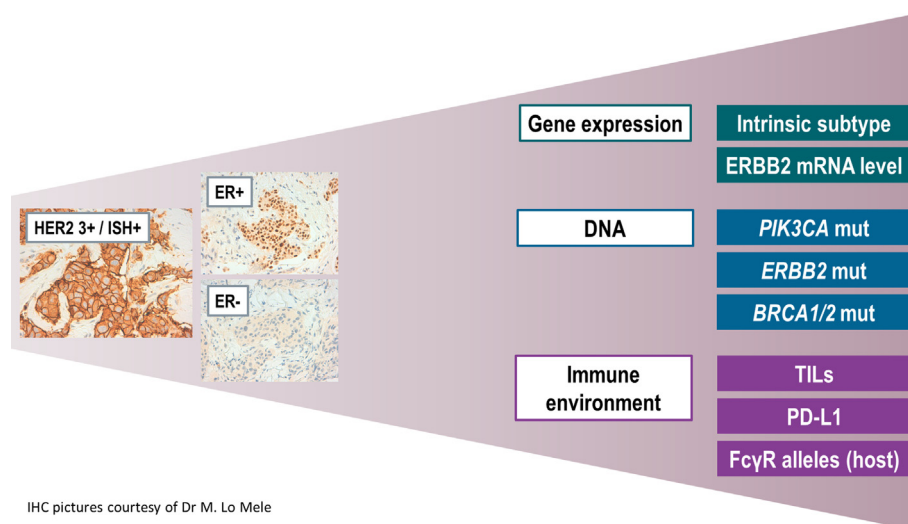

IHC pictures courtesy of Dr M. Lo Mele

Fig. 1. Sources of heterogeneity in HER2-positive breast cancer.

field is moving rapidly also fostered by the development of new anti-HER2 treatments. This review summarizes the updated evidence on biomarkers that hold the greatest promise to become potentially useful tools for optimizing HER2-positive metastatic BC patients' prognostic stratification and treatment in the next future.

Change of tumor phenotype from primary to metastasis

Since in clinical practice we base our decisions on HER2 and HR status, it is important to highlight that tumor phenotype may change from primary tumor to metastasis. A recent systematic review and metanalysis has collected the evidence from multiple studies assessing the receptor conversion during disease progression. For estrogen receptor, conversion rate was 22.5% from positive to negative and 21.5% from negative to positive. For progesterone receptor, conversion rate was 49.4% from positive to negative and 15.9% from negative to positive. HER2 loss occurred in 21.3% of cases with a HER2-positive primary tumor, HER2 acquisition was rare, occurring in 9.5% of cases with a HER2-negative primary tumor [12]. In some cases these changes may be due to technical issues, therefore it is recommended, whenever possible, to simultaneously re-assess the matched samples [13]. However, there are evidence also supporting a true change in tumor biology. Receptor loss leading to a triple-negative phenotype on metastasis has been associated with a worse survival [14]. Moreover, molecular intrinsic subtype can shift from primary tumor to metastasis. According to an analysis of 123 patients, the distribution of molecular intrinsic subtype in primary tumor vs metastasis was 39% vs 26% for Luminal A (p = 0.029), 26% vs 35.8% for Luminal B (p = 0.097), 11.4% vs 22% for HER2-enriched (p = 0.026) and 9.8% vs 12.2% for Basal-like (p = 0.540) [15]. In the same study, metastases were enriched for proliferation-related genes. Data from a prospective cohort of patients showed that clonal remodeling is associated to phenotype conversion from primary to metastasis. The cancer cell fraction of the different mutations of each sample was evaluated by deep sequencing in order to obtain a measure of tumor clonal heterogeneity. The authors reported a higher clonal heterogeneity (lower cancer cell fraction) for primary tumors as compared to metastases; moreover, significant changes in the cancer cell fraction were confined to matched samples that showed a conversion in tumor phenotype [16].

Metastasis biopsy is now endorsed by international guidelines whenever possible, especially when the disease course is unusual for the known phenotype of the primary tumor [17–19]. However, it is unknown which result should be used to drive therapeutic choices. According to the Advanced Breast Cancer 4 International Consensus

Conference, the recommendation is to consider the use of targeted therapy when receptors are positive in at least one biopsy, regardless of timing [19]. Nevertheless, the therapeutic scenario for metastatic BC patients is becoming more and more personalized and diversified according to tumor phenotype. Therefore, it should be encouraged to integrate the results of metastasis biopsy with clinical judgement, in order not to miss the opportunity of a more personalized treatment and the possibility to enroll patients in clinical trials.

HER2 and HER3

HER2 levels

One possible source of heterogeneity in HER2+ disease is the target itself. Although for therapeutic decisions HER2 status is commonly dichotomized in positive and negative according to immunohistochemistry (IHC) and fluorescent in situ hybridization (FISH), HER2 expression is a continuum. The levels of *ERBB2* mRNA progressively increase across samples classified as IHC score 0, IHC score 1+, IHC score 2+ non amplified by FISH, IHC score 2+ amplified by FISH and IHC score 3+ [20]. These considerations lead to two main questions:

1. What is the impact of HER2 levels in HER2-positive metastatic BC patients treated with standard therapy?

2. Is there room for targeting HER2-negative tumors with low HER2 expression (meaning cases with IHC score 1+ or 2+ not amplified by FISH)?

