

Preclinical Evaluation of Fatty Acid Synthase and EGFR Inhibition in Triple Negative Breast Cancer

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Running Title: Dual FASN and EGFR blockade in TNBC.

KeyWords: Triple Negative Breast Cancer (TNBC), FASN, Chemoresistance, TNBC Primary Tumor Samples, Orthoxenograft.

Finacial Support: This work was supported in part by the Spanish Instituto de Salud Carlos III (ISCIII) (FIS PI11/00692; TP and PI14/00329; TP), Fundación Ramón Areces (TP), the support of Catalonian Government (2014SGR00868), U.S. Department of Defense Breast Cancer Research Program Award (#W81XWH1210077, ALW) and Metavivor Research and Suport Inc (#50502690, DHL). The University of Girona awarded AG-P, SP and AB with a predoctoral grant.

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Competing Interests: None of the authors have any conflict of interest that can affect the impartiality of the research reported.

Manuscript Information: This manuscript contains 27 pages, 4944 words, 4 figures and 2 tables. The abstract contains 240 words.

Statement of Translational Relevance

Triple Negative Breast Cancer (TNBC) is the molecular subtype associated with the highest relapse rates and worse outcome. Patients lack targeted therapy and are generally treated with cytotoxic chemotherapy. There is a need to develop novel therapeutic strategies based on TNBC biology. Although EGFR overexpression is observed in 50-70% of TNBC, its inhibition alone or in combination with chemotherapy has resulted in minimal clinical benefit. The enzyme fatty acid synthase (FASN) has been shown to be a key therapeutic target in several human carcinomas. We found FASN is expressed in all TNBC primary tumor samples tested. We then showed therapeutic benefit of FASN inhibition in sensitive and chemoresistant TNBC preclinical models, as well as strong synergistic anti-tumor effect of FASN inhibitors in combination with cetuximab (an EGFR inhibitor). Our findings provide a rationale for further investigation of FASN inhibitors in combination with anti-EGFR signaling agents in TNBC.

Abstract

Purpose: Triple Negative Breast Cancer (TNBC) lacks an approved targeted therapy. Despite initial good response to chemotherapy, 30% of the patients relapse within 5 years after treatment. EGFR overexpression is a common marker in TNBC, and its expression has been correlated with poor outcome. Inhibition of Fatty Acid Synthase (FASN) activity leads to apoptosis of human carcinoma cells overexpressing FASN. We tested the hypothesis that blocking FASN in combination with anti-EGFR signaling agents would be an effective antitumor strategy in sensitive and chemoresistant TNBC.

Experimental Design: Several TNBC cell lines and 29 primary tumors were included to determine whether FASN is a potential target in TNBC. Doxorubicin-

resistant TNBC cell lines (231**DXR** and HCC**DXR**) have been developed and characterized in our laboratory. Cellular and molecular interactions of anti-FASN compounds (EGCG and C75) with cetuximab were analyzed. *In vivo* tumor growth inhibition was evaluated after cetuximab, EGCG or the combination in TNBC orthoxenograft models.

Results: TNBC cell lines showed overexpression of FASN enzyme and its inhibition correlated to FASN levels. FASN staining was observed in all of the 29 TNBC tumor samples. *In vitro*, EGCG and C75 plus cetuximab showed strong synergism in sensitive and chemoresistant cells. *In vivo*, the combination of EGCG with cetuximab displayed strong antitumor activity against the sensitive and chemoresistant TNBC orthoxenografts, without signs of toxicity.

Conclusions: Our results show that the simultaneous blockade of FASN and EGFR is effective in preclinical models of sensitive and chemoresistant TNBC.

Introduction

Breast cancer is the most prevalent and deadly cancer type in western women (1). Triple Negative Breast Cancer (TNBC) represents 15%-20% of the patients diagnosed with breast carcinomas and is characterized by the lack of expression of estrogen and progesterone receptors (ER/PR) and no amplification of the HER2 oncogene (2). TNBC runs an aggressive course and has a poor prognosis. As TNBC lacks a validated targeted therapy, patients are treated mainly with cytotoxic chemotherapy (anthracyclines and taxanes). Even though TNBC patients show a good initial response to chemotherapy, the recurrence rate within 5 years following diagnosis is about 30% (3,4).

Perou *et al.* defined five major molecular subtypes within breast cancer: luminal A, luminal B, HER2-enriched, basal-like and claudin-low or mesenchymal-like (5–7). The two major subtypes comprised in the TNBC are basal-like (BL) (~50%) followed by the

mesenchymal like (ML) (~20%) (8). Other molecular subtypes are also represented within TNBC subtype, because TNBC is such a heterogeneous disease. However, between 50-70% of TNBC have been shown to express the epidermal growth factor receptor (EGFR) (7,9), and its expression has been associated with poor prognosis (10). While EGFR inhibition has been considered a promising approach for TNBC, minimum benefit has been observed in the clinical settings alone or in combination with chemotherapy (11,12).

Alteration of metabolism is one of the hallmarks of cancer, providing advantages to cancer cells in the tumor environment (13,14). Fatty acid synthase (FASN), the major enzyme capable of de novo synthesis of fatty acids, has been described to be overexpressed in several carcinomas, such as breast, prostate and colon among others (15). This enzyme is not expressed in normal tissue, except in lipogenic tissues where its expression is tightly regulated by diet. In cancer, however, cells overexpress FASN enzyme to synthesize almost all their fatty-acids de novo (14,16). We and others have shown that blocking FASN activity has anticancer activity via the apoptotic pathway *in vitro* and *in vivo* (17–19), by disrupting lipid membrane synthesis, modification of protein palmitoylation and deregulating oncogenic signaling pathways (20,21).

The purpose of this study was to evaluate FASN as a target or co-target in sensitive- and chemoresistant-TNBC. First, we evaluated FASN expression in a panel of TNBC cell lines and in TNBC primary tumor samples. *In vitro*, C75 and EGCG (FASN inhibitors) were tested for anti-tumor efficacy in combination with chemotherapy and with cetuximab (an EGFR inhibitor). *In vivo* orthoxenografts of two different sensitive and resistant TNBC models were performed to test the anti-tumor effect of EGCG in combination with cetuximab. FASN, EGFR and downstream protein expression were

studied to determine cellular signaling pathways changes resulting from the different mono and dual treatments.

