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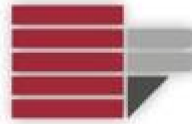

PhD in Translational Medicine

XXXVI cycle

Tamoxifen resistance in breast tumors relays on increased translation of lipid metabolism genes

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1- Breast Cancer and Tamoxifen Resistance

Breast cancer (BC), the most common malignancy in women worldwide, is a heterogeneous disease characterized by a broad spectrum of morphological, biological, and clinical features that profoundly affect the therapeutic response (Rakha et al., 2023).

1.1- Epidemiology and Risk Factors

BC patients account for as much as 36% of oncological patients (Nardin et al., 2020). The incidence of this disease is increasing worldwide, particularly in industrialized countries. This trend is mainly due to the so-called Western lifestyle, which includes high-calories and low-micronutrient food, poor physical activity, excessive exposure to stress and nicotinism (Bellanger et al., 2018). Furthermore, in developed countries, breast cancer diagnosis is often made at an early stage, with prognosis usually better than in low- and middle-income countries, where late diagnoses lead to higher mortality (Allemani et al., 2015).

In Italy, due to the availability of screening programs and greater awareness among women and general practitioners, most breast malignancies are diagnosed at an early stage of disease. This means that the 5-year survival rate is very high (87%, one of the highest recorded in Europe) (Coviello et al., 2017) but survival falls dramatically for metastatic disease to 34.2% or 30% (Mangone et al., 2021; G. Patel et al., 2021).

The unequivocal cause of breast cancer carcinogenesis has not yet been discovered, but numerous risk factors contribute to its onset. Among these, as is clear from epidemiological data described above, the gender, age and the economic development of countries are of particular importance.
The vast majority of breast cancer occur in women, with only 1% of malignant tumour affecting men (Religioni, 2020).
Age is also a crucial player in developing breast cancer; the global increase in breast cancer cases is mainly found in women under 50 and, even if this disease is not common in this age group, it represent a significant clinical and social problem, due to its worse course (Lima et al., 2021).
As mentioned above, the incidence and mortality of breast cancer is linked to the economic development of a country, and this relationship has been proven in several studies (Bellanger et al., 2018; Torre et al., 2017).

Alongside these risk factors, there are hormonal factors, mainly related to the time of exposure to estrogens, procreative factors, including the number of children born, the age of birth of the first child, or breastfeeding. Great importance in the development of breast cancer is attributed to genetic factors, the use of hormone replacement therapy, improper diet, and the resulting obesity (Smolarz et al., 2022).

1.2- Obesity and breast cancer

Obesity is a well-known risk factor for developing breast cancer. Studies show that both overweight and obesity increase the risk of developing breast cancer, particularly steroid-receptor-expressed breast cancer, in postmenopausal women who did not use hormone replacement therapy (Jiralerspong & Goodwin, 2016; Fortner et al., 2016). Surprisingly, overweight and obesity in premenopausal women reduce the risk of developing hormone-dependent breast cancer; however, literature data indicate a relationship between obesity in premenopausal patients and the risk of developing triple-negative breast cancer (Jiralerspong & Goodwin, 2016; Pierobon & Frankenfeld, 2013). Moreover, numerous studies highlight that overweight and obesity are associated with a worse prognosis in breast cancer patients before and after menopause (Wu et al., 2013; Chan et al., 2014). Obesity promotes cancer development and growth through several mechanisms. Hypertrophic adipose tissue is a source of several endocrine factors, cytokines, chemokines, especially proangiogenic and pro-mitogenic leptin, which affects the immune environment of the tissue (Gilbert & Slingerland, 2013). Moreover, excessive adipose tissue promotes the surrounding hypoxia, which leads to an increase in the secretion of leptin and VEGF factor, while inhibits the synthesis of antiangiogenic and antimitogenic adiponectin (Picon-Ruiz et al., 2017). At the molecular level, the NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway is responsible for the development and maintenance of the inflammatory process within excessive adipose tissue. Through the secretion of pro-inflammatory cytokines, adipose tissue exert an inhibitory effect on the apoptotic process and, at a later stage, promotes proliferation, invasion, and metastasis of breast cancer cells (Tornatore et al., 2012; Prasad et al., 2010). Adipose tissue is also the main source of sex hormones in postmenopausal women; in this tissue, adrenal androgens are converted in estrogens through the action of the aromatase enzyme, in a process known as aromatization (Picon-Ruiz et al., 2017). The metabolic syndrome that accompanies obesity is associated with insulin resistance,

hyperinsulinemia, increased synthesis of insulin-like growth factor 1 (IGF-1). Studies have shown that insulin resistance and hyperinsulinemia are associated with poorer survival of breast cancer patients (Arcidiacono et al., 2012). Obesity is a recognized risk factor not only for the development of breast cancer, but also for the development of relapses, even in the presence of adequate treatments. The effectiveness of systemic chemotherapy and hormone therapy is significantly lower in obese women. Furthermore, obese women are at a greater risk of local recurrence than women of normal weight (Lee et al., 2019).

1.3- Breast cancer classification and treatment options

1.3.1- Classification

Breast cancer is a highly heterogeneous neoplasm with distinct subtypes. The most common and widely accepted classification of breast cancer is from an immunohistochemical perspective, based on the expression of the following hormone receptors: estrogen (ER), progesterone (PR) and human epidermal growth factor (HER2). Accordingly, the following four subtypes of breast cancer are widely recognized: luminal A, luminal B, HER2-positive, and triple-negative (TNBC, which is characterized by the lack of expression of any of the above receptors) (Shaath et al., 2021). With the recent advances in cancer research, and an increased molecular understanding of breast cancer, the current clinical model for breast cancer classification may benefit from the addition of several molecular markers such as miRNAs (let-7, miR-155, miR-150, miR-153) and mutations (p53, BRCA 1 and 2 genes) (Erasmo Orrantia-Borunda et al., 2022).

Around 70-75% of invasive breast carcinomas are characterized by significantly high ER expression, making the ER an important diagnostic tool. The PR is expressed in more than 50% of ER-positive patients, but rarely in those with ER-negative breast cancer. PR expression is regulated by ER and the physiological PR expression inform about the functional ER pathway (DeSantis et al., 2019). Lower expression of PR is usually linked with a poor prognosis and a more aggressive disease, while higher expression of PR is associated with higher overall survival, time to recurrence, and time to treatment failure or progression (Purdie et al., 2014). HER2 is present in approximately 15–25% of breast cancers and it is essential to choose the appropriate treatment (Iqbal & Iqbal, 2014). The circulating HER2 levels are considered a promising real-time marker for recurrence. HER2 amplification provokes augmented activation of proto-oncogenic signalling

pathways, which lead to uncontrolled tumour cells growth that characterize worse clinical outcomes in HER2 positive cases. HER2 overexpression is also correlated with a significantly shorter disease-free interval (Krishnamurti et al., 2009). The need for a molecular classification is to categorize patients who may benefit from targeted therapy, such as hormone therapy and anti HER2 therapy (Sharma et al., 2021).

The luminal type A is characterized by high expression of ER, high expression of PR and low expression of HER2 (Smolarz et al., 2022). The proliferation rate is low as detectable by the expression of cell proliferation antigen, Ki-67, that is less than 20% (Harbeck et al., 2019). Clinically, these subtypes are low grade, slow growing, and have the best prognosis with less incidence of relapse and higher survival rate. These carcinomas benefit from treatment with hormone therapy (tamoxifen or aromatase inhibitors). (Higgins & Stearns, 2009). Bone relapse is more frequent while visceral and central nervous system (CNS) relapses have a lower rate. Likewise, relapsed patients have a longer survival rate (Matro et al., 2015).

Luminal type B represents 10-20% of luminal tumours. They are ER positive and can be PR negative, with a high expression of Ki67 (greater than 20%) that makes them grow faster and with a worse prognosis than Luminal A (Inic et al., 2014). Hormore therapy and chemotherapy are the treatment options for this subtype of breast.

The HER2-positive subtype represents 10-15% of breast cancers and is characterized by high HER2 expression and the lack of ER and PR. However, two subgroups can be distinguished among these tumours: luminal HER2 (ER+, PR+, HER2+ and Ki-67:15–30%) and HER2-enriched (HER2+, ER-, PR-, Ki-67>30%) (Krishnamurti & Silverman, 2014). They are more aggressive than the luminal subtypes, with a higher proliferation rate. The prognosis is worse compared to luminal tumours, with improvement achieved after the introduction of HER2-targeted therapy. The specific drugs targeting the HER2/neu protein include Trastuzumab, Trastuzumab combined with Emtasin (T-DM1), Pertuzumab, and tyrosine kinase inhibitors such as Lapatinib and Neratinib, among others (Figueroa-Magalhães et al., 2014). They have a high response rate to chemotherapy treatments (Wang & Xu, 2019). The bone is the most common site for metastatic disease, and visceral relapses are also more frequent in this subgroup compared to the previous group (Pulido et al., 2017; Grassini et al., 2022).

Triple-negative breast cancer is ER-negative, PR-negative, and HER2-negative, and constitutes about 20% of all breast cancers. The TNBC subtype can be classified into

several additional subgroups including basal-like (BL1 and BL2), claudin-low, mesenchymal (MES), luminal androgen receptor (LAR), and immunomodulatory (IM), with the first two representing the most frequent (50–70% and 20–30% of cases, respectively) (Kumar & Aggarwal, 2016). TNBC is characterized by an aggressive behaviour, high proliferation rate, early relapse and the tendency to present in advanced stages.

Breast cancer confined within the mammary tissue or only spread to the axillary lymph nodes (early breast cancer) is considered curable, with improvements in multimodal therapy allowing for the possibility of recovery in 70-80% of patients. On the contrary, metastatic (advanced) breast cancer is not considered curable using currently available therapeutic options, but just treatable with the primary aim of extending survival and control symptoms with low treatment-associated toxicity, in order to maintain or improve quality of life (Harbeck et al., 2019). In this case, the median survival time is 2-3 years (El Saghir et al., 2011; Bonotto et al., 2014) . Metastatic breast cancer is not very common at initial presentation, occurring in about 6–7% of newly diagnosed cases; however, approximately 30% of patients initially diagnosed with earlier stages of breast cancer eventually develop recurrent or metastatic disease (Berman et al., 2013). Data reported by the National Cancer Institute in relation to the "5-year relative survival rate", showing that the subtype with the most favourable prognosis is the luminal A subtype with a survival rate of 94.4%, followed by the luminal B with 90.7%, the HER2 subtype with 84.8% and the TNBC subtype, which has the worst survival, with 77.1%. It is worth emphasizing that, although the subtype influences survival, stage at diagnosis may be the most powerful factor in determining survival outcome. Also relapse may differ depending on the subtype (Smid et al., 2008). A study reported that HER2 and TNBC subtypes had the highest local and regional recurrence rate: 7.5 and 3.4% for HER2 and 7.6 and 3.3% for TNBC, respectively (Ignatov et al., 2018). Luminal A subtype was recurrent in 1.5 and 0.7% locally and regionally, respectively, while luminal B subtype was associated in 2.9 and 1.5% of cases with local and regional recurrence. Furthermore, the authors noted that, although the relapse rate for luminal subtypes was initially low, relapse can occur even after 10 years. Therefore, different breast cancer subtypes are associated with different patterns and times of recurrence, suggesting that these factors should be considered when making treatment decisions (Erasmo Orrantia-Borunda et al., 2022).

1.4- ER-positive Breast Cancer

Approximately 75% of Breast Cancers are HR-positive (Rozeboom et al., 2019). Within this population, several molecular subpopulations exist, including approximately 34% with altered PIK3CA expression, 3% altered mTOR expression, 28% *CCND1* amplification, 25% *TP53* mutations, 30% *ESR1* mutations, 85% BCL2 overexpression, and 8-12% amplified/overexpressed MDM2 (TGCA, 2012).

Anti-estrogen therapy is a key component of the treatment of both early and advanced-stage HR-positive breast cancer. The Estrogen Receptor is a steroid hormone nuclear receptor consisting of a DNA-binding domain (DBD), ligand-binding domain (LBD), and transcriptional activation function domains 1 (AF1) and 2 (AF2). Activated ER can interact with estrogen-responsive elements (EREs) within the DNA through its DBD or interactions with other transcription factors (R. Patel et al., 2023) and modulate the expression of genes involved in tumour formation and progression (Hartkopf et al., 2020). Of the two ER isoforms, ERα is the most expressed in BC and is the main target of endocrine therapy. The role of ERβ, although is not fully understood, seems to be opposite, and involved in the inhibition of estrogen-dependent cell proliferation (Green et al., 1986). ERα expression occurs in the normal ductal epithelium and invasive breast cancer, and immunohistochemistry can be used to semi-quantitatively measure the degree of ER and PR expression in tumour tissue (Mann et al., 2001). Approximately 70% of all breast cancers exhibit ER and/or PR expression and, therefore, potentially susceptible to molecules targeting the estrogen signalling pathway, also commonly referred to as “endocrine therapy” (ET) (Hanker et al., 2020). The binding of estrogen to the ligand-binding domain of ER provokes an activating conformational change enabling its dimerization and intranuclear localization. Activated ER can interact with estrogen-responsive elements (EREs), leading to gene transcription and, consequently, to cell survival and proliferation (**Fig.1.1**).

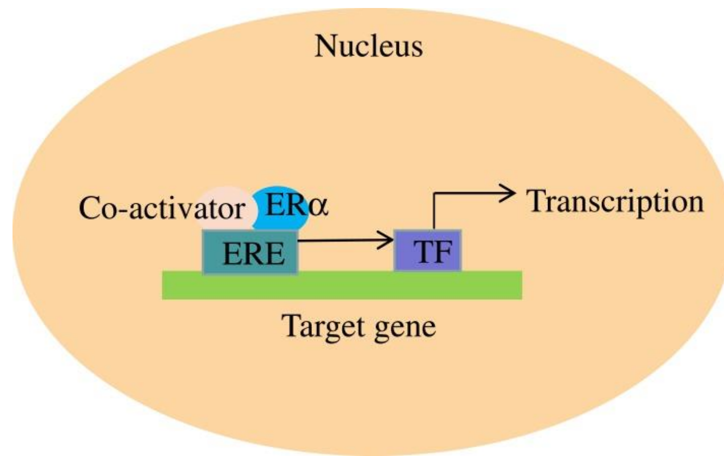

Fig. 1.1- ERα transcriptional activity (Y. Liu et al., 2020)

Classical endocrine therapies include selective ER modulators, (SERMs), like Tamoxifen, Raloxifene, Toremifene, or Selective ER degraders (SERDs), like Fulvestrant or Aromatase inhibitors (AIs) such as Letrozole, Anastrazole and Exemestane, with the aim to reduce estrogen levels (Harbeck et al., 2019). SERMs competitively inhibit the binding of estrogen to ER. SERM-bound ER dimers interact with chromatin at EREs of the DNA. In the breast, they are associated with co-repressors (CoR) leading to inhibition of ER transcriptional activity, but in other organ/tissues, such as bone and endometrium, they are associated with co-activators (CoA), allowing for gene transcription. SERDs are pure ER antagonists; the SERD–ER complex is prevented to translocate to the nucleus or undergo an open chromatin conformation that would allow transcription of ER-regulated genes. The SERD-ER complex, subsequently, undergoes proteosomal degradation. AIs, block estrogen production by inhibiting aromatase, the enzyme responsible for the conversion of androgens to estrogens (R. Patel et al., 2023). Novel anti-estrogen drug classes include complete estrogen receptor antagonists (CERANs), selective estrogen receptor covalent antagonists (SERCAs), and proteolysis-targeting chimerics (PROTACs) targeting ER. PROTACs are bifunctional molecules that consist of a ligand that binds to a target protein (ER) and a different ligand that binds to the E3 ubiquitin ligase. The interaction results in ubiquitination and degradation of the target protein through the ubiquitin-proteasome complex. CERANs block both transcriptional activation domains (AF1 and AF2) of ER by recruiting nuclear receptor corepressors (N-CoR) to inactivate AF1 and directly inhibit AF2. SERCAs covalently bind to a cysteine

residue (C530) on ER, resulting in ER inactivation and inhibition of gene transcription (R. Patel et al., 2023). However, a combination of endocrine therapy with other signalling pathways like PI3K/AKT/mTOR and Ras/Raf/MEK/ERK and coregulatory factors targeted agent should be the focus of future treatment.

