FATTY ACID SYNTHASE EXPRESSION AND INHIBITION IN CANCER

Adriana Blancafort Jorquera

Universitat de Girona

DOCTORAL THESIS

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Adriana Blancafort Jorquera

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Directed and tutorized by:

Dra. Teresa Puig Miquel

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Universitat de Girona

Dr. Teresa Puig Miquel, of University of Girona,

I DECLARE:

That the thesis titles "FATTY ACID SYNTHASE EXPRESSION AND INHIBITION IN CANCER", presented by Adriana Blancafort Jorquera to obtain a doctoral degree, has been completed under my supervision and meets the requirements to opt for an International Doctorate as compendium of articles.

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

Signature

Girona, August 31th 2015

A tu, al teu record,

i al valor que has donat a la nostra família.

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LIST OF PUBLICATIONS RESULTING FROM THE THESIS

This thesis is presented as a compendium of articles.

ARTICLE 1

Title: Fatty Acid Synthase Expression is Strongly Related to Menopause in Early-Stage Breast Cancer Patients

Authors: Rut Porta* and Adriana Blancafort*, Gemma Casòliva, Miquel Casas, Joan Dorca, Maria Buxo, Gemma Viñas, Glòria Oliveras and Teresa Puig (* Equal contributors)

Journal: Menopause. 2014, 21(2):188-91

Impact factor (2014): 3.361 (Q1, Obstetrics & Gynecology)

DOI: 10.1097/GME.0b013e31829d17dc

ARTICLE 2

Title: Different Fatty Acid Metabolism Effects of (−)-Epigallocatechin-3-Gallate and C75 in Adenocarcinoma Lung Cancer

Authors: Joana Relat* and Adriana Blancafort*, Glòria Oliveras, Sílvia Cufí, Diego Haro, Pedro F. Marrero and Teresa Puig (* Equal contributors)

Journal: BMC Cancer. 2012, 12:280

Impact factor (2012): 3.333 (Q2, Oncology)

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ARTICLE 3

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

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DOI: 10.1371/journal.pone.0131241

LIST OF ABBREVIATIONS

1+/2+/3+	*Level of expression: 1+, low expressed; 2+, medium expressed; 3+, high expressed*
ACC	*Acetyl-CoA carboxylase*
ACLY	*ATP citrate lyase*
ACP	*Acyl carrier protein*
ADC	*Antibody-drug conjugate*
ADCC	*Antibody-dependent cell mediated cytotoxicity*
Akt/PKB	*Protein kinase B*
AR	*Amphiregulin*
AR	*Androgen receptor*
BMI	*Body mass index*
BTC	*Betacellulin*
Cpi	*Cell proliferation inhibition*
CPT-1	*Carnitine palmitoyltransferase-1*
CTL	*Cytotoxic T lymphocytes*
DEPTOR	*Pleckstrin [DEP]-domain-containing mTOR interacting protein*
DH	*ß-hydroxyacyl dehydratase*
DNA	*Deoxyribonucleic acid*
EGCG	*(-)-epigallocatechin-3-gallate*
EGF	*Epithermal growth factor*
EGFR	*Epithermal growth factor receptor*
EMT	*Epithelial-mesenchymal transition*
EPR	*Epiregulin*
ER	*Enoyl reductase*
ER	*Estrogen receptor*
ERK	*Extracellular regulated kinase*
FA	*Fatty acids*
FASN	*Fatty acid synthase*
FASN+	*FASN-positive (overexpressed)*
FDA	*United States Food and Drug Administration*
HB-EGF	*Heparin binding EGF-like growth factor*
HER1	*Human epithermal growth factor receptor 1*

HER2	*Human epithermal growth factor receptor 2*
HER2+	*HER2-positive (overexpressed)*
HER2-PDX	*HER2 positive patient derived xenograft. Originally named as "Her2+ Grade 3" in Dr. Marta Palafox' Doctoral Thesis.*
HER2-PDX**R**	*HER2 positive patient derived xenograft resistant to trastuzumab and lapatinib. Originally named as "HCI-012" in DeRose YS et al. Nat Med. 2011.*
HER3	*Human epithermal growth factor receptor 3*
HER4	*Human epithermal growth factor receptor 4*
HNSCC	*Head and neck squamous cell cancer*
IC_{30}	*Concentration that produces 30% of cell viability inhibition*
IC_{50}	*Concentration that produces 50% of cell viability inhibition*
IGF	*Insulin growth factor*
IHQ	*Immunohistochemistry*
KR	*β-ketoreductase*
KS	*β-ketoacyl synthase*
Malonyl-CoA	*Malonyl-coenzyme A*
MAPK	*Mitogen activated kinase-like protein*
MAT	*Malonyl acetyl transferase*
MEK	*MAP kinse-ERK kinase*
MET	*Mesenchymal-epithelial transition*
mTOR	*Mammalian target of rapamycin*
mTORC1	*Mammalian target of rapamycin complex 1*
mTORC2	*Mammalian target of rapamycin complex 2*
n	*Number of patients/samples/cases*
NADPH	*Nicotinamide adenine dinucleotide phosphate*
NF-κB	*Nuclear factor kappa-B*
NK cells	*Natural killer cells*
NRG	*Neuregulin*
NSCLC	*Non-small cell lung cancer*
pCR	*Pathologic complete response*
PDX	*Patient derived xenografts*
PDX**R**	*Patient derived xenografts resistant to anti-HER2 drugs*
PFS	*Progression free survival*

PI3K	*Phosphatidylinositol 3-kinase*
PIP2	*Phosphatidylinositol 4,5-bisphosphate*
PIP3	*Phosphatidylinositol 3,4,5-trisphosphate*
PR	*Progesterone receptor*
PTEN	*Phosphatase and tensin homolog*
PUFA	*Polyunsaturated fatty acids*
RAPTOR	*Regulatory associated protein of mTOR*
RICTOR	*Rapamycin-insensitive companion of mTOR*
ROS	*Reactive oxygen species*
SCLC	*Small-cell lung cancer*
SH	*Steroid hormone*
SREBP-1	*Sterol regulatory element binding protein-1*
TE	*Thioesterase*
TGF-α	*Transforming growth factor a*
TGF-β	*Transforming growth factor β*
TK	*Tyrosine kinase*
TKI	*Tyrosine kinase inhibitor*
USP	*Ubiquitin-specific protease*
VEGF-A	*Vascular endothelial growth factor A*

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RESUM

La elevada i incontrolada proliferació cel·lular és una de les principals característiques que diferencien les cèl·lules tumorals de les no tumorals. Per això, les cèl·lules cancerígenes necessiten la fabricació de noves membranes cel·lulars i requereixen molta energia. La sintasa d'àcids grassos (FASN) és el principal enzim involucrat en la producció d'àcids grassos. Aquests, són els constituents principals de les membranes biològiques i també són elements necessaris per la producció d'energia. FASN està sobre-expressat en molts tipus de càncers (mama, colon, pròstata, ovari, pulmó, etc.). S'ha estudiat el paper d'aquest enzim i les implicacions de la seva inhibició en diferents models tumorals. No obstant, encara són necessaris molts estudis per acabar de desxifrar els mecanismes moleculars que regulen la seva expressió, que porten als efectes anti-tumorals a conseqüència de la seva inhibició i a les interrelacions amb altres rutes de senyalització, així com factors involucrats en el desenvolupament i progressió del càncer.

En aquesta tesi, per una banda, s'estudia la relació entre l'expressió de FASN i les característiques clinicopatològiques i antropomètriques en pacients amb càncer de mama, amb la finalitat d'esbrinar el paper de FASN com a pronòstic de càncer de mama d'estadis primerencs.

La ruta dels receptors de factor de creixement epidèrmic (EGFR/HER/ErbB) té un paper primordial en la senyalització del

creixement i la divisió de les cèl·lules tumorals. Aquesta ruta està sobreexpressada en varis tipus de càncers, i a més, s'ha descrit una relació entre l'expressió i activació d'aquesta ruta i l'expressió de FASN. Per altra banda, en aquesta tesi, es presenten resultats sobre l'expressió de FASN i les implicacions de la seva inhibició farmacològica (sola o en combinació amb la inhibició d'altres dianes relacionades amb la ruta de senyalització de HER) en models cel·lulars i animals (*xenografts* i *ortoxenopatients*) de càncer de pulmó HER1 i FASN-positiu, així com en càncer de mama HER2 i FASN-positiu. Per acabar, s'han desenvolupat models pre-clínics de càncer de mama HER2+ resistents a les actuals teràpies anti-HER2 (trastuzumab i lapatinib) per estudiar l'expressió de FASN i altres proteïnes involucrades en l'adquisició de la resistència i també, l'eficàcia anti-tumoral *in vivo* dels inhibidors de FASN, sols o en combinació.

Com a conclusió general, es descriu FASN com a possible nova diana anti-tumoral (sola o en combinació) per futurs estudis pre-clínics i clínics en models tumorals FASN positius.

RESUMEN

La elevada e incontrolada proliferación celular es una de las principales características que diferencian las células tumorales de las no tumorales. Por eso, las células tumorales necesitan la fabricación de nuevas membranas celulares y requieren mucha energía. La sintasa de ácidos grasos (FASN) es la principal enzima involucrada en la producción de ácidos grasos. Estos, son los constituyentes principales de las membranas biológicas y también son elementos necesarios para la producción de energía. FASN esta sobreexpresada en distintos tipos de cáncer (mama, colon, próstata, ovario, pulmón, etc.). Se ha estudiado el papel de FASN y las implicaciones de su inhibición en diferentes modelos tumorales. De todos modos, aun hacen falta estudios para terminar de descifrar los mecanismos moleculares que regulan su expresión, que llevan a los efectos antitumorales a consecuencia de su inhibición, a las interrelaciones con otras rutas de señalización y también, los factores involucrados en el desarrollo y progresión del cáncer.

En esta tesis, por un lado, se estudia la relación entre la expresión de FASN y las características clinicopatológicas y antropométricas en pacientes con cáncer de mama, con la finalidad de averiguar el papel de FASN como pronóstico de cáncer de mama de estadios tempranos.

La ruta de los receptores de factor de crecimiento epidérmico (EGFR/HER/ErbB) tiene un papel primordial en la señalización del

crecimiento y la división de las células tumorales. Esta ruta está sobreexpresada en varios tipos de cánceres, y además, se ha descrito una relación entre la expresión y activación de esta ruta y la expresión de FASN. Por otro lado, en esta tesis, se presentan resultados sobre la expresión de FASN y las implicaciones de su inhibición farmacológica (sola o en combinación con la inhibición de otras dianas relacionadas con la ruta de señalización de HER) en modelos celulares y animales (*xenografts* y *ortoxenopatients*) de cáncer de pulmón HER1 y FASN-positivo, y también en cáncer de mama HER2 y FASN-positivo. Para finalizar, se han desarrollado modelos preclínicos de cáncer de mama HER2+ resistentes a las actuales terapias anti-HER2 (trastuzumab y lapatinib) para estudiar la expresión de FASN y otras proteínas involucradas en la adquisición de resistencia, y también, la eficacia antitumoral *in vivo* de los inhibidores de FASN, solos o en combinación.

Como conclusión general, se describe FASN como posible nueva diana antitumoral (sola o en combinación) para futuros estudios preclínicos y clínicos en modelos tumorales FASN positivos.

ABSTRACT

The high and uncontrolled cell proliferation is one of the main features that distinguish tumoral cells from non-tumoral ones. For that, tumoral cells require the production of new cellular membranes and consume a lot of energy. Fatty acid synthase (FASN) is the main enzyme involved in fatty acids (FA) production. FA are the major constituents of biological membranes and elements for energy production. FASN is overexpressed in several types of cancers (breast, colon, prostate, ovary, lung, etc.). The role of this enzyme and implications of its inhibition in different tumor models has been studied, but more studies are necessary in order to elucidate the molecular mechanisms that regulate its expression, that bring the anti-tumoral effects of its inhibition and, the cross-link with other signaling pathways and agents involved in the development and progression of cancer.

In this thesis, on one side, the association of FASN expression with clinicopathological and anthropometric characteristics in breast cancer patients is studied in order to find out FASN role as a prognostic in early stage breast cancer. Epidermal growth factor receptors pathway (EGFR/HER/ErbB) has a capital role in signaling of growth and division on tumor cells. This pathway is overexpressed in several types of cancers, moreover, its expression and activation has been associated with FASN expression.

On the other side, we show results regarding FASN expression and implications of its pharmacological inhibition (alone or in combination with inhibition of other targets related to HER signaling pathway), in cellular and animal models (xenografts and patient derived xenografts) of HER1 and FASN-positive lung cancer and on HER2 and FASN-positive breast cancer. Finally, we have developed pre-clinical models of HER2+ breast cancer resistant to current anti-HER2 therapies (trastuzumab and lapatinib), to study expression of FASN and other proteins involved in acquisition of resistance, and, *in vivo*, the anti-tumoral efficacy of FASN inhibitors, alone or in combination.

As general conclusion, FASN is described as a new possible anti-tumoral target (alone or in combination) for future pre-clinical and clinical studies in FASN-positive tumor models.

GENERAL INTRODUCTION

Cancer is the principal pandemia, 1 in 8 deaths worldwide is caused by this illness[1]. 8.2 million people died from cancer in 2012, being the leading cause of death. Cancer is a generic term for a large group of diseases that can affect any part of the body. Lung, liver, stomach, colorectal and breast cancers cause the most cancer deaths each year[2]. Briefly, cancer is an **uncontrolled growth** of cells that escape from homeostatic systems of the body, and which can then invade adjoining parts of the body and spread to other organs. This process is referred to as **metastasis**. Metastases are the major cause of death from cancer[3].

Cancer cells acquire some features and **hallmarks** that provide them advantageous capabilities which allow tumor cells to survive, proliferate and disseminate. These functions are acquired in different tumor types, via distinct mechanisms (that can be co-regulated in some cases) and at various times; piling during tumorigenesis[4].

Genomic instability, achieved through increased sensitivity to mutagenic agents and through a breakdown in one or several components of the genomic maintenance machinery, generates random mutations that can orchestrate hallmark capabilities[4]. **Inflammatory state** of premalignant and malignant lesions, driven by cells of the immune system, can also promote tumor progression by supplying bioactive molecules to the tumor microenvironment; including growth factors that sustain proliferative signaling, survival factors that limit cell death, pro-angiogenic factors, extracellular matrix-modifying enzymes that facilitate angiogenesis, invasion, and metastasis, and inductive signals that lead to activation of EMT and other hallmark-facilitating programs[4,5].

Normal cells maintain tissue architecture and function by controlling cell cycle, growth and division with measured growth signals. Distinctly, cancer cells deregulate these growth signals to evade cell number homeostasis; thereby

maintain **chronic proliferation**. Growth signals are steered by growth signaling pathways. Binding of growth factors with their respective cell-receptors triggers the activation of intracellular signaling pathways that regulate cell cycle, growth, survival and energy metabolism. Cancer cells can outwit growth controls increasing growth signals by several ways[4]. This will be deeply explained below.

Cancer cells must also **evade growth suppressors** that negatively regulate cell proliferation. Tumor suppressor genes operate as central control nodes that govern the decisions of cells to proliferate or, alternatively, activate senescence and apoptotic programs if growth inhibitory signals are perceived. Even with apoptotic signals, cancer cells have mechanisms to **resist cell death**. Apoptosis is attenuated in some tumors that succeed in progressing to states of high-grade malignancy and resistance to therapy[4,6]. Tumor cells can avoid apoptosis by loss of tumor suppressors, increase of antiapoptotic or survival signals expression, downregulation of proapoptotic factors or by short-circuiting of the extrinsic ligand-induced death pathway. It is also possible to avoid other forms of programmed cell death, such as autophagy or necrosis[4].

Most normal cell types have a limited number of successive cell growth and division cycles due to the progressive shorten of telomeres that protect the ends of chromosomes from end-to-end fusions that can promote cell death[4,7]. Cancer cells can acquire **replicative immortality** through a DNA polymerase enzyme (telomerase) that extends telomers by adding repeated sequences[4].

Tumor cell mass formed by increased proliferation of cancer cells needs nutrients and oxygen availability and evacuation of metabolic wastes and carbon dioxide. During tumor progression **angiogenesis** is chronically activated forming new vessels that feed and clean cells within a tumor[8]. Some angiogenic regulators

are signaling proteins that bind to stimulatory or inhibitory cell-surface receptors displayed by vascular endothelial cells.

Even more, some tumor-cells acquire the capability of **invade** local tissues and **metastasize** distant ones. The multistep process of invasion and metastasis has been schematized as sequence of discrete steps, often termed the invasion-metastasis cascade[4,9]. This steps are regulated by the developmental regulatory program, referred to as the "epithelial-mesenchymal transition" (EMT) in which tumor-cells locally invade and then intravasate blood and lymphatic vessels to transit through the lymphatic and hematogenous systems. These cells may pass through the reverse process, termed the mesenchymal-epithelial transition (MET) to escape from the lumina of such vessels into parenchyma of distant tissues (extravasation), form small nodules of cancer cells (micrometastases), and finally grow into macroscopic tumors, this last step being termed "colonization"[4].

The increased cell proliferation in carcinomas also requires, in some cases, **adjustments of energy metabolism** in order to fuel cell growth and division[4]. Increased glycolysis, even in presence of oxygen (Warburg effect), allows the diversion of glycolytic intermediates into various biosynthetic pathways, including those generating nucleosides and amino acids; this facilitates, in turn, the biosynthesis of the macromolecules and organelles required for assembling increased amount of new cells.

The long-standing theory of immune surveillance proposes that cells and tissues are constantly monitored by an ever-alert immune system, and that such immune surveillance recognizes and eliminates the vast majority of incipient cancer cells and thus nascent tumors. According to this logic, solid tumors that do appear have somehow managed to **avoid detection by immune system** or have been able to limit the extent of immunological killing, thereby evading eradication.

Immunogenic cancer cells may well evade immune destruction by disabling components of the immune system that have been dispatched to eliminate them. For example, cancer cells may paralyze infiltrating CTLs and NK cells, by secreting TGF-β or other immunosuppressive factors[4], tumors can also recruit inflammatory cells that are actively immunosuppressive, including regulatory T cells and myeloid-derived suppressor cells, which can suppress the actions of cytotoxic lymphocytes[10,11].

HUMAN EPITHERMAL GROWTH FACTOR RECEPTOR (HER) FAMILY

Several hallmarks can guide cells to tumor status, but the leading disruption is **chronic proliferation** of cancer cells, which is acquired by means of growth signaling deregulation. **Growth/proliferation signaling** is directed by a convoluted and interconnected network of extracellular signals (such as environmental stresses, growth factors, neuropeptides or hormones) and their respective cell receptors that, through several intracellular pathways, command various cellular functions as diverse as growth, differentiation, cell motility or survival (reviewed by van der Geer, P. *et al*. [12]). One important arm of growth signaling is the **epidermal growth factor receptor (HER) family** that regulates cell growth and survival, as well as adhesion, migration, differentiation and other cellular responses[13].

1. HER/EGFR/ErbB Family

In humans, **HER/EGFR/ErbB family** receptors consists of four members: EGFR/HER1/ErbB1, Neu/HER2/ErbB2, HER3/ErbB3 and HER4/ErbB4[14]. This family of receptors is ubiquitously expressed in epithelial, mesenchymal, neuronal cells and their cellular progenitors. HER family is vital for **development, organogenesis and growth**. Impairment of any of the EGF receptors in mice produces embryonic or perinatal lethality. For instance, knockout of HER1 gene results in gastrointestinal, skin, and lung defects[15]. Disablement of HER2, HER3 and HER4, hinder cardiac and neuronal function and development[16-19].

Each HER receptor is a **transmembrane tyrosine kinase (TK) receptor** with partial homology that consists of an extracellular ligand-binding domain, a transmembrane lipophilic segment and (except for HER3) a functional intracellular TK domain. Ligand binding induces the formation of homo- or hetero-dimers which subsequently trigger the autophosphorylation and activation of cytoplasmic tyrosine residues[20,21]. Once activated, the signal transduction cascades of these receptors promote **cellular proliferation and survival** through a highly diverse repertoire of cellular signaling pathways, among them: the RAS-MAPK pathway and the phosphatidylinositol 3'-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway[22].

2. HER/EGFR Ligands

HER family members' signaling is activated by a large group of EGF-related growth factors or ligands. Common feature to all these growth factors is the EGF domain. This domain contains six conserved cysteine residues characteristically spaced to form three intramolecular disulphide bridges. Depending on their receptor affinities and specificities these **ligands** can be subdivided into four different categories (figure 1). While epidermal growth factor (EGF), amphiregulin (AR) and transforming growth factor α (TGFα) specifically bind to HER1; betacellulin (BTC), heparin binding EGF-like growth factor (HB-EGF) and epiregulin bind HER1 and HER4. Neuregulins (NRGs) or Neu differentiation factors (NDFs) bind and activate HER3 and HER4. Interestingly, despite the overlapping receptor specificity of the NRG1 and NRG2 isoforms they exhibit distinct biological activities depending on the cellular context. While NRG1 and NRG2 activate both HER3 and HER4, NRG3 and NRG4 exclusively bind HER4[14].

As a general rule, EGF family ligands are synthesized as single-pass transmembrane protein precursors which are proteolytically cleaved from the cell surface to yield the mature growth factor (reviewed in [23]). This process is called protein ectodomain shedding, and the proteolytic enzymes are sometimes referred to as sheddases. Each ligand has its particular cleaving course. While TGFα and NRG precursors require the cytoplasmic domains for efficient proteolytic processing[24], shedding of proHB-EGF and amphiregulin has been shown to be independent of their cytoplasmic moieties[25,26].The proteolytic cleavage of proTGFα had been found to be stimulated by serum factors, tetradecanoylphorbolacetate (TPA) and calcium ionophores[27]. Serine proteases and metalloproteases (specially the ADAMs [A disintegrin and metalloproteases] family) have been identified as potential mediators of ectodomain shedding[28,29]. Data obtained from transgenic animals lacking the ADAM17 (TACE) zinc-dependent transmembrane metalloprotease, revealed a critical contribution to proTGFα processing[30,31]. The involvement of another family member, ADAM9/MDC9, in TPA-induced proHB-EGF shedding further underlines the critical role of this metalloprotease family in the generation of mature EGF-like ligands[32]. The proteolytic processing and release of membrane proteins function as a post-translational switch that regulates the activity of the growth factor.

The HER/EGF/ErbB ligands generally act over short distances from their sites of generation. They may act in the same cell from which they are released (autocrine effect), on an adjacent cell (juxtacrine action) or in a nearby cell (paracrine communication)[33].

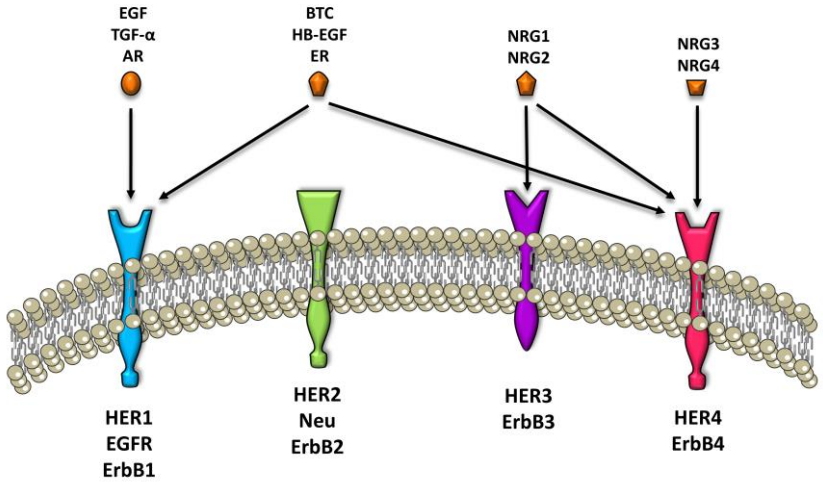

Figure 1. HER family receptors and their ligands. Each ligand binds to a specific HER receptor. Epithermal growth factor (EGF), transforming growth factor α (TGF-α) and amphiregulin (AR) ligands bind to HER1/EGFR/ErbB1 (Human epithermal growth factor receptor 1). Betacellulin (BTC), heparin binding EGF-like growth factor (HB-EGF) and epiregulin (EPR) ligands bind to both HER1/EGR/ErbB1 and HER4/ErbB4 (human epithermal growth factor receptor 4). Neuregulins 1 and 2 (NRG1, NRG2) ligands bind to human epithermal growth factor receptor 3 (HER3/ErbB3). And, Neuregulins 3 and 4 (NRG3, NRG4) ligands bind to human epithermal growth factor receptor 4 (HER4/ErbB4). Note that HER2 is ligand-binding domain impaired, and HER3 is kinase-domain impaired. Modified from Oliveras G. TDX, 2012[34].

3. HER Receptors Structure

Basic structure, contact regions and binding residues are conserved in all HER receptors. HER family receptors share from 53% to 64% of protein sequence[35]. All members of the HER family consist of an extracellular domain, a single transmembrane segment and an intracellular portion.

- The **extracellular domain** is divided into four parts: domains I and III, which are related leucine-rich segments that participate in ligand binding, and domains II and IV, which contain numerous cysteine residues that participate in

disulfide bond formation. Domain II participates in homo- and hetero-dimer formation with HER family members.

- The **transmembrane segment** of 19-25 amino acid residues that anchors the receptor to the cell membrane.

- The **intracellular domain** of about 550 amino acid residues contains a juxtamembrane segment, a protein kinase domain, and a carboxyterminal tail[35]. This intracellular part of the receptor is responsible for kinase activation of cell signaling pathways. All four members of the HER family possess a similar protein kinase domain. HER1/2/4 possess protein kinase activity while HER3 is not kinase death but, catalytically impaired. Autophosphorylation and phosphorylation of exogenous substrates driven by HER3 still need to be clarified. Lemmon *et al.* showed that human HER3 homodimer is able to undergo autophosphorylation at a rate of 1/1000th that of HER1 homodimer, but is unable to catalyze phosphorylation of exogenous protein substrates[36].

4. HER Activation

The general mechanism for HER receptors family activation entails that activating ligands (in this case growth factors) bind to the extracellular domain of HER receptors and induce them **activated dimerization state** that **signals through several cellular pathways**. There are a number of possible ways for which a **growth factor or ligand can induce receptor dimerization**. One possibility for growth-factor induced receptor dimerization involves a single ligand that interacts simultaneously with two receptor molecules and effectively cross links them to form a dimeric complex. Another possibility results when both ligands independently bind to both respective receptors and trigger their dimerization[35].

Before ligand-binding, the dimerization arm of HER1/3/4 is completely buried in domain IV, which stabilizes a **closed or inactive receptor conformation** that restricts its movement so that ligand binding, dimerization and activation are inhibited. Ligand binding breaks the intramolecular tie, exposing the dimerization arm and allowing interaction with another exposed or **open conformation-receptor**. As an exception, HER2 exists in an open conformation and the dimerization arm is always exposed and not buried[37]. Although unliganded HER2 is ready for dimerization with other HER family members, it does not form active dimers unless it is overexpressed.

HER2 fails to bind to any growth factor so that needs another partner of the HER family for activation. HER3 is kinase impaired so that also needs another HER family partner for signaling activation. HER2 is the preferred heterodimerisation partner within the HER family as it decreases ligand dissociation from the receptor heterodimer thus enhancing and prolonging receptors activation, and the HER2 heterodimer combinations with HER1 or HER3 exhibit robust signaling activity[38,39].

Macdonald-Obermann used luciferase fragment complementation imaging to analyze the interaction of HER1, HER2, and HER3[40]. Firefly luciferase can be split into amino-terminal and carboxyterminal fragments, neither of which exhibits enzyme activity alone, but they form a functional enzyme complex when they are brought together. They fused these fragments to the C-termini of the three receptors and stably expressed them in CHO cells. They found that Nrg-1 (HER3 ligand) leads to the following order of receptor pairing and stability: HER2/HER3 > HER1/HER3 > HER3/HER3. They found that EGF (HER1 ligand) leads to the following order of receptor pairing and stability: HER1/ HER1 ≈ HER1/HER2 > HER1/HER3. They also found evidence for the formation of dimers of HER1/HER2, HER1/HER3, and HER2/HER3 without ligand binding-activation.

In each of these cases, receptors dimerization brings the two cytoplasmic tyrosine kinase domains of the receptors close enough for **autophosphorylation** and to thereby **activate the intrinsic tyrosine kinase activity**. This phosphorylation is accomplished in trans, the first member of the dimer mediates the phosphorylation of the second and the second member mediates phosphorylation of the first. Autophosphorylation of receptor's kinase domain occurs in tyrosine residues, usually in the activation segment, that leads to protein kinase activation. The kinase domains also catalyze the phosphorylation of additional tyrosine residues that create docking sites for adaptor proteins or enzymes that result in downstream signaling.

5. HER Signaling

Multiple ligands and various combinations of homo- and heterodimerisation within the HER family couple to a complex and diverse set of biochemical pathways[14].

Ligand-induced receptor dimerization and subsequent autophosphorylation of distinct tyrosine residues creates **docking sites for various membrane-targeted proteins**. The docking proteins contain modular Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains (or both) that recognize phosphotyrosine sites in HER kinase domain receptors. Cytoplasmic mediators may either be adaptor proteins or enzymes. Adaptors such as Shc, Grb2, Crk or Dok-R proteins[41,42] show a modular structure containing protein–protein interaction domains and putative phosphorylation sites and act as signaling platforms, leading to colocalization of active signaling partners to extend the signals to intracellular pathways. Enzymes such as phospholipase Cγ (PLCγ) (which hydrolyzes PIP2 thus generating

diacylglycerol and inositol-trisphosphate) or the cytoplasmic tyrosine kinase c-src, link HER1 activation to second messenger generation and calcium metabolism or mitogenic signaling cascades respectively[14]. Other enzimes that bind to HER receptors are the Ras attenuator P120RasGAP, phosphatases PTB-1B and SHP1, and the tyrosine kinase Abl[35].

Downstream pathways that are **interconnected** and overlapped, such as the phosphatidylinositol 3-kinase (PI3K)/Akt (PKB) pathway, the Ras/Raf/MEK/ERK1/2 pathway, and the phospholipase C (PLCγ) pathway, serve as routes to **transmit information from cell surface receptors to the nucleus**[12,14,22,42].The PI3K/Akt pathway plays an important role in mediating **cell survival** and the Ras/ERK1/2 and PLCγ pathways participate in **cell proliferation**[22]. These and other HER signaling modules participate in **angiogenesis, cell adhesion, cell motility, development, and organogenesis**[43].

5.1. MAP Kinase Cascade

Several distinct MAP kinases have been identified as **targets of the HER family receptors**, among them the extracellular regulated kinases (Erks) 1 and 2, jun N-terminal kinases (Jnks), p38 and Erk5.

Erk1/2 signaling pathway is the most characterized MAP kinase cascade. Adaptors proteins (such as Shc, Grb2 and Crk) are recruited to the kinase domain of HER (among others) activated receptors. Grb2 adaptor links the receptor to the guanine nucleotide exchange factor SOS (Son of the sevenless), which activates the small G-protein Ras. Ras induction activates the dual specificity kinase MEK1/2, which in turn finally activates ERK1/2. ERK1/2 have several substrates including

protein kinases (such as p90RSK, MNK1/2) and transcription factors (such as Elk-1 and c-fos) that promote **cell division**[44].

This signaling pathway, like many others, is an **intricate network** with positive and negative regulators. Examples of negative regulators are the adaptors and HER1 binding proteins p66Shc and Dok-R[41,45] and the Abl interactor, Abi-1, that binds to the exchange factor SOS[46]. In contrast, SUR-8 (a scaffolding protein which complexes with Ras and Raf) and Shp-2 (the SH2 domain containing and Gab1-interacting protein tyrosine phosphatase) are positive regulators of the same pathway[47,48].

