Universitat de Girona

FATTY ACID SYNTHASE EXPRESSION IN TRIPLE-NEGATIVE BREAST CANCER

Cross-sectional study

FINAL DEGREE PROJECT

AUTHOR: Ariadna Roqué Lloveras
TUTORS: Dra. Teresa Puig and Dra. Gemma Viñas

Faculty of Medicine, January 2015

CONTENTS

Aquest treball de final de grau ha estat realitzat gaudint de la Beca de Col·laboració concedida pel Ministerio de Educación, Cultura y Deporte durant el curs 2014/2015 a títol personal per a dur-lo a terme amb el grup d'Oncologia Molecular del Department de Ciències Mèdiques de la Universitat de Girona (2014-2015/9863231).

Gràcies a tot el grup NEOMA, en especial a l'Adriana Blancafort, a l'Ariadna Giró i a la Sònia Palomeras, per endinsar-me en el món de la investigació i ajudar-me a la realització d'aquest projecte.

Gràcies a la Dra. Glòria Oliveras i al Dr. Ferran Pérez del Servei d'Anatomia Patològica de l'Hospital Dr. Josep Trueta, per la seva paciència i dedicació en tot el projecte de càncer de mama triple negatiu i per ajudar-me en la part histològica del treball.

Gràcies al Registre del Càncer de Girona, en especial al Dr. Rafael Marcos, per la seva col·laboració i ajuda en la completació de la recol·lecció de dades clíniques de les nostres pacients.

Gràcies a la Dra. Maria Buxó, per ajudar-me en la part metodològica i estadística i en l'anàlisis de resultats del treball.

I, finalment, agrair especialment a la Dra. Teresa Puig i a la Dra. Gemma Viñas, per la seva incansable implicació, dedicació i suport durant totes les etapes d'aquest projecte i per fer-me estimar l'Oncologia en totes les seves vessants.

"Wherever the art of Medicine is loved, there is also a love of humanity"
Hippocrates (460-370 a.C.)

Girona, January 2015

1. Abbreviations

cDNA Complementary deoxyribonucleic acid

CK Cytokeratin

CSC Cancer stem cell

EGCG Epigallocatechin gallate

EGFR Epidermal growth factor receptor

EMT Epithelial-mesenchymal transition

ER Estrogen receptor

FASN Fatty acid synthase

HER2 Human epidermal growth factor receptor-type 2

IHC Immunohistochemistry

MAPK Mitogen-activated protein kinase (also known as MEK)

MFS Metastasis-free survival

mTOR Mammalian target of rapamycin

OS Overall survival

PARP Poly ADP ribose polymerase

PI3K Phosphatidylinositol-3-kinase

PKB Protein kinase B (also known as AKT)

PR Progesterone receptor

PTEN Phosphatase tensin homolog

RB Retinoblastoma

siRNA Small interfering ribonucleic acid

TIC Tumor-initiating cell

TMA Tissue microarray

TN Triple-negative

TNBC Triple-negative breast cancer

VEGF Vascular endothelial growth factor

2. Abstract

Background: Triple-negative breast cancer (TNBC) is a heterogeneous breast cancer subgroup with lacking of tumor markers, which lead to limitations in its treatment options and a consequent adverse prognosis. For this, the absence of effective targeted therapies requires new biomarkers to develop therapeutic strategies in the future. Fatty acid synthase (FASN), which is overexpressed in several human carcinomas including triple-negative cell lines, could be a potential novel biomarker and a therapeutic target or co-target in TNBC.

Main objective: To characterize FASN expression levels in TNBC patient tissue samples, compared to FASN expression levels in non-tumoral breast tissue samples, obtained between 1990 and 2012 from oncologic patients of Institut Català d'Oncologia (ICO) in Hospital Universitari de Girona Dr. Josep Trueta.

Design: Cross-sectional study conducted between January 2014 and December 2014.

Methods: One hundred and four TNBC tissue samples have been analysed and graded according to FASN expression levels by immunohistochemistry methods, and subsequently classified in two categories: low (0+/1+) and high (2+/3+) expression of FASN. In parallel, clinical records from 90 TNBC patients have been collected, and clinical and histopathological characteristics of these patients have been described besides evaluating its association with the corresponding FASN expression results.

Results: In the TNBC group, 45 tumor samples (45%) presented a high FASN expression, and in the non-tumoral group, 22 samples (23,9%) presented a high FASN expression ($p<0,01$). No statistically significant differences have been found in the bivariate analysis where association between FASN expression levels and clinical characteristics (menopausal status, tumor stage, lymph node involvement, relapse, Ki-67 grade and CK 5/6 expression) has been evaluated.

Conclusions: Fatty acid synthase is moderately to highly expressed in TNBC tumors and could be considered as a novel tumor biomarker in triple-negative breast cancer.

3. Introduction

3.1. Epidemiology and impact of breast cancer

Breast cancer is the most common cancer in women both in the developed and the developing world, and the lifetime probability of acquiring it is estimated of 1 in 8. Despite recent advances in screening and treatment, breast cancer also remains the most deadly cancer in women worldwide: it is estimated that over 508.000 women died in 2011 due to this cancer type according to WHO Global Health Estimates in 2013 (1,2).

Breast cancer is still the most incident and prevalent neoplasm in European, Spanish and Catalan women. In Girona province, 383 new cases per year are reported, which represents a 27,5% of all oncologic diagnoses in 2009. Every year, breast cancer causes 74 deaths, a 13% of all oncologic deaths in this province, leading the first cause of oncology-related mortality in our women (3).

3.2. Molecular taxonomy and classification

Multiple systems have been proposed to classify breast cancer. Traditionally, clinical classification was based on criteria such as histological type, tumor grade, tumor size, presence or absence of lymph-node metastasis, estrogen and progesterone receptor expression and HER-2 amplification. But, in order to tackle the complexity of the disease and, thus, to improve the prognostic classification by identifying more homogeneous entities, the most recent approach to the classification of breast cancer is based on molecular profiling. Moreover, this molecular program described for the primary tumor may generally be retained in its metastasis, too (1,4–7).

Histologically, the epithelium of the mammary gland has two layers of cells. The inner (luminal layer) lines the lumen of the breast duct and lobule, and the outer layer is described as the

myoepithelial layer. Adjacent to the basement membrane, they are, sometimes confusingly called, basal cells, which are thought to be immature progenitors and stem cells (5).

Beyond this two-layer cell structure of the human breast biology (that can be differentiate by cellular biomarkers) and deepening in this distinction, breast cancer has been classified in the following different molecular groups using expression array techniques (tissue microarrays (TMA), such as cDNA microarrays): *luminal A, luminal B, HER2 positive, normal-like, basal-like* and, lately, *claudin-low.* It has been suggested that these different subtypes of breast cancer could originate from mammary stem or progenitor cells at different stages of lineage differentiation (Figure 1) (6,7).

- *Luminal A* and *luminal B* are both estrogen receptor (ER) positive and express the following low-weight cytokeratins: CK8 and CK18 as more important, but also CK17 and CK19 among others, in a similar pattern of a possible precursor differentiated luminal cell (7,8).

Luminal A demonstrated the highest expression of the ERα- gene and, also, can express progesterone receptor (PR). These tumors do not overexpress human epidermal growth factor receptor 2 (HER2) and are considered the best prognosis subtype. *Luminal B* was differentiated from the previous subgroup by PR and HER2 positive expression and showed low to moderate expression of the luminal-specific genes, including the ER cluster. A *Luminal C* subtype has also been identified and related to worst outcome in comparison to other *luminal* subtypes (9).

- *The Basal-like* subtype presents an expression pattern similar to the one observed in normal myoepithelial cells, which suggests that its origin may be in the outer layer of the breast epithelium (5,6). It represents a 15-25% of all breast cancers and it is mostly characterized by the lack of expression of ER, PR and HER2 amplification, and by the presence of myoepithelial cells that express high-weight basal cytokeratins –the *basal* cluster- such as CK5/6 as more

relevant, but also CK 14, CK 17, EGFR, moesin, α-basic crystalline and p-cadherine, which have been reported as independent poor-prognosis markers in breast cancer (5,7).

Triple-negative breast cancer (TNBC) is a unique subtype that represents a 15-20% of all diagnosed breast cancers and is also defined by the lack of expression of ER and PR and the absence of HER2 amplification. It has a heterogeneous molecular pattern, being the *basal-like* subtype the majority group (80%), although *claudin-low* subtype is also highly represented.

Because of the lack of ER, PR and HER2 receptor expression, the triple-negative definition has been widely used to refer to *basal* tumors. Nevertheless, there is not a total overlap between the clinical and the molecular classification. In fact, some not triple-negative (TN) tumors can present *basal-like* characteristics and vice versa, being a 75% overlap that could translate true differences in their biology (5,6,8).

- *The HER2 positive* subtype represents a 20-30% of all breast tumors and presents an increase of HER2-associated gene expression. It has association with P53 mutations, its ER expression is mostly negative, and its gene, HER2/*neu,* represents one of the most important oncogenes in breast cancer. Clinically, it is associated with a shorter survival time and a poorer relapse-free survival, correlating its expression with an unfavourable clinical outcome and corroborating HER2 overexpression as a prognostic factor (9).

- *The Normal-like* subtype shares characteristics with normal breast cancer (6). ER, PR and HER2 expression is usually negative and its gene expression pattern is typified by the high expression of genes characteristic of basal epithelial cells and adipose cells, besides low expression of genes characteristic of luminal epithelial cells (7).

- *The Claudin-low* subtype represents only approximately 5% and is characterized by a low to absent expression of many claudin genes (notably 3, 4 and 7), which are involved in epithelial

cell tight junctions. It presents low expression of differentiated luminal cell surface markers (such as CD24), enrichment of epithelial-to-mesenchymal transition (EMT) markers and immune response genes (1,10). These tumors also present proliferation-associated gene expression and increase of the $CD44^{+}/CD24^{-}$ relation, which has been previously found in breast tumor-initiating cells (TICs) and mammary stem cells (10). Some recent data suggest that these features (acquisition of EMT, TICs and/or stem cell-like biological processes) are also found in resistant post-treatment tumors, giving the *claudin-low* subtype an even poorer prognosis with less of a response to chemotherapy than other *basal-like* cancers, being an intermediate between the *basal-like* and *luminal* tumors (1,8,10). Immunohistochemically, they present ER, PR and HER2 negative expression and in the differentiation hierarchy that exists across all breast cancers, the *claudin-low* subtype most closely resembles the mammary epithelial stem cell.

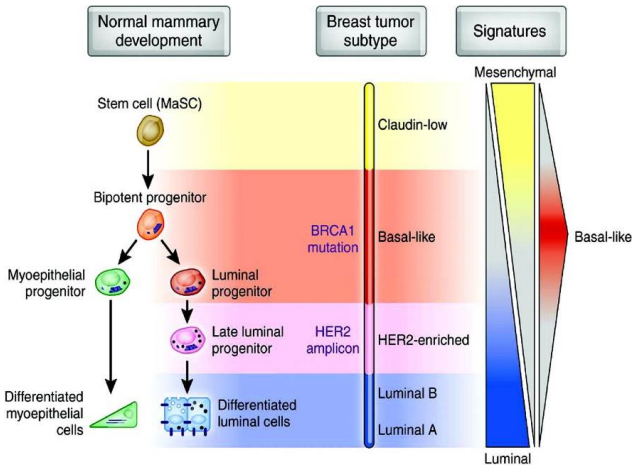


Figure 1. Different stages of cell differentiation during normal development of the breast and molecular subtypes of breast cancer associated (10).

Adapted to the routine clinical practice, the subtyping of breast cancer based on the described molecular profiling includes the following:

- ER status testing
- PR status testing
- HER2 receptor status testing

On the basis of these results, breast cancer is classified as:

- Hormone-receptor positive: ER and/or PR positive
- HER2 positive
- Triple-negative: ER, PR and HER2 negative

The importance of this classification is that ER, PR and HER2 are widely defined as prognostic biomarkers and present a capital importance in therapeutic decision making, treatment response prediction and relapse pattern determination, since this knowledge favours the use of selected therapeutic strategies with targeted agents (9).

Another additional classification can be performed with genetic tests, which can sort breast cancer on the presence of mutations of the tumor suppressor genes BRCA1 and BRCA2, since positive correlations with these germ line mutations have been found in TNBC: it has been reported that up to 20% of these patients are affected with the BRCA1 mutation subtype (1).

For all, breast cancer is considered a collection of different diseases and subtyping is regarded as essential to identify new molecular prognostic, predictive and/or therapeutic targets, which represent an important step toward tailoring the treatment of these patients (5–7).

3.3. Pathological characteristics of triple-negative breast cancer

Human breast tumors are histologically complex tissues, containing a variety of cell types in addition to the carcinoma cells: endothelial, stromal and adipose-enriched/normal breast cells, B lymphocytes, T lymphocytes and macrophages (7).

From the histopathological point of view, most *basal-like* tumors are invasive ductal cancers but, also, *in situ* ductal carcinomas are found, which can act as precursor lesions for invasive cases (5). Nevertheless, occasionally may be typical or atypical medullary, metaplastic, adenoid cystic, squamous-cell or mucoepidermoid tumors.

Classically, they have been described as high-grade tumors, with more than 75% being grade III, and most of them overexpress p53 protein according to a p53 mutation (5). Other particular pathological characteristics are a high mitotic index (Ki-67), high nuclear/cytoplasmic ratio, scarce stromal content, central necrosis, aggressive invasive edge, and finally, also present apoptotic cells and lymphocyte stromal response, which has been associated with a favourable prognostic impact (5,6).

Moreover, it is also known that correlation between pathological tumor size and axillary lymph node status is absent or weak in these tumors, which might reflect a preferentially haematogenous metastatic spread and/or a disproportionate relationship between the number of cancer cells with lymph metastatic potential and the size of the cancer (5,11).

3.4. Risk and prognostic factors

For breast cancers in general, the determination of clinical and pathological features is essential for staging the disease, determining treatments and estimating prognosis. However, for the majority of women presenting with breast cancer, it will not be possible to identify specific risk factors. Features to consider are patient tumor size, axillary node involvement, menopausal status, stage of the disease, the estrogen receptor and progesterone receptor status of the tumor, the histological type (favourable histological types include mucinous, medullary and tubular carcinomas), lymphovascular invasion, estrogen-inducible genes like cathepsin D, prootooncogens like HER2, mutations in P53 gene and histological grade, which have been all correlated to prognosis. Various patient factors have also been identified as independent risk factors for breast cancer in general, like age, hormone status, parity and ethnicity (1).

More specifically, *basal-like* breast cancer affects preferably younger premenopausal African American women or women with Hispanic descent, with menarche at an earlier age and

multiparity compared to *luminal A* (1,6). In fact, TNBC diagnosis under the age of 50, leads to recommend genetic tests even in absence of breast cancer familiar antecedents (1).

However, nowadays, histoclinical features translating prognosis in TNBC are far from being optimal but, fortunately, on-going research focused on describing in more detail these clinical characteristics is currently emerging.

3.5. Clinical characteristics and prognosis

As previously introduced, the *basal-like* phenotype is associated with poor outcome: it is related to an early age of cancer onset, a high chance of presentation with metastases, a high relapse rate, a shorter metastasis-free survival (MFS) and overall survival (OS) in TNBC population, suggesting an aggressive clinical history in this type of tumors and turning it in the breast cancer subtype with worse survival rate (6,12). Also, clinical studies described an specific lower 5-year survival, since tend to present in advanced stages (III and IV) (1).

On the contrary, axillary lymph node-positive cases were reported to be lower in TNBC group when comparing to the non-TNBC group in recent studies, and other differences on prognosis have also been characterized within the TNBC group itself (11,13).

Table 1. Response and survival rates related to triple-negative breast cancer and nontriple-negative breast cancer (1).

	Triple-negative breast cancer	Nontriple-negative breast cancer	*P* value (95% confidence interval)
Complete pathological response (%)	22	11	.034 (1.03-2.26)
3-Year overall survival (%)	74	89	<.0001 (1.77-3.57)
3-Year overall survival with complete pathological response (%)	94	98	.24 (0.7-4.2)
3-Year overall survival with residual disease (%)	68	88	<.0001 (1.3-1.8)

Some studies have shown that TNBC patients present more recurrence during the first 3 years after diagnosis, with a quickly decline after this period; had more incidence of visceral metastases, notably brain and lung; but develop less frequently bone and axillary lymph node

metastases in comparison to nonTNBC patients (8,11). They also tend to metastasize to soft tissues and, there, have a high rate of local relapse, especially after mastectomy, suggesting that TN breast cancers benefit less from radiation therapy after surgery (5,6,8).

