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PhD Thesis

Doctoral Programme in Biomedicine

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ABBREVIATIONS

3, 3´- diaminodbenzidine	DAB
A	
Antibody Dependant Cell mediated Cytotoxicity	ADCC
Aromatase Inhibitors	AI
Aldheyde Dehydrogenase isoform H1	ALDHA1
American Society of Clinical Oncology	ASCO
Artemin	ARTN
B	
Bone Marrow Derived Cells	BMDCs
Bovine Serum Albumin	BSA
Breast Cancer	BC
C	
Cancer Associated Fibroblasts	CAFs
Central Nervous System	CNS
Chromogenic *In Situ* Hybridization	CISH
Circulating Tumour Cells	CTCs
Cyclin Dependant Kinases	CDKs
Cytokeratin	CK
Cancer Stem Cell	CSC
D	
Dendritic Cells	DC
Differentially Expressed	DE
Deoxyribonucleic Acid	DNA
Disseminated Tumour Cells	DTCs
Ductal Carcinoma *In Situ*	DCIS
E	
Endocrine Neoplasia Type 2	MEN 2
Endothelial Growth Factor Receptor	EGFR
Endothelial Progenitor Cells	EPCs
Enzyme Linked Immunosorbent Assay	ELISA
Ephrin Receptors	EPH
Epidermal Growth Factor	EGF
Epidermal Growth Factor Receptor	EGFR
Extracellular Domain	ECD
Extracellular Matrix	ECM
F	
FGFR Substrate 2	FRS2
Fibroblast Activation Protein	FAP
Fibroblast Growth Factor 2	FGF2
Fibroblast Growth Factor Receptors	FGFRs
Fibroblast Growth Factors	FGFs
Fibroblasts Specific Protein-1	FSP-1
Foetal Bovine Serum	FBS
Fold Change	FC
Fluorescent *In Situ* Hybridization	FISH

Formalin-Fixed Paraffin Embedded	FFPE
G	
Glial Derived Neurotrophic Factor	GDNF
Glycosyl Phosphatidylinositol	GPI
G-protein-coupled Receptors	GPCRs
Growth Factor Receptor- Bound protein 2	GRB2
Guanosine Diphosphate	GDP
Guanosine Triphosphate	GTP
H	
Heat Shock Protein 90	Hsp90
Heparan Sulfate Proteoglycans	HPSGs
Hepatocyte Growth Factor	HGF
Hepatocyte Growth Factor Receptor	c-Met
Human Mammary Epithelial	HME
Atypical Ductal Hyperplasia	ADH
I	
Immunoglobulin-like	Ig
Immunohistochemistry	IHC
Including Nerve Growth Factor Receptor	TrkA
Ingenuity Pathway Analysis	IPA
Insulin	InsR
Insulin-like Growth Factor Receptor	IGFR
Insulin-like Growth Factor	IGF
Intercellular-adhesion Molecule 1	ICAM1
Invasive Breast Cancer	IBC
Invasive Ductal Carcinoma Not Otherwise Specified	IDC-NOS
M	
Mammalian Target of Rapamycin	mTOR
Matrix Metalloproteinases	MMPs
Medullary Thyroid Carcinoma	MTC
Mesenchymal Stem Cells	MSCs
Mitogen Activated Protein Kinase	MAPK
Myeloid Derived Cells	MDCs
Myeloid Derived Suppressor Cells	MDSCs
N	
National Comprehensive Care Network	NCCN
Natural Killer	NK
Nerve Growth Factor	NGF
Neutrophic Tyrosine Kinase Receptor A	NTRKA
Neurturin	NRTN
Non-Small Cell Lung Carcinoma	NSCLCs
O	
Oestrogen Receptor	ER
Overnight	ON
P	
Papillary Thyroid Carcinomas	PTCs
Paraformaldehyde	PFA
Persephin	PSPN

Phosphatidylinositol	PI
Phosphatidylinositol 3-Kinase	PI3K
Phosphatidylinositol Dependant Kinase-1	PDK1
Phosphor Tyrosine Binding	PTB
Platelet Derived Growth Factor	PDGF
Platelet Derived Growth Factor Receptor	PDGFR
Platelet Derived Growth Factor Receptor-β	PDGFR-β
Pleckstrin Homology	PH
Progesterone Receptor	PR
Propidium Iodide	PI
Protein and Gene Double Staining	PGDS
Protein Kinase B	PKB
Protein Kinase C	PKC
Phosphatase and Tensin Homolog Deleted on chromosome 10	PTEN
R	
Radio Immunoprecipitation Assay	RIPA
Reactive Oxygen Species	ROS
Receptor Tyrosine Kinase	RTKs
Rearranged during Transformation	RET
Receptor Tyrosine Kinase Like Orphan Receptor	ROR
Reverse Transcriptase Polymerase Chain Reaction	RT-PCR
Ribosomal Protein S6 Kinase 2	RSK2
Room Temperature	RT
S	
Signal Transducer and Activator of Transcription	STAT
Silver Enhanced *In Situ* Hybridization	SISH
Single Locus Short Tandem Repeat	STR
Src Homology 2 domain Containingn Transforming Protein	SHC
T	
Tyrosine Kinase with Ig and EGF homology domain	TIE
TIE2 Expressing monocytes	TEMs
Tyrosine Kinase Inhibitors	TKIs
Tissue Micro Array	TMA
Transforming Growth Factor β	TGF-β
Triple Negative Breast Cancers	TNBC
V	
Vascular Endothelial Growth Factor	VEGF
Vascular Adhesion Molecule 1	VCAM1

ABSTRACT

HER2 positivity defines a molecular subtype of breast cancer with an aggressive biological behaviour and poor prognosis. Its pharmacological targeting with the monoclonal antibody Trastuzumab, and later on with the small tyrosine kinase inhibitor Lapatinib, changed the prognosis for HER2-positive breast cancer patients. However, despite the positive results from many trials, resistance to anti-HER2 agents occurs in both metastatic and adjuvant settings, in part, because HER2 represent just a part of a more complex biological network, that when deregulated, plays a central role in sustaining the aggressive phenotype of tumour cells.

Moreover, the importance of the tumour microenvironment in drug resistance has been recognized during the last years, and it is now widely accepted that growth, survival and metastasis (as a consequence of treatment failure), are regulated by stromal-cancer cell interactions.

Then, it is likely that the tumour stroma, and cancer associated fibroblasts in particular, influence also the therapeutic outcome. Therefore, our hypothesis is that during the acquisition of drug resistance cancer and stromal cells co-evolve, and that the tumour cells can induce changes in the structure and composition of the microenvironment to support their own growth and progression or dissemination.

Our research has defined a coevolution process between breast cancer cells and fibroblasts where fibroblasts and BC cells crosstalk drives the drug resistant phenotype. We have identified RET and FGFR2 pathways as novel mechanisms of HER2 activation and we have demonstrated the importance of the interaction between these pathways *in vitro* and *in vivo*, in promoting breast cancer progression and resistance to Trastuzumab and Lapatinib.

Furthermore, we propose that inhibition of RET and FGFR2 pathways might become a promising strategy after Trastuzumab and Lapatinib failure in patients with HER2-positive breast cancer.

RESUM

El càncer de mama és una malaltia molt heterogènia que inclou diferents subtipus moleculars. El subtipus HER2+ suposa aproximadament el 15-20% dels diferents casos diagnosticats, i es caracteritza per presentar amplificació gènica en la regió 17q 12, que resulta en la sobrexpressió del receptor amb activitat tirosina cinasa HER2. Aquest subtipus presenta una pitjor evolució de la malaltia, estant associat a una major propensió de metàstasis a sistema nerviós, donant lloc a un fenotip més agressiu i de pitjor prognosi.

En els darrers anys la evolució clínica d'aquests pacients s'ha vist millorada gràcies al disseny de teràpies dirigides, que han permès el desenvolupament de nous fàrmacs com l'anticòs monoclonal Trastuzumab o l'inhibidor dual dels dominis tirosina quinasa de EGFR i HER2 Lapatinib. Tot i els resultats clínics inicials i l'avantatge que el Lapatinib va suposar pel tractament de pacients amb càncer de mama, alguns pacients no responen de inici al tractament, i de la mateixa manera que amb el Trastuzumab, pacients inicialment receptius desenvolupen resistència durant les primeres fases del tractament.

La interacció entre el tumor i un microambient favorable és essencial perquè les cèl·lules tumorals puguin progressar, i durant els darrers anys el paper del microambient en l'establiment de resistències has estat àmpliament estudiat i acceptat.

D'aquesta manera, és plausible, que l'estroma tumoral i en concret els fibroblasts influenciïn el pronòstic dels pacients. La nostre hipòtesi es basa en la assumpció que els diferents tractaments terapèutics podrien afavorir la selecció de cèl·lules tumorals amb propietats úniques amb una millor adaptació al microambient, i que aquestes mateixes cèl·lules podrien interaccionar de forma diferent amb el seu entorn, produint canvis en el propi microambient per tal d'afavorir el seu creixement, progressió i disseminació.

Els nostres resultats defineixen una coevolució entre les cèl·lules de càncer de mama i els fibroblasts. Hem identificat la via de RET i FGFR2 com un nou mecanisme d'activació de HER2, demostrant també la importància d'aquestes vies en l'establiment del fenotip resistent, així com en la progressió tumoral *in vitro* i *in vivo*. També proposem la inhibició de les vies de senyalització de RET i FGFR2 com a estratègia de rescat per aquells pacients amb càncer de mama HER2+ que han generat resistència a Trastuzumab i Lapatinib i que avui en dia presenten poques alternatives terapèutiques.

1. BREAST CANCER

1.1 ANATOMY OF THE BREAST

The breast is a bilateral organ that undergoes dramatic changes in size, shape, and function in females from intrauterine life to senescence (including infantile growth, puberty, pregnancy, lactation, and postmenopausal regression). In fact, the development of the human breast is a progressive process initiated during embryonic life, that experiences the largest growth during puberty with lobule formation. However, it is not until the end of the first full term pregnancy that the development and differentiation of the breast are completed [1].

The breast is composed mainly of adipose and glandular tissue with a surrounding dense fibrous stroma (**Figure 1A**). The glandular tissue is made up of glands called lobules, which are able to produce milk during lactation, and thin tubes called ducts that carry the milk from the lobules to the nipple. In the sexually mature breast, the glandular tissue consists of 15-20 lobules arranged radially around the nipple. Each lobe arises from multiple lobules, which connect to a common terminal interlobular duct that will then continue to their way out through the nipple [2].

Histologically, lobules and ducts are formed by two cell layers. In this way, ducts are lined by a single layer of luminal epithelial cells with secretory properties that are surrounded by transversely oriented myoepithelial cells (**Figure 1B**).

The myoepithelial cell layer is incomplete and for this reason some luminal cells could reach the basement membrane. Myoepithelial cells are attached to the adjacent luminal cells by desmosomes and to the basement membrane by hemi desmosomes [3].

These structures are separated from the surrounding tissue (stroma), by a basement membrane, whose breach is one of the hallmarks that distinguishes the transition to invasive carcinoma from carcinoma *in situ* [4].

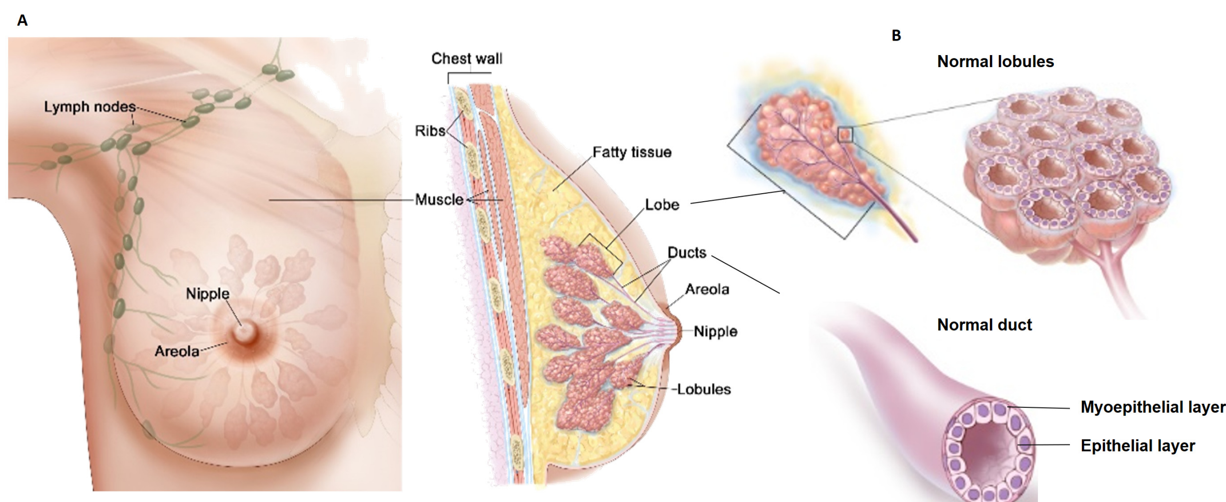


Figure 1. A. Anatomy of the female breast. B. Detail of a normal lobule and duct structure. *Taken from National Institute of Health webpage http://www.cancer.gov/types/breast*

The surrounding stroma comprises the extracellular matrix (ECM), discrete cells (fibroblasts, immune cells, and adipocytes), and organized structures (blood and lymph vessels), each of which contributes to the overall configuration of the local microenvironment [5].

1.2 EPIDEMIOLOGY

Breast cancer (BC) is recognized as the most frequently occurring cancer diagnosed among women, both in developing and developed regions, and more than 1.67 million new cases are annually diagnosed worldwide, representing the 25% of all cancers in 2012. In terms of mortality, it is estimated that around 95.357 women will die from breast cancer in 2015 in the Europe Union [6].

The World Cancer Report from 2014, published by the World Health Organization's International Agency for Research on Cancer, said that in 2012 the global incidence of cancer rose to an estimated 14 million new cases, a number expected to rise to 19.3 million by 2025 [7].

Female breast incidence and mortality rates vary among countries, the estimated age-standardized incidence and mortality rates for breast cancer in Spain are lower than in other countries like United States, Canada, United Kingdom, Belgium, Germany, France or Sweden, and are similar to those in Portugal and Slovenia [7], **Figure 2**.

Around new 26.000 breast cancer cases are diagnosed each year in Spain, which represent the 30% of all cancers diagnosed among women.

In Catalonia, there will be 26,455 incident cases diagnosed among men and 18,345 among women during 2020, which means an increase of 22.5 and 24.5 % comparing with the cancer incidence rates of 2010.

In women, the increase of cases (24.5 %) can be partitioned in three components: 9 % due to ageing, 8 % due to an increase in population size and 8 % due to cancer risk. 4.841 women will be diagnosed with breast cancer and around 932 patients will die from this disease in 2020 [8].

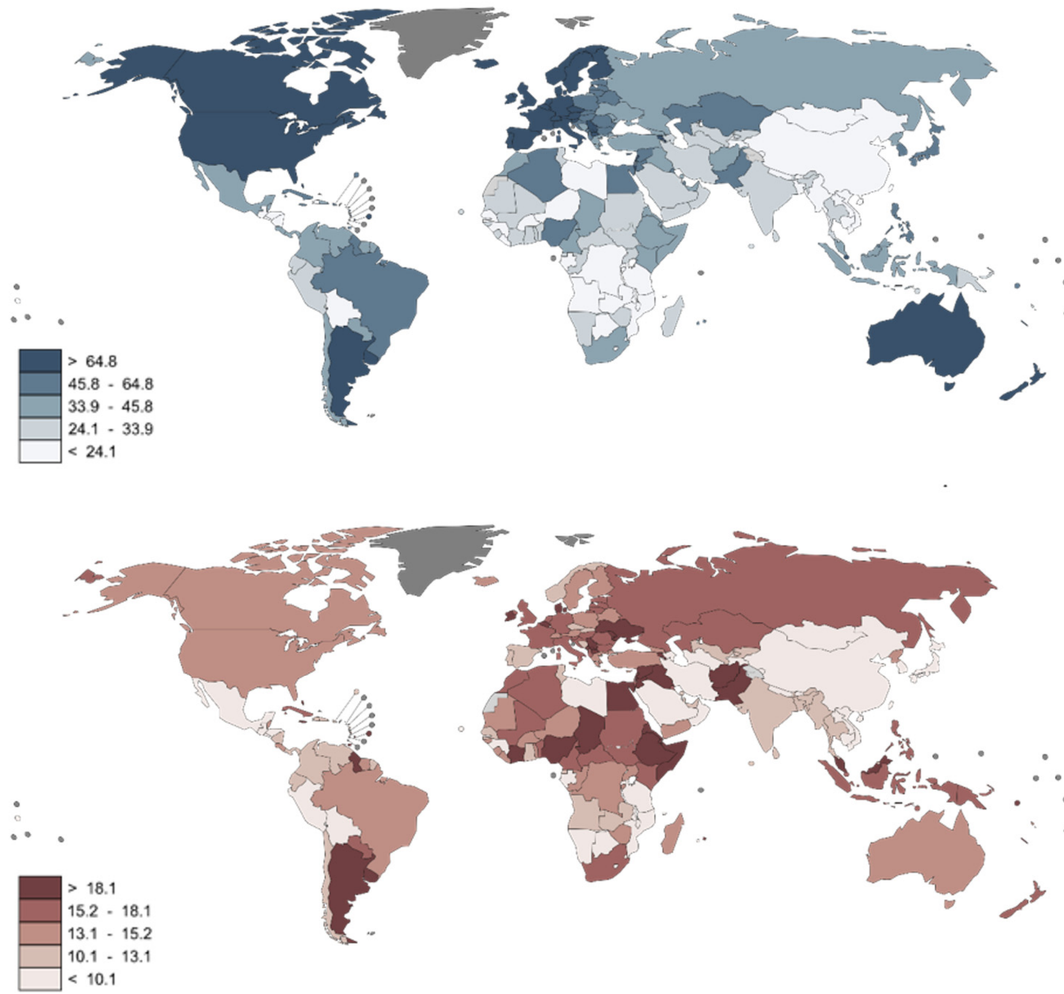


Figure 2. Estimated age-standarizated incidence (top map) and mortality (bottom map) distribution of breast cancer in women worldwide. *Adapted from http:globocan.iacr.fr*

1.3 PROGNOSIS

Prognostic markers are defined as tumour characteristics established at the time of diagnosis that determine the natural evolution of the disease in the absence of treatment and that are associated with outcome [9].

In breast cancer, the most common well-documented prognostic factors are: patient age, histological grade, tumour size, lymph node status, proliferation rate, hormone receptor status, and HER2 status.

1.3.1 Histological grade

The histological grade is based on the tumour's degree of differentiation and is a well-established prognostic factor in breast cancer [10]. A pathologist will determine the histological grade of a breast tumour by studying the tissue and cells in a sample from the lesion, obtained by biopsy, lumpectomy or mastectomy.

The histological grade takes into account three cellular features: degree of nuclear atypia, the mitotic activity, which determines the tumour's rate of proliferation, and to what extent the intraductal carcinoma has formed tubules. Each of these parameters is assigned with a numeric value from one to three. The most widely used system for grading is the Scarff, Bloom and Richardson (SBR) classification, latter modified by Elson and Ellis [11].

The histological grade is then categorized in well differentiated (grade 1), moderately differentiated (grade 2) or poor differentiated (grade 3), **Table 1**, **Figure 3.** Well differentiated tumours tend to show very good outcomes, whereas poorly differentiated tumours are usually associated with high risk of recurrence [12].

1.3.2 Tumour stage: TNM

While histological grade captures only cellular features, the tumour staging system is a way to classify the state of the disease as a whole.

The TNM breast cancer classification system was developed by the Union for International Cancer Control (UICC) and is a worldwide standard for staging breast tumours. This system divides the disease into groups taking into account three features: the size of the primary **t**umour, the **n**odal status and **m**etastasis (TNM) [13].

The **T**, **tumour feature**, is divided into three T-size categories: T1 tumours smaller than

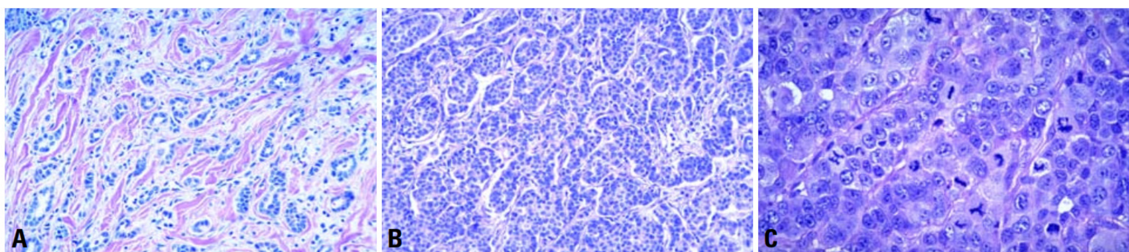


Figure 3. Representative images of different breast cancer tumour grades. A. Infiltrating ductal carcinoma, grade I. B. infiltrating ductal carcinoma, grade II. C. Invasive ductal NOS carcinoma, grade III with no evidence of glandular differentiation. *Taken from Rakha et al, 2010.*

20mm; T2 tumours between 20-50mm and T3 tumours larger than 50mm. In addition, TX is referred when the tumour size cannot be assessed; T0 if there is no primary tumour; Tis when there is a carcinoma *in situ*, and T4 when the tumour (any size) has extended to either the chest walls or skin.

The **N**, **regional nodal status**, categorizes the tumours according to the number of positive regional lymph nodes metastasized by tumour cells, in this context: pNX is used when regional lymph node cannot be assessed; pN0 when no metastatic lymph node is found; pN1 when metastasis is found in 1-3 axillary lymph nodes, pN2 when metastasis is found in 4-9 axillary lymph nodes, and pN3 when metastases are found in more than 10 axillary lymph nodes.

Finally, the **M**, **distant metastasis**, is divided into three categories: MX when presence of distant metastasis cannot be assessed, MO when no distant metastasis is detected and M1 when distant metastasis is detected.

The three TNM parameters are combined in the staging system (**Table 2**).

In addition, a prognostic index termed Nottingham Prognostic Index (NPI) was developed from a multiple-regression analysis of 387 patients with primary breast cancer. This system utilizes three parameters: tumour size (S), number of positive lesions (N) which is subdivided in three categories: 1 for no lesions, 2 for 1-3 positives, and 3 for more than 4 positives; and tumour grade (G).

The NPI score is divided in intervals and each interval represents a probability of 5 years survival [14].

1.3.3 Proliferation rate

Several biological markers have been assessed to evaluate cell proliferation in breast cancer, currently, one of the most widely used is the immunohistochemically assessment of the KI67 antigen which is informative as a predictive and prognostic marker.

The expression of KI67 varies in intensity throughout the cell cycle, reaching a pick during the mitosis, which makes KI67 a good marker for proliferation. A high KI67 score is associated with higher chance of response

FEATURE	SCORE
A. Tubule and gland formation	
• Majority of tumour (>75 %)	1
• Moderate degree (10-75 %)	2
• Little or none (<10 %)	3
B. Nuclear pleomorphism	
• Small, regular uniform cells	1
• Moderate increase in size and variability	2
• Marked variation	3
C. Mitotic counts	
• <10 mitoses in 10 microscopic fields	1
• 10-19 mitoses in 10 microscopic fields	2
• >20 mitoses in 10 microscopic fields	3
HISTOLOGICAL GRADE	**SCORE***
Grade I (well differentiated)	3-5
Grade II (moderately differentiated)	6-7
Grade III (poor differentiated)	8-9

Table 1. Semi-quantitative method for assessing histological grade in breast.
*Total score represented as A+B+C
Adapted from Elson and Ellis 2002.

to chemotherapy but also with poor prognosis, as it is a marker of tumour aggressiveness [15, 16].

STAGE	T	N	M
Stage 0	Tis	N0	MO
Stage I	T1	N0	MO
Stage IIA	T0	N1	MO
	T1	N1	MO
	T2	N0	MO
Stage IIB	T2	N1	MO
	T3	N0	MO
Stage IIIA	T0	N2	MO
	T1	N2	MO
	T2	N2	MO
	T3	N1	MO
	T3	N2	MO
Stage IIIB	T4	Any N	MO
	Any T	N3	MO
Stage IV	Any T	Any N	M1

Table 2. Breast cancer staging according to the TNM System. *Adapted from Sobin L. et al, 2010.*

1.3.4 Classification of cellular receptors

The presence of specific markers is routinely used to define subtypes with differential overall prognosis and also to identify tumours susceptible to targeted treatments, hence, breast tumours are often classified by their expression of the hormone receptors for, oestrogen (ER) and progesterone (PR), as well as for the growth factor receptor HER2 (**Figure 4**).

The **oestrogen receptors** (ERα and ERβ, encoded by the genes *ESR1* and *ESR2* respectively) are nuclear receptors activated by the binding of oestrogen. ER can function as a transcription factor and stimulate cellular functions such as proliferation, differentiation or angiogenesis among others [17].

ER positivity is usually accompanied with PR positivity, that is a predictive factor for better response to Tamoxifen. However, Tamoxifen is also administrated to the small group of patients with ER-/PR+ tumours [17].

Moreover, ER+/PR- tumours are usually methylated in the CpG Island of the *ESR1* gene promoter regions, expressing lower amount of ER and so responding less to the treatment [18].

The **HER2 receptor** is also routinely analysed in breast cancer. This cytoplasmic membrane receptor has tyrosine kinase activity, it is overexpressed in around 18-20% of all breast tumours, and it is a marker of poor prognosis [19]. HER2 can heterodimerize with other epidermal growth factors like EGFR, activating the PI3K/AKT pathway. HER2 can be targeted with Trastuzumab and Lapatinib, and treatment with these drugs has improved the relative poor prognosis of this group of patients [20].

Together, the three receptors and in particular ER and HER2 are important for the classification of tumours and are also used as prognostic and predictive markers, having key implications in deciding adjuvant treatments.

In addition to these prognostic factors, the development of new technologies during these last years has provided new tools for managing patient's outcome and prognosis. Gene expression profiling of breast tumours has identified gene signatures with prognostic value in overall survival and recurring disease [21, 22]. Mamma Print®

(Agendia) is a microarray based tool for assessing the risk of local recurrence by measuring gene expression in fresh tumour tissue from lymph node negative breast cancer patients with tumours smaller than 5 cm [22]. Oncotype DX® (Genomic Health) uses quantitative reverse transcription polymerase reaction (qRT-PCR) to predict the benefit from chemotherapy and the risk of recurrence in early stage ER+, lymph node negative breast cancers [23].

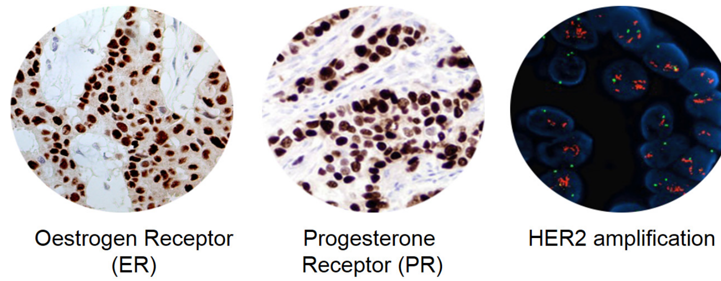


Figure 4. Representative images for ER and PR immunohistochemistry staining (left and middle panels) and FISH showing HER2 amplification (right panel). *From different sources (www.stonybrookmedicine.edu and HerceptTest™ Interpretation Manual Breast Cancer)*

1.4 THE NATURAL HISTORY OF BREAST CANCER

1.4.1 Breast cancer origin and tumour evolution models

The natural history of breast cancer is not completely well known, and different hypotheses have been suggested to explain breast carcinogenesis.

The linear model postulates that epithelial cells progressively evolve through the following (non obligatory) phases: normal healthy breast tissue, hyperplasia, and atypical hyperplasia, carcinoma *in situ* and invasive carcinoma [24], (**Figure 5**).

Thereby, cancer arises when the processes that control the multiplication and life span of normal cells are bypassed. As a result, cells start multiplying uncontrollably, fail to die and accumulate, finally forming a tumour mass.

Over time, tumours can enlarge as more cells accumulate, until some cells gain the ability to invade local tissues and spread, and metastasize to distant sites. This progression can take years or decades and also requires the accumulation of genetic alterations [25]. Cells reads the DNA code to produce functional proteins. As mutations in the DNA can result in altered protein amounts or functions, if these changes alter proteins that control certain critical cell functions, such as cell multiplication or survival, they can ultimately lead to cancer [26].

There is growing evidences that the carcinoma *in situ* is the direct precursor for most invasive breast cancers, and many of these cancers are indeed accompanied by an *in situ* component [27, 28].

Looking into the multi-step process model of breast carcinogenesis in more detail, as

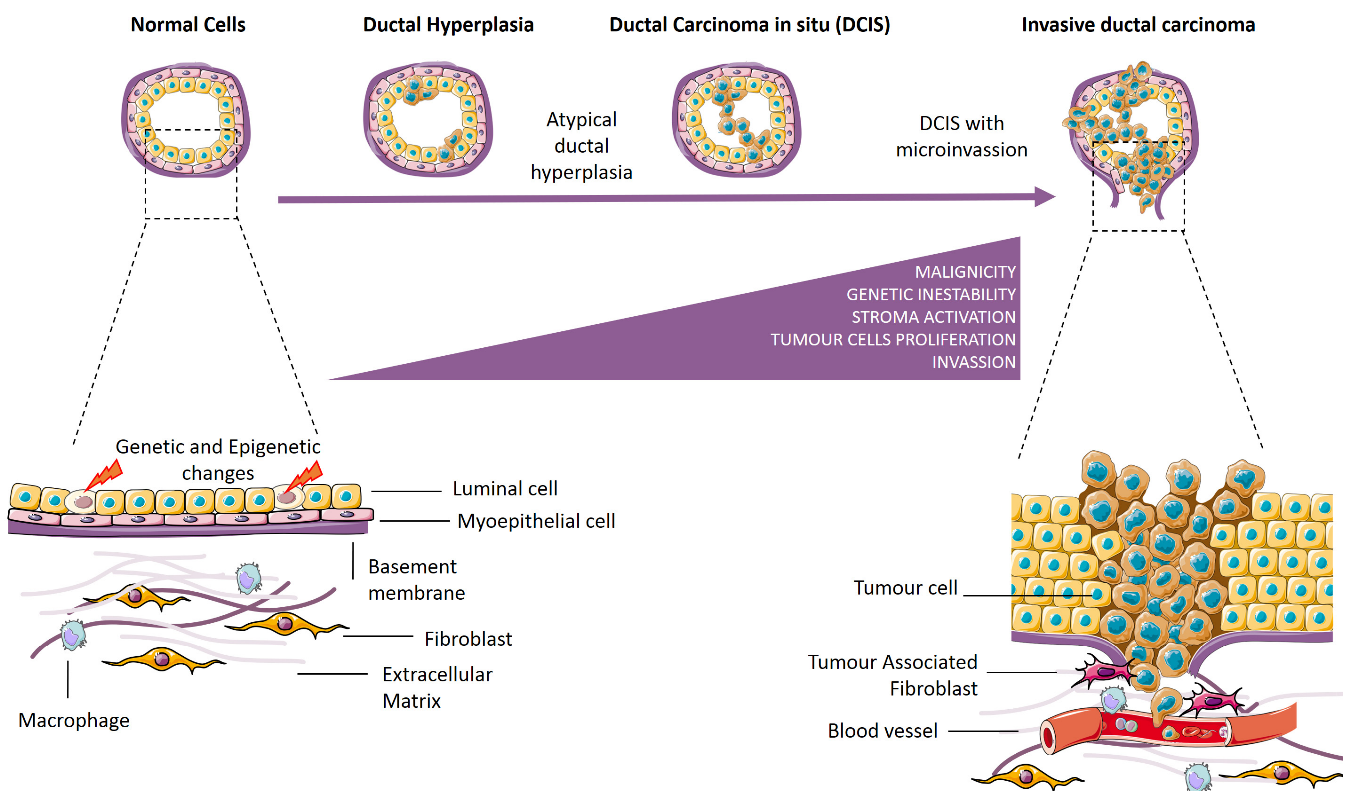


Figure 5. Hypothetical model of breast tumour progression. *Adapted from Vargo et al. 2007.*

mentioned before, the normal breast lobules and ducts consist of a bi-layered epithelium of luminal and myoepithelial cells. Hyperplasia and atypical ductal hyperplasia (ADH) are premalignant lesions characterized by abnormal cell layers within the duct or lobule. This lesions are considered premalignant because of their non-invasiveness and lack of ability to metastasize [24].

ADH is thought to be the precursor of ductal carcinoma *in situ* (DCIS), which is a non-invasive lesion that contains abnormal cells, and is defined as a proliferation of malignant epithelial cells without signs of invasion. With each stage, the risk of developing malignant or invasive breast cancer increases, in fact, approximately half of the patients with DCIS develop invasive breast cancer (IBC) [29, 30], (**Figure 5**). In IBC the myoepithelial cells layer is lost and invasive tumour cells can penetrate through the basement membrane into the stroma. There, they have the potential to invade the vasculature, survive without adhesion, spread through the vasculature system and thereby reach regional lymph nodes or other sites and invade the surrounding tissue causing distant metastasis in a foreign microenvironment [29, 31].

However, how tumours evolve is still an open question, and during the last years the linear simplistic model was challenged by an increasing amount of data showing molecular and genetic differences between certain specimens at the same progression level.

Furthermore, several parallelisms between progenitor cells of normal breast and cancer cells have lead the scientific community to propose that cancer cells with stem cell-like properties, called "tumour-initiating cells" drive breast cancer initiation, progression and recurrence (some of these characteristics include dormancy, self-renewal and differentiation capabilities) [30].

In 2007, Polyak proposed two hypothetical progression models: the "cell of origin" model, and the "tumour subtype-specific transforming event" model (**Figure 6**) [25].

In the **cell of origin model**, a bipotential stem cell has the ability of self-renewal and to give rise to more differentiated myoepithelial and luminal progenitor cells. Tumourigenesis starting in the bipotential stem cell will give rise to the basal like-subtype, while tumourigenesis in the luminal progenitor cells will give rise to the luminal-like subtypes. In the second model, the **tumour subtype-specific transforming event**, the cell of origin is the same for all subtypes, but specific genetic and epigenetic changes determine the subtype [25]. This model describes what a classic linear stepwise model cannot, the progression of the existing different subtypes that will be further described in **sections 1.6.1 and 1.7.2.**

Later on, in 2010, Navin and Hicks described five distinct tumour progression models: monoclonal evolution, polyclonal evolution, self-seeding, mutator phenotype and the cancer stem cell model [32].

Briefly, in the **monoclonal progression model**, a single dominant cell cone has an evolutionary advantage and gives rise to a monoclonal tumour, while in the **polyclonal model**, multiple cell clones co-exist and may draw advantage from other clones.

A tumour consisting of multiple clones can better handle changes in the microenvironmental conditions (lack of nutrients, hypoxia etc…). In the **self-seeding** model, a cell clone leaves the primary tumour and travels by the circulatory system to a distinct site where it lies dormant or develops before returning to the primary site and establishing new subpopulations. In the **mutator phenotype model** a tumour consists of a wide variety of small clones affected by random mutations or copy number changes.

In contrast to the previous models, the **cancer stem cell hypothesis** assumes that only a few cells of the tumour have indefinite proliferation ability. Cancer stem cells give rise to the cell population of the tumour, and are the main drivers of progression. Originally, it was thought that cancer stem cells derived from normal stem cells, but it has been proposed that somatic cells can also become a cancer stem cell [33].

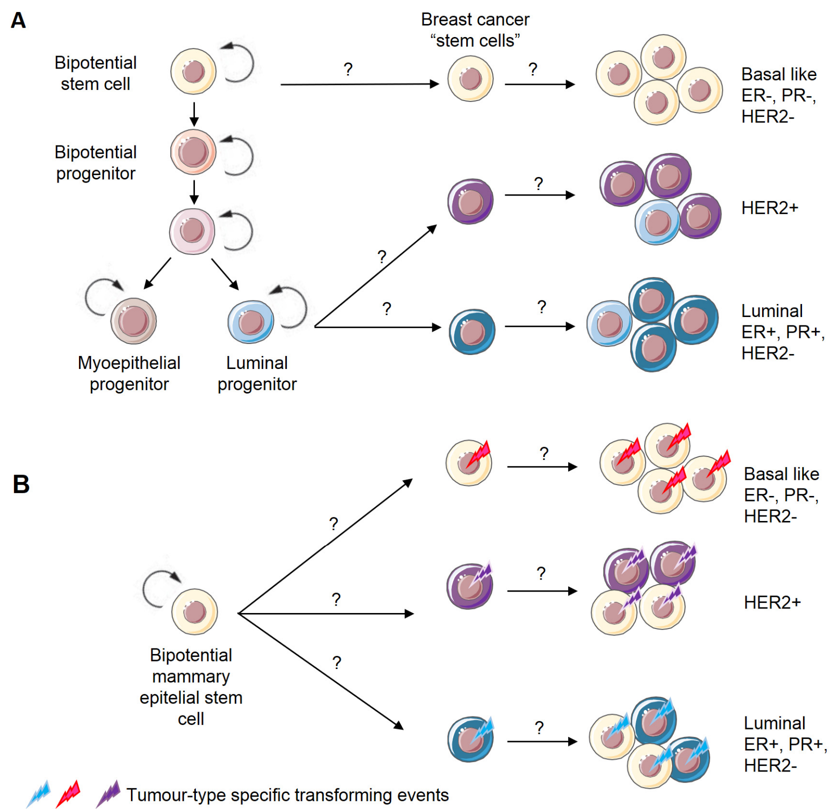


Figure 6. Hypothetical models explaining breast tumour subtypes. A. Cell of origin model. B. Tumour subtype–specific transforming event model. *Adapted from Polyak et al, 2007.*

1.4.2 Hallmarks of cancer

In the early 20th century the German biologist Theodor Boveri suggested that a malignant tumour was the result of single cell abnormalities [34]. Cancer is now considered the most common human genetic disease at a cellular level, and although there are many different types of cancer, one feature is common among all, they are driven by changes in the DNA of the cell that confers cells with the ability to continue growing and dividing in an uncontrolled and indefinite manner.

In this context, in order to be malignant, a cell has to overcome numerous mechanisms that limit and control normal cellular growth and proliferation. These physiological changes are often referred as “the hallmarks of cancer”. This concept was first presented in a review by Hanahan and Weinberg, published in Cell in 2000 [35], (**Figure 7**). They proposed 6 key changes or features that make cancer a cancer, and that represent the fundamental basis of malignancy. Four of the hallmarks are directly linked to the individual cell and its control of the cell cycle, while the last two are related to the tumour as an entity.

The first hallmark, **self-sufficiency of growth signals** describes the activation or loss of function of proto-oncogenes like *ERBB2*. Cancer cells constitutively activate signalling pathways to become self-sufficient in providing their own growth signals, in this way, they are no longer dependent on external signals to progress through the cell cycle. Cancers may also become resistant to anti-growth signals, ignoring the normal signalling that limits the growth of cells to prevent abnormal division [36-38], (**Figure 7**).

Similarly, insensitivity **to growth inhibitory signals** and **evasion of apoptosis**, are directly related to inactivation or gene loss of function, in this case of tumour suppressor genes such as *BRCA1* or *TP53*. In the face of aberrant and potentially cancerous growth signalling, normal cells activate programmed cell death (apoptosis). This is also activated in case of DNA damage and other cellular stresses, and so, apoptosis represents a crucial mechanism to avoid accumulation of damage and mutations that can evolve in cancer formation. Cancer cells acquire the ability to evade this induction of cell death, which is crucial for both maintaining tumour growth and allowing cancerous cells to originate first stages of disease [39-41] (**Figure 7**).

The fourth hallmark refers to **limitless replicative potential** of cancer cells. Most normal cells are unable to grow and divide indefinitely, as they are limited in the number of times they can effectively copy all of the cell’s DNA. As a cell divides more and more, its telomeres become shorter, which eventually causes the cell to enter into a permanent non-replicative state known as “senescence”. This mechanism that limits cellular replicative potential is known as the “end replication problem” [42-44] (**Figure 7**). Cancer cells must find ways of avoiding this induction of cellular senescence to be able to divide indefinitely and form tumours. In this context, many tumours have been found to contain mutations that lead to the reactivation of

telomerase, facilitating replicative immortality [45].

Like normal tissue, a tumour mass requires a blood supply supporting nutrients and oxygen, and so, to be able to grow bigger than around 1 mm^3 in size a tumour must induce the creation of its own blood supply. This process is called **angiogenesis**, and is not a change in the cells themselves, but a process which is promoted by the interactions between the tumour mass and its microenvironment [46, 47] (**Figure 7**).

Finally, cancer cells must acquire the ability to become motile and migrate from the original tumour site: acquiring an **invasive metastatic phenotype,** which is considered a late event in tumourigenesis. Changes promoting invasion happen at the cellular level, including changes in the expression of surface markers which allow the cells to adhere to the surrounding tissues [48] (**Figure 7**).

In a recent review published in Cell in 2011, Hanahan and Weinberg suggested four additional hallmarks for cancer development, including immune destruction evasion, altered cellular energetics, cancer-enabling inflammation, and cancer-enabling genetic instability [49], (**Figure 7**).

1.4.3 Microenvironment in cancer progression

The majority of the studies investigating breast cancer progression have been focused on the initial genetic and epigenetic processes that transforms an epithelial cell into a malignant tumour cell. However, during the last years it has been increasingly recognized that the microenvironment surrounding the epithelial cells plays a key role in tumour progression. Tumours, are now considered to function as complex tissues in which numerous recruited host cells play critical roles.

In 1993 Howelt and Bisell showed that the extracellular matrix together with cytokines plays an important role in the normal development of the mammary gland. They suggested that a “dynamic interaction between the microenvironment and the mammary epithelium regulates tissue specific gene expression in the mammary gland” [50].

The stromal microenvironment in breast cancer, also referred to as “reactive stroma”, is composed by the extracellular matrix (ECM) together with the non-malignant cells surrounding the tumour, including fibroblasts, immune and inflammatory cells, endothelial cells and adipocytes among others [51, 52]. Although the cells that populate the stroma are not neoplastic, they can influence tumour cell behaviour. It is then thought that the stroma not only plays an important role in the regulation of normal mammary gland development, but that it is also involved in the development and progression of breast cancer [53]. The neoplastic epithelial cancer cells and the cells of the tumour microenvironment are in constant crosstalk and there is an active recruitment of stromal cells into the tumour. Moreover, stromal cells produce additional tumour growth factors, cytokines, chemokines and matrix metalloproteinases that contribute to tumour progression [54].

The same way around, cancer cells can produce "stroma-modulating growth factors" that modify the stroma to provide themselves a supportive microenvironment for tumour progression [54].

In this context, Hanahan suggested that most of the hallmarks of cancer are enabled and sustained through contributions from cells in the stromal compartment (cancer associated fibroblasts, infiltrating immune cells and angiogenic vascular cells). These contributions are every day better understood and are summarized in **figure 7** [55].

Furthermore, a high number of studies have indeed recently shown changes in genomes of tumour associated stromal cells, postulating a "**tumour-stroma coevolution theory**" [56-58].

All these concepts will be further exposed and developed in **Section 4.**

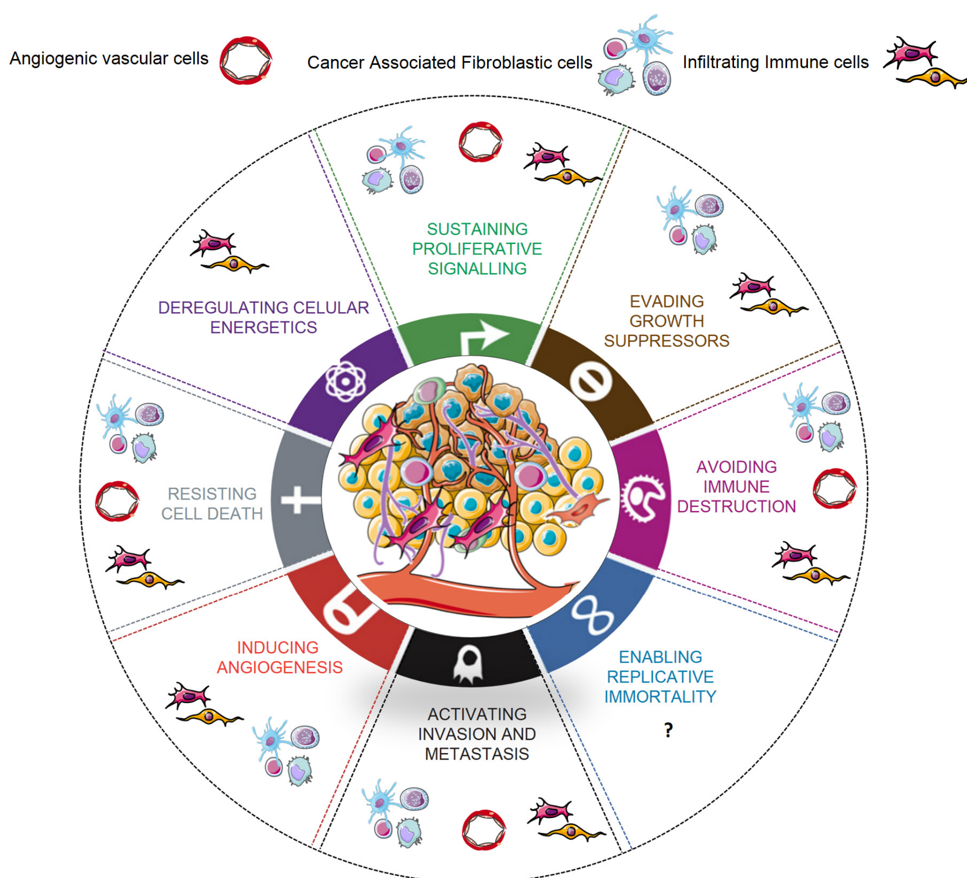


Figure 7. Multifactorial contributions of stromal cells to the Hallmarks of cancer. Of the eight acquired hallmarks capabilities, seven have demonstrated to be influenced by contributions of stromal cells from the tumour microenvironment. *Adapted from Hanahan et al, 2012.*

1.5 BREAST CANCER HETEROGENEITY

Breast cancer is a heterogeneous disease, with high degree of diversity within tumours (intratumour heterogeneity), as well as between breast cancer patients (intertumour heterogeneity), exemplified by the different molecular intrinsic subtypes and the histological classification of breast cancers (**section 1.6**).

1.5.1 Intertumour heterogeneity

Several hypotheses have been proposed to explain the origin of intertumoural heterogeneity, including subtype-specific tumour transforming events and the existence of different cell of origin models.

According to the **cell of origin hypothesis**, luminal and HER2+ tumours may have originated from luminal lineage progenitors, whereas basal-like tumours may arise from less differentiated stem cell-like cells. However, gene expression patterns and experimental models also suggest that luminal progenitors may also serve as precursors to basal-like tumours following genetic or epigenetic events that would later switch cellular phenotypes [59].

Moreover, one of the major confounding factors when using molecular portraits to stratify patients into treatment groups is the heterogeneous composition of the investigated sample.

For diagnostic purposes, histopathological evaluation of each biopsy is performed and heterogeneity among tumour cells is commonly observed. In patient samples from primary breast tumours, for example, both hormone receptor-positive and -negative tumour cells are seen routinely. Another example is the common phenomenon of a mixed diagnosis of primary tumours, where components of both ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC) can be found within the same specimen [60].

In this context, defining the exactly evolutionary pathway for breast cancer is a difficult task, as tumours are rarely able to be diagnose at their earliest stage in order to follow their molecular evolution. However, different reviews have suggested some hierarchical models for the mammary gland development and cancer initiation [21, 61, 62].

As mentioned before, the mammary gland is composed of a branching network of ducts and lobules, and the mammary epithelium contains a luminal layer of cells (ductal or lobular) that are surrounded by a basal layer of contractile myoepithelial cells, (cells with stem cell properties have been described in supra-basal location).

Different lineage-restricted progenitors also exist within the mammary epithelium, and will differentiate into the mature ductal, lobular and myoepithelial cells. The proposed epithelial hierarchy in the mammary gland states that during development, a BC stem cell will give rise to bi-potential progenitors, which will further differentiate into lineage-restricted progenitors to generate the ductal, lobular and myoepithelial cells. Based on the molecular similarities between normal and cancer cells, cells from the mammary gland at different stages of differentiation will be the cells of origin for the different BC

subtypes. Thus, the different molecular subtypes could arise from the same cell of origin that, due to the accumulation of additional genetic and epigenetic alterations, would evolve to one subtype or the other [21, 61, 62].

1.5.2 Intratumour heterogeneity

Besides the numerous differences detected between tumours, cancer cells within a tumour at any given time frequently display also some degree of heterogeneity.

This may be explained by clonal and cellular diversity due to genetic and epigenetic alterations, and also by non-hereditary mechanisms such as adaptive response or fluctuations in protein levels and signalling pathways activation. Then, from the single founder cell until the clinical detection of tumours, there are consecutive clonal expansions and a constant acquisition of genetic and epigenetic alterations events that contribute to the generation of intratumour heterogeneity [63].

The source of intratumour heterogeneity was initially explained by the existence of clonal selection and later by the stem cell hypothesis. The **clonal selection hypothesis** states that those clones with the best fitness for a particular microenvironment will be selected during tumour progression. On the other hand, **the cancer stem cell concept** proposes that, regardless of the cell of origin, only a minor fraction of tumour cells, termed "tumour stem cells", are responsible for the maintenance and progression of tumours because they possess the unique ability to both self-renew and differentiate into the bulk of tumour "non-stem" cells. Based on the experimental evidence supporting the existence of both processes in BC, it is becoming widely accepted that intratumour heterogeneity is the consequence of cancer stem-like cell differentiation along with the clonal selection of particular cell subpopulations [62-64]. In fact, it has also been postulated that intratumour heterogeneity may also underlie intertumour heterogeneity and help to explain breast tumour subtypes as tumours that are composed of different mixtures of cancer cells [65], **figure 8.**

In 2011, Polyak *et al.* proposed that intratumour heterogeneity is due to the presence of cancer cells with variable phenotypes such as different degrees of basal-like and luminal-like features. As a result, intertumour heterogeneity may also be explained by the presence of these different cell types within tumours at varying frequencies. Like so, cancer cells with a basal-like phenotype predominate in basal-like tumours, whereas luminal tumours are largely composed of luminal breast cancer cells. However, due to the variability in basal-like and luminal cell traits, not all basal-like and luminal tumours are the same, further contributing to heterogeneity even within tumour subtypes [65].

It is then evident that heterogeneity is a big challenge for the clinical management of the disease, and a better understanding of intratumour heterogeneity is crucial for the correct design of more effective therapies.

The coexistence of different clones within a tumour will influence the selection of a particular treatment, since the sampling of

a particular area of the tumour might not be representative of the whole, and even a minor subpopulation of cancer cells can be responsible for tumour relapse.
The main regulators for the generation of diversity are the genetic instability and the mutational rate, as well as the microenvironment, which acts as the selection force for tumour evolution. Then, clonal diversity could be an indicator of the risk for relapse, since a higher number of clones will provide the tumour with more chances to escape and adapt to any variations in the microenvironment induced by chemotherapeutic agents [62].

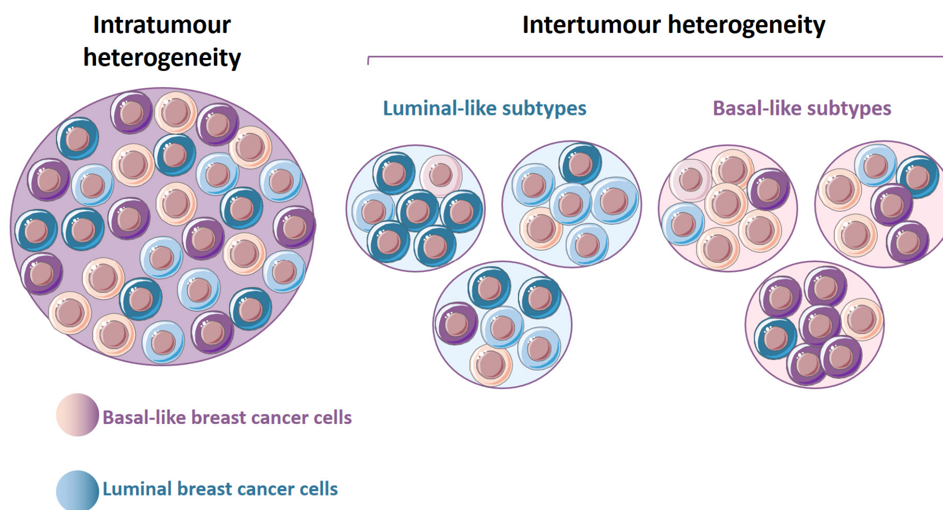


Figure 8. Hypothetical model explaining the origins of intertumour and intratumour heterogeneity in breast cancer. *Adapted from Polyak et al. 2011.*

1.6 BREAST CANCER CLASSIFICATION

As introduced in the previous section, breast cancer, rather than constituting a monolithic entity comprises heterogeneous tumours with different clinical characteristics, disease courses, and responses to specific treatments. In this wat, tumour intrinsic features, including classical histological and immunopathological classifications as well as recently described molecular subtypes, separate breast tumours into multiple groups [66].

1.6.1 World Health Organization histological classification

Classical pathology, based on the overall tumour morphology and structural organization, has divided breast tumours into multiple categories. The most common type observed and reported is invasive ductal carcinoma not otherwise specified (IDC-NOS) (about 75% of cases), while invasive lobular carcinoma represents the next most frequent histologic type of breast tumour (about 10% of cases). Together, these two categories and combinations make up the vast majority (about 90%) of all diagnosed breast cancers. Other types include the ductal carcinoma *in situ*, medullary, neuroendocrine, tubular, apocrine, metaplastic, mucinous (A and B), inflammatory, comedo, adenoid cystic, and micropapillary tumour types [67, 68].

Ductal carcinomas

As mentioned before they are the most common of all cancers, accounting for 75-80% of all breast carcinomas. Ductal carcinomas are normally separated into two groups, ductal carcinoma *in situ* (DCIS), and invasive ductal carcinoma (IDCs). The DCIS are non-invasive, as the tumour cells have not spread beyond the milk duct, while IDCs infiltrate the supporting breast tissue [4].

DCIS refers then to breast epithelial cells that have abnormal increased growth and accumulate within the ducts and lobules without evidence of invasion beyond the basement membrane. DCIS, also known as intraductal cancer, is the most common type of *in situ* carcinomas (80-90%) [24]. Calcifications represent the most common mammographic presentation of DCIS, and nearly 90% are diagnosed while they are clinically occult [26, 29]. The biology of DCIS is heterogeneous and poorly understood, several histopathological classifications have been proposed to distinguish different types of DCIS. These classifications are based on different aspects such as nuclear morphology, architectural pattern of tumour growth (solid, papillary, micropapillary or cribriform), and by the presence or absence of comedonecrosis (comedo, non-comedo) [26, 29, 31].

ICDs is defined as a malignant tumour that has the ability to penetrate the basement membrane, invade adjacent tissues and regional nodes to metastasize to distant organs [69]. Invasive carcinomas of the breast are associated with different clinical behaviour and prognosis according to histopathological characteristics (**section**

1.3) and could be also categorized based on histopathological and cytological features into medullary, mucinous, tubular, papillary, micropapillary or metaplastic ductal carcinomas, however, the majority of IDCs (80%) are normally not further classifiable and are referred to as ductal carcinomas of No Special Type or Not Otherwise Specified [69].

Ductal carcinomas are thought to arise from ductal hyperplasia that progress into ductal carcinoma in-situ (DCIS) and then to invasive ductal carcinoma (IDC).

As discussed before, ductal carcinomas can also be classified according to grade (**section 1.3.1**) and stage (**section 1.3.2**) with prognostic value, giving important information for guiding treatment.

Lobular carcinomas

Accounting for 10-15% of breast carcinomas, the invasive lobular carcinomas (ILCs) are the second most common type of breast cancer. During the last 20 years the incidence of ILCs has increased, and this increase has been linked to the hormone replacement therapy given to postmenopausal women [70].

This type of tumour is typically characterized by a low mitotic rate and little nuclear atypia, and is by definition a low-grade tumour. ILCs are associated with older age and a higher frequency of ER positive tumours compared to IDCs [67]. Although ILCs are genomically similar to low grade IDCs, and are therefore sometimes considered as IDC special type. However, the prognosis and overall survival for patients with ILs is better than for IDCs patients [71].

Inflammatory breast cancer

Diagnosis of IBC is based on a wide definition "characterized by diffuse erythema and edema of the breast, often without and underlying palpable mass" [72].

The IBC is an advanced form of breast cancer with an aggressive course, as a relatively high proportion of women with IBC have axillary lymph nodes and distant metastasis at the time of diagnosis.

A study of patients going through surgery alone or in combination with radiation therapy showed a median survival of 15 months, and local recurrence rate of 50% [73]. Efforts to introduce a multidisciplinary management approach with the use of pre-surgical chemotherapy, mastectomy and radiation therapy, have substantially increased the survival of these patients [74].

1.6.2 Breast cancer intrinsic molecular subtypes

BC is a heterogeneous disease regarding clinical and biological behaviour, and this complexity is partly reflected by the prognostic factors explained in **section 1.3**.

All these parameters are currently used by clinicians to predict prognosis and decide treatment strategies, but do not provide a complete understanding of the biology of the disease.

Over the past decade, the advance of high-throughput/high-content microarray-based gene expression profiling technologies have facilitated large-scale studies of BC cohorts, leading to the identification of multiple molecular subtypes of BC that may help to explain this heterogeneity.

In 1999 studies by Perou and colleagues described breast cancer molecular subtypes defined by distinct transcriptional signatures that partially recapitulate the original immunopathological classes adding an additional level of detail [75].

In 2000, further analyses of gene expression microarray data proposed the existence of at least four distinct subtypes of breast tumours. From a panel of 8102 human genes printed on the microarray, the genes for which expression varied the most between different individuals, and at the same time varied the least before and after treatment within the same individual were selected [61]. In this way, this list was considered to contain genes that distinguished and described the gene expression profile of each individual tumour. Hierarchical clustering methods results in a division of the tumours that could be classified in two main groups: ER-positive group and ER-negative group. The ER-negative group was further subdivided into basal-like, HER2+ group and the normal-like subtype, **figure 9** [21, 61].

In 2003, as a follow up of these studies, an intrinsic gene list of 534 genes was utilized to hierarchically cluster 115 breast tumour samples, and five gene expression subtypes were established: Luminal A, Luminal B, HER2+, Basal and Normal-like [76].

Tumours categorized as luminal give rise to the majority of breast tumours, being the **Luminal A** subtype the most prevalent. These tumours present high expression of cytokeratin (CK) 8 and 18, and also for the cluster of transcription factors that includes ER and PR [77].

The Luminal A subtype shows a low to absent expression of HER2 cluster genes, while contrary, the **Luminal B** subtype exhibits a greater proportion of HER2 positive tumours. Another difference between these subtypes of luminal tumours is their proliferation rate. Whereas Luminal A tumours generally present low expression of proliferative genes, proliferation rate in Luminal B is elevated [15]. In general, regarding treatment, luminal tumours are associated with endocrine sensitivity, and some Luminal B tumours may also get benefit from HER2-targeted therapies [77].

The **HER2 enriched subtype** is typified by a low expression of luminal and basal gene clusters but elevated expression of HER2 and proliferation gene clusters [21, 61].

Like HER2, the **Basal-like subtype** is less frequent than luminal tumours. It shows high expression of basal and proliferation genes, and low to absent expression of luminal and HER2 gene clusters. The basal cluster is composed of genes typically expressed in myoepithelial cells, such as CK 5/6/17, EGFR and vimentin, highly overlapping with the triple negative group [61].

Differences in patient outcome have also been described between intrinsic subtypes. Luminal A tumours show the highest survival rate among all subtypes measured as overall, breast-specific or relapse free survival [21].

In general terms, Luminal B is associated with intermediate prognosis and hormonal receptor negative with the worst. Outcomes for women diagnosed with HER2 enriched cancer have improved their

prognosis greatly in the recent decades due to the use of anti-HER2 therapies.

Basal-like tumours show the lowest survival, and identifying effective targets for this subtype remains a challenge in breast cancer treatment [76-79]

Also, other molecular subtypes have been proposed, most notably **Claudin-low** tumours which are characterized by low expression of genes involved in cell–cell adhesion, including CLDN3, CLDN4 and CLDN7 (claudin 3, 4 and 7) and CDH1 (E-cadherin). These tumours represent a rare type of triple negative breast cancers with mesenchymal features, which show high expression of immune system response genes and, in contrast to basal-like tumours, do not show high expression of proliferation-associated genes [80].

Finally, molecular **apocrine tumours** were more recently suggested to be another subtype, characterized by apocrine differentiation and increased androgen signalling [81].

Using statistical tools, the intrinsic molecular subtypes were later refined and a minimized gene set was built into a classifier

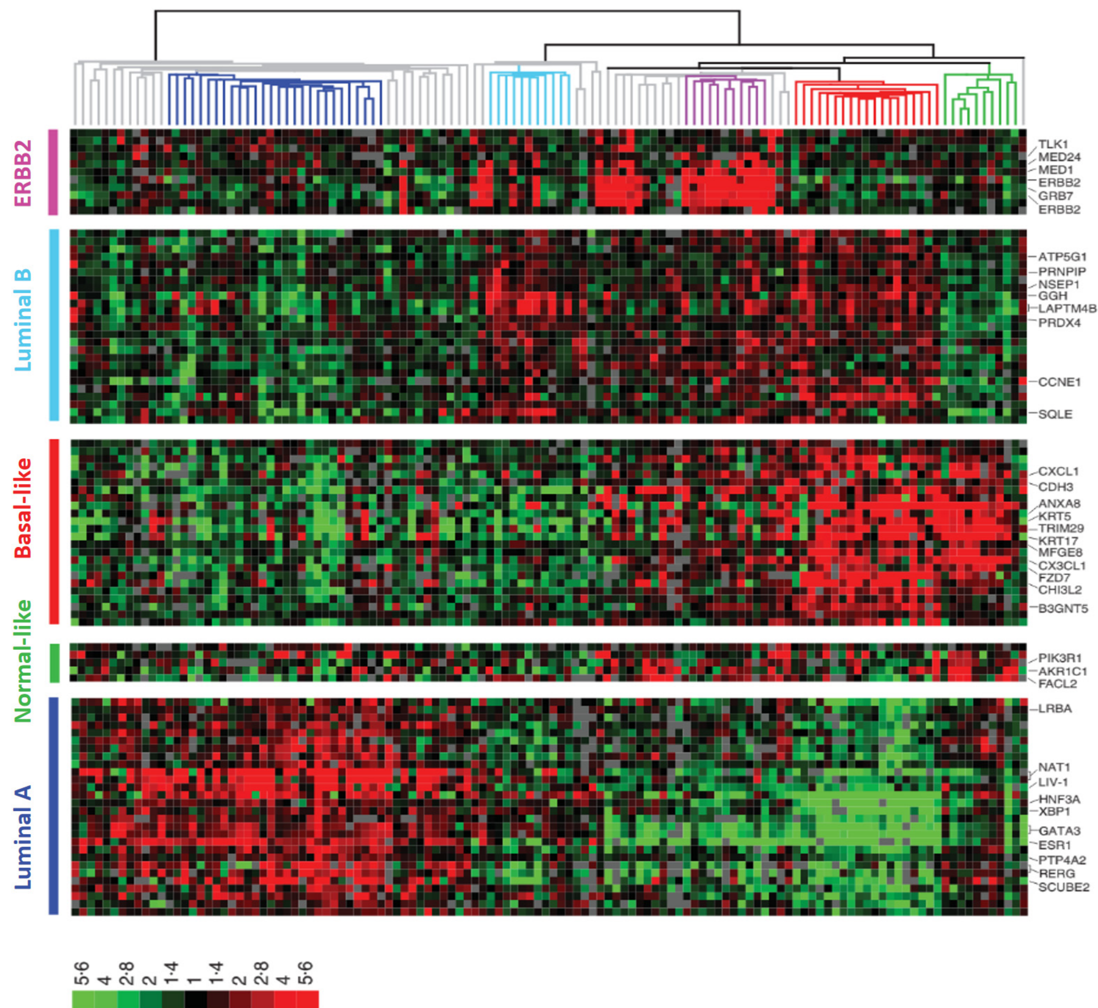


Figure 9. Hierarchical clustering of gene expression data from breast tumours, defining five subtypes with distinct gene expression patterns. *From Sorlie et al, 2001*.

termed PAM50 (prediction of picroarray 50 genes) [82].

A prognostic model with a *risk of relapse score* was also developed, this tool uses subtype information alone or along with clinical information to estimate prognosis or efficacy of chemotherapy.

PAM50 has increasingly gained attention and is now available as a standardized assay termed Prosigna™ Breast Cancer Prognostic Gene Signature Assay. PAM50 recently received approval from the US Food and Drug Administration to assess the risk of distant recurrence at 10 years in postmenopausal women with node-negative (stage I or II) or node-positive (stage II), hormone receptor-positive breast cancer [82].

A new area for studying the behaviour of breast cancers has also arisen with the discovery of microRNAs (miRNAs), short RNA molecules (approximately 22 nucleotides) that play important roles in transcriptional and posttranscriptional regulation of gene expression [83].

Analyses of miRNA profiles in breast cancer have determined that many miRNAs display expression patterns linked to molecular subtype, as well as ER status, tumour grade, and other tumour-related processes [84].

1.6.3 Breast cancer molecular subtypes explained by IHC markers

One of the main handicaps of intrinsic molecular subtype profiling is that microarray-based tests are expensive and so, not accessible for the vast majority of patients. This limits the use of gene expression patterns as a routine diagnostic tool in the public health setting.

For these reason, many efforts have been made to obtain accurate IHC surrogate biomarkers for subtyping breast cancer [77, 78, 85].

Molecular subtypes (*from here on in this section, the term molecular subtype refers to breast subtypes defined by IHC markers*) have been defined using a panel of three biomarkers: ER, PR and HER2.

In this context, Luminal A is defined by ER^+ and/or PR^+ and $HER2^-$, Luminal B by ER^+ and/or PR^+ and $HER2^+$, HER2-enriched by lack of ER and PR expression, but with HER2 amplification, and triple negative breast cancers (TNBC) are defined by lack of ER and PR positivity and lack of HER2 amplification. It was considered that TNBC would be equivalent to the basal-like subtype, however many studies have described biological heterogeneity within triple negative phenotypes [86].

In 2011 Prat found that ER and HER2 status do not entirely recapitulate the intrinsic subtypes [77]. In their data set, 83% of tumours classified as basal-like were ER^-/$HER2^-$, whereas 17% showed positivity for ER and/or HER2. In addition, 34% of the HER2-enriched cancers where $HER2^-$.

Distinguishing between Luminal A and Luminal B also represented a problem, as

both subtypes showed an elevated proportion of $ER^+/HER2^-$ tumours.

The St. Gallen International Expert Consensus has worked during recent years to better describe a classification of breast cancer molecular subtypes and associated treatment.

To avoid confusion between intrinsic molecular subtypes and those subtypes defined by IHC, the following terminology has been suggested for IHC defined molecular subtypes: Luminal A-like, Luminal B-like, HER2 positive and TNBC [87].

The most recent breast cancer molecular subtype classification, after 2013 updated is summarized in **Table 3** [85]. Compared to Luminal B-like, the Luminal A-like subtype was restricted to tumours showing positivity for PR and low expression of Ki67. Furthermore, the Luminal B-like subtype was separated into two subgroups according to their HER2 expression (Luminal B-like $HER2^-$, and Luminal B-like $HER2^+$). HER2 positive and TNBC were both defined by the absence of ER and PR expression, and HER2 was only overexpressed in tumours classified as HER2 positive.

MOLECULAR SUBTYPES	IHC DEFINITION
Luminal A-like	ER and PR positive HER2 negative KI67 low
Luminal B-like HER2 –	ER positive HER2 negative At least one: KI67 high or PR negative or low
Luminal B-like HER2+	ER positive HER2 overexpressed or amplified Any KI67 Any PR
HER2 positive	HER2 overexpressed or amplified ER and PR negative
Triple negative breast cancer	ER and PR negative HER2 negative

Table 3. Definition of breast cancer molecular subtypes suggested in the last St. Gallen recommendations, 2013. *Adapted from Goldhrish et al 2013.*

1.7 DIAGNOSIS, SCREENING AND PREVENTION

The reduction in the morbidity and mortality of cancer results from the advances in cancer prevention together with increased knowledge about risk factors. In this way, primary breast cancer prevention consists in avoiding or reducing exposure to risk factors.

Secondary prevention involves early detection and treatment of the disease and screening is the major component, as it can detect disease at an early stage increasing the probability that a cancer may be cured. Finally, tertiary prevention refers to curing cancers that have developed and preventing cancer death, and it's applied during the symptomatic phase through treatment and rehabilitation programs [88].

Early detection of breast cancer increases the probability of long term survival [89]. The first suspicious of breast cancer often comes from physical examination by breast palpation, and this is why women are encouraged to perform self-examination with regularity.

The second method is mammography, a method used to investigate suspicious lumps, and also a tool for screening. At the end of the last century, many developed countries, including Spain, implemented and organized breast cancer population based mammographic screening programs [90]. This mammographic program has been confirmed as the most effective method for breast cancer screening. Its objective is to reduce morbidity and mortality from the disease by detecting cancer at an early stage without any adverse effect on participants [91].

The mammogram is a type of X-ray image of the breast that enables detection of tumours at an earlier time point compared to self-examination**.**

Mammographic density, as seen on mammograms, relates to the differences in attenuation of the X-ray beam as it passes through the different components of the breast tissue. Stromal and epithelial tissues are radio dense and appear light, while fat tissue is radiolucent and appears dark on the mammogram. High density breast (in more than 60-75%) confer a 4-6 fold higher breast cancer risk, and sometimes high density breast tissue can make the interpretation of mammograms more difficult [92].

Another method for diagnosis is the fine needle aspiration biopsy (FNAB), when a lump or a cyst is evident, a small needle can be used to suction out some fluid from the lump, and the cellular content in the fluid will be assessed by a specialist trained pathologist. FNAB has shown an accuracy of breast cancer diagnosis of over 97% [93].

Most women who receive a breast cancer diagnosis after a mammogram are referred for further testing to assess more precisely the size of the breast tumour and to determine whether the cancer has invaded local tissue or spread to other parts of the body.

The results of these tests are important for providing the patient with an accurate diagnosis, which is crucial for deciding the best course of treatment.

For some women, one of these follow-up tests is magnetic resonance imaging (MRI)

that allows clinician to locate the tumour within the breasts. MRI can also be used to evaluate abnormalities seen by mammogram that were insufficiently clear for physicians to determine whether the patient has breast cancer or how large the tumour is.

In some cases, patients undergoing an MRI of the breasts are injected with a liquid called a contrast agent to help visualize abnormalities more clearly. In June 2014, the FDA approved a new contrast agent to use with MRI to assess the presence and extent of cancer within the breasts. This decision was made after the results of two large clinical trials showed that the new contrast agent, Gadobutrol (Gadavist), significantly improved the ability of MRI to clearly visualize cancer also in high density breasts [94].

1.8 THERAPEUTIC APPROACHES FOR BREAST CANCER

Early detection of cancer in any form can reduce the need of extensive treatments. Typically, once located, a solid tumour is surgically removed and, if determined that it has not spread to other parts of the body, a patient may not have to undergo chemotherapy.

In some cases, especially breast cancer, clinical experience suggests that adjuvant therapy with radiation and/or chemotherapy may additionally ensure that no undetected errant cancer cells have survived. However, if it is found that cancer has spread or is more locally advanced, a more intensive approach is indicated, with non-specific toxic chemotherapy application.

More recently, treatment agents have started to be more specific, targeting molecular drivers of cancer. Molecular aberrations like overexpression of HER2 is a good example, this receptor has been successfully targeted with the monoclonal antibody Trastuzumab, improving patient's outcome.

1.8.1 Surgery

Surgery is the most common treatment used in breast cancer and often the first to be applied if no distant metastases are detected. Breast cancer surgery either involves breast conservative surgery, where the tumour is removed and the breast is conserved, or mastectomy, where all of the breast tissue including the pectoral fascia is removed [95].

With breast conserving surgery (the preferred method for most patients), it is imperative that the tumour resection is complete, therefore, the margin between the tumour and the resection border should be at least 2 mm, to account for histopathological uncertainties. If the tumour measures more than 4 mm, is multifocal (two or more invasive foci separated by begin tissue), or has extensive DCIS, mastectomy is performed.

The **sentinel node hypothesis** states that tumour cells from the primary tumour migrate to a specific lymph node (the sentinel node) in the regional lymphatic chain, before migrating to other lymph nodes within the chain [96]. The tumour status of the sentinel node should therefore reflect the tumour status of the entire lymphatic system. Sentinel node biopsy identifies the sentinel node by using dye or radioactivity and investigate the presence of tumour cells. In patients where the sentinel node contains tumour cells, axillary lymph node dissection (removal of all lymph nodes within the lymphatic chain) is performed [96]. Patients with negative lymph node exploration will get no benefit from lymph node dissections, while in cases where the sentinel node is not localized axillary lymph node dissection is recommended.

1.8.2 Radiotherapy

Many patients who undergo conservative surgery are later treated with radiotherapy, although women undergoing radical surgery or no surgery can also receive it, as radiotherapy can be also used to shrink the

tumour before surgery (neoadjuvant therapy), to reduce the risk of local relapse or as a palliative treatment.

The ipsilateral (in same breast) recurrence rate in stage I or II patients treated with breast conserving surgery alone has been shown to be around 35-40%, whereas the recurrence rate decreased to 10-14% in patients treated with breast conservatory surgery and radiotherapy [95].

Radiotherapy consists in the use of controlled doses of high-energy radiation to damage the DNA of cancerous cells leading to apoptosis [97]. During radiotherapy administration, high doses of IR are directed towards tumour. The destructive events following IR exposure are the result of radiation depositing energy in the cell. IR-induced damaged is also due to indirect events caused by subsequent disturbances of metabolic oxidation and reduction reactions due to irradiation induced formation of free radicals, such as reactive oxygen species (ROS) [98].

Cells that are not directly targeted by radiation may also show radiation damage through cell-to-cell gap junction communication, and through exposure to secreted soluble factors by the resident irradiated cells. This effect is termed bystander effect [99]. Direct IR, bystander effect and ROS generation will induce breakage of chemical bonds, the production of new chemical bonds and the cross-linkage between macromolecules, will result in damage of DNA, ribonucleic acid (RNA) and proteins that regulate vital cellular processes.

1.8.3 Chemotherapy

Chemotherapy is the most common systemic treatment for cancer, and involves the use of cytotoxic drugs to stop the proliferation and growth of cancerous cells. Cancer cells divide more quickly than healthy cells, and chemotherapy drugs effectively target those cells The goal is to kill the remaining cancer cells present at the primary tumour area or metastatic cells elsewhere in the body. Unfortunately, fast-growing cells that are healthy can be damaged too.

