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# STUDY OF GENES INVOLVED IN HER2-POSITIVE BREAST CANCER PROGRESSION FOR IDENTIFICATION OF NEW THERAPEUTIC TARGETS

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# *Abstract*

HER2 overexpression is observed in 20-30% of invasive breast carcinomas and it is correlated with poor prognosis. Although targeted therapies have revolutionized the treatment of HER2-positive breast cancer, a high number of patients presented primary or acquired resistance to monoclonal antibodies and tyrosine kinase inhibitors. Tumor heterogenicity, epithelial to mesenchymal transition (EMT) and cancer stem cells are key factors in target therapy resistance and tumor progression.

The aim of this project was to discover alternative therapeutic strategies to overcome tumor resistance by harnessing immune system and looking for new targetable molecules.

The results reported in this thesis introduce a virus-like particles-based vaccine against HER2 as promising therapeutic approach to treat HER2-positive tumors. The high and persistent anti-HER2 antibody titers elicited by the vaccine significantly inhibited tumor growth and metastases onset over the time. Furthermore, the polyclonal response induced by the vaccine also inhibited human HER2-positive breast cancer cells resistant to trastuzumab *in vitro*, suggesting its efficacy also on trastuzumab resistant tumors.

To identify new therapeutic targets to treat progressed breast cancer, we took advantage from a dynamic model of HER2 expression obtained in our laboratory, in which HER2 loss and cancer progression were associated with the acquisition of EMT and stemness features. Targeting EMT-involved molecules, such as PDGFRβ, or the induction of epithelial markers, like E-cadherin, proved to be successful strategy to impair HER2-negative tumor growth.

Density alterations, which might be induced by anti-HER2 target therapies, in cell culture condition of a cell line with a labile HER2 expression, caused HER2 loss probably as consequence of more aggressive subpopulations which prevail over the others. These subpopulations showed an increased EMT and stemness profile, confirming that targeting EMT-involved molecules or antigen expressed by breast cancer stem cells together with anti-HER2 target therapies is a valid strategy to inhibit HER2-positive cells and simultaneously prevent selection of more aggressive clone.

# *Introduction*

# 1. BREAST CANCER

Breast cancer is the most commonly diagnosed, accounting for 11,7 % of all new annual cancer cases worldwide, and deadliest cancer among women worldwide (15.5% of all cancer deaths) (WHO 2020).

According to epidemiological surveys conducted by the Italian Cancer Register Association Italian (AIRTUM), in Italy approximately 55.700 new diagnoses were estimated in women in 2022. The development of new therapeutic strategies and the introduction of screening programs, which allow most breast cancers to be diagnosed at an early stage, have led to a five-years survival rate of 88% (AIOM 2021).

Breast cancer shows high geographical variability, with higher rates in more developed countries. This difference is explained by the different prevalence of mammography screening and the different distribution of risk factors (WHO 2020).

The first risk factor associated with this malignancy is the age: in all populations of the world, the risk of developing breast cancer increases with age. Looking at epidemiological data from Italy, the probability of developing breast cancer is 2.4% up to 49 years of age, 5.5% between 50 and 69 years, and 4.7% between 70 and 84 years. This correlation can be partially explained by the prolonged proliferative stimulus to which the mammary epithelium is subjected over the years, and in part by the progressive DNA damage and loss of balance between oncogenes and oncosuppressors caused by the accumulation of mutations (Collegio Italiano dei Senologi 2021).

The duration of childbearing age is also a risk factor for breast cancer: the longer a woman remains fertile (early menarche and/or late menopause), the more prolonged the hormonal stimulus and thus greater is the risk of developing this type of malignancy. In contrast, in women who have had one or more pregnancies, the risk is lower. Also other hormonal factors, such as endocrine replacement therapies in menopausal women and oral contraceptives, can contribute to increase the risk (Ban and Godellas 2014).

Other breast cancer risk factors are related to dietary and metabolic factors. For instance, adipose tissue, due to its aromatase activity, is responsible for peripheral estrogen

production, thus overweight and obesity are significant risk factors for breast cancer, as well as they are associated with poor prognosis (Patterson et al. 2010; Ban and Godellas 2014).

Among chemical carcinogens, in 2014 the European Prospective Investigation into Cancer and Nutrition (EPIC) study confirmed tobacco smoking as a risk factor for developing breast cancer. This study, consisting of a cohort of 300.000 women from 10 different European countries, compared the risk of developing breast cancer between women who had never smoked or been exposed to smoke and smokers (16% increase in risk), former smokers (14%) and women exposed to secondhand smoke (10%). Also from the EPIC study, it appears that alcohol is the only nutrient associated with an increased risk of breast cancer (Southgate et al. 2002; Slimani et al. 2007).

Finally, about 5-7% of breast cancers is related to hereditary factors, in particular the mutation of BRCA1 (Breast Cancer Type 1 susceptibility protein 1) and/or BRCA2 genes (Breast Cancer Type 1 susceptibility protein 2) (Tung et al. 2016).

### 1.1. Breast cancer intrinsic subtypes

The human mammary gland is a tubulo-alveolar gland formed by lobes surrounded by adipose tissue. The lobes consist of several lobules, whose physiological function is milk production. Milk is collected at the level of the lobules by the lobular ducts, which flow into the galactophore ducts; the latter terminate in the nipple. This type of gland is, therefore, composed of different cell types: lobular cells that make up the lobules, ductal cells constituting the ducts, myoepithelial cells that help excrete milk, and fat cells (Figure I).

There are many types of breast cancers as it can present in distinct areas of the breast, such as the ducts, the lobules, or the tissue in between. The type of breast cancer is determined by the specific cells that are affected. Based on which cell origin is involved, breast cancers can be divided into carcinomas and sarcomas. Carcinomas are breast cancers arising from the epithelial component of the breast, which consists of the cells that line the lobules and terminal ducts. Sarcomas are a much rarer form (<1% of primary breast cancer) arising from the stromal components of the breast, which include myofibroblasts and blood vessel cells. These groups are not always sufficient categories as, in some cases, a single

breast tumor can be a combination of different cells (Polyak 2007; Akram et al. 2017; Feng et al. 2018).

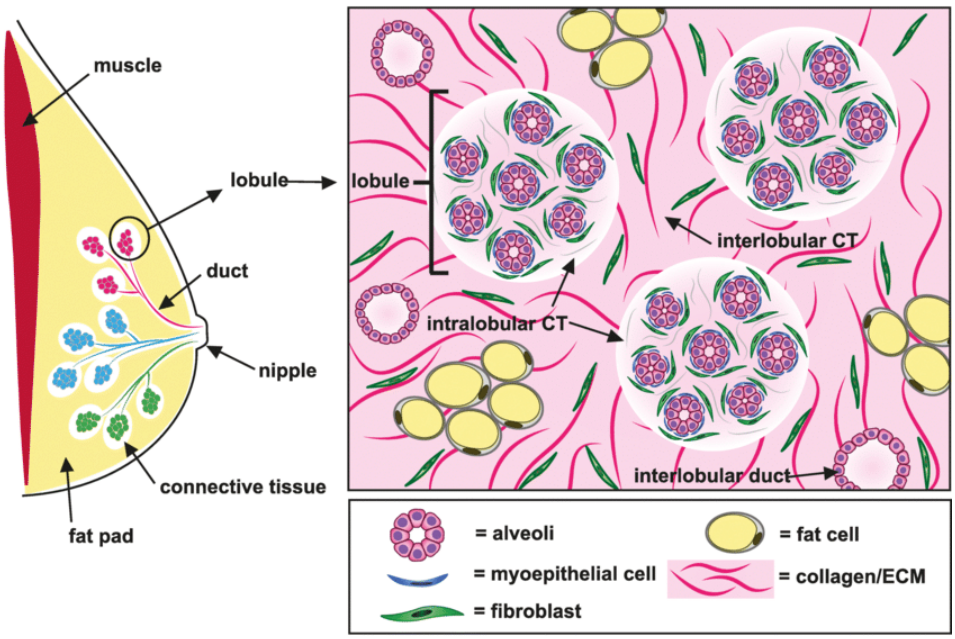


**Figure I. Human mammary gland.** Architecture of the human mammary gland. Ducts and lobules are located in an extracellular matrix-rich environment (Houthuijzen and Jonkers 2018).

A breast carcinomas classification can be done on anatomical basis and on the degree of invasiveness relative to the primary tumor. If tumor growth is restricted to the onset anatomic area (lobule or duct), the carcinoma is called "in situ"; on the other hand, it is called "invasive" breast cancer when it extends to different organs of the body (also recognized as metastatic breast cancer). Most common organ to which metastases are found are brain, bone, liver, and lungs (Akram et al. 2017).

Breast cancer is a heterogeneous tumor which includes many biologically different entities with distinct pathological features that lead to different treatment responses (Dai et al. 2015). Thus, accurate grouping of breast cancers into clinically relevant subtypes is necessary to choose the appropriate therapeutic strategy.

Breast cancer intrinsic subtypes were first described in 2000 by Perou and colleagues who analyzed more than 8000 genes in 65 breast tumor samples and 17 cell lines (Perou et al. 2000). Later, this gene list has been simplified and optimized for clinical practice and a

group of 50 genes was identified and called PAM50 (Prediction Analysis of Microarrays 50) (Wallden et al. 2015). Thanks to these studies, they identified 4 different intrinsic subtypes: Luminal-A, Luminal-B, HER2-enriched and Basal-like. Each of these subtypes has specific characteristics related to risk factors, incidence, biological behavior, and response to treatment and prognosis (Figure II) (Januškevičienė and Petrikaitė 2019).

Subsequent studies identified a new subtype classified as Claudin low (Prat et al. 2010).

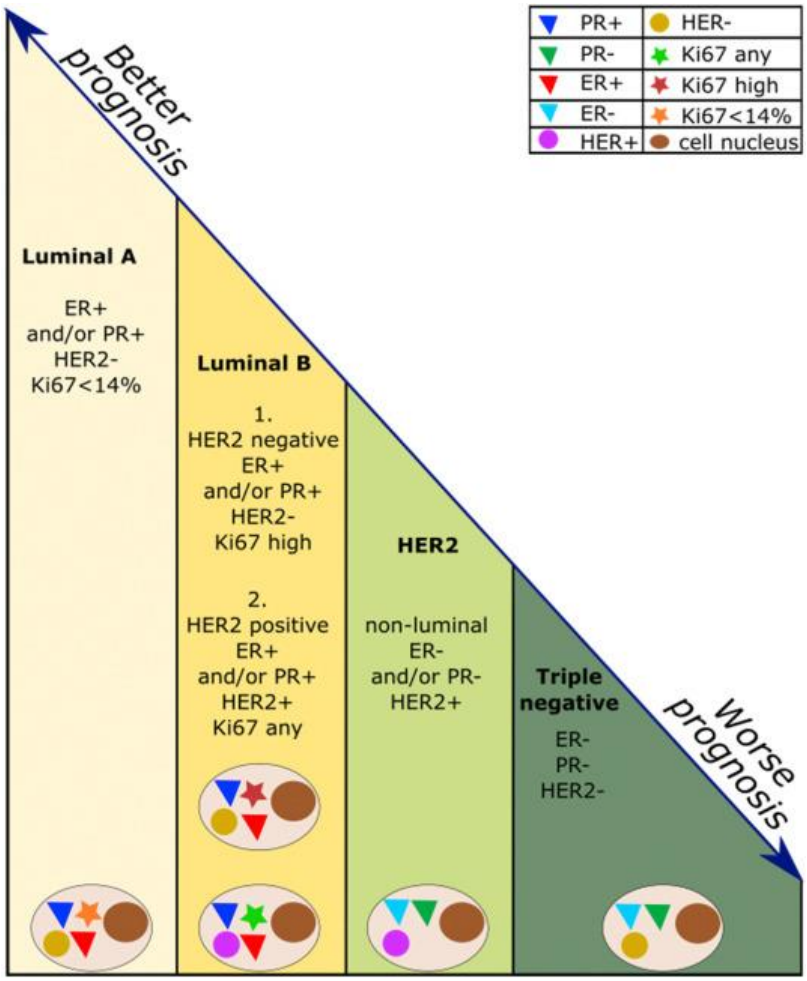


**Figure II. Breast cancer intrinsic subtypes.** Summary diagram of intrinsic subtypes, classified according to prognosis and major markers (Januškevičienė and Petrikaitė 2019).

Moreover, three molecular biomarkers: Estrogen Receptor (ER), Progesteron Receptor (PR), and Human Epidermal Growth Factor Receptor 2 (HER2) are used in the routine clinical management of patients with breast cancer. The presence of these three

receptors is generally assessed by immunohistochemistry (IHC) and by in situ hybridization (ISH) for the identification of HER2 gene amplification (Curigliano et al. 2019).

To learn more about the degrees of heterogeneity typical of breast cancer, the consequences that each individual gene alteration and/or mutation may have on prognosis and response to therapies, thousands of breast cancer samples have been analyzed and the data obtained have been collected in databases hosting information on the molecular and genetic characteristics of these samples such as "The Cancer Genome Atlas project" (TCGA) and "Molecular Taxonomy of Breast Cancer International Consortium" (METABRIC).

### *1.1.1. Luminal-A and Luminal-B subtypes*

Luminal-like A and B tumors express ER and PR hormone receptors.

Luminal A is the most common intrinsic subtype and accounts for about 50-60% of all breast carcinomas. These tumors frequently have low histologic grade, low nuclear pleomorphism and low mitotic activity. The immunohistochemical profile is characterized by high expression of ER, PR, luminal epithelial cytokeratins CK8/18 and estrogen receptor function-associated genes such as LIV1 (Solute Carrier Family 39 Member 6), FOXA1 (Forkhead Box A1), XBP1 (X-box binding protein 1), GATA3 (GATA binding protein 3), BCL2 (B cell lymphoma 2), erbB3 (Human epidermal growth factor receptor 3), erbB4 (Human epidermal growth factor receptor 4). It also presents negativity for the HER2 oncogene and low expression of Ki-67 (Yersal and Barutca 2014).

Patients with Luminal type A breast cancer generally have a favorable prognosis when compared with that of other subtypes: there is a 65% of reduction in the risk of metastasis and the risk of local recurrence is significantly lower. Where present, metastasis occurs mainly to the bone, while liver metastasis, lung and central nervous system occur in less than 10% of patients. The treatment of this intrinsic subtype is mostly based on hormonal therapies, while the response to chemotherapy is rather low (Laible et al. 2019).

On the other hand, Luminal subtype B accounts for 15-20% of all breast carcinomas and, compared with Luminal A, shows a more aggressive phenotype, high histologic grade, high proliferative index, high risk of recurrence, higher metastatization (especially to the bone) and lower disease-free survival (Figure III) (Lexe et al. 2009; Creighton 2012).

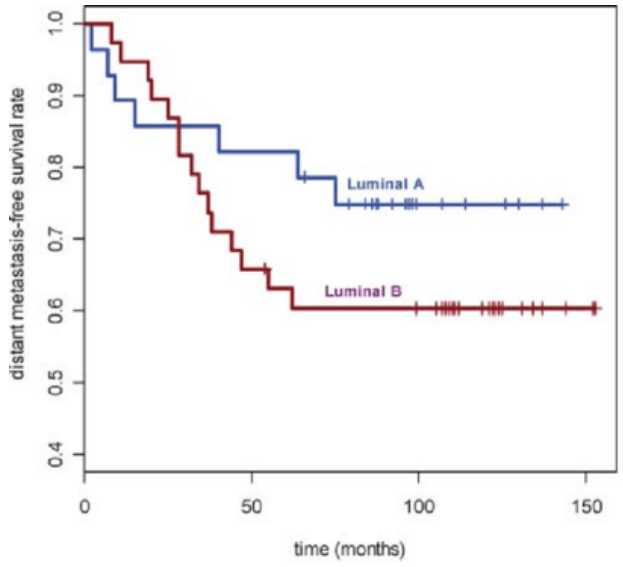


**Figure III. Kaplan-Meier survival curves for Luminal A vs Luminal B tumors** (Lexe et al. 2009).

While Luminal subtype A is HER2-negative, Luminal subtype B may express the receptor at varying levels, although not in all cases (Cheang et al. 2009).

Immunohistochemically, Luminal B can be defined in two ways:

- ER-positive, HER2-negative and with high Ki-67 expression;

- ER-positive, HER2-positive and with high expression of Ki-67.

However, this classification does not include all Luminal B tumors, as 6% of these are negative for both ER and HER2.

The main difference between the two Luminal subtypes concerns the increased expression in Luminal B of proliferation-related genes, such as v-MYB (Myeloblastosis viral oncogene homolog), GGH (Gamma glutamyl hydrolase), LAPTMB4 (Lysosome-associated transmembrane protein 4-beta), NSEP1 (Nuclease sensitive element binding protein) and CCNE1 (Cyclin E1) (Reis-Filho et al. 2010). To distinguish the two subtypes, Cheang and colleagues proposed Ki-67 protein expression as a marker: an index greater or less than 14% was set as a threshold to discriminate Luminal subtype B (Ki-67 index>14%) from Luminal A (Ki-67 index<14%) (Cheang et al. 2009).

The treatment of Luminal subtype B includes combining conventional chemotherapy, to which this subtype appears to be sensitive, with hormone therapy; in addition, in the presence of positivity for HER2 it is appropriate to also include targeted therapy against the oncogene (Goldhirsch et al. 2011).

### *1.1.2. HER2-enriched subtype*

HER2 overexpression and/or amplification occurs in 11-30% of all breast cancers (Cronin et al. 2010). In 2013, the American Society of Clinical Oncology (ASCO) established essential guidelines for characterizing the different subtypes of breast cancer. Indeed, on the correct identification of the tumor depends the choice of therapy and, therefore, also its success. HER2 positivity is defined today by IHC as complete and strong membrane staining (i.e. score of 3+) in ≥10% of cancer cells, and/or by in situ immunofluorescence (ISH) techniques with an HER2/CEP17 ratio ≥2.0 and an average HER2 gene copy number ≥ 4.0 signals per cell (Prat et al. 2015; Schettini et al. 2020; Schettini and Prat 2021).

The HER2-enriched subtype is characterized at the RNA and protein level by the high expression of HER2-related and proliferation-related genes and proteins, intermediate expression of luminal-related genes and proteins (e.g. ER1 and PR) and low expression of basal-related genes and proteins (e.g. keratin 5 and FOXC1). At the DNA level, these tumors show the highest number of mutations in the TP53 (Tumor protein p53) gene and the PIK3CA (Phosphatidylinositol-4,5-biphosphate 3-kinase catalytic subunit alpha) gene, 72% and 39% of HER2-enriched tumors respectively (Schettini et al. 2020).

Clinically HER2-positive breast cancer has been considered a single tumor entity for a long time; however, in 2012, TCGA project demonstrated that not all HER2-positive breast cancer had the same genomic profile (Comprehensive molecular portraits of human breast tumors 2012): only 50 % of HER2-positive tumors were HER2-enriched, while the other half were Luminal A or B, with high expression of typical luminal genes (Comprehensive molecular portraits of human breast tumours 2012; Schettini and Prat 2021). Recently, studies have revealed the presence of HER2-positivie Basal-like tumors, especially in hormone receptor-negative disease (Prat et al. 2014). Among the different subtypes within

HER2-positive disease, the HER2-enriched is characterized by the highest levels of ERBB2 mRNA, phosphorylated (p)HER2 and total HER2 protein, pEGFR and EGFR protein, suggesting that this group has the highest activation of the HER2 signaling pathway (Schettini and Prat 2021). Hormone receptor and HER2 status can influence the treatment efficacy; thus, the integration of multiple biomarkers might lead to different therapeutic strategies and better prognosis.

Histologically, these are highly proliferative tumors that tend to spread more aggressively than the other intrinsic subtypes. Therefore, HER2-enriched breast carcinomas have a poor prognosis due to the ability to metastasize to different visceral organs and to the brain (Arvold et al. 2012; Santa-Maria et al. 2016).

Because HER2 is overexpressed only by tumor cells, it has been possible to develop targeted therapies that can increase survival not only of patients in the early stage of disease but also in the metastatic phase (Yersal and Barutca 2014). There are several anti-HER2 therapeutic agents currently approved in clinical practice: trastuzumab, pertuzumab, lapatinib, trastuzumab emtansine, trastuzumab deretuxtecan, neratinib, tucatinib, margetuximab (Cardoso et al. 2018; Swain et al. 2022).

### *1.1.3. Basal-like subtype*

Approximately 8-10% of all breast cancer are classified as Basal-like. The basal subtype is composed of ER-, PR- and HER2- (triple negative) tumors and high expression of basal markers, such as cytokeratins (CK5/6, CK14, and CK17), caveolin 1 and 2, and EGFR (Perou et al. 2000; Dai et al. 2015; Milioli et al. 2017). On the other hand, these tumors express low levels of genes codifying for markers of the luminal epithelium such as CK8/18 and c-KIT (Eroles et al. 2012).

Basal tumors account for 60% to 90% of triple negative cases and show aggressive clinical course due to the metastatic pattern to visceral organs (excluding bone) and the lack of specific treatments. In fact, because of the triple negative receptor status these tumors do not benefit of conventional target therapies, leaving chemotherapy (e.g. anthracycline and taxane) the only therapeutic option (Dai et al. 2015; Milioli et al. 2017). Besides these

conventional treatments, many studies have suggested novel target such as EGFR and genes involved in matrix remodeling and angiogenesis, which have been shown to be associated with Basal-like subtype signature (Nielsen et al. 2004; Chang et al. 2005).

### *1.1.4. Claudin-low subtype*

The breast cancer intrinsic subtypes were initially identified by hierarchical clustering of genes differentially expressed by different breast tumors (Perou et al. 2000; Sørlie et al. 2001). Claudin-low breast tumors did not initially emerge as an independent group in this analysis, but this subtype was discovered 7 years later by Herschkowitz and colleagues comparing gene expression profile in an integrated analysis of human and murine mammary tumors (Herschkowitz et al. 2007). The existence of this subtype has later been observed in several independent breast cancer cohorts and an analogous claudin-low subtype has been identified in bladder cancer (Damrauer et al. 2014).

Claudin-low tumors account for 7-14% of all invasive breast cancer and, like Basal-like tumors, are reported to be mostly ER-negative, PR-negative, and HER2-negative (triple negative). This subtype is characterized by low expression level of cell–cell adhesion molecules, including claudins 3, 4, and 7, occludin, and E-cadherin, high expression of epithelial–mesenchymal transition (EMT) genes, stem cell-like less differentiated gene expression patterns, immune cell infiltration and gamma interferon (IFN-$\gamma$) activation (Prat et al. 2010; Dias et al. 2017; Fougner et al. 2020).

Despite their high variation in mutational burden and degree of copy number aberration (CNA), these tumors are genomically stable, probably due to their less differentiated state and a protective effect mediated by the EMT-related transcription factor ZEB1 which prevents replication and oxidative stress (Morel et al. 2017; Pommier et al. 2020).

Claudin-low is a highly heterogeneous subtype and Fougner and colleagues demonstrated that these tumors reflect the intrinsic subtype to which they are initially assigned (Fougner et al. 2020). Moreover, a recent study demonstrated the existence of three

main Claudin-low molecular subgroups, with different origin and distinct survival outcomes (Figure IV) (Pommier et al. 2020).

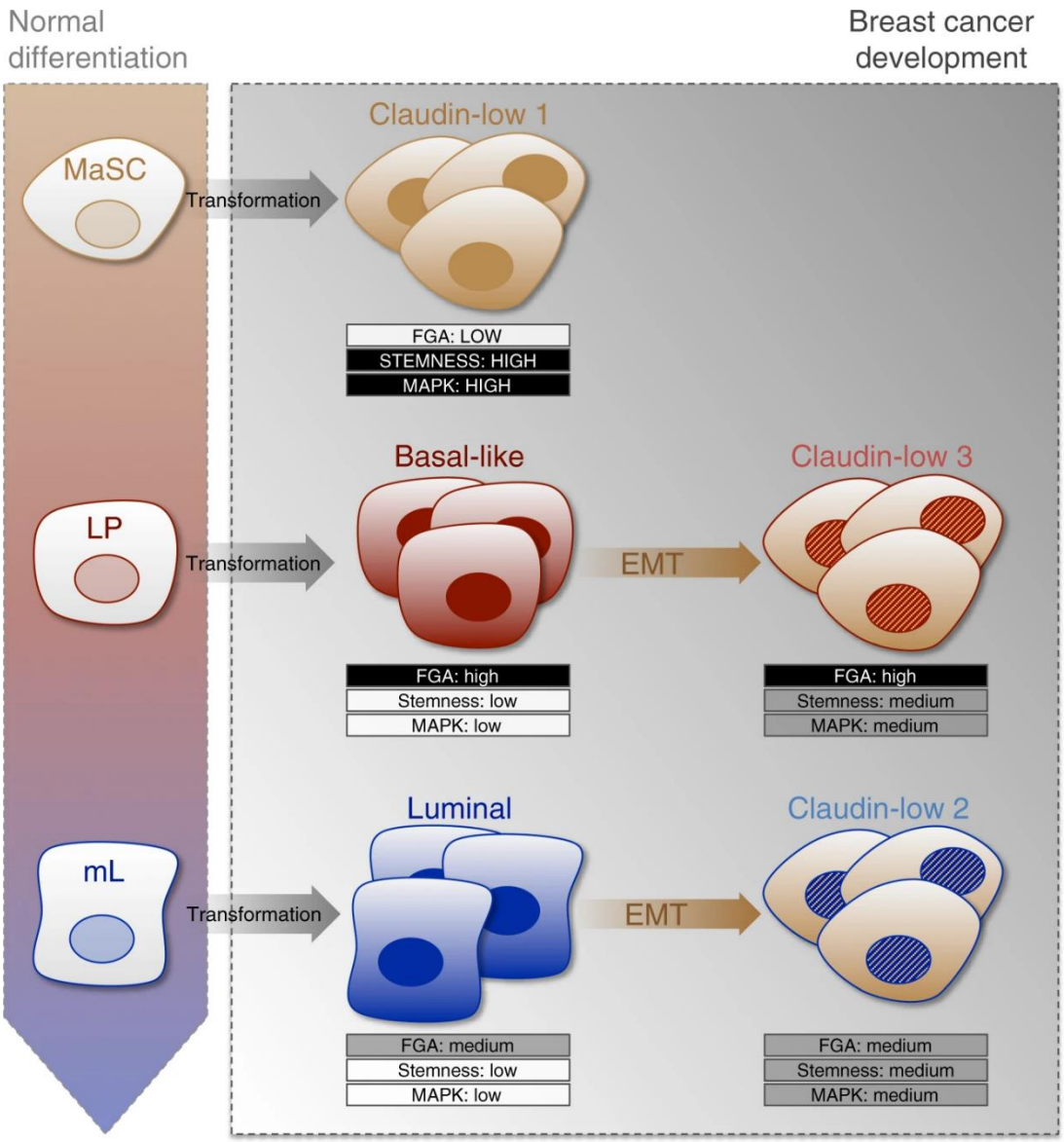


**Figure IV. Claudin-low molecular subtypes.** Transformation of a normal human mammary stem cells (MaSC) leads to Claudin-low 1. The activation of an EMT transdifferentiation process and the gain of mesenchymal features in a basal-like tumor promotes the development of a Claudin-low 3 tumor with extensive genomic aberrations. EMT commitment in a luminal-like breast cancer leads gradually to a Claudin-low 2 tumor with a moderate level of genomic aberrations. (LP=luminal progenitor, mL=mature luminal) (Pommier et al. 2020).

Claudin-low tumors are associated with higher tumor grade and extensive lymphocytic infiltrate. Clinically, they have been associated with a poor prognosis with

some evidence that they may be relatively resistant to conventional chemotherapeutic agents (Dias et al. 2017).

### 1.2. Human Epidermal Growth Factor Receptor family

The human epidermal growth factor receptor (ErbB-HER) family consists of four tyrosine-kinase receptors: HER1 (EGFR o ErbB1), HER2 (ErbB2 o Neu), HER3 (ErbB3) ed HER4 (ErbB4) (Yarden and Sliwkowski 2001).

Although the human ErbB genes are found on four different chromosomes, all members share a common structure and a high homology rate: *Caenorhabditis elegans* and *Drosophila melanogaster* have primordial linear versions of the ErbB signalling pathway which has evolved into a more complex network in higher organisms (Yarden and Sliwkowski 2001).

These receptors are ubiquitously localized in cell membrane of epithelial, mesenchymal, neuronal, and in their progenitor cells (Yarden 2001). ErbB receptors structure includes an extracellular domain (ECD, which can be divided in 4 subdomains I-IV), lipophilic transmembrane region, intracellular domain containing tyrosine kinase, and a carboxy-terminal region (Figure V) (Yarden and Sliwkowski 2001).

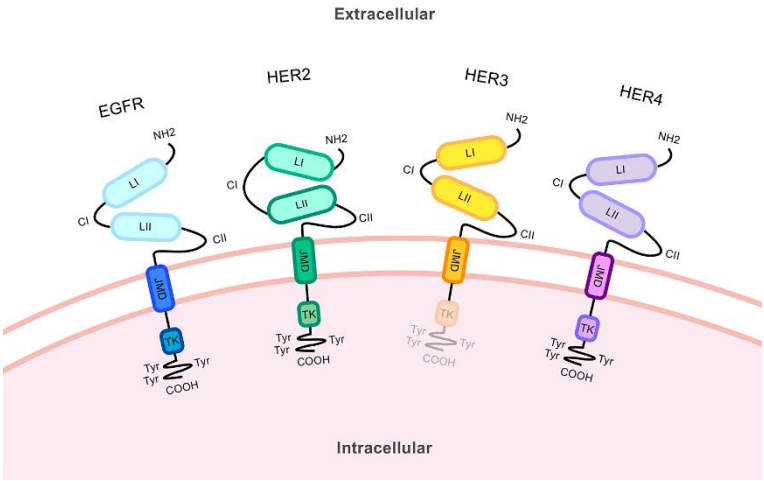


**Figure V. HER-ErbB family receptors**. The three domain regions (extracellular, transmembrane and intracellular domain) are represented (Hart et al. 2020).

Several growth factors (e.g. EGF, neuregulin, transforming growth factor $\alpha$) bind, with different affinity, the ErbB receptors. After the binding with the ligand, the ErbB receptor acquires an open conformation which allows the dimerization with another ErbB receptor member or with itself (forming heterodimers or homodimers respectively) (Garrett et al. 2003; Burgess et al. 2003). Thus, phosphorylation of receptors leads to activation of signaling pathways that control proliferation, survival, motility, and cell adhesion such as Ras-Raf-Mek-ERK1/2, PI3K-Akt-TOR or STAT (Yarden 2001).

EGF (epidermal growth factor) was discovered by Stanley Cohen in 1962 as the protein responsible for incisor and eyelid in mice (COHEN 1962). The evidence that EGF binds EGF receptor (EGFR/HER1) shed light on relationship between receptor overexpression and cell growth (Carpenter et al. 1978; Cohen 1983; COHEN 1986). In 1984, EGFR became the first receptor to be associated with an oncogene, v-ERBB, which was known to induce sarcomas and leukemias in chickens (Downward et al. 1984).

Later, in 1984 the *neu* gene was identified in malignant rat or mouse cell lines and this gene encoded for a 185.000-dalton protein (p185) that was able to induce cell transformation. The second ErbB receptor, HER2, presents high homology to *neu* and they are both located on chromosome 17, moreover they are closely related to HER1 (located on chromosome 7) (Schechter et al. 1984; Coussens et al. 1985). Regarding its physiological function, HER2 plays a key role during both embryonic and adult development. In embryonic development, the heterodimers formed between HER2 and HER4 through neuregulin binding are crucial for heart development (Meyer and Birchmeier 1995). Similarly, HER2-HER3 heterodimers are crucial for the proper formation of Schwann cells and ganglia in the peripheral nervous system (Morris et al. 1999). In addition, together with the other HER receptors, HER2 is responsible for mammary gland development (Andrechek et al. 2005). Despites the others ErbB receptors, HER2 ligands have never been identified, thus probably this receptor remains constitutively open and, therefore, active (Nami and Wang 2017); otherwise, HER2 appears to be the favored partner of the other receptors (Yarden and Pines 2012). Moreover, HER2 can also be activated by complexing with other membrane receptors such as insulin-like growth factor receptor 1 (Nahta et al. 2005).

Two additional ErbB family members were described: HER3 on chromosome 12 and HER4 on chromosome 2. ErbB3, unlike all other receptors in the ErbB family, shows reduced kinase activity and is unable to phosphorylate other proteins. For this reason, HER3 has mainly an allosteric activator function. Despite this, it still covers a role in cancer, mainly by promoting HER2 and EGFR signaling (Yarden and Sliwkowski 2001; Yarden and Pines 2012).

The ErbB4 receptor, on the other hand, is a fully functional tyrosine kinase receptor, either homodimer or heterodimer. Compared with other receptors belonging to this family, HER4 undergoes a proteolytic process after ligand binding. The domain released after cutting enters the nucleus and participates in the regulation of gene expression (Sardi et al. 2006; Yarden and Pines 2012).

### *1.2.1. HER2 full-length, HER2 isoforms and their role in cancer*

The first evidence for the role of HER2 as a protooncogene in cancer was inferred from the connection to its rat ortholog, *neu*, a mutant cDNA isolated from carcinogen-induced neuroblastomas (Schechter et al. 1984). Although rodent *neu* is activated by a point mutation, human HER2 is typically amplified. In HER2-positive tumors there can be 25 to 50 copies of the ErbB2 gene and an increase in gene expression up to 40-100 times, resulting in the expression of 2 million HER2 receptors on the tumor cell (Moasser 2007; Nami and Wang 2017). Overexpression of either rat or human wild-type ERBB2 was shown to transform diploid cells. Further evidence of its oncogenic activity is given by the overexpression of wild-type *neu* or HER2 under the control of a mammary-specific promoter leads to metastatic mammary tumors in transgenic mice (Finkle et al. 2004; Andrechek et al. 2005).

In 1987 Slamon and colleagues reported that HER2 was overexpressed in 20% of breast cancer and its amplification was associated with shorter disease-free and overall survival (Slamon et al. 1987; Ménard et al. 2001). Later, HER2 has also been found to be amplified in other malignancies such as colon, stomach, lung, ovarian and uterine cancer (Iqbal and Iqbal 2014). HER2 amplification is an early event in human breast tumorigenesis:

it is seen in nearly half of all in situ ductal carcinomas without any evidence of invasive disease and is maintained during progression to nodal and/or distant metastasis (Park et al. 2006).

HER2 oncogene overexpression only by tumor cells makes it an excellent therapeutic target. Targeting of HER2 with monoclonal antibodies, like trastuzumab and pertuzumab, has revolutionized the therapy of breast cancer, leading to significant improvements in patients' survival of both early and advanced HER2-positive breast cancers.

HER2 may be present in tumors not only in the full-length form (HER2 wild type) but also in other forms that originate as a result of splicing variants with contrasting roles in tumor cell biology (Figure VI) (Jackson et al. 2013; Hart et al. 2020).

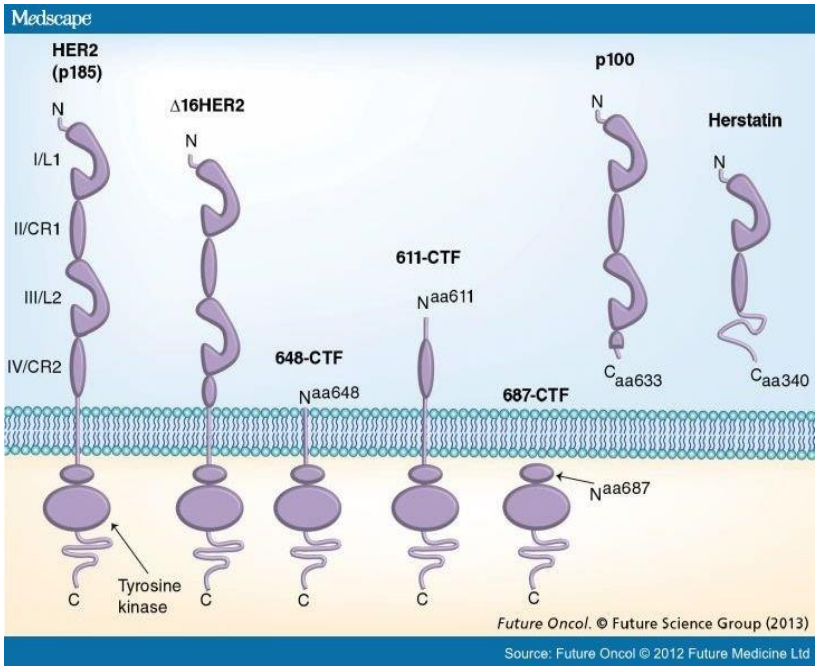


**Figure VI. HER2 and HER2 splicing variants.** 648-CTF, 611-CTF and 687-CTF are p95HER2 fragments (Wang et al. 2013).

The alternative splicing of HER2 leads to three identified isoforms: Delta16, that is related to an increased tumorigenesis, Herstatin and p100 which are associated to HER2 signaling inhibition (Jackson et al. 2013).

HER2Δ16 arises from the in-frame deletion of exon 16, which encodes a small region of the ECD of HER2, resulting in a conformational change which promotes homodimerization (Castiglioni et al. 2006). The overexpression of Delta16 in breast cancer

cell lines has been associated to increased mesenchymal markers, epithelial to mesenchymal transition involved molecules and a higher activation of PI3K-AKT, MAPK and Src pathways (Castiglioni et al. 2006; Mitra et al. 2009; Castagnoli et al. 2017). The Delta16 isoform has been reported in about 50% of all the human HER2-positive breast cancers and in 89% in whom disease progresses to local lymph nodes (Mitra et al. 2009). Although early studies have shown that the Delta16 variant reduced trastuzumab binding affinity (Castiglioni et al. 2006; Mitra et al. 2009), later results reported trastuzumab efficacy both in the murine model Delta16 and in cell lines, as well as in patients with HER2-positive breast carcinomas expressing the Delta16 isoform (Alajati et al. 2013; Castagnoli et al. 2014; Palladini et al. 2017).

Around 20-40% of HER2-positive mammary carcinomas expresses C-terminal HER2 fragments called p95HER2 (Arribas et al. 2010). The p95HER2 variant originates through two distinct mechanisms:

- proteolysis of the extracellular portion of HER2 full-length (by ADAM10 metalloproteases) that generates a 95-100 kDa fragment anchored to the cell membrane 648-CTF (Figure VI) (Arribas et al. 2011);
- the alternative initiation of translation by 2 internal codons, 611-CTF and 687-CTF (Figure VI), that are located before and after the transmembrane portion, respectively (Pedersen et al. 2009; Arribas et al. 2011).