1.4.1- Tamoxifen

Tamoxifen (Tam) is the most commonly used SERM, which allows a 31% reduction in the five-year mortality rate among hormone receptor positive women (R. Patel et al., 2023), (Abe et al., 2005). Tam (ICI 46474) (1-[4-(2-dimethylaminoethoxy)-phenyl]-1,2-diphenylbut-1(Z)-ene) is a nonsteroidal antiestrogen prodrug and, after oral administration, is activated by a member of the hemeprotein CYP450 family allowing the formation of 4-hydroxytamoxifen (4-OH-TAM) with higher ERα binding activity (**Fig. 1.2**). Moreover, its association rate is stronger and its dissociation rate is slower with ERα (Johnson et al., 2004) .

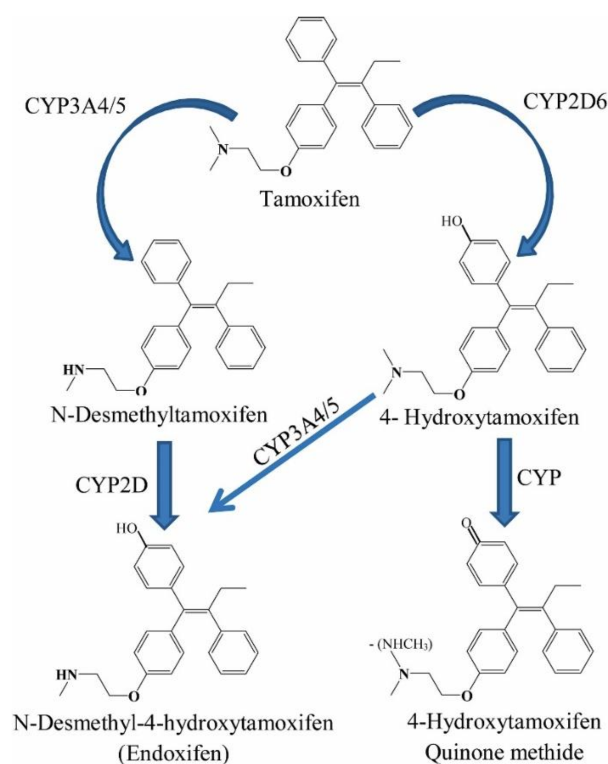

Fig. 1.2 – Tamoxifen metabolism. Tamoxifen is metabolized by cytochrome P450 2D6 and 3A4/5 enzyme, which results in the formation of hydroxylated TAM metabolites, 4-hydroxy-N-desmethyl-tamoxifen (endoxifen), and 4-hydroxytamoxifen (Mishra et al., 2021).

Once binding between 4-OH-TAM and ERα has occurred, conformational changes in the receptor inhibit the binding of coactivators and modify the transcriptional activity of the

ERα, resulting in reduced growth signals and, consequently, inhibition of breast cancer growth (Shiau et al., 1998). Despite these positive effects, around 40% of ERα-positive tumors showed an absence of response to endocrine therapy, and most of those who respond develop resistance (Ali & Coombes, 2000; Chanrion et al., 2008). Endocrine resistance occurs due to both de novo and acquired resistance. De novo resistance (defined as disease progression within 6 months of initiating ET or relapse within 2 years of initiating adjuvant ET for early breast cancer) establishes at the beginning of treatment. This type of resistance has been demonstrated in MCF-7, an ER-positive human breast cancer cell line, transfected with the HER2/neu gene which induced tumor growth in xenograft mice even during Tam treatment (Benz et al., 1992). The primary mechanism of de novo or intrinsic resistance to endocrine therapy, particularly to Tam, is due to lack of expression of ER (Rani et al., 2019). Recently, a second intrinsic mechanism has been documented in which patients carrying inactive alleles of cytochrome P450/2D6 (CYP2D6) fail to convert tamoxifen to its active metabolite, and are less responsive to Tam (Musgrove & Sutherland, 2009). Acquired resistance is an event occurring in patients initially responsive to therapy, and is characterized by the lack of response or eventually by stimulation of growth following a new round of treatment (Chang, 2012). Acquired resistance is well demonstrated when MCF-7 cells are inoculated into ovariectomized athymic mice treated with Tam. Most tumors in these mice initially respond to Tam and do not grow but some tumors begin to grow even in the presence of antiestrogen after about a year (O'Regan et al., 2006). Several mechanisms have been suggested to be involved in promoting resistance to Tam therapy, and include loss or alteration in ERα expression, development of resistance to apoptotic mechanisms, activation of autophagy for cell survival, alterations in the expression of coregulatory proteins, and deregulation of signaling pathways involved in survival, proliferation, and stress response (Viedma-RodríGuez et al., 2014) (**Fig. 1.3**).

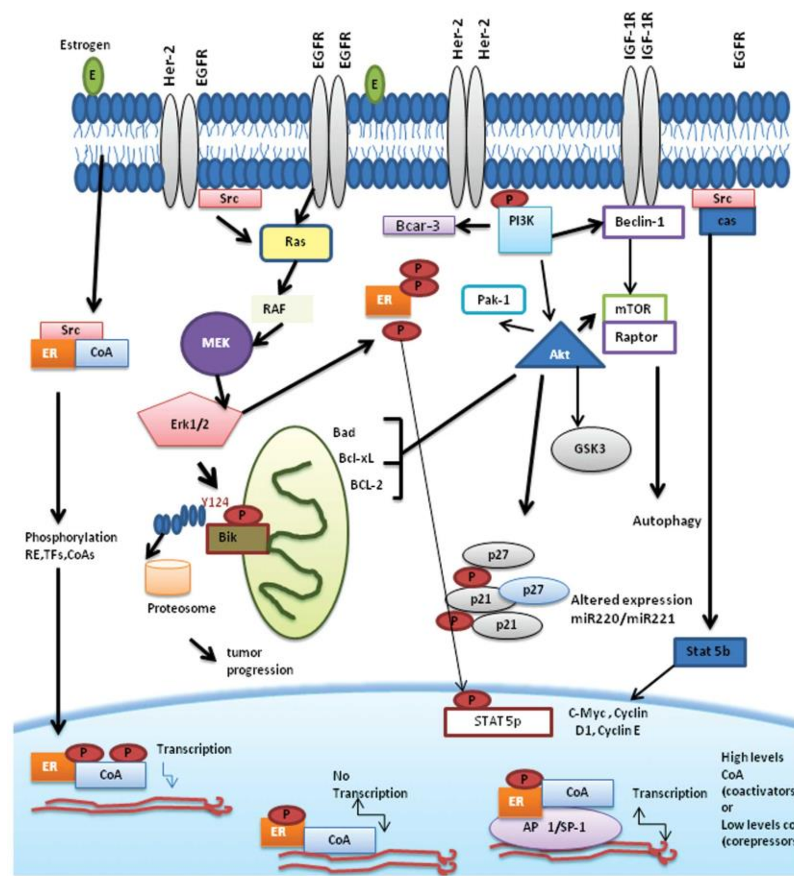

Fig. 1.3- Molecular mechanisms of tamoxifen resistance (Viedma-RodríGuez et al., 2014).

1.5- Mechanisms of Tamoxifen resistance

1.5.1- Loss and mutations of Estrogen Receptor

Lack of ERα expression is recognized as a primary mechanism for de novo Tam resistance. Moreover, the phosphorylation of specific residues of Serine on ERα has been related to Tam resistance in BC (de Leeuw et al., 2011). Even when the receptor is present, in about 20% of patients treated with Tam a loss of ER is observed over time. This phenomenon determines a change from an initially ER-positive to ER-negative phenotype and, as a consequence, the tumor is not suppressed by Tam and other endocrine agents targeting ERα (Giuliano et al., 2011). The mechanism responsible for the loss of ER expression is mainly the transcriptional repression (Chang, 2012). Transcriptional repression may be due to epigenetic changes, such as aberrant CpG island methylation of the ER promoter and histone deacetylation by histone deacetylase enzyme, resulting in a compact nucleosome structure that limits transcription (Viedma-RodríGuez et al., 2014). Inhibition of the histone deacetylase (HDAC) could restore ER transcription in BC cell

lines that have lost ER expression (Yang et al., 2000). As a consequence, a combination treatment with deacetylase inhibitor (HDACi) and DNA methyltransferase-1 (DNMT1) inhibitor can restore sensitivity to Tam in cell lines not expressing the ER, demonstrating that interfering with epigenetic modifications could represent a therapeutic strategy for a portion of patients resistant to endocrine therapy (J. Fan et al., 2008). Munster et al., in a recent Phase II clinical study, used Vorinostat (HDACi) in combination with Tam or Tam alone. The results showed that the combination treatment is effective in restoring Tam sensitivity, with 19% of objective response rate (ORR) and 40% of clinical benefit rate (CBR) (Munster et al., 2011).

Mutation of ERα gene is another key factor in determining the effectiveness of endocrine therapy. Studies demonstrate that these mutations are uncommon in primary tumors but relatively frequent in the progression of endocrine resistance and could be used as a prognostic/predictive tool to determine the effectiveness of endocrine therapy (Rani et al., 2019). The majority of these mutations are in the ERα LBD, leading to a constitutive activation of the ER (Toy et al., 2013; Jeselsohn et al., 2014). The most common hot spot mutation are Tyr537Ser, Tyr537Asn and Asp538Gly (Y537S, Y537N,D538G) (Jeselsohn, 2017). These residues and their phosphorylation are crucial in controlling the agonist state of the LBD domain of ER, conformational changes, and protein stability (Yudt et al., 1999; Harrod et al., 2017), thus mutations at these residues provokes alteration of the conformational dynamics of the loop connecting Helix 11 and Helix 12 in the LBD of ER, leading to a stabilized agonist state, even in the presence of endocrine treatment (Fanning et al., 2016). Targeting the transcriptional function of mutant ER proteins using Bromodomain and Extra-Terminal motif (BET) inhibitor OTX015 reverses the endocrine therapy resistance due to ER mutations. Thus, drugs that reverse ER mutation could be one of the possible treatment strategy in Tam resistant breast cancer resulting from ER mutation (Alluri et al., 2016).

1.5.2- Altered Expression Patterns of Co-Regulatory Proteins

The transcriptional activity of ER is controlled by co-regulatory proteins, which may activate (coactivators) or inhibit (corepressors) the ER-driven transcription (Belachew & Sewasew, 2021).

The SRC/p160 family of nuclear receptor coactivators are the best characterized coactivators and consist of three members. These are SRC-1/NCoA-1, SRC-2/GRIP1/TIF2/NCoA-2, and SRC-3 (p/CIP, RAC3, ACTR, AIB1, and TRAM-1) (Schiff

et al., 2003). SRC-1 regulates several signaling pathways and is important in Tam resistance (Browne et al., 2018). One of the possible mechanisms through which SRC can induce Tam resistance is by upregulating the NAD-dependent protein deacetylase sirtuin-1 (SIRT1) (J. Zhou et al., 2020). SRC-3 mRNA overexpression is also associated with Tam resistance and silencing SRC-3 can restore the antitumor effects of Tam (Su et al., 2008). The expression of SRC-3 is also controlled by General Control Nonderepressible 5 (GCN5), which leads to tamoxifen resistance by reducing p53 levels (Oh et al., 2020).
Nuclear corepressors NCOR1, NCOR2, and the nuclear receptor subfamily 2, group F, member 2 (NR2F2) are the best characterized corepressors linked to breast cancer (Légaré & Basik, 2016). The downregulation of NCOR1 has been related with Tam resistance (Lavinsky et al., 1998; Giuliano et al., 2011). In fact, the downregulation of NCOR1causes the upregulation MYC, CCND1 and SFD1 gene transcription, resulting in Tam behaving as a partial agonist for cell cycle progression. Similar to NCOR1, reduced levels of NCOR2 and NR2F2 are related to Tam resistance (Légaré & Basik, 2016).

1.5.3- miRNA and Extracellular Vesicles

miRNAs has been implicated in the development of Tam resistance. Cittelly and colleagues have shown that miRNA, and specifically miR-342, can induce endocrine resistance through several mechanisms such as upregulation of the drug efflux transporters, overexpression of anti-apoptotic proteins, promotion of Epithelial-Mesenchymal Transition (EMT) and formation of cancer stem cell (Cittelly et al., 2010). Specifically, the miR-21 induces Tam resistance by inactivating the onco-suppressor PTEN, whereas miR-221/222 provoke resistance through p27 and ER modulation, resulting in ER-indipendent tumor proliferation. The miR-155 also promotes Tam resistance via inhibition of cytokine signaling 6 (Dobre et al., 2020).
Extracellular vesicles (EVs) are cell-derived membrane-surrounded vesicles that carry bioactive molecules (like oncogenic material) and deliver them to recipient cells. EVs have been implicated in transferring Tam resistance from the Tam resistant cells to the Tam sensitive cells through the transfer of miR-221/222 (Wei et al., 2014).

1.5.4- Receptor Tyrosine Kinase (RTK)

Receptor Tyrosine Kinases (RTK) are a family of receptors including the Insulin-like growth factor-1 receptor (IGF-1R), the epidermal growth factor receptors (EGFR), the hepatocyte growth factor receptor (HGFR), the platelet-derived growth factor receptor (PDGFR), the vascular endothelial growth factor receptor (VEGFR) and the fibroblast growth factor receptor (FGFR). An upregulation of RTKs is observed in BC and is indicative of poor prognosis (Tomiguchi et al., 2016; Templeton et al., 2014). The main pathways activated by these receptor are the mitogen activated protein kinase (MAPK), janus kinase (JAK)/signal transducer and activator of transcription (STAT) and phosphoinositide (PI3K)/AKT pathways.

EGFR (also called ErbB1/HER1) belongs to the ErbB family of growth factor receptors, a family that includes also ErbB2/HER2, ErbB3/HER3 and ErbB4/HER4 and all of them are implicated in various degrees in BC. After being activated by ligands (such as EGF), these receptors undergo dimerization and autophosphorylation; these event activate signaling through several pathways, such as MAPK-ERK and PI3K-AKT pathways (Linggi & Carpenter, 2006). Studies suggested that one of the possible Tam resistance mechanism is the increased crosstalk between ER and HER2 coupled with high expression of SRC-3 (Chang, 2012). Tam acts as an estrogen agonist in BC cells with high expression level of HER2 and SRC-3 (Shou et al., 2004). High levels of HER2 and its downstream signal (such as MAPK) are directly linked to the loss of ER, one of the main mechanisms inducing endocrine resistance. Therefore, inhibition of SRC-3 and/or HER2 activity could represent a strategy to overcome Tam resistance and re-sensitize cells to Tam treatment (Chang, 2012). Interestingly, Mo et colleagues showed that long-term endocrine therapy facilitates translocation of GPR30 (G-protein coupled receptor 30, a seven-transmembrane domain protein identified as an estrogen receptor structurally distinguished from the classic ERα and Erβ (Prossnitz et al., 2008)) to cell membranes, resulting in inappropriate activation of the EGFR signaling pathway. Moreover, GPR30 attenuates the inhibitory effect of cAMP on MAPK. Combination treatment with the GPR30 specific antagonist G15 plus Tam induces both cytocidal action in vitro and antitumor progression in vivo. Thus, GPR30 might be a useful target in developing better treatments for Tam resistant BC patients (Mo et al., 2013).

Insulin-like growth factor-1 receptor (IGF-1R) is a trans-membrane tyrosine kinase protein activated by insulin-like growth factor-1 and -2 (IGF-1, IGF-2). Activation of this receptor results in proliferation, anti-apoptosis and in BC development, progression, and

metastasis through its role in Ras/Raf/MEK1/2/ERK1/2 and PI3K/AKT/ mTOR pathway (Christopoulos et al., 2015). This receptor provokes the redistribution of ER from the nucleus to extranuclear areas and increases ligand-independent activation of ER, which further activates Ras/Raf/MEK1/2/ERK1/2 and PI3K/AKT/mTOR pathway and results in acquired endocrine resistance (P. Fan & Craig Jordan, 2019). Tam-resistant cell growth is significantly reduced by using IGF-1R-specific inhibitors (like AG1024 and AEW541) or an IGF2 neutralizing antibody. (Fagan et al., 2012).

