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Inês de Paula Costa Monteiro

Targeting HER family in HER2-positive metastatic breast cancer: potential biomarkers and novel targeted therapies

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Targeting HER family in HER2-positive metastatic breast cancer: potential biomarkers and novel targeted therapies

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Para a minha avó.

Com um agradecimento sentido
aos meus pais.

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Pharmacogenomics

Targeting HER family in HER2-positive metastatic breast cancer: potential biomarkers and novel targeted therapies

HER2-targeted therapies have radically changed the prognosis of HER2-positive breast cancer over the last few years. However, resistance to these therapies has been a constant, leading to treatment-failure and new tumor progression. Recently, the kinase-impaired HER3 emerged as a pivotal player in oncogenic signaling, with an important role in both non-treated progression and treatment response. HER2/HER3 dimerization is required for full signaling potential and constitutes the key oncogenic unit. Also, when inhibiting PI3K/AKT pathway (as with anti-HER2 drugs) feedback mechanisms lead to a rebound in HER3 activity, which is one of the main roads to resistance. As current strategies to treat HER2-positive breast cancer are unable to inhibit this feedback response, two great promises emerged: the combination of targeted-therapies and drugs targeting HER3. In this article HER2 and HER3-targeted drugs and possible combinations between them, as well as the biomarkers to predict and monitor these drugs effect, are reviewed.

Keywords: antineoplastic drug resistance • biomarkers • breast cancer • erbB-2 receptor • erbB-3 receptor • molecular targeted therapies • monoclonal antibodies • protein kinase inhibitors

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Breast cancer (BC) is the most common cancer diagnosis and the main cause of cancer-related death in women worldwide [1]. HER2 is overexpressed in 10–34% of all breast cancers and is strongly associated with a lower time to disease relapse and overall survival in the absence of treatment [2,3]. Upon understanding its special biologic behavior, HER2-driven BC started to be classified as a phenotypically distinct BC subtype [4]. In 1998, a decade after linking HER2 positivity to adverse outcome, the approval of trastuzumab (the first HER2-targeted therapy) for the treatment of metastatic breast cancer (MBC) significantly improved BC prognosis [5]. Nowadays, new treatments targeting HER2 in the neoadjuvant, adjuvant and metastatic settings have significantly improved the panorama for BC patients.

Despite the progress achieved with HER2-targeted therapy, acquired resistance has revealed to be a difficult problem to overcome. In metastatic disease, the response to all of the approved therapies occurs only for a limited period of time after which tumor progression is again observed [6]. To continue the path of success achieved with HER2-targeted therapy understanding the network in which HER2 is integrated and discovering more effective treatment options is of utmost importance, especially in the metastatic setting. Thus, in this paper, the role played by ErbB family receptors in HER2-positive MBC and the strategies to optimize these cancers' treatment, as well as the molecular markers to guide and monitor these therapies, are reviewed.

ErbB family

Human EGFR family, or ErbB family, consists of four transmembrane tyrosine kinases: HER1 or EGFR, HER2, HER3 and HER4. They are encoded, respectively, by the *ERBB1*, *ERBB2*, *ERBB3* and *ERBB4* genes. These

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receptors are composed of four extracellular domains (I, II, III and IV), a transmembrane portion, a tyrosine kinase domain and a carboxyterminal tail containing tyrosine residues and docking sites for intracellular signaling molecules. Extracellular domains I and III are responsible for ligand binding and domains II and IV participate in dimer formation [7].

When inactive, these receptors exist as monomers in a tethered conformation that blocks the dimerization arm (included in domain II). Ligand-binding to domains I and III induces a conformational change that exposes the dimerization arm, allowing homo- or heterodimerization. The activated kinase domains then bind asymmetrically (ones carboxyterminal portion binds with others amino-terminal portion) and *trans*-phosphorylate the tyrosine residues in the carboxyterminal tails, creating docking sites for phosphotyrosine-binding proteins (such as Grb2, Shc and PLCγ). This process activates downstream pathways as the PI3K/AKT, Ras/Raf/MEK/MAPK and PLC-γ pathway [7]. The first has an important role in cell survival and the second and third ones mediate cell proliferation (Figure 1). These receptors participate in normal cell growth and development in several tissues, however, when overexpressed, this family is responsible for unregulated cell division, apoptosis, migration, adhesion and differentiation: all processes leading to tumorigenesis [8].

Despite sharing a common structure, there are some differences between the four receptors. The extracellular domain of HER2 has very unique characteristics: it is unable to bind a ligand or to assume a tethered configuration and, in addition, it has electronegative sequences in domain II. This makes it permanently available for dimerization with other receptors but unable to form homodimers due to electrostatic repulse [10]. Nevertheless, when overexpressed, this receptor forms ligand-independent functional homodimers [11]. HER2 is the preferred dimerization partner for all other receptors [12].

HER3 receptor can bind extracellular ligands and initiate a signaling transduction cascade (being its main ligand HRG) but it is described as having no kinase activity. Recent studies suggest that the kinase ability of HER3 is impaired, rather than inactive [13], however, more studies are needed to clarify this aspect. Once again, homodimers of this receptor are apparently nonfunctional [13]. HER3 presents six and HER4 one direct binding site for p85 subunit of PI3K, whereas HER1 and HER2 only interact with this protein indirectly. This makes dimers containing HER3 the ones capable of the strongest activation of the PI3K/AKT pathway [9]. The combination of the special features of HER2 and HER3 receptors mentioned are thought to be the reason why HER2/HER3 dimer is the most potent partnership regarding ligand-induced tyrosine phosphorylation and mitogenic signaling [14,15].

In HER2/HER3 dimers, HER3 phosphorylation is accomplished by HER2 kinase, however, how HER2 tyrosine residues are phosphorylated is not well established. A recent study suggests that HER2/HER3 heterodimers with a side-by-side orientation allow HER2 receptors to phosphorylate one another (a process termed proxy phosphorylation). Other high-order interactions among ErbB receptors have been hypothesized, hence this is a subject that requires further investigation [16].

ErbB family in HER2-positive breast cancer

HER2 positivity occurs mainly due to *ERBB2* gene amplification [4] but it can also occur as a result of transcriptional deregulation, leading to overexpression of the receptor [17]. HER2-activating mutations have been recently described as an alternative mechanism, but the impact of this fact is still unclear [17]. HER2 overexpression bias dimer formation toward HER2-containing dimers. Although overexpressed HER2 is able to homodimerize, heterodimers are formed preferentially and constitute stronger signaling complexes. These heterodimers can be formed in a ligand-dependent or ligand-independent way [8]. In HER2-driven BC, HER2 overexpression is an early event in neoplastic transformation [18] and is generally retained when tumor progresses and metastases are formed [4]. However, the vast interdependence between the ErbB family receptors highlights the crucial role of HER2 interactions with other ErbB receptors in neoplastic progression.

HER1 or HER2 overexpression is well established as predictive of decreased survival in invasive BC. More recently, HER3 overexpression was proved to decrease BC-specific survival independently of other prognostic factors [19]. It was also shown that HER3 expression in HER2-positive invasive BC is correlated with a significantly lower disease-free survival [20].

As for HER4, its role remains the most controversial. HER4 activity in BC cells has been linked to cellular responses as growth inhibition, differentiation, apoptosis and cell proliferation, being associated with both favorable and worse prognosis [21,22]. Recently, a unique property of HER4 has been suggested to underpin this controversial role: the formation of a soluble intracellular domain (ICD) which is pointed as the critical effector of HER4 signaling [22,23]. The localization of this ICD was found to determine the type of signaling response: while a mitochondrial localization leads to an apoptotic response, a nuclear translocation induces the ICD to act as an estrogen response

