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Mechanisms underlying Trastuzumab Resistance in HER2-positive (HER2+) Breast Cancer and potential therapeutic approaches

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ABSTRACT

HER2-positive (HER2+) breast cancer is characterized by overexpression of HER2 (Human Epidermal Growth Factor Receptor 2) protein on the surface of cells in the breast's ducts or lobes. HER2 overexpression creates an overabundance of homo- and/or heterodimers with other family members, and triggers activation of PI3K/Akt and MAPK signalling pathways, resulting in uncontrolled cell growth and proliferation. Past studies have shown that HER2 overexpression correlates with aggressiveness, higher relapse risk, and poor long-term survival. The incorporation of the monoclonal antibody trastuzumab into chemotherapy has dramatically improved treatments. Trastuzumab prevents HER2 homo- and/or heterodimerization, and promotes HER2 endocytosis, ubiquitination, and proteolytic degradation, resulting in inhibition of the HER2 signalling pathway and HER2-induced cellular responses. However, trastuzumab resistance which mainly develops in the metastatic setting, is a major clinical problem. This bibliographical review primarily focuses on presenting three molecular mechanisms, through which trastuzumab resistance arises leading to continuous activation of the HER2 signalling pathway. The first mechanism involves overexpression of CMTM6, leading to decreased HER2 ubiquitination, resulting in increased cell viability, proliferation and invasion, and decreased apoptosis. The second mechanism involves underexpression of CMTM7, caused by *miR-182-5p* and leading to Rab-5A inactivation, resulting in the prevention of HER2 degradation. The third mechanism involves the overexpression of TRAF4 E3 ubiquitin ligase that prevents SMURF2 E3 ubiquitin ligase from interacting and ubiquitinating HER2, resulting in continuous activation of the HER2 signalling pathway and increased cell viability and tumour volume. In addition, this review discusses several therapeutic approaches to overcome trastuzumab resistance, including a) the use of adavosertib to reduce CMTM6 expression, b) the use of the *miR-182-5p* inhibitor to prevent the reduction of CMTM7 expression and c) β-escin, as an alternative drug. Currently, approved therapies are focused on the use of trastuzumab in combination with pertuzumab or ado-trastuzumab emtansine (T-DM1) antibodies. This review showed that continued research to identify the optimal combined therapy holds promise for enhancing overall survival in HER2+ breast cancer patients.

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SEMINAR ANNOUNCEMENT

University of Cyprus
Department of Biological Sciences

BIO 680 Scientific Methodology in Molecular Biology

Student Presentation

Wednesday, 13 December 2023 at 09:00
Building FST02, Room B172, Panepistimioupoli Campus

This seminar is open to the public

Troodia Theodorou

Thesis Supervisor: Special Teaching Staff, Dr. Annita Charalambous

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1. INTRODUCTION

Cancer is one of the most life-threatening diseases worldwide (Siegel et al. 2023). Mounting evidence (e.g., GLOBOCAN, 2020) suggests that there were more than 19 million new cancer cases and roughly 10 million cancer deaths in 2020. With a reported incidence of 2.3 million new cases (11.7%), female breast cancer is the most often diagnosed type of cancer, followed by lung (11.4%), colorectal (10.0%), prostate (7.3%), and stomach (5.6%) cancers (Sung et al. 2021). Remarkably, since the middle of the 2000s, the incidence rates of breast cancer have been gradually rising by approximately 0.5% per year (Siegel et al. 2023).

1.1 Breast Cancer Epidemiology and Aetiology

Each year, the American Cancer Society counts the number of new cancer cases and deaths in the United States. Recently, 297,790 female residents of the United States were diagnosed with breast cancer, confirming this disease as the most frequent type of cancer among women and the second cause of cancer death behind lung cancer (Siegel et al. 2023). Women have one in eight lifetime risk of developing breast cancer, and despite substantial advancements in the diagnosis and treatment of breast cancer, 3% of women die from the disease (Giaquinto et al. 2022). Interestingly, the mortality rate of the disease peaked in 1989 and has since dropped by 43% (Siegel et al. 2023).

According to studies, modifiable risk factors cause 30% of breast cancer incidences. Breast cancer incidences are increased with age: 0.6% of women aged 40 years and younger have an increased risk of developing breast cancer, which rises to 4% between the ages of 40 and 59, and to 10.6% beyond the age of 60 (Giaquinto et al. 2022). Apart from age, risk factors for breast cancer development comprise smoking, excess body weight, alcohol consumption, poor diet, physical inactivity, delayed menarche, late childbirth age or lactation failure, late menopause age, Ultraviolet radiation, and infections (Feng et al. 2018, Kashyap et al. 2022). Breast cancer development is also associated with family history because of mutations in cancer-related genes inherited from a parent, such as ATM, TP53, CHEK2, PTEN, CDH1, STK11, PALB2, PIK3CA, MYC, CCND1, ERBB2, ZNF703/FGFR1 locus, GATA3, RB1, MAP3K1, and the most well-known BRCA1 and BRCA2 (Nass, Kalinski 2015, Feng et al. 2018).

1.2 Breast Cancer Biology and Classification

A British surgeon and anatomist, named Sir Astley Paston Cooper, was the first that studied the female breast anatomy. The breast that is fastened to the chest wall, is composed of the external skin and the internal structures (see description below). The physiological development and growth of the breast are associated with the primary female steroid hormones, estrogen and progesterone, to which femininity, fertility, and motherhood are due too. Throughout adolescence, the breast is scheduled to develop functional tissue, which is made up of adipose (fatty) tissue and the functional glandular tissue lobes, or terminal ductal lobular units (TDLU). The lobes are divided into smaller units, the lobules. These lobules produce milk during pregnancy and lactation, passing on out into a tubular network of ducts that flows into the nipple. In addition, the breast is comprised of lymph nodes, nerves, connective tissue, blood, and lymph vessels **(Figure 1)**. Each of these components constructs the area of tissue with a primary supporting role (McGhee, Steele 2020, Breast Cancer Prevention (PDQ®) – Patient Version, 2023).

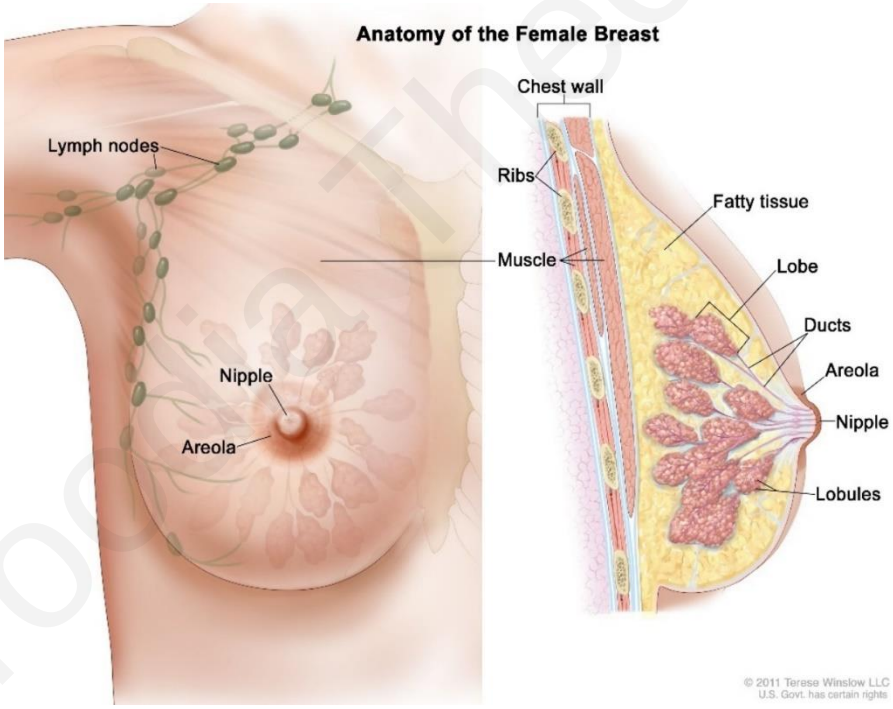

Figure 1. Anatomy of the Female Breast.

Anatomy of the female breast displaying the lymph nodes, nipple, and areola. Cross-section of the female breast displaying the fatty tissue, areola, nipple, ducts, lobes, and lobules that are arranged with respect to the chest wall. Adapted from: Breast Cancer Prevention (PDQ®)–Patient Version, 2023

While the minority of breast cancers (approximately 20%) are developed in the functional glandular tissue lobes or TDLU, the majority (approximately 80%) are developed in the ducts (Institute of Medicine, Committee to Review the Department of Defense's Breast Cancer Research Program 1997). Regarding how breast cancer subtypes are developed, there are presently two models. The first model contends that each tumour subtype develops from a unique cell of origin, whereas the second model contends that the cell of origin may be the same for distinct tumour subtypes (Polyak 2007). Also, breast cancer at any given time displays heterogeneity for a variety of tumour-related features, including angiogenic, invasive, and metastatic potential. Heterogeneity is divided into a) intratumor heterogeneity which refers to the different cell types within the same tumour; and b) intertumor heterogeneity which refers to the different cell types within the same subtype of tumour in various patients (Polyak 2011). Given the heterogeneity of breast cancer, this is classified according to four criteria: **stage**, **histopathology**, **grade**, and **receptor status**.

Similar to other types of cancer, the **stage** of breast cancer is determined by the TNM (tumour, node, metastasis) system published by the American Joint Committee on Cancer (AJCC). It incorporates the tumour size (T1 to T4), the number and location of lymph nodes involved (N0 to N3), and the metastatic capability (M0 or M1) (Singletary, Connolly 2006). Breast cancer has five stages, numbered from 0 to 4, or 0, I, II, III, and IV. Stage 0 is the non-invasive stage of the tumour, which means both cancerous and non-cancerous cells are in the region of the breast where the tumour first appears, and there has been no invasion to the tissues around that region. In contrast, stage 1 is the invasive stage of the tumour. It is divided into two categories: stage 1A and stage 1B. Stage 1A refers to tumours up to 2 cm in size with no lymph nodes implicated, whereas stage 1B refers to a small cluster of cancer cells larger than 0.2 mm in a lymph node (Akram et al. 2017). Likewise, stage 2 is divided into two categories: stage 2A and stage 2B. In stage 2A, the tumour is identified in the axillary lymph nodes and not in the breast; and the tumour size may range from less than 2 cm to more than 5 cm. However, in stage 2B the tumour may exceed 5 cm without reaching the axillary lymph nodes (Moran et al. 2014). Stage 3 is divided into three categories: stage 3A, stage 3B, and stage 3C. Stage 3A depicts a tumour that cannot be found in the breast but can be found in 4 to 9 axillary lymph nodes, whereas stage 3B depicts a tumour of any size that can cause an ulcer on the breast's skin and can be found up to 9 axillary lymph nodes. Stage 3C is the extension of the tumour to 10 or more axillary lymph nodes (Akram et al. 2017). Further extension of the tumour to other

body organs, such as the lungs, bones, liver, and brain, is the advanced stage of breast cancer, stage 4 (Neuman et al. 2010).

From a **histopathological** perspective, breast cancer is categorized as lobular carcinoma *in situ* (LCIS), invasive lobular carcinoma (ILC), ductal carcinoma *in situ* (DCIS), and invasive ductal carcinoma (IDC) according to the microanatomy of the breast tissue that provides evidence about the number, the organization, and the location of the cancer cells **(Figure 2)** (Nascimento, Otoni 2020, Hameed et al. 2022). DCIS is widely acknowledged as a precursor to IDC breast cancer type (GUMP et al. 1987, Allred et al. 2001) and it is characterized by the proliferation and the accumulation of malignant cells in the lumen of the ducts without penetrating the surrounding tissues (Schnitt et al. 1988, Burstein et al. 2004), opposed to IDC that is characterized by the invasion of cancer cells in the surrounding tissues (van't Veer et al. 2005, Siziopikou 2013). These traits are present respectively in the LCIS and ILC breast cancer types which are developed in the lobules (Hanby, Hughes 2008).

