Role Of d16HER2 Splice Variant In Breast Cancer Stem Cells

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Role of d16HER2 splice variant in breast cancer stem cells

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ABSTRACT

The transmembrane tyrosine kinase receptor HER2 is overexpressed in about 20% of human breast cancers (BCs). Evidence suggests that the co-existence of the full-length/wild-type HER2 oncoprotein (WTHER2) with its altered isoforms increases the heterogeneity of HER2 overexpressing (HER2+) disease thus affecting its biology and treatment response. We found that ~90% of HER2+ BC patients express a WHER2 isoform characterized by the lack of exon 16 (d16HER2), a peculiar absence promoting the generation of stable and constitutively activated HER2 homodimers. Since the impact of d16HER2 in HER2+ BC disease was debatable at the beginning of my Ph.D. project, I focused my studies to investigate and unravel d16HER2 implication in HER2+ BC aggressiveness, susceptibility to specific biotherapies and stemness. Our in vitro and in vivo findings made clearer the potent oncogenicity driven by d16HER2 variant vs WHER2 unveiling its direct functional interplay with the activated tyrosine kinase SRC that was found to be coupled with d16HER2 expression/activity in HER2+ BCs. Further, pre-clinical and clinical data indicated that d16HER2 activation determines the greatest benefits from trastuzumab administration in HER2+ BCs. Additionally, our results pointed to a crucial d16HER2 role in the regulation of the BC stem cells (BCSCs) activity through its functional interaction with the NOTCH family members. We also observed that the higher trastuzumab efficiency inhibits the growth/progression of HER2+ BCs characterized by an activated d16HER2 signature was consistent with its ability to target BCSCs effectively. Finally, our data revealed that d16HER2
expression/activity in driving tumour aggressiveness could be extended also to HER2+ gastric cancers.

The results of my Ph.D. thesis contribute to highlight the crucial role played by d16HER2 in the aggressiveness, sensitivity to trastuzumab and stemness of HER2+ BCs vs the WHER2 receptor.
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<th>Definition</th>
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<tbody>
<tr>
<td>pSRC</td>
<td>Activated SRC</td>
</tr>
<tr>
<td>pSTAT3</td>
<td>Activated STAT3</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis Coli</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cell-mediated cytotoxicity</td>
</tr>
<tr>
<td>ALDH</td>
<td>Aldehyde dehydrogenase</td>
</tr>
<tr>
<td>BC</td>
<td>Breast cancer</td>
</tr>
<tr>
<td>TCGA</td>
<td>Cancer Genome Atlas Network</td>
</tr>
<tr>
<td>CIC</td>
<td>Cancer initiating cell</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer stem cell</td>
</tr>
<tr>
<td>CTF</td>
<td>Carboxy-terminal truncated fragment</td>
</tr>
<tr>
<td>CRC</td>
<td>Colon-rectal cancer</td>
</tr>
<tr>
<td>DM-1</td>
<td>Emtansine</td>
</tr>
<tr>
<td>ECD</td>
<td>Extracellular domain</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>FDA</td>
<td>US Food and Drug Administration</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence <em>in situ</em> hybridization</td>
</tr>
<tr>
<td>GC</td>
<td>Gastric Cancer</td>
</tr>
<tr>
<td>GEJ</td>
<td>Gastro-esophageal junction</td>
</tr>
<tr>
<td>GSK-3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>HER2</td>
<td>Epithelial growth factor receptor 2</td>
</tr>
<tr>
<td>WHER2</td>
<td>Full-length/wild-type HER2</td>
</tr>
<tr>
<td>ICD</td>
<td>Intracellular domain</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IGF-R1</td>
<td>Insulin growth factor receptor 1</td>
</tr>
<tr>
<td>ISH</td>
<td>“in situ” hybridization</td>
</tr>
<tr>
<td>m.f.p.</td>
<td>Mammary fat pad</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal-epithelial transition</td>
</tr>
<tr>
<td>MAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MFI</td>
<td>Median fluorescence intensity</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mammary murine tumour virus</td>
</tr>
<tr>
<td>N-</td>
<td>Node negative</td>
</tr>
<tr>
<td>N+</td>
<td>Node positive</td>
</tr>
<tr>
<td>NICD</td>
<td>Notch-intracellular domain</td>
</tr>
<tr>
<td>OS</td>
<td>Overall survival</td>
</tr>
<tr>
<td>pCR</td>
<td>Pathological complete response</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression free survival</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>qPCR</td>
<td>Real-time PCR</td>
</tr>
<tr>
<td>RTK</td>
<td>Tyrosine kinase receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TKI</td>
<td>Tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>TN</td>
<td>Triple negative</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>TTP</td>
<td>Time to progression</td>
</tr>
<tr>
<td>d16HER2D</td>
<td>d16HER2 homodimers</td>
</tr>
<tr>
<td>d16HER2M</td>
<td>d16HER2 monomers</td>
</tr>
<tr>
<td>pd16HER2D</td>
<td>Activated d16HER2 homodimers</td>
</tr>
<tr>
<td>pd16HER2M</td>
<td>Activated d16HER2 monomers</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
</tbody>
</table>
Chapter 1 - INTRODUCTION
1.1 Breast Cancer

Breast cancer (BC) is the most common and frequently diagnosed cancer that accounts for 25% of all tumour cases and represents 15% of all tumour deaths in female (Torre et al. 2015). In fact, in 2012 an epidemiologic analysis reported about 1.7 million new cases of BC and 521,900 deaths (Torre et al. 2015) (Fig. 1).

![Figura 1: Incidence and mortality of different cancers in male and female populations](Adapted from Linsey et al, CA: A Cancer Journal for Clinicians, 2012)

In the past, BC was considered as a single disease and, the treatment schedules were exclusively guided by distinct clinicopathological variables, i.e presence of lymph node metastasis, histological grade, as well as expression of Estrogen (ER) and/or progesterone receptor (PR) or epithelial growth factor receptor 2 (HER2) respectively used as predictive markers of response to endocrine and HER2-target therapies. The introduction in the clinical practice of these pathological features was important to reduce the rate of BC mortality but they resulted not sufficient for implementation of personalized therapy (Reis-Filho & Pusztai 2011).

The mammary gland is defined as a tubular-alveolar gland constituted by 10-20 lobules incorporated in the adipose tissues and connected to the nipples through different ducts (Figure 2).
In physiological conditions, mammary gland cells are characterized by a normal proliferation rate necessary to the regular tissue growth. On the contrary, the BC tissue is formed by cells with uncontrolled proliferation rate and with altered survival. The molecular processes at the basis of the breast carcinogenesis are not yet completely defined. In this context, genetic alterations determining the generation of a small number of dysfunctional cells called Cancer Stem Cells (CSCs) or cancer initiating cells (CICs) were proposed as the initial step to the cancer development (Visvader & Lindeman 2012).

The anatomical breast structural units constituting the key components of the mammary gland define the different cancer oncotypes. Indeed, BC is classified as ductal or lobular and, in addition, as invasive or in situ (non-invasive) disease. (Sharma et al. 2010) In situ BC arises from the abnormal proliferation of epithelial cells that form the duct lumen or the lobule of origin without invading the basal membrane. On the other hand, the tumours invading the lobule or...
ductal edge are classified as invasive and can spread to distant organs developing cancer metastasis. (Sharma et al. 2010)

In the recent years, the advent of the high-throughput platforms for analysis of gene expression, such as microarrays, has allowed to overcome the concept of BC as a unique disease. In fact, the results collected using these molecular approaches confirmed that BC is a heterogeneous disease classified into different subtypes with peculiar risk factors, histopathological features and clinical outcome. In addition, also the response to different treatments could be influenced by intrinsic molecular characteristics of the tumours (Dai et al. 2015).

In light of these important advances, BC is now classified according to its own gene expression profile. This novel molecular BC classification is defined “intrinsic classification” and in its original generation it did not take into account the histopathological variables (Desmedt et al. 2012; Russnes et al. 2011; Sotiriou & Pusztai 2009). Perou and Sorlie and their research groups in the early 2000s through a hierarchical cluster analysis of global gene expression data from 143 sporadic BC revealed the existence of four different BC ‘intrinsic subtypes’ considered as an independent biological entity (Perou et al. 2000; Sorlie et al. 2001) each endowed of peculiar natural histories, capability to response to systemic and targeted treatments (Heiser et al. 2012) and defined luminal (A and B), HER2-positive, basal-like and normal-breast like (Table 1).
TABLE 1: BC INTRINSIC SUBTYPES WITH PREVALENT IMMUNE-HISTOCHEMICAL PROFILES AND OPTIONS OF TREATMENT (ADAPTED FROM TOSS A. ET AL, BC RESEARCH, 2015).

<table>
<thead>
<tr>
<th>Intrinsic subtype</th>
<th>cDNA microarrays</th>
<th>IHC</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A</td>
<td>The highest expression of the ER α gene, GATA-binding protein 3, X-box-binding protein 1, trefoil factor 3, hepatocyte nuclear factor 3 α, and estrogen-regulated LIV-1</td>
<td>ER- and/or PR-positive HER2-negative Ki-67 &lt; 14%</td>
<td>Endocrine therapy (chemotherapy for selected patients)</td>
</tr>
<tr>
<td>Luminal B</td>
<td>Low to moderate expression of the luminal-specific genes, including the ER cluster</td>
<td>ER- and/or PR-positive HER2-negative with Ki-67 ≥ 14%</td>
<td>Endocrine therapy ± chemotherapy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ER- and/or PR-positive HER2-positive with any Ki-67</td>
<td>Chemotherapy + anti-HER2 therapy + endocrine therapy</td>
</tr>
<tr>
<td>HER2-enriched</td>
<td>High expression of several genes in the ERBB2 amplicon at 17q22.24, including ERBB2 and GRB7</td>
<td>ER- and PR-negative HER2-positive</td>
<td>Chemotherapy + anti-HER2 therapy</td>
</tr>
<tr>
<td>Basal-like</td>
<td>High expression of keratins 5 and 17, laminin, and fatty acid-binding protein 7</td>
<td>ER- and PR-negative HER2-negative</td>
<td>Chemotherapy</td>
</tr>
</tbody>
</table>

In addition, further molecular analyses performed in a consistent number of BC cases unveiled the existence of other additional molecular subtypes defined as claudin-low (Prat et al. 2010) and molecular apocrine (Farmer et al. 2005) (Figure 3).
The luminal-A BC subtype includes about 50% of the total BC patients (Yersal & Barutca 2014). These tumours are characterized by low histological grade, reduced proliferative index and good prognosis with a relapse rate significantly lower than the other subtypes (Carey et al. 2016). Luminal A BCs are positive for expression of ER and/or PR and negative for HER2-expression and, for these reasons, they are mainly treated with 1) selective modulator of ER activity, as tamoxifen; 2) anti-estrogen agents, such as fulvestrant in premenopausal setting or 3) inhibitor of aromatase enzymatic activity in postmenopausal setting (Kumler et al. 2016).

Luminal B BC subtype accounts for about 15-20% of total BC patients and shows a more aggressive phenotype, higher histological grade and proliferative index and a worse prognosis with a higher recurrence and lower survival rates than
luminal A BC cases (Creighton 2012). Globally, luminal B BC subtype includes cases with a high expression of genes related to cell proliferation and an increased expression of tyrosine kinase growth factor receptors. In this context, about 30% of luminal B BC are HER2-positive according to their immunohistochemical staining for the HER2 oncoprotein (Loi et al. 2009). Generally, luminal B BC patients are treated with tamoxifen and aromatase inhibitors combined with specific anti-HER2 biodrugs either in the adjuvant as well as neoadjuvant settings. HER2-positive BC (about 20% of all BC) subtype is characterized by the overexpression/amplification of the HER2 gene (Kumler et al. 2014). About 50% of HER2-positive BC are classified as HER2 enriched (Toss et al. 2015) and are characterized by a high expression of cell proliferation-related genes (g. ERBB2/HER2 and GRB7) (Toss et al. 2015). Before the introduction in the clinical practice of the humanized mAb trastuzumab, currently the gold standard therapy for HER2-positive BC in combination with chemotherapy, such disease was significantly associated with a particularly poor prognosis (Maximiano et al. 2016; Ménard et al. 2002). Indeed, trastuzumab combined with chemotherapy has dramatically improved the outcome of HER2-positive BC patients despite about 50% of treated cases develop resistance and progressive disease within the first year of treatment (Callahan et al. 2011).

Basal-like BCs are characterized by high expression levels of genes promoting cellular proliferation, cell survival and invasion (Toss et al. 2015). This subtype and also includes hereditary BC cases carriers of BRCA1 and 2 mutations (26851). About 80-85% of the basal-like BCs are also triple negative breast cancers (TNBCs) which are negative for ER, PR and HER2 expression. In keeping
with such peculiar profile, the majority of TNBCs cannot be treated with the currently targeted therapies as trastuzumab and/or anti-hormonal treatments. The lack of specific biotherapies coupled with an intrinsic aggressive tumour phenotype renders such subtype particularly aggressive and characterized by worst prognosis.

The normal-like BC subtype represents about the 5-10% of total BC and is characterized by the expression of genes typical of the adipose tissue (Hou et al. 2006). This BC subtype is poorly investigated and its clinical significance remains undetermined. The lack of ER, PR and HER2 expression could identify normal-like BC as TNBC, but the absence of EGFR expression excludes such possibility (Hou et al. 2006). Furthermore, many researchers considered this BC subtype as a technical artifact due to a high contamination with normal tissue during the microarrays processing (Weigelt et al. 2010).

In this context, thanks to the advent of the next generation sequencing methodologies, in the last few years it has been possible to reveal single nucleotide polymorphism and mutations, small insertions/deletions and expression of alternative splicing variants thus overcoming the limitations of the first generation of molecular analyses (Previati et al. 2013). These analyses performed by the BC International Consortium (METABRIC) (Curtis et al. 2012) and the Cancer Genome Atlas (TCGA) Network (Cancer Genome Atlas Network 2012) that sequenced hundreds of BCs revealed the existence of a significant molecular heterogeneity in each BC subtype. The deep “knowledge” of the mechanisms underlying such intra- and inter-tumour BC heterogeneity, a pathobiological feature heavily implicated in tumour cell plasticity, and,
causative of treatment resistance, tumour invasion and metastasis could be very useful to improve the BC management (Norum et al. 2014).

1.2 HER2-positive BC

The HER2-positive BC subtype is characterized by the amplification/overexpression of HER2 at DNA/mRNA and protein levels. The HER2 oncogene is a member of the epithelial growth factor receptor (EGFR) gene family that includes EGFR, also named HER1, HER3 and HER4. Its intra-tumour identification and clinical diagnosis are determined through the investigation of the oncoprotein overexpression by immunohistochemistry (IHC) or DNA amplification by fluorescent in situ hybridization (FISH) analyses or mRNA upregulation by mRNA in situ hybridization (ISH), respectively (Perez et al. 2014).

The HER2-enriched molecular subtype characterized by high-level expression of the HER2 transcript, is enriched of genes whose expression is regulated by HER2 signaling activity (e.g. GRB7 and FGFR4), proliferation/cell-cycle-related genes (e.g. ribonucleotide reductase M2) and is coupled with a low expression of genes related to the luminal phenotype (e.g. ESR1, PGR, and forkhead box A1) (Prat et al. 2014). The clinical HER2-positive and molecular HER2-enriched classifications are not completely overlapping since, as outlined above, the HER2-positive BC subtype has also an intrinsic intra/inter-tumour heterogeneity (Prat et al. 2014).

In keeping with that, two different main HER2-positive BC subtypes have been identified so far and, specifically, only about 50% of total clinically diagnosed HER2-positive BCs are included in the HER2-enriched mRNA subtype while the remaining half is classified in the luminal B subset expressing a consistent luminal
genes cluster (Toss et al. 2015). In 2001, following a retrospective analysis of 1,200 primary BC cases surgically treated in our Institute in absence of any adjuvant chemotherapy, Dr. Menard and collaborators reported that the node-positive (N+) and HER2-overexpressing BC cases had a significant worse prognosis compared to the N+ and HER2-negative patients, as show in Figure 4 (Ménard et al. 2001). On the other hand, in the N-negative (N-) BC patients, the HER2-overexpression was not found associated with overall survival (Ménard et al. 2001).

Figure 4: Survival (%) of BC patients with primary BC surgically treated without systemic adjuvant chemotherapy stratified according to HER2-expression (Adapted from Menard S. et Al., Oncology, 2001).

In our lab, it was also demonstrated that one of the main mechanism determining the development of an early HER2-positive relapse could be referred to biological events occurring in the surrounding anatomic site of surgery as the expression and release of cytokines or growth factors that stimulate the recurrence of HER2-positive disease (Tagliabue et al. 2003).
In light of the crucial pathobiological role played by the altered expression of HER2 oncogene a deep evaluation to assess its expression levels/activation status in BC patients is mandatory to drive the choice of the most appropriate and efficient therapy. Consequently, the sensitivity, specificity, and reproducibility of the diagnostic techniques are key factors to select patients who can benefit from targeted therapies (Carney et al. 2007). Table 2 lists all the testing approaches to evaluate the HER2 status and discriminate HER2-positive from HER2-negative patients.

TABLE 2: FOOD AND DRUG ADMINISTRATION-APPROVED HER2 TESTING ASSAYS. THE FDA APPROVED THE USE OF THESE TESTS TO IDENTIFY HER2-POSITIVE PATIENTS CONSIDERED FOR THE TREATMENT WITH ANTI-HER2-TARGETED THERAPY (ADAPTED FROM PEREZ A, CANCER TREATMENT REVIEWS, 2014)

<table>
<thead>
<tr>
<th>Assay type</th>
<th>Trade name</th>
<th>Date of FDA approval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semi-quantitative IHC</td>
<td>HercepTest™</td>
<td>September, 1998</td>
</tr>
<tr>
<td>IHC</td>
<td>PATHWAY®</td>
<td>November, 2000</td>
</tr>
<tr>
<td>IHC</td>
<td>InSite®</td>
<td>December, 2004</td>
</tr>
<tr>
<td>Semi-quantitative IHC</td>
<td>Bond Oracle™</td>
<td>April, 2012</td>
</tr>
<tr>
<td>FISH</td>
<td>PathVysion®</td>
<td>December, 2001</td>
</tr>
<tr>
<td>FISH</td>
<td>PharmDx™ Kit</td>
<td>May 2005</td>
</tr>
<tr>
<td>CISH</td>
<td>SPoT-Light®</td>
<td>July, 2008</td>
</tr>
<tr>
<td>CISH</td>
<td>INFORM HER2 dual ISH DNA probe cocktail</td>
<td>June, 2011</td>
</tr>
<tr>
<td>CISH</td>
<td>PharmDx™</td>
<td>November, 2011</td>
</tr>
</tbody>
</table>

1.2.1 HER2 receptor: structure and intracellular signaling.

In 1984 and 1985 Dr. Schechter and Dr. King in two independent laboratories identified a human gene highly homologous to the EGFR that was called human EGFR-2 (HER2) (King et al. 1985; Schechter et al. 1984). Cytogenetic and biochemical analyses revealed that the HER2 gene is located on the chromosome
17q21-22 and encodes for a transmembrane glycoprotein constituted by 1255 amino acids. The \textit{HER2} gene is the human orthologue of the rat Neu gene (\textit{HER2/neu}) identified for the first time in a rat neuroblastoma induced by treatment with nitrosourea, a DNA alkylating agent (Schechter et al. 1984).

As already stated, \textit{HER2} is one of a tyrosine kinase receptor (RTK) of the EGFR genes family including also \textit{HER1}, \textit{HER3} and \textit{HER4}.

These proteins are type I transmembrane growth factor receptors able to respond to extracellular signals through the activation of a cascade of intracellular signaling pathways. HER-family members are constituted by a large extracellular domain (ECD) divided into 4 subdomains (I-IV), followed by a transmembrane (TM) and intracellular domain (ICD) that is composed of a small intracellular juxta-membrane domain and a kinase domain a C-terminal tail (Figure 5).

\textbf{Figure 5: Crystal structure of Human HER2 ECD.} The domains composing \textit{HER2} protein are represented with different color: domains I/I1 (Blue), II/I2 (Green), III/CR1 (Yellow) AND IV/CR2 (Red) (Adapted from Cho, Nature, 2003).
In the absence of any extracellular “growth” signal, the HER2 ECD is intrinsically in a “closed” inactive conformation even if “prone” to be activated. On the contrary, the binding between HER1, HER3 and/or HER4 ECD with their own specific ligand(s) determines the induction of a conformational change from a “closed” to an “open” active form. After its specific triggering, HER2 can homo or heterodimerize respectively with itself or another member of the HER family and, in turn, determine the transphosphorylation of the ICDs promoting downstream oncogenic signaling cascade (Burgess et al. 2003). Currently, 30 specific ligands for HER1, HER3 and HER4 receptors have been discovered. HER2 is the unique orphan-ligand receptor, and, as stated above, it is characterized by an intrinsic proper structural conformation resembling a ligand-activated RTK that favors its “spontaneous” dimerization (Yarden et al. 2001). In this context, the X-Rays based crystallographic studies unveiled that the HER2 ECD is composed of 630 amino acids organized in 4 subdomains: two β-barrel subdomains (I/L1; II/L2) and two cysteine-rich subdomains (III/CR1; IV/CR2) (Figure 5) (Cho et al. 2003). I/L1 and II/L2 ECDs are capable to interact each other determining the exposure of the HER2 receptor dimerization arm ready to generate either homodimers or heterodimers with the other EGFR family members. The homo and/or heterodimerization of HER2 and the consequential transactivation of their intrinsic tyrosine kinase activity determines the recruitment of different intracellular signaling proteins which, in turn, upon their activation regulate distinct cellular pathways related to tumourigenesis as proliferation, survival, migration, invasion, differentiation and angiogenesis. The involvement of the HER2 complex signaling network in tumourigenesis is not
limited to the BC but it also affects different epithelial tissues including ovarian, lung, gastric and colorectal driving other solid cancers (Jackson et al. 2013).

In light of the above described intrinsic activated structure, HER2 is the preferential partner for the heterodimerization with the other HER family members. Important biochemical studies suggest that dimers containing HER2 are more stable and prolong signaling activation when compared with non-HER2-containing dimers (Jackson et al. 2013). In addition, in vitro studies performed in HER2-overexpressing BC cells, have clearly demonstrated that HER2 can “autoactivate” itself generating homodimers in absence of any ligand(s) binding thus explaining the aggressive behavior of HER2-overexpressing BCs compared to others (Jackson et al. 2013).

The triggering of downstream pathways mediated by HER2 activation is complex. The canonical HER2 signaling associated with cell proliferation and survival is mediated by the activation of the mitogen-activated protein kinase axis (RAS-MAPK) and activated phosphatidylinositol-3-kinases-v-AKT (PI3K-AKT), respectively (Alimandi et al. 1995; Wallasch et al. 1995) (Figure 6) and the capability of anti-HER2 agents to impair HER2-driven tumour growth is strictly associated with their efficacy to halt the above indicated signaling pathways (Chakrabarty et al. 2013).
Figure 6: Canonical signalling pathways driven by HER-mediated activity. The autophosphorylation triggered by the heterodimerization between HER2 and other ErbB receptors stimulates the PI3K/AKT (survival mechanisms and inhibition of apoptosis) and the RAS/RAF/MEK/MAPK (cell proliferation) (Adapted from Jackson C, International Journal of Cell Biology, 2013)

Moreover, HER2 activity was associated with activation of other intracellular tyrosine kinases. In this context, in 2001 Allison and colleagues have demonstrated the capability of HER2 to interact with the SRC kinase thus increasing tumour growth, survival and cancer-related stemness features (Figure 7) (Belsches-Jablonski et al. 2001).
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Figure 7: interaction between HER2 activation and Src-kinase. HER2 activation leads to Src phosphorilation increasing cancer stem cell related features (Adapted from Shizhen Emily Wang, Cancer Research, 2009).

1.2.2 HER2 proteome

Pre-mRNA alternative splicing is a widespread molecular process contributing to structural transcripts variation and proteome diversity (Sveen et al. 2016). It is well established that the splicing variants deriving from the same full-length pre-mRNA transcript encoding for an oncogene could have different pathobiological impacts on tumour cells mediating either pro- or anti-tumourigenic effects (Oltean et al. 2014). In this context, it has been established that the HER2 pre-mRNA is processed in different splicing sites leading to the synthesis of various HER2 isoforms with contradicting roles in tumour cell biology (Jackson et al. 2013). Currently, three distinct HER2 splice variants driving peculiar effects on the HER2-positive BC cells have been identified and they are: d16HER2 (also identified in the literature as Δ16HER2, Delta-HER2, ErbB2ΔEx16, HER2Delta16), Herstatin and p100 (Table 3).
TABLE 3: HER2 SPLICE VARIANTS NATURALLY EXPRESSED IN HER2-POSITIVE BC.
DESCRIPTION OF ALTERNATIVE SPLICING MECHANISMS AT THE BASIS OF THE
GENERATION OF DIFFERENT HER2-ISOFORMS.

<table>
<thead>
<tr>
<th>HER2-splice variant</th>
<th>Alternatively spliced event</th>
</tr>
</thead>
<tbody>
<tr>
<td>d16HER2</td>
<td>Exon 16 skipped</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>p100</td>
<td>Intron 15 retained</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Herstatin</td>
<td>Intron 8 retained</td>
</tr>
</tbody>
</table>

The p100 isoform was the first described HER2 mRNA variant and is characterized from the retaining of intron 15. The result of this alteration is a HER2-protein of 633 amino acids constituted of only the HER2/ECD (subdomains I-IV) (Scott et al. 1993). p100 has been described to impair the capability of HER2 to mediate oncogenic activity, In fact, it was reported that it acts as an inhibitor of tumour cell proliferation (Aigner et al. 2001; Koletsa et al. 2008) through its activity to decrease downstream signals activation (Sasso et al. 2011).

