Cancer-associated fibroblasts and response to anti-HER2 monoclonal antibodies in breast cancer

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A mi familia.

Science, my boy, is made up of mistakes,
but they are mistakes which it is useful to make,
because they lead little by little to the truth

Jules Verne. Journey to the Center of the Earth

1. ABSTRACT

HER2-positive breast cancer (BC) is an aggressive subtype of this disease. The development of anti-HER2 targeted therapies, particularly the monoclonal antibody (Mab) trastuzumab, significantly improved its otherwise poor prognosis. More recently, another Mab called pertuzumab, has further improved the efficacy of trastuzumab, yet no all patients benefit from the combination of the two Mabs. A proportion of HER2-breast cancer patients will not benefit from anti-HER2 agents, and will ultimately die as a consequence of innate or acquired drug resistance mechanisms. For this reason, better understanding of the specific mechanisms involved in anti-HER2 drug sensitivity and resistance is crucial *to further advance towards its's cure.*

Tumours consist not only of heterogeneous populations of cancer cells, but also of the tumour microenvironment (TME). In recent years, increasing evidence has shown that cancer-associated fibroblasts (CAFs; an abundant stromal cell population within the TME), directly support tumorigenesis and promote therapy resistance. However, at the beginning of this PhD study, there was little published work on the role of CAFs on anti-HER2 targeted therapy resistance.

In this study we hypothesized that in HER2-positive BC, CAFs as well as paracrine secretion of soluble factors into the TME, would enable breast cancer cells to overcome the antitumor effects of anti-HER2 targeted treatments. To test this, we developed a co-culture system whereby HER2-positive BC cells were exposed to paracrine-released factors from immortalized CAFs, which had previously been derived from HER2-positive breast cancer patients (Fernandez-Nogueira et al., submitted). Interestingly, CAFs promote resistance to both trastuzumab and the chemotherapeutic agent paclitaxel, which are the cornerstone of most therapeutic schemes currently used for treating HER2-positive BC patients.

Among the repertoire of CAF-related proteins, we found that Neuregulin 1 (NRG-1), a HER3 ligand, is expressed by CAFs from HER2+ tumours. Paracrine signalling from CAFs activates the pro-survival PI3K/Akt and MAPK/Erk pathways in cancer cells. The implication of CAFs in sustaining tumorigenesis has been also highlighted in *in vivo* studies.
*E*xperiments carried out on mice suggested that CAFs may promote in vivo tumour growth of HER2+ breast cancer cells in an NRG-1 dependent manner. Importantly, stable depletion of NRG-1 in CAFs using a lentiviral vector-based shRNA system, was sufficient to abrogate paracrine CAF-effects in breast cancer cells *in vitro* and *in vivo*, as well as to restore drug sensitivity. We also found that crosstalk between cancer cells and CAFs occurs in a cooperative and bi-directional manner. NRG-1 increases the TGF-β gene expression programme in tumour cells, and this, in turn, may amply the activation loop in CAFs through Smads and Snail1 signalling network.

NRG-1 was also found in primary CAFs isolated from fresh HER2-positive BC patient biopsies from Hospital del Mar. In this context, paracrine signalling from primary CAFs also shaped the therapeutic response of cancer cells. Interestingly, the addition of pertuzumab, an anti-HER2 monoclonal antibody that prevents HER2 heterodimerization, prevented the paracrine effects derived from immortalized and primary CAFs and restored trastuzumab efficacy *in vitro*.

Furthermore, we found NRG-1 was mainly expressed in the stromal compartment of HER2-positive BC patients. Stromal NRG-1 expression in tumour tissue samples was associated with poor clinical outcome in a cohort of HER2-positive breast cancer patients treated with anti-HER2 based regimens in the neoadjuvant setting as well as in analysis performed in publically available gene data sets.

In summary, the work presented here supported a role of CAFs in tumour resistance to anti-HER2 targeted therapies in HER2+ breast cancer. In particular, NRG-1 was a main mediator for CAF-derived resistance to trastuzumab in HER2-positive BC. Importantly, the addition of pertuzumab overcomed this TME-derived resistance and its addition to trastuzumab may be particularly relevant for tumors enriched in CAFs.

2. RESUMEN

El cáncer de mama HER2-positivo es un subtipo muy agresivo. El desarrollo de terapias anti-diana HER2, particularmente el anticuerpo monoclonal (mAb) trastuzumab, supuso una mejora significativa en el pronóstico de esta enfermedad. De manera más reciente, otro mAb llamado pertuzumab, ha mejorado todavía más la eficiencia de trastuzumab. Aún y así, no todas las pacientes se beneficiarán de esta combinación de mAbs. Una proporción de estas pacientes no se beneficiarán de estas terapias anti-HER2, y fallecerán a causa de la presencia o el desarrollo de mecanismos de resistencia. Por este motivo, comprender los mecanismos moleculares implicados en la respuesta a las terapias anti-HER2, es de capital importancia para continuar hacia la cura de esta enfermedad.

Los tumores consisten no únicamente de una población heterogénea de células tumorales; si no también del conocido como microentorno tumoral (TME por sus siglas en inglés). En los últimos años, se ha evidenciado que los fibroblastos asociados al tumor (CAFs por sus siglas en inglés) (una población estromal muy abundante dentro del TME), directamente promueven los procesos tumorogénicos así como la resistencia a los fármacos. No obstante, al inicio de este proyecto de tesis doctoral, los estudios relativos sobre el papel de los CAFs en la resistencia a la terapia anti-HER2 eran escasos.

En este estudio, hemos hipotetizado que, en los tumores de mama HER2-positivos, los CAFs así como la secreción de factores solubles en el microentorno tumoral podrían promover la progresión de las células tumorales frente a los fármacos. Para aproximar esta hipótesis, hemos desarrollado un sistema de co-cultivo basado en el intercambio paracrino de factores solubles entre las células tumorales y CAFs. Estas poblaciones estromales, fueron previamente aisladas de pacientes con cáncer de mama HER2-positivo e inmortalizadas (Fernandez-Nogueira et al. enviado para publicación).

Significativamente, hemos observado que CAFs promueven resistencia no solo a trastuzumab si no también a paclitaxel. La combinación de ambos fármacos está incluida en los esquemas clásicos para el tratamiento de los pacientes con cáncer de mama HER2-positivos.

De entre el repertorio de potenciales mediadores secretados por los CAFs, hemos descrito que Neuregulina-1 (NRG-1) el ligando del receptor HER3, es expresado por estas poblaciones estromales de los tumores HER2+. Además, la señalización paracrina de estos fibroblastos promueve la activación de las vías de supervivencia celular: PI3K/AKT y MAPK/ERk en las células tumorales. Paralelamente, la relevancia de los CAFs en promover el crecimiento tumoral ha sido evidenciada en modelos murinos.

Los experimentos llevados a cabo en estos modelos sugieren que estas células estromales promueven el crecimiento tumoral a través, al menos en parte, de NRG-1. En esta línea, la depleción estable de NRG-1 en estas células mediante la modificación génica (mediante *short hairpin* RNAs) es suficiente para revertir los efectos protectores de los CAFs *in vitro* y en modelos animales, así como de restaurar la sensibilidad de las células tumorales a los fármacos.
No obstante, hemos observado además, que la comunicación entre las células tumorales y los CAFs se traduce en un proceso cooperativo y bidireccional. NRG-1 induce la expresión génica de TGF-β en las células tumorales. Esto promueve a su vez, la activación de programas celulares que incluyen proteínas de la familia de Smads así como del factor de transcripción Snail1 en los fibroblastos asociados al tumor.

NRG-1 ha sido también detectada en cultivos primarios de fibroblastos derivados de pacientes HER2-positivos del Hospital del Mar. En este contexto, la señalización paracrina de estas células estromales modula la respuesta de las células tumorales frente a los fármacos.

Notablemente, la adición de pertuzumab, un anticuerpo que inhibe de la dimerización de HER2, previene los efectos protectores tanto de los fibroblastos inmortalizados, así como de los primarios y reestablece la eficacia de trastuzumab.

En este trabajo se evidencia también que NRG-1 está principalmente expresada en el compartimento estromal de los pacientes HER2 y que tiene relevancia clínica en la respuesta a la terapia anti-HER2. La expresión de NRG-1 estromal en muestras tumorales de pacientes se asoció a una peor respuesta clínica en una cohorte de pacientes HER2 tratadas con terapia anti-HER2 neoadyuvante; así como en el análisis de datos públicos de expresión génica.

En resumen, nuestros hallazgos evidencian el papel de los fibroblastos asociado al tumor en la resistencia a la terapia anti-HER2. En este contexto, hemos descrito el papel de NRG-1, como un factor derivado de los CAFs que media en la resistencia a trastuzumab. Significativamente, la adición de pertuzumab revierte esta resistencia generada por las células del microentorno tumoral, y su adición a trastuzumab podría ser particularmente relevante en tumores enriquecidos con esta población estromal.

3. PREFACE

This doctoral work has been conducted in the Molecular Cancer Therapeutics unit at the *Hospital del Mar Medical Research Institute* (IMIM), which belongs to the Cancer Research Programme. The group is coordinated by Dr. Joan Albanell, director of the Medical Oncology Service at Hospital del Mar, and the preclinical laboratory is led by Dr. Ana Rovira. The group is a multidisciplinary team that includes oncologists, biologists, pathologists, immunologists, and technicians. The research team has been recognized as a consolidated excellence group by AGAUR/Generalitat de Catalunya, and CIBERONC/ Spanish Institute of Health Carlos III (ISCIII).

Since 2015, I have been working, as a predoctoral student, on various translational projects in the area of HER2-positive BC, all of them led by Dr. Albanell and Dr. Rovira. In the first year, I worked on the development of an FIS project (PI15/00146) "Role of tumour heterogeneity and dynamic tumour cell reprogramming in resistance to anti-HER2 antibodies in HER2 positive breast cancer". At that time I worked closely with a more senior member of the laboratory (Mohammad Ali Sabbaghi), using the drug-antibody conjugate T-DM1 (trastuzumab-emtansine). Sabbaghi was able to generate and deeply characterize HER2+ breast cancer cells resistant to T-DM1. I was in charge of genetically modifying the expression levels of the *cyclin B1* gene, through transfection experiments in sensitive and T-DM1 resistant cells, respectively, and examining the molecular and cellular consequences in the response to T-DM1. We provided unique insights into T-DM1 functions, describing the induction of cyclin B1 as a key event in the efficacy of T-DM1, and the fact that its deficiency causes resistance. This work has been published (Sabbaghi et al., Clin Cancer Res 2017), with me as a co-author.

From the second year up to the finalization of my PhD, I worked on two projects. In one, I worked closely with other researchers within the group, developing new combinations of anti-HER2 antibodies with other targeted therapies. I will be co-author of a manuscript showing that mTOR inhibition improves T-DM1 cytotoxicity by enhancing its targeting to the lysosomes and consequently increasing the amount of free intracellular DM1 (Casadevall et al., in prep.).

The second project was the object of my thesis work. My research *work* was focused on the role of the tumour microenvironment (TME), particularly cancer-associated fibroblasts (CAFs) in anti-HER2 targeted therapies, drug response, and resistance in HER2-positive BC. We have shown that paracrine crosstalk between tumour cells and CAFs impairs the drug efficacy of trastuzumab and paclitaxel. Moreover, from potential mediators, we identified NRG-1 from CAFs as being directly implicated in the modulation of drug response of HER2-positive BC cells, and we found stromal expression of NRG-1 was of promising clinical value. We are currently drafting a manuscript on this. The project was possible thanks to active collaborations with Dr. Rojo, Dr. Pandiella, Dr. Gascón, and Dr. Bragado, and has provided the opportunity of short interchanges leading to technological/conceptual advances.

The group has already received funding for a new FIS project (PI18/00006) with the overall objective of characterizing and defining the therapeutic repercussions of HER2-positive BC ecology, with a special focus on the role of the immune system in the therapeutic response of this breast cancer. I am currently collaborating in this project.

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4. ABBREVIATIONS

A

ADAM	A desintegrin and metalloproteinase
ADCC	Antibody-drug conjugate
AKT	Protein kinase B
ADCC	Antibody cell dependent citotoxicity
AR	Amphiregulin
αSMA	Alpha-smooth muscle actin
ATP	Adenosine triphosphate

B

BC	Breast cancer
BM	Basement membrane
BMSCs	Bone marrow stromal cells
BTC	Betacellulin

C

CAF	Cancer-associated fibroblasts
CAR-T	Chimeric antigen receptor T-cell
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CEP17	Chromosome 17 centromere
c-MET	Tyrosine-protein kinase Met
CSC	Cancer stem cell
CTGF	Connective tissue growth factor
CXCL7	Platelet-derived chemokine CXCL7

D

DCIS	Ductal carcinoma *in situ*

E

ECD	Extracellular domain
ECM	Extracellular matrix
EeF2	Eukaryotic elongation factor 2
EGF	Epidermal growht-factor
EGFR	Epidermal growht-factor receptor
4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1
eIF4B	Eukaryotic translation initiation factor 4E
EMT	Epithelial-to-mesenchymal transition
EPGN	Epigen
ER	Oestrogen receptor
EREG	Epiregulin
ERK	Extracellular regulated kinase

F

FAP	Fibroblast associated protein
FDA	Food and Drug Administration
FEC	neoadjuvant 5-fluorouracil, epirubicin and cyclophosphamide
FGF	Fibroblast growth factor
FISH	Fluorescence in situ hybridisation
FSP1	Fibroblast specific protein-1

G

GCSCs	Gastric cancer stem cells
GDP	Guanosine diphosphate
GEO	Gene expression omnibus
GSEA	Gene set enrichment analysis
GSVA	Gene set variation analysis
GS3Kβ	Glycogen synthase kinase 3 beta
GTP	Guanosine triphosphate

H

HB-EGF	Heparin-binding EGF
HER	Epidermal growht factor receptor
HER2	Human epidermal growth factor receptor 2
HGFR	Met/Hepatocyte growth factor receptor
HIF-1	Hypoxic inducible factor 1
HRG	Heregulin

I

ICC	Immunocytochemistry
IDCS	Invasive ductal carcinoma
IFNγ	Interferon gamma
IGF1R	Insuline-growth factor 1 receptor
IHC	Immunohistochemistry
IL-17A	Interleukin 17
IL-6	Interleukin 6
IL-8	Interleukin 8
ICC	Immunocytochemistry
IHC	Immunohistochemistry
IP	Immunoprecipitation

J

JAK	Janus activated kinase

M

mAb	Monoclonal antibody
MAPK	Mitogen-activaded protein kinase
mRNA	messenger RNA
MSCs	Mesenchymal stem cells
MsigDB	Molecular signatures database
mTOR	Mammalian target of rapamycin
MUC	Mucine

N

NAF	Normal associated fibroblasts
NF-kB	Nuclear factor *κ*B
NK	Natural Killer
NRG	Neuregulin

P

PAI-1	Plasminogen activator inhibitor 1
pCR	Partial complete response
PDCD4	Protein coding programmed cell death 4
PDGF	Platelet derived growth factor receptor
PDGFR	Platelet derived growth factor receptor
PDK1	Pyruvate Dehydrogenase Kinase 1
PFS	Progression-free survival
PGE2	Prostaglandin E2
PI3K	Phosphatidylinositol 3-kinase
PI3KCA	Phosphatidylinositol-4,5.biphosphate 3-kinase, catalytic subunit alpha
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKB	Protein kinase B
PR	Progesterone recpetor
PTEN	Phosphatase and tensin

R

RWD	Real world data
RT	Room temperature
RTK	Receptor tyrosine kinase

S

S6K	S6 kinase
SDF1	Stromal-cell derived factor 1
Ser	Serine
sh	Short Hairpin

T

TACE	Tumor necrosis factor-alpha converting enzyme
TAM	Tumour associated macrophage
T-DM1	Trastuzumab emtansine
TGF-α	Transforming growth factor alpha
TGF-β	Transforming growth factor beta
Thr	Threonine
TIL	Tumour infiltrating lymphocyte
TKI	Tyrosine kinase inhibitor
TME	Tumour microenvironment
TNBC	Triple negative breast cancer
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cell
Tyr	Tyrosine

V

VEGF	Vascular endothelial growth factor

W

WB	Western Blot

INTRODUCTION

BREAST CANCER

I1. BREAST CANCER OVERVIEW

Breast cancer (BC) is a highly prevalent disease. Every year, 2.1 million women are affected by BC, the most common type of cancer among women. Although incidence rates are higher in developed regions, they are increasing globally. Indeed, BC is the primary cause of cancer-related deaths in women. (1)

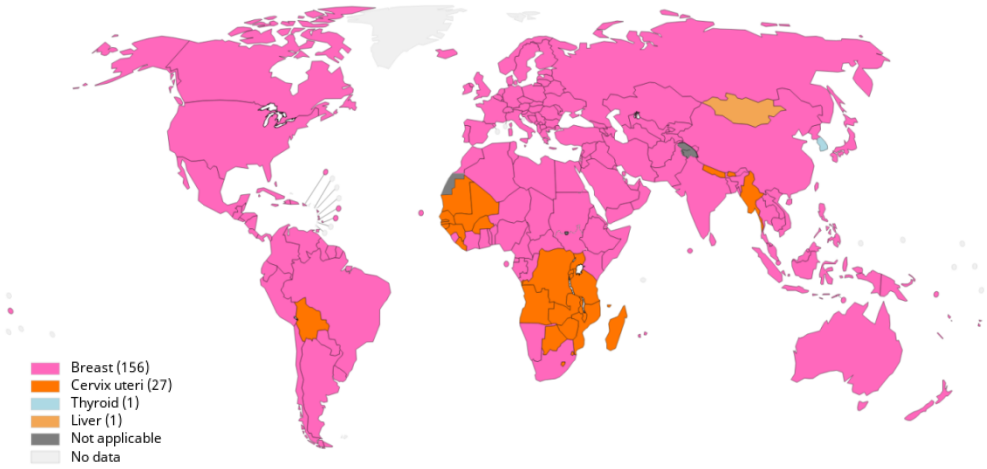


Figure 1. Top cancer per country, estimated age-standardized incidence rates (world) in 2018, females, all ages. Globocan Source

I2. BREAST CANCER CLASSIFICATION

BC is a complex and heterogeneous disease. Over the past decades, it has been classified into various subtypes. The diagnosis, prognosis, and therapeutic options for patients are significantly influenced by the intrinsic subtype. Briefly, BCs can be classified histologically, immunohistochemically, and molecularly, as explained below.

I2.1 Histological classification

BCs were first classified based on the pathological study of their morphology and structural organization. In this way, BCs can be sub-divided into:

- **Ductal carcinomas**

  Accounting for 75-80% of all BCs, this is the most common subtype. Principally, ductal carcinomas can be sub-classified into ductal carcinoma *in situ* (DCIS) and invasive ductal carcinoma (IDCS). The sprout of tumour cells into the milk duct is the main characteristic differentiating them. (2)

- **Lobular carcinomas**

  This BC subtype is the second most common cancer subtype (from 10-15% of all BCs). It is associated with older patients and has a better prognosis than ductal carcinoma.(3)

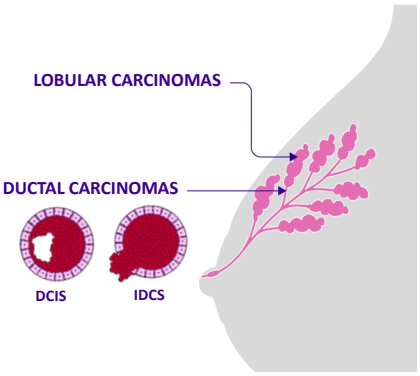


Figure 2. Histological classification of the main breast cancer subtypes. Adapted from Cancer Research UK.

- **Inflammatory breast cancer**

  Accounting for 1-5% of all BCs; this is a very rare and aggressive subtype. Its name refers to the way cancer cells block lymph vessels in the breast skin.(4)

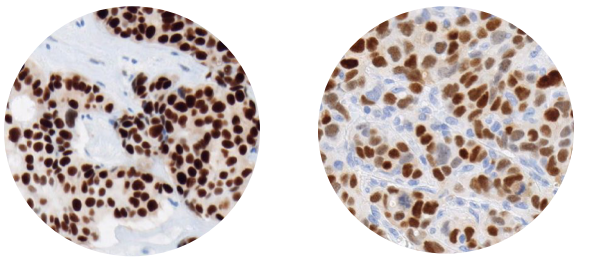
I2.2 Immunohistochemical (IHC) classification

Following histological phenotyping, breast tumours were later re-classified based on the expression of hormone receptors (oestrogen/progesterone receptors, ER/PR, respectively), and the human epidermal growth factor receptor 2 (HER2).

Based on this, three groups have been defined:

- **ER-positive breast tumours (ER+):**

  ERs are nuclear receptors that become activated upon binding oestrogen, promoting tumour cell proliferation and angiogenesis, among other things. Tumours overexpressing ER are the most prominent BC subtype, accounting for 60 to 70% of all breast tumours. ER positivity is usually accompanied by PR expression. From a clinical perspective, these tumours strongly benefit from anti-endocrine therapy(5).

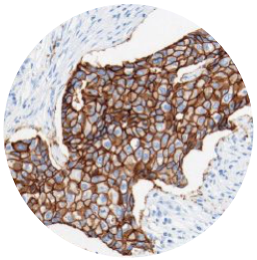


Oestrogen receptor (ER) Progesterone receptor (PR)

Figure 3. Representative immunohistochemical staining images from ER and PR. Adapted from the breast cancer IHC portfolio, from Roche.

- **HER2-positive breast tumours (HER2+):**

HER2 is a transmembrane tyrosine-kinase receptor from the Human Epidermal Receptor (HER) family. Representing 15-20% of all BCs, it was first described as a very aggressive subtype. These tumours tend to grow fast and are prone to metastasis, directly impacting the clinical outcome of these patients. However, their worst prognosis can be greatly improved by pharmacologically targeting HER2 with anti-HER2 targeted therapies(6).



HER2

Figure 4. Representative immunohistochemical staining image of HER2. Adapted from the breast cancer IHC portfolio, from Roche.

- **Triple-negative breast cancer (TNBC):**

This type is characterized by a lack of ER/PR and HER2 expression. This subgroup has a more aggressive phenotype and a poorer prognosis since it is prone to metastasis and does not benefit from specific targeted treatments(7).

I2.3 Molecular classification

Breast cancer is a collection of multiple diseases. The development of high-throughput microarray techniques in recent decades, has enabled the study of large-scale BC datasets. From these studies, breast tumours were initially hierarchically clustered into two main groups: ER-positive and ER-negative. Subsequent studies led to further subdivision of the ER-negative group into basal-like, HER2+, and normal-like subtypes.

In the early 2000s, BCs were classified further, into five gene expression subtypes(8):

- **Luminal breast cancer**

This subtype comprises the majority of breast tumours. They were defined as tumours with a similar gene expression profile to that of the luminal epithelia of the breast. This group can also be subdivided into two categories, A and B.

- **Luminal A tumours**, the most prevalent, are characterized by high expression of ER-related genes, low expression of HER2 genes, and low expression of proliferation-related genes(8).
- In contrast, **Luminal B** tumours are not enriched for ER-related genes, they present variable expression of HER2-related genes, and have high expression of proliferation-related genes. Luminal B tumours have a worse prognosis than luminal A tumours(9).

Luminal tumours are generally associated with endocrine sensitivity and it has been purported that some luminal B tumours may benefit from anti-HER2-targeted therapies(8).

- **Normal-like**

Similarly to Luminal A, these tumours are characterized by an enriched expression of ER-related genes, and low levels of HER2- and proliferation-related genes. The prognosis is slightly worse than for luminal A tumours(8).

- **HER2-enriched**

This subgroup is characterized by an enriched expression of HER2 and a proliferation of gene clusters. In contrast, luminal- and basal-related genes are poorly expressed(8,10).

- **Basal-like subtype/TNBC subtype**

Less frequent than the previous subgroup, this is characterized by poor expression of luminal- and HER2-related genes. In contrast, it shows high expression of basal- and proliferation-related genes.

In recent years, other molecular subtypes have also been postulated. Special mention should be given to the **Claudin-low** and **Apocrine** tumours, a special subgroup of basal-like tumours(8,11–13).

I3. BREAST CANCER THERAPY OPTIONS

Clinical options for BC patients vary depending on the tumour stage and BC subtype. Briefly, the tumour stage is scored according to:

I3.1 Histological grade

Determined by a pathologist, this refers to the degree of differentiation in the tumour biopsy sample. Tumours can be classified as: well differentiated (grade I), moderately differentiated (grade II), or poorly differentiated (grade III). A higher tumour-grading score is usually accompanied by a worse clinical outcome(14).

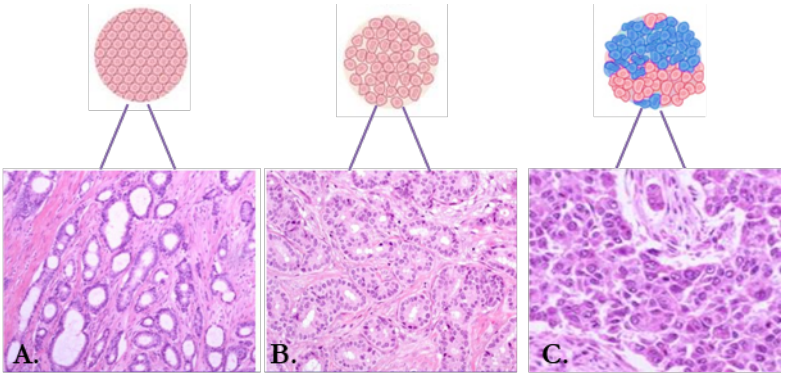


Figure 5. Representative images of breast cancer tumour grades. From left to right: A. well-differentiated tumour (grade I); B. moderately differentiated tumour (grade II); and C. poorly differentiated tumour (grade III)**.** Adapted from Rakha et al., 2010.

I3.2 Tumour size (TNM)

The TNM classification system divides tumours according to: their size, **T**; regional nodal status, **N**; and distant metastasis, **M.** Higher numbers for the T, N, or M criteria mean a tumour is more advanced(15).

In the early stages, whenever possible, solid tumours tend to be surgically removed. In some cases, neoadjuvant therapy is applied beforehand to reduce tumour volume. Moreover, evaluating the tumour biopsy after treatment also provides information on how the tumour is responding to the treatment and whether the patient is in pathological complete response, (pCR).
After surgical resection, adjuvant therapy with radiation and/or concomitant chemotherapy may also be applied to ensure that any cancer cells not previously detected are eradicated.
However, when a tumour is advanced or has spread into distant tissues, the treatment options may differ. With no curative endpoint, the main goal is to extend the life of the patient.

Briefly, the main therapeutic options for BC patients are:

I3.2 Chemotherapy

Anthracyclines (doxorubicin and epirubicin) and taxanes (paclitaxel and docetaxel) are the most common chemotherapy drugs used for BC. Whereas the first are topoisomerase II inhibitors, taxanes are antimitotic agents. Taxanes bind to cell microtubules and impair cell division, which in turn promotes cell death.

Despite chemotherapy being an effective treatment for solid tumours, many patients progress to these drugs. In BC, molecular subtyping and the subsequent development of gene profile tests has demonstrated that certain BC subtypes do not benefit from chemotherapy agents (16).

I3.3 Radiotherapy

Like chemotherapy, radiotherapy can be also used to shrink the tumour volume prior to surgery, as well as reduce the risk of local relapse and as a palliative treatment. Radiotherapy is based on the use of controlled doses of high-energy radiation to induce DNA damage in tumour cells(17).

I3.4 Anti-hormone therapy

As described above, a high percentage of BCs are ER+. In this case, the therapeutic options for these patients include endocrine therapy, via either tamoxifen or aromatase inhibitors (AI)(18).

I3.5 Targeted therapies

BC molecular subtype characterization, in addition to the identification of BC drivers, has led to the development of specific molecular targeted therapies. Unlike classic treatments, targeted therapies tend to be less toxic for patients, and incorporating them into clinics has changed the history of many tumour malignancies. Particularly in HER2+ BCs, the identification of this oncogene has enabled the development of specific anti-HER2 targeted therapies(19).

HER2-POSITIVE BREAST CANCER

I4. HER2 – POSITIVE BREAST CANCER (HER2+ BC)

As mentioned before, 15-20% of BCs are classified as HER2-positive (HER2+)(19).
HER2 (also known as c-erbB2/neu) is a transmembrane tyrosine kinase receptor (TKR) encoded by the *ERBB2* gene, located at chromosome 17 (position 17q21)(20). It was discovered in 1981 by Shih et al. and first described as a transforming oncogene. Indeed, it was found to be homologous to the v-erbB (avian erythoblastosis virus) viral oncogene and the epidermal growth factor receptor (EGFR)(21). In 1987, Slamon et al. demonstrated that HER2 was amplified in 30% of human primary breast cancer (BC) tumours, pointing out its biological relevance in carcinogenesis (22).

I4.1 Biology of the HER family of receptors

The HER2 receptor belongs to the Human Epidermal Receptor (HER) family (also called the ErbB family), which contains 3 further members: HER1 (ErbB1 or EGFR), HER3 (ErbB3), and HER4 (ErbB4)(23).
HERs comprise three major regions, as depicted in **Figure 6:**

- The **extracellular amino-terminal domain (ECD)**, formed by four sub-domains (I-IV). Domains I and III are responsible for peptide binding. Domain II promotes direct receptor homo- or hetero-interaction, and domain IV is involved in inactive status conformation(24).
- The **hydrophobic transmembrane domain** (24).
- The **carboxy-terminal kinase domain** which consists of the juxtamembrane domain, tyrosine kinase, and the c-terminal tail (containing multiple phosphorylation sites(24).

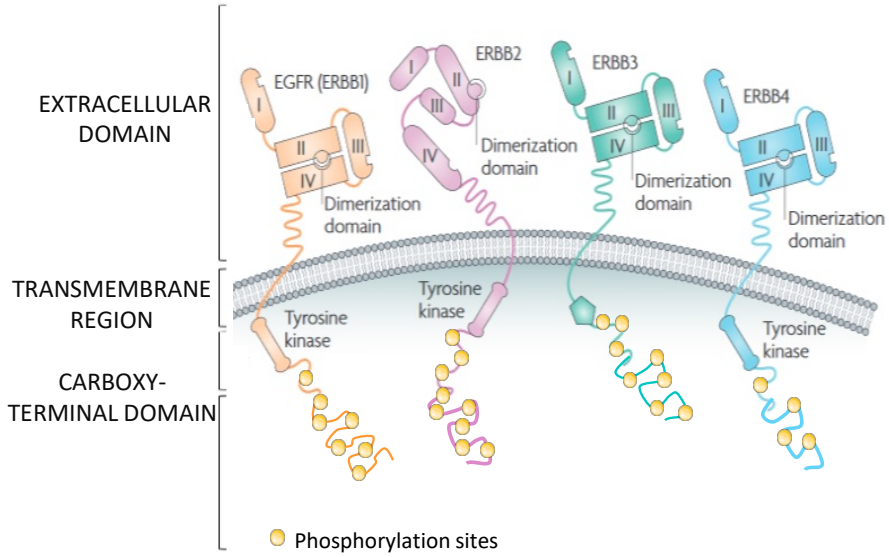


Figure 6. Structure of the HER family members. Adapted from Baselga et al., 2009.

Although all these family members share the same essential regions, the functional activity of each domain varies between them. EGFR and HER4 have functional tyrosine kinase domains and specific ligands. HER3, despite also having known ligands, lacks tyrosine kinase activity (it is incapable of binding adenosine triphosphate (ATP)). Being unable to undergo auto-phosphorylation after ligand binding, it only becomes activated when it dimerizes with other HERs(25).

HER2 deserves a special mention. Although it possesses tyrosine kinase activity, it is considered an orphan receptor. Studies of the HER2 crystal structure have demonstrated that no-ligand binds to this receptor(23,26).

In general, HER receptors exist in an inactive conformation, also called a tethered state. Upon ligand-binding, the folded structure changes, and the dimerization arm is exposed. This step is crucial for dimer formation and the functional activation of EGFR, HER3 and HER4. Subsequently, there is an asymmetrical interaction in the tyrosine kinase domains (the amino-terminal lobe of one receptor interacts with the carboxyl-terminal lobe of its partner)(25,27).

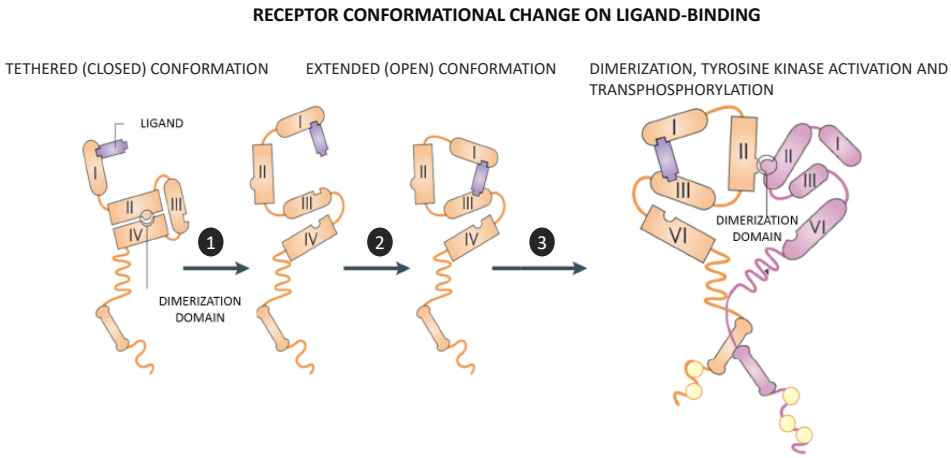


Figure 7. Conformational changes of the HER family members upon ligand binding and subsequent activation. Adapted from Baselga et al., 2009.

As mentioned, HER2 is always in an extended (open) state. This can be explained by the fact that there is no II-IV domain interaction in this receptor, meaning the dimerization loop in domain II is always exposed(23). Moreover, it has been described that although HER2 is unable to bind EGF-like ligands, when recruited into heterodimers, it strongly increases ligand-binding affinity(28).

All this makes HER2 a promiscuous receptor, and it is generally the preferred dimerization partner in this family(28,29).

I4.2 HER family ligands (ErbB family ligands)

As described above, with the exception of HER2, the ErbB family of receptors require ligand binding for subsequent activation. So far, 11 ligands of this family have been described. Epidermal Growth Factor (EGF) was the first to be reported, by Cohen et al. in 1975. Because of this, ligands from the HER family are also known as EGF-like ligands(30). HER family ligands can be classified into three groups:

- **EGF, Transforming growth factor alpha (TGF-α), amphiregulin (AR)** and **epigen (EPGN**). All these proteins bind exclusively to EGFR.
- **Betacellulin (BTC), heparin binding EGF (HB-EGF**) and **epiregulin (EREG)**. In this case, these proteins bind to either EGFR or HER4.
- **Neuregulins (NRGs).** These can be sub-classified into four types (NRG 1-4): NRG-1/2 bind to HER3; and NRG-3/4 bind to HER4.

ErbB ligands are expressed as single-pass integral precursor proteins. The structure of these immature forms contains: an extracellular component, a transmembrane segment, and a small intracellular region. Upon proteolytic cleavage by A desintegrin and metalloproteases proteins (ADAMs), the ErbB family of ligands can be released from the cell surface, acting as soluble growth factors in an autocrine and/or paracrine manner (31–33).

In particular, ErbB ligands have been reported to actively influence cancer biology and drug resistance. Studies on colorectal adenocarcinoma have reported that AREG and EREG correlate with poor response to cetuximab, an EGFR monoclonal antibody (mAb) inhibitor. Similarly, TGF-α and AREG paracrine signalling play a role in promoting *in vitro* resistance to anti-EGFR targeted therapy (34–36).

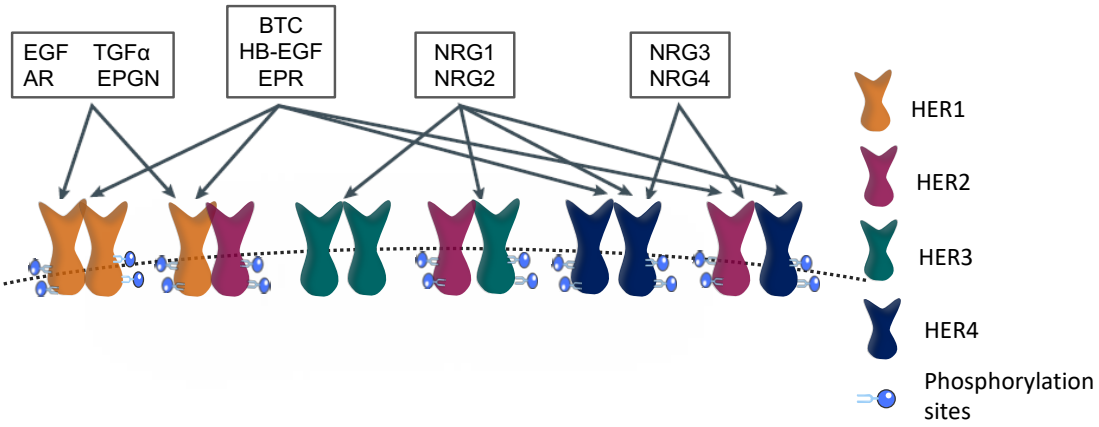


Figure 8. HER family receptors and ligands. Adapted from Hynes et al., 2005.

I4.3 HER2 signalling and regulation

Many pathways can be activated by HER2. Thus, HER2 signalling regulates many cellular processes, including cell proliferation and division, protein synthesis, cell death evasion, invasion, metastasis, and tumour vascularization, among others. Two major signalling cascades are strongly induced by HER2 activity, particularly when associated with HER3(37).

- **PI3K/AKT/mTOR pathway**

The PI3K/AKT/mTOR pathway controls many cellular functions, such as cell division, survival, growth, metabolism, angiogenesis, and migration, among other things. Indeed, this pathway is frequently dysregulated in cancer(38).

Several autocrine and paracrine molecules, like ErbB ligands, can initiate the PI3K cascade. Upon Tyrosine Kinase Receptor (TKR) activation, p85 mediates p110 translocation to the cell membrane. Once there, the p110 subunit phosphorylates and converts phospholipid 2 (PIP2) into PIP3. At this point, PTEN can act as a negative regulator of PI3K signalling by catalysing the conversion of PIP3 into PIP2. PIP3 in turn, fosters the recruitment of PDK1 and AKT to the membrane.

Subsequently, AKT is phosphorylated at the T308 and S473 sites. The latter enables full AKT activation(39–41).

AKT acts as a central node for many intracellular pathways and, in fact, is the main effector of the PI3K cascade. It is implicated in several processes, including cell survival by regulating apoptosis, cell cycle progression through GSK3β inhibition, and cell growth by activating the mTOR pathway. Regarding to the latest one, AKT regulates cell growth, activating the mTORC1 complex. In turn, this complex phosphorylates and activates S6 kinases (S6K) 1 and 2, which promote protein translation and ribosome biogenesis through the phosphorylation of ribosomal S6, eEF2 kinase, eIF4B and PDCD4. Moreover, the mTORC1 complex inactivates the 4E-binding protein (4EBP1), promoting translation of eIF4E. Using S6K and 4E-BP1 substrates, mTORC1 ultimately regulates protein synthesis(42,43).

Alterations in the PI3K pathway are often found in many solid tumours and these are largely associated with cancer. The PI3KCA gene is frequently mutated in many different types of cancer, including gastric, breast, prostate, colon, and endometrium cancer(44–46). Mutations are often located in the catalytic subunit p110α (especially exons 9 and 20, E542K and E545K, respectively). These mutations confer PI3K gain of function and AKT constitutive protein activation. Moreover, PTEN loss, usually through gene mutation or promoter hypermethylation, has also been linked with cancer development and progression(47,48).

- **MAPK/ERK pathway**

Also known as the Ras-Raf-MEK-ERK pathway, this cellular cascade controls many biological processes, such as cell proliferation, angiogenesis, migration, and invasion(37). HER2 activation results in the autophosphorylation of its tyrosine kinase domain. Subsequently, proteins with Src homology 2 (SH2)-containing domains bind to HER2 phosphorylated sites, and the signalling cascade is initiated. This, in turn, promotes the activation of the Small GTPase Ras family of proteins(49). There are three different Ras G proteins, each controlled by its GDP/GTP state.

Activated Ras binds to the MAPKKK of the Raf family (Raf-1, A-Raf and B-Raf), which then activates. MAPKKK directly phosphorylates and activates the MAPKK MEK1 and MEK2 proteins. These in turn, activate the MAPK ERK1/2 proteins through dual phosphorylation at their activation sites(50). ERK1/2 proteins are effector kinases that, once activated, phosphorylate diverse substrates in the cytosol and nucleus, controlling many biological processes(51).

In addition to the PI3K/AKT/mTOR pathway, the MAPK/ERK pathway is often dysregulated in tumorigenesis. In particular, the GTP-binding protein Ras is a potent oncogene(52). Indeed, B-Raf has attracted enormous interest, since the B-Raf gene has been found to be mutated in a high number of malignant melanomas, as well as other solid tumours, such as colon and ovarian cancers (53).

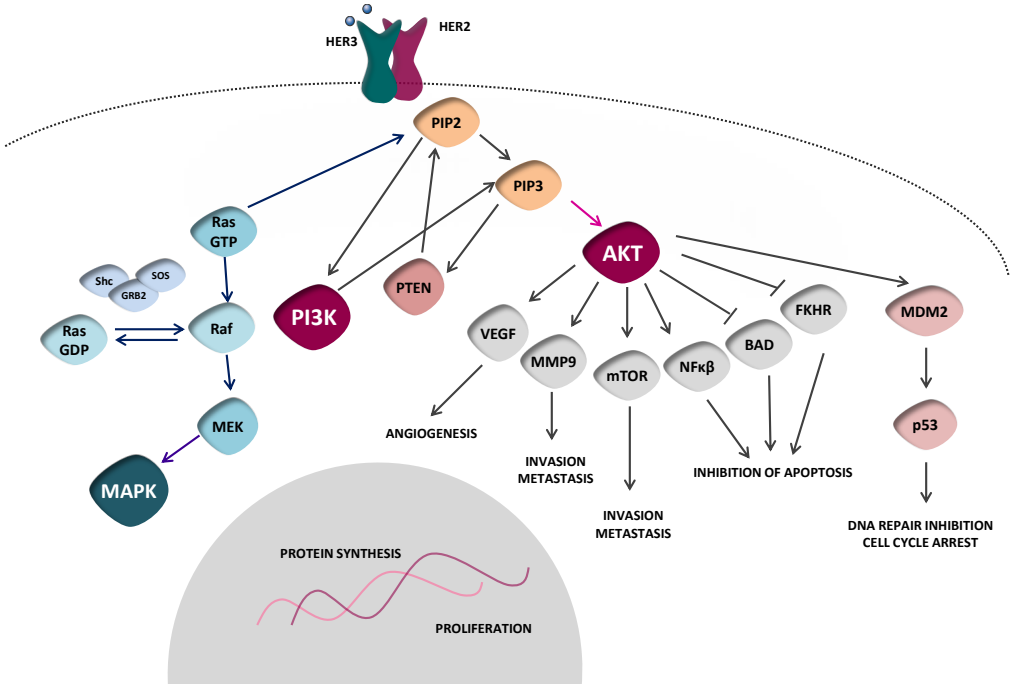


Figure 9. Signalling pathways and biological processes regulated by HER2 signalling, mainly through HER3. Adapted from Gringas et al., 2017.

I4.4 HER2 detection

HER2 overexpression and/or amplification accounts for 15-20% of BCs and has a prognostic, predictive, and therapeutic value(19). Because of this, HER2 status is routinely assessed in clinics. According to the 2018 ASCO/CAP guidelines, the methods for defining the status of HER2 in BC are Immunohistochemistry (IHC) and Fluorescence *In Situ* Hybridization (FISH)(54).

IHC evaluates HER2 expression on the tumour cell surface, classifying tumour samples with a score from 0 to 3+. The characteristics defining each score are given in **Figure 10.** Herceptest™ (Dako, Agilent Technologies) is one of the most commonly used IHC assays in routine diagnostic settings(54).

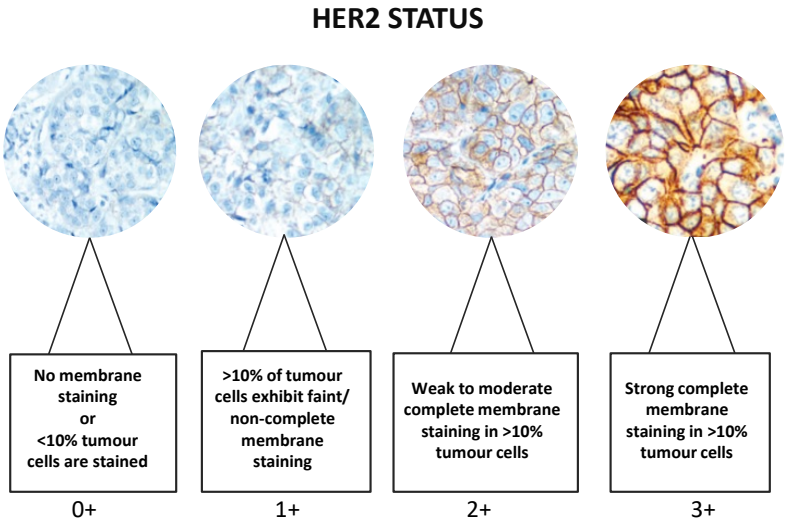


Figure 10. Illustrative examples of HER2 staining patterns for tissue scoring. Adapted from the breast cancer IHC portfolio from Roche.

In tumours scored as 2+, the HER2 status can be equivocal. In these cases, the FISH technique is needed to verify HER2 amplification. FISH determines the ratio between the gene copy number of *ERBB2* and the Chromosome 17 centromere (*CEP17)* within the nucleus, counting at least 20 tumour cells. If the ratio of *ERBB2/CEP17* is higher than 2.0, *ERBB2* is considered amplified, and the tumours are classified as HER2+ **(Figure 11)**(54)**.**

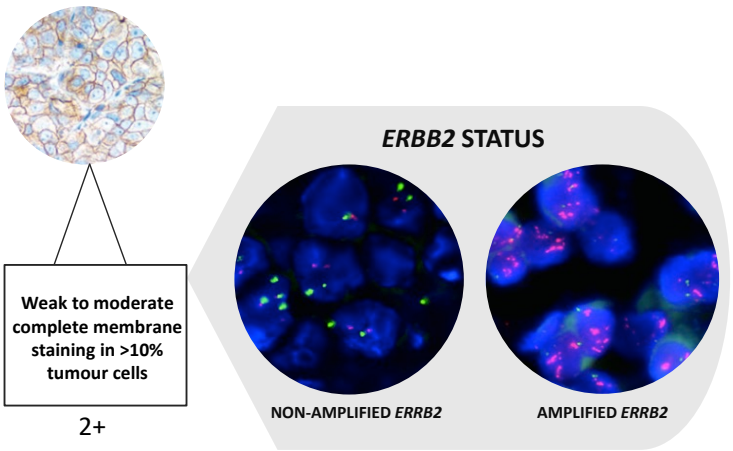


Figure 11. Example of HER2 amplification assessment using the FISH technique. Adapted from the breast cancer IHC portfolio from Roche.

I5. ANTI-HER2 TARGETED THERAPIES

Since the oncogenic role of HER2 was discovered, there has been intensive research into pharmacologically blocking it. These efforts crystalized almost two decades ago, when trastuzumab, the first specific anti-HER2 targeting agent, was developed. The introduction of trastuzumab into clinics led to outstanding improvements in patient outcomes(55). Despite its undeniable clinical benefit, many patients experienced relapse or non-response to this selective targeting agent. Due to this, over the past twenty years, further anti-HER2 targeted therapies have been developed. Currently, there are five FDA-approved HER2 targeting agents that are routinely used in the clinical practice; while others are still in the clinical-trial phase. This section involves a review of the main anti-HER2 targeted therapies.

I5.1 Monoclonal antibodies (mAb)

- **Trastuzumab**

Trastuzumab is a humanized murine monoclonal antibody (mAb) that targets domain IV of HER2 ECD, preventing it interacting with the transmembrane domain. However, it does not prevent dimerization with other HER receptors.

In vitro and *in vivo* preclinical studies showed potent antitumour effects in HER2+ cells(56–59). In addition, randomized clinical trials have demonstrated the clinical benefit of trastuzumab, in early stages, for HER2+ BCs. Currently, in combination with chemotherapy and/or other targeted therapies, it is the standard treatment for all HER2+ BC stages(19,55).

The reported functions of trastuzumab include: preventing ECD shedding of the truncated form of the receptor; HER2 receptor- and HER2-associated signalling downregulation; the inhibition of angiogenesis; and the activation of antibody-dependant cell mediated toxicity (ADCC) mechanisms.