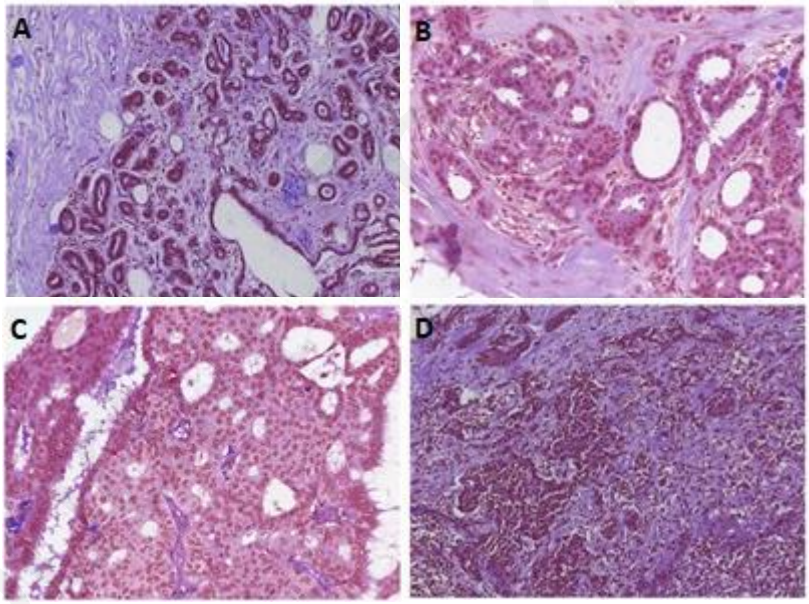

Figure 2. Histopathological characteristics of breast cancer.

Hematoxylin and Eosin (H/E) stained normal tissue (A), benign tumour (B), *in situ* carcinoma (C), and invasive carcinoma (D). Adapted from: Hameed et al. 2022

Based on histopathological parameters established by pathologists, the **grade** of breast cancer is a strong indicator of the tumour's aggressiveness. Tumour cells can be classified as being well-differentiated (low grade - I), moderately differentiated (intermediate grade - II), and poorly differentiated (high grade - III); and the latter has the worst prognosis. The gradual

decrease of differentiation grade leads cells to the loss of morphological architecture, the uniformity of the nucleus, and uncontrolled proliferation (Elston, Ellis 2002).

Lastly, breast cancer is categorized based on **receptor status** in five categories: Luminal A, Luminal B, HER2-positive (HER2+), triple-negative, and Claudin-low based on the presence of estrogen (ER), progesterone (PgR), and HER2 receptors expressed on cells' membrane, and the presence of claudins, a class of cell adhesion molecules expressed in tight junctions (Botstein et al. 2000, Sørlie et al. 2001, Herschkowitz et al. 2007). In Luminal A and Luminal B tumours, cells are closed to the duct lumen, thus named "luminal" (Lam et al. 2014). Both subtypes overexpress the estrogen and/or progesterone receptors, whilst only the Luminal B subtype expresses the HER2 receptors (Carey et al. 2006, Blows et al. 2010, Kennecke et al. 2010, Ades et al. 2014). Besides that, Luminal B tumours are extremely proliferative and aggressive compared to Luminal A tumours (De Azambuja et al. 2007). In comparison to Luminal subtypes, the HER2+ subtype lacks the expression of estrogen and progesterone receptors and overexpresses HER2 receptors. In the triple-negative subtype, estrogen, progesterone, and HER2 receptors are not expressed, thus named "triple-negative". Similarly, to the triple-negative subtype, the Claudin-low subtype lacks the expression of estrogen and progesterone receptors, and the overexpression of HER2 receptors (Blows et al. 2010). As its name implies, the Claudin-low subtype expresses less claudin (Perou 2011).

1.3 Breast Cancer Diagnosis and Therapy

Breast Cancer and its subtypes are diagnosed by combining a clinical examination, an ultrasound, mammography, or other imaging techniques, such as magnetic resonance imaging (MRI), with a histopathological analysis of a lesion biopsy (Gentilini et al. 2020). Immediately following a breast cancer diagnosis, the patient receives multiple therapies, including surgery combined with chemotherapy and/or radiation therapy (Shien, Iwata 2020). Both latter therapies exhibit high toxicity and low selectivity, thus the patient needs to receive a therapy combined with specific actions, such as hormone therapy, targeted therapy, or immunotherapy (Widakowich et al. 2007, Shien, Iwata 2020). Such therapies rely on the use of hormones, monoclonal antibodies, signal transduction inhibitors, gene expression modulators, and angiogenesis inhibitors (Tobin et al. 2015, Furukawa et al. 2020).

1.4 HER2-positive (HER2+) Breast Cancer

The HER2+ breast cancer subtype that is the focus of this dissertation; is an example of a breast cancer subtype that has been treated with targeted therapy. HER2 protein is normally expressed at low levels in the breast's ductal tissue or functional glandular tissue lobes, whereas when it is overexpressed, HER2+ breast cancer is developed (Dedić Plavetić et al. 2012). Evidence from immunological staining verifies the high levels of HER2 protein in the breast cancer tissue, as opposed to its low levels in the normal breast tissue (Monzen et al. 2020). In most cases, somatic mutations in the HER2 gene, or genes that regulate the expression of the HER2 gene, are linked to the mechanism by which HER2 is overexpressed (Rubin, Yarden 2001). More specifically, the somatic mutations within the Tyrosine Kinase domain of the HER2 gene cause HER2 overexpression and overactivation (Galogre et al. 2023). The majority of cases (50%) are in situ HER2+ carcinomas, while the minority of cases (20%) are invasive HER2+ carcinomas (Dedić Plavetić et al. 2012).

1.4.1 HER2 Biology and Clinical Significance

Human Epidermal Growth Factor Receptor 2 (HER2) is a member of the Human Epidermal Growth Factor Receptors (HER) family of cell surface receptor tyrosine kinases (RTKs), along with HER1, HER3, and HER4. As the HER family's amino acid sequences are very similar to those of the EGFR (epidermal growth factor receptor) family, the members of the HER family are also known as Erbb1 (HER1), Erbb2 (HER2), Erbb3 (HER3), and Erbb4 (HER4) (Davoli et al. 2010). The HER2 receptor, 185 kDa, is encoded by the HER2 proto-oncogene found on the long arm of chromosome 17q21 (Coussens et al. 1985). It is structured by the extracellular ligand binding domain, the transmembrane domain, and the intracellular tyrosine kinase domain **(Figure 3)** (Gaibar et al. 2020).

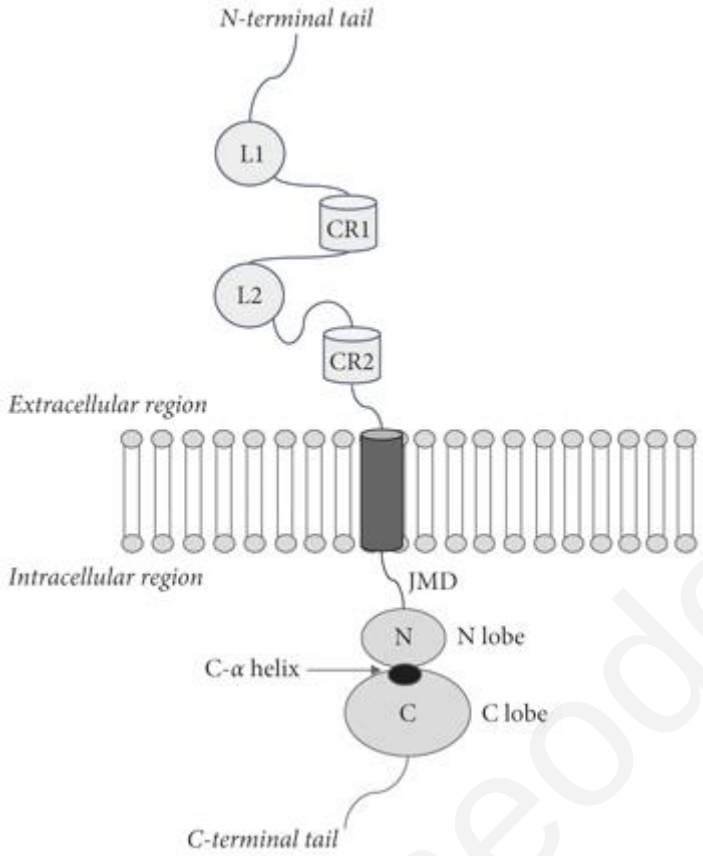

Figure 3. Structure of HER2 receptor protein.

HER2 receptor protein is structured by the N-terminal tail, the extracellular ligand binding domain (L1-L2), the extracellular cysteine-rich domain (CR1-CR2), the transmembrane domain, the intracellular tyrosine kinase domain, and the C-terminal tail. Adapted from: Gaibar et al. 2020

When the HER2 receptor exists as monomer is inactive (Galogre et al. 2023). Upon growth factor ligands binding to the extracellular ligand-binding domains, the HER2 receptor is activated by homo- and/or heterodimerization with other family members, followed by transphosphorylation of their intracellular tyrosine kinase domains (Dedić Plavetić et al. 2012, Du, Lovly 2018). Numerous intracellular proteins are docked at these phosphorylated tyrosine residues, activating an array of signalling pathways, such as the PI3K/Akt and MAPK, resulting in numerous cellular responses, such as cell growth, motility, cell proliferation, and survival (Tai et al. 2010, Du, Lovly 2018, Ding et al. 2019, Roviello et al. 2021). In detail, phosphoinositide 3-kinase (PI3K) phosphorylates phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)- triphosphate (PIP3), which serves as a docking site for proteins that possess the pleckstrin homology (PH) domain, such as Akt. Subsequent phosphorylation of Akt activates mTOR. Notably, PTEN is a negative regulator of PI3K/Akt signaling, because of PIP3 dephosphorylation to PIP2. Further to this,

the Mitogen-Activated Protein Kinase (MAPK) pathway is initiated by the Growth Factor Receptor-bound protein 2 (GRB2) binding to guanine nucleotide exchange factor Son of Sevenless (SOS), which exchanges guanosine diphosphate (GDP) from RAS to guanosine-5′-triphosphate (GTP), thus RAS becomes active. Then, RAS phosphorylates and activates Raf-1, which phosphorylates and activates MEK. MEK in turn phosphorylates and activates the extracellular signal-regulated kinase ERK **(Figure 4)** (Roviello et al. 2021).

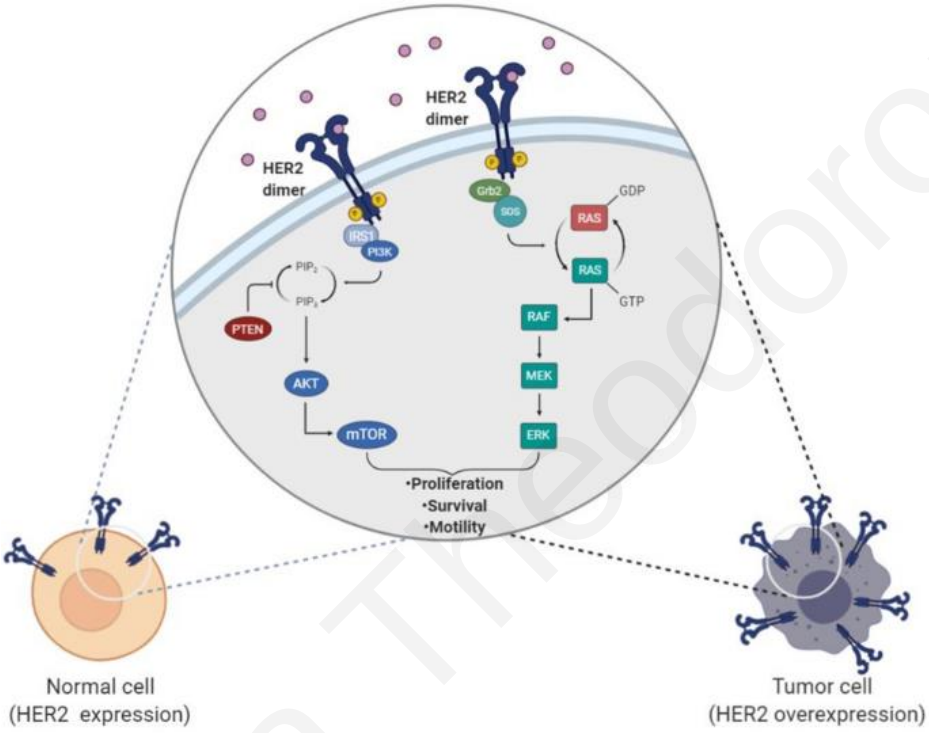

Figure 4. HER2 signaling pathway.

HER2 receptor activation by extracellular ligands, triggers PI3K/Akt and MAPK signaling pathways, resulting in cell proliferation, survival, and motility. Adapted from: Roviello et al. 2021

As previously stated, carcinogenesis is induced by the overexpression of the HER2 receptor, which creates an overabundance of homo- and/or heterodimers with other family members, resulting in increased activation of PI3K/Akt and MAPK signalling pathways, a fact that is verified by the overphosphorylation of themself and the cellular substrates. Consequently, it causes uncontrolled cell growth and proliferation (Galogre et al. 2023).