Fibroblast growth factor receptor (FGFR) families also play an important role in BC development and progression. Evidence shows that elevated levels of fibroblast growth factor receptor 3 (FGFR3) stimulate the activation of the Ras/Raf/MEK1/2/ERK1/ 2 and PI3K/AKT/mTOR signaling pathways and thereby inducing Tam resistance (Tomlinson et al., 2012). Amplification of fibroblast growth factor receptor 1(FGFR1) also promotes cyclin D1 expression in ER-positive BC, resulting in the failure of endocrine therapy (Y. Zhou et al., 2020).

1.5.5- Cell Cycle Regulators

Positive and negative cell cycle regulators can affect BC response to endocrine treatment (Musgrove & Sutherland, 2009). Cell cycle regulators like cyclins E1 and D, and oncogene c-MYC, are involved in endocrine resistance by activating Cyclin dependent-Kinases (CDKs) (Span et al., 2003; Belachew & Sewasew, 2021) . Moreover, the downregulation of the two G1 checkpoint CDK inhibitors, p21 and p27 (CDKN1A and CDKN1B), is also associated with endocrine resistance (Chu et al., 2008; Pérez-Tenorio et al., 2006). The lack of p21 allows cyclin-CDK complexes to excessively phosphorylate ER when bound to Tam, resulting in a growth-stimulatory phenotype (Abukhdeir & Park, 2008). Thus, Cyclin D1 and CDK4/6 inhibitors represent strategies to overcome endocrine resistant BCs and potent CDK4/6 inhibitors have become extensively available in the last decade. In fact, the addition of a CDK4/6 inhibitor to endocrine therapy as a combination therapy determines a prolonged PFS and is now included as a first line therapy in advanced hormone receptor-positive BC (Finn et al., 2016; Hortobagyi et al., 2016; Cristofanilli et al., 2016).

1.5.6- PI3K/AKT/mTOR

Activation of the PI3K pathway in breast tumors is associated with reduced ERa levels and endocrine resistance (Fox et al., 2012). Through GPER signaling, E2 can activate the

p110α subunit of PI3K, leading to FOXO3a inactivation and BC progression (Zekas & Prossnitz, 2015). Studies have demonstrated that the crosstalk between the PI3K and ER pathway is a very important event in the establishment of Tam resistance and suggested that a combination of a PI3K inhibitor with an anti-oestrogen is more effective than either of them alone (Fu et al., 2013). Two other factors, mTORC1 and mTORC2 are part of a feedback loop in the PI3K pathway. Upon inhibition of mTORC1, the mTORC2 complex provokes an activation of the PI3K pathway, highlighting that inhibition of one branch of the pathway can determine the activation of another branch, resulting in insufficient clinical benefits (Breuleux et al., 2009).

1.5.7- MAPK/ERK

Tam resistant BC cells display increased expression of activated MAPK and ERα (Britton et al., 2006). Activation of the RAS/MAPK/ERK pathway leads to phosphorylation of ERα at ser118 in the AF1 domain and increases its sensitivity to Estrogen, leading to cell proliferation and endocrine resistance (D. Chen et al., 2002; Chung et al., 2002; Osborne & Schiff, 2003). Bayliss et al. have shown that the inhibition of the MAPK/ERK pathways causes upregulation of ERα, with a subsequent improvement in Tam resistance (Bayliss et al., 2007). Moreover, another study has shown that Tam resistance can be mediated through CDK10 suppression following the activation of the MAPK/ERK1/2 pathway (Iorns et al., 2008).

These data demonstrate the possibility of restoring Tam responses and suggest that there exist BC patients who would benefit from a combined MAPK inhibition/hormonal therapy.

1.5.8- STATs

The STAT-family members (STAT1-4, STAT5a, STAT5b, and STAT6) represent a family of transcription factors involved in several cellular functions. Studies have shown that STAT3 and STAT5 signaling pathways are involved in Tam resistance (Wingelhofer et al., 2018; Silva & Shupnik, 2007). Yeh et al. demonstrated that the phosphorylation of STAT3 at serine 727 is negatively correlated with ER status, as demonstrated in breast cancer cell lines and in primary tumors. Cells treated with Tam have lower STAT3 phosphorylation level, suggesting an association between Tam sensitivity and decreased STAT3 transcriptional activity (Yeh et al., 2006). Moreover, another study has demonstrated that STAT5 is a predictive factor for endocrine therapy and a powerful

prognostic molecular marker in ER-positive BC, suggesting that expression of STAT5 can be exploited in selecting patients who may benefit from endocrine therapy (Yamashita et al., 2006).

1.5.9- NF-kB

The NFkB family of transcription factors includes five members (RelA/p65, RelB, cRel, NFkB1/p50, and NFkB2/p52). A study has shown that Tam resistant cells utilize NF-kB signaling to prevent or overcome the proliferation inhibitory actions of Tam. Using a specific siRNA to knock down p65 in Tam resistant MCF7 directly show that p65 contribute to the Tam resistant phenotype and that targeting p65 can restore the sensitivity to Tam (Yde et al., 2012). Another study demonstrated that parthenolide, a NF-kB inhibitor, restored sensitivity to Tam in resistant MCF7 cell lines. (Nehra et al., 2010).

All together, these data indicate that resistance to Tam (and in general to endocrine therapy) can be established with different mechanisms and through different molecular players; this highlights how the therapy to overcome resistance to Tam treatment must take into account the molecular characteristics of the individual patient's tumor, in order to obtain the best possible therapeutic response. Furthermore, the possibility of multiple mechanisms of resistance to Tam makes it clear that combination therapies of multiple drugs are more effective than monotherapies.

1.5.10- Metabolic reprogramming

Recently, metabolic reprogramming has sparked increasing interest for a possible contribution to Tam resistance; in particular, lipid metabolism could be involved in the molecular alterations that confer resistance to Tam (Mishra et al., 2021). In fact, BC patients treated with Tam, experience a decrease in blood cholesterol levels (C. L. Liu & Yang, 2003), an increase in hepatic fat content which can evolve to hepatic steatosis evidenced by an increase in neutral lipids and lipid droplets in hepatic cells (Nishino et al., 2003). Recent studies found a strong association between lipid metabolism and Tam resistance in breast cancer cells. Lipids are essential for cancer cells growth and proliferation, and can be obtained via two routes: de novo lipogenesis and increased lipid uptake (Zaidi et al., 2013). MCF-7 BC cell line, when grown for more than one year in

the presence of tamoxifen, acquires resistance to the SERM, and when grown in a fatty acid supplemented media manifests increased fatty acid uptake. Additionally, a significant increase in triglycerides stored in lipid droplets (LDs) was observed in Tam Resistant (Tam-R) BC cells (Hultsch et al., 2018). Free fatty acids excess is packaged in LDs to avoid lipotoxicity, but can be used as an energy source to quickly respond to any condition of higher energy needs (Aon et al., 2014). LDs are storage organelles at the center of lipid and energy homeostasis, consisting of a neutral lipids hydrophobic core, which is wrapped in a phospholipid monolayer adorned with specific proteins. A high number of LDs containing cholesterol esters is considered as hallmark of aggressive cancer (Beloribi-Djefaflia et al., 2016). Among lipid molecules, cholesterol is an important constituent of the plasma membranes of eukaryotic cells and plays a critical role in maintaining its integrity and fluidity. Cholesterol metabolism is deregulated in many malignancies, including BC. Tam resistance seems to be correlated with the overexpression of MUC1, an oncoprotein that upregulates the expression of several enzymes involved in cholesterol metabolism (Pitroda et al., 2009). Moreover, Tam, interacting with high affinity to the microsomal Anti-Estrogen Binding Site (AEBS, a hetero-oligomeric complex involved in cholesterol metabolism), can regulate the lipid balance (Sutherland et al., 1980; De Medina et al., 2009). This mechanism has been linked to control cell growth, differentiation and apoptosis in the presence of reactive oxygen species (ROS) and has been established as another mode by which Tam induces cytotoxicity (Hultsch et al., 2018).

2 - Aim of the study

Chemotherapy is one of the primary treatment options for human cancers. Although great advances have been made in cancer therapies and new classes of drugs have been introduced, chemotherapy continues to play an essential role in improving the survival of cancer patients, especially for those who have metastatic tumors or those who do not respond to immunotherapy. However, intrinsic or acquired chemoresistance results in tumor recurrence, which constitutes a major challenge in anti-cancer treatment. The high incidence of chemoresistant cancer makes it essential to understand the mechanisms underlying chemoresistance in order to develop new therapeutic strategies. Several mechanisms, including drug efflux, increased detoxifying enzymes expression, enhanced DNA damage reparability, presence of cancer stem cells (CSCs), epithelial mesenchymal transition (EMT), autophagy, ferroptosis and resistance to apoptosis, underlie the development of chemoresistance. Recently, accumulating evidence suggests that lipid metabolism reprogramming is strictly linked to drug resistance in tumor. Targeting lipid metabolism in combination with traditional chemotherapeutic drugs is a promising strategy to overcome drug resistance (R. Yang et al., 2022). Zhou et al. found that upregulation of ACLY induces resistance in colorectal cancer cells resistant to SN38, an active ingredient converted from irinotecan (Zhou et al., 2013). Dysfunction of \FASN is reported as a new characteristic in chemoresistance in malignant cells. Studies demonstrated that FASN levels were increased in paclitaxel-resistant hepatocellular carcinoma (HCC) (Meena et al., 2013), taxane-resistant prostate cancer (Souchek et al., 2017), cisplatin-resistant squamous cell carcinoma (Huang et al., 2012), paclitaxel-resistant laryngeal cancer (Xu et al., 2013) ,gemcitabine-resistant pancreatic cancer (Y. Yang et al., 2011) and carboplatin/paclitaxel-resistant ovarian cancer (Shih et al., 2010). In resistant BC cells, elevated levels of endogenous FA synthesis catalysed by FASN was related to HER2 overexpression (Ligorio et al., 2021). The high levels of CPT1A in pancreatic ductal adenocarcinoma (PDAC) patients was associated with chemoresistance by rewiring cancer lipid metabolism to escape from energy stress (Luo et al., 2016). These are just a few examples of how alterations in lipid metabolism are involved in drug resistance in tumors.

Recent studies found a strong association between lipid metabolism and Tam resistance in BC cells. A study by Hultsch and colleagues demonstrated that a significant increase

in triglycerides and cholesterol esters stored in lipid droplets (LDs) was observed in Tam Resistant (Tam-R) BC cells. Moreover, they demonstrated that, once resistance is achieved, lipid-altering compounds (C75, Bezafibrate, T 0070907, TO901317, and Orlistat) produce little or no effect on cell viability or lipid phenotype in TamR cells, indicating that BC cells are able to compensate for drug-induced lipid imbalance, and this occurs independently from the target of the pharmacological intervention. Besides, TamR cells contain an increased level of the enzyme stearoyl-CoA desaturase (SCD), which participates in the biosynthesis of monounsaturated fatty acids and supports their survival avoiding the accumulation of toxic polyunsaturated fatty acids (Hultsch et al., 2018).

This PhD research project aimed to define the major differences in lipid metabolism between Tam-sensitive and Tam-resistant MCF-7 cells. We investigated differences in lipid uptake, storage, mobilization from cellular stores, synthesis, and metabolism. Moreover, starting from the observation of Conn and colleagues, that demonstrated that a cap-recognition factor (eIF4E1) is necessary for the translation of genes controlling lipid processing, transport, storage and LD growth (Conn et al., 2021), we investigated whether a cap-recognition factors could be involved in the onset and/or maintenance of resistance to Tamoxifen.

3- Material and Methods

3.1- Cell Culture Conditions and Treatments

ERα+ human breast cancer epithelial cell lines MCF-7 were purchased from ATCC (LGC Standards S.r.l., Milan, Italy). MCF-7 cells were cultured in DMEM/F-12 medium, (Sigma-Aldrich, Merck, Milan, Italy), supplemented with 5% of fetal bovine serum (FBS), 100 IU/mL penicillin, 100 ng/mL streptomycin and 0.2 mM L-glutamine (growth medium; GM). The treatments utilized were: Etomoxir (HY-50202, MedChemExpress); Thioridazine (T9025, Sigma-Aldrich); BLT-1 (SML0059, Sigma-Aldrich); Avasimibe (PZ0190, Sigma-Aldrich); Cycloheximide (239763, Sigma-Aldrich); Briciclib (HY-16366, MedChemExpress).

3.2- Generation of Tamoxifen-Resistant Cell Lines

Tamoxifen-resistant MCF-7 (Tam-R) cells were obtained by growing cells GM supplemented with increasing concentrations of tamoxifen, starting from 1 nM and gradually going up to 1 µM by increasing concentration by 10-fold every month. Before using cells for experiments cells were exposed to Tam 1 µM for at least one year. The acquired resistance was periodically verified by checking the lack of Tam-dependent growth inhibition compared to the parental cells.

3.3- Migration Assay

For Transwell migration assays, cells were harvested in DMEM containing 0.5% FBS and replated onto the upper chamber of a Transwell filter with 8 µm pores (Corning Costar, Cambridge, MA, USA) and the chamber was placed in 24-well plate containinf GM. Cells were incubated at 37 °C with 5% CO_2 for 18 h. Non-migrated cells on the upper side of the filter were removed with a cotton swab, and cells on the underside of the filter were fixed and stained with a Comassie Brilliant Blue solution (Sigma) for 10 min. Images were captured using a Olympus CKX53 microscope. Cell migration was determined by the number of the migrated cells. For each experiment, the number of cells

in nine random fields on the underside of the filter was counted, and three independent filters were analyzed.

3.4- Tumor sphere forming efficiency

A single cell suspension was prepared using enzymatic (1x Trypsin-EDTA, Sigma Aldrich, #T3924), and manual disaggregation (25-gauge needle) to create a single cell suspension. Cells were plated at a density of 500 cells/cm^2 in mammosphere medium (DMEM-F12/ B27/ EGF (20ng/ml)/ Pen-Strep) in non-adherent conditions, in culture dishes coated with (2-hydroxyethylmethacrylate) (poly-HEMA, Sigma P3932). Cells were grown for 5 days and maintained at 37 °C in a 5% CO_2 humidified atmosphere. After 5 days in culture, spheres >50 µm were counted using an eye piece graticule.

3.5- SeaHorse Mitochondrial Stress Analysis

Real-time oxygen consumption rates (OCR) were determined using the Seahorse Extracellular Flux analyzer (XF96) (Agilent). MCF-7 cells were seeded into XF96-well cell culture plates (Seahorse Bioscience: North Billerica, MA, USA) and incubated overnight at 37 °C in a 5% CO_2 humidified atmosphere. At the end of treatment, cells were washed in warm XF assay media supplemented with glucose (10 mM), Pyruvate (1 mM), L-glutamine (2 mM) and adjusted at pH 7.4. Cells were then maintained for 1 h in 175 µL/well of XF assay media at 37 °C, in a non-CO_2 incubator. During the incubation time, 25 µL of a solution of XF assay media containing oligomycin (10 µM), FCCP (9 µM), rotenone (10 µM), antimycin A (10 µM) were loaded into the injection ports of the XFe-96 sensor cartridge. The dataset was analyzed by XFe-96 software (Agilent).