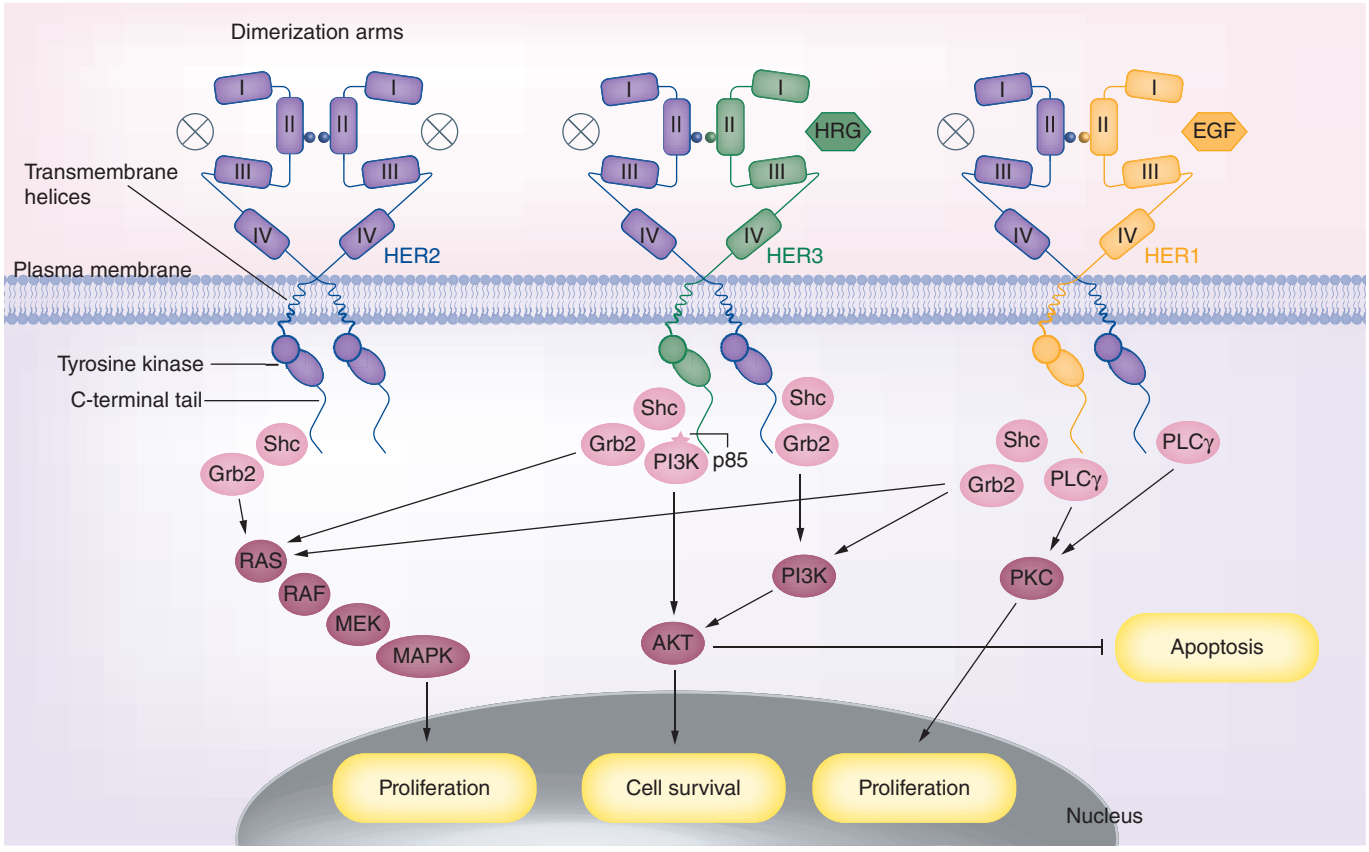

Figure 1. ErbB family receptors and ErbB signaling network. Only HER2/HER2, HER3/HER2 and HER1/HER2 dimers are represented due to their significance in HER2-driven breast cancer. Despite HER4 not being represented it shares the molecular structure of HER1–3. The four extracellular domains are represented as I–IV. Despite not being the exclusive ligands of HER1 and HER3, EGF and HRG are the only ligands shown as they are, respectively, HER1 and HER3 main activators. Most arrows portray processes that require several steps. For simplicity, only PI3K/AKT, RAS/RAF/MEK/MAPK and PLCγ/PKC pathways and their main signaling outcomes are shown. Proteins that directly bind phosphotyrosine residues are represented in light pink while subsequently activated proteins are represented in dark pink. HER3 tyrosine kinase domain is marked with a cross since it is kinase impaired. HER2 ligand binding sites are also associated with a cross since no ligand is known for this receptor. Note that only HER3 is able to bind PI3K p85 subunit (evidenced in the figure) and directly activate PI3K. HER1 and HER2 need other messenger proteins to indirectly activate this protein [7,9]. This figure is not drawn to scale.
Please see color figure at www.futuremedicine.com/doi/pdf/10.2217/pgs.14.133

element and to mediate proproliferative transcriptional activity [23]. The localization of the ICD is influenced by the type of cytoplasmic domain (ICD CYT-1 or CYT-2, formed by alternative splicing of HER4 pre-mRNA) [22] and by estrogen receptor-induced signaling [23]. As cycles of estrogen and progesterone during a woman's life lead to chromatin rearrangements, the nuclear ICD has different transcriptional potential in parous versus nonparous women [24]. This last group of women is more likely to present a BC cell proliferation response [24]. Thus, the activity of HER4 containing heterodimers, as in HER2-driven breast tumors, is hypothesized to lead to a better outcome in parous or lactating hosts when compared with nonparous women [24]. Even taking in account these different responses according to ICD localization the most accepted vision is that HER4-expressing tumors have an overall better prognosis, presenting a slight distinction among them: the ones expressing nuclear HER4 have a worse prognosis compared with the ones with membrane or cytoplasmic HER4 [21].

Comprehending ErbB signaling network is crucial for understanding major mechanisms leading to cancer progression and discovering new potential therapy targets. In this context, and since HER4's impact on survival is still under discussion, HER1 and HER3 were the most studied as HER2 coreceptors. It was shown that when HER2 is overexpressed, HER3 cooperates with it, enhancing its neoplastic transformation potential [25]. Two essays revealed that loss of functional HER3 in HER2-overexpressing BC cells has similar impact to the loss of HER2 function in cell proliferation [26,27]; on the contrary, HER1 knockdown has no significant biological relevance, whereby

it appears to be dispensable for proliferation [26]. In one of those studies the antiproliferative effect after HER3 function loss was reported in cell lines that expressed HER1 and HER4. This suggests that HER3 is essential for proliferation on HER2-driven BC and that neither HER1 nor HER4 can replace HER3 as HER2's partner [27]. In the other essay, phosphorylated HER3 was significantly higher in HER2-overexpressing BC tissues when compared with HER2-negative ones. Once again, this did not happen with HER1, suggesting that HER3 activity is important [26]. HER3 knockdown in HER2-positive cell lines also proved to inhibit growth in three-dimensional culture and to lead to rapid tumor regression *in vivo* [26]. In addition to this role as enhancer of HER2-induced progression, it was recently shown that HER3 also has a prominent role in the preneoplastic changes of breast epithelial cells and tumor formation, proving a more extensive time influence in HER2-positive breast cancers [28].

It is thought that what underpins HER3's special role is its capacity to directly activate the PI3K/AKT pathway. This is supported by the fact that a constitutively active AKT protein completely rescues the antiproliferative effect induced by the loss of HER2 and HER3 signaling [27]. It was shown that HER3-PI3K signaling is dispensable for HER2-driven tumorigenesis as HER2 and HER1 proven to be able to independently activate PI3K and rescue the tumorigenic response [29]. Nevertheless, the total absence of HER3 expression deeply affects tumor progression, suggesting that HER3 also has important PI3K-independent functions [29]. It is currently accepted that in HER2-overexpressing BC, HER2/HER3 dimerization is required for full signaling and carcinogenic potential. These data suggest that HER2/HER3 is the key oncogenic unit [26,27].

Targeting HER family

As mentioned above, this is a four-member family with multiple-domain receptors, which enables several targeting strategies. The two main strategies used to target these receptors are monoclonal antibodies (mAbs) that bind to the receptor's extracellular region and small-molecule tyrosine kinase inhibitors (TKIs) that block signal transduction (Figure 2) [30]. The first strategy attempted was the inhibition of HER2 with the mAb trastuzumab, an antibody that binds domain IV of the extracellular part of HER2 [5]. Since then, other options to inhibit this receptor were approved such as other anti-HER2 mAbs, a TKI that inhibits both HER2's and HER1's kinases activity and strategies that double-target HER2 at different sites. Despite the progress achieved, intrinsic and acquired resistances are a significant problem. Intrinsic, primary or *de novo* resistance defines a status of nonmeasurable response to a drug, in other words, apparent lack of drug activity. Acquired resistance is treatment-induced and defines the mechanism by which tumors that once had a favorable drug response stopped responding [31]. Hence, this resistance issues leave much room for improvement.

It is crucial to keep in mind that we are facing an interdependent network and not an isolated overexpressed receptor. The disruption of an axis integrated in an interconnected network like this one potentially leads to compensatory mechanisms [32]. Thereby, treatment-induced resistance is not a fixed problem; it is a dynamic response to a specific blockage that is different according to the blocked target.

When trastuzumab is used as treatment, there are several mechanisms described that may account for drug resistance: altered antibody-receptor interaction, cross-talk between HER2 and other receptors (like ErbB receptors and insulin-like growth factor 1 receptor – IGF1R) and modulation of the activity of intracellular signaling proteins [31]. When the choice is to target HER2 and HER1 kinases, new mechanisms are blamed for treatment-induced resistance. It was shown that lapatinib, the only TKI approved, is only capable of an incomplete inhibition of HER2 kinase. This allows HER3 *trans*-phosphorylation to occur and restores signaling activity sufficiently enough to evade the proapoptotic effect of this drug. This suggests that complete inactivation of kinase activity is needed to overcome this problem. However, a clinically viable drug with this feature is not available [33,34].