Material & Methods

Cell culture and development of doxorubicin-resistant triple negative breast cancer cells

All triple negative breast cancer cells were obtained from ATCC (Manassas, VA, USA). MDA-MB-231, MDA-MB-468 and MDA-MB-157 were routinely grown in DMEM (Gibco). HCC1806, DU4475 and BT549 were maintained with RPMI. Cell culture media were supplemented with 10% FBS (HyClone Laboratories) 1% L-glutamine (Gibco), 1% Sodium pyruvate (Gibco), 50 U/ml Pen/Strep (Linus). RPMI was additionally supplemented with 0.023 IU/ml insulin for BT549 culture. Cell lines were kept at 37°C and 5%CO₂ atmosphere. Doxorubicin-resistant cells MDA-MB-231 (231DXR) and HCC1806 (HCCDXR) were developed using a stepwise selection method. Increasing doses of doxorubicin (TEDEC-Meiji Farma) were performed until their corresponding IC₅₀ was reached. Briefly, initially cells were treated with a concentration of doxorubicin of 0,1xIC₅₀. After 48h, the treatment medium was replaced for fresh medium. When the cells were capable of growing and reached appropriate confluence, they were treated with double the previous doxorubicin concentration for 48h. The stepwise selection method was subsequently performed until the final concentration of doxorubicin achieved the parental IC₅₀. It took around 6 month for each cell line. Resistance was confirmed by cell viability assay.

Western blot analysis of cell and tumor lysates

Parental and resistant TNBC cells were synchronized by starvation in serum-deprived medium (0.5% FBS) for 24 hours. Cells were lysed in ice-cold lysis buffer (Cell Signaling Technology, Inc.) with 100 µg/mL PMSF by vortexing every 5min for 30 min. Frozen tumors were first ground (Dounce) in ice-cold lysis buffer and then vortexed as described above. Equal amounts of protein were heated in LDS Sample Buffer with Sample Reducing Agent (Invitrogen) for 10 min at 70°C, electrophoresed on SDS-polyacrylamide gel (SDS-PAGE), and transferred onto nitrocellulose membranes. Blots were incubated for 1h in blocking buffer (5% powdered-skim milk in Phosphate-buffered saline 0.05% Tween (PBS-T)) and incubated overnight at 4°C with the appropriate primary antibodies diluted in blocking buffer. Specific horseradish peroxidase (HRP)-conjugated secondary antibodies were incubated for 1h at room temperature. The immune complexes were detected using a chemiluminescent HRP substrate [Super Signal West Femto (Thermo Scientific Inc.) or Immobilon Western (Millipore)]. β-actin (Santa Cruz Biotechnology Inc.) was used as a control of protein loading. Western blot analyses were repeated at least three times and representative results are shown.

Cell viability assays

Parental and resistant cells were plated in 96-well plates at a cell density of 4×10^3 cells per well in their corresponding growth medium. After 24 hours, growth medium was removed and 100 µL of fresh medium containing the corresponding concentration of doxorubicin (TEDEC-Meiji Farma), EGFR inhibitor [cetuximab (erbitux®, Merck)], or FASN inhibitors [EGCG (Sigma) or C75 (Sigma)] were added to each well. For drug-combination experiments cells were treated with a fixed FASN inhibitor concentration

in combination with a serial of concentrations of (i) doxorubicin for 48 h or (ii) cetuximab for 4 days. Same treatments were assessed in monotherapy. Following treatment, cell viability was measured using the standard colorimetric MTT assay as previously described (19). Combinatorial effects were evaluated using the Interaction index (I_x) = \sum (% CPI drug alone) / % CPI combination. $I_x < 1$, synergism; $I_x = 1$, additivism and $I_x > 1$, antagonism.

Quantitative real-time PCR analysis

Cells were PBS washed, and then 1mL of Qiazol (Qiagen) was added. Total-RNA was isolated using RNeasy mini kit (Qiagen) following the instructions provided by the manufacturer. RNA was reverse-transcribed into complementary DNA (cDNA) using High Capacity cDNA Archive Kit (Applied Biosystems). Gene expression levels of EGFR and FASN were assessed using LightCycler® 480 Real-time PCR System (Roche) with LightCycler® 480 SYBR Green I Master (Roche). Primers were designed as follows: FASN (Fw 5'-CAGGCACACACGATGGAC-3' and Rv 5'-CGGAGTGAATCTGGGTTGAT-3'), EGFR (Fw 5'-CATGTCGATGGACTTCCAGA-3' and Rv 5'-GGGACAGCTTGGATCACACT-3') and β -actin (Fw 5'-ATTGGCAATGAGCGGTTC-3' and Rv 5'-CGTGGATGCCACAGGACT-3'). RT-PCR analyses were performed at least three times and each gene was run in triplicate. β -actin was used for normalization of EGFR and FASN expression levels.

Patients and tissue samples

FASN and EGFR tumor expression levels were retrospectively evaluated in paraffin-embedded core-biopsies of 29 patients with TNBC diagnosed in the Hospital Josep Trueta of Girona between 2007 and 2012. Tissue slides were previously treated with the

PT link (DAKO) solution at high pH as a deparaffination and antigen retrieval steps. Immunohistochemical staining was performed using an anti-Fatty Acid Synthase polyclonal antibody (dilution 1:100, Enzo Life Sciences) and an anti-EGFR monoclonal antibody (dilution 1:100, CellSignaling) with the detection kit EnVision™ (DAKO) in the AutostainerPlus Link (DAKO). FASN expression was graded from 0 to 2+: 0, no staining; 1+, moderate staining; 2+ high staining. EGFR expression was classified as positive when >5% of the cells showed specific membrane staining. Analysis was carried out by a board-certified pathologist. Written informed consent was obtained from all patients before the study was started. This study was approved by the ethical board of the Dr. Josep Trueta University Hospital.

***In vivo* studies: ortoxenografts experiments**

TNBC cell lines HCC1806 (HCC) and MDA-MB-231 (231) and their doxorubicin-resistant derivatives 231**DXR** and HCC**DXR** were orthotopically implanted (2×10^6 cells in 25 μ l matrigel) into both inguinal cleared mammary fat pads of NRG (NOD-Rag1^{<null>} IL2rg^{<null>}) mice (The Jackson Laboratory). When tumors reached 50 mm³, animals were randomized into four different treatment groups. Each group received intraperitoneal (i.p.) injection of control (vehicle alone, 3d/wk), 30 mg/kg EGCG 3d/wk, 0.5mg/mice cetuximab 1d/w, or combination of EGCG + cetuximab. Tumor xenografts were measured with calipers and tumor volumes were determined using the formula: $(\pi/6 \times (v1 \times v2 \times v2))$, where v1 represents the largest tumor diameter, and v2 the smallest one. Body weight was registered every two days. At the end of the experiment or when tumors reached 1 cm in diameter, animals were weighed and then euthanized using CO₂ inhalation. Tumors were stored at -80°C. Apoptosis in control and treated tumors was analyzed by Western-blot (PARP). *In vivo* mouse experiments were performed in Dr. A. Welm's laboratory at the Huntsman Cancer

Institute (University of Utah, Salt Lake City, Utah, USA) and at the Oklahoma Medial Research Foundation (ORMF, Oklahoma, USA). All protocols and experiments involving animals, including care and handling, were conducted in accordance with guidelines on animal care and use established by institutional guidelines. All mice were maintained in a specific pathogen-free facility with controlled light/dark cycle, temperature, and humidity. All surgery was performed under inhaled isoflurane anesthesia; burprenorphine was administered as analgesic to mice after surgery. All efforts were made to minimize pain and distress.