5.2. Phosphatidylinositol-3-Kinase (PI3K) pathway

In contrast to other HER receptors, HER3 contains six putative binding sites for **PI3K**[49]. The regulatory subunit (p85) of PI3K binds to phosphotyrosines in HER receptors that lead to the activation of PI3K activity. This enzyme catalyzes the phosphorylation of membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP2) to form phosphatidylinositol 3,4,5-trisphosphate (PIP3), which attracts Akt to the plasma membrane. **Akt**, which is also known as protein kinase B (PKB), is a protein-serine/threonine kinase that binds to phosphatidylinositol triphosphate (PIP3) with high affinity[50]. Akt downstream effectors promote **survival**, through **avoiding of apoptosis** by inhibition of FoxO1-Bim-Bcl-2-Bax[51] pro-apoptotic route and involving the transcription factor NF-κB among other mechanisms[50]. Akt **regulates cell cycle and cell proliferation** through its direct action on the CDK inhibitors p21 and p27, and its indirect effect on levels of cyclin D1 and p53[51]. Akt is also related with **glucose metabolism,** activating the enzyme PFKFB2 which is involved in glycolysis, and the GTPase AS160 or PIP5 kinase involved in glucose transport[51].

Not less important is the Akt downstream effector involved in protein synthesis, **mTOR** (mammalian target of rapamycin). mTOR is also a protein-serine/threonine kinase that has dozens of substrates and participates in **many cellular processes** including that of **cell survival**. Phosphoinositide-dependent protein kinase 1 (PDK1) and mammalian target of rapamycin complex 2 (mTORC2) catalyze the phosphorylation of Akt in two different sites, and the bisphosphorylated and activated Akt catalyzes the phosphorylation and activation of mTOR[50,52,53]. PI3K/Akt pathway is also a complex network with positive and negative regulators such as PTEN, a phosphatase that catalyzes the hydrolysis of PIP3 to form PIP2 and inorganic phosphate therefore executing a negative regulation[54].

Mammalian target of rapamycin (mTOR)

The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase with pivotal role in cell regulation, **integrating responses** to multiple stimuli such as amino acid availability, energy and oxygen stresses, and growth factor receptor signaling[55-57].

There are to forms of mTOR multiprotein complexes, mTOR complex 1 (mTORC1) and 2 (mTORC2)[53]. Both share some protein in their structures such as the mammalian lethal with SEC13 protein 8 (mLST8) and Egl-10, pleckstrin [DEP]-domain-containing mTOR interacting protein (DEPTOR). mTOR1 exclusively contains the scaffolding protein RAPTOR (shorthand for regulatory associated protein of mTOR) and the substrate competitor PRAS40 (proline-rich Akt substrate 40 kDa). On the other hand, mTOR2 contains RICTOR (rapamycin-insensitive companion of TOR), mSIN1(mammalian stress-activated protein kinase interacting protein 1) and PRR5/PROTOR (proline-rich protein 5/protein observed with RICTOR)[52,53]. mTORC1 **senses nutrient and energy** sufficiency, and promotes cell growth and

proliferation by controlling **translation**, **transcription** of ribosomal RNA (rRNA) and transfer RNA (tRNA), ribosome biogenesis, lysosome biogenesis, lipid synthesis and macro-autophagy (or protein breakdown). These mechanisms controlled by mTORC1 are needed to **increase cell biomass** prior to cell division. mTORC2 regulates **co-translational protein degradation, cytoskeletal rearrangement and cell survival**[53,58-60].

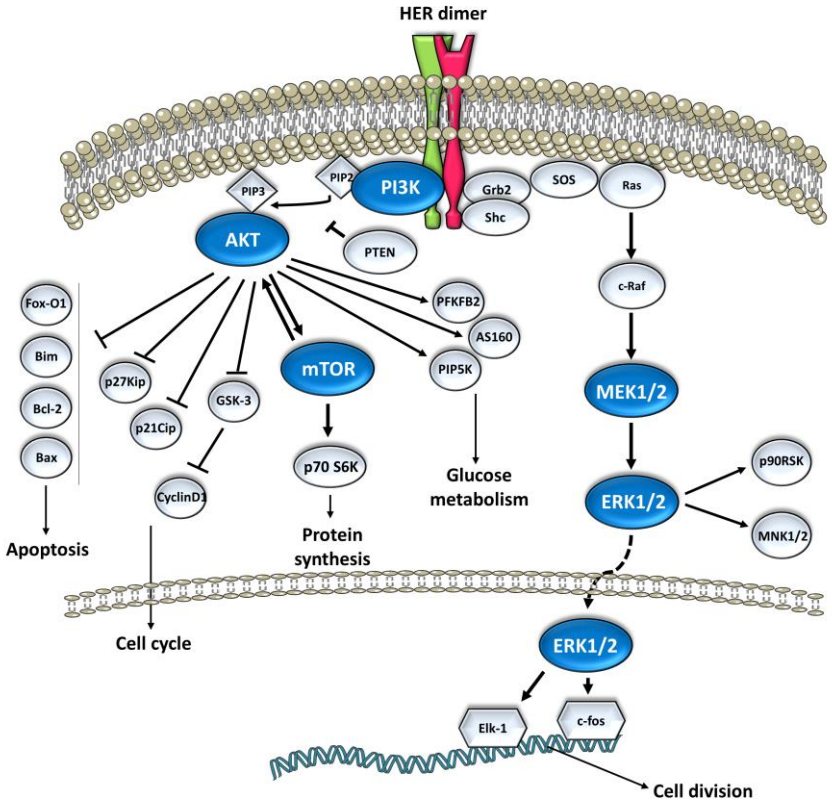

Figure 2. HER signaling pathways. Once HER-dimers are active, they signal through different cascades. Main signaling pathways related to HER are phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway that promotes cell cycle progression, protein synthesis, glucose metabolism and inhibition of apoptosis, and MAP kinase-ERK kinase1/2/extracellular regulated kinase (MEK1/2/ERK1/2) that signal for cell division and viability. Modified from Cell Signal Technology,2015[61].

5.3. Phospholipase C (PLCγ) Pathway

HER1, HER2, and HER4 possess several potential PLC γ phosphotyrosine binding sites. The nSH2 domain of PLCγ1 binds to the HER family phosphotyrosines. Then, PLCγ1 is phosphorylated leading to a conformational change and enzyme activation.

PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to form inositol1,4,5-trisphosphate (IP3) and diacylglycerol (DAG)[62]. Inositol trisphosphate promotes the release of Ca2+ from the endoplasmic reticulum and diacylglycerol activates the protein-serine/threonine kinase C (PKC). PKC has broad substrate specificity and **catalyzes the phosphorylation of dozens of proteins** and has many divergent physiological effects[63]. These effects include **angiogenesis, cell proliferation, cell death, increased gene transcription and translation, cell migration, and cell adhesion**. One of the downstream effectors of PKC is the Raf/MEK/ERK1/2 pathway leading to **cell proliferation** in a process that bypasses Ras[44,63].

6. HER Receptors Degradation

After stimulation, HER receptors will be ubiquitylated, endocytated and degradated to ensure **recycling** of used receptors for new and functional receptors[64]. Following EGF stimulation, Umebayashi reported that Cbl remains associated with HER1 and promotes receptor ubiquitylation along the endocytic route, thereby ensuring that the receptors are directed to multivesicular endosomes and targeted for lysosomal degradation[65].

Cbl family members are components of the ubiquitin ligation machinery involved in the targeting and degradation of phosphorylated proteins (HER

receptors among them). Cbl acts as an E3 ubiquitin-protein ligase, which accepts ubiquitin from specific E2 ubiquitin-conjugating enzymes, and then transfers it to substrates promoting their degradation by the proteasome.

7. HER Family Nuclear Localization

In addition to HER signaling, that governates nuclear functions through several kinase pathways, receptors also participate in cell signaling directly by them **translocation to the nucleus**. Presence of HER1/2/3/4 have been shown in nucleus[66]. Marti demonstrated that HER receptors are present in nucleus, but fewer (approximately 10%) when compared with the plasma membrane receptor[67].

Nuclear translocation occurs after endocytic vesicles fuse with early endosomes[66]. HER1 and HER2 are reported to translocate into the nucleus by importin α/ß-dependent mechanisms[68,69]. A positively charged sequence that interacts with importin-ß is conserved in all HER family members[68]. HER receptors bind to importin-ß, after that the binary complex binds to importin-α, and a ternary complex (importin-ß/importin-α/HER receptor) is translocated into the nucleus. Importin-ß interacts with the nucleoporins that constitute the nuclear pore complex. The ternary complex is disassembled on binding Ran-GTP in the nucleus. HER receptor is retained in the nucleus and the importins shuttle back into the cytoplasm.

Various functions have been ascribed to the HER family localized within the nucleus. These include **cell proliferation, DNA replication, DNA damage repair, transcription, development, and cancer growth or spread**[70]. The C-terminal regions of HER1/2/3/4 display **intrinsic transcription activity** of genes related with cell proliferation (CCND1, Cyclin D1, AURKA), signaling in inflammation,

cardiovascular system, and neoangiogenesis in cancer (NOS2), cell differentiation, proliferation and survival (BMYB)[70].

The protein kinase activity of the HER family also plays a role in its nuclear localization[70]. HER1 mediates the phosphorylation of PCNA, the chromatin-associated DNA proliferative cell nuclear antigen. PCNA participates in DNA synthesis and DNA damage repair. HER2 inhibits CDK2 by phosphorylation, what delays M-phase entry.

Nuclear HER1 has been detected in a variety of cancer types including breast, NSCLC, and head and neck squamous cell cancers[68,71]. Wang and Hun discuss the potential role of nuclear HER proteins in tumor metastasis, progression and resistance to radiation therapy[72]. The work of Sardi *et al*. demonstrated that HER4 participates in a biologically significant signaling mechanism mediated by direct nuclear action of an activated HER4 fragment that is transported from the cell membrane to the nucleus in a manner that is regulated by Nrg-1[73]. Several nuclear functions have been associated with HER receptors but translocation, mechanisms and other functions remain to be determined.

8. HER Family and Cancer

Aberrant signal transduction of **HER receptors is manifested in many types of solid human cancers**, including non-small cell lung cancer (NSCLC), breast cancer, bladder cancer, ovarian cancer, colorectal cancer, pancreatic cancer and head and neck squamous cell cancer (HNSCC)[74-76]. Hyperactivation of the pathway leads to downstream events that **stimulate five hallmarks of cancer**, including evasion of apoptosis, self-sufficient growth, insensitivity to anti-growth signals, sustained angiogenesis, and tissue invasion and metastasis[4]. Moreover, overexpression of

either HER1 or HER2 is related to a **poor prognosis** for many cancer patients. **Deregulation of HER family signaling system** is thought to be due to increased exposure to growth factor ligands, overexpression of receptors or critical downstream elements, and constitutive signaling of HER pathway sponsored by activating mutations in receptors or by defective down-regulation[22,74-77].

HER1 was the first tyrosine-kinase receptor to be linked directly to human tumors[77]. Several alterations that promote overexpression or hyperactivation of HER1 have been described in different types of cancer. Gene amplification leading to HER1 overexpression, overproduction of EGF-related growth factors leading to constitutive HER1 activation (reviewed in [78]), several deletions in the extra- and intracellular domain of the HER1[14], somatic mutations in the tyrosine-kinase domain of HER1[14], and other HER1 activating-alterations are often found in several types of human cancers, such as non-small cell lung cancers, breast, gastrointestinal stromal ovarian, prostate tumors and others.

Amplification of **HER2** leading to overexpression of the receptor has been detected in a variety of tumors, some examples are breast, ovarian, gastric and salivary cancers[79,80]. In human breast cancer HER2 gene amplification has been correlated with a poor prognosis (shorter overall survival and relapse-free survival)[80]. Mutations in HER2 have also been described in breast and non-small cell lung cancers[81,82].

FATTY ACIDS METABOLISM

Fatty acids (FA) are essential for cell viability since they are important constituents of biological **membrane lipids** and are metabolic substrates for **energy production** in cell metabolism, producing more energy than glucose. Fatty acids are used for the synthesis of many cellular lipids such as phospholipids, triglycerides and cholesterol esters, or for the acylation of proteins. Fatty acids can be oxidized through the ß-oxidation process in the mitochondria to obtain energy[83].

There are two sources for fatty acid procurement in animal's body, **exogenous** (from dietary) and **endogenous**, from hydrolysis of triglycerides (fatty acids are stored as triglycerides in adipose tissue) or from the lipogenic pathway (also named as ***de novo* synthesis**)[83].

Most normal cells and tissues, even those with high cellular turnover, seem to preferentially use dietary fatty acids for the synthesis of new structural lipids and energy production. In normal conditions *de novo* synthesis of fatty acids takes place in the liver, lactating mammary glands[84] and adipose tissue[85].

Fatty acids *de novo* synthesis is a large process that involves a **vast number of enzymes**. After pyruvate is synthesized in glucose metabolism, part of it is converted into acetyl-CoA in the mitochondria. To export acetyl-CoA from the mitochondria to the cytoplasm it is converted into citrate. In the cytoplasm citrate is converted back to acetyl-CoA by ATP citrate lyase (ACLY). Part of the acetyl-CoA is carboxylated to malonyl-CoA by acetyl-CoA carboxylase (ACC). Then, acetyl-CoA and malonyl-CoA are condensed into 16-carbon saturated FA palmitate and other saturated long-chain fatty acids by **fatty acid synthase enzyme (FASN)**. Nicotinamide adenine dinucleotide phosphate (NADPH) is used as an electron

donor[83]. Long-chain fatty acids can be disassembled for energy production in the mitochondria through **ß-oxidation**. **Carnitine palmitoyltransferase-1 (CPT-1)** is the rate-limiting enzyme of fatty acid oxidation, since allows for subsequent movement of the acyl part of the fatty acids from the cytosol into the intermembrane space of mitochondria. High levels of malonyl-CoA can inhibit CPT-1 and hence long-chain fatty acids oxidation[86].

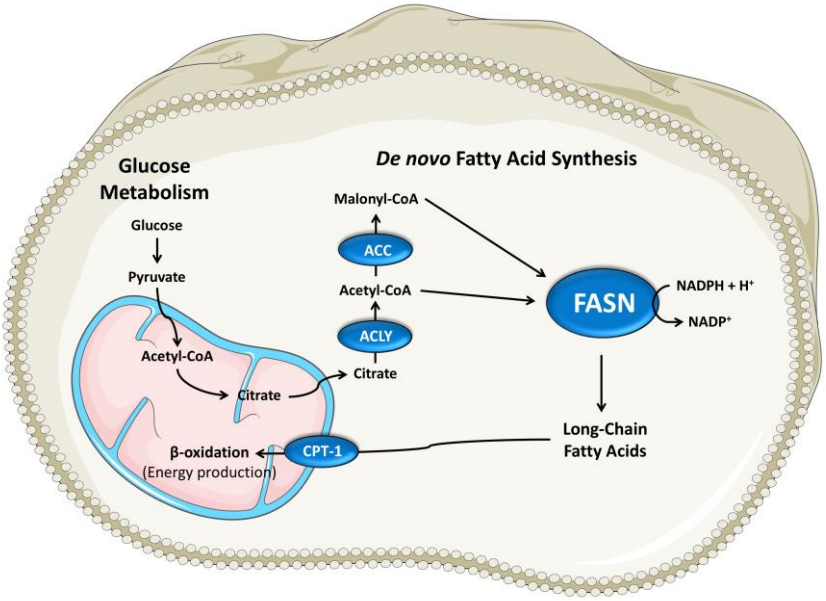

Figure 3. *De novo* synthesis of Fatty Acids. After glucose is converted into citrate in the mitochondria, ATP citrate lyase (ACLY) converts citrate into acetyl-CoA, which is transformed into malonyl-CoA by acetyl-CoA carboxylase (ACC). FASN condense acetyl-CoA and malonyl-CoA into 16-carbon saturated fatty acids. Fatty acids can be used for energy production through ß-oxidation in the mitochondria. Carnitine palmitoyl transferase-1 allows translocation of fatty acids in the mitochondria. Modified from Puig, T. *et al*. Med Clinica, 2009[87].

1. Fatty Acid Synthase Structure and Function

Two distinct fatty acid synthase (FASN) enzymes exist, **FASN type I** which is placed in the cytoplasm and **FASN type II**, which is in the mitochondria. Both participate in *de novo* fatty acid synthesis.

FASN type II is a complex of proteins with independent activities and is responsible for only less than 10% of fatty acid synthesis[88]. It is known to have important functions in mitochondria, but more investigations should to be done in order to fully understand the activity and functions of this enzyme[88].

FASN type I, the most studied and known FASN (called as FASN), is a **homodimeric multienzymatic polypeptide**, of 250-270 KDa, containing six catalytic domains in an "X" shape: ß-ketoacyl synthase (KS), malonyl acetyl transferase (MAT), ß-hydroxyacyl dehydratase (DH), enoyl reductase (ER), ß-ketoreductase (KR), and thioesterase (TE), and one elongating acyl chain-carrier domain, acyl carrier protein (ACP)[89,90].

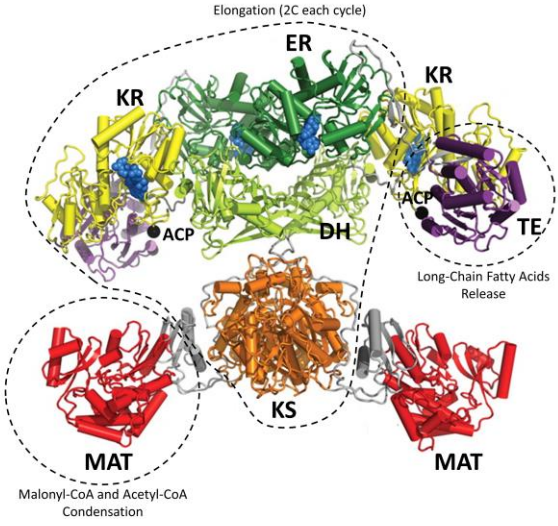

Figure 4. Fatty Acid Synthase complex structure. FASN domains and function in long chain fatty acids synthesis. Modified from Maier, T. *et al*. Science, 2008[1].

FASN uses acetyl-CoA as a primer, malonyl-CoA as two-carbon donor and NADPH as electron donor to produce long chain fatty acids. Fatty acids are synthesized as a production line: malonyl-CoA and acetyl-CoA are condensed in the MAT domain then, elongation is yielded by repeated cycles of reduction and dehydratation catalyzed by the KS, DH, ER, and KR domains. Two carbons are added in each cycle. Finally, new synthesized long-chain fatty acid is released from ACP carrier domain by the TE domain[83].

2. Fatty Acid Synthase Expression in Normal Cells

Most human tissues, even those with high proliferation rates, preferentially acquire **fatty acids from exogenous supplies**, such has diet or circulation. In this case, enzymes involved in lipogenesis (especially FASN) are low expressed.

As exceptions, *de novo* synthesis of fatty acids and **FASN** expression are **increased** in **lipogenic tissues** (liver and adipose tissue, especially with high-carbohydrate diets), in some **hormone-sensitive cells** (during embryogenesis or endometrial cell proliferation), in **mammary glands during lactation** or even in the **hypothalamus** (regulating food intake)[84,91-94].

FASN expression is **regulated** both **metabolically** and **hormonally**. Some elements related to up-regulation are: food intake (carbohydrates, glucose), amino acids, sterols, retinoic acid, hormones (such as insulin, cortisol, prolactin, triiodothyronine (T3), estrogens, progesterone and androgens), the proliferating antigen Ki-67, the transforming growth factor- ß (TGF-ß) and the lipogenesis-related nuclear protein SPOT14[95-101]. Down-regulation is stimulated, for instance, by polyunsaturated fatty acids (PUFA), leptin, cAMP, small amounts of fatty acids in the diet and fasting (glucagon), and progestin[84,102-105].

FASN gene (and other lipogenic genes) expression is mainly regulated by two distinct **transcription factors**, carbohydrate responsive element binding protein (**ChREBP**; in heptic cells) and sterol regulatory element binding protein-1 (**SREBP-1**; in hepatic and adipose cells)[94,106,107]. Glucose promotes post-translational modifications of ChREBP, resulting in its activation. Once ChREBP is activated, it moves from the cytoplasm to the nucleus, where it binds to carbohydrate responsive elements (ChoREs). Various lipogenic enzymes, FASN among them, contain ChoREs in their promoters[106]. Several mentioned hormones, such as insulin or progesterone, activate SREBP-1 by transcription and post-translational modifications. Once activated, SREBP-1 moves from endoplasmatic reticulum to the nucleus where bind to sterol regulatory elements, also present in some lipogenic enzymes such as FASN[83,107]. Contrary, leptin negatively contributes to lipogenesis regulation in adipose cells. High levels of leptin decrease SREBP-1 gene expression, what inhibits expression of lipogenic enzymes (FASN among others)[105,108].

3. Fatty Acid Synthase Expression in Tumor Cells

Highly proliferation rate of tumor cells requires large amount of new biological structures (such as biological membranes) and consumes a great deal of energy. Hence, **tumor cells require numerous amounts of fatty acids** for membrane lipid formation and energy production.

It has been observed that **in several cancer cells, 95% of their saturated and mono-unsaturated fatty acids derive mainly from their *de novo* synthesis**, even despite adequate nutritional lipid supply[83,86]. Consequently, **elevated expression and activity of lipogenic enzymes** (FASN among them) is reflected in tumor cells[83,87,109-114].

Overexpression and increased activity of FASN represents a common phenotype in cancer cells. Extremely high levels of **FASN are displayed in many human epithelial cancers and their pre-neoplastic lesions**, including breast, colorectum, prostate, bladder, ovary, oesophagus, stomach, lung, oral tongue, oral cavity, head and neck, thyroid and endometrium, mesothelioma, nephroblastoma, retinoblastoma, soft tissue sarcomas, Paget's disease of the vulva, cutaneous melanocytic neoplasms including melanoma, and hepatocellular carcinoma[83,86,87,98,109,114]. FASN is linked to risk of **recurrence, progression, malignity, aggressiveness and poor prognosis** in different cancers[83,87,114-117]. FASN also serves as a **prognostic marker** in oncogenic disease[83,86,87,109,110,114,116,117].

The molecular-signaling mechanisms by which FASN is overexpressed in cancer cells are not completely understood, but several pathways have been implicated. **Growth factors and their receptors** (such as HER family receptors) have been shown to stimulate FASN transcription. Particularly, **HER1 and HER2** have been highly correlated with FASN overexpression[105,118-121]. Alterations in HER-downstream pathways, PI3K/Akt/mTOR and MAPK cascades, have also been related to **FASN up-regulation** in prostate, ovarian, breast, lung and other carcinomas[119,120,122-125]. In **hormonally-responsive tumors**, steroid hormone (SH) receptors, including estrogens (ER), progesterone (PR) and androgen receptors (AR) also hyperactivate PI3K/Akt and MAPK cascades to stimulate FASN overexpression[126-128]. In fact, both PI3K/Akt/mTOR and MAPK interact with the FASN –promoter sterol regulatory element binding protein-1 (SREBP-1) and stimulate it to enter into the nucleus to promote FASN transcription.

Disturbances in **post-translational regulations of FASN** protein can also contribute to FASN overexpression and hyperactivation in cancer. Ubiquitin-specific protease USP2a removes ubiquitin from FASN protein, therefore stabilizes FASN avoiding its proteasome-degradation[129].

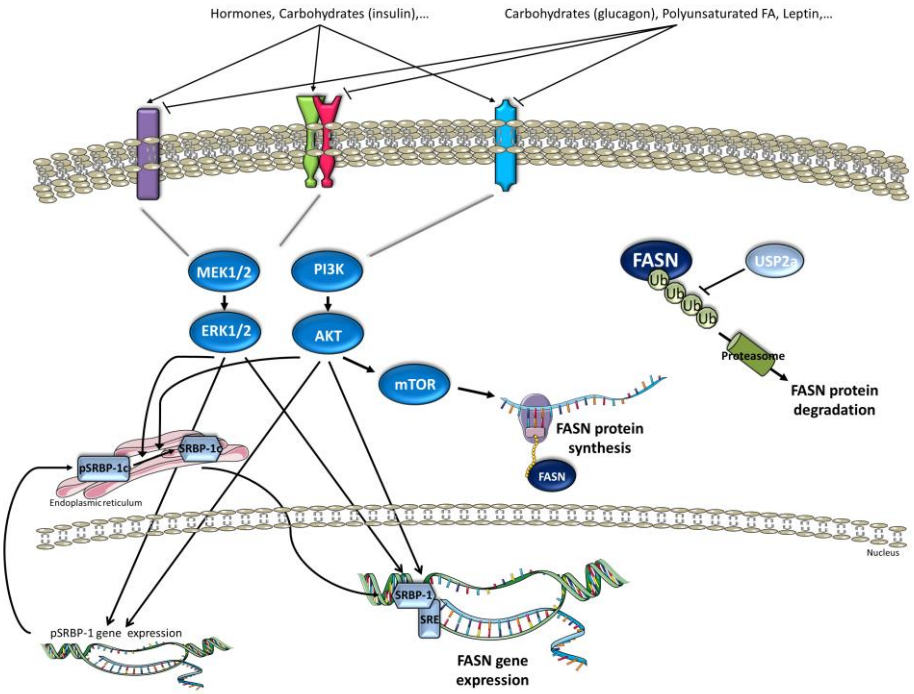

Figure 5. Fatty Acid Synthase regulation. Regulation of FASN gene expression, protein synthesis and degradation. Signaling pathways of membrane receptors (such as HER, hormone receptors, etc.) promote FASN gene expression and protein synthesis through, mainly, MEK1/2/ERK1/2 (MAP kinase-ERK kinase1/2/extracellular regulated kinase) and PI3K/AKT/mTOR (phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin) pathways. These pathways are stimulated by several inputs such as food intake (carbohydrates, glucose), amino acids, sterols, retinoic acid, hormones (such as insulin, cortisol, prolactin, triiodothyronine (T3), estrogens, progesterone and androgens), etc. And, can be inhibited by several elements, such as polyunsaturated fatty acids (PUFA), leptin, cAMP, etc. Once pathways are activated, ERK1/2 and AKT promote activation of sterol regulatory element binding protein-1 (SREBP-1), which is a transcriptor factor of FASN gene, and can also stimulate FASN gene expression directly. mTOR (mammalian target of rapamycin), which promotes protein synthesis, also stimulates FASN protein synthesis. FASN protein degradation is accomplished in the proteasome by an ubiquitination system. Ubiquitin-specific protease USP2a removes ubiquitin from FASN protein, avoiding FASN degradation. Modified from Menendez J. *et al*. Nature Reviews Cancer, 2007[114], and Relat J. *et al*. Frontiers in Drug Design & Discovery, 2010[83].

4. Fatty Acid Synthase Inhibition

FASN overexpression in some cancer cells, and not in normal cells, and its function as energy and structure-elements supplier for highly proliferating tumoral cells, makes FASN a promising target for anti-cancer therapy.

FASN inhibition triggers cancer **cell mortality** and, **decreases tumor growth** or **delays progression** of carcinomas in mice models[83,87,130-132]. FASN blockade also suppresses endothelial cell proliferation and **angiogenesis**[133]. FASN inhibition could be a novel strategy to **overcome drug-resistance**, since it has been shown that FASN overexpression confers resistance to Adriamycin and mitoxantrone in breast cancer cells[134].

Anti-tumoral effect of FASN inhibition may be explained by several mechanisms:

- **End-product starvation**: lack of fatty acids included in phospholipids for biological membrane construction, which induces apoptosis in cancer cells[135-137].
- **Disruption of lipid rafts assembling**: tyrosine kinase receptors HER family among others) are localized in lipid rafts (detergent-resistant membrane microdomains). Disruption of lipid rafts in the cell membrane impairs correct functioning of receptors[138,139].
- **Inhibition of DNA replication**: blocks cell cycle before G1 phase through cyclin-dependent kinase inhibitors p21 and p27, BRCA1, SKP2 and nuclear factor κB (NFκB), among others[140-142].
- **p53-regulated apoptosis or cytostatic responses**: FASN inhibition with non-functioning p53 initiates apoptosis, but with functioning p53 initiates growth arrest[143,144].

- **Toxic accumulation of malonyl-coenzyme A** (malonyl-CoA): accumulation of malonyl-CoA inhibits carnitine palmitoyltransferase 1 (CPT-1), what in turn inhibits ß- oxidation of fatty acids to produce energy, and also promotes the accumulation of the sphingolipid ceramide that induces pro-apoptotic genes involved in the ceramide-mediated apoptotic pathway (BNIP3, TRAIL and DAPK2)[145].
- **Downregulation of Akt**: inhibition of this pathway promotes apoptosis in tumor cells[128]. Reduction of HER2 expression in breast cancer cells and HER1 expression in ovarian cancer cells[146,147].

Several **compounds are known to inhibit FASN activity**, by acting into different domains of the enzyme:

Cerulenin:

Was isolated from *Cephalosporium caerulens* and is a **small molecule that covalently binds to the FASN** KS domain, thus preventing the elongating fatty acid chain cycles[120,148]. It was one of the first compounds to be shown to inhibit FASN in breast cancer cell lines by inducing programmed cell death, and to delay disease progression in a xenograft model of ovarian cancer[149,150]. Clinical application is limited because of the chemical instability caused by its very reactive epoxy group[130,149].

C75:

It is a **synthetic cerulenin-derived** designed to overcome chemical instability, that lacks the reactive epoxy group[115]. Is a small molecule that **inhibits KS, ER and TE domains of FASN enzyme**[151]. C75 showed tumor growth inhibition in xenograft prostate, breast, mesothelioma, lung and ovarian cancer models and chemopreventive activity for mammary cancer in Neu-N transgenic mice[125,152-154].

Clinical application is limited because induces rapid and profound **weight loss and affects food intake**[155,156]. Weight loss occurs through **activation of fatty acid oxidation** in the mitochondria via **stimulation of CPT-1** (even in the presence of inhibitory concentrations of malonyl-CoA), and through the inducement of **anorexia** via inhibition of neuropeptide Y production within the hypothalamus[155,157].

Other synthetic cerulenin-derived FASN inhibitors have been developed in order to ameliorate C75-side effects. A well-known example is C93, which have shown high *in vitro* and *in vivo* antitumoral effects in several types of cancer without C75-side effects[158-161].

Orlistat (Xenical®, tetrahydrolipstatin):

It is a FDA-approved anti-obesity drug as a pancreatic and gastrointestinal lipase inhibitor. It potently **inhibits FASN by blocking the TE domain**, and thus prevents long-chain fatty acid release from the enzyme[141,162]. It has shown apoptotic and antiproliferative activity against prostate, melanoma, gastric and in HER2-overexpressing breast cancer cell lines[162-164]. It has also shown *in vivo* anti-tumoral effects in xenograft prostate and gastric cancer model[162,164]. Unfortunately, it has **poor solubility, low cell permeability, lack of selectivity, low oral bioavailability and poor metabolic stability**[165-167].

Triclosan:

It is an antibiotic used in soaps and oral dentifrices. It **blocks the ER domain**, preventing the elongation phase[168]. Blockade in this domain increase the enoyl thiolester intermediate, which has similar structure to cerulenin and C75. Reduced tumor progression in a rat mammary chemical carcinogenesis model[132].

Epigallocatechin-3-gallate (EGCG):

It is a natural green tea component. EGCG is a **powerful antioxidant, anti-obesic, suppress angiogenesis, can inhibit cell growth and proliferation and induce apoptosis**[169-174]. It **blocks FASN's KS domain**, thus preventing the elongating fatty acid chain cycles[175]. Although EGCG is a **non-specific** inhibitor targeting multiple signaling pathways[176], its apoptosis-inducing effect seems to correlate with FASN inhibition[169]. EGCG induces **apoptosis and inhibit HER2, MAPK and Akt activity** in cancer cells[83,177-181]. EGCG does **not have side effects** in body weight because it does not stimulate CPT-1 activity[179,180]. Anyway, anti-tumoral effects **require high EGCG concentration** and, is has a **poor oral bioavailability and low stability** in physiological conditions[178,182].