Different chemotherapy drugs function in different manners to impair cell division, by effectively damaging the fast growing cancer cells [100].

Depending on when the treatment is administrated, chemotherapy can be classified as adjuvant (after surgery) or neoadjuvant (before surgery). The goal of adjuvant therapy is to eradicate possible occult, micrometastatic disease that, if left untreated, are a potential source of disease recurrence. Neoadjuvant chemotherapy is employed in patients with initially inoperable tumours, in order to shrink the tumour, thus making the patient later treatable with surgery or radiotherapy [100].

The guidelines for administration of chemotherapy are based on tumour phenotype (status of ER and HER2) and TNM staging together with individual patient characteristics. Then, although exact chemotherapy plans vary from person to person, treatment guidelines help to ensure quality care. These guidelines are based on the latest research and expert's consensus from The National

Comprehensive Care Network (NCCN) and American Society of Clinical Oncology (ASCO) [101].

Chemotherapy drugs used to treat early breast cancer include Taxanes and Anthracyclines. The principal mechanism of action of Taxanes is the disruption of microtubule function. This class of drugs includes Docetaxel and Paclitaxel (Taxotere and Taxol) [101]. Anthracycline class of drugs includes doxorubicin (Adriamycin) and Epirubicin (Ellence), with a broad action mechanism including inhibition of DNA/RNA synthesis and topoisomerase II [102]. These drugs are often used with others like Carboplatin, Cyclophosphamide (Cytoxan), and Fluorouracil (5 Fu), and women who have the HER2 gene overexpression may be further treated with Emtansine (Kadcyla), Lapatinib (Tykerb), Pertuzumab (Perjeta), or Trastuzumab (Herceptin) [101]. In many cases, chemotherapy medicines are given in combinations, known as chemotherapy regimens [101].

1.8.4 Hormonal therapy

Endocrine therapy, either tamoxifen or aromatase inhibitors (AI), is given to all patients with ER positive tumours. Patients that are younger than 55 years receive tamoxifen, while women older than 55 years receive either AI, or a combination of AI and tamoxifen [100].

Tamoxifen is metabolized into compounds which compete with oestrogen for binding to the oestrogen receptor in the breast tissue, thereby preventing oestrogen to activate cell growth [103].

AI inhibits the synthesis of oestrogen, by blocking the enzyme aromatase, which synthesizes oestrogen. Tamoxifen treatment for 5 years has shown to reduce the 15-year probability of recurrence to 12% [100], whereas treatment with AI in place of tamoxifen or AI after tamoxifen [104] increased the disease- free survival in postmenopausal ER-positive patients.

1.8.5. Targeted therapy

Research is continually expanding our understanding of cancer biology, and all this knowledge has allowed oncologists to treat cancer by targeting specific molecules involved in different stages of the cancer process. As a result, the standard of cancer care is changing from a one-size-therapy approach to one in which the molecular makeup of the patient and his or her tumour biology dictates the best therapeutic strategy.

Biological therapy tends to be less toxic than the treatments that have been the standard of patient care for decades. Thus, these new medicines are not only saving the lives of countless cancer patients but also improving their quality of life.

Some of the most important specific-targeted drugs for treating breast cancer are monoclonal antibodies or tyrosine kinase inhibitors.

Trastuzumab is a monoclonal antibody targeting the extracellular domain of HER2 protein. Administration of Trastuzumab for one year, following chemotherapy, has been shown to significantly improve disease-free survival in HER2 positive breast cancer patients [104, 105].

Lapatinib is a small tyrosine kinase inhibitor that simultaneously inhibits the tyrosine kinase activity of EGFR and HER2, and was approved by the FDA for the treatment of HER2+ metastatic BC refractory to Trastuzumab [106, 107].

These and other HER2 therapies, its mechanism of action and biology will be further explained in **section 3.**

2. ERBB SYSTEM

2.1. RECEPTOR TYROSINE KINASE SUPERFAMILY

Eukaryotic cells are constantly receiving and integrating a continuous flow of information coming from the extracellular medium. Evolution has then endowed cells with multiple mechanisms to cope with the complex net of messages coming from the extracellular medium as well as from neighbouring cells, and the Receptor Tyrosine Kinase (RTKs) are the main mediators of the signalling network that transmit extracellular signals into the cell, controlling cellular differentiation and proliferation [108].

In the same way, alterations in RTK signalling pathways have been linked to a number of diseases and diseases-related processes, such as cancer, inflammation and angiogenesis [109].

In particular, overexpression of RTK proteins or functional alterations caused by mutations in their corresponding genes, as well as abnormal stimulation caused by autocrine growth factor loops contribute to constitutive RTK signalling, resulting in deregulated cell growth and cancer [110].

Structurally, all RTKs contain a large, glycosylated, extracellular ligand-binding domain, a single transmembrane region and a cytoplasmic portion with a conserved protein tyrosine kinase domain. The extracellular domains of different RTKs also exhibit a variety of conserved elements, including immunoglobulin-like domains, cysteine-rich regions and fibronectin repeats. In fact, RTKs are classified according to their variable extracellular ligand-binding domain. RTK superfamily includes around 60 members and can be grouped into 20 subfamilies based on the kinase domain sequence, for instance, the Epidermal Growth Factor Receptor (EGFR), Ephrin Receptors (Eph), Fibroblast Growth Factor (FGFR), Insulin and Insulin-like Growth Factor Receptor (InsR/IGFR), Hepatocyte Growth Factor Receptor (c-Met), Platelet Derived Growth Factor Receptor (PDGFR), Tie (tyrosine kinase with Ig and EGF homology domain), RET (rearranged during transformation), Vascular Endothelial Growth Factor Receptor (VEGFR), TRK (neutrophic tyrosine kinase receptor), AXL, MuSK (muscle-specific receptor tyrosine kinase) and ROR (receptor tyrosine kinase like orphan receptor) [109].

Ligand binding to the extracellular domain leads to conformational changes and post-transcripcional modifications that induce and stabilize receptor dimerization [111]. From all ligands, growth factors mediate their diverse biological responses by binding to and activating cell-surface receptors with intrinsic protein kinase activity. In addition to the catalytic domain, a juxta membrane region and a carboxyl-terminal tail can be identified in the cytoplasmic portion [108].

RTK receptors may also further contain regulatory sub-domains which negatively or positively influence substrate-binding and phosphorylation, as well as sub-domains involved in the obligatory dimerization and/or structural changes during kinase activation after ligand-binding [109].

2.2 ERBB FAMILY

2.2.1 Structure and function

The ERBB subfamily consists of four members EGFR (ErbB1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). All partners have an extracellular ligand-binding domain containing cysteine rich regions and possible glycosylation sites, an amphipathic transmembrane region, a short intracellular juxta-membrane region, a tyrosine kinase domain and a carboxyl tail which contains critical tyrosine phosphorylation sites [112] (**Figure 10**).

The highest degree of sequence homology (approximately 80% amino acid identity) between HER2, EGFR and HER4 relies in the tyrosine kinase domain, suggesting that this region is essential for the signalling function of these molecules [113].

Due to its structure, the ERBB family proteins function mainly consist in the activation of intracellular signalling pathways in response to extracellular signals. Under normal physiological conditions, these receptors play important roles in cell growth and differentiation during embryonic development and also in adult life [114].

Activation of the ERBB receptors is controlled by the spatial and temporal expression of their ligands: EGFR binds different ligands including EGF and TGFα (6kDa), neuregulins bind to HER3 (however HER3 has no intrinsic tyrosine activity) and HER4, while no ligand has been identified for HER2 [115, 116].

Ligand binding to ERBB receptors induces the formation of receptor homodimers and heterodimers and activation of the intrinsic kinase domain, resulting in phosphorylation

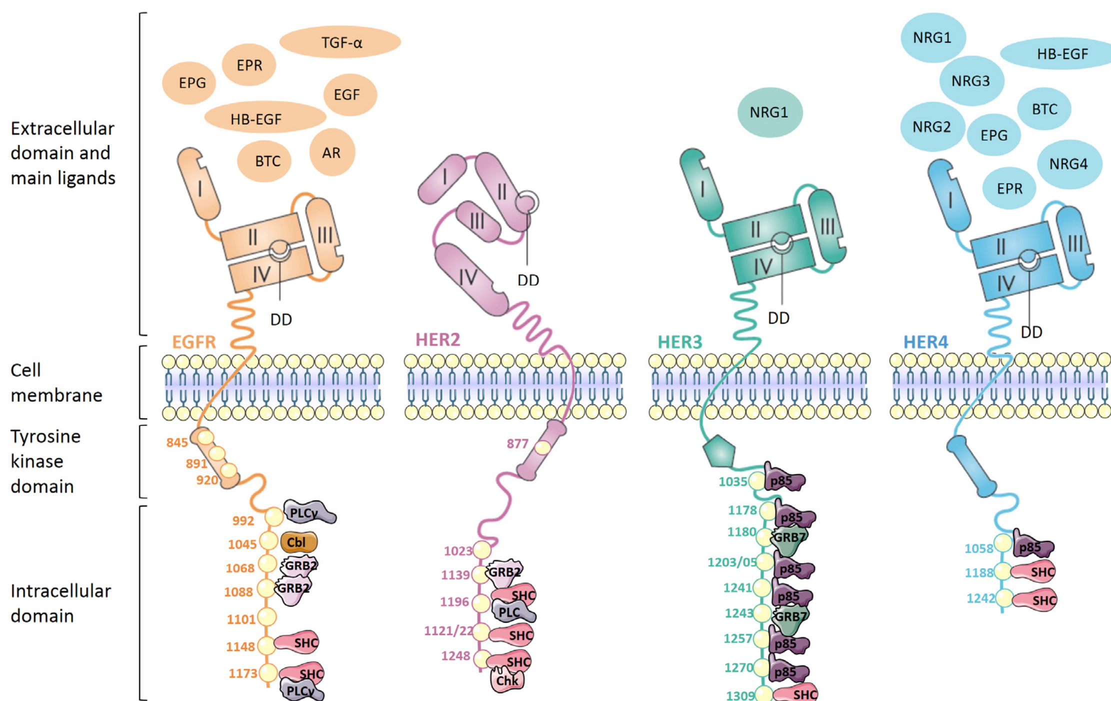


Figure 10. Schematic representation of structure domains, main known ligands, auto-phosphorylation sites and associated signalling molecules of ERBB members. *Adapted from Hynes et al., 2005 and Baselga et al., 2009.*

on specific tyrosine residues within the cytoplasmic tail. These phosphorylated residues serve as docking sites for a range of proteins, the recruitment of which leads to the activation of intracellular signalling pathways [117], summarized in **figure 21**.

HER2 has the strongest catalytic kinase activity among all family members, and HER2 containing heterodimers have the strongest signalling activity. Since a specific ligand for HER2 homodimers has not been identified, the role of HER2 within the cellular signalling network was largely unclear till it was described that EGFR activation induces transphosphorylation of HER2 through heterodimerization [118, 119]. In fact, HER2 has been shown to be the preferred dimerization partner for the other ERBBs [114].

Regarding the dimerization process, it's important to mention a few considerations:

- EGFR, HER3 and HER4 exist in a closed conformation in which the dimerization domain is not available to interact with the other ERBB partners in the absence of ligand [120].
- There is no known ligand for HER2, then this receptor exists in an active extended –opened conformation and is permanently available for dimerization [120, 121].

In general, ligands binding to ERBB receptors induce a conformational change in the folded structure of the molecule that exposes the dimerization domain, this step is required for dimer formation and functional activation of EGFR, HER3 and HER4 [120, 122, 123]. The kinase domain interaction is asymmetric, with the amino-terminal lobe of one tyrosine kinase interacting with the carboxyl-terminal lobe of the other [124] (**Figure 11**).

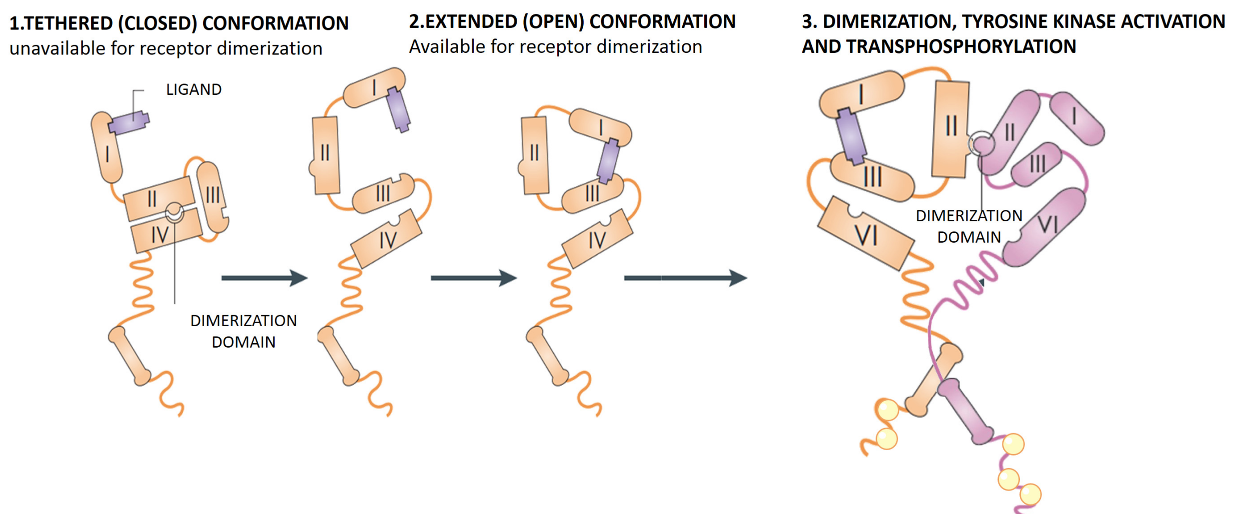


Figure 11. Schematic representation of ERBB general conformational change on ligand binding. *Adapted from Baselga et al., 2009.*

2.3 HER2 IN CANCER

2.3.1 ERBB activation in cancer

ERBB members have been found to be dysregulated in different types of tumours (summarized in **Table 5**). In the tumour cells context, ERBB receptor tyrosine kinases are constitutively activated by various mechanisms, including mutation, overexpression, and autocrine or paracrine production of epidermal growth factor (EGF) family ligands [125].

Furthermore, the EGF family of growth factors are produced as transmembrane precursors that can be cleaved by cell surface proteases a step that leads to the release of soluble ligands. This cleavage, referred to as ectodomain shedding, is an important step in the control of ligand availability and receptor activation [121, 125]. Therefore, it is essential to understand the mechanisms that control ligand processing, as novel therapeutic targets might be discovered.

Once active, ERBB receptors stimulate numerous signalling pathways by recruiting proteins to specific phosphorylated tyrosine residues in their carboxyl-terminal domain. The phosphatidylinositol 3-kinase (PI3K) −AKT pathway is activated through recruitment of the p85 adaptor subunit of PI3K to the receptor.

Mammalian target of rapamycin (mTOR) acts as a central sensor for nutrient/energy availability, and can also be modulated by PI3K–AKT-dependent mechanisms. In another hand the mitogen-activated protein kinase (MAPK) pathway is activated by recruitment of Growth Factor Receptor-Bound protein 2 (GRB2) or Containing Src Homology 2 Transforming Protein (SHC) to the receptor [126].

There are many nuclear effectors of ERBBs in tumour cells, one of these is the cyclin dependent kinase inhibitor p27, which has an important role in the control of proliferation. In tumour cells with overexpressed HER2, p27 is sequestered from cyclin E−CDK2 complexes and cells progress through the cell cycle [127]. Signal transducer and activator of transcription (STAT) is another nuclear effector, the binding of STAT to ERBB leads to its tyrosine phosphorylation, dimerization and nuclear entry, resulting in STAT binding to specific DNA sequences in the promoter regions of target genes encoding, for example, pro-survival factors [128].

Moreover, nuclear ER and oestradiol (E2) control transcription of cell-cycle regulators that are particularly important for breast cancer cell proliferation [129].

With high importance for tumour microenvironment, ERBB receptors also stimulate transcription of vascular endothelial growth factor (VEGF) through the MAPK pathway [130].

VEGF has a key role in induction of tumour associated angiogenesis, in this context, active EGFR receptors have been detected on tumour associated endothelial cells, which have also been proposed to release ERBB ligands [131].

Therefore, endothelial cells and tumour cells can communicate through ERBB ligands and receptors.

MOLECULE	DYSREGULATION	TYPE OF CANCER	COMMENT
EGFR	Overexpression	Head and neck, breast, bladder, prostate, kidney, non-small-cell lung cancer.	Significant indicator for recurrence in operable breast tumours; associated with shorter disease free and overall survival in advanced breast cancer, may serve as a prognostic marker for bladder prostate, and non-small-cell lung cancers.
	Overexpression	Glioma	Amplification occurs in 40 % of gliomas overexpression correlates with higher grade and reduced survival.
	Mutation	Glioma, lung, ovary, and breast.	Deletion of part of the extracellular domain yields a constitutively active receptor.
HER2	Overexpression	Breast, lung, pancreas, colon, oesophagu, endometriu, cervix.	Overexpressed owing to gene amplification in 15–30 % of invasive invasive ductal breast cancers. Overexpression correlates with tumour size, spread of the tumour to lymph nodes, high grade, high percentage of S-phase cells, aneuploidy and lack of steroid hormone receptors.
HER3	Expression	Breast, colon, gastric, prostate, other carcinomas	Co-expression of HER2 with EGFR or HER3 in breast cancer improves predicting power.
	Overexpression	Oral squamous	Overexpression correlates with lymph node cell cancer involvement and patient survival.
HER4	Reduced expression	Breast, prostate	Correlates with a differentiated phenotype.
	Expression	Childhood medulloblastoma	Co-expression with HER2 has a prognostic value.

Table 5. Dysregulation of ERBB members on cancer. *Adapted from Yarden et al., 2001.*

2.3.2 HER2 signalling and regulation

As mentioned before, the structure of HER2's extracellular region is radically different from other family members. HER2 has a fixed conformation that resembles the ligand-activated state: the domain II–IV interaction is absent and the dimerization loop in domain II is exposed [132]. This structure is consistent with the data that indicate that HER2 is the preferred partner for the other activated ERBBs, as its permanently predisposed for interaction with another ligand-bound receptor. Furthermore, this structure explains why no soluble EGF-related ligand has been found. It predicts that HER2 possesses a unique subdomain I–III interaction that makes ligand binding impossible because the predicting site is buried and not accessible for interaction [121, 127].

The levels and activity of HER2 are regulated by different mechanisms. Commonly, cell surface receptor levels are down-regulated by ligand-induced receptor endocytosis, whereby receptor dimers are internalized via clathrin-coated regions of the plasma membrane which form endocytic vesicles. Receptors are then either recycled back to the cell surface, or targeted to lysosomes for degradation. There are also evidences to suggest that internalized receptors might interact with effector molecules in pre-degradative intracellular compartments, thereby activating signalling pathways distinct from those that are activated at the cell surface [133, 134].

Heat shock protein 90 (Hsp90) also plays an important regulatory role in HER2 activity. Hsp90 and its complex of chaperone proteins promote the maturation of HER2 from its nascent form into its stable mature form. Hsp90 then interacts with the kinase domain of HER2, where it is responsible for maintaining the mature protein in a state competent for dimerization and activation [135].

G-protein-coupled receptors (GPCRs) comprise a large family of membrane receptors involved in signal transduction. These receptors are linked to a variety of physiological and biological processes such as regulation of neurotransmission, growth, cell differentiation and oncogenesis among others [136]. Some of the effects of GPCRs are known to be mediated by the activation of MAPK pathways, and it has also been shown that several GPCRs are able to transactivate receptors with tyrosine kinase activity (TKR) such as EGFR and HER2 and thus to control DNA synthesis and cell proliferation [137]. The interaction between these receptors not only plays an important physiological role but its dysregulation can also induce pathological states such as cancer. For this reason, the crosstalk between these two types of receptors can be considered a possible mechanism for cell transformation, tumour progression, reactivation of the metastatic disease, and the acquisition of resistance to therapies targeting TKR receptors [138]. The transactivation of some TKRs by GPCRs is related to the loss of response of TKRs to inhibitors of TK activity, mainly by the activation of the c-Src protein which can directly phosphorylate and activate the cytoplasmic domain of a TKR [139, 140]. Our group has recently described that the pain-associated tachykinin Substance P (SP)

contributes to the persistent transmodulation of the ERBB receptors, EGFR and HER2 in breast cancer, acting to enhance malignancy and therapeutic resistance. SP and its high-affinity receptor NK-1R were highly expressed in HER2+ primary breast tumours (relative to the luminal and triple-negative subtypes) and were overall correlated with poor prognosis factors. In breast cancer cell lines and primary cultures derived from breast cancer samples, we found that SP could activate HER2 [141], and we also investigated the involvement of Src and metalloproteinases in HER2/EGFR activation [142].

For these reason, the dual inhibition of GPCRs and TKRs in some types of cancer has been proposed as a better strategy to kill tumour cells. Increased understanding of the mechanisms that interconnect the two pathways regulated by GPCRs and TKRs may facilitate the design of new therapeutic strategies.

As the rest of the members of the ERBB family, the HER2 cytoplasmic tail of the ERBB receptors contain multiple tyrosine phosphorylation sites, 19 in the case HER2 (summarized in **figure 10**). These phosphorylated residues serve as docking sites for a range of signalling effector molecules containing Src homology-2 (SH2) and phospho-tyrosine binding (PTB) domains. Individual receptors have distinct patterns of tyrosines, and each tyrosine residue has different affinities for different molecules. Furthermore, a given receptor can be differentially trans-phosphorylated in distinct dimers [117]

Of the cellular proteins that interact with HER2, Shc is the most common, with at least five docking sites. Shc has three known isoforms, and mediates signalling from a number of other RTKs, including nerve growth factor receptor (TrkA) [143], platelet-derived growth factor receptor (PDGFR) [144], and InsR [145]. Phosphorylation of Shc causes its association with the adaptor protein GRB2. GRB2 can also bind directly to tyrosine 1139 of HER2. In the cytosol, GRB2 is bound to the guanine nucleotide exchange factor SOS [146], and recruitment of the GRB2-SOS complex to the plasma membrane stimulates the activation of guanosine triphosphate (GTP)-binding protein Ras, by exchanging its guanosine diphosphate (GDP) for GTP. RAS then activates various downstream signalling cascades including MAPK pathway. RAS associates with and activates the serine/threonine kinase RAF, which in turn phosphorylates MEK1 (also known as MAPK kinase). MEK1 then phosphorylates p42/44 MAPK [extracellular signal-related kinase 1 (ERK1 and ERK2)]. Once activated, MAPKs phosphorylate many cytoplasmic and nuclear substrates that include growth factor receptors, transcription factors and other proteins kinases, which mediate cellular processes such as proliferation, differentiation, cell survival and gene transcription [147].

Activation of PI3K occurs through binding of its p85 regulatory subunit to a phospho-tyrosine residue on a receptor, such as those of the HER family. Activated PI3K initiates a major signalling cascade involved in promoting cell growth and survival. The p110 subunit of active PI3K phosphorylates phosphatidylinositol (PI), producing lipids such as PI (3, 4, and 5) P3 and PI (3, 4) P2.

These lipids act as docking sites for proteins containing a pleckstrin homology (PH) domain. PH containing proteins then transmit signals from the plasma membrane into the cell.

Two of such proteins are phosphatidylinositol dependant kinase-1 (PDK1) and protein kinase B (PKB/AKT). Binding of AKT to the inositol lipids causes its translocation to the plasma membrane, where its conformation is altered, enabling its phosphorylation by PDK1 [148, 149]. Active AKT is released into the cytosol, where it phosphorylates target proteins, mediating multiple biological responses, including proliferation, metabolic responses and protection from apoptosis [150]. The tumour suppressor protein PTEN (phosphatase and tensin homolog deleted on chromosome 10) is known to regulate AKT signalling by dephosphorylating PIP2 and PIP3, thereby preventing AKT recruitment [151]. One of the downstream substrates of AKT is BAD, a member of the BCL-2 family of apoptotic proteins [152]. BAD neutralizes anti-apoptotic proteins BCL-2 and BCL-XL, promoting cell death. AKT signalling inhibits BAD thereby protecting cells from apoptosis [153]. HER2 and EGFR do not contain binding sites for PI3K, but active HER3, though kinase-deficient, contains six PI3K-binding sites, and HER2/HER3 or EGFR/HER3 dimers can therefore transmit potent survival signals through the AKT pathway [154].

Activation of PLC by HER2 occurs through the recruitment of its SH2 domain to HER2 phosphotyrosine sites, as well as through the binding of its PH-domain to PI3K products at the plasma membrane. Activated PLC hydrolyses PIP2 into IP3 and diacylglycerol leading to activation of calcium/calmodulin-dependant kinases and phosphatases, and protein kinase C (PKC) [155]. Other important signal effectors activated by the HER family include Src tyrosine kinase, which is activated upon binding to EGFR, and phosphorylates additional tyrosine docking sites, enhancing EGFR activity. Activated Src plays a role in multiple cellular processes, including endocytosis of RTKs such as EGFR, and the rearrangement of the cytoskeleton. Signal transducer and activator of transcription proteins 5 (STAT5), is also phosphorylated by HER proteins, and by activating protein kinases of the JAK family, it is translocated to the nucleus, where it binds to specific promoter sequences in target genes. The JAK/STAT pathway regulates the transcription of many genes involved in cell proliferation, differentiation and apoptosis [155].

One of the important downstream effects of HER family signalling is the promotion of cell cycle progression and cellular survival. Cyclin D1 is a key cell cycle regulator, and is modulated by HER2 through both the MAPK and PI3K pathways [156]. Cyclin D1 activates cyclin dependant kinases (CDKs) to promote G1/S phase cell cycle progression. Furthermore, MAPK and AKT signalling mediate additional cell cycle regulatory effects downstream of HER2 through p27 and p21, two key regulators of CDK function [157].

All these processes involving HER2 activation and HER2 signalling are summarized in **figure 12**.

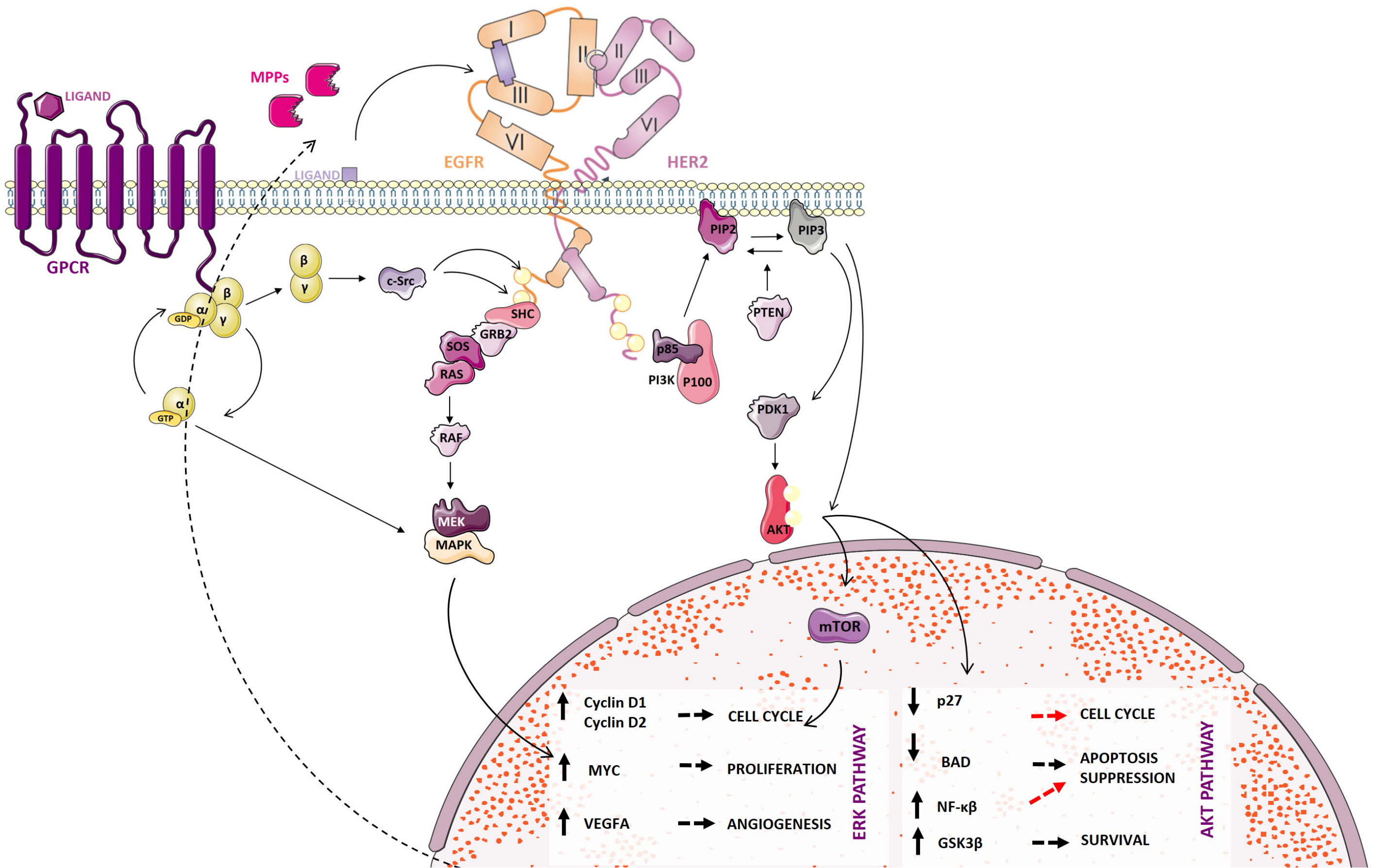


Figure 12. Signalling downstream ERBB dimer formation and receptor activation. *Adapted from Baselga et al., 2009, and Almendro et al., 2010.*

2.3.3 HER2 in transformation and tumourigenesis

Overexpression of HER2 has been fairly implicated in the transformation of epithelial cells, and in tumourigenesis in human tissues [158].

This overexpression is usually due to amplification of *HER2* gene by 2-fold to greater than 20-fold increasing number of copies. These genetic overexpression results in increased expression of the receptor in the cell surface, while normal breast cells have approximately 20.000 HER2 molecules, tumour cells have up to 2 million [159, 160].

Overexpression of HER2 in NIH/3T3 cells or NR6 mouse fibroblast induced cells was shown to induce transformation and tumourigenic growth [161-163]. Moreover, when simultaneously overexpressed, HER2 and EGFR synergistically act in the transformation of NIH/3T3 cells [164].

Transformation has also been showed to be induced by co-expressing HER2 or EGFR with HER3 or HER4 [165].

In MCF7 breast cancer cells, overexpression of HER2 resulted in elevated signalling through the PI3K pathway, and enhanced tumourigenesis when implanted into nude mice [166].

Moreover, in a 3-D model of MCF10A mammary epithelial acini, HER2 overexpression enhanced proliferation, suppressed apoptosis, and promoted the formation of solid acinar structures, resembling those seen during the early stages of epithelial transformation [167].

Overexpression of HER2 in tumours results in increased formation of HER2 containing heterodimers and HER2 homodimers [168]. Furthermore, HER2 homodimerization has also been associated with constitutive activation of its kinase domain [169].

As well as playing a role in tumourigenesis, HER2 has also been implicated in invasion, by regulating expression of integrins on the cell surface. In human mammary epithelial (HME) cells, HER2 overexpression downregulated α4-integrin, leading to increased invasiveness. This was mediated by signalling through RAC1- p38MAPK and PI3k-AKT-PKC-δ pathways [170, 171].

Comparing the roles of HER2 homodimers and HER2/EGFR heterodimers in normal MCF-10A breast cells transformation and invasion, it was observed that while activation of both types of dimers induced disruption of the epithelial acini-like structures, only the HER2/EGFR heterodimers promoted invasion of the cells through the extracellular matrix [172]. The ability of the heterodimers to induce invasion required HER2 kinase activity and was mediated through activation of PI3K, MAPK and phospholipase C1 signalling pathways. The synergistic effects of HER2 and EGFR may be explained, at least in part, by HER2 increasing affinity for EGFR ligand binding and interfering with the rate of EGFR internalization, resulting in prolonged activation of downstream signalling [116].

HER2 overexpression in breast cancer has also been implicated in the promotion of angiogenesis, another important prognostic indicator in breast cancer. In *in vitro* studies, activation of HER2 upregulated vascular endothelial growth factor (VEGF) levels, and treatment with anti-HER2 antibody 4D5 resulted in a dose-dependent reduction of VEGF expression [173].

Furthermore, it has been shown that when paired primary tumour and distant metastatic lesions are compared, approximately 94% and 93% of samples have a concordant HER2 status when analysed by IHC or FISH, respectively [174]. Therefore, taking into account all evidenced exposed before, HER2 over-expressing tumour cells would have higher ability to invade through the basement membrane, adhere to endothelial cells, extravasate, and migrate into normal organs.

High HER2 concordance between primary tumours and axillary lymph node or distant metastases has been also demonstrated in many studies. Among the discordant cases, it is more frequent to have HER2 positive metastases with negative primary tumours than the opposite [175, 176]. A reassessment of biomolecular status in residual tumours after neoadjuvant treatment or in metastatic sites is then advisable to modify the treatment schedule and to estimate the actual prognosis of patients [177].

In this context, the presence of circulating tumour cells (CTCs) in the blood as well as disseminated tumour cells (DTCs) in the bone marrow of breast cancer patients is associated with a worse prognosis in primary and metastatic situations [178].

As HER2 status may change during disease progression, CTCs/DTCs characterization may be useful for characterizing the phenotype of minimal residual disease in the adjuvant setting, serving as a "real time biopsy" of metastatic breast cancer and will help the scientific community to understand the mechanisms of resistance to HER2-directed therapy [179].

Regarding breast cancer DTCs and HER2, it was found that breast cancer patients with HER2 positive tumour cells in the bone marrow have a greater risk for subsequent metastatic relapse than patients with disseminated tumour cells lacking an immunocytochemically detectable expression of HER2 [180].

Moreover, HER2 overexpression in DTCs found in the bone marrow predicts poor clinical outcome [180]. Then, the high incidence of HER2 expression on micrometastatic breast cancer cells in the bone marrow suggested that these cells might have been positively selected during early stages of metastasis [181]. Other authors also stated that HER2 status on disseminated tumour cells after adjuvant therapy may differ from initial HER2 status on primary tumour, and HER2 positive DTCs could be detected in patients with HER2 negative tumours, in agreement with the previous statement [175].

Moreover, it was also recently found a discordance between HER2 status in DTCs when comparing them to CTCs. From a 78 patients study the overall discordance rate in HER2 status was 15% between primary tumour and CTCs, and 28.2% between primary tumour and DTCs, suggesting that HER2 expression is more critical in the lately phases of dissemination [178].

Therefore, it is clear that the HER2 oncogenic pathways have important implications in all different tumour stages.

2.3.4 HER2 detection

The 2007 ASCO guidelines recognized that HER2 is an important prognostic, predictive and therapeutic marker in invasive breast cancer [182]. Therefore, HER2 should be evaluated in every primary breast cancer either at the time of diagnosis or recurrence to guide therapy [176].

Most reports find that the HER2 gene is either amplified or overexpressed at the protein level in 15-30 % primary invasive breast cancers [124], however percentages regarding HER2 status have varied among different reports, probably due to the different techniques used to assess HER2 expression, then it is crucial to standardize testing techniques to accurately assess HER2 status.

Southern blotting was first used to assess HER2 gene amplification, while other studies have used Western blot, reverse transcriptase-polymerase chain reaction (RTPCR), enzyme-linked immunosorbent assay (ELISA), immunohistochemistry (IHC), and fluorescent in situ hybridization (FISH) methods to measure gene amplification or overexpression [183].

Of all of these tests, IHC and FISH are the most frequently used tests to evaluate the HER2 status in breast cancer [184] and the majority of HER2 testing begins with and IHC screening, using FISH to confirm HER2 status in cases with IHC scores of 2+, following the HER2 testing algorithm, **figure 13.**

As mentioned before, IHC has been most commonly used, and this technique measures the amount of HER2 protein expressed on the surface of cells, using a qualitative scoring system from 0 to 3+, with 3+ regarded as overexpression, according to the recently issued 2013 ASCO/CAP guidelines [185] (**Table 6, figure 14**).

Inconsistencies in results can occur due to variations in sample storage, fixation techniques, intensity of antigen retrieval, the type of antibody used, and scoring

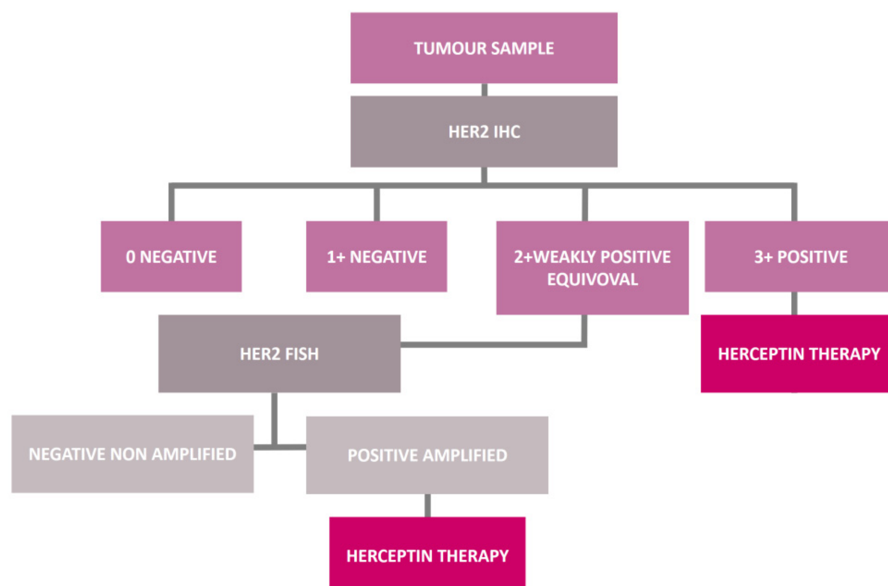


Figure 13. HER2 Testing algorithm. *Adapted from HercepTest™ Interpretation Manual Breast Cancer.*

systems. Two commercially available HER2 IHC tests, the HercepTest^{TM} and the Ventana Pathway^{TM} have been approved by the US Food and Drug Administration (FDA) for use in the clinic determining the eligibility of patients to receive Trastuzumab (HerceptinTM). Different studies have shown an excellent correlation between gene copy number and protein expression levels when 15 different standardized IHC techniques were used [183].

In another hand, FISH technique detects amplification of the HER2 gene and tumours are thus interpreted as negative or positive based on the number of HER2 gene copies. This method is also commonly used, and is probably a more sensitive and accurate method for measuring HER2 status [186]. FISH also has the advantage of having a more objective scoring system, however, this test is also more expensive and time-consuming. Two FISH assay kits have been approved by the FDA; the Ventana Inform^{TM} and the Abbott Vysis Path Vysion^{TM} [187] .

A novel method of hybridization, chromogenic in situ hybridization (CISH), uses a chromogen-labelled probe and offers the advantage of using a light microscope. Studies comparing CISH and FISH analysis of breast cancer samples report a strong correlation between the two techniques [188, 189]. A different study used silver enhanced in situ hybridization (SISH), and reported this technique to be as accurate and reliable as FISH [190].

Finally, another study combined IHC and CISH, this new technique called "protein and gene double staining" (PGDS), is proposed to improve IHC scoring, and increase the sensitivity and specificity of HER2 testing [191].

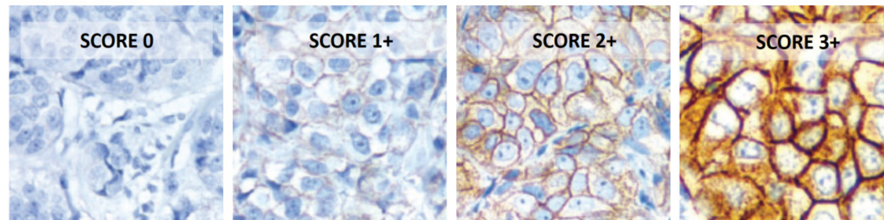


Figure 14. Examples of HER2 staining patterns for tissue scored 0, 1+, 2+, and 3+. *Taken from HercepTest^{TM}*

SCORE TO REPORT	HER2 PROTEIN ASSESSMENT	STAINING PATTERN
0	Negative	No staining is observed, or membrane staining is observed in <10 % of the tumour cells.
1+	Negative	A faint/barely perceptible membrane staining is detected in >10 % of the tumour cells. The cells exhibit incomplete membrane staining.
2+	Weakly Positive	A weak to moderate complete staining is observed in > 10 % of the tumour cells.
3+	Positive	A strong complete membrane staining is observed in > 10 % of the tumour cells.

Table 6. HER2 staining scoring guidelines. *Adapted from HercepTest™ Interpretation Manual Breast Cancer.*

2.3.5 HER2 as a prognostic factor

Amplification of HER2 leading to overexpression of the receptor was originally detected in a subset of breast tumours accounting for 15-30 % of breast cancers [37], but also occurs in other human cancers such as ovarian, gastric and salivary cancers [159]. Moreover, mutations in the kinase domain of HER2 have been identified also in a small number of non-small cell lung carcinoma (NSCLCs) [192].

In 1987, Slamon *et al* reported that in 86 patients with lymph node-positive breast cancer, HER2 amplification correlated significantly with both time to relapse and overall survival [37]. Univariate analysis demonstrated that HER2 amplification was equally strong as the number of lymph node metastases as a prognostic marker, but stronger than hormone receptors expression or tumour size. In multivariate analysis, the prognostic impact of HER2 gene amplification was also significant and independent of number of metastatic axillary nodes [37]. Subsequent studies confirmed the adverse prognostic impact of HER2 in lymph node positive breast cancer patients. In a review of 16 different reports, Revillion *et al* showed, using univariate analysis with disease-free interval as an endpoint, that overexpression of HER2 correlated with poor outcome in 11 studies but failed to correlate in 2 reports. In the remaining 3 studies, HER2 lacked prognostic impact in the overall population but predicted outcome in specific subgroups [158].

As well as tissue levels, levels of serum HER2 extracellular domain (ECD), have also been shown to correlate with high histological grade, lymph node involvement, hormone receptor negativity, disease recurrence, metastasis, and shortened survival [193, 194]. Most studies used ELISAs to quantify the soluble ECD of HER2, which is cleaved from the cell surface by a matrix metalloproteinase-like protein [193].

Proteolytic shedding of the HER2 ECD also generates an N-terminal truncated fragment p95HER2, which has also been related with patient prognostic [195, 196]. Patient studeis have found that the presence of p95HER2 in primary breast tumours correlates with the extent of lymph node metastasis, while the full length

receptor was unrelated to nodal disease. p95HER2 was also detected more frequently in lymph node metastasis than primary tumours, and high levels of p95HER2 significantly correlated with reduced five-year survival in primary breast cancer patients. This truncated form of HER2, therefore, is an independent predictor of patient outcome and may define a group of patients with HER2-positive cancer with significantly worse outcome [195-197].

It's also important to mention that the level of HER2 protein does not necessarily reflect the level of activity of the receptor. Some studies have been performed therefore, to examine the levels of phosphorylated HER2 (pHER2) in breast tumour samples, and to assess the prognostic value of pHER2 in patients.

In a study of invasive breast cancers, pHER2 was detected in 12 % of 307 HER2-positive samples [198]. pHER2 levels correlated with a higher percentage of HER2 positive cells, higher number of positive lymph nodes, and elevated cellular proliferation. Both HER2 and pHER2 were associated with poor prognosis in node-positive patients. In a similar, although smaller, study of DCIS patients, pHER2 was detected at a higher frequency (58 %) compared to HER2 positive invasive cancers [199]. A different study of 70 primary breast cancers also found that pHER2 correlated inversely with hormone receptor status, and was associated with poor clinical outcome [200]. All these results suggest the potential use of phosphorylated HER2 as a prognostic marker.

Patients with cancer whose tumours have alterations in ERBB receptors tend to have a more aggressive disease, usually also associated with factors that predict a poor clinical outcome, so ERBB receptors have been intensely tracked as therapeutic targets. There are two major classes of anti-ERBB therapeutics: ectodomain-binding antibodies and small-molecule tyrosine-kinase inhibitors (TKIs) that compete with ATP in the tyrosine-kinase domain [112]. Several antibodies directed against the extracellular domain of ERBBs and TKIs that target the kinase domain are in clinical use or at advanced developmental stages. The treatment of tumour cells with these agents affects many of the intracellular pathways that are essential for cancer development and progression.

2.4 FGFR FAMILY

2.4.1 Structure and function

The four-member subfamily of the fibroblast growth factor receptors (FGFR1-4) mediates signalling cascades that induce cell growth, differentiation, migration and chemotaxis, angiogenesis, and cell survival [201].

These receptors play a critical role in the development of the skeletal system, and have also been involved in many other cellular processes, such as stemness, anti-apoptosis, and drug resistance [202].

The tyrosine kinases receptors are comprised of an extracellular domain, a transmembrane domain, and a cytoplasmic domain. The extracellular portion contains three immunoglobulin-like (Ig) folds (Ig-I, Ig-II, and Ig-III) with a stretch of eight consecutive acidic residues between Ig-I and Ig-II (the acidic box).

While the Ig-II and Ig-III domains are necessary and sufficient for ligand binding, the amino-terminal portion of the receptor containing Ig-I and the acidic box has an auto-inhibitory function. Alternative splicing of the Ig-III extracellular fragment of FGFR1, 2, or 3 may generate isoforms that differ on their ligand-binding specificity, with Ig-IIIb and Ig-IIIc specifically expressed in the epithelium and mesenchyme, respectively.

The intracellular region of FGFRs contains a juxta-membrane domain, a split kinase domain with the classical tyrosine kinase motifs, and a carboxy-terminal tail [203].

Ligands for these receptors, fibroblast growth factors (FGFs) are secreted glycoproteins sequestered by the ECM and the cell surface by heparan sulfate proteoglycans (HPSGs). These cell surface HPSGs stabilize the FGF ligand–receptor interaction by protecting FGFs from protease-mediated degradation [204].

Upon ligand binding, FGFR substrate 2 (FRS2) functions as a key adaptor protein that associates with the receptor and initiates downstream signalling via activation of MAPK and PI3K-AKT pathways [205]. FGFR signalling also couples to PLC-γ in an FRS2-independent manner and stimulates PKC, which partly reinforces the MAPK pathway activation by phosphorylating RAF [205]. Depending on the cellular context, several other pathways are also activated by FGFRs including the p38 MAPK and Jun N-terminal kinase pathways, and the signal transducer and activator of transcription signalling and ribosomal protein S6 kinase 2 (RSK2) [203, 204, 206].

2.4.2 FGFR in cancer

As mentioned before FGFR signalling plays crucial roles in cancer cell proliferation, migration, angiogenesis, and survival. The FGFR pathway was primarily studied in cancer as a direct promoter of endothelial cell proliferation and tumour neo-angiogenesis, having complementary and synergistic effects with VEGF signalling [204, 207].

The majority of FGFs bind receptors in a trimeric complex with heparins, triggering a conformational change in the receptor that

leads to activation of the FGFR that results in phosphorylation of multiple sites on the intracellular domain, adapter protein binding and intracellular signalling. The deregulation of FGF signalling in cancer results in activation of the pathway without appropriate regulation leading to cancer development, by promoting cancer cell proliferation, survival and migration. FGFR family members are frequently overexpressed in breast cancer, and this is often accompanied by increased, or altered, expression of FGF ligands [208].

Different FGFR pathway aberrations have been identified in cancer. Genomic alteration of FGFR that leads to ligand-independent signalling and can occur through three mechanisms. First, activating mutations that result in ligand-independent dimerization or constitutive activation of the kinase [209]. Second, chromosomal translocations can also lead to ligand-independent signalling. Intragenic translocations generate fusion proteins, usually with the amino-terminal of a transcription factor fused to the carboxy-terminal FGFR kinase domain, resulting in dimerization of the fusion protein and constitutive signalling [210]. Third, receptor gene amplification, which results in supra-physiological receptor overexpression [211].

Moreover, altered FGFR expression on a cancer cell can potentially occur by splicing, which alters FGFR specificity or accumulation, or by amplification of an FGFR gene to express FGFR out of context, which is activated by FGF expressed by a stromal component. Tumour cells can stimulate stromal cells to release FGF ligands and increase the release of ligands from the extracellular matrix. FGF released from stromal cells or cancer cells can act on endothelial cells to promote angiogenesis [204].

A variety of FGFR abnormalities have been identified in breast cancer. The 8p11- 12 amplicon, which contains *FGFR1*, is observed in about 10 % of breast cancer patients, predominantly hormone receptor positive disease [208, 212, 213], and 16–27 % of luminal type B breast cancer [204]. In addition, both cytoplasmic and nuclear expression of FGFR2 are elevated in invasive ductal carcinoma compared to normal tissue, predicting worse outcomes and decreased overall survival and disease free survival [214]. Thus, *FGFR* amplification and mutations in breast cancer are associated with poor prognosis [204, 212].

FGFR2 amplification and enrichment also occurs in approximately 4 % of triple negative breast tumours [204]. In two triple negative *FGFR2* amplified cell lines, constitutive signalling appeared to confer a survival advantage over *FGFR2* non-amplified cell lines [204]. The role of FGFR2 in these cancers has been confirmed by *in vitro* studies using a FGFR-targeted small molecule tyrosine kinase inhibitor (PD173074) or RNAi treatment, which reduced cell survival, blocked PI3K/AKT signalling and induced apoptosis [204].

Mutations in *FGFR4* have been linked to breast and ovarian cancers [215]. In addition, more than 50 mutations in *FGFR1, FGFR2* and *FGFR3* have been identified in patients exhibiting two major classes of human developmental syndromes like craniosynostosis and dwarfism [216].

FGFRs are reasoned highly druggable for various reasons: they are cell surface receptors that are more accessible to most drug-like molecules; they reside at the apical end of the signalling cascade so that their inhibition can achieve a more complete blockade of the intended pathway. Moreover, FGFRs have several pockets that allow binding of small molecules for the inactivation of the kinase activities [217].

FGFR inhibitors are explored in therapeutics with early candidates developed as competitors for the ATP-binding pocket in the kinase domain. However, the high homology among FGFR1–4 and also with other kinase subfamilies creates an additional challenge in developing selective inhibitors [217].

FGFR-targeted drugs exert direct as well as indirect anticancer effects, as FGFRs are also expressed on endothelial cells and fibroblasts and thereby their inhibitors affect the stroma, effecting angiogenesis as well as tumourigenesis [218]. In this setting, several pharmaceutical companies have developed FGFR TKIs targeting FGFR signalling that are in the early phases of clinical trials [204, 219].

2.5 RET FAMILY

2.5.1 Structure and function

The RET oncogene was first identified by Takahashi et al., who reported a novel gene re-arrangement with transforming activity in NIH-3T3 cells transfected with human lymphoma DNA [220].

RET, encoded by the *RET* gene localized on human chromosome 10q11.2, is a receptor tyrosine kinase protein of 150 kDa that becomes 170 kDa when fully glycosylated [220].

The mature RET protein displays an extracellular region which contains four cadherin-like domains and a cysteine-rich region, a single membrane-spanning region, and an intracellular region containing a tyrosine kinase domain [220]. RET is the common signalling element of a macromolecular receptor complex containing dimerized RET receptor, two co-receptors molecules and a bound ligand [221].

The glial derived neurotrophic factor (GDNF) family ligands (GFLs) comprise GDNF, neurturin (NRTN), artemin (ARTN) and persephin (PSPN) that bind RET in association with one of the four glycosyl phosphatidylinositol (GPI) anchored GDNF family α-receptors (GFRα). GDNF mainly associates with GFRα1, whereas NRTN, ARTN and PSPN preferentially bind RET in conjunction with GFαa2, GFRα3 and GFRα4, respectively [222].

Ligand binding to its corresponding GFRα co-receptor triggers RET dimerization and subsequent trans-phosphorylation of intracellular tyrosines leading to the activation of different intracellular

signalling cascades, which have a pivotal role in regulating cellular survival, differentiation, proliferation, migration and chemotaxis [223].

RET encodes a receptor tyrosine kinase expressed primarily on neural crest-derived and urogenital cells [224, 225]. It is required for maturation of several cell lineages of the peripheral nervous system, kidney morphogenesis, and spermatogenesis [226, 227].

Signalling through complexes of RET plus one of the four different glycosyl-phosphatidylinositol linked receptors, is of critical importance for survival of many classes of neurons, including peripheral sympathetic and sensory neurons, as well as midbrain dopamine neurons [109].

2.5.2 RET in cancer

The first clinical manifestation of oncogenic RET was identified in papillary thyroid carcinomas (PTCs) where genomic rearrangements resulted in the fusion of the RET intracellular domain to different 50 N-terminal coding sequences led to the formation of chimeric RET/PTCs oncoproteins [228].

RET is mutated by different mechanisms in different types of thyroid carcinoma and Hirschsprung's disease (HSCR) that displays incomplete penetrance [229, 230].

In contrast, activating germline mutations of *RET* cause multiple endocrine neoplasia type 2 (MEN 2), an inherited cancer syndrome characterized by medullary thyroid carcinoma (MTC) (a malignant tumour from thyroid C cells), pheochromocytoma (a malignancy of adrenal medullary cells), and parathyroid adenomas [231].

Recently two independent studies have identified RET overexpression in a subset of ER positive breast cancers, suggesting an important role of RET in this breast cancer subtype. By *in situ* hybridization, in a cohort of 245 invasive breast cancers, RET and GFRα1 mRNA were detected in 29.7 % and 59.4 % of the tumours, respectively, and preferentially expressed in ER-positive cases [232]. Subsequent studies in the same cohort of patient samples corroborated that increased *RET* mRNA levels correlated with increased RET protein expression [233].

Other studies have demonstrated that the receptor tyrosine kinase RET is overexpressed in a subset of ER-positive breast cancers and that crosstalk between RET and ER is important in responses to endocrine therapy [221]. The development of small molecular inhibitors that target RET allows the opportunity to consider combination therapies as a strategy to improve response to treatment and to prevent and combat endocrine resistance. Combining Tamoxifen treatment with therapies targeting signalling pathways that interact with ER is a promising strategy to improve endocrine therapy response and prevent resistance [234].

3. TARGETED THERAPIES

The use of targeted therapies has come to the forefront of oncology in the past decades and breast cancer was the first solid tumour where targeted therapy was applied. The discovery of the dependence of some breast cancers on oestrogens and oestrogen receptor signalling led to the development of hormone therapy which radically changed the survival and quality of life for women with hormone-sensitive breast cancer [235].

Moreover, molecular pathways involved in cancer growth and progression have been elucidated and molecular targeted agents are being developed to block these signalling pathways. Targeted therapies tend to be less toxic compared to chemotherapy due to their selectivity and are often more effective [236].

The discovery of HER2 overexpression and its role in aggressive cancers led to efforts to develop agents targeting HER2 for anti-cancer therapy.

Targeted therapies can be broadly divided into 2 categories: monoclonal antibodies that block receptors or ligands extracellularly and small molecules which either inhibit intracellular protein kinases or other pathways relevant to cellular proliferation, growth and metastatic potential. The effectiveness of targeted therapies relies on the importance and uniqueness of the selected molecular pathway for tumour development and progression.

Although anti-HER2 targeted therapies are currently approved for breast, gastric, and gastroesophageal cancers overexpression of the HER2 protein or amplification of the HER2 gene, and HER2 aberrations (gene amplification, gene mutations, and protein overexpression) are reported in other diverse malignancies. Indeed, about 1-37 % of tumours of the following types harbour HER2 aberrations: bladder, cervix, colon, endometrium, germ cell, glioblastoma, head and neck, liver, lung, ovarian, pancreas, and salivary duct [237].

Five HER2-targeted therapies have been approved for HER2-positive breast cancer: two antibodies (Trastuzumab and Pertuzumab) [238, 239], an antibody-drug conjugate (Trastuzumab Emtansine) [240], and a small molecule kinase inhibitor (Lapatinib) [241]. In addition, Afatinib, a small molecule kinase inhibitor that causes irreversible inhibition of EGFR and HER2, was recently approved for EGFR-mutated non-small cell lung cancer (NSCLC) [242] **(Table 7)**.

ANTI HER2 AGENT	FIRST APPROVED BY FDA	COMPOUND	TARGET	MECHANISM
Trastuzumab	1998	Humanized murine monoclonal antibody	HER2	Binding to HER2extracellular domain, antibody dependent cell mediated cytotoxicity
Lapatinib	2007	Small molecule tyrosine kinase inhibitor	EGR/HER2	Reversely blocks ATP-binding site on kinase domain of EGFR and HER2
Pertuzumab	2012	Humanized murine monoclonal antibody	HER2	Binding to domain II of HER2, blocking ligand dependent dimerization of HER2 with other ERBB members
T-DM1	2013	Trastuzumab linked with a non-reducible linker to DM1	HER2	Anti-tumour properties of Trastuzumab combined with cytotoxic microtubule depolymerizing DM effects
Afatinib	2013	Small molecule tyrosine kinase inhibitor	EGFR/HER2	Irreversible blocking kinase domain of EGFR/HER2, including the Erlotinib resistant EGFR T790M variant

Table 7. Approved anti-HER2 agents. *Adapted from Yan et al., 2014.*

3.1 MONOCLONAL ANTIBODIES

3.1.1 Trastuzumab

The monoclonal antibody, Trastuzumab (Herceptin^{TM}, Genentech), was the first monoclonal antibody approved for breast cancer treatment, it was developed by Genentech (San Francisco, CA), and due to its anti-tumour effects in vitro and *in vivo*, this antibody was humanized for clinical use. Trastuzumab binds to the ectodomain of HER2, preventing its interaction with the transmembrane domain, however, it does not prevent heterodimerization of HER2, as the specific binding site for Trastuzumab is not involved in dimerization [243].

Trastuzumab demonstrated higher binding affinity for HER2 than the murine 4D5, and potent anti-tumour effects in human tumour xenografts [244].

The most significant advancement in the treatment of HER2 positive breast cancer came from five randomized trials demonstrating the benefit of Trastuzumab treatment also for early stage HER2 positive breast cancer. Addition of Trastuzumab to adjuvant therapy resulted in 39-52 % reduction in disease recurrence [245]. Furthermore, a meta-analysis of the five adjuvant trials, comparing adjuvant Trastuzumab plus chemotherapy versus chemotherapy alone, showed a significant reduction in mortality, recurrence and rates of metastasis for the Trastuzumab containing regimens [246].

In the clinic, Trastuzumab used in adjuvant therapy achieved overall responses of between 11 and 15 %, as a single agent, in

HER2 positive metastatic breast cancer patients who had progressed after chemotherapy [247-249]. As a first-line treatment, Trastuzumab showed response rates of about 26-34 % in HER2 positive metastatic breast cancer patients [248].

The pivotal phase III trial of Trastuzumab in combination with chemotherapy, in HER2 positive metastatic breast cancer, demonstrated improved overall response rate (50 % versus 32 %), longer duration of response (time to progression: 7.4 versus 4.6 months), longer survival (overall survival: 25.1 versus 20.3 months) and a 20 % reduction in risk of death, compared to chemotherapy alone [250].

Moreover, Trastuzumab has been tested in combination with a variety of chemotherapy agents, including taxanes, platinum salts, vinorelbine and gemcitabine, with response rates ranging from 24% to 84% [251].

Finally, in the neo-adjuvant setting, addition of Trastuzumab to chemotherapy has achieved pathological complete response (pCR) rates of 12-65% and clinical complete response rates of 30-86% [251].

A randomised trial to compare Trastuzumab plus chemotherapy to chemotherapy alone in the neo-adjuvant setting was performed in 42 patients but was stopped early by the Data Monitoring Committee because of the superior results observed for the Trastuzumab plus chemotherapy group (pCR 66.7 % versus 25 %) [252].

The mechanisms of action of Trastuzumab are not yet fully understood, however, several models have been proposed, some of which are discussed below.

Downregulation of HER2

It is still unclear to what extent Trastuzumab causes down-modulation of HER2. As seen in section 2, generally, ligand binding to ERBB family members causes ligand-receptor complexes internalization within endosomes. Once there, the ligand-receptor complex dissociates and the receptor can either be transported to lysosomes for degradation, or recycled back to the cell surface [109].

It has been shown that when HER2 is overexpressed, its signalling is mediated preferentially by heterodimers containing HER2 [253], and that dimers containing HER2 preferentially result in receptor recycling, thereby efficiently sending prolonged proliferative signals to the nucleus [254]. Some authors have demonstrated that Trastuzumab does not downregulate HER2, but recycles passively with the receptor after endocytosis. Moreover, other groups have also demonstrated that membrane receptor levels do not change in response to Trastuzumab [255, 256].

Reduced HER2 signalling

One of the further proposed mechanism of action is the reduction of HER2 signalling through AKT and MAPK pathways, thereby promoting apoptosis and inhibiting proliferation [255, 257]. Nagata *et al* have demonstrated that Trastuzumab specifically disrupts HER2- Src interactions, leading to inactivation of Src, which in turn causes reduced PTEN phosphorylation, and increasing PTEN phosphatase activity, that finally will lead to rapid AKT de-

phosphorylation and cell proliferation inhibition [255].

Serra *et al*. also found using HER2-overexpressing breast cancer cells, that PI3K inhibitors abolished AKT activation. This PI3K inhibition also resulted in a compensatory activation of the ERK signalling pathway, as a result of the activation of ERBB family receptors as evidenced by induction of HER receptors dimerization and phosphorylation, increased expression of HER3 and binding of adaptor molecules to HER2 and HER3. The activation of ERK was prevented with either MEK inhibitors or anti-HER2 monoclonal antibodies and tyrosine kinase inhibitors [258].

Inhibition of HER2 extracellular domain (ECD) shedding

It is known that when overexpressed, HER2 undergoes proteolytic cleavage by matrix metalloproteinase-like enzymes, which results in the shedding of its ECD, and the production of a truncated membrane-bound fragment named p95 [259]. Furthermore, ECD cleavage was shown to be a ligand-independent method of HER2 activation *in vivo* [260].

In vitro studies have demonstrated that this p95HER2 fragment has kinase activity, can form heterodimers with HER3, and can further be tyrosine phosphorylated by neuregulin-1 [196, 261]. Trastuzumab has been shown to inhibit ECD shedding in SKBR-3 and BT-474 breast cancer cell lines, by blocking matrix-metalloproteinase-mediated cleavage of the ECD [261].

The ECD can be detected in cell culture medium, and in the serum of breast cancer patients, and clinical studies have also reported a decline in serum ECD levels during Trastuzumab treatment [262], suggesting that serum levels prior to treatment may be a predictor of response [263, 264].

Antibody dependant cell mediated cytotoxicity (ADCC)

Animal studies and clinical trials have demonstrated that Trastuzumab possesses cytotoxic as well as cytostatic properties, and these properties may be due to the induction of an immune response. Single agent Trastuzumab reduces tumour size in patients with HER2-overexpressing metastatic breast cancer [248]. Whether this cytotoxic effect is a direct effect on the cancer cells or whether it is mediated by indirect mechanisms (by the immune system or anti-angiogenic activity) has yet to be defined.

ADCC has been described to be caused by the activation of natural killer (NK) cells, which express the Fcγ receptor (FcγR), and can be bound by the Fc domain of Trastuzumab IgG1 [265].

Clynes *et al.* used FcγR-knock-out mice models with BT-474 xenografts, and showed that while Trastuzumab reduced tumour volume by 90 % in FcγR+/+ mice, its cytotoxic activity was significantly impaired in FcγR-/- mice, where it reduced tumour volume by only 29 % [266]

Mohsin *et al.* report that reduction of tumour size after three weeks of Trastuzumab treatment in 35 breast cancer

patients was accompanied by an average 35 % increase in apoptosis [267].

In another small pilot study (n=11), Gennari *et al.* found no decrease in tumour size following treatment with Trastuzumab, but reported a strong increase in infiltrating lymphoid cells [256]. The authors suggested that the response seen by Mohsin may be due to an inflammation response caused by the core biopsy method used, rather than an early apoptotic response to Trastuzumab. Trastuzumab has also been shown to sensitize breast cancer cells to apoptosis inducing agents, for instance Paclitaxel and Etoposide by reducing the inhibitory phosphorylation of p34, down-regulating p21, and reducing expression of the anti-apoptotic Bcl2 family member MCl-1 [268, 269].

Inhibition of angiogenesis

Overexpression of HER2 in breast cancer cells correlates with elevated vascular endothelial growth factor (VEGF) expression and increased angiogenesis [270, 271]. Trastuzumab induced normalization and regression of vasculature in a mouse model by reducing the expression of VEGF, and increasing the expression of the anti-angiogenic factor thrombospondin-1 [272]. Klos *et al.* also showed that the combination of Trastuzumab and Paclitaxel inhibited angiogenesis to a greater degree than either drug alone in breast cancer xenografts [273].

3.1.2 Other monoclonal antibodies

A second anti-HER2 antibody, **Pertuzumab** (Omnitarg), was developed by Genentech and is currently in phase II clinical trials in breast cancer patients [274]. Contrary to Trastuzumab, when Pertuzumab binds to HER2, blocks the dimerization with other HER receptors, and in addition, Pertuzumab is also able to inhibit the growth of tumours with low levels of HER2 by preventing ligand induced activation of HER2 heterodimers [243].

Furthermore, the combination of Pertuzumab and Trastuzumab synergistically inhibited the survival of BT-474 breast cancer cells *in vitro*, partly due to increased apoptosis [275].

Trastuzumab-Emtansine (T-DM1) is an antibody-drug conjugate and is composed of Trastuzumab covalently linked to Maytansine, a cytotoxic agent that acts as a microtubule polymerization inhibitor [240]. T-DM1 binds to HER2 with similar affinity as Trastuzumab, and it has been postulated that after binding, the T-DM1/HER2 complex is internalized, degraded in the lysosome, which leads to further release of DM1, and subsequent cell lysis [276]. Although used at lower doses and frequency than Trastuzumab, T-DM1 retains the ability to inhibit HER2 signalling and engaging immune effectors that mediate ADCC, and is also active against Lapatinib-resistant xenografts [276]. Moreover, phase I–II studies of T-DM1 demonstrated mild, reversible toxicity and a remarkable clinical response rate in additional 25 % of patients that were

heavily pre-treated and who had progressed after Trastuzumab and Lapatinib [277, 278]. T-DM1 was recently approved by the FDA for advanced breast cancer treatment, and is nowadays being further evaluated in two large phase III randomized studies [279, 280].

3.2 TYROSINE KINASE INHIBITORS

3.2.1 Lapatinib

Another approach to targeting HER2 is to directly target its tyrosine kinase activity. Small molecule tyrosine kinase inhibitors (TKIs) have been extensity developed during the last decades. One class of TKIs are the quinazolines, which mimic ATP, reversibly binding to the ATP binding site, thereby inhibiting their kinase activity [281].

Lapatinib (GW572016, Tykerb) is a quinazoline TKI, and is a dual inhibitor of both EGFR and HER2, which binds reversibly to the ATP-binding site of both receptors and blocks receptor phosphorylation and activation [282].

HER2 and EGFR are co-expressed in approximately 30 % of breast cancers, and increased EGFR signalling may compensate for the loss of HER2 activity in some Trastuzumab-resistant tumours [264].

In the clinic, Lapatinib has also been demonstrated to have activity in HER2-positive patients and is currently approved as the second line treatment of metastatic breast cancer when used in combination with the chemotherapeutic agent Capecitabine, a pro-drug that is enzymatically converted to 5-Fluorouracil in the tumour [283].

Due to the fact that Trastuzumab and Lapatinib have complementary mechanisms of action, a number of studies have been undertaken to evaluate the synergistic effects of combining Trastuzumab with Lapatinib for the treatment of HER2 positive metastatic breast cancer [280, 284-286]. As a result of these studies, this treatment regime has been approved for patients suffering from HER2 positive, HR negative metastatic breast cancer by the European Medicines Agency (EMA). It has also been approved in combination with the aromatase inhibitor, Letrozole, in HER2 positive, HR metastatic breast cancer patients, showing an increased progression free survival as well as the clinical benefit rate [287].

Lapatinib was extensively studied in the neoadjuvant setting, with multiple trials demonstrating an improved pathologic complete response rate [288, 289]. Regrettably, these neoadjuvant trials were not followed by success in the adjuvant setting, the recent presentation of the ALTTO adjuvant trial last year at the 2014 American Society of Clinical Oncology meeting did not pass statistical boundaries for positivity, and even a trend favouring improved disease-free survival is balanced by significantly increased toxicity [290].

It has also been suggested that TKIs may have an advantage over antibody therapy due to their ability to cross the blood brain barrier, and may therefore reduce the risk of central nervous system (CNS) metastasis. In a study of 39 HER2-positive advanced

breast cancer patients with CNS metastatic disease, Lapatinib showed clinical benefit in 5 % of patients [186], however investigation with larger cohorts are warranted to explore this clinical benefit.

Regarding its mechanism of action, dual TK inhibitors like Lapatinib offer several potential advantages compared with a monoclonal antibody like Trastuzumab that targets extracellular HER2 only:

The combined blockade of 2 receptors involved in tumour proliferation may result in a synergistic inhibition of tumour cell growth [291]. Then the simultaneous inhibition of EGFR and HER2 is thought to be more effective in preventing the formation of heterodimers involving EGFR and HER2 [292], that will result in a more complete inhibition of different signal transduction pathways of the redundant intracellular signalling network [293].

Lapatinib may overcome Trastuzumab resistance linked to the overexpression of a truncated HER2 (p95). Because Lapatinib does not target the extracellular domain of HER2, and it is able to inhibit p95HER2 as has been shown in preclinical models [196, 294].

Furthermore, it has been shown that Lapatinib inhibits breast cancer cell proliferation *in vitro*, and downregulates HER2, RAF, AKT, and ERK phosphorylation [295] . The combination of Lapatinib and Trastuzumab had synergistic anti-proliferative effects in HER2-overexpressing breast cancer cell lines [286].

Moreover, Lapatinib inhibited the proliferation of HER2-overexpressing breast cancer cell lines and xenografts [286], and also had synergistic inhibitory effects when combined with Trastuzumab in four breast cancer cell lines, and significantly inhibited the proliferation of cell lines with acquired resistance to Trastuzumab [296].

3.2.2 Other tyrosine kinase inhibitors

Afatinib is also a tyrosine kinase dual inhibitor of HER2 and EGFR [297]. This agent, was developed by Boehringer Ingelheim, and has been shown to irreversibly inhibit HER2 and EGFR receptors [298].

The drug has been shown to be more potent than Lapatinib and, as a result, a dose of 50 mg per day has been identified as being effective in comparison to the 1250 mg twice a day that is required for Lapatinib [299, 300]. Clinical trial data has indicated that Afatinib may have activity in metastatic breast cancer patients that have developed resistance to Trastuzumab [301].

Due to inhibition of the EGFR receptor, the inhibitor has been proven to be also effective in the treatment of NSCLC and lung adenocarcinomas and a number of phase II and III clinical trials have been undertaken to study these malignancies [302, 303].

Neratinib (HKI-272), a dual kinase inhibitor, developed by Pfizer, targets both HER2 and EGFR [304]. By irreversibly binding to the HER2 and EGFR receptors, this TKI reduces auto-phosphorylation within the cell which in turn prevents the activation of downstream pathways [305, 306]. Phase I clinical trials within patients with solid tumours indicated that the most suitable dose of Neratinib was between 240 and 320 mg per day [302]. Initial phase I trials also

indicated that treatment of patients with Neratinib that had been pre-treated with Trastuzumab, anthracyclines or taxanes showed therapeutic potential and justified further studies [306]. In fact, a breast cancer recent study showed that Neratinib improve disease-free survival in a post-Trastuzumab adjuvant seeting in the ExteNET trial [307].

As Afatinib, due to its ability to target EGFR, Neratinib has also been investigated for the treatment of NSCLC [308], however, phase I [306] and phase II [302] trials have been limited and have shown disappointing results, with little to no response rate evident in the study.

Supplementary information regarding current clinical trials with anti HER2 targeted therapies could be found in the **Annex section A1**.

3.3 RESISTANCE TO TARGETED THERAPIES

Despite the proven clinical benefits of Trastuzumab and Lapatinib therapies, either as a single agents or in combination, not all patients that overexpress HER2 respond to Trastuzumab and Lapatinib. In fact, less than 35 % of patients with HER2 overexpressing metastatic breast cancer respond to Trastuzumab as a single agent [248] and the majority of those who initially response, acquire resistance within the first 1-2 years [255, 309]. Furhtermore, multiple phase II trials have revealed that only 20 % to 35 % of patients with HER2+ metastatic breast cancer respond to Lapatinib [107].

Several mechanisms have been proposed in an attempt to explain both intrinsic and acquired resistance to Trastuzumab and Lapatinib.

HER2 is the preferred dimerization partner of the HER family and has been described as a non-autonomous amplifier of the HER signalling network. Alterations at any stage of this complex network may confer resistance to Trastuzumab and Lapatinib [310].

Usually, resistance mechanisms are classified according to genetic or environmental alterations on RTKs and their downstream effectors (termed *de novo* resistance) or to the activation of alternative pathways, to bypass the HER-2 inhibition after anti-HER-2 exposure (known as acquired resistance) [311].

Different mechanisms of resistance have been defined for anti-HER2 therapies, and may be summarized by acting at three levels.

Impaired access to the receptor or altered receptor-antibody binding

Trastuzumab binds to the extracellular domain of the HER2 receptor; disruption of this binding is therefore a potential mechanism of resistance. Nagy *et al.* studied the properties of Trastuzumab-resistant cell line, JIMT-1, derived from a HER2 overexpressing patient with primary resistance to Trastuzumab. They found that even though HER2 expression, internalisation and down-regulation were similar to those found in Trastuzumab-

sensitive cell lines, there was reduced number of antibody binding sites, indicating that HER2 was partially masked in these cells [312]. Expression of MUC4, a membrane-associated mucin that contributes to the masking of membrane proteins, was higher in JIMT-1 cells compared to sensitive cell lines, and high levels of MUC4 correlated with decreased Trastuzumab binding capacity. Thus, elevated MUC4 may promote resistance to Trastuzumab by masking the antibody-binding epitopes of HER2 [312]. Moreover, a feedback activation of STAT3 via upregulation of MUC1 and MUC4 expression has been recently described as a mediator of Trastuzumab resistance [313]. Raina and colleagues also recently defined that targeting MUC-1, downregulated HER2 activation and affected Trastuzumab resistance [314].

The extent of enzymatic cleavage of HER2 may also be a contributing factor in Trastuzumab resistance. Scaltriti *et al.* showed in a small-scale study (n = 46), that breast cancer patients expressing p95 were less likely to respond to Trastuzumab than those expressing the full length receptor, and that cells expressing p95 were not sensitive to Trastuzumab but were responsive to the tyrosine kinase inhibitor Lapatinib, which targets the ATP binding pocket of the intracellular domain of HER2 [315].

There is also evidence that the metalloprotease ADAM 10 may play a role in the cleavage of the HER2 ECD. Hence, inhibition of ADAM 10 reduced shedding of HER2 ECD and increased cell line sensitivity to Trastuzumab [316]. Previous work from this group have indicated that p95HER2-positive tumours were resistant to Trastuzumab monotherapy [317].