Statistical analysis

Data were analyzed by Student's t-test when comparing two groups or ANOVA using a Bonferrony post-hoc test when comparing more than 2 groups. Non-Parametric analysis by Kruskal Wallis was used when data did not follow normal distribution. 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 \pm standard error of the mean (SEM). All observations were confirmed by at least three independent experiments.

Results

Triple negative breast cancer cell lines express fatty acid synthase (FASN) enzyme and are sensitive to FASN inhibitors

We screened several TNBC cell lines for FASN, EGFR and downstream protein expression and activation status (Fig. 1A). Molecular classification of TNBC cells was assessed based on Lehman *et al.* (7). Interestingly, FASN was detected in all TNBC cells examined, and showed higher protein expression levels in the basal-like than in the mesenchymal-like molecular subtype. Basal-like cell lines Du4475 and MDA-MB-468

showed the higher mRNA levels, while the other cell lines showed lower and similar FASN mRNA levels (Supplementary Fig. S1A). Activation of the EGFR (as assessed by phosphorylation) was also observed in almost all cell lines. The EGFR mRNA and protein expression showed that MDA-MB-468 had the highest levels and Du4475 the lowest (Supplementary Fig. S1B).

To further investigate the role of FASN as a target in TNBC, a cell proliferation inhibition (CPI) assay was performed using C75 and EGCG as FASN inhibitors (Fig. 1b). The most sensitive cell lines to C75 were the BL HCC1806 (IC_{50} : $29.4 \pm 1.4 \mu M$) and MDA-MB-468 (IC_{50} : $31.38 \pm 2.5 \mu M$). Higher values of IC_{50} were obtained in the ML subgroup for the lines MDA-MB-231 (IC_{50} : $46.6 \pm 2.2 \mu M$) and MDA-MB-157 (IC_{50} : $58.3 \pm 2.75 \mu M$). EGCG showed IC_{50} values ranging from 149 ± 6.7 in MDA-MB-231 to 222 ± 6.7 in BT549.

Doxorubicin-resistant TNBC cellular models are sensitive to FASN inhibitors

As preclinical models of acquired resistance to chemotherapy, we developed two TNBC cell lines resistant to doxorubicin. MDA-MB-231 (231) from the ML subgroup and the BL cell line HCC1806 (HCC) were chosen as two different models for FASN expression levels. 231 doxorubicin-resistant (231**DXR**) and HCC doxorubicin-resistant (HCC**DXR**) cells were developed in our laboratory by dose-increasing treatments of doxorubicin as described in 'Material and Methods' section. 231 and HCC were significantly more sensitive to doxorubicin for doses ranging from 10nM to 350nM compared to the resistant models 231**DXR** and HCC**DXR** respectively (Supplementary Fig. S2). As shown in Fig. 1C, 231**DXR** cells showed no apparent changes in either total protein or activation level in any of the proteins we examined related to the EGFR pathway. HCC**DXR**, however, showed increased expression levels of total EGFR, and

the downstream proteins p-AKT and p-ERK1/2. On the other hand, p-EGFR levels decreased compared to the parental, consistent with previous findings showing doxorubicin directly decreases EGF expression (22). FASN showed similar levels between the parental and resistant cells. Gene expression for FASN and EGFR proteins did not show any significant differences in 231 or HCC versus 231**DXR** and HCC**DXR**, respectively (Supplementary Fig. S3). Interestingly, no changes in sensitivity to FASN inhibitors alone (C75 or EGCG) were observed between parental and doxorubicin resistant cell lines (Supplementary Fig. S4).

FASN inhibition resensitizes doxorubicin resistant models to chemotherapy

Resistant cell lines 231**DXR** and HCC**DXR** showed similar FASN expression levels and FASN inhibitor sensitivity when compared to parental cells (Fig. 1C and Supplementary Figure S4). Therefore, we tested the effects of EGCG and C75 in combination with doxorubicin in parental and doxorubicin-resistant models. The results of CPI ratios induced by the mono treatments versus the dual treatments are shown in Table 1 (for MTT results see Supplementary Fig. S5). Doxorubicin (50-100-150nM) combined with C75 (40 μ M) exhibited synergic interaction index (Ix) in 231 and 231**DXR**. Furthermore, resistant cells 231**DXR** showed increased synergic interaction compared to 231. Similar results were observed in HCC**DXR**, because the addition of C75 (20 μ M) to the doxorubicin (50-100-150nM) treatment improved the effect of the combination compared to the parental cell line. The combination of doxorubicin (50-100-150nM) and EGCG (100 μ M) in 231 and 231**DXR** showed an additive effect in both cell lines. Interestingly, EGCG (120 μ M) combined with doxorubicin (50-100-150nM) also showed a major benefit in HCC**DXR** when compared to the parental.

FASN inhibitors shows strong synergism with cetuximab in sensitive and resistant cell lines

Several studies reported that EGFR is a common receptor expressed in TNBC (23–24). *In vitro*, EGFR activation is observed in almost all TNBC cell lines (Fig 1A), and 70% of the TNBC tumors evaluated in this study were positively stained for EGFR protein (Fig 1B). Furthermore, we have shown that the expression of EGFR is either maintained or increased in our doxorubicin-resistant models (Fig 1C). Therefore, the effect of combining cetuximab and the FASN inhibitors C75 and EGCG was studied.

The combination of cetuximab (0.5-1-2 μM) with EGCG or C75 showed a strong synergistic effect in all sensitive and resistant cells (Table 2). 231**DXR** showed lower I_x with both C75 (30 μM) and EGCG (50 μM) compared to parental 231 cells. However, HCC and HCC**DXR** showed similar ratio values for both C75 (20 μM) or EGCG (25 μM) when combined with cetuximab.

Interestingly, parental and resistant cells showed no differences in cell proliferation inhibition when treated with anti-FASN compounds alone. Cetuximab otherwise significantly increased CPI *in vitro* in the HCC**DXR** cell line (Supplementary Fig. S6 S5).

Synergistic antitumor activity of EGCG in combination with cetuximab in sensitive and doxorubicin resistant TNBC xenografts

To validate our *in vitro* findings, we then tested the antitumor activity of EGCG, cetuximab and the combination treatment *in vivo*. We developed orthotopic xenograft models for 231, its chemoresistant derivative 231**DXR**, HCC and its resistant derivative

HCCDXR. EGCG (30mg/Kg for 3d/w) and cetuximab (0.5mg/mice 1d/w) as single agents reduced tumor growth in 231 xenografts over 12 days of treatment (Fig. 2A). Control animals achieved a relative volume growth of 3.3 ± 0.33 , whereas EGCG reduced it to 3.0 ± 0.27 and cetuximab to 2.5 ± 0.29 . Interestingly, dual FASN and EGFR blockade showed significantly higher tumor growth inhibition (2.0 ± 0.07) compared to the control ($p < 0.01$) or EGCG alone ($p < 0.05$, Fig. 2A). Under the same schedule, **231DXR** xenograft showed a similar relative volume growth as the sensitive model (3.41 ± 0.4 , Fig. 2B). EGCG showed tumor growth inhibition by day 12 with a relative volume growth of 2.66 ± 0.33 , but cetuximab treatment did not inhibit growth. By 12 days of treatment, the relative volume growth for cetuximab was 3.52 ± 0.48 , similar to the control group. Again, the combination of EGCG and cetuximab induced significant tumor growth inhibition (1.98 ± 0.17) compared to control ($p < 0.01$) and to cetuximab alone ($p < 0.05$). Despite the absence of tumor shrinkage, the combination treatment (EGCG plus cetuximab) significantly reduced tumor growth in both 231 and **231DXR** orthotopic xenografts.