It soon became clear that the ErbB family-mediated signaling was more resilient than initially thought and that avoiding the acquisition of resistance would require further study. Clinical trials evidenced that combined anti-HER2 therapies were superior to single-agent strategies and that drugs targeting different domains of the HER2 receptor have a synergistic effect when combined [35]. Hence, dual HER2-targeting strategies became a great promise to overcome resistance [36]. In fact, a combination of trastuzumab and pertuzumab – a mAb with a mechanism of action complementary to trastuzumab – proved to improve HER2-positive MBC outcome when compared with trastuzumab alone [37]. This successful double-targeting scheme proved the suspicion about the great potential of dual HER2-targeted approaches.

When further assessing the mechanisms underpinning treatment response and acquired resistance, HER3 emerged as a key receptor. It was shown that when directly or indirectly inhibiting PI3K/AKT pathway, a negative feedback response leads to a multitude of redundant mechanisms to preserve tumorigenic signaling: among them, an increased transcription of receptors like the HER3 receptor [33,34,38,39] and

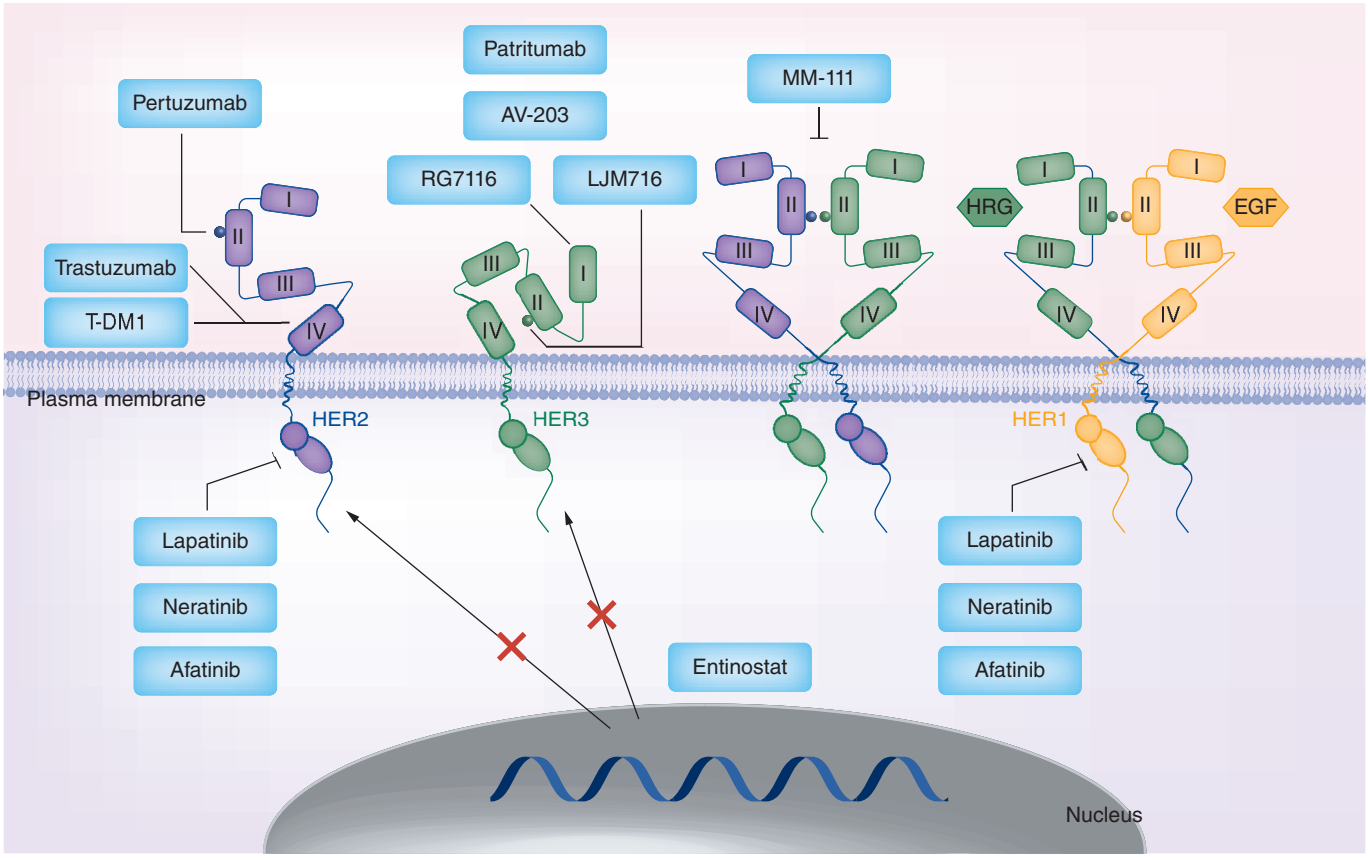

Figure 2. Drugs targeting HER2 and/or HER3. There are two main groups of drugs represented: monoclonal antibodies targeting the extracellular domains and tyrosine kinase inhibitors that bind to the receptor kinases in their intracellular portions. There are no monoclonal antibodies targeting the ligand-binding domains of HER2 once that no ligand is known for this receptor. There are no tyrosine kinase inhibitors targeting HER3 considering that its kinase is impaired. In its monomeric state, HER2 presents in an extended conformation. On the contrary, HER3 (as HER1 and HER4) assumes a tethered conformation, where its domains II and IV contact, maintaining the dimerization arm unavailable [7]. This figure is not drawn to scale.
T-DM1: Ado-trastuzumab emtansine.

the modulation of HER3 localization (locating it in the plasma membrane) [33]. This allows an increased phosphorylation of HER3. Also, when TKIs are used to inhibit this pathway, the protein tyrosine phosphatases activity is diminished and the dephosphorylation rate of this receptor is reduced [33]. These mechanisms all contribute to a rebound in HER3 activity, amplification of PI3K/AKT signaling and acquisition of resistance [33,34,38,39]. Evidence emerged that even the dual blockade of HER2 couldn't eliminate HER3 compensatory up-regulation and that drugs targeting HER3 could enhance the power of dual HER2-targeted approaches [40]. As evidenced in this paper, HER3 is a pivotal player in oncogenic signaling and it has an important role in both nontreated progression and treatment response. Thus, interest in anti-HER3 therapies is being raised and new drugs targeting this receptor are being tested.

The ultimate goal in HER-directed therapies is the complete inhibition of downstream signaling to avoid or at least delay the acquisition of resistance. To achieve this aim, the combination of targeted therapies has increasingly been pointed out as the road to follow [36]. Despite the existence of other possible targets in this network and due to the crucial role of HER2 and HER3 in neoplastic transformation, a particular focus will be given to drugs that interfere with HER2 and/or HER3 activity.

Biomarkers

Almost as important as developing targeted agents is the identification of biomarkers to allow treatment-decision and clinical application of the drug. Also, during treatment, biomarkers are tools of utmost importance for prediction and monitoring the drug's effect. At this point, HER2 overexpression as defined by *ERBB2* amplification (determined by FISH) or HER2 protein overexpression (determined by immunohistochemistry) is the only biomarker approved for HER2-targeted therapy [41]. *PIK3CA* is an oncogene frequently

mutated in BC [42] and oncogenic-*PIK3CA* mutations are emerging as a potential marker of worse prognosis in patients being treated with anti-HER2 drugs, except for the ones being treated with T-DM1, as documented in the NeoSphere, CLEOPATRA and EMILIA biomarker analyses [43–45]. p95HER2 expression (a truncated form of the HER2 receptor that lacks the extracellular domain) has been shown to be associated with intrinsic resistance to trastuzumab in the metastatic setting [46]. Nuclear imaging methods like PET (positron emission tomography) and SPECT (single photon emission computed tomography) are being tested for the detection of HER2-positive lesions [47,48]. These techniques allow a noninvasive way to assess metastases' HER2 status, which is particularly relevant for the metastases that are not accessible for biopsy. Moreover, as sampling errors cannot be ruled out with repeated biopsies, this provides a method to exclude sampling error [47]. Blood-based markers require samples that are easier to obtain and are also being evaluated as predictors of treatment response. Several blood biomarkers are currently being tested: circulating HER2-free DNA [49] and *PIK3CA*-activating mutations [42].