However, recent studies have shown that tumours expressing the most active form of these fragments, p95HER2/611CTF, respond to Trastuzumab plus chemotherapy [318].

ADCC has also been suggested to play a role in the antitumour activity of Trastuzumab. In ADCC, the antibody (Trastuzumab) first binds to tumour cells and then is engaged by effector cells through their receptors for IgG. Polymorphisms on the FccRIIIα are constitutively expressed on killer cells [319], in particular the 131 H/R and 158 V/F polymorphisms which appear to exert a strong effect on IgG1 affinity for FccR, and consequently on the ADCC-related activity [320]. In metastatic breast cancer, the FccRIIIα-58 V/V and 131 H/H genotypes were significantly correlated with better objective response rates (ORRs) and progression-free survival (PFS) in patients treated with Trastuzumab [321]. However, a large genotyping analysis failed to confirm these results [311].

Alternative HER2 signalling

Mediated by HER2 family members

HER2 is the preferred dimerization partner for each of the other ERBB family receptors. Trastuzumab does not inhibit ligand-induced heterodimerization [322, 323], therefore, increased activity of other ligands and receptors of the ERBB network could maintain HER2 signalling despite the presence of Trastuzumab. Ritter *et al.* reported increased levels of EGFR ligands such as TGF-α and heparin binding EGF in

Trastuzumab resistant BT-474 cells [324]. Increased levels of EGFR, phospho-EGFR and EGFR/HER2 heterodimers were also detected in the drug resistant cells. Co-expression of EGFR determined EGFR/HER2 homo- and heterodimerization in BT-474 and SK-BR-3 cells, and was further related to reduced response to Trastuzumab [325].

Other studies have also shown that increased expression of specific ERBB ligands, such as TGF-α, made cells less responsive to Trastuzumab, and in fact TGF-α was induced by Trastuzumab treatment of metastatic breast tumours [264, 326]. Furthermore, inhibition of EGFR was found to restore sensitivity to the Trastuzumab resistant cells [324].

Sergina *et al.* first showed that reactivation of HER3 mediated resistance to EGFR and HER2 inhibition. While EGFR (Gefitinib) and HER2 (Tyrophostin) selective TKIs efficiently inhibit auto-phosphorylation of EGFR, HER2 and downstream MAPK and JNK signalling in HER2 positive breast cancer cells, HER3 signalling resumes after 12-24 hours of treatment. Reactivation of HER3 leads to reactivation of AKT signalling and is believed to be mediated by AKT-driven negative feedback signalling. Although Lapatinib was not tested in this study, the HER3 mediated escape from EGFR and HER2 inhibition may apply to all ERBB TKIs [327].

An *in vitro* model of acquired resistance to Lapatinib was developed by chronic exposure of Lapatinib-sensitive BT-474 cells to Lapatinib [328]. In this model, acquired resistance was attributed to a switch in cell survival dependence from HER2 alone to co-dependence upon ER and HER2. The increase in ER signalling was mediated, at least in part, by de-repression of the transcription factor FOXO3a causing inactivation of AKT by Lapatinib [328].

Initially it was thought that the anti-HER2 monoclonal antibody Herceptin inhibited HER2 signalling, but different studies have shown that Herceptin does not decrease HER2 phosphorylation. Its failure to abolish HER2 phosphorylation may be a key to understand why acquired resistance inevitably occurs for all responders if Herceptin is given as monotherapy. Gijsen *et al.* determined that HER2 phosphorylation was maintained by ligand-mediated activation of EGFR, HER3, and HER4 receptors, resulting in their dimerization with HER2, and that the release of HER ligands was mediated by ADAM17 through a PKB negative feedback loop [329].

Mediated by other RTKS

Other potential mechanism of Trastuzumab resistance have been postulated to involve also RTKs outside of the ERBB family.

For instance, Shattuck *et al*. defined that the RTK MET (HGF receptor) was implicated in Trastuzumab resistance. They showed that HER2 overexpressing cells upregulate MET following exposure to Trastuzumab, and activation of MET protected cells against Trastuzumab by abrogating the induction of p27 [330].

AXL is an RTK with a kinase domain closely resembling MET and an extracellular domain resembling neural cell adhesion molecules, Hafizi *et al*., showed that in BT-474 cells rendered drug resistant by chronic exposure to Lapatinib exhibited increased expression and activation of AXL [331].

Using HER2-positive cells selected in culture, another study by Liu *et al.*, identified overexpression of AXL as a mechanism of resistance to Lapatinib [332]. Evidences of the cross-talk between IGF-IR and HER2 signalling pathways have led to investigations of the role of IGF-IR in the response or resistance to Trastuzumab. Lu *et al.* reported that IGF-IR signalling interfered with the action of Trastuzumab in breast cancer cell lines. Trastuzumab inhibited the growth of HER2 transfected MCF-7/HER2- cell lines, which express high levels of IGF-IR, only when IGF-IR signalling was minimized by reduction of serum concentration, treatment with IGF32 IR antibody or with recombinant human RH-IGFBP3 [333]. Later on it was reported that Trastuzumab regulates IGFBP-2 and IGFBP-3 to mediate growth inhibition [334].On the other hand, the growth of SKBR-3 cells, which have low IGF-IR levels, was inhibited by 42 % by Trastuzumab. However, transfection of IGF-IR into these cells conferred almost complete resistance to Trastuzumab. Again, RH-IGFBP3 restored sensitivity to Trastuzumab [333].

Nahta *et al.* demonstrated that HER2 and IGF-IR heterodimerize in Trastuzumab resistant SK-BR-3 cells, and not in parental SK-BR-3 cells, suggesting that this heterodimerization contributes to Trastuzumab resistance [255].

Further studies have reported enhanced activation of downstream signalling pathways originating from HER2, HER3, IGF-IR in Trastuzumab-resistant breast cancer cells by defining heterotrimeric complexes in these drug resistant cells [335].

Aberrant downstream signalling activation

Alterations in the intracellular signalling of the ERBB network have also been implicated in the development of Trastuzumab resistance. Increased PI3K/AKT activity has been reported in Trastuzumab resistant cells [336] There are a number of independent studies which suggests that loss of the protein phosphatase PTEN may be responsible for this deregulated signalling in drug resistant cells, and PTEN status has in fact been associated to patient outcome [337, 338]. PTEN functions as a tumour suppressor gene that negatively regulates the AKT signalling pathway via direct inhibition of PI3K signalling [339]. Downregulation of PTEN by antisense in breast cancer cells conferred resistance to Trastuzumab *in vitro* and *in vivo* [340]. Inhibition of proteosomal degradation of PTEN also restored sensitivity to Trastuzumab resistant cells [333]. However, not all studies have linked PTEN loss with Trastuzumab resistance. Neve *et al.* showed in a panel of nine HER2 positive cell lines that there was no association between PTEN protein levels and Trastuzumab response [341]. Another mechanism by which increased activation of AKT signalling can occur is by activating mutations in the gene encoding the p110α subunit of PI3K. When patients were grouped as either PTEN lost or PI3K mutant there was a strong significant association with poor Trastuzumab response in both groups, suggesting that increased activation of AKT signalling, either by loss of PTEN or by

activating mutations in PI3K, plays a role in Trastuzumab resistance [342]. The activated PI3K/AKT signalling association with Trastuzumab failure has been further studied, and also PI3K activity has also been related to upregulation of the HER3/HER4 ligand heregulin (HRG) and HER3 activation [343, 344].

Activation of the AKT pathway has been shown to increase tumour cell proliferation via direct phosphorylation of the cell cycle inhibitor p27. Phosphorylation of p27 by AKT prevents its translocation to the nucleus [345]. Trastuzumab mediated growth arrest is dependent on p27, and its inhibitory interaction with CDK2 [346]. Nahta *et al.* demonstrated that p27 levels were reduced in Trastuzumab resistant SK-BR-3 cells, while expression of CDK2 was increased. Exogenous addition of p27 into these cells restored sensitivity to Trastuzumab [347].

Evidence from preclinical and clinical testing of Lapatinib suggests that Lapatinib and Trastuzumab have non-overlapping mechanisms of resistance. In three cell line models of Trastuzumab resistance, Lapatinib retained significant activity [286]. Biomarkers studies in tumour samples from patients treated with Lapatinib suggest that neither loss of PTEN nor expression of IGF-IR preclude response to Lapatinib [296].

As happened with Trastuzumab, activating PIK3CA mutations, loss of PTEN and alternative signalling pathways that activate PI3K-AKT have been reported as Lapatinib escape mechanisms. Using a large-scale loss-of-function short hairpin RNA screen to identify novel modulators of resistance to Lapatinib, Eichhorn *et al*. identified PTEN as a gene whose loss reduced the sensitivity to the TKI both *in vitro* and *in vivo* [348].

In addition, two dominant activating mutations in PIK3CA (E545K and H1047R), which are prevalent in breast cancer, also conferred resistance to Lapatinib.

These authors also showed that the resistance to Lapatinib induced by the PI3K mutants can be abrogated through the use of BEZ235, a dual inhibitor of PI3K/mTOR currently tested in phase I/II clinical trials [348].

Some other proposed mechanisms of resistance to Lapatinib also involve de-repression and/or activation of compensatory survival pathways. For example, in HER2 overexpressing BT-474 cells selected for acquired resistance to Lapatinib, Xia *et al*. showed that the drug resistant cells still showed inhibition of HER2, HER3, MAPK and AKT phosphorylation upon treatment with Lapatinib. In these cells, inhibition of AKT with Lapatinib resulted in de-repression of the transcription factor FOXO3 thus leading to increased ER transcription and signalling [328, 349].

Current clinical data support that Trastuzumab resistant tumours, even in advanced stages, continue to be dependent on the HER2 pathways to survive [106, 107, 186, 350].

Altought Trastuzumab and Lapatinib have already been proven to be very effective in patients with HER2 positive breast cancer, *de novo* and acquired resistance still represent a major problem limiting their efficacy. Furthermore, the lack of clinical validated biomarkers predictive for anti-HER2 therapy resistance makes it

impossible to select the best therapeutic strategy for a specific patient.

There is an overabundance of agents that either target HER2 by different mechanisms or inhibit the described mechanisms of resistance, then potential strategies to overcome resistance to anti-HER2 treatment have been investigated and several drugs are currently in different phases of clinical development [237].

Data from two neoadjuvant studies (Neo-ALTTO [290] and the NeoSphere trial [105]) and one study in the MBC setting (Cleopatra [351]) clearly show that different HER2 targeted agents working with different mechanisms of action may improve the outcome in patients with HER2- positive breast cancer.

4. TUMOUR MICROENVIRONMENT

4.1 GENERAL CONCEPTS OF BREAST CANCER STROMA

Classically cancer research has focused mainly on the neoplastic cells within tumours. However, especially in the last years it has become obvious that while tumour epithelial cells that have undergone genetic and epigenetic events are essential for the initiation of BC, a variety of populations from the surrounding microenvironment also influence tumour progression actively (proliferation, migration and invasion) [352].

Therefore, in recent years, tumours have come to be understood to function as a complex tissue, where numerous host cells are recruited to play critical roles [58].

4.1.1 Breast stroma: protective role and tissue homeostasis

The mammary gland is a complex tissue composed of an epithelial parenchyma embedded in an array of stromal cells that regulate its proliferation, differentiation and survival [353].

The mammary gland also undergoes dynamic changes over the lifetime of a woman (expansion and development during puberty, hormonally controlled proliferation and apoptosis during menstrual cycle, and full lobule-alveolar development during lactation).

Then, homeostasis in this kind of dynamic tissues requires a tightly controlled balance of cell proliferation and death, which is achieved and maintained through intercellular communication. An important regulator of normal cell behaviour and tissue homeostasis is the extracellular matrix (ECM). Maintaining organ homeostasis can prevent neoplastic transformation in normal tissues by ensuring stable tissue structure, mediated by tight junction proteins and cell adhesion molecules such as β1 integrins and epithelial E-cadherin [354].

Another important feature of the normal stroma is the generation and maintenance of epithelial-cell polarity. Epithelial cells receive a variety of orientation cues from the environment that helps them establish cellular apical and basal surfaces as well as maintain their differentiated state. The basal surface of epithelial cells associates with the basement membrane, a specialized form of the ECM that provides both structural support and polarization signals to the epithelia. The basement membrane is a dynamic structure and changes in its composition lead to changes in cell shape and behaviour, altered binding affinity or redistribution of cell-surface receptors, and cellular responses to soluble molecules. Loss of polarity has been shown to lead to increased cell proliferation and tumourigenesis [355].

The normal tissue microenvironment also acts as a barrier to tumourigenesis. Then, under conditions of normal tissue homeostasis, the microenvironment exerts suppressive forces to keep tumours occult [356] (**Figure 15A**).

However, the intercellular signals that define the normal tissue context may be disrupted, and then the microenvironment can become permissive to tumour growth, and the combination of mutagens, inflammation, growth factors and other

tissue-associated promotional forces can breach the barrier to tumour formation [356] (**Figure 15B**).

For instance, under persistent inflammatory conditions, continual upregulation of enzymes such as matrix metalloproteinases (MMPs) by stromal fibroblasts can disrupt the ECM, and invading immune cells can overproduce factors that promote abnormal proliferation. As this process progresses, the normal organization of the organ is replaced by a functional disorder. In this context, if there are pre-existing epithelial cells within this changing context that possess tumourigenic potential, they will start to proliferate [355].

The proliferating cancer cells can then interact with their microenvironment and enhance the abnormal interactions. At this point, the tumour has become a different organ, with a distinct context that now defines all its cellular responses.

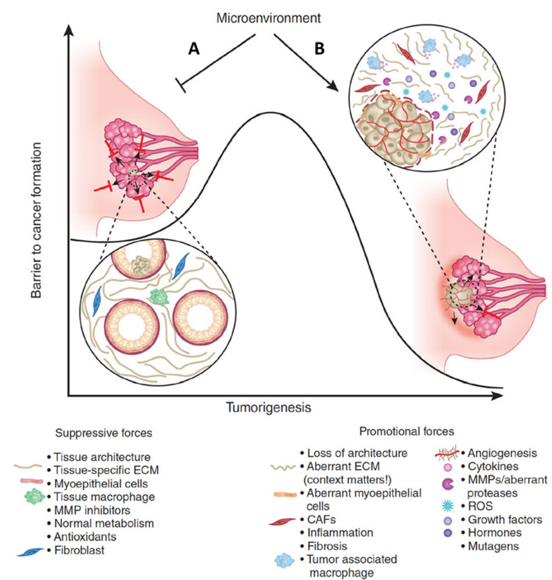


Figure 15. Microenvironment balance in normal breast and in breast cancer initiation and progression. *Taken from Bissell et al. 2011.*

4.1.2 Tumour stroma components

It is well established that the stroma associated with the normal mammary gland development is strikingly different from the one associated with carcinomas.

In the normal breast, the luminal epithelial microenvironment includes myoepithelial cells, basement membrane and the collective complex referred as stroma (fibroblasts, vasculature, immune cells, interstitial ECM and nerve fibers). Contrary, in invasive breast cancer, the myoepithelial cells and the basement membrane are essentially lost, and tumour cells are in direct contact with a remodelled interstitial stroma, which now include a higher number of infiltrating immune cells, such as lymphocytes, macrophages and mast cells [357].

In general, the breast cancer stromal microenvironment is also referred to as **reactive stroma**, and is composed of the ECM together with the non-malignant cells surrounding the tumour and recruited by tumoural cells.

Extracellular matrix

The ECM is composed of a large collection of biochemically distinct components including proteins, glycoproteins, proteoglycans, and polysaccharides with different physical and biochemical properties.

Structurally, these components make up both **basement membrane**, which is produced jointly by epithelial, endothelial, and stromal cells to separate epithelium or endothelium from stroma, and **interstitial matrix**, which is primarily made by stromal cells [358].

The basement membrane is a specialized ECM, which is more compact and less porous than the interstitial matrix. It has a distinctive composition containing type IV collagen, laminins, fibronectin, and linker proteins such as nidogen and entactin, which connect collagens with other protein components [358]. In contrast, the interstitial matrix is rich in fibrillar collagens, proteoglycans, and various glycoproteins such as tenascin C and fibronectin and is thus highly charged, hydrated, and contributes greatly to the tensile strength of tissues [359].

The ECM has many functions, including acting as a physical scaffold, facilitating interactions between different cell types, and providing survival and differentiation signals [360]. It's physical properties such as rigidity, porosity, insolubility, spatial arrangement and orientation determine its role in scaffolding to support tissue architecture and integrity [354]. Additionally, by functioning as a barrier, anchorage site, or movement track, the ECM's physical properties play both negative and positive roles in cell migration [361].

However, its biochemical properties allow cells to sense and interact with the microenvironment through various signal transduction cascades resulting in gene expression changes, altering cell behaviour [354].

Stromal cells

As exposed before, the tumour stromal compartment consists of an extracellular matrix and different types of cells which includes fibroblasts, immune and inflammatory cells, endothelial cells and adipocytes among others.

When compared to normal tissues, the stroma accompanying breast tumours contains an increased number of fibroblasts and immune cell infiltrates, enhanced capillary density, increased collagen I and fibrin deposition. All these changes alter the structure and stiffness of the ECM, inducing changes in the signalling within the adjacent epithelium [355].

Stromal populations may be subdivided in three groups: **angiogenic vascular cells** (including endothelial cells of the blood and lymphatic circulation), **infiltrating immune cells** (that also include a variety of bone marrow-derived cells (BMDCs), including macrophages, myeloid-derived suppressor cells (MDSCs), TIE2-expressing monocytes (TEMs) and mesenchymal stem cells (MSCs)) and finally **cancer associated fibroblastic cells** [55], **figure 16.**

Angiogenic vascular cells

The physiological process through which new blood vessels form from pre-existing vessels is named angiogenesis, and is an essential process for tumour growth and progression. First of all, biological signals as angiogenic growth factors activate receptors on endothelial cells present in the pre-existing blood vessels. Second, the activated endothelial cells begin to release enzymes called proteases that degrade the basement membrane to allow endothelial cells to escape from the original vessel walls. The endothelial cells then proliferate into the surrounding matrix and form solid sprouts connecting neighbouring vessels. Finally, as

sprouts extend toward the source of the angiogenic stimulus, endothelial cells migrate in tandem, using adhesion molecules called integrins [362].

Pericytes are contractile cells that wrap around the endothelial cells of capillaries and veins thorough the body. Recruitment of pericytes into tumours is crucially dependent on PDGFRβ expression, as well as PDGFβ production by endothelial cells [363].

Infiltrating immune cells (IIC)

It is known that most solid tumours contain infiltrates of diverse leukocyte subsets including both myeloid and lymphoid lineage cells, whose complexity and activation status vary depending on the tissue/organ location, stage and malignancy. In fact, many tumours show infiltration of immune cells that do not meet the classical definition of an inflammatory immune response. IIC supply the tumour with direct and indirect mitogenic growth mediators that stimulate proliferation of neoplastic cells, as well as other stromal cell types [55].

Macrophages function is to phagocytise cellular debris and foreign particles (including aberrant cancer cells), presenting antigens to T-cells during normal immune responses. Even though the macrophages in a normal tissue should be responsible for removing tumourigenic cells, a lot of evidences revealed that in tumours, macrophages act as active conspirators in cancer progression. Furthermore, it has also been shown that tumours can activate macrophages to take advantages from their abilities and functions [364]. In agreement with this, several studies have found that a high density of tumour associated macrophages (TAMs) correlates with poor prognosis and reduced survival in breast and other types of cancer [365].

Cancer associated fibroblastic cells.

Fibroblasts are the most abundant and active population of the breast stroma, and is becoming increasingly clear that fibroblasts are also prominent modifiers of cancer progression.

In a healthy mammary gland, fibroblasts functions include the deposition of ECM [366], regulation of epithelial differentiation [367] regulation of inflammation [368] and involvement in wound healing [369]. Interestingly, during wound healing they acquire a modified phenotype and become activated in a reversible manner. This activation results in a phenotype switch to a myofibroblast, an increased proliferation and secretion of ECM constituents [370]. Fibroblasts also play a crucial role in carcinomas, which Harold Dvorak firstly referred to as “wounds that never heal” [371].

These activated fibroblasts are known as cancer-associated fibroblasts (CAFs) and are thought to be involved in tumour growth and metastasis.

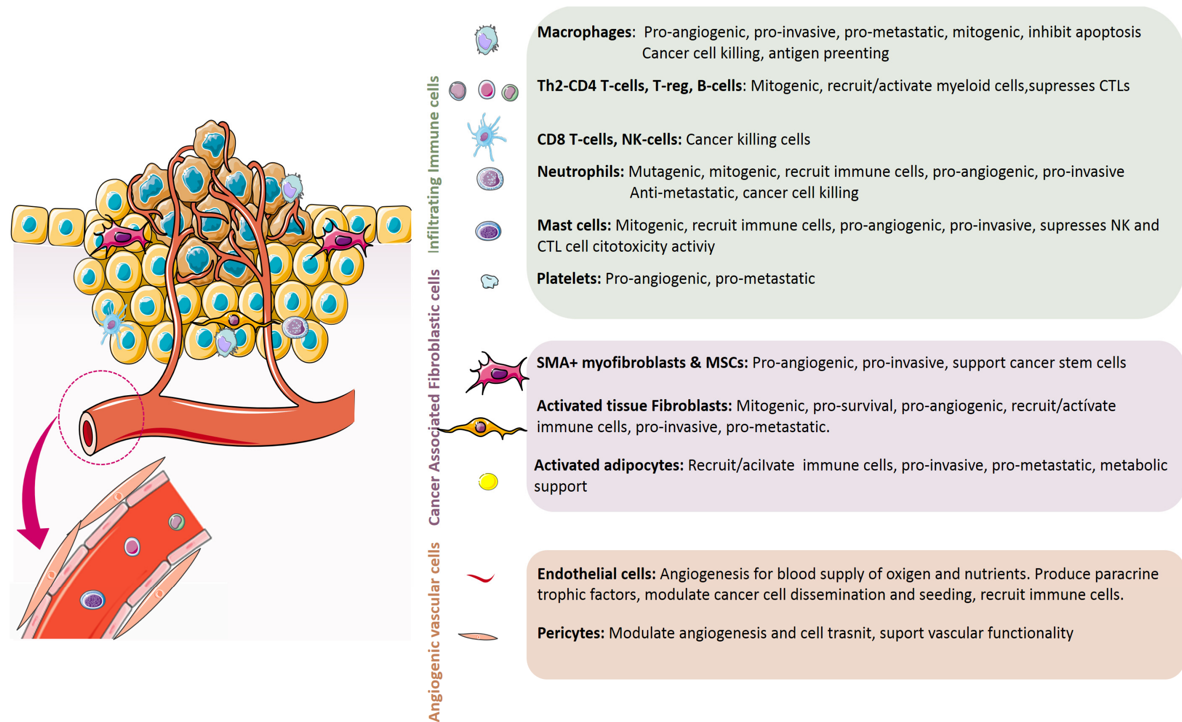


Figure 16. Primary tumour microenvironment representation, components and functions. *Adapted from Hanahan et al, 2012.*

4.1.3 Role of tumour stroma in cancer progression

It is known that all stroma components communicate with each other, and also with the neoplasic cells, contributing to tumourigenesis, and in some cases, it is thought that the trigger for neoplastic progression may come from signals within the stromal microenvironment [372].

Therefore, as mentioned above even though the normal stroma may protect the epithelium from tumourigenesis, aberrant stroma can also initiate tumourigenesis when the intercellular signals that define the normal context become disrupted. Alterations in epithelial tissues can lead to movement of epithelial cells and proliferation, for example, after activation of mesenchymal fibroblasts due to wounding [373].

A classic example of a stromal signal that can trigger neoplasms is chronic inflammation. In the past, the presence of leukocytes in tumours was generally thought to be a consequence of a failed attempt of cancer cells destruction. However, tumours are not only effective in escaping immune-mediated rejection, they also modify certain inflammatory cell types to render them tumour promoting rather than tumour suppressive [374]. In fact, many of these infiltrating immune cells may not be associated with the detection of cancer cell antigens, but may alternatively be associated with tissue disruption. [375].

For instance, TAMs support diverse phenotypes within the primary tumour, including growth, angiogenesis and

invasion, by secreting pro-tumourigenic proteases, cytokines and growth factors (for example, EGF, which participates in a paracrine signalling loop through tumour-secreted CSF-1). As tumours grow, immune-suppressor cells, including MDSCs and T-reg cells are mobilized into the circulation in response to activated cytokines that are induced by tumourigenesis (for example, TGF-β and CXCL5-CXCR2). MDSCs and T-reg cells infiltrate the growing tumour to disrupt immune surveillance through multiple mechanisms, including disruption of antigen presentation by DCs, inhibition of T and B cell proliferation and activation or inhibition of NK cell cytotoxicity [376].

Furthermore, it has been shown that VEGF has a central role in the emergence of the reactive stroma. VEGF can be released by cancer cells themselves, but fibroblasts and inflammatory cells are the principal host-derived source. VEGF induces microvascular permeability, which leads to the extravasation of plasma proteins such as fibrin, which in turn attract an influx of fibroblasts, inflammatory cells and endothelial cells. These cells produce an ECM that is rich in fibronectin and type I collagen, both of which activate tumour angiogenesis [377].

Some authors have proposed that stromal cells coevolved with cancer cells and undergo alterations in both, the genotype and the phenotype [378]. However, recent evidences suggest that mutations are limited to the tumourigenic cells, and that these modify the epigenetic program of other non-tumourigenic cells in the tumour microenvironment [378]. The cells in the microenvironment in turn produce epigenetic changes in the tumour cells, which will be reflected in their pattern of differentiation. These reciprocal interactions are illustrated by changes in the tumour microenvironment that occur during evolution of pre-invasive ductal carcinoma *in situ* to invasive carcinoma of the breast, and involve sequential epigenetic changes in the tumour stroma microenvironment as well [379, 380].

Then, is obvious that all these particular interactions between tumour cells and the associated stroma represent a powerful relationship that influences disease initiation and progression and patient prognosis.

CAFs, which become activated by tumour-derived factors (for example, TGF-β, FGF or PDGF, among others), secrete ECM proteins and basement membrane components that regulate differentiation, modulate immune responses and contribute to deregulated homeostasis. CAFs are also a key source of VEGF, which supports angiogenesis during tumour growth. In addition to cellular contributions, several extracellular properties contribute to tumour progression, including low oxygen tension, high interstitial fluid pressure and changes in specific components of the ECM [376], **figure 17**.

There are also accumulative evidences suggesting that the microenvironment has also an important role in supporting metastatic dissemination and colonization at secondary sites. Organ tropism, more classically known as **the seed-soil hypothesis,** was first proposed by Stephen Paget in 1889, when he concluded that the distribution of metastases was not random,

displaying instead a clear organ preference. Paget's hypothesis later evolve into the idea that before metastatic dissemination, primary tumours secrete factors that contribute to the development of a pre-metastatic niche, which is characterized by an abundance of BM-derived cell types, increased numbers of fibroblasts and secreted oncoproteins and cytokines that render the secondary environment receptive to tumour growth [372].

In addition, several components of the tumour stroma can modulate migration and invasion. For instance, macrophages and platelets and myeloid derived cells (MSCs) contribute to the EMT at primary sites, allowing tumour cells to separate from neighbouring epithelial cell-cell contacts and acquire a mobile and invasive phenotype. One major mediator of this event is transforming growth factor β (TGF-β), which is secreted by the tumour stroma and participates in a paracrine signalling loop with tumour cells [381]. TAMs, CAFs and myeloid progenitor cells also tend to cluster at the invasive or leading edge of the primary tumour, where they have an immunosuppressive role by interfering with dendritic cells (DC) differentiation [382].

During intravasation of tumour cells into the circulation, intravital imaging studies have shown that macrophages are localized in perivascular areas within tumours, where they help tumour cells to cross vessel barriers [365, 374]. In circulation, platelets and other components of the coagulation system support tumour cell survival by protecting them from cytotoxic immune cell recognition [46]. Platelets guide tumour cells in circulation to the site of extravasation, where they bind to areas of vascular retraction and help tumour cells exit the circulation into secondary organs [362]. At secondary sites such as the lung, fibroblasts upregulate fibronectin, which serves as a docking site for endothelial progenitor cells (EPCs) and the subsequent arrival of tumour cells [383-385].

Immunosuppressive cell types, MDSCs and NK cells, also populate pre-metastatic niches, where they help direct metastatic dissemination by creating a niche that is permissive to tumour colonization [382].

Furthermore, recent studies have demonstrated that primary and secondary sites can communicate through exosomes, shed not only by primary tumour cells but also by immune and stromal cells such as NK cells, CAFs and DCs [386]. Factors contained in exosomes have the capacity to direct organ tropism, modulate immune evasion and support the MET and are also predictive of metastasis and patient outcome [372].

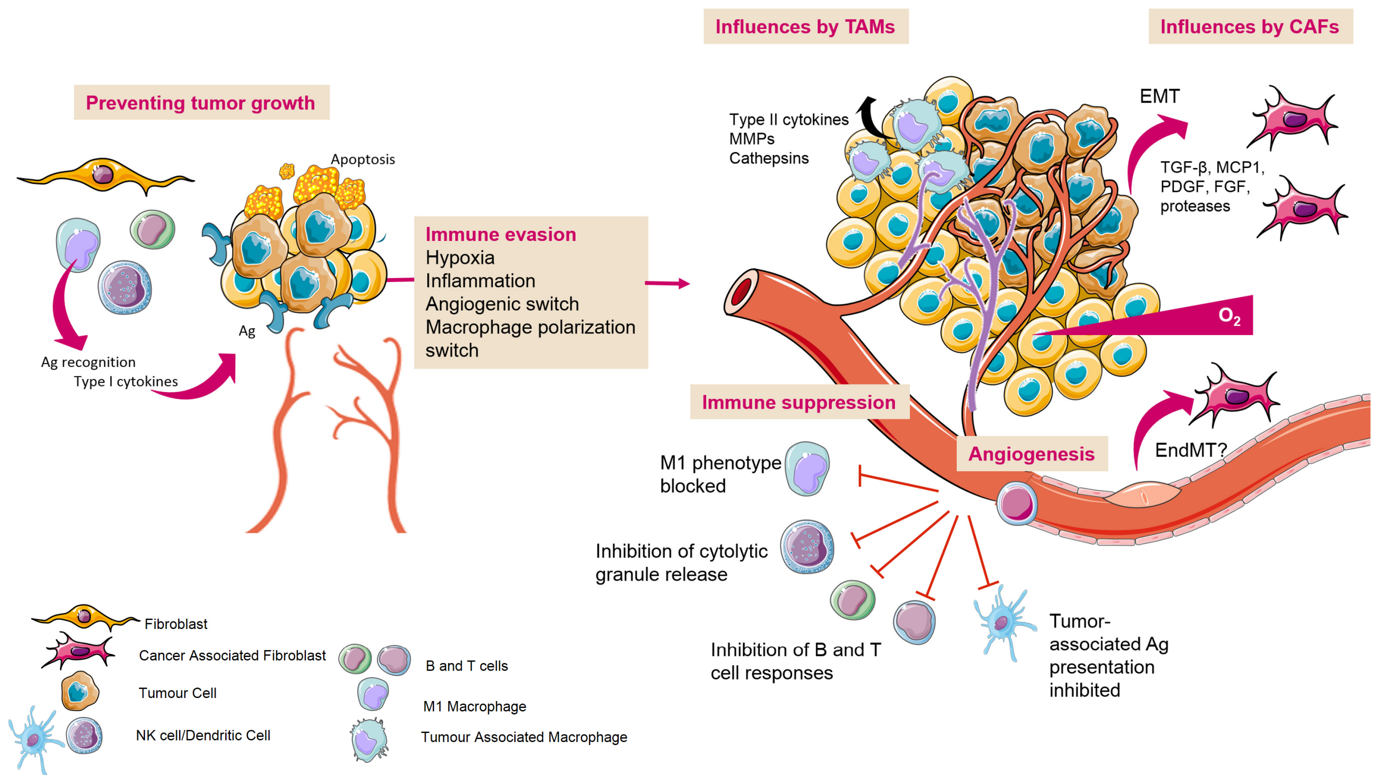


Figure 17. Representation of the tumourigenic primary niche. multiple stromal cell types converge to support the tumourigenic primary niche. After avoiding cell-intrinsic mechanisms of apoptosis, tumour cells are subject to elimination pressures by the immune system. Tumour cell specific antigens have an important role during this process, which are recognized by cytotoxic immune cells, leading to their destruction. Fibroblasts and macrophages within the TME also contribute to a growth-suppressive state; however, these cells may later become educated by the tumour to acquire pro-tumourigenic functions *Adapted from Joyce et al, 2013*.

4.2 FIBROBLASTS AND BREAST CANCER

4.2.1 Structure and function of fibroblasts

In normal conditions, fibroblasts are embedded within the fibrillary ECM of connective tissue and interact with their surrounding microenvironment through integrins such as α1β1 integrin. Fibroblasts are to a large extent, responsible for the synthesis of the constituents of the fibrillary ECM such as type I, type III and type V collagen, and fibronectin [373].

Fibroblasts more important functions include the deposition of ECM, regulation of epithelial differentiation and regulation of inflammation [387].

Moreover, they also contribute to the formation of basement membranes by secreting type IV collagen and laminin [388]. Fibroblasts are also an important source of ECM-degrading proteases such as MMPs, which highlights their crucial role in maintaining the ECM homeostasis by regulating the ECM turnover [389].

In addition, fibroblasts are important in maintaining the homeostasis of adjacent epithelia through the secretion of growth factors and direct mesenchymal–epithelial cell interactions [390].

Furthermore, fibroblasts have also a prominent role in wound repair. They invade lesions, generate new ECM that serves as a scaffold for other cells, and have cytoskeletal elements that facilitate contractions of healing wounds [373].

Fibroblasts are elongated cells with extended cell morphologic progressions that show a fusiform or spindle-like shape, being easily identified in tissue sections (however, fibroblasts are poorly defined in molecular terms), and unlike other cell lineages, we lack molecular markers for fibroblasts. There are several well-established indicators of the fibroblast phenotype, but none of them is both exclusive for fibroblasts and present in all fibroblasts [391] (**Table 8**).

Fibroblast specific protein-1 (FSP1), seems to provide the best specificity for detecting fibroblasts *in vivo,* while α-smooth muscle actin (αSMA) stained for activated fibroblast or myofibroblasts [384].

Sugimoto and colleagues identified several subpopulations of CAFs with varying overlap of expression of markers such as FSP1, αSMA, the platelet-derived growth factor receptor-β (PDGRβ) and NG2 [392]. Moreover, some markers can be considered as site-specific indicators, as desmin, a specific marker for skin fibroblasts [393, 394].

Fibroblasts in mammals are then highly heterogeneous, and those isolated from different sites reflect a substantial topographic diversity. Chang H *et al*. showed that the molecular profile among fibroblasts populations from 50 human fibroblasts cultures isolated form 16 different sites were distinct [384, 390]. Such diversity is evident due to the specific and differential secretion of ECM constituents, growth factors or differentiation factors in each anatomical site [390].

MARKER	FUNCTION	FIBROBLASTS TYPE IN WHICH IT IS FOUND	OTHER CELL TYPES EXPRESSING IT
Vimentin	Intermediate filament associated protein	Miscellaneous	Endothelial cells, myoepithelial cells and neurons
α-Smooth muscle actin (αSMA)	Intermediate filament associated protein	Myofibroblasts and activated fibroblasts	Vascular smooth muscle cells, pericytes and myoepithelial cells
Desmin	Intermediate filament associated protein	Skin fibroblasts	Muscle cells and vascular smooth muscle cells
FSP1	Intermediate filament associated protein	Miscellaneous	Invasive carcinoma cells
Fibroblast activation protein (FAP)	Serine protease	Activated fibroblasts	Active melanocytes
Discoidin-domanin receptor 2	Collagen receptor	Miscellaneous	Endothelial cells
α1β1 integrin	Collagen I biosynthesis	Miscellaneous	Monocytes and endothelial cells
Procollagen I α2	Collagen receptor	Miscellaneous	Osteoblasts and chondroblasts

Table 8. Fibroblasts markers. *Adapted from Kalluri et al, 2006.*

4.2.2 Origin and heterogeneity of cancer associated fibroblast and fibroblast activation

The fact that fibroblasts within the tumour stroma acquire a modified phenotype, similar to fibroblasts associated with wound healing, has been studied since 1970s. As mentioned before, such activated fibroblasts within the tumour stroma have been termed cancer associated fibroblasts (CAFs) [53]. CAFs are the most prominent cell type within the tumour stroma of many cancers, most notably breast, prostate and pancreatic carcinoma [363]. In fact, in breast carcinomas, about 80 % of stromal fibroblasts are thought to acquire this activated phenotype [395].

In culture, phenotypic features of CAFs can be induced by TGFβ, which mediates fibroblast activation during wound healing and organ fibrosis [396]. Fibroblasts activation is induced by various stimuli that arise when tissue injury occurs, and a part

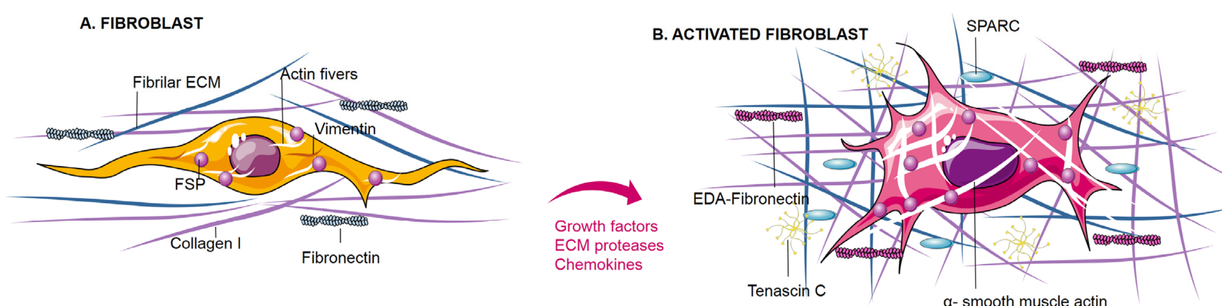


Figure 18. Fibroblast activation. *Adapted from Kalluri et al, 2007.*

from TGFβ, other growth factors like EGF, PDGF and FGF2 play an important role in this activation. These factors are also released from injured epithelial cells and infiltrating mononuclear cells such as monocytes or macrophages [397].

In addition, fibroblasts are activated by direct cell-cell communication and contacts with leukocytes through adhesion molecules such as the intercellular-adhesion molecule 1 (ICAM1) or the vascular-adhesion molecule 1 (VCAM1) [384], **figure 18.**

Fibroblast activated phenotype is associated with an increased proliferative activity and enhanced secretion of ECM proteins (type I collagen, tenascin C, fibronectin and SPARC). Phenotypically, activated fibroblast are often characterized by αSMA expression. Activated fibroblasts often secrete increased amounts of growth factors such as hepatocyte growth factor (HGF), insulin-like growth factor (IGF), nerve growth factor (NGF), WNT1, EGF and FGF2, which can induce proliferative signals within adjacent epithelial cells [383].

CAFs remain activated even when the initial inducement signal has cessed. In this sustained state of activation, fibroblasts continue to secrete ECM constituents growth factors and cytokines, which result in a self-perpetuating autocrine loop, which stimulates other fibroblasts [384].

In tumours, CAFs are proposed to originate from different cell types including pre-existing fibroblasts, pre-adipocytes, smooth muscle cells, endothelial cells, epithelial cells and bone morrow derived progenitors [398-401].

Orimo *et al*. and Bissell *et al.*, have proposed different models to explain the origin of CAFs within the tumour that are illustrated in **figure 19.**

1. Resident CAFs may be originated by activation of local fibroblasts, by cancer-derived growth factors. This trans-differentiation is completed without the acquisition of any genetic alteration.
2. Bone marrow derived cells, such as fibrocytes and mesenchymal stem cells (MSCs), are recruited into the tumour stroma where they are able to differentiate into CAFs [402-404].
3. It has been also proposed that either stromal epithelial cells or carcinoma epithelial cells can go through an EMT process, adopting a mesenchymal cell phenotype, characterized by an enhanced migratory capacity and invasiveness [400]. This process is characterized by the loss of endothelial markers such as CD31, and the expression of mesenchymal markers like FSP1 and αSMA, again under the influence of TGFβ [405].
4. Finally, acquisition of genetic alterations (*TP53* and *PTEN* mutations) within a small population of fibroblasts and/or progenitors may allow their clonal selection and expansion. In this context, p53 inactivation in stromal fibroblasts, as well as the genetic inactivation of PTEN in CAFs enhances tumour progression in breast carcinomas [378]. Some studies have reported high frequency (30 %) of somatic alterations in tumour juxtaposed fibroblasts [56], while recent studies have shown that genetic alterations were detected only in cancer epithelial cells but not in the stroma [406].

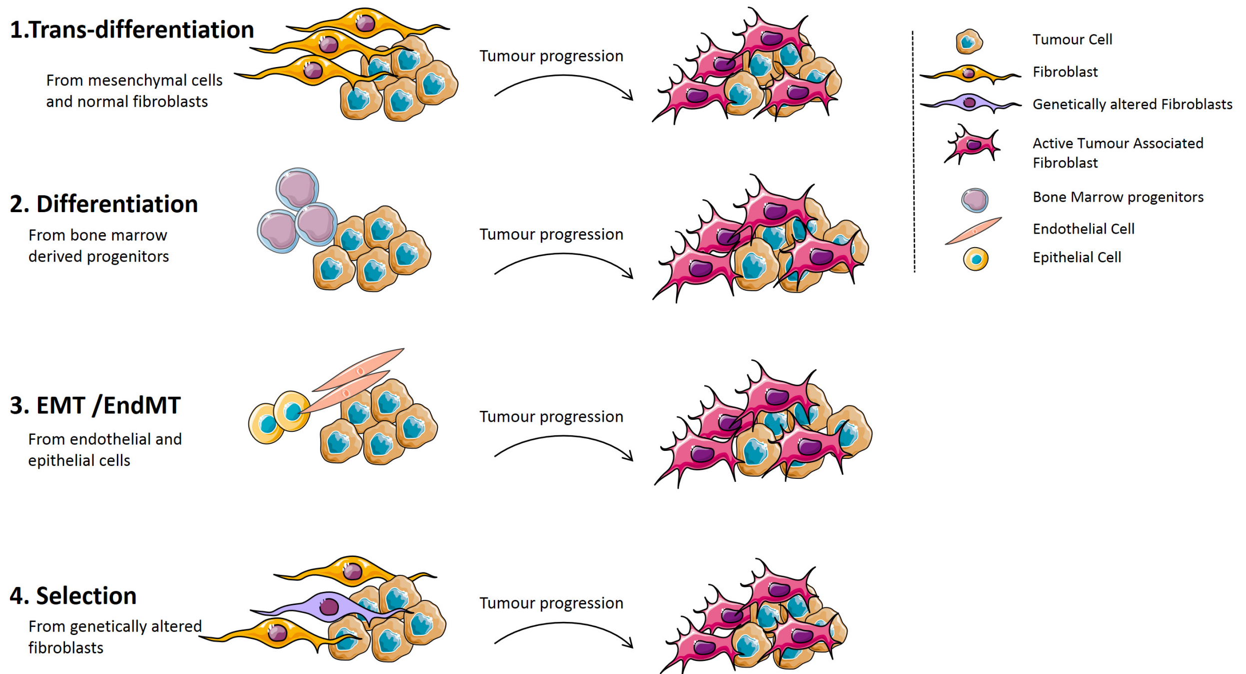


Figure 19. Possible models for generation of carcinoma associated fibroblasts. *Adapted from Orimo et al, 2010 and Bisell et al, 2006.*

4.2.3 Role of CAFs in breast cancer progression

As exposed before, the neoplastic epithelial cancer cells and the cells of the tumour microenvironment are in constant crosstalk and there is an active recruitment of stromal cells into the tumour. Stromal cells produce additional tumour growth factors, cytokines, chemokines and matrix metalloproteinases that contribute to tumour progression [365], and in the same way, cancer cells can produce "stroma-modulating growth factors" that modify the stroma to provide themselves with a supportive microenvironment for tumour progression [373].

All this suggests that the tumour stroma can have prognostic and therapeutic significance in breast cancer.

Orimo *et al.* and Weinberg *et al.* demonstrated that fibroblasts present in mammary carcinomas are very different from mammary stromal fibroblasts located outside the tumour masses and from fibroblasts from healthy stroma in several important functional aspects [407]:

1. CAFs from invasive human breast carcinomas are more competent than normal fibroblasts in enhancing tumour growth.
2. CAFs include larger populations of myofibroblasts, which exhibit high levels of SMA expression and increased collagen contractibility.
3. CAFs release increased levels of stromal cell-derived factor 1 (SDF-1) which is responsible for recruiting endothelial progenitor cells (EPCs) into the tumour mass, thereby boosting tumour angiogenesis. SDF1 secreted from CAFs

also enhances tumour growth by direct paracrine stimulation via the CXCR4 receptor in breast cancer cells [408].

4. Both, the tumour-enhancing and myofibroblastic properties of CAFs are stably retained by CAFs in the absence of direct and continous contact with breast carcinoma cells.

Then is obvious that fibroblasts can regulate different aspects of tumour biology and therefore play an important role in the different stages of breast tumour progression. Similarly to immune cells, which initially repress malignant growth, fibroblasts inhibit early stages of tumour progression, mainly through the formation of gap junctions between activated fibroblasts [409].

Conversely, later on fibroblasts become activated and CAFs promote tumour growth and progression, by the secretion of growth factors, cytokines and proteases, which leads to immune cell infiltration that in turn promotes angiogenesis and metastasis [410].

Classical mitogens for epithelial cancer cells, such as HFG, EGF, FGF2, as well as cytokines such as SDF1 and IL6 are vastly expressed by CAFs when contacting tumour cells. Also pro-inflammatory cytokines, such as interleukins, interferons and members of the tumour necrosis factor family are produced both by stromal and cancer cells [411]. Furthermore, CAFs also are able to secrete plasminogen activators as well as several members of the MMP family. This enzymes may be then exploited for mainly two purposes: directly degradation of ECM (associated with tumour expansion, invasion and angiogenesis), or cleavage of growth factors, pro-inflammatory cytokines and their receptors (commonly associated with their activations), as well as cell adhesion molecules leading to increase motility and EMT processes [412].

Moreover, the presence of larger numbers of CAFs in the stroma of different human cancers is associated with an increased risk of invasion and metastasis, and poor clinical prognosis [413].

4.3 THERAPEUTIC IMPLICATIONS OF TUMOUR STROMA

4.3.1 Tumour stroma as a prognostic factor: mammographic density

Mammographic density (MD) refers to the relative abundance of low-density adipose tissue to high–density glandular and fibroblastic stromal tissue within the breast. Since the concept was first described in the 1970's, it has become clear that MD is an important risk factor for the development of cancer [414].

Each type of breast tissue reacts differently to x-rays. Fatty breast tissue is relatively translucent, allowing x-rays to pass through showing dark areas on a mammogram. Epithelial and stromal tissues, on the other hand, block x-rays and appear as white areas. Breast lesions are not easily discernible in these areas since dense tissue and tumours both look white on the film (**Figure 20**) [415].

Breast density is positively associated with tumour size, lymph node status and lymphatic or vascular invasion, and it is thought that in women with dense breast tissue, detection of tumours is more difficult, and also tumours might grow quickly between examinations. In fact, patients with mammographically dense tissues (with 60 % or more dense component) have three to five increased relative risk for breast cancer [92].

Mammographically dense tissues are associated with increased collagen I tissue deposition, and a recently described mouse model indicated that higher collagen levels in the mammary gland increases tumour formation and invasive behaviour, suggesting a manner in which areas of dense tissue may be tumour promoting [389].

Moreover, expression profiling has been used to identify stromal signatures with predictive value for breast cancer. By using laser-capture microdissection to isolate tumour-associated stroma, a 26-gene stroma-derived prognostic predictor was generated and found to be predictive for relapse-free survival. Genes associated with poor survival were involved in hypoxic and angiogenic responses within the tumour as well as tumour-associated macrophage immune response [379].

Furthermore, another microarray study of the tumour stroma showed an association of a reactive stromal gene signature (suggestive for large stromal content within the tumour) with resistance to neoadjuvant chemotherapy [416].

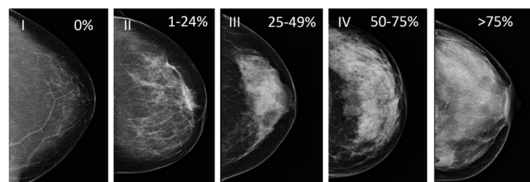


Figure 20. Mammographic images representing different density degrees. I. Almost entirely fatty, II. Scattered areas of fibroglandular density. III. Heterogeneously dense, IV. Extremely dense. *From www.mcancer.org*

4.3.2 Tumour stroma and drug resistance

Ideally, cancer treatment would eliminate all malignant cells in order to avoid relapse. However, even after complete responses, (evidenced by the absence of macroscopic lesions), a small but significant number of cancer cells often survive chemotherapy. These surviving cells constitute minimal residual disease and represent a potential valuable diagnostic test to predict therapeutic outcomes, and especially the probability of relapse [417].

There are two concepts, that highlight the role of the genetic variability in the generation of drug resistant subpopulations within the tumour mass and their subsequent selection and enrichment after treatment, and both of them may be occurring in the same tumour.

The intrinsic resistance model, can be explained by the asymmetrically division of cancer stem cells. This will produce a heterogeneous population of cells that compose a tumour while maintaining a drug resistant population of stem-like cells through self-renewal. Cancer stem cells may be then the source of this heterogeneity, and thus the cancer stem cells constitute the so-called drug resistant population [418].

***De novo* acquired resistance** suggests that cancer cells can undergo an epithelial-mesenchymal transition leading to the acquisition of stem cell properties [419], also epigenetic changes caused by extrinsic microenvironmental, persistent or temporary pressures may induce DNA methylation changes or alterations in some chromatin binding proteins leading to modifications in gene expression patterns that facilitate tumourigenesis and/or a drug resistant phenotype [420].

Some models propose that this stepwise acquisition and accumulation of mutations take a long time, suggesting that cells destined to develop genetic alterations first require a sustained protection from the toxic effects of the drug through a non-genetic mechanism prior to the acquisition of a drug resistant phenotype. Within this concept, the drug resistant cell population will then be protected by the tumour microenvironment (or selected by the environmental pressures during treatment) that will provide a nurturing niche in which the cells could undergo mutagenesis or epigenetic changes to become drug resistant [418].

Then, the microenvironment is expected to play a role in assisting these cells to survive until the tumour becomes effectively drug resistant. These resistance is known as **environment mediated drug resistance (EMDR).** EMDR can be divided in two categories: soluble factors mediated resistance and cell adhesion-mediated resistance (CAM-DR). This two interact in a cooperative way, as for instance, soluble factors may induce the expression of cellular adhesion molecules initiating a positive feedback loop and amplifying the resistance [421].

EMDR development process includes three stages, where the two concepts described before take part. The first step involves homing or attraction and requires specific cell-cell or cell-extracellular matrix interactions (**Figure 21A**). Soluble stromal factors, such as SDF-1 and IL-6, as well as

receptors mediating adhesion, will contribute to attract tumour cells to the stromal niche where the tumour will be established. This step is often seen in primary bone marrow tumours as well as in secondary metastatic tumour establishments, but may not be necessary for primary solid tumour development [422].

In the second stage, *de novo* resistance (first stage for primary tumours that are not established in the bone marrow), the stress from the treatment is applied to the naïve tumour (**Figure 21B**). This step is characterized by a series of cell responses and the modification of the composition of the ECM creating a positive feedback loop that amplifies the pro-survival and anti-apoptotic signals. Finally, the last step is the acquired resistance, where also the microenvironment can act as a barrier that physically or biochemically prevents the effective access of drugs to the tumour cells (**Figure 21C**) [417].

In view of the central role that CAFs play in the biology of breast cancer, it is likely that CAFs may also be important for the survival of tumour cells after treatment. It is known that stromal fibroblasts can influence chemosensitivity of tumour cells indirectly, by producing and activating ECM molecules. For instance, this activated ECM confers chemoresistance by integrin mediated adhesion to fibronectin [423].

In addition, co-cultured experiments and xenograft models have demonstrated that the efficacy of chemotherapy-induced cell cycle arrest or senescence in stromal fibroblasts is critical for the sensitivity of the tumour compartment to chemotherapy [424].

Increasing evidences revealed that CAFs can cause endocrine, chemotherapy and targeted therapeutic resistance [425-427]. For example, collagen type I secretion by CAFs decrease chemotherapeutic drug uptake in tumours which leads to increases intratumoural drug intake and inhibits primary tumour cell proliferation and metastasis in multidrug-resistant murine breast cancer models [428]. Also pre-clinical studies indicate that CAFs might also

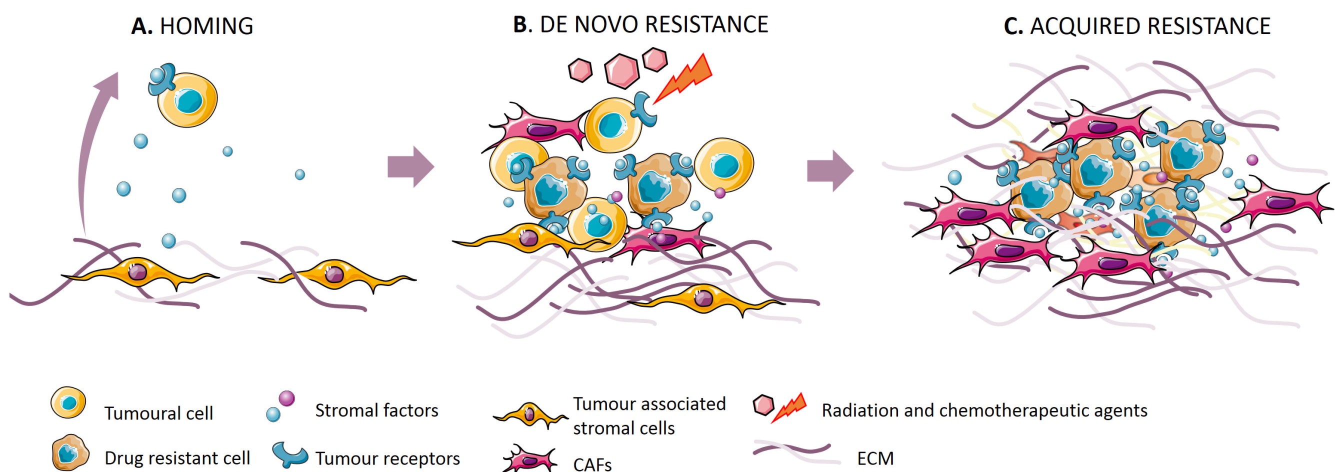


Figure 21. Stages during the development of environment-dependent drug resistance. *Adapted from Cuckierman et al, 2012.*

mediate resistance to anti-angiogenic therapy and tyrosine kinase inhibitors targeted therapy [377, 429].

Better understanding of the effects of the tumour microenvironment on cancer cells before, during and immediately after chemo- or radiotherapy is imperative to design new therapies aimed at targeting this tumour-protective niche.

4.3.3 Tumour stroma as a targeted therapy

As seen before, the development of drug resistance can depend on the genetic stability of target cells, and so, targeting stromal cells, that are genetically more stable than tumour cells has been proposed to be more beneficial therapeutically, since the possibility of acquiring drug resistance would be lower [430]. Then, a better understanding of the features underlying tumour heterogeneity regarding the tumour microenvironment, as well as the mechanisms and consequences of their interactions is essential to improve existing therapies and to develop novel agents.

One of the most active targeted microenvironment component has been the angiogenic system, with monoclonal antibodies like Bevacizumab, and tyrosine kinase inhibitors such as Sorafenib or Sunitinib. In fact, targeting VEGF in the clinic is showing clinical efficacy in metastatic breast and colon cancers in combination with chemotherapy [431]. In this context, multiple strategies to target the TME are either currently in clinical use, and some others are at different stages of clinical development. Some of them are summarized in **figure 22** [376].

Focusing on **fibroblasts**, several aspects of the biology of CAFs suggest that targeting these cells (in conjunction with the malignant epithelial cells) may be helpful to make anti-cancer therapy more effective.

In this context, different strategies have been proposed, some of them are summarized in **figure 23**, and include:

1. Targeting signals responsible for the activation of fibroblasts: the key role of PDGF and TGFβ signalling pathways in fibroblast activation has resulted in the development of inhibitors against these molecules. Imatinib inhibits PDGF-receptor tyrosine kinase and is used in the treatment of chronic myeloid leukaemia and other types of cancers [432]. Promising inhibitors of TGFβ signalling (ligand, ligand-receptor interaction and intracellular signalling level) have entered in clinical trials and shown encouraging results [381].

2. Targeting fibroblasts specific molecules that initiate and promote tumour growth: an example of this approach is the use of the humanised monoclonal antibody Sibrotuzumab against the fibroblast activation protein (FAP). FAP is a membrane bound glycoprotein with serine protease activity, that is highly expressed in the tumour stroma, and that has been shown to enhance tumour growth *in vivo* [433]. Efficacy has been shown in phase I studies in colorectal cancer [434]. The serine-protease activity of FAP has been also exploited to activate pro-toxins in the vicinity of the tumour, and vaccines have been developed to generate an immune reaction to the FAP antigen [428, 435].

3.Eliminating activated CAF subpopulations: efforts are being made to determine the differential transcriptional profile of normal fibroblast compared to CAFs in a variety of malignancies in order to identify unique transcriptional signatures. This will then provide new therapies aimed towards CAFs specific elimination.

The recognition of the active role that CAFs play in carcinogenesis adds a new level of complexity to cancer biology but also brings an opportunity for new therapeutic strategies. At the same time, pharmacological and biological agents that interfere with the signalling between the malignant epithelial cells and the supporting CAFs will likely continue to be tested [432].

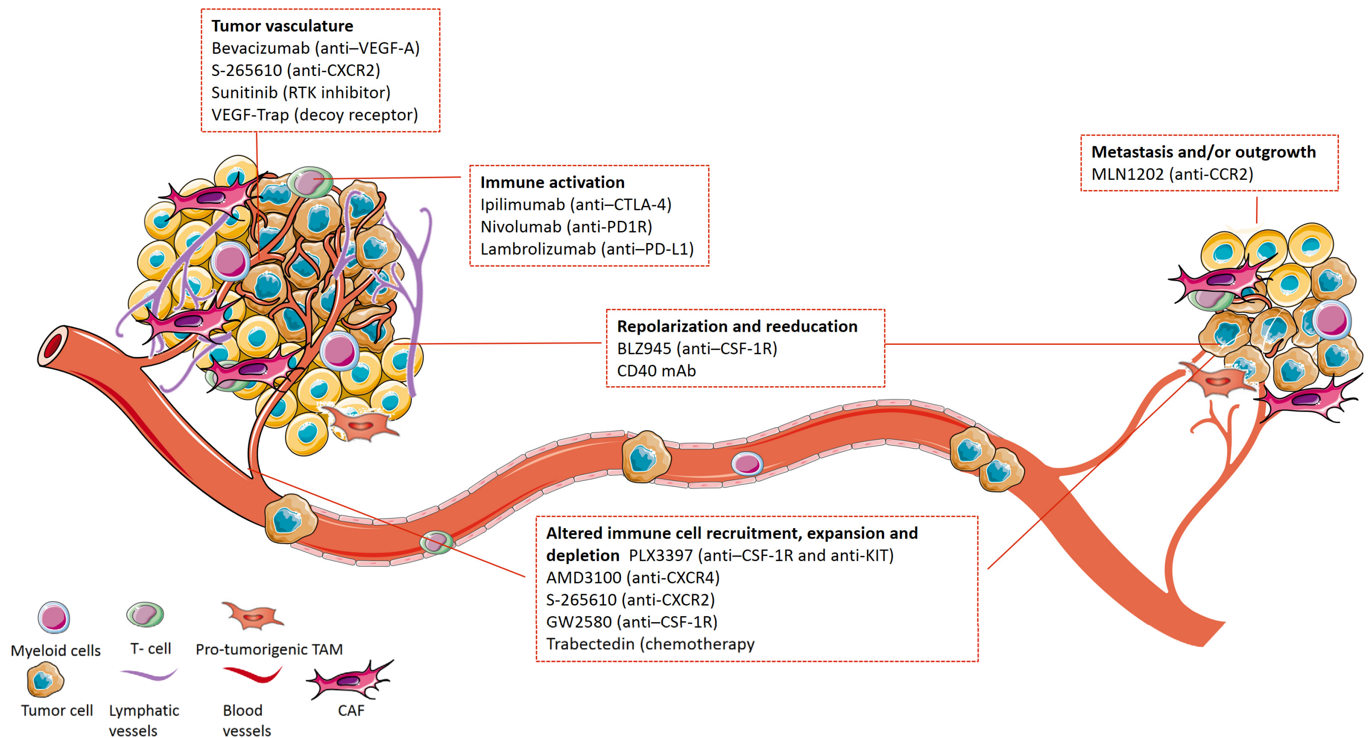


Figure 22. Stroma targeting current strategies. Immune activation, marked by an induction of T cells, is also a promising road of therapeutic intervention. This can be achieved through blockade of CTLA-4 (Ipilimumab), PD1 receptor (Nivolumab) or PD-L1 (Lambrolizumab). Repolarization or re-education of cells within the TME, in particular, macrophages or other myeloid cells, can be achieved by CSF-1R inhibition (for example, BLZ945) or monoclonal antibodies that activate CD40. Alternatively, immune cell recruitment and expansion can be blocked through inhibition of critical cytokine axes, such as CXCR4 (AMD3100), CXCR2 (S-265610), CSF-1R and/or KIT (PLX3397), and the chemotherapeutic agent Trabectedin, whose anti-tumour activity is proposed to be the result of selective depletion of monocytes and/or macrophage. Equally, metastatic seeding and outgrowth can be blocked by inhibition of key cytokine receptors, such as CCR2 (MLN1202). *Adapted from Joyce et al, 2013.*

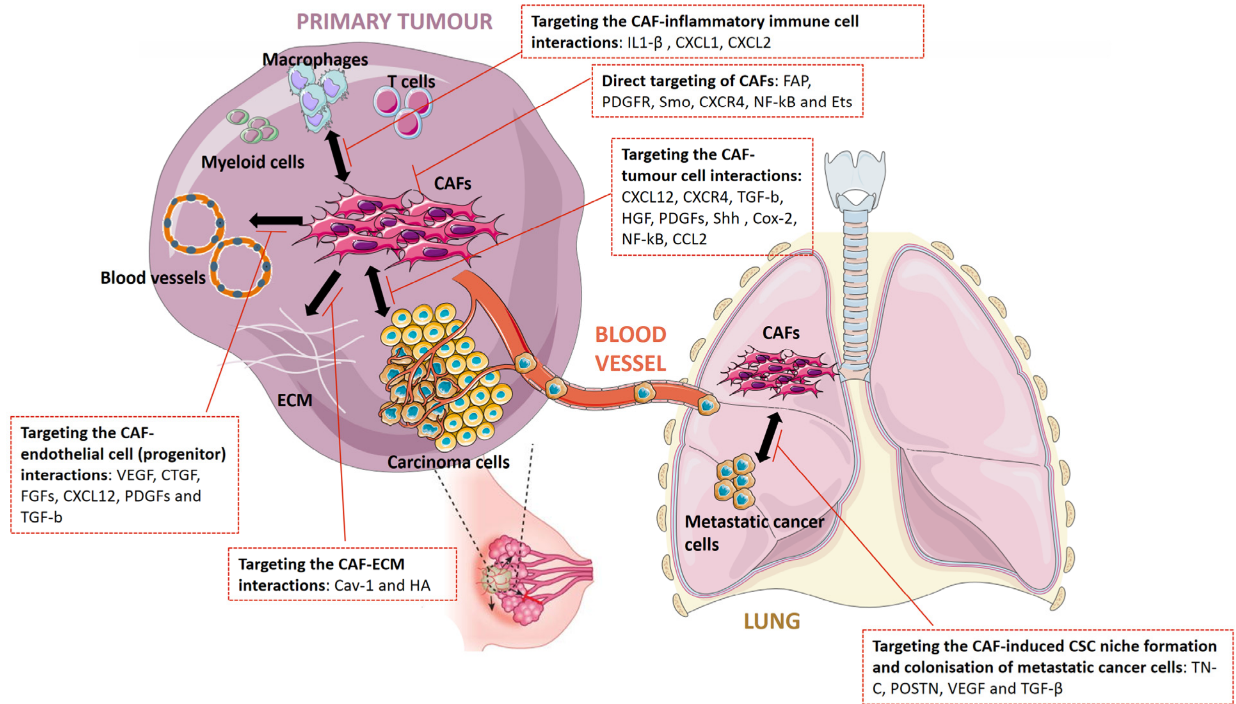


Figure 23. Schematic representation of approaches targeting interactions of CAFs with other tumour-constituting cells during tumour progression. *Adapted from Orimo et al, 2014.*

5. AIM OF THE STUDY

As exposed before HER2 positivity defines a molecular subtype of breast cancer (BC) with an aggressive biological behaviour and poor prognosis. Initially the pharmacological targeting of the tyrosine kinase receptor (TRK) HER2 with the monoclonal antibody Trastuzumab, and later on with the small tyrosine kinase inhibitor Lapatinib, changed the prognosis for HER2-positive breast cancer patients.

However, despite the positive results from many trials, resistance to anti-HER2 agents occurs in both metastatic and adjuvant settings, in part, because HER2 represent just a part of a more complex biological network, that when deregulated, plays a central role in sustaining the aggressive phenotype of tumour cells.

For these reasons there is an urgent need to identify new markers of response for these drugs as well as for discovering new therapeutic targets for those non-responding patients.

Moreover, the importance of the tumour microenvironment in drug resistance has been recognized during the last years, and it is now widely accepted that cancer develops as a result of genetic and epigenetic alterations in clonal cells, but that growth, survival and metastasis (as a consequence of treatment failure), are regulated by stromal-cancer cell interactions [425, 428].

Then, it is likely that tumour stroma, and cancer associated fibroblasts (CAFs) in particular, also influence therapeutic outcome, as well as provide new molecular targets with therapeutic potential. In fact, pre-clinical studies have indicated that CAFs can mediate resistance to anti-angiogenic therapy and targeted therapy using tyrosine kinase inhibitors [426], and further evidences of their important role were provided by the publication of a CAF gene signature predictive of response to neoadjuvant therapy in BC [53, 416].

Therefore, our hypothesis is that therapeutic treatments can favour the clonal selection of cells with unique properties and different fitness for a given microenvironment, and that the tumour cells can induce changes in the structure and composition of the microenvironment to support their own growth, progression and dissemination.

Our main aim is to study the role of the microenvironment and in particular, cancer associated fibroblasts in the acquisition of HER2 therapy resistance and to identify new therapeutic targets of recurrence. The main objective of the present work is subdivided in two main sections and objectives:

1. Identification of HER2-independent pathways related to tumour escape to anti-HER2 therapies (Trastuzumab and Lapatinib), as well as new therapeutic targets for non-responding patients.

2. Study the role of tumour stroma in therapy resistance acquisition:

2.1 Study if clonal selection induced by Trastuzumab and Lapatinib favours the outgrowth of cells with different microenvironmental crosstalk capabilities.

2.2 Study the role of fibroblasts in the establishment of the drug resistant phenotype, as well as in tumour progression and evolution.

6. MATERIALS AND METHODS

6.1 CELL CULTURES

6.1.1 Cell lines culture

The following BC cell lines with different HER2 background were purchased from the American Type Culture Collection (ATCC, Rockville, MD): MDA-MB-453 (metastatic carcinoma from pericardial effusion, HER2 overexpression, without amplification of 17q12), SK-BR-3 (adenocarcinoma from pleural effusion with 17q12 amplification) and BT-474 (ductal carcinoma with 17q12 amplification, and positive for oestrogen and progesterone receptors). Cells were cultured following ATCC instructions: incubated at 37 ºC in a humidified 5 % CO_2 atmosphere in DMEM-F12 growing medium, supplemented with foetal bovine serum (FBS) 10 %, Glutamax 5 % and Fungizone-Penicillin-Streptomycin mixture 5 %. For the BT-474 cell line, 10 µg/ml of insulin was added to the media.

The authenticity of all cell lines used in this study was validated by single locus short tandem repeat (STR) typing (Bio-Synthesis, Inc.).

All cell lines were infected with a lentiviral pBabe-Puro construct encoding a puromycin resistant gene, using Extremegene, in a relation 1:6 (DNA: transfection reagent). After infection, cells were cultured in the presence of puromycin (range of concentration summarized in **Table 9**) to select the cells that have incorporated the vector, and finally mCherry-LUC+ cells were sorted by flow cytometry.

CELL LINE	PUROMYCIN µg/mL)
SK-BR-3	2,5
SK-BR-3 TRASTUZUMAB resistant	3
SK-BR-3 LAPATINIB resistant	3
BT-474	4
BT747 TRASTUZUMAB resistant	4
BT-474 LAPATINIB resistant	4
MDA-MB-453	2,5
MDA-MB-453 TRASTUZUMAB resistant	3
MDA-MB-453 LAPATINIB®	3

Table 9. Puromycin resistant concentration

6.1.2 Generation of drug resistant cell lines

The generation of resistance was achieved by continuous exposure of the different mCherry/LUC+ cell lines MDA-MB-453, BT-474 and SK-BR-3 to increasing concentrations of different drugs (50-200 µg/ml for Trastuzumab and 0.5-4 µM for Lapatinib) and by the selection of the cells surviving after each round of treatment for over at least 5 months. Experiments were performed with the drug resistant population obtained from this process instead of using single clones to better mimic the normal process by which a primary tumour develops resistance and to

maintain some degree of intratumour heterogeneity.

The drug resistant cell lines were cultured as mentioned before, in DMEM-F12 media supplemented with Trastuzumab 100 µg/ml or Lapatinib 2 µM) to avoid the expansion of non-resistant cells (**Figure 24**).

Cells were drug-starved overnight or for 24 h before running experiments unless otherwise specified.

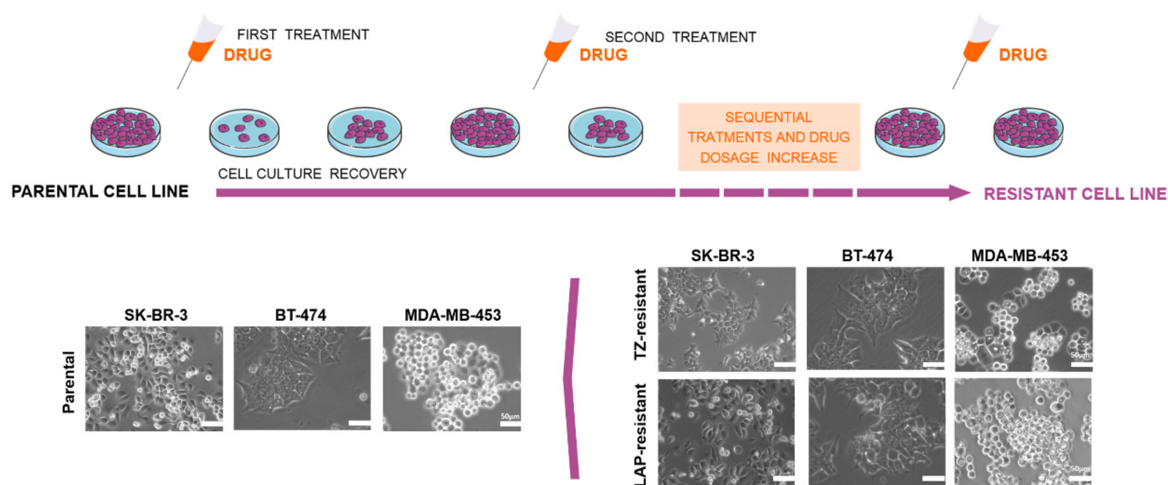


Figure 24. Drug resistance generation model. A. The diagram shows the sequential process for SK-BR-3, BT-474 and MDA-MB-453 Trastuzumab and Lapatinib resistant cell lines generation. Parental cell lines were initially treated once a week with Trastuzumab (50 µg/ml), and the medium was replaced every two days. During the 5 months process, the dose was sequentially increased to 200 µg/ml. For the Lapatinib resistant model, a low dose of 0,5 µM was used in the first treatment, and medium was changed after 24 hours. Due to the cytotoxic effect of the drug, only about 20% of the cells survive the treatment, and the culture took a couple of weeks to recover. Again, the same dose of 0,5 µM was added and this time the percentage of drug resistant population was higher, once recovered, a higher concentration of 1 µM was used, the same protocol was repeated for around 5 months until reaching a final drug concentration of 4 µM. B. Representative phase contrast microscopy images showing the cell lines morphology of the three cell lines used before and after the acquisition of the drug resistant phenotype.

6.1.3 Generation of fibroblast derived cell lines

For the establishment of non-tumour associated fibroblasts cell lines we obtained fresh healthy mammary tissue from women who underwent a reduction mammoplasty. For the development of tumour associated fibroblasts, a piece of tumour after tumourectomy surgery was collected from 6 patients with breast cancer (two HER2+++ patients, two HER2+ patients, and two HER2- patients). All samples were collected under the approval of the Institutional Review Board of Clinica Planas and Hospital Clinic in Barcelona.

Tissue from all tissue samples was minced into small pieces with the help of sterile tweezers, scissors and razor blades, and placed in a 250 mL sterile glass bottle, together with supplemented DMEM-F12 medium with 200 U/ml of collagenase type IV and 100 U/ml of hyaluronidase in a total volume of 3 times the initial tissue volume. The glass bottle was placed in a shaker

approximately at 50 rpm and 37 ºC for 1-2 hours for tissue digestion [436].

The disaggregation process was controlled every 30 min under the microscope, and once small organoids and single cells were observed the digestion process was interrupted and the samples were transferred into a canonical tube to centrifuge the stromal fraction (5 min at 1200 rpm).

The pellet was resuspended in 10 % FCS-DMEM medium and placed in a plastic plate to be cultured in regular conditions (37 ºC in a humidified 5 % CO_2 atmosphere).

After 1-2 days of culture, fibroblasts were present in the 5 cm petri dish, and in order to select them and separate them from all the other cell populations present in the sample, we applied differential trypsinization [437], which consists in the faster trypsinization of fibroblasts compared to epithelial and myoepithelial cells. Briefly, cells were incubated with trypsin for a short period of time (less than 1 minute), until detachment of fibroblasts was observed under the microscope. At this point, fibroblasts were collected with 10 % FCS-DMEM media and placed in a new plate to grow.

To immortalize the primary human normal mammary fibroblasts from reduction mammoplasties (RMF), and tumour associated fibroblasts (TAF) a retroviral vector pMIG (MSCV-IRES-GFP) expressing both hTERT and GFP was introduced, again using Extremegene transfection reaction (same conditions than **section 1.1**). Fibroblasts were cultured for 4-5 days and then the GFP+ cells were sorted using flow cytometry.