The expression of p95HER2 fragments correlated with an increased metastatic involvement at the lymph node level; moreover, this variant is also called "truncated" variant because of the absence of the extracellular portion, which is the binding site for monoclonal antibodies, thus lead to resistance to target therapies. However, the p95HER2 protein retains tyrosine kinase activity and is therefore sensitive to the action of tyrosine kinase inhibitor (TKI) drugs (Scaltriti et al. 2007).

### 1.3. Mechanisms behind HER2-positive breast cancer progression

Breast cancer is a highly heterogeneous disease, with differences in biology, gene expression profiles, epithelial to mesenchymal state, and treatment sensitivity often coexisting in the

same tumor. The co-existence of all these different features might influence therapy efficacy and lead to cancer progression.

### *1.3.1. HER2-positive breast cancer as a heterogeneous society: intra-tumoral heterogenicity*

Most HER2-positive tumors show a homogeneous pattern of amplification of ErbB2 and overexpression of the protein. However, in a variable percentage of these tumors (ranging from 1% to 40% depending on the methods used) there are sub-populations with a different pattern of HER2 amplification and expression. In some cases, tumors may also contain an HER2-negative subpopulation. This phenomenon is called intra-tumor heterogeneity (Ng et al. 2015). There are two different forms of intra-tumor heterogeneity: the coexistence of focal clones (cluster heterogeneity) or the presence of scattered single cells within a group with different characteristics (mosaic heterogeneity) (Ng et al. 2015).

In clinical practice the presence of cluster heterogeneity can be detected if a phenotypically different group covers 10% of the area evaluated; conversely, mosaic heterogeneity is confirmed if the proportion of heterogeneous cells is between 5 and 50% of all cancer cells identified (Nguyen et al. 2019). Thus, there are several HER2-positive tumors that exhibit mosaicism of expression. Tumor subpopulations may differ from each other by the presence of amplification of ErbB2, passenger mutations in other genes, or by driver mutations (Ng et al. 2015; Nguyen et al. 2019).

In addition, the exposure to anti-HER2 therapy affects these cells differently, killing some cell populations and clonally selecting others. HER2 heterogeneity can thus result in HER2 loss due to treatment exposure, with HER2-positive subpopulations killed by treatment and resistant HER2-negative cells surviving (Janiszewska et al. 2021; Morganti et al. 2022).

As better discussed in the following paragraphs, these heterogeneous subpopulations represent a problematic issue from a clinical point of view since their presence is often associated with a worse prognosis, greater resistance to therapies targeting HER2, and poorer survival (Ng et al. 2015; Swain et al. 2015).

### *1.3.2. Receptor discordance and loss of HER2 expression*

Local or distal tumor recurrences have long been considered biologically similar to the primary tumor. More recently, studies have shown that the status of hormone receptors and HER2 can change over time during breast cancer progression. This phenomenon is known as receptor discordance (McAnena et al. 2018).

A metanalysis conducted by Schrijver and colleagues reported the direction of change, positive-to-negative or negative-to-positive, of hormone receptors and HER2 between primary tumor and metastasis. For ER, PR, and HER2 were 22.5%, 49.4%, and 21.3%, respectively, of positive-to-negative conversion rates. On the other side, the conversion rates form negative to positive for ER, PR, and HER2 were 21.5%, 15.9%, and 9.5%, respectively. Furthermore, authors found that ER discordance was more common in brain (20.8%) and bone (29.3%) than in liver (14.3%) metastases. PR discordance was more frequent in bone (42.7%) and liver (47.0%) than in brain (23.3%) metastases. Whereas, there was no significant difference in HER2 discordance between brain, bone, and liver metastases (Schrijver et al. 2018; Sperduto et al. 2020).

There are many factors underlying receptor discordance. One of them is the intra-tumoral heterogenicity; the receptor discordance might be the results of a clonal selection where the most aggressive clone started the metastatic process. Furthermore, there could be a biological drift due to the presence of drug-selective pressure. Although the relation between cancer therapies and receptor conversion is still debated, some studies have shown an association between the use of therapy hormone or chemotherapy and the disappearance of ER- or PR-positive cells; similarly, treatment with trastuzumab can also cause the loss of HER2 receptor or the selection of HER2-negative clones (Morganti et al. 2022).

Typically, breast carcinomas that go through receptor discordance and loss of HER2 in metastases are associated with poorer overall survival compared with whose HER2 status remains unchanged (Niikura et al. 2012).

### *1.3.3. Epithelial to mesenchymal transition*

Epithelial-mesenchymal transition is a reversible cellular process during which epithelial cells acquire a mesenchymal phenotype and associated functional changes. Mesenchymal cells can reverse this phenomenon and return to the epithelial phenotype through the reverse process, the mesenchymal-epithelial transition (Dongre and Weinberg 2019).

During the epithelial to mesenchymal transition, epithelial cells acquire mesenchymal characteristics. Normally, the epithelial phenotype shows apical-basal polarity and cells are held together laterally by tight junctions and adherents junctions, the latter are formed by epithelial cadherin (E-cadherin) molecules. This organization is crucial for preserving the structural integrity of the epithelium (Dongre and Weinberg 2019; Yang et al. 2020). In the early stages of the EMT, E-cadherin expression is inhibited, resulting in the loss of the polygonal phenotype, loss of apical-basal polarity, and general destabilization of all junctions. The cells thus acquire a more mesenchymal phenotype, resulting in remodeling of the cytoskeleton, and exhibit back-front polarity. Mesenchymal cells contain intermediate vimentin filaments and use focal adhesions containing integrins to adhere to the extracellular matrix, features that confer increased migratory capacity and invasiveness (Figure VII) (Yang et al. 2020).

The main transcription factors responsible for the epithelial to mesenchymal transition are ZEB1 and ZEB2 (zinc-finger E-box binding homeobox factors), SNAI1 or SNAIL together with SNAI2 or SLUG (Snail family transcriptional repressor), TWIST1 and TWIST2 (basic helix-loop-helix factors). All these genes contribute to the downregulation of E-cadherin (Dongre and Weinberg 2019) and induction of vimentin, $\alpha$-smooth muscle actin (SMA) and fibronectin which lead to pseudopodia formation, cytoskeleton remodeling and promotion of cell motility (Christiansen and Rajasekaran 2006). Other important markers associated with transition are metalloproteases (MMPs): SNAIL and ZEB2 induce their expression, and their action leads to basement membrane degradation, promoting neoplastic cell invasiveness (Dongre and Weinberg 2019). IGF-1 and IGF-2 (insulin-like growth factor) also take part to EMT process: the binding between IGF1/2 and the respective receptors activates two main pathways: IRS-1/PI3K/Akt and Ras/Raf/ERK. Both lead to the

expression of specific transcription factors of the ZEB, SNAI and TWIST families. The bioavailability and half-life of IGF is regulated by seven IGF-binding proteins (IGFBPs). Excessive production of these proteins can induce tumor growth (Li et al. 2017). IGFBP4, for example, can lead to overexpression of factors involved in EMT and invasiveness, as well as downregulation of E-cadherin (Figure VII) (Praveen Kumar et al. 2014).

E-cadherin is undoubtedly one of the main EMT markers: its loss is associated with the destruction of cell junctions. Moreover, during the transition process, the cadherin switch occurs and meanwhile E-cadherin is down-regulated there is an increase in N-cadherin, which is typical of the mesenchymal phenotype. Down-regulation of the *cdh1* gene (encoding E-cadherin) helps tumor cells to detach from their onset site (Thompson et al. 2005).

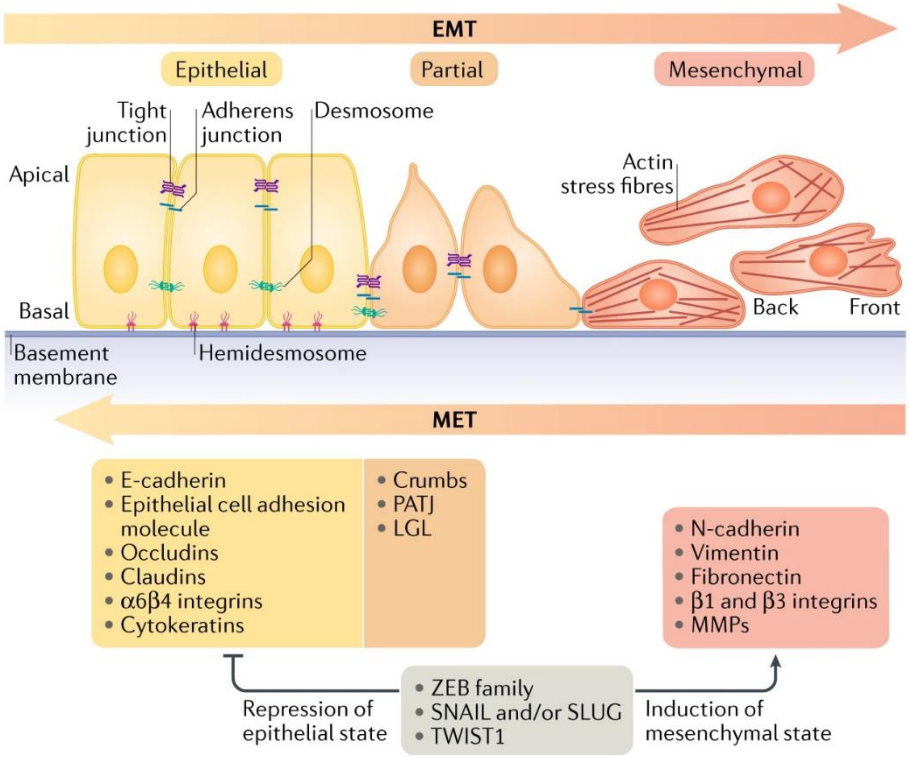


**Figure VII. Epithelial to mesenchymal transition.** Schematic view of EMT and MET processes by presenting the mail molecules involved (Dongre and Weinberg 2019).

The EMT concept was firstly developed in embryology field, in fact EMT is essential at various stages of embryonic development, including embryo implantation, gastrulation, and somitogenesis; in these processes, EMT enables epithelial cells to acquire a motile phenotype that allows them to migrate and take part in the formation of different embryonic

tissues (Yang et al. 2020). In adults, EMT plays a key role in maintaining tissue homeostasis, regeneration and repair (Dongre and Weinberg 2019). Later, EMT has shown to be involved also in pathological processes such as cancer. The acquisition of mesenchymal traits allows tumor cells to move and migrate, first locally and later also to other tissues, for these reasons EMT is involved in metastatic dissemination and cancer progression (Dongre and Weinberg 2019).

For a long time, the transition was regarded as a binary process: cells would lose their main epithelial marker, E-cadherin, and instead begin to express vimentin. Recent studies show that, on the contrary, the transition is a gradual process involving several intermediate stages in which there is expression of both epithelial and mesenchymal markers. Thus, high phenotypic heterogeneity can be identified within tumors: various epithelial, mesenchymal and hybrid cell subpopulations, i.e., in an intermediate transition state, have been identified in primary breast and skin tumors (Pastushenko et al. 2018; Pastushenko and Blanpain 2019).

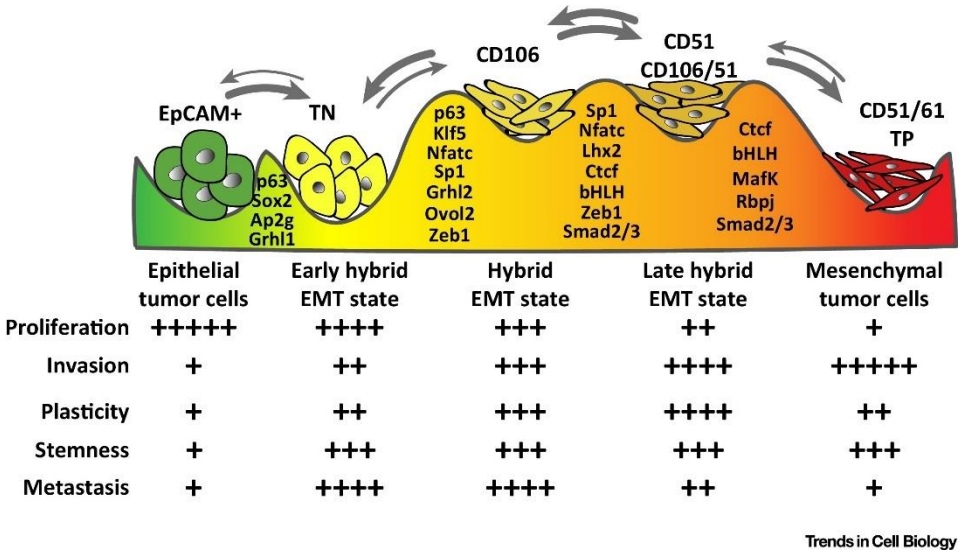


**Figure VIII. Epithelial to mesenchymal transition intermediate states.** Proliferation, invasion, plasticity, stemness, and metastatic capacity related to the different EMT transition states are summarized (+: low to ++++: very high) (Pastushenko and Blanpain 2019).

This phenotypic variability can generate extremely adaptable and resistant cells which are able to travel through the blood and/or lymphatic circulation and colonize distant organs different from the one of origin (Figure VIII) (Gooding and Schiemann 2020).

In addition, all these different EMT states contribute to tumor heterogenicity and an association between EMT and acquisition of stem-like features has been documented in several cancer type (e.g. breast, lung and colon carcinoma) (Shibue and Weinberg 2017).

HER family receptors are involved in the induction of EMT. The transforming growth factor (TGF)-β signaling pathway plays a key role in the epithelial to mesenchymal transition and is therefore overexpressed in HER2-positive tumors (Voutsadakis 2019). The crosstalk that exists between HER2-dependent signaling and the signaling pathways that regulate stemness moreover leads mammary epithelial cells to go into epithelial-mesenchymal transition (Nami and Wang 2017).

In breast cancer, EMT contributes to the formation of cells resistant to trastuzumab therapy in HER2-enriched subtypes: in fact, one of the main mechanisms of resistance is due to cleavage of the extracellular portion of HER2 by metalloproteases (Nami and Wang 2017). In particular, during EMT process there is a significant up-regulation of metalloproteases, which are required for proteolysis of cell-cell adhesion molecules and extracellular matrix. Consequently, HER2 also undergoes proteolytic cleavage: this negative correlation between HER2, EMT and stemness explains the phenomenon of trastuzumab resistance. Dual inhibition of HER2 and metalloproteases could be a winning treatment choice (Nami and Wang 2017).

### *1.3.4. Breast cancer stem cells*

Stem cells are essential for the preservation of tissue homeostasis. They perpetuate their own population through the self-renewal, a type of division where one (asymmetric division) or both (symmetric division) daughter cells remain stem, thus able to maintain the pool. In the case of asymmetric division, the non-stem cell undergoes differentiation to maintain tissue homeostasis (Prieto-Vila et al. 2017; Bagnara et al. 2020). Stem cells also can switch to different phenotypic states without altering the DNA (Kong et al. 2020).

Recent studies have shown that cancer stem cells (CSC) - a small population of cells endowed with stem-like properties, tumor-initiating and immune regulatory abilities – play a key role in tumor development, progression and resistance to therapy (Ruiu et al. 2019).

It is possible that CSC arise by mutation from normal stem cells or more differentiated progenitor cells that regain self-renewal properties (Jordan et al. 2006). Moreover, CSC can also originate from normal non-stem epithelial cells that undergo transformation and acquire self-renewal abilities, or from de-differentiated cancer cells. Studies suggested a commonality between CSCs and EMT states; in particular, agents generated in the tumor microenvironment, like TGF-β and IL6, can induced EMT in human breast cancer tumor cells and lead to an increase in breast cancer stem cells (BCSCs) number (Liu et al. 2014; Quaglino et al. 2020b).

BCSCs constitute about 1-5% of all tumor mass and are mostly found locked in the G0 phase of the cell cycle (quiescent state). Consequently, they are inherently immune to the action of all those drugs that exploit proliferation as a target, such as chemotherapy. Current therapeutic strategies are able to induce general tumor regression but do not directly target BCSCs: although they represent only a small percentage of the total mass, their presence acts as a reservoir for disease recurrence.

CSC are not necessary rare and they may exist in different states; thus, it is critically important to look at new therapeutic strategies for targeting cancer stem cells (Quaglino et al. 2020a).

These cells are identified through several markers, the main ones are CD44, CD24 and ALDH1 (aldehyde dehydrogenase 1). High CD44 expression combined with low CD24 expression defines a stem/mesenchymal (CD44+/CD24-) population (Nami and Wang 2017; Park et al. 2019), whereas a CD44-/CD24+ population is generally characterized by an epithelial phenotype (Nami and Wang 2017). Together with the epithelial to mesenchymal state, ALDH1 allows to distinguish between two different sub-populations of BCSCs: the CD44+/CD24-/ALDH1low phenotype identifies a group of mesenchymal and quiescent cells, called EMT-BCSC, while ALDH1+ is associated with an epithelial and proliferative subtype, called MET-BCSC (MET: mesenchymal to epithelial transition) (Al-Hajj et al. 2003).

Several therapeutic strategies, aimed both at impairing the self-renewal ability and/or at stimulating the host immune system, to inhibit BCSCs are currently under investigation (Quaglino et al. 2020b; Kong et al. 2020).

### 1.4. Therapies approved for the treatment of HER2-positive breast cancer

HER2 turns out to be a good therapeutic target because its levels in tumor tissue are much higher compared with normal tissue, thus decreasing toxicity and increasing the specificity of the drugs used. Therefore, HER2-positive breast cancers depend on the oncogene HER2 to sustain their malignant phenotype, this behavior is also known as "oncogene addiction" (Weinstein and Joe 2006). Targeted therapies exploit these two features to hinder tumor growth (Swain et al. 2022).

During the last 30 years, newer and novel therapeutic strategies have dramatically changed the management of HER2-positive breast cancer and significantly improved survival outcomes (Kunte et al. 2020; Swain et al. 2022).

Nowadays, there are two main therapy groups approved for the HER2-positive breast cancer treatment: the first one is represented by monoclonal antibodies (mAb) against HER2 to selectively deliver drugs against tumor cells, the second group includes drugs that inhibit the HER2 signaling pathway, called small tyrosine kinase inhibitors (Figure IX) (Escrivá-de-Romaní et al. 2018; Kunte et al. 2020).

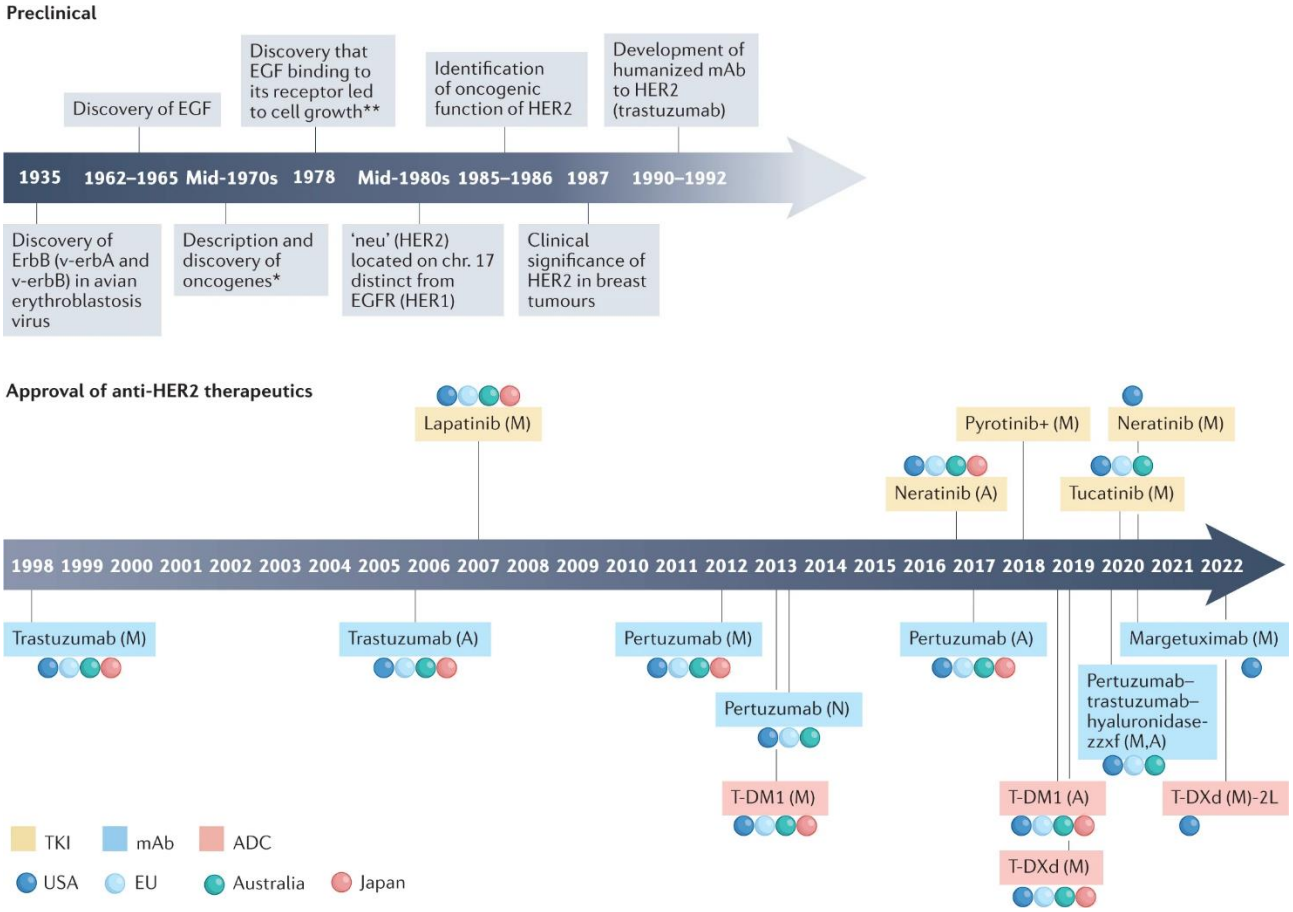


**Figure IX. Timeline of preclinical discovery milestones for regulatory approval of anti-HER2 therapies**. A, adjuvant setting; M, metastatic setting; N, neoadjuvant setting; +, approved in China only; *, Bishop and Varmus awarded Nobel Prize in 1989 for this discovery; **, Cohen and Levi-Montalcini awarded Nobel Prize in 1986 for discovery of growth factors and their receptors. (Swain et al. 2022).

Despite the significant improvement in the prognosis of patients with HER2-positive malignancy, a substantial number of patients develop forms of resistance to therapy over time, or manifest intrinsic tumor resistance and tumor progression (Swain et al. 2022). For this reason, it is critically important to find new therapeutic strategies against HER2.

### *1.4.1. Monoclonal antibodies*

For about a decade HER2 was measured simply to predict prognosis. In 1990s a murine anti-HER2 monoclonal antibody, m4D5, has been shown to be capable of inhibiting cell proliferation; this evidence prompted the development of a humanized mAb to HER2, known as trastuzumab (Herceptin) (Fendly et al. 1990; Carter et al. 1992).

Slamon and colleagues demonstrated that trastuzumab was able to inhibit the cell growth of tumor cells overexpressing HER2 (Slamon et al. 1987) and, thanks to his efforts, Genentech decided to produce Herceptin (Oostra and Macrae 2014). Trastuzumab was approved in 1998 by the Food and Drug Administration (FDA) for the treatment of metastatic HER2-positive breast cancer and later for the treatment of early-stage HER2-positive breast carcinomas (Ahmed et al. 2015; Kreutzfeldt et al. 2020). Trastuzumab is a humanized IgG1 antibody that binds the IV domain (in the extracellular region) of HER2 and works by following several mechanisms of action. It:

- by binding the ECD IV domain, induces the internalization and degradation of HER-2, thereby inhibiting, downstream signaling pathways of the receptor; it has been shown that the monoclonal antibody halts the interaction between HER-2 and Src, leading to the activation of PTEN (Vu and Claret 2012);
- induces cell cycle arrest and, thus, decreases cell proliferation; in fact, reduced expression of cyclin-dependent kinases (CDKs), including cyclin D1, is noted; this leads to the release of p27, which binds and inhibits the cyclin E/cdk2 complex (Kreutzfeldt et al. 2020);
- mediates ADCC (antibody-dependent-cell-mediated cytotoxicity); specifically, the Fc fragment of the antibody attracts immune system effector cells, such as Natural Killer (NK) cells or dendritic cells (DCs) (Vu and Claret 2012; Swain et al. 2022).

Actually, its combination with chemotherapy represents the standard care in metastatic setting, adjuvant and neoadjuvant setting (Senkus et al. 2015).

Although trastuzumab improves responses and outcomes, in neoadjuvant setting around 15% of patients showed resistance and up to 70% of metastatic patients developed resistance (Nahta and Esteva 2006; Vu and Claret 2012).

Thus, to overcome the resistance that some HER2-positive tumors show toward trastuzumab, a second humanized mAb, pertuzumab (Perjeta), which binds to ECD II, was developed. While trastuzumab is more effective in inhibiting tumor cells in absence of HER3 ligands, pertuzumab showed efficacy in preventing HER2 heterodimerization with HER1, HER3 and HER4 and blocking the downstream signalling (Ishii et al. 2019). These

complementary mechanisms of action and the ability of the two drugs to induce ADCC, complement-mediated cytotoxicity (CDC), antibody dependent cellular phagocytosis and complement dependent cellular phagocytosis ensured that these two drugs had a synergistic action (Nahta et al. 2004; Scheuer et al. 2009; Tsao et al. 2022). The efficacy of pertuzumab alone in the treatment of HER2-positive metastatic breast cancer is low; however, the phase III clinical trial CLEOPATRA (a study to evaluate pertuzumab + trastuzumab + docetaxel vs placebo + trastuzumab + docetaxel in previously untreated HER2–positive metastatic breast cancer) showed a significant improvement in median overall survival (OS) of 16 months with the addition of pertuzumab. Thus, currently the standard of care in metastatic setting is based on dual-HER2 antibody therapy (Swain et al. 2013; Swain et al. 2015). Thereafter, NEOSPHERE and TRYPHAENA clinical trials lead to the approvement of pertuzumab in combination with trastuzumab as neoadjuvant therapy to treat patients at high risk of metastasis or progression (Gianni et al. 2011; Schneeweiss et al. 2013).

Patients whose NK cells or DC cells bound more tightly to the Fc domain of trastuzumab (which mediates the ACDD response) showed stronger therapy response. This evidence inspired the development of a new anti-HER2 IgG1 mAb, margetuximab (MGAH22), with an engineered Fc domain to increase the affinity for the activating Fcγ receptor (CD16A) expressed on immune effector cells and induce an enhanced ADCC activity against HER2-positive cancer cells (Musolino et al. 2008; Nordstrom et al. 2011; Liu et al. 2019). The phase III clinal trial SOPHIA evaluated margetuximab plus chemotherapy compared with trastuzumab with chemotherapy in patients with advanced HER2-positive breast cancer or metastatic breast cancer. This trial showed that margetuximab was associated with a significantly longer median progression-free survival (PFS) compared to trastuzumab (Rugo et al. 2021). Based on SOPHIA results, margetuximab was approved for the treatment of metastatic HER2-positive breast cancer by FDA.

### *1.4.2. The dark side of anti-HER2 monoclonal antibodies: mechanisms of resistance and possible side effects*

There are several mechanisms of resistance to anti-HER2 therapies and some of these appear to be shared between different agents:

- HER2 mutations (3% of breast cancer) can drive tumor growth also in the absence of HER2 overexpression. Moreover, HER2 mutations frequently co-occur with HER3 mutations. Alterations in HER family lead to activation of downstream signaling pathways such as P13K–AKT and RAS–MAPK pathways (Figure Xa). This activating HER2 mutation has also conferred resistance to dual blockade by trastuzumab and pertuzumab (Swain et al. 2022);
- The HER2Δ16 isoform, which constitutive activates the downstream signaling, and generation of truncated p95HER2, that lacks the ECD recognized by anti-HER2 antibodies may harm the efficacy of target therapies. However, the role of HER2Δ16 isoform and p95HER2 in anti-HER2 therapy resistance is still debated (Figure Xb) (Pohlmann et al. 2009; Castagnoli et al. 2019);
- Epitope masking has also been investigated as a mechanism of resistance to trastuzumab. Some examples are MUC4 (mucin 4) and CD44–hyaluronan polymer complex, their overexpression led to HER2 epitope masking and consequent decreased antibody-binding capacity (Figure Xb) (Pohlmann et al. 2009; Swain et al. 2022);
- The activation of compensatory signaling pathways to overcome the effects of anti-HER2 treatments is also involved in target therapy resistance. The loss of function of PTEN caused by mutation of PTEN itself, or by transcriptional regulation, results in the constitutive upregulation of PI3K/Akt. Hyperactivation of PI3K/Akt signaling is linked to trastuzumab resistance (Figure Xc) (Chandarlapaty et al. 2012). Similarly, the overexpression of Cyclin D1, a protein involved in cell cycle control and found to interact with Rb (retinoblastoma protein) is associated with trastuzumab resistance (Figure Xc) (Vernieri et al. 2019). Another transmembrane TK receptor that stimulates cell proliferation, IGF-1R (insulin-like growth factor-I receptor), was found to interact

with HER2 in trastuzumab-resistant cell lines, inducing its phosphorylation. IGF-1R-mediated resistance to trastuzumab treatment seems to involve the PI3K pathway, leading to enhanced degradation of p27 (a Cdk inhibitor) (Lu et al. 2004);

- The heterogeneous HER2 expression within the tumor can decrease sensitivity to HER2-targeted therapies that are dependent on overexpression of HER2 (Figure Xd) (Swain et al. 2022).
- The immunosuppressive tumor microenvironment (TME) (Swain et al. 2022) and a genomic polymorphism that alters the FcγRIII receptor influencing the binding affinity of IgG1 to the Fcγ receptor, thus hampering the anti-tumor ADCC-mediated efficacy of trastuzumab (Koene et al. 1997).

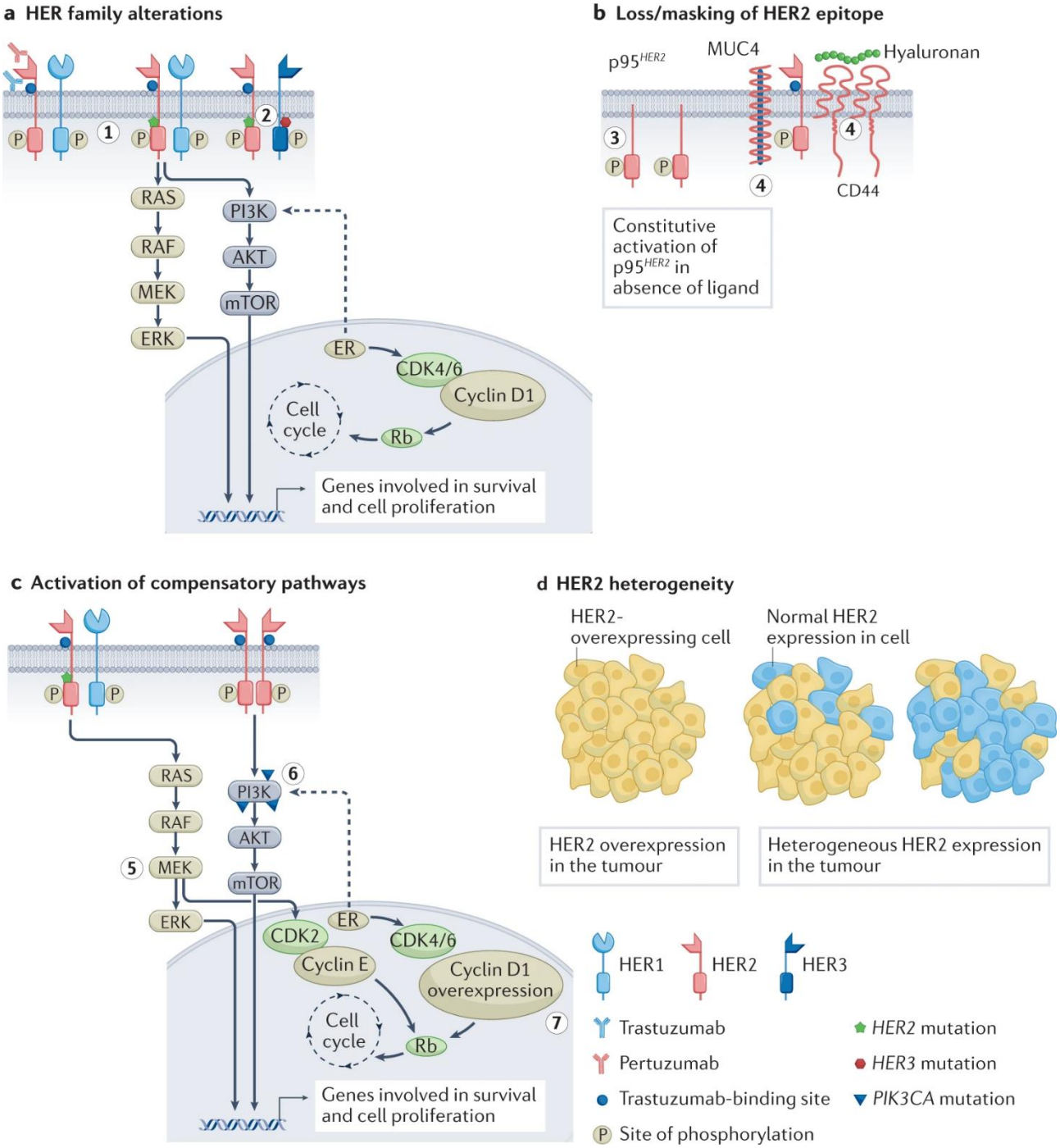


**Figure X. Mechanisms of resistance to anti-HER2 therapies**. A. Mutations and/or alterations in the HER family of receptors that lead to activation of downstream signalling pathways. B. Loss or masking of the trastuzumab-binding site. C. Activation of compensatory pathways. D. Heterogeneous expression of the HER2 receptor in tumour cells (Swain et al. 2022).

Although their high efficacy in improving patient survival, anti-HER2 mAb are not without potential side effect. The main one is the cardiotoxicity shown by patients treated in the metastatic breast cancer and early breast cancer setting with trastuzumab, especially

if combined with anthracyclines (Slamon et al. 2016). The discovery of trastuzumab-specific cardiotoxicity has paved the way for several lines of research aimed at characterizing the role of HER2 in adult cardiomyocytes, and the mechanisms consequent to its drug-specific blockade. Currently, the data available have not fully elucidated the mechanisms underlying cardiac toxicity, but they suggest how trastuzumab may result in cardiac damage through several mechanisms (Mohan et al. 2018). Neuregulin 1 (NR-1), a member of the EGF-like growth factor family, constitutes one of the natural ligands of Erb receptors. NR1/ErbB binding results in the activation of ERK1/2 and PI3K/Akt pathways with cardioprotective action, especially in stressful situations. In the presence of trastuzumab this pathway is blocked in the upstream. Oxidative stress induced by NR1/ErbB inhibition induces overexpression of angiotensin II (AT-2). AT-2 in turn represents an inhibitor of NR-1, triggering a cycle that increases intracellular oxidative stress (Kuramochi et al. 2006). The proapoptotic role of AT-2 is also determined by the activation of the NADPH oxidase, which induces mitochondrial dysfunction and cell death. Taken together, these evidences suggest the pivotal role of HER2 in cardiomyocyte survival under stressful situations. Binding to trastuzumab prevents the implementation of these defence mechanisms, thus justifying also clinical evidence about the synergistic cardiotoxic effect of administering of anthracyclines and anti-HER2, attributable to a biological pattern termed "2-hit" (Bowles et al. 2012). The cellular "first hit" is given by anthracyclines and induces the formation of reactive oxygen species (ROS) and inhibition of topoisomerase 2 resulting in DNA double-strand breaks. The simultaneous inhibition of the HER2 pathway represents the "second hit". Through the disruption of cardioprotective signaling it decreased the ability of cardiomyocytes to recover (Cote et al. 2012; Zhang et al. 2012).

Dual HER2-targeted therapy with pertuzumab and trastuzumab in HER2-positive metastatic breast cancer (MBC) has not been shown to exacerbate cardiotoxicity or lead to increased cardiac events (Piccart et al. 2021).

To sum up, anti-HER2 drugs have revolutionized the manage of HER2-positive breast cancer but further efforts are needed to identify new therapeutic strategies to

overcome resistance to targeted therapies, block tumor progression, and to reduce side effects.

### *1.4.3. Antibody-drug conjugates (ADC)*

T-DM1 (trastuzumab emtansine) is an immunoconjugate consisting of trastuzumab and emtansine (DM1), a chemotherapeutic agent with cytotoxic activity on microtubules. The antibody component selectively binds to HER2, which is exposed on the surface cell, mediating internalization of the complex, with subsequent release of the emtansine within the cell. This mechanism enables the selective performance of antitumor activity at the level of HER2-positive cells, minimizing systemic exposure to the chemotherapeutic component (Oostra and Macrae 2014).

In phase III EMILIA clinical trial T-DM1 showed greater efficacy than lapatinib and capecitabine, thus it has been approved by FDA in 2013 as standard second-line treatment, after taxane and trastuzumab, in metastatic or rapid progression (≥6 months) breast cancer (Verma et al. 2012).

There are several antibody-drug conjugates in clinical developed. The most relevant one is trastuzumab deruxtecan (T-DXd) which showed a benefit over T-DM1 in DESTINY-Breast03 clinical trial. At 12 months, the 75.8% of patients treated with T-DXd were alive without disease progression compared with 34.1% with T-DM1. An overall response occurred in 79.9% of the patients who received T-DXd as compared with 34.2% of those treated with T-DM1 (Cortés et al. 2022; Hurvitz et al. 2022).

### *1.4.4. Tyrosine kinase inhibitors (TKIs)*

To overcome the problem of resistance and activation of HER2 downstream signaling pathways by other receptors, another class of drugs was developed, the small tyrosine kinase inhibitors. TKI are small synthetic molecules which binds HER2 intracellular domain and blocks its tyrosine-kinase activity, by competition with ATP, promoting apoptosis and cell cycle arrest. Due to their small size, they are, unlike monoclonal antibodies, able to cross the blood-brain barrier and act against brain metastases (Xuhong et al. 2019).