Novel EGCG structurally-related inhibitors:

The need to improve FASN inhibitors as anti-cancer agents prompted our group to **synthesize new EGCG-related molecules**[183-186]. Structure-activity relation has been studied with distinct parts of EGCG, it has been shown **that galloyl group is essential** for inhibition of FASN[187]. New compounds, derivatives of EGCG, that maintain galloyl group with **modifications** in other parts of the structure, should **behave similarly to EGCG** (regarding FASN inhibition without inducing weight loss). We synthesized a panel of EGCG structure related compound, maintaining two galloyl moieties linked by a cyclic system[186]. We screened new compounds for selective growth inhibition of a panel of human breast cancer cell lines with distinct levels of FASN expression[186]: SKBr3 (FASN+++), MCF-7 (FASN++) and MDA-MB-231(FASN+/-).

In general, compounds with a naphthalene ring in the aromatic system were more potent than analogues containing a benzene ring. Specially two compounds

showed **potent FASN inhibition activity and high and FASN-selective apoptotic and anti-tumoral activity** in the three different breast cancer cell lines mentioned and also in a **HER2 positive breast cancer cell line resistant to trastuzumab**[185,186] (improving in more than 5 folds EGCG *in vitro* effects). Moreover, both compounds did **not stimulate CPT-1 activity**. In our work, we showed that anti-tumoral activity is accomplished by induction of **PARP cleavage, and inhibition of HER2, ERK1/2 and Akt activity** (demonstrated by reduction reduction in p-HER2, p-ERK1/2 and p-Akt protein levels)[186].

G28UCM was selected because it displayed **90% of FASN inhibition** and had the most **potent effect against breast cancer cells**[185,186]. Moreover, some insights pointed that G28UCM also have *in vivo* effect, **decreasing tumor growth** in a HER2-positive/FASN-positive breast cancer xenograft model[185]. We also showed that G28UCM **synergistically improves anti-HER2 drugs** (trastuzumab and lapatinib) and small molecule **HER1 inhibitors** (gefitinib and erlotinib) when administered in combination in a HER2+/FASN+ breast cancer cell line[185] (improving combinatorial effects of EGCG with same anti-HER1/2 drugs). G28UCM does **not display *in vivo* side effects** (does not induce weight loss), explained by non-stimulation of CPT-1.

Data support FASN inhibition as a good target for cancer therapy, especially for those cancers without direct and successful treatment, or for those resistant to current therapies. More studies should be done in order to understand the molecular mechanisms of action of FASN inhibition and to develop new FASN effective inhibitors that could be applied in patients. In fact, other groups have developed FASN inhibitors with marked anti-tumoral effects. 3-V Bioscience, in California, has started the first phase 1 clinical study with a FASN-inhibitor (TVB-2640) in patients with advanced solid tumors, whose cancer has become refractory to standard therapy, and for whom no useful treatment exists.

Our results and those from other groups, such as the first clinical study, encourages us to continue and deeply study novel FASN inhibitors to improve its effects in anti-tumoral *in vitro*, *in vivo* and even *in patients* models for those cancers related to FASN with poor clinical outcome.

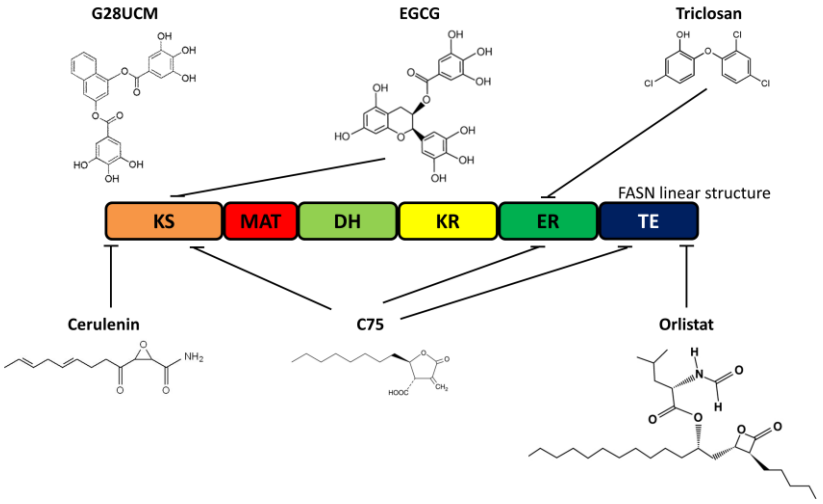

Figure 6. Fatty Acid Synthase inhibitors. Structure of some natural and synthetic FASN inhibitors and their effects in FASN domains. (-)-epigallocatechin-3-gallate (EGCG) and cerulenin inhibit ß-ketoacyl synthase (KS) domain. Triclosan inhibits enoyl reductase (ER) domain. C75 inhibits KS, ER and thioesterase (TE) domains. And, orlistat inhibits TE domain. Modified from Maier, T. *et al*. Science, 2008[90].

FASN EXPRESSION IN CANCER

Several carcinomas have overexpression or/and overactivation of FASN (FASN-positive) and can have also overexpression or/and overactivation of HER family receptors (HER-positive), since we and others have shown that both pathways are related. Thus, targeting FASN, alone or in combination with inhibitors of the HER family receptors pathway, could be a promise therapy for those cancers.

The presented thesis consist in two preclinical studies using two different types of cancer which are FASN-positive: non-small cell lung cancer and HER2-positive breast cancer, and a clinical study with early-stage breast cancer patients.

1. Non-Small Cell Lung Cancer

Lung cancers are classified clinically into two major groups: **non-small cell lung cancer (NSCLC)**, which accounts for about **85% of all lung cancers**, and small-cell lung cancer (SCLC), which accounts for the remainder[188]. The most common types of NSCLC include squamous cell carcinoma (≈35% of total lung carcinomas), large cell carcinoma (≈10%) and adenocarcinoma (≈45%)[189].

1.1. HER Family in NSCLC

HER1/EGFR/ErbB1 plays an important role in the **pathogenesis and prognosis** of more than half of non-small cell lung cancers. HER1 protein is present in approximately 80-85% of patients with NSCLC[190].

Both **mutation and amplification or overexpression** of HER1 have been described in lung cancers. 10-40% of this type of cancer harbor HER1 mutations in

the kinase domain (10% in Caucasian and 30–40% in Asian populations)[188]. More than 200 HER1 activating mutations have been described in NSCLC, but the deletion of five exon-19 residues (^{746}Glu-Leu-Arg-Glu-Ala^{750}) that occur immediately before the αC-helix and the exon-21 substitution of an arginine for leucine (Leu^{858}Arg) in the activation segment correspond to more than 90% of the **activating HER1 mutations** observed in NSCLC[191].

HER1 is frequently overexpressed in NSCLC (60% of NSCLCs), and has been associated with poor prognosis[188,192]. HER1 gene amplification occurs in about 15% of adenocarcinomas and 30% of squamous cell carcinomas[188].

1.2. Treatment of NSCLC

In non-small cell lung cancer, results of standard **anti-cancer therapy are poor** except for localized cancers. Treatment options depend on stage of disease and include surgery, radiation, platinum-based doublet chemotherapy, and targeted therapies in some cases[191]. The most potentially curative treatment is surgical removal[189]. Unfortunately, only 37% of lung cancers are diagnosed before the tumor has spread from its site of origin[193]. Chemotherapy can provide additional benefit to patients with resected NSCLC, but the median **survival rarely exceeds 10 months** in unselected patients with metastatic NSCLC disease treated with conventional chemotherapy[194]. Combined radiotherapy and chemotherapy improves survival of metastatic lung cancer, but the overall five-year survival is less than 15%[195]. Patients with advanced-stage disease can gain modest improvements in overall survival with chemotherapy or HER1 receptor kinase inhibitors.

A variety of chemotherapeutic regimens exist for all types of NSCLC[190]. Paclitaxel and carboplatin are the most used[196]. Paclitaxel enhances microtubule polymerization and thus interferes with microtubule breakdown during cell division. Carboplatin is a platinum-based antineoplastic agent that interferes with DNA synthesis and function. The angiogenesis inhibitor bevacizumab (Avastin®, a monoclonal antibody that binds to vascular endothelial growth factor A, or VEGF-A), in combination with paclitaxel and carboplatin, improves the efficacy, but with only minimal improvements in clinical outcomes and severe side effects[196,197].

An increase in HER1 levels or activation in a large percentage of NSCLCs prompted the development of therapies that **inhibit HER1 activity**[198]. Two classes of HER1 inhibitors, monoclonal antibodies (e.g., cetuximab) and small-molecule quinazoline derivatives TKIs (e.g., erlotinib, gefitinib, afatinib), have been studied in phase III clinical trials and are currently in **clinical use** in NSCLC[74,199-201]. **Monoclonal antibodies** are directed against the extracellular domain of HER1, block ligand binding and receptor dimerization and activation. **Small-molecule** HER1 TKIs compete reversibly (or irreversibly those which are new generation TKIs) with ATP to bind to the catalytic domain of the intracellular kinase domain to inhibit its activity.

The United States Food and Drug Administration (FDA) approved **gefitinib** in 2003 as monotherapy after failure of both platinum and taxane-based therapies[202]. Gefitinib is a small molecule reverse tyrosine kinase inhibitor (TKI). About 10% of unselected patients with NSCLC exhibit **rapid and often dramatic responses (tumor shrinkage)** to gefitinib. Responses are more frequent in females and non-smokers, and the median duration of response is 7.0 months. There is no correlation between response to gefitinib and HER1 expression, but with mutations in the kinase domain of HER1 receptor[203].

The FDA approved **erlotinib** (another small molecule reverse TKI) in 2004 for the treatment of locally advanced or metastatic NSCLC after failure of at least one prior chemotherapeutic regimen[204]. The median survival duration is 6.7 months compared with 4.7 months for placebo-treated patients. Never smokers and those with HER1-positive tumors **survive longer**. Patients who respond to erlotinib possess HER1 mutations in the kinase domain[205]. Several clinical trials demonstrated that gefitinib as first-line treatment would be superior to chemotherapy in HER1-mutant lung cancer. Response rate was increased from 47% with carboplatin–paclitaxel to 71%[206]. Median progression-free survival was increased from 6.3 months with cisplatin *plus* docetaxel treatment to 9.2 months with gefitinib treatment[206]. However, erlotinib is only recommended for second- and third-line therapy for NSCLC[190].

Afatinib is an ATP-competitive that belongs to a family of new small molecules which bind covalently and irreversibly to the tyrosine kinase domain of HER receptors acting as tyrosine kinase inhibitors (TKIs). Afatinib inhibits HER1, but also HER2 activation[207]. Nearly all NSCLC patients with HER1-activating mutations develop resistance to gefitinib or erlotinib after a median duration of 10–13 months[208]. The most common mechanism for resistance is the development of a new $Thr^{790}Met$ gatekeeper mutation in exon 20 that occurs in 50–60% of patients with disease progression[209]. Afatinib is able to inhibit the $Thr^{790}Met$ mutant and overcome **erlotinib or gefitinib resistance**[207]. Afatinib is approved by the FDA for the first-line treatment of NSCLC in patients harboring the activating exon-19 deletions or the $Leu^{858}Arg$ mutation.

1.3. Fatty Acid Synthase (FASN) in NSCLC

Fatty acid inhibition has been little studied, even with an imperative need for new treatments for this aggressive type of cancer. Orita *et al*. showed that the majority of human non-small cell lung cancer patient samples and cell lines studied **significantly express high levels of FASN**. They also proved that **C93** (a synthetic FASN inhibitor that not stimulate CPT-1) **inhibited tumor growth** in xenografts models of human non-small cell lung cancer, without causing anorexia and weight loss[159]. In another work, they also showed a chemopreventive action of FASN inhibition in chemically induced lung cancer[160]. More studies should to be done in order to understand FASN role in non-small cell lung cancer, and to find new regiment treatments targeting FASN, alone or in combination.

2. Breast Cancer

Breast cancer is the **second most common cancer** in the world and, by far, the most frequent cancer among women with an estimated 1.67 million new cancer cases diagnosed in 2012 (25% of all cancers). Incidence rates vary nearly four-fold across the world regions, with rates ranging from 27 per 100,000 in Middle Africa and Eastern Asia to 96 per 100,000 in Western Europe[2].

Breast cancer is the **fifth cause of cancer deaths** (522,000 deaths in 2012). It is the most frequent cause of cancer death in women in less developed regions (324,000 deaths, 14.3% of total) and the second cause of cancer death in more developed regions (198,000 deaths, 15.4%) after lung cancer[2].

For purposes of therapy, breast cancers are grouped into three categories, which are not mutually exclusive[210]:

- **Estrogen and progesterone hormone receptors positive.** This type of breast cancer cells overexpress estrogen and/or progesterone receptors in their cell surface membrane. This group is the most numerous and diverse, it has been reported that 79% of breast cancers express estrogen, progesterone or both hormone receptors[211]. Endocrine therapy is quite successfully administered in hormone-positive breast cancer patients[212].
- **HER2/Neu/ErbB2 positive.** Whose cells have amplification of HER2 (ErbB2) gene and/or overexpression of HER2 receptor protein. HER2 overexpression or amplification occurs in 20-30% of breast cancers and is correlated with a more aggressive phenotype and poor prognosis[80]. Effective therapeutics targeting HER2 exists for this group.
- **Triple negative breast cancer.** Lacking estrogen and progesterone receptors and HER2 amplification or overexpression[213]. Only 10-20% of breast cancers are triple negative. Treatment in this group of patients only admits chemotherapy, any specific target have been discovered yet.

Treatment efficacy and survival rates also depend on the stage of breast cancer of each patient. Stage classification depends on the size of the tumor and the dissemination from the place of origin[214]:

- **Stage 0:** also called carcinoma in situ. Abnormal cells are found in the duct or the lobule.
- **Stage I:** tumor is formed but has not spread outside the breast.
    - In stage IA, the tumor is 2 centimeters or smaller.
    - In stage IB, there are small clusters of breast cancer cells in the lymph nodes (larger than 0.2 millimeter but not larger than 2 millimeters); with or without tumor in the breast.

- **Stage II:**
  - In stage IIA, no tumor is found in the breast or the tumor is 2 centimeters or smaller. Tumor (larger than 2 millimeters) is found in 1 to 3 axillary lymph nodes or in the lymph nodes near the breastbone; or tumor is between 2 and 5 centimeters but has not spread to the lymph nodes.
  - In stage IIB, the tumor is between 2 and 5 centimeters, and there are small clusters of breast cancer cells in the lymph nodes (between 0.2 and 2 millimeters); or the tumor is between 2 and 5 centimeters, and tumor has spread to 1 to 3 axillary lymph nodes or to the lymph nodes near the breastbone; or the tumor is larger than 5 centimeters, but has not spread to the lymph nodes.
- **Stage III:**
  - In stage IIIA, no tumor is found in the breast or the tumor may be any size, but tumor is found in 4 to 9 axillary lymph nodes or in the lymph nodes near the breastbone; or the tumor is larger than 5 centimeters, and there are small clusters of breast cancer cells in the lymph nodes (between 0.2 and 2 millimeters); or the tumor is larger than 5 centimeters, and tumor has spread to 1 to 3 axillary lymph nodes or to the lymph nodes near the breastbone.
  - In stage IIIB, the tumor may be any size and cancer has spread to the chest wall and/or to the skin of the breast and caused swelling or an ulcer. Also, cancer may have spread up to 9 axillary lymph nodes or to lymph nodes near the breastbone.
  - In stage IIIC, no tumor is found in the breast or the tumor may be any size. Tumor may have spread to the skin of the breast and caused swelling or an ulcer and/or has spread to the chest wall. Also, cancer has

spread to 10 or more axillary lymph nodes, to lymph nodes above or below the collarbone or to axillary lymph nodes and lymph nodes near the breastbone.

- **Stage IV:** cancer has spread to other organs of the body, most often the bones, lungs, liver, or brain.

2.1. HER Family in Breast Cancer

All HER family receptors are present, differently, in breast cancers. Growth factors dysregulation, and amplification, overexpression and/or activating-mutations of one or more receptors of the HER family have been described in different breast tumors.

HER1. The presence of this receptor is strong in only **2.7% of patients** with breast cancer, but HER1 is highly associated with **poor prognosis** in this type breast cancer[215]. Moreover, HER1 overexpression is highly associated with basal-like breast carcinoma, being present in 54% of the cases[216]. It is a potential target for breast cancer overexpressing HER1.

HER2. 20-30% of breast tumors show overexpression of HER2 protein, which is associated with **reduced overall survival**[80,215]. HER2 protein is strongly linked to **metastatic and aggressive phenotype**[217]. 1–2% breast cancers show HER2 protein overexpression without gene amplification. This occurrence may take place due to modifications in systems controlling gene expression[218,219]. Amplification of the HER2 gene results in overexpression of the protein and also hyperactivity either without ligand binding[220].

Mutations in HER2 have also been described to hyperactivate tumoral-signaling to the cell machinery. Bose *et al.* estimated that about 1.6% of breast cancer

patients possess an HER2 mutation[82]. In a cohort of patients without HER2 gene amplification they found 5 different mutations in the extracellular domain of HER2 receptor, 1 mutation in the carboxyterminal tail and 12 different mutations occurred in the kinase domain. The most common mutation was the $Leu^{755}Ser$ mutation. From these 18 mutations, 7 were shown to activate the protein receptor and the downstream signaling, and to increase cell growth and tumor formation in mouse xenografts. One of these mutations ($Leu^{755}Ser$) confers resistance to Lapatinib, a tyrosine kinase HER2 inhibitor[82].

HER3. 17.5% of breast cancer patients show high expression of HER3, which is associated with **poor prognosis**[215].

HER4. HER4 protein expression is present in **11.9% of breast cancers**. Correlation with good or poor prognosis is **not clarified**. Some studies have correlated HER4 expression with a good prognosis, longer cancer-specific survival and disease free intervals[215,221]. Contrary, other studies correlated HER4 gene expression with poorer prognosis in breast cancer[222,223].

2.1.1. HER2 Positive Breast Cancer

HER2 is the most important and studied HER family receptor, it has been considered as a feature for treatment grouping of breast cancer. It is recommended to assess HER2 status for all invasive breast cancers, because it influences prognosis and selection of therapy[190,210].

HER2 positive breast cancer subtype is characterized by amplification and overexpression of HER2 (ErbB2) gene. An immunohistochemistry (IHQ) staining result of 3 or more, a fluorescence in situ hybridization (FISH) result of more than six HER2 gene copies per nucleus, or a FISH ratio greater than 2.2 is considered a

positive HER2 result[224], hence such tumor is considered HER2 positive breast cancer[80,215].

Patients diagnosed with HER2 positive breast cancer have a **poor prognosis, with reduced overall survival, related survival, recurrence-free survival and high risk of metastasis**[80,215,217,225-227].

2.1.2. Treatment of HER2 Positive Breast Cancer

The principal and most effective treatment for localized breast cancer is surgery, more than 90% of breast cancer patients undergo surgical excision of the tumor[193]. Secondly, other treatment options are chemotherapy, radiotherapy, and adjuvant hormonal therapy (for hormone receptor-positive tumors)[193]. Treatments can be co-administered, about 30% of patients are treated with surgery and radiation, 15% are treated with surgery and various drugs, and 21% are treated with surgery, radiation, and drugs. Chemotherapy may be used before surgery (neoadjuvant therapy), after surgery (adjuvant therapy), or instead of surgery for those cases in which surgery is considered unsuitable.

Against breast cancer, the most used chemotherapeutic drugs are doxorubicin, cyclophosphamide, docetaxel, and paclitaxel[190,228]. Other drugs also used in breast cancer are capecitabine, gemcitabine, pemetrexed, and vinorelbine[190].

In HER2 positive breast cancers, HER2 receptor has the driving role in HER signaling and tumor cells are addicted to the presence and activity of this protein. Such tumors are highly sensitive to **anti-HER2 treatments**[13,229].

Trastuzumab

Trastuzumab is a **humanized monoclonal antibody directed against the extracellular portion of HER2**, particularly on domain IV, and prevents the activation of its intracellular tyrosine kinase domain[37,230]. Trastuzumab is FDA-approved for the treatment of breast cancer overexpressing HER2, to be administered in different combinatorial regiments with chemotherapeutic drugs (such as doxorubicin, cyclophosphamide, carboplatin, docetaxel and paclitaxel), as a single agent after anthracycline-based therapy or other chemotherapy, or even as first-line treatment in combination with paclitaxel[190].

The mechanism of action of trastuzumab is not fully understood. In breast cancer cells that overexpress HER2, trastuzumab **down regulates HER2 receptor** and **increases endocytic destruction** and, as a consequence **arrest cell cycle** progression[231]. Trastuzumab **induces an immune response** called antibody-dependent cell mediated cytotoxicity (ADCC) in human patients and experimental animals. When trastuzumab binds to HER2, the monoclonal antibody is recognized by stromal immune natural killer (NK) cells which lead killing of tumor cell mediated by the release of perforin, granzyme, and cytokines[232]. HER2 can be cleaved into the formation of two truncated forms a 110 kDa extracellular domain and a 95 kDa membrane-bound carboxyterminal domain which is constitutively active. Trastuzumab also **inhibits this HER2 cleavage**[232,233].

Several clinical trials reported that addition of trastuzumab to the standard cytotoxic chemotherapy produces far better response rates than chemotherapy alone in patients with metastatic HER2 positive breast cancer[234-237]. In Slamon's study combination therapy increased median time to disease progression (7.4 *vs* 4.6 months), rate of objective response (50% *vs* 32%), median duration of response (9.1

vs 6.1 months), median survival (25.1 *vs* 20.3 months) and decreased rate of death at one year (22% *vs* 33%)[234].

Since HER2 is also expressed in cardiomyocytes, trastuzumab anti-HER2 treatment have important **cardiac dysfunction** adverse effect when administered with anthracyclines[238]. Cardiotoxicity increased from 8% to 27% of patients when adding trastuzumab to anthracycline therapy, and from 1% to 13% when adding trastuzumab to palitaxel therapy[235-237].

Lapatinib

Lapatinib is a **reversible HER1 and HER2 tyrosine kinase inhibitor**[239] FDA-approved drug for administering in second-line treatment, combined with capecitabine, in HER2 positive patients previously treated with cytotoxic drugs or trastuzumab and, combined with letrozole, for those post-menopausal hormone receptor-positive patients[190]. Lapatinib is a quinazoline derivative that most likely **binds to HER2 but also to other HER family receptors**.

In cell line and animal HER2-overexpressing models, lapatinib **inhibits HER2 activation and downstream Erk1/2 pathway, inhibits EGF-stimulated Akt activation and increase apoptosis**[239]. In HER1-overexpressing models, lapatinib **inhibits HER1** and Akt activation[239]. Differently from trastuzumab, lapatinib inhibits Erk1/2 phosphorylation in both HER1 and HER2 overexpresing models[239].

In clinical trials, patients who had **progressed to anthracycline, taxane or trastuzumab** were treated with capecitabine without or with lapatinib[240]. Lapatinib increased median time to progression from 4.4 to 8.4 months compared with capecitabine alone. The overall response rate was increased from 14 to 22% when adding lapatinib. But, skin rash also increased from 15% (in the capecitabine

group) to 27% (in the combination group) and non-serious cardiac events occurred in 2.5% of the combination group compared with 0.6% of the monotherapy group[240]. Lapatinib also improves letrozole anti-tumoral effects[241].

Synergistic activity between lapatinib and trastuzumab has been examined in clinical trials[242]. A Phase III trial involving 296 heavily pretreated, trastuzumab-refractory metastatic breast cancer patients randomized to treatment with lapatinib alone or with trastuzumab has been reported. Combination therapy significantly improved progression-free survival (PFS) (12 versus 8.4 weeks) compared with lapatinib alone. In fact, the NCCN Guidelines for Breast Cancer include trastuzumab *plus* lapatinib treatment for recurrent or metastatic HER2 positive breast cancer based on Blackwell's clinical study[190]. Other deeply clinical trials of lapatinib *plus* trastuzumab combination compared with monotherapy are currently being tested. The NeoALTTO trial in HER2 positive breast cancer patients showed that the pathologic complete response (pCR) rate in lapatinib *plus* trastuzumab combination was 51.3% compared to 24.7% for lapatinib monotherapy and 29.5% for trastuzumab monotherapy. These results confirm the potential of dual HER2-targeted therapy in the neoadjuvant setting[243].

Pertuzumab

Pertuzumab is a humanized monoclonal antibody directed against the domain II of HER2 and sterically blocks a binding pocket necessary for receptor dimerization and signaling, thereby blocking the formation of a heterodimer[244]. Pertuzumab and trastuzumab bind to different epitopes of the HER2 receptor, combinatorial administration improves anti-tumoral effects than either alone[245,246]. Pertuzumab is indicated for neoadjuvant use in combination with trastuzumab and

docetaxel, for the treatment of patients with HER2-positive metastatic breast cancer with tumors greater than 2 cm (≥T2) or node positive (≥N1) early-stage HER2-positive breast cancer[190].

In the CLEOPATRA clinical trial, Baselga *et al*. studied 808 patients with HER2-positive metastatic breast cancer who were treated with trastuzumab and docetaxel without or with pertuzumab as first-line therapy[246]. The median progression-free survival was 12.4 months in the control group and 18.5 months in the pertuzumab group. The safety profile was similar in the two groups. No additional cardiac toxicity was observed with the addition of pertuzumab to trastuzumab (and paclitaxel). Skin rash occurred in 34% of patients receiving three drugs and in 24% of those receiving trastuzumab and docetaxel[246].

In vitro and *in vivo* studies with 2C4[247] (the pertuzumab precursor) showed that pertuzumab is much more effective than trastuzumab in disrupting ligand-mediated HER2–HER3 dimer formation and that pertuzumab, but not trastuzumab, inhibits ligand-stimulated phosphorylation of HER2–HER3 and activation of ERK1/2 and PI3K[248].

Other anti-target therapies

Other therapies targeting HER2 or its downstream pathway are also FDA-approved or in clinical trials such as:

Ado-trastuzumab-DM1: FDA-approved HER2-targeter composed of trastuzumab, a stable thioether linker, and a derivative of maytansine (which is a potent antimitotic agent that inhibits the assembly of microtubules)[249].

Neratinib: irreversible tyrosine kinase inhibitor of HER1 and HER2 in clinical trials[250].

mTOR inhibitors: mTOR is a downstream of the HER2/Pi3K/Akt pathway[251]. Several mTOR inhibitors exist, but only everolimus is approved for breast cancer treatment.

- Rapamicin, the inhibitor that puts name to mTOR, selectively inhibits mTORC1. But, prolonged exposure of rapamicin also inhibits mTORC2[252].
- **Temsirolimus,** is a derived drug of rapamicyn. It is approved for advanced renal cell carcinoma. A phase II clinical trial studied the effects of temsirolimus in locally advanced or metastatic breast cancer[253]. They reported a response rate of 9.2% and a median time to progression of 12 weeks. Combination of temsirolimus with letrozole (a nonsteroidal aromatase inhibitor) amended median progression-free survival in a phase II trial, therefore a phase III trial is deeply evaluating this combination[254]. For metastatic HER2-positive and triple-negative breast cancer, temsirolimus is being investigated in combination of neratinib in a phase I-II trial (reviewed in [255]).
- Everolimus, another rapamicyn-derived drug, has been approved in combination with exemestane (an aromatase inhibitor) for estrogen receptor-positive, HER2-negative breast cancer previously treated by a nonsteroidal aromatase inhibitor[190].

2.1.3. Resistance to anti-HER2 therapies

Despite the considerable success of anti-HER2 therapies in the treatment of HER2-positive breast cancer, a proportion of patients who receive **trastuzumab and/or lapatinib** do not initially respond to anti-HER2 treatments, called **primary resistance**. And, for patients that initially respond to anti-HER2 treatments some develop resistance over time, called **secondary or acquired resistance**[256,257].

Regarding primary resistance, 74% of patients with HER2-positive metastatic breast cancer do not respond to first-line trastuzumab monotherapy and about 50% do not respond to trastuzumab with anthracycline and cyclophosphamide[234,258]. Regarding acquired resistance, trastuzumab containing adjuvant therapy treated patients will relapse and nearly all patients receiving trastuzumab for metastatic disease will progress after a year of treatment[256]. Metastatic breast cancer patients treated with lapatinib become refractory with tumor growth or spread[257]. **Resistance to trastuzumab *plus* lapatinib** combination is also observed[257].

The molecular **mechanisms** leading to trasuzumab and/or lapatinib resistance have been extensively studied[259]. These include those either affecting the HER2 receptor, other HER family receptors or its downstream signaling pathways, and even other diverse mechanisms. Some examples are:

Changes in HER2 receptor

Two main mechanisms involving HER2 receptor have been described, especially in trastuzumab resistance. The first mechanism is *in vivo* conversion of HER2+ to HER2- carcinoma after neoadjuvant trastuzumab[260], the second mechanism is shedding of the extracellular receptor domain leaving behind the constitutively active truncated form ($p95^{HER2}$)[261].

Regarding HER2 changes as mechanism of resistance to lapatinib, Thr^{798}Met gatekeeper mutation has been described in HER2 receptor[262].

Changes in HER family

HER family receptors have redundant pathways that converge in almost same downstream effectors to promote tumor cell proliferation and survival. Cross-talk

between HER2 and other HER family members such as HER1 or HER3[263-265], overexpression or hyperactivation of other HER family receptors, or its ligands, can compensate inhibition of HER2 and promote tumor cell proliferation even in the presence of anti-HER2 drugs[266].

Changes in HER downstream or other signaling

Desregulation of PI3K/Akt pathway is the most common mechanism of trastuzumab and lapatinib resistance. Decreased levels of PTEN expression or activity[267,268], overexpression and overactivation of the PI3K/Akt/mTOR signaling proteins[266,268,269], Akt activating-mutations[270], gain-of-function mutation in PI3KCA (encoding the PI3K catalytic isoform p110α)[271] are some resistant-related alterations described regarding this important pathway.

Cross-talk with other signaling pathways

Activation (or overexpression) of alternative signaling pathways to maintain cell proliferation has also been proposed as responsible for anti-HER2 drug resistance[272,273]. This includes, among others, the insulin-like growth factor (IGF) receptor and the hepatic growth factor receptor (c-Met) pathways[274]. IGF signaling through IGF-Insulin receptor (IGF-IR) has been shown to activate the MAPK and PI3K/AKT pathways and thus protect tumor cells from damage due to cytotoxic anti-HER2 agents.

2.2. Fatty acid synthase (FASN) in Breast Cancer

Fatty acid synthase inhibition in breast cancer has been extensively studied. FASN expression in breast cancer has been found to be high in various cell lines, including hormone-dependent, hormone-independent, HER2-dependent and HER2-independent[116,120,126,128,138,147,275]. Hormone-independent and HER2-positive SKBr3 breast cancer cell line expressed higher levels (~2.5-fold) of FASN compared with hormone-dependent breast cancer cell lines[115,275]. FASN levels increased with tumor stage[115].

Several inhibitors, mentioned before, have been developed and tested in different types of breast cancer. Promising results have and are emerging, but more studies should to be done in order to establish FASN as a target and find successful anti-FASN drugs for breast cancer, especially for those cancers without target-therapy or with poor prognosis (such as those resistant to anti-HER drugs).

OBJECTIVES OF THE WORK

Hypothesis

Inhibition of lipogenesis, through blockade of FASN activity, is a pharmacological strategy (alone or in combination) for the treatment of FASN positive cancers both, sensitive and resistant to standard therapies.

Objectives

The main objective of this thesis is to determine the role of fatty acid synthase (FASN) expression and inhibition (using common and novel FASN-inhibitors) in different preclinical models of cancer (breast and lung cancer) and in breast cancer tumor patient' samples.