6.1.4 Generation of xenograft-tumour derived cell lines

Mouse harbouring human breast cancer xenografts were euthanized after 90 days post tumour implantation, and tumours were surgically removed using a sterile biosafety cabinet.

Tumours were minced into small tissue fragments using sterile razor blades and digested as explained before in **section 1.3**. After centrifugation, samples were placed in a 5 cm petri dish with 10 % FBS-DMEM-F12 in the presence of puromycin (2.5 µg/ml) in regular conditions (37 ºC in a humidified 5 % CO_2 atmosphere) in order to eliminate any fibroblast and/or murine stromal cell. Puromycin resistant cells were propagated until confluence (2-3 weeks) and mCherry-LUC+ cells were sorted for by flow cytometry.

6.1.5 Generation of metastatic lung derived cell lines

Mouse harbouring human breast cancer xenografts were euthanized after 90 days post tumour implantation. In a sterile biosafety cabinet, the animal abdominal cavity was opened to expose the diaphragm, and after retracting the two halves of the ribs the heart and lung were exposed.

Carefully lungs were dissected and placed in a petri dish containing PBS supplemented with 1 mM magnesium chloride, 0.5 mM calcium chloride, 100 U/mL penicillin, and

100 µg/mL streptomycin (PBS++). Lungs were minced using sterile razor blades to obtain small fragments (5 mm), and enzymatically dissociated into single cell suspension by incubation with 4 ml of type 1A collagenase solution (0.15 g collagenase, 2.5 g BSA in 100 mL PBS++) for 30 min at 37 ^{o}C.

The tissue suspension was homogenized by gentle pipetting during the incubation, and after the incubation time, collagenase activity was inhibited by adding 8 ml of FBS, and finally the sample was centrifuged (1200 rpm, 5 min), and subsequently resuspended in 10 % FBS-DMEM-F12 and plated in a 10 cm petri dish. After 48 h, attached cells were washed with PBS, selected with puromycin 2.5 µM and expanded with DMEM-F12 medium.

6.2 PROTEIN ASSAYS

6.2.1. Cell Lysis

Frozen cell plates were enzymatically and physically lysed with ice-cold radio-immunoprecipitation assay (RIPA) buffer (Tris-HCl 5 mM, pH 7.4, NP-40 1 %, Na-deoxycholate 0.25 %, NaCl 150 mM, EDTA 1 mM, PMSF 1 mM, proteinase inhibitors, Na3VO4 1 mM and NaF 1 mM) for protein extraction. After centrifugation (13000 rpm for 5 min at 4 ^{o}C) supernatants containing the protein fraction were collected and quantified by Lowry quantification method using the DC™ Protein Assay Reagent (following manufacturer's instructions).

6.2.2 RTK phosphoprotein arrays

In order to study the activation of the tyrosine kinase proteome we used the Human Phospho-Receptor Tyrosine Kinase (RTK) Array Kit.

This array represent a screening tool designed to simultaneously detect the relative phosphorylation of 42 different RTKs. Briefly, 42 selected capture antibodies were spotted in duplicate on nitrocellulose membranes. Protein lysates from our samples were diluted and mixed with a cocktail of biotinylated detection antibodies. The sample/antibody mixture was then incubated within the array, and finally, any protein/antibody complex present was bound by its cognate immobilized capture antibody on the membrane.

Quantification levels of phospho-RTK were then assessed using a horsereadish peroxidase (HRP) conjugated pan phospho-tyrosine antibody followed by chemoluminescence detection: after receptors capture by antibodies were spotted on a nitrocellulose membrane, peroxidase and chemoluminescent detection reagents were carefully added, and so the signal produced was proportional to the amount of cytokine bound. Chemilumonescence was detected in the same manner as in a Western blot, by a Luminiscent Image Analyzer (LAS 4000 Imaging System, Fujifilm, Japan). Image

Gauge software was used for densitometric quantification.

6.2.3 Protein network study using IPA-Ingenuity software

Ingenuity Pathway Analysis (IPA), is a licensed software enabling the analysis of biological data by providing molecular and chemical interaction information, cellular and biological phenotype information, and disease process information for given sets of genes (*http://bioinformatics.fcrb.es)*. The information in IPA is based on manually reviewed, automatic text mining or manual text mining of the literature and databases. For the graphic representation of the phylogenic tree for the RTK selected for the study the online freely available Kinase Mapper tool was used.

6.2.4 Western blot

For the protein analysis equal amounts of total protein for each sample were separated in a SDS-PAGE gel and electrophoretically transferred to polyinylidene difluoride membranes, blocked with PBS-milk 5 % for 30 min at room temperature and incubated overnight (ON) at 4 ^{o}C with the corresponding primary antibodies (Summarized in **Table 11**, **Section 14**). The following day, the membranes were incubated for 1 hour at room temperature (RT) with HRP-conjugated secondary antibodies (Summarized in **Table 11**, **Section 14**). To confirm equal protein loading, the membranes were incubated with α-Tubulin or α-Actin antibodies as internal housekeeping controls.

Membrane chemoluminiscence was detected after incubation with ECL and image capture was performed with a Luminiscent Image Analyzer (LAS 4000 Imaging System, Fujifilm, Japan). Image Gauge software was used for densitometric quantification.

6.3 IMMUNOCYTOCHEMISTRY PROCESS

6.3.1 Cell Block from cell lines cultures

Cell blocks were generated by embedding cells in paraffin after fixation in agar cell blocks. Briefly, after trypsinization cells were washed twice in PBS and fixed in 2 % paraformaldehyde (PFA) for 30 min at RT. Cells were then washed twice again with PBS and centrifuged, and the cell pellets were resuspended with 10-20 µl of PBS and carefully mixed with 100 µl of melted agar. After its solidification (30 min in ice) the agar plugs were fixed 1h at RT in PFA 4 % and processed for paraffin embedding as regular biopsies.

The obtained blocks were used to construct slide samples containing sections of 2µm thickness that were stored at 4 ^{o}C until used

6.3.2 Immunocystochemistry staining

Tissues were dewaxed in xylene and hydrated in an ethanol series (100 %-70 %-50 %). After heat-induced antigen retrieval in citrate buffer (pH 6), the samples were blocked with 10 % goat serum and stained ON at 4 ^{o}C with antibodies against HER2, ER, PR, RET, FGFR2, or SMA (dilutions indicated in **Table 11, Section 14**).

Samples were then incubated with a goat anti-mouse or rabbit IgG biotinylated antibody for 1h at RT followed by the ABC peroxidase System (using manufacturer's instructions). DAB (3, 3´-diaminodbenzidine) was used as the colorimetric substrate and the slides were finally dehydrataed in ethanol seires (50 %-70 %-100 %) and counterstained with hematoxilyn.

Negative control specimens were incubated with goat serum and without primary antibody, and positive controls included tissues with known expression of each protein under evaluation.

6.3.3 Quantification of immunocytochemistry

HER2

Expression of HER2 was scored following the HercepTestTM Interpretation Manual for breast cancer [438], in summary: 0, no staining is observed or membrane staining is observed in < 10 % of the tumour cells; 1+, a barely perceptible membrane staining is detected in > 10 % of tumour cells, or the cells exhibit incomplete membrane staining; 2+, weak to moderate complete membranous staining in > 10 % of the tumour cells; and 3+, strong and complete membranous staining is observed in ≥ 10 % of the tumour cells.

For more information, see Introduction Section 2.3.

Oestrogen and Progesterone Receptors

ER and PR were regarded as positive if there were at least 1 % positive tumour nuclei, according to the ASCO/CAP guidelines [439], and receptor expression was scored semi-quantitatively depending on the percentage of positive cells regardless staining intensity. Intensity score was then determined as: 0 <1 of staining; 1+ for 1-50 % of positive cells; 2+ for 50-75 % of positive cells; and 3+ for >75 % of positive cells.

6.4 FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

Slides with formalin fixed paraffin embedded (FFPE) containing sections from agar cell blocks from our cell lines under study were dewaxed in xylene, and subsequently pretreated with 2 mM citrate buffer, pH 6, for 1 hour at 80 ^{o}C. After pepsin digestion (8 mg/ml 10 min at 37 ^{o}C), slides were dehydrated in ethanol and air-dried before hybridization. HER2 probes (PathVision, LSI DNA HER2 kit, Abbott Molecular, figure 25A) were added to air-dried slides and probes and tissue were codenatured at 71 ^{o}C for 5 min.

Hybridization was carried out ON at 37 ^{o}C. After hybridization slides were washed to

remove the cover slides at 37º C in SCC buffer, followed by 2 min at 73 ºC. Air-dried slides were finally mounted with DAPI and mounting medium.

The same protocol was used for EGFR FISH (Vysis LSI EGFR SO/CEP7 Kit, Abbott Molecular, **figure 25B**) only denaturizing conditions were different, 5 min at 71 ºC.

In both cases evaluation of signals was performed in an epifluorescence microscope. Selected cells were photographed in a Zeiss Axioplan 2 microscope equipped with an Axio Cam MRM CCD camera and Axio Vision software. Twenty cells from different areas of the individual samples were selected for thorough examination and quantification. As probes were placed on either side of the centromere, not fused signals but a gap between probes on normal or derivative chromosomes are to be expected.

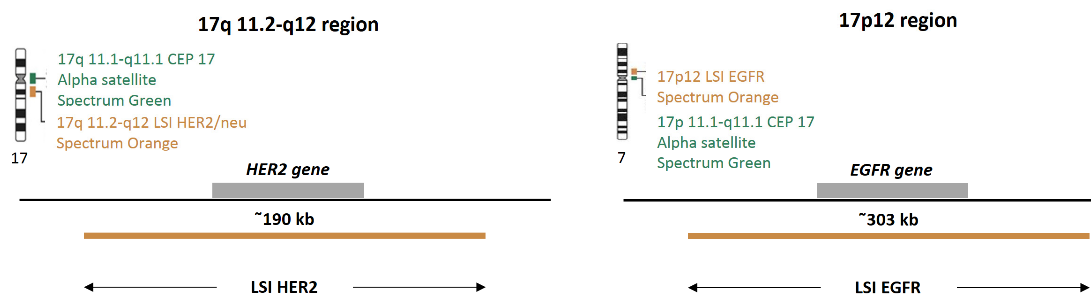


Figure 25. HER2 and EGFR probes design.

6.5 IMMUNOFLUORESCENCE, FLUORESCENCE MICROSCOPY AND IMAGE ACQUISITION

For the detection of the expression of the different RTK analysed in this study, different cell lines were seeded in coverslips until sub-confluence and treated with the corresponding doses of each drug for every particular experiment, for 24-72 h.

Cells were then washed twice with cold PBS and fixed for 15 min with PFA 4 % at 4 ºC, followed by 6 min in methanol at -20º C. After two washes with PBS, cells were blocked for 10 min with 1 % bovine serum albumin (BSA) on cold PBS and then incubated ON at 4 ºC with primary antibody (**Table 11, section 14**).

Then, coverslips were washed twice with PBS and incubated for 1 h at RT with anti-mouse or anti-rabbit secondary antibody labelled with Alexa 488 or Alexa 555 (**Table 11, Section 14**). Nucleus were counterstained with Hoechst for 15 minutes, and coverslips were mounted on glass with Prolong Gold Antifade Reagent.

Samples were visualized using an inverted epifluorescence microscope (Olympus Bx4I). Image assembly and processing was performed using the Cell Senes Standard software.

6.6 FLOW CYTOMETRY AND FLUORESCENT ACTIVATED CELL SORTING (FACS)

6.6.1 CD24 and CD44 analysis by flow cytometry

For the detection of cell surface CD24 and CD44 expression in basal conditions, cells were cultured until sub-confluence. Cells were concentrated (the supernatant was collected, and cells attached to the plate were trypsinizated) and centrifuged 5 min at 1500 rpm at 4 ^{o}C, the pellets were then washed with PBS and resuspended in blocking buffer (BSA 1 %-PBS) and placed on ice for 30 min. Cell surface staining of tumour cells was done with the primary monoclonal antibodies, anti-CD24 and anti-CD44 in blocking buffer for 1 h at 4°C (**Table 11, section 14**). Cells were then washed 3 times with BSA 1 %-PBS and stained with FITC-conjugated anti-mouse IgG antibody for 1 h at 4 ^{o}C.

Control cells without staining were used to determine the settings of autofluorescence, and cells stained only with secondary antibody were used to ensure the specificity of the primary antibodies. Stained cells were then analysed on a FAC scan flow cytometer using FACS Calibur (BD Biosciences) and data was processed by the Cell Quest software. Ten thousand cells were analysed for each sample, and every experiment was performed in triplicate. Results are expressed in arbitrary units.

6.6.2 Sorting of GFP^{+}/mCherry-LUC^{+} cells

Fibroblasts overexpressing GFP or breast cancer cell lines overexpressing mCherry-LUC were trypsinized, washed twice in PBS + 1 % FBS and resuspended in sorting buffer (EDTA 1 mM, HEPES 25 mM and FBS1 %). The cells were sorted using a BD Biosciences FACS Aria III cell sorter. Control cells without hTERT-GFP / mCherry vector were used to determine the autofluorescence settings. Live cells were gated on the basis of forward and side scatter, and single cells were gated on the basis of forward scatter height (FSC-H) and forward scatter area (FSC-A). Gates were determined by analysis of stained and single cells. Sorted cells were collected in FBS and kept on ice until plated.

6.7 RNA ASSAYS

6.7.1. Isolation and quantification of RNA

Total RNA from tumour cells was isolated with an RNeasy Mini Kit according to the manufacturer's instructions, and quantified using a NanoDrop® Spectrophotometer.

6.7.2 Quantitative real time PCR

1 µg of RNA was reverse transcribed in a final volume of 100 µL using High Capacity cDNA Reverse Transcription Kit according to manufacturer's recommendations. For

gene expression analysis, quantitative PCR was done on the 7900HT Fast Real-Time PCR System (Applied Biosystems) using Assay-on-demand primers and probes from Applied Biosystems **(Table 11, Section 14**). The PCR reaction mixture was done in a final volume of 10 µl for each sample, including 0.5 µl of each assayed probe, 5 µl of master mix (TaqMan Universal PCR Master Mix) 2.5 µl of H_2O DEPC and 2 µl of cDNA. Transcript levels were normalized to those of β-actin that was used as endogenous control. Gene expression levels were analysed in triplicate and calculated using the 2ΔCT method [440].

6.8 GENE EXPRESSION ANALYSES

6.8.1 Microarray technology

The classic principle behind the technology is to print and fix on a surface a set of probes that are complementary to the molecules of interest, enabling these molecules to hybridize the probes, in our case, oligonucleotide-probes for mRNA expression analysis. The molecules in the sample are labelled with a signal-emitting substance, and hybridized to the array. After hybridization the microarray is scanned, and the amount of signal from the probes is quantified. The data is then normalized and made comparable across multiple samples using the same common reference. The array also contains numerous internal control probes for monitoring the experimental steps in the amplification, labelling and hybridization procedures [441].

To obtain the molecular profiles of the different cell lines, the gene expression arrays G4851A Sure Print G3 Hmn GE 8X60k Microarray Kit (Agilent Technologies) were performed in duplicate for each cell line (in collaboration with the group of Prof. Anne-Lise Borresen-Dale at the Deptartment of Genetics located in the Oslo University Hospital, following manufacturer's instructions (main steps are summarized in **figure 26)**. Regarding methodology, RNA was isolated as explained before in **section 7.1**, total RNA was reversed transcribed into cDNA and the fluorescent dye Cy3-dUTP was incorporated during the labelling reaction. Hybridization was performed ON, and the microarrays were scanned using an Agilent microarray laser scanner (Agilent Technologies, Palo Alto, CA).

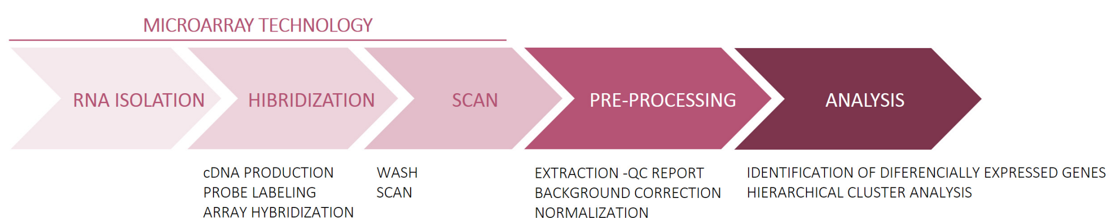


Figure 26. Array technology diagram

6.8.2 Pre-processing of microarray: data extraction and normalization

After the scan, the data was extracted using the Feature Extraction Software®. With this software we obtained a QC Report for each one of the arrays performed. QC reports include statistical results that help us to evaluate the reproducibility and reliability of the microarray data.

In our study, all check-up points were correct and the arrays passed successfully. Next step in the data analysis include data normalization using the Gene Spring® software.

Normalization of the fluorescent intensities is important to minimize systemic non-biological variance from the microarrays results. Common variations include differences in the quantity of initial RNA, variability in hybridization performance and differences in scanner parameters. Main steps in normalization process include: background correction, threshold raw values to 1, Log base 2 transformation and normalization algorithm, in our case a percentile shift to 75th percentile.

Applying quantile normalization all transcripts values on each array are sorted according to their intensity and then matched between the different arrays, consequently the smallest value on each array is identical and so forth, what ensures and identical signal distribution for all the arrays in the study.

A Box Whisker plot results from this process, which summarizes the normalized intensities, and where we can found several information as median values, outliers, quantiles and the distribution for particular samples or conditions [441].

6.8.3 Microarray data analysis: differentially expressed genes definition

Hierarchical clustering

Hierarchical clustering is an unsupervised method which groups together samples and genes, based on similarities in gene expression patterns, resulting in a tree where short branches join items (genes or samples) with high similarity, and as the similarity decreases the branch length increases. The clustering method defines all items as clusters and calculates the distance between all pairs of clusters [442].

Identifying differentially expressed genes via statistic synthesis

This approach, defined by Yang *et al*. 2005, addresses both ranking and selection of differentially expressed genes and integrates differing statistics via a distance synthesis scheme, including six statistics (t-test, SAM, fold change (FC), Moderated-t, Moderated-F, and B-statistics) which provides robustness properties lacked by individual statistics [443].

6.8.4 Generation of a gene list associated with tissue microenvironment

We define different microenvironment-related gene lists by searching key terms in the public data bases GeneGo (*www.portal.genego.com*), GeneCards (*www.genecards.org*) and Eugenes (*www.eugenes.org*), including genes codifying for soluble factors known to induce stromal changes, like chemokines,

cytokines, matrix remodeling-related enzymes, angiogenic and neurogenic factors.

6.9 CELL PROLIFERATION ASSAYS

To determine cell proliferation in parental and drug resistant cell lines the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay Kit was used as previously described [444].

Briefly, subconfluent cultures of the different cell lines were incubated for 72 h in the presence Lapatinib (0 to 5 µM), Trastuzuamb (0 to 250 µg/ml), Ponatinib (0 to 10 µM) and BGJ-398 (0 to 10 µM).

For Lapatinib assays, the corresponding dose of DMSO used as drug solvent (never above 0.02 % v/v) was added to the control wells as dose 0. Assay values for these points were taken as 100 % of viability, and the viability for each treatment point was calculated relative to the controls.

For colour development, 20 µl of the tetrazole MTT were added to each well, after an incubation time of 30-45 min (37 ^{0}C) the plate was read on a microplate spectrophotometer (Molecular Dynamics) at 490 nm (test wavelength) and 690nm (reference wavelength).

Different doses were assessed in sixtuplicate and every experiment was performed at least twice.

Determination of half maximal inhibitory concentration (IC50), curve fitting and statistical analysis were performed according to the extra sum of squares F-test principle with GraphPad Prism® 5.0 software, p-values <0.05 were considered statistically significant.

6.10 COLONY FORMATION ASSAY

For colony formation assay, cells were plated at a concentration of 500 cells/plate concentration in 6 cm diameter cell culture dishes with complete growing media, treated with Trastuzumab 200 µg/ml, and Lapatinib 5 µM and allowed to grow in a 37 ^{0}C humidified 5 % CO_2 atmosphere for 21 days (medium and drugs were replaced every three days). After three weeks the plates were washed twice with PBS and colonies were stained with Cristal Violet 1 % Aqueous Solution (Sigma) and scanned to obtain representative images. To obtain a more detailed image of single colonies, contrast microscopy images were taken by using an Invert Fluo Zeiss Axiovert 200 microscope.

Experiments were performed in triplicate to ensure reproducibility of the data.

6.11 ANNEXIN V STANING

Cells were seeded in 6-well tissue culture plates in 10 % FBS-DMEM-F12 medium, grown until sub-confluence and treated with the drugs for the corresponding time and dose in each particular experiment.

In all studies apoptosis was determined using Annexin-V-FLUOS Staining Kit (Biovision), according to the manufacturer's instructions. Briefly, cells from the culture supernatants and those attached to the plate were blocked for 30 min with BSA 1 % and then incubated for 15 min at 4 ^{o}C with Annexin V-FITC solution and propidium iodide (PI). Controls (binding buffer only, PI only, and annexin-V only) were used to set appropriate detector gains, compensation, and quadrant gates in the FACSCalibur flow cytometer. Twenty thousand cells for each sample were analysed by the CellQuest software. Every experiment was performed in triplicate.

This double staining allows us to differentiate live cells from those cells in early apoptosis, and those cells that already are in late stages of apoptosis.

6.12 *IN VIVO* EXPERIMENTS

All animal experiments were performed in accordance with the regulations of our institution's ethics commission, following the guidelines established by the regional authorities of Catalonia, Spain. The mice were bred at the Medical School (University of Barcelona) animal facility laboratory, and kept under specific pathogen-free conditions at constant ambient temperature (22° C-24 °C) and humidity (30 %–50 %). The mice were given sterilized food and tap water ad libitum. After each experiment, the animals were anesthetized and euthanized in accordance with the regulations of our institution's ethics commission.

6.12.1 *In vivo* 1. Efficacy study on a HER2+ breast cancer xenograft model

Five-week-old female athymic nude Foxn 1nu nu/nu mice were obtained from Janvier (Europe).

Xenograft tumours were established by orthotopical injection of a mixture (1:1 ratio) of 1.5x10^{6} MDA-MB-453 BC cells (parental, Trastuzumab resistant and Lapatinib resistant) in PBS:Matrigel (total volume 150 µl) into two mammary fat pad per mouse.

Once tumours reached volumes of 100 mm^{3}, mice were randomized into groups including five mouse per group, and treatments started (**Table 10**).

Tumour growth was measured twice weekly with a digital caliper during the 21 days of treatment, and tumour volume was calculated as tumour volume $V= (D*d^{2})/2$. Changes of tumour volume between groups were analysed by Anova statistical test. P values < 0.05 were considered statistically significant.

Once treatment was finished, animals were anesthetized and 1 mL of peripheral blood was extracted directly from the heart and kept on ice. For plasma isolation samples were centrifuged during 10 min at 13000 rpm, 4 ^{o}C, and plasma was collected from the supernatant and stored at -80 ^{o}C. Tumours were surgically recovered and frozen in liquid nitrogen and stored at -80°C until

protein and RNA extraction, or fixed in PFA 4 % and embedded in paraffin for further immunocytochemistry analysis, following standard procedures. In the same way, potentially metastatic organs as lungs, were surgically removed and embedded in paraffin after PFA 4% fixation.

DRUG	DOSE	ADMINISTRATION	DRUG SOLVENT
BGJ-398	10mg/kg	Oral - daily	Dextrose 5 %
Lapatinib	10mg/kg	Oral - twice daily	0.5 % hidroxipropil metilcelulosa
Ponatinib	10mg/kg	Oral - daily	Citrate buffer
Trastuzumab	10mg/kg	Intraperitoneal - twice a week	Saline

Table 10. *In vivo* tested drugs dose, administration and solvent.

6.12.2 *In vivo* 2. Co-injection of human mammary (RMF) and tumour associated (TAF) fibroblasts with HER2+ breast carcinoma cells into an immunodeficient mouse

Five-week-old female CB17/Icr Hand-PrKdcscid mice were obtained from Harlan (Europe).

Xenograft tumours were established by orthotopical injection of a mixture (1:1 ratio) of cells mixture in PBS: Matrigel (total volume 150 µl) into two mammary fat pad per mouse. Cells mixture consists in 1x106 MDA-MB-453 cells plus 1x106 immortalized human fibroblasts from different origin (RMF and TAF). For the control group only 1x106 MDA-MB-453 cells were injected.

Tumour growth was measured twice weekly with a digital caliper during 90 days, and tumour volume was calculated as explained in section 6.12.1. Luminescence from MDA-MB-453 mCherry-LUC+ cells were used to track tumours at experiment endpoint after Luciferin injection (15 mg/ml) in a Hamamatsu Aequorea bioluminescence system. At this time, animals were anesthetized and 1 mL of peripheral blood was extracted directly from the heart for plasma isolation (same procedure as explained before in section 6.12.1)

Tumours were surgically recovered and half of them were frozen in liquid nitrogen and stored at -80°C until protein and RNA extraction, or fixed in PFA 4 % and embedded in paraffin for further immunocytochemistry analysis following standard procedures. The other half of the tumour was used to generate primary cultured cells from xenografts (section 2.4). In the same way potentially metastatic organs such as lungs were surgically removed, part of the tissue was used to develop metastatic lung derived primary cell lines (section 2.5), and the rest of the tissue was embedded in paraffin after PFA 4% fixation.

6.13 PATIENTS STUDIES

6.13.1 Tissue Microarray (TMA) analyses

TMA containing 31 cases in duplicate of breast cancer with matched normal tissue and metastatic lymph node carcinoma tissue was purchased from US Biomax Inc, EEUU. Clinical and pathological information included: sex, age, tumour size, pathological diagnosis grade (I-III regarding well-differentiated, moderately-differentiated or poorly differentiated), tumour histological grade (Nottingham modification of Bloom-Richard system), pTNM and stage (AJCC Cancer Staging Manual 6th Edition), lymph node infiltration positivity, interval between primary and metastatic cancer (months), ER, PR, p53, follow-up moths was available. Hematoxylin and eosin-stained consecutive sections were also available and purchased. TMA slides were not coated with extra layer of paraffin (tissue cores could be easily seen on the glass), so there was no need to bake, and were directly putted into xylene for de-paraffin procedure and HER2, FGR2 and RET immunohistochemistry were performed as explained before in **section 3.2.**

6.14 REAGENTS USED IN THE STUDY

6.14.1 Antibodies

Table 11. Antibodies and fluorescent dyes used in this study, brand, catalogue number, origin and particular condition for each technical application are listed below:

Primary antibody	Brand	Cat#	Origin	Dilution	App*
α-Actin	Sigma Aldrich	A2066	Rabbit	1:2000	WB
α –Sooth muscle actin (SMA)	DAKO	M0851	Mouse	1: 200	IF
	DAKO	M0851	Mouse	1:100	IHQ
EGFR	Cell Signaling	2232	Rabbit	1:1000	WB
FGFR2	Sigma Aldrich	HPA035305	Rabbit	1:100	IHQ
	Sigma Aldrich	HPA035305	Rabbit	1:100	IF
Fibroblast surface protein (FSP)	Sigma Aldrich	F4771	Mouse	1:100	IF
HER2	Biogenex	MU134-UCE	Mouse	1:1000	WB
	Biogenex	MU134-UCE	Mouse	1:100	IHQ
Green Flourescent Protein (GFP)	Abcam	Ab290	Rabbit	1:100	IF
mCherry	Molecular Probes	M11217	Rat	1:100	IF
Phospho-EGFR Tyr1068	Cell Signaling	2234	Rabbit	1:1000	WB
Phospho-FGFR2 Tyr653/654	R&D Systems	AF3285	Rabbit	1:100	IF
Phospho-HER2 Tyr1248	Abcam	Ab47755	Rabbit	1:100	IF
Phospho-RET Tyr905	R&D Systems	AF3285	Rabbit	1:100	IF
RET	Sigma	HPA008356	Rabbit	1:100	IF
	Sigma	HPA008356	Rabbit	1:100	IHQ
S100A4	Abcam	Ab27957	Rabbit	1:100	IF
Secondary antibody	**Brand**	**Cat#**	**Origin**	**Dilution**	**Appl***
Alexa Fluor 555 anti-mouse IgG	Invitrogen	A21422	Goat	1:200	IF
Alexa Fluor 555 anti-rat IgG	Invitrogen	A21434	Goat	1:200	IF
Alexa Fluor 555 anti-rabbit IgG	Invitrogen	A21422	Goat	1:200	IF
Alexa Fluor 488 anti-mouse IgG	Invitrogen	A21121	Goat	1:200	IF
Alexa Fluor 488 anti-rabbit IgG	Invitrogen	A11034	Goat	1:200	IF
Alexa Fluor 488 anti-rat IgG	Invitrogen	A11006	Goat	1:200	IF
Anti-CD24 PE - conjugated	BD Bioscience	555428	Mouse	1:100	FC
Anti-CD44 FITC - conjugated	BD Bioscience	555478	Mouse	1:100	FC
Anti-mouse HRP-conjugated	Amersham	XA931	Goat	1:2000	WB
Anti-rabbit HRP-conjugated	Amersham	NA934V	Goat	1:2000	WB

Application*
FC: *Flow Citometry*
IHQ: *Immunohistochemistry*
IF: *Immunofluorescence*
WB: *Western Blot*

6.14.2 Probes

Table 12. Quantitative PCR probes used in this study, brand and catalogue number are listed below:

Gene	Brand*	Cat#	Gene	Brand*	Cat#
ALDH1	AP	Hs00946916_m1	ERBB2	AP	Hs01001595_m1
β-Actin	AP	Hs99999903_m1	ERBB3	AP	Hs00176538_m1
CD24	AP	Hs02379687_s1	ERBB4	AP	Hs00955525_m1
CD44	AP	Hs01075861_m1	FGFR2	AP	Hs01552926_m1
EGFR	AP	Hs01076090_m1	RET	AP	Hs01120030_m1

Brand*

***AP:** Applied Biosystems*

6.14.3 Drugs and Inhibitors

Table 13. Different drugs and inhibitors used in this study, brand and catalogue number are listed below:

Compound	Brand	Cat#
BGJ-398	Selleckchem	S2183
Herceptin®- Trastuzumab	Generously provided by Hospital Clinic	
Lapatinib	Selleckchem	S2111
Ponatinib	Selleckchem	S1490

All reagents were prepared and storage according to the manufacturer's instructions.

6.14.4 Cell Culture Reagents

Table 14. All cell culture reagents used in this study, brand and catalogue number are listed below:

Reagent Name	Brand	Cat#
DMEM-F12	Gibco	21331-046
DMEM	Gibco	41966-052
Foetal Bovine Serum	Gibco	10082-147
Fungizone	Gibco	15290-018
Glutamax	Gibco	35050-038
Insulin Human Solution	Sigma-Aldrich	I9278
PBS	Gibco	14190-169
Penicillin- Streptomycin	Invitrogen	15070
25 % Trypsin-EDTA	Gibco	25200-72

All reagents were prepared and storage according to the manufacturer's instructions.

6.14.5 Other Reagents and Kits

Table 15. Other reagents and kits different from the listed above used in this study, brand and catalogue number are summarized below:

Reagent Name	Brand	Cat#
Albumin, from Bovine Serum	Sigma Aldrich	A7906
Agar	Sigma Aldrich	A7002
BRDU (5-Bromo-2’Deoxyuridine)	Sigma Aldrich	B5002
Cell Titer 96® Cell Proliferation Assay	Promega	G3581
Cristal Violet 1 % Aqueous Solution	Sigma Aldrich	V5265
DAB	Sigma Aldrich	DA4168
DC™ Protein Assay Reagent A, B and S	Bio Rad	0114,0115
Dimethyl Sulfoxide - DMSO	Sigma Aldrich	D2650
ECL Western Blotting Detectin Kit	GE Healthcare	RPN2209
Extremgene 9 DNA Transfection Reagent	Roche	XTG9-RO
FGF2	Gold Bio	1140-02
FGF5	R&D systems	237-F5050/CF
GDNF	Gold Bio	1170-14
Goat Serum	Sigma	G9023
High Capacity cDNA Reverse Transcription Kit	BD Bioscience	4368813
Hoechst	Life Technologies	H3570
Luciferin-D	Anaspec	82252
Matrigel	BD Bioscience	356235
NRTN	R&D systems	1297-NE025/CF
Phospho RTK Array Kit	R&D Systems	ARY001
Precision Plus Standard	Bio Rad	161-0374
Prolong Gold Antifade Reagent	Life Technologies	P3693
Propidium Iodade from Annexin V-FITC Detection Kit	eBioscience	BMS500FI
Proteinase Inhibitor	Sigma Aldrich	P8340
Ribonuclease A, from Bovine Pancreas	Sigma Aldrich	R5503
Rneasy Mini Kit	Quiagen	74106
Sigma Fast™ 3,3’-Diaminobezidine	Sigma Aldrich	D4168-50ET
TaqMan Universal PCR Master Mix	Applied Biosystems	4352046
Vectastin Elite	Vector Lab	PK6100

All reagents were prepared and storage according to the manufacturer´s instructions.

All figures in this thesis were made by using Medical Servier Art Gallery powerpoint image bank.

7. RESULTS

1. RESISTANCE MODEL AND HER2 STATUS

1.1 Development and characterization of BC cell lines resistant to Trastuzumab and Lapatinib

As an experimental model to identify new potential targets related to tumour escape to anti-HER2 therapies, we developed a panel of several BC cell lines resistant to the current HER2 targeted therapies used in the clinic, the monoclonal antibody Trastuzumab and the small molecule Lapatinib (a tyrosine kinase inhibitor).

All the three cell lines selected to be used in the study are representative of the HER2 positive group of patients with different patterns of HER2 overexpression or amplification. These cell lines also reflect the molecular heterogeneity found in BC patients in terms of hormonal receptors and CKs (basal status and modulation within these markers with the acquisition of the drug resistant phenotype are shown in **Table 16,** see materials and methods section 6.3.3 for cuantification details).

Related to the HER2 receptors status, MDA-MB-453 cell lines show HER2 overexpression with no amplification of 17q12, while BT-474 and SK-BR-3 display 17q12 copy number gain (*ATCC source*).

To make sure that our model of drug resistance was recapitulating the degree of heterogeneity found in primary tumours we adjusted drug doses to avoid reductions in the cellular population of more than the 80-90 %, avoiding massive reductions in the cellular population and very narrow clonal selection processes.

One of the main objectives in our study was to identify new compensatory pathways other than HER2 that were activated as a result of cell adaptation to drug treatments, and so, it was important to ensure some degree of heterogeneity in the residual population.

On the contrary, if just individual clones found the drug resistant population, the sort of heterogeneity present in the culture, resembling tissue and tumour heterogeneity found in patients, would have been lost.

CELL LINE	ER	PR	CK5
SK-BR-3 parental	0	0	0
SKBR-3 Trastuzumab resistant	0	0	0
SKBR-3 Lapatinib resistant	0	0	0
BT-474 parental	2+	2+	0
BT-474 Trastuzumab resistant	3+	3+	0
BT-474 Lapatinib resistant	3+	3+	0
MDA-MB-453 parenal	0	0	0
MDA-MB-453 Trastuzumab resistant	0	0	0
MDA-MB-453 Lapatinib resistant	0	0	0

Table 16. Typical breast cancer markers modulation with the resistant phenotype. ER (oestrogen receptor), PR (progesterone receptor) and CK5 (cytokeratin 5).

The drug resistant phenotype was developed by continuous exposure of the parental cell lines to increases concentrations of Trastuzumab or Lapatinib for over 5 months, and by the selection of the cell population surviving after each round of treatment (**Figure 24 materials and methods section)**.

Final doses were set up at 200 µg/ml for Trastuzumab and 4 µM for Lapatinib. Different approaches were used to confirm drug resistance and to characterize the drug resistant phenotype. Cell proliferation and colony formation ability was determined by MTT and colony formation assays upon drug treatment (**Figure 27** and **28** respectively). While the parental cell lines died under Trastuzumab and Lapatinib treatment, the resistant cell lines were insensitive to low doses, and showed higher IC50 compared to parental cell lines (**Figure 27**). Moreover, the drug resistant cell lines displayed a more aggressive behaviour as evidenced by their increased colony formation ability that persisted even under drug pressure (**Figure 28**).

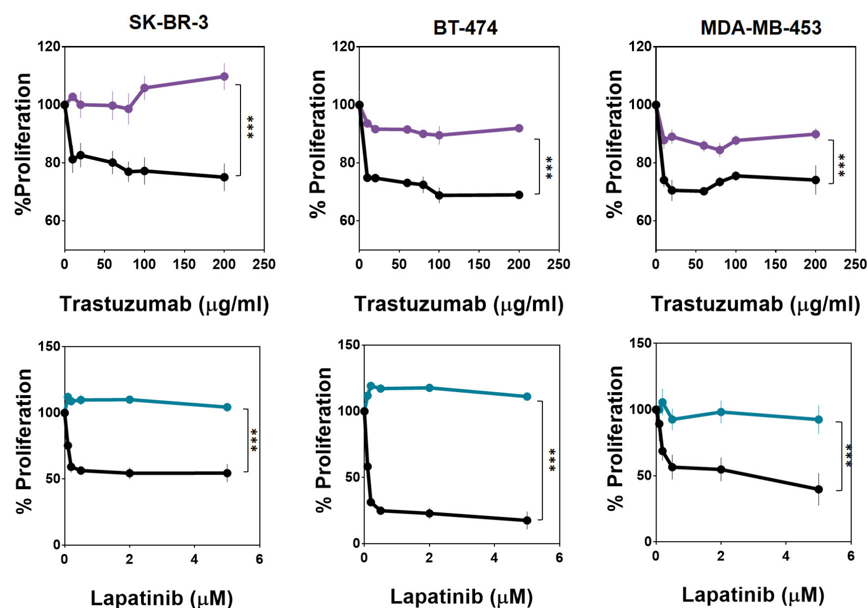


Figure 27. Assesment of differential drug responses to Trastuzumab and Lapatinib by MTT assay. Cell viability was quantified in SK-BR-3, BT-474 and MDA-MB-453 cells treated for 72h with increased doses of Trastuzumab (0-200 µg/ml, upper panels) or Lapatinib (0-5 µM, lower panels). Parental cell lines are represented with a black lines, while Trastuzumab and Lapatinib resistant cell lines are represented with turquoise and purple lines respectively. Graphs show the percentage of cell proliferation for each dose. Data represent mean ± S.D. for a minimum of 3 replicates. Significant differences by ANOVA with Sidak Multiple Comparison post-hoc test are indicated as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

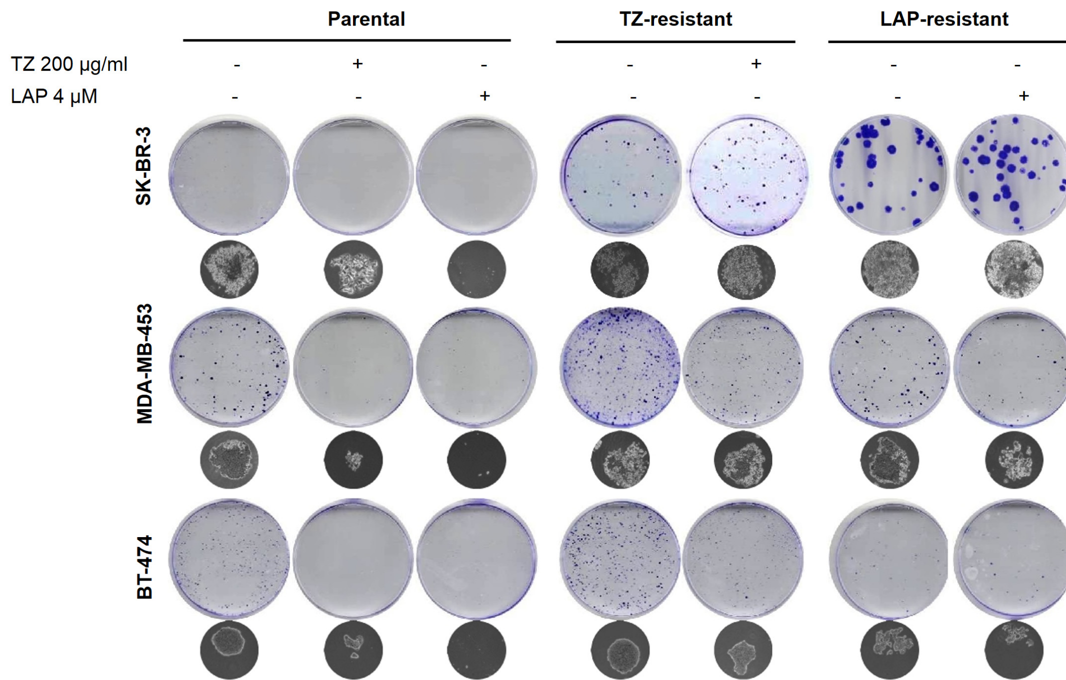


Figure 28. Validation of the Trastuzumab and Lapatinib resistant phenotype by colony formation assay. Colony formation ability was measured in SK-BR-3, BT-474 and MDA-MB-453 parental and resistant cells lines treated or not with Trastuzumab (200 µg/ml) and Lapatinib (4 µM). Treatments were done three times a week for three weeks. Images are representative of at least 3 independent experiments.

The stem cell phenotype has been widely associated in the literature with the intrinsic resistance phenotype. According to these models cancer stem cells (CSC) will be responsible for the outgrowth of the drug resistant population that will further drive tumour progression. The cell surface glycoproteins CD24 and CD44 are standard markers for stem cell-like cells in breast cancer [445], and also the aldehyde dehydrogenase isoform 1A1 (ALDHA1) has been vastly used as a CSC marker in breast cancer [446]. The differential expression of these set of markers was analysed by FACS and quantitative PCR in our panel of drug resistant and sensitive cell lines (**Figure 29A** and **29B)**. The SK-BR-3 cell line showed no changes in the expression of any of the molecular markers, not even in their phenotype profile. There was a significant increase in the gene expression of CD44 in both BT-474 drug resistant cell lines, but that was not translated into an enrichment in the stem-like population, as $CD44^{+}$ cells could not be detected by FACs. Conversely, for the MDA-MB-453 model, a selection for the $CD44^{+}$ population was detected in the drug resistant lines (more evident in the Lapatinib model).

Although this population enrichment did not correlate with significant gene overexpression, we found that the drug resistant cell lines showed higher CD44 expression and lower CD24 expression compared to the equal expressed levels of CD24 and CD44 in the parental cell line. The fact that the drug resistant lines downregulated CD24 expression at the

same time that overexpress CD44 may be responsible for this CD44^{+} population enrichment. Regarding ALDHA1, just the MDA-MB-453 drug resistant cell lines show a significant rise in gene expression.

All together, these results validate the drug resistant phenotype as well as the resultant heterogeneity in our drug resistant experimental model and showed that our drug resistant cell lines were mainly composed of non stem-like cells. However, some kind of stemness degree was also present for some of the drug resistant cell lines, which is in agreement with the idea that tumour drug resistance is closely related to many intrinsic or acquired properties of CSCs, such as quiescence, specific morphology, DNA repair ability and overexpression of antiapoptotic proteins, drug efflux transporters and detoxifying enzymes [447].

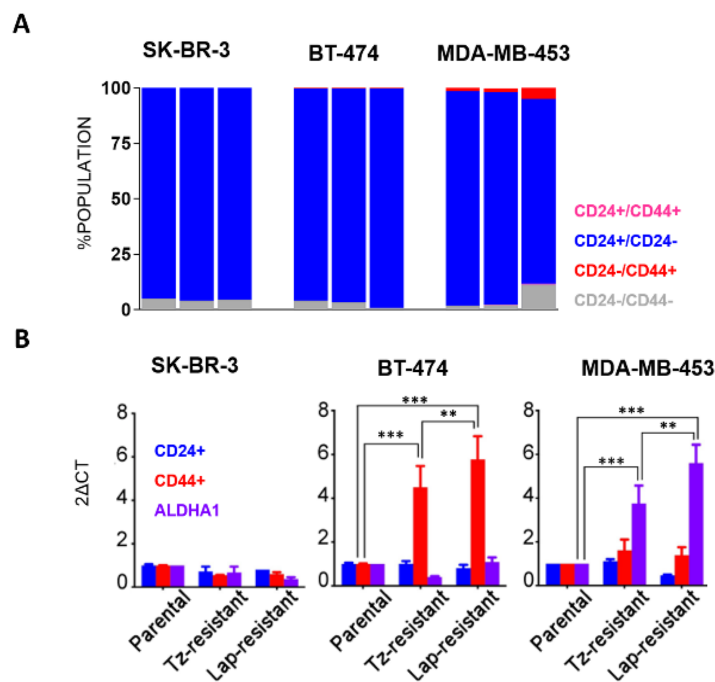


Figure 29. Breast cancer stem-like phenotype markers study. A. Graph showing the percentage for CD24 and CD44 surface markers populations analysed by FACS. The double negative population is shown in grey, the CD44 positive population in red, the CD24 positive population in blue and the double positive population in pink. Data represent mean ± S.D. for a minimum of 3 replicates. B. Graphs show the relative expression values (2ΔCT fold change) for CD24, CD44 and ALDHA1 measured by quantitative PCR. The expression changes are normalized with the parental cell line expression. From left to right, SK-BR-3 (parental, Trastuzumab (TZ) resistant, Lapatinib (LAP) resistant), BT-474 (parental, TZ resistant, LAP resistant) and MDA-MB-453 (parental, TZ resistant, LAP resistant). Data represent mean ± S.D. for a minimum of 3 replicates. Significant differences by ANOVA with Sidak Multiple Comparison post-hoc test are indicated as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

1.2 HER2 oncogenic addiction in the drug resistant cell lines

Our next step was focused on the study of the main common Trastuzumab and Lapatinib target receptor, in order to identify alterations in HER2 expression due to the drug resistant phenotype acquisition. We performed HER2 immunocytochemistry in parental and drug resistant cell line cell blocks, and found that HER2 expression remained stable after the acquisition of the drug resistant phenotype with few variations among drug resistant and parental cell lines (**Figure 30A**).

Similarly, FISH analysis of the chromosomal region 17q12 encoding the *ERBB2* gene, and also 17p12 region codifying the *EGFR* gene amplifications, showed for both cases few alterations within the drug resistant populations (**Figure 30B**).

As HER2 protein activation also depend on the receptor heterodimerization with other RTKs, we checked the genetic expression status for all ERBB family members by quantitative PCR analysis (**Figure 30C**). In general, we found changes in nearly all genes in the drug resistant cell lines when compared with parental gene expression, however most changes did not exceed 1-fold induction variations.

Only HER4 presented a variation greater than 2-fold induction in BT-474 Lapatinib

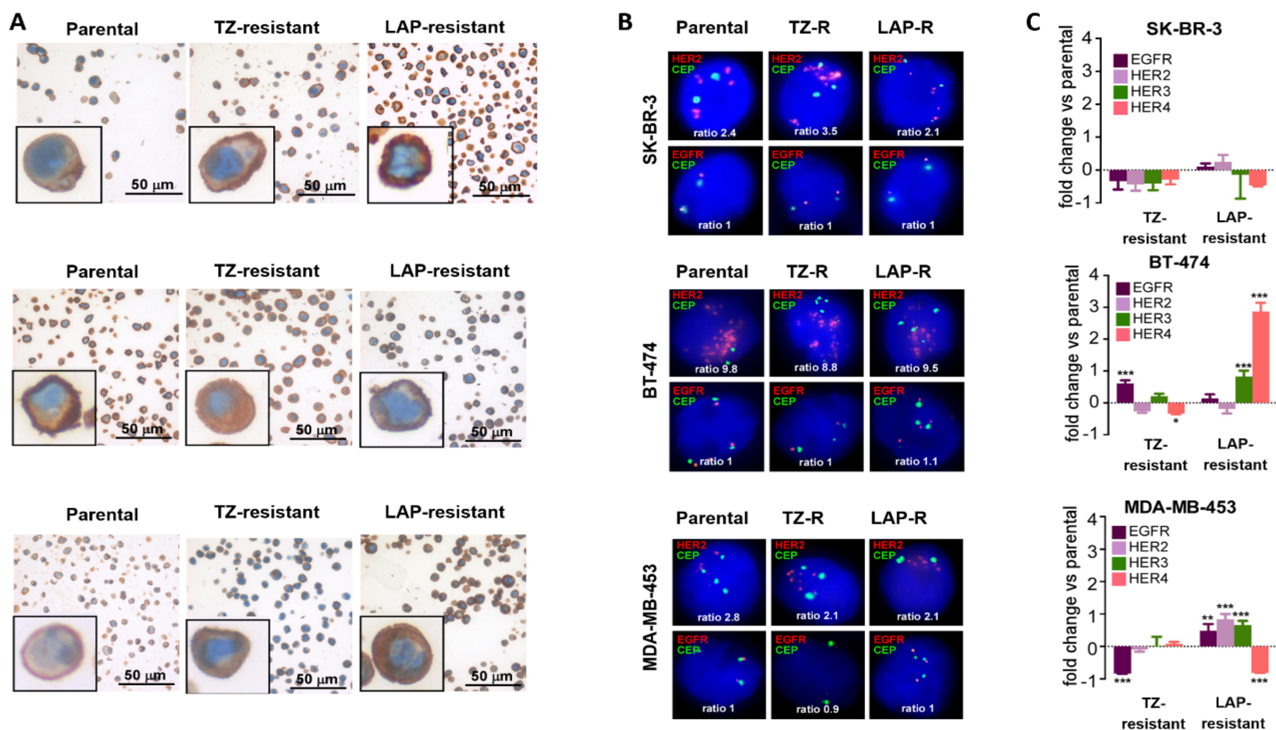


Figure 30. HER2 status in the resistant cell lines. A. HER2 immunohistochemistry representative images in parental and drug resistant cells lines SK-BR-3, BT-474 and MDA-MB-453 (Trastuzumab resistant, TZ –resistant, and Lapatinib resistant, LAP-resistant). Scale bar 50 µm. B. Representative images for HER2 (upper panels) and EGFR (lower panels) FISH for copy number gain analyses. HER2 and EGFR probes are shown in red, centromere probe (CEP) in green. Every image represents a single nucleus and the ratio was calculated as the mean of 20 cell counts for each cell line. C. Graphs show ERBB members (EGFR, HER2, HER3 and HER4) expression measured by quantitative PCR. The data represent the expression changes in the drug resistant cell lines *vs* parental cell line expression in terms of fold change Data represent mean ± S.D. for a minimum of 3 replicates. Significant differences by ANOVA with Sidak Multiple Comparison post-hoc test are indicated as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

resistant cell lines. The up-regulation of this receptor has been recently defined as a mechanism of acquired resistance to HER2 inhibitors in breast cancer cells [448], however we did not find this variation consistent within the other two cell line models.

Slightly differences were then found regarding HER2 over-expression in our drug resistant model, being evident that the drug resistant lines maintain high membrane HER2 protein expression, which suggests that the drug resistant lines may still depend on its oncogenic addiction to HER2.

To determine to what extend the drug resistant cell lines still depend on the HER2 pathway we analysed HER2 activity under drug pressure, denoted as HER2 phosphorylation analysed by immunofluorescence (**Figure 31A**). We found that in regular conditions the drug resistant cell lines showed similar levels of phosphorylation than the parental cell lines. Interestingly, under drug pressure drug resistant cell lines overactivated the HER2 pathway, which confirms that with the drug resistant phenotype HER2 oncogenic addiction is not lost, and further suggesting that this activation may be regulated through indirect transactivation, as the pathway is still active even when the receptor is being inhibited by the drugs. Moreover, parental cell lines showed a similar behaviour, and interestingly, the few cells that survive despite Trastuzmab and Lapatinib treatment also show high levels of HER2 phosphorylation, which is consistent with the natural evolutionary process underlying resistance development. Those cells with higher capability of HER2 transactivation under drug pressure conditions will be the ones surviving and supporting the drug resistant population. Similar results were also found when staining for total HER2 (**Figure 31B**), although basal levels in standard conditions did not vary considerably (**Figure 31A**).

These results suggest that in unfavourable conditions not only HER2 activity will drive the drug resistant fate, but it would also be dependent to some extent on the amount of total HER2 that latter cells will be able to transactivate in order to promote cell survival and proliferation.

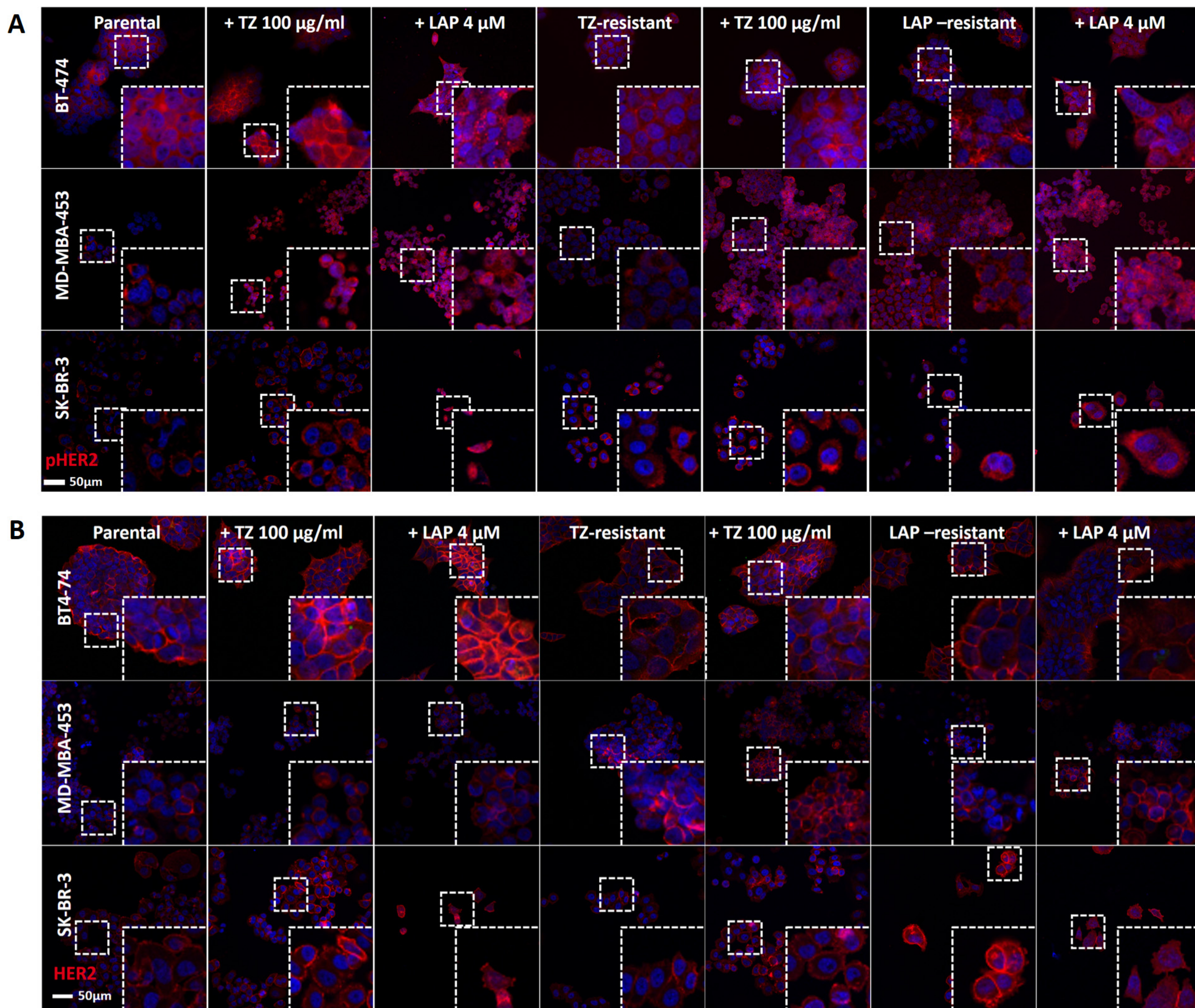


Figure 31. HER2 modulation under HER2-targeted therapies treatment. Representative immunofluorescence images showing HER2 activation (A), (Y1248, phosphorylated levels represented as p-) and total HER2 levels (B), in parental and drug resistant cells lines SK-BR-3, BT-474 and MDA-MB-453 (Trastuzumab resistant, TZ –resistant, and Lapatinib resistant, LAP-resistant), treated with Trastuzumab and Lapatinib (100 µg/ml and 4 µM respectively) for 72 hours. Images are representative of at least 3 independent experiments. Scale bar 50 µm.

2. DRUG RESISTANT GENE SIGNATURE DEFINITION

With the introduction of targeted therapy, drug sensitivity has been shown to be dependent upon the inhibition of different protein networks, that sometimes are redundant in the activation of proliferative and survival pathways.

It is presumable that resistant cells to targeted therapy overactivate compensatory pathways, relying from this moment on in other network nodes.

Keeping in mind the hypothesis that additional genes may play a role in drug sensitivity it becomes urgent in the clinical setting to also extend the knowledge about the genomic patterns driving these protein alterations, that may also be associated with clinical outcome and drug response, and that can be used as powerful and accurate predictive biomarkers. The identification of such powerful predictive biomarkers would provide clinical tools to stratify patients that would benefit from particular therapies, and avoid unnecessary toxicities to those patients that would not respond.

To better explore the genome wide changes associated with the acquisition of resistance to anti-HER2 therapies in breast cancer, we next wanted to determine the molecular profiles of the Trastuzumab and Lapatinib resistant cell lines by developing genomic expression arrays in collaboration with the group of Professor Anne-Lise Børresen-Dale, and Dr. Hege Russnes, at the Genetics Department of the Oslo University Hospital (OUS) in Oslo, Norway, as this group has been pioneer in studying the molecular profiles of breast cancer.

2.1 Genetic arrays development and pre-processing

In order to identify molecular profiles and gene signatures able to predict sensitivity to Trastuzumab and Lapatinib we explored the genome wide changes associated with the acquisition of resistance to these drugs.

To obtain the molecular profiles of the different cell lines, the gene expression arrays were performed in triplicate for each cell line (27 arrays total, with randomized sample distribution, **figure 32A**).

We used the Agilent G4851A Sure Print G3 Human GE 8x60K Microarray Kit, following manufacturer's instructions (see **materials and methods Section 8.1** for further details).

After the scan, data was extracted using the Feature Extraction software. With this software we obtained a QC Report for each one of the arrays performed. QC reports include statistical results that help us evaluate the reproducibility and reliability of the microarray data. All the check-up points for all the microarrays were correct and the arrays passed successfully.

The next step in the data analysis included data normalization that was performed using the Gene Spring software (see procedure details in **Section 8.2** from the materials and method section). From this process a Box Whisker plot was derived, where normalized intensities are represented, and also median values, outliers, quantiles and the distribution of particular samples or conditions are shown **(Figure 32B)**.

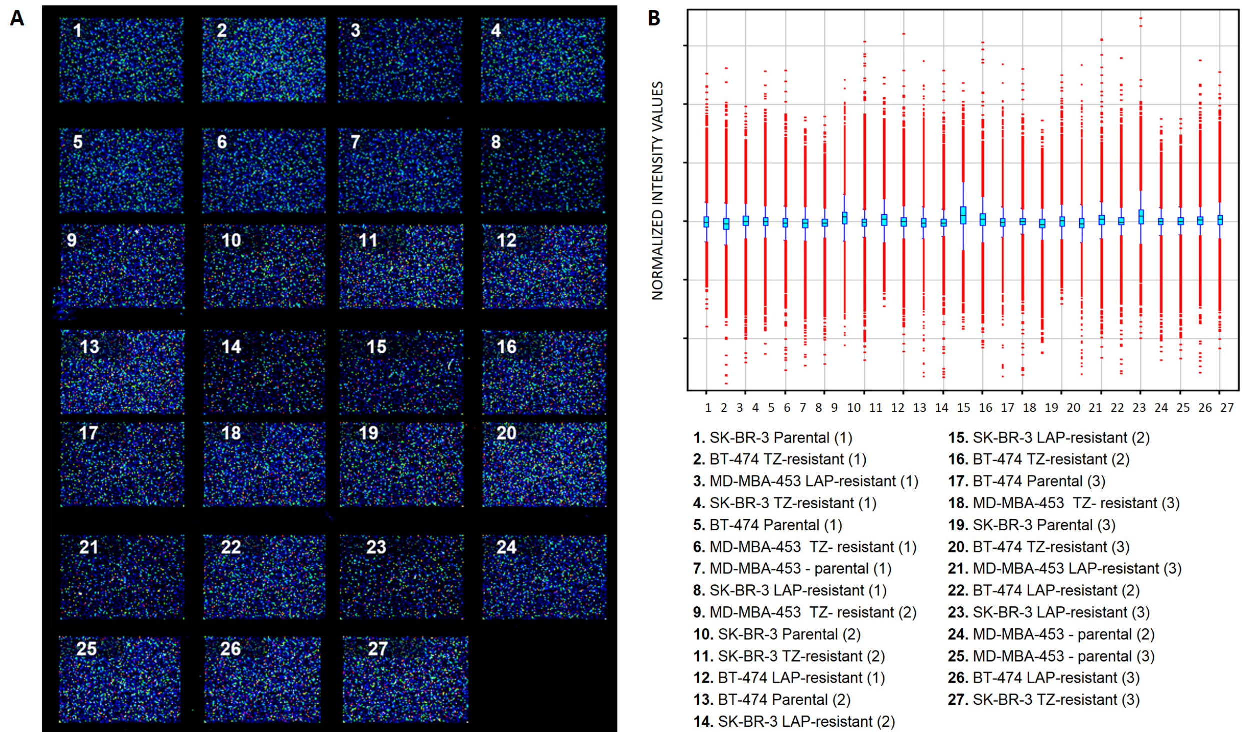


Figure 32. Gene expression arrays pre-processing. A. Resulting images from the scanning process for all 27 gene array expression performed, showing different levels of intensity for each probe in the array. B. Box Whisker plot diagram after intensity normalization (probe intensities are represented as red dots). Blue boxes represent the median and the 25% of genes above and below the median.

2.2 Array analysis: gene signature generation

For the gene signature generation and with a statistician assistance, two statistical methods were independently applied to the expression gene list files derived from the arrays, in order to identify those genes that were differentially expressed between parental and drug resistant cell lines.

We applied ABS (Δmean), a standard values comparative procedure, and DEDS methods, which is a method to measure differentially expressed (DE) genes by aggregating six statistics: t-test, SAM, FC, moderated-t, moderated-F, and B-statistics [443].

We applied both statistic procedures to each cell line, obtaining two lists of differentially expressed genes in each drug resistant cell line compared to their parental cell line for each one of the treatments **(Figure 33A).**

In the next step we identified those genes that overlapped between the two statistical methods, in order two have a more robust list of genes altered during resistance, and we also identified the ones that overlap between drug resistance to Trastuzumab or Lapatinib for each cell line independently, which are represented in the heatmaps **(Figure 33B).**

In a first attempt to investigate the potential clinical relevance of our signatures we first of all analysed the expression of those genes in the MicMa breast cancer cohort [449], in order to observe if in general those genes are typically expressed in breast cancer tumours **(Figure 33C).**

The MicMa cohort database includes clinical and pathological data from 112 women with early-stage breast cancer from the Oslo Micro Metastasis Project [449]. From the heatmaps we observed that in all three cell lines derived signatures some genes were upregulated while others remain downregulated in patients, and all lists showed genes that were highly differentially expressed between groups of clinical samples being able to define clusters of patients (**Figure 33C**).

To better define a list of genes distinctive of HER2 targeted therapy failure, we re-filtered the gene lists again, this time sorting by treatment instead of by cell line. We compared this time all three Trastuzumab resistant cell lines derived differentially expressed genes lists (filtered by ABS, and DEDS independently), obtaining 43 genes that were commonly differentially expressed in all three Trastuzumab resistant cell lines, and that overlapped among both statistical screening methods. Same approach was used for the Lapatinib resistant lines, obtaining this time a final list of 170 genes (**Figure 34).**

In order to get our final HER2 therapies-related resistance signature we fused both lists, excluding those entries representing probes with no known gene associated, obtaining a final list of 194 genes **(Figure 34).**

Complete lists of all gene names included in HER2 therapies resistance signature can be found in **Annex section-A2**.

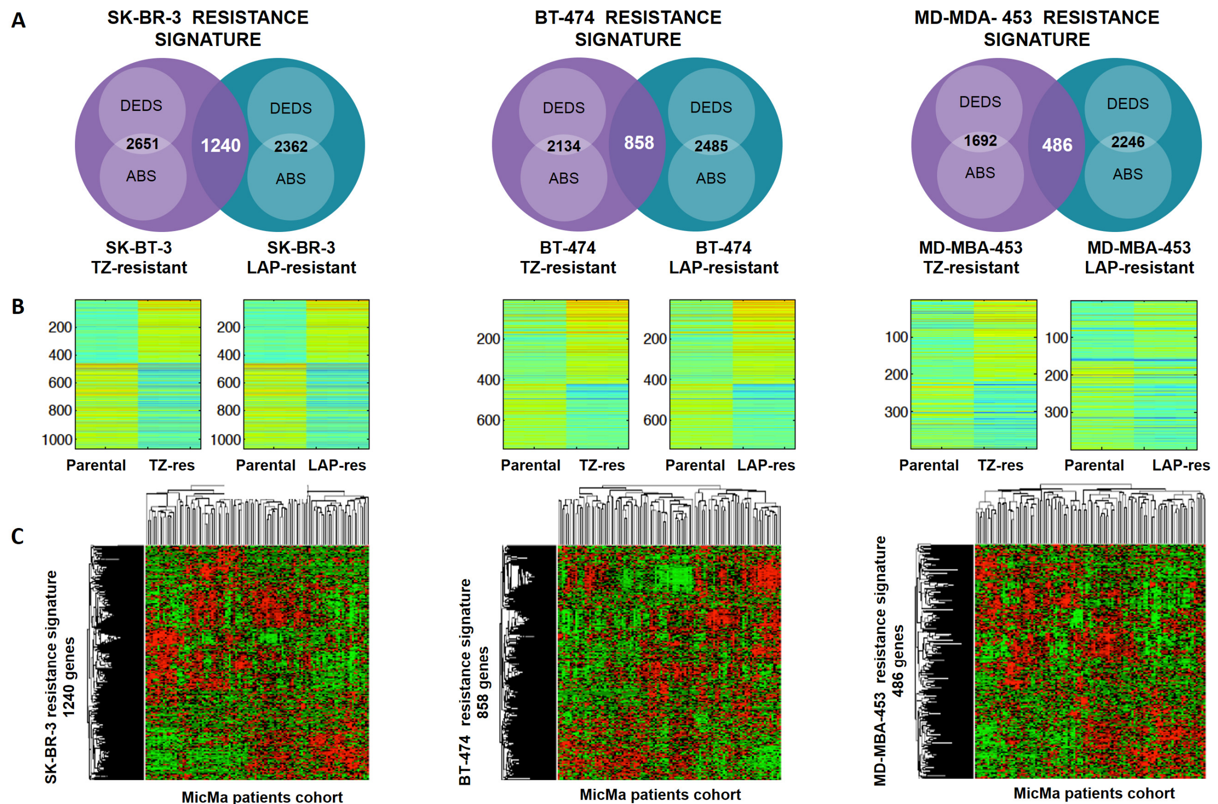


Figure 33. Drug resistant gene signature generation. A. Venn Diagrams showing the overlapping between DEDS and ABS derived differentially expressed genes for each one of the treatments in each one of the cell lines, SK-BR-3 (left), BT-474 (middle) and MDA-MB-453 (right). Also common differentially expressed genes among Trastuzumab and Lapatinib for each cell lines are shown (white numbers in the intersection). B. Heatmap showing the expression of the common HER2 resistance genes for each cell line (Trastuzumab resistant, TZ-resistant, TZ-res and Lapatinib resistant, LAP-resistant, LAP- res). C. For each cell line, the heatmap shows HER2 resistance signature genes expression and distribution in the MicMa BC patient cohort.

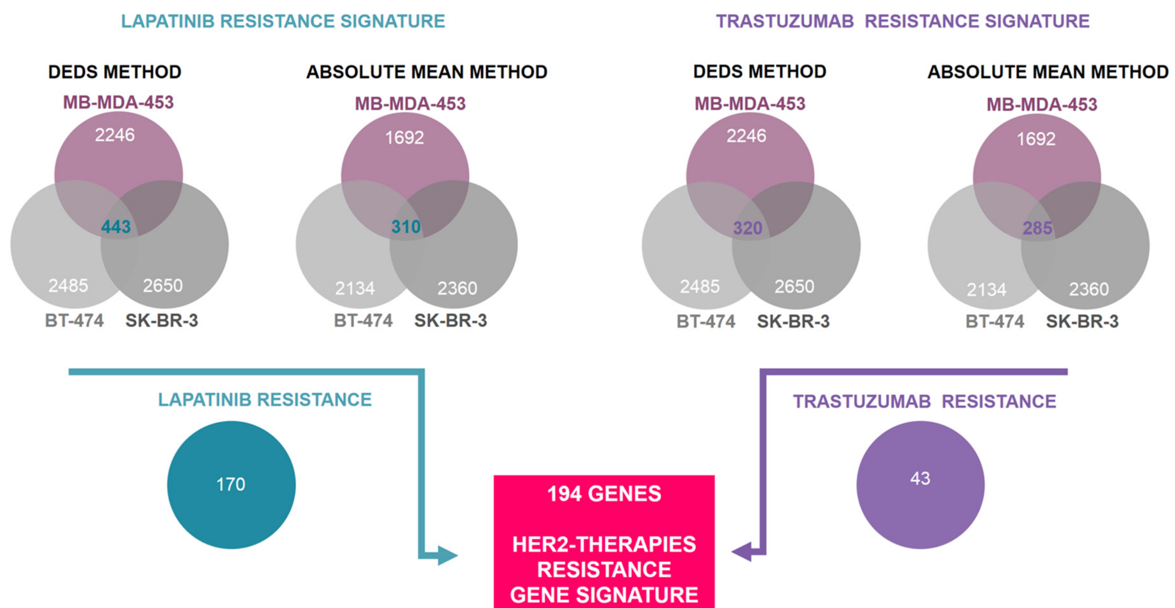


Figure 34. HER2 therapies resistance related gene signature generation. Venn diagrams showing overlap between the different statistical methods used to define the differentially expressed genes for each treatment in all three cell lines under study. The final accomulative HER2 therapies resistance gene signature contains 194 genes.

2.3 Patient correlation: MicMa cohort exploration

As done before as an exploratory method, we explored again the MicMa cohort with our 194 gene list to determine if the derived gene list was able to identify breast cancer patients with poor clinical outcome. At this time we firt selected the HER2+ patients, who may potentially have received Trastuzumab and/or Lapatinib treatments **(Figure 35A)**.

Patient information in combination with genetic expression values from our HER2 signature genes stratified patients in 4 groups with different prognostic outcome. The correlation with survival for these 4 groups is represented in the Kaplan-Meier plots depicting survival **(Figure 35B)**.

Looking at the heatmap area representing the group of patients with poor outcome (group 4) we were able to detect a set of overexpressed genes (SET-1) which may be responsible for poor prognosis and recurrence. In the other hand, looking at the heatmap representing group 3 (the one with higher survival rates) we were also able to identify a set of overexpressed genes, that in this case may define a good prognosis and better outcome group of patients (SET-2).

This preliminary data supports the plausible predicting value of our gene signature, and encouraged us to continue with the study. Further analysis will be done to further filter our gene list to identify those genes able to predicting survival more strongly, and we will also reanalyse our signature within another bigger HER2+ independent cohort as a validation set for our study.

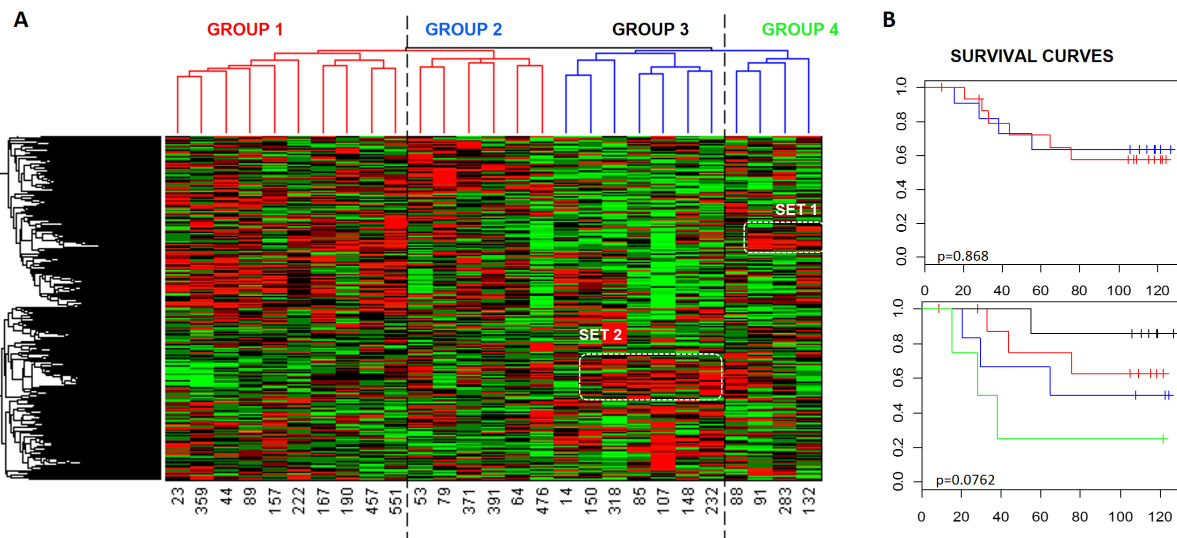


Figure 35. HER2 therapies resistance related gene signature correlation with patient outcome. A. Heatmap representing the expression and distribution of the 194 genes defined as HER2 therapy resistance gene signature. B. Kaplan-Meier plots showing the overall survival in months identify different subsets of genes capable of classifying patients from the MicMa cohort with different clinical outcome.

2.4 Microenvironment exploration

Under the hypothesis that therapeutic treatments can favour clonal selection of cells with unique properties and different fitness for a given microenvironment, and in order to explore if the drug resistant signature previously described also defined a drug resistant genotype with higher microenvironment crosstalk capability, we generated four different microenvironment-gene-related lists including genes codifying for soluble factors known to induce stromal changes, like chemokines, cytokines, matrix remodeling-related enzymes, angiogenic and neurogenic factors (cytokine and chemokine factors-related list, metastasis-related list, angiogenesis-related list, and neurogenic–related list), see **Material and Method section 8.4** for more details in the gene list generation process.

The analysis consisted in crossing each cell line differentially expressed (DE) list of genes (for Trastuzumab and Lapatinib independently) with each one of our microenvironment-related gene list **(Figure 36).**

The resulting lists from each cell line were overlapped, in order to obtain those DE genes related with each one of the microenvironments for each one of the treatments (numbers in the intersection correspond to the number of DE genes for each treatment for a particular microenvironment). We further analysed the common differentially expressed genes for each treatment in each one of our gene lists, and explored the different pathways associated with those selected genes **(annex section -A3).**

The pathway analysis of the sets of common receptors identified, indicated a main relevance for processes related with cell cycle, cellular growth and proliferation, cellular assembly and organization, and more interesting with axonal guidance signalling, agrin interactions at neuromuscular junctions, VEGF ligand-receptor interactions, endothelin-1 signalling and or leucocyte extravasation signalling.

