DNA METHYLOME IN HER2-POSITIVE RESISTANT BREAST CANCER

Sònia Palomeras Pairet

Doctoral thesis

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This doctoral thesis contains Annexes (Annex I and II)

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Presented in partial fulfillment of the requirements for a doctoral degree from the University of Girona

Universitat de Girona

Dr. Teresa Puig Miquel, associate professor at the University of Girona and Director of the Targets Lab group,

Certifies:

That the thesis titled “DNA methylome in HER2-positive resistant breast cancer”, presented by Sònia Palomeras Pairet to obtain a doctoral degree, has been completed under my supervision and meets the requirements to opt for a Doctorate.

For all intents and purposes, I hereby sign this document.

Signature

Girona, december 2nd of 2018

Per tu àvia, seguirem lluitant incansablement com vas fer-ho tu,
perquè algun dia des d'on siguis, celebrem que l'hem vençut.

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ARTICLE

Title: Epigenetic Silencing of TGFBI Confers Resistance to Trastuzumab in Human Breast Cancer.
Authors: Palomeras S*, Diaz-Lagares A*, Viñas G, Setien F, Ferreira HJ, Oliveras G, Crujeiras AB, Hernández A, Lum DH, Welm AL, Esteller M and Puig T.
Submitted.

Results have also been presented in the following scientific congresses:

43rd FEBS congress, Biochemistry Forever, Prague, Czech Republic, July 2018. S. Palomeras, Á. Díaz-Lagares, F. Setien, H.J. Ferreira, A. Hernandez, G. Viñas, A. Welm, M. Esteller, T. Puig . "DNA methylation pattern to identify trastuzumab resistance in HER2-positive breast cancer models". Poster presentation.

IMPAKT Breast Cancer Conference, Brussels, Belgium, May 2017. S. Palomeras, Á. Díaz Lagares, A. Blancafort, A. Sarrats, A.B. Crujeiras, J. Sandoval, M. Rabionet, A.L. Welm, M. Esteller, T. Puig. "DNA methylation signature to identify trastuzumab response in HER2 breast cancer models". Poster presentation. Published in *Annals of Oncology*, 2017;28 suppl_1. doi:10.1093/annonc/mdx138.010.

American Association for Cancer Research (AACR) Annual Meeting, New Orleans, Louisiana, USA, April 2016. Palomeras S, Díaz-Lagares A, Sarrats A, Crujeiras AB, Giró A, Sandoval J, Rabionet M, Esteller M* and Puig T*. "Epigenetic silencing of TGFBI, BCL6, KILLIN and CTSZ is related to Trastuzumab resistance in HER2-positive breast cancer models". Poster presentation. Published in *Cancer Research*, 2016;76 14 Supplement:2130A–2130A. doi:10.1158/1538-7445.AM2016-2130A.

Additional unrelated studies performed in fulfillment of the Ph.D degree will not be presented in this thesis and are represented in the following publication (Annex II):

ARTICLE 1

Title: Dual fatty acid synthase and HER2 signaling blockade shows marked antitumor activity against breast cancer models resistant to Anti-HER2 drugs.
Authors: Adriana Blancafort, Ariadna Giró-Perafita, Glòria Oliveras, Sònia Palomeras, Carlos Turrado, Òscar Campuzano, Dolors Carrión-Salip, Anna Massaguer, Ramon Brugada, Marta Palafox, Jorge Gómez-Miragaya, Eva González-Suárez and Teresa Puig.
Journal: PLoS One. 2015, 10(6):e0131241.
Impact factor (2014): 2.766 (Q1, Multidisciplinary Sciences/ Position 15/64).
DOI: 10.1371/journal.pone.0131241.

ARTICLE 2

Title: Preclinical Evaluation of Fatty Acid Synthase and EGFR Inhibition in Triple

Negative Breast Cancer.

Authors: Ariadna Giró-Perafita, Sònia Palomeras, David H. Lum, Adriana Blancafort, Gemma Viñas, Glòria Oliveras, Ferran Pérez-Bueno, Ariadna Sarrats, Alana L. Welm and Teresa Puig.

Journal: Clinical Cancer Research (2016) 1-11.

Impact factor: 10.199 (Q1, Oncology / Position 12 of 223).

DOI: 10.1158/1078-0432.CCR-15-3133.

ARTICLE 3

Title: Breast Cancer Stem Cell Culture and Enrichment Using Poly(ϵ-Caprolactone) Scaffolds.

Authors: Palomeras, Sònia*; Rabionet Díaz, Marc*; Ferrer Real, Inés; Sarrats Carbó, Ariadna; Garcia-Romeu, Maria Luisa; Puig i Miquel, Teresa; Ciurana, Quim de Ciurana.

Journal: Molecules 21, (2016). Special Issue "Biomaterials and Bioprinting".

Impact factor: 3.098 (Q2, Biochemistry & Molecular Biology, chemistry, Multidisciplinary science/ Position 131/293).

DOI: 10.3390/molecules23051160.

ARTICLE 4

Title: (−)-Epigallocatechin 3-Gallate Synthetic Analogues Inhibit Fatty Acid Synthase and Show Anticancer Activity in Triple Negative Breast Cancer.

Authors: Crous-Masó, J.*; Palomeras, S*.; Relat, J.; Camó, C.; Martínez-Garza, Ú.; Planas, M.; Feliu, L.; Puig, T.

Journal: Molecules 23, 1160 (2018).

Impact factor: 3.098 (Q2, Biochemistry & Molecular Biology, chemistry, Multidisciplinary science/ Position 131/293).

DOI: 10.3390/molecules23051160.

ARTICLE 5

Title: Targeting Breast Cancer Stem Cells to Overcome Treatment Resistance

Authors: Sònia Palomeras, Santiago Ruiz-Martínez and Teresa Puig.

Journal: Molecules 23, 1160 (2018).

Impact factor 3.098 (Q2, Biochemistry & Molecular Biology, chemistry, Multidisciplinary science/ Position 131/293).

DOI: 10.3390/molecules23092193.

Abbreviations

A

ADCC: Antibody-dependent cell mediates cytotoxicity
AKT: Protein kinase B
ANXA3: Annexin A3
ANGPTL4: Angiopoietin like 4
AmR: Amphiregulin
AU: AU565
AUC: Area under the curve

B

BC: Breast cancer
BCNU: Bis-chloroethylnitrosourea
BMP: Bone morphogenetic protein
BMP4: Bone morphogenetic protein 4
BS: Bisulfite
BTC: Betacellulin
BRCA1*:* Breast cancer 1

C

CGI: CpG island
CL: Claudin-low
CpG: Cytosine-phosphate-Guanine
ctDNA: Circulating tumor DNA
CTSZ: Cathepsin Z
CXCL2: C-X-C motif chemokine ligand 2
CYP4X1: Cytochrome P450 family 4 subfamily X member 1

D

DNMT: DNA methyltransferase
DSF: Disease-free survival

E

ECM: Extracellular matrix
EFS: Event-free survival
EGF: Epidermal growth factor
EGFR: Epidermal growth factor receptor
EID3: EP300 interacting inhibitor of differentiation 3
EMT: Epithelial-mesenchymal transition
ENTPD3: Ectonucleoside triphosphate diphosphohydrolase 3
ER: Estrogen receptor
EREG: Epiregulin
ERK: Extracellular regulated kinase

F

FACS: Fluorescence-activated cell sorting
FDA: United States Food and drug administration
FDR: False discovery rate

FFPE: Formalin-fixed paraffin-embedded tissues
FISH: Fluorescent in situ hybridization

G

GEO: Gene expression omnibus
GO: Gene ontology
GSTP1: Glutathione S-transferase pi 1

H

HATs: Histone acetyltransferases
HB-EGF: Heparin-binding EGF
HDAC: Histone deacetylase
HDM: Histone demethylase
HER1: Human epithermal growth factor receptor 1
HER2: Human rpithermal growth factor receptor 2
HER2+: HER2-positive
HER3: Human rpithermal growth factor receptor 3
HER4: Human epithermal growth factor receptor 4
HMT: Histone methyltransferase
HSD17B4: Hydroxysteroid 17-beta dehydrogenase 4

I

IGF2: Insulin-like growth factor 2
IGF-1R: Insulin-like growth factor 1 receptor
IHC: Immunohistochemistry
ITGB8: Integrin subunit beta 8
IRX6: Iroquois homeobox 6

K

KCNJ12: Potassium voltage-gated channel subfamily J member 12
KILLIN (or KLLN): P53 regulated DNA replication inhibitor

L

LINE-1: Member 1 of long interspersed nucleotide elements LINE family
LOI: Loss of imprinting
LincRNA: Intergenic RNAs
LncRNA: Long non-coding RNA

M

mAB: Monoclonal antibody
MAPK: Mitogen-activated protein kinase
MBC: Metastatic breast cancer
MBD: Methyl-CpG binding protein
MGMT: O^6-methylguanine-DNA methyltransferase
miRNA: MicroRNA
ML: Mesenchymal-like
MLH1: MutL homologue 1

MSP: Methyl-specific polymerase chain reaction
MUC: Mucin

N

ncRNA: Non-coding RNA
NK: Natural killer
NSCLC: Non-small cell lung cancer
NSG: Next-generation-sequencing
NRGs or NDFs: Neuregulins or neu differentiation factors

O

OS: Overall survival
OTX1: Orthodenticle homeobox 1

P

pCR: Pathological complete response
PCR: Polymerasa chain reaction
PFS: Progression-free survival
PITX2: Paired-like homeodomain 2
PI3K: Phosphatidylinositol-3 kinases
PTM: Multiple post-transcriptional modification
POU4F1: POU class 4 homeobox 1
PPARG: Peroxisome proliferator activated receptor gamma
PR: Progesterone receptor
PTEN: Phosphatase and tensin homolog
PTRF: Polymerase I and transcript release factor

R

RASSF1A: Ras association domain family member 1
RB: Retinoblastoma gene
RIN3: Ras and Rab Interactor 3
RNA-Seq: RNA sequencing
ROC: Receiver operating characteristic
R-RAS: Related RAS viral oncogene
rRNA: Ribosomal RNA
RTK: Receptor tyrosine kinases

S

SAM: S-adenosyl-methionine
SERD: Selective estrogen receptor downregulator
SERM: Selective estrogen receptor modulator
ShRNA: Small hairpin RNA
SK: SKBr3
SKTR: SKBr3 trastuzumab-resistant
SKLR: SKBr3 lapatinib resistant
SKTLR: SKBr3 trastuzumab *plus* lapatinib-resistant
SKLTR: SKBr3 lapatinib *plus* trastuzumab-resistant
SLC38A1: Solute carrier family 38 member 1

SLC16A3: Solute carrier family 16 member 3
sncRNA: Small non-coding RNA
SPOCK1: SPARC/osteonectin, cwcv and kazal like domains proteoglycan 1
SRA: SET and RING-associated domain
S100A4: 100 calcium binding protein A4

T

TDG: Thymine DNA glycosylase
T-DM1: Trastuzumab-DM1
TET: Ten-eleven translocation
TGF-α: Transforming growth factor alpha
TGF-β: Transforming growth factor beta
TGFBI: Transforming growth factor beta induced
TKI: Tyrosine kinase inhibitors
TNBC: Triple negative breast cancer
TRAIL: TNF-related apoptosis-inducing ligand
TTD: Tandem tudor domain
TXNRD1: Thioredoxin reductase 1

U

UHRF1: Ubiquitin-like with PHD and ring finger domains1

V

VEGF: Vascular endothelial growth factor

Others

5hmC: 5-hydroxymethylcytosine
5mC: 5-methylcytosine
5fC: 5- formylcytosine
5caC: 5-carboxylcytosine
5-aza-dC or AZA: 5-aza-2'-deoxycytidine

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Abstract
Resum
Resumen

Approximately 15% to 20% of patients with breast cancer overexpress the human epidermal growth factor receptor 2 (HER2). Although the development of anti-HER2 treatments has significantly improved breast cancer outcomes, a large percentage of patients display primary or acquired resistance to the drug. Several efforts have focused on identifying and understanding the molecular and cellular mechanisms involved in treatment resistance. DNA methylation is the most well-characterized epigenetic modification in humans and is involved in regulating the expression of a great variety of critical genes in cancer. For this reason, DNA methylation status has emerged as one of the most promising epigenetic biomarkers for several types of cancer. This epigenetic marker can be useful in detecting tumors earlier or identifying patients with an increased risk of cancer, as well as evaluating disease progression or predicting the response to anticancer drugs. In the last few years some significant genes that are inactivated by promoter methylation in breast cancer have been identified, including *BRCA1* and *RASSF1A*.

In this thesis, we have focused on the DNA methylation analysis in HER2+ breast cancer resistant to current therapies (trastuzumab and lapatinib). In a first approach, global DNA methylation pattern was analyzed in trastuzumab and lapatinib-sensitive and -resistant models (SK, SKTR, SKLR and SKTLR) using the Infinium HumanMethylation450 BeadChip (450k array). Each sensitive and resistant model was clearly differentiated according to their methylation pattern, demonstrating DNA methylation involvement in trastuzumab and lapatinib resistance. Based on the global analysis, our study focused on identifying potential biomarkers for trastuzumab and lapatinib resistance. To this purpose, we performed an integrative analysis of promoter and island methylation and expression pattern (RNA-Seq) comparing our sensitive and resistant models. In each comparison different genes with methylation and expression pattern correlations were identified as potential biomarkers for trastuzumab, lapatinib and trastuzumab *plus* lapatinib resistance. Unfortunately, due to the difficulties in obtaining tumor samples similar to our *in vitro* models from human patients, some of the potential biomarkers identified could not be validated in subsequent studies.

For this reason, we focused our study on analyzing trastuzumab resistance biomarkers; the basis of anti-HER2 therapies. Although four epigenetically regulated genes were identified (*TGFBI*, *KILLIN*, *CXCL2*, and *SLC38A1*), *TGFBI* was the only gene validated in

the two trastuzumab-sensitive (SK and AU) and -resistant (SKTR and AUTR) HER2+ breast cancer models analyzed. Furthermore, functional analyses revealed that TGFBI re-expression induced greater sensitivity to trastuzumab in our SKTR model, probably through its integrin-binding domains (EPDIM, NKDIL, YH and RGD). Independent of integrin-binding domains, TGFBI overexpression showed activation of some protein of HER pathway similar to the SK cells. Finally, the *in vivo* results corroborated the previous *in vitro* results. When compared to their pre-treatment samples, a significant increase in *TGFBI* methylation levels was identified in the post-treatment samples from patients who had developed resistance to neoadjuvant anthracycline-taxane-based chemotherapy *plus* trastuzumab treatment. Moreover, *TGFBI* methylation analysis in pre-treatment samples of responder and non-responder patients, as well as in normal breast samples, allowed us to suggest the role TGFBI plays as a treatment response biomarker in HER2+ breast cancer.

In summary, we demonstrated the role DNA methylation has in trastuzumab- and lapatinib-resistant HER2+ breast cancer. Furthermore, we identify the epigenetic inactivation of the *TGFBI* gene by promoter CpG island hypermethylation in trastuzumab resistance *in vitro* and in small cohort of HER2+ human samples. Our results suggest for the first time *TGFBI* hypermethylation as a biomarker for trastuzumab response in HER2+ breast cancer. Although further studies are required to identify the specific role TGFBI plays in trastuzumab resistance, the combination of *TGFBI* hypermethylation analysis with standard clinical markers may help stratify HER2+ patients according to the response to the trastuzumab treatment.

Keywords: HER2+ breast cancer, resistance, lapatinib, trastuzumab, cellular models, epigenetics, DNA methylation, biomarkers.

Aproximadament el 15-20% dels pacients amb càncer de mama presenten sobreexpressió i/o amplificació del receptor 2 del factor de creixement epidèrmic humà (HER2). Tot i els grans avanços terapèutics desenvolupats al llarg dels anys pel tractament d'aquest tipus de càncer, un nombre important de pacients desenvolupen resistència primària o adquirida al tractament. Durant els últims anys molts esforços s'han centrat a buscar i entendre els diferents mecanismes moleculars i cel·lulars implicats en la resistència. La metilació de l'ADN és la modificació epigenètica més ben caracteritzada en humans i està involucrada en la regulació de l'expressió d'una gran varietat de gens crítics en càncer. Per aquest motiu, l'estat de metilació de l'ADN ha esdevingut un dels biomarcadors epigenètics més prometedors per a diversos tipus de càncers. Aquesta marca epigenètica pot ser útil en la detecció de tumors inicials o en la identificació de pacients amb alt risc de desenvolupar un càncer així, com avaluar la progressió de la malaltia o predir la resposta al tractament. En els últims anys s'han identificat diferents gens inactivats epigenèticament per hipermetilació de la regió promotora en càncer de mama, com per exemple els gens *BRCA1* i *RASSF1A*.

En aquesta tesi, ens hem centrat en l'anàlisi de la metilació de l'ADN en càncer de mama HER2+ resistent a les teràpies actuals (trastuzumab i/o lapatinib). En una primera aproximació, es va analitzar la metilació global de l'ADN en models sensibles i resistents a trastuzumab i/o lapatinib desenvolupats al laboratori (SK, SKTR, SKLR i SKTLR), mitjançant l'*array* de metilació Infinium HumanMethylation450 BeadChip. El patró de metilació de l'ADN va permetre poder diferenciar clarament cada model sensible i resistent al tractament, reforçant el paper d'aquest mecanisme epigenètic en processos de resistència. Partint de l'anàlisi global, l'estudi es va centrar en la identificació de potencials biomarcadors de resistència a trastuzumab i/o lapatinib. Per fer-ho, es va realitzar una anàlisi integrativa del patró de metilació de la regió promotora i el patró d'expressió (RNA-Seq) comparant els diferents models sensibles i resistents a les teràpies anti-HER2. Diferents gens epigenèticament regulats van ser identificats en cada comparació com a possibles biomarcadors de resistència a

trastuzumab i lapatinib. Malauradament, no es va poder validar, en posteriors estudis, gran part dels possibles biomarcadors identificats per les dificultats en l'obtenció de mostres de pacients similars a cada model *in vitro*.

Per aquest motiu, l'estudi es va centrar en l'anàlisi de biomarcadors de resistència a trastuzumab, la base de les teràpies anti-HER2 actuals. Tot i que quatre gens epigenèticament regulats van ser identificats (*TGFBI, KILLIN, CXCL2 i SLC38A1*), només *TGFBI* va ser validat en les dues línies cel·lulars analitzades de càncer de mama HER2+ sensibles (SK i AU) i resistents (SKTR i AUTR) a trastuzumab. Les anàlisis funcionals van revelar que la re-expressió de TGFBI en el model resistent, induïa a una major sensibilització de les cèl·lules al tractament amb trastuzumab a través dels seus dominis d'interacció amb integrines (EPDIM, NKDIL, YH i RGD). A més, es va observar com la re-expressió de TGFBI, independentment d'aquests dominis d'interacció amb integrines, mostrava una activació de certes proteïnes de la via d'HER similars a la línia sensible. Per últim, els nostres resultats *in vitro* es van corroborar *in vivo.* Una elevada metilació del promotor de *TGFBI* va ser detectada en mostres post-tractament de pacients amb càncer de mama HER2+ resistents al tractament (trastuzumab més quimioteràpia en règim neoadjuvant) en comparació amb les mostres pre-tractament. A més, l'anàlisi d'aquesta metilació en les mostres pre-tractament de pacients resistents i sensibles així, com mostres de mama normal, ens va permetre determinar el rol de la metilació de *TGFBI* com a biomarcador de resposta al tractament en càncer de mama HER2+.

En resum, els nostres resultats mostren com la metilació de l'ADN té un paper en la resistència a trastuzumab i lapatinib en càncer de mama. A més, s'ha identificat la hipermetilació de *TGFBI* com a possible biomarcador de resistència a trastuzumab *in vitro* i en una petita cohort de pacients amb càncer de mama HER2+. Tot i que són necessaris més estudis per identificar el rol del gen *TGFBI* en resistència a trastuzumab, la combinació de l'anàlisi de la metilació d'aquest gen, juntament amb marcadors clínics estàndards, podria ser d'utilitat en la clínica, ajudant a estratificar els pacients en funció de la resposta al tractament amb trastuzumab.

Paraules clau: Càncer de mama HER2+, resistència, lapatinib, trastuzumab, models cel·lulars, epigenètica, metilació de l'ADN, biomarcadors.

Aproximadamente el 15-20% de los pacientes con cáncer de mama presentan sobreexpresión y/o amplificación del receptor 2 del factor de crecimiento epidérmico humano (HER2). A pesar de los grandes avances terapéuticos desarrollados a lo largo de los años para el tratamiento de este tipo de cáncer, un número elevado de los pacientes desarrollan resistencia primaria o adquirida al tratamiento. Durante los últimos años muchos esfuerzos se han centrado en buscar y entender los diferentes mecanismos moleculares y celulares implicados en la resistencia. La metilación del ADN es la modificación epigenética mejor caracterizada en humanos y está involucrada en la regulación de la expresión de una gran variedad de genes críticos en cáncer. Por este motivo, el estado de metilación del ADN se ha convertido en uno de los biomarcadores epigenéticos más prometedores en diferentes tipos de cáncer. Esta marca epigenética puede ser útil en la detección de tumores iniciales o en la identificación de pacientes con mayor riesgo de padecer cáncer, así como evaluar la progresión de la enfermedad o predecir la respuesta al tratamiento. En los últimos años se han identificado diferentes genes inactivados epigenéticamente por metilación en la región promotora en cáncer de mama, como *BRCA1* y *RASSF1A*.

En esta tesis, nos hemos centrado en el análisis de la metilación del ADN en cáncer de mama HER2+ resistente a las terapias actuales (trastuzumab y/o lapatinib). En una primera aproximación, se analizó la metilación global del ADN en modelos sensibles (SK) y resistentes a trastuzumab y/o lapatinib desarrollados en el laboratorio (SK, SKTR, SKLR y SKTLR) mediante el *array* de metilación Infinium HumanMethylation450 BeadChip. El patrón de metilación del ADN permitió diferenciar claramente cada modelo sensible y resistente, reforzando el papel que este mecanismo epigenético presenta en procesos de resistencia. Partiendo de este análisis global, el estudio se centró en la identificación de potenciales biomarcadores de resistencia a trastuzumab y lapatinib en nuestros modelos celulares. Para ello, se realizó un análisis integrativo del patrón de metilación de la región promotora y el patrón de expresión (RNA-Seq) comparando los diferentes modelos sensibles y resistentes a las terapias anti-

HER2. Diferentes genes regulados epigenéticamente fueron identificados como posibles biomarcaodres de resistencia a trastuzumab y lapatinib. Desafortunadamente, no se pudieron validar en posteriores estudios parte de los posibles biomarcadores identificados debido a la dificultad en la obtención de muestras de pacientes para cada modelo *in vitro*.

Por este motivo, el estudio se centró en el análisis de biomarcadores de resistencia a trastuzumab, la base de todas las terapias anti-HER2 actuales. Aunque se identificaron cuatro genes (*TGFBI, KILLIN, CXCL2 y SLC38A1*) epigeneticamente regulados, solamente el gen *TGFBI* fue validado en las dos líneas celulares analizas de cáncer de mama HER2+ sensibles (SK y AU) y resistentes (SKTR y AUTR) a trastuzumab. Los análisis funcionales revelaron que la re-expresión de TGFBI en las líneas resistentes inducía a una mayor sensibilización de las células al tratamiento con trastuzumab a través de sus dominios de interacción con integrinas (EPDIM, NKDIL, YH y RGD). Además, se observó como la re-expresión de TGFBI, independientemente de los dominios de interacción con integrinas, mostraba una activación de ciertas proteínas de la vía de HER similares a la línea sensible. Por último, nuestros resultados *in vitro* se corroboraron *in vivo*. Una elevada metilación de la región promotora de *TGFBI* fue identificada en las muestras post-tratamiento de pacientes con cáncer de mama HER2+ resistentes al tratamiento (trastuzumab más quimioterapia en règimen neoadyuvante) en comparación con la muestra pre-tratamiento. Además, el análisis de esta metilación en las muestras pre-tratamiento de pacientes resistentes y sensibles, así como muestras de mama normal, nos permitió determinar el rol de la metilación de *TGFBI* como biomarcador de respuesta al tratamiento en cáncer de mama HER2+.

En resumen, nuestros resultados muestran como la metilación del ADN tiene un papel en la resistencia a trastuzumab y lapatinib en cáncer de mama HER2+. Además, se identificó la hipermetilación de *TGFBI* como posible biomarcador de resistencia a trastuzumab *in vitro* y en una pequeña cohorte de pacientes HER2+. Aunque son necesarios más estudios para identificar el rol de TGFBI en la resistencia a trastuzumab, la combinación del análisis de la metilación de este gen

juntamente con marcadores clínicos estándares podría ser útil en clínica, ayudando a estratificar los pacientes en función de la respuesta al tratamiento con trastuzumab.

Palabras clave: Cáncer de mama HER2+, resistencia, lapatinib, trastuzumab, modelos celulares, epigenética, metilación del ADN, biomarcadores.

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Chapter I: Introduction

1.- CANCER

Every day, thousands of people around the world are diagnosed with cancer. Cancer is the most difficult human health affliction to prevent, control or eradicate[1]. All this leads to a great feeling of helplessness not only for the patients themselves, but also for clinicians and researchers.

Cancer is one of the leading causes of morbidity and mortality worldwide. In 2018 alone, there was an estimated 18.1 million new cases and 9.6 million deaths from cancer (latest worldwide data available by GLOBOCAN projects)[2]. The increase in the number of new cases is not only because of an increase in the population, but also because of the development of early detection techniques and the increase in life expectancy. The tumors responsible for the highest number of deaths worldwide are lung cancer (1.76 million deaths), colorectum cancer (880,792 deaths), stomach cancer (782,685 deaths), liver cancer (781,631 deaths), breast cancer (626,679 deaths), oesophagus cancer (508,585 deaths) and pancreatic cancer (432,242 deaths). In Spain, the total number of new invasive cancer cases in 2017 was 228,482, while in 2018 there were 270,363 estimated cases. Globally, the five most common cancer are colon-rectum (37,172 new cases), breast (32,825 new cases), prostate (31,728 new cases), lung (27,351 new cases), and urinary bladder (18,268 new cases). By gender, the four most common cancer in men is prostate cancer (31,728 cases) followed by colorectum (22,744 cases), lung (20,437 cases), and urinary bladder (14,793 cases). In women, the four most common are breast (32,825 cases), colon-rectum (14,428 cases), lung cancer (6914 cases) and corpus uteri (6784 cases)[3]. Moreover, in 2016 cancer was the most frequent cause of death in men (responsible for 68,619 deaths), ahead of cardiovascular and respiratory disease. In contrast, for women the second most common cause of death, behind cardiovascular diseases, was breast cancer[2,4].

Although cancer is now considered the most difficult human health affliction, it is as old as humanity itself. Curiously, some findings have determined that tumors were in fact already present in animals in prehistoric times before humans

even appearance. Cancer is a generic term for the large group of numerous and different diseases associated to the abnormal growth of cells that escape from homeostatic control mechanisms and initiate tumorigenesis. Each one has different multifactorial causes including genetic, environmental or nutritional triggers. A primary tumor can progress to a higher pathological degree of malignancy, causing an invasion into adjacent tissue or spreading to other organs and eventually establishing secondary tumors, a process known as metastasis. Unfortunately, metastasis has been described as the main factor responsible for cancer deaths[5].

As explained above, tumors initiate from normal cells which have specific functions into the organism. There are more than 100 types of cancer. The cellular origin allows us to classify cancer into the following five main categories: **Carcinomas** formed in epithelial cells, which are the cells that line or cover internal organs, **sarcomas** formed in connective or mesenchymal tissue, including bone, cartilage, fat or muscle, **lymphomas** and **myelomas** which are tumors that begin in the cells of the immune system, **leukemia** which affects the production of leukocytes in the bone marrow and **central nervous system cancers,** that are originated in the brain or spinal[5].

1.1.- The hallmarks of cancer

In recent decades, cancer research has focused on understanding the laws that govern the transformation of normal cells to malignant cells, a multi-step process called tumorigenesis or carcinogenesis. In their landmark article entitled "The Hallmarks of Cancer" (Figure 1), Hanahan and Weinberg described the phenotypic differences between normal and cancer cells to understand the high complexity the process of cancer is. These differences are what give malignant cells the ability and the conditions to survive, proliferate and disseminate. In 2000, Hanahan and Weinberg described six essential alterations in cell physiology that explain the malignant growth: **increase proliferation capabilities**, **decreased death**, **evasion of programmed cell death** (apoptosis), **enabling replicative immortality**, **inducing angiogenesis** and **tissue invasion**

and metastasis[6]. **Reprogramming of energy metabolism** and **evasion of immune destruction** were the two new hallmarks to be included in their revised work in 2011. In the same update, they also included two enabling characteristics or properties neoplastic cells have: genome instability and tumor-promoting inflammation.

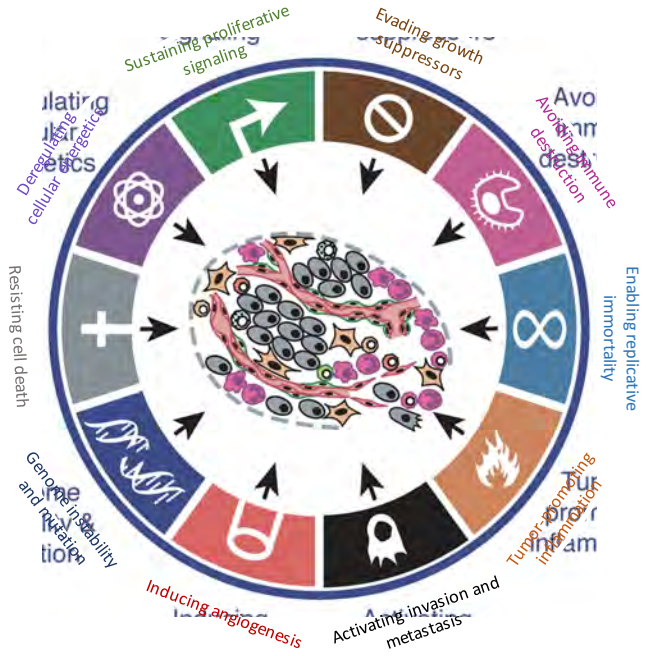

Figure 1. Hallmarks of cancer. Schematic representation of the ten hallmarks described in cancer. Adapted from Hanahan, D., 2011[7].

All these features together allow cancer cells to survive, divide and colonize neighboring and distant tissues. The accumulation of genetic and epigenetic changes, hereditarily maintained across cancer cell divisions, are implicated in the acquisition of this malignant phenotype[8,9].

Breast cancer (BC) is a prevalent human malignancy and a widespread cause of cancer-related death among women worldwide. Over the last few decades, the number of cancer survivors has increased thanks to early detection and the development of novel treatment strategies such as cytotoxic agents and radiation therapy techniques[10]. However, the incidence has increased in most countries as a consequence of the increase in the number of women with major BC risk factors. The main non-modifiable risk factors include age (an increase in incidence from age 35 followed by a stabilization from age 55, coinciding with menopause), age of menarche (the risk increases if menarche occurs at an early age), late period of first pregnancy (increases the risk by 20%), hormonal status, a later menopause (high age of the onset of menopause increases the risk by 30%) and family history of BC (10% of cases have a hereditary component due to mutation of a gene like breast cancer 1 (*BRCA1*)[11]. The main modifiable risk factors are fewer pregnancies (because giving birth reduces the risk by 10%) and shorter or no periods of breastfeeding (risk is reduced by 2% for every 5 months of lactation)[12].

BC is the most commonly diagnosed malignant tumor in women in most countries around the world with 2.08 million new cases in 2018, and this represents 24.2% of all cancers in women. Interestingly, female BC incidence and mortality rates vary between countries[13]. It is the most common cancer in women both in developed (666,731 cases) and less developed regions (105,620 cases). In less developed regions, BC is the most second cause of cancer death (52,846 deaths, 19.7% of total) after cervix uteri cancer and the second cause of cancer death in more-developed areas (184,014 deaths, 11.5%) after lung cancer. In 2018 in Spain, 32,825 (28.7%) women were diagnosed with BC, of which 6,421 (14.4%) women died of this disease[13].

2.1.- Breast cancer classification

BC is a multifactorial disease which involves an interaction between environmental, hormonal and genetic influences, differences in lifestyle or

nutritional exposure[14]. Consequently, patients with BC could have an extensive range of clinical, pathologic and molecular characteristics[15,16]. Although BC is described as a heterogeneous disease, it has been classified into three categories according to the therapeutic options:

Hormone Positive Breast Cancer characterized by the overexpression of estrogens (ER) and/or progesterone (PR) receptors[15]. Women with this kind of BC represent approximately 60-70% of BC patients, and they have a better prognosis than for other cancer types[15]. Currently, available therapies are the selective estrogens receptor modulators (SERMs) and downregulators (SERDs), and the inhibitors of estrogens biosynthesis[17,18].

HER2-Positive Breast Cancers (HER2+) characterized by the overexpression and/or amplification of the human epithermal growth factor receptor 2 (HER2, also known as ErbB-2, ERBB2 or HER2/neu)[19,20]. This represents the 20-30% of BC patients, and it is associated with a more aggressive phenotype,poorly prognosis and high risk of metastasis[20]. The anti-HER2 therapies, including monoclonal antibodies and small molecules TKIs, are the principal treatment options for this type of BC[21]. About half of HER2+ patients express ER, and their treatment also includes endocrine therapy (reviewed in [22]).

Triple-negative breast cancer (TNBC) characterized by the lack of expression of ER/PR and no amplification of the HER2 oncogene[23]. This type of cancer represents approximately 15-20% of patients diagnosed with BC, and is described as being aggressive and with poor prognosis. As triple negative lacks a validated directed therapy, patients are treated with chemotherapy (anthracyclines and taxanes)[24].

2.1.1.- Breast cancer intrinsic subtypes

In recent years, new BC classification based on gene expression patterns has emerged in addition to histopathological characterization. This classification has become an essential tool for clinicians as it provides complementary information

to the standard classification for tailoring treatment and predicting prognosis. In addition, it is helping to elucidate the molecular basis of BC. In 2000, *Perou et al.* explained how the phenotypic diversity of breast tumor might be accompanied or explained by the diversity in gene expression. cDNA microarray had been used to further classify BC subtypes based on gene expression pathways of an intrinsic gene list of 496 genes[25]. The original classification was re-defined by the same group and others, which contributed to the characterization of new molecular subtypes. Initially, four phenotypically distinct subgroups that correlate with clinical outcomes have been identified, including ER+/Luminal, basal-like, Erb-B2+ and normal like[25]. In the study that followed, the luminal subgroup was divided into two groups, Luminal A and Luminal B[26]. Years later, a new molecular subgroup, the so-called claudin-low (CL) was identified. This rare molecular subtype has poor prognosis and is related to mammary stem cells and epithelial-mesenchymal transition (EMT) biologic properties[27].

The **luminal** subtype represents the majority of BC tumors. They share features with luminal epithelial cells arising from the inner layer of the duct lining[16,26]. They are characterized for positively expressing ER and PR receptors. Usually these tumors are classified as low-grade. **HER2-enriched** represents about 10% of BC. They are characterized by high expression levels or loci amplification of the oncogene HER2, low expression of the luminal, hormone receptor-regulated gene cluster and low expression of basal-like genes. The **basal subtype** exhibits low to absent expression of HER2 gene clusters, ER and PR expression, high expression of basal and proliferation genes, presenting the poorest prognosis compared to other subtypes[28]. **Claudin-low** is characterized by a low expression of genes involved in cell-cell adhesion and tight junctions (Claudin), highly enriched with immune system response genes, a negative hormonal receptors expression, and an absence HER2 overexpression. The CL subtype is also known as mesenchymal-like (ML) because of its highest expression of mesenchymal genes[27,29]. **Normal-like** group is similar to Luminal A subtype, whith low proliferation rates and high expression of the luminal cluster. Some research argues that the normal-like group represents tumor samples contaminated with healthy breast tissue as the gene expression profile

has similarities to adipose tissue. While luminal A has the best prognosis among the five molecular subgroups, the normal-like and HER2-positive subtypes have been associated with the poorest one. An intermediate prognosis has been established for luminal B[26].

Transferring this molecular subtype classification as a routine diagnostic tool in public health is difficult due to the high cost of the microarray-based tests. Therefore, these molecular subtypes have been correlated with classical subtypes obtained by immunohistochemistry (Table 1).

Table 1. Molecular subtypes of breast cancer according to the immunohistochemical profile[16,25,27,30,31].

Molecular subtypes	Classical IHC classification			Cellular composition	Outcome
	ER	PR	HER2		
Luminal A	+	+	-	Luminal	Good
Luminal B (HER2-)	+	+	-	Luminal	Poor
Luminal B (HER2+)	+	+	+	Luminal	Poor
HER2-enriched	-	-	+	Luminal/basal	Poor
Basal-like	-	-	-	Basal	Poor
Claudin-low	-	-	-	Basal	Poor

2.2.- Prognosis factors and treatment options of breast cancer

As explained above, BC is a heterogeneous disease with distinct clinical behavior, histopathological features, and responses to therapy. Therefore, staging of BC allows grouping patients according to the extent of their disease and helps in determining the choice of treatment and estimate their prognosis[32,5]. Prognostic markers are defined as tumor characteristics established at the time of diagnosis and might determine the natural disease course in the absence of treatment and are associated with the outcome. There are several prognosis factors, here the

most common, well-documented and currently used in the clinic are described.

TNM classification is a widely accepted system of cancer staging based on Tumor size (T), lymph Node involvement (N) and Metastasis (M).

Tumor size (T): This refers to the size of the original primary tumor and can be classified in different groups: **TX** when primary tumor cannot be assessed, **T0** no evidence of primary tumor, **Tis** means "*in situ*", no healthy tissue is involved, **T1** tumor 20mm or less in greatest dimensions, **T2** tumor more than 20mm but less than 50mm in greatest dimension, **T3** tumor more than 50mm in greatest dimensions and **T4** tumor of any size with direct extension to chest wall below the breast and/or to the skin (ulceration or macroscopic nodules). Inflammatory BC is also included.

Lymph Node involvement (N): Lymph nodes are small organs scattered along the lymphatic system that act as filters and immune monitors, removing fluids, bacteria, or even cancer cells that travel through the lymph system. Nowadays, the axillary lymph node affection is the most important prognostic factor for BC[33]. The risk of cancer spreading is higher when cancer cells can be detected in the axillary lymph nodes. Node involvement can be classified into five different groups: **NX** when regional nodes cannot be measured or found, **N0** when cancer has not spread to adjacent lymph nodes, and **N1-3** describe the number of lymph nodes involved. The higher the N number, the greater the extent of lymph node involvement.

Metastasis (M): Metastasis is the term used when cells escape from the primary tumor through the circulatory system and generate a new tumor in a distant site from the original tumor. The four main sites of metastasis are bone, lung, brain and liver. Metastasis can be classified into three groups: **MX** when metastasis cannot be assessed, **M0** no distant metastasis detected and **M1** when distant metastasis is detected.

Stage: Tumor stage provides information about size, localization and tumor invasiveness. According to TNM classification, five-stages are recognized in BC (Table 2). **Stage 0** corresponds to *in situ* carcinomas, **stage I** (IA, IB) is associated with localized tumors, **stage II-III** (IIA, IIB and IIIA, IIIB, IIIC) associated with regional metastasis and large tumors and **stage IV**: associated with tumors with distant metastasis. In general, a lower stage is associated with less cancer invasion and a higher stage with more advanced cancer. Moreover, A means better stage and C worse stage[5].

Histological Grade: This is based on the degree of differentiation of the tumor, which is representative of the aggressive potential of the tumor[5]. It can be classified in **G1** (well-differentiated), **G2** (moderately-differentiated) and **G3** (poorly-differentiated).

Proliferation rate: Currently one of the most widely-used marker to assess the proliferation rate is the evaluation of the Ki-67 antigen by immunohistochemistry (IHC). Ki-67 is highly expressed during mitosis. It is both a predictive and prognostic marker for breast cancer. High values of Ki-67 are associated with poor prognosis, but at the same time, they show a good response to chemotherapy[34].

Therefore, BC can be divided into three different groups depending on their treatment options. This classification is based in the IHC of the primary tumor biopsy performed to determine the levels of hormonal or HER2 receptors. Currently, ER and PR are recognized as prognostic factors and the most important predictive factors for endocrine treatment. The prescence of at least >1% of cells positive for ER/PR is enough to consider tumors as hormone positive. Hormonal cancer is associated with better prognosis and patients can benefit from endocrine therapy[26,35]. For tumors that overexpress the HER2 receptor, HER2 blocking therapies such as trastuzumab or lapatinib are effective[36].

Table 2. Breast cancer stage according to the TNM classification[37].

Stage	T	N	M
0	T*is*	N0	M0
I	T1	N0	M0
IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
IIB	T2	N1	M0
	T3	N0	M0
IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
IIIB	T4	N0	M0
		N1	M0
		N2	M0
IIIC	T0	N3	M0
	T1	N3	M0
	T2	N3	M0
	T3	N3	M0
	T4	N3	M0
IV	T0	N0/1/2/3	M1
	T1	N0/1/2/3	M1
	T2	N0/1/2/3	M1
	T3	N0/1/2/3	M1
	T4	N0/1/2/3	M1

3.- ErbB/HER FAMILY OF RECEPTORS

The ErbB/HER protein-tyrosine kinases, which include the epidermal growth factor receptor, are among the most-studied cell signaling family in biology. This family is ubiquitously expressed in epithelial, mesenchymal, cardiac, and neuronal cells and their cellular progenitors[38]. These receptors work together as critical mediators of healthy cell growth and development and play an essential role in different kinds of cancers, such as BC[22]. The deregulation of this tightly controlled system by overexpression, amplification or mutation on critical pathway elements alteration can lead to a hyperproliferative disease such as cancer[39].

This family consists of four known members (Figure 2A): HER1 (epidermal growth factor receptor; EGFR or ERBB1) was the first family member to be

discovered, followed by HER2 (ERBB2 or NEU), HER3 (ERBB3) and HER4 (ERBB4)[40]. Each receptor structure consists of an extracellular domain, that has four parts. Domains I and III, which are involved in ligand binding, and domains II and IV, with numerous cysteine residues involucrate in disulfide bone formation. Domain II also participates in homo and heterodimer formation with ErbB family members. The transmembrane segment of 19-25 amino acid residues is α-helical transmembrane segment, that anchors the receptor to cell membrane. The intracellular domain with about 550 amino acid residues contains a juxtamembrane segment, a protein kinase domain, and a carboxyterminal tail. This intracellular domain is responsible for kinase activation and subsequent activation of cell signaling pathways[41,42]. All four members possess a similar protein kinase domain. However, HER3 has its TK domain inactivated (reviwed in [43]).

Broadly, the general activation mechanism of these HER receptors involves the ligands or growth factors binding to the extracellular domain (Figure 2B). For EGFR, HER3 and HER4 receptors an extended family of ligands are well-known, which include epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), amphiregulin (AmR), betacellulin (BTC), epiregulin (EREG), heparin-binding EGF (HB-EGF) and neuregulins (NRGs) or Neu differentiation factors (NDFs)[44–46]. Despite HER1, HER3, and HER4 having a natural ligand, HER2 has no known natural ligand to date, so it is recognized as an orphan receptor. This receptor presents a fixed active conformation and therefore, it is permanently available for dimerization (reviewed in[36]). Nevertheless, the HER2 receptor can act as a co-receptor with high affinity for HER1, HER3 or HER4, thus forming heterodimers[22,47]. The HER2-HER3 heterodimer is considered to be the most active HER signaling dimer concerning strong interaction, ligand-induced tyrosine phosphorylation and downstream signaling[48,49].

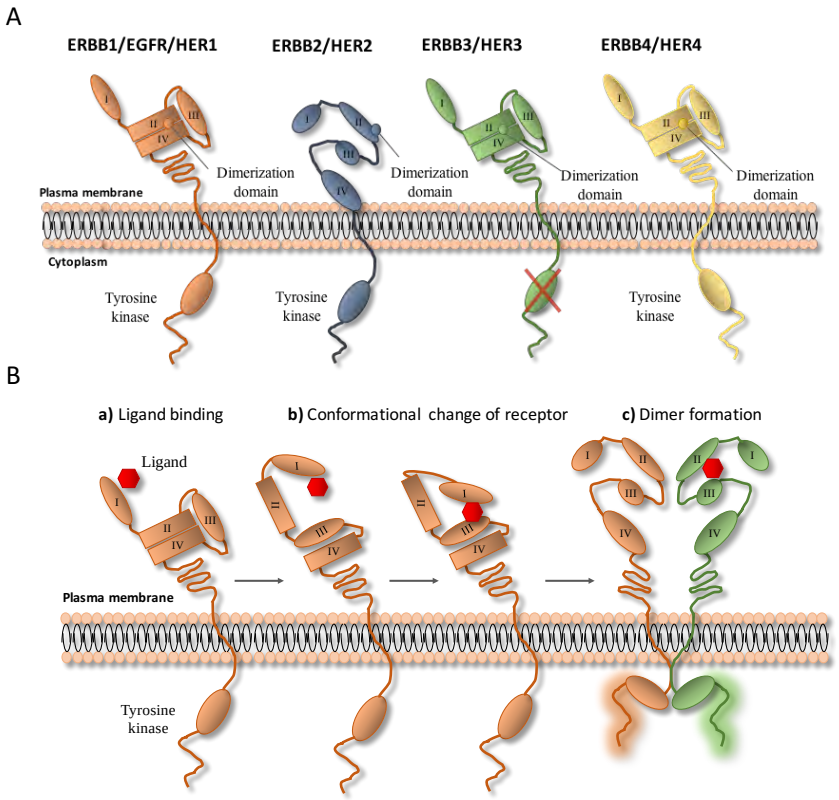

Figure 2. HER family of receptors. (A) Schematic representation of the structural basis for the four HER family members: ERBB1 (EGFR or HER1), ERBB2 (HER2), ERBB3 (HER3) and ERBB4 (HER4). Each receptor is composed of three functional regions: an extracellular region, a transmembrane segment, and an intracellular protein tyrosine kinase domain. In normal conditions, ERBB1, ERBB3, and ERBB4 present a closed conformation. ERBB2 has no known ligand and therefore it is fixed in the active conformation, always available to interact with other HER receptors. In addition, ERBB3 has an inactive kinase domain (TK). **(B)** The ligand binding to receptor produces a conformational change in the folded structure of the molecule and the dimer formation, an essential step for receptor activation. After dimerization, the intracellular kinase domains' cross-phosphorylate residues are found in the C-terminal receptor tail. Once HER-dimers are activated, their signal goes through different cascades. Adapted from Baselga and Swain 2009[50].

The ligand binding produces a receptor conformational change that facilitates the homo- or heterodimerization with another HER family member. Consequently, the dimerization gives rise to TK domains phosphorylation and the subsequent activation of different intracellular pathways, including the mitogen-activated protein kinase (RAS/RAF/MEK/MAPK) which mainly regulates cell

proliferation and the PI3K-activated AKT (PI3K/AKT) pathway, which is vital for cell survival (reviewed in [36]). These and other HER signaling modules participate in angiogenesis, cell adhesion, cell motility, development, and organogenesis[51]. This dimerization process is an essential requirement for the function and signaling activity of these receptors[52,53].

Even though the activation mechanisms of these receptors have been established, some ligand-independent mechanisms can activate the HER pathways. Some examples are (i) the alterations in some HER downstream proteins which can activate the pathway without the ligand binding[31], (ii) the dimerization of HER with other receptors like the insulin-like growth factor 1 receptor (IGF-1R)[54] or (iii) the homodimerization and downstream signaling activation due to high levels of HER2[55]. These new receptor activation routes may account for resistance to therapies that target only the extracellular domains of the receptor.

3.1.- Epidermal growth factor receptor 2 (HER2)

HER2, also known as ErbB2 or NEU in rats, was described by Schechter *et al.* in 1984[56]. It is a 185KDa transmembrane receptor with tyrosine kinase activity encoded by the *ERBB2* gene and located on chromosome 17 (17q12)[57].

3.1.1.- The role of HER2 in normal development

The HER2 receptor is widely expressed and functionally important in multiple tissues such as the central nervous system, bone, muscle, skin heart, lung and intestinal epithelium, apart from its highly-studied role in mammalian development [58,59]. One essential role of this receptor has been described in the cardiovascular and nervous system. Ozcelik *et al.* described the role of HER2, enriched in T-tubules, for adult heart function. The authors observed that mice with an HER2 conditional mutation in ventricular cardiomyocytes, displayed multiple independent parameters of dilated cardiomyopathy, with signs of cardiac dysfunction[60]. In regards to the sympathetic nervous system, HER2 plays a role

in neuregulin signaling and is expressed in the development of the nervous system[61]. Its role has been described in Schwann cell myelination control[62]. Besides this, another crucial role was also described in *in vivo* oligodendrocyte differentiation by Kim *et al.*[63].

3.1.2.- The role of HER2 in breast cancer

HER2 was first identified as the *neu* oncogene mutant in chemically-induced rat neuroblastomas or Schwannomas[56]. A few years later, the human homolog was identified[64]. After its identification as an oncogene, HER2 was found to be overexpressed in some mammary carcinoma cells as well as in a variety of other cancers including ovarian, gastric and salivary cancers[20,59,65,66]. In recent years, different activating mutations in the HER2 gene have been described in tumors do not overexpress HER2 in breast and non-small cell lung cancers[67–69].

As explained earlier, HER2 is frequently overexpressed in approximately 15-30% of BCs. Slamon and co-workers described a significant association between HER2 overexpression and relapse as well as patient survival[20]. Tumors are classified as HER2+ if they achieve an IHC staining result of 3+ or more, fluorescent *in situ* hybridization (FISH) results in more than six *HER2* gene copies per nucleus or a FISH ratio greater than 2.2[70]. Besides HER2 overexpression, a mutation of the *HER2* gene at codon 655 was identified, which modifies the HER2 receptor structure resulting in an active form of this receptor[71]. Cells that possess this mutation or an overexpressed HER2 protein have a high possibility of showing HER2 homodimerization promoting uncontrolled growth, division and apoptosis avoidance[72].

4.- TREATMENT OF HER2+ BREAST CANCER

BC treatments are determined by different prognostic and predictive factors like age, family history of BC, the pathological and molecular characteristics of the tumor, its location, the extent of disease and general condition of patients. The methods to estimate the risk of relapse and to plan complementary treatment have evolved in recent years in step with the evolving knowledge of the biology of tumors.

Currently, BC treatment can be categorized as either conventional or targeted therapy. Conventional therapy includes surgery, radiation and chemotherapy, while targeted therapy uses specific target drugs against cancerous cells. Depending on the time the treatment is administrated, this is classified as **adjuvant therapy** (after surgery) or **neoadjuvant therapy** (before the surgery). The neoadjuvant systemic therapy is commonly used to downstage the primary tumor and regional lymph nodes preoperatively, in order to increase operability and to enable breast-conserving surgery in patients with large primary tumors or locally advanced disease. It is considered a standard option, especially for patients with triple-negative or HER2+ BC[73]. Meanwhile, adjuvant therapy attempts to prevent the recurrence of BC.

4.1.- Conventional Therapy

Surgery is the principal and most effective treatment for early, localized or operable BC and often the first treatment to be applied if metastases are not detected. The surgical modalities are the breast-conservative surgery (BCT; tumorectomy, quadrantectomy or lumpectomy) and mastectomy. More than 90% of BC patients undergo surgical excision of the tumor. Other treatment option is **radiotherapy**, which consist in damaging the DNA with controlled doses of high-energy radiation by the generation of reactive chemical species with the aim to control and kill the cancerous cells, thus leading them to apoptosis[5]. Complementary radiotherapy is generally indicated after conservative surgery and in patients with high-risk treated with mastectomy. Breast irradiation reduces

the risk of local recurrence[5,74]. **Chemotherapy** is the most common systemic treatment for cancer. Briefly, chemotherapy involves the use of chemical agents to stop the proliferation and growth of cancerous cells. A lot of different chemotherapeutic agents have been developed and are administered in different combinations[5].

Chemotherapy and radiotherapy can also attack healthy cells causing important side effects. Therefore, the administration doses are crucial. Surgery and radiation therapy can often eradicate primary or localized tumors but they are not effective for metastasized cancer, a systemic therapy like chemotherapy is required[75].

4.2.- Target Therapy

Taking into account the role HER2 has in BC oncogenesis and progression, a large number of studies have focused on developing different therapeutic approaches against HER2. Currently, monoclonal antibodies and tyrosine kinase inhibitors (TKIs) are the most commonly-used treatments for HER2+ BC[76,77].

4.2.1.- Monoclonal antibodies

Around 1984 the growth-inhibitory effects of antibodies targeting the surface of HER2/*neu* transformed cells were discovered[78]. However, it would not be until a few years later that multiple laboratories would verify these observations and develop the monoclonal antibodies as a method to treat BC cancer patients[79].

Trastuzumab

Herceptin® (Trastuzumab; Genentech) is a recombinant humanized monoclonal antibody (rhumAb 4D5) approved in 1998, which acts against the extracellular domain of the HER2 protein (domain IV; Figure 3A)[80]. It is the first HER2-targeted therapy to be approved by the U.S. Food and Drug Administration (FDA) for the treatment of HER2-overexpressing BC. Before the humanized antibody developed by Carter and coauthors[81], several murine monoclonal antibodies (muMAbs) against the extracellular domain of HER2 with

anti-tumor effects had been developed[78,82,83]. Several, *in vitro* and *in vivo* studies in HER2+ BC cell lines and xenograft models demonstrated the anti-tumor activity of trastuzumab as a monotherapy and its enhanced activity in combination with chemotherapeutic agents[81,84–86].

Initially, trastuzumab was first approved by the FDA to treat HER2+ metastatic breast cancer (MBC). A phase II trial demonstrated the positive effects of trastuzumab as a single agent in HER2+ MBC patients who had progressed after chemotherapy treatment[79,87]. Furthermore, a phase II trial, which included women with HER2+ MBC, who had not received previous chemotherapy, treated with trastuzumab plus chemotherapy or trastuzumab/chemotherapy alone, demonstrated the efficacy of trastuzumab in combination with chemotherapy[88]. Based on these clinical trials, the FDA approved the use of trastuzumab in patients with HER2+ MBC as first-line therapy in combination with paclitaxel, and as a single agent for patients who have undergone a chemotherapy regimen. After the FDA approval, trastuzumab has become a fundamental therapeutic tool in HER2+ BC patients[80,81,89].

Trastuzumab as adjuvant therapy: Following the improvements of trastuzumab in MBC treatment, different clinical trials were developed to test this monoclonal antibody in adjuvant settings for HER2+ early BC. The National Surgical Adjuvant Breast and Bowel Project (NSABP) trial B-31 and the North Central Cancer Treatment Group (NCCTG) trial N9831 resulted in the FDA approving trastuzumab as a part of a treatment regime containing doxorubicin, cyclophosphamide, and paclitaxel for the adjuvant treatment of HER2+ node-positive BC patients. The HERA clinical trial (phase III) showed that one year of trastuzumab adjuvant treatment is still the standard care for people with HER2+ early-stage BC[90,91]. However, in a 10-year follow-up, 28.8% of patients were described as having experienced disease progression[91].

Trastuzumab as neoadjuvant therapy: Trastuzumab is also administered in the neoadjuvant regime. Different randomized trials were designed to evaluate the effects of trastuzumab in combination with chemotherapy. Trastuzumab in

combination with sequential anthracycline-taxane-based chemotherapy in the neoadjuvant setting showed an improvement in pathological complete response (pCR) as well as a lower relapse rate[92]. In phase III of a NeOAdjuvant Herceptin (NOAH) trial in patients with locally advanced BC, the addition of trastuzumab to chemotherapy treatment in neoadjuvant regime and the continued administration of trastuzumab after surgery significantly increased the pCR compared to patients who only received neoadjuvant chemotherapy alone (38.5% vs. 19.5%; HR 0.29, *P=0.0135*). Furthermore, these patients remained event-free longer (5-year event-free survival (EFS) 58% vs. 43%; HR 0.64, *P=0.016*)[93]. The results of five prospective adjuvant phase III trials have led to trastuzumab being selected as first-line treatment in the adjuvant therapy for HER2+ early BC patients[94–97].

The **trastuzumab action mechanisms** are numerous and complex: (i) down-regulation of the HER2 expression by accelerating receptor endocytosis and degradation and, as a consequence, arrest cell cycle progression[98], (ii) the induction of antibody-dependent cell-mediated cytotoxicity (ADCC) via recruitment of natural killer (NK) cells which will initiate the lysis of cancer cells[99], (iii) vascular endothelial growth factor (VEGF) and angiogenesis decrease[100], and (iv) reduction of phosphatase and tensin homolog (PTEN) phosphorylation, which results in an AKT dephosphorylation and cell proliferation inhibition[101]. Furthermore, trastuzumab can inhibit the formation of p95 HER2, by inhibiting the extracellular domain-shedding *in vitro*. HER2 can be cleaved into two different forms, a 110KDa extracellular domain and a 95KDa membrane-bound carboxyterminal domain which is constitutively active (p95-HER2). Serum extracellular domain levels of HER2 are lower in patients who have received trastuzumab than in those who have not[102–104].

Trastuzumab is generally well-tolerated, but unexpected cardiotoxicity has been described when it is administered in combination with anthracyclines[105,106].

Despite trastuzumab development having significantly improved the outcome in BC, a significant fraction of patients does not respond to the treatment, while

others will eventually progress. The *de novo* or acquired treatment resistance mechanisms are not fully understood, but several potential mechanisms have been proposed in the last few years[107,108]. Several studies have focused on developing strategies to overcome anti-HER2 resistance in both early and metastatic BC. For this reason, new therapies such as lapatinib, pertuzumab, trastuzumab-DM1 and neratinib have been developed.

Pertuzumab

Perjeta® (Pertuzumab: Genentech) is the second generation of humanized monoclonal anti-HER2. Pertuzumab binds to the domain II of the HER2 receptor and blocks its dimerization along with other HER family members such as HER2-HER3 heterodimerization and the subsequent activation of different downstream signaling cascades (Figure 3B)[109,110].

Pertuzumab was first approved by the FDA in 2012, in combination with trastuzumab and docetaxel, for HER2+ MBC patients who had not previously received anti-HER2 or chemotherapy treatment[5,89]. This was in accordance with the final results from a large phase III clinical trial CLEOPATRA (The Clinical Evaluation of Pertuzumab and Trastuzumab), which assessed the efficacy and safety of a pertuzumab *plus* trastuzumab *plus* docetaxel combination when used as first-line treatment for HER2+ MBC. The results of this trial demonstrated a significantly prolonged progression-free survival (PFS) from 12.4 months in the control group to 18.5 months in the pertuzumab group[111]. Since this study, pertuzumab *plus* trastuzumab has replaced trastuzumab *plus* chemotherapy treatment as the standard of care for first-line MBC[112].