In the basal-like HCC xenograft model, EGCG (30mg/Kg for 3d/w) and cetuximab (0.5mg/mice 1d/w) as single agents reduced tumor growth after 10 days of treatment (Fig. 2C). Compared to the tumor volume increase observed in the control group (17.79 ± 2.76), EGCG treatment achieved a relative volume growth of 13.03 ± 2.58 , while cetuximab significantly reduced the ratio to 6.33 ± 1.25 ($p < 0.05$). The combination treatment significantly reduced the tumor growth compared to the control ($p = 0.001$) and to EGCG alone ($p < 0.01$), with a relative volume growth of 4.87 ± 1.06 . In the **HCCDXR** xenograft model, control animals reached a relative volume growth of 5.02 ± 1.38 over the same period of time, significantly lower compared to the parental model ($p < 0.05$, Fig. 2D). EGCG and cetuximab as single agents had higher activity in the **HCCDXR**

model compared to HCC, with a relative volume growth of 3.04 ± 0.76 and 0.14 ± 0.04 , respectively (cetuximab vs control $p < 0.01$; cetuximab vs EGCG $p < 0.01$). The relative volume growth in the combination setting decreased up to 0.19 ± 0.1 compared to control ($p < 0.05$) and EGCG alone ($p < 0.05$). In the HCC**DXR** model, cetuximab used as a single agent quickly (day 4) displayed strong antitumor activity, leading to effective tumor regression (0.76 ± 0.12). Thus, the combination of EGCG plus cetuximab was not synergic at these treatment doses in the HCC**DXR** xenograft. For total volume results refer to Supplementary Fig. 7.

Tumor samples from the *in vivo* TNBC models were collected to evaluate drug response signaling mechanisms. 231 and 231**DXR** tumor samples were collected after 12 days of treatment. For HCC and HCC**DXR**, samples were collected after 1 cycle (4 days) because in cetuximab and EGCG plus cetuximab groups total tumor shrinkage was observed after 10 days of treatment. 231, HCC and HCC**DXR** tumor samples showed increased apoptosis (assessed by cleavage of PARP) in the combination treatment settings compared to single treatments and control groups (Fig. 2E). 231**DXR** however showed increased apoptosis in both single and combination treatments compared to control. Total EGFR and p-EGFR levels decreased in 231, 231**DXR** and HCC**DXR** after cetuximab and EGCG plus cetuximab co-treatment. HCC, however, showed a decrease in p-EGFR after cetuximab and combination treatment, but total EGFR levels decreased only after cetuximab treatment. No changes in FASN protein levels were observed among mono- and co-treatments in 231, 231**DXR**, HCC and HCC**DXR** tumors.

NRG mice treated with EGCG (30mg/Kg for 3d/w) plus cetuximab (0.5mg/mice 1d/w) were weighed daily to evaluate *in vivo* body weight effect. With respect to initial

weight, no significant changes on food and fluid intake or body weight after treatment were identified (Fig. 2F).

Dual blockade of FASN and EGFR changes EGFR downstream activated proteins

We have shown that EGCG combined with cetuximab was synergic in several cellular and animal models of TNBC. Therefore, we planned to study the effect of the EGCG plus cetuximab on FASN, EGFR, and downstream protein expression and activation, alone or in combination, in sensitive (231 and HCC) and resistant (231**DXR** and HCC**DXR**) cells (Fig. 3).

Cetuximab as a single agent induced a decrease in total EGFR in 231, 231**DXR** and HCC**DXR** cellular models. On the other hand, the same treatment increased total EGFR in HCC. While cetuximab treatment decreased p-EGFR levels in HCC and HCC**DXR**, 231 and 231**DXR** showed overactivation of p-EGFR after 72h of treatment. Previously described studies suggest that this activation may be likely to receptor homodimerization and autophosphorylation, and does not activate downstream proteins (22). Protein levels of AKT, ERK1/2 were maintained after cetuximab treatment in all cellular models. Regarding p-AKT activation levels, lower levels were observed compared to control in 231**DXR** and HCC**DXR** after 72h of treatment. 231 and HCC, however, showed similar or increased p-AKT levels compared to control after 72h of treatment respectively. Cetuximab treatment induced apoptosis in 231 and 231**DXR** after 72h, while cleaved PARP is observed already after 48h in HCC**DXR**. Total PARP increases after cetuximab treatment in HCC, but cleaved PARP was not observed.

EGCG reduced the expression and activation of EGFR and slightly decreased FASN levels after 24h of treatment in all cellular models studied. Downstream proteins AKT

and ERK1/2 showed similar levels at 24h of treatment respect to the control. While p-AKT decreased after 24h of treatment, p-ERK1/2 increased considerably after both 12h and 24h in all cellular models. PARP cleavage is observed in all cell lines after EGCG treatment.

The combination of both drugs led to the down regulation after 24h of total EGFR and p-EGFR in 231, 231**DXR** and HCC**DXR**. Otherwise, p-EGFR levels decreased in HCC while total EGFR did not change during the treatment. Regarding to FASN levels, a slight decrease is observed in HCC**DXR**, while the expression level is maintained in the other cell lines. Total ERK1/2 and AKT levels were maintained during the treatment in all cell lines, except for HCC**DXR**, which showed decreased levels of total AKT after 48h of treatment. p-ERK1/2 activation status increased in 231 and 231**DXR** after 48h, while decreased in HCC and HCC**DXR**. Truncated PARP was observed in all cell lines at 24h and 48h for the combination treatment.

In summary, the combination of cetuximab and EGCG enhances the inhibitory effect observed in the EGFR pathway by single treatments, with a general decreasing of EGFR, p-EGFR and p-AKT levels in all cellular models. The activation of ERK1/2 observed after EGCG treatment was decreased or inhibited after combination treatment.

FASN and EGFR are expressed in TNBC primary tumor samples

To further validate FASN as a potential target in TNBC, FASN expression was evaluated by Immunohistochemistry (IHC) in paraffin-embedded core-biopsies from 29 TNBC tumor samples. The clinicopathological characteristics of the patients included in the study are shown in Supplementary Table S1. FASN staining was positive in all 29 TNBC samples with tumor specific staining (Fig. 4). FASN expression positivity was graded from 0-2+ and scored into three categories, as described in 'Material and

Methods' section. Staining was observed in all 29 tumor samples and 31% of them with high positivity (2+). Cellular positivity within the tumor was between 66-100% in 26 (89.7%) of the samples, and between 33-66% in 3 of them (10.3%). The EGFR receptor was also evaluated in the same 29 TNBC core-biopsies with a positivity of 75.9%.