Interestingly, we found genes represented in more than one microenvironment related pathways, and some of them are detailed below.

Lamini beta-1, *LAMB1,* is implicated in a wide variety of biological processes including cell adhesion, differentiation, migration, signalling, neurite outgrowth and metastasis and has been recently described as a potential serological biomarker for colorectal cancer [450]. This gene was found up-regulated in the metastasis-related gene list, as well as in the neurogene and the angiogenesis-related gene lists.

Serpin F1, *SERPINF1,* is responsible of neuronal differentiation [451] and was found upregulated in all four gene lists. Mutations in this gene have been related to osteogenesis imperfecta type VI [452].

Neurophilin 1, *NRP1,* was also found up-regulated in most of the drug resistant cell lines, and is represented in all four microenvironment gene lists. This gene codifies for the Neuropilin-1 protein, a transmembrane glycoprotein with large extracellular domains that interact with both class 3 semaphorins and VEGF, and is involved in the regulation of many

physiological pathways, including angiogenesis. The neuropilins also interact directly with the classical receptors for VEGF, VEGF-R1 and -R2, mediating signal transduction, and has been found over-expressed in pathological angiogenesis in tumours [453].

Validation of the role of some of these genes in the microenvironment crosstalk and further analysis of the different networks in more detail will be done in future studies.

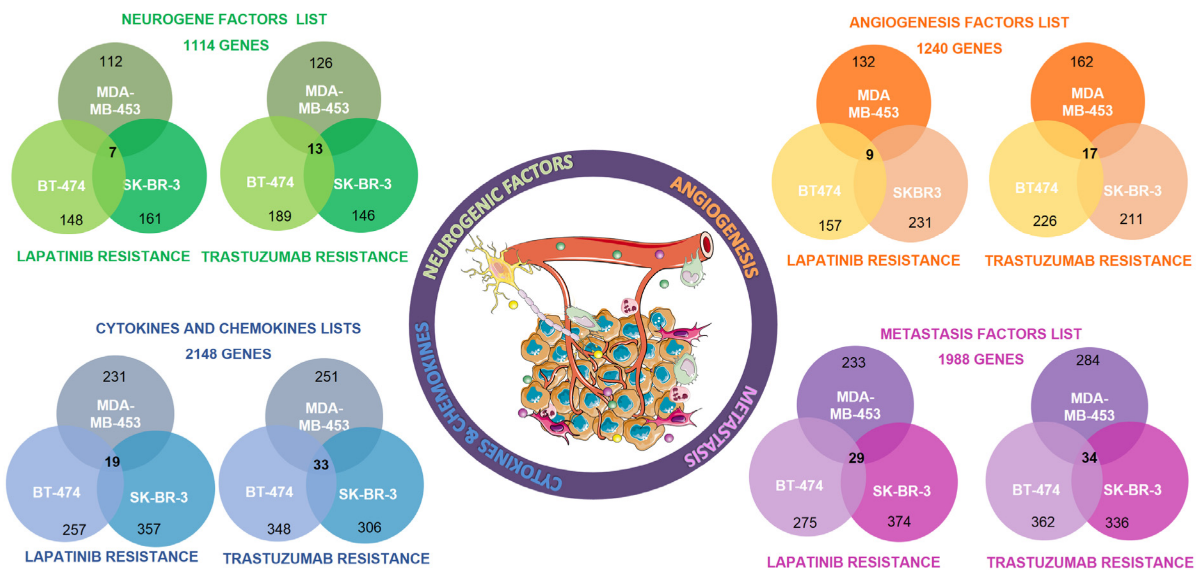


Figure 36. Microenvironment related signatures. Venn diagrams representing the resulting number of genes from each drug resistant cell line differentially expressed gene list that were also found in each one of the generated microenvironment related gene lists: neurogene factors gene list (green, upper left), angiogenesis gene list (orange, upper right), cytokine and chemokine gene list (blue, low left) and metastasis gene list (purple, low right). The number of common genes among the three cell lines for each treatment in every microenvironment is represented in the intersection of each diagram.

3. DIFFERENT PATTERNS OF RTK ACTIVATION IN THE DRUG RESISTANT CELL LINES: DEFINITION OF NEW POTENTIAL TARGETS

3.1 The drug resistant phenotype relies on the compensatory activation of a RTK panel

In order to identify new RTKs that may be responsible for HER2 transactivation, and that could be defined as new therapeutic targets for patients who are resistant to anti-HER2 therapies, we performed a high throughput proteomic screening to identify tyrosine kinase receptors differentially overactivated in the drug resistant cell lines. A complete list of the 42 RTK present in the array is compiled in **Annex section –A4).**

We identified some receptors overactivated in the Trastuzumab and Lapatinib resistant cell lines, compared with the parental cell lines profile, that have been previously related to failure to anti-HER2 therapies such as IGFR, HGFR and some Ephrin receptors, what suggests that our system reproduces some already known resistance mechanisms. Interestingly, we also identified other receptors with not previously known relation with HER2 resistance **(Figure 37A).**

For each treatment we selected as potential target receptors to continue with the study those receptors that were at least differentially overactivated in two out of the three drug resistant cell lines in our model, identifying 13 and 7 receptors commonly overactivated in the Trastuzumab and Lapatinib resistant cell lines respectively, 4 of them were commonly overactivated in both models **(Figure 37B).**

In order to explore a bit more the biology of this receptors we used the Ingenuity Pathway Analysis (IPA), to explore molecular and chemical interactions, cellular and biological phenotype information, and disease processes related to our set of receptors.

The analysis of the protein pathways associated with the signalling of those receptors indicated a major relevance for PTEN and NF-κβ pathways, cell signalling and cell cycle, cellular growth and proliferation, and interestingly an association with axonal guidance signalling and human embryonic stem cell pluripotency **(Figure 38).**

Consequently, with our objective, and taking into account the biological significance of each receptor we continue our study focusing on the Fibroblast Growth Factor Receptor 2 (FGFR2) and the RET proto-oncogene from the glial cell line-derived neurotrophic factor (GDNF) family. From the graphic representation of the phylogenic tree for the RTK selected for the study we observed that these two receptors have closed origins **(Figure 38).**

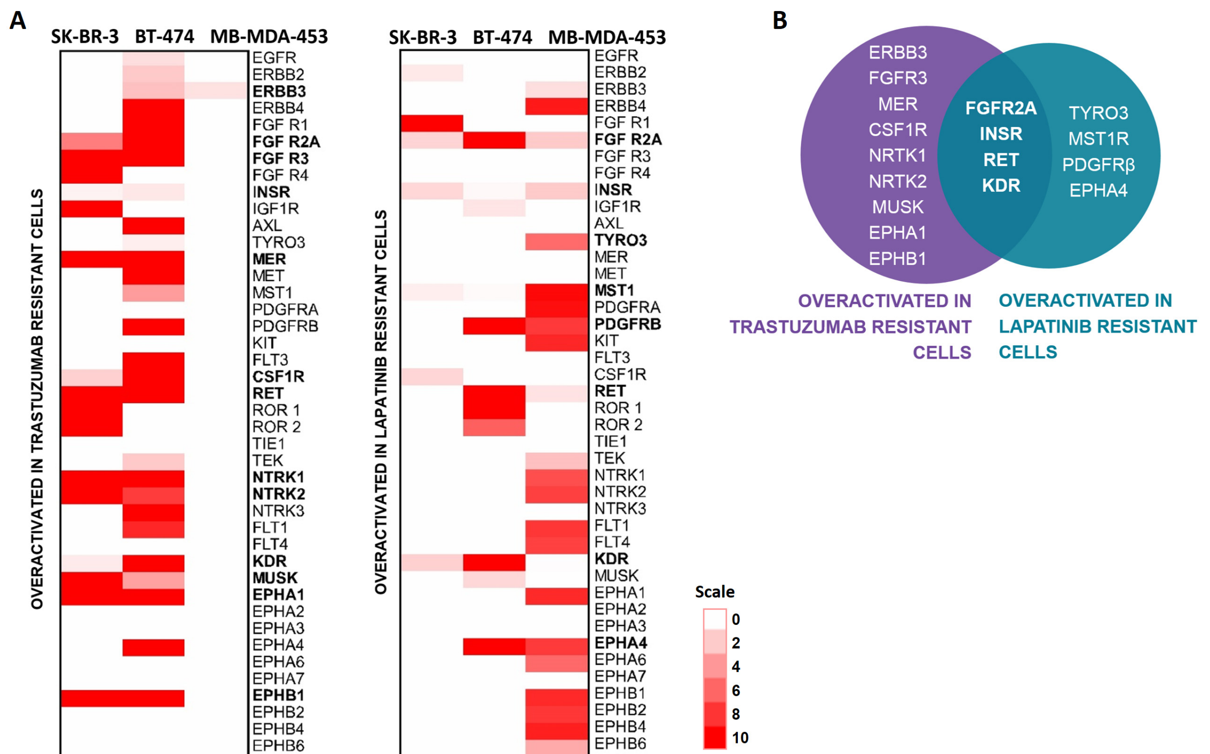
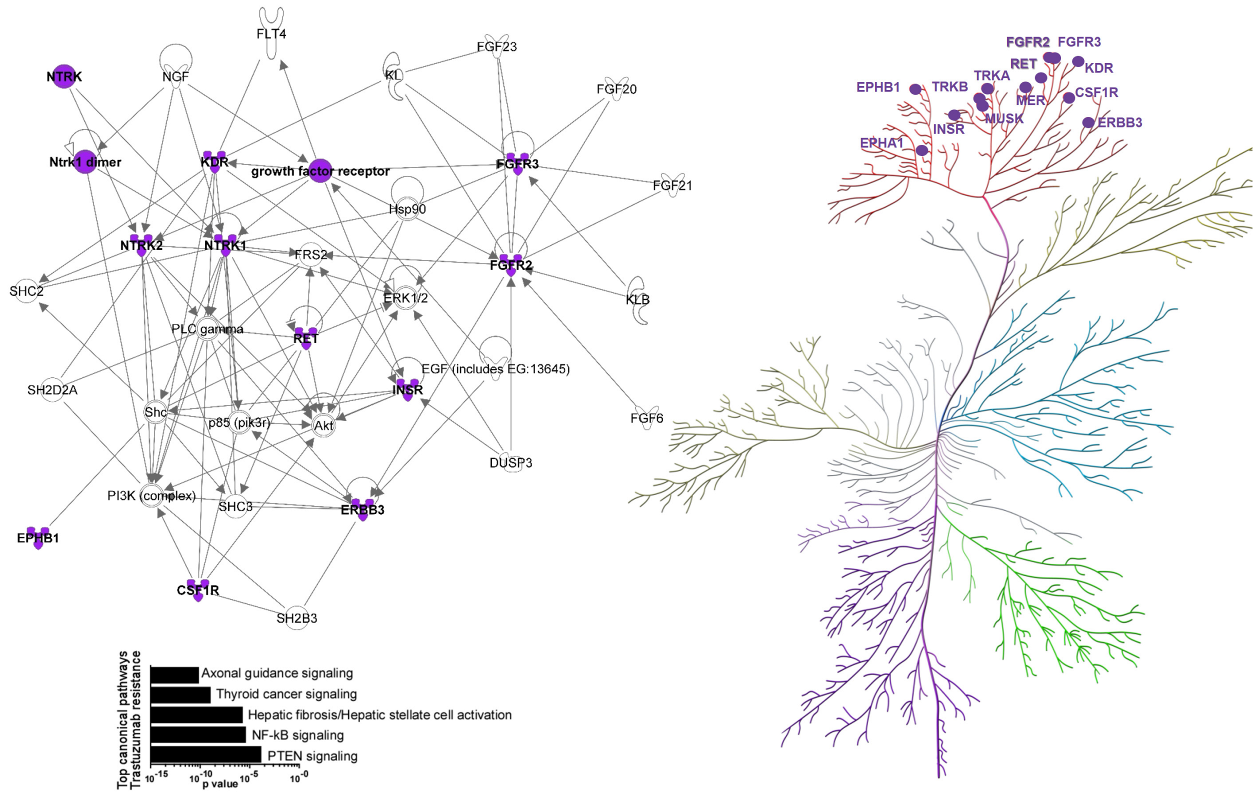


Figure 37. RTK screening. A. Heatmap depicting the relative active status for the 42 RTKs in the study. For each receptor, the mean value of the densitometric quantification in the resistant cell lines was referred to the parental. The data represent the mean from 3 independent experiments. B. Venn Diagram representing those RTKs that were overactivated at least in three out of the six resistant cell lines. Common differentially expressed genes among Trastuzumab and Lapatinib are shown in the intersection.

A



B

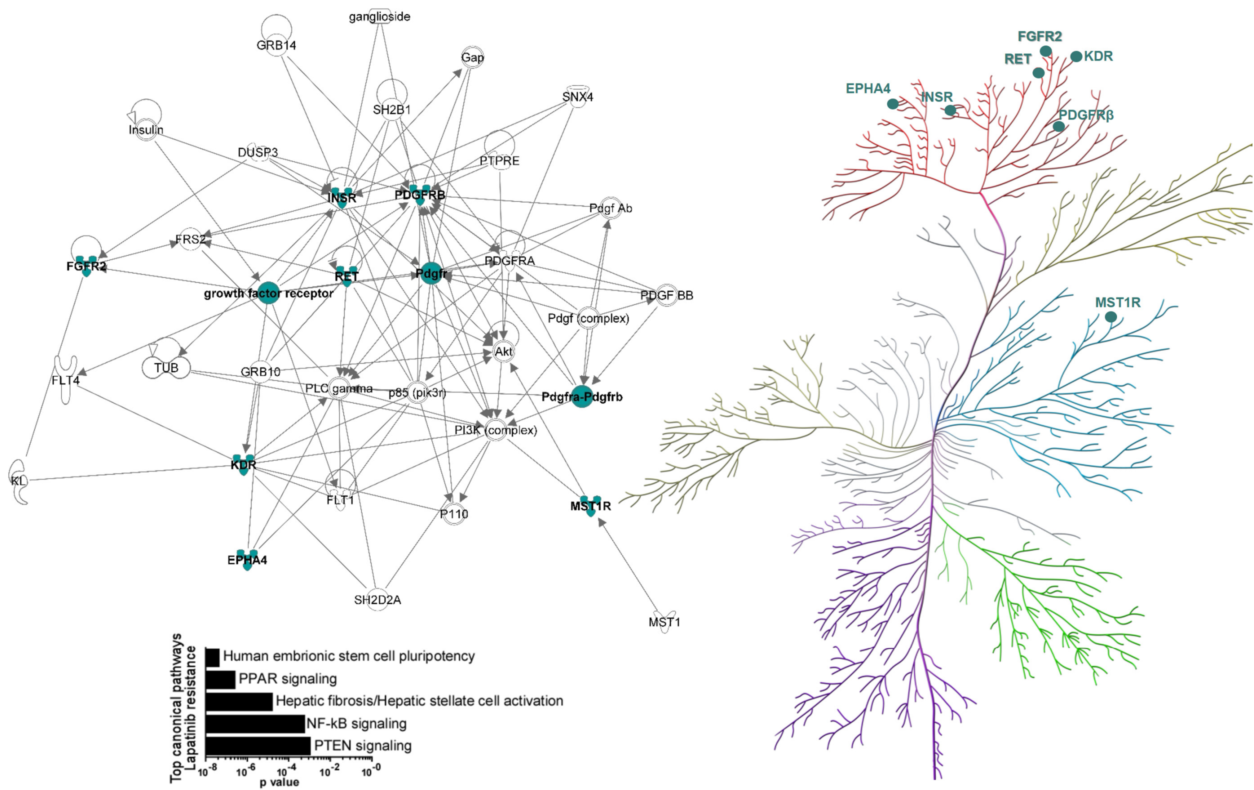


Figure 38. HER2 therapies resistance RTK related receptors. Representative functional network study and top canonical pathways for the main Trastuzumab resistance (A) and Lapatinib resistance (B) related RTKs using IPA software ,and phylogeny tree distribution using Kinase Mapper tool.

3.2 RET and FGFR2 activation under drug stress

In order to confirm that RET and FGFR2 have an important role in the drug resistant phenotype stablishment, we first analysed the response of these two receptors in the different cell lines in terms of receptor activation under drug stress (**Figure 39 A-B**). As expected, we found that in basal conditions drug resistant cell lines express equal or higher levels of basal protein phosphorylation than parental cells. Furthermore, after Trastuzumab and Lapatinib treatment, FGFR2 and RET receptors were further activated both in the parental and drug resistant cell lines but always at a higher degree in the drug resistant lines, (being the Lapatinib resistant cell lines the most responsive ones), which suggest a higher dependence of the drug resistant cell lines on those receptors (**Figure 39**).

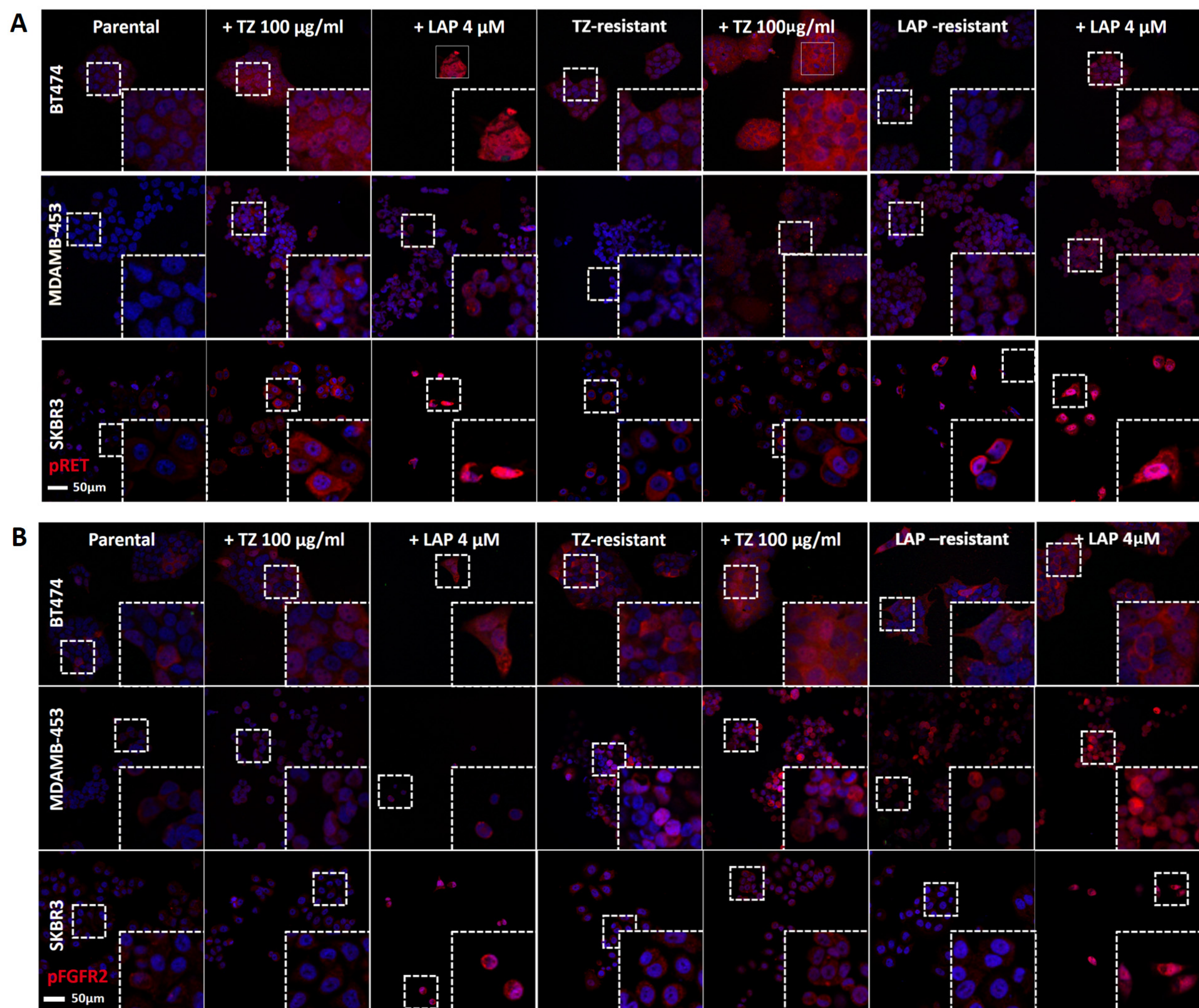


Figure 39. RET and FGFR2 response under HER2-target therapies treatment. Representative immunofluorescence images showing the expression of activated RET levels (A), (Y905, phosphorylated levels represented as p-) and FGFR2 levels (B) (Y653-654, phosphorylated levels represented as p-), in parental and drug resistant cells lines SK-BR-3, BT-474 and MDA-MB-453 (Trastuzumab resistant, TZ –resistant, and Lapatinib resistant, LAP-resistant), treated with Trastuzumab and Lapatinib (100 µg/ml and 4 µM respectively) for 72 hours. Images are representative of at least 3 independent experiments. Scale bar 50 µm.

These results demonstrate the implication of the signalling pathways related with the biology of these receptors in the drug resistant mechanism, contributing somehow to the drug resistant phenotype described before.

Interestingly, the response activation pattern of RET and FGFR2 correlates with the pattern seen for HER2 (**Figure 31**), which adds evidences for the proposed transactivation process.

In this way, in our resistance model, we proposed that drug pressure, that initially inhibits HER2 cell's dependence, would result in an activation of alternative pathways that after being activated are capable of transactivating HER2 to bypass drug inhibition.

4. RET AND FGFR2 AS NEW TARGETS FOR RECURRENCE

4.1 *In vitro* inhibition and molecular mechanism: effect on survival and proliferation

Following with our study, we next evaluated the effect of FGFR2 and RET inhibition on cell survival by using two tyrosine kinase comertial chemical inhibitors.

Ponatinib is an oral ATP-competitive multitargeted tyrosine kinase inhibitor that is in advanced clinical experimentation in leukaemia.

Its kinase selectivity profile characterization showed that it exhibits potent *in vit*ro biochemical activity with IC50 values <20nmol/L against all 4 FGFR, and also inhibited immunopurified RET kinase at an IC_{50} of 25.8 nM [454-457].

BGJ-398 is also a pan FGFR kinase inhibitor, acting as a potent inhibitor of human FGFRs with potential antiangiogenic and antineoplastic activities [458, 459].

First of all we evaluated the effect of these two inhibitors in all cell lines *in vitro* by performing proliferation assays (**Figure 40**). We found that both inhibitors were efficient in inhibiting cell survival, and that FGFR2 and RET dual inhibitor Ponatinib was more potent inhibiting proliferation inhibition than the pan-FGFR specific inhibitor BGJ-398, in agreement with the synthetic lethality concept nowadays applied also in the context of cancer therapy [460].

From the classical genetic concept, the word synthetic is used in the sense of “synthesis or coming together”, defines a situation where inhibiting one single target do not have a lethal effect, but combining the inhibition of more than one target will result in cell death. Here we can see how similar anti-FGFR drug concentrations produce a survival reduction of less than 50 % while dual inhibition of RET and FGFR2 with the same drug concentration reduces cell survival in more than the 80 % **(Figure 40, red boxes).**

Furthermore, in the MDA-MB-453 model we found that the drug resistant cell lines present lower IC50 compared to the parental suggesting that these cell lines are more sensitive to FGFR2/RET inhibition. This is in agreement with the assumption that in the drug resistant cell lines, the FGFR2 and RET pathways are more active and critic for their survival.

To further characterize the mechanism of action of these drugs in our model we performed and apoptotic annexin-V assay. The analysis of the annexin-V population in the parental and drug resistant MDA-MB-453 showed the ability of Ponatinib and BGJ-398 in promoting cell death in the drug resistant cell lines, while Trastuzumab and Lapatinib had no effect. Induction of apoptosis by Ponatinib and BGJ-398 was found also in the parental line (with similar effect due to treatment with Trastuzumab) but again, in concordance with the proliferation assay, was more effective in the drug resistant lines **(Figure 41).**

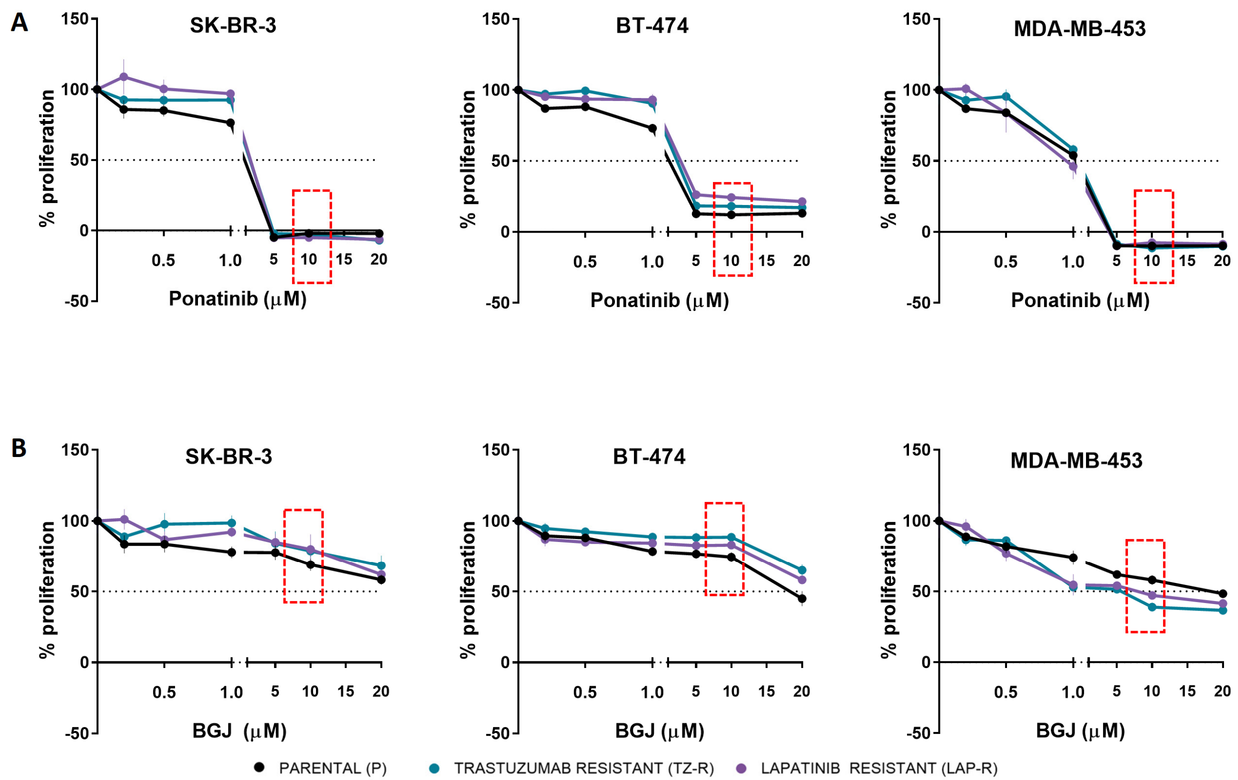


Figure 40. RET and FGFR2 *in vitro* inhibition. Cell viability quantification for SK-BR-3, BT-474 and MDA-MB-453 cells treated for 72h with increased doses of Ponatinib (0-20 μM, A. and BGJ-398 (0-20 μM, B. For each cell line, parental cell lines are represented by a black line, while Trastuzumab and Lapatinib resistant cell lines are represented with the turquoise and purple lines respectively. Graphs show the percentage of cell proliferation for each dose. Data represent mean ± S.D.

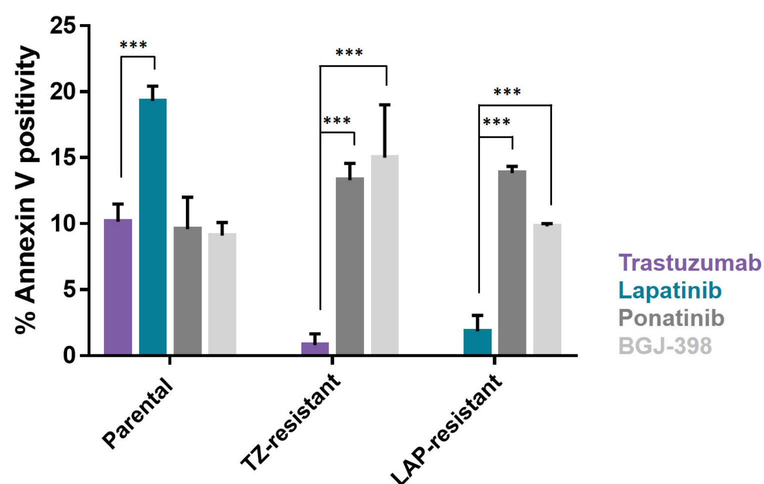


Figure 41. RTK inhibitors effect on apoptosis induction. Graph representing the percentage of Annexin-V positive cells in MDA-MB-453 parental and drug resistant (Trastuzumab resistant, TZ –resistant, and Lapatinib resistant, LAP-resistant) cell lines treated for 72 h with Trastuzumab (100 μg/ml, purple bars), Lapatinib (4 μM, turquoise bars), Ponatinib (2 μM), deep grey bars) and BGJ-398 (2 μM, light grey bars). Data represent mean ± S.D. for a minimum of 3 replicates. Significant differences by ANOVA with Sidak Multiple Comparison post-hoc test are indicated as * p<0.05, ** p<0.01 and *** p<0.001.

In agreement with these results, the immunoblotting analysis of AKT protein activation (pAKT) reflected the dependence of the drug resistant lines in those RTK alternative pathways **(Figure 42).** Treatments with Ponatinib and BGJ-398 in the parental line induced survival pathways activation as reflected by increased AKT phosphorylation levels, while in the drug resistant lines pAKT levels were reduced under drug treatments **(Figure 42).** This could be explained by the fact that in the parental cell lines survival and proliferation depends mainly on HER2 downstream pathways, and the inhibition of RET and/or FGFR2 pathways is considered a minor disturbance that could be balanced through HER2 signalling. On the contrary, the drug resistant lines, that deeply depends on FGFR2 and RET, are not able to overcome the effect of RET/FGFR2 inhibition since these pathways are vital for their survival. In the Lapatinib resistant model, (the one that more intensely depends on the alternative pathways) receptor inhibition also resulted in the inhibition of proliferative instigating signals, measured as ERK phosphorylation levels **(Figure 42).**

Finally, when combining Ponatinib and BGJ-398 inhibitors with Trastuzumab and Lapatinib in *in vitro* models we found an interesting additive cytotoxicity in all cell lines, parental and drug resistant **(Figure 43)**, which encouraged us to test the effect of these drugs combinations *in vivo* using xenograft models.

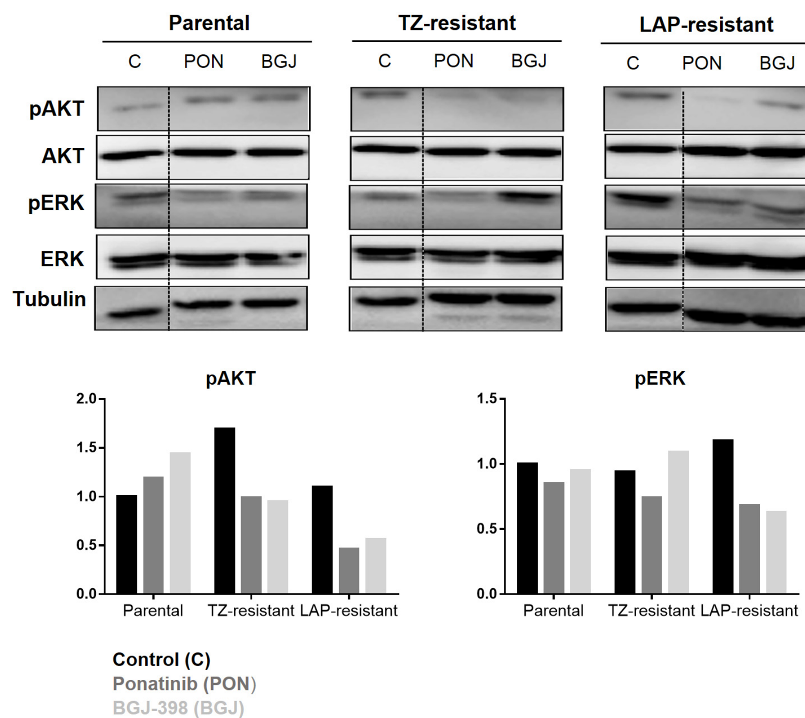


Figure 42. RET and FGFR2 inhibition effect on proliferative and survival pathways. Representative images of western blot analyses showing the phosphorylation (denoted by p-) and total levels of p42/44-MAPK and AKT, in MDA-MB-453 parental and drug resistant cell lines (Trastuzumab resistant, TZ –resistant, and Lapatinib resistant, LAP-resistant), treated with Ponatinib (PON, 2 μM) and BGJ-398 (BGJ, 2 μM) for 72 hours. Graphs below show the densitometric quantification of the western blot normalized with tubulin, which was used to ensure equal protein loading.

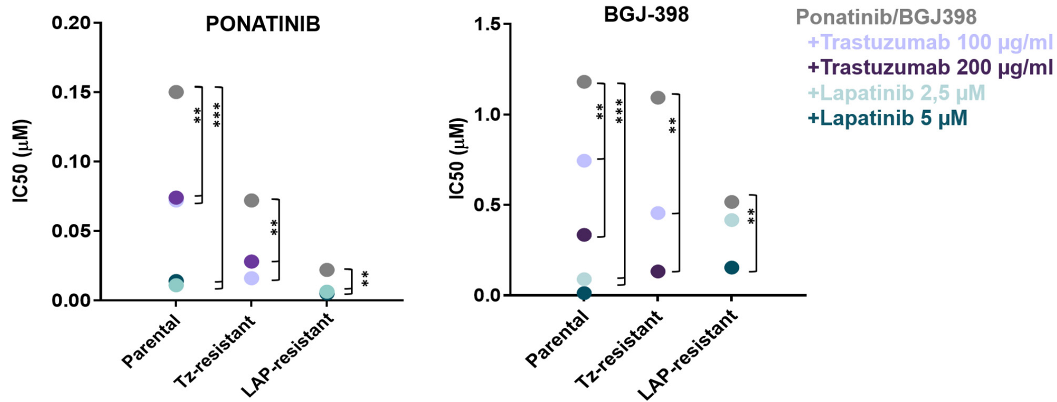


Figure 43. Ponatinib and BGJ-398 additive effect with current anti HER2 therapies. MTT assays comparing the IC50 for Ponatinib (left graph) and BGJ-398 (right graph), (both represented by grey dots) treated with different doses of Trastuzumab (purple dots, 100 µg/ml light purple and 200 µg/ml darck purple) or Lapatinib (turquoise dots, 2,5 µM light turquoise, 5 µM dark turquoise). Dots represent IC50 (95% confidence interval for each treatment) in MDA-MB-453 parental and resistant (Trastuzumab resistant, TZ –resistant, and Lapatinib resistant, LAP-resistant) upon 72 hours of treatment. Significant P values are indicated as * p<0.05, ** p<0.01 and *** p<0.001.

4.2 *In vivo* combination of Trastuzumab and Lapatinib with FGFR2 and RET inhibitors in BC xenograft models

In order to study if the inhibition of the FGFR2 and/or RET pathways also causes a cytotoxic effect in xenograft derived tumours, we orthotopically injected MDA-MB-453 BC cells (parental, Trastuzumab resistant and Lapatinib resistant) into two mammary fat pad per mouse. Once tumours reached volumes of $100mm^3$, randomized groups including five mice per group were defined to start with treatments for 21 days (**Figure 44 A**). As shown in the animal weight curves, all tested drug combinations had no toxic effect on animal's health (**Figure 44 B**).

Drug resistant derived tumours growth curves showed an important effect on tumour reduction for the tested drugs **(Figure 45).** Interestingly, Ponatinib and BGJ-398 alone have stronger effects on cell survival and tumour reduction than current drugs Trastuzumab and Lapatinib monotherapy, in parental and drug resistant cell lines derived tumours, confirming again that the drug resistant cell lines depend on FGFR2/RET pathways for survival and proliferation, and suggesting that RET/FGFR2 inhibition could be used as a second line treatment for those patients that progress after HER2 targeted therapies.

From these results we also observed that these drugs seem to have stronger effects *in vivo* than the ones we had seen with the *in vitro* models, where Ponatinib and BGJ-398 showed similar effect as Trastuzumab. This appreciation suggests a possible influence from the tumour microenvironment (absent in the *in vitro* procedures).

In addition, the drugs also add in some way efficacy to HER2 therapies when combined, evidenced by the last day volume graphs in the drug resistant models, were the more efficient treatment was in both cases a combinatory setting.

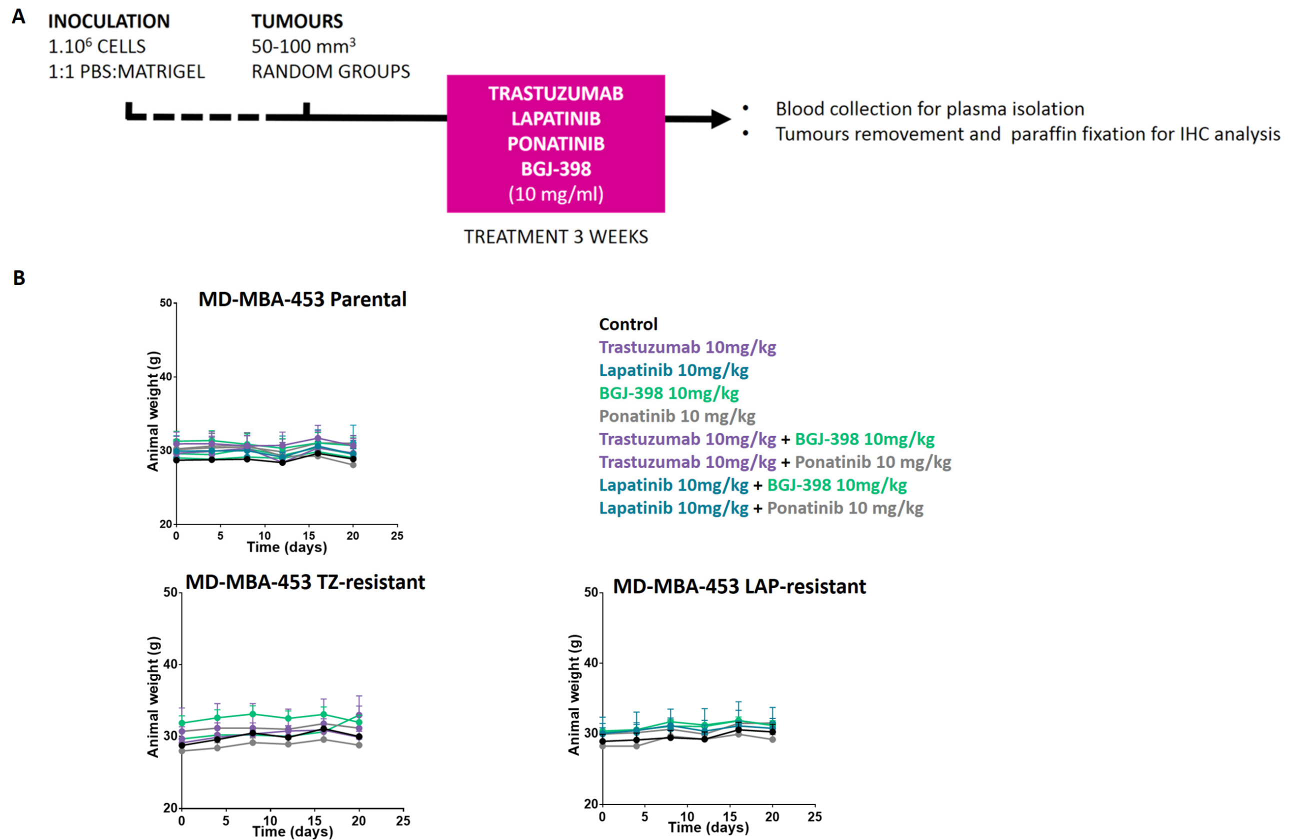


Figure 44. *In vivo* combination of Trastuzumab and Lapatinib with FGFR2 and RET inhibitors in BC xenograft models. A. Diagram showing the *in vivo* approach, including timing and drug dosage and administration for each drug used in the study. B. Graphs represent animal weight over time for each group of treatment during the 21 days of treatment, showing no systemic toxicity neither for the treatments or for drug solvents. Data represent mean ± S.D.

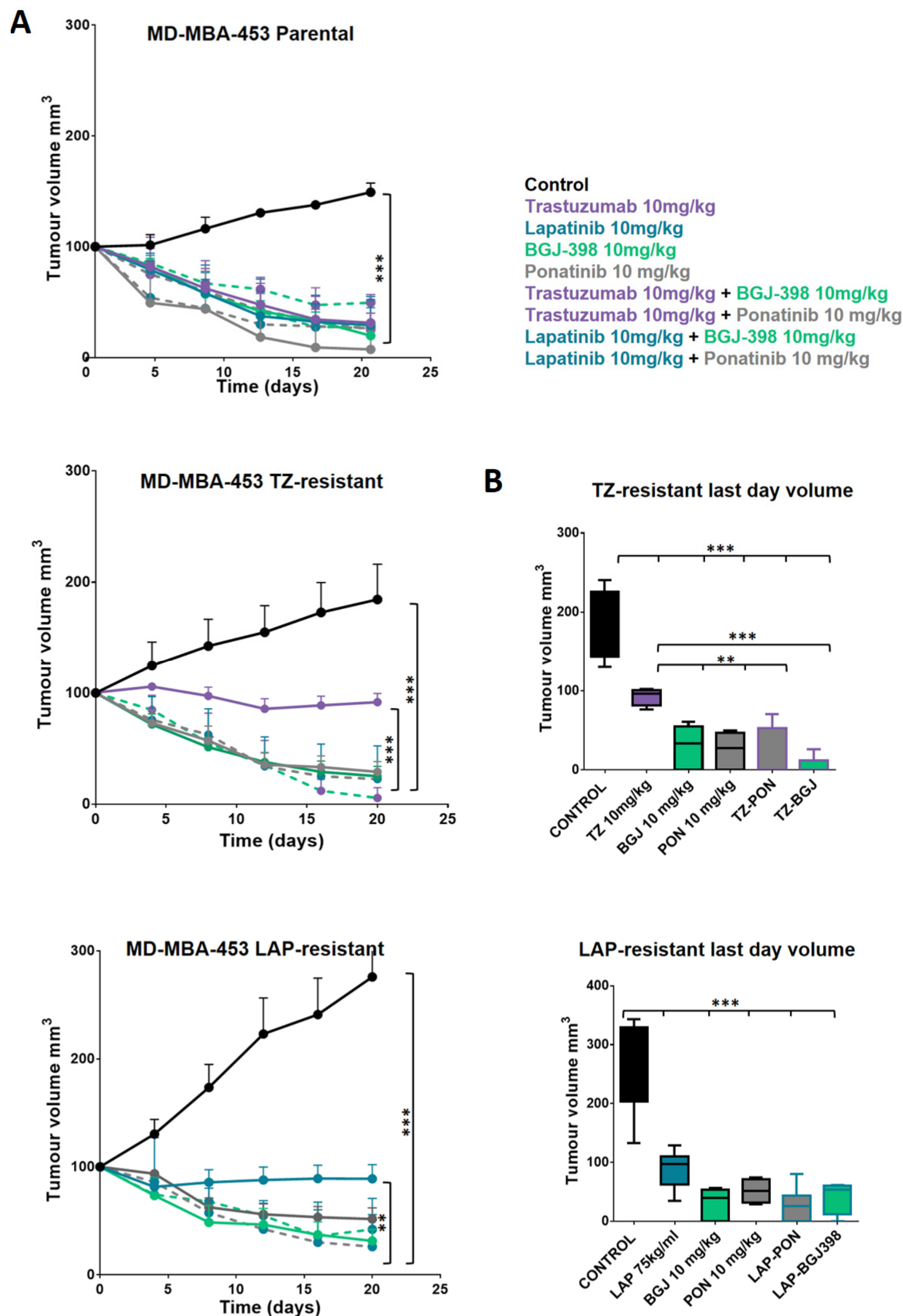


Figure 45. Modulation of tumour growth *in vivo* by FGFR2 and RET inhibitors alone or in combination with anti HER2 therapies in MDA-MB-453 BC xenograft models. A. Graphs showing tumour growth *in vivo* in control or treated animals with either Trastuzumab (10mg/kg, purple), Lapatinib (10mg/kg, turquoise), BGJ-398 (10mg/kg, green) or Ponantinib (10mg/kg, grey) or a combination for 21 days. The data are expressed as tumour volume reduction with day 0 treatment as a reference. Data represent mean of each group (five animals) ± S.D. B. Graphs showing the tumour volume at day 21 for each group of treatment. Data represent mean of each group (five animals) ± S.D. Significant differences by ANOVA with Sidak Multiple Comparison post-hoc test are indicated as * p<0.05, ** p<0.01 and *** p<0.001.

Once treatment was finished, animals were anesthetized and tumours were surgically recovered, fixed in PFA 4 % and embedded in paraffin for further immunocytochemistry analysis of HER2, and our targets under study, FGFR2 and RET.

Regarding HER2 total protein expression, we found that treatments with Ponatinib and BGJ-398 had little effect on HER2 expression (**Figure 46**). Contrary, in the same way to what happened with tumour reduction, we found a consistent higher inhibition in HER2 expression within the panels showing the combinatory administration with Trastuzuamb and Lapatinib (**Figure 46**).

FGFR2 staining was stronger in drug resistant tumours as well as in those parental line derived tumours treated with HER2 therapies, and also after Ponatinib and BGJ-398 treatments (**Figure 47**).

The effect of the inhibitors on p-FGFR2 was further studied in cell lines, and we found that both inhibitors decreased p-FGFR2 levels in Trastuzumab and Lapatinib resistant cell lines (**Figure 48A**).

In the drug resistant tumours, the small amount of cells that were able to survive after chronic exposure to drug, were the ones with higher receptor expression (reduced when drugs were combined with anti-HER2 therapies), were the drug was non efficient enough.

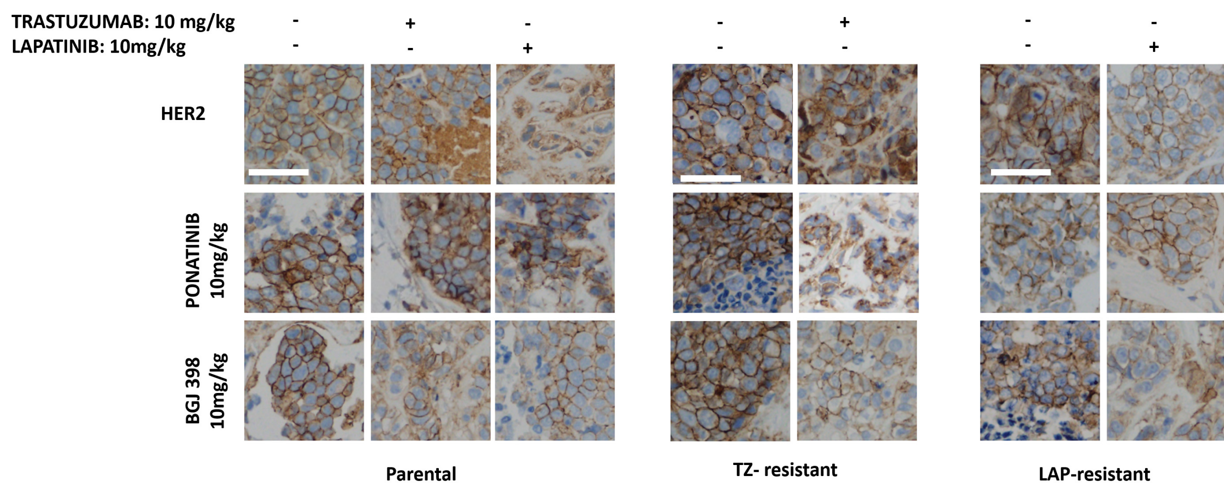


Figure 46. Modulation of HER2 expression in tumours by treatment with FGFR2 and RET inhibitors alone or in combination with anti HER2 therapies in MDA-3B-453 BC xenograft models. HER2 immunohistochemistry representative images in the tumours from the MDA-MB-453 xenograft model from control animals or treated with either Trastuzumab (10mg/kg, 2nd and 5th column), Lapatinib (10mg/kg, 3rd and 7th column), BGJ-398 (10mg/kg, 3rd row) or Ponantinib (10mg/kg, 2nd row) or a

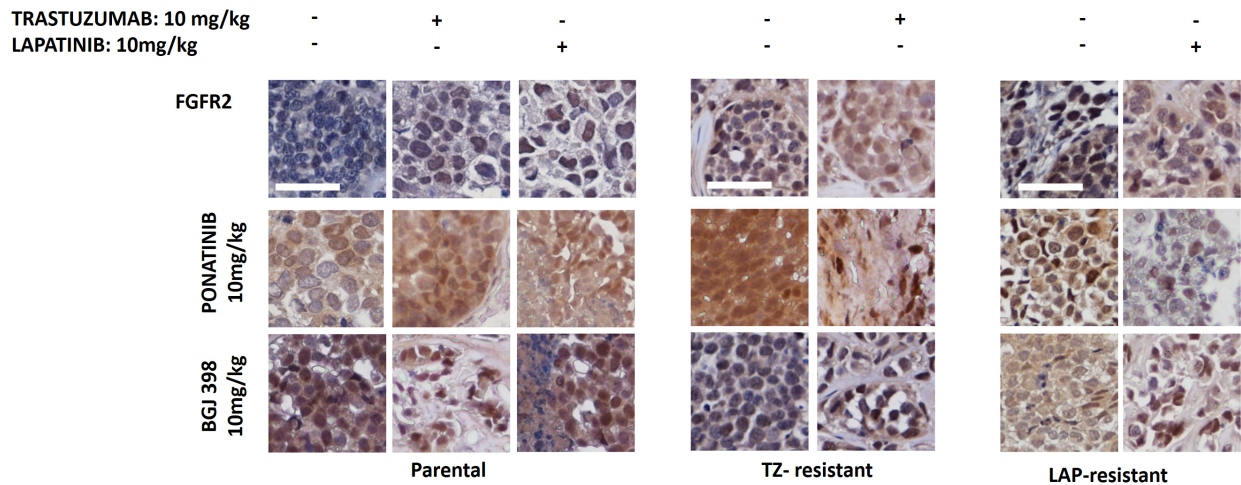


Figure 47. Modulation of FGFR2 expression in tumours by treatment with FGFR2 and RET inhibitors alone or in combination with anti HER2 therapies in MDA-3B-453 BC xenograft models. FGFR2 immunohistochemistry representative images in the tumours from the MDA-MB-453 xenograft model from control animals or treated with either Trastuzumab (10mg/kg, 2nd and 5th column), Lapatinib (10mg/kg, 3rd and 7th column), BGJ-398 (10mg/kg, 3rd row) or Ponantinib (10mg/kg, 2nd row) or a combination for21 days. Scale bar 50 µm.

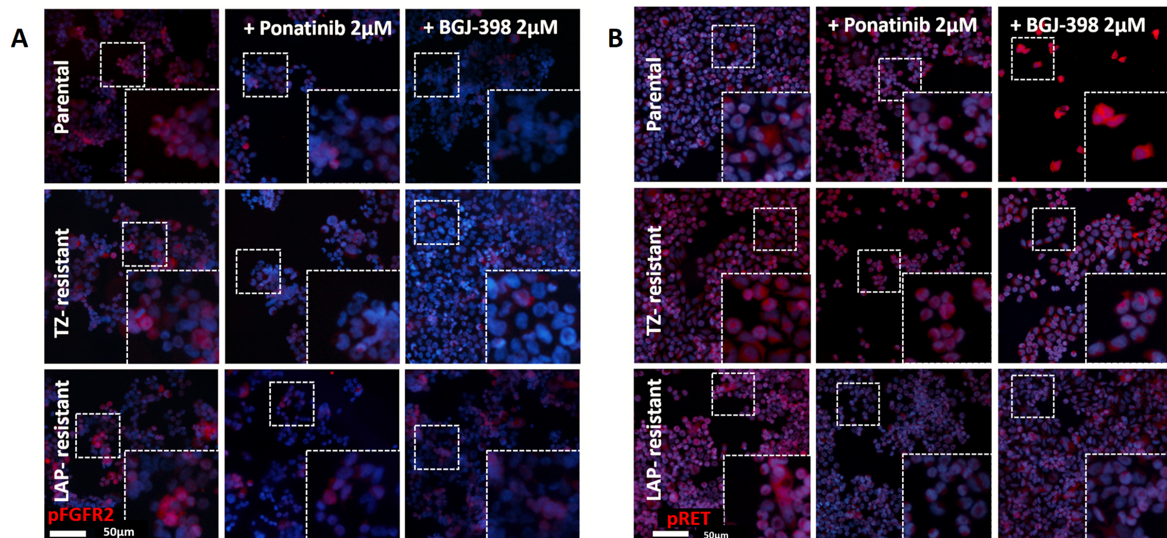


Figure 48. Inhibitory effect of Ponatinib and BGJ-398 effect on RET and FGFR2 activity. Representative immunofluorescence images showing the expression of activated FGFR2 levels (A) (Y653-654, phosphorylated levels represented as p-) and activated RET levels, (B) (905, phosphorylated levels represented as p-) in parental and resistant cells lines MDA-MB-453 (Trastuzumab resistant, TZ –resistant, and Lapatinib resistant, LAP-resistant), treated with Ponatinib and BGJ-398 (2 µM) for

Same pattern was also found when RET levels were evaluated (**Figure 49**). Again*, in vitro*, RET phosphorylation levels decreased in all cell lines when treated with Ponatinib and BGJ-398 (**Figure 48 B**)**.** As a summary, these results showed that the inhibition of FGFR2 and RET pathways in the drug resistant dependent cell lines derived tumours resulted in a reduction of the tumour mass *in vivo* (or cell death *in vitro*), because in this context the activation of the proliferative and survival pathways by FGFR2 and/or RET is compromised. However, under chronic exposure, those clones with higher expression of the targeted receptors will be the ones able to bypass protein inhibition and survive.

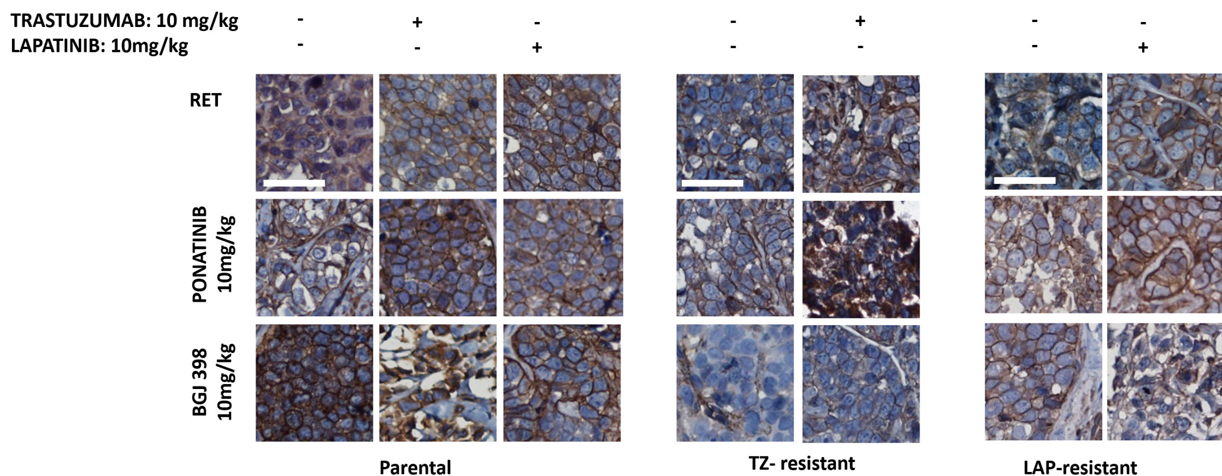


Figure 49. Modulation of RET expression in tumours by treatment with FGFR2 and RET inhibitors alone or in combination with anti HER2 therapies in MDA-3B-453 BC xenograft models. RET immunohistochemistry representative images from control animals or treated with either Trastuzumab (10mg/kg, 2nd and 5th column), Lapatinib (10mg/kg, 3rd and 7th column), BGJ-398 (10mg/kg, 3rd row) or Ponantinib (10mg/kg, 2nd row) or a combination for 21 days. Scale bar 50 µm.

Under the hypothesis that the tumour microenvironment may be influencing tumour cells behaviour and drug response, and keeping in mind that one of our targets belonged to the fibroblast growth factor receptor family, we also stained for αSMA in order to analyse the main microenvironment population in breast tissue fibroblasts.

As mentioned, FGFR2 is a receptor known to be also expressed in fibroblast, and interestingly, when we analysed the fibroblast population in tumours we found a reduction in fibroblasts infiltration upon FGFR2 inhibitory treatments (which was also more evident when combined with Trastuzumab and Lapatinib, **figure 50**)**.**

These results suggest that fibroblast may exert an important function in tumour biology, and in our case in the maintenance of the HER2 phenotype.

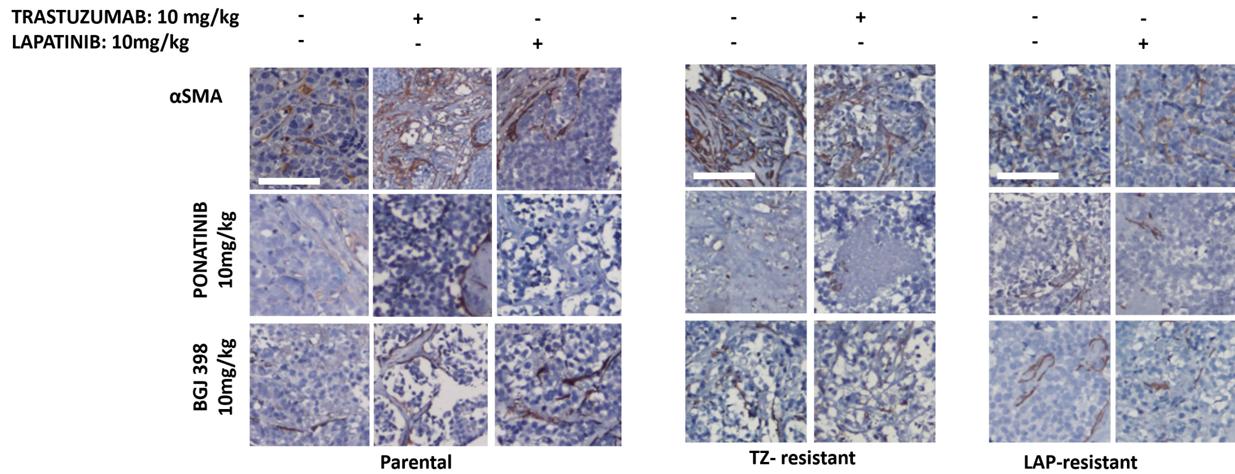


Figure 50. Modulation of αSMA expression *in tumours* by treatment with FGFR2 and RET inhibitors alone or in combination with anti HER2 therapies in MDA-3B-453 BC xenograft models. αSMA Immunohistochemistry images in the tumours from animals control or treated with either Trastuzumab (10mg/kg, 2nd and 5th column), Lapatinib (10mg/kg, 3rd and 7th column), BGJ-398 (10mg/kg, 3rd row) or Ponantinib (10mg/kg, 2nd row) or a combination for21 days. Scale bar 50 µm.

4.3 Preliminary patient RET and FGFR2 expression exploration

With the previous data from the *in vitro* and *in vivo* experiments we have seen that upregulation of HER2 correlates with RET/FGFR2 overexpression and with the acquisition of a drug resistant phenotype in the BC cell lines and the xenograft derived tumours.

In order to explore if this is also found in patients we stained a small group of breast cancer samples (n=30) for HER2, RET and FGFR2 (**Figure 51**).

All samples show positive staining for RET and FGFR2. From all the samples analysed, we found that RET and FGFR2 high expression was more often found in HER2+ tumours rather than in HER2- .

83% of the HER2+ samples expressed elevated amounts of RET and FGFR2 proteins (**Figure 51A**), while for the HER2- samples the same number of tumours expressed high and low RET and FGFR2 levels (**Figure 51B**).

From the HER2+ samples, it is also important to mention that the only sample showing low levels of RET and FGFR2 expression was the one with lower levels of HER2 (**Figure 51A**).

Despite the small sample size, and although we acknowledge that a bigger tumour sample collection will be needed in order to get further conclusions, these data suggests that RET and FGFR2 expression is also associated with HER2 expression in breast cancer tumours.

Although no treatment information was taken into account for the analysis, this preliminary data supports the role of RET and FGFR2 in HER2 cell biology. However further analysis taking into account treatments, or even better in recurrent cohorts should be done to validate these hypotheses.

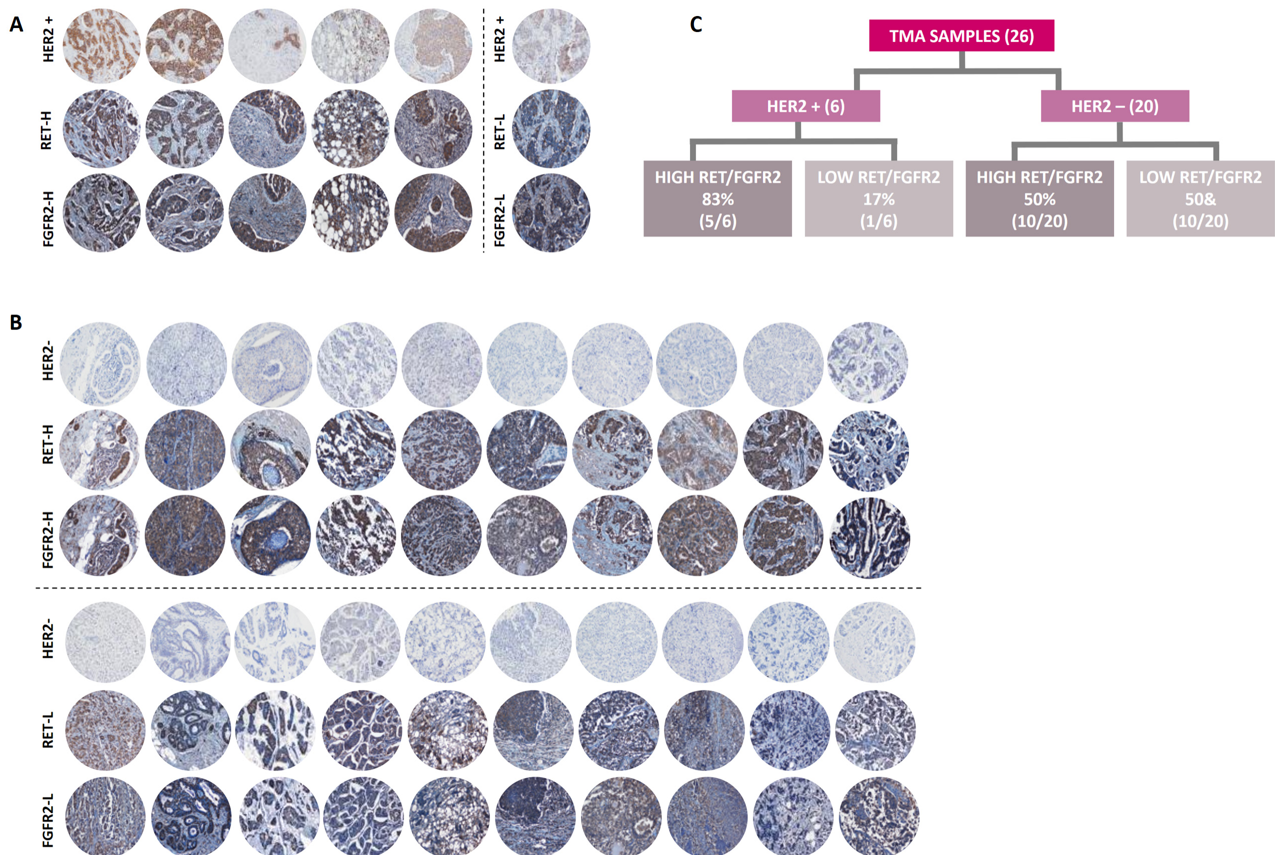


Figure 51. Preliminary RET and FGFR2 expression in BC patient tumours. A. RET, FGFR2 and HER2 Immunohistochemistry images in HER2+ patient samples from a commercial TMA. B. RET, FGFR2 and HER2 Immunohistochemistry images in HER2- patient samples from a commercial TMA. For RET and FGR2: H: high, L: low; for HER2: +: positive, -: negative. C. Diagram showing the quantification of the different expression levels of RET and FGFR among the samples tested.

5. SECRETOME STUDY

5.1 Effect of the drug resistant secretome in the drug resistant phenotype

As shown in **results section 2**, drug resistant cell lines showed a different genetic profile able to predict patient outcome that should further be validated as a response predictive gene set. Among those differentially expressed genes, we found that some of them were implicated on microenvironment related functions that usually define more aggressive tumour phenotypes, with metastatic capability and drug evasion properties.

Moreover, we have also verified that under drug pressure, drug resistant cells are forced to activate compensatory pathways, such as FGFR2 and RET, to overcome the inhibition of the proliferative pathways downstream HER2.

Bearing in mind the hypothesis that the crosstalk between the tumour cells and the surrounding microenvironment is mainly driven by secreted factors, we next analysed if these drug resistant cell lines also secreted soluble factors able to enhance BC cells resistance.

First of all, we performed MTT assays within the parental cell lines treated with the conditioned media collected from drug resistant lines cultures (after 72h) (**Figure 52**). Interestingly, we found that exposure to the soluble factors secreted by the drug resistant cells induced resistance to Trastuzumab and Lapatinib in parental cells lines in all three models (evidenced by the green line).

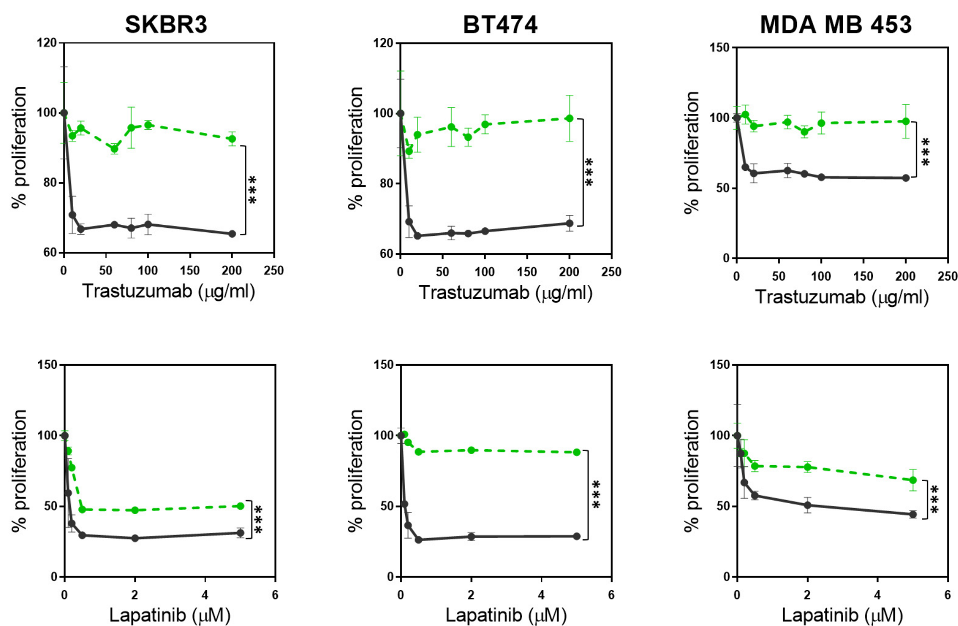


Figure 52. Resistant phenotype induction by the resistant cell lines secretome. Cell viability quantification of SK-BR-3 (left), BT-474 (middle) and MDA-MB-453 (right) cells treated for 72h with increased doses of Trastuzumab (0-200 µg/ml, upper panels) and Lapatinib (0-5 µM, lower panels), after incubation with the resistant cell lines conditioned media (green lines) for 72 hours *vs* normal conditions (black lines). Graphs show the percentage of cell proliferation for each dose. The data represent mean ± S.D. Significant differences by ANOVA with Sidak Multiple Comparison post-hoc test are indicated as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Moreover, when parental cell lines were incubated with the same drug resistant derived conditioned media, FGFR2 and RET activation was induced (**Figure 53**). These results support our data, and reinforce the proposed FGFR2 and RET activation as a HER2 targeted therapies resistance mechanism.

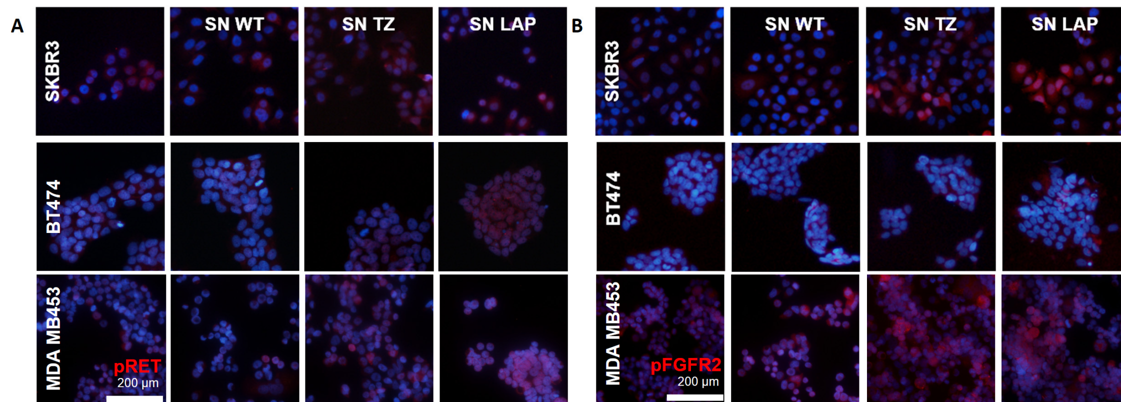


Figure 53. Resistant cell lines secretome effect on RET and FGFR2 activation. Representative immunofluorescence images showing the expression of activated RET levels (A), (Y905, phosphorylated levels represented as p-) and FGFR2 activated levels (B) (Y653-654, phosphorylated levels represented as p-), in parental SK-BR-3(upper panels), BT-474 (middle panels) and MDA-MB-453 (lower panels), after incubation with the conditioned media form the resistant cell lines for 72 hours. Images are representative of at least 3 independent experiments. Scale bar 200 µm.

5.2 Main target ligands exploration and HER2 transactivation by compensatory pathways as a mechanism of resistance

More interestingly, and in agreement with the proposed hypothesis that RET and FGFR2 would transactivate HER2, we also determined phosphorylated HER2 levels in the parental cell lines after treatment with these conditioned media from the drug resistant cell lines.

As expected, in all cases we found a clear activation of HER2 (**Figure 54**), that together with the activation found when analysing RET and FGFR2 phosphorylated levels, supported the drug resistant phenotype induction in the parental cell lines after being treated with the drug resistant cell lines conditioned media.

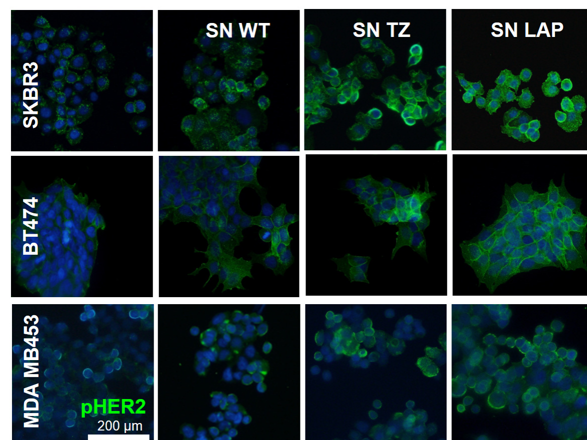


Figure 54. Resistant cell line secretome effect on HER2 activation. Representative immunofluorescence images showing the expression of activated HER2 levels (Y1248, phosphorylated levels represented as p-) in parental SK-BR-3 (upper panels), BT-474 (middle panels) and MDA-MB-453 (lower panels), after incubation with the conditioned media form the drug resistant cell lines for 72 hours. Images are representative of at least 3 independent experiments Scale bar 200 µm.

Therefore, we propose that HER2 activation was dependent on FGFR2 and RET prior activation. Furthermore, this suggest the presence of FGFR2 and/or RET ligands in the drug resistant cell lines conditioned media.

We explored the drug resistant and parental cell lines expression arrays data for the expression of genes codifying for the main ligands for RET and FGFR2, and in a similar way that we did with the RTKs phosphorylation in the first part of the study, we represented as a heat map the drug resistant cell lines expression compared to the expression in the parental cell lines in terms of fold induction (**Figure 55A**). From the list we choosed two ligands for each receptor that were overexpressed at least in three out of the six drug resistant cell lines: FGF2 and FGF5 for FGFR2 and NRTN and GDNF for RET.

First of all, we confirmed the capability of those ligands to activate the corresponding receptors, as seen in the ligand time course (**Figure 55 B**), and that allowed us to stablished an activation window from 1 to 12 hours after ligands treatment.

Next, in order to determine if HER2 transactivation really depends on FGFR2 /RET activation we treated the parental cell lines with the two main ligands for each receptor and excitingly found a clear HER2 activation with a pick at 6 hours after treatment (**Figure 56**).

Finally, the inhibition of RET and FGFR2 with the already tested inhibitors Ponatinib and BGJ-398 resulted in a reduction in phospho-HER2 levels in the parental and drug resistant cell lines (**Figure 57**), which added further evidences to the proposed transactivation model.

