



# Dissecting the biological heterogeneity of HER2-positive breast cancer

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## ARTICLE INFO

### Article history:

Received 31 March 2021

Received in revised form

19 July 2021

Accepted 29 July 2021

Available online 5 August 2021

### Keywords:

HER2

Breast cancer

Intrinsic subtypes

HER2-enriched

PAM50

TILs

## ABSTRACT

HER2-positive (HER2+) breast cancer (BC) is a heterogenous and multifaceted disease, with interesting therapeutic implications. First, all intrinsic molecular subtypes can be identified in HER2+ tumors, with the HER2-enriched being the most frequent. Such subtypes do not differ much from their counterparts in HER2-negative disease, apart for the high expression of genes in/near the HER2 amplicon on chromosome 17. Intrinsic subtyping, along with the quantification of *ERBB2* mRNA levels, is associated with higher rates of pathologic complete response across neoadjuvant trials of dual HER2 blockade and might help select patients for de-escalation and escalation treatment strategies. Secondly, HER2+ tumors have a broad range of DNA alterations. *ERBB2* mutations and alterations in the PI3K/Akt/mTOR pathway are among the most frequent and might predict benefit from potent pan-HER, PI3K and mTOR inhibitors. Moreover, HER2+ tumors are usually infiltrated by lymphocytes. These tumor infiltrating-lymphocytes (TILs) predict response to neoadjuvant anti-HER2-based treatment and exert a prognostic role. PD-L1, detected in ~42 % of HER2+ BC, might also be useful to define patients responding to novel anti-PD1/PD-L1 immunotherapies. New multiparametric clinicopathologic and genomic tools accounting for this complexity, such as HER2DX, are under development to define more tailored treatment approaches. Finally, HER2-targeted antibody-drug conjugates (ADC) such as trastuzumab deruxtecan might be active in tumors with low expression of HER2. Overall, there is a need to molecularly characterize and develop novel targeted therapies for HER2+ disease.

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## Contents

|   |     |
|---|-----|
| 1. Introduction .....   | 340 |
| 1.1. Clinically HER2+ disease based on hormone receptor status .....                    | 340 |
| 1.2. The intrinsic subtypes within cHER2+ disease .....                                 | 340 |
| 1.3. Intrinsic subtype, <i>ERBB2</i> mRNA and treatment response .....                  | 341 |
| 1.4. Intrinsic subtypes and survival outcome .....                                      | 342 |
| 1.5. Molecular alterations: focus on <i>ERBB2</i> , <i>PIK3CA</i> and <i>PTEN</i> ..... | 342 |
| 1.6. TILs and immune system .....   | 344 |
| 1.7. Beyond HER2 overexpression: HER2-low breast tumors and HER2 heterogeneity .....    | 345 |
| 1.8. HER2DX .....   | 346 |
| 2. Conclusions .....  | 347 |
| Author contributions .....  | 347 |
| Fundings .....  | 347 |

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|   |     |
|---|-----|
| Data availability .....                 | 347 |
| Declaration of competing interest ..... | 347 |
| Acknowledgements .....                  | 347 |
| References .....                        | 347 |

## 1. Introduction

HER2-positive (HER2+) breast cancer (BC) represents 10–20 % of all breast tumors [1] and are characterized by the amplification of the *ERBB2*/HER2 gene and/or overexpression of its related kinase receptor protein [2]. This confers a more aggressive behavior, leading to a reduced disease-free survival (DFS) and overall survival (OS) [2,3]. However, the introduction of the humanized anti-HER2 monoclonal antibody trastuzumab in clinical practice and the subsequent development of several other anti-HER2 targeted agents (i.e. lapatinib, pertuzumab and T-DM1), have lowered the risk for relapse in the early stage disease and/or significantly improved survival in the metastatic setting [4]. Despite therapeutic improvements, around 4–23 % of patients with localized disease still experience relapse after (neo)adjuvant anti-HER2-based regimens and become metastatic [5,6]. When systemic, the disease remains incurable, though median survival rates have improved consistently over the years, now reaching a median of ~57 months [7].

HER2+ BC is clinically defined, according to the ASCO/CAP guidelines [8], when a complete and intense circumferential membrane staining for the HER2 protein in >10 % of tumor cells (3+ score) is found at immunohistochemistry (IHC) and/or the HER2 gene (*ERBB2*) is amplified at *in situ* immunofluorescence (ISH) techniques, with an HER2/CEP17 ratio  $\geq 2.0$  and an average HER2 gene copy number  $\geq 4.0$  signals/cell. This definition is based on the one adopted for HER2+ clinical trials evaluating trastuzumab, pertuzumab and T-DM1. However, under the curtain of clinical (c) HER2+ disease lies a more complex and heterogenous disease that needs to be appropriately dissected, to develop and validate novel and more effective diagnostic and therapeutic approaches.

### 1.1. Clinically HER2+ disease based on hormone receptor status

Breast tumors are usually classified in 4 IHC subtypes (Luminal A-like, Luminal B-like, HER2-positive and triple-negative) that mostly overlap with four different corresponding molecular subtypes (Luminal A, Luminal B, HER2-Enriched [HER2-E] and Basal-like, respectively) [9,10]. The IHC-surrogate classification of cHER2+ disease endorsed by the St. Gallen Expert Consensus divides cHER2+ tumors into two. More precisely, a Luminal B-like subtype that features estrogen (ER) and/or progesterone receptor (PgR) expression (roughly 40–50 % of cHER2+ tumors) [11], and an HER2-E-like, which does not express both hormone receptors (HR) [9].

The IHC-based categorization of HR-positive (+) vs. HR-negative cHER2+ disease is undoubtedly useful in the clinical setting to indicate the need for endocrine therapy (ET). Nonetheless, HR status within cHER2+ BC is relevant from other points of view. For example, several meta-analyses regrouping neoadjuvant trials in cHER2+ disease have demonstrated a strong association between HR-negative status and pathologic complete response (pCR) [12–14] and better outcomes in HR-negative tumors compared with HR + disease [14]. Moreover, albeit pCR was found to be associated with more favorable survival outcome compared to non-pCR, the strength of the association was more evident in the HR-

negative disease than in the HR+ [12].

According to several studies, cHER2+ tumors also differ in main clinicopathological features and natural history of disease depending on HR expression. More specifically, cHER2+/HR-negative tumors are more likely to present with high histologic grade and higher tumor stage, less likely to first relapse in bone and more likely to recur in brain, than cHER2+/HR + disease. Moreover, patients with cHER2+/HR-negative tumors vs. cHER2+/HR + seem to be at increased risk of early death (i.e. first 5 years of follow-up) [15]. An exploratory analysis from the ALTO adjuvant phase III trial of CT combined with trastuzumab, lapatinib or both, compared survival outcomes periods 0–5 years versus >5 years based on HR status. Patients with HR + tumors had better DFS, distant disease-free survival (DDFS) and OS for years 0–5 compared to HR-negative disease. Nevertheless, survival outcomes at 8 years were similar between the two groups [16].

