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Disclosing FATP1 as a therapeutic target in breast cancer

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Abstract

Breast cancer is the most frequent malignant neoplasia in women worldwide, with an estimated 1.7 million cases and 521,900 deaths in 2012, accounting for 25% of all cancer cases and 15% of all cancer deaths among females. In western countries, incidence rates have been declining, however, in Portugal breast cancer age-adjusted incidence rates have been increasing for years. Indeed, breast carcinoma is the leading cause of cancer mortality among Portuguese women, with an estimated 6088 new cases and 1570 deaths in 2012.

Cancer is a complex group of diseases in which several cellular and molecular components of the tumor microenvironment contribute to the survival of cancer cells. The non-neoplastic cells of the tumor microenvironment including fibroblasts, adipocytes, immune and endothelial cells are determinant in cancer biology since they act as a functional network in which soluble factors and organic molecules are transiently shared. To fulfill the biosynthetic demands associated with proliferation, a cell must increase the import of nutrients that sustain survival and fuel cell growth, supported by a metabolic remodeling. In a proliferative tumor niche, fatty acids (FA) are crucial since they are both fuel and construction blocks, maintaining cell renewal and division.

Conceptually, adipocytes are the main suppliers of FA, however our team published that cancer associated fibroblasts (CAFs) also function as hubs of FA to supply the needs of cancer cells. Breast cancer cells exposed to CAFs-conditioned media increased their lipid uptake and the expression of *FATP1/SLC27A1* (FA transport protein 1), promoting FA transfer. FATP1 is an integral membrane protein known to enhance the uptake of long-chain and very long-chain FA into cells. Considering FA transfer from CAFs to breast cancer cells, FATP1 appears to be a suitable candidate to treat breast cancer and a possible marker of disease outcome.

The present thesis aims to prove the role of FATP1 in breast cancer cells survival and behavior in order to validate its use as a therapeutic target. The main objective was divided in 3 specific aims: The first aim is to confirm that estrogen and FA regulate the expression of *FATP1/SLC27A1*, which is crucial for breast cancer cells. The second aim emerged from the evaluation of the results obtained in the first aim, and it is to evaluate the role of ER-β in *FATP1/SLC27A1* regulation. The third aim is to target FATP1 with arylpiperazines, which were characterized as effective inhibitors of FATP1, testing a putative therapeutic approach.

RQ-PCR and *western blotting* analysis showed that FA (linoleic acid (C18:2)) and estradiol stimulated *FATP1/SLC27A1* expression, especially in MDA-MB-231, which has higher basal levels of *FATP1/SLC27A1* mRNA in comparison with MCF7. Interestingly, we found that estradiol stimulated the binding of ER-β to the *FATP1/SLC27A1* promoter. These findings prompted us to investigate the role of ER-β in *FATP1/SLC27A1* regulation using an ER-β agonist, ERB-041 and an ER-β antagonist, PHTPP. Results revealed that PHTPP affects cell viability, the uptake of FA and increases the levels of FATP1 in the cell membrane in both cell lines. Also indicating that, besides ER-α, ER-β is a pro-survival effector in breast cancer cells and it should be tested as a prognostic marker. Wound healing and cell cycle analysis showed that FA and estradiol induced cell migration and proliferation.

Our *in vitro* findings were supported by patients' data showing a higher expression of *FATP1/SLC27A1* in more aggressive and invasive breast carcinomas. Furthermore, the inhibition of FATP1 with arylpiperazines interfered with the uptake of FA and cell proliferation, validating the importance of FATP1 as a putative therapeutic target in breast cancer.

Our study paves the path in order to elect FATP1 a suitable marker for breast cancer prognosis and a putative therapeutic target to better treat breast cancer patients.

Keywords: breast cancer, tumor microenvironment, cancer metabolism, fatty acids (FA), *FATP1/SLC27A1,* FATP1, metabolic fitness, ER-β, arylpiperazines.

Resumo

A neoplasia maligna da mama é a neoplasia mais frequente nas mulheres a nível mundial com cerca de 1.7 milhões de casos e 521,900 mortes em 2012, representando 25% de todos os casos de cancro e 15% de todas as mortes por cancro entre mulheres. Os países mais desenvolvidos são os que apresentam as maiores taxas de incidência enquanto que a mortalidade relativa é maior nos países menos desenvolvidos. A redução do uso de terapia de reposição hormonal na pós-menopausa e a participação em programas de rastreio através de mamografias baixaram as taxas de incidência nos países ocidentais, no entanto, em Portugal, estas taxas têm vindo a aumentar nos últimos anos. Entre as mulheres portuguesas o carcinoma da mama é a neoplasia maligna mais comum e a principal causa de morte por cancro, com 6088 novos casos estimados e 1570 mortes em 2012. Este aumento pode estar também relacionado com alterações nos padrões reprodutivos, obesidade e inactividade física. O cancro da mama é dividido em vários subtipos com características moleculares intrínsecas: luminal A, luminal B, sobre-expressão de HER2 e basal. Os tipos luminal A e B são caracterizados pela expressão de receptores hormonais, enquanto que os restantes subtipos são menos propensos a expressar o receptor de estrogénio (ER) ou de progesterona (PgR), sendo que o basal não expressa ER, PgR e HER2 (tumores triplo negativos). A heterogeneidade biológica bem como a plasticidade dinâmica do microambiente tumoral são importantes desafios no estudo e o tratamento do carcinoma da mama. Exemplo disso, é a importância de conhecer o *status* do RE e HER2, uma vez que constituem indicadores de prognóstico e alvos terapêuticos relevantes. A acção do estrogénio é mediada pela ligação a receptores de estrogénio (ER-α e/ou ER-β) que são expressos em vários tecidos. Actualmente, apenas o ER-α tem sido usado na clínica como marcador de prognóstico e alvo terapêutico devido à sua elevada expressão proteica em lesões mamárias malignas em comparação com o tecido normal. A administração de fármacos antagonistas de estrogénios, como o tamoxifen, é eficaz no tratamento de cancro da mama, no entanto, estes fármacos têm diversos efeitos secundários e após tratamentos prolongados os tumores podem tornar-se resistentes.

A doença oncológica é considerada mundialmente como um problema de saúde pública e é a segunda maior causa de morte em termos globais. A acumulação de alterações genéticas e epigenéticas permite às células escaparem à rede de controlo que regula o equilíbrio homeostático entre a proliferação celular e morte celular, levando a uma proliferação anormal de células. Uma neoplasia é considerada maligna se as suas células tiverem adquirido independência mitogénica e a capacidade de invadir os tecidos adjacentes. Durante a carcinogénese, 6 propriedades celulares fundamentais são alteradas: aumento da sinalização proliferativa, elevado potencial replicativo, insensibilidade aos sinais de anti-mitogénese, capacidade de invasão tecidular e metastização, angiogénese sustentada e resistência à apoptose. Estas capacidades biológicas adquiridas durante esta transformação gradual e complexa são as chamadas *hallmarks* do cancro que foram recentemente revisitadas, emergindo 2 novas *hallmarks*, a reprogramação do metabolismo energético e evasão ao controlo imunitário.

Para além das células tumorais, as células não malignas do microambiente tumoral, incluindo fibroblastos, adipócitos, células imunes e endoteliais são determinantes na biologia do cancro visto que actuam como uma rede funcional na qual factores solúveis e moléculas orgânicas são continuamente partilhados. Para cumprir os requisitos biossintéticos associados à proliferação, uma célula deve aumentar a importação de nutrientes que suportam a sobrevivência e alimentam o crescimento celular ocorrendo uma remodelação metabólica. Num nicho tumoral proliferativo, os ácidos gordos são essenciais visto que podem ser usados simultaneamente como combustível e como blocos de construção de forma a manter a renovação e divisão celular.

A desmoplasia é relativamente frequente em carcinoma da mama, apesar de não ser característica exclusiva, e consiste numa alteração estromal caracterizada pela presença de estroma fibrótico que tem como principal componente celular os fibroblastos. Para além das células tumorais, os fibroblastos associados a cancro (CAFS) são a componente principal do microambiente tumoral e pensa-se que eles desempenhem um papel determinante no início e na progressão da doença.

Conceptualmente, os adipócitos são os principais fornecedores de ácidos gordos, no entanto, o nosso grupo de investigação demonstrou, em modelos *in vitro* e *in vivo,* que os CAFs cooperam com células de cancro da mama ao produzirem e fornecerem ácidos gordos a estas. As células de carcinoma da mama expostas a meio condicionado por CAFs aumentavam a absorção de lípidos bem como a expressão de *FATP1/SLC27A1* (FA transport protein 1), promovendo a transferência de ácidos gordos. FATP1 é uma proteína membranar responsável pela absorção de ácidos gordos de cadeia longa e muito longa. A regulação da síntese, absorção e degradação de ácidos gordos é essencial para sustentar a fisiologia celular. Assim, limitar a disponibilidade de ácidos gordos às células tumorais de forma a perturbar o crescimento tumoral é uma possível abordagem terapêutica que poderá usar o FATP1 como alvo. Considerando a transferência de ácidos gordos dos CAFs às células de carcinoma da mama, o FATP1 parece ser um candidato adequado para tratar o cancro da mama e um possível marcador de prognóstico. Em 2013, foram desenvolvidos uns fármacos que foram caracterizados como inibidores eficazes do FATP1, as arilpiperazidinas.

Este projecto tem como objectivo demonstrar o papel do FATP1 na sobrevivência e comportamento das células de cancro da mama, de forma a validar o seu uso como factor de prognóstico e alvo terapêutico. Para atingir este objectivo foram delineados 3 objectivos específicos: o primeiro é confirmar que o estrogénio e os ácidos gordos regulam a expressão de *FATP1/SLC27A1* e que esta proteína é crucial para as células de carcinoma da mama; o segundo objectivo surgiu aquando da análise dos resultados obtidos no primeiro objectivo e consiste em esclarecer o papel do ERβ na regulação de *FATP1/SLC27A1*, e o terceiro objectivo é testar a inibição de FATP1 recorrendo à exposição a arilpiperazidinas 5k e 12a na tentativa de delinear uma possível estratégia terapêutica.

Os resultados de RQ-PCR e *western blotting* mostraram que os ácidos gordos (ácido linoleico (C18:2)) e estradiol estimulam a expressão de *FATP1/SLC27A1*, especialmente nas MDA-MB-231 que possui níveis basais mais elevados de mRNA *FATP1/SLC27A1* em comparação com MCF7. Curiosamente, observámos que o estradiol estimula a ligação do ER-β ao promotor do *FATP1/SLC27A1*. Isto levou-nos a investigar o papel do ER-β na regulação do *FATP1/SLC27A1* utilizando um agonista do ER-β, o EPB−041 e um antagonista do ER-β, o PHTPP. Os resultados revelaram que o PHTPP afecta a viabilidade celular, a absorção de ácidos gordos e aumenta os níveis de FATP1 na membrana celular em ambas as linhas celular indicando que para além do ER-α, o ER-β actua como um factor de pro-sobrevivência em células de carcinoma da mama e deveria ser investigado como eventual marcador de prognóstico. Observámos igualmente que os ácidos gordos e o estradiol induzem a migração e a proliferação celular. Os resultados *in vitro* estão de acordo com dados de doentes que mostram uma maior expressão de *FATP1/SLC27A1* em carcinomas da mama mais agressivos e invasivos. Por conseguinte, a inibição do FATP1 com as arilpiperazidinas interferiu com a absorção de ácidos gordos e com a proliferação celular validando a importância do FATP1 como um potencial alvo terapêutico em cancro da mama.

O nosso estudo inicia um percurso de investigação que acreditamos que permitirá eleger o FATP1 como um marcador de mau prognóstico do cancro da mama e um potencial alvo terapêutico.

Palavras chave: cancro da mama, microambiente tumoral, metabolismo tumoral, ácidos gordos, *FATP1/SLC27A1*, FATP1, adaptação metabólica, ER-β, arilpiperazidinas.

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List of abbreviations, acronyms and symbols

AA - Antibiotic-antimycotic solution
ACP - Acyl carrier protein
ACS1 - Acyl-CoA synthetase
AKT - Protein kinase B
ATCC - American Type Culture Collection
ATP - Adenosine Triphosphate
ALCY - ATP-citrate lyase
BSA - Bovine serum albumine
cAMP - Cyclic adenosine monophosphate
CAAs - Cancer Associated Adipocytes
CAFS - Cancer associated fibroblasts
cDNA - Complementary DNA
ChIP - Chromatin immunoprecipitation
CO_2 - Carbon dioxide
CoA - Coenzyme A
CREB – Responsive element binding protein
ddH2O – Sterile ultra-pure water
DMEM – Dulbecco's modified Eagle media
DNA – Deoxyribonucleic acid
dNTPs – Deoxynucleotides
DTT – Dithiothreitol
ECM – Extracellular matrix
ER - Estrogen receptor
EREs - Estrogen receptor elements
ERα – Estrogen receptor α
ERβ – Estrogen receptor β
ESR1 – Estrogen receptor 1
ESR2 – Estrogen receptor 2
FA – Fatty acids
FABP - FA Binding Protein
FASN - FA synthase
FATP - FA transport proteins
FADH2 - Flavin adenine dinucleotide
FAT - Fatty-acid translocase
FBS – Fetal bovine serum
G0 – Cell cycle "resting phase"
G1 – Gap 1 cell cycle phase
G2 – Gap 2 cell cycle phase
GEO – Gene Expression Omnibus
GLUT1 – Glucose transporter 1
GLUT3 – Glucose transporter 3
GLUT4 – Glucose transporter 4
GPER - G protein-coupled estrogen receptor 1
HER-2 – Human epidermal growth factor receptor 2
HIF-1α - Hypoxia inducible factor-1 alpha
HPRT – Hypoxanthine-guanine phosphoribosyltransferase
HRP – Horse raddish peroxidase
HSP – Heat shock protein
IDC – Invasive ductal carcinoma
IL-6 – Interleukin-6
IGF - Insulin Growth factor
IL-10 - Interleukin-10
LDH - Lactate dehydrohgenases
LCFA - Long-chain fatty acids
MAPK – Mitogen-activated protein kinase

min – Minutes
MCTs - Monocarboxylate transporters
mRNA – messenger ribonucleic acid
mTOR – Mammalian target of rapamycin
NADPH - Nicotinamide adenine dinucleotide phosphate
NF-kB – Factor nuclear kappa B
ON – Overnight
OS – Overall survival
OXPHOS – Oxidative phosphorylation
PBS – Phosphate buffered saline
PCR - Polymerase chain reaction
PgR – Progesterone receptor
PI – Propidium iodite
PI3K – Phosphatidylinositol 3-kinase
PKA - protein kinase A
PS – Phosphatidyl serine
qRT-PCR – Quantitative real time polymerase chain reaction
Rev – Reverse
RFS – Relapse free survival
RIPA – Radio-Immunoprecipitation Assay
RNA – Ribonucleic acid
ROR - Registos Oncológicos Regionais
ROS – Reactive oxygen species
RQ-PCR – Relative quantifying polymerase chain reaction
S – Synthesis cell cycle phase
RT – Room temperature
RT-PCR – Reverse transcription polymerase chain reaction
SLC27 - Solute carrier family 27
TCA cycle – Tricarboxylic acid cycle
TGF-β – Transforming growth factor-β
TGS – Tris-glycine-sodium dodecyl sulfate
TGs - triacylglycerols
TAMs - Tumor associated macrophages
VEGF - Vascular Endothelial Growth Factor

1. Introduction

1.1 Cancer Biology

Cancer is a major public health problem worldwide and is the second leading cause of death globally [1]. The accumulation of genetic and epigenetic changes allows cells to escape the network of controls that regulate the homeostatic balance between cell proliferation and cell death, leading ultimately to an abnormal division of cells [2]. A tumor is considered cancer only if it is malignant, that is, only if its cells have acquired the ability of dividing relentlessly and invade surrounding tissues [3]. These features of malignancy drive cancer cells to break loose, enter blood or lymphatic vessels and form secondary tumors, called metastases, at other sites in the body.

During carcinogenesis, six fundamental cellular properties are altered: sustaining proliferative signaling, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis and cell death resistance [2]. These biological capabilities acquired during this multistep transformation are the so-called hallmarks of cancer that were recently revisited, emerging two new hallmarks, reprogramming of energy metabolism and evading immune destruction [4]. Beside cancer cells, the non-neoplastic cells of the tumor microenvironment including stromal and endothelial cells are determinant in cancer biology since they act as a functional network in which soluble factors and organic molecules are transiently shared [5].

1.2 Breast cancer

Breast cancer is the most frequent malignant neoplasia in women worldwide, with an estimated 1.7 million cases and 521,900 deaths in 2012, accounting for 25% of all cancer cases and 15% of all cancer deaths among females [6]. The same authors described that breast cancer in more developed countries represents one-half of all breast cancer cases and 38% of deaths. Northern America, Northern and Western Europe countries have higher incidence rates, while relative mortality is greatest in less developed countries [6,7,8]. Breast incidence rates vary internationally as a result of the differences in the availability of early detection as well as risk factors including reproductive and hormonal factors [9,10]. Reductions in the use of postmenopausal hormone replacement therapy and plateaus in participation of mammography screening led to declining incidence rates in western countries [11–13], however, in Portugal breast cancer age-adjusted incidence rates have been increasing for years (ROR-Sul, 2015). Indeed, breast cancer is the most common cancer and the leading cause of cancer mortality among Portuguese women, with an estimated 6088 new cases and 1570 deaths in 2012 [14]. This increasing trend might be explained by changing reproductive patterns, obesity and physical inactivity [10].

Additionally, although therapeutic approaches such as surgery, chemotherapy, radiation and endocrine therapy have reduced mortality rates, there are still many therapeutic failures resulting in cancer recurrence, metastasis and death. Metastatic breast carcinoma remains an incurable disease by current treatment strategies, accounting for 90% of breast cancer mortality [15]. More recently, the potential harms of screening mammography have been discussed and it has been shown that this method may cause false-positive results, overdiagnosis and overtreatment of some breast carcinomas [6,16,17]. In fact, women with large breasts may be at higher risk of radiation-induced breast cancer, however, the benefits of mammography outweigh these risks [18].