Starting from the first question, the level of HER2 expression evaluated either by IHC or mRNA has been shown to be prognostic for metastatic BC patients treated with chemotherapy and anti-HER2 monoclonal antibodies in the CLEOPATRA trial. Patients from this study showed a worse PFS in case of low HER2 levels (HR 0.77, 95% CI 0.63–0.93, p = 0.0080 for high vs low *ERBB2* mRNA; HR 0.83, 95% CI 0.69–1.00, p = 0.0502 for high vs low HER2 by IHC). In the same study, HER2 levels were not predictive for the benefit of dual blockade (trastuzumab and pertuzumab) over single blockade (trastuzumab) in combination with docetaxel [21].

Intuitively, the impact of HER2 levels in HER2-positive disease may be more relevant when dealing with chemotherapy-free regimens or in case of treatment with T-DM1 that specifically delivers the cytotoxic to those tumor cells expressing the target. Table 1 summarizes the median PFS according to level of HER2 expression for patients treated with T-DM1 or chemotherapy combined with anti-HER2 drugs in randomized clinical trials [22–24]. According to absolute median PFS, the

Table 1
PFS according to HER2 expression level in patients treated with T-DM1 or chemotherapy + anti-HER2 agent for HER2-positive metastatic BC (data from randomized phase III trials).

EMILIA [22]	HER2 level	T-DM1, mPFS (months)	CL, mPFS (months)	Δ mPFS (months)	HR (95% CI)
ERBB2 mRNA	High	10.6	6.9	+3.1	0.65 (0.50–0.85)
	Low	8.2	6.4	+1.8	0.64 (0.50–0.82)
TH3RESA [24]	**HER2 level**	**T-DM1, mPFS (months)**	**TPC, mPFS (months)**	**Δ mPFS (months)**	**HR (95% CI)**
ERBB2 mRNA	High	7.2	3.4	+3.8	0.40 (0.28–0.59)
	Low	5.5	3.9	+1.3	0.68 (0.49–0.92)
MARIANNE [23]	**HER2 level**	**T-DM1, mPFS (months)**	**T + Tax, mPFS (months)**	**Δ mPFS (months)**	**HR (95% CI)**
ERBB2 mRNA	High	18.6	15.9	+2.7	0.90 (0.65–1.25)
	Low	10.2	12.4	−2.2	1.00 (0.74–1.34)
HER2 IHC	IHC 3 +	14.6	14.4	+0.2	0.93 (0.75–1.16)
	IHC 2 +	7.3	12.6	−5.3	1.13 (0.55–2.32)

Abbreviations: mPFS, median progression-free survival; HR, hazard ratio; CI, confidence interval; CL, capecitabine and lapatinib; TPC, treatment of physician's choice; T, trastuzumab; Tax, taxane; IHC, immunohistochemistry.

performance of T-DM1 was worse in those patients with a low HER2 expression as compared to patients with a high HER2 expression. Moreover, in each trial, the difference in absolute median PFS between the T-DM1 and the chemotherapy + anti-HER2 arms was reduced in patients with low HER2 expression as compared to patients with high HER2 expression. However, the relative effect of T-DM1 was not or only marginally affected, therefore no interaction between HER2 levels and treatment can be claimed.

New anti-HER2 antibody-drug conjugates (trastuzumab deruxtecan - already approved by Food and Drug Administration for HER2-positive metastatic BC patients based on the phase II DESTINY-Breast01 trial [25] - and trastuzumab duocarmazine) have demonstrated clinical activity in patients presenting a low HER2 expression although categorized as HER2-negative as per standard definition (IHC score 1+ or 2+/ not amplified) [26]. These new antibody-drug conjugates present structural differences as compared to T-DM1 that may account for a distinct activity profile. trastuzumab deruxtecan shows a higher drug to antibody ratio (7.7) as compared to T-DM1 (3.5) that allows to effectively deliver a higher amount of drug to targeted cells without negative implications on the structure of the molecule. Both trastuzumab deruxtecan and trastuzumab duocarmazine present a cleavable linker between the cytotoxic and the antibody that confers a more pronounced ability to kill bystander cells as compared to T-DM1 (non-cleavable linker). Moreover, the payload of T-DM1, being an inhibitor of microtubules assembly, is active in specific phases of the cell cycle, whereas the payloads of trastuzumab deruxtecan (topoisomerase I inhibitor) and trastuzumab duocarmazine (duocarmycin analogue with alkylating activity) may have a broader effect on tumor cells [27].