3.6- Western Blot Analysis

Total cell lysates were prepared in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM EDTA, 0.1% SDS and a mixture of protease inhibitors). Western blot analysis was performed using the same amount of protein for each sample. Protein concentration was determined by the Bradford

(Sigma-Aldrich, St. Louis, MO, USA) method and equal quantities of proteins were subjected to Western blot analysis. SDS-PAGE-separated proteins were electroblotted onto a nitrocellulose membrane. Blots were incubated overnight at 4 °C with the following primary antibodies: anti-SR-BI (Ab52629; 1:1000; Abcam), anti-LDLR (10007665, 1:1000, Cayman Chemical), anti-SLC27A2 (sc-393906, 1:500, Santa Cruz), anti-FASN (sc-48357, 1:1000, Santa Cruz), anti-ACOX-1 (sc-517306, 1:500, Santa Cruz), anti-CPT1 (sc-393070, 1:500, Santa Cruz), anti-ATGL (sc-36578, 1:500, Santa Cruz), anti-SLC27A4 (sc-393309, 1:500, Santa Cruz), anti-Catalase (sc-365738, 1:500, Santa Cruz), anti-ABCD3 (sc-514728, 1:500, Santa Cruz), anti G0S2 (sc-518067, 1:500, Santa Cruz), anti-ELOVL5 (sc-374138, 1:500, Santa Cruz), anti-eIF4E1 (sc-9976, 1:500, Santa Cruz), anti-eIF4E2 (sc-100731, 1:500, Santa Cruz). Anti-βactin (ab8226; 1:1000; Abcam) was used as a loading control. IRDye secondary Abs, the Odyssey FC Imaging System imager and the Image Studio™ Lite v5.2 software were all from LI-COR Biosciences (GmbH, Bad Homburg, Germany).

3.7- RNA extraction, reverse transcription, and real-time PCR

Total RNA was extracted with PureLink™ RNA Mini Kit (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA was reverse-transcribed in a final volume of 50 µL using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher, Foster City, CA, USA); cDNA was diluted 1:3 in nuclease free water. Quantitative PCR was performed using the following primer sequences:

Gene Name	Genbank accession number	Forward Primer	Reverse Primer
LDLR	NM_000527.5	GAGGTGGCCAGCAATAGAATCT	GCCGTGGGCTCTGTCAAG
SR-BI	NM_005505.5	GGGCTCTTCACGGTGTTCAC	ACATTTGCCCAGAAGTTCCATT

SLC27A2	NM_003645.4	GATATTGCGTCAGAGT TCCC	CTTCTCTGTCTGAG CCTTTG
SLC27A4	NM_005094.4	GGTGCACAGCAGGTA TTA	CCGATTCCCACGAT GTTT
ACAT1	NM_003101.6	GCAGCTGTGCTGAGA ATAC	CTTCCCATGCTGCT TTACTT
CD36	NM_001001548 .3	GGCTGTGTTTGGAGGT ATT	GTACCTTCTTCGAG GACAAC
ACACA	NM_198834.3	TTCACTCCACCTTGTC AGCGGA	GTCAGAGAAGCAG CCCATCACT
FASN	NM_004104.5	TTCTACGGCTCCACGC TCTTCC	GAAGAGTCTTCGTC AGCCAGGA
ELOVL5	NM_021814.5	ACCGCAGGAGAATCA GATA	CTTGCGCAGGATGA AGAA
ATGL	NM_020376.4	GAGAAGACGTGGAAC ATCTC	ATGTTGGAGAGGGT GGT
G0S2	NM_015714.4	GATGGTGAAGCTGTA CGTG	TGCACACAGTCTCC ATCA
CPT1A	NM_001876.4	GACCGGGAGGAAATC AAAC	CTGGGTCCGGGAA GTATTA
ABCD3	NM_002858.4	TCCGAGTAAGGCTCA CTAAA	AGCAGCTGGTCTGG ATTA
ACOX1	NM_004035.7	CAACCATCGTATTCCC AGAG	CCGTAAGTCAGCTT GTTACTC
CAT	NM_001752.4	ACATGGTCTGGGACTT CT	CCATTCATGTGGCG ATGT
eIF4E1	NM_001968.5	CAAACAGCAGAGACG AAGT	CAACAGCGCCACAT ACA
eIF4E2	NM_004846.4	CACAGTGACTTCCATC TCTTC	CTTCCGCAGCCGAA TAAT
18S	M10098.1	CGGCGACGACCCATT CGAAC	GAATCGAACCCTGA TTCCCCGTC

PCR reactions were performed in the QuantStudioTm 3, Real Time PCR System (Thermo Fisher) using 0.2 μM of each primer. PowerUp™ SYBR™ Green Master Mix (Thermo Fisher) with the dissociation protocol was used for gene amplification; negative controls contained water instead of first-strand cDNA. Each sample was normalized to its 18S rRNA (18S) content. Final results were expressed as n-fold differences relative to a calibrator and calculated using the ΔΔCt method.

3.8- Cell Viability Assay and Cell Proliferation Assays

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) colorimetric assay, which measures mitochondrial activity in viable cells. Cells were plated in 48-well plates and, after 24 h, were treated. Fresh MTT (Sigma), resuspended in phosphate buffered saline (PBS), was added to each well (final concentration 0.33 mg/mL) and the plate was incubated at 37 °C for 1 h in an incubator humidified to 5% of CO_2. Media were then removed, and the formazan crystals were dissolved in 200 µL of DMSO (Sigma-Aldrich). Absorbance at 570 nm was evaluated with a spectrophotometer (Synergy H1 plate reader, BioTek Instruments, Inc., Winooski, VT, USA). Each condition was in sextuplicate and experiments were performed three times.

For cell proliferation assay, cells were plated in 96-well plates and after 24h treated. Cell proliferation was measured using CyQUANT® Cell Proliferation Assay Kit (Invitrogen, C7026). The Proliferation rate was measured following the manufacturer's instructions. The number of cells at the time of treatment was set as 100%.

3.9- Generation of stable MCF-7 clones

MCF-7 cells were grown in a complete, antibiotic-free medium in 6 well plates (5×10^5 cells/well). After 48 h, cells were infected with Lentiviral particles containing short hairpin RNA (shRNA) construct encoding a scrambled sequence (shCTR) (Santa Cruz, sc108080), or targeted eIF4E2 shRNA (sheIF4E2) (Santa Cruz, sc94498-V), according to the manufacturer’s protocol (Santa Cruz Biotecnology). After 72 h, infected cells were selected by the addition of puromycin (10 µg/mL) (Sigma). Cells resistant to the antibiotic

formed clones that were isolated and amplified. In about three weeks the concentration of antibiotics was gradually decreased from 10 µg/mL to 1 µg/mL.

3.10- Immunofluorescence Assay

Breast cancer cells were seeded on coverslips in 6-well plates at a density of 1×10^5 cells/well, and cultured overnight in complete medium. The following day cells were were fixed with 4% Formaldehyde for 10 min at room temperature, washed three times with Phosphate buffered saline (PBS, Sigma-Aldrich), and incubated for blocking with 3% BSA (Sigma-Aldrich) in PBS for 30 min at 37 °C. Then, cells were incubated overnight with anti-ABCD3 monoclonal antibody (sc-514728, Santa Cruz), at 1:200 dilution. The next day, coverslips were washed three times for 5 min with PBS to discard excess of primary antibody, then incubated for 30 min at 37 °C in anti-mouse IgG-secondary antibody (Alexa Fluor 594) at 1:1000 dilution and washed in PBS. DAPI (Sigma-Aldrich, Milan, MI, IT) was used to stain nuclei.

3.11- Bodipy Staining

MCF-7 cells were seeded on coverslips in 6-well plates at a density of 1×10^5 cells/well, and cultured overnight in complete medium. After treatment had ended, cells were fixed with 4% Formaldehyde for 10 min at room temperature, washed three times with Phosphate buffered saline (PBS, Sigma-Aldrich), and incubated for 30 min with BODIPY™ 493/503 (2 µM, for Neutral Lipids staining) or CholEsteryl BODIPY™ FL C12 (2 µM, for Cholesterol Esters staining) or BODIPY™ 558/568 C12 (2 µM, for fatty acids staining). Next, the coverslips were washed three times for 5 min with PBS to discard excess of dye. DAPI (Sigma-Aldrich, Milan, MI, IT) was also used to stain nuclei.

3.12- Statistical analysis

All experiments were performed at least three times. Data are expressed as mean values ± standard deviation (SD). The statistical significance was analyzed using GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA). Normality was assessed using the Kolmogorov–Smirnov, D'Agostino & Pearson omnibus and Shapiro–

Wilks test, with a p value < 0.05. When the results satisfied the normality (Gaussian distribution and equal variance), unpaired t-tests with Welch correction or ANOVA (analysis of variance) with post hoc Bonferroni test were used. When data did not meet normality, the non-parametric Mann–Whitney's test (for independent comparisons), and Kruskal–Wallis test (for multiple comparisons) with post hoc Dunns test were used.

4- Results

4.1- Lipid metabolism sustains breast cancer aggressiveness

A first comparison between Tamoxifen-sensitive (WT) and Tamoxifen-Resistant (Tam-R) MCF-7 cells aimed to define differences in terms of proliferation rates in adherent and non-adherent (3D-spheroids) culture condition and cell migration. Tam-R cells manifest a more aggressive behavior, evidenced by increased ability to migrate (**Fig. 4.1A)** and increased efficiency to grow in non-adherent conditions, forming larger spheroids **(Fig. 4.1B**). This latter result indicate that Tam-R cells contain a larger number of stem-like cells, a feature of more aggressive tumors. Accordingly, when grown in adherent conditions Tam-R cells manifest a growth rate similar to WT cells when grown in the presence of serum, however, Tam-R have a better metabolic flexibility, that allow them to continue growing when switched to nutrient-depleted conditions (**Fig. 4.1C**).

Metabolic parameters were evaluated by Seahorse XF Analyzers, demonstrating that Tam-R cells display a higher energetic profile compared to WT cells (**Fig. 4.2**), a greater ability to adapt to stressful conditions, such as nutrient-depletion.

The more aggressive tumor cells survive under stressful conditions by using metabolic reservoirs where crucial energy-producing molecules are stored. Lipid droplets (LDs) represent long lasting energy deposits, where fatty acids (FA) and cholesterol are stored as triglycerides (TAG) and cholesterol esters (CE). WT and Tam-R MCF-7 cells were stained with a lipid-specific dye, Bodipy, for neutral lipids (NLs) (CE and TAG) and CE. Tam-R cells displayed a dramatic increase in LDs content compared to WT cells (**Fig. 4.3A, 4.3B**). Additionally, Bodipy staining for free fatty acids (FFAs), evidenced a similar pattern of distribution between the visualized cell lines (**Fig. 4.3C**). These results are in agreement with the literature data (Hultsch et al., 2018).

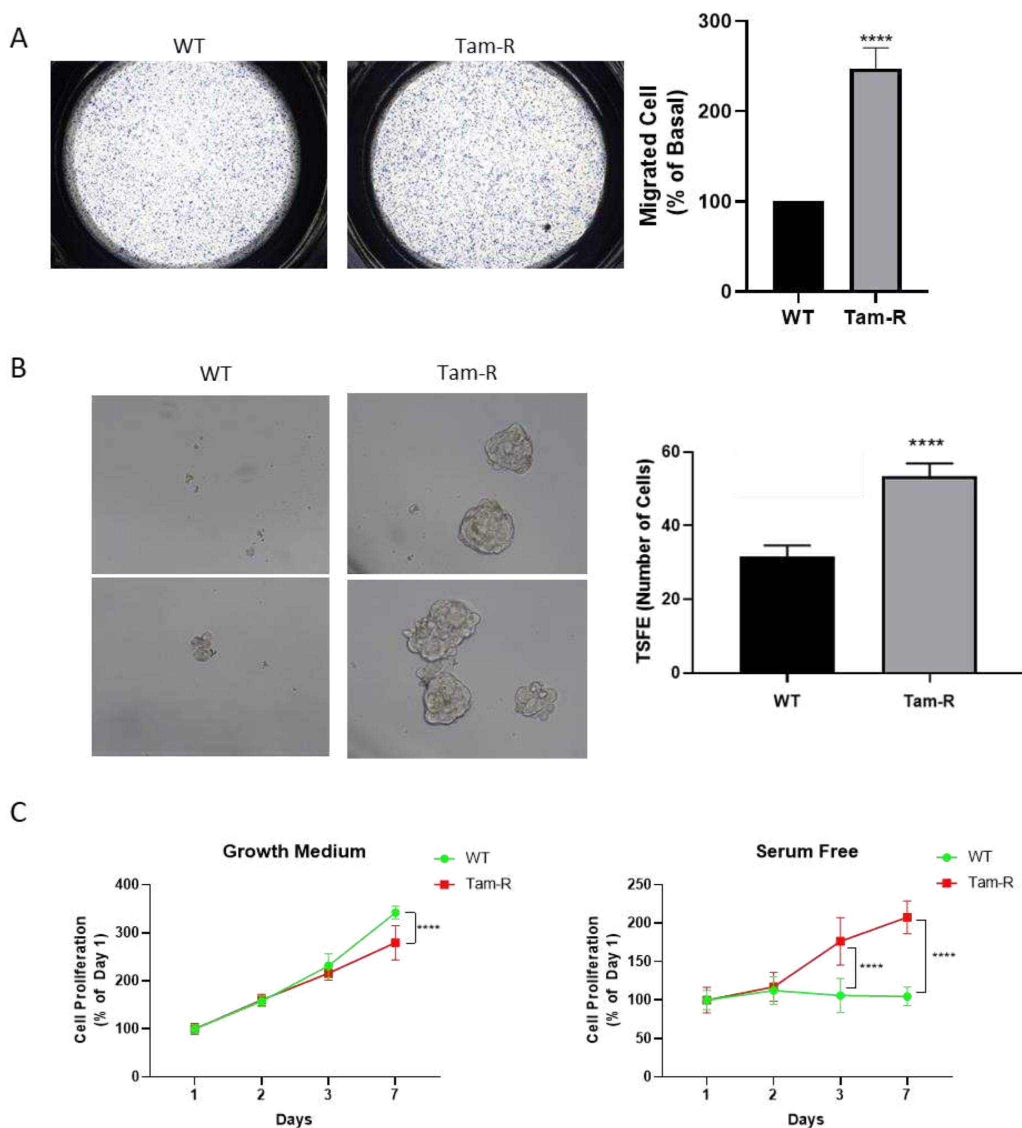

Fig. 4.1- Tamoxifen resistant BC cells have an aggressive phenotype and grow in nutrient-depleted conditions. **(A)** MCF7 WT and Tam-R migration evaluated by boyden chambers. Graph represents the number of migrated cells expressed ad % of WT cells. P value <0.0001. **(B)** Tumor sphere formation efficiency (TSFE) was evaluated for WT amd Tam-R cells over 5-days. Spheres >50 µm were counted using an eye piece graticule, the graph represents the average number of spheroids from 3 separate experiments. ****P <0.0001. **(C)** Time course proliferation assay for WT and Tam-R cells grown in complete medium or in serum-free medium. At the indicated times, proliferation was evaluated by CyQUANT proliferation Assay kit. For each cell line, the number of cells at day 1 was set as 100%. Graph is the average of data from three independent experiments. ****P <0.0001.

