Universitat de les Illes Balears

MASTER'S THESIS

MITOCHONDRIA RESPONSE TO OBESITY-RELATED INFLAMMATION IN A BREAST CANCER 3D CULTURE APPROACH

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Master's Degree in Biomedical Research

(Specialisation/Pathway *Cancer Research*)

Centre for Postgraduate Studies

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Key words:

obesity-related inflammation, cancer stem cell (CSC), mitochondria, epithelial-to-mesenchymal transition (EMT), estrogen receptor beta (ERβ), mammospheres, breast cancer cell lines.

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Abstract

Mitochondria are closely connected to breast cancer and its progression. Obesity influences breast cancer cells by the production of hormones and proinflammatory cytokines. Here, we analyzed gene expression of different biomarkers associated to mitochondria response, epithelial-mesenchymal transition and stemness and evaluated estrogen receptor ratio (ERα/ERß) in human breast cancer cell lines (T47D, MCF7 and MDA-MB-231), with a great variety in this ratio, after ELIT (17β-estradiol, leptin, IL-6 and TNFα) treatment. This treatment mimics the hormonal situation and the obesity-related inflammation state of a postmenopausal obese woman. Furthermore, breast cancer stem cells (BCSCs), which have a role in stemness, tumor growth and metastasis, can be studied in detail using 3D cultures. To analyze the effect of ELIT in BCSCs subpopulations, mammospheres cultures were performed. We observed that in T47D cells mitochondria response to ELIT was to enhance autophagy which could be as a quality control to promote stemness and self-renewal, that was stimulated in this cell line, as well as an increase in the *ERα*/*ERß* ratio. An insufficient or absent mitochondria response was linked to an activation of the epithelial-mesenchymal transition mechanisms in MCF7 cell line, which also suffered a reduction in *ERα*/*ERß* ratio, and in MDA-MB-231 cells, while were not induced in T47D cell line. Moreover, ELIT increased mammosphere forming efficiency (MFE) in T47D and MCF7 cell lines, especially in T47D cells. Thus, our results propose a relationship between mitochondrial state and migration or stemness activity in breast cancer cell lines with differences in the expression of the estrogen receptor subtypes, especially ERα and ERß, in an obesity-related inflammation condition.

1. Introduction

Breast cancer is the most commonly tumor and with the highest mortality among women according to GLOBOCAN 2020 (https://gco.iarc.fr/) [1]. For this reason, it is important to understand the biological processes which are involved in this pathology to improve prevention, diagnosis, and therapy. Breast cancer is a multifactorial disease and obesity is the major risk factor, especially in postmenopausal women [2–4]. Moreover, obese women have a worse prognosis and more difficulties associated to the efficacy of surgery or chemotherapy [5]. In fact, some hormones, such as estrogen and leptin, and inflammatory cytokines are postulated to have a role in the relationship between breast cancer and obesity [6]. ELIT treatment is a cocktail, composed by 17β-estradiol (10 nM), leptin (100 ng/mL), IL-6 (50 ng/mL) and TNFα (10 ng/mL), which has been used to imitate the hormonal and inflammatory conditions of a postmenopausal obese woman in cell cultures [7].

It has been postulated an important role of estrogen receptor (ER) in breast cancer [8,9]. Different subtypes of the ER, alpha and beta vary in the affinity for the ligand, 17β-estradiol. It has been reported that ERα (also known as ESR1) could be related to the proliferation of breast cancer cells, whereas ERß (or ESR2) could have a protective effect against proliferation or migration and invasion [9,10]. Nonetheless, in some cases contradictory results have been observed with an involvement of ERß in cancer progression, and this receptor has been considered as a possible therapeutic target [11,12]. T47D, MCF7 and MDA-MB-231 human breast cancer cell lines have a different ERα/ERß ratio. T47D present a higher expression of ERß, while in MCF7 cells dominate the other receptor. MDA-MB-231 it is a triple negative cell line, but with a poor expression of ERß [7,13]. G protein-coupled estrogen receptor 1 (GPER1) is also an estrogen receptor with some implications in breast cancer as maintaining stemness properties, inducing migration and invasion or hormone therapy resistance [14–16]. Progesterone receptor (PGR) is another receptor implicated in breast cancer and it has described that can regulate ERα activity [17].

Mitochondria are highly dynamic organelles responsible for cell energy production. They are linked to breast cancer development and aggressiveness and are affected by its hormonal, as estrogen stimulation, and inflammatory environment [7,8,18]. ERα and ERß activated receptors are involved in the regulation of the mitochondrial function and can be located in the mitochondria modulating cell mechanisms such as mitochondrial respiration, apoptosis or therapy resistance [19–21]. Mitochondrial dynamics control the number of mitochondria that are present in a cell, through their division or combination, which are called as fission and fusion, respectively. It has been reported that alterations in mitochondrial dynamics are associated to cancer progression and metastasis [22–24]. The major fission regulator is dynamin-related protein 1 (DRP1) which can be recruited by other proteins as FIS1. OPA1 drives the inner mitochondrial membrane (IMM) fusion, while Mitofusin (MFN) 1 and 2 are the main proteins responsible for the outer mitochondrial membrane (OMM) fusion [22,25]. Mitochondrial dynamics are also related to the mitochondria degradation, a mechanism based in macroautophagy, known as mitophagy. PTEN-induced putative kinase 1 (PINK1) initiated this process with the recruitment of other necessary proteins for the autophagosome generation [25].

Epithelial-mesenchymal transition (EMT) consist of the passage from epithelial cells to mesenchymal and is common in tumors as breast cancer, especially in triple-negative cells, and associated to metastasis. Inhibition of e-cadherin, by some repressors as ZEB1, Snail 1 and 2; is required to carry out this transition [16,26]. A connection between mitochondrial metabolism and EMT has been suggested [24].

Traditionally, most cancer studies were conducted using monolayers of cell cultures. Nevertheless, new methods based in three-dimensional (3D) cultures are being established as a better model to

study the biological basis of cancer. Mammospheres are 3D cultures that allow the cancer stem cells (CSCs) enrichment [27,28]. CSCs are implicated in tumor generation and there are a correlation between stemness or self-renewal genes; such as Sry-related HMG box 2 (*SOX2*), *NANOG* or octamer-binding transcription factor 4 (*OCT4*), and epithelial-mesenchymal transition (*EMT*) genes as matrix metallopeptidase 9 (*MMP9*) [29]. Some hormones as estradiol and leptin can regulate breast cancer stem cell (BCSCs) proliferation and activity [30,31].

As shown in **Figure 1**, this master's thesis aimed to determine the effect of ELIT treatment on gene expression levels of different hormone receptors and genes involved in mitochondrial function, EMT and stemness in breast cancer cell lines (T47D, MCF7 and MDA-MB-231) with different estrogen receptor ratio. Likewise, the influence of obesity-related inflammation in the mammosphere forming efficiency (MFE) of 3D breast cancer cell cultures has been analyzed.