Pertuzumab as adjuvant therapy: On December 2017, the FDA approved the combination of pertuzumab *plus* trastuzumab and chemotherapy as adjuvant treatment for patients with HER2+ early BC at high risk of recurrence. This decision was made based on data from the APHINITY trial (NCT01358877)[89]. The phase III trial demonstrated how pertuzumab *plus* a trastuzumab and docetaxel regime as adjuvant treatment, helped people with an aggressive type of early BC, live longer without their disease returning compared to the standard

trastuzumab *plus* chemotherapy treatment. Gunther von Minckwitz presented the results at the 2017 ASCO Annual Meeting. Side effects like diarrhea, nausea, alopecia, fatigue, peripheral neuropathy, and vomiting, were reported in at least 30% of patients receiving this combination[113].

Pertuzumab as neoadjuvant therapy: Recently, the dual HER2 inhibition with pertuzumab *plus* trastuzumab in combination with chemotherapy as neoadjuvant treatment was approved for patients with locally advanced, inflammatory or early-stage BC with a high risk of relapse. This approval is based on the NeoSphere trial, which demonstrated significant improvement in the pCR rate with this combination of either trastuzumab *plus* chemotherapy or pertuzumab *plus* chemotherapy. At 5 years, 86% of the patients who had received the combinations were alive and disease-free, compared to 81% of the patients who had received trastuzumab plus chemotherapy[114]. Similar results were obtained in phase II of the cardiac safety study TRYPHAENA[115].

Trastuzumab-DM1

Kadcyla® (Trastuzumab-DM1 (T-DM1): Genentech) a novel antibody-drug conjugate, is a combination of trastuzumab with the microtubule-inhibiting chemotherapeutic agent DM1 and higher activity of T-DM1 compared to trastuzumab alone has been identified (Figure 3C)[116].

In 2013 the T-DM1 was approved by the FDA as a single agent for HER2+ MBC patients who had previously received trastuzumab *plus* taxane treatment, separately or in combination[89,117]. T-DM1 approval was based on the results of phase III of the randomized and multicenter EMILIA trial, where T-DM1 as single-agent was compared with a lapatinib and capecitabine combination in HER2+ locally advanced or metastatic BC patients previously treated with trastuzumab, anthracyclines and taxanes[118].

T-DM1 as adjuvant therapy: Nowadays, different phase III trials are currently involved in the ongoing study of the efficacy of T-DM1 in the adjuvant setting, such as the KATHERINE (NCT01772472) and KAITLIN (NCT01966471) trials.

The first trial, KATHERINE (NCT01772472), is studying the T-DM1 treatment over one year in women who have residual disease in the breast or axillary lymph nodes following neoadjuvant therapy and comparing this to treatment with trastuzumab. The second trial, KAITLIN (NCT01966471), is examining the combination of T-DM1 and pertuzumab to trastuzumab, pertuzumab, and a taxane component of chemotherapy following 3 cycles of anthracycline-based chemotherapy[112].

T-DM1 as neoadjuvant therapy: So far there have not been any successful trials with T-DM1 in the neoadjuvant setting[112]. The phase III KRISTINE trial (NCT02131064) showed no benefit in pCR when HER2+ BC patients were treated with the T-DM1 combined with pertuzumab compared to pertuzumab *plus* trastuzumab with chemotherapy[112].

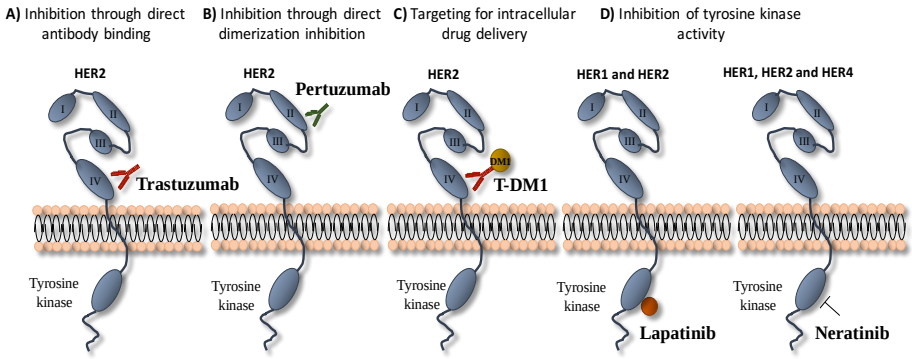

Figure 3. **Anti-HER2 target therapy.** **(A)** Trastuzumab binds directly to domain IV of the extracellular region of the HER2 receptor and marks tumor cells that overexpress HER2 for a further immunological attack through antibody-dependent cell-mediated cytotoxicity. **(B)** Pertuzumab, another anti-HER2 antibody, acts against domain II of the extracellular region and prevents the dimerization with signaling partners. **(C)** T-DM1 is an antibody-drug conjugate which consists of trastuzumab linked to a cytotoxic agent emtansine (DM1). The antibody is internalized and DM1 can exert their cytotoxic effects. **(D)** Lapatinib (against HER1 and HER2 receptors) and neratinib (against HER1, HER2 and HER4 receptors) inhibit the tyrosine kinase domain and block its downstream signaling pathway. Adapted from Baselga and Swain 2009[50].

4.2.2.- Tyrosine Kinase Inhibitors (TKIs)

As explained earlier, HER receptors require TK domain phosphorylation for activation. For this reason, different TK inhibitors have been developed as another mechanism to block HER receptors. The TKIs compete against ATP to bind onto an ATP binding site reducing downstream pathway activation[119]. These TKIs emerged as an alternative treatment for those patients who developed anti-HER2 antibody treatment resistance or present mutant HER2 activity[112]. Among the group of TKIs, the most clinically advanced anti-HER2 TKI is lapatinib[120].

Lapatinib

Tykerb® (Lapatinib; Glaxo-SmithKline) is an orally active, low molecular weight TKI. It is a selective, reversible inhibitor of HER2 and HER1 (EGFR) receptors (Figure 3D). Lapatinib works intracellular and directly targets the TK domain. It binds to the cytoplasmic ATP-binding site of both receptors preventing the subsequent activation of MAPK or PI3K/AKT downstream signaling pathways, leading to an increase in apoptosis and decrease in cellular proliferation[121–123]. Lapatinib is generally well-tolerated, but some adverse events have been described such as skin rash, diarrhea, nausea and fatigue[124].

In 2007, the FDA approved the lapatinib treatment in combination with capecitabine (Xeloda®) for treating patients with advanced or HER2+ MBC who have previously been treated with trastuzumab and anthracycline or taxane[5,125]. In a phase II trial, lapatinib *plus* capecitabine showed superior time to progression compared to capecitabine alone for MBC that had progressed on from trastuzumab-based therapy[125]. In 2010, the FDA also approved the lapatinib treatment in combination with letrozole (Femara®, Novartis Pharmaceuticals Corp.) for the treatment of post-menopausal women with hormone receptor-positive MBC that overexpresses the HER2 receptor and for whom hormonal therapy is indicated[5,126].

Lapatinib as adjuvant therapy: two phase III trials examined lapatinib treatment in the adjuvant setting for HER2+ BC. The TEACH trial, which

compared lapatinib to placebo in women with HER2+ early BC who had previously received adjuvant chemotherapy but not trastuzumab, demonstrated no significant benefit of lapatinib as a single agent[127]. The ALTTO trial (Adjuvant Lapatinib and/or Trastuzumab Treatment Optimization) evaluated the activity of trastuzumab alone, lapatinib alone, trastuzumab *plus* lapatinib (T+L) and the sequential trastuzumab and lapatinib (T→L) treatment[90]. The ALTTO results showed that disease-free survival (DFS) had no significant improvement in lapatinib *plus* trastuzumab treatment in adjuvant settings compared to single-agent trastuzumab with modest clinical benefits and added toxicity[90]. Nowadays, lapatinib in the adjuvant setting, either alone or in combination with trastuzumab, has been ruled out and there are no further studies in progress.

Lapatinib as neoadjuvant therapy: As with the adjuvant setting, the efficacy of lapatinib has been investigated in the neoadjuvant setting both as a single agent and in a combination with trastuzumab. The NeoALTTO trial, a phase III study, showed that the pCR rate was significantly higher in the combination of lapatinib and trastuzumab *plus* chemotherapy (51.3%) compared to either lapatinib *plus* chemotherapy (24.7%) and trastuzumab *plus* chemotherapy (29.5%)[128]. Unfortunately, the lapatinib *plus* trastuzumab combination had no significant improvement in overall survival (OS) or event-free survival. For these reasons, there is not much interest in the lapatinib *plus* trastuzumab combination in the neoadjuvant setting[129,112].

Neratinib

Nerlynx® (Neratinib; HKI-272, Puma Biotechnology Inc.) is a second-generation tyrosine kinase inhibitor[130]. It is an irreversible inhibitor of EGFR, HER2, and HER4, which covalently binds to the ATP-binding sites of the receptors and produces the blockage of the downstream pathways (Figure 3D).

Neratinib as adjuvant therapy: Recently the FDA and the European Medicines Agency approved the use of neratinib for the extended adjuvant treatment of adult patients with early-stage HER2+ BC previously treated with trastuzumab-based therapy[2]. Approval was based on phase II of the ExtNET trial, which

compared one year of neratinib to placebo in women with stage I-III HER2+ BC who had completed neoadjuvant and adjuvant trastuzumab up to two years before randomization. The neratinib treatment improved invasive DFS (94.2%) compared to the placebo treatment (91.1%). This improvement was most pronounced in hormone-positive patients, suggesting that neratinib might provide additional treatment for this subset of patients[90].

Neratinib as neoadjuvant therapy: Neratinib in the adjuvant setting has been examined in two phase II trials, the NSABP FB-7 and the I-SPY 2. The FB-7 study compared neratinib, trastuzumab or the combination of both drugs, all given with chemotherapy in early HER2+ BC. In this study, the combination of neratinib *plus* chemotherapy did not present benefits compared to trastuzumab *plus* chemotherapy (pCR 33.3% vs. 38.1%). However, an improvement in pCR (12%) with the neratinib *plus* trastuzumab combination compared to trastuzumab *plus* chemotherapy was observed[131]. In the I-SPY 2 study, the combination of neratinib and chemotherapy resulted in a higher rate of pCR than trastuzumab and chemotherapy did (39% vs. 23%)[132]. The investigation of neratinib in the neoadjuvant setting is ongoing.

Table 3. Monoclonal antibodies and small molecule clinical trials[76,112].

	Drug name	Drug class	Target(s)	Current status in HER2+ BC	Clinical trial examples	Regime
Monoclonal Antibodies	**Trastuzumab** *Herceptin*	mAb	HER2	Approved for MBC	Phase III	Single agent for patients who have undergone a chemotherapy regimen and in combination with paclitaxel
				Approved for node positive BC	Phase III B-31, N9831 or HERA	Trastuzumab+doxorubicin+cyclophosphamide+paclitaxel in adjuvant regime
				Approved for early BC	Phase III NOAH, BCIRG 006 or ISRCTN76560285	Trastuzumab+ anthracycline-taxane-based chemotherapy in neoadjuvant regime
	T-DM1 *Kadcyla*	mAb conjugate	HER2	Approved for MBC	Phase III EMILIA	Single agent in patients who had previously received trastuzumab and taxane treatment, separately or in combination
				Clinical trials for early HER2+ BC (ongoing)	Phase III KATHERINE or KAITLIN	T-DM1 vs. trastuzumab in adjuvant regime T-DM1+pertuzumab vs. trastuzumab+pertuzumab+taxane in adjuvant regime
	Pertuzumab *Perjeta*	mAb	HER2	Approved for MBC	Phase III CLEOPATRA	Pertuzumab+trastuzumab+docetaxel
				Approved for early HER2+ BC	Phase III APHINITY, NeoSphere or TRYPHAENA	Pertuzumab+trastuzumab+chemotherapy in adjuvant or neoadjuvant regime
Small molecules	**Lapatinib** *Tykerb*	TKI (rev)	EGFR and HER2	Approved for MBC	NCT00078572	Lapatinib+capacitabine in patients who had previously received trastuzumab+ anthracycline or taxane
				Approved for hormone-positive MBC that overexpresses HER2	EGF30008	Lapatinib+Letrozole
	Neratinib *Nerlynx*	TKI (irrev)	EGFR, HER2 and HER4	Approved for early HER2+ BC	Phase II ExteNET trial	Single agent in adjuvant regime in adult patients with early-stage HER2+ BC previously treated with trastuzumab-based therapy for 1 year
				Clinical trial for early HER2+ BC (ongoing)	Phase II NSABP FB-7 or I-SPY 2	Neratinib+trastuzumab+chemotherapy as neoadjuvant treatment

MBC, metastatic breast cancer; mAb, monoclonal antibody; TKI (rev), tyrosine kinase reversible inhibitor; TKI (irr

4.3.- Resistance to HER2+ breast cancer treatment

As explained earlier, despite the clinical benefits of anti-HER2 treatment in HER2+ BC, a large percentage of patients display primary or acquired (also known as secondary) resistance to drugs such as trastuzumab or lapatinib[88,133]. In the last few years, several studies have focused on identifying the molecular mechanisms of trastuzumab and lapatinib resistance.

Alterations of HER2 receptor

Accumulation of the truncated forms of the HER2 receptor that lack the extracellular trastuzumab-binding domain has been described as a trastuzumab-resistant mechanism. As explained earlier (5.2.1.-Monoclonal antibodies; trastuzumab), the HER2 receptor can be cleaved by different metalloproteases, creating a truncated receptor known as p95HER2 or C-terminal fragments. The p95HER2 is the membrane-bound portion that remains constitutively active due to the loss of the extracellular domain recognized by trastuzumab[134]. Interestingly, lapatinib, as monotherapy or in combination with capecitabine, was equally effective in patients with p95HER2 positive and p95HER2 negative HER2+ BC tumors[135]. Probably, the lapatinib efficacy in these patients is because p95 fragment retains the TKI domain recognized by lapatinib[136]. Another resistant mechanism described is the HER2 splice variant (HER2Δ16) with enhanced transforming activity in BC cell lines and tumors. HER2Δ16 increases the stabilization of the HER2 homodimers[137].

Another potential mechanism of acquired trastuzumab resistance described is the loss of HER2+ status in metastatic tumors in patients with primary HER2+ BC during or after trastuzumab therapy[138].

Masking of membrane proteins

Another potential resistance mechanism is the blocking or “masking” of the HER2 receptor. Several preclinical studies suggest that the membrane-associated mucin glycoproteins, mucin 4 (MUC4) and mucin 1 (MUC1), are implicated in resistance to trastuzumab and potentially to additional anti-HER2 therapies. MUC4 forms protective barriers on epithelial cells and can impede the

binding of trastuzumab to its epitope on HER2, rendering trastuzumab less able to inhibit the HER2 pathway[139,140]. Another preclinical study demonstrated that a breast cancer cell line acquired trastuzumab resistance through the upregulation of a cleaved form of MUC1 (MUC1* or MUC1-C), which contributes to HER2 constitutive activation. Furthermore, they demonstrated that MUC1* antagonist treatment reverses trastuzumab-resistance in these cells[141,142].

Loss of PTEN/PI3K

Resistance to trastuzumab or lapatinib might also arise through the downstream signaling pathways of the HER2 receptor, including PI3K/AKT and RAS/MAPK. Preclinical evidence suggests that constitutive activation of the PI3K/AKT pathway[143,144], AKT activating-mutations[145], the loss of the tumor suppressor gene PTEN[146] or mutations in the PIK3CA gene (encoding the PI3K catalytic isoform p110α)[147] are the most common mechanisms of trastuzumab and lapatinib resistance. The loss of PTEN, which functions to prevent the PI3K/AKT pathway from being activated, has been implicated in trastuzumab resistance *in vitro* and *in vivo* and is a predictive marker for trastuzumab resistance[146,148] The *in vitro* expression of mutant PI3KCA in HER2+ cells is also associated with trastuzumab resistance[143,149]. However, some studies suggest that lapatinib resistance is not related to PTEN loss or PIK3CA mutations[150,151]. Other published studies have linked the amplification of PI3K/AKT with an increase in Src tyrosine kinase activity (which regulates the phosphorylation of PTEN) in both trastuzumab and lapatinib resistant cells[152,153].

Cross-talk with other signaling pathways

The HER2 receptor can interact with other HER receptors or other receptor tyrosine kinases (RTKs) and lead to trastuzumab and lapatinib resistance. The overexpression or hyperactivation of HER1[154,155], HER3[156] or other HER family receptors as well as an excess of its ligands [157] have been exposed as being able to compensate the HER2 inhibition by trastuzumab. As explained earlier, trastuzumab reduces HER2-mediated signaling through a PI3K/AKT or MAPK pathway, but not signaling mediated from other HER receptors. Thus, cells with EGFR/HER3 heterodimers or EGFR homodimers, lacking HER2, could

potentially bypass the trastuzumab blockade[101].

Other studies suggest the interaction between HER2 and other RTKs leads to the activation of alternative signaling pathways responsible for anti-HER2 drug resistance. The most widely-studied receptor in this group is the IGF-1R which activates the same pathways as the HER family receptors, such as PI3K/AKT and RAS/MAPK do[158]. Overexpression of IGF-1R or an increase in the levels of IGF-1R/HER2 heterodimers can potentially activate PI3K/AKT signaling and confer resistance to trastuzumab[54,159]. Conversely, lapatinib blocked IGF-IR signaling and the initiation of apoptosis in trastuzumab-resistant cells even in the presence of IGF-I[160]. The hepatic growth factor receptor (c-MET) and ephrin-type-A receptor 2 (EphA2) are other RTK examples that not only can interact directly with the HER receptor, but can also directly activate downstream proteins like PI3K/AKT[157,161,162]. Another well-known crosstalk is HER2/ER, where ER acts as an escape mechanism to bypass HER2 inhibition, probably via PI3K[107,163]. HER2 inhibition has been described as triggering an increase in ER signaling in HER2+/ER+ tumor models[164]. Furthermore, the role the ER receptor plays has been demonstrated not only in trastuzumab resistance, but also in lapatinib resistance. Different preclinical studies and neoadjuvant trials showed an increased expression of several genes related to ER signaling, such as FOXO3a, as a mechanism to negate lapatinib inhibition[107,164–166].

Tumor microenvironments

Extrinsic signals from the tumor microenvironment, such as paracrine growth factors or extracellular matrix protein (ECM) may be involved in treatment resistance in breast cancer[167–170]. The ECM and integrin signaling are the most common tumor microenvironment components related to HER2-drugs resistance. Several *in vitro* studies suggest that both β1 and β4 integrins, members of a large family of receptors that mediate the interaction between cytoskeletal elements and the ECM, can interact with EGFR/HER2 to negate their inhibition or can provide alternative survival signals to protect cells from apoptosis induced by anti-HER2 treatment[169,171].

In conclusion, although different trastuzumab and lapatinib resistance

mechanisms have been described in several papers, they are not yet fully understood. Besides, the lapatinib and trastuzumab resistance mechanisms do not overlap so a number of resistant mechanisms proposed for trastuzumab do not seem to apply to lapatinib.

5.- EPIGENETICS

The epigenetic concept (from Greek term means "over" or "upon" genetics) was introduced by Conrad Waddington in 1942 to describe the molecular mechanisms that convert genetic variations into observable traits or phenotype as well as to explain how genes interact with their environment[172]. This initial definition allowed different researchers, like Hadorn and Goldschmidt, to expand this field[173–175]. Currently, the term "epigenetics" can be defined as, "*the study area of those traits that are inherited in a stable manner resulting from changes in the chromosome without alterations in the nucleotide sequence*"[176]. The discovery of epigenetics has evolved remarkably, allowing us to increase our knowledge about the molecular mechanisms that regulate the gene expression in eukaryotes[177].

The epigenetic process is essential during normal development and differentiation of distinct cell lineages throughout the lifetime of the organism. In other words, epigenetics explains how cells with the same DNA can differentiate into different cell type and also maintain the differentiated state[178–180]. Several environmental factors, including nutrition[181], tobacco[182] and lifestyle[183], can modulate epigenetic modifications causing effects on gene expression during early life and adulthood.

This field of study consists of three types of mechanisms that cooperate in an integrated manner: post-translational modifications of histone proteins, DNA methylation and noncoding RNAs (Figure 4). These processes are not mutually exclusive and together control chromatin accessibility and modulate transcriptional activity[184].

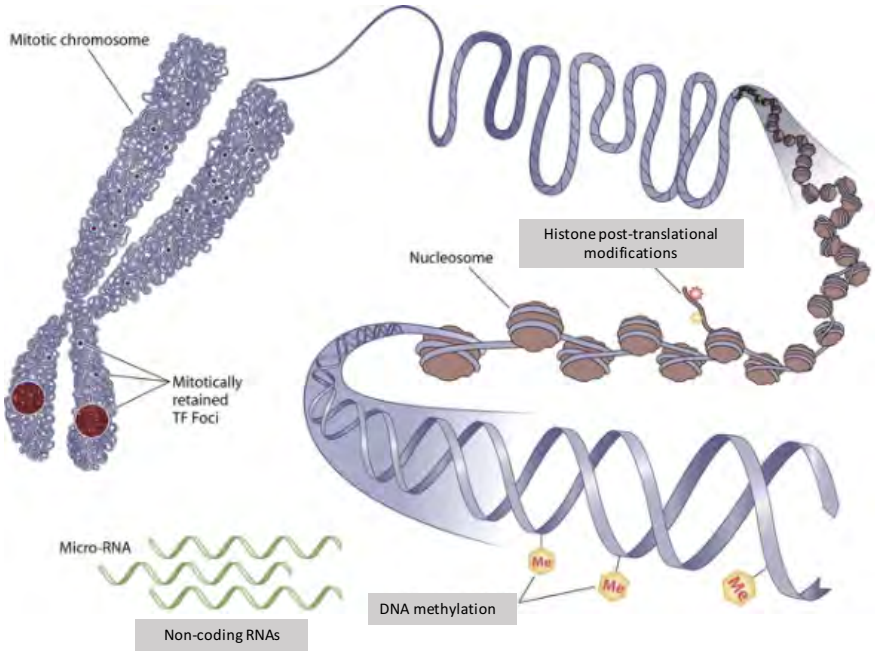

Figure 4. **Epigenetic mechanisms of gene regulation**. Schematic representation of epigenetic mechanisms which play an essential role in the modulation of chromatin structure and consequently in gene expression: DNA methylation, histone post-transcriptional modifications and non-coding RNAs (ncRNAs). Adapted from Zaidi *et al*. 2010[185].

In conclusion, the epigenetic mechanisms work collectively to regulate many cellular processes, including the DNA-protein interaction, suppression of transposable element mobility, X-chromosome inactivation, genomic imprinting, cellular differentiation and embryogenesis. Thus, epigenetic is an essential regulatory mechanism, above genetics, that control gene expression in a potentially heritable way[178].

5.1.- Histone modification

Vincent Allfrey was the first to expose the possible role histone modifications may have in transcription regulation[186]. Nowadays, the role they play has been described not just for transcription but in all DNA-template process[187].

Genomic DNA is packaged into a highly compacted DNA-protein complex, called chromatin, in the nucleosome of eukaryotic cells. The nucleosome is the

basic functional unit of the chromatin, allowing chromatin packaging and chromosome formation[188,189]. The nucleosome contains 145-147 base pairs of DNA wrapped around a histone octamer composed of one H3-H4 tetramer histone flanked by two separate H2A-H2B dimers[190–192]. Histones can undergo up to 16 classes of post-transcriptional and reversible covalent modifications (PTMs), which occur mainly in its N-terminal tails, modulating nucleosome dynamics and chromatin structure by altering noncovalent connections within and among nucleosomes[193]. The most studied histone modifications are acetylation, methylation and phosphorylation, but other PTMs have been described such as ubiquitylation and SUMOylation (Figure 5A)[192]. The histones not only have a purely structural role as packaging proteins, but the multiple PTMs have a direct impact on the chromatin conformation[193]. As mentioned earlier, post-transcriptional histone modifications play an essential role in different processes including transcriptional regulation, DNA repair, DNA replication, alternative splicing and chromosome condensation[187,194,195]. Accordingly, their dysregulation is linked to various diseases such as cancer, cardiovascular disease or neurodegenerative disorders among others[196,197].

A simplified view of chromatin recognizes two basic states: a relaxed chromatin state, transcriptionally competent **euchromatin** and a more condensed, transcriptionally silent **heterochromatin** (Figure 5B)[192,198]. The histone modification associated with euchromatin are high levels of acetylation and di- or trimethylation of H3K4, H3K36 and H3K79[198]. A large part of the genome presents a euchromatin state[187]. The histone modifications associated with heterochromatin are low levels of acetylation and high levels of H3K9, H3K27 and H4K20 methylation, among other histone covalent modification[198].

Different combinations of histone modifications in a specific genomic region, the so-called “histone code”, can change dynamically according to the cellular requirements in a particular moment to assist or block gene transcription[199]. There are several epigenetic modifier enzymes responsible for recognizing, catalyzing and removing histone modifications that induce a specific cellular response, popularly known as “readers”, “writers” and “erasers”, respectively (Figure

5C)[200]. The most well-known enzymes that modify histones are the histone acetyltransferases **(HATs)**, histone methyltransferases **(HMTs),** histone deacetylases **(HDACs)**, histone demethylases **(HDMs),** kinases and phosphatases, SUMO ligases and proteases, among others[187,201]. Interestingly, depending on the residue in which they are found, the type and the number of modifications are associated with an active or repressed state of genes.

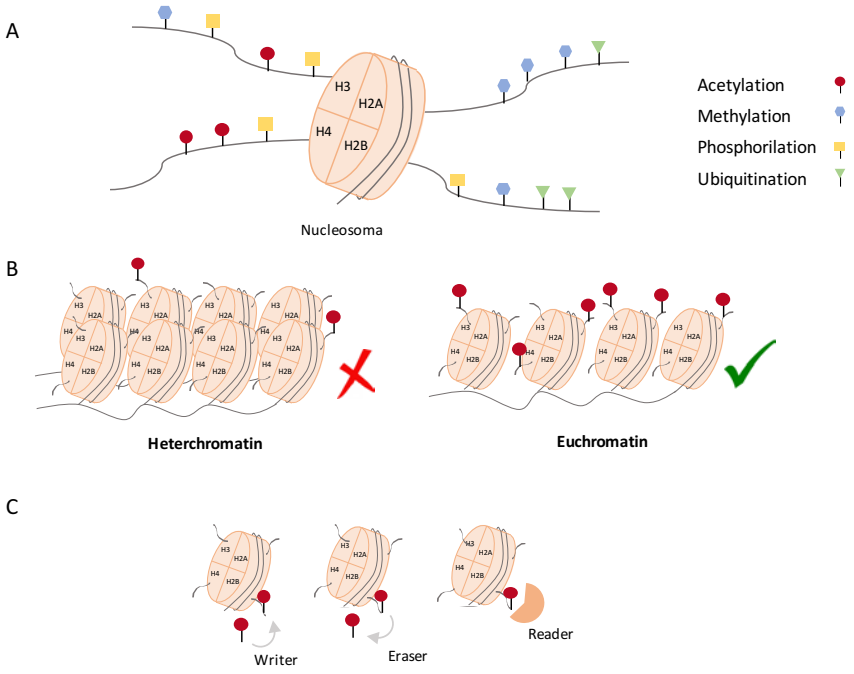

Figure 5. **Schematic representation of histone modifications.** **(A)** Nucleosome representation, which is composed of DNA wrapped around histones (H2A, H2B, H3 and H4). Core histone tails are subjected to post-transcriptional modifications (PTMs) mainly methylation, acetylation, phosphorylation and ubiquitination. **(B)** Chromatin presents two different states: the heterochromatin condensed state and transcriptional repression and euchromatin with an open state and transcription activation. **(C)** The epigenetic modifier enzymes are popularly known as "writers", "erasers" and "readers". The "writers" are the enzymes that add the histone mark, the "erasers" are those that remove the histone modifications and the "readers" role is to bind to a specific type or combination of histone modification and translate it into the biological function. Adapted from L. Simón-Riudalbas and M. Esteller 2015[178].

5.2.- DNA methylation

In 1975 Riggs and Holliday suggested that DNA methylation might play a role in gene expression regulation[202,203]. Nowadays is the most well-characterized covalent modification of DNA in humans[192].

DNA methylation entails the covalent addition of a methyl group from *S*-adenosyl-methionine (SAM) to the 5-carbon position of the cytosine ring (5-methylcytosine, 5mC; Figure 6). It occurs almost exclusively at cytosine residues that are followed immediately by guanine, the so-called **CpG dinucleotides**. This CpG dinucleotide tends to cluster in CpG-rich regions, frequently associated to gene promoters, called **CpG islands** (**CGIs**: regions of more than 200 bases with a G+C content of at least 50%)[204,205]. The human genome contains roughly 29,000 CGIs that are not randomly distributed in the human genome, but are preferentially found near the transcriptional start sites (TSSs) in the promoter and first exon regionsof approximately 72% of mammalian genes[206–208]. Although most of the epigenetic studies on DNA methylation and gene expression have focused on the CpG island regions, it is known that the DNA methylation does not occur exclusively in these specific sites. There are lower CpG density regions located a short distance from the CpG island (~2kb) called the **CpG island shores** (CGIs shore). Generally, the tissue-specific DNA methylation occurs on the CGIs shore and not the CpG islands[209,210]. The DNA methylation at the CGI and CGI shore is generally associated with transcriptional silencing of genes[207,211].

In healthy cells, DNA methylation plays a crucial role in cell growth and development through the regulation of germline- and tissue-specific genes. About 60% of human gene promoters are associated with CpG islands and are usually unmethylated and transcriptionally active. For instance, housekeeping or tumor suppressor genes[212]. However, some genes are customarily methylated, including those associated with X-chromosome inactivation or genomic imprinting[213]. Genomic imprinting is a particular form of transcriptional silencing associated with the hypermethylation of one of the parental alleles during the gametogenesis and maintained throughout the development[214,215]. Similar, mono-allelic

repression of genes also occurs on the X-chromosome in female cells in a process called the X-chromosome inactivation[216]. Besides controlling gene expression, the DNA methylation of repetitive genomic sequences prevents chromosomal instability, translocations and gene disruption by the reactivation of parasitic sequences[217].

The addition of a methyl group is carried out by **DNA methyltransferases (DNMTs)**. This epigenetic mark is inherited over the mitotic and meiotic cell division[218], through four main enzymes: DNMT1, DNMT3a, DNMT3b and DNMT3L[178]. Only DNMT1, 3a and 3b have methylase activity, DNMT3L acts as a cofactor of the previous ones. DNMT1 allows the "maintenance" of DNA methylation patterns through mitosis, from the parent strand to the daughter. It has the higher affinity for hemi-methylated double-strands DNA (methylated on one DNA strand) and restores the entire methylated CpG dinucleotides after DNA replications[219]. DNMT-3a and 3b carry out the *de novo* DNA methylation, crucial during mammalian development at very early stages[220]. All the DNMTs have a pivotal role and a lack will result in embryonic lethality or impaired embryonic development[221].

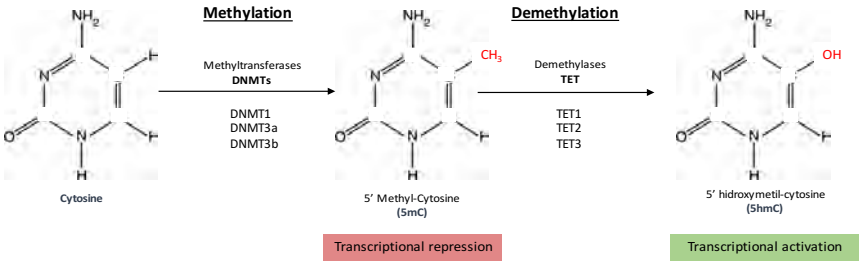

Figure 6. **DNA methylation in mammals.** DNA methylation entails the covalent addition of a methyl group from S-adenosyl-methionine (SAM) to the 5-carbon position of the cytosine ring (5-methylcytosine, 5mC) by the DNA methyltransferases (DNMTs). The DNA demethylation consists of the conversion of 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC) by the ten-eleven translocation proteins (TET). The 5mC is associated with a repressed chromatin structure and therefore transcriptional repression, whereas the 5hmC is associated with an open active chromatin structure and transcriptional activation. Adapted from Day and Sweatt 2010[222].

Contrary to the initial idea, DNA methylation is not a relatively stable epigenetic modification, and it can be actively reversed. This reversion process of the methylation state of the DNA is known as **DNA demethylation** and is necessary to maintain the balance of the DNA methylation level throughout the genome. The active DNA demethylation process in mammalian cells involves a set of reactions catalyzed by the **ten-eleven translocation (TET)** 1-3 (TET1, TET2 and TET3) family proteins that are 2-oxoglutarate and Fe(II)-dependent dioxygenases. The TET proteins possess an enzymatic activity that transforms the 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) through consecutive oxidation. The 5fC and 5acC can be converted back to cytosine by thymine DNA glycosylase (TDG) in base excision repair[223,224]. The role TET proteins have has recently been demonstrated in several physiological and pathological processes like leukemia[225].

In mammals, the DNA methylation in the CpG dinucleotide context has been described and characterized extensively. However, DNA methylation in a non-CpG context has also been identified. In humans, the CHG and CHH sites (where H is adenine, cytosine or thymine) were found to be exclusive for stem cells. Non-CpG methylation is lost while cells become differentiated and restored in induced pluripotent stem cells, suggesting an essential role in the origin and maintenance of pluripotent state[226].

The correlation between promoter CGIs hypermethylation and the transcriptional silencing in different cell types and its implication in cancer has been described[213,227,228]. According to the literature, this repression is based on the spatial impediment for the accessibility of transcription factors to their binding sites and on a decreased affinity of DNA to enroll into nucleosomes, changing the nucleosome positioning[229,230]. The first mechanisms involve the recruitment of the **methyl CpG-binding domain protein (MBDs)** family, which recognizes the 5-methyl CpG dinucleotide, translating the DNA methylation information into a function or activity[231]. This protein family is composed of the methyl binding protein 2 (MeCP2) and methyl-CpG binding domain protein 1-6: MBD1, MBD2,

MBD3, MBD4, MBD5, and MBD6[232]. Besides the MBD family, other proteins can preferentially or specifically bind methylated DNA through zinc-finger domains such as the Kaiso family (Kaiso/ZBTB33, ZBTB4 and ZBTB38) or the ring finger domain protein 1 (UHRF1)[233–235]. Despite MBD3, MBD5 and MBD6 presenting a conserved methyl binding domain, they are unable to bind efficiently to methylated DNA *in vitro*, unlike MeCP2, MBD2 and UHRF1 which bind strongly to methylated CpG dinucleotides[233]. The MBD family members recruit histone-modifying enzymes such as HDACs or HMTs and other co-repressors to methylated sites in order to change the chromatin structure into more condensed form[178]. Mutations in the MBD family are related to different disorders like MeCP2 and are the cause of Rett syndrome (RTT)[236].

In conclusion, the demethylation process and *de novo* DNA methylation can occur, and different specific cellular processes can be activated or not in response to physiological or environmental stimuli[237]. As previously mentioned, disruption of these DNA methylation patterns in developmental programming or during adult life have been linked to aging and contributing to the development of human diseases including neurodevelopmental (RETT- and ATRX syndrome), neurological (Parkinson's and Huntington's disease), autoimmune (immunodeficiency, centromeric instability and facial anomalies), lupus erythematosus or rheumatoid arthritis disorders as well as cancer [238–241].

5.3.- Non-coding RNAs

In late 1960 it was discovered that approximately less than 2% of the sequence in the entire human genome is translated into protein. Indeed 98% of the transcriptional output has non-coding potential[242]. Historically, these non-coding regions were thought to be non-functional, and they were called "junk DNA" or experimental artifact[243]. In recent years, the "junk DNA" concept has changed a lot, and the RNA that does not encode for a protein product is classified as a putative non-coding RNA (ncRNA), with an essential role in both physiological and pathological conditions[244–246]. Furthermore, the strong correlation between the organismal complexity and size of the non-coding, rather

than protein-coding genome, highlighted their relevance[247]. This idea was reinforced by the National Human Genome Research Institute (NHGRI) which launched the Encyclopedia of DNA Elements (ENCODE) which assigns "biochemical functions for 80% of the genome", in particular, outside of the well-studied protein-coding regions[248].

As explained, for a number of years it had been generally assumed that genes were synonymous with proteins except for those that are required, directly or indirectly, for messenger RNA (mRNA) processing and translation, such as ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), and small nuclear (spliceosomal) RNAs[249,250]. Since this idea has now been identified as erroneous, the number of new putative functional ncRNAs that regulate gene expression in different ways has dramatically expanded[251]. The ncRNA has been divided into two main groups (excluding the rRNAs and tRNAs) based on their length: the **small non-coding RNAs (sncRNAs)**, less than 200 nucleotides in length and the **long non-coding RNAs** (**lncRNAs**), which are typically more than 200 nucleotides long[252]. The **sncRNAs** are divided into different subclasses and characterized by different lengths: small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), piwi-interacting RNAs (piRNAs), small interfering RNAs (siRNAs) and, to date, the most extensively studied are the microRNAs (miRNAs)[192]. These specific ncRNAs are involved in transcriptional and post-transcriptional gene silencing through specific base pairing with their targets[253]. The **lncRNAs** comprise a limited but fast-growing number of lncRNAs such as long intergenic RNAs (lincRNAs), natural antisense transcripts (NATs), telomeric repeat-containing RNAs (TERRA), transcribed ultraconserved regions (T-UCRs), and enhancer RNAs (eRNAs; reviewed in [254]). The lncRNAs play critical regulatory roles in different cellular processes like the epigenetic modification of DNA by recruiting chromatin remodeling complexes to specific loci[255,256].

5.4.- Interplay between the components of the epigenetic machinery

There is extensive and coordinated crosstalk among the different epigenetic processes to precisely regulate the expression of the genome, forming a complex "epigenetic network" through the entire cell[257]. This complex network can regulate the "on" or "off" of several genes implicated in multiple cellular process[258].

During cell division, it is essential to keep the pattern of histone modification, and there is some evidence that DNA methylation plays a critical role in this process[259,260]. This methylation pattern is maintained in mammals by DNMT1 aided by UHRF1. The UHRF1 presents different structural motifs such as the SET- and RING-associated domain (SRA) or the tandem tudor domain (TTD) that allows it to establish a relationship between DNA methylation and different histone tails[234,261–263]. As explained before, another interaction between the DNA methylation process and the chromatin modifying enzymes might be partially mediated by the MBD proteins, such as MeCP2 and MBD2, which can recruit HDACs and induce the transcriptional gene's repression[264–267]. Furthermore, ncRNAs can bind and recruit histone modifying complexes or modulate the activity of DNA methyltransferases, regulating gene expressing[252,268]. For example, the lncRNA *XIST* is involved in chromosome X inactivation in females through the interaction with methyltransferase and histone ubiquitinase, and lncRNA *HOTAIR* represses genes by recruiting the histone methyltransferase PRC2[269–271].

Interestingly, DNA methylation and histone modifications localized in special ncRNAs sequences often participate in the expression regulation of these ncRNAs[272,273]. For example, DNA hypermethylation of miRNA-874 or the miR-200 family, a critical EMT regulator, has been identified as being hypermethylated in breast and colorectal tumors, respectively[274–276]. The list of miRNAs undergoing promoter CpG island hypermethylation in human cancer is rapidly expanding[277].

5.5.- Epigenetic drugs

In contrast to genetic mutations, epigenetic alterations are reversible, either in physiological context or by treatment with DNMT or HDAC inhibitors, allowing the restoration of the normal epigenetic landscape in cancer cells[199,278,279]. To our knowledge, seven epigenetic drugs have been approved by FDA. The demethylating agents **5-Aza-2'-deoxycytidine** (Dacogen®) and **5-Azacytidine** (Vidaza®) were approved for myelodysplastic syndrome (MDS). Both agents block DNMT activity, returning to normal levels after successive replication rounds[280,281]. Currently, these agents are being tested in an extensive list of clinical trials for leukemia and solid tumors[282]. **Vorinostat** (Zolinza®) or **SAHA, belinostat** (Beleodaq®), **romidepsin** (Istodax®) and **panobinostat** (Farydak®) HDAC inhibitors are approved for cutaneous T cell lymphoma (CTCL) and/or peripheral T cell lymphoma (PTCL) and multiple myeloma (MM)[283–285]. Finally, **ruxolitinib** (Jakafi®) a JAK1/2 inhibitor has been approved for myelofibrosis[283]. DNMT inhibitors present some cytotoxicity to normal cycling cells, causing a decrease in methylation levels, which can reactivate genes at random such as genes with deleterious effects. Therefore, they are usually used in low doses[286]. HDAC inhibitor treatment as single agents in solid tumors has been shown to have low therapeutic effects, probably due to the stability of the inhibitors. Therefore, they are now being tested in combination with DNMTi[284,285].

6.- DNA METHYLATION AND CANCER

The epigenetic equilibrium described for normal cells is dramatically disturbed during tumorigenesis. The piece of first evidence of the role DNA methylation played in cancer was in 1983 when a reduction in DNA methylation of specific genes in human colon cancer was identified when compared with normal tissues[287]. During tumor progression, the specific DNA methylation pattern of cancer cells can change according to the original tissue and tumor stages[288]. Hence, DNA methylation is considered a hallmark of cancer[289].

Generally, the methylated pattern of cancer cells is characterized by a global decrease in methylated CpG content (**hypomethylation**) and CGI

hypermethylation in promoter regions of specific genes (Figure 7)[227,290,291]. The discovery of DNA methylation as a mechanism for gene inactivation required the re-formulation of the Knudson hypothesis, which explains the necessity of two mutations (*two hits*) for gene inactivation to include the epigenetic inactivation as a *second hit*[292,293].

6.1.- DNA hypomethylation

Global DNA hypomethylation was one of the first epigenetic abnormalities discovered in cancer[287]. This overall loss of methylation is observed in the early stages of cancer, and increases in parallel with the development of the tumor, from a benign proliferation of cells to an invasive cancer[294].

Global hypomethylation occurs mainly at repetitive sequences, promoting chromosomal instability, translocations, gene disruption and the reactivation of endoparasitic sequences[291,295,296]. A well-studied example of the **reactivation of transposable elements** by CpG island hypomethylation is the member 1 of the long interspersed nucleotide elements LINE family (LINE-1 or L1). Under normal conditions, *LINE-1* is often heavily methylated in normal somatic cells and contributes to maintaining genomic stability and integrity[35]. In cancer, the loss of *LINE-1* methylation has been described as increasing genomic instability, which is frequently found in different cancers including colorectal[297,297], urothelial[298], ovarian[299], prostate[300], breast, lung ,bladder and liver[301,178].

Furthermore, the aberrant DNA hypomethylation can also account for the **activation of some oncogenes** and lead to **loss of imprinting (LOI)**[293]. Several genes or oncogenes have been described to undergo this epigenetic re-expression. The first hypomethylated oncogene was the related RAS viral oncogene homolog (*R-RAS)* discovered in 1983 in gastric cancer[302]. Since then, the association between hypomethylation and overexpression has been demonstrated in different genes such as the S100 calcium binding protein A4 (*S100A4)* in colon cancer[303]. Currently, the number of genes identified with this hypomethylation pattern is

shorter when compared to those genes inactivated by DNA hypermethylation. However, the gene reactivation process is a key factor during carcinogenesis.

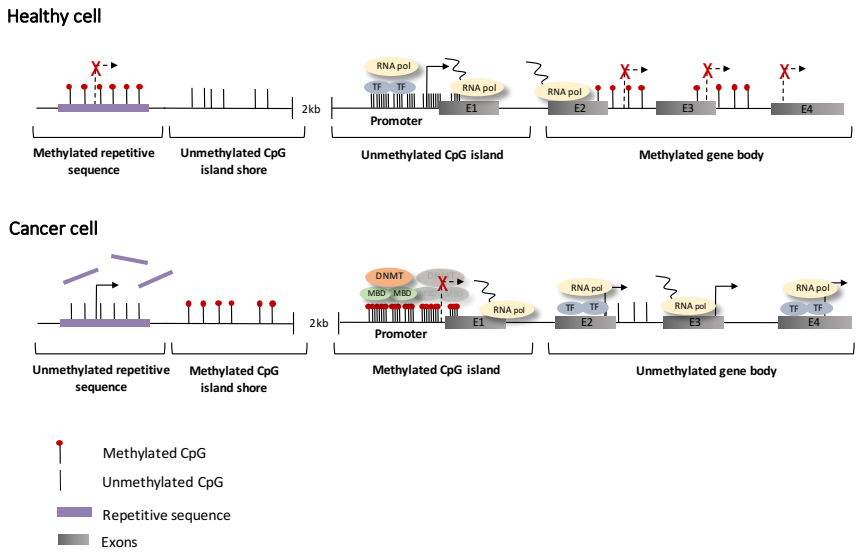

Figure 7. DNA methylation patterns. Schematic representation of healthy and malignant cell methylation patterns are represented. In healthy cells, the CpG islands in the promoter region are usually unmethylated, allowing transcription while the gene body is methylated. The same pattern is observed for island shores located up to 2Kb upstream from the CpG island. In contrast, cancer cells are characterized by global hypomethylation and promoter hypermethylation. The repetitive sequence hypomethylation leads to chromosomal instability, translocations and the activiation of repetitive sequences generally repressed by hypermethylation. The legend is in the lower left panel of the image. Adapted from Portela and Esteller 2010[178].

Genomic hypomethylation can also induce **LOI,** activating the transcription of maternal or parental imprinted loci. The most common LOI event in cancer is the hypomethylation of the insulin-like growth factor 2 (*IGF2*), necessary for embryonic development in humans. A constitutive loss of *IGF2* imprinting has been observed in different cancers such as colorectal, breast and prostate, among others[304–310].

6.2.- DNA hypermethylation

DNA hypermethylation is not a random process. In cancer, the CGI promoter hypermethylation is involved in tumor suppressor inactivation, affecting different cellular pathways. In general, this gene repression results in an adaptive

advantage for the cells, allowing them to adopt a more aggressive and invasive phenotype[311].

The first promoter hypermethylated gene discovered in human cancer was the calcitonin gene in 1986[312]. However, a link between CpG island promoter hypermethylation and transcriptional inactivation was not found until 1989 during the analyses of the retinoblastoma gene (*RB1*)[313]. Nowadays, the DNA hypermethylation of CGI and CpG shores is the most widely studied epigenetic alteration in cancer[178,293]. A large number of tumor suppressor genes are known to be inactivated by DNA promoter hypermethylation, causing alterations in different cellular processes including the cell cycle (*CDKN2A/p16-INK4, CDKN2B/p15-INK4B, RB1)*, apoptosis (*TMS1, DAPK, SFRP1, TP73*), DNA repair (*hMLH1, MGMT, BRCA1),* or cell adhesion (*E-cadherin, H-cadherin, FAT-cadherin)* (reviewed in [314]). Interestingly, the same tumor suppressor gene may be hypermethylated or hypomethylated in different types of tumor. For example, *MASPIN* (or *SERPINB5*) is a tumor suppressor gene that could be hypermethylated in breast and prostate cancer and hypomethylated in other tumor types[315,316].

In BC, more than 100 genes have been reported to be hypermethylated, of which two are the well-known genes *BRCA1* or *PTEN*[317]. The BC susceptibility *BRCA1* gene is a classical tumor suppressor gene implicated in DNA repair, homologous recombination, cell cycle control and transcription[318,319]. *BRCA1* inactivation by promoter methylation has been identified in breast and ovarian cancer[320]. Specifically, *BRCA1* inactivation was significantly associated with basal-like/TNBC[321,322]. Another hypermethylated tumor suppressor gene described in BC is *PTEN*[323]. This gene regulates negatively the PI3K/AKT pathway. Therefore, a decrease in *PTEN* expression produces the AKT pathway activation which, in turn, induces apoptosis inhibition and cell survival[324]. Ras association domain family member 1 *(RASSF1A)* is another tumor suppressor gene associated with BC development as well as among other cancers[325,326].

The disruption of the epigenetic machineries by mutations, deletion or altered expression of any of their components are also involved in aberrant gene expression patterns in cancer[293]. Different DNMT mutations have been identified in cancer such as DNMT1 in colorectal cancer[327] and DNMT3a in acute myeloid leukemia[328]. Apart from these enzymes' mutations, DNMT overexpression has also been described as a mechanism for aberrant methylation pattern in cancer[329,330]. For example, DNMT1 overexpression in colorectal[331], lung[332] and breast cancer[333], DNMT1, DNMT3a and DNMT3b overexpression in gastric cancer[334,335]or DNMT3b overexpression in BC[336]. Some MBD polymorphisms have also been reported to be associated with cancer risk. For instance, while MBD1 polymorphism increases the overall risk of lung cancer it, on the other hand, reduced the risk of BC in premenopausal women[337,338]. *TET* overexpression or downregulation has been observed in different cancers such as breast[339,340], liver[339,341], colon[342], lung[339] and gastric[339,343], among others[339]. Furthermore, chromosomal deletions has been described in *TET2* locus, producing an inefficient conversion of 5mC to 5hmC in myeloid malignancies[344,345].

In conclusion, this epigenetic mechanism plays an important role in the transcriptional regulation of critical tumor suppressor and growth regulatory genes in cancer.

6.3.- Genome-wide DNA methylation in cancer

Since DNA methylation plays an essential role in both physiologic and pathologic conditions, the significance of profiling DNA marks to answer biological questions is being emphasized. Moreover, DNA methylation marks are modifiable through pharmacological intervention and easy to measure in liquid biopsies, accentuating further the importance of epigenetic mark study for translational research[346,347]. As such, the interest in DNA methylation has rapidly grown and expanded across new areas of research thanks to the advancement of various profiling approaches, both experimental and computational[348,349]. Methylation analysis techniques have evolved from being able to analyze the amount of 5mC within a particular genome in the

early 1980s, to today's genome-wide DNA methylation analysis thanks to new technologies such as microarrays and next-generation-sequencing (NSG). These technologies allow for DNA methylomes (the sequencing of all the methylated nucleotides of an organism's genome or particular cell) in higher organisms to be analyzed[351,352].

In conclusion, the development of new epigenetic approaches appears to be highly promising for decoding the nature and patterns of DNA modifications, their distribution in the human genome, as well as their implications in different physiologic and pathologic processes. Moreover, DNA methylation analysis is also enormously important for the development of personalized epigenomic-based therapy[352].

7.- DNA METHYLATION AND TREATMENT RESISTANCE IN CANCER

Standard care for tumor treatment is based on conventional and target therapies. It is generally accepted that recent advances in anticancer drugs have contributed significantly to improving disease-free survival and the quality of life of cancer patients. However, in many cases patients developed a resistance to these drugs within a few years. As explained earlier, the molecular mechanisms that lead to drug resistance can be heterogeneous and complex[353]. However, by analyzing epigenetic profiles, tumor-specific drug-response markers that are capable of predicting response to therapeutic treatment can be identified[354–357]. Therefore, many efforts are focused on studying the epigenetic patterns that occur during the acquisition of the drug-resistant phenotype[213].

Drug treatment induces epigenetic changes that can affect different pathways such as those related to apoptosis, cell adhesion, angiogenesis, cell-cycle regulation and DNA repair[199]. The best example of an epigenetic biomarker indicating a response to a chemotherapeutic agent is the hypermethylation of the DNA repair gene O^6-methylguanine-DNA methyltransferase (*MGMT*) in gliomas. MGMT protects cells against transition mutations by removing alkyl groups, which are introduced by carcinogens such as nitrosamides from guanine

bases. In cancer, *MGMT* hypermethylation produces a deactivation of this mechanism, which then leads to a greater sensitivity to the alkylating agents in gliomas[351]. Furthermore, *MGMT* hypermethylation has also been described in non-small cell lung cancer (NSCLC), lymphomas and head and neck carcinomas[358–360]. In another instance, the promoter hypermethylation of mutL homologue 1 (*hMLH1)*, leads to an increase in chemotherapeutic drug resistance (cisplatin and carboplatin) in ovarian cancer[361]. TNF-related apoptosis-inducing ligand (*TRAIL*) is a further another example of how promoter hypermethylation can trigger treatment resistance in lung cancer[362]. Currently there is a long list of genes with aberrant DNA methylation patterns related to resistance or sensitivity to drug treatment. Table 4 provides a list of examples of methylated genes involved in treatment response. In breast and ovarian cancer, the promoter hypermethylation of *BRCA1,* a gene involved in DNA repair, has been associated with an increase in sensitivity to cisplatin and carboplatin treatment[355,356,363]. More recently, the association between hydroxysteroid 17-beta dehydrogenase 4 (*HSD17B4)* promoter methylation and trastuzumab response in HER2+ BC was also identified. The promoter hypermethylation of *HSD17B4* could be used as a marker to select HER2+ BC patients who would achieve pCR only through trastuzumab therapy and might not need additional surgery[364].

Table 4. Examples of hypermethylated genes involved in sensitivity and resistance to drug treatment. Adapted from Heyn and Esteller 2012[288].

	Gene	Treatment	Cancer	Example refs
Sensitivity	***BRCA1***	PARP inhibitors and alkylating agents	Breast and ovary	355,356,365
	MGMT	Temozolomide, BCNU, ACNU, procarbazine	Glioma, colon, lung, lymphoma, and heat and neck carcinomas	351,358–360
	GSTP1	Doxorubicin	Prostate, breast and kidney	366
	CHFR	Paclitaxel and docetaxel	Ovary, endometrium and stomach	367–369
	ABCB1	Doxorubicin	Breast	370
Resistance	***MLH1***	Cisplatin	Colon, stomach, endometrium and ovary	354,361,369,371
	SLC19A1	Methotrexate	Lymphomas	372
	PITX2	Tamoxifen	Breast	357
	IGBP3	Cisplatin	Lung and colon	373,374
	LINE-1	Fluoropyrimidines	Colon	375

BRCA1, breast cancer 1*; MGMT*, O^6-methylguanine-DNA methyltransferase; *GSTP1*, glutathione S-transferase pi 1; *CHFR*, checkpoint with forkhead and ring finger domains; *ABCB1*, ATP-binding cassette subfamily B member 1; *MLH1*, mutL homologue 1; *SLC19A1*, solute carrier family 19; *PITX2*, paired-like homeodomain 2; *IGBP3*, insulin-like growth factor binding protein 3; *LINE-1*, member 1 of long interspersed nucleotide elements LINE family, BCNU, bis-chloroethylnitrosourea; ACNU (1-4-amino-2-methyl-5-pyrimidinyl)-methyl-3-(2-chloroethyl)-3nitrosourea hydrochloride.

8.- DNA METHYLATION BIOMARKERS IN CANCER

In 1998, the National Institutes of Health Biomarkers Definition Working Group defined a biomarker as "*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic process or pharmacologic response to a therapeutic intervention*"[376]. While different factors can be used as a biomarker, they are mainly proteins or metabolites, DNA,

mRNA and epigenetic alterations, which can be found in various fluids (e.g., blood or sputum), frozen tissues, formalin-fixed paraffin-embedded tissues (FFPE) and cell lines[363,377,378].

For a biomarker to be considered clinically useful, it needs to be very sensitive and specific. In clinical terms, biomarker sensitivity is described as the proportion of individuals with confirmed disease i.e., those, who present a positive biomarker screening test. Biomarker specificity is described as the proportion of control subjects for whom the screening test is negative. Hence, the optimum sensitivity is when the number of false negative approaches zero and the optimum specificity is when the number of false positives is low[379]. Bigbee W and Herberman R, described the general requirements that a biomarker must fulfill such as (i) specific production by malignant or premalignant tissue early in the progression of disease, (ii) produced at detectable levels in all patients with the a specific malignancy, (iii) expression in an organ site-specific manner, (iv) evidence of presence in bodily fluids obtained noninvasively or in easily accessible tissue, (v) levels related quantitatively to tumor volume, biological behavior, or disease progression, (vi) relatively short half-life, reflecting temporal changes in tumor burden and response to therapy and (vii) standardized and reproducible detection methodologies[380].

Biomarkers can be classified into several categories according to their potential for **diagnosis** (identification and categorization of disease), for **prognostication** (assessment of outcome), to **predict response to treatment** (identification of patient subsets which are more likely to respond to a certain type of therapy) and to **monitor disease** (identification of disease relapse during follow-up)[377].

Currently, the epigenetics filed are revolutionizing biomedicine and clinical diagnosis, contributing to the development of personalized and precision medicine by providing special relevance biomarkers for the clinical management of diseases. Their stability, frequency, reversibility and accessibility in body fluids make epigenetic alterations major candidates for becoming clinically useful

biomarkers[381]. Furthermore, thanks to the new technologies able to detect DNA methylation changes in different biological fluids, this study area is now considered extremely promising for the field of precision oncology in the foreseeable future[199,288]. In recent decades, a large number of epigenetic biomarkers have already been identified in cancer.

Below, some biomarkers with clinical applicability have been outlined. The glutathione S-transferase pi 1 (*GSTP1*) is the best DNA methylation marker for prostate **cancer detection**[382]. *GSTP1* hypermethylation was identified in 80-90% of men with prostate cancer, with an 82% sensitivity and an a 95% specificity[381,383]. *GSTP1* can be evaluated in urine and can discriminate between the different degrees of tumor malignancy analyzed[384]. However, it is hypermethylated at a lower percentage in other tumor types like breast, liver and kidney[382]. In BC detection, promoter hypermethylation of some tumor suppressor genes such as *RASSF1A, BRCA1* or *PTEN* has been described[385–388]. Moreover, other genes like *RARβ, CDH1, GSTP1* or *BRCA1* were identified in different studies and showed reasonably consistent results (reviewed in[389]). Several epigenetic biomarkers have been described to indicate **disease prognosis** or **outcome** in cancer patients. Some examples are *NSD1, DAPK1* or *CDKN2A* hypermethylation, which are related to a poor outcome in neuroblastoma, lung and colorectal cancer, respectively[293,390–392]. In BC, the methylation of *RASSF1A* in plasma and *PITX2* promoter hypermethylation assessed in tissue or blood have also demonstrated their potential as prognostic biomarkers[357,393]. As explained in the last section (*7.- DNA methylation and treatment resistance in cancer*), some biomarkers can **predict treatment response** (see Table 4). The most well-known examples are *MGMT* and *GSTP1*, as explained before[288,351,382,383]. In BC, the methylation of *PSAT1* (predicts response to tamoxifen) or *BRCA1* (predicts response to the PARP inhibitor) have also been identified[365,394,395].

All these epigenetic biomarkers not only allow us to increase the sensitivity and specificity of cancer detection, but also to develop a more personalized treatment as well[288].