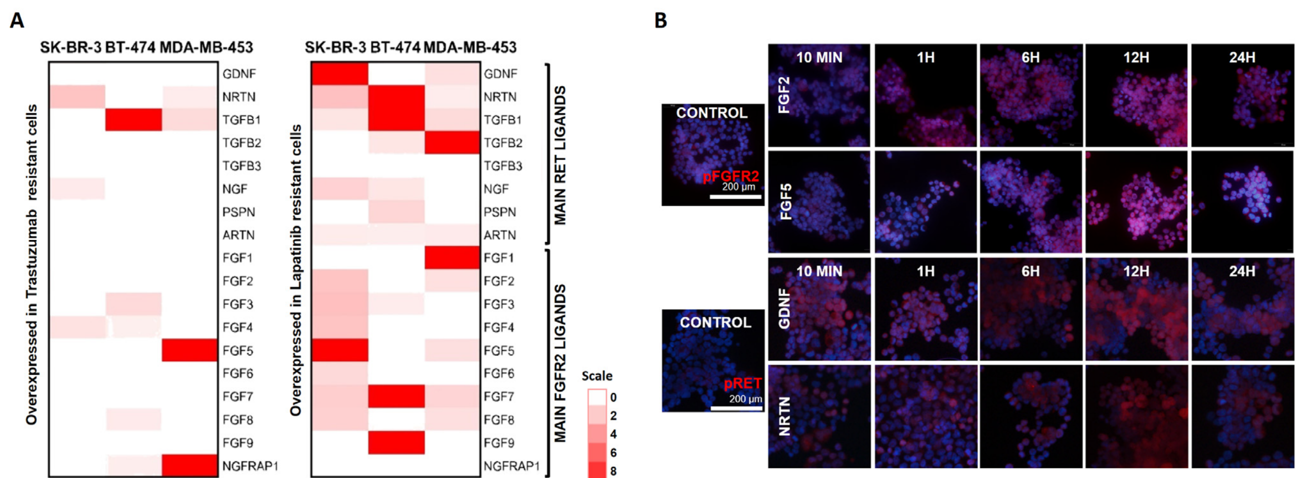


Figure 55. Analysis of FGFR2 and RET main ligands expression on the drug resistant cell lines. A. Heatmap depicting the relative expression of the main ligands for RET and FGFR2. For each gene, the expression in the drug resistant cell lines SK-BR-3, BT-474 and MDA-MB-453 (Trastuzumab resistant, TZ –resistant, and Lapatinib resistant, LAP-resistant) was normalized to the parental. The data represent the mean values from 3 independent experiments. B. Representative immunofluorescence images showing the expression of activated FGFR2 and RET levels (Y653-654 and Y905 respectively, phosphorylated levels represented as p-) in parental MDA-MB-453 cell line, after incubation with FGF2 and FGF5 (100 ng/ml) for FGFR2 activation (upper panels), and GDNF and NRTN (100 ng/ml) for RET activation (lower panels), for 10 minutes, 1-6-12 and 24 hours. Images are representative of at least 3 independent experiments Scale bar 200 µm.

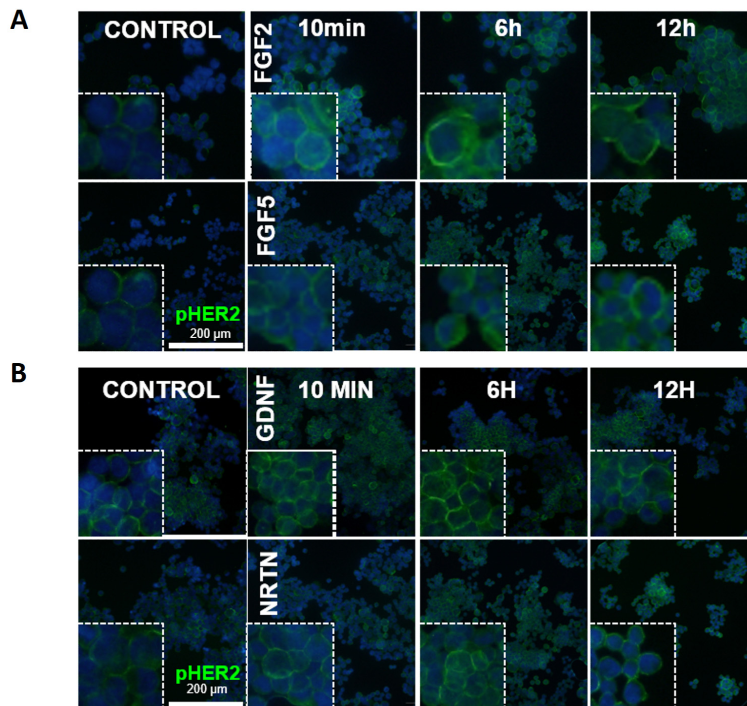


Figure 56. FGFR2 and RET main ligands effect on HER2 activation. Representative immunofluorescence images showing the expression of activated HER2 (Y1248, phosphorylated levels represented as p-) in parental MDA-MB-453 cell line, after incubation with FGF2 and FGF5 (100 ng/ml) (A), or GDNF and NRTN (100 ng/ml) (B) for 10 minutes, 6 and 12 hours. Images are representative of at least 3 independent experiments. Scale bar 200 µm.

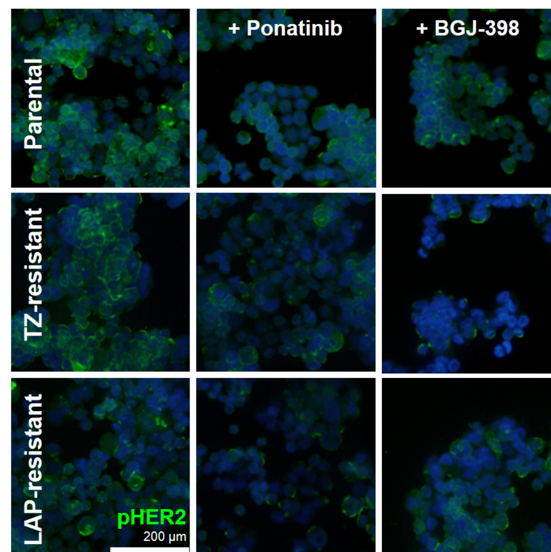


Figure 57. FGFR2 and RET inhibitors effect on HER2 activation. Representative immunofluorescence images showing the expression of activated HER2 (Y1248, phosphorylated levels represented as p-) in MDA-MB-453 parental and drug resistant cell lines (Trastuzumab resistant, TZ –resistant, and Lapatinib resistant, LAP-resistant), after treatment with RET and FGFR2 inhibitors (Ponatinib and BGJ-398, 2µM) for 72 hours. Images are representative of at least 3 independent experiments. Scale bar 200 um.

5.3 Effect on cell survival

Immunoblotting analysis showed that treatments with the ligands slightly activated classical survival pathways typically downstream of HER2, such as phospho-AKT, which were more activated in the resistant cell lines in basal conditions.

In addition, proliferation inductor proteins like ERK were activated after treatments with some of the ligands under study (**Figure 58**).

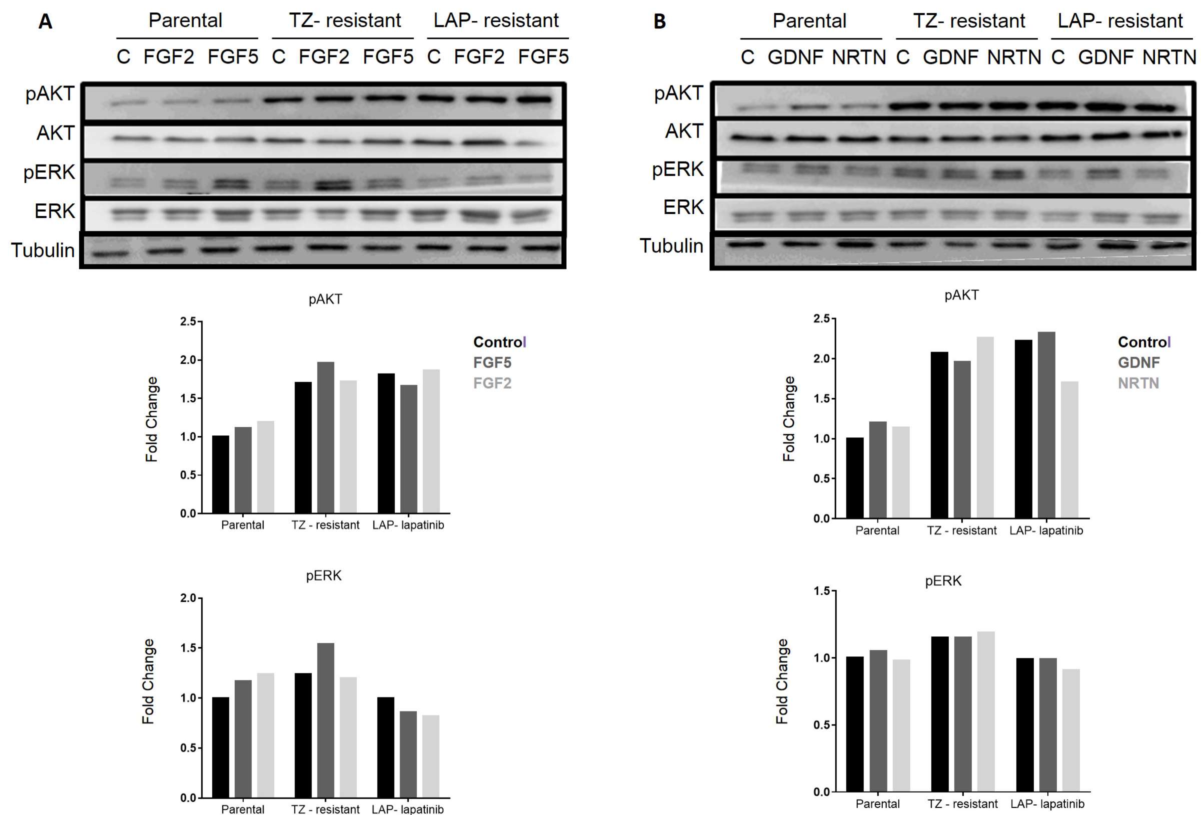


Figure 58. FGFR2 and RET main ligands effect on proliferative and survival pathways. Representative images of western blot analyses showing the phosphorylation (denoted by p-) and total levels of ERK-MAPK and AKT, in MDA-MB-453 parental and drug resistant cell lines (Trastuzumab resistant, TZ –resistant, and Lapatinib resistant, LAP-resistant), treated with with FGF2 and FGF5 (100 ng/ml) (A), or GDNF and NRTN (100 ng/ml) (B), for 72 hours (ligands were added into the media every 24 hours). Graphs show the densitometric quantification of the western blot analyses normalized with tubulin (which was used to ensure equal protein loading), and refered to the control situation in the parental cell line.

We next evaluated the direct effect of RET and FGFR2 ligands in the induction of the resistant phenotype, by performing MTT assays in the parental cell lines that were pre-treated with the different ligands before drug addition (**Figure 59**).

The results show a slightly although really interesting general effect on survival promotion when ligands were added.

The resistant cell lines displayed a consistent survival rescue when pre-treated with the soluble factors, while parental cell lines were not capable of overcoming Ponatinib and BGJ-398 effect, in fact, in this case the treatment with ligands reduce survival compared to the basal situation in absence of ligands.

This may somehow be reflecting the different efficiency that parental and resistant cell lines take from the alternative FGFR2/RET pathways activation. As showed, resistant cell lines depend on these new pathways and its activation imply HER2 activation, and in consequence, the activation of survival and proliferative pathways that will allow cells to overpass Ponatinib and BGJ-398 drug pressure.

Contrary, parental cell lines survival and proliferation do not depend on RET/FGFR2 pathways, as its oncogenic addiction to HER2 is the one that really drive its tumourigenesis. Then, when RET and FGFR2 are activated by the ligands there is an imbalance between the survival activation that these pathways should promote, and the fact that the cells are now more sensible to the drugs, which will finally result in cell death.

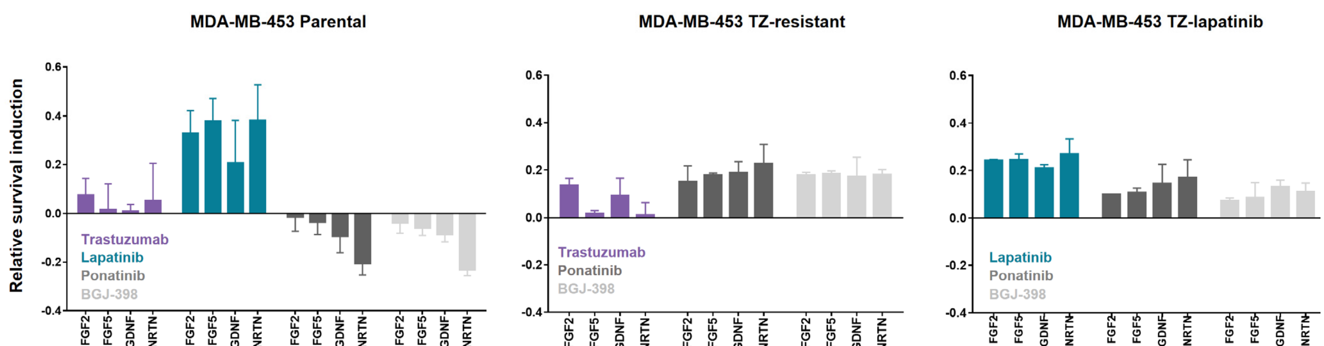


Figure 59. FGFR2 and RET main ligands effect on cell survival. Cell viability quantification of MDA-MB-453 parental and drug resistant cell lines (Trastuzumab resistant, TZ –resistant, and Lapatinib resistant, LAP-resistant) treated for 72h with increased doses of Trastuzumab (100 µg/ml, purple bars), Lapatinib (4 µM, turquoise bars), Ponatinib (2 µM, deep grey bars) and BGJ-398 (2 µM, light grey bars) for 48h, after FGF2, FGF5, GDNF and NRTN incubation for 24 hours (100ng/ml). Graphs show survival induction *vs* control situation (were no ligand was added) Data represent mean ± S.D. for a minimum of 3 replicates.

6. FIBROBLAST ROLE IN THE ESTABLISHMENT OF THE DRUG RESISTANT PHENOTYPE

6.1 Immortalized fibroblast cell lines development and characterization

We have previously shown that treatment with FGFR2 inhibitors decreases fibroblast infiltration in BC xenograft tumours (**Figure 50**). It is well known that although cells that populate the stroma are not neoplastic, they can influence tumour cell behaviour [461]. Fibroblasts are the most abundant and active population of the breast stroma, and it has been widely documented that this stromal cell population is activated by chemokines, cytokines and growth factors secreted by the stromal microenvironment, acting in an autocrine and paracrine way under the influence of the tumour [462]. In the same way, fibroblasts can stimulate tumour cell proliferation, growth and progression through production of various growth factors, hormones and cytokines [54].

In this context, we next wanted to study the crosstalk between the drug resistant cell lines and the main mammary stromal population, in order to explore if the fibroblasts reduction found in BC xenograft treated with FGFR2 and RET inhibitors may have additional implications in tumour progression and drug sensitivity.

As explained in detail in the **material and methods section 1.3,** we developed several fibroblast cell lines from different origin:

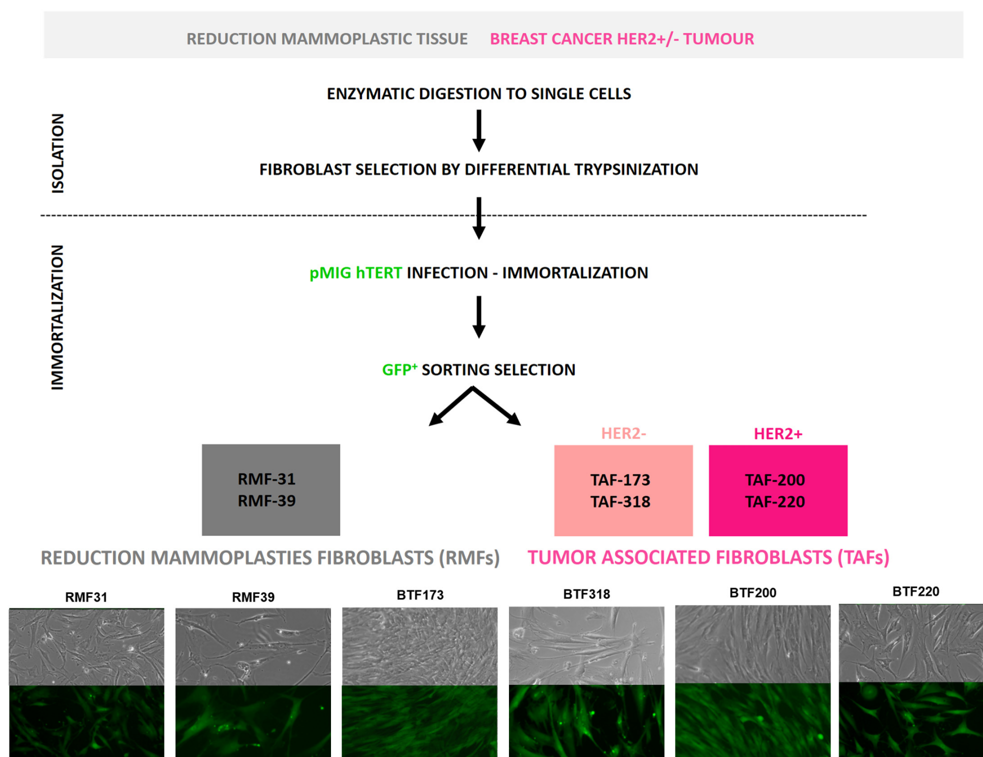


Figure 60. Immortalized fibroblast cell lines development. The diagram shows the sequential process for fibroblast isolation and immortalization. Representative phase contrast and GFP fluorescence (in green) images of the cell lines after sorting selection are shown at the bottom. Scale bar 200 µm.

fibroblast associated to healthy tissue (obtained from reduction mammoplasties) and fibroblasts associated to breast tumours (obtained from different BC patients tumourectomies with different profiles in terms of HER2 positivity).

The process is summarized in **figure 60,** and briefly could be divided in two main steps: fibroblasts isolation from the different tissues of origin, and fibroblasts immortalization by retroviral infection.

Our hypothesis is that fibroblast may also play an important role in the drug resistant phenotype establishment by enhancing HER2 transactivation in the drug resistant cells and promoting tumour progression, as one of the receptors described in this work belongs to the fibroblast growth factor receptor family.

In order to characterize the different fibroblasts lines, we first of all examined in the BC tumours of origin the levels of RET and FGFR2 (HER2 positivity status was kindly reported from the anatomical pathology department from the Hospital Clínic). Interestingly we found that the RET and FGFR2 expression correlated with HER2 status, being higher in the HER2+ tumours **Figure 61A** (in accordance with the patient cohort study, **figure 51**). Looking at fibroblasts derived cell lines FGFR2 expression, we did not found a correlation with FGFR2 expression levels regarding fibroblast tumoural origin, as for all tumour associated fibroblasts (TAF173, TAF318, TAF200 and TAF220) similar expression levels of FGFR2 where found. Surprisingly, reduction mammoplasty fibroblasts (RMF31 and RMF39) displaied the lowest levels, far from TAFs expression levels (**Figure 61B**).

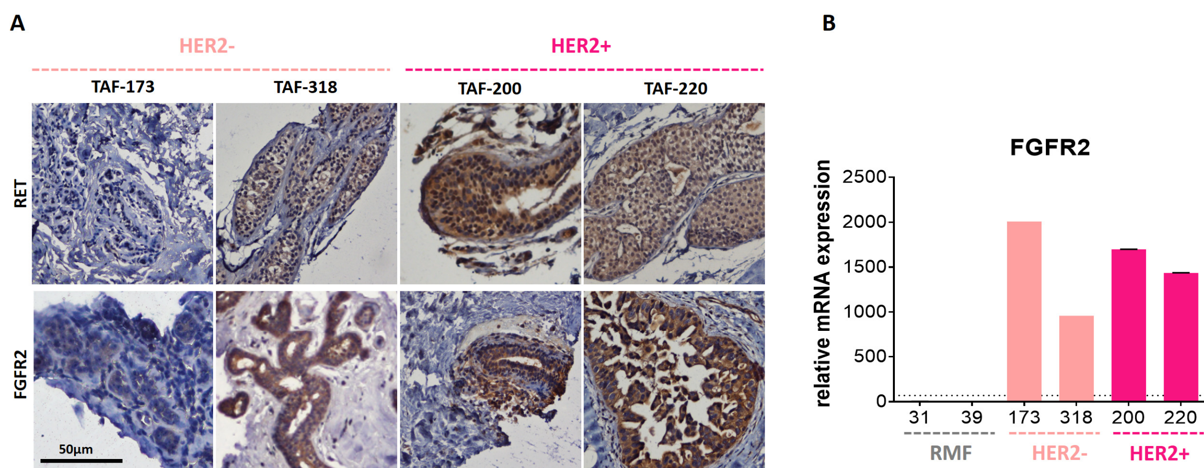


Figure 61. Immortalized fibroblast cell lines characterization. A. Representative immunohistochemistry images of RET (upper panels) and FGR2 (lower panels) in the primary tumours from where fibroblasts were isolated. Scale bar 50 µm. B. Graphs show the expression values for FGFR2 measured by quantitative PCR. Data represent mean ± S.D. for a minimum of 3 replicates.

6.2 Tumour associated fibroblasts promote BC resistance to anti-HER2 therapies *in vitro* through HER2/FGFR2 activation

To explore the crosstalk between drug resistant cells and fibroblasts, we first wanted to determine if in the same way that drug resistant cells do, fibroblasts can also secrete soluble factors able to enhance BC cells resistance.

To do so we cultured MDA-MB-453 parental cell lines with the collected conditioned media from fibroblast with different origin (tumoural activated fibroblast derived from tumours with different status, HER2- (TAF173 and TAF318) and HER2+ (TAF200 and TAF220), *vs* non-activated healthy tissue derived fibroblasts (RMF31 and RMF39).

We found that the conditioned media from tumour derived fibroblast was able to induce HER2 activation. Moreover, supporting our transactivation model phospho-FGFR2 was also found overactivated, and to our impression this

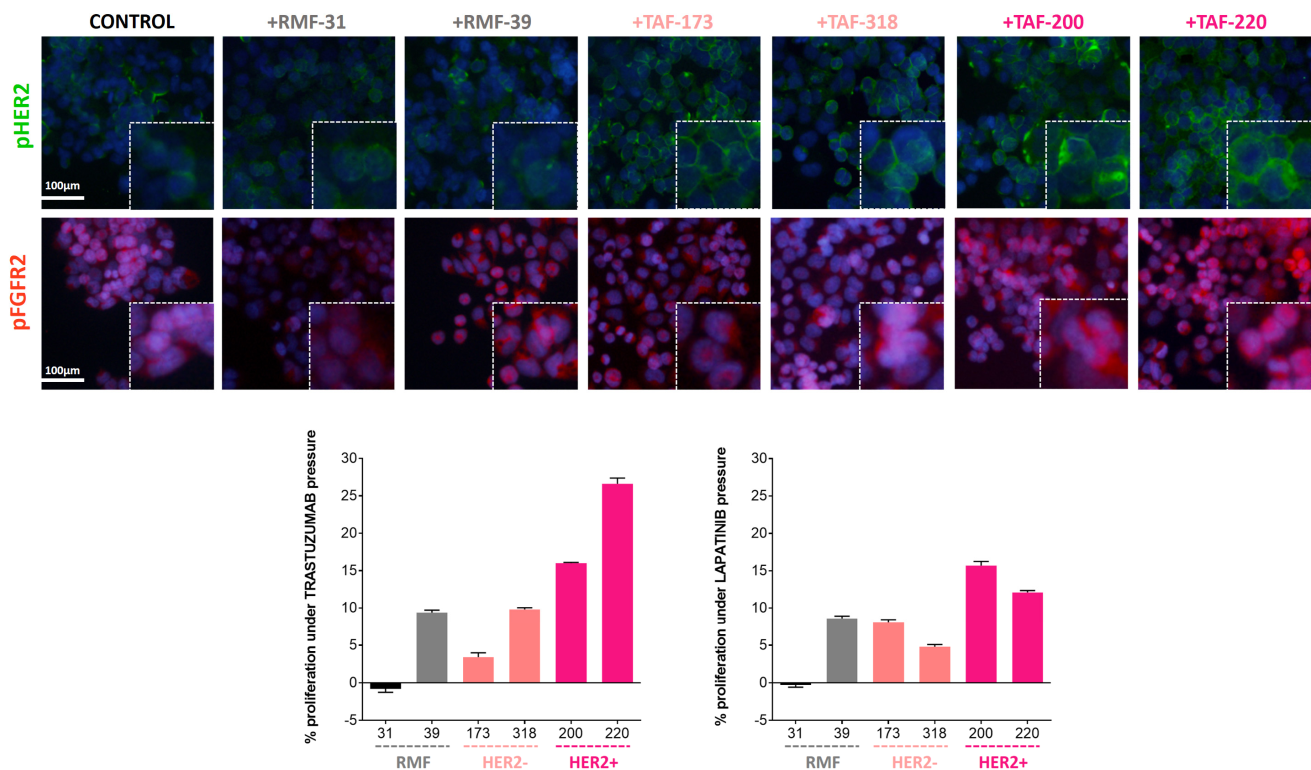


Figure 62. Tumour associated fibroblasts promote BC resistance to anti-HER2 therapies *in vitro* through HER2/FGFR2 activation. A. Representative immunofluorescence images showing the expression of activated HER2 levels (Y1248, phosphorylated levels represented as p-) and activated FGFR2 levels (Y653-654, phosphorylated levels represented as p-), in parental MDA-MB-453 cell lines, after culturing them in the presence of fibroblast derived conditioned media for 72 hours. Images are representative of at least 3 independent experiments. Scale bar 100 µm. B. Parental MDA-MB-453 cells were cultured with fibroblast derived conditioned media for 72 hours. After 24 hours of fibroblast conditioned media addition, cells were treated for 48h with increased doses of Trastuzumab (0-200 µg/ml) and Lapatinib (0-5 µM), and cell viability was quantified. Grey bars represent fibroblasts from healty tissue origin, light pink bars represent tumour associated fibroblast from HER2 negative tumours, and dark pink represent tumour associated fibroblasts from HER2 positive tumours. Graphs show the percentage of cell proliferation for each fibroblast conditioned media relative to the control. Data represent mean ± S.D. for a minimum of 3 replicates. Significant differences by ANOVA with Sidak Multiple Comparison post-hoc test are indicated as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

activation may be the one mediating HER2 activation.

Interestingly, we also found that the conditioned media from HER2+ tumours derived fibroblasts was the most efficient in activating HER2 and FGFR2 pathways (**Figure 62A**).

Our previous experiments have shown that activation of these pathways mediated the drug resistant phenotype, then, we performed MTT assays in the same conditions using MDA-MB-453 parental cell line cultured with the conditioned media from fibroblasts of different origin, and followed by Trastuzumab or Lapatinib treatments.

As shown in **figure 62B**, we found that the soluble factors secreted by tumour associated fibroblasts were able to induce resistance to Trastuzumab and Lapatinib in the parental cell line, as evidenced by a sensitivity reduction under drug pressure.

Again, we also observed that the conditioned media derived from fibroblasts isolated from HER2+ tumours (TAF200 and TAF220) had a stronger effect, further supporting the FGFR2/HER2 crosstalk.

6.3 Drug resistant cell lines promote fibroblast activation *in vitro*

In order to further study the crosstalk between fibroblasts and the drug resistant cells, we next wanted to test if the drug resistant cell lines have any effect on the fibroblasts phenotype.

First of all, we characterized our fibroblasts in terms of fibroblast activation well known markers expression such as αSMA and S100A4 (**Figure 63**). We found a beautiful distribution pattern for both markers, being as expected, higher expressed on TAFs, and interestingly, even more on those TAFs with a HER2+ origin. In order to get no saturated images for αSMA staining for those samples with the highest expression, microscope settings and laser intensity and exposure time were reduced, therefore, from the images it looks like RMFs did not expressed

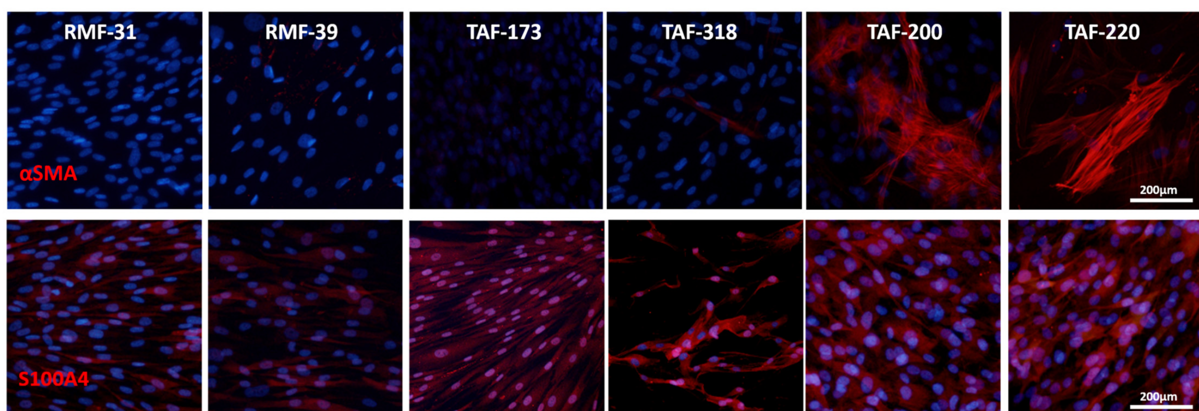


Figure 63. Fibroblast activated markers characterization. Representative immunofluorescence images showing the expression of typical fibroblast activated markers, αSMA (upper panels) and S100A4 (lower panels) on the fibroblast cell lines generated by us. Normal fibroblasts: RMF-31, RMF-39. Tumour associated fibroblasts from HER2 negative tumours: TAF-173, TAF-318. Tumour associated fibroblasts from HER2 positive tumours: TAF-200, TAF-220. Scale bar 200 µm.

αSMA staining at all, when some degree of αSMA staining could be reported under other microscope conditions.

Next, we cultured immortalized fibroblast derived from healthy mammary tissue (the ones without an active phenotype) with the conditioned media derived from Trastuzumab and Lapatinib resistant cell lines.

To determine fibroblast activation, we stained for αSMA and S100A4, and in both cases we observed an increase in the activated-fibroblast markers, not only in terms of expression levels, but also in terms of the percentage of positive population, being the whole population of fibroblasts positive for αSMA and S100A4 after co-cultured them with the resistant enriched media. This activation profile was more evident after the treatment with the Lapatinib resistant cell lines derived media (**Figure 64**).

Interestingly, regarding αSMA we also observed a modification in protein distribution, being more perinuclear in the basal situation, and completely cytoplasmic after the treatment with the conditioned media from the drug resistant cell lines.

These results suggest that the acquisition of chemoresistance renders BC cells with more capacity to activate fibroblast.

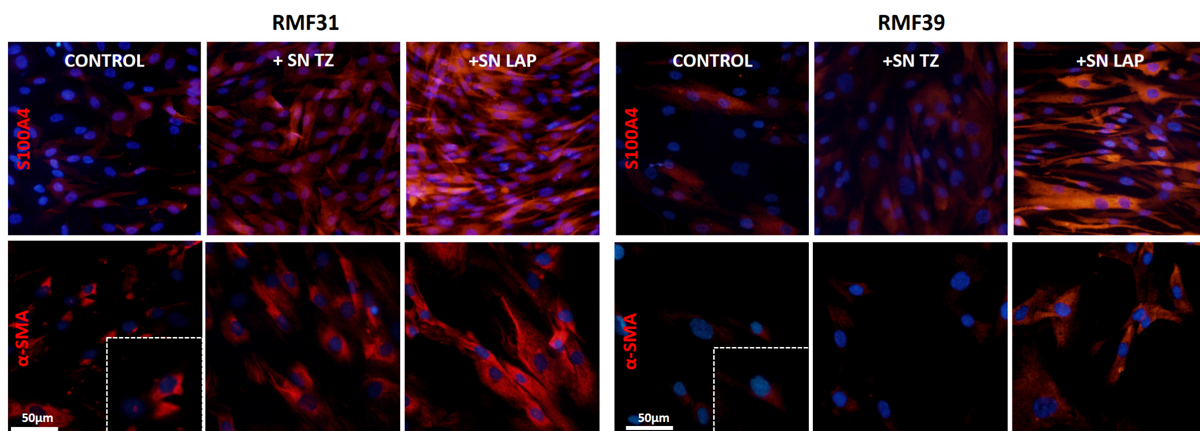


Figure 64. Drug resistant cell lines promote fibroblast activation *in vitro*. Representative immunofluorescence images showing the expression of typical fibroblast activated markers, αSMA (lower row) and S100A4 (upper row), in health tissue associated fibroblast, RMF31 left panel, and RMF39 right panel, after culturing them with drug resistant cell lines conditioned media for 72h. Images are representative of at least 3 independent experiments. Scale bar 50 µm.

Having demonstrated on one hand the ability of HER2+ associated fibroblasts to induce resistance through FGFR2 and HER2 activation, and on the other hand that the drug resistant cell lines are capable of promoting an activated phenotype in non-tumour associated fibroblasts, we next wanted to test if these fibroblasts activated as a result of the crosstalk with the drug resistant cell line, have now more potential to induce the drug resistant phenotype *in vitro*.

To do so, we first collected the conditioned media from the HER2+ cell lines resistant to Lapatinib and Trastuzumab, and after media filtration, we add these enriched solutions to fibroblast cultures from different origin (RMFS and TAFs) incubating them for 24 h. After the incubation time, we collected the fibroblasts conditioned media that was

immediately added to the parental cell lines for 72hours, and we finally performed MTT assays on those parental cell lines cultured with conditioned media under the presence of Trastuzumab or Lapatinib (**Figure 65A**).

For the Lapatinib assay, we found that in all cases, the conditioned media collected from activated fibroblasts (previously cultured with the conditioned media of the Lapatinib resistant cell lines) had a stronger capability to increase Lapatinib resistance compared with the conditioned media from non-activated fibroblasts (**Figure 65B**). Furthermore, we also found that the conditioned media from tumour associated fibroblasts pre-cultured with conditioned media from BC resistant cell lines showed a higher capability to enhance resistance in the parental cell lines, compared with the conditioned media of fibroblasts derived from normal tissue.

The conditioned media from the Trastuzumab resistant cell lines was not so efficient in promoting the Trastuzumab resistant phenotype, and few changes were seen with the MTT assay (**Figure 65C**).

Taking together these results revealed the capability of the drug resistant cell lines to not only activate fibroblasts, but also to modulate its tumour promoting factors secretion pattern.

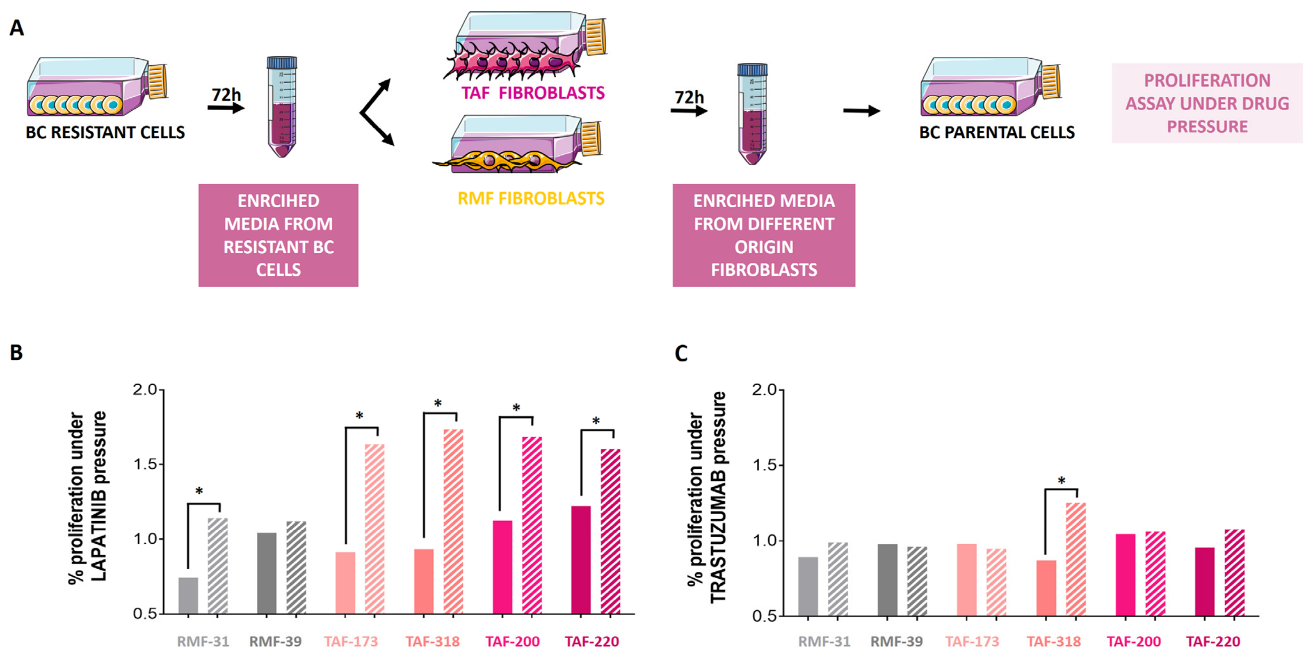


Figure 65. *In vitro* fibroblast activation promoted by resistant cell lines induces changes in fibroblast resistant phenotype induction capability.

A. Diagram showing the *in vitro* approach for fibroblast activation and induction of secretome alteration by resistant cell lines conditioned media. B-C The graphs show the survival induced by fibroblast activated conditioned media *vs* non activated fibroblasts conditioned media, in MDA-MB-453 cells treated for 72h with Trastuzumab (100 µg/ml, B) or Lapatinib (4 µM, C). Graphs show the cell viability induction relative to controls with no treatment. Grey bars represent fibroblasts from healty tissue origin, light pink bars represent tumour associated fibroblast from HER2 negative tumours, and dark pink represent tumour associated fibroblasts from HER2 positive tumours. Data represent mean ± S.D. for a minimum of 3 replicates. Significant differences by ANOVA with Sidak Multiple Comparison post-hoc test are indicated as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

6.4 Fibroblast crosstalk with BC cell lines *in vivo* induces a more aggressive and drug resistant phenotype

Taking into account our previous data, we finally wanted to test if the crosstalk between breast cancer cells and fibroblasts defined *in vitro,* has also an important role in the *in vivo* establishment of tumours, contributing in some way to resistance.

Xenograft tumours were established by orthotopical injection of a mixture (1:1 ratio) of cells into two mammary fat pad per mouse. Cells mixture consisted in $1x10^6$ MDA-MB-453 cells plus $1x10^6$ immortalized human fibroblasts from different origin (RMFs and TAFs). As control group MDA-MB-453 cells were injected alone.

Interestingly we found that the co-inoculation of HER2+ associated fibroblast and BC MDA-MB-453 cells, lead to the development of more aggressive tumours compared to the tumours derived from the co-inoculation of BC cells with fibroblast associate to HER2- (BT-318 and BT-173) tumours or to the parental cells alone, as represented in the volume curves and the cherry LUC+ fluorescent *in vivo* images (**Figure 66A-B** respectively).

Moreover, the co-inoculation with healthy tissue associated fibroblasts (RMF-31 and RMF-39) resulted in smaller and less aggressive tumours, suggesting that non activated fibroblast might have an inhibitory role in tumour growth.

Analysing the derived xenograft tumours, we interestingly found that those tumours derived from the co-inoculation with HER2+ associated fibroblasts (BT-200 and BT-220), presented higher HER2 expression (**Figure 67, upper panel**), represented by a higher and more homogenous membrane staining. We also stained for FGFR2, and found that all tumour coming from fibroblast co-injection display higher amounts of FGFR2 regardless of the fibroblast origin (**Figure 67, lower panel**).

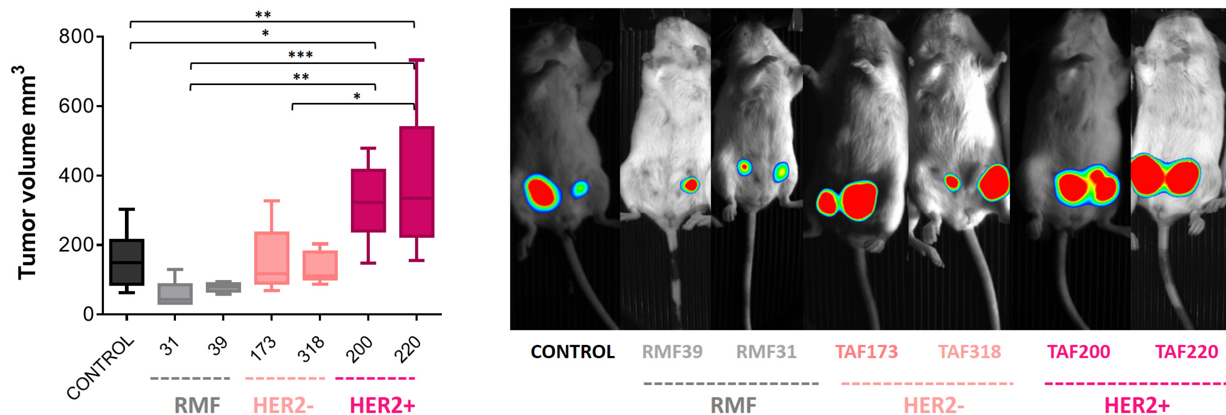


Figure 66. Fibroblast crosstalk with BC cell lines *in vivo* induces a more aggressive phenotype. MDA-MB-453 parental cell lines were co-injected with the different fibroblasts cell lines orthotopically in the mammary gland for 90 days. A. Graph shows the tumour volume at day 90 for each group. Grey bars represent fibroblasts from healthy tissue origin, light pink bars represent tumour associated fibroblast from HER2 negative tumours, and dark pink represent tumour associated fibroblasts from HER2 positive tumours. Data represent mean of each group (n=5) ± S.D. Significant differences by ANOVA with Sidak Multiple Comparison post-hoc test are indicated as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. B. Representative images showing the luciferase intensity signal from the Cherry-Luc positive MDA-MB-453 cells.

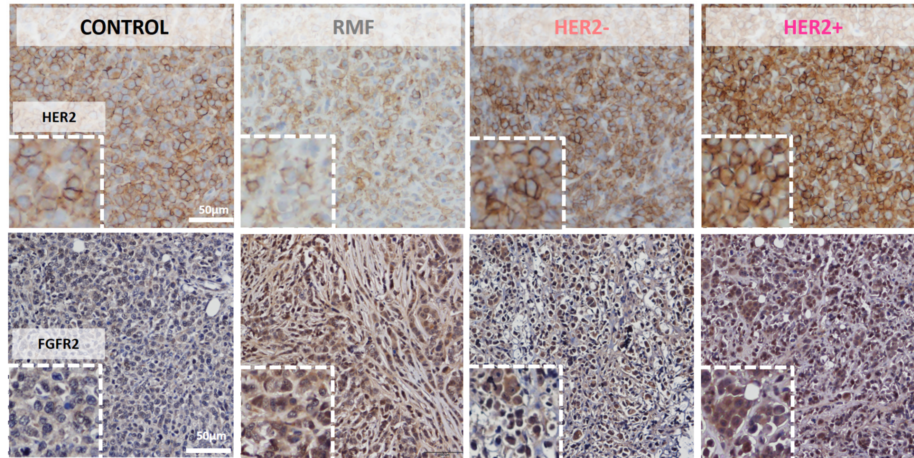


Figure 67. Modulation of tumour HER2 and FGFR2 expression *in vivo* by fibroblasts. Representative immunohistochemistry images of HER2 (upper panel) and FGFR2 (lower panel) in tumours sections from animals injected with MDA-MB-453 parental cell lines (control) or co-injected with fibroblast from different origin (RMF: healthy tissue origin; HER2-: tumour associated fibroblast from HER2 negative tumours; HER2+: tumour associated fibroblasts from HER2 positive tumours. Scale bar 50 µm.

Regarding αSMA we observed that those tumours derived from the co-inoculation with HER2+ associated fibroblasts were more fibroblast infiltrated. Moreover, the fibroblasts present in xenografts derived from the co-inoculation with TAFs show a more mesenquimal morphology, typical from activated fibroblasts, more evident in the HER2+ derived fibroblasts co-injected xenografts. In contrast, fibroblasts infiltrating the xenograft tumours derived from the co-inoculation with RMFs present a typical fibroblastic spindle-like morphology (**Figure 68, upper panel**).

With these results we next wanted to determine if those fibroblasts that we found in the xenograft derived tumours where the same population of immortalized fibroblast that we had co-injected three months before. To do so we performed double immunofluorescence for αSMA and GFP, under the assumption that all fibroblast will stain for αSMA, but only double positive cells will correspond to human immortalized fibroblasts. We were able to found double positive cells in all samples analysed except for the control group, however this double positive cells represents a really minor population, representing less than the 3-4 % of the total fibroblasts population, so we assumed that almost the entire fibroblast population was from mice origin (**Figure 68, lower panel**).

The fact that tumours derived from TAFs co-injected xenografts were more enriched in mice fibroblasts suggests that those fibroblasts coming from breast tumours, especially those with a HER2+ profile, may have a higher potential capability to recruit stromal cells, in particular fibroblast, that will later help in tumour promotion.

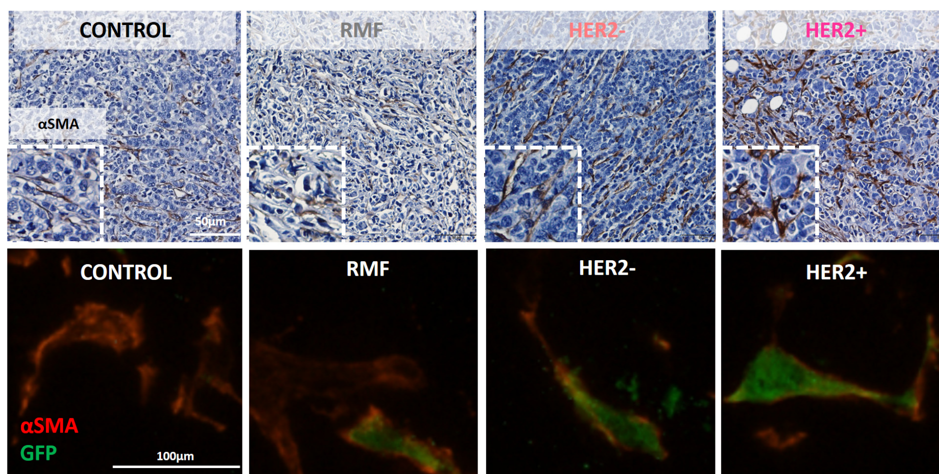


Figure 68. Tumour associated fibroblast infiltration *in vivo*. αSMA representative immunohistochemistry images (upper panel, scale bar 50 µm) and magnifications from representing immunofluorescence images for double αSMA (red) and GFP (green) fibroblast staining (lower panel, scale bar 50 µm) in xenograft sections from animals injected with MDA-MB-453 parental cell lines (control) or co-injected with fibroblast from different origin (RMF: healthy tissue origin; HER2-: tumour associated fibroblast from HER2 negative tumours; HER2+: tumour associated fibroblasts from HER2 positive tumours).

One small fraction from each tumour was disaggregated after surgery, and MDA MB-453 cells were isolated and cultured, mCherry-LUC fluorescence in the resulting population confirmed the population reliability (**Figure 69**). Complete procedure explanation in **material and method section 1.4**.

The analyses of HER2 in these cultures showed that the HER2 overexpression found in the tumours was maintained overtime and excitingly, FGFR2 activation expression was also higher in those cells coming from tumours that were initially co-injected with fibroblast derived from HER2+ tumours (**Figure 70A**).

Consequently, we finally evaluated the resistance of the isolated cell lines to HER2 and FGFR2 inhibitory therapies. We performed MTT assays with these cells and found that cells derived from tumours co-injected with HER2+ tumours derived fibroblasts display a more resistant phenotype to anti-HER2 therapies, and interestingly, this group was also more resistant against FGFR2 inhibitors, (**Figure 70B**).

These results demonstrate the effect of fibroblast in promoting tumour growth, and also revealed the fact that tumours that coevolved with HER2+ tumours associated fibroblast were more resistant to HER2 and FGFR2 targeted therapies.

Taking together, these data support the hypothesis that tumour associated fibroblasts crosstalk with BC cells (especially those derived from HER2+ tumours), activate FGFR2 which in turn upregulates HER2, leading to increased tumour aggressiveness and resistance.

All these evidences also reinforce the idea that the acquisition of resistance to anti-HER2 therapies increases BC cells microenvironment crosstalk capability and highlights the importance of fibroblasts in supporting tumour maintenance, progression and drug resistant phenotype establishment.

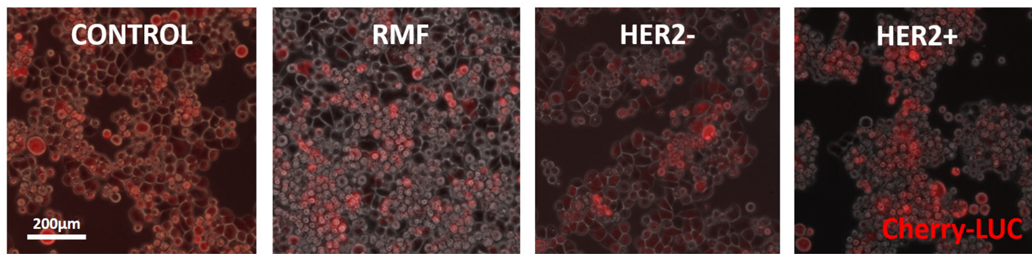


Figure 69. MDA-MB-453 BC cells isolation from BC xenografts. Representative immunofluorescence images for the MDA-MB-453 mCherry LUC+ cultures from the isolated xenograft cells from animals injected with MDA-MB-453 parental cell lines (control) or co-injected with fibroblast from different origin (RMF: healthy tissue origin; HER2-: tumour associated fibroblast from HER2 negative tumours; HER2+: tumour associated fibroblasts from HER2 positive tumours). Scale bar 200 µm.

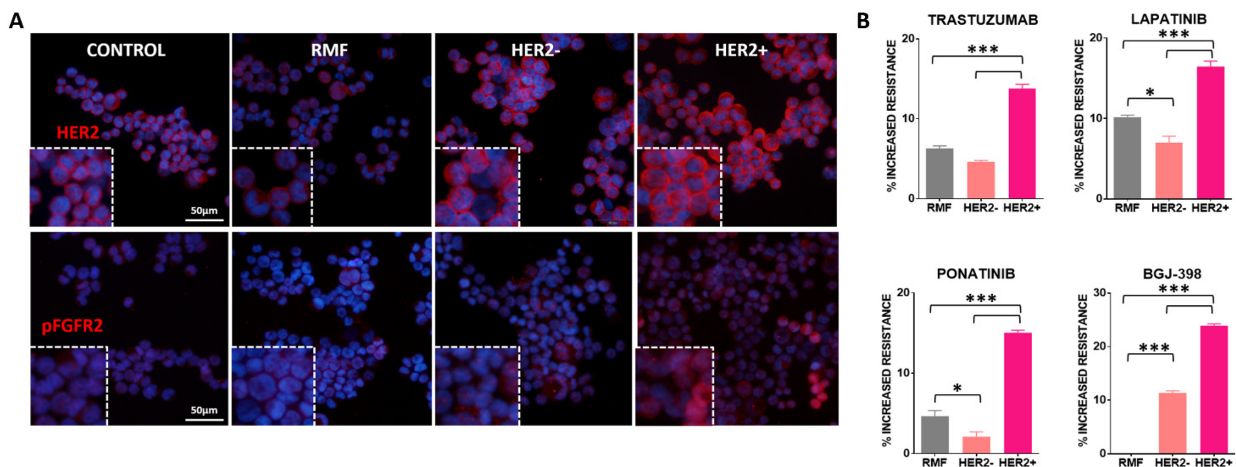


Figure 70. Fibroblast crosstalk with BC cell lines *in vivo* induces a more resistant phenotype.
A. Representative immunofluorescence images for HER2 and activated FGFR2 (Y653-654, phosphorylated levels represented as p-) in the MDA-MB-453 Cherry Luc+ primary cultures from the isolated xenograft cells from animals injected with MDA-MB-453 parental cell lines (control) or co-injected with fibroblast from different origin (RMF: healthy tissue origin; HER2-: tumour associated fibroblast from HER2 negative tumours; HER2+: tumour associated fibroblasts from HER2 positive tumours. Scale bar 50 µm. B. Cell viability quantification of MDA-MB-453 Cherry Luc+ cells treated for 72h with Trastuzumab (100 µg/ml) and Lapatinib (4 µM), Ponatinib (1 µM) and BGJ-398 Lapatinib (1 µM). Grey bars represent MDA-MB-453 Cherry Luc+ cells isolated from healthy tissue fibroblast and MDA-MB-453 co-injected xenografts, while light and dark pink bars represent MDA-MB-453 Cherry Luc+ cells isolated from tumour associated fibroblast isolated from HER2 negative tumours and MDA-MB-453 co-injected xenografts and from tumour associated fibroblast isolated from HER2 positive tumours and MDA-MB-453 co-injected xenografts (HER2- and HER2+ respectively). The graphs show the cell viability induction relative to controls where no fibroblast were co-injected. Data represent mean ± S.D. for a minimum of 3 replicates Significant differences by ANOVA with Sidak Multiple Comparison post-hoc test are indicated as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

6.5 Metastasis studies

Drug resistance and tumour aggressiveness are properties broadly associated with tumour progression and metastatic potential. In breast cancer, advanced disease tends to typically metastasize to bone, lung, brain and liver [174, 351].

In order to explore as a preliminary study, the implication of fibroblasts on metastasis, we collected lungs and brains from all mice in the study. Organs were fixed in PFA for immunohistochemistry procedures, and from at least two lungs of each group we were also capable to isolated the BC cells obtaining metastatic lines *in vitro*. Also BC disseminated circulating cells were isolated from the bone marrow**,** and together with brains, will be further studied in the future (data not shown).

Focusing on lungs, we first of all stained lung sections with HER2.

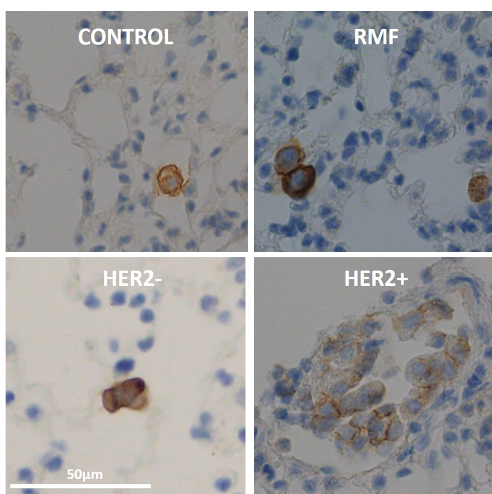


Figure 71. Breast cancer disseminated cells identification. HER2 immunohistochemistry representative images of BC disseminated cells in the lungs isolated from animals injected with MDA-MB-453 parental cell lines (control) or co-injected with fibroblast from different origin (RMF: healthy tissue origin; HER2-: tumour associated fibroblast from HER2 negative tumours; HER2+: tumour associated fibroblasts from HER2 positive tumours. Scale bar 50 µm.

All section analysed, representative for all groups in the study, contained disseminated tumour cells and micro-metastasis (**Figure 71**). A preliminary frequency study on the metastatic cells found in each section suggested a high metastatic rate on those lungs derived from TAFs co-injected xenografts, however further analysis with a bigger number of cases should be performed to confirm these results.

Furthermore, in one case from the HER2+ TAFs co-injected group we found a lung completely invaded by BC cells, where the immunohistochemistry staining for HER2 also suggested evidences of blood vessels invasion, which was further corroborate with a double staining for HER2 and Vimentin (**Figure 72**). These images manifested the metastatic capability of those cells to invade blood vessels and progress to different organs.

In order to evaluate if this metastatic behaviour was accompanied by changes in cells profile, we compare the primary tumour cell population, with the lung metastatic population for the same mice. With this purpose total and phosphorylated FGFR2 immunofluorescence was performed in tumour and lung sections (**Figure 73**), to further study if the pathways that we have already defined as important for drug resistance were also altered in metastatic populations.

Interestingly we found that total levels of FGFR2 were upregulated in the macrometastasis compared with the primary tumour. Remarkably those metastatic cells presented lower levels of

phospho-FGFR2 compared to the primary tumour.

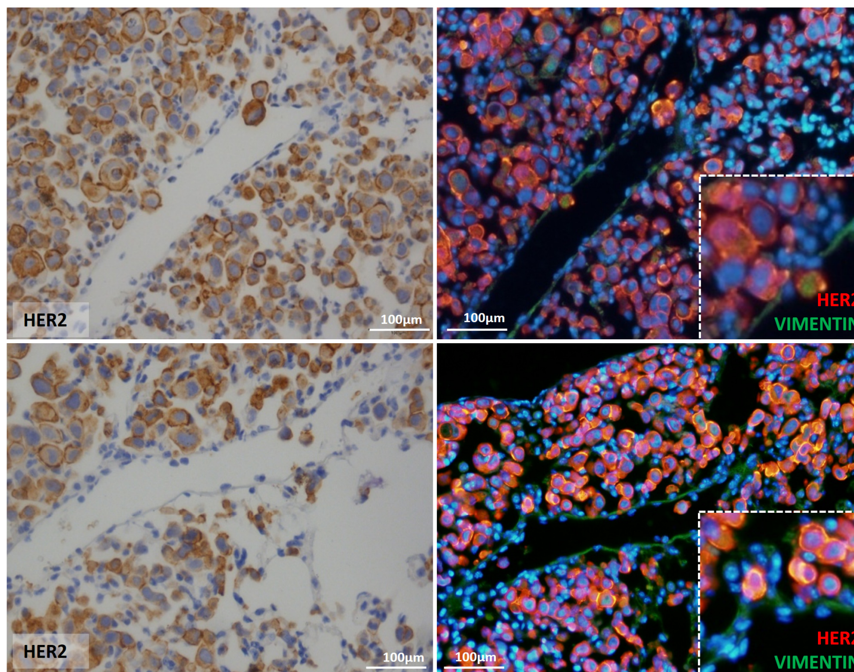


Figure 72. Breast cancer lung macrometastasis and blood vessel invasion identification. HER2 immunohistochemistry representative images in lung BC macrometastasis (left panels) and HER2 (red) and vimentin (green) dual immunofluorescence representative images in the same lung section showing blood vessels endothelial cells. Scale bar 100µm.

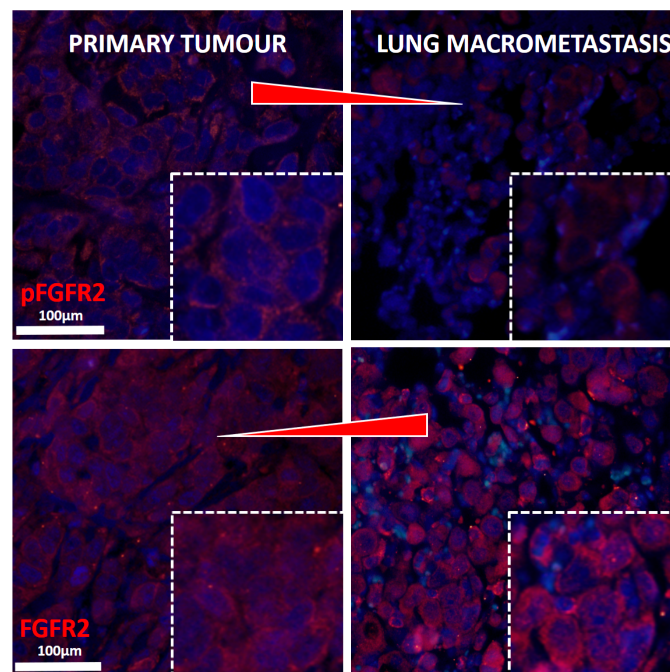


Figure 73. FGFR2 expression in primary tumour and BC macrometastasis analysis. Representative immunofluorescence images for activated FGFR2 upper panels (Y653-654, phosphorylated levels represented as p-) in primary tumour sections (left) and lung macrometastasis (right). Lower panels show total FGFR2 expression. Scale bar 100µm.

These results may suggest that during tumour cell progression, those clones with higher FGFR2 expression will be the ones with better metastatic potential, as will be able to take advantage from the FGFR2 signalling in terms of drug resistance and survival as well as microenvironment crosstalk. Once in the metastatic site, when the tumour cells have overpass all barriers and are proliferating without restrictive pressures, the FGFR2 pathway may not be so relevant anymore, and cells do not need to have it active.

As a summary, our results highlight the importance of fibroblasts in supporting tumour maintenance, progression and the HER2-resistant phenotype establishment, and strengthen the hypothesis that the acquisition of resistance to anti-HER2 therapies increases BC cells direct crosstalk capability with the microenvironment. We provided several *in vitro* evidences for fibroblasts crosstalk with breast cancer cells, through FGFR2 activation. We also demonstrated the capability of fibroblast to enhance HER2 expression and activation, as well as BC drug resistant cell lines capability to promote fibroblasts activation, and that can be also summarized in a beautiful image taken from one section from a BC cells and TAFs co-injection xenograft (**Figure 74**).

Firstly observed when analysing HER2 staining, we found a region where a group of cells expressed higher levels of HER2 than the neighbouring tumoural cells. From the histopathology analysis of the sample, it looked as this group of cells were surrounded by a circle of fibroblast. Immunofluorescence analysis of αSMA in the consecutive section of this tumour was done to confirm our suspicion, and surprisingly we also found that those fibroblasts that were wrapping the group of cells with higher HER2 expression were also showing a more active phenotype, evidenced by higher amounts of αSMA.

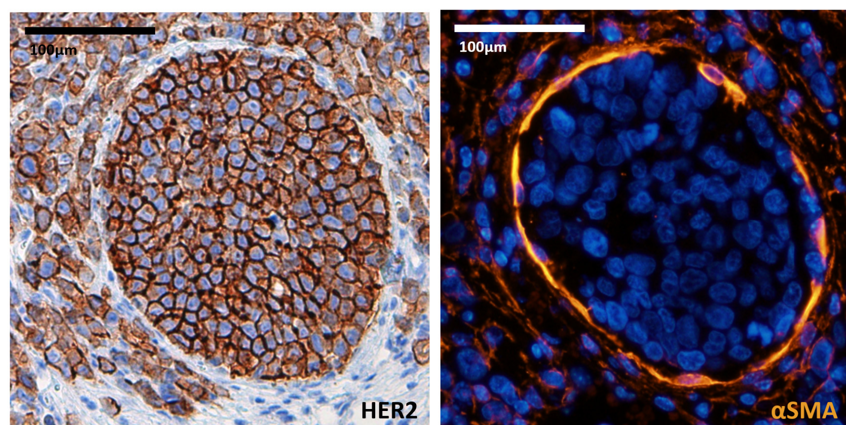


Figure 74. BC cells and fibroblast crosstalk *in vivo*. Representative HER2 immunohistochemistry of a BC xenograft section showing a group of cells with higher HER2 staining than the surrounding cells. In the middle panel, same section was stained by immunofluorescence for αSMA and shows that the fibroblast surrounding the same group of BC cells displayed higher αSMA staining intensity. Scale bar 100 µm.

8. DISCUSSION

Breast cancer is the leading cause of death among women worldwide, with about one million new cases diagnosed each year [463]. The development of resistance represents a major obstacle towards effective cancer treatment, accounting for treatment failure in the majority of cases. Given the lack of accurate biomarkers of response, it is unknown which percentage of these failures are due to acquired or intrinsic resistance to treatment.
Nowadays, the selection of patients for a particular adjuvant treatment is based on the characteristics of the patient and the tumour, in terms of hormone receptors (oestrogen and progesterone) and HER2 expression, tumour grade, lymph node status, and age. However, the high rate of acquired resistance and relapse after treatment highlights the major clinical need to develop tools that allow a more accurate identification *a priori* of patients who will benefit or not from the currently available therapies [464], as well as the identification of additional targets that could be therapeutically used to treat those patients who relapse.

In breast cancer, HER2 amplification is used to identify patients that could benefit from anti-HER2 therapies, like Trastuzumab and Lapatinib [465]. However, as mentioned before, despite the clinical benefit observed, a significant number of patients do not respond to these drugs or develop resistance after an initial response. For example, poor response rates to targeted drugs like Herceptin monotherapy reach the 26 % of the cases [465], and Multiple phase II trials have revealed that only 20 % to 35 % of patients with HER2+ metastatic breast cancer respond to Lapatinib [107].
Some preclinical models have reported that HER2 gene amplification is maintained in Trastuzumab-resistant clones [333, 347]. However, the high proportion of patients with HER2+ breast cancers that failed to respond to anti-HER2 therapies [463, 466] suggests that the mechanisms of resistance might rely in part in other HER2-independent pathways.

As mentioned in the introduction of this thesis, the resistance to anti-HER2 therapies can be mediated by multiple molecular mechanisms including impaired access to the receptor or altered receptor-antibody binding, activation of alternative ERBB members signalling, and aberrant HER2 downstream signalling activation. Then, several mechanisms related to the acquisition of resistance to anti-HER2 therapies have been described including PI3K or PTEN mutations, or the overexpression of the transmembrane receptor MUC4 that allosterically affects the interaction between the antibody and the receptor, causing poor immune system recognition [465]. In addition, the cleavage of HER2 into the p95 form has been extensively described and correlated with poor prognosis outcome and higher lymph node metastasis risk [467]. Furthemore, crosstalk with other tyrosine kinase receptors like HER3, AXL or IGF-R1 has also been widely studied [332, 468].
In addition to mutations and/or changes in the main target, intra-tumour heterogeneity for target expression, and the activation of alternative routes of cell

survival leading to adaptive resistance are the major cell autonomous mechanisms by which tumours avoid the inhibitory action of targeted therapies. **In this thesis we have shown that activation of RET and FGFR2 is necessary for Trastuzumab and Lapatinib resistance.**

The importance of the stromal microenvironment in drug resistance has been recognized during the last years, and it is now widely accepted that cancer survival and metastasis (as a consequence of treatment failure), are also regulated by stromal-cancer cell interactions [383, 469].
As described before, the cancer stroma is characterized by a modified ECM composition, increased microvessel density, inflammatory cells and activated fibroblasts. The cancer-associated fibroblast (CAF) are the most prominent cell type within the tumour stroma of many cancers, especially in breast and pancreatic carcinoma [384]. These fibroblasts are activated by chemokines, cytokines, and growth factors secreted by the stromal microenvironment that act in an autocrine and paracrine way under the influence of the tumour, and in the same way, CAFs can stimulate tumour cell proliferation, growth, and progression through production of various growth factors, hormones, and cytokines [384]. Moreover, in the recent years there have been some articles involving CAFs in the development of resistance.
Our results highlight the importance of fibroblasts in supporting tumour maintenance, progression, and in the acquisition of the HER2-targetes therapies resistant phenotype. In addition, they support the hypothesis that the acquisition of resistance to anti-HER2 therapies increases BC cells direct crosstalk capability with the microenvironment.

8.1 Identification of HER2-independent pathways related to tumour escape to anti-HER2 therapies, as well as new therapeutic targets for non-responding tumours

Understanding tumour progression and the dynamics of signalling pathways is very challenging given the multiple factors that will ultimately influence cell survival. However, better strategies for recurrent patient treatment will also require the prediction of kinome changes that allowing the rational design of new combination therapies. Moreover, drug sensitivity has been shown to be dependent on the inhibition of protein networks.

We hypothesize that additional genes may play an important role in Trastuzumab and Lapatinib resistance. Therefore, in order to identify HER2-independent pathways related to tumour scape to anti-HER2 therapies that could be used as potential new markers of response to these drugs and that might have therapeutic potential for non-responding patients, we developed BC cell lines resistant to Trastuzumab and Lapatinib.

We found that breast cancer Trastuzumab and Lapatinib resistant cell lines, which displayed a more aggressive phenotype, are still dependent on HER2 oncogenic addiction, as HER2 pathway is still modulated and active even when the receptor is being targeted by HER2 targeted therapies treatments.

This is in agreement with current clinical data supporting that Trastuzumab resistant tumours, even in advanced stages, continue to be dependent on the HER2 pathways to survive [106, 107, 186, 350].

In these drug resistant tumours, selective pressure leads to the emergence of drug resistant variants and/or to compensations in other receptors signalling in order to continue growing. This further suggests that HER2 activation under Trastuzumab and Lapatinb pressure may be regulated through indirect transactivation. In fact, a major challenge in targeted drug development is the activation of compensatory pathways. As an example, the reprogramming of the RTK network, that will lead RTK co-expression alterations in response to treatments is a dynamic mechanism of acquired resistance in many cancers [470].

EGFR, HER3 or HER4, are members of the ERBB family that can form heterodimers with HER2 and participate in the regulation downstream pathways. In the case of Trastuzumab and Lapatinib it has been shown that signalling initiated by EGFR, HER3 or HER4 can rescue the anti-proliferative effect of HER2 targeted therapies [471]. For instance, a study in a cohort of 76 HER2 positive metastatic breast cancer (MBC) treated with Trastuzumab, showed that patients carrying mutations did not achieve partial response, whereas the 32 % of patients with no mutations in EFGR, HER2 and HER4 achieved partial response ($p = 0.029$), demonstrating that activation of other HER proteins may result in a worse response to Trastuzumab [472].

In the same way, it has been shown that HER3 signalling can be activated via

amplification of the *MET* gene [473]. In fact, upregulation of *MET* expression in breast cancer cells has been also related to resistance to Trastuzumab [330]. Moreover, Sergina *et al*. also revealed that the HER2-HER3 signalling complexes represent the principal oncogenic unit in HER2-amplified breast cancers [327] . They proposed that in these tumours there is an AKT-driven feedback signalling that establishes HER3 signalling by inducing a forward-shift in HER3 phosphorylation-dephosphorylation steady state equilibrium [327].

HER4 overexpression has been also identified in 38-54 % of BC patients but its role in patient outcome is controversial. Co-overexpression of HER2 and HER4 receptors has been found to be significantly associated with delayed onset of metastases following neoadjuvant Trastuzumab therapy [474]. However, other studies have demonstrated that nuclear HER4 expression may be a mechanism of acquired resistance to Trastuzumab. In 73 HER2 positive BC patients HER4 expression after 21 days of Trastuzumab monotherapy was shown to be higher than basal levels, and also statistically associated with worse overall survival (OS) and progression free survival (PFS), increased tumour size and Ki67 levels [475].

Also other RTKs besides the ERBB family have been identified to be involved in the transactivation of HER2 or EGFR [332, 467, 468]. For these reasons, and the fact that RTKs are the main mediators of the signalling network that transmit extracellular signals into the cell, and control cellular differentiation and proliferation [110], we performed a phosphoproteomic analysis to assess the level of activation of receptors with tyrosine kinase activity in Trastuzumab and Lapatinib resistant cells after HER2 inhibition.

The proteomic profile analysis of the drug resistant cell lines revealed 21 RTKs differentially activated in the drug resistant cell lines.

Some of these receptors such as IGFR [476], HGFR [330], and some Ephrin receptors [477, 478] have been previously described to mediate HER2 resistance. For example, in the case of IGF1R, it has been described that its overexpression can increase the levels of IGF1R/HER2 heterodimers, activating the PI3K/AKT pathway and therefore producing resistance to Trastuzumab in BC patients [476].

EPHA2 has also been shown to be overexpressed in HER2 positive BC patients, potentially conferring resistance to Trastuzumab [478]. Zhuang *et al*. also showed, in a screening of a large cohort of human breast cancers, that *EPHA2* overexpression correlated with a decrease in disease-free and overall survival of HER2 overexpressing patients [477]. Moreover, they also showed that Trastuzumab resistant cell lines overexpressed EphA2, and that EphA2 inhibition was sufficient to restored Trastuzumab treatment sensitivity *in vivo* [477].

The overlap of our hit receptors with previously identified receptors in the literature gave us confidence of the clinical relevance of our experimental system. We next decided to focus on alternative RTK signalling with no previous relation to HER2 targeted therapies, like RET and FGFR2.

In the drug resistant cell lines, the cellular activation of FGFR2 and RET increased after Trastuzumab or Lapatinib treatment, suggesting that under drug pressure, the survival cells rely on those pathways as main drivers of cell survival and proliferation, in order to overcome HER2 inhibition.

The fact that HER2 total and active protein levels also increased upon Trastuzumab and Lapatinib treatment in the drug resistant cells, showing similar patterns to RET and FGFR2 activation, suggested that FGFR2 and/or RET may be capable of transactivating HER2.

In agreement with our model, Azuma *et al*. found that FGFR2 was involved in UACC812 cells resistance to Lapatinib. However, contrary to our findings, in their model FGFR2 gene was highly amplified, and this correlated with a reduced expression of HER2. In this model FGFR2 appeared to be a pivotal molecule for the survival of UACC812 drug resistant cell lines when they became independent of the HER2 pathway. In fact, PD173074 (a small-molecule inhibitor of FGFR tyrosine kinase) decreased the phosphorylation of FGFR2 and substantially induced apoptosis in UACC812 drug resistant cell line while no effect was found in the parental cells. This suggests that the switch from addiction to HER2 to addiction to the FGFR2 pathway enabled UACC812 cells to become resistant to Lapatinib [479].

Although we didn't found FGFR2 gene amplification in any of our Trastuzumab or Lapatinib resistant cell lines, we neither found a decrease in HER2 oncogenic dependence. In our model, drug resistant cell lines continue to depend on HER2 signalling, and FGFR2 may represent a main pathway of HER2 transactivation. However, it is possible that in a different model where the main oncogenic driver has been lost, cells overexpressed other PI3K/AKT activators as compensatory mechanisms.

FGFRs have been shown to be upregulated in various tumour cell types and have been described to be involved in tumour cell differentiation and proliferation, tumour angiogenesis, and tumour cell survival [480]. In addition, their ligands, fibroblast growth factors (FGFs), are also involved in mediating many other cellular processes, such as stemness, anti-apoptosis and drug resistance [202].

Recent studies have uncovered increasing evidence of their role as an escape mechanism to anti-VEGF therapies. In these studies it has been shown that deregulated FGFRs can function as driving oncogenes in certain tumour types, acting in a cell autonomous fashion to maintain the malignant properties of cancer cells [481].

FGFR1 has been found to be amplified in 10 % of BC, and FGFR2 amplifications are observed in approximately 4 % of BC, and 3 % to 25 % of gastric and colon cancers. FGFR2 and FGFR3 have also been found in 10 % of endometrial cancers [482].

Several multitargeted tyrosine kinase inhibitors have been shown to have activity against one or more FGFRs in preclinical models. These inhibitors selectively bind to and inhibit the activities of FGFRs, which may result in the inhibition of tumour angiogenesis and tumour cell proliferation,

and the induction of tumour cell death [455].

In our study we evaluated the effect of two tyrosine kinase inhibitors. Ponatinib is an oral multitargeted kinase inhibitor, and the characterization of its kinase selectivity profile showed that it exhibits potent *in vitro* biochemical activity (with IC50 values <20 nmol/L) against all 4 FGFR (33), and also has shown to be effective on RET inhibition [454]. BGJ398 is also a pan FGFR kinase inhibitor, acting as a potent inhibitor of human FGFRs with potential antiangiogenic and antineoplastic activities [483].

Inhibition of RET and FGFR2 pathways in the drug resistant cell lines with the chemical inhibitors (Ponatinib and BGJ-398) resulted in enhanced cell death *in vitro*. Furthermore, we found that these agents also decreased tumour growth *in vivo*, showing interestingly additive effects when combined with Trastuzumab and Lapatinib. Moreover, we also found that inhibition of FGFR2 and RET reduced HER2 levels and activity.

These results provide interesting evidences suggesting that FGFR2 and RET are capable of transactivating HER2, and all together these data support the idea that RET and FGFR2 could be considered as new targets for the treatment of tumours that have relapsed to anti-HER2 therapies.

