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Metabolic reprogramming of oestrogen receptor positive breast cancer in endocrine therapy resistance

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Abstract

The majority of breast tumours express oestrogen receptor (ER) and are dependent on oestrogen (E2) for their growth and survival. Endocrine therapy is the standard of care for this breast cancer subset and acts by targeting ER pathway in different ways: selective ER modulators compete with E2 to bind ER (e.g. tamoxifen), selective ER downregulators promote ER degradation (e.g. fulvestrant) and aromatase inhibitors (AI) block E2 biosynthesis. Despite the efficacy of these endocrine agents, a large proportion of women relapse with endocrine-resistant disease. In this study, we investigated the link between altered breast cancer metabolism and endocrine therapy resistance. We found that AI-resistance cells can adapt to metabolic stress and switch *ad hoc* between OXPHOS and glycolysis. In particular, we identified the miR-155/hexokinase-2 (HK2) axis as an important regulator of this tumour plasticity. In addition to central carbon metabolism, we found a deregulated node between miR-23b-3p and the amino acid transporter SLC6A14 in endocrine therapy resistant cells, which leads to an impairment of amino acids metabolism in the resistant cells with subsequent activation of autophagy. Furthermore, the miRNA characterised have prognostic (miR-155 and miR-23b-3p) and predictive (miR-155) value in ER positive breast cancer. These results suggest that high metabolic plasticity is involved in acquiring adaptive features that allow breast cancer cell survival even in the presence of endocrine therapy.

Abbreviations used in thesis

2-DG	2-Deoxy-Glucose
ACC	Acetyl-CoA carboxylase
ACLY	ATP citrate lyase
ACSS2	Acyl-CoA synthetase 2
AF	Activation Function
Ago	Argonaute protein
AI	Aromatase inhibitors
AKT	Protein Kinase B, PKB
AMPK	AMP-activated protein kinase
ASS	Arginosuccinato synthase
ATGs	Autophagy-related genes
ATP	Adenosine 5-triphosphate
Bcl-2	*B-cell lymphoma 2*
BECN1	Beclin-1 gene
BIF-1	BAX-interacting factor 1
BSA	Bovine serum albumin
CBP	CREB-binding protein
CCND1	Cyclin D1
CDK	Cyclin Dependent Kinases
CI	Combination index
CIC	Protein citrate carrier
CK	Cytokeratins
CLL	Chronic lymphocyte leukemia
c-Met	Hepatocyte Growth Factor Receptor
CV	Cristal Violet
DCC	Dextran charcoal treated
DCIS	Ductal Carcinoma *in situ*
DMSO	Dimethyl sulfoxide
dsRBD	Double-strand RNA-binding domain

E1	Oestrone
E2	Oestradiol, or 17β-oestradiol
E3	Oestriol
ECL	Enhanced chemiluminescence
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-Mesenchymal Transition
ER	Oestrogen Receptor
EREs	Oestrogen Response Elements
ERK	Extracellular-signal-regulated kinase
FA	Fatty Acids
FANS	Fatty acid synthase
FBP	Fructose-1-6-biphosphate
FBS	Foetal bovine serum
FDG	^{18}F-fluorodeoxyglucose
FOXO3a	Forkhead box O3A
FSH	Follicle-Stimulating Hormone
GAB3	GRB2 Associated Binding Protein 3
GAPDH	Glyceraldeid-3-phosphate dehydrogenase
GDH	Glutamate Dehydrogenase
GDP	Guanosin-Di-Phosphate
GLS	Glutaminase
GLUTs	Glucose transporters
GSA	Glutamic-γ-semi-aldehyde
GTP	Guanosine Tri-Phosphate
HAT	Histone Acetyltransferase
HCC	Hepatocellular carcinoma cell
HER2	Human Epidermal Growth Factor 2
HIF1-α	Hypoxia-inducible factor 1 α
HK	Hexokinase
HRP	HorseRadish Peroxidase
HTR	Hormone Replacement Therapy

ICI	Fulvestrant
LBD	Ligand Binding domain
LCIS	lobular carcinoma *in situ*
LDH	Lactate Dehydrogenase
LDs	Lipid droplets
LH	Luteinising Hormone
LKB1	Liver Kinase B1
MAPK	Mitogen-Activated Protein Kinase
MCTs	Monocarboxylate transporters
Met	Metformin
miRNA	microRNA
MPC	Mitochondrial Pyruvate Carrier
mTOR	Mammalian target of rapamycin
mTORC1	Protein kinase complex mTOR complex 1
NADH	Nicotinamide Adenine Dinclueotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCOA1	Nuclear-Receptor Co-activator 1
NCOR	Nuclear-Co-Repressor
NISCH	Nischarin
ORF	Open Reading frame
OXPHOS	Oxidative phosphorylation
P5C	α-pyrroline-5-carboxylate
PAK2	P21 (RAC1) Activated Kinase 2
PBS	Phosphatase Buffered Saline
PCAF	p300/CBP-associated factor
PCR	Polymerase Chain Reaction
PDAC	Pancreatic Ductal Adenocarcinoma
PDCD4	Programmed cell death protein 4
PDGH	Phosphoglycerate Dehydrogenase
PDH	Pyruvate Dehydrogenase complex
PDK1	Pyruvate Dehydrogenase Kinase 1

PE	Phopshatidyl ethanolamine
PEP	Phosphoenolpyruvate
PET	Positron Emission Tomography
PFK1	6-phosphofructokinase
PI3K	Phosphoinositide 3 Kinase
PI3P	Phopshatydilinositol-3-phosphate
PKM1	Pyruvate Kinase M1
PKM2	Pyruvate Kinase M2
PR	Progesterone Receptor
PRODH/POX	Proline dehydrogenase (oxidase)
PTEN	Phosphatase and tensin homolog
qRT-PCR	Quantitative real-time reverse transcription PCR
RAB6A	Ras-Related Protein Rab-6A
Rb	Retinoblastoma protein
RhoA	Ras homolog family member A
SDS	Sodium Dodecyl Sulphate
SERMs	Selective ER Modulators
SHMT	Serine Hydroxymethyl Transferase
SOCS1	Suppressor of cytokine signalling 1
SREBP-1	Sterol regulatory element-binding protein 1
STAT3	Signal transducer and activator of transcription 3
SWI/SNF	Switch/Sucrose Non-Fermenting
TAM	Tamoxifen
TCA	Tricarboxylic Acid Cycle
TIMP3	Metallopeptidase Inhibitor 3
TP53INP1	Tumour protein 53-induced nuclear protein 1
TRAP/DRIP/SMCC	Thyroid-Hormone-Receptor-Associated Protein
TSC2	Tuberous Sclerosis Complex 2
ULK1	Autophagy activating kinase 1
uPA	Urokinase-Type Plasminogen Activator
VDAC	Voltage Dependent Anion Channel

WB	Western Blotting
α-KG	α-ketoglutarate

Introduction

Breast cancer is the second most common cancer in the world and the most frequent cancer among women with an estimated 1.67 million new cancer cases diagnosed in 2012, representing the 25% of all cancers. Incidence rates are very different across the world regions, with rates ranging from 27 per 100000 people in Middle Africa and Eastern Asia to 96 in Western Europe. In Europe, approximately 464000 new cases were diagnosed in 2012, in particular ~50000 cases in Italy. Breast cancer ranks as the fifth cause of all the cancer related death with 522000 cases/year; it is the leading cause of cancer death in women in less developed regions (324.000 deaths, 14.3% of total) and the second cause of cancer death in more developed region (198000 deaths, 15.4%) after lung cancer. In Europe, breast cancer deaths were ~130000 in 2012 of which 12000 in Italy. In Western Europe and United States, breast cancer mortality rate is lower than incidence rate with respect to undeveloped regions, because of the majority availability of diagnostic technologies and cares in developed regions, which allow an increase of survival and better prognosis of breast cancer patients (1, 2).