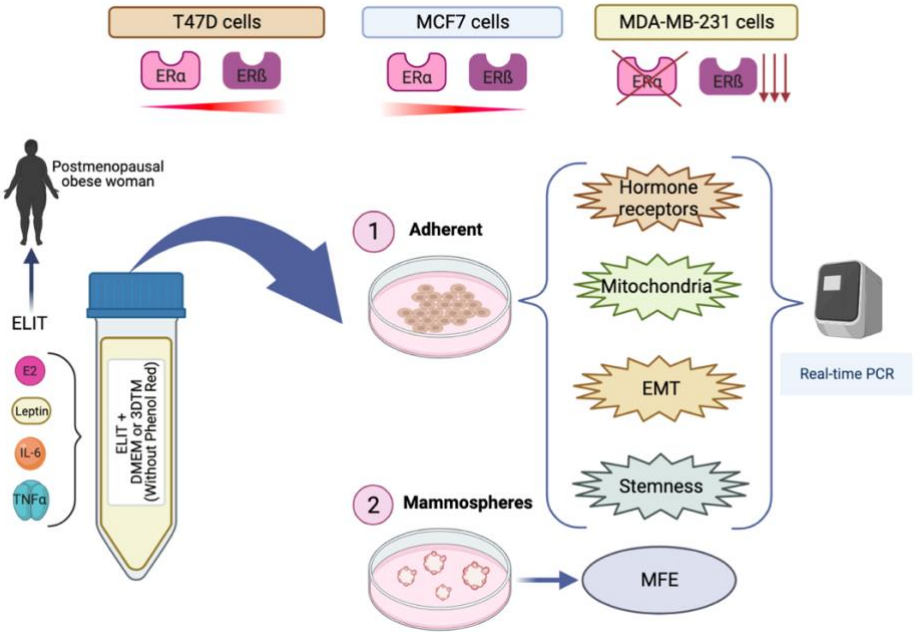

Figure 1. *ELIT, a cocktail which imitate the inflammatory and hormonal conditions of a postmenopausal obese woman, has been used to evaluate the influence of obesity-related inflammation on gene expression levels of hormone receptors and mitochondria, epithelial-mesenchymal transition (EMT) and stemness markers in adherent cell cultures, with different estrogen receptor ratio, via real-time quantitative PCR. Mammosphere forming efficiency of ELIT or vehicle-treated mammospheres were also studied. Created with Biorender.com*

2. Materials and methods

2.1. Reagents

Dulbecco's Modified Eagle's medium (DMEM) high glucose without phenol red was purchased from GIBCO (Paisley, UK). 17β-estradiol, leptin, interleukin-6 (IL-6), and TNF-α were supplied by Sigma-

Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Biological Industries (Kibbutz Beit Haemek, Israel). Phenol red-free 3D Tumorsphere Medium XF (3DTM) was purchased from Promocell (Heidelberg, Germany). Routine chemicals used were procured from Sigma–Aldrich (St Louis, MO, USA) and Bio-Rad Laboratories (Hercules, CA, USA).

2.2. Cell Culture and Treatments

T47D, MCF7 and MDA-MB-231 human breast cancer cell lines were obtained from American Type Culture Collection ATCC (Manassas, VA, USA) and cultured in DMEM supplemented with 10% FBS at 37 °C with 5% CO_2. Cells were seeded (6×10^5 cells/well for T47D cell line, 4×10^5 cells/well for MCF7 cell line and 3×10^5 cells/well for MDA-MB-231 cell line) in 6-well plates in DMEM w/o phenol red containing 10% FBS. The next day, to obtain 2D-3DTM cell cultures, DMEM was replaced by 3DTM, and cells, that reached about 70–80% confluence, were treated with vehicle (0.1% DMSO) or ELIT treatment (10 nM 17β-estradiol, 100 ng/mL leptin, 50 ng/mL IL-6 and 10 ng/mL TNFα) for 48 h.

2.3. RNA isolation and Real-time quantitative PCR (RT-qPCR)

Total RNA from 2D-3DTM cell cultures was isolated using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's protocol. RNA was quantified using a BioSpec-nano spectrophotometer (Shimadzu Biotech, Kyoto, Japan) set at 260 nm. 260/280 nm and 260/230 nm ratios were measured to assess RNA purity. Samples (700 ng of RNA) were denaturalized (at 90°C for 1 min), were reverse transcribed to cDNA, at 37°C for 50 min, and again, suffered another denaturation step (at 70°C for 15 min). The transcription reaction mixture was composed by random hexamers 50 µM, dNTP 10 mM, RNAse inhibitor 20 U/µL, DTT, Buffer 5x, and M-Mlv 200 U/µL. Then, cDNA obtained was diluted 1/10 in free-RNAse water. Quantitative real-time PCR (qRT-PCR) was carried out on LightCycler480 System II Rapid Thermal cycler (Roche Diagnostics, Basel, Switzerland) with SYBR Green Premix ExTaq (TAKARA, RR420A). Final reaction volume was 10 µl, containing 7.5 µl SYBR GreenMaster MixI (with 0.5 µM forward and reverse specific primers) and 2.5 µl of the cDNA template. Quantitative real-time PCR is based on one cycle of denaturation of cDNA template (5 min at 95 °C), 50 cycles of amplification and a cooling period. The cycles of amplifications were composed by three steps: denaturation; 10 s at 95 °C, annealing; 10 s at a certain temperature (depending on primers; showed in Table 1) and elongation; 12 s at 72 °C. Ct values of qRT-PCR were normalized with control and relativized using GenEx Standard Software (Multi-DAnalises, Sweden). The genes, primers, and temperatures for the annealing step are indicated in **Table 1**. 18S was used as a housekeeping gene and a free-cDNA template negative control was added in each run.

Table 1. *Primers sequences and conditions used for Real-time quantitative PCR.*

GENE	Forward primer (5'–3') / Reverse primer (5'-3')	Ann. Tº (ºC)	GENE	Forward primer (5'–3') / Reverse primer (5'-3')	Ann. Tº (ºC)
18S	GGACACGGACAGGATTGACA ACCCACGGAATCGAGAAAGA	60	*CDH1*	GTCACTGACACCAACGATAATCCT TTTCAGTGTGGTGATTACGACGTTA	61
ALDH	CGGGAAAAGCAATCTGAAGAGGG GATGCGGCTATACAACACTGGC	60	*ESR1*	AATTCAGATAATCGACGCCAG GTGTTTCAACATTCTCCCTCCTG	61
CD24	TGAAGAACATGTGAGAGGTTTGAC GAAAACTGAATCTCCATTCCACAA	60	*ESR2*	TAGTGGTCCATCGCCAGTTAT GGGAGCCACACTTCACCAT	60
CD44	CACACCCTCCCCTCATTCAC TGGATGGCTGGTATGAGCTG	60	*GPER1*	CATCATCGGCCTGTGCTACT GATGAAGACGTTCTCCGGCA	61
DRP1	GTTCACGGCATGACCTTTTT AAGAACCAACCACAGGCAAC	51	*MMP9*	CGCAGACATCGTCATCCAGT AAACCGAGTTGGAACCACGA	59
FIS1	GCTGAAGGACGAATCTCA CTTGCTGTGTCCAAGTCCAA	55	*NANOG*	CAGCTACAAACAGGTGAAGAC TGGTGGTAGGAAGAGTAAAGG	61
MFN1	TTCGATCAAGTTCCGGATTC TTGGAGCGGAGACTTAGCAT	52	*OCT4*	CTTGAATCCCGAATGGAAAGGG GTGTATATCCCAGGGTGATCCTC	60
MFN2	GCAGAACTTTGTCCCAGAGC AGAGGCATCAGTGAGGTGCT	56	*SNAI1*	ACCCCAATCGGAAGCCTAAC TCCCAGATGAGCATTGGCAG	60
OMA1	TTGGATTGCTCTTTGTGGTG GGTATCGGGCATCTTTCTCA	51	*SNAI2*	ACGCCTCCAAAAAGCCAAAC ACTCACTCGCCCCAAAGATG	60
OPA1	ACAATGTCAGGCACAATCCA GGCCAGCAAGATTAGCTACG	51	*SOX2*	GCCGAGTGGAAACTTTTGTCG GGCAGCGTGTACTTATCCTTCT	60
PINK1	GTCCAGCTCCACAAGGATGTT CATGCCTACATTGCCCCAGA	60	*PGR*	CTCAGCTGCCACCACACTAA GACAGAAGCCTCCAGCACAT	60
ZEB1	CTGCTGGGAGGATGACACAG ATGACCACTGGCTTCTGGTG	61			