Preventing ECD shedding from the truncated form, p95

Through proteolytic cleavage, the ECD of HER2 can be released from the cell surface, resulting in the production of the truncated membrane-bound fragment, p95. This p95 form is phosphorylated and retains signalling activity(60).
Studies led by Molina et al. demonstrate that trastuzumab can prevent the release of this truncated form by inhibiting metalloproteinase activity(61). Moreover, a decline in p95 HER2 levels in the serum of patients after treatment with trastuzumab, indirectly supports this hypothesis, since it has been associated with tumour response prediction and improved tumour progression-free survival(62)(63).

HER2 receptor-, HER2-associated signalling downregulation, and G1 arrest

Firstly, downregulation of the HER2 receptor by mediated-endocytosis has been reported in pre-clinical studies. However, this trastuzumab mechanism of action still remains controversial. On the one hand, Yarden et al. propose that antibodies like trastuzumab may form large antigen-antibody structures at the cell surface, which then collapse in the cytoplasm and undergo lysosomal degradation(64,65). On the other hand, studies led by Hommelgaard report a preferential association of HER2 with plasma membrane protrusions in a HER2+ BC cell line that makes HER2 resistant to internalisation processes(66).
Secondly, by inhibiting the PI3K/AKT and MAPK pathways, trastuzumab has been reported to inhibit cell proliferation, promote apoptosis, and induce cell cycle arrest(56,67–69). These effects may result from trastuzumab-mediated internalisation and degradation of the HER2 receptor. Nagata et al. also demonstrated that trastuzumab was effective at inhibiting PI3K signalling by reducing PTEN phosphorylation, through increasing PTEN localization in the membrane and its phosphatase activity. This resulted in rapid AKT dephosphorylation and the inhibition of cell proliferation(70).

With regard to cell cycle arrest, it has been described that in HER2+ BC cell lines trastuzumab induced G1 phase arrest *in vitro*. These effects are accompanied by an induction of the cyclin-dependent kinase (Cdk) inhibitor $p27^{Kip1}$/Cdk2 complex, arresting the cells in the G1 phase(71).

Inhibition of angiogenesis

Studies carried out using an engineered HER2-expressing mice model showed that trastuzumab induces normalization and regression of the tumour vasculature(72).

Moreover, in another study performed by Klos et al., immunodeficient mice were injected with HER2+ BC cells and treated with trastuzumab and paclitaxel either alone or in combination. Interestingly, a nice correlation between tumour response and reduced micro-vessel density was found in the combination arm. These observations may reflect that trastuzumab-inhibition of aberrant vasculature may enhance drug delivery to the tumour(73).

Immune-mediated response (ADCC)

In addition to being cytostatic, trastuzumab has been described as a cytotoxic agent due to its recruitment of immune effector cells, which trigger the ADCC response. ADCC is mainly mediated through the activation of natural killer (NK) cells, expressing the Fc gamma receptor, which can be bound by the Fc domain of trastuzumab. This event triggers the release of cytotoxic granules from effector cells that, in turn, induce the lysis of tumour cells bound to trastuzumab. This has been demonstrated in several xenograft models and in HER2+ BC patients(56,67,74–77).

- **Pertuzumab**

Complementary to trastuzumab, pertuzumab is a second-generation humanized mAb. It was developed in an attempt to improve pharmacological targeting of HER2 and overcome trastuzumab resistance(19,23). Pertuzumab binds to extracellular domain II of HER2, and thus inhibits ligand-dependent HER2-dimerization (mainly through HER3). As a result, the associated signalling of downstream intracellular pathways, such as PI3K/AKT and MAPKs is reduced. As a mAb, it also presents ADCC activity(78–80).

In particular, preclinical studies have reported that pertuzumab is more effective than trastuzumab at inhibiting Neuregulin- (NRG), also called Heregulin- (HRG) mediated morphogenesis in breast cancer cell lines(80,81). Moreover, in studies performed by Agus et al., pertuzumab was better at disrupting NRG-induced signalling(79). Similarly, breast tumours developed from the TNBC cell line MDA-MB-175, which depend on a paracrine loop of NRG, showed pertuzumab response *in vitro*, but no response to trastuzumab(82).

Xenograft data also supports the *in vitro* studies. Synergistic effects have been observed when pertuzumab is combined with other agents, particularly trastuzumab. These effects are superior to those exerted by pertuzumab alone(83). Sustained complete responses have been observed in breast xenograft models when trastuzumab and pertuzumab are combined(81,84,85). Moreover, drug combination has also been shown to be effective on tumours progressing to trastuzumab. Additionally, these studies demonstrate the sustained prevention (<99 days) of metastatic tumours spreading to the lungs and liver; these effects have not been observed with either of these anti-HER2 agents on their own(84).

Interestingly, pertuzumab has also shown preclinical activity, inhibiting tumour growth independently from the level of HER2 expression(84). In xenograft models of MCF7 cells with low HER2 expression levels, pertuzumab causes inhibition of tumour growth(79). This suggests that pertuzumab, either alone or in combination, may have potential therapeutic benefit in non-HER2+ tumours.

In clinical practice, when combined with trastuzumab and chemotherapy, pertuzumab has shown antitumour activity in both metastatic and neoadjuvant settings. Because of this, together with trastuzumab and chemotherapy, it is currently approved in the neoadjuvant and as a first-line therapy in the metastatic settings of HER2+ BC(85–88).

I5.2 Antibody-drug conjugate (ADC)

Trastuzumab-emtansine (T-DM1) is an antibody-drug conjugate (ADC) comprising the mAb trastuzumab covalently linked with DM1, an antimitotic agent. DM1 is a derivative of maytansine, a potent antimitotic drug. The T-DM1 mechanism of action retains trastuzumab-related antitumour effects in addition to those associated with DM1 catabolites. Once T-DM1 binds to HER2+ tumour cells, the HER2-T-DM1 complexes are endocytosed and processed in lysosomes. This results in the cleavage of T-DM1 and the intracellular release of the active catabolite Lys-MCC-DM1. Active DM1 binds to the beta subunit of tubulin, modifying its assembly properties. As a result, the formation of the mitotic spindle necessary for chromosome segregation during mitosis is impaired. The consequence is mitotic arrest, cell collapse, and cell death(89–91).

T-DM1 was the first antibody drug conjugated for HER2+ BC and is currently the standard second-line treatment for HER2+ metastatic BC patients who have progressed to trastuzumab-containing regimens(92,93).

I5.3 Small molecule tyrosine kinase inhibitors

- **Lapatinib**

Lapatinib is a dual EGFR/HER2 small tyrosine kinase inhibitor. By mimicking ATP, it reversibly binds to the ATP binding sites of both receptors, blocking receptor phosphorylation and activation(94).

In clinical settings, lapatinib has shown antitumour activity and is currently approved for HER2+ BC metastasis after T-DM1 progression, in combination with capecitabine (a chemotherapeutic agent)(95).

- **Neratinib**

Neratinib is another dual EGFR/HER2 small tyrosine kinase inhibitor. However, it irreversibly binds to both receptors, reducing auto-phosphorylation and subsequent downstream signalling pathway activation. Neratinib is currently approved for extended adjuvant treatment in early-HER2+ BC stages after trastuzumab-based therapies(96–98).

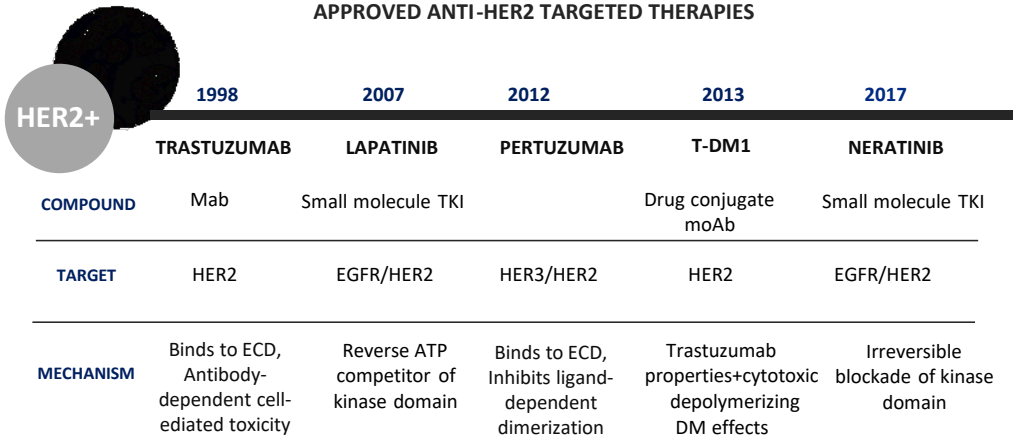


Figure 12. Approved anti-HER2 agents for HER2+ BC. Adapted from Yan et al., 2014.

I6. MECHANISMS OF RESISTANCE TO ANTI-HER2 TARGETED THERAPIES

As mentioned, trastuzumab was the first anti-HER2 targeting agent. Subsequently, a second-generation of anti-HER2 targeted therapies was developed. Although this involved an enormous clinical benefit, many patients presented *de novo* or acquired resistance mechanisms. The main reported mechanisms of resistance to trastuzumab, as well as to other anti-HER2 targeted agents, are reviewed below.

I6.1 Mechanisms of resistance to trastuzumab

- **Impaired trastuzumab-HER2 binding**

As previously mentioned, the p95 form of HER2 is phosphorylated and constitutively active. Studies led by Scaltriti revealed a strong correlation between breast tumours expressing the p95 form and trastuzumab resistance(60,69).

In line with this, another mechanism is membrane-associated glycoprotein mucine-4 (MUC4) interaction with HER2. This has been reported to increase HER2 phosphorylation and physically prevent trastuzumab binding to the receptor(99–101).

Additionally, impaired ADCC response due to the FCyR binding capacity has been linked to trastuzumab resistance. Musolino et al. demonstrated that FCyRIII polymorphism modulates trastuzumab response in HER2+ BC patients(102).

- **Alternative HER2 signalling mediated by ErbB-family receptors and ligands**

The activation of alternative HER family members and their downstream-associated signalling has been linked with trastuzumab resistance in HER2+ BC(56). Narayan et al. found that EGFR and HER3 expression was greater in HER2+ BC cells after long-term exposure to trastuzumab(103).

In line with this, our group has recently described that, in a model of trastuzumab-resistant HER2+ gastric cancer (GC) cells, trastuzumab treatment induced compensatory upregulation of HER3. Interestingly, this acquired resistance involved greater basal activation of the downstream kinase SRC, as well as the MAPK and PI3K/AKT/mTOR signalling pathways (A. Sampera *et al.*Molecular Cancer Therapeutics. In press.)
In addition, upregulation of ErbB-family ligands has also been linked to trastuzumab resistance in preclinical models; increased expression of TGF-α conferred trastuzumab resistance in breast cancer cells(104). Moreover, treatment with EGF and NRG-1 impaired the effects of trastuzumab in HER2+ BC cell lines(105). In line with this, preclinical trastuzumab-resistant BC and GC models upregulate the mRNA expression of EGF, TGF-α HB-EGF, and NRG-1(104,106).

In accordance with this, in our study on trastuzumab-resistance in HER2+ GC, we also observed that the expression of ErbB-family ligands, EGF, AREG, and HB-EGF were increased in the trastuzumab-resistance-acquired cells (A. Sampera *et al.* Molecular Cancer Therapeutics. In press.).

- **Alternative HER2 signalling mediated by other TKRs**

Other tyrosine kinase receptors, such as insulin-like growth factor receptor (IGF1R) and hepatocyte growth factor receptor (HGFR or c-Met) have been found to be alternatively activated in trastuzumab-resistant preclinical models(107–109). In line with this, Nahta et al. showed that IGF1R, through HER2 heterodimerization can induce HER2 phosphorylation and contribute to trastuzumab resistance in HER2+ BC cells(110).

Furthermore, preclinical studies from Shattuck et al. show that the c-MET receptor is also upregulated during trastuzumab treatment in HER2+ BC cells(109).

- **Aberrant downstream signalling activation**

Several mechanisms of aberrant downstream signalling activation have been elucidated from preclinical studies. Firstly, increased PI3K/AKT activity has been found in trastuzumab-resistant cells(56,111). PI3K activation can be caused by hyperactivating PIK3CA mutations, reducing PTEN expression, or increasing AKT phosphorylation(70,112,113). Indeed, Berns et al. found that, in a cohort of BC patients, PIK3CA mutations or low PTEN expression was associated with poor prognosis after trastuzumab treatment(112). Moreover, Carpten et al. described a point mutation in AKT (E17K) in breast, colorectal, and ovarian tumour samples(114).

Secondly, in HER2+ GC, a positive-feedback loop of STAT3 activation was found to promote upregulation of fibronectin (FN), EGF, and interleukin-6 (IL-6), accompanied by increased MUC1 and MUC4 expression, which promoted trastuzumab resistance(115).
Thirdly, a study by Nogueria et al., described the fact that RET and FGFR2 pathways promote *in vitro* and *in vivo* resistance to trastuzumab and lapatinib in HER2+ BC pre-clinical models *(Fernandez-Nogueira et al., Clin Cancer Res, second revision).*

Finally, a recently published work from Diaz-Rodríguez et al. demonstrates that trastuzumab-resistant cell lines had a deregulation in cell death pathways. Specifically, alterations in the response to the death factor TRAIL, and in the TRAIL-related components of the pathway were observed in both these resistant- cell lines as well as in HER2+ BC patient samples(116).

I6.2 Mechanisms of resistance to pertuzumab

Until to date, no mechanisms underlying pertuzumab resistance, even in combination therapy, have yet been described.

I6.3 Mechanisms of resistance to T-DM1

To date, few reported T-DM1 resistance mechanisms have been described. On the one hand, it has been proposed that trastuzumab-related mechanisms of resistance may also account for T-DM1 unresponsiveness(89). On the other, defects in internalisation and cellular trafficking, as well as alteration in extrusion bombs or tubulin β structures, may be linked with T-DM1 resistance. Moreover, a work performed by Rios-Luci et al. has demonstrated that resistance to T-DM1 is based on reduced lysosomal proteolytic activity(117).

Specifically, our group has defined a novel mechanism of T-DM1 resistance. We have observed that defective cyclin B1 induction by T-DM1 promotes resistance in T-DM1 acquired resistance cell lines. Defective cyclin B1 induction has also been observed in *in vivo* models and in a cohort of HER2+ BC patient samples. Indeed, we are currently studying a potential pharmacodynamic assay to test cyclin B1 as a biomarker for early-patients T-DM1 response screening (91).

I7. BEYOND HER2 POSITIVITY: THE ABSENCE OF ADDITIONAL PREDICTIVE OR PROGNOSTIC BIOMARKERS IN CLINICAL PRACTICE

The development of high-throughput platforms over recent years has allowed better stratification and characterization of breast cancer patients at a molecular level. HER2 positivity defines a breast cancer molecular subtype that is aggressive and has a poor prognosis. Indeed, along with hormone receptors, HER2 is the most important prognostic and predictive marker for breast cancer(19).
The development of effective strategies that pharmacologically inhibit HER2 has dramatically changed the natural course of this disease. However, resistance to anti-HER2 agents occurs in both metastatic and adjuvant settings, prompting intensive research(25). Nevertheless, most of the aforementioned potential biomarkers derived from preclinical studies have not translated into the clinical setting. Thus, HER2 positivity itself still remains the unique criteria for treating patients with anti-HER2 targeted therapies(19).

There are many reasons for this. On the one hand, the absence of specific biomarkers within HER2+ BC can be explained by the fact that HER2 is just part of an intricate biological network that is yet to be fully understood. Alternative forms of HER2, as well as HER2 mutations, have been evaluated. As previously mentioned, preclinical data on p95HER2 suggests that this aberrant form can compete with HER2 in binding with anti-HER2 antibodies and, therefore, promote trastuzumab resistance. However, high levels of p95HER2 in serum have been associated with a poor prognosis in the metastatic setting, whereas the opposite correlation has been found in early stage HER2+ BC(19).

The downstream effectors of HER2 associated signalling have also been evaluated. Alterations in the PI3K pathway have been associated with trastuzumab resistance in preclinical and early clinical studies. However, this data is still controversial(19).

Moreover, the HER family members, as well as most of the ErbB family ligands, have been assessed in clinical settings. In this regard, EGFR and HER3 expression has been evaluated as predicting trastuzumab resistance in retrospective, single cohort studies, but without consistent results. In the CLEOPATRA trials, patients with higher HER3 mRNA expression levels had a better prognosis, but these did not predict any benefit with regard to pertuzumab, a HER2-HER3 dimerization inhibitor; EGFR mRNA expression levels, showed neither prognostic nor predictive value(58,63,118–121).

Since HER2 is a promiscuous receptor, it can dimerize with other tyrosine kinase receptors, such as Estrogen Receptor (ER) and Insulin Growth Factor 1 Receptor (IGF1R). Indeed, 10-20% of HER2+ tumours co-express IGF1R, although the active form of this receptor is found in only around half these cases. Although more data is needed, few studies have yet reported that increased IGF1R activity may correlate with trastuzumab resistance(19).

Furthermore, the heterogeneity underlying HER2 *per se* has been reported as another potential issue. This was highlighted in 2016, in work by Ferrari et al, when 64 HER2+ tumour samples were subjected to whole genome sequencing. Four different subgroups were identified based on distinct genome aberrations (somatic mutations, copy-number changes, and structural variations). The results suggested that, although clinically classified by HER2 gene amplification, these tumours were distributed throughout the luminal–basal breast cancer spectrum rather than standing apart(122).

The fact that all the biomarkers previously described could not be translated into clinical practice, as well as the increasing evidence of the role of the tumour microenvironment (TME) and the interplay with the immune system, have opened new research windows. In this vein, over the past few years, some studies have assessed the role of tumour-infiltrating lymphocytes (TILs) in anti-HER2 therapy response. Indeed, a recent study led by Dr. Muntasell in collaboration with our group has demonstrated that numbers of circulating $CD57^+$ NK cells inversely correlate with pathologic complete response (pCR) to HER2-specific antibody treatment in patients with primary breast cancer, independently of age, traditional clinical-pathologic factors, and CD16A 158F/V genotype(123).

In addition to this, other studies have recently reported that the study of the tumour microenvironment (TME) could be an opportunity to uncover potential mechanisms of response or resistance to cancer therapies.
Cancer cells gain resistance through a variety of mechanisms in both primary and metastatic sites, involving cell intrinsic and extrinsic factors, but the latter often remains overlooked. Mounting evidence suggests critical roles played by the tumour microenvironment (TME) in multiple aspects of cancer progression particularly therapeutic resistance(124,125). The TME decreases drug penetration, confers proliferative and antiapoptotic advantages to surviving cells, facilitates resistance without causing genetic mutations and epigenetic changes, collectively modifying disease modality and distorting clinical indices. Recent studies have set the baseline for future investigation on the intricate relationship between cancer resistance and the TME in pathological backgrounds(125). Thanks to this, it is expected that, in the coming years, potential biomarkers will arise from these studies.

In line with this, a study performed by Nogueira *et al*, in which we collaborated, reports that CAFs promote resistance to anti-HER2 targeted therapies through a novel paracrine HER2-FGFR2 activation mechanisms (Fernandez-Nogueira *et al.,* Clin Cancer Res, second revision).

I8. THE ROLE OF HER3 IN HER2+ BC AND THERAPY RESISTANCE

Unlike other HER family members, HER3 is the only one with no intrinsic catalytic function(25). However, recently, there has been increasing evidence found suggesting that its non-catalytic function is critically important in many cancers driven by other HER receptors(126). Indeed, enhanced expression and/or amplification of this receptor has been reported in many solid tumours, including ovarian, gastric, and breast cancer(127–129).

In line with this, studies by Xue et al. show that HER3 signalling promotes tumour cell motility and intravasation in breast cancer lung metastasis, as well as the fact that a HER3-incRNA axis was implicated in bone metastasis from breast tumours(130,131). Furthermore, elevated HER3 expression and activity was also found in brain breast tumour metastasis(132).

HER2+ BC commonly includes co-expression of HER3. Experiments carried out on erbB2/neu-transgenic mice have shown that the expression of endogenous HER3 promotes the formation of mammary tumours(133). Although HER3 has no catalytic activity, receptor hetero-dimerization results in a potent activation of mitogenic signalling, mainly through the PI3K/AKT pathway. Indeed, among all possible homo- or hetero-conformations, the HER2/HER3-containing heterodimers foster the most potent oncogenic activity. Thus, HER3 directly influences the neoplastic transformation of HER2 and orchestrates tumour cell growth and the proliferation of HER2+ BC cells(25).

HER3 has also been implicated in cancer drug therapy failure, involving chemotherapeutic and targeted therapy agents. Several studies have indicated that compensatory upregulation of HER3 along with sustained PI3K/AKT activation is a mechanism of EGFR-targeted therapy resistance (25,79,126,134–137). Studies by Liu et al. demonstrate that in Luminal B BCs, HER2/HER3 structures activate αER principally through the aforementioned PI3K/AKT and MEK/MAPK pathways, impairing tamoxifen efficacy(138).

As an attempt to pharmacologically inhibit HER3 activity, several anti-HER3 targeted agents are currently in clinical development, some of which are already being tested in phase I/II clinical trials (e.g., seribantumab and patritumab). In addition, some bi-specific antibodies, targeting either HER2-HER3 (MM-111) or HER3-IGF1R (MM-141), are also in phase I and II clinical trials. In particular, the latter has shown to be efficient at binding to HER3 and IGF-IR, and preventing NRG- and IFG- associated signalling(126).

Besides this, the potential prognostic value of HER3 protein expression has also been evaluated. However, despite the fact that elevated HER3 expression has been found in 50-70% of BCs(139–141), its role as a prognostic marker remains controversial(126,139,140). On the one hand, HER3 protein expression seems to correlate with the worst clinical features in relation to tumour size, metastasis, and recurrence(142,143). In line with this, some studies have suggested that HER3 expression is associated with a poorer clinical outcome in these patients(127,144–147). On the other hand, different studies have reported the contrary role for HER3 expression, as a marker of good prognosis(148,149).

Multiple factors may explain these controversial findings. Firstly, the fact that there is no standardised method for detecting HER3 expression in clinical samples(150). Secondly, that breast cancer is a heterogeneous disease composed by different intrinsic subtypes that dramatically influence in the biological behaviour of these tumours. Therefore, it may be reasonable that the influence of HER3 on patient's survival may be diverse depending on the molecular context of this tumours(126,150). Thirdly, the influence of the HER3 ligand-Neuregulin (NRG, also called Heregulin or HRG). In this direction, NRG is vital to induce activation of the HER3 signalling and aberrant production and/or maturation will affect on tumour proliferation and survival. Therefore, it is reasonable that the differential expression of this ligand could also impact on the clinical value of HER3.

I9. NRGs, HER3 LIGANDS: BIOLOGICAL IMPLICATIONS IN CANCER

The extreme importance of HER3 in carcinogenesis cannot be understood without taking into consideration the family of Neuregulins (NRGs), the HER3 ligands. Indeed, the role of these proteins in solid tumours and therapy resistance has attracted substantial interest over recent years(151).

As described in Section I.4.2, NRGs represent the largest subgroup of proteins from the EGF growth factor family. Also known as Heregulins, (HRGs), they were discovered in 1992 by Peles et al.: in an attempt to find specific HER2 ligands, a 44 kDa protein was isolated and cloned from a RAS-transformed fibroblast from a rat. This protein was capable of inducing phosphorylation of HER2/p185neu and differentiation of human breast cancer cells(152). Almost at the same time, another group purified and cloned a 45 KDa protein from a human cancer cell line. This protein induced HER2/p185neu phosphorylation and was named HRG(153).

It was subsequently reported that these proteins were not capable of stimulating phosphorylation in the tyrosine kinase domain of HER2 in fibroblast cells. Instead, NRGs demonstrated capacity to interact with HER3 and HER4(154,155).

I9.1 Classification, structure and biological functions of NRGs

NRGs are signalling proteins that participate in cell-cell interactions in the nervous system, heart, and breast, as well as other organs. They are codified by four different genes: NRG 1-4. At least 30 isoforms have been reported, most generated by alternative splicing mechanisms(156).

The NRG subtypes differentially bind to the HER family of receptors. Specifically, NRG-1 and NRG-2 bind and activate HER3 and HER4. However, NRG-1 binds to HER3 with a higher affinity than NRG-2. NRG-3 and NRG-4 exclusively bind to HER4(157).

These proteins are commonly synthesized as membrane-bound precursor forms; generally known as proNRGs(31,158). The proNRG structure has three different domains:

- The **C-terminal intracellular part**: variable in terms of length across the different subtypes. It has been proposed that it may be important in anchoring proNRG to the plasma membrane.
- The **transmembrane region:** a hydrophobic domain.
- The **N-terminal extracellular domain**: this region contains the EGF-like module as well as the Ig-like or kringle-like modules.

The EGF-like domain is essential for HER binding and activation. Differences in the EGF-like domain have led to the subdivision of the NRG-1-4 subtypes into α- and β- isoforms. Of note, β- isoforms generally display a higher receptor binding affinity than α- isoforms and, thus, are associated with more potent signalling activity(159).

The role of Ig-like domains remains controversial. Some studies have reported that Ig-like domains may act by favouring the interaction of soluble NRG with its cognate receptors(160). Others in contrast, have suggested that this domain may act by inducing receptor downregulation to attenuate NRG-associated signalling(161).

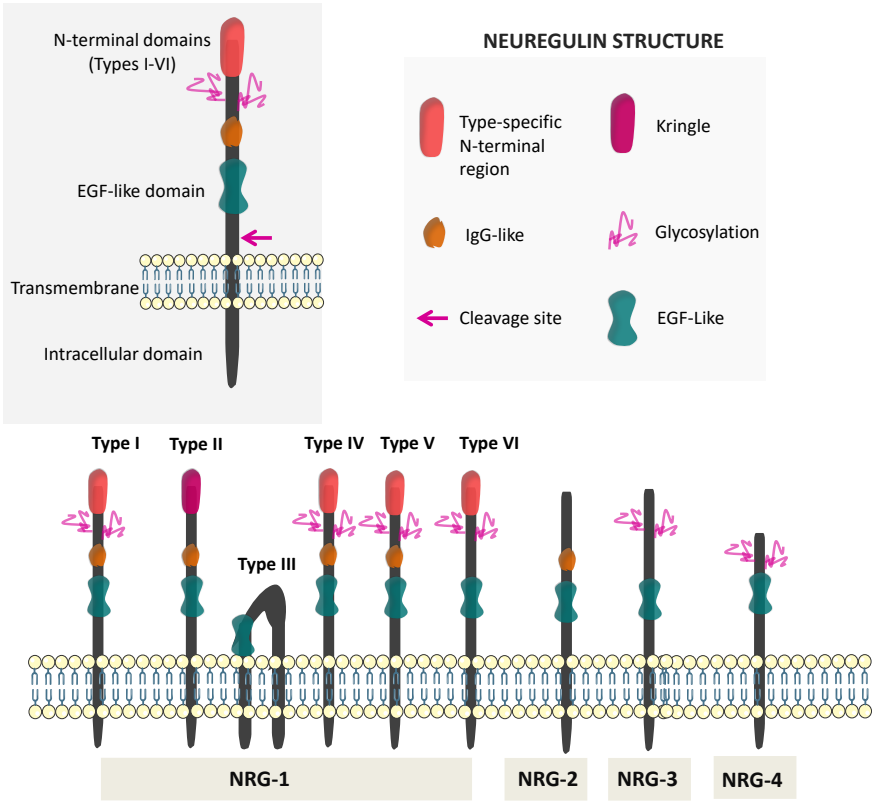


Figure 13. Neuregulins types and structure. Adapted from Gumà et al., 2010.

NRGs are commonly single-pass proteins that are anchored to the cell membrane in a precursor state. Once at the plasma membrane, they may remain there or undergo proteolytic processing and be solubilized. ADAM proteins can process them, and once released to the extracellular space, they can act in an autocrine or paracrine manner, as well as travelling to distant tissues(151).

In particular, two proteases have been implicated in the shedding mechanisms of pro-NRGs. The first is the tumour necrosis factor-a converting enzyme (TACE) also named ADAM17(156). Studies performed on TACE-deficient cells have shown that the shedding of the isoform pro-NRGa2c is impaired in these cells(162). Moreover, the expression of wild-type (WT) TACE in these cells results in pro-NRG protein cleavage.

In line with these, another protease, ADAM19/meltrin-h has been reported to play a role in NRG membrane-bound shedding(163).

Neuregulins are expressed mainly by cells with an endothelial, mesenchymal, or neuronal origin. They are critical in both development and adult tissue homeostasis. The role of NRGs in the mammary gland deserves a special mention: several studies carried out in mice models have reported that NRG-1 is involved in mammary gland formation and differentiation(164).

In summary, NRGs are implicated in the proliferation, differentiation, migration, and survival of many different cell types(31). However, NRGs have also been reported to play a role in the development and/or progression of certain types of human cancers(151).

I9.2 NRGs and cancer

As previously mentioned, the dysregulation of the HER signalling pathway is involved in many solid tumours, including breast, ovarian, and lung cancers(151). This phenomenon can be explained by ErbB gene amplification, the gain of function/mutations, transactivation via other receptors, or by the presence of autocrine or paracrine loops induced by ErbB family ligands(165).

In particular, the role of NRGs in cancer has garnered research interest in recent years. In colorectal cancer, HER2/HER3 heterodimers and NRG have been reported to promote resistance to cetuximab (an EGFR inhibitor) in preclinical models(156). In melanoma, too, NRG-1 has been found to promote resistance to vemurafenib (a BRAF inhibitor)(166).
In non-small cell lung cancer (NSCLC), upregulation of HER2/HER3 expression has also been reported in resected tumours from these patients(167). However, no clinical association of NRG expression and NSCLC prognosis has been described(168). Also in lung cancer, gene fusions involving NRG-1 have been identified as drivers of these malignancies(169).

In breast cancer, NRG-1 expression has been reported to account for 30% of tumours(151). Furthermore, *in vitro* and *in vivo* studies suggest that NRGs, especially NRG-1, are involved in inducing tumorigenesis and BC cell metastasis. Studies by Atlas et al. have demonstrated that overexpression of NRGs in the mammary tissue promotes adenocarcinoma formation in mice. Moreover, these studies also show that NRGs enhance the metastatic capacity of cancer cells *in vivo*(170). In addition, Tsai et al. report that the downregulation of NRG-1 by antisense oligonucleotides reduces tumorigenesis and BC cell metastasis in *in vitro* and *in vivo* models(171).

In line with this, studies performed by Seoane et al. have revealed that NRG-1 expression is associated with a worse outcome in a subset of BC patients. Indeed, they were able to determine that collagenase 3 facilitates the dissemination of tumour cells through NRG-1 signalling(172).

Besides this, NRGs have also been reported to play a role in mediating BC cell response or resistance to various drug agents. Studies by Leung et al. have shown that in HER2+ BC, exogenous NRG-1 partially rescued cells against lapatinib treatment(173). Interestingly, in other studies performed by Phillips et al., exogenous NRG-1β reduced *in vitro* cytotoxic effects of T-DM1 in a subset of HER2+ BC cells(82).

Taking all of this into account, the preclinical data supports the role of NRGs in cancer generation/progression and therapy resistance. However, when translated into a clinical setting, the predictive or prognostic value of NRG-1 remains controversial. Whereas in some tumours the expression of NRG proteins has been associated with a poor prognosis, in others, certain isoforms have been correlated with better clinical outcomes(174).

Three main issues may explain this, at least in part: firstly, the inherent complexity of the neuregulin genes and the multiple splicing isoforms; secondly, the fact that the expression and distribution of NRGs in solid tumours is still not well clarified; thirdly, there is a lack of reliable and useful antibodies for testing tissue specimens for neuregulin expression(174).

In summary, despite the fact that many aspects of NRGs are still poorly understood, their contribution to several solid malignancies is undeniable. Although challenging, it is expected that in the future, all these preclinical findings will translate into effective therapies. In line with this, some NRG-targeting agents are being developed. These mainly consist of anti-metalloproteinase agents and NRG-fusion proteins. On the one hand, two specific ADAM17 metalloproteinase inhibitors (INCB3619 and D1(A12)) have been generated. These have shown preclinical efficacy in inhibiting HER3 signalling in gefitinib-resistant NSCLC cell lines, as well as in reducing cell proliferation and motility in head and neck squamous cell carcinoma (HNSCC) and in ovarian cancer cells *in vivo*(156). On the other hand, chimeric NRG-toxic fusion proteins have been demonstrated to have cytotoxic activity in BC cell lines(156).

In particular, in a work published last year, Zuo et al. engineered T cells with a CAR consisting of the extracellular domain of HRG-1β. They showed that the HRG1β-CAR was effective at killing HER2+ BC cells *in vitro* and in xenograft *in vivo* models. Thus, these authors suggested that HRG1β-based CAR-T cells effectively target BCs driven by HER family members and that this fact may be the basis of a novel strategy for overcoming resistance to anti-HER2 targeted agents(175).

THE TUMOUR MICROENVIRONMENT

I10. THE ROLE OF TUMOUR STROMA IN BREAST CANCER

Classically, cancer research has focused on the intrinsic properties of tumour cells. However, and especially over recent years, a deeper and more complex perspective has been sought. Tumours have come to be understood as complex tissues composed of multiple cell types, and it has become evident that, while genetic and epigenetic changes in epithelial cells are essential in tumour initiation, the surrounding cell populations and the interplay with these also orchestrates the features of a tumour(176).

I10.1 Breast stroma: from a protective- to permissive-tumour tissue

The mammary gland is a complex tissue. Epithelial cells are embedded in a matrix of stromal cells that tightly control their proliferation, survival, and differentiation(177).
Adult homeostasis involves hormones, immune cell surveillance, extracellular matrices, stromal cells, and mechanical forces. As a dynamic tissue, the mammary gland undergoes architectural and biological changes over its lifetime(178).

It is, therefore, reasonable that in the breast, cellular processes are governed by regulatory programmes that maintain tissue balance. The breast microenvironment plays a crucial role in this by providing essential signalling for tissue architecture maintenance, to inhibit cell growth, and supress cellular aberrations(179).

However, this microenvironment can be a double-edged sword. When incorrect signals disrupt tissue homeostasis, aberrant stroma may become a permissive niche allowing tumour growth(179,180).

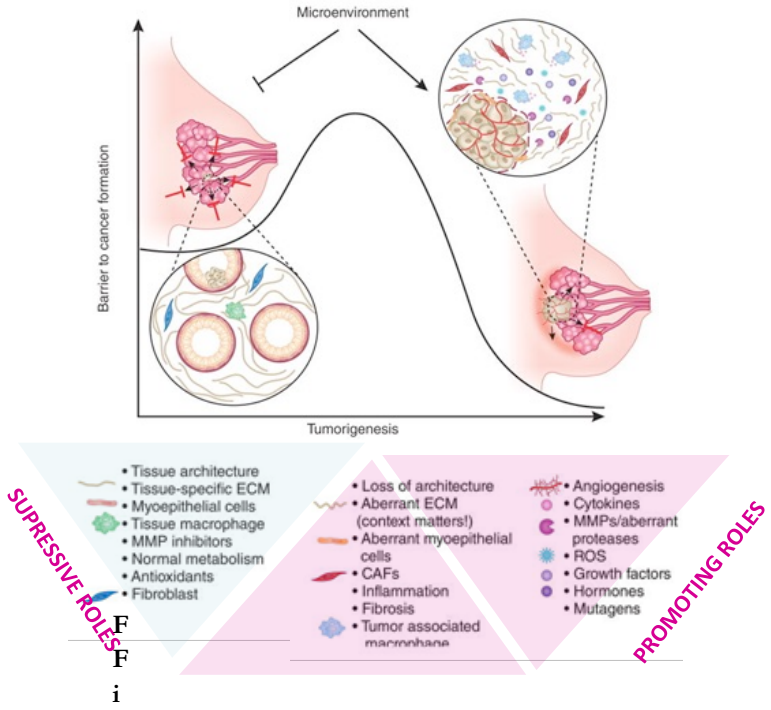


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Figure. 14. Changes in the breast microenvironment from tissue homeostasis to tumorigenesis. Modified from Bissell et al., 2011.

I10.2 The role of breast stroma in tumour promotion

The concept of the tumour microenvironment was first defined by Stephen Paget in 1889 in the context of the seed and soil hypothesis. Paget analyzed more than 700 data from women who had died from breast cancer and realized that metastases were not randomly distributed. Paget suggested that cancer cell sprouts in distant organs (seeds) were based on the tissue microenvironment of that tissue (soil)(181).

These studies were the basis for subsequent observations on the role the tissue microenvironment plays in tumour initiation and promotion. In the latest 1980s, Dvorak defined tumours as "wounds that do not heal". In that definition, he referred to the fact that all solid tumours, regardless of

their site of origin require stroma if they are to grow beyond a minimal size of 1-2 mm(182).

Almost two decades later, it has become evident that tumour development is an intricate process in which bidirectional crosstalk between epithelial cells and their surrounding microenvironment plays a critical role(178,183).

In breast cancer, studies led by Boyd et al. (2007) emphasized the link between stromal biology and tumour progression. In this context, they described the fact that women with mammographically dense breasts had a 2 to 6-fold increase in the risk of developing breast cancer. They associated breast density with an increase in cellularity, as well as increased concentrations of collagen in breast stroma(184).
In line with this, an innovative study led by Allinen et al. isolated single cells of normal breast tissue, DCIS, and invasive carcinoma, showing that all the cell types underwent extensive changes in gene expression during cancer progression. Indeed, the greatest differences were found in the myoepithelial cells when analysing the genes that are differentially expressed between normal breast tissue and DCIS. This highlights the dramatic changes that also occur in the tumour microenvironment during tumorigenesis(185).

I10.3 Tumour breast stroma components and their role in tumorigenesis

As mentioned previously, normal breast tissue is highly-structured and complex. The epithelial parenchyma is embedded in the myoepithelium, separated from the surrounding stroma by an intact basement membrane (BM). However, in the process of tumorigenesis, this hierarchical organization is dysregulated(186). In addition to the transformation of epithelial cells, the myoepithelial cells and BM are often lost. At this point, the stroma is subjected to profound structural and functional changes. Resident fibroblasts are converted into myofibroblasts and other stromal cells may be recruited from the vasculature to promote direct epithelial-stromal interactions. This results in an invasive breast cancer, in which aberrant tumour cells are surrounded by desmoplastic and fully activated stroma(180).

The main components of this reactive stroma are detailed below.

- **EXTRACELLULAR MATRIX (ECM)**

The ECM is a complex molecular network comprising numerous structural proteins such as collagens, fibronectins, laminins, and glycans, among others. As a physical scaffold, the ECM has many different functions, including allowing cell-cell interactions and providing survival and differentiation signals. Moreover, as a barrier, the ECM also allows or impedes cell migration(187).
Particularly in breast tumorigenesis, the ECM has been reported to undergo profound remodelling. Interestingly, it has also been shown that in BC, the ECM is very similar to the matrix present in tissues undergoing wound healing processes. Because of this, the significance of ECM in tumorigenic processes and therapy resistance has attracted substantial interest over the past few years(188).

- **Stromal cells**

Besides ECM remodelling, the stromal cell population also undergoes dramatic changes during tumorigenesis. Indeed, when compared to normal breast stromal tissue, tumour-associated stroma presents a large number of fibroblastic and immune cells as well as increased capillary density(189).

The main populations in the tumour stroma are detailed below.

ANGIOGENIC VASCULAR CELLS

Tumours require the formation of blood vessels to support nutritional and metabolic growth needs. This process, called angiogenesis, is engineered by the endothelial cells, either using pre-existing blood vessels or newly formed ones. Vascular Endothelial Growth Factor (VEGF) is the main factor involved in this process. Secreted by tumour cells, immune cells and/or stromal fibroblasts, it induces tumours to switch to angiogenic pathways, resulting in the generation of new vasculature.

However, tumour vessels formed through VEGF stimulation are aberrant, with an abnormal distribution, irregular shapes, and leaky. All this, in turn, contributes to a further increase in tissue hypoxia and stimulates VEGF production(190,191).

INFILTRATING IMMUNE CELLS

Several lineages of immune cells are recruited and reside within the tumour stroma. These include mainly monoyctes/macrophages, neutrophils, and lymphocytes(192).
Under normal conditions, resident immune cells are responsible for preserving tissue homeostasis and organ integrity. However, in cancer, immune cells play a dual role, either supressing or promoting tumorigenesis. Premalignant tissues contain aberrant cells that typically activate prosurvival pathways. In this situation, the chronic activation of immune and stromal cells may foster the survival of aberrant cells, further supporting the expansion of pre-malignant lesions. If inflammatory-type events are sustained, pre-malignant lesions may progress to neoplasia(193).

Monocytes migrate into tumours following chemotaxis gradients. Once recruited, they differentiate into tumour-associated macrophages (TAMs). TAMs are thought to contribute to tumour progression in many different ways: by promoting genetic instability; by feeding the cancer stem cell (CSC) population; or by contributing to immune evasion, among other things. Moreover, TAMs also release a number of factors such as VEGF that directly influence the behaviour of endothelial cells(194).

Neutrophils are other angiogenic stimulator cells in the tumour stroma. Like TAMs, they can also release factors such as VEGF, HGF or IL-8 to enhance vascularization processes(192,193).

In relation to lymphocytes, it is thought that, in tumours, multiple immune cell types with immunosuppressive activity may co-exist(193). In particular, Tregs are a subtype of CD4+ T lymphocytes that function as potent immunosuppressors. By producing IL-10 and transforming growth factor beta (TGF-β), among other factors, they inhibit the recognition and clearance of tumour cells by the immune system(195). Indeed, high numbers of Tregs in the TME have been associated with a worse prognosis in many types of cancers(196,197).
Natural killer (NK) and natural killer T (NKT) cells are also innate cytotoxic lymphocytes with important functions in tumours. They also infiltrate the tumour stroma, but in this case, they are not thought to be in contact with tumorigenic cells. However, their potential value as a prognostic marker still remains unclear. Whereas in some malignancies such as colorectal, gastric or lung cancers, they have been associated with a better prognosis, other studies have predicted the opposite effect(198). Studies led by Fridman et al. suggest that NK cells present in tumour stroma may have an anergic phenotype induced by the secretion of TGF-β from cancer cells(199).

CANCER-ASSOCIATED FIBROBLASTS (CAFs)

CAFs constitute the major and most active population in the tumour stroma. By secreting a variety of soluble factors, including chemokines and growth factors, they closely modulate tumour stroma behaviour and promote tumorigenesis(200). The role of CAFs in cancer is reviewed in the following section.

Taking all of this together, it is clear that tumours are formed by a myriad of cells in addition to neoplastic cells. Cancer cells and stromal cells cooperate closely and co-evolve together. In this process, the intercellular communication between cell populations is driven by a complex network of chemokines and cytokines, as well as growth and inflammatory factors, and matrix remodelling enzymes(185).

Thus, tumour initiation and progression is, therefore, profoundly influenced by the interaction of cancer cells with their surrounding environment. This interaction ultimately determines whether premalignant abnormalities are eradicated or tumorigenesis is allowed, in turn enhancing malignant processes and tumour metastasis.

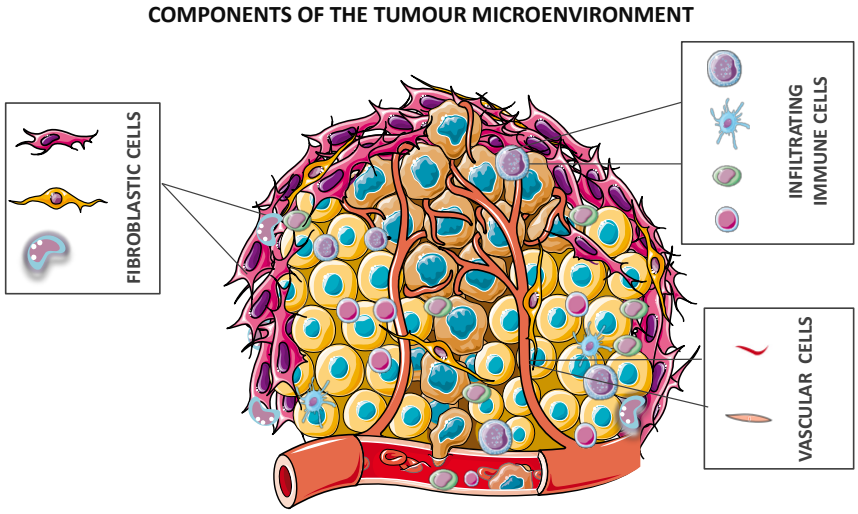


Figure 15. Components of the tumour microenvironment. Adapted from Balkwill et al., 2012.

I11. CANCER-ASSOCIATED FIBROBLASTS (CAFs)

Activated fibroblasts, best-known as cancer-associated fibroblasts (CAFs), represent the most abundant and active population of the tumour microenvironment(201). Through reciprocal crosstalk between cancer cells, CAFs undergo several biological and morphological transitions in response to tumour progression(202). Despite the fact that there are many unanswered questions relating to CAFs, it is obvious that they are crucial for maintaining an optimal microenvironment for cancer cell survival and proliferation. Indeed, they have also been linked with therapy resistance, and, in recent years, CAFs have emerged as an attractive molecular target(200).

I11.1 Origin, structure and function of CAFs

CAFs are a heterogeneous population with multiple origins. Most are derived from resident fibroblasts, but they can also be derived from other cell lines, such as mesenchymal stem cells (MSCs), epithelial cells that undergo epithelial-to-mesenchymal transition (EMT), pericytes, adipocytes, and endothelial cells(203).

Under normal conditions, when tissues are injured, resident fibroblasts become activated and differentiate into myofibroblasts. Once tissue homeostasis is restored, fibroblasts return to their basal status. However, in tumorigenesis, CAFs are perpetually activated. In this sustained state of activation, fibroblasts continue to secrete growth factors and cytokines, resulting in a self-perpetuating autocrine feedback loop that in turn, transactivates other fibroblasts(204).

Morphologically, CAFs are long spindle-shaped cells. Although they have a characteristic morphology, fibroblasts are poorly defined in molecular terms. Several CAF-related markers have been described to date, but none are either exclusively for activated fibroblasts or expressed in all fibroblastic cells. In line with this, the activated fibroblast phenotype is associated with alpha-smooth muscle actin (αSMA) expression, and, in fact, αSMA is the most widely accepted marker for CAFs(205).

Other markers such as fibroblast associated protein (FAP), Vimentin, fibroblast-specific protein (FSP-1), and platelet-derived growth factor receptor-β (PDGRβ) are also widely accepted as CAF markers(204).

FIBROBLAST MARKERS

	FUNCTION	FIBROBLAST TYPE	OTHER CELL TYPES DESCRIBED
α-SMA	Intermediate filament-associated protein	Myofibroblastd and activated fibroblasts	Vascular smooth muscle, pericytes and myoepithelial cells
VIMENTIN	Intermediate filament-associated protein	Myofibroblastd and activated fibroblasts	Endothelial, myoepithelial and neuronal cells
FSP1	Intermediate filament-associated protein	Miscellaneous	Invasive tumour cells
FAP	Serine protease	Activated fibroblasts	Active melanocytes
DESMIN	Intermediate filament-associated protein	Skin fibroblasts	Muscle and vascular smooth muscle cells
DISCOIDIN-DOMAIN RECEPTOR II	Collagen receptor	Miscellaneous	Endothelial cells
α1β1 INTEGRIN	Collagen I biosynthesis	Miscellaneous	Monocytes and endothelial cells
PROCOLLAGEN I α2	Collagen receptor	Miscellaneous	Osteoblasts and chondroblasts

Figure 16. Main well-established fibroblast and CAF-related markers. Adapted from Kalluri et al., 2006.

With regard to their role in solid tumours, a large list of CAF functions has been reported. On the one hand they are responsible for the synthesis of the fibrillary components of the ECM, such as fibronectins and collagens. Moreover, they are also an important source of ECM-degrading proteases, a crucial factor for cancer cell invasion and metastasis(206,207).

On the other hand, they constitute a feeder layer for cancer cells. CAFs secrete increased quantities of growth factors, pro-inflammatory cytokines, and chemokines that, in turn, induce the proliferation of adjacent epithelial cells, stimulate angiogenic processes, or promote tumour immune evasion(192). Of note, among several growth factors and cytokines that have been reported to be secreted by CAFs, there are classical mitogens for cancer cells, such as TGF-β, HGF, EGF and fibroblast growth factor 2 (FGF2), as well as SDF1 and IL6(183,192,208,209).