The breasts are composed of fat, connective tissue and gland tissue and are divided into lobes. A network of tubular structures (ducts) originates from the lobes and collectively culminates into the nipple (Figure 1). The breasts composition changes during lifetime: pre-menopausal women have more glandular tissue, whereas in post-menopausal women the glandular tissue is gradually replaced by fat. Breast tissue covers a large area of the chest. It extends from just below the clavicle to the axilla and across to the sternum. The breast is characterised by many blood and lymph vessels. The lymph vessels collect and move lymph fluid away from the breast into the small bean-shaped masses of lymphatic tissue, called lymph nodes, in the area around the breast. Lymph nodes are located all-round the breast tissue, but the axillary lymph nodes are most important and are divided into

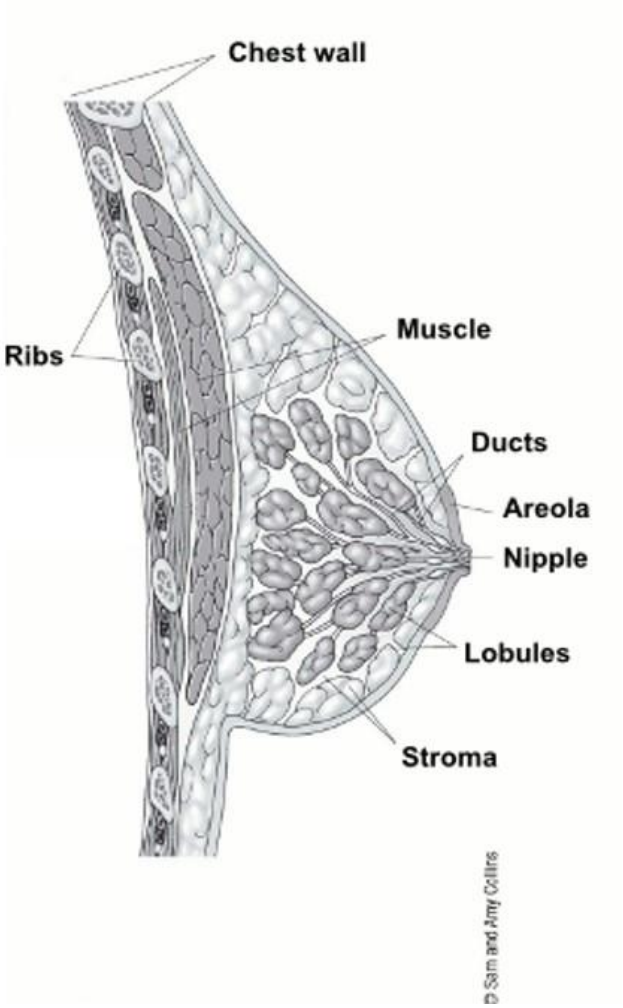


Figure 1. Anatomy of the female breast

three levels according to how close they are to the pectoral muscle. The lymph vessels and lymph nodes are part of the lymphatic system, which has a crucial role during extravasation of cancer cells. Once cancer cells leave the primary site, they can arrive to the axillary lymph nodes through the lymph vessels and from there they can invade to other tissues and metastasize. (Figure 2).

Adenocarcinoma is the most common type of breast cancer and originates from breast glandular tissue. Depending on the site of origin, breast tumours are classified as ductal carcinoma and lobular carcinoma, localized in the breast ducts and lobules, respectively. Ductal carcinoma can be defined *in situ* (DCIS) when the tumour is localised inside glandular tissue and invasive ductal carcinoma when cancer cells invade the proximal

lymph nodes and metastasise to other part of the body. In addition, lobular carcinoma can be classified as lobular carcinoma *in situ* (LCIS or lobular

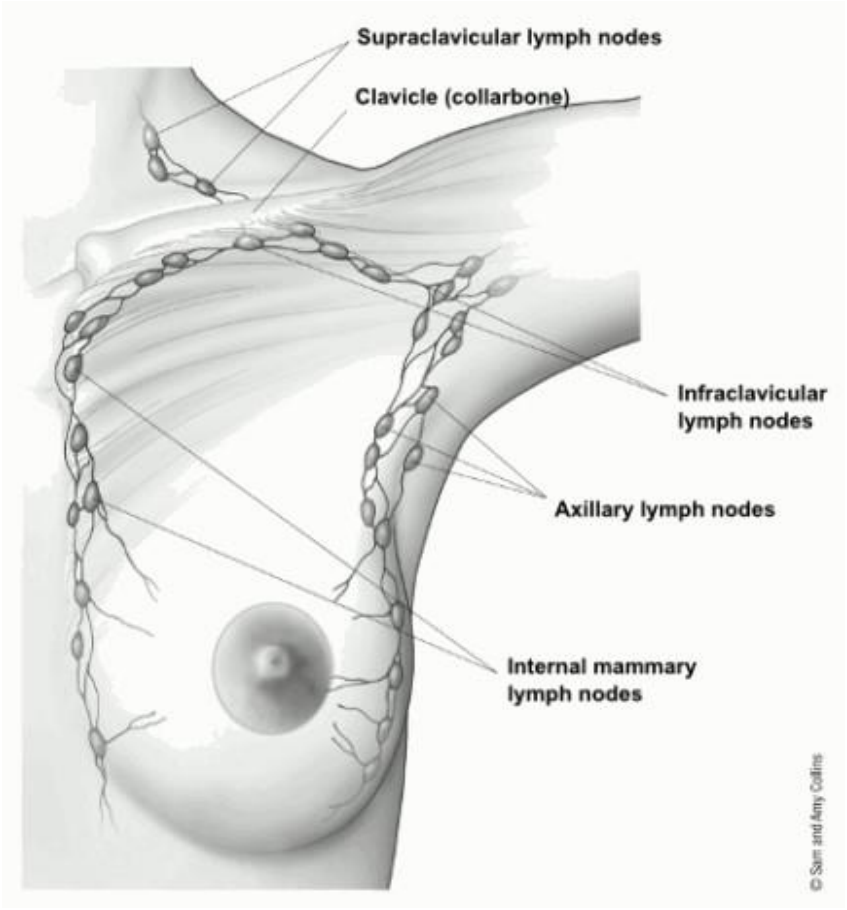


Figure 2. Breast lymphatic system.

neoplasia) and invasive lobular carcinoma, when the cancer cells have already invaded the surrounding tissue. Around 90% of breast carcinomas diagnosed are ductal carcinomas, and around 10% are invasive lobular carcinoma, which is most common in women between 45 and 55 years old. Furthermore, there are other less common malignant breast tumours, such as inflammatory breast cancers, Paget's disease of the nipple and phyllodes tumours.