2.4. Mamosphere formation

T47D, MCF7 and MDA-MB-231 cell lines were seeded (6×10^5 cells/well, 4×10^5 cells/well and 3×10^5 cells/well, respectively) in 6-well plates in phenol-red free DMEM containing 10% FBS 24 h prior to treatment. Cells were treated, with vehicle or ELIT treatment for 48 h, as it explained in 2D-3DTM culture, but maintaining DMEM. Then, to perform mammospheres generation, cells (1×10^3 cells/well) were cultured in an ultra-low attachment (ULA) 96-well plates (Promocell, Heidelberg, Germany) in 3DTM phenol free supplemented with 10% FBS (v/v) for 4 days (37°C with 5% CO_2).

2.5. Mammosphere forming efficiency and size determination

After mammospheres formation, spheres > 40 µm were counted using an inverted microscope at 100x magnification. Size and area determination were measured with ImageJ software. Mammospheres forming efficiency (MFE) was calculated using the following equation:

$$MFE(\%) = \frac{(number\ of\ mammospheres\ generated\ per\ well)}{(number\ of\ cells\ seeded\ per\ well)} \times 100$$

2.6. Statistical analysis

The statistical analyses were conducted with the Statistical Programme for the Social Sciences software for Macintosh (SPSS, version 25.0; SPSS Inc, Chicago, IL, USA). All data in this study are presented as means ± their respective standard error of the mean (SEM). Equality of variances between control and ELIT-treated cells were evaluated via Levene test and statistical differences were determined using a Student's t-test. Differences with $p < 0.1$, $p < 0.05$, $p < 0.01$ and $p < 0.001$ were considered as statistically significant and were indicated in results as #, *, ** and ***; respectively.

3. Results

3.1. Obesity-related inflammation altered Estrogen Receptor Ratio in breast cancer cell lines

Estrogen receptor alpha (*ESR1*), estrogen receptor beta (*ESR2*) and G protein-coupled estrogen receptor 1 (*GPER1*) mRNA expression levels were assessed **(Table 2)**. *ERα* mRNA levels presented a significant reduction in cell lines that normally express these receptors, with a huge difference in MCF7 cells. However, *ERß* expression was modified differently in breast cancer cell lines, with a statistically significant decrease in ELIT-treated T47D cells ($p < 0.1$) and a higher expression in MCF7 cells, whereas no changes were observed in MDA-MB-231. So, *ERα*/*ERß* ratio was affected inversely in T47D (1.63 ± 0.23 vs 1.00 ± 0.09) and MCF7 cells (0.05 ± 0.01 vs 1.00 ± 0.23), triggered by changes in estrogen receptor beta. Moreover, *GPER1* suffered a significant reduction in all cell lines analyzed, while *PGR* expression was only decreased in MCF7 cell line.

Table 2. *mRNA relative expression levels of ESR1, ESR2, GPER1, PGR and ESR1/ESR2 ratio*

Cell line	mRNA relative expression								*ESR1/ESR2* ratio	
	ESR1		*ESR2*		*GPER1*		*PGR*			
	Control	ELIT	Control	ELIT	Control	ELIT	Control	ELIT	Control	ELIT
T47D	1.00 ± 0.09	0.81 ± 0.05 *	1.00 ± 0.07	0.68 ± 0.32 #	1.00 ± 0.07	0.79 ± 0.05 *	1.00 ± 0.09	1.06 ± 0.05	1.00 ± 0.09	1.63 ± 0.23 **
MCF7	1.00 ± 0.03	0.12 ± 0.02 ***	1.00 ± 0.27	1.90 ± 0.58 *	1.00 ± 0.03	0.11 ± 0.02 ***	1.00 ± 0.05	0.23 ± 0.03 ***	1.00 ± 0.23	0.05 ± 0.01 ***
MDA-MB-231	UD	UD	1.00 ± 0.09	0.95 ± 0.14	1.00 ± 0.06	0.55 ± 0.03 ***	1.00 ± 0.28	1.28 ± 0.26	UD	UD

*Data are presented as means ± SEM (n = 6). Statistically significant difference among vehicle and treated cells are represented as # (p < 0.1), * (p < 0.05), ** (p < 0.01) and *** (p < 0.001). UD means undetected mRNA expression levels.*

3.2. mRNA expression of mitochondria response gene markers was considerably reduced in MCF7 ELIT-treated cells

The effect of obesity-related inflammation to mitochondria response was evaluated **(Figure 2)**. A reduction was observed in mRNA expression of a mitochondrial fusion-related gene; *OPA1* in ELIT-treated T47D cells, nevertheless, higher mRNA expression levels were found in *DRP1* and *PINK1*, especially in this mitophagy-related gene **(Figure 2a)**. In all mitochondrial dynamics (*DRP1, FIS1, MFN1, MFN2, OMA1* and *OPA1*), and mitophagy genes (*PINK*) assessed, mRNA expression presented a significant reduction in MCF7 breast cancer cell line **(Figure 2b)**. Only a modest increase in the mitophagy gene, *PINK1* ($p < 0.1$), and a reduction in DRP1 were seen in MDA-MB-231 cells **(Figure 2c)**.