Discussion

Triple Negative Breast Cancer (TNBC) lacks an approved targeted therapy, and it is mainly treated with a combination of anthracyclines and taxanes (25). The high rate of relapse after treatment urges for the development of new targeted therapies for these patients. TNBC breast cancer is well known for its heterogeneity (8). Here we show that FASN, the key enzyme for novo lipogenesis, is not only expressed in all TNBC cell lines included in the study, but also in TNBC patient samples. *In vitro*, basal-like cell lines express more FASN than mesenchymal-like cell lines (both at gene and protein level) and are more sensitive to the FASN inhibitor C75. Immunostaining of FASN in 29 core-biopsies from TNBC patients shows that FASN is overexpressed in all tumor samples included in the study compared to their non-tumoral, surrounding breast tissues. Recent studies performed in breast and ovarian cancers have demonstrated the overexpression and specificity for FASN staining in neoplastic tissues, and also its relationship with cancer aggressiveness (26,27).

After four decades, doxorubicin is still the most used and effective chemotherapy agent in the clinic for the treatment of breast cancer (28). For this reason, two doxorubicin-resistant models representative of the two major molecular subtypes in TNBC, 231**DXR** (ML) and HCC**DXR** (BL) were developed in our laboratory. Both models showed significant resistance when exposed to high doses of doxorubicin compared to the

parental cell lines. Several mechanisms have been described for doxorubicin resistance in the last few years (28,29), some of them regarding changes in the EGFR pathway. **HCCDXR** showed overexpression of EGFR and overactivation of AKT and ERK1/2 when compared to its parental cell line. Overexpression of EGFR in breast cancer patients after chemotherapy has been already described to be related with higher local recurrence, metastasis and so poor outcome (30,31). On the other hand, AKT and ERK1/2 have been shown to be overactivated and also linked to chemoresistance in several cancers (32,33). Interestingly, similar levels of FASN were observed in parental cell lines compared to their resistant derivate (**DXR**). Recent studies pointed out that FASN activity plays an important role in drug resistance because it allows fast synthesis of new phospholipids for membrane remodeling and plasticity (34,35). Here, we performed combinatorial experiments with doxorubicin and the FASN inhibitors C75 or EGCG in our sensitive and resistant cell lines to determine whether FASN inhibition may be useful to overcome doxorubicin resistance in TNBC. Although the EGCG antitumor effects may be due to binding probably with other cellular targets than FASN (36), we and others have previously identified EGCG as an inhibitor of FASN activity, able to induce apoptosis in several tumor cell lines and also to reduce the size of mammary tumors in animal models (17-19,37,38). Our results showed that the Interaction index for both combinations was improved in both resistant models (**231DXR** and **HCCDXR**) compared to the parental lines. Interestingly, C75 and EGCG showed same CPI between parental or **DXR** derivate when treated alone, suggesting FASN activity could play an important role in doxorubicin resistance in TNBC. In addition, FASN inhibition has been described to reverse chemoresistance in ovarian and hepatocellular cancer cells (39,40).

Several studies have shown that EGFR is overexpressed in TNBC (22,23). Our results showed a high percentage of immunostaining positivity for EGFR in the core biopsies from TNBC patients, in concordance with previous findings. The anti-EGFR compound cetuximab showed low levels of CPI *in vitro* in all cell lines except for HCC**DXR**, which showed significantly increased CPI and apoptosis compared to the parental cell line. *In vivo*, while none, low or moderate antitumor effect was observed in 231**DXR**, 231 and HCC xenografts, respectively, cetuximab treatment obtained almost complete tumor shrinkage after two cycles of treatment in HCC**DXR**. These results are consistent with the EGFR overexpression in HCC**DXR** cell line, probably as an adaptation to overcome doxorubicin-induced apoptosis. Paradoxically, this mechanism of chemoresistance could sensitize the cell line to anti-EGFR treatments. This is in agreement with the results of a phase II study combining cetuximab with cisplatin in metastatic TNBC, where the overall response rate was doubled and the progression free survival increased from 1.5 to 3.7 months when compared to cisplatin monotherapy (41).

The common overexpression of EGFR in TNBC and its relationship with cancer progression after therapy, together with FASN expression in all TNBC and in **DXR** models, provided the rationale to test combined FASN inhibitors and EGFR blockade in these settings. We found that simultaneous treatment of cetuximab and EGCG or C75 resulted in strong synergism in both parental and resistant models. Strong cooperative growth inhibition between FASN and EGFR have been also observed *in vitro* for ovarian cancer cell lines (42). The synergism between FASN and EGFR may be due to the mechanisms involving FASN blockade that results in blockage of lipid synthesis, lipid raft destabilization, and further EGFR degradation (42). Furthermore, EGCG has been described to be closely associated with destabilization and inhibition of

EGFR receptor and downstream signaling pathways (43,44). In addition, Bollu et al. recently found that FASN-dependent palmitoylation of EGFR is required for EGFR dimerization and kinase activation (45). The same authors, in a previous study, described that FASN-dependent palmitoylation also activates mitochondrial EGFR and promotes cell survival, while its inhibition leads to apoptosis (46). Furthermore, phosphorylation and activation of FASN by direct interaction with members of the EGFR family have already been described (46).

These *in vitro* results together with TNBC patient sample study, supported the evaluation of the antitumor efficacy of cetuximab in combination with the FASN inhibitor EGCG in doxo-sensitive and -resistant TNBC orthoxenografts *in vivo*. We and others have demonstrated that EGCG displays anti-tumor activity without affecting food intake or weight loss, which are common side effects of other FASN inhibitors (37,38). We have previously shown that EGCG produces apoptosis *in vitro* and *in vivo* in breast cancer (19,37,38). Here, EGCG showed strong CPI and high apoptosis *in vitro*, and moderate anti-tumour activity *in vivo*. EGFR inactivation and downregulation is observed in all cell lines after EGCG treatment *in vitro*, consistent with previous studies (43,44,48). At the same time, slightly decreases in FASN levels were observed in all cell lines, suggesting that the EGFR pathway could be implicated in FASN expression regulation in TNBC (38,49). Furthermore, p-AKT decreased its activation levels, while p-MAPK increased considerably after EGCG treatment, maybe due to a negative crosstalk between these two proteins (33,42,38).

Here, we clearly validate our *in vitro* results by showing that the combination of EGCG with the EGFR inhibitor cetuximab results in synergistic tumor growth reduction in both doxorubicin-sensitive and resistant xenograft models. Tumor analysis showed decreased EGFR and p-EGFR in the cetuximab and combination settings in all models.