Breast cancer is not a single disease process but rather a compilation of several and unique subtypes as defined via gene expression analysis [19]. Microarray techniques have divided breast cancer into various intrinsic subtypes: luminal A, luminal B, HER2-enriched and basal. Luminal A and B are characterized by the expression of hormone receptor-related genes while both HER2-enriched and basal subtypes are less likely to express Estrogen Receptor (ER) or Progesterone Receptor (PgR). Regarding

prognosis, tumors of the ER positive luminal subtype have been shown to exhibit a better outcome as well as higher relapse-free and overall survival time comparing with those of the ER negative basal subtype [20]. The morphological heterogeneity of breast carcinoma constitutes the basis for the histopathological classification of breast cancer. The WHO (world health organization) currently recognizes the existence of at least 18 distinct histological types of invasive breast cancer, however the large majority (50-80%) are invasive ductal carcinomas (IDC) [21]. The biological diversification of breast cancer phenotypes accompanied by dynamic plasticity of the tumor microenvironment make tumor categorization an imperative task since it relates to therapeutic responses and disease progression [22].

1.2.1 Estrogen receptors in breast cancer

Besides being essential for normal growth and differentiation in the mammary gland, estrogens play a major role in promoting the proliferation of neoplastic breast epithelium [23]. High levels of serum estrogen correlate with an increased risk of breast cancer [24]. Estrogen action is mediated by binding to estrogen receptors (ER-α and/or ER-β) which are expressed in a number of tissues, forming functional homodimers or heterodimers. ER-α and ER-β are encoded by distinct genes located on different chromossomes, respectively, chromosome 6 (*ESR1*) and chromosome 14 (*ESR2*), and act as transcription factors after estrogen binding [25].

The classical pathway of estrogen action states that in the absence of estrogen, ER binds to Heat-Shock Proteins (HSP 50, 70 and 90), however, when estrogen is present, the HSPs are liberated from the ligand-receptor complex, inducing a conformational change in the receptor and promoting dimerization. ER, as a homodimer, subsequently binds to estrogen receptor elements (EREs) in the regulatory regions of target genes to either activate or repress gene expression [26].

Presently, only ER-α has been used in a clinical setting, since its protein levels are elevated in malignant breast lesions compared with normal tissue, allowing its use as a predictive, prognostic marker and a therapeutic target. Administration of estrogens antagonists, such as tamoxifen, is effectively useful in the treatment of breast cancer [27]. However, these drugs have unwanted side effects in non-target tissues and after prolonged treatment, tumors become resistant [28]. Only few studies have investigated the potential prognostic role for ER-β in breast cancer until now. Speirs et al., found ER-β mRNA as being significantly upregulated in tamoxifen-resistant breast cancers compared with tamoxifen-sensitive tumors, suggesting a link between ER-β overexpression and tamoxifen resistance. On the other hand, further studies reported that high expression of ER-β protein was associated with a good outcome in breast cancer patients treated with tamoxifen [30]. Accordingly, ER-β prognostic and predictive value remains controversial, however, targeting this receptor in some cases could offer new treatment options for breast cancer patients where previously only aggressive chemotherapies were available [31].

1.3 Cancer metabolism

Although cancer metabolism is one of the oldest areas of research in cancer biology, the study of metabolic alterations in tumors has grown exponentially over the past decade [32,33]. To fulfill the biosynthetic demands associated with proliferation, a cell must increase the import of nutrients such as glucose and glutamine that support survival and fuel cell growth [34].

The link between cancer and metabolism was first made by Otto Warburg in 1923 with the observation that most cancer cells predominantly produce energy through a high rate of glycolysis followed by acid lactic fermentation, rather than through oxidative phosphorylation (OxPhos) in the mitochondria [35]. Thus, tumors exhibit an increased rate of glucose uptake with lactate production, even in the presence of oxygen, through aerobic glycolysis with the production of carbon skeletons, NADPH and ATP [36]. Warburg thought cells would prefer fermentation as a source of energy instead of the more

efficient OXPHOS- the Warburg Effect. However, this view was challenged as several studies found that defects of mitochondrial OXPHOS are not common in spontaneous tumors and the function of mitochondrial OXPHOS in most cancers is intact [37–39].

One of the most frequently altered pathways in cancer is the PI3K-Akt-mTOR signaling pathway that contributes to the uptake and utilization of several nutrients including glucose, glutamine, nucleotides and lipids [34], some of them are used in OXPHOS instead of glucose in highly glycolytic cells. Monocarboxylate transporters (MCTs) and lactate dehydrogenases (LDH) allow the production and export of lactate after glycolysis and subsequently the import and its conversion into pyruvate to supply tricarboxylic acid (TCA) cycle and OXPHOS for energy and biomass production [40–43]. Glutamine, an important metabolic substrate and energy source for tumor cells, is converted to glutamate and channeled into the TCA Cycle as α-ketoglutarate, accounting for ATP and carbon skeleton production, essential for amino-acid, nucleotide and lipid biosynthesis [44]. Glutamine can be converted to citrate, by the reversal of the Krebs cycle reactions, which in turn can be used for the production of acetyl-groups for fatty acids (FA) synthesis [45,46,47]. An increased rate of lipid synthesis in malignant tissues has also been recognized as an important aspect of the rewired metabolism of transformed cells[48].

1.3.1 Fatty acids (FA) metabolism and cancer

Synthesis of fatty acids (FA) is a fundamental cellular process, which converts nutrients into metabolic intermediates for membranes biosynthesis, energy storage and the production of signaling molecules [49]. Being cellular proliferation the main characteristic of all cancers, FA are crucial in the microenvironment since they can be used as fuel and construction blocks, maintaining cell renewal and division [50,51]. FA are composed by a terminal carboxyl group and a hydrocarbon chain, occurring frequently in even numbers of carbons which can be saturated or unsaturated [45].

While most normal human cells prefer exogenous sources, tumors generate almost all their cellular FA through *de novo* synthesis [52]. The metabolic intermediate that provides the substrate for the synthesis of FA is cytoplasmic acetyl-CoA that can be produced through different ways. Glucose or glutamine generates citrate that is cleaved by ATP-citrate lyase (ALCY) to oxaloacetate and acetyl-CoA, here ALCY is phosphorylated and activated by AKT [53,54]. Then, acetyl-CoA is carboxylated to malonyl-CoA and condensed by FA synthase (FASN), attaching them to acyl carrier protein (ACP) to generate subsequentially palmitate. Palmitate is a 16-carbon saturated FA (16:0) that is elongated to produce other molecules of various lengths. Together with the uptake of essential FA from the environment, they form a pool of intracellular FA that can be used for the generation of triacylglycerols (TGs) for energy storage, glycerophospholipids and sphingolipids for membrane synthesis and eicosanoids for signaling processes [49]. FA are sequentially degraded to acetyl-CoA, which can subsequently be oxidized by the TCA cycle to produce ATP in a process called mitochondrial β-oxidation. In this process, NADH and FADH2 are used by the electron transport chain to generate ATP for many cellular mechanisms [55].

1.3.2 Fatty acids (FA) synthesis

The regulation of the synthesis, uptake and degradation of FA is essential for sustaining cellular physiology and perturbation of the processes controlling lipid provision can impair cell survival. Limiting FA availability to cancer cells in order to disturb tumor growth is therefore a potential therapeutic target in cancer. Studies have proposed the inhibition of FASN as a strategy to treat cancer, being some inhibitors evaluated in preclinical trials [56–59]. However, FASN inhibitors have several side effects [60] and FASN was shown to be essential for adult neuronal stem cell function, raising additional concerns [61]. Another issue is the potential metabolic flexibility of cancer cells which could lead to adaptation and resistance, for example, cancer cells could switch from *de novo* FA synthesis to FA

uptake in the presence of FASN inhibitors [49]. Hence, the design of new soluble drugs that can be applied systemically is needed. The role of FA transporters in the control of cellular FA uptake under normal conditions and their implication in metabolic alterations occurring in cancer suggest that the blockade of these transporters could also work as an attractive therapeutic approach.

1.3.3 Fatty acids (FA) transporters

In terms of FA transport, it is acknowledged that membrane-associated FA-binding proteins, known as FA transporters, facilitate the cellular entry of FA across the cell membrane instead of passive diffusion [62]. To date, four groups of FA transporters have been identified: Plasma Membrane FA Binding Protein (FABP) family, FA transport proteins (FATP), FA translocase/CD36 and Caveolin-1 [63]. Although these proteins are commonly referred as "FA transporter" little is known about their exact biological functions and mechanisms by which each one of these proteins participate in the transport process across the cell membrane [63–66]. These proteins differ in molecular mass and degree of post-translational modifications, some have characteristic patterns of tissue distribution and others are expressed ubiquitously [63].

Given their important roles in FA transport, the FATP proteins have been considered as viable targets to prevent aberrant accumulation of fat in cells and tissues leading to lipotoxic disease [67]. In particular, FATP1 was chosen as a therapeutic target for the treatment of insulin resistance and several arylpiperazines were selected as inhibitors by high throughput screening [68].

1.4 Metabolic remodeling in breast cancer

Metabolic reprogramming is one of the emerging hallmarks of cancer [4,5] and it is well acknowledged that cancer cells have a vast metabolic plasticity in order to support continuous cell growth and proliferation meeting their energetic and biomass demands [41]. The metabolic adaptation of tumor cells not only allows the development and establishment of a tumor in a certain microenvironment but also influences the response to therapy [69]. Metabolic remodeling in the tumor niche is not exclusive to cancer cells, since stromal and endothelial cells share the same microenvironment [69].

Therefore, the microenvironment surrounding cancer cells cannot be ignored as normal cells contribute for this adaptive process by sharing soluble factors and organic molecules that support cellular viability and proliferation [55]. Indeed, a tight metabolic synergy occurs between epithelial cancer cells and stromal fibroblasts in which the normal cells act as suppliers of energy sources and precursors for macromolecules synthesis [41,70]. The microenvironment is composed of both cellular (fibroblasts, adipocytes, endothelial and immune cells) and non-cellular components (proteins, proteases, cytokines - the extracellular matrix (ECM)) which synergistically play a role in cancer progression [69,71].

Desmoplasia, a tissue phenomenon characterized by the presence of a fibrotic rich stroma around a tumor has long been associated with a poor clinical outcome in patients with cancer [72]. In desmoplastic tissues, an increased proliferation of fibroblasts takes place causing the deposition of fibrotic stroma surrounding neoplastic cells. Although this stromal alteration is not an exclusive characteristic of breast cancer, it is rather frequent [50,73].

Thus, besides cancer cells, cancer associated fibroblasts (CAFS) are the predominant cellular component of the tumor microenvironment, being thought to promote cancer initiation, progression and therapeutic response [74]. In comparison with normal fibroblasts within the microenvironment, the higher proliferative rate of CAFs is sustained by increased glycolysis, glutaminolysis, autophagy, ketogenesis and FA synthesis [41,50,75–78].

In breast carcinoma, tumor cells induce the down-regulation of caveolin-1, a known breast cancer stromal biomarker, reprogramming normal fibroblasts to CAFs concomitant with autophagic/lysosomal degradation [72]. Hence, CAFs are reprogrammed by cancer cells and tumor

microenvironment [69]. In the tumor niche, to sustain the metabolic demands there is a glycolytic switch in CAFs induced by a decreased oxygen availability, Reactive Oxygen Species (ROS) disequilibrium, activity of growth factors (TGF-β, IL-6) and hypoxia inducible factor-1 alpha (HIF-1α) stabilization [79–81]. It has been shown that CAFs isolated from breast cancer patients with poor prognosis express high levels of glycolytic enzymes and that the increased glycolytic rate in CAFs results in an enriched lactate microenvironment being lactate further imported and catabolized by cancer cells [82,83]. Lactate which is converted to pyruvate provides a mechanism for tumor growth as these metabolites can enter into the TCA cycle in cancer cells which are using oxidative metabolism promoting efficient energy production and higher proliferative capacity [83]. There is also the activation of GPER/cAMP/PKA/CREB and PI3K/AKT signaling pathways that fuel mitochondrial biogenesis and oxidative metabolism of cancer cells [41,83,84].

Autophagy is a survival mechanism in which cells degrade and recycle their own structural components to obtain sufficient energy to stay alive at critical times, however if it lasts too long cells will undergo cell death [85]. Autophagy seems to play a key role in supporting CAFs phenotype as active suppliers of energy sources for cancer cells: autophagic CAFs favor the pro-tumorigenic nutrient-rich microenvironment and tumor cells, in nutrient detriment or energy restraint, can be fed by products resulting from autophagic CAFs [69,86,87].

In breast cancer, adipocytes are an essential source of FA for cancer cells [88]. Cancer Associated Adipocytes (CAAs) have an important role in the tumor microenvironment and exhibit phenotypical and biological changes that support cancer demands. CAAs secret adipokines such as leptin and Insulin Growth factor (IGF) that act on cancer cells activating tumor progression and metastasis [89,90]. Other relevant intervenients are the tumor associated macrophages (TAMs) which are associated with tumor initiation, growth, invasion and metastasis. TAMs secrete Tumor Growth Factor B (TGFβ) and Interleukin-10 (IL-10) that stimulate cancer cells to switch to a metastatic phenotype [91]. Both CAAs and TAMs are responsible for the release of Vascular Endothelial Growth Factor (VEGF) stimulating angiogenesis [41,92,93]. Blood vessel supply is crucial for tumor survival not only by providing oxygen and nutrients for anabolism but also by removing waste products from cellular metabolism, contributing for the metabolic adaptation of the tumor [94].

1.5 Metabolic crosstalk between breast cancer cells and CAFs mediated by FA Transporter 1 (FATP1)

Conceptually, the only hub of lipids within the tumor microenvironment is adipocytes. However, recently, our team [50] demonstrated that CAFs cooperate with breast cancer cells as FA producers and suppliers, in *in vitro* and *in vivo* models. Breast cancer cells exposed to CAFs-conditioned media increased their lipid uptake and the expression of FATP1, promoting FA transfer. These results disclose, for the first time, FATP1 as a putative target to suppress lipid symbiosis between CAFs and breast cancer cells, thereby disturbing tumor progression.

FA transport proteins or solute carrier family 27 (SLC27) form a highly conserved family of six integral membrane proteins FATP1-6 [95]. FATPs enhance the uptake of long-chain and very long-chain FA into cells and are differentially expressed in a tissue-specific manner [63]. FATP1 is expressed in different tissues such as brain, kidney, lung, skin, adipose tissue, heart and skeletal muscle. It is encoded by the *SLC27A1* gene and has approximately 63 kDa. Interestingly, FATP1 is an insulin-sensitive transporter as it was reported that FATP1 translocates from an intracellular compartment to the plasma membrane in response to insulin, in adipocytes and skeletal myocytes [96].

FA are thought to be transported by FATP1 across the membrane and activated by plasma membrane acyl-CoA synthetase (ACS1) to form acyl-Co esters. The rapid esterification of FA to CoA thioesters by ACS1 is a process known as vectorial acylation [97]. FATPs function have been shown to be determinant in metabolic disease context, motivating the development of drugs to abrogate the transport

of FA across cell membranes [68,98]. Drugs targeting ACS1 which is associated to FATP1 were developed but showed reduced efficacy [50,98]. In a tumoral context, a possible role of FATPs in tumor metabolism has been described, demonstrating that *FATP1/SLC27A1* mRNA expression was increased in rat hepatomas in comparison with normal liver tissue which correlated with FA uptake rates [55,99,100]. FATP1 expression is elevated in adipose tissue of obese patients, as well as in the heart, skeletal muscle and adipose tissue of mice models of obesity [55], consequently, it is possible that this protein might have a role in obesity-sensitive cancers. As mentioned before, FATP1 is an insulin-sensitive transporter which fundaments the use of anti-diabetic drugs in the treatment of breast cancer that is described in some studies. Epidemiological and clinical studies showed that metformin, a drug prescribed for type 2 Mellitus diabetes, reduces the risk of cancer in patients with diabetes and improves prognosis and survival rate of cancer patients [101].

1.6 Arylpiperazines as a therapeutic approach in breast cancer

Considering FA transfer from CAFs to breast cancer cells, FATP1 appears to be a suitable candidate to treat breast cancer and a possible marker of disease outcome. In 2013, arylpiperazines were pointed out as effective inhibitors of FATP1[68]. Through a derivatization process, arylpiperazines derivatives 5k and 12a were identified as maintaining potent chemical inhibitory effect against human and mouse FATP1 and also showed excellent pharmacokinetic properties. Although in this study arylpiperazines were tested for the treatment of insulin resistance, they could perfectly be applied in a breast cancer context. Moreover, these compounds are soluble and can be applied systemically which make them suitable to be used in cell culture. The destabilization of the metabolic crosstalk between CAFs and breast cancer cells, in order to inhibit tumor growth, could be achieved with these drugs. Therefore, the utilization of arylpiperazines in *in vitro* models of breast carcinoma might be a good strategy to test this innovative therapeutic approach in breast cancer.

2. Hypothesis and aims of the project

Our hypothesis is that FATP1 is crucial for breast cancer cells biology. To accomplish the main objective of this thesis, which is to prove the role of FATP1 in breast cancer cells survival and behavior in order to validate its use as a therapeutic target, this study was divided in three aims. The first aim is to confirm that estrogen and FA regulate the expression of *FATP1/SLC27A1* and that this protein is crucial for breast cancer cells. The second aim emerged from the evaluation of the results obtained in the first aim, and it is to evaluate the role of ER-β in *FATP1/SLC27A1* regulation. The third aim is to target FATP1 with arylpiperazines, testing a putative therapeutic approach.

3. Materials and methods

3.1 Cell lines and culture conditions

Human cell lines from triple-negative breast cancer, MDA-MB-231 (HTB-26™, ATCC) and from luminal-A breast cancer, MCF7 (HTB-22™, ATCC) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained at 37°C in a humidified environment of 5% CO2 in Dulbecco's modified essential medium 1X (DMEM) (41965-039, Gibco, Life Technologies) containing 4.5g/L of D-glucose and 0.58 g/L L-glutamine. Medium was supplemented with 10% fetal bovine serum (FBS; S 0615, Merck), 1% Antibiotic-Antimycotic (AA; P06-07300, PAN Biotech) and 50 µg/mL Gentamicin (15750-060, Gibco, Life Technologies). Cells were cultured until an optical confluence of 75% - 100% before they were detached with 0.05% trypsin-EDTA 1X (25300-054, Invitrogen). For *in vitro* assays, cell number was determined using a Bürker counting chamber.

For gene expression analysis, mRNA was quantified by RT plus RQ-PCR (3.2) and protein quantification by western blotting (3.3) was performed. Cells ($5x10^5$ cells/well) were seeded in 6-well plates and synchronized under starvation (FBS free), for 8h. After starvation, cells were cultured in the presence and/or absence of FA (C18:2 - linoleic acid water-soluble, 96 µM; L5900, Sigma Aldrich; from now on called C18 in this thesis) and/or estradiol (1 nM; E4389, Sigma Aldrich) for 16h. To examine gene expression over time, a pulse chase assay was performed. Cells were seeded and cultured as described above and collected after 15 min, 30 min, 1h, 1h30min, 2h, 4h and 6h.