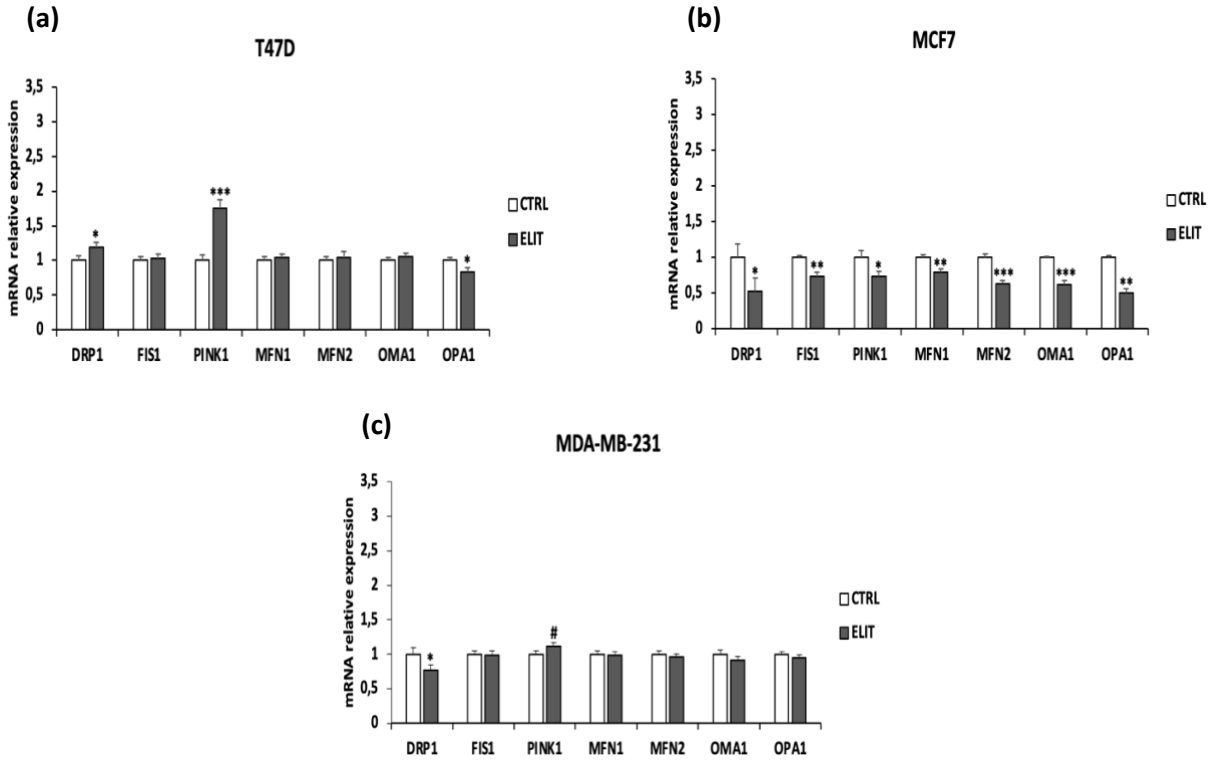

Figure 2. *mRNA relative expression of mitochondrial dynamics and mitophagy gene markers in T47D* ***(a)****, MCF7* ***(b)*** *and MDA-MB-231* ***(c)*** *ELIT-treated cells compared to control cells. mRNA relative expressions were normalized using 18S as housekeeping gene and relativized respect to control cells. Data are expressed as means ± SEM (n = 6). Statistically significant difference between treated and control cells (Student's t-test) are represented as # ($p < 0.1$), * ($p < 0.05$) and *** ($p < 0.001$).*

3.3. ELIT treatment increased the expression of epithelial-mesenchymal transition-related genes in MCF7 and MDA-MB-231 cell lines

mRNA expression of epithelial-mesenchymal transition (EMT) markers was analyzed in vehicle (DMSO) and ELIT-treated cells (**Figure 3 and Table 3)**. T47D cells only presented a higher *SNAI1* relative expression after ELIT treatment **(Figure 3a)**. A modest reduction in *SNAI2* expression and a quite

significant decrease in e-cadherin (*CDH1*) were found in MCF7 treated cells **(Figure 3b)**. MDA-MB-231 experienced changes in all EMT markers analyzed. ELIT significantly decreased e-cadherin, *SNAI1* and *ZEB1* gene expression and induced an overexpression of *SNAI2* **(Figure 3c)**.

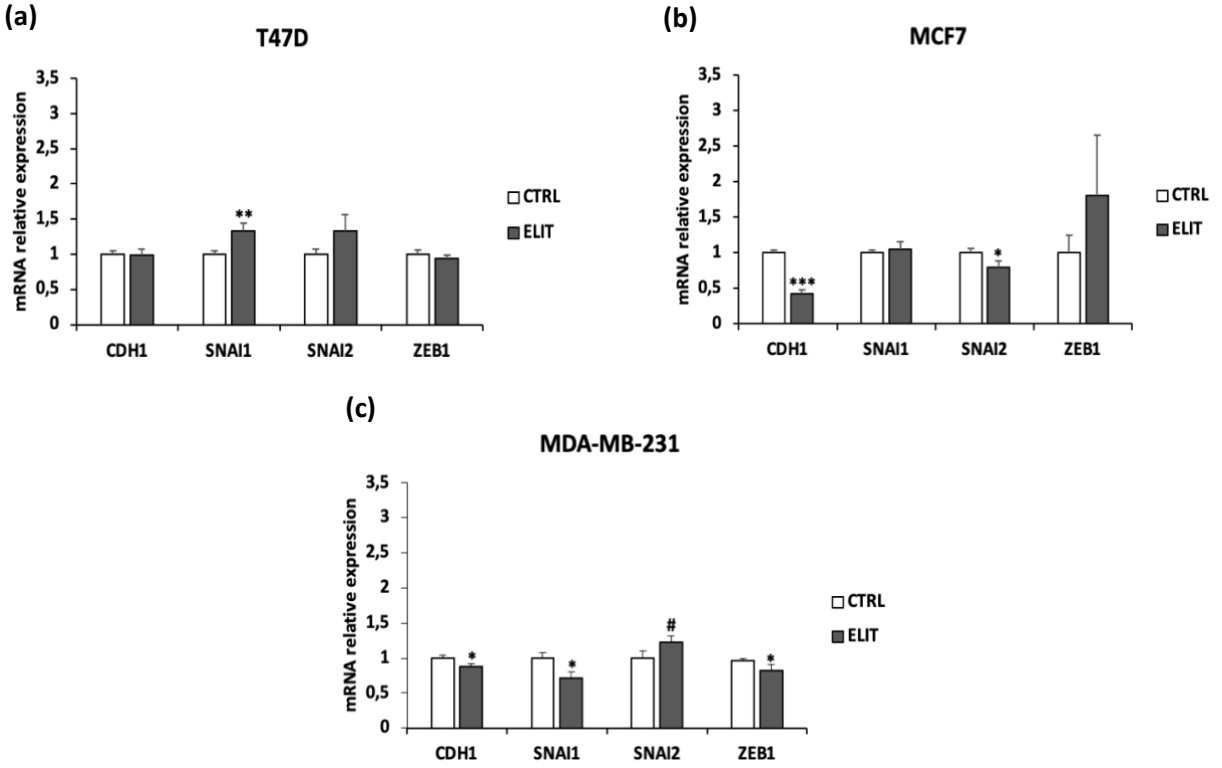

Figure 3. *Epithelial-mesenchymal transition-related genes and their mRNA relative expression in T47D* ***(a)****, MCF7* ***(b)*** *and MDA-MB-231* ***(c)*** *ELIT-treated cells compared to vehicle cells. mRNA relative expressions were normalized using 18S as housekeeping gene and relativized respect to control cells. Data are expressed as means ± SEM (n = 6). Statistically significant difference between both experimental groups (Student's t-test) are represented as # (p < 0.1), * (p < 0.05), ** (p < 0.01) and *** (p < 0.001)*

As shown in **Table 3**, MCF7 and MDA-MB-231 cells suffered a substantial increase (significant in both cell lines with p < 0.001), near to 10-fold, in *MMP9* gene expression after ELIT treatment, whereas in T47D cell line these changes were not observed.