8.1.- DNA methylation biomarkers in clinical practice

A PubMed Central database search (24th October 2018) shows 1844 articles for the term “epigenetic biomarkers” and 915 for “DNA methylation biomarkers” (Figure 8). A long list of articles providing new potential biomarkers for a wide range of diseases and disorders can be found. However, the number of epigenetic biomarkers accepted and implemented in clinical practice is relatively small. According to the *ClinicalTrials.gov (https://clinicaltrials.gov)*, there are currently 12 ongoing trials related to “DNA methylation biomarkers”. The limitations of measurement methods such as study design, small sample sizes and lack of independent validation, are some explanations that allow us to understand the gap between the number of promising biomarkers reported and their actual application in the clinic[396].

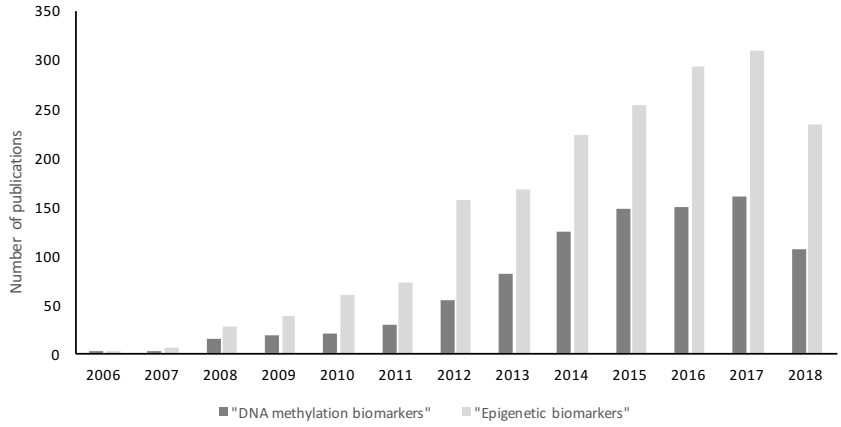

Figure 8. **Rate of increase in publications related to “Epigenetic biomarkers” and “DNA methylation biomarkers” from 2006 to 2018.** Data are derived from PubMed Central’s citation analysis using the terms “Epigenetic biomarkers” and “DNA methylation biomarkers” in the 2006-2018 period.

The most well-established biomarkers to date include the hypermethylation of *MGMT* in serum, used in the clinic as a biomarker for glioblastoma classification and in the decision of treatment[288]. Currently, the Septin 9 (*SEPT9*) methylation has received approval from the FDA for use as a blood methylated biomarker for colorectal cancer in Epi proColon® plasma test (Epigenomics AG)[397–399]. Recently, the EPICUP® an epigenetic diagnostic test has been commercialized,

which is based on the analysis of DNA methylation profiles in tumor samples with utility to identify the primary tumor in patients with cancer of unknown origin).

Chapter II: Hypothesis and Objectives

HYPOTHESIS

Primary resistance and the acquisition of secondary resistance are the main clinical problems encountered when treating patients with HER2+ BC. Many cellular defects contribute to treatment resistance, but epigenetic changes can also be a cause. Analyzing the DNA methylation pattern in HER2+ resistant BC could be useful for a more detailed understanding of the changes in resistance event and in identifying potential epigenetic biomarkers of therapeutic response in HER2+ BC; something essential for precision oncology.

OBJECTIVES

The main objective of this thesis was to evaluate the DNA methylation patterns of the trastuzumab, lapatinib and trastuzumab *plus* lapatinib HER2+ resistant breast cancer model. And the ultimate objective was to identify epigenetically regulated genes with potential clinical value as biomarkers for trastuzumab and lapatinib resistance in HER2+ BC patients.

In order to fulfill the main objective, three specific objectives were pursued:

1. Genome-wide DNA methylation analysis in trastuzumab, lapatinib and trastuzumab *plus* lapatinib-sensitive and -resistant breast cancer models.

- Develop and characterize a new HER2+ cell line resistant to both trastuzumab and lapatinib treatments (TL).

- Analyze the DNA methylation pattern for trastuzumab (T), lapatinib (L) and trastuzumab *plus* lapatinib (TL)-sensitive and -resistant HER2+ breast cancer models.

- Carry out transcriptomic analysis of trastuzumab (T), lapatinib (L) and trastuzumab *plus* lapatinib (TL)- sensitive and -resistant HER2+ breast cancer models.

2. Identification of genes responsible for trastuzumab, lapatinib and trastuzumab *plus* lapatinib resistance in our HER2+ resistant breast cancer models.

- Identify which genes epigenetically regulated by DNA promoter methylation in trastuzumab (T), lapatinib (L) and trastuzumab *plus* lapatinib (TL)-resistant models.

- Validate the DNA promoter methylation and expression patterns of selected genes using different epigenetic approaches.

- Determine the potential role of candidate genes in trastuzumab or lapatinib resistance in our *in vitro* models.

3. Biomarker candidate gene validation in HER2+ breast cancer human samples.

- Evaluate the DNA promoter methylation of candidate genes in a cohort of HER2+ breast cancer patients before and after anthracycline-taxane-based chemotherapy *plus* trastuzumab with complete or non-response to the treatment.

- Evaluate the diagnostic value of potential biomarker candidates in our cohort.

- Determine the association between the DNA methylation status of selected genes and their clinical-histopathological features in HER2+ BC.

Chapter III: Materials and Methods

1.- CELL CULTURE AND HUMAN HER2+ BREAST CANCER SAMPLES

1.1.- Cell culture

SKBr3 (SK) and AU565 (AU) HER2+ breast carcinoma cells were obtained from Eucellbank (University of Barcelona)[400] and from the American Type Culture Collection (ATCC), respectively. SKBr3 and AU565 cells were routinely grown in McCoy's (Gibco) and Dulbecco's Modified Eagle's Medium (DMEM, Gibco), respectively, supplemented with 10% FBS (HyClone Laboratories), 1% L-glutamine (Gibco), 1% sodium pyruvate (Gibco), 100 U/mL penicillin/streptomycin (HyClone Laboratories). Cells lines were kept at 37ºC and 5% CO_2 atmosphere. The resistant models (SKTR, SKLR and AUTR) were developed and characterized previously in our laboratory[401,402]. The double-resistant cell model (SKTLR) was developed later using the SKTR model co-cultured with trastuzumab 2μM and lapatinib 1.5μM and after one month in culture, the dose of lapatinib was increased up to 3μM. Cells were co-cultured with trastuzumab and lapatinib for 12 months. Trastuzumab *plus* lapatinib resistance was confirmed by dose-response studies using the standard colorimetric MTT assays as we described in *6.7. Cell viability assays* section.

1.2.- Patients and tissue samples

TGFBI promoter methylation levels were retrospectively evaluated in tumor samples from 24 patients with HER2+ BC diagnosed at the Dr. Josep Trueta University Hospital, Girona, Spain between 2007-2015. The patients were selected from the hospital's pharmacy database. The selection criterion included: a patient with early or locally advanced HER2+ BC who had received neoadjuvant treatment with trastuzumab and chemotherapy. Twenty patients had no-response or partial response and four patients had complete response to trastuzumab *plus* chemotherapy. For all patients, hematoxylin and eosin (H&E) stained slides from FFPE tumor blocks were examined to determine the representative areas of the invasive tumor. Normal breast tissue was obtained from non-tumoral regions of the breast. Estrogen receptor (ER), progesterone receptor (PR) and HER2 expression were previously analyzed on

tumors by IHC. For each patient, clinical and histopathological features were obtained: age, stage (TNM classification[403]), histological grade (Bloom-Richarson grading system), menopause status, type of surgery and relapse. The protocol was approved by the Institutional Review Board of the Dr. Josep Trueta Hospital and an informed written consent was obtained from the patients included in the study.

2.- DNA METHYLATION ANALYSIS

2.1.- DNA isolation procedures

Genomic DNA was extracted from cell lines or FFPE core biopsies (10μm) and tissue sections (5μm). Tissues section from each tumor block were collected on a coverslip glass or directly to sterile 1.5mL microcentrifuge tubes. The sections collected in coverslips were attempted to remove the surrounding paraffin as much as possible before proceeding to deparaffinization process. The sections were deparaffinated using the Deparaffinization Solution with QIAamp DNA FFPE Tissue Kit (Qiagen) used following the manufacturer's instructions. The cell DNA was isolated using the QIAamp DNA Mini Kit. Total DNA was quantified using the NanoDrop 2000 spectrophotometer (Thermo Scientific) or Qubit 2.0 (Invitrogen) using the Quant-iTTM Pico Green Kit (Invitrogen) following the manufacturer's specifications.

2.2.- Demethylating agent treatment

To determine the correlation between DNA methylation and mRNA expression of selected genes, the tumor cell lines that showed hypermethylation of candidate genes were treated with demethylating agent 5-aza-2'-deoxycytidine (5-aza-dC; Sigma-Aldrich). AZA is a cytosine analog which is incorporated into the DNA forming an irreversible covalent complex with DNMT1. In turn, this union causes the DNMT depletion in the DNA replication, triggering passive demethylation and genes reactivation[404]. Cell lines were incubated in culture medium containing 3μM or 5μM of 5-aza-dC for 72 hours, medium and demethylating agent were replaced every day to promote DNA demethylation.

2.3.- DNA bisulfite conversion

Bisulfite-modified genomic DNA allows translating the methylation event to a genetic change. This specific treatment induces chemical conversion of unmethylated cytosine but not methylated cytosine[77]. Therefore, for the unmethylated DNA, the resultant sequence after DNA treated with sodium bisulfite presents uracil, which is converted to thymine after PCR cycle, instead of the cytosine of the original sequence. For the methylated DNA sequence, the cytosine would remain unchanged after treatment. In all cases, 500ng or all extracted DNA was chemically modified to convert all unmethylated cytosine to uracil by EZ DNA Methylation-Gold Kit (Zymo Research) according to the manufacturer's recommendations. The DNA was incubated for 16 hours at 50ºC through different denaturalization phases every hour. The DNA-bs was eluted at a final concentration of 20ng/μL.

2.4.- Genome-wide DNA methylation array

Whole-genome DNA methylation was analyzed in trastuzumab-sensitive and -resistant models using the Illumina Infinium HumanMethylation450 BeadChip (450K; Illumina Inc., CA, USA) as previously described[277]. The Infinium450K interrogates the methylation status of more than 450.000 CpGs through the genome. It covers 96% of the CpG island, with additional coverage in island shores and the regions flanking them. Interestingly, the 99% of the genes annotated in the NCBI Reference Sequence database (RefSeq) are covered in all its regions (5'UTR, promoter, gene body and 3'UTR). The annotation of the CG island (CGIs) used the following categorization: (i) *Shore*, for each of the 2-kb sequences flanking a CGI; (ii) *shelf*, for each of the 2-kb sequences next to a shore; and (iii) *opean sea*, for DNA not included in any of the previous sequences or in CGIs[277]. In relation to their position over the gene, probes can be classified in: (i) inside the promoter (TSS1500/TSS200), if they are localized between 1500pb upstream and transcription initiation side; (ii) in the 5'UTR region; (iii) in the 3'UTR region; (iv) in gene body and (v) intragenic regions.

A total of 500ng of bisulfite-converted DNA from SK and SKTR models were used to hybridize on Infinium Human Methylation 450 BeadChip, following

the Illumina Infinium HD Methylation protocol. Briefly, samples were whole-genome amplified step followed by enzymatic end-point fragmentation, precipitation and resuspension. The resuspended samples were hybridized on HumanMethylation 450 BeadChips at 48ºC for 16 hours and then, the unhybridized and non-specifically hybridized DNA were washed away. After hybridization, a single nucleotide extension using the hybridized bisulfite-treated DNA as a template was performed. The nucelotides incorporated were labeled with biotin (ddCTP and ddGTP) and 2,4-dinitrophenol (DNP; ddATP and ddTTP). Finally, Illumina iScan System, scans the BeadChip, at two wavelengths (532nm/660nm). The methylated (green) and unmethylated (red) signals are generated in different color channels. The methylation level of each cytosine was expressed as a β-value, which represents ratios of the fluorescence intensity of the methylated to the unmethylated version of the probes. It is expressed as a continuous variable that ranges from 0 (unmethylated) to 1 (fully methylated) according to a combination of the two fluorescence intensities (Cy3 and Cy5 fluorescence). Color balance adjustment and normalization were performed to normalize the samples between the two-color channels. β-values with a detection *p*-value>0.01 are considered to fall below the minimum intensity and threshold, and these values were consequently removed from further analysis. Additionally, the probes that were localized to the sex chromosomes and those CpGs that contain single-nucleotide polymorphisms (SNPs) were filtered out. For their analysis, the samples were grouped according to the sensitivity or resistance to lapatinib and trastuzumab, and the medians of their methylation values were calculated for each probe. To carry out the comparisons between groups, the differences between methylation medians by groups were calculated, and those with the highest changes (≥60%) were selected. By the analysis false discovery rate (FDR) below 5% were considered statistically significant. Data were deposited into the NCBI Gene Omnibus.

2.5.- Bisulfite Pyrosequencing

DNA methylation of selected genes was analyzed in our cellular models and human samples by pyrosequencing. This methylation analysis method quantifies the degree of methylation status of CpG positions in a short fragment of

amplified DNA after bisulfite treatment. The procedure can be divided into two major steps. To generate a sufficient amount of template bisulfite-treated DNA extracted from cell lines and FFPE samples was amplified by PCR under standard conditions to convert the PCR product to single-stranded DNA templates, using a pair of primers in which one of them is biotinylated. A large number of amplification cycles are needed (50) to exhaust de primers and prevent the interference of biotinylation with the subsequent sequencing reaction. PCR products were verified on 2% agarose gel (Conda agarose in TBE (Tris-Borate Edta) 0.5x) and SYBER Safe before pyrosequencing analyze. The single-stranded DNA template is added to sepharose beads and a subsequent alkali treatment, with the aim to remove salts that inhibit the subsequent enzymatic reaction. This preparation step is carried out with Vaccum Prep Tool (Biotage, Sweden), which captures the beads and holds them during the different purification steps. Pyrosequencing reactions were performed in a PyroMark Q96 System version 2.0.6 (Qiagen) using appropriate reagents and protocols. Methylation values were calculated as an average of high-quality CpG sites (determined a *'passed'*) included in the sequences analyzed using Pyro Q-CpG 1.0.9 (Qiagen). The level of methylation at each CpG position is then expressed (%) as the ratio of methylated cytosine over the sum of total thymine and methylated cytosine. Graphic representation of DNA methylation values shows the averaged values over multiple CpG sites. The primers for PCR amplification and sequencing were designed with PyroMark assay design software version 2.0.01.15. Primers (Table 5) were chosen based on the methylated CpG detected in the Genome-wide DNA methylation DNA analysis.

Table 5. PCR and pyrosequencing primers.

	Forward	Reverse	PyroSeq
KILLIN	ATGTGGGTGTTTGTGTAAT	CCTTTACTCRAAATACTCTTACC	CCCCAACCCTTCCTA
CXCL2	GGTTTTTTAGTTTTAATTATGTATAAAAGG	ACCTATAACCCRAACTCTATAACT	AGTTTTAATTATGTATAAAAGGG
SLC38A1	TGTAGGTTGTGTTTAGGGTTAGT	AAAAAACRAAAAAATTACATTATA	AAAATAACCRCRAAAATAACAAATC
CTSZ	GGTAGGAGTAGYGATGGGATAGTT	ACCCCYCACTTTAAACCC	GGAGTAGYGATGGGATAGTTT
TGFBI	TGGGTGTTTAGGGTAGTTA	CCCRAACCAAATTAAATAA	GGTGTTTAGGGTAGTTAG
NR2F2	GGAGGTTTAAATTATAAATGG	ACCCTTTCCTACTTCCTAT	AATTATAAATGGTAATTTTATGTA

2.6.- Methylation-specific PCR (MSP)

DNA methylation in cellular models was also analyzed by methylation-specific PCR (MSP) using a set of primers designed for each analyzed gene by Methyl Primer Express program. This specific PCR provides qualitative information on the methylation status of a particular sequence[405]. For this reason, specifics primers for modified DNA were used (Table 6). The first pair of primers amplifies a sample in which cytosine followed by guanine are not methylated. The second pair of primers amplifies the sequences if the cytosines are methylated. Therefore, for each sample, two PCR reactions were carried out using the same DNA template: one reaction with methylated primers (M: methylated) and another with unmethylated primers (U: unmethylated) (Figure 9). Commercial methylated human male genomic DNA (CpGenome Universal Methylated DNA, Millipore) was used as a methylated positive control (IVD: *in vitro* methylated DNA), and DNA from normal lymphocytes (NL) as a positive control for unmethylated alleles. The following differences can be visualized by UV irradiation following SYBR Safe staining and 2% agarose gel (agarose in TBE (Tris-Borate Edta) 0.5X).

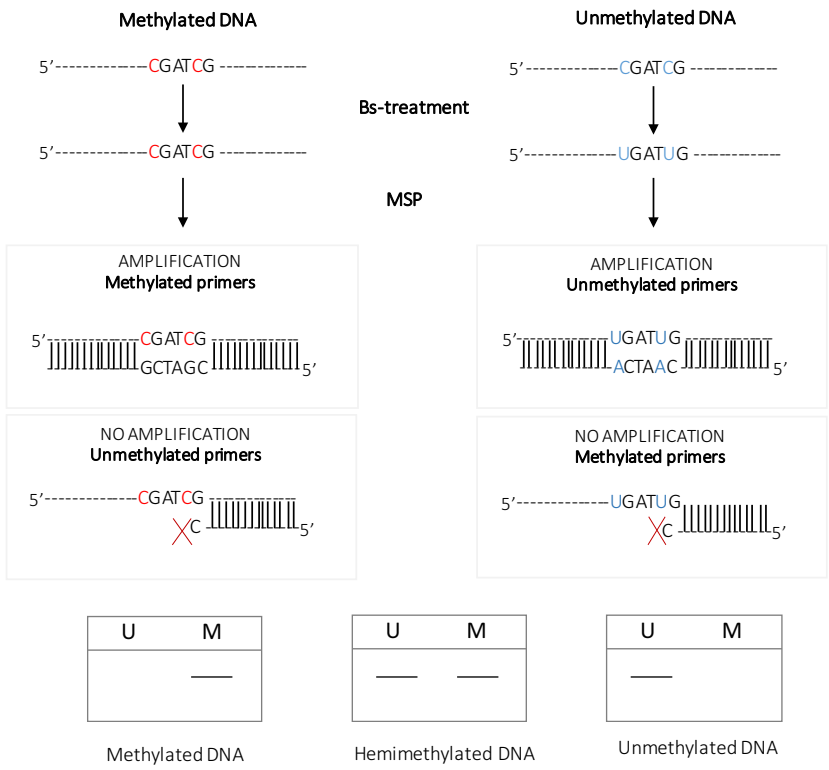

Figure 9. **Schematic process of methylation-specific polymerase chain reaction (MSP).** Adapted from Zhang *et al.* 2009[406].

The conditions for individual MSP reactions should be carefully optimized. Annealing temperature and elongation time may affect the efficiency of PCR and the specificity of primer sets. The unmethylated and methylated primer pairs were optimized individually tested (see Table 6). Based on the theoretical melting temperature of the primer pairs, a range of temperatures for the annealing step was.

Table 6. MSP primers

	Forward	Reverse
MSP_KILLIN_Methylation	GAGTTTTTGTTTTCGTCGC	CGTCTTCTTCCTTTACTCGAA
MSP_KILLIN-Unmethylation	GAGTTTTTGTTTTTGTTGT	CATCTTCTTCCTTTACTCAAA
MSP_CXCL2_Methylation	TTAAGGGATTTGATTTACGAC	CGAATCCCTAAAACGAAA
MSP_CXCL2-Unmethylation	GTTTAAGGGATTTGATTTATGAT	CCCAAATCCCTAAAACAAAA
MSP_SLC38A1-Methylation	GTTTTTCGGGTTGCGTTCG	TTTTAAATACGCCAAAACCCCG
MSP_SLC38A1-Unmethylation	GGTTTTTTGGGTTGTGTTTG	ATTTTAAATACACCAAAACCCCA
MSP_CTSZ-Methylation	AGCGATGGGATAGTTTCGTTTC	TCGACCTCGACCCAACAC
MSP_CTSZ-Unmethylation	AGTAGTGATGGGATAGTTTTGTTTT	CTCAACCTCAACCCAACACCC
MSP_TGFBI-Methylation	AGGGTAGTTAGGGGCGTAC	CCAAATTAAATAAACTACGAACG
MSP_TGFBI-Unmethylation	TTTAGGGTAGTTAGGGGTGTAT	AAACCAAATTAAATAAACTACAAACA
MSP_NR2F2-Methylation	TGGTAATTTTATGTATTTCGTCG	CGAAAAATCACATAAACGCT
MSP_NR2F2-Unmethylation	AAATGGTAATTTTATGTATTTTGTTG	CCACAAAAAATCACATAAACACT

3.- GENE EXPRESSION ANALYSIS

3.1.- RNA isolation procedures

For RNA extraction, cells were PBS washed, and then 1mL of Qiazol (Qiagen) was added. Total RNA was treated with RNase-Free DNase Set (Qiagen) and isolated using RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. All RNA samples were quantified using NanoDrop 2000 spectrophotometer (Thermo Scientific).

3.2.- RNA sequencing

Sequencing technology used for the RNA-Seq data in this thesis was from Illumina Truseq analysis in Dr. A. Welm's laboratory at the Huntsman Cancer Institute (University of Utah, Salt Lake City, Utah, USA).

Illumina TruSeq Stranded mRNA Sample Prep with oligo dT selection 3.1. Library construction is performed using the Illumina TruSeq Stranded mRNA Sample Preparation Kit (cat# RS-122-2101, RS-122-2102) as described below. Briefly, total RNA (100ng to 4μg) is poly-A selected using poly-T oligo-attached magnetic beads. Poly-A RNA eluted from the magnetic beads is fragmented and

primed with random hexamers in preparation for cDNA synthesis. First strand reverse transcription is accomplished using Superscript II Reverse Transcriptase (Invitrogen, cat#18064-014). Second strand cDNA synthesis is accomplished using DNA polymerase I and Rnase H under conditions in which dUTP is substituted for dTTP, yielding blunt-ended cDNA fragments in which the second strand with dUTP. An A-base is added to the blunt ends to prepare the cDNA fragments for adapter ligation and block concatamer formation during the ligation step. Adapters containing a T-base overhang are ligated to the A-tailed DNA fragments. Ligated fragments are PCR-amplified (12-15 cycles) under conditions in which the PCR reaction enables amplification of the first strand cDNA product, whereas attempted amplification of the second strand product stalls at dUTP bases and therefore is not represented in the amplified library. The PCR-amplified library is purified using Agencourt AMPure XP beads (Beckman Coulter Genomics cat#A63881). Following amplification, the library is purified by bead-based methodologies. The concentration of the amplified library is measured with a NanoDrop spectrophotometer and an aliquot of the library is resolved on an Agilent 2200 Tape Station using a D1K (cat# 5067-5361 and 5067-5362) or a High Sensitivity D1K (cat# 5067-5363 and 5067-5364) assay to define the size distribution of the sequencing library. Libraries are adjusted to a concentration of approximately 10nM and quantitative PCR is performed using the KapaBiosystems Kapa Library Quant Kit (cat# KK4824) to calculate the molarity of adapter ligated library molecules. The concentration is further adjusted following qPCR to prepare the library for Illumina sequence analysis using HiSeq 101 Cycle paired-end sequencing. Sequencing libraries (25 pM) were chemically denatured are applied to an Illumina HiSeq v4 paired end flow cell using an Illumina cBot. Hybridized molecules were clonally amplified and annealed to sequencing primers with reagents from an Illumina HiSeq PE Cluster Kit v4-cBot (PE-401-4001). Following transfer of the flowcell to an Illumina HiSeq instrument, a 101-cycle paired-end sequence run was performed using HiSeq SBS Kit v4 sequencing reagents (FC-401-4002). Sequencing data are posted in the Gene Expression Omnibus (GEO) database under accession number GSE114575.

Reader 1 Adapter: AGATCGGAAGAGCACACGTCTGAACTCCAGTCA
Reader 2 Adapter: AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

3.3.- Real-time quantitative reverse transcription PCR

In order to establish the correlation between the DNA methylation status of candidate's genes and the expression levels, we carried out quantitative real-time PCR (qRT-PCR). RNA was reverse-transcribed into cDNA using a High Capacity cDNA Archive Kit (Applied Biosystems). Gene expression levels of selected genes were assessed using a LightCycler 480 Real-time PCR System (Roche) with a LightCycler 480 SYBR Green I Master (Roche). qRT-PCR analyses were performed at least four times and each gene was run in triplicate. *GAPDH* was used as an endogenous control to enable normalization. The quantification cycle values were normalized according to the 2^{-dCt} methods. For a detailed list of the designed primers, please refer to Table 7.

Table 7. Primers for qRT-PCR with SYBER Green.

	Forward	Reverse
KILLIN	TGGTTCTGTGCTTGAGGGTA	GCAACATCGGAGAATGCAC
CXCL2	GTCATTTGTTAATATTTCTTCGTGATG	CACTGGCCATTTTCTTGGA
SLC38A1	CTTTTGCCACCTTTCCCTTT	GAGAGAAACAAACATGCTCCAA
CTSZ	CCAGCACATCCCCCAATA	CGCTCCCTTCCTCTTGATG
TGFBI	GTGTGTGCTGTGCAGAAGGT	CATATCCAGGACAGCACTCG
NR2F2	CCATAGTCCTGTTCACCTCAGA	AATCTCGTCGGCTGGTTG
SPDYA	AGTATGGTGGACTGAATTCTGGA	GACCCTCAGAAATTTCCCTTC
TXNDR1	GACCCGGTCACACAAAGC	CAATTCCGAGAGCGTTCC
POU4F1	CTGCTTTCTTTTGCGGTAGG	CCTCTTTTCTGGGATTTAGGTG
BMP4	TCCACAGCACTGGTCTTGAG	TGGGATGTTCTCCAGATGTTCT
ITGB8	CTACCCGCAGGTCTGGAG	CGGCTAGGATGCGAGAGA
OTX1	AGACGCATCAGACCCTGAAGGACT	CCAGACCTGGACTCTAGACTC
PPARG	TGAATGTGAAGCCCATTGAA	GAGGACTCAGGGTGGTTCAG
SLC16A3	TCACCTCCTCCCTGATTTTG	CTTTGGGCTTCTTCCTAATGC
ANXA3	CCCATCAGTGGATGCT	TCACTAGGGCCACCATGAGA
PTRF	CCCTGCCACCAAGTTGAG	CTGGCTGCTCTGTGATGTTC
SPOCK1	GGCCCACATATCTATCTCTTGC	TTTATGTGGCTTTTGTGTGGA
GAPDH	ATCATCCCTGCCTCTACTGG	GTCAGGTCCACCACTGACAC

4.- PROTEIN EXPRESSION ANALYSIS

4.1.- Western blot analysis

Parental (SK and AU) and resistant (SKTR and AUTR) models were synchronized by starvation in serum-deprived medium (0.5% FBS) for 24 hours. Cells were lysed in ice-cold lysis buffer (Cell Signaling Technology, Inc.) with 100µg/mL phenylmethylsulfonylfluoride (PMSF). Protein concentration was determined with Lowry (DC Protein Assay, Bio-Rad). Equal amounts of protein were heated in LDS Sample Buffer with Sample Reducing Agent (Invitrogen) for 10 minutes at 70°C, separated on SDS-PAGE, and transferred to nitrocellulose

membranes. Blots were incubated for 1 hour in blocking buffer [5% powdered-skim milk in PBS 0.05% Tween (PBS-T)] and incubated overnight at 4°C with the primary antibody diluted in blocking buffer (Table 8). Secondary antibodies conjugated to horseradish peroxidase were incubated for 1 hour at room temperature. The immune complexes were detected using a chemiluminescent HRP substrate [Super Signal West Femto (Thermo Scientific Inc.) or Immobilon Western (Millipore)] and in a Bio-Rad ChemiDocTM MP Imaging System. β-actin and α-tubulin (Cell Signaling Technology, Inc) were used as a control of protein loading. The protein bands density was quantified using the ImageJ program v.1.51 and the relative quantification was calculated based on the β-Actin or α-tubulin signal. Western blot analyses were repeated at least three times and representative results were shown.

Table 8. Antibody description.

Antibody	#Ref	Supplier	Dilution	Source
KILLIN	ab110756	Abcam	1:200	Rabbit
CXCL2	ab9841	Abcam	1:500	Rabbit
SLC38A1	ab60145	Abcam	1:200	Rabbit
TGFBI	BAF2935	R&D System	1:500	Goat
NR2F2	ab50487	Abcam	1:200	Rabbit
CTSZ	ab89777	Abcam	1:200	Mouse
EGFR	2231	Cell Signaling Technology	1:1000	Rabbit
p-EGFR	2234	Cell Signaling Technology	1:500	Rabbit
HER2	2165	Cell Signaling Technology	1:1000	Rabbit
p-HER2	6942	Cell Signaling Technology	1:500	Rabbit
AKT	9272	Cell Signaling Technology	1:1000	Rabbit
p-AKT	4058	Cell Signaling Technology	1:1000	Rabbit
ERK1/2	9102	Cell Signaling Technology	1:1000	Rabbit
p-ERK1/2	9106	Cell Signaling Technology	1:1000	Rabbit
α-tubulin	3873	Cell Signaling Technology	1:1000	Mouse
β-actin	3700	Cell Signaling Technology	1:1000	Mouse

5.- FUNCTIONAL ENRICHMENT ANALYSIS

To understand the biological relevance of the selected genes identified in the methylation array, Gene Ontology test (GO)[407] (http://www.geneontology.org) test was performed. The genes are grouped depending on the biological process in which they are involved. GO terms with an adjusted *p*-value<0.05 were considered significant.

6.- *IN VITRO* FUNCTIONAL ASSAYS

TGFBI depletion was carried out in the SK model with considerable endogenous levels of *TGFBI*. TGFBI overexpression and site-directed mutagenesis was performed in the SKTR model, which express low levels. TGFBI overexpression vectors were cloned in the laboratory, while the RNA interference vectors were designed and acquired from Sigma.

6.1.- Loss-of-function assays

For the *TGFBI* long-term knockdown in the SK model, five different shRNAs were specifically designed against TGFBI mRNA (NM_000358) in five different loci, considering a 19-base target sequence. For comparison purposes and to evaluate their knockdown efficiency targeting TGFBI-expressing cells, a shRNA against MSS2 yeast protein (NM_001180166), absent in mammals, was considered as a Scramble[408]. The shRNA vectors were created by oligonucleotide-based cloning. Briefly, the designed shRNA oligos were annealed and inserted between the BamHI and EcoRI restriction sites in pLVX-shRNA2 plasmid, purchased from Clontech, by T4 ligase enzyme (New England Biolabs, 400.000U/µl) over-night (o/n) at 4ºC. For shRNA oligonucleotides, please refer to Table 9.

6.2.- Gain-of-Function assays

For stable overexpression experiments, *TGFBI* ORF (ENSG00000120708, 2052pb) was amplified from SK cDNA, using the oligo-dT kit (Invitrogen), following the manufacturer's instructions. Specific primers with end-adaptors containing XhoI and NotI restriction enzyme sites and a Kozak sequence were

used (Table 9). This construction (wild-type) was then used as a template to fuse a FLAG-tag in the protein C-terminal region. After purification of the amplification product, the restriction enzymes were used to digest both PCR fragment and the pLVX-IRES-tdTomato bicistronic expression vector (Clontech), that was subsequently ligated by T4 ligase (New England Biolabs, 400.000U/ µl) at the same conditions as shRNA, and verified by Sanger-sequencing. An empty vector (Mock) was used as a control.

6.3.- Site-directed mutagenesis

Site-directed mutagenesis was performed using the TGFBI overexpression construction (wild-type) as a template to create its mutated version following a PCR based strategy. TGFBI is composed by multimeric domains – four FAS1 domains and RGD motif. Mutations were performed in three different binding motifs of FAS1 domains the **NKDIL motif** (amino acids 354-358), the highly conserved tyrosine and histidine residues **YH motif** (amino acids 563-580) in the four FAS1 domain, and the **EPDIM motif** (amino acids 617-621) in the second FAS1 domains as well as in the C-terminus **RGD motif**[409]. These motifs effects on adhesion mediated through interactions with various integrin's including α1β1, α3β1, αvβ3, and αvβ5. This construction was then also used as a template to fuse a FLAG-tag in the C-terminal of its mutated version (tagged and not tagged) following a PCR based strategy. The oligonucleotides used for site-directed mutagenesis were designed according to the following standard (Table 9). The amino acid to be mutated in each domain were selected according to their polarity, molecular mass (weight), and human frequency mainly. Seven mutagenic primers were designed, containing the desired mutation proximal to 5' end. Multiple mutations were introduced at the same time into the *TGFBI* gene through different overlapping primers followed by PCR. Then, all fragments were assembly with a linearized vector. Inserted mutations are checked by Sanger sequencing.

6.4.- Lentiviral infection

To perform the transfection process, 50µl of jetPRIME® (Polyplus-transfection S.A., Illkirch) was added to a mixture of 10µg of each plasmid

construction, 7.5µg of psPAX2 and 2.5µg of pMD2.G plasmids in 1mL of jetPRIME® buffer. Each transfection mix was then vortexed and incubated at RT for 10min and subsequently added drop-wise to a 10-cm dish containing Lenti-X™ 293T cells (Clontech) at 80% confluence. After culturing the cells for 4h, the culture medium was replaced by 10mL of fresh pre-heated medium (DMEM). Viral supernatants were collected and 0.45-µm filtered at 72 hours. SK and SKTR models were seeded in 6-well plates 24 hours before infection. Cells were infected with viral supernatants for three days and then were checked for infection efficiency. After lentiviral transduction, ZsGreen1- positive cells and tdTomato-positive cells were sorted by flow cytometry (FACS). The loss-of-function, overexpression and site-directed mutagenesis of TGFBI were confirmed by qRT-PCR and Western blot, following the previously indicated protocol.

6.5.- Confocal immunofluorescence microscopy

Transfected cells were cultured on adherent coverslips and glass slides were mounted using mowiol (Sigma-Aldrich). Multi-color immunofluorescence images were then analyzed under a Leica SP5 laser scanning confocal spectral microscope (Leica Microsystems) equipped with Argon, DPSS561, HeNe633 and 405 Diode and a × 63 oil-immersion objective lens (NA 1.32).

6.6.- Flow cytometry analysis

Cells were detached by trypsinization process, washed in PBS and then 10.000 cells/mL were analyzed by flow cytometric analysis using a MoFlo™ XDP High-Performance Cell Sorter (Beckman Coulter) to quantify the green- and red-positive cell population. Data were analyzed using Summit v.5.2 software. Side-scatter (SSD) and forward scatter (FSC) dot plots were performed, and only single cells were selected, excluding debris and cells aggregates.

Table 9. Oligonucleotides used to create the lentiviral vectors.

Name	Sequence
Scramble-F	gatccGCGCAGAACAAATTCGTCCATTCAAGAGATGGACGAATTTGTTCTGCGTTTTTTacgcgtg
Scramble-R	aattcacgcgtAAAAAACGCAGAACAAATTCGTCCATCTCTTGAATGGACGAATTTGTTCTGCGCg
TGFBI-Sh1-S	gatccGGTTATTGGCACTAATAGGTTCAAGAGACCTATTAGTGCCAATAACCTTTTTTg
TGFBI-Sh1-AS	aattcAAAAAAGGTTATTGGCACTAATAGGTCTCTTGAACCTATTAGTGCCAATAACCg
TGFBI-Sh2-S	gatccGCACGATGCTTGAAGGTAACTTCAAGAGAGTTACCTTCAAGCATCGTGTTTTTTg
TGFBI-Sh2-AS	aattcAAAAAACACGATGCTTGAAGGTAACTCTCTTGAAGTTACCTTCAAGCATCGTGCg
TGFBI- Sh3-S	gatccGTGGCGTGGTCCATGTCATCTTCAAGAGAGATGACATGGACCACGCCATTTTTTg
TGFBI- Sh3-AS	aattcAAAAAATGGCGTGGTCCATGTCATCTCTCTTGAAGATGACATGGACCACGCCACg
TGFBI-Sh4-S	gatccGCAGTCATCAGCTACGAGTGTTCAAGAGACACTCGTAGCTGATGACTGTTTTTTg
TGFBI-Sh4-AS	aattcAAAAAACAGTCATCAGCTACGAGTGTCTCTTGAACACTCGTAGCTGATGACTGCg
TGFBI-Sh5-S	gatccGGAAGGCGATCATCTCCAATTCAAGAGATTGGAGATGATCGCCTTCCTTTTTTg
TGFBI-Sh5-AS	aattcAAAAAAGGAAGGCGATCATCTCCAATCTCTTGAATTGGAGATGATCGCCTTCCg
sh tdTOMATE1-S	gatccGCGCTGATCTACAAGGTGAATTCAAGAGATTCACCTTGTAGATCAGCGTTTTTTACGCGTg
sh tdTOMATE1-AS	aattcACGCGTAAAAAACGCTGATCTACAAGGTGAATCTCTTGAATTCACCTTGTAGATCAGCGCg
TGFBI-XhoI-S	aaaaaaCTCGAGCCGCCACCATGGCGCTCTTCGTGCGGCTGC
TGFBI-mut1-AS	GTGGCTAGGATAATAATAATGGAGATGATCGCCTTCCCGTTGATAGTG
TGFBI-mut1-S	GATCATCTCCATTATTATTATCCTAGCCACCAACGGGGTGATCCACTACATTGATGAGC
TGFBI-mut2-AS	CATCACCAATGAAGAATTTCAGGATGTTGGCAAGTTCCTTGGCATC
TGFBI-mut2-S	CATCCTGAAATTCTTCATTGGTGATGAAATCCTGGTTAGCGGAGGCATCGGGG
TGFBI-mut3-AS	GTGGCCATGATAATAGGAATGGCAACAGGCTCCTTGTTGACACTCACCACATTGTTTTTC
TGFBI-mut3-S	GAGCCTGTTGCCATTCCTATTATCATGGCCACAAATGGCGTGGTCCATGTCATCACCAATGTT
TGFBI-mut4-AS	CTGCAAGTTCAATGGCAATTTCCTGAGGTCTGTTGGCTGGAGGCTGCAGAACATTG
TGFBI-mut4-S	GACCTCAGGAAATTGCCATTGAACTTGCAGACTCTGCGCTTGAGATCTTCAAACAAG
TGFBI-NotI-AS	aaaaaaGCGGCCGCCTAATGCTTCATCCTCTCTAATAACTTTTGATAGACAGGGG
TGFBI-FLAG-NotI-AS	aaaaaaGCGGCCGCCTACTTgTCGTCgTCCTTGTAgTCgCCgctgCCATGCTTCATCCTCTCTAATAACTTTTGATAGACAGGGGC

6.7.- Cell viability assays

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assay. Briefly, parental and resistant cells were plated in 96-well plates at a cell density of $1{,}5x10^3$ cells per well in their growth medium. After 24 hours, growth medium was removed and 100µL of fresh medium containing the corresponding concentration of trastuzumab was added to each well for five days. Following treatment, media was replaced by drug-free medium (100µL/well) containing 10µL of 5mg/mL MTT solution, and incubation was prolonged for 2 hours a 37ºC. Formazan crystals formed by metabolically viable cells were dissolved in DMSO

(100µL/well) and absorbance was determined at 570nm in a multi-well plate reader Benchmark Plus, Bio-Rad). Using control OD values (C) and test OD values (T), % cell proliferation inhibition (% CPI) was calculated from the equation 100 - [(T x 100) / C]. Data presented are from three separate wells per assay and the assay was performed at least three times.

7.- STATISTICAL ANALYSIS

7.1.- Treatments and *in vitro* studies

All data are expressed as mean±standard error of the mean (SEM). Normality and homoscedasticity were evaluated using Shapiro-Wilks and Levene tests, respectively. For normally distributed continuous variables Student's t-test were performed. Parametric analysis by ANOVA using a Bonferroni or Tamhane T2 *post hoc* test was used when comparing more than two groups. Data were analyzed by the Mann-Whitney U tests for non-normally independent variables when comparing two groups or Kruskal Wallis when comparing more than two groups. The level of significance was set at $p<0.05$ and are represented by asterisks, as follows: $p < 0.05$ (denoted as *), $p < 0.01$ (denoted as **) and $p < 0.001$ (denoted as ***). All observations were confirmed by at least three independent experiments. The statistical analysis was performed using the IBM SPSS (Version 21,0; SPSS Inc.).

7.2.- Patients related studies

In the patient cohort we analyzed *TGFBI* promoter methylation status and its association with the clinical-histopathological characteristics. Patient data were summarized as median (first quartile-third quartile) for continuous variables, and frequencies and percentages for categorical variables. The potential association between clinical-histopathological characteristics and levels of *TGFBI* methylation (low ≤20%, high ≥20%) or differences in *TGFBI* methylation before and after treatment were analyzed using the Mann-Whitney U and Kruskal-Wallis tests for continuous variables and the Chi-square or Fisher exact tests, as appropriate, for categorical variables. The correlation between variables was observed using Spearman's Rho coefficient. Characteristic (ROC) curves were

used to assess the predictive capacity of the TGFBI marker. Levels of significance was set at $p<0.05$ and are represented by asterisks, as follows: $p < 0.05$ (denoted as *), $p < 0.01$ (denoted as **) and $p < 0.001$ (denoted as ***). The statistical analysis was performed using the IBM SPSS software (Version 21,0; SPSS Inc.).

Chapter IV: Results

1.- GENOME-WIDE DNA METHYLATION ANALYSIS IN TRASTUZUMAB, LAPATINIB AND TRASTUZUMAB *PLUS* LAPATINIB-SENSITIVE AND RESISTANT BREAST CANCER MODELS

Trastuzumab and lapatinib resistance is a huge challenge when treating HER2+ BC patients. For this reason, it is necessary to explore the mechanisms of each type of resistance and develop a feasible treatment strategy for it. The DNA methylation and expression pattern analysis using different microarray and sequencing technology, may help us to discover which genes are involved in drug resistance and could be used to determine potential biomarkers and to explore novel diagnostic and therapeutic strategies. To determine which genes epigenetically regulated contribute to trastuzumab, lapatinib and trastuzumab *plus* lapatinib-resistant phenotype, the preclinical models of acquired resistance to trastuzumab (TR), lapatinib (LR) and trastuzumab *plus* lapatinib (TLR), developed by our group, were analyzed.

SKTR and SKLR were developed previously in our laboratory using the long-term method (12 months) and a high drug concentration exposure of trastuzumab or lapatinib in keeping with the Nahta R *et al.* methodology[401,410]. As a preclinical model of acquired resistance to both anti-HER2 drugs, was similar to a metastatic BC patient's treatment, the double-resistant model (SKTLR) was developed later and derived from SKTR following the same methodology as mono-resistant models. The SKTLR model treated with trastuzumab (10^{-4}μM) *plus* increasing concentrations of lapatinib (0.05-10μM) for five days showed a significantly more resistant pattern to both drugs compared with the SKTR and SK models (Figure 10A).

Some HER family protein receptors and their downstream proteins related to PI3K/AKT/mTOR and MAPK/ERK1/2 pathways were analyzed to elucidate the molecular mechanisms regarding the acquired resistance in our SKTLR model (Figure 10B), as was performed previously for mono-resistant models[401]. SKTLR showed a significant increase in expression levels of p-EGFR, p-HER4 and p-

AKT and to a lesser extent in EGFR and p-HER2, whereas levels of p-HER3 were decreased compared with the parental (SKTR). Similar levels between SKTR and SKTLR were observed for the other downstream proteins.

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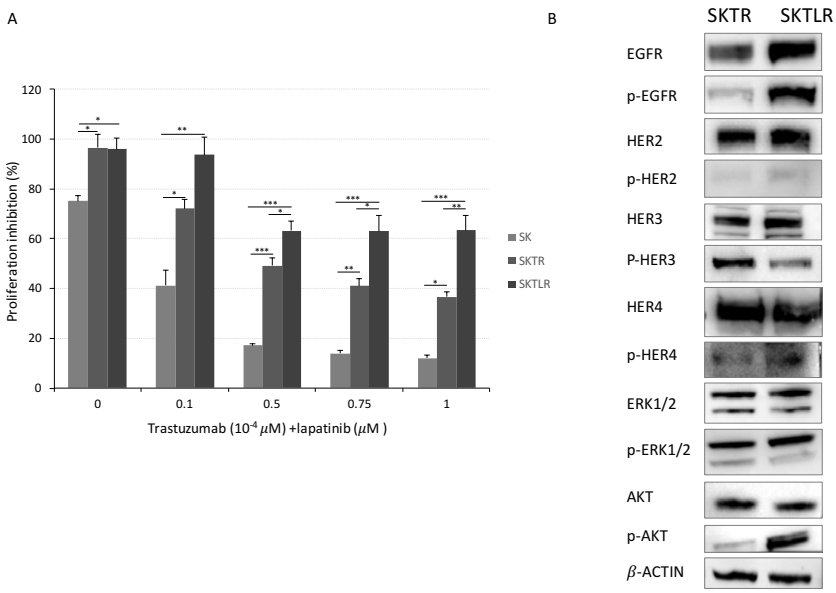


Figure 10. **Characterization of trastuzumab *plus* lapatinib-resistant model (SKTLR).** **(A)** Cell proliferation inhibition in trastuzumab-sensitive (SK), -resistant (SKTR) and trastuzumab *plus* lapatinib-resistant (SKTLR) models. SK, SKTR and SKTLR were treated with trastuzumab (10^{-4}µM) *plus* increasing concentrations of lapatinib (0.1–1µM) for five days. Experiments were performed at least three times in triplicates. *($p<0.05$), **($p < 0.01$) and ***($p < 0.001$) indicate levels of statistically significance. **(B)** Characterization of SKTLR and its corresponding parental model (SKTR) for the HER receptor family and downstream proteins expression and activation status by Western blot. Results shown are representative of those obtained from three independent experiments.

1.1.- DNA methylation landscape in trastuzumab, lapatinib and trastuzumab *plus* lapatinib-sensitive and -resistant breast cancer models

Analyzing the methylation profile of the human genome is an epigenomic approach that can be addressed using the Infinium HumanMethylation450 BeadChip (450k array). This technology is a validated tool for carrying out epigenomic projects because it allows the methylation status of approximately 450,000 CpGs located throughout the human genome to be detected[277]. This approach has recently been employed for several groups and aims at identifying

genes regulated by DNA methylation for clinical applications such as biomarkers[411,412]. Therefore, we took advantage of 450K array methodology to characterize the DNA methylation profile associated with trastuzumab and lapatinib resistance in HER2+ BC and compare the sensitive (SK) and -resistant (SKTR, SKLR and SKTLR) BC models (Annex I, Supplementary Figure 1). The results were normalized and analyzed following the established protocol for this platform and sex chromosomes were excluded from the analysis due to the fact that they usually represent a high source of variation on DNA methylation levels[277,413].

The global analysis of the DNA methylation (β-values) corresponding to the CpG sites with *p*-value<0.01 (469,927 CpGs) showed some differences in the scatter plot (Figure 11A) between SKTR, SKLR and SKTLR compared to SK. The scatter plot of SKTR (r^2=0.9313) revealed 27,314 differentially methylated CpGs (Δβ≥0.20) between the BC models that were 'sensitivity' and the 'resistance to trastuzumab'. In particular, the number of CpGs that gained (red triangle in scatter plot) and lost (green triangle in scatter plot) a methylation level ≥0.20 in SKTR with respect to SK were 14,845 and 12,469, respectively (Figure 11A, *Upper panel*). The SKLR model presented the highest correlation methylation CpGs levels with SK (r^2=0.9425), revealing 21,135 differentially methylated CpGs (Δβ≥0.20), with 13,944 CpGs that gained (red triangle in scatter plot) and 7,191 CpGs that lost (green triangle in scatter plot) a methylation level ≥0.20 in SKLR respect to SK (Figure 11A, *Middle panel*). Finally, the SKTLR model presented the most different methylation pattern (r^2=0.9014) compared to SK, with 38,636 differentially methylated CpGs (Δβ≥0.20) between SK and SKTLR models. In particular, the number of CpGs that gained (red triangle in scatter plot) and lost (green triangle in scatter plot) a methylation level ≥0.20 in SKTLR with respect to SK were 28,544 and 10,094, respectively (Figure 11A, *Lower panel*).

Supervised hierarchical clustering of the 5000 most variable CpGs confirmed distinctly different methylation patterns between our developed sensitive and resistant models (Figure 11B). However, similar average values of DNA

methylation levels were observed between different analyzed samples. Presenting the SKTLR model slightly higher methylation average levels compared to the other resistant models (Figure 11C).

As expected, all cellular models analyzed presented the typical features of cancer methylation patterns, with a more poorly methylated CpGs ($\Delta\beta<0.33$) and less highly methylated CpGs ($\Delta\beta>0.66$; Figure 11D).

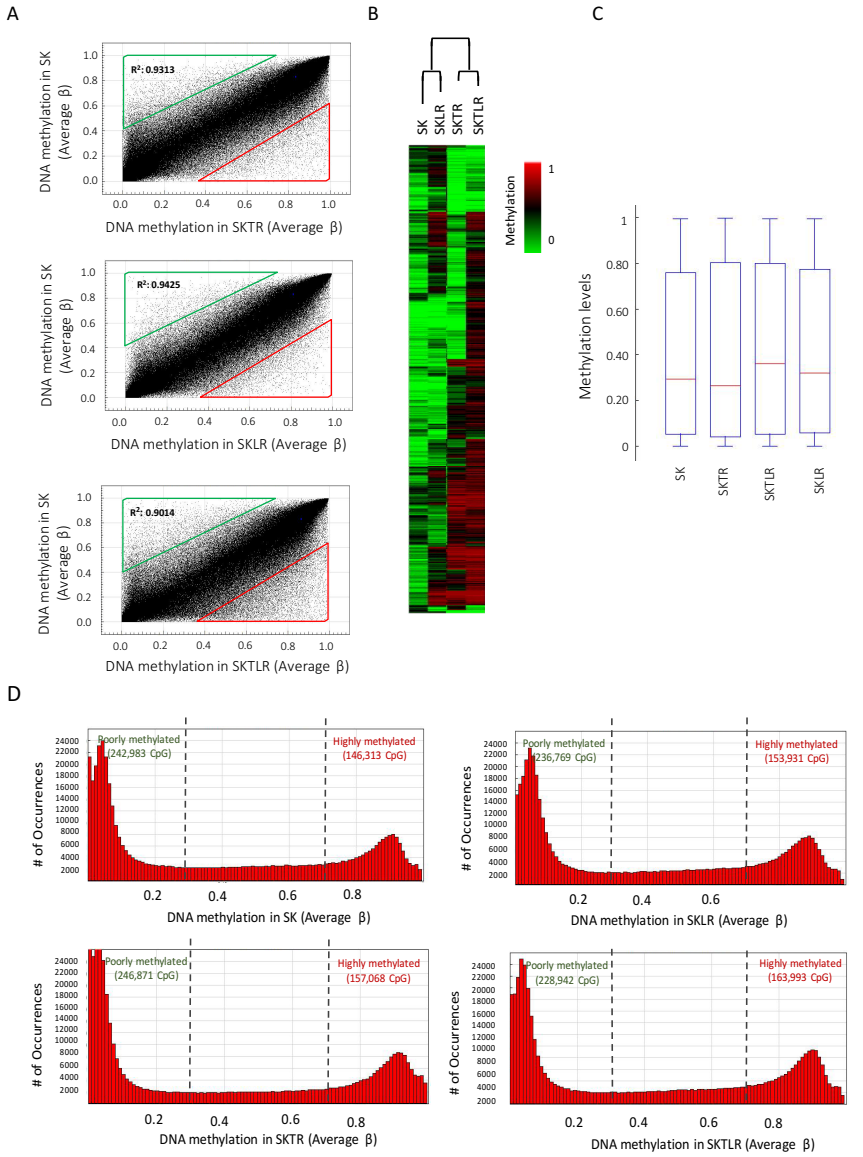


Figure 11. Analysis of the DNA methylation profile associated with anti-HER2 treatment resistance in HER2+ breast cancer models. (A) Scatter plot representing DNA methylation normalized levels (β-values) of trastuzumab- and lapatinib-resistant models (SKTR, SKLR and SKTLR) compared to the sensitive model (SK). Red and green triangles indicate the CpGs that gained and lost, respectively, a methylation level ≥0.20 in resistant models compared to the sensitive. **(B)** Supervised clustering of the 5000 most variables CpGs between the sensitive and resistant models. Green indicates 0% methylated and red 100% methylated. **(C)** Average global methylation levels for each model analyzed. The central solid line indicates the median and the limits of the vertical lines show the upper and lower percentiles. **(D)** Histograms show bimodal distribution pattern of the DNA methylation profile in sensitive and resistant models. The frequency of CpGs according to DNA methylation levels are represented in the graph.

DNA methylation is an epigenetic mechanism that usually affects CpG islands and promoters regulating the expression of the genes[311,414]. Therefore, the CpG sites representing promoter regions, defined as a region of 200 or 1500 bases upstream from transcription start site (TSS200 and TSS1500, respectively), the 5'UTR region as well as 1st Exon, were selected to investigate the biological relevance. This selection yielded 89,168 CpGs and 13,508 genes. Like the global methylation analysis, the supervised clustering of the 5000 most variable CpGs from promoter and islands regions showed a methylation pattern that clearly discriminated between SK and SKTR, SKLR and SKTLR models (Figure 12A). To verify whether the methylation observed in these genes had an impact on their transcriptional expression or not, we also performed a transcriptomic analysis using Illumina TruSeq (RNA sequencing; RNA-Seq) in all the models analyzed. Transcriptomic sequencing analysis revealed the expression pattern of 16,100 genes. As with the methylation array, the hierarchical clustering of all genes analyzed showed an expression pattern that could differentiate between sensitive and resistant models (Figure 12B). Like the DNA methylome analysis, the SKLR model presented the most similar expression pattern to the SK model. Interestingly, the SKTR model presented the most different expression pattern compared to the other models.

The combination of the two comprehensive analyses, (i.e. the DNA methylation array (450K) and the RNA sequencing analysis (RNA-Seq)), has been used as a powerful tool to appropriately detect some epigenetically regulated genes that could be used as potential trastuzumab and lapatinib resistance biomarkers in the clinic.

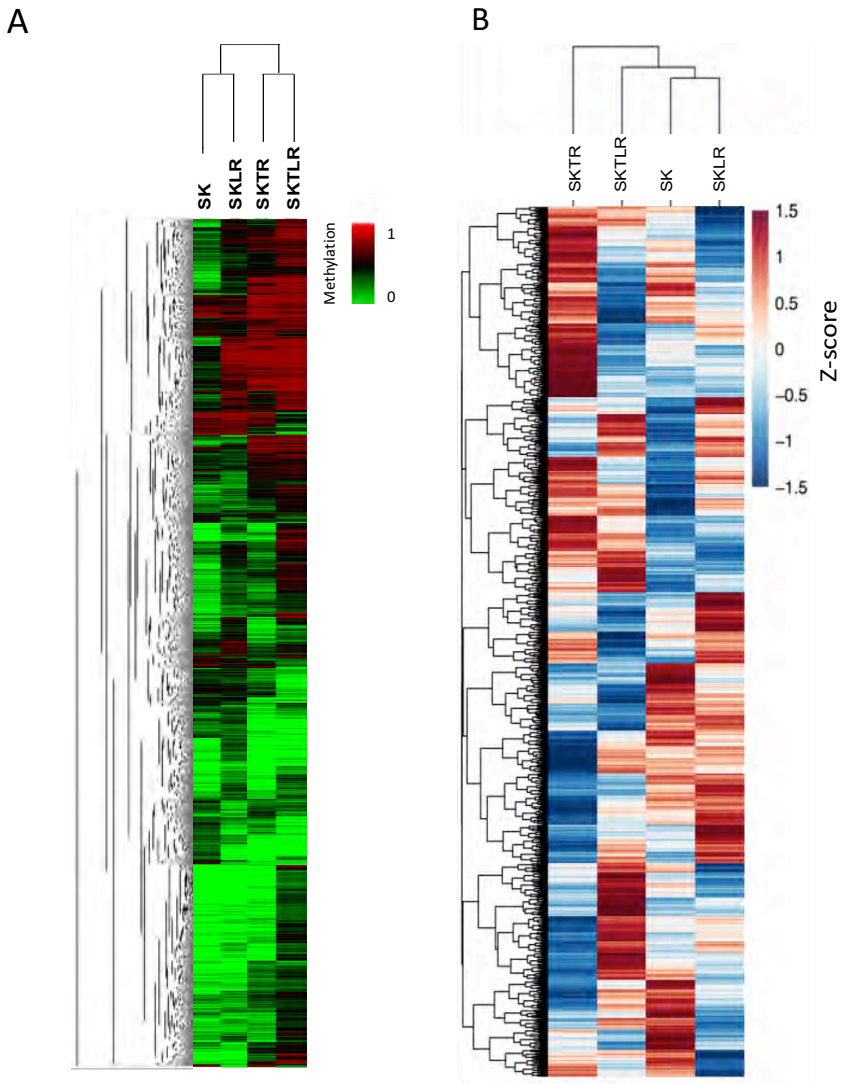


Figure 12. DNA methylation profile of promoter and island region and expression pattern of trastuzumab, lapatinib and trastuzumab plus lapatinib-sensitive and -resistant HER2+ breast cancer models. (A) Supervised clustering of the 5000 most variable CpGs between the sensitive (SK) and resistant models (SKTR, SKLR and SKTLR). Green is 0% methylated and red 100% methylated. **(B)** Hierarchical clustering of 16100 genes identified in our sensitive and resistant models. The heatmap was clustered by Euclidean distance of expression. Colors indicate the range of each gene's expression with the lowest expression in blue and the highest in orange

2.- THE IDENTIFICATION OF GENES RESPONSIBLE FOR TRASTUZUMAB, LAPATINIB AND TRASTUZUMAB *PLUS* LAPATINIB RESISTANCE IN OUR HER2+ BREAST CANCER MODELS

2.1.- Identifying potential epigenetic biomarkers in trastuzumab and lapatinib- resistant HER2+ models

After comparing the DNA methylome and transcriptomic analysis in our resistant models to the sensitive one, further comparisons between the sensitive and resistant models were performed to address whether epigenetic changes in some genes can be associated with trastuzumab, lapatinib and trastuzumab *plus* lapatinib resistance. Each mono-resistant model was compared to the sensitive model from which they derive. Contrary to this, SKTLR was compared with its parental line SKTR as well as with the SKLR model to determine the characteristic double-resistance pattern (Annex I, Supplementary Figure 1).