There are no current biomarkers to predict HER3-targeted response. As in most HER3-driven tumors HER3 is not mutated or amplified it is much more difficult to define this receptor as responsible for tumor progression. So far, high levels of *ERBB3* mRNA in patients with HER2-positive MBC treated with trastuzumab, pertuzumab and docetaxel were significantly associated with better prognosis in CLEOPATRA biomarker analyses [44]. However, no significant association was found in other studies [43,50]. HER3 protein expression has also been evaluated: no correlation was found regarding neoplasias treated with association of anti-HER2 drugs [43,44,50] but high levels of HER3 were significantly associated with poor prognosis on MBCs treated with trastuzumab [46]. It was also shown that an anti-HER3 antibody was more effective in cancers with ligand-dependent activation of HER3. This suggests that expression of HER3 ligands could be a marker for successful HER3 targeting [51]. *Trans*-phosphorylated HER3 (or pHER3) has been referred as one of the most promising biomarkers [33,40,52]. Receptor phosphorylation is a marker for HER3-mediated cellular activation, contrary to the presence of markers such as HER3 ligands, which represent an indirect evidence of HER3-mediated signaling. This biomarker could be useful for predicting which cancers would benefit from anti-HER3 therapies and as a pharmacodynamics marker for HER2 or HER3-targeting therapies, in other words, for assessing the drugs' efficacy and monitor their effect through time [33,40,52]. pHER3/HER3 ratio is pointed out as other promising pharmacodynamics marker [52]. Proximity-based immunoassays are being investigated as instruments to quantitatively measure HER3 activation in breast tumor samples [53] and may be useful tools if the discussed biomarkers reveal to be effective. As described for HER2, nuclear imaging methods are also a future possibility for detecting HER3-expressing neoplasias [54].

Although several targets are being studied and new drugs against HER2 and/or HER3 receptors are being tested, target populations that may benefit from these therapies are still to be found. Moreover, effective treatment-monitoring is not yet a reality. It is urgent that some biomarkers emerge as good predictors of response to help decision making during clinical trials and, maybe in the future, to be used in clinical practice, allowing an effective therapy for those patients who can benefit of it and less adverse events to those who cannot.

Targeting HER2

Trastuzumab is a humanized mAb that has high affinity for domain IV of the extracellular part of the HER2 receptor (Figure 2) [55]. The several mechanisms by which trastuzumab is believed to act include: prevention of ligand-independent dimerization, induction of endocytotic destruction of the receptor, induction of antibody-dependent cellular cytotoxicity (ADCC) and inhibition of extracellular domain cleavage [55]. Trastuzumab efficiently disrupts the formation of ligand-independent dimers but fails to block ligand-induced dimers [56].

The approval of trastuzumab for HER2-positive MBC in combination with chemotherapy was based in a clinical trial that demonstrated that, when compared with chemotherapy alone, the combination was associated with a higher rate of objective response (ORR; 50 vs 32%; $p < 0.001$), a longer time to disease progression (TTP; 7.4 vs 4.6 months; $p < 0.001$), a longer duration of response (9.1 vs 6.1 months; $p < 0.001$) and a longer median overall survival (OS; 25.1 vs 20.3 months; $p = 0.046$) [57]. Trastuzumab is also approved for the treatment of HER2-overexpressing MBC without chemotherapy [58]. Therapeutic schemes hat include trastuzumab are the preferred first-line treatment for these cancers in Europe, however, in the US a dual HER2-targeting approach that combines trastuzumab and pertuzumab is preferred [59,60].

It is also established that continuing HER2-targeting even in HER2-positive MBC that progressed during trastuzumab treatment is beneficial. Trastuzumab plus capecitabine is superior to capecitabine alone in these patients as it is associated with a significantly improved TTP (8.2 vs 5.6 months; $p = 0.0338$) and

ORR (48.1 vs 27.0%; p = 0.0115) and does not increase toxicity [61]. Hence, the continued use of trastuzumab plus chemotherapy is a second-line treatment for MBC (Table 1) [59,60].

The main concern regarding the use of trastuzumab is cardiac dysfunction. Hence, cardiac monitoring is recommended for patients receiving treatments with this mAb [60]. Despite this adverse event is potentially severe, it seems to be reversible with standard medical management in the majority of cases [57,62].

Lapatinib is an oral, TKI that reversibly competes with ATP for the ATP-binding site in HER2 and HER1 tyrosine kinases, downregulating these receptors' phosphorylation (Figure 2) [63]. It targets the intracellular domain of HER2 whereby it is capable of inhibiting p95HER2 activity [64]. Also, as it uses a different molecular pathway of interfering with this network, it is still effective against trastuzumab-resistant BC cells [65]. The combination of lapatinib plus capecitabine has proven to be superior when compared with capecitabine alone in women with HER2-positive advanced or metastatic BC that progressed after treatment with trastuzumab. TTP was shown to be significantly improved (8.4 vs 4.4 months; p < 0.001) without an increase in serious adverse events [66]. Thus, this combination is approved for tumors that progressed on/after trastuzumab-based therapies, constituting a second-line treatment in the metastatic setting in the US and in Europe (Table 1) [59,60].

Pertuzumab is a humanized mAb that binds to the subdomain II of the extracellular part of HER2, preventing ligand-dependent dimerization and inducing ADCC (Figure 2) [67]. Hence, pertuzumab and trastuzumab exhibit complementary mechanisms of action and, when combined, act synergistically to inhibit BC cells survival [35]. Pertuzumab has been approved by the US FDA and by the EMA but it is only used in combination with trastuzumab (Table 1). Combination of therapies will be discussed in more detail later on this article.

Ado-trastuzumab emtansine (T-DM1) is an antibody-drug conjugate incorporating trastuzumab and DM1, a microtubule-disrupting drug (Figure 2). DM1 has the ability to be delivered directly in the intra-

Table 1. HER2-targeted drugs in single anti-HER2 therapies for the treatment of HER2-positive metastatic breast cancer.

Agent	Molecular target and mechanism of action	Licensed indication (therapy line) or development status (NCT)	Efficacy
Trastuzumab	HER2 (domain IV): prevents ligand-independent dimerization, induces HER2 endocytotic destruction, ADCC and inhibits HER2 cleavage	HER2-positive recurrent or MBC (first line) HER2-positive recurrent or MBC (second line)	T + CT vs CT [57]: ORR 50 vs 32% (p < 0.001); TTP 7.4 vs 4.6 months (p < 0.001); OS 25.1 vs 20.3 months (p = 0.046) T + C vs C [61]: ORR 48.1 vs 27.0% (p = 0.0115); TTP 8.2 vs 5.6 months (p = 0.0338); OS 25.5 vs 20.4 months (p = 0.257)
Lapatinib	HER1, HER2 TKI	HER2-positive recurrent or MBC (second line)	L + C vs C [66]: ORR 22 vs 14% (p = 0.09); TTP 8.4 vs 4.4 months (p < 0.001)
Pertuzumab	HER2 (domain II): inhibits dimerization, induces ADCC	Approved only in combination regimens (see Table 2)	
T-DM1	HER2 (domain IV): all the referred for trastuzumab plus targeted delivery of an anti-microtubule agent	HER2-positive recurrent or MBC (second line)	T-DM1 vs L + C [68]: ORR 43.6 vs 30.8% (p < 0.001); PFS 9.6 vs 6.4 months (p < 0.001); OS 30.9 vs 25.1 months (p < 0.001)
Neratinib	HER1, HER2 TKI	III (NCT01808573) II (NCT00915018) II (NCT01494662)	N (prior T) vs N (T-naive) [69]: ORR 24 vs 56%; 16-week PFS 59 vs 78%; PFS 22.3 vs 39.6 weeks
Afatinib	HER1, HER2 TKI	II (NCT00431067) [70] III (NCT01125566) II (NCT01271725) II (NCT01441596)	A (single-arm) [70]: PFS 15.1 weeks OS 61.0 weeks

A: Afatinib; ADCC: Antibody-dependent cellular cytotoxicity; C: Capecitabine; CT: Chemotherapy; L: Lapatinib; MBC: Metastatic breast cancer; N: Neratinib; NCT: Clinicaltrials.gov identifier; ORR: Overall response rate; OS: Overall survival; PFS: Progression-free survival; T: Trastuzumab; TKI: Tyrosine kinase inhibitor; TTP: Time to tumor progression; T-DM1: Ado-trastuzumab emtansine.

cellular compartment of HER2-overexpressing cells, which minimizes exposure of normal tissues and improves the therapeutic index. T-DM1 retains all the mechanisms of action of trastuzumab and is still active against heavily pretreated HER2-positive MBC, namely in cancers that progressed on trastuzumab [68]. Also, it seems to be the only drug that is still efficient even when oncogenic activations of *PIK3CA* are present [45]. When compared with lapatinib and capecitabine, as in the EMILIA study, T-DM1 significantly improves ORR (43.6 vs 30.8%; $p < 0.001$), PFS (9.6 vs 6.4 months; $p < 0.001$) and OS (30.9 vs 25.1 months; $p < 0.001$) with less toxicity (41 vs 57%) [68]. This drug was approved by the FDA and the EMA for use as a single agent in HER2-positive MBC that recurred after treatment with trastuzumab and a taxane. It is the preferred second-line treatment for MBC in the US; however, its use is not contemplated in European guidelines yet (Table 1) [59,60].