In early phase clinical trials the objective response rate with trastuzumab deruxtecan [25] and trastuzumab duocarmazine [26] in HER2-low pretreated patients was > 30% (37% for trastuzumab deruxtecanand 32% for trastuzumab duocarmazine). With trastuzumab deruxtecan, the median PFS was 11.1 months and the median duration of response was 10.4 months, which is remarkable for a heavily pretreated population (median number of prior regimens: 7.5). The DESTINY-Breast04 phase III trial, comparing trastuzumab deruxtecan vs treatment of physician's choice in HER2-low metastatic BC patients is currently ongoing (NCT03734029). The results of this trial, if positive, will open the opportunity to expand the pool of patients that could benefit from anti-HER2 therapies. Indeed, HER2-low tumors represent a high proportion of BC cases, estimated around 45–55% [28]. In a series of 534 metastatic BC patients from our Institution, the prevalence of HER2-low cases is 32% (36% in the HR+ and 34% in the HR− subgroup; unpublished personal data). The biology, clinical landscape and therapeutic implications of HER2-low tumors have been recently reviewed in detail [28].

HER2 heterogeneity

HER2 expression and amplification may also show intratumoral heterogeneity. This heterogeneity may present in three main patterns: the "clustered" type, with two distinct areas of the same tumor showing different HER2 status; the "mosaic" type, displaying either diffuse intermingling of cells with different HER2 statuses; the "scattered type", with positive and/or amplified cells dispersed within a negative tumor area [29]. The prevalence of HER2 genetic heterogeneity has been described in the range of 1–34% [30–39]. The majority of data on the impact of intratumoral HER2 heterogeneity on response to anti-HER2 therapy comes from the neoadjuvant setting. In a retrospective series of 64 cases of HER2-positive BC patients treated with neoadjuvant chemotherapy and anti-HER2 monoclonal antibodies (either trastuzumab or trastuzumab and pertuzumab), significantly more cases with HER2 intratumoral heterogeneity were found in patients not achieving a pathological complete response (pCR) as compared to the pCR group (56% vs 13%, p < 0.001). The presence of HER2 intratumoral heterogeneity was independently associated with non-pCR (OR 0.21, p = 0.021) [38]. A recent study has prospectively evaluated the impact of HER2 heterogeneity on the efficacy of neoadjuvant treatment with T-DM1 and pertuzumab for 6 cycles. Among 157 patients with centrally confirmed HER2-positive BC, 10% showed HER2 heterogeneity (most were HR+) defined as either FISH amplified in > 5% and < 50% of tumor cells or an area of tumor that tested HER2-negative. None of the patients in the heterogeneous group achieved a pCR as compared to 55% of patients with non heterogeneous tumors (p < 0.001) [40]. Interestingly, there was a partial overlap between heterogeneous cases and tumors with HER2 IHC score 2+: of the 16 heterogeneous tumors, 12 (75%) had a IHC score 2+ and 4 an IHC score 3+. Patients with a HER2 2+ IHC score achieved significantly lower pCR rates vs patients with HER2 3+ tumors (27% vs 56%, p = 0.002), however the statistical significance was lost when heterogeneous tumors were excluded (pCR 40% vs 58%, p = 0.10), suggesting that HER2 heterogeneity is a major driver of reduced sensitivity to T-DM1 and pertuzumab.

HER2 heterogeneity was also evaluated in the KRISTINE trial. This study randomized patients to receive neoadjuvant T-DM1 and pertuzumab vs trastuzumab, pertuzumab, docetaxel and carboplatin. In the T-DM1 and pertuzumab arm, patients who experienced a locoregional progression before surgery were enriched for cases with heterogeneous HER2 IHC expression (80%), whereas 85% of patients without locoregional progression had a homogeneous HER2 IHC expression [41].

In the metastatic setting, a post-hoc analysis of the MARIANNE trial evaluated the impact of HER2 heterogeneity. The performance of the TDM1 containing arms was poorer in those patients with a heterogeneous HER2 expression (< 80% of IHC 2+/3+ tumor cells) as compared to patients with a homogeneous HER2 expression (≥80% of IHC 2+/3+ tumor cells). Patients with HER2 heterogeneity achieved a median PFS < 10 months with T-DM1 and T-DM1 and pertuzumab

whereas patients with homogeneous HER2 IHC expression achieved median PFS of 14.7 months with T-DM1 and 17.8 months with T-DM1 and pertuzumab. The outcome of patients in the taxane + trastuzumab arm was less affected by HER2 heterogeneity [23].

These data do not support at the moment the choice to discard T-DM1 treatment for metastatic BC patients in case of HER2 intratumoral heterogeneity. Although neoadjuvant data strongly suggest that T-DM1 may be less effective in these patients, such data cannot be directly transferred to the metastatic setting. Indeed, it is unknown how HER2 intratumor heterogeneity may evolve during progression and which is the role of intermetastases heterogeneity. The only data in the metastatic setting are based on few events from a post-hoc analysis of a randomized trial conducted in a setting that is not the current indication for T-DM1. Nevertheless, it is likely that reporting the proportion of positive cells within a HER2-positive tumor will be required in the next future if HER2 heterogeneity will be confirmed as a negative predictive factor for T-DM1. Functional imaging may also play a role in this context: according to the ZEPHIR study, the presence of intrapatient heterogeneity by HER2-PET/CT (positron emission tomography/computed tomography scan) combined with a poor early metabolic response was able to discriminate metastatic HER2-positive patients not responding to T-DM1 [42]. Moreover, the role of new anti-HER2 antibody-drug conjugates in HER2 heterogeneous tumors needs to be clarified in order to define the best treatment option for these patients.