A DNA promoter and island methylation and expression analysis were performed for each comparison. To obtain a broader insight into the molecular mechanisms of drug resistance, an integrative analysis of the methylation array and RNA-Seq data (Venn Diagram) was carried out to find the genes which had a correlation between promoter and island methylation and their expression pattern.

2.1.1.- Mono-resistant models comparison

Trastuzumab-sensitive and -resistant models comparison

A DNA methylation profile characterization of trastuzumab resistance in HER2+ BC by comparing the SK and SKTR models was performed (Annex I, Supplementary Figure 1). With the aim of identifying epigenetically regulated genes as potential resistance biomarkers, we focused our study on analyzing the methylation levels of the CpG sites located at regulatory regions. The supervised clustering of the most variable CpGs from promoters and islands ($\Delta\beta \geq 0.20$), showed a methylation pattern that clearly discriminated between the SK and SKTR models, thus corroborating the previous results (Figure 13A). Next, we

used more stringent criteria to determine the most differentially methylated CpGs among the promoters and islands. In SKTR, we considered the CpGs with a methylation level (β) in SK<0.20 and in SKTR>0.60 as hypermethylated and as hypomethylated the CpGs with a methylation level (β) in SKTR<0.20 and in SK>0.60. This analysis revealed 184 differentially methylated CpGs corresponding to 152 genes which, according to a gene ontology (GO) analysis, were significantly associated (false discovery rate (FDR)<0.05) with several biological processes related to cancer, such as cell adhesion pathways (GO:0007155), regulation of transcription (GO:0006355), development (GO:0007275) and control of apoptosis (GO:0043065) (Figure 13B). To verify whether the methylation observed in these genes had an impact on their transcriptional expression or not, we performed a transcriptomic analysis with RNA-Seq comparing the SK and SKTR models and obtained 1,995 genes overexpressed and 3,191 downregulated in SKTR displaying a fold change ≥1.5 over the SK model. Then we correlated the genes differentially methylated at the promoter and island, with those differentially expressed between SK and SKTR (Figure 13C). We identified 31 hypermethylated and downregulated genes and no gene hypomethylated and overexpressed in SKTR with respect to the SK model (Table 10). From the list of 31 genes, we selected three genes: *TGFBI* or *BIGH3* (Transforming growth factor β induced), *CXCL2* (C-X-C motif chemokine ligand 2) and *SLC38A1* (Solute carrier Family 38 Member 1) for further analysis. *TGFBI, CXCL2* and *SLC38A1* were selected because of the high number of differentially methylated CpGs (≥3 CpG sites) that they presented and their previous implications in BC[415–417]. Besides, *KILLIN* or *KLLN* (P53 regulated DNA replication inhibitor) derived from the methylation array were also selected due to its relation with PTEN (i.e., share the same transcription start site) and to its previous implication in BC[418–420].

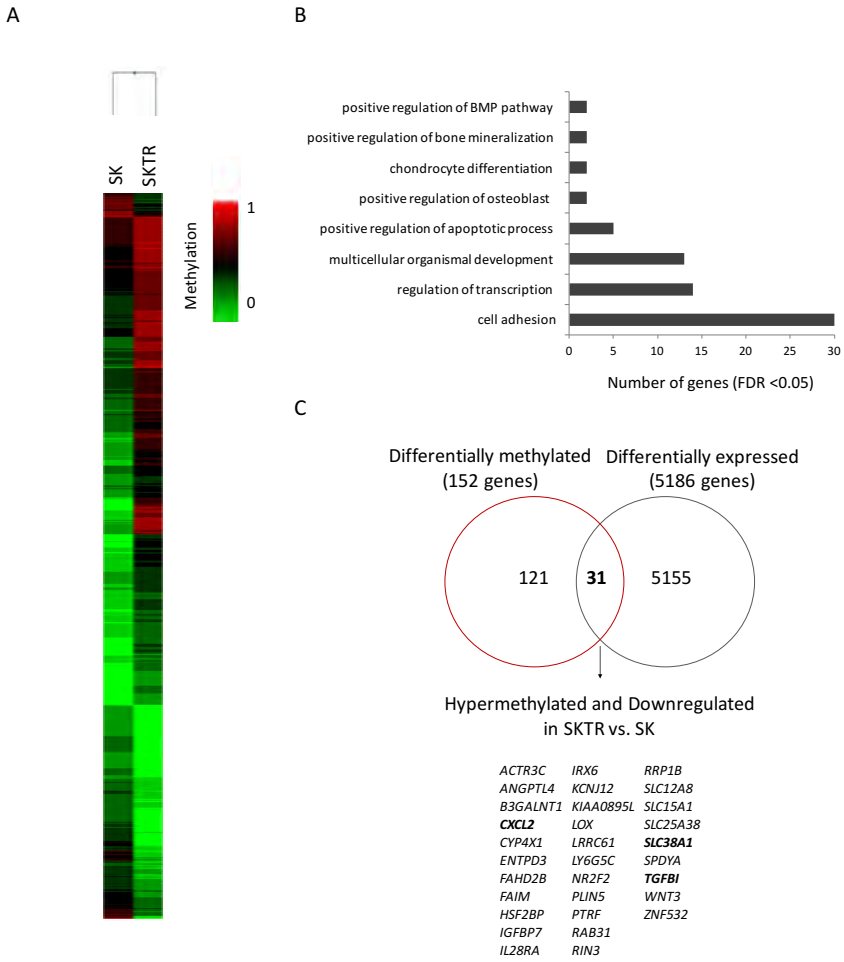


Figure 13. **Analysis of the DNA methylation profile associated with trastuzumab resistance in breast cancer models**. (**A**) Supervised clustering of the most variable CpGs ($\Delta\beta \geq 0.20$) from island and promoter regions between the SK and SKTR models. Green is 0% methylated and red 100% methylated. (**B**) Summary of gene ontology (GO) analysis of the biological process categories for the 152 differentially methylated genes at CpG island and promoter levels between the SK and SKTR models. (**C**) Venn diagram showing the differentially methylated and differentially expressed genes (obtained by RNA-Seq) between the SK and SKTR models. The names of the 31 hypermethylated and downregulated genes in SKTR compared to SK model are indicated.

Table 10. Thirty-one differentially methylated and expressed genes, between the SK and SKTR models, in the CpG island and promoter region. Bold denotes the three final genes with methylation and expression pattern correlation selected for further analysis (*KILLIN* is not included). CpGs were defined as hypomethylated (negative Δβ values) or hypermethylated (positive Δβ values).

TargetID	Chr	Position	Gene name	Gene region	SK	SKTR	SKTR vs SK
cg22893248	7	150020751	*ACTR3C;ACTR3C;LRRC61;LRRC61*	1stExon;5'UTR;5'UTR;5'UTR	0.19	0.73	0.54
cg06837791	19	8429491	*ANGPTL4;ANGPTL4*	1stExon;1stExon	0.17	0.61	0.44
cg02505409	19	8429160	*ANGPTL4;ANGPTL4;ANGPTL4;ANGPTL4*	5'UTR;1stExon;5'UTR;1stExon	0.16	0.79	0.63
cg20841906	3	160822911	*B3GALNT1;B3GALNT1;B3GALNT1;B3GALNT1;B3GALNT1;B3GALNT1*	5'UTR;5'UTR;5'UTR;5'UTR;TSS1500	0.06	0.7	0.64
cg00630212	4	74965135	***CXCL2***	TSS200	0.16	0.65	0.49
cg18804985	4	74965226	***CXCL2***	TSS1500	0.18	0.96	0.78
cg19031658	4	74964856	***CXCL2;CXCL2***	1stExon;5'UTR	0.05	0.89	0.85
cg22847221	4	74964920	***CXCL2;CXCL2***	1stExon;5'UTR	0.13	0.61	0.48
cg26558485	1	47489282	*CYP4X1;CYP4X1*	1stExon;5'UTR	0.14	0.69	0.54
cg17264618	3	40429014	*ENTPD3*	5'UTR	0.14	0.61	0.46
cg14798656	2	97760745	*FAHD2B*	TSS200	0.17	0.62	0.45
cg15860013	3	138327718	*FAIM;FAIM;FAIM;FAIM;FAIM;FAIM;FAIM*	5'UTR;TSS200;1stExon;5'UTR;1stExon;5'UTR;1stExon	0.18	0.68	0.5
cg20986370	4	57976171	*IGFBP7*	1stExon	0.19	0.69	0.49
cg08757148	1	24513722	*IL28RA;IL28RA;IL28RA*	1stExon;1stExon;1stExon	0.11	0.67	0.56
cg03470088	1	24513939	*IL28RA;IL28RA;IL28RA*	TSS200;TSS200;TSS200	0.05	0.79	0.75
cg01549404	16	55358636	*IRX6;IRX6*	5'UTR;1stExon	0.11	0.61	0.5
cg01666600	17	21279561	*KCNJ12*	TSS200	0.05	0.7	0.65
cg03928539	17	21279613	*KCNJ12*	TSS200	0.08	0.62	0.54
cg01637175	17	21281507	*KCNJ12*	5'UTR	0.08	0.78	0.7
cg01568244	16	67218584	*KIAA0895L;EXOC3L*	TSS1500;Body	0.14	0.82	0.69
cg01429321	5	121413797	*LOX;LOX*	5'UTR;1stExon	0.1	0.62	0.52
cg07753583	7	150020206	*LRRC61;ACTR3C;LRRC61*	TSS200;5'UTR;TSS200	0.17	0.86	0.69
cg10348193	7	150020240	*LRRC61;ACTR3C;LRRC61*	TSS200;5'UTR;TSS200	0.15	0.97	0.82
cg11026333	7	150020269	*LRRC61;ACTR3C;LRRC61*	TSS200;5'UTR;TSS200	0.06	0.98	0.92
cg01270001	7	150020401	*LRRC61;ACTR3C;LRRC61;LRRC61;LRRC61*	1stExon;5'UTR;5'UTR;5'UTR;1stExon	0.13	0.94	0.81
cg07151644	6	31649089	*LY6G5C*	TSS1500	0.16	0.6	0.44
cg18614734	15	96876248	*NR2F2;NR2F2;NR2F2;MIR1469;NR2F2*	Body;Body;5'UTR;TSS1500;TSS1500	0.18	0.65	0.47
cg22932336	19	4535070	*PLIN5*	5'UTR	0.1	0.88	0.78
cg04532834	19	4535188	*PLIN5;PLIN5*	1stExon;5'UTR	0.17	0.64	0.46
cg11804833	17	40575289	*PTRF;PTRF*	1stExon;5'UTR	0.08	0.62	0.54
cg24441185	18	9708096	*RAB31*	TSS200	0.06	0.92	0.86
cg03485262	14	92980031	*RIN3*	TSS200	0.04	0.72	0.68
cg08114373	14	92980204	*RIN3;RIN3*	5'UTR;1stExon	0.17	0.61	0.44
cg12831261	21	45078437	*RRP1B;HSF2BP*	TSS1500;5'UTR	0.01	0.93	0.92
cg09363539	3	124931746	*SLC12A8*	TSS200	0.01	0.82	0.81
cg03859162	13	99404887	*SLC15A1;SLC15A1*	1stExon;5'UTR	0.12	0.66	0.55
cg25999267	3	39424992	*SLC25A38;SLC25A38*	1stExon;5'UTR	0.19	0.75	0.56
cg09327770	12	46663270	***SLC38A1;SLC38A1***	TSS200;TSS200	0.02	0.83	0.81
cg20463033	12	46663274	***SLC38A1;SLC38A1***	TSS200;TSS200	0.01	0.77	0.76
cg24795297	12	46663281	***SLC38A1;SLC38A1***	TSS200;TSS200	0.07	0.77	0.71
cg06816106	2	29033352	*SPDYA;SPDYA*	TSS1500;TSS1500	0.18	0.64	0.46
cg21034676	5	135364552	***TGFBI***	TSS200	0.11	0.61	0.51
cg14120129	5	135364575	***TGFBI***	TSS200	0.06	0.61	0.55
cg09873933	5	135364580	***TGFBI***	TSS200	0.09	0.63	0.54
cg06093379	17	44896080	*WNT3;WNT3*	5'UTR;1stExon	0.01	0.83	0.82
cg17289202	18	56530789	*ZNF532*	5'UTR	0.13	0.8	0.67

TargetID: Column represents the name of each methylation probe.
Chr: Chromosome.
Position: Position in the genome (hg19).
SK and SKTR column: β-values indicate the levels of methylation for each probe.
SKTR vs. SK column: Differences in the β values for a given CpG site between models (Δβ values).

Lapatinib-sensitive and -resistant models comparison

Like the SKTR model, the DNA methylation of promoter and island profile characterization of lapatinib resistance in HER2+ BC had been performed comparing the SK and SKLR models (Annex I, Supplementary Figure 1). The supervised clustering of the most variable CpGs from promoters and islands ($\Delta\beta \geq 0.20$), showed a methylation pattern that clearly discriminated between the SK and SKLR models (Figure 14A). As in the SKTR and SK comparison, we used more stringent criteria to identify the most differentially methylated CpGs in the analyzed regions, i.e., considering in SKLR the CpGs with a methylation level (β) in SK<0.20 and in SKLR>0.60 as hypermethylated, while in SKLR the CpGs with a methylation level (β) in SKLR<0.20 and in SK>0.60 were considered as hypomethylated. This analysis revealed 120 differentially methylated CpGs corresponding to 82 genes which, according to the GO analysis, the differentially methylated genes were significantly associated (FDR<0.05) with several biological processes related to cancer, including cell proliferation (GO:0008283), regulation of transcription (GO:0006355), and morphogenesis (GO:0042472) (Figure 14B). The RNA-Seq between SK and SKLR models showed 1,748 genes overexpressed and 2,160 genes downregulated in SKLR displaying a fold change ≥1.5 over the SK model. The correlation between expression and methylation patterns allowed us to obtain 15 genes (Table 11), 12 of which were hypermethylated and downregulated and 3 hypomethylated and overexpressed in SKLR model (Figure 14C). From the list of 15 genes, we ultimately selected the *TXNRD1* (Thioredoxin reductase 1) gene for further analysis because of the high number of differentially methylated CpGs (≥3 CpG sites) that it presented and its previously described interaction with the HER2 receptor and implications in HER2+ BC[421,422].

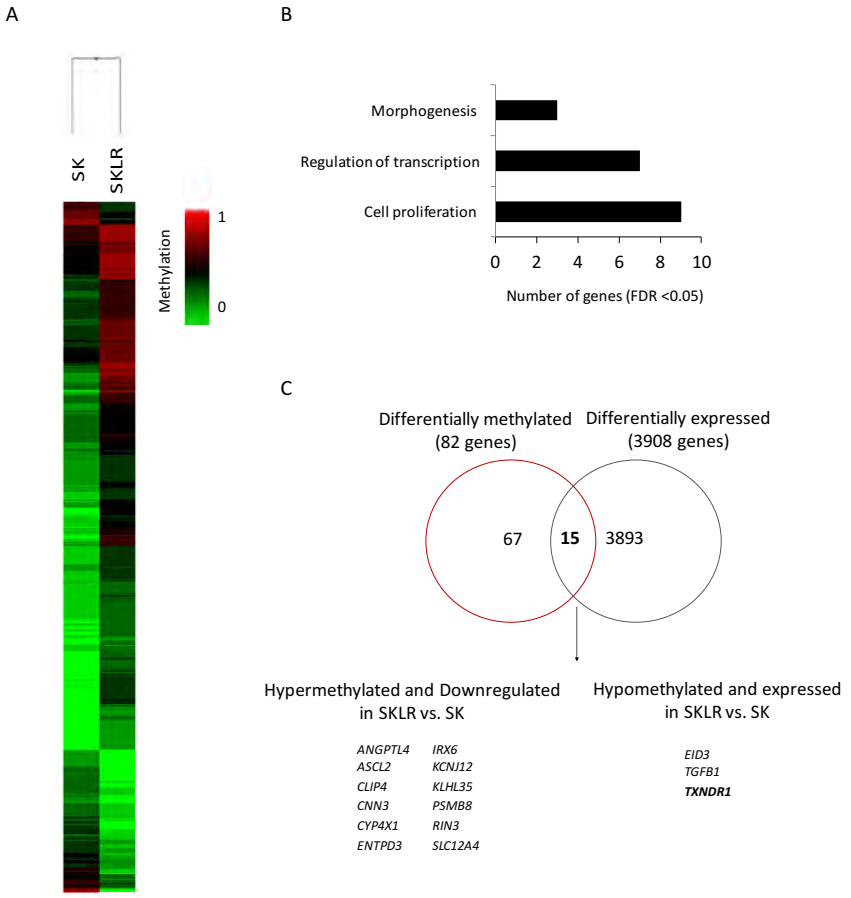


Figure 14. **Analysis of the DNA methylation profile associated with lapatinib resistance in breast cancer models.** **(A)** Supervised clustering of the most variable CpGs ($\Delta\beta \geq 0.20$) from island and promoter regions between SK and SKLR models. Green is 0% methylated and red 100% methylated. **(B)** Summary of gene ontology (GO) analysis of the biological process categories for the 82 differentially methylated genes at CpG island and promoter levels between the SK and SKLR models. **(C)** Venn diagram showing the differentially methylated and expressed genes (obtained by RNA-Seq) between the SK and SKLR models. The names of the 15 genes, (12 hypermethylated and downexpressed genes and 3 hypomethylated and overexpressed genes in SKLR compared to SK model) are indicated.

Table 11. Fifteen differentially methylated and expressed genes between the SK and SKLR models in the CpG island and promoter region. Bold denotes the gene finally selected for further analysis. CpGs were defined as hypomethylated (negative Δβ values) or hypermethylated (positive Δβ values).

TargetID	Chr	Position	Gene name	Gene region	SK	SKLR	SKLR vs. SK
cg02505409	19	8429160	*ANGPTL4;ANGPTL4;ANGPTL4;ANGPTL4*	5'UTR;1stExon;5'UTR;1stExon	0.16	0.83	0.67
cg13930892	11	2292000	*ASCL2;ASCL2*	1stExon;5'UTR	0.07	0.62	0.55
cg02774855	2	29338636	*CLIP4*	5'UTR	0.16	0.62	0.45
cg07823585	1	95392682	*CNN3;CNN3*	5'UTR;1stExon	0.06	0.71	0.64
cg18158385	1	95392678	*CNN3;CNN3*	5'UTR;1stExon	0.05	0.63	0.58
cg22045340	1	95392675	*CNN3;CNN3*	5'UTR;1stExon	0.12	0.65	0.52
cg26558485	1	47489282	*CYP4X1;CYP4X1*	1stExon;5'UTR	0.14	0.72	0.58
cg17264618	3	40429014	*ENTPD3*	5'UTR	0.14	0.63	0.48
cg04853843	16	55358461	*IRX6*	TSS200	0.12	0.64	0.53
cg10250663	16	55358458	*IRX6*	TSS200	0.03	0.72	0.69
cg01549404	16	55358636	*IRX6;IRX6*	5'UTR;1stExon	0.11	0.76	0.64
cg01666600	17	21279561	*KCNJ12*	TSS200	0.05	0.61	0.56
cg03934926	11	75141835	*KLHL35*	TSS200	0.17	0.73	0.56
cg24767041	6	32811733	*PSMB8;PSMB8*	Body;1stExon	0.12	0.75	0.63
cg24893844	6	32811694	*PSMB8;PSMB8*	Body;1stExon	0.17	0.60	0.43
cg03485262	14	92980031	*RIN3*	TSS200	0.04	0.64	0.60
cg03498383	16	68002044	*SLC12A4;SLC12A4;SLC12A4;SLC12A4*	Body;Body;Body;TSS1500	0.08	0.73	0.65
cg00588621	12	104697620	*EID3;**TXNRD1;TXNRD1;TXNRD1;EID3;TXNRD1;TXNRD1***	1stExon;5'UTR;5'UTR;Body;5'UTR;Body;5'UTR	0.90	0.07	-0.83
cg05057777	12	104697631	*EID3;**TXNRD1;TXNRD1;TXNRD1;EID3;TXNRD1;TXNRD1***	1stExon;5'UTR;5'UTR;Body;5'UTR;Body;5'UTR	0.88	0.03	-0.85
cg01848457	12	104697983	*EID3;**TXNRD1;TXNRD1;TXNRD1;TXNRD1;TXNRD1***	1stExon;5'UTR;5'UTR;Body;Body;5'UTR	0.74	0.07	-0.67
cg24767336	19	41860095	*TGFB1*	TSS1500	0.66	0.17	-0.49
cg03817911	12	104697389	***TXNRD1;TXNRD1;TXNRD1;EID3;TXNRD1;TXNRD1***	5'UTR;5'UTR;Body;TSS200;Body;5'UTR	0.97	0.02	-0.95
cg09477407	12	104697545	***TXNRD1;TXNRD1;TXNRD1;EID3;TXNRD1;TXNRD1***	5'UTR;5'UTR;Body;TSS200;Body;5'UTR	0.86	0.06	-0.80
cg18633684	12	104697387	***TXNRD1;TXNRD1;TXNRD1;EID3;TXNRD1;TXNRD1***	5'UTR;5'UTR;Body;TSS200;Body;5'UTR	0.87	0.16	-0.71
cg20923245	12	104697419	***TXNRD1;TXNRD1;TXNRD1;EID3;TXNRD1;TXNRD1***	5'UTR;5'UTR;Body;TSS200;Body;5'UTR	0.97	0.02	-0.95
cg21234561	12	104697526	***TXNRD1;TXNRD1;TXNRD1;EID3;TXNRD1;TXNRD1***	5'UTR;5'UTR;Body;TSS200;Body;5'UTR	0.96	0.04	-0.92
cg26614816	12	104697532	***TXNRD1;TXNRD1;TXNRD1;EID3;TXNRD1;TXNRD1***	5'UTR;5'UTR;Body;TSS200;Body;5'UTR	0.97	0.09	-0.89
cg27205904	12	104697514	***TXNRD1;TXNRD1;TXNRD1;EID3;TXNRD1;TXNRD1***	5'UTR;5'UTR;Body;TSS200;Body;5'UTR	0.91	0.10	-0.80

TargetID: Column represents the name of each methylation probe.
Chr: Chromosome.
Position: Position in the genome (hg19).
SK and SKLR column: β-values indicate the levels of methylation for each probe.
SKLR vs. SK column: Differences in the β values for a given CpG site between models (Δβ values).

2.1.2.- Double-resistant model comparison

As explained before, the combination of different therapies directed against the extracellular and intracellular domains of the HER2 receptor, known as the vertical blockade, has been described to achieve a better inhibition of HER2 pathway. Currently, lapatinib is administered in combination with capecitabine in advanced or HER2+ MBC patients who had previously received trastuzumab in combination with chemotherapy as first-line treatment. Consequently, we wanted to identify specific genes altered during the double-resistant acquisition as potential biomarkers of HER2+ resistant MBC using the SKTLR model we developed.

Like the mono-resistant model comparisons, the global DNA methylation (β-values) characterization of trastuzumab *plus* lapatinib resistance comparing the SKTLR and SKTR or SKLR models were performed first (Annex I, Supplementary Figure 1). Moreover, the DNA promotor and island methylation and the expression pattern were also analyzed to identify genes epigenetically regulated in each comparison.

Trastuzumab plus lapatinib-resistant (SKTLR) and trastuzumab-resistant (SKTR) models comparison

To characterize the DNA methylation profile associated with double-resistance in HER2+ BC, we first performed the global DNA methylation (β values) analysis by comparing the SKTLR and SKTR model. The scatter plot indicated a different global methylation pattern between the SKTLR and SKTR (r^2=0.9389), revealing 25,426 differentially methylated CpGs (Δβ≥0.20) between both models. In particular, the number of CpGs that gained (red triangle in scatter plot) and lost (green triangle in scatter plot) a methylation level ≥0.20 in SKTLR with respect to SKTR were 20,417 and 5,009, respectively (Figure 15A).

After the global methylation pattern analysis, we focused our study on analyzing the methylation levels of the CpG sites located at the regulatory regions. The supervised clustering of the most variable CpGs (Δβ≥0.20) from

promoter and island was clearly differentiated in the SKTLR and the SKTR models (Figure 15B). Next, we used more stringent criteria to determine the most differentially methylated CpGs in these regions, considering the CpGs with a methylation level (β) in SKTR<0.20 and in SKTLR>0.60 as hypermethylated in SKTLR and the CpGs with a methylation level (β) in SKTLR<0.20 and in SKTR>0.60 as hypomethylated. This analysis revealed 211 differentially methylated CpGs corresponding to 166 genes. According to the GO analysis, these genes were significantly associated (FDR<0.05) with different biological processes including cell adhesion pathways (GO:0007155), regulation of transcription (GO:0045944), transport (GO:0006810), regulation of cell proliferation (GO:0008284), regulation of actin cytoskeleton organization (GO:0032956), regulation of phosphatidylinositol 3-kinase activity (GO:0043552), regulation of cell adhesion (GO:0007162), DNA methylation (GO:0010424) and regulation of EMT (GO:0010424) (Figure 15C). The RNA-Seq comparing the SKTLR and the SKTR models showed 3,882 genes downregulated and 2,807 genes overexpressed in SKTLR displaying a fold change ≥1.5 over the SKTR model. Venn diagram analysis revealed 35 genes (Figure 15D), 18 of which are hypomethylated and overexpressed and 17 hypermethylated and downregulated genes in SKTLR compared to SKTR (Table 12). *POU4F1* (POU class 4 homeobox 1) and *TXNDR1* hypomethylated and overexpressed in SKTLR and *OTX1* (Orthodenticle homeobox 1), *ITGB8* (Integrin subunit beta 8), *BMP4* (bone morphogenetic protein 4), *ANXA3* (Annexin A3) and *PPARG* (Peroxisome proliferator-activated receptor γ) hypermethylated and downregulated in SKTLR were finally selected because of their previous implications in BC[422–428]. Genes with a high number of differentially methylated CpGs (≥3 CpG sites) but without any previous links to resistance or BC were not selected.

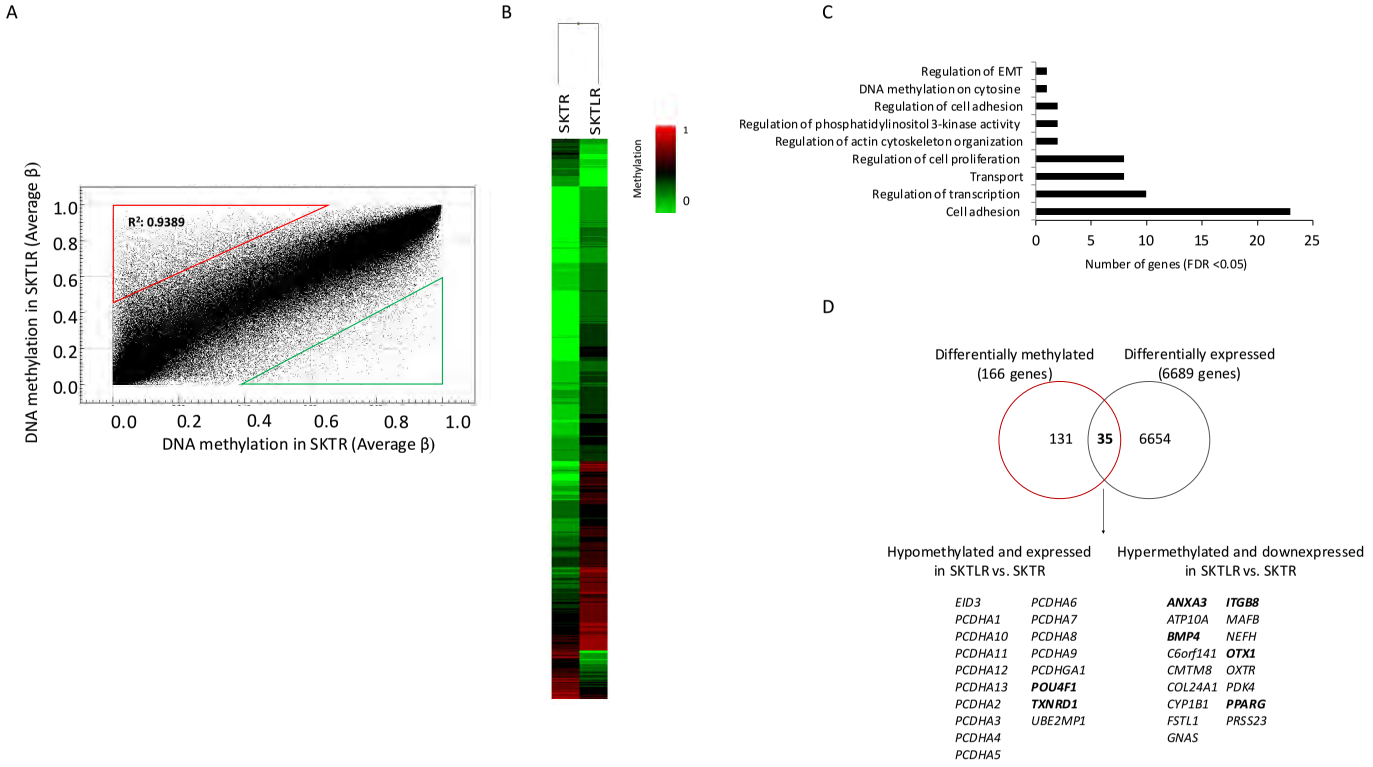


Figure 15. **Analysis of the DNA methylation profile associated with dual lapatinib and trastuzumab resistance in the breast cancer model (SKTLR vs. SKTR).** (**A**) Scatter plot of global DNA methylation normalized levels (β-values) of SKTR and SKTLR models. Red and green triangles indicate the CpGs that gained and lost, respectively, a methylation level ≥ 0.20 in SKTLR with respect to SKTR model. **(B)** Supervised clustering of the most variable CpGs (Δβ≥0.20) from island and promoter regions between SKTLR and SKTR models. Green is 0% methylated and red 100% methylated. **(C)** Summary of the gene ontology (GO) analysis of the biological process categories for the 166 differentially methylated genes at CpG island and promoter levels between the SKTR and SKTLR models. **(D)** Venn diagram showing the differentially methylated and expressed genes (obtained by RNA-Seq) between the SKTR and SKTLR models. The names of the 35 genes, (17 hypermethylated and downexpressed genes and 18 hypomethylated and overexpressed genes in SKTLR compared to SKTR model) are indicated.

Table 12. Thirty-five differentially methylated and expressed genes between the SKTLR and SKTR models in the CpG island and promoter region. Bold denotes the seven genes finally selected for further analysis. CpGs were defined as hypomethylated (negative Δβ values) or hypermethylated (positive Δβ values).

TargetID	Chr	Position	Gene name	Gene region	SKTR	SKTLR	SKTLR vs. SKTR
cg02245837	15	26108739	***ANXA3;ANXA3***	5'UTR;1stExon	0.15	0.76	0.61
cg03430923	10	63422668	*ATP10A*	TSS1500	0.15	0.67	0.51
cg03601952	16	15489968	*ATP10A*	TSS1500	0.19	0.60	0.41
cg03703356	14	103989368	*ATP10A*	TSS1500	0.16	0.60	0.44
cg04367345	11	44332636	*ATP10A*	TSS1500	0.12	0.72	0.60
cg04689720	9	2622584	***BMP4;BMP4;BMP4***	5'UTR;5'UTR;TSS1500	0.14	0.61	0.47
cg04981088	16	15489614	***BMP4;BMP4;BMP4***	TSS200;5'UTR;5'UTR	0.01	0.74	0.72
cg06000963	21	33245114	*C6orf141;C6orf141*	5'UTR;1stExon	0.10	0.76	0.66
cg07791578	6	24358308	*CMTM8*	TSS1500	0.12	0.66	0.54
cg07904983	20	17511988	*COL24A1*	TSS1500	0.02	0.74	0.72
cg08169864	14	54421413	*COL24A1*	TSS1500	0.07	0.67	0.60
cg08196359	3	32279722	*COL24A1*	TSS1500	0.19	0.60	0.42
cg09054633	5	136834294	*CYP1B1*	TSS1500	0.18	0.71	0.53
cg09130556	2	38304030	*CYP1B1*	TSS1500	0.05	0.68	0.63
cg09138115	6	5085177	*CYP1B1*	5'UTR	0.16	0.66	0.50
cg09168604	10	88127188	*CYP1B1*	TSS1500	0.11	0.67	0.56
cg13814950	9	90112515	*FSTL1;FSTL1*	5'UTR;1stExon	0.18	0.85	0.67
cg14775114	7	93520036	*GNAS;GNAS;GNAS;GNAS;GNAS;GNAS;GNAS*	3'UTR;TSS1500;TSS1500;Body;TSS1500;3'UTR;TSS1500;Body	0.18	0.65	0.47
cg17296166	19	46974567	*ITGB3*	TSS200	0.12	0.60	0.48
cg17299935	17	74534077	***ITGB8;ITGB8***	5'UTR;1stExon	0.19	0.69	0.50
cg19329121	7	95226035	*MAFB*	1stExon	0.10	0.66	0.56
cg20680592	17	68164914	*NEFH*	1stExon	0.18	0.67	0.49
cg21913319	1	86622628	***OTX1***	TSS1500	0.06	0.73	0.67
cg21978924	11	44332756	***OTX1***	TSS1500	0.02	0.63	0.61
cg22418909	8	41166738	*OXTR*	5'UTR	0.10	0.63	0.52
cg22639895	17	21279621	*PDK4*	TSS200	0.15	0.62	0.47
cg22639895	17	21279621	*PDK4*	TSS200	0.15	0.62	0.47
cg23100152	11	10472156	*PRSS23*	TSS200	0.08	0.64	0.56
cg04632671	3	12329826	***PPARG;PPARG;PPARG***	TSS1500;5'UTR;5'UTR	0.04	0.62	0.58
cg05057777	12	104697631	*EID3;**TXNRD1;TXNRD1;TXNRD1**;EID3;**TXNRD1;TXNRD1***	1stExon;5'UTR;5'UTR;Body;5'UTR;Body;5'UTR	0.82	0.20	-0.63
cg21264189	13	79177782	***POU4F1***	TSS200	0.78	0.06	-0.72
cg27398263	13	79177700	***POU4F1***	TSS200	0.73	0.01	-0.72
cg22459739	5	140201153	*PCDHA2;PCDHA1;PCDHA1;PCDHA3;PCDHA4;PCDHA5;PCDHA5*	Body;Body;Body;Body;Body;TSS1500;TSS1500	0.67	0.10	-0.57
cg21915910	5	140182627	*PCDHA2;PCDHA1;PCDHA3;PCDHA3;PCDHA1*	Body;Body;1stExon;1stExon;Body	0.87	0.10	-0.77
cg25967418	5	140261759	*PCDHA7;PCDHA12;PCDHA6;PCDHA10;PCDHA4;PCDHA11;PCDHA8;PCDHA1;PCDHA2;PCDHA6;PCDHA1;PCDHA9;PCDHA5;PCDHA13;PCDHA3;PCDHA13;PCDHA10*	Body;Body;Body;Body;Body;Body;Body;Body;Body;Body;Body;Body;Body;Body;TSS200;Body;TSS200;Body	0.65	0.10	-0.55
cg17852221	5	140263655	*PCDHA7;PCDHA13;PCDHA12;PCDHA6;PCDHA10;PCDHA4;PCDHA11;PCDHA8;PCDHA1;PCDHA2;PCDHA6;PCDHA1;PCDHA9;PCDHA13;PCDHA5;PCDHA3;PCDHA10*	Body;1stExon;Body;Body;Body;Body;Body;Body;Body;Body;Body;Body;1stExon;Body;Body;Body	0.70	0.03	-0.67
cg18835078	5	140712209	*PCDHGA1;PCDHGA1*	1stExon;1stExon	0.87	0.19	-0.68
cg07215693	5	140215806	*PCDHA6;PCDHA2;PCDHA1;PCDHA7;PCDHA1;PCDHA6;PCDHA5;PCDHA3;PCDHA4;PCDHA7*	Body;Body;Body;1stExon;Body;Body;Body;Body;Body;1stExon	0.91	0.19	-0.72
cg10205431	5	140215762	*PCDHA6;PCDHA2;PCDHA1;PCDHA7;PCDHA1;PCDHA6;PCDHA5;PCDHA3;PCDHA4;PCDHA7*	Body;Body;Body;1stExon;Body;Body;Body;Body;Body;1stExon	0.88	0.10	-0.79
cg01857475	12	104697193	***TXNRD1;TXNRD1;TXNRD1;EID3;TXNRD1;TXNRD1***	5'UTR;5'UTR;Body;TSS1500;Body;5'UTR	0.87	0.17	-0.70
cg01298284	16	34404774	*UBE2MP1*	TSS200	0.68	0.09	-0.59

TargetID: Column represents the name of each methylation probe.
Chr: Chromosome.
Position: Position in the genome (hg19).
SKTR and SKTLR column: β-values indicate the levels of methylation for each probe.
SKTLR vs. SKTR column: Differences in the β values for a given CpG site between models (Δβ values).

Trastuzumab plus lapatinib-resistant (SKTLR) and lapatinib-resistant (SKLR) models comparison

As with the SKTLR vs. SKTR comparison, we first analyzed the global methylation (β-values) in the SKTLR vs. SKLR comparison (Annex I, Supplementary Figure 1). The scatter plot revealed clearly methylation differences between both analyzed models (r^2=0.9247), showing 28,556 differentially methylated CpGs (Δβ≥0.20) between them. The number of CpGs that gained (red triangle in scatter plot) and lost (green triangle in scatter plot) a methylation level ≥0.20 in SKTLR respect SKTR were 19,191 and 9,365, respectively (Figure 16A).

Next, we focussed our analysis on the DNA methylation pattern of the promotor and island regions to identify which genes are epigenetically regulated in the double-resistant model. The supervised clustering of the most variable CpG from promoter and island (Δβ≥0.20), showed a methylation pattern that clearly differentiates the SKTLR and the SKLR models (Figure 16B). Next, we used more stringent criteria to determine the most differentially methylated CpGs in the promoters and islands, considering as hypermethylated in SKTLR the CpGs with a methylation level (β) in SKLR<0.20 and in SKTLR>0.60, and as hypomethylated the CpGs with a methylation level (β) in SKTLR<0.20 and in SKLR>0.60. This analysis revealed 306 differentially methylated CpGs corresponding to 224 genes which, according to the GO analysis, were significantly associated (FDR<0.05) with several biological process related to cancer such as cell adhesion (GO:0007162), signal transduction and intracellular transduction (GO:0007165 and GO:0035556, respectively), cell proliferation (GO:0008283), regulation of apoptosis (GO:0043065), MAPK cascade (GO:0000165), extracellular matrix organization (GO:0030198), regulation of BMP (Bone morphogenetic protein) pathway (GO:0030513) DNA methylation (GO:0006306), regulation of endothelial cell migration (GO:0010595) and regulation of cell cycle (GO:0045787) (Figure 16C). The RNA-Seq analysis comparing both resistant models revealed 1,424 downregulated genes and 1,550 overexpressed genes in SKTLR displaying a fold change ≥1.5 over the SKLR

model. The correlation analysis of differentially methylated at the promoter and island regions with those differentially expressed between both models by Venn diagram analysis identified 27 genes, 14 of them hypomethylated and overexpressed and 13 hypermethylated and downregulated in the SKTLR compared to the SKLR model (Figure 16D). The *SLC16A3* gene (Solute carrier family 16 member 3), a hypomethylated and overexpressed gene in SKTLR, was selected for further validations. For hypermethylated and downregulated genes, four of them *ANXA3*, *PTRF* (Polymerase I and transcript release factor also known as caveolae associated protein 1), *SPOCK1* (SPARC/osteonectin, cwcv and kazal like domains proteoglycan 1) and *PPARG*, were selected according to their previous implications in BC[422–428].

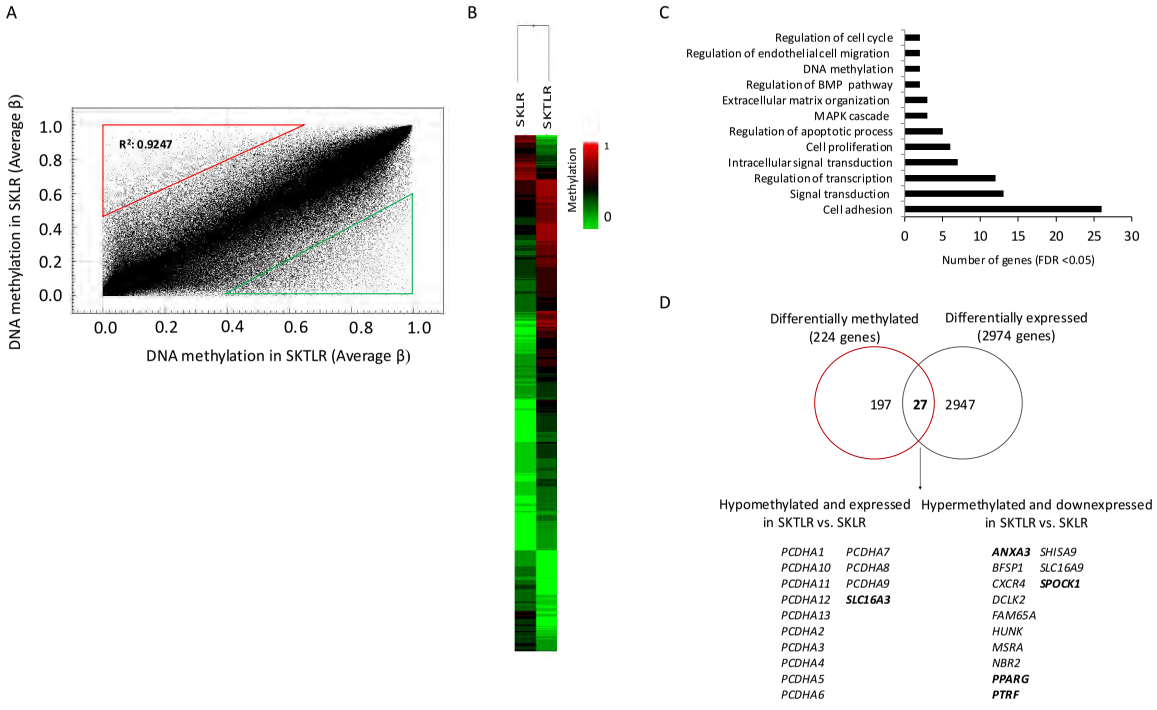


Figure 16. **Analysis of the DNA methylation profile associated with dual lapatinib and trastuzumab resistance in breast cancer model (SKTLR vs. SKLR).** (**A**) Scatter plot representing DNA promoter and island methylation normalized levels (β-values) of SKTLR and SKLR models. Red and green triangles indicate the CpGs that gained and lost, respectively, a methylation level ≥0.20 in SKLR and SKTLR models. (**B**) Supervised clustering of the most variable CpGs (Δβ≥0.20) from island and promoter regions between SKLR and SKTLR models. Green is 0% methylated and red 100% methylated. (**C**) Summary of gene ontology (GO) analysis of biological process categories for the 224 differentially methylated genes at CpG island and promoter levels between the SKTLR and SKLR models. (**D**) Venn diagram showing the differentially methylated and expressed genes (obtained by RNA-Seq) between SKTLR and SKLR models. The names of the 27 genes, 13 hypermethylated and downregulated and 14 hypomethylated and overexpressed genes in SKTLR compared to SKLR model, are indicated.

Table 13. Twenty-seven differentially methylated and expressed genes between the SKLR and SKTLR models in the CpG island and promoter region. Bold denotes the five final selected genes for further analysis. CpGs were defined as hypomethylated (negative Δβ values) or hypermethylated (positive Δβ values).

TargetID	Chr	Position	Gene name	Gene region	SKLR	SKTLR	SKTLR vs. SKLR
cg26919459	4	79472874	***ANXA3;ANXA3***	5'UTR;1stExon	0.08	0.63	0.54
cg07217075	20	17511826	*BFSP1;BFSP1*	Body;1stExon	0.05	0.66	0.61
cg07904983	20	17511988	*BFSP1;BFSP1;BFSP1*	Body;5'UTR;1stExon	0.00	0.74	0.73
cg06679534	2	136877039	*CXCR4*	TSS1500	0.10	0.62	0.52
cg17398233	2	136876887	*CXCR4*	TSS1500	0.19	0.96	0.77
cg22606658	4	150999251	*DCLK2;DCLK2*	TSS1500;TSS1500	0.09	0.84	0.75
cg03784054	16	67564076	*FAM65A*	5'UTR	0.11	0.87	0.76
cg06000963	21	33245114	*HUNK*	TSS1500	0.10	0.76	0.66
cg00958676	8	9911641	*MSRA;MSRA*	TSS200;TSS200	0.05	0.63	0.58
cg25288140	17	41278341	*BRCA1;NBR2;BRCA1;BRCA1;BRCA1;BRCA1*	TSS1500;Body;TSS1500;TSS1500;TSS1500;TSS1500	0.17	0.97	0.79
cg04632671	3	12329826	***PPARG;PPARG;PPARG***	TSS1500;5'UTR;5'UTR	0.01	0.62	0.61
cg11804833	17	40575289	***PTRF;PTRF***	1stExon;5'UTR	0.07	0.70	0.63
cg09229683	16	12995011	*SHISA9;SHISA9*	TSS1500;TSS1500	0.14	0.65	0.51
cg26752526	16	12994998	*SHISA9;SHISA9*	TSS1500;TSS1500	0.16	0.71	0.55
cg04215870	10	61469151	*SLC16A9*	5'UTR	0.09	0.61	0.52
cg12583927	10	61469753	*SLC16A9*	TSS200	0.09	0.76	0.68
cg20627835	10	61469748	*SLC16A9*	TSS200	0.07	0.79	0.72
cg02243437	10	61469599	*SLC16A9;SLC16A9*	1stExon;5'UTR	0.18	0.67	0.50
cg09054633	5	136834294	***SPOCK1***	5'UTR	0.10	0.71	0.61
cg14146100	4	134069236	*PCDH10;PCDH10*	TSS1500;TSS1500	0.86	0.15	-0.71
cg19712603	13	53422777	*PCDH8;PCDH8*	TSS200;TSS200	0.78	0.02	-0.75
cg09583939	5	140166213	*PCDHA1;PCDHA1;PCDHA1*	1stExon;1stExon;1stExon	0.64	0.11	-0.53
cg16234335	5	140188119	*PCDHA2;PCDHA1;PCDHA1;PCDHA4;PCDHA3;PCDHA4*	Body;Body;Body;1stExon;Body;1stExon	0.61	0.14	-0.47
cg21915910	5	140182627	*PCDHA2;PCDHA1;PCDHA3;PCDHA3;PCDHA1*	Body;Body;1stExon;1stExon;Body	0.91	0.10	-0.81
cg01416563	5	140237151	*PCDHA6;PCDHA2;PCDHA1;PCDHA10;PCDHA9;PCDHA7;PCDHA1;PCDHA6;PCDHA5;PCDHA3;PCDHA4;PCDHA10;PCDHA10;PCDHA8*	Body;Body;Body;1stExon;Body;Body;Body;Body;Body;Body;Body;1stExon;1stExon;Body	0.83	0.07	-0.76
cg07215693	5	140215806	*PCDHA6;PCDHA2;PCDHA1;PCDHA7;PCDHA1;PCDHA6;PCDHA5;PCDHA3;PCDHA4;PCDHA7*	Body;Body;Body;1stExon;Body;Body;Body;Body;Body;1st Exon	0.74	0.19	-0.56
cg10205431	5	140215762	*PCDHA6;PCDHA2;PCDHA1;PCDHA7;PCDHA1;PCDHA6;PCDHA5;PCDHA3;PCDHA4;PCDHA7*	Body;Body;Body;1stExon;Body;Body;Body;Body;Body;1st Exon	0.92	0.10	-0.83
cg07507057	5	140223107	*PCDHA6;PCDHA2;PCDHA1;PCDHA7;PCDHA1;PCDHA8;PCDHA6;PCDHA5;PCDHA8;PCDHA3;PCDHA4*	Body;Body;Body;Body;Body;1stExon;Body;Body;1stExon;Body;Body	0.63	0.14	-0.48
cg17852221	5	14026365	*PCDHA7;PCDHA13;PCDHA12;PCDHA6;PCDHA10;PCDHA4;PCDHA11;PCDHA8;PCDHA1;PCDHA2;PCDHA6;PCDHA1;PCDHA9;PCDHA13;PCDHA5;PCDHA3;PCDHA10*	Body;1stExon;Body;Body;Body;Body;Body;Body;Body;Body;Body;Body;Body;1stExon;Body;Body;Body	0.92	0.03	-0.89
cg17752015	17	80186763	***SLC16A3;SLC16A3***	5'UTR;TSS200	0.62	0.02	-0.60

TargetID: Column represents the name of each methylation probe.
Chr: Chromosome.
Position: Position in the genome (hg19).
SKLR and SKTLR column: β-values indicate the levels of methylation for each probe.
SKTLR vs. SKLR column: Differences in the β values for a given CpG site between models (Δβ values).

2.1.3.- Differentially methylated and expressed genes identified in the different comparisons

Once the differentially methylated and expressed genes were identified in each comparison, we wanted to observe if some of these genes had also been found in other comparisons. Focusing on both the trastuzumab and lapatinib mono-resistant comparisons, six hypermethylated and downexpressed genes were identified in SKTR and SKLR compared to SK model: *ANGPTL4* (Angiopoietin like 4), *CYP4X1* (Cytochrome P450 family 4 subfamily X member 1), *ENTPD3* (Ectonucleoside triphosphate diphosphohyrdrolase 3), *IRX6* (Iroquois homeobox 6), *KCNJ12* (Potassium voltage-gated channel subfamily J member 12) and *RIN3* (Ras and Rab ineractor 3). This observation suggests the possible role these six genes play in resistance mechanisms independent of the anti-HER2 agents to which it is resistant. For double-resistant comparisons, *ANXA3* and *PPARG* hypermethylated and downexpressed in SKTLR and the protocadherin gene family (*PCDHA 1-13*) hypomethylated and overexpressed in SKTLR were found to be differentially methylated and expressed in the SKTLR vs. SKTR and SKTLR vs. SKLR comparison. Therefore, these genes are probably explicitly involved in the resistant mechanisms of trastuzumab *plus* lapatinib treatment. Meanwhile, *EID3* (EP300-interacting inhibitor of differentiation 3) and *TXNDR1* from the SK vs. SKLR and SKTR vs. SKTLR comparisons, as well as the *PTRF* gene from the SK vs. SKTR and SKLR vs. SKTLR comparisons, suggest that their methylation pattern could be regulated by the effect of lapatinib or trastuzumab treatment alone, respectively. For all of the potential epigenetic biomarkers identified in the different resistant models, a summary of selected genes in each comparison is presented in Table 14. Although all these genes may present an interesting role in treatment resistance mechanisms of HER2+ BC, they could not be studied in more detail in this doctoral thesis.

Table 14. Schematic table of all differentially methylated and expressed genes from each comparison. Genes that are common in more than one comparison have been indicated in different colors. In light blue, genes that were found in both the SKTR and SKLR models compared to SK. In green, genes that were detected in both the SKTLR vs. SKTR and the SKLR models. In yellow, genes that were detected in SKTR vs. SK and SKTLR vs. SKLR. Finally, genes detected in the SKLR vs. SK and SKTLR vs. SKTR comparisons are in red. Bold denotes the final genes selected from each comparison for further analysis.

Mono-resistant comparison			Double-resistant comparison			
SKTR vs. SK	**SKLR vs. SK**		**SKTR vs. SKTLR**		**SKLR vs. SKTLR**	
Hypermethylated/downexpressed SKTR Hypomethylated/overexpressed in SK	Hypermethylated/downexpressed SKLR Hypomethylated/overexpressed in SK	Hypomethylated/overexpressed in SKLR Hypermethylated/downexpressed SK	Hypermethylated/downexpressed SKTR Hypomethylated/overexpressed in SKTLR	Hypomethylated/overexpressed in SKTR Hypermethylated/downexpressed SKTLR	Hypermethylated/downexpressed SKLR Hypomethylated/overexpressed in SKTLR	Hypomethylated/overexpressed SKLR Hypermethylated/downexpressed in SKTLR
ACTR3C	*ANGPTL4*	*EID3*	*EID3*	***ANXA3***	*PCDHA1*	***ANXA3***
ANGPTL4	*ASCL2*	*TGFB1*	*PCDHA10*	*ATP10A*	*PCDHA10*	*BFSP1*
B3GALNT1	*CLIP4*	***TXNRD1***	*PCDHA11*	***BMP4***	*PCDHA11*	*CXCR4*
CXCL2	*CNN3*		*PCDHA12*	*C6orf141*	*PCDHA12*	*DCLK2*
CYP4X1	*CYP4X1*		*PCDHA13*	*CMTM8*	*PCDHA13*	*FAM65A*
ENTPD3	*ENTPD3*		*PCDHA2*	*COL24A1*	*PCDHA2*	*HUNK*
FAHD2B	*IRX6*		*PCDHA3*	*CYP1B1*	*PCDHA3*	*MSRA*
FAIM	*KCNJ12*		*PCDHA4*	*FSTL1*	*PCDHA4*	*NBR2*
HSF2BP	*KLHL35*		*PCDHA5*	*GNAS*	*PCDHA5*	***PPARG***
IGFBP7	*PSMB8*		*PCDHA6*	***ITGB8***	*PCDHA6*	***PTRF***
IL28RA	*RIN3*		*PCDHA7*	*MAFB*	*PCDHA7*	*SHISA9*
IRX6	*SLC12A4*		*PCDHA8*	*NEFH*	*PCDHA8*	*SLC16A9*
KCNJ12			*PCDHA9*	***OTX1***	*PCDHA9*	***SPOCK1***
KIAA0895L			*PCDHGA1*	*OXTR*	***SLC16A3***	
LOX			***POU4F1***	*PDK4*		
LRRC61			***TXNRD1***	***PPARG***		
LY6G5C			*UBE2MP1*	*PRSS23*		
NR2F2						
PLIN5						
PTRF						
RAB31						
RIN3						
RRP1B						
SLC12A8						
SLC15A1						
SLC25A38						
SLC38A1						
SPDYA						
TGFBI						
WNT3						
ZNF532						

2.2.- CpG island hypermethylation-associated silencing of potential biomarkers in HER2+ resistant breast cancer models

Although DNA promoter hypermethylation is closely related to expression regulation, other mechanisms can regulate gene expression, such as non-coding RNA or histone modifications[258]. For this reason, it is important to validate the gene expression regulation by DNA promoter methylation using different methods. In this doctoral thesis, after having identified specific genes epigenetically regulated in each comparison, qRT-PCR was used to validate the expression pattern. Furthermore, epigenetic signatures are characterized by a very dynamic nature, whereas DNA methylation has often been shown as a reversible mechanism of transcriptional control by inhibition of enzymes such as the DNA methyltransferases[429]. In this sense, the demethylating agent 5-aza-2'-deoxycytidine (5-aza-dC) treatment at 3μM and 5μM, was used to validated if the epigenetic methylation mechanisms had a functional role in the transcriptional control of all the selected genes.

2.2.1.- Mono-resistant comparisons

Like the previous expression analysis with RNA-Seq, the SKTR model also showed a significant reduction in the transcript expression levels of *TGFBI* ($p<0.01$), *KILLIN*, *CXCL2* and *SLC38A1* ($p<0.05$) when compared to SK (Figure 17A, *Upper panel*). Interestingly, *TGFBI* was the gene that showed the greatest decrease (fold change=5.7) in transcriptional levels in the SKTR model. These results confirmed that hypermethylation at the promoter and CpG island region in SKTR correlated inversely with their corresponding transcriptional level for all four selected genes. After treating the SKTR model with the demethylating agent 5-aza-dC at 3μM and 5μM, the transcriptional levels of *TGFBI*, *KILLIN*, *CXCL2* and *SLC38A1* were significantly restored ($p<0.05$), indicating that methylation is an epigenetic mechanism that has a functional role in the transcriptional control of the four previously selected genes (Figure 17A, *Lower panel*).

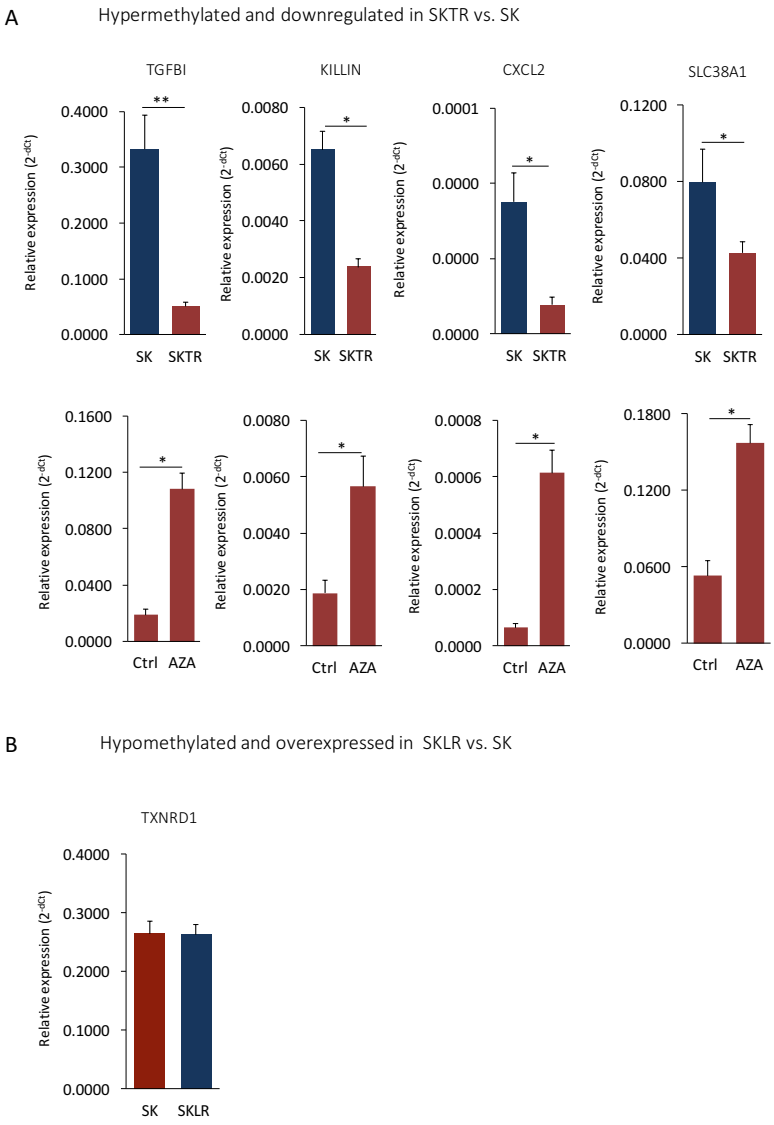


Figure 17. DNA methylation-associated silencing of selected genes comparing the trastuzumab and lapatinib-resistant (SKTR and SKLR) and -sensitive models (SK). **(A)** Expression levels of *TGFBI*, *KILLIN*, *CXCL2* and *SLC38A1* in the unmethylated (SK) and methylated (SKTR) models determined by qRT-PCR (*Upper panel*). Restored expression of selected genes (*TGFBI*, *KILLIN*, *CXCL2* and *SLC38A1*) after the DNA demethylating agent 5-aza-2'-deoxycytidine (5-aza-dC; AZA) in the SKTR methylated model by qRT-PCR (*Lower panel*). **(B)** Expression levels of *TXNRD1* in the methylated (SK) and unmethylated (SKLR) models determined by qRT-PCR. No significant differences of *TXNDR1* expression levels were observed between both models analyzed. Values from qRT-PCR were determined from triplicates, normalized against the *GAPDH* gene and are expressed as the mean ±SEM. Significance of the Mann-Whitney U test, **P<0.01; *P<0.05.