Regarding the benefit to trastuzumab, the joint analysis of two large adjuvant randomized controlled trials (RCT), the NCCTG N9831 and the NSABP B-31, comparing different chemotherapy (CT) regimens  $\pm$  trastuzumab in cHER2+ BC, revealed that the 10-year DFS and OS rates were similar between HR-negative vs. HR + tumors [17]. Concordant results were observed also in the adjuvant trastuzumab pivotal trial HERA [18]. Yet, the levels of ER protein might help to identify a subgroup of patients with cHER2+/HR + disease who might not benefit much from trastuzumab. In fact, a retrospective population-based study on 872 HR + cHER2+ BC patients treated with adjuvant CT  $\pm$  trastuzumab, revealed a clear benefit from its addition, in the overall population; however, the effect on BC specific survival (BCSS) in tumors expressing ER in >30 % of cells was not statistically significant ( $p = 0.26$ ). Furthermore, adjuvant trastuzumab did not improve both Relapse-Free Survival (RFS) and BCSS in tumors with ER overexpressed in >50 % of cells, as opposite to the significant effect observed in case of ER staining in  $\leq 50$  % of tumor cells [19]. A possible explanation might reside in a higher dependency on the ER rather than HER2 pathway for tumor growth and survival, which might limit the therapeutic efficacy of agents specifically blocking the HER2 downstream signaling pathway as their main mechanism of action.

Although these findings will require further validation, they do suggest that a subgroup of HR + tumors with high luminal features and hormone-dependency might not benefit much from (neo) adjuvant trastuzumab.

### 1.2. The intrinsic subtypes within cHER2+ disease

The four BC molecular subtypes, also called intrinsic subtypes, have shown different outcome patterns and response to therapy [20–22]. Clinically HER2+ BC has been considered a single tumor entity for a long time; however, in 2012, The Cancer Genome Atlas (TCGA) project demonstrated that not all cHER2+ BC had the same genomic profile. More precisely, only 50 % of cHER2+ tumors were HER2-E, while the other half were Luminal A or B, with high expression of typical luminal genes such as *ESR1*, *GATA3* and *BCL2*, among others [23]. More recent studies have revealed the presence of cHER2+ Basal-like tumors, especially in HR-negative disease [24–30]. Among the different subtypes within cHER2+ disease, the

HER2-E is characterized by the highest levels of *ERBB2* mRNA, phosphorylated (p)HER2, total HER2 protein, pEGFR and EGFR protein, suggesting that this group has the highest activation of the HER2 signaling pathway [23–25].

In summary, cHER2+ tumors comprises all of the 4 BCE intrinsic subtypes, with the HER2-E being the most frequent (~47%), followed by Luminal B (~18–28%), Luminal A (11–23%) and Basal-like (7–14%) [10,24]. Yet, the distribution seems to be heavily influenced by HR status, with HER2-E subtype representing 30% of molecular subtypes within HR+/cHER2+ BC and 75% in HR-negative/cHER2+ tumors [10,26–30] (Fig. 1). Although related, the concordance rate between the pathology-based subtypes and the intrinsic subtypes is moderate (67.4%; kappa statistic: 0.50) [28].

An important question to address was how different an intrinsic subtype is when it is cHER2+ versus cHER2-negative. The answer is that a given subtype within cHER2+ disease is largely undistinguishable, biologically speaking, from its cHER2-negative counterpart, except for the high expression of genes in, or near, the HER2 amplicon on chromosome 17 in cHER2+ disease [24,31]. Furthermore, HER2 status is an independent prognostic factor beyond clinical-pathological variables, but such prognostic relevance disappears when intrinsic subtypes are considered [24].

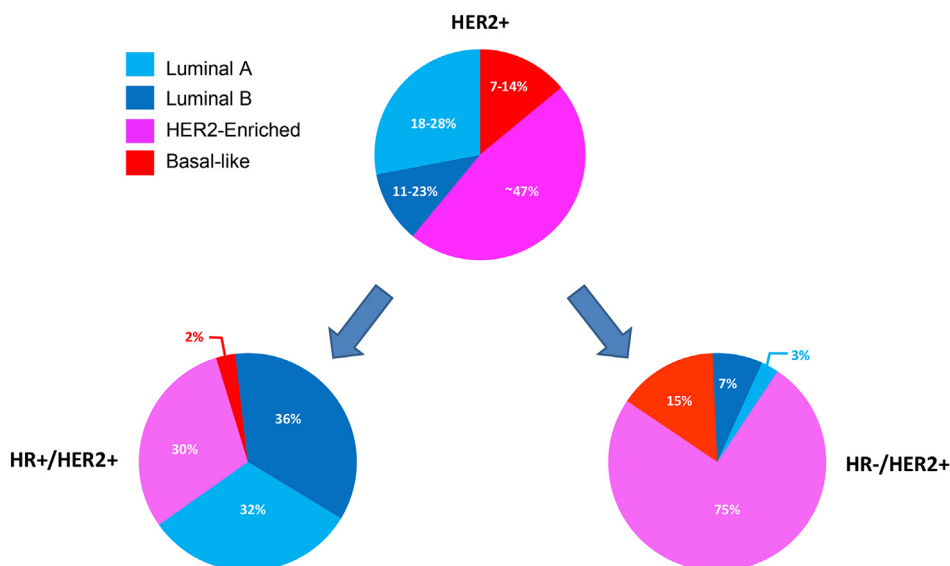
### 1.3. Intrinsic subtype, *ERBB2* mRNA and treatment response

Results from 6 randomized phase III, 5 randomized phase II, 3 single arm phase II, 2 non-randomized neoadjuvant clinical trial and 1 retrospective observational study evaluating several anti-HER2-based treatment regimens, +/- CT, concordantly suggested the association between the HER2-E subtype and pCR [25–30,32–42]. A subsequent trial-level joint analysis of such studies confirmed a strong association between the HER2-E molecular subtype with pCR (pooled odds ratio [OR]: 3.50,  $p < 0.001$ ), also beyond HR status ( $p < 0.001$ ) and CT use ( $p < 0.001$ ) [13]. These results suggest that intrinsic subtypes might identify a subgroup of cHER2+ tumors that benefit the most from anti-HER2 therapies,