In order to accomplish the main objective, the following specific objectives (which are sorted by articles) were posed:

Fatty Acid Synthase Expression is Strongly Related to Menopause in Early-Stage Breast Cancer Patients

- To determine the association of FASN tumor tissue expression with clinicopathological features in early-stage breast cancer patients.
- To determine the association of FASN tumor tissue expression with anthropometrical features in early-stage breast cancer patients.

Objectives

Different Fatty Acid Metabolism Effects of (−)-Epigallocatechin-3-Gallate and C75 in Adenocarcinoma Lung Cancer

- To analyze the anti-tumoral effect of two FASN inhibitors (C75 and EGCG) in FASN-positive lung cancer cell models.
- To compare the effects of C75 and EGCG on FASN activity enzyme (lipogenesis), CPT activity (fatty acid oxidation), cellular proliferation, induction of apoptosis and cell signaling (HER1, ERK1/2, AKT and mTOR) in lung carcinoma cells.
- To study the efficacy and toxicity effects of C75 and EGCG on lung cancer xenografs.

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

- To develop HER2-positive breast cancer (SKBr3) cells resistant to anti-HER2 treatments (trastuzumab and/or lapatinib) and to study molecular mechanisms of resistance and their implication in FASN expression.
- To determine the implications of FASN inhibition (using EGCG and its novel derivative, G28UCM) on cell viability of sensitive HER2-positive breast cancer (SKBr3) and on trastuzumab and/or lapatinib resistant (SK**TR**, SK**LR** and SK**LTR**) cells.
- To study the cellular and molecular effects of inhibiting FASN together with other anti-HER2 pathway inhibitors (pertuzumab and temsirolimus) in sensitive and resistant HER2-positive cells.
- To evaluate the antitumor activity of EGCG, temsirolimus and pertuzumab (alone and in combination) in a HER2+ patient derived xenograft (PDX) model and in a trastuzumab *plus* lapatinib-resistant HER2+ PDX model.

RESULTS

Article 1:

Fatty Acid Synthase Expression is Strongly Related to Menopause in Early-Stage Breast Cancer Patients

Porta R, Blancafort A, Casòliva G, Casas M, Dorca J, Buxo M, Viñas G, Oliveras G, Puig T. Fatty acid synthase expression is strongly related to menopause in early-stage breast cancer patients. Menopause. 2014 Feb;21(2):188-91.

https://doi.org/10.1097/GME.0b013e31829d17dc

Abstract

Objective: Overexpression of fatty acid synthase (FASN), the enzyme involved in the de novo synthesis of fatty acids, has been reported in several human carcinomas, including breast cancer, and has been related to poor prognosis. Our aim was to analyze the association of FASN tumor tissue expression with clinicopathological and anthropometrical features in early-stage breast cancer patients.

Methods: We prospectively studied 53 women with early-stage breast cancer who were treated with surgical operation and postoperative chemotherapy.

Results: Menopause status and age were strongly associated with higher levels of FASN tumor expression ($P < 0.005$ and $P = 0.038$, respectively). Body mass index and pathological stage were also related to FASN tumor expression.

Conclusions: Our findings suggest that FASN could be a potential therapeutic target in postmenopausal breast cancer patients. However, further studies are needed.

Keywords

Fatty acid synthase , Breast cancer , Early stage , Menopause

Article 2:

Different Fatty Acid Metabolism Effects of (−)-Epigallocatechin-3-Gallate and C75 in Adenocarcinoma Lung Cancer

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http://www.biomedcentral.com/1471-2407/12/280

BMC Cancer

RESEARCH ARTICLE Open Access

Different fatty acid metabolism effects of (–)-Epigallocatechin-3-Gallate and C75 in Adenocarcinoma lung cancer

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Abstract

Background: Fatty acid synthase (FASN) is overexpressed and hyperactivated in several human carcinomas, including lung cancer. We characterize and compare the anti-cancer effects of the FASN inhibitors C75 and (–)-epigallocatechin-3-gallate (EGCG) in a lung cancer model.

Methods: We evaluated *in vitro* the effects of C75 and EGCG on fatty acid metabolism (FASN and CPT enzymes), cellular proliferation, apoptosis and cell signaling (EGFR, ERK1/2, AKT and mTOR) in human A549 lung carcinoma cells. *In vivo*, we evaluated their anti-tumour activity and their effect on body weight in a mice model of human adenocarcinoma xenograft.

Results: C75 and EGCG had comparable effects in blocking FASN activity (96,9% and 89,3% of inhibition, respectively). In contrast, EGCG had either no significant effect in CPT activity, the rate-limiting enzyme of fatty acid β-oxidation, while C75 stimulated CPT up to 130%. Treating lung cancer cells with EGCG or C75 induced apoptosis and affected EGFR-signaling. While EGCG abolished p-EGFR, p-AKT, p-ERK1/2 and p-mTOR, C75 was less active in decreasing the levels of EGFR and p-AKT. *In vivo*, EGCG and C75 blocked the growth of lung cancer xenografts but C75 treatment, not EGCG, caused a marked animal weight loss.

Conclusions: In lung cancer, inhibition of FASN using EGCG can be achieved without parallel stimulation of fatty acid oxidation and this effect is related mainly to EGFR signaling pathway. EGCG reduce the growth of adenocarcinoma human lung cancer xenografts without inducing body weight loss. Taken together, EGCG may be a candidate for future pre-clinical development.

Keywords: Lung cancer, Xenograft, Fatty acid synthase, EGCG, C75, Inhibitors, Weight loss, Fatty acid metabolism, EGFR

Background

Fatty acid synthase (E.C.2.3.1.85; FASN) is a homodimeric multienzymatic protein that catalyzes de novo synthesis of long-chain fatty acids from acetyl-CoA, malonyl-CoA, and NADPH precursors [1]. In most human tissues the diet supplies the fatty acids needs and FASN expression is low or undetectable. In contrast, in many human solid carcinomas, lipogenic enzymes (mainly FASN) are highly expressed [2-7] and *de novo* fatty acids biosynthesis supplies the needs of long chain fatty acids (LCFA) for energy production, protein acylation, synthesis of biological membranes, DNA synthesis and cell cycle progression among other biological processes, providing an advantage for tumour growth and progression [3-5].

FASN inhibition that blocks lipogenic pathway and impedes fatty acid synthesis, entails apoptosis in tumour cells that overexpress FASN, without affecting non-malignant cells (reviewed in ref. [8]). In this context, FASN enzyme has became a promising target for anti-cancer therapy, a putative biomarker of malignancy and an indicative of prognosis for many cancers, including lung carcinomas [5-7,9].

The oncogenic properties of FASN seem to be the result of an increased activation of HER2 and its downstream

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signaling cascades: phosphoinositide-3 kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR), mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK1/2) pathways [10-18].

The use of FASN inhibition as anticancer therapy was first described with Cerulenin (a natural antibiotic from *Cephalosporium ceruleans)* that causes apoptotic cancer cell death *in vitro* [19]. More recently, C75, a synthetic analogue of cerulenin or (−)-epigallocatechin-3-gallate (EGCG), the main polyphenolic catechin of the green tea, have been identified as FASN inhibitors, able to induce apoptosis in several tumour cell lines and also to reduce the size of mammary tumours in animal models [8,20-24]. Although its selective cytotoxicity, C75 has been discarded in many cancer models due to its side effects: anorexia and body weight loss. In contrast, we have demonstrated that in SKBr3 breast cancer cells EGCG has similar effects as C75 in inhibiting FASN and it does not induce CPT activity *in vitro,* neither weight loss *in vivo* [11,25,26], opening new perspectives in the use of green tea polyphenols or its derivatives as anti-cancer drugs alone or in combination with other therapies.

Here we compare the effects of C75 and EGCG on lipogenesis (FASN activity), fatty acid oxidation (CPT activity), cellular proliferation, induction of apoptosis and cell signaling (EGFR, ERK1/2, AKT and mTOR) in A549 lung carcinoma cells. We also evaluated their anti-cancer activity and their effect on body weight with a mice model of A549 lung cancer xenograft. We examined EGCG as a potential drug for clinical development in adenocarcinoma of lung cancer that accounts for 40% of non-small-cell lung cancers (NSCLC), the most common type of lung cancer [27].

Methods

Cell Lines and Cell Culture

A549 lung cancer cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), and were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Berlin, Germany) containing 10% heat-inactivated fetal bovine serum (FBS, HyClone Laboratories, Utah, USA), 1% L-glutamine, 1% sodium pyruvate, 50 U/mL penicillin, and 50 μg/mL streptomycin (Gibco). Cells were routinely incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

Growth Inhibition Assay

EGCG, C75 and 3–4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dose–response studies were done using a standard colorimetric MTT reduction assay. Briefly, cells were plated out at a density of 3×10^3 cells/100 μL/well in 96-well microtiter plates. Following overnight cell adherence fresh medium along with the corresponding concentrations of EGCG and C75 were added to the culture. Following treatment, media was replaced by drug-free medium (100 μL/well) and MTT solution (10 μL of a 5 mg/mL), and incubation was prolonged for 2,5 h at 37°C. After carefully removing the supernatants, the MTT-formazan crystals formed by metabolically viable cells were dissolved in DMSO (100 μL/well) and absorbance was determined at 570 nm in a multi-well plate reader (Spectra max 340PC (380), Bio-Nova Cientifica s.l., Madrid, Spain). Using control optical density OD values (OD_{CTRL}) and test OD values (OD_{T-EST}), the agent concentration that caused 50% growth inhibition (IC_{50} value) was calculated from extrapolating in the trend line obtained by the formula (OD_{CTRL} - OD_{T-EST})*100/OD_{CTRL}.

Fatty Acid Synthase Activity Assay

Cells were plated out at a density of $1x10^5$ cells/500 μL/well in 24-well microtiter plates. Following overnight cell adherence media was replaced by DMEM supplemented with 1% lipoprotein deficient Fetal Bovine Serum (Sigma) along with the corresponding IC_{50} concentrations of C75 (72 μM) and EGCG (265 μM) or DMSO. For the last 6 h of the treatment, ([1,2-^{14}C] Acetic Acid Sodium salt (53,9 mCi/mmol) (Perkin Elmer Biosciences, Waltham, MA, USA) was added to the media (1 μCi/mL). Cells were harvested and washed twice with phosphate-buffered saline (PBS) (500 μL) and once with Methanol:PBS (2:3) (500 μL). The pellet was resuspended in 0,2 M NaCl (100 μL) and broke with freeze-thaw cycles. Lipids from cell debris were extracted by centrifugation (2000 g, 5 min) with Chloroform:Phenol (2:1) (350 μL) and KOH 0,1 M (25 μL). The organic phase recovered is then washed with Chloroform:Methanol:Water (3:48:47) (100 μL) and evaporated in a Speed-vac plus SC110A (Savant). The dry-pellets were resuspended in ethanol and transferred to a vial for radioactive counting.

Mitochondria Isolation of A549 Cells

Cells were grown to confluence in 10 mm dishes and collected in PBS (100 μL/dish). The pellet was resuspended in Buffer A (150 mM KCl, 5 mM Tris–HCl, pH 7.2) (125 μL/dish), and disrupted using a glass homogenizer (10 cycles with tight fitting pestle and 10 cycles with light one). Mitochondria were collected by centrifugation (16000 g, 5 min at 4°C), resuspended in Buffer A and quantified using Bradford-based Bio-Rad assay (BioRad Laboratories, Hercules, CA, USA). At this step mitochondria could be used for total CPT activity measurement.

Carnitine Palmitoyltransferase (CPT) Activity Assay

CPT activity was assayed by the forward exchange method using L- [methyl-^{3}H] Carnitine hydrochloride (82 Ci/mmol) (Perkin Elmer Biosciences) as we previously

described [25]. Briefly, reactions (were performed in the standard enzyme assay mixture (1 mM L-[^{3}H]carnitine (~5000 dpm/nmol), 80 μM palmitoyl-CoA (Sigma), 20 mM HEPES (pH 7.0), 1% fatty acid-free albumin (Roche Sciences, Mannheim, Germany), 40–75 mM KCl and the corresponding IC_{50} concentrations of C75 (72 μM) and EGCG (265 μM) or DMSO when indicated. Reactions were initiated by addition of A549 isolated mitochondria (100 μg) and all incubations were done at 30°C for 3 min. Reactions were stopped by addition of 6% Perchloric Acid and then the product [^{3}H]-palmitoylcarnitine was extracted with butanol at low pH and was transferred to a vial for radioactive counting.

Western Blot Analysis of Tumour and Cell Lysates

The primary mouse monoclonal antibody for FASN was from Assay designs (Ann Arbor, MI, USA). Monoclonal anti–β-actin mouse antibody (clone AC-15) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against poly-(ADP-ribose)-polymerase (PARP), AKT, phospho-AKT^{Ser473}, ERK 1/2, EGFR, phospho-EGFR^{Tyr1068}, mTOR, phospho-mTOR-^{Ser2448} and mouse monoclonal antibody against phospho-ERK1/2$^{Thr202/Tyr204}$, were from Cell Signaling Technology, Inc (Danvers, MA, USA). A549 cells were harvested following treatment of A549 cells with EGCG or C75. Tumour tissues were collected from A549 human lung cancer xenografts at the end of the *in vivo* experiment. Cells and tumour tissues were lysed with ice-cold in lysis buffer (Cell Signaling Technology, Inc.) containing 1 mM EDTA, 150 mM NaCl, 100 μg/mL PMSF, 50 mM Tris–HCl (pH 7.5), protease and phosphatase inhibitor cocktails (Sigma). Protein content was determined by the Lowry-based Bio-Rad assay (BioRad Laboratories). Equal amounts of protein were heated in LDS Sample Buffer and Sample Reducing Agent from Invitrogen (California, USA) for 10 min at 70°C, separated on 3% to 8% or 4% to 12% SDS-polyacrylamide gel (SDS-PAGE) and transferred to nitrocellulose membranes. After blocking, membranes were incubated overnight at 4°C with the corresponding primary antibody. Blots were washed in PBS-Tween, incubated for 1 hour with corresponding peroxidase-conjugated secondary antibody and revealed using a commercial kit (Super Signal West Pico or Super Signal West Femto chemiluminescent substrate from Thermo scientific (Illinois, USA) or Immobilon Western HRP Substrate from Millipore (Massachusetts, USA)). Blots were re-proved with an antibody against β-actin as control of protein loading and transfer.

In vivo Studies: Human Lung Tumour Xenograft and Long-term Weight Loss Experiments

Experiments were conducted in accordance with guidelines on animal care and use established by Biomedical Research Institute of Bellvitge (IDIBELL) Institutional Animal Care and Scientific Committee (AAALAC unit 1155). Tumour xenograft were established by subcutaneous injection of 10 x 10^6 A549 cells mixed in Matrigel (BD Bioscience, California, USA) into 4–5 week old athymic nude BALB/c female's flank (Harlan Laboratories, Gannat, France). Female mice A549 (12 wk, 23–25 g) were fed ad libitum with a standard rodent chow and housed in a light/dark 12 h/12 h cycle at 22°C in a pathogen-free facility. Animals were randomized into three groups of five animals in the control and four animals in the C75 and EGCG-treated groups. When tumours' volume were palpable (reached around 35–40 mm^3) each experimental group received an i.p. injection once a week of C75 or EGCG inhibitor (40 mg/kg) or vehicle alone (DMSO), dissolved in RPMI 1640 medium. Tumour volumes and body weight were registered the days of treatment and four days after every treatment until 33 days after first administration. Tumours were measured with electronic calipers, and tumour volumes were calculated by the formula: $\pi/6 \times (v1 \times v2 \times v2)$, where v1 represents the largest tumour diameter, and v2 the smallest one. At the end of the experiment, all mice were euthanized and tumour tissues were collected.

Statistical Analysis

In vitro results were analysed by Student's *t*-test or by one-way ANOVA using a Bonferroni test as a post-test. All data are mean ± standard error (SE). All observations were confirmed by at least three independent experiments. *In vivo* drug efficacy experiment results were analyzed using the non-parametric Wilcoxon test comparing repeated measurements (tumour volume). Data are the median of tumour volume of 4 or 5 animals. Statistical significant levels were $p < 0.05$ (denoted as *) and $p < 0{,}001$ (denoted as **).

Results

Effect of EGCG and C75 on FASN and CPT Activities in A549 Cells

In order to evaluate the specificity of EGCG and C75 for FASN, we analyzed their effect on FASN and CPT system activities. A549 cells were treated for 24 hours with IC_{50} concentration values of C75 (72 ± 2,8 μM) or EGCG (265 ± 7,1 μM) [Additional file 1: Figure S1]. As shown in Figure 1, C75 and EGCG significantly reduced FASN activity in A549 cells compared to control cells (remaining FASN activity of 3,1 ± 0,6% and 10,7 ± 1,5%, $p = 0{,}000$; both). Significant changes in FASN protein levels were also observed in EGCG-treated cells but not in control or C75-treated cells, as assessed by Western blotting (Figure 2). The effect of both compounds on CPT enzymatic activity was assayed in A549 isolated mitochondria, as described in the Material and Methods section. EGCG had no effect on CPT activity (115 ± 12%, respect

to control; p = 0,006), in contrast to C75, which produced a significant activation of CPT system (131 ± 11%, respect to control; p = 0,294).

Analysis of the Effect of EGCG and C75 on Apoptosis and Cell Signaling in A549 Cells

Apoptosis and induction of caspase activity were checked with cleavage of PARP in Western blotting analysis. Apoptosis was not detected in A549 non-treated cells. In A549 cells treated for 6, 12 and 24 hours with IC_{50} concentration values of C75 or EGCG (Additional file 1: Figure S1), there was an increase in the levels of 89 kDa PARP product in a time-dependent manner (Figure 3). We examined the effects of EGCG and C75 on the phosphorylated and the total levels of EGFR (p-EGFR), HER2 (p-HER2), HER3 (p-HER3), HER4 (p-HER4) and its related downstream AKT, ERK1/2 and mTOR proteins. Results in Figure 3 confirmed that A549 cells treated with EGCG showed a marked decrease in the phosphorylated forms of EGFR, AKT, ERK1/2 and mTOR within 6 hours of EGCG treatment, with no changes in the total levels of the corresponding proteins. In contrast, C75 treatment needs up to 48 hours just to detect a partial decrease on total levels of EGFR protein and on p-AKT protein. Phosphorylated and total protein levels of HER2 (p-HER2), HER3 (p-HER3) and HER4 (p-HER4) did not change after C75- or EGCG-treatment (Data not shown).

In Vivo Analysis of EGCG and C75 on Human Lung Cancer Xenografts

To explore the potential effectiveness of EGCG and C75 for lung cancer treatment *in vivo*, we treated athymic nude mice with A549 human lung cancer xenograft. In control animals, on final day the median of the tumour volume (519 mm^3 on day 33) was significantly different from the starting median tumour volume (33 mm^3 on day 0, p = 0,04) and this trend (was similar from days 12 to 33 in control animals' group (Data not shown). In the experimental animals, the median of the tumour volume of C75- and EGCG-treated animals on day 33 (290 and 224 mm^3, respectively) wasn't significantly different from the median of the tumour volume on the starting day (40 and 36 mm^3, respectively; p = 0,07 both), those pointing out that the treatment with the anti-FASN compounds C75 and EGCG prevents the growth of A549 xenografts (Figure 4A). C75 and EGCG-treated tumours showed apoptosis by induction of PARP cleavage without any change in the total levels of FASN protein (Figure 4A). In EGCG-treated animals we do not find significant changes on fluid, food intake, body weight or other toxicity parameters (data not shown) versus control animals, after 33 days of weekly treatment with 40 mg/Kg of EGCG (Figure 4B). C75-treated animals showed a marked decrease of body weight (close to 6%) after each i.p. administration, which was especially remarkable in the first 20 days of treatment (Figure 4B).

Discussion

Levels of FASN expression in different human carcinomas attracted considerable interest of this enzyme as a target for therapy [10,11]. In this study, we show that adenocarcinoma of lung cancer, is among the foremost of cancers that could potentially be treated by inhibiting FASN.

C75 has been studied in A549 lung cancer xenografts [28] where it induces a transient and reversible growth inhibition. EGCG anti-cancer effects in lung cancer have

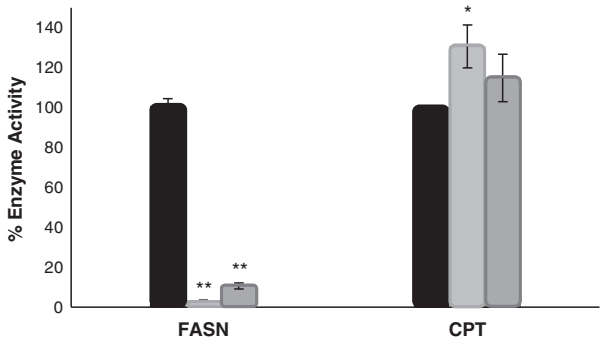

Figure 1 EGCG inhibits FASN activity in A549 cancer cells with no change on CPT system activity. A549 Cells were treated for 24 hours with C75 (72 μM) and EGCG (265 μM) and FASN activity was assayed by counting radiolabelled fatty acids synthesized *de novo*. Isolated mitochondria from A549 cells were assayed for CPT activity in the presence of DMSO (control), C75 (72 μM) or EGCG (265 μM), as described in Material and Methods. Bars represent the remaining enzyme activity in A549 treated cells or mitochondria. Data are means ± SE from at least 3 separate experiments. ** p < 0,001 versus control, by one-way ANOVA or Student's *t*-test.

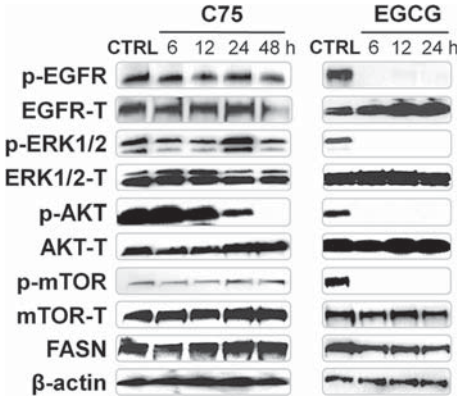

Figure 2 EGCG blocks phosphorilation of EFGR, HER2, ERK1/2, AKT and mTOR in A549 cells. A549 cells were treated for 6, 12, 24 and 48 hours with C75 (72 μM) and 6, 12 and 24 hours with EGCG (265 μM), and equal amounts of lysates were immunoblotted with anti-EGFR, anti-HER2, anti-ERK1/2, anti-AKT, anti-mTOR and anti-FASN antibodies. Activation of the protein under study was analyzed by assessing the phosphorylation status using the corresponding phospho-specific antibody. Total amounts of HER2 and AKT proteins remain unchanged. Blots were reproved with an antibody for β-actin to control for protein loading and transfer. Gels shown are representative of those obtained from three independent experiments.

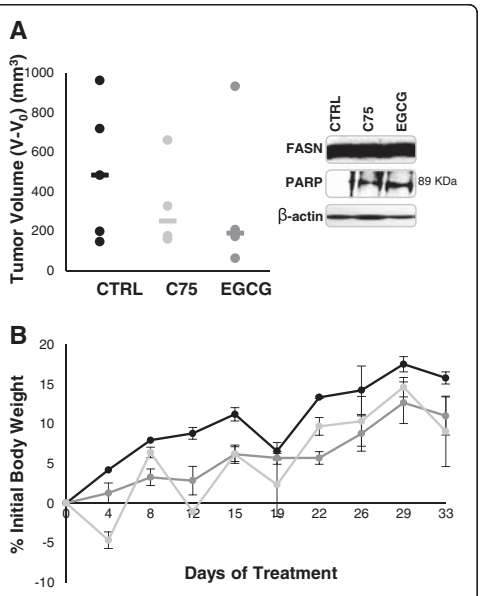

Figure 4 EGCG inhibits A549 xenograft growth and do not induce *in vivo* weight loss. A, once a week i.p. administration of 40 mg/kg of C75 (●) or EGCG (●) during 33 days blocked the growth of A549 lung cancer xenografts compared to control animals (●). Circles represent individual increase in tumour volume at final day (day 33) and horizontal lines represent the median value for each experimental group. C75 and EGCG-treated tumours showed apoptosis, whereas FASN protein levels did not change. Treated and control tumours were lysed and equal amounts of lysates were subjected to Western blot analyses with anti-PARP and anti-FASN. Blots were reprobed for β-actin as loading control. Gels shown are representative of those obtained from two independent experiments. **B**, EGCG treatment does not induce weight loss. The body weight of each mouse was measured before and weekly after treatment with C75 or EGCG (40 mg/Kg/day for 33 days) or vehicle control. Data are expressed as percentage of initial body weight and represent mean values ± SE for each experimental group.

also been evidenced and, besides FASN-inhibition, several mechanisms of action have been proposed, such as G3BP1 (GTPase activating protein (SH3 domain) binding protein) inhibition [29], generation of Reactive Oxygen Species (ROS) [30] or induction of p53-dependent transcription [31].

To further investigate the implications of FASN inhibition in lung adenocarcinoma, we have analyzed the blockage of FASN by EGCG and C75 in A549 lung cancer cells. Firstly, we ensured similar levels of FASN inhibition by C75- and EGCG-treatment (96,9% and 89,3% of control, respectively). As C75 had no effect on the abundance of FASN protein levels and EGCG diminished the levels of this enzyme, it is probable that in the EGCG-treated cells, the reduction of FASN activity could be in part consequence of the reduced FASN protein levels.

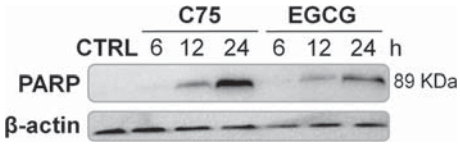

Figure 3 EGCG and C75 induce apoptosis in A549 cells. Induction of caspase activity was confirmed by PARP cleavage. A549 cells were treated with C75 (72 μM) or EGCG (265 μM) for 6, 12 and 24 hours, and equal amounts of lysates were immunoblotted with anti-PARP antibody, which identified the 89 kDa (cleavage product) band. Blots were reproved for β-actin as loading control. Gels shown are representative of those obtained from three independent experiments.

The inhibition of FASN activity by EGCG and C75 was accompanied by an induction of apoptosis, and changes in cell growth and proliferation signaling pathways. The active phosphorylated form of EGFR (p-EGFR) was completely abolished after 6 hours of exposure to EGCG. Consequently, phosphorylated forms of ERK1/2 (p-ERK1/2), AKT (p-AKT) and mTOR (p-mTOR) were also markedly decreased. It is remarkable that

comparable concentrations of C75, even with prolonged exposure (48 hours), only partially decreased total levels of EGFR and phosphorylated levels of AKT (p-AKT). Several data supported a relationship between HER2 and FASN in breast cancer, head and neck carcinomas, HER2-overexpressed fibroblasts and other carcinomas [11,32-35]. Furthermore, some authors have demonstrated the blocking effects of the FASN inhibitor EGCG on all members of epidermal growth factor receptor (ErbB) family [11,36-38].

This is the first evidence that EGFR is involved in the regulation of FASN expression in a lung cancer model with EGFR-overexpression. EGFR may be another EGCG-direct target that through inhibition of its downstream signalers (Akt, ERK1/2 and mTOR) is able to down-regulate FASN expression at two different levels: 1, at the transcriptional level through the sterol response element-binding proteins 1c (SREBP-1c), the FASN-transcription factor mediated by PI3K/Akt and MAPK/ERK1/2 pathways [39]; 2, at the translational level, through Akt-mTOR-signaling and its downstream effectors, eIF4G and S6K (reviewed in ref [40]) as seen in breast cancer [41] and in human hepatoma cells [42].

In addition, we corroborate a FASN-ErbB loop, described in breast cancer. The FASN disruption impedes synthesis of lipids, which are integrated in membrane lipid raft in which cell surface receptors, ErbB among others, accommodate and sense to tumourigenic pathways [43]. C75 is a direct and competitive inhibitor of FASN [21]. Consequently, we have seen a strong and fast inhibition of FASN activity with C75 treatment and a later effect on levels of EGFR and phosphorylation of it downstream effector Akt (p-Akt), what brings us to corroborate the idea of a FASN-lipid rafts-ErbB inhibition loop.

An important result of our study is the *in vivo* drug-efficacy study and long-term body weight evaluation. EGCG and C75 markedly blocked the growth of A549 lung cancer xenografts while the tumour volumes of control animals growth significantly until the final day study. C75-treated mice showed a marked decrease in body weight after each administration (close to 6% of initial body weight). This result accords to the data that C75 is able to stimulate CPT system and fatty acid β-oxidation, which has been related to the severe decrease of food intake and induction of weight loss in rodents [44]. In contrast, we have not observed a significant decrease in body weight in the animals treated for 33 days with EGCG.

A key feature of EGCG is that does not affect CPT activity (as it is shown in vitro in Figure 1) and, consequently, it does not induce weight loss in experimental animals. This result in a lung cancer model are in agreement with our previous findings in a mouse breast cancer model [11] and reinforces the hypothesis that CPT-activation is the cause of weight loss in xenografts models. Our data also reveal for the first time that the effects of EGCG in lung carcinoma involve different pathways than C75 but also that the undesirable side effects observed in C75 treated-mice are not produced in EGCG-treated mice.

Conclusions

In conclusion, the work reported here supports the development of EGCG as a FASN inhibitor for adenocarcinoma lung cancer treatment. EGCG acts as potent and lipogenic-selective inhibitor of FASN, and do no exhibit adverse effects on body weight, therefore holding promise for further target-directed anti-cancer drug studies either alone or co-administered with other antitumoural drugs.

Additional file

Additional file 1: Figure S1. EGCG and C75 show cytotoxic activity in A549 human lung carcinoma cells. A549 cells were treated with different concentrations of C75 (20 – 200 μM) or EGCG (40 – 300 μM) for 48 hours. Pale gray (●) and dark grey (●) circles represent the percentage of A549 cell proliferation inhibition after C75 and EGCG treatment respectively, which was determined using an MTT assay. Results are expressed as mean percentage of inhibition in cell proliferation from three independent experiments performed in triplicate ± SE. PDF File Format.

Abbreviation

FASN: inhibition in lung cancer.

Competing interests

None of the authors has any potential conflict of interest regarding this work.

Authors' contributions

JR carried out the activity assays, participated in the design of the study, performed the statistical analysis and drafted the manuscript. AB carried out the immunoassays, performed the statistical analysis and drafted the manuscript. GO carried out the immunoassays. SC carried out the *in vivo* assays. TP conceived of the study and drafted the manuscript. TP, DH and PM participated in the design and coordination of the study. All authors have approved the final version of the manuscript.

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Article 3:

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

PLOS ONE

RESEARCH ARTICLE

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

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of FASN and HER2 pathways is effective in cells and in breast cancer models refractory to anti-HER2 therapies.

Introduction

The human epidermal growth factor receptor 2 (HER2) is amplified or overexpressed in ~ 20% of human breast carcinomas and is associated with a more aggressive phenotype and worse prognosis [1].

HER receptors family is composed of four closely related tyrosine kinase (TK) receptors: HER1 (EGFR), HER2, HER3, and HER4. Dimerization of HER receptors, induced by ligand binding or receptor overexpression in the case of HER2, leads to the recruitment of several adaptor proteins that mediate the activation of downstream signaling pathways [2, 3]. Among them, the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB/AKT)/mammalian target of rapamycin (mTOR) protein and the mitogen activated protein kinases (MAPK or ERK1/2) pathways promote cell proliferation, transformation, and survival [4, 5].