For the SKLR model, the *TXNRD1* gene had similar expression levels between the sensitive and resistant models with no significant differences (Figure 17B). Consequently, it was not selected as a potential candidate biomarker of lapatinib resistance.

2.2.2.- Double-resistant model comparisons

The double-resistant model comparisons showed some interesting candidate genes for further validations. *POU4F1* and *TXNRD1* genes, hypomethylated and overexpressed in the SKTLR model compared to SKTR, did not present an inverse correlation between their expression levels and promoter methylation status (Figure 18A). With regards to the five selected genes (*BMP4, ITGB8, PPARG, ANXA3* and *OTX1*) hypermethylated and downregulated in SKTLR model, *BMP4, ITGB8, PPARG* and *ANXA3* had significantly ($p<0.05$) decreased expression levels in SKTLR, with *ITGB8* being the gene that showed the greatest decrease (fold change=130.5) in transcriptional levels. The *OTX1* gene presented a slight expression decrease with no significant differences between models (Figure 18B, *Upper panel*). Therefore, DNA promoter methylation of the *BMP4, ITGB8, PPARG* and *ANXA3* genes in SKTLR correlates inversely with their transcriptional levels. Besides, the transcriptional levels of all four selected genes were significantly restored ($p<0.05$) after 5-aza-dC treatment (Figure 18B, *Lower panel*), indicating that methylation is an epigenetic mechanism that has a functional role in the transcriptional control of these genes. Meanwhile, *POU4F1, TXNDR1* and *OTX1* were not selected for further validations as biomarkers for trastuzumab *plus* lapatinib resistance.

In the SKTLR vs. SKLR comparison, the expression levels of the hypomethylated and overexpressed *SLC16A3* gene in the SKTLR model was not validated (Figure 19A). Furthermore, from the four previously hypermethylated and downexpressed genes selected (*ANXA3, PPARG, PTRF* and *SPOCK1*) in the SKTLR model, only *ANXA3* and *PPARG* presented an expression correlation with their promoter methylation pattern (Figure 19B, *Upper panel*). From the two genes validated, *ANXA3* was the one that showed the most significant decrease (fold change=24.7) in the transcriptional levels in the SKTLR model.

Furthermore, the transcriptional levels of *ANXA3* and *PPARG* were significantly restored ($p<0.05$) after 5-aza-dC treatment in the methylated SKTLR model (Figure 19B, *Lower panel*). Hence, for the *ANXA3* and *PPARG* genes, their expression regulation by promoter methylation were confirmed. *SLC16A3, PTRF*, and *SPOCK1* were discarded.

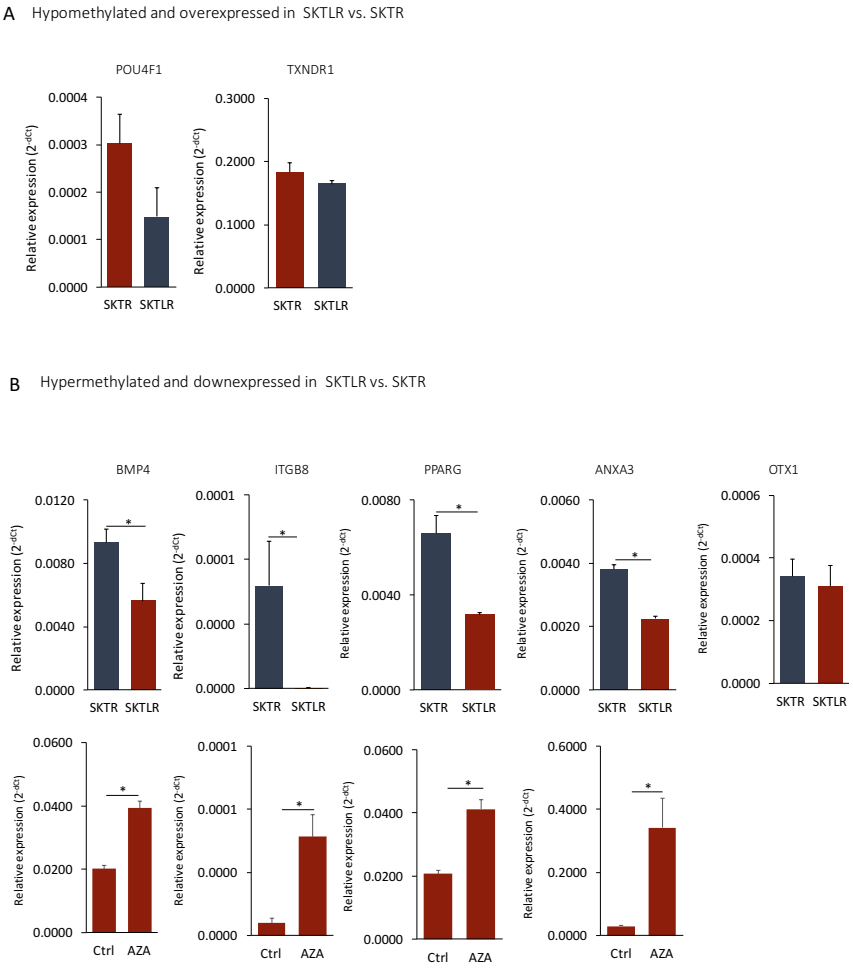


Figure 18. **DNA methylation-associated silencing of selected genes comparing the trastuzumab-resistant (SKTR) and trastuzumab plus lapatinib double-resistant (SKTLR) model.** **(A)** Expression levels of *POU4F1* and *TXNDR1* in the methylated (SKTR) and unmethylated (SKTLR) models determined by qRT-PCR. The expression of both selected genes was not validated. **(B)** Expression of *BMP4*, *ITGB8*, *PPARG*, *ANXA3* and *OTX1* in the unmethylated (SKTR) and methylated (SKTLR) models determined by qRT-PCR (*Upper panel*). *OTX1* expression was not validated. Restored expression of selected genes (*BMP4*, *ITGB8*, *PPARG*, and *ANXA3*) after DNA demethylating agent 5-aza-2'-deoxycytidine (5-aza-dC; AZA) in the SKTLR methylated model by qRT-PCR (*Lower panel*). Values from qRT-PCR were determined from triplicates, normalized against the *GAPDH* gene and are expressed as the mean ±SEM. Significance of the Mann-Whitney U test, *P<0.05.

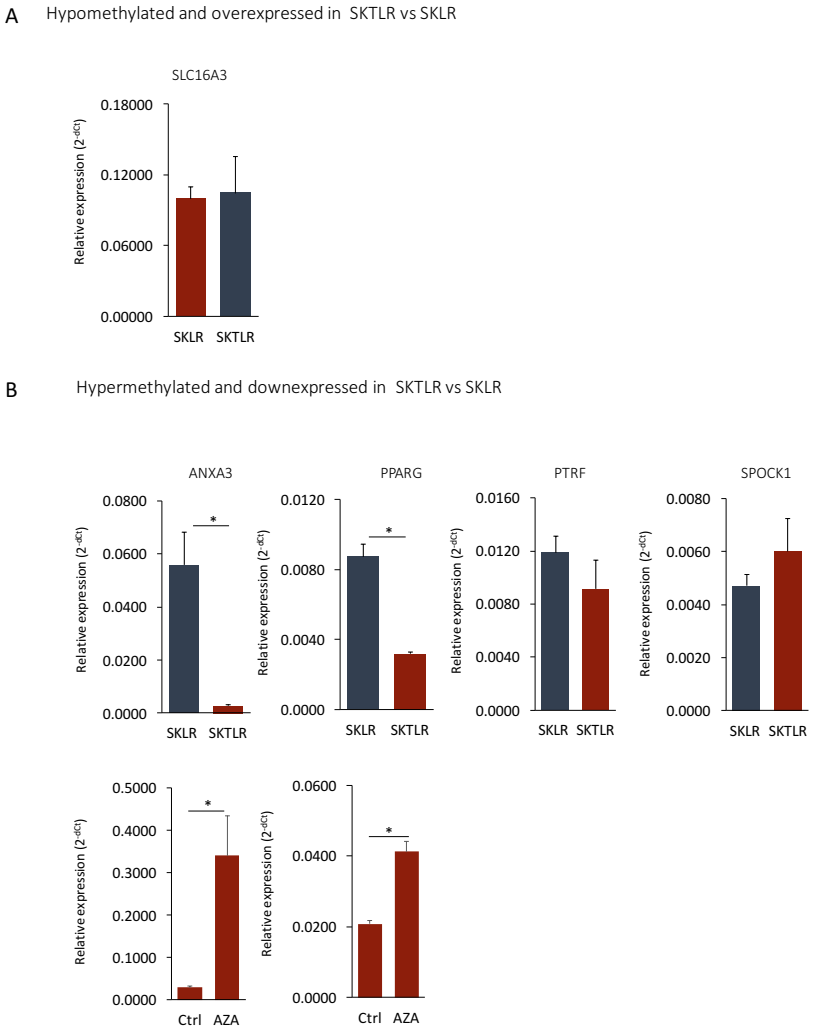


Figure 19. DNA methylation-associated silencing of selected genes comparing the lapatinib mono-resistant (SKLR) and trastuzumab plus lapatinib double-resistant (SKTLR) model. (A) Expression levels of *SLC16A3* in the methylated (SKLR) and unmethylated (SKTLR) models determined by qRT-PCR. *SLC16A3* gene expression was not validated. **(B)** Expression levels of *ANXA3*, *PPARG*, *PTRF* and *SPOCK1* in the unmethylated (SKLR) and methylated (SKTLR) models determined by qRT-PCR. Neither *PTRF* nor *SPOCK1* expression was validated (*Upper panel*). Restored expression of selected genes (*ANXA3* and *PPARG*) after DNA demethylating agent 5-aza-2'-deoxycytidine (5-aza-dC; AZA) in the SKTLR methylated model by qRT-PCR (*Lower panel*). Values from qRT-PCR were determined from triplicates, normalized against the *GAPDH* gene and are expressed as the mean ±SEM. Significance of the Mann-Whitney U test, *P<0.05.

Once the different potential biomarkers for trastuzumab, lapatinib and trastuzumab *plus* lapatinib resistance has been selected, we were interested in determining if the same effect could be observed in clinical samples. Therefore, patients treated in neoadjuvant settings were selected. Lapatinib has a limited response as a single agent and is not given as a first-line treatment. This drug is currently used only as a secondary treatment for BC that has progressed after treatment with trastuzumab[125]. The trastuzumab *plus* lapatinib combinatorial treatment is administered in specific cases of HER2+ MBC, which is why we could only obtain human samples from patients who had received trastuzumab *plus* chemotherapy in neoadjuvant settings. Although the selected genes epigenetically regulated in the SKLR and SKTLR models could not be validated in human samples in this doctoral thesis, we present them as potential epigenetic biomarkers of lapatinib and trastuzumab *plus* lapatinib resistance for future studies. Determining epigenetic biomarkers for lapatinib and the combination of trastuzumab *plus* lapatinib resistance could be very useful in the clinic.

2.3.- Validation of DNA promoter methylation of *TGFBI, KILLIN, CXCL2*, and *SLC38A1* in the trastuzumab-resistant model (SKTR)

The DNA promoter methylation and the transcriptomic analysis in the SKTR model allowed us to identify four epigenetically regulated genes (*TGFBI*, *KILLIN*, *CXCL2* and *SLC38A1*) as potential biomarkers of trastuzumab resistance. After expression validation, we decided to confirm the promoter and island DNA methylation of all four selected genes using gold standard methodologies for DNA methylation. First, we validated the DNA methylation of selected genes by bisulfite pyrosequencing, which is a technique that allows single genes to be analyzed on a single CpG level[430]. We observed significantly higher methylation levels for the four genes in the SKTR model relative to the SK model ($p<0.05$ for *TGFBI* and *SLC38A1*; $p<0.01$ for *KILLIN* and *CXCL2*) (Figure 20A). Similar results were obtained with methylation-specific polymerase chain reaction (MSP) analysis, which is a very specific and sensitive method[405]. The MSP results showed higher methylation in SKTR with respect to the SK model for the *TGFBI*, *KILLIN* and *CXCL2* genes and hemi-methylation

amplification (both methylated and unmethylated alleles) for the *SLC38A1* gene (Figure 20B).

To ascertain which selected gene was the best candidate to be subsequently validated in human samples, we wanted to analyze whether the transcriptional repression was also confirmed at protein level before and after demethylating agent treatment by Western blot. Importantly, this epigenetic silencing was also confirmed at protein level in three (*TGFBI*, *KILLIN* and *CXCL2)* of the four selected genes, showing a reduction of the protein levels in SKTR with respect to the SK model. Interestingly, *TGFBI* and *CXCL2* expression was significantly restored in hypermethylated SKTR model after 5-aza-dC treatment at 3μM and 5μM (Figure 20C). The change observed in the protein levels after the 5-aza-dC treatment was especially drastic in *TGFBI*, where the absence of protein expression induced by promoter hypermethylation in SKTR was completely recovered after the *in vitro* demethylation. Taken together, these results suggest that *TGFBI* is the most epigenetically regulated gene of the four selected.

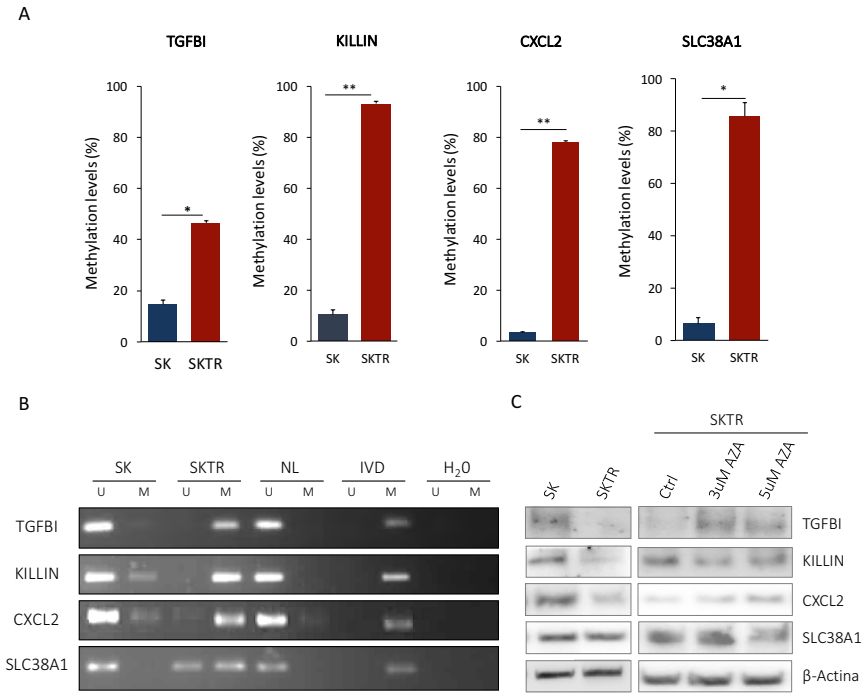


Figure 20. **DNA methylation validation of selected genes in trastuzumab-resistant (SKTR) and –sensitive (SK) models.** **(A)** DNA methylation levels of *TGFBI*, *KILLIN*, *CXCL2* and *SLC38A1* in SKTR and SK models by bisulfite pyrosequencing. **(B)** DNA promoter methylation validation of selected genes (*TGFBI*, *KILLIN*, *CXCL2* and *SLC38A1*) by methylation-specific polymerase chain reaction (MSP) analysis. **(C)** Protein expression of all selected genes (*TGFBI*, *KILLIN*, *CXCL2* and *SLC38A1*) in SK and SKTR models before and after 5-aza-2'-deoxycytidine (5-aza-dC; AZA) treatment by Western blot. In MSP, the presence of visible polymerase chain reaction products in lanes marked U, indicate unmethylated sequences, while the presence of products in lanes marked M indicate methylated sequences. *In vitro* methylated DNA (IVD) was used as a positive control for methylated sequences. DNA from normal lymphocytes (NL) was used as a negative control for methylated sequences. Results shown are representative of those obtained from three independent experiments and β-actin was used as a control. Values from pyrosequencing were determined from triplicates and are expressed as the mean ±SEM. Significance of the Mann-Whitney U test, **P<0.01; *P<0.05.

2.4.- Epigenetic silencing of *TGFBI, KILLIN, CXCL2* and *SLC38A1* in another trastuzumab-resistant model (AUTR)

To verify that the results obtained for these genes (*TGFBI, KILLIN, CXCL2* and *SLC38A1*) were not specific to the SK and SKTR models, we extended their epigenetic analyses to other trastuzumab-sensitive (AU) and -resistant (AUTR) human HER2+ BC cell models (AU565), developed previously in our laboratory (Figure 21)[402]. The *TGFBI, KILLIN, CXCL2* and *SLC38A1* genes were validated using the qRT-PCR before and after 5-aza-dC treatment, bisulfite pyrosequencing and MSP as suitable methods for their analysis in these models.

Similar to the SK and SKTR models, the AUTR also showed a significant reduction in the transcript expression levels of *TGFBI, KILLIN* and *CXCL2* ($p<0.05$) compared to the AU model (Figure 21A, *Upper panel*). For *SLC38A1*, similar expression values were observed in both models. *TGFBI* was the gene that showed the greatest decrease (fold change=3.8) in transcriptional levels in the AUTR model. After treating the AUTR model with demethylating agent 5-aza-dC treatment at 3μM and 5μM, the transcriptional levels of *TGFBI, KILLIN, CXCL2* and *SLC38A1* were significantly restored ($p<0.05$), also indicating the functional role DNA methylation has in the transcriptional control of these genes in this model (Figure 21A, *Lower panel*).

DNA promoter methylation was firstly validated with bisulfite pyrosequencing. We observed significantly ($p<0.05$) higher methylation levels for *CXCL2* and *SLC38A1* of the previously selected genes. Although *TGFBI* and *KILLIN* show a slightly higher methylation percentage in the trastuzumab-resistant model, no significant differences were observed (Figure 21B). The MSP showed hemi-methylated DNA amplification for *TGFBI* and *CXCL2* genes in AU compared to higher methylated band amplification in the AUTR model. *KILLIN* showed a hemi-methylated DNA in both models. The DNA promoter methylation of the *SLC38A1* gene was not validated, with unmethylated lane amplification in both models analyzed (Figure 21C). Therefore, only *TGFBI* and *CXCL2* showed a correlation between bisulfite pyrosequencing and MSP results. Finally, protein

levels were also evaluated for each gene before and after 5-aza-dC treatment by Western blot (Figure 21D). Interestingly, only the epigenetic silencing of the *TGFBI* gene was confirmed at protein level, where the low protein expression induced by promoter hypermethylation in AUTR was recovered after the 5-aza-dC at 3μM and 5μM.

Taking into account all the results obtained, the most strongly validated gene in both resistant HER2+ models using the different methylation and expression techniques was the *TGFBI* gene (also known as Big-H3 or keratoepithelin), which is an ECM protein whose secretion is induced by the transforming growth factor β (TGF-β). Therefore, it was selected as a candidate biomarker for further *in vitro* functional studies and HER2+ BC human sample validation analysis.

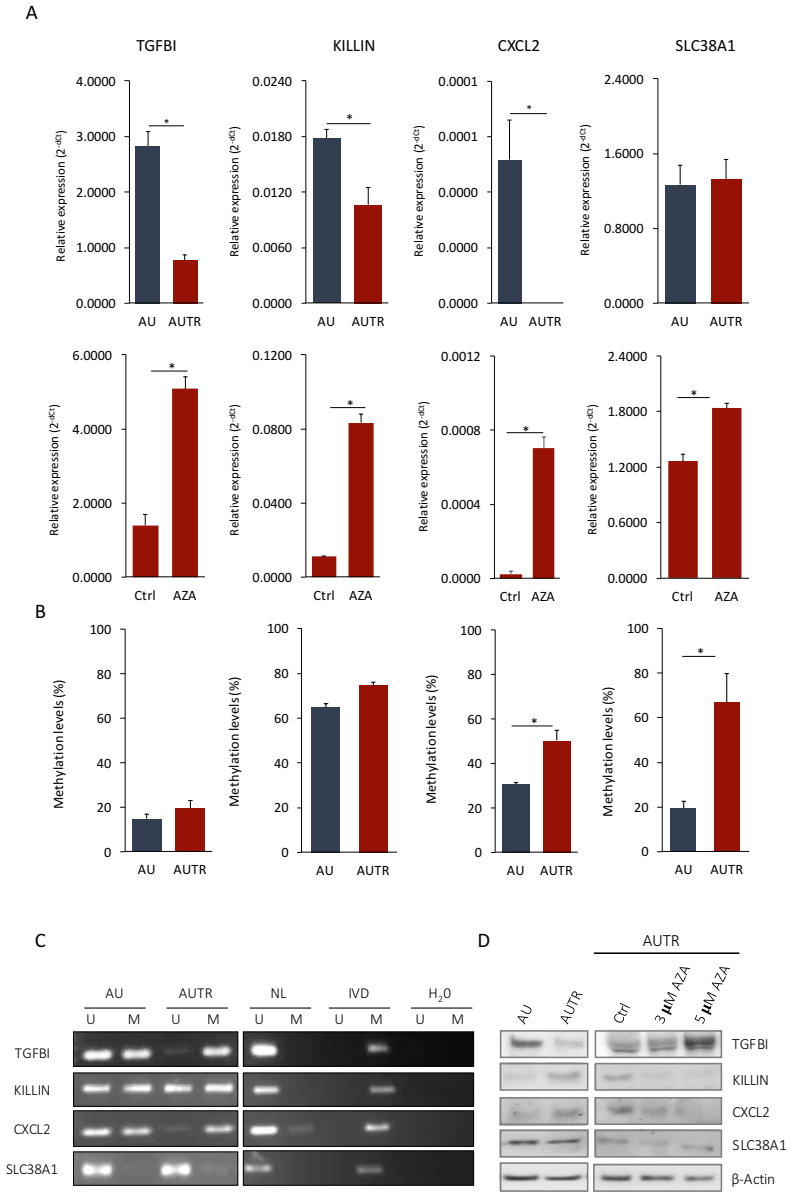


Figure 21. **DNA methylation-associated silencing of selected genes comparing the trastuzumab-resistant (AUTR) and -sensitive (AU) HER2+ cell models. (A)** Expression levels of *TGFBI*, *KILLIN*, *CXCL2* and *SLC38A1* in the unmethylated (AU) and methylated (AUTR) models (*Upper panel*). Restored expression of selected genes (*TGFBI*, *KILLIN*, *CXCL2* and *SLC38A1*) after DNA demethylating agent 5-aza-2'-deoxycytidine (5-aza-dC; AZA) in the AUTR methylated model (*Lower panel*). Expression levels were obtained by qRT-PCR and normalized against the *GAPDH* gene. **(B)** DNA methylation levels of *TGFBI*, *KILLIN*, *CXCL2* and *SLC38A1* in AU and AUTR models by bisulfite pyrosequencing and **(C)** methylation-specific polymerase chain reaction (MSP) analysis **(D)** Protein expression of all selected genes (*TGFBI*, *KILLIN*,

CXCL2 and *SLC38A1*) in AU and AUTR models before and after 5-aza-dC treatment by Western blot. In MSP, the presence of visible polymerase chain reaction products in lanes marked U, indicate unmethylated sequences, while the presence of products in lanes marked M indicate methylated sequences. *In vitro* methylated DNA (IVD) was used as a positive control for methylated sequences. DNA from normal lymphocytes (NL) was used as a negative control for methylated sequences. Results shown are representative of those obtained from three independent experiments and β-actin was used as a control. Values from bisulfite pyrosequencing and qRT-PCR were determined from triplicates and are expressed as the mean ±SEM. Significance of the Mann-Whitney U test, **P<0.01; *P<0.05.

2.5.- The role TGFBI expression plays in the trastuzumab-resistant HER2+ model (SKTR)

We have shown that *TGFBI* promoter hypermethylation is highly associated with the downregulation of its transcript and protein levels in two trastuzumab-resistant models (SKTR and AUTR) when compared to the corresponding sensitive models (SK and AU). Therefore, we next sought to demonstrate whether the epigenetic inactivation of the *TGFBI* gene functionally contributed to trastuzumab resistance. We depleted endogenous *TGFBI* gene expression in the SK model by stable transfection with two different short hairpin RNAs (Sh2 and Sh4). Previously, five sh-sequences targeting different regions of *TGFBI* RNA transcript were designed and evaluated by Western blot and qRT-PCR, but only two of them (Sh2 and Sh4) had shown good efficiency (Figure 22A). Scrambled shRNA (scramble) was used as a control. Furthermore, we also rescued *TGFBI* expression in hypermethylated *TGFBI* and in the downregulated SKTR model by stable transfection with a plasmid encoding the full-length of *TGFBI* cDNA (TGFBI). The empty vector (Mock) was used as a control.

TGFBI is an ECM and plays a role in mediating cell adhesion to the ECM and integrin-mediated signaling[431,432]. According to the bibliography, TGFBI protein is composed by 683 amino acids containing a secretory signal (SP) in the *N*-terminal cysteine rich domain (CRD), and four fasciclin-1 domains (FAS1-1, FAS1-2, FAS1-3 and FAS1-4), which contain several known integrin binding motifs, including NKDIL, YH and EPDIM as well as an Arg-Gly-Asp (RGD) domain. The RGD motif is found in many extracellular

matrix proteins modulating cell adhesion and serves as a ligand recongnition sequence for several integrins[409]. Furthermore, the four fasciclin-1 domains and the RGD-motif has been reported to function as an ECM protein to mediate cell adhesion and migration through interacting with collagen, fibronectin and laminin and several integrins including α1β1, α3β1, αvβ3, and αvβ5[432–434]. This integrin binding properties of TGFBI have been related to cell proliferation, adhesion, migration and differentiation[434]. For this reason, we became interested in determining whether this interaction between TGFBI with the ECM and integrins could be involved in the trastuzumab resistance in our models. For this reason, we became interested in determining whether this interaction between TGFBI with the ECM and integrins could be involved in the trastuzumab resistance in our models. With this objective in mind, we also expressed a mutated form of TGFBI with four different altered integrin binding motifs: the NKDIL motif (amino acids 354-358), the EPDIM motif (amino acids 617-621) and the YH motif (amino acids 563-580) in the second and fourth FAS1 domains, as well as the RGD motif in the C-terminus, affecting cellular adhesion through the integrin interactions (Figure 22B)[409,434,435]. The transfection and validation protocols were the same as the overexpression vector.

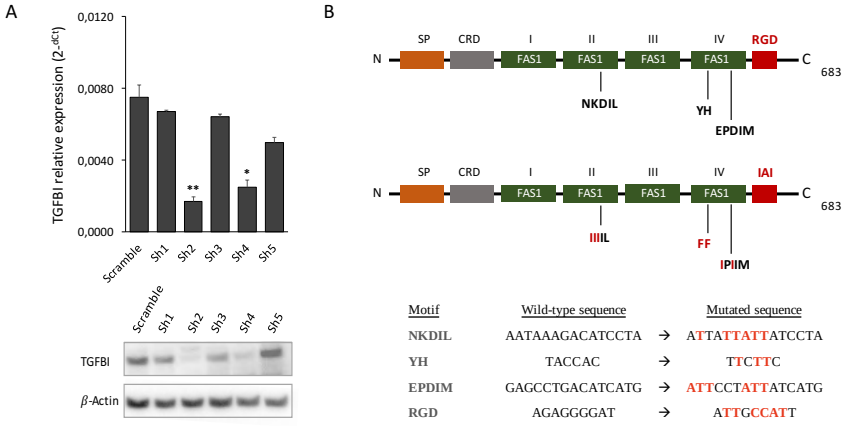


Figure 22. TGFBI short hairpin and mutagenesis vector designs. (A) Expression analysis (qRT-PCR) and protein levels (Western blot) showing the *in vitro* stable transfection of the five short hairpin designs, showing a greater inhibition efficiency for sh2 and sh4 compared to the other short hairpins designed. **(B)** Schematic diagram of the transforming growth factor-beta-induced (TGFBI or βig-H3) protein structure (68KDa). Secretory signal (SP) in the N-terminus cysteine-rich domain (CRD), four fasciclin-1 domains: FAS1-1(I), FAS1-2(II), FAS1-3(III), and FAS1-4(IV) and RGD motif are represented. Selected mutated motifs are represented in black and mutated amino acids in red. Values of qRT-PCR were determined from triplicates, normalized against the *GAPDH* gene and are expressed as the mean±SEM. ANOVA using a Bonferroni post-hoc test, ***P<0.001; **P<0.01; *P<0.05 indicate levels of statistical significance. Image modified from Miranda P. Ween *et al.*[409].

Before carrying out further studies, a silencing test experiment was performed to verify the short hairpin effectiveness and the overexpression vector design using a new methodology. After overexpressed TGFBI cell sorting, these cells were transfected with four different short hairpins: ShScramble (control), Shtomato1 (direct against the tdTomato fluorescent protein), Sh2 (short hairpin with better *TGFBI* inhibition efficiency) and Sh5 (short hairpin with lower *TGFBI* inhibition efficiency). The pLVX-IRES-tdTomato1 is a bicistronic vector that translates the *TGFBI* gene and the *tdTomato1* from a single mRNA. Therefore, we can induce the vector degradation either with a shRNA against the RNA sequence of encoding *TGFBI* or with shRNA directed against the RNA sequence of encoding *tdTomato1*. Upon transfection, cells were examined by fluorescence-activated cell sorting (FACS) and confocal immunofluorescence microscopy analysis. In addition, endogenous and exogenous TGFBI silencing

proteins were validated using the anti-FLAG antibody against the FLAG tag of exogenous TGFBI protein and anti-TGFBI against TGFBI protein by Western blot. These results corroborated the high efficiency an optimal design of the Sh2 and TGFBI overexpression vector (Figure 23A, B and C).

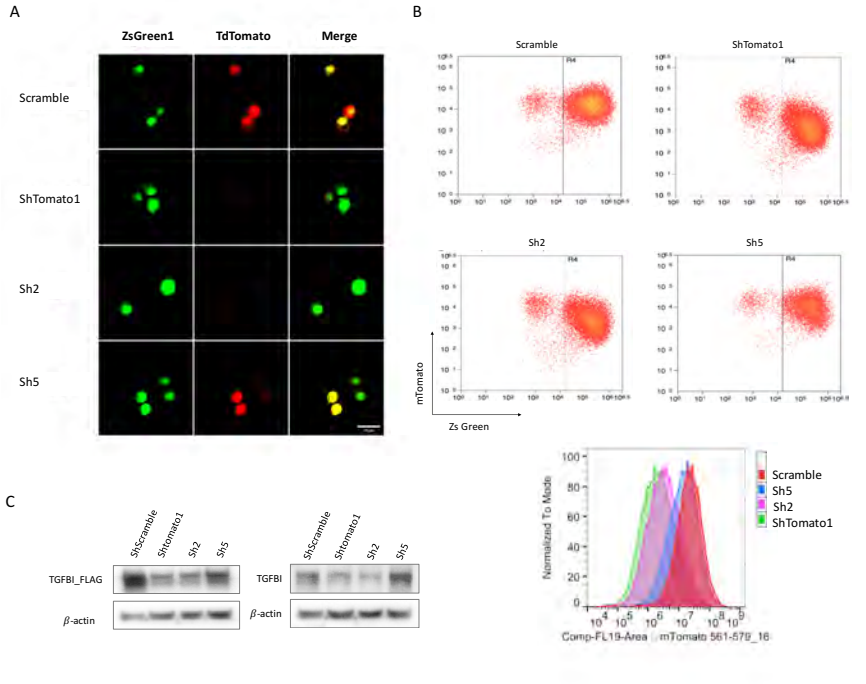


Figure 23. **Validation of short hairpin and overexpression vectors of TGFBI.** **(A)** Confocal images of TGFBI overexpressed models (red; tdTomato1 fluorescents protein) transfected with two different short hairpin RNA vectors (green; ZsGreen1 fluorescents protein). The ShScramble was used as a control. The shTomato1 directed against the tdTomato1 fluorescent protein produced the inhibition of tdTomato1 and TGFBI, disappearing the red fluorescence. Sh2 (short hairpin with better *TGFBI* inhibition efficiency) presented the same fluorescence pattern as ShTomato1. Sh5 had the same fluorescence pattern as the control demonstrating also its worst silencing efficiency. **(B)** ZsGreen1 fluorescent protein and tdTomato1 were used as markers for selection and analysis by flow cytometry. Like the confocal images, a lower percentage of red fluorescents cells were observed in ShTomato1 and Sh2 compared to the Scramble control. **(C)** Validation of TGFBI protein expression decreasing using anti-FLAG antibody against FLAG tag of exogenous TGFBI protein and anti-TGFBI against TGFBI protein by Western Blot.

Despite its known TGFBI function in cell adhesion and integrin-mediate signaling, we did not observe any morphological changes after depletion,

overexpression or mutagenesis of the *TGFBI* gene (Figure 24A). The efficiency of the transfection was assessed by measuring *TGFBI* expression using qRT-PCR and Western blot (Figure 24B). A significant change in its levels was detected both after depletion and overexpression. Upon TGFBI transfection, we analyzed the resistance or sensitivity to trastuzumab in comparison to the parental and empty vector-transfected cells through an MTT assay over five days. In the SK model, we observed that TGFBI depletion did not affect the trastuzumab response (Figure 24C, *Upper panel*). In contrast, overexpression of TGFBI in SKTR led to a significantly higher sensitivity at a trastuzumab concentration of 10 μM compared to the resistant empty-vector cell line (Mock: $p<0.05$) and the SKTR model ($p<0.01$; Figure 24C, *Middle panel*). The mutated TGFBI showed the same response to trastuzumab treatment as the mock vector did, suggesting that TGFBI-mediated trastuzumab sensitivity requires the NKDIL, YH, EPDIM and RGD binding motifs (Figure 24C, *Lower panel*).

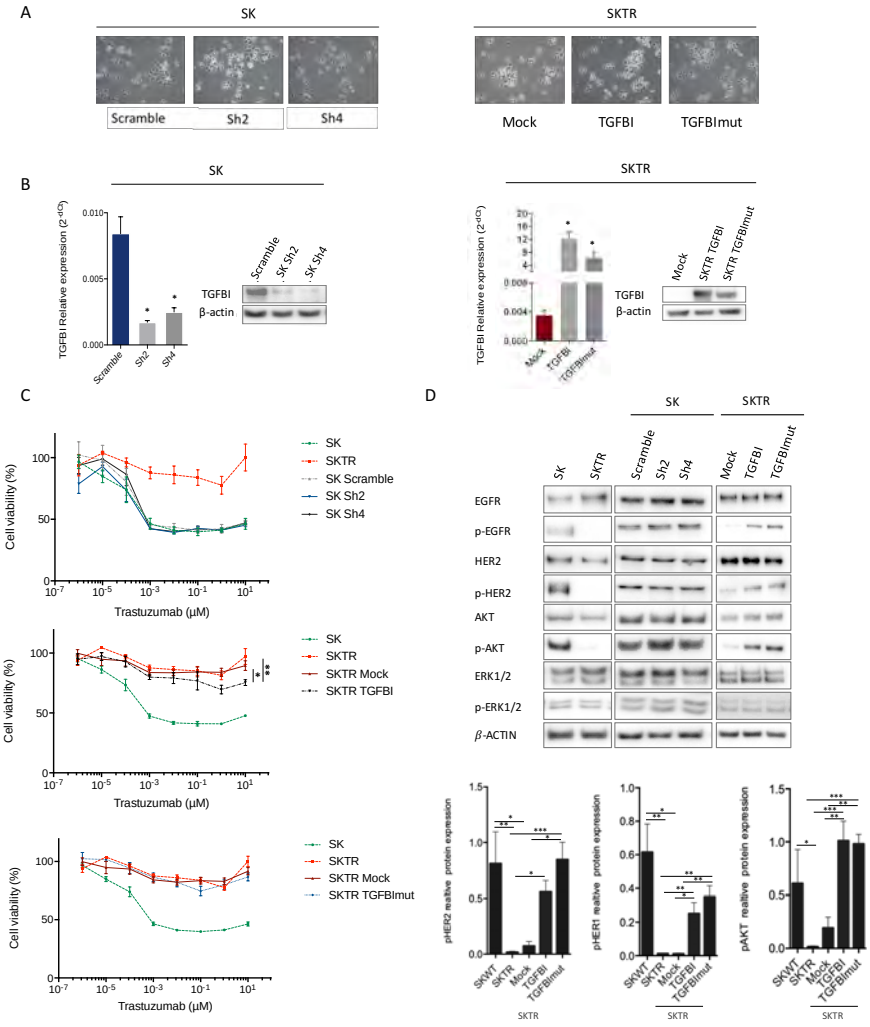


Figure 24. Impact of TGFBI expression in trastuzumab-resistant model. (A) Representative bright field microscopy images of TGFBI depletion (Scramble, sh2 and sh4), overexpression (Mock and TGFBI) and mutagenesis (TGFBImut) in SK and SKTR cells. **(B)** Expression analysis by qRT-PCR and Western blot showing the *in vitro* stable depletion of TGFBI in SK cells (left) and overexpression or mutagenesis of TGFBI in SKTR cells (right). Values of qRT-PCR were determined from triplicates, normalized against the *GAPDH* gene and are expressed as the mean±SEM. **(C)** Cell viability determined by MTT assays upon the use of increasing concentrations of trastuzumab (10^{-6} to 10 μM) for 5 days. (*Upper panel*) The TGFBI depletion in SK did not affect cell viability upon trastuzumab treatment. (*Middle panel*) The TGFBI overexpression in SKTR cells (TGFBI) give rise to a major sensitivity to trastuzumab at 10μM. (*Lower panel*) The TGFBI-mutagenesis in SKTR cells (TGFBImut) did not affect cell viability after trastuzumab treatment. Results are expressed as a percentage of surviving cells after drug treatment (mean±SEM). **(D)** HER family receptors and their downstream proteins related to PI3K/AKT and MAPK/ERK1/2 pathway characterization in TGFBI-depletion SK and

TGFBI-overexpression SKTR models. The protein analysis shows a significant enhancement of phosphorylation levels of HER1, HER2 and AKT upon overexpression and mutagenesis of TGFBI in SKTR cells. TGFBI depletion did not produce any change in the HER receptors and their related downstream proteins. Western blot band intensities were quantified using ImageJ. Results shown are representative of those obtained from three independent experiments and β-actin was used as a control. ANOVA using a Bonferroni post hoc test, $^{***}P<0.001$ $^{**}P<0.01$; $^{*}P<0.05$ indicates levels of statistical significance.

A previously published study by our group demonstrated how different activation patterns of some HER receptors and their downstream signaling proteins were involved in the molecular mechanisms of acquired trastuzumab resistance (SK and SKTR)[401]. For this reason, we extended this study to include evaluating the possible changes in HER family protein receptors and their downstream proteins related to the PI3K/AKT and MAPK/ERK1/2 pathways after TGFBI depletion, overexpression and mutation. Consistent with the results from the trastuzumab cell viability, TGFBI depletion in SK cells showed no apparent changes in either total protein or activation levels for any of the proteins examined. Unlike TGFBI depletion, TGFBI overexpression and mutation (TGFBImut) in the SKTR model produced some changes in the activation levels of some of the HER receptors and downstream signaling proteins in comparison with the mock control vector (Figure 24D). In particular, overexpression of either wild type TGFBI or its mutated form resulted in a higher or significant increase in the activation levels of HER1 (pHER1), HER2 (pHER2) and AKT (pAKT) compared to the SKTR model (Mock) that contains hypermethylated *TGFBI* without change in total levels of the respective proteins. Hence, TGFBI overexpression and its mutated form allow the trastuzumab-resistant model to adopt similar activation levels for HER1, HER2, and AKT as the trastuzumab sensitive cells do.

Although additional functional studies' validation is required, the results suggest that selective overexpression of TGFBI in the SKTR model (hypermethylated for *TGFBI*) induces an increased sensitivity to trastuzumab and the activation of HER1 and HER2 receptors as well as the AKT downstream protein. Furthermore, the mutated domains from the second and fourth FAS1 regions of TGFBI are involved in its response to trastuzumab treatment, but not

in its activation or interaction with HER2 downstream proteins. In summary, the trastuzumab-resistant model with overexpression of TGFBI presents a behavior similar to that of the trastuzumab sensitive model.

3.- BIOMARKER CANDIDATE GENE VALIDATION IN HER2+ BREAST CANCER HUMAN SAMPLES

Given the previous *in vitro* results obtained, we wanted to determine whether the presence of the *TGFBI* promoter CpG island hypermethylation-associated to trastuzumab resistance was also occurring in BC patients. Therefore, we evaluated the *TGFBI* gene methylation in tumors from 24 HER2+ early BC patients as a preliminary study. We determined by pyrosequencing analysis the DNA methylation levels of 3 consecutive CpG sites in the 5'-end promoter CpG island of *TGFBI* for all collected human samples. As with our sensitive and resistant models, we analyzed *TGFBI* methylation in pre-treatment samples from patients who were complete responders and non-responders to trastuzumab-based therapy. With the non-responder patients, we also evaluated *TGFBI* methylation after treatment (post-treatment samples). For the non-responder patients, we obtained paired samples (pre-treatment and post-treatment samples) from 10 patients, pre-treatment samples only from 3 patients, and post-treatment samples only from 7 patients. For the patients with a pathological complete response, only pre-treatment samples could be evaluated (Figure 25A). The limited number of samples obtained was, in part, due to exhausting the sample during diagnostic procedures and the poor preservation of the DNA in FFPE blocks.

Our results showed similar pre-treatment *TGFBI* methylation levels ($p>0.05$) in the patients with complete response to trastuzumab (6.45%±1.90) and in the non-responders (6.08%±1.51; Figure 25B). These results indicate that the *TGFBI* methylation levels of the pre-treatment samples are not associated with the absence of response to trastuzumab. In contrast, the non-responsive patients showed significantly higher ($p<0.001$) methylation levels of *TGFBI* in tumors following treatment with trastuzumab (30.26%±3.52) than before starting the therapy (6.08%±1.51), suggesting that acquired resistance to trastuzumab is

associated with increased *TGFBI* methylation levels. In particular, when considering the non-responsive patients with pre- and post-treatment paired samples, we observed a significant ($p<0.001$) *TGFBI* promoter hypermethylation after trastuzumab (8 out of 10 = 80%) compared to the pre-treatment samples, suggesting that the increase in *TGFBI* methylation levels is associated with trastuzumab resistance (Figure 25C). Importantly, the ROC curve analysis with an area under the curve (AUC) of 0.9502 ($p<0.0001$; 95% CI: 0.8716 to 1.029) allowed us to clearly differentiate the methylation levels of *TGFBI* between the pre- and post-treatment samples of the non-responsive patients. This result suggests that TGFBI could be a potential biomarker for identifying and monitoring resistance to trastuzumab during HER2+ BC patients' treatment (Figure 25D). On the other hand, no significant association between *TGFBI* methylation levels before and after treatment and the clinical-histopathological characteristics was identified (Table 15).

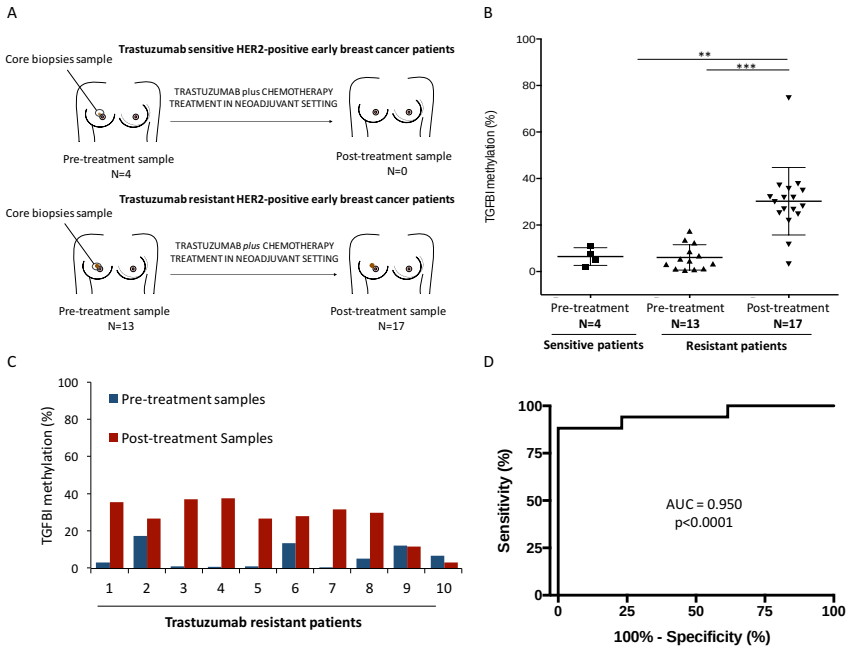


Figure 25. ***TGFBI* promoter hypermethylation in HER2-positive breast cancer patients with sensitivity and resistance to trastuzumab. (A)** Schematic representation of selected patient samples. *TGFBI* methylation levels were evaluated in tumor samples of 24 HER2+ breast cancer samples. From this cohort, after trastuzumab plus chemotherapy in neoadjuvant regimen, 20 patients developed partial or no response and 4 patients presented complete treatment response. Of the 20 patients with non-response, 10 patients had pre-treatment and the post-treatment samples, 3 patients with pre-treatment only samples and 7 with post-treatment only samples (*Upper panel*) The 4 patients with complete response to treatment only had pre-treatment samples (*Bottom panel).* **(B)** *TGFBI* methylation of 3 consecutive CpG sites in the 5'-end promoter CpG island in HER2+ breast cancer treated with trastuzumab analyzed by bisulfite pyrosequencing. The central solid line indicates the median and the limits of the vertical lines show the upper and lower percentiles. **(C)** *TGFBI* methylation of 3 consecutive CpG sites in the 5'-end promoter CpG island in resistant patients with paired pre- and post-treatment samples analyzed by bisulfite pyrosequencing. **(D)** Diagnostic accuracy of *TGFBI* hypermethylation for resistant HER2+ breast cancer samples. ROC analysis was applied to the *TGFBI* methylation levels analyzed by pyrosequencing for trastuzumab-resistant samples (pre- and post-treatment samples). Area under the curve (AUC) was 0.9502 ($p<0.0001$). *TGFBI* showed great potential for monitoring trastuzumab response in HER2+ breast cancer patients. Significance of the Mann-Whitney U test is indicated as ***P<0.001; **P<0.01.

Table 15. Clinical and pathological characteristics in HER2+ early breast cancer according to *TGFBI* promoter methylation levels between pre- and post-treatment human samples.

Characteristics	% N=10		p-Value*
	n	Median (p25-p75)	
Age	10	-0.383*	0.275*[1]
Menopause			0.151*[2]
Premenopausal	5	32.13 (24.58 – 35.86)	
Postmenopausal	5	14.58 (-0.68 – 25.77)	
ER			0.089*[2]
Negative	2	2.85 (-3.57 – 9.28)	
Positive	8	28.50 (19.58 – 34.00)	
HER2 status			-
FISH	0	-	
IHC +	10	25.18 (9.28 – 32.13)	
Histological Grade			-
Grade 1 - 2	10	25.18 (9.28 – 32.13)	
Grade 3	0	-	
Clinical Stage			0.804*[3]
IIB	2	16.15 (-3.57 – 25.86)	
IIIA	2	19.58 (14.58 – 24.58)	
IIIB	6	28.50 (9.28 – 32.13)	
Pathological response			-
No response	1	9.28	
Partial response	9	25.77 (14.58 – 32.13)	
Miller & Payne			1.000*[2]
G2	1	0.68	
G3	2	22.57 (9.28 – 35.86)	
G4	7	25.77 (19.58 – 31.68)	
Type of Surgery			0.267*2
Lumpectomy	2	10.51 (-3.57 – 24.58)	
Mastectomy	8	28.50 (11.93 – 34.00)	

[1] Spearman correlation
*[2] Mann-Whitney test
*[3] Kruskal-Wallis test

Chapter V: Discussion and future perspectives

1.- GENOME-WIDE DNA METHYLATION ANALYSIS IN TRASTUZUMAB, LAPATINIB AND TRASTUZUMAB *PLUS* LAPATINIB-SENSITIVE AND RESISTANT BREAST CANCER MODELS

HER2 is overexpressed in 20-30% of BC and is associated with the aggressive phenotype and poor prognosis[20]. HER2-targeted therapy has transformed the outlook for both early and metastatic HER2+ BC with the development of trastuzumab and the different anti-HER2 agents such as lapatinib[112]. Despite the good initial response to the treatment, a large fraction of patients presents *de novo* or acquired treatment resistance. Currently, HER2 detection is the only validated biomarker for predicting the benefit of anti-HER2 therapies[88]. While different cellular and molecular mechanisms involved in trastuzumab resistance have been described, none of them are used to predict, detect or monitor BC treatment in a clinical routine[54,108,134]. DNA promoter methylation is considered a potential epigenetic biomarker that possesses valuable information regarding resistance and prognosis. In recent years, alterations in DNA promoter methylation have been associated with several diseases including BC[288,351,436]. Therefore, it is of great interest to find potential epigenetic biomarkers for trastuzumab and lapatinib resistance that are sufficiently accurate to stratify patients and save costs and toxicities for the patients who do not respond to treatment.

For several years, trastuzumab and lapatinib have been the most commonly used treatment for HER2+ BC. For this reason, three trastuzumab and lapatinib-resistant models, SKTR, SKLR and the patented SKLTR[437], were developed and published previously in our laboratory[401]. Here, a novel dual trastuzumab *plus* lapatinib (T→L) long-term exposure model was developed and characterized (SKTLR) as an *in vitro* model of HER2+ MBC patients. Several mechanisms have been described for trastuzumab and lapatinib resistance in the last few years, some of them concerning changes in the HER receptor family and HER2 pathway. The SKTLR model showed overexpression of p-EGFR, p-HER4 and p-AKT and, to a lesser extent, in EGFR and p-HER2 (compared to its control SKTR) to overcome the anticancer effects of both anti-HER2 agents. Conversely,

p-HER3 presented decreased levels compared to SKTR. The SKTLR model presented a similar HER receptor and HER2 downstream pattern to the previously developed SKLTR. The increased expression and activation of HER1 and the PI3K/AKT signaling pathway has been demonstrated to have an essential role in the resistance acquisition of both anti-HER2 drugs[143,144,438,439]. It has been identified that HER3 overexpression leads to HER2-HER3 heterodimer formation consequently activating the PI3K/AKT pathway. However, the overactivation of HER2 besides HER1, and the decreased p-HER3 expression in the SKTLR model could be accomplished through dimerization of the HER2 receptor with other HER receptors such as HER1 or HER4, when cells acquired the resistance[401]. HER4 activation has been detected in both the SKTLR and SKLTR models, suggesting its activation is a molecular mechanism of double-resistance to anti-HER2 therapies. Moreover, an increased expression of HER1-HER4 ligands including EREG and HB-EGF has been identified in SKLTR model[401]. In fact, another report has been described HER4 role in the development and sustaining of anti-HER2 (trastuzumab and lapatinib) treatment resistance in BC cells[440].

DNA methylation alteration contributes to neoplastic development through its involvement in the initiation, promotion, invasion, metastasis and treatment resistance[227,293,441,442]. For example, Lindqvist BM *et al.* demonstrated a different methylation profile for HER2+ BC tissue samples compared with normal breast tissue using a whole genome DNA methylation signature[423]. Interestingly, DNA methylation has also been described to be involved in treatment resistance mechanisms in cancer[443–445]. Therefore, we wanted to evaluate the role of DNA methylation in trastuzumab and lapatinib resistance in our models (SK, SKTR, SKLR and SKTLR) using the 450k methylation array. Taking into account all the CpGs analyzed, SKTR, SKLR and SKTLR presented differentially methylated CpGs with respect to the sensitive model (SK), highlighting the role the DNA methylation pattern has in treatment resistance phenotype. The SKLR model presented the highest correlation methylation CpGs levels compared to the SK. Probably, other non-epigenetic factors are more critical for the lapatinib resistance phenotype, such as post-translational mechanisms or other cytoplasmic

mechanisms. In contrast, the SKTLR model presented the most diverse methylation pattern compared to SK, probably because the acquisition of multidrug resistance alters a greater number of molecular pathways compared to mono-resistance. As expected, all resistant models analyzed presented the typical features of cancer methylation patterns with a more poorly methylated CpGs and less highly methylated CpGs. It is well-known that during carcinogenesis, a site-specific DNA hypermethylation and a global DNA hypomethylation (associated with genetic instability in cancer cells) take place[295].

In summary, we demonstrated the involvement the DNA methylation pattern has in trastuzumab, lapatinib and trastuzumab *plus* lapatinib resistance in our HER2+ BC models, reinforcing the role of this epigenetic modification in cancer treatment resistance disclosed in other papers.

2.- IDENTIFICATION OF GENES RESPONSIBLE FOR TRASTUZUMAB, LAPATINIB AND TRASTUZUMAB *PLUS* LAPATINIB RESISTANCE IN OUR HER2+ BREAST CANCER MODELS

Epigenetic biomarker studies are focused on analyzing the methylation changes in the promoter regions of candidate genes in specific tumor types for their implication in gene silencing[54,293]. In general, this gene repression results in an adaptive advantage for the cells, allowing them to adopt a more aggressive and invasive phenotype.

The DNA methylation analysis in promoter and island regions of our resistant models allow us to differentiate each model clearly. The identified differential promoter methylated genes between mono-resistants and double-resistant models were associated to several biological processes related to cancer mainly cell adhesion, cell proliferation pathways or transcription regulation, among others. Our results are in accordance with the results obtained by Zhang L. *et al.* who studied the expression pattern between the lapatinib-sensitive and -resistant models. They observed that most differentially expressed genes between both cellular models were mainly related to different biological functions like cell

adhesion, proliferation, and motion[446]. Currently, tumor microenvironment and their cross-talk have been described as playing an important role in cancer development and progression, regulating cell-cell and cell-matrix communications, cell migration, invasion and metastatic dissemination and, at the same time, can also condition the efficacy of antitumor therapies[447–449]. Several types of cancer cells have been shown to use their interactions with the surrounding environment to acquire drug resistance, either within the primary tumor or the context of disseminating metastases to distant organs (reviewed in [450]). In fact, several reports have linked trastuzumab and lapatinib resistance to the extracellular matrix proteins[451,452]. Therefore, it is not surprising that the genes with the highest DNA methylation differences were related to cell adhesion or cell proliferation in our resistant models. Our data also revealed other enriched categories, including items associated with multiple different cellular pathways and processes, demonstrating a large number of pathways and process that could be altered during trastuzumab and lapatinib resistance.

In this doctoral thesis, the DNA promoter and island methylation analysis and the RNA-Seq data were integrated to create a powerful tool with which to detect genes epigenetically regulated and involved in trastuzumab or lapatinib resistance in our cellular models. Interestingly, the SKTR vs. SK and SKLR vs. SK model comparison showed a high number of hypermethylated and downexpressed genes compared with the hypomethylated and overexpressed genes, which is consistent with the common promoter methylation pattern described in cancer cells[453]. As explained earlier, some reports of promoters who were hypomethylated in cancer have been published; however, it is generally accepted that the balance weight leans toward hypermethylation of tumor controlling genes[287]. Our study shows that promoter hypomethylation is as broadly distributed as promoter hypermethylation in our SKTLR vs. SKTR and SKLR comparison, suggesting a role for promoter hypomethylation in the double-resistance acquisition. Although promoter hypermethylation has been mainly associated with gene silencing, a low number of genes with methylation and expression correlation were identified in our study. A previous study described that only 15% of the significantly differentially methylated genes using the methylation array 450K showed a

correlated change in mRNA expression in pediatric acute lymphoblastic leukemia[454]. Different reasons may be able to explain this low number of genes with methylation and expression correlation. For instance, the interaction between the epigenetic machinery components (Chapter I, section *5.4.- Interplay between the components of the epigenetic machinery*). Gene expression can be regulated by a complex network of DNA methylation, histone modification and ncRNA processes[252,257,258,268]. Therefore, some gene expression could be regulated by epigenetic mechanisms other than DNA methylation. On the other hand, the non-direct expression control by CpG methylation in the promoter region could be another hypothesis, suggesting gene expression regulation by enhancers that impact the transcription while lying distal to the transcription start site[455]. Therefore, maybe the DNA methylation differences in enhancer sequences cannot be correctly detected with the 450K analysis[456]. Currently, a new platform for DNA methylation studies (Methylation EPIC BeadChip Infinium) has been developed. It can analyze 853,307 CpG (850K) and appears to be a promising tool with which to address these issues as it incorporates 33,265 CpG sites located in the enhancer regions[457].

The subsequent qRT-PCR and demethylating agent treatment validation techniques allow us to identify epigenetically regulated genes as potential epigenetic biomarkers in each comparison. *TGFBI, KILLIN, CXCL2* and *SLC38A1* were the four genes validated for the SKTR vs. SK comparison for both methodologies. The downregulation of *TGFBI* and its epigenetic regulation have been described in a variety of human tumor cell lines[458–460]. Meanwhile, *CXCL2* up-regulation has been linked to chemotherapy resistance in BC[461,462]. Contrary to our results, the overexpression of *SLC38A1* (also known as *ATA1/SNAT1/SAT1)* has been associated with an advanced tumor stage and nodal metastasis. Probably, the positive correlation between SLC38A1 and AKT expression demonstrated previously, could explain the downregulation of *SLC38A1* identified in our SKTR model (present low p-AKT levels)[417]. Unlike other genes, *KILLIN* or *KLLN* was selected from the methylation array analysis because of their previously described implications in BC. *KILLIN* is located in the 10q23.31 chromosomal region, proximal to PTEN. *PTEN* and *KILLIN* share

the same transcription start site and are assumed to be regulated by the same promoter but transcribed in opposite directions[418]. The DNA promoter methylation and epigenetic regulation of *KILLIN* had been previously described in renal cancer[463] and Cowden Syndrom[464]. More interestingly, Ng E. *et al.* observed increased DNA methylation of *KILLIN/PTEN* in the plasma of patients with thyroid and BC[418]. Furthermore, the loss of PTEN has been well described as an underlying mechanism that increases after trastuzumab treatment[146].