Table 3. *mRNA relative expression levels of MMP9 in breast cancer cell lines*

Cell line	mRNA relative expression of *MMP9*	
	Control	ELIT
T47D	1.00 ± 0.25	0.86 ± 0.21
MCF7	1.00 ± 0.11	9.29 ± 2.92 **
MDA-MB-231	1.00 ± 0.06	9.12 ± 0.83 ***

*Data are presented as means ± SEM (n = 6). Statistically significant difference between ELIT-treated cells and control cells area represented as ** (p < 0.01) and *** (p < 0.001)*

3.4. Expression of stemness-related genes were modified after ELIT treatment in breast cancer cell lines

Gene expression of stemness markers was analyzed in all control or ELIT-treated cells **(Figure 4)**. After ELIT treatment, *CD24*, *CD44* and *SOX2* experimented a marked increase in T47D cell line, while mRNA expression of *OCT4* suffered a modest decrease **(Figure 4a)**. ELIT-treated cells presented higher *ALDH* mRNA expression and a significant reduction in the mRNA levels of *CD24*, *OCT4* and *SOX2* **(Figure 4b)**. Some stemness markers (*CD24, NANOG, OCT4* and *SOX2*) presented lower levels in MDA-MB-231 after ELIT treatment (**Figure 4c)**. ELIT enhanced the *CD44*/*CD24* ratio in MCF7 and MDA-MB-231 cells, especially in MCF7 **(Figure 4d)**.

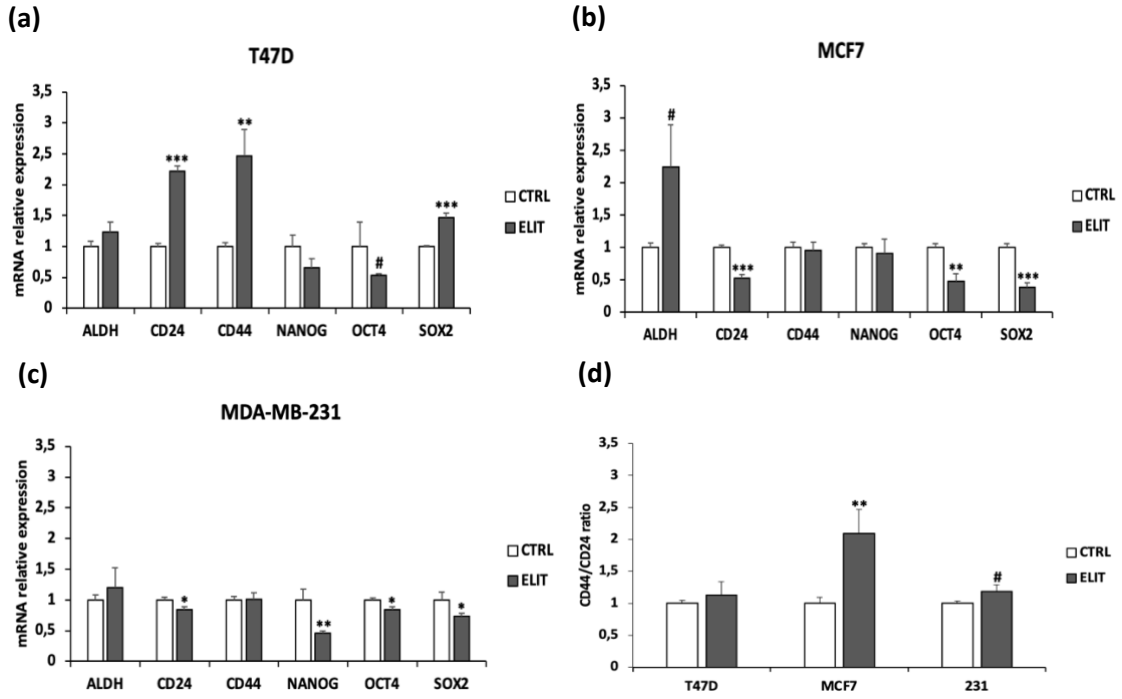

Figure 4. *mRNA relative expression of stemness-related genes in T47D* ***(a)****, MCF7* ***(b)*** *and MDA-MB-231* ***(c)*** *ELIT-treated cells compared to vehicle cells. mRNA relative expressions were normalized using 18S as housekeeping gene and relativized respect to control cells. CD44/CD24 ratio was measured* ***(d)****. Data are expressed as means ± SEM (n = 6). Statistically significant difference between both experimental groups (Student's t-test) are represented as # (p < 0.1), * (p < 0.05), ** (p < 0.01) and *** (p < 0.001).*

3.5. ELIT treatment induced an enrichment in cancer stem cells in T47D and MCF7 cell lines

In T47D, MCF7 and MDA-MB-231 vehicle and ELIT-treated cells mammosphere forming efficiency (MFE) and size of spheres were analyzed **(Figure 5)**. On all cell lines examined, area of mammospheres was clearly reduced **(Figure 5a)**. However, ELIT affected differently to the MFE **(Figure 5b)**. The number of spheres was significantly higher in ELIT-treated T47D cells compared to control (32 ± 2 vs 20 ± 3 spheres and 2.68 ± 0.18 vs 1.68 ± 0.24 in MFE). A modest increase (with p-value < 0.1) of spheres was observed in MCF7 cells with ELIT treatment (16 ± 4 vs 9 ± 1 spheres and 1.33 ± 0.30 vs 0.75 ± 0.10 in

MFE). No significant differences were found in the number of mammospheres in MDA-MB-231 cells (28 ± 2 vs 24 ± 2 spheres and 2.33 ± 0.14 vs 2.00 ± 0.21 in MFE). In summary, ELIT generated mammospheres with a lower size, and in T47D and MCF7 cell lines increased their number, as shown in the representative images in **Figure 5c.**