Herstatin derives by the retention of intron 8 in the HER2 pre-mRNA. This modification determines the formation of a secreted protein of 68kDa that consists of the first 340 aminoacidic residues HER2/ECD-related. It has been demonstrated that when expressed in HER2-positive cellular models and normal
and tumour breast tissues, Herstatin can induce growth-inhibitory properties through some mechanisms such as the impairment of its dimerization, reduced tyrosine kinase phosphorylation and, in turn, the decrease of downstream signaling (Justman et al. 2002; Koletsa et al. 2008).

d16HER2 is a splice variant of the full-length HER2 (WTHER2) oncoprotein characterized by the in-frame skipping of exon 16 that causes the loss of 16 aminoacids (634-649) in the HER2 ECD (domain IV) including two relevant cysteine residues very close to the juxtamembrane region (Kwong et al. 1998). The consequential cysteine residues imbalance plays a primary role in HER2 catalytic activity regulation because it induces receptor activation, promoting constitutive homodimerization through stable intermolecular disulfide bonds formation, enhances multi-signaling activity and accelerates transformation suggesting a causal role of d16HER2 in cancer. To make the HER2 proteome more complex, a subgroup of HER2-positive patients express a series of carboxyl-terminal membrane-anchored or soluble HER2 fragments that derives from two different biological mechanisms as the proteolytic cleavage the ECD fragment also referred as soluble HER2 ECD or p100 (Carney et al. 1991; Pupa et al. 1993), or the alternative initiation of the translation (Pedersen et al. 2009; Yuan et al. 2003). As concern the HER2 variants generated by the proteolytic shedding ADAM and MMP metalloproteases were found to cause the cleavage of HER2 ECD generating a 95 to 100 kDa anchored fragment in cell membrane named -648-CTF (p95) with the peculiar capability to activate several intracellular signal transduction pathways related to the aggressive and metastatic phenotype (Pupa et al. 1993; Wang et al. 2013). In addition, clinical studies indicate that high
Role of d16HER2 splice variant in BC Stem Cells

Circulating levels of HER2ECD can represent a useful biomarker to monitor HER2 status and also for prognosis (Tse et al. 2012). On the other hands, the alternative initiation of translation of HER2 mRNA occurs from two different starting codons at positions 611 and 687 generating two additional p95 fragments named 611CTF (transmembrane) and 687CTF (intracellular), respectively (Christianson et al. 1998). The role of 611CTF fragment in the HER2-positive BC biology has been deeply characterized (Arribas et al. 2011). This HER2 variant is constitutively activated being able to form disulfide bound homodimers and, in turn, to stimulate the downstream signaling cascade linked to MAPK and AKT “nodes”. In keeping with these reported data, the ectopic expression of the human 611CTF variant in a transgenic mouse model determines the development of mammary adenocarcinomas within 4 months of age (Pedersen et al. 2009). Furthermore, the altered expression of 611CTF in advanced HER2-positive BC determines an intrinsic resistance to the anti-tumour Trastuzumab activity (Pedersen et al. 2009; Scaltriti et al. 2007). In this context, in 2010 Scaltriti and colleagues reported data from two HER2-positive BC cohorts showing that altered intratumour expression of the p95HER2 variant was coupled with lapatinib efficacy (Scaltriti et al. 2010). On the contrary, the soluble intracellular 687CTF isoform does not activate its own kinase domain, and, according to this feature, the mouse model transgenic for the 687CTF does not develop spontaneous mammary gland (Pedersen et al. 2009).

To add further complexity to the HER2 proteome, 27 somatic mutations were identified in BCs lacking HER2 amplification and were found spread out on all HER2 sequence (Figure 8) (Bose et al. 2013).
Figure 8: HER2 somatic mutations observed in 25 BC patients. Characterization and location of HER2 somatic mutations identified in 25 patients with BC (Adapted from Bose R, Cancer Discovery, 2013).

Thirteen of these mutations were functionally characterized using different experimental approaches (Bose et al. 2013). In particular, 7 of them were classified as activating mutations (G309A, D769H, D769Y, V777L, P780ins, V842I, and R896C), one was able to stimulate the activity of HER1 (in-frame deletion 755–759), and one conferred lapatinib resistance (L755S). In addition, all mutations were sensitive to treatment with neratinib, a TKI or EGFR-family members (Bose et al. 2013). These findings provided the evidence that HER2 somatic mutations could be an alternative biological mechanism to activate HER2 oncoprotein in BC and they validate HER2 somatic mutations as druggable for BC treatment.

1.2.3 Treatment of HER2-positive BCs.

The HER2 receptor is one of the first molecules overexpressed in BC to be targeted with specific biological therapies. Indeed, its selective overexpression only on the BC cells membrane coupled with its implication in cell proliferation
Role of d16HER2 splice variant in BC Stem Cells

survival and progression makes HER2 an ideal target to design specific anti-HER2 agents. The introduction of HER2-targeted therapies has represented a significant progress in the clinical management of HER2-positive BC cancer (Campiglio et al. 2013). In order to generate anti-HER2 specific therapeutic approaches, two different strategies were followed: 1) the production of humanized mAbs designed to bind the HER2/ECD (i.e trastuzumab and pertuzumab) 2) the synthesis of TKIs that compete with the ATP binding site of the HER2 receptor halting the capability to activate a downstream signaling cascade (i.e Lapatinib and neratinib).

1.2.3.1 Trastuzumab

The recombinant humanized MAb trastuzumab (Herceptin, Genentech, South San Francisco, CA, USA) binds the subdomain IV of the HER2/ECD. Trastuzumab was derived by the molecular engineering of the mouse monoclonal antibody (mAb) 4D5 reported to mediate a strong anti-tumour activity against HER2-overexpressing BC cells both in vitro and in vivo settings. Trastuzumab was introduced in clinic for the treatment of HER2-positive metastatic BC in 1998 in combination with the systemic chemotherapy (https://www.cancer.gov/about-cancer/treatment/drugs/fda-trastuzumab). The results of a Phase III trial demonstrated that the addition of trastuzumab extended the progression free survival (PFS) from 4.6 to 7.4 months and reduced the risk of death by 20%. (Slamon et al. 2001). Currently, trastuzumab is approved with or without systemic chemotherapy in the treatment of primary or metastatic HER2-positive disease either in neoadjuvant or adjuvant settings. In addition, since HER2
overexpression/amplification is also found in about 20% of gastric and gastroesophageal junction cancers, trastuzumab was also approved for the treatment of this oncotype. The Trastuzumab for Gastric Cancer (ToGA) trial provided a clear evidence that the addition of trastuzumab to standard chemotherapy prolonged the median survival from 11 to 13.5 months (Bang et al. 2010). Moreover, in the last year, data from HERACLES trial demonstrated that the combination of trastuzumab plus lapatinib in the treatment of HER2-positive colon-rectal cancer (CRC) (about 5% of total CRC) extended the median PFS from 16 to 29 weeks (Sartore-Bianchi et al. 2016). Several potential mechanisms of action were proposed to explain the exceptional clinical benefits mediated by trastuzumab in HER2-positive neoplasia. Globally, pre-clinical and clinical studies suggest that trastuzumab mediates distinct anti-tumour “cytostatic” activities based either on the inhibition of HER2-driven signaling or arrest of HER2/ECD shedding and/or tumour neo-angiogenesis. (Spector et al. 2005); anti-tumour “cytotoxic” activity mainly determined by antibody (Ab)-dependent cellular cytotoxicity (ADCC) through the intratumour recruitment of effector cells (Tagliabue et al. 2011), induction of both innate and/or adaptive anti-tumour immune responses (Triulzi et al. 2016); capability to target and debulk the CSC compartment (Magnifico et al. 2009); impairment of the receptor internalization into the cytoplasm and promotion of HER2 degradation (Klapper et al. 2000); inhibition of the DNA-damaged repair induced by conventional chemo and/or radiotherapy (Tagliabue et al. 2011).

Despite the introduction of trastuzumab as current gold standard therapy has revolutionized the management of HER2-positive BC patients, less than 35% of
HER2-positive BCs are addicted to HER2 signaling and only initially respond to the Ab administration (Wolff et al. 2007). Indeed, a consistent number of patients initially responding to the biodrug administration dynamically develop an acquired drug resistance and evolves to a metastatic disease within one year from the beginning of trastuzumab administration (Gajria et al. 2011). Different potential mechanisms of trastuzumab resistance have been proposed and demonstrated both in vitro and in vivo models (Figure 9).

![Figure 9: Molecular resistance mechanisms to trastuzumab treatment.](image)

Figure 9: Molecular resistance mechanisms to trastuzumab treatment. Molecular characterization of the different mechanisms implicated in HER2-positive BC trastuzumab resistance: 1) steric effects or masking of trastuzumab binding sites; 2) overexpression and enhanced activity of other tyrosine kinase receptors; 3) intracellular alterations, e.g. lost of function of PTEN or constitutively activation of PI3K and/or AKT (Adapted from Thuy Vu, Front Oncol, 2012).

These include the overexpression of the truncated p95HER2-648CTF isoform that cannot bind trastuzumab since lacking of its binding site (Scott et al. 1993).
Another proposed mechanism of trastuzumab resistance is the alteration of the intracellular signaling induced by the overexpression of other redundant RTKs as HER1, HER3 and HER4. In particular, it was observed that overexpression of HER3, insulin growth factor receptor 1 (IGF-R1) or ER can compensate the inhibition of HER2 signaling mediated by Trastuzumab (Vu et al. 2012). In this context, the expression and activation of the non-receptor tyrosine kinase SRC has been proposed as a crucial mechanism of trastuzumab resistance in the advanced stage of HER2-positive BC metastatic setting in keeping with SRC capability to hyperactivate signaling axis downstream of HER2 (Zhang et al. 2011).

Another biological event related to trastuzumab resistance is the downregulation of the oncosuppressor phosphatase and tensin homolog (PTEN) protein expression was observed in 36% of HER2-positive primary breast tumour specimens (Vu et al. 2012) and such intracellular alteration was associated with a remarkably lower overall response rates to trastuzumab than patients expressing the physiological level of PTEN (Berns et al. 2007).

1.2.3.2 Lapatinib

Lapatinib is an orally active RTK inhibitor able to block HER2 signaling. This drug competes with ATP for binding to its “pocket” in the f HER2/ICD. Specifically, Lapatinib halts HER2 signaling promoting both the arrest of the cell cycle and the activation of apoptosis-related mechanisms. In pre-clinical studies, Lapatinib has demonstrated a strong anti-tumour activity against HER2-positive BC lines resistant to trastuzumab (Moy et al. 2006) and these findings were confirmed
also in the clinical setting. In fact, the combination of lapatinib with systemic chemotherapy improve time to progression (TTP) in HER2-positive BC patients progressed after trastuzumab-based treatment (Gomez et al. 2008). However, the phase III of MA.3.1 clinical trial provided evidence that Lapatinib in combination with chemotherapy has failed to improve PFS vs trastuzumab plus chemotherapy in HER2-positive metastatic BC patients (Gelmon et al. 2015). These results suggest that lapatinib should be introduced in the clinical treatment of metastatic HER-positive BC patients only in combination with trastuzumab (Eroglu et al. 2014).

1.2.4.3 Pertuzumab.

Pertuzumab is a fully humanized MAb able to selectively bind the HER2/ECD (subdomain II) thus inhibiting the formation of HER2-HER3 dimers even in presence of Heregulin and, in turn, reducing the oncogenic downstream signaling (Capelan et al. 2013). Similarly to trastuzumab, the ADCC was proposed as one of the major potential mechanism of action of pertuzumab. Since pertuzumab binds a different domain vs that targeted by trastuzumab (subdomain IV), their therapeutic combination was tested in pre-clinical setting. Scheuer W and colleagues showed a synergistic effect in the inhibition of HER2-positive BC and non–small cell lung cancer growth both in in vitro and in vivo settings (Scheuer et al. 2009). These results were also confirmed by different clinical trials and currently, the combination of pertuzumab and trastuzumab plus chemotherapy is approved for the treatment of advanced HER2-positive BC patients who have not received previous anti-HER2 therapy or chemotherapy for metastatic disease.
(Swain et al. 2015). In addition, the combination of these two anti-HER2 mAbs is also approved in clinic for the treatment of locally advanced, inflammatory or early HER2-positive disease (Baselga et al. 2010; Gianni et al. 2010).

**1.2.4.4 trastuzumab-emtansine.**

Trastuzumab emtansine (DM1) is an Ab-drug conjugate in which the potent cytotoxic agent DM1 is stably linked to trastuzumab. The emtansine mediates its anti-tumour effects by binding to the tubulin protein thus preventing the formation of microtubules, intracellular protein structures necessary during cell mitosis, inhibiting tumour cell proliferation. However, this compound is toxic causing neuropathy, diarrhea and systemic weakness (Cassady et al. 2004). Trastuzumab DM-1 binds HER2 with the same efficiency of trastuzumab alone and specifically delivers the cytotoxic DM1 to cancer cells (Barok et al. 2014). After its binding, the conjugated mAbs is internalized into the tumour cytoplasm and is degraded by activation of the lysosomal activity that permits the release of the cytotoxic drug (Figure 10).
Trastuzumab-DM1-mediated anti-BC activity was mainly demonstrated in the EMILIA study (Verma et al. 2012) whose clinical data led to approval of trastuzumab-DM1 in the second-line treatment of HER2-positive BC resistant to trastuzumab.

1.2.4.5 Neratinib

Neratinib is an orally available, small, irreversible pan-HER kinase inhibitor that targets a conserved cysteine residue at the ATP binding site in the intracellular region of the EGFR family members (Echavarria et al. 2017). Neratinib covalently
binds its targets halting the ATPase related properties of the EGFR family members and blocking the intracellular signaling cascade thus inhibiting cancer cell proliferation (Echavarria et al. 2017; Singh et al. 2011). Also neratinib displays its high anti-tumour activity by promoting an inhibition of HER2-driven downstream signaling cascade. Its clinical efficacy in HER2-positive BC patients was also tested in some clinical trials. The study ExteNet has provided evidence of the therapeutic advantage to use neratinib after an adjuvant treatment with trastuzumab. However longer follow-up is needed to assess the capability of neratinib to improve HER2-positive BC outcome (Chan et al. 2016).

1.3 Cancer stem cells

The first evidence of the existence of the cancer stem cells (CSC), a small subset of tumour cells able to drive tumourigenesis and, also, at the basis of tumour heterogeneity and metastatization, was reported in the middle of the 1990s when a great deal of literature showed their intra-tumour presence in different hematopoietic, neurological and epithelial oncotypes (Vinogradova et al. 2015). The CSC theory suggests that tumours are characterized by a cellular hierarchy in which CSCs are placed at the top (Fulawka et al. 2014). CSCs possess peculiar characteristics compared to their matched differentiated tumour counterparts such as slow proliferation rate, ability for limitless self-renewal and to differentiate into heterogeneous cancer cells lineages (Clarke et al. 2006). Two hypotheses have been formulated in the attempt to explain intra-tumour heterogeneity: CSCs and clonal evolution (Shackleton et al. 2009). Both CSC and clonal evolution models are based on the existence of subsets of cells able to
initiate and sustain the tumours. CSC model expects the tumour stem cells differentiate into phenotypically diverse progenitors (Shackleton et al. 2009) (Figure 11 A). On the other hand, the clonal evolution model is based on the assumption that tumour originates from an ancestral clone that dynamically evolves into different clones guided by the activity over time of “driver” and/or “passengers” mutations. (Tysnes 2010) (Figure 11 B).

**Figure 11: Basic tumour heterogeneity models. A) CSC model: CSCs represent a particular cell subset capable of unlimited number of divisions. The heterogeneity of tumor cell population is determined from the presence of phenotypically diverse populations in different stages of cell maturation; B) Clonal evolution model: the high proliferation rate and the genomic instability of tumor cells are responsible for the generation of large number of cells differing in genotype and phenotype (Adapted from Fulawka L, Biological Research, 2014).**

Despite the CSC compartment is composed of a very small number of tumour elements, such cells contribute to different pathobiological properties strictly related with an aggressive tumour phenotype. In fact, CSCs participate to
metastasis and cancer dissemination into distant organs (Chang et al. 2016). Furthermore, CSCs, in keeping with their quiescent status, are resistant to chemo and radiotherapy (Chang et al. 2016) versus (vs) highly proliferating differentiated tumour cells. For all features listed above the study of CSC subset is a “hot” field in cancer research in order to better understand the biological nature of cancer and refine the current clinical therapeutic approaches.

1.3.1 CSC related signaling

The peculiar biological CSC features such as self-renewal, cell fate decisions, survival, proliferation, and differentiation are driven by specific signaling pathways. In particular, Notch, Wnt and Hedgehog are the three signaling hubs with a strict implication in the regulation of CSCs properties (Figure 12) (Matsui 2016).
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Figure 12: Schematic representation of Wnt, Hedgehog and Notch signaling pathways. Molecular mechanisms involved in the activation and intracellular signaling driven by (a) Notch, (b) Wnt, and (c) Hedgehog pathways (From Malhotra GK, Journal of Carcinogenesis, 2011).

1.3.1.1 Notch signaling pathway.

The Notch pathway is highly conserved in mammals. The activation of NOTCH-related signaling molecules is triggered by the binding occurring between the specific Notch ligands (DLL1, DLL3, DLL4, JAG1 and JAG2) and their corresponding transmembrane receptors consisting of four members (NOTCH1-4) (Matsui 2016). This functional interaction leads to the unfolding of the juxtamembrane region of the Notch receptor and permits the access of the protease ADAM10 which cleaves the Notch ECD. As shown in Figure 11, this structural modification is recognized by the γ-secretase complex that cleaves Notch within its transmembrane region to release the Notch ICD (NICD) portion (Kopan 2012).
Following these two cleavage passages, the NICD is released into the cytoplasm and then translocates into the nucleus to accomplish its transcriptional function. An important class of genes strictly regulated by the Notch transcriptional activity is represented by the nuclear HES and HEY gene family members. Indeed, the analysis of the expression of such molecules that act as downstream effectors of Notch signaling is a “mirror” of NOTCH pathway activation (Iso et al. 2003).

Notch signaling is involved in many physiological developmental events and regulates stem cell differentiation and self-renewal (Yuan et al. 2015). In this context, a great deal of literature in BC field reports the relation occurring between Notch-signaling with carcinogenesis and tumour progression (Gallahan et al. 1997). In particular, a critical and functional cross-talk between HER2 expression and activation, Notch signaling and stemness was originally shown in our lab by Magnifico et al. and, successively by others (Baker et al. 2014; Magnifico et al. 2009). In keeping with the important role played by Notch in the wide range of critical processes regulating the CSCs activity, different specific drugs were produced and pre-clinically tested in the attempt to halt this signaling pathway. In particular, different γ-secretase inhibitors (GSIs) were developed to prevent the final cleavage of NICD and its translocation into the nucleus. The clinical efficacy of these compounds is also currently under investigation in several trials for the treatment of the advanced BCs (Olsauskas-Kuprys et al. 2013).
1.3.1.2 Wnt signaling pathway

Alteration of the Wnt signaling is a common feature of many tumour types and is commonly identified as canonical β-Catenin-dependent or non-canonical β-Catenin-independent pathways (Zhan et al. 2017). The main event in canonical Wnt signaling is the cytoplasmatic accumulation of the transcriptional factor β-Catenin followed by its translocation into the nucleus where it mediates the direct or indirect up-regulation of transcriptional target molecules mainly involved in the EMT/CSCs regulation such as Twist, Snail1 and Snail2. In physiological condition, β-Catenin is degraded by a protein complex constituted by different molecules including Axin, adenomatous polyposis Coli (APC) and glycogen synthase kinase (GSK-3) whose activity maintains low levels of β-Catenin. The binding between the Wnt ligand and its specific Frizzled receptor inhibits the Axin-APC-GSK-3 protein complex leading to the block of β-Catenin degradation via its ubiquitination (Gordon et al. 2006). The non-canonical Wnt-signaling is independent by the transcriptional activity of β-Catenin and its role is strictly implicated with the embryonic development and in the regulation of the cell-cell adhesion. (Valenta et al. 2012)

Globally, Wnt signaling has a significant role in the initiation of carcinogenesis, in the regulation of CSC self-renewal and in the resistance to the systemic chemotherapy (De Sousa et al. 2016). The involvement of Wnt signaling in a broad spectrum of cancer biological mechanisms renders such pathway a primary target for its pharmacological inhibition. Recently, Novartis started with a Phase I trial using a specific inhibitor of Wnt signaling cascade in a variety of
malignancies as melanoma, BC and pancreatic adenocarcinoma, but the results are not yet public (Kahn 2014).

**1.3.1.3 Hedgehog signaling**

Hedgehog signaling is considered one the most important regulators of the embryonic stem cells activity and its proper activation by specific secreted factors as Sonic, Indian and Desert Hedgehog is essential for the cell differentiation. The binding of these ligands with the cell-membrane protein PTCH1 triggers the Hedgehog signaling through the activation of the GLI1 and GLI2 transcriptional activity and the induction of the Hedgehog target genes expression. Aberrant Hedgehog signaling is associated with the carcinogenesis and the progression of different tumours thus sustaining its closed link with the existence/activity of the CSCs. In fact, the genes whose expression is directly regulated by this pathways Nanog, Oct4, Bmi and Sox 2 are crucial for the self-renewal and CSCs differentiation into different cell lineages (Cochrane et al. 2015)). Currently, Vismodegib, a small-molecule inhibitor of the Hedgehog signaling, is approved for the treatment of basal-cell carcinoma (Chang et al. 2016) and different undergoing trials are validating its therapeutic activity for metastatic colon-rectal, lung and gastric cancers (Verline J, Clinical.Cancer.Research, 2016).

**1.3.2. Breast Cancer Stem Cells**

The strategic importance of the BCSC in sustaining tumour growth as well as their implication in the anti-cancer therapy resistance is still a topic extensively
under investigation. An important limitation in the CSC research is the capability to specifically recognize this small cell subset inside the bulk differentiated tumour mass. In order to solve this experimental problem and facilitate the study of the molecular pathways regulating CSC growth and survival, different peculiar markers of BCSCs were identified and found specifically expressed on their cell membrane. In particular, Al-Hajj et al demonstrated the possibility to distinguish breast cancer stem cells (BCSC) from the differentiated tumour cells counterpart on the basis of CD44+/CD24- phenotype (Al-Hajj et al. 2003). Furthermore, another CSCs surface- associated marker identified a decade ago was the aldehyde dehydrogenase (ALDH) (Ginestier et al. 2007). Recent studies demonstrated that both the CD44+/CD24- phenotype and the ALDH protein were able to distinguish two independent BCSC subsets. In particular, ALDH+ CSCs are characterized by an epithelial-like phenotype with an enrichment of mesenchymal to epithelial transition (MET) associated genes expression (i.e Cadherin and Occludin), high proliferative rate and were found located in the central area of the tumours (Liu et al. 2014). On the other hand, the CD44+/CD24- CSC subset biologically defined as mesenchymal-like CSCs, are characterized by higher expression of epithelial to mesenchymal transition (EMT) related genes (ZEB1, ZEB2 and vimentin), a quiescent phenotype and were found located in the tumour invasive front (Liu et al. 2014). These different CSC states were not static, but endowed of an intrinsic plasticity being able to transit from an EMT to a MET status and vice versa (Liu et al. 2014). In addition, it was also reported that tumour cells characterized by an ALDH+/CD44+/CD24- phenotype displayed the greatest tumour-initiating capability thus suggesting that this small overlapping
cell fraction could identify the “real” undifferentiated CSC compartment (Liu et al. 2014).

1.3.2.1 HER2 and Breast Cancer Stem Cells

The existence of a close relationship between HER2 overexpression/activity and CSCs regulation was unveiled by different studies (Korkaya H, Oncogene, 2008; Magnifico A, Clinical.Cancer.Res, 2009; Shad D, Genes & Disease, 2016). Korkaya et colleagues reported the first evidence supporting the capability of HER2 to regulate the CSCs subset thus driving tumourigenesis and invasion (Korkaya et al. 2008). In particular, they observed that HER2 overexpression/activity on tumour cells surface induced an enrichment of intratumour CSCs. A further study performed in our laboratory pointed out that “canonical intratumour CSCs” of HER2-overexpressing BCs were characterized by high levels expression of the HER2 oncoprotein (Magnifico et al. 2009) and, also, that the HER2 expression levels in CSCs were not exclusively related to the HER2 gene copy number, but, rather, depend on NOTCH1 protein activity, at least in part. Indeed the pharmacological inhibition of NOTCH1 activation significantly reduced the HER2 mRNA levels providing the evidence that such stemness-related signaling pathway regulates HER2 expression in the HER2-positive BCSCs. In this context, Baker et al reported the existence of a complex form of cross-talk between NOTCH1 and HER2 oncoprotein in HER2-positive BC (Baker et al. 2014). In addition, Pradeep et al have also shown that HER2 up-regulation induced the transcription of Notch3, another member of the Notch family. Conversely, the specific Notch3 inhibition achieved a reduction of HER2-positive cell proliferation
and survival, impairing also the formation of mammospheres (Pradeep et al. 2012).

Recent evidence underlines the importance of HER2 role not only in the CSCs of HER2-positive BC but also in the absence of HER2 amplification, i.e. luminal BC. In particular, Ithimakin and colleagues found a selective expression of HER2 in the CSCs derived from ER+/HER2- luminal BCs (Ithimakin et al. 2013). In addition, data from literature provided evidence for HER2 activity also in the CSC compartment of other epithelial HER2-overexpression cancers such as gastric cancer (Jiang et al. 2012).