For wound healing assay (3.6), $2x10^5$ cells/well were seeded in 12-well plates and cultured until reaching a confluent monolayer. Cell proliferation was inhibited with Mitomycin-C (5 µg/mL; M4287, Sigma), 3h prior the starting of the experiment. After, cells were exposed to FA and/or estradiol (as described above).

In cell death analysis (3.7), $1.5x10^5$ cells/well were plated in 24-well plates. After starvation, cells were exposed to arylpiperazines (12a and 5k) in a range of concentrations from 0.125 to 125 µM, for 16h.

For both cell cycle analysis (3.8) and lipid quantification/Nile red staining (3.9) by flow cytometry, $5x10^5$ cells/well were plated in 6-well plates. After 8h of starvation, cells were exposed to FA and/or estradiol with and without arylpiperazines, 5k (0.125 µM and 12.5 µM) and 12a (12.5 µM and 125 µM), for 16h.

For FATP1 levels quantification (3.5) by flow cytometry, $1.5x10^5$ cells/well were plated in 24-well plates and synchronized under starvation for 8h. After starvation, cells were exposed to estradiol (1 µM) and/or C18 (96 µM) and/or PHTTP (50 µM, ERβ antagonist; SML1355, Sigma Aldrich) and/or ERB-041 (1 µM, ERβ agonist; PZ0183, Sigma Aldrich), for 16h.

Additionally, cells were exposed to estradiol (1 µM) and/or C18 (96 µM) and/or PHTTP (50 µM, ER-β antagonist) and/or ERB-041 (10 µM, ER-β agonist), for 16h and cell death and lipid quantification assays were performed as described above.

In all *in vitro* assays, control cells were maintained in culture medium free of FA, estradiol, arylpiperazines, ERB-041 and PHTTP.

3.2 RNA extraction, reverse transcription (RT) and relative quantifying real-time polymerase chain reaction (RQ-PCR)

In the present thesis, the modulation of *FATP1/SLC27A1*, *ESR1* and *ESR2* genes expression by FA (C18) and/or estradiol was evaluated by RQ-PCR. Gene transcription can be evaluated by mRNA quantification in biological samples, using reverse transcription (RT). This technique is based in a single-stranded complementary DNA copy (cDNA) from a RNA template, through the action of the enzyme reverse transcriptase. Quantifying real-time polymerase chain reaction (qRT-PCR) measures

gene expression with accuracy and sensitivity, allowing both absolute (AQ-PCR) or relative (RQ-PCR) quantification of cDNA (Derveaux et al., 2010; Freeman et al., 1999). The RQ-PCR analysis is normalized for a housekeeping gene, which is constitutively expressed in cells (Gubern et al., 2009).

RNA was extracted using RNeasy Mini Kit Qiagen® (74104, Qiagen), according to the manufacturers' protocol. RNA concentration in samples were measured at 260nm, in a NanoDrop 2000 (ND-2000, Thermo Scientific). cDNA was synthesized from 1μg of total RNA by incubation at 70 °C for 10 min with random primers (11034731001, Roche) and with bidistilled water to complete a final volume of 8uL. Then, cDNA was reversely-transcribed using SuperScript™ II reverse transcriptase (18080-44, Invitrogen), RNAse OUT™ (10777-019, Invitrogen) and deoxynucleotides (dNTPs) mix (10mM; 28-4065-22V, 28-4065-02V, 28-4065-12V and 28-4065-32V, GE Healthcare), according to the manufacturers' protocol.

RQ-PCR was performed using cDNA, specific pairs of primers for genes of interest (*Supplementary Table 1)* and SYBR® Green Master Mix (04707516001, Roche), according to manufacturer's instructions, using a Lightcycler® 480 System instrument (05015243001, Roche). Hypoxanthine-guanine phosphoribosyltransferase 1 gene (*HPRT1*) was used as a housekeeping gene.

3.3 Western blotting

Western blotting is one of the gold standard technique used to evaluate protein levels, allowing the detection of antibody-targeted proteins in biological samples (Y. Liu et al., 2011).

Cell pellets were lysed in Radio-Immunoprecipitation Assay (RIPA) buffer (Appendix) and stored at -20ºC. Cell lysates were centrifuged at 11000 g (in a fixed angle rotor, Eppendorf, f45-30-11) for 4 min at 4ºC and supernatants were collected. Protein concentration was established by Bradford method, using Bio-Rad protein assay reagent (500-0006, Bio-Rad) through spectotrophometric quantification (595 nm). After protein quantification, loading buffer 5x (Appendix) with 10% β-mercaptoethanol (M3148, Sigma) was added to each cell lysate and boiled at 95-100ºC for 10 min, to denature proteins. Then, samples were centrifuged at 11000 g for 2 min at 4ºC and placed on ice.

The same amount of total protein in each sample was loaded in 15% polyacrylamide gel (Tris-glycine SDS-Polyacrylamide gel) (Appendix) and electrophoresis was carried out in MINI-PROTEAN Tetra Electrophoresis System (Bio-Rad) at 140V, into 1X TGS buffer (Tris-Glycine-SDS 10x (TGS); 161-0772, Bio-Rad). After, proteins from the gel were transferred to an Immun-Blot® PVDF membrane with Trans-Blot® Turbo ™ Blotting system, at 25 V, 2.5 A for 13 min.

To avoid non-specific antibody binding, membranes were blocked using 3% (w/v) BSA in phosphate buffered saline 1x (PBS) 0.1% (v/v) Tween 20, for 2 h at room temperature (RT), with gentle shaking. For protein detection, membranes were incubated with primary specific antibodies (mouse anti-human FATP1, MAB3304, R&D systems; rabbit anti-human ERα, ab75635, Abcam; rabbit anti-human ERβ, ab196787, Abcam) at appropriate concentrations (1:1000 in 3% (w/v) BSA in PBS 0.1% (v/v) Tween 20 (Appendix), 1:200 and 1:500 in 5% (w/v) non-fat milk in PBS 0.1% (v/v) Tween 20 (Appendix), respectively, at 4ºC, ON, with shaking.

After, to remove unbound primary antibody membranes were rinsed 3 times, for 5-10 min, with PBS 1x 0.1% (v/v) Tween 20, followed by an incubation for 2h at RT with secondary antibody IgG-conjugated Horse raddish peroxidase (HRP; anti-mouse, 31430, Thermo Scientific; 1:5000 in 3% (w/v) BSA in PBS 0.1% (v/v) Tween 20). Membranes were rinsed 3 x, for 5 min with PBS 1x 0.1% (v/v) Tween 20 and immunoreactive bands were detected by using ECL western blotting substrate (SuperSignal® West Pico Chemiluminescent Substrate (34080, Thermo Scientific) in a ChemiDoc XRS System (Bio-Rad) with Image Lab software. To normalize the protein levels in samples, membranes were re-incubated using mouse anti-human β-actin (A5441, Sigma; 1:5000 in 3% (w/v) BSA in PBS1x

0.1% (v/v) Tween 20), at 4ºC, ON. Protein levels were detected as described above. Bands were quantified using Image J software (rsb.info.nih.gov/ij/).

3.4 Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) is used to investigate interactions between proteins and chromatin in a cell (Collas, 2010) In the context of the current thesis, this technique was employed to analyze putative interactions between ER-α and β transcription factors and Estrogen Responsive Elements (ERE) sequences in *FATP1/SLC27A1* promoter. Those regions localize in 5'UTR (unstranslated reagion) of *FATP1/SLC27A1* gene between nucleotides –179 to –406. To crosslink proteins and DNA cells were treated with 37% formaldehyde at a final concentration of 1% (v/v), followed by an incubation with 125mM glycine (pH 9). Subsequently, cells were scraped, centrifuged at 150 g for 2 min and ChIP lysis buffer (Appendix) was added. Cell lysates were sonicated and the size of the chromatin fragments (expected size between 1000bp and 500bp) was evaluated by electrophoresis, in a 1.2% (w/v) agarose gel. ChIP assay was performed using OneDay ChIP kit (kch-onedIP-060, Diagenode) according to the manufacturer's protocol. The chromatin complexes were immunoprecipitated with 1µl (~1µg/mL) of specific antibodies: rabbit anti-human ERα (ab75635; Abcam) and rabbit anti-human ERβ (ab196787; Abcam). The relative occupancy of the immunoprecipitated factors at a specific promoter region was performed by RQ-PCR as described in section 3.3 and calculated using the following formula:

$$\text{Relative occupancy}=2^{(\text{CtNegCtl} - \text{CtTarget})}$$

3.5 FATP1 levels by flow cytometry

To evaluate the effects of estradiol, PHTPP and ERB-041, with and without FA in FATP1 expression, FATP1 levels were quantified using flow cytometry. Briefly, $1.5x10^5$ cells/mL were plated in 24-well plates. After starvation, cells were exposed to PHTPP and ERB-041 with or without estradiol and FA. Cells were detached with PBS-EDTA, centrifuged at 230 g for 5 min, at 4ºC and washed with PBS-BSA (0,1%). Cells were incubated with 100 µL of FATP1 (1:500 in PBS-BSA 0,5%) with and without 0.1% saponin (47036, Sigma Aldrich), for 1h, at 4ºC. After, PBS-BSA 0,1% was added and cells were centrifuged at 230 g for 5 min, at 4ºC, followed by an incubation of 30 min with anti-mouse FITC (1:1000 in in PBS-BSA 0,5%; A11059, Invitrogen), with and without 0.1% saponin. After, cells were washed, centrifuged and resuspended in 300 µL PBS-BSA 0,1%. Samples were analysed in FACScalibur (Becton Dickinson) and data analyzed with FlowJo 8.7 software.

3.6 Wound healing assay

Wound healing assay is a method used to measure directional cell migration *in vitro*, mimicking cell migration during wound healing *in vivo*(Liang et al., 2007). This assay was performed in order to identify a potential role of FA and estradiol on cancer cell migration.

In each cell monolayer, a scratch was made to the diameter of the well, and the wound closure was followed by acquiring phase-contrast images (x200 field) at the following time points: 0, 1, 3, 6, 9, 12, 24 and 48 h. Images were analyzed and quantified using ImageJ software.

3.7 Cell death analysis by flow cytometry

Apoptotic cells, among other typical features, are characterized by DNA fragmentation and loss of nuclear DNA (Riccardi and Nicoletti, 2006). In normal viable cells, phosphatidyl serine (PS) is located on the inner surface of the cell membrane, however, in apoptotic cells, PS is translocated to the outer leaflet of the plasma membrane exposing PS to the external environment. Labeled Annexin V allows the identification of apoptotic cells by binding to the exposed PS, and propidium iodide (PI), which is not permeant to live cells, binds to double stranded DNA by intercalating between base pairs, thus staining necrotic cells.

In the context of the present thesis, apoptosis assay was performed to analyze the effect of arylpiperazines in breast cancer cells. After experimental conditions, supernatants were collected and cells were harvested with trypsin, centrifuged at 150 g for 2 min. Cell pellets were stained with 0.5 µl fluorescein isothiocyanate (FITC)-Annexin V (640906, BioLegend) plus 2.5 µl PI (50µg/mL; P4170, Sigma-Aldrich) in annexin V binding buffer 1X (Appendix) and incubated at RT, in dark for 15 min. After incubation, samples were resuspended in 200 µl PBS 1x 0.1% (v/w) BSA (Appendix) and centrifuged at 150 g for 2 min. Cells were resuspended in 100 µl of annexin V binding buffer 1X and analyzed by flow cytometry (FACScalibur – Becton Dickinson). Sample data was analyzed using FlowJo 8.7 software (https://www.flowjo.com).

3.8 Cell cycle analysis by flow cytometry

The cell cycle are ordered events involving cell growth and cell division, resulting in two new daughter cells. It is divided in four sequential stages: Gap 1 (G1), Synthesis (S), Gap 2 (G2), and Mitosis (M). In the G1 phase, metabolic changes prepare the cells for the synthesis of DNA which is then replicated in the S phase that is followed by the G2 phase, in which the cell prepares for division and ultimately divides in mitosis (M phase). In the quiescent phase, G0, cells are not dividing. The DNA content in the cells at the G0/G1, S and G2/M phases can be accurately quantified using DNA staining dyes such as PI [108]. After exposure to FA, estradiol and FA plus estradiol, cells were harvested and fixed in 70% ethanol, at 4ºC. Then, cells were centrifuged at 150 g for 5 min and the cell pellet were incubated with 100 µL of PI solution (50µg/mL PI; 0.1mg/mL RNase A, 0.05% Triton X-100) for 40 min, at 37ºC. After the incubation, PBS 1X was added, cells were centrifuged at 230 g for 5 min at 4ºC and then resuspended in 200 µL of PBS-BSA 0.1% (v/w). Samples were analysed in FACScalibur (Becton Dickinson) and data was analyzed with FlowJo 8.7 software.

3.9 Nile red staining by flow cytometry

Nile red staining assay was performed to study the role of arylpiperazines in the uptake of FA by breast cancer cells. Neutral and polar lipids in cells were quantified using Nile red, a fluorescent dye. Nile red staining was performed based on (Lopes-Coelho et al., 2017). Briefly, cells were detached with trypsin, washed with PBS 1x and stained with Nile Red (1 mg/mL in PBS 1x) for 15 min, at RT. After, cells were centrifuged and PBS-BSA-0.1% (v/w) was added. Samples were analysed on FACSCalibur flow cytometer (Becton-Dickinson) and data was analyzed with FlowJo 8.7 software.

3.10 Bioinformatics analysis

Data from Gene Expression Omnibus (GEO) was used to analyze the gene expression of FATP1 in human samples. Kaplan-Meier curves of overall survival (OS) and recurrence-free survival (RFS) of breast cancer patients were obtained from http://kmplot.com/analysis/.

3.11 Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 software (www.graphpad.com). Sample data were presented as the mean (normal distribution) or median (non-normal distribution) ± SD. All assays were performed in independent triplicates. Comparisons between data from each group were statistically analyzed by a two-tailed unpaired Student's *t*-test. Differences between experimental conditions were statistically significant at $p<0.05$.

4. Results

4.1 Linoleic acid (C18) and estradiol stimulate FATP1 expression

In order to evaluate the modulation of FATP1 by estradiol and FA (C18), MDA-MB-231 and MCF7 cells were exposed to C18 and estradiol, in separate and in combination, using as control cells cultured in medium without both C18 and estradiol and supplemented with 1% FBS. The expression of FATP1 was analyzed by a RQ-PCR assay (Figure 4.1) and by western blotting (Figure 4.2).

FATP1/SLC27A1 mRNA levels were significantly increased after 30 minutes of exposure to C18+estradiol in MDA-MB-231 cells (Figure 4.1 A) and after 15 minutes of exposure to C18 in the MCF7 cell line (Figure 4.1 B), comparing to cells cultured in control conditions. In both cases, *FATP1/SLC27A1* mRNA levels suffered a boost in expression in the first minutes of exposure but then decreased with time. In figure 4.1 C, control cells from both cell lines were compared to assess the basal mRNA levels of *FATP1/SLC27A1* and different starvation times were tested. The results revealed a significant different expression of *FATP1/SLC27A1* between cell lines, being higher in MDA-MB-231.

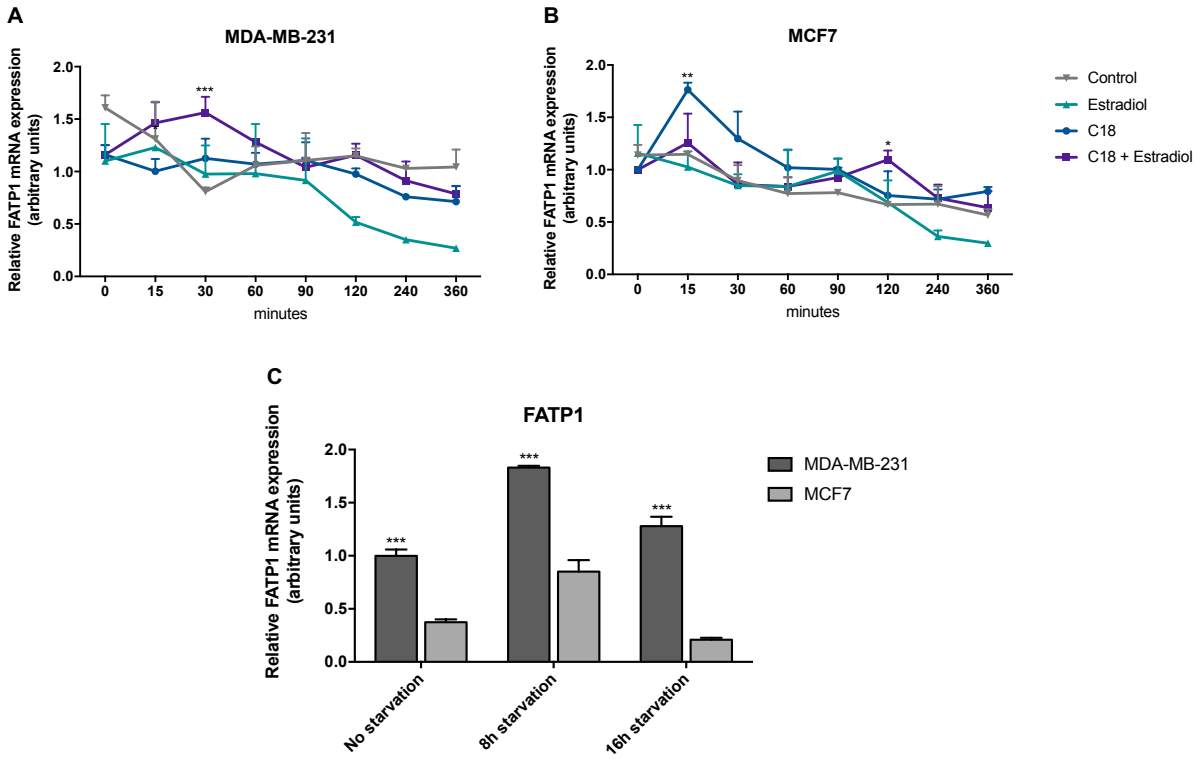


Figure 4.1 - Relative gene expression of *FATP1/SLC27A1* in MDA-MB-231 and MCF7 cells. (a,b) In a pulse chase assay, cells were cultured in medium and exposed to estradiol, C18 and C18+estradiol over 360 minutes. **(a)** MDA-MB-231 cells presented higher levels of *FATP1/SLC27A1* mRNA expression in the C18+estradiol condition after 30 minutes of exposure. **(b)** C18 stimulated FATP1 expression in MCF7 cells at 15 minutes of exposure. **(c)** Cells were submitted to different starvation times (0h, 8h and 16h) and the basal levels of *FATP1/SLC27A1* mRNA expression were analyzed. FATP1 levels were significantly higher in MDA-MB-231 cells in all cases. HPRT gene was used as endogenous control. Expression levels were normalized to control condition. Data are mean ± error bars of triplicates. *p≤0.05 **p ≤0.01 ***p ≤0.001.