Neratinib is an irreversible TKI of HER1, HER2 and HER4 (Figure 2) [69] and appears to be active in cells with HER2 mutations that are resistant to lapatinib [17]. It is currently being tested in combination with paclitaxel as a first-line treatment for HER2-positive advanced BC (NCT00915018), with capecitabine as a second-line treatment for HER2-positive MBC (NCT01808573) and as a single-agent for patients with HER2-positive BC and brain metastases (NCT01494662). A Phase II trial compared the effect of this TKI in patients with advanced BC with and without prior trastuzumab treatment: 16-week PFS rates were 59 and 78%, PFS was 22.3 and 39.6 weeks and ORR were 24 and 56%, respectively. This study demonstrated that neratinib is clinically active and reasonably well tolerated in patients that received prior trastuzumab treatment and in trastuzumab-naive patients (Table 1) [69].

Afatinib is also an irreversible TKI of HER1, HER2 and HER4 (Figure 2) [70]. It was studied as a single-agent in a Phase II trial in patients with HER2-positive MBC that progressed on trastuzumab (NCT00431067), where a PFS of 15.1 weeks (95% CI: 8.1–16.7) and an OS of 61.0 weeks (95% CI: 56.7-not evaluable) were shown [70]. Afatinib is currently being tested in the Phase III trial LUX-Breast 1 (NCT01125566) in combination with vinorelbine in patients with HER2-overexpressing MBC that failed prior treatment with trastuzumab. Also, two Phase II trials are evaluating afatinib for HER2-driven MBC: LUX-Breast 2 trial (NCT01271725), which is testing afatinib alone and in combination with paclitaxel or vinorelbine in patients that failed HER2-targeted treatment in the neoadjuvant or adjuvant setting; and LUX-Breast 3 trial (NCT01441596), that is studying afatinib alone and in combination with vinorelbine in patients with progressive brain metastases after treatment with trastuzumab and/or lapatinib (Table 1). This drug was approved by the FDA and the EMA but only for non-small-cell lung cancer.

Dual HER2-targeted approaches

Due to their complementary mechanisms of action, different strategies to combine trastuzumab and pertuzumab have been tested (Table 2). The combination of trastuzumab, pertuzumab and docetaxel or paclitaxel is currently the preferred first-line treatment for HER2-positive MBC in the US. In Europe, despite pertuzumab has been approved for MBC, this regimen is not yet part of the guidelines used [59,60]. The approval of pertuzumab in this therapeutic scheme followed the results of Phase III CLEOPATRA, where the use of pertuzumab, trastuzumab and docetaxel compared with the use of placebo, trastuzumab and docetaxel showed a significant improvement in PFS (18.5 vs 12.4 months; HR 0.62; $p < 0.001$) and OS (not reached vs 37.6 months; HR 0.66; $p = 0.0008$) without an increase in cardiac toxic effects [37,67].

The combination of trastuzumab and lapatinib proved to be superior in terms of PFS (11.1 vs 8.1 weeks; HR 0.74; $p = 0.011$) and OS (14 vs 9.5 months; HR 0.74; $p = 0.026$) in comparison with lapatinib alone in patients with HER2-positive trastuzumab-refractory MBC. This combination was found to have an acceptable safety profile and offers a chemotherapy-free regimen [71]. Combined trastuzumab and lapatinib constitutes a second-line treatment for recurrent or MBC in the US and, despite not being in European guidelines yet, it is approved by the EMA (Table 2) [59,60].

A single-arm Phase Ib/II trial (TDM4373g) studied T-DM1 and pertuzumab as a first and second-line treatment in patients with HER2-overexpressing locally advanced or metastatic BC. An ORR of 57.1% (95% CI: 34.0–78.2%) in the first-line setting and of 34.8% (95% CI: 22.2–50.0%) in the refractory setting was obtained. The clinical benefit rate was 61.9% (95% CI: 39.8–80.3%) and 45.7% (95% CI: 30.9–60.2%), respectively. This trial showed that this combination is clinically active and has an acceptable safety and tolerability profile [72] and encouraged further studies. The ongoing Phase III MARIANNE trial (NCT01120184) is studying T-DM1 plus pertuzumab versus T-DM1 versus trastuzumab plus a taxane as a first-line treatment for metastatic and locally advanced BC (Table 2).

A study in Phase I/II (NCT00398567) is evaluating the combination of trastuzumab and neratinib for patients with HER2-positive MBC that progressed on trastuzumab (Table 2). In preliminary results this

Table 2. Dual HER2-targeted approaches for the treatment of HER2-positive metastatic breast cancer.

Combination of anti-HER2 agents (therapy line)	Efficacy or primary end point	Clinical trial (reference or NCT)	Licensed indications or development status
T + P + docetaxel (first line)	T + P + docetaxel vs T + docetaxel: PFS 18.5 vs 12.4 months (HR 0.62; p < 0.001); OS not reached vs 37.6 months (HR 0.66; p = 0.0008); ORR 80.2 vs 69.3% (p = 0.001)	CLEOPATRA [37,67]	HER2-positive recurrent or MBC
T + L (second line)	T + L vs L: PFS 11.1 vs 8.1 weeks (HR 0.74; p = 0.011); OS 14 vs 9.5 months (HR 0.74; p = 0.026)	EGF104900 [71]	HER2-positive recurrent or MBC
T-DM1 + P (first line)	PFS AEs	MARIANNE (NCT01120184)	Phase III
T + N (second line)	ORR 27% (95% CI: 13–46%)†; PFS 16-week 47% (95% CI: 29–63%)†; PFS 19 weeks (95% CI: 15–32 weeks)†	(NCT00398567) [73]	Phase I/II

For each combination of anti-HER2 agents only the clinical trial with the highest development status was considered in this table.
†Preliminary results.
AE: Adverse event; HR: Hazard ratio; L: Lapatinib; MBC: Metastatic breast cancer; N: Neratinib; NCT: Clinicaltrials.gov identifier; ORR: Overall response rate; OS: Overall survival; P: Pertuzumab; PFS: Progression-free survival; T: Trastuzumab; T-DM1: Ado-trastuzumab emtansine.

combination appeared to be generally well tolerated and clinically active (ORR 27% (95% CI: 13–46%); 16-week PFS 47% (95% CI: 29–63%); PFS 19 weeks (95% CI: 15–32 weeks) [73].

Targeting HER3

Monoclonal antibodies

As HER3 is kinase impaired, targeting this receptor has been a challenge. The majority of drugs capable of effectively targeting HER3 are antibodies that bind to its extracellular domain, however, even among them, not all have revealed effective in HER2-amplified BC cell lines. In this article only anti-HER3 antibodies included in clinical trials assessing their efficacy in patients with HER2-positive BC or that proved efficient in HER2-positive BC cell lines will be discussed (Table 3).

Patritumab (U3-1287 or AMG-888), a fully humanized mAb, was the first HER3-targeted antibody. It binds to the extracellular domain of HER3, promoting its internalization and inhibiting its basal and ligand-induced signaling (Figure 2) [74]. In a Phase I study in advanced solid tumors (NCT00730470) a favorable safety profile and preliminary evidence of antitumor activity were observed [74], encouraging studies that are currently assessing this drug's activity in combination with anti-HER2 drugs (Table 4).

Another antibody against HER3 is the humanized mAb AV-203 that prevents ligands to bind with HER3 and induces receptor degradation (Figure 2). It was shown that this antibody potently inhibits both ligand-dependent and ligand-independent activation of HER3 and also inhibits tumor growth in human BC representing xenografts. AV-203 was able to restore sensitivity to lapatinib in a HER2-positive BC model, suggesting that its combination with HER2-targeted therapies could prevent the emergence of HER3-induced resistance [75]. Thus, AV-203 is currently being evaluated in a Phase I study for the treatment of advanced solid tumors (NCT01603979).

LJM716 is a fully humanized mAb that possesses a novel mechanism of action: it binds to domains II and IV of HER3, locking it in its inactive conformation (Figure 2). It is capable of inhibiting ligand-dependent and ligand-independent HER3 activation and HER2-overexpressing cancer cells proliferation. Also, it revealed to be active as a single-agent in tumor xenografts and demonstrated a synergic antitumor effect when combined with trastuzumab [76]. This drug is being tested in a Phase I study in HER2-overexpressing MBC (NCT01598077).

RG7116 (GE-huMab-HER3 or RO5479599) binds to the domain I of HER3, preventing HRG-binding to this receptor and downregulating its cell surface expression (Figure 2). Also, as it is a glycol-engineered antibody, it is a more potent activator of ADCC than conventional antibodies [77]. In a preclinical study, this mAb activity showed to be increased in combination with pertuzumab [77]. A Phase I clinical trial is testing this drug as a single-agent and in combination with anti-HER1 agents (NCT01482377). In a preliminary analysis, RG7116 monotherapy was well tolerated and showed signs of clinical activity [78].