Lapatinib (Tykerb) is a reversible inhibitor capable of binding to both HER2 and EGFR. It was first approved by the FDA in 2007 in combination with capecitabine for the treatment of patients with advanced or metastatic HER2-overexpressing breast cancers who have received prior therapy (trastuzumab, taxanes and anthracyclines) (Ryan et al. 2008). The addition of this drug to standard therapy results in more frequent complete remission of the disease, but without improving overall survival, and it is for this reason that it is used as second-line therapy (Ocaña and Amir 2009). This molecule can interact with the ATP binding site of the receptors through the formation of a reversible bond; this binding goes on to inhibit the activation of the three major pathways activated by EGFR and HER2: MAPK, PI3K/AKT, and PLC. Lapatinib has also been shown to be effective on trastuzumab-resistant cells: underlying this phenomenon appears to be the ability of the TKI to inhibit truncated forms of the receptors (particularly p95HER2) or the effects of the drug on IGF-1 signaling (Medina and Goodin 2008). On the other hand, inhibition of EGFR and HER2 leads to the activation of compensatory mechanisms including the increased expression of HER3, which is able to activate signaling sufficient to allow tumor growth. This constitutes one of the main mechanisms of resistance to lapatinib therapy (Ruiz-Saenz and Moasser 2018).

Neratinib (Nerlynx) is a small tyrosine-kinase inhibitor that binds irreversibly to a cysteine residue placed inside the binding pocket for ATP of HER2. Neratinib can also acts with the catalytic domain of EGFR, HER3 and HER4 and consequently block, their phosphorylation and downstream signal transduction pathways: this gives it greater antitumor activity than lapatinib (Feldinger and Kong 2015). In the phase III ExteNET clinical trial, it was shown that the use of neratinib for 12 months significantly reduced, compared with placebo, the risk of recurrence and mortality in women with HER2-positive early breast cancer previously treated with adjuvant or neoadjuvant trastuzumab (Chan et al. 2016). Thanks to these results, neratinib was approved in 2017 by FDA for the extended adjuvant treatment of adult patients with early stage HER2-positive breast cancer, following adjuvant trastuzumab-based therapy. In Europe, neratinib is approved for the extended adjuvant treatment of adult patients with early-stage hormone receptor-positive HER2-overexpressed/amplified breast cancer and who are less than one year from completion of

prior adjuvant trastuzumab-based therapy. In 2020, FDA has also approved neratinib for the treatment of patients with advanced or metastatic HER2-positive breast cancer who have received two or more prior anti-HER2 based regimens in the metastatic setting. A unique feature of neratinib is its activity in cell lines with somatic HER2 mutations in the absence of HER2 amplification, suggesting that it can overcome possible resistance to other anti-HER2 therapies (Canonici et al. 2013). Moreover, the SUMMIT basket trial showed that neratinib can be effective also in HER2-positive breast cancer with HER2 mutations or alterations (Hyman et al. 2018).

Tucatinib (Tukysa) is a tyrosine kinase inhibitor of HER2 which inhibits both HER2 and HER3 phosphorylation and downstream signal transduction through the MAPK and PI3K pathways. In 2020, based on the HER2CLIMB clinical trial FDA approved tucatinib in combination with trastuzumab and capecitabine for the treatment of patients with advanced unresectable or metastatic HER2-positive breast cancer, including patients with brain metastases, who have received one or more prior anti-HER2-based regimens in the metastatic setting. Tucatinib demonstrated efficacy compared with placebo in progression-free survival (PFS) and overall survival (OS). The 48% of patients enrolled in the study presented stable or active brain metastases. Also patients with brain metastases showed benefit in PSF after treatment with tucatinib. Thus, this is the first FDA approval which specifies patients with brain metastases (Shah et al. 2021).

# 2. CANCER VACCINES AS THERAPEUTIC STRATEGIES FOR HER2-POSITIVE BREAST CANCER

Immunization has been practiced for hundreds of years starting from 1796 when Edward Jenner discovered the first vaccine to prevent smallpox infection. In the early 20th century, immunotherapy, as a method of cancer treatment, was born in America by William Coley, who began using bacteria to stimulate patients' immune systems and push them to fight the tumor (DeMaria and Bilusic 2019). Later, in 1950s Lloyd Old developed a similar approach with Bacillus Calmette–Guérin (BCG) (OLD et al. 1959). Thanks to these two pioneers, the immunotherapy has evolved the treatment of several cancers (Grimmett et al. 2022).

Cancer vaccines are a complete antitumoral immunological approach thanks to their ability to stimulate both innate and acquired immunity. Despites passive immunization strategies, such as monoclonal antibodies, cancer vaccines are able to induce an immune memory which led to a long-lasting protection (Baxter 2014).

In 1980 Hoover and colleagues developed one of the first cancer vaccines based on tumor cells and lysates to treat colorectal cancer (Hoover et al. 1985). Around 10 years later, the first human tumor antigen to be recognized by T lymphocytes was identified by van der Bruggen and colleagues. Later they provided the first identification of a real molecular target through cloning the melanoma antigen encoding gene (MAGE), a gene encoding an antigen recognized by the cytotoxic T cells (van der Bruggen et al. 1991).

Nowadays, there are only two vaccines approved by FDA to prevent malignancies caused by viruses, the hepatitis B vaccine and the HPV (Human Papilloma Virus) vaccine (Liu et al. 2022). Except for tumor induced by virus infection that can be addressed to the entire population, it is difficult to image a vaccination against an oncogene to be use in large scale on healthy people. Perhaps, people who have a specific genetic risk of cancer, or have been exposed to an exogenous carcinogen might be the more appropriate cohort for a preventive vaccine based on a specific target antigen (Lollini et al. 2006; Lollini et al. 2015).

Another promising use of cancer vaccine might be on therapeutic setting. However, the development of a therapeutic cancer vaccine is more complicated than a prophylactic vaccine mostly because tumors, in order to progress, have already escaped the

immunosurveillance (Lollini et al. 2006). Thus, a good therapeutic cancer vaccine has to induce a strong T cell response against tumor cells and elicit high and persistent antibody titers able to inhibit cancer cells survival and proliferation. Although the good preclinical results, these are unlikely to be seen in clinical trials (Lollini et al. 2006; Grimmett et al. 2022). Sipuleucel-T (Provenge), based on dendritic cells, is the only cancer vaccine approved by FDA (in 2012) for the treatment of prostate cancer (Gardner et al. 2012).

As better presented in the following paragraphs, HER2 is an excellent candidate as target antigen for cancer vaccine but, until now, any antitumor vaccine has been approved for the treatment of HER2-positive breast cancer (Swain et al. 2022).

### 2.1. Target antigens for cancer vaccines

The choice of antigen is crucial to the efficacy of the vaccine in the clinic. The ideal antigen should be specifically expressed on cancer cells with no expression on normal cells, it should be necessary for cell survival and be highly immunogenic (Paston et al. 2021). Targeting overexpressed molecules is a promising strategy but must be careful to possible autoimmunity toward normal tissue expressing low levels of the molecule (for example HER2 expression on heart cells).

Neoantigens are new protein specifically expressed on tumor cells and are formed in cancer cells when certain mutations occur in tumor DNA, they are the result of the genetic instability of cancer cells. Thus, they can trigger a tumor specific T-cell response by limiting "off-target" effects (Blass and Ott 2021). One of the most studied neoantigen in vaccine and immunotherapy is KRAS in non-small cell lung cancer (NSCLC) (Paston et al. 2021). Other examples of tumor specific antigens are the cancer germline antigens, that have been widely studied due to their expression only on germ cells of immune-privileged organs and high expression on tumor cells.

Oncoantigens, overexpressed antigens, cancer-testicular antigens (MAGE), and viral-original "non-self" antigens can be used for cancer vaccines (Plaen et al. 1994; Chen et al. 1997; Paston et al. 2021). Oncoantigens are a class of persistent tumor antigen which support tumor growth, are generally expressed at low levels on normal cells but are overexpressed

and/or amplified in tumor cells and do not escape from immune recognition, some examples are EGFR, HER2, the mucin MUC1 and the idiotype of B and T cell malignancies (Lollini and Forni 2003; Lollini et al. 2005). They can be classified on the basis of their location in cancer cells in class I, class II or class III (Lollini et al. 2011). Class I antigens are expressed at the level of the plasma membrane of tumor cells, class II antigens are not expressed directly by cancer cells, but are found at the level of the tumour microenvironment and class III antigens are intracellular antigens expressed by cancer cells (Lollini et al. 2010; Cavallo et al. 2014; Conti et al. 2014). Not all tumor antigens can be classified as oncoantigens because of their intracellular localization (e.g. MAGE) or because they appear not to be directly related to tumorigenic capacity, e.g. carcinoembryonic antigen, CEA (Cavallo et al. 2007).

Tissue lineage and differentiation antigens are expressed on normal and tumor cells from the same tissue, targeting these antigens require attention to any potential toxicity on normal cells. The most studied differentiation antigens are prostatic acid phosphatase (PAP), prostate-specific antigen (PSA), glycoprotein 100 (gp100) and melanoma antigen recognized by T cells 1 (MART-1) (DeMaria and Bilusic 2019; Paston et al. 2021).

A new category of targetable antigens includes molecules involved in EMT and stemness, such as OCT-4, CD44 and CD133, and breast cancer stem cells, like xCT and teneurin-4 (Bolli et al. 2018; Quaglino et al. 2020b; Ruiu et al. 2021).

### 2.2. Cancer vaccine platforms

Cancer vaccines differ not only in target antigens but also in the platforms chosen. Vaccine platforms can be divided in 4 main categories: cell-based vaccines (subdivided in cancer cell and dendritic cell vaccines), peptide-based vaccines, virus/bacteria-based vaccines, gene-based vaccine (divided into DNA and RNA vaccine) (Lopes et al. 2019).

One of the earliest approaches to cancer vaccine is based on the use of whole tumor cells or products from tumor cells lysis. The whole tumor cell vaccine is a simple and direct approach to tumor immunotherapy, by containing the whole tumor associated antigens it can potentially induce a polyclonal immune response. However, cellular vaccine production is expensive and difficult and this technology needs a co-stimulatory molecule

to trigger antigen-specific immune response (Ladjemi et al. 2010; Al-Awadhi et al. 2018). Kim and coworkers demonstrated that combined administration in mice of HER2/neu-specific mAb and a HER-2/neu-expressing, GM-CSF-secreting whole tumor cell vaccine enhanced induction of neu-specific CD8+ T cells through Fc-mediated activation of dendritic cells (Kim et al. 2008). Nanni and colleagues demonstrated that a recombinant cell vaccine based on cells expressing p185 target antigen combined with two adjuvants (Triplex vaccine), IL-12 and allogenic class I MHC molecules, was effective in inhibiting metastasis outgrowth in 90% of HER2/neu transgenic mice (Nanni et al. 2007).

The use of dendritic cells (DCs) as vaccine platform allows to take advantage of the ability of these cells to promote antigen presentation to the other immune cells (Ladjemi et al. 2010). Preclinical studies showed that DCs engineered to produce HER2 protein delayed the onset of spontaneous HER2/neu overexpressing mammary tumors in BALB/c transgenic mice and DCs transfected with an adenovirus encoding both HER2 and IL-12 induce tumor protection in FVB mice challenged with syngeneic HER2 over-expressing tumor cells by stimulating both CD4+ and CD8+ T lymphocytes response (Chen et al. 2001; Sakai et al. 2004). Unfortunately, DCs vaccine cannot be produced on large scale since they are based on autologous dendritic cells.

Protein/peptide vaccines are composed of known or predicted tumor antigen epitopes. These vaccines are relatively simple to produce and several studies have tested the efficacy of vaccine composed of peptides derived from HER2 ECD or intracellular domain and many protein or peptide vaccines against HER2-positive breast cancer are under clinical trials (Ladjemi et al. 2010; Swain et al. 2022). By the way, this platform is poorly immunogenic and requires adjuvants and clinical application is limited by HLA restrictions (Paston et al. 2021).

Gene-based vaccine can be divided in DNA-based or RNA-based vaccine. Early gene-based vaccine primarily focused on DNA vaccines due to the stability and long-time presence in the body of this nucleic acid. DNA molecules need to enter the cell nucleus to initiate transcription, while mRNA enters the cytoplasm to translate and express antigens directly. Therefore, mRNA antigen production is instantaneous and efficient. DNA vaccines

need an extra step to go into the cell nucleus, leading to a lower immune response than mRNA vaccines. Preclinical studies on a human HER2 transgenic mouse model demonstrated that a DNA cancer vaccine was effective to break the immunological tolerance to human HER2 and elicit immune response, that inhibited the in vivo growth of human HER2-positive cancer cells (DeGiovanni et al. 2014). Internalization of these vaccine platforms by patient's cells could be not complete in many cases leading to low efficacy (Paston et al. 2021). Electroporation and chimeric or xenogenic antigens can be used to improve DNA vaccine effectiveness (Cavallo et al. 2014; Riccardo et al. 2017).

Virus-based cancer vaccine mainly use viruses as vectors to treat and prevent tumors (the approved cancer vaccine to prevent HPV infection is based on this technology) (Sander and Lollini 2018; Mohsen et al. 2020; Liu et al. 2022). Virus-like particles (VLP) are an interesting option to maximize cancer vaccine immunogenicity without compromising safety and efficacy (Sander and Lollini 2018; Caldeira et al. 2020). Antigens can be incorporated on virus capsid by genetic modification or by chemical binding, allowing immunization against peptides, parts of peptides or even whole proteins (Caldeira et al. 2020; Mohsen et al. 2020). Antigens are arranged on the surface of the virus-like particle to form repetitive and highly organized geometric patterns that are capable, not only of stimulating B lymphocytes, but also of attracting components of the humoral immune system such as antibodies and proteins of the complement system. In this way, both the innate and adaptive components of the immune system are stimulated (Sander and Lollini 2018; Mohsen et al. 2020). A study performed by Prof. Lollini group reported that a HER2-VLP vaccine was effective to break the immunological tolerance to human HER2 and induced higher antibody titres than a DNA-based vaccine in human HER2 transgenic mouse models. Moreover, the anti-HER2 virus-like particles-based vaccine prophylactic vaccination reduced spontaneous development of mammary carcinomas by 50%-100% in human HER2 transgenic mice and inhibited the growth of HER2-positive tumors implanted in wild-type syngeneic mice (Palladini et al. 2018).

Several bacteria are highly motile in the prevalent anaerobic/hypoxic conditions of the tumor cells and can be attracted by the high amounts of metabolic nutrients in the tumor

microenvironment. Furthermore, it has been reported that several conserved bacterial ligands act as agonists for innate immune system receptors like TLRs, and when binding, initiates an intracellular signaling cascade thereby, leading to the production of proinflammatory cytokines. Due to these features, bacteria are considered as potential candidates for delivery of anticancer agents or gene-based vaccines for treating tumors. (Shanmugaraj et al. 2020).

Gram-negative bacteria release outer membrane vesicles (OMVs), which are double-layer membrane vesicles with a lipid-based spherical structure. OMVs can be recognized by the host immune system through surface proteins such as toxoid factors, DNA, RNA and this recognition can inhibit the immune response, promoting infection and triggering inflammation (Wang et al. 2022). By the help of gene technologies antigens can be successfully introduced into OMVs and, together with OMVs inherent ability to elicit the immune response, they may represent a potential platform for cancer vaccine.

### 2.3. Therapeutic strategies to improve cancer vaccines efficacy

Vaccine efficacy can be hindered by an immunosuppressive microenvironment consisting of immunosuppressive type 2 macrophages (M2 macrophages), myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Treg cells) that impair the action of effector T cells (Saxena et al. 2021). An impaired or downregulated antigen presentation to T cells may also affect vaccine efficacy (Saxena et al. 2021).

The first and easier strategy to improve vaccine immunogenicity is the appropriate choice of the adjuvant. The choice of adjuvants should be optimized for each vaccine formulation, as well as for each patient, in order to attract immune cells to the site of injection and to break immune tolerance for achieve maximum immune responses and clinical efficacy (Cuzzubbo et al. 2020). Based on their function adjuvants can be divided into formulation (or "depot") and immunostimulatory. The first group include Alum, Addavax or emulsion, such as water-in-oil emulsions. The incomplete Freund's adjuvant or Montanide (Montanide ISA 720 or Montanide ISA 51) is an example of water-in-oil emulsion commonly used in clinical trials, it forms a depot at the injection site, resulting in the

catching of the antigen and prevents their rapid trafficking to local lymph nodes, this induces inflammation and the gradual release of the antigen. In a clinical trial Montanide ISA 51 was shown to induce both CD4+ and CD8+ T cell responses in patients (Kenter et al. 2009; Paston et al. 2021). Immunostimulatory adjuvants exploit the intrinsic functions of cytokines (e.g. INF$\alpha$, INF$\gamma$, IL2, IL12 or GM CSF) and toll-like receptor ligands to improve vaccine efficacy. An immunostimulatory adjuvant widely used is GM-CSF, a cytokine that stimulates the bone marrow to produce granulocytes and macrophages and release them into the bloodstream (Tay et al. 2017). In preclinical studies, this adjuvant proved to be a promising candidate, able to recruit DC cells to the injection site, induce their maturation and facilitate antigen presentation. However, in clinical trials the results have been discordant (Cuzzubbo et al. 2020).

The vaccine delivery system could be also considered as an adjuvant, in fact they can enhance cellular uptake (Paston et al. 2021). Electroporation is a strategy to improve DNA vaccine uptake by forming pores in cell membrane. In this way DNA uptake increase by 1000-fold and there is an adjuvant effect due to local tissue damage and the resulting stimulation of proinflammatory cytokines (van Drunen Littel-van den Hurk and Hannaman 2010). However, this kind of system may experience reduced compliance from patients (Aurisicchio and Ciliberto 2012).

A combination of a delivery system adjuvant and an immunostimulant adjuvant is commonly chosen as strategy to enhance cancer vaccine efficacy. For instance, Montanide (for its depot effect) and a TLR ligand (that can induce APC stimulation) constitutes a common combination of adjuvants for anti-cancer vaccines (Ahonen et al. 2004; Cuzzubbo et al. 2020).

Treg cells are known for inhibiting cytotoxic T-cells (CTL) functions through the release of anti-inflammatory factors as well as the depletion of IL2 in the microenvironment, thereby reducing its availability for T cells. Combining cancer vaccines with molecules that can reduce the number of Treg cells, such as chemotherapeutic agents (e.g. cyclophosphamide) constitutes a possible approach to improve the efficacy of CTLs induced by cancer vaccine (Ghiringhelli et al. 2007; Cuzzubbo et al. 2020).

Tumor irradiation that occurs during radiotherapy can stimulate immunogenic cell death (ICD) and thus lead to tumor regression (Golden et al. 2012). Additionally, radiotherapy has been reported to stimulate the expression of several molecules in cancer cells that promotes CTL functions, such as MHC class I, death receptors and adhesion molecules (Reits et al. 2006). Therefore, combining radiotherapy and therapeutic cancer vaccine also represent an attractive treatment option (Cuzzubbo et al. 2020).

Immune checkpoint inhibitors (ICIs), including antibodies against programmed cell death protein-1 (PD-1) or its ligand (PDL-1) and cytotoxic T-lymphocyte antigen-4 (CTLA-4) have proven to enhance anti-tumor immunity and efficacy in several cancers. However, many patients do not benefit from ICI therapy due to a limited specific T cell response developed against cancer cells (Esfahani et al. 2020). Thus, combining a cancer vaccine, which can elicit specific T cell responses, and ICIs represents an attractive therapeutic option. Based on positive results in pre-clinical models, several clinical trials are now evaluating novel personalized vaccines in combination with ICIs.

### 2.4. Anti-HER2 cancer vaccines under clinical trials: an overview

In breast cancer patients humoral and cellular immune responses have been recorded against HER-2 because of its high immunogenicity (Disis et al. 2000). Despite this, these immune responses fail to contain tumor growth. Thus, therapeutic vaccines against HER-2 need to overcome this immune tolerance and promote a durable and effective response (Zhu and Yu 2022).

A key consideration regarding cancer vaccines is the therapeutic setting, for example the immunosuppressive tumor microenvironment in metastatic disease might limit T cell activity and reduce cancer vaccine efficacy (Swain et al. 2022).

Nowadays, there are 60 clinical trials in which cancer vaccines are being evaluated for the treatment of HER2-positive tumors, and 49 of these use strategies directed against HER2, the remainder are aimed at boosting the immune response to enhance treatments with monoclonal antibodies or chemotherapeutics. The 27% of these cancer vaccines is based on cell technology (both tumor or immune cells), the 51% is testing peptide/protein

vaccine, the 14% is looking for viral-based vaccines efficacy and around 6% is evaluating DNA vaccines (ClinicalTrials.gov accessed on January 28th, 2023).

As with other cancer types, one of the first approaches to breast cancer vaccination was based on the administration of whole tumor cells or their lysates. Whole cell vaccines can be constituted of autologous or allogenic (based on established cancer cell lines) tumor cells. In a phase I clinical trial with an allogenic HER2 cell vaccine in combination with GM-CSF and administered together with cyclophosphamide and trastuzumab, authors observed a clinical benefit rate of 55% at 6 months, with progression-free survival and overall survival of 7 months and 42 months respectively, without recording increased side effect (Chen et al. 2014). Unfortunately, cell-based vaccine presents a high variability and a difficult production process (Swain et al. 2022).

The largest part of anti-HER2 vaccination strategies is based on peptide platforms due to their high cost-effectiveness and ease of production (Swain et al. 2022). Unfortunately, this type of vaccine can be given to a segment of patients who have HLA for which the vaccine is restricted. E75 vaccine (Nelipepimut-S) is a peptide vaccine derived from HER2 ECD and is widely studied in preclinical and clinical trials for the treatment of HER2-positive breast cancer (Mittendorf et al. 2008). Clinical studies used E75 combined with incomplete Freund's adjuvant or GM-CSF and showed that vaccination induced a specific anti-peptide immune response with no associated toxicity (Knutson et al. 2002; Murray et al. 2002). Clinical trial results reported that vaccine induced a T cell response in 60-70% of treated patients. In a phase I/II trial (PRESENT trial), 5-year disease-free survival was 89.7% in the vaccinated group versus 80.2% in the control group. The following phase III PRESENT clinical trial demonstrated the synergy between active immunotherapy (E75 vaccine) and passive immunotherapy (trastuzumab) (Peoples et al. 2008). In this study, Peoples and coworkers reported that E75 administration significantly increased peptide-specific CD8+ immunity in 65% of patients but this immunity was maintained in only 43% of them 6 months after the last vaccination (Peoples et al. 2008; Ladjemi et al. 2010). The E75 peptide vaccine is the only one that, to date, has reached the phase III for the treatment of HER2-positive breast cancers (ClinicalTrials.gov accessed on January 28th, 2023).

Protein-based vaccines contain the entire HER2 protein and, despites peptide vaccines, they have both HLA class I and class II epitopes, then they do not present HLA restrictions. In a phase I clinical trial (NCT00058526) was evaluated the efficacy, safety, and immune response elicitation ability of a vaccine containing a recombinant HER2 protein combined with the adjuvant AS15. This trial highlighted the correlation between the dose used, the vaccination schedule, and the prevalence of HER2-specific humoral responses. Immunity against HER2 persisted 5 years after vaccination in 6 of 8 patients who received the highest dose (Disis et al. 2004).

Several preclinical studies have attested the efficacy of DNA vaccines to treat HER2-positive breast cancers (Marchini et al. 2013; DeGiovanni et al. 2014). Actually, some DNA vaccines are under clinical trials evaluation both expressing HER2 intracellular domain alone or multiple epitopes (HER2, IGFBP2 and IGF1R) (Salazar et al. 2009; Swain et al. 2022).

Finally, breast cancer vaccines can be based on DC cells. Generally, these cells are obtained ex vivo from the patient and are transfected with the gene encoding the tumor antigen. Although DC vaccines are a promising individualized strategy, their production can be challenging (Zhu and Yu 2022). A randomized trial conducted by Lowenfeld et al., showed that a DC vaccine was well tolerated by patients and induced a T cell specific response. The pathologic complete response rate was higher in ductal carcinoma *in situ* patients compared with early invasive breast cancer patients (28.6% vs. 8.3%) (Lowenfeld et al. 2017).

# *Aim of the work*

The aim of this work is to discover new therapeutic strategies against HER2-positive breast cancers to overcome tumor resistance by harnessing immune system and looking for new targetable molecules.

Due to its overexpression only in cancer cells, HER2 is an excellent target antigen for immunotherapeutic strategies, such as cancer vaccines. In the first half of this project, the efficacy of a novel virus-like particles-based (VLP) vaccine (re-engineered for human administration) for the treatment of HER2-positive breast cancer tumors was evaluated in different mouse models both in prevention and therapeutic setting.

The second part of the project aimed to find new therapeutic targets to the treatment of HER2-positive breast cancers that undergo HER2 loss and that are resistant to target therapies. Through a preclinical model of HER2 loss, were investigated new promising targets in the absence of HER2 and the role of epithelial to mesenchymal transition in HER2-positive breast cancer progression. Furthermore, the balance between populations with different HER2 expression levels coexisting within a heterogeneous tumor was studied in order to understand mechanisms behind target therapies resistance.

# *Results*

# 3. ANTI-HER2 CANCER VACCINES

The introduction of monoclonal antibodies targeting HER2 in clinical practice (like trastuzumab and pertuzumab) led to significant improvements in patient survival (Baselga et al. 2005). However, the treatment of HER2-positive breast cancer patients with monoclonal antibodies is frequently linked to intrinsic or acquired resistance, leading to the therapy failure (Luque-Cabal et al. 2016). Moreover, treatments with monoclonal antibodies are often expansive. These huge limitations have challenged the investigation of new therapeutic approaches, including the development of anti-HER2 vaccines able to induce a strong and persistent immune response against the tumor.

Anti-HER2 cancer vaccines might boost anti-HER2 monoclonal antibody activity by inducing both a comprehensive anti-HER2 polyclonal antibody response and the immunological memory.

A proof-of-concept study on VLP-based vaccine against HER2 was performed in our laboratory thanks to a collaboration with Prof. Adam Sander (University of Copenhagen, Copenhagen, Denmark) (Palladini et al. 2018). Recently, this vaccine has been re-engineered by Expres$^2$ion Biotechnologies for clinical administration.

In this chapter the prevention and therapeutic activity of the new HER2-VLP (called ES2B-C001) vaccine was investigated in human HER2-positive mammary carcinoma mouse models. These data have been included in the manuscript of Ruzzi and colleagues (Ruzzi et al. 2022).

### 3.1. ES2B-C001 as anti-HER2 cancer vaccine

ES2B-C001 is a VLP-based vaccine against HER2 developed for human breast cancer therapy.

Vaccine's technology employs a tag/catcher conjugation system: the full HER2 ECD (subdomains I-IV) was genetically fused with Catcher (highly reactive protein) and the fusion antigen (Catcher-HER2) attached to the surface of Acinetobacter phage (AP205) phage capsid derived VLPs, each presenting 180 Tag peptides (Figure 1).

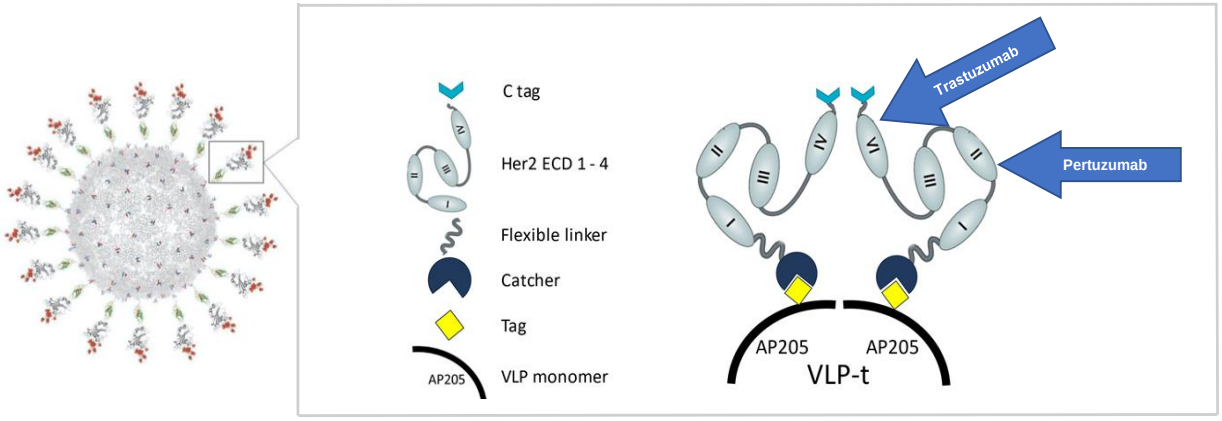


**Figure 1. VLP platform.** Vaccine's technology: the AP205 capsid conjugated with a tag/catcher system. Arrows indicate HER2 subdomains targeted by pertuzumab and trastuzumab.

While VLP act as intrinsic adjuvant, the vaccine can be also formulated with conventional adjuvants in a 50/50 ratio. The adjuvant chosen for our preclinical study is Montanide ISA 51 (Montanide for short).

### 3.2. Therapy of local tumor and lung metastasis in FVB mice

Therapeutic vaccinations were performed in syngeneic FVB female mice challenged in the mammary fat pad with QD cells. QD cell line was obtained in the Laboratory of Biology and Immunology of Metastasis directed by Prof. Lollini from a mammary carcinoma arose in a human HER2 transgenic mouse. Vaccine, alone or with Montanide, was administered bi-weekly starting from 2 weeks after cell challenge. Mice received 7 administrations, mimicking clinical schedule.

All mice vaccinated with ES2B-C001+Montanide were tumor-free for more than 1-year post-challenge, whereas all untreated mice developed progressive tumors within 1-2

months. Furthermore, vaccination without adjuvant blocked tumor growth in 70% of mice (Figure 2A). The strong tumor growth inhibition induced by the vaccine, both with and without adjuvant, began as early as 49 days after cell injection and ES2B-C001+Montanide tumor inhibition was significant also if compared with vaccine alone, starting from day 76 (Figure 2B).

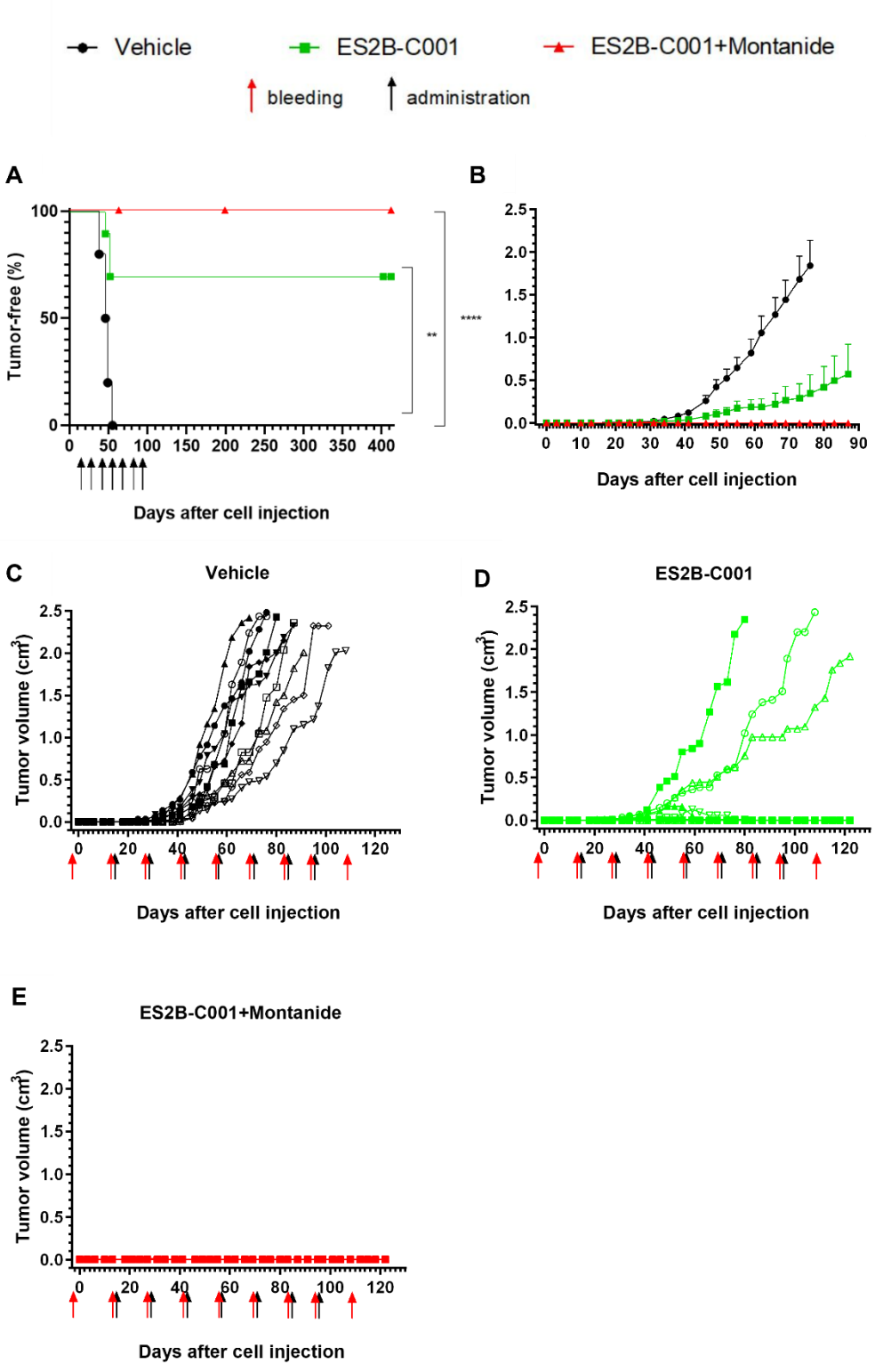

**Figure 2. ES2B-C001 therapeutic efficacy in FVB mice challenged in the mammary fat pad with QD cells.** (A) Kaplan–Meier tumor-free survival curves. ***p < 0.001, **** p < 0.0001 by log-rank (Mantel–Cox) test; (B) tumor growth curves, each point represents the Mean ± SEM of all tumors in each group (n=10), the curve interrupts when the first mouse of a group was sacrificed. *p < 0.05 at least vehicle vs. ES2B-C001 and ES2B-C001+Montanide from day 49 and *p < 0.05 ES2B-C001 vs. ES2B-C001+Montanide from day 76 by Tukey's test; (C–E) individual tumor growth curves.

A major clinical application of anti-HER2 vaccines would be against breast cancer metastases, for example to prevent metastasis outgrowth in an adjuvant setting. To model this situation, we injected QD cells intravenously (i.v.) in FVB mice and 1 week after challenge were vaccinated bi-weekly (5 times) and sacrificed 12 weeks after QD cells injection. ES2B-C001, with or without adjuvant, completely blocked metastases outgrowth; in contrast all control mice presented hundreds of lung nodules (Figure 3).

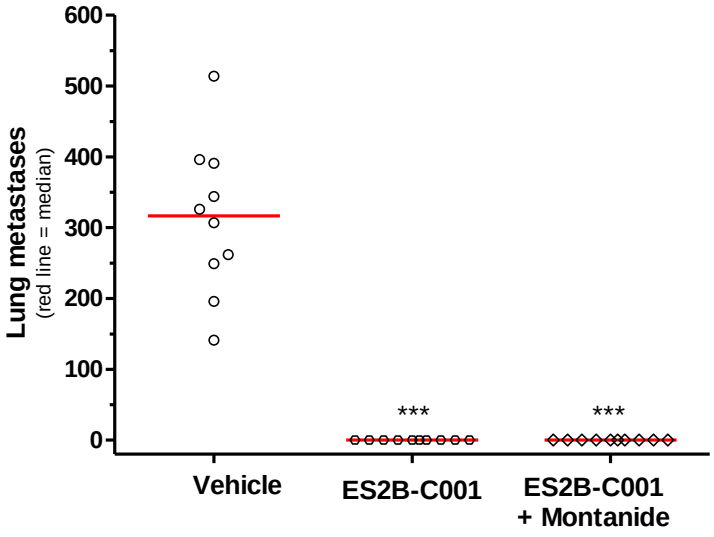


**Figure 3. ES2B-C001 therapeutic vaccinations inhibited lung metastases in FVB mice challenged i.v. with QD cells.** Lungs were perfused with black India ink to contrast metastatic nodules; pictures show one representative lung for each experimental group, blue arrows indicate some metastatic nodules (to the left: vehicle; in the middle: ES2B-C001; to the right: ES2B-C001+Montanide). In the graph, each point represents the total number of lung nodules of one mouse, as counted under a dissection microscope; *** p < 0.001 vs. vehicle by the Dunn's non-parametric test.

Thus, ES2B-C001 vaccine successfully inhibited the onset, growth, and metastatic spread of HER2 positive mammary carcinomas in mice.

### 3.3. Anti-HER2 antibody, cytokine and T cell responses

Mice sera were collected before QD cell challenge, before each vaccination, two weeks after last vaccination and then periodically to monitor how long vaccine-induced antibody titers remained stable.

Anti-HER2 immunoglobulins (Ig) were analyzed for binding to native HER2 molecules on the surface of human breast cancer cells BT-474 by means of indirect immunofluorescence followed by flow cytometry or by binding to HER2 ECD in ELISA. Both assays showed that vaccinations elicited strong anti-HER2 antibody responses, in particular when administered with Montanide (Figure 4A-B). Anti-HER2 Ig titers plateaued in the 1-10 milligram/mL range. Moreover, antibodies levels persisted in this range for many months after the last vaccination (Figure 4C), suggesting that vaccinations induced long-term immunological memory against HER2.