The clinical significance of the HER2 receptor was defined in the early 1980s. Evidence suggests that the *neu* oncogene discovered in rats is the mutationally active homologous to the HER2 receptor protein (Shih et al. 1981). Afterwards, the human homolog was discovered (Coussens et al. 1985, Semba et al. 1985) in a breast cancer cell line, where it was

overexpressed (King et al. 1985). Based on this evidence, Slamon and teammates examined the HER2 expression in multiple human breast cancer samples, concluding that HER2 overexpression is correlated with aggressive disease, higher relapse risk, and poor long-term survival (Slamon et al. 1987, Pegram et al. 2000).

1.4.2 Monoclonal Antibody Trastuzumab (Herceptin®)

Given the crucial role of HER2 in breast cancer development, extensive research has focused on HER2-targeted therapy (Tai et al. 2010). The United States Food and Drug Administration (FDA) and Health Canada agencies have approved the use of a recombinant humanized monoclonal antibody (mAb), named Trastuzumab (also known as Herceptin), for HER2-targeted therapy (Leahy et al. 2003, Blumenthal et al. 2013). Earlier than that, *in vitro* and *in vivo* experiments of the murine monoclonal antibody (muMAb) precursor 4D5 revealed the therapeutic efficacy of trastuzumab for HER2+ breast cancer (Fendly et al. 1990, Michael Shepard et al. 1991, Park et al. 1992). Though, a preclinical study in monkeys, showed that the pharmacokinetics of muMAb4D5, the monkey anti-mouse antibody, were affected (Shepard et al. 2008). A further preclinical study in humans showed that the muMAb4D5, the human anti-mouse antibody response was effective (Slamon 2000). Subsequently, the humanized version of muMAb4D5, named Trastuzumab, was developed (Carter et al. 1992). Particularly, trastuzumab binds to the juxtamembrane domain of the HER2 receptor protein. Even though its mechanism of action is unknown, proposed mechanisms include the prevention of HER2 homo- and/or heterodimerization with other family members, resulting in cell growth and proliferation inhibition, induced by the HER2 signalling pathway; and the HER2 endocytosis, ubiquitination, and proteolytic degradation, resulting in downregulation of HER2 activity (Magnifico et al. 1998, Pohlmann et al. 2009, Scaltriti et al. 2011, Dedić Plavetić et al. 2012, Wang et al. 2014, Wymant et al. 2020). Evidence suggests that the overall survival rate is improved while the recurrence risk is reduced, both by 33%, when trastuzumab is added to chemotherapy in patients with early-stage HER2+ breast cancer. Overall, trastuzumab reacts positively with multiple chemotherapy drugs (Dedić Plavetić et al. 2012).

1.4.3 Trastuzumab Resistance

Trastuzumab has significantly improved the clinical outcome, in both early-stage and metastatic HER2+ breast cancer (Mazzotta et al. 2019). However, an intrinsic or *de novo* trastuzumab resistance which mainly is developed in the metastatic setting, is a major clinical problem (Dedić Plavetić et al. 2012, Lavaud, Andre 2014). Therefore, it is important to investigate the mechanisms of trastuzumab resistance, by examining samples from HER2+ breast cancer patients that received HER2-targeted therapy, or by building *in vitro* cell line models that are resistant to the drug. This dissertation presents three molecular mechanisms by which trastuzumab resistance arises and discusses several therapeutic approaches to overcome trastuzumab resistance.

2. OVERVIEW

Several potential mechanisms of trastuzumab resistance have been described in the literature (Zazo et al. 2016). These include resistance due to HER2 mutations (Sun et al. 2015) and dimerization of the HER2 receptor with either Insulin-like Growth Factor-1 Receptor (IGF1R) (Lu et al. 2001) or c-Met receptor (Shattuck et al. 2008). Resistance has also been shown to arise following the binding of co-chaperones cell division cycle 37 (Cdc37) and heat shock protein 90 (Hsp90), or SH3 domain-binding glutamic acid-rich (SH3BGR) protein to HER2, resulting in the prevention of HER2 degradation and internalization (Pearl 2005, Li et al. 2020). An alternative mechanism leading to trastuzumab resistance via the CMTM6 protein has been described recently by Cao et al. (Cao et al. 2023). In the next sections, three mechanisms by which trastuzumab resistance arises, including the one that is linked to the CMTM6 protein, will be described in detail.

2.1 Mechanism 1: CMTM6 promotes trastuzumab resistance

2.1.1 CMTM6 expression in breast cancer and its subtypes

CMTM6 (chemokine-like factor – like MARVEL transmembrane domain-containing 6) as its name implies, is a member of the chemokine-like factor (CKLF) – like MARVEL transmembrane domain-containing (CMTM) family, that is expressed in almost all tissues. It can also be located in the cytoskeleton, cytoplasm, lysosomes, and endosomes (Burr et al. 2017, Mezzadra et al. 2017, Mamessier et al. 2018, Ribas, Wolchok 2018, Wu et al. 2020). CMTM6 is overexpressed in multiple types of cancer, such as glioma (Guan et al. 2018), head and neck cancer (Chen et al. 2020), lung cancer (Wang et al. 2020), liver cancer (Liu et al. 2021), colon cancer, and prostate cancer (Xing et al. 2023). A recent study, comparing non-tumour breast tissues and breast tumour tissues, indicated that CMTM6 is also overexpressed in breast tumour tissues. A follow-up analysis in Luminal A, Luminal B, HER2+, trastuzumab-resistant HER2+, and triple-negative breast cancer cell lines, indicated that mRNA and protein levels of CMTM6 were higher in trastuzumab-resistant HER2+ breast cancer cell line than its mRNA and protein levels in other breast cancer cell lines. Remarkably, mRNA and protein levels of CMTM6 were greatly lower in the HER2+ breast cancer cell line, than its mRNA and protein levels in the trastuzumab-resistant HER2+ breast cancer cell line. mRNA levels of CMTM6 were quantified by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and its protein levels were identified by Western Blot **(Figure 5A, B)** (Xing et al. 2023).

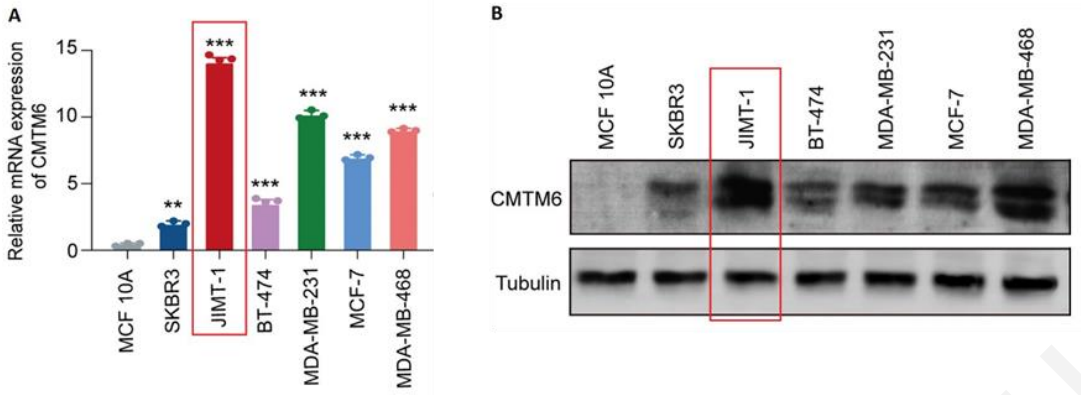

Figure 5. CMTM6 mRNA and protein levels in Luminal A, Luminal B, HER2+, trastuzumab-resistant HER2+, and triple-negative breast cancer cell lines, by qRT-PCR and Western Blot.

A. mRNA levels of CMTM6 were higher in the trastuzumab-resistant HER2+ breast cancer cell line (JIMT-1) than its mRNA levels in other breast cancer cell lines. mRNA levels of CMTM6 were greatly lower in HER2+ breast cancer cell line (SKBR3), than its mRNA levels in JIMT-1 cell line (**$P < 0.01$, ***$P < 0.001$). MCF 10A is a breast epithelial cell line; BT-474 is a Luminal B breast cancer cell line; MDA-MB-231 is a triple-negative breast cancer cell line; MCF-7 is a Luminal A breast cancer cell line; and MDA-MB-468 is a triple-negative breast cancer cell line. **B.** Protein levels of CMTM6 were higher in the JIMT-1 cell line than its protein levels in other breast cancer cell lines. Protein levels of CMTM6 were greatly lower in the SKBR3 cell line than its protein levels in the JIMT-1 cell line. Tubulin was used as a loading control for protein normalization. Adapted from: Xing et al. 2023

The mRNA and protein levels of CMTM6 were also high in triple-negative breast cancer cell lines **(Figure 5A, B)** (Xing et al. 2023). Similarly, Shi et al. showed the high expression of CMTM6 by immunohistochemical (IHC) analysis, in triple-negative breast cancer (Shi et al. 2022). CMTM6 in triple-negative breast cancer is required for the preservation of programmed death-ligand 1 (PD-L1) expression (Burr et al. 2017). PD-L1 binds to the programmed cell death protein 1 (PD1) on T cells, to suppress T cells proliferation, activation, and cytotoxic secretion (Gou et al. 2020). Shi et al. also showed the high expression of PD-L1, which was positively related to the high expression of CMTM6. Both proteins were correlated with tumour size, lymph node metastasis, and proliferation of triple-negative breast cancer cells (Shi et al. 2022). Therefore, CMTM6 regulates anti-tumour immunity, preserving the PD-L1 expression in triple-negative breast cancer (Burr et al. 2017).

2.1.2 JIMT-1 cell line as a trastuzumab-resistant HER2+ breast cancer model

As mentioned, the highest CMTM6 expression was observed in the trastuzumab-resistant HER2+ breast cancer cell line (JIMT-1) (Xing et al. 2023), established by Tanner et al. The JIMT-1 cell line was the first suitable experimental model for studies of trastuzumab resistance mechanisms. It was developed from a tumour originating from a 62-year-old patient who was clinically resistant to trastuzumab. JIMT-1 cells are medium-sized epithelial cells with alterable nuclear size. Along with these characteristics, these cells display continuous growth as an adherent monolayer (Tanner et al. 2004). In addition, by qRT-PCR and IHC analyses, authors indicated that these cells present high mRNA levels of HER2 receptor, and low mRNA levels of HER1, HER3, and HER4 receptors; and they lack expression of estrogen and progesterone receptors (Junttila et al. 2003, Tanner et al. 2004). By injecting JIMT-1 cells into nude mice, they noted that 90% of animals developed xenograft tumours. Xenograft tumours were developed equally well with or without trastuzumab treatment (Tanner et al. 2004).

2.1.3 CMTM6 role in JIMT-1 cell line

Given that CMTM6 is overexpressed in the JIMT-1 cell line, it becomes vital to investigate its role (Xing et al. 2023). The role of a protein in the cell can be investigated by silencing its gene (Banaszynski et al. 2006). Hence, Xing et al. using lentiviral vectors which were able to integrate the control shRNA or CMTM6-specific shRNA into the genome of JIMT-1 cells, silenced the CMTM6 gene. The CMTM6 gene silencing was validated by qRT-PCR and Western Blot analyses **(Figure 6A)**. Following cell viability, proliferation, apoptosis, and invasion assays, they assessed the effect of CMTM6 silencing on JIMT-1 cells. All assays were performed following trastuzumab treatment. In particular, the Cell Counting Kit-8 (CCK-8) assay indicated that the viability of CMTM6-silenced JIMT-1 cells was decreased, compared to the viability of control JIMT-1 cells **(Figure 6B)**. Likewise, the ethynyl-2′-deoxyuridine (EdU) assay showed that the proliferation of CMTM6-silenced JIMT-1 cells was decreased, compared to the proliferation of control JIMT-1 cells **(Figure 6C)**. The terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay indicated that the number of apoptotic CMTM6-silenced JIMT-1 cells was higher than the number of apoptotic control JIMT-1 cells **(Figure 6D)**. Moreover, the cell invasion assay showed that the invasive CMTM6-silenced JIMT-1 cells were less than the invasive control JIMT-1 cells **(Figure 6E)** (Xing et al. 2023).