However, this event was not observed in the EGCG treatment *in vivo* possibly due to the unstable nature of EGCG in serum (50). Nevertheless, increased apoptosis was observed in the combination settings in all models compared to the single treatments, even after only one cycle in the HCC and HCCDXR xenograft. *In vitro* study interactions also reveal that p-ERK1/2 levels decreased in the combination settings in the HCC and HCCDXR cell lines, and reduced the loop effect in p-ERK1/2 in the combination treatment in 231 and 231DXR.

In summary, we found FASN is expressed in all TNBC primary tumor samples tested. We then showed therapeutic benefit of FASN inhibition in sensitive and chemoresistant TNBC preclinical models, as well as strong synergistic anti-tumor effect of FASN inhibitors in combination with cetuximab. Our findings provide a rationale for further investigation of FASN inhibitors in combination with anti-EGFR signaling agents in TNBC and TNBC who progressed after chemotherapy.

Acknowledgments

We would like to thank Dr. F. Soler, Division of Pharmacy of Catalan Institute of Oncology (Girona, Spain), for kindly supplying doxorubicin. We are grateful to Oklahoma Medical Research Foundation (OMRF) PDX-PCT Core (Oklahoma, USA) for their support in the animal experiments. We also would like to thank Dr. E. López (Pathology Department Director, Hospital Josep Trueta, Girona, Spain) and Dr. J. Brunet (Medical Oncology Director, Catalan Institute of Oncology, Girona, Spain) for their support in the patient studies. Work supported by the Xarxa de Bancs de Tumors de Catalunya sponsored by Pla Director d'Oncologia de Catalunya (XBTC).

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Analysis and interpretation of data: A.Giró-Perafita, F. Pérez-Bueno, A.Sarrats, G.Viñas, AL. Welm and T. Puig.

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Study supervision: T.Puig

Tables

Table 1. Interaction index between doxorubicin and C75 and EGCG in TNBC cells sensitive or doxo-resistant.

plus	doxorubicin			
	231	231 DXR	HCC	HCC DXR
C75	0,84 ± 0,07 synergism*	0,67 ± 0,04 synergism***	1,23 ± 0,12 antagonism*	0,77 ± 0,05 synergism*
EGCG	1,03±0,05 additivism	0,99±0,09 additivism	1,36 ±0,16 antagonism**	0,93 ±0,08 additivism

Cells were treated with doxorubicin (50-100-150nM) and C75 (20µM for HCC, HCC**DXR**; 40µM for 231, 231**DXR**) or EGCG (100µM for 231, 231**DXR**; 120µM for HCC, HCC**DXR**) for 48h. Results were determined using an MTT assay and are expressed as the Ix obtained from the inhibition of cell proliferation induced for both treatments separately *versus* inhibition for the co-treatment. Experiments were performed at least three times in triplicate. *(p < 0.05), **(p < 0.01) and ***(p < 0.001) indicate levels of statistical significance.

Table 2. Interaction index between C75 and EGCG and cetuximab in TNBC cells sensitive or doxo-resistant.

plus	cetuximab			
	231	231 DXR	HCC	HCC DXR
C75	0,69± 0,08 synergism**	0,52±0,04 synergism***	0,78±0,08 synergism*	0,79±0,09 synergism*
EGCG	0,71± 0,06 synergism**	0,56±0,07 synergism***	0,69±0,08 synergism**	0,74±0,04 synergism***

Cells were treated with cetuximab (0.5-1-2 μ M) and C75 (20 μ M for HCC, HCC**DXR**; 30 μ M for 231, 231**DXR**) or EGCG (50 μ M for 231, 231**DXR**; 25 μ M for HCC, HCC**DXR**) for 4 days. Results were determined using an MTT assay and are expressed as the Ix obtained as the ratio from the inhibition of cell proliferation induced for both treatments separately *versus* inhibition for the co-treatment. Experiments were performed at least three times in triplicate. *(p < 0.05), **(p < 0.01) and ***(p < 0.001) indicate levels of statistical significance.

Legends for Figures

Figure 1. Characterization of different TNBC subtypes and doxorubicin-resistant TNBC cell models. (A) FASN, EGFR and downstream proteins expression and its activation status where analyzed by western-blot. Results shown are representative of those obtained from 3 independent experiments. **(B)** IC₅₀ values for the FASN inhibitors C75 and EGCG. Results shown are mean \pm SEM from 3 independent experiments. **(C)** Characterization of MDA-MB-231 and HCC1806 doxor resistant models (231**DXR**, HCC**DXR**) and their corresponding parental cells (231, HCC) for FASN, EGFR and downstream proteins expression and its activation status by western-blot. Results shown are representative of those obtained from 3 independent experiments.

Figure 2. EGCG plus cetuximab in sensitive and resistant TNBC ortoxenograft.

(A) Mice bearing 231 and (B) 231DXR xenografts were treated with saline (Control), EGCG (30mg/Kg, 3 days a week), cetuximab (0.5mg/mice 1 day a week) or the combination (EGCG plus cetuximab) for 12 days. (C) Mice bearing HCC and (D) HCCDXR xenografts were treated with saline (Control), EGCG (30mg/Kg, 3 days a week), cetuximab (100uL/mice 1 day a week) or the combination (EGCG plus cetuximab) for 10 days. (E) Western-blot analysis for FASN, EGFR, p-EGFR and PARP in 231, 231DXR after 12 days of treatment. HCC and HCCDXR tumor samples were collected after one cycle of treatment (4 days). (F) Body weight from NGR mice treated with the combination of EGCG (30mg/Kg, 3 days a week) and cetuximab (100uL/mice 1 day a week) for 10 days. Relative volume growth is expressed as (Final Volume / Initial Volume). Dots are mean of each experimental group and bars, SEM. *($p < 0.05$), **($p < 0.01$) and ***($p < 0.001$) indicate levels of statistically significance.

Figure 3. *In vitro* interactions between EGCG and cetuximab in 231, 231DXR, HCC and HCCDXR. FASN, EGFR, p-EGFR, AKT, p-AKT, ERK1/2, p-ERK1/2 and PARP expression were analyzed by western-blot at different times after treatments of EGCG (200 μ M for 12h and 24h), cetuximab (1 μ M at 48h and 72h), and the combination of both (for 24h and 48h) in (A) 231 and 231DXR and (B) HCC and HCCDXR.

Figure 4. Immunohistochemistry (IHC) staining of FASN and EGFR in paraffin-embedded core-biopsies from TNBC patients. (A) Adipocytes. (B) Renal tissue. (C) Ductal carcinoma. (D) Ductal carcinoma. (E) Skin. (F) Muscular tissue (G) Ductal carcinoma. (H) Ductal carcinoma.