HER2-overexpressing tumors are sensitive to monoclonal antibodies (mAb) and small-molecule TK inhibitors (TKI) that interfere with HER2 function and signaling [6–8]. Trastuzumab, a humanized mAb directed against the extracellular domain of the receptor, was the first approved therapy for the treatment of HER2-positive (HER2+) breast cancer. Despite the considerable clinical benefit provided, a large fraction of HER2+ tumors display primary or acquired resistance to trastuzumab [9]. Lapatinib, a small-molecule TKI targeting the intracellular tyrosine kinase domain of EGFR and HER2, was found to improve time to progression in HER2 breast cancer patients who had progressed to tratuzumab [7]. Lapatinib is administered alone or in combination with trastuzumab to abolish the activation of HER2-downstream pathway. But unfortunately, some tumors develop lapatinib resistance and also resistance against the combination of both drugs [10]. The molecular mechanisms leading to trastuzumab and lapatinib resistance has been extensively studied [11]. These include for example *in vivo* conversion of HER2+ to HER2- carcinoma after neoadjuvant trastuzumab [12], predominance of the constitutively active HER2 form ($p95^{HER2}$) [8], overexpression or hyperactivation of other HER family receptors or its ligands [13], amplification of the PI3K/AKT/mTOR pathway by loss of phosphatase and tensin homolog (PTEN) [14], gain-of-function mutation in PI3KCA (encoding the PI3K catalytic isoform p110α) [15] and AKT mutations or amplifications [16].

Fatty acid synthase (FASN) is a homodimeric multienzymatic protein that catalyzes de novo synthesis of long-chain fatty acids [17]. Blocking FASN activity causes *in vitro* and *in vivo* anticancer activity in several overexpressing FASN human carcinomas [18, 19]. The proposed oncogenic properties of FASN seem to be the result of an increased activation of HER2 and its dowstream related PI3K/AKT/mTOR and MAPK signaling pathways [18–20]. FASN can also inhibit the intrinsic pathway of apoptosis [21], may also contribute to modulation of the membrane lipid rafts that anchor HER2 [22] and has been recently proposed as a direct target of p53 family members, including p63 and p73 [23]. In the past, FASN inhibitors with antitumour activity have been limited by either cross-activation of β-oxidation, which produces *in vivo* anorexia and body weight loss [24, 25], or low potency [26, 27]. We have developed new polyphenolic anti-FASN compounds that exhibit *in vitro* and *in vivo* anticancer activity improving the antitumor efficacy and the toxic effects of classical FASN inhibitors, in HER2+ breast cancer cells and mouse models [19, 28, 29]. Among of them, G28UCM has shown a strong

antitumor effect, alone or in combination with anti-HER drugs, in HER2+ breast cancer cells and on breast cancer cells resistant to trastuzumab [29].

In this study, we have investigated the anticancer activity of the classical FASN inhibitor epigallocathequin-3-gallate (EGCG) and G28UCM, as single agents or in combination with pertuzumab and temsirolimus, in our developed trastuzumab (SK**TR**), lapatinib (SK**LR**) and trastuzumab *plus* lapatinib (SK**TLR**) resistant HER2+ breast cancer models. In addition, we analyzed the antitumor activity of EGCG, alone or in combination, in two *in vivo* xenografts: one HER2+ patient and another from a HER2+ patient who fail to respond to trastuzumab and lapatinib therapies.

Materials and Methods

Cell culture and development of long-term resistant breast cancer cells

SKBr3 (SK) breast carcinoma cells were obtained from Eucellbank (University of Barcelona) [30]. SKBr3 cells were routinely grown in McCoy's (Gibco) supplemented with 10% FBS (HyClone Laboratories), 1% L-glutamine, 1% sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco). Trastuzumab-resistant SK cells (SK**TR**) were developed by exposing SK cells continuously to trastuzumab (Herceptin, Hoffmann-La Roche Pharma), starting with 1μM concentration for three months of exposure and increasing the concentration up to 2 μM for a 12 months period, as we previously described [29]. Thus, cells resistant to trastuzumab were maintained in 2 μM trastuzumab, a concentration at which SK parental cells were not viable. To develop lapatinib-resistant cells (SK**LR**), SK cells were treated for one month with an initial dose of 1.5 μM of lapatinib (GW572016; Tykerb, GlaxoSmithKline) and after one month the dose of lapatinib was increased up to 3 μM for 12 months as we described [29], a concentration at which SK parental cells were not viable. To develop lapatinib plus trastuzumab resistant cells (SK**LTR**), SK**LR** were co-cultured with lapatinib 3 μM and trastuzumab 1μM and after one month in culture the dose of trastuzumab was increased up to 2 μM. Cells were co-cultured with lapatinib and trastuzumab for 12 months. SK**LTR** cells were mantained with 3 μM of lapatinib and 2 μM of trastuzumab. Trastuzumab, lapatinib and trastuzumab plus lapatinib resistance was confirmed by dose-response studies using the standard colorimetric MTT assay as we describe in S1 File. Cell line authentication was performed with STR analysis in an external laboratory (Genetica DNA Laboratories) (S2 File). Parental and resistant cells shared 100% STR profile with SKBr3 cell line.

HER2-Fluorescent *in situ* hybridization (FISH)

HER2 FISH pharmDX Kit (Dako) was used to quantify HER2 gene copy number in parental and resistant cells as previously described [29]. The ratio of average HER2 to average CEN17 copy number was calculated for twenty nuclei. Gene amplification was defined when the FISH ratio HER2 signal / CEN17 signal was > 2.

Western blot analysis of tumor and cell lysates

Parental (SK) and resistant (SKTR, SKLR and SKLTR) cells were serum-deprived for 24 hours in 0.5% FBS-medium, then were lysed with ice-cold in lysis buffer (Cell Signaling Technology, Inc.) containing 1 mM EDTA, 150 mM NaCl, 100 μg/mL PMSF, 50 mM Tris-HCl (pH 7.5), protease and phosphatase inhibitor cocktails (Sigma). Equal amounts of protein were heated in LDS Sample Buffer and Sample Reducing Agent (Invitrogen) for 10 min at 70°C, separated on SDS-polyacrylamide gel (SDS-PAGE), and transferred to nitrocellulose membranes. Blots were incubated overnight at 4°C with the following primary antibodies: rabbit polyclonal antibodies

against FASN (Assay Designs; 905-069; dilution 1:1500), HER2/ErbB2/Neu (C-18) (Santa Cruz Biotechnology Inc.; SC-284; dilution 1:1000), EGFR (Cell Signaling Technology Inc.; #2232; dilution 1:200), phospho-EGFR^{Tyr1068} (Cell Signaling Technology Inc.; #2234; dilution 1:200), AKT (Cell Signaling Technology Inc.; #9272; dilution 1:500), p44/42 MAPK (Erk 1/2) (Cell Signaling Technology Inc.; #9102; dilution 1:500), and phospho-mTOR^{Ser2448} (Cell Signaling Technology Inc.; #2971; dilution 1:500); rabbit monoclonal antibodies against HER3/ErbB3 (Cell Signaling Technology Inc.; #4754; dilution 1:200), phospho-HER3/ErbB3^{Tyr1289} (Cell Signaling Technology Inc.; #4791; dilution 1:200), HER4/ErbB4 (Cell Signaling Technology Inc.; #4795; dilution 1:200), phospho-HER4/ErbB4^{Tyr1284} (Cell Signaling Technology Inc.; #4757; dilution 1:200), phospho-AKT^{Ser473} (Cell Signaling Technology Inc.; #4058; dilution 1:200) and mTOR (Cell Signaling Technology Inc.; #2983; dilution 1:500), and mouse monoclonal antibodies against phospho-p44/42 MAPK (Erk 1/2)$^{Thr202/Tyr204}$ (Cell Signaling Technology Inc.; #9106; dilution 1:500) and phospho-c-erbB-2 (HER-2/neu)^{Tyr1248} (Thermo Scientific Inc.; MS-1072-P1; dilution 1:200). Antibodies were diluted in blocking buffer (2.5% powdered-skim milk in phosphate buffered saline solution with 0.05% Tween 20, PBS-T (10 mM Tris-HCL pH 8,0 and 150 mM NaCl). Then, blots were incubated with mouse and rabbit peroxidase-conjugated secondary antibody and revealed using a commercial kit (Super Signal West Pico or Super Signal West Femto chemiluminescent substrate (Thermo Scientific Inc.) or Immobilon Western HRP Substrate (Millipore). Blots were re-proved with a mouse monoclonal antibody against β-actin (Santa Cruz Biotechnology Inc.) as control of protein loading and transfer. Western blot analyses were repeated at least three times and representative results are shown (S3 File).

Genetic analysis of PI3K mutations

DNA was extracted from SK, SK**TR**, SK**LR** and SK**LTR** cells following commercial protocols (QIAamp DNA blood Mini kit, Qiagen). Subsequently, polymerase chain reaction (PCR) was used to amplify the *PI3K* gene (NM_006218) (NCBI-National Center for Biotechnology Information). PCR products were purified using ExoSAP-IT (Isogen Life Science), and the analysis of the exonic and intron-exon regions was performed forward/reverse by direct sequencing (Genetic Analyzer 3130XL, Applied Biosystems).

Quantitative real-time PCR analysis of HER ligands

Parental and resistant cells were washed with PBS and trypsinized. Total-RNA from each sample was isolated using RNeasy mini kit (Qiagen). RNA was reverse-transcribed into complementary DNA (cDNA) using High Capacity cDNA Archive Kit (Applied Biosystems). HER ligands expression (EGF, TGF-α, AR, BTC, EREG, NRG-1 and HB-EGF) was quantified by real-time PCR using a pre-designed, gene-specific TaqMan probe and primer sets (TaqMan Gene Expression assays, Applied Biosystems). Quantitative PCR was performed using TaqMan One-Step Universal Master Mix (Applied Biosystems) and 7300 Real-Time PCR system (Applied Biosystems). All samples were tested in triplicate. Relative quantification of the mRNA level (μg/ml) of HER ligands was carried out. Then, mRNA level was normalized to the housekeeping gene TATA box binding (TBP) protein.

Cell invasion and adhesion assays

Parental and resistant cells were overnight FBS-starved (0.5% FBS-medium) before carrying the CytoSelect 24-well cell invasion assay and the CytoSelect 48-well cell adhesion assay (Cell Biolabs), following the manufacturer's instructions.

Growth inhibition and dose-response studies

Parental and resistant cells were plated out at a density of 5 x 10^3 cells/100 μL/well in 96-well microtitre plates. Following overnight cell adherence fresh medium along with the corresponding concentrations of HER2 inhibitors (trastuzumab and pertuzumab [2C4, Perjeta, Genentech]), FASN inhibitors (EGCG [Sigma] and 1,3-bis((3,4,5-thilhydroxybenzoil)oxy) naphthalene (G28UCM) synthesized as we previously described [19]) or mTOR inhibitor (temsirolimus; CCI-779, Torisel, Pfizer) was added to the cultures. Pertuzumab (5 μg/ml) was combined with trastuzumab (20 μM) or FASN inhibitors (60 μM of EGCG or 5 μM of G28UCM) for 5 days. For temsirolimus drug-combination experiments cells were treated for 2 days with a dose curve concentration of EGCG (5–300 μM) or G28UCM (0.1–15 μM) plus fixed concentrations of temsirolimus (0.05, 0.1, 0.5 and 1 μM). Same treatments were assessed in monotherapy. Following treatment, media was replaced by drug-free medium (100 μL/well) containing MTT (3,4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide, Sigma) solution, and incubation was prolonged for 3 h at 37°C. Formazan crystals formed by metabolically viable cells were dissolved in DMSO (100 μL/well) and absorbance was determined at 570 nm in a multi-well plate reader (Model Anthos Labtec 2010 1.7). Using control OD values (*C*) and test OD values (*T*), % of 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. Combinatorial effects were evaluated using the ratio of % cpi produced by each drug alone *vs* % cpi produced by drug combination (% cpi drug / % cpi combination), average of both ratios was calculated to know the effect of combination compared with both compounds alone. Interactions of G28UCM and EGCG with temsirolimus were also evaluated by the isobologram method as we previously published [29]. Briefly, the concentration of one agent producing a 30% inhibitory effect is plotted on the horizontal axis, and the concentration of another agent producing the same degree of effect is plotted on the vertical axis; a straight line joining these two points represents zero interaction (addition) between two agents. The experimental isoeffect points were the concentrations (expressed relative to the IC_{30} concentrations) of the two agents that when combined kill 30% of the cells. When the experimental isoeffect points felt below that line, combination effect of the 2 drugs was considered to be supra-additive or synergistic, whereas antagonism occurs if the experimental isoeffect points lie above it. Within the designed assay range, a set of isoeffect points was generated because there were multiple FASN inhibitors and anti-target agent concentrations that achieved the same isoeffect. A quantitative index of these interactions was provided by the equation Ix = (A/a) + (B/b), where, for this study, a and b represent the respective concentrations of FASN inhibitors (EGCG or G28UCM) and anti-mTOR agent (temsirolimus) required to produce a fixed level of inhibition (IC_{30}) when administered alone, and A and B represent the concentrations required for the same effect when the drugs were administered in combination, and Ix represents an index of drug interaction (interaction index). Ix values of <1 indicate synergism, a value of 1 represents additivism, and values of >1 indicate antagonism. For all estimations of Ix, we used only isobolos where intercept data for both axes were available.

In vivo studies: human breast tumor PDX (patient-derived xenografts) experiments

Tumor chunks from HER2+ breast cancer patient and HER2+ patient who relapsed after trastuzumab and lapatinib-based treatment were orthotopically implanted into both inguinal cleared mammary fat pads of NOD/SCID (Harlan Laboratories, Inc.) or NSG (NOD/SCID; IL2Rγ−/−) mice (Charles River Laboratories). When tumors reached 30–60 mm^3, animals were randomized into treatment-groups. Each group received a single intraperitoneal (i.p.)

injection (maximum of 0.2 mL) of control (vehicle alone 3d/wk), 30 mg/kg EGCG 3d/wk, 30 mg/kg pertuzumab 1d/w, 10 mg/kg temsirolimus 1d/w, or combination of EGCG + pertuzumab and EGCG + temsirolimus. Tumor xenografts were measured with calipers and tumor volumes were determined using the formula: (π/6 x (v1 x v2 x v2)), where v1 represents the largest tumor diameter, and v2 the smallest one. Body weight was registered 2–3 d/wk. At the end of the experiment, animals were weighed and then euthanized using CO_2 inhalation. Tumors and serum were stored at -80°C. Lung, heart, liver, kidneys and tumor were fixed with formalin (S1 File). Apoptosis in control and treated tumors was analyzed by fluorescent TUNEL assay. Briefly, DNA fragmentation by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nickend labeling (TUNEL) was performed according to the manufacturer's instructions (in situ cell death detection, Roche). Nuclei contrast was performed using fluorescent DAPI staining (4′-6′-Diamidino-2- phenylindole, Sigma). Pictures shown are representative of two samples per each treatment group.

Ethics Statement

Experiments were conducted in accordance with guidelines on animal care and use established by Biomedical Research Institute of Bellvitge (IDIBELL) Institutional Animal Care and Scientific Committee (protocol EST-FOR-070.03). All mice were maintained in a specific pathogen-free AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) International accredited facility in accordance with Spanish and European regulations with controlled light/dark cycle, temperature, and humidity. As approved by the above-mentioned committee, all surgery was performed under inhaled isoflurane anesthesia, burprenorphine was administered as analgesic to mice after surgery, all mice were euthanized with CO_2 asphyxiation at the end of the experiment or when tumors reached 1 cm of diameter, and all efforts were made to minimize suffering.

Statistical analysis

Data were analyzed by Student *t* test when comparing two groups or ANOVA using a Bonferrony test as post-test when comparing more than 2 groups. Statistical significant levels were $p < 0.05$ (denoted as *), $p < 0.01$ (denoted as **) and $p < 0.001$ (denoted as ***). *p-value* is shown in results when significance is reached ($p < 0.05$). All data are means ± standard error (SE). All observations were confirmed by at least three independent experiments.

Results

Characterization of trastuzumab (SKTR), lapatinib (SKLR) and lapatinib plus trastuzumab-resistant (SKLTR) breast cancer cells

As preclinical models of acquired resistance to anti-HER2 drugs, we developed a panel of resistant HER2+ breast cancer cells (SK) with long-term (12 months) and high drug concentration exposure of trastuzumab (SK**TR**), lapatinib (SK**LR**) and lapatinib *plus* trastuzumab (SK**LTR**) (S1 Fig), following Nahta R *et al*. methodology [31]. To elucidate molecular mechanisms regarding acquired resistance in our developed resistant cells (SK**TR**, SK**LR** and SK**LTR**) we first examined HER2 gene amplification by fluorescence *in situ* hybridation. The ratio of the average HER2 gene copy number to the average CEP17 gene copy number in SK was 3.53 and in SK**TR**, SK**LR** and SK**LTR** was 6.32, 3.99 and 4.84, respectively (Fig 1A). These results showed that resistant cells possess HER2 amplification, similar as parental cells.

We next analyzed changes in HER family protein receptors and their downstream proteins related to PI3K/AKT/mTOR and MAPK/ERK1/2 pathways. As shown in Fig 1B, trastuzumab

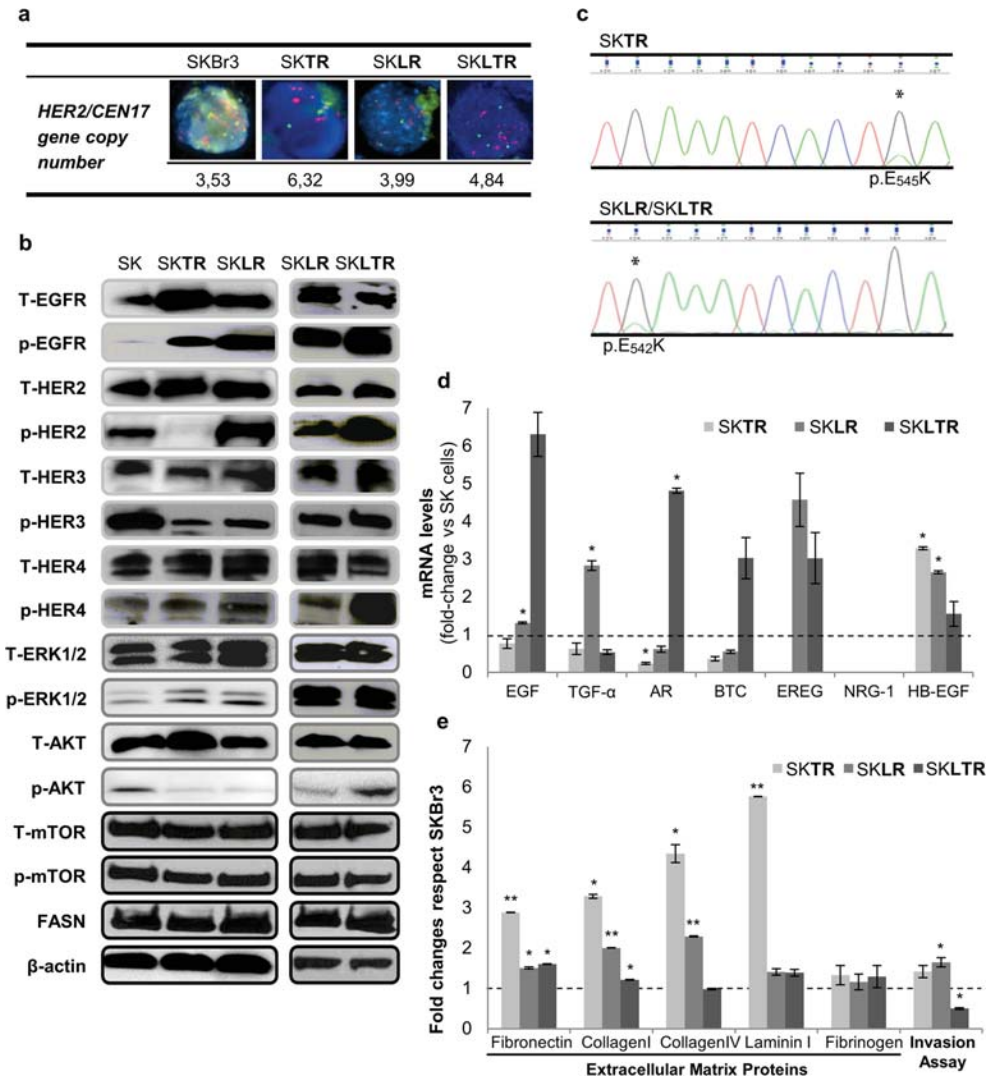

Fig 1. Characterization of parental (SK) and resistant (SKTR, SKLR and SKLTR) cells. (a) HER2 gene copy number is maintained in resistant cells. FISH, fluorescence in situ hybridization; HER2/CEN17 > 2 indicates HER2 gene amplification. **(b)** Resistant cells showed changes in the expression and activation of EGF family receptors but maintained downstream pathways activation (ERK1/2/AKT/mTOR) without affecting FASN protein expression levels. Protein expression and activation of EGFR family receptors pathways was analyzed by Western Blot. Gels shown are representative of those obtained from 3 independent experiments. **(c)** Mutational status of PIK3CA gene in resistant cells. Trastuzumab-resistant cells (SK**TR**) acquire the activating PIK3CA_E545K mutation and lapatinib- and lapatinib plus trastuzumab-resistant cells (SK**LR** and SK**LTR**) acquire the activating PIK3CA_E542K mutation. DNA sequencing of PI3K exon 9 of the resistant cells compared with the parental cells. **(d)** EGFR ligands are increased in resistant cells. Changes in the expression of each ligand by acquisition of resistance were assessed by real-time PCR and values were normalized against the corresponding mRNA expression of TBP constitutive gene. Then, ligands expression of trastuzumab-, lapatinib- and trastuzumab plus lapatinib-resistant cells (SK**TR**, SK**LR**, SK**LTR**) was compared to parental cells (SK). The bars indicate the mean fold change ± SE of two independent quantifications. Bars over the dotted line indicate an increase in the gene expression compared to the control cells, while bars under the dotted line represent impaired gene expression after the

treatment. **(e)** Cellular adhesion and invasion capacity are increased in resistant cells. Fold-changes of resistant cells (SK**TR**, SK**LR** and SK**LTR**) respect to wild type SKBr3 cells (SK) in adhering to extracellular matrix proteins or in invasion capacity. Fold changes were assessed with adhesion or invasion kit assays. Experiments were performed at least twice. * ($p \leq 0.05$) and ** ($p \leq 0.01$) indicate levels of statistically significant difference compared with parental cells.

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resistant cells (SK**TR**) had a significant increase in EGFR and phosphorylated EGFR proteins and to a lesser extent in p-ERK1/2 and AKT, with a noticeably decreased p-HER2 protein and reduced levels of HER3, p-HER3 and p-AKT compared to SK control cells. Cells resistant to lapatinib (SK**LR**) showed a great increase in p-EGFR and p-HER2 proteins and a slightly increase in p-ERK1/2, whereas levels of HER3, p-HER3 and p-AKT were decreased compared to SK control cells. Lapatinib *plus* trastuzumab-resistant cells (SK**LTR**) showed increased expression of phosphorylated forms of HER2, EGFR, HER4 and AKT compared to its control lapatinib-resistant cells (SK**LR**). Interestingly, no significant changes in mTOR and p-mTOR protein levels were observed in any resistant cells compared to SK cells. Regarding FASN, which transcription and translation is mediated by HER2 signaling pathway [29, 32], protein expression levels showed no changes in resistant cells.

Mutation of PI3K is another described mechanism of resistance to anti-HER2 treatments. We found low incidence of activating mutations in the p110α catalytic subunit of PI3K (*PIK3CA)* in all resistant cells. We detected the PIK3CA_E545K mutation in SK**TR** cells and the PIK3CA_E542K mutation in SK**LR** and its derivative SK**LTR** (Fig 1C).

Changes in crosstalk between receptors of HER family prompted us to investigate the mRNA expression profile of several HER activating ligands by real-time PCR in resistant and parental cells (Fig 1D). HB-EGF (heparin-binding EGF-like growth factor) mRNA expression was significantly up-regulated (more than 3-fold; *p-value*: *0.012*) in trastuzumab resistant cells (SK**TR)** compared to SK control cells. In contrast, AR (amphiregulin; *p-value*: *0.024*) was down-regulated, and TGF-α (transforming growth factor-α) and BTC (beta-cellulin) expression was slightly down-regulated in SK**TR** *versus* SK cells. Otherwise, SK**LR** cells up-regulated EGF (epidermal growth factor) (1.5 folds compared with SK; *p-value*: *0.045*), TGF-α (nearly 3 folds; *p-value*: *0.044*), EREG (epiregulin) (4.5 folds) and HB-EGF (more than 2.5 folds; *p-value*: *0.014*), but down-regulated AR and BTC expression in about 0.5 folds, comparing with SK. Double-resistant (SK**LTR**) cells showed a great up-regulation in almost all ligands (EGF in more than 6 folds, AR in almost 5 folds (*p-value*: *0.011)*, BTC and EREG in 3 folds and HB-EGF almost 2 folds), only TGF-α was slightly down-regulated in 0.5 folds, comparing with SK cells.

Since some studies reported that HER2 mediates tumor growth and metastasis [33, 34], and several molecular changes (that could alter tumor aggressiveness) occurred on HER2 and its downstream pathways, we conducted experiments to evaluate such hallmark by measuring cell invasion and adhesion to extracellular matrix (opening metastatic event) capacities of our developed resistant cells (Fig 1E). Moreover, several types of resistance such as some chemotherapeutic drugs and multidrug resistance (combining several natural chemotherapeutic drugs) have been associated with cancer invasion and metastasis [35, 36]. Hence, we wanted to know if mechanisms of resistance in our anti-HER2 resistant models also turned to a more aggressive phenotype of tumor cells. SKTR cells showed a huge significantly increased capacity to adhere to extracellular matrix proteins: fibronectin (2.9 fold-change; *p-value*: *0.002*), collagen I (3.3 fold-change; *p-value*: *0.013*), collagen IV (4.4 fold-change; *p-value*: *0.043*), laminin I (5.8 fold-change; *p-value*: *0.001*) and a slightly increase in fibrinogen adherence (1.3 fold-change) compared with SK parental cells. SK**LR** cells had a remarkable increased adherence to fibronectin (1.5 fold-change; *p-value*: *0.031*), collagen I (2 fold-change; *p-value*: *0.003*) and collagen IV

(2.3 fold-change; *p-value*: *0.005*) and to a lesser extent to laminin I (1.4 fold-change) and fibrinogen (1.1 fold-change) compared with SK cells. SK**LTR** showed a significant increased capacity to adhere to fibronectin (1.6 fold-change; *p-value*: *0.011*) and collagen I (1.2 fold-change; *p-value*: *0.030*), a slight adherence to laminin I (1.4 fold-change) and fibrinogen (1.3 fold-change), whereas adhesion to collagen IV was almost unchanged, compared to SK. Regarding the invasion capacity, both SK**TR** and SK**LR** cells showed relevant increased capacity than SK cells [1.4 and 1.6 (*p-value*: *0.011*) folds more, respectively]. Conversely, SK**LTR** cells showed half invasion capacity (0.5 folds; *p-value*: *0.025*) than SK cells (Fig 1E).

In vitro cell growth interactions between HER2 inhibitors, trastuzumab and pertuzumab, in SK, SKTR, SKLR and SKLTR

SK**TR**, SK**LR** and SK**LTR** cells maintained HER2 protein expression levels similar to parental cells (Fig 1B). Therefore, we first checked the effects of the HER2-dimerization inhibitor, pertuzumab, in comparison with trastuzumab in resistant cells. As shown in Fig 2, 5 μg/ml of pertuzumab and 20 μM of trastuzumab were needed to achieve 50% inhibition of cell proliferation (% cpi) of SK parental cells. Same drug concentrations in SK**TR**, SK**LR** and SK**LTR** showed significant resistance to pertuzumab and trastuzumab, compared with SK cells. Pertuzumab only reached 27.8% (*p-value*: *0.004*), 38.8% (*p-value*: *0.041*) and 19.6% (*p-value*: *0.001*) of cpi in SK**TR**, SK**LR** and SK**LTR**, respectively. Similar inhibitory pattern displayed trastuzumab in SK**TR** (19.9%; *p-value*: *0.000*), SK**LR** (32.1%; *p-value*: *0.022*) and SK**LTR** (15.4%; *p-value*: *0.000*) cells.

Co-treatment of resistant cells with trastuzumab *plus* pertuzumab did not increase cytotoxic effect in SK**TR** (25.7% cpi; *p-value*: *0.008*), neither in SK**LR** (38.3% cpi; *p-value*: *0.023*) cells. But, co-treatments effect in SK and SK**LTR** significantly improved the inhibitory effect up to 63.1% in SK cells (*p-value*: *0.007* compared with trastuzumab), and 52.5% in combined treatments ($p\text{-value}_{Pertuzumab}$: *0.001* compared with pertuzumab and $p\text{-value}_{Trastuzumab}$: *0.000* compared with trastuzumab).

In vitro cell growth interactions between FASN inhibitors and pertuzumab in SK, SKTR, SKLR and SKLTR

Since targeting only HER2 was far from obtaining desired results in resistant models, we decided to explore dual targeting pharmacological strategies. Because FASN showed similar expression levels in parental and resistant cells (Fig 1B), this could be a candidate target to overcome anti-HER2 resistance. Thus, we conducted series of combinatory experiments to evaluate the inhibitory effect of EGCG and G28UCM alone and in combination with pertuzumab in SK**,** SK**TR,** SK**LR** and SK**LTR** cells. The natural anti-FASN compound EGCG had similar cytotoxic effect in parental and resistant cells. IC_{50} values ranged from 206 ± 18.7 μM to 229 ± 29.4 μM in SK, SK**TR**, SK**LR** and SK**LTR** cells. G28UCM, the synthetic derivative of EGCG, improved the cytotoxic effect of EGCG in all cell lines. IC_{50} value of G28UCM in parental and resistant cells ranged from 9 ± 1.5 μM to 19 ± 2.1 μM (S1 Table). In addition and according to our previous results in HER2+ parental cells, EGCG and G28UCM induced apoptosis (cleavage of PARP) in resistant cells (S2 and S3 Figs).

Results regarding pertuzumab combinatory experiments with EGCG and G28UCM are shown in Fig 3A. Pertuzumab (5 μg/ml) combined with anti-FASN compounds (60 μM of EGCG or 5 μM of G28UCM) increased cpi in parental and resistant cells. Ratios of cpi induced for treatments alone *versus* inhibition induced for co- treatment was less than 1 in all combinatory experiments. In SK cells, ratio of mono-treatments/combination was 0.34. In SK, SK**LR** and SK**TR**, ratio of mono-treatments/combination was 0.34, 0.82 and 0.62, respectively. In

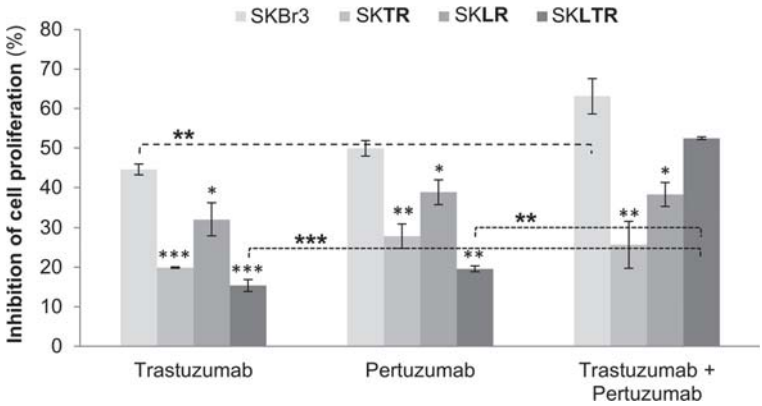

Fig 2. Pertuzumab plus trastuzumab combination improves effects in SK and SKLTR. Cells were treated with trastuzumab (20 μM), pertuzumab (5 μg/ml) and the combination of both for 5 days. Results were determined using an MTT assay and are expressed as the percentage of cell proliferation inhibition from three independent experiments performed in triplicate. Columns represent % of cell proliferation inhibition after trastuzumab or pertuzumab exposure and bars SE. * ($p \leq 0.05$), ** ($p \leq 0.01$) and *** ($p \leq 0.001$) indicate levels of statistically significant difference compared with effect of the same drug in SKBr3 cells or compared with drugs administered alone (dashed line).