Bispecific antibody

Bispecific antibodies started to be designed based on the hypothesis that co-targeting two tumor-associated antigens would increase tumor-targeting specificity

Table 3. Drugs targeting HER3 and their mechanism of action.

Agent	Molecular target	Mechanism of action	Development status (NCT)
Patritumab (U3-1287 or AMG-888)	HER3 (ECD)	Promotes HER3 internalization and inhibits basal and ligand-induced signaling	I (NCT00730470) [74]
AV-203	HER3 (ECD)	Prevents ligand binding, inhibits ligand-dependent and -independent activation and induces HER3 degradation	I (NCT01603979)
LJM716	HER3 (domains II and IV)	Locks HER3 in its inactive conformation and inhibits ligand-dependent and -independent signaling	I (NCT01598077)
RG7116 (GE-huMab-HER3 or RO5479599)	HER3 (domain I)	Prevents HRG binding, reduces HER3 cell surface expression and mediates enhanced ADCC	I (NCT01482377) [78]
MM-111 (ALM)	HER2 and HER3 (ECD)	Forms a trimeric complex with HER2 and HER3, blocking HER3 binding with HRG	I (NCT00911898) [81]
Entinostat (SNDX-275)	HDACs	Inhibits the HDACs inducing the transcription of miRNAs that silence the expression of HER2 and HER3	I (NCT00020579) [85]

ADCC: Antibody-dependent cellular cytotoxicity; ECD: Extracellular domain; HDAC: Histone deacetylase; NCT: Clinicaltrials.gov identifier.

and decrease the toxic effect on normal tissues. In fact, MM-111 (ALM), an antibody that targets both HER2 and HER3, exhibits selective targeting of tumor cells that co-express these receptors [79]. MM-111 binds to HER2 and subsequently to HER3, forming a trimeric complex and blocking its ligand-induced activation (Figure 2). In preclinical studies, the inhibition of ligand-mediated HER3 phosphorylation was greater with MM-111 than with pertuzumab and, when combined with trastuzumab, MM-111 is more effective at inhibiting tumor cell growth than trastuzumab plus pertuzumab. Also, MM-111 showed to be more effective at blocking ligand-induced HER3 phosphorylation than lapatinib and proved to increase PIK3-AKT pathway suppression when added to lapatinib [80]. Hence, this antibody was studied in HER2-positive cancers in a Phase I trial (NCT00911898) revealing to be well tolerated (Table 3) [81]. Currently, it's being studied in combination with several anti-HER2 regimens (Table 4).

Histone deacetylase inhibitor

Entinostat (SNDX-275) is a histone deacetylase inhibitor (HDACi) that dual-targets HER2 and HER3. It is believed that its effect is due to an ability to induce the transcription of microRNAs that silence the expression of HER2 and HER3 (Figure 2) [82]. It acts preferentially

Table 4. Dual targeting: HER2 and HER3.

Combination of anti-HER2 and anti-HER3 agents	Setting (therapy line)	Primary end point	Development status (NCT)
T + patritumab + paclitaxel	HER2-positive MBC (first line)	DLT, PFS	Ib/II (NCT01512199)
MM-111 + T	HER2-positive ABC (second line)	Safety	I (NCT01097460)
MM-111 + T + CT MM-111 + T ± L	HER2-positive AST (second line)	Safety, DLT, MTD	I (NCT013047849)
LJM716 + T	HER2-positive MBC (second line)	DLT	I (NCT01602406)
RG7116 + T + paclitaxel	HER2-positive MBC expressing HER3	DLT, AEs, PK, HAHAs	I (NCT01918254)
Entinostat + T + L	HER2-positive MBC (second line)	RP2D	I (NCT01434303)

ABC: Advanced breast cancer; AE: Adverse event; AST: Advanced solid tumors; CT: Chemotherapy; DLT: Dose limiting toxicity; HAHA: Human anti-human antibody; L: Lapatinib; MBC: Metastatic breast cancer; MTD: Maximum tolerated dose; NCT: Clinicaltrials.gov identifier; PFS: Progression-free survival; PK: Pharmacokinetics; RP2D: Recommended Phase II dose; T: Trastuzumab.

in HER2-overexpressing cell lines inhibiting downstream signaling and reducing the levels of pHER2, pHER3, pAKT and pMAPK [83]. This HDACi showed to enhance trastuzumab-mediated growth inhibition in trastuzumab-sensitive and resistant BC cells that overexpressed HER2 (Table 3) [84]. Entinostat revealed to be well tolerated in a Phase I study (NCT00020579) [85] and is currently being studied in combination with HER2-inhibitors (Table 4).

As HER2-overexpression is the main driver of HER2-positive BC, the greater promise regarding HER3-targeted drugs is not their action as single-agents but the potential of dual targeting HER2 and HER3 to potentiate the inhibition of tumor growth and reduce the acquisition of resistance. Hence, several dual targeting strategies with HER2 and HER3-targeted drugs are being evaluated. Clinical trials currently evaluating dual HER2/HER3-targeted strategies in HER2-positive BC are summarized in Table 4.

Conclusion

As knowledge about this type of cancer evolved, remarkable progress was made that significantly improved its prognosis: HER2-targeted therapies and strategies to double-target HER2 are two great examples of success regarding HER2-positive BC. Still, overcoming resistance to targeted therapies in HER2-positive MBC remains an incredible challenge. The ErbB network is now recognized as interdependent and resilient and as having several mechanisms con-

Executive summary

ErbB family

- When overexpressed, this family is responsible for unregulated cell division, apoptosis, migration, adhesion and differentiation: all processes leading to tumorigenesis.
- HER3 is a particular receptor as it is kinase-impaired and capable of the strongest activation of the PI3K/AKT pathway.

ErbB family in HER2-positive breast cancer

- HER2 overexpression is an early event in neoplastic transformation. Nevertheless, HER2 interactions with other ErbB receptors have a crucial role in neoplastic progression.
- HER2/HER3 dimerization is required for full signaling potential and this dimer is the key oncogenic unit.

Targeting HER family

- Resistance to targeted therapies remains an enormous challenge. When inhibiting the PI3K/AKT pathway (as with anti-HER2 drugs), a negative feedback response leads to a rebound in HER3 activity, which is one of the main mechanisms contributing to resistance.
- The combination of targeted therapies is superior to single-agent strategies. Strategies double-targeting HER2 proved to improve these tumors' outcome, however, they are still unable to fully block this feedback response. Strategies simultaneously inhibiting HER2 and other targets are being thoroughly studied.

Biomarkers

- HER2 overexpression as defined by FISH or immunohistochemistry remains the only biomarker approved to guide HER2-targeted therapy.
- pHER3 is one of the molecular markers believed to have the greatest potential both to monitor treatment response to anti-HER2 and/or anti-HER3 drugs and to predict which patients might benefit from HER3-targeted therapy.
- It is urgent that some biomarkers emerge for a better prediction and monitoring of anti-HER2 therapies' effect and to guide decision making in clinical trials testing HER3-targeted drugs.

Targeting HER2

- Trastuzumab, lapatinib and T-DM1 are approved as single anti-HER2 agents for the treatment of HER2-positive metastatic breast cancer (MBC). Strategies double-targeting HER2 in MBC with trastuzumab plus pertuzumab or trastuzumab plus lapatinib are also approved.
- Dual targeting approaches enhance progression-free survival and overall survival but still cannot avoid tumor progression.

Targeting HER3

- Targeting this receptor is a great promise to further inhibit neoplastic progression. Monoclonal antibodies, bispecific antibodies and one inhibitor of the histone deacetylases are being studied in clinical trials. Combinations of these therapies with anti-HER2 drugs are also being evaluated.

Conclusion

- Further studies are required for a realistic understanding of these drugs' utility, to set the optimal combinations between them and to establish novel biomarkers to guide their use.
- This new approach is hoped to become another step toward individualized and enhanced treatment for patients with HER2-positive MBC.

tributing to the acquisition of resistance [33]. Among them HER3 has emerged as a potential target. HER3 possess an intracellular domain that is at the same time kinase-impaired [13] and capable of the strongest activation of the PI3K/AKT pathway [9]. HER3 is now established as a crucial coreceptor for treatment response and acquired resistance. Therefore, HER3-targeted drugs and the association between them and anti-HER2 therapies are currently in clinical development. In spite of being in early stages of development, these combinations showed great potential in preclinical studies and are believed to be capable of improving HER2-positive MBC prognosis [80].