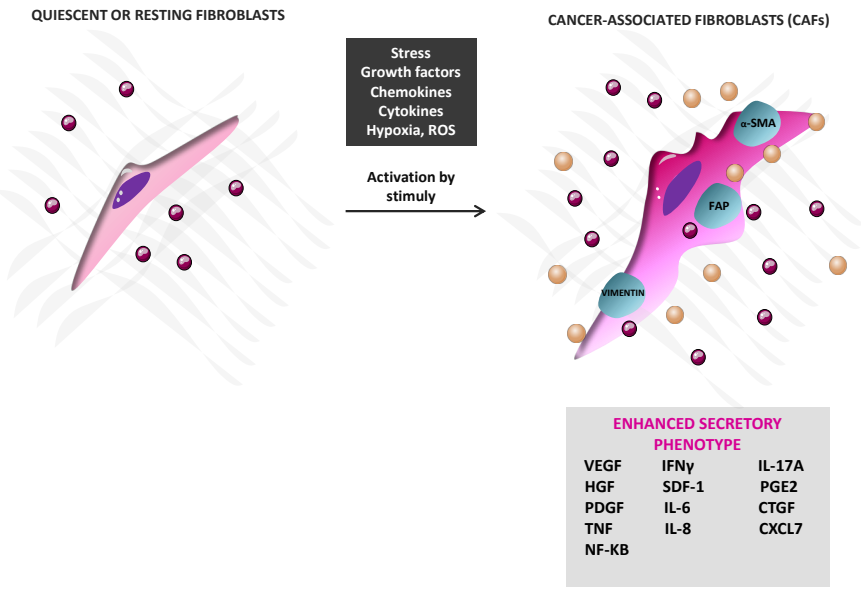


Figure 17. Illustration of CAF secretory features. Adapted from Kalluri et al., 2016.

I11.2 Stromal heterogeneity of CAFs

As previously explained, CAFs are the major and most active population of the tumour stroma. They are found in almost all solid tumours. However, their abundance varies between tumours. For example, breast, prostate and pancreatic cancers contain large proportions of CAFs, whereas their number is much reduced in other types, such as renal, ovarian and brain tumours(210).

Although CAFs were first considered a single entity, they are by far one of the most heterogeneous tumour cell populations. The main, currently accepted reasons for this are: the multiple origins of CAFs; the specificity of the tissues in which they reside; and their interaction with cancer cells(211,212).
Studies by Chang et al. show that fibroblasts from different anatomical sites display different transcriptional patterns and, thus, may be considered distinct, differentiated cell types. Moreover, CAF heterogeneity appears not to be tissue-specific; recent studies have reported that different populations of CAFs may-coexist within a single tumour(212,213). In line with this, it has been reported that a subset of CAFs from oral carcinomas have a low expression of αSMA and that they differentially support cell proliferation but supress the stemness of oral cancer cells(214). A study performed by Brechbuhl et al., showed that in ER+ BC, two different populations of CAFs co-existed based on the expression of CD146. CD146-positive CAFs conferred sustained ER-dependent proliferation and tamoxifen sensitivity to these tumours(215). Indeed, some researchers have proposed that certain subtypes of CAFs may negatively influence tumorigenesis, either by supressing initial malignant cells or by modulating the immune response(216).

There is, therefore, no consensus on their molecular definition. As previously explained, several markers have been proposed to define CAFs. Since CAFs are genetically stable entities, they have emerged as potential targets in cancer. However, recent data suggests that the widely-accepted CAF markers are neither expressed by all CAF stromal populations nor even uniquely restricted to them(217).

Because of this, it is hoped that, in the near future, a better understanding of the role of CAFs in tumorigenesis, as well as their molecular characteristics, may aid in the development of effective tumour-treatments.

I11.3 TGF-β: a pleiotropic cytokine with a pivotal role in CAFs

- **The TGFβ family of proteins: structure and associated signalling**

TGF-beta (TGF-β) proteins are a family of pleiotropic cytokines. They play important roles in cell growth, development, tissue repair, and host immunity, among other things. Mainly existing as three major isoforms (TGFβ1, TGFβ2, and TGFβ3), this family of cytokines are secreted as latent precursor forms and can act in an autocrine or paracrine manner.(218)

TGF-β signals via specific complexes of TβRI and TβRII Ser/Thr kinase receptors. Upon binding, these complexes become activated and TGFβ-canonical signalling is initiated. This comprises mainly phosphorylation through the SMAD family of receptor proteins, mainly SMAD2 and SMAD3. These in turn, associate with SMAD4, and this trimeric complex shuttles to the nucleus(218,219).
Once there, by binding to DNA-cofactors, the expression or repression of a particular set of genes is selectively targeted. Because of this, a common TGF-β signal can repress the activation of hundreds of genes at once, enhancing or inhibiting multiple biological processes(218).
However, TGFβ signalling can be initiated by alternative Smad-independent pathways. Among these, MAPK cascades and PI3K pathways have been reported to crosstalk potently with the TGFβ cascade(218).

A series of studies has shown that HER2-associated signalling, which results in PI3K/AKT and MAPK pathway activation, intimately communicates with TGFβ/Smad in controlling breast cancer development and that HER2/Ras signalling may enhance the pro-migratory and pro-invasive functions of TGF-β(209,220,221).

With regard to PI3K-TGF-β signalling crosstalk, previous studies have speculated that these two pathways might counteract each other to ensure cell-survival balance. However, emerging evidence has pointed out that far from balancing each other, these two pathways crosstalk closely and collaborate in driving cancer progression(220).

- TGF-β: From a tumour suppressor to a tumour-promoting factor

As previously explained, the TGF-β signalling crucially drives many cell processes. Indeed, in tissue homeostasis or in the early stages of tumour malignancies, TGF-β displays a tumour-suppressive role. In this context, TGF-β prevents tumour formation by inducing cell-cycle arrest and apoptosis. However, cancer cells can activate alternative mechanisms to circumvent this effect. Basically, two major mechanisms have been described so far: on the one hand, by inactivating receptor mutations, cancer cells can become blind and unresponsive to TGF-β signalling; on the other hand, they selectively inhibit the tumour-suppressive arm of the pathway(209).

Either through one mechanism or the other, dysregulated TGF-β signalling increases the tumour progression potency of cancer cells. In this context, TGF-β stimulates cell growth, epithelial to mesenchymal transition (EMT), enhancement of metastatic capacity, stemness, and the promotion of autocrine mitogenic factors(209,217,222).

- **The role of TGF-β in the tumour microenvironment and CAFs**

However, TGF-β does not only play a role in epithelial cells. As a potent pleiotropic cytokine, TGF-β plays a pivotal role in the tumour stroma and immunoevasion. It is known that TGF-β promotes the differentiation of resident stromal fibroblasts into activated fibroblasts(209,223,224).
Activated fibroblasts, in turn, secrete large quantities of TGF-β, which amplifies the signal loop that differentiates the fibroblasts into myofibroblasts. In line with this, several *in vitro* studies have reported the extreme significance of TGF-β for the biology of CAFs. Enhanced TGF-β signalling has been found in CAFs from colorectal cancer subtypes, as part of a stromal signature that clinically confers poor prognosis(223).

With regard to its immunosuppressive role, it has been proposed that tumours which produce high levels of TGF-β may escape immune surveillance. Moreover, defective TGF-β responsiveness in immune cells may result in chronic inflammation and the production of a pro-tumorigenic environment(209).

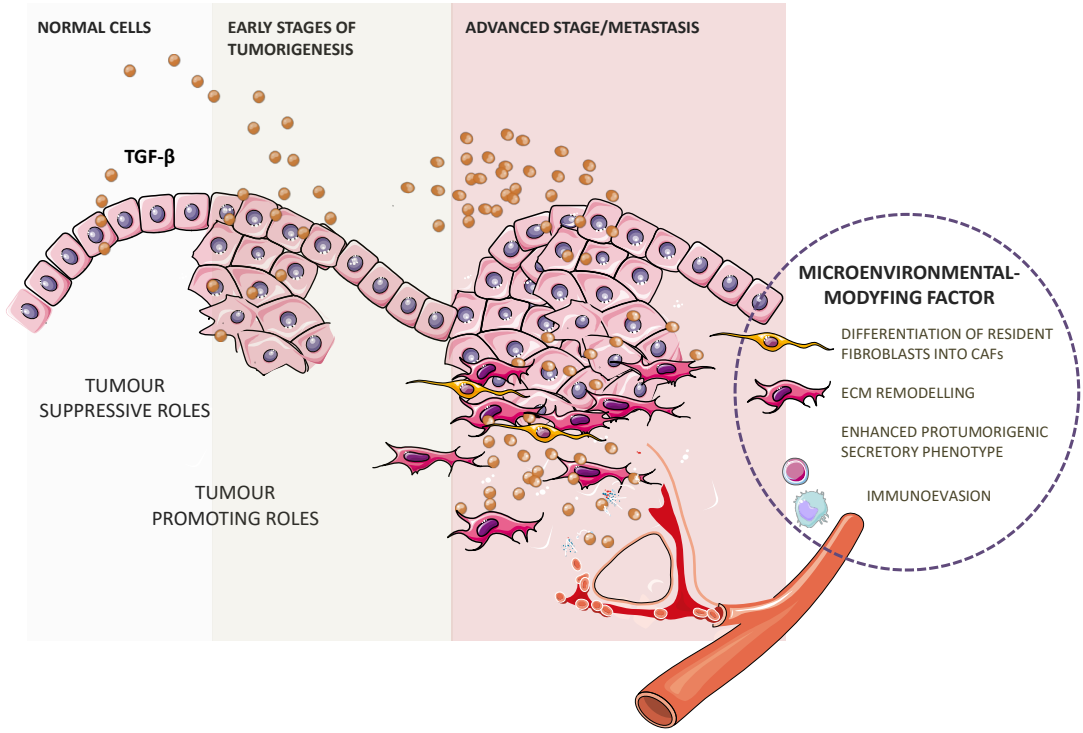


Figure 18. **TGF-β roles in tumorigenesis, and in stromal fibroblasts.** Adapted from Massagué et al., 2008/ Blobe et al., 2000).

I12. THE ROLE OF TUMOUR STROMA IN DRUG RESISTANCE

So far, the role of CAFs in tumour initiation and promotion is undeniable. As previously mentioned, CAFs closely control many tumorigenic processes(204,225). In line with this, in recent years, several studies have described how CAFs are also intimately involved in promoting cancer therapy resistance(226–228).
Despite the fact we do not fully understand the mechanisms behind how CAFs promote drug resistance, the main mechanisms reported to date are reviewed in this section.

One of the proposed mechanisms of therapy resistance is the modulation of processes involving cancer cell-ECM interactions, CAF-ECM adhesion, and cytokine- or chemokine-associated signalling. Moreover, it has also been reported that CAFs may contribute to increasing intratumoral interstitial fluid pressure, therefore indirectly inhibiting the uptake of anticancer drugs. Promotion of immunoevasion, induction of angiogenic processes, induction of EMT in cancer cells, and metabolic reprogramming of the TME, are other reported mechanisms of CAF-led therapy resistance(204).

Moreover, CAFs provide cancer cells the fuel needed for many biological processes through the paracrine secretion of soluble factors, such as growth factors and cytokines. In this context, TGF-β, IL-6 and HGF secretion by CAFs has emerged as a potential drug resistance mediator(204,226,229).
Specifically, it has been reported that CAFs induce chemoresistance as well as endocrine and targeted therapy failure. Studies led by Crawford et al. show that CAFs promote resistance to anti-VEGF therapy through the PDGF axis(230). Other studies also report that HGF secretion from fibroblasts promotes chemoresistance in lung cancer by activating c-MET in tumour cells(229). Indeed, subsequent studies have also shown that, in addition, HGF is involved in tumour resistance to targeted therapies in BRAF mutant melanoma(231).

Due to this, over recent years, the co-targeting of CAF and cancer cells has emerged as a potential cancer therapy. Various strategies have been proposed: the targeting of signals involved in fibroblast activation, such as PDGF or TGF-β signalling pathways, have shown promising results in some malignancies. Indeed, the PDGF inhibitor Imatinib, is used to treat chronic myeloid leukaemia and other types of cancers(232). In addition, the targeting of fibroblast-specific molecules involved in supporting tumour growth such has FAP is already being clinically trialled(233).

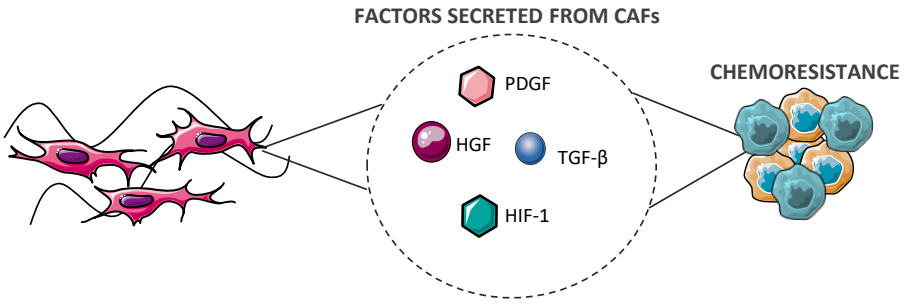


Figure 19. The role of CAFs in promoting tumour resistance. Illustration of the main reported CAF-secreted factors that have been linked with therapy resistance in solid tumours.

In light of the information contained in this chapter, it is evident that tumours function as complex tissues. Multiple cell types co-exist in them, and the complex interactions between these dramatically influence the features of the tumour. Among all this complexity, CAFs have emerged as crucial players in tumour initiation, maintenance, and progression. Despite the active role of CAFs adding a new layer of complexity to cancer biology, they also represent an opportunity for finding new therapeutic strategies. Although the role of CAFs in supporting tumorigenesis is undeniable, emerging data underlines the fact that CAFs are extremely heterogeneous and that certain subtypes of CAFs may also restrict tumorigenesis(216).

A better understanding of TME complexity, as well as the biology of CAFs and their underlying heterogeneity, could therefore translate into more effective and specific therapeutic treatments for cancer patients.

HYPOTHESIS

To date, HER2 positivity itself stands as the unique criteria for treating HER2-positive BC patients with anti-HER2 targeted therapies. Several biomarkers predictors of response or resistance to anti-HER2 targeted agents have been described in preclinical research. However, their translation into clinical practice has failed up to now. The fact that these studies have been generally based exclusively on intrinsic tumour cell properties stands as a potential factor behind this.

Herein, the hypothesis of this doctoral thesis stands on the fact that: **Secreted factors from cancer-associated fibroblasts, commonly present in the tumour microenvironment, may shape resistance of HER2-positive breast cancer to anti-HER2 targeted therapies. Hence, a better understanding of the crosstalk between tumour cells and CAFs, may help to identify novel targets or biomarkers ro prevent or revert resistance to currently available anti-HER2 targeting drug agents.**

OBJECTIVES

I. To study the crosstalk between cancer-associated fibroblasts (CAFs) and HER2+ breast cancer (BC) cells in tumour cell biology and in therapy response.

a. To establish an *in vitro* model of paracrine interaction between cancer cells and CAFs.

b. To study the paracrine effects of soluble factors secreted by CAFs in cancer cell response to anti-HER2 targeted therapies.

II. To identify potential paracrine signalling molecules from CAFs that may mediate drug response or resistance of HER2+ breast cancer cells.

III. To generate a collection of primary CAFs cultures isolated from fresh surgical tumour specimens from breast cancer patients.

a. To establish a protocol of isolation as well as immunohistochemical and genetic characterization of primary CAFs.

b. To assess their impact on HER2+ breast cancer response to anti-HER2 based therapy.

IV. To study the role of CAFs in *in vivo* models of HER2+ breasr cancer

V. To analyse the relationship between CAFs (via expression of potential resistance markers previously identified) and patient outcome in large-scale public datasets and in HER2+ breast cancer patient's samples.

RESULTS

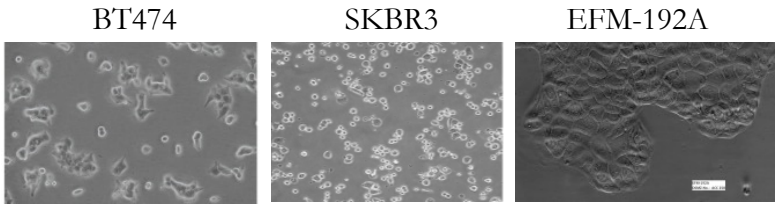
R1. CHARACTERIZATION OF *IN VITRO* HER2+ BREAST CANCER MODELS

R1.1 HER2+ BC cell lines

The aim of this project was to study the crosstalk between cancer cells and CAFs in order to find potential mediators of breast cancer (BC) response or resistance to anti-HER2 targeted therapies.

To approach this, we used a panel of BC cell lines. HER2 status in cell lines was previously characterized by our group by using the same criteria as that applied in clinic settings. Specifically, these methods comprised: the HercepTest™ (DAKO), a semi-quantitative immunohistochemistry (IHC) assay for evaluating HER2 protein overexpression; and Fluorescence *In Situ* Hybridization (FISH), for evaluating HER2 gene amplification. Only 3+ HER2 positive cells were selected for further studies. The cell lines selected were: BT474, SKBR3, and EFM-192A **(Figure 1A)**.

Since many HER2+ BCs co-express oestrogen and/or progesterone receptors (ER/PR, respectively) we also assessed the expression of these proteins in our cell line panel. Whereas SKBR3 and EFM192A cells were negative for the expression of both receptors, BT474 cells were positive for ER and PR. **(Figure 1B).**



	HER2 - IHC	HER2- FISH (Mean)	ER status	PR status
BT474	3+	24 *HER2*: 3.5 *CEP17*	+	+
SKBR3	3+	35 *HER2*: 2.5 *CEP17*	-	-
EFM-192A	3+	30 *HER2*:1.8 *CEP17*	+	+

Figure 1. Characterization of HER2+ BC cell lines. A. Morphology of the BT474, SKBR3 and EFM192A cell lines under a light microscope. **B.** HER2 status was evaluated using IHC and FISH techniques and ER/PR expression status by IHC. All the experiments were performed in previous studies by our group, by M. Sabbaghi and S. Menendez.

R.1.2 Drug sensitivity of HER2+ BC cells

As previously mentioned, trastuzumab was the first anti-HER2 targeted agent developed for targeting HER2+ BCs. Almost two decades later, trastuzumab is included in the standard scheme for HER2+ BCs, in combination with chemotherapy and/or other targeted therapies.
Because of this, we decided to focus on the study of potential mediators of drug response and resistance to trastuzumab, and to study whether these were also relevant in the presence of chemotherapy.

To do this, we first characterized the trastuzumab and paclitaxel drug sensitivity in our panel of HER2+ BC cell lines. This involved treating the BT474 and SKBR3 cells with trastuzumab [15μgr/mL] for seven days. Cell viability was assessed using Scepter, an automated cell counter, and crystal violet staining. Trastuzumab drug concentration was based on prior experiments from the group, and the dose approved for patient treatment was escalated.

The results are shown in **Figure 2.** On day seven, trastuzumab significantly impaired cell viability in both cell lines. No differences were found between the Scepter counting and 0.1% crystal violet staining. For this reason, to reduce both the time and costs involved, subsequent cell viability experiments were performed using only crystal violet staining.

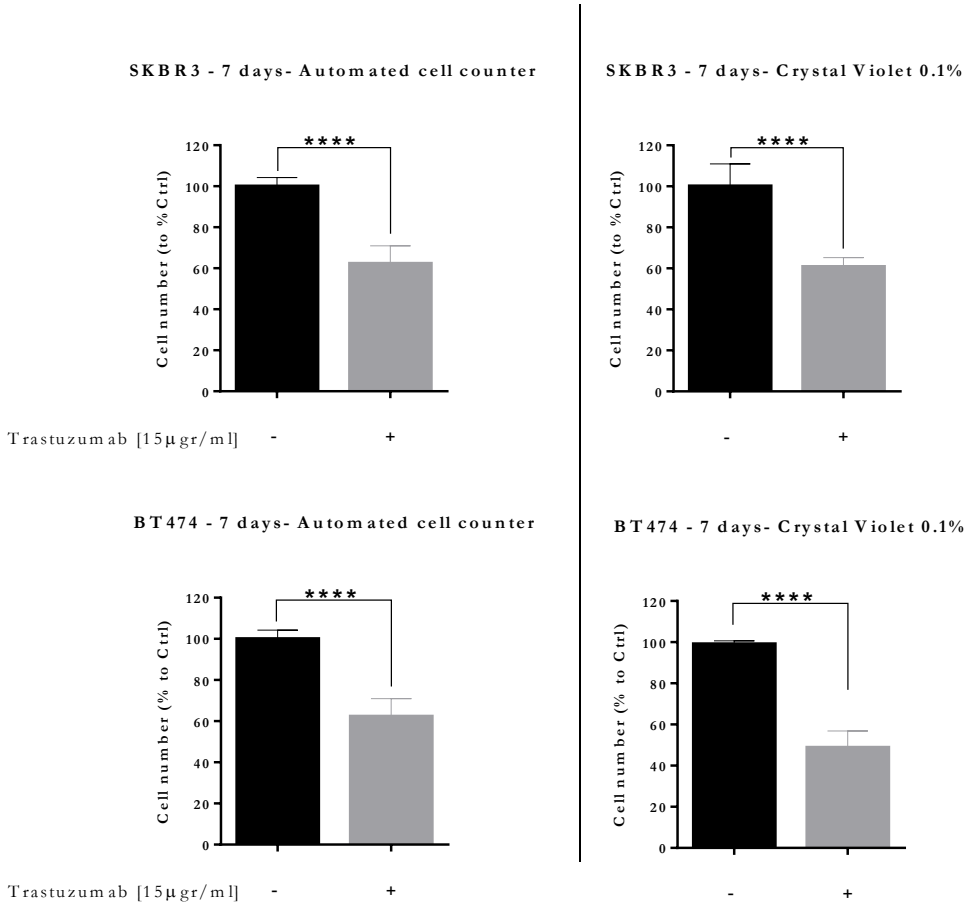


Figure 2. Characterization of *in vitro* drug response to trastuzumab. BT474 and SKBR3 cell lines were treated for seven days with trastuzumab at [15µgr/mL]. Cell viability was analysed using Scepter and 0.1% crystal violet staining. The graphs show the percentage of remaining cells in respect to non-treated cells. The data represents the average ± standard deviation (SD) of at least 3 replicates of each cell line. The statistical differences were calculated using an unpaired t test and indicated as * ($p<0.05$).

Drug sensitivity to paclitaxel was also assessed in BT474, SKBR3, and EFM-192A cells. The cells were plated and treated for 72 hours with increasing drug concentrations. Cell viability was measured using an MTT assay. Paclitaxel reduced the cell viability of all the BC cell lines in a dose-dependent manner. In particular, the most sensitive of the three cell lines assessed were the SKBR3 cells **(Figure 3).**

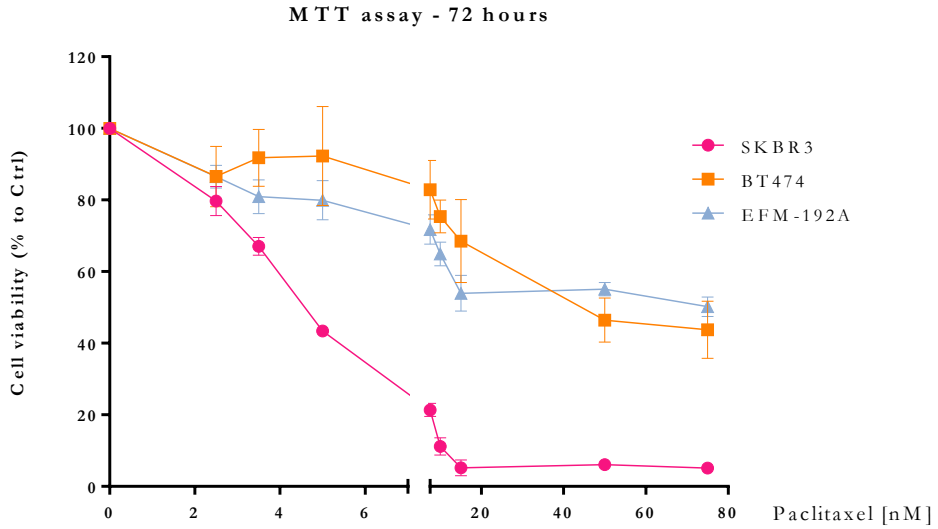


Figure 3. Characterization of *in vitro* drug response to paclitaxel. BT474, SKBR3, and EFM-192A cell lines were treated for 72 hours with paclitaxel at different concentrations. Cell viability was analysed through MTT assays. The data represents average ± standard deviation (SD) for at least 3 replicates of each cell line. The statistical differences were calculated using an unpaired t test and indicated as * ($p<0.05$).

R2. ESTABLISHING A CAF AND CANCER CELL *IN VITRO* CROSSTALK MODEL

To study the potential modulatory effects of CAFs on BC drug response, a paracrine crosstalk model was set up. As we were interested in the effects of paracrine signalling between CAFs and cancer cells we decided to first move to an indirect *in vitro* model. This involved studying potential paracrine signalling exchange through the derived secretomes.

The HER2+ BC cell lines used were those described above: BT474, SKBR3, and EFM-192A cells. For the stromal fibroblasts, two CAF cell lines, CAF200 and CAF220 were kindly provided by Dr. Gascón and Dr.Bragado. These cells were originally derived from HER2+ BC patients who had undergone a tumour biopsy. They were isolated though enzymatic digestion, selected via differential trypsinization, immortalized by retroviral infection with hTERT-GFP, and sorted using flow cytometry **(Figure 4)**.

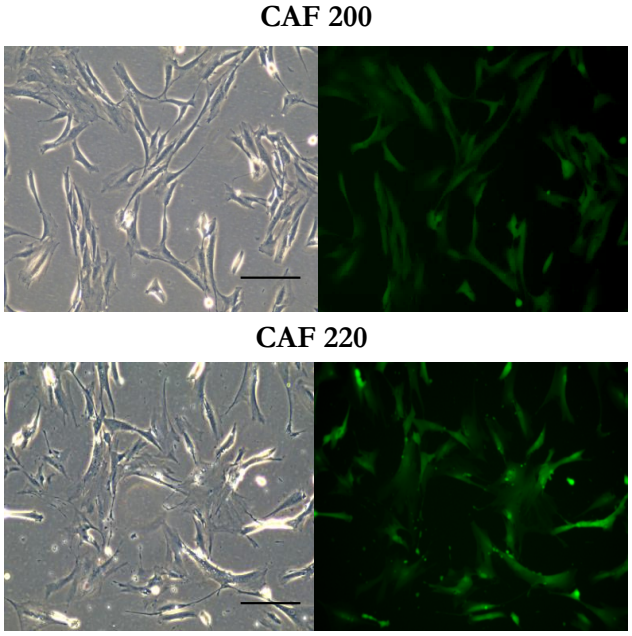


Figure 4. Immortalized CAF cell lines. Representative CAF200 and CAF220 light microscope and GFP fluorescence (in green) images (20X).

The model is briefly detailed in **Figure 5.** Either cancer cells or CAFs were cultured following their specific cell growth requirements (see Material and methods). When the cells reached a confluence of 70-80%, the derived conditioned media (also referred to as secretome or CM) was collected, centrifuged for 5 minutes at 1500 rpm to discard potential cell debris or detached cells; and filtered.

To set up the protocol, we mixed conditioned media from SKBR3 cells or CAFs with fresh cell growth media in different proportions (1:1, 1:3, 1:10). The resulting mix was placed on top of the cells and these were cultured for seven days. We decided to choose a 1:3 ratio of conditioned media to fresh media, since cell viability was preserved at seven days (data not shown).

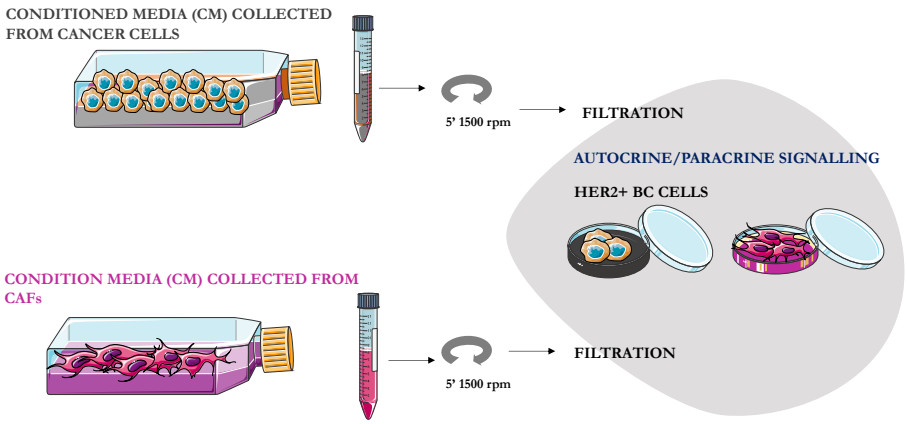


Figure 5. *In vitro* model set up for *in vitro* cancer cell-CAF crosstalk. Illustrative image of the protocol set up. Cancer cells and CAFs were cultured and their derived secretome was collected. The secretomes were centrifuged and filtered. The enriched CM was mixed with cell growth culture media in different proportions. The resulting autocrine or paracrine mixes were placed on the top of SKBR3 cells. On day seven, the cell viability was assessed using 0.1% crystal violet staining.

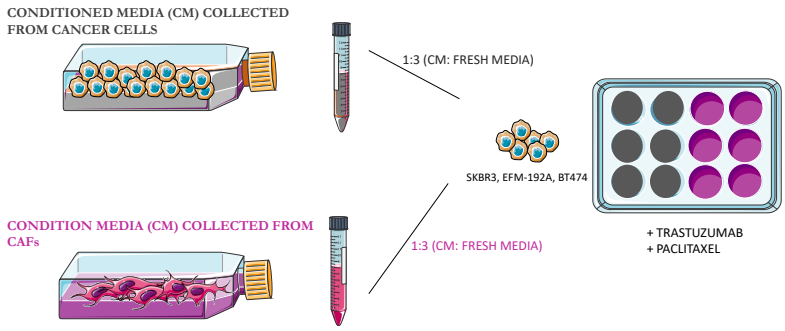
R3. CAF SECRETOME-IMPAIRED TRASTUZUMAB AND PACLITAXEL *IN VITRO* EFFICACY IN HER2+ BC CELLS

As previously mentioned, the first objective of this doctoral work was to determine whether paracrine soluble factors derived from CAFs may modify the drug phenotype of HER2+ BC cells. To study this, we performed cell viability experiments on HER2+ BC cells in the presence of both the derived CM of CAF200 cells and their own derived CM.

The protocol is summarized in **Figure 6A.** The reason for culturing cancer cells with their own derived CM was to rule out the effect of any potential autocrine soluble mediators on cancer cell drug response. As depicted, either cancer cell or CAF CM was prepared in a 1:3 ratio with fresh cell culture media. The cells were then treated with trastuzumab as previously described. At day fourth, paclitaxel was added, and cells were cultured for 72 hours more.

We found that the autocrine secretome of cancer cells did not alter the efficacy of either trastuzumab or paclitaxel, as single agents or in combination, in any of the three cell lines assessed. Interestingly, the secretome from CAFs induced a different drug phenotype in all the BC cell lines. The presence of paracrine signalling from CAFs significantly compromised the drug efficacy of trastuzumab and paclitaxel both as single agents and in combination in all the models assessed **(Figure 6B)**.

A.



B.

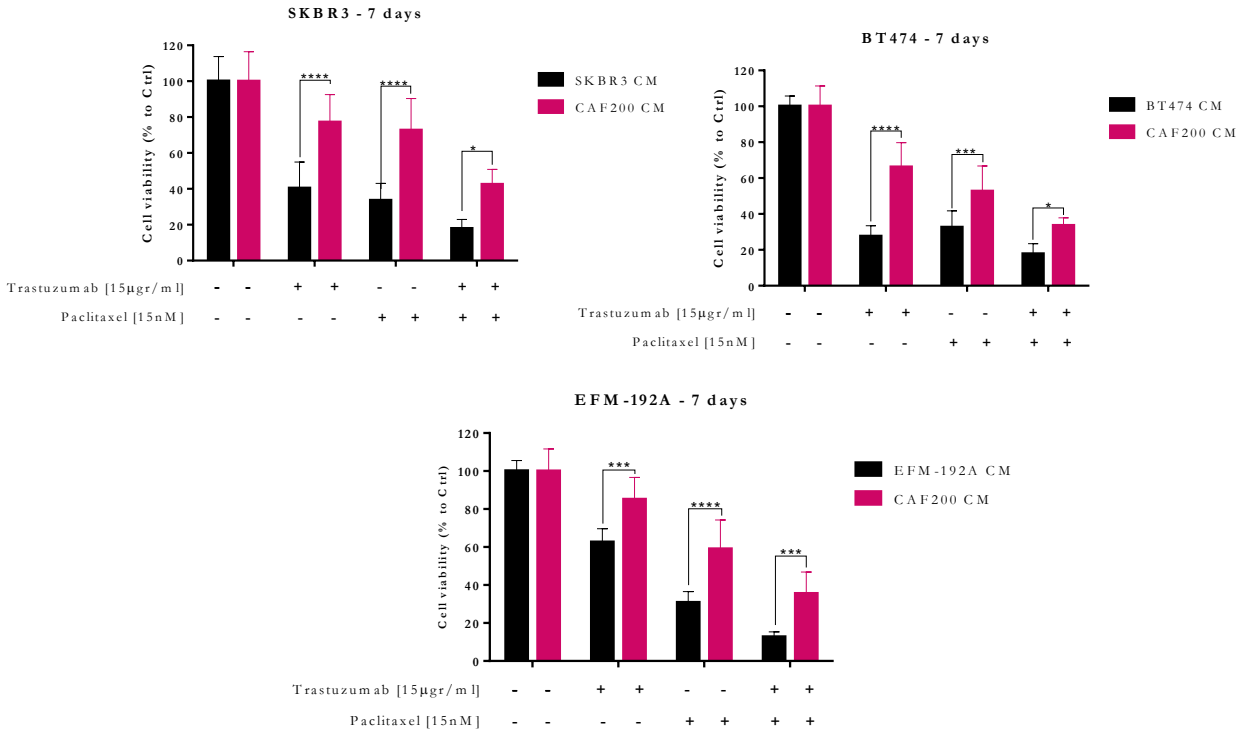


Figure 6. Secretome from CAF200 reduced *in vitro* trastuzumab and paclitaxel drug efficacy in HER2+ BC cell lines. A. Diagram of cell viability experiments with trastuzumab and paclitaxel in the presence of CAF200/HER2+ cancer cell CM. **B.** The graphs show the survival (in % to untreated cells; first two columns from the left) of cancer cells when treated with trastuzumab and paclitaxel in the presence of autocrine or paracrine secretomes. The data represents the average ± SD of at least 3 replicates. The statistical analysis was performed using a one-way Anova test, * $p<0.05$.

R4. CAF SECRETOME-INDUCED TRANSACTIVATION OF THE PI3K AND MAPK PATHWAYS IN HER2+ BC CELLS

The PI3K-AKT and MAPK cascades are two major pathways that regulate many cell processes, such as proliferation or protein synthesis, and are commonly dysregulated in cancer(37,38,53). Indeed, reactivation of these cellular pathways has been linked to therapy resistance. Our group has recently reported that trastuzumab-acquired resistant gastric cancer cell lines alternatively reactivate these two pro-survival pathways to overcome therapeutic drug pressure. The mechanism relies on enhanced autocrine ErbB ligands as well as increased ErbB receptor plasticity in these cells (A.Sampera et al. Molecular Cancer Therapeutics.*In press).*

Taking this into account, we wanted to explore whether paracrine signalling from CAFs activates these pro-survival means as potential drug evasion mechanisms.
To study this, we plated SKBR3 cells and let them attach. The cells were then starved (SS) at 1% FBS overnight (O/N). After this, the cells were cultured for different times with either their own CM or CAF CM. Protein was then extracted and immunoblotted **(Figure 7A).**

Interestingly, we observed that, CAF secretome strongly activated the PI3K/ AKT and MAPK/ERK pathways. In the PI3K pathway, we observed that potential autocrine secreted factors from cancer cells had a modest or null effect on inducing AKT activity. However, this was significantly upregulated in the presence of paracrine signalling from CAFs. In this context, phosphorylation of AKT on the Serine 473 residue was observed at early short time intervals (5 minutes) **(Figure 7B).** Notably, phosphorylation of this specific residue is reported to allow full AKT activation that, in turn, is involved in regulating many signalling pathways(234).

Concomitant to PI3K/AKT pathway activation, the MAPK/ERK cascade was also activated by paracrine secreted factors from CAFs. In this case, activation occurred transiently, reaching a peak between 5 and 30 minutes, after which it started to decrease. ERK1/2 activity was measured through increased phosphorylation at Threonine 202 and 204 sites. In line with previous results, no potential autocrine molecules induced the same molecular response from cancer cells as that exerted by CAF-associated signalling **(Figure 7B**).

These results besides the relative data reported above suggested that, despite no direct contact in our model, the secretome from CAFs was sufficient to strongly modify cancer cell dynamics and the drug phenotype.

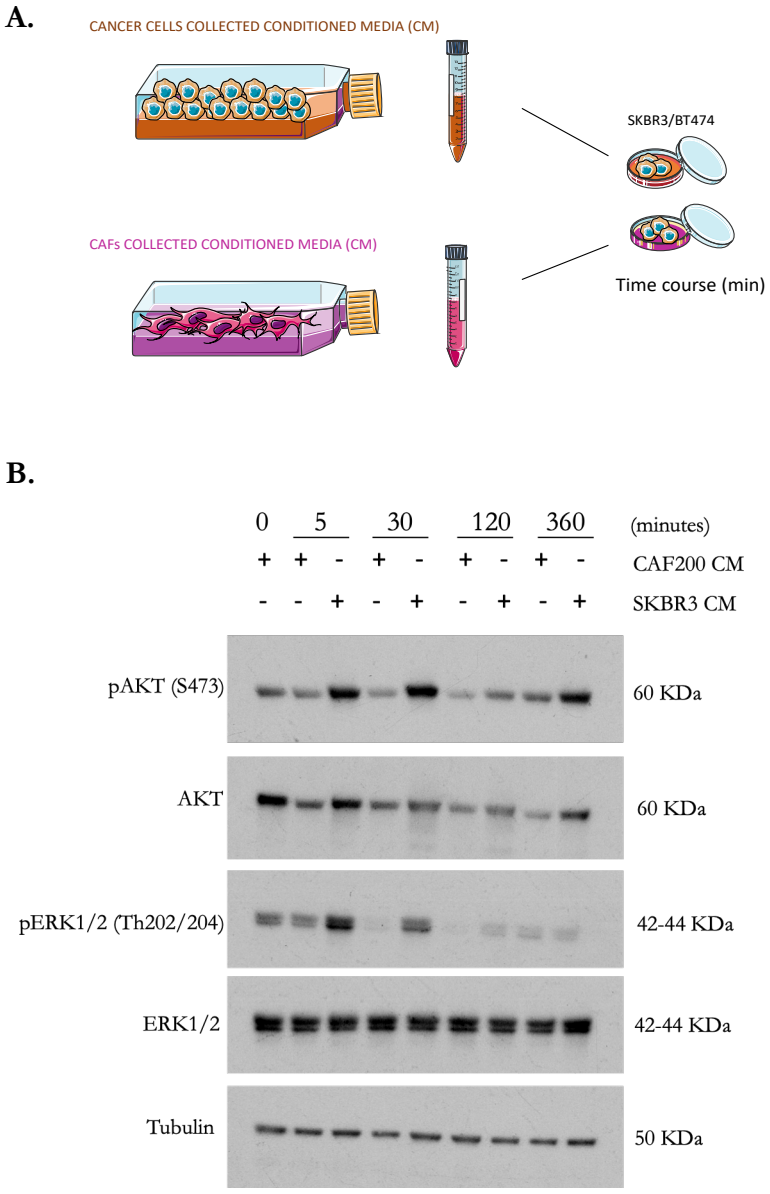


Figure 7. Paracrine effects of CAF secretome on SKBR3 and BT474 cell signalling. A. Diagram of the protocol for signal transduction experiments. **B.** WB analysis of AKT and MAPK activity (AKT S473, and ERK1/2 T202/204 phosphorylated motifs, respectively). The immunoblot images are representative of at least 3 replicates of each cell line.

R5. THE ROLE OF CAF SECRETOME IN ANTI-HER2 TARGETED THERAPIES: IDENTIFYING POTENTIAL TARGETS

Tumour stroma, and particularly CAFs, secrete large amounts of cytokines, chemokines, and growth factors that support cancer cell biology and tumour progression(204). So far, we have demonstrated that CAFs strongly modify the cell signalling and drug response of HER2+ BC cells. Our next step was to elucidate which potential factors secreted from fibroblasts could be behind the acquired phenotype in cancer cells. Amongst all possibilities, it was rational to assume that the ErbB family of ligands could be involved. In line with this, two assumptions encouraged us: firstly, the literature shows that CAFs constitute a biological source of growth factors such as HGF, IGFs, and EGF(225); secondly, the heterodimerisation of the HER family members requires ligand binding(25). Since HER2 is an orphan receptor and HER2-HER3-containing heterodimers are especially significant in HER2+ BC cell survival(25), we decided to focus on the study of NRG-1 expression, the HER3 ligand, in CAFs.

R5.1 NRG-1, the HER3 ligand, is expressed by stromal fibroblasts

Firstly, we analysed the expression of *NRG1* at mRNA and protein level in stromal CAFs and HER2+ BC cell lines. The results of the RTqPCR and IP experiments are shown in **Figure 8A.** With regard to NRG-1 expression at mRNA level, we observed that CAF200 and CAF220 cells expressed higher levels of NRG-1 than the HER2+ BC cell lines (BT474 and SKBR3). The MDA-MB-231 cell line, a TNBC cell line, was used as a positive control.

For protein detection, IP experiments were performed in collaboration with Dr. A. Pandiella. In this case, cell lysates were incubated with an anti-NRG antibody. When NRG was pulled down, a band of 100 KDa appeared in CAF200 and in CAF220 (highlighted with an arrow). This corresponds to the pro-membrane protein NRG-1 (the transmembrane non-cleaved form). In this case, BT549 (another TNBC cell line) was used as an experimental positive control **(Figure 8B).**

Notably, mRNA expression correlated with protein expression in fibroblasts. CAF200 expressed higher levels of NRG-1 by mRNA than CAF220, and this ratio was maintained at a protein level. **(Figures 8A and 8B)**.

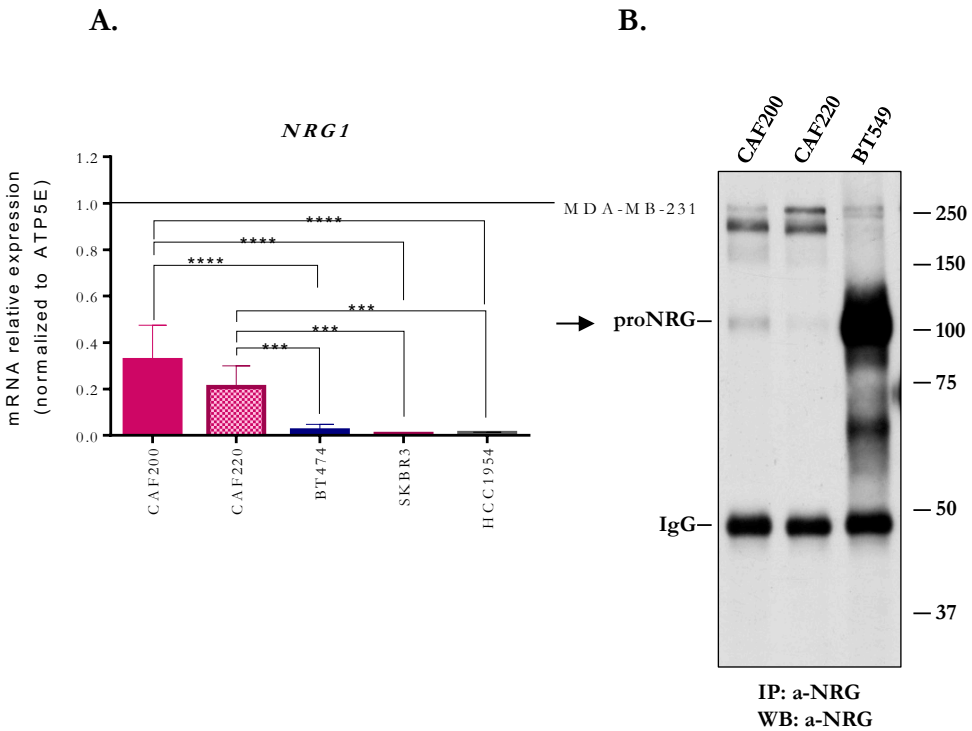
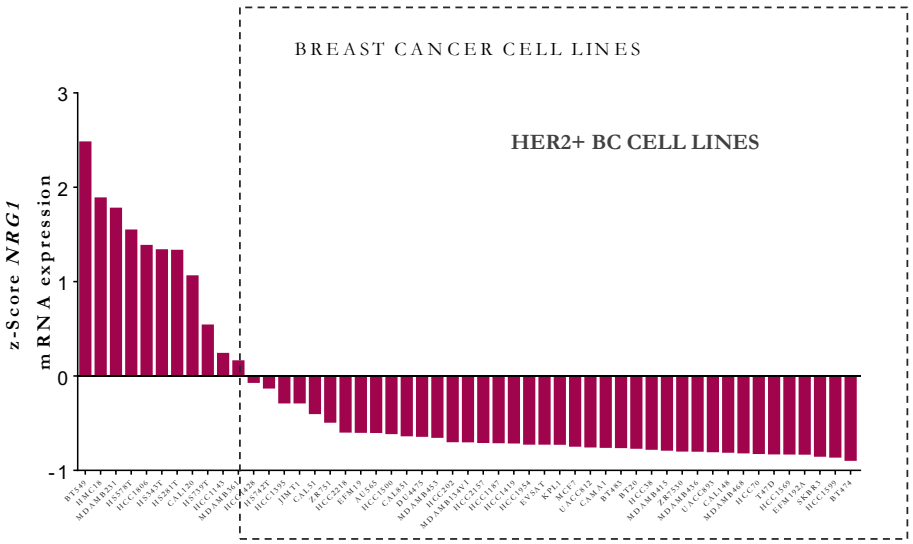


Figure 8. NRG-1 expression in CAFs and HER2+ BC cells. ***A.*** *NRG1* mRNA expression in CAFS: CAF200, CAF220 and in HER2+ BC cells: BT474, SKBR3. mRNA relative expression was calculated in reference to MDA-MB-231 *NRG1* expression. The data represents the average ± SD of at least 3 replicates of each cell line. **B.** Pro NRG-1 protein expression in CAF200 and CAF220 cells. The IP experiments were performed in collaboration with A. Pandiella.

The differences found in terms of *NRG1* mRNA expression between CAFs and HER2+ BC cells drove us to look at whether they were restricted to certain cell lines or a common trait in HER2+ BC cell lines. To address this question, we performed an exploratory analysis for *NRG1* mRNA expression in BC cell lines by using the open-access platform cBioportal. This tool provides visualization and analysis of large-scale cancer genomics datasets from significant cancer genomic projects, such as TCGA and TARGET, as well as from publications from individual labs(235,236).

The relative mRNA expression levels were represented by z-scores. This measurement indicates the number of standard deviations the sample is away from the mean of expression in the reference population (in this case, all the cancer cell lines included in this study). It therefore indicated whether a target gene (in this case, *NRG1*) was up- or down-regulated relative to all the samples.

Unexpectedly, we observed that when we molecularly classified BC cell lines into HER2+, ER/PR + or TN, the *NRG1* mRNA expression varied. As shown in **Figure 9** in general, TNBC cell lines expressed the highest levels of *NRG1* mRNA compared with the other subtypes. In line with our previous data, HER2+ BC cells lines expressed relatively low levels of *NRG1*.



GENE_ID	*NRG1*
BT549	2,4621
HMC18	1,869
MDAMB231	1,76
HS578T	1,5281
HCC1806	1,3659
HS343T	1,3191
HS281T	1,3138
CAL120	1,0425
HS739T	0,522
HCC1143	0,2216
MDAMB361	0,1426
HCC1428	-0,0485
HS742T	-0,1078
HCC1395	-0,266
JIMT1	-0,2664
CAL51	-0,3785
ZR751	-0,4698
HCC2218	-0,5751

GENE_ID	*NRG1*
EFM19	-0,5779
AU565	-0,5787
HCC1500	-0,5908
CAL851	-0,6141
DU4475	-0,6196
MDAMB453	-0,6312
HCC202	-0,6772
MDAMB134VI	-0,6782
HCC2157	-0,6851
HCC1187	-0,6879
HCC1419	-0,6909
HCC1954	-0,7032
EVSAT	-0,7032
KPL1	-0,7046
MCF7	-0,7239
UACC812	-0,7332
CAMA1	-0,7365

GENE_ID	*NRG1*
BT483	-0,7392
BT20	-0,7458
HCC38	-0,7567
MDAMB415	-0,766
ZR7530	-0,7758
MDAMB436	-0,7771
UACC893	-0,7828
CAL148	-0,7891
MDAMB468	-0,7954
HCC70	-0,8015
T47D	-0,8061
HCC1569	-0,8077
EFM192A	-0,8088
SKBR3	-0,831
HCC1599	-0,8406
BT474	-0,8739

Figure 9. *NRG1* mRNA expression in BC cell lines. Z-score mRNA values for *NRG1* expression in BC cell lines. HER2+ BC cells are in the pink-highlighted area. Data obtained from c-Bioportal online database. Upper graph shows the distribution of breast cancer cell lines in terms of NRG1 mRNA expression. Lower table shows the exact values (in Z-score) for each cell line. Those highlighted in grey are cell lines in which amplification or overexpression of HER2 has been reported.