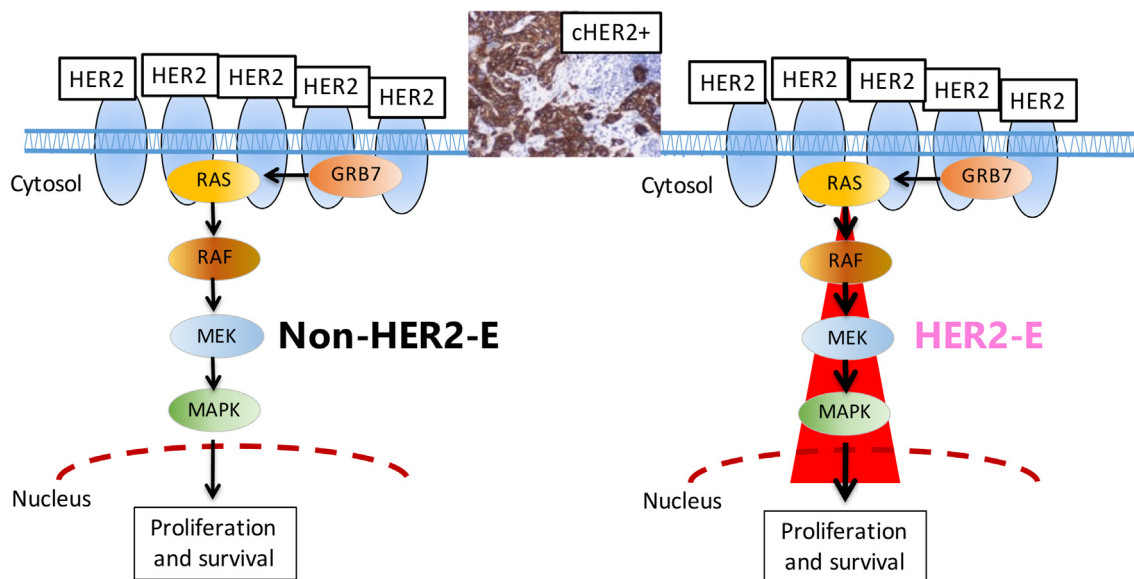
irrespective of CT and HR status.

Furthermore, 3 of those trials reported *ERBB2* mRNA-high tumors achieving more frequently a pCR, compared to mRNA-low tumors (41.9–79.3% vs. 25.5–51.72% pCR rates) [26,39,43], albeit with different methodologies and cut-offs for defining high vs. low levels. Another secondary analysis from the GeparQuattro neoadjuvant trial evaluating anthracycline/taxane-based CT + trastuzumab, showed that the response to the combination correlated to *ERBB2* mRNA levels [44]. Finally, high *ERBB2* mRNA levels were found associated with better tumor responses and progression-free survival (PFS) in the metastatic setting and higher rates of pCR in the neoadjuvant setting, when treated with the antibody-drug conjugate (ADC) ado-trastuzumab-emtansine (T-DM1) [45].

It is interesting to note that the combination of the HER2-E subtype with *ERBB2* mRNA expression levels might better predict response to anti-HER2-based treatments than each variable alone. In fact, in a combined retrospective analysis of the TBCRC006 and PAMELA CT-free neoadjuvant trials of double anti-HER2 blockade (lapatinib + trastuzumab ± ET) in cHER2+ BC, our group evaluated intrinsic subtypes and *ERBB2* mRNA levels of expression, dichotomizing the latter as low or high. As expected, pCR was more likely to occur in HER2-E tumors compared to non-HER2-E (35.1% vs. 9.9%;  $p < 0.001$ ), but also in tumors with *ERBB2* mRNA high compared to low levels (36.1% vs. 8.2%;  $p < 0.001$ ). Notably, HER2-E/*ERBB2* mRNA high BC showed pCR rates higher than the rest (adjusted OR: 6.0;  $p < 0.001$ ), suggesting that combining HER2-E subtype and *ERBB2* mRNA levels better identifies anti-HER2 sensitivity than each variable alone [46]. The most likely explanation is that the HER2-E subtype is more indicative of the activation of the HER2 signaling pathway than the expression of the HER2 protein or mRNA, which are more representative of the presence of the therapeutic target (Fig. 2). This might open up the possibility of de-escalating neoadjuvant systemic therapy to avoid CT, at least for a small proportion of patients carrying cHER2+ BC and with high “molecular addiction” to HER2 (i.e. HER2-E/*ERBB2* mRNA high) and low-risk clinicopathological prognostic parameters (e.g. node



**Fig. 1.** Intrinsic subtype distribution in cHER2+ tumors. Legend. HR: hormone receptors; +: positive; -: negative. Number of samples: 3390 HR+/HER2+ tumors and 2567 HR-/HER2+ tumors.



**Fig. 2.** HER2-dependent pathway for proliferation and survival in HER2+ breast cancer. Legend. cHER2+: clinically HER2-positive; HER2-E: HER2-Enriched. The red cone visually suggests a higher activation of the HER2-related pathway in cHER2+/HER2-E tumors, compared to cHER2+/non-HER2-E.

negative, small primary tumor). Ultimately, more data are needed to draw definitive conclusions.

Differently, no evidence of an added predictive value for the *ERBB2* mRNA levels beyond molecular subtypes has been evaluated in the context of CT + anti-HER2 therapy, so far.

Interestingly, an ongoing single arm phase II trial in cHER2+ BC cases with high molecular HER2 addition as previously defined and clinical low risk (clinically node negative, unifocal primary lesion of  $\leq 2$  cm), is evaluating the omission of surgery and sentinel lymph-node dissection, in case of achievement of a radiologic complete response at magnetic resonance, following neoadjuvant therapy with trastuzumab, pertuzumab and paclitaxel (ELPIS trial, NCT04301375). Results might be important to prove for the first time that in cHER2+ early stage BC, a combination of molecular and clinical tumor characteristics might be useful to de-escalate the therapeutic strategy, this time focusing on surgery, rather than systemic treatments. Recruitment is currently ongoing.

#### 1.4. Intrinsic subtypes and survival outcome

Retrospective analyses from (neo)adjuvant trials evaluated the association of the intrinsic molecular subtypes with survival outcomes within the cHER2+ disease. By using archived tumor blocks of cHER2+ disease from the NSABP B-31 adjuvant trial, 47 % samples were classified as HER2-E. Similar DFS benefit was observed for trastuzumab in HER2-E and non-HER2-E tumors [47]. Differently, a retrospective analysis from the N9831 trial showed that HER2-E and Luminal tumors derived benefit from adjuvant trastuzumab in terms of relapse-free survival (RFS), while Basal-like did not [48]. In addition, another analysis from the adjuvant pertuzumab APHINITY trial showed that the Luminal A subtype was associated with strikingly better outcomes, mostly when compared to patients with a Basal-like subtype. However, no significant interaction was observed between intrinsic subtypes and treatment [49].

As opposite to previous evidence, a novel molecular analysis from the phase III Short-HER study of 1 year vs 9 weeks of adjuvant trastuzumab demonstrated that the HER2-E subtype was associated with worse metastasis-free survival (MFS) compared to the other intrinsic subtypes taken together, both in the short and standard arm [50]. Within the NeoALTO trial there was no

association between the HER2-E subtype and event-free survival (EFS), although the authors accounted for an overall low statistical power to perform this analysis [33]. The NSABP B-41 trial demonstrated that OS was increased in patients who achieved pCR, with no significant differences in pCR according to treatment arm. With respect to intrinsic subtyping, HER2-E patients on trastuzumab-based arms had higher pCR than those on the lapatinib arm, though no differences in terms of EFS and OS were observed [30]. Finally, albeit demonstrating better pCR rates for the HER2-E subtype compared to the others, a post-hoc analysis from the CALGB 40601 trial showed that the Luminal A subtype was favored over the HER2-E in terms of invasive DFS (iDFS). Moreover, within HR + disease, the Luminal A subtype was associated to a longer iDFS compared to those with Luminal B or HER2-E tumors [51].