Biomarkers to predict and monitor the response to anti-HER2 therapies and to predict which patients may benefit from HER3-targeted agents in clinical trials are of utmost importance. Besides emerging as a potential target, HER3 has also emerged as a promising biomarker. Despite no molecular markers besides HER2 overexpression have been approved, biomarkers such as pHER3, *ERBB3* mRNA, p95HER2 and *PIK3CA*-activating mutations, amid others, are being studied.

Future perspective

The progress of targeted therapies directed against HER2 has been astonishing. Nevertheless, there is still room for improvement. A better understanding of the optimal use of the combinations of anti-HER2 drugs and of the potential biomarkers is needed. Regarding this last subject, pHER3 has been one of the greatest promises to monitor the response to HER2 or HER3-targeted therapies and to predict which patients would benefit from HER3-targeted treatment [33,40,52]. It is expected that future studies can clarify its role.

When it comes to HER3-targeted therapy, there is still a long way to go before some of these possibilities become real treatment options in clinical practice. It is predicted that in the next few years there will be a more realistic awareness of the effect and utility of the available drugs and of the optimal combinations between them. The only ongoing Phase II study assessing a combination between HER2 and HER3-targeted therapies (NCT01512199) is estimated to be complete in the present year. Also, if these drugs actually prove to be efficient, we can anticipate that the molecular markers to guide and monitor its use will be assessed more extensively in future biomarkers analysis.

Individualized therapies are the ultimate goal for cancer treatment. As the combination of targeted therapies has been proving to be a successful strategy and HER3 is established as a pivotal oncogenic receptor in the ErbB network, this new approach is hoped to become another step toward individualized and enhanced treatment for patients with HER2-positive MBC.

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Future Medicine Author Guidelines

Contents

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The audience for Future Medicine titles consists of clinicians, research scientists, decision-makers and a range of professionals in the healthcare community. Authors should bear in mind the multidisciplinary status of the readership when writing the article.

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Key formatting points

Please ensure your paper concurs with the following article format:

Title: concise, not more than 120 characters.

Author(s) names & affiliations: including full name, address, phone & fax numbers and e-mail.

Abstract/Summary: approximately 120 words. No references should be cited in the abstract.

Keywords: approximately 5–10 keywords for the review.

Body of the article: article content under relevant headings and subheadings.

Conclusion: analysis of the data presented in the review.

Future perspective: a speculative viewpoint on how the field will evolve in 5–10 years time.

Executive summary: bulleted summary points that illustrate the main topics or conclusions made under each of the main headings of the article.

References:
For full details on formatting see References section above.

- Primary literature references, and any patents or websites, should be numerically listed in the reference section in the order that they occur in the text.
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- Any references that are cited in figures/tables/boxes that do not appear in the text should also be numerically listed in the reference section in the order that they occur in the text.
- Quote first six authors' names. If there are more than six, then quote first three *et al.*
- The Future Medicine Endnote style can be downloaded from our website at: www.futuremedicine.com/page/authors.jsp
- A maximum of 20 references are allowed for Editorials, Priority Paper Evaluations and Conference Scenes.
- A maximum of 80 references is recommended for Reviews, Perspectives and Special Reports.

Reference annotations: please highlight 6–8 references that are of particular significance to the subject under review as "* of interest" or "** of considerable interest" and provide a brief (1–2 line) synopsis.

Figures/Tables/Boxes: Summary figures/tables/boxes are very useful, and we encourage their use in reviews/perspectives/special reports. The author should include illustrations and tables to condense and illustrate the information they wish to convey. Commentary that augments an article and could be viewed as 'stand-alone' should be included in a separate box. An example would be a summary of a particular trial or trial series, a case study summary or a series of terms explained.

If any of the figures or tables used in the manuscript requires permission from the original publisher, it is the author's responsibility to obtain this. Figures must be in an editable format.

No figures/tables/boxes are permitted in Editorials and Conference Scenes.

Article types

Reviews 

Reviews aim to highlight recent significant advances in research, ongoing challenges and unmet needs. Authors should strive for brevity and clarity. Each article should concentrate on the most recent developments in the field and should aim for concise presentation of relevant information.

Word limit: 4000–6000 words (excluding Abstract, Executive Summary, References and Figure/Table legends)
Required sections (for a more detailed description of these sections see Article sections):

- Summary
- Keywords
- Future perspective
- Executive summary
- References: target of approximately 80 references
- Reference annotations
- Financial disclosure/Acknowledgements

Perspectives

Perspectives have the same basic structure and length as review articles, however they should be more speculative and very forward looking, even visionary. They offer the author the opportunity to present criticism or address controversy. Authors of perspectives are encouraged to be highly opinionated. The intention is very much that these articles should represent a personal perspective. Referees will be briefed to review these articles for quality and relevance of argument only. They will not necessarily be expected to agree with the authors' sentiments.

Word limit: 4000–6000 words (excluding Abstract, Executive Summary, References and Figure/Table legends)
Required sections (for a more detailed description of these sections see Article sections):

- Summary
- Keywords
- Future perspective
- Executive summary

- References: target of approximately 80 references
- Reference annotations
- Financial disclosure/Acknowledgements

Special reports

Special reports are short review-style articles that summarize a particular niche area, be it a specific technique or therapeutic method.

Word limit: 1500–3000 words (excluding Abstract, Executive Summary, References and Figure/Table legends)
Required sections (for a more detailed description of these sections see Article sections):

- Summary
- Keywords
- Future perspective
- Executive summary
- References: target of approximately 50 references
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- Financial disclosure/Acknowledgements

Original research articles

When submitting a primary research article, please also submit a covering letter, stating the novelty and interest of the article to our readers.

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- Structured abstract (broken down into Aims, Materials & Methods, Results and Conclusions)
- Keywords
- Introduction
- Patients & methods/Materials & methods
- Results
- Discussion
- Conclusions
- Summary points – 8–10 bullet point sentences highlighting the key findings and conclusions of the research study
- References
- Reference annotations
- Financial disclosure/Acknowledgements

Three types of research article are accepted:

Full research article

Original research articles must present novel science that represents a substantial advancement in the field under investigation.

Preliminary communication

Preliminary communication articles are intended to be short reports of studies that present promising improvements or developments on existing areas of research.

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Methodology articles should provide an overview of a new experimental or computational method related to research. The method described may be either completely novel, or may offer a demonstrable improvement on an existing method.

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- Keywords
- Introduction
- Patients & methods/Materials & methods
- Results
- Discussion
- Conclusions
- Summary points – 8–10 bullet point sentences highlighting the key findings and conclusions of the research study
- References
- Reference annotations
- Financial disclosure/Acknowledgements

Short Communications

Short Communications are short, peer-reviewed articles that build on a previously published study, document partial research results from an ongoing study, or discuss results from studies limited in scope.

Word limit: 3000–5000 words (excluding Abstract, Summary Points, References and Figure/Table legends)

Required sections (for a more detailed description of these sections see Article sections):

- Structured abstract (broken down into Aims, Materials & Methods, Results and Conclusions)
- Keywords
- Introduction
- Patients & methods/Materials & methods
- Results
- Discussion
- Conclusions
- Summary points – 8–10 bullet point sentences highlighting the key findings and conclusions of the research study
- References
- Reference annotations
- Financial disclosure/Acknowledgements

Case Studies/Case Series

Case studies/series present a notable medical case or series of related cases of interest, and aim to further the reader's understanding of the issues relating to such situations.

Word limit: 1500–3000 words
Required sections:

- Executive Summary
- Summary
- Body of the article. A suggested structure could be:
    - Presentation of case – setting and patient details/history
    - Initial diagnosis/assessment
    - Treatment/management
    - Outcome and implications
- Discussion/conclusion
- References
- Reference annotations
- Financial disclosure/Acknowledgements (including appropriate patient consent)

Editorials

Editorials are short articles on issues of topical importance. We encourage our editorial writers to express their opinions, giving the author the opportunity to present criticism or address controversy. The intention is very much that the article should offer a personal perspective on a topic of recent interest.

Word limit: 1500 words maximum (excluding summary, keywords and references).
Required sections:

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- Photo (headshot) of authors (including all co-authors)
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- Financial disclosure/Acknowledgements

Research Highlights

Research highlights discuss a number of recent primary research papers, summarizing and commenting on each paper to give readers a real sense of the cutting edge of research in the field.

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Commentaries

Commentaries are short articles that are similar to Editorials, yet provide a more detailed discussion of a topic.

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- Keywords
- Photo (headshot) of authors (including all co-authors)
- **Please note:** No figures, tables or boxes are permitted in commentaries
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Opinions

Opinion articles should typically be informed, agenda-setting and authoritative. If addressing a problem, they should also present coherent argued solutions. They can address issues relating to scientific research, or peripheral areas of debate affecting industry and academia of concern to the journal's scope.

Word limit: 1500 words maximum (excluding summary, keywords and references).
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- Keywords
- Photo (headshot) of authors (including all co-authors)
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- **Please note:** A maximum of **20 references** are permitted
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Interviews

Interviews are conducted with key opinion leaders in the field, and can include a look back over their career and achievements to date, a discussion on their current research, and their thoughts and observations on the field as a whole.