Following breast cancer diagnosis and staging of tumour based on the cancer size and the presence of cancer cells in lymph nodes, breast cancer patients are treated with specific therapy. When possible, the patient undergoes surgery, followed by radiotherapy, chemotherapy (e.g.

antracycline), biological therapy (antibody against epidermal growth factor receptor HER2, trastuzumab), hormonal therapy (endocrine therapy) or a combination of treatments, depending on the genetic and/or molecular profile of the cancer.

1- Molecular subtypes of breast cancers

Breast cancer is a highly heterogeneous disease. Over the last decades, genomic, transcriptomic and proteomic analyses were applied to identify new molecular markers with prognostic and predictive value to better determine the appropriate therapy.

Two distinct types of epithelial cells compose the human mammary gland: basal (and/or myoepithelial) cells in contact with the basement membrane and luminal epithelial cells that are in continuum with the basal cells and are polarized culminating/facing the lumen. These breast cells are characterised by specific cytokeratins (CK) expression. In particular, luminal epithelial cells are characterised by the expression of CK 8, 18 and 19, while basal cells express CK 5/6, 14 and 17 (3). Expression profile studies showed two main groups of breast cancer based on oestrogen receptor α (ER) expression: ER positive (ER+) breast cancer characterised by high ER expression and ER negative (ER-) breast cancer characterised by low or absence of ER levels (4). Moreover, breast cancer can be classified into four different phenotypes based on the gene expression profile. These subtypes are associated to specific tumour characteristics and clinical outcomes. Accordingly, they are defined as ER+/ Luminal-like, basal like, HER2 positive (HER2+), characterised by overexpression of HER2neu/ ERBB2 oncogene, and Normal like. It is important underling that the clinical designation of ER- breast carcinoma encompasses at least two biologically distinct subtype of tumours, basal like and HER2+ (4). Subsequent studies demonstrated that the ER+ luminal subtype can be divided into additional different subgroups, according to their distinct expression profiles: luminal A, characterised by high expression levels of ER and ER-related genes; luminal B that show, in addition to ER, high expression of a set of genes related to proliferation and the cell cycle; and a new heterogeneous subtype denominated luminal C, which show a more

aggressive evolution than luminal A or B subtypes (5). Moreover, the biological behaviour of breast cancer is correlated with their gene expression profile, indicating that ER- tumours have a worse prognosis with reduced overall and clinical survival compared to ER+ tumours (5). In the clinical practice, three biomarkers are usually analysed to evaluate the subtype of a given breast cancer, that is, ER, progesterone receptor (PR) and HER2 (6). Taking these markers into consideration, we can correlate ER, PR and HER2 expression to different molecular subtypes. Indeed, luminal A are ER+ and/or PR+, HER2-; luminal B are ER+ and/or PR+, HER2+; HER2 type are ER-, PR- and HER2+; basal like are negative for ER-, PR- and HER2- and are also called triple negative. In addition to ER, PR and HER2, the basal like group can be defined more precisely by antibody staining against to typical basal CK 5/6 and epidermal growth factor receptor (EGFR) (7). These molecular subtypes have specific prognosis and clinical outcome, described in figure 3 (8).

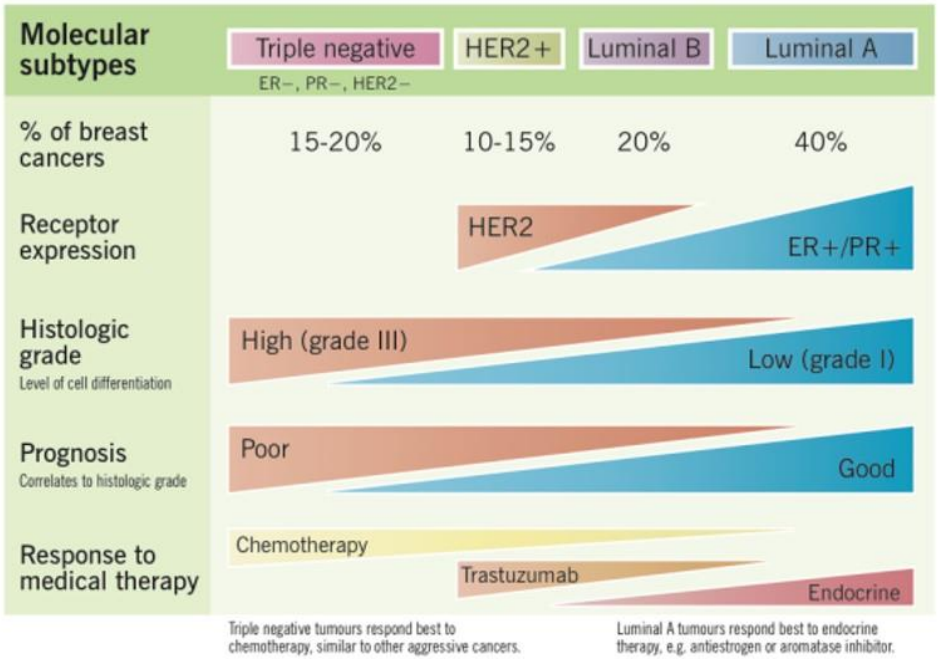


Figure 3. Molecular subtypes and clinical outcome of breast cancer.

2- ER positive breast cancer

Oestrogens stimulate the proliferation and the growth of the epithelial cells of normal human breast (9). Approximately 10-15% of luminal epithelial cells of the mammary epithelium express ER at detectable levels. In normal human breast, ER+ cells do not proliferate, although they are often in close proximity to proliferating cells. Interestingly, oestrogens stimulate the proliferation of ER- epithelial cells through the secretion of paracrine factors by surrounding ER+ cells (10, 11). In contrast, in human breast tumours, ER+ cells are proliferating and their proliferation is directly regulated by oestrogens (10). Approximately 75% of breast cancers are ER+ (12) and they are dependent on oestrogens for their survival and proliferation. About two-third of ER+ tumours regress after oestrogen deprivation by endocrine therapy (13).

2.1 Oestrogen and breast cancer risk

Oestrogens have a key role in the aetiology of breast cancer due to their proliferative effects. Exposure to oestrogens is associated with an increased risk of breast cancer (14). Factors that correlate with increased risk include early menarche, late first full-term pregnancy, late menopause and the use of hormone replacement therapy (HRT), all of which likely enhance lifetime breast cancer risk by increase of exposure to oestrogens (15, 16). The molecular aspects underlying increased breast cancer risk due to oestrogen exposure are not fully understood. This prolonged exposure could increase cell proliferation, thus enhancing the errors associated with DNA replication. Additionally, oestrogen metabolites can have a genotoxic effects (17). In addition, prolonged exposure to other hormones involved in the oestrogen signalling, such as prolactin (18), progesterone (14) and testosterone (19) may have a role in the increased breast cancer risk.

2.2 Oestrogens synthesis

Oestrogens are a class of steroid hormones synthesised from cholesterol. Oestrogen physiological effects are mediated by ER, which acts as transcription factor regulating the expression of different genes (20). There are three major forms of physiological oestrogens in females: oestrone

(E1), oestradiol (E2, or 17β-oestradiol), and oestriol (E3). E2 is the major product from the whole biosynthesis process and is the most potent oestrogen during the premenopausal period in a woman's life. Oestrogens play key role in the development and maintenance of female sexual and reproductive function and regulate physiological process in the cardiovascular, skeletal, immune and central nervous system (21). In addition to these roles, oestrogens are also involved in the development and progression of breast cancer.