HER3

HER3, another member of the EGFR receptor family, has been shown to play a crucial role in driving oncogenic cellular proliferation in several human tumors [43]. The HER2–HER3 dimer is crucial for HER2-mediated signalling in tumours containing amplifications of HER2 and, in fact, HER2–HER3 is considered the most active signalling dimer [43]. In preclinical studies, HER3 has been reported to play a pathophysiological role in resistance to anti-HER therapies [44–46]. However, the evaluation of HER3 mRNA levels in patients enrolled in recent randomized clinical trials led to inconsistent results in terms of association with prognosis. The expression of HER3 was not prognostic according to translational analyses of the EMILIA, TH3RESA and MARIANNE trials [22–24]. In the CLEOPATRA trial, high HER3 expression was independently associated with improved prognosis [21]. In the same trial HER3 did not predict for the benefit of trastuzumab + pertuzumab vs trastuzumab combined with docetaxel [21].

It is possible that the evaluation of HER3 expression may not fully recapitulate the biology and state of activation of the receptor. Indeed, a secreted isoform of HER3 able to capture circulated neuregulins, thus preventing their binding with transmembrane receptors, has been described [47]. Moreover, recent evidence suggests that the biological activity of HER3 depends on its subcellular distribution. The re-localization from the intracellular compartment to the plasma membrane may depend on the phosphorylation level and the presence of ligands [48]. Interesting anti-HER3 drugs are under development. U3-1402 is a novel HER3-targeted antibody-drug conjugate carrying a topoisomerase I inhibitor payload, with a high drug-to-antibody ratio (~8:1). An ongoing phase 1/2, multicenter, open-label, first-in-human study is evaluating the safety and efficacy of U3-1402 in HER2-negative, HER3-expressing advanced breast cancer (NCT02980341/JapicCTI-163401). In the dose-escalation and dose-findings parts of the trial, among 41 evaluable patients, the objective response rate was 46.3% and the disease control rate was 90.2% [49]. The maximum tolerated dose was not reached and dose limiting toxicities included thrombocytopenia and transaminase increase [49]. The phase 2, dose expansion part of this trial is currently ongoing [50]. MCLA-128 is a bi-specific antibody directed against HER2 and HER3 that is undergoing evaluation in a phase II study (NCT03321981) after encouraging data from a phase I/II trial [51].

Gene expression

We now know that all the four main intrinsic subtypes (PAM50) are represented within HER2-positive disease, with a distribution that varies according to HR co-expression. Among HR−/HER2+ BC, around 75% pf cases are HER2-enriched, 15% basal-like and 10% Luminal A or B; in HR+/HER2+ positive BC around 35% of the cases are Luminal A, 31% Luminal B, 30% HER-2 enriched, and 3% Basal-like [52,53].

A number of studies have established the role of intrinsic molecular subtype as a biomarker in the neoadjuvant setting for HER2-positive BC [54–57]. There is also evidence that PAM50 subtypes may have implication on prognosis and treatment for metastatic HER2-positive BC patients.

The EGF3008 randomized trial compared letrozole-placebo vs letrozole-lapatinib for HR-positive metastatic BC patients. In HR+/HER2+ patients (n = 219), addition of lapatinib to letrozole significantly reduced the risk of disease progression versus letrozole-placebo (median PFS 8.2 vs 3.0 months, HR 0.71; 95%CI, 0.53–0.96; p = 0.019) [9]. PAM50 subtype was evaluated for 157 HR+/HER2+ patients, showing that those patients with a Luminal A tumor experienced the longest PFS among other subtypes (both arms combined). Median PFS was: 11.07 months for Luminal A, 5.55 months for Luminal B, 4.37 months for HER2-enriched and 3.58 months for basal-like (p < 0.001). Similar results were obtained for overall survival. No interaction between treatment and molecular subtype was observed [58].

A major interest is the evaluation of treatment regimens including CDK4/6 inhibitors for HR+/HER2+ patients. In the phase II randomized MonarcHER trial 237 patients with metastatic HR+/HER2+ patient metastatic BC were randomized to abemaciclib + trastuzumab + fulvestrant vs abemaciclib + trastuzumab vs trastuzumab + chemotherapy of physician's choice. Patients receiving abemaciclib + fulvestrant + trastuzumab showed a PFS improvement over patients treated with trastuzumab + chemotherapy (median PFS 8.3 vs 5.7 months, HR 0.67, p = 0.0506) [59].