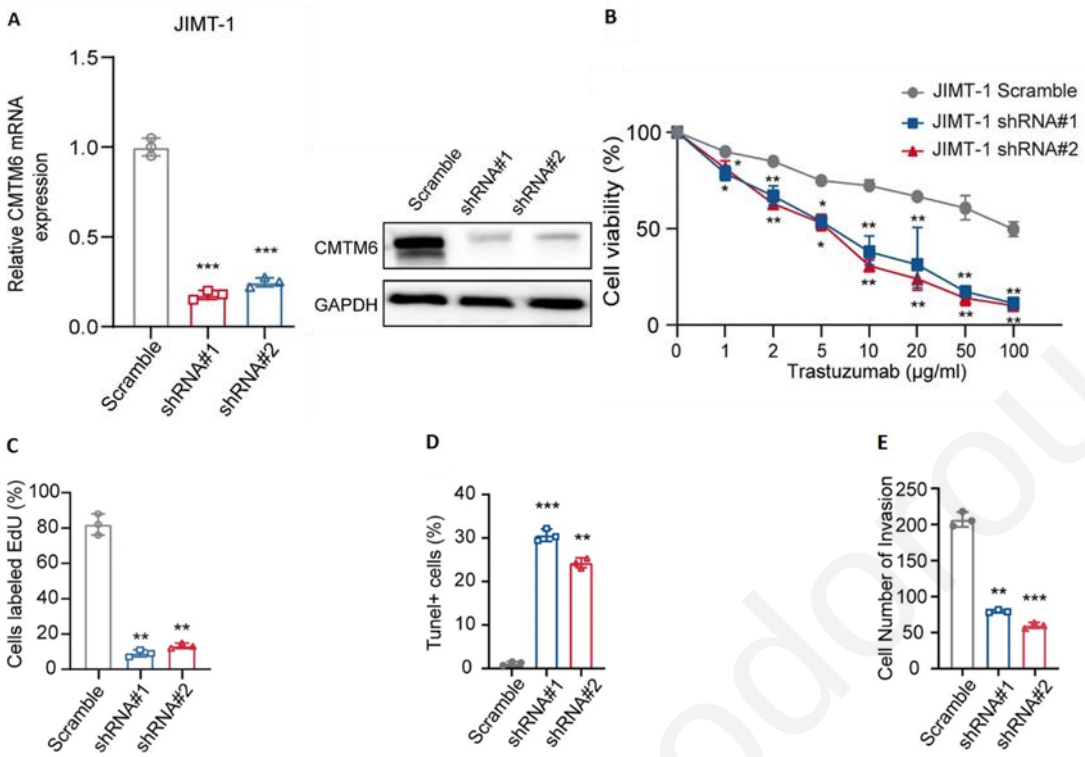

Figure 6. The viability, proliferation, apoptosis, and invasion of control JIMT-1 cells and CMTM6-silenced JIMT-1 cells, following trastuzumab treatment.

A. qRT-PCR and Western Blot validated CMTM6 gene silencing in JIMT-1 cells (***$P < 0.001$). shRNA#1 and shRNA#2 are two distinct CMTM6-specific shRNA sequences used for CMTM6 gene silencing. GAPDH was used as a loading control for protein normalization. **B.** CCK-8 assay showed that the viability of CMTM6-silenced JIMT-1 cells was decreased, compared to the viability of control JIMT-1 cells (*$P < 0.05$, **$P < 0.01$). **C.** EdU assay showed that the proliferation of CMTM6-silenced JIMT-1 cells was decreased, compared to the proliferation of control JIMT-1 cells (**$P < 0.01$). **D.** TUNEL assay showed that the number of apoptotic CMTM6-silenced JIMT-1 cells was higher than the number of apoptotic control JIMT-1 cells (**$P < 0.01$, ***$P < 0.001$). **E.** Cell invasion assay showed that the invasive CMTM6-silenced JIMT-1 cells were less than the invasive control JIMT-1 cells (**$P < 0.01$, ***$P < 0.001$). Adapted from: Xing et al. 2023

As mentioned, activation of PI3K/Akt and MAPK signalling pathways induced by the HER2 receptor, results in numerous cellular responses, such as cell proliferation and survival (Roviello et al. 2021). As Xing et al. demonstrated, these cellular responses are affected by the CMTM6 expression. This evidence suggests that CMTM6 may be correlated with the HER2 receptor, and the signalling pathways induced by it. Immunofluorescence (IF) assay followed by confocal imaging, indicated the co-localization of HER2 and CMTM6 on the cell membrane **(Figure 7A)** (Xing et al. 2023). Following that, by performing a co-immunoprecipitation (Co-IP) assay followed by Western Blot, the authors demonstrated that these proteins interact with each other **(Figure 7B)** (Xing et al. 2023).

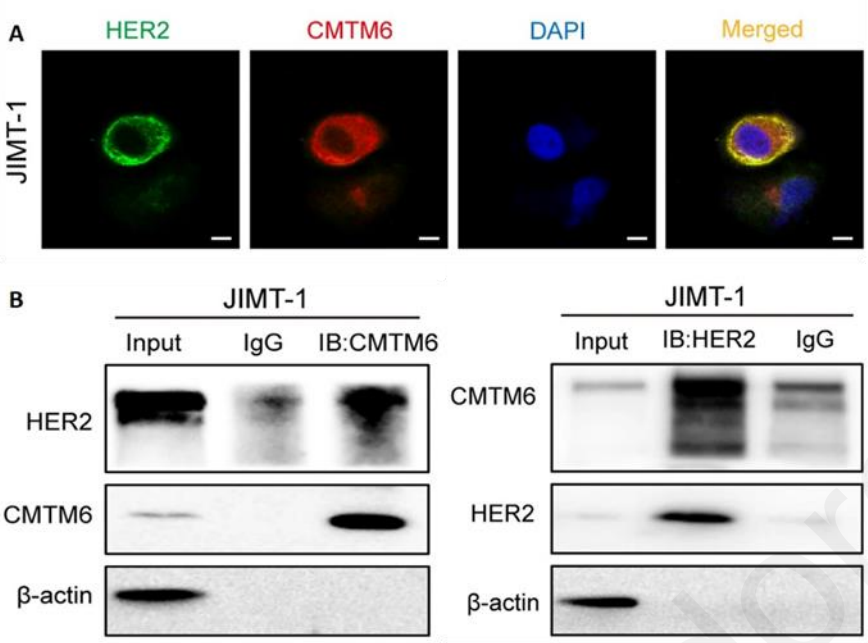

Figure 7. Co-localization and interaction of HER2 and CMTM6 proteins in JIMT-1 cells.

A. IF assay followed by confocal imaging showed that HER2 (green) and CMTM6 (red) proteins were co-localized on the cell membrane of JIMT-1 cells. DAPI is a blue fluorescent DNA stain. **B.** Co-IP assay followed by Western Blot showed that the anti-HER2 antibody precipitated CMTM6 protein (left panel) and the anti-CMTM6 antibody precipitated HER2 protein (right panel). β-actin was used as a loading control for protein normalization. Adapted from: Xing et al. 2023

Based on the observations so far, CMTM6 may be correlated with the PI3K/Akt and MAPK signalling pathways that are induced by the HER2 receptor. To investigate this, studies showed the total and phosphorylated levels of HER2 protein, and the total and phosphorylated levels of the main proteins of PI3K/Akt and MAPK signalling pathways, which are Akt and ERK respectively, in SKBR3 and JIMT-1 cells, which express CMTM6 to a high and low levels, respectively (Damiano et al. 2009, Bon et al. 2020, Xing et al. 2023). On the one hand, in SKBR3 cells, the levels of pHER2, pAkt, and pERK were lower in the presence of trastuzumab than in the absence of trastuzumab (Bon et al. 2020). On the other hand, in JIMT-1 cells, the levels of pHER2, pAkt, and pERK were high, either with or without trastuzumab (Damiano et al. 2009). An additional study in CMTM6-silenced JIMT-1 cells showed that the levels of pHER2, pAkt, and pERK were low in the presence of trastuzumab. Together, these findings suggest that CMTM6 is correlated with the HER2 signalling pathway (Xing et al. 2023).

A recent study indicated that programmed death-ligand 1 (PD-L1) is highly expressed in HER2+ breast cancer cells (Duro-Sánchez et al. 2023). As mentioned, PD-L1 binds to the programmed cell death protein 1 (PD1) on T cells, to suppress T cells proliferation, activation, and cytotoxic secretion. Consequently, HER2+ breast cancer cells switch off T cells (Zitvogel, Kroemer 2012, Gou et al. 2020). Knowing that CMTM6 interacts with PD-L1 to prevent its ubiquitination (Mamessier et al. 2018, Duro-Sánchez et al. 2023), Xing et al. examined whether the interaction of CMTM6 and HER2 results in the prevention of HER2 ubiquitination. By performing an IP assay followed by Western Blot, authors showed that HER2 was ubiquitinated less in control JIMT-1 cells than in CMTM6-silenced JIMT-1 cells **(Figure 8A)**. A follow-up Western Blot analysis of the control JIMT-1 cells and CMTM6-silenced JIMT-1 cells with MG132 proteasome inhibitor treatment (Harhouri et al. 2022, Xing et al. 2023), showed that HER2 protein levels were lower in CMTM6-silenced JIMT-1 cells than in control JIMT-1 cells, and even lower without MG132 treatment. These analyses were followed by trastuzumab treatment **(Figure 8B)** (Xing et al. 2023).

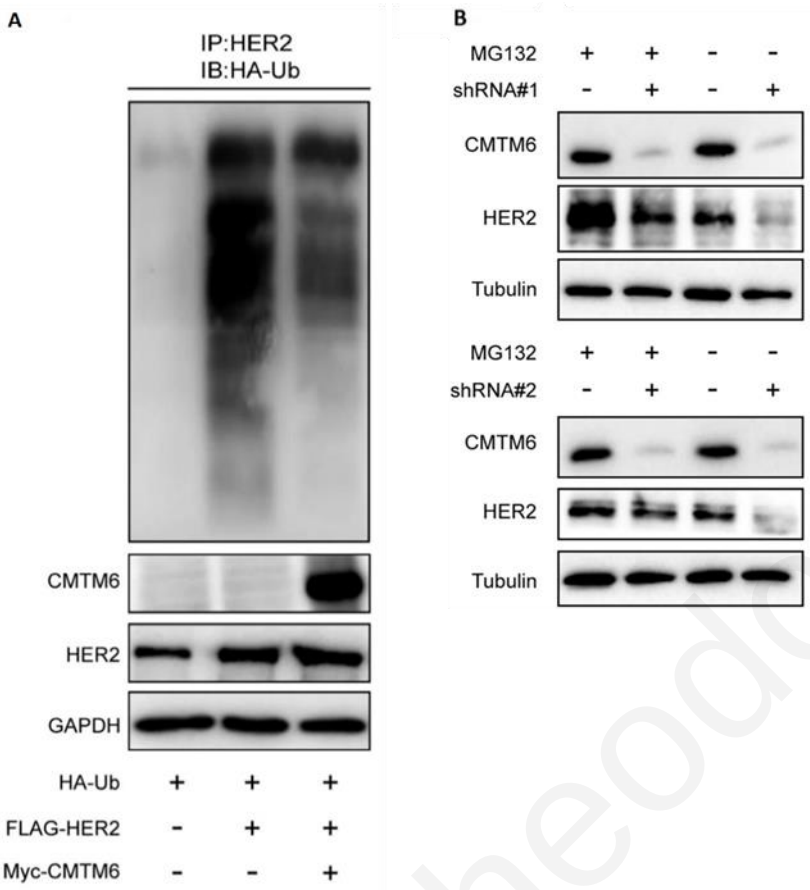

Figure 8. CMTM6 decreases HER2 ubiquitination.

A. IP assay followed by Western Blot showed that HER2 was ubiquitinated less in control JIMT-1 cells than in CMTM6-silenced JIMT-1 cells. HER2 ubiquitination was observed as a ladder pattern. HA-tag to the Ub allows the Ub to be studied with an antibody against the HA-tag sequence. FLAG-tag to the HER2 allows the HER2 to be studied with an antibody against the FLAG-tag sequence. Myc-tag to the CMTM6 allows the CMTM6 to be studied with an antibody against the Myc-tag sequence. GAPDH was used as a loading control for protein normalization. **B.** Western Blot analysis showed that HER2 protein levels were lower in CMTM6-silenced JIMT-1 cells than in control JIMT-1 cells, and even lower without MG132 treatment. shRNA#1 and shRNA#2 are two distinct CMTM6-specific shRNA sequences used for CMTM6 gene silencing. Tubulin was used as a loading control for protein normalization. Adapted from: Xing et al. 2023

To summarize, HER2 endocytosis, induced by trastuzumab, results in its ubiquitination and proteolytic degradation (Wymant et al. 2020). HER2 ubiquitination can be induced by the c-Cbl E3 ubiquitin ligase, which is recruited to the HER2 intracellular tyrosine kinase domain (Klapper et al. 2000). CMTM6, which is overexpressed in trastuzumab-resistant HER2+ breast cancer cells, decreases HER2 ubiquitination, resulting in the continuous activation of the HER2 signalling pathway and the cellular processes induced by it. Therefore, CMTM6 silencing increases apoptosis, while decreasing cell viability, proliferation, and invasion.