1.3.2.3 CSC sensitivity to anti-HER2 targeted therapies

A great deal of data indicate that the complete eradication of the CSCs subset is strictly correlated with the therapeutic success of cancer therapies (Kumar et al. 2017; Yang et al. 2015). Taking into consideration the crucial role played by HER2 in the regulation of the CSCs in different BC subtypes, many groups have extensively studied the capability of specific anti-HER2 therapies to hit not only the bulk differentiated cell population but also the CSC counterpart. In fact, reported evidence clearly showed that HER2-targeted therapies, i.e. trastuzumab and lapatinib, can successfully target BC-associated CSCs (Farnie et al. 2014; Korkaya et al. 2008; Korkaya et al. 2013; Magnifico et al. 2009). A research conducted in our lab by Dr. Magnifico provided a clear evidence of the CSC sensitivity to the trastuzumab activity thus revealing that the clinical efficacy mediated by this biodrug is also related to its ability to target CSCs i.e. the “root” (population) of cancer growth, survival and metastatization (Magnifico et al. 2019).
2009). A further study has provided a clear evidence that trastuzumab mainly targets the ALDH+ CSC compartment whereas the CD44+/CD24- CSC population is not susceptible to this biodrug effects (Martin-Castillo et al. 2015). Then, to improve the beneficial targeting of HER2-positive CSCs, the combination of trastuzumab with pertuzumab has been found to eradicate more efficiently also the CD44+/CD24- CSCs subset compared to each monotherapy alone (Diessner et al. 2013).

In addition, Lapatinib, the TKI designed to halt the HER2 signaling, has demonstrated anti-CSC effects. In fact, Farnie et al showed that lapatinib inhibits the proliferation of CSCs, thus suggesting its use to reduce both the recurrence and distant metastatization of HER2-positive disease (Ablett et al. 2014).

1.4. d16HER2 splice variant

Different studies revealed that HER2-overexpressing BCs also express an alternative splicing of WTHER2 characterized by an in-frame skipping of 48 nucleotides (634-649) corresponding to exon 16 (d16HER2) (Figure 13). The exon 16 skipping leads to the loss of two crucial cysteine residues that, in turn, causes the disruption of the normal disulfide bonds structure promoting the formation of stable and constitutively activated d16HER2 homodimers. (Kwong et al. 1998; Siegel et al. 1999). The expression and the biological role of d16HER2 splice variant have been studied only in human HER2-positive BC models and, at present, no study in other different HER2-positive oncotypes or in other species has been reported.
Figure 13 – Schematic representation of human WTHER2 and d16HER2 isoforms. (From Ghedini et al, Atlas Genet Cytogenet Oncol Haematol, 2014).

1.4.1 d16HER2 role in breast cancer

The molecular analysis of d16HER2 expression performed in 46 BC samples immunohistochemically ranging from 0 to 3+ HER2 revealed that the d16HER2 isoform represents about 4-9% of total HER2 mRNA transcript (Castiglioni et al. 2006). In the same study published in our lab, Castiglioni and colleagues showed that d16HER2-transfected NIH3T3 cells proliferate “in vitro” faster vs WHER2 engineered cell counterparts. Furthermore, HEK293 cells, an immortalized human embryonic kidney cell line, ectopically expressing the human d16HER2 transgene developed tumours in athymic mice compared to HEK293 transduced with the human WHER2 isoform, significantly suggesting that only the d16HER2 variant is tumourigenic per se (Castiglioni et al. 2006).
In the attempt to better understand the tumourigenic pathways driven by the d16HER2 expression/activity, Mitra and colleagues, compared the signaling cascades activated by the ectopic expression of human d16HER2 vs WHER2 transgenes plasmidically transfected in the luminal HER2-negative BC cell line MCF7 (Mitra et al. 2009). They observed that only the d16HER2 overexpression promoted HER2 homodimerization thus enhancing the activation of tumour-related signaling pathways including FAK, PI3K/Akt and MAPK kinases. Furthermore, they also found that the d16HER2-driven signaling was mediated through its direct coupling with the non-receptor tyrosine kinase SRC (Mitra et al. 2009). In addition, in human HER2-positive BC samples the same authors found a significant correlation between the intratumour d16HER2 expression levels and positive nodal status. This finding represented a further important observation reflecting also a potential clinical involvement played by d16HER2 in tumour progression and metastatization (Mitra et al. 2009). In keeping with these data, Alajati and colleagues deeply investigate the biological role displayed by d16HER2 in BC models. In particular, they observed that the d16HER2 overexpression in the MCF10A cells, an immortalized human normal epithelial breast cell line, was associated with the activation of a distinct phosphoproteome that was found to include also tyrosine phosphorylated proteins not previously associated with the canonical WHER2-driven signaling. This peculiar “activated” signaling profile defined an aggressive phenotype associated with a reduced apoptosis and enhanced proliferation and tumour invasiveness (Alajati et al. 2013).
Further, they also provided a biochemical evidence that d16HER2 up-regulation increased the expression of a peculiar mesenchymal marker as N-Cadherin and, further, mediated an in vivo higher tumourigenic potential in comparison with the WTHER2-transfected cells (Alajati et al. 2013). Additionally, Turpin and collaborators confirmed the existence of a close link between d16HER2 overexpression and mesenchymal tumour phenotype and showed that the aberrant expression of d16HER2 variant promoted a pro-metastatic tumour microenvironment (Turpin et al. 2016).

To better understand the in vivo implications of d16HER2 expression, in collaboration with Dr. A. Amici (Department of Molecular, Cellular and Animal Biology of the University of Camerino, Camerino), using the bicistronic expression vector pMMTV-Δ16HER2-IRES, we generated a FVB transgenic mouse model overexpressing the human d16HER2 transgene (Marchini et al. 2011) originally excised from our plasmid vector pcDNA3/ΔHER2 (Castiglioni et al. 2006). The mouse mammary tumour virus (MMTV) promoter allows the expression of d16HER2 only in the mammary gland. A deep molecular analysis revealed that three-five d16HER2 transgene copies were inserted in a single site into the murine chromosome 5. The overexpression of d16HER2 induced a development of multiple asynchronous mammary lesions between 12 and 19 weeks of age (Marchini et al. 2011). The mean onset was established at 15.11±2.5 weeks (mean ± SD) indicating a rapid carcinogenesis and a bioluminescence analysis allowed to unveil the presence of pre-neoplastic lesions one month before tumours became palpable (Marchini et al. 2011).

Noteworthy, while 30-50 copies of the human WTHER2 transgene under the
control of MMTV-WHER2 were necessary to induce mammary carcinogenesis in about 80% of FVB transgenic mice (Finkle et al. 2004), only 5 copies of the human d16HER2 transgene were sufficient to generate neoplastic transformation in 100% of the female transgenic mice (Marchini et al. 2011). In 2016 Turpin, using an inducible transgenic mice model capable to express d16HER2 variant when treated with doxycycline, addressed the capability of the d16HER2 variant to drive an aggressive tumour phenotypes and found that mammary tumours developed in these peculiar transgenic mice were characterized by an enrichment of genes implicated in the EMT process, as outlined above (Turpin et al. 2016).

In a recent study, Gautrey provided evidence of the implication of two splicing regulator factors in the differential expression of the HER2 spliced transcripts. In particular, this study identified SRSF3 and hnRPH1 as the main splice factors implicated in the regulation of the d16HER2 expression (Gautrey et al. 2015). Using different experimental approaches, Gautrey and colleagues showed that SRSF3 activity increased the levels of d16HER2 variant. On the other hand, hnRPNH1 factor negatively regulates d16HER2 expression and increased the levels of the p100HER2 isoform (Hsu et al. 2013).

1.4.2. d16HER2 susceptibility to anti-tumour therapies

The sensitivity of d16HER2 to anti-HER2 targeted therapies was firstly addressed by Castiglioni et al using the engineered NIH3T3 and HEK293 cell lines respectively transfected with the human d16HER2 and WHER2 transgenes (Castiglioni F, 2006). This study was the first to reveal in in vitro setting the lower
efficiency of trastuzumab to bind d16HER2-positive cells vs those engineered with WHER2 transgene thus implying that d16HER2 expression could represent a possible mechanism of trastuzumab resistance (Castiglioni et al. 2006).

In a following study, Mitra et colleagues reported an intrinsic in vitro resistance of d16HER2-positive MCF7 transfectants to trastuzumab activity in comparison with the same cells engineered to express the WHER2 form (Mitra et al. 2009). They also suggested that the aggressive phenotype mediated by d16HER2 was due to its direct physical and functional interaction with the SRC kinase suggesting that dasatinib, a small TKI able to halt SRC kinase activity, promoted d16HER2 dephosphorylation thus impairing the tumourigenic potential guided by the splice variant (Mitra et al. 2009). In contrast with these in vitro results, Alajati and co-workers reported in vivo for the first time that trastuzumab mediated a strong anti-tumour effect against MCF10 cells engineered to express the human d16HER2 and injected orthotopically in the MFP of immunodeficient SCID mice. Finally, Turpin et al evaluated the therapeutic effects mediated by trastuzumab-DM1 vs the NMuMG cells, a murine cell line derived from the normal mammary gland tissue, transduced to ectopically express d16HER2 or WHER2 forms. The obtained results pointed out the capability to d16HER2 variant to mediate Trastuzumab-DM1 higher susceptibility vs the WHER2 counterpart cells (Turpin et al. 2016).

1.5. Aims of the thesis

d16HER2 splice variant is expressed in about 90% of total BCs and accounts for 4-9% of total full-length HER2 transcript. Taking the advantage of the availability of
the tg mouse models expressing the human d16HER2 variant and WHER2 isoform, my PhD thesis focused on the deep investigation of the biological roles exerted by d16HER2 in HER2-positive BC aggressiveness, sensitivity to HER2-targeted therapies and regulation of the CSC activity. In particular, to evaluate the genetic penetrance of the d16HER2 variant and to analyze its susceptibility to specific anti-HER2 biotherapies, we firstly performed a comparative analysis of the two available tg mouse models, by comparing the d16HER2- or WHER2-driven tumourigenicity and, successively, by investigating the downstream signalling pathways (Chapters 3 and 4). Different in vitro d16HER2- and WHER2-positive primary mammary tumour cell lines were generated and characterized (Chapters 3 and 4). We evaluated trastuzumab and lapatinib activity in the tg mouse models and, also, in mice orthotopically transplanted with tg mammary tumour cell lines expressing both d16HER2 and WHER2 (Chapter 3). In addition, based on the observed co-localization of activated d16HER2 and SRC (pSRC) on the same HER2-positive tumour cells of pre-clinical models, we evaluated in human HER2-positive BC cases the occurrence of such potential signalling axis and its impact on HER2-positive patients’ prognosis (Chapter 3). To investigate whether d16HER2 might be the predominant HER2 form involved in the regulation of BCSCs in HER2-positive BC, we compared the capability of d16HER2 and WHER2 isoforms to guide the enrichment of CSCs in both tg and properly engineered human tumour cell lines (Chapter 4). Further, through the analysis of the gene expression profiles of our tg pre-clinical models and HER2-positive BC cases, we performed a comparison analysis of the potential enrichment of CSC-related pathways (Wnt and Notch) in d16HER2 vs WHER2-positive mammary
tumour cell lines and in HER2-positive BC cases characterized by high vs low d16HER2 expression and activation (Chapter 4). Finally, we explored the possible involvement of d16HER2 in the biological mechanisms regulating CSC and at the basis of tumour aggressiveness in HER2-negative luminal BC (Luminal A) and in HER2-positive GC (Chapter 5).

The results reported in my PhD thesis demonstrate that d16HER2 splice variant is: 1) the main HER2 form governing maintenance/expansion of HER2-positive BCSCs, 2) significantly linked to HER2-positive BC tumourigenesis and aggressiveness and that 3) its expression and activation could be considered a marker of HER2-addiction and, in turn, susceptibility to specific anti-HER2 treatment. In addition, our data provide the first evidence of the expression and implication of d16HER2 variant also in another different epithelial oncotype characterized by the HER2-amplification.
Chapter 2 – MATERIALS AND METHODS
2.1 Cell biology

2.1.1. Tumour cell lines

2.1.1.1. Murine mammary d16HER2- and Wther2-positive tumour cell lines

Primary mammary adenocarcinomas spontaneously grown in FVB d16HER2-positive transgenic female mice were resected, weighed and conserved in RPMI 1640 medium culture (Lonza, Verviers, Belgium) supplemented with 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA), and 100 µg/ml streptomycin (Sigma) until use. Tumour samples were mechanically processed to generate tumour cell suspensions, which were then incubated in erythrocyte lysis buffer (0.15 M NH₄Cl, 15 mM NaHCO₃, 1 mM EDTA, pH 7.4), enzymatically digested with 300 U/ml collagenase and 100 U/ml hyaluronidase (StemCell Technologies, Vancouver, Canada) for 1 h at 37°C and then extensively washed in standard RPMI culture medium before being counted. Under sterile conditions, d16HER2-positive cells were selected using an AutoMACS™ separator (Miltenyi Biotec, Bergisch Gladbach, Germany). In particular, cells were probed with phycoerythrin (PE)-conjugated mouse mAb anti-human CD340 (erbB2/HER2) IgG1 (Biolegend, San Diego, CA, USA) and, successively, with magnetic microbeads conjugated with an anti-PE IgG1 MAb (Miltenyi Biotec) to perform an immune-magnetic isolation using a proper cell sorter. The magnetically labeled tumour cells were collected as the positive d16HER2-positive fraction whereas the undesired infiltrating cells were removed. The isolated transgenic d16HER2-
positive primary mammary tumour cell cultures named MI6 and MI7 were used throughout this Ph.D. project.

WHER2_1 and WHER2_2 mammary tumour cell lines, isolated from two distinct FVB-huHER2 transgenic female mice (Finkle et al. 2004), were kindly provided by Prof. PL Lollini and Prof. P. Nanni (Laboratory of Immunology and Biology of Metastasis, Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Bologna, Italy). These cell lines exclusively overexpress the full-length WHER2 form. Both d16HER2- and WHER2-positive mammary tumour cell lines were cultured in MammoCult medium (StemCell Technologies) supplemented with 1% fetal bovine serum (FBS, Sigma) and penicillin-streptomycin (Sigma) cultured at 37°C in a humidified 5% CO₂.

2.1.1.2. Human cancer cell lines

The human BC cell lines MCF7, T47D, BT474, MDAMB361, MDAMB453, HCC1954, SKBR3 and ZR75.30 and GC cell lines MKH45, 23132/87, OE19 and NCI-N87 were purchased from the American Type Culture Collection (Rockville, MD, USA). The HER2-negative BC cell lines MCF7 and T47D, HER2-overexpressing BC cell lines HC1954, SKBR3 and ZR75.30, HER2-negative GC cell lines MKH 45 and 23132/87 and HER2-positive GC cell lines EO19 and NCI-N87 were cultured in RPMI 1640 (Lonza) supplemented with 10% FBS (Sigma). HER2-positive BC cell lines BT474 and MDAMB361 and MDAMB453 were cultured in DMEM (Lonza) supplemented with 10% FBS (Sigma). Engineered MCF7 and T47D cells were cultured in RPMI1640 (Lonza). All tumour cell lines were maintained with 10%
FBS in a humidified 5% CO₂ atmosphere at 37°C and routinely tested for mycoplasma contamination.

2.1.1.3 Generation of human engineered cell lines

Lentiviral vectors coding for d16HER2 and WHER2 were constructed using a third-generation self-inactivating lentiviral system that, being based on four different plasmids, offers maximal biosafety. The backbone consisted of pRRL-sin-cPPt.CMV-GFP.WPRE (kindly provided by Dr. Ferrari, HSR, Milan) in which the GFP sequence was replaced by either d16HER2 or WHER2 transgenes. The cDNA for d16HER2 and WHER2 was excised using the *XhoI* restriction enzyme from the pCDNA3-d16HER2 (Castiglioni et al. 2006) and pCDNA1-Neo-WHER2 (Di Fiore et al. 1987), respectively, followed by blunting with T4 DNA polymerase (New England Biolabs, Ipswich, MA, USA). The inserts were cloned into a viral backbone and sequenced for confirmation. Empty vector (obtained by excision of the GFP sequence) was used to infect target cells and to obtain Mock-transduced cell lines, respectively named MCF7-Mock and T47D-Mock. Lentiviral particles were produced using standard methods. Briefly, lentiviral particles were generated by transient cotransfection of the transfer vector constructs pRRL-SIN-CMV-d16HER2 or pRRL-SIN-CMV-WHER2 with the VSV-G expressing construct pMDG and the third-generation packaging constructs pMDLg/pRRE and pRSVRev (Dull et al. 1998) into 293T cells. The medium was changed 20 h later and replaced with fresh medium. The supernatant was collected 24 h later and used for target cell infection at 1:2 in presence of 8 µg/ml of polybrene to facilitate viral entry into the cells. The medium was changed the next day, and the
efficiency of transduction was evaluated by FACS analysis using anti-HER2 antibody after 4 days. The same experimental approach was performed to produce shRNA in order to generate stables clones silenced for d16HER2 expression. In this context, a lentivirus vector coding for the 5’-CTCCCCTCTGACGTCCA – 3’ sequence was used. MCF7 and T47D cells infected with pRRL-SIN-CMV-d16HER2 and pRRL-SIN-CMV-WHER2 were respectively named MCF7_d16, T47D_d16, MCF7_WT and T47D_WT and were always tested in parallel with corresponding mock cells as control. Human transduced cell lines were enriched for d16HER2 and WHER2 expression by an immune-based cytofluorimetric cell sorting performed under sterile conditions before each bioassay, except for the Mammosphere Forming Efficiency evaluation. Briefly, d16HER2- and WHER2-positive cells were incubated for 30 min at 0°C with anti PE-anti-human CD340 (erbB2/HER-2) antibody (BioLegend, San Diego, CA, USA), washed twice with PBS 1X and sorted using a FACS Aria cytometer (BD Bioscience, San Jose, CA, USA). The HER2-positive engineered cells were identified using the basal expression of HER2 in MCF7-Mock and T47D-Mock cells as threshold.

2.1.2 Western blot analysis

Transgenic d16HER2 and WHER2-positive lesions, MI6, MI7 WHER2_1 and WHER2_2 cell lines, lentivirus-engineered BC and OE19 and N87 GC cell lines were solubilized for 40 min at 0°C with lysis buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 2 mM Na-orthovanadate, and protease inhibitor cocktail (Complete Mini, Roche, Basel, Switzerland). The samples were-
mixed with gel sample buffer under non-reducing and reducing conditions, heated for 5 min at 95°C, and resolved by electrophoresis on pre-cast 3-8% Tris-acetate polyacrylamide gels and 4-12% bis-tris gels (Invitrogen, Thermo Fisher, Waltham, MA, USA). The separated proteins were electrophoretically transferred onto nitrocellulose filters, stained with Red Ponceau to evaluate protein loading, washed with 0.5 % Tween-20 1X TBS, and saturated for 1 h at room temperature in blocking solution (5 % low-fat milk in 0.1 % Tween-20 1X TBS) with (5 % low-fat milk in 0.1 % Tween-20 1X TBS) before addition of primary antibodies in 3 % low-fat milk in 0.1 % Tween-20 1X TBS for 1 h at room temperature or overnight at 4°C with gentle shaking. The following primary mouse MAbs were used:
- 1:100 Ab3 c-erbB-2/HER2/neu IgG1 (Calbiochem, Darmstadt, Germany);
- 1:300 Anti-SRC, clone GD11, IgG1 Ab (Millipore Corp., Billerica, MA, USA);
- 1:1000 phospho-STAT3 (B-7 clone) IgG2b (Santa Cruz Biotechnology, Santa Cruz, CA, USA);
- 1:1000 Anti-VINCULIN IgG1 Ab (Sigma);
- 1:50.000 anti-β-ACTIN-peroxidase (clone AC-15) (Sigma).

The following primary rabbit polyclonal antibodies were used:
- 1:2000 anti-phospho-HER2 p-Neu (tyr 1248) IgG (Santa Cruz Biotechnology)
- 1:200 STAT3 (C-20) IgG Ab (Santa Cruz Biotechnology);
- 1:2000 anti-phospho-p44/42 MAPK (ERK1/2) (Cell Signalling Technology, Danvers, MA, USA);
- 1:1000 anti-p44/42 MAPK (ERK1/2) (Cell Signaling Technology);
- 1:1000 anti-phospho-AKT (Ser473) (Cell Signaling Technology);
- 1:1000 anti-AKT (Cell Signaling Technology);
- 1:1000 anti-phospho-SRC family (Tyr416) Ab (Cell Signaling Technology).
- 1:1000 anti-SOX10 (Abcam, Cambridge, UK)
- 1:500 anti-FAK (Abcam)
- 1:1000 Anti-phospho-FAK (Tyr397) (Abcam)
- 1:1000 Anti-TGF-β (Abcam)
- 1:2000 Anti-NOTCH4 (Millipore Corp. Fox Ridge CT Boulder, CL, USA)
- 1:200 Anti-WNT5A (R&D Systems, Minneapolis, MN, USA)

Filters were washed with 0.5 % Tween-20 1X TBS and probed with 1:5000 HRP-conjugated goat anti-mouse Ig or 1:10000 HRP-conjugated donkey anti-rabbit Ig (Amersham GE Healthcare, Little Chalfont, UK) for 1 h at room temperature (RT). The signals were detected using enhanced chemiluminescence (ECL, Amersham GE Healthcare). Levels of phosphorylated and basal protein expression were assessed relative to those of housekeeping genes in the same sample; phosphorylated protein expression was further normalized relative to basal protein expression by Quantity One (Bio-Rad, Hercules, CA, USA). Linear regression analyses were calculated between pd16HER2D (under non-reducing conditions) and pSRC (under reducing conditions) levels and between pd16HER2M and pSRC (both under reducing conditions) levels in d16HER2 tg tumour samples and between pHER2M and pSRC levels (under reducing conditions) in WHER2 tg tumour samples.
2.1.3. Immunohistochemistry analyses of transgenic tumour samples

Murine lesions were excised and successively fixed in 10% neutral buffered formalin for 1 to 2 days. In collaboration with Dr. M. Iezzi (University G. D’Annunzio, Chieti, Italy), paraffin blocks were prepared and slides were stained with hematoxylin and eosin (H&E) for histological examination. For immunohistochemistry, slides were deparaffinized, serially rehydrated and, after the appropriate antigen retrieval procedure, stained with primary antibodies against the following molecules:

- HER2 (polyclonal rabbit anti-human, A0485) (Dako);
- pSRC-Tyr 416 (polyclonal rabbit Ab 2101) (Cell Signalling Technology).

Then, slides were incubated with the appropriate secondary antibodies (Jackson ImmunoResearch, LiStarFish, Milan, Italy). Immunoreactive antigens were detected using streptavidin-peroxidase (Lab Vision Corp. – Thermo Scientific) and the DAB Chromogen System (Dako). Image acquisition and analysis were performed using Zeiss LSM 510 Meta confocal microscope (Zeiss, Oberkochen, Germany).

2.1.4. Immunofluorescence analyses of murine/human tumour samples and tumour cell lines

In collaboration with Dr. M. Iezzi (University G. D’Annunzio, Chieti, Italy), primary d16HER2 transgenic tumours and 84 formalin-fixed paraffin-embedded (FFPE)
primary human breast lesions were analyzed by immunofluorescence (IF) and confocal microscopy to assess HER2 and pSRC (%) expression. All tumour samples were probed with the same primary antibodies used in the immunohistochemical analysis (paragraph 2.1.3). Secondary antibodies conjugated with Alexa 488 and Alexa 546 (Invitrogen, Life Technologies) were used. Nuclei were stained with DRAQ5 (Alexis, Life Technologies). Image acquisition and analysis were performed using Zeiss LSM 510 Meta confocal microscope (Zeiss). Ten visual fields from different areas of each tumour (magnification 200X) were evaluated for each marker. Sections stained with pSRC-Y416 were scored based on the percentage of positive tumour cells within the whole tissue section. Tumour-specific pSRC expression was evaluated exclusively on HER2-positive tumour cells as pSRC (%). Cases with pSRC-Y416 > 20% were considered to have high pSRC-Y416 staining.

IF analyses were also performed on transduced tumour cells grown in adherent condition. T47D-Mock, T47D-d16, T47D-WT, MCF7-Mock, MCF7-d16 and MCF7-WT cell populations were seeded on 1μ-Slide 8 well (ibiTreat, Ibidi, Munich, Germany). Tumour cells were fixed with 4 % (w/v) paraformaldehyde for 7 minutes at room temperature, incubated for 90 minutes in 10 % mouse serum. The following antibodies were used:

- 1:5 phycoerythrin (PE)-conjugated anti-human CD340 (erbB2/HER-2) IgG1 (Biolegend, San Diego, California, U.S)
- 1:10 Allophycocyanin (APC) anti-human CD44 (BD Bioscience, Franklin LAKES, New Jersey, U.S)
The slides were examined using a Leica TCS SP8 X confocal laser scanning microscope (Leica Microsystems GmbH, Mannheim, Germany) and images were acquired in a single plane (1024x1024 pixels) using an HC PL APO CS2 63X/1.40 oil-immersion objective and a pinhole set to 1 Airy unit. The data were analyzed using Leica LASX rel. 1.1 software (Leica Microsystems GmbH) with a tool that measures gray-scale values in images inside linear regions of interest (ROI). The results are evaluated by calculating number of pixels and the arithmetical mean of the measured gray-scale values.