Concerning FATP1 protein levels assessed by western blotting, the same dynamic was observed in MDA-MB-231. After 16h hours of exposure, FATP1 protein levels were visibly higher in the C18+estradiol condition in MDA-MB-231 (Figure 4.2 A) whereas in MCF7, no significant differences were observed (Figure 4.2 B).

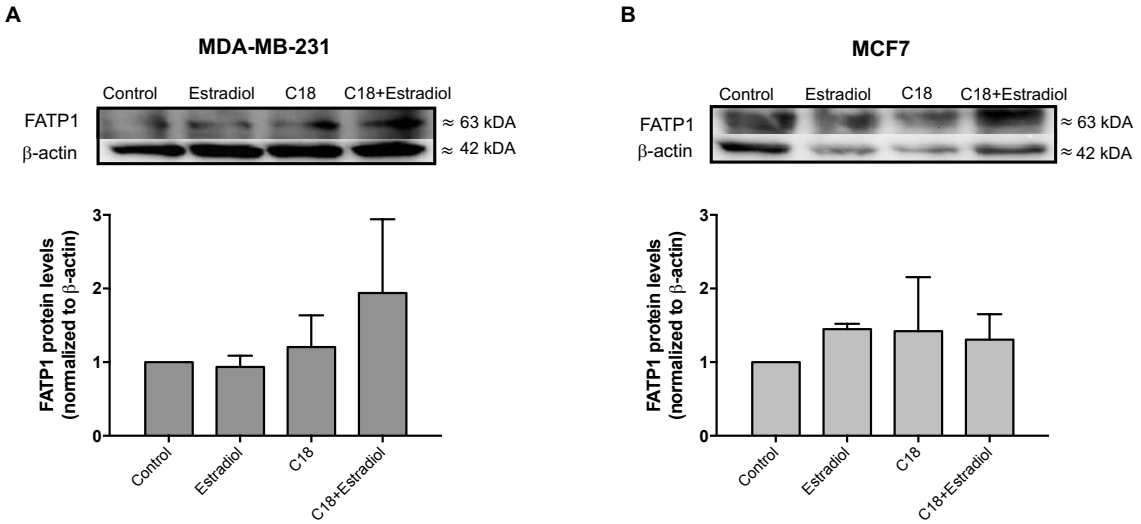
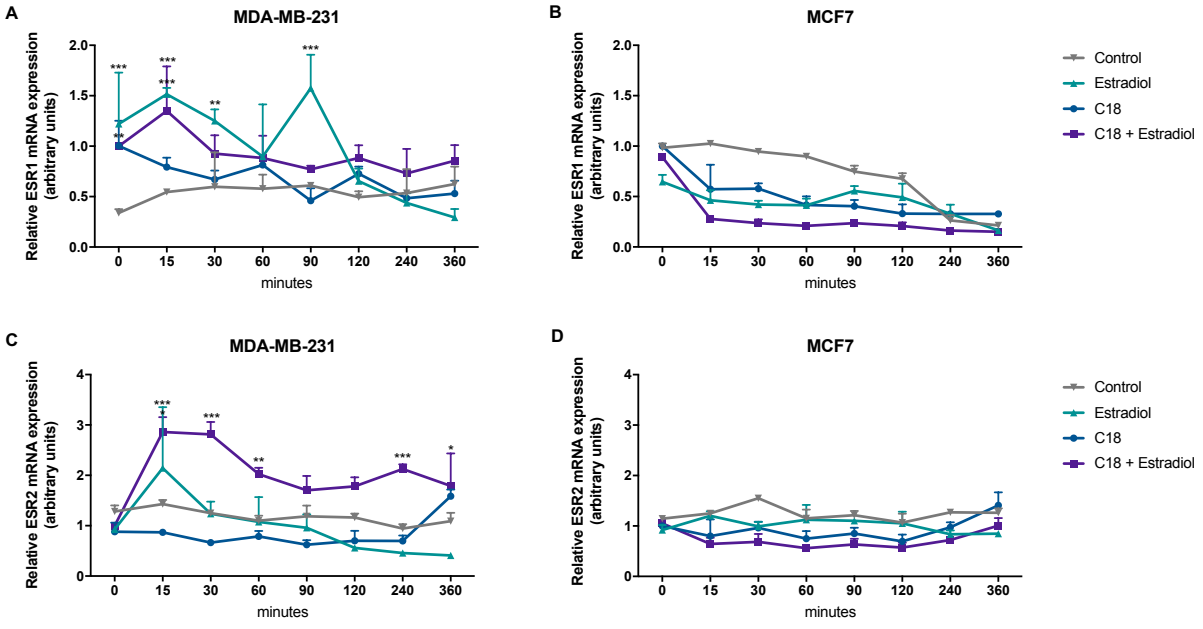


Figure 4.2 - FATP1 protein levels assessed by Western blotting. Cells were cultured in control, estradiol, C18 and C18+estradiol conditions. Protein levels were normalized to β-actin and are relative to those obtained for cells cultured in control conditions. **(a)** MDA-MB-231 cells showed higher levels of FATP1 protein in the C18+estradiol condition; **(b)** while in MCF7 there were no major differences between conditions

As estrogen is known to have an essential role in the development and progression of breast cancer and since our previous results showed that estradiol in combination with C18 modulates *FATP1/SLC27A1* expression, we analyzed the expression of estrogen receptors genes 1 and 2 (ESR1 and ESR2) by RQ-PCR (Figure 4.3) and western blotting (Figure 4.4). In MDA-MB-231, an ER- cell line, the expression of ESR1 was significantly higher when cells were exposed to estradiol, in comparison with the control condition in the first 30 minutes of exposure and then at 90 minutes. At 15 minutes of exposure, C18+estradiol condition also showed increased levels of ESR1 (Figure 4.3 A). On the other hand, in MCF7, ER+ cell line, ESR1 mRNA levels did not increase with any of the stimulus (Figure 4.3 B). A statistically significant increase of ESR2 mRNA levels was observed with C18+estradiol exposure over time in MDA-MB-231 (Figure 4.3 C) whereas in MCF7 no differences between conditions were observed (Figure 4.3 D). In terms of basal expression of ESR1, it is significantly upregulated in the MCF7 cell line (Figure 4.3 E) comparing with MDA-MB-231. ESR2 mRNA expression was significantly more elevated in MDA-MB-231 cells, especially with 8h of starvation (Figure 4.3 F).



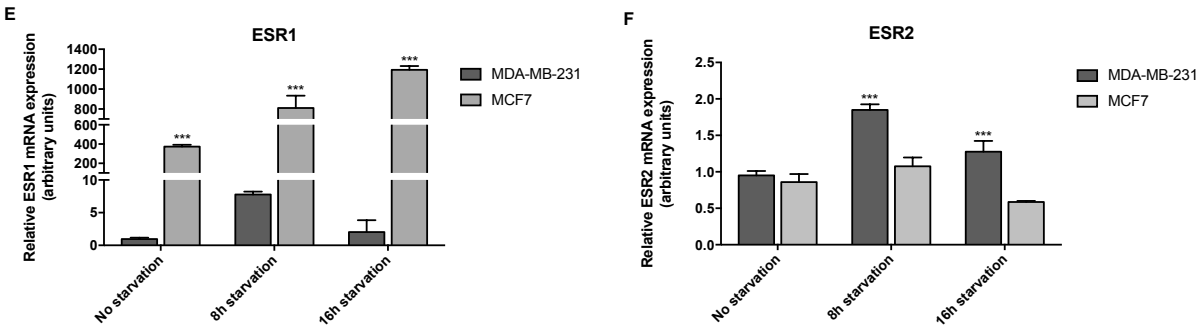
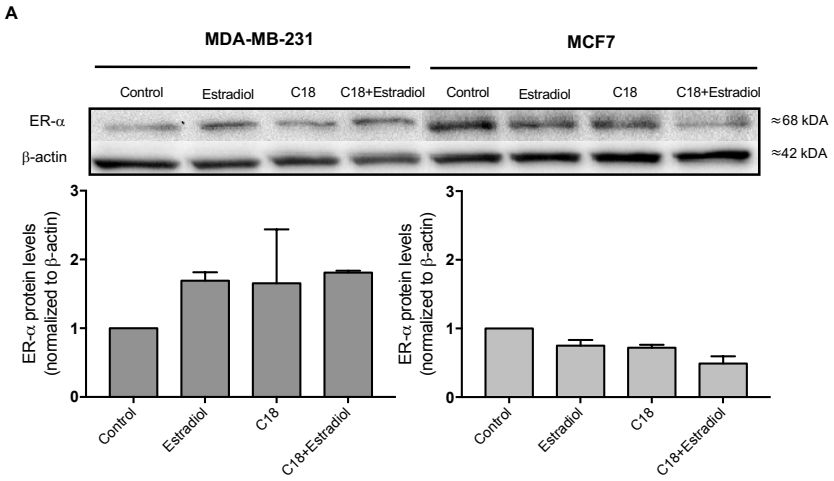


Figure 4.3 - Relative gene expression of ESR1 and ESR2 in MDA-MB-231 and MCF7 cells. (a, b, c, d) In a pulse chase assay, cells were cultured in medium and exposed to estradiol, C18 and C18+estradiol over 360 minutes. **(a)** MDA-MB-231 cells presented higher levels of ESR1 mRNA expression in the estradiol condition in the first 30 minutes of exposure and at 90 minutes. **(b)** the addition of C18 or estradiol had no effect in ESR1 expression in MCF7 cells, being higher in the control condition. **(c)** ESR2 expression was significantly upregulated in MDA-MB-231 cells while in MCF7 no differences were observed **(d)**. **(e)** Cells were submitted to different starvation times (0h, 8h and 16h) and the basal levels of ESR1 and ESR2 mRNA expression were analyzed. ESR1 levels were significantly higher in MCF7 cells and ESR2 levels were greater in MDA-MB-231. HPRT gene was used as endogenous control. Expression levels were normalized to control condition. Data are mean ± error bars of triplicates. *p≤0.05 **p ≤0.01 ***p ≤0.001.

Western blotting analysis showed that both cell lines express ER-α (codified by ESR1) and ER-β (codified by ESR2) proteins despite being MDA-MB-231 reported as ER-. Regarding ER-α, C18 and estradiol increased ER-α protein levels in MDA-MB-231 (Figure 4.4 A) which is in agreement with the RQ-PCR results. In MCF7 no major differences were observed in both receptors corroborating with the mRNA levels (Figure 4.4 A and B). MCF7 cell line also exhibited higher levels of ER-α levels in comparison with MDA-MB-231 cells, which is also in concordance with the mRNA levels. In MDA-MB-231, ER-β protein levels were not altered with the addition of C18 or estradiol, in fact, they were higher in the cells cultured in control medium which does not correspond with the RQ-PCR results (Figure 4.4 B). ER-β protein levels were greatest in the MCF7 cell line, which was not the case at the mRNA level.



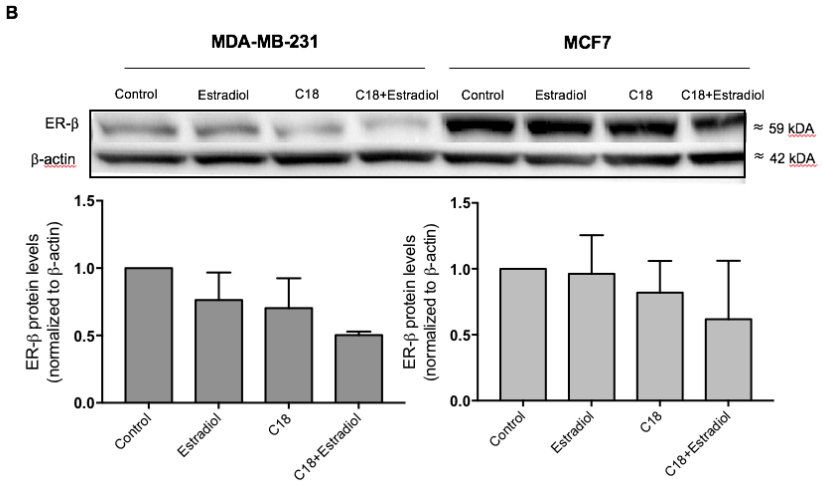


Figure 4.4 - ER-α and ER-β levels assessed by Western blotting. Cells were cultured in control, estradiol, C18 and C18+estradiol conditions. Protein levels were normalized to β-actin and are relative to those obtained for cells cultured in control conditions. **(a)** MDA-MB-231 cells showed higher levels of ER-α protein in the C18 and estradiol condition; while in MCF7 there were no major differences between conditions. **(b)** ER-β protein levels decreased when cells were treated with estradiol and/or C18 in MDA-MB-231. In MCF7 C18+estradiol also led to a reduction in ER-β expression.

4.2 Estradiol stimulates the binding of ER-β to *FATP1/SLC27A1* promoter

The involvement of estrogen signaling pathway in *FATP1/SLC27A1* regulation was assessed by chromatin immunoprecipitation (ChIP). The relative occupancies of ER-α and ER-β on the *FATP1/SLC27A1* promoter were measured. These transcription factors were immunoprecipitated by ChIP assay and then a RQ-PCR was performed to amplify the fragment of *FATP1/SLC27A1* promoter in which Responsive Elements (ERE) sequences are located. Figure 4.5 shows that ER-β binding to FATP1 promoter increased upon estradiol stimulation, being statistically significant in MCF7 cells. Regarding ER-α, it only was observed a trend to increase in both cell lines.

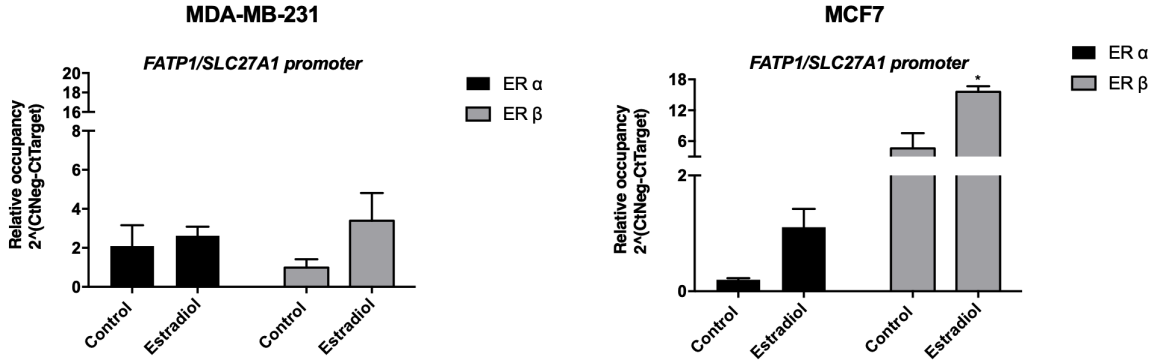


Figure 4.5 - Estradiol stimulates the binding of ER-β to promoter *FATP1/SLC27A1*. Cells were cultured in control and in estradiol conditions. The FATP1 promoter showed a significant increase of relative occupancy of ER-β after exposure to estradiol. Results are shown as mean ± SD. *p<0.05, **p<0.01, ***p<0.001.

4.3 *FATP1/SLC27A1* expression is regulated by ER-β

In order to evaluate the role of ER-β in *FATP1/SLC27A1* regulation, cells were exposed to ERB-041, an ER-β agonist, and to PHTPP, an ER-β antagonist. A cell viability assay was performed to evaluate the effect of these compounds in cells cultured in the presence or absence of estradiol with or without C18. Figure 4.6 shows that PHTPP, with and without C18, caused a significant increase in cell

death in both cell lines while the other conditions did not affect cell viability. Comparing with DMSO condition, MDA-MB-231 cells display higher levels of cell death upon exposure to PHTPP than MCF7. ERB-041 did not affect cell viability in neither cell lines.