Several genes with methylation and expression correlation were identified as potential candidate biomarkers for trastuzumab, lapatinib, and trastuzumab *plus* lapatinib resistance. Due to the retrospective study design, we could not obtain samples from patients with advanced or MBC or tumor samples treated with lapatinib as single agent to validate our selected genes. That said, their validation as biomarkers of lapatinib and trastuzumab *plus* lapatinib resistance in future studies could be relevant to stratify patients for whom treatment with T+L or L could be useful or not.

For the SKLR comparison the only gene selected, *TXNDR1* (hypomethylated and overexpressed in SKLR), was not validated by qRT-PCR. TXNDR1 (which plays a key role in oxidative stress control) has been identified overexpressed in different cancers including node-negative breast[422], lung[465–467] and oral squamous cell carcinomas[468]. Furthermore, Cadenas C. *et al.* suggest that HER2 could influence the expression of TXNDR1[422]. Although it has been identified as a good correlation between RNA-Seq and qRT-PCR methods, the qRT-PCR primers design or the biased results analysis produced from the RNA-Seq analysis could explain the differences between both methods[469,470].

Different genes were identified as potential biomarkers for the double-resistant model. Taking into account both comparisons (SKTR vs. SKTLR and SKLR vs. SKTLR) the *ANXA3* and *PPARG* genes appear to be epigenetically regulated in double-resistant acquisition. *ANXA3* is a membrane associated protein from the calcium-dependent phospholipid-binding proteins family and plays a crucial role in a diverse range of molecular and cellular processes such as

cell differentiation, migration, proliferation, immune regulation, anti-inflammation processes, and bone formation[471–474]. Furthermore, it has been described as being involved in tumor progression, metastasis and drug resistance processes[473,475]. The *ANXA3* gene was epigenetically repressed during trastuzumab *plus* lapatinib resistance in our models, suggesting its possible role as a tumor suppressor gene. These results were correlated with its role in prostate[476] and renal carcinomas[477,473]. In contrast, ANXA3 overexpression has been related to a tumor promoter role in other cancer types such as ovarian[478], colorectal[479] and lung cancer[480], as well as in hepatocarcinoma[481]. Hence, all this evidence revealed that ANXA3 might specifically function either as a tumor suppressor or tumor promoter gene depending on the tumor and tissues type. *PPARG* (or *PPARγ*) is a transcription factor gene with the same pattern as *ANXA3* and was found to be hypermethylated and downexpressed in SKTLR in both double-resistant comparisons. PPARG is a member of a family of three PPARs that control the expression of a network of genes involved in adipogenesis, lipid metabolism, inflammation and the maintenance of metabolic homeostasis and has been linked to human BC[482–484]. Furthermore, PPARG expression alteration or mutation has been associated with tumorigenesis[485–487]. Jiang WG *et al.* showed that lower levels of PPARG were associated with local recurrence of BC when compared to those who remained disease free[488]. Besides this, a recently published paper revealed in a lapatinib-resistant model the up-regulation of PPARG similar to the PPARG expression in our resistant model (SKLR)[446]. Hence, according to our results and the bibliography, *ANXA3* and *PPARG* genes could have a potential role as trastuzumab *plus* lapatinib resistance biomarkers in HER2+ BC.

Apart from the genes selected from each comparison, other different epigenetically regulated genes were identified in more than one comparison and will be relevant for future studies. The *ANGPTL4*, *CYP4X1*, *ENTPD3*, *KCNJ12*, *IRX6* and *RIN3* genes were found to be hypermethylated and downexpressed in both mono-resistant models suggesting their possible role in resistance mechanisms independent of the anti-HER2 agents to which it is resistant. *ANGPTL4* has been described as a genetically and epigenetically inactivated

tumor suppressor gene that inhibits tumor angiogenesis, which is in correlation with our results[489]. To our knowledge, this is the first time that the *CYP4X1, ENTPD3, KCNJ12, IRX6* and *RIN3* genes have been linked to a tumor suppressor function in BC. Interestingly, *PTRF* (also known as *Cavin1*) was hypermethylated and downexpressed in SKTLR compared to SKLR and in SKTR compared to SK. This observation suggests that *PTRF* promoter hypermethylation is probably due to the trastuzumab treatment. In accordance with our results, the *PTRF/cavin-1* mRNA levels have previously been described as being associated with DNA methylation and loss of expression in other cancer such as prostate[490], lung cancer[491] and also BC[492,493] and have been demonstrated to be related with tumor progression[494]. The hypomehtylated and overexpressed *EID3* and *TXNDR1* genes in SKTLR and SKLR from SKTLR vs. SKTR and SKLR vs. SK comparisons, suggest possible promoter hypomethylation due to the lapatinib treatment. Little bibliography information has been obtained on *EID3* in BC. EID3 has been described as a negative regulator of cellular differentiation by binding to HDACs or HATs (CREB-binding protein/p300) and suppressing transcription[495–497]. Furthermore, *EID3* is involved in cell differentiation inhibition and cancer stem cell formation in colorectal cancer[498]. Therefore, as far as we know, this is the first time the possible role *EID3* has in BC and lapatinib BC treatment resistance has been described. The role *TXNDR1* plays in cancer has been previously described in the SKLR vs. SK comparison.

2.1.- Potential epigenetic biomarkers for trastuzumab resistance in HER2+ breast cancer

Taking trastuzumab as the basis of HER2+ BC treatment, we focused our analysis on potential biomarkers for trastuzumab resistance. As explained before, biomarker development involves multiple processes, i.e., linking initial discoveries in cell lines, validating these and clinical implementation. Therefore, all four selected (*TGFBI, KILLIN, CXCL2* and *SLC38A1*) genes identified were validated employing different methylation and expression approaches. Additionally, the selected genes were subsequently analyzed in another long-term trastuzumab-sensitive (AU) and -resistant (AUTR) human HER2+ BC model

developed previously by our group[402]. Although both cell lines analysed derive from the same patient and have similar genetic and transcriptomic profiles, they have some differences in transcriptional levels[499]. Moreover, SKBr3 and AU565 have been described as having distinct responses to drug treatment depending on culture conditions[452]. From all the genes selected, *TGFBI* was the only one with a consistent methylation, expression and protein pattern in both resistant models. This epigenetic regulation by promoter hypermethylation of the *TGFBI* gene observed is in line with previous studies in other cancer cell lines including lung and prostate[460,500]. In addition, Shao *et al*. showed that mammary cell line MCF-7, MDA-MB231 and MDA-MB361 do not present a *TGFBI* promoter hypermethylation, which is in correlation with our results in the SK model.

TGFBI (also known as Betaig-H3 or Keratoepithelin) was first identified during the 1990s, when it was isolated from human lung adenocarcinoma cell line (A549) which had been treated with transforming growth factor-β (TGF-β)[501]. *TGFBI* encodes a 68KDa secretory protein induced by TGF-β in human adenocarcinoma cells as well as other human cell types[502]. Briefly, TGFBI protein contains four fasciclin-1 (FAS1) domains and the RGD-motif and has been reported to function as an ECM protein to mediate cell adhesion and migration through interacting with collagen, fibronectin and laminin and several integrins including α1β1, α3β1, αvβ3, and αvβ5[432,433,503]. Mutations of the *TGFBI* gene have been shown to be involved in several corneal dystrophies[504,505]. Furthermore, TGFBI is involved in cell growth, cell differentiation, wound healing, apoptosis and tumorigenesis[458,459,506,507]. While TGFBI has also been reported to be involved in tumorigenesis, its role is not clear. In our study, we have linked the promoter DNA methylation-associated silencing of the *TGFBI* gene with a possible tumor suppressor function in the trastuzumab-resistant model. Several previous reports have indicated that TGFBI plays the role of a tumor suppressor gene in various cancers such as lung, breast and ovarian[415,460,500,508,509]. However, in other cancers, such as colon or pancreas, it has been described as having a tumor promoting function[510,511]. These opposing effects of TGFBI suggest that its expression and function are dependent on cell type[409].

As mentioned before, TGFBI regulates cell adhesion by interacting with different integrins which, in turn, has been shown to play an important role in tumor progression in humans[512–514]. Different integrins have been related to trastuzumab resistance such as β1 or α6β[171,515]. Furthermore, a direct interaction between the HER2 receptor and different integrins has also been described in multiple reports[171,516–520]. This interaction is carried out through the integrin binding motifs in TGFBI protein, including the well-characterized RGD motif along with the NKDIL, EPDIM and YH motifs in the second and fourth FAS1 domains[409]. For this reason, different functional studies were carried out to determine whether TGFBI has a potential role in trastuzumab resistance or not. In our hands, TGFBI overexpression in SKTR significantly sensitizes the cells to the treatment, and activates some downstream HER2 protein pathways (pAKT, pHER1, and pHER2), adopting a similar pattern to SK. In contrast, TGFBI knockdown in endogenous TGFBI expressed in the SK cells did not produce any effect. In addition, an analysis of TGFBI with integrin-binding mutated motifs was performed to investigate the role of the RGD and NKDIL, EPDIM and YH integrin-binding domains in trastuzumab response[409]. Like TGFBI overexpression, the TGFBI mutated form induced the activation of different HER2 proteins downstream but there were no differences in trastuzumab treatment response. Therefore, it is likely that the four mutated domains of TGFBI are required for it to function in trastuzumab response; which is consistent with other studies that described the involvement of FAS1 domains in tumor angiogenesis, tumor growth inhibition and in promoting apoptosis[509,521]. Moreover, the RGD motif has also been described as mediating TGF-β induced apoptosis in ovarian cancer[507]. For example, it has been described that FAS1 and αvβ3 integrin interaction exerts anti-angiogenic activity *in vitro* and *in vivo*, leading to tumor inhibition[521,522].

The different HER downstream pathway changes observed in TGFBI overexpression or mutagenesis suggest interaction between TGFBI and some of the HER receptors and downstream proteins independent of the altered integrin-binding motif. Little information has been found about the possible TGFBI and HER pathway interaction. Wen G. *et al.* described the involvement of TGFBI in

mesothelioma progression through AKT/mTOR pathway[508]. The authors hypothesized that the absence of TGFBI, which may bind to the extracellular domain of the receptor, can produce enhanced autophosphorylation of the receptor, which results in more activated downstream signaling pathways. In summary, our study suggests that TGFBI expression may promote the effectiveness of trastuzumab treatment.

Although more *in vitro* functional analysis is necessary to elucidate the role of TGFBI in trastuzumab resistance, we suggest some hypothetical situations taking into account all the action mechanisms TGFBI has. TGFBI has been described as possibly being able to inhibit cell adhesion to various ECM proteins, inhibiting cell proliferation and invasion in neuroblastoma[523]. Cell adhesion to ECM has been consistently reported as one of the mechanisms used by tumor cells to resist chemotherapy[524]. These observations are in correlation with previous studies in our group, where our SKTR model presented a highly significant adhesion capacity to bind to extracellular matrix proteins like fibronectin, collagen I, collagen IV or laminin I compared to SK (data not shown)[401]. Therefore, we hypothesized that in the absence of TGFBI, the integrins could bind to the ECM providing attachment sites for cells and inducing more invasion, or acting as a physical drug barrier, restricting drug transport and limiting their efficacy (Figure 26A). The intratumoral diffusion and/or physical masking of HER receptors by ECM proteins have been described as a mechanism that can affect the therapeutic efficacy of some drugs including trastuzumab[451]. This hypothesis also correlates with the non-sensitivity to trastuzumab treatment observed in TGFBI with mutated integrin-binding motifs. On the other hand, when TGFBI has secreted, its union with integrins could inhibit the ECM-integrin interaction. As a consequence, not only is focal adhesion kinase (FAK) activated, but also the HER2 and EGFR receptors and their downstream pathways.

Another hypothesis could be related with the ability of integrins to regulate HER2 traffic to the membrane (Figure 26B). The αv-integrin expression has been described as possibly being able to modulate HER2 localization. Sangeet Lal *et al.* showed that αv-integrin knockdown altered HER2 localization from its

normal membrane position to a predominantly lysosome localization[525]. Hence, we hypothesize that TGFBI-integrin interaction may regulate the correct localization of HER2 into the membrane where it can be recognized by trastuzumab. This hypothesis corroborates that TGFBI overexpression in the SKTR model sensitizes the cells to the treatment, whereas the TGFBImut does not. In both hypotheses, the HER2 receptors and their downstream pathway activation in TGFBImut, could be related to a TGFBI-HER2 interaction independent of the integrin-binding domains or the involvement of TGF-β. In BC, the functional cross-talk between HER2 and TGF-β signaling has been identified previously and is known to be involved in increased cancer cell proliferation, survival and invasion, accelerating metastasis in animal models and resistance to chemotherapy and anti-HER2 therapy[526,527]. Briefly, TGF-β through a Smad complex regulates the expression of different genes or can activate directly other pathways including ERK, p38MAPK or PI3K[528–530]. In cancer, TGF-β has been described as playing a dual role as an oncogene and tumor-suppressor gene[530].

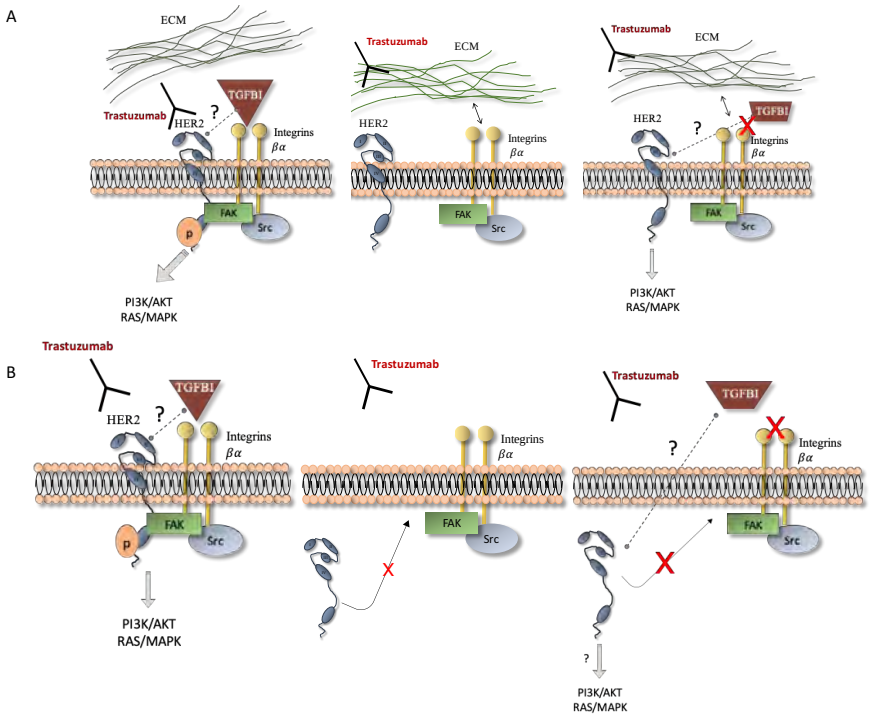


Figure 26. Schematic representation of hypothetical TGFBI roles in our trastuzumab-sensitive and –resistant cellular model. (A) Schematic representation of our first hypothesis in which TGFBI regulates the ECM-integrin interaction. Without TGFBI, the ECM-integrin interaction provides attachment sites for cells, inducing more invasion or masking the HER2 receptor and trastuzumab cannot recognize it. If TGFBI presents the integrin-binding domains as mutated, ECM-integrin interaction can occur along with the TGFBI and HER2 cross-talk (not described), activating the AKT-PI3K pathway. (**B)** Schematic representation of our second hypothesis in which TGFBI induces through its integrin interaction, the correct localization of HER2 into the membrane. If TGFBI is not present, HER2 remains incorrectly localized and could be activated by the TGFBI itself regardless of the integrin binding domains or other pathways such as TGF-β.

In summary, we identified different epigenetically regulated genes as potential biomarkers of trastuzumab and/or lapatinib resistance in HER2+ BC and would like to highlight the identification and validation of the *TGFBI* gene as a potential epigenetic biomarker for trastuzumab response in HER2+ BC. In addition, we also showed that ectopic expression of TGFBI in the SKTR model increased its sensitivity to trastuzumab treatment, suggesting that the TGFBI integrin-binding domains are involved. Our findings provide a rationale for the further investigation of *TGFBI* epigenetic silencing by DNA methylation in trastuzumab resistance in HER2+ BC.

3.- *TGFBI* HYPERMETHYLATION AS AN EPIGENETIC BIOMARKER IN HER2+ BREAST CANCER HUMAN SAMPLES

Biomarker validation in human samples is a necessary step in biomarker discovery. Hence, *TGFBI* promoter methylation has been analyzed in the HER2+ BC patient cohort who were treated with neoadjuvant anthracycline-taxane-based chemotherapy *plus* trastuzumab. *TGFBI* hypermethylation was analyzed before (*core biopsies*) and after (*tumor samples*) trastuzumab treatment in FFPE tumor samples. The FFPE is an invaluable resource for oncology studies because it is the most widespread method used to store tissue in hospitals. The fixation process and long-term storage of tissue embedded in paraffin can lead to DNA damage due to fragmentation, nucleotide base lesions, and/or modified bases, among others, resulting in poor quality DNA[531,532]. For these reasons, because of its easy standardization and lower false positive rate, the pyrosequencing method was chosen as an affordable and quantitative method that counterbalances some weaknesses of previous and extensively-used methodology such as MSP[533]. Furthermore, different groups have shown that pyrosequencing-based assay is a suitable methodology for analyzing short DNA sequences (usually <150bp) such as those extracted from FFPE tissue[412,534–536]. Therefore, we used pyrosequencing methodology to measure *TGFBI* DNA promoter methylation in our human tumor samples.

In our preliminary study, a higher *TGFBI* methylation level after treatment was observed in patients who had developed treatment resistance when compared to their pre-treatment samples. These results are in accordance with other studies which demonstrated an association between *TGFBI* hypermethylation and poor prognosis in prostate and lung cancer[537]. Consistently, Calaf, G. M., *et al.* identified a decreased *TGFBI* protein expression from bening tissues to invasive ductal breast carcinoma (the most malignant tissue analyzed) in 192 cases of breast tumors by IHC[538].

Due to the small size of the patient cohort and the retrospective design, the role of *TGFBI* methylation as a biomarker requires further validation in a larger

and independent cohort. Nevertheless, the present study brings to light for the first time that *TGFBI* methylation has a significant discriminative value between pre- and post-treatment samples, as demonstrated by the ROC curve analyses. Interestingly, the *TGFBI* promoter methylation analysis in responder patients showed similar methylation levels to the pre-treatment samples of non-responders. This observation suggests the possible role *TGFBI* has as a monitoring biomarker for trastuzumab response in patients with HER2+ BC. Pajares M J *et al.* also described TGFBI as a biomarker of treatment response in lung cancer. The authors identified high levels of TGFBI protein in patients with stage IV NSCLC who respond to chemotherapy, meanwhile patients who did not respond showed low levels[539].

Although no significant association was found between *TGFBI* methylation before and after treatment and patients' clinical-histopathologic characteristics, a high number ER-positive patients (80%) was observed. These results are consistent with other preclinical data that showed an increase of ER activity as an escape pathway (probably via PI3K) in ER+/HER2+ cells exposed to trastuzumab and lapatinib[107]. Furthermore, ER status has been known to be associated with pCR in HER2+ BC[107,128,540,541]. In addition, although the type of surgery is not associated with a higher *TGFBI* methylation level in post-treatment samples, 73% of our patients were treated with mastectomy versus 27% with lumpectomy, probably due to the high prevalence of stage IIIB. The low histological grade (grade 1-2) and high clinical stage (IIIB) of treatment were probably due to most of the patients having non-operable cancers including inflammatory tumors. Currently, neoadjuvant treatment is generally used for operable HER2+ BC thanks to an improvement in the efficacy of the drugs used for treatment[114]. At the time our cohort was treated, none of these drugs were yet available. That said, trastuzumab remains the gold-standard treatment for HER2+ BC.

In summary, even though our results are preliminary and more approaches should be performed to evaluate the role of TGFBI in trastuzumab resistance, our work suggests for the first time *TGFBI* promoter methylation as a biomarker for

trastuzumab response in patients with HER2+ BC. The combination of *TGFBI* hypermethylation analysis with standard clinical markers may help stratify HER2+ patients according to the response to the trastuzumab treatment.

4.- GENERAL DISCUSSION

Cancer has traditionally been regarded as a genetic disease, but recently it is becoming apparent that the deregulation of epigenetic mechanisms greatly contributes to tumor development[7,227,542]. Furthermore, it has also been described that different genetic and epigenetic events are probably involved in the *de novo* or primary cellular mechanisms to escape the anti-tumoral effects mediated by the anticancer compounds[288]. The main objective of this work was the evaluation of the DNA methylome in different cellular models resistant to the main treatments for HER2+ BC used in clinical practice. Through the application of the most recent epigenomic and expression techniques, potential biomarkers for trastuzumab and lapatinib treatment resistance have been identified. We highlight in particular, the identification and validation of the *TGFBI* gene as a possible indicator of trastuzumab resistance in patients with HER2+ BC. This work has been performed using pre-clinical models and a small cohort of HER2+ tumor samples.

In the first part of this thesis, we identified the DNA methylome in trastuzumab and lapatinib sensitive and resistant HER2+ BC, analyzing our pre-clinical models using Infinium HumanMethylation 450 BeadChip. We demonstrated that DNA methylation mechanisms are involved in trastuzumab and lapatinib treatment resistance in our models. As expected, a common cancer methylation pattern was observed in our resistant models.

The aim to identify new biomarkers to detect, predict or monitor BC is the spotlight of many studies. Despite promising epigenetic biomarkers such as *MGMT* hypermethylation for temozolomide treatment in gliomas, there are fewer biomarkers focused on anti-HER2 treatment resistance[355,370,543–545]. Herein, we

focused our DNA methylation analysis on the promoter and island regions of the candidate genes to determine its implication in gene expression regulation[227,293]. In analyzing the promoter methylation profile of our sensitive and resistant models, we found that the majority of differentially methylated genes are related to cell adhesion processes, which are critical to the preservation of normal tissue architecture, and disruption of the cell adhesion system can lead to tumor infiltration and metastatsis[546]. Therefore, our results reinforce previous studies demonstrating the role tumor microenvironment and cellular adhesion process in tumor cell drug resistance has[448,451,452,547]. The integrative analysis of methylation and RNA-Seq data for each sensitive and resistant model comparison, showed different genes with methylation and expression pattern correlations. For instance, *TGFBI*, *KILLIN*, *CXCL2* and *SLC38A1* from the SKTR vs. SK comparison, *ITGB8* and *BMP4* from SKTLR vs. SKTR and, *ANXA3* and *PPARG* from SKTLR compared to SKTR and SKLR were identified as potential biomarkers of trastuzumab and lapatinib resistance. However, only the selected genes in the trastuzumab-resistant models could be validated in another resistant model and the patient cohort.

By analyzing the promoter methylation profile of long-term trastuzumab-sensitive and -resistant models, four genes (*TGFBI*, *KILLIN*, *CXCL2* and *SLC38A1*) were identified and subsequently validated using different methylation and expression approaches. Interestingly, only the *TGFBI* gene presented a correlation between methylation, expression and protein levels in two different trastuzumab-sensitive and -resistant models (SKBr3 and AU565). While TGFBI has been reported to be involved in tumorigenesis, its role is still not clear. Similar to other previous reports, we have linked the promoter DNA methylation-associated silencing of *TGFBI* with a possible tumor suppressor function[415,537,538,548]. However, in other cancers like colon or pancreas, *TGFBI* has been described as an oncogene[510,511]. These opposing effects suggest that TGFBI expression and function are dependent on cell type[409].

Similar results to the SKTR and SK models were obtained when, using bisulfite pyrosequencing, we analyzed *TGFBI* promoter hypermethylation in 24 HER2+ human BC samples with complete response or no-response to

trastuzumab-based treatment in neoadjuvant settings. Our preliminary *in vitro* study identified a significant increase of *TGFBI* methylation levels in the post-treatment samples of patients who had developed resistance to treatment compared to their pre-treatment samples. These results are in accordance with other studies which demonstrated an association between *TGFBI* hypermethylation and poor prognosis in prostate and lung cancer as well as with a decreased *TGFBI* expression in advanced stages of BC and in NSCLC tumors[537–539]. Interestingly, the *TGFBI* promoter methylation analysis in responder patients showed similar methylation levels with the pre-treatment samples of non-responder's. Although further validation in a large and independent cohort is required, the present study suggests that *TGFBI* methylation has a significant discriminative value between pre- and post-treatment samples, as demonstrated by ROC curve analyses. This observation suggests the possible role of *TGFBI* promoter hypermethylation as an epigenetic biomarker for trastuzumab response in patients with HER2+ BC.

TGFBI is described as a "linker" participating in the interaction between ECM and integrins, affecting different traits of malignant tumors[432]. It has been demonstrated that expression of TGFBI ectopically reduced the metastatic ability of MCF-7 and H522, lung and breast cancer cell lines, respectively, *in vitro* and *in vivo*[415]. Consistently, a previous study in our group demonstrated a greater invasive capacity for SKTR model compared to SK[401]. Therefore, our results reinforce that the *TGFBI* hypermethylation in the SKTR model could be related to a more aggressive and invasive phenotype. Attempting to identify the role of TGFBI in trastuzumab resistance and its involvement in cell adhesion and tumor microenvironment, we demonstrated that TGFBI overexpression in the SKTR model significantly sensitizes the cells to the treatment by interacting with integrins through its integrin-binding domains (EPDIM, NKDIL, YH and RGD). These results were reinforced after the expression of TGFBI with different mutated integrin-binding domains. The TGFBI mutated form induced the activation of different HER2 proteins downstream but there were no differences in trastuzumab treatment response. Additionally, TGFBI overexpression and mutated TGFBI showed some downstream HER2 protein activation, independent

of its integrin-binding domains. Although further studies are needed to elucidate the TGFBI and HER2 downstream protein interaction, we suggest a possible direct interaction between the TGFBI and HER receptors or the involvement of the TGF-β pathway. Together, these findings suggest that TGFBI promotes trastuzumab sensitivity through its interaction with integrins, probably by blocking the ECM-integrin cross-talk. Other studies also described the involvement of FAS1 domains and RGD motifs in tumor growth inhibition and promoting apoptosis[507,509,521]. In contrast, TGFBI knockdown in endogenous TGFBI expressed in the SK cells did not produce any effect. Further studies are required to elucidate the molecular pathways determining how TGFBI repression causes trastuzumab resistance and to determine how integrins are involved.

In summary, the results of this thesis have provided an overview of the DNA methylation pattern in HER2+ resistant BC. Furthermore, different epigenetically regulated genes have been identified as potential biomarkers for trastuzumab, lapatinib and trastuzumab *plus* lapatinib to be validated in future studies. Interestingly, *TGFBI* promoter hypermethylation has been identified and validated using different methylation and expression techniques such as potential methylation monitoring biomarker for trastuzumab response. The *in vitro* results were validated *in vivo* in a small cohort of HER2+ BC patients treated with trastuzumab *plus* chemotherapy in a neoadjuvant setting. However, further prospective studies are required to identify the specific role TGFBI plays in trastuzumab resistance in the neoadjuvant settings.

5.- LIMITATIONS OF THE STUDY

At the conclusion of the present research, it is necessary to point out some limitations that have not been possible to control in both *in vitro* and *in vivo* analysis.

We used the SKBr3 HER2+ BC cell model to identify potential genes involved in trastuzumab, lapatinib and trastuzumab *plus* lapatinib resistance. Although the genes involved in trastuzumab resistance in SKTR were validated in another resistant model (AUTR), the other genes identified in lapatinib and trastuzumab *plus* lapatinib resistance could not be validated. Biomarker validation in different cellular models allows the reproducibility to be established and demonstrates that selected genes are not an *in vitro* culture artifact. However, the availability of resistant models and the development of long-term resistant models make this process difficult. To our knowledge, the SKTLR model (together with the SKLTR) is the only existing double-resistant models.

Another significant limitation of our study is the small number of HER2+ human BC samples. As explained before, the poor preservation of the DNA in FFPE tumor blocks was one of the most important limitations. The fixation process and long-term storage of FFPE tissue led to DNA damage resulting in poor-quality DNA resulting in the fact that all the previously selected samples could not be finally analyzed. Besides this, the difficulty of obtaining and extracting DNA from pre-treatment samples (core biopsies) and the lack of post-treatment samples from patients who achieved complete response, were one of the most critical limitations that we found. Finally, the clinical-histopathologic characteristics of our selected samples differs slightly from what was expected, with a low histological grade and high clinical stage. These differences are probably due to most of the patients having non-operable cancers, including inflammatory tumors, and that most of the samples were collected between 2007 and 2014. Currently, because of an improvement in the efficacy of treatments, neoadjuvant treatment is generally used in operable HER2+ BC. Finally, taking into account that patient selection is not random since they are obtained in our

hospital, our *in vitro* study requires a further validation in a more extensive and independent cohort.

6.- FUTURE PERSPECTIVES

Despite new advances in medicine and public health, early diagnosis of BC remains a worldwide public health dilemma as it is currently the most common tumor found in women[1]. The astonishing advance in our understanding of BC has allowed for the development of more competent treatments such as trastuzumab or lapatinib for HER2+ early-stage and metastatic BC[549,88]. That said, in the last few years, oncology has been focusing on the discovery of new biomarkers capable of detecting cancer at the very early stages and predicting or monitoring treatment response. These biomarkers can help in different processes such as new pharmacological treatment development, the discovery and characterization of new resistance mechanisms and, interestingly, can efficiently stratify patients which would allow for a more personalized therapy for each patient to be developed.

Currently, there has been an explosion in cancer research focused on circulating tumor DNA (ctDNA) analysis. ctDNA is a double-stranded nucleic acid that is shed from tumor cells into the blood and contains all the gene information detected in the tumor tissue[550]. Hence, it contains a great deal of information about the genetic and epigenetic profiles associated with cancer development, progression and response to therapy[550,551]. For this reason, ctDNA can be considered as a valuable complement to conventional diagnostic procedures and relevant for early disease detection in cancer patients, especially for tumor types where symptoms arise in late stages or when biopsies are not available[552]. For example, Fiegl *et al.* identified the *RASSF1A* DNA methylation in the plasma of patients with BC who had received adjuvant tamoxifen. The authors demonstrated that *RASSF1A* methylation was related to tamoxifen treatment resistance[553]. Hence, we suggest that *TGFBI* methylation analysis in the plasma of HER2+ BC treated with trastuzumab *plus* chemotherapy in neoadjuvant setting at different times once the treatment has been administered,

could be a validated method. This non-invasive analysis could allow the limitations out study had working with FFPE tissue to be overcome. Besides, it could also be an alternative method to validate the *ANXA3* and *PPARG* methylation identified as potential biomarkers of trastuzumab and lapatinib resistance, as it is less invasive than performing an additional core biopsy after lapatinib treatment.

Chapter VI: Conclusions

The results obtained from the current thesis led us to conclude that:

1. DNA methylation analysis allowed us to differentiate our trastuzumab and lapatinib-sensitive and -resistant HER2+ models, demonstrating the involvement this epigenetic alteration has in trastuzumab and lapatinib HER2+ resistant breast cancer. The SKLR model presented the most similar and the SKTLR the most different DNA methylation pattern compared to the SK model.

2. The DNA promoter and island methylation analysis allowed us to identify several differently methylated genes between our sensitive and resistant models. Furthermore, these differently methylated genes identified were mainly related to cell adhesion and cell proliferation events, suggesting tumor microenvironment plays an essential role in trastuzumab and lapatinib resistance.

3. The integrative analysis of DNA promoter and island methylation and RNA-Seq data suggest *ANXA3* and *PPARG* genes as potential biomarkers for trastuzumab *plus* lapatinib resistance in HER2+ breast cancer.

4. *TGFBI* promoter hypermethylation and downexpression was identified and validated using gold standard methodologies for DNA methylation and gene expression in trastuzumab-resistant models (SKTR and AUTR) compared to trastuzumab-sensitive models (SK and AU), suggesting its tumor suppressor role.

5. The ectopic expression of TGFBI in our trastuzumab-resistant model increased its sensitivity to trastuzumab treatment. In addition, our *in vitro* results suggest the role TGFBI integrin-binding domains (EPDIM, NKDIL, RGD and YH motifs) play in trastuzumab response in our resistant

model, providing insight into the possible mechanism through which TGFBI might contribute to resistance.

6. Our *in vitro* results suggest a possible cross-talk between the TGFBI and HER pathway independent of its integrin-binding domains.

7. Like the trastuzumab-resistant model (SKTR), higher *TGFBI* promoter methylation has been identified in post-treatment samples of HER2+ resistant breast cancer patients treated with neoadjuvant anthracycline-taxane-based chemotherapy *plus* trastuzumab compared with their pre-treatment samples.

8. *TGFBI* promoter methylation in pre-treatment samples of patients with complete treatment response, non-response and healthy breast tissue, showed similar methylation levels, suggesting *TGFBI*'s possible role as a monitoring biomarker for trastuzumab response in patients with HER2+ BC.

The general conclusion of this thesis is that the DNA methylation mechanism is involved in trastuzumab- and lapatinib-resistant HER2+ breast cancer. Our *in vitro* and preliminary *in vivo* studies encourage us to think that *TGFBI* promoter hypermethylation could be a potential methylation biomarker for trastuzumab response. However, further prospective studies are required to identify the specific role TGFBI plays in trastuzumab resistance in neoadjuvant settings. Future studies based on *TGFBI* promoter methylation analysis in circulating DNA during neoadjuvant treatment could be a potential strategy with which to confirm our findings.

Chapter VII: References

1. Akram, M., Iqbal, M., Daniyal, M. & Khan, A. U. Awareness and current knowledge of breast cancer. *Biological research* **50**, 33 (2017).
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, J. A., Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., *et al.* Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* **68**, 394–424 (2018).
3. Galceran, J., Ameijide, A., Carulla, M., Mateos, A., Quirós, J. R., Rojas, D., *et al.* Cancer incidence in Spain, 2015. *Clin. Transl. Oncol.* **19**, 799–825 (2017).
4. Spanish cancer registries. REDECAN. Available at: http://redecan.org/es. (Accessed: 3rd April 2018)
5. National Cancer Institute (NIH). Comprehensive Cancer Information. (2017). Available at: https://www.cancer.gov/types/breast. (Accessed: 25th November 2017)
6. Hanahan, D., Weinberg, R. A. & Francisco, S. The Hallmarks of Cancer. *Cell* **100**, 57–70 (2000).
7. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646–74 (2011).
8. Flavahan, W. A., Gaskell, E. & Bernstein, B. E. Epigenetic plasticity and the hallmarks of cancer. *Science (80-.).* **357**, (2017).
9. Timp, W. & Feinberg, A. P. Cancer as a dysregulated epigenome allowing cellular growth advantage at the expense of the host. *Nat. Rev. Cancer* **13**, 497–510 (2013).
10. Miller, K. D., Siegel, R. L., Lin, C. C., Mariotto, A. B., Kramer, J. L., Rowland, J. H., *et al.* Cancer treatment and survivorship statistics, 2016. *CA. Cancer J. Clin.* **66**, 271–289 (2016).
11. Reeves, G. K., Pirie, K., Green, J., Bull, D., Beral, V. & Million Women Study Collaborators. Reproductive factors and specific histological types of breast cancer: prospective study and meta-analysis. *Br. J. Cancer* **100**, 538–544 (2009).
12. Howell, A., Anderson, A. S., Clarke, R. B., Duffy, S. W., Evans, D. G., Garcia-Closas, M., *et al.* Risk determination and prevention of breast cancer. *Breast Cancer Res.* **16**, 446 (2014).
13. Ferlay, J., Steliarova-Foucher, E., Lortet-Tieulent, J., Rosso, S., Coebergh, J. W. W., Comber, H., *et al.* Cancer incidence and mortality patterns in Europe: Estimates for 40 countries in 2012. *Eur. J. Cancer* **49**, 1374–1403 (2013).
14. Dieterich, M., Stubert, J., Reimer, T., Erickson, N. & Berling, A. Influence of lifestyle factors on breast cancer risk. *Breast Care (Basel).* **9**, 407–14 (2014).
15. Carey, L. A., Perou, C. M., Livasy, C. A., Dressler, L. G., Cowan, D., Conway, K., *et al.* Race, breast cancer subtypes, and survival in the Carolina breast cancer study. *JAMA* **295**, 2492 (2006).
16. Sørlie, T., Tibshirani, R., Parker, J., Hastie, T., Marron, J. S., Nobel, A., *et al.* Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc. Natl. Acad. Sci.* **100**, 8418–8423 (2003).
17. Lumachi, F., Santeufemia, D. A. & Basso, S. M. Current medical treatment of estrogen receptor-positive breast cancer. *World J. Biol. Chem.* **6**, 231 (2015).
18. Lumachi, F., Brunello, A., Maruzzo, M., Basso, U. & Basso, S. M. M. Treatment of estrogen receptor-positive breast cancer. *Curr. Med. Chem.* **20**, 596–604 (2013).
19. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., *et al.* Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* **244**, 707–12 (1989).
20. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A. & McGuire, W. L. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **235**, 177–82 (1987).
21. Maughan, K. L., Lutterbie, M. A. & Ham, P. S. Treatment of breast cancer. *Am.*

Fam. Physician **81**, 1339–1346 (2010).
22. Rimawi, M. F., Schiff, R. & Osborne, C. K. Targeting HER2 for the treatment of breast cancer. *Annu. Rev. Med.* **66**, 111–28 (2015).
23. Bauer, K. R., Brown, M., Cress, R. D., Parise, C. A. & Caggiano, V. Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype. *Cancer* **109**, 1721–1728 (2007).
24. Giro-Perafita, A., Palomeras, S., Lum, D. H., Blancafort, A., Vinas, G., Oliveras, G., *et al.* Preclinical evaluation of fatty acid synthase and EGFR inhibition in triple-negative breast cancer. *Clin. Cancer Res.* **22**, 4687–4697 (2016).
25. Perou, C. M., Sørlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., *et al.* Molecular portraits of human breast tumours. *Nature* **406**, 747–752 (2000).
26. Sørlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., *et al.* Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 10869–74 (2001).
27. Prat, A., Parker, J. S., Karginova, O., Fan, C., Livasy, C., Herschkowitz, J. I., *et al.* Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. *Breast Cancer Res.* **12**, R68 (2010).
28. Cheang, M. C. U., Voduc, D., Bajdik, C., Leung, S., McKinney, S., Chia, S. K., *et al.* Basal-Like Breast Cancer Defined by Five Biomarkers Has Superior Prognostic Value than Triple-Negative Phenotype. *Clin. Cancer Res.* **14**, 1368–1376 (2008).
29. Perou, C. M. Molecular Stratification of Triple-Negative Breast Cancers. *Oncologist* **15**, 39–48 (2010).
30. Prat, A. & Perou, C. M. Deconstructing the molecular portraits of breast cancer. *Mol. Oncol.* **5**, 5–23 (2011).
31. Koboldt, D. C., Fulton, R. S., McLellan, M. D., Schmidt, H., Kalicki-Veizer, J., McMichael, J. F., *et al.* Comprehensive molecular portraits of human breast tumours. *Nature* **487**, 61–70 (2012).
32. Weigel, M. T. & Dowsett, M. Current and emerging biomarkers in breast cancer: prognosis and prediction. *Endocr. Relat. Cancer* **17**, R245-62 (2010).
33. Hilsenbeck, S. G., Ravdin, P. M., de Moor, C. A., Chamness, G. C., Osborne, C. K. & Clark, G. M. Time-dependence of hazard ratios for prognostic factors in primary breast cancer. *Breast Cancer Res. Treat.* **52**, 227–37 (1998).
34. Urruticoechea, A., Smith, I. E. & Dowsett, M. Proliferation Marker Ki-67 in Early Breast Cancer. *J. Clin. Oncol.* **23**, 7212–7220 (2005).
35. Hoffmann, M. J. & Schulz, W. A. Causes and consequences of DNA hypomethylation in human cancer. *Biochem. Cell Biol.* **83**, 296–321 (2005).
36. Hynes, N. E. & Lane, H. A. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat. Rev. Cancer* **5**, 341–354 (2005).
37. DeVita, V. T., Lawrence, T. S. & Rosenberg, S. A. *DeVita, Hellman, and Rosenberg's cancer : principles & Practice of Oncology*.
38. Lemmon, M. A. & Schlessinger, J. Cell signaling by receptor tyrosine kinases. *Cell* **141**, 1117–1134 (2010).
39. Prenzel, N., Fischer, O. M., Streit, S., Hart, S. & Ullrich, A. The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. *Endocr. Relat. Cancer* **8**, 11–31 (2001).
40. Carpenter, G., King, L. & Cohen, S. Epidermal growth factor stimulates phosphorylation in membrane preparations *in vitro*. *Nature* **276**, 409–410 (1978).
41. Roskoski, R. ErbB/HER protein-tyrosine kinases: Structures and small molecule inhibitors. *Pharmacol. Res.* **87**, 42–59 (2014).
42. Roskoski, R. The ErbB/HER family of protein-tyrosine kinases and cancer. *Pharmacological Research* **79**, 34–74 (2014).
43. Brennan, P. J., Kumogai, T., Berezov, A., Murali, R. & Greene, M. I. HER2/Neu:

mechanisms of dimerization/oligomerization.
44. Yarden, Y. The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities. *Eur. J. Cancer* **37 Suppl 4**, S3-8 (2001).
45. Zhang, D., Sliwkowski, M. X., Mark, M., Frantz, G., Akita, R., Sun, Y., *et al.* Neuregulin-3 (NRG3): a novel neural tissue-enriched protein that binds and activates ErbB4. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 9562–7 (1997).
46. Harari, D., Tzahar, E., Romano, J., Shelly, M., Pierce, J., Andrews, G., *et al.* Neuregulin-4: a novel growth factor that acts through the ErbB-4 receptor tyrosine kinase. *Oncogene* **18**, 2681–2689 (1999).
47. Singh, B., Carpenter, G. & Coffey, R. J. EGF receptor ligands: recent advances. *F1000Research* **5**, (2016).
48. Pinkas-Kramarski, R., Soussan, L., Waterman, H., Levkowitz, G., Alroy, I., Klapper1, L., *et al.* Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. *EMBO J.* **15**, 2452–2467 (1996).
49. Tzahar, E., Waterman, H., Chen, X., Levkowitz, G., Karunagaran, D., Lavi, S., *et al.* A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. *Mol. Cell. Biol.* **16**, 5276–87 (1996).
50. Baselga, J. & Swain, S. M. Novel anticancer targets: revisiting ERBB2 and discovering ERBB3. *Nat. Rev. Cancer* **9**, 463–475 (2009).
51. Seshacharyulu, P., Ponnusamy, M. P., Haridas, D., Jain, M., Ganti, A. K. & Batra, S. K. Targeting the EGFR signaling pathway in cancer therapy. *Expert Opin. Ther. Targets* **16**, 15–31 (2012).
52. Olayioye, M. A., Neve, R. M., Lane, H. A. & Hynes, N. E. The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J.* **19**, 3159–3167 (2000).
53. Ferguson, K. M., Berger, M. B., Mendrola, J. M., Cho, H. S., Leahy, D. J. & Lemmon, M. A. EGF activates its receptor by removing interactions that autoinhibit ectodomain dimerization. *Mol. Cell* **11**, 507–17 (2003).
54. Nahta, R., Yuan, L. X. H. H., Zhang, B., Kobayashi, R. & Esteva, F. J. Insulin-like growth factor-I receptor/human epidermal growth factor receptor 2 heterodimerization contributes to trastuzumab resistance of breast cancer cells. *Cancer Res.* **65**, 11118–11128 (2005).
55. Citri, A. & Yarden, Y. EGF–ERBB signalling: towards the systems level. *Nat. Rev. Mol. Cell Biol.* **7**, 505–516 (2006).
56. Schechter, A. L., Stern, D. F., Vaidyanathan, L., Decker, S. J., Drebin, J. A., Greene, M. I., *et al.* The neu oncogene: an erb-B-related gene encoding a 185,000-Mr tumour antigen. *Nature* **312**, 513–516 (1984).
57. Yamamoto, T., Ikawa, S., Akiyama, T., Semba, K., Nomura, N., Miyajima, N., *et al.* Similarity of protein encoded by the human c-erb-B-2 gene to epidermal growth factor receptor. *Nature* **319**, 230–234 (1986).
58. Press, M. F., Bernstein, L., Thomas, P. A., Meisner, L. F., Zhou, J. Y., Ma, Y., *et al.* HER-2/neu gene amplification characterized by fluorescence in situ hybridization: poor prognosis in node-negative breast carcinomas. *J. Clin. Oncol.* **15**, 2894–2904 (1997).
59. Coussens, L., Yang-Feng, T. L., Liao, Y. C., Chen, E., Gray, A., McGrath, J., *et al.* Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. *Science* **230**, 1132–9 (1985).
60. Ozcelik, C., Erdmann, B., Pilz, B., Wettschureck, N., Britsch, S., Hü Bner, N., *et al.* Conditional mutation of the ErbB2 (HER2) receptor in cardiomyocytes leads to dilated cardiomyopathy.
61. Morris, J. K., Lin, W., Hauser, C., Marchuk, Y., Getman, D. & Lee, K. F. Rescue

of the cardiac defect in ErbB2 mutant mice reveals essential roles of ErbB2 in peripheral nervous system development. *Neuron* **23**, 273–83 (1999).

62. Garratt, A. N., Voiculescu, O., Topilko, P., Charnay, P. & Birchmeier, C. A Dual role of erbB2 in myelination and in expansion of the schwann cell precursor pool. *J. Cell Biol.* **148**, 1035–1046 (2000).
63. Kim, J. Y., Sun, Q., Oglesbee, M. & Yoon, S. O. The role of ErbB2 signaling in the onset of terminal differentiation of oligodendrocytes *in vivo*. *J. Neurosci.* **23**, 5561–71 (2003).
64. Semba, K., Kamata, N., Toyoshima, K. & Yamamoto, T. A v-erbB-related protooncogene, c-erbB-2, is distinct from the c-erbB-1/epidermal growth factor-receptor gene and is amplified in a human salivary gland adenocarcinoma. *Proc. Natl. Acad. Sci. U. S. A.* **82**, 6497–501 (1985).
65. King, C. R., Kraus, M. H. & Aaronson, S. A. Amplification of a novel v-erbB-related gene in a human mammary carcinoma. *Science* **229**, 974–6 (1985).
66. Heiser, L. M., Sadanandam, A., Kuo, W.-L., Benz, S. C., Goldstein, T. C., Ng, S., *et al.* Subtype and pathway specific responses to anticancer compounds in breast cancer. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 2724–9 (2012).
67. Bose, R., Kavuri, S. M., Searleman, A. C., Shen, W., Shen, D., Koboldt, D. C., *et al.* Activating HER2 mutations in HER2 gene amplification negative breast cancer. *Cancer Discov.* **3**, 224–237 (2013).
68. Stephens, P., Hunter, C., Bignell, G., Edkins, S., Davies, H., Teague, J., *et al.* Lung cancer: Intragenic ERBB2 kinase mutations in tumours. *Nature* **431**, 525–526 (2004).
69. Lee, J. W., Soung, Y. H., Seo, S. H., Kim, S. Y., Park, C. H., Wang, Y. P., *et al.* Somatic Mutations of ERBB2 Kinase Domain in Gastric, Colorectal, and Breast Carcinomas. *Clin. Cancer Res.* **12**, 57–61 (2006).
70. Wolff, A. C., Hammond, M. E. H., Schwartz, J. N., Hagerty, K. L., Allred, D. C., Cote, R. J., *et al.* American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *Arch. Pathol. Lab. Med.* **131**, 18–43 (2007).
71. Watrowski, R., Castillo-Tong, D. C., Wolf, A., Schuster, E., Fischer, M. B., Speiser, P., *et al.* HER2 codon 655 (Ile/Val) polymorphism and breast cancer in Austrian women. *Anticancer Res.* **35**, 6667–70 (2015).
72. Witton, C. J., Reeves, J. R., Going, J. J., Cooke, T. G. & Bartlett, J. M. Expression of the HER1–4 family of receptor tyrosine kinases in breast cancer. *J. Pathol.* **200**, 290–297 (2003).
73. Gralow, J. R., Burstein, H. J., Wood, W., Hortobagyi, G. N., Gianni, L., von Minckwitz, G., *et al.* Preoperative therapy in invasive breast cancer: pathologic assessment and systemic therapy issues in operable disease. *J. Clin. Oncol.* **26**, 814–819 (2008).
74. Clarke, M., Collins, R., Darby, S., Davies, C., Elphinstone, P., Evans, V., *et al.* Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials. *Lancet* **366**, 2087–2106 (2005).
75. Chabner, B. A. & Roberts, T. G. Chemotherapy and the war on cancer. *Nat. Rev. Cancer* **5**, 65–72 (2005).
76. Baselga, J. Treatment of HER2-overexpressing breast cancer. *Ann. Oncol.* **21**, vii36-vii40 (2010).
77. Frommer, M., McDonald, L. E., Millar, D. S., Collis, C. M., Watt, F., Grigg, G. W., *et al.* A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 1827–31 (1992).
78. Drebin, J. A., Stern, D. F., Link, V. C., Weinberg, R. A. & Greene, M. I. Monoclonal antibodies identify a cell-surface antigen associated with an activated

cellular oncogene. *Nature* **312**, 545–8

79. Baselga, J., Tripathy, D., Mendelsohn, J., Baughman, S., Benz, C. C., Dantis, L., *et al.* Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. *J. Clin. Oncol.* **14**, 737–744 (1996).
80. Cho, H.-S., Mason, K., Ramyar, K. X., Stanley, A. M., Gabelli, S. B., Denney, D. W., *et al.* Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. *Nature* **421**, 756–760 (2003).
81. Carter, P., Presta, L., Gorman, C. M., Ridgway, J. B., Henner, D., Wong, W. L., *et al.* Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 4285–4289 (1992).
82. Hudziak, R. M., Lewis, G. D., Winget, M., Fendly, B. M., Shepard, H. M. & Ullrich, A. p185HER2 monoclonal antibody has antiproliferative effects *in vitro* and sensitizes human breast tumor cells to tumor necrosis factor. *Mol. Cell. Biol.* **9**, 1165–72 (1989).
83. Kasprzyk, P. G., Song, S. U., Di Fiore, P. P. & King, C. R. Therapy of an animal model of human gastric cancer using a combination of anti-erbB-2 monoclonal antibodies. *Cancer Res.* **52**, 2771–6 (1992).
84. Baselga, J., Norton, L., Albanell, J., Kim, Y. M. & Mendelsohn, J. Recombinant humanized anti-HER2 antibody (Herceptin) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/neu overexpressing human breast cancer xenografts. *Cancer Res.* **58**, 2825–31 (1998).
85. Pegram, M., Hsu, S., Lewis, G., Pietras, R., Beryt, M., Sliwkowski, M., *et al.* Inhibitory effects of combinations of HER-2/neu antibody and chemotherapeutic agents used for treatment of human breast cancers. *Oncogene* **18**, 2241–2251 (1999).
86. Pietras, R. J., Pegram, M. D., Finn, R. S., Maneval, D. A. & Slamon, D. J. Remission of human breast cancer xenografts on therapy with humanized monoclonal antibody to HER-2 receptor and DNA-reactive drugs. *Oncogene* **17**, 2235–2249 (1998).
87. Cobleigh, M. A., Vogel, C. L., Tripathy, D., Robert, N. J., Scholl, S., Fehrenbacher, L., *et al.* Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J. Clin. Oncol.* **17**, 2639–48 (1999).
88. Slamon, D. J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., *et al.* Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N. Engl. J. Med.* **344**, 783–92 (2001).
89. U S Food and Drug Administration Home Page. Available at: https://www.fda.gov/default.htm. (Accessed: 17th December 2017)
90. Piccart-Gebhart, M., Holmes, E., Baselga, J., de Azambuja, E., Dueck, A. C., Viale, G., *et al.* Adjuvant lapatinib and rrastuzumab for early human epidermal growth factor receptor 2–positive breast cancer: Results from the randomized phase III adjuvant lapatinib and/or trastuzumab treatment optimization trial. *J. Clin. Oncol.* **34**, 1034–1042 (2016).
91. Cameron, D., Piccart-Gebhart, M. J., Gelber, R. D., Procter, M., Goldhirsch, A., de Azambuja, E., *et al.* 11 years' follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive early breast cancer: final analysis of the HERceptin Adjuvant (HERA) trial. *Lancet* **389**, 1195–1205 (2017).
92. Buzdar, A. U., Ibrahim, N. K., Francis, D., Booser, D. J., Thomas, E. S., Theriault, R. L., *et al.* Significantly Higher Pathologic Complete remission rate after neoadjuvant therapy with trastuzumab, paclitaxel, and epirubicin chemotherapy: Results of a randomized trial in human epidermal growth factor

receptor 2–positive operable breast cancer. *J. Clin. Oncol.* **23**, 3676–3685 (2005).
93. Gianni, L., Eiermann, W., Semiglazov, V., Lluch, A., Tjulandin, S., Zambetti, M., *et al.* Neoadjuvant and adjuvant trastuzumab in patients with HER2-positive locally advanced breast cancer (NOAH): follow-up of a randomised controlled superiority trial with a parallel HER2-negative cohort. *Lancet Oncol.* **15**, 640–647 (2014).
94. Piccart-Gebhart, M. J., Procter, M., Leyland-Jones, B., Goldhirsch, A., Untch, M., Smith, I., *et al.* Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. *N. Engl. J. Med.* **353**, 1659–1672 (2005).
95. Romond, E. H., Perez, E. A., Bryant, J., Suman, V. J., Geyer, C. E., Davidson, N. E., *et al.* Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *N. Engl. J. Med.* **353**, 1673–1684 (2005).
96. Joensuu, H., Kellokumpu-Lehtinen, P.-L., Bono, P., Alanko, T., Kataja, V., Asola, R., *et al.* Adjuvant Docetaxel or Vinorelbine with or without Trastuzumab for Breast Cancer. *N. Engl. J. Med.* **354**, 809–820 (2006).
97. Slamon, D., Eiermann, W., Robert, N., Pienkowski, T., Martin, M., Rolski, J., *et al.* Phase III randomized trial comparing doxorubicin and cyclophosphamide followed by docetaxel (AC→T) with Doxorubicin and Cyclophosphamide gollowed by Docetaxel and Trastuzumab (AC→TH) with Docetaxel, Carboplatin and Trastuzumab (TCH) in Her2neu positive ea. *Cancer Res.* **69**, 62–62 (2009).
98. Sarup, J. C., Johnson, R. M., King, K. L., Fendly, B. M., Lipari, M. T., Napier, M. A., *et al.* Characterization of an anti-p185HER2 monoclonal antibody that stimulates receptor function and inhibits tumor cell growth. *Growth Regul.* **1**, 72–82 (1991).
99. Clynes, R. A., Towers, T. L., Presta, L. G. & Ravetch, J. V. Inhibitory Fc receptors modulate *in vivo* cytoxicity against tumortargets. *Nat. Med.* **6**, 443–446 (2000).
100. Petit, A. M., Rak, J., Hung, M. C., Rockwell, P., Goldstein, N., Fendly, B., *et al.* Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells *in vitro* and *in vivo*: angiogenic implications for signal transduction therapy of solid tumors. *Am. J. Pathol.* **151**, 1523–30 (1997).
101. Nahta, R. & Esteva, F. J. Herceptin: mechanisms of action and resistance. *Cancer Lett.* **232**, 123–138 (2006).
102. Xia, W., Liu, L.-H., Ho, P. & Spector, N. L. Truncated ErbB2 receptor (p95ErbB2) is regulated by heregulin through heterodimer formation with ErbB3 yet remains sensitive to the dual EGFR/ErbB2 kinase inhibitor GW572016. *Oncogene* **23**, 646–653 (2004).
103. Köstler, W. J., Schwab, B., Singer, C. F., Neumann, R., Rücklinger, E., Brodowicz, T., *et al.* Monitoring of serum Her-2/neu predicts response and progression-free survival to trastuzumab-based treatment in patients with metastatic breast cancer. *Clin. Cancer Res.* **10**, 1618–24 (2004).
104. Molina, M. A., Codony-Servat, J., Albanell, J., Rojo, F., Arribas, J. & Baselga, J. Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells. *Cancer Res.* **61**, 4744–9 (2001).
105. Baselga, J. Clinical trials of Herceptin(R) (trastuzumab). *Eur. J. Cancer* **37 Suppl 1**, 18–24 (2001).
106. Keefe, D. L. Trastuzumab-associated cardiotoxicity. *Cancer* **95**, 1592–1600 (2002).
107. Wang, Y.-C., Morrison, G., Gillihan, R., Guo, J., Ward, R. M., Fu, X., *et al.* Different mechanisms for resistance to trastuzumab versus lapatinib in HER2-positive breast cancers - role of estrogen receptor and HER2 reactivation. *Breast Cancer Res.* **13**, R121 (2011).