Finally, in order to explain the treatment failure and recurrence, it has been suggested that a small population of cells within tumors, often designated and previously described as TICs, which may display mesenchymal features, could be resistant to therapy and hence may reinitiate tumor growth after treatment (14). Currently, TICs, also described as stem-like cells, are highly susceptible of research, in a theory that is called *cancer stem cell theory*. These cancer stem cells (CSCs) may generate tumors through the stem cell processes of self-renewal and differentiation into multiple cell types (13–15).

For all, this EMT-rich TNBC needs to be taken into account and more specific targeted therapies are necessary to overcome this particular chemoresistance.

Table 2. Characteristics of *basal*/TN breast cancers (5).

Epidemiological features	Younger age
	Premenopausal status
	African-American race
	High body mass index (BMI)
	Younger age at menarche
Histoclinical features	Ductal carcinoma (and medullary)
	High-grade
	High mitotic index
	Nuclear pleomorphism
	Pushing margins of invasion
	Central necrosis
	Negative ER, PR, HER2 in IHC staining
	Poor correlation between pathological tumor size and axillary lymph node status
Molecular features	TP53 mutations
	BRCA1 deficiency
	RB inactivation
	Genome instability
Prognosis	Poor prognosis
	Early relapses (first 3 years)
	Visceral metastases (brain and lung)
Therapeutic response	Sensitive to primary chemotherapy
	No validated targeted therapy (on-going trials)

3.6. Potential new targeted therapies

Nowadays, basic cytotoxic chemotherapy (anthracyclines and taxanes) is the only systemic treatment used since the triple-negativity does not render the patients candidate to hormone therapy and anti-HER2 therapies (1). Advances in chemotherapy have particularly benefited this patient group but, although the initial response is satisfactory, and indeed, possibly more responsive to certain forms of chemotherapy than nonTN breast cancer, relapse occurs in a 45% of treated patients (chemoresistance), which occurs in a higher proportion than in other breast cancer subtypes (what is called "triple-negative paradox"). Currently, there are no preferred chemotherapy regimens, and treatments are being unspecifically selected with similar considerations as with other breast cancers.

Chemotherapical strategies exploit the defect in double-strand DNA break repair mechanisms, like DNA-damaging agents such as platinum agents (cisplatin and carboplatin). In the case where the tumor become resistant to anthracyclines and taxanes, and besides platinum agents, other drugs including capecitabine, vinorelbine, nab-paclitaxel, ixabepilone, eribulin and gemcitabine are available, instead remaining unclear their efficiency. Today, there is no regimen specifically recommended for metastatic TN patients and larger studies are needed to validate or not the predictive value of *basal* subtype for tumor chemosensitivity (5).

However, the recent insights in the pathogenesis of these tumors allow the development of new therapeutic strategies targeting molecular alterations. The following targets have been identified as possible therapeutic options for this subtype of cancer but, unfortunately, they have not been proved effective until now, probably due to high heterogeneity and variability both intra and inter tumor, and remain experimental (Figure 2).

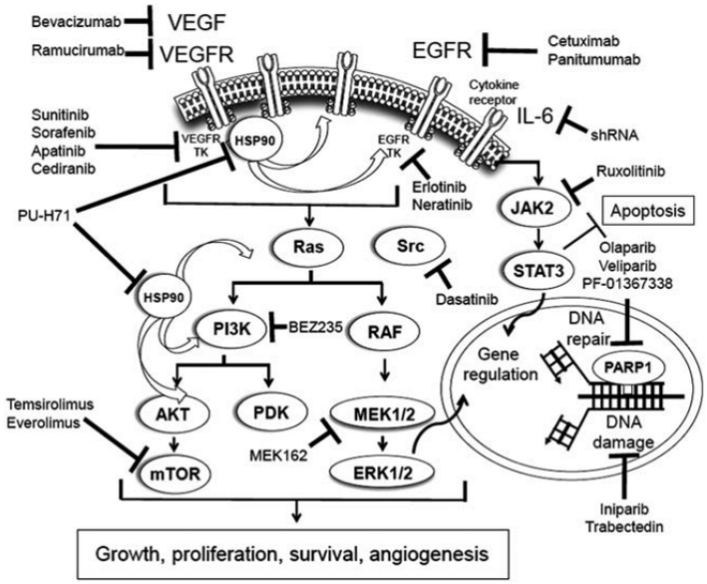


Figure 2. Different pathways, targets and directed agents undergoing clinical research in TNBC therapeutics (16).

- Anti-VEGFs: TNBC tumors are highly proliferative and require constant angiogenesis during their development, invasive and metastatic processes. Anti-vascular endothelial growth factor (anti-VEGFs) such as bevacizumab (but also ramucirumab) are currently being studied alone and in combination in metastatic TNBC and in the neoadjuvant setting; but, for now, it remains unclear whether TN cancers are more sensitive than others to anti-angiogenic drugs (5,17). Moreover, diverging decisions in their approval between the Food Drug Administration (FDA) and the European Medicines Agency (EMA) reflect differences in the perceived benefit:risk ratio of this therapy (17).

- Kinase inhibitors: Other agents undergoing clinical trials in TNBC include kinase inhibitors such as erlotinib and gefitinb as tyrosine kinase inhibitors against epidermal growth factor receptor (EGFR); and sorafenib and sunitinib as tyrosine kinase inhibitors against VEGF, but clinical trials are disappointing (4,5). Dasatinib, that inhibits proto-onocogene tyrosine-protein kinase SRC, has also been studied with even less mature results.

- EGFR inhibitors: The inactivation of EGFR pathway by specific inhibitors such as cetuximab or lapatinib has low efficiency, suggesting an alternative activation of the EGFR pathway in TN tumors. Moreover, EMT has been implicated in resistance to EGFR inhibition in lung cancer and may contribute to its therapy resistance also in TNBC (4,8,12).

- mTOR inhibitors: The mammalian target of rapamycin (mTOR) is an effector of the PI3K signalling pathway regulated by AKT and the tumor-suppressor PTEN. Proteins of PI3K pathway are frequently mutated in general breast cancer. In TNBC in particular, a PTEN loss has been described, which involves a mTOR activation (Figure 2). Examples of mTOR inhibitors include everolimus and temsirolimus, which are currently being studied (13).

- PARP-1 inhibitors: Genomic integrity and cell survival are critically dependent on coordinated pathways of DNA repair. Poly ADP ribose polymerase (PARP) enzymes play a key role in these pathways by mediating the repair of single-strand DNA breaks via base excision repair (BER). Consequently, loss of PARP activity results in the accumulation of single-strand breaks, which are normally repaired by double-strand homologous recombination pathways that include the important tumor-suppressor proteins BRCA1 and BRCA2 (16).

Researchers have long recognized that women who carry germ-line mutations in BRCA1 have a higher risk to develop breast cancer, and most of the time those breast cancers are, in fact, the *basal-like* molecular subtype (8). Sporadic *basal* breast cancers and hereditary BRCA1-asociated breast cancers share several morphological, immunohistochemical and biological features. All these similarities suggest a fundamental defect in the BRCA1 DNA-repair pathway also in sporadic *basal* breast cancers, too (5).

PARP-1 inhibitors, such as olaparib and veliparib are potential therapies in development. Iniparib was first developed also as a PARP inhibitor, but its exact mechanism of action remains to be elucidated (16).

Whereas most BRCA1 carriers have *basal-like* breast cancer, most *basal-like* breast cancers arise in women who do not have inherited BRCA1 mutations and it still remains to be seen whether BRCA status can be used as a surrogate for response in triple-negative patients (8). While initial data with PARP inhibitors is promising, it is important to stress the need for proper patient selection (BRCA1 or BRCA2 mutations as possible criteria) (1).

Finally, PI3K inhibitors and an investigational antibody designed to block the Met receptor, MetMab, are currently undergoing clinical trials, and other research lines are based on the favourable prognostic impact of the lymphocyte activation in *basal* breast cancer. The identification of new antigens suggests that strategies aimed at stimulating the immune system should also be tested.

3.7. FASN tissue expression

Metabolism has been recently incorporated as a *hallmark of cancer*, since rapidly growing tumor cells require lipid as a source for membrane biosynthesis as well as for energy supply. However, evidence that cancer cells presented *de novo* activated FASN started at the 90s. Nowadays, many studies reported that high expression of FASN is common in a wide variety of solid human tumors, including breast, prostate, colon, lung, bladder, ovary, stomach, endometrium, kidney, skin, oesophagus, tongue and soft tissue carcinomas (18–22). Moreover, FASN expression is also present in premalignant atypical duct proliferations and in *in situ* ductal carcinomas, although the responsible pathways for FASN overexpression are not yet well understood in these cases (23).

FASN is a 250 kDa sole mammalian multienzymatic complex in charge of *de novo* synthesis of long-chain fatty acids (basically palmitate in a 80% and myristate and stearate in a both 10%) from acetyl-CoA condensation and NADPH-dependent malonyl-CoA, in seven sequential reactions.

The primary physiological function of FASN-catalysed *de novo* fatty acid biogenesis seems to differ between normal and cancer cells. In normal conditions, fatty acid synthesis takes place in lypogenic tissues (adipose tissue and liver) with the aim to store energy in a triglyceride form. In most human tissues, diet supplies the needs of fatty acids and FASN expression is low or undetectable. By contrast, in tumor tissues, fatty acids from endogenous synthesis (a procedure that acquires a capital importance in this situation) are not stored in the standard form but in a phospholipid form, since FASN activity plays a major role in the synthesis of phospholipids for the cell membrane.

These fatty acids are also implicated in key cellular processes and vital functions including proliferation, energetic metabolism through β-oxidation, signal transduction, DNA synthesis, intracellular trafficking, protein acylation, cell polarization, cell cycle progression and cell migration (21,22,24,25). Furthermore, lipid rafts of the cell membrane localize proteins (EGFR, HER2 and hormone receptors) to correct cell signalling (Ras, RAF, AKT, MEK…) (Figure 2). Therefore, the inhibition of FASN could block these signalling pathways by modifications in the membrane lipid content.

Fatty acid synthesis regulation in normal cells is basically controlled by dietetic factors. However, in tumor cells, is regulated in a transcriptional level (hormones, growth factors and microenvironment stress factors), suggesting an epigenetic basis of increased FASN expression (Figure 3) (21,22). In accordance with others, the group has demonstrated that this overexpression occurs through a modification of the sterol regulatory element-binding protein-1c (SREBP-1c) transcription factor, which is the major factor involved in the regulation of FASN in liver and adipose tissue and is known to be regulated by the MEK/ERK1/2 and PI3K/AKT pathway (Figure 3) (22).

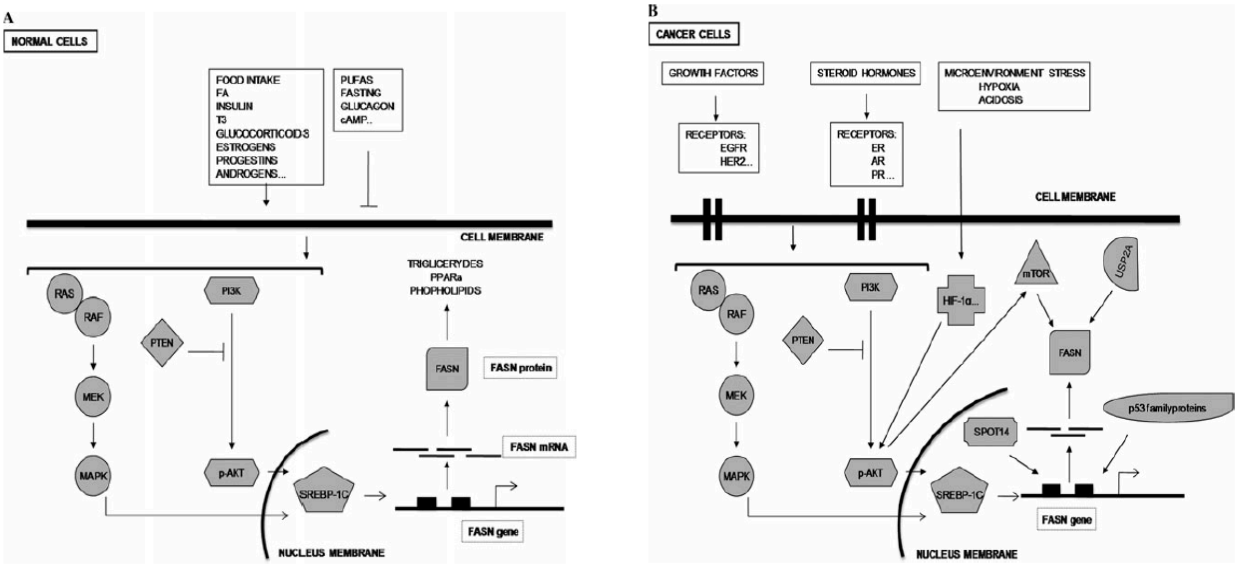


Figure 3. FASN regulation in normal cells (A) and in tumor cells (B). *A) In normal cells, FASN regulation and expression mostly depend on hormonal and nutritional status. Major pathways that regulate FASN transcription are PI3K/AKT and MAPK (ERK 1/2). B) FASN expression is insensitive to the nutritional status. Growth factors, hormones and tumor microenvironment (hypoxia and acidosis) activate the pathways described above, which modulate the FASN expression (26).*

Studies have reported the physiological effect of FASN on the cell cycle status. FASN complex can inhibit the intrinsic pathway of apoptosis. Our group and others have identified some of the molecules involved in the proliferation and tumor growth dependent of FASN expression and activity, such as p21, p53 and p27, which are also accumulated in tumor cells after FASN inhibition.

It also has been observed in cell lines that there is a correlation between HER2 and FASN overexpression. FASN contributes to the growth advantage promoted by the HER2 and FASN activity is required to maintain the malignant transformation induced by HER2 overexpression, revealing a bidirectional connection between HER2 and FASN-dependent neoplastic lipogenes. Moreover, FASN inhibition may disrupt the membrane lipid rafts that anchor HER2 and, taking into account that possession of high levels of HER2/*neu* oncogene may be a molecular determinant for hypersensitivity to FASN blocker-induced cytotoxicity in human breast cancer

cells, FASN also could be a targeted therapy in breast carcinomas with acquired resistance to anti-HER2 therapies (19,21,22).

Clinically, it is suggested that patients with high levels of FASN expression experience both shortened disease-free and overall survival. FASN serum concentrations were significantly higher as it advanced the tumor stage in comparison to healthy controls in a study, which reveal a necessary role of FASN in the development, maintenance and/or enhancement of the malignant cancer phenotype (18). Also, FASN as a prognostic factor was found to be statistically independent of important clinical parameters including tumor size, lymph node involvement, assessment of estrogen and progesterone receptor, HER2 and cathepsin D in another study (23). Menopausal status, age, body mass index and pathological stage are also shown to be associated with higher levels of FASN tumor expression (24).

Furthermore, FASN inhibition is highly cytotoxic in human tumor cells (pharmacologically or with siRNA), which is correlated with its expression levels. It produces an inhibition of DNA replication in consequence of a delayed S-phase progression due to insufficient phospholipids from fatty acid synthesis to synthetize a cellular membrane, as well as apoptotic death by activation of pro-apoptotic genes (20,24). FASN inhibition also has been shown to induce apoptosis in EGFR positive lung cancer, where the EGFR signal pathway has a key paper (19,27,28). This has significant importance because, as previously described, EGFR is also overexpressed in 70-78% of all TNBC.

Finally, in recent reports, immunohistochemistry has detected FASN expression in TN cell lines *in vitro*, being higher in *basal-like* phenotypes and lower in *mesenchymal-like* cells. This fact has encourage to lead to new research lines to study FASN expression in the TNBC field.

3.8. FASN inhibitors

As previously described, FASN inhibition that blocks the lypogenic pathway and impedes fatty acid synthesis, entails apoptosis in tumor cells that overexpress FASN, without affecting non-malignant cells (28).