These results were subsequently confirmed by RTqPCR experiments performed on a panel of HER2+ BC cell lines. For this, we plated BT474, SKBR3, HCC1954, AU565, EFM192A, and JIMT1 cells. The MDA-MB-231 cell line was also included as a positive control. After 48 hours, RNA was extracted. In line with previous analyses, we observed that HER2+ BC cells expressed low levels of *NRG1*.

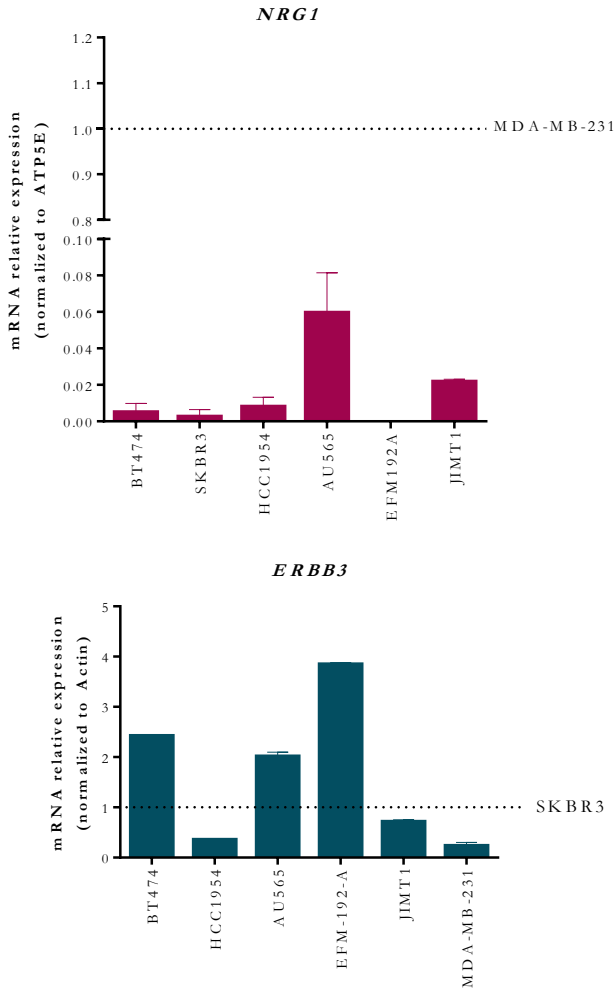


Figure 10. *NRG1 and ERBB3* mRNA expression in a panel of HER2+ BC cell lines. BT474, SKBR3, HCC1954, AU565, EFM-192A, JIMT1, and MDA-MB-231 cells were plated and after 48 hours RNA was extracted and quantified. RTqPCR was performed. Upper graph; N*RG1 mRNA* relative expression. (normalized to MDA-MB-231). Lower graph: *ERBB3* mRNA relative expression (normalized to SKBR3). The data represents the average ± SD of two replicates of each cell line.

R5.2 Study of the CAF secretome

First, we decided to analyse the CAF secretome by mass spectrometry. Our main objective was to find out whether we could detect soluble NRG-1 in the secretome derived from CAFs. For this, we decided to use the same experimental conditions as those we described previously for looking at the paracrine modulatory effects of CAFs in the drug response of cancer cells. In other words, we plated CAFs at a confluence of 70-80%, and 24 hours after this we collected this media. The derived secretome was centrifuged for 5 minutes at 1500 rpm to discard potential cell debris or detached cells, then filtered, concentrated with Amicons for 40 minutes at 40000 Gs at 4ºC, and processed for digestion by mass spectrometry. However, to maintain the same experimental conditions, we had to maintain normal growing conditions, which meant growing cells in 10% FBS. This decision was made due to the fact that we had no relative data on how depleting FBS in CAFs could affect NRG shedding. However, within this decision, two issues were taken into consideration. Firstly, FBS contains very-abundant and complex structural proteins that may hinder NRG-1 soluble detection. Secondly, FBS-derived soluble factors may potentially interfere in the results by generating false positives. To resolve this last issue, in parallel we also processed the cell culture media used for growing CAFs, containing 10% FBS. This sample was centrifuged, filtered and processed for mass spectrometry just like the previous one. After digestion and sampling analysis, a list of proteins was obtained from the two experimental conditions. Most of the proteins were growth factors, vitamins, and cytokines already present in the cell culture growing media supplemented with 10% FBS **(see Annex).** Interestingly, among the exclusively secreted proteins from CAFs we found: ECM components, such as collagens (including collagen type IV which has been linked with tumorigenesis); structural proteins, such as Beta-2-microglobulin, Vimentin, Decorin, and Versican; proteases, such as Catepsin B and secreted growth factors; and cytokines, such as the latent form of transforming-growth factor β (TGF-β), Interleukin-36, and Hepatocyte growth factor (HGF). Interestingly, some of these molecules have been reported to mediate trastuzumab resistance in BC, as well as in other targeted therapies for other tumours, such as lung cancer(227,229,231,237).

Unfortunately, we were not able to detect NRG-1. This may be due to the fact that NRGs may be secreted by CAFs in lower concentrations than other factors, or because NRG-1 may be below the detection limit. Supporting our hypothesis was the fact that there were large magnitude differences between FBS containing proteins (those identified from *Bos Taurus* orgin) and those secreted by CAFs. Thus, it may be reasonable to assume that FBS proteins were so abundant in our samples that they limited the spectra of proteins we were able to detect. Also, in line with this, we did not detect soluble fibroblast-growth factors ligands (FGFs). In this direction, in the study led by Dr. Gascón and Dr. Bragado, in which we collaborated, we observed that HER2-CAFs (the same CAF200 cells used in this project) were implicated in trastuzumab and lapatinib resistance through paracrine secretion of FGF ligands.

R6. NRG-1 EXPRESSION IN CAFs AFTER TRASTUZUMAB AND PACLITAXEL DRUG PRESSURE

To this point, we have demonstrated how CAFs strongly modify cancer cell dynamics and drug phenotypes. Moreover we have hypothesised that this may be due to a potential NRG-1 paracrine loop. However, in a tumour, it is not only cancer cells that are impacted by therapeutic drugs. Unlike tumour cells, CAFs are considered genetically stable. However, upon extracellular stimuli, such as drug pressure, they can undergo dynamic architectural and biological changes. In line with this, recent studies have suggested that chemotherapeutic agents can modify the cellular responses of CAFs and modulate their secretory repertoire(238,239). Because of this, we wondered whether NRG-1 expression could be affected by paclitaxel, either alone or in combination with trastuzumab.

To study this, we used the same experimental scheme as before. CAF200 cells were plated and treated for seven days with trastuzumab at a concentration of 15μgr/ml. On day four, paclitaxel was added for 72 hours at a concentration of 5 nM (the same as that applied to the SKBR3 cells), either alone or in combination with trastuzumab. The cells were stained with 0.1% crystal violet and the cell viability of each condition was calculated in relation to untreated cells (referred to as Ctrl). As shown in **Figure 11B**, the CAFs were resistant to trastuzumab alone and low sensitive to paclitaxel on its own or in combination. Moreover, we also evaluated HER2 expression in CAFs by comparing HER2 expression in CAFs with the non HER2- positive breast MCF-7 cells. Interestingly, CAFs expressed at protein level higher levels than MCF-7 cells **(Figure 11A)**.

To evaluate the NRG-1 expression, the CAF200 cells were plated and treated as before. Then, RNA was extracted and quantified, and RTqPCR experiments were performed. *NRG1* mRNA expression was not significantly changed in any of the experimental conditions. We therefore concluded that after receiving the drugs, the CAFs were able to survive and NRG-1 expression, at least by mRNA, was preserved. **(Figure 11C).** We also assessed *FAP* mRNA expression in this context. Interestingly, paclitaxel significantly enhanced the expression of *FAP*.

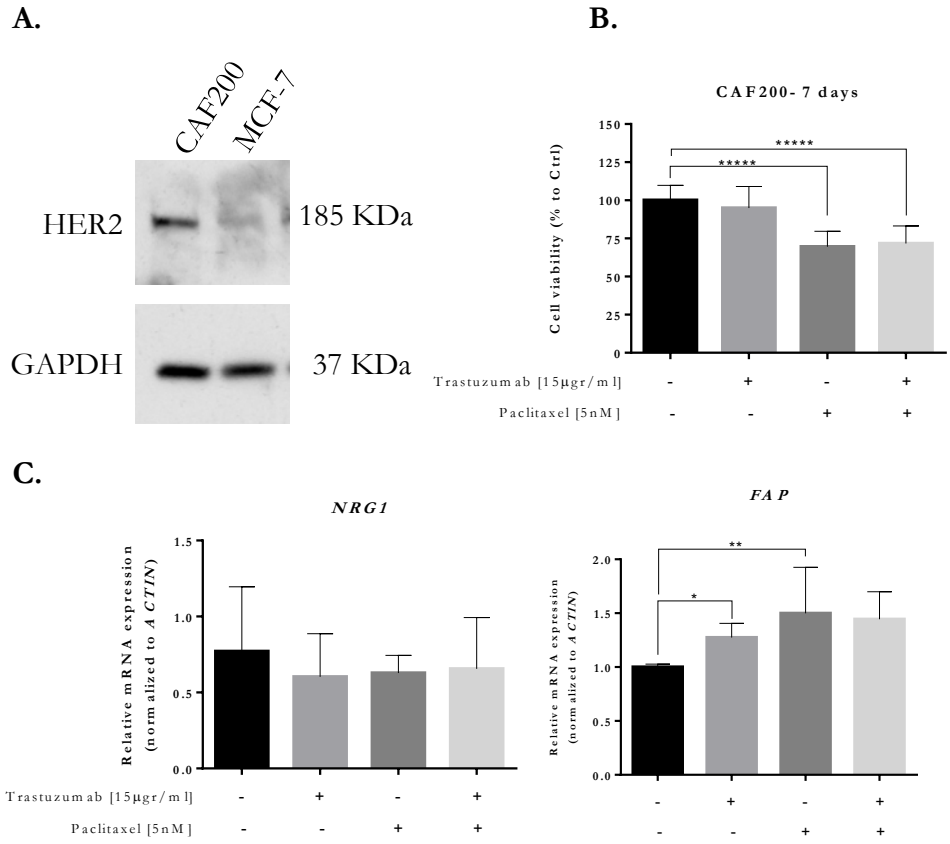


Figure 11. Cell viability and gene expression effects of CAF200 on paclitaxel and trastuzumab treatment A. WB for assessing HER2 expression in CAFs using MCF-7 a non-HER2 breast cancer cell line**.** Cell viability of CAF200 when treated with paclitaxel, alone or in combination with trastuzumab. **B.** cell viability of CAF200 with paclitaxel and trastuzumab treatment. The right graph shows the comparative cell viability of SKBR3 and CAF200 cells in the aforementioned conditions. **C.** *NRG1* and *FAP* mRNA relative expression after paclitaxel treatment, alone or in combination with trastuzumab. The data from Figure 13 A and B is representative of the average ± SD of at least 3 replicates of each cell line. The statistical analysis was performed using the one-way Anova test, * $p<0.05$.

R7. THE ROLE OF NRG-1 IN HER2+ BC CELLS

R7.1 NRG-1 effects in cell proliferation and signal transduction

With the hypothesis that NRG-1 could explain, at least in part, paracrine effects from CAFs in tumour cell biology and drug phenotypes, we decided to mimic previous experiments by adding recombinant NRG-1 instead of the derived CM from CAFs. We used the β isoform as it binds to HER3 with the highest affinity(159).

We treated BT474 and SKBR3 cells with different concentrations of recombinant NRG-1β for seven days. At low concentrations (pM range), cell growth was induced. **(Figure 12)**. However, at higher concentrations (nM range) the growth factor induced cell death over long periods (seven days). The dual role of NRG-1 in promoting cell proliferation and inducing cell death has already been reported by other authors as being dose and time dependent(240).

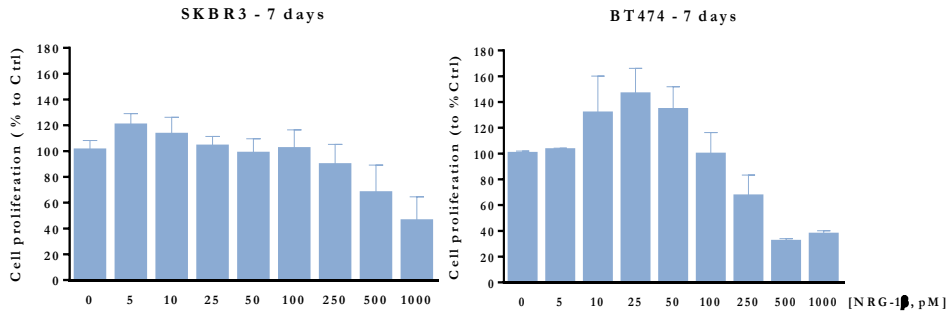
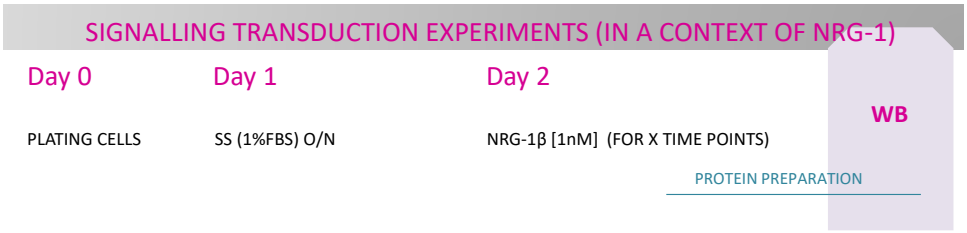


Figure 12. Effects of NRG-1β on SKBR3 and BT474 cell proliferation. NRG-1β was added at different concentrations (pM range) and cell proliferation was assessed at 7 days using crystal violet staining. The data is representative of the average ± SD of at least 3 replicates of each cell line.

Moreover, we also studied the role of this ligand on HER2-HER3 signalling transduction through the PI3K and the MAPK pathways. For this, we performed time-course experiments by stimulating HER2+ BC cells with the recombinant protein at a dose at which cell viability was preserved. As shown in **Figure 13,** NRG-1β rapidly enhanced the activation of HER2 and HER3, measured by an increased phosphorylation of these receptors at residues T1248 and T1289, respectively. However, receptor activation was transient and decreased through time. Indeed, the HER2 and HER3 total- and phospho-protein levels were strongly downregulated at 24 hours, probably due to compensatory mechanisms.

In line with previous data, NRG-1β potently induced AKT activation through an increased and sustained phosphorylation of the Serine 473 site. Concomitantly with PI3K, the MAPK pathway was also activated by NRG-1β. In this case, the activation was transient, reaching a peak 30 minutes after NRG-1β stimulation. ERK1/2 activity was measured by increased phosphorylation at the Threonine 202 and 204 sites.

A



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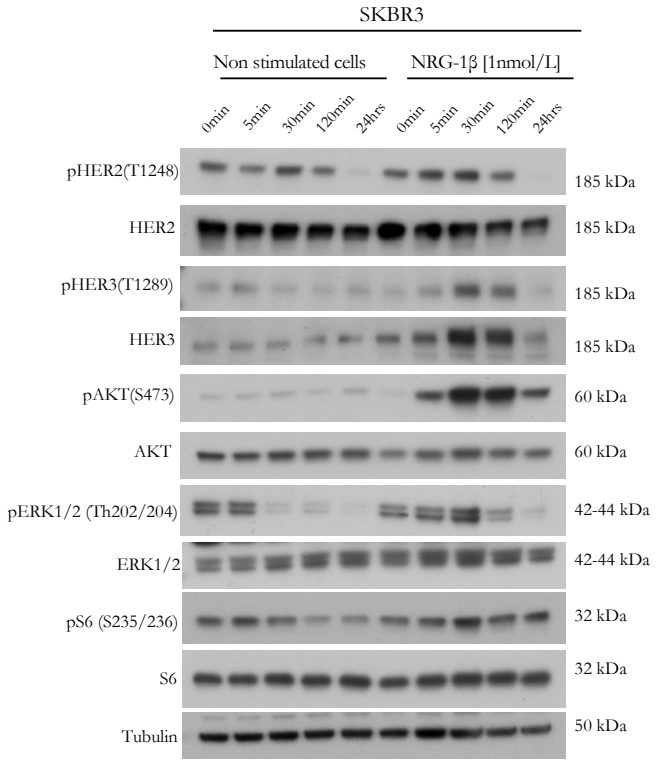


Figure 13. NRG-1β effects on PI3K/ AKT/mTOR and MAPK signal transduction cascades. A. Illustration of the experimental conditions. **B.** WB analysis of HER2, HER3 (and their phosphorylation status at the T1248 and T1289 sites, respectively), AKT/mTOR and MAPK activity (phospho-AKT S473/-S6 S235/236, and -ERK1/2 T202/204 motifs, respectively). The immunoblot images are representative of at least 3 independent replicates.

R7.3 The role of NRG-1 on anti-HER2 targeted therapies

We also assessed the paracrine effects of NRG-1β on the HER2+ BC response to trastuzumab and paclitaxel, either alone or in combination. For this, BT474 and SKBR3 cells were treated with NRG-1β [10pM] trastuzumab. Interestingly, NRG-1β significantly decreased trastuzumab effects in both cell lines, as shown in **Figure 14.**

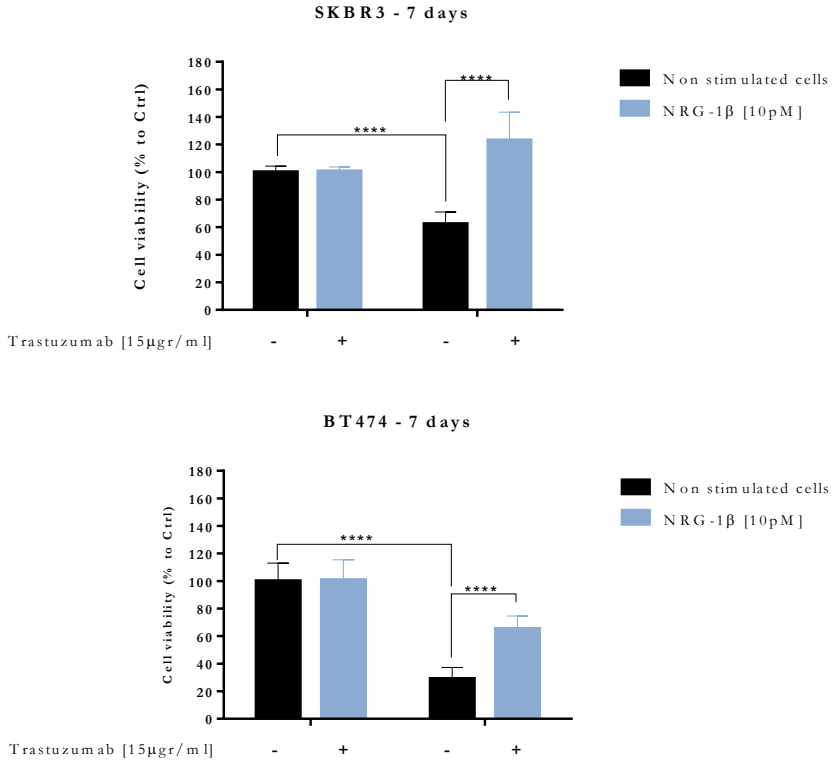


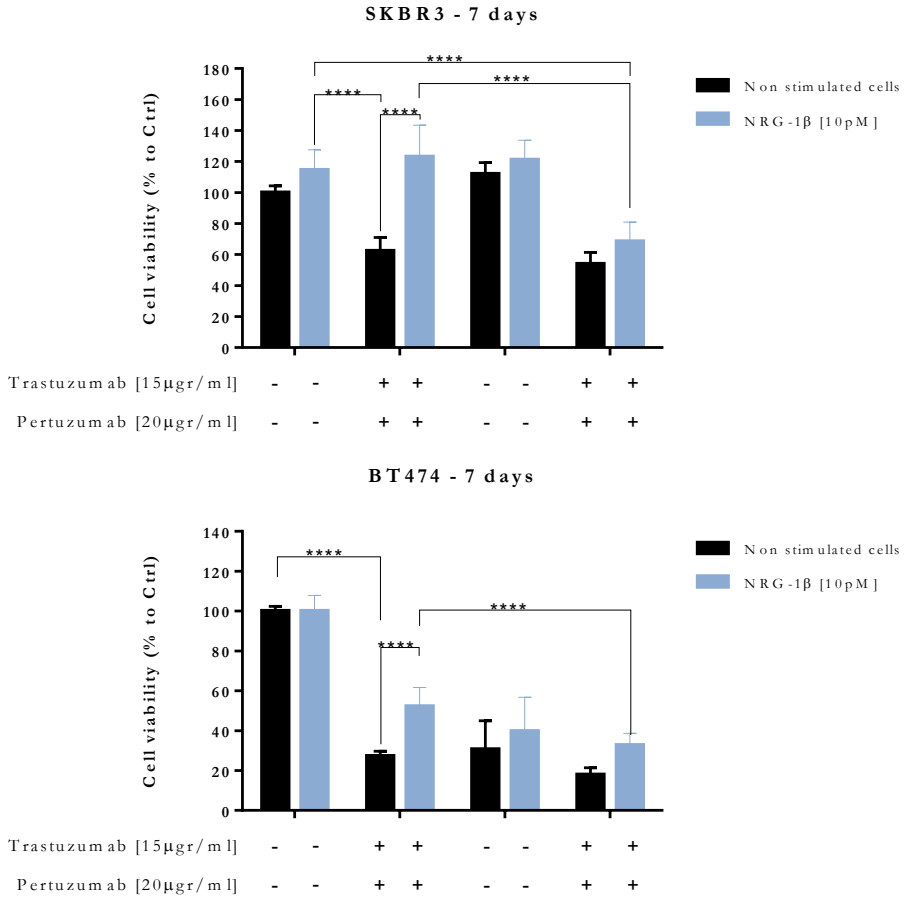
Figure 14. NRG-1β effects on trastuzumab drug effects in SKBR3 and BT474 cell lines. The cells were seeded and treated for 7 days either with NRG-1β [10pM], or trastuzumab [15µgr/ml], or a combination of the two. The cell media and treatments were replaced every 3 days. Cell viability was measured using 0.1% crystal violet staining. The data represents the cell viability for each condition (calculated in % to untreated cells). The results depicted are the average ± SD of at least 3 replicates for each condition and cell line. The statistical analysis was performed using the one-way Anova test, * $p<0.05$.

R8. PERTUZUMAB ADDITION TO OVERCOME PARACRINE CAF AND NRG-1-ACQUIRED DRUG PHENOTYPE

Our next objective was to overcome paracrine NRG-1-derived effects on HER2+ BC cells and to restore the response to trastuzumab. For this, we took different approaches, detailed here and in Section 9. On the one hand, we decided to pharmacologically abrogate the NRG-1-derived effects on cancer cells. For this, we used pertuzumab, since it binds to HER2 in a distinct trastuzumab domain and prevents ligand-dependent association with other HER family members, mainly HER3(16).

The data generated is shown in **Figure 15**. In this case, the SKBR3 and BT474 cells were treated with trastuzumab, pertuzumab, and NRG-1β, and all the possible combined treatments. In a context of no ligand, pertuzumab had almost no effect on its own. Different responses were observed in the cell lines for the combination with trastuzumab: whereas in BT474 cells the drug combination had a greater cell viability inhibition effect than trastuzumab alone, no statistical differences were found in SKBR3 cells.

As expected, NRG-β1 strongly impaired trastuzumab effects on cancer cells but the addition of pertuzumab fully restored the trastuzumab response in both cell lines.



***F*igure 15. Pertuzumab addition reverses the effect of NRG-1ß on trastuzumab drug efficacy in SKBR3 and BT474 cells.** The graphs show the cell survival (in % to untreated cells) of cancer cells with trastuzumab and pertuzumab, alone or in combination, in a context of ± NRG-1β. The data represents the average ± SD of at least 3 replicates. The statistical analysis was performed with the one-way Anova test, * $p<0.0$

We also studied the effects of NRG-1β, trastuzumab and pertuzumab on cancer cells at a molecular level. For this, SKBR3 and BT474 cells were pre-incubated with and without trastuzumab and/or pertuzumab for 1 hour. After that, NRG-1β was added for 24 hours and WB experiments were performed **(Figure 16).**

With regard to the status of HER2 and HER3, we observed differences between the two cell lines. Whereas after 24 hours of NRG-1β stimulation, no detectable phosphorylation of HER2 was observed in SKBR3 cells, BT474 cells retained the HER2 phosphorylation levels (although this was less than in untreated cells). As previously explained, this may be due to regulatory mechanisms. In SKBR3 cells, trastuzumab alone did not induce significant changes in either phosphorylated or total protein HER2/HER3 levels.
However, in the case of BT474 cells, the trastuzumab pre-treatment slightly reduced the phosphorylation, but not the total levels of HER2. The phosphorylation status of HER3 was markedly reduced with trastuzumab treatment in this cell line. With regard to the combination of NRG-1β and trastuzumab, we observed, on the one hand, in SKBR3 cells pHER2 and pHER3 levels were reduced. On the other hand, the combination of NRG-1β and trastuzumab in the BT474 cells induced a decrease of pHER2 but not pHER3 levels.

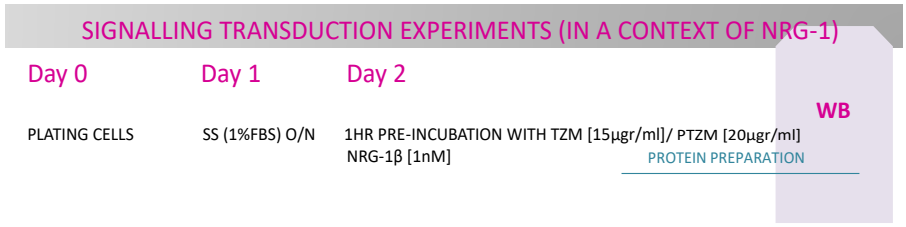
In relation to downstream associated signalling, NRG-1β strongly induced PI3K pathway activation in both cell lines. Moreover, in a context of no ligand stimulation, trastuzumab alone was sufficient to impair basal AKT signalling activity but these effects were reduced when the cells were stimulated with NRG-1β.
Indeed, in the case of SKBR3, we observed that NRG-1β also induced downstream activation of the S6 protein (measured by an increase in phospho-S6 protein levels) and, again, trastuzumab was not able to decrease the PI3K/mTOR pathway activation led by NRG-1β.

With regard to the effects of NRG-1β on the MAPK pathway, we observed differences between the cell lines. In the case of SKBR3, NRG-1β enhanced the phosphorylation of ERK1/2 and this was sustained despite pre-treatment with trastuzumab. However, in BT474 cells at the same time point, ERK1/2 activity was not upregulated by NRG-1β and, in fact, there was a slight down-regulation of phospho-protein levels, probably due to compensatory feedback mechanisms.
As shown in prior data, the activation of ERK family proteins by NRG-1 is rapid and transient, with residual activation after 24hrs. To evaluate drug effects on this cellular cascade, earlier time points are needed.

This data led us to the conclusion that despite slight differences in cell signalling transduction between cell lines, NRG-1β strongly activated the PI3K/mTOR pathway at these time points and significantly impaired trastuzumab efficacy at the cellular and molecular levels.

In contrast, pertuzumab, either alone or in combination with trastuzumab, significantly abrogated NRG-1β-derived effects in HER2+ BC cells. Although slight differences were found between the cell lines, in a context of paracrine stimulation with NRG-1β, pertuzumab efficiently reduced HER3 activation, and the combination was even better at restoring basal levels.
Looking at the PI3K/ AKT/mTOR downstream signalling pathway, AKT and S6 activities were supressed by the addition of pertuzumab, either alone or in combination with trastuzumab. These effects were particularly evident in SKBR3 cells.

A.



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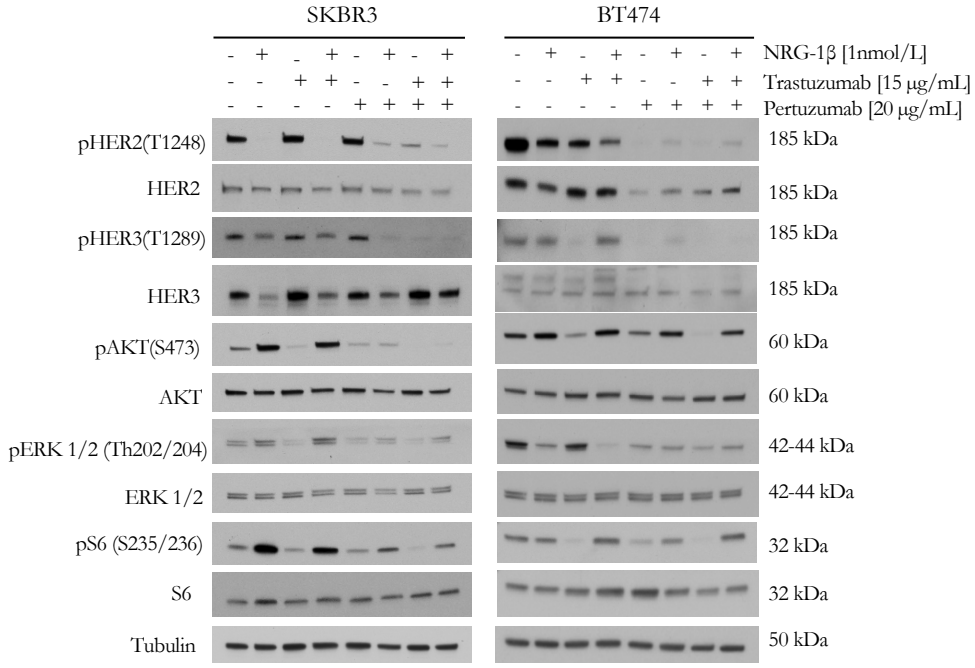


Figure 16. Trastuzumab and pertuzumab effects on PI3K/ AKT/mTOR and MAPK signalling activity by paracrine NRG-1. A. Illustration of the experimental conditions. **B.** WB analysis of HER2, HER3 (and their phosphorylation status at the T1248 and T1289 sites, respectively); AKT/mTOR and MAPK activity (pAKT S473/pS6 S235/236, and ERK1/2 T202/204, respectively). The immunoblot images are representative of at least 3 independent replicates.

We next performed the same cell viability experiments with pertuzumab but in the presence of paracrine secretome from CAFs. The results are depicted in **Figure 17.** In this case, SKBR3 and BT474 were cultured with either their own autocrine derived CM or CAF200 derived CM. The cells were treated with trastuzumab, pertuzumab, and paclitaxel, alone or in combination.

In line with what happened in the previous trastuzumab experiments, no potential autocrine molecules changed their sensitivity to pertuzumab, either alone or when combined with trastuzumab.

Interesting results were seen when the cells were cultured in the presence of paracrine secretome from CAFs. As expected, the CAFs reduced trastuzumab and paclitaxel sensitivity in all the cell lines, either alone or in combination. We observed that adding pertuzumab to any of these individual agents was sufficient to significantly impair cell viability in SKBR3 and BT474 cells. Moreover, the triple combination of trastuzumab, pertuzumab and paclitaxel showed a combined benefit towards trastuzumab plus chemotherapy.

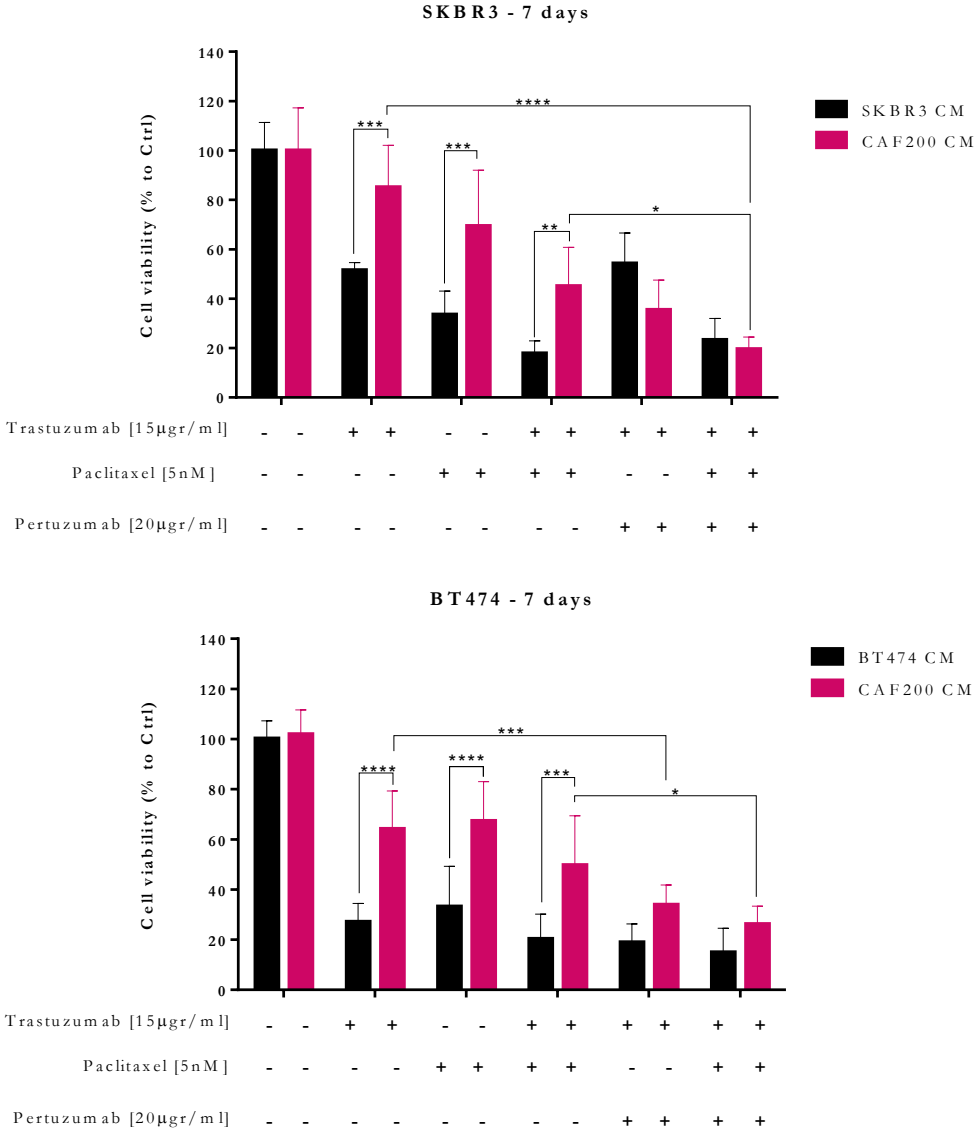


Figure 17. Pertuzumab addition overcomes paracrine effects from CAFs and restores drug sensitivity in SKBR3 and BT474 cells. The graphs show the cell survival (in % to untreated cells) of cancer cells to trastuzumab, paclitaxel and pertuzumab, alone or in combination in a context of autocrine/paracrine signalling from cancer cells and CAFs, respectively. The data represents the average ± SD of at least 3 replicates. The statistical analysis was performed using the one-way Anova test, * $p<0.05$.

R9. KNOCK DOWN OF NRG-1 IN CAF-RESTORED TRASTUZUMAB EFFECTS ON HER2+ BC CELLS

To further validate our hypothesis, we decided to abrogate the function of NRG-1 using different approaches. On the one hand, we tried to neutralize soluble recombinant NRG-1β and block HER3 receptor activity. Unfortunately, several attempts to set up the two techniques were performed with only modest success in both cases (data not shown).
On the other hand, we decided to deplete NRG-1 expression in CAFs. To do so, NRG-1 expression from CAF200 was knocked down using short-hairpin RNA technology. These experiments were performed in collaboration with Dr. Pandiella. The protocol is detailed in the Materials and Methods section M6. Short hairpin RNA efficiency was evaluated by performing immunoprecipitation (IP) experiments. As shown in **Figure 18,** short hairpin RNA technology efficiently down-regulated NRG-1 protein expression. Lentiviral infection with scrambled shRNA (shSCR) did not affect NRG-1 expression.

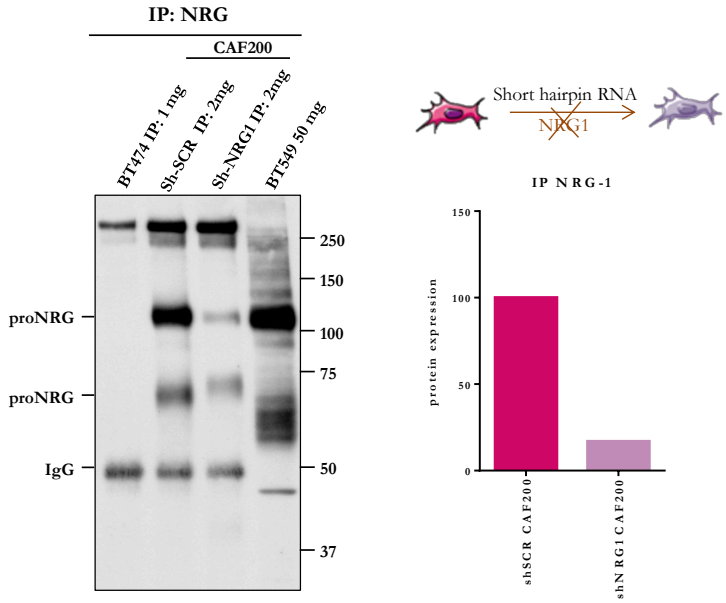


Figure 18. NRG-1 knock down in CAF200 cells. On the left, representative IP for proNRG in shSCR CAF200 and shNRG-1 CAF200 cells. BT474 and BT549 were included as negative and positive experimental controls, respectively. On the right, the quantification corresponding to the proNRG band for each condition.

To further corroborate the efficency of the knockdown, shSCRCAFs and shNRG-1 CAFs cells were fixed and stained for NRG-1 by Immunocytochemistry (IHC). SKBR3 cells were also included as a negative control. In correlation with IP results, shSCR CAFs expressed at protein level (more evidenced in the cell membrane of the cells) whereas shNRG-1 CAFs displayed lower levels in counterpart. SKBR3 were almost negative for NRG-1 staining.

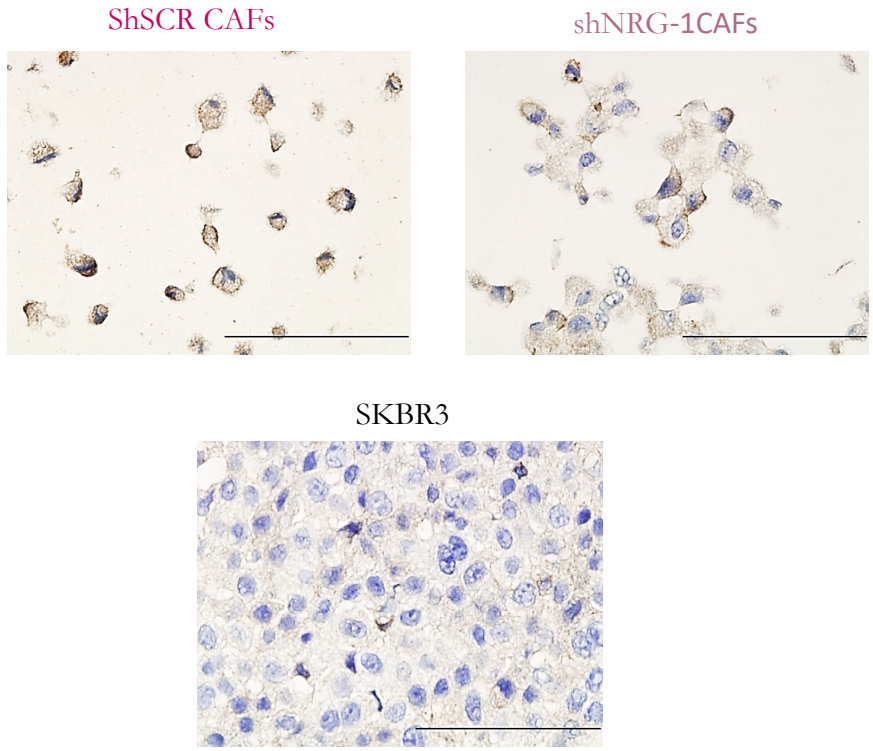


Figure 19. NRG-1 protein evaluation in CAF200 cells wild-type (shSCR) and with stable depletion for NRG-1 (shNRG-1). Representative images from IHC stained cells for NRG-1. Cells were seeded in P150 plates and when they reached a confluence of 70-80% they were harvested, formalin-fixed and paraffin-embedded. Samples were stained for NRG-1. SKBR3 was used as a negative control.

We next assessed cancer cell drug response to trastuzumab in the presence of the media collected from shSCR CAFs and NRG-1-depleted cells. The data generated is summarized in **Figure 20.** As expected, secretome from shSCR CAFs significantly reduced trastuzumab effects on both cancer cell lines. Interestingly, these effects were prevented when the cancer cells were cultured in the presence of paracrine secretome from shNRG1 CAFs.

In summary, we observed that paracrine signalling from CAFs strongly compromised *in vitro* trastuzumab and paclitaxel drug efficacy in HER2+ BC cell lines. Under the hypothesis that NRG-1 secreted from CAFs could be, at least in part, responsible for the acquired drug phenotype in cancer cells, we reproduced same *in vitro* experiments but in the presence of recombinant NRG-1β. In this context, trastuzumab drug efficacy was also heavily impaired. To further validate our hypothesis, we took two different approaches: firstly, we pharmacologically inhibited NRG-1-derived effects on cancer cells using pertuzumab; secondly, we prevented NRG-1 paracrine effects from CAFs by downregulating NRG-1 protein expression. In both cases, we observed that the strategies significantly restored drug sensitivity in HER2+ BC cells.

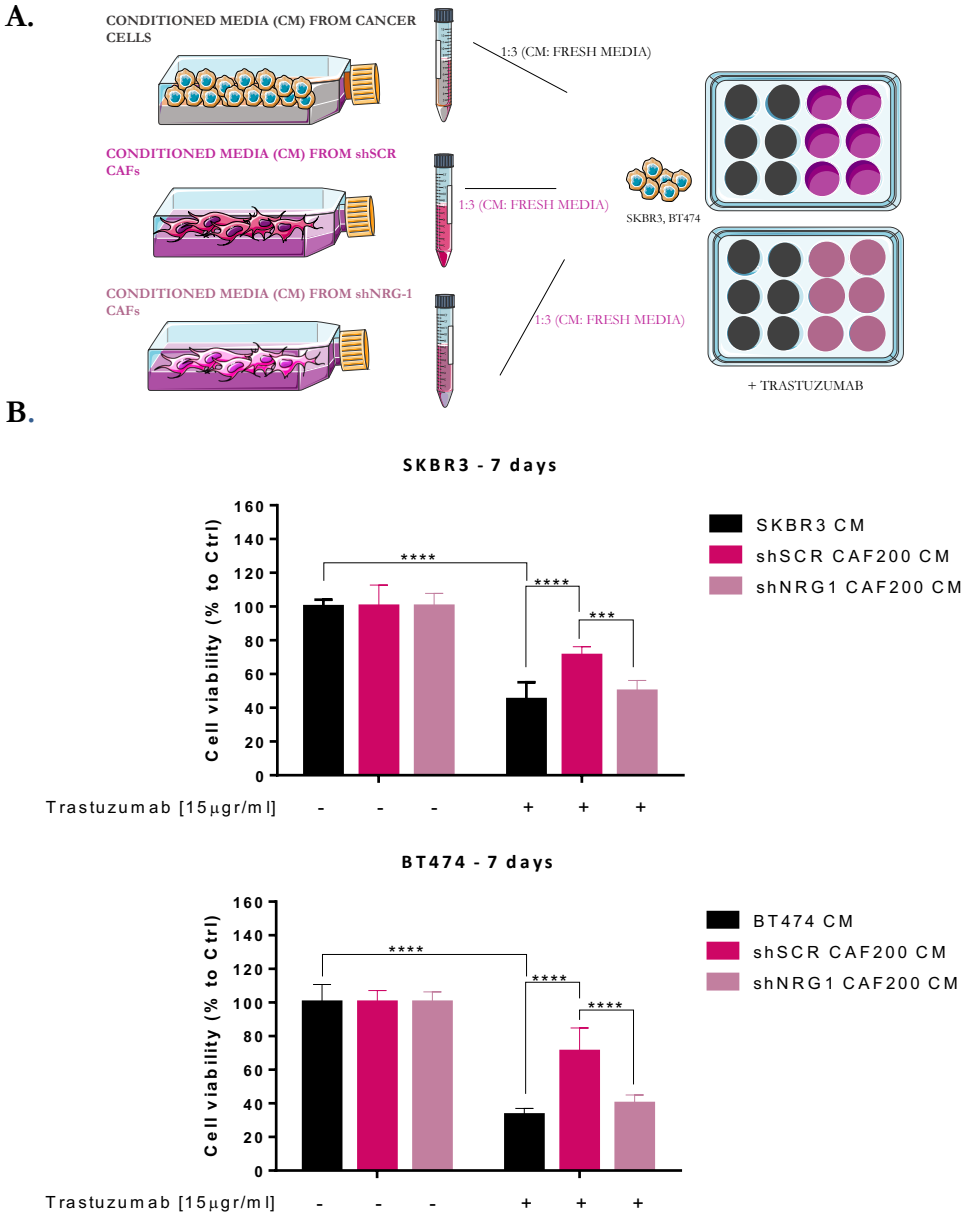


Figure 20. Depletion of NRG-1 expression in CAFs prevented the paracrine effects of CAFs affecting trastuzumab response in SKBR3 and BT474 cells. Diagram of cell viability experiments with trastuzumab in the presence of shSCRCAF200/shNRG-1/HER2+ cell CM. **B.** The graphs show the survival (in % to untreated cells) of cancer cells to trastuzumab in the presence of autocrine or paracrine shSCR/shNRG-1 CAF200 secretomes. The data represents the average ± SD of at least 3 replicates. The statistical analysis was performed using the one-way Anova test, * $p<0.05$.

R10. MICROENVIRONMENTAL NRG-1 ENGAGED ACTIVATION OF ALTERNATIVE MOLECULAR PROGRAMMES IN HER2+ BC CELLS

R10.1 NRG-1β upregulated TGFβ1, TGFβ2 and Snail expression in HER2+ BC cells

We demonstrated previously that recombinant NRG-1β and secretome from CAFs activated the PI3K and MAPK pathways. Interestingly, these cellular cascades have been closely related to TGFβ signalling. In line with this, it has been reported that the activation of these two pathways may result in the activation of non-canonical TGFβ signalling(220). Because of this, we wondered if activating PI3K and MAPK with NRG-1β could modulate TGF-β gene expression in HER2+ BC cells.
To study this, we stimulated SKBR3 and BT474 cells with recombinant NRG-1β at different time points: 6, 12, and 24 hours (with prior O/N serum starvation at 1% FBS), and *TGFβ1* and *TGFβ2* mRNA expression was evaluated through RTqPCR experiments.

As shown in **Figure 21A**, we observed that, in a context of no ligand stimulation, *TGFβ1* and *TGFβ*2 mRNA expression levels remained almost unchanged through time. Interestingly, NRG-1β strongly induced the upregulation of *TGFβ1* and *TGFβ2* mRNA expression in SKBR3 cells.

Moreover, we also analysed the expression of the transcription factor Snail1. Snail1 is normally retained in the cytoplasm by GSK3β and ubiquitin-labelled for proteasome degradation. However, upon PI3K activation, such as growth factor stimulation, AKT becomes activated; this in turn phosphorylates and inactivates GSK3β. Snail1 then becomes stable and accumulates; it translocates to the nucleus and regulates many target genes and cell processes(220). Among these, two processes are critically influenced by the activity of Snail1: the TGFβ signalling pathway and the epithelial-to-mesenchymal transition (EMT) programme(220,241).
In line with this, we performed signal transduction experiments by stimulating cancer cells with recombinant NRG-1β for several time points: 0, 0.5, 2, 24 and 48hrs (prior SS O/N at 1% FBS). After this, protein was extracted, quantified, and immunoblotted.