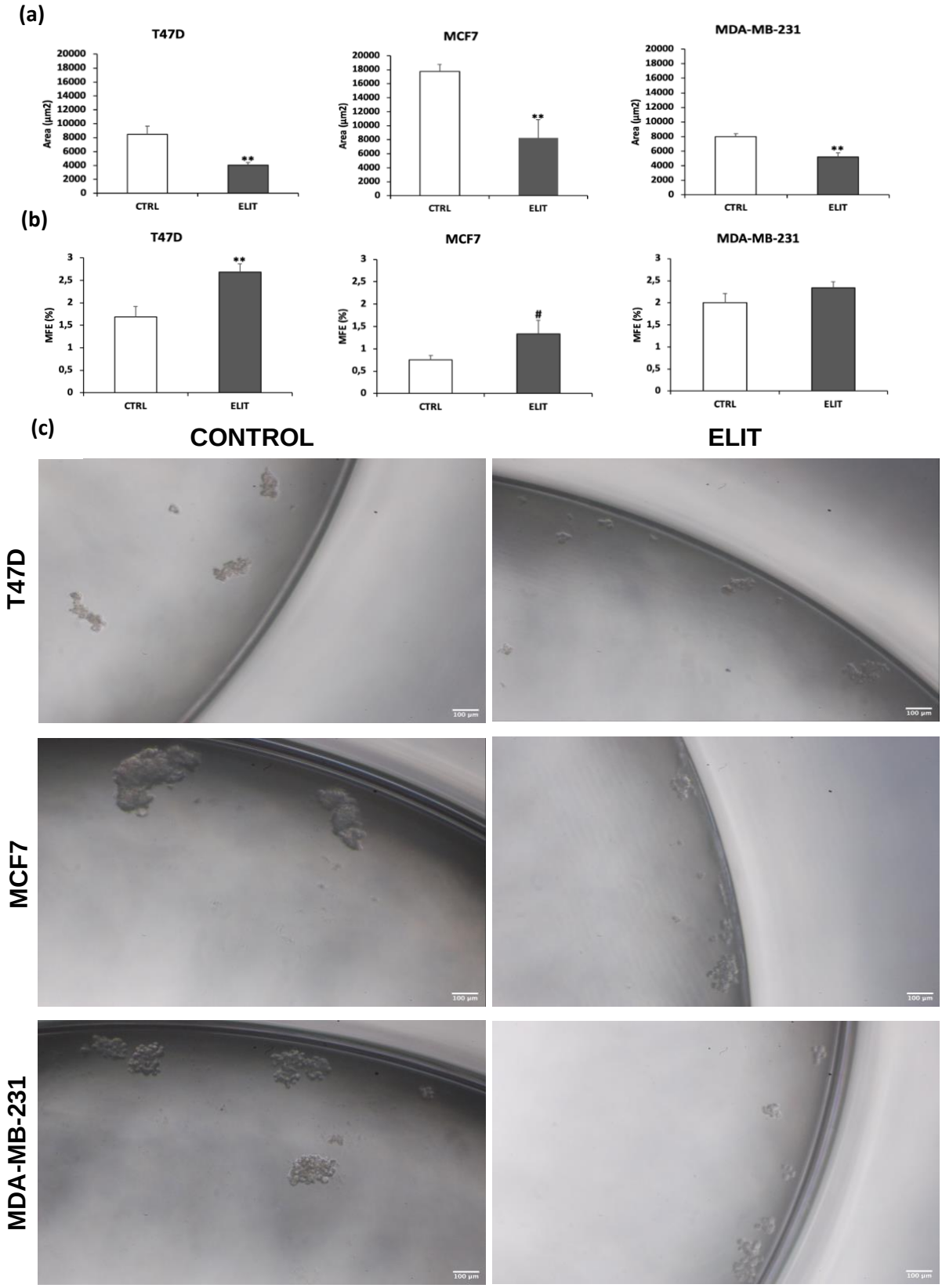

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Figure 5. *Size determination* ***(a)*** *and mammospheres counting in breast cancer cell lines treated with vehicle (DMSO) and ELIT cells. Cells were incubated for 48 h with control or ELIT. Mammosphere forming efficiencies* ***(b)*** *were calculated as (number of mammospheres/ number of seeded cells) * 100. Spheres over 40 µm were counted at 4 days after been cultured. Area analysis was performed with ImageJ software Representative images* ***(c)*** *(100x magnification and scale bar = 100 µm) were taken using an inverted microscope. Data are presented as means ± SEM (n = 6). Statistically significant difference between both experimental groups (Student's t-test) are represented as # (p < 0.1), ** (p < 0.01) and *** (p < 0.001).*

4. Discussion

In this study, the relative expression of estrogen receptors and mitochondria response, EMT and stemness gene markers were assessed in ELIT or vehicle-treated human breast cancer cell lines. We found that ELIT promoted a shift in the *ERα*/*ERß* ratio in T47D and MCF7 cells, with an increase in cell lines with a major expression of ERß and a lower ratio in cell lines that ERα predominates, caused by an important reduction in gene expression of the main expressed estrogen receptor of each cell line. Also, in MCF7 cells ELIT overexpressed *ERß*, which is known to increase its expression in breast cancer stem cells [11]. However previous studies did not observe this higher ratio in T47D cells and greater *ERß* expression in MCF7 cell line [7]. Additionally, we observed a clear reduction in all cell lines of GPER1 expression by ELIT and a decrease in progesterone receptor only in MCF7, which showed that obesity-related inflammation diminishes hormone receptor expression except *ERß*, whose enhance its importance, in MCF7 cell line.

Referring to mitochondria response, estradiol alone has been described as stimulant for mitochondrial fusion and as inhibitor for mitochondrial fission in the MCF7 cell line [32]. Nevertheless, later studies evaluated the role of ELIT in mitochondrial function and observed a lower expression of the different mitochondrial dynamics markers that reflected mitochondrial disfunction and oxidative stress, as well as an association with a higher invasion and a need to scape. On the other hand, T47D could maintain mitochondrial network and with a better quality [7]. This reduction in mitochondria markers in MCF7 cells after ELIT treatment was also found in this work. Otherwise, in T47D cell line mitochondrial fusion markers were conserved their gene expression levels, but with an overexpression in fission and, specially, in mitophagy. This could be due to a possible mechanism to remove damaged mitochondria and promoting cell survival in these cells, since a role of mitophagy in cell death protection has been described [33]. No marked differences were seen respect to mitochondrial dynamics in MDA-MB-231 which suggest that these cells did not modify their mitochondria response to ELIT.

Moreover, EMT markers gene expression correlated with the above. T47D ELIT-treated cells did not undergo great changes compared to the other cell lines. However, MCF7 experimented a high

induction of EMT, specially observed with a significant downexpression of e-cadherin and a dramatic increase in *MMP9* gene expression. Considering the desire of MCF7 cells to scape and develop EMT, possibly due to the appearance of mitochondrial dysfunction, as well as the suitable mitochondrial profile of T47D against ELIT stress, mentioned before, these results could be explained. In MDA-MB-231 there was also a stimulation of EMT due to ELIT treatment similar than in MCF7 cells. In this case it was expected that a triple-negative cell line could incline to activate the migration mechanisms under stress generated by ELIT. Noteworthy, other authors described that leptin promoted MMP9 expression in MCF7 and it has observed that in MDA-MB-231 this adipokine increased the gene expression of EMT markers [34,35]. Also, TNF-α induced EMT by the activation of Snail and ZEB1 proteins and could decrease e-cadherin expression in breast cancer cells [36]. Thus, these compounds present in ELIT could be especially implicated with the promotion of EMT in MCF7 and MDA-MB-231 cells.