#### 1.5. Molecular alterations: focus on *ERBB2*, *PIK3CA* and *PTEN*

Recent studies have addressed the molecular complexity that characterize cHER2+ and HER2-E tumors. Analysis of somatic mutations from the TCGA showed that, other than *ERBB2* amplification, cHER2+ primary tumors were characterized by a broad range of infrequent mutations (<10 %), including in *ERBB2* (3.6 %). However, the most frequent mutations were observed in *PIK3CA* (~31 % cases) and in *TP53* (40 % cases) (Fig. 3).

Similarly, the HER2-E subtype, showed frequent *TP53* (72 %) and *PIK3CA* mutations (39 %), as well as other mutated genes (i.e. *PTEN*, *PIK3R1*), albeit at significantly lower frequencies (usually <10 %) and *ERBB2* amplification in 80 % cases [23,52].

*ERBB2* mutations are infrequent with respect to amplifications; however, they can exert an oncogenic effect by constitutively activating HER2 tyrosine kinase activity or by increasing HER2 dimerization with other member of the EGFR family. These mutations have been found to cluster in the tyrosine kinase and extra-cellular domains of the HER2 protein and might be counterbalanced by irreversible TKI inhibitors like neratinib [53]. The most frequent activating mutations identified are the G309A, D769H, D769Y, V777L, P780ins, V842I, del755–759 and R896C, while the L755S, despite being not activating, seem to induce lapatinib resistance [53]. These mutations were mostly characterized in HER2-non-amplified tumors. Interestingly, in a recent study, the mutational

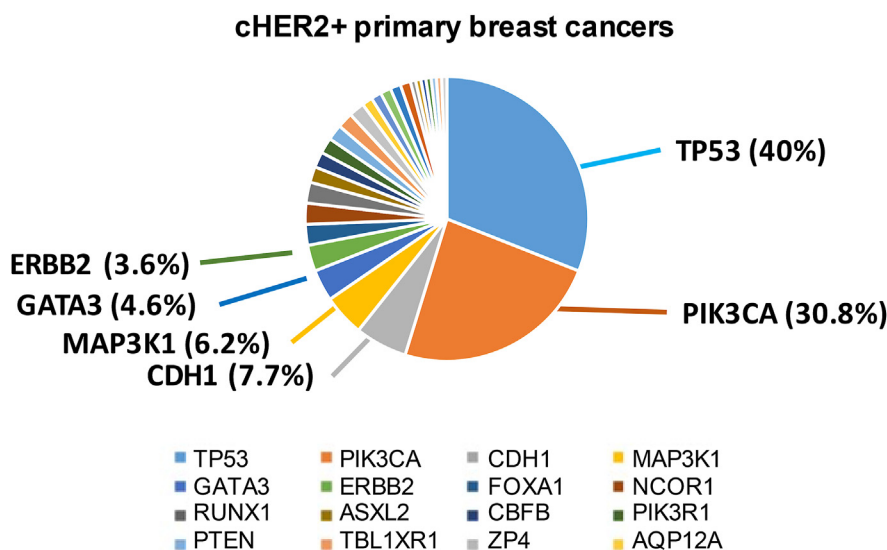


Fig. 3. Mutational spectrum of cHER2+ primary tumors. Legend. cHER2+: clinically HER2-positive.

landscape of *ERBB2* was also assessed in HER2 amplification-positive patients. The study showed a higher *ERBB2* mutation frequency in HER2-amplified tumors than in HER2-negative (19.5 % vs. 4.8 %;  $p < 0.001$ ) [54]. One-hundred-ninety-four *ERBB2* variants were predicted as driver mutations and 192 variants were predicted as passenger mutations. The top three frequency mutations were represented by the already known V777L, L755S and D769Y and were all predicted as driver mutations [54]. These rare mutations, at least in part, seem to be induced by HER2-targeted therapy and ET, and prevails in metastatic tumors, compared to primary ones [54–56]. Furthermore, in case of concomitant HER2 mutations and amplification, resistance to trastuzumab has been detected [54]. Conversely, in this cHER2+ subset, the tyrosin kinase inhibitor (TKI) neratinib showed promising activity [57], such as the TKI pyrotinib in BC with HER2 mutations without amplification [58]. However, randomized studies are needed to validate these preliminary findings.

*PIK3CA*, which codifies for the phosphatidyl-inositol-3-kinase (PI3K), is on the top of a cascade that positively modulates cell survival, proliferation and metabolism. The pathway is negatively regulated by *PTEN*, which codifies for a protein/lipid phosphatase [59]. Usually, oncogenic *PTEN* and *PIK3CA* mutations do not occur together [23,60]. In cHER2+ tumors *PIK3CA* mutations have been shown to be associated with trastuzumab resistance in preclinical models [61–63]. Therefore, *PIK3CA* and *PTEN* potential role as prognostic and predictive biomarkers of response to treatments was further investigated in cHER2+ BC.

Retrospective studies on tumor samples deriving from adjuvant trastuzumab trials found *PIK3CA* mutations in ~25 % cHER2+ BC, but failed to find an association with trastuzumab benefit [47,64]. At the same time a prognostically favorable effect was observed, albeit this effect disappeared after 3 years [64]. On the other hand, *PIK3CA* mutational status might be relevant in the neoadjuvant setting. Indeed, a patient-level pooled-analysis involving 5 neoadjuvant RCT investigating trastuzumab-/lapatinib-based treatments proved that *PIK3CA*-mutant/cHER2+ tumors had significantly lower pCR rates compared to wild-type tumors (16.2 % vs. 29.6 %;  $p < 0.001$ ). However, such results seemed to be mostly driven by the HR+/cHER2+ cohort, for which the pCR rates differences were significantly more pronounced (7.6 % in *PIK3CA* mutant vs. 24.2 % in the wild-type group;  $p < 0.001$ ) than in the HR-

negative/cHER2+ cohort ( $p = 0.125$ ) [65]. Mutational status did not affect the outcome in terms of DFS and OS in the overall population examined, though. However, HR+/*PIK3CA*-mutant patients seemed to have significantly worse DFS ( $p = 0.050$ ) [65].

A possible explanation for *PIK3CA* influence on response to anti-HER2 agents might lie in the fact that the rates of *PIK3CA* mutation seem to be inversely proportional to *ERBB2* mRNA levels (raw data obtained from a TCGA-derived dataset of 94 cHER2+ patients).

Interestingly, in advanced setting, *PIK3CA* mutational status proved to be a relevant prognostic marker for patients treated with pertuzumab + trastuzumab + docetaxel, in a biomarker analysis of the first-line CLEOPATRA trial. More specifically, longer median PFS was observed for patients whose tumors expressed wild-type versus mutated *PIK3CA* in the pertuzumab groups (21.8 v 12.5 months), as well as in the control group (13.8 v 8.6 months). However, pertuzumab showed a PFS benefit independently from *PIK3CA* mutational status, making it not suitable for selecting patients that might benefit from this drug [66]. Similar results were observed in a post-hoc biomarker analysis of the EMILIA and TH3RESA phase III RCT, where T-DM1 proved its effectiveness in both cHER2+ *PIK3CA*-mutant and wild-type patients over the therapeutic standard of second and subsequent lines [47,67]. Interestingly, *PIK3CA*-mutant patients appeared to better respond to lapatinib + capecitabine in the control arm of the EMILIA trial, both in terms of median PFS (mutant vs. wild type: 4.3 vs. 6.4 months) and OS (17.3 vs. 27.8 months) [67].