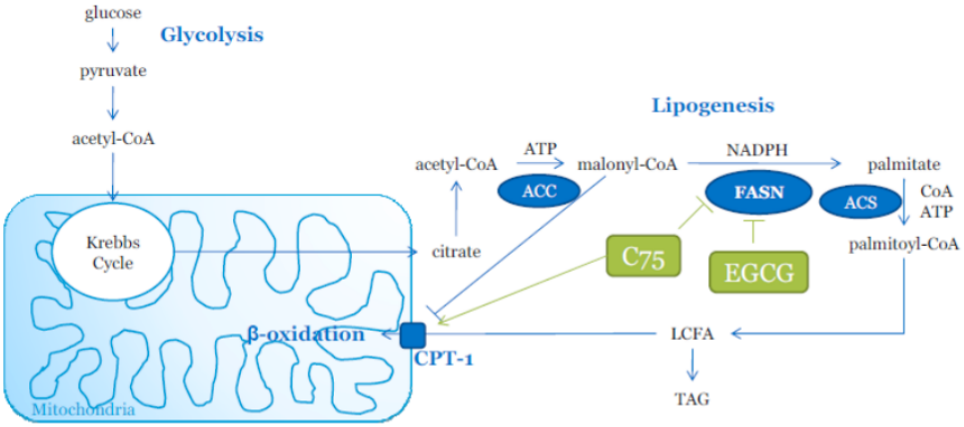


Figure 4. Fatty acid synthesis and oxidation pathways. *ACC, acetyl-CoA carboxylase; ACS, acyl-CoA synthetase; LCFA, long-chain fatty acids; TAG, triacylglycerides (29).*

First FASN inhibitory molecule with cytotoxic effects in tumor cells was **cerulenin**, (2R,3S)-2,3-epoxy-4-oxo-7,10-trans (transdodecadienamide), a natural product of *Cephalosporium ceruleans* fungus. It inhibits FASN by binding covalently to the active cysteine site of the β-ketoacyl-synthase (KS) moiety, which performs the condensation reaction mentioned previously that leads to apoptotic cell death (Figure 5). Unfortunately, the clinical relevance of these results is limited because the cerulenin structure harbours a very reactive epoxy group that may interact with other proteins and may affect other processes than FASN activity, including palmitoylation, cholesterol synthesis and/or proteolysis.

Later, **C75** was developed, an α-methylene-γ-butirolactone with a C7 hydrocarbon side chain, a synthetic cerulenine analogous, which is more chemical stable and has similar cytotoxic effects *in vitro* and in *vivo* by blocking the ketoacyl synthase (KS) domain of FASN (Figure 5). The compound C75 provided the first evidence *in vivo* of tumor reduction by FASN inhibition,

although no therapeutic development continued due to the weight loss that suffered experimental animals together with anorectic effects. From this, it was discovered that C75 not only inhibits FASN, but stimulates carnitine palmitoyltransferase-1 (CPT-1), a transporter enzyme of long-chain fatty acids from the cytoplasm to the mitochondrial matrix (where the β-oxidation occurs), and in consequence of that, increases fatty acid oxidation, which explains the weight loss that occurs in *in vivo* C75 administration (26,27,29).

New research has focused on polyphenols, which constitute a wide group of different molecular structures: catechins, flavones, anthocyanidins, anthraquinones, lignans, coumarins and tannins. **(-)-Epigallocatechin-3-gallate (EGCG)**, the main polyphenolic catechin of green tea, is an antitumor compound whose effect, in part, is due to modulation of multiple signalling pathways, including FASN. It acts through a competitive inhibition of NADPH for the same binding site in ketoacyl synthase (KS) domain of FASN (Figure 5). The group and others have reported that EGCG has comparable effects to C75 in blocking FASN activity, in reduction in cell proliferation, inducing apoptosis in tumor cells and, also, in significant reduction in the active forms of HER2 oncoprotein and MAPK and AKT kinases; although unlike C75, EGCG does not affect the activity of CPT-1, fatty acid oxidation nor produces *in vivo* weight loss. It has been reported that induces apoptosis *in vitro* and reduces the size of tumors in animal models *in vivo*. The therapeutic use of EGCG as an antitumor agent is limited by its high value of half maximal inhibitory concentration IC_{50} (149 μM), poor oral availability and relative instability under physiological conditions (26–31).

Other novel compounds related to green tea epigallocatechin have also been tested with promising results and could be even more active than the parent EGCG molecule, such as **G28UCM** (26,30).

Orlistat ((-)-tetrahydrolipstatin), as a β-lactone, has also been preliminarily studied as a FASN inhibitor besides its anti-obesity effects, but no conclusive results have arisen because of its

poor solubility and bioavailability; and **resveratrol**, a phytochemical abundant in natural foods, has been suggested to induce also apoptosis through modulation of FASN expression (21,25).

Finally, the group and others have described that inhibition of lipogenesis via the inhibition of FASN enzyme can increase the effectiveness of current treatments like chemotherapy and monoclonal antibodies (trastuzumab), plus reverse the resistance to anthracyclines (19). Interestingly, it has also recently been demonstrated that inhibition of FASN acts on growth cancer stem cell population, too (24,25). Moreover, loss of P53 function increased the sensitivity of tumor cells to FASN inhibitors with no effect on fatty acid synthesis level, which hold special appeal as an experimental therapeutic target in cancer cells afforded by both elevated fatty acid synthesis and loss of P53 function (20).

A) **B)**

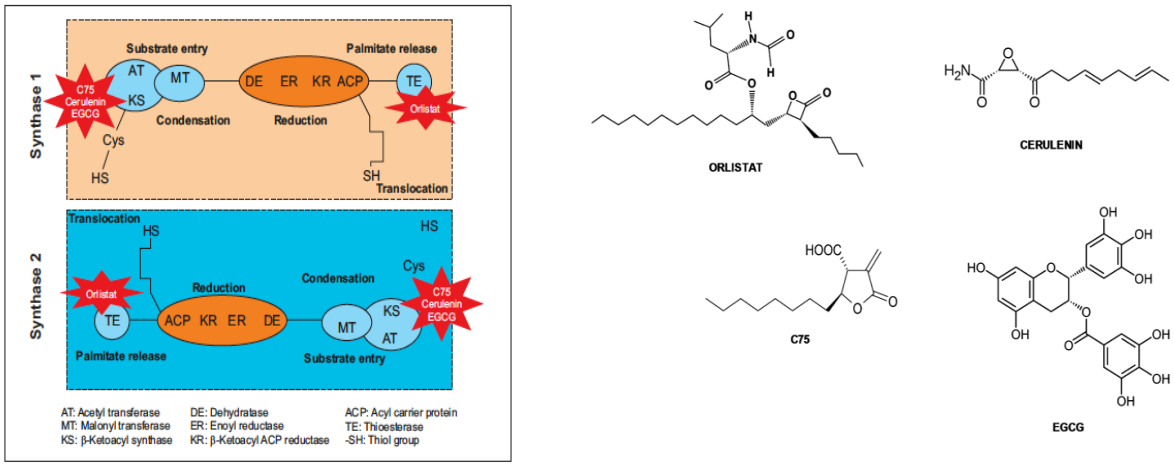


Figure 5. A) Schematic representation of fatty acid synthesis (FASN) enzymatic complex and target sites of chemical FASN blockers. FASN is divided into seven functional domains, assembled in two homodimers (21). **B) Chemical structure of most used FASN inhibitors** (26).

4. Justification

In developed countries, there has been a remarkable improvement in mortality from breast cancer, although almost all of that benefit has occurred in ER+ and HER2+ subtypes (8). Triple-negative tumors differ from other breast cancers in gene expression, response to chemotherapeutic and biological therapies, prognosis, and display epidemiological and clinicopathological features distinct from other subtypes (4).

Despite their relative scarcity, TN tumors cause a disproportionate mortality among breast cancer patients while having a specific high impact, since TNBC affect premenopausal young women, who have worse prognosis due to the lack of specific targeted therapies, such as hormonal and anti HER-2 treatments (1,6).

For that, TNBC remains today the biggest challenge moving forward in oncology, that leads us to the goal of a more personalized medicine.

However, a tailored therapeutic approach could be limited without the characterisation of specific markers for each breast cancer subtype. Such markers will allow developing novel targeted therapies for specific breast cancer patients to ensure the greatest benefit.

Therefore, metabolism as a new *hallmark of cancer* and, specifically, endogenous fatty acid metabolism have been recently studied, since are shown to be crucial to maintain the cancer cell malignant phenotype in a wide variety of human carcinomas. FASN enzyme is also considered to have a causative effect on tumorigenesis rather than merely a consequence of tumor growth, besides having an inherent association with a worse prognosis. The differential expression of this enzyme between normal and tumor tissues has launched research lines searching for FASN as a useful tumor marker for cancer diagnosis and prognosis, and *in vitro* results also suggest that FASN could be a promising target for anti-tumor therapy, too.

For all these reasons and taking into account the preliminary results of FASN expression in TN cell lines in preclinical studies, a clinical study phase could provide new evidence on FASN role in TNBC. Our assumptions focus on a first preliminary analysis and study of FASN expression patterns in TN patient breast samples, that could lead to research lines to study and validate FASN role as a novel tumor biomarker in TNBC population and a possible new therapeutic target or co-target for this malignant breast cancer subtype.

5. Bibliography

1. Griffiths CL, Olin JL. Triple negative breast cancer: a brief review of its characteristics and treatment options. J Pharm Pract [Internet]. 2012 Jun [cited 2014 Nov 4];25(3):319–23. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22551559

2. WHO. Breast cancer: prevention and control [Internet]. World Health Organisation. Geneve; 2014 [cited 2014 Dec 10]. Available from: http://www.who.int/cancer/detection/breastcancer/en/index1.html

3. CanGir. El càncer a Girona 2007-2009. Projecció incidència 2013-2014 [Internet]. Departament de Salut. Generalitat de Catalunya. Barcelona; 2014 [cited 2014 Dec 11]. Available from: http://ico.gencat.cat/web/.content/minisite/ico/professionals/documents/registre_cancer_girona/arxius/cangir_2007-9_projeccions_de_la_incidencia_2013-14.pdf

4. Duffy MJ, McGowan PM, Crown J. Targeted therapy for triple-negative breast cancer: where are we? Int J Cancer [Internet]. 2012 Dec 1 [cited 2014 Nov 4];131(11):2471–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22581656

5. Bertucci F, Finetti P, Birnbaum D. Basal breast cancer: a complex and deadly molecular subtype. Curr Mol Med [Internet]. 2012 Jan [cited 2014 Nov 4];12(1):96–110. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3343384

6. Guzmán EJO. Cáncer de mama triple negativo, estado actual. Rev Espec Médico-Quirúrgicas [Internet]. 2010 [cited 2014 Nov 6];15(4):228–36. Available from: http://www.redalyc.org/pdf/473/47316086008.pdf

7. Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature [Internet]. 2000 Aug 17 [cited 2014 Sep 23];406(6797):747–52. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10963602

8. Carey LA. Directed therapy of subtypes of triple-negative breast cancer. Oncologist [Internet]. 2011 Jan [cited 2014 Nov 4];16 Suppl 1:71–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21278443

9. Sørlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A [Internet]. 2001 Sep 11 [cited 2014 Aug 13];98(19):10869–74. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC58566

10. Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI, et al. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res [Internet]. 2010 Jan [cited 2014 Nov 2];12(5):R68. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3096954

11. Yuan N, Meng M, Liu C, Feng L, Hou L, Ning Q, et al. Clinical characteristics and prognostic analysis of triple-negative breast cancer patients. Mol Clin Oncol [Internet]. 2014 Mar [cited 2014 Nov 11];2(2):245–51. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3917786

12. Corkery B, Crown J, Clynes M, O'Donovan N. Epidermal growth factor receptor as a potential therapeutic target in triple-negative breast cancer. Ann Oncol [Internet]. 2009 May [cited 2014 Nov 4];20(5):862–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19150933

13. Yunokawa M, Koizumi F, Kitamura Y, Katanasaka Y, Okamoto N, Kodaira M, et al. Efficacy of everolimus, a novel mTOR inhibitor, against basal-like triple-negative breast cancer cells. Cancer Sci [Internet]. 2012 Sep [cited 2014 Nov 4];103(9):1665–71. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22703543

14. Creighton CJ, Li X, Landis M, Dixon JM, Neumeister VM, Sjolund A, et al. Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. Proc Natl Acad Sci U S A [Internet]. 2009 Aug 18 [cited 2014 Oct 27];106(33):13820–5. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2720409

15. Lim S, Becker A, Zimmer A, Lu J, Buettner R, Kirfel J. SNAI1-mediated epithelial-mesenchymal transition confers chemoresistance and cellular plasticity by regulating genes involved in cell death and stem cell maintenance. PLoS One [Internet]. 2013 Jan [cited 2014 Oct 29];8(6):e66558. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3684605

16. Crown J, O'Shaughnessy J, Gullo G. Emerging targeted therapies in triple-negative breast cancer. Ann Oncol [Internet]. 2012 Aug [cited 2014 Oct 30];23 Suppl 6:vi56–65. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23012305

17. Thomssen C, Pierga J-Y, Pritchard KI, Biganzoli L, Cortes-Funes H, Petráková K, et al. First-line bevacizumab-containing therapy for triple-negative breast cancer: analysis of 585 patients treated in the ATHENA study. Oncology [Internet]. 2012 Jan [cited 2014 Nov 4];82(4):218–27. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22508241

18. Wang Y, Kuhajda FP, Li JN, Pizer ES, Han WF, Sokoll LJ, et al. Fatty acid synthase (FAS) expression in human breast cancer cell culture supernatants and in breast cancer patients. Cancer Lett [Internet]. 2001 Jun 10 [cited 2014 Nov 12];167(1):99–104. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11323104

19. Puig T, Aguilar H, Cufí S, Oliveras G, Turrado C, Ortega-Gutiérrez S, et al. A novel inhibitor of fatty acid synthase shows activity against HER2+ breast cancer xenografts and is active in anti-HER2 drug-resistant cell lines. Breast Cancer Res [Internet]. 2011 Jan [cited 2014 Nov 5];13(6):R131. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3326573

20. Li JN, Gorospe M, Chrest FJ, Kumaravel TS, Evans MK, Han WF, et al. Pharmacological inhibition of fatty acid synthase activity produces both cytostatic and cytotoxic effects modulated by p53. Cancer Res [Internet]. 2001 Feb 15 [cited 2014 Nov 12];61(4):1493–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11245456

21. Menendez JA, Lupu R, Colomer R. Targeting fatty acid synthase: potential for therapeutic intervention in her-2/neu-overexpressing breast cancer. Drug News Perspect [Internet]. 2005 [cited 2014 Nov 12];18(6):375–85. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16247515

22. Menendez JA, Mehmi I, Verma VA, Teng PK, Lupu R. Pharmacological inhibition of fatty acid synthase (FAS): a novel therapeutic approach for breast cancer chemoprevention through

its ability to suppress Her-2/neu (erbB-2) oncogene-induced malignant transformation. Mol Carcinog [Internet]. 2004 Nov [cited 2014 Nov 12];41(3):164–78. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15390078

23. Kuhajda FP, Jenner K, Wood FD, Hennigar RA, Jacobs LB, Dick JD, et al. Fatty acid synthesis: a potential selective target for antineoplastic therapy. Proc Natl Acad Sci U S A [Internet]. 1994 Jul 5 [cited 2014 Oct 21];91(14):6379–83. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8022791

24. Porta R, Blancafort A, Casòliva G, Casas M, Dorca J, Buxo M, et al. Fatty acid synthase expression is strongly related to menopause in early-stage breast cancer patients. Menopause [Internet]. 2014 Feb [cited 2014 Nov 12];21(2):188–91. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23982110

25. Pandey PR, Okuda H, Watabe M, Pai SK, Liu W, Kobayashi A, et al. Resveratrol suppresses growth of cancer stem-like cells by inhibiting fatty acid synthase. Breast Cancer Res Treat [Internet]. 2011 Nov [cited 2014 Nov 3];130(2):387–98. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3404809

26. Oliveras G, Blancafort A, Urruticoechea A, Campuzano O, Gómez-Cabello D, Brugada R, et al. Novel anti-fatty acid synthase compounds with anti-cancer activity in HER2+ breast cancer. Ann N Y Acad Sci [Internet]. 2010 Oct [cited 2014 Nov 11];1210:86–92. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20973802

27. Puig T, Vázquez-Martín A, Relat J, Pétriz J, Menéndez JA, Porta R, et al. Fatty acid metabolism in breast cancer cells: differential inhibitory effects of epigallocatechin gallate (EGCG) and C75. Breast Cancer Res Treat [Internet]. 2008 Jun [cited 2014 Oct 25];109(3):471–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17902053

28. Relat J, Blancafort A, Oliveras G, Cufí S, Haro D, Marrero PF, et al. Different fatty acid metabolism effects of (-)-epigallocatechin-3-gallate and C75 in adenocarcinoma lung cancer. BMC Cancer [Internet]. 2012 Jan [cited 2014 Nov 5];12:280. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3500220

29. Puig T, Relat J, Marrero PF, Haro D, Brunet J, Colomer R. Green tea catechin inhibits fatty acid synthase without stimulating carnitine palmitoyltransferase-1 or inducing weight loss in experimental animals. Anticancer Res [Internet]. 2008 [cited 2014 Nov 12];28(6A):3671–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19189648

30. Puig T, Turrado C, Benhamú B, Aguilar H, Relat J, Ortega-Gutiérrez S, et al. Novel Inhibitors of Fatty Acid Synthase with Anticancer Activity. Clin Cancer Res [Internet]. 2009 Dec 15 [cited 2014 Nov 12];15(24):7608–15. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20008854

31. Turrado C, Puig T, García-Cárceles J, Artola M, Benhamú B, Ortega-Gutiérrez S, et al. New synthetic inhibitors of fatty acid synthase with anticancer activity. J Med Chem [Internet]. 2012 Jun 14 [cited 2014 Nov 12];55(11):5013–23. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22559865

32. Zhang C, Schmidt LA, Hatanaka K, Thomas D, Lagstein A, Myers JL. Evaluation of napsin A, TTF-1, p63, p40, and CK5/6 immunohistochemical stains in pulmonary neuroendocrine tumors. Am J Clin Pathol [Internet]. 2014 Sep [cited 2015 Jan 15];142(3):320–4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25125621

6. Hypotheses

6.1. Main hypothesis

Based on the preliminary results of fatty acid synthase (FASN) expression observed in several human breast carcinomas and to confirm the *in vitro* FASN expression results in TN cell lines, the main hypothesis of this study is:

- FASN, that has currently been considered as a metabolic oncogene, is specifically overexpressed in triple-negative breast cancer (TNBC) patient samples and could be a novel biomarker and a potential therapeutic target or co-target in TNBC.