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SK**LTR**, monotreatments/combination ratio was 0.31. In this case, pertuzumab *plus* EGCG combination significantly improved effects of each treatment alone (which is graphed as 1 in Fig 3; $p\text{-value}_{\,compared\ with\ 1}$: *0.036*). G28UCM *plus* pertuzumab slightly improved EGCG *plus* pertuzumab inhibitory effects in SK**TR** cells (0.69 when combined with G28UCM compared with 0.82 in EGCG case) (Fig 3A).

Together, these data show that the co-exposure of the FASN inhibitors, EGCG and G28UCM, with pertuzumab in parental and resistant HER2+ breast cancer cells is more active than either of the drugs used as a single agent.

In vitro cell growth interactions between FASN inhibitors and temsirolimus in SK, SKTR, SKLR and SKLTR

In our resistant cells we showed changes in EGF family receptors expression and activation without changes in mTOR expression and activation, neither in FASN expression. Since resistant cells express similar levels of mTOR and FASN as same as parental cells, we tested the apoptotic (PARP cleavage) effect of inhibiting mTOR (temsirolimus) and FASN (EGCG) in combination with trastuzumab and/or lapatinib. Temsirolimus or EGCG did not recovered trastuzumab and/or lapatinib sensitivity in resistant cells (S3 Fig). Therefore we conducted experiments to evaluate the inhibitory effect of temsirolimus (mTOR inhibitor) alone and in combination with anti-FASN compounds (EGCG and G28UCM) in the developed resistant HER2+ models (SK**TR,** SK**LR** and SK**LTR**).

Temsirolimus alone displayed a potent anti-proliferative effect in parental and resistant cells. IC_{50} concentration ranged from 9 ± 0.9 μM to 11 ± 0.4 μM in resistant models (S1 Table). Temsirolimus (0.05, 0.1, 0.5 and 1 μM) combined with anti-FASN compounds (60 μM of EGCG or 5 μM of G28UCM) for 2 days increased cpi in parental and resistant cells. Mean ratios of cpi induced for each treatment alone *versus* inhibition induced for co-treatment is shown in Fig 3B EGCG plus temsirolimus ratio was similar in parental and resistant cells

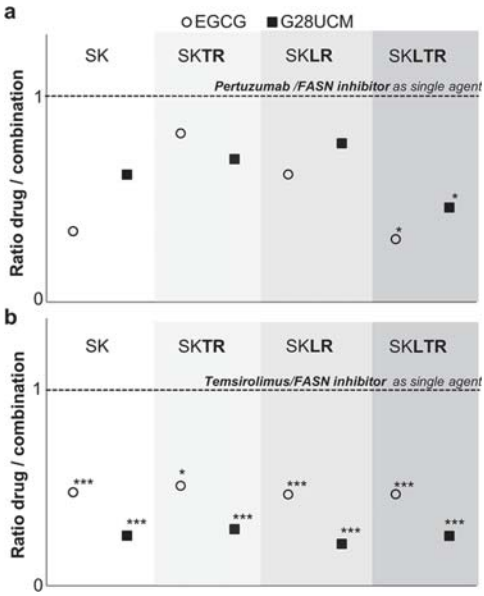

Fig 3. FASN inhibitors improve pertuzumab and temsirolimus activity in parental and resistant cells. **(a)** Cells were treated with pertuzumab (5 μg/ml) combined with EGCG (60 μM) or G28UCM (5 μM) for 5 days. Results were determined using an MTT assay and are expressed as ratio of inhibition of cell proliferation induced for each treatment alone versus inhibition induced for co- treatment from three independent experiments performed in triplicate. Dashed lines represent the effect of each drug alone, ratio 1. **(b)** Cells were treated with temsirolimus (0.05, 0.1, 0.5 and 1 μM) combined with EGCG (60 μM) or G28UCM (5 μM) for 2 days. Results were determined using an MTT assay and are expressed as ratio of inhibition of cell proliferation induced for each treatment alone versus inhibition induced for co- treatment from three independent experiments performed in triplicate and with several temsirolimus concentrations. Dashed lines represent the effect of each drug alone, ratio 1. * ($p \leq 0.05$), ** ($p \leq 0.01$) and *** ($p \leq 0.001$) indicate levels of statistically significant difference compared with drugs administered alone.

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(from 0.48 to 0.52 and all significantly different from mono-treatments effect; graphed as 1). When temsirolimus was combined with G28UCM, combinatorial effect was almost doubled (ratios were from 0.22 to 0.30; all *p-values* < 0.000).

These results were confirmed by the isobologram method, using a series of isobologram transformations of multiple dose-response curves at an effect level of 30% (IC_{30}), an statistical analysis that we have used previously [29]. Simultaneous treatment of SK, SK**TR**, SK**LR** and SK**LTR** cells with EGCG and temsirolimus resulted in a strong synergistic interaction index ($0.84 < Ix < 0.94$). Combination of G28UCM *plus* temsirolimus had an enhanced synergistic interaction index in parental and resistant cells ($0.36 < Ix < 0.58$), shown in S1 Table.

Effects of EGCG plus temsirolimus compared with mono-treatments and other combinations (EGCG plus pertuzumab and pertuzumab plus trastuzumab and/or lapatinib) on FASN and mTOR expression were also assessed by western blot analysis (see S4 Fig). No significant inhibition in FASN and mTOR protein levels was seen in any treatment except in the case of EGCG plus temsirolimus combination. EGCG plus temsirolimus completely abolished mTOR expression in SK**TR**, SK**LR** and SK**LTR** resistant cells.

These data show that co-exposure of temsirolimus with FASN inhibitors, EGCG and G28UCM, display a more potent synergistic effect in HER2+ parental and resistant cells than either of the drugs used as a single treatment.

Antitumor activity of EGCG in combination with pertuzumab and temsirolimus in HER2+ sensitive and resistant patient derived xenografts

To better recapitulate the clinical setting we extent our findings *in vivo* evaluating the antitumor activity of pertuzumab, temsirolimus and EGCG and the combination in a HER2+ PDX model (HER2-PDX) and in a trastuzumab *plus* lapatinib-resistant HER2+ PDX (HER2-PDX**R**) model. Both PDX models showed similar HER2, mTOR and FASN expression levels as the *in vitro* parental and resistant cellular models (S5 Fig). EGCG (30 mg/kg for 3d/w) and pertuzumab (30 mg/kg/once weekly), as single agents, reduced tumor growth in the HER2-PDX model after 24 days of treatment. Control animals achieved a median tumor growth of 461.0 ± 65.6 mm^3 whereas EGCG significantly reduced tumor growth to 247.6 ± 45.0 mm^3 (*p-value*: *0.017)*, and pertuzumab reduced to 301.0 ± 62.9 mm^3. Interestingly, superior (and more rapid) tumor regression was achieved by dual FASN and HER2 blockade (87.2 ± 38.2 mm^3; $p\text{-}value_{vsEGCG}$: *0.017 and* $p\text{-}value_{vsPertu}$: *0.010*), compared with EGCG or pertuzumab as single agents (Fig 4A, *left panel*). Despite the absence of complete tumor shrinkage, combinatorial treatment significantly reduced tumor growth in the HER2-PDX model. Under the same schedule, in the HER2-PDX**R** model EGCG (30 mg/kg for 3d/w) and pertuzumab (30 mg/kg/once weekly) also reduced tumor growth after 24 days of treatment (Fig 4A, *right panel*), but *in vivo* efficacy of the dual FASN and HER2 blockade was also superior (and more rapid) compared with EGCG and pertuzumab as a single agents. Compared with the control group (393.9 ± 95.5 mm^3), EGCG and pertuzumab decreased tumor growth to 285.9 ± 36.5 mm^3 and 310.4 ± 34.5 mm^3, respectively. The combination of EGCG with pertuzumab significantly reduced tumor growth up to 177.64 ± 34.5 mm^3 ($p\text{-}value_{vsEGCG}$: *0.030 and* $p\text{-}value_{vsPertu}$: *0.008)*.

Regarding mTOR and FASN inhibition *in vivo*, EGCG (30 mg/kg for 3d/w) reduced tumor growth in the HER2-PDX model after 21 days of treatment (Fig 5A, *left panel*). Control animals achieved a median tumor growth of 386.4 ± 66.7 mm^3 whereas EGCG median tumor growth was significantly reduced to 183.3 ± 15.1 mm^3 (*p-value*: *0.017*). Despite the strong antitumor activity exhibited by temsirolimus when used as a single agent (18.0 ± 15.1 mm^3; *p-value*: *0.000*), its activity was little enhanced (day 21) by the addition of EGCG. Combination of temsirolimus with EGCG not only reduced tumor ratio of growth, but also achieved tumor shrinkage compared with the initial tumor volume (-8.2 ± 6.0 mm^3). In the trastuzumab *plus* lapatinib-resistant HER2-PDX model EGCG treatment decreased the median tumor growth (231.8 ± 38.4 mm^3) compared with control group (314.8 ± 81.1 mm^3) at the end of the experiment (Fig 5A, *left panel*). In the HER2-PDX**R** model, temsirolimus significantly decreased tumor growth when used as a single agent (114.3 ± 27.1 mm^3; *p-value*: *0.045*), and its activity was enhanced by the addition of EGCG (94.9 ± 33.1 mm^3). These results show that temsirolimus could have a relevant effect in patients with HER2 breast cancer, even those that have progressed to anti-HER2 therapies, and combination with FASN-inhibitors could even assist temsirolimus to achieve tumor depletion.

Tumor samples from HER2-PDX and HER2-PDX**R** treated tumors showed an increased apoptosis compared with HER2-PDX and HER2-PDX**R** control tumors, assessed by fluorescent TUNEL assay (Figs 4B and 5B). EGCG, pertuzumab and temsirolimus used as single agents induced apoptosis in HER2-PDX (133±14 TUNEL+ cells/mm^2, 122±16 TUNEL+ cells/mm^2 and 333±19 TUNEL+ cells/mm^2, respectively) and HER2-PDX**R** (337±19 TUNEL+ cells/mm^2,

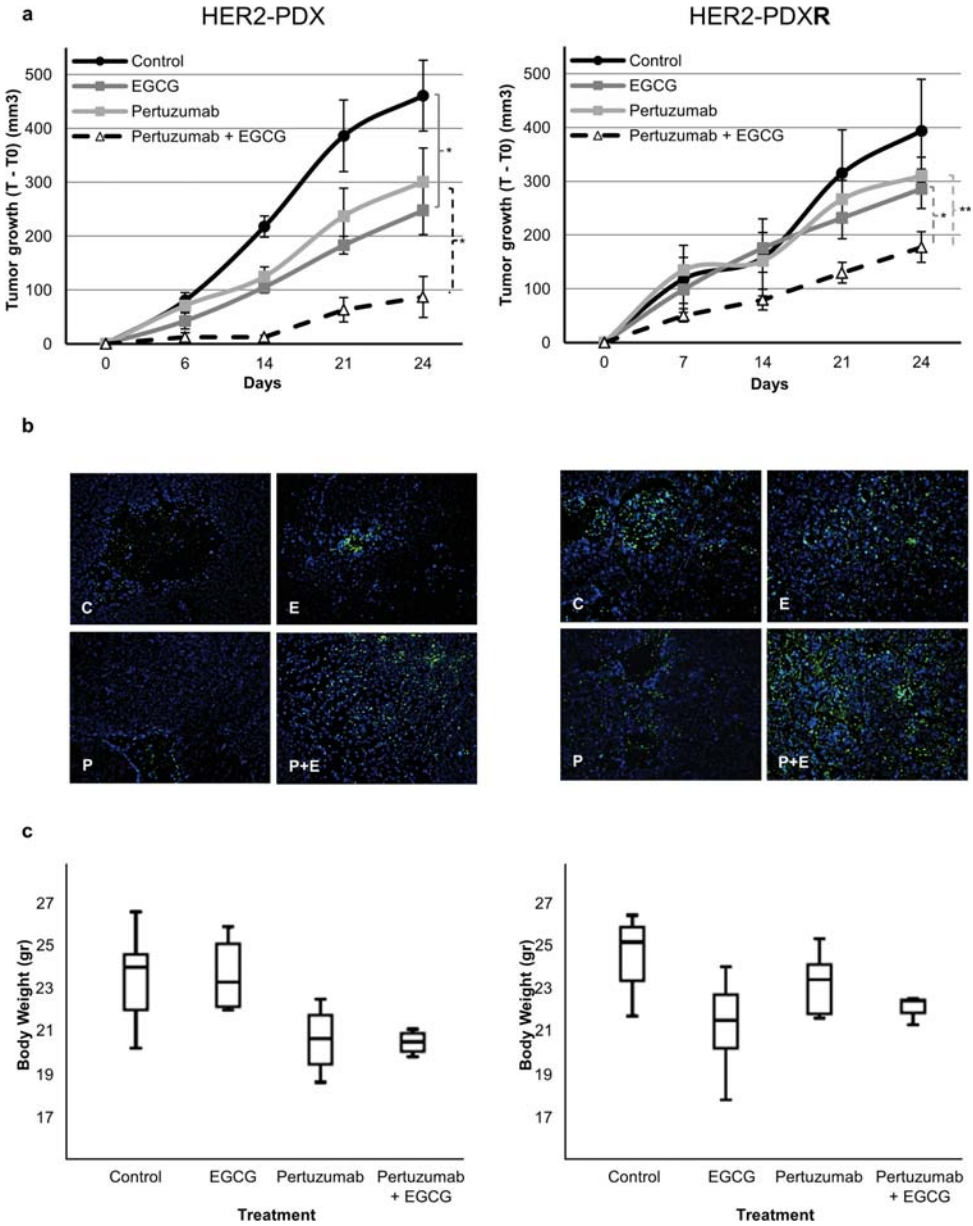

Fig 4. EGCG, alone or combined with pertuzumab, inhibits tumor growth of sensitive and resistant HER2+ orthoxenopatients. (a) Mice bearing HER2-PDX and resistant HER2-PDX (HER2-PDXR) were treated with control (C), EGCG (30 mg/kg, 3 days a week), pertuzumab (30 mg/kg, 1 day a week) or the combination (EGCG plus pertuzumab) for 24 days. Dots are mean of each experimental group and bars, SE. * ($p \leq 0.05$), ** ($p \leq 0.01$) and ***

($p \leq 0.001$). **(b)** Apoptosis, by TUNEL fluorescent assay, was perfomed in control (C), EGCG (E), pertuzumab (P) and combination (P+E) treated as in (A) tumors. Tumors were collected at the end of the experiment and fixed in paraffin. Pictures are representative of two samples of each group. **(c)** Body weight of the mice treated as in (A). Data are expressed as body weight at the end of the experiment and boxes show the 25th to 75th percentiles, whereas whiskers extend to the 5th and 95th percentiles.

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352±18 TUNEL+ cells/mm^2 and 803±36 TUNEL+ cells/mm^2, respectively) tumors compared with apoptosis showed by untreated HER2-PDX (66±8 TUNEL+ cells/mm^2) and HER-PDX**R** (287±23 TUNEL+ cells/mm^2) tumors. Combinatory treatments (pertuzumab *plus* EGCG and temsirolimus *plus* EGCG) increased the apoptosis in HER2-PDX (933±40 TUNEL+ cells/mm^2 and 1265±51 TUNEL+ cells/mm^2, respectively) and HER2-PDX**R** (866±40 TUNEL+ cells/mm^2 and 1197±55 TUNEL+ cells/mm^2, respectively) tumors compared with each single treatment alone.

Previous first-generation of FASN inhibitors such as C75 and cerulenin have been limited by inducing severe body weight loss, which is thought to be related to a parallel stimulation of fatty acid oxidation by these inhibitors [24, 37]. But we have previously reported that animals treated with EGCG didn't display neither change on body weight nor on hepatic, renal and haematological function serum markers compared to control animals [19]. In this study, animals treated with EGCG and also with pertuzumab and temsirolimus (alone or in combination) were weighed daily to evaluate *in vivo* body weight effect. With respect to control animals, we identified no significant changes on food and fluid intake or body weight after treatment with EGCG, pertuzumab and temsirolimus alone or in combination (Figs 4C and 5C).

Histological studies (Hematoxylin-Eosin) of liver, lung, kidney and heart showed no tissue structural abnormalities between control and treated animals in both HER2-PDX models (S6, S7 and S8 Figs).

Discussion

Despite the remarkable success of anti-HER2 therapies, patients with advanced HER2-positive breast cancer frequently display primary resistance and, in patients initially sensitive to these agents, acquired resistance may emerge over time [9, 10]. To date, even several mechanisms of resistance to anti-HER2 agents are known, this clinical problem is not fully understood. Here, we have developed and characterized stable cell lines derived from the HER2-positive SKBr3 cells that are resistant to either trastuzumab (SK**TR**), lapatinib (SK**LR**) or both (SK**LTR**). Some molecular mechanisms of resistance in our developed anti-HER2 resistant models are consistent with the previously described [8–16]. One of the commonly described mechanisms of anti-HER2 therapies is the overexpression of other RTKs or their ligands. Thus, it has been reported that HER3 overexpression leads to HER2/HER3 heterodimer formation consequently activating the PI3K/AKT/mTOR pathway [6]. Conversely, our trastuzumab, lapatinib and trastuzumab *plus* lapatinib resistant models show a decrease in HER3 expression and activation, whereas an overexpression of EGFR and increased expression levels of EGFR (EGF and TGF-α) and EGFR-HER4 (EREG and HB-EGF) ligands. This is consistent with several reports that show EGFR overexpression (and its ligands) in trastuzumab resistant SKBr3 cells and in xenograft models of acquired trastuzumab and lapatinib resistance [38–40]. Even more, after dual trastuzumab and lapatinib long-term exposure, our patented SK**LTR** [41] cells overexpressed HER4 besides EGFR, and increased the expression of EGFR (EGF and AR) and EGFR-HER4 (BTC, EREG and HB-EGF) ligands to overcome the anticancer effects of both anti-HER2 agents. In a clinical study, constitutive presence of HER4 has been directly related with sensitivity to anti-HER2 drugs in breast cancer [42] whereas in prostate cancer an increase of HER4 expression has been shown to be responsible of resistance to the EGFR inhibitor erlotinib [43].

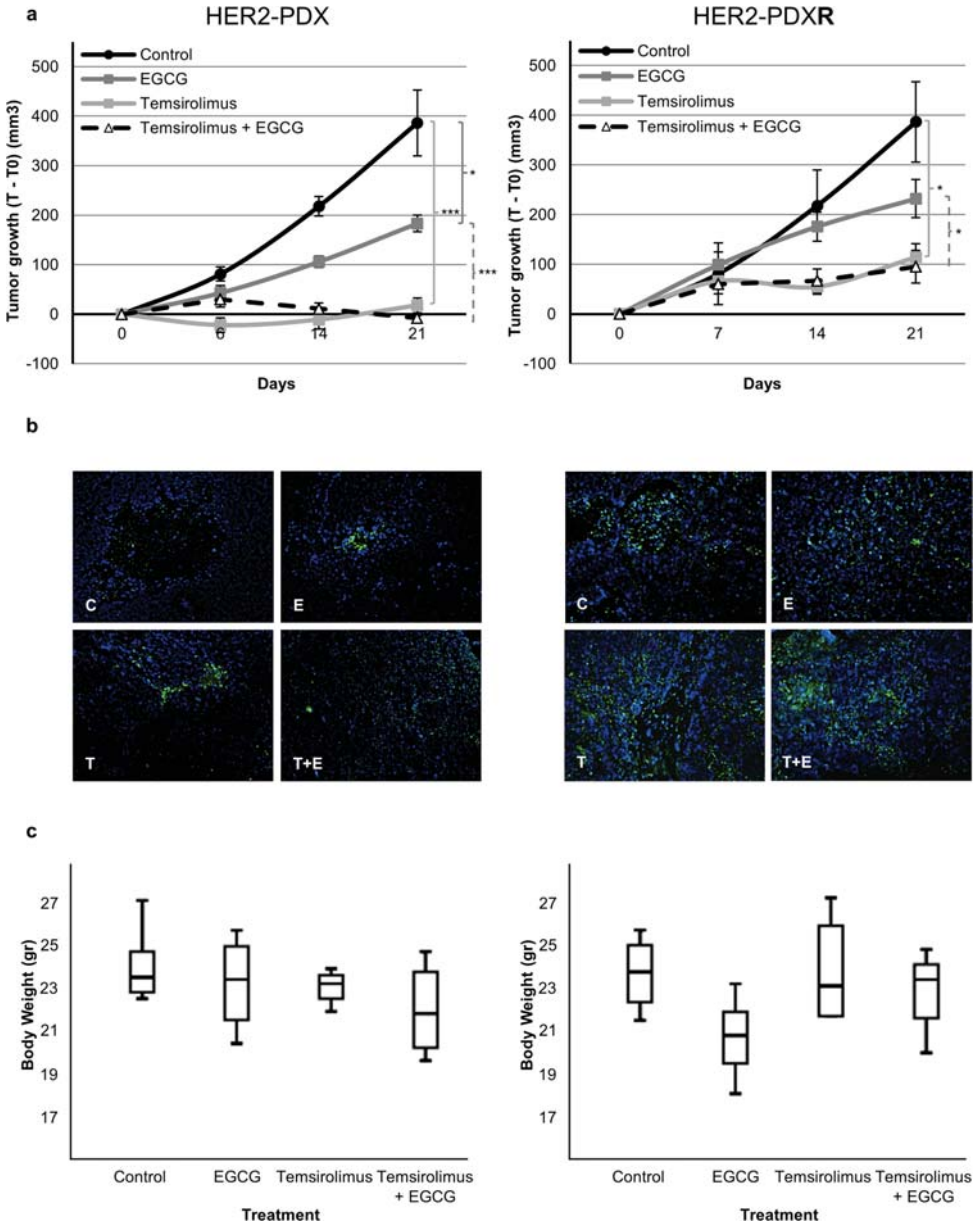

Fig 5. EGCG, alone or combined with temsirolimus, inhibits tumor growth of sensitive and resistant HER2+ orthoxenopatients. (a) Mice bearing HER2-PDX and resistant HER2-PDX (HER2-PDXR) were treated with control (C), EGCG (30 mg/kg, 3 days a week), temsirolimus (10 mg/kg, 1 day a week) or the combination (EGCG plus temsirolimus) for 21 days. Dots are mean of each experimental group and bars, SE. * ($p \leq 0.05$), ** ($p \leq 0.01$) and

*** ($p \leq 0.001$). **(b)** Apoptosis, by TUNEL fluorescent assay, was perfomed in control (C), EGCG (E), temsirolimus (T) and combination (T+E) treated as in (A) tumors. Tumors were collected at the end of the experiment and fixed in formalin. Pictures are representative of two samples of each group. **(c)** Body weight of the mice treated as in (A). Data are expressed as body weight at the end of the experiment and boxes show the 25th to 75th percentiles, whereas whiskers extend to the 5th and 95th percentiles.

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Our findings highlight that HER4 overexpression and activation could be a new molecular mechanism of resistance to anti-HER2 therapies.

Changes in HER2 downstream proteins (such as loss of PTEN, PI3K mutations/hyperactivation, AKT overexpression and hyperactivation) have also been identified as resistant mechanisms to trastuzumab and lapatinib therapies [14–16]. In patients treated with trastuzumab, activating mutations of PI3K (*PI3KCA*) were associated with poor response and survival [44]. Eichhom PJ *et al* also reported *PI3KCA* mutations as responsible of lapatinib resistance [15]. In this study, we have shown that although *PI3KCA* mutations (PIK3CA_E545K mutation in SK**TR** cells and PIK3CA_E542K mutation in SK**LR** and SKLTR cells) have not enough incidence to show effects in AKT activation, they collaborate with RTKs changes and downstream loops in order to maintain PI3K/AKT/ mTOR pathway activation in trastuzumab, lapatinib and also in a trastuzumab *plus* lapatinib resistant cells. Lapatinib effects have been described to be mediated preferentially through the MAPK/ERK pathway through Ras overexpression or mutation [45]. Accordingly, in our study ERK1/2 overactivation is also shown to be another downstream change that leads to cell proliferation signaling of the resistant cells. No significant changes in mTOR and p-mTOR proteins were observed in our long-term resistant cells. Although the main pathway related to mTOR is the PI3K/AKT axis, mTOR is a downstream in which several signaling pathways converge. In addition, this pathways act as a complex network, and activation of one important effector can be accomplished by several emissaries. Even with decreased activation of AKT in our resistant models, maintenance of mTOR activation can be accomplished by direct signaling of PI3K to mTOR, bypassing AKT [46]. Also, it is described that MAPK inhibit the tuberous sclerosis complex (TSC1/TSC2), which in turn inhibits mTOR activation [47].

Together, overactivation of HER2 in SK**LR** and SK**LTR** cells and maintenance of mTOR, p-mTOR and FASN expression in all the resistant models provided the rationale to test combined FASN and HER2 or mTOR pathways blockade in this setting. We found that the simultaneous treatment of parental and resistant (SK**TR**, SK**LR** and SK**LTR**) cells with anti-FASN compounds (EGCG and the novel derivative G28UCM) *plus* pertuzumab improved the effects of each drug alone in SK**LTR**. But, inhibiting mTOR, the downstream target of HER2 pathway in combination with FASN inhibition resulted in a strong synergistic interaction in all parental and resistant cells. G28UCM, the novel FASN inhibitor, also improved combinatorial effect of EGCG, producing much more synergism between temsirolimus and G28UCM. We had previously shown that G28UCM improved EGCG effects, alone and in several combinatorial regiments with anti-HER2 drugs and chemotherapy, in parental and trastuzumab- or lapatinib-resistant AU565 HER2 breast cancer cells [29]. Several studies have used mTOR inhibition to overcome resistance to HER2-targeted therapies [48] and it has already been assayed in women with trastuzumab-resistance [49]. It has also been shown the synergism between mTOR and FASN inhibition to induce cytotoxicity in ER/HER2-positive breast cancer cell lines [50]. These *in vitro* results support the rationale to test *in vivo* the antitumor efficacy of these agents in a combination regimen in tumors resistant to anti-HER2 therapies.

In previous preclinical studies conducted in nude mice bearing HER2 cells, we and others showed that EGCG displayed *in vivo* antitumor activity without decreasing food intake and induction of weight loss [24, 37]. This is the first study that attempt to evaluate the *in vivo*

efficacy and feasibility of dual blockade of FASN and the HER2 signaling pathway in HER2-positive patient tumor samples (HER2-PDX) and in HER2 samples of a patient who relapsed after trastuzumab and lapatinib therapies (HER2-PDX**R**). Here, we report the validity of this approach clearly showing that the combination of EGCG with pertuzumab and temsirolimus resulted in synergistic reduction of HER2-PDX and HER2-PDX**R** tumors, without signs of toxicity (weight loss) related to *in vivo* antitumor efficacy experiments using anti-FASN compounds [24, 28, 29]. Reduction of tumor growth could be accomplished, in part, by an apoptotic event. Increase of apoptosis has been seen in parental and resistant tumor samples treated with EGCG, pertuzumab and temsirolimus. As synergism in tumor reduction after combinatorial treatments, apoptosis has also been synergistically increased when combining EGCG with pertuzumab or temsirolimus. These similar profiles in tumor growth reduction and apoptosis suggest that apoptosis is responsible for tumor growth inhibition. In fact, we had previously shown that EGCG produces apoptosis *in vitro* and *in vivo* [18, 19, 28, 37]. Apoptosis is also consistent with other studies of pertuzumab in cells and mouse models [51, 52]. Temsirolimus has been shown to produce apoptosis in a resistant oropharyngeal carcinoma cell line [53], colorectal cancer cells [54] and other cancers. But little, if any, apoptosis has been seen in different breast cancer cell lines treated with temsirolimus [55]. In this study, we demonstrate that tumor growth inhibition in HER2 breast cancer PDX (non-resistant and resistant) occurs by apoptotic event in tumoral cells, and this is consistent what have been found in other types of cancer. These findings, accordingly with those obtained *in vitro*, encourages us to think that combining FASN inhibitors with temsirolimus or pertuzumab could be one example of a potential combinatorial available in the clinical management of HER2-positive breast cancer patients who progressed to standard treatments.

In this study, we have developed novel mono- and dual- long term trastuzumab *plus* lapatinib resistant breast cancer models to find out new pharmacological strategies to overcome this setting. Then, we have showed *in vitro* and *in vivo* that the inhibition of FASN, alone or in combination with anti-HER2 signaling drugs (temsirolimus and pertuzumab), could have relevant clinical implications in patients who fail to respond to current therapies.

In summary, our findings provide a rationale for the preclinical development of inhibitors of FASN activity in combination with anti-HER2 signaling agents in breast cancer refractory to anti-HER2 therapies.

Supporting Information

S1 File. Additional Materials and Methods. Checking resistance of the developed cells and histological analysis of mice organs.
(DOCX)

S2 File. STR analysis of parental and resistant cells.
(DOCX)

S3 File. Full length Western Blots.
(DOCX)

S1 Table. Synergy analysis between FASN inhibitors and temsirolimus in parental and resistant cells. Drug cytotoxicity was calculated as the concentration of drug needed to produce 50% of cell death (IC_{50}) when parental SKBr3 (SK) or trastuzumab-, lapatinib- and trastuzumab *plus* lapatinib-resistant cells (SK**TR**, SK**LR** and SK**LTR**). Values represent the mean ± SE from at least three independent experiments performed in triplicate. The interaction index (Ix) for temsirolimus plus FASN inhibitors effect was calculated using isobologram analysis. The Ix parameter indicate whether the doses of the two drugs required to produce a

given degree of cytotoxicity are greater than (Ix > 1 or antagonism) equal to (Ix = 1 or additivism) or less than (Ix < 1 or synergism) the doses that would be required if the effect of two agents were strictly synergic. Ix mean values ± SE for the two drug treatment were obtained from triplicate studies with different combination treatments and performed at least twice independently. * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) indicate the level of statistical significance of the Ix compared with an Ix of 1.0.
(DOCX)

S1 Fig. Checking resistance of the developed resistant cells. (a) SKBr3 (SK) parental (o) and trastuzumab-resistant SKBr3 (SK**TR**, ●) cells where both treated with increasing concentrations of trastuzumab (1–30 μM) for 5 days. **(b)** SKBr3 (SK) parental (with o) and lapatinib-resistant SKBr3 (SK**LR**, ●) cells where both treated with increasing concentrations of lapatinib (2–30 μM) for 2 days. **c,** Lapatinib-resistant cells (SK**LR**, ●) and trastuzumab plus lapatinib-resistant SKBr3 (SK**LTR**, ●) cells where both treated with 3 μM lapatinib plus increasing concentrations of trastuzumab (1–30 μM) for 5 days. Results are expressed as percentage of surviving cells after drug treatment (mean ± SE), which was determined using an MTT assay. Experiments were performed at least twice in triplicate. * ($p < 0.05$) and ** ($p < 0.01$) indicate statistical difference compared with parental cells.
(DOCX)

S2 Fig. G28UCM induces apoptosis in parental and resistant cells without affecting FASN expression. Apoptosis and induction of caspase activity were assessed as cleavage of PARP. SKBr3 (SK) parental, trastuzumab-resistant SKBr3 (SK**TR**), lapatinib-resistant SKBr3 (SK**LR**) and lapatinib plus trastuzumab-resistant SKBr3 (SK**LTR**) cells were treated with G28UCM (28 μM) for 24 hours. Control cells were cultured under the same conditions, without treatment for 24 hours. Equal amounts of lysates were immunoblotted with anti-PARP antibody which identified the 116 KDa (intact PARP) and the 89 KDa (cleavage product) bands. Same lysates were also immunobloted with FASN antibody to check G28UCM effect on expression of FASN. Blots were reproved for β-actin as loading control.
(DOCX)

S3 Fig. EGCG and temsirolimus improve trastuzumab, lapatinib and trastuzumab plus lapatinib treatment in parental and resistant cells. Apoptosis and induction of caspase activity were assessed as cleavage of PARP. a) SKBr3 (SK) parental cells and b) trastuzumab-resistant SKBr3 (SK**TR**), lapatinib-resistant SKBr3 (SK**LR**) and lapatinib plus trastuzumab-resistant SKBr3 (SK**LTR**) cells were treated with trastuzumab (T; 2 μM), lapatinb (L; 3 μM), EGCG (250 μM) and temsirolimus (Temsi; 12 μM) for 12 and 24 hours. Control cells were cultured under the same conditions, without treatment for 12 or 24 hours. Equal amounts of lysates were immunoblotted with anti-PARP antibody. Blots were reproved for β-actin as loading control.
(DOCX)

S4 Fig. Effect of EGCG with pertuzumab and temsirolimus combinations in parental and resistant cells. a) SKBr3 (SK) parental cells and **b)** trastuzumab-resistant SKBr3 (SK**TR**), **c)** lapatinib-resistant SKBr3 (SK**LR**) and **d)** lapatinib plus trastuzumab-resistant SKBr3 (SK**LTR**) cells were treated with trastuzumab (T; 2 μM), lapatinb (L; 3 μM), EGCG (250 μM), pertuzumab (5 μg/ml) and temsirolimus (Temsi; 12 μM) for 12 and 24 hours. Control cells were cultured under the same conditions, without treatment for 12 or 24 hours. Equal amounts of lysates were immunoblotted with anti-FASN and anti-mTOR antibodies. Blots were reproved for β-actin as loading control.
(DOCX)

S5 Fig. HER2 PDX-tumors characterization. SKBr3 (SK) parental cells and tumors from HER2-PDX and HER2-PDX**R** were lysed and equal amounts of lysates were immunoblotted with anti-HER2, anti-FASN and anti-mTOR antibodies.
(DOCX)

S6 Fig. EGCG, alone or combined with pertuzumab, does not induce liver and heart toxicity in xenografts. Histological analysis studies (Hematoxylin-Eosin) of liver and heart showed no tissue structural abnormalities between control and treated animals in both non-resistant and resistant HER2-PDX models. At least 2 mice per group were analyzed and image shown is representative of each group.
(DOCX)

S7 Fig. EGCG, alone or combined with temsirolimus, does not induce liver and heart toxicity in xenografts. Histological analysis studies (Hematoxylin-Eosin) of liver and heart showed no tissue structural abnormalities between control and treated animals in both non-resistant and resistant HER2-PDX models. At least 2 mice per group were analyzed and image shown is representative of each group.
(DOCX)

S8 Fig. EGCG, alone or combined with pertuzumab and temsirolimus, does not induce kidney and lung toxicity in xenografts. Histological analysis studies (Hematoxylin-Eosin) of kidney and lung showed no tissue structural abnormalities between control and treated animals in both non-resistant and resistant HER2-PDX models. At least 2 mice per group were analysed and image shown is representative of each group.
(DOCX)

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Author Contributions

Conceived and designed the experiments: TP. Performed the experiments: AB AG-P SP GO CT AM OC DC-S MP JG-M. Analyzed the data: AB TP OC EGS RB AM. Contributed reagents/materials/analysis tools: TP EGS RB AM. Wrote the paper: AB TP EGS RB.