2.1.5. Flow cytometry analyses

Direct IF was performed on MI6 and MI7 cells to evaluate d16HER2 expression levels before and after AutoMACS procedure and on Wther2_1 and Wther2_2 cells to evaluate the full-length HER2 expression. Tumour cells were stained for 30 min at 0°C with:

- 20 μl/10⁶ cells PE-conjugated mouse anti-human CD340 (erbB2/HER-2) (Biolegend);
- 2.5 μg/ml APC-conjugated rat anti-mouse CD326 (Ep-CAM) IgG2a (Biolegend);
- Fluorescein (FITC)-conjugated mouse anti-CD45 IgG1 (BD Pharmingen).

Murine MI6, MI7, Wther2_1, Wther2_2 and human MCF7_d16, MCF7_WT, MCF7-Mock, T47D_d16, T47D_WT, aT47D-Mock tumour cell lines were analyzed through direct IF to determined basal and ectopic expression of both d16HER2 and Wther2 isoforms. Tumour cells were stained for 30 min at 0°C with:

- 1:20 PE-conjugated mouse anti-human CD340 (erbB2/HER-2) (Biolegend)
MI6 and WHER2_1 cells and freshly isolated (ex-vivo) cell suspensions from d16HER2- and WHER2-positive transgenic primary lesions were analyzed by multiparametric flow cytometry to evaluate the expression of molecular markers related to cancer stemness. Tumour cells were stained for 30 min at 0°C with:

- 1:150 PerCP-Cyanine5.5 rat anti-mouse CD24 (eBioscience, San Diego, CA, USA)
- 1:150 PE-Cyanine7 Armenian hamster anti-mouse/rat CD29 (Integrin beta 1) (eBioscience, San Diego, CA, USA)
- 1:100 FITC-conjugated rat anti-mouse Ly-6A/E (Sca-1) (eBioscience, San Diego, CA, USA).

MCF7_d16, MCF7_WT, MCF7-Mock, T47D_d16, T47D_WT, T47D-Mock tumour cells were stained for 30 min at 0°C with:

1:10 APC-conjugated mouse APC anti-Human CD44 (BD Bioscience)

In all IF experiments, the data were processed with FlowJo software (Tree Star, Inc., Ashland, OR, USA).

2.1.6. ALDEFLUOR assay

ALDH activity was measured using the ALDEFLUOR assay kit (StemCell Technologies) in MCF7_d16, MCF7_WT, MCF7-Mock, T47D_d16, T47D_WT, T47D-Mock cell lines as per the manufacturer’s instructions. First, 10^6 cells were suspended in ALDEFLUOR assay buffer that contains an ALDH substrate, bodipy-aminoacetaldehyde (BAAA), and incubated for 1 h at 37°C. A specific inhibitor of ALDH, diethylaminobenzaldehyde (DEAB), was used to distinguish ALDH-positive and -negative cells. The same samples were incubated with PE-anti-human
CD340 (erbB2/HER-2) Ab (1:20) (BioLegend, San Diego, CA, USA) for 1 h at 37°C to analyze HER2 expression. The analysis of ALDH-activity was performed only on HER2-expressing cells using the basal expression of HER2 in MCF7-Mock and T47D-Mock cells as the threshold.

2.1.7 Mammosphere-forming efficiency (MFE) assay

Dissociated MI6, WTHER2_1, MCF7_d16, MCF7_WT, MCF7-Mock, T47D_d16, T47D_WT, T47D-Mock single tumour cells were plated in 6-well, ultra-low attachment plates (Corning) at a density of 500 cells/cm² and grown for 7 days as described (Dontu et al. 2003). Subsequent cultures after dissociation of primary spheres were replated at the same density. Mammospheres were grown in serum-free MammoCult Medium (StemCell Technologies). MFE was calculated as number of spheres (diameter 60 µm) formed in 7 days/initial number of single cells seeded and given as a percentage.

MFE inhibition was assessed in MI6 and WTHER2_1 cells initially plated at a density of 500 cells/cm² and treated at day 0 with trastuzumab (50 µg/ml) (Roche, Basel, Switzerland), 0.5 and 1 µM of GSI DAPT agent (Calbiochem-Merck, Darmstad, Germany), 2 and 4 µM of GSI RO4929097 (Roche), or with 0.1% of their diluent DMSO; GSI treatments were refreshed after 72 h of culture. MFE inhibition (%) was calculated as {(untreated MFE-treated MFE)/(untreated MFE)}X100.

2.1.8 siRNA transfection

To knock-down the WHER2 gene in the human HER2-overexpressing BC cells
BT474, specific siRNA oligonucleotides directed against the WHER2 receptor were designed (siRNA sequence: 5’-CTGGATGACAAGGGCTGCC-3’) (Life Technologies) and properly tested. Cells were incubated with siRNA constructs in presence of the Lipofectamine RNAi Max transfection reagent (Life Technologies) following manufacturer’s instructions, and then harvested after 72 h for RNA analyses.

2.2 Molecular Biology

2.2.1 FISH analysis

The status of HER2 was investigated in ex vivo d16HER2 and WHER2-positive cells derived from corresponding spontaneous transgenic tumours, d16HER2- and WHER2-positive mammary tumour cell lines and human MCF7_d16, MCF7_WT, MCF7-Mock T47D_d16, T47D_WT, and T47D-Mock cells using PathVision HER2/neu DNA probe kit (Vysis, Abbot Molecular, Abbot Park, IL, USA) in collaboration with Patrizia Gasparini (Tumour Genomics Unit, Dept. of Research, Fondazione IRCCS Istituto Nazionale dei Tumori). FISH analysis was performed in 100 nuclei and at least 10 metaphase spreads as described (Castagnoli et al. 2014). Briefly, slides were pretreated with 2X SSC/0.5% (v/v) NP40 at 37°C for 30 min and co-denatured at 70°C for 2 min and 37°C overnight using Hybrite kit (Vysis). HER2 amplification was determined based on the presence of clusters in at least 10% of analyzed cells. FISH hybridized slides were analyzed with an Olympus BX51 microscope coupled to a charge-coupled device camera COHU 4912 (Olympus, Milan, Italy). Captured images were analyzed using Mac Probe software (PowerGene, Olympus).
2.2.2 RNA extraction and quantitative RT-PCR (qPCR)

cDNAs were reverse-transcribed from 1 µg of total RNA in a 20-µl volume with SuperScript III (Invitrogen) using oligo-(dT) primers and examined by qRT-PCR using Applied Biosystems SYBR® Green dye-based PCR assay on the ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA). Supplementary Table 4 lists the primers used to detect genes. All primer sets were used under identical qRT-PCR cycling conditions with similar efficiencies to obtain simultaneous amplification in the same run. The data were normalized to GAPDH. The mRNA transcripts levels of gene analyzed in MI6 and WHER2_1 cells was calculated by the comparative Ct method and represented as the ratio between MI6 and WHER2_1 relative expression.

Total RNA from murine and human tumour cell lines and frozen human HER2-positive BC was extracted with Qiazol reagent (Qiagen, Valencia, CA, USA) according to manufacturer’s instructions. Frozen tissues were first grinded with a mixer mill MM 200 unit (Retsch, Hann, DK). Briefly, tumours were disaggregated in Qiazol with a bead-mediated disaggregation protocol (frequency 30/s for 2 minutes); the suspension was centrifuged at 1500 rpm for 5 minutes and Qiazol supernatant was recovered for RNA extraction. For gene quantification, cDNAs were reverse-transcribed from 1 µg of total RNA in a 20-µl volume with SuperScript III (Invitrogen) using oligo-(dT) primers and examined by qRT-PCR using Applied Biosystems SYBR® Green dye-based PCR assay on the ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA). The data were normalized to GAPDH. Table 4 lists the primers used to detect genes. All primer sets were used under identical qRT-PCR cycling conditions with
similar efficiencies to obtain simultaneous amplification in the same run (Table 4).

**TABLE 4. SEQUENCES OF CUSTOM PRIMERS FOR SYBR GREEN-BASED QRT-PCR.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH Human Fw</td>
<td>5'- GCTCACTGGCATGGCCTTC-3</td>
</tr>
<tr>
<td>GAPDH Human Rev</td>
<td>5'- CCTTCTTAGTAGTACCATATTTGGC-3’</td>
</tr>
<tr>
<td>d16HER2 Human Fw</td>
<td>5'- CACCCACTCCCCCTGAC-3’</td>
</tr>
<tr>
<td>d16HER2 Human Rev</td>
<td>5'- GCTCCACCCAGCTCGGTTTCTG -3’</td>
</tr>
<tr>
<td>WTHER2 Human Fw</td>
<td>5'- GTGTGGGACTGACGACAGGACAGG -3’</td>
</tr>
<tr>
<td>WTHER2 Human Rev</td>
<td>5'- GCTCCACCCAGCTCGTTTCTG -3’</td>
</tr>
<tr>
<td>Gapdh Mouse Fw</td>
<td>5'- GCTCAGGCTGATGGCCCTTC -3’</td>
</tr>
<tr>
<td>Gapdh Mouse Rev</td>
<td>5'- CCTTCTTAGTAGTACCATATTTGGC -3’</td>
</tr>
<tr>
<td>Hey2 Mouse Fw</td>
<td>5'- TGAGAAGACTAGTGCCCAACAG -3’</td>
</tr>
<tr>
<td>Hey2 Mouse Rev</td>
<td>5'- TGGGCATCAAAGTAGCCCTTA -3’</td>
</tr>
<tr>
<td>Frizzled 5 Mouse Fw</td>
<td>5'- CCGAAGGAGAGAGGACAGG -3’</td>
</tr>
<tr>
<td>Frizzled 5 Mouse Rev</td>
<td>5'- ACAAGGCTAGTGCTGTGGGC -3’</td>
</tr>
<tr>
<td>Notch 3 Mouse Fw</td>
<td>5'- AAGCAGCTAGTGCCCAACAG -3’</td>
</tr>
<tr>
<td>Notch 3 Mouse Rev</td>
<td>5'- TCCGACTTTTAAAGGGCAAC -3’</td>
</tr>
<tr>
<td>Notch 4 Mouse Fw</td>
<td>5'- TTCCCATGCTGCTGTGGCCTCT -3’</td>
</tr>
<tr>
<td>Ptk 2 Mouse Fw</td>
<td>5'- GCCCTCGACTTACGACGGATTAG -3’</td>
</tr>
<tr>
<td>Ptk 2 Mouse Rev</td>
<td>5'- GCTCAGGCTAGTGCTGTGGGC -3’</td>
</tr>
<tr>
<td>Sox10 Mouse Fw</td>
<td>5'- CTTGGCTGCACAACCTCAGAG -3’</td>
</tr>
<tr>
<td>Sox10 Mouse Rev</td>
<td>5'- GCCCTCGACTTACGACGGATTAG -3’</td>
</tr>
<tr>
<td>Tgfβ1 Mouse Fw</td>
<td>5'- GCAACATGTGGAACCTCTACAAGGA -3’</td>
</tr>
<tr>
<td>Tgfβ1 Mouse Rev</td>
<td>5'- GACGTCAAAAGACAGCCACTCA -3’</td>
</tr>
<tr>
<td>Wnt5a Mouse Fw</td>
<td>5'- CTACGAGAAGAGAGGACAGGAGG -3’</td>
</tr>
<tr>
<td>Wnt5a Mouse Rev</td>
<td>5'- TGCTTGTGCACTTCAATG -3’</td>
</tr>
<tr>
<td>Wnt5b Mouse Fw</td>
<td>5'- ATGCCAGAAGAGAGGACAGGAGG -3’</td>
</tr>
<tr>
<td>Wnt5b Mouse Rev</td>
<td>5'- ACTTTCGAGAGGACAGGACAGG -3’</td>
</tr>
</tbody>
</table>

The relative expression was calculated using the comparative $2^{-\Delta\Delta C_t}$ method. The relative expression of d16HER2 mRNA compared with that of WTHER2 was calculated by the comparative $C_t$ method with d16HER2 transcript levels indicated as the ratio $2^{-(\Delta C_t) d16HER2}/2^{-(\Delta C_t) WTHER2}$. To correlate d16HER2 transcript and pSRC expression levels in human breast cancers, gene expression
data were split in two groups according to tertiles: low, containing values under the first tertile, and high, containing values greater than the first tertile. The mRNA transcripts levels of gene analyzed in MI6 and WETHER2_1 cells was calculated by the comparative Ct method and represented as the ratio between MI6 and WETHER2_1 relative expression.

2.2.3 RT² Profiler PCR Array

The mouse EMT RT² Profiler PCR Array, which profiles the expression of 84 key genes, was purchased from SABiosciences (Qiagen). Total RNA from MI6 and WITHER2_1 cells was used for screening by qRT-PCR following the manufacturer's instructions.

2.2.4 Gene expression profiles of mammary tumour cell lines

Gene expression profiles were generated using the Illumina MouseWG-6 v2.0 Expression BeadChip (Illumina, Inc., San Diego, CA, USA), according to Illumina protocol in collaboration with the Functional Genomics and Bioinformatics Core Facility, Department of Applied Research and Technological Development, Fondazione IRCCS Istituto Nazionale dei Tumouri. Illumina BeadScan software was used for image acquisition. Raw data were log2-transformed and quantile normalized using the Bioconductor lumi package (18467348). Probes were annotated using the illuminaMousev2.db package. Probes without HUGO gene symbol annotation were filtered out. Multiple probes mapping to the same gene were collapsed, selecting the probe with the highest mean expression across samples using the “collapseRows” function from the WGCNA package.
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(19114008).

2.3. In vivo experiments

Animal care and experimental procedures were approved by the Ethics Committee for Animal Experimentation of the Fondazione IRCCS Istituto Nazionale dei Tumouri and performed in accordance with Italian law.

2.3.1. Transgenic mice

A breeding colony of d16HER2-LUC transgenic mice, previously reported (Marchini et al. 2011), was bred in the Animal Facility of Fondazione IRCCS Istituto Nazionale dei Tumouri. Animal care and experimental procedures were approved by the Ethics Committee for Animal Experimentation of the Institute according to Italian law. DNA purification from tail biopsies was used for genotyping by PCR analysis (primers: F: 5′-GGCTCAGTGACCTGTTTTGG-3′ and R: 5′-TGATGAGGATCCAAAGACC-3′), with an expected amplicon length of 231 bp. Mice were inspected twice weekly by palpation. FVB-huHER2 (WHER2) transgenic mouse line MMTV.f.hu.HER2#5 (Fo5) carries the full-length normal huHER2 gene under the control of the MMTV promoter on an FVB background (Finkle et al. 2004) and was obtained from Genentech, Inc. (South San Francisco, CA, USA). FVB-huHER2 mice were bred in animal facilities of the DIMES Department of the University of Bologna and genetically screened by PCR using a primer set specific to human growth hormone exons 4 and 5 included in the transgene backbone (Finkle et al. 2004). Mice were inspected weekly by palpation. In vivo experiments were performed in compliance with the Italian and European
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guidelines and were approved by the institutional review board of the University of Bologna. Progressively growing masses ≥50 mm³ were scored as tumours in both transgenic models.

2.3.1.1. In vivo therapy of transgenic mice

d16HER2 tg mice were injected intraperitoneally (i.p) with trastuzumab (Roche) or 0.9% NaCl diluent solution in a short (n=8/group) and prolonged (n=7-8/group) administration protocol. In the short treatment, transgenic mice were treated i.p. with 8 mg/kg trastuzumab (Roche) once per week for 5 weeks starting from 8 weeks, when only microscopic tumour lesions are present (Marchini et al. 2011), until 12 weeks of age. The study was terminated at 29 weeks of age when all d16HER2 mice developed the first spontaneous tumour. In the prolonged protocol, d16HER2 transgenic mice received 4 mg/kg trastuzumab (Roche) twice weekly from 8 until 42 weeks of age. In each experiment, tumours were calibrated twice weekly and tumour volume was calculated as 0.5 X d1² X d2, where d1 and d2 are the smaller and larger diameters, respectively. The use of the two different dosing schedules of trastuzumab administration is based on the reliable results we previously obtained (Ghedini et al. 2010; Magnifico et al. 2009).

2.3.2. In vivo tumour growth of d16HER2- and WHER2-positive tumour cell lines

To verify the orthotopic setting as suitable model to assess drug sensitivity of d16HER2 vs WHER2, FVB female mice (6-8 weeks old, body weight 20-25 g)
were purchased (Charles River, Calco, Italy). Mice (n=3/group) were injected into the mammary fat pad (m.f.p.) with $1 \times 10^6$ MI6 or W Ther2_1 tumour cells. Tumours were calibrated twice weekly and tumour volume was calculated as described above (2.3.1.1). At the end of in vivo experiments, tumours and lungs were harvested and fixed overnight in 10 % neutral-buffered formalin and, following, transferred into 70 % ethanol before processing and paraffin-embedding for histopathological analyses. Paraffin sections (5-µm thick) were stained as indicated in paragraph 2.1.3.

2.3.2.1. In vivo therapy of m.f.p-injected mice

In all the experiments performed in the orthotopic setting, mice were injected into the m.f.p. with $1 \times 10^6$ MI6 or W Ther2_1 tumour cells. In the first experiment, when tumours reached 50 mm$^3$, 20 mice injected with MI6 or W Ther2_1 cells were randomized into 2 groups (n=10 group) to receive biweekly i.p. injections of 4 mg/kg trastuzumab (Roche), or diluent NaCl solution (0.9 %). Mice were sacrificed when tumour volumes reached $\sim 2,000$ mm$^3$. In the second experiment, when tumours reached 50 mm$^3$, 14 mice injected with MI6 or W Ther2_1 cells were randomized into 2 groups (n=7/group) to receive daily per os administration of 100 mg/kg lapatinib (LC Laboratories, Woburn, MA, USA), or per os administration of DMSO. Mice were sacrificed when tumour volumes reached $\sim 2,000$ mm$^3$. In the third experiment, when the tumours reached 150, 100 or 50 mm$^3$, 5 mice for each tumour volume received biweekly i.p. injections of 4 mg/kg trastuzumab (Roche). The control group (indicated as untreated) was treated i.p. with 0.9 % NaCl diluents solution. Mice were sacrificed when tumour
volumes reached ~2,000 mm$^3$.

2.3.2.2 In vivo tumourigenicity

Eight-week-old female FVB mice (n=5 or 6/group), purchased from Charles River Laboratories, were injected with serial dilutions of MI6 or WHER2_1 cells from 10 to 10$^6$ bilaterally into the m.f.p. Tumour onset was set at 50 mm$^3$, and the mice were monitored twice weekly for up to 6 months. The frequency of stem cells between groups was determined using the ELDA web tool for limiting dilution analysis (Hu et al. 2009)

2.4. Patient cohort

The 84 HER2-positive BC patients included in this study were part of the observational retrospective multicenter Italian study GHEA (Campiglio et al. 2013). Patients were treated with trastuzumab (Roche) in an adjuvant setting in Fondazione IRCCS Istituto Nazionale dei Tumouri, Milan, from 2005 to 2009. BC first relapse events were registered and relapse-free survival (RFS) was defined as the time from start of trastuzumab treatment to the first event. Table 6 lists the pathobiological and clinical characteristics of the HER2-positive cohort. The Independent Ethics Committee of Fondazione IRCCS Istituto Nazionale Tumouri, Milan, approved the molecular characterization of material from patients included in this observational study.
2.4.1. In silico analyses

In collaboration with Dr. Triulzi (Molecular Targeting Unit, Department of Research, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan) gene pathways differentially enriched in activated d16HER2-expressing human BCs were evaluated by Gene Set Enrichment Analysis using GSEA v2.0.13 (Subramanian et al. 2005) on a 193-cancer-related gene set (Triulzi et al. 2013). Permutation type was applied 1,000 times. Core members of each significantly (p<0.05) enriched gene set were extracted and their mean expression levels were considered as the ‘activated-d16HER2 metagene’ value. Data for the ‘activated-d16HER2 metagene’, constructed based on the Illumina Whole-Genome DASL® gene expression profiling of 21 HER2-positive BCs characterized for pSRC and d16HER2 expression (GSE55348) (Table 5), were quantile-normalized using BeadStudio software and filtered with a data matrix containing 22,121 probes, corresponding to 15,715 Entrez Ids.

Table 5 – List of cases of the GSE55348.

<table>
<thead>
<tr>
<th>Case</th>
<th>GEO_accn</th>
<th>d16HER</th>
<th>pS</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA</td>
<td>GSM1334487</td>
<td>high</td>
<td>lo</td>
</tr>
<tr>
<td>AZ7</td>
<td>GSM1334489</td>
<td>low</td>
<td>hig</td>
</tr>
<tr>
<td>AZ6</td>
<td>GSM1334490</td>
<td>low</td>
<td>lo</td>
</tr>
<tr>
<td>BQ</td>
<td>GSM1334493</td>
<td>high</td>
<td>lo</td>
</tr>
<tr>
<td>BA</td>
<td>GSM1334498</td>
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<td>lo</td>
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<tr>
<td>BD</td>
<td>GSM1334508</td>
<td>high</td>
<td>lo</td>
</tr>
<tr>
<td>AZ8</td>
<td>GSM1334530</td>
<td>high</td>
<td>lo</td>
</tr>
</tbody>
</table>
The metagene was calculated in HER2-positive BC biopsies of two publically available datasets, GSE22358 (Gluck et al. 2012) and GSE41656 (de Ronde et al. 2013), for which pathological complete response information was available. Differences in ‘activated-d16HER2 metagene’ values between responders and non-responders were evaluated by unpaired t-test. Area under the ROC curve was calculated by nonparametric ROC analysis (Hajian-Tilaki et al. 1997).

To classify human HER2-positive BCs as “activated-d16HER2 metagene” High or Low, we assessed the expression of the metagene in GSE55348 (Triulzi et al. 2015) and NKI (Van de Vijver et al. 2002) gene expression datasets. Microarray and clinical data of the NKI cases were retrieved from the Computational Cancer Biology website present at The Netherlands Cancer Institute.
Gene annotation was performed using the SOURCE Database (https://source-search.princeton.edu/). Briefly, for each sample, we first calculated the mean expression of the 73 genes included in the “activated-d16HER2 metagene”, a transcriptional signature found up-regulated in HER2-positive BCs expressing activated d16HER2. We labeled samples as d16HER2-High if the metagene expression exceeded the upper tertile. GSEA between d16HER2-High and –Low human samples was run ranking genes according to the t statistic. Notch gene expression was calculated as the mean of log2 expression of genes belonging to the Notch gene list in both datasets.

2.5. Statistical analyses

Differences in tumour multiplicity curves in both d16HER2 and WHER2 transgenic models and differences in trastuzumab antitumour activity in orthotopic MI6 and WHER2 models were calculated, by two-tailed unpaired t-test. Overall, differences between groups were tested using a two-tailed unpaired t-test. Differences were considered significant at p<0.05. Linear regression and Pearson’s correlation coefficient r were calculated to estimate the correlation of: 1) pd16HER2M and pd16HER2D with pSRC and of pWHER2 with pSRC levels both under non-reducing and reducing conditions in protein extracts from both d16HER2 and WHER2 transgenic models; 2) d16HER2 with WHER2 gene expression levels; and 3) pSRC (%) with d16HER2 transcript levels in human primary BCs. Survival was assessed using the Kaplan-Meier estimator, while log-rank test was used to compare survival distributions. Survival analysis was carried out using Cox proportional hazards regression models, and the effects of
explanatory variables on event hazard were quantified by hazard ratios (HR) (Cox 1972).
Chapter 3 – RESULTS: ANALYSIS OF THE ROLE OF d16HER2 ON TUMOUR AGGRESSIVENESS AND TRASTUZUMAB SUSCEPTIBILITY.
In order to unveil the pathobiological role of d16HER2 variant in BC and investigate its potential implication in the sensitivity to HER2-targeted therapies, we performed a series of experiments aimed at investigating the capability of d16HER2 to activate specific signalling pathways and to evaluate its susceptibility to trastuzumab and lapatinib anti-HER2 therapies.

### 3.1 Impact of d16HER2 expression in BC tumourigenicity

To evaluate the oncogenic activity mediated by d16HER2 expression in HER2-positive BC, in collaboration with Dr. Augusto Amici (Department of Bioscience and Biotechnology, University of Camerino, Camerino, Italy) we generated a FVB transgenic mouse model specifically expressing the human d16HER2 variant in the mammary glands \((FVB/NCrl \times MMTV-\Delta 16HER2-LUC)\), under the transcriptional control of the MMTV promoter (Gabrielli et al. 2013). Thanks to the availability of the FVB transgenic mouse model expressing the human WHER2 gene under the control of the MMTV-promotor \((FVB \times MMTV.f.huHER2)\) (Finkle et al. 2004), we compared the \textit{in vivo} d16HER2-guided tumourigenic potential vs that mediated by the WHER2 form. This model has been kindly provided by Prof. Lollini and Prof. Nanni (Department of Experimental, Diagnostic and Specialty Medicine (DIMES), University of Bologna, Bologna, Italy) (Figure 14).
We observed in a large cohort of d16HER2 transgenic animals (n=87) that all mice stochastically and asynchronously developed spontaneous mammary adenocarcinomas with a shorter latency period (8 to 32 weeks of age, p<0.001, Figure 14 A) and higher tumour incidence (5 lesions for mouse in d16HER2 mouse models at 30 weeks of age vs 2 nodules in WHER2 expressing mice at 60 weeks of age, p<0.001, Figure 14) vs the WHER2-positive transgenic animals (n=40). In light of these in vivo data, we concluded that the d16HER2 variant is able to hasten and increase the mammary tumourigenesis vs the WHER2 form.

3.2 d16HER2-driven signaling in HER2-positive mammary cancer cells.