6.2. Secondary hypotheses

Age, menopausal status, tumor stage, lymph node involvement, relapse, Ki-67 grade, CK 5/6 expression, histological type, tumor size, histological grade and P53 expression of triple-negative breast cancer patients will be described, although formal hypotheses will not be tested for this descriptive objective.

Neverthless and derived from this, a secondary hypothesis of this study is:

- Menopausal status, tumor stage, lymph node involvement, relapse, Ki-67 grade and CK 5/6 expression could be associated with high levels of FASN expression, which would generate new hypotheses for future research lines.

Moreover, since high FASN expression has been reported to be associated with a worse prognostic outcome, our main secondary hypothesis is:

- High FASN expression levels in TN patient breast samples are associated with a lower clinical overall survival of this breast cancer population in comparison to low FASN expression levels.

7. Objectives

7.1. Main objective

The aim of this project is:

- To characterize the expression levels of fatty acid synthase (FASN) in triple-negative breast cancer patient tissue samples, compared to FASN expression levels in non-tumoral breast tissue samples obtained between 1990 and 2012 from TNBC patients of Institut Català d'Oncologia (ICO) in Hospital Universitari de Girona Dr. Josep Trueta.

7.2. Secondary objectives

- To describe specific clinical and histopathological features such as age, menopausal status, tumor stage, lymph node involvement, relapse, Ki-67 grade, CK 5/6 expression, histological type, tumor size, histological grade and P53 expression of a TNBC women population.

- To ascertain whether tumor FASN expression levels are associated with clinical and histopathological features such as menopausal status, tumor stage, lymph node involvement, relapse, Ki-67 grade and CK 5/6 expression, in the described population of women diagnosed with TNBC.

- To preliminary determine whether association exists between FASN expression levels and the overall survival in women diagnosed with TNBC.

8. Methods

8.1. Study design

A cross-sectional population-based study conducted from January 2014 to December 2014 in Hospital Universitari de Girona Dr. Josep Trueta, in which we analyse FASN expression levels in tumor and non-tumoral patient tissue samples, and specific clinicopathological features from women diagnosed of triple-negative breast cancer.

8.2. Participants

The study population is women diagnosed with triple-negative breast cancer by a health professional such as a gynaecologist, a medical oncologist or an anatomopathologist.

Triple-negative breast cancer is considered, as in clinical routine, by immunohistochemistry (IHC) techniques carried out in the Pathology Department, observing low or null expression of estrogen receptors, progesterone receptors and HER2 amplification in a patient tumor tissue sample. These specimens have been obtained through a biopsy (BAG) done by a radiologist or a surgeon during the diagnostic process of the patient.

More specifically detailed, in clinical routine, tumors with ER and PR expression being 0 (negative) are considered negative hormone receptor breast cancer subtypes. HER2 is measured using the following algorithm: 3+ HER2 expression in IHC is considered positive, 0 or 1+ HER2 expression is considered negative, but if tumors present 2+ HER2 expression, fluorescent in situ hybridization (FISH) technique considers positivity or not for this parameter. Patients with ER, PR and HER2 negative are classified as triple-negative and have been, consequently, potential candidates to our study.

8.3. Inclusion and exclusion criteria

Inclusion criteria are the following:

- Confirmed diagnosis of triple-negative breast cancer subtype by IHC methods (Section 8.2).

- Minimum age of 18 years.

- Patients referred to Hospital Universitari de Girona Dr. Josep Trueta.

- Patients with enough surplus tumoral material from the healthcare process to perform tissue microarrays (TMA)

- Informed patients who have signed the informed consent form from the Tumor Bank of Hospital Universitari de Girona Dr. Josep Trueta during their clinical diagnostic, therapeutic or following-up process (Annex 1).

Exclusion criteria are the following:

- Breast cancer tissues with ER, PR or HER2 positive expression or amplification in IHC methods.

- Breast cancer with receptor status unknown or missing for at least one receptor type.

- Patients of whom the Tumor Bank has not enough tumoral material to perform TMA.

8.4. Setting

This study has been set in the specific research area of the Institut Català d'Oncologia (ICO) and the Pathology Department in Hospital Universitari de Girona Dr. Josep Trueta.

8.5. Sample size

The sample size and power calculator GRANMO has been used. Accepting an alpha risk of 0,05 and a beta risk lower than 0,2 in a two-sided test, 61 samples are needed in the first group (non-tumoral tissue samples) and 61 samples in the second one (tumor tissue samples) to detect a statistically significant difference between two proportions (percentage of ≥2+ in

FASN staining), which is expected to be 0,25 in group 1 and 0,50 in group 2. It has been estimated a rate of following loss of 5%. The ARCSINUS approach has been used.

It is estimated that 5 patients per year are diagnosed with triple-negative breast cancer in Hospital Universitari de Girona Dr. Josep Trueta. Thus, to guarantee the enough sample calculated, the study period has to include patients diagnosed during a minimum of 13 years. Taking into account clinical data loss, existence of enough tumor tissue samples in the Tumor Bank and availability of signed informed consent forms to carry up the study, women diagnosed between 1990 and 2012 have been included in this study, with a difference of 22 years from the first to the last patient studied (Section 8.6).

8.6. Sampling

Tissue sample collection has taken place at the Tumor Bank from the Hospital Universitari de Girona Dr. Josep Trueta, which is part of Institut d'Investigació Biomèdica de Girona (IdibGi), in a non-probabilistic sampling. They have been obtained from the surplus material of the diagnostic or therapeutic process of the patient and form part of the health care archive, which is considered Pathology Tumor Bank (Biobank) when applying for investigational purposes. Samples were started to be collected in 2012, when triple-negative project began. Successively, patients from previous years who meet all inclusion criteria have been also selected in order to achieve a sample size greater than 100, reaching up to 1990.

A final total of 104 microarrays containing the corresponding tumor samples and normal controls of 104 TNBC patients have been carried out and analysed in the Pathology Department (Section 8.8). From that, 100 tumor tissue samples and 92 normal tissue samples have been able to be graded and validated, whereas the remainder samples have not been found to be tumor or normal tissues, respectively, in a second histopathological analysis before applying IHC, and, consequently, have not been included in the study.

Also, from the codification of these 104 tumor samples and from additional clinical records obtained from TNBC patients referred to the Hospital Universitari de Girona Dr. Josep Trueta from 1990 to 2012, 90 TNBC patients have been selected, and their clinical and histopathological features of their oncologic processes have been collected. Summarazing, for 70 of these 90 patients, both FASN expression gradations from the corresponding tumor samples and clinical and histopathological data from clinical records have been compared and analysed.

8.7. Variables

Since this is a cross-sectional study, independent and dependent variables cannot be identified. Our variables have been approximately defined as potentially explanatory variable and primary endpoint variables.

8.7.1. Potentially explanatory variable

- The main variable is **FASN expression** in tissue samples, which is considered as ordinal and categorical. FASN expression has been graded as described below by IHC methods (Section 8.8) from 0 being null FASN expression to 3+ being maximum FASN expression.

8.7.2. Primary endpoint variables

- The **overall survival** is the main primary endpoint variable. It has been defined as the period from the diagnosis date to the death date of TNBC patients for any cause, or from the diagnosis date to date of control for living patients, which has been estimated as at 30th June 2014 for all of them. This death date has been obtained from the Índice Nacional de Defunción, and patients who did not appear in this source have been considered alive in this mentioned cut-off point. It has been considered as a continuous quantitative variable since it has been measured in years of survival and a nominal categorical variable since it has been measured into alive/dead/missed and, consequently, expressed as a proportion.

Other analysed primary endpoint variables have been the following:

- Tumor stage. It defines the extension of cancer in the patients' body and it is strongly related to prognosis and survival. It has been considered as an ordinal categorical variable and has been measured from I to IV based on the TNM status according to the American Joint Committee of Cancer (AJCC).

Table 3. Tumor stage validated by the American Joint Committee of Cancer (AJCC) and the International Union against Cancer (UICC) in its 7th edition from 2009 of AJCC Cancer staging manual.

Stage	T	N	M
0	Tis	N0	M0
I	T1	N0	M0
	T0	N1mi	M0
	T1	N1mi	M0
IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
IIB	T2	N1	M0
	T3	N0	M0
IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
IIIC	Any T	N3	M0
IV	Any T	Any N	M1

- Menopausal status. Menopause has been considered when, at the time of diagnosis, 12 months have passed after last patient menstrual period. It has been defined as a dichotomous nominal categorical variable and has been measured as yes/no.

- Lymph node involvement has been considered according to pathological criteria in the histological analysis of the tumor samples obtained within the surgery treatment process, which is carried out by the Pathology Department. It has been measured as a dichotomous nominal categorical variable since it has been collected as yes/no.

- **Relapse** has been defined as the return of the disease or the appearance of signs and symptoms of illness after a disease-free period. It has been considered a dichotomous nominal categorical variable and has been measured as yes/no.

- **Ki-67 grade** analysis determines the growth fraction of a studied cell population in a tumor tissue sample, and is carried out in the Pathology Department taking into account histological criteria. It is considered a discrete quantitative variable and has been measured as a percentage between 0 and 100.

- **CK 5/6 expression**. Since CK 5/6 positive expressions are characteristic of the *basal-like* subtype, these cytokeratins have been measured in these tumor samples and this variable has been categorized into negative/positive/positive focal according to their absence or presence, respectively. This variable is defined as a nominal categorical variable.

- **Histological subtype** variable is defined by pathological criteria according to cell resemblance and tissue pattern that constitutes the breast cancer neoplasy. It is considered as a nominal categorical variable.

- **Tumor size** is measured taking into account the biggest diameter that contains neoplastic cells in the quirurgical breast tissue sample. It is considered a continuous quantitative variable.

- **Histological grade** is a measure of cell anaplasia deviations in the growth rate of the tumor sample and is based on the resemblance of the tumor to the tissue of origin. It is considered as an ordinal categorical variable and has been grade from I to III; being I the most differentiated tumor cells and III the most undifferentiated cells, based on Bloom-Richardson grading system.

- **P53 expression.** P53 is a protein crucial in multicellular organisms, which regulates the cell cycle and has important functions as a tumor suppressor. It has been measured with IHC and

has been classified into positive/negative expression, which is related to the presence or not of mutations, respectively. It is considered as a dichotomous nominal categorical variable.

8.7.3. Covariates

Clinical and histopathological variables that can contribute to the overall survival results and, probably, FASN expression, have been also collected as covariates: age, TNM and tumor stage, histological grade and lymph node involvement:

- Age has been measured in years and has been considered as a discrete quantitative variable.

- TNM (tumor, nodes and metastases) has been measured according to the American Joint Committee of Cancer (AJCC). It has been considered as an ordinal categorical variable.

8.8. Measurements and data collection

In order to determine FASN tissue expression, formalin-fixed paraffin-embedded tissue sections obtained from the tumorectomy of the patient's primary tumor have been performed on tissue microarrays (TMA) in the Pathology Department of Hospital Universitari de Girona Dr. Josep Trueta. The pathologist have run the following design (Figure 6): four spots of tumor samples and one spot of normal tissue sample for each patient, used as a negative expression of FASN (control), have been placed to form one block. Each array contained 8 blocks from 8 patients, and has been histologically assessed by the pathologist for a second time to verify tumoral and non-tumoral spots with haematoxylin-eosin before applying immunohistochemistry.

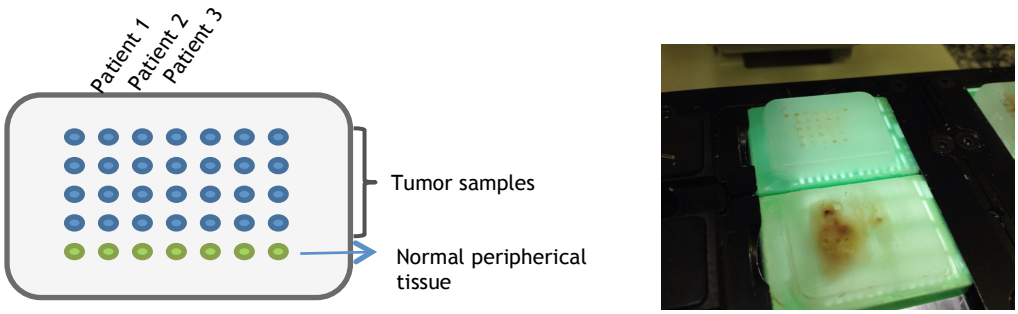


Figure 6. Tissue microarray design (TMA). Figure courtesy of Ariadna Giró (PhD student from NEOMA Research Group).

Immunohistochemical staining for FASN has been performed using a polyclonal antibody anti-FASN (Assay Designs, Enzo Life Sciences, Exeter, United Kingdom) at a dilution of 1:250. It has been used the detection kit EnVision™ (DAKO) using the AutostainerPlus Link (Dako). Negative control, using mouse IgG at a comparable concentration in place of the primary antibody has been included.

Briefly detailing the procedure, tissue sections of 3 μm thick have been deparaffinised, rehydrated, and blocked with 2% hydrogen peroxide for endogenous peroxidase. Slides have been washed with phosphate-buffered saline (PBS) and blocked with 20% horse serum (JRH Bioscience, Lexena, KS, USA). Slides have been, then, incubated with anti-FASN antibody overnight at 4°C. After additional PBS washes, sections have been sequentially incubated at room temperature for 45 minutes with biotin-labeled antirabbit IgG (Envision + R system Labelled Polymer- HRP anti-rabbit, Dako, Aachen, Germany). Slides have been washed again with PBS and incubated with diaminobenzidine (DAB, Sigma Chemical, St. Louis, MO). Finally, they have been counterstained with haematoxylin-eosin, dehydrated, cleared and cover-slipped. All tumor specimens analysed contained more than 50% tumor cells.