Concerning *PTEN*, retrospective analyses from the NeoALTTO and N9831 trials respectively showed no correlation between low levels of *PTEN* and pCR [75] and that the benefit from adjuvant trastuzumab was not affected by *PTEN* levels [76]. On the contrary, a retrospective analysis from the neoadjuvant TBCRC006 trial, where double blockade therapy with lapatinib + trastuzumab was administered without CT, showed that high levels of *PTEN* were associated to higher rates of pCR, compared to low *PTEN* levels (32 % vs. 9 %;  $p = 0.04$ ). Moreover, in case of a combination of *PTEN* loss/low expression and *PIK3CA* mutation, pCR was achieved significantly less than in case of wild-type *PIK3CA* and high *PTEN* expression levels (4 % vs. 39 %;  $p = 0.006$ ) [77].

In advanced setting, low *PTEN* levels were associated with better PFS outcome but, surprisingly, with worse OS in the CLEOPATRA trial. However, as observed with *PIK3CA* mutational status, a

consistent PFS benefit from pertuzumab was shown independently from the expression levels of any biomarker [66]. Within the EMILIA, T-DM1 was associated with longer median PFS and OS compared to lapatinib + capecitabine, regardless of *PTEN* expression. Anyway, in case of low/absent *PTEN*, the PFS benefit obtained with T-DM1 appeared to be more pronounced than in case of normal/high *PTEN* expression. This relationship was not observed for OS [67].

Two recent phase III trials, BOLERO-1 and BOLERO-3 evaluated the addition of the mTOR inhibitor everolimus to trastuzumab and CT (paclitaxel or vinorelbine, respectively) in cHER2+ advanced BC. The serine/threonine kinase mTOR is a downstream effector of the PI3K pathway. A joint analysis on 377 samples from both trials was performed to evaluate genomic alterations. The overall genetic landscape was similar between the 2 trials. Nevertheless, *PTEN* was altered in 1.5 % of the BOLERO-1 samples, compared to the 8 % in BOLERO-3. Differently from pertuzumab and T-DM1, a statistically significant PFS benefit from the addition of everolimus was only observed in patients with *PIK3CA* mutations, *PTEN* loss or hyperactive PI3K pathway (namely, low *PTEN* expression/mutation and/or known *PIK3CA* and/or other activating mutations in the downstream pathway) [60]. These findings suggest that *PIK3CA* and/or *PTEN* mutational status might be considered a predictive biomarker for everolimus efficacy in this BC subtype. However, the clinical development of this drug in cHER2+ BC in first-line setting has been somewhat stopped by the introduction of pertuzumab-based regimens in first-line and T-DM1 in second-line, given the outstanding performances observed in their pivotal trials [68–70]. Its role might be revalued in more advanced treatment lines, in patients with hyperactivation of the *PIK3CA* pathway, albeit trials designed to prospectively assess the efficacy and safety of this drug in patients with alterations in the PI3K pathway are needed to confirm this hypothesis.

### 1.6. TILs and immune system

A constantly growing body of evidence strongly suggests that cancer can induce an immune response by producing new antigens and modulate such immune response through the induction of microenvironmental changes and interplay with immune cells and immune response (cancer immunoediting) [71,72]. Being this process heavily related to T cells activity, tumor-infiltrating lymphocytes (TILs) have been claimed to be a morphological representation of the complex interplay between cancer and immune system [73]. Interestingly, there are several evidences reporting a previously unacknowledged interaction between anticancer treatments and immune system. For example, CT and radiotherapy seem to elicit an immune system activation against cancer cells [74], while trastuzumab anticancer activity seems to heavily rely also on antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity, rather than merely blocking the HER2 pathway. Such activity might be in turn modulated by the innate or adaptive cancer immune status [75–77]. Therefore, the biological complexity of cHER2+ BC not only lies in its molecular heterogeneous profile, but also in its microenvironmental features. Indeed, cHER2+ tumors are characterized by differential levels of TILs, which seems to play a role in modulating the responsiveness to CT and anti-HER2 treatments. In fact, it has been reported that in early stage, around 50 % of cHER2+ BC present with  $\geq 10$  % TILs, around 20 % shows  $\geq 20$  % and about 10 %  $\geq 40$  % TILs (Fig. 4) [78–80].

A recent study showed that cHER2+ HER2-E and Basal-like subtypes were also characterized by higher levels of PD-L1, compared to the Luminal A and B [80]. PD-L1 is a transmembrane protein that is implied in suppressing the adaptive immune response by interacting with the PD1 on the surface of T cells,

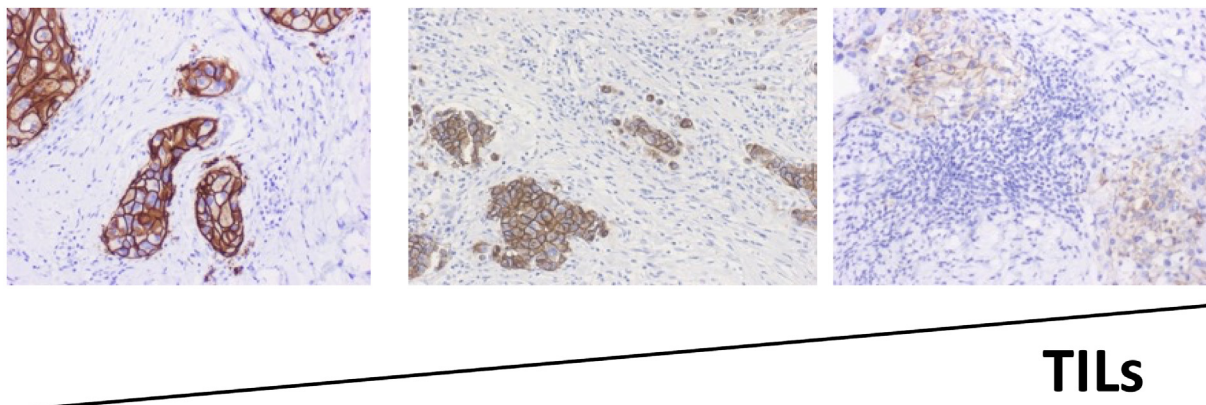
reducing the proliferation of antigen-specific T-cells in lymph nodes, while simultaneously reducing apoptosis in immunosuppressive T-regulatory (Treg) cells [81]. Recent studies have observed the presence of PD-L1 in ~42 % of cHER2+ BC, with different detecting assays [82,83]. PD-L1 levels correlate with response to anti-PD-L1 immune-checkpoint inhibitors (ICI), as observed in the phase III trial IMPASSION130 in advanced triple negative breast cancer (TNBC), as well as in other trials in different solid tumors [81,84]. In cHER2+ disease, the phase I/II trial PANACEA showed that only PD-L1+ tumors responded to the combination of trastuzumab with the anti-PD1 pembrolizumab, in a group of trastuzumab-pretreated metastatic cHER2+ BC patients [82]. Similarly, the randomized phase II KATE-2 showed a trend for a PFS benefit in pretreated HER2+ PDL1+ tumors with T-DM1 and the anti-PD-L1 atezolizumab, with no benefit for the overall population enrolled, or in the PD-L1-negative subset [83].