In premenopausal women, oestrogens synthesis occurs predominantly in the ovaries and is stimulated by follicle stimulating hormone (FSH) and luteinising hormone (LH), the pituitary gonadotropins (22). Androgen hormones produced by theca cells are transported to the granulosa cells where they are converted into oestrogens, in a reaction catalysed by the aromatase enzyme. Ovarian synthesis of oestrogen ceases at menopause, when the main source of oestrogens is no longer the ovaries. In post menopause the major oestrogens synthesis occurs in distal organs, including bone, adipose tissue, the vascular endothelium, aortic smooth muscle and the brain. This localised production has an important role in tumour progression in post-menopausal women (23). Indeed, in these type of patients, intratumoral concentration of E2 are more than 20-fold higher than those present in the plasma. This is probably because also breast tumour tissue concurs with the other tissues in converting androgens into oestrogens (24, 25).

2.3 Mechanisms of oestrogen action

Oestrogens action is mediated by two oestrogen receptors, respectively receptor α (ERα) and receptor β (ERβ). ERs belong to the nuclear receptor superfamily and act as ligand dependent transcription factors. ERs contains six structural domains, which are defined by the putative functions contained in each region (Figure 4) (26).

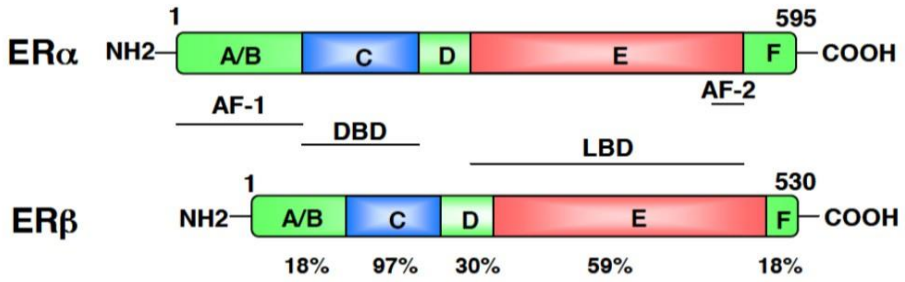


Figure 4. Schematic representation of human ERα and ERβ. Both receptors contain six functional domains (A-F) including the DNA Binding Domain (DBD), the Ligand Binding Domain (LBD) and both Activating Function domains 1 and 2 (AF1, AF2). The percentage amino acid similarity between ERα and ERβ is indicated for ERβ.

The domains are: the highly conserved *DNA-binding domain* (DBD), which contains two zinc finger motifs that permit ER binding to DNA, and the *ligand binding domain* (LBD) which mediates oestrogen binding. Moreover, there are two additional domains with transcriptional *activation functions* (AFs), known as AF1 and AF2. The first regulates the ligand-independent transcriptional activation in response to phosphorylation mediated by downstream signalling events orchestrated by growth factors, including Mitogen-Activated Protein Kinase (MAPK) and Protein Kinase B (PKB or AKT). Conversely, AF2 is ligand dependent and regulates the transcriptional activation upon oestrogen binding (26, 27). ERα and ERβ show the 96% of amino acid identity in their DBD and only 53% homology in their LBD, the latter could explain the difference in the response of the two receptors. The characterisation of ERα and ERβ in knockout mice has revealed distinct, non-redundant, role for ERβ. Particularly, ERβ seems to have opposing proliferation related effects when compared to ERα (28-30). In particular, some ERβ splice variants act as dominant-negative effectors of ERα (31). In this context, it is interesting to note that during the proliferative phase of pregnancy in rats, mammary epithelial cells express only one of the two ERs isoforms, whereas up to 60% of epithelial cells co-express the two receptors during the non-proliferative, oestrogen insensitive lactational phase (32). Despite these differences, both ERs seem to have similar affinity for oestrogens and bind to the same DNA response elements (33). Several studies show that ERβ is expressed in

breast cancer, but its role is highly controversial (34, 35). Since it is well characterised the role of ERα and its mechanism of activation, here I will limit my discussion to ERα, hereafter called ER.

After entering cells, oestrogen binds to and activates ER. Oestrogen binding results in a conformational change that enables oestrogen-regulated genes to be activated. The ER binds as a dimer to small palindromic DNA motifs, known as oestrogen response elements (EREs), in the promoters of specific genes, through the action of two zing fingers (36). Two distinct activation domains, AF1 and AF2, mediate transcription activation. AF2 is integral to the ligand-binding domain (LBD) and its activity requires the binding between LBD and oestrogen, whereas AF1 activity is regulated by phosphorylation (Figure 5) (37, 38). AF1 and AF2 activate the transcription independently and/or synergistically and there is evidence that AF1 and AF2 activities are influenced by the promoter and cell type (39). ER activates gene expression by stimulating recruitment of the general transcription machinery to the transcription start site through the action of its activation domains.

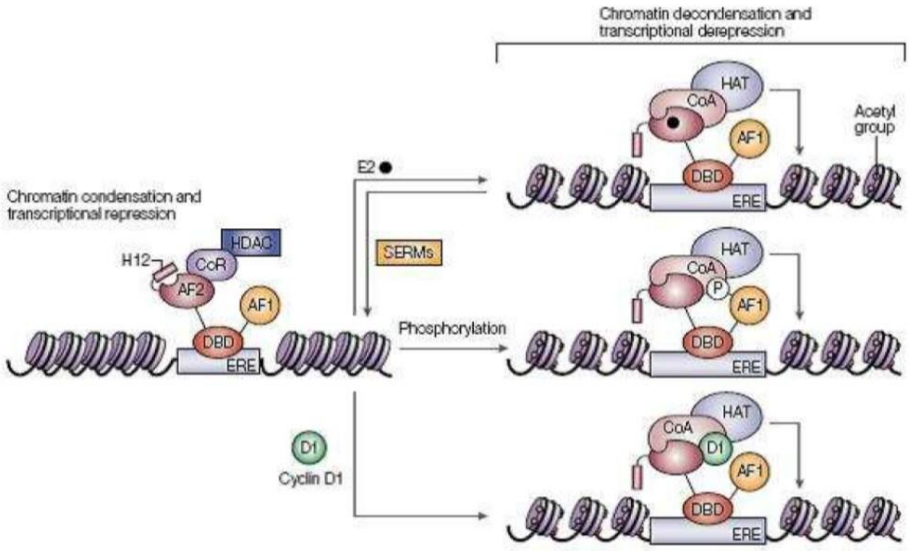


Figure 5. Mechanisms of oestrogen-receptor activation. The oestrogen receptor (ER) has three domains: AF1, which is regulated by phosphorylation; AF2, which is regulated by oestogen binding; and a DNA binding domain (DBD). In the inactivated state, the ER is bound to corepressor (CoR) complexes, which recruit histone deacetylases (HDACs). HDACs maintain histones in a deacetylated state, which favours chromatin condensation. Oestrogen binding results a conformational change in AF2 that facilitates interaction with co-activators (CoA), which bind histone acetyltransferases (HATs). Acetylation of histones by HATs leads to chromatin decondensation, facilitating transcriptional activation. Modulation of

ER activity by selective ER modulators (SERMs) is likely to be achieved by a balance between coactivator and corepressor complex recruitment to AF2, depending on the conformation induced by the SERM, as well as tissue-specific differences in co-activator/corepressor availability. Other factors, such as cyclin D1 and growth-factor-induced phosphorylation of AF1, might facilitate ligand-independent recruitment of co-activators. ERE, oestrogen response element; H12, helix 12 (taken from 40).