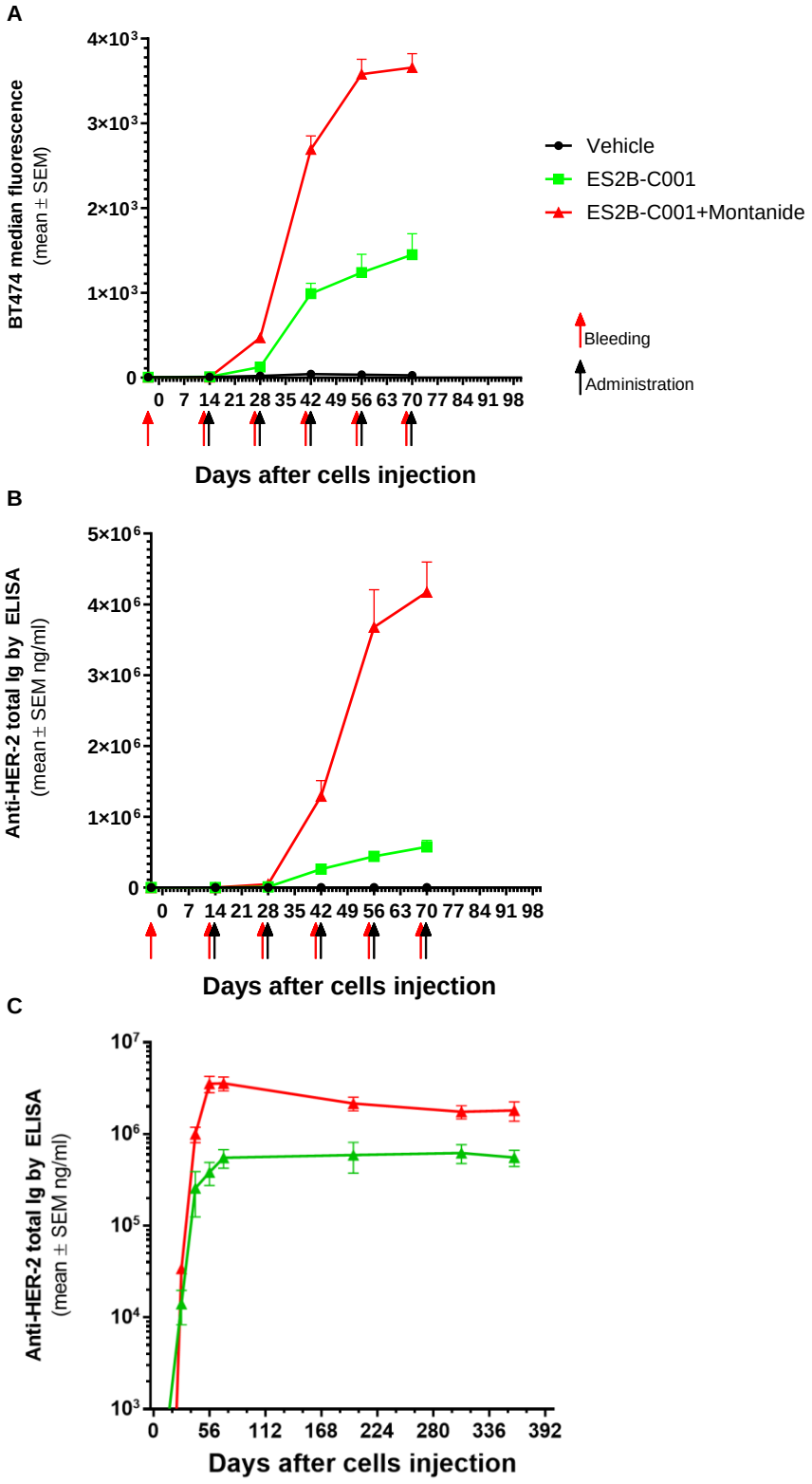


**Figure 4. Antibodies elicited in FVB mice by ES2B-C001 therapeutic vaccinations.** (A) Anti-HER2 antibodies measured by flow cytometry of human HER2+ BT-474 breast cancer cells and (B) by ELISA on human HER2. Each point represents the Mean ± SEM of the Ig titers of each mouse group shown in Figure 2. (C) Long-term persistence of anti-HER2 antibodies in vaccinated mice after the complete vaccination schedule. Each point represents the Mean ± SEM of the IgG titers of 3 representative long-surviving mice tested over a period of 1 year.

All the IgG subclasses were detected in vaccinated mice sera, which appear to follow the order reported in the literature for other HER2 vaccines, i.e. IgG1 > IgG2a ≈ IgG2b > IgG3, with a sizeable induction of cytotoxic, "Th1" isotypes, IgG2a and IgG2b, which are known to have anti-tumor activities (Vidarsson et al. 2014). Therefore, the isotype analysis confirmed the strong antibody response elicited by vaccination (Figure 5).

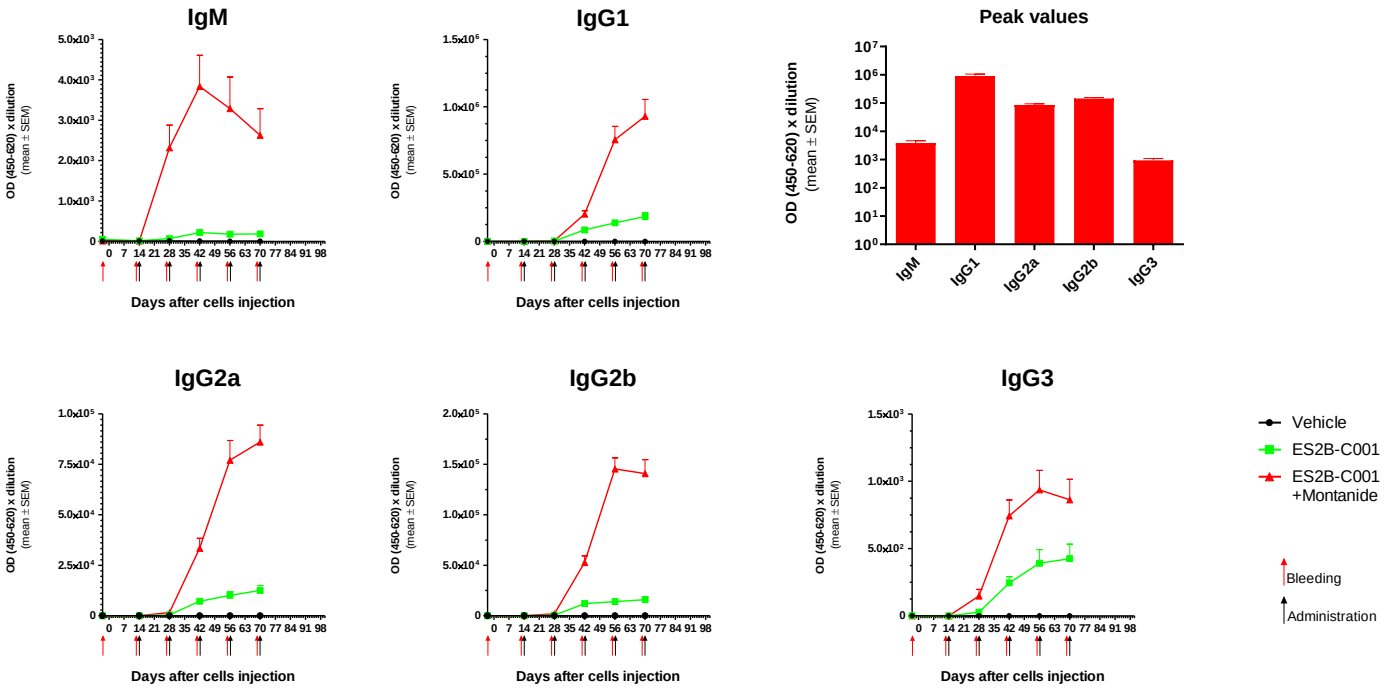


**Figure 5. HER2 specific IgM and IgG isotypes elicited by ES2B-C001 therapeutic vaccinations**. Each point represents the Mean ± SEM of mouse groups shown in Figure 2. Histograms represent the higher isotype titer point.

T cell responses were less evident than antibody ones. Serum cytokine profiles after one or two vaccine administrations was evaluated by Bioplex, including IFN-γ, TNF-α, and several interleukins like IL-1, 2, 4, 5, 6, 10 and 12, and differences between control and treated groups were not detected (data not shown).

The presence of IFN-γ secreting splenocytes was instead revealed by ELISpot assay (20.7 ± 2.9 spots / 2x10$^5$ cells) after two in vivo vaccinations followed by *in vitro* restimulation with a pool of HER-2 ECD peptides (Figure 6). Overall, the data suggests that the vaccine did not induce a cytokine storm.

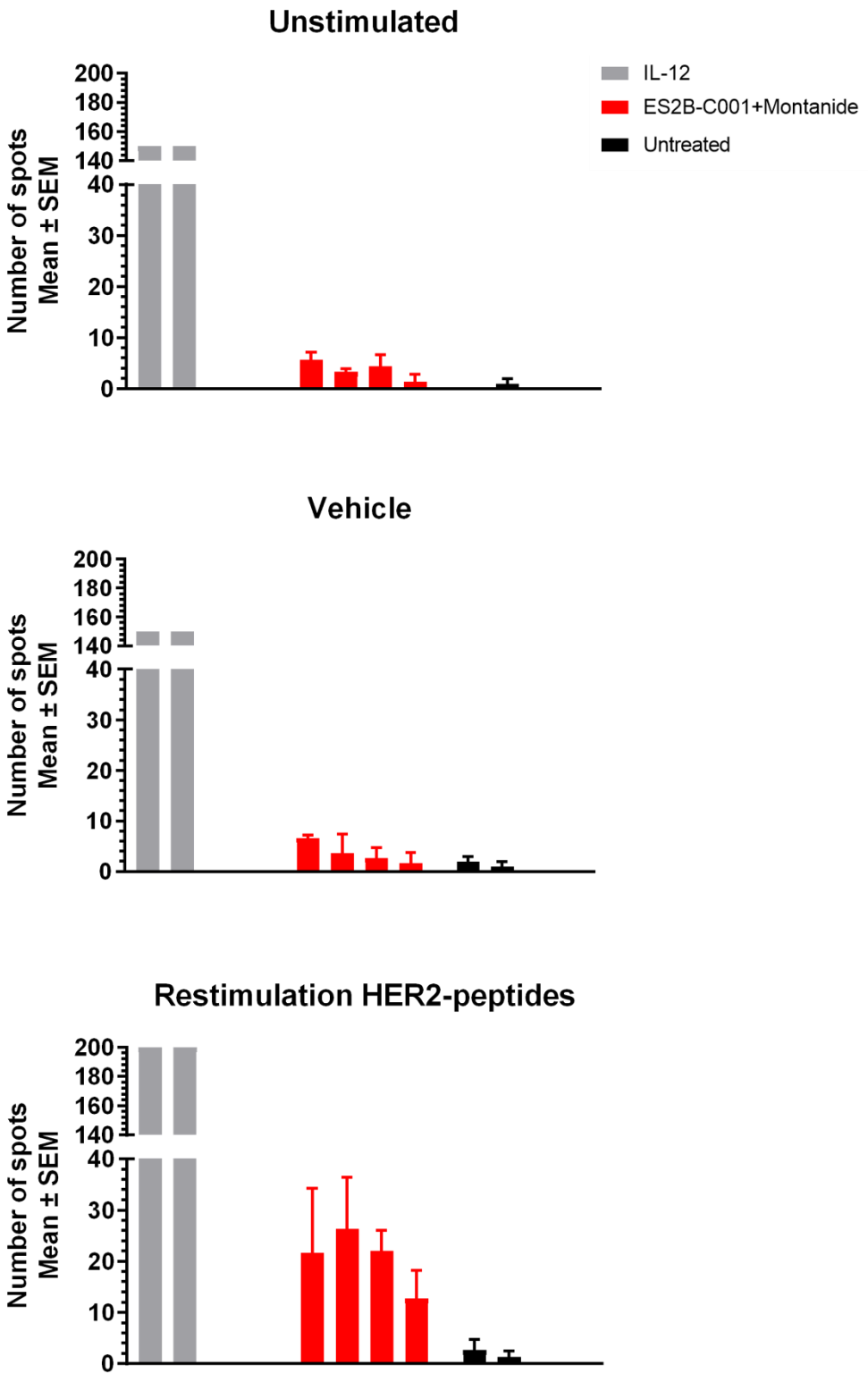


**Figure 6. IFN-γ ELISpot assay.** INF-γ released by splenocytes from unchallenged FVB mice untreated (n = 2, black bars) or previously vaccinated twice with ES2B-C001+Montanide (n = 4, red bars) and restimulated *in vitro* with a pool of human HER2 peptides. Positive controls were splenocytes of mice treated three times intaperitoneally (i.p.) with recombinant mouse interleukin 12 (IL-12(p70)) (n = 2, gray bars). Each panel represents a different *in vitro* restimulation. Each bar represents the Mean ± SEM of three wells.

### 3.4. Prevention of mammary carcinoma onset and therapy of metastases in Delta16 transgenic mice

Delta16 mice are prone to the onset of mammary carcinomas driven by the expression of the HER2 Delta16 transgene, an activated isoform of human HER2 derived from the skipping of exon 16, located under the transcriptional control of a mouse mammary tumor virus (MMTV) long terminal repeat. This transgene induces a faster carcinogenesis leading to the development of multiple mammary carcinomas in all female mice during the first year of life. Delta16 mice are immunologically tolerant to human HER2, hence the induction of anti-HER2 immunity in these mice entails a break of tolerance similar to what must occur in human patients to develop protective immune responses against HER2-positive tumors.

Young Delta16 female mice were vaccinated twice with varying doses of ES2B-C001 and Montanide (5, 10, 20 and 40 μg/mouse). Vaccination completely prevented spontaneous tumor onset for more than one year of age in 95% of mice, in contrast all untreated mice develop tumors within 4-8 months of age. The difference is highly significant (Figure 7A) and all the doses tested significantly prevent tumor onset, indicating that the immune response elicited by only two vaccinations effectively prevents HER2-driven carcinogenesis in Delta16 mice.

Anti-HER2 antibody levels of Delta16 mice were lower (0.1-1 mg/ml) than those of FVB mice (1-10 mg/ml) (Figures 4B and 7B, respectively), in keeping with the immunological tolerance to human HER2 characteristic of the model. Antibody titers remained at high levels for more than one year after the last immunization, showing that ES2B-C001 induced long-lasting anti-HER2 immunity also in this mouse model (Figure 7B).

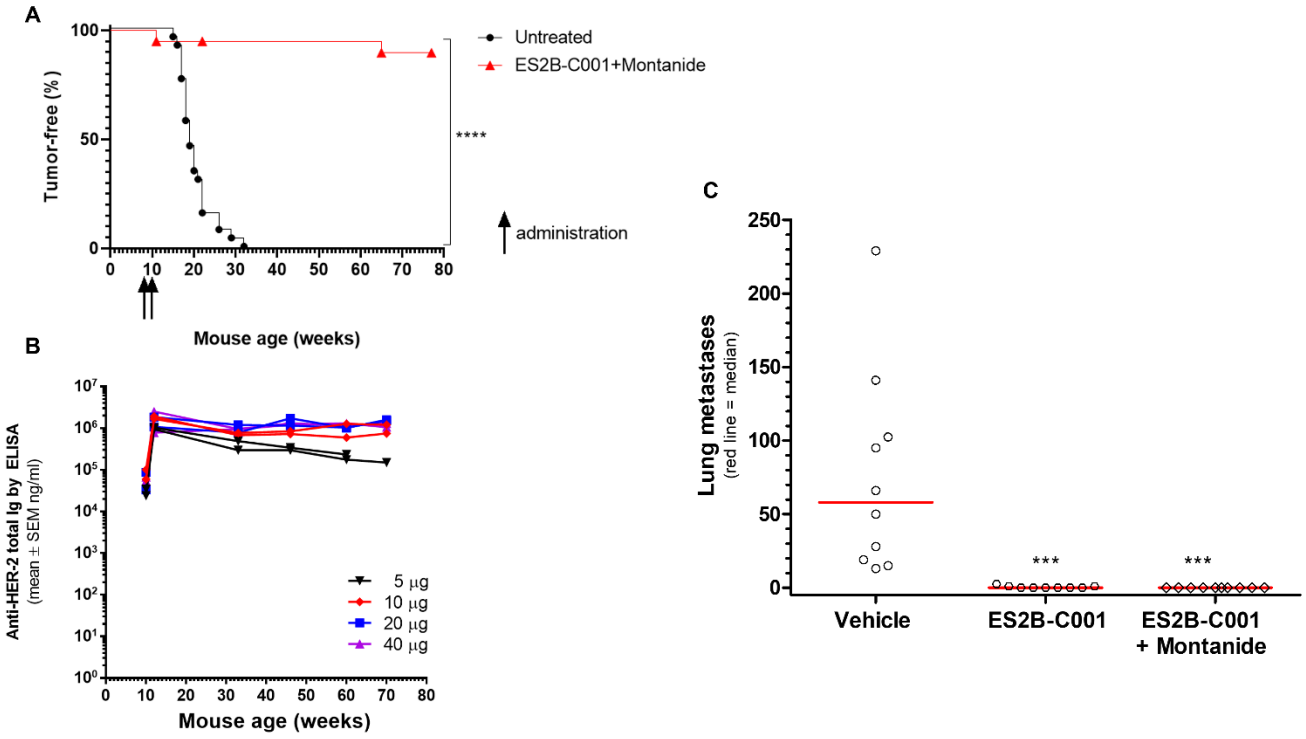


**Figure 7. Prevention of mammary carcinoma onset and therapy of lung metastases in HER2 transgenic Delta16 mice.** (A) The Kaplan–Meier tumor-free survival curve of pooled Delta16 mice vaccinated twice with ES2B-C001 and Montanide (5-40 µg/mouse) (n=20) was significantly different from untreated (n=26) Delta16 mice, **** $p < 0.0001$ by the log-rank test. (B) Long-term anti-HER2 antibody response elicited in Delta16 mice by ES2B-C001 vaccinations, as measured by ELISA; each line represents one mouse. (C) Therapeutic vaccination of young, tumor-free Delta16 mice challenged i.v. with $0.25x10^6$ QD cells; each point represents the total number of lung nodules of one mouse, as counted under a dissection microscope; *** $p < 0.001$ vs. vehicle by the Dunn's non-parametric test.

Furthermore, to determine if ES2B-C001 could be therapeutically effective in Delta16 mice, young females were challenged i.v. with QD cells and then vaccinated bi-weekly starting from 7 days after cell challenge. Metastasis outgrowth was blocked by vaccination. All control mice had a mean of 68 lung nodules at 13 weeks after challenge, while all mice vaccinated with E2SB-C001+Montanide and 73% of mice vaccinated with E2SB-C001 without adjuvant were metastasis-free, the remaining had just 1-2 lung nodules (Figure 7C).

These results confirm the strong anti-metastatic activity of ES2B-C001, as previously demonstrated in FVB mice (Figure 3).

### 3.5. Inhibition of human HER2-positive cancer cells in 3D culture

To better evaluate the therapeutic activity of antibodies induced by the vaccine, we studied their inhibitory activity on human HER2-positive breast cancer cells BT-474 (trastuzumab-sensitive) and on its trastuzumab-resistant clone BT-474 C5, growing as three-dimensional (3D) colonies in agar. As expected, trastuzumab, applied in a similar concentration as the immune sera, inhibited the growth of BT-474, but not of BT-474 C5. In contrast, the antibodies elicited by ES2B-C001+Montanide strongly inhibited both trastuzumab-sensitive (Figure 8A and 8C) and trastuzumab-resistant breast cancer cells (Figure 8B and 8C), thus providing strong evidence of the potential advantages of the polyclonal antibody response elicited by the ES2B-C001 vaccine.

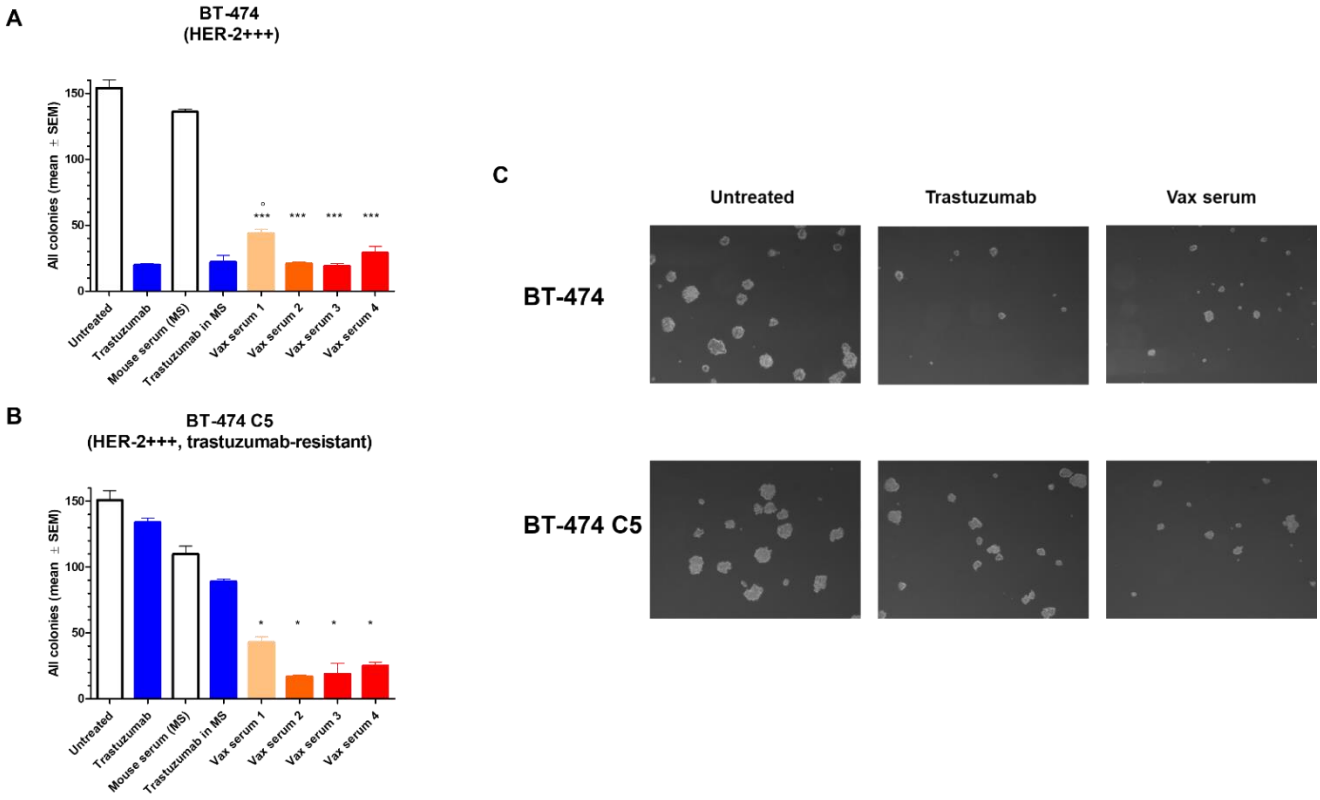


**Figure 8. Inhibition of human breast cancer cell 3D agar colony growth by antibodies elicited by ES2B-C001 vaccinations.** (A) BT-474 is a HER2 positive (+++) trastuzumab-sensitive cell line, and (B) C5 is a trastuzumab-resistant BT-474 clone. Each bar represents the mean ± SEM number of colonies larger than 90 μm as counted in two independent cultures with the aid of a micrometer. Vax sera 1–4: sera of 4 previously unchallenged Delta16 mice vaccinated twice with ES2B-C001 (Vax serum 1: 10 μg, Vax serum 2: 20 μg, Vax serum 3 and 4: 40 μg /mouse i.m. with Montanide) were compared with a pool of naïve mouse sera (MS) as a negative control and benched-marked up against trastuzumab applied in a comparable concentration. *** $p < 0.001$ vs. MS; $p < 0.05$ at least vs. both MS and trastuzumab, Tukey's test. (C) Representative micrographs of live agar colonies were taken with an inverted microscope (dark-field, 25X).

The sera of four vaccinated mice from the prevention study were used to analyze also the inhibition of gastric cancer cell lines in the agar colony assay (Figure 9). All sera of vaccinated mice strongly inhibited colony formation of NCI-N87, a cell line expressing high levels of HER2; the inhibitory activity of vaccinated sera was stronger than that of trastuzumab at comparable concentrations (Figure 9). In contrast, KATO III gastric cell line, with a much lower HER2 expression than NCI-N87, was partially inhibited by only two sera (Figure 9).

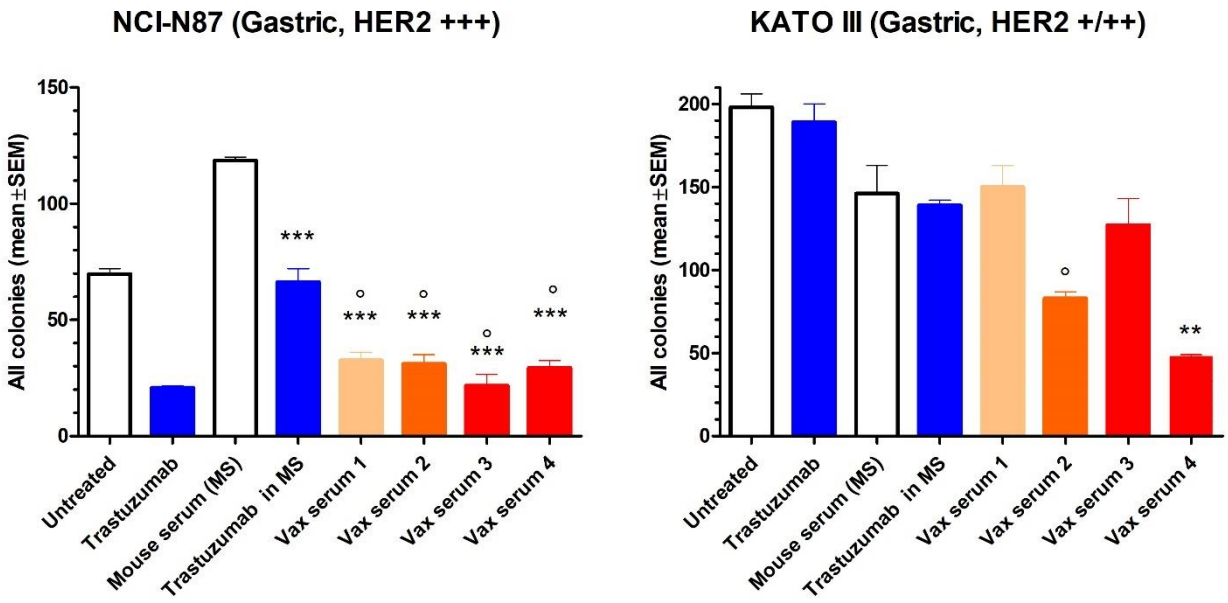


**Figure 9. Inhibition of gastric cancer agar colony growth by sera of vaccinated Delta16 mice.** NCI-N87 HER2-positive (+++) (left panel) and KATO III HER2-positive (+/++) (right panel) cell lines. Each bar represents the mean ± SEM number of colonies larger than 90 μm as counted in two independent cultures with the aid of a micrometer. Vax sera 1–4: sera of 4 previously unchallenged Delta16 mice vaccinated twice with ES2B-C001 (Vax serum 1: 10 μg, Vax serum 2: 20 μg, Vax serum 3 and 4: 40 μg /mouse i.m. with Montanide) were compared with a pool of naïve mouse sera (MS) as a negative control and benched-marked up against trastuzumab applied in a comparable concentration. * $p < 0.05$ at least vs. MS; °$p < 0.05$ at least vs. trastuzumab in MS, Tukey's test.

The results indicate that the inhibitory activity of vaccine-elicited anti-HER2 antibodies is not limited to breast cancer cells, suggesting that the vaccine might be used for the treatment of different types of human tumors.

# 4. DISCOVERY OF NEW THERAPEUTIC STRATEGIES THROUGH A PRECLINICAL MODEL OF BREAST CANCER PROGRESSION AND HER2 LOSS

In the Laboratory of Immunology and Biology of Metastasis directed by Prof. Pier-Luigi Lollini a preclinical model of HER2 loss, consisting of cell lines and their clones, has been developed.

In this model the HER2 loss was linked to EMT features, in fact HER2-negative cell lines acquire a fibroblast-like phenotype, increase stemness traits and in vivo malignancy. Thus, some oncogenic transformations allowed the cell lines to overcome the HER2 oncogene addiction and sustain the malignant HER2-negative phenotype.

Therefore, these cell lines mimic well what happens in progressing patients in whom the tumor was no longer addicted to HER2 or had lost the oncogene expression.

Taking advantage of the characteristics of this model, we began to investigate possible alternative targets for HER2-positive breast cancer in progression.

Some data reported in this section were included in Giusti, Ruzzi, Landuzzi and colleagues' manuscript (Giusti et al. 2021).

## 4.1. Characterization of HER2 loss model

As reported in Giusti et al. manuscript, our dynamic model of HER2 expression is based on cell lines derived from spontaneous mammary tumors arose in two human HER2 transgenic mice (FVBhHER2). MamBo89HER2$^{stable}$ cell line displayed high and stable HER2 expression, both *in vitro* and *in vivo*, whereas MamBo43HER2$^{labile}$ despite its high HER2 expression undergo HER2 loss upon *in vitro* treatment with trastuzumab or *in vivo* tumor growth, HER2-negative cell line MamBo38HER2$^{loss}$ was obtained from a MamBo43HER2$^{labile}$ derived tumor (Figure 10).

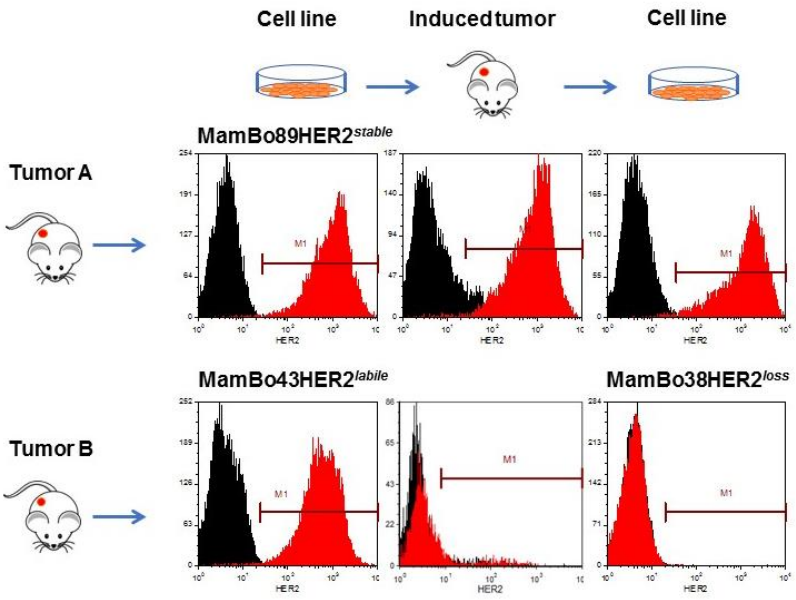


**Figure 10. Dynamic HER2 expression in MamBo cell lines**. Tumor A and Tumor B gave rise to MamBo89HER2$^{stable}$ and MamBo43HER2$^{labile}$ cell lines respectively**.** The MamBo38HER2$^{loss}$ cell line was derived from a tumor induced via the *in vivo* injection of the MamBo43HER2$^{labile}$ cell line**.** Panels show HER2 level measured by cytofluorometric analysis. Black profile, refers to secondary antibody alone; red profile, refers to anti-HER2 antibody.

HER2-positive cell lines, MamBo89HER2$^{stable}$ and MamBo43HER2$^{labile}$, grew *in vitro* as a monolayer of polygonal cells, in contrast the HER2-negative cell line acquired a spindle-like phenotype (Figure 11A).

Along with the change of morphology, the loss of the oncogene expression was accompanied by the acquisition of a more stem-like phenotype. The HER2 loss cells showed an increased ability to develop mammospheres *in vitro* compared to HER2 positive cell lines (Figure 11B), together with a highly staminal profile (95% of CD24$^{low}$/CD44$^{high}$ cells). On the opposite, MamBo89HER2$^{stable}$ and MamBo43HER2$^{labile}$ presented only a small population of CD24$^{low}$/CD44$^{high}$ cells, 3% and 1%, respectively (Figure 11C).

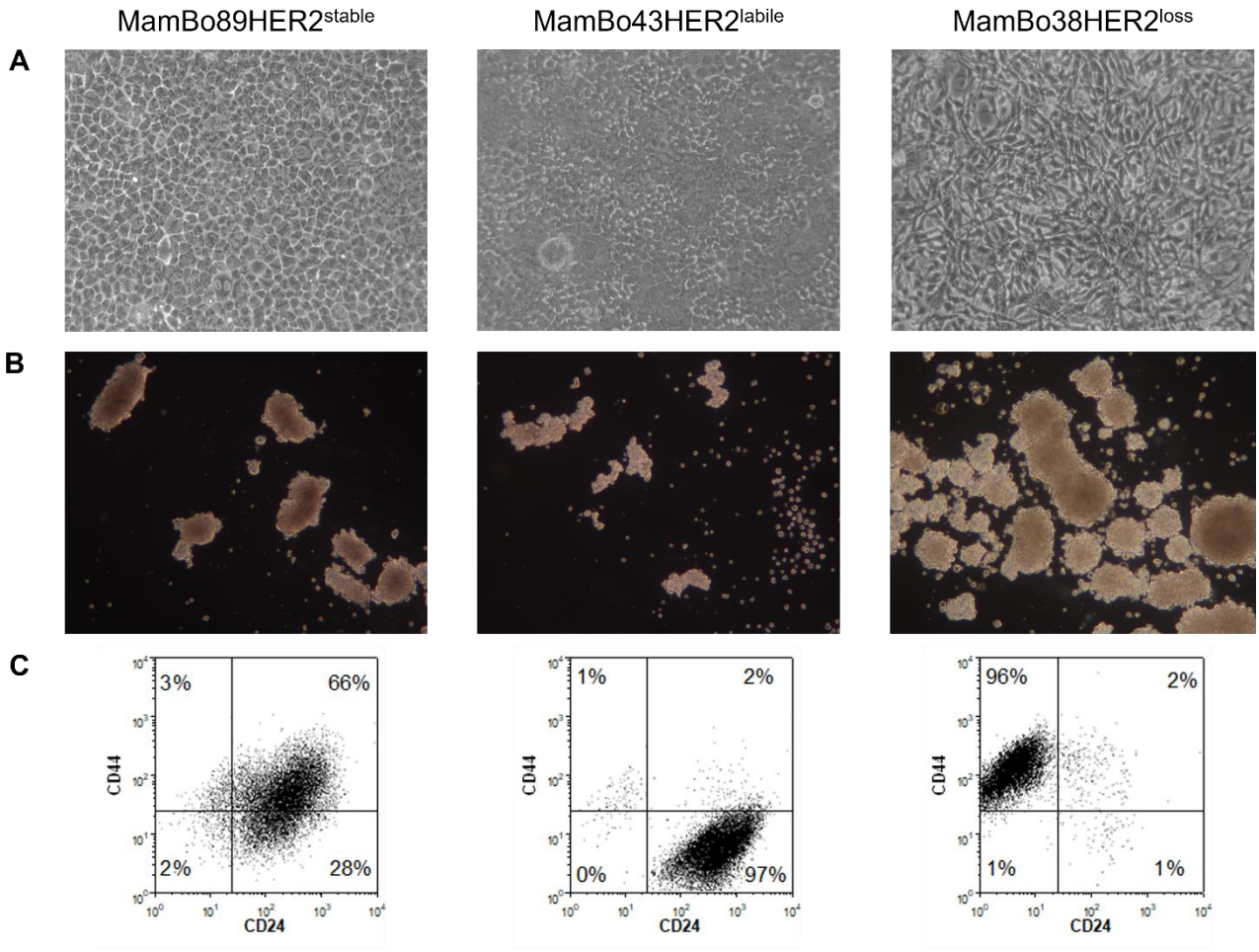


**Figure 11. MamBo cell lines morphology, mammospheres and stemness markers (A-C)**. (A) HER2-positive cell lines (left and middle panels), showing an epithelial morphology, and the HER2-negative cell line (right panel) grown as a multilayer of spindle-like cells. (B) Dark-field micrographs of mammosphere formation assay. Number of mammospheres (n=4): MamBo89HER2$^{stable}$, 23±1; MamBo43HER2$^{labile}$, 17±1; MamBo38HER2$^{loss}$, 69±9; MamBo38HER2$^{loss}$ *vs* MamBo89HER2$^{stable}$ and MamBo43HER2$^{labile}$ cell lines, $p<0.01$ by Student's *t*-test. (C). Expression of stemness markers CD24 and CD44 in cells cultured under 2D-adherent conditions, measured by cytofluorimetric analysis.

The morphology and stemness of the MamBo38HER2$^{loss}$ cell line resembled some of the typical features of the EMT. To further investigate the EMT state of our cell lines, the transcription level of genes involved in different stages of the EMT were evaluated by RT-PCR (Figure 12). In the epithelial and HER2-positive cells the presence of EMT drivers or E-cadherin down-modulators, such as *Zeb1*, *Zeb2* and *Igfbp4*, was not detected; as expected these cell lines had high expression of *Cdh1* (epithelial marker) and low levels of *Vim* (mesenchymal marker). Instead, MamBo38HER2$^{loss}$ cells showed an EMT molecular profile with the loss of *Cdh1* expression linked to higher levels of *Zeb1*, *Zeb2*, *Igfbp4* and *Vim*. There were no differences in *Twist* levels between HER2-positive and HER2-negative cell lines.

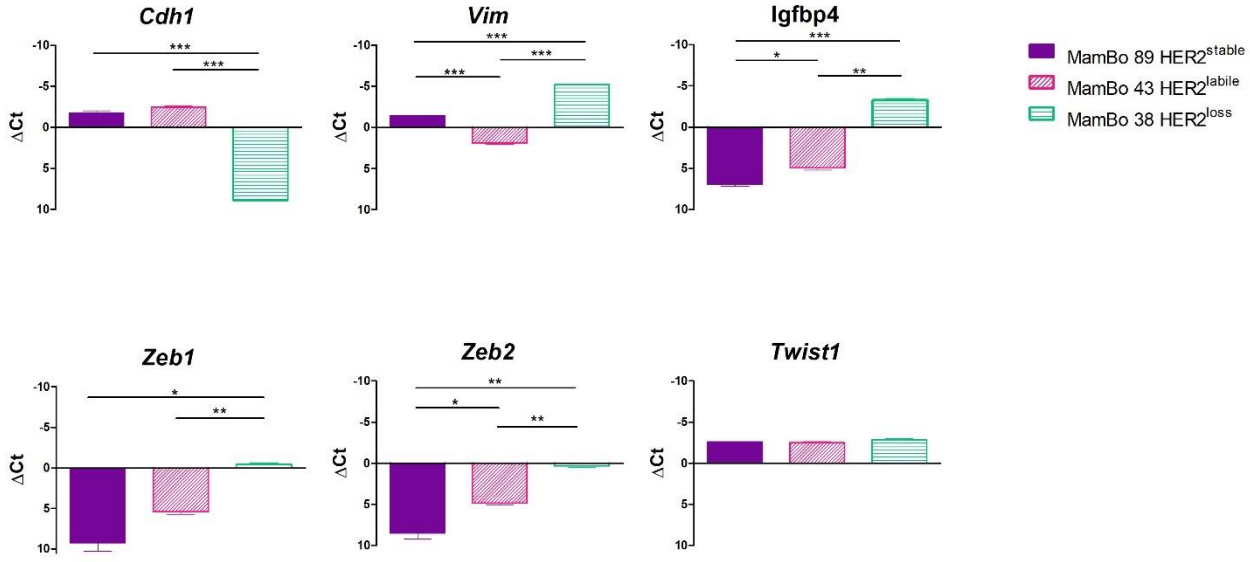


**Figure 12. EMT profile measured by Real-Time PCR of MamBo cell lines**. ΔCt values were reported on y axis. Expression was determined by RT-PCR analysis, normalized over TBP (total binding protein) expression (ΔCt= $Ct_{gene}$ - $Ct_{TBP}$). Each bar represents Mean ± SEM of n=2 experiments, *p<0.05, **p<0.01, ***p<0.001 by Student's *t*-test.