Then, FASN expression positivity has been graded from 0 to 3+ and scored into four categories, considering the average result of the 4 tumor spots (Figure 7):

- **0** absence of staining
- **1+** low staining
- **2+** moderate staining
- **3+** high staining

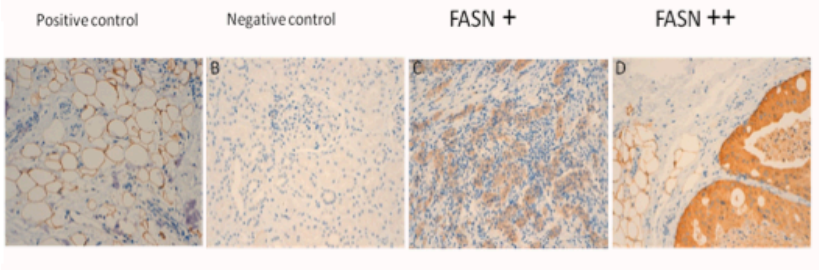


Figure 7. Immunohistochemical staining for FASN. A) Adipocytes, B) Renal tissue, C and D) Ductal carcinomas. *Brown corresponds to FASN staining.*

For analysis purposes, 0+ and 1+ have been categorized as low FASN expression, and 2+ and 3+ have been categorized as high FASN expression. Two pathologists blinded to other data have interpreted all immunohistochemically stained slides.

In parallel, clinical and pathological characteristics have been collected from medical records in paper format or from SAP (systems, applications, products) clinical management program in a database, CRF (Case Report Form), specifically created for the study. This collection has been conducted from January to November 2014 by three independent investigators blinded to FASN expression results and the CRF has been completed, designed and trained by the study personnel. The identification of patients has been encoded with their clinical history number in order to preserve the anonymity, and a second reassessment has been performed when data has been in disagreement between two independent investigators. All data from the histological variables have been collected from reports made by the Pathology service during routine diagnostic and therapeutic pathological analysis of oncologic samples.

9. Statistics

In the univariate analysis, patient and tumor characteristics have been descriptively summarized and assessed for normality before analysis using normal probability plots and Kolmogorov-Smirnov test statistics. Categorical variables have been expressed as absolute (number of cases) and relative frequencies (percentages) and represented with bar charts, while quantitative variables have been expressed as mean ± standard deviation if normal distribution has been assumed, or median and percentiles if not, and represented in box-plots.

In the bivariate analysis, dichotomous categorical variables have been compared in a 2x2 contingency table and evaluated by χ^2 test or Fisher's exact test. The strength of association among ordinal categorical variables could be analysed by Kendall's τ or γ coefficient, although it has not been performed in this project. For the main objective, a 2-sample test for equality of proportions with continuity correction has been used, and to analyse continuous and normally distributed data with categorical variables, a two-sided Student's τ test has been performed, or a U-Mann-Whitney test, if normality has not been assumed. Finally, the Log-Rank test and the Kaplan-Meier method have been applied for the survival analysis.

In the multivariate analysis and in order to adjust for overall-survival confounders, the Cox proportional hazards model could be used, although it is yet to be done in this current project. For that, survival results will be assumed carefully and, in the case where new hypotheses can be generated, these associations will be adjusted for the mentioned confounders in further studies.

All tests have been two-tailed and P values below 0,05 have been considered significant. Data analysis has been performed using the IBM Statistical Package for the Social Sciences (SPSS) V22, Intercooled Stata 8.0 for Windows® and R Statistical Package 2.15.

10. Study limitations

First limitation of this study is the involvement of only one institution. Results can be extrapolated to the population of the province of Girona but more studies are needed in different geographic areas to predict the role of FASN in all triple-negative breast cancer cases.

Secondly, a more thorough study of the patient medical records is required to have more clinical data, so that they can be correlated with FASN expression of the corresponding tumor sample in a higher number of patients, which will increase the reliability and internal validity of the study results and improve the inference of association between FASN expression and these features.

Thirdly, patients included in the study are those who have given their consent to donate their tumor samples to the Biobank for investigational purposes. A selection bias of patients must be taken into account when extrapolating results. A prospective study including analysis of tumor samples during the medical process of all triple-negative breast cancer patients of Girona, after being widely informed and having signed the appropriate informed consent form, may have a higher rate of participation and can improve the reliability of the results.

Fourthly, to increase validity of the hypothesis contrasts for secondary objectives, specifically design studies for this purpose are needed: in order to confirm association between FASN expression and a lower overall survival, a longitudinal and prospective study designed *ad hoc* is required. A possible information bias has to be taken into account since specific cause of death is not collected, so we are not able to inference results due to oncological reasons. Also, calculation of the sample is only performed to pay off the main objective. A proper calculation must be performed to better respond to secondary objectives.

To finish, given that the study is cross-sectional, no causality can be inferred and confusion factors must be taken into account. It is necessary, thus, a multivariate analysis to complete the statistical analysis (Section 9).

Finally, and despite all mentioned limitations, this study is the first project presenting results about FASN expression evaluation in a significative population of triple-negative breast cancer patients and set the stage for a larger and more elaborate study in which many additional breast tumors need to be examined and combined with detailed clinical information, which will provide, then, a means for validating new biomarkers, and specifically FASN, in TNBC.

11. Ethical aspects

This study has the conformity of Institut Català d'Oncologia (ICO), the Hospital de Girona Dr. Josep Trueta consent and the AEMPS authority. Also, it has been subjected to the evaluations of the Comitè Ètic d'Investigació Clínica (CEIC) and the Scientific Comitee of the Biobank of Institut d'Investigació Biomèdica de Girona (IdibGi).

It has been carried out in accordance to the ethical principles for medical research involving human subjects established by the most recent version of the Declaration of Helsinki by the World Medical Association and it has respected the Good Clinical Practice guidelines.

Furthermore, and more specifically to this study, it also complies existing local laws and regulations, such as the Ley Orgánica 15/1999 of 13th of December "Personal data protection", the Royal Decree 994/1999 of Security measures for automated files containing personal data, and the Royal Decree 1720/2007 of the Development of the organic law on data protection. Their main aim is to guarantee and protect personal data, freedom and fundamental rights of physical people, protecting the privacy of all the participants as well as the confidentiality of

their personal information, both in data collection and processment.

For that, patient identifications have been codified and patient identities will remain confidential in any presentation of the results of this study at meetings or in publications.

Also, it complies the Ley Orgánica 14/2007 of 3rd of July "Biomedical investigation: invasive procedures, biological samples and biobanks" and its Royal Decree 1716/2011, of 18 November, by which the basic requirements for authorization and operation of biobanks for biomedical research and treatment of biological samples of human origin are established, and also, the functioning and organization of the regulated National Registry of Biobanks for biomedical research.

Taking as reference the latter law, only patients diagnosed before its implantation are exempt from signing the informed consent form, when it comes to surplus material of the care process, but all patients diagnosed after that, have been widely informed and invited to sign the informed consent form agreeing that their corresponding tumor samples will be used for strictly research purposes, as well as their clinical data from their oncologic process may be consulted for the described purposes (Annex 1). Thereby, the principle of autonomy has also been respected.

Finally, all tumor samples have been obtained from the surplus material of the medical treatment and no invasive processes have been needed to perform this study, so not many ethical problems are either involved in the data obtaining process.

12. Results

12.1. Main objective

Expression of FASN in TNBC tumor samples and non-tumoral samples

From among 100 TNBC tumor tissue samples and 92 normal tissue samples analysed, 45 samples in the first group had a high (≥2+) staining for FASN expression (45%) and 22 samples in the second group had a high (≥2+) staining for FASN expression (23,91%). This difference of proportions has been considered statistically significant with a p value of 0,003604 ($p<0,05$ and $p<0,01$) (Figure 8).

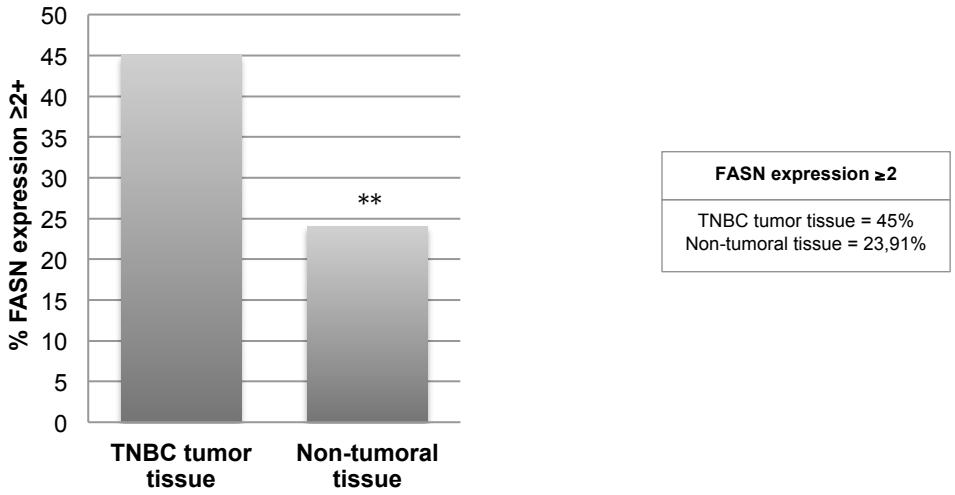


Figure 8. FASN expression in tumor and non-tumoral tissue from TNBC patients. *FASN expression has been graded with IHC methods, being 0+ absence of FASN staining, 1+ low FASN staining, 2+ moderate FASN staining and 3+ high FASN staining. 0/1+ have been categorized as low FASN expression and 2+/3+ have been categorized as high FASN expression, as described in Methods section. Normal tissue has been obtained from peripherical non-tumoral breast tissue from TNBC patients. Data are relative frequencies, expressed as percentages (n=100) ** p<0,01 versus control, by a 2-sample test for equality of proportions with continuity correction.*

12.2. Secondary objectives

Description of clinical and histopathological features of patients with TNBC (n=90)

Clinical and histopathological characteristics have been summarized in Table 4. In our study, mean age has been 57 years, proportion of premenopausal and postmenopausal women within TNBC population has found to be similar, the most prevalent tumor stage at diagnosis has been IIA, closely followed by stage I, and a predominance of node affection cases has been found. Thirty four percent of TNBC patients have had relapse, Ki-67 grade have had an average of nearly 60% and 54,4% of tumors have shown CK 5/6 negative expression whereas another 15,6% have shown positive expression. An 81,1% of the tumors have been infiltrating ductal carcinomas, the mean tumor size has been 27 mm and > 50% have been grade III. Finally, a predominance of p53 positive expression has been found.

Table 4. Clinical and histopathological characteristics of TNBC patients (n=90).

Characteristics		Values	
Age, mean (range) [median] y		57,67	(26-98) [53]
Menopausal status, n (%)	Premenopausal	33	(36,7)
	Postmenopausal	32	(35,6)
	Unknown	25	(27,8)
AJCC tumor stage, n (%)	I	17	(18,9)
	IIA	22	(24,4)
	IIB	12	(13,3)
	IIIA	11	(12,2)
	IIIB	11	(12,2)
	IIIC	4	(4,4)
	IV	3	(3,3)
	Unknown	10	(11,3)
Lymph node involvement, n (%)	Yes	42	(46,7)
	No	38	(42,2)
	Unknown	10	(11,1)
Relapse, n (%)	Yes	31	(34,4)
	No	40	(44,4)
	Unknown	19	(21,1)
Ki-67 grade, mean (SD)[a]		59,75	(23,517)
CK 5/6 expression, n (%)	Negative	49	(54,4)
	Positive	14	(15,6)
	Focal positive	8	(8,9)
	Unknown	19	(21,1)
Histological type, n (%)	Infiltrating ductal carcinoma	73	(81,1)
	Infiltrating lobular carcinoma	6	(6,7)
	Infiltrating tubular carcinoma	1	(1,1)
	Unknown	10	(10,10)
Tumor size, mean (SD) mm		27,56	(21,074)
Histological grade, n (%)[b]	I	0	(0)
	II	12	(13,3)
	III	51	(56,7)
	Unknown	27	(30)
P53 expression, n (%)	Positive	30	(33,3)
	Negative	28	(31,1)
	Unknown	32	(35,5)

[a] Normal distribution for this variable has been assessed with Kolmogorov-Smirnov test
[b] According to Bloom-Richardson grading system
AJCC, American Joint Cancer Committee

Association between FASN expression levels and clinicohistological characteristics

- Menopausal status: Among women with low FASN expression, 14 (56%) were premenopausal and 11 (44%) were postmenopausal, and among women with high FASN expression, 12 (50%) were premenopausal and 12 (50%) were postmenopausal, in a total of 49 subjects. Premenopausal women were more likely to have high expression of FASN levels [14 (53,8%) vs 12 (46,2%)] and postmenopausal women were more likely to have low expression of FASN levels [12 (52,2%) vs 11 (47,8%)]. Although, no statistically significant differences have been found between these two variables since p value provided by χ^2 = 0,674 (p value > 0,05).

- Tumor stage: Low FASN expression levels were higher in early stages, since a cumulative relative frequency of 71,4% has been found adding stage I, IIA and IIB. However, high FASN expression levels were found in later stages, since a 49,6% of them were found adding intermediate stages IIB, IIIA and IIIB. In IIIC stage, more high FASN expression levels have been found in comparison to low levels of FASN expression, in a final total of 65 subjects. However, no statistically significant differences have been found since Fisher's exact test has provided a p value = 0,635 (p value > 0,05).

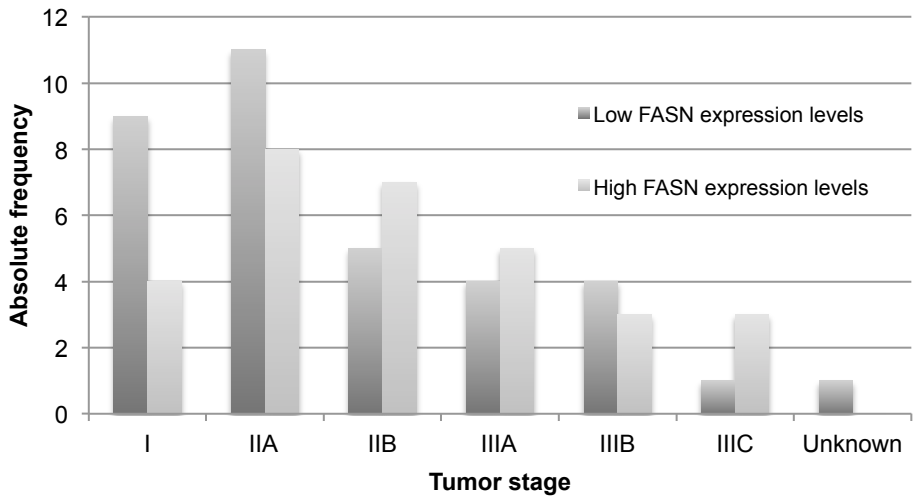


Figure 9. FASN expression levels in different tumor stages in TNBC patients. *Tumor stages have been classified according to the American Joint Committee of Cancer (AJCC). FASN expression has been graded with IHC methods, being 0+ absence of FASN staining, 1+ low FASN staining, 2+ moderate FASN staining and 3+ high FASN staining. 0/1+ have been categorized as low FASN expression and 2+/3+ have been categorized as high FASN expression, as described in Methods section. Dara are absolute frequencies. Fisher's exact test has been applied (n=90).*

- Lymph node involvement: Among women with low FASN expression levels, less proportion of lymph node involvement has been found [21 (58,3%) vs 15 (41,7%)] and among women with high FASN expression levels, more proportion of lymph node involvement has been found [18 (58,1%) vs 13 (41,9%)] in a total of 67 subjects. However, χ^2 test has not found statistically significant differences between these two variables, finding a p value = 0,181 (p value > 0,05).