Different studies have identified co-activator complexes that mediate trans-activation by nuclear receptors. These co-activators facilitate recruitment of the general transcription machinery through direct interaction but, importantly, several co-activator complexes also mediate chromatin remodelling (41, 42). These include: the SWI/SNF complexes (switch/sucrose non-fermenting), which facilitate transcription-factor binding to nucleosomal DNA by ATP dependent chromatin remodelling (43, 44); the TRAP/DRIP/SMCC complex, these alternative names stand for thyroid-hormone-receptor-associated protein (TRAP); vitamin-D-receptor-interacting protein (DRIP), and SRB and mediator-protein containing complex (SMCC), which associates with RNA polymerase II63; CREB-binding protein (CBP), and p300/CBP-associated factor (PCAF), which are histone acetyltransferase (HAT) complexes. Hyperacetylation of histones seems to correlate with more actively transcribed regions of the genome than to hypoacetylated regions. Therefore, histone acetylation through recruitment of HATs might be crucial in overcoming the repressive effects of chromatin on transcription (45, 46). Three related co-activators, collectively known as the p160 co-activators, stimulate ER activity following ligand stimulation, through direct interaction with AF2 (41, 42). These three proteins, known as nuclear-receptor co-activator 1 (NCOA1; also known as SRC1) NCOA2 (also known as TIF2 or GRIP1) and NCOA3 (also known as P/CIP, ACTR, AIB-1, RAC3 or TRAM1) associate with the transcription factor CBP to facilitate histone acetylation (47, 48). Furthermore, NCOA1 and NCOA3 can themselves acetylate histones (43, 44, 49). Several nuclear receptors that are not bound to the cognate ligands, such as the thyroid hormone receptor, repress gene expression. This repression is mediated, at least in part, through recruitment of histone deacetylase complexes (HDACs) by interaction of nuclear receptors with nuclear-receptor corepressor 1 (NCOR1) or NCOR2 (also known as SMRT) (50-52). NCOR recruitment, like that of co-activators, is mediated by the

LBD/AF2, albeit in the absence of ligand. Therefore, ligand binding results in dissociation of co-repressors and recruitment of co-activator complexes. Other histone-modifying proteins, such as arginine methyltransferases, act as co-activators for nuclear receptors, including ER, either through direct interaction with the LBD (53) or through association with co-activators (54, 55). The LBD is encoded by about 300 amino acids. Structural studies have shown that it is a wedge-shaped structure that contains the ligand-binding pocket. Ligand binding results in a remarkable conformational change in the LBD, that induces the exposition of a surface for the recruitment of co-activators. Co-activators recruitment to the LBD is mediated by a short motif, characterised by the amino-acid sequence leucine–X–X–leucine–leucine (where X is any amino acid), which forms an α-helix (56-58). Different studies identified *cis*-regulatory domains that augment transcription of these ER gene targets. Specifically, ER association with gene targets results from an association with the pioneer factor FoxA1, responsible for recruitment of ER to the genome. Recruitment of ER to the genome does not seem to occur at the promoter proximal regions, but instead involves distal enhancer elements that function together with the ER complex at the promoter of the target genes (59).

Many of the genes regulated by oestrogen signalling promote tumorigenic phenotypes, including cell proliferation, inhibition of apoptosis, invasion and metastasis, and angiogenesis (60). Of particular importance are Cyclin D1 (61, 62) and c-Myc (63, 64), both of which are essential drivers of oestrogen stimulated cellular proliferation and tumorigenesis. Cyclin D1 binds to and activates cyclin dependent kinases (CDK) 4/6, which phosphorylate the retinoblastoma protein (Rb), resulting in release of the E2F transcription factor, and progression through the restriction point within the G1 phase of the cell cycle (65, 66). Inhibition of Cyclin D1, either with antibodies or by expression of its inhibitor, CDK4 p16INK, prevents oestrogen stimulation of cellular proliferation and progression through the G1 checkpoint (67). c-Myc is a proto-oncogene transcription factor that regulates the expression of a large number of target genes that promote cell growth and cell cycle progression (68, 69). Similarly to Cyclin D1, inhibition of c-Myc prevents oestrogen stimulated cell proliferation (70). Conversely, overexpression of Cyclin D1 or c-Myc can mimic the effects of

oestrogen by reinitiating cell cycle progression in anti-oestrogen arrested cells (71). These results indicate that Cyclin D1 and c-Myc are essential drivers of oestrogen stimulated cellular proliferation.

Since ER positive breast cancers are dependent upon oestrogen for their growth and progression, this type of tumour can be treated with endocrine therapies that deprive cells of ER signalling, resulting in tumour inhibition.

3- Endocrine therapy

ER+ breast cancer cells are dependent to oestrogens for their growth and proliferation. The hypothesis that oestrogen could have a crucial role in the tumour progression dates back to 1936. In that year, Lacassagne demonstrated in mice with high incidence of mammary cancer that ovariectomy or oestrogen replacement prevents or enhance tumorigenesis, respectively (72). However, the mechanism of tumour inhibition was not elucidated until the role for the ovarian hormone oestrogen in stimulating breast cancer growth was discovered (73). The discovery of ER and the development of an assay quantifying ER expression in patients with breast cancer made possible to identify women likely to respond to endocrine therapy (20, 74). Endocrine ablation by ovariectomy in pre-menopausal patients has now been replaced by pharmacological agents, generally called selective ER modulators (SERMs). SERMs are structurally different compounds that interact with intracellular ERs in target organs as oestrogen receptor agonists or antagonists. These drugs have been intensively studied over the past and have been proven to be a highly versatile group for the treatment of different conditions associated with post-menopausal women's health including osteoporosis and hormone responsive cancer (75, 76). The first SERM developed was MER25, non-steroidal antioestrogen capable of blocking oestrogen action (77). However, MER25 was unsuccessful due to toxicity issue (hallucination) and the first successful oestrogen antagonist to enter the clinic was tamoxifen (ICI 46474) (78). Although SERMs have many benefits, they also have some potentially serious adverse effects, such as thromboembolic disorder and, in the case of tamoxifen, uterine cancer.

3.1 Tamoxifen

Tamoxifen binds to ER and inhibits ER signalling, limiting cellular proliferation of breast cancer cells (79, 80). The major metabolites of tamoxifen in human are *N*-desmethyltamoxifen and trans-4-hydroxytamoxifen; the affinity of the latter for ER is equivalent to that E2 (81). The anti-tumour effects of tamoxifen are mediated by competitive inhibition of oestrogen binding to ER (82) and by recruitment transcriptional co-repressor (e.g. NCoR) instead of co-activator (83). As a consequence, tamoxifen inhibits the expression of oestrogen-dependent genes, including growth factor and angiogenic factor secreted by cancer cell that in turn may stimulate tumour growth by autocrine or paracrine mechanism (84). The net result is a block in the G1 phase of cell cycle and subsequent reduction of the cell proliferation rate. Furthermore, it has been shown that tamoxifen may also directly induce apoptosis (85).