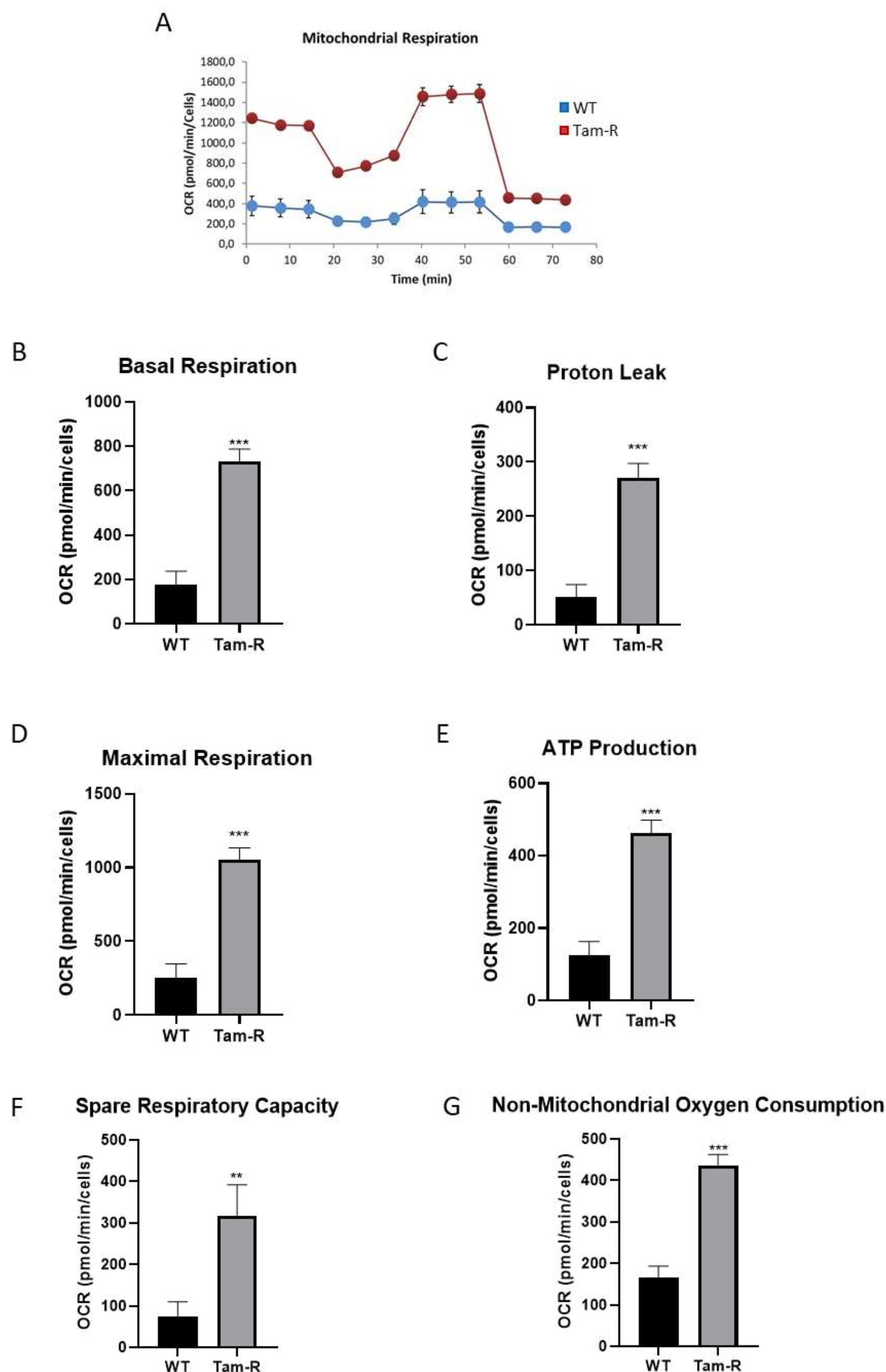

Fig. 4.2- Tam-R MCF7 cells display a higher energetic profile than Tam-sensitive cells. A) Real-time oxygen consumption rate (OCR) from MCF-7 WT and Tam-R assessed by Seahorse XF96 analyzer. **(B–G)** Bar chart showing mitochondria functional parameters: **(B)** Basal respiration; **(C)** Proton Leak; **(D)** Maximal respiration; **(E)** ATP production**; (F)** Spare capacity; **(G)** Non-Mitochondrial Oxygen Consumption. Graph is the average of data from three independent experiments. ** P <0.01. *** P <0.001.

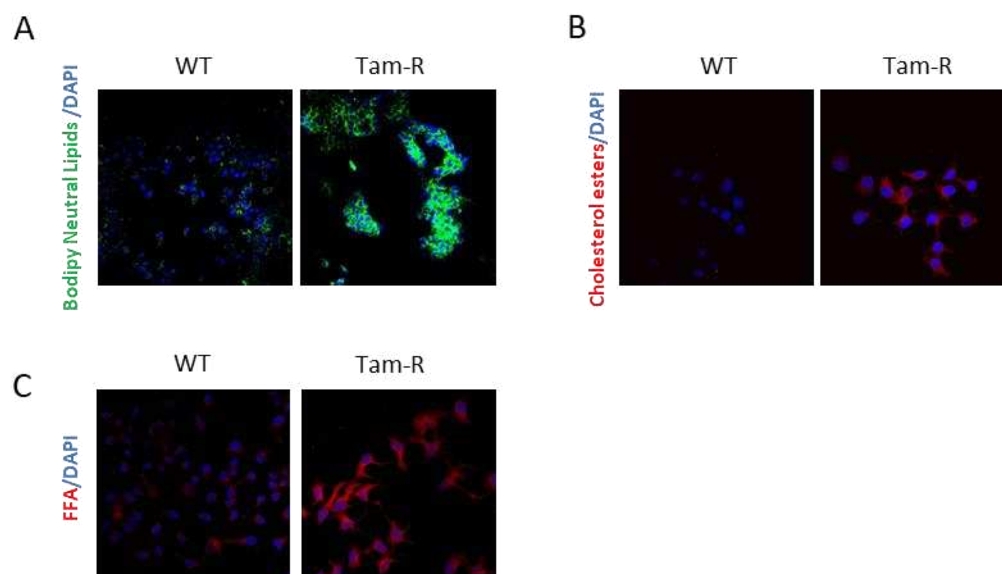

Fig. 4.3- Tam-R cells display high levels of lipid content. MCF-7 WT and Tam-R were plated in regular growth medium and 24h later were used to evaluate abundance of neutral lipids by BODIPY™ 493/503 **(A)**, cholesterol esters by CholEsteryl BODIPY™ FL C12 **(B)** and free fatty acids (FFA) by BODIPY™ 558/568 C12 **(C).** DAPI staining was used for nuclei detection.

Lipid droplets are ubiquitous in cells and have a unique ultrastructure, consisting of a core of neutral lipids surrounded by a phospholipid monolayer that presents integral and peripheral proteins. LDs are highly dynamic organelles, modifying their size and number in relation to fasting and growing conditions. These changes require a modulation of lipolysis, through lipases activity, or lipophagy, a selective form of autophagy. During extended periods of starvation, the lipids stored in LDs can supply membrane building blocks and serve as energy source. Lipid droplets also buffer cellular amounts of potentially toxic lipids and have prominent roles in preventing lipotoxicity and oxidative stress. During their life cycle, lipid droplets establish contacts with several other cellular organelles. First and foremost, they make contacts with the endoplasmic reticulum (ER), an organelle that supplies lipid droplets with most of their constituent molecules and has a central role in their biogenesis. Lipid droplets also make contacts with peroxisomes, mitochondria and lysosomes. All these interactions are tightly orchestrated and essential for the normal life cycle of lipid droplets and their diverse functions (Olzmann & Carvalho, 2019).

Interestingly, when Tam-R cells were cultured for 72 hours in serum-free medium a reduction in lipid droplets and FFA was observed (**Fig. 4.4A**), concomitant to the reported increase in cell proliferation (**Fig. 4.1C**). These data further confirm a greater metabolic adaptability of Tam-R cells, promoted by the large abundance of LDs, which fuel

bioenergetic and biosynthetic processes particularly in harsh conditions. Accordingly, the use of a specific inhibitor of mitochondrial FA oxidation (FAO), etomoxir, reverted Tam-R phenotype, forcing cells to behave and grow as WT cells (**Fig. 4.4B**).

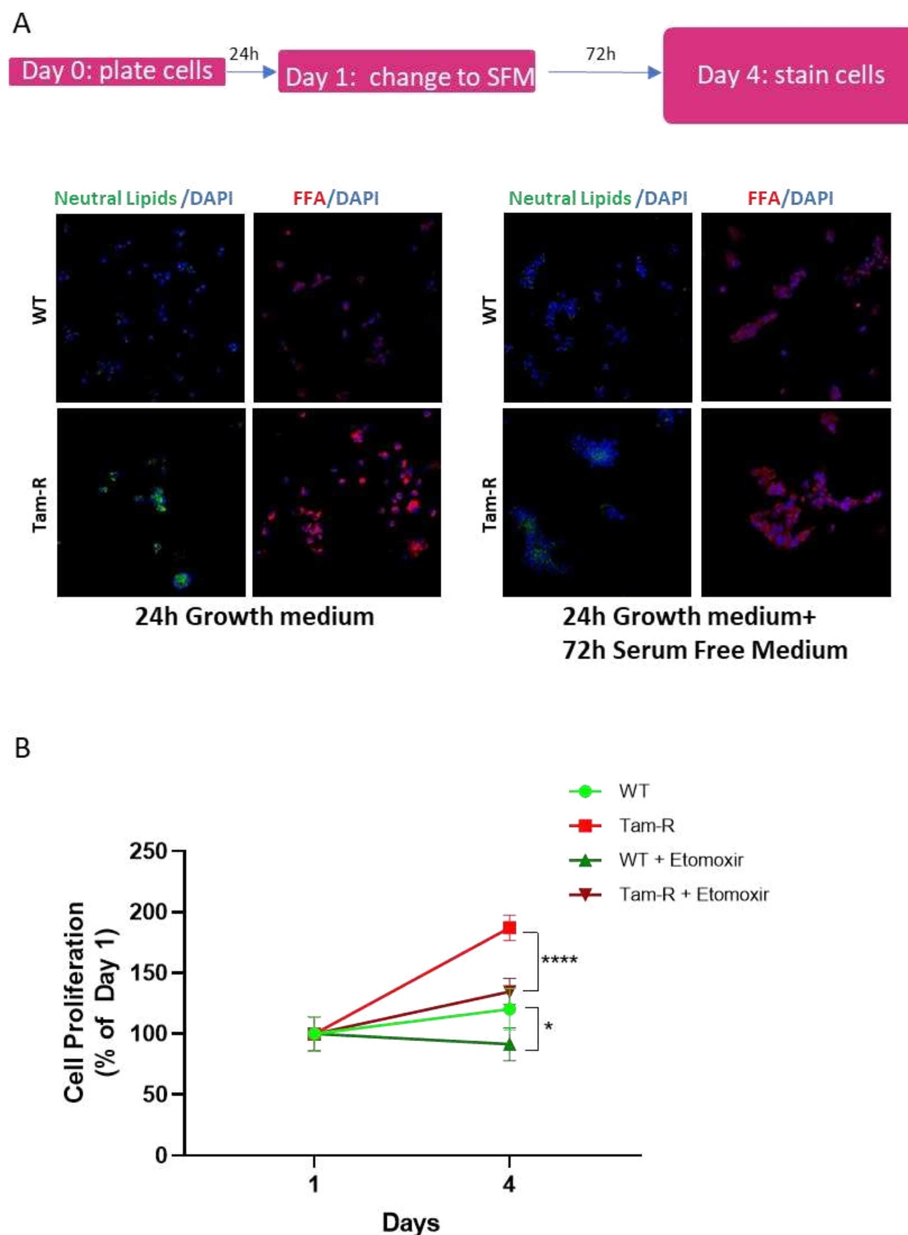

Fig. 4.4- LDs fuel bioenergetic processes and sustain proliferation particularly in harsh conditions in Tam-R cells. (A) MCF-7 WT and Tam-R cells were plated in regular growth medium and 24h later were stained with specific dyes for NL (BODIPY™ 493/503) and FFA (BODIPY™ 558/568 C12). A second set of cells were grown for 24h in growth medium and then switched to serum-free medium for additional 72h, and then stained with specific dyes for NL (BODIPY™ 493/503) and FFA (BODIPY™ 558/568 C12). DAPI staining was used for nuclei detection. **(B)** Cells plated in regular growth medium for 24h were switched to serum-free medium, and grown for 72 hours with or without Etomoxir (20 µM) as indicated. Proliferation was evaluated by CyQUANT proliferation Assay kit. For each cell line, the number of cells at day 1 was set as 100%. Graph is the average of data from three independent experiments. *P <0.05; ****P <0.0001.

4.2- The increased lipid content in Tam-R cells relies on uptake, de novo synthesis and reduced mobilization from cellular stores

Several mechanisms are behind cancer cell ability to increase their lipid content: uptake, de novo synthesis, reduced mobilization and catabolism. Key genes are involved in these mechanisms, and their expression was evaluated at both mRNA and protein levels. We found that Tam-R cells have higher protein expression of the two main lipoprotein receptors, LDL Receptor (LDLR) and Scavenger Receptor class B type 1 (SR-BI) **(Fig 4.5A,B)**. Furthermore, FA transporters such as SLC27A2 and SLC27A4 were found increased **(Fig 4.5C,D).** These transporters modulate the levels of long-chain fatty acids (LCFA) in the cell, by facilitating their import across cell membranes (Falcon et al., 2010). They also function as an acyl-CoA ligase, catalyzing the ATP-dependent formation of fatty acyl-CoA using LCFA and very-long-chain fatty acids (VLCFA) as substrates, preventing FA efflux from cells. Surprisingly, mRNA levels for these proteins evidenced opposite trends compared to protein expression, with LDLR, SR-BI and SLC27A2 downregulated (**Fig. 4.5E-G**) and only SLC27A4 upregulated (**Fig. 4.5H**). To further increase FA uptake, Tam-R cells have an increased expression of CD36 (**Fig. 4.5I**), which has been associated with lipid metabolism reprogramming, cancer cell proliferation, epithelial-mesenchymal transition, poor prognosis and reduced survival (Guerrero-Rodríguez et al., 2022). In addition we observed an increased mRNA expression of the microsomal enzyme acyl-coenzyme A:cholesterol acyltransferase-1 (ACAT1, also known as SOAT1) (**Fig. 4.5J**), which prevents the accumulation of excess unesterified, or free, cholesterol (Tabas, 2002), toxic for cells. This result is in accordance with CE abundance illustrated in **Figure 4.3C**.

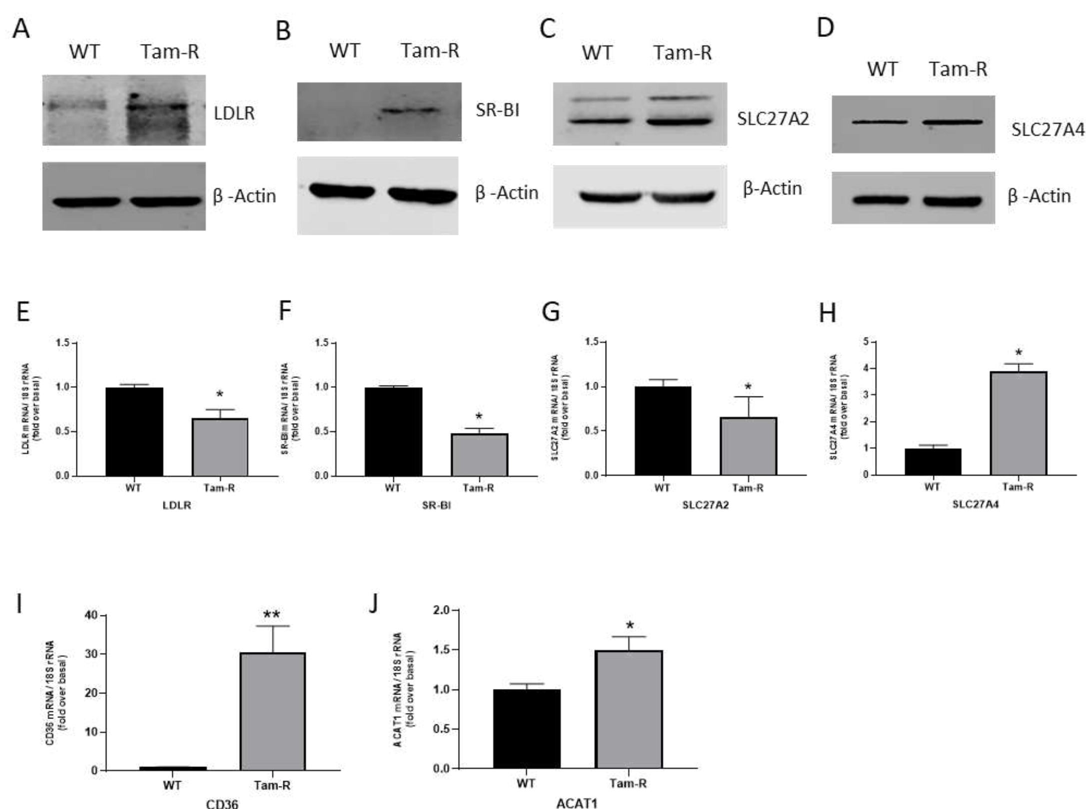

Fig. 4.5- Tam-R cells have high expression of lipids transporter. MCF-7 WT and Tam-R were plated in growth medium and 24h later assayed for mRNA and protein expression. Western blotting analysis for **(A)** LDL-R, **(B)** SR-BI, **(C)** SLC27A2, and **(D)**SLC27A4. β-Actin was used as a loading control. RT-PCR analysis for **(E)** LDLR, **(F)** SR-BI, **(G)** SLC27A2, **(H)** SLC27A4, **(I)** CD36, **(J)** ACAT1. Results are from at least three independent experiments. *P <0.05. **P <0.01.