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DISCUSSION

This section is intended as a global discussion of the manuscripts referred to in the present doctoral thesis. We have studied the role of FASN expression and inhibition in breast and lung human carcinomas. First, we aimed forward to study whether expression levels of FASN in early-stage breast cancer patients could be related to clinicopathological characteristics of prognostic relevance (cross-sectional study, *article 1*). Then, we focused in studying the preclinical inhibition of FASN in adenocarcinoma of lung cancer as a FASN and HER1-positive model (*article 2*). Finally, based on the previous background of our research group (and others) studying the linkage between FASN and HER2, we studied the importance of blocking FASN (alone or in combination) in HER2-positive breast cancer preclinical models resistant to current anti-HER2 (*article 3*). FASN inhibition in lung and breast preclinical cancer models are discussed together in order to provide a global scenario to analyze the effects of FASN inhibition in different types of cancer overexpressing HER family receptors.

FASN

For the high duplication rate and increased metabolism, tumor cells have a prevailing requirement of structural and metabolic elements that give them enough suppliers for new membrane construction, energy production and metabolic pathways processors. *De novo* lipogenesis or *de novo* fatty acid synthesis is the mechanism by which tumor cells can obtain enough quantity of fatty acids, which are one of the resources needed for the assembly of new cells. *De novo* fatty acid synthesis is the metabolic pathway that synthesizes fatty acids from the excess of carbohydrates. These fatty acids can then be incorporated into triglycerides for energy storage and membrane precursors. In normal conditions *de novo* fatty acid synthesis mainly takes place in liver and adipose tissue and it is considered to be a

minor contributor in the maintenance of serum triglycerides homeostasis[85]. The conversion from glucose to fatty acids, which is modulated by the lipogenic pathway, includes a coordinated series of enzymatic reactions (*see figure 3 in general introduction*). Fatty acid synthase (FASN) is the key rate-limiting enzyme that performs the conversion of malonyl-CoA into palmitate (as the main product) (*see figure 4 in general introduction*). After that, a series of reactions turn palmitate into elaborated fatty acids. Deregulations in the lipogenic pathway are observed in certain pathological or physiological conditions. In addition to that the non-lipogenic tissues (where *de novo* fatty acid synthesis is suppressed under normal conditions, since most of the fatty acids and triglycerides are obtained from dietary sources) could exhibit up-regulation of this pathway. This inapt activity of lipogenesis in ordinary non-lipogenic tissues could be caused by viral infections or by malignant transformation of normal cells[276].

FASN in cancer

1. FASN expression in breast cancer patients (*article 1*)

Selective FASN overexpression has been related to several carcinomas, such as breast, colorectal, prostate, bladder, ovary, esophagus, stomach, lung, oral tongue, oral cavity, head and neck, thyroid and endometrium, mesothelioma, nephroblastoma, retinoblastoma, soft tissue sarcomas, Paget's disease of the vulva, cutaneous melanocytic neoplasms including melanoma, and hepatocellular carcinoma[83,86,87,98,109,111,114]. Moreover, FASN expression has been associated to poor prognosis in several types of cancer and has been proposed as a possible cancer progression biomarker[277-282]. FASN expression has also been related to several clinicopathological features, such as histological grade, risk of recurrence, tumor

aggressiveness, lymphatic permeation, perineural infiltration and nodal metastasis in several types of carcinomas[83,87,114-117,283,284]. Particularly in breast cancer, it has also been associated with invasion and metastasis[285], with different breast cancer subtypes and with epidermal growth factor receptors expression[286]. Studies of the anthropometrical characteristics related to FASN have affiliated some fatty acid or glucose metabolic diseases or features to fatty acid synthase expression, such as obesity and type 2 diabetes, body mass index and nonalcoholic fatty liver disease[92,99,105,287-292].

1.1. FASN and clinicopathological characteristics

In our study of FASN expression related to clinicopathological and anthropometrical characteristics of early-stage breast cancer patients (a*rticle 1*), we found a correlation between FASN expression and tumor stage (*p-value* = 0.024). We did not found any other association with clinicopathological features (multifocality, histological subtype and grade, tumor size, vascular invasion, lymph nodes metastasis, pathological stage, estrogen and progesterone receptors, HER2 status, p53 mutation, and Ki-67 level). Since several data supporting the association of FASN with histological subtype, invasion and metastasis, HER2 status or other clinicopathological characteristics have been stated, we assumed that the low number of patients in the study (n = 53) impacted in the correlational results. For example, FASN has been extensively correlated with early events in human cancers, such as squamous cell carcinoma of the lung, in situ or poorly differentiated breast carcinoma, prostate cancer and colorectal neoplasia[110,111,277,281,293]. Consistent with our findings, FASN serum levels have been associated with tumor stage and progression in colorectal and prostate cancer[278,279] and with increased risk of recurrence and poor prognosis in lung carcinoma, stage I

breast carcinoma, ovarian neoplasm, soft tissue sarcoma, endometrial carcinoma and renal cell carcinoma[116,117,280,282,283]. The relation between FASN and HER2 status and expression, with estrogenic activity, progestins, p53 modulation and with Ki-67 has also been described[119,120,126,143,147,294]. Silva, S.D. *et al*. correlated FASN immunohistochemical expression with well-differentiated oral squamous cell carcinoma and with some clinicopathological features (lymphatic permeation, perineural infiltration and regional lymph node metastasis)[284]. Myometrial invasion, lymnphatic and vessel permeation and nodal metastasis were associated with FASN in endometrial cancer[295]. In infiltrating ductal breast cancer, Zhou, L. *et al*., also studied the clinicopathological characteristics related to FASN. This study correlated FASN with lymph node metastasis and tumor size[285].

1.2. FASN and anthropometric characteristics

Our results correlated FASN expression with some anthropometric characteristics related to fatty acid metabolism or its regulation. Menopause was strongly related to higher levels of FASN tumor expression (*p-value* = 0.005), 67.9 % of postmenopausal patients presented tumors overexpressing FASN (3+). We were the first to evidence that postmenopausal early-stage breast cancer patient have higher FASN tumor expression than premenopausal patients. Contrary to our findings, it has been shown by several authors that FASN expression is higher in premenopausal patients than in postmenopausal ones[294,296]. Moreover, estrogen levels decrease with menopausal stage. This should decrease FASN levels since estrogen receptor is one of the pathways that regulate FASN expression (*see figure 5 in general introduction*) in hormonal dependent cancers through SREBP pathway[126,128]. Our findings are supported by the fact that estrogen therapy in postmenopausal women decreased FASN levels in abdominal adipose tissue, which

decipher a negative-loop between estrogens and FASN[297]. Furthermore, 83 % of patients in our study were positive for estrogen receptor (ER) in tumor samples. Growth and hormonal receptors of tumor cells can be independent of its ligands and downstream pathways of those receptors are complex and involve activation by cross talk between multiple signal transduction pathways[12,20]. Hence, FASN could bypass depression of estrogen levels in postmenopausal stage by overactivating ER without ligand stimulation, or by cross talks with other signaling pathways. Further investigations should be done in this setting in order to elucidate ER and FASN mechanisms and cross talks with other pathways.

Age was also correlated with FASN expression (*p-value* = 0.038), the patient's median age of highest FASN expression (3+) was 54 years, whereas the age for lowest FASN expression (1+) was 46 years old. This phenomenon could be explained by the fact that age is closely related to menopausal stage.

In our results, body mass index (BMI) was linked to FASN tumor expression (*p-value* = 0.018). BMI and body fat distribution change in postmenopausal women[298]. Obesity has been associated with an increased risk of postmenopausal breast cancer and the risk of breast cancer and mortality increases with increasing BMI[299]. Moreover and consistent with our results, FASN expression has been linked to BMI and trunk-leg fat ratio in endometrial cancer[295].

Results found in the preliminary study of *paper 1* encouraged us to continue analyzing FASN expression and regulation in several subtypes of breast cancers with different expression patterns of hormonal and growth receptors expression. Nowadays, several projects of our group include clinical and patient samples sections to elucidate FASN mechanisms in the clinical field.

2. FASN inhibition in preclinical models of cancer (*articles 2* and *3*)

High levels of FASN expression in different human carcinomas attracted considerable interest of this enzyme as a target (or co-target) for cancer treatment[114,130-132,300]. Our results showed that, blocking FASN activity has apoptotic anticancer activity in FASN-overexpressed non-small cell lung cancer (*article 2*) and HER2-positive breast cancer resistant to trastuzumab and lapatinib in cellular and mice models (*article 3*). In these experiments, molecular effects of FASN-inhibition were linked to HER family downstream pathways (Akt, MAPK/ERK1/2 and mTOR) (as discussed in the next section).

FASN overexpression had been studied in different types of human lung carcinomas[112,293]. For instance, Orita *et al* studied FASN expression in patients with non–small cell lung cancers of various histological types and showed that 88.4% of tumor samples expressed significantly increased levels of the FASN protein compared with normal lung bronchial epithelial tissues. Then, they showed that C93, a synthetic FASN inhibitor structurally related to C75, inhibited FASN causing apoptosis in lung cancer cells and blocked the growth of orthotopic xenograft models of non-small cell lung cancer[159]. Some natural and synthetic compounds have been assayed as FASN inhibitors. C75 is a specific FASN inhibitor, which has demonstrated highly anti-tumoral effects in several types of cancer cells, including A549 lung cancer xenografts[301].

EGCG is a natural polyphenol catechin which also inhibits FASN and has shown anti-tumoral effects in different kind of carcinomas. EGCG is not a FASN-specific inhibitor and, besides FASN-inhibition, several mechanisms of action have been attributed to its anticancer activity. For example, it blocks activation of several

epithermal growth factor receptors (HER1, HER2 and HER3) and the IGF-1R receptor; and, as an indirect consequence, their tumoral stimulatory pathways[181,302-306]. Anyway, EGCG can directly inhibit effectors and transcription factors of proliferation and survival cell signaling pathways. EGCG downregulates activity of PI3K/AKT, ERK and NF-κß[307-309]. It can inhibit the expression of oncogenic genes, such as MMP-9, MMP-2, EGFR, Stat3, cyclin D1, bcl-2 and NF-κß[310-314]. Not only through affection in lipid metabolism (in which FASN-inhibition can be involved), but also through 67-kDa laminin receptor (67-LR), EGCG can disturb the membrane lipid rafts formation[315-318]. EGCG is known to inhibit DNA methyltransferases (DNMTs) (such as DNMT1, DNMT3a, and DNMT3b) through different mechanisms, playing a role in epigenetic anti-tumoral control[319].

Particularly in lung cancer, some of the anti-tumoral mechanisms of EGCG are G3BP1 (GTPase activating protein [SH3 domain] binding protein) inhibition[313], generation of Reactive Oxygen Species (ROS)[320], induction of p53-dependent transcription[321], reduction of cyclin D1 and bcl-2 and increase of p53 and p27 expression levels[313,314], attenuation DNMT1, p-AKT, and γ-H2AX induction[322].

2.1. FASN inhibition in FASN+/HER1+ lung cancer cell model

In our study (*article 2*), we compared the anticancer effects of C75 and EGCG (FASN inhibidors) in adenocarcinoma of lung cancer that accounts for 40% of non-small-cell lung cancers (NSCLC), the most common type of lung cancer[190,193]. We treated A549 cells and A549 lung cancer xenograft with FASN inhibitors, C75 and EGCG, and we evaluated the effects on lipogenesis (FASN activity), fatty acid oxidation (CPT activity), induction of apoptosis, signaling and their anti-cancer effects and toxicity *in vivo*.

Regarding C75- and EGCG-effects on FASN expression in our experiments, C75 (72 μM) had no effect on the abundance of FASN protein levels, but comparable treatment with EGCG (265 μM) diminished the amount of this enzyme (*see western blott figure 2 in article 2*). Therefore, it is probable that in the EGCG-treated cells, the reduction of FASN activity (remaining FASN activity was 10.7 ± 1.5 %, *p-value* = 0.000) could be in part a consequence of the reduced FASN protein levels. EGCG had similar effect as C75 specific FASN inhibitor on the activity of this enzyme. The remaining activity of FASN after treating with C75 was 3.1 ± 0.6% (*p-value* = 0.000 compared with control cells). Pan, M.H. *et al*. had previously linked EGCG-treatment to the reduction of FASN induced protein levels through inhibition of HER1, HER2 and HER3 signaling pathways in breast cancer cells[323]. Huang, C.H. *et al*. also reported inhibition of FASN induced expression with EGCG-treatment in human hepatoma cells[324]. As far as we know, we were the first to demonstrate that EGCG is able to reduce FASN expression in non-small cell lung cancer *in vitro,* and this feature could be, partially responsible for the anti-tumor effect of EGCG in this kind of carcinoma. Further discussion about the possible molecular mechanism involved in FASN downregulation will be displayed in the "*FASN and HER"* section.

2.2. FASN inhibition in parental and resistant FASN+/HER2+ breast cancer cell model

In our second study (*article 3*) and in consistence with other studies in our group and others[119,120,146,179,184,185,325], we showed maintenance of FASN overexpression in HER2-positive breast cancer cells and patient derived xenografts (PDX) even when acquiring resistance to nowadays anti-HER2 treatments (trastuzumab and/or lapatinib). Causes of this maintenance will be discussed in the

"FASN and HER family relationship" section. FASN inhibition by EGCG or G28UCM (the novel EGCG-derivative) displayed anti-tumoral effects in both parental and resistant FASN- and HER2-positive breast cancer models. The natural anti-FASN compound EGCG had similar cytotoxic effects in parental and resistant cells. IC_{50} values ranged from 206 ± 18.7 μM to 229 ± 29.4 μM. Anti-cancer results displayed with EGCG were improved when treating with G28UCM. IC_{50} value of G28UCM in parental and resistant cells ranged from 9 ± 1.5 μM to 19 ± 2.1 μM. In previous works of our group[34,184,185,326] we synthesized and biologically evaluated a new series of polyphenolic derivatives in order to improve EGCG's IC_{50} values as well as its relative instability under the slightly neutral or alkaline physiological conditions. Among them, G28UCM was selected because of its high FASN activity inhibition, its potent and selective cancer cell cytotoxicity, its ability to induce apoptosis in a FASN/HER2+ breast cancer model and its marked inhibition of HER2-related signaling pathways compared to EGCG. Moreover, G28UCM showed strong anti-tumoral and apoptotic activity in another trastuzumab- or lapatinib-resistant HER2- breast cancer cell model (AU565), anti-tumoral effects in HER2-xenografts without signs of toxicity and EGCG-improved synergistic effects when combined with anti-HER2 drugs[184-186].

In *article 3* study, G28UCM did not diminish FASN protein levels in parental and resistant HER2-breast cancer cell lines (see figure S2 in *article 3*). This was consistent with molecular analysis performed in previous works in which FASN protein levels were not affected by G28UCM-treatment, but in which FASN activity was disturbed; behaving similar to EGCG in breast cancer models (without FASN stimulation by epithermal factors)[179,180]. As we knew, G28UCM induced apoptosis (as assessed by PARP cleavage) in SKBr3 breast cancer cell lines, included those resistant to trastuzumab, lapatinib or both (see figure S2 in *article 3*). Hence,

apoptosis could be one of the mechanisms by which G28UCM exerts its anti-tumoral effects.

Further investigations should be done to elucidate the exact mechanism of action of different FASN inhibitors in different cancers.

2.3. FASN inhibition in parental and resistant FASN+/HER1+ lung cancer xenograft

In the *in vivo* side, EGCG and C75 markedly blocked the tumor growth of A549 lung cancer xenografts while tumor volumes of control group growth significantly (*see figure 4A in article 2*). On final day the median tumor volume (519 mm^3) was significantly different from the starting median tumor volume (33 mm^3, *p-value* = 0.04). The median tumor volume of C75- and EGCG-treated animals on the final day of the experiment (290 and 224 mm^3, respectively) was not significantly different from the median tumor volume on the starting day (40 and 36 mm3, respectively; *p-value* = 0.07 both). C75 and EGCG-treated tumors showed apoptosis by induction of PARP cleavage without any change in total levels of FASN protein (*see figure 4A* in *article 2*). Maintenance of FASN expression, assayed in mice model after treatment with EGCG, differs from reduction observed in lung cells. Since, for experimental requirements time of both experiments enormously differs (24 hours *in vitro vs* 33 days *in vivo*), inter-pathway cross talks and effects on FASN expression regulatory pathway could have been compensated over time, and moreover with other systems (such as immune system) involved in mice's body. Anyway, *in vivo* results on apoptosis are consistent with those obtained with cell models (*see figure 3 in article 2*) and also the anti-tumoral effect.

2.4. FASN inhibition in parental and resistant FASN+/HER2+ breast cancer patient derived xenograft (PDX)

In two separate experiments in article 3, tumor growth was also significantly blocked when we treated FASN- and HER2-positive breast cancer-PDX with EGCG, and was slightly blocked in FASN- and HER2-positive breast cancer-PDX resistant to trastuzumab and lapatinib when treated equally. Control (non-treated) FASN+/HER2+ PDX animals achieved a median tumor growth of 461.0 ± 65.6 mm^3 whereas EGCG significantly reduced tumor growth to 247.6 ± 45.0 mm^3 (*p-value* = 0.017). EGCG also had anti-tumoral effects on resistant FASN+/HER2+ PDX. Compared to the control group (393.9 ± 95.5 mm^3), EGCG decreased tumor growth to 285.9 ± 36.5 mm^3 (*see figures 4a and 4b in article 3*). Similarly, in the second experiment, values of tumor growth of FASN+/HER2+ PDX and resistant FASN+/HER2+ PDX control groups were 386.4 ± 66.7 mm^3 and 314.8 ± 81.1 mm3, respectively. In this case, EGCG reduced tumor growth up to 183.3 ± 15.1 mm^3 (*p-value* = 0.017) in non-resistant PDX model and, up to 231.8 ± 38.4 mm^3 in the resistant-PDX (PDX**R**) model (*see figures 5a and 5b in article 3*). In order to evaluate the mechanism by which EGCG induce tumor-regression, tumor samples from PDX and PDX**R** treated tumors were evaluated for apoptosis, assessed by fluorescent TUNEL assay. In both models, EGCG-treated samples showed an increase in apoptosis compared with control tumors (*figs 4B and 5B, article 3*). EGCG induced apoptosis in HER2-PDX was 133 ± 14 TUNEL+ cells/mm^2 and 337 ± 19 TUNEL+ cells/mm^2 in the HER2-PDX**R**, compared with untreated HER2-PDX (66 ± 8 TUNEL+ cells/mm^2) and HER-PDX**R** (287 ± 23 TUNEL+ cells/mm^2) tumors.

2.5. *In vivo* side effects of FASN inhibition

EGCG and C75 on lung cancer xenografts

Side effects of both FASN inhibitors were evaluated in lung xenografts and analyzed *in vitro*. C75-treated mice showed a marked decrease in body weight after each administration (close to 6% of initial body weight, *see figure 4B in article 2*). In contrast, we did not observe a significant decrease in body weight in the animals treated with EGCG. As fatty acids ß-oxidation has been related to the severe decrease of food intake and induction of weight loss in rodents and we and others showed that C75 is able to stimulate CPT-I enzyme of CPT system (which controls the entry of long-chain fatty acids into the mitochondria for subsequent oxidation) and produce weight loss in animal models[157,327], we analyzed the effects of both compounds on CPT enzymatic activity in A549 isolated mitochondria. EGCG had no effect on CPT activity (115 ± 12%, *p-value* = 0.294) compared to non-treated cells. In contrast, C75 produced a significant activation of CPT system (131 ± 11%, *p-value* = 0.006) (*see figure 1* in *article 2*). This was consistent with the fact that EGCG does not affect CPT-I activity and, consequently, it does not induce weight loss in experimental animals. This result in a lung cancer model reinforces the hypothesis that CPT activation is the cause of weight loss in xenografts models and are in agreement with our previous findings in a mouse breast cancer model[179] and results found in *article 3* presented in this thesis.

EGCG on breast cancer PDX

EGCG treatment on breast cancer patient derived xenografts, even in combinatorial regiments with the dimerization inhibitor of HER2 (pertuzumab) or the mTOR inhibitor (temsirolimus), did not show signs of toxicity (nor significant weight loss, neither organs histological abnormalities) (*see figures 4c, 5c, S6, S7 and S8 in article 3*). At the end of two separate experiment, the mean body weight of control PDX animals ranged from 23.5 ± 1.1 g to 24.1 ± 0.8 g whereas EGCG-treated group's weight was from 23.2 ± 1.1 g to 23.6 ± 0.9 g. EGCG behaved similarly in the resistant-PDX model, the control's mean weight was from 23.7 ± 0.9 g to 24.6 ± 1.0 g and when treated with EGCG, mean weight ranged from 20.7 ± 0.9 to 21.2 ± 1.1 g. *p-values* were > 0.05 in all cases, showing no significant difference between treated and non-treated groups. These results, together with the fact that any histological disturbances were seen in lung, heart, liver and kidneys of EGCG-treated animals, reinforces our previous results that showed none side effects of EGCG *in vivo*[34,179,180].

Our data reveals that FASN inhibition (with C75, EGCG or G28UCM) has anti-tumoral effects, handled by apoptosis, in FASN overexpressed carcinomas that also overexpress HER1 (assessed in lung cancer model) and HER2 (assessed in breast cancer and resistant-breast cancer model). The inhibition of FASN by EGCG in animal models, accomplish C75 anti-cancer effects without causing side effects. Also that, EGCG-derivatives, such as G28UCM, improve anti-tumoral effects in resistant and non-resistant HER2 breast cancer that overexpress FASN. Therefore, FASN could be a potential target (alone or in combination with other targets) and could be part of new pharmacological strategies for FASN-positive cancers.

FASN and HER

Regulation of FASN expression has been, in part, attributed to the HER receptors family, especially HER2 pathway. A HER2-FASN relationship has been described in breast cancer, head and neck carcinomas, HER2-overexpressed fibroblasts and other carcinomas[119,138,179,325]. HER1 receptor has also been linked to FASN expression in some carcinomas, such as ovarian and prostate carcinomas[118,146]. Other HER family receptors have also been linked to FASN expression and regulation. Furthermore, in order to prove FASN-HER loop, our group and others has shown that FASN inhibition has blocking effects on all members of epidermal growth factor receptor (HER) family[179,181,302,328].

The signaling pathway by which HER receptors family governs FASN transcription and translation has been studied. Briefly, HER receptors, through its intercellular pathway (AKT/mTOR and ERK1/2), stimulate SREBP-1 to enter into the nucleus and promote FASN transcription [94,104,107,123]. Besides transcription, AKT and mTOR also promote FASN translation[83] *(for more information see figure 5* in *general introduction)*. Hence, those carcinoma cells overexpressing any of the HER receptors family should also overexpress FASN enzyme. Anyway, other pathways or cross talks can interact with downstream signalers to modify HER receptors signals[272-274]. We and others have deciphered this linkage between both proteins, but further studies should deepen into this complex and intricate connection, especially when FASN or HER pathways inhibitors take a role in it.

1. FASN and HER1 non-small cell lung cancer

We used non-small cell lung cancer (A549 cells) as a model for HER1 and FASN overexpressing carcinoma to study the relationship between both tumoral proteins and to assess FASN-inhibition as an anticancer treatment in this type of carcinoma with a FASN-HER1 loop (*article 2*).

The inhibition of FASN activity by EGCG produced a completely abolishment of the active phosphorylated form of EGFR (p-EGFR) from 6 hours of exposure. Consequently, phosphorylated forms of ERK1/2 (p-ERK1/2), AKT (p-AKT) and mTOR (p-mTOR) were also markedly decreased. Comparable concentrations of C75, even with prolonged exposure (48 hours), only partially decreased total levels of EGFR and phosphorylated levels of AKT (p-AKT) (*see figure 2 in article 2*).

EGFR may be another EGCG-direct target that through inhibition of its downstream signalers (Akt, ERK1/2 and mTOR) is able to down-regulate FASN transcription (by SREBP-1c and PI3K/Akt and MAPK/ ERK1/2 pathways) and translation (by AKT-mTOR-signaling and its downstream effectors, eIF4G and S6K)[83], as seen in breast cancer[329] and in human hepatoma cells[324].

Since, C75 specifically inhibits FASN and has no other targets, we have seen a later effect on levels of EGFR and phosphorylation of it downstream effector AKT (p-AKT) (*see figure 2 in article 2*). FASN inhibition in lung cancer cells disrupted EGFR signaling, demonstrating the existence of a loop-direction from FASN to EGFR receptor.

Our results of EGCG and C75 in HER1 pathway corroborate a FASN-HER loop, described in breast cancer. The FASN disruption impedes the synthesis of lipids, which are integrated in membrane lipid raft. Lipid rafts are sphingolipids and gangliosides enriched structures that act as molecular platforms for the

accommodation of various receptors and non-receptor proteins[139,330-333] and influence HER receptors signaling by altering the activity of protein kinases[334] and changing the association state of membrane proteins[335]. Disruption of lipid rafts affects the function of membrane receptors, such as HER1[336,337] or HER2[139,338]. In our experiments, FASN inhibition could impair formation of long-chain fatty acids that participate in lipid rafts structures. Hence, disrupting lipid rafts and as a consequence, HER receptors stability and signaling.

2. FASN and HER2 breast cancer

Another interesting FASN-positive model to deepen inside to find molecular mechanisms involved in the connection between FASN and HER family receptors, is the HER2-positive breast cancer model and its resistant secondary models.

Extensive studies have shown the interconnection between HER2 and FASN[146,179,180,185,280], and the consequences of inhibiting FASN in HER2 pathway or vice versa[146,179,180,183-186,325]. Our contribution in this field was to analyze the possible synergistic effects of inhibiting FASN together with some players in the HER2 pathway signaling (HER2 and mTOR inhibition). In order to find new pharmacological strategies for those HER2-breast cancers that have progressed to anti-HER2 treatments and as a comparison between HER2-breast cancers, we developed HER2-positive breast cancer cell lines resistant to trastuzumab and/or lapatinib, which changed the HER family receptors pathway's profile (changes in expression and activation of all HER family receptors and changes in downstream executors) (*article 3*).

2.1. Resistance to anti-HER2 treatments

Although HER2-positive breast cancers have an aggressive phenotype, these tumoral cells possess an *Achilles heel* to battle against. HER2 receptor is selectively overexpressed in this type of breast carcinoma, which allows a direct treatment against this tumoral protein without affecting non-tumoral cells. Some anti-HER2 therapies are: trastuzumab, a monoclonal antibody directed against the extracellular domain of HER2[37,230]; pertuzumab, a monoclonal antibody that blocks the extracellular domain needed for receptor dimerization[244]; lapatinib, a reversible HER1 and HER2 tyrosine kinase inhibitor[239]; neratinib, another HER1 and HER2 tyrosine kinase inhibitor[250]. Anti-HER2 therapies have had a valuable success for HER2-postivie breast cancer patients. Anyway, a percentage of these patients do not initially respond to anti-HER2 treatments, called primary resistance. For those patients that respond to anti-HER2 treatments some develop resistance over time, called secondary or acquired resistance[256,257]. Several mechanisms of resistance to anti-HER2 agents have been described (see table 1) but more studies are needed in order to fully understand these problems and to find new strategies to treat this occurrence.

Table 3. Summary of the mechanisms of resistance to anti-HER2 treatments.

Mechanism of Resistance	Reference
Changes in HER2	
Conversion to HER2-negative carcinoma	[260]
Conversion to constitutively active truncated form of HER2 (p95HER2)	[261]
Gatekeeper mutation in HER2	[262]
Overexpression of other RTKs or their ligands	
Ligands and receptor HER1 overexpression	[263,264,266,339,340]
Ligands and receptor HER3 overexpression	[264-266,341]
Ligands and receptor HER4 overexpression	[263,342]
Changes in HER2 downstream proteins	
Loss of PTEN	[267,268,343]
Overexpression and overactivation of ERK1/2 and PI3K/Akt/mTOR signaling proteins	[266,268-271]
PI3K activating mutations and hyperactivation	[271,344,345]
AKT activating mutations	[270]
Ras overexpression or mutation	[346]
Cross talk with other signaling pathways	
Insulin-like growth factor (IGF) receptor pathway	[272-274]
Hepatic growth factor receptor (c-Met) pathway	[265]

In *article 3* and in order to first study the molecular mechanisms that derived to resistance and its relation to FASN expression, we developed and characterized stable cell lines derived from HER2-positive SKBr3 cells that were long term-resistant to either trastuzumab (SK**TR**), lapatinib (SK**LR**) or both (SK**LTR**; patented in [347]). Some molecular mechanisms of resistance in our developed anti-HER2 resistant models were consistent with the previously described (see table 2 below)[256,257,259-261,266,268,270,271]. Our trastuzumab, lapatinib and trastuzumab *plus* lapatinib resistant models showed a decrease in HER3 expression and activation,

whereas an overexpression and overactivation of HER1, and increased expression levels of HER1 (EGF and TGF-α) and HER1-HER4 (EREG and HB-EGF) ligands were seen. Even more, after dual trastuzumab and lapatinib long-term exposure, SK**LTR** cells overexpressed HER4 besides HER1, and increased the expression of HER1 (EGF and AR) and HER1-HER4 (BTC, EREG and HB-EGF) ligands (*see figures 1b and 1d in article 3*). Overactivation of HER2 in SK**LR** and SK**LTR** could be accomplished through dimerization with other HER receptors such as HER1 and HER4 (which are overexpressed and overstimulated by their ligands).