Up to now, the role of TILs as prognostic/predictive factor has been more extensively studied than PD-L1 in several clinical trials involving cHER2+ BC. In the adjuvant setting, contrasting results have been reported so far, based on retrospective analyses from adjuvant trastuzumab trials [85,86]. Conversely, more interesting results have come from neoadjuvant anti-HER2-based trials. A retrospective analysis of the NeoALTO from Salgado et al. showed that levels of TILs greater than 5 % were associated with higher pCR rates independent of treatment group ( $p = 0.01$ ). Moreover, every 1 % increase in TILs was associated with a 3 % decrease in the rate of an event ( $p = 0.002$ ) across all treatment groups [78]. In the CHERLOB trial, both stromal (Str)TILs and intratumoral (It-)TILs were significantly associated with pCR, also independently from ER status [29]. However, continuous TILs levels significantly predicted pCR only for patients in lapatinib-containing arms, while no statistically significant effect was observed in the CT + trastuzumab group. In a dichotomous comparison between low-TILs BC and high-TILs BC, with a cut-off for TILs levels of 60 %, pCR rates were 64.7 % for high-TILs BC and 25 % for low-TILs BC patients, respectively ( $p < 0.001$ ). Of note, molecular intrinsic subtypes outperformed TILs evaluation in predicting pCR at the multivariate analysis [29].

Opposite results were observed in the NeoSphere trial, where both TILs and PD-L1 did not associate with pCR [35]. Conversely, TILs as continuous variable and high vs low TILs were significantly linked to pCR in the HER2+ cohort of the neoadjuvant GeparSixto trial, where an anthracycline/taxane-based CT regimen with trastuzumab + lapatinib  $\pm$  carboplatin was tested [87].

A retrospective analysis on 175 patients with primary cHER2+ BC receiving neoadjuvant CT  $\pm$  trastuzumab failed to demonstrate a correlation between pCR rates and basal TILs levels. However, TILs levels decreased during treatment in 78 % of the patients and TILs' variation was strongly associated with pCR ( $p < 0.001$ ). Furthermore, post-neoadjuvant TILs were strongly associated with post-neoadjuvant tumor residue parameters and were higher in tumors with aggressive characteristics ( $p < 0.001$ ), high nodal involvement ( $p = 0.054$ ) and large nodal metastases ( $p = 0.001$ ). In the population of patients that failed to achieve pCR, levels of TILs in residual tumor higher than 25 % were an independent poor prognostic factor at the multivariate analysis [88].

In a secondary analysis of the PAMELA trial, the rates of pCR in high-level TILs BC vs. non-high-TILs BC were 58.3 % and 27.2 %, respectively ( $p = 0.024$ ). However, basal TILs lost their association with pCR at the multivariable analysis. Interestingly, the higher the TILs at day 15 (D15), the higher the odds of achieving a pCR and the correlation between D15 TILs and pCR was retained at the multivariate analysis. This study also proposed a mathematic model based on tumor cellularity and TILs at D15 (the CelTIL) to predict benefit from anti-HER2 therapy. The CelTIL was significantly



**Fig. 4.** Representative sections of TILs in early-stage HER2+ breast tumors. Legend. TILs: tumor-infiltrating lymphocytes. TILs levels increase from left to right.

associated with pCR in univariate and multivariate analysis either as a continuous variable or using a pre-specified cut point [89].

Notably, there are accumulating data on the capability of TILs or specific TILs subpopulations (e.g. CD8<sup>+</sup>) to predict clinical benefit of ICI in solid tumors like melanoma [90–92]. Whether this might be the case for cHER2+ BC is yet to be assessed. Moreover, the prognostic impact of specific immune cell infiltration (e.g. monocytes, B lymphocytes, T lymphocytes) is still a matter of debate. In this context, a more favorable outcome was observed in cHER2+ BC with lymphocyte infiltration and expression of lymphocyte-related genes [93]. Other groups discovered several immune-system-related metagenes regrouping specific clusters of genes that seem to be representative of different tumor-infiltrating immune cell subpopulations (e.g. the IgG metagene related to B lymphocytes, the LCK metagene related to T lymphocytes, the HCK metagene related to macrophages and monocyte/myeloid lineage), with different prognostic implications according to HR status [94,95]. More recently, a cluster of immune-related genes contained in a bigger six-metagene signature (138 genes), was found to be associated with higher pCR rates ( $p = 0.019$ ) following neoadjuvant CT and better prognosis in cHER2+/HR-negative BC ( $p = 0.026$ ). Immunity metagene expression was also associated with the presence of tumor-infiltrating lymphocytes (TILs) in the same study [96]. Overall, there is an accumulating evidence supporting the prognostic and predictive roles of immune gene signatures, especially in primary BC [97]. However, the published signatures are numerous and partially overlapping. Their prognostic and/or predictive role has been tested in different therapeutic contexts, as well. Therefore, a better harmonization and characterization of these signatures is required to properly understand their clinical utility and applicability [97]. Furthermore, whether the prognostic and predictive information captured by gene-expression-based signatures can be predicted by the assessment of cheaper and easy-to-detect immunologic peripheral blood markers is also yet to be determined. At present, it is also unknown the capability of immune metagenes to predict any benefit from novel immunotherapies.

Importantly, multiple evidences show that the HER2-E intrinsic BC subtype is associated with higher levels of TILs infiltration, compared to the others [29,75,89]. A possible explanation might reside in the fact that HER2-E subtype is frequently characterized by the lack of co-amplification of the 17q12 chromosomal region, which contains genes encoding chemokines, and is located proximal to the HER2 gene amplicon [71]. Such lack of co-amplification, which as opposite is relatively frequent in cHER2+ Luminal tumors, results in higher levels of T-cells infiltration [98]. Additionally, several data confirm a link between APOBEC-mediated

mutagenesis and the acquisition of subclonal mutations, leading to genomic instability, potential neoantigens expression and subsequently more immune tumor infiltration, as well as better response to ICI [99–101]. This might be particularly relevant for cHER2+ BC, considering that APOBEC genetic signature is frequently associated to the HER2-E subtype [22,102].