The resulting data is shown in **Figure 21B**. Upon NRG-1β stimulation, pHER3 activity (measured by an increased phosphorylation in the T1289 motif) was significantly upregulated over short time periods, reaching a peak after 2 hours. As happened previously in other signal transduction experiments, this increased activity was transient and after a longer time (24 and 48 hours) less phospho- and total HER3 levels were detected, probably due to a compensatory signal regulation. Interestingly, enhanced pHER3 activity was also accompanied by an increase in Snail1 protein levels. Similarly, Snail1 protein expression was heavily upregulated at even short times (30 minutes), lasting until 24 hours, when it started to return to basal levels

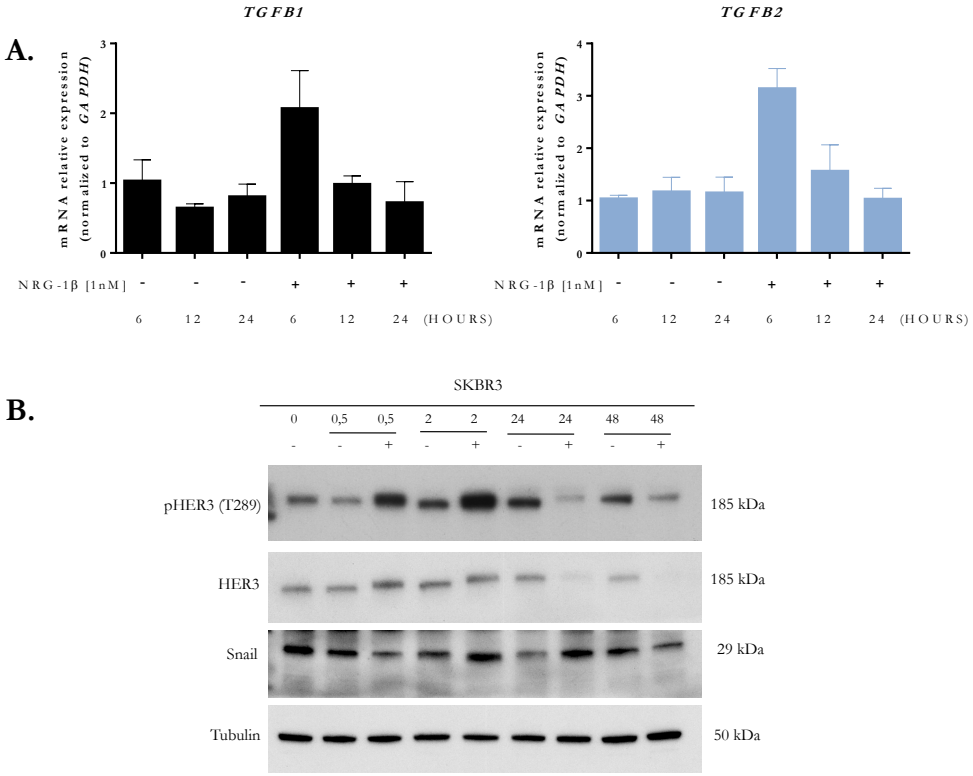


Figure 21. NRG-1 effects on TGFβ1, TGFβ2 mRNA expression and Snail1 at protein level. A. *TGFβ1* and *TGFβ2* mRNA expression in SKBR3 cells ± NRG-1 [1nM]. mRNA relative expression was calculated in relation to time 0. **B.** Representative immunoblot for pHER3 (T1289), HER3 and Snail1 under ± NRG-1β stimulation at different time points. The data in Figures 23A and B is representative of the average ± SD of at least 3 replicates of each cell line. The statistical analysis was performed using the one-way Anova test, * $p<0.05$

As we observed Snail1 protein accumulation up to 24 hours, we decided to perform immunofluorescence (IF) experiments to study the cellular distribution of Snail1. For this, cells were plated in cover-slides and left to attach. After this, the cells were cultured O/N in serum starvation at 1% FBS. Recombinant NRG-1β was then added for 24 hours. The cells were then washed with PBS and fixed with PFA%, blocked with BSA and stained for Snail1, Tubulin, and DAPI. In line with previous results, we observed that in basal conditions (i.e., non-stimulated cells, referred to as Ctrl cells) no Snail1 induction was observed. In contrast, NRG-1β stimulation strongly enhanced the accumulation/upregulation of nuclear Snail1 in SKBR3 cells.

Interestingly, tubulin staining revealed slight morphological changes after NRG-1β stimulation. In this context, some SKBR3 cells looked bigger and enlarged, with protrusions, more similar to a contractile phenotype.
With these results together with the previous ones, we concluded that NRG-1 strongly activated PI3K and MAPK pathways through AKT and ERK1/2 respectively in HER2+ BC cells Following the activation of these two pro-tumorigenic pathways, TGFβ1 and TGFβ2 mRNA expression was enhanced in addition to Snail1 stabilisation and nuclear accumulation.

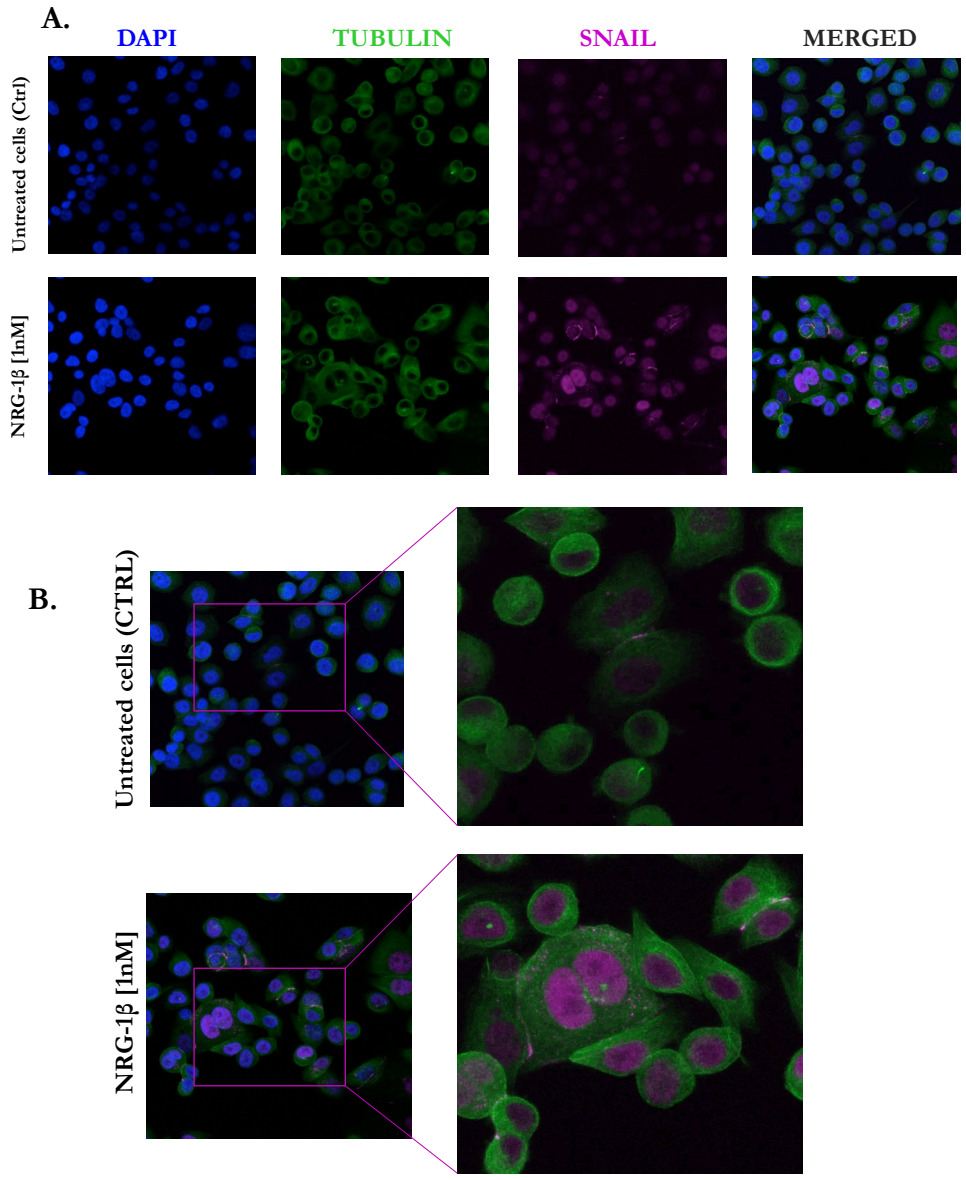


Figure 22. Cellular distribution of Snail1 after NRG-1β stimulation in SKBR3 cells. Cells were plated, left to attach and starved at 1% FBS O/N. NRG-1β was added to the media for 24 hours. The cells were then fixed**,** permeabilized and anti-tubulin (in green) and anti-Snail1 (in magenta) antibodies were incubated. The cells were also stained with DAPI (blue). **A.** From left to right: representative images from split channels for Tubulin, Snail1, DAPI and the merged result. **B.** Snail1 (in magenta) and Tubulin (in green) merged channels for the two experimental conditions. These confocal microscope images are representative of at least 3 independent experimental replicates.

R11. POTENTIAL BI-DIRECTTIONAL FEEDBACK LOOP BETWEEN HER2+ BC CELLS AND CAFs THROUGH NRG-1

So far, we have focused on understanding how CAFs modulate cancer cell biology and drug response. Nonetheless, it is known that in tumours, CAFs establish a strong relationship with cancer cells and that this in turn, is advantageous to both cell types(208).

Epithelial cancer cells secrete growth factors and cytokines into their surroundings that in turn leads to the recruitment and activation of fibroblasts. In particular, the cytokine transforming growth factor beta (TGF-β) has been reported as being one of the key players in fibroblast activation as it promotes ECM synthesis and deposition, matrix remodelling and enhancement of tumour-promoting soluble factor release(208). Having demonstrated that NRG-1β enhanced the expression of the TGF-β programme in cancer cells, we wanted to elucidate whether this could, in turn, result in enhanced CAF activation.

R11.1 Tumour cells differentially modulate signalling transduction in CAFs through paracrine NRG-1

To study this, we performed signal transduction experiments on CAFs by culturing them with the secretome from either non-stimulated cancer cells (referred to as parental (P)) or the secretome from NRG-1β stimulated cells (referred to as NRG-1β-treated cells (NRG-1+)). Negative and positive controls were also included: CAFs cultured with their own CM; and CAFs treated with recombinant TGF-β (1/1000), respectively. The first was a negative experimental control to rule out autocrine TGFβ contributing to activate canonical TGFβ signalling. The second was a positive experimental control. The protocol followed is illustrated in **Figure 23A**. Briefly, CAF200 cells were plated and left to attach. Prior to culturing CAFs with autocrine/paracrine secretomes, they were cultured in SS at 1% FBS O/N. After this, CAFs were cultured for 120 minutes in the presence of CAF200 CM (negative experimental control), SKBR3 non-stimulated CM, or SKBR3 NRG-1+ CM.

With regard to SKBR3 (±NRG-1) and CAF200 CM, in the case of SKBR3 cells, prior to NRG-1β stimulation the cells were starved at 1% FBS O/N; NRG-1 was then added for 24 hours and after which the derived CM was collected, centrifuged and filtered. For autocrine CAF CM, when CAF200 reached a confluence of 70-80%, they were starved at 1% FBS O/N and the derived CM was collected, centrifuged and filtered.

The data relating to these experiments is provided in **Figures 23B** and **23C.** The expression of two of the main components of the TGFβ/Smad dependant pathway, Smad 2 and 3, was evaluated. Moreover, the transcription factor Snail1, one of the target genes of the TGF-β pathway, was also studied.

As shown, the secretome from non-stimulated BC cells (SKBR3 cells, (P)) was able to activate the canonical TGFβ signalling pathway in CAFs. Upregulation of phospho Smad 2/3 and Snail1 protein levels was observed compared to control cells (i.e., CAFs cultured with their own CM).

However, the most interesting results were from SKBR3 cells stimulated with NRG-1β. We observed that the derived CM from NRG-1β stimulated SKBR3 cells was more efficient at inducing the canonical TGF-β pathway. Phospho Smad 2/3 and Snail1 were significantly upregulated under these conditions. Indeed, the levels achieved were similar to those expressed by recombinant TGF-β.

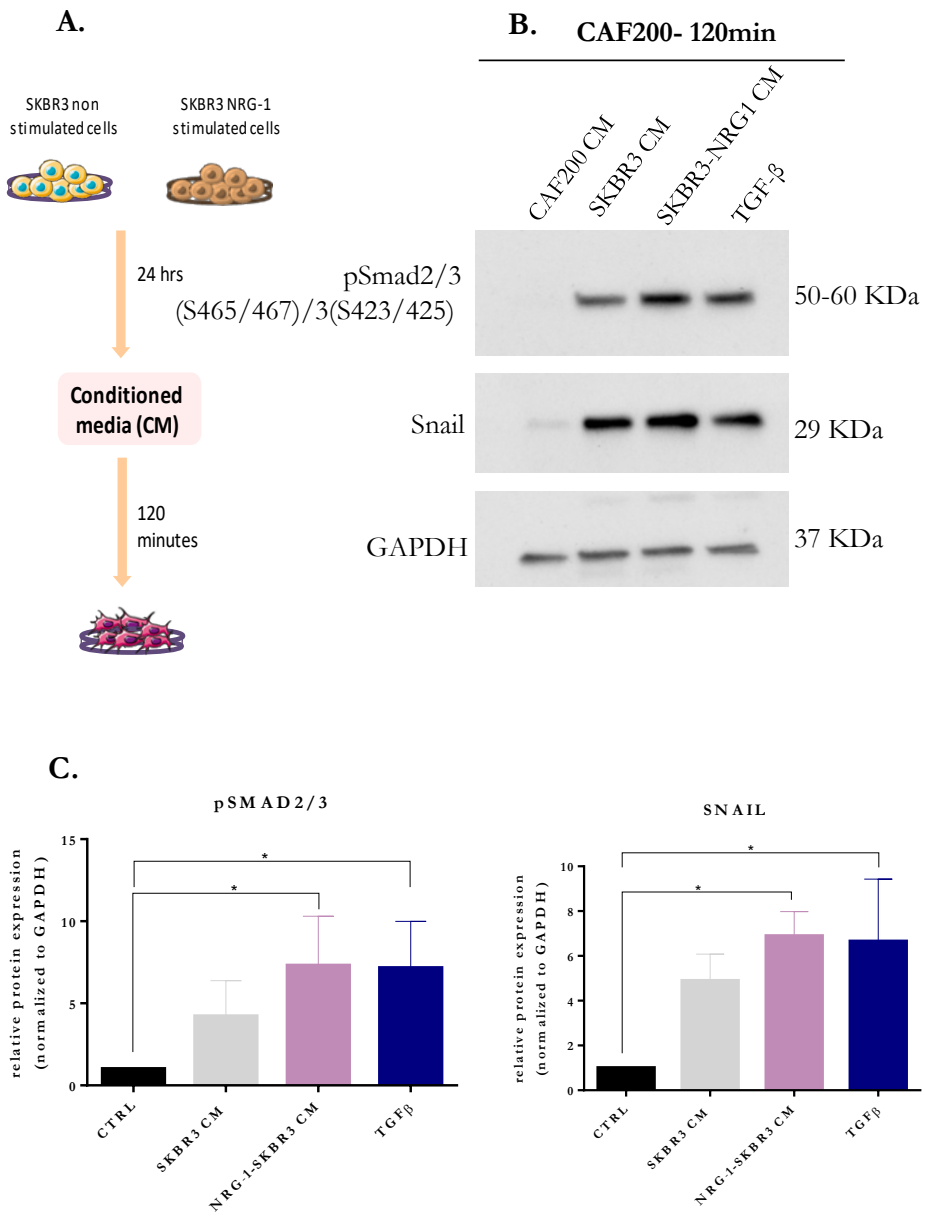


Figure 23. Paracrine effects on TGF-β signalling in CAFs were enhanced by NRG-1 stimulation of cancer cells. A. Diagram of the experimental conditions. **B.** Representative immunoblots for pSmad2/3 and Snail1 in CAF200. **C.** Quantification of the protein expression detected for each condition. The data is representative of the average ± SD of at least three replicates of each cell line.

All this information enabled us to conclude that, as reported, paracrine signalling from cancer cells sustains the TGF-β dependant loop in CAFs. However, upon NRG-1 stimulation of tumour cells, an alternative signalling may occur. In this context, we demonstrated that paracrine NRG-1β is able to induce the TGF-β expression programme in BC cells.

We then hypothesised that, if the expression of the TGFβ programme was upregulated, the derived secretome of these cells may be enriched for TGF-β, although we did not directly quantify TGFβ proteins in these experimental conditions. To test this, we collected the derived CM of either untreated cells or NRG-1-stimulated ones and performed signal transduction experiments on CAFs. Interestingly, we observed that the derived secretome of NRG-1-stimulated cells was the most efficient activator of the TGF-β canonical pathway.

In summary, although there was no physical contact between the tumour cells and CAFs in our *in vitro* model, both cell populations were strongly influenced by paracrine molecules. Indeed, we found that crosstalk between cancer cells and CAFs was present in our *in vitro* model and that this may occur via a NRG-1/TGF-β axis.

R11.2 Pertuzumab may bi-directional crosstalk through a NRG-1/TGFβ feedback loop

Since paracrine crosstalk between cancer cells and CAFs may be mediated through a bi-directional NRG-1/TGFβ axis, we next wanted to determine whether anti-HER2 targeted therapies may be effective at disrupting this. To test this, signal transduction experiments were performed as previously described. In this case however, prior to NRG-1β stimulation, the tumour cells were pre-incubated for 1 hour with either trastuzumab alone or in combination with pertuzumab. The experimental scheme followed was the same as the previous one **(Figure 24A)**.

In line with prior data, the derived secretome from NRG-1+ SKBR3 cells activated the TGFβ/Smad-dependent pathway in CAFs. Interestingly, these effects were not supressed by pre-incubating cells with trastuzumab. However, when cancer cells were treated in combination with pertuzumab, different results were observed. As shown in the immunoblots and the quantification below, the drug combination was effective at attenuating paracrine signalling from tumour cells in CAFs **(Figure 24B and 24C).**

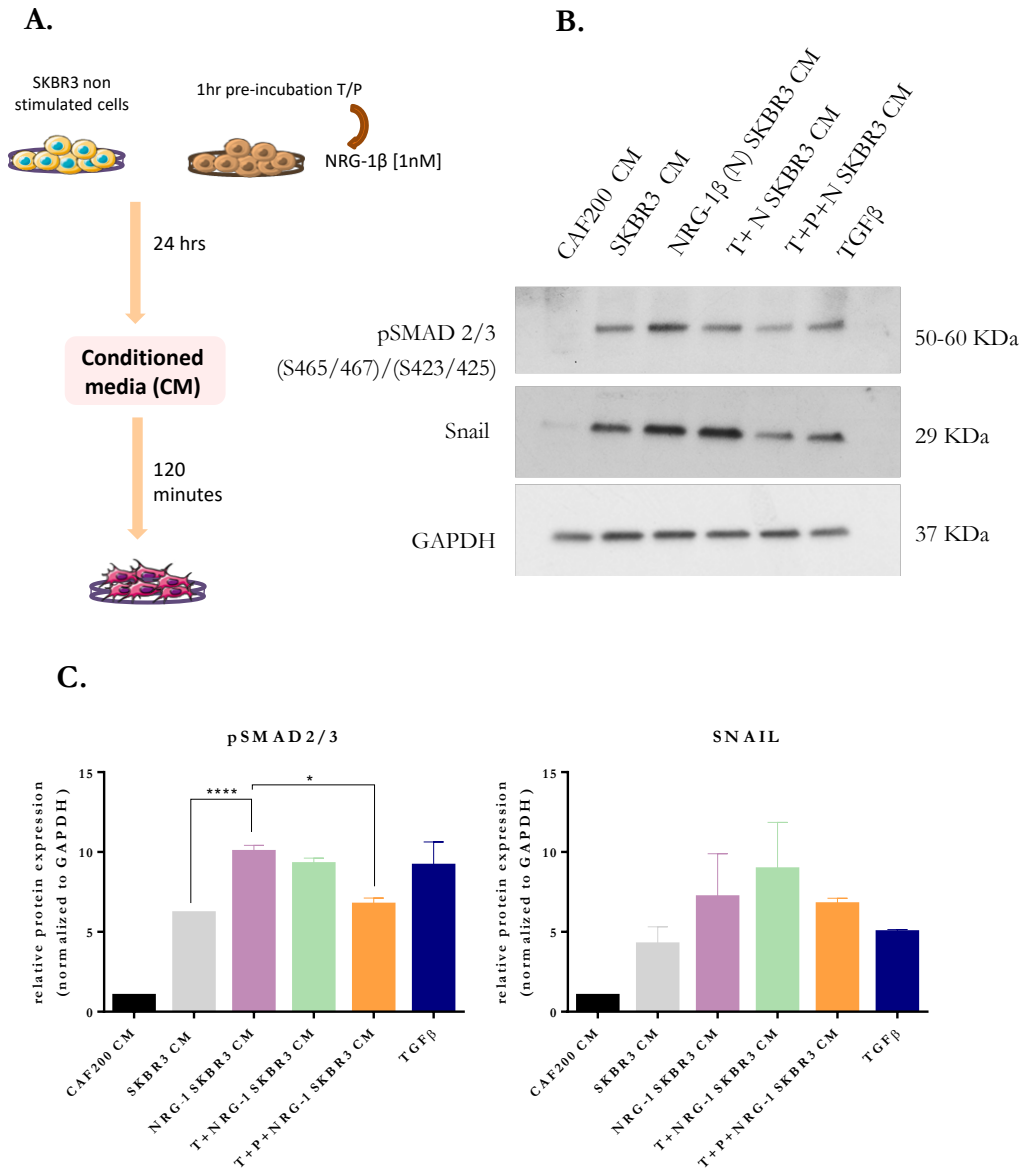


Figure 24. Paracrine effects on TGFβ signalling in CAFs led by NRG-1 were attenuated by treating cancer cells with trastuzumab and pertuzumab. A. Diagram of the experimental conditions. **B.** Representative immunoblots for phosphor Smad2/3 and Snail1 in CAF200. **C.** Quantification of the detected protein expression for each condition. The data is representative of the average ± SD of at least three replicates of each cell line.

R12. GENERATION AND CHARACTERIZATION 3D HETEROTYPIC SCAFFOLD-FREE MODELS

As shown in our study, CAF played important roles in response to anti-HER2 monoclonal antibodies in breast cancer. For this reason, in the last year of this doctoral thesis, our group started to set up new techniques for studying direct cell-to-cell contact interactions between tumour cells and CAFs. These would allow us to further understand paracrine crosstalk through NRG-1 in heterotypic models, in which the immune system could be also incorporated. Only preliminarly data regarding these experiments were included in this manuscript.

Firstly, we studied the effects of direct crosstalk between cancer cells and CAFs by co-culturing them *in vitro*. For this, we plated HER2+ BC cells either on their own or by mixing them with CAF200 cells in a 1:1 ratio in ultra-low attachment round bottom 96-well plates. The 3D culture morphology was assessed 48 hours later. As shown in **Figure 25A**, HER2+ cancer cells displayed *per se,* differential 3D formation capacity. Whereas BT474 cells formed round-compact spheroids, either by their own or when mixed with CAFs; SKBR3 cells were not able to aggregate in 3D. Nonetheless, this capacity was slightly improved when SKBR3 cells were culturd with CAFs (in green, GFP+).

Secondly, we also performed IHC staining of these homotypic and heterotypic 3D-scaffold free cultures. For this, we stained these tumours with Hematoxylin eosin (HE) as well as with Vimentin (a mesenchymal marker) and with Cytokeratin 19 (CK19, an epithelial marker) to distinguish both cell populations. We observed different morphologies and different patterns of tumour cell aggregation with CAFs **(Figure 25B).** As previously, the poor 3D-formation capacity of SKBR3 cells was evidenced in the IHC staining, and as describe above, this capacity was improved when cultured with CAFs **(Figure 25B).**

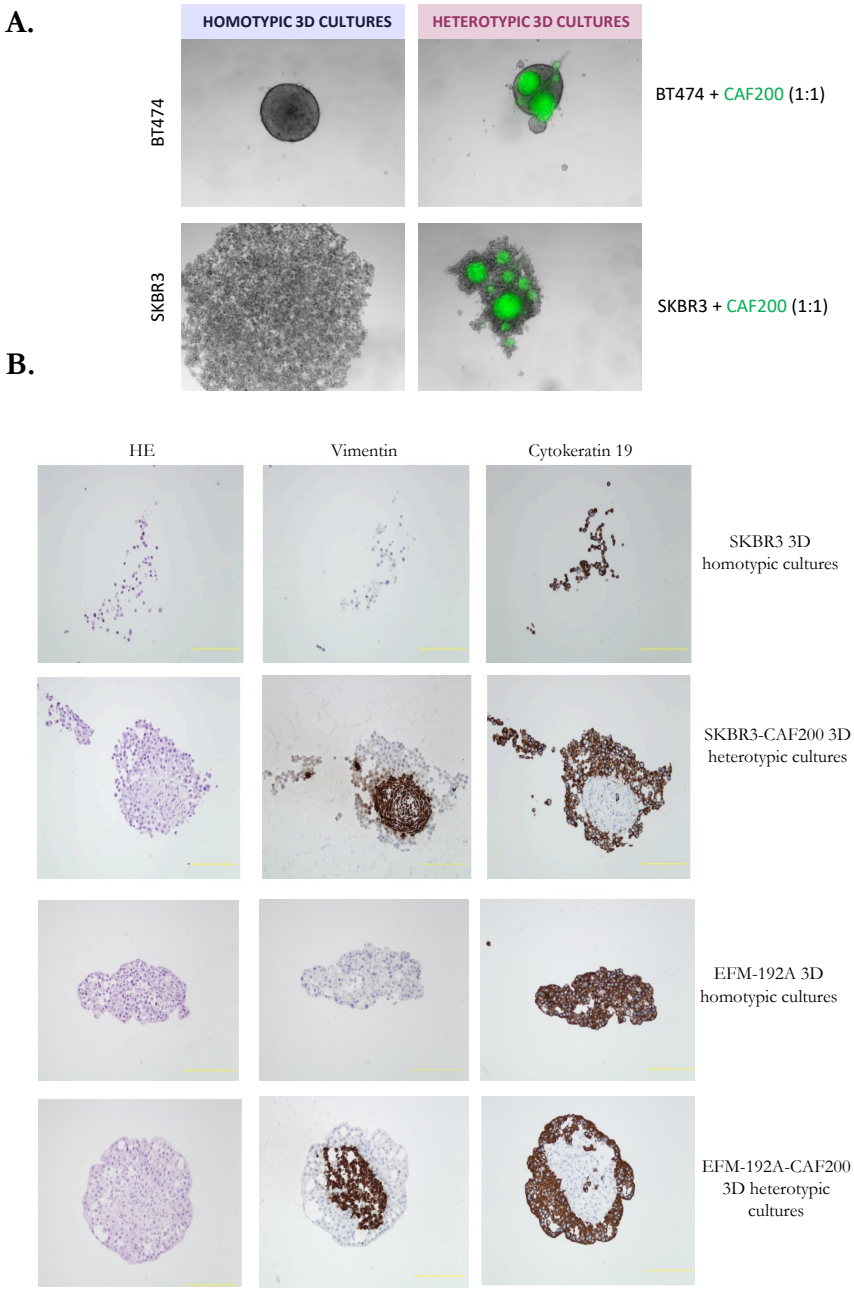


Figure 25. ***In vitro*** **model of 3D direct cancer cell-CAF crosstalk. A.** Representative images from homotypic and heterotypic 3D cultures (1:1). On the left: light microscope images from homotypic 3D cultures. On the right: representative images from heterotypic 3Ds. The cells in green are CAF200 (GFP+). The images are the result of merging the bright and fluorescence fields. **B.** Representative images of HE staining, and Vimentin and CK19 staining by IHC.

R13. GENERATION AND CHARACTERIZATION OF PRIMARY CANCER-ASSOCIATED FIBROBLASTS (CAFs) DERIVED FROM HER2+ BC PATIENTS

The third objective of this thesis was to translate potential findings derived from objectives I and II into HER2+ BC patient samples. To do this, a panel of primary CAF cultures derived from BC patients was generated. From June 2018 until the submission date of this doctoral thesis, tumour biopsies from BC patient biopsies at Hospital del Mar were collected. To date, a total of 100 primary CAF cultures have been derived, and these are still being characterized. Moreover, since primary CAF cultures cannot be extended for many passages, some of these were selected for hTERT immortalization. The relative data on this cannot be shown as the work is still on-going.

Of all the primary BC cultures, for this project we considered only those deriving from HER2+ BC patients: a total of 25.

The protocol is summarized in **Figure 26A**. Tumour samples were plated on a culture dish, poorly adhering cells were removed, and the remaining cells were left to grow. As shown in the images in **Figure 26B,** sprouting cell outgrew from the tumour bulk and started to attach to the plate surface. These cells attached to the petri dish and proliferated, filling the plate. Our protocol resulted in an enriched fibroblast population. Macroscopically, the attached cells were long and spindly with small nuclei and fine chromatin, corresponding to a typical mesenchymal phenotype. In this step, a low-passage cellular stock was generated, and the remaining cells were sub-cultured for molecular and phenotypic characterization.

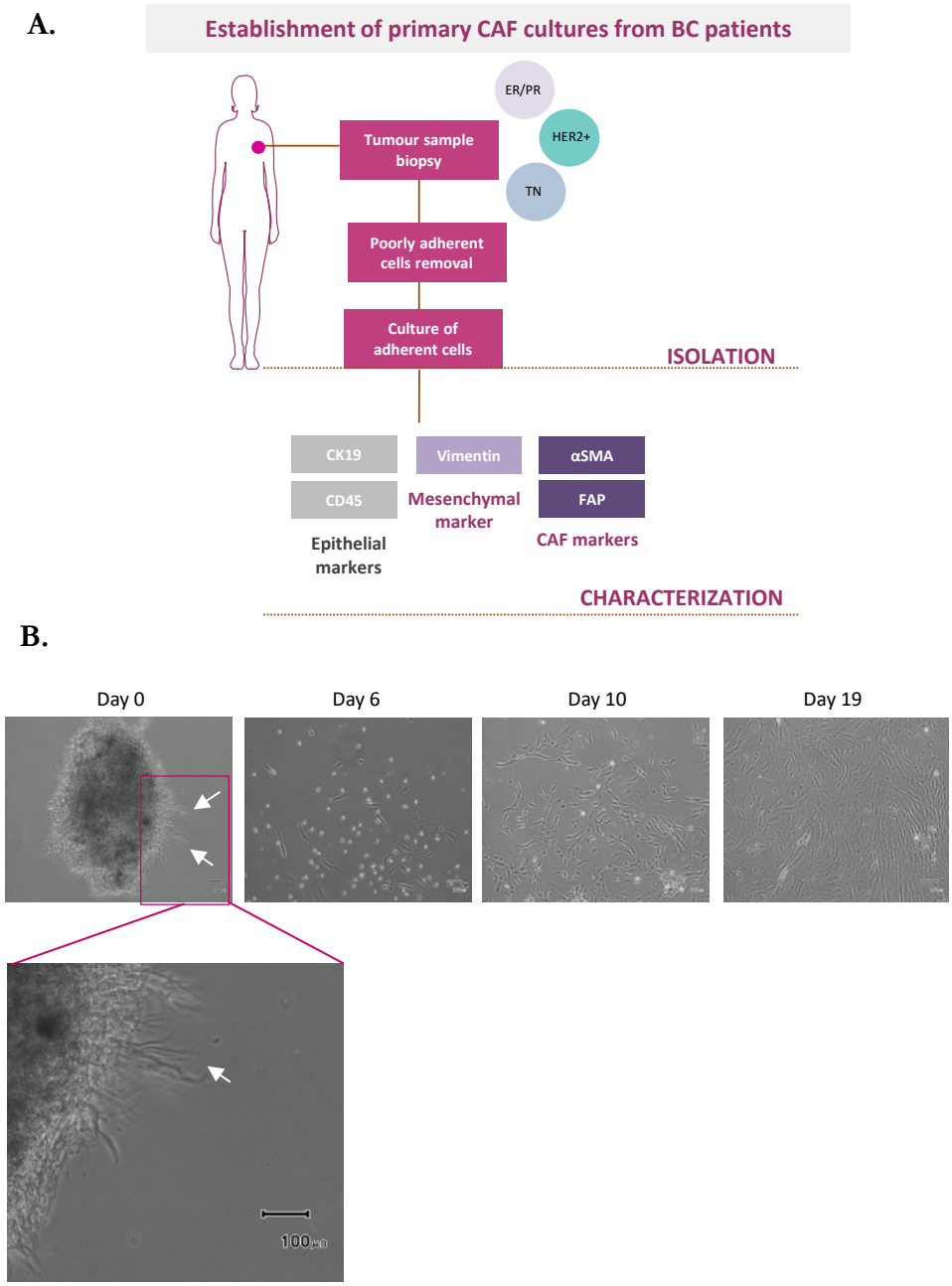


Figure 26. CAF primary culture generation and characterization. A. Description of the isolation and characterization protocol. **B.** Example of a primary CAF culture establishing through time. The images were taken under a light microscope (scale 100µm). The white arrows in the day 0 image (on the left) indicate long sprouting cells outgrowing from the tumour.

To phenotypically and molecularly characterize our collection of primary CAFs, a panel of different cell-lineage markers was analysed. First, we analysed the expression of Cytokeratin 19 (CK19) and αSMA by WB **(Figure 27A)**. On the one hand, CK19 is a well-established epithelial marker and was used to rule out the potential presence of tumour cells. On the other hand, αSMA is typically expressed by activated fibroblasts, and, therefore, by CAFs. As shown, the primary cultures were negative for CK19 and strongly positive for αSMA. Interestingly, we observed that αSMA protein expression levels varied among all the primary CAFs evaluated, also highlighting the extreme heterogeneity of CAFs.

Moreover, we also analysed HER2 and HER3 expression in these primary cultures. Interestingly, we observed non HER2 nor HER3 detectable expression levels in any of them **(Figure 27A).**
Additionally, two primary CAFs were selected for immunocytochemistry (ICC) analysis. BC-T50 and BC-T41 were plated on specific slides and stained for Vimentin and αSMA (mesenchymal markers) and CK19 (epithelial marker)). As shown, both primary CAFs were strongly positive for Vimentin and αSMA and negative for CK19. **(Figure 27B).**

Through this, we determined that although we applied no cell sorting techniques, our method resulted in highly enriched CAF primary cultures with apparently minimal cross contamination from other cell types.

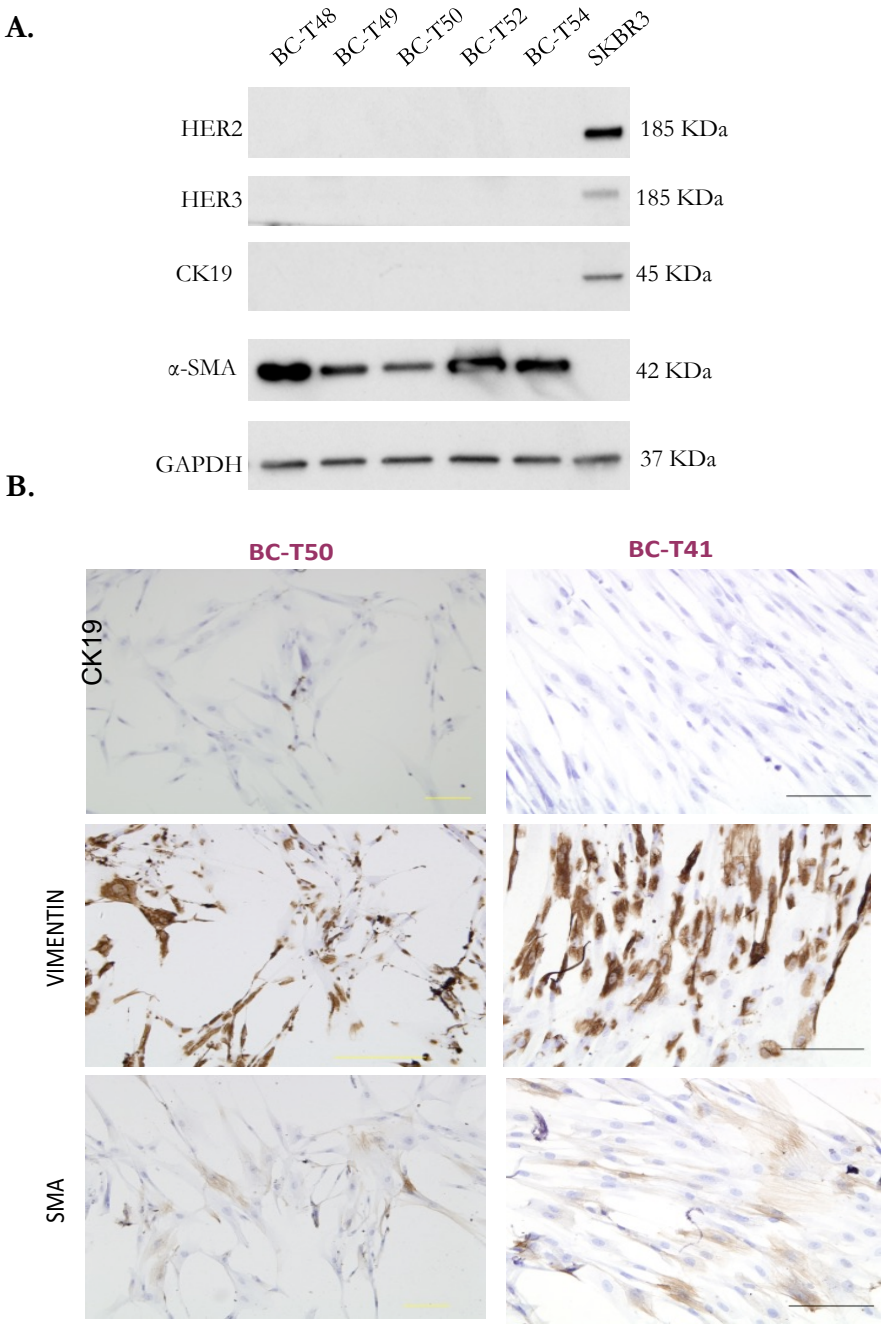


Figure 27. Molecular and phenotypic characterization of primary CAF cultures derived from HER2+ BC patients. A. Immunoblot images for HER2, HER3, CK19, αSMA, and GAPDH. **B.** Representative IHC images for BC-T50 and BCT-41 primary CAFs stained for CK19, Vimentin, and αSMA,**.** The data from Figures 28 A and B is representative of 1 replicate of each cell line.

R13.1 HER2+ primary CAFs display heterogeneous expression of αSMA, FAP, and CXCL12

Our collection of primary CAFs expressed variable levels of αSMA, one of the main accepted CAF markers. In recent years, the concept of stromal heterogeneity has gained research interest in the oncological field. Despite being more genetically stable, CAFs are a highly heterogeneous population and differences in αSMA expression, as well as other markers, have been linked to pro- or anti-tumour progression effects or drug resistance.

In line with this, FAP and CXCL12 were analysed in our panel of primary CAFs. For this, when the primary CAF cultures reached a 70-80% confluence, RNA was extracted and RTqPCR experiments were performed.

The results are depicted in **Figure 28** As shown, FAP and CXCL12 (SDF-1) mRNA expression levels also varied between primary CAFs. Although preliminary, these results suggested to us that primary CAFs recapitulate stromal heterogeneity, as reported by other authors for breast and other solid tumours.

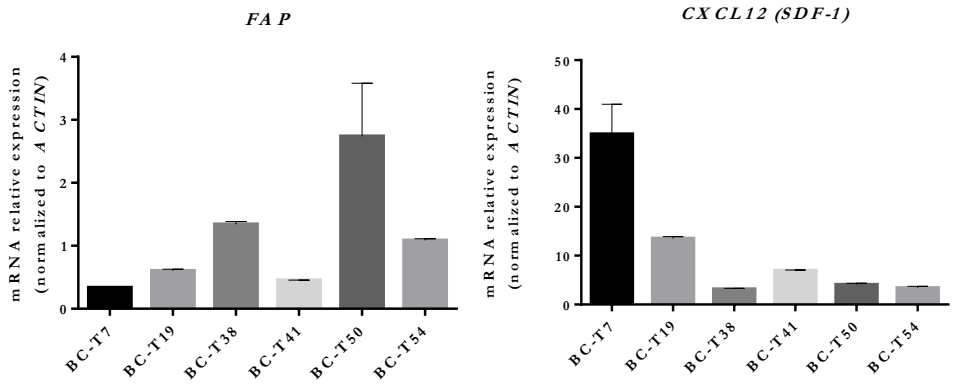


Figure 28. FAP and CXCL12 (SDF-1) mRNA expression in the collection of primary CAFs derived from HER2+ BC patients. The left graph corresponds to FAP mRNA expression The right one refers to CXCL12 mRNA expression. The data is representative for 1 replicate of each primary CAF, except for BC-T50 where 4 replicates were included. The data is represented as mRNA relative expression (referring to CAF200) and normalized with Actin as housekeeping.

R13.2 NRG-1, the HER3 ligand, is expressed by primary CAFs derived from HER2+ BC patients

We analysed the expression of *NRG1* at mRNA and protein level. The results of the RTqPCR experiments are shown in **Figure 29.** We observed that in our collection of primary CAFs, NRG-1 mRNA expression was heterogeneous and lower than in CAF200, our reference cell line for fibroblasts. However, all of them showed higher levels of NRG-1 than SKBR3 and BT474 cells, the HER2+ BC cell lines. Interestingly, we observed that BC-T50, showed at mRNA level, similar levels of *NRG1* expression as CAF200.

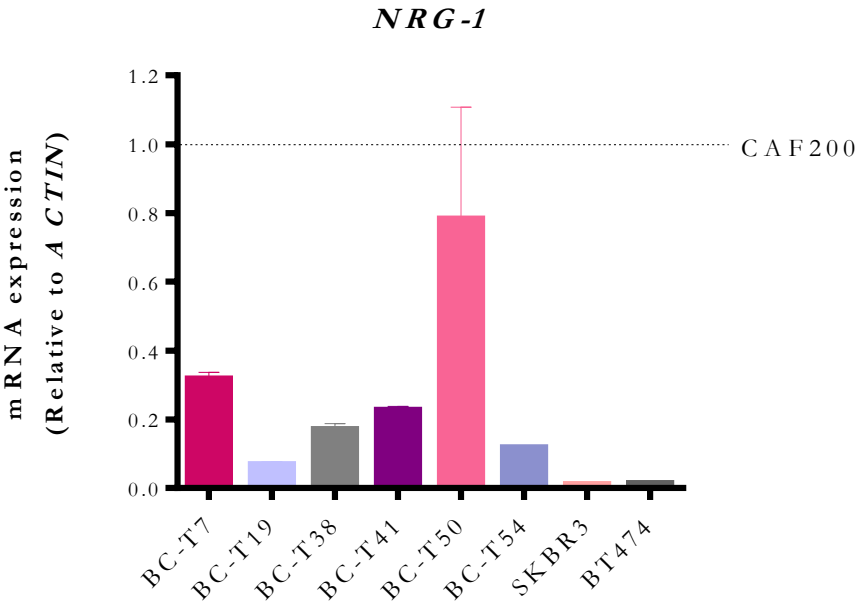


Figure 29 *NRG1* mRNA distribution across primary CAF cultures. Primary CAF cultures from HER2+ BC patients were plated at low passages. When the cell confluence reached 70-80%, RNA was extracted and subjected to RTqPCR experiments. The data is representative of 1 replicate for each primary CAF, except for BC-T50 where 4 replicates were included. The data is represented as mRNA relative expression (referring to CAF200) and normalized with Actin as the housekeeping gene.

R13.3 Primary NRG-1+ CAFs reduce trastuzumab and paclitaxel response in HER2+ BC cells

As reported, *NRG1* mRNA distribution varied across our panel of primary CAFs. To validate our hypothesis that paracrine NRG-1 from CAFs reduces trastuzumab and paclitaxel response in HER2+ BC cells, we selected BC-T50 primary CAF cultures and analysed the modulatory effects of these primary CAFs in the drug response of HER2+ BC cells.

SKBR3, BT474, and EFM192A cells were plated and cultured for 7 days in the presence of either autocrine or BCT-50 CM, both prepared in a ratio of 1:3 (CM:fresh media). The cells were treated with trastuzumab and on day 4 of the treatment, paclitaxel was added for 72 hours **(Figure 30A)**.

In line with previous results, we found that autocrine molecules did not alter either trastuzumab or paclitaxel drug sensitivity in any of the BC cell lines. In the case of the drug combination, no contribution from autocrine molecules was observed. Interestingly, when the cells were cultured with the derived CM from BC-T50 primary CAFs, the drug phenotype of the BC cell lines changed. In 2 out of 3 cell lines (BT474 and EFM192A), trastuzumab effects were significantly reduced. Similarly, in SKBR3 and EFM192A cells, the effects of paclitaxel were significantly abrogated in the presence of autocrine secretome from primary CAFs.

Furthermore, we also observed that in all cell lines drug combination efficacy was reduced and in two out of the three cell lines (SKBR3 and EMF-192A), the differences were statistically significant **(Figure 30B).**

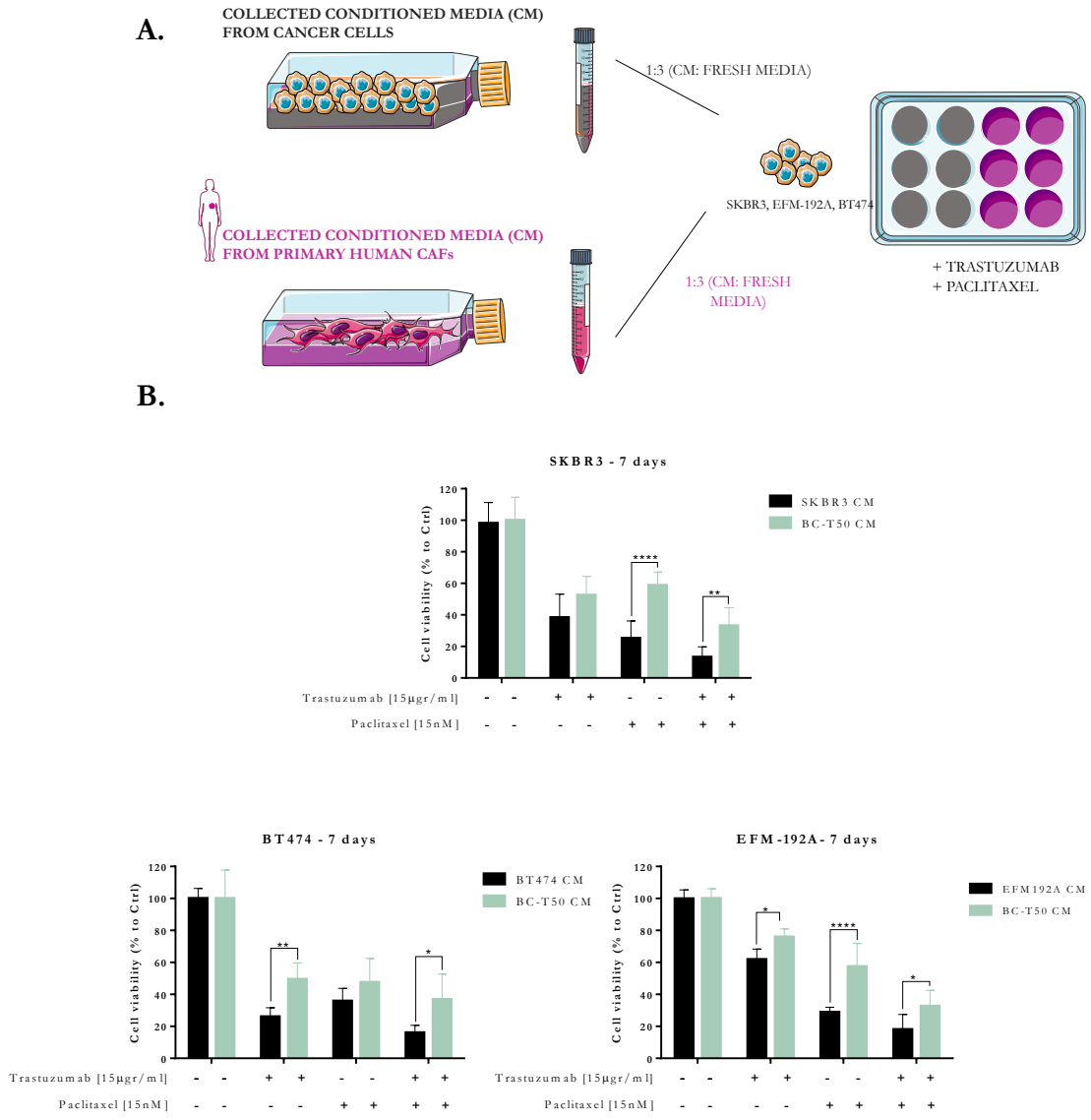


Figure 30. Primary CAFs derived from HER2+ BC patients reduced HER2+ BC cell line drug response. NRG-1 expressing CAFs derived from HER2+ BC patients reduced trastuzumab and paclitaxel response in HER2+ BC cell lines. Diagram of cell viability experiments with trastuzumab in the presence of shSCRCAF200/shNRG-1/HER2+ cell CM. **B.** The graphs show the survival (in % to untreated cells, referred to as CTRL) of cancer cells to trastuzumab in the presence of autocrine or paracrine shSCR/shNRG-1 CAF200 secretomes. The data represents the average ± SD of at least 3 replicates. The statistical analysis involved the one-way Anova test, * $p<0.05$.