Moreover, we have shown that PI3KCA mutations (PIK3CA_E545K mutation in SK**TR** cells and PIK3CA_E542K mutation in SK**LR** and SK**LTR** cells) collaborate with HER changes to maintain PI3K/AKT/mTOR pathway activation in all resistant cells. ERK1/2 overactivation is another downstream produced change that leads to cell proliferation signaling of the resistant cells.

Table 4. Summary of the mechanisms of resistance to anti-HER2 treatments in SKTR, SKLR and SKLTR cells.

Mechanism of Resistance described	Mechanisms of Resistance observed		
	SKTR	SKLR	SKLTR
Changes in HER2			
Conversion to HER2-negative carcinoma	No, but abolishment of HER2 activation	No	No
Conversion to constitutively active truncated form of HER2 (p95HER2)	No assessed	No assessed, but overactivation of HER2	No assessed, but overactivation of HER2
Overexpression of other HER receptors or their ligands			
Ligands and receptor HER1 overexpression and overactivation of receptor	Yes. Overexpression and overactivation of HER1, but decrease in HER1 ligand (AR)	Yes. Overactivation of HER1 and increased expression of HER1 ligands (EGF and TGF-α and HER1-HER4 (HB-EGF) ligand	Yes. Overactivation of HER1 and increased expression of HER1 (AR) and HER1-HER4 (HB-EGF) ligands
Ligands and receptor HER3 and overactivation of receptor	No. Decrease of HER3 expression and activation	No. Decrease of HER3 expression and activation	No. Decrease of HER3 expression and activation
Ligands and receptor HER4 and overactivation of receptor	No	Yes, increased expression of HER1-HER4 ligand (HB-EGF)	Yes. Overactivation of HER4 and increased expression of HER1- HER4 ligands (EREG and HB-EGF)
Changes in HER2 downstream proteins			
Overexpression and overactivation of ERK1/2 and PI3K/Akt/mTOR signaling proteins	Yes. Slighly increase in ERK1/2 activation and AKT expression	Yes. Slighly increase in ERK1/2 activation. But, decrease in AKT activation	Yes. Slighly increase in ERK1/2 activation and increase in AKT activation
PI3K activating mutations and hyperactivation	Yes, but low incidence	Yes, but low incidence	Yes, but low incidence

Anyway, no significant changes in mTOR and p-mTOR proteins were observed in our long-term resistant cells. mTOR is a downstream protein in which several signaling pathways converge. mTOR pathway is a complex network in which an important effector can be regulated by distinct emissaries. Hence, even with changes in PI3K/AKT (which are the main upstreamers of mTOR), mTOR expression and activity could be compensated by other pathways and regulators. For example, maintenance of mTOR activation (even with decrease in AKT activation) can be accomplished by direct signaling of PI3K to mTOR, bypassing AKT[251]. Also, it is described that MAPK inhibit the tuberous sclerosis complex (TSC1/TSC2), which in turn inhibits mTOR activation[348]. As a consequence, at the end of the HER2 pathway, mTOR expression and activation and FASN expression were maintained.

2.2. Combination of FASN inhibition with HER2 pathway inhibition

Overactivation of HER2 in SK**LR** and SK**LTR** cells and maintenance of mTOR, p-mTOR and FASN expression in all the resistant models, provided the rationale to test combined FASN and HER2 or mTOR inhibition for HER2-positive breast cancer patients non-responsive to anti-HER2 treatments. Firstly, and to corroborate resistance to anti-HER2 drugs, we checked the effects of the HER2-dimerization inhibitor, pertuzumab, compared with trastuzumab in all parental and resistant cells (see figure 2 in *article 3*). Since SK**TR** and SK**LTR** are resistant to trastuzumab, and SK**LR** cells are resistant to lapatinib and have managed to overcome HER2-activation inhibition, we expected no significant effect when treating with two directed and selective HER2 inhibitors, trastuzumab and pertuzumab.

Dual HER2 inhibition

Several multi-target or combinatorial strategies have been studied in order to surpass trastuzumab and/or lapatinib resistance in HER2-positive breast cancer. Dual monoclonal antibody blockade is one of the strategies used to block HER2 downstream signaling more completely than targeting HER2 alone. This approach is aimed to prevent HER2 activation through hetero-dimerization with other HER family receptors, such as HER1 and HER3[248]. Pertuzumab is a monoclonal antibody directed against the HER2 domain responsible for its homo- and hetero-dimerization. The combination of pertuzumab and trastuzumab has been shown to be effective in disrupting HER2-HER3 heterodimers and hence, their downstream PI3K signaling[248,349]. Moreover, combinatorial regiment was more effective, than trastuzumab or pertuzumab alone, in inhibiting tumor growth in *in vivo* studies[245,350]. The addition of pertuzumab in trastuzumab therapy has also been studied in patients. CLEOPATRA and NeoSphere clinical studies demonstrated increased overall survival, progression-free survival and rate of tumor disappearance when pertuzumab was added to trastuzumab *plus* chemoterapy treatment[246,351,352]. In consequence, pertuzumab *plus* trastuzumab combination has been approved by the FDA, for the treatment of HER2-positive breast cancer in both the neoadjuvant and metastatic settings[353]. Anyway, it has been shown that trastuzumab, lapatinib, and pertuzumab combination is not effective in inhibiting heregulin-induced HER3 activation in HER2-positive breast cancer cells[354]. Our trastuzumab resistant cells showed inhibition of HER2 activation, suggesting that the mechanism of resistance bypass, in part, HER2 pathway. Since pertuzumab inhibits HER2 dimerization, its anti-tumoral effect was not expected in SK**TR** cells.

As far as we know, any trial has been performed using pertuzumab as a treatment to overcome lapatinib- or trastuzumab *plus* lapatinib-resistance. As a

tyrosine kinase inhibitor, lapatinib is able to inhibit HER2 independently of its mechanism of receptors dimerization. Hence, we expected poor response of the dimerization inhibitor, pertuzumab, in our lapatinib or lapatinib *plus* trastuzumab resistant cells.

On agreement, SK**TR**, SK**LR** and SK**LTR** showed significant resistance to pertuzumab and trastuzumab, compared with SK cells. Trastuzumab only reached 19.9% (*p-value* = 0.000), 32.1% (*p-value* = 0.022) and 15.4% (*p-value* = 0.000) of cell proliferation inhibition (cpi) in SK**TR**, SK**LR** and SK**LTR**, respectively. Pertuzumab, showed a similar effect in SK**TR** (27.8%; *p-value* = 0.004), SK**LR** (38.8%; *p-value* = 0.041) and SK**LTR** (19.6%; *p-value* = 0.001) cells. When using both anti-HER2 drugs in combination, cytotoxic effect in SK**TR** was not increased (25.7% cpi; *p-value* = 0.008), neither in SK**LR** (38.3% cpi; *p-value* = 0.023) cells. But, co-treatments effect in SK and SK**LTR** significantly improved the inhibitory effect. In SK cells, cpi was up to 63.1% (*p-value* = 0.007 compared with trastuzumab), and 52.5% in SK**LTR** ($p\text{-value}_{Pertuzumab}$ = 0.001 compared with pertuzumab and $p\text{-value}_{Trastuzumab}$ = 0.000 compared with trastuzumab). SkBr3 cells have not developed mechanisms of resistance to anti-HER2 treatments and their proliferation is dependent on HER2 signaling. SK**LTR** cells display significant p-HER2 overexpression together with overactivation of other HER family receptors (HER1, HER4), indicating a reactivation of the HER2 pathway. For that reason, inhibiting HER2 dimerization, with pertuzumab, in combination with HER2 direct inhibition, with trastuzumab, is more effective than each treatment alone in these cells, which are dependent to HER2 signaling.

Co-treatment with two HER2 inhibitors overcomes anti-HER2 drugs resistance, but the inhibitory effect is far from desired results. Moreover, it has been demonstrated that inhibiting more than one target in the same pathway produces a

better anti-tumoral effect since it can bypass compensatory loops and it achieves higher blocking effect in the tumor cell proliferating pathway[341,355-359].

FASN *plus* HER2 pathway inhibition

Because inhibiting FASN alone showed anti-tumoral effects in parental and resistant HER2-positive breast cancer *in vitro* and *in vivo* (*see "FASN inhibition in preclinical models of cancer" paragraph*), we conducted a series of combinatory experiments to evaluate the inhibitory effect of EGCG and G28UCM in combination with the anti-HER2 inhibitor, pertuzumab, and the mTOR inhibitor, temsirolimus, in parental and resistant cell models and patient derived xenografts (PDX).

EGCG or G28UCM *plus* pertuzumab

Pertuzumab combined with anti-FASN compounds (EGCG or G28UCM) increased anti-tumoral effects in parental and resistant cells (*see figure 3A in article 3*) compared with drugs used as a single agent. Ratios of cpi induced by treatments alone (which is graphed as 1) versus inhibition induced by co- treatment was less than 1 in all combinatory experiments. When combining pertuzumab with EGCG, the ratiso of mono-treatments/combination were 0.34, 0.82, 0.62 and 0.31 in SK, SK**LR**, SK**TR** and SK**LTR,** respectively. In SK**LTR** cells, pertuzumab *plus* EGCG combination significantly improved the effects of each treatment alone (*p-value*$_{\text{compared with 1}}$ = 0.036). Combination of G28UCM *plus* pertuzumab slightly improved EGCG co-treatment inhibitory effects in SK**TR** cells (0.69 when combined with G28UCM compared with 0.82 in EGCG). This is consistent with previous studies of our group in which we showed that G28UCM improved EGCG effects in several

combinatorial regiments with anti-HER2 drugs and chemotherapy, in parental and trastuzumab- or lapatinib-resistant AU565 HER2 breast cancer cells[185].

To corroborate our results with an *in vivo* model and to evaluate side effects of combinatorial treatment in animals, we evaluated the antitumor activity of pertuzumab combined with EGCG in a HER2+ PDX model (HER2-PDX) and in a trastuzumab *plus* lapatinib-resistant HER2+ PDX (HER2-PDX**R**) model. Both PDX models showed similar HER2, mTOR and FASN expression levels as the *in vitro* parental and resistant cellular models (*see figure S5 in article 3*). EGCG and pertuzumab, as single agents, reduced tumor growth in the HER2-PDX model, but superior (and more rapid) tumor regression was achieved by dual FASN and HER2 blockade (see figure 4a, left panel, in *article 3*). In HER2-PDX model, control animals achieved a median tumor growth of 461.0 ± 65.6 mm^3 whereas EGCG significantly reduced tumor growth to 247.6 ± 45.0 mm^3 (*p-value* = 0.017), and pertuzumab reduced to 301.0 ± 62.9 mm^3. Combination of EGCG with pertuzumab had a potent and significant effect in reducing tumor growth compared with treatments alone, the median tumor growth in this group was 87.2 ± 38.2 mm^3 ($p\text{-}value_{vsEGCG}$ = 0.017 and $p\text{-}value_{vsPertu}$ = 0.010). In the HER2-PDX**R** model, EGCG and pertuzumab decreased tumor growth to 285.9 ± 36.5 mm^3 and 310.4 ± 34.5 mm^3, respectively, compared with the control group (393.9 ± 95.5 mm^3). The combination of EGCG with pertuzumab significantly reduced tumor growth up to 177.64 ± 34.5 mm3 ($p\text{-}value_{vsEGCG}$ = 0.030 and $p\text{-}value_{vsPertu}$ = 0.008) (*see figure 4a, right panel, in article 3*).

Since apoptosis has been described as a mechanism of action of pertuzumab and EGCG[179,183,186,349,360] and we have corroborated the induction of caspase activity in parental and resistant HER2-positive breast cancer cells with EGCG (see figure S3 in *article 3*), we assessed if apoptosis could be responsible for the reduction of

tumor growth in HER2-PDX and HER2-PDX**R** when treating with this combinatorial regimen. Tumor samples from HER2-PDX and HER2-PDX**R** treated tumors showed increased apoptosis compared with HER2-PDX and HER2-PDX**R** control tumors, assessed by fluorescent TUNEL assay (*see figure fig 4b in article 3*). EGCG and pertuzumab used as single agents induced apoptosis in HER2-PDX (133 ± 14 TUNEL+ cells/mm^2 and 122 ± 16 TUNEL+ cells/mm^2, respectively) and in HER2-PDX**R** (337 ± 19 TUNEL+ cells/mm^2 and 352 ± 18 TUNEL+ cells/mm^2, respectively) tumors compared with apoptosis showed by control HER2-PDX (66 ± 8 TUNEL+ cells/mm^2) and HER-PDX**R** (287 ± 23 TUNEL+ cells/mm^2) tumors. As synergism in tumor reduction after combinatorial treatments, apoptosis has also been synergistically increased when combining EGCG with pertuzumab in HER2-PDX (933 ± 40 TUNEL+ cells/mm2) and HER2-PDX**R** (866 ± 40 TUNEL+ cells/mm2) tumors. These similar profiles in tumor growth reduction and apoptosis suggest that apoptosis is responsible for tumor growth inhibition. These results corroborate our previous studies showing that EGCG produces apoptosis *in vitro* and *in vivo*[179,180,183,185] and are consistent with other studies of pertuzumab in cells and mouse models[349,360].

We have previously shown in *article 2* and in other studies that EGCG displays *in vivo* antitumor activity without decreasing food intake and induction of weight loss[179,180,183,185]. In *article 3*, we show that the combination of EGCG with pertuzumab resulted in synergistic reduction of HER2-PDX and HER2-PDX**R** tumors, without signs of toxicity (weight loss and organs histological abnormalities) *in vivo*. EGCG *plus* pertuzumab treatment on HER2-PDX and HER2-PDX**R** did not show signs of toxicity (nor significant weight loss, neither organs histological abnormalities) (*see figures 4c, S6 and S8 in article 3*). At the end of the experiment, the mean body weight of control HER2-PDX and HER2-PDX**R** animals was 23.5 ± 1.1 g and 24.6 ± 1.0 g, respectively, whereas even with EGCG *plus* pertuzumab treatment the mean body weight of animals only decreased down to 20.5 ± 0.3 g (*p-value* = 0.05)

in HER2-PDX and 22.1 ± 0.4 g (*p-value* = 0.1) in HER2-PDX**R**, showing no significant difference between treated and non-treated groups. Moreover, no histological disturbances were seen in lung, heart, liver and kidneys of EGCG-treated animals. We had previously shown that EGCG does not stimulate CPT-I enzyme and thus fatty acids ß-oxidation (what produces weight loss in animal models)[183,326,361,362]. Now, we demonstrate that even with pertuzumab combination, EGCG has no side effects *in vivo*, strengthening the use of this combination strategy for those HER2-positive breast cancer patients, even for those resistant to trastuzumab and/or lapatinib.

EGCG or G28UCM *plus* temsirolimus

mTOR expression and activation was also maintained in SK**TR**, SK**LR** and SK**LTR** resistant cells, and treatment with mTOR inhibitors have displayed strong anti-tumoral effects in several types of carcinomas[251,253,363-366]. We also tested EGCG combined with the mTOR inhibitor, temsirolimus, in parental and resistant cell and PDX models.

First, in order to assess the combinatorial effect of inhibiting mTOR or FASN together with lapatinib and/or trastuzumab, we tested the apoptotic (PARP cleavage) effect of temsirolimus and EGCG in combination with trastuzumab and/or lapatinib. Temsirolimus or EGCG did not significantly improve trastuzumab and/or lapatinib apoptotic effects (assessed by cleavage of PARP) on SK, SK**TR** and SK**LR** cells. In SK**LTR** cells, the combination of temsirolimus *plus* trastuzumab and lapatinib had a significantly apoptotic effect compared with each treatment alone (*see figure S3 in article 3*). Temsirolimus has been shown to enhance the growth inhibition effect of trastuzumab in SKBr3 HER2-positive breast cancer cell lines[367] and it has been investigated in combination with neratinib (HER2 tyrosine kinase

inhibitor) of in a phase II trial of HER2-positive breast cancer patients[358]. Moreover, mTOR inhibition has been assessed *in vitro*, *in vivo* and in clinical trials of HER2 positive breast cancer with advantageous results to beat resistance to anti-HER2 drugs[251,266,271,355,364,368]. Specific studies of temsirolimus in combination with lapatinib or lapatinib *plus* trastuzumab in naïve or resistant HER2 positive breast cancer have not been performed. We were the first to study this elaborated combination in resistance, but more studies should be done in order to elucidate the efficacy of this regiment.

Inhibition of mTOR, in combination with FASN inhibition, resulted in a strong synergistic interaction in all parental and resistant HER2-positive breast cancer cells. Even alone, temsirolimus had a potent inhibitory effect on parental and resistant cells, IC_{50} concentration ranged from 9 ± 0.9 μM to 11 ± 0.4 μM (*see table S1 in article 3*). But, when co-administered with EGCG there was a synergistic effect (assessed with the isobologram analysis) in all cell lines. The interaction index (Ix) obtained in the isobologram analysis indicates whether the doses of the two drugs required to produce a given degree of cytotoxicity are greater than (Ix > 1 or antagonism) equal to (Ix = 1 or additivism) or less than (Ix < 1 or synergism) the doses that would be required if the effect of two agents were strictly synergic[185]. The interaction index (Ix) in SK, SK**TR**, SK**LR** and SK**LTR** was from 0.84 to 0.94, *p-values* in all cases were less than 0.01 compared with 1. The same effect was assessed by induction of cell proliferation inhibition (cpi), mean ratio of cpi induced for each treatment alone versus cpi induced for co-treatments was from 0.48 to 0.52 (*p-values* < 0.05 compared with monotreatment) in parental and resistant cells (see figure 3b in *article 3*). To analyze the synergic effect in mTOR and FASN protein levels after its inhibition, we performed a western blott panel

(*see figure S4 in article 3*). No significant inhibition in FASN protein levels was seen in any mono-treatment or EGCG *plus* temsirolimus combination in parental and resistant cells. But, co-treatment completely abolished mTOR expression in SK**TR**, SK**LR** and SK**LTR** resistant cells. Although FASN protein levels were not affected, FASN activation was not analyzed and, regarding previous works, it should be inhibited. Nowadays, our group is extending this analysis in order to elucidate the exact mechanism of action. What is clear is that, even with the potent anti-proliferative action of temsirolimus, combination is needed in order to completely abolish mTOR expression. mTOR is an important tumoral protein and several cancer cells are dependent on its expression and signaling to continue proliferating[53,59,60,251,369]. It has been shown that mTOR inhibition overcomes resistance to HER2-targeted therapies in pre-clinical and in clinical studies with trastuzumab-resistance[355,364,368]. Synergism between mTOR and FASN inhibition induce cytotoxicity in ER/HER2-positive breast cancer cell lines[370]. With our experiments, we corroborate that anti-HER2 resistant breast cancer cells are also dependent on mTOR signaling. These *in vitro* results support the rationale to test the antitumor efficacy of FASN *plus* mTOR inhibition in mice models with tumors resistant to anti-HER2 therapies.

G28UCM improves the effects of EGCG when treated alone, as explained in "*FASN inhibition in preclinical models of cancer*" paragraph, but it also improves combinatorial effects with anti-HER2 drugs and chemotherapy in parental and trastuzumab- or lapatinib-resistant AU565 HER2 breast cancer cells, as we have seen in previous works[185]. Here, we corroborate that G28UCM also enhance EGCG combinatorial effects when co-administered with the mTOR inhibitor, temsirolimus. In this case, the interaction index in parental and resistant cells

decreased halfway (0.36 < Ix < 0.58) (see table S1 in *article 3*). A similar decrease was seen when assessed in cpi ratios (from 0.22 to 0.30; all p-values < 0.000). Since G28UCM improves the EGCG anti-tumoral effect and its synergism in combination with other drugs, it encourages us to continue studying this compound as a possible clinical drug for FASN-positive cancers. It has been difficult for us to synthesize it in a large scale. For this reason, we have a new ongoing project to elucidate mechanisms of large scale synthesis of G28UCM and to find out the exact mechanism of action and possible implications.

FASN inhibition together with mTOR inhibition was also studied *in vivo* in HER2-PDX and HER2-PDX**R** mice models (see figure 5a in *article 3*). In control animals, it achieved a median tumor growth of 386.4 ± 66.7 mm^3 whereas EGCG median tumor growth was significantly reduced to 183.3 ± 15.1 mm^3 (*p-value* = 0.017). Similar as *in vitro* results, temsirolimus had a strong antitumor activity even when used alone (18.0 ± 15.1 mm^3; *p-value* = 0.000) and its activity was slightly increased with EGCG combination, mean tumor volume displayed complete shrinkage (-8.2 ± 6.0 mm^3) (*see figure 5a, left panel, in article 3*).

In HER2-PDX**R** model, EGCG treatment decreased the median tumor growth (231.8 ± 38.4 mm^3) compared with control group (314.8 ± 81.1 mm^3). Temsirolimus alone also significantly decreased tumor growth when used as a single agent (114.3 ± 27.1 mm^3; *p-value* = 0.045), and its effects on tumor volume were also improved by adding EGCG (94.9 ± 33.1 mm^3).

Apoptosis was responsible for tumor reduction with EGCG and/or mTOR (see figure 5b in *article 3*). EGCG and temsirolimus induced apoptosis in HER2-PDX (133 ± 14 TUNEL+ cells/mm^2 and 333 ± 19 TUNEL+ cells/mm^2, respectively) and HER2-PDX**R** tumors (337 ± 19 TUNEL+ cells/mm^2, and 803 ± 36 TUNEL+ cells/mm^2,

respectively) compared with untreated HER2-PDX (66 ± 8 TUNEL+ cells/mm^2) and HER-PDX**R** (287 ± 23 TUNEL+ cells/mm^2) tumors. The combinatory treatment of temsirolimus *plus* EGCG increased apoptosis in both parental and resistant HER2-PDX tumors (1265 ± 51 TUNEL+ cells/mm^2 and 1197 ± 55 TUNEL+ cells/mm^2, respectively). It has been shown that temsirolimus produces apoptosis in a resistant oropharyngeal carcinoma cell line, colorectal cancer cells and other cancers[363,365]. But it is not clear that apoptosis is produced by temsirolimus in breast cancer cell lines[371]. In this experiment, we demonstrate that tumor growth inhibition in non-resistant and resistant HER2 breast cancer PDX occurs by apoptotic event in tumoral cells when treating with EGCG or its combinations, and this is consistent with what have found in lung cancer.

Regarding toxicological effects in mice, EGCG, temsirolimus and combination of EGCG *plus* temsirolimus did not show any side effect (weight loss and organs histological abnormalities) *in vivo* (*see figures 5c, S7 and S8 in article 3*). As seen in EGCG *plus* pertuzumab and in other EGCG-studies previously done[179,180,183,185]; EGCG (alone or in combination with temsirolimus) does not produce weight loss in HER2+-PDX, nor in HER2+-PDX**R** models. At the end of the experiment, the mean body weight of control HER2-PDX and HER2-PDX**R** animals was 23.5 ± 1.1 g and 24.6 ± 1.0 g, respectively. Even with the most severe treatment, EGCG *plus* temsirolimus, the mean body weight of animals only decreased up to 22 ± 1.1 g (*p-value* = 0.16) in HER2-PDX and up to 22.8 ± 0.9 g (*p-value* = 0.5) in HER2-PDX**R**, showing no significant difference between treated and non-treated groups. As seen in EGCG *plus* pertuzumab combination, lung, heart, liver and kidneys were not histologically affected when treating with EGCG, temsirolimus, or combination of both.

Together, *in vitro* and *in vivo* results in parental and resistant HER2-positive breast cancer, demonstrate that FASN inhibition (alone or in combination with other targets in the HER2 pathway, such as pertuzumab or temsirolimus), could be a promising strategy for those patients with advanced HER2-positive breast cancer and even for those that have progressed to standard anti-HER2 therapies.

GENERAL CONCLUSIONS

The general conclusion of this thesis is that fatty acid synthase (FASN) inhibition, alone or in combination with anti-HER2 drugs, displays anti-tumoral effects in breast and lung models overexpressing FASN and HER receptors. Moreover, FASN could be a potential target in postmenopausal breast cancer patients.

The specific conclusions that support the general conclusion are:

Fatty Acid Synthase Expression is Strongly Related to Menopause in Early-Stage Breast Cancer Patients

- FASN tumor expression levels are associated with tumor pathological stage in early-stage breast cancer patients.
- FASN expression in early-stage breast cancer tumor tissue is strongly associated to menopause status, age and body mass index.

Different Fatty Acid Metabolism Effects of (−)-Epigallocatechin-3-Gallate and C75 in Adenocarcinoma Lung Cancer

- C75 and EGCG (FASN inhibitors) display strong cytotoxicity on A549 lung cancer cells.
- C75 and EGCG have different mechanisms of action on fatty acid metabolism and on signaling pathways:
    - C75 and EGCG block FASN activity, but EGCG also reduces its protein expression levels.
    - C75 stimulates fatty acid ß-oxidation (CPT-1 enzyme) and EGCG does not.
    - C75 has slight effect on HER1/ERK1/2/AKT/mTOR signaling pathway, whereas EGCG markedly inhibits their activation. We corroborate the existence of a FASN-HER1 loop in lung cancer.

Conclusions

- Both, C75 and EGCG, induce apoptosis in A549 lung cancer cells.

- Both, C75 and EGCG, reduce the growth of human adenocarcinoma lung cancer xenografts. But, C75 induces strong body weight loss and EGCG does not.

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

- Mechanisms of acquired resistance to trastuzumab and lapatinib include several changes in HER pathways (HER receptors, ERK1/2, AKT) but mTOR activation and FASN overexpression are maintained in trastuzumab and lapatinib acquired resistance.
- EGCG is cytotoxic in both parental (SK) and resistant (SK**TR**, SK**LR** and SK**LTR**) cell lines. G28UCM, a novel EGCG derivative, is more potent and cytotoxic than EGCG in parental and resistant cells.
- Combination of anti-FASN (C75 and EGCG) compounds together with HER2-pathway inhibitors (pertuzumab or temsirolimus) improves the effects of each compound alone, in sensitive and resistant cells. These synergistic effects are strongly improved when using G28UCM as FASN inhibitor in combination with pertuzumab and temsirolimus.
- In parental (HER2-PDX) and resistant (HER2-PDX**R**) HER2-positive breast cancer patient derived xenografts, simultaneous treatment of EGCG with pertuzumab or temsirolimus results in increased reduction of tumor growth compared with each treatment alone, with any signs of body weight loss and other side effects.

CONTRIBUTION TO THE FIELD AND NEXT STEPS

Cancer research has developed several therapies (surgical, chemotherapy and directed) and has progressed in diagnostic methods which have improved the prognosis of several types of cancer. Anyway, some cancers (such as lung, among others) continue having a bad prognosis. For instance, lung cancer is the most common cause of death from cancer worldwide. The ratio of mortality to incidence is as high as 0.87. The median survival rarely exceeds 10 months in unselected patients with metastatic NSCLC disease treated with conventional chemotherapy. Other cancers, such as HER2+ breast cancer, develop resistance to nowadays treatments, even for those treatments directed against HER family receptors. For instance, trastuzumab containing adjuvant therapy treated patients relapse and nearly all patients receiving trastuzumab for metastatic disease progress after a year of treatment. Metastatic breast cancer patients treated with lapatinib become refractory with tumor growth or spread. Thus, it is required the study and development of new biomarkers and new targeted therapies for those and other human carcinomas.

Although expression and inhibition of fatty acid synthase have been studied in some types of cancers, it is needed to go in depth in the molecular mechanisms that promote the anti-tumoral effects of FASN inhibition. Also, in order to take advantage of FASN inhibition in clinical practice, it is required to develop new and more powerful and stable FASN inhibitors, which do not exhibit toxicity effects.

The contribution of this thesis into the field provides new arguments to continue studying FASN as a therapeutic target (alone or in combination) for non-small cell lung cancer and HER2-breast cancer (sensitive and resistant to anti-HER2 drugs).

Contribution to the field and next steps

We were the first group describing that (-)-epigallocatechin-3-gallate reduces the expression of FASN protein and at the same time abolish the activation of HER1 and its downstream pathway in lung cancer. Differently, C75 inhibits the activation of FASN and, p-HER1 inhibition is lately produced. With these results, we corroborated a FASN/HER1 and HER1/FASN loop that had been previously described in other types of cancer, such as breast cancer.

Moreover, we showed that EGCG has the same anti-tumoral effects as the specific FASN-inhibitor, C75, in cellular and xenograft models of HER1/FASN lung cancer and HER2/FASN breast cancer (sensitive and resistant to trastuzumab and/or lapatinib). In addition, EGCG did not show toxicity in vivo.

Combinations of drugs targeting epidermal growth factor receptors family with EGCG have showed synergistic effects against tumoral proliferation in cells and patient derived xenograft of HER2-positive breast cancer (both in sensitive and in resistant to anti-HER2 drugs models). This supports the combination of FASN-inhibition together with the inhibition of other targets from HER pathway to deeper abolish proliferation and viability signaling of tumoral cells.

G28UCM, a novel EGCG derivative previously developed by our group, has improved the in vitro anti-tumoral effects of EGCG and has increased the synergism in combination with pertuzumab and temsirolimus.

We also included a preliminary study of early-stage breast cancer patient samples in which we observed association between FASN expression and tumoral stage and anthropometric characteristics. These results demonstrate that FASN expression could be a biomarker of tumoral stage and the anthropometric characteristics that could bring to the development of breast cancer.

As a whole, the results obtained in the presented thesis aim as to continue studying FASN as a possible biomarker and target for those FASN-positive cancers with bad prognosis. The studies presented in this thesis are preliminary results that bring us to the extension to new projects in order to deep inside in the molecular mechanisms of the anti-tumoral effects of FASN inhibition.

We are conscious that EGCG is a multi-target compound and its anti-tumoral effects could be exerted for the inhibition of other targets apart from FASN. In previous studies of our group, the anti-tumoral effects produced by EGCG (and G28UCM) treatment were associated with FASN expression. Showing that, at least in part, the anti-tumoral effects of EGCG are related to FASN inhibition. Anyway, our group is absorbed in a new project to decipher the molecular mechanisms that distinct FASN inhibitors (C75, EGCG and G28UCM) use to produce tumoral inhibition in different types of cancer with different profiles (including lung, HER2-breast cancer and triple negative breast cancer). This project includes, among other experiments, siRNA inhibition of several proteins involved in HER-FASN pathway and other pathways to figure out the effect of FASN inhibition in each of them.

This project also includes an extensive evaluation of FASN in breast and lung cancer patients' tissue samples to determine its correlation with clinicopathological data and potential use as biomarker or therapeutic target. After our preliminary study of early-stage breast cancer patient samples, in order to obtain significant results, we have increased the number of patients' tissue samples. Also, different types of carcinomas have been included in the new patients study, including sensitive and resistant HER2 breast cancer, triple negative breast cancer and lung cancer.

On the other hand, we have also bet on the large scale synthesis of the new inhibitor, G28UCM. In the presented thesis we made an attempt to study G28UCM, alone and in combination, in HER2-positive breast cancer cells (sensitive and resistant). Unfortunately, the study of G28UCM in vivo was not possible due to its difficult, inefficient and costly large scale synthesis. The new project includes, in collaboration with a chemical group of the University of Girona, the efficient development of large scale synthesis of G28UCM. This will allow us to continue studying the anti-tumoral effects of G28UCM, in vitro and in vivo.

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