Breast cancer stem cells (BCSCs) are an important subpopulation present in tumor with a role in its growth maintaining and progression [31]. BCSCs are characterized by a certain expression of some markers; CD44, CD24 and ALDH. Estrogen decreased the expression of *CD24* in ER-α^+ breast cancer cell lines and it has been observed as an inductor of CSC phenotype [37,38]. In other case, IL-6 induce more $CD44^+$ T47D cells due to EMT activation, but ELIT did not replicate this scenario [39]. Some studies described that CD44 high was associated to self-renewal, cell proliferation and migration and ALDH high was linked to stemness, migration, invasion, and metastasis. However, CD24 low was mainly associated to cell migration and metastases [11,30]. Our results correlated with these phenomena, because *CD24* was only reduced in MCF7 and MDA-MB-231 cell lines, the possible two cell lines that ELIT enhanced EMT expression, while in T47D cells was substantial increased after ELIT treatment. In fact, breast cancer cell subpopulations with $CD24^-$ (low) and $CD44^+$ (high) were presented a higher expression of EMT-related genes as *MMP9* [40]. This is connected to that we observed, because we only found a greater *CD44*/*CD24* ratio in cell lines with an increase in EMT gene expression, MCF7 and MDA-MB-231. Furthermore, *ALDH* was significant overexpressed in MCF7 by ELIT and could promote migration in this cell line, since is a stronger maker of migration than CD44/CD24 ratio [30,41].

Regarding to the other stemness markers, *SOX2*, which had a marked increase in its gene expression in T47D and a lower expression in MCF7 and in MDA-MB-231, is known to be involved in the self-renewal of BCSCs, so SOX2 could contribute to the higher proliferation and stemness of the T47D after ELIT treatment. Also, *NANOG*, that can regulate CSCs properties acting together with SOX2 and OCT4 was clearly reduced in MDA-MB-231 [29,42]. Nonetheless, *OCT4* gene expression was reduced in all cell lines examined. In mammospheres, estrogen treatment produced a lower expression of *SOX2, OCT4*

and *NANOG*, so this could be linked with a decrease in the expression of some of them in adherent cultures by ELIT [43].

With respect to the mamospheres forming efficiency, our results showed that ELIT enhanced the appearance of more spheres in T47D and MCF7 cell lines, especially in T47D cells, which means that these cells have a greater number of cancer stem cells. This is similar than the higher stemness in T47D observed in 2D cultures. Several studies mentioned that some cytokines and adipokines, as Leptin and TNF-α, can increase cancer stem cell enrichment [35,44]. Additionally, it has been observed that Leptin increased MFE and a subpopulation with a higher CD44/CD24 ratio [31]. Again, these compounds could influence cancer stem cell subpopulation as affected EMT.

Together these results, suggest that ELIT induced a mitochondria reaction in T47D cells that could enhance their stemness potential and increased their *ERα*/*ERß* ratio. However, in MCF7, which suffered a shift in estrogen receptor ratio, given importance to *ERß*, cells did not develop this response, maybe due to mitochondrial damaged, and cells intends to activate EMT expression and downexpressed self-renewal markers **(Figure 6)**. Otherwise, MDA-MB-231 response were not linked to mitochondria. These triple-negative cells maintained the expression of their mitochondria marker levels, but are cells habituated to experiment EMT. Nonetheless this master's thesis let future perspectives to focus on, such as evaluating protein activity, analyze cell migration and invasion with an obesity-related inflammation and considering new markers to differentiate stemness and migration.

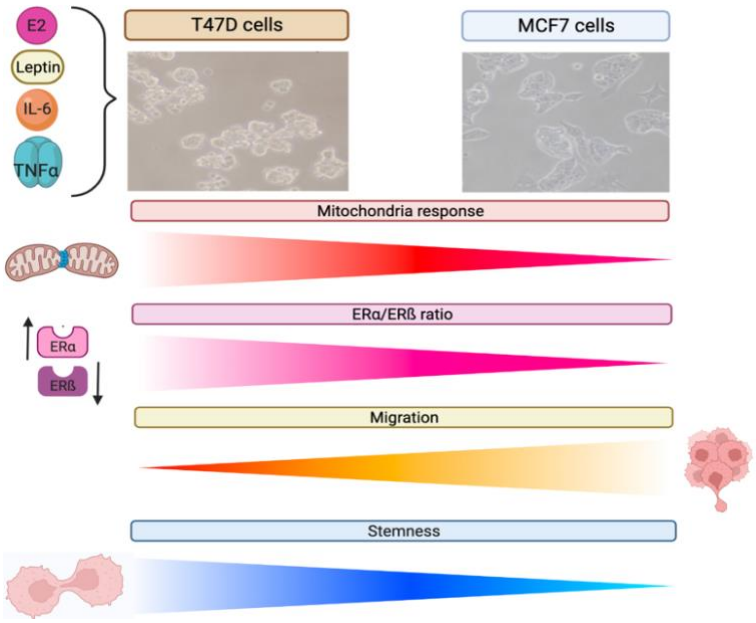

Figure 6. *Differences in mitochondria response, migration, self-renewal and ERα/ERß ratio in T47D and MCF7 cell lines after ELIT treatment. Created with Biorender.com*

5. Conclusion

To sum up, the present study was focused on evaluating the mitochondria response to obesity-related inflammation and to identify a connection with ERα/ERß ratio and the expression of EMT and BCSCs markers in breast cancer cell lines. Also, the influence of ELIT in cancer stem cell subpopulations was assessed in mammospheres. Our findings suggest that T47D cell line induced a higher mitophagy and promoted a self-renewal state instead of cell migration against ELIT stress, while the opposite was found in the other cell lines, MDA-MB-231 cells, that usually tend to migrate, and MCF7 cells, which suffered a change in mitochondria response that was not sufficient to promote self-renewal. Furthermore, ELIT treatment altered differently the estrogen receptor ratio in T47D cells and in MCF7 cells and increased mammosphere formation in both cell lines.

However, further studies are needed to assess the relationship between BCSCs and EMT promotion in an obesity-related inflammation state and the role of mitochondria to induce these mechanisms, as well as give more importance to both estrogen receptors, alpha and beta.

6. References

1. Sung, H. *et al.* Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA. Cancer J. Clin.* **71**, 209–249 (2021).
2. Mohanty, S. S. & Mohanty, P. K. Obesity as potential breast cancer risk factor for postmenopausal women. *Genes and Diseases* vol. 8 117–123 (2021).
3. Kabat, G. C. *et al.* Metabolic obesity phenotypes and risk of breast cancer in postmenopausal women. *Cancer Epidemiol. Biomarkers Prev.* **26**, 1730–1735 (2017).
4. Gravena, A. A. F. *et al.* The obesity and the risk of breast cancer among pre and postmenopausal women. *Asian Pacific J. Cancer Prev.* **19**, 2429–2436 (2018).
5. Lee, K., Kruper, L., Dieli-Conwright, C. M. & Mortimer, J. E. The Impact of Obesity on Breast Cancer Diagnosis and Treatment. *Current Oncology Reports* vol. 21 (2019).
6. Sánchez-Jiménez, F., Pérez-Pérez, A., de la Cruz-Merino, L. & Sánchez-Margalet, V. Obesity and Breast Cancer: Role of Leptin. *Front. Oncol.* **9**, 596 (2019).
7. Martinez-Bernabe, T. *et al.* Estrogen receptor beta (Erβ) maintains mitochondrial network

regulating invasiveness in an obesity-related inflammation condition in breast cancer. *Antioxidants* **10**, (2021).