Word limit: 1500 words
Required sections:

- Summary/biographical paragraph
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- Response from the author to each point
- Additional reference sources for the interested reader

Priority paper evaluations

Priority paper evaluations review significant, recently published original research articles carefully selected and assessed by specialists in the field (not a paper from the author's own group). The original research detailed in the chosen paper is discussed with the aim of keeping readers informed of the most promising discoveries/breakthroughs relevant to the subject of the journal through review and comment from experts.

Priority Paper Evaluations are intended to extend and expand on the information presented, putting it in context and explaining why it is of importance.

The ideal article will provide both a critical evaluation and the author's opinion on the quality and novelty of the information disclosed.

Word limit: 1500 words maximum (excluding summary, keywords and references).
Required sections (for a more detailed description of these sections see Article sections):

- Summary
- Keywords
- Summary of methods and results
- Discussion
- Future perspective
- Executive summary
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Conference scenes

Conference scenes aim to summarize the most important research presented at a recent conference in the subject area of the journal.

It is not usually feasible to attempt comprehensive coverage of the conference, as presentations are frequently too numerous for each to be done justice. The author should focus on those presentations that are most topical, interesting or thought-provoking.

Word limit: 1500 words maximum (excluding abstract, conference details and references).
Required sections:

- Conference details (title, date, location)
- Abstract/overview of meeting of approximately 100 words (120 words max)
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Company profiles

Company profiles allow representatives from pharmaceutical, biotechnology, etc. companies to describe the work currently being carried out within their particular organization, relevant to the field of the journal in question.

These reports are intended to provide an insight into the history and strategy of a company and profile its corporate capabilities, advanced technologies and future potential.

Word limit: 2000 words
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- Introduction – brief factual account of the history and strategy of the company including background information e.g., the year the company was founded, number of employees etc.
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Letters to the Editor

Readers may submit Letters to the Editor, commenting on an article published in the journal.

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Separate author guidelines for the submission of this article type are available.

Clinical trial commentaries

Separate author guidelines for the submission of this article type are available.

Manuscript preparation

Spacing & headings

Please use double line spacing throughout the manuscript. No more than four levels of subheading should be used to divide the text and should be clearly designated.

Abbreviations

Abbreviations should be defined on their first appearance, and in any table and figure footnotes. It is helpful if a separate list is provided of any abbreviations.

Spelling

US-preferred spelling will be used in the finished publication.

Article sections

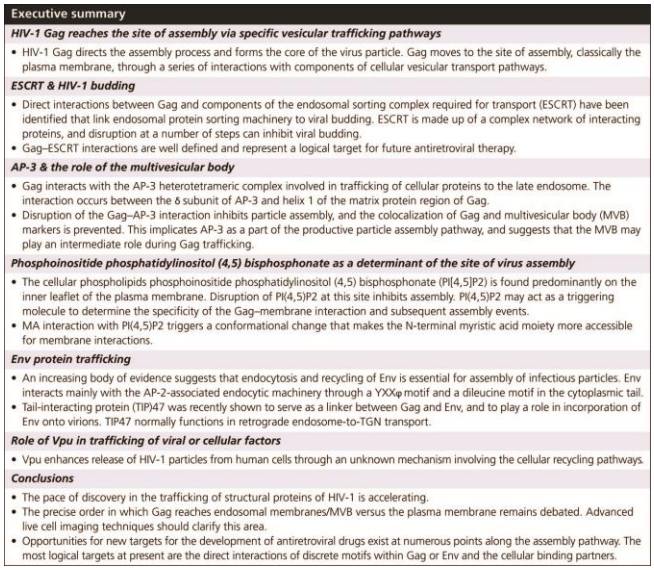
Summary
Not more than 150 words, this should not be an abstract but merely a scene-setting summary outlining the article scope and briefly putting it in context. The role of the summary is to draw in the interested casual browser.

Keywords
Up to 10 keywords (including therapeutic area, mechanism(s) of action etc.) plus names of drugs and compounds mentioned in the text.

Future perspective
The author is challenged to include speculative viewpoint on how the field will have evolved 5–10 years from the point at which the article was written.

Executive summary
A series of bulleted statements representing key conclusions, unresolved issues and points for emphasis of work in future, under the main headings of the article.

Example:



Executive summary

HIV-1 Gag reaches the site of assembly via specific vesicular trafficking pathways

- HIV-1 Gag directs the assembly process and forms the core of the virus particle. Gag moves to the site of assembly, classically the plasma membrane, through a series of interactions with components of cellular vesicular transport pathways.

ESCRT & HIV-1 budding

- Direct interactions between Gag and components of the endosomal sorting complex required for transport (ESCRT) have been identified that link endosomal protein sorting machinery to viral budding. ESCRT is made up of a complex network of interacting proteins, and disruption at a number of steps can inhibit viral budding.
- Gag–ESCRT interactions are well defined and represent a logical target for future antiretroviral therapy.

AP-3 & the role of the multivesicular body

- Gag interacts with the AP-3 heterotetrameric complex involved in trafficking of cellular proteins to the late endosome. The interaction occurs between the δ subunit of AP-3 and helix 1 of the matrix protein region of Gag.
- Disruption of the Gag–AP-3 interaction inhibits particle assembly, and the colocalization of Gag and multivesicular body (MVB) markers is prevented. This implicates AP-3 as a part of the productive particle assembly pathway, and suggests that the MVB may play an intermediate role during Gag trafficking.

Phosphoinositide phosphatidylinositol (4,5) bisphosphonate as a determinant of the site of virus assembly

- The cellular phospholipids phosphoinositide phosphatidylinositol (4,5) bisphosphonate (PI[4,5]P2) is found predominantly on the inner leaflet of the plasma membrane. Disruption of PI(4,5)P2 at this site inhibits assembly. PI(4,5)P2 may act as a triggering molecule to determine the specificity of the Gag–membrane interaction and subsequent assembly events.
- MA interaction with PI(4,5)P2 triggers a conformational change that makes the N-terminal myristic acid moiety more accessible for membrane interactions.

Env protein trafficking

- An increasing body of evidence suggests that endocytosis and recycling of Env is essential for assembly of infectious particles. Env interacts mainly with the AP-2-associated endocytic machinery through a YXXφ motif and a dileucine motif in the cytoplasmic tail.
- Tail-interacting protein (TIP)47 was recently shown to serve as a linker between Gag and Env, and to play a role in incorporation of Env onto virions. TIP47 normally functions in retrograde endosome-to-TGN transport.

Role of Vpu in trafficking of viral or cellular factors

- Vpu enhances release of HIV-1 particles from human cells through an unknown mechanism involving the cellular recycling pathways.

Conclusions

- The pace of discovery in the trafficking of structural proteins of HIV-1 is accelerating.
- The precise order in which Gag reaches endosomal membranes/MVB versus the plasma membrane remains debated. Advanced live cell imaging techniques should clarify this area.
- Opportunities for new targets for the development of antiretroviral drugs exist at numerous points along the assembly pathway. The most logical targets at present are the direct interactions of discrete motifs within Gag or Env and the cellular binding partners.

References

Authors should focus on recent papers and papers older than 5 years should not be included except for an over-riding purpose.

References should be denoted numerically and in sequence in the text, using Arabic numerals placed in square brackets, i.e., [12].

Please note: A maximum of 20 references are permitted in Editorials, Priority Paper Evaluations and Conference Scenes.

Format

- Author's names should appear without full stops in their initials
- Quote first six authors' names. If there are more than six, then quote first three *et al*
- A full stop follows authors' names.
- Journal name should be in italics and abbreviated to standard format
- Volume number followed by comma, not bold
- Page number range separated by a hyphen with no spaces, followed by the year in brackets, and then a full stop

Examples

Journal example:
Fantl JA, Cardozo L, McClish DK *et al*. Estrogen therapy in the management of urinary incontinence in postmenopausal women: a meta-analysis. *Obstet. Gynecol.* 83(1), 12–18 (1994).

Book example:

De Groat WC, Booth AM, Yoshimura N. Neurophysiology of micturition and its modification in animal models of human disease. In: *The Autonomic Nervous System (Volume 6)*. Andrews WR (Ed.), Harwood Academic Publishers, London, UK, 227–289 (1993).

Meeting abstract example:

Smith AB, Jones CD. Recent progress in the pharmacotherapy of diseases of the lower urinary tract. Presented at: *13th International Symposium on Medicinal Chemistry*. Atlanta, GA, USA, 28 November–2 December 1994.

Patent example:

Merck Frosst Canada, Inc. WO9714691 (1997).
(Use the following formats for patent numbers issued by the World, US and European patent offices, respectively: WO1234567, US1234567, EP-123456-A).

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