The phase II PATRICIA trial enrolled 45 postmenopausal pre-treated HER2-positive metastatic patients in 3 cohorts: HR− receiving trastuzumab + palbociclib; HR+ receiving trastuzumab + palbociclib; HR+ receiving trastuzumab + palbociclib + letrozole. The analysis of PFS according to PAM50 subtype (n = 40, all cohorts combined) showed that patients with a Luminal profile had a better outcome as compared to non-Luminal patients (median 12.4 vs 4.1 months, HR 0.37, 95%CI 0.14–1.00, p = 0.052) [60]. These results are mainly driven by HR+/HER2+ patients since no Luminal case was detected within the HR− group. Based on these results, the PATRICIA II study was initiated, enrolling patients with HR+/HER2+ and a Luminal profile by PAM50. Patients are allocated 1:1 to palbociclib + trastuzumab + endocrine therapy vs treatment of physician's choice (NCT02448420).

We know from the neoadjuvant setting that HER2-enriched tumors are the most sensitive to anti-HER2-based treatments [61]. Moreover, a high expression of *ERBB2* is able to discriminate among HER2-eriched tumors those that are the most HER2-addicted. Indeed, HER2-enriched/*ERBB2* high patients can achieve rates of pCR of 45% with dual HER2 blockade without chemotherapy [57]. This combined biomarker has been also evaluated in the metastatic setting in the context of the phase III EGF104900 trial comparing lapatinib vs lapatinib + trastuzumab for HER2-positive patients. The final overall survival analysis showed an improvement in PFS for the combination of lapatinib and trastuzumab in the intention-to-treat population (n = 291; HR 0.74, 95%CI 0.57–0.97, p = 0.026). According to HR status, the significant improvement in overall survival was limited to HR− patients [6]. Analysis according to PAM50 subtype showed that those patients with HER2-enriched subtype and *ERBB2* high who were randomized to lapatinib + trastuzumab achieved the best overall survival (p = 0.007). The benefit of lapatinib + trastuzumab was maintained in both HER2-

enriched/*ERRB*-high and other patients [57]. These results suggest that this combined biomarker could indicate a population of patients that may be suitable from effective chemotherapy-free anti-HER2 regimens, a hypothesis that needs further validation.

DNA mutations

PIK3CA mutations

PIK3CA gene mutations are frequent in HER2+ breast cancer, occurring in 20% to 30% of patients [55,56,62–4], with a similar rate according to HR status [55,56,62,63]. In HER2+ disease, exon 20 mutations occur more frequently than exon 9 [63,64]. According to PAM50 subtypes, *PIK3CA* mutations are mainly present in Luminal and HER2-enriched subtypes [62].

PIK3CA mutations have been proposed as a potential mechanism of resistance to anti-HER2 therapies [65]. Indeed, in the neoadjuvant setting, the presence of a *PIK3CA* mutation is associated with a lower rate of pCR after chemotherapy and anti-HER2 treatment [63].

In the metastatic setting, data from phase III studies show that *PIK3CA* mutations are associated with a worse prognosis as compared to *PIK3CA* wild-type status in patients treated with standard treatment regimens [21–24] (Table 2). However, in the same trials, the presence of a *PIK3CA* mutation was not predictive for treatment benefit. There was a trend from the EMILIA study for a larger magnitude of benefit from T-DM1 over capecitabine and lapatinib in *PIK3CA* mutated patients; the interaction test was p = 0.22 for PFS and p = 0.05 for overall survival [22].

The main interest is to evaluate *PIK3CA* mutation in relation with treatments targeting the Pi3k/Akt/mTOR pathway. The addition of everolimus to chemotherapy and trastuzumab has been evaluated in two phase III trials. In BOLERO-1, patients were randomized to receive trastuzumab + paclitaxel vs trastuzumab + paclitaxel + everolimus as first-line treatment [66]; in BOLERO-3 patients progressing on prior trastuzumab and taxane were randomized to receive trastuzumab + vinorelbine vs trastuzumab + vinorelbine + everolimus [67]. The results of BOLERO-1 showed no benefit for the everolimus-containing arm (median PFS 15.0 vs 14.5 months in the standard arm) [66]. In BOLERO-3 a modest absolute improvement in PFS that resulted statistically significant was observed for the everolimus-containing arm

(median PFS 7.0 vs 5.8 months, p = 0.0067) [67]. The safety profile and the availability of more effective options for patients resistant to trastuzumab and taxane precluded the implementation of this regimen in clinical practice. In terms of biomarkers, a pooled analysis of these two trials demonstrated that the benefit of adding everolimus was confined to patients with a *PIK3CA* mutation, whereas no effect was seen in *PIK3CA* wild type patients [68]. In *PIK3CA* mutated patients, median PFS with everolimus was 12.0 months vs 7.6 months in the control group for BOLERO-1 and 6.9 vs 5.7 months in BOLERO-3 (pooled analysis HR 0.67, 95%CI 0.45–1.00, p = 0.05). Similar results were obtained for PTEN loss and Pi3k pathway activation [68].