To evaluate the activation status mediated by d16HER2 vs the WHER2 receptor, Western blot analyses of protein extracts from different spontaneous mammary lesions of d16HER2 and WHER2 transgenic mouse models were performed (Figure 15).
Role of d16HER2 splice variant in BC Stem Cells

Figure 15: Western blot analysis of primary mammary tumours derived from spontaneous lesions of d16HER2 and WHER2-transgenic mice. **A)** Protein lysates purified from d16HER2-positive spontaneous tumours (n=8) were separated using 3-8% SDS-PAGE in non-reducing condition and probed with proper Abs binding HER2 (d16HER2D and d16HER2 M) and phosphoHER2 (pd16HER2D and pd16HER2M). **B)** The same protein lysates were separated by 4-12% gradient SDS-PAGE in reducing condition to analyze the basal and activation status of d16HER2M, SRC, STAT3, AKT and MAPK. Actin expression was used to normalize the protein loading. **C)** Linear regression correlation analysis of pd16HER2D vs pSRC expression in protein extracts from d16HER2-positive spontaneous lesions. **D)** Linear regression correlation analysis of pd16HER2M vs pSRC expression in protein extracts from d16HER2-positive spontaneous lesions. **E)** Protein lysates obtained from WHER2-positive spontaneous lesions were separated by 4-12% gradient SDS-PAGE in reducing condition to analyze the basal and status of pd16HER2M, SRC, STAT3, AKT and MAPK. Vinculin expression was used to normalize the protein loading. **F)** Linear regression correlation analysis of pWHER2 vs pSRC expression in protein extracts from d16HER2-positive spontaneous lesions (Adapted from Castagnoli et al. 2014).
d16HER2-positive spontaneous mammary lesions (n=8) were tested through WB assay in non-reducing condition to preserve the disulfide bond structures responsible for the formation of the stable and constitutively activated d16HER2 homodimers (d16HER2D). As shown in figure 15 A, d16HER2D was heterogeneously expressed in different spontaneous lesions, a biological event probably due to the acid condition of the tumour macroenvironment, while the monomeric form was homogeneously distributed. d16HER2D were found highly activated in 4 mammary tumours (samples 3,6,7 and 8) whereas in 3 samples were less (samples 1,4,5) or no activated (sample 2). The same protein extracts were analyzed in reducing condition to evaluate the downstream signaling cascade mediated by the d16HER2 activation (Figure 15 B). Activated d16HER2 triggered the canonical cancer-related proliferation pathway, mediated by the mitogen-activated protein kinase (MAPK) and the survival pathway, mediated by protein kinase B (AKT). In addition, we also observed a closed association between pd16HER2 levels and the activation of the non-receptor kinase SRC (pSRC) and STAT3 (pSTAT3), a signal transducer implicated in “in vivo” SRC-mediated tumourigenesis. In this context, a densitometric analysis revealed a significant positive direct correlation between pd16HER2D (r=0.8787, p=0.0041, Figure 15 C) and pd16HER2M (r=0.8199, p=0.0127, Figure 15 D) and pSRC levels.

The signaling cascade driven by WHER2 was studied exclusively under reducing condition since HER2 stable homodimers were never evidenced in the WHER2 experimental models. The WHER2-positive spontaneous lesions do not reveal any significant link between WHER2 phosphorylation and MAPK and AKT activation even though STAT3 was more activated in WHER2 vs d16HER2
mammary tumours (Figure 15 E). In addition, pSRC was found activated in 6 of 9 examined samples and its activation was not correlated with the levels of pWHER2M (Figure 15 F) providing evidence that the link between HER2 and pSRC is mainly regulated by the d16HER2 variant activity.

To further prove this speculation and test whether HER2 and pSRC could be co-expressed in the same tumour cells, IHC and immunofluorescence on FFPE samples derived from d16HER2 spontaneous mammary tumours were performed in collaboration with the pathologist Dr. M. Iezzi (University G. D’Annunzio, Chieti, Italy).

Figure 16 – Representative analyses of pSRC and HER2 expression in primary tumour from a transgenic d16HER2 mouse. IHC showed pSRC and HER2 expression in the same tumour areas (outer and inner zone of the tumour). Confocal microscopy revealed colocalization of the two proteins (pSRC, green and HER2, red) on mammary tumour cell membranes (Adapted from Castagnoli et al. 2014).

IHC and confocal microscopy analyses showed tumour areas constituted of cells co-expressing both d16HER2 and pSRC. IHC staining also revealed that d16HER2-positive mammary lesions are constituted by 3 different contiguous tumour areas: an outer zone composed of epithelial cells highly positive for d16HER2 and pSRC; an intermediate zone formed of fusiform/spindle cells less reactive to both
anti-HER2 and anti pSRC staining; an inner zone of epithelial cells positive for HER2 and pSRC (Figure 16). These data combined with the biochemical findings (Figure 15) provide a further evidence of the existence of a signaling crosstalk between d16HER2 and pSRC and prompted us to hypothesize that the expression of pSRC in HER2-positive tumour cells could be considered as a surrogate marker of the d16HER2 expression and activation.

3.3 Generation and characterization of d16HER2 and WHER2-positive mammary tumour cell lines

As useful tools for pre-clinical in vitro studies, mammary tumour cell lines were directly established starting from spontaneous tumours arisen in d16HER2 transgenic mice. To accomplish this aim, isolation of d16HER2-positive tumour cells were performed using an immune-magnetic purification device (autoMACS®). In particular, d16HER2-positive lesions developed in two distinct transgenic mice, respectively at 18th and 16th weeks of age, were mechanically and enzymatically dysgregated and, successively, tumour cell suspensions probed with an anti-HER2 Ab bound to proper designed magnetic beads. d16HER2-positive tumour cells were purified from the bulk ex-vivo tumour samples using the autoMACS® under sterile conditions. In particular, through this experimental strategy, we specifically depleted the CD45-positive infiltrating cells thus obtaining two tumour samples containing purified epithelial cells positive for both HER2 and EPCAM biomarkers. In details, as shown in Figure 17, we generated 2 mammary tumour cell lines called MI6 and MI7 respectively.
Figure 17: Generation of the d16HER2 expressing mammary tumour cell lines MI6 (A) and MI7 (B). Flow cytometry analyses of d16HER2 mammary tumour cells before (Upper A and B, blue panels) and after (Upper A and B, red panels) immunomagnetic-based sorting with PE-anti-HER2 MAb and anti-PE-conjugated microbeads. To confirm the epithelial nature of the purified tumour cells, we evaluated the EPCAM membrane expression. All multiparametric analyses were performed on live cells gated according to side scatter (SSC) and forward scatter (FSC) parameters.

As stated above, after mechanical tumour disaggregation and enzymatic digestion of the d16HER2-positive nodules, we respectively recovered 76.9% (MI6) and 67% (MI7) epithelial cells positive for both HER2 and EPCAM expression (Figure 15 A and B, blue squares). The tumour cell purifications by the immune-magnetic procedure determined an enrichment of double-positive tumour cells, indeed, specifically, we obtained two distinct primary cell cultures of 88.3% and 87.6% HER2-positive and EPCAM positive cells that MI6 and MI7 respectively called (Figure 17 A and B, red squares). MI6 and MI7 cells were cultured in vitro in Mammocult, a medium optimized for primitive mammary cell cultures, additioned with 1% FBS and were routinely processed by flow
cytometry and western blot to evaluate d16HER2 expression and activation status.

In order to investigate the biological functions mediated by d16HER2 variant in tumour aggressiveness and sensitivity to anti-HER2 targeted therapies, MI6 and MI7 cells were characterized by comparison with other two murine primary mammary tumour cell lines overexpressing WHER2, WHER2_1 and WHER2_2 which were kindly provided by Prof. Nanni and Prof. Lollini (Department of Experimental, Diagnostic and Specialty Medicine (DIMES), University of Bologna, Bologna, Italy). These cells were directly isolated from spontaneous mammary lesions of FVB mice transgenic for human WHER2 transgene. We firstly started tumour cell characterization by evaluating the expression of the d16HER2 and WHER2 transcripts in all MI6, MI7 WHER2_1 and WHER2_2 cell targets by qPCR analyses.

![Figure 18 - mRNA levels of d16HER2 and WHER2 transgenes in MI6, MI7, WHER2_1 and WHER2_2 primary mammary tumour cell lines.](image-url)
As expected, d16HER2 mRNA was found exclusively expressed in MI6 and MI7 cells, while very high levels of WTHE2 were revealed only in WTHE2_1 and WTHE2_2 cells (Figure 18). Furthermore, in collaboration with Dr. P. Gasparini (Tumour Genomics Unit, Department of Experimental Research, Fondazione IRCCS Istituto Nazionale Dei Tumouri), we performed FISH analyses to test the HER2 genetic status in MI6, MI7, WTHE2_1 and WTHE2_2 cells. We found that MI6 and MI7 cell lines were cytogenetically characterized by a diploid karyotypes (about 40 chromosomes) (Figure 19 Ai and Aii) whereas WTHE2_1 and WTHE2_2 cells revealed a marked chromosome instability reflected by a near-tetraploid karyotypes (76–88 chromosomes) (Figure 19 Aiii, Aiv) in keeping with the hypothesis that WTHE2–driven carcinogenesis is characterized by a “chaotic” genetic pattern (Shiu et al. 2014). Additionally, we revealed a single HER2 signal from both metaphase and interphase nuclei on 2 chromosomes in MI6 and MI7 cells. On the other hand, WTHE2_1 and WTHE2_2 cells were characterized by clusters of HER2 amplification signals in 2 or 3 chromosomes. In keeping with these data, flow cytometry analyses showed that MI6 and MI7 cell lines displayed significantly lower expression of HER2 (MFI: 30±2 and 21±2 respectively) vs WTHE2_1 and WTHE2_2 cells (MFI: 72±9 and 58±2 respectively) (MI6 vs WTHE2_1, p=0.0103; MI6 vs WTHE2_2, p= 0.0006; MI7 vs WTHE2_1, p=0.0052; MI7 vs WTHE2_2, p=0.0002) (Figure 19 B) still corroborating the evidence that HER2 genetic amplification is reflected by higher levels of the HER2 oncoprotein.
Role of d16HER2 splice variant in BC Stem Cells

Figure 19: Cytogenetic, HER2-amplification status and cell surface expression in MI6, M7, WTHE_2.1, and WTHE_2.2 cells. A) Fish analyses of metaphase nuclei from MI6 (i), MI7 (ii), WTHE_2.1 (iii) and WTHE_2.2 (iv) cells. FACS analyses of HER2 expression in MI6, MI7, WTHE_2.1 and WTHE_2.2 tumour cells. Results are mean±SEM (n=3) (Adapted from Castagnoli et Al, Oncogene, 2017).

To establish whether d16HER2- and WTHE2-positive cell lines conserved and reflected the same biochemical features associated with the spontaneous tumours of origin (see Figure 15), we performed a biochemical characterization of MI6, MI7, WTHE_2.1 and WTHE_2.2 cell targets (Figure 20 A and B).

Figure 20: Western blot analyses of MI6, MI7, WTHE_2.1 and WTHE_2.2 mammary tumour cell lines. A) Protein extracts from MI6 and MI7 cells were separated in non-reducing conditions by 3-8 % gradient SDS-PAGE and probed with
anti-HER2 (d16HER2D and d16HER2M) and anti-phosphoHER2 (pd16HER2D and pd16HER2M) Abs. B left panel) The same protein extracts were separated by 4-12% SDS-PAGE under reducing conditions to evaluate the basal and activation status (indicated with p) of SRC, STAT3, AKT and MAPK. Actin was used to normalize the protein loading. B right panel) protein extracts from WHER2_1 and WHER2_2 cells were separated by 4-12% SDS-PAGE under reducing conditions to evaluate the basal and pSRC, pSTAT3, pAKT and pMAPK expression. Actin was used to normalize protein loading (Adapter from Castagnoli et Al, Oncogene, 2017).

Western blot analyses of MI6 and MI7 cells, performed under non-reducing conditions, provided the clear evidence of the expression of both active homodimers and monomers forms of d16HER2 (pd16HER2D and pd16HER2M) as previously observed in the d16HER2-positive spontaneous lesions (Figure 14 A - B). Again, also in “in vitro” models, pd16HER2D was mainly coupled to elevated levels of pAKT and pSRC (Figure 19 B – left panel), whereas WHER2 activation was linked to high levels of pMAPK (Figure 19 B – right panel). Overall, MI6, MI7, WHER2_1 and WHER2_2 cells revealed the same pattern of signal transduction already observed in the corresponding ex-vivo d16HER2 and WHER2 lesions thus supporting the appropriateness of the “in vitro” models for the following pre-clinical studies. To obtain a functional experimental evidence of the hypothesized direct link between pd16HER2 and pSRC, we impaired the HER2-driven downstream signaling specifically treating MI6 cells with two different anti-HER2 ECD mAbs respectively named MGR2, generated in UO12-molecular target unit lab (Tagliabue et al. 1991) and 4D5 (the murine counterpart of trastuzumab) (Shepard et al. 1991).
**Figure 21: Functional link between d16HER2 and SRC kinase.**

1. **A)** Protein extracts from MI6 cells treated with the MGR2 and 4D5 MAbs at different time points (5, 30 minutes, 4, 24 hours) were separated by 3-8% gradient SDS-PAGE under non-reducing conditions and probed with anti-HER2 (d16HER2D and d16HER2M) and anti-phosphoHER2 (pd16HER2D and pd16HER2M) Abs. **B)** The same protein extracts of panel A were separated by 4-12% SDS-PAGE under reducing conditions to evaluate the basal and pSRC expression. Actin was used to normalize the protein loading (Adapted from Castagnoli et al, Oncogene, 2017).

The biochemical analyses of treated cells revealed the capability of the two different anti-HER2 mAbs to decrease the pd16HER2D and pd16HER2M levels. Further, the decreased levels of pd16HER2D and pd16HER2M were coupled with a proportional reduction of the activation status of SRC kinase (Figure 21), thus supporting the existence of a functional link between d16HER2 and SRC in agreement with their co-localization on the tumour cell membrane (Figure 15, 16, and 20).

### 3.4 Susceptibility of d16HER2- and WHER2-positive pre-clinical models to the anti-HER2 target therapies

To investigate *in vivo* the susceptibility of d16HER2 vs WHER2-positive cells to the anti-HER2 targeted therapies, we first analyzed the up-take of MI6 and WHER2_1 cells injected in the m.f.p. of the parental FVB strain. As shown in
figure 21, we observed that \(1 \times 10^6\) MI6 cells developed palpable mammary nodules 10 days after their orthotopic implant. On the other hand, WHER2_1 cells started to generate palpable lesions at 30 days after the injection of \(10^6\) cells in the m.f.p. (Figure 22).

![Figure 22: Tumour growth of MI6 and WHER2_1 cells after orthotopic implant. Tumour growth curves of MI6 (red) and WHER2_1 (black) mammary cell lines. Data are mean ± SD (n=3)](image)

Histological and IHC analyses revealed that MI6 and WHER2_1 cells injected into the m.f.p. of FVB mice developed tumours with the same morphologic features of the spontaneous lesions of origin (Figure 23), confirming the suitability of the orthotopic setting to perform drug sensitivity studies.

![Figure 23: IHC analyses of MI6 and WHER2_1 orthotopic and spontaneous lesions. H&E and HER2 staining of MI6 and WHER2_1 tumour cells injected in the](image)
m.f.p. of parental FVB females (left panel) and of their spontaneous transgenic primary mammary tumour of origin (right panel) (Adapted from Castagnoli et al. 2014).

We first tested the capability of trastuzumab (4 mg/kg of the biodrug once a week) to impair MI6 and WHER2_1 tumour cell growth when orthotopically implanted in the m.f.p of FVB mice upon their randomization (n=10) at the development of palpable tumours. Trastuzumab mediated a strong and significant reduction of the volume of the MI6 tumour when compared to untreated (NaCl 0.9%) animals. (p=0.0005) (Figure 24 A). On the contrary, WHER2_1 tumours were less responsive to trastuzumab, indeed, no significant therapeutic effects were observed upon the humanized Ab administration (Figure 24 B).

Figure 24: Trastuzumab-mediated antitumour activity in d16HER2 and WHER2 orthotopic in vivo models. Tumour growth curves of MI6 (A) and WHER2_1 (B) orthotopic tumours treated with trastuzumab (black triangle) and NaCl 0.9% (black circle)(n=10). Data are mean±SD (Adapted from Castagnoli et al. 2014).

These data provided the first evidence that MI6 cells were strictly dependent on HER2-signaling and that d16HER2 variant could be crucial in sustaining HER2-addiction of HER2-positive BC vs the WHER2 form. To further corroborate this hypothesis, we tested the capability of lapatinib to block MI6 and WHER2_1
tumours growth upon their injection in the m.f.p of FVB mice, following the same schedule described for trastuzumab treatment. As shown in figure 25, lapatinib was able to significantly halt MI6 tumour growth (p<0.001) (Figure 25 A), while WHER2_1 tumours do not demonstrate any therapeutic benefit from the TKI administration(Figure 25 B).

Figure 25: Lapatinib-mediated anti-tumour activity in d16HER2 and WHER2 orthotopic in vivo models. Tumour growth curves of MI6 (A) and WHER2_1 (B) orthotopic tumours treated with lapatinib (black triangle) and DMSO (empty square). Data are mean±SD.

Overall these results highly supported our working speculation suggesting a crucial role played by d16HER2 in the HER2-addiction of HER2-positive BC and, in turn, a high sensitivity to HER2 inhibitor. To further sustain this hypothesis, d16HER2-positive transgenic mice were administered with Trastuzumab 8 mg/kg, i.p., once a week for a short time (5 weeks ) starting from the 8th weeks of age of the mice, when only microscopic tumour lesions are present (Marchini et al. 2011).
Figure 26: Trastuzumab-induced anti-tumour activity in d16HER2 transgenic mice (short schedule). A) Tumour-free survival (%) and B) tumour multiplicity of d16HER2-positive transgenic mice treated for 5 weeks (from 8th to 13th) with trastuzumab and NaCl 0.9% solution. Data are mean ± SEM. Differences were assessed by log-rank test (A) and by unpaired t-test (B) (Adapted from Castagnoli et al, Cancer Research, 2014).

As shown in Figure 26 A and B, in keeping with findings obtained in orthotopic setting, trastuzumab determined a significant delay in the spontaneous tumour onset (p=0.0038) and, also, a significant reduction of tumour multiplicity (p=0.0004, n=1±0.19), as compared with the untreated control groups (n=4.25±0.67). In a further experiment, d16HER2-positive transgenic mice were treated with trastuzumab (4 mg/kg, i.p., twice a week) for 34 weeks (from the 8th to the 42nd week of age).
Figure 27: Trastuzumab anti-tumour activity in d16HER2 transgenic mice (long schedule). A) Tumour-free survival (%) and B) tumour multiplicity of transgenic d16HER2 mice treated for 34 weeks (from 8th to 42nd) with trastuzumab and NaCl 0.9% solution. Data are mean ± SEM. Differences were assessed by log-rank test (A) and by unpaired t-test (B) (Adapted from Castagnoli et al, Cancer Research, 2014).

Such prolonged trastuzumab administration not only led to a marked antitumour activity (p=0.0065) (Figure 27 A) but also significantly reduced the tumour multiplicity (p=0.0002, n=0.8±0.2, Figure 27 B) as compared with the control untreated group (n=5.25±0.85). In addition, 1 out of 7 treated transgenic mice was completely protected until the 42nd week of age when all the mice in the control group had already developed tumours within the 20 weeks. Altogether “in vivo” data revealed the susceptibility of d16HER2-positive cells to trastuzumab both in the orthotopic and spontaneous transgenic models and strongly indicate an important role played by d16HER2 variant in HER2-dependency and trastuzumab susceptibility of human HER2-positive BC.

3.5 Implication of d16HER2 expression and activation in trastuzumab sensitivity of human HER2-positive BC patients

To confirm the strong evidence for the potential association of d16HER2 and pSRC in the clinical setting, we evaluated the possible existence of the
pd16HER2D/pSRC signalling axis also in a series of HER2-positive primary BC specimens derived from patients treated with trastuzumab in adjuvant setting (GHEA study) (Campiglio et al. 2013) whose pathological and clinical features are described in table 6

**TABLE 6 – PATHOBIOLOGICAL AND CLINICAL CHARACTERISTICS OF HER2-POSITIVE BC PATIENTS** (ADAPTED FROM CASTAGNOLI ET AL. 2014).

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In collaboration with Dr. M Iezzi (Dipartimento di Medicina e Scienza dell’invecchiamento, Università Gabriele D’Annunzio, Chieti), 84 FFPE histological
samples were evaluated for pSRC expression in the tumour cells (identified by HER2 staining) by confocal microscopy (Figure 28).

We found a heterogeneous expression of pSRC in the analyzed HER2-positive BC specimens which were classified as pSRC-low (from 0 % to < 20 % of positive tumour cells, n=50/84, Figure 26 A) or pSRC-high (≥ 20 % of positive tumour cells, n=34/84, Figure 26 B) or. In addition, 43 of the 84 HER2-positive BC were tested in qPCR to correlate the d16HER2 transcript levels with pSRC expression gene expression data were split in two groups according to tertiles: low, containing values under the first tertile, and high, containing values greater than the first tertile
Figure 29: Association between d16HER2 transcript and pSRC activation. **A)** Association between d16HER2 transcript levels measured by qPCR and pSRC (%) expression by confocal microscopy in 43 human HER2-overexpressing BCs. *p=0.0482 by unpaired t-test. **B)** Pearson correlation between d16HER2 transcript levels and pSRC (%) levels in 18 cases positive for pSRC expression (>0) (Adapted from Castagnoli et al. 2014).

Interestingly, as shown in Figure 29, d16HER2 expression split in two groups according to tertiles, low (containing values under the first tertile), and high (containing values greater than the first tertile), was found significantly associated with pSRC (Figure 29 A). Moreover, HER2-positive BC characterized by pSRC > 0% revealed a significant direct correlation (p=0.0016) between d16HER2 transcript levels and the pSRC expression positive cells (Figure 29 B). These data strongly suggest that the presence of pd16HER2D in primary HER2-positive BC is reflected by high SRC activation thus sustaining the existence of a direct link between d16HER2 and pSRC. To corroborate the evidence of a potent trastuzumab activity in impairing d16HER2-driven tumourigenicity, we compared the relapse-free survival (%) of HER2-positive BC patients stratified in two groups according to the pSRC expression levels (pSRC-high vs pSRC-low). We observed that HER2-positive BC with high expression of pSRC levels had a better outcome vs those scored pSRC-low (HR, 0.28; 95 % confidence interval (CI), 0.09-0.83; p=0.022) (Figure 30).
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Figure 30: Risk of relapse in human HER2-overexpressing BC patients treated adjuvantly with trastuzumab. Association between pSRC levels (low <20%; high ≥20%) with relapse-free survival in 84 HER2-positive BC patients treated with trastuzumab (Adapted from Castagnoli et al. 2014).

This result provides a clear evidence that high levels of pd16HER2D could predict benefit from trastuzumab treatment in HER2-positive BC, still sustaining our hypothesis that pSRC may be a potential surrogate marker of pd16HER2D in BC addicted to HER2-driven signaling. In this context, to investigate the possibility that d16HER2-driven signaling could be used as biological in silico tool to identify the BC cases more susceptible to trastuzumab-mediated antitumour effects, we exploited the available gene expression profiles (GSE55348), (Triulzi et al. 2015) of 21 BC cases included in the GHEA patient cohort, previously analyzed by qPCR and confocal microscopy to determine d16HER2 and pSRC expression levels. GSEA analyses revealed that tumours with active d16HER2 variant (d16HER2- High; pSRC High; n=5) were characterized by a significant enrichment in genes related to hypoxia, tumour metastasis and cell motility pathways vs inactive or negative d16HER2 tumours (d16HER2-high, pSRC-low, d16HER2-low; n=16). These findings indicate that the activation of d16HER2 is combined with an
increase of genes expression related to features of tumour aggressiveness (Figure 31).

Figure 31: GSEA plots of the comparison between gene expression profiles of active d16HER2 and inactive or negative d16HER2 tumours (Adapted from Castagnoli et al, 2014).
In light of these data, in collaboration with Dr. Triulzi (Molecular Targeting Unit, Department of Research, Fondazione IRCCS Istituto Nazionale dei Tumouri), to further examine whether patients with high d16HER2 signaling are those more susceptible to trastuzumab, we generated an “activated-d16HER2 metagene” by comparing the gene expression profiles of the 21 HER2-positive cases stratified on the d16HER2 and pSRC expression levels. The “activated-d16HER2 metagene” is composed of 73 leading genes of hypoxia, tumour metastasis and cell motility pathway. “In silico” application of the “activated d16HER2-metagene” in two different datasets of HER2-positive BC treated or not with trastuzumab in neo-adjuvant setting “GSE22358” (Gluck et al. 2012) and “GSE41656” (de Ronde et al. 2013) respectively, revealed that patients more sensitive to trastuzumab therapeutic effects were significantly characterized by higher levels of the “activated-d16HER2 metagene” expression (p=0.0305) (Figure 32 A). Conversely, responders and non-responders to neoadjuvant therapy consisting of chemotherapy alone revealed no difference in the "activated-d16HER2 metagene" expression level (Figure 32 B).

Figure 32: d16HER2 metagene and trastuzumab anti-tumour effects. A) Association between “activated d16HER2-metagene” expression and response to trastuzumab-based
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neoadjuvant therapy in the GSE22358 dataset (p=0.0305 by unpaired t-test)

**B)** Association between “activated d16HER2-metagene” expression and response to neoadjuvant chemotherapy in the GSE41656 dataset. CR, complete response; PR, partial response (Adapted from Castagnoli et al. 2014).