Based on the increased expression of the studied genes, we evaluated the effect of specific inhibitors on cell proliferation. Specifically, we tested BLT-1 (SR-BI inhibitor) and Avasimibe (ACAT1 inhibitor) demonstrating that in Tam-R cells a significant inhibition was achieved by the lower doses of all inhibitors (**Fig. 4.6A,B**). These data highlight that in Tam-R cells the increased expression of the studied genes supports proliferative mechanisms. We next investigated the contribution of de novo lipogenesis (DNL) to the higher amounts of LDs observed in Tam-R cells (**Fig. 4.3**). The key enzymes involved in this process are Acetyl-CoA Carboxylase Alpha (ACCalpha), encoded by ACACA gene and responsible for the condensation of two molecules of acetyl-CoA into malonyl-CoA, and Fatty Acid Synthase (FASN), which catalyzes C16:0 palmitate synthesis. Analysis of protein (**Fig. 4.7A**) and mRNA expression (**Fig. 4.7B**) evidenced again opposite trends. While Protein levels were increased, mRNA was unchanged or down-regulated. Additionally, also ELOVL5 (Very long chain fatty acid elongase 5), the enzyme that catalyzes the first and rate-limiting reaction of the four reactions that constitute the long-

chain fatty acids elongation cycle necessary to elongate fatty acids, presents increased protein levels with no mRNA change (**Fig. 4.7C**).

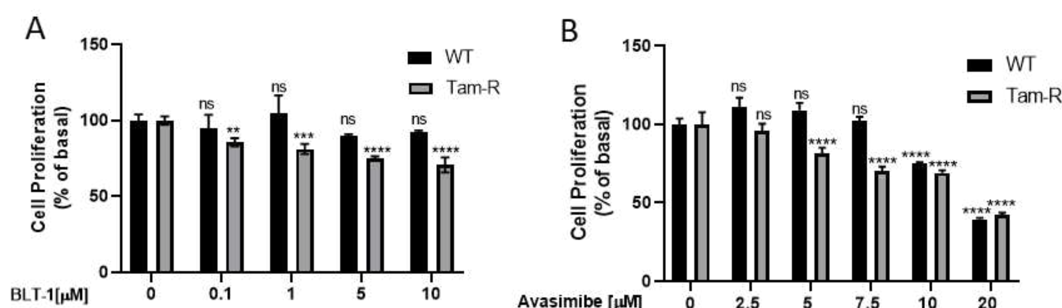

Fig. 4.6- Tam-R cells are more sensitive to BLT-1 and Avasimibe compared to WT. (**A, B**) MCF-7 WT and Tam-R were plated in growth medium and 24h later were treated with the indicated concentrations of BLT-1 (0, 0.1 μM, 1 μM, 5 μM, 10 μM) and Avasimibe (0, 2.5 μM, 5 μM, 7.5 μM, 10 μM, 20 μM). After 72h, proliferation was evaluated by CyQUANT proliferation Assay kit. Graphs are the average of data from three independent experiments. **P <0.01. *** P <0.001. **** P <0.0001.

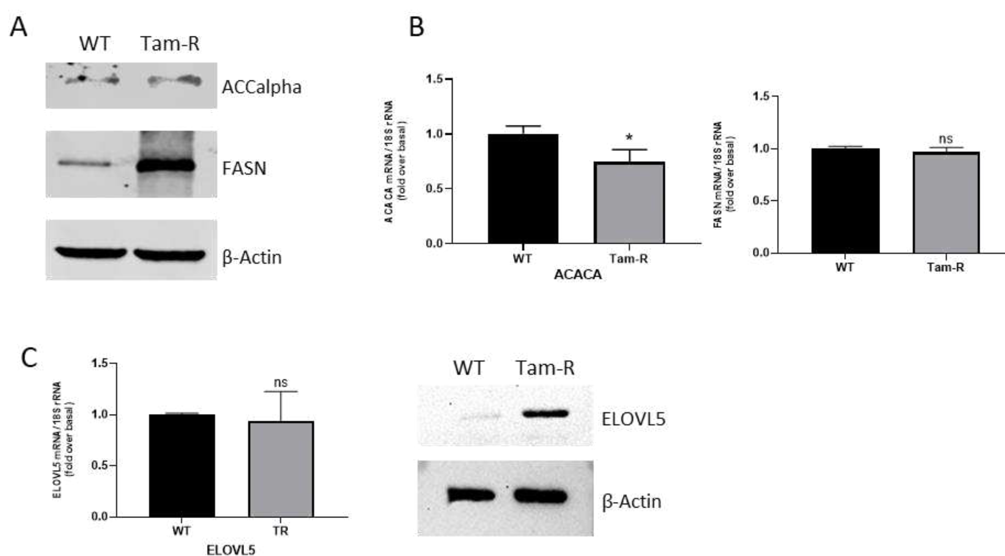

Fig. 4.7- Tam-R cells have high expression of protein involved in FAs synthesis. MCF-7 WT and Tam-R were plated in growth medium and 24h later assayed for mRNA and protein expression. **(A)** Western Blot analysis for ACCalpha and FASN. **(B)** RT-PCR analysis for ACACA and FASN. **(C)** RT-PCR and Western Blot Analysis for ELOVL5. Results are from at least three independent experiments. *P <0.05; **P <0.01; ns: not significant.

Potentially a reduced lipid mobilization and altered lipid metabolism could also contribute to the higher amounts of LDs (**Fig. 4.3**) and bioenergetics profile (**Fig. 4.2**) observed in Tam-R cells. Transcript and protein levels of Adipose triglyceride lipase (ATGL), involved in TAG hydrolysis, and its inhibitor G0S2 which prevents TAG

binding to ATGL, were increased in Tam-R cells (**Fig. 4.8A, 4.8B**), leading to the hypothesis that despite ATGL expression is higher, its activity is unchanged. Additionally, Carnitine Palmitoyl Transferase 1 (CPT1), representing the rate-limiting enzyme of the mitochondrial FAO was also upregulated in Tam-R cells (**Fig. 4.8C**), justifying the dramatic differences in OCR values between WT and Tam-R reported in the seahorse graph (**Fig. 4.2**).

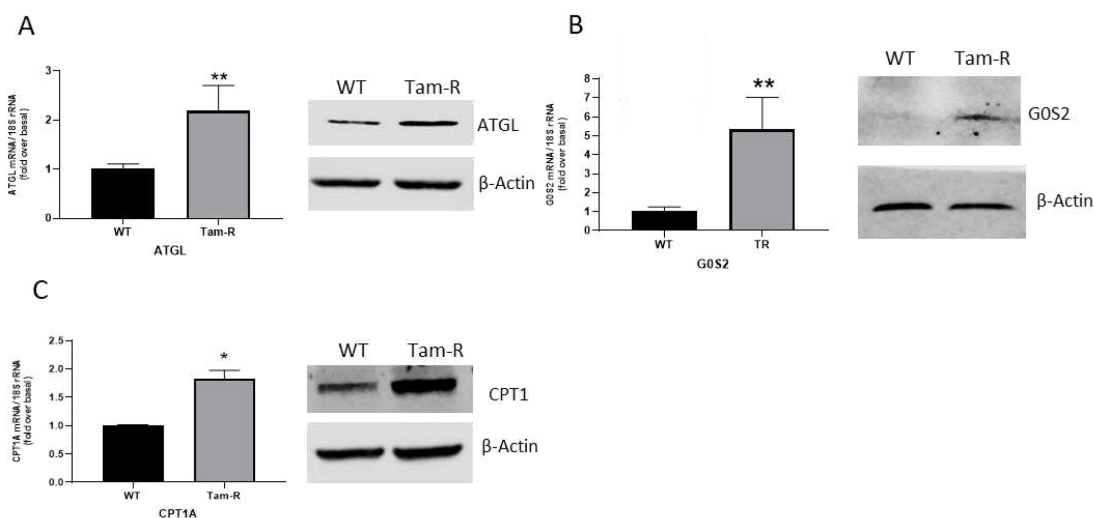

Fig. 4.8- Tam-R cells have higher expression of mitochondrial FAO enzyme than WT. MCF-7 WT and Tam-R were plated in growth medium and 24h later assayed for mRNA and protein expression. RT- PCR and Western Blot Analysis for **(A)** ATGL, **(B)** G0S2, and **(C)** CPT1. Results are from at least three independent experiments. *P <0.05; **P <0.01.

In addition to mitochondria, FAO can also occur in peroxisomes. The peroxisome is a single membrane-enclosed organelle that plays an important role in lipid metabolism, ensuring β-oxidation of very long chain fatty acids, α-oxidation of branched chain fatty acids, synthesis of bile acids and ether-linked phospholipids and removal of reactive oxygen species (Lodhi & Semenkovich, 2014). ATP Binding Cassette Subfamily D Member 3 (ABCD3) is involved in peroxisomal import of fatty acids and/or fatty acyl-CoAs, which undergo oxidation by Acyl-CoA Oxidase 1 (ACOX1), representing the rate-limiting enzyme of peroxisomal fatty acid β-oxidation pathway. ACOX1, catalyzes the desaturation of acyl-CoAs to 2-trans-enoyl-CoAs, donating electrons directly to molecular oxygen, thereby producing hydrogen peroxide (H_2O_2), an unstable molecule rapidly converted into H_2O and oxygen by catalase, a key antioxidant enzyme. Analysis of ABCD3, ACOX1 and Catalase expression evidenced a clear increase in protein levels, which are not paralleled by their transcripts (**Fig. 4.9**). More specifically, transcript levels

of ABCD3 were down-regulated, ACOX1 was modestly upregulated and catalase was unchanged. Once again evidencing a discrepancy between transcriptional and translational events.

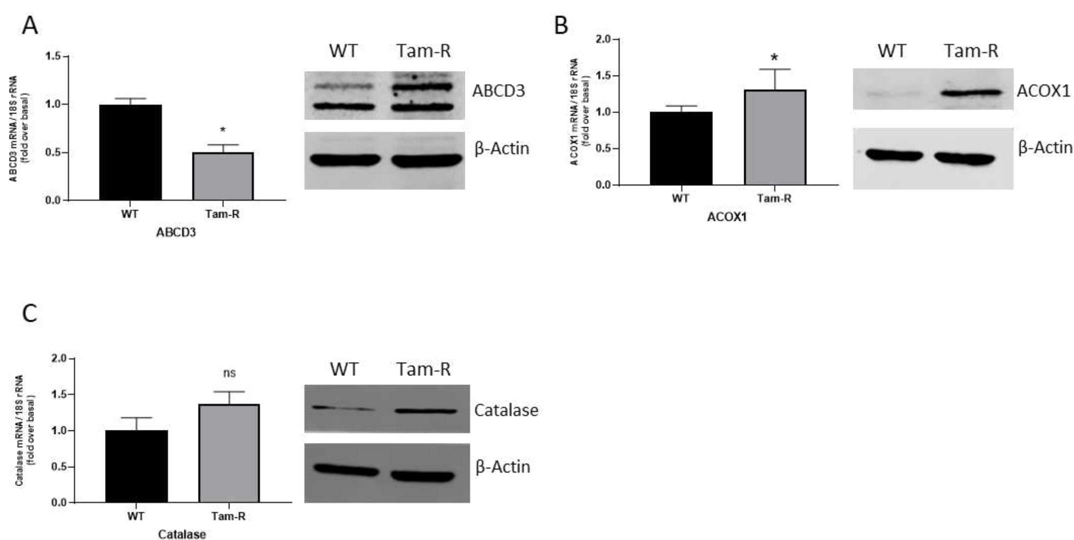

Fig. 4.9- Tam-R cells display high expression of peroxisomal protein involved in lipid metabolism. MCF-7 WT and Tam-R were plated in growth medium and 24h later assayed for mRNA and protein. RT-PCR and Western Blot analysis for **(A)** ABCD3, **(B)** ACOX1 and **(C)** Catalase. β-Actin was used as a loading control. Results are from at least three independent experiments. *P <0.05; ns: not significant.

To prove the relevance of lipid metabolism in sustaining Tam-R cells survival and growth, selective inhibitors for mitochondrial and peroxisome activity were used. Specifically, FAO inhibitors etomoxir, for CPT1, and thioridazine, for ACOX1, were used in cell viability and proliferation assays. Cell viability assay by MTT is based on the ability of cells to metabolize a specific substrate, the amount of product formed is tightly dependent on mitochondria health. Our data demonstrate that Tam-R cells are more sensitive than WT cells to FAO inhibition (**Fig. 4.10A**), and when both mitochondrial and peroxisome FAO are inhibited the effects on cell viability effect were more pronounced. Effects on cell proliferation paralleled data on cell viability (**Fig. 4.10B**). Accordingly, metabolic data evidenced that Tam-R cells are more sensitive to FAO inhibition, achieved by doses of Etomoxir and Thioridazine not influencing cell growth, and the most effective inhibition was reached with the combined treatment (**Fig. 4.10C-F**). Indeed, peroxisome FAO seems to provide a larger contribution to the energetic status of Tam-R cells, since thioridazine reduced all metabolic parameters to a higher extent than etomoxir (**Fig. 4.10C-F**). These data suggest the importance of further studying the contribution of cell

organelles in the establishment and maintenance of tamoxifen resistance.

Collectively, these data indicate that lipid metabolism plays a central role in maintaining features of aggressiveness in the endocrine resistant breast cancer phenotype. Identification of a master regulator will allow development of novel therapies aimed to prevent or reverse endocrine resistance.

4.3- Protein synthesis is mandatory to maintain high lipid content in Tam-R cells

A relevant observation coming from our experiments is that for many of the lipid metabolic genes the levels of transcripts did not always parallel protein expression. Considering the possible mechanisms that can justify the discrepancy between transcripts and proteins, we first evaluated the contribution of protein synthesis using cycloheximide, a specific inhibitor of eukaryotic translational elongation (**Fig. 4.11**). Our results show how protein synthesis plays a fundamental role in the regulation of key enzymes of lipid metabolism as the levels of proteins such as LDLR (**Fig. 4.11A**), SR-BI (**Fig. 4.11B**), FASN (**Fig. 4.11C**) and ACOX1 (**Fig. 4.11D**) are significantly decreased after treatment with Cycloheximide both in WT and Tam-R cells (with the exception of ACOX1 in the WT), although the relative levels of transcript are unchanged or even increased.

Protein synthesis requires a well-orchestrated contribution of translation factors operating in each step of the process: initiation, elongation and termination. Translation initiation, the rate limiting step in protein synthesis, is dependent on the activity of eukaryotic initiation factors (eIFs). Translation initiation in eukaryotes is the most highly regulated phase in the translation of most mRNAs, leading to the assembly of an elongation-competent 80S ribosome through the join of the large (60S) ribosomal subunits to the small (40S) ribosomal subunits with the Met-tRNAi positioned around the start codon. Initiation begins with the formation of the eIF2-GTP-Met-tRNAi ternary complex, which then assembles with the 40S ribosomal subunit, eIF1, eIF1A, eIF3 and probably eIF5 to form a 43S PIC (pre-initiation complex). Then the PIC is recruited to the 5’ end of mRNA, labeled with an inverted m7GpppN cap. Prior to the attachment of PIC to this mRNA region, mRNA needs to be unwound or activated by the eIF4F complex consisting of the cap-binding protein (eIF4Es), RNA helicase (eIF4A) and eIF4G with the assistance of eIF4B, eIF3 and polyA binding protein (PABP).