These data, together with the efficacy of alpelisib in *PIK3CA* mutated HR+/HER2- metastatic BC demonstrated by the SOLAR-1 trial (randomized phase III trial of fulvestrant + alpelisib vs fulvestrant + placebo in HR−positive/HER2-positive advanced BC patients previously treated with endocrine therapy [69]), constitute the rationale to evaluate Pi3k inhibitors in *PIK3CA* mutated HER2-positive metastatic BC. A summary of the results of early phase clinical trials of Pi3k inhibitors conducted mostly in PIK3CA-unselected HER2-positive patients is provided in Table 3.

ERBB2 mutations

Mutations in the *ERBB2* gene have been described in 2–3% of BC, mostly occurring in HER2-negative tumors [70]. These mutations generally affect the kinase domain and harbor an oncogenic potential [70].

Patients with pretreated, *ERBB2* mutant, non-amplified BC were enrolled in the phase II SUMMIT trial and received neratinib monotherapy (if HR−) or neratinib and fulvestrant (if HR+) [71,72]. Results showed an objective response rate of around 30% in both groups [71,72]. Thanks to these results, *ERBB2* hot-spot activating missense mutations and in-frame insertions (exon 20) are classified as Tier IIB by the European Society of Medical Oncology Scale for Clinical Actionability of molecular Targets (ESCAT) [73]. Studies with other tyrosine kinase inhibitors are currently ongoing (NCT02544997, NCT03412383).

BRCA1/2 germline mutations

It is recognized that the majority of BC diagnosed in *BRCA1* mutation carriers are triple negative, whereas *BRCA2* mutations are frequently associated with HR+/HER2- BC [74,75]. However, a small proportion of *BRCA*-associated BC are HER2-positive: 10% for *BRCA1*-associated BC and 13% for *BRCA2* associated BC [74]. In the absence of family history, genetic testing in young patients (< 41 years) diagnosed with HER2-positive BC patients found a 4% rate of *BRCA1/2* mutations [76].

Unfortunately, HER2-positive patients with a germline *BRCA* mutation have been excluded from large phase III trials testing PARP inhibitors [77–79]. In the phase II BROCADE trial 15 HER2-positive patients were enrolled but no data was specifically reported for this group. In the phase II ABRAZO study of talazoparib in non-platinum resistant advanced BC patients with germline *BRCA1/2* mutation, 6 patients with HER2-positive disease considered refractory to previous HER2-targeted therapies were enrolled. All HER2-positive patients were also HR+. In these patients, an objective response was confirmed in 2 of 6 patients by the independent radiology facility [80].

Although the placement of PARP inhibitors in the treatment algorithm of HER2-positive BC patients would be complicated, this reason should not preclude their evaluation in HER2-positive, *BRCA*-associated BC.

Table 2
Impact of PIK3CA mutations on PFS in randomized phase III trials of current standard therapies for HER2-positive metastatic BC patients.

Trial	PIK3CA mut	PIK3CA wt
CLEOPATRA [21]		
T + P + D, mPFS	8.6	13.8
T + D, mPFS	12.5	21.6
HR (95%CI) for T + P + D vs T + D	0.64 (0.43–0.93)	0.67 (0.50–0.89)
EMILIA [22]		
T-DM1, mPFS	10.9	9.8
C + L, mPFS	4.3	6.4
HR (95%CI) for T-DM1 vs C + L	0.45 (0.25–0.82)	0.74 (0.40–1.10)
TH3RESA [24]		
T-DM1, mPFS	6.2	6.8
TPC, mPFS	3.1	3.4
HR (95%CI) for T-DM1 vs TPC	0.44 (0.26–0.73)	0.47 (0.33–0.67)
MARIANNE [23]		
T-DM1 + P, mPFS	11.0	18.8
T-DM1, mPFS	8.3	16.6
T + Tax, mPFS	12.4	14.6
HR (95%CI) for T-DM1 vs T + Tax	1.12 (0.75–1.66)	0.90 (0.69–1.17)
HR (95%CI) for T-DM1 + P vs T + Tax	0.88 (0.58–1.32)	0.85 (0.62–1.11)
HR (95%CI) for T-DM1 + P vs T-DM1	0.80 (0.54–1.17)	0.94 (0.72–1.24)

Abbreviations: T, trastuzumab; P, pertuzumab; D, docetaxel; C, capecitabine, L, lapatinib; TPC; treatment of physician's choice; Tax, taxane; mPFS, median progression-free survival; HR, hazard ratio; CI, confidence interval; mut, mutated; wt, wild type.

Table 3
Summary of results of early phase clinical trials of Pi3k inhibitors combined with anti-HER2 therapy for HER2-positive advanced breast cancer patients.