Tamoxifen has been the mainstay endocrine therapy in breast cancer for the last 25 years. Compelling data have demonstrated a significant overall survival benefit; in patients with ER+ breast cancer, tamoxifen treatment results in a 51% reduction in recurrence and a 28% reduction in death as well as improved quality of life for patients with metastatic disease (86). Tamoxifen has also been shown to be effective in reducing the incidence of breast cancer in patients at risk for developing the disease (87) and in women with ductal carcinoma *in situ* (88).

Despite the documented benefits of ER-targeted therapy in breast cancer, it is known that not all patients who have ER expressing tumours respond to endocrine manipulation (*de novo* resistance) and a substantial number of patients who do respond will develop disease progression or recurrence while on therapy (acquired resistance): all patients with metastatic disease and 40% of early stage breast cancer patients treated with adjuvant tamoxifen, eventually relapse with tamoxifen resistant disease (89).

Tamoxifen is classified as a SERM; although tamoxifen acts as an ER antagonist in the breast, it exerts agonistic effects in some tissues such as the endometrium and the vascular system. This agonistic activity is associated with rare yet life-threatening side effects such as thromboembolic events and uterine cancer (87, 90). Whether tamoxifen

acts as an antagonist or agonist of ER signalling depends on the cellular context. As discussed previously, ER contains two domains that regulate transcriptional activation, AF1 and AF2 (Figure 5). AF2 acts in a ligand dependent manner, whereas AF1 is largely controlled by phosphorylation and as such tamoxifen only inhibits AF2 activation. Since ER activity in the breast is mainly AF2 driven, tamoxifen acts largely as an antagonist. This is in contrast to other tissues, such as the uterus, where ER activity is also controlled by AF1, resulting in greater agonistic activity of tamoxifen (91).

3.2 Aromatase inhibitors

Aromatase is an enzyme belonging to the cytochrome P-450 superfamily (92) and is highly expressed in the placenta and in the granulosa cells of ovarian follicles, where its expression depends on cyclical gonadotropin stimulation. Aromatase is also present, at lower levels, in several non-glandular tissue, including subcutaneous fat, liver, muscle, brain, normal breast and breast cancer tissue (93, 94). After menopause, oestrogen source derived exclusively from non glandular tissues, in particular from subcutaneous fat. In postmenopausal patients, an alternative to oestrogen antagonists for endocrine therapy are aromatase inhibitors (AI), which block the conversion of androgen to oestrogen by aromatase inhibition. Therefore, treatment of postmenopausal women with AI results in oestrogen deprivation and reduced ER signalling (95). Three generations of AI have been developed. The first- (aminoglutethimide) and second-generation AI (e.g., fadrozole and vorozole) were less selective and in addition to aromatase, they decreased aldosterone and cortisol production. These drugs were poorly tolerated and had limited clinical efficacy (96). Third-generation AI such as letrozole, anastrozole or exemestane, are highly selective for the enzyme aromatase and are well tolerated from the patients. Anastrozole and letrozole are nonsteroidal inhibitors that reversibly bind aromatase, whereas exemestane is a steroidal AI that irreversibly binds aromatase (97). These drugs are effectively challenging tamoxifen for use in postmenopausal patients with ER+ breast cancer (98). The clinical benefits associated with AI include significantly greater disease-free survival, a longer median time to recurrence, and a reduced incidence of contralateral breast cancer. Indeed, AI markedly supress

plasma oestrogens levels in postmenopausal women and in contrast with tamoxifen, AI have no partial agonist activity reducing incidences of thromboembolic events, vaginal bleeding, and endometrial cancer (99). As a consequence, aromatase inhibitors are increasingly being used for the endocrine treatment of postmenopausal ER+ breast cancer patients. Despite clinical benefits of AI, up to 50% of treated patients develop resistance to AI (100), again limiting effectiveness.

3.3 Fulvestrant

Oestrogen ablation therapy has been intensively used as a mean of treating ER+ breast cancer: antioestrogen (e.g. tamoxifen) and AI are now established as first–line agents in adjuvant endocrine therapy of ER+ breast cancer patients. Although adjuvant endocrine therapy is an effective treatment for breast cancer, most patients with advanced disease will eventually exhibit resistance to individual therapy (101). However, an initial response to endocrine treatment is generally indicative of a positive response to further alternative endocrine agents (102). Consequently, therapeutic options against ER+ breast cancer have expanded tremendously. In contrast to tamoxifen, which exhibits partial antagonist activity, fulvestrant (or ICI 182,780) is a “full” or “pure” anti-oestrogen that has no known oestrogen agonist effects (WO 2001051056 A1, Astrazeneca 2001). Fulvestrant exerts its antitumor activity preventing the oestrogen-ER interaction, thus abrogating the oestrogen–regulated transcription pathway (103, 104). Its binding affinity for ER is higher than tamoxifen (103, 105). Following binding to ER, fulvestrant blocks dimerization of the receptor and limits its nuclear translocation (106-108). Furthermore, fulvestrant-ER complex is instable and more susceptible to degradation by proteasome (109). Fulvestrant also blocks the recruitment of both transcriptional activating factors, AF1 and AF2 (109). As a result, in contrast to tamoxifen, which blocks recruitment of AF-2 only, fulvestrant exhibits full ER antagonist and no agonist effects (109). Fulvestrant has similar efficacy to tamoxifen as a first line therapy in patients with advanced ERα positive breast cancer (110) and has been shown to be as effective as the aromatase inhibitor anastrozole as a second line therapy in patients whose disease has progressed on prior endocrine therapy (110). It has been

demonstrated that fulvestrant monotherapy may be superior to AI in patients who have not received adjuvant endocrine therapy and in patients that present inoperable locally or advanced cancer and treated in first line setting (111). Fulvestrant monotherapy is associated with less arthralgia, but the combination with AI increase the risk of hot flashes and gastrointestinal disturbance (111). Despite its usefulness, resistance to fulvestrant also occurs frequently (112). These findings have stimulated the search for new mixed SERMs, which are anti-oestrogenic for the breast, but oestrogenic for other tissues, in which the protective actions of oestrogen are desirable (113).

4- Endocrine therapy resistance

Despite the relative safety and significant anti-neoplastic activities of endocrine therapies, the major limitation remains *de novo* and acquired resistance to endocrine agents. Although clinicians are encouraged from positive effects of second- and third-line endocrine therapy for patients who initially benefited from first line treatment (114), the clinical response rate declines from approximately 70% for first line-therapy fulvestrant or AI to around 30% in the successive lines of treatments (115, 116). Several approaches have been used to elucidate the mechanisms that drive endocrine therapy resistance to discover predictive markers and/or alternative targets that could be investigate for therapeutic approach. However, the molecular mechanisms that underlie resistance are not fully understood and as such, definitive approaches for preventing and overcoming resistance are not yet available. Several mechanism have been associated with endocrine therapy resistance, such as mutations of *ER* (*ESR1*), growth factors driven signalling cross talk, cell cycle alteration, enhanced autophagy or epigenetic aberration (Figure 6).