Together with the fibroblast-like morphology, the EMT molecular profile and the increased stemness, MamBo38HER2$^{loss}$ cell line showed higher *in vivo* malignancy. In fact, upon orthotopic cell injection into the mammary fat pad (m.f.p.) the HER2 loss cell line displayed a faster tumorigenicity compared to MamBo89HER2$^{stable}$ and MamBo43HER2$^{labile}$ cell lines (Figure 13).

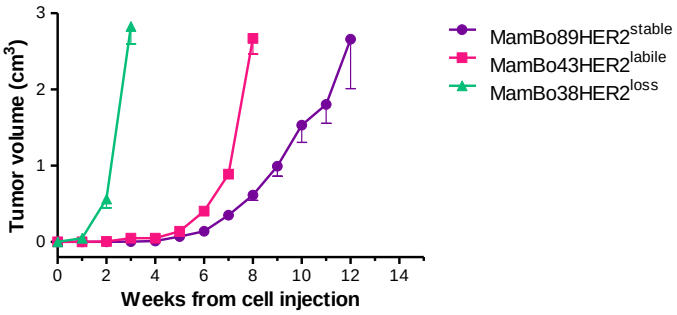


**Figure 13.** ***In vivo*** **growth of MamBo cell lines in FVBhHER2 female mice.** Mean and SEM from 3–8 mice per group is shown. MamBo38HER2$^{loss}$ growth was significantly faster from 1 week after cell injection than MamBo43HER2$^{labile}$ and MamBo89HER2$^{stable}$ cell lines, p< 0.01 by unpaired *t* test with Welch's correction. From 6 weeks after cell injection, MamBo43HER2$^{labile}$ cells also grew faster compared to the MamBo89HER2$^{stable}$ cell line, p< 0.001 at 6-7 weeks and p = 0.068 at 8 weeks by unpaired *t* test with Welch's correction.

MamBo43HER2$^{labile}$ cells gave rise to HER2-negative tumors regardless of the injection dose and the presence or absence of the immune system in mice. So, B, T and NK

cells are not involved in clearance of HER2-positive cells from injected MamBo43HER2$^{labile}$ population (Giusti et al. 2021).

In this model the HER2 loss was also linked to an amplified metastatic ability. After i.v. injection in immunocompetent mice, MamBo38HER2$^{loss}$ cells gave rise to more than 200 lung nodules within 3 weeks. In contrast, HER2-positive cell lines induced few (MamBo89HER2$^{stable}$ cells, median number of metastasis 2 and incidence 4/5 mice) or no (MamBo43HER2$^{labile}$ cells) lung metastases 18 weeks after cell injection.

### 4.2. Mechanisms of HER2 loss *in vitro*: HER2 copy number analysis and role of the DNA methylation

We performed a copy number analysis on MamBo89HER2$^{stable}$, MamBo43HER2$^{labile}$ and MamBo38HER2$^{loss}$ cell lines to find out whether MamBo38HER2$^{loss}$ cells had lost the transgene. In MamBo89HER2$^{stable}$ cell line 51 copies of HER2 transgene were detected and this number of copies was comparable with human mammary HER2-positive cancer BT474 cell line and with normal tissue (ear fragment) of FVBhHER2 transgenic mice. Surprisingly, we found that MamBo38HER2$^{loss}$ had a similar HER2 copy number to MamBo43HER2$^{labile}$, 18 and 19 copies respectively (Table 1).

| Cell line | ΔCt | hHER2 copy number |
|---|---|---|
| MamBo89HER2$^{stable}$ | -7.27 | 51 |
| MamBo43HER2$^{labile}$ | -5.83 | 19 |
| MamBo38HER2$^{loss}$ | -5.79 | 18 |
| TS/A | 7.26 | 0 |
| Non-transgenic normal tissue | 4.25 | 0 |
| HER2-transgenic normal tissue | -5.96 | 26 |
| HER2-transgenic mammary tumor | -6.60 | 32 |
| HCC1954 | -9.34 | 215 |
| BT474 | -7.53 | 61 |
| SKBr3 | -5.25 | 13 |
| MDA-MB-453 | -4.14 | 6 |

**Table 1. Number of hHER2 gene copies.** HER2 copy number was analyzed by Real Time-PCR. $\Delta Ct = Ct_{huHER2} - Ct_{hu/mPTGER2}$ (PTGER2: prostaglandin E receptor 2). Expression level of $2^{-(\Delta ct\ MDA\text{-}MB\text{-}231 + \Delta ct\ MCF7)/2}$ was associated to two HER2 copies. Ear fragments were used as normal tissue.

This result confirmed that MamBo38HER2$^{loss}$ cells harbor HER2 transgene and it might be silenced by other mechanisms at transcriptional or translational level.

The level of HER2 transcript was about 1000-times less in MamBo38HER2$^{loss}$ cells as compared to MamBo89HER2$^{stable}$ and MamBo43HER2$^{labile}$ cells (Figure 14A). Moreover, we can exclude a post-translational event due to the lack of HER2 protein showed by western blot (Figure 14B).

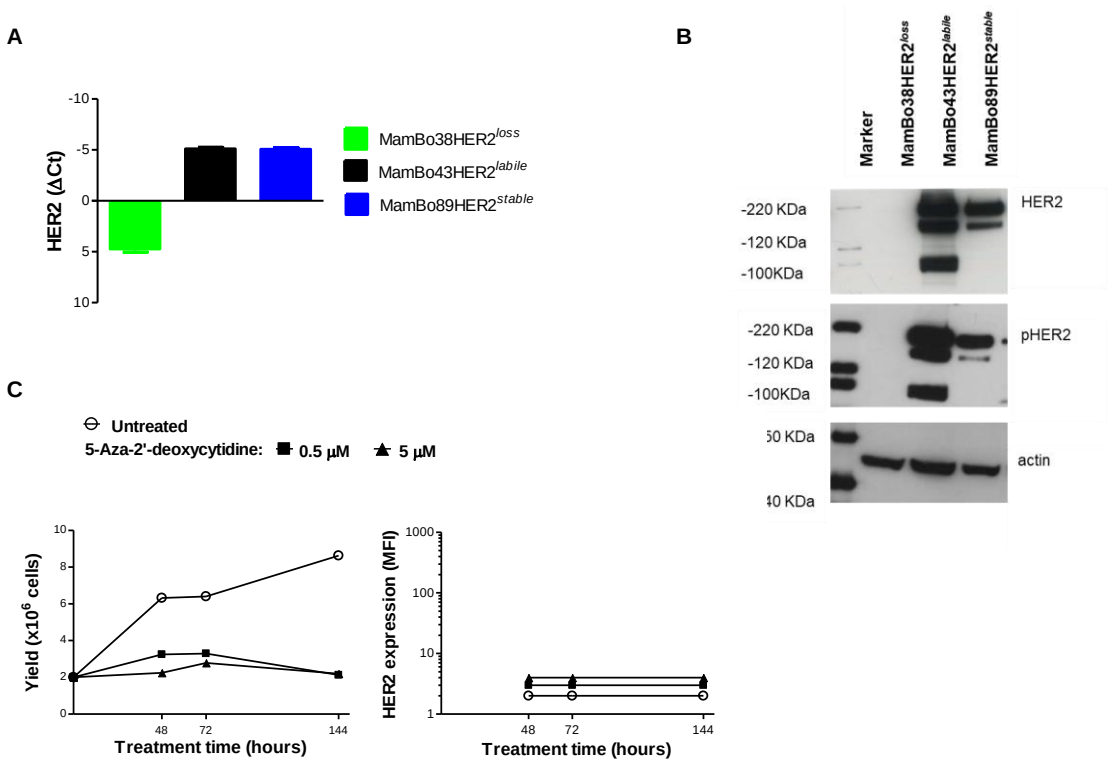


**Figure 14. HER2 expression by MamBo cell lines and the effect of demethylation on HER2 loss cells.** (A) Level of HER2 transcript measured by Real-Time PCR; n=2-4. Each bar shows mean and SEM of the different ΔCt. $\Delta Ct = Ct_{huHER2} - Ct_{mTBP}$. (B) Levels of HER2 protein and of its phosphorylated isoform pHER2 detected by Western blot (panel is representative of at least n=5) experiments. Actin was used as housekeeping protein for protein normalization. (C) MamBo38HER2$^{loss}$ cells growth curve (left panel). HER2 expression of untreated and AZA treated MamBo38HER2$^{loss}$ cells evaluated by flow-cytometry and reported as fluorescence intensity (MFI) (right panel).

DNA hyper-methylation could determine gene silencing, so MamBo38HER2$^{loss}$ cell line was treated with 5-Aza-2'-deoxycytidine (a DNA methyl-transferase inhibitor) for six consecutive days to determine if DNA methylation could be involved in HER2 loss. 5-Aza-2'-deoxycytidine had a cytostatic effect on MamBo38HER2$^{loss}$ cells but did not induce any changes in HER2 expression level (Figure 14C).

Unfortunately, the mechanisms behind the absence of HER2 expression in cells with multiple copies of HER2 was still unclear in our preclinical model, the loss of HER2 expression may be the consequence of genomic events affecting the transgene. Thus, further studies will be focused on genome sequencing to clarify whether the genes are intact. Moreover, additional experiment will be performed to confirm that HER2 loss was not related to DNA methylation events.

### 4.3. Mechanisms behind the HER2 loss *in vitro*

The advent of trastuzumab has revolutionized HER2-positive breast cancer therapy by significantly increasing patient survival. Unfortunately, this success has been hampered by patients that are either intrinsically resistant to treatment with target therapy or develop resistance after treatment. In addition, initial treatment with trastuzumab and pertuzumab could induce loss of HER2 expression which is led to poor prognosis (Morales et al. 2020). Even if the induction of HER2 loss by trastuzumab neoadjuvant therapy is still debated, we decided to take advantage of MamBo43HER2$^{labile}$ cell line to understand how trastuzumab may affect the loss of HER2 expression.

*In vitro* studies in three-dimensional (3D) culture conditions demonstrated that trastuzumab at the dose of 10 µg/ml inhibited the 80% of MamBo43HER2$^{labile}$ colonies formation (Figure 15A). On the contrary, under adherent culture condition (2D), trastuzumab showed little inhibition efficacy after 120 hours of treatment at different concentration (Figure 15B). After 2D treatment with trastuzumab (10 µg/ml), an increasing in HER2-negative population was detected by flow cytometry (Figure 15C).

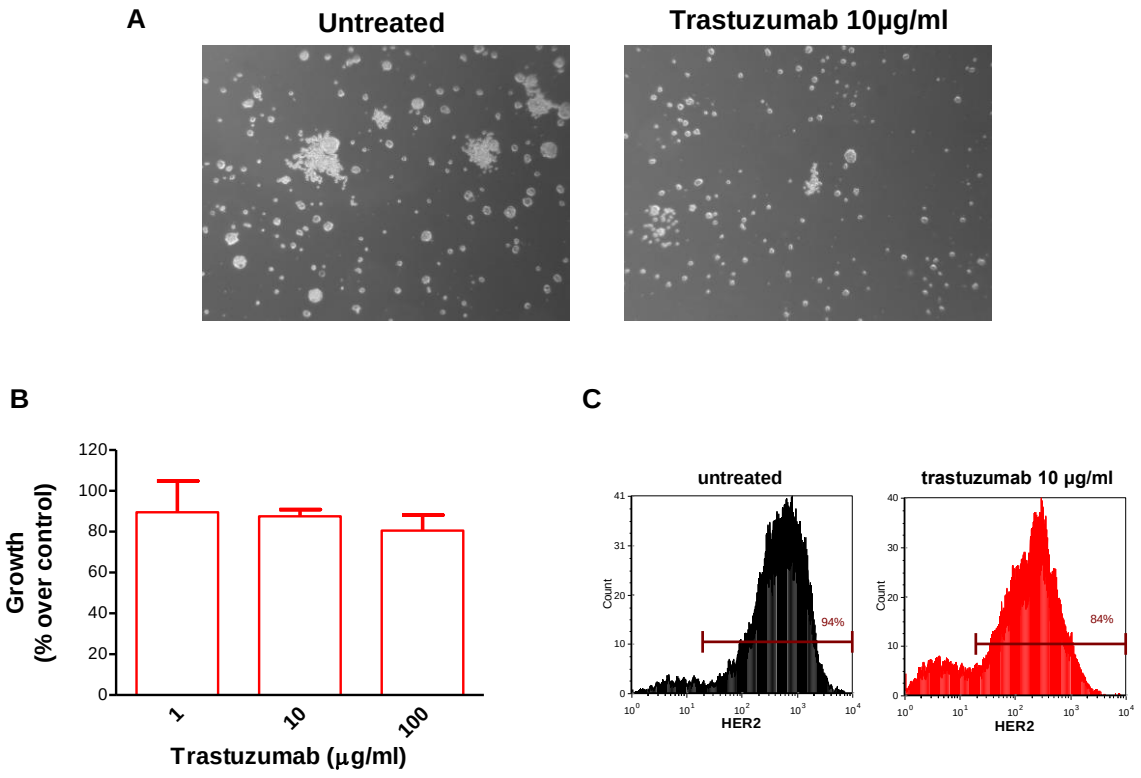

**Figure 15. Trastuzumab treatment in MamBo43HER2$^{labile}$ cells *in vitro*.** (A) Representative images of MamBo43HER2$^{labile}$ 3D agar colony (>90 μm) formation in trastuzumab (10 μg/ml); number of mammospheres (n=2): untreated 22±4, trastuzumab 7±1. (B) *In vitro* growth of MamBo43HER2$^{labile}$ cells after 120h of treatment of different trastuzumab doses. Each bar shows the Mean and SEM of n=4 experiments. (C) HER2 expression of untreated (black profile) and trastuzumab-treated (10 μg/ml) (red profile) cells measured by cytofluorometric analysis.

Starting from this evidence, a continuous adherent culture with trastuzumab 30 μg/ml was conducted up to 60 days to verify if persistent exposure to trastuzumab can lead to the loss of HER2 expression. While untreated cells grew as a polygonal HER2-positive monolayer, trastuzumab HER2$^{labile}$-treated cells showed a progressive reduction (10%) in the HER2-positive population accompanied by an increase in the CD44$^{high}$/CD24$^{low}$ population rising from 1% to 65% within 2 months (Figure 16A-B).

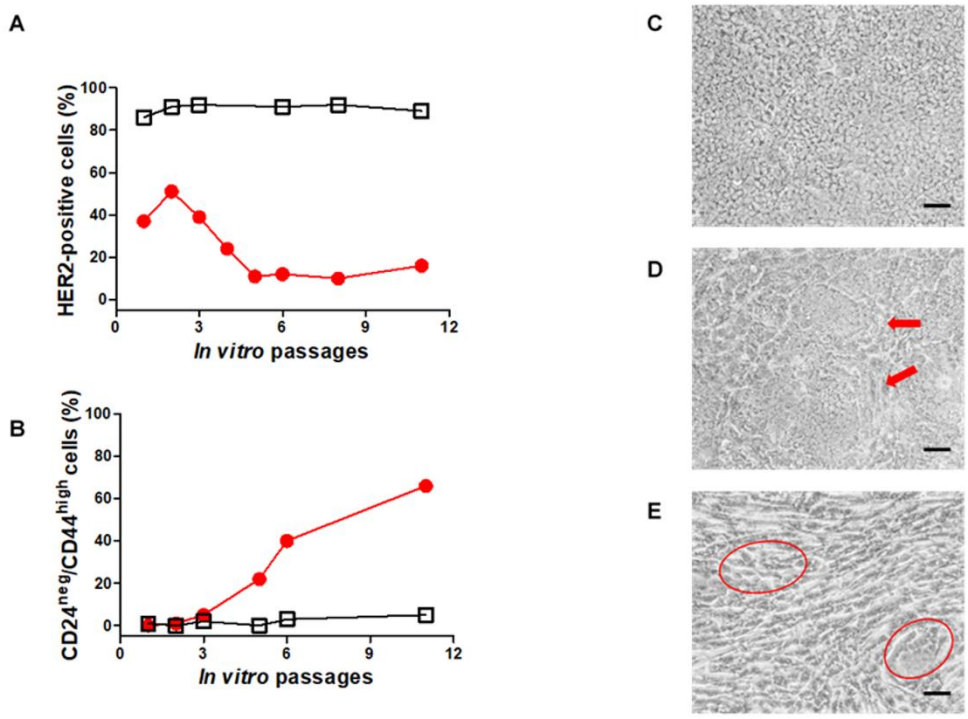


**Figure. 16. Phenotypic evaluation of MamBo43HER2$^{labile}$ cells maintained in culture with and without trastuzumab.** (A-B) Cytofluorometric analysis of cell culture at different time points. Black open square, untreated cells; red solid circle, cells cultured in presence of 30 μg/ml of trastuzumab. Percentage of HER2-positive (A) and CD24$^{low}$/CD44$^{high}$ (B) MamBo43HER2$^{labile}$ cells. (C-E) Photo of untreated cells after 4 *in vitro* passages (C) or trastuzumab-treated cells after 4 (D) and 8 *in vitro* passages (E). The red arrows indicate spindle-like areas after 4 *in vitro* passages. The red circles indicate the spindle-like population after 8 *in vitro* passages. Black bars correspond to 200 μm.

Also in this case, the HER2 loss and the increased stemness profile were linked to a morphology change, in fact spindle-like cells had gradually taken over the polygonal cells that remain confined to small areas (Figure 16C-E).

However, the loss of HER2 expression was not specifically related to trastuzumab treatment but it was a consequence of any treatment that reduces cell density. In fact, MamBo43HER2$^{labile}$ cells were seeded at lower cell doses ($4 \times 10^4$ cells/cm$^2$ versus $1.6 \times 10^5$ cells/cm$^2$) and treated with trastuzumab at 30 µg/ml for 60 days (Figure 17). Surprisingly, similar to what happened to trastuzumab-treated cells, even untreated cells gradually acquired spindle-like morphology at lower seeding density (data not shown), stemness phenotype and HER2 expression was detectable in less than 25% of cells. A further reduction in cell seeding dose ($10^4$ cells/cm$^2$) induced a faster (within 30 days) acquisition of the fibroblast-like morphology (data not shown), the loss of HER2 expression and an improvement in stemness, even in the absence of trastuzumab treatment (Figure 17).

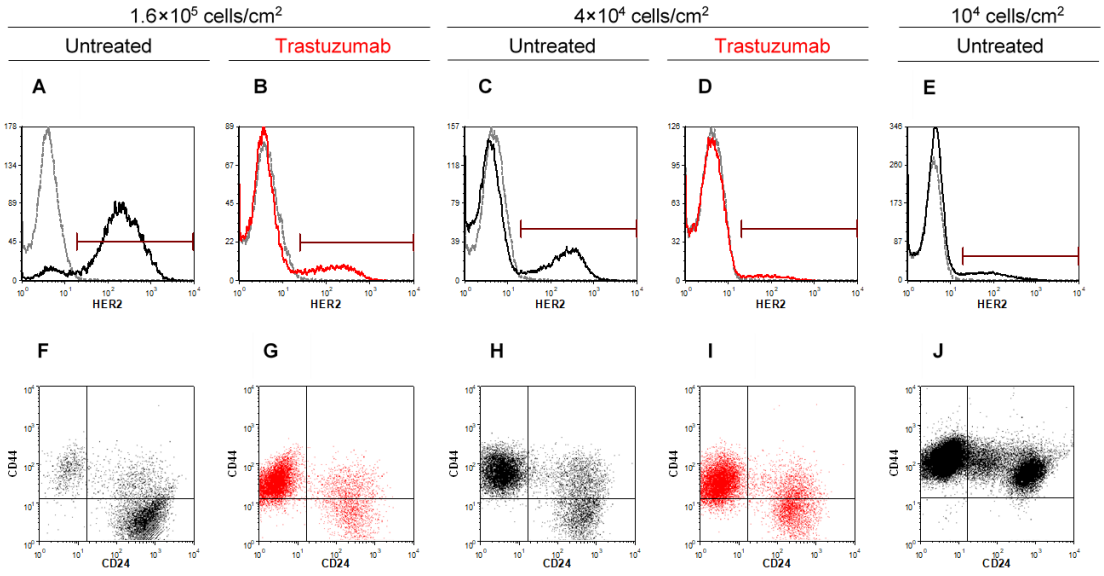


**Figure 17. Effect of cell seeding and trastuzumab treatment on MamBo43HER2$^{labile}$ cells**. Continuous cultures in control medium (A, C, E, F, H, J) or in medium supplement with trastuzumab (30 µg/ml) (B, D, G, I). Percentage of HER2 (A-E), CD24 and CD44 (F-J) cells measured by cytofluorometric analysis in different seeding conditions and in the presence of absence (black profile) of trastuzumab (red profile).

In addition, it is interesting to note that these cells do not recover the initial phenotype either once trastuzumab treatment was suspended or when low-density seeding was

stopped, but remain negative for HER2, predominantly $CD24^{-}/CD44^{+}$ and with mostly spindle morphology (data not shown).

*In vivo* treatment with trastuzumab significantly accelerated tumor growth of MamBo43HER2$^{labile}$ cells starting from 25 days after cell injection (Giusti et al. 2021).

Thus, the HER2 loss in MamBo43HER2$^{labile}$ cell line appeared to be density-related, nevertheless trastuzumab showed to play a key role in enhancing the spontaneous trend of HER2 expression loss in HER2 labile cell line.

### 4.4. MamBo43HER2$^{labile}$ cells as a heterogeneous society

The loss of HER2 observed *in vitro* was probably the result of a selection among several sub-populations coexisting within MamBo43HER2$^{labile}$ cells. In this contest HER2$^{labile}$ cell line could be a good example of the tumor as a heterogeneous society.

Several clones were isolated from MamBo43HER2$^{labile}$ with different morphology and molecular profile. Five of them were selected to further investigate the HER2 and stem markers expression, EMT profile and tumorigenic capacity. We focused our attention on:

- 2 HER2-negative clones with spindle-like morphology and a stemness profile close to MamBo38HER2$^{loss}$ cell line (AG11F and AG24F);
- 1 HER2-negative clone with mixed morphology and a CD44/CD24 profile resembling that of MamBo89HER2$^{stable}$ cell line instead of MamBo38HER2$^{loss}$ (AD56 IOTA);
- 2 HER2-positive and polygonal clones, similar to MamBo89HER2$^{stable}$ and MamBo43HER2$^{labile}$ cell lines (AD56 C1 and AD56 C5) (Figure 18).

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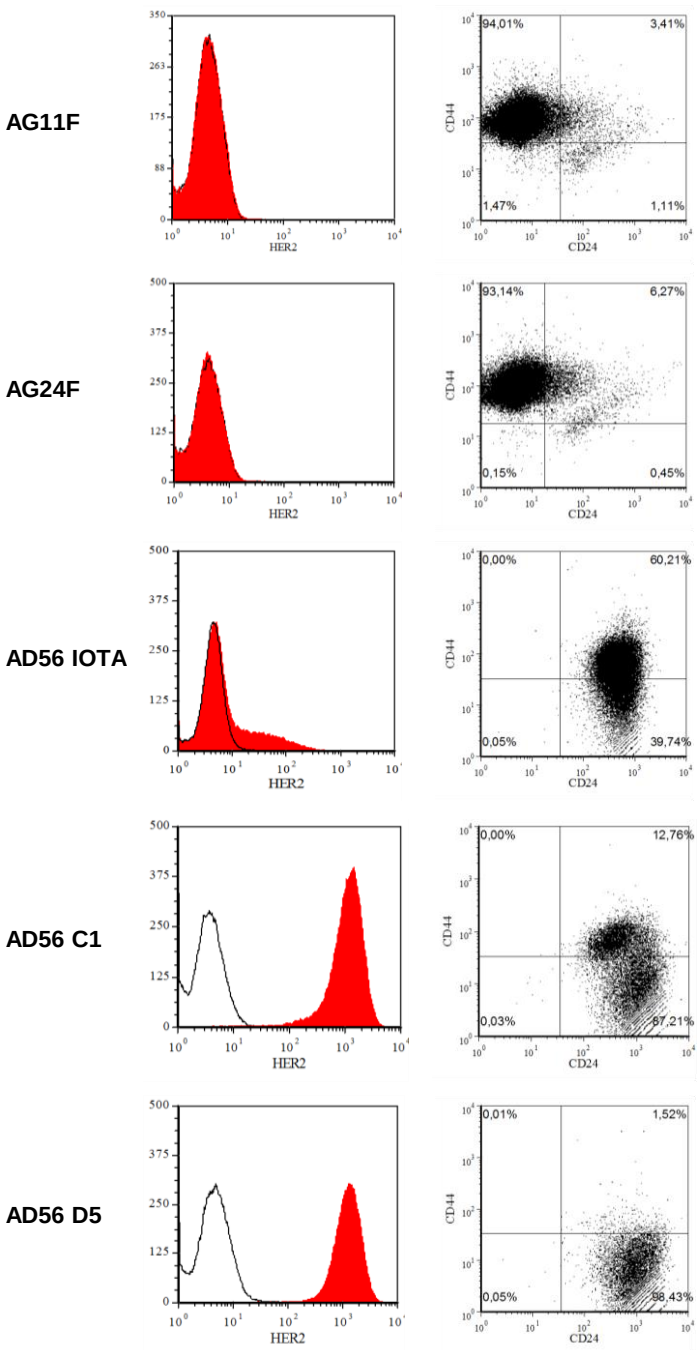


**Figure 18. HER2, CD24 and CD44 expression in MamBo43HER2*labile* derived clones.** HER2 expression of MamBo43HER2$^{labile}$ clones measured by cytofluorimetric analysis (left panels). Black profile, secondary antibody alone; red profile, anti-HER2 antibody. Stemness markers CD24 and CD44 measured by flow cytometry (right panels).

Differences between MamBo43HER2$^{labile}$ clones were also found by analysis of several genes associated with the EMT. Polygonal clones showed an EMT gene expression profile comparable to the MamBo43HER2$^{labile}$ parental cell line, and the profile of the spindle clones reminded that of the MamBo38HER2$^{loss}$ cell line. In contrast, in AD56 IOTA clone the expression of the *Mmp2*, *Igfbp4* and *Fgfbp1* genes was intermediate between the two cell

lines, for the *Dsp* and *Col5a2* genes the expression is comparable to that of HER2$^{labile}$ cells, while in all the other genes it was closer to HER2 loss cell line. Consequently, despite having a spindle component within it, AD56 IOTA also retained characteristics of the parental cell line and did not acquire a full EMT associated profile but presented a partial EMT state (Figure 19).

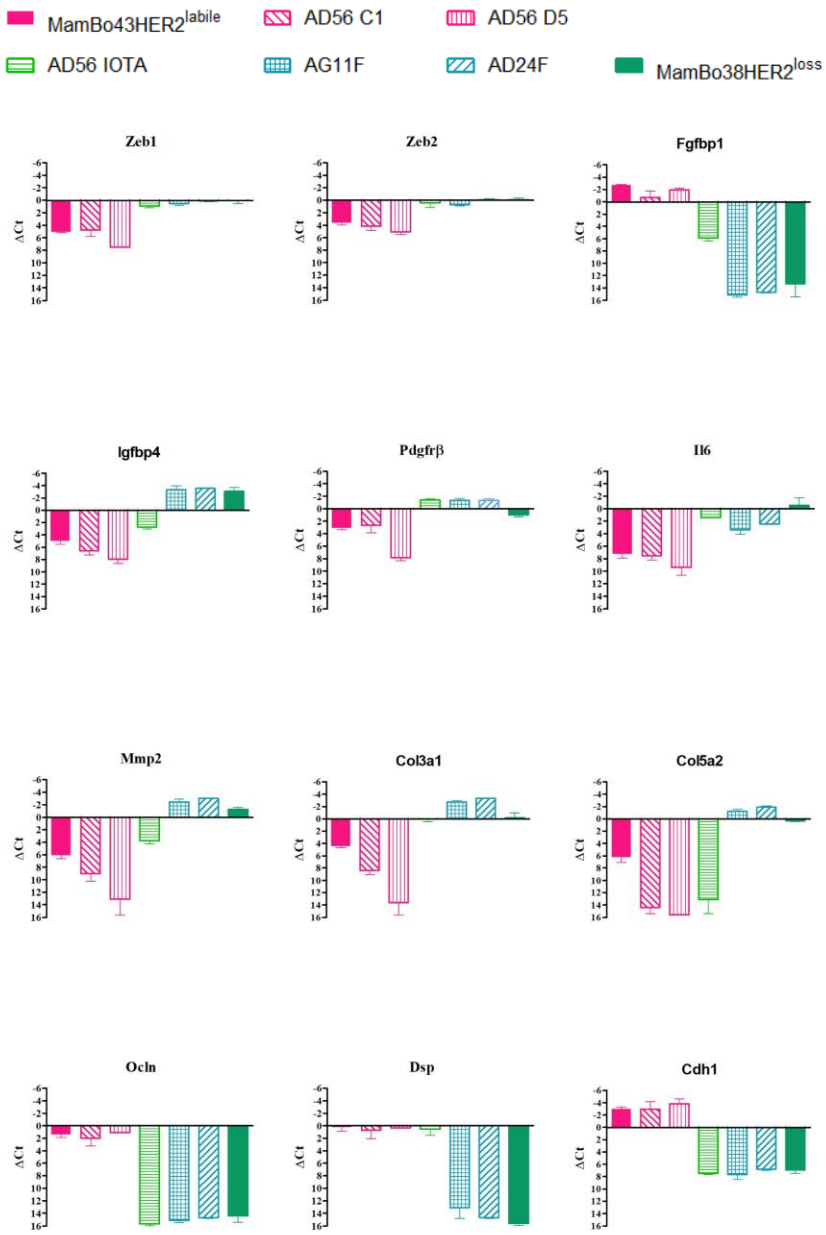


**Figure 19. EMT related genes expressed by MamBo43HER2$^{labile}$ parental cell line and clones and MamBo38HER2$^{loss}$ cell line.** Bar graphs represent expression of each indicated gene in MamBo43HER2$^{labile}$, AD56 C1, AD56 D5, AD56 IOTA, AG11F, AG24F, MamBo38HER2$^{loss}$ cells (n=2, each bar shows Mean ± SEM). Expression was determined by RT-PCR analysis, normalized over TBP (total binding protein) expression ($\Delta Ct = Ct_{gene} - Ct_{TBP}$).

To evaluate how the different morphology, HER2 expression, CD24/CD44 level and EMT markers influenced tumorigenicity, $10^5$ s.c. MamBo43HER2$^{labile}$ clones were inoculated in NOD-SCID-Il2rg-/- (NSG) female mice and their ability and speed of growth was compared to that of MamBo43HER2$^{labile}$ parental cell line and MamBo38HER2$^{loss}$ cells.

As reported in Table 2, all mice developed tumors with 100% incidence, except for AD56 C1 clone (67%). AG11F and AG24F HER2-negative clones, according to the EMT features and the high stemness profile, showed short latency and time to sacrifice comparable to that of MamBo43HER2$^{labile}$ (which undergo HER2 loss *in vivo*) ones. Despite the mesenchymal-like morphology and the lack of HER2 expression, AD56 IOTA gave rise to tumors 20 weeks after cell challenge, thus confirmed that the reduced EMT state and stemness profile impaired the tumorigenic capacity. The polygonal HER2-positive clones, AD56 C1 and D5, presented the longest latency and slow tumor growth.

| *Tumorigenicity* | | | | |
|---|---|---|---|---|
| | **Injection dose** | **Incidence (%)** | **Weeks of latency (median) [range]** | **Weeks at sacrifice after cell injection (median) [range]** |
| MamBo43HER2$^{labile}$ | $10^5$ s.c. | 100 | 5 [5-5] | 7 [7-7] |
| AD56 C1 | $10^5$ s.c. | 67 | 25 [23-26] | 35 [30-40] |
| AD56 D5 | $10^5$ s.c. | 100 | 40 [36-52] | 51 [46-55] |
| AD56 IOTA | $10^5$ s.c. | 100 | 20 [17-25] | 22 [22-22] |
| AG11F | $10^5$ s.c. | 100 | 4 [4-4] | 7 [7-7] |
| AG24F | $10^5$ s.c. | 100 | 5 [4-5] | 7 [7-7] |
| MamBo38HER2$^{loss}$ | $10^5$ s.c. | 100 | 2 [2-2] | 3 [3-3] |

**Table 2.** *In vivo* tumorigenicity of MamBo38HER2$^{loss}$, MamBo43HER2$^{labile}$ and its polygonal and spindle-like clones.

The cell lines and clones also differed in spontaneous metastatic capacity to the lung. Despite the high tumorigenicity, MamBo38HER2$^{loss}$, MamBo43HER2$^{labile}$, AG11F and AG24F cells induced a low number of lung nodules probably due to the short latency and rapid tumor growth rate (Table 3). While the spontaneous metastatic ability was almost absent in AD56 C1 (incidence: 0%) and AD56 D5 (incidence: 33%) clones according to their low *in vivo* malignancy, AD56 IOTA cells showed the stronger metastasis outgrowth (Table 3). In this

clone, it appears that the partial EMT state simultaneously slowed tumor latency and helped the cells to take advantage of the increased time available to succeed in colonizing the lung and to induce a higher number of metastases than in the other cell lines.

| *Lung metastasis* | | | | |
|---|---|---|---|---|
| | **Injection dose** | **Incidence (%)** | **Number of lung metastasis (median)** | **Number of lung metastasis (range)** |
| MamBo43HER2$^{labile}$ | $10^5$ s.c. | 100 | 1 | 1-6 |
| AD56 C1 | $10^5$ s.c. | 0 | 0 | 0 |
| AD56 D5 | $10^5$ s.c. | 33 | 0 | 0-10 |
| AD56 IOTA | $10^5$ s.c. | 100 | 3 | 2->500 |
| AG11F | $10^5$ s.c. | 100 | 13 | 11-18 |
| AG24F | $10^5$ s.c. | 0 | 0 | 0 |
| MamBo38HER2$^{loss}$ | $10^5$ s.c. | 100 | 1 | 1-8 |

**Table 3.** Spontaneous metastatic ability of MamBo38HER2$^{loss}$, MamBo43HER2$^{labile}$ and its polygonal and spindle-like clones

The expression of HER2 and the stemness markers CD24 and CD44 was evaluated by cytofluorimetric analysis on tumor cells grown s.c.. Together with MamBo38HER2$^{loss}$ and MamBo43HER2$^{labile}$ cell lines, also AG11F, AG24F and AD56 IOTA gave rise to HER2-negative tumors with comparable levels of CD24 and CD44 expressed by MamBo43HER2$^{labile}$ ones (data not shown).

Interesting, AD56 C1 clone induced tumor growth in 2/3 of mice and HER2 expression was found only in one out of two tumors (Figure 20). The HER2-positive and HER2-negative tumors had both high expression of CD24 and low expression of CD44, but HER2-positive tumor showed a longer latency and slower tumor growth than the HER2-negative one (Figure 20). Moreover, all tumors derived from AD56 D5 were HER2-positive.

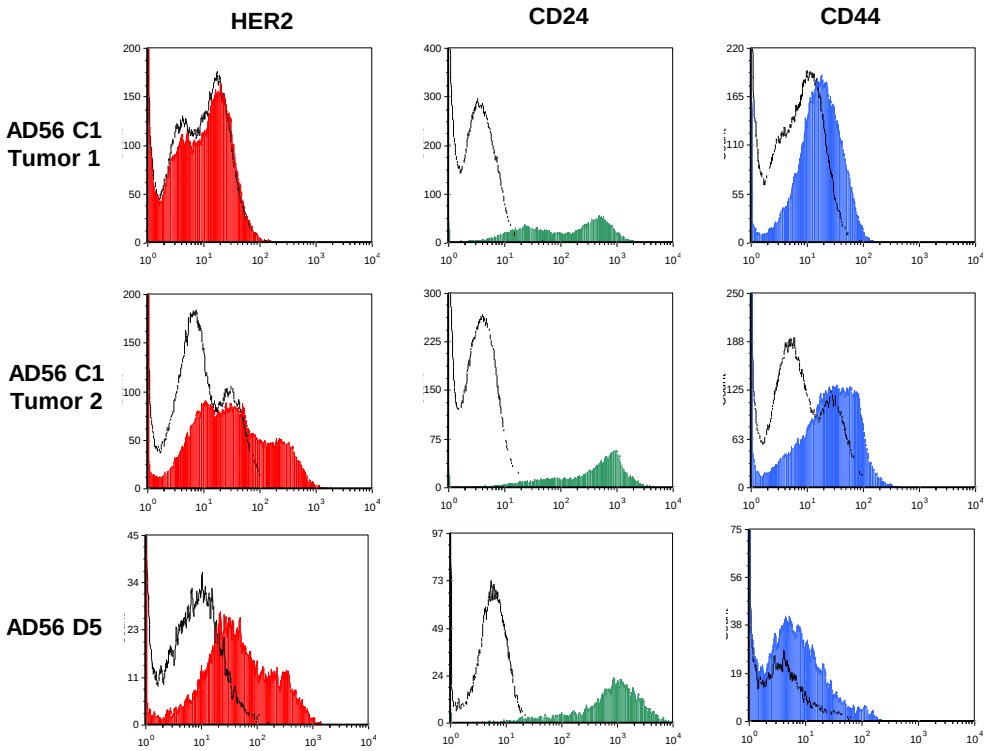


**Figure 20. HER2 and stem markers CD24 and CD44 expression of tumors induced by *in vivo* injection of HER2-positive clones AD56 C1 and AD56 D5.** Graphs show HER2 (red), CD24 (green) and CD44 (blue) expression in two different AD56 C1 induced tumors and in a representative AD56 D5 tumor. Protein expression levels were detected by flow cytometry.