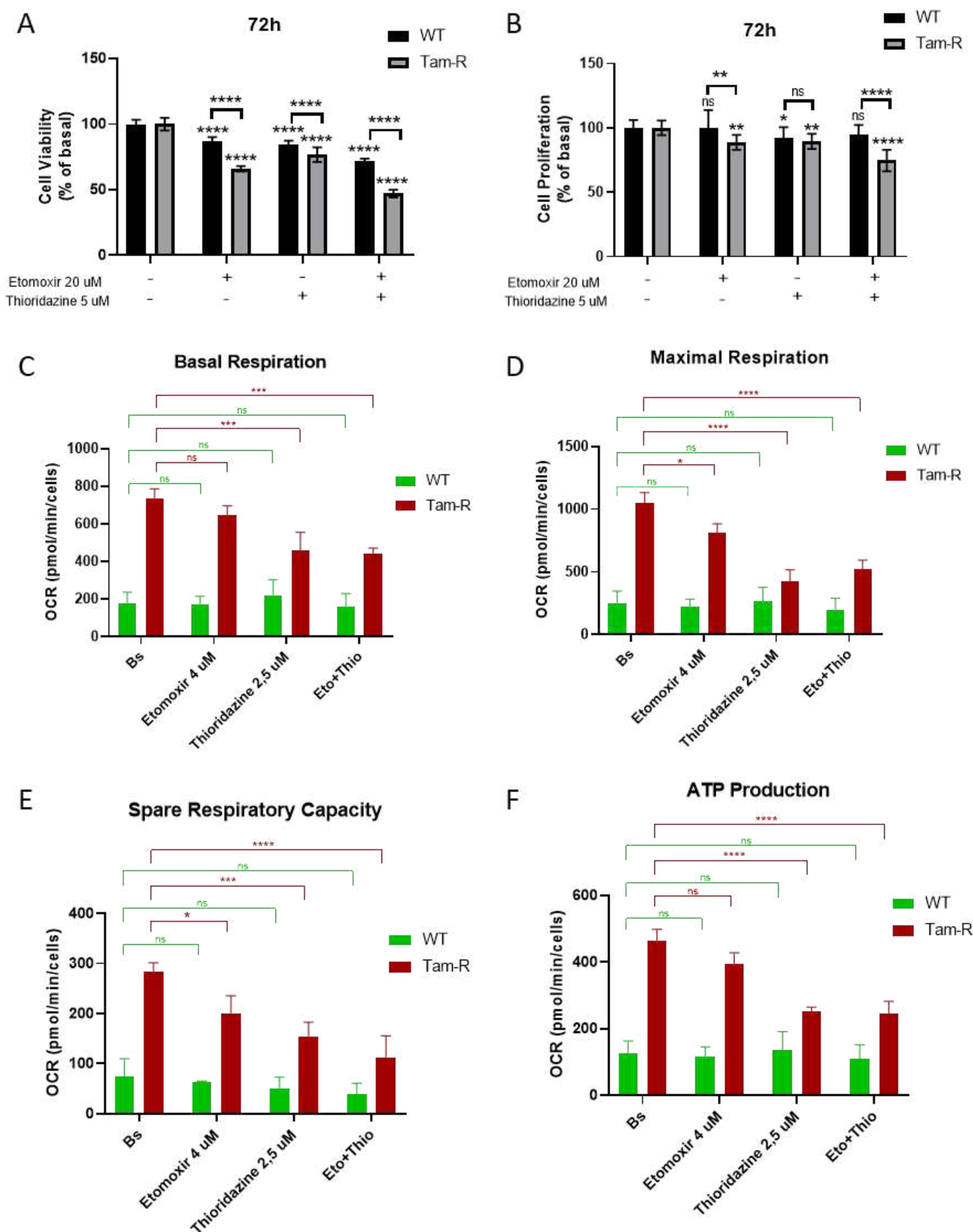

Fig. 4.10- Tam-R cells survival and growth is sustained by lipid metabolism. **(A,B)** MCF-7 WT and Tam-R were left untreated (Basal, Bs), treated with Etomoxir (20 µM), Thioridazine (5 µM) or combination of Etomoxir and Thioridazine for 72h. **(A)** Cell viability was evaluated by MTT assay and **(B)** Proliferation was evaluated by CyQUANT proliferation Assay kit. Graphs are the average of data from three independent experiments. **(C-F)** Real-time oxygen consumption rate (OCR) assessed by Seahorse XF96 analyzer from MCF-7 WT and Tam-R cells untreated (Bs), or treated for 16h with Etomoxir (4 µM), Thioridazine (2,5 µM) or combination of Etomoxir and Thioridazine. Bar chart showing mitochondria functional parameters: **(C)** Basal respiration; **(D)** Maximal respiration; **(E)** Spare Respiratory Capacity; **(F)** ATP Production. Graphs are the average of data from three independent experiments. * P <0.05. ** P <0.01. *** P <0.001. **** P <0.0001.

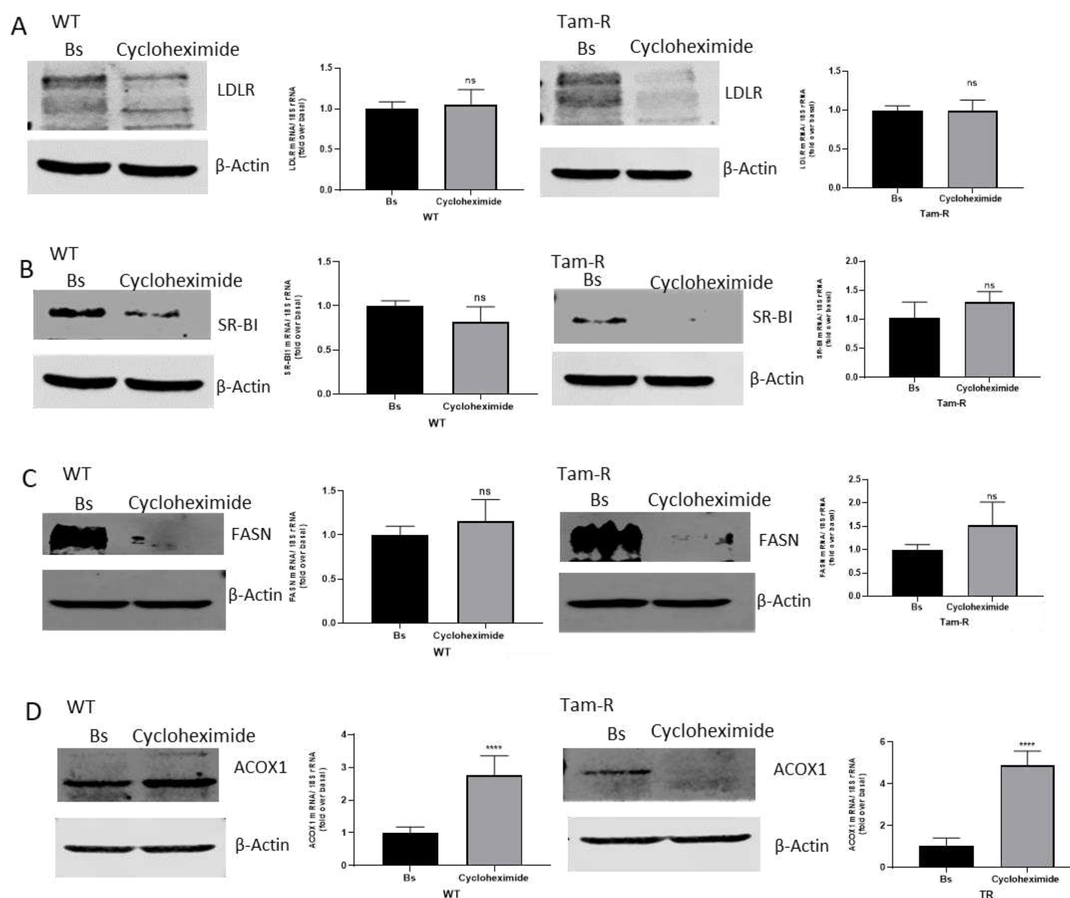

Fig. 4.11- Protein synthesis plays a key role in the expression of lipid metabolism enzymes. mRNA and protein expression were assessed for MCF-7 WT and Tam-R treated for 48h with Cycloheximide (35 µM). Western Blot analysis and RT-PCR for **(A)** LDLR, **(B)** SR-BI, **(C)** FASN, **(D)** ACOX1. β-Actin was used as a loading control. Data and images are from at least three independent experiments. **** P <0.0001.

The association of regulating factor eIF4B with eIF4A significantly enhances its helicase activity. Once attached to the mRNA, the 43S PIC is considered to scan on the 5'-untranslated region (5'UTR) in the 5' to 3' direction until the start codon is recognized and the 48S initiation complex formed. Once PIC recognizes the start codon, eIF1 is released, allowing the hydrolysis of eIF2-bound GTP and phosphate (Pi) release mediated by eIF5. These processes prompt the transition of PIC from an 'open' conformation to a 'closed' conformation, the latter stabilizing the interaction of PIC with mRNA. Then eIF5B-GTP collaborates with the eIF1A to assist in the formation of 80S ribosomal initiation complex through the recruitment of the 60S subunit to the 48S initiation complex, a process which is accompanied by the release of eIF1, eIF2-GDP, eIF3, eIF4B, eIF4F and eIF5. Subsequent hydrolysis of GTP by eIF5B and the displacement of eIF5B-GDP and eIF1A from assembled 80S ribosome make the complex ready to enter the

elongation phase of protein synthesis (Hao et al., 2020). As described above, mRNA 5'-end is recognized by eIF4E, which, in synergy with proteins such as the helicase eIF4A and the scaffolding protein eIF4G, allow the recruitment of ribosomes and translation initiation (Amorim et al., 2018). There are three different known cap-recognition factors: eIF4E, alias eIF4E1, eIF4E2 and eIF4E3.

An interesting study by Conn and colleagues (Conn et al., 2021) reported that eIF4E1 knock-out mice have enhanced metabolic fitness, preventing obesity while feeding a diet enriched in lipids. Importantly, the authors demonstrated that eIF4E1 is necessary for the translation of genes controlling lipid processing, transport, storage and LD growth. Specifically, among the protein regulated by eIF4E1, the authors found CD36 (fatty acid uptake), ACCalpha, FASN (Fatty Acid synthesis), ELOVL5 (Fatty Acid Elongation). Importantly, they found that eIF4E+/− livers are de-repressed for mitochondrial FAO (mFAO) and have an increased mitochondrial density. Related to FAO, authors demonstrated that the transport proteins for mFAO (CPT1, CPT2 and SLC25a20) remained unaltered, while one of the four main mFAO enzymes (Hsd17b10) was significantly increased. These data suggest an increase in lipid breakdown for energy, which is a feature of Tam-R cells. However, authors did not study the role of eIF4E1 in synthesis of enzymes for peroxisomal FAO (pFAO). This publication leaves open questions: can an alternative cap-recognition factor compensate for eIF4E1? In particular, can this alternative translation factor drive both mFAO and pFAO enzyme synthesis? Can this factor be involved in the lipid reprogramming observed in our model of Tam-R cells? We performed western blot analysis of eIF4E1, comparing WT and Tam-R cells, evidencing similar levels of protein expression (**Fig. 4.12A**); on the contrary we found that eIF4E2 is significantly overexpressed in Tam-R cells (**Fig. 4.12B**).

4.4- The translation initiation factor eIF4E2 drives lipid-related Tamoxifen resistant features of BC cells

To establish a role for eIF4E2 in the Tam-R BC phenotype, its gene expression was silenced in WT and Tam-R cells by using a specific sh-RNA. A significant downregulation of eIF4E2 level was achieved in both cell lines (**Fig. 4.12C**). Interestingly, cells with silenced eIF4E2 manifest an increased expression of eIF4E1 (**Fig. 4.12D**), suggesting that cap-binding factors must keep established levels of protein

synthesis or alternatively eIF4E2 is responsible for the translation of a selective inhibitor of eIF4E1 translation.

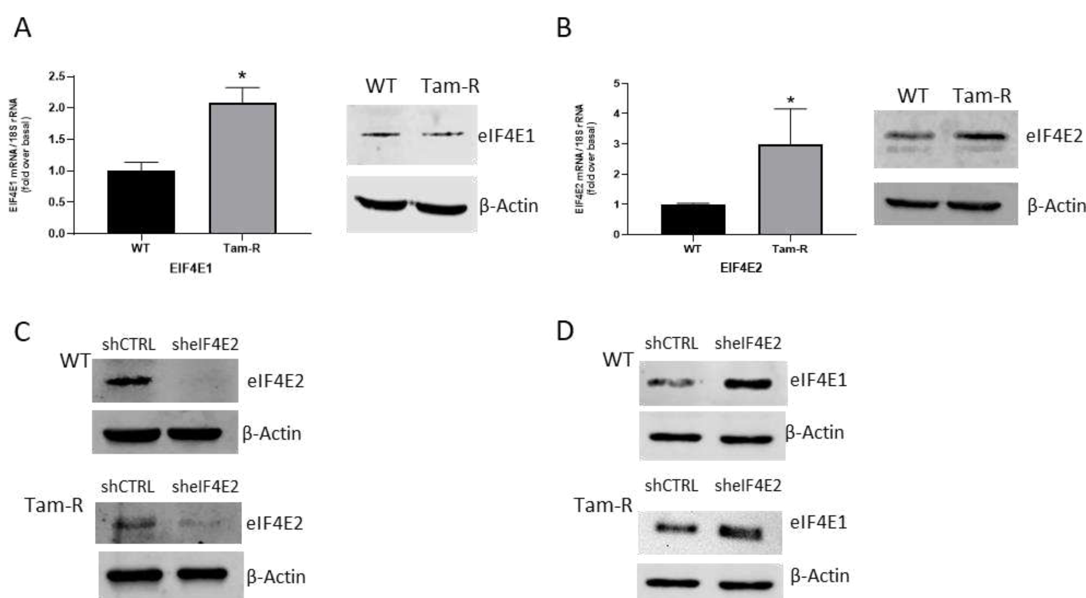

Fig. 4.12- Evaluation of the expression of eIF4E1 and eIF4E2 in MCF-7 WT and Tam-R and Silencing of eIF4E2. (**A,B**) MCF-7 WT and Tam-R were plated in regular growth medium and 24h later were lysed for mRNA and protein expression by real-time RT-PCR and Western blotting analysis. RT-PCR and Western Blot analysis in WT and Tam-R cells for **(A)** eIF4E1 and **(B)** eIF4E2. **(C,D)** MCF-7 WT shCTRL, WT sheIF4E2, Tam-R shCTRL and Tam-R sheIF4E2 were plated in regular growth medium and 24h later were lysed for protein expression by Western blotting analysis in order to evaluate the silencing of **(C)** eIF4E2 and **(D)** relative expression of eIF4E1. β-Actin was used as a loading control. Images are representative of data from three independent experiments. * P <0.05.

MCF-7 sheIF4E2 cells have a lower tumor-sphere forming efficiency (TSFE) in both cell lines (**Fig. 4.13A, B**). Moreover, Bodipy, for neutral lipids (NLs) demonstrated that eIF4E2 levels are determinant for the dramatic increase in LDs content observed in Tam-R cells, since Tam-R sheIF4E2 appear similar to WT shCTRL cells, meaning that they lost the ability to accumulate NLs (**Fig. 4.13C**). Evaluation of cell proliferation in serum-free conditions evidenced for sheIF4E2 cells a slower growth rate compared to Tam-R shCTRL cells, resembling WT shCTRL cells after 3 days of culture (**Fig. 4.13D**).

We then tried to understand if eIF4E2 is responsible for the upregulation of lipid-metabolism gene translation found in Tam-R cells (see **Fig. 4.5, 4.7-4.9**). Western blot analysis for SR-BI, FASN, ATGL and ACOX-1 demonstrated that their translation is strictly controlled by eIF4E2, since protein levels are decreased in both WT and Tam-R sheIF4E2 (Fig. **4.14B,C, D, F**). On the other hand, expression of SLC27A2 and CPT1 was further increased in Tam-R sheIF4E2 cells, but interestingly, their levels decreased in WT sheIF4E2 cells (Fig. **4.14A-E**). Immunofluorescence analysis of ABCD3 further

confirmed a decreased peroxisome activity in sheIF4E2 cells, in both WT and TR cells (**Fig. 4.14G**).