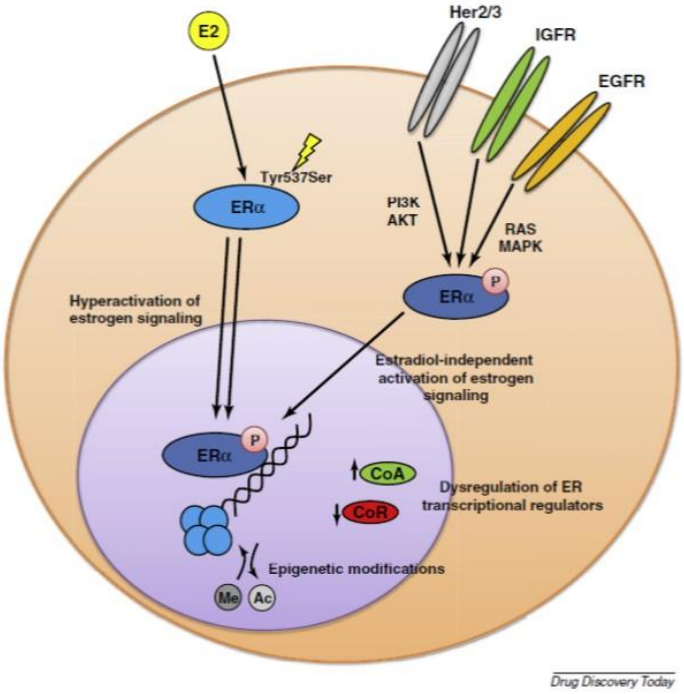


Figure 6. Schematic representation of major endocrine-resistance mechanisms. Several mechanisms have been shown to contribute to endocrine therapy resistance, which encompass hypersensitivity to E2 stimulation, phosphorylation of the ER by several kinase cascades, such as phosphoinositide 3 kinase (PI3K) or mitogen-activated protein kinase (MAPK), which in turn can be activated by tyrosine kinase receptors. Furthermore, changes in the expression of transcriptional regulators of the ER transcriptional complex are responsible for increased expression of ER-responsive genes (taken from 117).

4.1 *ESR1* mutations

Although loss of ER expression may be a reasonable explanation for the emergence of endocrine therapy resistance, loss of ER occurs in only 10% of primary and metastatic tumours that show the resistance (118). Therefore, ER remains a potential target in the majority of the endocrine resistant cancers. It has been reported that in solid tumours, the resistance to "oncoreceptor"-targeted therapies, such as tyrosine kinase inhibitors, is frequently driven by the emergence of additional mutations in the target oncogene (119). A similar molecular mechanism might underpin resistance to therapies targeting the ER. Specifically, attention has recently focused on mutations in the gene *ESR1*. A recent work has revealed that *ESR1* mutations are more frequent in patients bearing metastatic breast cancers or that have already received a therapeutic intervention (120). Highly

recurrent mutations were noted at two residues in the LBD of the receptor: Y357S and D538G (Figure 7) (121-123).

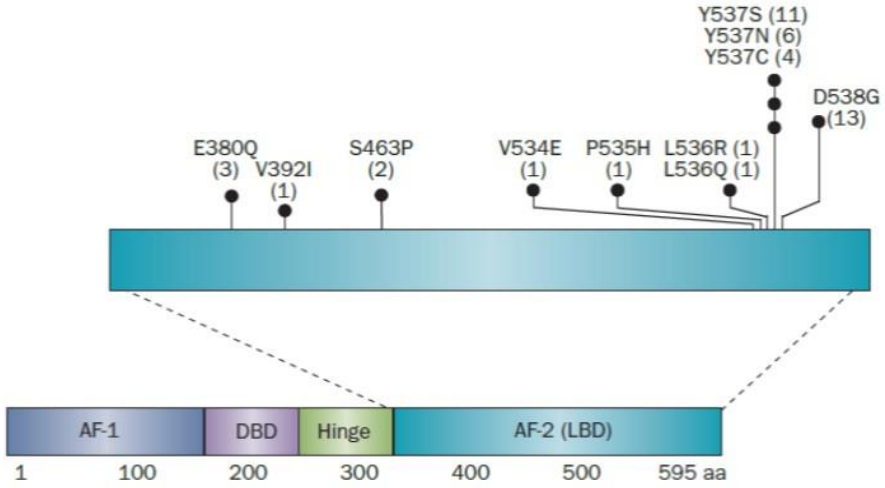


Figure 7. Structural diagram of the ERα protein encoded by the *ESR1* gene. Schematic representation of the ER protein and its functional domains; the position and number of cases (*n*) of the *ESR1* (ER) LBD point mutations reported in metastatic ER+ breast cancers are indicated. Black circles indicate each mutation at the specific amino acid residue; numbers in parentheses indicate the total number of samples reported to harbour the specific indicated mutations. Abbreviations: AF-1, activation function-1; AF-2, activation function-2; DBD, DNA-binding domain; ERα, oestrogen receptor α; LBD, ligand-binding domain (taken from reference (124).

These mutations generally are observed in 10-30% of all endocrine-resistant advanced breast cancers and have been linked to enhanced sensitivity to oestrogen as well as to the constitutive activation of transcriptional activity of ER in the absence of an ER agonist (121-123, 125-127). These mutations seem to be more common only after exposure to one or more lines of endocrine treatments (in particular AI) (121, 128), as highlighted by paired analysis of primary tumours and their metastatic therapy-resistant counterparts (128, 129). Furthermore, gene expression of *ESR1*-harboring breast cancer cells show dysregulation of both ER-dependent and ER-independent genes, suggesting that *ESR1* mutants alter the natural landscape of ER interaction network, or arise together with other resistance mechanisms (128). However, since *ESR1* mutations could be the indication of the emergence of endocrine therapy resistant clones, monitoring *ESR1* mutation status in patients that are undergoing endocrine

therapy might help clinicians to prevent and combat therapy resistance (128-130).

4.2 Growth factor receptors: PI3K/AKT/mTOR and MAPK pathway activation

Overexpression and/or amplification of growth factor receptor including FGFR1, HER2, HER3, EGFR, IGF1R and RET are associated with the emergence of endocrine therapy resistance (131-135). The activation of growth factor receptors can induce the phosphorylation of ER and AIB1 through cross talk mechanism, which have been shown to empower oestrogen signalling and induce tamoxifen resistance (136-139). Furthermore, their signalling pathways converge on the Phosphoinositide 3 kinase (PI3K)/AKT/ mammalian target of rapamycin (mTOR) and MAPKs pathway. Several kinases belonging to MAPK family, such as ERK1/2 and ERK3, can phosphorylate ER (e.g. Ser-118), prompting ligand-independent activation of the receptor and altering the response to endocrine therapies (89, 136, 140, 141). PI3K and AKT also have a role in the activation of ER in absence of oestrogen trough of the AF-1 (PI3K) and AF-2 (PI3K and AKT) domains of the receptor ((142). *PI3K* gene is mutated in ~40% of human breast cancers (143, 144). These mutations promote a PI3K pathway hyper-activation that induces oestrogen-independent ER transcriptional activation (145). mTOR is a key downstream effector of PI3K/AKT pathway involved in important cellular processes, such as protein synthesis and cellular metabolism (146). Therefore, mTOR has become an attractive target for therapies attempting to reverse the endocrine therapy resistance. Accordingly, randomized Phase III BOLER2 trial has shown that the combination of mTOR inhibitor everolimus with the AI exmestane improve progression-free survival in ER+ breast cancer patients previously treated with non-steroidal AI (BOLERO2 clinical trial.gov number NCT00863655, (147). However, it has been reported that combinatorial treatment can promote several toxicity issues in breast cancer patients. Therefore, we should consider the real benefits of the combination in relation to toxicity before that patients undergo to these treatments. Other approaches to revert endocrine therapy resistance are based on combination of endocrine agents with selective PI3K inhibitors