#### 1.7. Beyond HER2 overexpression: HER2-low breast tumors and HER2 heterogeneity

Based on IHC assays, current ASCO/CAP guidelines recommend to classify tumors characterized by an HER2 IHC score of 1+ or 2+ (with the latter accompanied by an ISH negative result) as HER2-negative BC, for which there is no clinical recommendation for anti-HER2 targeted agents. However, 2 of the pivotal trials that demonstrated the efficacy of trastuzumab-based adjuvant therapies were found to contain a cohort of patients without amplification/overexpression of HER2 on tissue submitted for central testing. These so-called HER2-low cohorts appeared to benefit from the addition of trastuzumab, retrospectively [103], but the phase III RCT NSABP B-47 failed to demonstrate prospectively this benefit [103]. Similarly, the 3-year results of the KRISTINE trial demonstrated that replacing systemic CT with T-DM1 in a pertuzumab-containing neoadjuvant regimen leads to a greater risk of relapse, mostly locoregional. Interestingly, tumors from these early-relapsing patients had lower HER2 expression (2+ vs. 3+) and HER2 2+/3+ heterogeneity (focal vs. heterogeneous), compared to those from other patients in the same treatment arm [104]. However, recent phase I trials of potent ADC trastuzumab deruxtecan and trastuzumab duocarmazine showed impressive response rates and median PFS in heavily pretreated metastatic HER2-low BC [105,106], leading to their further clinical development, along with other promising anti-HER2 ADC (e.g. MEDI4276 and XMT-1522) [107].

Our group conducted a large retrospective study on more than 3500 patients and comprehensively dissected HER2-low clinicopathological and molecular features [108]. Overall, we observed that HER2-low disease within HER2-negative BC is frequent (65 % of HR+/HER2-negative tumors and 37 % of TNBC) and mostly characterized by Luminal A/B tumors. Nevertheless, TNBC/HER2-low showed higher levels of Basal-like tumors, compared to HR+/HER2-low. However, while intrinsic subtypes' distribution, Basal- and proliferation-related genes levels of expression were higher in TNBC, with no differences between HER2 0 and HER2-low tumors, Luminal genes, as well as *ERBB2* and its companion gene *GRB7* were significantly more expressed in HR+/HER2-low than in both HR+/

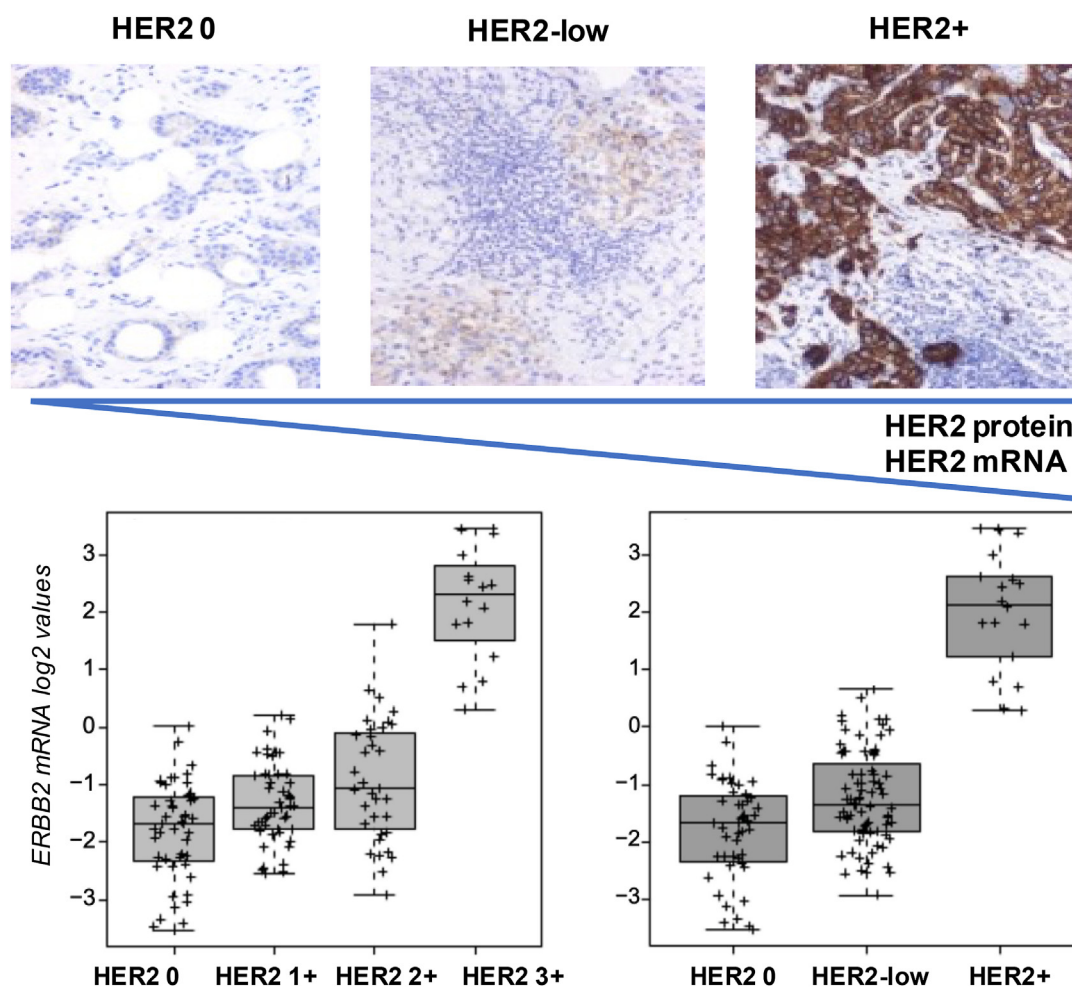
HER2 0 and TNBC, irrespectively of HER2 status. These findings were also accompanied by a higher prevalence of Luminal A/B subtypes in HR+/HER2-low compared to the rest [108]. Interestingly, the higher the HER2 IHC score, the higher the *ERBB2* mRNA levels [108]. This finding was also confirmed in a following study where we showed a significant positive correlation between *ERBB2* mRNA levels and HER2 protein levels (Spearman Correlation = 0.531,  $p < 0.001$ ) [109]. Finally, we found no survival differences between HER2-low vs 0 tumors, however the large biological heterogeneity observed, confirmed by a following study based on TCGA data, might have potential therapeutic implications [108,110]. An exemplification of the correlation between HER2 protein and mRNA levels is reported in Fig. 5.

Notably, about 1–34 % of breast tumors are characterized by heterogeneity in HER2 expression levels, which is responsible for poor response to anti-HER2-based regimens and worse prognosis, compared to cHER2+ BC [108,111]. This feature is more common in tumors with an HER2 equivocal status [111]. A recent single arm phase II trial of neoadjuvant T-DM1+pertuzumab in centrally confirmed cHER2+ BC, tested tumor tissues for HER2 intratumor heterogeneity (ITH-HER2), defined as the presence in at least one tumor area of either 1) HER2 positivity by ISH in >5 % and <50 % of tumor cells or 2) an area of tumor that tested HER2 negative. ITH-HER2 was more frequent in ER + tumors compared to ER-negative (81 % vs 19 %) and no pCR was observed among cases classified as

heterogeneous. A significant association between ITH-HER2 and pCR stratified by ER status ( $p < 0.0001$ ) was observed. This study suggests that, if further validated, a routine ITH-HER2 assessment might be an additional biomarker for selecting patients for (de)escalated therapeutic approaches [112].