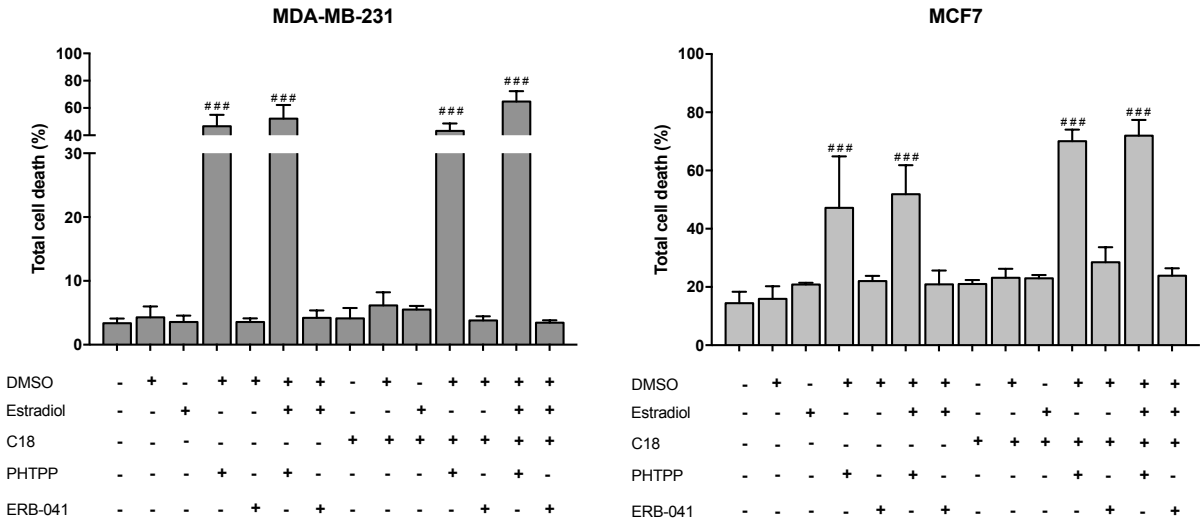
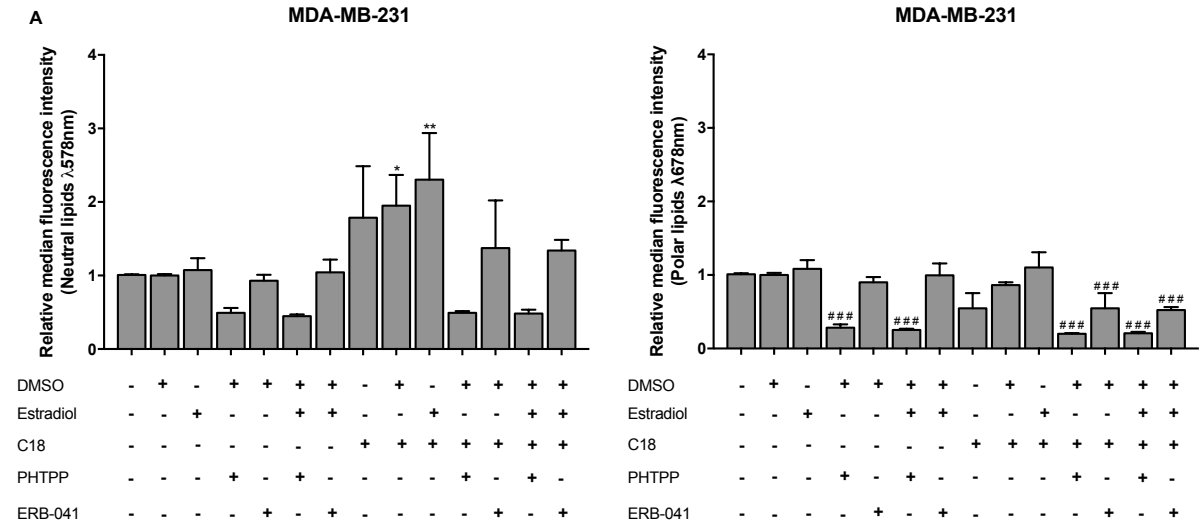


Figure 4.6 – PHTPP affects cell viability of MDA-MB-231 and MCF7 cells. Cells were cultured in control, control DMSO, estradiol and/or C18 conditions, in the presence or absence of PHTPP and ERB-041.PHTPP caused a significant increase in cell death in both cell lines. Results are shown as mean ± SD. *p<0.05, **p<0.01, ***p<0.001. (#) represents the statistical analysis in relation to control DMSO condition.

To evaluate the FA uptake upon estradiol, PHTPP and ERB-041, with and without C18, a flow cytometry assay was performed using Nile Red (Figure 4.7). MDA-MB-231 cells exposed to C18+estradiol showed a significant uptake of neutral lipids (Figure 4.7 A). When cells were exposed to PHTPP (ER-β antagonist), the uptake of FA was decreased, being statistically significant in the polar lipids in both cell lines (Figure 4.7 A and B). In MCF7 cells, ERB-041 (ER-β agonist) induces a significant increase in the uptake of both neutral and polar lipids (Figure 4.7 B). However, when ERB-041 was combined with C18 the uptake decreased. C18 and estradiol separate exposure also led to a higher uptake of neutral lipids in MCF7 cells.



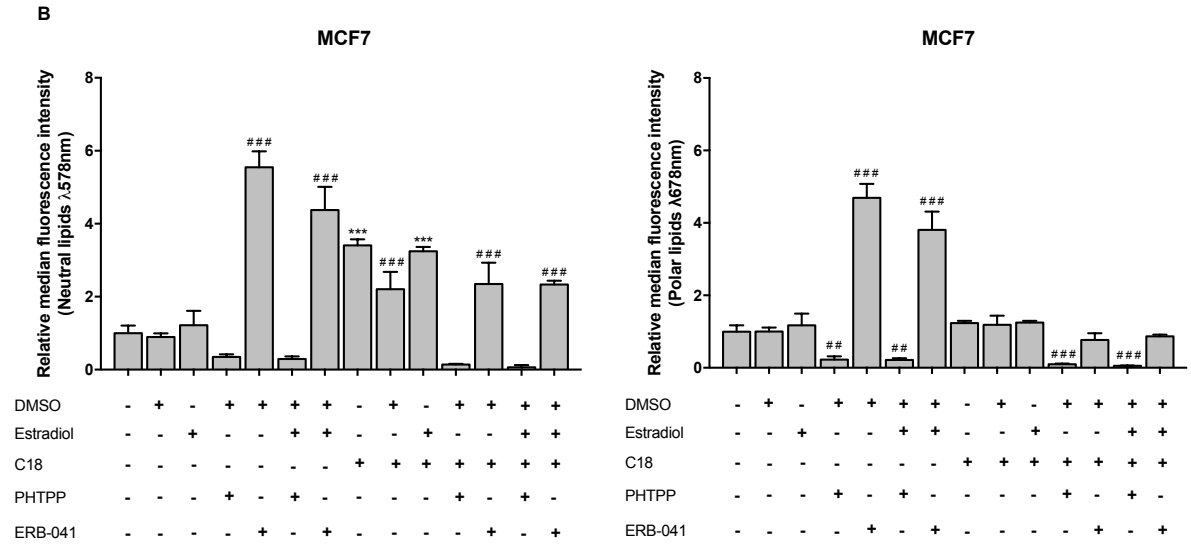
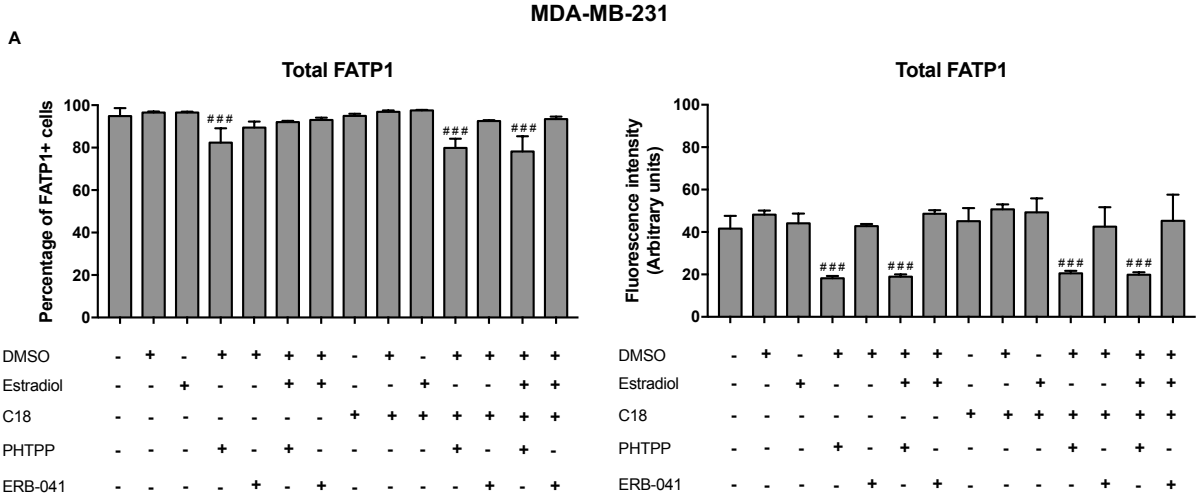


Figure 4.7 - MDA-MB-231 and MCF7 cells exposed to PHTPP accumulate less FA. Cells were cultured in control, control DMSO, estradiol and/or C18 conditions, in the presence or absence of PHTPP and ERB-041. **(a)** MDA-MB-231 cells displayed a higher uptake of neutral lipids when exposed to C18 and estradiol but when they were treated with PHTPP they accumulated less FA. **(b)** MCF7 cells showed a significant increase in the uptake when exposed to ERB-041 and when treated with the ER-β antagonist the uptake of FA was dramatically decreased. Results are shown as mean ± SD. *p<0.05, **p<0.01, ***p<0.001. (*) represents the statistical analysis in relation to control condition and (#) represents the statistical analysis in relation to control DMSO condition.

Since ER-β antagonist (PHTPP) interfered with cell viability and both ER-β agonist (ERB-041) and antagonist (PHTPP) interfered with the uptake of FA, we then analyzed the modulation of *FATP1/SLC27A1* expression by estradiol, PHTPP and ERB-041, in the presence and absence of C18, by flow cytometry. Figure 4.8 A shows that the total percentage of FATP1 positive cells was significantly lower when cells were treated with the ER-β antagonist (PHTPP), the same happened for the fluorescence intensity in both MDA-MB-231 and MCF7 cells (Figure 4.8 A and C). At the same time, the percentage of FATP1 positive cells in the cell membrane was significantly upregulated when cells were exposed to PHTPP in both cell lines (Figure 4.8 B and D). In this assay, the ER-β agonist (ERB-041) did not have effects except in the membranar percentage of FATP1 positive cells in MCF7 that was significantly enhanced ERB-041 (Figure 4.8 D). Cells exposed to C18 also had more FATP1 positive cells in the membrane (Figure 4.8 B and D). MCF7 cells displayed a significant amount of membranar FATP1 positive cells when treated with C18 in combination with estradiol (Figure 4.8 D).



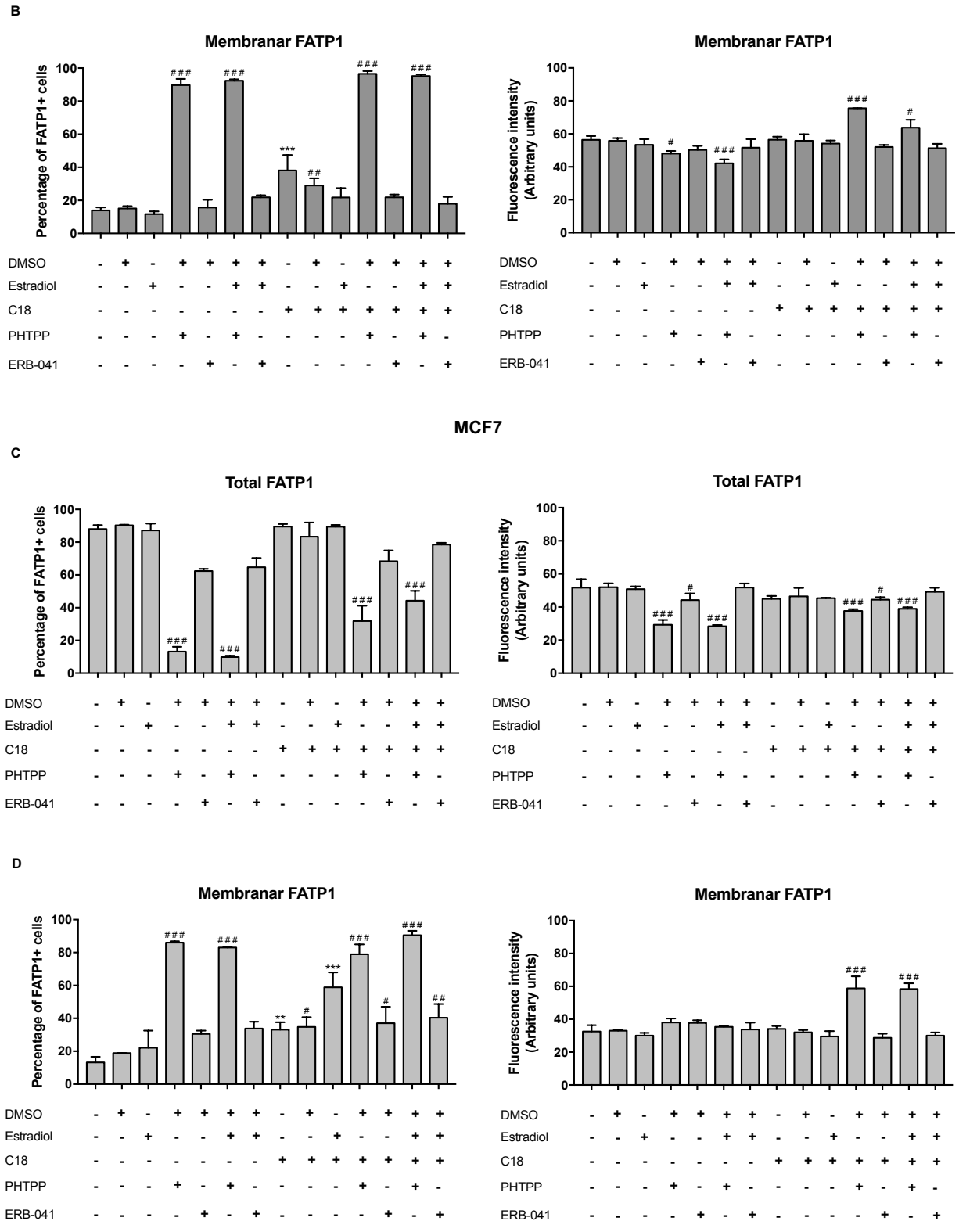


Figure 4.8 – FATP1 is upregulated in the membrane of cells treated with PHTPP. Cells were cultured in control, control DMSO, estradiol and/or C18 conditions, in the presence or absence of PHTPP and ERB-041. **(a, c)** MDA-MB-231 and MCF7 cells exposed to PHTPP had a significant lower percentage of total FATP1 positive cells, as for fluorescence intensity of total FATP1, it was also significantly decreased in cells treated with PHTPP. **(b)** FATP1 positive cells in the membrane were significantly elevated after exposure to PHTPP and to C18 comparing with control conditions. **(d)** MCF7 cells cultured in C18 and C18+estradiol, with ERB-041 and with PHTPP exhibited a significant increase in the percentage of FATP1 positive cells, however, only cells treated with PHTPP and C18 with and without estradiol displayed a higher fluorescence intensity. Results are shown as mean ± SD. *p<0.05, **p<0.01, ***p<0.001. (*) represents the statistical analysis in relation to control condition and (#) represents the statistical analysis in relation to control DMSO condition.

4.4 FA and estradiol increase cell migration and proliferation

As FA are metabolically crucial for the synthesis of membranes and signaling molecules, we investigated the effects of FA and estradiol in the migration and proliferation of breast cancer cells. For the migration analysis, a wound healing assay was performed over 48h. Both cell lines had a higher percentage of wound closure when cells were exposed to C18+estradiol condition, being this increase most significant after 48h of exposure (Figure 4.9 A and B). Estradiol alone also increased significantly migration however only in MCF7 cells (Figure 4.9 B). Proliferation was assessed by cell cycle analysis using flow cytometry. MDA-MB-231 cells displayed a higher percentage of cells dividing (S + G2/M phases) when they were exposed to C18 in combination with estradiol (Figure 4.9 C). The opposite happened in MCF7 cells when exposed to C18 with and without estradiol (Figure 4.9 D).

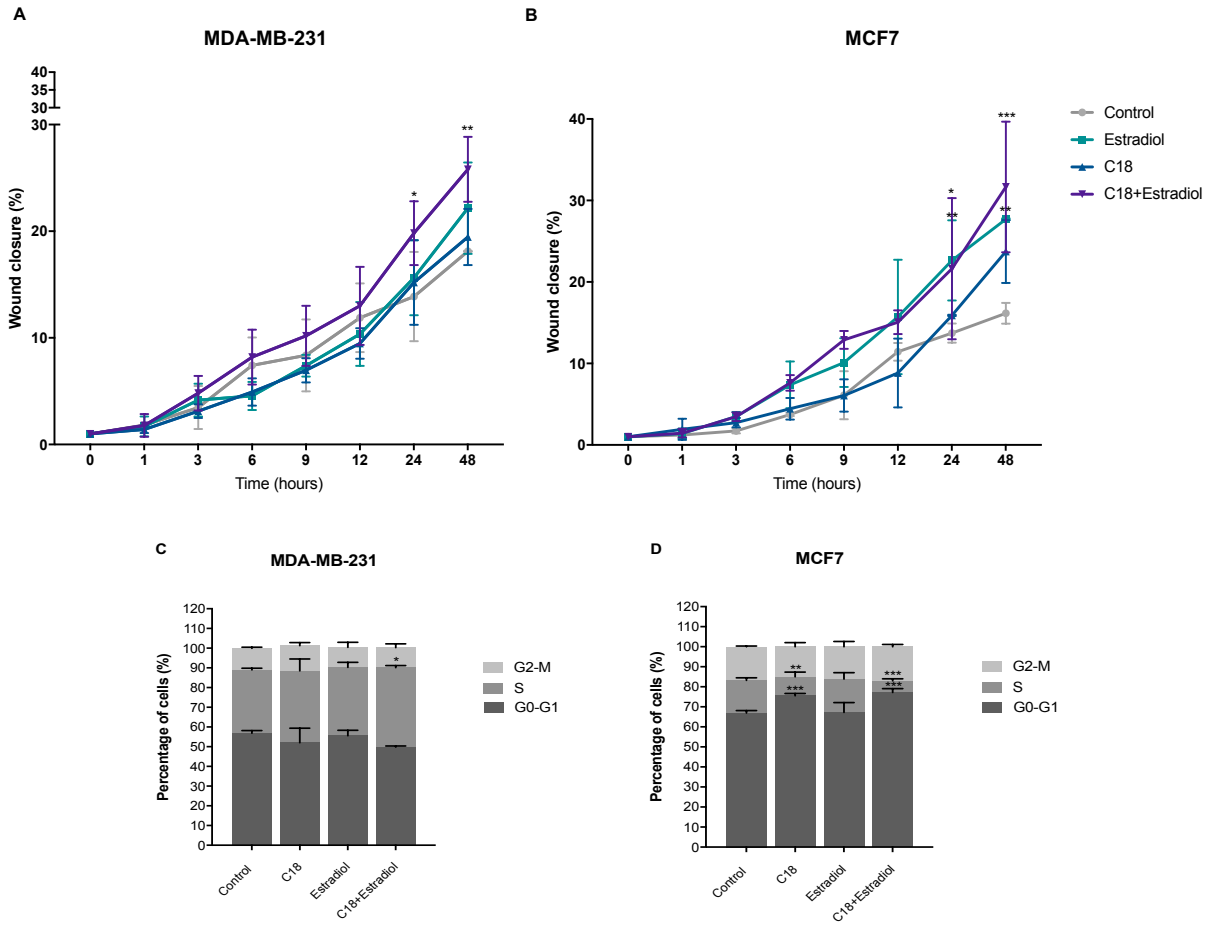


Figure 4.9 - MDA-MB-231 cells exposed to C18 and estradiol migrate and proliferate more. Cells were cultured in control, estradiol, C18 and C18+estradiol conditions. **(a), (b)** C18 in combination with estradiol led to a higher migration in both cell lines. **(c)** MDA-MB-231 exposed to C18 and estradiol proliferate more. **(d)** MCF7 cells do not proliferate. Data are means of triplicates. Results are shown as mean ± SD. *p<0.05, **p<0.01, ***p<0.001.