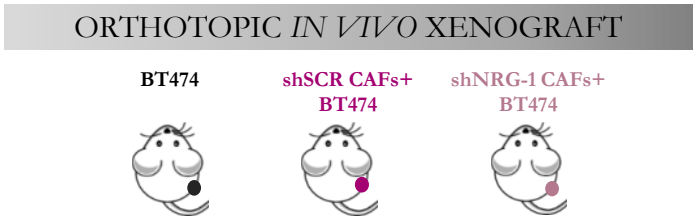
R14. NRG-1 EXPRESSING CAFs ENHANCED *IN VIVO* ORTHOTOPIC TUMOUR GROWTH

Taking into account our previous data we decided to study the crosstalk between CAFs and cancer cells *in vivo*.

Xenograft tumours were established by injecting orthotopic cancer cells alone (1.5x10^6 cells/mice) or co-injecting them with either shSCR CAF200 or shNRG-1 CAF200 cells, in a ratio of 1:1, into the mammary fat pad of 7-week-old SCID-BEIGE JY female mice. Moreover, as BT474 cells were HR+, an estradiol pellet was subcutaneously implanted into each animal **(Figure 31A).**

For the *in vivo* implantation rate, we observed no statistical differences in any of the conditions. Therefore, at the end of the experiment (day 82), same proportion of animals developed tumours (values around 60-65% for each condition **(Figure 31B).**

A.



B.

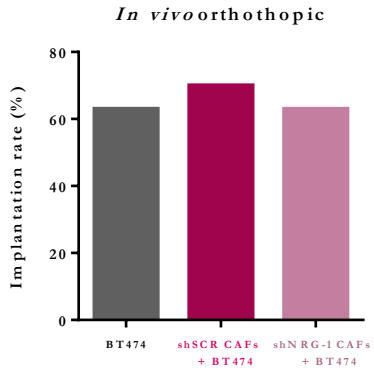
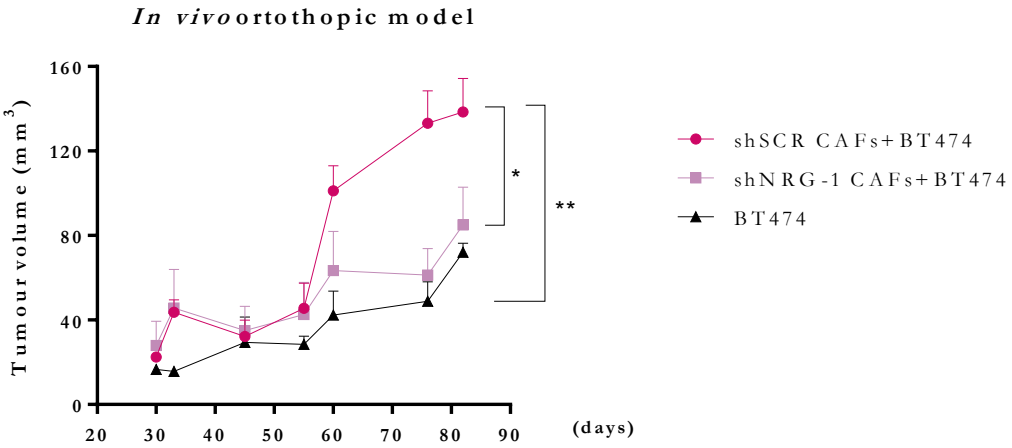


Figure 31. Orthotopic xenograft model of BT474 cells co-injected with NRG-1 expressing CAFs (shSCR) or NRG-1 knock down CAFs (shNRG-1). A. Diagram illustrating the experimental design. **B.** Orthotopic tumour implantation rates. The data represents the average (in %) for each experimental condition.

The tumours were followed up throughout the experiment. Once a week, tumour volume was measured in all the animals. As shown in **Figure 32A**, no differences were observed in the beginning between the three experimental arms. However, on day 55, BT474 cells co-injected with shSCR CAFs started to differentiate from the others. In terms of volumes, these tumours started to look bigger when measured, and this effect increased through time, becoming statistically significant **(Figure 32C)**. Interestingly, co-injection of BT474 with NRG-1-depleted CAFs resulted in reduced tumour growth compared to WT CAFs. Indeed, the growth behaviour resembled that of BT474 cells co-injected alone.

A.



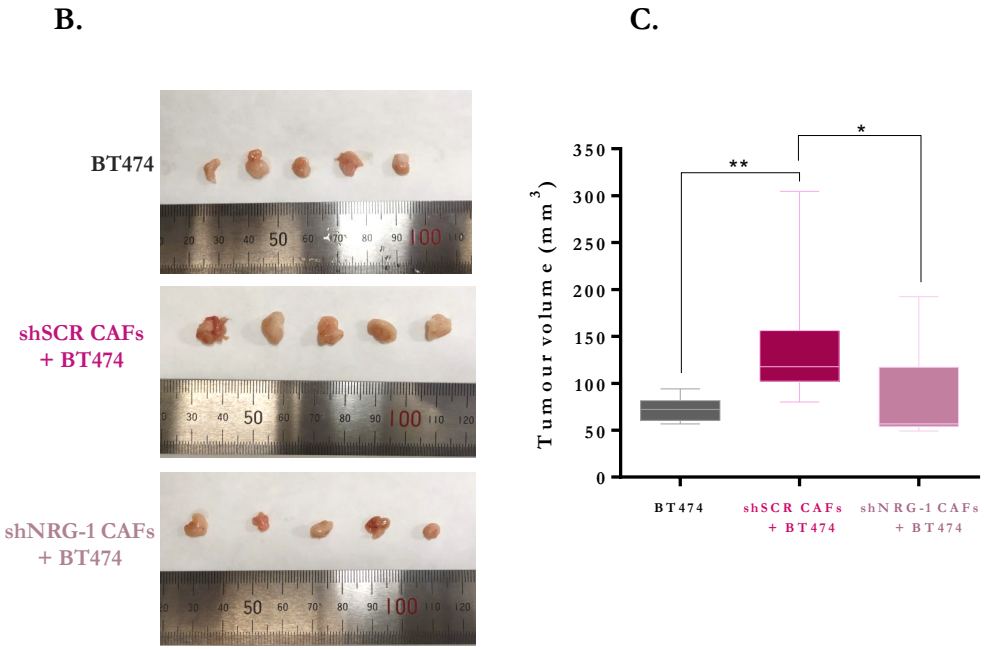


Figure 32. NRG-1 expressing CAFs enhanced BT474 orthotopic *in vivo* tumour growth. A. Tumour volume growth over 11 weeks. shSCRCAFs+ BT474 (in pink), shNRG-1CAFs+BT474 (in grey), and BT474 cells (in violet). **B.** Representative images of excised tumours from each experimental arm. **C.** Box plot of tumour volume for each condition at the end-point of the experiment. The statistical analysis involved the one-way Anova test, * $p<0.05$.

At the end of the experiment, tumours were excised and subjected to histological analysis by hematoxylin and eosin (HE), for morphological evaluation of the tumours, and by IHC staining for assessing cell proliferation. As shown in **Figure 33.** the histology of the tumours was similar, although tumour cellularity of co-injected shSCR CAFs-BT474 tumours was slightly higher than in the other groups. pHistone H3 (pH3) was used for assessing cell proliferation. With regard to this marker, pH3 is specifically phosphorylated during both mitosis and meiosis, being heavily phosphorylated in metaphase chromosomes(242). As depicted in the images, differences were found between the different experimental groups.

All of this suggested to us that, CAFs were able to foster *in vivo* growth of tumour cells. Since the absence of NRG-1 in CAFs was able to attenuate or delay this effect, we hypothesized that NRG-1 expressing CAFs may transform tumour cells *in vivo*, by conferring greater proliferation potential.

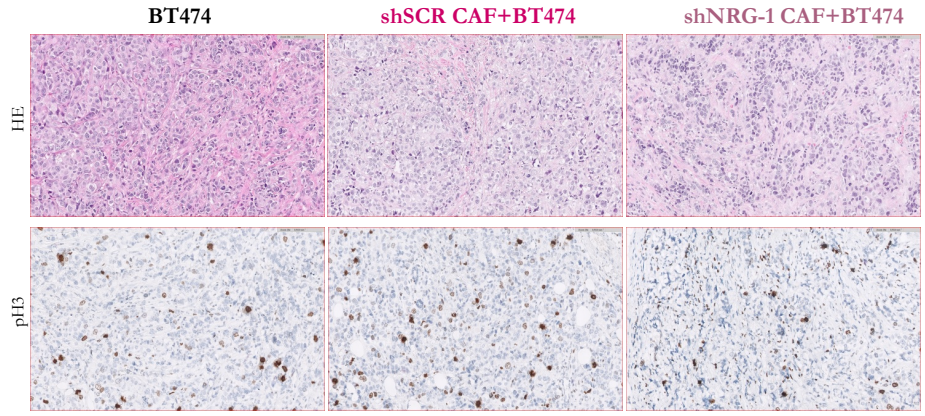


Figure 33. IHC analyses of *in-vivo* excised BT474 tumour cells alone or co-injected with SCR/shNRG-1 CAFs. A. Histological analysis of tumour sections from excised BT474; shSCR CAF+BT474; and shNRG-1 CAF+BT474 tumours. HE and pH3 have been used for assessing cell morphology and cell proliferation, respectively. Data are representative of independent tumours harvested from 7 animals of each group.

For evaluating protective effects of NRG-1 in trastuzumab drug efficacy in HER2-positive tumours, ex-vivo experiments were performed, according to our own experience(91,243). When animals were sacrificed, fresh BT474 excised tumours were minced and immediately cultured for 5 days upon differential experimental conditions **(see Figure 34)**. After this, samples were formalin-fixed and paraffin-embedded (FFPE) and analysed by IHC. As previously, pH3 was used for assessing cell proliferation, whereas cl-caspase 3 was used as a marker of cell death. Interestingly, NRG-1 significantly induced proliferation of tumour cells. Trastuzumab by alone showed opposite effects.

However, antiproliferative effects of trastuzumab were counteracted by paracrine NRG-1. In line with this, whereas trastuzumab in its one was able to induce apoptosis in tumours, these effects were impaired in the presence of NRG-1. As we previously evidenced, pertuzumab addition in this context, potently restored trastuzumab drug efficacy. Indeed, drug combination was greatly superior on inducing higher anti-proliferative and pro-apoptotic effects in tumours than trastuzumab alone, despite the presence of NRG-1.

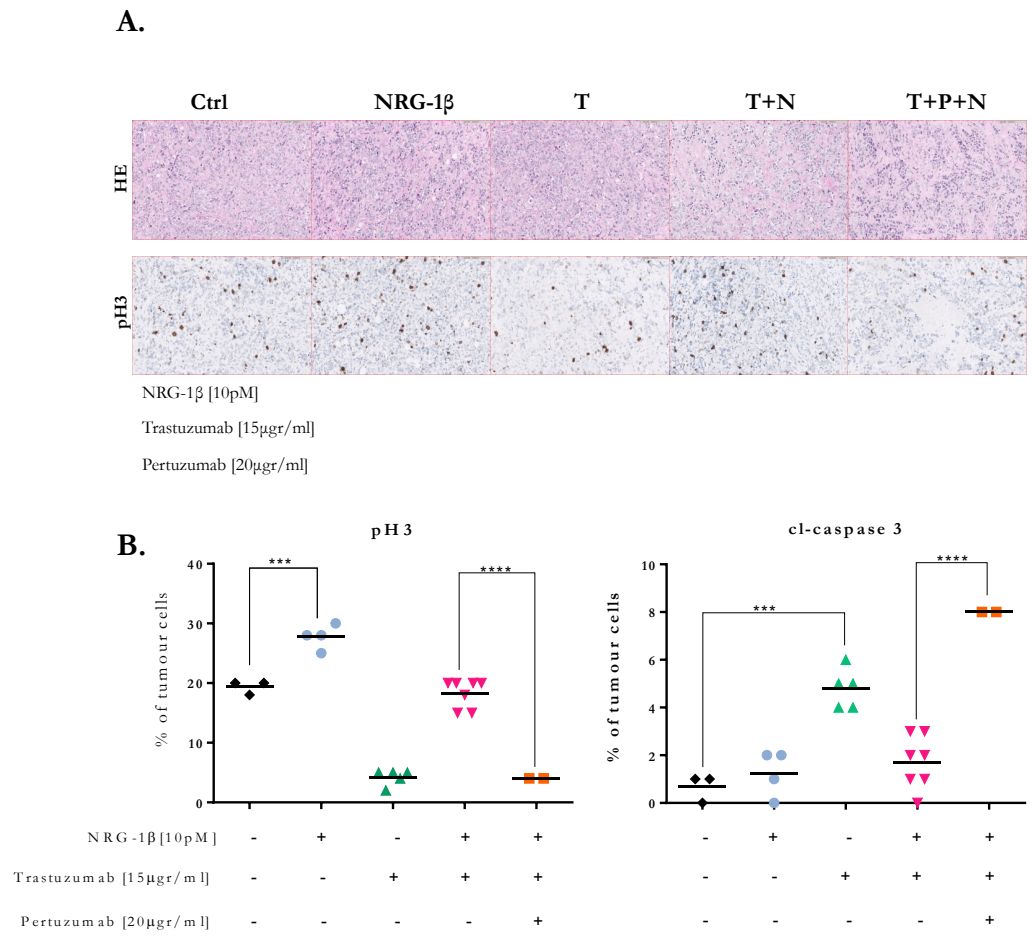


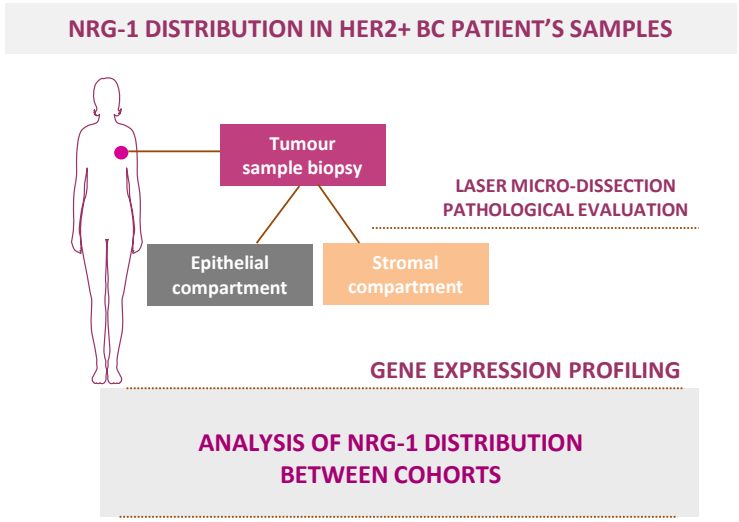
Figure 34. NRG-1β, trastuzumab and pertuzumab effects in ex-vivo BT474 tumours. A.Representative images of HE and pH3 for each one of the experimental conditions. From left to right, respectively: Untreated (CTRL), NRG-1b, Trastuzumab (T), T+NRG-1, T+pertuzumab (P)+NRG-1.**B.** Scatter dot plots showing the % of positive tumour cells for pH3 (in the left graph) and cl-caspase 3 (in the right graph).

R15. EXPRESSION OF NRG-1 IN TUMOUR-ASSOCIATED STROMA FROM HER2+ BC PATIENTS

So far, we have seen that immortalized CAFs derived from HER2+ BC patients express NRG-1. Moreover, we have corroborated these results in a panel of primary CAFs derived from HER2+ BC patients from Hospital del Mar. In contrast, HER2+ BC cells display very low levels of NRG-1. Armed with this information, our next step was to study NRG-1 distribution in BC tumours.

To address this question, we took advantage of public RNA datasets and bioinformatically analysed these.
A work by Liu et al., published in 2017, compared the tumour epithelium and tumour-associated stroma from HER2+ BC patients. The study included 40 samples of tumour epithelium and 39 samples of tumour stroma. The relative raw data from this study was downloaded from the GEO website (GSE83591, hereinafter referred to as the "Liu Dataset"). A comparative analysis for NRG-1 expression in both tissue specimens (the epithelial and the stromal compartments) was performed. In line with our previous results, the epithelial compartment expressed significantly lower levels of NRG-1 than the tumour associated stroma from these patients ($p < 0.01$, **Figure 35B**).

A.



B.

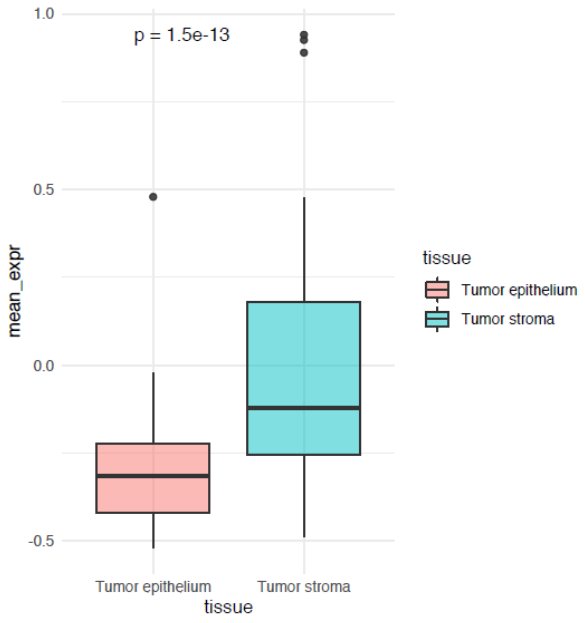


Figure 35. NRG-1 distribution in HER2+ BC tumours. ***A.*** Illustrative scheme for NRG-1 screening in tumour epithelium and tumour stroma datasets from *Liu et al.* **B.** Box plot showing normalized NRG-1 expression levels across the tumour epithelium and the tumour-associated stroma. Horizontal line within the box represents the median expression in each compartment. The area of the box extends from the first quartile (bottom) to the third quartile (top) of the expression distribution. The whiskers extend to 1.5x the interquartile range (1st quartile - 3rd quartile) and the dots represent outliers. All p-values shown have been adjusted for multiple comparisons (see Material and methods).

Next, we sought to assess if NRG1 expression was higher in the tumour stroma when compared with non-tumour associated stroma. For that, we first compared the NRG-1 expression between both stromal samples included in the Liu Dataset **(Figure 36)**. Interestingly, NRG-1 expression levels were significantly higher in the tumour-associated stroma from HER2+ BC patients.

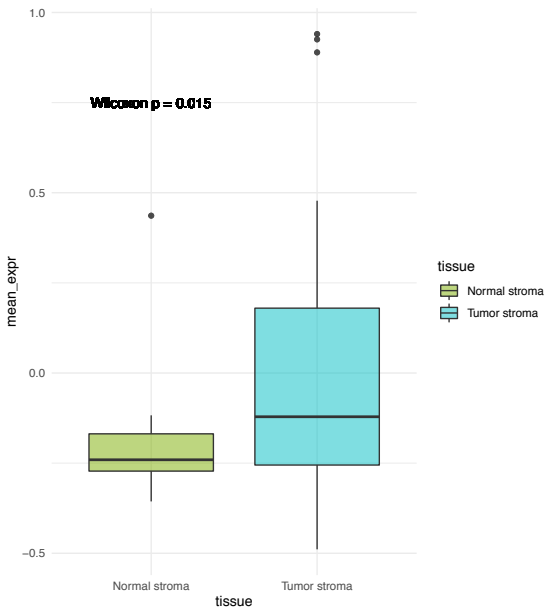


Figure 36. NRG-1 distribution in HER2+ BC tumours. Box plot showing normalized NRG-1 expression levels across tumour- and non-tumour-associated stroma. Horizontal line within the box represents the median expression in each compartment. The area of the box extends from the first quartile (bottom) to the third quartile (top) of the expression distribution. The whiskers extend to 1.5x the interquartile range (1st quartile - 3rd quartile) and the dots represent outliers. All p-values shown have been adjusted for multiple comparisons (see Methods).

This question was also evaluable in another independent dataset from a study by Finak et al. (GSE9014, hereinafter referred to as the "Finak Dataset"). In this case, *Finak et al.* published a work in 2008 about how stromal gene expression predicted clinical outcome in breast cancer patients. No information regarding sample-compartment pairing was available. In this study, all types of breast cancer patients were included (that means: HR+, HER2 and TNBC patients).

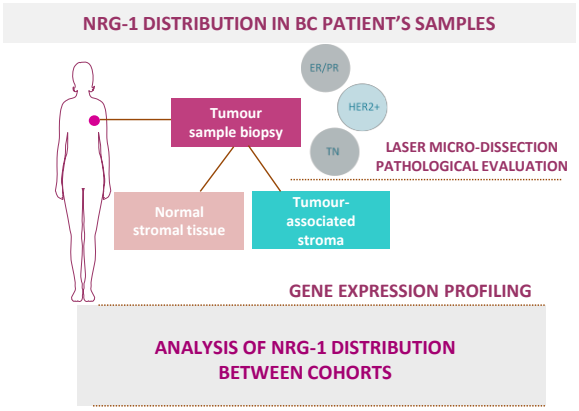
In total, 53 samples of tumour stroma (of which 11 belonged to HER2+ tumours) from non-previously treated invasive breast carcinoma and 6 normal breast tissue samples were collected from laser capture micro-dissection, evaluated by a pathologist and subjected to RNA expression profiling by microarray techniques.

Since the study also contained information about the BC subtype, we just considered HER2+ BC patients. Coherent with our findings in the Liu Dataset, the tumour-associated stroma expressed significantly higher levels of NRG-1 than the distant associated stroma (referred to as normal) from this subtype of BC patient's. In contrast to the Liu dataset, the Finak dataset also contained information about HR status of these patients. As many of breast tumours classified as HER2+ also co-express Oestrogen and/or Progesterone receptors (ER/PR, respectively) we then examined if this could influence on the stromal distribution of NRG-1. As depicted in **Figure 37B.** no differences where observed when HER2+ BC patients were further subdivided into HER2+/ER- and HER2+/ER+.

The main limitation of the aforementioned studies is their small sample size. Furthermore, the number of normal stromal tissue specimens in the Finak dataset was only 6, which may be not representative of the total spectrum of NRG-1 expression in normal breast stroma. As mentioned, there was no information about if normal and tumoral stromal tissue came from the same patient (paired samples) or for different patients (unpaired). However, only one normal stroma sample came from a HER2+ patient, meaning that tumour and normal samples can be virtually considered two separate cohorts.

Notwithstanding the potential limitations listed above, the results of these analyses reinforce our hypothesis that NRG-1 is overexpressed in the stromal compartment of HER2+ breast tumours, and that this appears to be independent of HR status.

A.



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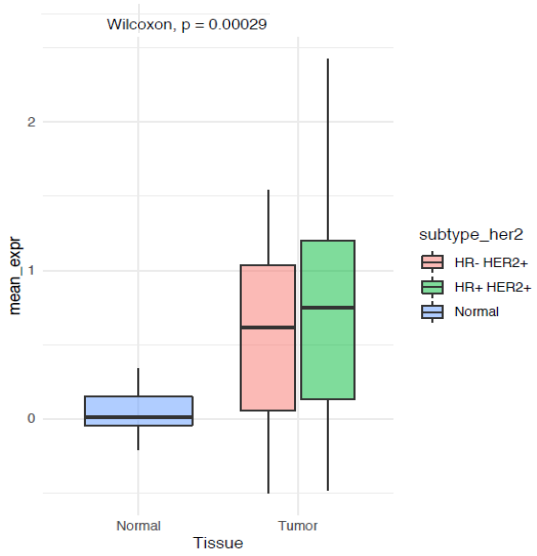

Figure 37. NRG-1 distribution along normal breast stroma or tumour-associated stromal tissue in HER2+ BC patients. A. Experimental reported approach performed by *Finak et al.* **B.** NRG-1 distribution across normal and tumour-associated stroma, taking HR status into account. Data was taken from GEO Datasets (GSE9014). Horizontal line within the box represents the median expression in each compartment. The area of the box extends from the first quartile (bottom) to the third quartile (top) of the expression distribution. The whiskers extend to 1.5x the interquartile range (1st quartile - 3rd quartile) and the dots represent outliers. All p-values shown have been adjusted for multiple comparisons (see Methods).

R16. NRG-1 IS EXPRESSED IN THE TUMOUR STROMA FROM HER2+ BC PATIENTS

Considering the relative data on NRG-1 expression from the public RNA data-sets involving HER2+ BC patients, we decided to stain HER2+ BC patient samples from Hospital del Mar. The formalin-fixed paraffin-embedded (FFPE) samples were obtained from the same patient biopsies that the primary CAF cultures were generated from. This was for us, an important step towards translating our *in vitro* findings. Few published articles have previously reported the expression of NRG-1 in stromal populations. Moreover, most of those studies were done in preclinical *in vitro* models and there is no relative information about NRG-1 staining or distribution within the stromal tissue from cancer patients. Thus, apart from proper positive and negative internal controls for the setup of the technique, the possibility of having primary CAFs and their associated paired FFPE samples both provided us further evidence and increased the reliability of the resulting data.

Apart from little background on NRG-1 expression in the stromal compartment, the absence of proper commercial antibodies hindered the setting up of the protocol. Two commercial antibodies were used initially, but neither produced either reliable or reproducible results (data not shown). Dr. A. Pandiella kindly provided us with an anti-NRG antibody generated in his lab. All the experiments in this subsection were performed using this antibody.

The protocol for the IHC technique is detailed in Materials and methods section 9.1. Of note, all the staining in this subsection and the following one was evaluated by a pathologist. In the BC-T50 FFPE sample, in which we previously detected NRG-1 expression in the primary fibroblasts isolated from this tumour patient, NRG-1 was found in the tumour-associated stroma. Moreover, the staining pattern was heterogeneous, but the pathological analysis identified the NRG1-expressing cells as fibroblasts **(Figure 38).** In contrast, no positive staining was seen in the tumour epithelium

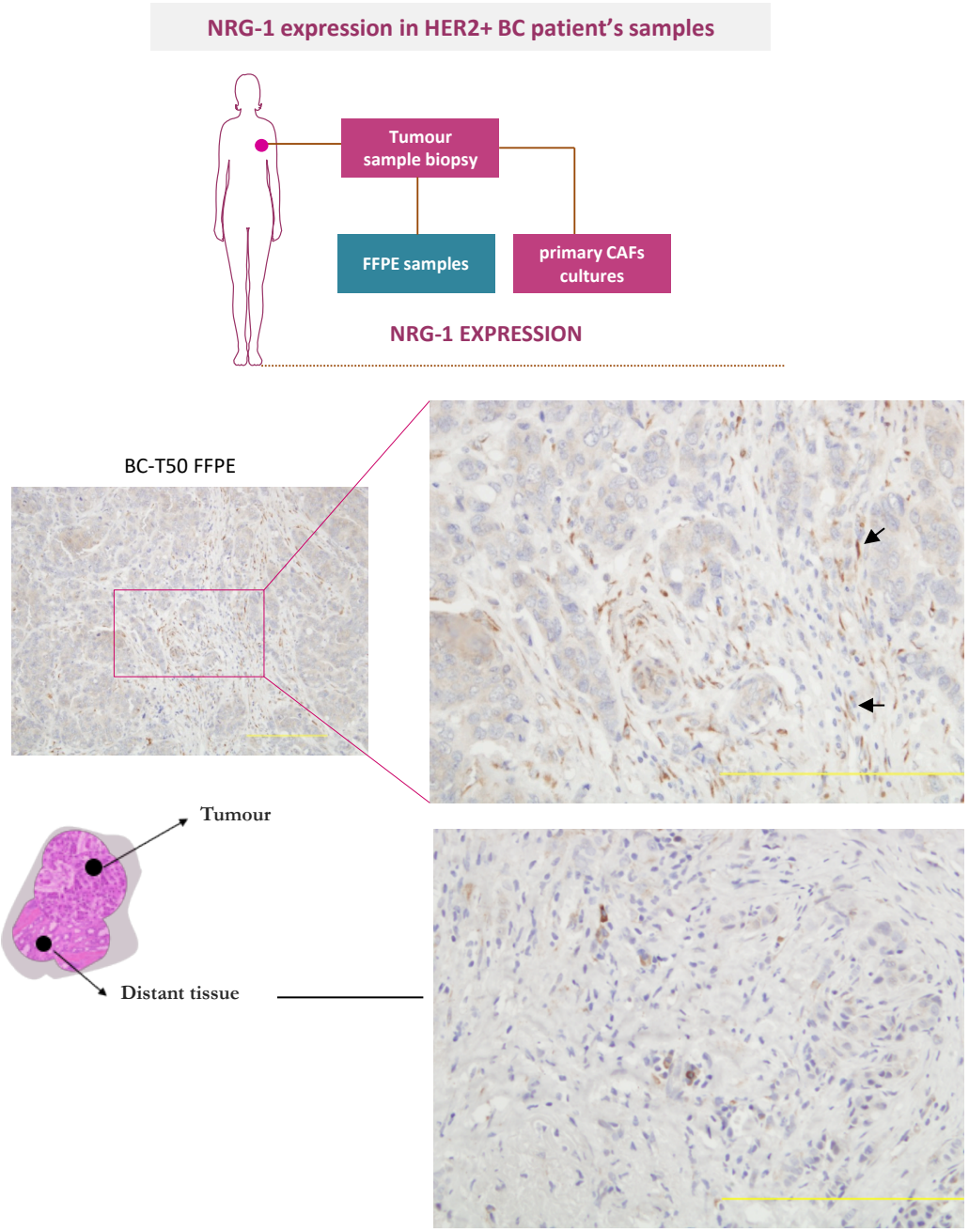

Figure 38. Stromal NRG-1 expression in HER2+ BC patient FFPE samples. Representative images of IHC staining of NRG-1 in BC-T50 FFPE tumour patient's biopsy. From IHC images, upper image shows stromal NRG-1 staining in the intratumoral stroma. Positive cells (highlighted with an arrow) have been identified as fibroblasts by pathologist evaluation. Lower image corresponds to a distant section from the same patient biopsy and shows almost non NRG-1 staining.

As mentioned, CAFs are in a permanently activated state. Although not exclusively, one of the main accepted markers for defining CAFs, and thus, a reactive stroma, is the presence of FAP protein expression. Because of this, we also stained the BC-T50 FFPE sample for FAP. As shown in **Figure 39**, the peri-tumoral stroma of this patient positively stained for FAP.

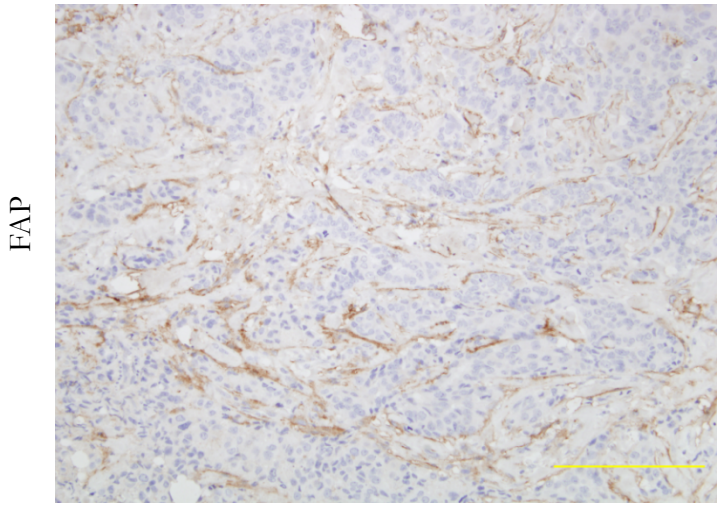

Figure 39. FAP staining in a BC-T50 FFPE sample. A. NRG-1 IHC in BC-T50 FFPE distant stroma (non-tumoral). **B.** FAP IHC in BC-T50 FFPE tumour-associated stroma.

Taking all this into account, although preliminary, this data suggests that NRG-1 expression may not be homogeneous within the stromal compartment of a tumour. Indeed, it may be restricted to certain CAF subpopulations or to ones that crosstalk closely with tumour cells.

R17. NRG-1 STROMAL EXPRESSION CORRELATES WITH POOR RESPONSE TO NEOADJUVANT TRASTUZUMAB PLUS CHEMOTHERAPY IN HER2+ BC PATIENTS

Having observed that NRG-1 was expressed in FFPE tumour samples from BC patients, we wanted to expand our studies to a small cohort of HER2+ BC patients. On the one hand, we wondered whether NRG-1 stromal expression in HER2+ BC is a common trend or restricted to a subset of patients. Moreover, we also wanted to study whether NRG-1 stromal expression potentially correlates with clinical response. To this end, since we wanted to avoid potential bias effects for previous-line treatments, we decided to stain NRG-1 in naïve HER2+ BC patient samples and correlate these with the clinical response in the neoadjuvant setting.

To do this, we selected 53 samples from HER2+ BC patients treated with trastuzumab plus taxanes in neoadjuvancy, and we performed IHC against NRG-1. The results were evaluated by a pathologist and clinical correlations were made.

Firstly, with regard to NRG-1 stromal expression, we observed that, of the 53 tumour samples included in the study, in 5, NRG-1 was detected in the stromal compartment. Interestingly, NRG-1 localized in the intertumoural stromal fibroblasts (those inside the tumour mass) with a major presence in the growing border of tumours and less in the central area. Occasionally, NRG-1 was detected in the extracellular matrix (ECM) itself, probably the secreted form.

In summary, despite the small size of our cohort, for NRG-1 stromal expression in HER2+ BCs, we considered that NRG-1 would be restricted to a subgroup of patients, at least, in the early stages of the disease. Unexpectedly, 3 of the 53 patients also showed NRG-1 staining in the tumour epithelial compartment. Moreover, in 1 of these 3 tumour samples, NRG-1 was found in both the epithelial and stromal compartments.

We next evaluated the clinical response of these patients. We classified the patients based on their clinical response according to the Miller Payne criteria. This guide, used in BC in the neoadjuvant setting, also takes into consideration clinical information from breast tumours, as well as from the lymphatic nodes in the armpit.
Briefly, the Miller Payne criteria classify tumours into grades (G), from G1 to G5.

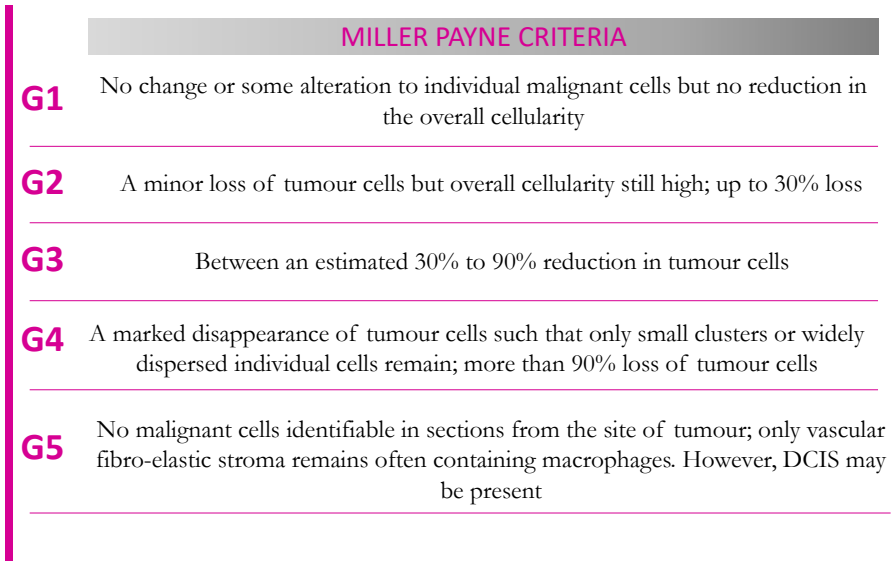

Figure 40. Miller Payne grading system for evaluating clinical response after trastuzumab plus chemotherapy treatment in neoadjuvancy in HER2+ BC.

We considered patients classified as G1-G4 as having non-pathological complete response (pCR) (referred to as non-complete in Figure 40) and patients classified as G5 as having pathological complete response (referred to as complete in Figure 40). Interestingly, we found that all HER2+ BC patients who expressed NRG-1 in the stromal fraction had residual disease after trastuzumab plus chemotherapy neoadjuvant treatment. The differences were statistically significant. Moreover, the clinical response in those tumours in which NRG-1 was found in the tumour epithelium was mixed and did not reach statistical significance.

Taking all of this together, this data suggests that NRG-1 stromal expression in HER2+ BC may be restricted to a subset of patients. However, the data further corroborated our *in vitro* results and highlighted the relevance of stromal NRG-1 expression in this subset of patients. Since NRG-1 expression is clinically associated with a reduced response to trastuzumab and chemotherapy in the neoadjuvant setting, evaluating stromal NRG-1 expression may be a potential biomarker for better selecting patients prior to them receiving anti-HER2 targeted therapies.

STROMA

NRG-1 AND CLINICAL RESPONSE CORRELATION

			NRG1_ stroma		
			Negative	Positive	Total
RESPONSE	Non complete (G1-G4)	Count	27	5	32
		% within response	84,4 %	15,6 %	100,0 %
		% within NRG1_stroma	56,3 %	100,0 %	60,4 %
	Complete (G5)	Count	21	0	21
		% within response	100,0 %	,0%	100,0 %
		% within NRG1_stroma	43,8 %	,0% %	39,6 %
TOTAL		Count	48	5	53
		% within response	90,6 %	9,4 %	100,0 %
		% within NRG1_stroma	100,0 %	100,0 %	100,0 %

P=0.043

TUMOUR

NRG-1 AND CLINICAL RESPONSE CORRELATION

			NRG1_ tumour		
			Negative	Positive	Total
RESPONSE	Non complete (G1-G4)	Count	30	2	32
		% within response	93,8 %	6,3 %	100,0 %
		% within NRG1_tumour	60,0%	66,7 %	60,4 %
	Complete (G5)	Count	20	1	21
		% within response	95,2 %	4,8 %	100,0 %
		% within NRG1_tumour	40,0 %	33,3% %	39,6 %
TOTAL		Count	50	3	53
		% within response	94,3 %	5,7 %	100,0 %
		% within NRG1_stroma	100,0 %	100,0 %	100,0 %

P=0.259

Figure 41. Association of NRG-1 stromal and epithelial expression with clinical response in a cohort of HER2+ BC patients. The upper graph shows NRG-1 expression in the stromal fraction of HER2+ BC patients. The lower graph shows NRG-1 expression in the epithelial compartment. In both cases, the tables show the expression of NRG-1 and its correlation with tumour response according to the Miller Payne criteria. Statistical analysis was performed by usying Chi-squared test.

R18. THE NRG-1 GENE SIGNATURE CORRELATES WITH A POOR RESPONSE TO NEOADJUVANT TRASTUZUMAB IN LARGE-SCALE DATASETS FROM HER2+ BC PATIENTS

To provide more evidence on the clinical significance of NRG-1 stromal expression in HER2+ BC patients, we tried to translate our findings into a large-scale patient dataset. For this, we screened public RNA datasets derived from clinical trials on HER2+ BC. For this study, we only considered those where the relative data was properly documented.

The first included trial was the transNOAH dataset, which included samples from a phase III randomized trial (NOAH) designed to assess clinical benefit from adding neoadjuvant trastuzumab to chemotherapy vs chemotherapy alone in women with HER2-positive locally advanced or inflammatory breast cancer. This study also included a HER2-negative cohort which received chemotherapy alone. For our purposes, we performed the analyses only on the HER2+ cohort. Moreover, aware by the fact that the presence of ER/PR also clinically associated with reduced response to trastuzumab, the HR status was also included in the study.

However, when we analysed the data for these parameters, we found no statistical correlation between NRG-1 expression and pCR in any of the subgroups. However, one of the main limitations of analysing bulk RNA from tumour samples is that it generally has no information about tumour purity (i.e. the amount of stromal compartment or normal breast tissue that may be present in a given sample). Furthermore, the analysis of the expression of one single gene across such heterogeneous mixtures (tumour-stroma-normal tissue) may be insufficient for capturing biological differences.

Because of this, we decided to take an alternative approach. By exploring the GSEA MSigDB dataset of gene signatures, we found two NRG-1 associated gene signatures. Both signatures were developed in a preclinical study conducted by Nagashima and colleagues(244,245). In this study, MCF-7 cells were stimulated for several time points and different doses with NRG-1. Total RNA was extracted at each time point and GeneChip experiments were performed. Signals were processed by following the GeneChip expression analysis algorithm. Taking into account gene expression differences in the different time points and through complex mathematical modelling, the investigators provided a final list of 180 and 59 genes that were respectively up-regulated and down-regulated after NRG-1 stimulation.

Taking these two gene sets, we then computed a summarized score of NRG-1 activation for each sample included in the trasNOAH dataset using the GSVA tool developed by Castelo *et al*(246). Briefly, we calculated the expression score of each gene set (up-regulated and down-regulated genes) separately for each sample and ranked the patients according to these scores. The final, combined score was defined as the sum of ranks in each score for each patient.

Thus, we studied if NRG-1 score was associated with clinical response to neoadjuvant therapy in HER2+ patients. Interestingly, HER2+ breast cancer patients in the overall study cohort who achieved a pathological complete response (pCR) presented a lower NRG-1 combined score (p=0,046). However, this was only statistically significant in patients that received trastuzumab associated to their chemotherapy (p=0.029), while no difference was observed in patients receiving only chemotherapy. Our *in vitro* data demonstrated that paracrine NRG-1 reduced both trastuzumab and chemotherapy drug efficacy. However, our analysis of clinical samples suggested that NRG-1 may be particularly relevant in the patient's drug response to trastuzumab.

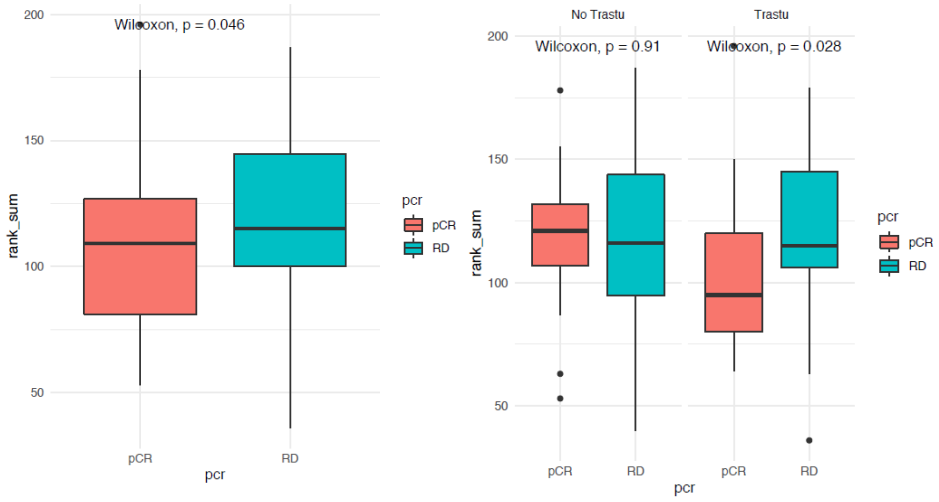

Figure 42. NRG-1 gene signature in HER2+ BC patient samples and correlation with clinical response. The left graph shows pCR between NRG-1 low-score signature (red boxplot) and NRG-1 high-score (green boxplot). The boxplots represent the average for NRG-1 low score and NRG-1 high- score for HER2+ BC patients. On the right is the same analysis but filtered by treatment arm: chemotherapy alone or chemotherapy plus trastuzumab in neaoadjuvancy. The statistical analysis was performed using the Wilcoxon test. P<0.05.

Although these findings reinforced our *in vitro* data and the results derived from HER2+ BC patient's samples, they should be evaluated with caution because of several potential limitations. First, the NRG-1 gene signature was derived from a non-HER2+ BC cell line, and as we know, the expression of the ErbB family receptors significantly impacts on the genetic expression programs in the cells. Second, the monitoring and processing of the raw data was inaccessible to us (only the data previously normalized by investigators was analysed), so statistical confounding effects may also be present. In line with this, we are currently considering the possibility of designing our own NRG-1 gene signature to fully translate our results into large-scale datasets from patients as well as to see if NRG-1 stromal expression also influences on therapy response of other HER2-addicted tumours.

WORKING MODEL

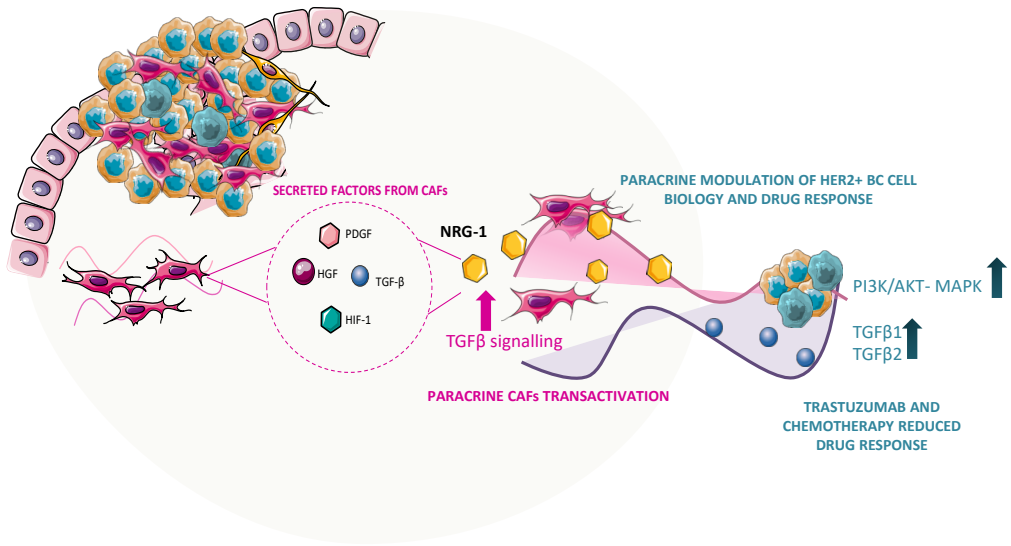

Figure 43. Hypothesised working model. We propose that in HER2+ BC, NRG-1 is secreted from stromal fibroblasts (CAFs) and that this secretion dramatically influences BC cell biology and therapy response. The paracrine effect of NRG-1 causes tumour cells to activate pro-survival pathways that, in turn, may promote drug evasion. Moreover, NRG-1 may also activate the TGF-β programme in these cells that, subsequently, may enhance the activation of stromal fibroblasts, resulting in a cooperative feedback loop between cancer cells and CAFs. Pharmacological inhibition of HER2/HER3 dimerization and its associated downstream signalling, with pertuzumab, abrogates paracrine NRG-1 derived effects. In tumours in which NRG-1 feeds the tumour tissue, the addition of pertuzumab may circumvent NRG-1-derived effects, resulting in clinical benefit for these patients.

DISCUSSION

D1.CONTEXTUALIZATION OF THE STUDY

HER2 gene amplification and receptor overexpression, which accounts for 15 to 20% of breast cancers (BC) patients, defines an aggressive and poor prognosis breast cancer subtype (HER2+ BC). The introduction of anti-HER2 targeted therapies, particularly trastuzumab, led to dramatic improvements in survival in both early and advanced settings(247).Notwithstanding, nearly all metastatic patients will ultimately progress and die on standard treatment with anti-HER2 therapies due to the appearance of resistance mechanisms(248).

With the ultimate goal of finding personalized strategies and effective treatments for these patients; biomedical research efforts have centred on understanding the molecular basis that may account for clinical response variations between patients or during the different stages of the disease. Thanks to this, potential biomarkers predictors of response or resistance, mainly to trastuzumab, have been purposed. Some of the reported mechanisms include: a constitutively active truncated form of HER2 (p95-HER2) that lacks the trastuzumab-binding domain; activation of the downstream PI3K/AKT/mTOR signalling pathway (i.e. by activating mutations in the *PI3KCA* gene or by PTEN loss); crosstalk with other growth factor receptors; increased expression or compensatory signalling trough other receptor tyrosine kinases of the HER family members (such as HER3) or with other related families, including insulin-like growth factor-1 receptor (IGF-R1), or MET; as well as modulation of some of the ErbB family ligands such as AREG, EGF or TGFα)(248,249). Very recently, a study leaded by Dr. Pandiella has identified that trastuzumab resistant cells exhibit a deregulation of the tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) pathway that confer sensitivity to TRAIL in resistant cells. These findings offer a therapeutic option to fight trastuzumab resistance in the clinics with TRAIL-R agonists that are currently being developed(116).