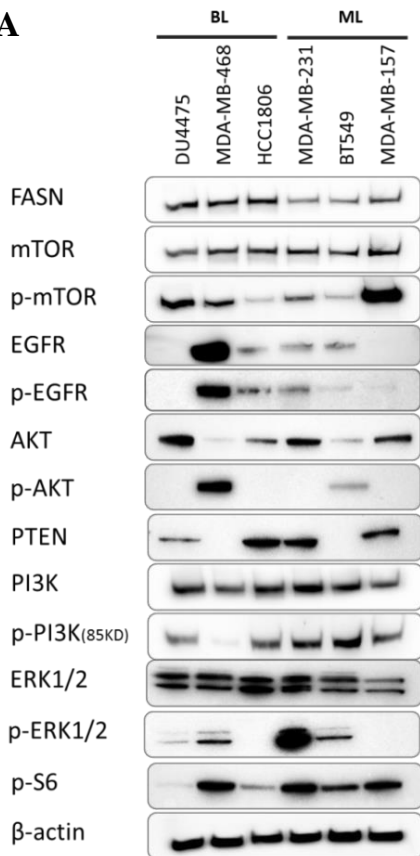
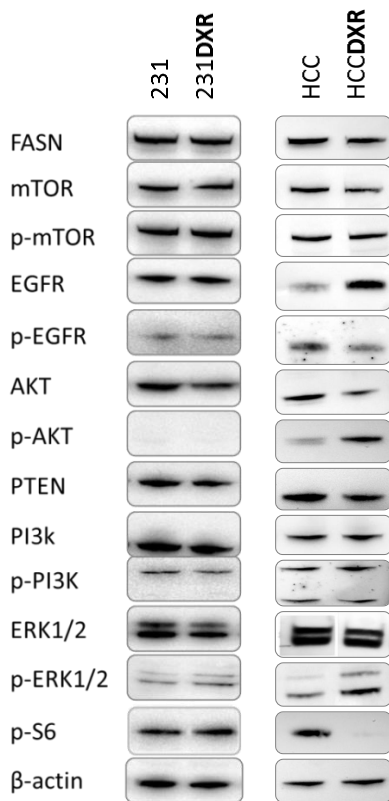
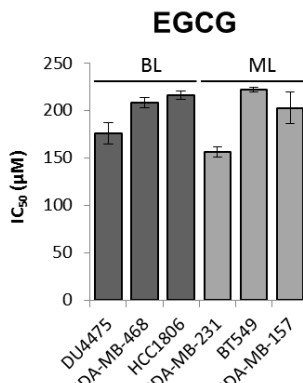
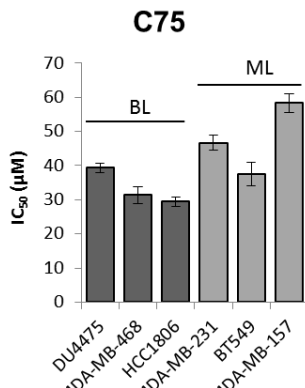
Figure 1**A****C****B**