4.5 Patients with upregulated expression of *FATP1/SLC27A1* have lower overall survival and relapse free survival times

To determine the relevance of the expression levels of the *FATP1/SLC27A1* gene on the clinical outcome in untreated and treated breast cancer patients, a Kaplan-Meier analysis was employed. Overall survival (OS) was determined in grade 1 (26 patients), grade 2 (64 patients) and grade 3 (204 patients) breast carcinomas with high and low expression of FATP1. OS in patients with high *FATP1/SLC27A1* expression (red line) was lower than that in patients with low *FATP1/SLC27A1* expression (black line) and that was even more apparent in more advanced stages of the disease (grade 3) although not

significant (Figure 4.10 A). In Figure 4.10 B, grade 3, basal and ER- breast cancer cases (26) were evaluated and they are representative of the MDA-MB-231 cell line; and grade 3, luminal and ER+ breast cancer cases (36) were analyzed being representative of MCF7 cell line. OS in grade 3 triple negative breast cancer patients (basal, ER-) with high *FATP1/SLC27A1* expression was significantly lower than that in patients with low expression. This trend was also observed for grade 3, luminal, ER+ patients, however with no statistical significance.

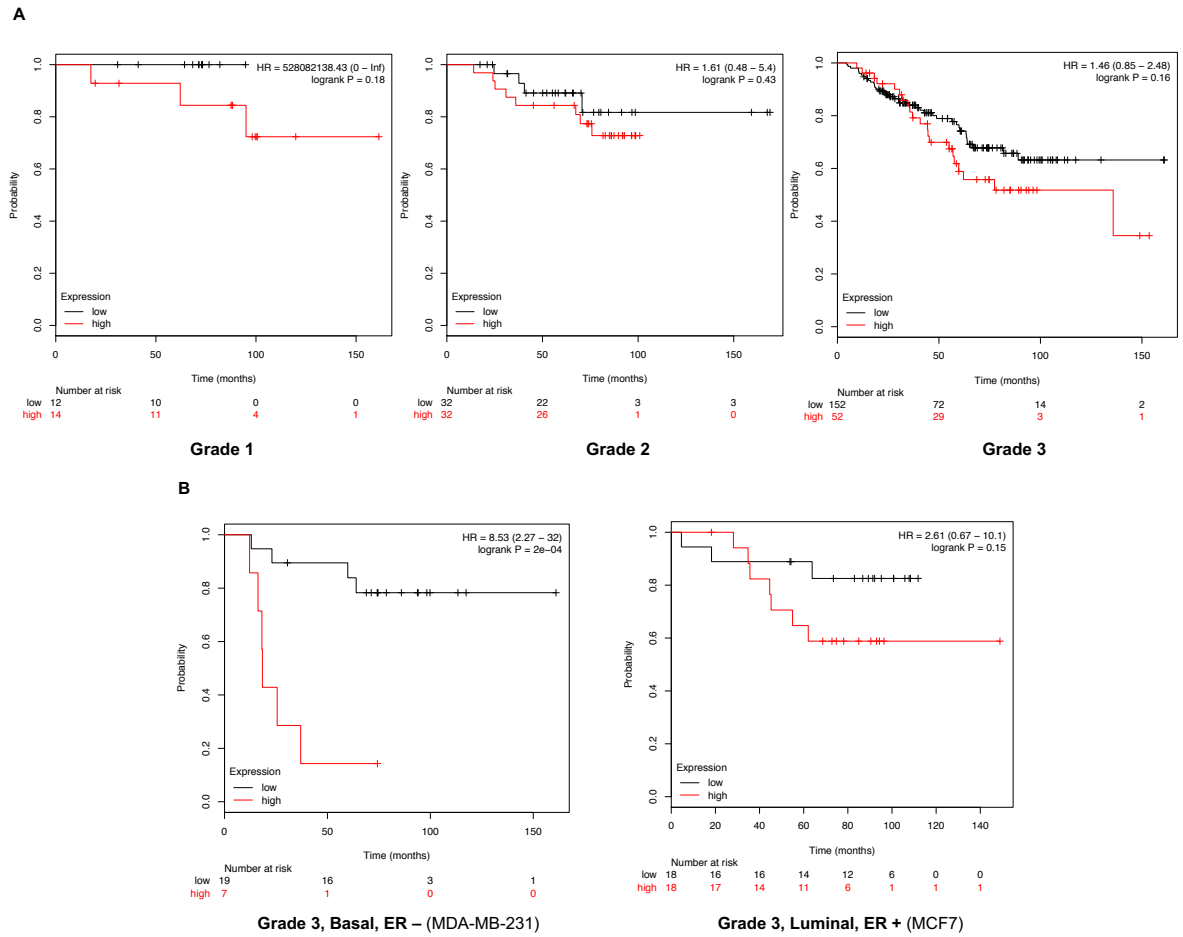


Figure 4.10 – Patients with high levels of *FATP1/SLC27A1* expression have a lower OS. Comparison of the overall survival curves of GEO patients with high levels of FATP1 (red line) and low levels of FATP1 (black line) expression using Kaplan-Meier method. **(a)** Kaplan-Meier survival curves for patients with breast cancer grade 1 (n=26), grade 2 (n=64) and grade 3 (n=204). **(b)** Kaplan-Meier survival curves for patients with grade 3, basal and ER – breast cancer (n=26) and grade 3, luminal and ER + breast cancer (n=36).

Regarding relapse free survival time (RFS) analysis was assessed in grade 1 (106 patients), grade 2 (227 patients) and grade 3 (444 patients) breast carcinomas with high and low expression of *FATP1/SLC27A1* (Figure 4.11 A). Patients in more advanced stages and with high expression of *FATP1/SLC27A1* showed a lower RFS comparing with grade 1 and grade 2 patients.

As shown in figure 4.11 B, grade 3 breast cancer patients, with a basal and ER – subtype (108), with high *FATP1/SLC27A1* expression (red line) displayed a significantly lower RFS than patients with low *FATP1/SLC27A1* expression (black line). A similar observation was made for grade 3, luminal, ER+ patients (86) but with no statistical significance.

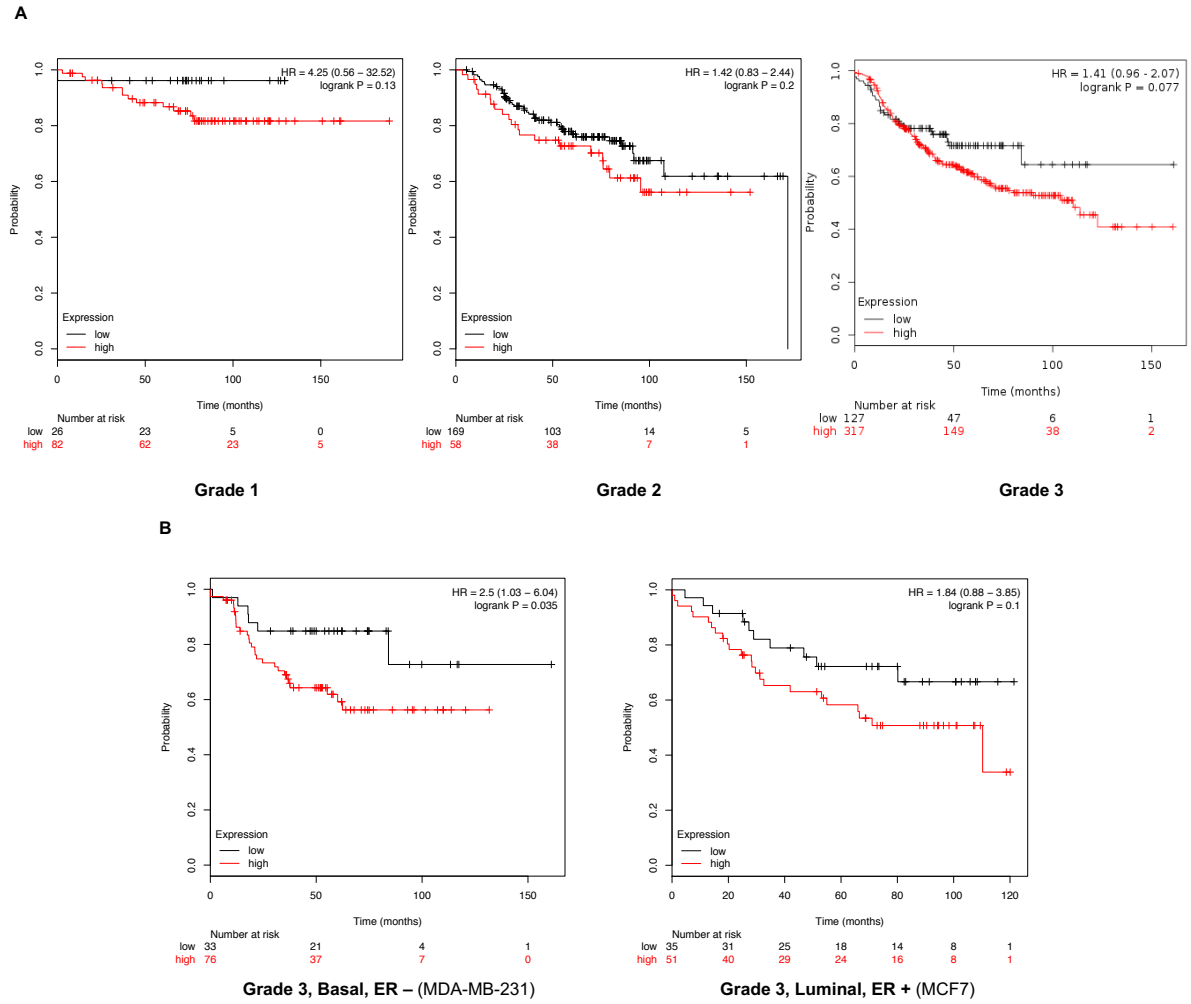


Figure 4.11 - Patients with high levels of *FATP1/SLC27A1* expression have a lower RFS. Comparison of the relapse free survival curves of GEO patients with high levels of FATP1 (red line) and low levels of FATP1 (black line) expression using Kaplan-Meier method. **(a)** Kaplan-Meier RFS curves for patients with breast cancer grade 1 (n=106), grade 2 (n=227) and grade 3 (n=444). **(b)** Kaplan-Meier survival curves for patients with grade 3, basal and ER – breast cancer (n=108) and grade 3, luminal and ER + breast cancer **(**n=86).

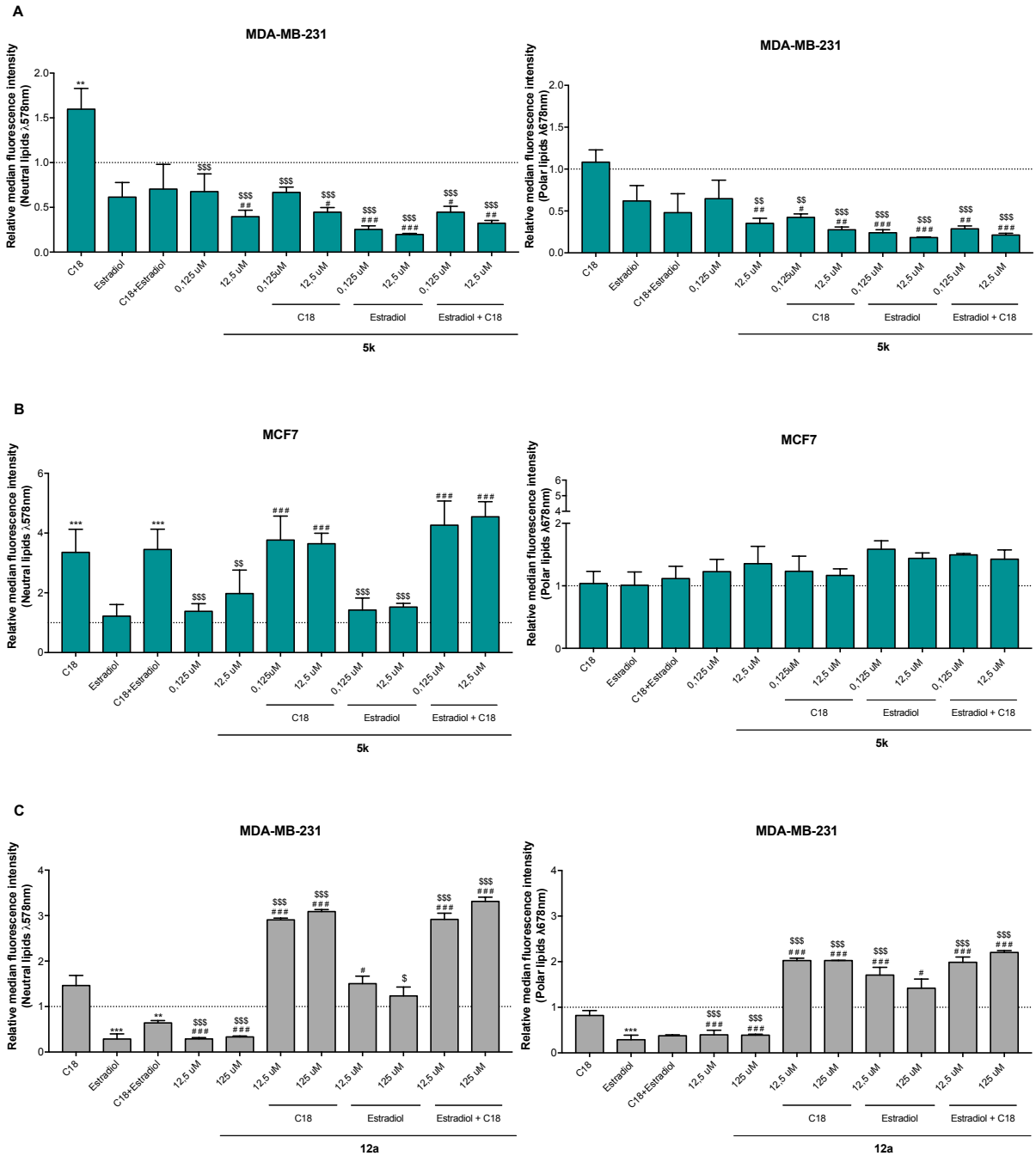
4.6 Arylpiperazines interfere with FA uptake

Our previous results proved that FA and estradiol are important in the regulation of *FATP1/SLC27A1* and that this gene is upregulated in more aggressive and invasive breast carcinomas. Thus, the pharmacological inhibition of FATP1 might be a strategy to fight cancer. To test this therapeutic approach, cells were exposed to arylpiperazines, compounds that act as FATP1 inhibitors, interfering with the transport of FA across the cell membrane.

The uptake of FA in cells exposed to arylpiperazines was assessed by flow cytometry using Nile red. Two arylpiperazines derivatives were tested, 5k and 12a in MDA-MB-231 and MCF7 cells cultured in control, control DMSO and estradiol with and without C18. For each derivative, two concentrations were used: for 5k, 0.125 and 12.5 µM, and for 12a, 12.5 and 125 µM. In figure 4.12 A, the uptake of neutral and polar lipids in MDA-MB-231 cells was significantly reduced by arylpiperazines when compared to the uptake in control cells (represented by the dot line) and in cells that were only exposed to C18. Both cell lines exhibited a lower uptake of neutral lipids when cells were cultured in estradiol and treated with 5k (4.12 A and 4.12 B). In MCF7, 5k alone did cause a lower uptake of FA but when

combined with C18 with and without estradiol an accumulation of lipids was observed (Figure 4.12 B). In terms of polar lipids, no differences were detected.

When cells were exposed to the 12a derivative alone, the uptake of lipids was effectively impaired comparing with control and C18 conditions (Figure 4.12 C). However, it caused a significant accumulation of both neutral and polar FA when MDA-MB-231 cells were cultured in C18, in the presence or absence of estradiol. In figure 4.12 D, MCF7 cells exhibited high levels of uptake of lipids when they were exposed to C18 in the presence or absence of estradiol, even in the presence of 12a. Neutral lipids were not affected with the addition of arylpiperazines. Hence, the 12a derivative had a strong effect in the accumulation of FA.



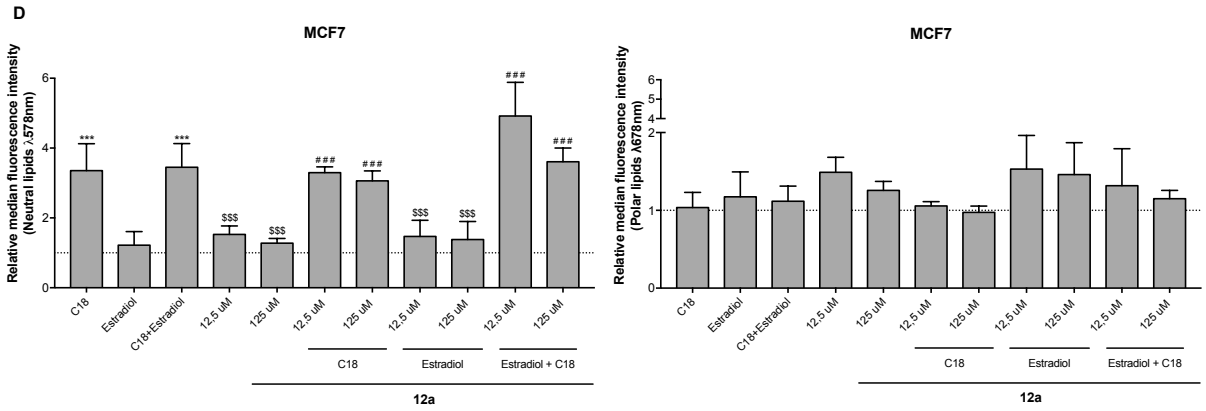
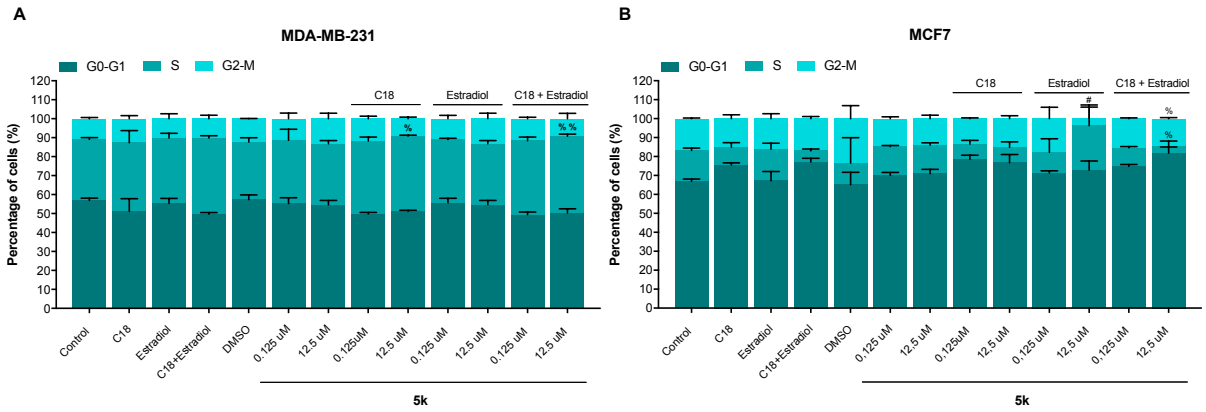


Figure 4.12 - Arylpiperazines interfere with FA uptake. Cells were cultured in control, control DMSO, estradiol with and without C18 and exposed to arylpiperazines (5k and 12a). (a) in MDA-MB-231 cells, 5k decreased the lipid uptake while in MCF7 (b) an accumulation of lipids was observed. (c) 12a compound led to an increased uptake of neutral and polar FA in MDA-MB-231. (d) Cells cultured in C18 condition accumulated significantly more FA, even in the presence of arylpiperazines. Data are means of triplicates. Results are shown as mean ± SD. *p<0.05, **p<0.01, ***p<0.001. (*) represents the statistical analysis in relation to the control condition; (#) statistical analysis in relation to the control (DMSO) condition; ($) statistical analysis in relation to the C18 condition. The dot line defines the relative median fluorescence intensity for the control conditions.