In agreement with our results, it has been reported that FGFR inhibitors enhance tumour sensitivity to conventional anticancer drugs such as 5-Fluoracil, Irinotecan, and Paclitaxel in human cancers with aberrant FGFR activation [484, 485]. Additionally, Lieu *et al*. showed that FGF signalling inhibition attenuates revascularization, and reduces tumour burden in human tumours that acquired autocrine FGF signalling due to FGF2 upregulation after VEGFR2-targeted therapy [207].

FGFR inhibitors are predicted to be effective on relapsed tumours, based on the clonal evolution of a FGFR-minor activated subpopulation after targeted therapy to VEGFRs o EGFRs [204]. Furthermore, BGJ398 has also been started to be studied in clinical trials for patients with advanced solid tumours with FGFR1 or FGFR2 amplification or FGFR3 mutation. The most frequently observed adverse events were diarrhoea, fatigue, nausea and hyperphosphatemia (about one-third of the patients) and dose-limiting toxicities included grade 3 elevations in transaminase levels and grade 2 corneal events [486].

Regarding RET, there are no reports indicating an involvement of RET signalling in resistance to anti-HER2 therapies. However, several publications have associated RET and its main ligand, the glial-derived neurotrophic factor (GDNF) in resistance to aromatase inhibitors. RET and its co-receptor GFRα1 have been found to be upregulated in a subset of ER+ breast cancers. Morandi *et al*. reported that in a model of breast cancer cells resistant to aromatase inhibitors, GDNF-mediated RET signalling was enhanced in two-dimensional and three-dimensional cultures.

Furthermore, GDNF-RET signalling promoted the survival of aromatase

inhibitor resistant cells and elicited resistance in aromatase inhibitor sensitive cells. Both of these effects were selectively reverted by the RET kinase inhibitor, NVP-BBT594 [221]. Moreover, gene expression profiling of ER + cancers identified a proliferation-independent GDNF response signature that correlated with poor patient outcome and, more importantly, predicted poor response to aromatase inhibitor treatment with the development of resistance. Furthermore, they validated these findings by showing increased RET protein expression levels in an independent cohort of aromatase inhibitor resistant patient specimens [221].

In relation with these data, we found that in the ER/PR+ BT-474 cell lines, the acquisition of resistance to HER2 therapies (Trastuzumab and Lapatinib) was also accompanied with a higher expression of hormonal receptors (from 2+ to 3+). Moreover, we also reported that GDNF was also effective on activating HER2 pathway. In fact, the relation between ER and HER2 has also been associated with failure to Tamoxifen treatments [103].

Massarweh *et al*. showed that although EGFR and HER2, increased slightly after Tamoxifen treatment, they were markedly increased when tumours became drug resistant. Hence, Gefitinib, which inhibits EGFR/HER2, improved the antitumour effect of Tamoxifen and delayed resistance acquisition [103].

Moreover, in a panel of Trastuzumab and Lapatinib resistant cell lines, where ER levels were further evaluated, Wang *et al*. showed that under sustained HER2 inhibition by HER2 targeted therapies, ER functions as a key escape and survival pathway in ER/HER2+ cells [487].

These data suggested that complete blockade of the ERBB network, together with ER inhibition, may provide optimal therapy in selected patients.

Our data highlights the relevance of the GDNF/RET pathway in BC resistance, and together with the previous mentioned data suggests that combination of HER2 targeted therapies and Tamoxifen or other aromatase inhibitors, as well as with RET inhibitors may provide optimal therapy for some HER2 Trastuzumab and Lapatinib recurrent subset of patients.

8.2 Effect of the drug resistant cell lines secretome on the drug resistant phenotype and HER2 activation

We have demonstrated that Trastuzumab and Lapatinib resistant cell lines showed a different genetic profile able to predict patient outcome and that should further be validated as a response predictive gene set. We have also found that the drug resistant cell lines have some differentially expressed genes that are implicated on microenvironment related functions that usually define more aggressive tumour phenotypes, with metastatic capability and drug evasion properties.

Under the hypothesis that the crosstalk between the tumour cells and the surrounding microenvironment may be driven by secreted factors, we also studied if the drug resistant cells secreted soluble factors could induce drug resistance.

We found that the conditioned media of the drug resistant cell lines was able to induce a change in the phenotype of the drug-sensitive cell lines towards a more drug resistant phenotype. Moreover, co-culture of parental cell lines with the conditioned media from the drug resistant cell lines enhanced HER2, RET and FGFR2 activation. These data suggest that the drug resistant cell lines that overactivated RET and FGFR2 pathways to maintain HER2 activation, may also up-regulate ligands secretion.

We next confirmed that the drug resistant cell lines upregulated the main RET and FGFR2 ligands codifying genes. Therefore, we tested the effects of FGF and RET ligands on HER2 activation. Interestingly, we found that FGF2, FGF5, GDNF, and NRTN were able to induce HER2 activation.

In agreement with our data, overproduction of ligands is one of the mechanisms that can activate ERBB receptors. It is known for instance, that TGFβ receptor activation triggers ADAM17 to release more TGFα, amphiregulin and heregulin, that are well known HER3, HER4, and EGFR ligands, which leads to the activation of these receptors, and consequently the PI3K/AKT pathway [488]. Moreover, Wang *et al*. developed cell line models of acquired resistance to FGFR inhibition by exposure of cell lines harbouring FGFR3 gene amplification and translocation, to the selective FGFR inhibitor BGJ398 and multi-targeted FGFR inhibitor Ponatinib which are the same inhibitors we used in our study. Interestingly, they found that the acquisition of resistance is fast, reversible and characterized by an epithelial to mesenchymal transition and a switch from FGFR3 to ERBB family member's dependency [458]. According to its model, acquired resistance was associated with changes in gene expression including increased production of ERBB2/3 ligands, which were sufficient to drive resistance in the setting of FGFR3 dependency but not on other FGFR family member's dependency [458].

These data support the concept that activation of ERBB family members is necessary to bypass dependency on FGFR3, while our model suggests that activation of FGFR2 is sufficient to bypass HER2 dependence, and together they suggest

that concurrent inhibition of these two pathways may be desirable when targeting FGFR3/2 or HER2 dependent cancers. Interestingly, both studies suggest that FGFR and ERBB ligands play an important role in the switch from FGFR to ERBB or ERBB to FGFR oncogenic dependence.

Moreover, pathway activation as a result of FGFs released in the tumour microenvironment has been implicated in resistance to Gefitinib and Erlotinib in NSCLC, Cetuximab in KRAS wild-type squamous carcinoma cells and to Vemurafenib in BRAF V600E melanoma cells [489-491].

This data is in agreement with our data suggesting that FGF ligands play a role on the acquisition of resistance to anti-ERBB therapies in BC. Therefore, we propose that combination of FGFR inhibitors with other targeted agents, as we did in our study, might be a rationale clinical testing to prevent resistance in several types of cancers.

As mentioned before, somatic alterations of FGFRs have been described in a wide range of malignancies, and a number of anti-FGFR therapies are currently under investigation in clinical trials for subjects with FGFR gene amplifications, mutations and translocations [209]. Most FGFR inhibitors currently in development are small molecules kinase inhibitors of the ATP-binding domain. As the catalytic domains of various kinases exhibit significant structural homology, TKIs generally demonstrate activity against more than one kinase. This is a common feature of the first generation of FGFR inhibitors, which generally have activity also against VEGFR and/or PDGFR, two structurally related receptor tyrosine kinases [492, 493]. As a consequence, multikinase inhibition may increase effectiveness in the treatment of a particular tumour type by disrupting redundant pathways that drive resistance. In addition, therapeutic monoclonal antibodies can be highly specific for a particular FGF ligand or FGFR isoform, hence displaying a narrower range of toxicity when compared with pan-FGFR inhibitors [494].

The first agent to move into the clinic was FP-1039, a soluble fusion protein consisting on the extracellular domain of human FGFR1 linked to the Fc portion of human IgG1. It was engineered to spare the metabolic hormone FGFs (including FGF23 involved in phosphate and vitamin D metabolism) and to bind tightly to all of the mitogenic FGF ligands (final report of the phase I trial with FP-1039 is still pending) [495].

Moreover, RNA interference targeting of FGF2 in basal-like cell lines significantly reduced growth *in vitro.* Notably, small-molecule tyrosine kinase inhibitors (TKIs) of FGFR induce tumour shrinkage in xenogafts models with an active autocrine FGF2 signalling loop [496].

Furthermore, some preclinical studies have uncovered potential mechanisms of intrinsic or acquired resistance to FGFR inhibitors, including: mutations in the tyrosine kinase domain (FGFR2 N550K) that decrease the affinity of Dovitinib to its binding domain [497]; mutations on the ATP binding cleft of FGFR3 V555M; [497, 498]); and activation of the ERBB family members

resulting in a switch from dependency from FGFR signalling to ERBB signalling, which can be overcome with a combination of FGFR and EGFR inhibitors [499].

The variability of resistance mechanisms added to a context of tumour heterogeneity justifies the consideration of biopsy in secondary drug resistant tumours to individualize subsequent treatment, as well as discovery of novel resistance mechanisms. In fact, combination strategies have been proposed to prevent or delay the emergence of resistance. For example, the combination of Ponatinib and Ridaforolimus in an endometrial xenograft model with an FGFR2-activating mutation resulted in superior efficacy and tumour regression [500].

8.3 Role of fibroblasts in the establishment of the drug resistant phenotype and the selection of the drug resistant cells, tumour progression and evolution

In the *in vivo* study with RET and FGFR2 inhibitors we observed that the treatment with Ponatinib and BJG-398 inhibit HER2 activation as well as CAFs recruitment in the tumours derived from drug resistant cell lines xenografts, suggesting that FGFR2 and/or RET may be responsible for HER2 status in these tumours. Furthermore, our results also support the hypothesis that fibroblasts are involved in the cell fate of the drug resistant cells.

Some models have proposed that a stepwise acquisition and accumulation of mutations that will lead to the acquisition of a drug resistant phenotype might take a long time, suggesting that cells destined to develop genetic alterations first require a sustained protection from the toxic effects of the drug through a non-genetic mechanism prior to the acquisition of a drug resistant phenotype [356]. Cell populations from the tumour microenvironment are then expected to play a role in assisting tumour cells to survive until the tumour becomes effectively drug resistant. This process is known as environment-mediated drug resistance (EMDR) and protects tumour cells from the initial effects of diverse therapies [421].

The role of stromal factors in aiding cancer initiation, growth and progression, has been

well described [5, 373, 383, 407], and during the last years it has also been suggested that the stromal compartment is likely to influence therapeutic outcome, as well as provide ample opportunities for targeting [501].

In fact, a stroma derived gene expression signature prognostic of clinical outcome has been described for breast cancer [502]. Moreover, Farmer *et al*. provided further evidences defining a CAF gene signature predictive for response to neoadjuvant chemotherapy in ER negative breast cancers treated with 5-Fluoracil, Epirubicn and Cyclophosphamide [416].

In terms of targeted therapy, pre-clinical studies indicate that CAFs also mediated resistance to anti-angiogenic therapy [481]. Additionally, Wang *et al*. also defined fibroblast implication in a tyrosine kinase resistance model of lung cancer to Genitinib [429].

To further understand the crosstalk between cell lines resistant to anti-HER2 therapies and the microenvironment, and the role of fibroblasts in particular, we developed different fibroblast cell lines with different origin: fibroblast associated to healthy tissue and fibroblasts associated to breast tumours (obtained from different BC patients tumourectomies with different levels of HER2).

We first determined if similarly, to the drug resistant cancer cells, fibroblasts could also secrete soluble factors able to enhance BC cells drug resistance. We found that the conditioned media derived from CAFs was able to induce HER2 activation and switch the cancer cell phenotype from drug-sensitive to drug resistant. This effect was not found when the cells were treated with the conditioned media of healthy tissue-derived fibroblasts conditioned media. In addition, among the tumour derived fibroblasts, those coming from HER2+ tumours produced the strongest effect. This data suggested that fibroblasts originated from different tumours had a different capability to communicate with breast cancer cells lines favouring drug resistance.

In agreement with our findings, Tchou *et al*. compared the gene expression profiles of early passages fibroblast isolated from twenty human breast cancer samples representing the three main subtypes (HER2+, ER+ and triple negative). The data demonstrated that in addition to the distinct molecular profiles that characterize the neoplastic cells in a tumour, the CAFs gene expression was also differentially regulated between distinct subtypes of breast cancer [503]. Moreover, the signalling pathways from fibroblast with HER2+ origin, upregulated in CAFs contribute to enhanced migration of breast cancer cells in transwell assays [503].

We haven't assessed migration in our model, but with our results and the supporting bibliography we can hypothesize that the conditioned media of fibroblasts derived from HER2+ tumours will have also an effect on breast cancer cells migration and metastatic capability. This together with the impact that HER2+ derived CAFs have on HER2 pathway activation and survival induction upon targeted therapies pressure may contribute to an unfavourable prognosis.

Interestingly, we also found evidences that the conditioned media of Trastuzumab and Lapatinib resistant cell lines was able to induce an activated phenotype on normal fibroblasts.
Together these results suggest the existence of a crosstalk between drug resistant cells and fibroblasts and the importance of these population's communication in tumour fate and evolution in terms of resistance and progression.

We further identified *in vivo* that the co-injection of fibroblasts with breast cancer cells lead to the development of larger and more aggressive tumours, being the strongest effect observed when the cancer cells were co-injected with fibroblasts derived from HER2+ tumours. Interestingly, the co-inoculation with healthy tissue derived fibroblast exerted a repressive effect on tumour growth and development, in concordance with the known normal tissue microenvironment protective function, acting as a barrier to tumourigenesis. It is well known that under normal tissue homeostasis conditions , the microenvironment exerts suppressive forces to keep tumours occult [356].
Similar results have been described in the context of prostate cancer. Co-implantation of pre-malignant prostate epithelial cells with prostate tumour CAFs, but not normal prostate fibroblasts, leads to malignant transformation and proliferation of epithelial cells [504].
We also observed that the tumours derived from the co-injection with fibroblasts derived from HER2+ tumours actually displayed higher HER2 expression. Interestingly, also a higher infiltration of mice origin fibroblasts on those tumours derived from the co-inoculation groups, was also found.

It is known that co-injected fibroblasts do not survive long term in xenografts immunodeficient mice [505, 506], which suggests that its presence might be important at the time of xenograft tumour initiation, but not necessary for the subsequent tumour growth.
In this scenario different possibilities are plausible to explain our results. It could happen that at first, fibroblasts with different origins recruit and activate mice fibroblasts in different ways concerning its origin. In this way, HER2+ tumours derived fibroblast would be able to activate mice fibroblasts at higher degree, while fibroblasts isolated from healthy tissues would not be so efficient in activating and recruiting mice fibroblasts.
Another possibility is that fibroblasts from different origin co-injected with BC cells in mice modulate in a paracrine way BC cells phenotype in a different way depending on the fibroblasts origin. As a consequence, BC cells with different properties will drive progression of tumours with different properties and phenotype.
In the first case, mice fibroblast with different activation phenotypes will co-evolve with BC cells leading to different tumour evolution. In the second situation, co-injected fibroblasts will select those cells with different tumourigenic capability that will finally drive tumour growth. To better asses this hypothesis, it would be

interesting to develop an *in vivo* time course study with a similar approach in order to better define how the selective process takes place in the initial phase of tumour establishment.

It has been shown that fibroblasts sub-lethally irradiated before mice injection enhanced tumour growth more potently than did unirradiated cells, due to their acquisition of an activated phenotype [507]. This pattern, toghether with the tumour-promoting role of irradiated stromal cells has raised concerns about the possible undesirable side-effects of radiotherapy during breast cancer treatment [380]. The different capability of tumour growth induction by fibroblast with different pattern of activation by irradiation resemble in some way the proposed different effect on tumour growth by fibroblasts from different origin in terms of HER2 status of our study.

Furthermore, in our model, the different tumour growth and HER2 status in the derived xenografts also resulted in different sensitivity to HER2 therapies. BC cell lines derived from tumours co-inoculaed with HER2+ fibroblasts were more resistant to Trastuzumab and Lapatinib. Interestingly, these cells also showed higher levels of FGFR2 activation that also conferred resistance to FGFR2 inhibitors (Ponatinib and BGJ-398).

Then, the drug resistant phenotype was evaluated in isolated cells lines from xenograft derived tumours, but it would be really interesting to further test if those populations are also resistant in an *in vivo* setting, to further characterize fibroblast crosstalk implications in BC resistance. In relation to this, Lotti *et al*., showed that tumour response to treatment is associated with increased frequency of CAFs infiltrating the tumour, being chemotherapy the responsible of CAFs recruitment, as fibroblasts were found significantly increased after treatment [508].

Similarly, in a lung cancer model, Wang *et al*. showed that both human fibroblast cell lines and primary cultured fibroblasts produced HGF. This, lung cancer cells became resistant to EGFR-TKIs when co-cultured *in vitro* with HGF-producing fibroblasts and co-injected into severe combined immunodeficient mice. Importantly, combined use of Gefitinib plus anti-HGF antibody or the HGF antagonist, NK4, successfully overcame the fibroblast induced EGFR-TKI resistance both *in vitro* and *in vivo* [429].

Our data provide more evidences to the field, indicating that the microenvironment (fibroblast) mediated drug resistance arises from an adaptive, reciprocal signalling dialogue between tumour cells and the surrounding microenvironment.
Then, application of anti-FGFR2 therapies in the clinical setting will not only be effective as a treatment for the drug resistant patients, and should also be considered to use these therapies during the initial phases of treatment to prevent the emergence of acquired fibroblast mediated resistance.

Although we did not further characterize fibroblast implication in RET signalling, in MCF7 xenograft experiments, GDNF was associated with a higher levels of infiltrating fibroblasts and invasive tumour margins [232]. These data suggest the interesting possibility that oestrogen-induced upregulation of RET and ARTN in tumour cells may promote tumourigenesis resulting in recruitment of stromal cells which, in turn, induce GDNF expression. Together this would create a vicious loop of RET signalling in ER positive breast cancers that impacts on tumour cell survival and resistance to therapy [482].

Finally, *in vivo* preliminary data from our study also suggested that the FGFR2 pathway may confers metastatic capacity.
90 % of cancer deaths from solid tumours are being caused by metastasis which is preceded by local invasion [509].
A key feature of carcinoma-associated myofibroblasts is their ability to actively promote the invasion of cancer cells thereby facilitating metastasis [385]. In breast cancer these myofibroblasts showed extensive gene expression changes in genes encoding invasion-associated factors and receptors [406]. Therefore, we propose the hypothesis that during tumour cell progression those cells with higher FGFR2 will take advantage from the FGFR2 signalling, promote drug resistance and survival as well as increase the microenvironment crosstalk with fibroblasts, favouring the metastatic potential. However further experiments are needed to confirm this.

In summary, our results highlight the dual contribution of the FGFR2 pathway in cancer cell resistance to anti-HER2 therapies, and that FGFR2 inhibitors might decrease both FGFR2- and HER2-mediated signalling pathways in cancer cells, inhibiting also tumour-supporting signalling pathways from CAFs.

In agreement with our data, Wei W. *et al.* recently proposed evidences of another mechanism of FGFR2-HER2 interaction, defining that FGFR2 signalling promotes HER2 shedding through the metalloprotease ADAM 10 leading to intracellular accumulation of the truncated p95HER2 protein. This was accompanied by enhanced HER2 signalling and diminished sensitivity to Trastuzumab [510].

CONCLUDING REMARKS

According to our work, it is evident that factors secreted by fibroblast can support cancer growth and progression by influencing and promoting a drug resistant phenotype. In our proposed model, HER2 positive BC cell lines resistant to Trastuzumab and Lapatinib have an active FGFR2/RET signalling pathway, able to maintain HER2 signalling by receptor transactivation. This bypass HER2 inhibition and contributes to the maintenance of the cellular addiction to HER2 pathways even when the protein is being targeted, promoting cell proliferation and survival (**Figure 75**).
Moreover, drug resistant cell lines secreted factors that induced fibroblast activation and also modulated the secretion pattern of

tumour promoting factors from fibroblasts. As a result, after exposure to secreted factors from drug resistant cell lines, fibroblasts become more efficient on promoting BC cell lines resistance.

Similarly, the conditioned media from fibroblasts isolated from HER2+ tumours was able to induce the activation of HER2 and FGFR2 pathways. The same effect was found *in vivo*, where co-injection with HER2+ tumour associated fibroblasts promoted tumour aggressiveness and resistance through HER2 and FGFR2 activation (**Figure 75**).

All this data together, suggests a coevolution process between BC cells and fibroblasts. Moreover, it identifies FGFR2 pathway as a novel mechanism of HER2 activation mediated by fibroblasts and BC cells crosstalk.

This demonstrates the importance of the interaction between these two pathways *in vitro* and in *vivo*, in promoting breast cancer resistance and progression. Furthermore, we propose that inhibition of RET and FGFR2 pathways might become a promising salvage strategy after Trastuzumab and Lapatinib failure in patients with HER2-positive breast cancer.

Moreover, the addition of anti-FGFR therapies in the early stages of breast cancer treatment may prevent resistance development.

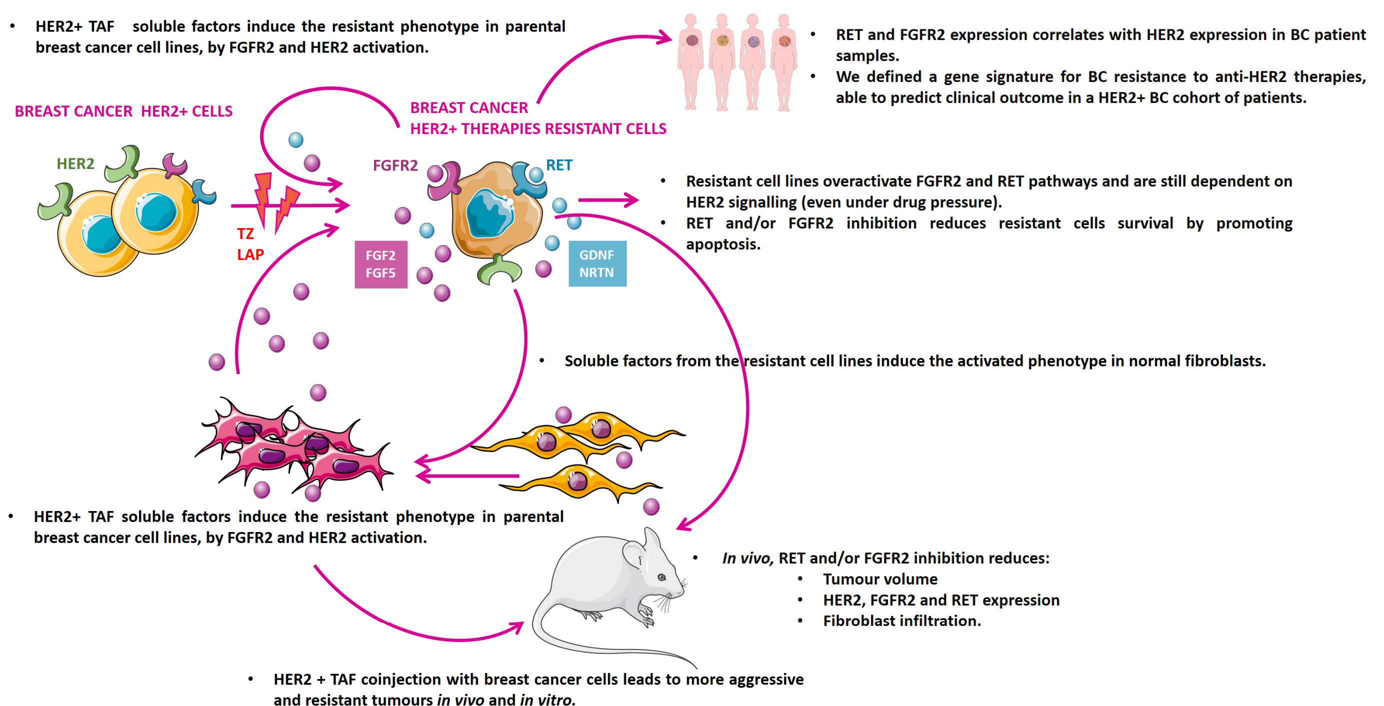


Figure 75. Image summarizing HER2 resistance model and fibroblast crosstalk with the resistant cells.

9. CONCLUSIONS

1. Breast cancer cell lines resistant to Trastuzumab and Lapatinib, which displayed a more aggressive phenotype *in vitro*, are still dependent on HER2 oncogenic addiction, as they are still activating the HER2 pathway under drug pressure.

2. Breast cancer cell lines resistant to Trastuzumab and Lapatinib rely on alternative RTK signalling, like RET and FGFR2, as main drivers of tumourigenicity, cell survival and proliferation. Inhibition of these pathways results in tumour reduction *in vivo*, showing also additive effects when combined with current anti-HER2 therapies. Therefore, RET and FGFR2 can be considered as new therapeutic targets for Trastuzumab and Lapatinib resistant patients.

3. We defined a 194 gene signature for breast cancer resistance to HER2 therapies, that was able to predict clinical outcome in a HER2+ breast cancer cohort of patients.

4. FGFR2 and RET ligands are capable of activate HER2 through RET and FGFR2 transactivation and inducing Trastuzumab and Lapatinib resistance. Moreover, the drug resistant cell lines secretome induced the drug resistant phenotype in parental cell lines through HER2, RET and FGFR2 activation.

5. Resistant cell lines secreted factors induce fibroblast activation. Furthermore, Lapatinib resistant cell lines – mediated fibroblast activation, increases fibroblast capability to promote the drug resistant phenotype in sensitive breast cancer cells, by modulating its tumour promoting factors secretion pattern.

6. HER2+ tumour associated fibroblasts secretome is able to induce the drug resistant phenotype in breast cancer sensitive cells by FGFR2 activation and consequent HER2 transactivation *in vitro.*

7. *In vivo,* HER2+ tumour associated fibroblasts co-injection with breast cancer cells induce more aggressive tumours. Moreover, breast cancer cells isolated from co-injected tumours showed increase resistance levels to Trastuzuamb, Lapatinib, Ponatinib and BGJ-398 by HER2 and FGFR2 activation.

8. Our results suggest a coevolution process between breast cancer cells and fibroblasts, and defines FGFR2 pathway as a novel mechanism of HER2 activation, mediated by fibroblasts.

9. We highlight the importance of the interaction between these two pathways in *vitro* and in *vivo*, in promoting breast cancer resistance and progression. Furthermore, we propose that inhibition of RET and FGFR2 pathways might become a promising salvage strategy after Trastuzumab and Lapatinib failure in patients with HER2-positive breast cancer. Moreover, the addition of anti-FGFR therapies in the early stages of breast cancer treatment may prevent resistance development

10. SCIENTIFIC ACTIVITY

10.1 PUBLICATIONS

Differential expression of neurogenes among breast cancer subtypes identifies high risk patients.
Patricia Fernandez-Nogueira, Paloma Bragado, Vanessa Almendro, Elisabet Ametller, Mario Mancino*, Pedro Gascon*. *Oncotarget, 2015 Dec 10. doi: 10.18632.*

The new antitumour drug ABTL0812 inhibits Akt/mTORC1 axis by upregulating Tribbles-3 pseudokinase. Tatiana Erazo, Mar Lorente, Anna López, Pau Muñoz-Guardiola, **Patricia Fernandez-Nogueira**, José A García-Martínez, Paloma Bragado, Gemma Fuster, María Salazar, Jordi Espadaler, Javier Hernández-Losa, Jose R Bayascas, Marc Cortal, Laura Vidal, Pedro Gascón, Mariana Gómez-Ferreria, José Alfón, Guillermo Velasco, Carles Domènech, Jose M Lizcano. *Clinical Cancer Research, 2015 Dec 15*.

Fibroblast role in the acquisition and maintenance of anti-HER2 targeted therapies resistance in breast cancer.
Fernandez-Nogueira P, Mancino M, Fuster G, Enreig E, Bragado P, Bill A, Rye I, Lopez A, Ametller E, Russnes H, Gaither A, Almendro V*, Gascón P*. *Manuscript in preparation.*

Substance P Autocrine Signaling Contributes to Persistent HER2 Activation That Drives Malignant Progression and Drug Resistance in Breast Cancer.
Garcia-Recio S, Fuster G, **Fernandez- Nogueira P**, Pastor-Arroyo EM, Park SY, Mayordomo C, Ametller E, Mancino M, Gonzalez-Farre X, Russnes HG, Engel P, Costamagna D, Fernandez PL, Gascón P, Almendro V. *Cancer Research, 2013 Nov 1;73(21):6424-34.*

Targeting of substance P induces cancer cell death and decreases the steady state of EGFR and HER2. Cristina Mayordomo, Susana García-Recio, Elisabet Ametller, **Patricia Fernandez-Nogueira**, Ignasi Casas, Pablo Engel, Eva María Pastor-Arroyo, Pedro Gascón, Vanessa Almendro. *Journal of Cellular Physiology 2012 Apr;227(4).*

Tumour promoting effects of CD95 signaling in chemoresistant cells.
Elisabet Ametller, Susana García-Recio, Domizziana Costamagna, Cristina Mayordomo, **Patricia Fernandez-Nogueira**, Neus Carbó, Eva María Pastor-Arroyo, Pedro Gascón, Vanessa Almendro**.** *Mol Cancer. 2010 Jun 23; 9:16.*

11. ANNEX

A1. HER2 TARGETED THERAPIES COMBINATIONS

COMBINATION	REGIMEN	TESTED DOSE	DISEASE	COMMENT	REF.
HER2-targeted therapies	Trastuzumab + lapatinib	Trastuzumab 2 mg/kg IV weekly Lapatinib 1000 mg PO daily	Advanced HER2-positive breast cancer	This regimen significantly improved PFS in a phase III trial as compared to lapatinib alone. Recommended by NCCN for Trastuzumab exposed HER2-positive disease	[511]
	Pertuzumab + trastuzumab	Pertuzumab 420 mg IV Q3 weeks Trastuzumab 2 mg/kg IV weekly	HER2-positive metastatic breast cancer that has progressed on trastuzumab-based therapy	Phase II trial ORR was 24 % in 66 patients	[512]
	T-DM1 + pertuzumab	T-DM1 3.6 mg/kg IV Q3 weeks Pertuzumab 420 mg IV Q3 weeks	HER2-positive breast cancer	4/9 Patients in phase Ib trial had PR. Phase II trial completed and not reported yet	[513]
HER2-targeted therapies with other biological therapies	Trastuzumab + lapatinib + bevacizumab	Trastuzumab 6 mg/kg IV Q3 weeks Lapatinib 1000 mg PO daily Bevacizumab 15 mg/kg IV Q3 weeks	Advanced HER2-positive lung cancer and salivary duct carcinoma	This regimen achieved durable partial response in one NSCLC patient with HER2 mutation and resolution of measurable disease in a patient with salivary duct cancer	[514, 515]
	Trastuzumab + erlotinib	Trastuzumab 2 mg/kg IV weekly Erlotinib 100 mg PO daily	Metastatic HER2-positive breast cancer	Phase I/II study. Four out of 12 patients had PR (duration > 6 months) 7/47(15 %) Had PR	[516, 517]
	Pertuzumab + erlotinib	Pertuzumab 420 mg IV Q3 weeks Erlotinib 150 mg PO daily	Advanced NSCLC	Phase Ib study 3/15 patients had PR. One responder had wildtype EGFR	[518]
	Lapatinib + bevacizumab	Lapatinb 1500 mg PO daily Bevacizumab 10 mg/kg IV Q2 weeks	HER2-positive advanced breast cancer	Phase II study 6/45 (13 %) had PR	[519]

COMBINATION	REGIMEN	TESTED DOSE	DISEASE	COMMENT	REF
HER2-targeted therapies with chemotherapy	Trastuzumab + fluorouracil + cisplatin	Trastuzumab 6 mg/kg IV Q3 weeks Cisplatin 80 mg/m2 IV on day 1 Fluorouracil 800 mg/m2 IV daily on days 1–5 of 3-week cycle	HER2-positive advanced gastric and gastroesophageal junction cancer	Adding trastuzumab significantly increases OS in the phase III trial as compared to chemotherapy alone	[520]
	Lapatinib + capecitabine	Capecitabine 1000 mg/m PO BID 14 of 21-days cycle Lapatinib 1250 mg PO daily	HER2-positive metastatic breast cancer that has progressed on trastuzumab-based therapy HER2-positive advanced breast cancer	Phase III trial. Superior to capecitabine alone	[106]
	Pertuzumab + trastuzumab + docetaxel	Pertuzumab 420 mg IV Q3 weeks. Trastuzumab 6 mg/kg IV Q3 weeks. Docetaxel 75 mg/m2 IV Q3 weeks	HER2-positive advanced breast	Phase III trial Adding pertuzumab significantly increased PFS. Recommended by NCCN as first line breast cancer treatment for HER2-positive advanced	[521]
	Trastuzumab + gefitinib + docetaxel	Trastuzumab 6 mg/kg IV Q3 weeks Docetaxel 60 mg/m2 IV Q3 weeks Gefitinib 250 mg PO daily 2 out of 3 weeks	HER2-positive advanced breast	Phase I/II trial in 29 patients, single arm CR:18 % PR:46 % SD:29 % Phase II study ORR was 73 % in 66 patients	[522, 523]
	Pertuzumab + gemcitabine	Pertuzumab 420 mg IV Q3 weeks Gemcitabine 800 mg/m2 IV day 1 and 8 of a 21-day cycle	Advanced, platinum-resistant ovarian cancer	Phase II study 14 % ORR in 65 patients	[524]
	Lapatinib + nab-paclitaxel	Lapatinb 1000 mg PO daily Nab-paclitaxel 100 mg/m2 IV on day 1,8,15 Q28 days	HER2-positive advanced breast cancer	Phase II study ORR was 53 % (n = 32)	[525]
	Trastuzumab + paclitaxel	Trastuzumab 2 mg/kg IV weekly Paclitaxel 60 mg/m2 IV weekly	HER2-positive NSCLC	First report of successful use of trastuzumab in HER2-mutated NSLCL	[526]

A1. Selected studies of tested combinations of targeted anti-HER2 therapy. Abbreviations: EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor; NCCN, national comprehensive cancer network; NSCLC, non-small cell lung cancer; ORR, overall response rate; OS, overall. Adapted from Min Yan *et al.* 2014 [527].

A2. HER2 GENE SIGNATURE GENES

MUC19	BIRC5	FAM72A	KIF20A	HOTAIR	RSAD2	ABCC3	SLPI
SUSD4	GABARAPL1	SERPINF1	VCX2	MPP2	LOC400578	CXorf27	STAU2
ABCC3	RFTN1	CKAP2L	SATB1	HSPA2	PRDX2	SPRY4	SLC1A5
ADAM20	FBXW10	C15orf52	PLCE1	LOC100128838	CIB2	RFTN1	IGFBP3
ALHX1	PLA2G2A	DEPDC1	FOSB	LY6E	LIPH	UBE2T	SDC4
ASPM	DNAH5	COL4A6	KANK4	CIT	PDE8B	SLC25A6	ESCO2
BRF2	IFIT2	LOC728276	PTCH1	CYBRD1	SLCO2A1	CYP1A1	S100P
C11orf92	SPC25	TFF1	UQCRFS1	HERC5	MEIS2	STIL	SGOL2
CACNA2D4	ANP32E	ESCO2	RAPGEF3	CASC5	ESPL1	LOC200609	TARP
CASP10	APOBEC3F	GUCY1B2	CYP27A1	CPAMD8	PPP1R3E	BBOX1	MESP1
CENPE	MUCL1	C15orf42	PHGDH	PDE4A	AKR7A2	STX19	FLNA
EDN1	LRRC39	SYTL5	NLGN4X	PARM1	FTCD	KANK4	APOL6
EGR2	MAFB	CDCA2	UBE2C	SLC16A5	AGPAT9	C8orf46	FLJ36031
FAM95B1	GALNTL1	SLC13A3	SBSN	TP53INP2	SEPP1	LAMB1	GAL3ST1
FOS	PCK2	NFIL3	KCNK2	NR4A2	NRP1	CXCR4	ASPH
MECOM	FMOD	POLQ	NANOS1	EGR4	NR2F1	SNIP	C1orf54
NTN4	hCG_1994695	MAL2	PLAC1	N4BP2L1	KIF14	ULBP1	BUB1
PALMD	ABCC8	PBK	SLC7A11	GPER	DDIT4	NUF2	CEP55
RGS16	TROAP	HMMR	NR4A2	DPYD	SPAG5	EML5	RAD54L
SCGB2A2	OASL	CAPN8	ARCN1	CITED2	C14orf73	PIF1	
SGOL1	CADM1	GULP1	TRIM32	DIRC3	DUSP6	N4BP2L1	
SUMO3	MECOM	CENPI	ZNF226	DACH1	ARHGAP26	RCAN2	
TBC1D1	ANTXR1	TP53INP1	PJA1	UNC5B	GPNMB	RGS16	
UGT2B11	GARS	TSPAN1	SESN2	CCNA2	IGFBP3	PDE4DIP	
WNT5A	CLCA2	CDC2	ANKRD20A2	CHAC1	AVIL	HJURP	

A3. MICROENVIRONEMENT RELATED GENE LISTS

NEUROGENE FACTORS RELATED PATHWAYS

TRASTUZUMAB	LAPATINIB
GAL3ST1	CXCR4
LAMB1	DACH1
PDGFD	EGR2
RIMS3	GAD1
SERPINF1	GAL3ST1
TAC3	GNAS
	LHX1
	NR4A2
	NRP1
	PDE4A
	PHGDH
	PTCH1
	RAPGEF3

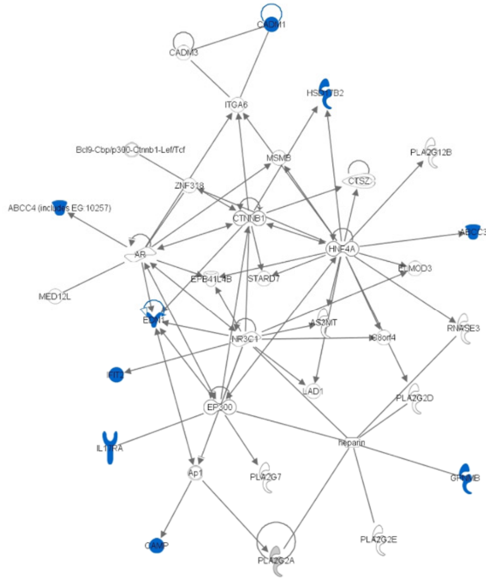
LAPATINIB RESISTANCE TRASTUZUMAB RESISTANCE

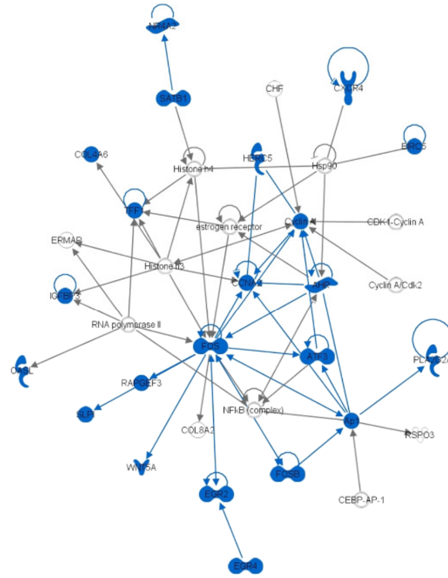
ANGIOGENESIS FACTORS RELATED PATHWAYS

TRASTUZUMAB	LAPATINIB
SERPINF1	NRP1
CEACAM1	BIRC5
PDGFD	CXCR4
GUCY1B2	RCAN2
EDN1	RAPGEF3
LAMB1	HMMR
HSPG2	NTN4
IGFBP3	IGFBP3
PLA2G2A	FOS
AACSL	COL4A6
	PDE4A
	PTCH1
	SLPI
	AHR
	CCNA2
	GNAS
	PLA2G2A
	ABCA17P

LAPATINIB RESISTANCE TRASTUZUMAB RESISTANCE

CYTOKINES AND CHEMOKINES RELATED PATHWAYS

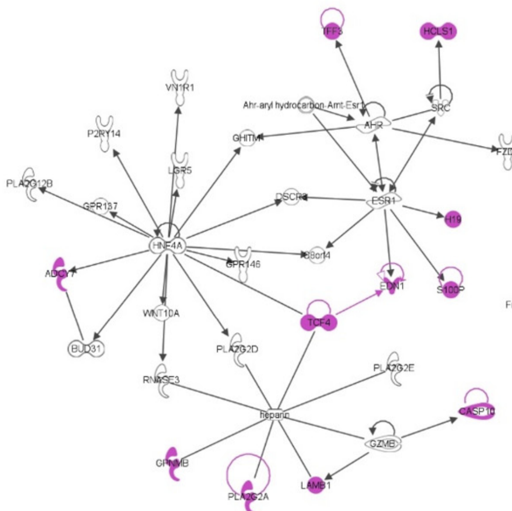


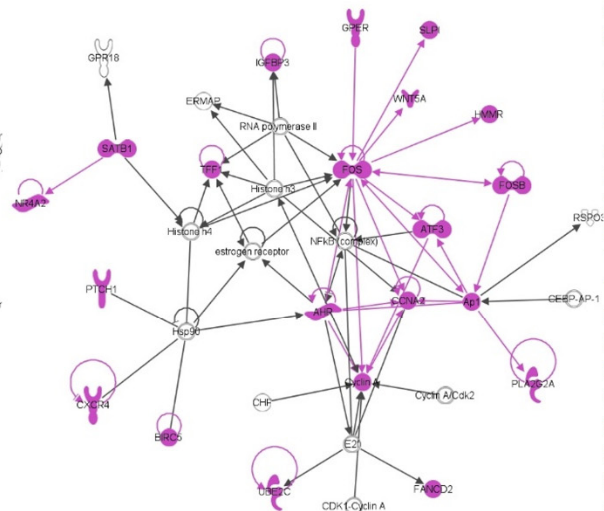


LAPATINIB RESISTANCE TRASTUZUMAB RESISTANCE

TRASTUZUMAB	LAPATINIB	
IL27RA	CCNA2	EGR2
IL17RA	CENPE	SDC4
CAMP	CDC2	RAPGEF3
EDN1	CXCR4	ABCC3
IFIT2	HERC5	AHR
CEACAM1	PDE4A	NRP1
GPNMB	CITED2	NR4A2
PLA2G2A	SLPI	SATB1
IGFBP3	LY6E	ATF3
ABCC3	EGR4	COL4A6
CA2	CMTM3	TFF1
SERPINF1	MUC19	ABCC8
HSD17B2	FOS	BIRC5
ST6GAL1	SEPP1	DPYD
TFF1	FOSB	
COL1A1	PCK2	
CADM1	OASL	
CYP1A1	PLA2G2A	
ABCC4	IGFBP3	

METASTASIS FACTORS RELATED PATHWAYS





LAPATINIB RESISTANCE TRASTUZUMAB RESISTANCE

TRASTUZUMAB	LAPATINIB	
LAMB1	AGPAT9	FOS
CEACAM1	ASPH	IGFBP3
CEACAM7	CXCR4	PTCH1
SERPINF1	HMMR	FANCD2
TFF3	SCGB2A2	AHR
SCGB2A1	SLPI	GNAS
MUCL1	MUCL1	
AOC3	STC1	
MGAT3	ATF3	
CA2	PRDX2	
ST6GAL1	BIRC5	
TFF1	CCNA2	
TCF4	NRP1	
H19	ESPL1	
CADM1	DPYD	
PLA2G2A	TFF1	
CYP1A1	FLNA	
S100P	PLA2G2A	
GPNMB	HOTAIR	
GPER	FOSB	
HCLS1	CLCA2	
ADCY7	SATB1	
WNT3	DIRAS3	
NR4A3	S100P	
CASP10	RGS16	
IDH1	EOMES	
COL1A1	GPER	
IGFBP3	NR4A2	
EDN1	SLC7A11	

A4. RTK INFORMATION

ID	FULL NAME	CROMOSOMAL REGION	
AXL	AXL receptor tyrosine kinase	chr19:46459235-46459294	hs|19q13.2
MERTK	c-mer proto-oncogene tyrosine kinase	chr2:112447572-112447631	hs|2q13
TYRO3	TYRO3 protein tyrosine kinase	chr15:39658551-39658610	hs|15q15.1
EPHA1	EPH receptor A1	chr7:142801432-142801044	hs|7q35
EPHA2	EPH receptor A2	chr1:16328559-16324387	hs|1p36.13
EPHA3	EPH receptor A3	chr3:89582092-89582151	hs|3p11.1
EPHA4	EPH receptor A4	chr2:221992385-221992326	hs|2q36.1
EPHA6	EPH receptor A6	chr3:98850014-98850073	hs|3q11.2
EPHA7	EPH receptor A7	chr6:94008778-94008719	hs|6q16.1
EPHB1	EPH receptor B1	chr3:136460804-136460863	hs|3q22.2
EPHB2	EPH receptor B2	chr1:23113178-23113237	hs|1p36.12
EPHB4	EPH receptor B4	chr7:100240736-100239102	hs|7q22.1
EPHB6	EPH receptor B6	chr7:142278499-142278558	hs|7q34
EGFR	epidermal growth factor receptor	chr7:55242335-55242394	hs|7p11.2
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2	chr17:35120176-35120235	hs|17q12
ERBB3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3	chr12:54768980-54769039	hs|12q13.2
ERBB4	v-erb-b2 erythroblastic leukemia viral oncogene homolog 4	chr2:211948819-211948760	hs|2q34
FGFR1	fibroblast growth factor receptor 1	chr8:38400769-38400710	hs|8p12
FGFR2	fibroblast growth factor receptor 2	chr10:123227843-123227830	hs|10q26.13
FGFR3	fibroblast growth factor receptor 3	chr4:1778852-1778911	hs|4p16.3
FGFR4	fibroblast growth factor receptor 4	chr5:176455727-176455786	hs|5q35.2
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	chr4:55301268-55301327	hs|4q12
MET	hepatocyte growth factor	chr7:81167004-81166945	hs|7q21.11
MST1	macrophage stimulating 1 receptor (c-met-related tyrosine kinase)	chr3:49696614-49696555	hs|3p21.31
IGF1R	insulin-like growth factor 1 receptor	chr15:97318690-97318749	hs|15q26.3
INSR	insulin receptor-related receptor	chr1:155078886-155078827	hs|1q23.1
MUSK	muscle, skeletal, receptor tyrosine kinase	chr9:112602896-112602955	hs|9q31.3
NTRK1	neurotrophic tyrosine kinase, receptor, type 1	chr1:155112056-155112528	hs|1q23.1
NTRK2	neurotrophic tyrosine kinase, receptor, type 2	chr9:86615799-86615858	hs|9q21.33
NTRK3	neurotrophic tyrosine kinase, receptor, type 3	chr15:86221248-86221189	hs|15q25.3
CSFR1	colony stimulating factor 1 receptor,	chr5:149413113-149413054	hs|5q33.1
FLT3	fms-related tyrosine kinase 3	chr13:27476051-27475993	hs|13q12.2
PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	chr4:054857372-054857313	hs|4q12
PFGFRB	platelet-derived growth factor receptor, beta polypeptide	chr5:149473716-149473657	hs|5q33.1
RET	ret proto-oncogene	chr10:42937418-42939131	hs|10q11.21
ROR1	receptor tyrosine kinase-like orphan receptor 1	chr1:64419380-64419439	hs|1p31.3
ROR2	receptor tyrosine kinase-like orphan receptor 2	chr9:93525347-93525288	hs|9q22.31
TIE1	tyrosine kinase with immunoglobulin-like and EGF-like domains 1	chr1:43560915-43560974	hs|1p34.2
TEK	TEK tyrosine kinase, endothelial	chr9:27220070-27220129	hs|9p21.2
FLT1	fms-related tyrosine kinase 1	chr13:27869188-27869129	hs|13q12.3
FLT4	fms-related tyrosine kinase 4	chr5:179961798-179961739	hs|5q35.3
KDR	kinase insert domain receptor (a type III receptor tyrosine kinase)	chr4:55640213-55640154	hs|4q12

12. BIBLIOGRAPHY

1. Russo, J. and I.H. Russo, *Development of the human breast.* Maturitas, 2004. **49**(1): p. 2-15.
2. Lakhani, S.R. and M.J. O'Hare, *The mammary myoepithelial cell--Cinderella or ugly sister?* Breast Cancer Res, 2001. **3**(1): p. 1-4.
3. Thorarinn Gudjonsson, M.C.A., Mark D. Sternlicht, Ole W. Petersen, and Mina J. Bissell, *Myoepithelial Cells: Their Origin and Function in Breast Morphogenesis and Neoplasia.* J Mammary Gland Biol Neoplasia 2005. **10(3): 261–272**.
4. Pinder, S.E. and I.O. Ellis, *The diagnosis and management of pre-invasive breast disease: ductal carcinoma in situ (DCIS) and atypical ductal hyperplasia (ADH)--current definitions and classification.* Breast Cancer Res, 2003. **5**(5): p. 254-7.
5. Arendt, L.M., et al., *Stroma in breast development and disease.* Semin Cell Dev Biol, 2010. **21**(1): p. 11-8.
6. Ferlay J, S.H., *Early Breast Cancer: From Screening to Multidisciplinary.* 3th edition, 2013.
7. http://globocan.iarc.fr/Pages/Map.aspx, accessed on September 2015.
8. Ribes, J., et al., *Cancer incidence and mortality projections up to 2020 in Catalonia by means of Bayesian models.* Clin Transl Oncol. **16**(8): p. 714-24.
9. DeVita, H., *Cancer: Principles & Practice of Oncology.* 10th ed, 2015.
10. Hopton, D.S., et al., *Histological grading of breast cancer; significance of grade on recurrence and mortality.* Eur J Surg Oncol, 1989. **15**(1): p. 25-31.
11. Elston, C.W. and I.O. Ellis, *Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up.* Histopathology, 2002. **41**(3A): p. 154-61.
12. Rakha, E.A., et al., *Breast cancer prognostic classification in the molecular era: the role of histological grade.* Breast Cancer Res. **12**(4): p. 207.
13. Sobin, L.H. and C.C. Compton, *TNM seventh edition: what's new, what's changed: communication from the International Union Against Cancer and the American Joint Committee on Cancer.* Cancer. **116**(22): p. 5336-9.
14. Willett, W.C., *Diet and cancer.* Oncologist, 2000. **5**(5): p. 393-404.
15. Cheang, M.C., et al., *Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer.* J Natl Cancer Inst, 2009. **101**(10): p. 736-50.
16. Urruticoechea, A., I.E. Smith, and M. Dowsett, *Proliferation marker Ki-67 in early breast cancer.* J Clin Oncol, 2005. **23**(28): p. 7212-20.
17. Welboren, W.J., et al., *Genomic actions of estrogen receptor alpha: what are the targets and how are they regulated?* Endocr Relat Cancer, 2009. **16**(4): p. 1073-89.
18. Bardou, V.J., et al., *Progesterone receptor status significantly improves outcome prediction over estrogen receptor status alone for adjuvant endocrine therapy in two large breast cancer databases.* J Clin Oncol, 2003. **21**(10): p. 1973-9.
19. Yarden, Y., *Biology of HER2 and its importance in breast cancer.* Oncology, 2001. **61 Suppl 2**: p. 1-13.
20. Murphy, C.G. and M. Fornier, *HER2-positive breast cancer: beyond trastuzumab.* Oncology (Williston Park). **24**(5): p. 410-5.
21. Sorlie, T., et al., *Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications.* Proc Natl Acad Sci U S A, 2001. **98**(19): p. 10869-74.
22. Buyse, M., et al., *Validation and clinical utility of a 70-gene prognostic signature for women with node-negative breast cancer.* J Natl Cancer Inst, 2006. **98**(17): p. 1183-92.
23. van de Vijver, M.J., et al., *A gene-expression signature as a predictor of survival in breast cancer.* N Engl J Med, 2002. **347**(25): p. 1999-2009.
24. Allred, D.C., *Ductal carcinoma in situ: terminology, classification, and natural history.* J Natl Cancer Inst Monogr. **2010**(41): p. 134-8.
25. Polyak, K., *Breast cancer: origins and evolution.* J Clin Invest, 2007. **117**(11): p. 3155-63.
26. Burstein, H.J., et al., *Ductal carcinoma in situ of the breast.* N Engl J Med, 2004. **350**(14): p. 1430-41.
27. Virnig, B.A., et al., *Ductal carcinoma in situ of the breast: a systematic review of incidence, treatment, and outcomes.* J Natl Cancer Inst, 2010. **102**(3): p. 170-8.
28. Leonard, G.D. and S.M. Swain, *Ductal carcinoma in situ, complexities and challenges.* J Natl Cancer Inst, 2004. **96**(12): p. 906-20.
29. Claus, E.B., M. Stowe, and D. Carter, *Breast carcinoma in situ: risk factors and screening patterns.* J Natl Cancer Inst, 2001. **93**(23): p. 1811-7.
30. Vargo-Gogola, T. and J.M. Rosen, *Modelling breast cancer: one size does not fit all.* Nat Rev Cancer, 2007. **7**(9): p. 659-72.
31. Virnig, B.A., et al., *Ductal carcinoma in situ of the breast: a systematic review of incidence, treatment, and outcomes.* J Natl Cancer Inst. **102**(3): p. 170-8.
32. Navin, N.E. and J. Hicks, *Tracing the tumor lineage.* Mol Oncol. **4**(3): p. 267-83.
33. Clarke, M.F., et al., *Cancer stem cells--perspectives on current status and future*

directions: AACR Workshop on cancer stem cells. Cancer Res, 2006. 66(19): p. 9339-44.

34. Boveri, T., *Concerning the origin of malignant tumours by Theodor Boveri. Translated and annotated by Henry Harris.* J Cell Sci, 2008. 121 Suppl 1: p. 1-84.
35. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. 100(1): p. 57-70.
36. Holland, J.F.B., R.C.; Morton, D.L.; Frei, E.; Kufe, D.W.; Weichselbaum, R.R., *Growth factors. In: Cancer Medicine.* Williams and Wilkins Fedi, P., Tronick, S.R. and Aaronson, S.A, 1997. pp 41–64. .
37. Slamon, D.J., et al., *Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene.* Science, 1987. 235(4785): p. 177-82.
38. Yarden, Y., and Ullrich, A and *EGF and erbB2 receptor overexpression*

in human tumors. Growth factor recepor tyrosine kinases. Annu. Rev. Biochem., 1988. 57, 443–478.

39. Ishizaki, Y., Cheng, L., Mudge, A.W., and Raff, M.C. , *Programmed cell death by default in embryonic cells, fibroblasts, and cancer cells.* Mol. Biol. Cell, 1995. 6, 1443–1458.
40. McDonnell, T.J. and S.J. Korsmeyer, *Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14; 18).* Nature, 1991. 349(6306): p. 254-6.
41. Wyllie, A.H., J.F. Kerr, and A.R. Currie, *Cell death: the significance of apoptosis.* Int Rev Cytol, 1980. 68: p. 251-306.
42. Counter, C.M., et al., *Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity.* Embo j, 1992. 11(5): p. 1921-9.
43. Hayflick, L., *Mortality and immortality at the cellular level. A review.* Biochemistry (Mosc), 1997. 62(11): p. 1180-90.
44. Wright, W.E., O.M. Pereira-Smith, and J.W. Shay, *Reversible cellular senescence: implications for immortalization of normal human diploid fibroblasts.* Mol Cell Biol, 1989. 9(7): p. 3088-92.
45. Bryan, T.M. and T.R. Cech, *Telomerase and the maintenance of chromosome ends.* Curr Opin Cell Biol, 1999. 11(3): p. 318-24.
46. Hanahan, D. and J. Folkman, *Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis.* Cell, 1996. 86(3): p. 353-64.
47. Bouck, N., V. Stellmach, and S.C. Hsu, *How tumors become angiogenic.* Adv Cancer Res, 1996. 69: p. 135-74.
48. Sporn, M.B., *The war on cancer.* Lancet, 1996. 347(9012): p. 1377-81.
49. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell. 144(5): p. 646-74.
50. Howlett, A.R. and M.J. Bissell, *The influence of tissue microenvironment (stroma and extracellular matrix) on the development and function of mammary epithelium.* Epithelial Cell Biol, 1993. 2(2): p. 79-89.
51. Sobin, L.H. and C.C. Compton, *TNM seventh edition: what's new, what's changed: communication from the International Union Against Cancer and the American Joint Committee on Cancer.* Cancer, 2010. 116(22): p. 5336-9.
52. Rakha, E.A., et al., *Breast cancer prognostic classification in the molecular era: the role of histological grade.* Breast Cancer Res, 2010. 12(4): p. 207.
53. Mueller, M.M. and N.E. Fusenig, *Friends or foes - bipolar effects of the tumour stroma in cancer.* Nat Rev Cancer, 2004. 4(11): p. 839-49.
54. Arendt, L.M., et al., *Stroma in breast development and disease.* Semin Cell Dev Biol. 21(1): p. 11-8.
55. Hanahan, D. and L.M. Coussens, *Accessories to the crime: functions of cells recruited to the tumor microenvironment.* Cancer Cell. 21(3): p. 309-22.
56. Moinfar, F., et al., *Concurrent and independent genetic alterations in the stromal and epithelial cells of mammary carcinoma: implications for tumorigenesis.* Cancer Res, 2000. 60(9): p. 2562-6.
57. Weber, F., et al., *Microenvironmental genomic alterations and clinicopathological behavior in head and neck squamous cell carcinoma.* JAMA, 2007. 297(2): p. 187-95.
58. Weinberg, R.A., *Coevolution in the tumor microenvironment.* Nat Genet, 2008. 40(5): p. 494-5.
59. Proia, T.A., et al., *Genetic predisposition directs breast cancer phenotype by dictating progenitor cell fate.* Cell Stem Cell. 8(2): p. 149-63.
60. Perez, E.A., *Breast cancer management: opportunities and barriers to an individualized approach.* Oncologist. 16 Suppl 1: p. 20-2.
61. Perou, C.M., et al., *Molecular portraits of human breast tumours.* Nature, 2000. 406(6797): p. 747-52.
62. Almendro, V. and G. Fuster, *Heterogeneity of breast cancer: etiology and clinical relevance.* Clin Transl Oncol. 13(11): p. 767-73.
63. Marusyk, A. and K. Polyak, *Tumor heterogeneity: causes and consequences.* Biochim Biophys Acta. 1805(1): p. 105-17.
64. Michor, F. and K. Polyak, *The origins and implications of intratumor heterogeneity.* Cancer Prev Res (Phila). 3(11): p. 1361-4.
65. Polyak, K., *Heterogeneity in breast cancer.* J Clin Invest. 121(10): p. 3786-8.
66. Weigelt, B., F.C. Geyer, and J.S. Reis-Filho, *Histological types of breast cancer: how*

special are they? Mol Oncol. 4(3): p. 192-208.
67. Li, C.I., D.J. Uribe, and J.R. Daling, *Clinical characteristics of different histologic types of breast cancer.* Br J Cancer, 2005. 93(9): p. 1046-52.
68. Yang, F. and J. Li, *[WHO classification of tumors of the breast].* Zhonghua Wai Ke Za Zhi. 52(1): p. 1-3.
69. Yoder, B.J., E.J. Wilkinson, and N.A. Massoll, *Molecular and morphologic distinctions between infiltrating ductal and lobular carcinoma of the breast.* Breast J, 2007. 13(2): p. 172-9.
70. Li, C.I., et al., *Relationship between long durations and different regimens of hormone therapy and risk of breast cancer.* JAMA, 2003. 289(24): p. 3254-63.
71. Pestalozzi, B.C., et al., *Distinct clinical and prognostic features of infiltrating lobular carcinoma of the breast: combined results of 15 International Breast Cancer Study Group clinical trials.* J Clin Oncol, 2008. 26(18): p. 3006-14.
72. Greene, F.l., *Aajcc cancer staging manual* 6th edition, 2002.
73. Jaiyesimi, I.A., A.U. Buzdar, and G. Hortobagyi, *Inflammatory breast cancer: a review.* J Clin Oncol, 1992. 10(6): p. 1014-24.
74. Bozzetti, F., et al., *Inflammatory cancer of the breast: analysis of 114 cases.* J Surg Oncol, 1981. 18(4): p. 355-61.
75. Perou, C.M., et al., *Distinctive gene expression patterns in human mammary epithelial cells and breast cancers.* Proc Natl Acad Sci U S A, 1999. 96(16): p. 9212-7.
76. Sorlie, T., et al., *Repeated observation of breast tumor subtypes in independent gene expression data sets.* Proc Natl Acad Sci U S A, 2003. 100(14): p. 8418-23.
77. Prat, A. and C.M. Perou, *Deconstructing the molecular portraits of breast cancer.* Mol Oncol. 5(1): p. 5-23.
78. Kaufmann, M. and L. Pusztai, *Use of standard markers and incorporation of molecular markers into breast cancer therapy: Consensus recommendations from an International Expert Panel.* Cancer. 117(8): p. 1575-82.
79. Sotiriou, C., et al., *Breast cancer classification and prognosis based on gene expression profiles from a population-based study.* Proc Natl Acad Sci U S A, 2003. 100(18): p. 10393-8.
80. Prat, A., et al., *Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer.* Breast Cancer Res. 12(5): p. R68.
81. Farmer, P., et al., *Identification of molecular apocrine breast tumours by microarray analysis.* Oncogene, 2005. 24(29): p. 4660-71.
82. Parker, J.S., et al., *Supervised risk predictor of breast cancer based on intrinsic subtypes.* J Clin Oncol, 2009. 27(8): p. 1160-7.
83. van Wolfswinkel, J.C. and R.F. Ketting, *The role of small non-coding RNAs in genome stability and chromatin organization.* J Cell Sci. 123(Pt 11): p. 1825-39.
84. Bockmeyer, C.L., et al., *MicroRNA profiles of healthy basal and luminal mammary epithelial cells are distinct and reflected in different breast cancer subtypes.* Breast Cancer Res Treat. 130(3): p. 735-45.
85. Goldhirsch, A., et al., *Strategies for subtypes--dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011.* Ann Oncol. 22(8): p. 1736-47.
86. Metzger-Filho, O., et al., *Dissecting the heterogeneity of triple-negative breast cancer.* J Clin Oncol. 30(15): p. 1879-87.
87. Goldhirsch, A., et al., *Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013.* Ann Oncol. 24(9): p. 2206-23.
88. Marmot, M.G., et al., *The benefits and harms of breast cancer screening: an independent review.* Br J Cancer. 108(11): p. 2205-40.
89. Elwood, J.M. and W.P. Moorehead, *Delay in diagnosis and long-term survival in breast cancer.* Br Med J, 1980. 280(6227): p. 1291-4.
90. Ascunce, N., et al., *[Breast cancer screening program. Results of the process and impact indicators (1990-2002)].* An Sist Sanit Navar, 2004. 27(3): p. 319-33.
91. Committee., W.G.A.b.t.G.R., *WHO Position Paper on Mammography Screening.* Geneva: World Health Organization; 2014. , 2014.
92. Boyd, N.F., et al., *Mammographic density and the risk and detection of breast cancer.* N Engl J Med, 2007. 356(3): p. 227-36.
93. Al-Sindi, K., N. Kamal, and J. Golbahar, *Efficacy of fine-needle aspiration biopsy in diagnosis of breast cancer: a retrospective study of 303 cases in Bahrain.* Diagn Cytopathol, 2009. 37(9): p. 636-40.
94. Arteaga, C.L., et al., *AACR Cancer Progress Report 2014.* Clin Cancer Res. 20(19 Suppl): p. S1-S112.
95. Fisher, B., et al., *Twenty-year follow-up of a randomized trial comparing total mastectomy, lumpectomy, and lumpectomy plus irradiation for the treatment of invasive breast cancer.* N Engl J Med, 2002. 347(16): p. 1233-41.
96. Kim, T., A.E. Giuliano, and G.H. Lyman, *Lymphatic mapping and sentinel lymph node biopsy in early-stage breast carcinoma: a metaanalysis.* Cancer, 2006. 106(1): p. 4-16.

97. www.cancer.gov, accessed on September 2015.
98. Begg, A.C., F.A. Stewart, and C. Vens, *Strategies to improve radiotherapy with targeted drugs.* Nat Rev Cancer. **11**(4): p. 239-53.
99. Arnold, H., *Basic Clinical Radiobiology.* 4th edition, 2009.
100. *Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials.* Lancet, 2005. **365**(9472): p. 1687-717.
101. http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0032825/-BreastCancerTreatment, *(PDQ®) Patient Version PDQ Adult Treatment Editorial Board.* . Last Update: August 13, 2015.
102. Takimoto CH, C.E., *Principles of Oncologic Pharmacotherapy. Cancer Management: A Multidisciplinary Approach.* 11st edition, 2008.
103. Massarweh, S., et al., *Tamoxifen resistance in breast tumors is driven by growth factor receptor signaling with repression of classic estrogen receptor genomic function.* Cancer Res, 2008. **68**(3): p. 826-33.
104. Piccart-Gebhart, M.J., et al., *Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer.* N Engl J Med, 2005. **353**(16): p. 1659-72.
105. Gianni, L., et al., *Treatment with trastuzumab for 1 year after adjuvant chemotherapy in patients with HER2-positive early breast cancer: a 4-year follow-up of a randomised controlled trial.* Lancet Oncol. **12**(3): p. 236-44.
106. Geyer, C.E., et al., *Lapatinib plus capecitabine for HER2-positive advanced breast cancer.* N Engl J Med, 2006. **355**(26): p. 2733-43.
107. Gomez, H.L., et al., *Efficacy and safety of lapatinib as first-line therapy for ErbB2-amplified locally advanced or metastatic breast cancer.* J Clin Oncol, 2008. **26**(18): p. 2999-3005.
108. Lemmon, M.A. and J. Schlessinger, *Cell signaling by receptor tyrosine kinases.* Cell, 2010. **141**(7): p. 1117-34.
109. Choura, M. and A. Rebai, *Receptor tyrosine kinases: from biology to pathology.* J Recept Signal Transduct Res, 2011. **31**(6): p. 387-94.
110. Bennasroune, A., et al., *Tyrosine kinase receptors as attractive targets of cancer therapy.* Crit Rev Oncol Hematol, 2004. **50**(1): p. 23-38.
111. Heldin, C.H., *Dimerization of cell surface receptors in signal transduction.* Cell, 1995. **80**(2): p. 213-23.
112. Holbro, T. and N.E. Hynes, *ErbB receptors: directing key signaling networks throughout life.* Annu Rev Pharmacol Toxicol, 2004. **44**: p. 195-217.
113. Earp, H.S., et al., *Heterodimerization and functional interaction between EGF receptor family members: a new signaling paradigm with implications for breast cancer research.* Breast Cancer Res Treat, 1995. **35**(1): p. 115-32.
114. Schlessinger, J., *Cell signaling by receptor tyrosine kinases.* Cell, 2000. **103**(2): p. 211-25.
115. Olayioye, M.A., et al., *The ErbB signaling network: receptor heterodimerization in development and cancer.* Embo j, 2000. **19**(13): p. 3159-67.
116. Yarden, Y. and M.X. Sliwkowski, *Untangling the ErbB signalling network.* Nat Rev Mol Cell Biol, 2001. **2**(2): p. 127-37.
117. Olayioye, M.A., et al., *ErbB-1 and ErbB-2 acquire distinct signaling properties dependent upon their dimerization partner.* Mol Cell Biol, 1998. **18**(9): p. 5042-51.
118. Stern, D.F. and M.P. Kamps, *EGF-stimulated tyrosine phosphorylation of p185neu: a potential model for receptor interactions.* Embo j, 1988. **7**(4): p. 995-1001.
119. King, C.R., et al., *Egf binding to its receptor triggers a rapid tyrosine phosphorylation of the erbB-2 protein in the mammary tumor cell line SK-BR-3.* Embo j, 1988. **7**(6): p. 1647-51.
120. Burgess, A.W., et al., *An open-and-shut case? Recent insights into the activation of EGF/ErbB receptors.* Mol Cell, 2003. **12**(3): p. 541-52.
121. Hynes, N.E. and G. MacDonald, *ErbB receptors and signaling pathways in cancer.* Curr Opin Cell Biol, 2009. **21**(2): p. 177-84.
122. Ferguson, K.M., et al., *EGF activates its receptor by removing interactions that autoinhibit ectodomain dimerization.* Mol Cell, 2003. **11**(2): p. 507-17.
123. Cho, H.S. and D.J. Leahy, *Structure of the extracellular region of HER3 reveals an interdomain tether.* Science, 2002. **297**(5585): p. 1330-3.
124. Zhang, X., et al., *An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor.* Cell, 2006. **125**(6): p. 1137-49.
125. Hynes, N.E. and D.F. Stern, *The biology of erbB-2/neu/HER-2 and its role in cancer.* Biochim Biophys Acta, 1994. **1198**(2-3): p. 165-84.
126. Bjornsti, M.A. and P.J. Houghton, *The TOR pathway: a target for cancer therapy.* Nat Rev Cancer, 2004. **4**(5): p. 335-48.
127. Lane, H.A., et al., *ErbB2 potentiates breast tumor proliferation through modulation of p27(Kip1)-Cdk2 complex formation: receptor overexpression does not determine growth dependency.* Mol Cell Biol, 2000. **20**(9): p. 3210-23.

128. Ishizawar, R. and S.J. Parsons, *c-Src and cooperating partners in human cancer.* Cancer Cell, 2004. **6**(3): p. 209-14.

129. Doisneau-Sixou, S.F., et al., *Estrogen and antiestrogen regulation of cell cycle progression in breast cancer cells.* Endocr Relat Cancer, 2003. **10**(2): p. 179-86.

130. Yen, L., et al., *Differential regulation of tumor angiogenesis by distinct ErbB homo- and heterodimers.* Mol Biol Cell, 2002. **13**(11): p. 4029-44.

131. Baker, C.H., et al., *Blockade of epidermal growth factor receptor signaling on tumor cells and tumor-associated endothelial cells for therapy of human carcinomas.* Am J Pathol, 2002. **161**(3): p. 929-38.

132. Garrett, T.P., et al., *The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors.* Mol Cell, 2003. **11**(2): p. 495-505.

133. Wiley, H.S., *Trafficking of the ErbB receptors and its influence on signaling.* Exp Cell Res, 2003. **284**(1): p. 78-88.

134. Burke, P., K. Schooler, and H.S. Wiley, *Regulation of epidermal growth factor receptor signaling by endocytosis and intracellular trafficking.* Mol Biol Cell, 2001. **12**(6): p. 1897-910.

135. Citri, A., B.S. Kochupurakkal, and Y. Yarden, *The achilles heel of ErbB-2/HER2: regulation by the Hsp90 chaperone machine and potential for pharmacological intervention.* Cell Cycle, 2004. **3**(1): p. 51-60.

136. H, N.E., *eterotrimeric G proteins: organizers of transmembrane*

signals. Cell, 1995. **80(2): 249-57.**

137. Bhola NE, G.J.F. and *Crosstalk between G-protein-coupled*

receptors and epidermal growth factor receptor in cancer. Biosci, 2008. **13: 1857-65**.

138. Dorsam RT, G.J., *G-protein-coupled receptors and cancer.* Nat Rev Cancer, 2007. **(2): 79-94**.

139. Almendro, V., S. Garcia-Recio, and P. Gascon, *Tyrosine kinase receptor transactivation associated to G protein-coupled receptors.* Curr Drug Targets, 2010. **11**(9): p. 1169-80.

140. Ma YC, H.J., Ali S, Lowry W, Huang XY. , *Src tyrosine kinase is a novel direct effector of G proteins.* . Cell, 2000. **102(5): 635-46.**

141. Garcia-Recio, S., et al., *Substance P autocrine signaling contributes to persistent HER2 activation that drives malignant progression and drug resistance in breast cancer.* Cancer Res, 2013. **73**(21): p. 6424-34.

142. Garcia-Recio, S., et al., *The Transmodulation of HER2 and EGFR by Substance P in Breast Cancer Cells Requires c-Src and Metalloproteinase Activation.* PLoS One, 2015. **10**(6): p. e0129661.

143. Obermeier, A., et al., *Identification of Trk binding sites for SHC and phosphatidylinositol 3'-kinase and formation of a multimeric signaling complex.* J Biol Chem, 1993. **268**(31): p. 22963-6.

144. Yokote, K., et al., *Direct interaction between Shc and the platelet-derived growth factor beta-receptor.* J Biol Chem, 1994. **269**(21): p. 15337-43.

145. Pronk, G.J., et al., *Insulin-induced phosphorylation of the 46- and 52-kDa Shc proteins.* J Biol Chem, 1993. **268**(8): p. 5748-53.

146. Craparo, A., T.J. O'Neill, and T.A. Gustafson, *Non-SH2 domains within insulin receptor substrate-1 and SHC mediate their phosphotyrosine-dependent interaction with the NPEY motif of the insulin-like growth factor I receptor.* J Biol Chem, 1995. **270**(26): p. 15639-43.

147. De Meyts, P., et al., *Structural biology of insulin and IGF-1 receptors.* Novartis Found Symp, 2004. **262**: p. 160-71; discussion 171-6, 265-8.

148. Chan, T.O. and P.N. Tsichlis, *PDK2: a complex tail in one Akt.* Sci STKE, 2001. **2001**(66): p. pe1.

149. Chan, T.O., S.E. Rittenhouse, and P.N. Tsichlis, *AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation.* Annu Rev Biochem, 1999. **68**: p. 965-1014.

150. Kulik, G., A. Klippel, and M.J. Weber, *Antiapoptotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt.* Mol Cell Biol, 1997. **17**(3): p. 1595-606.

151. Maehama, T. and J.E. Dixon, *The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate.* J Biol Chem, 1998. **273**(22): p. 13375-8.

152. Datta, S.R., et al., *Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery.* Cell, 1997. **91**(2): p. 231-41.

153. Zha, J., et al., *Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L).* Cell, 1996. **87**(4): p. 619-28.

154. Schulze, W.X., L. Deng, and M. Mann, *Phosphotyrosine interactome of the ErbB-receptor kinase family.* Mol Syst Biol, 2005. **1**: p. 2005.0008.

155. Marmor, M.D., K.B. Skaria, and Y. Yarden, *Signal transduction and oncogenesis by ErbB/HER receptors.* Int J Radiat Oncol Biol Phys, 2004. **58**(3): p. 903-13.

156. Lenferink, A.E., et al., *ErbB2/neu kinase modulates cellular p27(Kip1) and cyclin D1*

through multiple signaling pathways. Cancer Res, 2001. **61**(17): p. 6583-91.

157. Citri, A., K.B. Skaria, and Y. Yarden, *The deaf and the dumb: the biology of ErbB-2 and ErbB-3.* Exp Cell Res, 2003. **284**(1): p. 54-65.

158. Revillion, F., J. Bonneterre, and J.P. Peyrat, *ERBB2 oncogene in human breast cancer and its clinical significance.* Eur J Cancer, 1998. **34**(6): p. 791-808.

159. Slamon, D.J., et al., *Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer.* Science, 1989. **244**(4905): p. 707-12.

160. Pegram, M.D., G. Konecny, and D.J. Slamon, *The molecular and cellular biology of HER2/neu gene amplification/overexpression and the clinical development of herceptin (trastuzumab) therapy for breast cancer.* Cancer Treat Res, 2000. **103**: p. 57-75.