2.2 Mechanism 2: CMTM7 may lead to trastuzumab resistance

CMTM7 (chemokine-like factor – like MARVEL transmembrane domain-containing 7) as its name implies, is a member of the chemokine-like factor (CKLF) – like MARVEL transmembrane domain-containing (CMTM) family, that is expressed in normal tissues. In contrast, CMTM7 is underexpressed in multiple types of cancer, such as lung cancer, esophageal cancer, stomach cancer, liver cancer, and pancreatic cancer (Miyazaki et al. 2012, Wu et al. 2020). A recent study indicated that CMTM7 is also underexpressed in breast cancer (Wu 2020). A follow-up analysis in Luminal A, HER2+, and triple-negative breast cancer cell lines, indicated that mRNA and protein levels of CMTM7 were much lower in Luminal A and HER2+ breast cancer cell lines than its mRNA and protein levels in other breast cancer cell lines. mRNA levels of CMTM7 were quantified by qRT-PCR and its protein levels were identified by Western Blot **(Figure 9A, B)** (Chen et al. 2023).

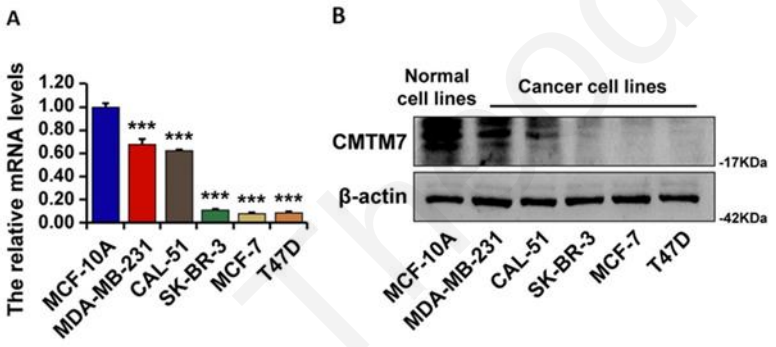

Figure 9. CMTM7 mRNA and protein levels in Luminal A, HER2+, and triple-negative breast cancer cell lines, by qRT-PCR and Western Blot.

A. mRNA levels of CMTM7 were much lower in Luminal A and HER2+ breast cancer cell lines (MCF-7 and T47D; and SK-BR-3 respectively), than its mRNA levels in other breast cancer cell lines (***$P < 0.001$). MCF 10A is a breast epithelial cell line; MDA-MB-231 and CAL-51 are triple-negative breast cancer cell lines. **B.** Protein levels of CMTM7 were much lower in MCF-7, T47D, and SK-BR-3 breast cancer cell lines, than its protein levels in other breast cancer cell lines. β-actin was used as a loading control for protein normalization. Adapted from: Chen et al. 2023

In Luminal A and HER2+ breast cancer cell lines, the low CMTM7 mRNA and protein levels result from the high levels of *miR-182-5p* (Krishnan et al. 2013, Lu et al. 2021), a 22-nucleotide-long microRNA (miRNA) loaded into the RNA-induced silencing complex (RISC) to induce CMTM7 mRNA degradation and suppress its translation (Lu et al. 2021, Samuels et al. 2023).

According to Wu et al., CMTM7 promotes the degradation of an endocytosed epidermal growth factor receptor (EGFR) by activating Rab-5A, which regulates the endosome-lysosome fusion. Thus, the EGFR-induced PI3K/Akt and MAPK signalling pathways are suppressed. In contrast, CMTM7 underexpression causes Rab-5A inactivation, resulting in the prevention of EGFR degradation and activation of PI3K/Akt and MAPK signalling pathways (Wu et al. 2019). A recent study indicated that the phosphorylated levels of the EGFR (pEGFR) and Akt (pAkt) were higher in cells expressing low levels of CMTM7 than in cells expressing high levels, because of the *miR-182-5p* inhibitor **(Figure 10)** (Lu et al. 2021).

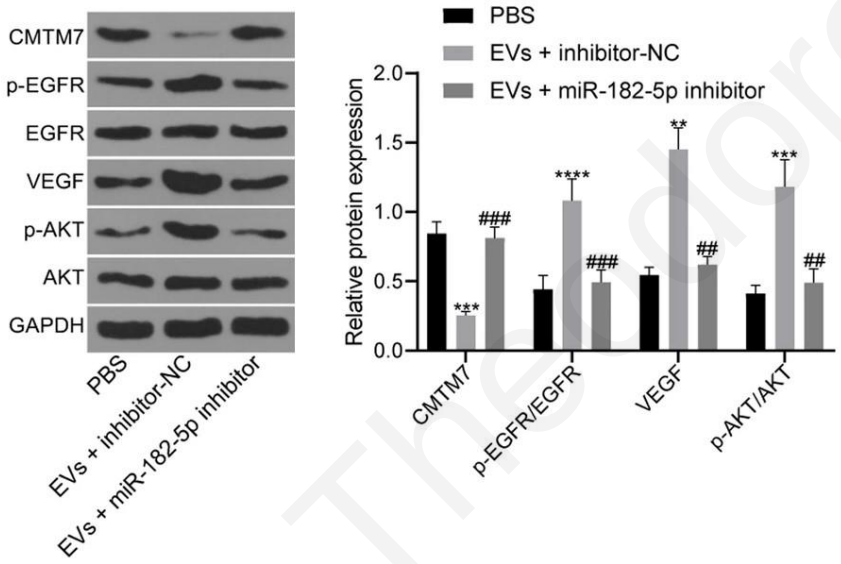

Figure 10. The phosphorylated levels of the EGFR (pEGFR) and Akt (pAkt) in cells expressing low or high levels of CMTM7, because of the *miR-182-5p* inhibitor.

Western blot analysis showed that the phosphorylated levels of the EGFR (pEGFR) and Akt (pAkt) were higher in cells expressing low levels of CMTM7 than in cells expressing high levels of CMTM7, because of the *miR-182-5p* inhibitor (**$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$). Adapted from: Lu et al. 2021

In conclusion, HER2 endocytosis that is induced by trastuzumab results in its ubiquitination and proteolytic degradation (Wymant et al. 2020). HER2 degradation can be mediated by the CMTM7. Thus, the underexpression of CMTM7 by *miR-182-5p* prevents HER2 degradation, resulting in the continuous activation of the HER2 signalling pathway. As CMTM6 decreases HER2 ubiquitination and leads to trastuzumab resistance, we hypothesize that CMTM7 may also lead to trastuzumab resistance.

2.3 Mechanism 3: TRAF4 contributes to trastuzumab resistance

2.3.1 TRAF4 expression in breast cancer and its subtypes

TRAF4 (tumour necrosis factor receptor-associated factor 4) as its name implies, is a member of the tumour necrosis factor (TNF) receptor-associated factor (TRAF) family (Wajant et al. 2001). It is structured by the carboxyl terminus, nuclear localization signal (NLS), and amino terminus. Next to the amino terminus, there is the zinc and RING finger domain; the latter finger domain has E3 ubiquitin ligase activity (Park 2018, Ruan et al. 2022). Given that, TRAF4 can cause ubiquitination followed by proteolytic degradation, resulting in the regulation of numerous cellular responses, such as cell proliferation, immunity, and apoptosis (Régnier et al. 2002, Wei Li et al. 2013). Typically, TRAF4 is expressed during embryogenesis because it is essential for neurogenesis (Régnier et al. 2002).

It is well-known that TRAF4 is overexpressed in multiple types of cancer, such as glioma (Shi et al. 2018), lung adenocarcinoma (He et al. 2022), esophageal carcinoma (Li et al. 2019), hepatocellular carcinoma (Yang et al. 2018), colorectal carcinoma (Yang et al. 2015), endometrial carcinoma (Xie et al. 2019), and prostate adenocarcinoma (Singh et al. 2018). Zhang et al., comparing non-tumour breast tissues and breast tumour tissues, indicated that TRAF4 is also overexpressed in breast tumour tissues (Zhang et al. 2014). A follow-up analysis in Luminal A, Luminal B, HER2+, and triple-negative breast cancer subtypes, indicated that mRNA levels of TRAF4 were higher in the HER2+ breast cancer subtype than its mRNA levels in other breast cancer subtypes **(Figure 11)** (Gu et al. 2022). Several studies demonstrated that TRAF4 is co-expressed with HER2 to a high level because TRAF4 and HER2 genes are close together in the q11.2 region of the long arm of chromosome 17 (Régnier et al. 1995, Tomasetto et al. 1995, Bieche et al. 1996, Zhang et al. 2013, Gu et al. 2022).

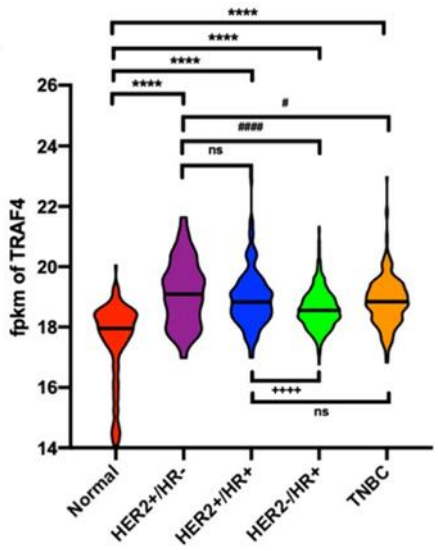

Figure 11. TRAF4 mRNA levels in breast cancer subtypes.

mRNA levels of TRAF4 were higher in the HER2+ subtype (HER2+/HR-) than its mRNA levels in other subtypes, such as Luminal B (HER2+/HR+), Luminal A (HER2-/HR+), and triple-negative (TNBC) (*$P <$ 0.05, ****$P <$ 0.0001). HR: Hormone Receptor. Adapted from: Gu et al. 2022

2.3.2 SMURF1/2 expression in breast cancer

SMAD ubiquitination regulatory factors 1 (SMURF1) and 2 (SMURF2) are two E3 ubiquitin ligases (David et al. 2013), which are expressed to a higher level in breast tumour tissues than in non-tumour breast tissues (Liu et al. 2014, Yang et al. 2018). SMURFs are structured by the carboxyl terminus HECT domain, two to three tryptophan-containing WW domains, and amino terminus C2 domain (Scheffner, Kumar 2014). Because of their structure, SMURFs interact with other proteins, via their WW domains, and with intracellular membranes via their C2 domains (Zou et al. 2015). Based on the data obtained from UbiBrowser (http://ubibrowser.ncpsb.org/), which is a bioinformatics platform for examining the human E3 ubiquitin ligase-targeted substrates, SMURF1 and SMURF2 were predicted to interact with TRAF4 and HER2 (Li et al. 2017, Gu et al. 2022).

2.3.3 TRAF4 – SMURF1/2 – HER2 interaction

A recent study performing Co-IP assay followed by Western Blot demonstrated that SMURF1 interacts with HER2 **(Figure 12)**. SMURF1 ubiquitinates HER2 at the K716 site, resulting in the reduction of HER2 protein levels (data not shown) (Ren et al. 2021). Another study performing IP assay followed by Western Blot demonstrated that SMURF1 interacts with TRAF4. SMURF1 ubiquitinates TRAF4, resulting in the reduction of TRAF4 protein levels (data not shown). Even though TRAF4 is an E3 ubiquitin ligase, there is no evidence to support that TRAF4 ubiquitinates SMURF1 (Zhang et al. 2013).

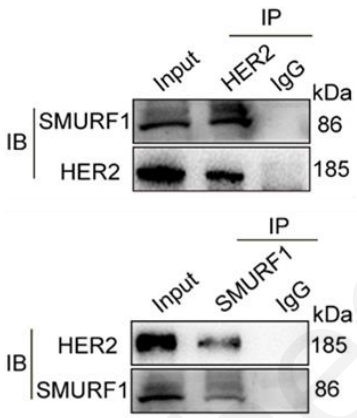

Figure 12. SMURF1 – HER2 interaction.

Co-IP assay followed by Western Blot showed that SMURF1 interacts with HER2. The anti-HER2 antibody precipitated SMURF1 protein (upper panel) and the anti-SMURF1 antibody precipitated HER2 protein (lower panel). Adapted from: Ren et al. 2021

Zhang et al. performing an IP assay followed by Western Blot demonstrated that SMURF2 interacts with TRAF4 **(Figure 13A)**. A follow-up analysis, separately deleting SMURF2's HECT, WW, and C2 domains, or combining the deletion of two of them, indicated that the SMURF2 – TRAF4 interaction is achieved through the WW and C2 domains **(Figure 13B)**. Importantly, by performing another IP assay followed by Western Blot, the authors showed that SMURF2 was ubiquitinated by TRAF4 **(Figure 13C)** (Zhang et al. 2013). TRAF4 ubiquitinates SMURF2 at the K48 site (Gu et al. 2022). In contrast, SMURF2 was not ubiquitinated by TRAF4, when TRAF4 was silenced **(Figure 13C)** (Zhang et al. 2013).