4.7 Arylpiperazines interfere with cell proliferation

In order to understand if arylpiperazines have an effect in cell proliferation, a cell cycle assay was performed by flow cytometry. MDA-MB-231 cells exposed to 5k did not suffer many alterations, only a slight increase in dividing cells (S + G2 phases) in cells cultured in C18 and 12.5 µM of 5k and also in cells cultured in C18 and estradiol exposed to 12.5 µM when compared to cells exposed to arylpiperazines alone (Figure 4.13 A). MCF7 cells did not divide as much as the MDA-MB-231, having a high percentage of cells in the G0/G1 phase. When exposed to 12.5 µM of 5k cells cultured in estradiol and C18 exhibited a decrease in proliferating cells (Figure 4.13 B). Thus, cells in which FATP1 was inhibited proliferated less. In figure 4.13 C, MDA-MB-231 cells cultured in C18 and/or estradiol with 125 µM of 12a displayed a dramatic reduction in proliferating cells. In MCF7 cells treated with 12a, no meaningful differences were observed (Figure 4.13 D).



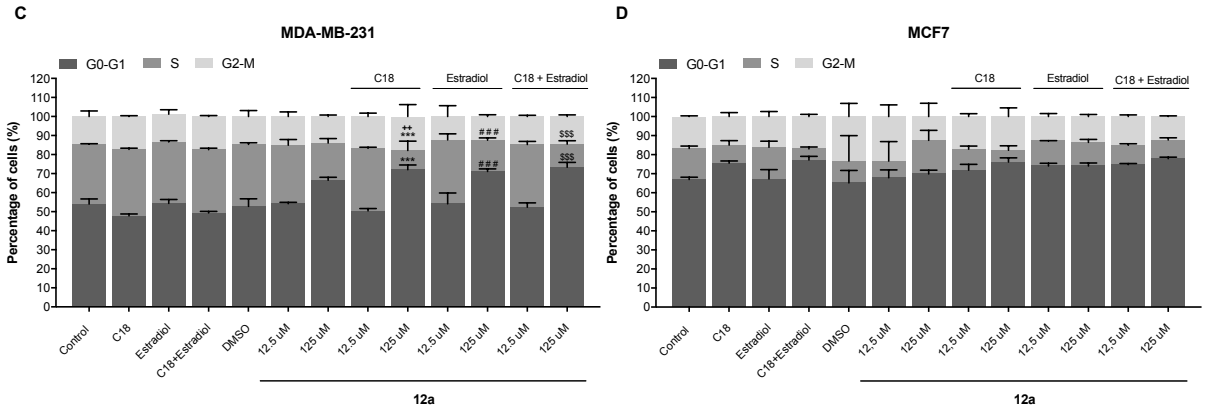
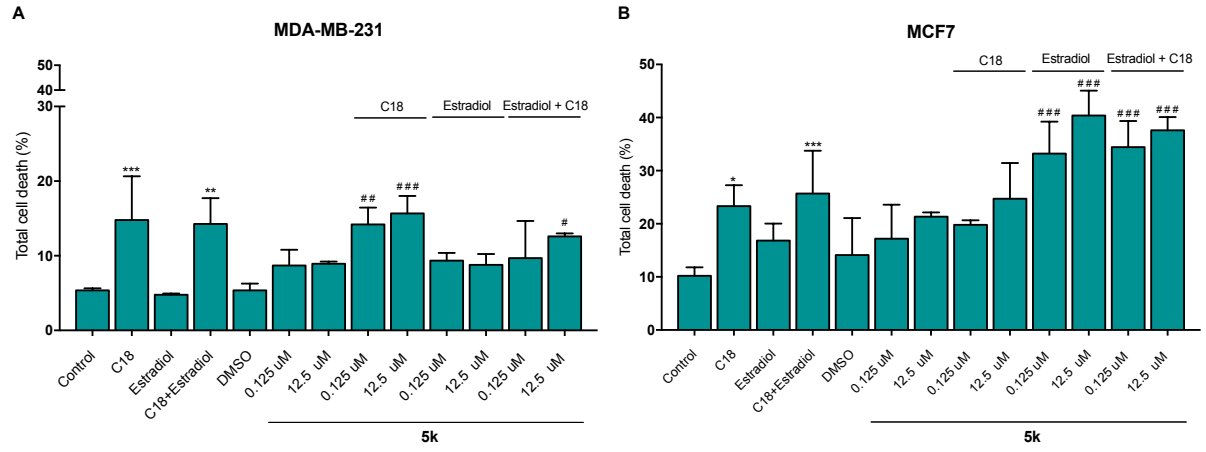


Figure 4.13 – Arylpiperazines interfere with cell proliferation. Cells were cultured in control, control DMSO, estradiol with and without C18 and exposed to arylpiperazines (5k and 12a). **(a)** 5k (12.5 μM) led to a slight increase in proliferation in MDA-MB-231. **(b)** In MCF7 cells exposed to C18 in combination with estradiol displayed a lower percentage of proliferative cells when treated with 12.5 μM 5k. **(c)** MDA-MB-231 cells exposed to 125 μM of 12a showed a significant reduction in cell proliferation when they were cultured in C18 and/or estradiol. **(d)** No differences were detected in terms of proliferation. Data are means of triplicates. Results are shown as mean ± SD. *p<0.05, **p<0.01, ***p<0.001. (*) represents the statistical analysis in relation to the C18 condition; (#) statistical analysis in relation to the estradiol condition; ($) statistical analysis in relation to the C18+estradiol condition; (%) statistical analysis in relation to the 5k 12.5 μM condition; (+) statistical analysis in relation to the 12a 125 μM condition.

4.8 Arylpiperazines effect on cell viability

With the intent of analyzing the effect of arylpiperazines in cell viability, a cell death assay was carried out. In figure 4.14 A, MDA-MB-231 cells, the total percentage of cell death was significantly higher in cells cultured in C18 and C18 plus estradiol, as well as with C18 in combination with the two concentrations of 5k. In the MCF7 cell line, similar results were obtained but the highest percentage of cell death was with 12.5 μM 5k in combination with estradiol, in the presence or absence of C18 (Figure 4.14 B). Concerning the 12a derivative, only higher concentrations (125 μM) caused increased cell death in MDA-MB-231 cells, which were cultured with C18 in the combination of estradiol and in separate (Figure 4.14 C). As for MCF7 cells, the exposition of C18 with and without 12a 125 μM also induced an increase in the levels of total cell death (Figure 4.14 D). Overall, MDA-MB-231 had a lower percentage of total cell death in comparison with MCF7.



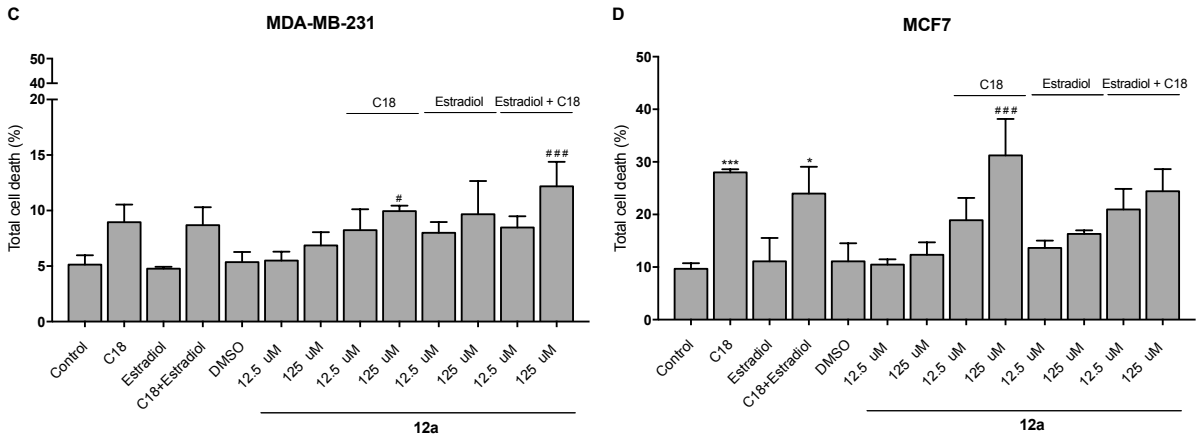


Figure 4.14 - Arylpiperazines effect on cell viability. Cells were grown in control, control DMSO, estradiol with and without C18 and exposed to arylpiperazines (5k and 12a). **(a)** MDA-MB-231 cells displayed higher levels of cell death in the C18 and C18+estradiol conditions, as well as C18 plus arylpiperazines. **(b)** MCF7 showed increased cell death in cells cultured in C18 in combination with arylpiperazines and estradiol. **(c)**12a effect was not very noticeable in MDA-MB-231 cells, being cell death most increased in cells exposed to C18+estradiol and 125 μM of 12a. **(d)** Cell death in MCF7 was upregulated in the C18, C18 plus estradiol and C18 plus 12a 125 μM conditions. Data are means of triplicates. Results are shown as mean ± SD. *p<0.05, **p<0.01, ***p<0.001. (*) represesents the statistical analysis in relation to the control condition; (#) statistical analysis in relation to the control (DMSO) condition.

5. Discussion

Cancer metabolic reprogramming has been recognized as one of the ten hallmarks by Hanahan and Weinberg in 2011 [4]. Metabolic remodeling leads to changes in biosynthetic and bioenergetic pathways not only in neoplastic cells but also in non-cancerous cells that share the same microenvironment [41]. This cellular and molecular network sustains the high demanding of energy and biomass production which are essential for cancer initiation and progression [69]. In breast and ovarian cancer cells it was stated that tumor microenvironment promotes tumor growth and provides a rationale for the development of targeted therapies that block cancer metabolism fueled by the microenvironment [83,109].

Cancer associated fibroblasts (CAFs) are the predominant cellular component in the tumor microenvironment [41,69,110], and have been shown to fuel tumor cells by producing and exporting high energy metabolites such as lactate, pyruvate and ketone bodies, which are used by adjacent cells [111]. Recently, our team [50] have shown that CAFs cooperate with breast cancer cells as FA suppliers, in *in vitro* and *in vivo* models, through the FA transport FATP1. Aberrant lipid metabolism has long been recognized as a major metabolic event during cancer development [48]. However, most studies have been focused on *de novo* lipogenesis as the source for FA required for tumor cell growth, being the role of exogenous FA often disregarded [47,112,113]. FA can also be acquired by alternate mechanisms as some studies have shown that cancer cells are able to internalize exogenous free FA to fuel their growth [114,115]. The present work focuses on the uptake of exogenous FA by breast cancer cells rather than on the *de novo* FA synthesis that has been extensively investigated. Furthermore, the main objective of this thesis was to disclose the role of FATP1 in breast cancer cells survival and behavior and to validate its use as a therapeutic target.

Our team [50] have shown that MDA-MB-231 exposed to CAFs exhibited a decreased FASN activity and an increased *FATP1/SLC27A1* transcriptional expression suggesting that breast cancer cell change their metabolic feature from FA producers to FA gatherers. Hence, our first approach was to verify the transcriptional expression of the *FATP1/SLC27A1* gene in MDA-MB-231 and MCF7 breast

cancer cells. We found that *FATP1/SLC27A1* mRNA levels were significantly upregulated within 30 minutes of exposure to C18+estradiol in MDA-MB-231 (Figure 4.1 A) and within 15 minutes of exposure to C18 in MCF7 cells (Figure 4.1 B) comparing to cells grown in control conditions. *FATP1/SLC27A1* mRNA levels suffered a boost in expression in the first minutes of exposure but then decreased with time, thus, this gene seems to be able to respond very quickly to exogenous stimulus. It was also found that the highly aggressive and invasive MDA-MB-231 cells exhibit a significant higher expression of FATP1 than the poorly aggressive and non-invasive MCF7 cells (Figure 4.1 C). In such manner, this result suggests that enhanced *FATP1/SLC27A1* expression is correlated with more aggressive phenotypes. Most studies have been investigating FATP1 in the context of diet induced obesity, insulin resistance hyperglycemia, hyperinsulinemia and hypertension [116–119]. To our knowledge, this is the first study that investigates the role of FATP1 in breast cancer. It was found that metabolic genes such as *SLC27* genes, including *FATP1/SLC27A1*, may be altered in metastatic cancer cells and are associated with poor prognosis [120]. Moreover, an expression array analysis demonstrated that *FATP1/SLC27A1* expression is up-regulated in human intrahepatic cholangiocarcinoma samples being these results validated by RQ-PCR analysis [115].

Concerning FATP1 protein levels, we found that they were visibly higher in MDA-MB-231 cells exposed to C18+estradiol (Figure 4.2 A) while in MCF7 there were no repercussions with the addition of estradiol or FA (Figure 4.2 B). The protein expression of FATP1 was evaluated in human melanoma patient biopsies and it was found that 44% of patients overexpressed FATP1 specifically in the tumor cell compartment [121]. *In vitro*, we showed that in CAFs, FATP1 and FAT/CD36 were expressed in higher levels than in breast cancer cells, contributing for the functioning metabolic network in cancer microenvironment [50]. On the contrary, a study showed that FATP1 alongside FAT/CD36 was downregulated in endometrial carcinomas as compared to healthy tissue, being accompanied by an upregulation of GLUT1, GLUT3 and GLUT4, suggesting that these carcinomas favor glucose metabolism and are less dependent of FA uptake [122].

The discovery of the estrogen receptor (ER) provided us with a powerful, predictive and prognostic marker and an efficient target for the treatment of hormone-dependent breast cancer with antiestrogens [123]. The analysis of the expression of estrogen receptors genes 1 and 2 (*ESR1* and *ESR2*) by RQ-PCR (Figure 4.3) showed that MDA-MB-231 cells, an ER- cell line, exhibited a significant expression of *ESR1* and *ESR2* when cells were exposed to estradiol in combination with C18 and in separate. MCF7 cell line (ER+) did not respond to the stimulus as the expression levels were higher in the control condition (Figure 4.3 B, D). We also found that MDA-MB-231 showed a significant upregulated expression of *ESR2* while MCF7 cells exhibited significant enhanced levels of ESR1. With these findings we can assume that FATP1 and *ESR2* seem to be a pro-survival factor in MDA-MB-231, whereas *ESR1* seems to be a pro-survival factor in MCF7 as its expression is higher and increases progressively with the starvation time.

Protein analysis of these receptors showed that ER-α (codified by *ESR1*) expression was higher after C18+estradiol exposure in MDA-MB-231 (Figure 4.4 A) which is in agreement with the RQ-PCR results. ER-β (codified by *ESR2*) protein levels were not altered with the addition of C18 or estradiol, not corroborating with the relative gene expression (Figure 4.4 B). In MCF7 no major differences were observed in both receptors between conditions and this cell line exhibited higher levels of ER-α levels in comparison with MDA-MB-231 cells, which is also in concordance with the mRNA levels (Figure 4.4 A and B). ER-β protein levels were greatest in the MCF7 cell line, which was not the case at the mRNA expression. These discrepancies may be due to possible post-translational modifications such as phosphorylation [124], acetylation [125], ubiquitination [126], sumoylation [127] and methylation [128] that have been identified as event modifying ER expression and stability, subcellular localization as well as sensitivity to hormonal response [129]. Particularly, two studies have shown that the phosphorylation of ER-β stimulates its degradation, possibly explaining our findings relatively to the poor protein levels of ER-β in MDA-MB-231 [130]. These results prove that MDA-MB-231 considered as a triple negative cell line

in the literature [131] expresses both estrogen receptors, especially *ESR2* and that its expression is influenced by the addition of FA. Other studies have already shown that this cell line under certain culture conditions express *ESR1* gene [132].

Interestingly, a study that investigated the role of estrogen on FA release in humans showed that estrogen had direct effects on adipose tissue lipolysis with the finding that estrogen deficiency increased systemic FA release/lipolysis [133]. Insulin was found to inhibit lipolysis of stored fat in the adipose tissue and gluconeogenesis in the liver [134] and to induce long-chain fatty acids (LCFA) uptake in adipocytes [135]. It has been shown that exposure to insulin stimulated FATP1 accompanied by an increased LCFA uptake in fat [96,136]. In such manner, as insulin inhibits lipolysis in the adipose tissue and stimulates the FATP1 protein, estrogen exposure might enhance the uptake of FA through FATP1.