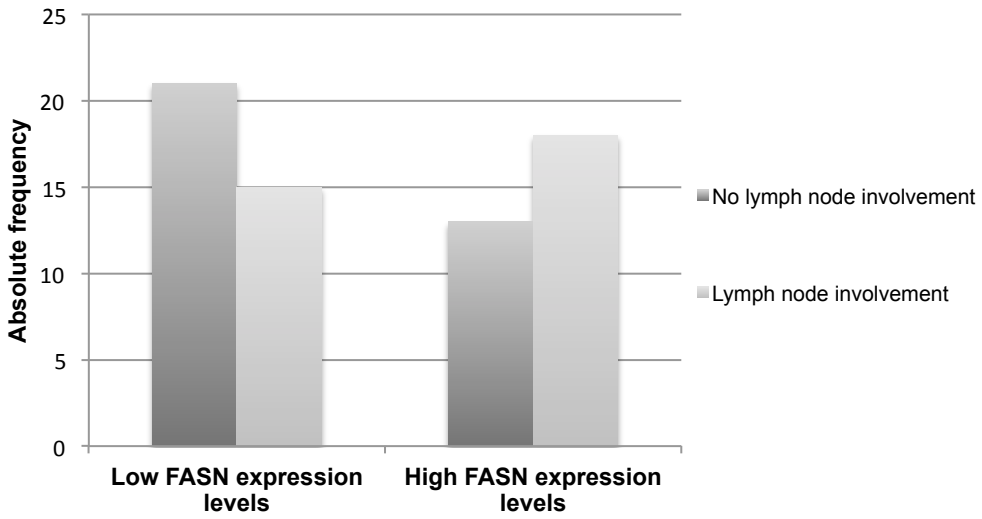


Figure 10. Lymph node involvement according to FASN expression levels in TNBC patients. *Lymph node involvement has been considered according histological analysis in the surgical specimen during treatment process of TNBC patients. FASN expression has been graded with IHC methods, being 0+ absence of FASN staining, 1+ low FASN staining, 2+ moderate FASN staining and 3+ high FASN staining. 0/1+ have been categorized as low FASN expression and 2+/3+ have been categorized as high FASN expression, as described in Methods section. Data are absolute frequencies. χ^2 test has been applied (n=90).*

- Relapse: Among women with low FASN expression 39,4% had a relapse, and among women with high FASN expression 41,7% had a relapse, in a final total of 57 subjects. No statistically significant differences have been found since p value provided by χ^2 test has been equal to 0,863 (p value > 0,05).

- Ki-67 grade: Normality for this variable has been assessed with Kolmogorov-Smirnov test before analysing it. In the low FASN expression group, Ki-67 grade had a mean of 57,72 with a standard deviation of 21,946; while in high FASN expression group, Ki-67 had a mean of 57,67 with a standard deviation of 26,815. In a τ test, equal variances have been assumed and p

value has been equal to 0,995, which concludes that no statistically significant differences exist between Ki-67 means regarding FASN expression levels.

- CK 5/6 expression: It has been analysed in 83 patients. The majority of the subjects [64 (77,1%)], taking into account both high FASN expression group and low FASN expression group, had negative staining for CK 5/6, since in both groups the majority of cases have also separately shown negative staining. Fisher's exact test have found no statistically significant differences between different levels of FASN expression and CK 5/6 expression.

Survival analysis and survival curves according to FASN expression levels

In the low FASN expression group, 17 events occurred of a total of 38 followed cases (55,3%) and in the high FASN expression group, 16 events occurred of a total of 32 followed cases (50%); in a final total of 70 subjects. Means, medians and percentiles are shown in table 5A and 5B. Low FASN expression curve remains above high FASN expression curve and shows more accumulate survival in the first group for a majoritarian period. Log-Rank test have not found statistically significant differences since p value is 0,355 (p value > 0,05) in this test for equality of survival distributions for different FASN levels.

Table 5A. Survival analysis according to FASN expression levels in TNBC patients.

FASN in tumor tissue	Mean				Median			
	Estimation	Standard deviation	95% Confidence interval (CI)		Estimation	Standard deviation	CI 95%	
			Lower limit	Upper limit			Lower limit	Upper limit
Low FASN expression	13,549	1,689	10,239	16,859	13,148	-	-	-
High FASN expression	11,999	2,024	8,032	15,967	8,003	4,042	0,080	15,926

Low FASN expression is considered when FASN staining is 0+ (absence) or 1+ (low) in IHC methods. High FASN expression is considered when FASN staining is 2+ (moderate) or 3+ (high) in IHC methods, as described in Methods section (n=107).

Table 5B. Survival analysis according to FASN expression levels in TNBC patients.

FASN in tumor tissue	Percentile 50		Percentile 75	
	Estimation	Standard deviation	Estimation	Standard deviation
Low FASN expression	13,148	-	3,468	0,876
High FASN expression	8,003	4,042	2,282	0,315

Low FASN expression is considered when FASN staining is 0+ (absence) or 1+ (low) in IHC methods. High FASN expression is considered when FASN staining is 2+ (moderate) or 3+ (high) in IHC methods, as described in Methods section (n=107).

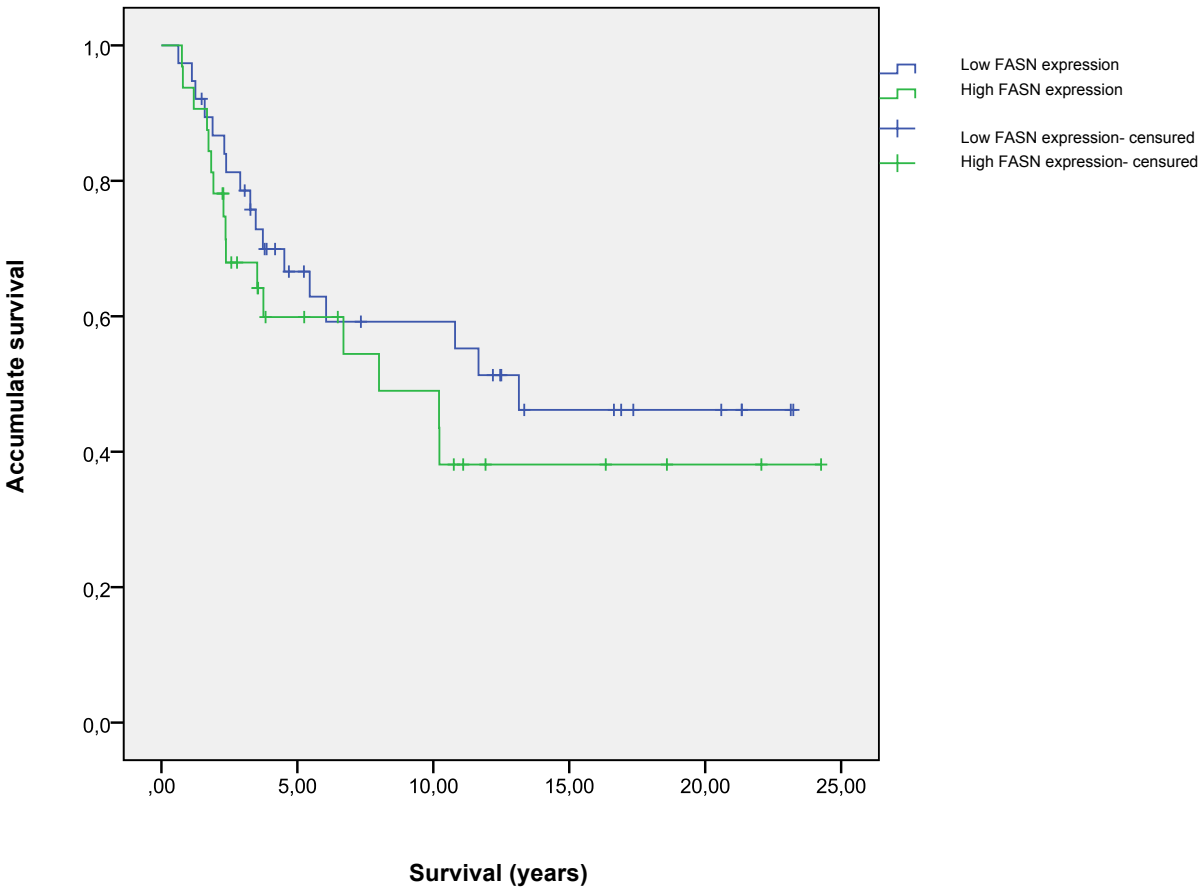


Figure 11. Survival curves of TNBC patients according to FASN expression levels. *For survival analysis, diagnosis date and death date for deceased patients or diagnosis date and date of control for living patients (that has been estimated as at 30th June 2014) has been considered. Death date has been obtained from the Índice Nacional de Defunción provided by the Registre del Càncer de Girona, as described in Methods section. Data are relative frequencies for accumulate survival and survival is measured in years. The Kaplan-Meier method has been applied (n=107).*

13. Discussion

Expression of FASN in TNBC tumor samples and non-tumoral samples

The main result of this study is that FASN expression levels differ between tumoral and non-tumoral TNBC patient tissues since statistically significant differences (p value= 0,003604) have been found between these two groups, being FASN expression higher in tumor samples.

This finding is in accordance to previous studies from our research group and from others, which demonstrate FASN overexpression in a wide variety of human carcinomas, including breast cancer (24). But this is the first study performed in a significative triple-negative breast cancer population, although FASN expression has been previously evaluated in TN breast cell-lines in pre-clinical studies and in serum of breast cancer patients (18,23). This strong association provides new evidence about the role of FASN as a tumor biomarker, not only in breast cancer in general, but specifically in triple-negative breast cancer, where the lack of tumor biomarkers entails a worrying lack of therapeutic targets. Further studies are required in order to define the biological significance and to validate the role of FASN in this specific breast cancer subtype.

Description of clinical and histopathological features of patients with TNBC

Mean and median age are in accordance to other breast cancer studies (around 50-60 years), although our values are quite older in comparison to TNBC specific studies whereas the age range is quite wider (5,24). Also, premenopausal and postmenopausal proportions of women affected with triple-negative breast cancer are very similar, which contrasts to the opposite results published in other studies where detect more prevalence of TNBC in premenopausal young women (1,5). This can be explained by the fact that population is aging and prevalence of older population is increasing, who also present postmenopausal characteristics, and thus,

breast cancer cases have increased in this particular age range in absolute numbers, equalling the total proportion of premenopausal women with postmenopausal women among TNBC patients. A proper correction for age should minimalize these differences.

Tumor stage shows accordance with previous studies, being stage II the most prevalent, followed by stage III. This can be explained by the advancement of the techniques for the early diagnosis but, also, that advanced stages are more frequently found in TNBC subtype than in other breast cancer subtypes, which equals the major proportion in intermediate stages (1).

The proportion of patients with lymph node involvement and no lymph node involvement is found to be quite similar. However, some studies reported lower axillary lymph node-positive cases in TNBC group in comparison to other breast cancer subtypes; for this, further studies designed for this purpose would validate or reject this hypothesis since our results are not concordant, probably due to the limited number of patients (8,11,13).

According to relapse results, a 34,4% of TNBC patients present relapse criteria. This finding is in accordance to previous studies, which present a higher rate of TNBC relapse cases in comparison to other breast cancer subtypes, whose relapse case percentage sorrounds the 30% (5).

Ki-67 grade mean is 59,75, which shows agreement with other pathological studies that present this high mitotic index as a solid characteristic in TNBC patients (5,6), and CK 5/6 expression is mostly negative, which differs from most molecular studies, since CK 5/6 expression is characteristic of the *basal-like* phenotype (7). However, the *basal-like* phenotype is usually defined as CK 5/6 and/or EGFR positivity by IHC methods, so EGFR determination is needed to classify TNBC tumors with a *basal-like* phenotype. Moreover, the cut-off point to

establish positivity to CK 5/6 has been considered when > 10% of cells present CK 5/6 staining above the background staining, but some studies establish this cut-off point at 1%, which will increase CK 5/6 positive expression in our results (32). Formol problems should also be considered (in some of the tumor samples formol precipitates have been found), since tumorectomy specimens have been preserved in formol for more than 10 years in many samples, which can mask IHC results. For that, EGFR determination and improved CK 5/6 IHC studies are needed to determine the *basal-like* phenotype in our TNBC tumor sample.

The most prevalent histological subtype is the infiltrating ductal carcinoma, which is in accordance to other studies about both breast cancer and TNBC subtype, and histological grade III remains the most frequently found, which shows consistence with the aggressive characteristics described in this type of tumors (5,6).

Finally, P53 has more positive than negative cases, which suggests that this overexpression is due to P53 mutations, which is in accordance with previous TNBC studies (5).

Association between FASN expression levels and clinicohistological characteristics

We also wanted to analyse association between FASN expression levels and a selection of clinical and pathological characteristics, as a first study to generate new hypothesis in a clinical phase, since some of these items have been related before in pre-clinical studies. However, no statistically significant differences have been found regarding menopausal status, tumor stage, lymph node involvement, relapse, Ki-67 grade or CK 5/6 expression.

Postmenopausal status was related to high FASN tumor expression levels in a previous study from the group (24), which has not been able to verify with this current project, although most of the patients included were hormone receptor positive or HER2 positive. Another possible

explanation could be the lack of sufficient number of patients to detect this association, since only data for 49 subjects has been analysed for this parameter.

FASN serum concentrations were significantly higher as it advanced the tumor stage in a study, but not FASN tissue expression was analysed, as it is in the current study (18). Low FASN expression levels are found in early tumor stage phases, high FASN expression levels are found to be higher in intermediate stages, and in the most advanced stage (IIIC), high FASN expression levels predominate over low FASN expression levels, which could suggest a potential role of FASN as a prognostic biomarker that needs to be further studied. For that, it is necessary to increase the sample size of the study to reinforce this hypothesis.

Also, a shortened disease-free survival, which can be approximated to our relapse variable, has been reported in patients with high levels of FASN expression, but methods to analyse disease-free survival differ in these studies, since it is measured according to radiological criteria and and not clinical criteria. Thus, further analyses are needed to validate these associations (23).

As far as we know, none of the other parameters (lymph node involvement, Ki-67 grade and CK 5/6) had been studied before according to FASN expression levels.

Regarding lymph node involvement, our results express that, among the low FASN expression group, less lymph node involvement is found and vice versa: among the high FASN expression group, more lymph node involvement is found. This suggests that tumors with high expression of FASN are more aggressive, which is consistent with previous studies that provide more evidence about the potential role of FASN as a prognostic marker (23).

Regarding Ki-67 grade, no differences have been found in the two FASN groups, since the high mitotic index is an inherent characteristic of triple-negative breast cancer and it is found to be independent of FASN levels, and thus, appears high in both groups separately.

Finally, CK 5/6 results are not completely reliable, as has been discussed previously, and no inferences should be taken between CK 5/6 expression and FASN expression levels in our tumor samples. A second revision about CK 5/6 staining and EGFR analysis is strongly needed in further TNBC studies.

Despite these results, a more exhaustive search is needed to collect the maximum possible missing clinical data that could not be analysed in this current project. Then, some of these associations could become statistically significant.

Survival analysis and survival curves according to FASN expression levels

In the survival analysis, median and mean times of survival in low FASN expression group are longer (about 13 years in both measurements) in comparison to the high FASN expression group (about 8 and 12 years, respectively). This interesting result supports the idea that high FASN expression in tumor samples may be considered as a poor prognostic biomarker regarding survival parameters, and more specifically designs for this purpose should be carried out in future studies.

As far as we know, FASN tissue expression levels have not been related to overall survival in clinical studies before. There, these results could generate new hypotheses for future research lines. However, no statistically significant differences have been found in the bivariate analysis between these two parameters. Explaining this, in Figure 11, we can see how the survival curve corresponding to patients who had tumors with low levels of FASN expression is always kept above the survival curve corresponding to patients who had tumors with high levels of

FASN expression, except at one point, around the period of 6 years. This junction could be explained by the low number of patients, who had high FASN expression levels and died around this period in comparison to the number of patients in other time periods and, thus, the corresponding survival curve at this point does not have enough cases to descend at the same way as the other curve. A possible explanation is that it is probably due to fewer subjects in high FASN expression group than in low FASN expression group and we venture that a few more cases would restructure this curve, remaining in all moments of time under the low-FASN expression curve. However, new studies are needed to reaffirm these assumptions.

14. Conclusions

Our project provides the basis for upcoming studies and research lines to validate the enzyme fatty acid synthase (FASN) as a novel tumor biomarker in triple-negative breast cancer patients and proporcionate the bases for a more exhaustive study to approximate FASN as a new prognosis indicator and a potential therapeutic target or co-target in triple-negative breast cancer population.

15. Scientific history of the research group

I have developed my final degree project in the Molecular Oncology (NEOMA) group from Medical Science Department (Faculty of Medicine, University of Girona), which Dra. Teresa Puig is the Principal Investigator.