### 3.6 Summary of results

The data reported in this chapter indicated that d16HER2 variant is tumourigenic “per se” and was able to increase HER2-driven tumour aggressiveness. Further, we observed that d16HER2 activation was significantly linked/co-localized to SRC kinase phosphorylation in murine and human mammary cancer tissues and, in addition, the provided results denote that this signaling axis could represent a marker of HER2-addiction supporting the candidacy of d16HER2 expression and activation as a new clinical biomarker of increased trastuzumab benefits.
Chapter 4 – RESULTS: ANALYSIS OF THE ROLE OF d16HER2 IN CANCER STEM CELL SUBSET OF HER2-POSITIVE BREAST CANCER
In keeping with the reported data indicating the capability of full-length HER2 to regulate the CSCs population in HER2-positive tumours and in light of our data strongly supporting the link between d16HER2 expression and activation and HER2-positive tumour aggressiveness, we analyzed the possible implication of d16HER2 splice variant in the regulation of CSC features and evaluated its potential candidacy as the predominant HER2 form governing maintenance and expansion of BCSCs in HER2-positive tumours.

4.1 Analysis of the stemness of d16HER2- and WHER2-positive tumour cells

Taking into consideration the association between d16HER2 activation with a significant enrichment of genes related to hypoxia, tumour metastasis and cell motility pathway, we hypothesized a possible involvement of d16HER2 in tumour stemness features. Thus, we performed a series of experiments aimed to analyze the relationship between d16HER2 expression, activation and regulation of CSCs in HER2-positive BC models. Firstly, we examined the whole gene expression profiles of previously characterized and described MI6, MI7, WHER2_1 and WHER2_2 mammary tumour cell lines (Chapter 1). Unsupervised hierarchical clustering of these mammary tumour cell lines based on the expression of the top 5000 most variable genes revealed that d16HER2 and WHER2-positive cells clustered in two well defined and distinct branches (Figure 33).
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Figure 33: Dendrogram obtained from hierarchical clustering of d16HER2- and WHER2-positive mammary tumour cell lines according to the expression of the top 5000 most variables genes. (Adapted from Castagnoli et al, Oncogene, 2017)

Analysis of genes differentially expressed highlighted 992 and 1137 genes significantly up- (green plot) and down-regulated (red plot) in MI6 and MI7 cells compared with WHER2-positive mammary cell lines (Figure 34).

Figure 34: Volcano plot of log2 fold changes vs -log10 false discovery rate exhibiting the differences at transcriptional level between d16HER2 and WHER2 cells. Up- and downregulated genes are highlighted in red and green, respectively.

A functional classification using DAVID gene-annotation enrichment analysis (Huang et al. 2009) of genes found differentially expressed between d16HER2- and WHER2-positive cells, unveiled that MI6 and MI7 cells were significantly enriched in pathways related to stemness, EMT, migration and invasion, whereas WHER2_1 and WHER2_2 cells up-regulated genes included in p53 signaling, inflammation and the immune response gene related pathways (Table 7).
Table 7 – Genes of KEGG pathways found upregulated in d16HER2- vs WHER2- or in D16HER2-positive cells (Adapted from Castagnoli et al, Oncogene, 2017).

<table>
<thead>
<tr>
<th>TUMOUR CELL LINE</th>
<th>KEGG PATHWAY</th>
<th>UPREGULATED GENES</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Focal Adhesion</strong></td>
<td>COMP; COL1A1; FYN; RAC3; PARVA; ACTB; PTK2; ROCK2; BCL2; ITGAV; PRKCA; ITGAV; ITGA6; PPP1CB; ACTG1; LAMB2; VAV3; MAPK8; FLNB; ACTN4; ITGAV; ITGB6; TNC; PDGFC; THBS1; PK2; VCL; LAMC2; ITGA3; ARHGAP35; CND1; ITGB4; PIK3CB</td>
<td></td>
</tr>
<tr>
<td><strong>Reactome Cell-Cell Communication</strong></td>
<td>FERM2; ARHGEF6; FYN; IQGAP1; CLDN1; FBLIM1; CTNN1A; PARVA; PTK2; SPTBN1; CTNN1D; SPTAN1; PTK2; MPFS; PVR; MAPK8; ACTN4; CLDN7; LAMC2; CLDN10; ITGB4; PIK3CB</td>
<td></td>
</tr>
<tr>
<td><strong>Peroxisome</strong></td>
<td>NUDT19; GSTK1; EPHX2; CAT; DHR54; ACOX2; PEX7; ABCD3; NUDT12; PEX11G; ACS14; PEX2; PXMNP4; FAR1; PEX26; PAXX; IDH1</td>
<td></td>
</tr>
<tr>
<td><strong>Tight Junction</strong></td>
<td>PRK2; AMOT1L1; CLDN1; PRKCH; CTNN1A; ACTB; GNA11; OCLN; TJP3; SPTAN1; IGSF5; MPFS; PRKCA; ACTG1; EPB41L1; ACTN4; CLDN7; CLDN23; RARSA; CLDN10; MYH14; PRKCD</td>
<td></td>
</tr>
<tr>
<td><strong>Regulation of Actin Cytoskeleton</strong></td>
<td>ARHGAP4; TMSB4X; IQGAP1; CHRM3; RAC3; ACTB; PTK2; ROCK2; ITGAV; ITGA6; ACTB; PPP1CB; IQGAP2; ACTG1; VAV3; ACTN4; ITGAV; ITGB6; PDGFC; PRK2; KLC; F2R; PIP5K1B; MYH14; ITGB3; ARHGAP35; ITGB4; PIK3CB</td>
<td></td>
</tr>
<tr>
<td><strong>Leukocyte Transendothelial Migration</strong></td>
<td>CXXCR4; CLDN1; CTNN1A; ACTB; PTK2; ROCK2; GNA11; OCLN; CTNN1D; PTX2; PRKCA; ACTG1; VAV3; ACTN4; CLDN7; CLDN23; VCL; CLDN10; ARHGAP35; PIK3CB</td>
<td></td>
</tr>
<tr>
<td><strong>Apoptotic Execution Phase</strong></td>
<td>PTK2; OCLN; STX24; SATB1; SPTAN1; APC; HIST1H1C; DSP; LMBNB; PAK2; PKP1; PRKCD</td>
<td></td>
</tr>
<tr>
<td><strong>Apoptotic Cleavage of Cellular Proteins</strong></td>
<td>PTK2; OCLN; STX24; SATB1; SPTAN1; APC; DSP; LMBNB; PKP1; PRKCD</td>
<td></td>
</tr>
<tr>
<td><strong>Adherens Junction</strong></td>
<td>FYN; IQGAP1; RAC3; FARF2; CTNN1A; ACTB; CTNN1D; TCF7L2; ACTG1; PVR; ACTN4; CREBBP; VCL; IN5A</td>
<td></td>
</tr>
<tr>
<td><strong>Arrhythmogenic Right Ventricular Cardiomyopathy Arvc</strong></td>
<td>CTNN1A; ACTB; DAG1; ITGAV; TCF7L2; ITGA6; DSP; ACTG1; ACTN4; ITGAV; ITGB6; PKP2; ITGAV; ITGB4</td>
<td></td>
</tr>
<tr>
<td><strong>Small Cell Lung Cancer</strong></td>
<td>TRAF6; PTK2; BCL2; CDK2; IRKB; ITGAV; RARB; ITGA6; LAMB2; ITGA2; LAMC2; ITGA3; CCND1; PIK3CB</td>
<td></td>
</tr>
<tr>
<td><strong>ECM Receptor Interaction</strong></td>
<td>COMP; COL11A1; CD44; DAG1; ITGAV; ITGA6; LAMB2; ITGA2; ITGB6; TNC; THBS1; LAMC2; ITGA3; ITGB4</td>
<td></td>
</tr>
<tr>
<td><strong>Cell Junction Organization</strong></td>
<td>FERM2; ARHGEF6; CLDN1; FBLIM1; CTNN1A; PARVA; CTNN1D; MPFS; PVR; CLDN7; LAMC2; CLDN10; ITGB4</td>
<td></td>
</tr>
<tr>
<td><strong>Metabolism of Polyamines</strong></td>
<td>SAT1; SMOX; ODC1; AMO1; PAXX</td>
<td></td>
</tr>
<tr>
<td><strong>Notch-HLH Transcription Pathway</strong></td>
<td>NOTCH4; KAT2B; MAML2; NOTCH3; CREBBP</td>
<td></td>
</tr>
<tr>
<td><strong>N-Glycan Antenna Elongation in the Medial Trans Golgi</strong></td>
<td>B4GALT6; MAN2A1; ST8SIA6; ST8SIA2; FUT8; MGAT4A</td>
<td></td>
</tr>
<tr>
<td><strong>Signaling by Rho GTPases</strong></td>
<td>ARHGAP4; RAC3; ARHGAP6; FG2d; ARAP3; SRGAP3; NGEF; ARHGP4; ARHGP8; VAV3; STARD8; ROHBB2; ROHVC; ARHGID1; ARHGAP35</td>
<td></td>
</tr>
<tr>
<td><strong>Nephrin Interactions</strong></td>
<td>FYN; IQGAP1; SPTBN1; SPTAN1; ACTN4; PIK3CB</td>
<td></td>
</tr>
<tr>
<td><strong>Platelet Activation Signaling and Aggregation</strong></td>
<td>GN67; MGLL; GNA14; ARBB1; FYN; TMSB4X; PRK2; ROCK2; DGK6; PTX2; GNA11; WDR1; PRKCA; DGK6; CLI; VAV3; PROS5; ACTN4; THBS1; VCL; F2R; PRKCD; PIK3CB</td>
<td></td>
</tr>
<tr>
<td><strong>Glycolysis - Gluconeogenesis</strong></td>
<td>ALDH1A3; PKP1; TPI1; DLAT; PFK1; ACS5; ALDOC; LDHB; ALDH3A2; ACSS2</td>
<td></td>
</tr>
<tr>
<td><strong>Pathways in Cancer</strong></td>
<td>FZD5; TRAF6; RAC3; CTNN1A; FZD3; PTK2; BCL2; CDK2; IKBKB; EMLN3; ITGAV; RALA; TCF7L2; PRKCA; RARB; ITGA6; APC; FOS; FADD; LAMC2; EMLN1; MAPK8; ITGAV; EPAS1; CREBBP; FOXO1; F2D2; LAMC2; WNT5B; ITGA3; CCND1; PIK3CB</td>
<td></td>
</tr>
<tr>
<td><strong>Pre Notch Transcription and Translation</strong></td>
<td>NOTCH4; KAT2B; MAML2; NOTCH3; CREBBP; CCND1</td>
<td></td>
</tr>
<tr>
<td><strong>Wnt signaling pathway</strong></td>
<td>FZD5; DAKA1; PRKCB8; PPP2R5A; RAC3; FZD3; ROCK2; TCF7L2; PRKCA; APC; MAPK8; LRP6; CREBBP; FRAT2; FZD2; WNT5B; CTNBP1; CCND1</td>
<td></td>
</tr>
</tbody>
</table>
In light of these molecular results, we tested whether d16HER2-positive cells were also characterized by an additional enrichment of genes related to tumour-initiating properties vs the WHER2 cellular counterpart. To accomplish this aim, four *ad hoc* generated gene lists, including genes implicated in the regulation of
normal and malignant stem cells as those of *Wnt, mTOR, Notch* and *Hedgehog* pathways (Table 8), were tested.

Table 8 – Genes in the four gene lists (*Wnt, mTOR, Hedgehog, Notch*) used for GSEA analyses in gene profiling deriving from murine tumour cell lines (Adapted from Castagnoli et al, Oncogene, 2017).

<table>
<thead>
<tr>
<th>GENE LIST</th>
<th>WNT</th>
<th>MTOR</th>
<th>HEDGEHOG</th>
<th>NOTCH</th>
</tr>
</thead>
<tbody>
<tr>
<td>GENES IN THE FOUR GENE LISTS (<em>WNT, mTOR, HEDGEHOG, NOTCH</em>) USED FOR GSEA ANALYSES IN GENE PROFILING DERIVING FROM MURINE TUMOUR CELL LINES (Adapted from Castagnoli et al, Oncogene, 2017).</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WNT</td>
<td>PPN; WNT2; WNT13; WNT7; WNT3A; WNT4; WNT5A; WNT5B; WNT6; WNT7A; WNT7B; WNT8A; WNT8B; WNT9A; WNT9B; WNT10A; WNT10B; WNT11; MMP7; WNT16; CER1; WIF1; APC; CTNNB1; AXIN1; DKK; SFRP1; SFRP2; SFRP4; SFRP5; FZD2; DALLY; FZD3; DVL1; DVL2; DVL3; FZD4; FZD5; FZD6; FZD7; TCF7; PRKCA; PRKCB; PRKCG; FZD9; FZD10; LRPS; LRPS; FRAT1; GSK3B; SKP1; FRAT2; Csnk2a1; Csnk2a2; Csnk2b; Wnt1; NKD1; NKD2; Cxxc4; Senp2; Cul1; Fbxw11; Btrc; Bxix; Prkaa1; Prkaa2; Prkaca; Prkacb; Prkacg; Ppp3ca; Ppp3cb; Ppp3cc; Myc; Axin2; Rac1; Map3k7; Mapk8; Mapk9; Mapk10; Nfatc1; Tps5; Nlk; Tcf7l1; Tcf7l2; Lef1; Ctnnb1p1; Chd8; Sox17; Ctbp1; Ctbp2; Groucho; ep300; Crebbp; Ruvbl1; Smad4; Fosl1; Ccnd1; Pparg; Psen1; Siah1; Cacybp; Tbl1x; Tbl1xr1; Tbl1y; Vang1; Vang2; Prickle1; Prickle2; Daam1; Daam2; Rhod; Cnmk2; Fzd3; Plocb1; Plocb2; Plocb3; Plocb4; Pp3r1; Pp3r2; Rac2; Rac3; Gpc4; Csnk1a1; Csnk1a2; Csnk1d; Ccnd3; Bambi; Sost; Nfatc2; Nfatc3; Nfatc4; Rock2</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>MTOR</td>
<td>Pk3cb; Pk3cd; Pk3cg; Pten; Pk3rs; Pk3rs; Pk3rs; Pk3rs; Pk3rs; Pk3rs; Pk3rs; Prkaa1; Prkaa2; Prkaca; Prkacb; Prkacg; Rps6; Tnfa; Eif4b; Eif4e; Braf; Mapk1; Mapk3; Rps6ka6; Rps6ka1; Rps6ka2; Rps6ka3; Akt1; Akt2; Akt3; Ins; Rps6kb1; Rps6kb2; Vegfa; Igif1; Pdcd1; Prkaa1; Prkaa2; Mtor; Riptor; Eif4ebp1; Tsc1; Tsc2; Rheb; Kbnb1; Stk11; Mlst8; Rictor; Hif1a; Ulk1; Ulk2; Ulk3; Ddit4; Strada; Cab39; Cab39l; Iris1; Akt1; Rrag3; Rraga; Rragc; Rragd</td>
<td></td>
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<tr>
<td>HEDGEHOG</td>
<td>Wnt2; Wnt13; Wnt3; Wnt3a; Wnt4; Wnt5a; Wnt5b; Wnt6; Wnt7a; Wnt7b; Wnt8a; Wnt8b; Wnt9a; Wnt9b; Wnt10a; Wnt10b; Wnt11; Wnt16; Csnk1j; Gsk3b; Wnt1; Btrc; Fbxw1; Pka; Prkaa1; Prkaa2; Prkaca; Prkacb; Prkacg; Ppax; Bmp2; Bmp4; Ptp1; Smo; Coss2; Sftk3; Sfj1u; Gli3; Hhip; Gas1; Lrps; Rab23; Zic2; Csnk1a; Csnk1g; Csnk1g; Csnk1g; Csnk1d; Csnk1e; Ptpch2; Shh; Ihh; Dhx9; Gli1; Gli2; Ci</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOTCH</td>
<td>Dvl; Notc1h; Notc2h; Notc3h; Notc4h; Ctbph1; Ctbph2; Groucho; Crebbp; Ep300; Psen1; Psen2; Lfng; Mfng; Rfng; Dll1; Dll2; Dll3; Jag1; Jag2; Rpp8; Rpp8; Hes1; Hes5; Ptcra; Numbl; Dtx2; Dtx2; Dtx3; Dtx4; Dtx3l; Adam17; Psen1; Maml1; Maml2; Maml3; Kat2a; Kat2b; Hairless; Ncor2; Cir1; Hdac1; Hdac2; Pen2; Ncstn; Aph1a; Aph1b</td>
<td></td>
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</tr>
</tbody>
</table>

GSEA analysis using these four gene sets revealed a significant enrichment mainly of *Wnt* and *Notch* gene sets in d16HER2 vs WHER2 counterparts (p=0.013, NES=1.53; p=0.013, NES=1.59) (Figure 35 A). (Figure 35 B).
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Figure 35: A) GSEA enrichment plots of Wnt and Notch gene sets in d16HER2- vs WHER2 positive cell lines. B) Heatmap displaying in d16HER2- and WHER2-positive cells differentially expressed genes included in the Wnt and Notch gene sets (Adapted from Castagnoli et al, Oncogene, 2017).

In light of these results and taking into consideration the close interaction occurring between Wnt and Notch pathways with the EMT program, we used a commercial array to evaluate the expression of 84 EMT-related genes in MI6 and WHER2_1 cells. This array revealed an up-regulation of 19 genes in MI6 cells at levels of 4-fold vs WHER2_1 cells that includes transcripts encoding crucial proteins implicated in the EMT program and in CSC regulation such as Sox10, Tgfb1, Ptk2, Zeb2 and Wnt5b (Fufa et al. 2015) (Figure 36 A). On the other hands, only 6 mRNA transcripts were found up-regulated in WHER2_1 vs MI6 cells as Vim, Bmp7, Fgfbp1, Tmeff1, Wnt11 and Gsc (Figure 36 B).
Figure 36: Differential expression of EMT-related genes in MI6 vs WHER2_1 mammary tumour cell lines. A) Analysis of EMT and stem-cell related genes differentially expressed (fold) in MI6 vs WHER2_1 mammary tumour cells using the mouse EMT RT² Profiler PCR Array. B) Analysis of EMT and stem-cell related genes differentially expressed (fold) in WHER2_1 vs MI6 mammary tumour cells using the mouse EMT RT² Profiler PCR Array. Changes in gene expression were analyzed by SABioscience software using GAPDH to normalize the results (Adapted from Castagnoli et al. Oncogene, 2017).

To validate these data, we analyzed the expression of the following genes as Sox10, Tgfb1, Hey2, Wnt5a, Notch4, Ptk2, Notch3, Wnt5b and Fdz5, known to be implicated in the regulation of both EMT and CSC processes, and found to be up-regulated both in the gene expression profile and in the EMT array. As shown in figure 37, qPCR analyses confirm the previously described data (Figure 37).
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Figure 37: Quantitative PCR validation of the differential expression of genes involved in EMT and stemness. The data are the mean ± s.d. (n=2) and represented as fold increase of relative expression in MI6 vs WHER2_1 cells (MI6/WHER_1) (Adapted from Castagnoli et al, Oncogene, 2017).

Furthermore, these results were validated at the protein levels by Western blot analyses. Indeed, we found higher levels of SOX10, TGFβ1, WNT5A, FAK (Ptk2) and pFAK, NOTCH4 and its activated (cleaved) form in MI6 vs WHER2_1 cells (Figure 38).

Figure 38: Western blot analyses of MI6 and WHER2_1 protein extracts separated by 4-12% SDS-PAGE under reducing conditions to evaluate SOX10, basal FAK, pFAK, TGFβ, basal and cleaved NOTCH4 and WNT5A proteins (Adapted from Castagnoli et al, Oncogene, 2017).

To investigate the possibility that the higher expression of factors implicated in EMT and stemness regulation could be a mirror of an enrichment of the stem cell population in d16HER2-driven mammary cells vs those WHER2-positive, we
analyzed and compared the efficiency of MI6 and WHER_1 cells to form mammospheres (MFE (%)), spheroid structures known to be enriched in CSCs (Dontu et al. 2003). We revealed a significant higher MFE (%) in MI6 vs WHER_1 cells both in the first (1st) and second (2nd) mammosphere generation passages (Figure 39 A) strongly indicating a more potent efficiency of MI6 cells to undergo self-renewal than WHER2_1 cells (Figure 39 B).

Figure 39: Evaluation of stemness features in MI6 and WHER2_1 mammary tumour cell lines. A) First and second mammosphere generation by MI6 and WHER2_1 cells evaluated as MFE (%). B) Self-renewal in MI6 and WHER2_1 mammary tumour cell lines calculated as the ratio between 2nd/1st MFE (%). Data are mean ± SD of 3 independent experiments. Significance was calculated by two-tailed unpaired t-test (Adapted from Castagnoli et al, Oncogene, 2017).

To sustain the enrichment of CSCs in d16HER2- vs WHER2-driven tumours, we tested by multiparametric flow cytometry analysis the frequency of the cells characterized by the peculiar CD29\(^{\text{High}}\)/CD24\(^{\text{High}}\)/Sca-1\(^{\text{Low}}\) phenotype, a cell subset reported enriched in murine mammary CSCs (Shackleton et al. 2006). We observed that the % of cells with CD29\(^{\text{High}}\)/CD24\(^{\text{High}}\)/Sca-1\(^{\text{Low}}\) was 3.7% ± 0.6% in MI6 vs 0.2% ± 0.2% in WHER2_1 cells (Figure 40), still confirming a higher frequency of CSCs within MI6 in comparison with WHER2_1 cells.
Figure 40 Multiparametric FACS analysis of the CD29<sup>High</sup>/CD24<sup>+</sup>/Sca-1<sup>Low</sup> stem cell subset in MI6 and WHER2_1 cells gated according to SSC and FSC parameters. Data are mean ± SD of 3 independent experiments. Significance was calculated by two-tailed unpaired t-test (Adapted from Castagnoli et al, Oncogene, 2017).

In parallel, we performed the same experiment in the HER2-positive tumour cell suspensions derived after mechanical disaggregation and enzymatic digestion of spontaneous mammary lesions developed in d16HER2 (n=4) and WHER2-positive (n=3) transgenic (tg) mice. The results of this experiment revealed a mean frequency of cells with CD29<sup>High</sup>/CD24<sup>+</sup>/Sca-1<sup>Low</sup> phenotype of 11.6% ± 1.3% in d16HER2 vs 6.8 ±2.4% in WHER2-positive samples (Figure 41) corroborating the “in vitro” data obtained with MI6 and WHER2_1 cell lines.
To further prove that d16HER2 expression and activation drives a more consistent enrichment of the CSC population than the WHER2 form, we analyzed the tumour-forming ability of MI6 and WHER2_1 cells injected in limiting dilution conditions (from $10^3$ to $10^6$ cells) in the m.f.p. of FVB mice. We observed a significantly higher “in vivo” tumour uptake of MI6 vs WHER2_1 cells. In particular, $10^3$ MI6 cells were able to form tumours in 33% of the injected mice, whereas only 17% of mice injected with $10^4$ WHER2_1 cells developed tumours (Figure 42 and Table 7).

Figure 41: Multiparametric FACS analysis of the CD29$^{\text{High}}$/CD24$^+$/$\text{Sca-1}^{\text{Low}}$ stem cell subset in spontaneous primary mammary lesions form transgenic d16HER2 (n=4) and WHER2_1 (n=3) mice. All analyses were performed after gating live cells according to SSC and FSC parameters only in the HER2-positive tumour cell subset. Data are Mean ± SD of 3 independent experiments. Significance was calculated by two-tailed unpaired t-test (Adapted from Castagnoli et al, Oncogene, 2017).
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Figure 42: Tumour-forming ability (outgrowths/injections (%)) of MI6 and WHER2_1 cells injected at different serial dilutions starting from $10^6$ to $10^3$ cells into the m.f.p. of parental FVB mice (n=12 for each animal group except for $10^5$ and $10^3$ WHER2_1 dilutions, n=10) (Adapted from Castagnoli et al, Oncogene, 2017).

This gold standard stemness bioassay outlines and sustain our data showing that d16HER2-positive tumour models are enriched in CSC compared to those WHER2-positive (Figures 41 and 42). These findings open a new perspective on the candidacy of the “real/true” HER2 isoform governing the HER2-positive BCSC subset vs the full-length HER2 receptor whose candidacy was sustained by a great deal of previous literature (Korkaya et al. 2008; Korkaya e al. 2013; Magnifico et al. 2009).

In addition, the results of the “in vivo” tumour-forming ability evaluated using the Extreme Limiting Dilution Assay software (ELDA), a free bioinformatic tool created to estimate the CSC frequency starting from limiting dilution data (Hu et al. 2009), estimated a >10-fold increase in BCICs frequency in MI6 (1/4,601) vs WHER2_1 cells (1/61,573) (Table 7), providing a further and significant
evidence for the enrichment of a CSC sub-population in d16HER2- vs WHER2-positive cells.