However, all of these potential biomarkers have failed when analysed in large-scale datasets from patients and HER2 positivity status itself (amplification/overexpression) stands as the unique valid criteria for tailoring patients candidate to anti-HER2 drug therapies.

Nonetheless, the poor translation of biomarker discovery into clinical utility is a global issue that commonly accounts in other non-HER2 addicted tumours(250). Of the numerous tumour markers identified, described and extensively researched, only a handful of them are currently used in the routine clinical practice; and even of these, only a few have served for establishing consensus guidelines for the use in day-to-day care of patients. With regard to this, apart from HER2, hormone receptors (ER/PR) status clinically stratify patients candidate to receive endocrine treatment in breast cancer(251); mutated EGFR predicts sensitivity to EGFR tyrosine kinase inhibitors such as gefitinib in non-small lung cancer(252) and mutated BRAF tailors patients for anti-BRAF drug treatments in melanoma(253).Other biomarkers have shown clinical utility on identifying resistance to treatment, such as RAS mutations; which define a group of colorectal cancer patients not susceptible to treatment with the anti-EGFR- targeted therapies, cetuximab and panitumumab(254).

In the context of HER2-BC three main reasons may explain the fact that although all the body of evidence, preclinical findings have not successfully been translated into clinical practice. Firstly, the intricate biology and promiscuity of the HER2 receptor. Secondly the intra-tumoral heterogeneity underlying HER2-breast cancer. Thirdly, the fact that most of the aforementioned studies have traditionally been focused in autonomous cancer cell-based mechanisms.

In line with this, in the past decade, the complex interplay between tumour cells and the tumour microenvironment (TME) to drive cancer progression and therapy scape has collectively risen in prominence. Tumours consist not only of heterogeneous populations of cancer cells but also of the tumour microenvironment (TME).

The TME is composed by multiple stromal cell types embedded in an extracellular matrix in which a complex and dynamic network of cytokines, chemokines, growth factors and matrix-remodelling enzymes takes part. Particularly, cancer-associated fibroblasts (CAFs) (the most abundant stromal cell population in the TME of many solid tumours) are critically involved in cancer progression(217). CAFs also play a role in resistance to both chemotherapy and targeted therapies (183,204,255,256), and recently have been linked to immune evasion and immunotherapy failure(257).

Nonetheless, while much of CAF contributions on tumorigenesis have been largely reported (217,258), their role on facing therapy failure remains poorly understood. This is particularly relevant in HER2+ breast cancer, in which potential contribution of CAFs in anti-HER2 therapy resistance has not been widely explored Communication between cancer cells and CAFs occur at multiple levels. CAFs can influence cancer cell-therapy response behaviour via cell–cell contact, by remodelling the extracellular matrix (ECM)(259), and by releasing numerous regulatory factors. Particularly, release of microenvironmental factors from CAFs have been linked with drug resistance in other solid malignancies, also known as soluble factor-mediated drug resistance (SF-MDR)(183,204,260). Because of this, we hypothesised that **in HER2+ breast cancer, anti-HER2 drug responses of cancer cells would not be exclusively determined by their intrinsic characteristics, but they may also be alternatively regulated by paracrine signals derived from CAFs within the TME.**

D2. CROSSTALK WITH CAFS DESENSITIZES HER2+ BREAST CANCER CELLS TO TRASTUZUMAB AND PACLITAXEL

In this PhD thesis we focused mainly on studying paracrine crosstalk between cancer cells and CAFs *in vitro,* and to translate our findings in i*n vivo,* and in HER2-positive BC patient samples. For hypothesis generation, we have used a conventional method such conditioned media transferring between CAF and tumour cells(261) that has allowed us in the identification of novel soluble factors from CAFs with may modulate therapeutic response to anti-HER2 therapies of HER2+ BC cells.

Soluble-factor mediated drug resistance (SF-MDR) was firstly reported in relation to conventional treatment such as chemotherapeutic agents and later on, in resistance to targeted therapies. In most cases, release of bioactive factors from CAFs promotes activation of stemness-related programs, metabolic reprogramming, induction of the epithelial-to-mesenchymal transition (EMT) and activation of pro-survival pathways in tumour cells(204,217), as we have also proven. Although soluble factors also comprise exosomes and other type of microvessel-secreted factors, we have focused in potential growth factors secreted from CAFs that ultimate may modulate response to HER2 targeted therapy. The relative data of SF-MDR mediated by soluble factors and the relevance of our findings will be discussed below.

In relation to conventional therapies, CAFs promote cisplatin and 5-fluorouracil resistance in esophaegal squamous cell carcinoma, by secreting IL-6, which in turns mediates upregulation of CXCR7 through the STAT3/NKκβ pathway in tumour cells(262). In breast cancer, secretion of Insulin-like growth factors 1 and 2 (IGF-1, IGF-2, respectively) by CAFs has been shown to promote gemcitabine and paclitaxel drug resistance in murine models.

Regarding to targeted therapies, CAF-secreted growth factors have been implicated in resistance to b-RAF- and EGFR-inhibitors in various tumour types(231). HGF has been described as the main mediator of stroma-induced resistance to BRAF inhibitors in BRAF mutated melanoma, colorectal cancer and glioblastoma. Experiments of co-culturing tumour cells and CAFs have evidenced how CAF-derived HGF promotes resistance to RAF inhibitors through activation of the MAPK and the PI3K/AKT signalling in tumour cells via HGF/c-MET pathway. HGF has also found especially active in supporting resistance to EGFR inhibition in various tumours. MET signalling cascade triggered by CAFs-HGF is responsible for promoting drug resistance to EGFR inhibition in $KRAS^{wt}$ colon cancer stem-like cells(263). These cells, isolated from xenografts, are intrinsically sensitive to EGFR targeting, however exposure to CAF-conditioned medium impairs the pro-apoptotic effect of cetuximab and gefitinib. In lung cancer also, the malignant crosstalk between cancer cells and stroma mediated by HGF induces resistance to EGFR-tyrosine kinase when tumour cells and CAFs are co-injected in vivo inhibitors(264)

Similarly, cholangiocarcinoma cells chronically treated with the EGFR inhibitor erlotinib, upregulate the Insulin receptor (IR)/Insulin-like growth factor 1 receptor (IGF1R) signalling(265). Mechanistically, a positive feedback circuitry involving IR/IGF1R signalling and CAF-secreted insulin-like growth factor 2 (IGF2) fuels both erlotinib resistance in cholangiocarcinoma cells and activation of hepatic myofibroblasts. Accordingly, combined treatment with erlotinib and an IR/IGF1R inhibitor impairs growth of resistant tumour xenografts and reduces their stromal content(265).

In regard to breast cancer, CAFs have been implicated in tamoxifen resistance through various mechanisms such as activation of the PI3K/AKT and MAPK/ERK pathways and phoshphorylation of estrogen receptor-alfa (at Ser118 site)(266,267), or by metabollic reprogramming of CAFs leaded by cancer cells. Upon this, CAFs supply nutrients to cancer cells that promote tamoxifen resistance(268).

In triple negative breast cancer cells, HGF-derived from fibroblasts have shown to activate Met, which promotes cancer cell survival and gefitinib drug evasion(227).

In relation to HER2-BC instead, the specific contribution of CAFs in therapy resistance remains, in comparison to other malignancies, largely unexplored. In this direction, 3D co-culture of fibroblasts and breast cancer cells has shown to protect tumour cells from lapatinib drug treatment, an EGFR/HER2 inhibitor. In line with our results other authors have referred that CAFs promote trastuzumab resistance. In 2015, Mao *et al.* firstly reported CAF protective effects against trastuzumab drug efficacy by promoting expansion of cancer stem cells population through activation of multiple pathways such as NFkB, JAK/STAT3, and PI3K/AKT pathways. In the study, the authors isolated primary CAF cultures derived from invasive mammary ductal carcinomas and HER2-BC cancer cells were cultured in the presence of the derived CM. The authors evidenced that IL-6 secreted from CAFs was behind the acquired phenotype of cancer cells(269). In this line, a study by Nogueria *et al.,* has described that RET and FGFR2 pathways promote *in vitro* and *in vivo* resistance to trastuzumab and lapatinib in HER2+ BC pre-clinical models (Fernandez-Nogueira *et al.*, Clin Cancer Res, second revision).

In the work presented in this doctoral thesis we have described that secretome from HER2-immortalized CAFs *per se* is sufficient to significantly by-pass trastuzumab drug efficacy in HER2+ BC cell lines. Moreover, therapeutic protective effects from CAFs have also been evidenced in relation to the classical agent paclitaxel and also in drug combination, which represents two classical standard treatments in HER2-positive BC. Further reinforcing the concept that CAFs play pivotal roles in HER2-cancer drug evasion is the fact that none of these effects have been observed when tumour cells are cultured in the presence of their own secretome.

D3. PARACRINE NRG-1 IS A POTENTIAL MEDIATOR OF TRASTUZUMAB AND PACLITAXEL DRUG RESPONSE IN HER2+ BC CELLS

In the process of drug evasion, tumour cells activate alternative mechanisms in response to tyrosine kinase inhibitors (TKIs) and to other targeted therapies(270). Among them, reactivation of MAPK and PI3K/AKT cellular pathways have been extensively proven(271). Our group has recently shown that in HER2+ gastric cancer, trastuzumab acquired resistant cell lines upregulate PI3K/AKT/mTOR and MAPK/Erk cell signalling to evade trastuzumab drug effects. Taking this into account, we have explored how CAFs may alternatively modulate these two major cellular cascades. Interestingly, we have seen that in the events that ultimately may increase drug tolerance capacity in tumour cells, the PI3K/AKT and the MAPK/Erk pathway are strongly activated by paracrine signalling crosstalk from CAFs.

Yet to be considered as single entities, the PI3K/AKT and MAPKs pathways intersect and regulate each other, as well as other downstream functions(271). Although multiple stimuli can trigger their activation, mitogenic growth factors are powerful inducers of these intracellular cascades. Particularly, the ErbB family ligands potently trap the activation of these pro-survival pathways. In the context of HER2+ BC, co-expression of HER3 commonly occurs. Although HER3 lacks innate kinase function, it is the preferred dimerization partner when signalling occurs through the PI3K pathway(23). HER2-HER3 dimer is crucial for HER2-mediated signalling in tumours with HER2-oncogenic addition (i.e.HER2-amplified)(272). Pivotal roles of HER3 in tumorigenesis have been reported, further evidenced by the fact that loss of HER3 function eradicates the transforming capability of HER2(272). Notwithstanding, the biological role of HER3 cannot be understood without taking in consideration its ligand, the family of NRGs. Overexpression of NRG results in increased transformation and tumorigeneicity (170,273), whereas blockade of NRG expression inhibits tumorigeneicity and metastasis in TNBC cells(171).

Hence, tumorigenic roles of NRGs have already been evidenced by other authors. However, most of the aforementioned studies have been based on exploring autonomous-NRGs roles in tumour cells either based on overexpressing NRG-1 tumour cells or by ectopic forced expression of these family of ligands(171,274). In our study, we have demonstrated that in two CAF cell lines previously derived from HER2+ BC patients, NRG-1 is highly expressed at mRNA and protein level and that stromal cells express relative higher levels of NRG-1 than HER2+ tumour cells. The relative low expression of NRG-1 in HER2+ cancer cells has also been evidenced by the analysis of large-scale public datasets as well as in a panel of HER2+ BC cells, further reinforcing our hypothesis that in HER2-BCs, stromal CAFs may act on feeding the tumoral tissue for NRG-1.

In line with our findings, in the recent years, microenvironmental NRG-1 has also been reported by other authors and it has been linked with tumour promotion and drug resistance in other types of solid cancers. In colorectal cancer, bone marrow derived mesenchymal stem cells (BMSCs) promote cancer progression through NRG(275). In melanoma, CAFs derived from cancer patients express higher levels of NRG-1 than normal dermal fibroblasts. Furthermore, these CAFs promote resistance to vemurafenib, a BRAF inhibitor(166). In gastric cancer, NRG-1 secreted from CAFs increase self-renewal of gastric cancer stem cells (GCSCs) through the NF-kß pathway(276). In pancreatic cancer, tumour cells and CAFs express NRG-1 and 7E3, an original antibody directed to NRG-1, efficiently targets both pancreatic stromal and cancer cells(277). Recently, human umbilical cord-derived mesenchymal stem cells (MSCs) have been reported to impair paclitaxel efficacy in ErbB2/ErbB3-coexpresing breast cancer cells. The authors purpose that this could be attributed to upregulation of Survivin, via paracrine effect of NRG-1/ErbB3/PI3K/AKT signalling(278).

D4. NRG-1β MIMICS PARACRINE MODULATORY EFFECTS FROM CAFS IN HER2+ BREAST CANCER CELLS

To validate the potential value of microenvironmental NRG-1 on promoting drug evasion in HER2+ BC cells, different *in vitro* approaches have been performed. Firstly, with the soluble recombinant form of NRG-1. We have seen that *in vitro*, NRG-1β is able to induce cell proliferation of HER2+ BC cell lines. Moreover, we have also observed that, at higher concentrations, NRG-1β is able to affect cell viability. This is in line with some studies which suggest that besides its pro-tumorigenic functions, NRGs can act as pro-apoptotic factors. In a study by Weinstein *et al*, NRG-1 forced expression causes apoptosis in various cell lines, including MCF-7, an ER+ breast cancer cell line.
However, this study proposes that, pro-apoptotic functions of NRG-1 are independent of ErbB-family receptors and require the C-terminal part, which is not present in our recombinant form of the protein(279). In the same way, already in 1992, Bacus *et al*., demonstrated that NRGs induced differentiation of breast cancer cell lines. In line with our results, this study showed that exogeneous NRG-1 may exert opposite functions in a dose- and time-dependent manner(280). Whereas lower doses of NRG resulted in proliferation, higher doses promoted growth inhibition after 6 days.

Moreover, we have seen that at a ligand concentration in which cell viability is preserved, exogeneous NRG-1β is able to significantly reduce trastuzumab effects in HER2+ BC cell lines. Moreover, it also fosters activation of the pro-survival PI3K/AKT and MAPK/Erk pathways, as we previously observed in the context of paracrine signalling from CAFs. Our results are in line with previous data. In esophaegal cancer, both HER3 and the metalloprotease ADAM10 are simultaneously upregulated in response to trastuzumab. This in turn, promotes release of NRGs from cell surface. Through HER3 activation, resistance to trastuzumab occurs(281). In the same way, Schwarz *et al.* have reported that plasticity of the ErbB receptor network is implicated in the development of acquired resistance to anti-HER2 therapies(282).

To prove this, they treat HER2+ cancer cell lines and xenograft mice with different combinations of anti-HER2 therapies: trastuzumab plus pertuzumab (P) or plus lapatinib (L); T-DM1 alone and a pan-HER ErbB1-ErbB3 neutralizing antibody mixture. Interestingly, xenograft post-trastuzumab-P/L or T-DM1 recurrent tumours, express high levels of NRG-1 as well as total and phosphorylated HER3 levels. Upregulation of NRG-1 and p-HER3/HER3 levels are also observed in HER2+ breast tumours progressing after trastuzumab and T-DM1. The authors propose that escape from anti-HER2 therapies may be mediated by upregulation of a NRG1-HER3 axis. In line with this, other authors have also reported NRG-1 implications in trastuzumab resistance as well as in other HER2-targeted agents or TKIs(82).

As previously mentioned, we have confirmed that immortalized CAFs derived from HER2-positive breast cancer patients express NRG-1 at mRNA and protein level. NRGs are expressed as pro-membrane forms that upon proteolytic cleave are relesead into the extracellular space. With regard to this, we have tried to detect the CAF-secreted form of the ligand. Unfortunately, technical limitations have limited us on succeeding. Different commercial antibodies, as well as to optimize our experimental conditions have been approached but we have not trusted on the reproducibility of the generated data. Following this, we have also tried to detect the protein by mass spectrometry, but we have also failed on this. NRG-1 has not been able to be detected in the secretome from CAF200 cells, probably because of in the experimental conditions approached; NRG-1 is out of the detection rank. The difficulty on detecting secreted NRG-1 has limited to go deeper on studying how CAFs may modulate paracrine NRG-1 secretion in other potential biological contexts, in which we were also interested in. Our difficulties have also been reported by other authors and indeed, they have been proposed as a potential issue that may explain the controversial data about NRGs and the inconsistent translation of preclinical findings into clinical practice, which will be discussed below(174).

Secondly, we have performed pharmacological targeting of HER2/HER3 dimerization to inhibit paracrine NRG-1 effects in cancer cells. Pertuzumab is a monoclonal antibody directed against the ECD of HER2. Upon binding to the dimerization domain of the receptor, it blocks dimerization with other HER family members, mainly with HER3(23). Thus, differentially to trastuzumab, pertuzumab is effective on inhibiting ligand-dependent HER2 signalling. Interestingly, we have observed that pertuzumab addition significantly prevents both NRG-1β and CAF paracrine effects in tumour cells, in terms of dug sensitivity. In this context, sensitivity to trastuzumab alone or in combination with paclitaxel is retained. Additionally, we have seen that trastuzumab is not effective on attenuating PI3K/AKT activation leaded by NRG-1β, whereas addition of pertuzumab efficiently supresses it.

In line with our findings, other authors have also evidenced that pertuzumab constitutes a therapeutic strategy to overcome NRG-1 derived effects. In HER2+ BC cells, drug combination of lapatinib and pertuzumab efficiently overcome autocrine NRG-1 mediated resistance *in vitro* and *in vivo* models(173). In the same way, treatment of HER2-overexpressing tumour cells with NRG-1β reduces cytotoxic activity of T-DM1 in a subset of breast cancer cell lines *in vitro*. In this context, addition of pertuzumab reverts these effects(82).

Recently, Watson *et al*. have described an innovative platform for the discovery of microenvironmental signals which may influence on drug responses. The method is based on monitoring TKIs-treated breast cancer cell lines grown on microenvironment microarrays. These platforms are composed by printed extracellular matrix proteins supplemented with soluble proteins. The authors have demonstrated that NRG-1β and HGF supress both responses to lapatinib and neratinib in luminal-like HER2 cells (L-HER2) and basal-like HER2 cells (HER2E), respectively. In case of NRG-1β resistance, this is effectively reversed by adding pertuzumab. Moreover, they hypothesise that the differentially responses observed, are due to differential epigenomic and regulatory pathway mechanisms between L-HER2 and HER2E breast cancers. Whereas HER2E resistance may rely on alternative MET signalling, L-HER2 may overcome therapeutic effects by HER2-HER3 related mechanisms(283).

Thirdly, to fully confirm that microenvironmental NRG-1 is shifting drug phenotype of HER2-positive breast cancer cells, we have knock-down NRG-1 expression in CAFs. By genetically engineering CAFs with short hairpin RNA technology, we have been able to demonstrate that stable depletion of NRG-1 in CAFs is sufficient to prevent paracrine derived effects in cancer cells in terms of drug response. In this context, trastuzumab sensitivity is preserved.

This data is particularly significant to us. It is known that CAFs secrete multiple growth factors, cytokines and soluble molecules which potentially may interfere in drug efficacy(202,205,217).This has also evidenced by us when secretome from CAF200 cells has been analysed by mass spectrometry. Secretome from these cells contains bioactive molecules such as HGF or PAI-1, which have been previously implicated by other authors in drug resistance.(238,283). The fact that stable depletion of NRG-1 in CAFs potently restores drug efficacy of trastuzumab in HER2+ BC cell lines, reinforces our hypothesis that, although other potential mediators such as HGF or PAI-1 would also contribute to the acquired phenotype, microenvironmental NRG-1 is potently orchestrating trastuzumab drug response in HER2-BC cells.

Notwithstanding, it must be taken in consideration that in tumours, not only neoplastic cells receive the impact of the drugs and the potential implications of this remain largely unexplored. Despite targeted-therapies were conceived to selectively attack neoplastic cells, drug side-effects are indicative that non-tumoral cells are also potentially affected. A mention apart deserves chemotherapeutic agents. Still remaining as one of the most effective anticancer drugs, they are highly unspecific. With regard to this, emerging data is pointing out how CAFs remodel their secretory repertoire in response to chemotherapy(284). Pancreatic CAFs promote a proinflammatory response upon chemotherapy treatment driven by stress-associated MAPKs. This in turn, enhances tumour cell growth and invasiveness(239). In the same line, upon cisplatin treatment, CAFs enhance secretion of the cytokine Plasminogen activator inhibitor-1(PAI-1) which in turn, reduces cisplatin effects in esophageal squamous cell carcinoma cells (ESCC)(238).

In line with this, we have also started to characterize the impact of the drug therapies used for treating HER2-positive breast cancer, and the potential impact on CAFs. On the one hand, we have shown that CAFs are appreciably, low sensitive to paclitaxel alone or in combination with trastuzumab. On the other hand, we have observed no significant changes in terms of NRG-1 mRNA expression, although we cannot discard that NRG-1 shedding may be affected in response to drug treatment. Interestingly, although very preliminary, CAFs significantly upregulate FAP mRNA expression under drug pressure. FAP has been involved in driving proinflammatory phenotype in CAFs and in promoting immunosuppression in the TME(285). In line with this, in collaboration with Dr. Rojo, we are currently studying how chemotherapy alone or in combination with anti-HER2 targeted therapies may modulate cellular programmes in CAFs, and if this in turn may potentially shape tumour cell progression or drug therapy efficacy.

D5. PARACRINE NRG-1 IMPLICATIONS IN ORTHOTOPIC AND IN EX-VIVO BREAST CANCER TUMOURS

Orthotopical co-injection of CAFs with breast cancer cells enhances tumour growth. With regard to this, NRG-1 may be playing important roles since stable depletion of NRG-1 in CAFs is sufficient to counteract CAF derived effects on enhancing tumour growth. This data is particularly relevant. Although other authors have referred stromal contribution of NRGs in the acquisition of tumour cell capacities that will ultimately enhance tumour progression and therapy resistance in other types of cancers, no data is available of the specific contribution of stromal NRGs in tumorigenesis in *in vivo* models.

However, it may be taken in consideration that, co-injected fibroblasts do not survive long-term in xenografted immunodeficient mice(286,287).

Notwithstanding, the significant differences observed between both experimental arms, has driven us to hypothesise which potential issues could explain our results. It may be plausible that in this context, paracrine NRG-1 would modify tumour cells at early time points on tumour initiation. This in turn, may provide advantages for subsequent tumour growth, even when human CAFs were not already present. Among this, it could be rational that in the early-stages, wild type (WT) CAFs could help cancer cells on surviving, on enhancing tumour cell proliferation as well as on promoting formation of new vasculature, which is required for *in vivo* tumour growth. In this context, depletion of microenvironmental NRG-1 may delay these effects. We are currently designing new experimental approaches to clarify these open questions.

The fact that co-injected CAFs do not survive long-term *in vivo*, besides the slow tumour growth rate of BT474 cells *in viv*o, has limited subsequent studies and we have not been able to study if CAFs may differentially modulate *in vivo* drug efficacy depending on the expression of NRG-1. Alternatively, we have performed ex-vivo experiments, which have been previously set up in the lab in other HER2-positive and bladder cancer projects (91,243). BT474 excised tumours have been minced and treated for 5 days with trastuzumab and NRG-1 either by alone or combined or both combined with pertuzumab. In line with prior data, paracrine NRG-1 is able to significantly neutralize trastuzumab drug effects in BT474 ex-vivo tumours, whereas the addition of pertuzumab potently inhibits NRG-1 protective drug effects.

D6. MOLECULAR MECHANISMS UNDERLYING MICROENVIRONMENTAL NRG-1 AND THEIR POTENTIAL IMPLICATION IN DRUG RESISTANCE

The work presented here supports that microenvironmental NRG-1 modulates cancer cell biology and therapy response of HER2-positive breast cancer cells. In line with this, we have started to characterize the molecular contributors that may explain the acquired phenotype. At a molecular level, we have seen that paracrine NRG-1 results in a potent induction of the PI3K/AKT and the MAPK/Erk cellular cascades. These pathways are the chief mechanism for controlling cell survival, differentiation, proliferation, metabolism, and motility in response to extracellular cues; and they have been largely involved in therapy resistance in most of solid tumours(271,288). Among all the multiple cellular cascades which they have been reported to crosstalk with, we have decided on focusing in the transforming growth factor-beta (TGF-β) signalling cascade.

In relation with HER2-positive breast cancer, accumulating evidence indicates a functional crosstalk between the HER2 tyrosine kinase and the TGF-β signalling pathway(289,290). In animal models, this crosstalk results in increased cancer progression and metastasis as well as on resistance to chemotherapy and HER2-targeted therapy(291). Preclinical evidence support that HER2 gene amplification or overexpression converts TGF-β from a tumour suppressor to a promoting factor(291,292).

With regard to this, we have described that stimulation of the HER2-positive, SKBR3 breast cancer cells with recombinant NRG-1β promotes activation of PI3K/AKT and MAPK/Erk pathways. Concomitantly, it results in an upregulation on the mRNA isoforms of TGFβ1 and TGFβ2 in a time-dependent manner. Moreover, we have also observed that NRG-1 promotes an increased expression/stabilisation of the transcription factor Snail1 at a protein level.

Other authors have reported that upon AKT activation, the Glycogen synthase kinase (GSK)-3β is inactivated being unable to retain Snail in the cytoplasm. In these circumstances, Snail translocates to the nucleus. Besides PI3K/AKT activity in enhancing TGF-β oncogenic signalling and on regulating activity of Snail1; in 2003, Peinado *et al.* described that transforming growth factor beta-1 (TGF-β1) induced Snail1 expression in canine kidney cells and triggered epithelia-to-mesenchymal transition by a mechanism dependent on the MAPKs signalling pathway. Furthermore, the authors demonstrated that TGF-β induced the activity of the Snail promoter in a MEK1/2 activity dependent manner(241).

Nuclear Snail acts as a key inducer of EMT, and it can also foster cell survival and migration of tumour cells(220). Hence, it may be reasonable to think that microenvironmental NRG-1 may be able to foster cancer progression in a manner that not simply resolves on activating cell survival programs; but also on promoting motility and invasion of cancer cells. Although the data has not been included in this doctoral thesis, we have observed that paracrine NRG-1 promotes migration of HER2-positive breast cancer cells *in vitro* by performing wound-healing assays. We are currently working on finishing these experiments to fully confirm these NRG-1 roles.

With regard to therapy resistance, TGF-β has been reported to promote drug evasion to conventional therapies such as chemotherapeutic agents and also, to targeted therapies(293–295). Interestingly, in a work leaded by Dr. Arteaga, TGF-β has been linked with promoting trastuzumab resistance in HER2-positive breast cancer cells as a result of PI3K activation(290). Hence, having observed that microenvironmental NRG-1 enhances TGF-β mRNA expression, it could be interesting to corroborate if a switch in the TGF-β signalling in cancer cells could be a potential NRG-1-induced mechanism to bypass trastuzumab and paclitaxel drug efficacy in HER2-BC cells.

D7. NRG-1 MAY ACT IN A BIDIRECTIONAL MANNER BY SUSTAINING CAF ACTIVATION

As previously mentioned, communication between cancer cells and CAFs occurs at multiple levels. Upon this, they closely interact with each other, in a process that confers benefits to both populations. Because of this, in this project we have wondered if paracrine crosstalk leaded by microenvironmental NRG-1, may also result in a bi-directional feedback loop that may confer advantages for both cell types. We have seen that NRG-1β enhances TGF-β expression in tumour cells. Latent form of TGF-β is released into the extracellular space where it can act in an autocrine or paracrine manner(218). Although we have not validated the presence of soluble latent form of TGF-β in the experimental conditions approached; our results show that tumour cells *per se* modulate cell signalling in CAFs but that, NRG-1β accentuates this phenomenon. Upon NRG-1β, tumour cells modulate their secretory repertoire and increase their capacity to trans-activate the canonical TGF-β pathway in CAFs. In this context, enhanced activation of two of the main downstream effectors in this process, the phospho-Smads 2 and 3, is observed in CAFs.

Additionally, under these circumstances, enhanced Snail1 protein accumulation and/or stabilisation in CAFs occur. This is particularly interesting since Snail1 has been purposed to be required for CAF's activity in promoting tumour progression. In line with this, Snail1 levels have been correlated with myofibroblast markers; with CAF's activity on the extracellular matrix as well as on promoting tumour cell invasion in a paracrine manner(296–298).

Moreover, we have also observed that trastuzumab is not effective on preventing this bidirectional crosstalk leaded by NRG-1β, but combination with pertuzumab in tumour cells slightly prevents it. These data reinforce our idea that microenvironmental NRG-1 strongly modifies tumour cell dynamics through HER2/HER3 associated signalling in a way that not simply resolves on modulating cancer cells itself but also on affecting other cell populations such as CAFs.

In this vein, the fact that microenvironmental NRG-1 may result in enhanced shedding of TGF-β is particularly interesting. Tumour-derived TGF-β can affect several cell types that are in proximity to the growing tumours such as stromal fibroblasts, endothelial cells and immune cells. All of this in turn, results in tumour microenvironment remodelling to a more permissive niche(209). With regard to stromal fibroblasts, TGF-β promotes differentiation of resident stromal fibroblasts into activated fibroblasts. Indeed, TGF-β signalling is enhanced in CAFs from several cancer subtypes, and clinically correlates with poor prognosis and locally advanced disease(223,224,299–301).

To us, no prior reports are relative about paracrine-crosstalk between cancer cells and CAFs through a potential bi-directional NRG-1/TGF-β feedback loop. However, although exciting, this data has to be taken carefully. We have not proven that NRG-1β promotes shedding of latent active TGF-β isoform. In the same line, we have not demonstrated that residual recombinant NRG-1β that could remain in the conditioned media of tumour cells is innocuous to CAFs. Besides this, TGF-β signalling is highly complex and can be alternatively activated by other mechanisms. Thus, we cannot assure if this paracrine crosstalk is due to NRG-1 direct effects on the TGF-β program or by an indirect effect on the shedding of other potential soluble molecules in cancer cell's secretome that would ultimately crosstalk with the canonical TGF-β pathway in CAFs. We are currently working on designing new experimental approaches to clarify these open questions.

In line with microenvironmental roles of TGF-β, our group is participating in a project leaded by Dr. Muntasell, which has the objective of identifying molecular mechanisms involved in immunosuppressive TGF-β roles in NK cells. Derived from this, we have recently reported that CD57+ NK cells levels inversely correlate with pCR in HER2-positive breast cancer patients treated with anti-HER2 monoclonal antibodies, such as trastuzumab and pertuzumab(123). One of the main mechanisms of action of monoclonal antibodies is triggering ADCC cell response mediated by NK cells.

Therefore, exploring potential contribution of NRG-1/TGF-β axis in immunoevasion would provide further evidence about the role of stromal NRG-1 in anti-HER2 therapy resistance in a more complex point of view. In this context of TME immunoremodelling, a recent paper has shown that stimulation of macrophages with NRG-1 enhance breast cancer cells transendothelial migration and intravasation through paracrine JAG1 secretion, a ligand of the Notch receptor(274).

With regard to this, we have recently started a new project about tumour ecology of HER2 and its contribution on anti-HER2 targeted therapies. These include the use of more-complex systems such as heterotypic 3D scaffold-free *in vitro* models composed by CAFs and tumour cells; in which, we have also started to incorporate immune cells (A. Hernández-Prat *et al.*/L. Soria.Combining immume check point inhibitors with innovative mTOR inhibitors through PD-L1 up-regulation in bladder cancer model. I ASEICA- ASPIC International meeting; Salamanca, september 2019. Abstract #2).

The work presented in this doctoral thesis has been generated in the landmark of studying resistance to anti-HER2 targeted therapy by incorporating new areas of research in our group, such as the interplay with stromal fibroblasts. Hence, as a matter of time, the new experimental approaches (i.e. 3D-heterotipic models, microfluidic devices or *in vivo* immunocompetent models) in which we are currently working in, have preliminarily started to be explored. In this direction, we are aware that our 2D paracrine system has some limitations, and that, cell-to-cell contact either by 3D cultures or microfluidic devices will help us in the future to better understanding microenvironmental NRG-1 roles in HER2-positive breast cancer, not only in regard to tumour cells but also in studying the tumour as a global, in which the potential modulation and contribution of other stromal populations such as immune cells will be captured.

D8. PARACRINE NRG-1 FROM PRIMARY CAFS DERIVED FROM HER2+ BREAST CANCER PATIENTS REDUCE TRASTUZUMAB AND PACLITAXEL DRUG EFFICACY

Notwithstanding, the project has also comprised the generation of a collection of primary CAF cultures derived from breast cancer patients from Hospital del Mar. To us, no prior reports from other authors are relative about the generation of a primary CAF cultures bank derived from breast cancer patients (not only to HER2, but also to the other BC subtypes that we are also stablishing). This collection represents to us an opportunity to study the underlying heterogeneity of these stromal populations, and to correlate potential findings in human primary patient samples.

Primary HER2-CAFs analysed are, morphologically and phenotypically, a highly heterogeneous population. At a molecular level, they express variable levels of α-SMA and FAP. This is in line with previous studies in which molecular analysis have revealed distinct populations of CAFs in terms of α-SMA(214). Besides this, although expression of these two markers are widely accepted for determining CAFs, increasing data is demonstrating that they are not uniquely expressed by this type of cells nor expressed by all of them(212).This in turn adds a layer of complexity for the identification and review of the literature.

Bearing in mind the complexity for CAF's analysis as well as the underlying heterogeneity and plasticity of these stromal cells, NRG-1 has also been analysed. In the same way, primary CAFs are heterogeneous in terms of NRG-1 mRNA expression. As mentioned, recent studies have suggested that distinct subpopulations of CAFs co-exist within tumours and that they differentially regulate tumour cell progression and therapy response. aSMA low and high CAFs have been reported to differentially impact on cell proliferation and self-renewal capacity of oral tumour cells(214,302). Taking this into account, it may be reasonable to think that NRG-1 could be differentially expressed by certain subpopulations of breast cancer CAFs. In this context, it may be reasonable that NRG-1^{high} and NRG-1^{low} CAFs, would differentially regulate tumour cell dynamics and therapy response.

With regard to the latest aspect, we have also assessed if primary CAFs with similar NRG-1 expression levels than CAF200 cells are able to reduce trastuzumab and paclitaxel *in vitro* drug efficacy. Interestingly, secretome from BC-T50 primary CAFs, significantly shapes trastuzumab and paclitaxel drug effects as single agents or even in drug combination in a similar manner than immortalized CAFs.

D9. NRG-1 IS EXPRESSED IN THE TUMOUR-ASSOCIATED STROMA FROM HER2+ BREAST CANCER PATIENTS

We have analysed the distribution of NRG-1 expression in HER2+ breast cancer patients by different approaches. On the one hand, we have performed an extensive search from curated gene expression datasets. Of note, in this search we have just considered those studies in which raw data was well-indexed and informed. Upon selection, two studies have satisfied our demands. In the first one, Liu *et al.* performed gene expression profiling from the tumour epithelium and the tumour associated stroma of HER2+ breast cancer patients.

The authors referred that tissue specimens were obtained by laser micro dissection and evaluated by a pathologist, which provides us reliability on the data. The number of samples in each cohort was similar between groups.

Unfortunately, the authors did not refer if they were paired samples or not, so we have just been able to study each cohort as a global, and no potential association between the tumour epithelia and the tumour stroma of a particular patient's sample has been performed.

By performing R statistics, we have analysed both cohorts by separate and the mean for NRG-1 expression has been calculated. Interestingly, NRG-1 is highly expressed in the tumour associated stroma than in the tumour epithelia of these patients. This data, besides with our previous *in vitro* results about NRG-1 expression in HER2+ breast cancer cell lines as well as in primary and immortalized HER2-CAFs, provides stronger evidence that in HER2+ breast cancer patients, NRG-1 is predominantly found in the tumour microenvironment. In the second work analysed, Finak *et al.* performed gene expression profiling from the tumour stroma and the normal distant stroma of breast cancer patients.

By analysing each group by separate, we have observed that NRG-1 expression is higher in the tumour-associated stroma than in the normal breast tissue.

However, two aspects may be considered. Firstly, the N size of the normal stromal tissue group was notably low, so potential variability in this group may not be captured in our study. Secondly, the fact that the normal breast stroma was also obtained from breast cancer patients. Authors referred that it was obtained from a distant area from where the tumour was located and evaluated by a pathologist. Nonetheless, potential differences with tumour-free normal breast stroma may be considered.

With all of these external limitations, our study is in the same way than two previous work published in 2008 and 2009, respectively(303,304). In both studies, the authors explored molecular distinctions between normal breast associated fibroblasts (NAFs) and CAFs. As a result, they defined a list of genes differentially expressed between both stromal populations.
The relative genes encoded soluble and matrix-bound molecules that are associated with tumour growth, invasion or migration and extra-cellular matrix remodelling (ECM), among others. From both lists of differentially expressed genes, NRG-1 appeared highly expressed in CAFs. Besides exploring NRG-1 distribution in public datasets, we have also stained a cohort of HER2+ breast cancer patient's samples from Hospital del Mar.
This has been a challenging part of the project. As mentioned, the absence of reliable commercial antibodies has hindered the process. Moreover, few data are reported about NRG-1 expression in the stromal compartment of cancer patient's samples. Thus, we could not speculate if NRG-1 distribution within the TME should be homogeneous or restricted to certain areas. To solve this, we have trained ourselves by staining NRG-1 in some of the associated paraffin samples (FFPE) from the same patients from which we have generated our primary CAF cultures. NRG-1 has been detected in the stromal compartment of some of these patients.

Interestingly, NRG-1 localized in the intertumoural stromal fibroblasts (those inside the tumour mass) with a major NRG-1 staining in the growing border of tumours and less in the central area. Altough we have not gone deeply on that this may suggest that stromal NRG-1 may associate with the growing neoplastic areas.

D10. NRG-1 STROMAL EXPRESSION IS ASSOCIATED WITH POORER RESPONSE TO TRASTUZUMAB AND CHEMOTHERAPY IN HER2+ BREAST CANCER PATIENTS

A cohort of 53 HER2-positive breast cancer patient's samples treated with neoadjuvant trastuzumab plus chemotherapy has been stained for NRG-1. NRG-1 has been found in the tumour-associated stroma from a subgroup of these breast cancer patients. Approximately, the 9% of them. Therefore, in HER2+ breast cancer patients, NRG-1 stromal expression may define a subset of breast cancer patients with different biological features with poorer response to trastuzumab.

To further validate our findings, we have expanded our analysis into large-scale datasets from HER2+ breast cancer patients. For this, patient dataset from the TransNoah phase III clinical trial has been analysed. The objective of this study was to evaluate benefit from adding neoadjuvant trastuzumab to chemotherapy in HER2+ breast cancer patients. NRG-1 expression *per se* has no statistical correlation with any of the parameters studied. This may be explained by the fact there is no information about tumour purity and the analysis of the expression of one single gene across such heterogeneous mixtures could be insufficient for capturing biological differences. In this vein, translation of tumour microenvironment-related studies into large-scale datasets from patients is still challenging. In line what with other authors have referred, future gene expression studies should continue to incorporate close pathology evaluation of the source material. It may be reasonable then that full representation of the entire tumour-bearing region would most closely match completed gene expression studies(305).

Because of this, we have indirectly assessed potential clinical value of stromal NRG-1 through an NRG-1 gene derived signature. Interestingly, higher score for NRG-1 signature significantly correlates with residual disease in these patients. In particular, higher expression of NRG-1 gene signature may particularly define a subgroup of breast cancer patients with intrinsic poorer response to trastuzumab.

Although these bioinformatic analyses are in line with our prior data derived from HER2+ breast cancer patient's samples, they should be interpreted carefully. The NRG-1 gene signature we have used has not been generated by us. Moreover, it has been derived from a non HER2- breast cancer *in vitro* model so, it must be taken in consideration that, NRG-1 may differentially modulate other gene expression programs in a context of HER2-oncogenic addition.
In relation with our findings, few studies have assessed clinical value of stromal NRG-1. De Boeck *et al.* have described that transmembrane NRG-1 (t-NRG1) in tumour mesenchymal cells (t-MC) clinically correlates with poor prognosis in colorectal cancer (CRC) patients. In this study, the authors have studied the presence of the ligand in frozen sections of primary CRC and of adjacent normal colorectal tissues. tNRG-1 has been found highly expressed in the stromal compartment of these patients, mainly at the invasive front. Stromal t-NRG1 expression correlates with a decreased 5-years progression-free survival (PFS)(275). The authors propose that t-NRG1 may be a novel prognostic marker in CRC and that, t-NRG1 derived from tumour-mesenchymal cells (t-MC) may support metastatic outgrowth. However, although the study highlights that, stromal expression of t-NRG1 confers poorer prognostic features, it does not provide experimental evidence about its potential value in predicting clinical response to CCR standard treatments.
In the same line, Han *et al.* have shown that stromal expression of NRG-1 positively correlates with TNM-stage lymph node metastasis and reduced gastric cancer patient's survival but does not inform about NRG-1 role as potential drug biomarker(306).

Besides this, and adding another layer of complexity, special mention deserves two studies about NRG-1 role in non-HER2 addicted tumours. In 2005, Yuste *et al.*, describe that *in vitro*, NRG-1 forced expression in MCF7 cells cause higher proliferation of tumour cells as well as constitutive activation of ErbB2, ErbB3 and ErbB4 receptors. Unexpectedly, trastuzumab shows inhibitory effects on proliferation of these cells. The authors suggested then that trastuzumab may also be an effective treatment in tumours in which NRGs feed the tumoral tissue(307). Later on, in 2007, extended data from the same group shows preclinical trastuzumab efficacy in xenograft mice bearing MCF7-NRG2c tumours. Moreover, in a retrospective study of 124 patients with early-stage or metastatic breast cancer, transmembrane NRG-1 is frequently found expressed in tumour cells(308). Overexpression of transmembrane NRG significantly correlates with a longer event-free survival and OS in patients with low or normal HER2-expression who are treated with trastuzumab-based therapies (that means, patients that after HER2 re-evaluation are classified as HER2-negative). Interestingly, this correlation is not observed in patients with HER2-overexpression.

The authors already suggested that in breast cancer patients, besides the determination of HER2, other parameters such as ligand expression may better select patients who potentially would benefit from trastuzumab-based therapies(308).

Taking all of this together, this data, side by side our results generate us two potential new hypotheses: one the one hand that, NRGs distribution between tumour cells and/or the surrounding tissue may be tissue-type or tumour type-specific. On the other hand, that, autocrine or paracrine expression of NRGs may dramatically determine innate trastuzumab response or resistance.

Whereas in low-HER2 expressing tumours, NRG-1 expression would predominantly come from the tumour epithelia, in HER2+ breast cancer, NRG-1 would predominantly come from the stromal compartment. In other words, NRG cellular distribution besides the oncogenic addition to HER2 may also define differential innate responses to trastuzumab of breast cancers. In low-HER2 expressing tumours, NRG-1 tumoral expression would be associated with trastuzumab responses(308).

On the contrary, in HER2+ breast cancers, stromal expression of NRG-1 would define a subset of patients which poorer response to trastuzumab.

This is also putting in context the relevance of our findings. NRGs have emerged as attractive drug targets in last years and some synthetic inhibitors are currently in preclinical phases(151). However, in this context, the tumour-type and/or the specific distribution of NRGs may be critical. In this scenario, our work provides strong evidence that in breast cancers with HER2-oncogenic addiction, NRG-1 is mostly expressed by stromal CAFs and that this stromal expression of NRG-1 clinically associates with poorer response to trastuzumab and chemotherapy, which have classically been the standard treatment for HER2+ breast cancer patients.

D11. CONCLUDING REMARKS AND POTENTIAL CLINICAL RELEVANCE OF THE STUDY

Nowadays, oncologists rely on a rich arsenal of drugs that efficiently target HER2, as well as on optimal drug sequencing guidelines for treating patients diagnosed with HER2-positive breast cancer(19). Currently, dual HER2-targeted therapy with trastuzumab and pertuzumab in combination with chemotherapy is routinely indicated for treating patients with early HER2 BC in the neoadjuvant setting and is the first line of therapy for patients with metastatic disease(19,248). Although this drug combination has led to a significant improvement in response rates and patient survival in both early and metastatic settings, a significant subset of patients (35-60%) still show fail to show a pathological complete response (pCR) and nearly all metastatic patients eventually progress(87).

Despite multiple research efforts focused on identifying clinically useful biomarkers, positivity of HER2 (defined by immunohistochemical or *in situ* hybridation techniques) remains the only valid criterion for patient selection(19,248). Translational research on this field has classically focused on understanding the canonical HER2 signalling pathway and the crosstalk of HER2 with other receptors and their ligands. More recently, the importance of the underlying heterogeneity, as well as the identification of intrinsic or acquired mutations and host-gene polymorphisms have become important research areas(19,135,248).

Notwithstanding all these efforts, a complete understanding of the mechanism of action of most anti-HER2 targeted therapies is still lacking. Furthermore, the important role of the tumour microenvironment (TME) and the immune system still remain largely unexplored. In this regard, a growing number of investigations is highlighting that the tumour context dramatically influences on HER2-BC biology and in therapy response(124,180,192,248,284). However, the specific interaction mechanisms between HER2+ tumour cells and their microenvironment are yet to be characterized.

Our present work has shown that the tumour microenvironment (TME), particularly CAFs, alternatively promote drug therapeutic evasion through the secretion of paracrine NRG-1. Our original hypotheses have been validated by different cellular and molecular *in vitro* and *in vivo* approaches and in primary cultures derived from HER2+ BC patients. Also, findings from phase III randomized clinical patient datasets as well as from a small cohort of patient biopsies treated in with neoadjuvant trastuzumab and taxane chemotherapy suggest an important role for NRG-1 in trastuzumab-based therapy resistance. Mechanistically, we have provided insights regarding how stromal NRG-1 may not only by-pass HER2-drug response but that it also participates in co-evolving interactions between tumour cells and stromal CAFs through, potentially through activation of an NRG-1/TGF-b axis.

Our findings suggest that, at least in part, stromal expression of NRG-1 may identify a subset of patients in which trastuzumab is potentially less effective. The true prevalence of high NRG-1 stromal expression in HER2-positive cancers is yet unknown, but it may help to identify clinical contexts in which the addition of pertuzumab would be especially useful, while trastuzumab may be sufficient in those patients whose stromas are poor in NRG-1 expression and lack other features that potentially confer pertuzumab sensitivity. Of note, this may have some further relevance in clinical contexts in which the addition of pertuzumab as part of the anti-HER2 therapy backbone may be beneficial, especially in 3rd and subsequent lines of therapy.

Together with recent evidence(82), our findings this suggests that evaluation of stromal NRG-1 may help to better define a subset of patients who especially benefit from dual anti-HER2/HER3 targeted therapies.

In summary, the data presented in this doctoral thesis provides evidence about the role of the tumour microenvironment, and particularly of CAFs, in HER2+ breast cancer biology and in therapy resistance through NRG-1. Our study is in line with the emerging concept that the role of the TME has to be taken into account in translational efforts pursuing the development of personalized medicine strategies. Ultimately, a more precise identification of patients who benefit from pertuzumab will spare others unnecessary toxicities and, at the same time, contribute to the sustainability of our public healthcare system.

CONCLUSIONS

1. Immortalized human CAFs derived from HER2-positive BC patients promoted resistance to trastuzumab. In this context, the drug efficacy of paclitaxel, alone or in combination with trastuzumab, was also impaired.

2. *In vitro*, CAFs foster paracrine activation of the pro-survival PI3K/Akt and MAPK/ERK pathways in cancer cells.

3. Among the repertoire of CAF-secreted factors, Neuregulin 1 (NRG-1) was expressed by CAFs at mRNA and protein level.

4. Recombinant human NRG-1β mimicked the paracrine effects of CAFs in terms of PI3K/Akt and MAPK/Erk pathway activation, as well as impairing drug efficacy.

5. Stable depletion of NRG-1 in CAFs restored trastuzumab drug efficacy in HER2-positive BC cells. Hence, stromal NRG-1 was a key growth factor that mediated resistance to anti-HER2-targeted therapy.

6. *In vivo*, the orthotopic co-injection of HER2-positive BC cells with CAFs enhanced tumour growth. These effects were attenuated when cancer cells are co-injected with NRG-1-depleted CAFs.