- Group components: Dr. T Puig (IP), Dr. A Sarrats (postdoctoral researcher), A Blancafort, A Giró and S Palomeras (pre-doctoral researchers), Dr. G Oliveras (research technician and responsible for the Tumor Bank), Dr. G Viñas and Dr. R Porta (medical oncologists), Dr. F Pérez (pathologist), M Rabionet and A Roqué (final degree students).
- Researchers (groups) associated with the project:

Product, Process and Production Engineering Research Group (GREP) at the University of Girona: Dr. J Ciurana (full professor).

Processes and Products of Organic Synthesis Research Group (LIPPSO) at the University of Girona: Dr. M Planas (professor) and Dr. L Feliu (professor).

The multicenter, multidisciplinary research team [Faculty of Medicine and Science (University of Girona), Catalan Institute of Oncology-Institute for Biomedical Research (Girona), Institute of Biomedical Research of Bellvitge (IDIBELL) in Barcelona and University Hospital Dr. Josep Trueta] initiated the joint activity in the area of endogenous fatty acid synthesis in breast cancer in 2008. The resulting projects have been recognized and funded by various public and private calls, as well as awards (2007, 2008, 2010 and 2014).

Currently, the research group has focused on the study of the antitumor activity of FASN inhibitors, characterization of the molecular mechanisms generated by inhibition of FASN in breast cancer, analysis of drug interactions with other drugs and models of chemosensitive and chemoresistant breast cancer. Fruit of the results, several publications in journals of high

impact index, book chapters (3) and scientific presentations (oral and posters) in several national and international conferences have been generated.

It has also led to the characterization of lead-compounds with anti-tumor activity and, as a result, three patents have been applied (P201030239, EP2008/058099 and P201231228), one of which has been transferred to the pharmaceutical industry, and another one allowed to LIPPSO group to create a spin-off. Also, three collaboration contracts with the pharmaceutical industry (Wyeth, Pfizer and Italfármaco) and a contract with AbilityPharma, the developing company drugs (Bellaterra, Barcelona) have been signed.

In order to translate these findings into clinical practice, clinical trials are required, which include the clinical experience of group members conducting clinical trials at the University Hospital Dr. Josep Trueta.

At a training level, the group has directed two doctoral theses, 4 more are in progress and counts with a master student and two final grade students. In addition, several national and international collaborations with the intention of promoting synergies and promote talent have been established: Dr. Alana L Welm (Huntsman Cancer Institute, University of Utah, Salt Lake City, USA), Dr. Balazs Gyorffy (Szentágothai János Knowledge Centre, Semmelweis University Budapest, Hungary), Dr. Ramon Brugada (Centre for Cardiovascular Genetics-IdIBGi, Girona), Manel Esteller (IDIBELL, Barcelona) and Dra. González-Suárez (IDIBELL, Barcelona).

Researchers (groups) associated with the project also have a proven track record. The group Product, Process and Production Engineering Research (GREP) at the University of Girona has published over 100 articles about methodology designs, planning manufacturing processes, characterization of machining processes, additive-manufacturing processes, and design and prototyping of medical devices. It has been funded by more than 30 research projects and has directed 15 doctoral theses. Currently, its research focuses on the application of additive

manufacturing technologies to the development of medical devices in metallic or polymeric biodegradable, biocompatible and bioimplantable materials.

The research group Innovation Lab Processes and Products of Organic Synthesis (LIPPSO) of the University of Girona, is recognized as Consolidated Research Group (2014 SGR 639) by the Generalitat de Catalunya. Their main research focuses on developing compounds with biological activity, and is currently involved in the design and synthesis of polyphenolic compounds with activity against breast cancer. The group has published over 40 research articles, several patents and doctoral theses.

Finally, last years financing in competitive grant funding is the following:

- In 2006, Ministry of Science and Innovation (MCINN). Juan de la Cierva (JCI/2005/1616/1; 2006-2009). TPuig.

- In 2013, Ministry of Education, Culture and Sport. Collaboration grant (2013-2014/7979206). ZAranda.

- In 2013, AGAUR predoctoral scholarship of international mobility (BE-DGR2012-9015-64418/2012). ABlancafort.

- In 2014, Instituto de Salud Carlos III. FIS. Preclinical characterization of fatty acid metabolism inhibition in chemoresistant triple-negative breast cancer models (PI1400329).

- In 2014, “La Marató de TV3” foundation. RANK signalling as a novel therapeutic target in HER2+ and HER2+ resistant breast cancer.

- In 2014, RETOS/INNPACTO (MINECO). Development of new high efficiency and specificity, and low toxicity cancer therapies for oral administration (RTC-2014-2589-1).

- In 2014, Ministry of Education, Culture and Sport. Collaboration grant (2014-2015/10108860) MRabionet.

- In 2014, Ministry of Education, Culture and Sport. Collaboration grant (2014-2015/9863231). ARoqué.

16. Index of tables and figures

---

17. Annexes

17.1. Annex 1. Information sheet for patients and informed consent form

Hospital Universitari de Girona Doctor Josep Trueta · ICO Institut Català d'Oncologia · IAS Institut d'Assistència Sanitària · Bio Banc IDIBGI

FULL D'INFORMACIÓ AL PACIENT

UTILITZACIÓ DE DADES CLÍNIQUES I MATERIAL BIOLÒGIC EXCEDENT DEL PROCÉS ASSISTENCIAL PER A INVESTIGACIÓ BIOMÈDICA I LA SEVA CONSERVACIÓ EN UN BIOBANC

A l'Hospital Universitari de Girona Josep Trueta (HUGJT) i/o altres Centres Hospitalaris adscrits, igual que en la majoria d'hospitals, a més de l'assistència als pacients, es realitza investigació biomèdica. La finalitat d'aquesta investigació és progressar en el coneixement de les malalties i en la seva prevenció, diagnòstic i tractament. Aquesta investigació biomèdica requereix recollir dades clíniques i mostres biològiques de pacients i donants sans per a analitzar-los i obtenir conclusions amb l'objectiu de conèixer millor les malalties i avançar cap al seu diagnòstic i/o tractament.
Les mostres i dades clíniques obtingudes per al diagnòstic o control de les malalties, una vegada utilitzades amb aquesta finalitat, resulten també útils i necessàries per a la investigació. De fet, molts dels avenços científics obtinguts en aquests últims anys en medicina són fruit d'aquest tipus d'estudis. Si no fossin cedides per a investigació, aquestes mostres biològiques sobrants o excedents del procés assistencial serien destruïdes.

Sol·licitem la seva autorització per a incorporar al Biobanc IDIBGI el material biològic sobrant de les proves que, com a part de l'actual procés assistencial, se li han realitzat o se li realitzaran en aquest centre, amb la finalitat que puguin ser utilitzades en investigació biomèdica.

Seguint el que estableix la Llei 14/2007, d'Investigació Biomèdica, la Llei Orgànica 15/1999, de Protecció de Dades Personals, i les seves normes de desenvolupament, li sol·licitem que llegeixi detingudament aquest document d'informació i el consentiment informat que se li adjunta al final per a la seva firma, si està d'acord en participar en aquesta proposta.
Un Biobanc és una institució regulada per lleis específiques que facilita la investigació biomèdica, és a dir, aquella destinada a promoure la salut de les persones. Les mostres incloses en un Biobanc poden ser cedides per a la investigació en Medicina, sempre sota la supervisió d'un comitè científic i un altre d'ètica. Les mostres es cediran generalment sense informació personal associada, encara que a vegades podrà ser necessari l'accés a la història clínica o al resultats d'altres proves per a completar la investigació.

FINALITAT DE LA INVESTIGACIÓ: progressar en el coneixement de les malalties

La finalitat de la investigació és millorar el nostre coneixement de les malalties. Les mostres, les dades clíniques i analítiques i les proves d'imatge s'utilitzaran per a la recerca biomèdica.

MOSTRES BIOLÒGIQUES I INFORMACIÓ ASSOCIADA: es custodiaran i conservaran al Biobanc IDIBGI fins la seva extinció.

Es guardarà i disposarà de material biològic sobrant que se li extregui durant el procés assistencial (mostres de sang, líquids biològics i/o teixits) per a realitzar estudis d'investigació biomèdica, sense que aquest fet li causi molèsties addicionals. La donació de mostres excedents d'aquest procés assistencial no impedirà que vostè o la seva família puguin utilitzar-les, quan sigui necessari per motius de salut, sempre que estiguin disponibles. Les mostres i la informació associada a les mateixes es custodiaran i/o guardaran en el Biobanc (banc de mostres biològiques) IDIBGI fins a la seva extinció.
Aquest Biobanc és un establiment sense ànim de lucre i inscrit en el *Registro Nacional de Biobancos* dependent de l'*Instituto de Salud Carlos III* amb la referència B.0000872, que acull col·leccions organitzades de mostres biològiques i informació associada a les condicions i garanties de qualitat i seguretat que exigeix la legislació anteriorment referida i els codis de conducta aprovats per els Comitès d'Ètica. Les esmentades mostres i la seva informació associada queden disponibles per aquells investigadors que ho sol·licitin oficialment al Biobanc IDIBGI.
Qualsevol estudi d'investigació per al qual se sol·liciti la utilització d'aquestes dades o mostres haurà de disposar sempre de l'aprovació del Comitè d'Ètica de la Investigació Clínica (CEIC) competent, que vetllarà per a què els investigadors desenvolupin els seus estudis seguint sempre les més estrictes normes ètiques i legals. A més, el comitè científic del Biobanc garantirà que els projectes siguin d'excel·lència científica. La investigació biomèdica és actualment un fenomen global, de manera que ocasionalment aquestes mostres podran ser cedides a grups d'investigació fora d'Espanya, sempre que compleixin els requisits de la legislació espanyola i ho aprovin els corresponents comitès.

A partir de les mostres donades, en els casos en que la investigació ho requereixi, es realitzaran estudis genètics, i a partir d'ells es pot obtenir informació sobre la seva salut i la dels seus familiars. Sempre s'actuarà vetllant per la protecció d'aquesta informació (apartat protecció de dades).
Per aquest consentiment, els responsables del Biobanc IDIBGI podran consultar el seu historial clínic, només en el cas que això sigui imprescindible per a la investigació del projecte per al qual se sol·liciten les mostres i prèvia autorització per part del Comitè d'Ètica corresponent.
En el cas de ser necessària alguna mostra addicional, la institució sanitària es podria posar en contacte amb vostè per a sol·licitar-li novament la seva col·laboració. En aquest cas se li informarà dels motius i se li sol·licitarà de nou el seu consentiment.

PROTECCIÓ DE DADES I CONFIDENCIALITAT: les mostres es conservaran codificades.

Les dades personals que es recullin seran obtingudes, tractades i emmagatzemades complint en tot moment el deure del secret, d'acord amb la legislació vigent en matèria de protecció de dades de caràcter personal.
La identificació de les mostres biològiques del Biobanc serà sotmesa a un procés de codificació. A cada mostra se li assigna un codi d'identificació, que serà l'utilitzat per els investigadors. Només el personal autoritzat per el Biobanc podrà relacionar la seva identitat amb els citats codis. Mitjançant aquest procés els investigadors que sol·licitin mostres al Biobanc no podran conèixer cap dada que reveli la seva identitat. De la mateixa manera, encara que els resultats obtinguts de la investigació realitzada amb les seves mostres es publiquin en revistes científiques, la seva identitat no serà facilitada. En aquells estudis en els quals no es prevegin resultats potencialment útils per a la seva salut, i d'acord amb el corresponent Comitè d'Ètica, les mostres i dades podran ser anonimitzades, és a dir, no hi haurà cap possibilitat de tornar a associar la mostra amb la seva identitat.
Les seves mostres i dades clíniques associades a les mateixes passaran a formar part del fitxer del Biobanc, inscrit en l'Agència de Protecció de Dades. La persona responsable de la custòdia és el Director del Biobanc.
Vostè podrà exercir els seus drets d'accés, rectificació, cancel·lació i objecció, així com obtenir informació sobre l'ús de les seves mostres i dades associades, dirigint-se a la Direcció del Biobanc IDIBGI per correu electrònic (Biobanc@IDIBGI.org) o via postal a la següent adreça:

DIRECCIÓ DEL BIOBANC IDIBGI	Avinguda de França s/n
Hospital Universitari de Girona Dr Josep Trueta	17007 Girona
Biobanc@IDIBGI.org	Tfn 972 940 282

CARÀCTER ALTRUISTA DE LA DONACIÓ: La cessió de mostres biològiques que vostè realitza al Biobanc IDIBGI és gratuïta.

La donació té per disposició legal caràcter altruista, per la qual cosa vostè no obtindrà ni ara ni en el futur cap benefici econòmic de la mateixa, ni tindrà drets sobre possibles beneficis comercials dels descobriments que es puguin aconseguir com a resultats de la investigació biomèdica.

PARTICIPACIÓ VOLUNTÀRIA: la seva negativa NO repercutirà en la seva assistència mèdica, present o futura.

La seva participació és totalment voluntària. Si firma el consentiment informat, confirmarà que desitja participar. Pot negar-se a participar o retirar el seu consentiment en qualsevol moment posterior a la firma sense haver d'explicar els motius i que això repercuteixi en la seva assistència mèdica, present o futura.

REVOCACIÓ DEL CONSENTIMENT: si vostè decideix firmar aquest consentiment podrà també cancel·lar-lo lliurement. Això comportarà la destrucció de les mostres.

Si en un futur vostè volgués anul·lar el seu consentiment, les seves mostres biològiques serien destruïdes i les dades associades a les mateixes serien retirades del Biobanc. També podria sol·licitar l'anonimització de les mostres, de manera que en aquest cas s'eliminaria la relació entre les seves dades personals (que revelen la seva identitat) i les seves mostres biològiques i dades clíniques associades. Els efectes d'aquesta cancel·lació o anonimització no es podrien estendre a la investigació que ja s'hagi realitzat. Si desitgés cancel·lar el consentiment, ho hauria de sol·licitar per escrit a la Direcció del Biobanc IDIBGI, a l'adreça anteriorment mencionada.

INFORMACIÓ SOBRE ELS RESULTATS DE LA INVESTIGACIÓ: se li proporcionarà informació si vostè la desitja rebre.

En el cas que vostè ho demani expressament, el Biobanc podrà proporcionar informació sobre quines són les investigacions en què s'han utilitzat les seves mostres i dels resultats globals d'aquestes investigacions, excepte en el cas de cancel·lació o anonimització.
Els mètodes utilitzats en investigació biomèdica solen ser diferents dels aprovats per a la pràctica clínica, per el que no han de ser considerats amb valor clínic per a vostè. Malgrat això, en el cas que aquestes investigacions proporcionin dades que poguessin ser clínica o genèticament rellevants per a vostè i interessar a la seva salut o a la seva família, li seran comunicats si així ho estima oportú. Així mateix, podria donar-se el cas que s'obtingui informació rellevant per a la seva família. En aquest supòsit, li correspondrà a vostè decidir si vol o no que aquesta informació li sigui comunicada. En cas afirmatiu, ha de consignar-ho a la casella que apareix al final d'aquest document.
Si vostè no desitja aquesta informació, tingui en compte que la llei estableix que, quan la informació obtinguda sigui necessària per a evitar un greu perjudici per a la salut dels seus familiars biològics, un Comitè d'experts estudiarà el cas i haurà de decidir si és convenient informar als afectats o als seus representants legals.

Si us plau, pregunti al personal sanitari que li ha comunicat aquesta informació sobre qualsevol dubte que pugui tenir, ara o en el futur, en relació a aquest consentiment. Així mateix, pot comentar els seus dubtes al seu metge, que el posarà en contacte amb el personal sanitari autoritzat.

Moltes gràcies per la seva col·laboració.

BIOBANC IDIBGI

Li agraïm la seva desinteressada col·laboració amb l'avenç de la ciència i la medicina.
D'aquesta manera està col·laborant a vèncer les malalties i ajudar a multitud de malalts actuals i futurs.