108. Nahta, R., Yu, D., Hung, M.-C., Hortobagyi, G. N. & Esteva, F. J. Mechanisms of Disease: understanding resistance to HER2-targeted therapy in human breast cancer. *Nat. Clin. Pract. Oncol.* **3**, 269–280 (2006).
109. Agus, D. B., Akita, R. W., Fox, W. D., Lewis, G. D., Higgins, B., Pisacane, P. I., *et al.* Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth. *Cancer Cell* **2**, 127–37 (2002).
110. Franklin, M. C., Carey, K. D., Vajdos, F. F., Leahy, D. J., de Vos, A. M. & Sliwkowski, M. X. Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. *Cancer Cell* **5**, 317–28 (2004).
111. Baselga, J., Cortés, J., Kim, S.-B., Im, S.-A., Hegg, R., Im, Y.-H., *et al.* Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer. *N. Engl. J. Med.* **366**, 109–19 (2012).
112. Baselga, J., Coleman, R. E., Cortés, J. & Janni, W. Advances in the management of HER2-positive early breast cancer. *Crit. Rev. Oncol. Hematol.* **119**, 113–122 (2017).
113. von Minckwitz, G., Procter, M., de Azambuja, E., Zardavas, D., Benyunes, M., Viale, G., *et al.* Adjuvant pertuzumab and trastuzumab in early HER2-positive breast cancer. *N. Engl. J. Med.* **377**, 122–131 (2017).
114. Gianni, L., Pienkowski, T., Im, Y.-H., Tseng, L.-M., Liu, M.-C., Lluch, A., *et al.* 5-year analysis of neoadjuvant pertuzumab and trastuzumab in patients with locally advanced, inflammatory, or early-stage HER2-positive breast cancer (NeoSphere): a multicentre, open-label, phase 2 randomised trial. *Lancet. Oncol.* **17**, 791–800 (2016).
115. Schneeweiss, A., Chia, S., Hickish, T., Harvey, V., Eniu, A., Hegg, R., *et al.* Pertuzumab plus trastuzumab in combination with standard neoadjuvant anthracycline-containing and anthracycline-free chemotherapy regimens in patients with HER2-positive early breast cancer: a randomized phase II cardiac safety study (TRYPHAENA). *Ann. Oncol.* **24**, 2278–2284 (2013).
116. Phillips, G. D. L., Li, G., Dugger, D. L., Crocker, L. M., Parsons, K. L., Mai, E., *et al.* Targeting HER2-positive breast cancer with trastuzumab-DM1, an antibody-cytotoxic drug conjugate. *Cancer Res.* **68**, 9280–9290 (2008).
117. Michel, L. L., Bermejo, J. L., Gondos, A., Marmé, F. & Schneeweiss, A. T-DM1 as a new treatment option for patients with metastatic HER2-positive breast cancer in clinical practice. *Anticancer Res.* **35**, 5085–90 (2015).
118. Verma, S., Miles, D., Gianni, L., Krop, I. E., Welslau, M., Baselga, J., *et al.* Trastuzumab emtansine for HER2-positive advanced breast cancer. *N. Engl. J. Med.* **367**, 1783–1791 (2012).
119. Xia, W., Mullin, R. J., Keith, B. R., Liu, L.-H., Ma, H., Rusnak, D. W., *et al.* Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. *Oncogene* **21**, 6255–6263 (2002).
120. Spector, N. L., Xia, W., Burris, H., Hurwitz, H., Dees, E. C., Dowlati, A., *et al.* Study of the biologic effects of lapatinib, a reversible inhibitor of ErbB1 and ErbB2 tyrosine kinases, on tumor growth and survival pathways in patients with advanced malignancies. *J. Clin. Oncol.* **23**, 2502–2512 (2005).
121. Xia, W., Gerard, C. M., Liu, L., Baudson, N. M., Ory, T. L. & Spector, N. L. Combining lapatinib (GW572016), a small molecule inhibitor of ErbB1 and ErbB2 tyrosine kinases, with therapeutic anti-ErbB2 antibodies enhances apoptosis of ErbB2-overexpressing breast cancer cells. *Oncogene* **24**, 6213–6221 (2005).
122. Rusnak, D. W., Lackey, K., Affleck, K., Wood, E. R., Alligood, K. J., Rhodes, N., *et al.* The effects of the novel, reversible epidermal growth factor receptor/ErbB-2 tyrosine kinase inhibitor, GW2016, on the growth of human normal and tumor-derived cell lines *in vitro* and *in vivo*. *Mol. Cancer Ther.* **1**, 85–

94 (2001).
123. Konecny, G. E., Pegram, M. D., Venkatesan, N., Finn, R., Yang, G., Rahmeh, M., *et al.* Activity of the dual linase inhibitor lapatinib (GW572016) against HER-2-overexpressing and trastuzumab-treated breast cancer cells. *Cancer Res.* **66**, 1630–1639 (2006).
124. Frankel, C. & Palmieri, F. M. Lapatinib side-effect management. *Clin. J. Oncol. Nurs.* **14**, 223–233 (2010).
125. Geyer, C. E., Forster, J., Lindquist, D., Chan, S., Romieu, C. G., Pienkowski, T., *et al.* Lapatinib plus capecitabine for HER2-positive advanced breast cancer. *N Engl J Med* **26355**, 2733–43 (2006).
126. Johnston, S., Pegram, M., Press, M., Pippen, J., Pivot, X., Gomez, H., *et al.* Lapatinib combined with letrozole vs. letrozole alone for front line postmenopausal hormone receptor positive (HR+) metastatic breast cancer (MBC): first results from the EGF30008 Trial. *Cancer Res.* **69**, 46 (2009).
127. Goss, P. E., Smith, I. E., O'Shaughnessy, J., Ejlertsen, B., Kaufmann, M., Boyle, F., *et al.* Adjuvant lapatinib for women with early-stage HER2-positive breast cancer: a randomised, controlled, phase 3 trial. *Lancet Oncol.* **14**, 88–96 (2013).
128. Baselga, J., Bradbury, I., Eidtmann, H., Di Cosimo, S., de Azambuja, E., Aura, C., *et al.* Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): a randomised, open-label, multicentre, phase 3 trial. *Lancet* **18**, 633–640 (2012).
129. de Azambuja, E., Holmes, A. P., Piccart-Gebhart, M., Holmes, E., Di Cosimo, S., Swaby, R. F., *et al.* Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): survival outcomes of a randomised, open-label, multicentre, phase 3 trial and their association with pathological complete response. *Lancet Oncol.* **15**, 1137–1146 (2014).
130. Wissner, A. & Mansour, T. S. The development of HKI-272 and related compounds for the treatment of cancer. *Arch. Pharm. (Weinheim).* **341**, 465–477 (2008).
131. Jacobs, S. A., Robidoux, A., Garcia, J. M. P., Abraham, J., La Verde, N., Orcutt, J. M., *et al.* NSABP FB-7: A phase II randomized trial evaluating neoadjuvant therapy with weekly paclitaxel (P) plus neratinib (N) or trastuzumab (T) or N+T followed by AC with postoperative T in women with locally advanced HER2-positive breast cancer. *Cancer Res.* **76**, PD5-04 (2016).
132. Park, J. W., Liu, M. C., Yee, D., Yau, C., van 't Veer, L. J., Symmans, W. F., *et al.* Adaptive randomization of neratinib in early breast cancer. *N. Engl. J. Med.* **375**, 11–22 (2016).
133. Esteva, B. F. J., Valero, V., Booser, D., Guerra, L. T., Murray, J. L., Pusztai, L., *et al.* Phase II study of weekly docetaxel and trastuzumab for patients with HER-2–overexpressing metastatic breast cancer. *J. Clin. Oncol.* **20**, 1800–1808 (2002).
134. Scaltriti, M., Rojo, F., Ocaña, A., Anido, J., Guzman, M., Cortes, J., *et al.* Expression of p95HER2, a truncated form of the HER2 receptor, and response to Anti-HER2 therapies in breast cancer. *J. Natl. Cancer Inst.* **99**, 628–638 (2007).
135. Scaltriti, M., Chandarlapaty, S., Prudkin, L., Aura, C., Jimenez, J., Angelini, P. D., *et al.* Clinical benefit of lapatinib-based therapy in patients with human epidermal growth factor receptor 2-positive breast tumors coexpressing the truncated p95HER2 receptor. *Clin. Cancer Res.* **16**, 2688–2695 (2010).
136. Pedersen, K., Angelini, P.-D., Laos, S., Bach-Faig, A., Cunningham, M. P., Ferrer-Ramon, C., *et al.* A naturally occurring HER2 carboxy-terminal fragment promotes mammary tumor growth and metastasis. *Mol. Cell. Biol.* **29**, 3319–3331 (2009).
137. Castiglioni, F., Tagliabue, E., Campiglio, M., Pupa, S. M., Balsari, A. & Ménard, S. Role of exon-16-deleted HER2 in breast carcinomas. *Endocr. Relat. Cancer* **13**, 221–232 (2006).

138. Niikura, N., Liu, J., Hayashi, N., Mittendorf, E. A., Gong, Y., Palla, S. L., *et al.* Loss of human epidermal growth factor receptor 2 (HER2) expression in metastatic sites of HER2-overexpressing primary breast tumors. *J Clin Oncol* **30**, 593–599 (2011).
139. Price-Schiavi, S. A., Jepson, S., Li, P., Arango, M., Rudland, P. S., Yee, L., *et al.* Rat Muc4 (sialomucin complex) reduces binding of anti-ErbB2 antibodies to tumor cell surfaces, a potential mechanism for herceptin resistance. *Int. J. Cancer* **99**, 783–791 (2002).
140. Nagy, P., Friedländer, E., Tanner, M., Kapanen, A. I., Carraway, K. L., Isola, J., *et al.* Decreased accessibility and lack of activation of ErbB2 in JIMT-1, a herceptin-resistant, MUC4-expressing breast cancer cell line. *Cancer Res.* **65**, 473–82 (2005).
141. Raina, D., Uchida, Y., Kharbanda, A., Rajabi, H., Panchamoorthy, G., Jin, C., *et al.* Targeting the MUC1-C oncoprotein downregulates HER2 activation and abrogates trastuzumab resistance in breast cancer cells. *Oncogene* **33**, 3422–3431 (2014).
142. Fessler, S. P., Wotkowicz, M. T., Mahanta, S. K. & Bamdad, C. MUC1* is a determinant of trastuzumab (Herceptin) resistance in breast cancer cells. *Breast Cancer Res. Treat.* **118**, 113–124 (2009).
143. Berns, K., Horlings, H. M., Hennessy, B. T., Madiredjo, M., Hijmans, E. M., Beelen, K., *et al.* A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. *Cancer Cell* **12**, 395–402 (2007).
144. Brady, S. W., Zhang, J., Seok, D., Wang, H. & Yu, D. Enhanced PI3K p110 Signaling Confers Acquired Lapatinib Resistance That Can Be Effectively Reversed by a p110 -Selective PI3K Inhibitor. *Mol. Cancer Ther.* **13**, 60–70 (2014).
145. Clark, A. S., West, K., Streicher, S. & Dennis, P. A. Constitutive and inducible Akt activity promotes resistance to chemotherapy, trastuzumab, or tamoxifen in breast cancer cells. *Mol. Cancer Ther.* **1**, 707–17 (2002).
146. Nagata, Y., Lan, K.-H. H., Zhou, X., Tan, M., Esteva, F. J., Sahin, A. A., *et al.* PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. *Cancer Cell* **6**, 117–127 (2004).
147. Miller, T. W., Rexer, B. N., Garrett, J. T. & Arteaga, C. L. Mutations in the phosphatidylinositol 3-kinase pathway: role in tumor progression and therapeutic implications in breast cancer. *Breast Cancer Res.* **13**, 224 (2011).
148. O'Brien, N. A., Browne, B. C., Chow, L., Wang, Y., Ginther, C., Arboleda, J., *et al.* Activated phosphoinositide 3-Kinase/AKT signaling confers resistance to trastuzumab but not lapatinib. *Mol. Cancer Ther.* **9**, 1489–1502 (2010).
149. Razis, E., Bobos, M., Kotoula, V., Eleftheraki, A. G., Kalofonos, H. P., Pavlakis, K., *et al.* Evaluation of the association of PIK3CA mutations and PTEN loss with efficacy of trastuzumab therapy in metastatic breast cancer. *Breast Cancer Res. Treat.* **128**, 447–456 (2011).
150. Xia, W., Husain, I., Liu, L., Bacus, S., Saini, S., Spohn, J., *et al.* Lapatinib antitumor activity is not dependent upon phosphatase and tensin homologue deleted on chromosome 10 in ErbB2-overexpressing breast cancers. *Cancer Res.* **67**, 1170–5 (2007).
151. Dave, B., Migliaccio, I., Gutierrez, M. C., Wu, M.-F., Chamness, G. C., Wong, H., *et al.* Loss of phosphatase and tensin homolog or phosphoinositol-3 kinase activation and response to trastuzumab or lapatinib in human epidermal growth factor receptor 2-overexpressing locally advanced breast cancers. *J. Clin. Oncol.* **29**, 166–73 (2011).
152. Zhang, S., Huang, W.-C., Li, P., Guo, H., Poh, S.-B., Brady, S. W., *et al.*

Combating trastuzumab resistance by targeting SRC, a common node downstream of multiple resistance pathways. *Nat. Med.* **17**, 461–469 (2011).

153. Rexer, B. N., Ham, A.-J. L., Rinehart, C., Hill, S., de Matos Granja-Ingram, N., González-Angulo, A. M., *et al.* Phosphoproteomic mass spectrometry profiling links Src family kinases to escape from HER2 tyrosine kinase inhibition. *Oncogene* **30**, 4163–4174 (2011).
154. Cheng, H., Ballman, K., Vassilakopoulou, M., Dueck, A. C., Reinholz, M. M., Tenner, K., *et al.* EGFR expression is associated with decreased benefit from trastuzumab in the NCCTG N9831 (Alliance) trial. *Br. J. Cancer* **111**, 1065–71 (2014).
155. Gschwantler-Kaulich, D., Hudelist, G., Koestler, W. J., Czerwenka, K., Mueller, R., Helmy, S., *et al.* EGFR activity in HER-2 over-expressing metastatic breast cancer: evidence for simultaneous phosphorylation of Her-2/neu and EGFR. *Oncol. Rep.* **14**, 305–11 (2005).
156. Frolov, A., Schuller, K., Tzeng, C.-W. D., Cannon, E. E., Ku, B. C., Howard, J. H., *et al.* ErbB3 expression and dimerization with EGFR influence pancreatic cancer cell sensitivity to erlotinib. *Cancer Biol. Ther.* **6**, 548–54 (2007).
157. Rexer, B. N. & Arteaga, C. L. Intrinsic and acquired resistance to HER2-targeted therapies in HER2 gene-amplified breast cancer: mechanisms and clinical implications. *Crit. Rev. Oncog.* **17**, 1–16 (2012).
158. Surmacz, E. Growth factor receptors as therapeutic targets: strategies to inhibit the insulin-like growth factor I receptor. *Oncogene* **22**, 6589–6597 (2003).
159. Lu, Y., Zi, X., Zhao, Y., Mascarenhas, D. & Pollak, M. Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin). *J. Natl. Cancer Inst.* **93**, 1852–7 (2001).
160. Nahta, R., Yuan, L. X. H., Du, Y. & Esteva, F. J. Lapatinib induces apoptosis in trastuzumab-resistant breast cancer cells: effects on insulin-like growth factor I signaling. *Mol. Cancer Ther.* **6**, 667–674 (2007).
161. Shattuck, D. L., Miller, J. K., Carraway, K. L. & Sweeney, C. Met receptor contributes to trastuzumab resistance of Her2-overexpressing breast cancer cells. *Cancer Res.* **68**, 1471–7 (2008).
162. Zhuang, G., Brantley-Sieders, D. M., Vaught, D., Yu, J., Xie, L., Wells, S., *et al.* Elevation of receptor tyrosine kinase EphA2 mediates resistance to trastuzumab therapy. *Cancer Res.* **70**, 299–308 (2010).
163. Nahta, R. & O'Regan, R. M. Therapeutic implications of estrogen receptor signaling in HER2-positive breast cancers. *Breast Cancer Res. Treat.* **135**, 39–48 (2012).
164. Xia, W., Bacus, S., Hegde, P., Husain, I., Strum, J., Liu, L., *et al.* A model of acquired autoresistance to a potent ErbB2 tyrosine kinase inhibitor and a therapeutic strategy to prevent its onset in breast cancer. *Proc. Natl. Acad. Sci.* **103**, 7795–7800 (2006).
165. Guo, S. & Sonenshein, G. E. Forkhead Box Transcription Factor FOXO3a regulates estrogen receptor alpha expression and is repressed by the Her-2/neu/Phosphatidylinositol 3-Kinase/Akt Signaling pathway. *Mol. Cell. Biol.* **24**, 8681–8690 (2004).
166. Rimawi, M. F., Mayer, I. A., Forero, A., Nanda, R., Goetz, M. P., Rodriguez, A. A., *et al.* Multicenter phase II study of neoadjuvant lapatinib and trastuzumab with hormonal therapy and without chemotherapy in patients with human epidermal growth factor receptor 2-overexpressing breast cancer: TBCRC 006. *J. Clin. Oncol.* **31**, 1726–31 (2013).
167. Wilson, T. R., Fridlyand, J., Yan, Y., Penuel, E., Burton, L., Chan, E., *et al.* Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors. *Nature* **487**, 505–509 (2012).
168. DeNardo, D. G., Brennan, D. J., Rexhepaj, E., Ruffell, B., Shiao, S. L., Madden,

S. F., *et al.* Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discov.* **1**, 54–67 (2011).
169. Huang, C., Park, C. C., Hilsenbeck, S. G., Ward, R., Rimawi, M. F., Wang, Y.-C., *et al.* β1 integrin mediates an alternative survival pathway in breast cancer cells resistant to lapatinib. *Breast Cancer Res.* **13**, R84 (2011).
170. Acerbi, I., Cassereau, L., Dean, I., Shi, Q., Au, A., Park, C., *et al.* Human breast cancer invasion and aggression correlates with ECM stiffening and immune cell infiltration. *Integr. Biol. (Camb).* **7**, 1120–34 (2015).
171. Guo, W., Pylayeva, Y., Pepe, A., Yoshioka, T., Muller, W. J., Inghirami, G., *et al.* β4 Integrin Amplifies ErbB2 Signaling to Promote Mammary Tumorigenesis. *Cell* **126**, 489–502 (2006).
172. Waddington, C. H. The epigenotype. 1942. *Int. J. Epidemiol.* **41**, 10–13 (2012).
173. Van Speybroeck, L. From epigenesis to epigenetics: the case of C. H. Waddington. *Ann. N. Y. Acad. Sci.* **981**, 61–81 (2002).
174. Holliday, R. Epigenetics: a historical overview. *Epigenetics* **1**, 76–80
175. Urnov, F. D. & Wolffe, A. P. Above and Within the Genome: Epigenetics Past and Present. *J. Mammary Gland Biol. Neoplasia* **6**, 153–167 (2001).
176. Berger, S. L., Kouzarides, T., Shiekhattar, R. & Shilatifard, A. An operational definition of epigenetics. *Genes Dev.* **23**, 781–783 (2009).
177. Felsenfeld, G. A brief history of epigenetics. *Cold Spring Harb. Perspect. Biol.* **6**, (2014).
178. Portela, A. & Esteller, M. Epigenetic modifications and human disease. *Nat. Biotechnol.* **28**, 1057–1068 (2010).
179. Handel, A. E., Ebers, G. C. & Ramagopalan, S. V. Epigenetics: molecular mechanisms and implications for disease. *Trends Mol. Med.* **16**, 7–16 (2010).
180. Wolffe, A. P. & Matzke, M. A. Epigenetics: regulation through repression. *Science* **286**, 481–6 (1999).
181. Choi, S.-W. & Friso, S. Epigenetics: A new bridge between nutrition and health. *Adv. Nutr.* **1**, 8–16 (2010).
182. Zeilinger, S., Kühnel, B., Klopp, N., Baurecht, H., Kleinschmidt, A., Gieger, C., *et al.* Tobacco smoking leads to extensive genome-wide changes in DNA methylation. *PLoS One* **8**, e63812 (2013).
183. Fraga, M. F., Ballestar, E., Paz, M. F., Ropero, S., Setien, F., Ballestar, M. L., *et al.* Epigenetic differences arise during the lifetime of monozygotic twins. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 10604–9 (2005).
184. Jones, P. A. & Laird, P. W. Cancer epigenetics comes of age. *Nat. Genet.* **21**, 163–7 (1999).
185. Zaidi, S. K., Young, D. W., Montecino, M., Lian, J. B., Stein, J. L., van Wijnen, A. J., *et al.* Architectural epigenetics: mitotic retention of mammalian transcriptional regulatory information. *Mol. Cell. Biol.* **30**, 4758–66 (2010).
186. Allfrey, V. G., Faulkner, R. & Mirsky, A. E. Acetylation and methylation of histone and their possible role in the regulation of RNA synthesis. *Proc. Natl. Acad. Sci. U. S. A.* **51**, 786–94 (1964).
187. Kouzarides, T. Chromatin modifications and their function. *Cell* **128**, 693–705 (2007).
188. Kornberg, R. D. Chromatin structure: a repeating unit of histones and DNA. *Science* **184**, 868–71 (1974).
189. Kornberg, R. D. & Thomas, J. O. Chromatin structure; oligomers of the histones. *Science* **184**, 865–8 (1974).
190. Eickbush, T. H. & Moudrianakis, E. N. The histone core complex: an octamer assembled by two sets of protein-protein interactions. *Biochemistry* **17**, 4955–4964 (1978).
191. Luger, K., Mäder, A. W., Richmond, R. K., Sargent, D. F. & Richmond, T. J. Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* **389**,

251–260 (1997).
192. Dawson, M. A. & Kouzarides, T. Cancer epigenetics: From mechanism to therapy. *Cell* **150**, 12–27 (2012).
193. Dillon, N. Gene regulation and large-scale chromatin organization in the nucleus. *Chromosom. Res.* **14**, 117–126 (2006).
194. Huertas, D., Sendra, R. & Muñoz, P. Chromatin dynamics coupled to DNA repair. *Epigenetics* **4**, 31–42 (2009).
195. Luco, R. F., Pan, Q., Tominaga, K., Blencowe, B. J., Pereira-Smith, O. M. & Misteli, T. Regulation of alternative splicing by histone modifications. *Science (80-.).* **327**, 996–1000 (2010).
196. Abi Khalil, C. The emerging role of epigenetics in cardiovascular disease. *Ther. Adv. Chronic Dis.* **5**, 178–87 (2014).
197. Landgrave-Gómez, J., Mercado-Gómez, O. & Guevara-Guzmán, R. Epigenetic mechanisms in neurological and neurodegenerative diseases. *Front. Cell. Neurosci.* **9**, 58 (2015).
198. Li, B., Carey, M. & Workman, J. L. The Role of Chromatin during Transcription. *Cell* **128**, 707–719 (2007).
199. Rodríguez-Paredes, M. & Esteller, M. Cancer epigenetics reaches mainstream oncology. *Nat. Med.* **17**, 330–339 (2011).
200. Simó-Riudalbas, L. & Esteller, M. Targeting the histone orthography of cancer: drugs for writers, erasers and readers. *Br. J. Pharmacol.* **172**, 2716–2732 (2015).
201. Allis, C. D., Berger, S. L., Cote, J., Dent, S., Jenuwien, T., Kouzarides, T., *et al.* New Nomenclature for Chromatin-Modifying Enzymes. *Cell* **131**, 633–636 (2007).
202. Riggs, A. D. X inactivation, differentiation, and DNA methylation. *Cytogenet. Cell Genet.* **14**, 9–25 (1975).
203. Holliday, R. & Pugh, J. E. DNA modification mechanisms and gene activity during development. *Science* **187**, 226–32 (1975).
204. Gardiner-Garden, M. & Frommer, M. CpG islands in vertebrate genomes. *J. Mol. Biol.* **196**, 261–82 (1987).
205. Takai, D. & Jones, P. A. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 3740–5 (2002).
206. Saxonov, S., Berg, P. & Brutlag, D. L. A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 1412–7 (2006).
207. Ball, M. P., Li, J. B., Gao, Y., Lee, J.-H., LeProust, E. M., Park, I.-H., *et al.* Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. *Nat. Biotechnol.* **27**, 361–368 (2009).
208. Vavouri, T. & Lehner, B. Human genes with CpG island promoters have a distinct transcription-associated chromatin organization. *Genome Biol.* **13**, R110 (2012).
209. Irizarry, R. A., Ladd-Acosta, C., Wen, B., Wu, Z., Montano, C., Onyango, P., *et al.* The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. *Nat. Genet.* **41**, 178–186 (2009).
210. Doi, A., Park, I.-H., Wen, B., Murakami, P., Aryee, M. J., Irizarry, R., *et al.* Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. *Nat. Genet.* **41**, 1350–1353 (2009).
211. Jones, P. A. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat. Rev. Genet.* **13**, 484–492 (2012).
212. Wu, H. & Zhang, Y. Mechanisms and functions of Tet proteinmediated 5-methylcytosine oxidation. *Genes and Development* **25**, 2436–2452 (2011).
213. Baylin, S. B. DNA methylation and gene silencing in cancer. *Nat. Clin. Pract.*

Oncol. Oncol. **2 Suppl 1**, S4-11 (2005).
214. Reik, W. & Lewis, A. Co-evolution of X-chromosome inactivation and imprinting in mammals. *Nat. Rev. Genet.* **6**, 403–410 (2005).
215. Kacem, S. & Feil, R. Chromatin mechanisms in genomic imprinting. *Mamm. Genome* **20**, 544–556 (2009).
216. Heard, E. & Disteche, C. M. Dosage compensation in mammals: fine-tuning the expression of the X chromosome. *Genes Dev.* **20**, 1848–1867 (2006).
217. Bestor, T. H. Transposons Reanimated in Mice. *Cell* **122**, 322–325 (2005).
218. Bird, A. DNA methylation patterns and epigenetic memory. *Genes Dev.* **16**, 6–21 (2002).
219. Pathania, R., Ramachandran, S., Elangovan, S., Padia, R., Yang, P., Cinghu, S., *et al.* DNMT1 is essential for mammary and cancer stem cell maintenance and tumorigenesis. *Nat. Commun.* **6**, 6910 (2015).
220. Okano, M., Bell, D. W., Haber, D. A. & Li, E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* **99**, 247–57 (1999).
221. Li, E., Bestor, T. H. & Jaenisch, R. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* **69**, 915–26 (1992).
222. Day, J. J. & Sweatt, J. D. DNA methylation and memory formation. *Nature Neuroscience* **13**, 1319–1323 (2010).
223. Tahiliani, M., Koh, K. P., Shen, Y., Pastor, W. A., Bandukwala, H., Brudno, Y., *et al.* Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* **324**, 930–5 (2009).
224. Ito, S., D'Alessio, A. C., Taranova, O. V, Hong, K., Sowers, L. C. & Zhang, Y. Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature* **466**, 1129–33 (2010).
225. Cimmino, L., Abdel-Wahab, O., Levine, R. L. & Aifantis, I. TET family proteins and their role in stem cell differentiation and transformation. *Cell Stem Cell* **9**, 193–204 (2011).
226. Lister, R., Pelizzola, M., Dowen, R. H., Hawkins, R. D., Hon, G., Tonti-Filippini, J., *et al.* Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature* **462**, 315–322 (2009).
227. Sandoval, J. & Esteller, M. Cancer epigenomics: Beyond genomics. *Curr. Opin. Genet. Dev.* **22**, 50–55 (2012).
228. Taby, R. & Issa, J.-P. J. Cancer Epigenetics. *CA. Cancer J. Clin.* **60**, 376–392 (2010).
229. Rodriguez, C., Borgel, J., Court, F., Cathala, G., Forné, T. & Piette, J. CTCF is a DNA methylation-sensitive positive regulator of the INK/ARF locus. *Biochem. Biophys. Res. Commun.* **392**, 129–134 (2010).
230. Pérez, A., Castellazzi, C. L., Battistini, F., Collinet, K., Flores, O., Deniz, O., *et al.* Impact of methylation on the physical properties of DNA. *Biophys. J.* **102**, 2140 (2012).
231. Wade, P. A. Methyl CpG-binding proteins and transcriptional repression. *BioEssays* **23**, 1131–1137 (2001).
232. Spruijt, C. G. & Vermeulen, M. DNA methylation: old dog, new tricks? *Nat. Struct. Mol. Biol.* **21**, 949–954 (2014).
233. Bogdanović, O. & Veenstra, G. J. C. DNA methylation and methyl-CpG binding proteins: developmental requirements and function. *Chromosoma* **118**, 549–65 (2009).
234. Bostick, M., Kim, J. K., Estève, P.-O., Clark, A., Pradhan, S. & Jacobsen, S. E. UHRF1 plays a role in maintaining DNA methylation in mammalian cells. *Science* **317**, 1760–4 (2007).
235. Filion, G. J. P., Zhenilo, S., Salozhin, S., Yamada, D., Prokhortchouk, E. & Defossez, P.-A. A Family of human zinc finger proteins that bind methylated

DNA and repress transcription. *Mol. Cell. Biol.* **26**, 169–181 (2006).
236. Amir, R. E., Van den Veyver, I. B., Wan, M., Tran, C. Q., Francke, U. & Zoghbi, H. Y. Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. *Nat. Genet.* **23**, 185–188 (1999).
237. Smith, Z. D. & Meissner, A. DNA methylation: roles in mammalian development. *Nat. Rev. Genet.* **14**, 204–220 (2013).
238. Urdinguio, R. G., Lopez-Serra, L., Lopez-Nieva, P., Alaminos, M., Diaz-Uriarte, R., Fernandez, A. F., *et al.* Mecp2-null mice provide new neuronal targets for Rett Syndrome. *PLoS One* **3**, e3669 (2008).
239. Urdinguio, R. G., Sanchez-Mut, J. V & Esteller, M. Epigenetic mechanisms in neurological diseases: genes, syndromes, and therapies. *Lancet Neurol.* **8**, 1056–1072 (2009).
240. Jin, B., Tao, Q., Peng, J., Soo, H. M., Wu, W., Ying, J., *et al.* DNA methyltransferase 3B (DNMT3B) mutations in ICF syndrome lead to altered epigenetic modifications and aberrant expression of genes regulating development, neurogenesis and immune function. *Hum. Mol. Genet.* **17**, 690–709 (2008).
241. Javierre, B. M., Esteller, M. & Ballestar, E. Epigenetic connections between autoimmune disorders and haematological malignancies. *Trends Immunol.* **29**, 616–623 (2008).
242. Amaral, P. P., Dinger, M. E., Mercer, T. R. & Mattick, J. S. The Eukaryotic Genome as an RNA Machine. *Science (80-.).* **319**, 1787–1789 (2008).
243. Ohno, S. So much 'junk' DNA in our genome. *Brookhaven Symp. Biol.* **23**, 366–70 (1972).
244. Alexander, R. P., Fang, G., Rozowsky, J., Snyder, M. & Gerstein, M. B. Annotating non-coding regions of the genome. **11**, 559–571 (2010).
245. Wei, S. & Wang, K. Long noncoding RNAs: pivotal regulators in acute myeloid leukemia. *Exp. Hematol. Oncol.* **5**, 30 (2015).
246. Deniz, E. & Erman, B. Long noncoding RNA (lincRNA), a new paradigm in gene expression control. *Funct. Integr. Genomics* **17**, 135–143 (2017).
247. Liu, G., Mattick, J. S. & Taft, R. J. A meta-analysis of the genomic and transcriptomic composition of complex life. *Cell Cycle* **12**, 2061–2072 (2013).
248. Dunham, I., Kundaje, A., Aldred, S. F., Collins, P. J., Davis, C. A., Doyle, F., *et al.* An integrated encyclopedia of DNA elements in the human genome. *Nature* **489**, 57–74 (2012).
249. Mattick, J. S. Challenging the dogma: the hidden layer of non-protein-coding RNAs in complex organisms. *BioEssays* **25**, 930–939 (2003).
250. Mattick, J. S. & Makunin, I. V. Small regulatory RNAs in mammals. *Hum. Mol. Genet.* **14**, R121–R132 (2005).
251. Palazzo, A. F. & Lee, E. S. Non-coding RNA: what is functional and what is junk? *Front. Genet.* **6**, 2 (2015).
252. Peschansky, V. J. & Wahlestedt, C. Non-coding RNAs as direct and indirect modulators of epigenetic regulation. *Epigenetics* **9**, 3–12 (2014).
253. Shahandeh, A. Molecular mechanisms of oncogenic long non-coding RNAs. *Biosci. Res.* **10**, 38–54 (2013).
254. Prensner, J. R. & Chinnaiyan, A. M. The emergence of lncRNAs in cancer biology. *Cancer Discovery* **1**, 391–407 (2011).
255. Gupta, R. A., Shah, N., Wang, K. C., Kim, J., Horlings, H. M., Wong, D. J., *et al.* Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. *Nature* **464**, 1071–1076 (2010).
256. Esteller, M. Non-coding RNAs in human disease. *Nat. Rev. Genet.* **12**, 861–874 (2011).
257. Rose, N. R. & Klose, R. J. Understanding the relationship between DNA methylation and histone lysine methylation. *Biochimica et Biophysica Acta* -

Gene Regulatory Mechanisms **1839**, 1362–1372 (2014).
258. Huang, B., Jiang, C. & Zhang, R. Epigenetics: the language of the cell? *Epigenomics* **6**, 73–88 (2014).
259. Cedar, H. & Bergman, Y. Linking DNA methylation and histone modification: patterns and paradigms. *Nat. Rev. Genet.* **10**, 295–304 (2009).
260. Eden, S., Hashimshony, T., Keshet, I., Cedar, H. & Thorne, A. W. DNA methylation models histone acetylation. *Nature* **394**, 842–842 (1998).
261. Du, J. & Patel, D. J. Structural biology-based insights into combinatorial readout and crosstalk among epigenetic marks. *Biochim. Biophys. Acta - Gene Regul. Mech.* **1839**, 719–727 (2014).
262. Sharif, J., Muto, M., Takebayashi, S., Suetake, I., Iwamatsu, A., Endo, T. A., *et al.* The SRA protein Np95 mediates epigenetic inheritance by recruiting Dnmt1 to methylated DNA. *Nature* **450**, 908–912 (2007).
263. Karagianni, P., Amazit, L., Qin, J. & Wong, J. ICBP90, a novel methyl K9 H3 binding protein linking protein ubiquitination with heterochromatin formation. *Mol. Cell. Biol.* **28**, 705–717 (2008).
264. Hendrich, B. & Bird, A. Identification and characterization of a family of mammalian methyl-CpG binding proteins. *Mol. Cell. Biol.* **18**, 6538–47 (1998).
265. Nan, X., Ng, H.-H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N., *et al.* Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* **393**, 386–389 (1998).
266. Jones, P. L., Jan Veenstra, G. C., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., *et al.* Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat. Genet.* **19**, 187–191 (1998).
267. Laherty, C. D., Yang, W. M., Sun, J. M., Davie, J. R., Seto, E. & Eisenman, R. N. Histone deacetylases associated with the mSin3 corepressor mediate mad transcriptional repression. *Cell* **89**, 349–56 (1997).
268. Morris, K. V. & Mattick, J. S. The rise of regulatory RNA. *Nat. Rev. Genet.* **15**, 423–437 (2014).
269. Khalil, A. M., Guttman, M., Huarte, M., Garber, M., Raj, A., Rivea Morales, D., *et al.* Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. *Proc. Natl. Acad. Sci.* **106**, 11667–11672 (2009).
270. Chow, J. & Heard, E. X inactivation and the complexities of silencing a sex chromosome. *Curr. Opin. Cell Biol.* **21**, 359–366 (2009).
271. Rinn, J. L., Kertesz, M., Wang, J. K., Squazzo, S. L., Xu, X., Brugmann, S. A., *et al.* Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell* **129**, 1311–1323 (2007).
272. Jones, P. A. & Baylin, S. B. The fundamental role of epigenetic events in cancer. *Nat. Rev. Genet.* **3**, 415–428 (2002).
273. Weber, B., Stresemann, C., Brueckner, B. & Lyko, F. Methylation of Human MicroRNA Genes in Normal and Neoplastic Cells. *Cell Cycle* **6**, 1001–1005 (2007).
274. Zhang, L., Yan, D.-L., Yang, F., Wang, D.-D., Chen, X., Wu, J.-Z., *et al.* DNA methylation mediated silencing of microRNA-874 is a promising diagnosis and prognostic marker in breast cancer. *Oncotarget* **8**, 45496–45505 (2017).
275. Davalos, V., Moutinho, C., Villanueva, A., Boque, R., Silva, P., Carneiro, F., *et al.* Dynamic epigenetic regulation of the microRNA-200 family mediates epithelial and mesenchymal transitions in human tumorigenesis. *Oncogene* **31**, 2062–2074 (2012).
276. Park, S.-M., Gaur, A. B., Lengyel, E. & Peter, M. E. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. *Genes Dev.* **22**, 894–907 (2008).
277. Sandoval, J., Heyn, H., Moran, S., Serra-Musach, J., Pujana, M. A., Bibikova, M.,

et al. Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. *Epigenetics* **6**, 692–702 (2011).
278. Chun, P. Histone deacetylase inhibitors in hematological malignancies and solid tumors. *Arch. Pharm. Res.* **38**, 933–949 (2015).
279. Nervi, C., De Marinis, E. & Codacci-Pisanelli, G. Epigenetic treatment of solid tumours: a review of clinical trials. *Clin. Epigenetics* **7**, 127 (2015).
280. Howell, P. M., Liu, Z., Khong, H. T. & Khong, H. T. Demethylating agents in the treatment of cancer. *Pharmaceuticals (Basel).* **3**, 2022–2044 (2010).
281. Kaminskas, E., Farrell, A. T., Wang, Y.-C., Sridhara, R. & Pazdur, R. FDA Drug Approval Summary: Azacitidine (5-azacytidine, Vidaza™) for Injectable Suspension. *Oncologist* **10**, 176–182 (2005).
282. Home - ClinicalTrials.gov. Available at: https://clinicaltrials.gov/. (Accessed: 12th February 2018)
283. Verstovsek, S., Mesa, R. A., Gotlib, J., Levy, R. S., Gupta, V., DiPersio, J. F., *et al.* A Double-Blind, placebo-controlled trial of Ruxolitinib for myelofibrosis. *N. Engl. J. Med.* **366**, 799–807 (2012).
284. Mann, B. S., Johnson, J. R., Cohen, M. H., Justice, R. & Pazdur, R. FDA Approval summary: Vorinostat for treatment of advanced primary cutaneous T-cell lymphoma. *Oncologist* **12**, 1247–1252 (2007).
285. Qiu, T., Zhou, L., Zhu, W., Wang, T., Wang, J., Shu, Y., *et al.* Effects of treatment with histone deacetylase inhibitors in solid tumors: a review based on 30 clinical trials. *Futur. Oncol.* **9**, 255–269 (2013).
286. Pfister, S. X. & Ashworth, A. Marked for death: targeting epigenetic changes in cancer. *Nat. Rev. Drug Discov.* **16**, 241–263 (2017).
287. Feinberg, A. P. & Vogelstein, B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature* **301**, 89–92 (1983).
288. Heyn, H. & Esteller, M. DNA methylation profiling in the clinic: applications and challenges. *Nat. Rev. Genet.* **13**, 679–692 (2012).
289. Paz, M. F., Fraga, M. F., Avila, S., Guo, M., Pollan, M., Herman, J. G., *et al.* A systematic profile of DNA methylation in human cancer cell lines. *Cancer Res.* **63**, 1114–21 (2003).
290. Gaudet, F., Hodgson, J. G., Eden, A., Jackson-Grusby, L., Dausman, J., Gray, J. W., *et al.* Induction of yumors in mice by genomic hypomethylation. *Science (80-.).* **300**, 489–492 (2003).
291. Howard, G., Eiges, R., Gaudet, F., Jaenisch, R. & Eden, A. Activation and transposition of endogenous retroviral elements in hypomethylation induced tumors in mice. *Oncogene* **27**, 404–408 (2008).
292. Garinis, G. A., Patrinos, G. P., Spanakis, N. E. & Menounos, P. G. DNA hypermethylation: when tumour suppressor genes go silent. *Hum. Genet.* **111**, 115–127 (2002).
293. Esteller, M. Epigenetics in Cancer. *N. Engl. J. Med.* **358**, 1148–1159 (2008).
294. Fraga, M. F., Herranz, M., Espada, J., Ballestar, E., Paz, M. F., Ropero, S., *et al.* A Mouse Skin Multistage Carcinogenesis Model Reflects the Aberrant DNA Methylation Patterns of Human Tumors. *Cancer Res.* **64**, 5527–5534 (2004).
295. Eden, A., Gaudet, F., Waghmare, A. & Jaenisch, R. Chromosomal instability and tumors promoted by DNA hypomethylation. *Science (80-.).* **300**, 455–455 (2003).
296. Rodriguez, J., Frigola, J., Vendrell, E., Risques, R.-A., Fraga, M. F., Morales, C., *et al.* Chromosomal instability correlates with genome-wide DNA demethylation in human primary colorectal cancers. *Cancer Res.* **66**, 8462–9468 (2006).
297. Ogino, S., Kawasaki, T., Nosho, K., Ohnishi, M., Suemoto, Y., Kirkner, G. J., *et al.* LINE-1 hypomethylation is inversely associated with microsatellite instability and CpG island methylator phenotype in colorectal cancer. *Int. J. cancer* **122**, 2767–73 (2008).

298. Jürgens, B., Schmitz-Dräger, B. J. & Schulz, W. A. Hypomethylation of L1 LINE sequences prevailing in human urothelial carcinoma. *Cancer Res.* **56**, 5698–703 (1996).
299. Pattamadilok, J., Huapai, N., Rattanatanyong, P., Vasurattana, A., Triratanachat, S., Tresukosol, D., *et al.* LINE-1 hypomethylation level as a potential prognostic factor for epithelial ovarian cancer. *Int. J. Gynecol. Cancer* **18**, 711–717 (2008).
300. Cho, N.-Y., Kim, B.-H., Choi, M., Yoo, E., Moon, K., Cho, Y.-M., *et al.* Hypermethylation of CpG island loci and hypomethylation of LINE-1 and Alu repeats in prostate adenocarcinoma and their relationship to clinicopathological features. *J. Pathol.* **211**, 269–277 (2007).
301. Wilson, A. S., Power, B. E. & Molloy, P. L. DNA hypomethylation and human diseases. *Biochim. Biophys. Acta - Rev. Cancer* **1775**, 138–162 (2007).
302. Feinberg, A. P. & Vogelstein, B. Hypomethylation of ras oncogenes in primary human cancers. *Biochem. Biophys. Res. Commun.* **111**, 47–54 (1983).
303. Nakamura, N. & Takenaga, K. Hypomethylation of the metastasis-associated S100A4 gene correlates with gene activation in human colon adenocarcinoma cell lines. *Clin. Exp. Metastasis* **16**, 471–9 (1998).
304. Cui, H., Cruz-Correa, M., Giardiello, F. M., Hutcheon, D. F., Kafonek, D. R., Brandenburg, S., *et al.* Loss of IGF2 imprinting: A potential marker of colorectal cancer risk. *Science (80-.).* **299**, 1753–1755 (2003).
305. Bhusari, S., Yang, B., Kueck, J., Huang, W. & Jarrard, D. F. Insulin-like growth factor-2 (IGF2) loss of imprinting marks a field defect within human prostates containing cancer. *Prostate* **71**, 1621–1630 (2011).
306. Wu, H.-K., Weksberg, R., Minden, M. D. & Squire, J. A. Loss of imprinting of human insulin-like growth factor II gene, IGF2, in acute myeloid leukemia. *Biochem. Biophys. Res. Commun.* **231**, 466–472 (1997).
307. van Roozendaal, C. E., Gillis, A. J., Klijn, J. G., van Ooijen, B., Claassen, C. J., Eggermont, A. M., *et al.* Loss of imprinting of IGF2 and not H19 in breast cancer, adjacent normal tissue and derived fibroblast cultures. *FEBS Lett.* **437**, 107–11 (1998).
308. Kim, H. T., Choi, B. H., Niikawa, N., Lee, T. S. & Chang, S. I. Frequent loss of imprinting of the H19 and IGF-II genes in ovarian tumors. *Am. J. Med. Genet.* **80**, 391–5 (1998).
309. Kim, K. S. & Lee, Y. I. Biallelic expression of the H19 and IGF2 genes in hepatocellular carcinoma. *Cancer Lett.* **119**, 143–8 (1997).
310. Ito, Y., Koessler, T., Ibrahim, A. E. K., Rai, S., Vowler, S. L., Abu-Amero, S., *et al.* Somatically acquired hypomethylation of IGF2 in breast and colorectal cancer. *Hum. Mol. Genet.* **17**, 2633–43 (2008).
311. Esteller, M. Epigenetic gene silencing in cancer: The DNA hypermethylome. *Hum. Mol. Genet.* **16**, 50–59 (2007).
312. Baylin, S. B., Höppener, J. W., de Bustros, A., Steenbergh, P. H., Lips, C. J. & Nelkin, B. D. DNA methylation patterns of the calcitonin gene in human lung cancers and lymphomas. *Cancer Res.* **46**, 2917–22 (1986).
313. Greger, V., Passarge, E., Höpping, W., Messmer, E. & Horsthemke, B. Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma. *Hum. Genet.* **83**, 155–8 (1989).
314. Cheung, H.-H., Lee, T.-L., Rennert, O. M. & Chan, W.-Y. DNA methylation of cancer genome. *Birth Defects Res. C. Embryo Today* **87**, 335–50 (2009).
315. Futscher, B. W., O'Meara, M. M., Kim, C. J., Rennels, M. A., Lu, D., Gruman, L. M., *et al.* Aberrant methylation of the Maspin promoter Is an early event in human breast cancer. *Neoplasia* **6**, 380–389 (2004).
316. Bettstetter, M., Woenckhaus, M., Wild, P. J., Rümmele, P., Blaszyk, H., Hartmann, A., *et al.* Elevated nuclear maspin expression is associated with microsatellite instability and high tumour grade in colorectal cancer. *J. Pathol.*

205, 606–614 (2005).
317. Jovanovic, J., Rønneberg, J. A., Tost, J. & Kristensen, V. The epigenetics of breast cancer. *Mol. Oncol.* **4**, 242–254 (2010).
318. Hasan, T. N., Leena Grace, B., Shafi, G. & Syed, R. Association of BRCA1 promoter methylation with rs11655505 (c.2265C>T) variants and decreased gene expression in sporadic breast cancer. *Clin. Transl. Oncol.* **15**, 555–562 (2013).
319. Venkitaraman, A. R. Cancer susceptibility and the functions of BRCA1 and BRCA2. *Cell* **108**, 171–82 (2002).
320. Esteller, M., Silva, J. M., Dominguez, G., Bonilla, F., Matias-Guiu, X., Lerma, E., *et al.* Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. *J. Natl. Cancer Inst.* **92**, 564–9 (2000).
321. Stefansson, O. A., Jonasson, J. G., Olafsdottir, K., Hilmarsdottir, H., Olafsdottir, G., Esteller, M., *et al.* CpG island hypermethylation of BRCA1 and loss of pRb as co-occurring events in basal/triple-negative breast cancer. *Epigenetics* **6**, 638–649 (2011).
322. Hsu, N. C., Huang, Y.-F., Yokoyama, K. K., Chu, P.-Y., Chen, F.-M. & Hou, M.-F. Methylation of BRCA1 promoter region Is associated with unfavorable prognosis in women with early-stage breast cancer. *PLoS One* **8**, e56256 (2013).
323. Khan, S., Kumagai, T., Vora, J., Bose, N., Sehgal, I., Koeffler, P. H., *et al.* PTEN promoter is methylated in a proportion of invasive breast cancers. *Int. J. Cancer* **112**, 407–410 (2004).
324. Lu, Y., Lin, Y.-Z., LaPushin, R., Cuevas, B., Fang, X., Yu, S. X., *et al.* The PTEN/MMAC1/TEP tumor suppressor gene decreases cell growth and induces apoptosis and anoikis in breast cancer cells. *Oncogene* **18**, 7034–7045 (1999).
325. Connolly, R. & Stearns, V. Epigenetics as a therapeutic target in breast cancer. *J. Mammary Gland Biol. Neoplasia* **17**, 191–204 (2012).
326. Park, S. Y., Kwon, H. J., Lee, H. E., Ryu, H. S., Kim, S.-W., Kim, J. H., *et al.* Promoter CpG island hypermethylation during breast cancer progression. *Virchows Arch.* **458**, 73–84 (2011).
327. Kanai, Y., Ushijima, S., Nakanishi, Y., Sakamoto, M. & Hirohashi, S. Mutation of the DNA methyltransferase (DNMT) 1 gene in human colorectal cancers. *Cancer Lett.* **192**, 75–82 (2003).
328. Ley, T. J., Ding, L., Walter, M. J., McLellan, M. D., Lamprecht, T., Larson, D. E., *et al.* DNMT3A mutations in acute myeloid leukemia. *N. Engl. J. Med.* **363**, 2424–2433 (2010).
329. Di Croce, L. Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. *Science (80-.).* **295**, 1079–1082 (2002).
330. Miremadi, A., Oestergaard, M. Z., Pharoah, P. D. P. & Caldas, C. Cancer genetics of epigenetic genes. *Hum. Mol. Genet.* **16**, R28–R49 (2007).
331. el-Deiry, W. S., Nelkin, B. D., Celano, P., Yen, R. W., Falco, J. P., Hamilton, S. R., *et al.* High expression of the DNA methyltransferase gene characterizes human neoplastic cells and progression stages of colon cancer. *Proc. Natl. Acad. Sci.* **88**, 3470–4 (1991).
332. Belinsky, S. A., Nikula, K. J., Baylin, S. B. & Issa, J. P. Increased cytosine DNA-methyltransferase activity is target-cell-specific and an early event in lung cancer. *Proc. Natl. Acad. Sci.* **93**, (1996).
333. Agoston, A. T., Argani, P., Yegnasubramanian, S., De Marzo, A. M., Ansari-Lari, M. A., Hicks, J. L., *et al.* Increased Protein Stability Causes DNA Methyltransferase 1 Dysregulation in Breast Cancer. *J. Biol. Chem.* **280**, 18302–18310 (2005).
334. Chen, J., Wei, X., Wu, Q., Xu, Z., Gu, D., Jin, Y., *et al.* Clinical significance of the expression of DNA methyltransferase proteins in gastric cancer. *Mol. Med. Rep.* **4**, 1139–43 (2011).

335. Etoh, T., Kanai, Y., Ushijima, S., Nakagawa, T., Nakanishi, Y., Sasako, M., *et al.* Increased DNA methyltransferase 1 (DNMT1) protein expression correlates significantly with poorer tumor differentiation and frequent DNA hypermethylation of multiple CpG islands in gastric cancers. *Am. J. Pathol.* **164**, 689–99 (2004).
336. Roll, J. D., Rivenbark, A. G., Jones, W. D. & Coleman, W. B. DNMT3b overexpression contributes to a hypermethylator phenotype in human breast cancer cell lines. *Mol. Cancer* **7**, 15 (2008).
337. Zhu, Y., Brown, H. N., Zhang, Y., Holford, T. R. & Zheng, T. Genotypes and haplotypes of the methyl-CpG-binding domain 2 modify breast cancer risk dependent upon menopausal status. *Breast Cancer Res.* **7**, R745 (2005).
338. Jang, J.-S., Lee, S. J., Choi, J. E., Cha, S. I., Lee, E. B., Park, T. I., *et al.* Methyl-CpG Binding domain 1 gene polymorphisms and risk of primary lung cancer. *Cancer Epidemiol. Biomarkers Prev.* **14**, 2474–2480 (2005).
339. Yang, H., Liu, Y., Bai, F., Zhang, J.-Y., Ma, S.-H., Liu, J., *et al.* Tumor development is associated with decrease of TET gene expression and 5-methylcytosine hydroxylation. *Oncogene* **32**, 663–669 (2013).
340. Hsu, C.-H., Peng, K.-L., Kang, M.-L., Chen, Y.-R., Yang, Y.-C., Tsai, C.-H., *et al.* TET1 Suppresses Cancer Invasion by Activating the Tissue Inhibitors of Metalloproteinases. *Cell Rep.* **2**, 568–579 (2012).
341. Liu, C., Liu, L., Chen, X., Shen, J., Shan, J., Xu, Y., *et al.* Decrease of 5-Hydroxymethylcytosine Is associated with progression of hepatocellular carcinoma through downregulation of TET1. *PLoS One* **8**, e62828 (2013).
342. Rawłuszko-Wieczorek, A. A., Siera, A., Horbacka, K., Horst, N., Krokowicz, P. & Jagodziński, P. P. Clinical significance of DNA methylation mRNA levels of TET family members in colorectal cancer. *J. Cancer Res. Clin. Oncol.* **141**, 1379–1392 (2015).
343. Frycz, B. A., Murawa, D., Borejsza-Wysocki, M., Marciniak, R., Murawa, P., Drews, M., *et al.* Decreased expression of ten-eleven translocation 1 protein is associated with some clinicopathological features in gastric cancer. *Biomed. Pharmacother.* **68**, 209–212 (2014).
344. Delhommeau, F., Dupont, S., Valle, V. Della, James, C., Trannoy, S., Massé, A., *et al.* Mutation in *TET2* in Myeloid Cancers. *N. Engl. J. Med.* **360**, 2289–2301 (2009).
345. Ko, M., Huang, Y., Jankowska, A. M., Pape, U. J., Tahiliani, M., Bandukwala, H. S., *et al.* Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. *Nature* **468**, 839–43 (2010).
346. Heitzer, E., Ulz, P. & Geigl, J. B. Circulating Tumor DNA as a Liquid Biopsy for Cancer. *Clin. Chem.* **61**, 112–123 (2015).
347. Issa, J.-P. J. & Kantarjian, H. M. Targeting DNA methylation. *Clin. Cancer Res.* **15**, 3938–3946 (2009).
348. Yong, W.-S., Hsu, F.-M. & Chen, P.-Y. Profiling genome-wide DNA methylation. *Epigenetics Chromatin* **9**, 26 (2016).
349. Pelizzola, M. & Ecker, J. R. The DNA methylome. *FEBS Lett.* **585**, 1994–2000 (2011).
350. Harrison, A. & Parle-McDermott, A. DNA methylation: a timeline of methods and applications. *Front. Genet.* **2**, 74 (2011).
351. Esteller, M., Garcia-Foncillas, J., Andion, E., Goodman, S. N., Hidalgo, O. F., Vanaclocha, V., *et al.* Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *N. Engl. J. Med.* **343**, 1350–1354 (2000).
352. Barros-Silva, D., Marques, C. J., Henrique, R. & Jerónimo, C. Profiling DNA methylation based on next-generation sequencing approaches: New insights and clinical applications. *Genes* **9**, 429 (2018).

353. Galluzzi, L., Vitale, I., Michels, J., Brenner, C., Szabadkai, G., Harel-Bellan, A., *et al.* Systems biology of cisplatin resistance: Past, present and future. *Cell Death and Disease* **5**, e1257–e1257 (2014).
354. Wei, S. H., Chen, C.-M., Strathdee, G., Harnsomburana, J., Shyu, C.-R., Rahmatpanah, F., *et al.* Methylation microarray analysis of late-stage ovarian carcinomas distinguishes progression-free survival in patients and identifies candidate epigenetic markers. *Clin. Cancer Res.* **8**, 2246–52 (2002).
355. Stefansson, O. A., Villanueva, A., Vidal, A., Martí, L. & Esteller, M. BRCA1 epigenetic inactivation predicts sensitivity to platinum-based chemotherapy in breast and ovarian cancer. *Epigenetics* **7**, 1225–9 (2012).
356. Xu, Y., Diao, L., Chen, Y., Liu, Y., Wang, C., Ouyang, T., *et al.* Promoter methylation of BRCA1 in triple-negative breast cancer predicts sensitivity to adjuvant chemotherapy. *Ann. Oncol. Off. J. Eur. Soc. Med. Oncol.* **24**, 1498–505 (2013).
357. Nimmrich, I., Sieuwerts, A. M., Meijer-van Gelder, M. E., Schwope, I., Bolt-de Vries, J., Harbeck, N., *et al.* DNA hypermethylation of PITX2 is a marker of poor prognosis in untreated lymph node-negative hormone receptor-positive breast cancer patients. *Breast Cancer Res Treat* **111**, 429–437 (2008).
358. Inno, A., Fanetti, G., Di Bartolomeo, M., Gori, S., Maggi, C., Cirillo, M., *et al.* Role of MGMT as biomarker in colorectal cancer. *World J. Clin. cases* **2**, 835–9 (2014).
359. Gu, C., Lu, J., Cui, T., Lu, C., Shi, H., Xu, W., *et al.* Association between MGMT promoter methylation and non-small cell lung cancer: A meta-analysis. *PLoS One* **8**, e72633 (2013).
360. Esteller, M., Gaidano, G., Goodman, S. N., Zagonel, V., Capello, D., Botto, B., *et al.* Hypermethylation of the DNA repair gene O(6)-methylguanine DNA methyltransferase and survival of patients with diffuse large B-cell lymphoma. *J. Natl. Cancer Inst.* **94**, 26–32 (2002).
361. Strathdee, G., MacKean, M. J., Illand, M. & Brown, R. A role for methylation of the hMLH1 promoter in loss of hMLH1 expression and drug resistance in ovarian cancer. *Oncogene* **18**, 2335–2341 (1999).
362. Tang, X., Wu, W., Sun, S.-Y., Wistuba, I. I., Hong, W. K. & Mao, L. Hypermethylation of the death-associated protein kinase promoter attenuates the sensitivity to TRAIL-induced apoptosis in human non-small cell lung cancer cells. *Mol. Cancer Res.* **2**, 685–91 (2004).
363. Hanash, S. M., Baik, C. S. & Kallioniemi, O. Emerging molecular biomarkers—blood-based strategies to detect and monitor cancer. *Nat. Rev. Clin. Oncol.* **8**, 142–150 (2011).
364. Fujii, S., Yamashita, S., Yamaguchi, T., Takahashi, M., Hozumi, Y., Ushijima, T., *et al.* Pathological complete response of HER2-positive breast cancer to trastuzumab and chemotherapy can be predicted by HSD17B4 methylation. *Oncotarget* **8**, 19039–19048 (2017).
365. Veeck, J., Ropero, S., Setien, F., Gonzalez-Suarez, E., Osorio, A., Benitez, J., *et al.* BRCA1 CpG island hypermethylation predicts sensitivity to poly(adenosine diphosphate)-ribose polymerase inhibitors. *J. Clin. Oncol.* **28**, e563–e564 (2010).
366. Dejeux, E., Rønneberg, J., Solvang, H., Bukholm, I., Geisler, S., Aas, T., *et al.* DNA methylation profiling in doxorubicin treated primary locally advanced breast tumours identifies novel genes associated with survival and treatment response. *Mol. Cancer* **9**, 68 (2010).
367. Gao, Y., Lou, G., Zhang, G.-M., Sun, X.-W., Ma, Y.-Y., Yang, Y.-M., *et al.* CHFR promoter hypermethylation and reduced CHFR mRNA expression in ovarian cancer. *Int. J. Biol. Markers* **24**, 83–9
368. Wang, X., Yang, Y., Xu, C., Xiao, L., Shen, H., Zhang, X., *et al.* CHFR suppression by hypermethylation sensitizes endometrial cancer cells to paclitaxel.