Figure 2

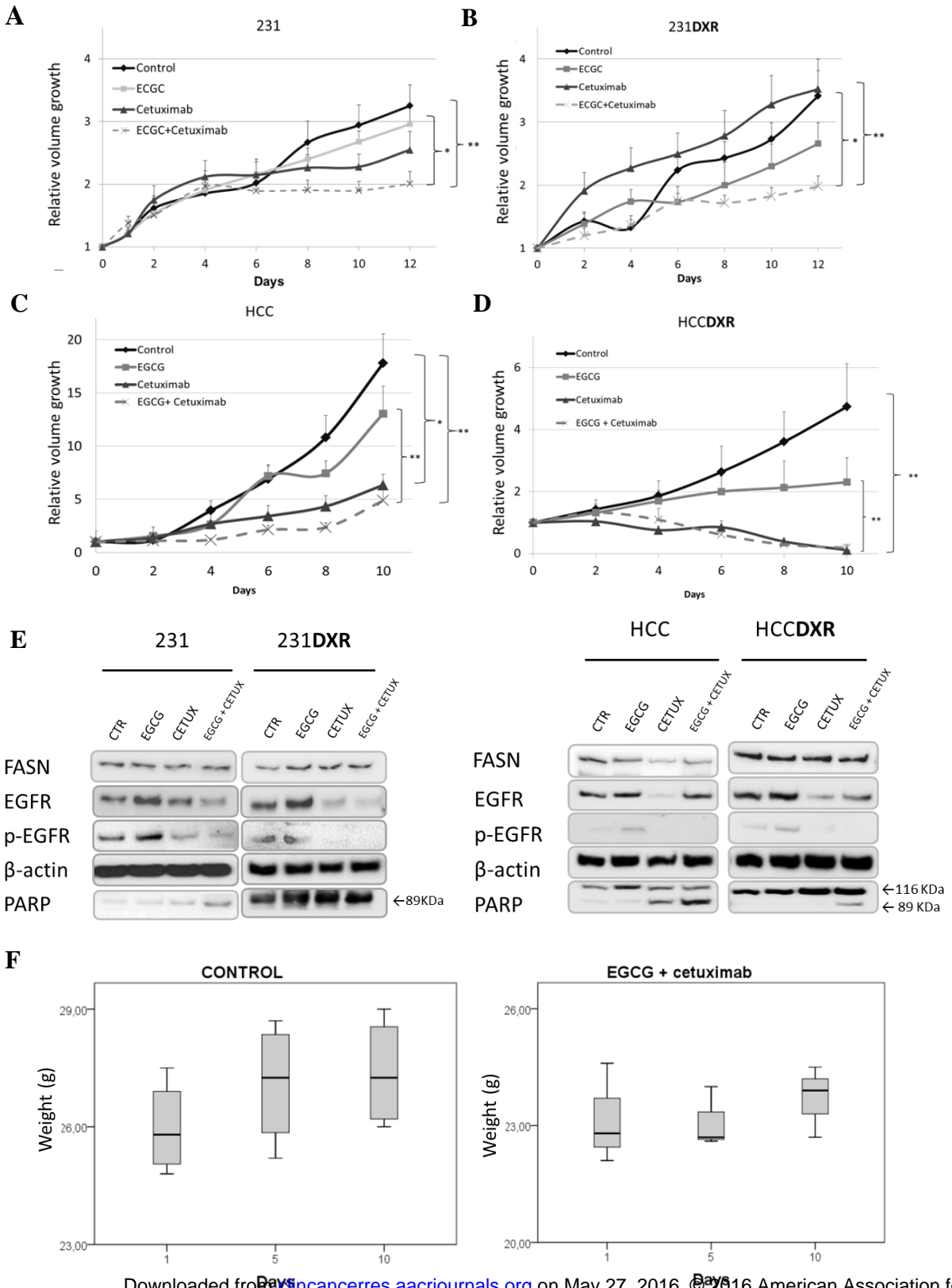


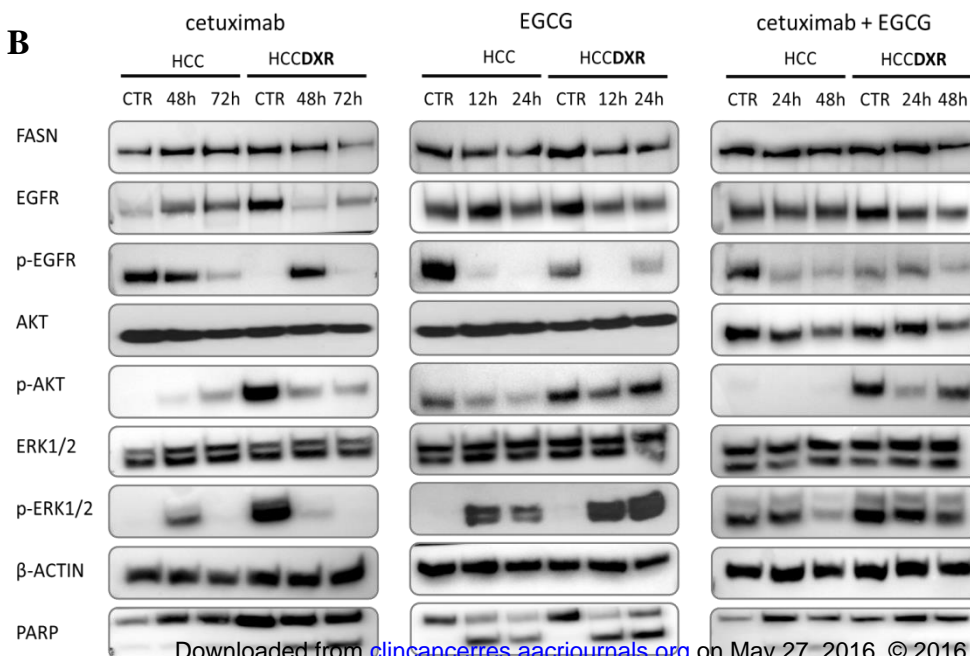
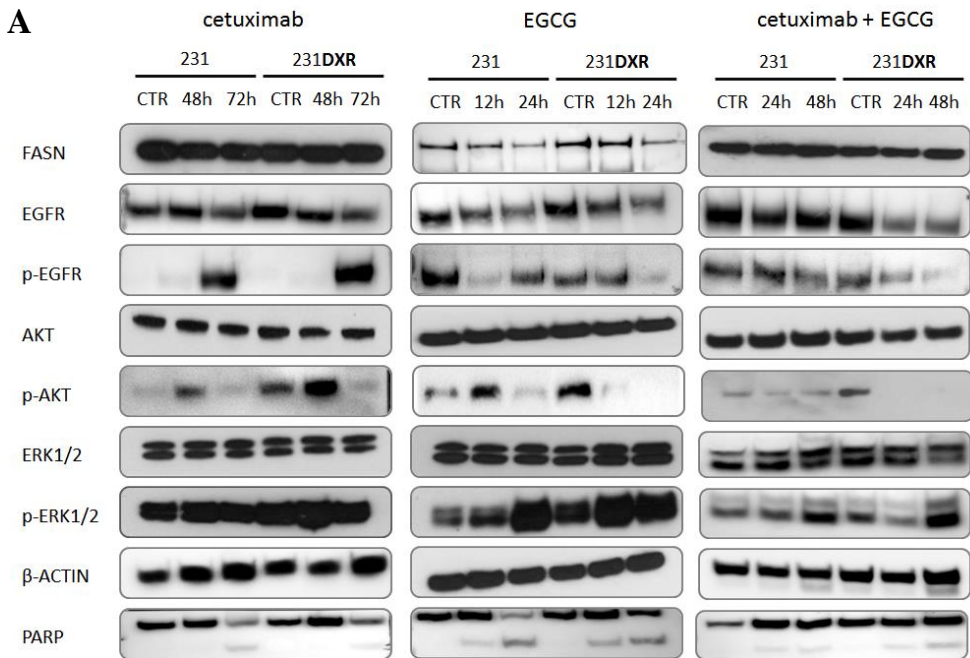
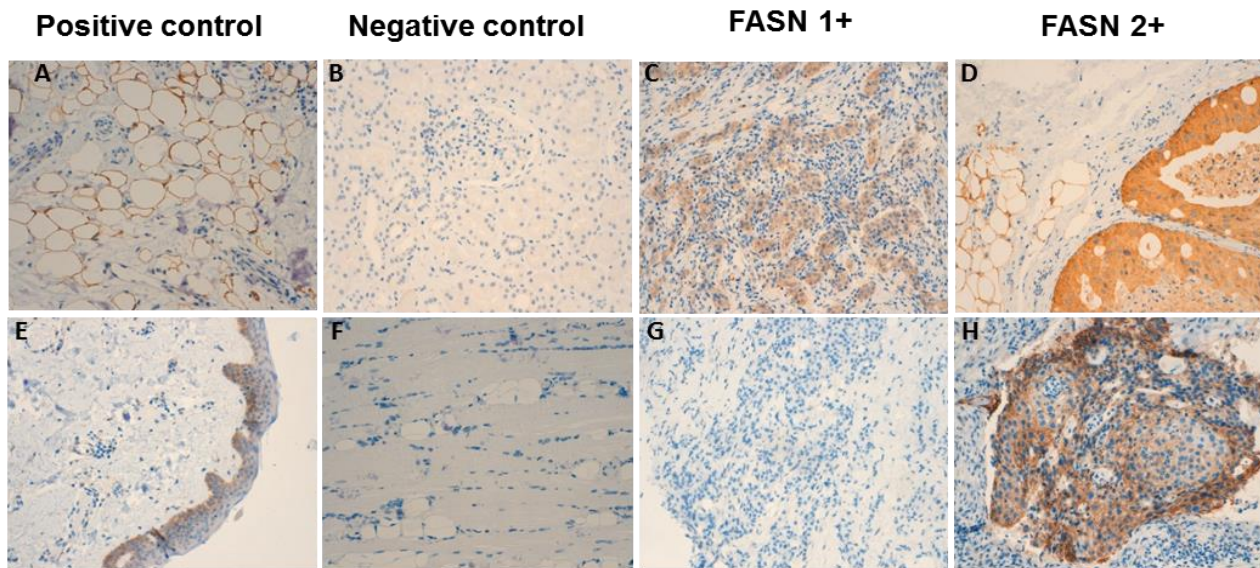
Figure 3

Figure 4



Positive control

Negative control

EGFR -

EGFR +

	Total (n=29)(%)		Total (n=29)(%)		Cellular rank (n=29) (%)		
	Positive	Negative	1+	2+	0-33%	33-66%	66-100%
FASN	29(100)	0(0)	20(68,9)	9(30,1)	0(0)	3(10,3)	26(89,7)
EGFR	22(75,9)	7(24,1)	-	-	-	-	-

Clinical Cancer Research

Preclinical Evaluation of Fatty Acid Synthase and EGFR Inhibition in Triple Negative Breast Cancer

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Clin Cancer Res Published OnlineFirst April 22, 2016.

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