8. Gandhi, N. & Das, G. M. Metabolic reprogramming in breast cancer and its therapeutic implications. *Cells* vol. 8 (2019).

9. Bhardwaj, P. *et al.* Estrogens and breast cancer: Mechanisms involved in obesity-related development, growth and progression. *Journal of Steroid Biochemistry and Molecular Biology* vol. 189 161–170 (2019).

10. Song, P. *et al.* Estrogen receptor β inhibits breast cancer cells migration and invasion through CLDN6-mediated autophagy. *J. Exp. Clin. Cancer Res.* **38**, (2019).

11. Ma, R. *et al.* Estrogen Receptor B as a therapeutic target in breast cancer stem cells. *J. Natl. Cancer Inst.* **109**, (2017).

12. Austin, D. *et al.* Estrogen receptor-beta is a potential target for triple negative breast cancer treatment. *Oncotarget* **9**, 33912–33930 (2018).

13. Datta, J. *et al.* Activity of Estrogen Receptor β Agonists in Therapy-Resistant Estrogen Receptor-Positive Breast Cancer. *Front. Oncol.* **12**, 1 (2022).

14. Lappano, R., Pisano, A. & Maggiolini, M. GPER function in breast cancer: An overview. *Frontiers in Endocrinology* vol. 5 (2014).

15. Chan, Y. T. *et al.* GPER-induced signaling is essential for the survival of breast cancer stem cells. *Int. J. Cancer* **146**, 1674–1685 (2020).

16. De Francesco, E. M., Maggiolini, M. & Musti, A. M. Crosstalk between notch, HIF-1α and GPER in breast cancer EMT. *International Journal of Molecular Sciences* vol. 19 (2018).

17. Mohammed, H. *et al.* Progesterone receptor modulates ERα action in breast cancer. *Nature* **523**, 313–317 (2015).

18. Lebok, P. *et al.* High mitochondrial content is associated with breast cancer aggressiveness. *Mol. Clin. Oncol.* **15**, (2021).

19. Klinge, C. M. Estrogenic control of mitochondrial function. *Redox Biology* vol. 31 101435 (2020).

20. Karakas, B. *et al.* Mitochondrial estrogen receptors alter mitochondrial priming and response to endocrine therapy in breast cancer cells. *Cell Death Discov.* **7**, (2021).

21. Song, I. S. *et al.* Modulation of mitochondrial ERβ expression inhibits triple-negative breast cancer tumor progression by activating mitochondrial function. *Cell. Physiol. Biochem.* **52**,

468–485 (2019).

22. Srinivasan, S., Guha, M., Kashina, A. & Avadhani, N. G. Mitochondrial dysfunction and mitochondrial dynamics-The cancer connection. *Biochimica et Biophysica Acta - Bioenergetics* vol. 1858 602–614 (2017).

23. Zhao, J. *et al.* Mitochondrial dynamics regulates migration and invasion of breast cancer cells. *Oncogene* **32**, 4814–4824 (2013).

24. Avagliano, A. *et al.* Mitochondrial flexibility of breast cancers: A growth advantage and a therapeutic opportunity. *Cells* vol. 8 401 (2019).

25. Bordi, M., Nazio, F. & Campello, S. The Close interconnection between mitochondrial dynamics and mitophagy in cancer. *Frontiers in Oncology* vol. 7 1 (2017).

26. Wang, Z., Jiang, Q. & Dong, C. Metabolic reprogramming in triple-negative breast cancer. *Cancer Biology and Medicine* vol. 17 44–59 (2020).

27. Weiswald, L. B., Bellet, D. & Dangles-Marie, V. Spherical Cancer Models in Tumor Biology. *Neoplasia (United States)* vol. 17 1–15 (2015).

28. Chaicharoenaudomrung, N., Kunhorm, P. & Noisa, P. Three-dimensional cell culture systems as an in vitro platform for cancer and stem cell modeling. *World Journal of Stem Cells* vol. 11 1065–1083 (2019).

29. Huang, T. *et al.* Stem cell programs in cancer initiation, progression, and therapy resistance. *Theranostics* vol. 10 8721–8743 (2020).

30. Giovannelli, P. *et al.* Breast cancer stem cells: The role of sex steroid receptors. *World Journal of Stem Cells* vol. 11 594–603 (2019).

31. Giordano, C. *et al.* Leptin as a mediator of tumor-stromal interactions promotes breast cancer stem cell activity. *Oncotarget* **7**, 1262 (2016).

32. Sastre-Serra, J., Nadal-Serrano, M., Pons, D. G., Roca, P. & Oliver, J. Mitochondrial dynamics is affected by 17β-estradiol in the MCF-7 breast cancer cell line. Effects on fusion and fission related genes. *Int. J. Biochem. Cell Biol.* **44**, 1901–1905 (2012).

33. Ferro, F. *et al.* Autophagy and mitophagy in cancer metabolic remodelling. *Seminars in Cell and Developmental Biology* vol. 98 129–138 (2020).

34. Juárez-Cruz, J. C. *et al.* Leptin induces cell migration and invasion in a FAK-Src-dependent manner in breast cancer cells. *Endocr. Connect.* **8**, 1539–1552 (2019).

35. Bowers, L. W. *et al.* Leptin signaling mediates obesity-associated CSC enrichment and EMT in

preclinical TNBC models. *Mol. Cancer Res.* **16**, 869–879 (2018).

36. Cruceriu, D., Baldasici, O., Balacescu, O. & Berindan-Neagoe, I. The dual role of tumor necrosis factor-alpha (TNF-α) in breast cancer: molecular insights and therapeutic approaches. *Cellular Oncology* vol. 43 (2020).

37. Kaipparettu, B. A. *et al.* Estrogen-mediated downregulation of CD24 in breast cancer cells. *Int. J. Cancer* **123**, 66–72 (2008).

38. Fillmore, C. M. *et al.* Estrogen expands breast cancer stem-like cells through paracrine FGF/Tbx3 signaling. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 21737–21742 (2010).

39. Xie, G. *et al.* IL-6-induced epithelial-mesenchymal transition promotes the generation of breast cancer stem-like cells analogous to mammosphere cultures. *Int. J. Oncol.* **40**, 1171–1179 (2012).

40. Liu, S. *et al.* Breast cancer stem cells transition between epithelial and mesenchymal states reflective of their normal counterparts. *Stem Cell Reports* **2**, 78–91 (2014).

41. Li, W. *et al.* Unraveling the roles of CD44/CD24 and ALDH1 as cancer stem cell markers in tumorigenesis and metastasis. *Sci. Rep.* **7**, (2017).

42. Jeter, C. R., Yang, T., Wang, J., Chao, H. P. & Tang, D. G. Concise Review: NANOG in Cancer Stem Cells and Tumor Development: An Update and Outstanding Questions. *Stem Cells* **33**, 2381–2390 (2015).

43. Simões, B. M. *et al.* Effects of estrogen on the proportion of stem cells in the breast. *Breast Cancer Res. Treat.* **129**, 23–35 (2011).

44. Liu, W. *et al.* TNF-α increases breast cancer stem-like cells through up-regulating TAZ expression via the non-canonical NF-κB pathway. *Sci. Rep.* **10**, (2020).