Trial	Pi3k inhibitor	Anti-HER2 backbone	Phase	Clinical activity	Most common G ≥ 3 AEs
Pistilli B, Breast Cancer Research and Treatment 2018[99]	Buparlisib	Trastuzumab	II	ORR 10%	increased ALT 16%, increased AST 12%, rash 10%
Guerin M, European Journal of Cancer 2017[100]	Buparlisib	Lapatinib	1b	ORR 4% CBR 29%	diarrhea 21%, skin toxicity 17%, liver toxicity 17%, amilase/lipase increase 12%
Tolaney, Breast Cancer Research and Treatment 2015[101]	Pilaralisib	Trastuzumab (arm 1) or Trastuzumab + Paclitaxel (arm 2)	I/II	ORR 0% (arm1) and 20% (arm 2)	Arm 1: increase in ALP 9.5%, rash 9.5% Arm 2: diarrhea 14.3%, peripheral neuropathy 14.3%, neutropenia 14.3%, anemia 9.5%, rash 9.5%
Jain S, Breast Cancer Research and Treatment 2018[102]	Alpelisib	T-DM1	I	ORR 43%, median PFS 8.1 months	maculopapular rash 41%, hyperglycemia 24%, thrombocytopenia 18%, anorexia 12%, hypertension 12%
Shah P, ASCO 2015*[103]	Alpelisib	Trastuzumab + LJM716	I	SD 83% (5/6 evaluable patients)	diarrhea (5/8 pts), hyperglycemia (2/8 pts), hypokalemia (2/8 pts), transaminitis (2/8 pts)
Metzger Filho O, ASCO 2017[104]	Taselisib	T-DM1	1b	ORR 33%, median PFS 7.6 months	thrombocytopenia 19.2%, diarrhea 15.4%

Abbreviations: G, grade; AEs, adverse events; ORR, objective response rate; CBR, clinical benefit rate; PFS, progression-free survival; SD, stable disease.
* PIK3CA mutated population.

Immune biomarkers

Tumor immune microenvironment

Another important source of heterogeneity in HER2-positive disease is the tumor immune microenvironment. Triple negative and HER2-positive BC present the highest levels of tumor infiltrating lymphocytes (TILs) [81,82]. In triple negative early BC, stromal TILs (located in the tumor stroma and described as the percentage of tumor stroma area occupied by mononuclear inflammatory cells) have reached level of evidence 1b as prognostic marker [83,84] and their evaluation is now endorsed by the 2019 St Gallen recommendations [85].

Within HER2-positive BC, the level of TILs varies according to the molecular intrinsic subtype, being higher in basal-like and HER2-enriched tumors [86].

In early HER2-positive BC, higher TILs have been associated with both increased likelihood of pCR after neoadjuvant therapy [84] and with improved prognosis [87]. TILs have also been shown a significant positive correlation with PD-L1 expression in both early and metastatic HER2-positive BC samples [86,88].

TILs have been evaluated on samples collected from 678 patients enrolled in the CLEOPATRA trial. In multivariate analysis, higher stromal TILs were associated with longer overall survival (HR 0.89, 95%CI 0.83–0.96, p = 0.0014). Patients with > 20% TILs had a median overall survival of 56.6 months vs 44.5 months for patients with lower TILs (log-rank p = 0.021). No significant association with PFS was observed. Benefit of trastuzumab + pertuzumab + docetaxel over trastuzumab + docetaxel was similar in high- and low-TILs patients [89].

The knowledge on the role of the immune microenvironment in HER2-positive BC, coupled with the immune-mediated mechanism of action of anti-HER2 monoclonal antibodies, has prompted the evaluation of the efficacy of immune checkpoint inhibitors for these patients. The PANACEA phase II study enrolled 48 HER2-positive metastatic BC patients with documented progression on trastuzumab or T-DM1. Patients received pembrolizumab combined with trastuzumab. PD-L1 was evaluated by the 22C3 assay (cut-off for positivity: combined positive score ≥1). Objective responses were reported only in the group of patients with a PD-L1+ tumor (15%) [90]. Although there was no difference in PFS according to PD-L1 status, PD-L1+ patients showed a longer overall survival as compared to PD-L1- patients (median not reached vs 7.0 months). The evaluation of TILs revealed a positive association between PD-L1 status and TILs level, with PD-L1+ cases showing significantly higher TILs vs PD-L1- patients (p = 0.0004). Higher TILs were also found in patients achieving an objective response (p = 0.006) or disease control (p = 0.0006) [90]. Moreover, when PD-L1 and TILs were combined, results showed that, within the PD-L1+ cohort, patients who achieved a response were enriched for cases with TILs ≥ 5% [91].

Another phase II study (KATE2) randomized 202 patients resistant to prior taxane and trastuzumab to receive T-DM1 + placebo or T-DM1 + atezolizumab. In this study, 42% of pts resulted PD-L1 positive by the SP142 assay, according to the cut-off of at least 1% of positively stained immune cells. In the intention-to-treat population there was no substantial difference in PFS between the two arms (HR 0.82, 95%CI 0.55–1.23). When the analysis was limited to the PD-L1+ population, there was a signal for a numerical benefit from T-DM1 + atezolizumab over T-DM1 + placebo both in PFS (median PFS 8.5 vs 4.1 months, HR 0.60, 95%CI 0.32–1.11) and in overall survival (1-year rate 94.3% vs 87.9%, HR 0.55, 95%CI 0.22–1.38) [92]. TILs were also evaluated and the results confirmed higher TILs in PD-L1+ patients. In subgroup analysis, patients with high TILs derived a non significant PFS benefit from T-DM1 + atezolizumab (HR 0.55, 95%CI 0.26–1.12) [93].