To sum up, these data confirmed that MamBo43HER2$^{labile}$ cell line could be compared to a heterogeneous society in which multiple sub-populations with different characteristics coexist. The balance between them can be altered by continuous low-density culture or by the presence of trastuzumab *in vitro* or after its *in vivo* injection. All these conditions promote the expansion of the spindle-like, HER2-negative and stem component which overwhelms the polygonal component. The mechanisms underlying the dynamic equilibrium governing the coexistence of these populations remain unclear and they will be the object of future studies.

Besides, AD56 C1 clone characteristics will be further investigated to shed light on what elements lead to giving rise tumors with different HER2 expression starting from the same clone.

### 4.5. Transcriptome analysis of a preclinical model of HER2 dynamic expression

In parallel with the ongoing research for the mechanisms behind the loss of the HER2 oncogene, we decided to exploit the model's ability to mimic HER2-positive tumors with a

stable expression of the oncogene and progressing tumors which are no longer addicted to HER2 or give rise to metastasis with different or absent HER2 level of expression. Thanks to these characteristics, our preclinical model of HER2 loss is a powerful tool to discover new therapeutic strategies for HER2-positive breast cancer in progression.

For these reasons, in collaboration with Prof. Calogero and coworkers (University of Turin, Italy), an RNA-sequencing analysis was performed on MamBo89HER2$^{stable}$, MamBo43HER2$^{labile}$ and MamBo38HER2$^{loss}$ cell lines. The analysis also included MamBo43HER2$^{labile}$ treated with trastuzumab (30 μg/ml) for 30 days and then removed (TRNT) or maintained for further 30 days (TRT).

To make the analysis more comprehensive, samples of MamBo89HER2$^{stable}$ and MamBo38HER2$^{loss}$ treated with trastuzumab in the same manner as MamBo43HER2$^{labile}$ cells were included. Despite of HER2$^{labile}$ cell line, the treatment with trastuzumab of MamBo89HER2$^{stable}$ and MamBo38HER2$^{loss}$ did not induce any changes in HER2 expression, stemness profile and morphology (data not shown).

Comparison between HER2-negative and HER2-positive cell lines revealed 751 differentially expressed genes: 409 of them are found to be up-regulated in HER2-negative cell lines and 349 down-regulated.

Using the Enrichr platform, the biological processes and pathways, in which the differentially expressed genes appear to be involved, were identified (Figure 21A-B). The biological processes and KEGG pathways found to be up-regulated in HER2-positive cell lines are: positive regulation of angiogenesis (*Ecm1; Jup; Serpinf1; Vegfb; Hmga2; Foxo4; Thbs2; Hspg2; Hif1a; Pgf; Dcn; Aqp1; Mtdh; Vegfa; Ceacam1; Stim1; Gpnmb; Adgra2; Zc3h12a; Erbb2; Hmox1*), membrane protein localization (*Slc9a3r1; Jup; Cdh1; Tesc; Pkp3; F11r; Atp1b1; Ezr; Ramp1; Nkd2*), proteoglycans in cancer, cell junction assembly processes as well as epithelial cell differentiation and development. In HER2-negative cell lines, on the other hand, the following biological processes and KEGG pathways were found to be up-regulated: PI3K-Akt signaling pathways, pathways related to proteoglycans in cancer and focal adhesions (*Pdgfrβ; Pdgfrα; Ppp1r12a; Cav1; Fn1; Vegfb; Lamb1; Parvb; Thbs2; Vegfa; Col1a2; Col6a2; Fyn; Flnc; Itga5; Vcl*) , organization of the ECM, positive regulation of migration (*Pdgfrβ; Pdgfrα;*

*Ccl7; Gpnmb; Plau; Sema3a; Pdpn; Lamb1; Elp3; Vegfa*), angiogenesis, exocytosis, biosynthesis, PkB signaling (*Pdgfrβ; Pdgfrα; Fgf7; Ddit3; C1qbp; Itgb1bp1; Fyn; Park7; Xdh; Ereg; Mtdh*), cellular biosynthesis processes (*Pdgfrβ; Pdgfrα; Zeb2; Myc; Eif3e; Park7; Vim; Ptgs2; Hif1α; Eif3d; Ereg*). In addition, some genes involved in EMT and promotion of cancer cell proliferation and aggressiveness, such as *Dcn*, *Cav1*, *Cdkn1a*, *Myc* and *Pdgfrβ*, were also found to be up-regulated in cells that have lost HER2 (Figure 21A-B).

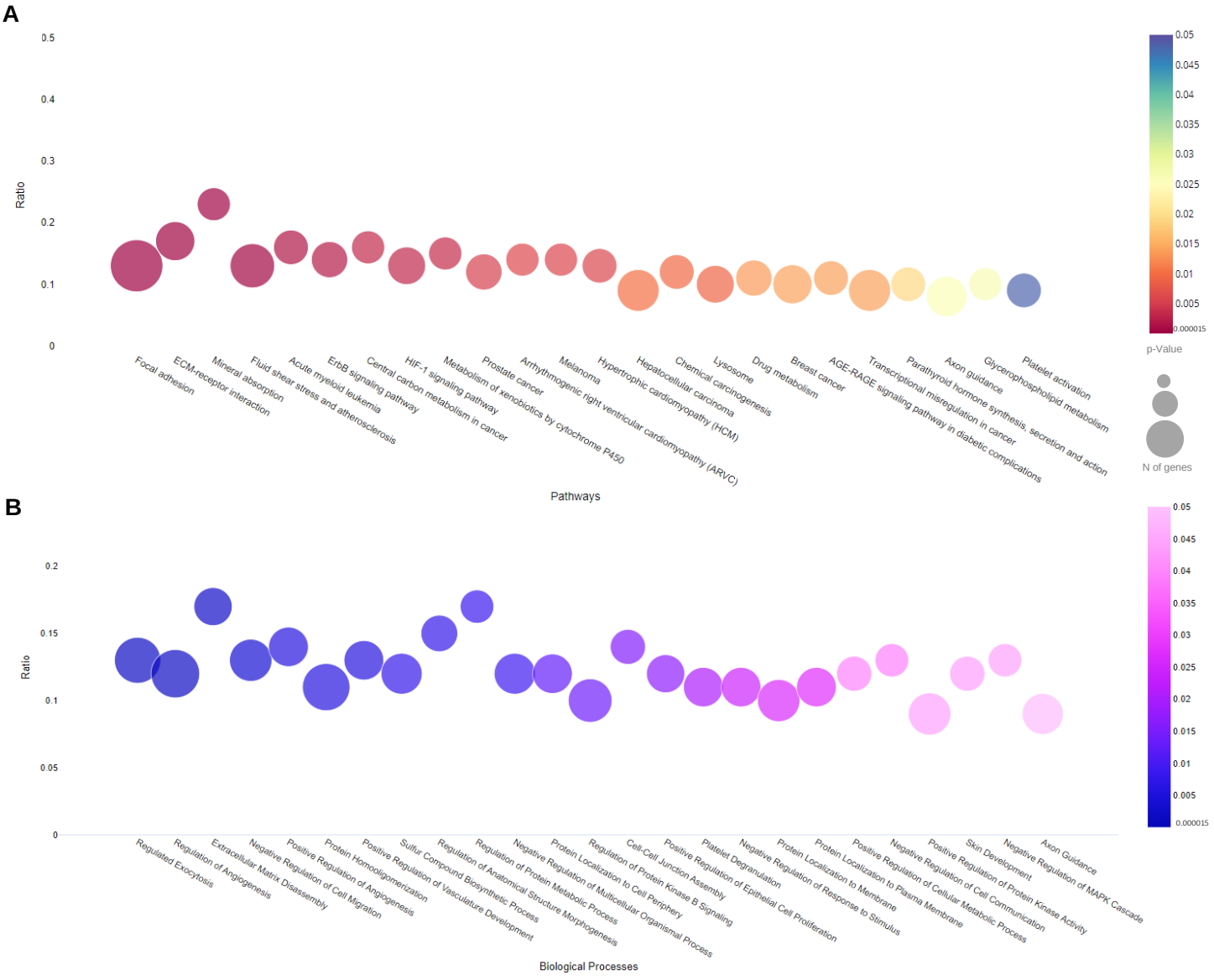


**Figure 21. Biological processes and pathway differentially modulated in HER2-negative *vs* HER2-positive cell lines.** (A) KEGG pathways and (B) biological processes with adjusted p-value<0.05, gathering genes >10 and excluding biological processes and pathways with a number of genes >200. The size of the dots represents the absolute number of differentially expressed genes, between HER2-positive and HER2-negative cells.

Thus, we can state that in HER2-positive cells there is evidence of a molecular profile associated with increased epithelial differentiation and rich in cell-cell communication, whereas cells that have lost expression of the oncogene exhibit genes associated with increased migratory capacity and behavior typical of cells that have undergone an EMT.

As better shown in Figure 22, which reports the principal component analysis (PCA) of EMT related genes of MamBo43HER2$^{labile}$ cell line, MamBo43HER2$^{labile}$ cells treated with trastuzumab (TRT and TRNT) and MamBo38HER2$^{loss}$ cells, the HER2 loss and the HER2-positive cells formed two different clusters according to the different EMT state (Figure 22).

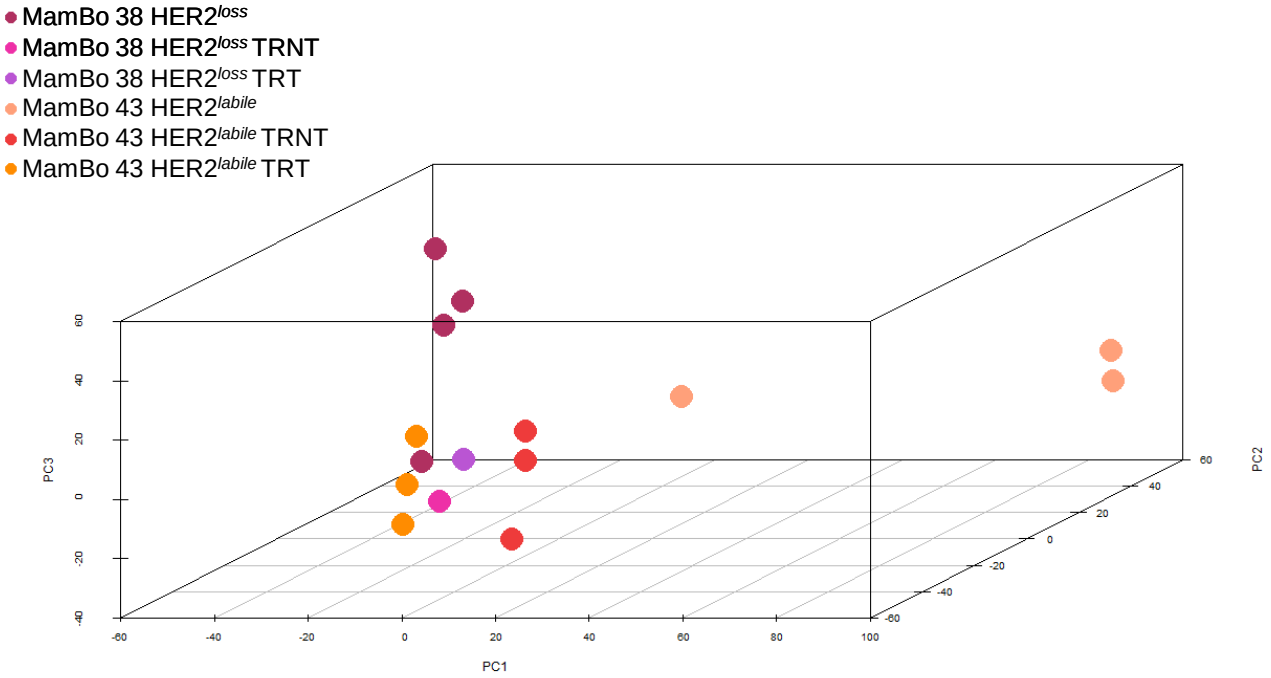


**Figure 22. Principal component analysis (PCA) of EMT related genes of MamBo43HER2$^{labile}$, MamBo38HER2$^{loss}$ and of the same cells treated with trastuzumab (TRT and TRNT).** PC1, PC2 and PC3 represent, respectively, 45.3%, 39.4% and 22,6% of the sample variance.

In order to identify the mechanisms behind the loss of HER2 oncogene expression, the differential gene expression of the MamBo89HER2$^{stable}$ and MamBo43HER2$^{labile}$ cell lines was also compared (Figure 23 A-B).

The comparison revealed 225 differentially expressed genes: 77 were up-regulated in the MamBo89HER2$^{stable}$ line and 148 down-regulated.

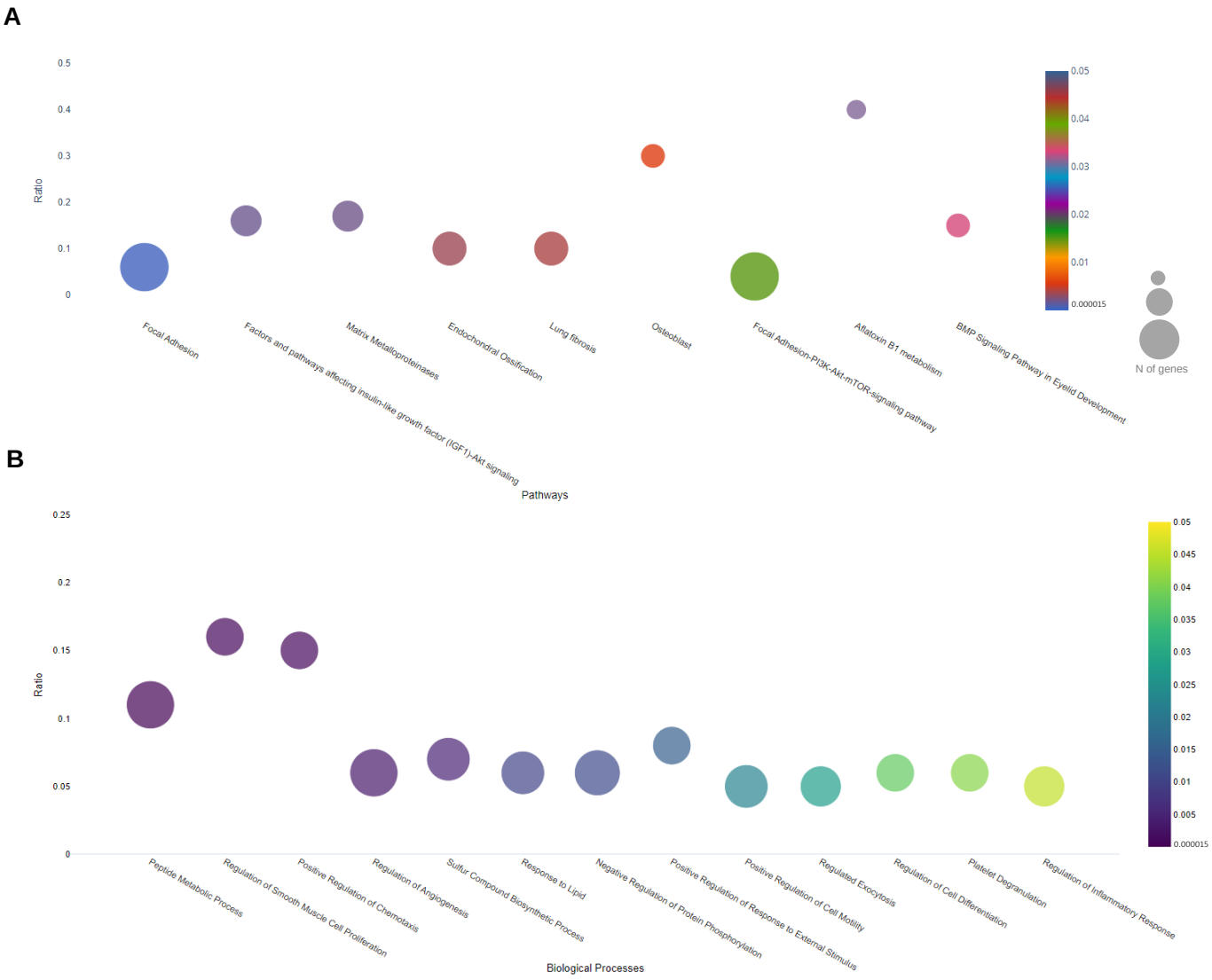


**Figure 23. Biological processes and pathway differentially modulated in MamBo89HER2$^{stable}$ *vs* MamBo43HER2$^{labile}$ cell lines.** (A) KEGG pathways and (B) biological processes with adjusted p-value<0.05, gathering genes >10 and excluding biological processes and pathways with a number of genes >200. The size of the dots represents the absolute number of differentially expressed genes, between HER2$^{stable}$ and HER2$^{labile}$ cells.

Analysis of biological processes and pathways revealed that the 225 genes are involved in factors and pathways affecting insulin-like growth factor (IGF1)-Akt signaling (*Igfbp4; Igfbp2; Igfbp6; Igf1; Pdk1*), focal adhesion (*Pdgfrβ; Cav1; Col4a2; Selenop; Col11a2; Tnc; Col4a6; Itga8; Lamc2; Igf1; Thbs1; Pgf*), positive regulation of cell motility (*Pdgfrβ; Mmp14; Tgfb1; Plau; Ccl5; Ctsh; Igf1; Lgr6; Thbs1*) and response to lipid (*Socs2; Tgfb1; Cited1; Alpl; Ctsh; Tnfrsf11b; Csn1s1; Cxcl2; Cxcl5*). Notably, many of these pathways and processes are downmodulated in the cell line with stable HER2 expression compared with the HER2$^{labile}$

cells. Genes up-regulated in HER2$^{labile}$ cells are mainly involved in the positive regulation of epithelial cell proliferation (*Bmp4, Cav1, Nr4a1, Osr1, Tgfb1, Tnfaip3*), negative regulation of cell-cell adhesion (*B4galnt2, Bmp4, Cav1, Cx3cl1, Ephb6, Fstl3, Klf4, Tgfb1, Tnfaip3*) and in the regulation of Bmp signaling pathway (*Bmp4, Cav1, Fstl3, Msx2*), which is often involved in EMT process.

To shed light on potential genes involved in the loss of HER2 oncogene expression, a gene expression correlation analysis was conducted in the MamBo89HER2$^{stable}$, MamBo43HER2$^{labile}$ and MamBo38HER2$^{loss}$ cell lines, aimed at identifying groups of genes with a similar or inverse expression trend to HER2. R software, which can calculate co-expression with the *prcomp()* function and Spearman's correlation index, was chosen to perform the analysis.

HER2 expression appears to be closely associated ($\varrho>0.55$ or $\varrho<-0.55$ and $p<0.05$) with the expression of molecules involved in the pathways of focal adhesions, integrin-mediated cell adhesion, cytoskeletal actin regulation, and the EGFR signaling pathway. Some of these genes are shared by more than one pathway such as *Cav1*, which is involved in the focal adhesion pathway, integrin-mediated cell adhesion and EGFR signaling pathway. In our model *Cav1* expression negatively correlates with HER2 expression in the HER2-positive cell lines, whereas its gene expression level is positively associated with the HER2 absence in MamBo38HER2$^{loss}$. It indicates that Cav1 might be one of the genes involved in HER2 dynamic expression in our preclinical model (Figure 24).

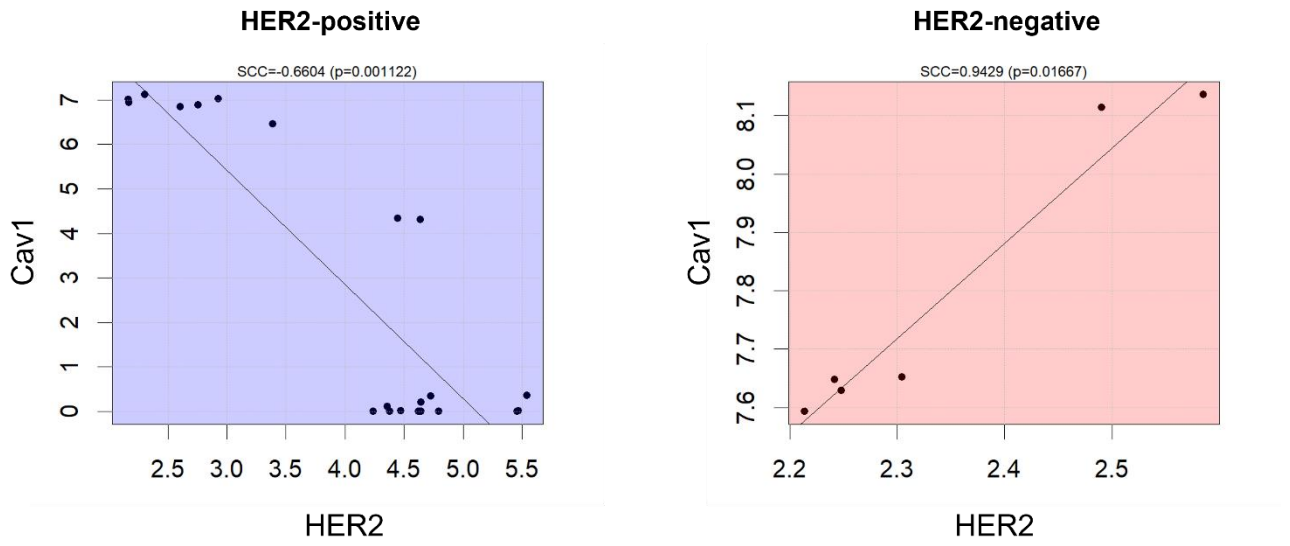


**Figure 24. Gene expression correlation analysis between *HER2* and *Cav1* expression in HER2-positive (MamBo89HER2$^{stable}$ and MamBo43HER2$^{labile}$) and HER2-negative (MamBo38HER2$^{loss}$).**

### 4.6. Targeting PDGFR-β: a therapeutic strategy for HER2 loss cells

RNA-sequencing analysis revealed that *Pdgfrβ* is involved in several pathways and biological processes which might sustain HER2-negative cells malignancy. Together with *Pdgfrβ*, also *Vegfr* expression is up-regulated in MamBo38HER2$^{loss}$ cell line and involved in different pathways like regulation of cell migration, focal adhesion and angiogenesis.

For this reason, we tested the ability of sunitinib, a pan TKI inhibitor that acts both on VEGFR and PDGFR-β, to counteract the growth of MamBo38HER2$^{loss}$ cells both *in vitro* and *in vivo*.

PDGFR-β protein level was measured by flow cytometry and, as predicted by gene expression analysis, PDGFR-β was detected only in HER2-negative cell lines (Figure 25A). MamBo38HER2$^{loss}$ appeared sensitive to sunitinib under adherent condition in the dose range of 1-10 μM, showing a IC50 equal to 4.66 μM (Figure 25B). Three-dimensional (3D) agar colonies growth was dramatically inhibited by sunitinib 5 μM and slightly reduced (20%) by sunitinib 1 μM (Figure 25C). Moreover, HER2-negative cells treated with sunitinib 5 μM acquired a more polygonal morphology and showed an increased *Cdh1* expression level (Figure 25D-E).

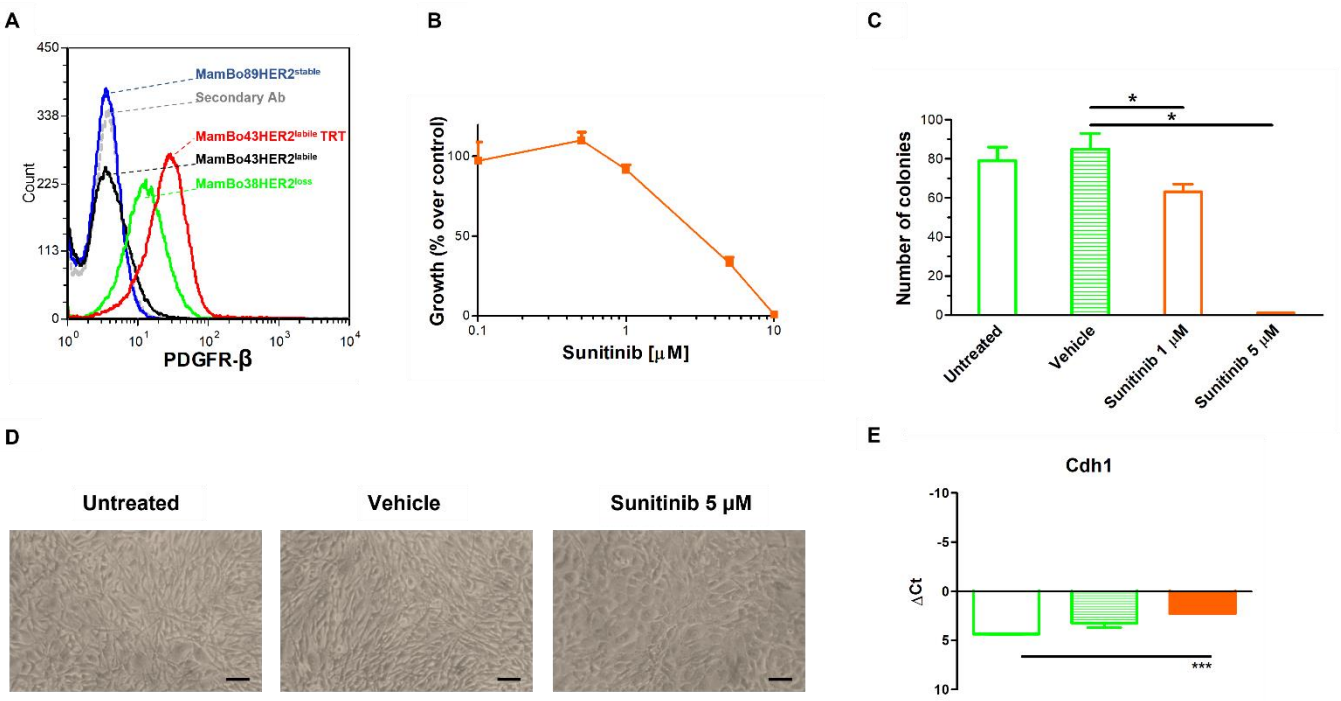


**Figure 25**. **Sunitinib activity *in vitro* on MamBo38HER2$^{loss}$ cells**. (A) Expression level of PDGFR-β by cytofluorometric analysis. (B) Effect of sunitinib on the growth MamBo38HER2$^{loss}$ under 2D-adherent conditions, n=3. (C) Colonies growth inhibition under 3D conditions by sunitinib, * $p<0.05$ versus vehicle by Student's *t* test. n=2-4 (D) Representative images of untreated (left panel), vehicle-treated (middle panel) and sunitinib-treated (right panel) MamBo38HER2$^{loss}$ cells. Bar corresponds to 200 μm. (E) E-cadherin expression by Real-Time PCR of untreated (green), vehicle (green with rows) and sunitinib- (5 μM) treated cells (solid orange). Expression was determined by RT-PCR analysis, normalized over TBP (total binding protein) expression ($\Delta Ct = Ct_{gene} - Ct_{TBP}$). Data reported are Mean ± SEM of the ΔCt, n=2. *$p<0.05$ by Student's t test

Furthermore, sunitinib 5 μM significantly inhibited mammospheres formation as well as cell migration ability of MamBo38HER2$^{loss}$ cells (Figure 26A-B).

Interestingly, after 4 days of treatment with sunitinib a reduction of interleukin-6 (IL-6) was detected in cells supernatants. IL-6 reduction was also accompanied by the inhibition of its downstream pathway through down-modulation of pStat3 (Figure 26C-D).

Sunitinib efficacy in reducing the aggressiveness of HER2-negative cells was confirmed *in vivo*, where it significantly slowed down tumor growth induced by of MamBo38HER2$^{loss}$ cells starting from 14 days after cell challenge (Figure 26E).

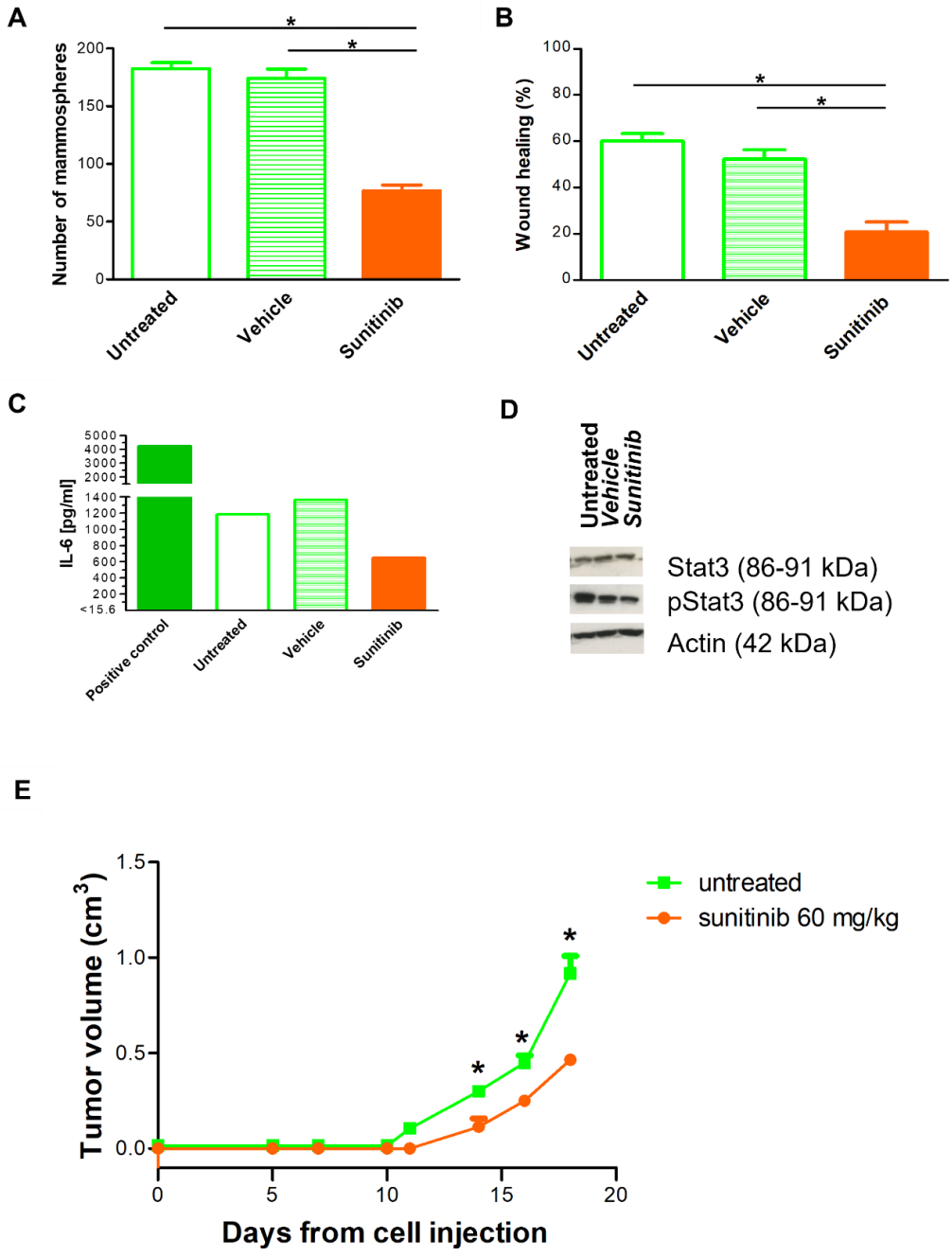


**Figure 26. Targeting PDGFR-β *in vitro* (A-D) and *in vivo* (E) on MamBo38HER2$^{loss}$ cell line.** (A) Number of mammospheres formed by MamBo38HER2$^{loss}$ cells after treatment with sunitinib (5 μM). Data shown are the Mean ± SEM, n= 2-4 for each group; *p<0.01, sunitinib vs untreated or vehicle by Student's *t* test. (B) Wound-healing assay. Data shown are the Mean ± SEM of the area covered by cells 24 hours after the wound formation, n= 6-8 for each experimental group. *p<0.01, vs untreated or vehicle by Student's *t* test. (C) IL-6 production detected by ELISA in MamBo38HER2$^{loss}$ cells treated or not with sunitinib (5 μM). (D) Western blotting analysis for STAT3 and pSTAT3 on MamBo38HER2$^{loss}$ cells treated or not with sunitinib (5 μM). (E) Tumor growth of MamBo38HER2$^{loss}$ cells in vehicle-(green line) and sunitinib-(orange line) treated mice. Sunitinib significantly reduced tumor growth from the 14$^{th}$ day after cell injection onward, *p<0.05, at least, by Student's *t*-test. Data shown are the mean and SEM of tumor volumes from n=5 mice per group.

The impaired MamBo38HER2$^{loss}$ malignancy in cells treated with sunitinib should be connectable to the inhibition of inducer molecules of EMT, like PDGFR-β and IL-6, and the induction of the expression of epithelial marker E-cadherin.

Taken together these data suggest that targeting PDGFR-β by using multi-targeted molecules like sunitinib might be a valid strategy in the treatment of patients with HER2 receptor conversion.

### 4.7. Role of *Cdh1* gene in a preclinical breast cancer model of HER2 loss

As reported in the previous paragraphs, the changes in morphology and the epithelial to mesenchymal transition are two important players in our preclinical model of HER2 loss.

To better evaluate how the acquisition of epithelial features can affect the malignant phenotype of the MamBo38HER2$^{loss}$ cell line, the expression of the main epithelial marker, *Cdh1*, was induced in these cells and its influence on the *in vitro* and *in vivo* cells growth was investigated.

#### *4.7.1. Validation of epithelial to mesenchymal markers E-cadherin and Vimentin*

According to the data derived from the sequencing analysis and the RT-PCR results shown in Figure 27, the protein expression of the E-cadherin, an epithelial marker, and vimentin, a mesenchymal marker, were validated in MamBo89HER2$^{stable}$, MamBo43HER2$^{labile}$ and MamBo38HER2$^{loss}$ cell lines.

Vimentin was detected by immunofluorescence on adherent cells and protein expression confirmed the gene expression data. MamBo89HER2$^{stable}$ cell line did not present vimentin expression, just a few MamBo43HER2$^{labile}$ cells were positive for vimentin, whereas MamBo38HER2$^{loss}$ cell line showed high vimentin expression according to its spindle-like morphology (Figure 27A-C).

Cytofluorimetric analysis confirmed the E-cadherin expression in MamBo89HER2$^{stable}$ and MamBo43HER2$^{labile}$ cell lines, which had a median fluorescence intensity (MFI) of 115 and 35. In contrast, E-cadherin protein (MFI=4) and mRNA expression was not detected in HER2-negative cells (Figure 27D-E).

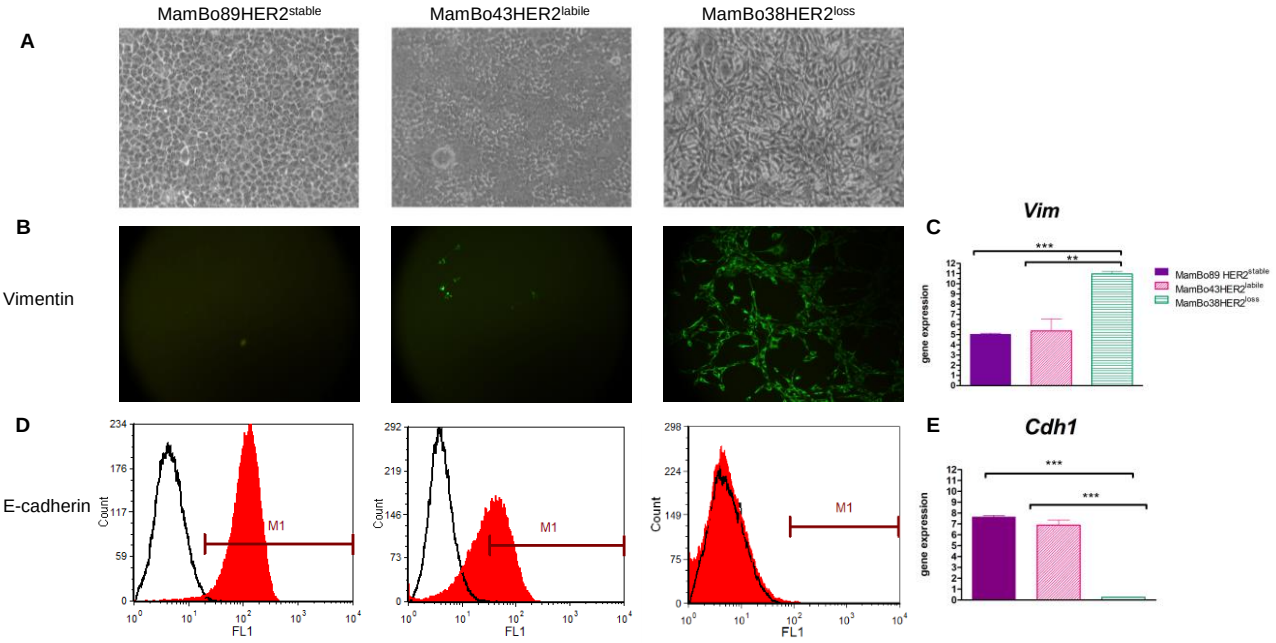


**Figure 27. E-cadherin and vimentin expression in MamBo cell lines.** (A) Representative images of HER2-positive cell lines (left and middle panels), showing an epithelial morphology, and the HER2-negative cell line (right panel) grown as a multilayer of spindle-like cells. (B) Vimentin protein expression detected by immunofluorescence on adherent cells and (C) Vimentin gene expression level from RNA-seq analysis. Gene expression unit reported on y axis is FPKM (Fragments per kilo base of transcript per million mapped fragments) (D)Representative images showing data of indirect immunofluorescence and cytofluorimetric analysis of E-cadherin on MamBo89HER2$^{stable}$, MamBo43HER2$^{labile}$ and MamBo38HER2$^{loss}$. White profile, secondary antibody alone; red profile, anti-CDH1 antibody. (E) *Cdh1* gene expression level from RNA-seq analysis. Gene expression unit reported on y axis is FPKM (Fragments per kilo base of transcript per million mapped fragments). Statistical analysis was performed using Student's *t* test, ***p<0.001.