### 1.8. HER2DX

The increasingly complex and rich molecular and clinicopathological heterogeneity that characterizes cHER2+ BC, is negatively counterbalanced by a substantial absence of clinically validated biomarkers other than HER2 and HR status. In order to help predict treatment benefit and better personalize the therapeutic approaches, we and others developed a prognostic assay that integrates multiple data types for predicting survival outcome in patients with newly diagnosed HER2-positive breast cancer. This so-called HER2DX combined prognostic model, was built on retrospective clinicopathological and genomic data from patients who participated in the previously mentioned Short-HER phase 3 trial. The final prognostic model was then evaluated in an independent combined dataset of patients with early-stage cHER2+ BC treated with different neoadjuvant and adjuvant anti-HER2-based combinations from four other studies with available DFS data [113]. The model included tumor dimension, nodal status, StrTILs, PAM50 subtypes, and expression of 13 genes [113].



**Fig. 5.** HER2 protein levels detected by immunohistochemistry and *ERBB2* mRNA levels according to HER2 IHC status. Legend. Representative pathological images of cHER2+ and cHER2-negative tumors courtesy of the Hospital Clinic of Barcelona Pathology Department and *ERBB2* mRNA expression levels (log<sub>2</sub> values) across HER2 IHC categories, as observed in 146 metastatic samples analyzed at the Translational Genomics and Targeted Therapies in Solid Tumors laboratory at IDIBAPS. HER2 protein and mRNA levels increase from left to right. HER2+: HER2-positive; IHC immunohistochemistry.



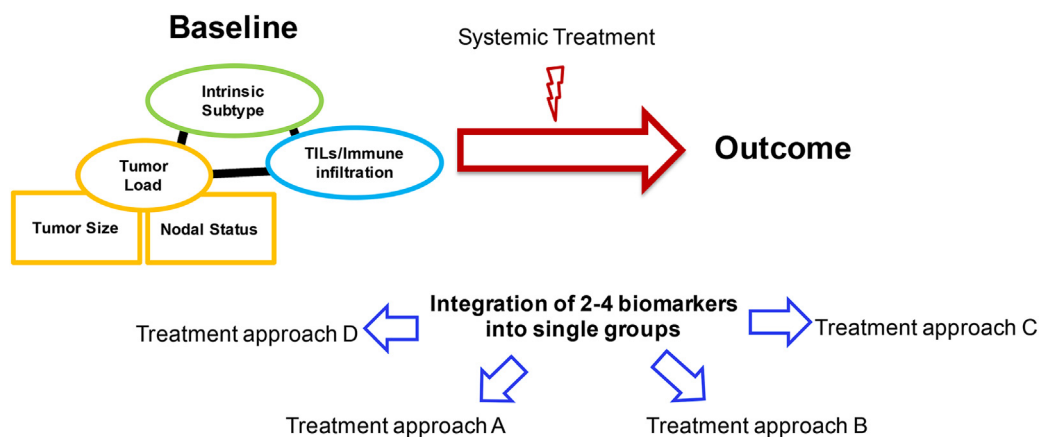


Fig. 6. Personalized medicine in cHER2+ breast cancer. Legend. TILs: tumor-infiltrating lymphocytes.

HER2DX score as a continuous variable was significantly associated with DFS and distant metastasis-free survival (DMFS) but not with pCR. The model could also stratify patients in low-, intermediate- and high-risk categories. Study results suggested that a substantial proportion of early-stage cHER2+ BC might not need additional treatments (e.g. pertuzumab, neratinib, T-DM1) other than CT + trastuzumab (and ET in HR + tumors) [113].

## 2. Conclusions

cHER2+ BC is a complex and multifaceted disease. Molecular profiling, gene expression patterns, clinicopathological tumor features and microenvironmental features altogether provide a faceted portrait of a nosological entity that no longer we should consider as one. Importantly, the integration of multiple biomarkers, also depending on the clinical setting, might lead towards different therapeutic strategies, depending on the presence/absence/value of several tumor/microenvironmental features or scores/categories identified by their combination (Fig. 6). In fact, in the era of personalized medicine [114], a multiple biomarker-driven diagnostic and therapeutic approach is progressively replacing the old “one-size-fit-all” formula, and single-biomarker-driven approaches, when possible.

For example, the multiparametric score HER2DX might be useful in the (neo)adjuvant setting to select candidates for (de)escalated therapeutic approaches, while the identification of HER2-E/ERBB2 mRNA-high cHER2+ tumors might be useful to select candidates for CT-free anti-HER2-based regimens, in early and advanced setting [25,45,113]. Similarly, the detection of TILs and PD-L1 might be crucial to identify candidates for ICI with anti-HER2 combinations [82,83]. Alterations of the *PIK3CA*/Akt/mTOR pathway might play a role in selecting patients that might gain benefit with PI3K-, Akt- and mTOR-inhibitors [60,66,67]; similarly, *ERBB2* mutations might be overcome with potent anti-HER family TKI such as neratinib and pyrotinib [57,58]. Finally, the novel category of HER2-low tumors seems to benefit from new potent anti-HER2 ADC, such as trastuzumab deruxtecan [105,106]. All these strategies are worthy of further investigation and need to be tested in prospective clinical trials.

The final goal will be to avoid or reduce treatment toxicity where unnecessary, without compromising patient's outcome at the same time, or correctly use escalated regimens to gain the maximum benefit at the cost of higher toxicity only in the most appropriate candidates. These tools might also help to correctly allocate financial resources, in a context where novel targeted agents are reaching unsustainable costs and their use is substantially unfeasible for numerous countries all over the world

[115–117].

## Author contributions

The authors equally contributed in the conception, drafting, revision and final approval of the manuscript.

## Fundings

This article did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## Data availability

No new datasets were generated or analyzed for this report.

## Declaration of competing interest

F.S. declares no conflict of interest. A.P. declares an immediate family member being employed by Novartis; fees for Non-CME Services Received Directly from Commercial Interest or their Agents (e.g. speakers' bureaus); personal honoraria from Pfizer, Novartis, Roche, MSD Oncology, Lilly and Daiichi Sankyo; travel, accommodations and expenses paid by Daiichi Sankyo; research funding from PUMA, Nanostring Technologies, Roche and Novartis; consulting/speaker/advisory role for NanoString Technologies, Amgen, Roche, Novartis, Pfizer, PUMA, Seattle Genetics, AstraZeneca, Guardant Health, Foundation Medicine and Bristol-Myers Squibb; patent PCT/EP2016/080056; HER2 AS A PREDICTOR OF RESPONSE TO DUAL HER2 BLOCKADE IN THE ABSENCE OF CYTOTOXIC THERAPY and HER2DX (filed); founder of Reveal Genomics.

## Acknowledgements

We thank Dr. Pedro L. Fernández Ruiz, from the Department of Pathology of Hospital Clinic of Barcelona, for the anatomopathological images shown in Figs. 4 and 5.

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