Hospital Universitari de Girona Doctor Josep Trueta · ICO Institut Català d'Oncologia · IAS Institut d'Assistència Sanitària · Bio Banc IDIBGI

CONSENTIMENT INFORMAT

Exemplar DONANT

UTILITZACIÓ DE DADES CLÍNIQUES I MATERIAL BIOLÒGIC EXCEDENT DEL PROCÉS ASSISTENCIAL PER A INVESTIGACIÓ BIOMÈDICA I LA SEVA CONSERVACIÓ EN UN BIOBANC

Si ha comprès la informació que se li ha proporcionat en el document informatiu, resolt qualsevol dubte que pogués tenir i decideix col·laborar amb el Biobanc IDIBGI en els termes abans explicats, si us plau, llegeixi i firmi a continuació aquest full:

Qui signa el present document autoritza al Biobanc IDIBGI a emmagatzemar i utilitzar científicament tant la informació clínica i assistencial del seu historial mèdic com les proves d'imatge i les mostres biològiques sobrants de les proves que se li han realitzat o se li realitzaran com a part de l'actual procés assistencial en l'HUGJT i/o altres Centres Hospitalaris adscrits, amb la finalitat de dur a terme projectes de recerca biomèdica, sempre que aquests comptin amb l'obligada aprovació del Comitè d'Ètica d'Investigació competent. Aquesta autorització la concedeix després d'haver estat informat verbalment i haver llegit la informació adjunta.

Confirmo que:

1. Autoritzo que l'excedent de material biològic utilitzat per a proves diagnòstiques i la informació clínica associada s'utilitzin per a investigació:
   Nacionals: ☐ SÍ ☐ NO Internacionals: ☐ SÍ ☐ NO

2. Desitjo que se'm comuniqui la informació derivada de la investigació que realment sigui rellevant i aplicable per a la meva salut o la de la meva família:
   ☐ SÍ ☐ NO Telèfon o email de contacte.....

3. Autoritzo a ser contactat en el cas de necessitar més informació o mostres biològiques addicionals:
   ☐ SÍ ☐ NO Telèfon o email de contacte.....

4. He expressat el meu desig de que se'm respectin les següents excepcions respecte a l'objectiu i mètodes de les investigacions:
   .....
   .....

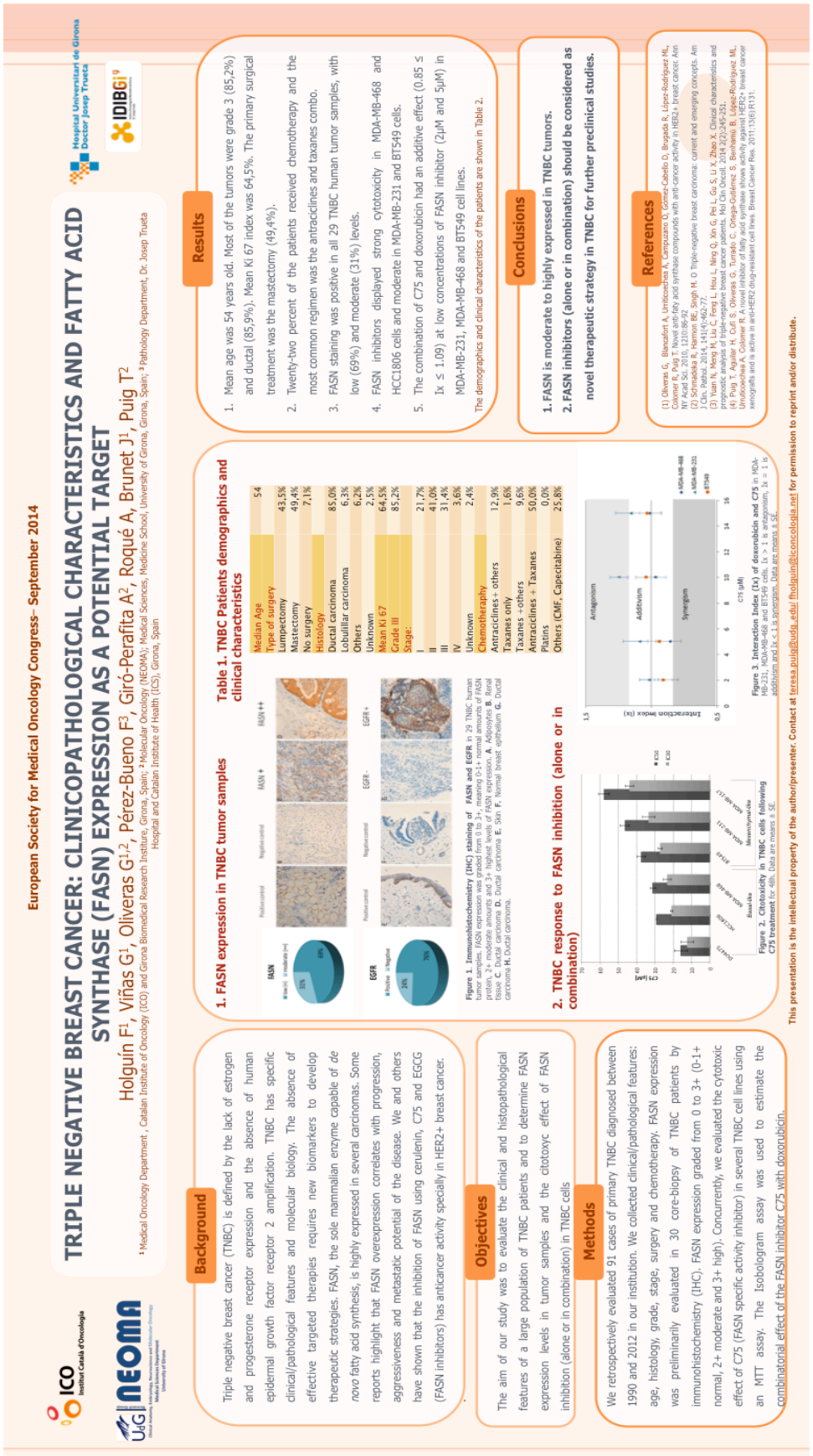
DONANT	PERSONA QUE INFORMA	☐ TESTIMONI[1] / ☐ TUTOR[2]
Nom *Cognoms* *DNI* *Edat* *Signatura*	*Nom* *Cognoms* *DNI* *Signatura*	*Nom* *Cognoms* *DNI* *Relació amb el donant:* *Signatura*

[1] Autoritzat pel donant
[2] Representant legal

A ....., a .....de.....de.....

Arribada la majoria d'edat, el donant té dret a l'anul·lació del consentiment. En cas que no l'exerceixi, es considerarà que l'actual document de consentiment continua vigent.

17.2. Annex 2. Poster. European Society for Medical Oncology Congress. Abstract 2014



European Society for Medical Oncology Congress- September 2014

TRIPLE NEGATIVE BREAST CANCER: CLINICOPATHOLOGICAL CHARACTERISTICS AND FATTY ACID SYNTHASE (FASN) EXPRESSION AS A POTENTIAL TARGET

Holguín F[1], Viñas G[1], Oliveras G[1,2], Pérez-Bueno F[3], Giró-Perafita A[2], Roqué A, Brunet J[1], Puig T[2]

[1]Medical Oncology Department , Catalan Institute of Oncology (ICO) and Girona Biomedical Research Institute, Girona, Spain; [2] Molecular Oncology (NEOMA); Medical Sciences, Medicine School, University of Girona, Girona, Spain; [3] Pathology Department, Dr. Josep Trueta Hospital and Catalan Institute of Health (ICS), Girona, Spain

Background

Triple negative breast cancer (TNBC) is defined by the lack of estrogen and progesterone receptor expression and the absence of human epidermal growth factor receptor 2 amplification. TNBC has specific clinical/pathological features and molecular biology. The absence of effective targeted therapies requires new biomarkers to develop therapeutic strategies. FASN, the sole mammalian enzyme capable of *de novo* fatty acid synthesis, is highly expressed in several carcinomas. Some reports highlight that FASN overexpression correlates with progression, aggressiveness and metastatic potential of the disease. We and others have shown that the inhibition of FASN using cerulenin, C75 and EGCG (FASN inhibitors) has anticancer activity specially in HER2+ breast cancer.

Objectives

The aim of our study was to evaluate the clinical and histopathological features of a large population of TNBC patients and to determine FASN expression levels in tumor samples and the citotoxyc effect of FASN inhibition (alone or in combination) in TNBC cells

Methods

We retrospectively evaluated 91 cases of primary TNBC diagnosed between 1990 and 2012 in our institution. We collected clinical/pathological features: age, histology, grade, stage, surgery and chemotherapy. FASN expression was preliminarily evaluated in 30 core-biopsy of TNBC patients by immunohistochemistry (IHC). FASN expression graded from 0 to 3+ (0-1+ normal, 2+ moderate and 3+ high). Concurrently, we evaluated the cytotoxic effect of C75 (FASN specific activity inhibitor) in several TNBC cell lines using an MTT assay. The Isobologram assay was used to estimate the combinatorial effect of the FASN inhibitor C75 with doxorubicin.

1. FASN expression in TNBC tumor samples

Table 1. TNBC Patients demographics and clinical characteristics

Median Age	54
Type of surgery	
Lumpectomy	43,5%
Mastectomy	49,4%
No surgery	7,1%
Histology	
Ductal carcinoma	85,0%
Lobulillar carcinoma	6,3%
Others	6,2%
Unknown	2,5%
Mean ki 67	64,5%
Grade III	85,2%
Stage:	
I	21,7%
II	41,0%
III	31,4%
IV	3,6%
Unknown	2,4%
Chemotheraphy	12,9%
Antraciclines+ others	1,6%
Taxanes only	9,6%
Taxanes +others	50,0%
Antraciclines + Taxanes	0,0%
Platins	25,8%
Others (CMF, Capecitabine)	

Figure 1. Immunohistochemistry (IHC) staining of FASN and EGFR in 29 TNBC human tumor samples. FASN expression was graded from 0 to 3+, meaning 0-1+ normal amounts of FASN protein, 2+ moderate amounts and 3+ highest levels of FASN expression. **A.** Adenocytes **B.** Renal tissue **C.** Ductal carcinoma **D.** Ductal carcinoma **E.** Skin **F.** Normal breast epithelium **G.** Ductal carcinoma **H.** Ductal carcinoma.

2. TNBC response to FASN inhibition (alone or in combination)

Figure 2. Citotoxicity in TNBC cells following C75 treatment for 48h. Data are means ± SE.

Figure 3. Interaction Index (Ix) of doxorubicin and C75 in MDA-MB-231, MDA-MB-468 and BT549 cells. Ix > 1 is antagonism, Ix = 1 is additivism and Ix < 1 is synergism. Data are means ± SE.

Results

1. Mean age was 54 years old. Most of the tumors were grade 3 (85,2%) and ductal (85,9%). Mean Ki 67 index was 64,5%. The primary surgical treatment was the mastectomy (49,4%).
2. Twenty-two percent of the patients received chemotherapy and the most common regimen was the antracilines and taxanes combo.
3. FASN staining was positive in all 29 TNBC human tumor samples, with low (69%) and moderate (31%) levels.
4. FASN inhibitors displayed strong cytotoxicity in MDA-MB-468 and HCC1806 cells and moderate in MDA-MB-231 and BT549 cells.
5. The combination of C75 and doxorubicin had an additive effect (0.85 ≤ Ix ≤ 1.09) at low concentrations of FASN inhibitor (2μM and 5μM) in MDA-MB-231; MDA-MB-468 and BT549 cell lines.

The demographics and clinical characteristics of the patients are shown in Table 2.

Conclusions

1. **FASN is moderate to highly expressed in TNBC tumors.**
2. **FASN inhibitors (alone or in combination) should be considered as novel therapeutic strategy in TNBC for further preclinical studies.**

References

(1) Oliveras G, Blancafort A, Lorrio-Cerezo A, Campuzano O, Gomez-Cabello D, Brugada R, López-Rodríguez ML, Colomer R, Puig T: Novel anti-fatty acid synthase compounds with anti-cancer activity in HER2+ breast cancer. Ann NY Acad Sci. 2010; 1210:86-92.
(2) Sidhanabala K, Hamon RE, Singh M. O Triple-negative breast carcinoma: current and emerging concepts. Am J Clin Pathol. 2011; 136:407-17.
(3) Yuan N, Meng M, Liu C, Feng L, Hou L, Ning Q, Xin G, Pei L, Gu S, Li X, Zhao X. Clinical characteristics and prognostic analysis of triple-negative breast cancer patients. Mol Clin Oncol. 2014 2(2):245-251.
(4) Puig T, Aguilar H, Cufí S, Oliveras G, Turrado C, Ortega-Gutiérrez S, Benhamú B, López-Rodríguez ML, Urruticoechea A, Colomer R. A novel inhibitor of fatty acid synthase shows activity against HER2+ breast cancer xenografts and is active in anti-HER2 drug-resistant cell lines. Breast Cancer Res. 2011; 13(6):R131.

17.3. Annex 3. Personal experience in the laboratory. TN cell-lines treated with FASN inhibitors

During July 2014, I made a stay in the laboratory of the NEOMA group in Parc Científic i Tecnològic de la Universitat de Girona (UdG) in order to learn cell culture techniques, cell culture methodology and treatment of tumor cells with FASN inhibitors. In this annex, an exemple of an own therapeutic experiment treating a doxorubicin resistant TN cell line (231-DxR) with a variable concentration of EGCG (a FASN inhibitor) and a fixed concentration of temsirolimus (an mTOR inhibitor) is provided (Figure 12).

Results shown an I_x parameter < 1 with a p value < 0,0001, which indicates synergism between EGCG and temsirolimus effects. Other replicas of the experiment are necessary to validate these results.

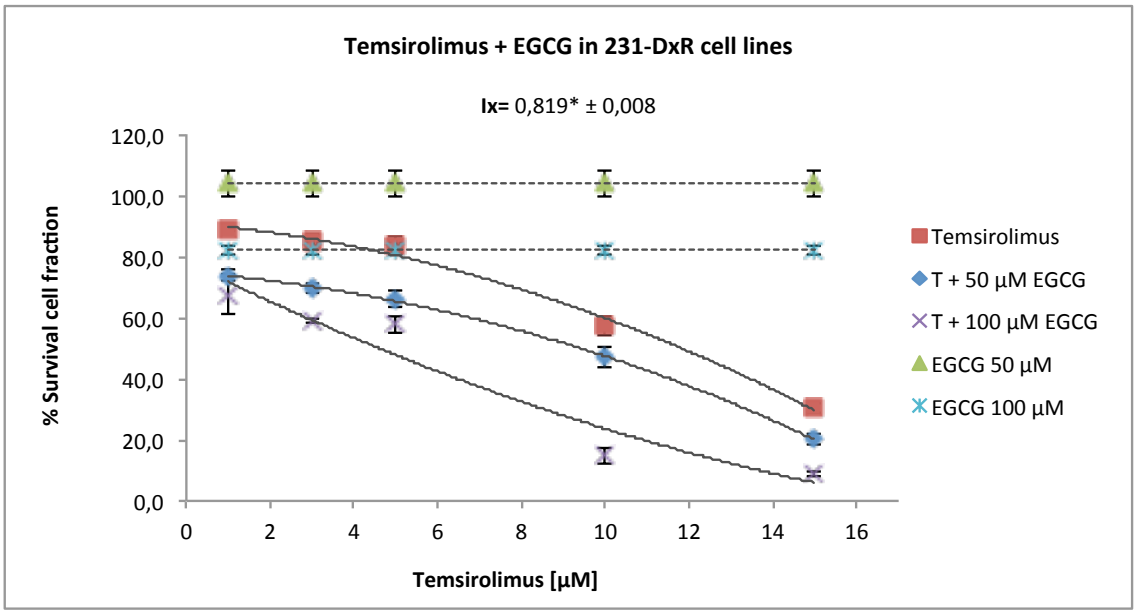


Figure 12. Citotoxicity in 231-Doxorubicin resistant (DxR) cells following temsirolimus and EGCG combination treatment. *231-DxR cells were treated with different concentrations of EGCG (50-100 µM) and a fixed temsirolimus concentration (1, 3, 5, 10 and 15 µM) for 48h. Green triangles represent the percentage of surviving cells after 48h in EGCG 50 µM treatment and blue asterisks represent the percentage of surviving cells after 48h in EGCG 100 µM treatment, which was determined using the MTT assay. Results are expressed as percentage of surviving cells from one experiment. The interaction index (I_x) for the two-drug effect in 231-DxR cells was calculated using isobologram analysis. The I_x parameter indicate whether the doses of the two drugs required to produce a given degree of cytotoxicity are greater than (I_x > 1 or antagonism), equal to (I_x = or additivism) or less than (I_x < 1 or synergism) the doses that would be required if the effect of two agents were strictly additive. * (p< 0,0001) indicate the level of statistical significance of the I_x compared with an I_x of 1.*