Overall, data from PANACEA and KATE2 show that immunotherapy warrants further evaluation in advanced HER2-positive BC especially in patients with PD-L1+ and/or high TILs. The results also claim for a

combined evaluation of both biomarkers in prospective clinical trials.

An issue that should be taken into account is that the tumor immune microenvironment may change from primary to metastasis. An analysis of 20 patients from the CLEOPATRA study with matched primary and metastatic samples showed lower level of TILs in the metastases [89]. In another series of samples from HER2-positive metastases, CD8+ cells were significantly lower in case of prior treatment for metastatic disease as compared to samples collected at the time of first diagnosis of advanced BC (p = 0.011) [88]. Moreover, TILs and PD-L1 levels in metastatic samples show variability according to anatomical site, with higher TILs in lung samples and lower TILs in liver and skin samples [89,90,95]. Although the most recent sample may be more representative of the actual immune microenvironment, data from the IMPASSION130 trial for triple negative breast cancer have demonstrated that PD-L1 expression ($> 1\%$ of total tumor area occupied by positive immune cells) on either primary breast or metastatic samples was predictive of benefit from atezolizumab + nab-paclitaxel [94]. All these considerations should encourage a broad collection of tumor samples from both primary tumor and metastasis in trials of immunotherapy for HER2-positive BC.

Host immune factors

Finally, heterogeneity in host immune factors may affect the efficacy of anti-HER2 therapies. All currently approved monoclonal anti-HER2 antibodies are of the IgG isotype, comprising a crystalline fragment (Fc) linked to the antigen-binding fragments. The Fc domain interacts with Fc gamma receptors (FcγRs) expressed on a variety of immune cells. FcγRIIa and FcγRIIIa are activating FcγRs that mediate antibody-dependent cell-mediated cytotoxicity (ADCC) [95]. Some single nucleotide polymorphisms in the extracellular component of FcγRIIa and FcγRIIIa have been associated with differential antibody-binding affinity and ADCC [95]. In particular, the *FcγRIIIa*- 158 V allele showed high antibody-binding affinity and higher trastuzumab-mediated ADCC as compared to other genotypes. An analysis conducted in the adjuvant NSABP B-31 trial showed that patients with the low affinity 158 F/F genotype received less benefit from trastuzumab in comparison with patients with 158 V/F or 158 V/V [96].

Margetuximab is a Fc-engineered anti-HER2 antibody with a higher affinity for FcγRIIIa; in particular, the binding to the low-affinity *FcγRIIIa*- 158 F is increased [97]. In the phase III SOPHIA trial, margetuximab + chemotherapy was compared to trastuzumab + chemotherapy in pre-treated metastatic HER2-positive patients. Margetuximab was associated with better PFS (HR 0.76, 95%CI 0.59–0.98, p = 0.033) especially in those patients carrying the *FcγRIIIa*-158 F allele (HR 0.68, 95%CI 0.52–0.90, p = 0.005) [98].

Conclusions

In conclusions, treatment decisions for metastatic BC patients remain based on HER2 and HR status. However, even though not ready for clinical implementation at this time, novel potentially useful biomarkers for anti-HER2 therapies are emerging. Moreover, in terms of clinical feasibility, most of the discussed biomarkers require testing methods that are already part of the current routine practice (such as HER2-low or HER2 heterogeneity, *PIK3CA* mutation currently used to select HR-positive/HER2-negative patients for alpelisib, PD-L1 and TILs for triple negative BC, *BRCA1/2* mutations) or have the potential to be implemented in clinical practice (such as *ERBB2* mutation and PAM50 subtypes).

The efficacy of new antibody-drug conjugates in HER2-negative patients with low HER2 expression and the encouraging results with neratinib in patients with *ERBB2*-mutation may contribute to expand, in the next future, the pool of patients suitable for anti-HER2 therapies. PAM50 molecular subtypes refine the HER2 classification and should be used to stratify patients in clinical trials. In particular, HER2+/HR+

patients are the ideal population for the development of more effective endocrine-based combinations and HER2-enriched/*ERRB2*-high patients are the ideal setting to focus on chemotherapy-free regimens. *PIK3CA* mutations are frequently detected in HER2-positive disease and data from trial exploring Pi3k inhibitors in this subset of patients are highly awaited. Finally, immunotherapy is promising for advanced HER2-positive BC patients and will probably be of more value in earlier lines of treatment, since the tumor immune microenvironment of heavily pretreated patients may be less favorable. PD-L1+ and high-TILs patients seem to derive the greatest benefit and the combined evaluation of both biomarkers in clinical trials is recommended.

Author contribution

Concept and design (MVD, VG); acquisition of data (FM, GG); article draft and revision (all authors); final approval (all authors).

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