Int. J. Gynecol. Cancer **21**, 996–1003 (2011).
369. Li, Y., Yang, Y., Lu, Y., Herman, J. G., Brock, M. V, Zhao, P., *et al.* Predictive value of CHFR and MLH1 methylation in human gastric cancer. *Gastric Cancer* **18**, 280–7 (2015).
370. Chekhun, V. F., Kulik, G. I., Yurchenko, O. V., Tryndyak, V. P., Todor, I. N., Luniv, L. S., *et al.* Role of DNA hypomethylation in the development of the resistance to doxorubicin in human MCF-7 breast adenocarcinoma cells. *Cancer Lett.* **231**, 87–93 (2006).
371. Bruegl, A. S., Djordjevic, B., Urbauer, D. L., Westin, S. N., Soliman, P. T., Lu, K. H., *et al.* Utility of MLH1 methylation analysis in the clinical evaluation of Lynch Syndrome in women with endometrial cancer. *Curr. Pharm. Des.* **20**, 1655–63 (2014).
372. Ferreri, A. J. M., Dell'Oro, S., Capello, D., Ponzoni, M., Iuzzolino, P., Rossi, D., *et al.* Aberrant methylation in the promoter region of the reduced folate carrier gene is a potential mechanism of resistance to methotrexate in primary central nervous system lymphomas. *Br. J. Haematol.* **126**, 657–664 (2004).
373. Ibanez de Caceres, I., Cortes-Sempere, M., Moratilla, C., Machado-Pinilla, R., Rodriguez-Fanjul, V., Manguán-García, C., *et al.* IGFBP-3 hypermethylation-derived deficiency mediates cisplatin resistance in non-small-cell lung cancer. *Oncogene* **29**, 1681–1690 (2010).
374. Perez-Carbonell, L., Balaguer, F., Toiyama, Y., Egoavil, C., Rojas, E., Guarinos, C., *et al.* IGFBP3 methylation is a novel diagnostic and predictive biomarker in colorectal cancer. *PLoS One* **9**, e104285 (2014).
375. Mima, K., Nowak, J. A., Qian, Z. R., Cao, Y., Song, M., Masugi, Y., *et al.* Tumor LINE-1 methylation level and colorectal cancer location in relation to patient survival. *Oncotarget* **7**, 55098–55109 (2016).
376. Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework. *Clin. Pharmacol. Ther.* **69**, 89–95 (2001).
377. Prensner, J. R., Rubin, M. A., Wei, J. T. & Chinnaiyan, A. M. Beyond PSA: the next generation of prostate cancer biomarkers. *Sci. Transl. Med.* **4**, 127rv3 (2012).
378. Duffy, M. J., Napieralski, R., Martens, J. W. M., Span, P. N., Spyratos, F., Sweep, F. C. G. J., *et al.* Methylated genes as new cancer biomarkers. *Eur. J. Cancer* **45**, 335–346 (2009).
379. Kulasingam, V. & Diamandis, E. P. Strategies for discovering novel cancer biomarkers through utilization of emerging technologies. *Nat. Clin. Pract. Oncol.* **5**, 588–599 (2008).
380. Bigbee, W. & Herberman, R. Characteristics of the ideal tumor marker. in *Holland-Frei cancer medicine. 6th edition.* (BC Decker, 2003).
381. Van Neste, L., Herman, J. G., Otto, G., Bigley, J. W., Epstein, J. I. & Van Criekinge, W. The Epigenetic promise for prostate cancer diagnosis. *Prostate* **72**, 1248–1261 (2012).
382. Esteller, M., Corn, P. G., Urena, J. M., Gabrielson, E., Baylin, S. B. & Herman, J. G. Inactivation of glutathione S-transferase P1 gene by promoter hypermethylation in human neoplasia. *Cancer Res.* **58**, 4515–8 (1998).
383. Jerónimo, C., Usadel, H., Henrique, R., Oliveira, J., Lopes, C., Nelson, W. G., *et al.* Quantitation of GSTP1 methylation in non-neoplastic prostatic tissue and organ-confined prostate adenocarcinoma. *J. Natl. Cancer Inst.* **93**, 1747–52 (2001).
384. Henrique, R. & Jerónimo, C. Molecular Detection of Prostate Cancer: A Role for GSTP1 Hypermethylation. *Eur. Urol.* **46**, 660–669 (2004).
385. Karsli-Ceppioglu, S., Dagdemir, A., Judes, G., Ngollo, M., Penault-Llorca, F., Pajon, A., *et al.* Epigenetic mechanisms of breast cancer: an update of the current

knowledge. *Epigenomics* **6**, 651–664 (2014).
386. Muggerud, A. A., Rønneberg, J. A., Wärnberg, F., Botling, J., Busato, F., Jovanovic, J., *et al.* Frequent aberrant DNA methylation of ABCB1, FOXC1, PPP2R2B and PTEN in ductal carcinoma in situ and early invasive breast cancer. *Breast Cancer Res.* **12**, R3 (2010).
387. Lo, P.-K. & Sukumar, S. Epigenomics and breast cancer. *Pharmacogenomics* **9**, 1879–1902 (2008).
388. Birgisdottir, V., Stefansson, O. A., Bodvarsdottir, S. K., Hilmarsdottir, H., Jonasson, J. G. & Eyfjord, J. E. Epigenetic silencing and deletion of the BRCA1gene in sporadic breast cancer. *Breast Cancer Res.* **8**, R38 (2006).
389. Terry, M. B., McDonald, J. A., Wu, H. C., Eng, S. & Santella, R. M. Epigenetic biomarkers of breast cancer risk: Across the breast cancer prevention continuum. in *Advances in Experimental Medicine and Biology* **882**, 33–68 (2016).
390. Berdasco, M., Ropero, S., Setien, F., Fraga, M. F., Lapunzina, P., Losson, R., *et al.* Epigenetic inactivation of the Sotos overgrowth syndrome gene histone methyltransferase NSD1 in human neuroblastoma and glioma. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 21830–5 (2009).
391. Tang, X., Khuri, F. R., Lee, J. J., Kemp, B. L., Liu, D., Hong, W. K., *et al.* Hypermethylation of the death-associated protein (DAP) kinase promoter and aggressiveness in stage I non-small-cell lung cancer. *J. Natl. Cancer Inst.* **92**, 1511–6 (2000).
392. Maeda, K., Kawakami, K., Ishida, Y., Ishiguro, K., Omura, K. & Watanabe, G. Hypermethylation of the CDKN2A gene in colorectal cancer is associated with shorter survival. *Oncol. Rep.* **10**, 935–8
393. Martins, A. T., Monteiro, P., Ramalho-Carvalho, J., Costa, V. L., Dinis-Ribeiro, M., Leal, C., *et al.* High RASSF1A promoter methylation levels are predictive of poor prognosis in fine-needle aspirate washings of breast cancer lesions. *Breast Cancer Res. Treat.* **129**, 1–9 (2011).
394. Stefansson, O. A. & Esteller, M. Epigenetic modifications in breast cancer and their role in personalized medicine. *Am. J. Pathol.* **183**, 1052–1063 (2013).
395. Martens, J. W. M., Nimmrich, I., Koenig, T., Look, M. P., Harbeck, N., Model, F., *et al.* Association of DNA Mmethylation of phosphoserine aminotransferase with response to endocrine therapy in patients with recurrent breast cancer. *Cancer Res.* **65**, 4101–4117 (2005).
396. Ioannidis, J. P. A. & Bossuyt, P. M. M. Waste, leaks, and failures in the biomarker pipeline. *Clin. Chem.* **63**, 963–972 (2017).
397. Lofton-Day, C., Model, F., DeVos, T., Tetzner, R., Distler, J., Schuster, M., *et al.* DNA methylation biomarkers for blood-based colorectal cancer screening. *Clin. Chem.* **54**, 414–423 (2008).
398. Grützmann, R., Molnar, B., Pilarsky, C., Habermann, J. K., Schlag, P. M., Saeger, H. D., *et al.* Sensitive detection of colorectal cancer in peripheral blood by septin 9 DNA methylation assay. *PLoS One* **3**, e3759 (2008).
399. Lamb, Y. N. & Dhillon, S. Epi proColon® 2.0 CE: A Blood-based screening test for colorectal cancer. *Mol. Diagn. Ther.* **21**, 225–232 (2017).
400. Trempe, G. L. Human breast cancer in culture. *Recent Results Cancer Res.* 33–41 (1976).
401. Blancafort, A., Giró-Perafita, A., Oliveras, G., Palomeras, S., Turrado, C., Campuzano, Ò., *et al.* Dual fatty acid synthase and HER2 signaling blockade shows marked antitumor activity against breast cancer models resistant to Anti-HER2 drugs. *PLoS One* **10**, (2015).
402. Puig, T., Aguilar, H., Cufí, S., Oliveras, G., Turrado, C., Ortega-gutiérrez, S., *et al.* A novel inhibitor of fatty acid synthase shows activity against HER2 + breast cancer xenografts and is active in anti-HER2 drug-resistant cell lines. *Breast Cancer Res.* **13**, R131 (2011).

403. Edge, S. B. & Compton, C. C. The American joint committee on cancer: the 7th edition of the AJCC Cancer Staging Manual and the Future of TNM. *Ann. Surg. Oncol.* **17**, 1471–1474 (2010).
404. Christman, J. K. 5-Azacytidine and 5-aza-2′-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. *Oncogene* **21**, 5483–5495 (2002).
405. Herman, J. G., Graff, J. R., Myohanen, S., Nelkin, B. D. & Baylin, S. B. Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands (DNA methylation/tumor suppressor genes/pl6/p15). *Proc Natl Acad Sci U S A* **93**, 9821–9826 (1996).
406. Zhang, Y., Bailey, V., Puleo, C. M., Easwaran, H., Griffiths, E., Herman, J. G., *et al.* DNA methylation analysis on a droplet-in-oil PCR array. *Lab Chip* **9**, 1059 (2009).
407. Falcon, S. & Gentleman, R. Using GOstats to test gene lists for GO term association. *Bioinformatics* **23**, 257–258 (2007).
408. Vizoso, M., Ferreira, H. J., Lopez-Serra, P., Carmona, F. J., Martínez-Cardús, A., Girotti, M. R., *et al.* Epigenetic activation of a cryptic TBC1D16 transcript enhances melanoma progression by targeting EGFR. *Nat. Med.* **21**, 741–750 (2015).
409. Ween, M. P., Oehler, M. K. & Ricciardelli, C. Transforming growth factor-beta-induced protein (TGFBI)/(βig-H3): A matrix protein with dual functions in ovarian cancer. *Int. J. Mol. Sci.* **13**, 10461–10477 (2012).
410. Nahta, R. & Esteva, F. J. *In vitro* effects of trastuzumab and vinorelbine in trastuzumab-resistant breast cancer cells. *Cancer Chemother. Pharmacol.* **53**, 186–190 (2004).
411. Diaz-Lagares, A., Crujeiras, A. B., Lopez-Serra, P., Soler, M., Setien, F., Goyal, A., *et al.* Epigenetic inactivation of the p53-induced long noncoding RNA TP53 target 1 in human cancer. *Proc. Natl. Acad. Sci.* **113**, E7535–E7544 (2016).
412. Diaz-Lagares, A., Mendez-Gonzalez, J., Hervas, D., Saigi, M., Pajares, M. J., Garcia, D., *et al.* A novel epigenetic signature for early diagnosis in lung cancer. *Clin. Cancer Res.* **22**, 3361–3371 (2016).
413. Naumov, V. A., Generozov, E. V, Zaharjevskaya, N. B., Matushkina, D. S., Larin, A. K., Chernyshov, S. V, *et al.* Genome-scale analysis of DNA methylation in colorectal cancer using Infinium HumanMethylation450 BeadChips. *Epigenetics* **8**, 921–34 (2013).
414. Baxter, E., Windloch, K., Gannon, F. & Lee, J. S. Epigenetic regulation in cancer progression. *Cell Biosci.* **4**, 45 (2014).
415. Wen, G., Partridge, M. A., Li, B., Hong, M., Liao, W., Cheng, S. K., *et al.* TGFBI expression reduces *in vitro* and *in vivo* metastatic potential of lung and breast tumor cells. *Cancer Lett.* **308**, 23–32 (2011).
416. Kronski, E., Fiori, M. E., Barbieri, O., Astigiano, S., Mirisola, V., Killian, P. H., *et al.* miR181b is induced by the chemopreventive polyphenol curcumin and inhibits breast cancer metastasis via down-regulation of the inflammatory cytokines CXCL1 and -2. *Mol. Oncol.* **8**, 581–595 (2014).
417. Wang, K., Cao, F., Fang, W., Hu, Y., Chen, Y., Ding, H., *et al.* Activation of SNAT1/SLC38A1 in human breast cancer: correlation with p-Akt overexpression. *BMC Cancer* **13**, 343 (2013).
418. Ng, E. K., Shin, V. Y., Leung, C. P., Chan, V. W., Law, F. B., Siu, M. T., *et al.* Elevation of methylated DNA in KILLIN/PTEN in the plasma of patients with thyroid and/or breast cancer. *Onco. Targets. Ther.* **7**, 2085–92 (2014).
419. Wang, Y., He, X., Yu, Q. & Eng, C. Androgen receptor-induced tumor suppressor, KLLN, inhibits breast cancer growth and transcriptionally activates p53/p73-mediated apoptosis in breast carcinomas. *Hum. Mol. Genet.* **22**, 2263–2272 (2013).

420. Wang, Y., Yu, Q., He, X., Romigh, T., Altemus, J. & Eng, C. Activation of AR sensitizes breast carcinomas to NVP-BEZ235's therapeutic effect mediated by PTEN and KLLN upregulation. *Mol. Cancer Ther.* **13**, 517–27 (2014).
421. Giraud, M.-N., Flück, M., Zuppinger, C. & Suter, T. M. Expressional reprogramming of survival pathways in rat cardiocytes by neuregulin-1β. *J. Appl. Physiol.* **99**, 313–322 (2005).
422. Cadenas, C., Franckenstein, D., Schmidt, M., Gehrmann, M., Hermes, M., Geppert, B., *et al.* Research article Role of thioredoxin reductase 1 and thioredoxin interacting protein in prognosis of breast cancer. *Breast Cancer Res.* **12**, R44 (2010).
423. Lindqvist, B. M., Wingren, S., Motlagh, P. B. & Nilsson, T. K. Whole genome DNA methylation signature of HER2-positive breast cancer. *Epigenetics* **9**, 1149–1162 (2014).
424. Faryna, M., Konermann, C., Aulmann, S., Bermejo, J. L., Brugger, M., Diederichs, S., *et al.* Genome-wide methylation screen in low-grade breast cancer identifies novel epigenetically altered genes as potential biomarkers for tumor diagnosis. *FASEB J.* **26**, 4937–4950 (2012).
425. Cao, Y., Slaney, C. Y., Bidwell, B. N., Parker, B. S., Johnstone, C. N., Rautela, J., *et al.* BMP4 inhibits breast cancer metastasis by blocking myeloid-derived suppressor cell activity. *Cancer Res.* **74**, 5091–5102 (2014).
426. Landemaine, T., Jackson, A., Bellahcène, A., Rucci, N., Sin, S., Abad, B. M., *et al.* A six-gene signature predicting breast cancer lung metastasis. *Cancer Res* **68**, 6092–9 (2008).
427. Terrinoni, A., Pagani, I. S., Zucchi, I., Chiaravalli, A. M., Serra, V., Rovera, F., *et al.* OTX1 expression in breast cancer is regulated by p53. *Oncogene* **30**, 3096–3103 (2011).
428. Pagani, I. S., Terrinoni, A., Marenghi, L., Zucchi, I., Chiaravalli, A. M., Serra, V., *et al.* The mammary gland and the Homeobox gene Otx1. *Breast J.* **16**, S53–S56 (2010).
429. Bender, C. M., Pao, M. M. & Jones, P. A. Inhibition of DNA methylation by 5-aza-2'-deoxycytidine suppresses the growth of human tumor cell lines. *Cancer Res.* **58**, 95–101 (1998).
430. Dejeux, E., Abdalaoui, H. El, Gut, I. G. & Tost, J. Identification and quantification of differentially methylated loci by the Pyrosequencing™ technology. *Methods Mol Biol.* **507**, 189–205 (2009).
431. Jeong, H.-W. & Kim, I.-S. TGF-beta1 enhances betaig-h3-mediated keratinocyte cell migration through the alpha3beta1 integrin and PI3K. *J. Cell. Biochem.* **92**, 770–780 (2004).
432. Billings, P. C., Charles Whitbeck, J., Adams, C. S., Abrams, W. R., Cohen, A. J., Engelsberg, B. N., *et al.* The transforming growth factor-β-inducible matrix protein βig-h3 interacts with fibronectin. *J. Biol. Chem.* **277**, 28003–9 (2002).
433. Hashimoto, K., Noshiro, M., Ohno, S., Kawamoto, T., Satakeda, H., Akagawa, Y., *et al.* Characterization of a cartilage-derived 66-kDa protein (RGD-CAP/βig-h3) that binds to collagen. *Biochim. Biophys. Acta - Mol. Cell Res.* **1355**, 303–314 (1997).
434. Kim, J. E., Kim, S. J., Lee, B. H., Park, R. W., Kim, K. S. & Kim, I. S. Identification of motifs for cell adhesion within the repeated domains of transforming growth factor-beta-induced gene, betaig-h3. *J. Biol. Chem.* **275**, 30907–15 (2000).
435. Park, S. J., Park, S., Ahn, H.-C., Kim, I.-S. & Lee, B.-J. Conformational resemblance between the structures of integrin-activating pentapetides derived from βig-h3 and RGD peptide analogues in a membrane environment. *Peptides* **25**, 199–205 (2004).
436. Paluszczak, J. & Baer-Dubowska, W. Epigenetic diagnostics of cancer — the

application of DNA methylation markers. *J. Appl. Genet.* **47**, 365–375 (2006).
437. A B, T. P. Trastuzumab and lapatinib-resistant cell lines as an screening method for testing the drug antitumor activity in this setting. (2013).
438. Dua, R., Zhang, J., Nhonthachit, P., Penuel, E., Petropoulos, C. & Parry, G. EGFR over-expression and activation in high HER2, ER negative breast cancer cell line induces trastuzumab resistance. *Breast Cancer Res. Treat.* **122**, 685–697 (2010).
439. Ritter, C. A., Perez-Torres, M., Rinehart, C., Guix, M., Dugger, T., Engelman, J. A., *et al.* Human breast cancer cells selected for resistance to trastuzumab *in vivo* overexpress epidermal growth factor receptor and ErbB ligands and remain dependent on the ErbB receptor network. *Clin. Cancer Res.* **13**, 4909–19 (2007).
440. Canfield, K., Li, J., Wilkins, O. M., Morrison, M. M., Ung, M., Wells, W., *et al.* Receptor tyrosine kinase ERBB4 mediates acquired resistance to ERBB2 inhibitors in breast cancer cells. *Cell Cycle* **14**, 648–55 (2015).
441. Kinzler, K. W. & Vogelstein, B. Lessons from hereditary colorectal cancer. *Cell* **87**, 159–70 (1996).
442. Ma, X.-J., Salunga, R., Tuggle, J. T., Gaudet, J., Enright, E., McQuary, P., *et al.* Gene expression profiles of human breast cancer progression. *Proc. Natl. Acad. Sci.* **100**, 5974–5979 (2003).
443. Chang, X., Monitto, C. L., Demokan, S., Kim, M. S., Chang, S. S., Zhong, X., *et al.* Identification of hypermethylated genes associated with cisplatin resistance in human cancers. *Cancer Res.* **70**, 2870–2879 (2010).
444. Zhang, X., Li, W., Li, H., Ma, Y., He, G. & Tan, G. Genomic methylation profiling combined with gene expression microarray reveals the aberrant methylation mechanism involved in nasopharyngeal carcinoma taxol resistance. *Anticancer. Drugs* **23**, 856–864 (2012).
445. He, D.-X., Gu, F., Gao, F., Hao, J., Gong, D., Gu, X.-T., *et al.* Genome-wide profiles of methylation, microRNAs, and gene expression in chemoresistant breast cancer. *Sci. Rep.* **6**, 24706 (2016).
446. Zhang, L., Huang, Y., Zhuo, W., Zhu, Y., Zhu, B. & Chen, Z. Identification and characterization of biomarkers and their functions for Lapatinib-resistant breast cancer. *Med. Oncol.* **34**, 89 (2017).
447. Son, B., Lee, S., Youn, H., Kim, E., Kim, W. & Youn, B. The role of tumor microenvironment in therapeutic resistance. *Oncotarget* **8**, 3933–3945 (2017).
448. Meads, M. B., Hazlehurst, L. A. & Dalton, W. S. The Bone marrow microenvironment as a tumor sanctuary and contributor to drug resistance. *Clin. Cancer Res.* **14**, 2519–2526 (2008).
449. Damiano, J. S. Integrins as novel drug targets for overcoming innate drug resistance. *Curr. Cancer Drug Targets* **2**, 37–43 (2002).
450. Holle, A. W., Young, J. L. & Spatz, J. P. *In vitro* cancer cell–ECM interactions inform *in vivo* cancer treatment. *Adv. Drug Deliv. Rev.* **97**, 270–279 (2016).
451. Beyer, I., Li, Z., Persson, J., Liu, Y., van Rensburg, R., Yumul, R., *et al.* Controlled extracellular matrix degradation in breast cancer tumors improves therapy by trastuzumab. *Mol. Ther.* **19**, 479–89 (2011).
452. Weigelt, B., Lo, A. T., Park, C. C., Gray, J. W. & Bissell, M. J. HER2 signaling pathway activation and response of breast cancer cells to HER2-targeting agents is dependent strongly on the 3D microenvironment. **122**, 35–43 (2010).
453. Esteller, M., Corn, P. G., Baylin, S. B. & Herman, J. G. A gene hypermethylation profile of human cancer. *Cancer Res.* **61**, 3225–9 (2001).
454. Nordlund, J., Bäcklin, C. L., Wahlberg, P., Busche, S., Berglund, E. C., Eloranta, M.-L., *et al.* Genome-wide signatures of differential DNA methylation in pediatric acute lymphoblastic leukemia. *Genome Biol.* **14**, r105 (2013).
455. Pennacchio, L. A., Bickmore, W., Dean, A., Nobrega, M. A. & Bejerano, G. Enhancers: five essential questions. *Nat. Rev. Genet.* **14**, 288–295 (2013).

456. Heyn, H., Vidal, E., Ferreira, H. J., Vizoso, M., Sayols, S., Gomez, A., *et al.* Epigenomic analysis detects aberrant super-enhancer DNA methylation in human cancer. *Genome Biol.* **17**, 11 (2016).
457. Moran, S., Arribas, C. & Esteller, M. Validation of a DNA methylation microarray for 850,000 CpG sites of the human genome enriched in enhancer sequences. *Epigenomics* **8**, 389–99 (2016).
458. Kim, J. E. C., Kim, E. H., Han, E. H., Park, R. W., Park, I. H., Jun, S. H., *et al.* A TGF-beta-inducible cell adhesion molecule, betaig-h3, is downregulated in melorheostosis and involved in osteogenesis. *J. Cell. Biochem.* **77**, 169–78 (2000).
459. Zhao, Y., Shao, G., Piao, C. Q., Berenguer, J. & Hei, T. K. Down-regulation of Betaig-h3 gene is involved in the tumorigenesis in human bronchial epithelial cells induced by heavy-ion radiation. *Radiat. Res.* **162**, 655–9 (2004).
460. Shao, G., Berenguer, J., Borczuk, A. C., Powell, C. A., Hei, T. K. & Zhao, Y. Epigenetic inactivation of Betaig-h3 gene in human cancer cells. *Cancer Res.* **66**, 4566–4573 (2006).
461. Feliciano, P. CXCL1 and CXCL2 link metastasis and chemoresistance. *Nat. Genet.* **44**, 840–840 (2012).
462. Acharyya, S., Oskarsson, T., Vanharanta, S., Malladi, S., Kim, J., Morris, P. G., *et al.* A CXCL1 paracrine network links cancer chemoresistance and metastasis. *Cell* **150**, 165–78 (2012).
463. Bennett, K. L., Campbell, R., Ganapathi, S., Zhou, M., Rini, B., Ganapathi, R., *et al.* Germline and somatic DNA methylation and epigenetic regulation of KILLIN in renal cell carcinoma. *Genes Chromosom. Cancer* **50**, 654–661 (2011).
464. Bennett, K. L., Mester, J. & Eng, C. Germline epigenetic regulation of KILLIN in Cowden and Cowden-like syndrome. *JAMA* **304**, 2724 (2010).
465. Nguyen, P., Awwad, R. T., Smart, D. D. K., Spitz, D. R. & Gius, D. Thioredoxin reductase as a novel molecular target for cancer therapy. *Cancer Lett.* **236**, 164–174 (2006).
466. Fernandes, A. P., Capitanio, A., Selenius, M., Brodin, O., Rundlöf, A.-K. & Björnstedt, M. Expression profiles of thioredoxin family proteins in human lung cancer tissue: correlation with proliferation and differentiation. *Histopathology* **55**, 313–320 (2009).
467. Soini, Y., Kahlos, K., Näpänkangas, U., Kaarteenaho-Wiik, R., Säily, M., Koistinen, P., *et al.* Widespread expression of thioredoxin and thioredoxin reductase in non-small cell lung carcinoma. *Clin. Cancer Res.* **7**, 1750–7 (2001).
468. Uzawa, Uzawa, S., Yamano, Y., Takiguchi, Y., Tanzawa, H., Tatsumi, K., *et al.* Upregulation of thioredoxin reductase 1 in human oral squamous cell carcinoma. *Oncol. Rep.* **25**, 637–44 (2011).
469. Wang, Z., Gerstein, M. & Snyder, M. RNA-Seq: a revolutionary tool for transcriptomics. *Nat. Rev. Genet.* **10**, 57–63 (2009).
470. Griffith, M., Griffith, O. L., Mwenifumbo, J., Goya, R., Morrissy, A. S., Morin, R. D., *et al.* Alternative expression analysis by RNA sequencing. *Nat. Methods* **7**, 843–847 (2010).
471. Perron, B., Lewit-Bentley, A., Geny, B. & Russo-Marie, F. Can enzymatic activity, or otherwise, be inferred from structural studies of annexin III? *J. Biol. Chem.* **272**, 11321–6 (1997).
472. Gerke, V., Creutz, C. E. & Moss, S. E. Annexins: linking Ca2+ signalling to membrane dynamics. *Nat. Rev. Mol. Cell Biol.* **6**, 449–461 (2005).
473. Wu, N., Liu, S., Guo, C., Hou, Z. & Sun, M.-Z. The role of annexin A3 playing in cancers. *Clin. Transl. Oncol.* **15**, 106–110 (2013).
474. Gerke, V. & Moss, S. E. Annexins: From structure to function. *Physiol. Rev.* **82**, 331–371 (2002).
475. Mussunoor, S. & Murray, G. The role of annexins in tumour development and

progression. *J. Pathol.* **216**, 131–140 (2008).
476. Köllermann, J., Schlomm, T., Bang, H., Schwall, G. P., von Eichel-Streiber, C., Simon, R., *et al.* Expression and prognostic relevance of Annexin A3 in prostate cancer. *Eur. Urol.* **54**, 1314–1323 (2008).
477. Bianchi, C., Bombelli, S., Raimondo, F., Torsello, B., Angeloni, V., Ferrero, S., *et al.* Primary cell cultures from human renal cortex and renal-cell carcinoma evidence a differential expression of two spliced isoforms of Annexin A3. *Am. J. Pathol.* **176**, 1660–1670 (2010).
478. Yan, X., Yin, J., Yao, H., Mao, N., Yang, Y. & Pan, L. Increased expression of Annexin A3 is a mechanism of platinum resistance in ovarian cancer. *Cancer Res.* **70**, 1616–1624 (2010).
479. Madoz-Gúrpide, J., López-Serra, P., Martínez-Torrecuadrada, J. L., Sánchez, L., Lombardía, L., Casal, J. I., *et al.* Proteomics-based validation of genomic data. *Mol. Cell. Proteomics* **5**, 1471–1483 (2006).
480. Liu, Y., Xiao, Z., Li, M. M., Li, M. M., Zhang, P., Li, C., *et al.* Quantitative proteome analysis reveals annexin A3 as a novel biomarker in lung adenocarcinoma. *J. Pathol.* **217**, 54–64 (2009).
481. Baine, M. J., Chakraborty, S., Smith, L. M., Mallya, K., Sasson, A. R., Brand, R. E., *et al.* Transcriptional profiling of peripheral blood mononuclear cells in pancreatic cancer patients identifies novel genes with potential diagnostic utility. *PLoS One* **6**, e17014 (2011).
482. Feige, J. N., Gelman, L., Michalik, L., Desvergne, B. & Wahli, W. From molecular action to physiological outputs: Peroxisome proliferator-activated receptors are nuclear receptors at the crossroads of key cellular functions. *Prog. Lipid Res.* **45**, 120–159 (2006).
483. Knouff, C. & Auwerx, J. Peroxisome proliferator-activated receptor-γ calls for activation in moderation: Lessons from genetics and pharmacology. *Endocr. Rev.* **25**, 899–918 (2004).
484. Papadaki, I., Mylona, E., Giannopoulou, I., Markaki, S., Keramopoulos, A. & Nakopoulou, L. PPARgamma expression in breast cancer: clinical value and correlation with ERbeta. *Histopathology* **46**, 37–42 (2005).
485. Sarraf, P., Mueller, E., Smith, W. M., Wright, H. M., Kum, J. B., Aaltonen, L. A., *et al.* Loss-of-function mutations in PPAR gamma associated with human colon cancer. *Mol. Cell* **3**, 799–804 (1999).
486. Sabatino, L., Casamassimi, A., Peluso, G., Barone, M. V., Capaccio, D., Migliore, C., *et al.* A novel peroxisome proliferator-activated receptor gamma isoform with dominant negative activity generated by alternative splicing. *J. Biol. Chem.* **280**, 26517–25 (2005).
487. Capaccio, D., Ciccodicola, A., Sabatino, L., Casamassimi, A., Pancione, M., Fucci, A., *et al.* A novel germline mutation in Peroxisome Proliferator-Activated Receptor γ gene associated with large intestine polyp formation and dyslipidemia. *BBA - Mol. Basis Dis.* **1802**, 572–581 (2010).
488. Jiang, W. G., Douglas-Jones, A. & Mansel, R. E. Expression of peroxisome-proliferator activated receptor-gamma (PPARgamma) and the PPARgamma co-activator, PGC-1, in human breast cancer correlates with clinical outcomes. *Int. J. Cancer* **106**, 752–757 (2003).
489. Okochi-Takada, E., Hattori, N., Tsukamoto, T., Miyamoto, K., Ando, T., Ito, S., *et al.* ANGPTL4 is a secreted tumor suppressor that inhibits angiogenesis. *Oncogene* **33**, 2273–2278 (2014).
490. Gould, M. L., Williams, G. & Nicholson, H. D. Changes in caveolae, caveolin, and polymerase 1 and transcript release factor (PTRF) expression in prostate cancer progression. *Prostate* **70**, 1609–1621 (2010).
491. Gámez-Pozo, A., Sánchez-Navarro, I., Calvo, E., Agulló-Ortuño, M. T., López-Vacas, R., Díaz, E., *et al.* PTRF/Cavin-1 and MIF proteins are identified as Non-

Small Cell Lung Cancer biomarkers by label-free proteomics. *PLoS One* **7**, e33752 (2012).

492. Verma, P., Ostermeyer-Fay, A. G. & Brown, D. A. Caveolin-1 induces formation of membrane tubules that sense actomyosin tension and are inhibited by polymerase I and transcript release factor/cavin-1. *Mol. Biol. Cell* **21**, 2226–40 (2010).
493. Hashizume, T., Fukuda, T., Nagaoka, T., Tada, H., Yamada, H., Watanabe, K., *et al.* Cell type dependent endocytic internalization of ErbB2 with an artificial peptide ligand that binds to ErbB2. *Cell Biol. Int.* **32**, 814–826 (2008).
494. Rao, X., Evans, J., Chae, H., Pilrose, J., Kim, S., Yan, P., *et al.* CpG island shore methylation regulates caveolin-1 expression in breast cancer. *Oncogene* **32**, 4519–4528 (2013).
495. Ogryzko, V. V, Schiltz, R. L., Russanova, V., Howard, B. H. & Nakatani, Y. The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* **87**, 953–9 (1996).
496. Sasajima, Y., Tanaka, H., Miyake, S. & Yuasa, Y. A novel EID family member, EID-3, inhibits differentiation and forms a homodimer or heterodimer with EID-2. *Biochem. Biophys. Res. Commun.* **333**, 969–975 (2005).
497. Båvner, A., Matthews, J., Sanyal, S., Gustafsson, J.-A. & Treuter, E. EID3 is a novel EID family member and an inhibitor of CBP-dependent co-activation. *Nucleic Acids Res.* **33**, 3561–9 (2005).
498. Munakata, K., Uemura, M., Tanaka, S., Kawai, K., Kitahara, T., Miyo, M., *et al.* Cancer stem-like properties in colorectal cancer cells with low proteasome activity. *Clin Cancer Res* **22**, 5277–86 (2016).
499. Neve, R. M., Chin, K., Fridlyand, J., Yeh, J., Baehner, F. L., Fevr, T., *et al.* A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell* **10**, 515–27 (2006).
500. Wang, N., Zhang, H., Yao, Q., Wang, Y., Dai, S. & Yang, X. TGFBI promoter hypermethylation correlating with paclitaxel chemoresistance in ovarian cancer. *J. Exp. Clin. Cancer Res.* **31**, 6 (2012).
501. Gibson, M. A., Kumaratilake, J. S. & Cleary, E. G. The protein components of the 12-nanometer microfibrils of elastic and nonelastic tissues. *J. Biol. Chem.* **264**, 4590–8 (1989).
502. Skonier, J., Neubauer, M., Madisen, L., Bennett, K., Plowman, G. D. & Purchio, A. F. cDNA cloning and sequence analysis of βig-h3, a novel gene induced in a human adenocarcinoma cell line after treatment with transforming growth factor-β. *DNA Cell Biol.* **11**, 511–522 (1992).
503. Kim, J.-E., Jeong, H.-W., Nam, J.-O., Lee, B.-H., Choi, J.-Y., Park, R.-W., *et al.* Identification of motifs in the fasciclin domains of the transforming growth factor-β-induced matrix protein βig-h3 that interact with the αvβ5 integrin. *J. Biol. Chem.* **277**, 46159–46165 (2002).
504. Poulaki, V. & Colby, K. Genetics of anterior and stromal corneal dystrophies. *Semin. Ophthalmol.* **23**, 9–17 (2008).
505. Romero, P., Vogel, M., Diaz, J.-M., Romero, M.-P. & Herrera, L. Anticipation in familial lattice corneal dystrophy type I with R124C mutation in the TGFBI (BIGH3) gene. *Mol. Vis.* **14**, 829–35 (2008).
506. Zhao, Y. L., Piao, C. Q. & Hei, T. K. Downregulation of Betaig-h3 gene is causally linked to tumorigenic phenotype in asbestos treated immortalized human bronchial epithelial cells. *Oncogene* **21**, 7471–7477 (2002).
507. Kim, J.-E., Kim, S.-J., Jeong, H.-W., Lee, B.-H., Choi, J.-Y., Park, R.-W., *et al.* RGD peptides released from βig-h3, a TGF-β-induced cell-adhesive molecule, mediate apoptosis. *Oncogene* **22**, 2045–2053 (2003).
508. Wen, G., Hong, M., Li, B., Liao, W., Cheng, S. K., Hu, B., *et al.* Transforming growth factor-β-induced protein (TGFBI) suppresses mesothelioma progression

through the Akt/mTOR pathway. *Int. J. Oncol.* **39**, 1001–1009 (2011).

509. Zhao, Y. L., Piao, C. Q. & Hei, T. K. Overexpression of Betaig-h3 gene downregulates integrin alpha5beta1 and suppresses tumorigenicity in radiation-induced tumorigenic human bronchial epithelial cells. *Br. J. Cancer* **86**, 1923–8 (2002).
510. Kitahara, O., Furukawa, Y., Tanaka, T., Kihara, C., Ono, K., Yanagawa, R., *et al.* Alterations of gene expression during colorectal carcinogenesis revealed by cDNA microarrays after laser-capture microdissection of tumor tissues and normal epithelia. *Cancer Res.* **61**, 3544–9 (2001).
511. Schneider, D., Kleeff, J., Berberat, P. O., Zhu, Z., Korc, M., Friess, H., *et al.* Induction and expression of betaig-h3 in pancreatic cancer cells. *Biochim. Biophys. Acta* **1588**, 1–6 (2002).
512. Bae, J.-S., Lee, S.-H., Kim, J.-E., Choi, J.-Y., Park, R.-W., Yong Park, J., *et al.* βig-h3 supports keratinocyte adhesion, migration, and proliferation through α3β1 integrin. *Biochem. Biophys. Res. Commun.* **294**, 940–948 (2002).
513. Ohno, S., Noshiro, M., Makihira, S., Kawamoto, T., Shen, M., Yan, W., *et al.* RGD-CAP (βig-h3) enhances the spreading of chondrocytes and fibroblasts via integrin α1β1. *Biochim. Biophys. Acta - Mol. Cell Res.* **1451**, 196–205 (1999).
514. Jeong, H. W. & Kim, I. S. TGF-β1 enhances βig-h3-mediated keratinocyte cell migration through the α3β1 integrin and PI3K. *J. Cell. Biochem.* **92**, 770–780 (2004).
515. Huck, L., Pontier, S. M., Zuo, D. M. & Muller, W. J. 1-integrin is dispensable for the induction of ErbB2 mammary tumors but plays a critical role in the metastatic phase of tumor progression. *Proc. Natl. Acad. Sci.* **107**, 15559–15564 (2010).
516. Desgrosellier, J. S. & Cheresh, D. A. Integrins in cancer: biological implications and therapeutic opportunities. *Nat. Rev. Cancer* **10**, 9–22 (2010).
517. Soung, Y. H., Clifford, J. L. & Chung, J. Crosstalk between integrin and receptor tyrosine kinase signaling in breast carcinoma progression. *BMB Rep.* **43**, 311–8 (2010).
518. Shimizu, H., Seiki, T., Asada, M., Yoshimatsu, K. & Koyama, N. α6β1 integrin induces proteasome-mediated cleavage of erbB2 in breast cancer cells. *Oncogene* **22**, 831–839 (2003).
519. Kuwada, S. K., Kuang, J. & Li, X. Integrin alpha5/beta1 expression mediates HER-2 down-regulation in colon cancer cells. *J. Biol. Chem.* **280**, 19027–35 (2005).
520. Lesniak, D., Xu, Y., Deschenes, J., Lai, R., Thoms, J., Murray, D., *et al.* 1-Integrin Circumvents the Antiproliferative Effects of Trastuzumab in Human Epidermal Growth Factor Receptor-2-Positive Breast Cancer. *Cancer Res.* **69**, 8620–8628 (2009).
521. Nam, J.-O., Jeong, H.-W., Lee, B.-H., Park, R.-W. & Kim, I.-S. Regulation of tumor angiogenesis by Fastatin, the fourth FAS1 domain of βig-h3, via αvβ3 integrin. *Cancer Res.* **65**, 4153–4161 (2005).
522. Nam, J.-O., Kim, J.-E., Jeong, H.-W., Lee, S.-J., Lee, B.-H., Choi, J.-Y., *et al.* Identification of the alphavbeta3 integrin-interacting motif of betaig-h3 and its anti-angiogenic effect. *J. Biol. Chem.* **278**, 25902–9 (2003).
523. Becker, J., Volland, S., Noskova, I., Schramm, A., Schweigerer, L. L. & Wilting, J. Keratoepithelin reverts the suppression of tissue factor pathway inhibitor 2 by MYCN in human neuroblastoma: a mechanism to inhibit invasion. *Int. J. Oncol.* **32**, 235–40 (2008).
524. Bissey, P.-A., Law, J. H., Bruce, J. P., Shi, W., Renoult, A., Chua, M. L. K., *et al.* Dysregulation of the MiR-449b target TGFBI alters the TGFβ pathway to induce cisplatin resistance in nasopharyngeal carcinoma. *Oncogenesis* **7**, 40 (2018).
525. Lal, S., Kersch, C., Beeson, K. A., Wu, Y. J., Muldoon, L. L. & Neuwelt, E. A. Interactions between αv-Integrin and HER2 and their role in the invasive

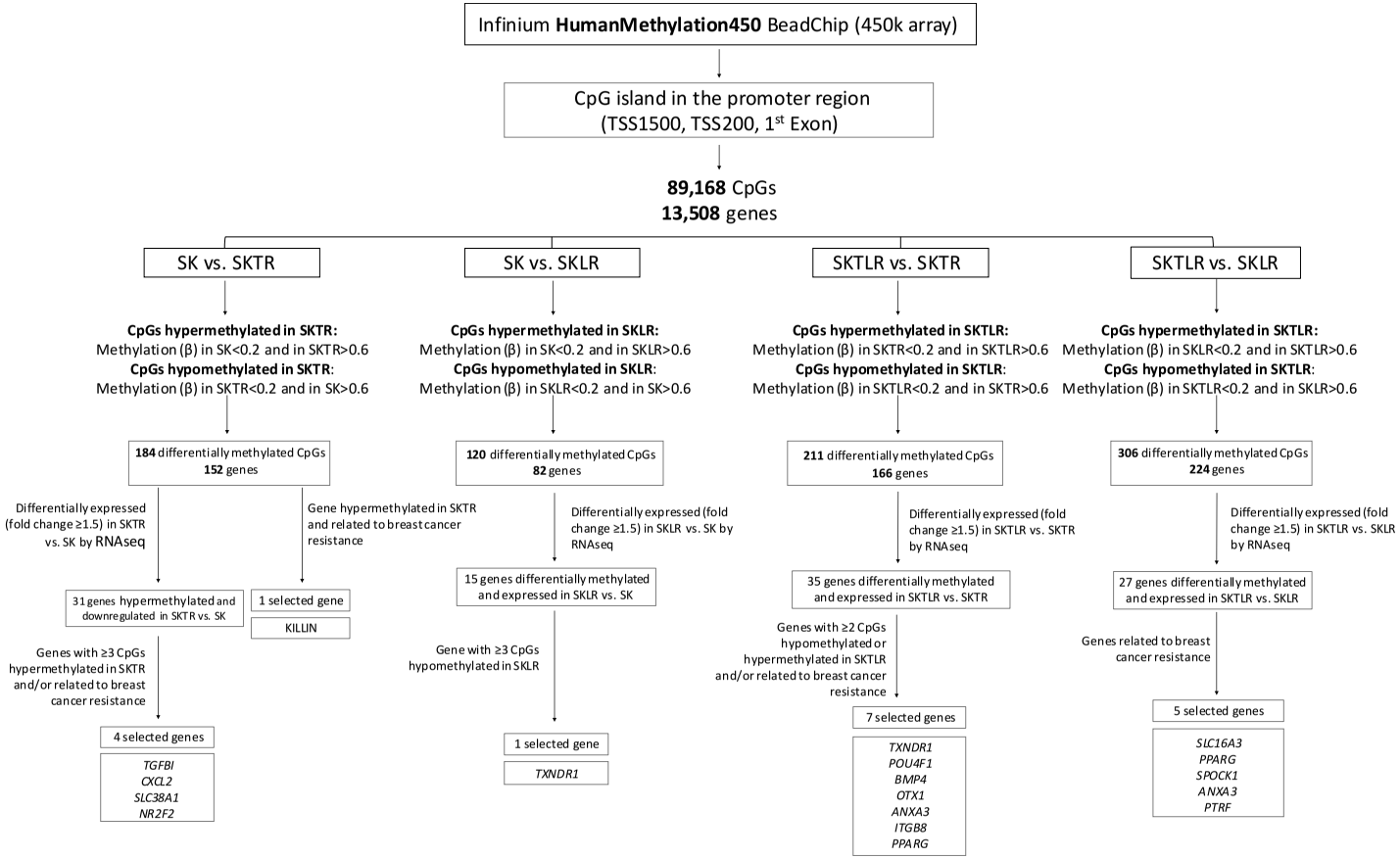
phenotype of breast cancer cells *in vitro* and in rat brain. *PLoS One* **10**, e0131842 (2015).
526. Chow, A., Arteaga, C. L. & Wang, S. E. When tumor suppressor TGFβ meets the HER2 (ERBB2) oncogene. *J. Mammary Gland Biol. Neoplasia* **16**, 81–8 (2011).
527. Wang, S. E. The Functional Crosstalk between HER2 Tyrosine Kinase and TGF-β Signaling in Breast Cancer Malignancy. *J. Signal Transduct.* **2011**, 1–8 (2011).
528. Wrana, J. L., Attisano, L., Wieser, R., Ventura, F. & Massagué, J. Mechanism of activation of the TGF-β receptor. *Nature* **370**, 341–347 (1994).
529. Massagué, J., Blain, S. W. & Lo, R. S. TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell* **103**, 295–309 (2000).
530. Derynck, R., Akhurst, R. J. & Balmain, A. TGF-β signaling in tumor suppression and cancer progression. *Nat. Genet. 2001 292* **29**, 117 (2001).
531. Iwamoto, K. S., Mizuno, T., Ito, T., Akiyama, M., Takeichi, N., Mabuchi, K., *et al.* Feasibility of using decades-old archival tissues in molecular oncology/epidemiology. *Am. J. Pathol.* **149**, 399–406 (1996).
532. Srinivasan, M., Sedmak, D. & Jewell, S. Effect of Fixatives and Tissue Processing on the Content and Integrity of Nucleic Acids. *Am. J. Pathol.* **161**, 1961–1971 (2002).
533. Doyle, B., O'Riain, C. & Appleton, K. Pyrosequencing of DNA extracted from formalin-fixed paraffin-embedded tissue. *Methods Mol Biol.* **724**, 181–190 (2011).
534. Dufort, S., Richard, M.-J. & de Fraipont, F. Pyrosequencing method to detect KRAS mutation in formalin-fixed and paraffin-embedded tumor tissues. *Anal. Biochem.* **391**, 166–168 (2009).
535. Mikeska, T., Bock, C., El-Maarri, O., Hübner, A., Ehrentraut, D., Schramm, J., *et al.* Optimization of quantitative MGMT promoter methylation analysis using pyrosequencing and combined bisulfite restriction analysis. *J. Mol. Diagnostics* **9**, 368–381 (2007).
536. Nosho, K., Shima, K., Irahara, N., Kure, S., Baba, Y., Kirkner, G. J., *et al.* DNMT3B expression might contribute to CpG island methylator phenotype in colorectal cancer. *Clin. Cancer Res.* **15**, 3663–71 (2009).
537. Shah, J. N., Shao, G., Hei, T. K. & Zhao, Y. Methylation screening of the TGFBI promoter in human lung and prostate cancer by methylation-specific PCR. *BMC Cancer* **8**, 284 (2008).
538. Calaf, G. M., Echiburú-Chau, C., Zhao, Y. L. & Hei, T. K. BigH3 protein expression as a marker for breast cancer. *Int. J. Mol. Med.* **21**, 561–8 (2008).
539. Pajares, M. J., Agorreta, J., Salvo, E., Behrens, C., Wistuba, I. I., Montuenga, L. M., *et al.* TGFBI expression is an independent predictor of survival in adjuvant-treated lung squamous cell carcinoma patients. *Br. J. Cancer* **110**, 1545–1551 (2014).
540. Schettini, F., Buono, G., Cardalesi, C., Desideri, I., De Placido, S. & Del Mastro, L. Hormone receptor/human epidermal growth factor receptor 2-positive breast cancer: Where we are now and where we are going. *Cancer Treat. Rev.* **46**, 20–26 (2016).
541. Houston, S. J., Plunkett, T. A., Barnes, D. M., Smith, P., Rubens, R. D. & Miles, D. W. Overexpression of c-erbB2 is an independent marker of resistance to endocrine therapy in advanced breast cancer. *Br. J. Cancer* **79**, 1220–1226 (1999).
542. Baylin, S. B. & Jones, P. A. A decade of exploring the cancer epigenome — biological and translational implications. *Nat. Rev. Cancer* **11**, 726–734 (2011).
543. Weller, M., Stupp, R., Hegi, M. E., van den Bent, M., Tonn, J. C., Sanson, M., *et al.* Personalized care in neuro-oncology coming of age: why we need MGMT and 1p/19q testing for malignant glioma patients in clinical practice. *Neuro. Oncol.* **14**, iv100-iv108 (2012).

544. Iorns, E., Turner, N. C., Elliott, R., Syed, N., Garrone, O., Gasco, M., *et al.* Identification of CDK10 as an important determinant of resistance to endocrine therapy for breast cancer. *Cancer Cell* **13**, 91–104 (2008).
545. Fan, M., Yan, P. S., Hartman-Frey, C., Chen, L., Paik, H., Oyer, S. L., *et al.* Diverse gene expression and DNA methylation profiles correlate with differential adaptation of breast cancer cells to the antiestrogens tamoxifen and fulvestrant. *Cancer Res.* **66**, 11954–11966 (2006).
546. Stetler-Stevenson, W. G., Aznavoorian, S. & Liotta, L. A. Tumor cell interactions with the extracellular matrix during invasion and metastasis. *Annu. Rev. Cell Biol.* **9**, 541–573 (1993).
547. Correia, A. L. & Bissell, M. J. The tumor microenvironment is a dominant force in multidrug resistance. *Drug Resistance Updates* **15**, 39–49 (2012).
548. Ahmed, A. A., Mills, A. D., Ibrahim, A. E. K. K., Temple, J., Blenkiron, C., Vias, M., *et al.* The extracellular matrix protein TGFBI induces microtubule stabilization and sensitizes ovarian cancers to Paclitaxel. *Cancer Cell* **12**, 514–527 (2007).
549. Filipits, M., Dafni, U., Gnant, M., Polydoropoulou, V., Hills, M., Kiermaier, A., *et al.* Association of p27 and cyclin D1 expression and benefit from adjuvant trastuzumab treatment in HER2-positive early breast cancer: A TransHERA study. *Clin. Cancer Res.* **24**, 3079–3086 (2018).
550. Wang, R., Li, X., Zhang, H., Wang, K. & He, J. Cell-free circulating tumor DNA analysis for breast cancer and its clinical utilization as a biomarker. *Oncotarget* **8**, 75742–75755 (2017).
551. Xu, R., Wei, W., Krawczyk, M., Wang, W., Luo, H., Flagg, K., *et al.* Circulating tumour DNA methylation markers for diagnosis and prognosis of hepatocellular carcinoma. *Nat. Mater.* **16**, 1155–1161 (2017).
552. Vaca-Paniagua, F., Oliver, J., da Costa, A. N., Merle, P., McKay, J., Herceg, Z., *et al.* Targeted deep DNA methylation analysis of circulating cell-free DNA in plasma using massively parallel semiconductor sequencing. *Epigenomics* **7**, 353–362 (2015).
553. Fiegl, H., Millinger, S., Mueller-Holzner, E., Marth, C., Ensinger, C., Berger, A., *et al.* Circulating tumor-specific DNA: A marker for monitoring efficacy of adjuvant therapy in cancer patients. *Cancer Res* **65**, 1141–5 (2005).

Chapter VIII: Annexes

Annex I

Supplementary Figure 1. Schematic flow chart used to identify epigenetically regulated genes associated to trastuzumab and lapatinib resistance after comparing the trastuzumab and lapatinib-sensitive (SK) and -resistant (SKLR, SKTR and SKTLR) HER2+ breast cancer models.



Annex II

ARTICLE 1

PLOS | ONE

Dual Fatty Acid Synthase and HER2 Signaling Blockade Shows Marked Antitumor Activity against Breast Cancer Models Resistant to Anti-HER2 Drugs

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Abstract

Blocking the enzyme Fatty Acid Synthase (FASN) leads to apoptosis of HER2-positive breast carcinoma cells. The hypothesis is that blocking FASN, in combination with anti-HER2 signaling agents, would be an effective antitumor strategy in preclinical HER2+ breast cancer models of trastuzumab and lapatinib resistance. We developed and molecularly characterized *in vitro* HER2+ models of resistance to trastuzumab (SK**TR**), lapatinib (SK**LR**) and both (SK**LTR**). The cellular interactions of combining anti-FASN polyphenolic compounds (EGCG and the synthetic G28UCM) with anti-HER2 signaling drugs (trastuzumab plus pertuzumab and temsirolimus) were analyzed. Tumor growth inhibition after treatment with EGCG, pertuzumab, temsirolimus or the combination was evaluated in two *in vivo* orthoxenopatients: one derived from a HER2+ patient and another from a patient who relapsed on trastuzumab and lapatinib-based therapy. SK**TR**, SK**LR** and SK**LTR** showed hyperactivation of EGFR and p-ERK1/2 and PI3KCA mutations. Dual-resistant cells (SK**LTR**) also showed hyperactivation of HER4 and recovered levels of p-AKT compared with mono-resistant cells. mTOR, p-mTOR and FASN expression remained stable in SK**TR**, SK**LR** and SK**LTR**. *In vitro*, anti-FASN compounds plus pertuzumab showed synergistic interactions in lapatinib- and dual- resistant cells and improved the results of pertuzumab plus trastuzumab co-treatment. FASN inhibitors combined with temsirolimus displayed the strongest synergistic interactions in resistant cells. *In vivo*, both orthoxenopatients showed strong response to the antitumor activity of the combination of EGCG with pertuzumab or temsirolimus, without signs of toxicity. We showed that the simultaneous blockade of FASN and HER2 pathways is effective in cells and in breast cancer models refractory to anti-HER2 therapies.

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Competing interests: The authors have declared that no competing interests exist.

ARTICLE 2

Published version cannot be used

Ariadna Giró-Perafita, Sonia Palomeras, David Lum, Adriana Blancafort, Gemma Viñas, Glòria Oliveras, Ferran Pérez-Bueno, Ariadna Sarrats, Alana L Welm and Teresa Puig. "Preclinical Evaluation of Fatty Acid Synthase and EGFR Inhibition in Triple Negative Breast Cancer". *Clinical cancer research* (2016) : p.: 1-11

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Abstract

Abstract Purpose: Triple Negative Breast Cancer (TNBC) lacks an approved targeted therapy. Despite initial good response to chemotherapy, 30% of the patients relapse within 5 years after treatment. EGFR overexpression is a common marker in TNBC, and its expression has been correlated with poor outcome. Inhibition of Fatty Acid Synthase (FASN) activity leads to apoptosis of human carcinoma cells overexpressing FASN. We tested the hypothesis that blocking FASN in combination with anti-EGFR signaling agents would be an effective antitumor strategy in sensitive and chemoresistant TNBC. Experimental Design: Several TNBC cell lines and 29 primary tumors were included to determine whether FASN is a potential target in TNBC. Doxorubicin-resistant TNBC cell lines (231DXR and HCCDXR) have been developed and characterized in our laboratory. Cellular and molecular interactions of anti-FASN compounds (EGCG and C75) with cetuximab were analyzed. In vivo tumor growth inhibition was evaluated after cetuximab, EGCG or the combination in TNBC orthoxenograft models. Results: TNBC cell lines showed overexpression of FASN enzyme and its inhibiton correlated to FASN levels. FASN staining was observed in all of the 29 TNBC tumor samples. In vitro, EGCG and C75 plus cetuximab showed strong synergism in sensitive and chemoresistant cells. In vivo, the combination of EGCG with cetuximab displayed strong antitumor activity against the sensitive and chemoresistant TNBC orthoxenografts, without signs of toxicity. Conclusions: Our results show that the simultaneous blockade of FASN and EGFR is effective in preclinical models of sensitive and chemoresistant TNBC.

ARTICLE 3

molecules

MDPI

Article

Breast Cancer Stem Cell Culture and Enrichment Using Poly(ε-Caprolactone) Scaffolds

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Abstract: The cancer stem cell (CSC) population displays self-renewal capabilities, resistance to conventional therapies, and a tendency to post-treatment recurrence. Increasing knowledge about CSCs' phenotype and functions is needed to investigate new therapeutic strategies against the CSC population. Here, poly(ε-caprolactone) (PCL), a biocompatible polymer free of toxic dye, has been used to fabricate scaffolds, solid structures suitable for 3D cancer cell culture. It has been reported that scaffold cell culture enhances the CSCs population. A RepRap BCN3D+ printer and 3 mm PCL wire were used to fabricate circular scaffolds. PCL design and fabrication parameters were first determined and then optimized considering several measurable variables of the resulting scaffolds. MCF7 breast carcinoma cell line was used to assess scaffolds adequacy for 3D cell culture. To evaluate CSC enrichment, the Mammosphere Forming Index (MFI) was performed in 2D and 3D MCF7 cultures. Results showed that the 60° scaffolds were more suitable for 3D culture than the 45° and 90° ones. Moreover, 3D culture experiments, in adherent and non-adherent conditions, showed a significant increase in MFI compared to 2D cultures (control). Thus, 3D cell culture with PCL scaffolds could be useful to improve cancer cell culture and enrich the CSCs population.

Keywords: breast cancer; cancer stem cell; scaffold; PCL; RepRap; tridimensional cell culture; mammospheres

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molecules

MDPI

Article

(−)-Epigallocatechin 3-Gallate Synthetic Analogues Inhibit Fatty Acid Synthase and Show Anticancer Activity in Triple Negative Breast Cancer

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Abstract: (−)-Epigallocatechin 3-gallate (EGCG) is a natural polyphenol from green tea with reported anticancer activity and capacity to inhibit the lipogenic enzyme fatty acid synthase (FASN), which is overexpressed in several human carcinomas. To improve the pharmacological profile of EGCG, we previously developed a family of EGCG derivatives and the lead compounds G28, G37 and G56 were characterized in HER2-positive breast cancer cells overexpressing FASN. Here, diesters G28, G37 and G56 and two G28 derivatives, monoesters M1 and M2, were synthesized and assessed in vitro for their cytotoxic, FASN inhibition and apoptotic activities in MDA-MB-231 triple-negative breast cancer (TNBC) cells. All compounds displayed moderate to high cytotoxicity and significantly blocked FASN activity, monoesters M1 and M2 being more potent inhibitors than diesters. Interestingly, G28, M1, and M2 also diminished FASN protein expression levels, but only monoesters M1 and M2 induced apoptosis. Our results indicate that FASN inhibition by such polyphenolic compounds could be a new strategy in TNBC treatment, and highlight the potential anticancer activities of monoesters. Thus, G28, G37, G56, and most importantly M1 and M2, are anticancer candidates (alone or in combination) to be further characterized in vitro and in vivo.

Keywords: triple-negative breast cancer; fatty acid synthase; FASN inhibition; polyphenolic FASN inhibitors; (−)-epigallocatechin 3-gallate; synthetic analogues; apoptosis; anticancer activity

ARTICLE 5

molecules

MDPI

Review

Targeting Breast Cancer Stem Cells to Overcome Treatment Resistance

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Abstract: Despite advances in breast cancer diagnosis and treatment, many patients still fail therapy, resulting in disease progression, recurrence, and reduced overall survival. Historically, much focus has been put on the intrinsic subtyping based in the presence (or absence) of classical immunohistochemistry (IHC) markers such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-related protein (HER2). However, it is widely understood that tumors are composed of heterogeneous populations of cells with a hierarchical organization driven by cancer stem cells (CSCs). In breast tumors, this small population of cells displaying stem cell properties is known as breast CSCs (BCSCs). This rare population exhibit a $CD44^+/CD24^{-/low}$ phenotype with high ALDH activity ($ALDH^+$), and possesses higher tolerability to chemotherapy, hormone therapy, and radiotherapy and is able to reproduce the bulk of the tumor after reduction of cell populations sensitive to first-line therapy leading to disease relapse. In this review, we present special attention to BCSCs with future directions in the establishment of a therapy targeting this population. Drugs targeting the main BCSCs signaling pathways undergoing clinical trials are also summarized.

Keywords: BCSCs; resistance; targeted therapy; breast cancer; BCSCs markers