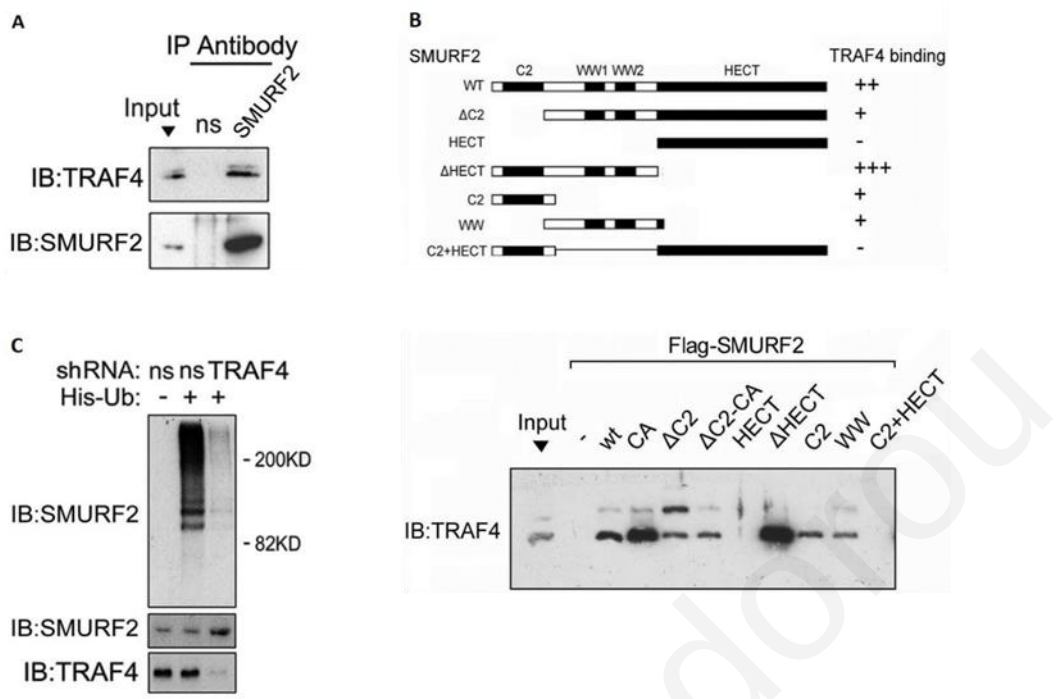

Figure 13. SMURF2 – TRAF4 interaction leads to SMURF2 ubiquitination.

A. IP assay followed by Western Blot showed that SMURF2 interacts with TRAF4. The anti-SMURF2 antibody precipitated TRAF4 protein. **B.** Truncation diagram of SMURF2 domains for TRAF4 binding assay (upper panel). IP assay followed by Western Blot showed that WW and C2 domains are required for SMURF2 – TRAF4 interaction. FLAG-tag to SMURF2 allows SMURF2 to be studied with an antibody against the FLAG-tag sequence. CA is a mutant form of SMURF2 (lower panel). **C.** IP assay followed by Western Blot showed that SMURF2 was ubiquitinated by TRAF4. SMURF2 ubiquitination was observed as a ladder pattern. SMURF2 was not ubiquitinated by TRAF4 when TRAF4 was silenced. His-tag to Ub allows Ub to be studied with an antibody against the His-tag sequence. Adapted from: Zhang et al. 2013

A recent study performing an IP assay followed by Western Blot demonstrated that SMURF2 ubiquitinates HER2 **(Figure 14)** at the K48 and K63 sites (Gu et al. 2022). Based on the above observations, the authors examined TRAF4, SMURF2, and HER2 localization. IF assay followed by confocal imaging showed that TRAF4 was located on the cell membrane and cytoplasm; SMURF2 was uniformly dispersed across the cell; and HER2 was located on the cell membrane **(Figure 15A)**. A further Co-IP assay followed by Western Blot demonstrated that these proteins interact with each other **(Figure 15B)** (Gu et al. 2022).

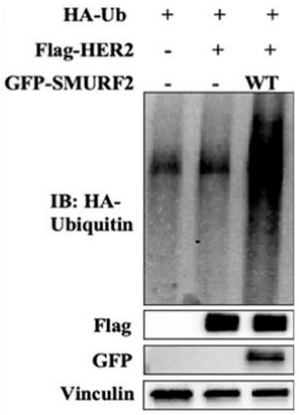

Figure 14. SMURF2 – HER2 interaction leads to HER2 ubiquitination.

IP assay followed by Western Blot showed that HER2 was ubiquitinated by SMURF2. HER2 ubiquitination was observed as a ladder pattern. HA-tag to Ub allows Ub to be studied with an antibody against the HA-tag sequence. FLAG-tag to HER2 allows HER2 to be studied with an antibody against the FLAG-tag sequence. GFP-tag to SMURF2 allows SMURF2 to be studied with an antibody against the GFP-tag sequence. Vinculin was used as a loading control for protein normalization. Adapted from: Gu et al. 2022

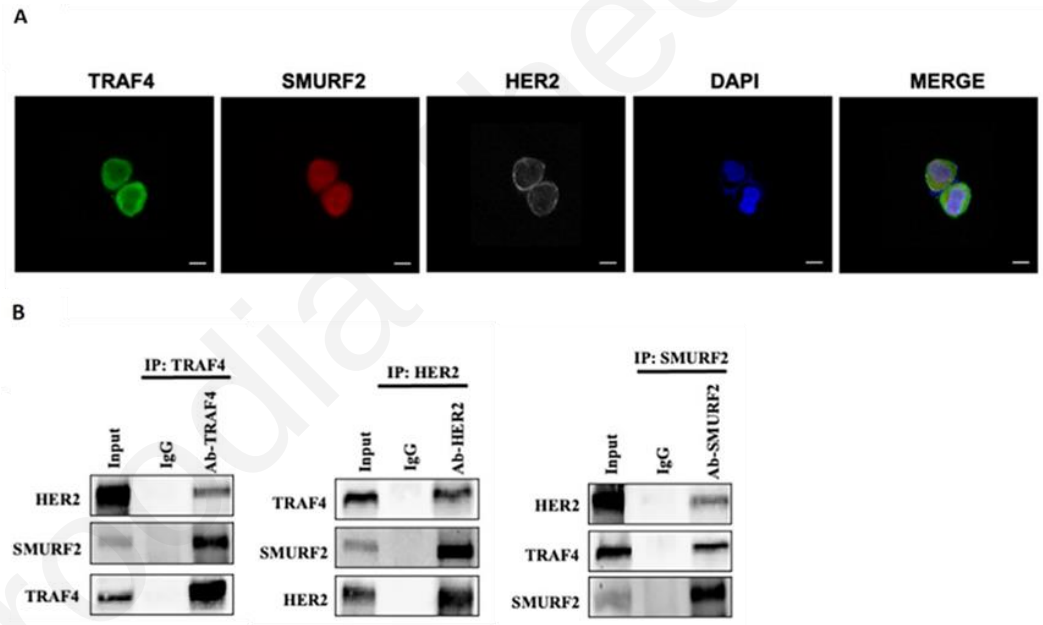

Figure 15. TRAF4 – SMURF2 – HER2 interaction on the cell membrane.

A. IF assay followed by confocal imaging showed that TRAF4 (green) was located on the cell membrane and cytoplasm; SMURF2 (red) was uniformly dispersed across the cell; and HER2 was located on the cell membrane. Blue-fluorescent dye stains DNA. **B.** Co-IP assay followed by Western Blot showed that TRAF4, SMURF2, and HER2 interact with each other. The anti-TRAF4 antibody precipitated HER2 and SMURF2 proteins, the anti-HER2 antibody precipitated TRAF4 and SMURF2 proteins, and the anti-SMURF2 antibody precipitated HER2 and TRAF4 proteins. Adapted from: Gu et al. 2022

Given these findings, a recent study examined the potential impact on signalling pathways induced by HER2 receptor. To examine this, the authors showed the total and phosphorylated levels of HER2 protein, and the total and phosphorylated levels of the main proteins of PI3K/Akt and MAPK signalling pathways, which are Akt and ERK respectively, in TRAF4-silenced and control cells. Gu et al. using lentiviral vectors that were able to integrate the control shRNA or TRAF4-specific shRNA into the genome of cells, silenced the TRAF4 gene. On the one hand, when TRAF4 was silenced, the levels of pHER2, pAkt, and pERK were low. On the other hand, when TRAF4 was expressed, the levels of pHER2, pAkt, and pERK were high (Gu et al. 2022).

Also, Gu et al. examined TRAF4-silenced and control cell viability, following trastuzumab treatment. TRAF4-silenced cell viability was lower than control cell viability **(Figure 16A)** (Gu et al. 2022). A recent study demonstrated that silencing of the TRAF4 gene in triple-negative Patient-Derived Xenograft (PDX) models, results in small tumour volume (Zhu et al. 2018). Similarly, Gu et al. demonstrated that the silencing of the TRAF4 gene in HER2+ PDX models, results in small tumor volume, which is even smaller following trastuzumab treatment **(Figure 16B)** (Gu et al. 2022).

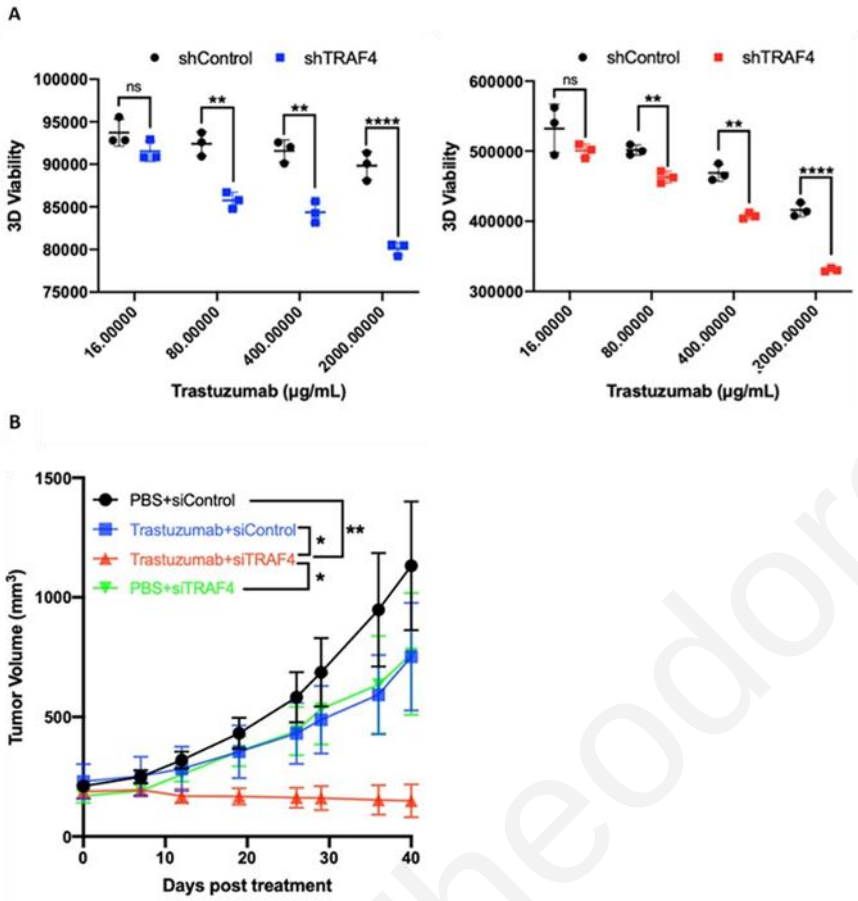

Figure 16. TRAF4 gene silencing impacts the viability of HER2+ breast cancer cells and tumour volume of HER2+ Patient-Derived Xenograft (PDX) models.

A. TRAF4-silenced cells viability (blue and red dots) was lower than control cells viability (black dots), following trastuzumab treatment (**$P < 0.01$, ****$P < 0.0001$). **B.** Tumour volume was smaller in TRAF4-silenced PDX models (green line) than tumour volume in control PDX models (black line). Tumour volume was even smaller following trastuzumab treatment (*$P < 0.05$, **$P < 0.01$). Adapted from: Gu et al. 2022

Overall, HER2 endocytosis that is induced by trastuzumab results in its ubiquitination and proteolytic degradation (Wymant et al. 2020). HER2 ubiquitination can also be induced by the SMURF2 E3 ubiquitin ligase. TRAF4 E3 ubiquitin ligase, which is overexpressed in HER2+ breast cancer cells, prevents SMURF2 from interacting and ubiquitinating HER2, resulting in continuous activation of the HER2 signalling pathway. TRAF4 silencing decreases the viability of HER2+ breast cancer cells and tumour volume of HER2+ Patient-Derived Xenograft (PDX) models.