The involvement of estrogen signaling pathway in *FATP1/SLC27A1* regulation demonstrated that ER-β binding to *FATP1/SLC27A1* promoter increased upon estradiol stimulation, being statistically significant in MCF7 cells, as for ER-α, only a trend to increase was observed in both cell lines (Figure 4.5). Our findings suggest that ER-β seems to be relevant in the modulation of *FATP1/SLC27A1* as well as a possible association with ER-β expression and mammary carcinogenesis. ER-α has been studied extensively in familial and sporadic breast cancers but there is limited information on ERβ and its isoforms. Studies have reported that ERβ2 expression was increased in invasive carcinomas in comparison with normal glands, in both ER-α-positive and ER-α-negative tumors [137] and that cytoplasmic ERβ2 expression correlated with shorter overall survival at 15 years [138]. Moreover, an association of ERβ1 expression with increased cell proliferation in ER-α-negative breast cancer was also described [139]. These evidences indicate that ER-β may have a role in the progression of breast carcinomas and could potentially serve as a prognostic marker.

With the intent of investigating further the role of ER-β in *FATP1/SLC27A1* regulation, cells were exposed to ERB-041, an ER-β agonist [140], and to PHTPP, an ER-β antagonist [141]. PHTPP, with and without C18, caused a significant increase in cell death in both cell lines (Figure 4.6). MDA-MB-231 cells displayed higher levels of apoptosis upon exposure to PHTPP than MCF7. A possible explanation for this is that MDA-MB-231 cells are more likely to depend on ER-β in order to function correctly since they have lower levels of ER-α, as mentioned before. The inhibition of ER-β strongly induced apoptosis suggesting the crucial role of ER-β in breast cancer cells. As expected, ERB-041 did not affect cell viability in neither cell lines. In terms of lipid uptake, MDA-MB-231 cells exposed to C18+estradiol showed a significant uptake of neutral lipids (Figure 4.7 A). When cells were exposed to PHTPP, the uptake of FA was decreased, being statistically significant in the polar lipids in both cell lines (Figure 4.7 A and B). In MCF7 cells, ERB-041 with and without estradiol induced a significant increase in the uptake of both neutral and polar lipids (Figure 4.7 B). However, when ERB-041 was combined with C18 the uptake decreased, indicating that in MCF7 the agonist of ER-β might be affected by estradiol but not by FA. C18 and estradiol separate exposure also led to a higher uptake of neutral lipids in MCF7 cells. These findings indicate that ER-β is important in the uptake of FA, since its inhibition decreased the levels of FA. We can also observe that C18 in combination with estradiol is associated with an enhanced uptake of FA that might be transported by FATP1.

The total percentage of FATP1 positive cells was significantly lower when cells were treated with the ER-β antagonist (PHTPP) in both MDA-MB-231 and MCF7 cells (Figure 4.8 A and C). At the same time, the percentage of FATP1 positive cells in the cell membrane was significantly higher when cells were exposed to PHTPP in both cell lines (Figure 4.8 B and D). These findings indicate that PHTPP is translocated to the membrane. In this assay, the ER-β agonist (ERB-041) did not have strong effects except in the membranar percentage of FATP1 positive cells in MCF7 that was significantly enhanced by ERB-041 (Figure 4.8 D). Cells exposed to C18 also showed more FATP1 positive cells in the membrane in both cell lines (Figure 4.8 B and D), which is in agreement with the higher levels of FA uptake observed in these conditions in figure 4.7. MCF7 cells displayed a significant amount of

membranar FATP1 positive cells when treated with C18 in combination with estradiol (Figure 4.8 D). These results demonstrate that a higher uptake of FA corresponds to an increased percentage of FATP1 positive cells, therefore, FA are likely to be transported by FATP1 across the cell membrane. When ER-β is impaired (after PHTPP treatment) it triggers apoptosis in breast cancer cells (Figure 4.6) and leads to a decreased uptake of FA (Figure 4.7) and to compensate, the percentage of FATP1 membrane positive cells increased significantly.

Cancer is essentially a mitogenic disorder, which requires cellular building blocks, such as nucleic acids, proteins, and lipids [45]. Wound healing results revealed an enhanced migration when cells were exposed to C18+estradiol (Figure 4.9 A and B). Estradiol alone also increased significantly migration however only in MCF7 cells (Figure 4.9 B). Moreover, MDA-MB-231 cells displayed a higher percentage of proliferating cells when they were exposed to C18 in combination with estradiol (Figure 4.9 C). The opposite happened in MCF7 cells when exposed to C18 with and without estradiol (Figure 4.9 D). All in all, these findings indicate that FA and estradiol induce migration and cell proliferation in MDA-MB-231. It was reported that adipocyte-derived FA enhanced breast cancer cells proliferation and migration [142], and that FA uptake into pancreatic cancer cells increased the migration and invasiveness [143] corroborating our results. Another study described that human LDL stimulated proliferation of ER- MDA-MB-231 cells, but had little effect on proliferation of ER+ MCF-7 cells [144] which is in agreement with our findings (Figure 4.9 C and D).

Kaplan-Meier curves revealed that patients with upregulated expression of *FATP1/SLC27A1* showed lower overall survival (OS) and relapse free survival (RFS) in comparison with patients with low expression of *FATP1/SLC27A1* and that was even more apparent in more advanced stages of the disease (grade 3) (Figure 4.10 and 4.11). Moreover, OS in grade 3 triple negative breast cancer patients (basal, ER-) with high *FATP1/SLC27A1* expression was dramatically lower than OS in grade 3, luminal, ER+ patients. These findings reveal that the *FATP1/SLC27A1* gene is undoubtedly relevant on the clinical outcome of breast cancer as more aggressive and invasive carcinomas (basal, ER-) exhibit a significantly upregulated expression of *FATP1/SLC27A1*. In that way, FATP1 could be considered as a marker for disease outcome.

Exploiting the features of cancer metabolism for cancer detection and treatment is a very promising strategy in cancer therapeutics, diagnosis and prevention [145]. In many tumors, lipids are provided via de novo lipogenesis, showing an upregulation of FASN particularly in melanoma, breast and prostate cancer [57,146,147]. Inhibition of FASN by either small molecules (C75, orlistat) or small interfering RNA can efficiently suppress tumor cell growth *in vitro* and xenograft models [148–150]. However, most FASN inhibitors show several adverse and side effects [60] and our group has found that MDA-MB-231 exposed to CAFs exhibited a decreased FASN activity and an increased FATP1 [50]. Accordingly, the pharmacological inhibition of FATP1 might be a strategy to fight cancer. Breast cancer cells were exposed to arylpiperazines (5k and 12a) that act as FATP1 inhibitors, interfering with the transport of FA across the cell membrane. We found that the uptake of neutral and polar lipids in MDA-MB-231 cells was significantly reduced by arylpiperazines (5k) when compared to the uptake in control cells and in cells that were only exposed to C18 (Figure 4.12 A). In MCF7, 5k alone did cause a lower uptake of FA but when combined with C18 with and without estradiol an accumulation of lipids was observed (Figure 4.12 B). When cells were exposed to the 12a derivative alone, the uptake of lipids was effectively impaired (Figure 4.12 C). However, it caused a significant accumulation of both neutral and polar FA when cells were cultured in C18, in the presence or absence of estradiol in both cell lines (Figure 4.12 C and D). Hence, the 12a derivative had a strong effect in the accumulation of FA while 5k significantly reduced the uptake of FA but only in MDA-MB-231. Therefore, the 5k compound might be a good inhibitor of FA uptake but only in cancer cells that express high levels of FATP1. However, more studies should be done to optimize 5k and to validate this finding. Understanding how free FA move across the cell membrane has proven to be a challenging biophysical and biochemical problem, which after 30 years of research is still only poorly understood and remains controversial [151]. FATP1 has been postulated to mediate bi-directional transport of LCFA across the plasma membrane of

adipocytes [152]. Hence, the 12a compound might be inhibiting the transport of FA to the extracellular *milieu* causing an accumulation of FA in the cell. Another possible explanation is that other FA transporters such as FATP4 or CD36 which are highly expressed in adipose tissue [153] might be upregulated since FATP1 function is impaired.

When exposed to 5k MCF7 cells cultured in estradiol and C18 exhibited a decrease in proliferating cells (Figure 4.13 B) and MDA-MB-231 cells cultured in C18 and/or estradiol with 12a displayed, as well, a dramatic reduction in proliferating cells (Figure 4.13 C). Therefore, cells in which FATP1 was inhibited proliferated less. Regarding cell viability, in MDA-MB-231 cells, the percentage of cell death was significantly higher in cells cultured in C18 and C18 plus estradiol, as well as with C18 in combination with arylpiperazines (5k). In the MCF7 cell line, similar results were obtained (Figure 4.14 B). Concerning the 12a derivative, only higher concentrations caused increased cell death in MDA-MB-231 cells, which were cultured with C18 in the combination of estradiol and in separate (Figure 4.14 C). As for MCF7 cells, the exposure to C18 with and without 12a 125 μM also induced an increase in the levels of total cell death (Figure 4.14 D). Overall, MDA-MB-231 had a lower percentage of total cell death in comparison with MCF7. Apart from adipocytes, most cells have a limited capacity for lipid storage and when this capacity is exceeded, as in the case of FA overload, cell death may result from a process called lipotoxicity [67]. This evidence might explain the higher cell death observed when only C18 was added. Despite the fact that the tested concentrations of arylpiperazines did not significantly induce apoptosis in these breast cancer cells, they could be used as an adjuvant therapy to sensitize breast cancer cells in combination with chemotherapy in order to kill cancer cells more efficiently.

In the literature, it was reported using a mouse model that the expression of FASN was frequently down regulated in intrahepatic cholangiocarcinoma (ICC) and a robust uptake of exogenous FA by ICC cells was found [115]. The same authors referred that in a pilot study, they found that suppression of *SLC27A1* by specific small interfering RNA decreased the *in vitro* growth of HUCCT1 and HuH28 ICC cell lines. In prostate cancer, increased FA uptake by cancer cells enhanced cell invasiveness *in vitro* and both primary tumor growth and distant metastasis *in vivo* [143]. Another very recent study demonstrated that adipocyte-derived lipids were taken up by FATP1 that are aberrantly expressed in melanoma [121]. They stained a series of freshly isolated tumors from both primary and metastatic sites (subcutaneous, lymph nodes, and brain) for both FATP1 and lipids using Oil Red O, which revealed a strong positive correlation for FATP1 versus Oil Red O staining meaning that FATP1 showed the ability to mediate the uptake of LCFA. Inhibition of FATPs with lipofermata (FATP2 inhibitor) decreased melanoma lipid uptake, invasion, and growth. These findings are in agreement with our results despite the use of a FATP2 inhibitor instead of arylpiperazines.

It has been hypothesized that a high-fat diet is associated with the development of postmenopausal breast cancer [154]. Our study also raises this question: do the dietary factors and obesity play a role in the progression of breast cancer? As FATP1 is an insulin sensitive transporter [96], it is in agreement with the use of anti-diabetic drugs in cancer treatment which is described by some studies [155]. Therefore, the use of arylpiperazines could eventually help with insulin resistance, diabetes or obesity.

In conclusion, in this thesis we unraveled the crucial role of ER-β in *FATP1/SLC27A1* regulation and modulation in the uptake of FA. Estradiol and an increased uptake of FA fuel migration and cell proliferation *in vitro*. Our *in vitro* findings are validated by patients' data showing a higher expression of *FATP1/SLC27A1* in more aggressive and invasive breast carcinomas which promotes the progression of the disease. The inhibition of FATP1 with arylpiperazines interfered with the uptake of FA and cell proliferation, consistent with the importance of FATP1 as a putative therapeutic target in breast cancer.

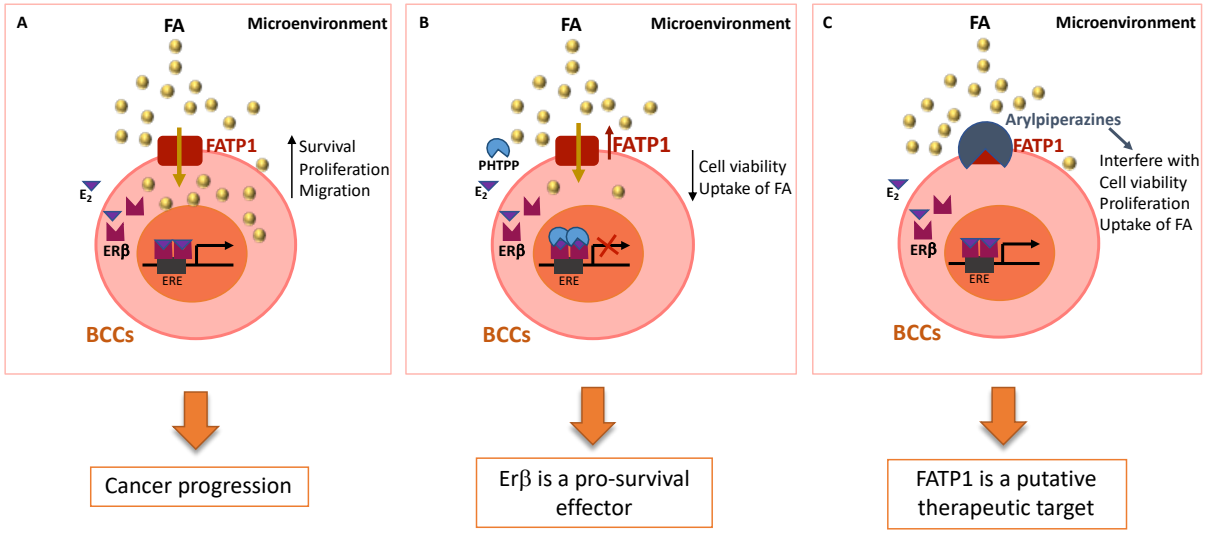


Figure 5.1 – Stimulation of estrogen receptor β (ER-β) by estradiol (E_2) contributes for *FATP1/SLC27A1* expression, cell survival, proliferation and migration of BCCs (breast cancer cells). FATP1 is also a pro-survival element, mediating the transport of fatty acids (FA), which are essential for BCCs **(a)**. BCCs exposed to PHTPP (ER-β antagonist) show a decreased cell viability and lower uptake of FA suggesting that ER-β controls *FATP1/SLC27A1* expression **(b)**. The inhibition of FATP1 with arylpiperazines interferes with cell viability, proliferation and with the uptake of FA, indicating that FATP1 is a putative therapeutic target in breast cancer **(c)**.

5.1 Future perspectives

As future perspectives, to understand the role of FATP1 in breast cancer biology and to eventually validate FATP1 as a marker for breast cancer prognosis and putative therapeutic target, it would be important to:

- perform a stable knockdown/knockout of *FATP1/SLC27A1*;
- perform a wound healing and proliferation assay with the ER-β antagonists (PHTPP) and agonists (ERB-041) and ER-α antagonists (fulvestrant and MPP);
- evaluate the percentage of FATP1 positive cells in the cell and in the membrane, lipid uptake and cell viability with ER-α antagonists (fulvestrant and MPP);
- validate of our results with a normal immortalized breast cell line (MCF10A);
- test the effect of other concentrations of arylpiperazines;
- test the effect of arylpiperazines in the cell cycle to assess the biological processes;
- perform a viability assay with arylpiperazines in combination with chemotherapeutic agents, and
- evaluate FATP1 expression in breast cancer sections by immunohistochemistry.

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Appendices

Solutions prepared for the experimental work:

10X PBS (pH 7.4-7.6)
For 1L: 80g NaCl (1.37M) (106404, Merck) 2g KH2PO4 (14.7mM) (104873, Merck) 11.1g Na2HPO4 (78.1mM) (S-0876, Sigma) 2g KCl (26.8mM) (104936, Merck) ddH2O to 1L

50 µg/mL Propidium Iodide (PI) solution – Cell cycle assay
For 50 mL: 1 mL of 2.5 mg/mL PI solution (P4170, Sigma) (prepared in 1X PBS) 49 mL 1X PBS 0.1 mg/mL RNase A (Easy spin kit, Citomed) 0.05% Triton X-100 (T8787, Sigma)

50 µg/mL Propidium Iodide (PI) solution – Apoptosis assay
For 50 mL: 1 mL of 2.5 mg/mL PI solution (P4170, Sigma) (prepared in 1X PBS) 49 mL 1X PBS

Annexin binding buffer 1X
0.01 M Hepes (pH 7.4) (391333, Millipore) 0.14 M NaCl (106404, Merck) 2.5 mM $CaCl_2$ (449709, Sigma)

RIPA buffer
For 10 mL: 20 mM Tris-HCl pH 7.5 150 mM NaCl (106404, Merck) 5mM KCl (104936, Merck) 5mM MgCl2 (M-8266, Sigma) 1% Triton X-100 (T8787, Sigma) ddH2O to 10 mL 1 Complete, Mini, EDTA-free Protease Inhibitor Cocktail Tablet (11836170001, Roche) 1 mM Orthovanadate (Na3VO4) 1 mM Sodium fluoride (NaF) (201154, Sigma)

Transfer buffer
For 5L: 75g glycine (US16407, USB) 15g Trizma-base (T-8524, Sigma) ddH2O to 4L 1L Metanol (107018, Merck) 5mL 10% SDS (V6551, Promega)

5X SDS gel loading buffer (Sambrook & Russel, 2001)
250 mM Tris HCl (pH 6.8) (0.5M 161-0799, Bio-rad) 10% SDS (V6551, Promega) 0.5% bromophenol blue

PBS 0.1% (v/v) Tween 20
For 1L: 1mL Tween 20 (20605, USB) 1X PBS to 1L

5% (w/v) skim milk in PBS 0.1% (v/v) Tween 20
5g skim milk (Molico, Nestlé) 100 mL PBS 0.1% (v/v) Tween 20

3% (w/v) skim milk in PBS 0.1% (v/v) Tween 20
3g skim milk (Molico, Nestlé) 100 mL PBS 0.1% (v/v) Tween 20

PBS 0.1% (w/v) BSA
0.1g BSA (A9647, Sigma) 100 mL 1X PBS

CHIP lysis buffer
50 mM Tris-HCL (pH 8.0) 10 mM EDT 1% SDS 1 Complete, Mini, EDTA-free Protease Inhibitor Cocktail

Supplementary Table 1 – Primers sequences used during the experimental work

Primer	Forward (5´-3´)	Reverse (5´-3')
FATP1	CAACATGGACGGCAAGGTC	CAGCAGCTCCATTGTGTCCTC
ESR1	GCCAGGCTTTGTGGATTTGAC	GGAGCAAACAGTAGCTTCCC
ESR2	GATCTTGTTCTGGACAGGGATG	GGAATTGAGCAGGATCATGGC
HPRT	TGACACTGGCAAAACAATGCA	GGTCGTTTTTCACCAGCAAGCT
FATP1 promoter	GACTGTTGTAAGATTGGCAGGG	CTGGGATTGGTCAACTCCTC