**TABLE 7: TUMOUR-FORMING ABILITY OF MI6 AND WHER2_1 CELLS**

<table>
<thead>
<tr>
<th>Injected cells (no.)</th>
<th>MI6 outgrowths/injections (%)</th>
<th>WT_1 outgrowths/injections (%)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^6$</td>
<td>12/12 (100)</td>
<td>12/12 (100)</td>
<td></td>
</tr>
<tr>
<td>$10^5$</td>
<td>12/12 (100)</td>
<td>8/10 (80)</td>
<td>0.1948$^1$</td>
</tr>
<tr>
<td>$10^4$</td>
<td>10/12 (83)</td>
<td>2/12 (17)</td>
<td>0.0033$^1$</td>
</tr>
<tr>
<td>$10^3$</td>
<td>4/12 (33)</td>
<td>0/10 (0)</td>
<td>0.0964$^1$</td>
</tr>
<tr>
<td>$10^2$</td>
<td>0/12 (0)</td>
<td>0/12 (0)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0/12 (0)</td>
<td>0/12 (0)</td>
<td></td>
</tr>
</tbody>
</table>

**ELDA (95% CI)$^2**

<table>
<thead>
<tr>
<th></th>
<th>MI6 ELDA</th>
<th>WHER2_1 ELDA</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/4601</td>
<td>1/61753</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>(95% CI)$^2$</td>
<td>(1/2468-1/8579)</td>
<td>(1/31463-1/121207)</td>
<td></td>
</tr>
</tbody>
</table>

In keeping with our results unveiling a significant increased expression of Notch-related genes (Figure 33) and with higher expression and activation of NOTCH4 in MI6 vs WHER2_1 cells (Figure 37), we analyzed the role mediated by the Notch pathway in the regulation of the activity of MI6 vs WHER1_1 CSC subsets. In this context, MI6 and WHER1_1 spheroids were treated with two different γ-secretase inhibitors such as DAPT ((N-)(3,5-difluorophenacetyl)-L-alanyl)-S-phenylglycine t-butyl ester) (Figure 42 A) or RO4929097(Figure 42 B), that specifically inhibit the activation of NOTCH family members. Notably, both drugs were found to significantly halt the MFE only in MI6 cells (Figure 43), indicating a main functional role of Notch in d16HER2-guided stemness.
Figure 43: Capability of two distinct γ-secretase inhibitors to impair MI6 and WHER2_1 mammospheres formation. A) MFE inhibition (%) in MI6 and WHER2_1 mammospheres treated with DAPT (0.5 μM and 1 μM) and B) RO4929091 (2 μM and 4 μM). Data are mean ± SD of 3 independent experiments. Significance was calculated by a two-tailed unpaired t-test (Adapted from Castagnoli et al, Oncogene, 2017).

4.2 Capability of trastuzumab to mediate anti-CSC effects in both d16HER2- and WHER2-positive cellular models.

In keeping with our evidence showing that d16HER2 expression and activation achieved a higher sensitivity to trastuzumab in HER2-positive BC patients (Chapter 1), we tested and compared its activity directed to MI6 and WHER2_1 mammospheres. Our data provided evidence that d16HER2-positive mammospheres (Figure 44 A) were significantly more susceptible to trastuzumab activity compared to those WHER2-positive (Fig. 44 B), suggesting that the benefits mediated by trastuzumab in HER2-positive BCs characterized by high expression of d16HER2 activated metagene can be induced by its anti-CSCs activity.
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Figure 44: Capability of trastuzumab to impair MI6 and WHER2_1 mammospheres formation. A) MFE inhibition (%) in MI6 and B) WHER2_1 cells treated with trastuzumab (T) (10 μM). Data are mean ± SD of 3 independent experiments. Significance was calculated by a two-tailed unpaired t-test.

To investigate the capability of trastuzumab to target CSCs also in the “in vivo” setting, we injected MI6 cells into the m.f.p. of FVB mice (n=5/animal group) and started to administrate the biodrug when the tumours reached the 50mm$^3$, 100mm$^3$ or 150 mm$^3$ of volume, respectively. We observed that the mAb was able to halt the “in vivo” growth of MI6 cells when administrated very early during d16HER2-driven tumourigenesis whereas no therapeutic effects were exerted when tumours reached 100 mm$^3$ or 150 mm$^3$ of volume (Figure 45).

Figure 45: Trastuzumab-mediated anti-CSCs activity into MI6 orthotopic models. MI6 tumour growth in syngeneic FVB mice treated or not (blue) with trastuzumab. The biodrug treatment started when the tumour volumes were considered palpable (orange) or reached 50mm$^3$ (Black) and 150 mm$^3$ (green).
These data support the hypothesis that the “in vivo” anti-tumour effects exerted by trastuzumab against d16HER2-positive tumours can be due to its capability to impair CSCs activity in the early phase of d16HER2-driven tumourigenicity when the initial CSCs contribution is crucial to tumour growth, progression and response to therapy. Furthermore, these results could also imply that trastuzumab activity in HER2-positive BC patients with higher “activated d16HER2-metagene” was mainly directed to the CSCs compartment.

4.3 Analysis of the stemness activity of d16HER2- and WHER2-engineered human BC cells

To address whether d16HER2 variant expression and activation could be the main regulator of HER2-driven stemness vs its full-length HER2 isoform, the luminal HER2-negative BC cell lines MCF7 and T47D were transfected with the pcDNA3.0 plasmid vector carrying either d16HER2 or WHER2 genes under the control of the cytomegalovirus (CMV) promoter. In parallel, MCF7 and T47D cells were also transfected with the pcDNA3.0 empty vector as internal negative controls (MCF7-empty and T47D-empty). The engineered tumour cell clones were selected in neomycin-containing cell medium. As preliminary step, the d16HER2 (Figure 46 A and 46 C) and WHER2 (Figure 46 B and 46 D) transcript levels were tested by qPCR in each transfectant and two of them were selected for further analyses and named MCF7-d16-cl1 and MCF7-d16-cl2; MCF7-WT-cl1 and MCF7-WT-cl2 and in T47D (T47D-d16-cl1 and T47D-d16-cl2; T47D-WT-cl1 and T47D-d16-cl2, respectively (Figure 46).
Figure 46: qPCR analysis of d16HER2 and WHER2 transcripts levels in selected MCF7 and T47D transfectants. A, C) evaluation of d16HER2 transcript levels in T47D-d16-cl1, T47D-d16-cl2, MCF7-d16-cl1 and MCF7-d16-cl2 clones. T47D- and MCF7-empty transfectants were used as negative control. B, D) evaluation of WHER2 transcript levels in the selected T47D-WT-cl1, T47D-WT-cl2, MCF7-WT-cl1 and MCF7-WT-cl2 clones. T47D and MCF7 empty transfectants were used as negative control. Data are mean ± SD of 3 technical replicates and normalized on the relative expression of d16HER2 or WHER2 genes assessed in cells transfected with the empty-vector.

As shown in Figure 47, despite the initial selection of properly expressing clones, we observed that the levels of d16HER2 transcript decreased at the increasing of the time of "in vitro" clones culturing. To overcome these technical problems, in collaboration with Dr. Claudia Chiodoni (Molecular Immunology Unit, Department of Research, Fondazione IRCCS Istituto Nazionale dei Tumouri), we...
transduced the same tumour cell targets with properly lentivirus vectors encoding d16HER2 or WHER2 genes and generated stably infected MCF7 and T47D cell populations ectopically expressing d16HER2 (MCF7-d16 and T47D-d16) (Figure 47 A and C) or WHER2 (MCF7-WT and T47D-WT) forms (Figure 47 B and C). This experimental approach guaranteed the insertion of the each transgene in the tumour cell genome and allowed a higher stable expression of either d16HER2 (Figure 47 A) or WHER2 (Figure 47 B) both at mRNA and protein levels (Figure 47 C) vs their basal levels expressed in the Mock cell counterparts (MCF7-Mock and T47D-Mock) (Figure 47 A-C).

**Figure 47:** Expression of d16HER2 and WHER2 isoforms in MCF7- and T47D-infected cells by qPCR and FACS analysis. A) qPCR evaluation of the relative expression of d16HER2 and B) WHER2 transcripts in properly infected MCF7 and T47D cells. Data are mean ± SD of 3 independent experiments. C) Representative FACS
analyses of properly d16HER2 and WTHERT2 infected MCF7 (left) and T47D (right) cells stained with the PE-conjugated anti-HER2 Ab CD340 (Adapted from Castagnoli et al, Oncogene, 2017).

Further, we performed cytogenetic analyses of the transduced cells to assess whether the lentivirus infection has lead to a possible modification of the karyotypes and, interestingly, we found the same triploid karyotypes in all tested tumour cell lines independently from the inserted transgene (Figure 48).

![Cytogenetic analyses of lentivirus-infected MCF7-Mock (i), MCF7_d16 (ii), MCF7_WT (iii), T47D-Mock (iv), T47D_d16 (v) and T47D_WT (vi) cell populations](image-url)

**Figure 48:** Cytogenetic analyses of lentivirus-infected MCF7-Mock (i), MCF7_d16 (ii), MCF7_WT (iii), T47D-Mock (iv), T47D_d16 (v) and T47D_WT (vi) cell populations (Adapted from Castagnoli et al, Oncogene, 2017).

Finally, in a preliminary Western Blot, we also analyzed the expression and activation of HER2-homodimers in d16HER2- or WHER2-engineered cells using both T47D-Mock and MCF7-Mock cells as internal negative controls. As shown in figure 47, d16HER2-positive cells were characterized by a sharp expression of d16HER2-homodimers (Figure 49 A) vs the WHER2-expressing cells (Figure 49 B) in which a very faint band at the same molecular weight was revealed.
Figure 49: Western blot analyses of MCF7 and T47D-engineered cell lysates. A) Protein lysates purified from MCF7-mock, MCF7-d16 and MCF7-WT engineered cells separated by 3-8% SDS-PAGE in non-reducing condition and probed with proper anti-HER2 MAb to visualize basal HER2D and HER2M; B) Protein lysates purified from T47D-WT, T47D-d16 and T47D-mock engineered cells separated by 3-8% SDS-PAGE in non-reducing condition and probed with proper anti-HER2 MAb to visualize basal HER2D and HER2M.

After the molecular and biochemical characterization of the engineered MCF7 and T47D cell populations, we carried out a series of experiments aimed to evaluate their enrichment in CSCs. To accomplish this goal, we tested and compared the capability of engineered MCF7 and T47D cells to generate mammospheres. As shown in Figure 50, we observed a significantly higher MFE % in MCF7-d16 (Figure 50 A) and T47D-d16 cells (Figure 50 B) vs their WHER2- and Mock- cell counterparts, supporting that the d16HER2 variant sustains a more consistent BCIC enrichment vs the WHER2 receptor also in the context of human cell lines.
To evaluate the self-renewal capability of the stem cell compartments from d16HER2 and WHER2-engineered cell lines, we evaluated the ratio of the MFE between the second and the first passages of their generation. In this context, we observed a higher self-renewal capability in MCF7-d16 (Figure 50 A) and T47D-d16 (Figure 50 B) cells vs their WHER2-positive and Mock cell counterparts, thus supporting the crucial implication of d16HER2 expression/activity in the regulation of the stem cell features.
Figure 51: Self-renewal capability of CSCs from engineered MCF7 and T47D cell lines. **A)** First and second passages of mammospheres generation by engineered MCF7 and **B)** T47D cell lines evaluated as MFE (%). **C)** Self-renewal of engineered MCF7 and **D)** T47D cell lines calculated as the ratio between 2nd/1st MFE (%). Data are mean ± SD of 3 independent experiments. Significance was calculated by a two-tailed unpaired t-test (Adapted from Castagnoli et al, Oncogene, 2017).

To support these functional data, we performed a comparative cytofluorimetric analysis to investigate the ALDH-positive cells, a cell subset enriched in epithelial CSCs (Liu et al. 2014), in the HER2-positive cell fraction of MCF7-d16 vs MCF7 WT cells (Figure 52 A and B) and of T47D-d16 vs T47D-WT cells (Figure 52 C and D) using MCF7-Mock and T47D-Mock as internal controls. The results of three
independent experiments clearly revealed a significantly higher frequency of ALDH-positive cells in the d16HER2-engineered models vs the WTHER2-positive and Mock-cells (Figure 52 A-D), still corroborating our hypothesis of the close interaction between d16HER2 expression and activation and the enrichment of the CSC subset.

Figure 52: ALDH expression in d16HER2- and WTHER2-engineered MCF7 and T47D cell lines. A) Representative plots and B) summary of the data showing the % of ALDH-positive cells in the HER2-positive cell subset of MCF7_d16 and MCF7_WT cells and in bulk MCF7-Mock cells. C) Representative plots and D) summary of the data showing the % of ALDH-positive cells in the HER2-positive cell subset of T47D_d16 and T47D_WT cells and T47D-Mock cells. The basal expression of HER2 in MCF7-Mock and T47D-Mock cells was established as the threshold to identify d16 and WT-positive cellular subsets in the bulk MCF7_d16, MCF7_WT, T47D_d16, and T47D-WT engineered cell lines. The results are the mean±SD of three independent experiments (n=3). Significance was calculated by two-tailed unpaired t-test (Adapted from Castagnoli et al, Oncogene, 2017).

To further corroborate the involvement of the d16HER2 variant in the enrichment of CSC subsets of HER2-positive BC, we analyzed and compared the expression of the CD44 protein, a peculiar marker of mesenchymal stem cells (Liu...
et al. 2014), in all the engineered cell targets, by FACS analyses. We found that MCF7-d16 (Figure 53 A) and T47D-d16 (Figure 53 B) cells expressed higher levels of CD44 compared with MCF7-WT and T47D-WT and their corresponding mock cell populations.

![Figure 53: Differential expression of CD44 in engineered human BC cell lines. A) Representative FACS analysis of CD44 expression in MCF7-d16, MCF7-WT and MCF7-Mock cell lines and B) in T47D-d16, T47D-WT, and T47D-Mock. The analysis of CD44 expression was performed only in HER2-positive cell subset. The basal expression of HER2 in MCF7-Mock and T47D-Mock cells was established as the threshold to identify d16 and WT-positive cellular subsets in the bulk counterpart (Adapted from Castagnoli et Al. Oncogene, 2017). To further support this evidence, we purified the cell populations expressing d16HER2 and WTER2 forms from the bulk cell populations of the transduced cell lines using an immune-based cytofluorimetric cell sorting. Afterwards, confocal microscopy analyses defined the occurrence of a higher expression of CD44 in d16HER2-engineered models compared with the WTER2-infected cells (Figure 54 A-D).]
In keeping with our working hypothesis, all together these data strongly sustain the candidacy of d16HER2 variant as the “real” HER2 variant driving the oncogenic activity of the HER2-positive CSCs inside the BC. To implement our evidence on the crucial role exerted by d16HER2 in the HER2-positive stem cell population, we tried to generate stable d16HER2-silenced cells transducing BT474 cells with shRNA sequences designed to specifically knock down d16HER2 expression. As shown in Figure 55, we observed that the amount of d16HER2

Figure 54: Immunofluorescence analysis of CD44 expression in d16HER2- and WHER2-engineered human BC cell lines. A) Representative immunofluorescence of expression and co-expression (merged) of HER2 (red) and CD44 (blue) markers in the HER2-positive cell subsets of MCF7-d16 and MCF7-WT and B) of T47D-d16- and T47D-WT-infected cells were evaluated by confocal microscopy. The nuclei were counterstained with DAPI-Prolong (cyan). C) Box plot representing CD44-stained pixel number of MCF7 and D) T47D engineered cells. Mock cell lines were used as internal control. Significance was calculated by a two-tailed unpaired t-test (Adapted from Castagnoli et al, Oncogene, 2017).
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(Figure 55 A) and WHER2 transcripts (Figure 55 B) in d16HER2-silenced BT474 cells decreased more or less to the same extent to that observed in the corresponding scrambles-transduced cells and the parental untreated BT474 cells. These findings strongly suggest that puromycin antibiotic selection or viral vector backbone could heavily interfere with transgenes expression levels. In addition, this experiment also revealed that two distinct d16HER2-shRNAs sequences were not able to selectively inhibit the expression of the only d16HER2 splice variant.

Figure 55: qPCR analysis of d16HER2 and WHER2 transcript levels after specific d16HER2 silencing of BT474 cells. A) qPCR evaluation of d16HER2 and B) WHER2 expression in BT474 tumour cell line infected with lentivirus vectors carrying two different sequence properly designed to exclusively silence the d16HER2 expression (six d16(1) and six d16 (2)). A scramble sequence (scr) was used as internal control.

To overcome this technical problem, we addressed the analysis of the d16HER2 implication in HER2-positive BC stemness through the exclusive silencing of the WHER2 form. To accomplish this aim, we used a siRNA sequence specifically designed to impair the expression of WHER2 form and, then, properly transfected BT474 cells. Notably, we observed a specific decrease of the WHER2 mRNA levels only in silenced cells compared to internal control, while no effects in d16HER2 expression in each BT474 cell subset was revealed (Figure 56).
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Figure 56: qPCR analysis of d16HER2 and Wther2 transcript levels after specific Wther2 silencing of BT474 cells. BT474 cells were transfected with siRNA scramble sequence (siSCR) or with 2 siRNAs designed to silence Wther2 expression (sil (1) and sil (2)).

However, despite the encouraging preliminary findings, in the subsequent silencing experiments we did not observe any specific d16HER2 and Wther2 decrease suggesting that such experimental approach was not achievable.

In the ultimate effort to accomplish such aim proposed in my Ph.D. project, we started to set the experimental conditions to perform a silencing of the Wther2 form through the use of CRISPR/Cas9 technology (Sander et al. 2014). In particular, molecular assays are still ongoing in the attempt to cleave the exon 16, by the use of guide strands that will drive the Cas9 toward two DNA sequences located in the intron region above and beyond the exon 16 of the HER2 gene, as illustrated in figure 57.
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Through the CRISPR/Cas9 approach, we will obtain engineered cell lines permanently silenced for the WTHE2 form and expressing only the d16HER2 splice variant.

### 4.4 Analysis of d16HER2 role in stem cell compartments of HER2-positive BC cells.

To further support the hypothesis that the d16HER2 variant is the main driver regulating the enrichment of the stem cell subsets in HER2-positive CSCs, we analyzed the expression of d16HER2 and WTHE2 mRNAs in a two different human HER2-positive cancer cell lines, BT474 and MDAMB361, cultured in adherent or in mammosphere promoting conditions (3D). As shown in Figure 58, d16HER2 expression was found significantly enriched in 3D cultured cells (Figure 58 A and C), whereas the WTHE2 transcript levels were significantly more elevated in the bulk cell populations (Figure 58 B and D). These results suggest a
potential enrichment of d16HER2 expression in CSC subsets confirming the implication of the splice variant in the regulation of the CSC activity in HER2-positive tumours.

Figure 58: Comparison of d16HER2 and WHER2 expression levels in MDAMB361 and BT474 cells cultured in 2D vs 3D conditions by qPCR analyses. A) d16HER2 variant expression in MDAMB361 and C) BT474 HER2-overexpressing human BC cell lines cultured in adhesion (2D) or mammosphere promoting conditions (3D). B) WHER2 expression in MDAMB361 and D) BT474 HER2-overexpressing human BC cell lines cultured in adhesion (2D) or mammosphere promoting condition (3D). The results are the mean±SD of three independent experiments. Significance was calculated by a two-tailed unpaired t-test.

To move these discoveries into the clinical setting and unveil the impact of d16HER2 activity in CSCs of human HER2-positive BC patients, we analyzed
human cases from two different HER2-positive datasets. In particular, 53 primary HER2-positive cases included in GHEA study (Campiglio et al. 2013) and 52 profiled HER2-positive samples derived by NKI study (Van de Vijver et al. 2002) were analyzed using gene lists that included genes associated with Noch, Wnt, Hedgehog and mTOR pathway (Table 7). GSEA analyses provided evidence of a significant enrichment of Notch pathway-related genes (p = 0.056, NES = 1.48 in the GHEA dataset and p = 0.015, NES = 1.68 in the NKI dataset) in HER2-positive BC stratified according to the levels of “activated d16HER2-metagene” both in GHEA (Figure 59 A) and NKI BC cohorts (Figure 59 B).

Figure 59: Association of activated d16HER2 metagene levels with the NOTCH pathway in human HER2-positive BCs. A) Notch pathway expression in human HER2-positive BCs of GHEA (GSE55348) and B) NKI datasets according to the “activated d16HER2 metagene” classification. The data are shown as the mean of log2 expression of genes belonging to the Notch gene list (Table 9). Significance was calculated by a two-tailed unpaired t-test (Adapted from Castagnoli et al, Oncogene, 2017).

The evaluation of the expression of the genes directly regulated by the transcriptional activity of the NOTCH receptors, whose expression is indicative of activation of the Notch pathway, unveiled higher levels of HEY1, HES5 and HES6 genes in GHEA cases with high levels of “activated d16HE2-metagene” vs those with low expression (Figure 60 A). Consistent with these data, analyses of NKI datasets revealed higher expression of HEY1, HEY2, and HES4 in BC samples with high levels of “activated d16HE2-metagene” (Figure 60 B) strongly supporting the
implication and cooperation of d16HER2 with Notch signaling in HER2-positive human CSCs.

Figure 60: Association of “activated d16HER2 metagene” levels with the expression of genes downstream to the transcriptional activity of NOTCH receptors. A) Expression levels of Notch-downstream genes in human HER2-positive BC included in the GHEA (GSE55348) or B) NKI datasets with high or low expression of “activated-d16HER2 metagene”. Significance was calculated by a two-tailed unpaired t-test (Adapted from Castagnoli et al, Oncogene, 2017).

4.5 Summary of results.

The results obtained in this Chapter showed that d16HER2-positive mammary tumour cell lines are enriched in the expression of *Wnt, Notch* and epithelial-mesenchymal transition pathways-related genes as compared to the WHER2-positive cells. In addition, we also observed that d16HER2 expression guides an enrichment of CSC population in comparison with the WHER2 form both in murine and human properly-engineered tumour cell lines. Finally, we observed that HER2-positive BC cases characterized by high levels expression of activated-d16HER2 metagene are significantly enriched of Notch family members and signal transducer genes vs those with low levels of the same metagene.
Chapter 5: RESULTS: ANALYSIS OF D16HER2 EXPRESSION AND ACTIVATION IN HER2-NEGATIVE LUMINAL BC AND OTHER HER2-POSITIVE GASTRIC CANCERS.
In light of the importance of the d16HER2 variant expression and activation in HER2-positive BC, we enlarged our investigation in other epithelial oncotypes as the Luminal A BC and the HER2-positive GC. In particular, we performed a series of preliminary experiments aimed to evaluate the implication of d16HER2 expression and activation in the CSC subset of these cancers.

5.1 Evaluation of the expression of d16HER2 in HER2-negative luminal BC

To evaluate the potential implication and activity of d16HER2 splice variant in the regulation of stem cell properties of HER2-negative and ER-positive MCF7 and T47D luminal A BC cell lines, we started to compare the expression of HER2 in CSCs sorted for high ALDH activity (ALDH-pos) vs those with low ALDH-activity (ALDH-neg). Indeed, in keeping with previously reported data (Ithimakin et al. 2013), we also observed in luminal cell models cultured in 3D conditions a higher HER2 expression in the ALDH-positive cell fraction vs the ALDH-negative cell compartment (Figure 61 A and B), thus supporting the implication of HER2 activity in the stemness of BC in absence of HER2 gene amplification. In addition, these findings strongly imply that a wider group of BC patients may benefit from trastuzumab whose ability to hamper and delete the CSC population is well established.
Figure 61: Evaluation of HER2-expression in the ALDH-positive and -negative cell subsets of luminal BC cell models. A) Representative FACS analysis of HER2 expression in ALDH-positive CSCs subset of the luminal BC cell lines MCF7 and B) T47D vs the ALDH-negative cell counterparts.

To further corroborate these data, we investigated the possible enrichment of HER2-expression also in the mesenchymal-like stem cell compartment identified as CD44-positive cells (CD44-pos). In particular, the data obtained with MCF7 and T47D cells cultured in 2D conditions unveiled a two-fold increased expression of HER2 in sorted CD44-positive (CD44-pos) vs CD44-negative (CD44-neg) cells (Figure 62), reinforcing the hypothesis that HER2 expression regulates both epithelial and mesenchymal-like CSC subsets also in the absence of HER2 expression.
Figure 62: Evaluation of HER2-expression in the CD44-positive and -negative CSC subsets of luminal BC cell models. A) Representative FACS analysis of HER2 expression in CD44+ CSCs fraction of the luminal BC cell lines MCF7 and B) T47D vs the CD44-negative cell counterparts.

In the attempt to evaluate whether d16HER2 splice variant can be expressed and play a role in the stemness of BC-negative for HER2 gene amplification, we are currently setting the proper experimental conditions to purify the small ALDH- and CD44-positive CSC fractions in both MCF7 and T47D cells. After their sorting performed according to the expression of BC stemness markers, we will analyze d16HER2 mRNA transcript levels in epithelial- (ALDH-positive) and mesenchymal-like (CD44-positive) CSC populations and compare the results with those obtained using MCF7 and T47D ALDH- and CD44-negative cell counterparts.
5.2 Evaluation of d16HER2 expression in HER2-positive gastric cancer (GC).

To evaluate the possible implication of d16HER2 expression in the pathobiological features of another HER2-positive epithelial oncotype, we focused our attention to HER2-positive gastric (GC) and gastroesophageal junction (GEJ) cancer that represent about 20% of total GC reported to be the second leading cause of cancer-related deaths world-wide (Cancer Genome Atlas Network 2012). The analyses of two different HER2-positive GC cell lines, EO19 (GEJ) and N87 (GC), kindly provided by Professor Silvia Giordano, Cancer Molecular Biology, University of Torino, Medical School, clearly demonstrated that d16HER2 variant is also up-regulated in HER2-positive GC, and, noteworthy, its expression is higher or, in some circumstances, comparable to that observed in HER2-amplified BC cell lines (Figure 63). All together these findings imply that d16HER2 variant can play an important pathobiological role also in HER2-positive GC.
Figure 63: qPCR analysis of d16HER2 mRNA expression in a series of HER2-positive BC and GC cell lines. Results are the mean±SD of three independent experiments (n=3).