2.4 Therapeutic approaches to overcome trastuzumab resistance

The understanding of trastuzumab resistance mechanisms in HER2+ breast cancer constitutes a key step for the identification of areas to target. A significant therapeutic target is the CMTM6 protein. A recent study indicated that tyrosine kinase WEE1 in combination with BRCA1, CREB, and TEAD1 transcription factors, are involved in the modulation of the CMTM6 expression (Jin et al. 2021). Given that, performing an IF assay followed by confocal imaging, the authors demonstrated that adavosertib (development code AZD1775) (Mortlock et al. 2017), a WEE1 inhibitor, significantly reduced the CMTM6 expression. Remarkably, a combination of trastuzumab and adavosertib eliminated the CMTM6 expression **(Figure 17)** (Jin et al. 2021).

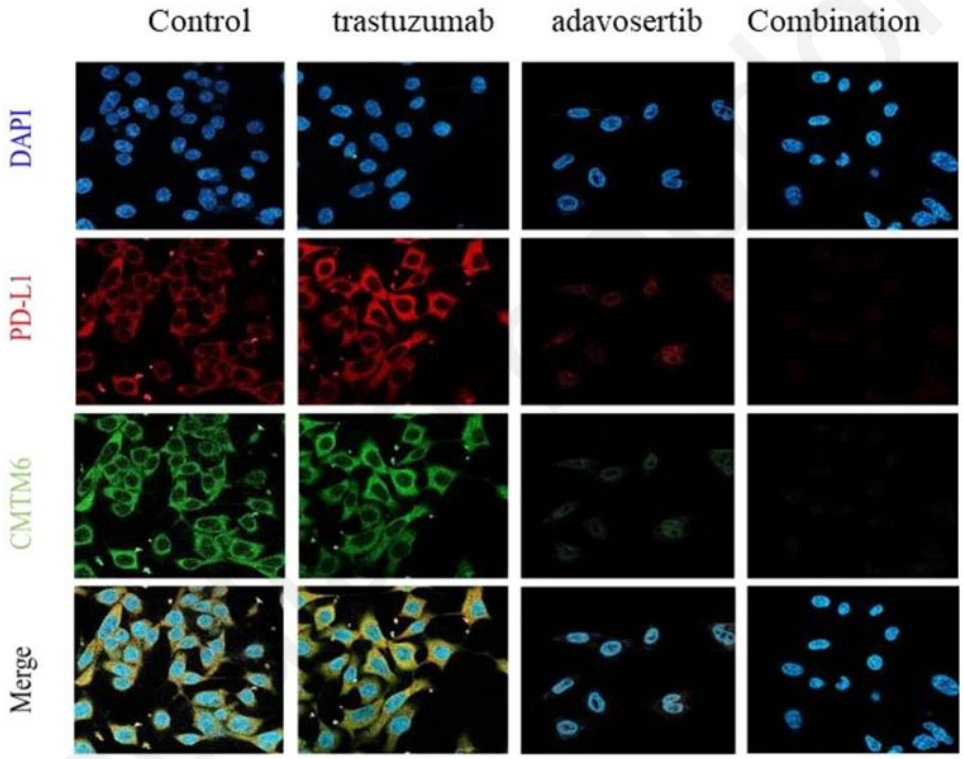

Figure 17. The expression of CMTM6 after treatment with adavosertib, or trastuzumab and adavosertib, in trastuzumab-resistant HER2+ breast cancer cells.

IF assay followed by confocal imaging showed that adavosertib significantly reduced CMTM6 expression. A combination of trastuzumab and adavosertib eliminated CMTM6 expression. Green-fluorescent dye stains CMTM6. Red-fluorescent dye stains PD-L1. Blue-fluorescent dye stains DNA. Adapted from: Jin et al. 2021

The authors also showed that adavosertib with trastuzumab significantly decreased tumour volume rather than adavosertib alone, providing additional evidence for the efficacy of this combined strategy (Jin et al. 2021).

Another significant therapeutic target is the *miR-182-5p*, which underexpresses the CMTM7 protein. A *miR-182-5p* inhibitor which is an oligonucleotide complementary to the *miR-182-5p* target, forms a duplex with it, preventing its binding to the CMTM7 target sequence (Tang et al. 2017). miRNA inhibitors can be loaded into extracellular vesicles (EVs) which enter cells via electroporation (Munir et al. 2020). Similarly, the *miR-29a* can be another significant therapeutic target. Ahmed et al. demonstrated that the up-regulation of the *miR-29a* reduced the expression of the TRAF4 E3 ubiquitin ligase, in prostate adenocarcinoma (Ahmed et al. 2013). The up-regulation of the *miR-29a* was achieved using chemically synthesised *miR-29a* mimics, which are small double-stranded oligonucleotides that match and upregulate the *miR-29a* target sequence (Ahmed et al. 2013, Caglayan et al. 2023). However, these data have not been proven in any studies of HER2+ breast cancer.

Beyond that, alternative therapeutic approaches to overcome trastuzumab resistance have been described in the literature. A recent study demonstrated that β-escin can be an attractive drug for trastuzumab-resistant HER2+ breast cancer (Park et al. 2022). β-escin isolated from Horse Chestnut (*Aesculus Hippocastanum*) has anti-inflammatory and anti-edematous effects (Gallelli 2019). In multiple types of cancer, β-escin has anti-cancer effects, by suppressing NF-kB activity, causing G2/M arrest, generating ROS, and inducing the intrinsic apoptotic pathway (Cheong et al. 2018, Paneerselvam, Ganapasam 2020, Akar et al. 2022). According to evidence, β-escin also induces the intrinsic apoptosis pathway in trastuzumab-resistant HER2+ breast cancer cells (JIMT-1), by generating ROS which causes damage to proteins, nucleic acids, lipids, membranes and organelles, resulting in the release of cytochrome c from mitochondria and the activation of caspase-3/-7 (Redza-Dutordoir, Averill-Bates 2016, Park et al. 2022). The caspase-3 controls DNA fragmentation and morphologic changes of apoptosis, whereas caspase-7 controls cell viability (Lakhani et al. 2006). Based on this evidence, Park et al. indicated that JIMT-1 cell viability was decreased while increasing β-escin concentration **(Figure 18)**. β-escin also decreased cell proliferation and tumour volume. A follow-up analysis examining the β-escin impact on the HER2 signalling pathway showed the total and phosphorylated levels of HER2 protein, and the total and phosphorylated levels of the main protein of the PI3K/Akt signalling pathway, which is Akt. The levels of both pHER2 and pAkt were decreased while increasing β-escin concentration (Park et al. 2022).

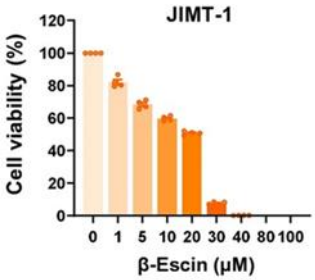

Figure 18. JIMT-1 cell viability followed β-escin treatment.

JIMT-1 cell viability was decreased while increasing β-escin concentration. Adapted from: Park et al. 2022

Recent studies indicated that the regulatory agencies have approved the use of pertuzumab or ado-trastuzumab emtansine (T-DM1), for overcoming trastuzumab resistance in HER2+ breast cancer (Li et al. 2019, Giordano et al. 2022). Pertuzumab which is a monoclonal antibody, binds to the HER2 extracellular domain to inhibit HER2/HER3 heterodimerization (Gradishar 2012). T-DM1 which is an antibody-drug conjugate consisting of trastuzumab and anti-microtubule agent DM1 (De Mattos-Arruda, Cortes 2013), binds to the HER2, resulting in the HER2-T-DM1 complex internalization, while DM1 exerts anti-cancer effects (Isakoff, Baselga 2011). Trastuzumab and pertuzumab's distinct mechanisms of action operate in concert to maximize the suppression of the HER2 signalling pathway (Scheuer et al. 2009). Bon et al. demonstrated that the combined use of trastuzumab and pertuzumab antibodies led to a greater reduction in cell viability than either antibody alone **(Figure 19)** (Bon et al. 2020). The combination of two antibodies also reduced cell proliferation (Blancafort et al. 2015) and tumour volume (Irie et al. 2020). Barok et al. demonstrated that T-DM1 led to a reduction in cell viability **(Figure 20)**, causing mitotic catastrophe (Barok et al. 2011). T-DM1 also reduced cell proliferation (Junttila et al. 2011), and tumour volume (Barok et al. 2011).

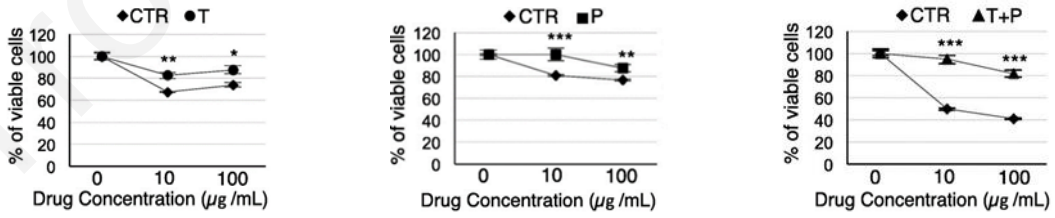

Figure 19. The viability of cells followed trastuzumab and pertuzumab treatment.

The combined use of trastuzumab and pertuzumab antibodies led to a greater reduction in cell viability than either antibody alone (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Adapted from: Bon et al. 2020

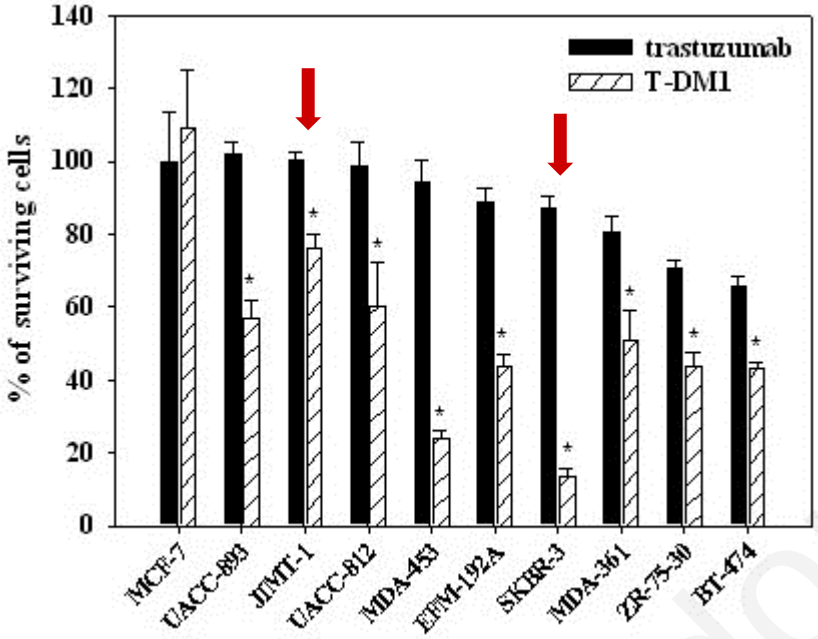

Figure 20. The viability of cells followed the ado-trastuzumab emtansine (T-DM1) treatment.