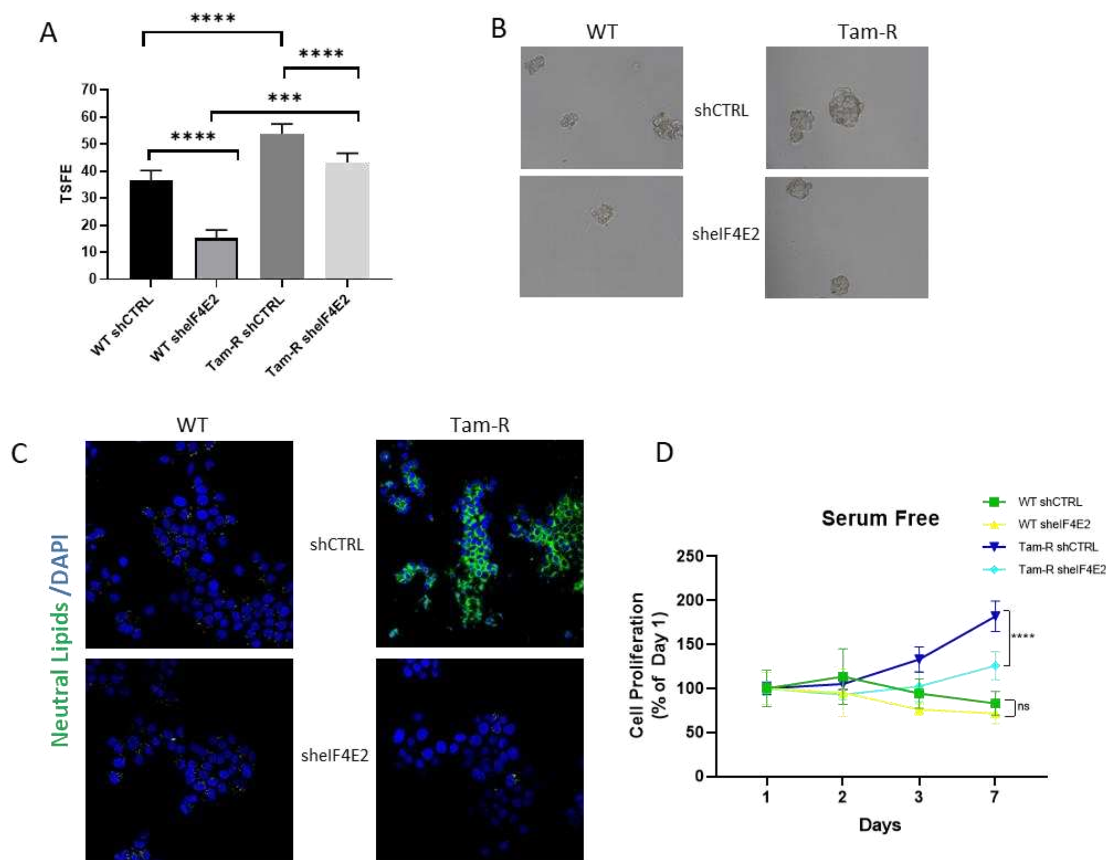

Fig. 4.13- Silencing of eIF4E2 disrupts lipid metabolism in BC cells. (A). Tumor sphere formation efficiency (TSFE) was evaluated for WT shCTRL, WT sheIF4E2, Tam-R shCTRL and Tam-R sheIF4E2 cells over 5-days, the graph represents the average number of spheroids from 3 separate experiments. **(B)** Spheres >50 µm were counted using an eye piece graticule. ***P <0.001;****P <0.0001. **(C)** MCF-7 WT shCTRL, WT sheIF4E2, Tam-R shCTRL and Tam-R sheIF4E2 cells were plated in regular growth medium and 24h later were stained with specific dyes for NL (BODIPY™ 493/503). DAPI staining was used for nuclei detection. **(D.)** Time course proliferation assay for WT shCTRL WTsheIF4E2, Tam-R shCTRL and Tam-R sheIF4E2 cells grown in serum-free medium. At the indicated times, proliferation was evaluated by CyQUANT proliferation Assay kit. Day 1 was set as 100% for each cell line. Graph is the average of data from three independent experiments. ****P value <0.0001; ns: not significant.

4.5- Briciclib a promising targeted therapy for Tam-R tumors

These data confirm that a subset of lipid metabolism genes is under the eIF4E2 translational control. In addition, the presented data lead to the hypothesis that eIF4E2 is a determinant factor in the establishment and maintenance of endocrine resistance,

through the modulation of peroxisome biogenesis and activity.

To further demonstrate that targeting eIF4E2 could represent a valid mean to counteract Tamoxifen resistance, a selective eIF4E2 inhibitor, briciclib, was tested on cell proliferation. Briciclib interfered with the proliferation of Tam-R cells, however, did not affect the growth of WT cells (**Fig 4.15**).

Western blot analysis of WT and Tam-R cells treated with briciclib confirmed that eIF4E2 inhibition causes a decrease in the expression of ABCD3 (**Fig. 4.16**).

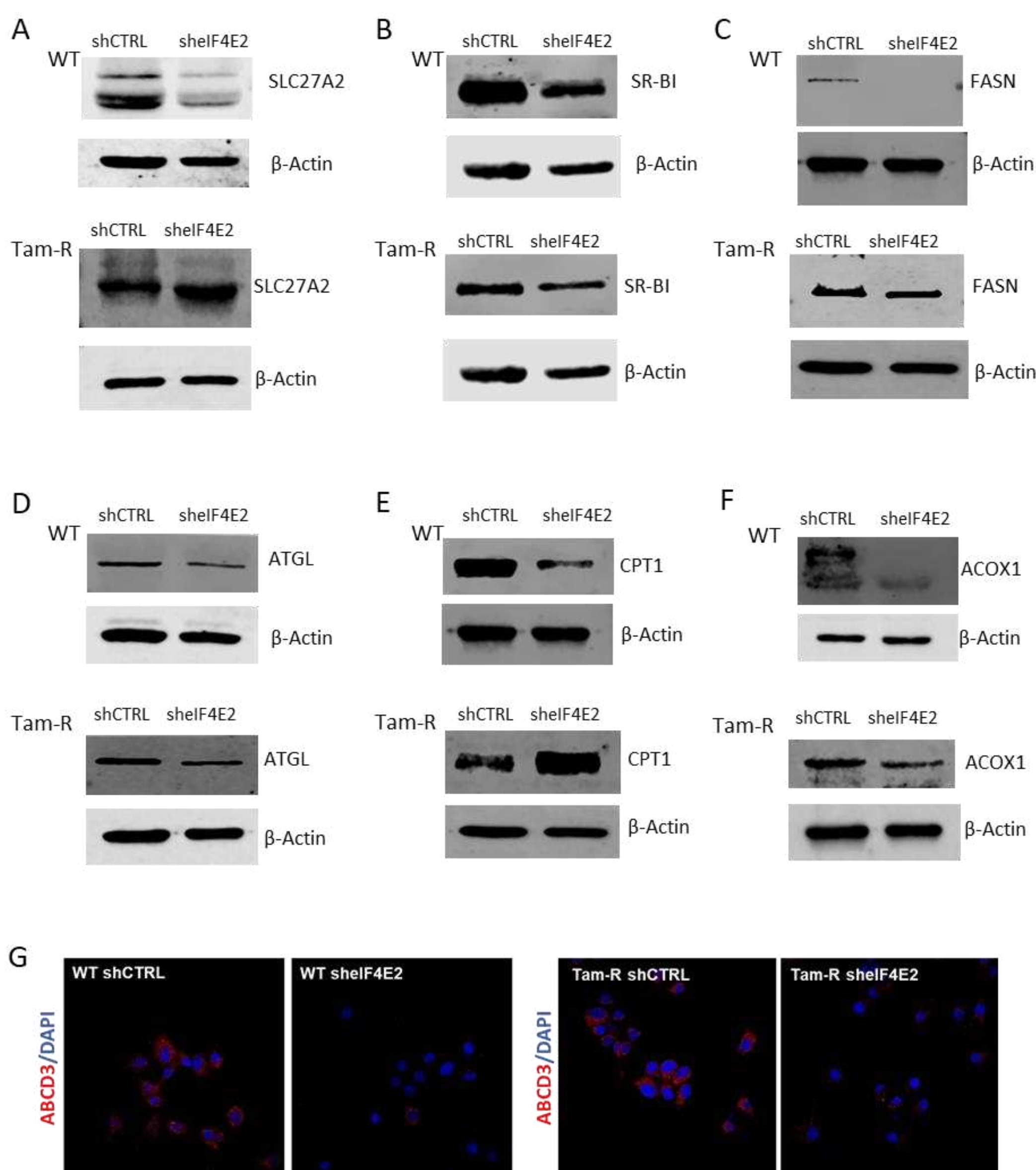

Fig. 4.14- eIF4E2 drives translation of many protein involved in lipid homeostasis. MCF-7 WT shCTRL, WT sheIF4E2, Tam-R shCTRL and Tam-R sheIF4E2 were plated in growth medium and 24h later were assayed for protein expression of **(A)** SLC27A2, **(B)** SR-BI, **(C)** FASN, **(D)** ATGL, **(E)** CPT1 and **(F)** ACOX1. β-Actin was used as a loading control. (**G**) MCF-7 WT shCTRL, WT sheIF4E2, Tam-R shCTRL and Tam-R sheIF4E2 were used for evaluating ABCD3 protein expression by immunofluorescence. DAPI staining was used for nuclei detection. Images are from at least three independent experiments.

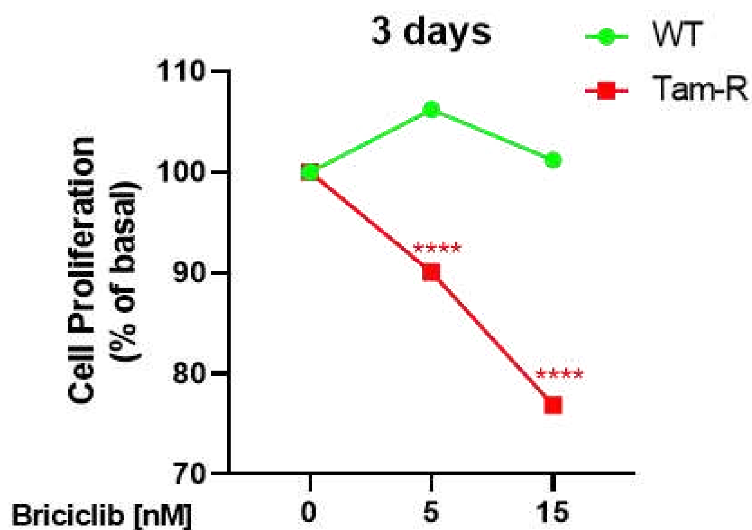

Fig. 4.15- Briciclib interfere with the proliferation of Tam-R cells but not affect the growth of WT cells. MCF-7 WT and Tam-R were plated in regular growth medium and after 24h treated with Briciclib (0, 5 nM or 15 nM). After 3 days of treatment, proliferation was evaluated by CyQUANT proliferation Assay kit. Graph is the average of data from three independent experiments. The untreated samples were set as 100% for each cell line. **** P <0.0001.

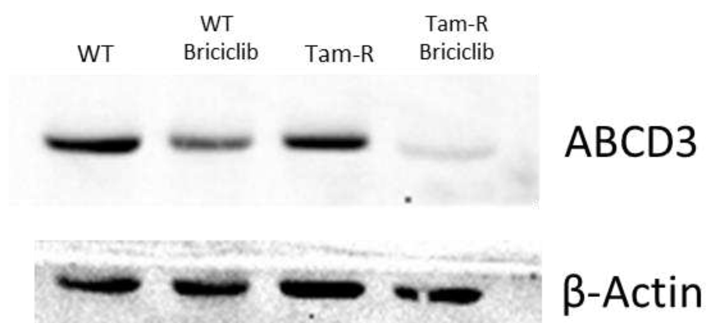

Fig. 4.16- eIF4E2 inhibition decreases the expression of ABCD3. MCF-7 WT and Tam-R cells were treated for 48h with Briciclib (5 nM) and ABCD3 protein expression was assessed Western blotting analysis. β-Actin was used as a loading control.

5- Conclusions

In the last years, metabolic reprogramming has emerged as a possible mechanism contributing to Tamoxifen resistance in Breast Cancer; in particular, lipid metabolism could be involved in the molecular alterations that confer resistance to Tam (Mishra et al., 2021).

Through this research project we have well characterized the differences in the lipid profile between MCF-7 WT and Tam-R cells once resistance is established, highlighting how these molecular differences lead to variations in the phenotypic behavior of Tam-R cells. We found that resistance to Tamoxifen leads to a more aggressive phenotype, with an increased ability of Tam-R cells to migrate, to grow in non-adherent conditions and to proliferate when switched to nutrient-depleted conditions.

These effects can be traced back, among other mechanisms, to greater metabolic flexibility and ability to store neutral lipid as Lipid Droplets (LDs). In fact, free fatty acids excess is packaged in LDs to avoid lipotoxicity, but can be used as an energy source to quickly respond to any condition of higher energy needs (Aon et al., 2014). Moreover, a high number of LDs containing cholesterol esters (as we observed in our model) is considered as hallmark of aggressive cancer (Beloribi-Djefaflia et al., 2016). In MCF-7 Tam-R cells, the accumulation of LDs appears to be responsible for the increased ability to survive under nutrient deprivation conditions through the mobilization of these reserves and the utilization of FAs through FAO. However, although this may offer an advantage in terms of survival, it could be exploited in therapeutic terms as the use of Etomoxir, an inhibitor of mitochondrial FAO, reduces the proliferation of Tam-R cells more than it does on WT. Furthermore, in addition to mitochondrial lipid metabolism, we observed that Tam-R also rely heavily on peroxisomal lipid metabolism. The peroxisome plays a key role in lipid metabolism, ensuring β-oxidation of very long chain fatty acids, α-oxidation of branched chain fatty acids, synthesis of bile acids and ether-linked phospholipids and removal of reactive oxygen species (Lodhi & Semenkovich, 2014). We discovered that thioridazine, a peroxisomal FAO inhibitor, is even more effective than etomoxir in reducing Tam-R cells proliferation. Indeed, peroxisomal FAO seems to provide a larger contribution to the energetic status of Tam-R cells, since in seahorse experiments, thioridazine reduced all metabolic parameters to a higher extent than etomoxir. These data suggest the importance of further studying the contribution of specific organelles in the establishment and maintenance of resistance to tamoxifen, and

in particular targeting the peroxisome, could represent a valid mean to counteract resistance.

Another relevant observation coming from our experiments was the opposite trend in mRNA and protein expression for many of the lipid metabolic genes. We found that protein synthesis is mandatory to maintain high lipid content in Tam-R cells, as the use of a protein synthesis inhibitor, Cycloheximide, downregulated many of the genes involved in the lipid reprogramming of Tam-R cells. This is in line with a recent study (Conn et al., 2021) demonstrating a key role of the translation initiation factor eIF4E1, in the control of lipid metabolism. eIF4E1 knock-out mice are resistant to high fat diet-induced obesity and maintain high levels of FAO genes, including CPT1, suggesting that eventually an alternative translation initiation factor could compensate for the lack of eIF4E1. In our study we observed that WT and Tam-R cells have similar protein levels of eIF4E1; while eIF4E2, an alternative cap-recognition factor, was significantly overexpressed in Tam-R cells. We questioned its involvement to sustain the increased expression of lipid metabolism observed in Tam-R cells, and therefore, decided to silence it expression by shRNA. We found that eIF4E2 drives the translation of many proteins responsible for the phenotypic features related to lipid metabolism observed in Tam-R cells. eIF4E2 can be targeted by a small molecule inhibitor, briciclib, which gives hope for a clinical translation of our in vitro data.

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