7. NRG-1β activated the TGF-β gene expression programme in HER2-positive BC cells. In this context, the tumour cells amplified the activation loop in CAFs by enhancing the Smads and Snail1 signalling network. Pre-treating cancer cells with trastuzumab and pertuzumab attenuated NRG-1 paracrine effects in CAFs.

8. Pertuzumab (an anti-HER2 hetero-dimerization inhibitor), significantly reversed the protective effects of CAFs, and restored drug efficacy in BC cells.

9. A collection of primary CAF cultures was derived from fresh tumour biopsies from HER2-positive patients. Primary CAFs showed distinct expression patterns for the tumour stromal markers αSMA, FAP, and SDF-1. The differences observed highlight the extreme heterogeneity in the stromal compartment of BC patients.

10. Primary CAFs displayed differential NRG-1 expression. Paracrine signalling from NRG-1-expressing CAFs reduced the *in vitro* drug efficacy of trastuzumab and paclitaxel.

11. NRG-1 was detected in tumour sections from HER2-positive BC patient biopsies from which primary CAFs have been derived. NRG-1 was mostly expressed in the tumour stromal compartment of these tumours.

12. In an analysis of public datasets on HER2-positive tumours in which the epithelia and the tumour stroma had been micro-dissected, NRG-1 was highly expressed in the stromal compartment. Moreover, in subsequent analysis of public datasets from BC patients, NRG-1 appeared to be highly expressed in the tumour-associated stroma in comparison to the normal breast stroma.

13. Stromal NRG-1 protein expression correlated with a poor clinical outcome in a cohort of HER2-positive BC patient treated with trastuzumab plus chemotherapy in the neoadjuvant setting. Similarly, an NRG-1 public gene signature was associated with a poor response to neoadjuvant trastuzumab in HER2-positive BC patients.

14. NRG-1 stromal expression may be a potential biomarker for defining a group of HER2-positive breast cancer patients resistant to trastuzumab based therapy. Likewise, strategies aiming to block NRG-1 paracrine effects may enhance the antitumor activity of currently available anti-HER2 targeted agents.

MATERIALS AND METHODS

M1. CELL LINES AND CELL CULTURE

A panel of breast cancer cell lines was used in this study. SKBR3, BT474, EMF-192A, HCC1954, AU565, and MDA-MB-231 were obtained from the ATCC (American Type Culture Collection). SKBR3, BT474, HCC1954 and AU565 were classified as HER2+ breast cancer cell lines, whereas MDA-MB-231 was classified as a triple negative breast cancer cell line. The SKBR3 and BT474 cells were grown in DMEM:F12 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12) (Sigma-Aldrich); HCC1954, AU565 and EFM-192A in RPMI media 1640 (Invitrogen); and MDA-MB-231 in DMEM. All the cell culture media were supplemented with 10% foetal bovine serum (FBS, Sigma-Aldrich), 2 mM/L L-glutamine (Live Technologies, Inc. Ltd.), penicillin (100 units/ml), and streptomycin (100 µg/ml) (Live Technologies, Inc. Ltd.). In the case of the EFM-192A cells, the cell culture media was supplemented with 20% FBS. All the cell lines were kept at 37ºC in a humidified atmosphere containing 5% CO_2.

Cancer-associated fibroblast cell lines (CAFs), CAF200 and CAF220, were kindly provided by Dr. Gascón's lab, at Hospital Clinic (Barcelona), and were generated from HER2+ patients who underwent tumourectomy surgery. These were immortalized, introducing a retroviral pMIG (MSCV-IRES-GFP) expressing both hTERT and GFP. The CAFs were grown in DMEM supplemented with 10% FBS, 2 mM/L L-glutamine, penicillin (100 units/ml), and streptomycin (100 µg/ml). The cells were kept at 37ºC in a humidified atmosphere containing 5% CO_2

M2. DRUGS AND GROWTH FACTORS

Trastuzumab (Herceptin, Genentech, Roche) and pertuzumab (Perjeta, Genentech, Roche) were purchased from the Hospital del Mar pharmacy at 20 mg/mL and 30 mg/mL, respectively, and stored at 4ºC. Paclitaxel was purchased from MerkMillipore, prepared at 10 mM stock solution in DMSO, and stored at -20ºC. Human recombinant Neuregulin-1β (NRG-1), also referred to as Heregulin (HRG-1β), and Human recombinant transforming growth factor beta (TGF-β) were purchased from SigmaAldrich Co (Merck KGaA).

They were dissolved in sterile DEPC-treated water, at a concentration of 100 µg/mL, aliquoted, and stored at -20ºC.

M3. PROTEIN DETECTION

M3.1 Protein extraction

For whole-cell lysates, the BC cells were cultured in P60, P100, and P150 plates, depending on the experiment to be undertaken. Cancer cells were plated at a cell density of between 6x10^5–8x10^5 cells/plate (depending on the cell growth rate of each cell line). Cancer-associated fibroblasts (CAFs) were plated in P100 plates at 2.5x10^5 cells/plate. This proportion was established based on 24-hour experiments, but the number of cells was scaled according to the experimental design. At the end of the experiment, the plates were washed with cold phosphate-buffered saline (PBS). The cells were mechanically lysed by scraping them with ice-cold lysis buffer (Tris-HCL (pH=7.4) 50 mM, NaCl 150 mM, 1% NP40, EDTA 5 mM, NaF 5 mM) supplemented with protease inhibitors (Complete Mini EDTA-free Protease Inhibitor Cocktail, Sigma, Merck KGaA). The cell lysates were shaken for 30 minutes at 4ºC, then the samples were centrifuged for 10 minutes at 13,200 rpm, and the supernatant was aliquoted and stored at -20ºC.

M3.2 Protein quantification

The DC Protein Assay (Bio-Rad) colorimetric method was used for total protein quantification. The method was based on the Lowry assay. An acidic dye was added to the whole-cell protein extracts, and absorbance was measured at 750 nm with a microplate reader. Comparing the generated reads to a standard curve of bovine serum albumin (BSA) provided a relative measurement of protein concentration.

M3.3 Western Blot protein detection

To determine the expression of specific proteins in particular experimental conditions, an equal quantity of protein for each sample, around 20-40 μg, was adjusted to the same volume with lysis sample buffer. An equal amount of 2x 5% β-mercaptoethanol Laemmly-sample buffer was added to each sample. The samples were then denatured for 5 minutes at 95ºC. Subsequently, the samples were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PDVF) membranes using pre-cast gels and membranes (Bio-Rad). The transfer was made using Trans-Blot Turbo and Transfer Packs (including optimized buffer, membrane, and filter paper) within 10 minutes.

Immunoblotting was carried out according to the standard procedure. The PDVF membranes were incubated with Ponceau S solution 0.2% (Serva) for 5 minutes at room temperature (RT) to confirm that the transfer had been carried out correctly, as well as to cut the membranes depending on the molecular weight of the target protein. After washing the membranes with distilled water, they were incubated with 5% tris-buffered saline with 5% tween (TBS-Tween) milk for 1h at RT to block non-specific detection. The membranes were then incubated overnight at 4ºC with the specific primary antibodies (listed below). After washing three times with TBS-T, horseradish peroxidise-conjugated secondary antibodies (Amersham) were used to detect primary antibodies. The membranes were later washed twice more with TBS-T and a final time with TBS. The targeted proteins were visualized after enhanced chemoluminescence treatment (Amersham) of membranes and subsequent exposure to X-ray film.

The following primary antibodies were used for protein detection: phosphoHER2 (Y1248), phosphoHER3 (1289), HER3, phosphoERK1/2 (T202/Y204), ERK1/2, phosphoAKT (S473), AKT, phosphoS6 (S235/236), S6, Snail1, phospho Smad2 (Ser465/467)/Smad3 (Ser423/425). These were purchased from Cell Signaling Technology. HER2 was bought from BioGenex (clone CB11); αSMA, Cytokeratin 19 and FAP from Dako; α-Tubulin and β-Actin from Sigma Aldrich Co (Merck KGaA); and GAPDH from Santa CRUZ.

Mouse and rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare Life Sciences) were used.

M3.4 Immunoprecipitation (IP) protein detection

Immunoprecipitation experiments were kindly performed by Dr J.C. Montero, from Dr. Pandiella's group. Between 0.4 and 2 mg of total cell protein- lysate was incubated for 2 hours at 4ºC with 1 μgram of an anti-NRG-1 antibody (chimeric, anti-rabbit) and with 60 μl of A-sepharose protein ®. The immunocomplexes were washed three times with 1 ml of cold-lysis buffer, and finally mixed with 30 μl of loading buffer. Subsequently, the samples were subjected to gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PDVF) membranes. The transfer was made using the Trans-Blot system from BioRad. The membranes were blocked with 5% non-fat milk for 1 hour at room temperature. The primary antibody were incubated overnight (O/N) at 4ºC. The membranes were washed three times with TBS-T, and, after this, horseradish peroxidise-conjugated secondary antibodies (anti-Rabbit-HRP, BioRad) were used to detect the primary antibodies. The proteins were visualized using a detection system based on luminol oxidation in the presence of hydrogen peroxide augmented with iodophenol (Ausbel and Gitler, 1987; Cabrera *et al*., 1996).

M3.5 Immunofluorescence (IF) protein detection

Cells were seeded on tissue culture treated slides. 24 hours later, the cells were serum starved O/N with 1% FBS. The cells were then treated with recombinant NRG-1β [1nM] for 24 hours, washed with cold PBS, and fixed with paraformaldeyde (PFA) at 4% (prepared in PBS) for 10 minutes. After fixation, the cells were washed twice with PBS and incubated with PBS/0.25%-Tryton X 100 for 10 minutes at RT. They were then incubated with 1% BSA. The cells were subsequently incubated for 1 hour at RT with primary anti-Snail1 antibody (1:200) (Cell Signalling). The slides were washed twice with PBS and incubated for 1 hour at RT (protected from light) with a secondary anti-rabbit Alexa 488 antibody (1:1000) and an anti-tubulin FITC-conjugated antibody (1:1000).

The secondary antibodies were prepared with PBST-BSA 1%. After this, the slides were washed twice with PBS and mounted on glass with SlowFade™ Diamond Antifade Mountant with DAPI (Thermo Fisher). DAPI was used to counterstain the nucleus.

M3.6 Western Blot and IP analysis

For the Western Blot analysis: X-ray films were scanned, the background of the films was subtracted, and the intensity of each band was measured using the ImageJ software. The anti-α-Tubulin, anti-β-Actin and GAPDH antibodies were used as loading controls to verify that there were equal quantities of protein among the samples. Values corrected for loading were expressed as fold change of the control condition. Moreover, for each WB, protein quantification was represented as the average and SD of three independent assays.
For the IP analysis, X-ray films were scanned, the background was also subtracted and the intensity of each band was measured using the ImageJ software.

M3.7 Mass spectrometry protein detection

Secretome from CAF200 cells was analysed through mass spectrometry. For this, 2x10^6 cells were plated on P150 plates and left to attach for 24 hours. After this, the cells were left for a further 24 hours to secrete factors into the cell culture media, and the derived secretome was collected. As a negative control, a cell culture growth medium sample was included. The objective was to eliminate potential background contributions from the cell culture media or FBS-containing proteins. Both samples were centrifuged for 5 minutes at 1500 rpm, to discard potential cell debris (or detached cells in the case of CAF secretome), filtered with Millex-HP Syringe Filter Unit, 0.45 μm (Merck, Millipore), concentrated with Amicon Ultra-4 /Ultracel-3 (Merck, Millipore) for 40 minutes at 40000 Gs, at 4ºC. The samples were precipitated O/N at -20ºC with six volumes of acetone HPLC grade (Sigma-Aldrich). After this, the precipitated products were centrifuged for 10 minutes at 16000g, at 4ºC.

The acetone was removed and the pellet was dried and resuspended in 6M Urea/200 mM ammonium bicarbonate buffer, for in-solution sample digestion.

The samples were quantified with Pierce™ BCA Protein Assay (ThermoFisher) and the protein concentration was calculated using a BSA standard dose curve. Sample absorbance was read at 565 nm with a microplate reader.

Mass spectrometry was performed in the Centre for Genomic Regulation (CRG) Proteomics Unit, at the PRBB. A total of 10 μgr of protein for each condition was digested for each sample by in-solution tryptic digestion. Subsequently, 2 μgr of the digested samples were injected into a Velos Mass Spectrometer. The samples were cleaned up by off-line phase reverse phase purification with C18 columns. The data generated was analysed with Proteome Discoverer v1.4 and the Mascot v2.6 search engine. The mass spectrometry data was matched with the Uniprot human and *Bos Taurus* databases.

M4. REAL TIME QUANTITATIVE PCR (RT-PCR)

To study mRNA expression by RT-PCR, total RNA was extracted using TRIzol reagent (Sigma) according to our lab protocol. The cells were incubated for 5 minutes with the reagent at RT and chloroform was then added to separate the RNA, DNA, and protein according to their solubility. After being incubated for 10 minutes at RT, the samples were centrifuged for 15 minutes at the maximum speed, at 4ºC. The upper aqueous phase was collected and mixed with isopropanol 100% to precipitate RNA. After being incubated for 10 minutes at RT, the samples were centrifuged for 10 minutes at the maximum speed, at 4ºC. The supernatant was discarded and the pellet was mixed with 75% ethanol. The samples were centrifuged for 5 minutes at the maximum speed, at 4ºC, the supernatant was discarded, and the pellet dried at RT. Finally, the RNA was dissolved with 100 μL ultrapure DEPC-treated water (ThermoFisher SCIENTIFIC) and stored at -80ºC. The amount of mRNA per sample was quantified using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

M4.1 cDNA synthesis

After extraction, 500 μgr of mRNA was reversed transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The conditions for cDNA reverse transcription performed in the thermal cycler Mastercycler ep (Eppendorf) were:

1- 10 min at 25ºC
2- 120 min at 37ºC
3- 5 min at 85ºC
4- Hold at 4ºC

M4.2 Primer design

To design primers, we searched for the cDNA sequence of the target genes on the web page https://www.ensembl.org/index.html. From the transcript variant, we selected just exons. Once we had the exon of interest, we pasted the sequence into http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi. The primer design included the following characteristics: length of the amplicon between 90-220 base pairs; length of each primer between 18-27 mer; melting temperature between 59-61ºC and equal or similar between primers; sequence amplified from two different exons, preferably ending in "C" or "G" at both 5' and 3' ends; and lack of capacity to bind to other sites in the template. Finally, a BLAST was performed to confirm that the primers generated were not homologous and did not have the capacity to bind non-specifically to other sequences in the human transcriptome and genome.
The following primers were designed:

NRG1β **Fw:** 5'-CAGCCCAAGAGCCTGTTAAG-3'
NRG1β **Rv:** 5'-ACTGCTCTGGGAGCTTGTGT-3'

HER3 **Fw:** 5'-GGGGAGTCTTGCCAGGAG-3'
HER3 **Rv:** 5'-CATTGGGTGTAGAGAGACTGGAC -3'

TGFB1 **Fw:** 5'-CACGTGGAGCTGTACCAGAA-3'
TGFB1 **Rv**: 5'-GAACCCGTTGATGTCCACTT-3'

TGFB2 **Fw:** 5'- ACCGGCGGAAGAAGCGTGCTTTGGA-3'
TGFB2 **Rv:** 5'-GGGCATGCTCCAGCACAGAAGTTGGCA-3'

TGFB3 **Fw:** 5'-GGAAAACACCGAGTCGGAATAC-3'
TGFB3 **Rv**: 5'-GCGGAAAACCTTGGAGGTAAT-3'

FAP **Fw:** 5'-GGCTCACGTGGGTTACTGAT-3'
FAP **Rv:** 5'-TCCTGGGTCTTTGGACAATC-3'

CXCL12 **Fw:** 5'-TCAGAGCCGTGGTCCTTTGGGGTGA -3'
CXCL12 **Rv:** 5'-AGCAGTGCTGGGCTTGGCCCTAGTT -3'

ACTIN **Fw:** 5'-GGGGTGTTGAAGGTCTCAAA-3'
ACTIN **Rv:** 5'-AGAAAATCTGGCACCCC-3'

GAPDH **Fw:** 5'- GGAGTCAACGGATTTGGTCGTA-3'
GAPDH **Rv:** 5'- GGCAACAATATCCACTTTACCAGAGT-3'

M4.3 RT-PCR

For the DNA amplification we used LightCycler® 480 SYBR Green I Master (Roche) and the QuantStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific) using 384-well plates. The samples were loaded in triplicate. Primer amplification was carried out at 60ºC. The experiments were performed in the genomics core facility at Pompeu Fabra University (UPF), at the PRBB.

The amplification cycle values of each sample and specificity of the amplified PCR product were analysed using the Quantstudio 12K Flex Real-time software. The relative expression of each gene in each condition was normalized to the expression of the housekeeping genes *GAPDH* or *ACTIN*. The data was calculated as 2 to the power of - $\Delta\Delta C_t$, where $\Delta\Delta C_t = (Ct_{Gene} - Ct_{ATP5E})_{Assay}$ for three independent assays for each cell line (except in some experimental approaches where only two independent assays were performed).

M5. *IN VITRO* PARACRINE CROSSTALK BETWEEN CANCER CELLS AND CAFs

M5.1 2D indirect crosstalk model

To study the modulatory effects of CAFs in cancer cell biology and drug response, an indirect interaction system was set up. For this, either cancer cells or CAFs were cultured following their specific cell growth requirements (see M1 from this section). When the cells reached a confluence of 70-80%, the derived conditioned media (also referred to as secretome or CM) was collected, centrifuged for 5 minutes at 1500 rpm to discard potential cell debris or detached cells, and passed through a 0.45 μm filter. The resulting enriched CM was diluted at 1:3 with fresh cell culture media and the mixture was placed onto the cells, for different times, depending on the *in vitro* experimental technique used.

M5.2 Homotypic and heterotypic 3D culture models

For the direct co-culture model, BT474 and SKBR3 cells were mixed with CAF200 cells in a ratio of 1:1. To do this, each cell line was trypsinized and counted. The cells were then mixed in the same ratio and plated in ultra-low attachment round bottom 96 well-plates (Corning). The plates were then centrifuged at 1000 g for 10 minutes. Spheroids were cultured for 48 hours and a widefield microscope was used to take bright-field and fluorescent images of the homotypic and heterotypic spheroids.

M5.3 Analysis of 3D- homotypic and heterotypic cultures through immunohistochemistry (IHC)

Spheroids were collected one by one and centrifuged at 12,000 rpm for 2 minutes. The supernatant was removed and the spheroids were washed once with PBS, before being centrifuged at 800 rpm for 8 minutes. The supernatant was carefully removed so that no spheroids were lost, and these were embedded in Histogel (Thermo Scientific).

The resulting mix was cooled down for 5 minutes at 4ºC in order to solidify the Histogel, and the spheroids were then fixed with 4% phormol for 10 minutes. For sample preparation, sections were obtained from FFPE-embedded spheroids, mounted onto charged slides, and then deparaffinized in xylene and hydrated. The samples were stained for haematoxylin and eosin (H&E). After describing the H&E staining, the samples were IHC stained with the epithelial marker Cytokeratin 19 (Dako), and Vimentin (Dako). The antigen-antibody reaction was detected by incubating the samples with anti-rabbit Ig-dextran polymer coupled with peroxidase (Flex+, Dako, Agilent Technologies).

The sections were then visualized with DAB and counterstained with haematoxylin. H&E and IHC were evaluated by the pathologist Dr. Federico Rojo, head of pathology at the Fundación Jimenez Díaz, in Madrid.

M6. SHORT-HAIRPIN RNA INTERFERENCE STUDIES

CAFs were genetically engineered using short hairpin RNA technology for NRG-1 knock down. NRG-1 knock down from CAFs was kindly performed by Dr. JC Montero, from Dr.Pandiella's group. HEK-293T cells were used for virus production. Cells were derived from HEK-293 and transformed with adenovirus E1a and E1b, and engineered to contain the antigen T SV40 virus insert. Transfection of HEK293T cells was performed with jetPEI™ (PolyPlus Transfection).

For transfection, 2.5x10^6 HEK-293T cells were seeded in P100 plates. 24 hours later, transfection was performed. On the one hand, 4 μgr of plasmids required for lentivirus production (pMDL-RRE, pRSV-REV, pMDG-VSV) were mixed with either 8 μgr of non-targeting pLKO-shControl, shRNA, or 8 μgr of pLKO-shNRG1 sequence #1, shNRG-1. The plasmids were mixed with NaCl to a final volume of 250 μl. On the other hand, 40 μl of jetPEI was mixed with the previous mix. The resulting product was incubated for 30 minutes at room temperature, and the solution was added onto HEK-293T cells. 24 hours later, the culture media was replaced with fresh media.

In parallel, target cells for infection, in other words CAF200 cells, were seeded. 24 hours later, the cell-culture-containing virus was collected, passed through 0.45 μm filters (Merck, Millipore), and 6 μgr/ml of polibrene (Hexadimethrine Bromide, Sigma Aldrich) was added.
Following this, the CAF200 cells were infected. 72 hours after infection, 3 μgr/ml of puromycin (Sigma Aldrich) was added, to select cells that had previously incorporated the plasmid (which contained a resistance cassette against puromycin). After 72 hours, the selected cells were lysated and the NRG-1 stable knock-down efficiency was analyzed by Western Blot.

M7. *IN VIVO* STUDIES

M7.1 Animals

Seven-week-old female SCID-Beige CB-17/IcrHsd-Prkdc^{scid}Lyst^{bg-J} mice were purchased from Envigo (Madison, WI) and hosted in the pathogen-free animal facility at the Barcelona Biomedical Research Park (PRBB).

M7.2 Orthotopic xenograft model in SCID/Beige mice

Xenograft tumours were established through the orthotopic injection of BT474 cells, either alone or in a 1:1 mixture of 1.5x10^^{6} of BT474 cells with either shSCR CAF or shNRG-1 CAF cells. Prior to *in vivo* inoculation, the cells were harvested, pelleted by centrifugation, counted and resuspended with sterile PBS in 50% Matrigel (BD Biosciences). The cells were inoculated orthotopically in the N4 or N5 of the mammary fat pad of the mice. Since the BT474 cells were ER/PR-positive, a pellet of 17β-estradiol 0.72 mg was subcutaneously implanted in all the animals prior to cell inoculation.
Tumour volume was determined from calliper measurements of the tumour length (L) and width (W), according to the formula (L*W2*π)/6. Tumour size was measured once a week. At the end of the experiment, the animals were euthanized and the tumours were excised for subsequent studies.

M7.3 *In vivo* tumour sample analysis through IHC

The excised tumours were subjected to H&E staining, and the IHC study was kindly evaluated by Dr. Federico Rojo. For sample preparation, tissue sections from formalin-fixed and paraffin-embedded samples were obtained, mounted onto charged slides, and then deparaffinized in xylene and hydrated. The samples were stained for H&E. Samples were also IHC stained with the proliferation marker phosphorylated (S10) Histone H3 (pHistoneH3) (9701, Cell Signaling Technology).
The antigen-antibody reaction was detected through incubation with anti-rabbit Ig-dextran polymer coupled with peroxidase (Flex+, Dako, Agilent Technologies).
The sections were then visualized with DAB and counterstained with haematoxylin. H&E and IHC were evaluated by the pathologist Dr. Federico Rojo. The data was presented as the percentage of positive cells for each staining.

M7.4 Ex-vivo studies and IHC analyses

BT474 excised tumours were cut into pieces and cultured for 5 days in RPMI cell culture media supplement with 10% foetal bovine serum, 2 mM/L L-glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml). The tumours were treated with trastuzumab at 15 μgr/ml, pertuzumab at 20 μgr/ml, and NRG-1β at 10 pM. The samples were stained for IHC with the proliferation marker phospho-H3 and with the apoptosis marker cleaved caspase 3. The antibodies used for IHC were the following: phosphorylated (Ser10) Histone 3 rabbit polyclonal antibody (1:100); and active caspase-3 (9664, Cell Signaling Technology) rabbit polyclonal antibody (1:100). The antigen-antibody reaction was detected through incubation with anti-rabbit Ig-dextran polymer coupled with peroxidase (Flex+, Dako, Agilent Technologies). The sections were then visualized with DAB and counterstained with haematoxylin. H&E and IHC were evaluated by the pathologist Dr. Federico Rojo. The data was presented as the percentage of positive cells for each staining.

M8. GENERATION OF PRIMARY CAF CULTURES DERIVED FROM BREAST CANCER PATIENTS

Primary CAF cultures were derived from BC patients who underwent a tumour biopsy at Hospital del Mar. The tissue samples were cut into small pieces, placed into P100 plates and cultured in DMEM cell culture media supplemented with 10% FBS, 2 mM/L L-glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml). The cells were kept at 37ºC in a humidified atmosphere containing 5% CO_2. Non-adherent cells were carefully removed, and fresh new media was added every 3-4 days. In most cases, after a few days in culture, long cells outgrew from the tumour bulk and started to attach to the cell surface. The cells proliferated and filled-up the plate. When the primary cultures reached a confluence of between 70-80%, they were trypsinized and stocked.

M8.1 Assessment of culture purity for Western Blot and immunohistochemistry (IHC)

The resulting populations were analysed for the expression of typically expressed epithelial mesenchymal and immune-related markers.

M9.1 Tumor patient's samples analysis through Immunohistochemistry (IHC)

Paraffin embeded tumour sections from HER2-positive breast cancer patients were subjected to H&E staining, as previously detailed in section (ex-vivo studies and IHC analyses). Samples were IHC stained with anti-NRG and anti-FAP antibody rabbit. Anti-NRG antibody that was kindly provided by Dr. Pandiella. Antigen-antibody reaction was detected by incubation with anti-rabbit Ig-dextran polymer coupled with peroxidase (Flex+, Dako, Agilent Technologies). Sectiones where then visualized with DAB and counterstained wih hematoxylin. H&E and IHC were evaluated by the pathologist Dr. Federico Rojo.

M9. PATIENT SAMPLES

HER2+ BC patient samples were obtained from BC patients from Hospital del Mar who underwent a tumour biopsy. The samples were obtained from a cohort of 54 patients diagnosed with early HER2+ breast cancer treated with neoadjuvant trastuzumab plus taxanes. The pre-treatment diagnostic core biopsies were assayed. Pathological complete response (pCR) was defined according to the Miller Payne criteria, which establishes a grading system from G1 to G5 as histological evidence and/or the absence of invasive disease both in breast and axillary lymph nodes.

The study was approved by the Ethics Committees of each of the hospitals and conducted according to institutional guidelines. All the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

M9.1 Analysis of patient tumour samples through immunohistochemistry (IHC)

Paraffin embedded tumour sections from HER2+ breast cancer patients were subjected to H&E staining, as previously described. The samples were IHC stained with anti-NRG and anti-FAP antibodies. The anti-NRG antibody was kindly provided by Dr. Pandiella. The antigen-antibody reaction was detected by incubation with anti-rabbit Ig-dextran polymer coupled with peroxidase (Flex+, Dako, Agilent Technologies). The sections were then visualized with DAB and counterstained with haematoxylin. H&E and IHC were evaluated by the pathologist Dr. Federico Rojo. The data was presented as the percentage of positive cells for each staining.

M10. *NRG1* TRANSCRIPTOMIC ANALYSES

Several public datasets containing microarray gene expression data on HER2+ BC patients were explored in order to address several questions arising from the preclinical experiments conducted as part of this doctoral work. The study data was downloaded via the GEO2R feature on the NCBI GEO Datasets Website (309).

For analyses of variations in *NRG1* expression between different compartments or conditions, *NRG1* expression was computed by obtaining the mean expression of all probes mapped to the *NRG1* gene for each sample. In one study (see below), we developed an *NRG1* signalling score based on the enrichment of genes upregulated and downregulated by *NRG1*. Briefly, these gene sets were published by Nagashima *et al.* and are the result of differential gene expression analyses performed on a BC cell line after treatment with increasing concentrations of NRG-1 at different time points (244). The authors report two lists of genes, one for significantly upregulated genes (n = 180 genes) and another for those significantly downregulated (n = 59 genes) after NRG-1 stimulation. These gene sets were downloaded from the curated gene set section (C2) of the GSEA MSigDB database portal (NAGASHIMA_NRG1_SIGNALING_UP/NAGASHIMA_NRG1_SIGNALING_DN). To obtain an NRG1 signalling score for each sample, we first computed the individual gene set enrichment scores for each sample using the GSVA R package. We first obtained enrichment scores for the upregulated and downregulated gene sets in each sample. Then, the samples were sorted by both enrichment scores (the upregulated gene score was sorted in decreasing order and the downregulated score in increasing order) and their ranks were obtained. Next, the final NRG1 signalling score was computed by obtaining the sum of the ranks for each score per sample.

All the analyses were performed using R version 3.5 or higher within the RStudio IDE. To statistically compare the two groups, the Wilcoxon Rank Sum Test was employed. When more than two groups were present, the Krusal-Wallis test with the Nemenyi *post-hoc* correction was used.

M10.1 Description of datasets used to quantify *NRG1* expression differences between epithelial and stromal tumour compartments (GSE83591)

- **GSE83591**(310)

Overall design of the Liu *et al.* study: 107 samples isolated by laser capture microdissection; in total, 40 samples of tumour epithelium, 39 samples of tumour stroma, 14 samples of normal epithelium and 14 samples of normal stroma (defined as normal breast tissue >2cm away from the tumour area); 2 samples possessed biological replicates; each was subjected to gene expression profiling in a common reference design using Agilent whole human genome oligonucleotide arrays.

M10.2 Description of the dataset used to quantify *NRG1* expression differences between tumour and non-tumour-associated stroma (GSE83591/ GSE9014)(245)

GSE83591(310)

See above.

- **GSE9014**(311)

Overall design of the Finak *et al.* study: 53 samples, with dye-swap replicates. Some samples replicated three or four times; a total of 111 arrays. Tumour-associated and non-tumour-associated breast stroma were laser-capture microdissected from IDC breast cancer cases. The samples were subjected to gene expression profiling in a common reference using the Agilent microarray platform. In this study, 22 tumour-associated stroma samples belonging to HER2+ BC patients were compared to 12 non-tumour associated stroma samples.

M10.3 Description of the dataset used to study the association between the *NRG1* signalling score and pCR in patients with eBC treated with trastuzumab in the neoadjuvant setting (GSE0948)(245)

- **TransNOAH breast cancer clinical trial (GSE0948)**

The Neoadjuvant Herceptin [NOAH] trial is a randomized, controlled, phase 3 trial in women with locally advanced or inflammatory HER2+ BC. The primary objective of the study was to assess differences in pCR rates with neoadjuvant chemotherapy + trastuzumab compared with neoadjuvant chemotherapy alone in HER2+ BC. A computer program running a minimisation technique randomly assigned the participants (1:1) to treatment with neoadjuvant chemotherapy alone or with 1 year of trastuzumab (concurrently with neoadjuvant chemotherapy, continued after surgery).

Gene expression profiling was performed using RNA from FFPE core biopsies from 114 patients with HER2+ tumours randomized to receive neoadjuvant doxorubicin/paclitaxel (AT) followed by cyclophosphamide/methotrexate/fluoracil (CMF), or the same regimen in combination with trastuzumab for 1 year.

M11. STATISTICAL DATA ANALYSIS

Statistical analyses were carried out using Prism 6.0 software (GraphPad®). The independent (unpaired) t-test was used to compare two separate sets of results: in the *in vitro* experiments; and to compare two independent variables. Additionally, we used analysis of variance (ANOVA) to test whether there were statistically significant differences between more than two groups of data. Tukey's *post hoc* analysis was used to perform pairwise comparisons among the groups of data. The ANOVA was used to assess differences in the effects between different treatments in the *in vitro, in vivo* and *ex-vivo* experiments.

To statistically correlate clinical results with pCR in the cohort of HER2+ breast cancer patient samples treated with trastuzumab in neoadjuvance, we used the Chi-squared test. All the statistical tests were performed at the two-sided 0.05 level of significance. Finally, the data was represented as the average ± the standard deviation (SD) of three independent replicates.

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ANNEX

A1. List of proteins exclusively detected in CAF200 secretome

Accession	Description	ΣCoverage	Σ# Proteins	Unique Peptic	Σ# Peptides	Σ# PSMs	Area
Q0VD16	MAP2K1 protein OS=Bos taurus GN=MAP2K1 PE=2 SV=2 - [Q0VD16_BOVIN]	2,04	1	1	1	1	1,541E9
E1BE11	Uncharacterized protein OS=Bos taurus GN=HMCN1 PE=4 SV=2 - [E1BE11_BOVIN]	0,13	2	1	1	1	1,533E9
F1MUT7	DAZ interacting zinc finger protein 1 OS=Bos taurus GN=DZIP1 PE=4 SV=2 - [F1MUT7_BOVIN]	0,71	2	1	1	1	7,842E8
E1BK15	Chloride channel protein OS=Bos taurus GN=CLCN6 PE=3 SV=1 - [E1BK15_BOVIN]	0,69	1	1	1	1	6,554E8
Q29RM2	Protein FAM53C OS=Bos taurus GN=FAM53C PE=2 SV=1 - [FA53C_BOVIN]	2,55	2	1	1	1	3,001E8
P35125	Ubiquitin carboxyl-terminal hydrolase 6 OS=Homo sapiens OX=9606 GN=USP6 PE=1 SV=2 - [UBP6_HUMAN]	0,43	1	1	1	1	2,227E8
Q9Y6R4	Mitogen-activated protein kinase kinase kinase 4 OS=Homo sapiens OX=9606 GN=MAP3K4 PE=1 SV=2 - [M3K4_HUMAN]	0,44	1	1	1	1	2,213E8
P01033	Metalloproteinase inhibitor 1 OS=Homo sapiens OX=9606 GN=TIMP1 PE=1 SV=1 - [TIMP1_HUMAN]	47,83	1	6	6	8	1,694E8
Q2M1P5	Kinesin-like protein KIF7 OS=Homo sapiens OX=9606 GN=KIF7 PE=1 SV=2 - [KIF7_HUMAN]	0,52	2	1	1	1	8,728E7
Q92820	Gamma-glutamyl hydrolase OS=Homo sapiens OX=9606 GN=GGH PE=1 SV=2 - [GGH_HUMAN]	1,89	1	1	1	1	6,878E7
E1B9X8	CD84 molecule OS=Bos taurus GN=CD84 PE=4 SV=2 - [E1B9X8_BOVIN]	2,40	2	1	1	1	6,842E7
Q9Y228	TRAF3-interacting JNK-activating modulator OS=Homo sapiens OX=9606 GN=TRAF3IP3 PE=1 SV=2 - [T3JAM_HUMAN]	1,45	2	1	1	1	5,645E7
E1B9S7	OTU deubiquitinase 7B OS=Bos taurus GN=OTUD7B PE=4 SV=1 - [E1B9S7_BOVIN]	2,14	1	2	2	2	5,413E7
P61769	Beta-2-microglobulin OS=Homo sapiens OX=9606 GN=B2M PE=1 SV=1 - [B2MG_HUMAN]	37,82	1	4	4	8	5,128E7
Q16270	Insulin-like growth factor-binding protein 7 OS=Homo sapiens OX=9606 GN=IGFBP7 PE=1 SV=1 - [IBP7_HUMAN]	46,81	2	8	8	13	4,840E7
P07093	Glia-derived nexin OS=Homo sapiens OX=9606 GN=SERPINE2 PE=1 SV=1 - [GDN_HUMAN]	25,38	1	4	8	10	4,449E7
Q8IVV2	Lipoxygenase homology domain-containing protein 1 OS=Homo sapiens OX=9606 GN=LOXHD1 PE=2 SV=4 - [LOXH1_HU	0,29	1	1	1	1	4,405E7
P03956	Interstitial collagenase OS=Homo sapiens OX=9606 GN=MMP1 PE=1 SV=3 - [MMP1_HUMAN]	20,26	4	9	9	10	4,385E7
Q12841	Follistatin-related protein 1 OS=Homo sapiens OX=9606 GN=FSTL1 PE=1 SV=1 - [FSTL1_HUMAN]	18,83	2	6	6	7	4,135E7
Q76FK4	Nucleolar protein 8 OS=Homo sapiens OX=9606 GN=NOL8 PE=1 SV=1 - [NOL8_HUMAN]	1,20	1	1	1	2	4,046E7
P08253	72 kDa type IV collagenase OS=Homo sapiens OX=9606 GN=MMP2 PE=1 SV=2 - [MMP2_HUMAN]	25,76	5	12	12	14	3,540E7
P05121	Plasminogen activator inhibitor 1 OS=Homo sapiens OX=9606 GN=SERPINE1 PE=1 SV=1 - [PAI1_HUMAN]	17,66	1	5	5	5	3,058E7
Q05BQ5	MBT domain-containing protein 1 OS=Homo sapiens OX=9606 GN=MBTD1 PE=1 SV=2 - [MBTD1_HUMAN]	0,96	2	1	1	1	2,985E7
F1MZX2	Serpin family E member 2 OS=Bos taurus GN=SERPINE2 PE=3 SV=2 - [F1MZX2_BOVIN]	14,86	1	1	5	6	2,874E7
Q8HXA6	Ankyrin repeat and SOCS box protein 15 OS=Bos taurus GN=ASB15 PE=2 SV=2 - [ASB15_BOVIN]	1,36	1	1	1	1	2,672E7
P08621	U1 small nuclear ribonucleoprotein 70 kDa OS=Homo sapiens OX=9606 GN=SNRNP70 PE=1 SV=2 - [RU17_HUMAN]	1,83	1	1	1	1	2,564E7
O00391	Sulfhydryl oxidase 1 OS=Homo sapiens OX=9606 GN=QSOX1 PE=1 SV=3 - [QSOX1_HUMAN]	8,57	1	4	4	5	1,592E7
P00736	Complement C1r subcomponent OS=Homo sapiens OX=9606 GN=C1R PE=1 SV=2 - [C1R_HUMAN]	10,64	4	6	6	6	1,549E7
P26022	Pentraxin-related protein PTX3 OS=Homo sapiens OX=9606 GN=PTX3 PE=1 SV=3 - [PTX3_HUMAN]	16,27	1	4	4	5	1,360E7
O76061	Stanniocalcin-2 OS=Homo sapiens OX=9606 GN=STC2 PE=1 SV=1 - [STC2_HUMAN]	7,95	2	2	2	3	1,313E7
Q8IW75	Serpin A12 OS=Homo sapiens OX=9606 GN=SERPINA12 PE=1 SV=1 - [SPA12_HUMAN]	1,45	1	1	1	1	1,295E7
A2VDU2	Target of rapamycin complex 2 subunit MAPKAP1 OS=Bos taurus GN=MAPKAP1 PE=2 SV=1 - [SIN1_BOVIN]	2,49	2	1	1	1	1,137E7
P80425	Fatty acid-binding protein, liver OS=Bos taurus GN=FABP1 PE=1 SV=1 - [FABPL_BOVIN]	11,02	2	1	1	2	1,108E7
P35555	Fibrillin-1 OS=Homo sapiens OX=9606 GN=FBN1 PE=1 SV=4 - [FBN1_HUMAN]	5,05	4	8	8	10	1,094E7
G3MZ30	Actin like 10 OS=Bos taurus GN=ACTL10 PE=3 SV=1 - [G3MZ30_BOVIN]	2,45	1	1	1	1	1,055E7
F1N430	Metalloproteinase inhibitor 2 OS=Bos taurus GN=TIMP2 PE=4 SV=1 - [F1N430_BOVIN]	3,64	3	1	1	1	1,043E7
Q05717	Insulin-like growth factor-binding protein 5 OS=Bos taurus GN=IGFBP5 PE=2 SV=2 - [IBP5_BOVIN]	12,18	2	3	3	4	9,978E6
G3MZZ1	Uncharacterized protein OS=Bos taurus PE=4 SV=1 - [G3MZZ1_BOVIN]	4,48	1	1	1	1	9,901E6
Q08380	Galectin-3-binding protein OS=Homo sapiens OX=9606 GN=LGALS3BP PE=1 SV=1 - [LG3BP_HUMAN]	11,11	1	5	5	5	9,713E6
F1MNQ4	Superoxide dismutase [Cu-Zn] OS=Bos taurus PE=3 SV=2 - [F1MNQ4_BOVIN]	23,03	3	3	3	3	9,411E6
P07858	Cathepsin B OS=Homo sapiens OX=9606 GN=CTSB PE=1 SV=3 - [CATB_HUMAN]	10,03	1	2	2	2	9,398E6
P26927	Hepatocyte growth factor-like protein OS=Homo sapiens OX=9606 GN=MST1 PE=1 SV=2 - [HGFL_HUMAN]	0,98	2	1	1	1	9,160E6
Q0VC53	Deoxyhypusine hydroxylase OS=Bos taurus GN=DOHH PE=1 SV=1 - [DOHH_BOVIN]	1,98	1	1	1	1	8,399E6
P07585	Decorin OS=Homo sapiens OX=9606 GN=DCN PE=1 SV=1 - [PGS2_HUMAN]	15,88	2	4	4	5	8,190E6
P20959	Insulin-like growth factor-binding protein 3 OS=Bos taurus GN=IGFBP3 PE=1 SV=3 - [IBP3_BOVIN]	7,22	2	2	2	2	7,529E6
A5PJT7	ECM1 protein OS=Bos taurus GN=ECM1 PE=2 SV=1 - [A5PJT7_BOVIN]	3,29	1	1	1	1	7,148E6
F1MND9	Collagen type IX alpha 1 chain OS=Bos taurus GN=COL9A1 PE=4 SV=2 - [F1MND9_BOVIN]	7,45	1	3	3	3	6,907E6
Q16610	Extracellular matrix protein 1 OS=Homo sapiens OX=9606 GN=ECM1 PE=1 SV=2 - [ECM1_HUMAN]	2,96	1	1	1	1	6,886E6
P08670	Vimentin OS=Homo sapiens OX=9606 GN=VIM PE=1 SV=4 - [VIME_HUMAN]	4,94	2	2	2	2	6,076E6
Q02818	Nucleobindin-1 OS=Homo sapiens OX=9606 GN=NUCB1 PE=1 SV=4 - [NUCB1_HUMAN]	6,29	2	2	2	3	5,753E6
G3X6J9	Uncharacterized protein OS=Bos taurus PE=4 SV=1 - [G3X6J9_BOVIN]	0,73	2	1	1	1	5,746E6
A5PK73	Fructose-bisphosphate aldolase OS=Bos taurus GN=ALDOB PE=2 SV=1 - [A5PK73_BOVIN]	4,67	3	1	1	1	5,726E6

E1B953	Uncharacterized protein OS=Bos taurus GN=LOC533307 PE=3 SV=2 - [E1B953_BOVIN]	2,70	5	1	1	1	5,457E6
F1MKP6	Cadherin-13 OS=Bos taurus GN=CDH13 PE=4 SV=2 - [F1MKP6_BOVIN]	1,81	3	1	1	1	5,419E6
O94907	Dickkopf-related protein 1 OS=Homo sapiens OX=9606 GN=DKK1 PE=1 SV=1 - [DKK1_HUMAN]	4,51	1	1	1	1	5,171E6
G3N2L2	Reticulocalbin 1 OS=Bos taurus GN=RCN1 PE=1 SV=1 - [G3N2L2_BOVIN]	10,98	1	1	1	1	4,927E6
Q8IVU3	Probable E3 ubiquitin-protein ligase HERC6 OS=Homo sapiens OX=9606 GN=HERC6 PE=1 SV=2 - [HERC6_HUMAN]	0,59	1	1	1	1	4,882E6
A6QLB7	Adenylyl cyclase-associated protein OS=Bos taurus GN=CAP1 PE=1 SV=1 - [A6QLB7_BOVIN]	3,40	2	1	1	1	4,840E6
P13611	Versican core protein OS=Homo sapiens OX=9606 GN=VCAN PE=1 SV=3 - [CSPG2_HUMAN]	1,56	6	4	4	6	4,753E6
A7YWG4	Gamma-glutamyl hydrolase OS=Bos taurus GN=GGH PE=2 SV=1 - [GGH_BOVIN]	6,92	1	2	2	2	4,568E6
Q5E9E3	Complement C1q subcomponent subunit A OS=Bos taurus GN=C1QA PE=2 SV=1 - [C1QA_BOVIN]	8,20	1	1	1	1	4,194E6
P07900	Heat shock protein HSP 90-alpha OS=Homo sapiens OX=9606 GN=HSP90AA1 PE=1 SV=5 - [HS90A_HUMAN]	1,37	2	1	1	1	4,034E6
Q99715	Collagen alpha-1(XII) chain OS=Homo sapiens OX=9606 GN=COL12A1 PE=1 SV=2 - [COCA1_HUMAN]	0,82	2	2	2	2	3,930E6
P26038	Moesin OS=Homo sapiens OX=9606 GN=MSN PE=1 SV=3 - [MOES_HUMAN]	1,73	2	1	1	1	3,863E6
Q2KJB3	Angiopoietin-like 3 OS=Bos taurus GN=ANGPTL3 PE=2 SV=1 - [Q2KJB3_BOVIN]	6,97	1	2	2	3	3,845E6
P09871	Complement C1s subcomponent OS=Homo sapiens OX=9606 GN=C1S PE=1 SV=1 - [C1S_HUMAN]	5,81	4	3	3	3	3,784E6
O00300	Tumor necrosis factor receptor superfamily member 11B OS=Homo sapiens OX=9606 GN=TNFRSF11B PE=1 SV=3 - [TR1	2,00	1	1	1	1	3,728E6
A0A140T828	Microfibrillar-associated protein 5 OS=Bos taurus GN=MFAP5 PE=4 SV=1 - [A0A140T828_BOVIN]	11,76	3	2	2	3	3,507E6
A6QM01	N-acetyl-alpha-glucosaminidase OS=Bos taurus GN=NAGLU PE=2 SV=1 - [A6QM01_BOVIN]	3,45	1	1	1	1	3,202E6
P50395	Rab GDP dissociation inhibitor beta OS=Homo sapiens OX=9606 GN=GDI2 PE=1 SV=2 - [GDIB_HUMAN]	4,27	4	1	1	1	2,983E6
P61223	Ras-related protein Rap-1b OS=Bos taurus GN=RAP1B PE=2 SV=1 - [RAP1B_BOVIN]	14,13	1	1	1	1	2,861E6
F1MPE1	CD109 molecule OS=Bos taurus GN=CD109 PE=4 SV=2 - [F1MPE1_BOVIN]	2,63	1	2	2	2	2,833E6
F1MQ37	Uncharacterized protein OS=Bos taurus GN=MYH9 PE=1 SV=2 - [F1MQ37_BOVIN]	1,58	3	2	2	2	2,790E6
F1MT21	Uncharacterized protein OS=Bos taurus PE=4 SV=2 - [F1MT21_BOVIN]	1,84	1	1	1	1	2,726E6
Q95122	Monocyte differentiation antigen CD14 OS=Bos taurus GN=CD14 PE=2 SV=2 - [CD14_BOVIN]	4,83	2	1	1	1	2,616E6
F1N0D3	AXL receptor tyrosine kinase OS=Bos taurus GN=AXL PE=3 SV=2 - [F1N0D3_BOVIN]	3,60	1	1	1	1	2,241E6
G3X7I5	Uncharacterized protein OS=Bos taurus PE=4 SV=1 - [G3X7I5_BOVIN]	10,09	3	1	1	1	2,187E6
Q8TF72	Protein Shroom3 OS=Homo sapiens OX=9606 GN=SHROOM3 PE=1 SV=2 - [SHRM3_HUMAN]	0,45	1	1	1	1	2,012E6
F1N2W0	Prostaglandin reductase 1 OS=Bos taurus GN=PTGR1 PE=4 SV=1 - [F1N2W0_BOVIN]	6,99	2	1	1	1	1,773E6
G3X6W0	MICOS complex subunit OS=Bos taurus GN=APOO PE=4 SV=1 - [G3X6W0_BOVIN]	3,03	2	1	1	1	1,369E6
Q14766	Latent-transforming growth factor beta-binding protein 1 OS=Homo sapiens OX=9606 GN=LTBP1 PE=1 SV=4 - [LTBP1_H	1,63	1	2	2	2	1,359E6
Q3ZBU7	Tubulin beta-4A chain OS=Bos taurus GN=TUBB4A PE=2 SV=1 - [TBB4A_BOVIN]	3,83	1	1	1	1	1,343E6
E1BB32	Glycoprotein Ib platelet alpha subunit OS=Bos taurus GN=GP1BA PE=4 SV=2 - [E1BB32_BOVIN]	1,69	1	1	1	1	1,339E6
F1MYE4	Chondroadherin OS=Bos taurus GN=CHAD PE=4 SV=2 - [F1MYE4_BOVIN]	3,60	2	1	1	1	8,095E5
Q9NQ30	Endothelial cell-specific molecule 1 OS=Homo sapiens OX=9606 GN=ESM1 PE=1 SV=2 - [ESM1_HUMAN]	6,52	1	1	1	1	4,877E5