To support these data, we also analyzed the ratio between d16HER2 and WHER2 expression (d16HER2/WHER2) in a small series of human HER2-positive GC (n=14) vs HER2-positive BC samples. In keeping with “in vitro” data, these preliminary results revealed that HER2-positive GC cases are significantly (p=0.0002) characterized by higher d16HER2 expression levels as compared to HER2-positive BC cases (Figure 64).
To verify the existence of both d16HER2M and d16HER2D expression also in HER2-positive GC, we performed Western blot analyses by processing under non-reducing conditions the cell lysates from the HER2-positive OE19 and N87 GC cell lines and, concomitantly, as d16HER2-positive/negative controls from the murine mammary transgenic MI6 cell lysate\textsuperscript{12,13} and MKN45 HER2-negative GC cells. In keeping with qPCR data (Figure 63), we observed stable d16HER2D migrating above 250 kDa, with markedly greater expression in both OE19 and N87 vs the mouse d16HER2-positive mammary MI6 protein extract, either as constitutive basal (Figure 65 left panel) or activated (Figure 65 right panel) forms. As expected, no band was found expressed at the same MW in negative controls (Figure 65, lanes 1).

Figure 64: Levels of d16HER2/WTHE2 ratio expression evaluated by qPCR in a series of HER2-positive BC (red plots; n=52) vs HER2-positive GC (blue plots; n=14) (p=0.0002). Significance was calculated by unpaired t-test.
Figure 65: HER2 expression and activation in HER2-positive BC and GC cell lines: Protein extracts from MKN45 (lane 1), M16 (lane 2), N87 (lane 3) and OE19 (lane 4) tumour cell lines were separated on 3-8% gradient SDS-PAGE under non-reducing conditions and probed with the MAb Ab3 (Oncogene Science) (left panel/constitutive basal form), and the PoAb anti-phospho-HER2 p-Neu (Santa Cruz Biotechnology) (right panel/activated form). anti-Actin AC-15 MAb (Sigma Chemical Co.) was used as a control for protein loading.

In keeping with the possible role of d16HER2 in the stemness of HER2-positive GC, FACS analysis of N87 GC cells sorted for the CSCs-associated marker ALDH revealed a consistent enrichment in the abundance of “total” HER2 in the ALDH-positive cell compartment vs the ALDH-negative (Fig. 66), strongly supporting the hypothesis that HER2 signaling/addiction can regulate the CSCs compartment in HER2-positive GC, similarly to previous evidence in BC.

Figure 66: Representative histograms of data showing HER2 expression (HER2 rMFI) in ALDH-positive (red line) vs ALDH-negative (blue line) cell subsets of the N87 GC cell line. Results are expressed as mean±SD.
5.3 Summary of results

In conclusion, the obtained data clearly indicated an enrichment of HER2 expression in the CSC population of both luminal A BC and HER2-positive GC. In addition, we provided the first evidence of d16HER2 expression and activation in HER2-positive GC suggesting the possibility that d16HER2 could play an important pathobiological role also in HER2-positive GC.
Chapter 6 – DISCUSSION
6.1 Role of d16HER2 in the HER2-driven BC tumourigenesis and susceptibility to specific biotherapies

6.1.1. Implication of d16HER2 in HER2-positive BC tumor aggressiveness

The HER2-positive BC molecular subtype is a particularly aggressive disease characterized by amplification or overexpression of HER2 receptor and accounts for about 20% of all BC (Pupa et al. 2005). The altered expression of the HER2 oncogene guides a high mitotic index, poor prognosis and higher risk of relapse (Pupa et al. 2005). In addition, the complex proteome encoded by HER2 (see paragraph 1.2.3 of Introduction Chapter) could biologically explain the intrinsic and “dynamic diversity” or heterogeneity observed in the HER2-positive disease. In this context, a great deal of evidence suggests that the HER2-positive BC intra- and inter-tumour heterogeneity is significantly enhanced by the co-existence of the full-length WHER2 receptor with different forms of HER2 that could drive selective advantages emerging at the expense of others, thus affecting both sensitivity to HER2-targeted therapies and clinical outcome (Amirouchene-Angelozzi et al. 2017). Recently, an alternative usage of transcript isoforms from the same gene has been hypothesized as an important feature in cancers (Vitting-Seerup et al. 2017), and, in turn, alternative gene products have a consistent and active role in cancer. Currently, many labs are actively investigating the multiple ways by which the pre-mRNA splicing machinery is pathologically altered to promote initiation and/or maintenance of cancer (Sveen et al. 2016). In particular, there is accumulating evidence that several molecular
subtypes of cancer are highly dependent on splicing function for cell proliferation, survival, migration and invasion (Chen et al. 2015; Lee et al. 2016).

In this peculiar pathobiological scenario, the splice variant of human full-length HER2 (WTHER2) lacking exon 16 (d16HER2), was found co-expressed with the full-length receptor in almost 90% of the total analyzed human BC cases accounting for about 10% of all WHER2 mRNA (Castiglioni et al. 2006). In our study, the comparison of the intrinsic tumourigenic potential guided by the two HER2 forms was tested in a consistent number of the proper mouse models respectively transgenic for the human deleted d16HER2 variant (Marchini et al. 2011) and the WHER2 form (Finkle et al. 2004). Findings from such in vivo analyses, performed in collaboration with the PL Lollini’s group (University of Bologna), highlighted the significantly shorter latency period and higher tumour multiplicity driven by the d16HER2 variant vs the full-length isoform demonstrating, for the first time, the powerful and constitutive oncogenic potential of the d16HER2 variant (Castagnoli et al. 2014). These findings prompted us to speculate that d16HER2 expression and activity could display a key role in the pathobiology of the HER2-positive BC disease (Castagnoli et al. 2014). The biochemical characterization of the spontaneous mammary nodules developed in the d16HER2 transgenic mice revealed that the d16HER2 variant was expressed either as monomeric or dimeric form promoting the formation of stable phosphorylated d16HER2 homodimers (pd16HER2D) which, in turn, triggered a downstream intracellular signalling cascade that was found different from that observed downstream the activated WHER2 (pWHER2) monomeric form (Castagnoli et al. 2014). In particular, the intracellular signalling driven by
pd16HER2 elicited multiple signaling circuitries including also the activation of the non-receptor tyrosine kinase SRC. In this context, data preliminarily developed by Mitra and co-authors in d16HER2-transfected cells unveiled a physical interaction between d16HER2 and SRC suggesting that SRC could functionally be the “master regulator” of the splice variant expression and activation. In line with Mitra’s findings, by biochemical analyses, we also found a significant direct correlation between high levels expression of pd16HER2D and pSRC both in tumour samples and the derived mammary tumour cell lines MI6 and MI7, strongly suggesting the existence of a d16HER2/pSRC signalling axis capable to sustain a strong oncogenic phenotype. This signaling axis was not unveiled downstream pWTWER2 (Castagnoli et al. 2014). The co-expression of d16HER2 and SRC kinase on the mammary tumours cell membrane was also confirmed by IHC and confocal microscopy analyses performed on spontaneous mammary lesions. Noteworthy, we also found that the anti-HER2 MGR2 and 4D5 MAbs were able to significantly decrease the activation levels of both d16HER2 and SRC kinase providing the major piece of evidence of the existence of a functional cross-talk between these two oncoproteins (Castagnoli et al. 2014). Additionally, we revealed a significant direct correlation between d16HER2 and pSRC in human HER2-positive BC samples (Castagnoli et al. 2014). Specifically, by confocal microscopy, we observed a heterogeneous expression of pSRC in different human HER2-positive BC patients. Interestingly, the expression levels of pSRC were found to significantly correlate with the levels of d16HER2 mRNA, strongly suggesting that pSRC levels could reflect the expression of pd16HER2D in human HER2-positive BCs. It is well established that aberrant expression and
activity of SRC is a common alteration observed in many tumours being associated with an aggressive and poorly differentiated phenotype and worst prognosis (Yeatman 2004). In particular, in keeping with our very recent data (Castagnoli 2017), pSRC enhances the motility and invasiveness of tumour cells through its close interaction with FAK kinase, another non-receptor tyrosine kinase involved in the regulation of cytoskeleton reorganization and necessary for the cell motility and also is the master regulator of EMT (Frisch et al. 2013).

6.1.2. Implication of d16HER2 in susceptibility to HER2-targeted therapies in HER2-positive BC tumors.

The different HER2 splice variant seems to be implicated in the trastuzumab susceptibility of HER2-positive BC. Scientific literature relating the role of d16HER2 in response to trastuzumab activity was controversial (Alajati et al. 2013; Castiglioni et al. 2006; Mitra et al. 2009) until it was tested in the proper mouse models (Castagnoli et al. 2014). Indeed, the in vitro findings obtained in 2009 by Mitra and colleagues using properly engineered BC cell models provided preliminary evidence suggesting that d16HER2-positive transfectants were resistant to trastuzumab activity compared to those HER2-positive. Successively, Alajati et al. reported the susceptibility of in vivo d16HER2-positive transfectants to trastuzumab activity (Alajati 2013). To make clearer the involvement of d16HER2 vs WHER2 in anti-HER2 biodrugs susceptibility, thanks to the availability of the proper in vivo and in vitro transgenic d16HER2- and WHER2-positive pre-clinical models, we started to investigate the capability of Trastuzumab and Lapatinib to impair the in vivo growth of MI6 and WHER2_1.
As opposite to in vitro data reported by Mitra (Mitra et al. 2009), but in keeping with those shown in vivo by Alajati and colleagues, we found that both anti-HER2 biodrugs significantly halted MI6 tumour growth vs the WTHER2-positive cell counterpart. Indeed, in mice injected with the WTHER2_1 cells, the therapeutic effects exerted by trastuzumab were found to be more modest compared to those observed with the d16HER2-positive cells. On the other hand, lapatinib did not exert any therapeutic effect on WTHER2_1 cells being the growth curves of treated and untreated mice completely overlapping. Thus, our in vivo data demonstrated that the in vitro findings obtained by Mitra were misleading probably due to the lack in in vitro bioassays of immunocompetent cells necessary to exert the trastuzumab-mediated anti-tumour therapeutic effects.

The marked capability of trastuzumab to inhibit d16HER2-driven mammary tumourigenesis was also observed in the d16HER2-positive transgenic mice following either a short or prolonged treatment protocols. Indeed, both the onset and tumour multiplicity of spontaneous neoplastic lesions were significantly impaired by both trastuzumab treatments, preventing tumour development at one year of observation in a minority of d16HER2 females (Castagnoli et al. 2014). Then, we went further with our study and evaluated whether d16HER2 expression and activity might have a significant impact to predict trastuzumab benefits also in HER2-positive BC patients. In particular, in absence of an antibody able to specifically discriminate d16HER2 variant from the WTHER2 form, and, in light of our findings previously described, we evaluated whether pSRC expression could mirror pd16HER2D expression/activity in HER2-positive BC cases treated with trastuzumab in adjuvant setting. To this
aim, we tested a series of 84 HER2-positive cases by confocal microscopy and stratified the patients according to pSRC-High (>20%) or –Low (<20%) expression levels (evaluated only on HER2-overexpressing cells), and, successively, we evaluated their prognosis according to pSRC reactivity. Interestingly, in agreement with the therapeutic findings collected in d16HER2-positive transgenic mice, we observed a significant better prognosis in BC patients characterized by high levels of pSRC, implying the existence of an activated d16HER2/pSRC signalling axis also in the human setting. To delineate the pathobiological tissue context of high expression levels of d16HER2/pSRC axis in HER2-positive BC, we performed an in silico analysis on 21 HER2-positive cases whose molecular profile and relative expression of intratumour d16HER2 transcript were already available and generate a specific d16HER2 signature named “activated-d16HER2 metagene (Castagnoli et al. 2014). Interestingly, we observed that the BC expressing high d16HER2 mRNA and pSRC levels were also characterized by a significant enrichment of genes implicated in tumour metastasis, hypoxia and cell motility pathways, thus indicating, for the first time, the direct involvement of the d16HER2 variant activation in the aggressiveness of HER2-positive BC patients and supporting the data obtained in d16HER2 transgenic mice. To support further the evidence that high levels of d16HER2 expression and activation could be indicative of a better response to trastuzumab, the “activated-d16HER2-metagene” was then tested in public datasets of two distinct HER2-positive BC series. Following these in silico analyses, we found that patients achieving a complete response to trastuzumab-based neo-adjuvant regimen were those characterized by a higher expression of
“d16HER2-activated metagene”. On the other hand, our metagene was unable to discriminate between complete and partial response to neo-adjuvant chemotherapy, thus providing a clear evidence that d16HER2 expression/activity is a marker indicative of HER2-positive BC patients benefitting from trastuzumab-based therapeutic regimens (Castagnoli et al. 2014). These clinical findings appear to be in direct contrast with those reported by Zhang and colleagues who identified in pSRC expression a key node of trastuzumab resistance (Zhang et al. 2011). However, it is important to underline that their HER2-positive BC series consisted of 57 patients treated with trastuzumab in the metastatic setting, whereas our cohort consisted of 84 HER2-positive BC patients treated with trastuzumab in the early disease. In this context, we hypothesize that this apparent controversial might rely on BC patients stage disease treated with trastuzumab. Indeed, at early stage disease HER2-positive BC cells characterized by high levels expression of d16HER2-SRC signaling axis are strongly addicted to the HER2-signalling and, in turn, trastuzumab response (Llombart-Cussac et al. 2017; Triulzi et al. 2015); conversely, at late stage disease the potent oncogenicity guided by high levels expression/activity of d16HER2 could determine a progressive and dynamic gain of many genetic alterations generating new genetic dependencies, causing tumour evasion from HER2 addiction, increasing genomic instability and favouring the development of trastuzumab resistance.
6.2 *Study of the molecular mechanisms underlying the aggressiveness guided by d16HER2 variant and analysis of its involvement in the activity of HER2-positive cancer stem cells activity.*

In order to unveil the biological mechanisms “orchestrated” by d16HER2 expression and activation closely implicated in increasing the tumour aggressiveness of HER2-positive BC, we investigated the gene expression profiles of MI6, MI7 and, for comparison, WTHER2_1 and WTHER2_2 transgenic mammary tumour cell lines. The molecular comparison between all the tumour cells indicated that the d16HER2 splice variant, rather than the WThER2 receptor, was involved in the enrichment of several genes expression related to EMT and CSCs regulation (Castagnoli et al. 2017), two hallmarks of tumour initiation and progression (Hanahan et al. 2011). The results obtained were in complete agreement with those previously reported by Alajati and Turpin (Alajati et al. 2013; Turpin et al. 2016), who, following different strategies and using other pre-clinical models, suggested the candidacy of d16HER2 variant as key regulator of a mesenchymal tumour phenotype. Consistently, our DAVID analysis of genes differentially expressed in d16HER2- and WT HER2-positive transcriptomes revealed the enrichment of several transcripts reportedly involved in migration and invasion as those included in the formation of tight junction (Latorre et al. 2000), focal adhesion, adherent junction (Farahani et al. 2014), Wnt signalling (Monteiro et al. 2014) and others in d16HER2 cells (Castagnoli et al. 2017). These
results prompted us to speculate the possibility that the d16HER2 variant could sustain tumour progression through its capability to up-regulate genes related to EMT and stemness vs the WTHE2 counterpart. Indeed, we also revealed a significant enrichment of Wnt (Monteiro et al. 2014) and Notch genes (Lindsay et al. 2008), pointing to a role for the d16HER2 variant not only in governing the EMT program but also in the maintenance and expansion of CSCs in HER2-positive BC models (Castagnoli et al. 2017). Then, we performed a series of experiments aimed to test and compare the enrichment of CSCs in MI6 vs WTHE2_1 cells. To the best of our knowledge, the significantly higher MFE and self-renewal capability observed in d16HER2-positive compared to WTHE2_1 cells provided the first functional “proof of principle” supporting d16HER2 candidacy instead of full-length HER2 as a key biomarker capable to significantly affect the enrichment and activity of the intratumour CSCs in HER2-positive BC (Castagnoli et al. 2017). Also, our findings showed a significantly higher expression of tumour cells characterized by a CD29^{High}/CD24^{+}/Sca1^{Low} phenotype which has been reported to identify an enrichment of peculiar CSCs (Visvader et al. 2014). Additionally, to support our observations, we carried out the stemness-sustaining “gold standard” bioassay by evaluating in vivo the self-renewal capability of our tumour cell targets. In this context, we injected MI6 and WTHE2_1 cells into the mammary fat pad of FVB mice in limiting dilution conditions and applied the Extreme Limiting Dilution Analysis (ELDA) software, an online tool specifically designed for the analysis of intratumour CSCs frequency. According to our in vitro data, ELDA estimated more than >10-fold increase in CSCs in MI6 vs WTHE2_1 cells (Castagnoli et al. 2017). All such
results provided a clear evidence of the interaction occurring between d16HER2 and cancer stemness in HER2-positive BC models. In keeping with these preclinical data collected using transgenic models, we also observed that the d16HER2 ectopically expressed in human HER2-negative BC cell lines elicited an enrichment of the epithelial and mesenchymal CSC compartments vs the same cell targets engineered to express the WThER2 form. Indeed, the comparison of the d16HER2- and WThER2-transduced cell models unveiling that the d16HER2 variant determined a higher MFE (%), self-renewal capability and an enrichment of ALDH and CD44 expression, thus unveiling a direct action of d16HER2 in human HER2-positive BCICs instead of the well established full-length HER2 (Castagnoli et al. 2017). In this context, in line with the literature (Korkaya et al. 2008; Korkaya et al. 2013; Magnifico et al. 2009), the comparison between WThER2-infected human BC cells with the mock cell lines revealing a greater capability to form mammospheres and a higher expression of ALDH and CD44 biomarkers in MCF7-WT and T47D-WT cells vs their corresponding mock cell populations, thus indicating that also the WThER2 form is capable to regulate BCICs in human BC, but to a lesser extent than the d16HER2 splice variant (Castagnoli et al. 2017). These data underline the capability of d16HER2 to increase both the epithelial and mesenchymal CSC populations and, again, strongly sustain the candidacy of the d16HER2 isoform as the master regulator of the CSC compartment of HER2-positive BC. In addition, in keeping with our results (Castagnoli et al. 2017), we have hypothesized that the enriched expression of the mesenchymal stemness-related biomarker CD44 observed upon d16HER2 lentiviral infection of MCF7 cells could sustain a biological
explanation for the findings obtained by Alajati who previously showed an increased expression of N-cadherin in MCF7 cells upon their plasmid engineering with d16HER2 (Alajati et al. 2013).

The clinical validation of our preclinical data was performed by analyzing two independent gene datasets of HER2-positive BC patients. After our in silico analysis, we evidenced a significant enrichment of Notch genes family and other genes downstream of Notch signaling only in the HER2-positive BC cases characterized by high levels expression of the “activated-d16HER2 metagene”, i.e. those cases expressing high levels of tumour metastasis, cell motility and hypoxia genes and highly responsive to Trastuzumab. The functional evidence that a dynamic cross-talk occurred between the pd16HER2D/pSRC axis and the NOTCH members came from the therapeutic efficiency displayed by the γ-secretase inhibitors DAPT- and/or RO4929097 which were both found to significantly inhibit mammosphere formation in MI6 vs the WHER2_1 counterpart (Castagnoli et al. 2017). Based on a well-established literature demonstrating a close interaction between HER2 and NOTCH signaling, our current pre-clinical and clinical findings provide a clear evidence that d16HER2, rather than WHER2, is the real driver of the functional link between these two pathways which are functionally active in the pathobiological context of HER2-positive cancer stemness (Baker et al. 2014).

Taking into consideration the capability of trastuzumab to effectively target the HER2-positive CSC compartment (Korkaya et al. 2008; Magnifico et al. 2009; Martin-Castillo et al. 2015), in our study we evaluated whether the higher sensitivity of d16HER2-positive tumour cells to trastuzumab vs those WHER2-
positive could find its biological basis in the ability of the humanized monoclonal antibody to target d16HER2-positive CSCs in the early stage of the disease. In keeping with our speculation, we found that trastuzumab significantly impaired the MFE% of only d16HER2-positive cells, whereas no therapeutic effect was exerted towards mammospheres derived from WHER2_1-positive cells. Furthermore, \textit{in vivo} experiments carried out to evaluate trastuzumab-mediated anti-CSCs activity revealed that the biodrug was effective only when the MI6 orthotopic tumours were treated at palpable dimension when the cancer stem cells contribution is crucial to sustain tumour growth and progression. These results also provided a biological and evident explanation of our clinical data indicating that the expression and activation of d16HER2 in HER2-positive BC patients treated with trastuzumab correlates positively with a better outcome.

6.3 \textbf{Implication of d16HER2 expression and activation in CSCs activity of HER2-negative luminal BC and HER2-positive gastric cancers.}

Taking into consideration the data published by Ithimakin and coworkers pointing out that the full-length HER2 oncoprotein is selectively expressed in HER2-negative luminal BC CSCs and regulates their self-renewal (Ithimakin et al. 2013), we performed preliminary experiments aimed to investigate whether d16HER2 expression and activation could affect to some extent the luminal HER2-negative MCF7 and T47D BC stemness. Our preliminary results confirmed the enrichment of HER2 expression in the CSC compartments of the tested HER2-negative BC cells. To highlight whether the d16HER2 splice variant can play a role
in the stemness of HER2-negative BC, we will assess the expression levels of the splice variant in the CSCs population of HER2-negative luminal BC cell lines sorted according to the expression of the epithelial CSC biomarker ALDH and, then, compare the d16HER2 mRNA levels with those obtained testing the ALDH-negative cells.

In order to extend the study of the d16HER2 variant to other epithelial oncotypes characterized by the HER2-overexpression/amplification, we started to analyze the CSC population of HER2-positive GCs that represent about the 20% of the total gastric neoplasms (Boku 2014). Our preliminary studies, still unpublished, show a significantly higher d16HER2 mRNA expression in the HER2-positive GCs vs the HER2-positive BCs, thus implying that the d16HER2 variant may play an important pathobiological impact also in GC. In keeping with the very high levels expression of the d16HER2 transcript in the HER2-positive GC vs relevant BC, we found a marked expression of stable pd16HER2D in both HER2-positive GC cell lines vs the negative one, suggesting that the high levels expression of d16HER2 transcript could reflect a proportional activation of pd16HER2D. The consistency of these preliminary biochemical findings was sustained in a small cohort of HER2-positive GC cases. Again, the comparison of the d16HER2 expression in HER2-positive GC vs BC patients revealed significantly higher expression levels of d16HER2 in GC vs BC cases. This last set of data on HER2-positive GC included in my PhD thesis, not only support the results already reported in HER2-positive BC (Castagnoli et al. 2014; Castagnoli et al. 2017), but heavily imply that d16HER2 expression and activation could be implicated in the aggressiveness of HER2-positive GC playing a key role also in the susceptibility to anti-HER2 targeted
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strategies and GC stemness. In this specific context, Jiang and colleagues have reported the capability of trastuzumab to target the CSC compartment of HER2-positive GC suggesting that ERBB2 signaling has a role in maintaining the CSC populations (Jiang et al. 2012). In keeping with these already reported findings, we very recently observed higher levels expression of the HER2 oncoprotein in the ALDH-positive stem cell compartment vs the ALDH-negative thus sustaining a functional role of d16HER2 also in HER2-positive GC. This evidence is preliminary to a new project aimed to unveil HER2 addiction in HER2-positive GC to respond to the health need of improving therapeutic strategies in this particularly aggressive oncotype.

6.4. Concluding remarks

Overall, the preclinical, clinical and in silico data reported in this PhD thesis demonstrate that: 1) the activated d16HER2 variant drives a constitutive oncogenic signalling vs the WTHER2 receptor through its functional interplay with the activated SRC kinase (pSRC); 2) HER2-positive BC patients characterized by high levels of d16HER2 expression and activation are significantly more responsive to trastuzumab therapeutic effects compared to those with low levels; 3) the d16HER2 variant rather than WTHER2 receptor plays a crucial role in the regulation of stemness/EMT of HER2+ BCs and 4) may play a potent oncogenic role in the aggressiveness of HER2+ gastric cancers. In particular, this study dissects the biological role exerted by the d16HER2 variant in HER2+ BCs making clearer its pathobiological role in the HER2-positive disease. In this context, we found that the d16HER2/pSRC signaling axis may be considered a
novel potential marker predicting BCs characterized by high responsiveness to trastuzumab. In keeping with the significant impact of d16HER2 expression and activation to determine mammary tumourigenesis and trastuzumab susceptibility, we revealed that the d16HER2 variant is enriched in the CSC subset of HER2+ BCs strongly sustaining its candidacy as the main driver of HER2+ BCSCs activity. This finding is clinically relevant according to the recent data supporting that cancer eradication/cure is strongly related to the capability of the anti-cancer therapy to completely silence the cancer stem cell compartment (Martin-Castillo B, Oncotarget, 2015). In addition, the results of my Ph.D thesis provide the first evidence of the expression of d16HER2 variant in HER2+gastric cancer, a neoplasia characterized by distinctive features of aggressiveness.

6.5. Future perspectives

Future perspectives that arise from this PhD thesis include further studies aimed to investigate the regulation of d16HER2 expression and, in particular, we will focus our efforts to unravel the HER2-related splicing machinery alterations that contribute to increase the cancer cell heterogeneity and whose consequences on the cancer cell biology are still not clear. The knowledge of the whole HER2-related splicing machinery modifications will contribute to modulate the expression and activity of d16HER2 variant thus impairing its driven aggressiveness. Furthermore, we are setting up a new molecular methodology aimed to identify intratumour d16HER2 mRNA expression in the attempt to identify the HER2-positive BC patients sensitive to trastuzumab therapeutic
effects. In this context the BaseScope™ assay is a high resolution RNA “in situ” hybridization (ISH) that allows the detection of specific transcripts in FFPE tissue samples. Such molecular strategy can be used to reveal and semi-quantitatively assess the mRNA expression. In particular, three different BaseScope™ ISH probes were designed to discriminate d16HER2 from the WHER2 form in HER2-positive oncotypes as BC, GC and colorectal cancers.
Chapter 7 – PUBLICATIONS
7.1. Publications related to data reported in this thesis


7.2. Other publications produced during my PhD program


Chapter 8 – References List

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