### *4.7.2. Induction of Cdh1 gene expression in MamBo38HER2$^{loss}$ cell line*

To determine if it was possible to revert the mesenchymal phenotype of MamBo38HER2$^{loss}$ cells, *Cdh1* gene expression was induced by transfection with lentiviral particles.

The transfection had a high efficacy and cytofluorimetric analysis showed that the 91% of cells were positive for E-cadherin expression with MFI=71 (Figure 28, left panel), a protein level comparable to the one expressed in HER2-positive cell lines MamBo89HER2$^{stable}$ and MamBo43HER2$^{labile}$.

E-cadherin expression induced a morphology change into transfection bulk which showed polygonal areas interspersed with areas of spindle-like cells (Figure 28, right panel).

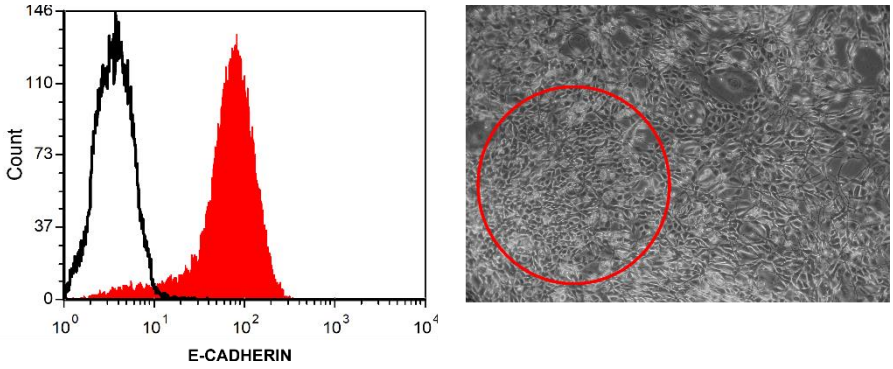


**Figure 28. Induction of *Cdh1* expression in MamBo38HER2$^{loss}$.** Left panel: representative image of E-cadherin level measured by cytofluorometric analysis in MamBo38HER2$^{loss}$ transfected cells. White profile, secondary antibody alone; red profile, anti-CDH1 antibody. Right panel: representative image of transfection bulk morphology, red circle highlighted polygonal cells area.

To identify subpopulation with different morphology and E-cadherin levels, the transfection bulk was cloned. The 100% of clones expressed E-cadherin showing different protein levels and cell morphologies.

To conduct our study three representative Cdh1-expressing clones with different morphology and E-cadherin levels were chosen (referred as M38_CDH1_*clone number*) (Figure 29A-B).

In parallel to Cdh1 transfection, MamBo38HER2$^{loss}$ cell line was also transfected with a control vector (referred as M38_CTRL_*clone number*). Control bulk and clones did not express E-cadherin and did not show significant morphology changes (Figure 29A-B).

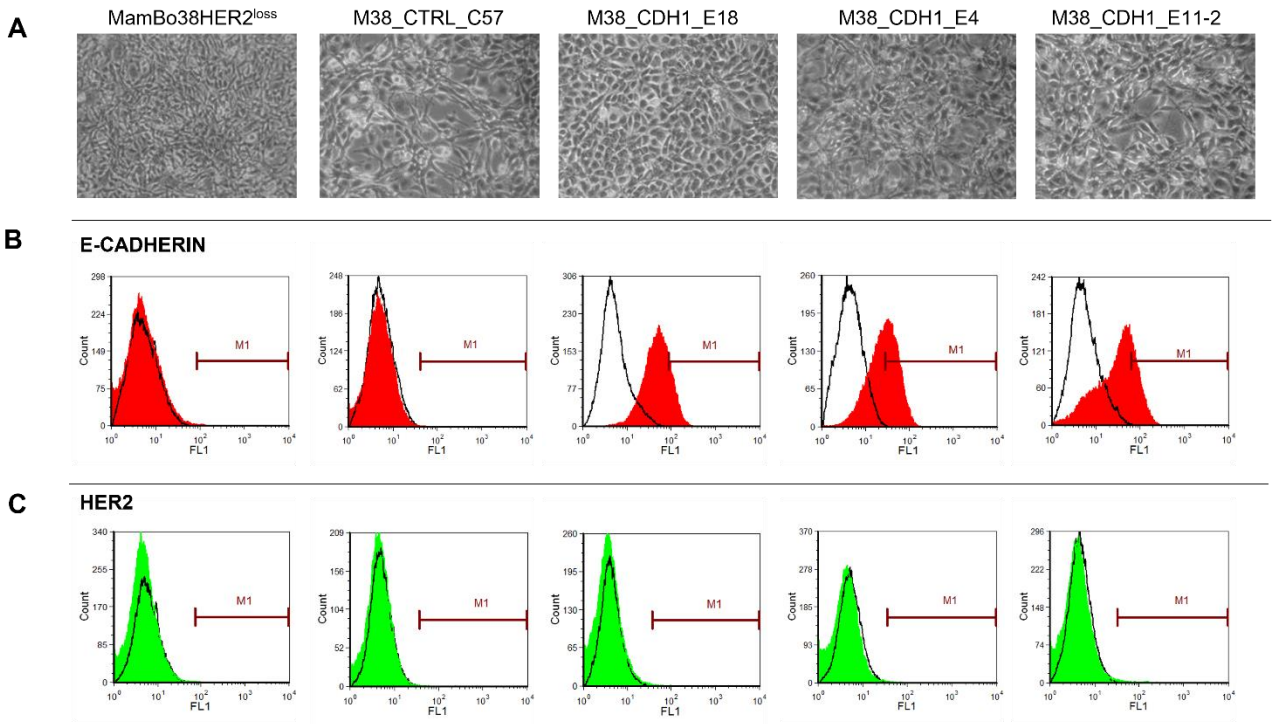


**Figure 29. Characterization of clones derived from MamBo38HER2$^{loss}$ transfection with *Cdh1*.** (A) Representative images of morphology of MamBo38HER2$^{loss}$, M38_CTRL_C57, M38_CDH1_E18, M38_CDH1_E4 and M38_CDH1_E11-2. (B and C) Representative images of CDH1 and HER2 expression level measured by cytofluorometric analysis. Black profile, secondary antibody alone; red profile, anti-CDH1 antibody; green profile, anti-HER2 antibody.

The clonal cellular lines derived from *Cdh1* induction in MamBo38HER2$^{loss}$ cells were cultured *in vitro* for several passages and E-cadherin expression remained stable during time.

M38_CDH1_E18 clone had the most polygonal and homogeneous morphology accompanied by the higher E-cadherin protein level (MFI=52) (Figure 29A-B), whereas M38_CDH1_E4 and M38_CDH1_E11-2 presented both polygonal and spindle-like cells and CDH1 expression (MFI=28) (Figure 29A-B) comparable to that of MamBo43HER2$^{labile}$ cell line (MFI=36) (Figure 27D-E).

Like MamBo38HER2$^{loss}$ parental cell line, M38_CTRL_C57 clone did not express E-cadherin (MFI=4) and presented a fibroblast-like morphology (Figure 29A-B).

Unfortunately, E-cadherin restoration in cell membrane and a newfound more epithelial phenotype did not induce the HER2 expression in MamBo38HER2$^{loss}$-derived cells (Figure 29C).

E-cadherin acquisition was followed by an increase in $CD44^{+}/CD24^{+}$ population, which went from 30%, in control cell lines (Figure 30A-B) and in the clones with the lower CDH1 expression (Figure 30C-D), up to 80% in M38_CDH1_E18 cells, with the higher E-cadherin level and polygonal morphology (Figure 30E).

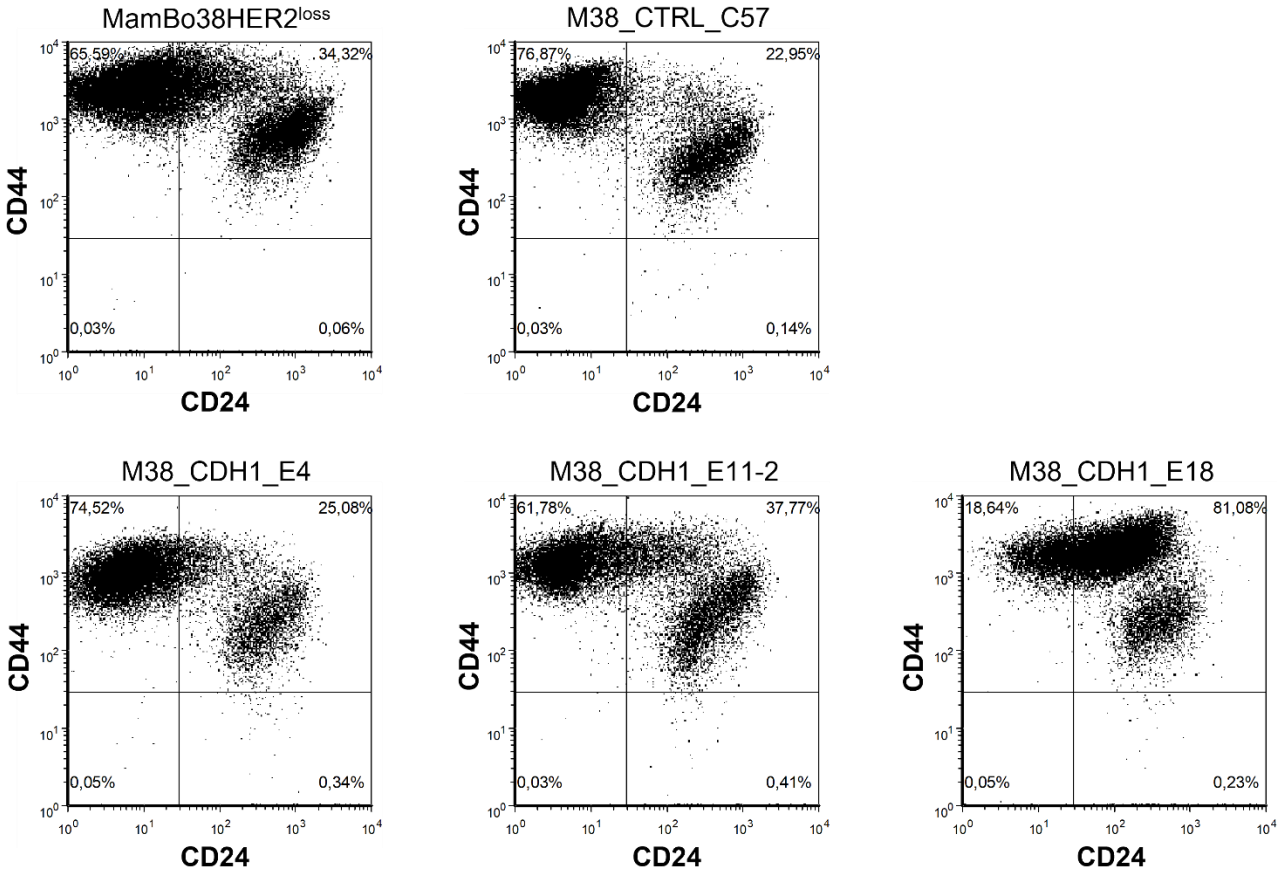


**Figure 30. CD44 and CD24 expression in MamBo38HER2$^{loss}$ and clones derived from transfection with *Cdh1*.** (A) MamBo38HER2$^{loss}$ parental cell line, (B) M38_CTRL_C57 transfection control clone and Cdh1 expressing clones (C) M38_CDH1_E4, (D) M38_CDH1_E11-2 and (E) M38_CDH1_E18. Representative dot plots wing the expression of the stem markers CD24 (x axis) and CD44 (y axis) measured by cytofluorometric analysis (n=2), each quadrant shows the percentage of cells positive for the markers.

### *4.7.3. Influence of Cdh1 on in vitro growth of MamBo38HER2$^{loss}$ cell line*

To evaluate the effects of E-cadherin induction on the proliferation ability of MamBo38HER2$^{loss}$ cell line, the *in vitro* growth rate of Cdh1-positive clones was studied and compared with hat of Cdh1-negative cells one.

Cells were seeded at 0.4x10$^5$ cells/cm$^2$ and the cellular yield was evaluated at 24, 48, 72 and 96 hours after seeding. Control clone M38_CTRL_C57 showed a similar growth trend to MamBo38HER2$^{loss}$ parental cell lines even if not reaching the same yield (Mean ± SEM: 6x10$^6$ ± 0.57 and 8x10$^6$ ± 0.63 respectively) (Figure 31A). While M38_CDH1_E18 showed an *in vitro* growth rate similar to M38_CTRL_C57, M38_CDH1_E4 and M38_CDH1_E11-2 presented a strongly reduced growth rate and a halved yield at 4 days after seeding if compared with control cell lines (Mean ± SEM: 2.8x10$^6$ ± 0.13 and 3.4x10$^6$ ± 0.21 respectively). Overall, *in vitro* cells growth was significantly reduced from day 3$^{rd}$ after seeding in cell lines transfected with Cdh1 compared to control groups (Figure 31A).

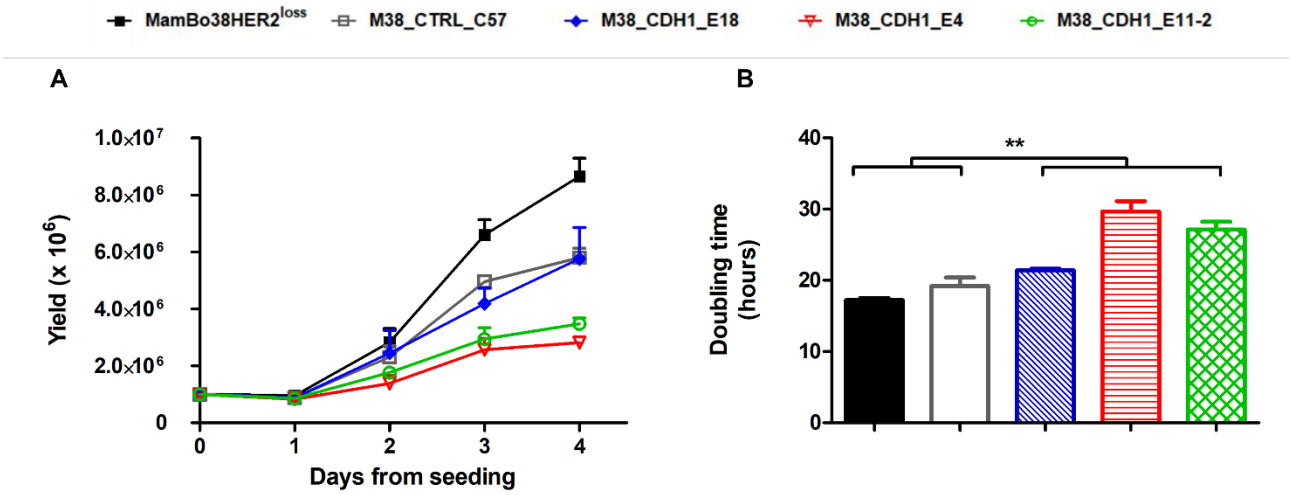


**Figure 31. *In vitro* growth rate of MamBo38HER2$^{loss}$ cell line and clones.** (A) *In vitro* growth curve of CDH1-positive clones (n=2) and control cell lines (n=2). ** p<0.001 control group vs CDH1-transfected cell lines from day 3 after seeding, Student's *t* test. (B) Cell lines doubling time calculated with Doubling Time Computing (Roth V. 2006). ** p<0.001 control group vs CDH1-transfected cell lines, Student's *t* test.

Moreover, cells doubling time was estimated by considering cell yield at 24 and 72 hours after seeding, when cells were in exponential growth phase. Similar to what the growth curves showed, CDH1-positive clones presented a doubling time significantly higher than MamBo38HER2$^{loss}$ and M38_CTRL_C57 cell lines (Figure 31B).

Bromodeoxyuridine (BrdU), a thymidine analog replace, incorporation assay was performed to detect the percentage of cells in active replication phase both in control and transfected cell lines. Indeed, E-cadherin expression induced a slowdown in proliferative capacity of MamBo38HER2$^{loss}$ cell line (p=0.09) (Figure 32A). Cell cycle analysis confirmed the growth rate data (Figure 32B)

These data suggest that E-cadherin might mediate, by contact inhibition, a slowdown of cell growth and proliferation in our model.

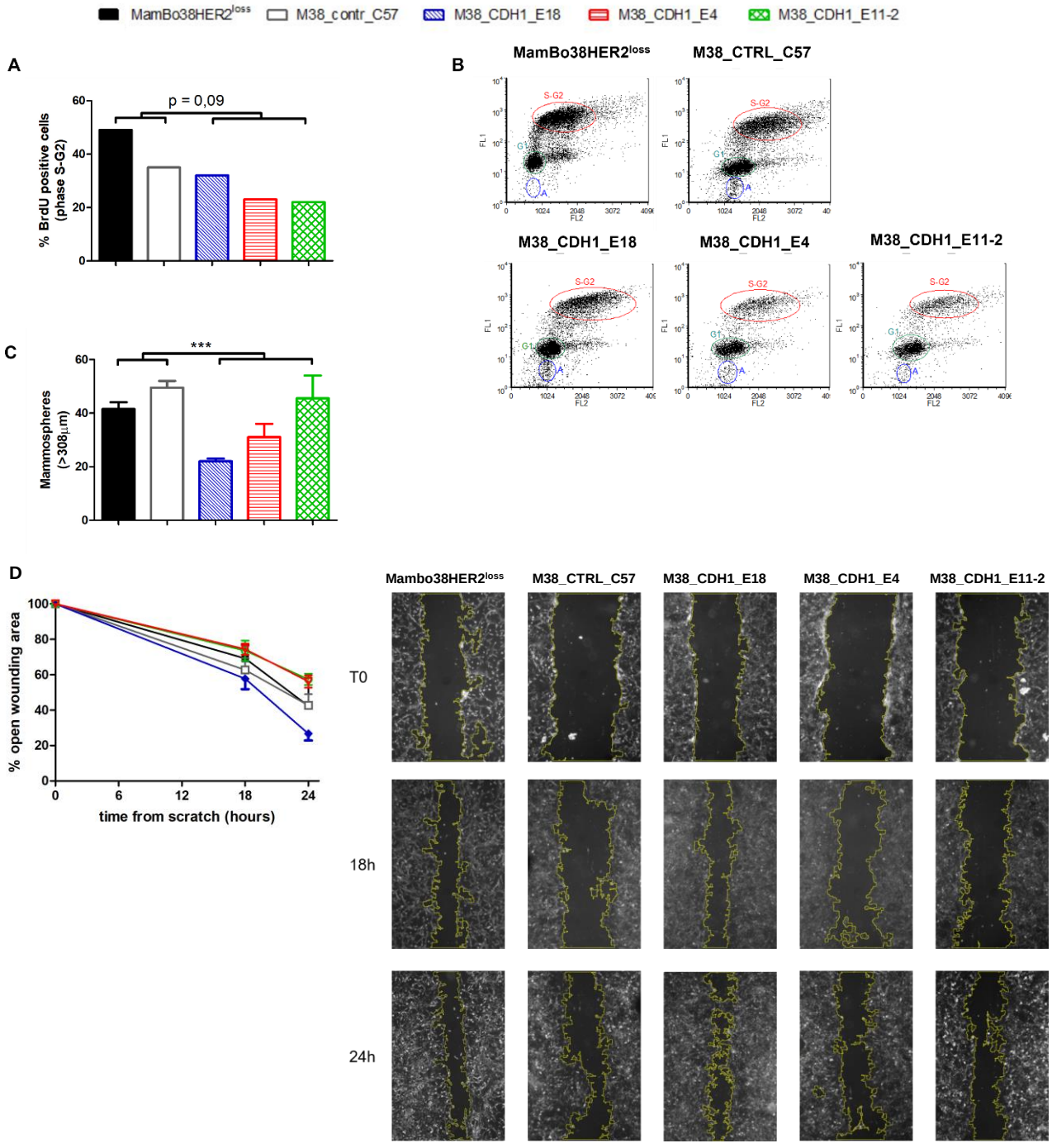

**Figure 32. Cell cycle analysis, mammospheres and migratory capacity of Cdh1-transfected clones**. (A) Percentage of cells in S-G2 phase detected by BrdU incorporation and cytofluorimetric analysis, p=0.09 by Student's *t* test (B) Representative dot plots show the percentage of cells in different cell cycle phases: S-G2, G1 and apoptosis (A) measured by cytofluorimetric analysis. (C) Numbers of mammosphere formed by each cell lines. Histograms reported spheres >309μm. ***p<0.001 by Student's *t* test. (D) Wound healing assay, open wounding area measured at 18 and 24 hours after scratch by ImageJ software. Panels on the right show representative images of the open wound healing areas at different time points for each cell lines.

The migratory ability of Cdh1-positive clones remarked the *in vitro* growth data reported above: M38_CDH1_E4 and M38_CDH1_E11-2 had a slightly reduced migratory capacity than MamBo38HER2$^{loss}$ and M38_CTRL_C57, in contrast M38_CDH1_E18 presented a greater ability to heal the wound than the other cell lines. Thus, E-cadherin expression appeared not to affect significantly the *in vitro* migratory capacity of MamBo38HER2$^{loss}$ cell line (Figure 32D).

The tendency of breast cancer cells to form mammospheres has been linked to stem-like properties and has been shown to be indicative of tumorigenicity in mice. Cdh1-positive cells showed a significantly impaired mammospheres formation ability compared to Cdh1-negative cell lines. Moreover, M38_CDH1_E18, with his polygonal morphology and increased percentage of CD44$^{+}$/CD24$^{+}$ cells, was the cell lines with the lower capacity to form spheres *in vitro* (Figure 32C).

Further confirmation that expression of E-cadherin led to partial reversion of EMT in the HER2 loss cell line was given by the restoration of E-cadherin/β-catenin complex in M38_CDH1_E4, M38_CDH1_E11-2 and M38_CDH1_E18 cells (Figure 33).

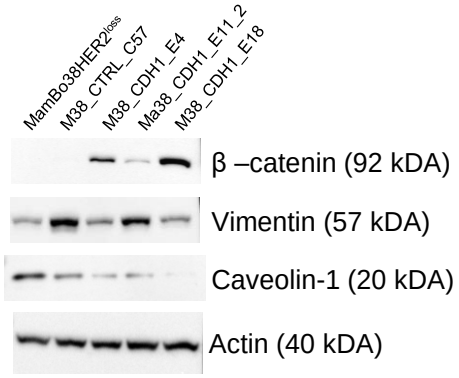


**Figure 33. Western blot analysis of genes related to EMT.** The immunoblot shows protein expression of markers related to EMT in MamBo38HER2$^{loss}$ and transfected clones. Actin was used as loading control.

The restored E-cadherin/β-catenin complex might also explain the reduced aggressiveness of these clones by E-cadherin recruiting of β-catenin from transcriptional complexes involved in cell proliferation.

Of note, is the reduction in Caveolin-1 observed in clones transduced with *Cdh1* (Figure 33*)*. The role of Caveolin-1 in cancer is controversial; however, recent studies have shown how this gene is implicated in various aspects of EMT, stemness and metastasis, thus driving breast cancer progression (Qian et al. 2019). Together with the β-catenin re-uptake mediated by E-cadherin, the Caveolin-1 reduction may have contributed to decrease the MamBo38HER2$^{loss}$ cell line EMT profile and aggressiveness. This evidence, together with its inverse correlation with HER2 expression in this model (Figure 24) suggested that Caveolin-1 could be an intriguing target for the treatment of HER2 loss breast cancer.

### *4.7.4. Tumorigenicity and metastatic ability of MamBo38HER2$^{loss}$ cells transfected with Cdh1*

The expression of E-cadherin has been implicated in tumor metastasis, often as a tumor suppressor, but also as a promoter of growth and metastasis. To shed light on the influence of this protein on MamBo38HER2$^{loss}$ tumorigenicity and metastatic ability, cell lines were injected s.c. and i.v. in immunocompetent or immunodeficient mice.

Cdh1-positive cells presented an increased latency and tumor growth was significantly impaired starting from 11 days after challenge if compared to MamBo38HER2$^{loss}$ and M38_CTRL_C57 control cell lines (Figure 34). The strong tumor growth inhibition has been highlighted both in the presence (Figure 34A) and absence (Figure 34B) of the immune system, thus suggesting that *in vivo* cell growth was not influenced by the immune system.

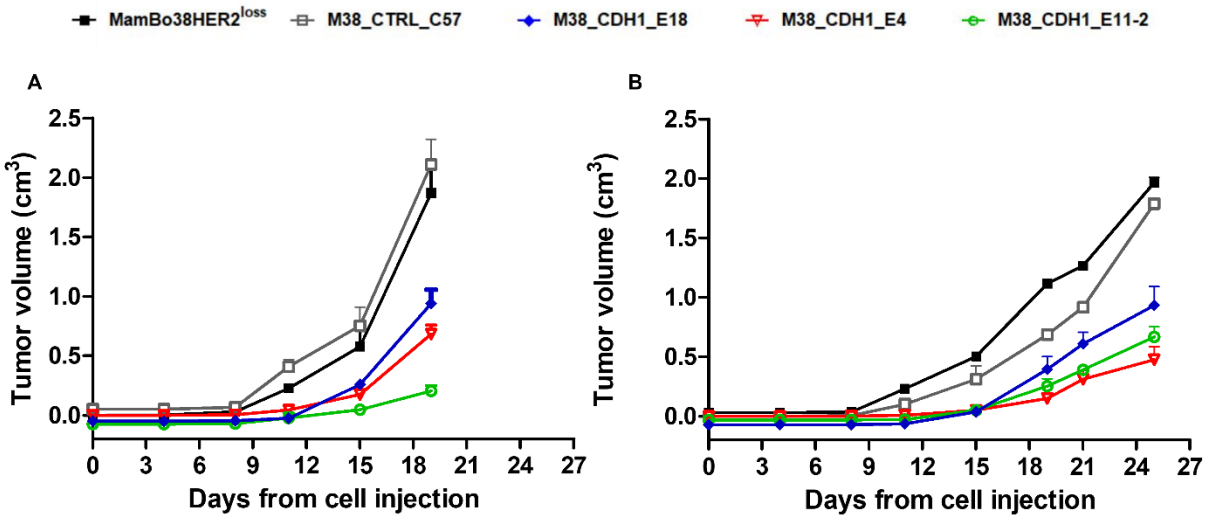


**Figure 34.** ***In vivo*** **growth of MamBo38HER2$^{loss}$ parental cell line and transfected clones.** (A) Tumor growth curve in FVB female mice injected $10^6$ s.c. *** p<0.0001 control group vs CDH1-transfected cell lines from day 11, Student's t test. Mean ± SEM from n=3 mice per group is shown. (B) Tumor growth curve in NSG female mice injected $10^5$ s.c. *** p<0.0001 control group vs Cdh1-positive cell lines from day 11, Student's t test. Mean ± SEM from n=3 mice per group is shown.

To study the E-cadherin influence on MamBo38HER2$^{loss}$ metastatic ability, control cell lines and CDH1-positive clones were injected ($0.25x10^6$) i.v. in NSG female mice. Mice were sacrificed 3 weeks after cell injection when mice injected with parental cell line showed the first metastases signs. The incidence of metastases was 100% in all groups, but the number of lung nodules was significantly impaired in mice challenged with M38_CDH1_E18, M38_CDH1_E4, M38_CDH1_E11-2. Together with the high reduction of the number of lung nodules, a significant decrease in metastasis dimension of Cdh1-expressing clones was observed (Figure 35).

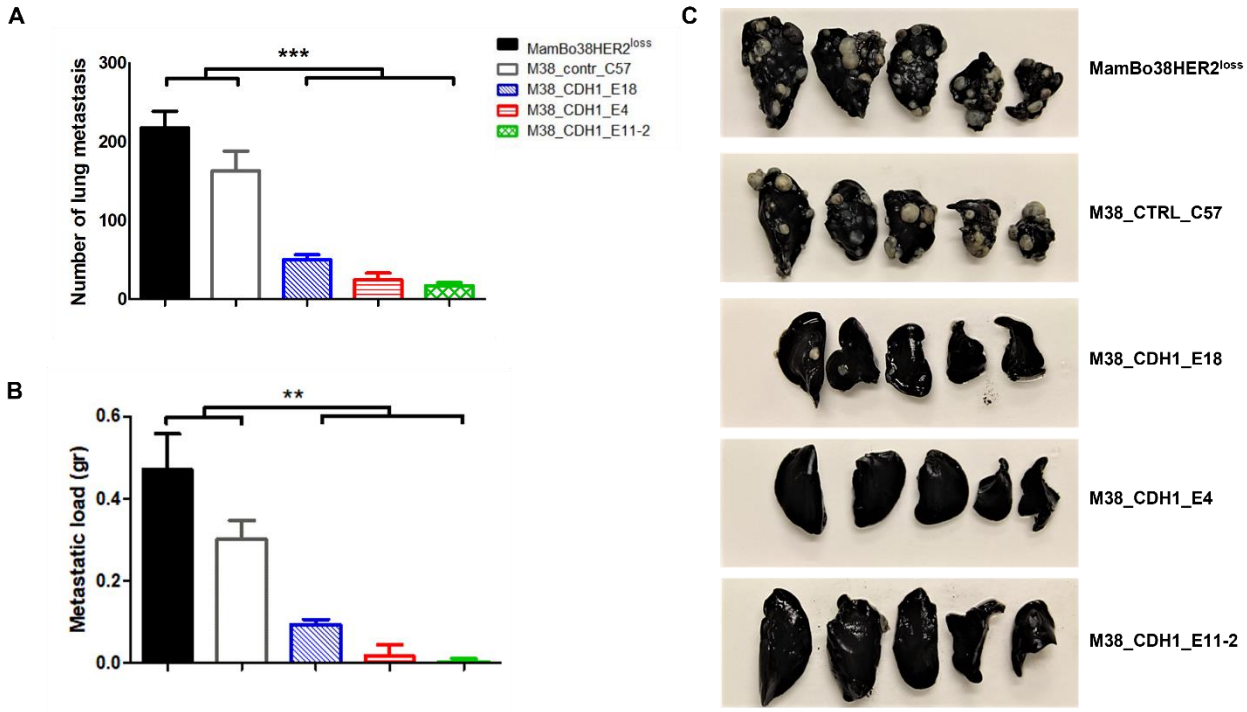


**Figure 35. Metastasis outgrowth of MamBo38HER2$^{loss}$ parental cell line and transfected clones injected i.v. in NSG female mice.** (A) Number of lung nodules counted under a dissection microscope. *** $p<0.001$ control group vs Cdh1-positive cells, by non-parametric Mann-Whitney test. Mean and SEM from n=3 mice per group is shown. (B) Metastatic load estimated by weighting lungs with an analytical balance. The weight of each lung was subtracted from the average weight of lungs from healthy NSG mice to normalize the data. ** $p<0.01$ control group vs Cdh1-positive cell lines, Student's *t* test. Mean and SEM from n=3 mice per group is shown. (C) Lungs perfused with black India ink and fixed in a modified Fekete's solution, a representative lung for each group was shown.

All together *in vivo* results advised that E-cadherin restoration significantly impaired MamBo38HER2$^{loss}$ tumorigenesis and metastatic ability by mediating cell-cell adhesion and contact inhibition and by decreasing extravasation from the circulation and target organ seeding abilities.

# *Discussion*

## 5. ANTI-HER2 CANCER VACCINES

ES2B-C001 vaccine displayed an impressive anti-tumor and anti-metastatic activity *in vivo* against mouse mammary carcinomas expressing human HER2 (Ruzzi et al. 2022). The results of the preclinical study illustrate one of the advantages of active immunotherapy compared with passive one (monoclonal antibodies), the duration of the antibody response. In fact, ES2B-C001 elicited an extremely high anti-HER2 response, larger than one that might be obtained by the administration of monoclonal antibodies (Milani et al. 2013). Anti-HER2 antibody titers remained stable for more than one year after the last vaccination protecting mice from tumor relapse. A persisting anti-tumor immune response would be especially relevant in the therapy of breast cancer, in which tumor and metastasis dormancy entails a risk of relapse, even after one or two decades after the therapy (Veronesi et al. 2002; Negoita and Ramirez-Pena 2022). For instance, patients with ER-positive breast cancer receive routinely administration of ER modulators and/or aromatase inhibitors for 5 to 10 years, to provide a continuing protection from tumor recurrence (Yersal and Barutca 2014).

The long-lasting protection induced by the vaccine was also observed in the prevention of spontaneous mammary carcinoma onset in HER2 transgenic mice. Despite control mice, which developed progressive mammary tumors within 4-8 months of age, only two vaccine administration prevent spontaneous tumor onset in the 90% of transgenic mice for more than one year, as previously demonstrated in this mouse model using another type of vaccine (Nanni et al. 2001; Piechocki et al. 2003).

In perspective, this may translate into a greater long-lasting protection than monoclonal antibody administration and in a consequent economic advantage of the vaccination over monoclonal antibodies treatment (Geynisman et al. 2014). Moreover, the use of vaccines affording long-term protective immune responses might be combined with monoclonal antibody, to prolong their efficacy over the time (Swain et al. 2022).

A further advantage of ES2B-C001 over monoclonal antibodies was the induction of a polyclonal immune response, comprising all IgG isotypes, including IgG2a, IgG2b and IgG3, which were previously found to be involved in the protection of HER2 transgenic mice from tumors (Quaglino et al. 2010; DeGiovanni et al. 2014).

The advantage of a polyclonal response induced by the vaccine was also evaluated by testing anti-HER2 antibody's ability to block the 3D growth of the trastuzumab-resistant BT-474 clone C5 *in vitro*, in the absence of Fc-mediated inhibitory activities. Antibodies induced by the vaccine were also effective in inhibiting 3D colonies formation of human gastric cell lines with different HER2 expression levels. Thus, suggesting that ES2B-C001 might be also used as treatment of HER2-positive gastric tumors, which present high resistance rates to target therapies in clinical practice (Okines and Cunningham 2010; Aprile et al. 2014).

ES2B-C001 showed a remarkable activity when used alone, for example preventing metastasis outgrowth in the lungs of all FVB mice; however, in therapy of local tumor the 30% of FVB mice developed progressive tumors and a few numbers of lung metastases were detected in a minor fraction of Delta16 mice treated with vaccine alone. Thus, suggesting that vaccine efficacy should be enhanced, for example adding an adjuvant. The addition of Montanide ISA 51 significantly boosted the levels of anti-HER2 total Ig and of all Ig isotypes, resulting in an immune response that completely inhibited tumor and metastasis growth both in FVB and in Delta16 mice. According to these results, Montanide, which is already used in clinical practice, could be attractive for the vaccine formulation in clinical administration (Ascarateil et al. 2015).

IFN-gamma ELISpot revealed a small T cell response in splenocyte of mice vaccinated twice and then restimulated *in vitro* with a pool of human HER2 peptides. Moreover, cytokine analysis in immune sera of vaccinated mice did not reveal systemic cytokine response; thus, recommending that vaccine might not induce cytokine storm. The absence of high cytokine levels and the stable mice weight suggest that the vaccine is well tolerated (Ruzzi et al. 2022).

We plan to further investigate T cell response induced by ES2B-C001 vaccine to better define the role and contribution of T cells subpopulations in the anti-tumor responses observed.

Finally, HER2 positive tumors could develop resistance to HER2 target therapy, such as antibodies, through the loss of HER2 expression or the selection of HER2-negative

subpopulation and the activation of alternative pathways (Cardoso et al. 2002). Of note, such phenomena occur also in HER2 transgenic mouse models, which are suitable systems to investigate the onset and evolution of resistance (Giusti et al. 2021; Landuzzi et al. 2021). We did not observe the emergence of resistant tumors in mice repeatedly vaccinated with ES2B-C001+Montanide, not even one year after the last vaccination. The results lead us to conclude that the strong and persistent immune responses elicited by the vaccine could prevent the onset of resistant variants.

Thus, our preclinical results suggest that ES2B-C001 is a promising candidate vaccine for the therapy of HER2-positive human tumors.

# 6. DISCOVERY OF NEW THERAPEUTIC STRATEGIES THROUGH A PRECLINICAL MODEL OF BREAST CANCER PROGRESSION AND HER2 LOSS

One of the mechanisms behind HER2-positive breast cancer progression and therapy resistance is the heterogenicity that characterized this breast cancer subtype. HER2-positive tumors generally show a homogeneous pattern of HER2 amplification. Indeed, from 1% to 40% of patients present tumors with variable level of HER2 expression (Ng et al. 2015) and around the 10% of patients undergoes receptor discordance, presenting HER2-negative metastases (Yeung et al. 2016; Sperduto et al. 2020).

The second aim of this thesis was to discover new therapeutic approaches to overcome tumor progression and treat tumors that lost the oncogene expression. In the meantime, we try to shed light on the breakable balance between subpopulation of the same tumor with different HER2 expression level.

To reach this goal we took advantage from a dynamic preclinical model of HER2 expression obtained in our laboratory. This preclinical model consists of three main cell lines (originally derived, directly or indirectly, from spontaneous mammary tumors arose in human HER2 transgenic mice) and clones derived from them and mimics tumor heterogeneity, as found in clinic and other preclinical models, such as human HER2-positive breast cancer cell lines (Nanni et al. 2000; Szöllösi et al. 1995; Smith et al. 2017) and breast cancer patient-derived xenograft (PDX) collections (Landuzzi et al. 2021). The main players of our model were: MamBo89HER2$^{stable}$ with its high and stable HER2 expression, MamBo43HER2$^{labile}$ that undergoes HER2 loss upon *in vitro* treatment with trastuzumab or *in vivo* tumor growth and the HER2-negative cell line (MamBo38HER2$^{loss}$) that was derived from one of MamBo43HER2$^{labile}$ tumors.

The mechanisms that underlie receptor conversion are mainly unknown due to the limited amount of information available on the patients' molecular profiles and the insufficiency of preclinical models that can reproduce this condition. According to the oncogene subtraction theory, the loss of the oncogene expression is mainly associated with tumor regression or impaired tumorigenicity (Moasser 2007; Weinstein and Joe 2008). On

the contrary, the HER2 loss in our model is associated with spindle-like morphology, a gene expression profile that refers to EMT traits, higher stemness and *in vivo* malignancy.