161. Hudziak, R.M., J. Schlessinger, and A. Ullrich, *Increased expression of the putative growth factor receptor p185HER2 causes transformation and tumorigenesis of NIH 3T3 cells.* Proc Natl Acad Sci U S A, 1987. **84**(20): p. 7159-63.

162. Di Fiore, P.P., et al., *erbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells.* Science, 1987. **237**(4811): p. 178-82.

163. Chazin, V.R., et al., *Transformation mediated by the human HER-2 gene independent of the epidermal growth factor receptor.* Oncogene, 1992. **7**(9): p. 1859-66.

164. Kokai, Y., et al., *Synergistic interaction of p185c-neu and the EGF receptor leads to transformation of rodent fibroblasts.* Cell, 1989. **58**(2): p. 287-92.

165. Zhang, K., et al., *Transformation of NIH 3T3 cells by HER3 or HER4 receptors requires the presence of HER1 or HER2.* J Biol Chem, 1996. **271**(7): p. 3884-90.

166. Benz, C.C., et al., *Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu.* Breast Cancer Res Treat, 1992. **24**(2): p. 85-95.

167. Muthuswamy, S.K., et al., *ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini.* Nat Cell Biol, 2001. **3**(9): p. 785-92.

168. Moasser, M.M., *The oncogene HER2: its signaling and transforming functions and its role in human cancer pathogenesis.* Oncogene, 2007. **26**(45): p. 6469-87.

169. Penuel, E., et al., *Structural requirements for ErbB2 transactivation.* Semin Oncol, 2001. **28**(6 Suppl 18): p. 36-42.

170. Woods Ignatoski, K.M., et al., *The role of phosphatidylinositol 3'-kinase and its downstream signals in erbB-2-mediated transformation.* Mol Cancer Res, 2003. **1**(7): p. 551-60.

171. Woods Ignatoski, K.M., et al., *p38MAPK induces cell surface alpha4 integrin downregulation to facilitate erbB-2-mediated invasion.* Neoplasia, 2003. **5**(2): p. 128-34.

172. Zhan, L., B. Xiang, and S.K. Muthuswamy, *Controlled activation of ErbB1/ErbB2 heterodimers promote invasion of three-dimensional organized epithelia in an ErbB1-dependent manner: implications for progression of ErbB2-overexpressing tumors.* Cancer Res, 2006. **66**(10): p. 5201-8.

173. Petit, A.M., et al., *Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors.* Am J Pathol, 1997. **151**(6): p. 1523-30.

174. Gancberg, D., et al., *Comparison of HER-2 status between primary breast cancer and corresponding distant metastatic sites.* Ann Oncol, 2002. **13**(7): p. 1036-43.

175. Krawczyk, N., et al., *HER2 status on persistent disseminated tumor cells after adjuvant therapy may differ from initial HER2 status on primary tumor.* Anticancer Res, 2009. **29**(10): p. 4019-24.

176. Krishnamurti, U. and J.F. Silverman, *HER2 in breast cancer: a review and update.* Adv Anat Pathol, 2014. **21**(2): p. 100-7.

177. Rossi, S., et al., *Hormone Receptor Status and HER2 Expression in Primary Breast Cancer Compared With Synchronous Axillary Metastases or Recurrent Metastatic Disease.* Clin Breast Cancer, 2015. **15**(5): p. 307-12.

178. Krishnamurthy, S., et al., *Discordance in HER2 gene amplification in circulating and disseminated tumor cells in patients with operable breast cancer.* Cancer Med, 2013. **2**(2): p. 226-33.

179. Hartkopf, A.D., M. Banys, and T. Fehm, *HER2-positive DTCs/CTCs in breast cancer.* Recent Results Cancer Res, 2012. **195**: p. 203-15.

180. Braun, S., et al., *ErbB2 overexpression on occult metastatic cells in bone marrow predicts poor clinical outcome of stage I-III breast cancer patients.* Cancer Res, 2001. **61**(5): p. 1890-5.

181. Freudenberg, J.A., et al., *The role of HER2 in early breast cancer metastasis and the origins of resistance to HER2-targeted therapies.* Exp Mol Pathol, 2009. **87**(1): p. 1-11.

182. Harris, L., et al., *American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer.* J Clin Oncol, 2007. **25**(33): p. 5287-312.

183. Ross, J.S., et al., *The Her-2/neu gene and protein in breast cancer 2003: biomarker and target of therapy.* Oncologist, 2003. 8(4): p. 307-25.
184. Carlson, R.W., et al., *HER2 testing in breast cancer: NCCN Task Force report and recommendations.* J Natl Compr Canc Netw, 2006. 4 Suppl 3: p. S1-22; quiz S23-4.
185. Wolff, A.C., et al., *Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update.* J Clin Oncol, 2013. 31(31): p. 3997-4013.
186. Press, M.F., et al., *Evaluation of HER-2/neu gene amplification and overexpression: comparison of frequently used assay methods in a molecularly characterized cohort of breast cancer specimens.* J Clin Oncol, 2002. 20(14): p. 3095-105.
187. Press, M.F., et al., *HER-2 gene amplification, HER-2 and epidermal growth factor receptor mRNA and protein expression, and lapatinib efficacy in women with metastatic breast cancer.* Clin Cancer Res, 2008. 14(23): p. 7861-70.
188. Sinczak-Kuta, A., et al., *Evaluation of HER2/neu gene amplification in patients with invasive breast carcinoma. Comparison of in situ hybridization methods.* Pol J Pathol, 2007. 58(1): p. 41-50.
189. Gupta, D., et al., *Comparison of fluorescence and chromogenic in situ hybridization for detection of HER-2/neu oncogene in breast cancer.* Am J Clin Pathol, 2003. 119(3): p. 381-7.
190. Dietel, M., et al., *Comparison of automated silver enhanced in situ hybridisation (SISH) and fluorescence ISH (FISH) for the validation of HER2 gene status in breast carcinoma according to the guidelines of the American Society of Clinical Oncology and the College of American Pathologists.* Virchows Arch, 2007. 451(1): p. 19-25.
191. Ni, R., et al., *PGDS, a novel technique combining chromogenic in situ hybridization and immunohistochemistry for the assessment of ErbB2 (HER2/neu) status in breast cancer.* Appl Immunohistochem Mol Morphol, 2007. 15(3): p. 316-24.
192. Stephens, P., et al., *Lung cancer: intragenic ERBB2 kinase mutations in tumours.* Nature, 2004. 431(7008): p. 525-6.
193. Carney, W.P., et al., *Potential clinical utility of serum HER-2/neu oncoprotein concentrations in patients with breast cancer.* Clin Chem, 2003. 49(10): p. 1579-98.
194. Ludovini, V., et al., *Evaluation of serum HER2 extracellular domain in early breast cancer patients: correlation with clinicopathological parameters and survival.* Ann Oncol, 2008. 19(5): p. 883-90.
195. Molina, M.A., et al., *NH(2)-terminal truncated HER-2 protein but not full-length receptor is associated with nodal metastasis in human breast cancer.* Clin Cancer Res, 2002. 8(2): p. 347-53.
196. Xia, W., et al., *Truncated ErbB2 receptor (p95ErbB2) is regulated by heregulin through heterodimer formation with ErbB3 yet remains sensitive to the dual EGFR/ErbB2 kinase inhibitor GW572016.* Oncogene, 2004. 23(3): p. 646-53.
197. Saez, R., et al., *p95HER-2 predicts worse outcome in patients with HER-2-positive breast cancer.* Clin Cancer Res, 2006. 12(2): p. 424-31.
198. Thor, A.D., et al., *Activation (tyrosine phosphorylation) of ErbB-2 (HER-2/neu): a study of incidence and correlation with outcome in breast cancer.* J Clin Oncol, 2000. 18(18): p. 3230-9.
199. DiGiovanna, M.P., et al., *Active signaling by HER-2/neu in a subpopulation of HER-2/neu-overexpressing ductal carcinoma in situ: clinicopathological correlates.* Cancer Res, 2002. 62(22): p. 6667-73.
200. Cicenas, J., et al., *Phosphorylation of tyrosine 1248-ERBB2 measured by chemiluminescence-linked immunoassay is an independent predictor of poor prognosis in primary breast cancer patients.* Eur J Cancer, 2006. 42(5): p. 636-45.
201. Eswarakumar, V.P., I. Lax, and J. Schlessinger, *Cellular signaling by fibroblast growth factor receptors.* Cytokine Growth Factor Rev, 2005. 16(2): p. 139-49.
202. Katoh, M. and H. Nakagama, *FGF receptors: cancer biology and therapeutics.* Med Res Rev, 2014. 34(2): p. 280-300.
203. Knights, V. and S.J. Cook, *De-regulated FGF receptors as therapeutic targets in cancer.* Pharmacol Ther, 2010. 125(1): p. 105-17.
204. Turner, N. and R. Grose, *Fibroblast growth factor signalling: from development to cancer.* Nat Rev Cancer, 2010. 10(2): p. 116-29.
205. Zhou, W., et al., *FGF-receptor substrate 2 functions as a molecular sensor integrating external regulatory signals into the FGF pathway.* Cell Res, 2009. 19(10): p. 1165-77.
206. Wesche, J., K. Haglund, and E.M. Haugsten, *Fibroblast growth factors and their receptors in cancer.* Biochem J, 2011. 437(2): p. 199-213.
207. Lieu, C., et al., *Beyond VEGF: inhibition of the fibroblast growth factor pathway and antiangiogenesis.* Clin Cancer Res, 2011. 17(19): p. 6130-9.
208. *Comprehensive molecular portraits of human breast tumours.* Nature, 2012. 490(7418): p. 61-70.
209. Dienstmann, R., et al., *Genomic aberrations in the FGFR pathway: opportunities for*

targeted therapies in solid tumors. Ann Oncol, 2014. **25**(3): p. 552-63.

210. Qing, J., et al., *Antibody-based targeting of FGFR3 in bladder carcinoma and t(4;14)-positive multiple myeloma in mice.* J Clin Invest, 2009. **119**(5): p. 1216-29.

211. Zhang, X., et al., *Receptor specificity of the fibroblast growth factor family. The complete mammalian FGF family.* J Biol Chem, 2006. **281**(23): p. 15694-700.

212. Reis-Filho, J.S., et al., *FGFR1 emerges as a potential therapeutic target for lobular breast carcinomas.* Clin Cancer Res, 2006. **12**(22): p. 6652-62.

213. Brunello, E., et al., *FGFR-1 amplification in metastatic lymph-nodal and haematogenous lobular breast carcinoma.* J Exp Clin Cancer Res, 2012. **31**: p. 103.

214. Sun, S., et al., *Increased expression of fibroblastic growth factor receptor 2 is correlated with poor prognosis in patients with breast cancer.* J Surg Oncol, 2012. **105**(8): p. 773-9.

215. Thussbas, C., et al., *FGFR4 Arg388 allele is associated with resistance to adjuvant therapy in primary breast cancer.* J Clin Oncol, 2006. **24**(23): p. 3747-55.

216. Wilkie, A.O., *Bad bones, absent smell, selfish testes: the pleiotropic consequences of human FGF receptor mutations.* Cytokine Growth Factor Rev, 2005. **16**(2): p. 187-203.

217. Ho, H.K., et al., *Current strategies for inhibiting FGFR activities in clinical applications: opportunities, challenges and toxicological considerations.* Drug Discov Today, 2014. **19**(1): p. 51-62.

218. Daniele, G., et al., *FGF receptor inhibitors: role in cancer therapy.* Curr Oncol Rep, 2012. **14**(2): p. 111-9.

219. Tenhagen, M., et al., *Fibroblast growth factor receptors in breast cancer: expression, downstream effects, and possible drug targets.* Endocr Relat Cancer, 2012. **19**(4): p. R115-29.

220. Takahashi, M., J. Ritz, and G.M. Cooper, *Activation of a novel human transforming gene, ret, by DNA rearrangement.* Cell, 1985. **42**(2): p. 581-8.

221. Morandi, A., I. Plaza-Menacho, and C.M. Isacke, *RET in breast cancer: functional and therapeutic implications.* Trends Mol Med, 2011. **17**(3): p. 149-57.

222. Airaksinen, M.S. and M. Saarma, *The GDNF family: signalling, biological functions and therapeutic value.* Nat Rev Neurosci, 2002. **3**(5): p. 383-94.

223. Ichihara, M., Y. Murakumo, and M. Takahashi, *RET and neuroendocrine tumors.* Cancer Lett, 2004. **204**(2): p. 197-211.

224. Pachnis, V., B. Mankoo, and F. Costantini, *Expression of the c-ret proto-oncogene during mouse embryogenesis.* Development, 1993. **119**(4): p. 1005-17.

225. Schuchardt, A., et al., *Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret.* Nature, 1994. **367**(6461): p. 380-3.

226. Durbec, P.L., et al., *Common origin and developmental dependence on c-ret of subsets of enteric and sympathetic neuroblasts.* Development, 1996. **122**(1): p. 349-58.

227. Meng, X., et al., *Regulation of cell fate decision of undifferentiated spermatogonia by GDNF.* Science, 2000. **287**(5457): p. 1489-93.

228. Imkamp, F., et al., *Rearrangement analysis in archival thyroid tissues: punching microdissection and artificial RET/PTC 1-12 transcripts.* J Surg Res, 2007. **143**(2): p. 350-63.

229. Edery, P., et al., *Mutations of the RET proto-oncogene in Hirschsprung's disease.* Nature, 1994. **367**(6461): p. 378-80.

230. Romeo, G., et al., *Point mutations affecting the tyrosine kinase domain of the RET proto-oncogene in Hirschsprung's disease.* Nature, 1994. **367**(6461): p. 377-8.

231. Hansford, J.R. and L.M. Mulligan, *Multiple endocrine neoplasia type 2 and RET: from neoplasia to neurogenesis.* J Med Genet, 2000. **37**(11): p. 817-27.

232. Esseghir, S., et al., *A role for glial cell derived neurotrophic factor induced expression by inflammatory cytokines and RET/GFR alpha 1 receptor up-regulation in breast cancer.* Cancer Res, 2007. **67**(24): p. 11732-41.

233. Plaza-Menacho, I., et al., *Targeting the receptor tyrosine kinase RET sensitizes breast cancer cells to tamoxifen treatment and reveals a role for RET in endocrine resistance.* Oncogene, 2010. **29**(33): p. 4648-57.

234. Johnston, S.R., *New strategies in estrogen receptor-positive breast cancer.* Clin Cancer Res, 2010. **16**(7): p. 1979-87.

235. Kelly, C.M. and A.U. Buzdar, *Using multiple targeted therapies in oncology: considerations for use, and progress to date in breast cancer.* Drugs, 2013. **73**(6): p. 505-15.

236. Zardavas, D., et al., *Beyond trastuzumab and lapatinib: new options for HER2-positive breast cancer.* Am Soc Clin Oncol Educ Book, 2013.

237. Yan, M., et al., *HER2 aberrations in cancer: implications for therapy.* Cancer Treat Rev, 2014. **40**(6): p. 770-80.

238. Franklin, M.C., et al., *Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex.* Cancer Cell, 2004. **5**(4): p. 317-28.

239. Hudis, C.A., *Trastuzumab--mechanism of action and use in clinical practice.* N Engl J Med, 2007. **357**(1): p. 39-51.

240. Lewis Phillips, G.D., et al., *Targeting HER2-positive breast cancer with trastuzumab-DM1, an antibody-cytotoxic drug conjugate.* Cancer Res, 2008. **68**(22): p. 9280-90.
241. Xia, W., et al., *Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways.* Oncogene, 2002. **21**(41): p. 6255-63.
242. Solca, F., et al., *Target binding properties and cellular activity of afatinib (BIBW 2992), an irreversible ErbB family blocker.* J Pharmacol Exp Ther, 2012. **343**(2): p. 342-50.
243. Kamath, S. and J.K. Buolamwini, *Targeting EGFR and HER-2 receptor tyrosine kinases for cancer drug discovery and development.* Med Res Rev, 2006. **26**(5): p. 569-94.
244. Tokuda, Y., et al., *In vitro and in vivo anti-tumour effects of a humanised monoclonal antibody against c-erbB-2 product.* Br J Cancer, 1996. **73**(11): p. 1362-5.
245. Piccart-Gebhart, M.J., *Adjuvant trastuzumab therapy for HER2-overexpressing breast cancer: what we know and what we still need to learn.* Eur J Cancer, 2006. **42**(12): p. 1715-9.
246. Viani, G.A., et al., *Adjuvant trastuzumab in the treatment of her-2-positive early breast cancer: a meta-analysis of published randomized trials.* BMC Cancer, 2007. **7**: p. 153.
247. Baselga, J., et al., *Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer.* J Clin Oncol, 1996. **14**(3): p. 737-44.
248. Vogel, C.L., et al., *Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer.* J Clin Oncol, 2002. **20**(3): p. 719-26.
249. Cobleigh, M.A., et al., *Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease.* J Clin Oncol, 1999. **17**(9): p. 2639-48.
250. Slamon, D.J., et al., *Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2.* N Engl J Med, 2001. **344**(11): p. 783-92.
251. Tokunaga, E., et al., *Trastuzumab and breast cancer: developments and current status.* Int J Clin Oncol, 2006. **11**(3): p. 199-208.
252. Buzdar, A.U., et al., *Significantly higher pathologic complete remission rate after neoadjuvant therapy with trastuzumab, paclitaxel, and epirubicin chemotherapy: results of a randomized trial in human epidermal growth factor receptor 2-positive operable breast cancer.* J Clin Oncol, 2005. **23**(16): p. 3676-85.
253. Tzahar, E., et al., *A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor.* Mol Cell Biol, 1996. **16**(10): p. 5276-87.
254. Yeon, C.H. and M.D. Pegram, *Anti-erbB-2 antibody trastuzumab in the treatment of HER2-amplified breast cancer.* Invest New Drugs, 2005. **23**(5): p. 391-409.
255. Nahta, R. and F.J. Esteva, *Herceptin: mechanisms of action and resistance.* Cancer Lett, 2006. **232**(2): p. 123-38.
256. Gennari, R., et al., *Pilot study of the mechanism of action of preoperative trastuzumab in patients with primary operable breast tumors overexpressing HER2.* Clin Cancer Res, 2004. **10**(17): p. 5650-5.
257. Delord, J.P., et al., *Selective inhibition of HER2 inhibits AKT signal transduction and prolongs disease-free survival in a micrometastasis model of ovarian carcinoma.* Ann Oncol, 2005. **16**(12): p. 1889-97.
258. Serra, V., et al., *PI3K inhibition results in enhanced HER signaling and acquired ERK dependency in HER2-overexpressing breast cancer.* Oncogene, 2011. **30**(22): p. 2547-57.
259. Christianson, T.A., et al., *NH2-terminally truncated HER-2/neu protein: relationship with shedding of the extracellular domain and with prognostic factors in breast cancer.* Cancer Res, 1998. **58**(22): p. 5123-9.
260. Hudelist, G., et al., *Her-2/neu-triggered intracellular tyrosine kinase activation: in vivo relevance of ligand-independent activation mechanisms and impact upon the efficacy of trastuzumab-based treatment.* Br J Cancer, 2003. **89**(6): p. 983-91.
261. Molina, M.A., et al., *Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells.* Cancer Res, 2001. **61**(12): p. 4744-9.
262. Kostler, W.J., et al., *Monitoring of serum Her-2/neu predicts response and progression-free survival to trastuzumab-based treatment in patients with metastatic breast cancer.* Clin Cancer Res, 2004. **10**(5): p. 1618-24.
263. Esteva, F.J., et al., *Clinical utility of serum HER2/neu in monitoring and prediction of progression-free survival in metastatic breast cancer patients treated with trastuzumab-based therapies.* Breast Cancer Res, 2005. **7**(4): p. R436-43.
264. Valabrega, G., F. Montemurro, and M. Aglietta, *Trastuzumab: mechanism of action,*

resistance and future perspectives in HER2-overexpressing breast cancer. Ann Oncol, 2007. **18**(6): p. 977-84.

265. Chowdhury, F., et al., *Development of immunomonitoring of antibodydependent cellular cytotoxicity against neuroblastoma cells using whole blood.* Cancer Immunol Immunother, 2014. **63**(6): p. 559-69.
266. Clynes, R.A., et al., *Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets.* Nat Med, 2000. **6**(4): p. 443-6.
267. Mohsin, S.K., et al., *Neoadjuvant trastuzumab induces apoptosis in primary breast cancers.* J Clin Oncol, 2005. **23**(11): p. 2460-8.
268. Lee, S., et al., *Enhanced sensitization to taxol-induced apoptosis by herceptin pretreatment in ErbB2-overexpressing breast cancer cells.* Cancer Res, 2002. **62**(20): p. 5703-10.
269. Henson, E.S., X. Hu, and S.B. Gibson, *Herceptin sensitizes ErbB2-overexpressing cells to apoptosis by reducing antiapoptotic Mcl-1 expression.* Clin Cancer Res, 2006. **12**(3 Pt 1): p. 845-53.
270. Konecny, G.E., et al., *Association between HER-2/neu and vascular endothelial growth factor expression predicts clinical outcome in primary breast cancer patients.* Clin Cancer Res, 2004. **10**(5): p. 1706-16.
271. Laughner, E., et al., *HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression.* Mol Cell Biol, 2001. **21**(12): p. 3995-4004.
272. Izumi, Y., et al., *Tumour biology: herceptin acts as an anti-angiogenic cocktail.* Nature, 2002. **416**(6878): p. 279-80.
273. Klos, K.S., et al., *Combined trastuzumab and paclitaxel treatment better inhibits ErbB-2-mediated angiogenesis in breast carcinoma through a more effective inhibition of Akt than either treatment alone.* Cancer, 2003. **98**(7): p. 1377-85.
274. Walshe, J.M., et al., *A phase II trial with trastuzumab and pertuzumab in patients with HER2-overexpressed locally advanced and metastatic breast cancer.* Clin Breast Cancer, 2006. **6**(6): p. 535-9.
275. Nahta, R., M.C. Hung, and F.J. Esteva, *The HER-2-targeting antibodies trastuzumab and pertuzumab synergistically inhibit the survival of breast cancer cells.* Cancer Res, 2004. **64**(7): p. 2343-6.
276. Junttila, T.T., et al., *Trastuzumab-DM1 (T-DM1) retains all the mechanisms of action of trastuzumab and efficiently inhibits growth of lapatinib insensitive breast cancer.* Breast Cancer Res Treat, 2011. **128**(2): p. 347-56.
277. Krop, I.E., et al., *Phase I study of trastuzumab-DM1, an HER2 antibody-drug conjugate, given every 3 weeks to patients with HER2-positive metastatic breast cancer.* J Clin Oncol, 2010. **28**(16): p. 2698-704.
278. Burris, H.A., 3rd, et al., *Phase II study of the antibody drug conjugate trastuzumab-DM1 for the treatment of human epidermal growth factor receptor 2 (HER2)-positive breast cancer after prior HER2-directed therapy.* J Clin Oncol, 2011. **29**(4): p. 398-405.
279. Dieras, V. and T. Bachelot, *The success story of trastuzumab emtansine, a targeted therapy in HER2-positive breast cancer.* Target Oncol, 2014. **9**(2): p. 111-22.
280. Verma, S., et al., *Trastuzumab emtansine for HER2-positive advanced breast cancer.* N Engl J Med, 2012. **367**(19): p. 1783-91.
281. Garrett, M.D. and P. Workman, *Discovering novel chemotherapeutic drugs for the third millennium.* Eur J Cancer, 1999. **35**(14): p. 2010-30.
282. Moy, B. and P.E. Goss, *Lapatinib: current status and future directions in breast cancer.* Oncologist, 2006. **11**(10): p. 1047-57.
283. Capri, G., et al., *An open-label expanded access study of lapatinib and capecitabine in patients with HER2-overexpressing locally advanced or metastatic breast cancer.* Ann Oncol, 2010. **21**(3): p. 474-80.
284. Baselga, J., et al., *Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): a randomised, open-label, multicentre, phase 3 trial.* Lancet, 2012. **379**(9816): p. 633-40.
285. Rimawi, M.F., et al., *Multicenter phase II study of neoadjuvant lapatinib and trastuzumab with hormonal therapy and without chemotherapy in patients with human epidermal growth factor receptor 2-overexpressing breast cancer: TBCRC 006.* J Clin Oncol, 2013. **31**(14): p. 1726-31.
286. Konecny, G.E., et al., *Activity of the dual kinase inhibitor lapatinib (GW572016) against HER-2-overexpressing and trastuzumab-treated breast cancer cells.* Cancer Res, 2006. **66**(3): p. 1630-9.
287. Schwartzberg, L.S., et al., *Lapatinib plus letrozole as first-line therapy for HER-2+ hormone receptor-positive metastatic breast cancer.* Oncologist, 2010. **15**(2): p. 122-9.
288. Robidoux, A., et al., *Lapatinib as a component of neoadjuvant therapy for HER2-positive operable breast cancer (NSABP protocol B-41): an open-label, randomised phase 3 trial.* Lancet Oncol, 2013. **14**(12): p. 1183-92.
289. Untch, M., et al., *Lapatinib versus trastuzumab in combination with neoadjuvant anthracycline-taxane-based chemotherapy (GeparQuinto, GBG 44): a randomised phase 3 trial.* Lancet Oncol, 2012. **13**(2): p. 135-44.

290. Gebhart, G., et al., *18F-FDG PET/CT for early prediction of response to neoadjuvant lapatinib, trastuzumab, and their combination in HER2-positive breast cancer: results from Neo-ALTTO.* J Nucl Med, 2013. **54**(11): p. 1862-8.
291. Burris, H.A., 3rd, *Dual kinase inhibition in the treatment of breast cancer: initial experience with the EGFR/ErbB-2 inhibitor lapatinib.* Oncologist, 2004. **9 Suppl 3**: p. 10-5.
292. Rusnak, D.W., et al., *The characterization of novel, dual ErbB-2/EGFR, tyrosine kinase inhibitors: potential therapy for cancer.* Cancer Res, 2001. **61**(19): p. 7196-203.
293. Nelson, M.H. and C.R. Dolder, *Lapatinib: a novel dual tyrosine kinase inhibitor with activity in solid tumors.* Ann Pharmacother, 2006. **40**(2): p. 261-9.
294. Anido, J., et al., *Biosynthesis of tumorigenic HER2 C-terminal fragments by alternative initiation of translation.* Embo j, 2006. **25**(13): p. 3234-44.
295. Medina, P.J. and S. Goodin, *Lapatinib: a dual inhibitor of human epidermal growth factor receptor tyrosine kinases.* Clin Ther, 2008. **30**(8): p. 1426-47.
296. Xia, W., et al., *Lapatinib antitumor activity is not dependent upon phosphatase and tensin homologue deleted on chromosome 10 in ErbB2-overexpressing breast cancers.* Cancer Res, 2007. **67**(3): p. 1170-5.
297. Li, D., et al., *BIBW2992, an irreversible EGFR/HER2 inhibitor highly effective in preclinical lung cancer models.* Oncogene, 2008. **27**(34): p. 4702-11.
298. Khelwatty, S.A., et al., *Growth response of human colorectal tumour cell lines to treatment with afatinib (BIBW2992), an irreversible erbB family blocker, and its association with expression of HER family members.* Int J Oncol, 2011. **39**(2): p. 483-91.
299. Reardon, D.A., et al., *A phase I/II trial of pazopanib in combination with lapatinib in adult patients with relapsed malignant glioma.* Clin Cancer Res, 2013. **19**(4): p. 900-8.
300. Murakami, H., et al., *Phase I study of continuous afatinib (BIBW 2992) in patients with advanced non-small cell lung cancer after prior chemotherapy/erlotinib/gefitinib (LUX-Lung 4).* Cancer Chemother Pharmacol, 2012. **69**(4): p. 891-9.
301. Lin, N.U., et al., *A phase II study of afatinib (BIBW 2992), an irreversible ErbB family blocker, in patients with HER2-positive metastatic breast cancer progressing after trastuzumab.* Breast Cancer Res Treat, 2012. **133**(3): p. 1057-65.
302. Sequist, L.V., et al., *Neratinib, an irreversible pan-ErbB receptor tyrosine kinase inhibitor: results of a phase II trial in patients with advanced non-small-cell lung cancer.* J Clin Oncol, 2010. **28**(18): p. 3076-83.
303. Katakami, N., et al., *LUX-Lung 4: a phase II trial of afatinib in patients with advanced non-small-cell lung cancer who progressed during prior treatment with erlotinib, gefitinib, or both.* J Clin Oncol, 2013. **31**(27): p. 3335-41.
304. Bose, P. and H. Ozer, *Neratinib: an oral, irreversible dual EGFR/HER2 inhibitor for breast and non-small cell lung cancer.* Expert Opin Investig Drugs, 2009. **18**(11): p. 1735-51.
305. Rabindran, S.K., et al., *Antitumor activity of HKI-272, an orally active, irreversible inhibitor of the HER-2 tyrosine kinase.* Cancer Res, 2004. **64**(11): p. 3958-65.
306. Wong, K.K., et al., *A phase I study with neratinib (HKI-272), an irreversible pan ErbB receptor tyrosine kinase inhibitor, in patients with solid tumors.* Clin Cancer Res, 2009. **15**(7): p. 2552-8.
307. http://www.pumabiotechnology.com/pr2014072202.html.
308. Majem, M. and C. Pallares, *An update on molecularly targeted therapies in second- and third-line treatment in non-small cell lung cancer: focus on EGFR inhibitors and anti-angiogenic agents.* Clin Transl Oncol, 2013. **15**(5): p. 343-57.
309. Nahta, R. and F.J. Esteva, *Trastuzumab: triumphs and tribulations.* Oncogene, 2007. **26**(25): p. 3637-43.
310. Citri, A. and Y. Yarden, *EGF-ERBB signalling: towards the systems level.* Nat Rev Mol Cell Biol, 2006. **7**(7): p. 505-16.
311. Thery, J.C., et al., *Resistance to human epidermal growth factor receptor type 2-targeted therapies.* Eur J Cancer, 2014. **50**(5): p. 892-901.
312. Nagy, P., et al., *Decreased accessibility and lack of activation of ErbB2 in JIMT-1, a herceptin-resistant, MUC4-expressing breast cancer cell line.* Cancer Res, 2005. **65**(2): p. 473-82.
313. Li, G., et al., *Feedback activation of STAT3 mediates trastuzumab resistance via upregulation of MUC1 and MUC4 expression.* Oncotarget, 2014. **5**(18): p. 8317-29.
314. Raina, D., et al., *Targeting the MUC1-C oncoprotein downregulates HER2 activation and abrogates trastuzumab resistance in breast cancer cells.* Oncogene, 2014. **33**(26): p. 3422-31.
315. Scaltriti, M., et al., *Expression of p95HER2, a truncated form of the HER2 receptor, and response to anti-HER2 therapies in breast cancer.* J Natl Cancer Inst, 2007. **99**(8): p. 628-38.
316. Liu, P.C., et al., *Identification of ADAM10 as a major source of HER2 ectodomain*

sheddase activity in HER2 overexpressing breast cancer cells. Cancer Biol Ther, 2006. **5**(6): p. 657-64.
317. Parra-Palau, J.L., et al., *Effect of p95HER2/611CTF on the response to trastuzumab and chemotherapy.* J Natl Cancer Inst, 2014. **106**(11).
318. Arribas, J., et al., *p95HER2 and breast cancer.* Cancer Res, 2011. **71**(5): p. 1515-9.
319. Koene, H.R., et al., *Fc gammaRIIIa-158V/F polymorphism influences the binding of IgG by natural killer cell Fc gammaRIIIa, independently of the Fc gammaRIIIa-48L/R/H phenotype.* Blood, 1997. **90**(3): p. 1109-14.
320. Shields, R.L., et al., *High resolution mapping of the binding site on human IgG1 for Fc gamma RI, Fc gamma RII, Fc gamma RIII, and FcRn and design of IgG1 variants with improved binding to the Fc gamma R.* J Biol Chem, 2001. **276**(9): p. 6591-604.
321. Musolino, A., et al., *Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/neu-positive metastatic breast cancer.* J Clin Oncol, 2008. **26**(11): p. 1789-96.
322. Cho, H.S., et al., *Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab.* Nature, 2003. **421**(6924): p. 756-60.
323. Agus, D.B., et al., *Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth.* Cancer Cell, 2002. **2**(2): p. 127-37.
324. Ritter, C.A., et al., *Human breast cancer cells selected for resistance to trastuzumab in vivo overexpress epidermal growth factor receptor and ErbB ligands and remain dependent on the ErbB receptor network.* Clin Cancer Res, 2007. **13**(16): p. 4909-19.
325. Dua, R., et al., *EGFR over-expression and activation in high HER2, ER negative breast cancer cell line induces trastuzumab resistance.* Breast Cancer Res Treat, 2010. **122**(3): p. 685-97.
326. du Manoir, J.M., et al., *Strategies for delaying or treating in vivo acquired resistance to trastuzumab in human breast cancer xenografts.* Clin Cancer Res, 2006. **12**(3 Pt 1): p. 904-16.
327. Sergina, N.V., et al., *Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3.* Nature, 2007. **445**(7126): p. 437-41.
328. Xia, W., et al., *A model of acquired autoresistance to a potent ErbB2 tyrosine kinase inhibitor and a therapeutic strategy to prevent its onset in breast cancer.* Proc Natl Acad Sci U S A, 2006. **103**(20): p. 7795-800.
329. Gijsen, M., et al., *HER2 phosphorylation is maintained by a PKB negative feedback loop in response to anti-HER2 herceptin in breast cancer.* PLoS Biol, 2010. **8**(12): p. e1000563.
330. Shattuck, D.L., et al., *Met receptor contributes to trastuzumab resistance of Her2-overexpressing breast cancer cells.* Cancer Res, 2008. **68**(5): p. 1471-7.
331. Hafizi, S. and B. Dahlback, *Signalling and functional diversity within the Axl subfamily of receptor tyrosine kinases.* Cytokine Growth Factor Rev, 2006. **17**(4): p. 295-304.
332. Liu, L., et al., *Novel mechanism of lapatinib resistance in HER2-positive breast tumor cells: activation of AXL.* Cancer Res, 2009. **69**(17): p. 6871-8.
333. Lu, Y., et al., *Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin).* J Natl Cancer Inst, 2001. **93**(24): p. 1852-7.
334. Dokmanovic, M., et al., *Trastuzumab regulates IGFBP-2 and IGFBP-3 to mediate growth inhibition: implications for the development of predictive biomarkers for trastuzumab resistance.* Mol Cancer Ther, 2011. **10**(6): p. 917-28.
335. Huang, X., et al., *Heterotrimerization of the growth factor receptors erbB2, erbB3, and insulin-like growth factor-i receptor in breast cancer cells resistant to herceptin.* Cancer Res, 2010. **70**(3): p. 1204-14.
336. Fujita, T., et al., *PTEN activity could be a predictive marker of trastuzumab efficacy in the treatment of ErbB2-overexpressing breast cancer.* Br J Cancer, 2006. **94**(2): p. 247-52.
337. Stern, H.M., et al., *PTEN Loss Is Associated with Worse Outcome in HER2-Amplified Breast Cancer Patients but Is Not Associated with Trastuzumab Resistance.* Clin Cancer Res, 2015. **21**(9): p. 2065-74.
338. Debska-Szmich, S., et al., *Prognostic value of HER3, PTEN and p-HER2 expression in patients with HER2positive breast cancer.* Postepy Hig Med Dosw (Online), 2015. **69**: p. 586-97.
339. Song, M.S., L. Salmena, and P.P. Pandolfi, *The functions and regulation of the PTEN tumour suppressor.* Nat Rev Mol Cell Biol, 2012. **13**(5): p. 283-96.
340. Nagata, Y., et al., *PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients.* Cancer Cell, 2004. **6**(2): p. 117-27.
341. Neve, R.M., et al., *A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes.* Cancer Cell, 2006. **10**(6): p. 515-27.
342. Berns, K., et al., *A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer.* Cancer Cell, 2007. **12**(4): p. 395-402.

343. Chakrabarty, A., et al., *H1047R phosphatidylinositol 3-kinase mutant enhances HER2-mediated transformation by heregulin production and activation of HER3.* Oncogene, 2010. **29**(37): p. 5193-203.
344. O'Brien, N.A., et al., *Activated phosphoinositide 3-kinase/AKT signaling confers resistance to trastuzumab but not lapatinib.* Mol Cancer Ther, 2010. **9**(6): p. 1489-502.
345. Liang, J., et al., *PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest.* Nat Med, 2002. **8**(10): p. 1153-60.
346. Le, X.F., et al., *The role of cyclin-dependent kinase inhibitor p27Kip1 in anti-HER2 antibody-induced G1 cell cycle arrest and tumor growth inhibition.* J Biol Chem, 2003. **278**(26): p. 23441-50.
347. Nahta, R., et al., *P27(kip1) down-regulation is associated with trastuzumab resistance in breast cancer cells.* Cancer Res, 2004. **64**(11): p. 3981-6.
348. Eichhorn, P.J., et al., *Phosphatidylinositol 3-kinase hyperactivation results in lapatinib resistance that is reversed by the mTOR/phosphatidylinositol 3-kinase inhibitor NVP-BEZ235.* Cancer Res, 2008. **68**(22): p. 9221-30.
349. Guo, S. and G.E. Sonenshein, *Forkhead box transcription factor FOXO3a regulates estrogen receptor alpha expression and is repressed by the Her-2/neu/phosphatidylinositol 3-kinase/Akt signaling pathway.* Mol Cell Biol, 2004. **24**(19): p. 8681-90.
350. Burstein, H.J., et al., *Neratinib, an irreversible ErbB receptor tyrosine kinase inhibitor, in patients with advanced ErbB2-positive breast cancer.* J Clin Oncol, 2010. **28**(8): p. 1301-7.
351. Errico, A., *Breast cancer: CLEOPATRA sheds light on how to tackle metastatic disease.* Nat Rev Clin Oncol, 2015. **12**(4): p. 188.
352. Liotta, L.A. and E.C. Kohn, *The microenvironment of the tumour-host interface.* Nature, 2001. **411**(6835): p. 375-9.
353. Ercan, C., P.J. van Diest, and M. Vooijs, *Mammary development and breast cancer: the role of stem cells.* Curr Mol Med, 2011. **11**(4): p. 270-85.
354. Nelson, C.M. and M.J. Bissell, *Of extracellular matrix, scaffolds, and signaling: tissue architecture regulates development, homeostasis, and cancer.* Annu Rev Cell Dev Biol, 2006. **22**: p. 287-309.
355. Bissell, M.J. and D. Radisky, *Putting tumours in context.* Nat Rev Cancer, 2001. **1**(1): p. 46-54.
356. Bissell, M.J. and W.C. Hines, *Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression.* Nat Med. **17**(3): p. 320-9.
357. Ronnov-Jessen, L. and M.J. Bissell, *Breast cancer by proxy: can the microenvironment be both the cause and consequence?* Trends Mol Med, 2009. **15**(1): p. 5-13.
358. Lu, P., V.M. Weaver, and Z. Werb, *The extracellular matrix: a dynamic niche in cancer progression.* J Cell Biol. **196**(4): p. 395-406.
359. Egeblad, M., E.S. Nakasone, and Z. Werb, *Tumors as organs: complex tissues that interface with the entire organism.* Dev Cell. **18**(6): p. 884-901.
360. Brizzi, M.F., G. Tarone, and P. Defilippi, *Extracellular matrix, integrins, and growth factors as tailors of the stem cell niche.* Curr Opin Cell Biol, 2012. **24**(5): p. 645-51.
361. Hynes, R.O., *The extracellular matrix: not just pretty fibrils.* Science, 2009. **326**(5957): p. 1216-9.
362. Mackey, J.R., et al., *Controlling angiogenesis in breast cancer: a systematic review of anti-angiogenic trials.* Cancer Treat Rev, 2012. **38**(6): p. 673-88.
363. Pietras, K. and A. Ostman, *Hallmarks of cancer: interactions with the tumor stroma.* Exp Cell Res. **316**(8): p. 1324-31.
364. Desmouliere, A., C. Guyot, and G. Gabbiani, *The stroma reaction myofibroblast: a key player in the control of tumor cell behavior.* Int J Dev Biol, 2004. **48**(5-6): p. 509-17.
365. Mantovani, A., et al., *Role of tumor-associated macrophages in tumor progression and invasion.* Cancer Metastasis Rev, 2006. **25**(3): p. 315-22.
366. Vlodavsky, I., et al., *Endothelial cell-derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix.* Proc Natl Acad Sci U S A, 1987. **84**(8): p. 2292-6.
367. Parrinello, S., et al., *Stromal-epithelial interactions in aging and cancer: senescent fibroblasts alter epithelial cell differentiation.* J Cell Sci, 2005. **118**(Pt 3): p. 485-96.
368. Buckley, C.D., *Why does chronic inflammation persist: An unexpected role for fibroblasts.* Immunol Lett, 2011. **138**(1): p. 12-4.
369. Bainbridge, P., *Wound healing and the role of fibroblasts.* J Wound Care, 2013. **22**(8): p. 407-8, 410-12.
370. Parsonage, G., et al., *A stromal address code defined by fibroblasts.* Trends Immunol, 2005. **26**(3): p. 150-6.
371. Ribatti, D., *The contribution of Harold F. Dvorak to the study of tumor angiogenesis and stroma generation mechanisms.* Endothelium, 2007. **14**(3): p. 131-5.
372. Fidler, I.J., *The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis*

revisited. Nat Rev Cancer, 2003. 3(6): p. 453-8.
373. Werb, Z. and P. Lu, *The Role of Stroma in Tumor Development.* Cancer J. 21(4): p. 250-3.
374. Dirkx, A.E., et al., *Monocyte/macrophage infiltration in tumors: modulators of angiogenesis.* J Leukoc Biol, 2006. 80(6): p. 1183-96.
375. Balkwill, F. and A. Mantovani, *Inflammation and cancer: back to Virchow?* Lancet, 2001. 357(9255): p. 539-45.
376. Joyce, J.A. and J.W. Pollard, *Microenvironmental regulation of metastasis.* Nat Rev Cancer, 2009. 9(4): p. 239-52.
377. Crawford, Y., et al., *PDGF-C mediates the angiogenic and tumorigenic properties of fibroblasts associated with tumors refractory to anti-VEGF treatment.* Cancer Cell, 2009. 15(1): p. 21-34.
378. Trimboli, A.J., et al., *Pten in stromal fibroblasts suppresses mammary epithelial tumours.* Nature, 2009. 461(7267): p. 1084-91.
379. Ma, X.J., et al., *Gene expression profiling of the tumor microenvironment during breast cancer progression.* Breast Cancer Res, 2009. 11(1): p. R7.
380. Polyak, K., I. Haviv, and I.G. Campbell, *Co-evolution of tumor cells and their microenvironment.* Trends Genet, 2009. 25(1): p. 30-8.
381. Calone, I. and S. Souchelnytskyi, *Inhibition of TGFbeta signaling and its implications in anticancer treatments.* Exp Oncol. 34(1): p. 9-16.
382. Quail, D.F. and J.A. Joyce, *Microenvironmental regulation of tumor progression and metastasis.* Nat Med, 2013. 19(11): p. 1423-37.
383. Bhowmick, N.A., E.G. Neilson, and H.L. Moses, *Stromal fibroblasts in cancer initiation and progression.* Nature, 2004. 432(7015): p. 332-7.
384. Kalluri, R. and M. Zeisberg, *Fibroblasts in cancer.* Nat Rev Cancer, 2006. 6(5): p. 392-401.
385. De, W.O., Demetter,P., Mareel,M., and Bracke,M. , *Stromal myofibroblasts are drivers of invasive cancer growth.* Int. J. Cancer 2008. 123, 2229-2238.
386. Peinado, H., S. Lavotshkin, and D. Lyden, *The secreted factors responsible for pre-metastatic niche formation: old sayings and new thoughts.* Semin Cancer Biol, 2011. 21(2): p. 139-46.
387. Smith, R.S., et al., *Fibroblasts as sentinel cells. Synthesis of chemokines and regulation of inflammation.* Am J Pathol, 1997. 151(2): p. 317-22.
388. Yun, Y.R., et al., *Fibroblast growth factors: biology, function, and application for tissue regeneration.* J Tissue Eng, 2010. 2010: p. 218142.
389. Provenzano, P.P., et al., *Collagen density promotes mammary tumor initiation and progression.* BMC Med, 2008. 6: p. 11.
390. Chang, H.Y., et al., *Diversity, topographic differentiation, and positional memory in human fibroblasts.* Proc Natl Acad Sci U S A, 2002. 99(20): p. 12877-82.
391. Tarin, D. and C.B. Croft, *Ultrastructural features of wound healing in mouse skin.* J Anat, 1969. 105(Pt 1): p. 189-90.
392. Mowen, K.A., et al., *Arginine methylation of STAT1 modulates IFNalpha/beta-induced transcription.* Cell, 2001. 104(5): p. 731-41.
393. Lazarides, E. and D.R. Balzer, Jr., *Specificity of desmin to avian and mammalian muscle cells.* Cell, 1978. 14(2): p. 429-38.
394. Scott, A.M., et al., *A Phase I dose-escalation study of sibrotuzumab in patients with advanced or metastatic fibroblast activation protein-positive cancer.* Clin Cancer Res, 2003. 9(5): p. 1639-47.
395. Sappino, A.P., et al., *Smooth-muscle differentiation in stromal cells of malignant and non-malignant breast tissues.* Int J Cancer, 1988. 41(5): p. 707-12.
396. Ronnov-Jessen, L. and O.W. Petersen, *Induction of alpha-smooth muscle actin by transforming growth factor-beta 1 in quiescent human breast gland fibroblasts. Implications for myofibroblast generation in breast neoplasia.* Lab Invest, 1993. 68(6): p. 696-707.
397. Zeisberg, M., F. Strutz, and G.A. Muller, *Role of fibroblast activation in inducing interstitial fibrosis.* J Nephrol, 2000. 13 Suppl 3: p. S111-20.
398. Direkze, N.C., et al., *Bone marrow contribution to tumor-associated myofibroblasts and fibroblasts.* Cancer Res, 2004. 64(23): p. 8492-5.
399. Mishra, P.J., et al., *Carcinoma-associated fibroblast-like differentiation of human mesenchymal stem cells.* Cancer Res, 2008. 68(11): p. 4331-9.
400. Radisky, D.C., P.A. Kenny, and M.J. Bissell, *Fibrosis and cancer: do myofibroblasts come also from epithelial cells via EMT?* J Cell Biochem, 2007. 101(4): p. 830-9.
401. Ronnov-Jessen, L., et al., *The origin of the myofibroblasts in breast cancer. Recapitulation of tumor environment in culture unravels diversity and implicates converted fibroblasts and recruited smooth muscle cells.* J Clin Invest, 1995. 95(2): p. 859-73.
402. Worthley, D.L., et al., *Bone marrow cells as precursors of the tumor stroma.* Exp Cell Res, 2013. 319(11): p. 1650-6.
403. Quante, M., et al., *Bone marrow-derived myofibroblasts contribute to the mesenchymal stem cell niche and promote*

tumor growth. Cancer Cell, 2011. **19**(2): p. 257-72.

404. Karnoub, A.E., et al., *Mesenchymal stem cells within tumour stroma promote breast cancer metastasis.* Nature, 2007. **449**(7162): p. 557-63.

405. Zeisberg, E.M., et al., *Discovery of endothelial to mesenchymal transition as a source for carcinoma-associated fibroblasts.* Cancer Res, 2007. **67**(21): p. 10123-8.

406. Allinen, M., et al., *Molecular characterization of the tumor microenvironment in breast cancer.* Cancer Cell, 2004. **6**(1): p. 17-32.

407. Orimo, A. and R.A. Weinberg, *Stromal fibroblasts in cancer: a novel tumor-promoting cell type.* Cell Cycle, 2006. **5**(15): p. 1597-601.

408. Orimo, A., et al., *Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion.* Cell, 2005. **121**(3): p. 335-48.

409. Hinz, B., et al., *The myofibroblast: one function, multiple origins.* Am J Pathol, 2007. **170**(6): p. 1807-16.

410. Cirri, P. and P. Chiarugi, *Cancer associated fibroblasts: the dark side of the coin.* Am J Cancer Res. **1**(4): p. 482-97.

411. Ostman, A. and M. Augsten, *Cancer-associated fibroblasts and tumor growth--bystanders turning into key players.* Curr Opin Genet Dev, 2009. **19**(1): p. 67-73.

412. Roy, R., J. Yang, and M.A. Moses, *Matrix metalloproteinases as novel biomarkers and potential therapeutic targets in human cancer.* J Clin Oncol, 2009. **27**(31): p. 5287-97.

413. Cardone, A., et al., *Prognostic value of desmoplastic reaction and lymphocytic infiltration in the management of breast cancer.* Panminerva Med, 1997. **39**(3): p. 174-7.

414. Wolfe, J.N., *Breast parenchymal patterns and their changes with age.* Radiology, 1976. **121**(3 Pt. 1): p. 545-52.

415. www.mcancer.org.

416. Farmer, P., et al., *A stroma-related gene signature predicts resistance to neoadjuvant chemotherapy in breast cancer.* Nat Med, 2009. **15**(1): p. 68-74.

417. Cukierman, E. and D.E. Bassi, *The mesenchymal tumor microenvironment: a drug-resistant niche.* Cell Adh Migr. **6**(3): p. 285-96.

418. Borovski, T., et al., *Cancer stem cell niche: the place to be.* Cancer Res, 2011. **71**(3): p. 634-9.

419. Mani, S.A., et al., *The epithelial-mesenchymal transition generates cells with properties of stem cells.* Cell, 2008. **133**(4): p. 704-15.

420. Sharma, S., T.K. Kelly, and P.A. Jones, *Epigenetics in cancer.* Carcinogenesis. **31**(1): p. 27-36.

421. Hazlehurst, L.A., T.H. Landowski, and W.S. Dalton, *Role of the tumor microenvironment in mediating de novo resistance to drugs and physiological mediators of cell death.* Oncogene, 2003. **22**(47): p. 7396-402.

422. Dankbar B, P.T., Leo R, Feldmann B, Kropff M, Mesters RM, et al. , *Vascular endothelial growth factor and interleukin-6 in paracrine tumor-stromal cell interactions in multiple myeloma.* Blood. , 2000. ;95:2630–6.

423. Miyamoto, H., et al., *Tumor-stroma interaction of human pancreatic cancer: acquired resistance to anticancer drugs and proliferation regulation is dependent on extracellular matrix proteins.* Pancreas, 2004. **28**(1): p. 38-44.

424. Lafkas, D., et al., *P53 mutations in stromal fibroblasts sensitize tumors against chemotherapy.* Int J Cancer, 2008. **123**(4): p. 967-71.

425. Sun, X., et al., *IL-6 secreted by cancer-associated fibroblasts induces tamoxifen resistance in luminal breast cancer.* Oncogene.

426. Mueller, K.L., et al., *Fibroblast-secreted hepatocyte growth factor mediates epidermal growth factor receptor tyrosine kinase inhibitor resistance in triple-negative breast cancers through paracrine activation of Met.* Breast Cancer Res. **14**(4): p. R104.

427. Amornsupak, K., et al., *Cancer-associated fibroblasts induce high mobility group box 1 and contribute to resistance to doxorubicin in breast cancer cells.* BMC Cancer. **14**: p. 955.

428. Loeffler, M., et al., *Targeting tumor-associated fibroblasts improves cancer chemotherapy by increasing intratumoral drug uptake.* J Clin Invest, 2006. **116**(7): p. 1955-62.

429. Wang, W., et al., *Crosstalk to stromal fibroblasts induces resistance of lung cancer to epidermal growth factor receptor tyrosine kinase inhibitors.* Clin Cancer Res, 2009. **15**(21): p. 6630-8.

430. Kerbel, R. and J. Folkman, *Clinical translation of angiogenesis inhibitors.* Nat Rev Cancer, 2002. **2**(10): p. 727-39.

431. Ellis, L.M. and D.J. Hicklin, *VEGF-targeted therapy: mechanisms of anti-tumour activity.* Nat Rev Cancer, 2008. **8**(8): p. 579-91.

432. Gonda, T.A., et al., *Molecular biology of cancer-associated fibroblasts: can these cells be targeted in anti-cancer therapy?* Semin Cell Dev Biol. **21**(1): p. 2-10.

433. Cheng, J.D., et al., *Promotion of tumor growth by murine fibroblast activation protein, a serine protease, in an animal*

model. Cancer Res, 2002. **62**(16): p. 4767-72.
434. Welt, S., et al., *Antibody targeting in metastatic colon cancer: a phase I study of monoclonal antibody F19 against a cell-surface protein of reactive tumor stromal fibroblasts.* J Clin Oncol, 1994. **12**(6): p. 1193-203.
435. Ostermann, E., et al., *Effective immunoconjugate therapy in cancer models targeting a serine protease of tumor fibroblasts.* Clin Cancer Res, 2008. **14**(14): p. 4584-92.
436. Speirs, V., et al., *Short-term primary culture of epithelial cells derived from human breast tumours.* Br J Cancer, 1998. **78**(11): p. 1421-9.
437. Halaban, R. and F.D. Alfano, *Selective elimination of fibroblasts from cultures of normal human melanocytes.* In Vitro, 1984. **20**(5): p. 447-50.
438. Yoon, N., I.G. Do, and E.Y. Cho, *Analysis of HER2 status in breast carcinoma by fully automated HER2 fluorescence in situ hybridization (FISH): comparison of two immunohistochemical tests and manual FISH.* Apmis, 2014. **122**(9): p. 755-60.
439. Hammond, M.E., et al., *American Society of Clinical Oncology/College Of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer.* J Clin Oncol, 2010. **28**(16): p. 2784-95.
440. Pfaffl, M.W., *A new mathematical model for relative quantification in real-time RT-PCR.* Nucleic Acids Res, 2001. **29**(9): p. e45.
441. Quackenbush, J., *Computational analysis of microarray data.* Nat Rev Genet, 2001. **2**(6): p. 418-27.
442. Eisen, M.B., et al., *Cluster analysis and display of genome-wide expression patterns.* Proc Natl Acad Sci U S A, 1998. **95**(25): p. 14863-8.
443. Yang, Y.H., Y. Xiao, and M.R. Segal, *Identifying differentially expressed genes from microarray experiments via statistic synthesis.* Bioinformatics, 2005. **21**(7): p. 1084-93.
444. Almendro, V., et al., *The role of MMP7 and its cross-talk with the FAS/FASL system during the acquisition of chemoresistance to oxaliplatin.* PLoS One, 2009. **4**(3): p. e4728.
445. Al-Hajj, M., et al., *Prospective identification of tumorigenic breast cancer cells.* Proc Natl Acad Sci U S A, 2003. **100**(7): p. 3983-8.
446. Koukourakis, M.I., et al., *Cancer stem cell phenotype relates to radio-chemotherapy outcome in locally advanced squamous cell head-neck cancer.* Br J Cancer, 2012. **106**(5): p. 846-53.
447. Vinogradov, S. and X. Wei, *Cancer stem cells and drug resistance: the potential of nanomedicine.* Nanomedicine (Lond), 2012. **7**(4): p. 597-615.
448. Canfield, K., et al., *Receptor tyrosine kinase ERBB4 mediates acquired resistance to ERBB2 inhibitors in breast cancer cells.* Cell Cycle, 2015. **14**(4): p. 648-55.
449. Wiedswang, G., et al., *Detection of isolated tumor cells in BM from breast-cancer patients: significance of anterior and posterior iliac crest aspirations and the number of mononuclear cells analyzed.* Cytotherapy, 2003. **5**(1): p. 40-5.
450. Lin, Q., et al., *Analysis of colorectal cancer glyco-secretome identifies laminin beta-1 (LAMB1) as a potential serological biomarker for colorectal cancer.* Proteomics, 2015.
451. Becerra, S.P., et al., *Pigment epithelium-derived factor behaves like a noninhibitory serpin. Neurotrophic activity does not require the serpin reactive loop.* J Biol Chem, 1995. **270**(43): p. 25992-9.
452. Al-Jallad, H., et al., *The effect of SERPINF1 in-frame mutations in osteogenesis imperfecta type VI.* Bone, 2015. **76**: p. 115-20.
453. Staton CA1, K.I., Reed MW, Brown NJ., *Neuropilins in physiological and pathological angiogenesis.*

J Pathol, 2007. **212(3):237-48.**
454. De Falco, V., et al., *Ponatinib (AP24534) is a novel potent inhibitor of oncogenic RET mutants associated with thyroid cancer.* J Clin Endocrinol Metab, 2013. **98**(5): p. E811-9.
455. Gozgit, J.M., et al., *Ponatinib (AP24534), a multitargeted pan-FGFR inhibitor with activity in multiple FGFR-amplified or mutated cancer models.* Mol Cancer Ther, 2012. **11**(3): p. 690-9.
456. O'Hare, T., et al., *AP24534, a pan-BCR-ABL inhibitor for chronic myeloid leukemia, potently inhibits the T315I mutant and overcomes mutation-based resistance.* Cancer Cell, 2009. **16**(5): p. 401-12.
457. Zhou, T., et al., *Structural mechanism of the Pan-BCR-ABL inhibitor ponatinib (AP24534): lessons for overcoming kinase inhibitor resistance.* Chem Biol Drug Des, 2011. **77**(1): p. 1-11.
458. Wang, J., et al., *Ligand-associated ERBB2/3 activation confers acquired resistance to FGFR inhibition in FGFR3-dependent cancer cells.* Oncogene, 2015. **34**(17): p. 2167-77.
459. Ye, Y., et al., *Silencing of FGFR4 could influence the biological features of gastric cancer cells and its therapeutic value in gastric cancer.* Tumour Biol, 2015.
460. Kaelin, W.G., Jr., *The concept of synthetic lethality in the context of anticancer therapy.* Nat Rev Cancer, 2005. **5**(9): p. 689-98.
461. Quail, D.F. and J.A. Joyce, *Microenvironmental regulation of tumor*

progression and metastasis. Nat Med. **19**(11): p. 1423-37.

462. Dumont, N., et al., *Breast fibroblasts modulate early dissemination, tumorigenesis, and metastasis through alteration of extracellular matrix characteristics.* Neoplasia, 2013. **15**(3): p. 249-62.
463. Jemal, A., et al., *Global cancer statistics.* CA Cancer J Clin, 2011. **61**(2): p. 69-90.
464. Tagliabue, E., et al., *The HER2 World: Better Treatment Selection for Better Outcome.* J Natl Cancer Inst Monogr, 2011. **2011**(43): p. 82-5.
465. Baselga, J. and S.M. Swain, *Novel anticancer targets: revisiting ERBB2 and discovering ERBB3.* Nat Rev Cancer, 2009. **9**(7): p. 463-75.
466. Guarneri, V., et al., *Anti-HER2 neoadjuvant and adjuvant therapies in HER2 positive breast cancer.* Cancer Treat Rev, 2010. **36 Suppl 3**: p. S62-6.
467. Arteaga, C.L., *Trastuzumab, an appropriate first-line single-agent therapy for HER2-overexpressing metastatic breast cancer.* Breast Cancer Res, 2003. **5**(2): p. 96-100.
468. Chen, F.L., W. Xia, and N.L. Spector, *Acquired resistance to small molecule ErbB2 tyrosine kinase inhibitors.* Clin Cancer Res, 2008. **14**(21): p. 6730-4.
469. Soon, P.S., et al., *Breast cancer-associated fibroblasts induce epithelial-to-mesenchymal transition in breast cancer cells.* Endocr Relat Cancer, 2013. **20**(1): p. 1-12.
470. Goltsov, A., et al., *Systems analysis of drug-induced receptor tyrosine kinase reprogramming following targeted mono- and combination anti-cancer therapy.* Cells, 2014. **3**(2): p. 563-91.
471. Madrid-Paredes, A., et al., *De novo resistance biomarkers to anti-HER2 therapies in HER2-positive breast cancer.* Pharmacogenomics, 2015. **16**(12): p. 1411-26.
472. Boulbes, D.R., et al., *HER family kinase domain mutations promote tumor progression and can predict response to treatment in human breast cancer.* Mol Oncol, 2015. **9**(3): p. 586-600.
473. Engelman, J.A., et al., *MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling.* Science, 2007. **316**(5827): p. 1039-43.
474. Portier, B.P., et al., *HER4 expression status correlates with improved outcome in both neoadjuvant and adjuvant Trastuzumab treated invasive breast carcinoma.* Oncotarget, 2013. **4**(10): p. 1662-72.
475. Nafi, S., et al., *Nuclear HER4 mediates acquired resistance to trastuzumab and is associated with poor outcome in HER2 positive breast cancer.* Oncotarget, 2014.
476. Nahta, R., et al., *Insulin-like growth factor-I receptor/human epidermal growth factor receptor 2 heterodimerization contributes to trastuzumab resistance of breast cancer cells.* Cancer Res, 2005. **65**(23): p. 11118-28.
477. Zhuang, G., et al., *Elevation of receptor tyrosine kinase EphA2 mediates resistance to trastuzumab therapy.* Cancer Res, 2010. **70**(1): p. 299-308.
478. Vaught, D., D.M. Brantley-Sieders, and J. Chen, *Eph receptors in breast cancer: roles in tumor promotion and tumor suppression.* Breast Cancer Res, 2008. **10**(6): p. 217.
479. Azuma, K., et al., *Switching addictions between HER2 and FGFR2 in HER2-positive breast tumor cells: FGFR2 as a potential target for salvage after lapatinib failure.* Biochem Biophys Res Commun, 2011. **407**(1): p. 219-24.
480. Ahmad, I., T. Iwata, and H.Y. Leung, *Mechanisms of FGFR-mediated carcinogenesis.* Biochim Biophys Acta, 2012. **1823**(4): p. 850-60.
481. Crawford, Y. and N. Ferrara, *Tumor and stromal pathways mediating refractoriness/resistance to anti-angiogenic therapies.* Trends Pharmacol Sci, 2009. **30**(12): p. 624-30.
482. Turner, N., et al., *FGFR1 amplification drives endocrine therapy resistance and is a therapeutic target in breast cancer.* Cancer Res, 2010. **70**(5): p. 2085-94.
483. Huang, W.S., et al., *Discovery of 3-[2-(imidazo[1,2-b]pyridazin-3-yl)ethynyl]-4-methyl-N-{4-[(4-methylpiperazin-1-y l)methyl]-3-(trifluoromethyl)phenyl}benzamide (AP24534), a potent, orally active pan-inhibitor of breakpoint cluster region-abelson (BCR-ABL) kinase including the T315I gatekeeper mutant.* J Med Chem, 2010. **53**(12): p. 4701-19.
484. Yashiro, M., et al., *Synergistic antitumor effects of FGFR2 inhibitor with 5-fluorouracil on scirrhous gastric carcinoma.* Int J Cancer, 2010. **126**(4): p. 1004-16.
485. Qiu, H., et al., *A FGFR2 inhibitor, Ki23057, enhances the chemosensitivity of drug-resistant gastric cancer cells.* Cancer Lett, 2011. **307**(1): p. 47-52.
486. Wolf J, L.P., Camidge RD et al, *A phase I dose escalation study of NVPBGJ398, a selective pan FGFR inhibitor in genetically preselected advanced solidtumours.* Proceedings of the103rd Annual Meeting of the American Association for Cancer Research (AACR), LB-122., 2012.
487. Wang, Y.C., et al., *Different mechanisms for resistance to trastuzumab versus lapatinib in HER2-positive breast cancers--role of estrogen receptor and HER2 reactivation.* Breast Cancer Res, 2011. **13**(6): p. R121.

488. Wang, S.E., et al., *Transforming growth factor beta engages TACE and ErbB3 to activate phosphatidylinositol-3 kinase/Akt in ErbB2-overexpressing breast cancer and desensitizes cells to trastuzumab.* Mol Cell Biol, 2008. **28**(18): p. 5605-20.
489. Ware, K.E., et al., *A mechanism of resistance to gefitinib mediated by cellular reprogramming and the acquisition of an FGF2-FGFR1 autocrine growth loop.* Oncogenesis, 2013. **2**: p. e39.
490. Terai, H., et al., *Activation of the FGF2-FGFR1 autocrine pathway: a novel mechanism of acquired resistance to gefitinib in NSCLC.* Mol Cancer Res, 2013. **11**(7): p. 759-67.
491. Ware, K.E., et al., *Rapidly acquired resistance to EGFR tyrosine kinase inhibitors in NSCLC cell lines through de-repression of FGFR2 and FGFR3 expression.* PLoS One, 2010. **5**(11): p. e14117.
492. Liang, G., et al., *Anticancer molecules targeting fibroblast growth factor receptors.* Trends Pharmacol Sci, 2012. **33**(10): p. 531-41.
493. Liang, G., et al., *Small molecule inhibition of fibroblast growth factor receptors in cancer.* Cytokine Growth Factor Rev, 2013. **24**(5): p. 467-75.
494. Greulich, H. and P.M. Pollock, *Targeting mutant fibroblast growth factor receptors in cancer.* Trends Mol Med, 2011. **17**(5): p. 283-92.
495. Harding, T.C., et al., *Blockade of nonhormonal fibroblast growth factors by FP-1039 inhibits growth of multiple types of cancer.* Sci Transl Med, 2013. **5**(178): p. 178ra39.
496. Sharpe, R., et al., *FGFR signaling promotes the growth of triple-negative and basal-like breast cancer cell lines both in vitro and in vivo.* Clin Cancer Res, 2011. **17**(16): p. 5275-86.
497. Byron, S.A., et al., *The N550K/H mutations in FGFR2 confer differential resistance to PD173074, dovitinib, and ponatinib ATP-competitive inhibitors.* Neoplasia, 2013. **15**(8): p. 975-88.
498. Chell, V., et al., *Tumour cell responses to new fibroblast growth factor receptor tyrosine kinase inhibitors and identification of a gatekeeper mutation in FGFR3 as a mechanism of acquired resistance.* Oncogene, 2013. **32**(25): p. 3059-70.
499. Herrera-Abreu, M.T., et al., *Parallel RNA interference screens identify EGFR activation as an escape mechanism in FGFR3-mutant cancer.* Cancer Discov, 2013. **3**(9): p. 1058-71.
500. Dieci, M.V., et al., *Fibroblast growth factor receptor inhibitors as a cancer treatment: from a biologic rationale to medical perspectives.* Cancer Discov, 2013. **3**(3): p. 264-79.
501. Pietras, K. and A. Ostman, *Hallmarks of cancer: interactions with the tumor stroma.* Exp Cell Res, 2010. **316**(8): p. 1324-31.
502. Finak, G., et al., *Stromal gene expression predicts clinical outcome in breast cancer.* Nat Med, 2008. **14**(5): p. 518-27.
503. Tchou, J., et al., *Human breast cancer associated fibroblasts exhibit subtype specific gene expression profiles.* BMC Med Genomics, 2012. **5**: p. 39.
504. Hayward, S.W., et al., *Malignant transformation in a nontumorigenic human prostatic epithelial cell line.* Cancer Res, 2001. **61**(22): p. 8135-42.
505. Yauch, R.L., et al., *A paracrine requirement for hedgehog signalling in cancer.* Nature, 2008. **455**(7211): p. 406-10.
506. Hu, M., et al., *Regulation of in situ to invasive breast carcinoma transition.* Cancer Cell, 2008. **13**(5): p. 394-406.
507. Cunha, G.R., S.W. Hayward, and Y.Z. Wang, *Role of stroma in carcinogenesis of the prostate.* Differentiation, 2002. **70**(9-10): p. 473-85.
508. Lotti, F., et al., *Chemotherapy activates cancer-associated fibroblasts to maintain colorectal cancer-initiating cells by IL-17A.* J Exp Med, 2013. **210**(13): p. 2851-72.
509. Gupta, G.P. and J. Massague, *Cancer metastasis: building a framework.* Cell, 2006. **127**(4): p. 679-95.
510. Wei, W., et al., *The breast cancer susceptibility FGFR2 provides an alternate mode of HER2 activation.* Oncogene, 2015.
511. Blackwell, K.L., et al., *Overall survival benefit with lapatinib in combination with trastuzumab for patients with human epidermal growth factor receptor 2-positive metastatic breast cancer: final results from the EGF104900 Study.* J Clin Oncol, 2012. **30**(21): p. 2585-92.
512. Baselga, J., et al., *Phase II trial of pertuzumab and trastuzumab in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer that progressed during prior trastuzumab therapy.* J Clin Oncol, 2010. **28**(7): p. 1138-44.
513. Phillips, G.D., et al., *Dual targeting of HER2-positive cancer with trastuzumab emtansine and pertuzumab: critical role for neuregulin blockade in antitumor response to combination therapy.* Clin Cancer Res, 2014. **20**(2): p. 456-68.
514. Falchook, G.S., et al., *Human epidermal receptor 2-amplified salivary duct carcinoma: regression with dual human epidermal receptor 2 inhibition and anti-vascular endothelial growth factor combination treatment.* Head Neck, 2014. **36**(3): p. E25-7.
515. Falchook, G.S., et al., *Non-small-cell lung cancer with HER2 exon 20 mutation: regression with dual HER2 inhibition and*

anti-VEGF combination treatment. J Thorac Oncol, 2013. 8(2): p. e19-20.

516. Britten, C.D., et al., *A phase I/II trial of trastuzumab plus erlotinib in metastatic HER2-positive breast cancer: a dual ErbB targeted approach.* Clin Breast Cancer, 2009. 9(1): p. 16-22.

517. Morrow, P.K., et al., *Phase I/II study of trastuzumab in combination with everolimus (RAD001) in patients with HER2-overexpressing metastatic breast cancer who progressed on trastuzumab-based therapy.* J Clin Oncol, 2011. 29(23): p. 3126-32.

518. Felip E, R.M., Cedres S, Dean E, Brewster M, Martinez P, et al. , *A phase Ib,*

dose-finding study of erlotinib in combination with a fixed dose of

pertuzumab in patients with advanced non-small-cell lung cancer. Clin

Lung Cancer 2012: p. 13:432–41.

519. Rugo, H.S., et al., *A phase II study of lapatinib and bevacizumab as treatment for HER2-overexpressing metastatic breast cancer.* Breast Cancer Res Treat, 2012. 134(1): p. 13-20.

520. Bang, Y.J., et al., *Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial.* Lancet, 2010. 376(9742): p. 687-97.

521. Baselga, J., et al., *Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer.* N Engl J Med, 2012. 366(2): p. 109-19.

522. Martin, M., et al., *Phase II study of bevacizumab in combination with trastuzumab and capecitabine as first-line treatment for HER-2-positive locally recurrent or metastatic breast cancer.* Oncologist, 2012. 17(4): p. 469-75.

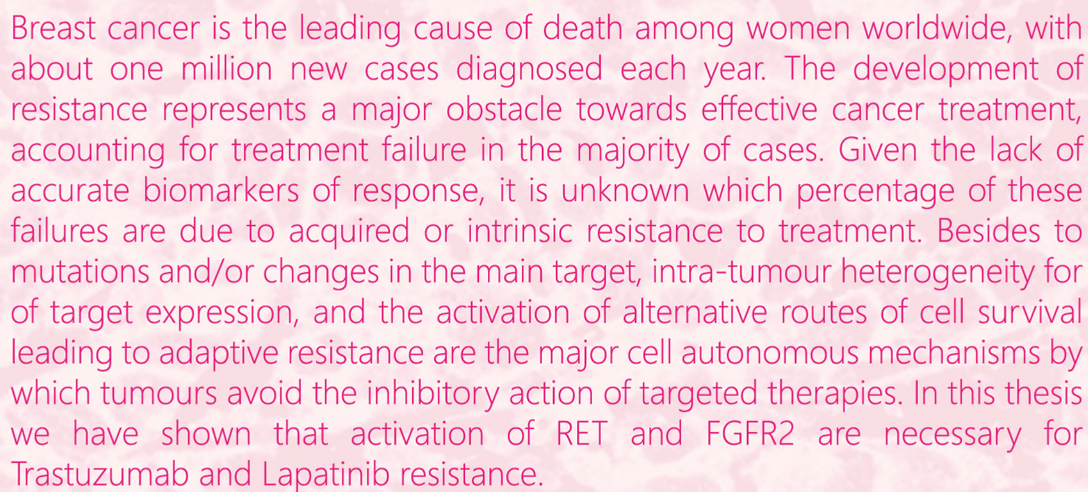
523. Somlo, G., et al., *A phase I/II prospective, single arm trial of gefitinib, trastuzumab, and docetaxel in patients with stage IV HER-2 positive metastatic breast cancer.* Breast Cancer Res Treat, 2012. 131(3): p. 899-906.

524. Makhija, S., et al., *Clinical activity of gemcitabine plus pertuzumab in platinum-resistant ovarian cancer, fallopian tube cancer, or primary peritoneal cancer.* J Clin Oncol, 2010. 28(7): p. 1215-23.

525. Yardley, D.A., et al., *Phase II study evaluating lapatinib in combination with nab-paclitaxel in HER2-overexpressing metastatic breast cancer patients who have received no more than one prior chemotherapeutic regimen.* Breast Cancer Res Treat, 2013. 137(2): p. 457-64.

526. Cappuzzo, F., L. Bemis, and M. Varella-Garcia, *HER2 mutation and response to trastuzumab therapy in non-small-cell lung cancer.* N Engl J Med, 2006. 354(24): p. 2619-21.

527. Yan M, P.B., Schwab R, Kurzrock R., *HER2 aberrations in cancer: implications for therapy.* Cancer Treat Rev. , 2014. 40(6):770-80.



Breast cancer is the leading cause of death among women worldwide, with about one million new cases diagnosed each year. The development of resistance represents a major obstacle towards effective cancer treatment, accounting for treatment failure in the majority of cases. Given the lack of accurate biomarkers of response, it is unknown which percentage of these failures are due to acquired or intrinsic resistance to treatment. Besides to mutations and/or changes in the main target, intra-tumour heterogeneity for of target expression, and the activation of alternative routes of cell survival leading to adaptive resistance are the major cell autonomous mechanisms by which tumours avoid the inhibitory action of targeted therapies. In this thesis we have shown that activation of RET and FGFR2 are necessary for Trastuzumab and Lapatinib resistance.

The importance of the stromal microenvironment in drug resistance has been recognized during the last years, and it is now widely accepted that cancer survival and metastasis (as a consequence of treatment failure), are regulated by stromal-cancer cell interactions. The cancer-associated fibroblast (CAF) are the most prominent cell type within the tumour stroma of breast cancer, and are activated by chemokines, cytokines, and growth factors secreted by the stromal microenvironment that act in an autocrine and paracrine way under the influence of the tumour, and in the same way, CAFs can stimulate tumour cell proliferation, growth, and progression through production of various growth factors, hormones, and cytokines.

Our research defines a coevolution process between breast cancer cells and fibroblasts. We have identified FGFR2 pathway as a novel mechanism of HER2 activation mediated by fibroblasts and breast cancer cells crosstalk. Moreover, we demonstrated the importance of the interaction between these two pathways in vitro and in vivo, in promoting breast cancer resistance and progression. Furthermore, we propose that inhibition of RET and FGFR2 pathways might become a promising salvage strategy after Trastuzumab and Lapatinib failure in patients with HER2-positive breast cancer, and that the addition of anti FGFR therapies in the early stages of breast cancer treatment may prevent resistance development.