T-DM1 antibody led to a reduction in trastuzumab-resistant HER2+ breast cancer cells (JIMT-1) viability; and an even greater reduction in trastuzumab-sensitive HER2+ breast cancer cells (SKBR3) viability (*$P < 0.05$). Adapted from: Barok et al. 2011

3. DISCUSSION

The development of trastuzumab resistance in HER2-targeted therapy remains a major clinical problem (Dedić Plavetić et al. 2012). Researchers have adopted two approaches to address this problem: a) repurposing existing drugs to improve their efficacy, and b) finding new targets to expand the range of therapeutic options (Cao et al. 2023). Focusing on the latter approach, this dissertation discussed three mechanisms by which trastuzumab resistance may arise. As mentioned, HER2 receptor endocytosis that is induced by trastuzumab results in its ubiquitination and proteolytic degradation (Wymant et al. 2020). HER2 ubiquitination can be mediated by the c-Cbl E3 ubiquitin ligase (Klapper et al. 2000). The first mechanism suggests that CMTM6 which is overexpressed in trastuzumab-resistant HER2+ breast cancer cells, decreases HER2 ubiquitination, resulting in the continuous activation of the HER2 signalling pathway. The levels of phosphorylated proteins such as pHER2, pAkt, and pERK were high with or without trastuzumab. Thus, CMTM6 increases cell viability, proliferation, and invasion, while decreasing apoptosis. However, it is not clear how CMTM6 decreases HER2 ubiquitination. It is hypothesized that CMTM6 may interact with c-Cbl, resulting in ineffective ubiquitination of HER2. Because there is no evidence in the literature to support this, we suggest that future experiments such as the Co-IP assay followed by Western Blot analysis should be conducted to demonstrate if CMTM6 interacts with c-Cbl. Assuming that both proteins interact with each other, a follow-up analysis might examine the HER2 ubiquitination when a) both proteins are expressed; b) c-Cbl is expressed while CMTM6 is silenced; and c) CMTM6 is expressed while c-Cbl is silenced. Investigating the potential relationships among HER2, PD-L1 and CMTM6 would also be of great interest. The second mechanism suggests that CMTM7 underexpression caused by the *miR-182-5p* leads to Rab-5A inactivation, resulting in the prevention of HER2 degradation, and the continuous activation of the HER2 signalling pathway. The levels of phosphorylated proteins were low in the presence of the *miR-182-5p* inhibitor. In the future, it is suggested to evaluate the cell viability, proliferation, apoptosis, and invasion in the presence of the *miR-182-5p* inhibitor. The third mechanism suggests that TRAF4 E3 ubiquitin ligase which is overexpressed in HER2+ breast cancer cells, prevents SMURF2 E3 ubiquitin ligase from interacting and ubiquitinating HER2, resulting in continuous activation of the HER2 signalling pathway and increased cell viability and tumour volume. Future studies should evaluate the cells' proliferation, apoptosis, and invasion. Paradoxically, a recent study indicated that trastuzumab increases TRAF4 protein levels in HER2+ breast cancer (Gu et al. 2022).

Therefore, future *in vitro* and *in vivo* experiments are required for the investigation of the mechanisms by which trastuzumab increases TRAF4 expression.

Previous studies indicated that the interaction of other proteins with HER2 receptor may lead to trastuzumab resistance. For example, the interaction of HER2 with c-Met or Insulin-like Growth Factor-1 Receptor (IGF1R), leads to the activation of the HER2 signalling pathway (Shattuck et al. 2008, de Groot et al. 2016). However, IGF1R silencing does not affect HER2+ breast cancer cell viability (Hernandez-Juarez et al. 2023). c-Met and IGF1R contribute to HER2+ signalling pathway activation rather than the prevention of trastuzumab-HER2 binding, or HER2 endocytosis and ubiquitination, thus we cannot consider these proteins as prognostic factors for trastuzumab resistance.

Given that, CMTM6, *miR-182-5p*, and TRAF4 are attractive therapeutic targets. The CMTM6 expression can be modulated by the tyrosine kinase WEE1 in combination with BRCA1, CREB, and TEAD1 transcription factors. Thus, CMTM6 expression can be reduced by the adavosertib (WEE1 inhibitor). Importantly, future experiments will be needed to examine the mechanism by which WEE1, BRCA1, CREB, and TEAD1 module CMTM6 expression. In addition, a *miR-182-5p* inhibitor can down-regulate *miR-182-5p* and prevent it from reducing the expression of CMTM7. Despite the lack of literature related to HER2+ breast cancer, a *miR-29a* mimic can up-regulate *miR-29a* and reduce the expression of TRAF4. We suggest that future experiments such as qRT-PCR assays should be conducted to demonstrate the alteration in their expression levels following the *miR-29a* mimic treatment.

Regardless of the mechanism underlying trastuzumab resistance, β-escin is an alternate drug that can be used. It reduces tumour volume, cell viability, and proliferation without causing any adverse effects on the liver and kidney of mice xenografts. Because these therapeutic targets and the associated drugs have recently been proposed, the regulatory agencies have approved the use of trastuzumab in combination with pertuzumab or ado-trastuzumab emtansine (T-DM1) antibodies, for overcoming trastuzumab resistance in HER2+ breast cancer patients. Although both therapeutic approaches strongly decrease tumour volume, cell viability, and proliferation, trastuzumab/pertuzumab has lower efficacy than T-DM1 (Bon et al. 2020).

The American Society of Clinical Oncology (ASCO) Guideline proposed a systematic HER2-targeted therapy based on the pertuzumab and T-DM1 antibodies. It is subdivided into first-line, second-line, and third-line therapies (Giordano et al. 2022). The first-line therapy refers to the use of trastuzumab antibody in combination with pertuzumab and taxane

antibodies (Celik et al. 2022). In case the cancer has progressed during or after first-line therapy, the patient receives second-line therapy. The second-line therapy refers to the use of trastuzumab deruxtecan (T-Dxd) or T-DM1 antibodies; the latter antibody is given when the patient has previously received T-Dxd (Cao et al. 2023). Likewise, in case the cancer has progressed during or after second-line therapy, the patient receives third-line therapy. The third-line therapy refers to the use of pertuzumab or T-DM1 if the patient has previously not received these. More options include tucatinib, trastuzumab, and capecitabine; T-Dxd; neratinib and capecitabine; lapatinib and trastuzumab; lapatinib and capecitabine; and a combination of chemotherapy with trastuzumab or margetuximab **(Figure 21)** (Giordano et al. 2022).

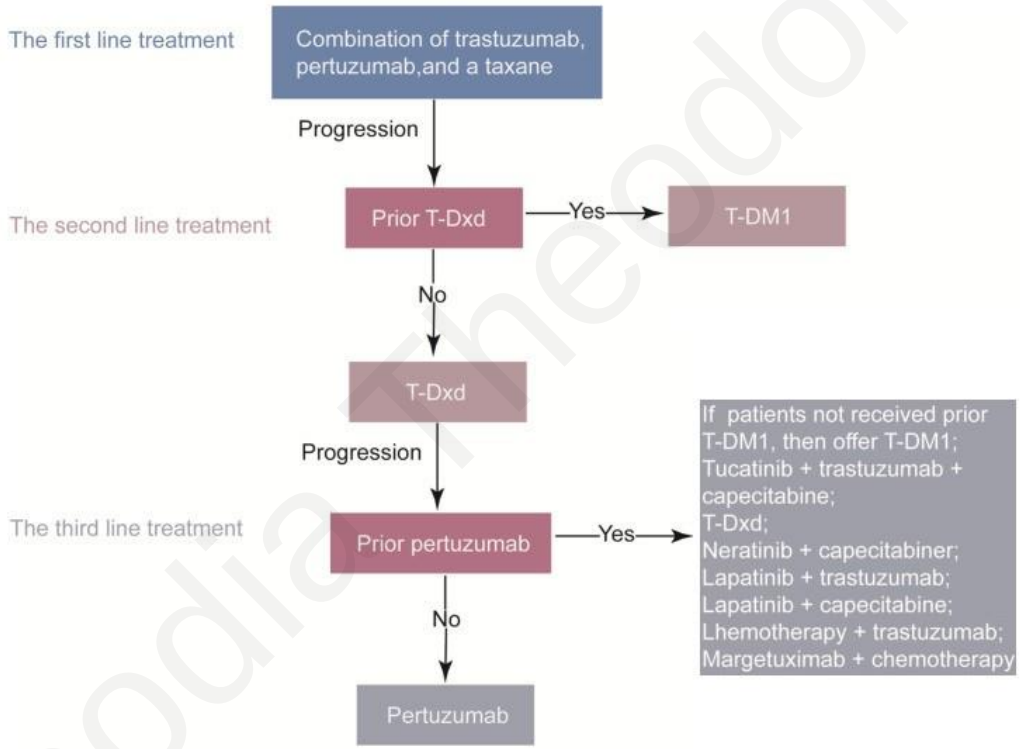

Figure 21. Systematic HER2-targeted therapy.

The systematic HER2-targeted therapy is subdivided into first-line, second-line, and third-line therapies. The first-line therapy refers to the use of trastuzumab in combination with pertuzumab and taxane antibodies. If cancer has progressed during or after first-line therapy, the patient receives the second-line therapy. The second-line therapy refers to the use of T-Dxd or T-DM1 antibodies. T-DM1 is given when the patient has previously received T-Dxd. If cancer has progressed during or after second-line therapy, the patient receives the third-line therapy. The third-line therapy refers to the use of pertuzumab or T-DM1 if the patient has previously not received these. More options include tucatinib, trastuzumab, and capecitabine; T-Dxd; neratinib and capecitabine; lapatinib and trastuzumab; lapatinib and capecitabine; and a combination of chemotherapy with trastuzumab or margetuximab. Adapted from: Cao et al. 2023

In conclusion, an acceptable HER2-targeted therapy may involve the use of combined antibodies or antibodies alone, along with the use of β-escin, and the inhibitors of WEE1 and *miR-182-5p*. We hope that the continuous effort to determine the most appropriate HER2-targeted therapy combination will improve the overall survival of HER2+ breast cancer patients.

ABBREVIATIONS

ABBREVIATION	MEANING
AJCC	American Joint Committee on Cancer
ATM	Ataxia Telangiectasia Mutated
BRCA1	BReast CAncer gene 1
BRCA2	BReast CAncer gene 2
CCK-8	Cell Counting Kit-8
CCND1	Cyclin D1
Cdc37	Co-chaperones cell division cycle 37
CDH1	Cadherin 1
CHEK2	Checkpoint kinase 2
c-Met	Mesenchymal-epithelial transition factor
CMTM6	Chemokine-like factor – like MARVEL transmembrane domain-containing 6
CMTM7	Chemokine-like factor – like MARVEL transmembrane domain-containing 7
Co-IP	Co-immunoprecipitation
CR1	Cysteine-rich domain 1
CR2	Cysteine-rich domain 2
CREB	cAMP Response Element-Binding Protein
DAPI	4',6-diamidino-2-phenylindole
DCIS	Ductal carcinoma *in situ*
EdU	Ethynyl-2′-deoxyuridine
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor

ERBB2	V-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2
ERK	Extracellular signal-regulated kinase
EV	Extracellular vesicle
FDA	Food and Drug Administration
FGFR1	Fibroblast growth factor receptor 1
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GRB2	Growth Factor Receptor-bound protein 2
GTP	Guanosine-5′-triphosphate
H/E	Hematoxylin/Eosin
HA-tag	Hemagglutinin-tag
HECT	Homologous to the E6-AP Carboxyl Terminus
HER	Human epidermal growth factor receptor
HER1	Human epidermal growth factor receptor 1
HER2	Human epidermal growth factor receptor 2
HER3	Human epidermal growth factor receptor 3
HER4	Human epidermal growth factor receptor 4
His-tag	Polyhistidine-tag
HR	Hormone receptor
Hsp90	Heat shock protein 90
IDC	Invasive ductal carcinoma
IF	Immunofluorescence
IGF1R	Insulin-like growth factor receptor-1
IHC	Immunohistochemistry

ILC	Invasive lobular carcinoma
LCIS	Lobular carcinoma *in situ*
mAb	Monoclonal antibody
MAP3K1	Mitogen-activated protein kinase kinase kinase 1
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
miRNA	MicroRNA
MRI	Magnetic Resonance Imaging
mTOR	Mammalian target of rapamycin
muMAb	Murine monoclonal antibody
muMAb4D5	Murine monoclonal antibody 4D5
NF-Kb	Nuclear factor kappa B
NLS	Nuclear localization signal
PALB2	Partner and Localizer of BRCA2
PD-L1	Programmed Death-Ligand 1
PDX	Patient-Derived Xenograft
PgR	Progesterone receptor
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PIP2	Phosphorylates phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)- triphosphate
PTEN	Phosphatase and TENsin homolog deleted on chromosome 10
qRT-PCR	Quantitative reverse transcription-polymerase chain reaction

Raf-1	Rapidly accelerated fibrosarcoma
RAS	Rat sarcoma virus
RB	Retinoblastoma
RING	Really Interesting New Gene
RISC	RNA-induced silencing complex
ROS	Reactive oxygen species
RTKs	Receptor tyrosine kinases
SH3BGR	SH3 domain-binding glutamic acid-rich
shRNA	Short hairpin RNA
SMURF1	Smad ubiquitination regulatory factor 1
SMURF2	Smad ubiquitination regulatory factor 2
SOS	Son of Sevenless
STK11	Serine/threonine kinase 11
TDLU	Terminal ductal lobular units
T-DM1	Ado-trastuzumab emtansine
TEAD1	TEA Domain Transcription Factor 1
TNBC	Triple-Negative Breast Cancer
TNM	Tumour, Node, Metastasis
TP53	Tumor protein p53
TRAF4	Tumour necrosis factor receptor-associated factor 4
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
ZNF703	Zinc finger protein 703

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