By observing these cell lines, many questions came to our minds: *what caused the loss of HER2 expression? What might be a good therapeutic target in cells that have lost expression of the HER2 oncogene?*

The HER2 copy number of MamBo38HER2$^{loss}$ cells was similar to that of the HER2-positive MamBo43HER2$^{labile}$ cells, ruling out a total loss of the transgene. However, the level of HER2 transcript was barely detectable in MamBo38HER2$^{loss}$ cells as compared to HER2-positive cells and western blot and cytofluorimetric analyses showed a complete lack of HER2 protein within the HER2-negative cells. Since DNA hyper-methylation could determine gene silencing, MamBo38HER2$^{loss}$ cell line was treated with a DNA methyl-transferase inhibitor but HER2 expression was not induced. We are now studying whether other epigenetic modulators could be effective in unblocking HER2 expression in MamBo38HER2$^{loss}$ cell line.

Several studies traced the origin of this heterogeneity to both clonal expansion and the presence of differentiation hierarchies such as that formed by cancer stem cells and non-stem cells, which are in dynamic equilibrium. These two populations would give rise to cells that are phenotypically and genotypically different since they can accumulate genetic and epigenetic mutations, thus generating great intra-tumor heterogeneity (Neelakantan et al. 2015). MamBo43HER2$^{labile}$ cell line might represent a valid instrument to study tumor heterogenicity by mirroring the HER2 loss or the clonal selection that can occur after target therapy treatments (Janiszewska et al. 2021; Morganti et al. 2022). Indeed, in i*n vitro* treatment with trastuzumab up to 60 days gradually showed a reduction in the HER2-positive population accompanied by an increase in the CD44$^{high}$/CD24$^{low}$ population rising from 1% to 65% within 2 months. However, the loss of HER2 expression was not specifically related to trastuzumab treatment but it was a consequence of any treatment that reduces cell density. In fact, when MamBo43HER2$^{labile}$ cells were seeded at low doses gradually acquired spindle-like morphology, stemness phenotype and HER2 expression was detectable in a tiny portion of cells. In addition, it is interesting to note that these cells do

not recover the initial phenotype either once trastuzumab treatment was suspended or when low-density seeding was stopped and cells were allowed to confluence, but remain negative for HER2, predominantly $CD24^{-}/CD44^{+}$ and with mostly spindle morphology. Thus, the HER2 loss in MamBo43HER2$^{labile}$ cell line appeared to be density-related, nevertheless trastuzumab showed to play a key role in enhancing the spontaneous trend of HER2 expression loss in HER2 labile cell line.

The loss of HER2 observed *in vitro* was probably the result of a selection among several sub-populations coexisting within MamBo43HER2$^{labile}$ cells, in this context HER2 labile cell line were cloned and sub-populations with different HER2 expression, stemness feature and EMT gene profile were obtained, confirming that MamBo43HER2$^{labile}$ cell line is a heterogeneous society. As expected, HER2-negative clones with higher stemness and EMT features showed the greater tumorigenicity and gave rise to HER2-negative tumors. In contrast, epithelial HER2-positive clones had the longer latency and induced HER2-positive tumors. These data suggests that density alterations, which might be induced by *in vivo* cell injection or treatment with trastuzumab, can lead the more aggressive (with EMT profile and high stemness) subpopulations to prevail over the others. Thus, combining anti-HER2 target therapies with therapeutic strategies against EMT or stem cell markers might be an interesting approach to enhance target therapy efficacy and prevent, at the same time, clonal selection and tumor progression.

Although the mechanism behind the HER2 loss is still under investigation, we decided to exploit our model in order to find new targeted therapies for patients with HER2 conversion. The RNA sequencing performed on MamBo89HER2$^{stable}$, MamBo43HER2$^{labile}$ and MamBo38HER2$^{loss}$ confirmed that HER2 loss cells present EMT and stemness gene expression profile that resembles the peculiar traits of Claudin-low tumors. Looking at possible genes and pathways that sustain the aggressive phenotype of HER2-negative cells, *Pdgfr-β*, known to be involved in EMT (Jechlinger et al. 2006; Meng et al. 2015), was found to be up-regulated in MamBo38HER2$^{loss}$ cell line. Sunitinib is a multi-targeted molecule capable of binding both PDGFR-β and VEGFR, another molecule up-regulated in HER2-negative cells (Motzer et al. 2017). Thus, we tried its efficacy against the HER2 loss cell line.

Sunitinib successfully impaired MamBo38HER2$^{loss}$ growth *in vitro* and *in vivo*; moreover, sunitinib-treated cells showed a transient reduction of EMT features and the partial acquisition of epithelial traits (such as *Cdh1* expression) both at morphological and gene expression level. Sunitinib promoted the acquisition of an EMT intermediate state accompanied also by the reduction of IL-6 level of expression. Even if sunitinib did not completely eradicate the growth of MamBo38HER2$^{loss}$ tumor cells, advising that PDGFR-β signaling pathway sustains the growth of HER2-negative cells but is likely not the only driver of their malignancy, these results suggest that the use of sunitinib or similar drugs might be useful in the treatment of patients with HER2 receptor conversion (Giusti et al. 2021). Hence, it might be interesting to combine other targets up-regulated in HER2-negative cell line and involved in EMT, such as IGFBP4 or caveolin-1.

The EMT is closely related to generation of cancer stem cells and therapy resistance in several cancers (Nieto et al. 2016; Shibue and Weinberg 2017). The gene expression profile and the subpopulation that coexist within the HER2 labile cell line highlighted that the EMT plays a key role in sustaining cancer progression in our preclinical model. Moreover, the partial EMT reversion (linked to an increased *Cdh1* expression) mediated by sunitinib in MamBo38HER2$^{loss}$ cells led to a malignancy reduction. Thus, we tried to completely revert the EMT state inducing E-cadherin expression by transfection in MamBo38HER2$^{loss}$ cell line. E-cadherin is an important epithelial marker and its loss in cancer is often associated with cancer progression since the loss of cell-cell adhesion allows cell detachment from the primary tumor, invasion of surrounding tissues, and migration to distant sites (Mendonsa et al. 2018; Bure et al. 2019). E-cadherin loss can be caused by either EMT transcription factor or by mutations or alterations in the *Cdh1* gene and is associated with poor prognosis in several tumors, such as invasive lobular breast cancer, triple negative breast cancer and gastric cancer (Berx and van Roy 2001; Corso et al. 2013).

E-cadherin expression in MamBo38HER2$^{loss}$ cell line induced changes in cell morphology leading the cells to have a more epithelial phenotype and an increased CD44$^{+}$/CD24$^{+}$ population compared to parental and control cell lines which suggest a partial reversion of EMT. The reduction of EMT features was also confirmed by the restoration of

E-cadherin/β-catenin complex, an impaired caveolin-1 and vimentin expression. Cdh1-positive clones showed significantly reduced growth *in vitro* associated with a slowdown in proliferative capacity. Therefore, promising results were obtained by *in vivo* studies where E-cadherin restoration significantly impaired MamBo38HER2$^{loss}$ tumorigenesis and metastatic ability.

The role of E-cadherin as tumor suppressor gene is already know and widely demonstrated by several preclinical models (Navarro et al. 1991; Onder et al. 2008; Corso et al. 2020). Together with E-cadherin, the restored β-catenin re-uptake and the downregulation of caveolin-1 may have contributed to the decreased EMT profile and aggressiveness of the MamBo38HER2$^{loss}$ cell line.

These data advise that therapeutic strategies aimed to enhance E-cadherin expression and adhesion, such as novel engineering antibodies (Xie et al. 2022), or to inhibit E-cadherin suppression genes (e.g. Zeb2, Snail, Igfbp4) might reduce tumor progression and metastases.

Combining therapies targeting molecules involved in EMT or antigen expressed by breast cancer stem cells with anti-HER2 target therapies are promising strategies to inhibit HER2-positive cells and simultaneously prevent selection of more aggressive clone. Due to their tolerability, polyclonal and long-lasting response, VLP-based cancer vaccines might represent an intriguing approach to enhance standalone or combined therapy efficacy.

# *Conclusions*

Despite all the advances of the last decades in the treatment of HER2-positive breast cancers, we have probably just scratched the surface. Additional efforts are needed to improve the survival rate of patients who show resistance, primary or acquired, to currently available therapies. To archive this goal, it is crucially important to look at new therapeutic approaches and/or targets to counterattack resistant cancer cells. The availability of more preclinical models that can reproduce tumor progression and heterogeneity is certainly an important weapon in achieving this milestone.

The results reported in this thesis introduce a VLP-based vaccine against HER2 as a new therapeutic approach to treat HER2-positive tumors. Vaccine showed a strong antitumoral activity against both local tumor and metastasis. Furthermore, we did not observe the onset and evolution of resistance, thus suggesting that the strong and persistent immune responses elicited by the vaccine might prevent the onset of resistant variants.

A dynamic model of HER2 expression obtained in our laboratory was exploited to find new promising targets. In this model, HER2 loss and cancer progression were strictly associated with the acquisition of epithelial to mesenchymal and stemness features suggesting us that targeting EMT-involved molecules, such as PBGFR-β or E-cadherin suppressor genes to restore the epithelial marker expression, could be an effective strategy to impair HER2-negative tumor growth.

$HER2^{labile}$ cell line is a powerful tool which mimics the unstable balance that governs the coexistence of different subpopulations within heterogeneous HER2-positive tumors. Density alterations, which might be induced by anti-HER2 target therapies can lead the more aggressive subpopulations to prevail over the others. Often these subpopulations have shown an increased EMT and stemness profile, thus targeting either EMT-involved molecules and/or antigen expressed by breast cancer stem cells with anti-HER2 target therapies are promising strategies to inhibit HER2-positive cells and simultaneously prevent selection of more aggressive clone.

# *Material and Methods*

# 7. IMMUNOLOGICAL STRATEGIES

Material and methods reported in this section were included Ruzzi et al 2022.

## 7.1. Mice

FVB female mice (6–8 weeks old) were purchased from Charles River (Calco, Lecco, Italy).

Delta16 mice (FVB background) harbor Δ16 transgene, an activated isoform of HER2 derived from the skipping of exon 16 which caused an in-frame loss of 16 amino acids in the ECD (Marchini et al. 2011; Castagnoli et al. 2019). Delta16 mice expressed human Δ16 transgene in the mammary glands under the transcriptional control of mouse mammary tumor virus long terminal repeats, leading to the development of mammary carcinomas (Marchini et al. 2011). Delta16 mice were kindly gifted by Dr. A. Amici (University of Camerino, Camerino, Italy) and Dr. S. Pupa (Fondazione IRCCS, Istituto Nazionale dei Tumori, Milano, Italy) and bred in our animal facilities by crossing FVB female mice and heterozygotic Delta16 male mice. Transgene-bearing mice were selected by polymerase chain reaction (PCR). Genomic DNA was isolated by digestion of ear tissue (1–2 mm) and extracted. To detect Δ16 transgene, two sets of primers were used: forward GGT CTG GAC GTC CCA GTC TGA and reverse GAT AGA ATG GCG CCG GGC CTT (Invitrogen, Milan, Italy). Gene expression was evaluated by PCR using Platinum TAQ Polymerase Reactions Kit (Thermo Fisher Scientific, Monza, Italy) and CFX96 Thermal Cycler (Bio-Rad Laboratories, CA, USA). Electrophoresis on a 2% agarose gel was performed to detect gene bands in the amplified DNA.

All mice were monitored daily and weighed twice weekly. All *in vivo* experiments were performed according to Italian and European laws and were authorized by the Italian Ministry of Health (letter 714-2017).

## 7.2. Formulation of ES2B-C001 vaccine

Design, expression and purification of Catcher-HER2-antigen and Tag-VLP was performed by Expres²ion Biotechnologies.

The Tag-VLP and the Catcher-HER2 were mixed in a 2:1 molar ratio (AP205 subunit per antigen) in PBS containing 10 mM Tris and 200 mM sucrose for 20 h at room temperature, resulting in covalent linkage of the HER-2 antigen to the VLP via the Catcher-Tag system. Conjugated HER2-VLP was then purified and unbound HER2 antigen was removed. To determine coupling efficiency, calculated as percentage conjugation (number of bound antigens divided by total available binding sites (=180) per VLP), densitometric analysis of SDS-PAGE gels were performed.

### 7.3. Vaccination

ES2B-C001 cVLP vaccine was formulated with Montanide ISA 51 (referred as Montanide) (Seppic, Courbevoie, France) or with PBS (Thermo Fisher Scientific) in a 50/50 volume ratio; control groups received PBS alone. Montanide was emulsified with ES2B-C001 according to the manufacturer's protocol.

The standard dose of vaccine was 10 μg per mouse per administration; in the prevention experiment on Delta16 mice graded doses ranging 5–40 μg were used. Vaccinations were administered i.m. in the hind left leg every two weeks. The total number of vaccinations is detailed in the Results section for each experiment.

### 7.4. Cell lines

D16-BO-QD cell line (QD for short) was established in our laboratory from a transgenic HER2-positive mammary carcinoma of a Delta16 female mouse. QD cell line was cultured in MammoCult medium (STEMCELL Technologies, Vancouver, Canada) supplemented with 1% fetal bovine serum (FBS), 100 U/mL penicillin and 10 _g/mL streptomycin (all from Thermo Fisher Scientific).

Human HER2-positive breast cancer cell line BT-474 (+++) and its trastuzumab-resistant clone C5 were routinely cultured in RPMI (Thermo Fisher Scientific) supplemented with 10% FBS, 100 U/mL penicillin and 10 μg/mL streptomycin. BT-474 cell line (from ATCC) was kindly provided by Dr. S. Pupa (Istituto Nazionale dei Tumori, Milan, Italy); BT-474 C5 (from ATCC) was kindly given by Prof. Adam Sander (University of Copenhagen, Denmark).

Human HER2-positive gastric cancer cell lines KATO III (from ATCC) and NCI-N87 (from ATCC, kindly given by Dr P. Ulivi and Dr M. Canale from Istituto Tumori della Romagna, Meldola, Italy) were cultured in RPMI supplemented with 10% or 20% FBS respectively.

Cells were cultured at 37 °C in a humidified 5% $CO_2$ atmosphere and were split once or twice a week according to density using 0.05% trypsin-EDTA (Thermo Fisher Scientific).

### 7.5. Local tumor and metastasis therapy models

In the local tumor therapy model, 7-week-old virgin FVB female mice were challenged with $10^6$ QD cells in the m.f.p of the fourth mammary area. Vaccine with or without adjuvant was administered starting two weeks after cell injection. Tumor dimensions were measured with calipers twice a week; tumor volume was calculated as $\pi/6*(\sqrt{ab})^3$ where a=maximal tumor diameter and b=maximal tumor diameter perpendicular to a. Mice were euthanized if they showed any sign of distress or if tumor volume exceeded 2.5 $cm^3$.

In the metastasis therapy model, 9-week-old FVB female mice were challenged i.v. with $10^6$ QD cells in a lateral tail vein, while Delta16 female mice (6–8 weeks old) received $0.25x10^6$ QD cells i.v.. Mice were euthanized 11–13 weeks after cell injection, an accurate necropsy was performed, lungs were perfused with black India ink and fixed in a modified Fekete's solution. Metastases were counted under a dissection microscope. Serum samples from vaccinated and control mice were collected periodically and stored at -80 °C.

### 7.6. Indirect immunofluorescence and flow cytometry

To evaluate the HER2 cell lines expression level, all the cell lines were incubated with mouse anti-human HER2 monoclonal antibody MGR2 (Enzo Life Sciences, Farmingdale, NY, USA), then with an anti-mouse IgG labeled with Alexa Fluor 488 (Thermo Fisher Scientific). For the relative quantitative evaluation of anti-HER2 antibodies elicited by vaccination in the differently treated mice groups, mouse sera were diluted 1:65 and applied to stain a standard human HER2-positive cell line (BT-474) followed by addition of anti-mouse IgG-AF488; a sample incubated with the anti-HER2 monoclonal antibody MGR2 was included in each session to normalize variations in HER2 expression of BT-474 cells.

Cytofluorimetric analysis was performed using CyFlow Space (Sysmex Partec, Germany) and data were analyzed with FCS Express 7 (De Novo Software, Los Angeles, CA, USA)

### 7.7. Enzyme-Linked Immunosorbent Assay (ELISA)

Serum anti-HER2 IgG were quantitatively measured in a specific ELISA.

Immunoplate Nunc Maxisorp 96-well microplates (Merck, Darmstadt, Germany) were coated overnight with the HER2 ECD (amino acids 22–652; ExpreS$^2$ion Biotechnologies) at 1 μg/mL in Carbonate Bicarbonate buffer (Merck). A standard curve (0.04 to 30 ng/mL) with mouse monoclonal antibody H2M5B against human HER-2 (IgG1, R&D Systems, Minneapolis, MN, USA) was run in parallel. The following horseradish peroxidase (HRP)-labeled goat anti-mouse Ig antibodies, all from Thermo Fisher Scientific, were used for detection: total Ig (1:30000 dilution), IgM (1:10000 dilution), IgG1 (1:10000 dilution), IgG2a (1:5000 dilution), IgG2b (1:10000 dilution), IgG3 (1:5000 dilution).

### 7.8. Enzyme-Linked Immunospot (ELISpot)

FVB female mice (n = 4) received two administrations of ES2B-C001+Montanide. The vaccine emulsion was administered bi-weekly at the dose of 10 μg/mouse, and spleens were resected 13 days after the last immunization. Positive control mice (n = 2) received three daily i.p. administrations of recombinant mouse IL-12 (provided by S. Wolf, Genetics Institute, Andover, MA). IL-12 was administered as previously reported (Nanni et al. 2001). Spleens were collected one hour after last IL-12 injection. Control mice (n = 2) were untreated. Red blood cells were removed using Red Blood Cell Lysis Solution (Miltenyi Biotec, Bergisch Gladbach, Germany). A total of $0.2x10^6$ splenocytes/well were seeded and stimulated with HER-2 peptide pool (PepMix, JPT Peptide Technologies GmbH, Berlin, Germany) or left untreated for 48h. HER2 peptide pool was solved in dimethyl sulfoxide (DMSO, Merck) and an *in vitro* splenocytes restimulation with DMSO was performed as control (referred to as vehicle). ELISpot Mouse IFN- kit (R&D systems) was used to perform the analysis and performed according to the manufacturer's protocol. Spots were counted under a dissection microscope.

### 7.9. Agar colony growth inhibition

*In vitro* sensitivity of human breast cancer BT-474 (trastuzumab-sensitive) and BT-474 C5 (trastuzumab-resistant) cells and human gastric cancer KATO III and NCI-N87 cells to HER2-VLP-induced antibodies was evaluated in three-dimensional cultures. Cells were seeded at 500 cells/well in 24-well plates in RPMI supplemented with 10% FBS + 0.33% agar (Lonza Bioscience Solutions, Siena, Italy) (BT-474, BT-474 C5 and KATO III) or 10000 cells/well (NCI-N87) in 24-well plates in MammoCult supplemented with 1% FBS + 0.33% agar with mouse sera diluted 1:100 or a pharmacologically relevant concentration of trastuzumab (18 μg/mL, kindly provided by Genentech).

Colonies (diameter > 90 μm) were counted under an inverted microscope in dark-field 18–30 days after seeding. Photos were taken with a Canon EOS 600D.

# 8. DISCOVERY OF NEW THERAPEUTIC STRATEGIES THROUGH A PRECLINICAL MODEL OF BREAST CANCER PROGRESSION AND HER2 LOSS

Some material and methods reported in this section were included in Giusti et al. 2021.

## 8.1. Mice

FVB female mice were purchased from Charles River (Calco, Lecco, Italy).

FVBhHER2 mice, transgenic for the human HER2 full-length, were obtained from Genetech (South San Francisco, CA, USA) and bred in our animal facilities.

NSG female mice (breeders received from Charles River) were bred and kept under sterile conditions in our animal facility and used as immunodeficient models since lacked B, T and NK immune components.

Experiments were approved by the institutional review board of the University of Bologna, authorized by the Italian Ministry of Health and done according to Italian and European laws and guidelines (letters 688/2015 and 502/2016).

## 8.2. Cell lines

MamBo89HER2$^{stable}$ and MamBo43HER2$^{labile}$ cell lines were established from mammary tumors arose in two human HER2 transgenic mice. MamBo38HER2$^{loss}$ was obtained from a tumor derived from MamBo43HER2$^{labile}$ *in vivo* cell injection. MamBo43HER2$^{labile}$ clones were derived by low density seeding in adherence (AD clones) or 3D (soft agar) conditions (AG clones). MamBo cell lines and clones were stabilized and cultured in DMEM (Thermo Fisher Scientific, Milan) that was supplemented with 20% FBS, 30 μg/ml bovine pituitary extract (Corning) and 0.5% v/v MITO Serum Extender (Corning).

All cell lines and clones were maintained at 37°C in a humidified 5% CO2 atmosphere and were split once or twice a week according to density using 0.05% trypsin EDTA (Thermo Fisher Scientific).

To induce *Cdh1* (encoding for mouse E-cadherin) gene expression, the MamBo38HER2$^{loss}$ cell line was transfected by lentiviral particles (Lenti-ORF particles, pLenti-C-Myc-DDK-P2A-Puro, OriGene Technologies, Rockville, MD, USA). The

multiplicity of infection (MOI) ratio chosen for cell infection was 15. To enhance transfection efficacy, polybrene (hexadimethrine bromide, Sigma Aldrich, Saint Louis, Missouri, USA) was added to the culture medium at the final concentration of 8 mg/mL. Antibiotic selection consisting of puromycin (Thermo Fisher Scientific, Waltham, MA, USA) at a concentration of 2.5 μg/μl was added to the culture medium after transfection. A transfection with a control vector was run in parallel. Transfection bulks were cloned by low density seeding in adherence.

### 8.3. *In vivo* studies

#### *8.3.1. Tumorigenicity and metastatic ability of MamBo cell lines*

To evaluate the tumorigenicity of the MamBo89HER2$^{stable}$, MamBo43HER2$^{labile}$ and MamBo38HER2$^{loss}$ cell lines, FVBhHER2 virgin (12–20-week-old) female mice received the injection of $10^6$ cells into the m.f.p.. Tumor volume was calculated as previously described. Mice were sacrificed before tumors reached 2.5 $cm^3$ or 10% of mouse weight.

Experimental metastatic potential was assessed in FVBhuHER2 virgin female mice (8–17-week-old) via the injection of $10^5$ cells into a caudal vein (n = 5). The general health status of the mice was checked weekly and the mice were either euthanized at any sign of lung metastasis, or 18 weeks after cell injection. Lungs were stained with ink, fixed in Fekete's solution and metastases were counted under a stereoscope.

#### *8.3.2. Tumorigenicity and spontaneous metastatic ability of MamBo43HER2$^{labile}$ clones*

Tumorigenicity and spontaneous metastatic ability of MamBo43HER2$^{labile}$ clones was evaluated and compared to MamBo43HER2$^{labile}$ and MamBo38HER2$^{loss}$ ones via s.c. cell injection in the hind leg of $10^5$ cells in NSG virgin female mice (n=3).

Tumor volume was calculated as previously described. Mice were sacrificed before tumors reached 2.5 $cm^3$ or 10% of mouse weight. HER2 expression and stemness markers were analyzed by cytofluorimetric analysis on tumors. Lungs were stained with ink, fixed in Fekete's solution and metastases were counted under a stereoscope.

### *8.3.3. MamBo38HER2$^{loss}$ tumor growth inhibition by sunitinib*

FVB female mice that harbored tumors that were induced by the s.c. injection of MamBo38HER2$^{loss}$ cells (0.25x10$^5$ cells) were treated daily with sunitinib 60 mg/kg per os by gavage starting from 1 day after cell injection (n=5). Animals in the vehicle group received Methylcellulose 0.5%+Tween80 0.4% (n = 5).

### *8.3.4. Tumorigenicity and metastatic ability of Cdh1-transfected MamBo38HER2$^{loss}$ cells*

To evaluate the difference in tumorigenicity of MamBo38HER2$^{loss}$, M38_CTRL_C57, M38_CDH1_E4, M38_CDH1_E11-2, M38_CDH1_E18 cells, NSG and FVB female mice received 10$^5$ and 10$^6$ cells s.c. respectively in the hind leg. Tumor volume was measured with a caliper and calculated as $\pi/12*(\sqrt{ab})^3$. Mice were sacrificed before tumors reached 2.5 cm$^3$ or 10% of mouse weight.

The metastatic ability was evaluated in NSG virgin female mice via i.v. injection in a lateral tail vein of MamBo38HER2$^{loss}$ parental cell line and transfection-derived clones at the dose of 0.25x10$^5$ cells. The general health status of the mice was checked weekly and the mice were either euthanized 3 weeks after cell injection. Lungs were stained with ink, fixed in Fekete's solution and metastases were counted under a stereoscope.

## 8.4. *In vitro* assay

### *8.4.1. Sensitivity to demethylating agent*

MamBo38HER2$^{loss}$ cells were seeded at 0.8x10$^5$ cells/cm$^2$. 24 hours after seeding, cells were treated with vehicle (DMSO 0.02%), 5' aza-2-deoxycytidine (Sigma Aldrich, Saint Louis, Missouri, USA) 0.5 μM or 5 μM. Treatment was renewed after 72 hours of treatment. Cells were harvested after 48, 72 and 144 hours from the first treatment and counted. HER2 expression was determined by cytofluorimetric analysis.

### *8.4.2. Induction of HER2 loss in vitro*

MamBo43HER2$^{labile}$ cells were kept in culture 120 h with trastuzumab (1 μM, 10 μM and 100 μM) (Herceptin, Roche) to evaluate the short-term growth inhibition and the HER2 expression level.

MamBo43HER2$^{labile}$ cells were cultured up to 60 days in the presence of 30 μg/ml trastuzumab. Cells were counted weekly and seeded at a concentration of either $1.6x10^5$ cells/cm$^2$ (high density) or $0.4x10^5$ cells/cm$^2$ (low density). In parallel, cells were harvested for molecular analysis, cytofluorimetric analysis of HER2 and stemness marker expression.

A low-density culture, without trastuzumab, was performed by seeding MamBo43HER2$^{labile}$ cells at $10^4$ cells/cm$^2$. Cells were harvested for HER2 and stemness marker expression in cytofluorimetric analysis.

### *8.4.3. Sensitivity to sunitinib in 2D adherent and 3D soft agar condition*

For the experiment under 2D adherent conditions MamBo38HER2$^{loss}$ cells were plated at $3.125x10^3$ cells/cm$^2$ in 96-well plates (Corning Life Sciences, USA). After 24 hours from seeding, cells were treated with sunitinib (sunitinib malate, LC Laboratories, MA, USA) 0.1, 0.5, 1, 5 and 10 μM (LC Laboratories, MA, USA), or DMSO 0.2% (Sigma) or fresh medium. Each treatment was evaluated in triplicate. After 72 hours of treatment, sensitivity to sunitinib was determined using the WST-1 reagent (Merck), according to the manufacturer's instructions.

For the experiment under 3D non-adherent conditions, MamBo38HER2$^{loss}$ cells ($0.5x10^3$ cells/cm$^2$) were seeded in a solution of agarose 0.33% (Sea Plaque Agarose, Lonza, Siena, Italy) in medium containing DMSO 0.02% (Sigma) or sunitinib 1 or 5 μM (LC Laboratories, MA, USA) or no drug. Colonies were counted after 14-22 days.

### *8.4.4. Mammosphere formation assay*

The ability of MamBo89HER2$^{stable}$, MamBo43HER2$^{labile}$, MamBo38HER2$^{loss}$ cell lines to form mammospheres *in vitro* was assessed seeding $4 \times 10^4$ cells in 4 ml complete MammoCult medium without serum in 6 well UltraLow Adherence plates (Corning). Mammospheres that were bigger than 60 μm were counted on day 7 (n=4).

The same protocol was used to evaluate the ability of MamBo38HER2$^{loss}$ to form mammospheres in a medium supplied with sunitinib 5 μM (untreated n=2; vehicle and sunitinib n = 4).

Mammospheres formation ability of Cdh1-transfected MamBo38HER2$^{loss}$ was evaluated by employing the same protocol (n=2).

### *8.4.5. Wound healing assay*

The migratory ability of MamBo38HER2$^{loss}$ cells in the presence of sunitinib was evaluated in a wound healing test. Cells were seeded in 24-well plates (Corning) in complete medium The cell monolayer was scratched when cells reached confluence with a pipette tip 200 μl, the medium was changed with or without sunitinib, 5 μM, and wound width was measured at times 0 and 24 h after scratching (untreated n = 8, vehicle n = 6, sunitinib 5 μM n = 8).

To investigate how Cdh1 expression influenced MamBo38HER2$^{loss}$ transfected cells, control and Cdh1-positive clones were seeded at 5000 cells/cm$^2$. When cells reached 80% confluence, the scratch was performed and wound width was measured at times 0, 18 and 24 h after scratching.

Wound width was analyzed using ImageJ software and migratory ability was calculated as width(24 h)/width(t0)x100.

### *8.4.6. mIL-6 production*

Cells were seeded 8x10$^4$ cells/cm$^2$ in medium containing either sunitinib 5 μM (LC Laboratories, MA, USA) or DMSO 0.05% (Sigma) or trastuzumab 30 μg/ml or no drug. After 4 days, supernates were collected and centrifuged for 20 min at 2000 RCF and 4°C, then filtered through 45 μm filters (Millipore, Burlington, Massachusetts, USA). Supernates were stored at -20°C. mIL-6 production was analyzed with Mouse IL-6 Quantikine ELISA Kit

(R&D Systems) according to manufacturer's protocol. Concentration of each sample was calculated interpolating values on a standard curve.

### 8.5. Immunofluorescence and cytofluorimetric analysis

Harvested cells and tumor samples, which had previously been dissociated to yield single-cell suspensions, were analysed by immunofluorescence and cytofluorimetric analysis. The antibodies used for indirect immunofluorescence included: rat anti-mouse CD16-CD32 antibody Fc block (clone 2.4G2; diluted 1:100 dilution; BD, Pharmingen, CA, USA); mouse anti-human HER2 (MGR2, diluted 1:100, Alexis, and also kindly provided by Dr. Elda Tagliabue; IRCCS, Istituto Nazionale dei Tumori, Milan, Italy); rat anti-mouse E-cadherin (clone DECMA-1, diluted 1:50, NeoMarkers, New Jersey, USA); Anti-mouse IgG AF488 (diluted 1:100; Thermo Fisher Scientific) and anti-rat IgG FITC (diluted 1:40; KPL) were used as secondary antibodies. Antibodies used for direct immunofluorescence: anti-mouse CD24AF488 (clone M1/69; diluted 1:10; Biolegend); anti-mouse-CD44PE (clone IM7; diluted 1:10, Biolegend); anti-mouse Sca1PE (clone E13-161.7, 1:100 dilution; Biolegend); and anti-mouse CD29PE (clone HMβ1-1; diluted 1:10; Biolegend).

5′-bromo-2′-deoxyuridine (BrdU) (Sigma Aldrich) 1mM was incorporated for 1 h by cells to perform a cell cycle assay and detect DNA synthesis. Cells were harvested, fixed and permeabilized and the DNA was denatured with 2N HCl for 30 min at room temperature. Cells were incubated with mouse anti-BrdU (diluted 1:4, clone B44, Becton Dickinson, CA, USA) and with anti-mouse IgG AF488 as secondary antibody.

Data were acquired using CyFlow Space, (Sysmex Partec) and analyzed using FCS Express 4 (De Novo Software, Glendale, CA, USA).

Immunofluorescence on adherent cells was performed on MamBo89HER2$^{stable}$, MamBo43HER2$^{labile}$ and MamBo38HER2$^{loss}$. Cells were seeded at 0.4x10$^5$ cells/cm$^2$ and fixed with a acetone-methanol solution. Primary antibody rat anti-vimentin (clone D21H3, diluted 1:1000, Cell Signaling) was incubated overnight at 4°C, then anti-rat IgG AF488 (diluted 1:500, Thermo Fisher Scientific) as secondary antibody was incubated 1 h at room temperature. Samples were observed with a fluorescence microscope.

### 8.6. Molecular analysis

#### *8.6.1. Real-Time PCR (RT-PCR)*

RNA was extracted from cellular pellets or tumor samples according to the TRIzol protocol (Total RNA Isolation Reagent; Life Technologies, Milan). Frozen tissue samples were mechanically dissociated by gentleMACS Octo Dissociator (Milteny Biotech GmbH, Bergisch Gladbach, Germany).

RNA was quantified by Qubit Assay Kit (Life Technologies). 1 μg of RNA was reverse transcribed with iScript cDNA Synthesis Kit (Bio-Rad Laboratories, CA, USA). 10 ng of cDNA were analyzed for each sample. cDNA was amplified using Sso Advanced SyBR Green Supemix or SsoAdvanced Universal Probes Supermix (Bio-Rad Laboratories, CA, USA) reagents. Reactions were performed by Thermal Cycler CFX96 (Bio-Rad Laboratories, CA, USA). Data were analyzed using Bio-Rad CFX Manager 3.1 Software and relative quantification was calculated as $\Delta Ct = Ct_{gene} - Ct_{housekeeping}$. TATA Box Binding Protein (TBP) was used as housekeeping gene.

We used the following Bio-Rad assays: Cdh1 (qMmuCID0006332); Col3a1 (qMmuCID0006332); Col5a2 (qMmuCID0011413); Dsp (qMmuCID0019458); Fgfbp1 (qMmuCID0007813); Igfbp4 (qMmuCID0006155); Il1rn (qMmuCID0009153); Mmp2 (qMmuCID00021124); Ocln (qMmuCID0005446); Pdgfrb (qMmuCID0025167); Sparc (qMmuCID0023536); Vcan (qMmuCID0005235); Snai1 (qMmuCID0024342); Zeb1 (qMmuCID0009095); Zeb2 (qMmuCED0046769); Twist1 (qMmuCED0004065); IL6 (qMmuCID0005613); Tbp (qMmuCID0040542).

DNA was extracted using a PureLinkTM Genomic DNA Mini kit (Thermo Fisher Scientific), according to the manufacturer's protocol. HER2 copy number was detected by Real-Time PCR using a HER2 primer qHsaCEP0052301 assay (Bio-Rad Laboratories) and was normalized over human/mouse Ptger2. Amplification was performed using Sso Advanced Universal Probes Supermix (Bio-Rad Laboratories). The copy number of the human and murine cell lines was inferred by considering that MCF7 and MDA-MB231 harbor 2 copies of HER2 in the genome.

### *8.6.2. RNA sequencing of MamBo cell lines*

MamBo cell lines were sequenced in collaboration with Prof. R. Calogero and coworkers (University of Turin, Italy).

Total RNA was extracted from cell pellets using Trizol Reagent (Thermo Fisher), according to the manufacturer's instructions. RNAseq libraries were generated using TruSeq RNA Sample Prep Kit v2 (Illumina), according to the manufacturer's recommendations. High-throughput sequencing was carried out on a NextSeq 500 (Illumina) using 75 nucleotides, in single-end mode. Reads were analyzed on a SeqBox (Beccuti et al. 2018). The generation of Demultiplexing (bcl2fastq Illumina tool version 2.17.1.14-2) counts using STAR (version 2.5) /RSEM (version 1.3.0), and differential gene expression analysis using DESeq2 (version 1.14.1, adjusted P-value < 0.1 and |log2 fold change| ≥1) were all performed within the SeqBox framework. Two distinct analyses were performed on differentially expressed genes. Functional enrichment analysis was performed using the EnrichR web tool (https://maayanlab.cloud/Enrichr/).

In the HER2-positive vs HER2-negative comparison, we also included samples from MamBo89HER2$^{stable}$ cells and MamBo38HER2$^{loss}$ cells that were treated long-term with trastuzumab for either 30 or 60 days, both maintaining the initial shape, HER2 expression level and stemness profile of correspondent untreated cells. In detail, the HER2-positive cell samples included MamBo89HER2$^{stable}$ parental cells, trastuzumab-treated and a HER2-positive clone plus MamBo43HER2$^{labile}$ cells. HER2-negative samples included MamBo38HER2$^{loss}$ parental cells and trastuzumab-treated cells plus trastuzumab-treated (30 and 60 days), MamBo43HER2$^{labile}$ cells having lost HER2 expression. The comparison of HER2 stable vs HER2 labile cells only included MamBo89HER2$^{stable}$ parental cell line and its HER2-positive clone, and MamBo43HER2$^{labile}$ samples.

Bubble charts were carried out using Plotly –Chart Studio (Plotly Technologies Inc. Collaborative data science. Montréal, QC, 2015. https://plot.ly), Biological processes were retrieved from Wiki Pathway database (ver. October 2020), KEGG pathways were retrieved

from KEGG (ver. October 2020) database, both from Enrichr web interface (Enrichr - https://maayanlab.cloud/Enrichr/).

PCA was carried out using R Studio (ver. 1.3.1093) on RNAseq normalized data, using the R function *prcomp()* that computes a singular value decomposition of the centered and scaled data matrix.

### 8.7. Western Blotting

Proteins were extracted from cellular pellets or frozen tissue samples, which were mechanically dissociated by gentleMACS Octo Dissociator (Milteny Biotech GmbH, Bergisch Gladbach, Germany). Extraction was performed in PhosphoSafe Extraction Reagent (Novagen) supplmented with protease inhibitors (Protease Inhibitor Cocktail, Sigma 100x). After 10 min incubation, suspensions were centrifuged (12800xg, 15 min, 4°C) and proteins harvested at -80°C for further analysis.

Protein concentration was quantified using DC™ Protein Assay (Bio-Rad Laboratories). Western blotting was performed as reported previously (Landuzzi et al. 2021). The following primary antibodies were used: anti-HER2 (clone 3B5, diluted 1:1000, Calbiochem, Merck), anti-pNeu (Tyr 1248) (diluted 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Stat3 (clone 124H6, diluted 1:1000, Cell Signaling, Danvers, Massachusetts, USA), anti-pStat3 (clone D3A7, diluted 1:2000, Cell Signaling), anti-Vimentin (clone D21H3, diluted 1:1000, Cell Signaling), anti-Caveolin1 (clone D46G3, diluted 1:1000, Cell Signaling), anti-βcatenin (clone D10A8, diluted 1:1000, Cell Signaling), anti-Actin (diluted 1:1000, Merck). Membranes were either incubated with polyclonal horse-radish-peroxidase (HRP) conjugated anti-rat IgG antibody (diluted 1:3000, Bio-Rad Laboratories), or anti-mouse IgG antibody (diluted 1:1000, Santa Cruz Biotechnology). Protein presence was detected by chemiluminescent reaction (Bio-Rad Laboratories) before film exposure.

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