(https://www.clinicaltrials.gov/ct2/show/NCT02340221, accessed online 18 January 2016) and FGFR, EGFR or IGFR inhibitors (Https://www.cliniclatrials,gov/ct2/results?term=%22FGFR+AND+%22breast+cancer%22&recr0Open accessed online 28Febraury 2016; https://www.clinicaltrials.gov/ct2/show/NCT02115282, accessed online 19 January 2016)

4.3 Cell cycle checkpoint alterations

Normal and cancerous cells receive a plethora of proliferative and antiproliferative signals and the balance of these inputs determines whether a cell will undergo cell division or will enter into quiescent phase (148). The deregulation of the cell cycle progression via alterations of key cell cycle checkpoints can also contribute to endocrine therapy resistance (149). Tumour suppressor Rb is a negative regulator of the cell cycle able to mediate antiproliferative signals. Rb itself is regulated by a complex of cyclin and CDK, a family of serine-threonine kinases (150). Progression through the G1-S phase requires the phosphorylation of Rb by CDK4 in complex with cyclin D1, D2 or D3 (151). Rb hyper-phosphorylation leads to an increase of genes synthesis whose products are essential for DNA replication and mitotic progression (152). Many tumours increase cyclin D-dependent activity and thereby escape senescence via multiple mechanisms such as CDK4 amplification, CDK4 mutations, cyclin D1 translocation, amplification or overexpression (153). Cyclin D1 amplification is a common event in ER+ breast cancer, identified in 58% of luminal B cancers and 29% luminal A cancers (154). Anti-oestrogen induced growth arrest in ER+ breast cancer cells is accompanied by decreased cyclin D1 expression, whereas there is a persistent cyclin D1 expression and Rb phosphorylation in the case of the endocrine therapy resistance (155, 156). The first drugs developed to target cell cycle progression abnormalities in human cancer were non-selective pan-CDK inhibitors (157, 158). The therapeutic potential of this strategy in breast cancer is increased by development of highly selective inhibitors of CDK. Accordingly, Palbociclib is a small-molecule inhibitor of CDK4 and CDK6, and preclinical data have shown that it was able to inhibit the growth of ER+ breast cancer and reverse endocrine therapy resistance (159). These results have led to a

clinical trial, the so-called PALOMA-1 (Palbociclib: Ongoing Trials in the Management of Breast Cancer). This trail has demonstrated that the combination of palbociclib with letrozole as first-line therapy in ER+ breast cancer is more effective when compared to letrozole alone, displaying a longer-progression free survival for patients subjected to such combinatorial treatment (160). Later, PALOMA-2 clinical trial has shown that palbociclib combined with letrozole treatment represent a good therapeutic approach for postmenopausal women with ER+/HER-2 advanced breast cancer. Indeed, PALOMA-2 has confirmed the same results for postmenopausal women with ER+/HER2-advanced breast cancer (PALOMA-2 clinicaltials.gov number NCT01740427, (161).

4.4 Enhanced autophagy

Autophagy is an intracellular process leading to the degradation of damaged or unnecessary subcellular organelles. This process represents a key mechanism for survival of normal and cancer cells during stress condition, such as nutrient deprivation. A recent work has demonstrated that autophagy inhibition is linked to endocrine therapy sensitivity restoration, promoting cell apoptosis in preclinical models of endocrine-resistant breast cancer (162). The inhibitors of autophagy are currently explored in early phase trials in breast cancer. In the advanced endocrine-resistant breast cancer setting, hydoxychloroquine is being evaluated in combination with endocrine therapy in a phase Ib/II study (https://www.clinicaltrials.gov/ct2/shoeìw/NCT02414776, accessed online 23 May 2016).

4.5 Epigenomic signature

Changes in gene expression are not uniquely dependent on the presence of mutations but can also derived from changes in DNA methylation patterns and histone modifications, a process named epigenetics (163). Endocrine therapy has been shown to alter the epigenetic landscape of tumour cells by downregulating oestrogen-responsive genes (164). Recent studies showed that compared to sensitive tumours, endocrine therapy resistant breast cancers are characterised by a differential gene methylation pattern in the enhancer regions of oestrogen-dependent genes, which are in turn involved in different cellular process, such as apoptosis

regulation, endoplasmic reticulum Golgi trafficking and DNA damage response (165). Studies investigating breast cancer epigenetic alterations after endocrine therapy have reported dysregulation in the expression of genes involved in key cellular pathways, including metabolic processes, nucleoside transport and development process (166, 167). These results show that epigenetic deregulation of ER and its responsive genes largely contribute to endocrine resistance, although other mechanisms exist, as previously described.

In conclusion, there are several mechanisms involved in endocrine therapy resistance and new pharmacological approaches are investigated. Clinical trials suggest that ER+/HER2+ tumours may benefit of the combining treatment of AI with HER2-targeting compounds (168, 169). Several clinical trials also suggest that subsets of patients with ER+/HER2- breast cancers may benefit from a combination of a growth factor pathway inhibitor with endocrine therapy agents (147). However, given the adaptability of cancer cells, targeting a single growth factor or downstream signalling hub can lead to compensatory mechanism and the fail of therapeutic approaches. Further studies are necessary to understand other mechanisms of resistance in order to develop other therapeutic strategy to limit endocrine therapy resistance challenge.

5- Tumour metabolism

The hallmarks of cancer are the different biological capabilities that cancer cells acquire during tumour development and progression (Figure 8) (170). Genomic instability is the most important driver of the cancer cells alterations, which affect several cellular process, such as proliferation, senescence, survival signalling and apoptosis (170, 171). Metabolic deregulation is an established hallmark of cancer. To support the enhanced proliferation and uncontrolled cell division, most cancer cells exhibit metabolic adaptations that promote their survival and progression under non physiological conditions. Therefore, although cellular transformation occurring in different cell type arises from many different pathways, the metabolic reprogramming of cancer cells is similar (172). The requirements

of proliferative cancer cells are essentially to generate energy, in the form of adenosine 5-triphosphate (ATP), and to sustain macromolecules biosynthesis, while managing the high oxidative stress levels that accompany a rapid cell growth. Cancer cells are surrounded by different cell components of the tumour microenvironment that contribute to the acquisition of hallmarks traits. The tumour microenvironment influences tumour metabolism by exerting additional selective pressure on the cancer cells to adapt to harsh conditions, such as hypoxia, acidity and/or nutrient starvation.

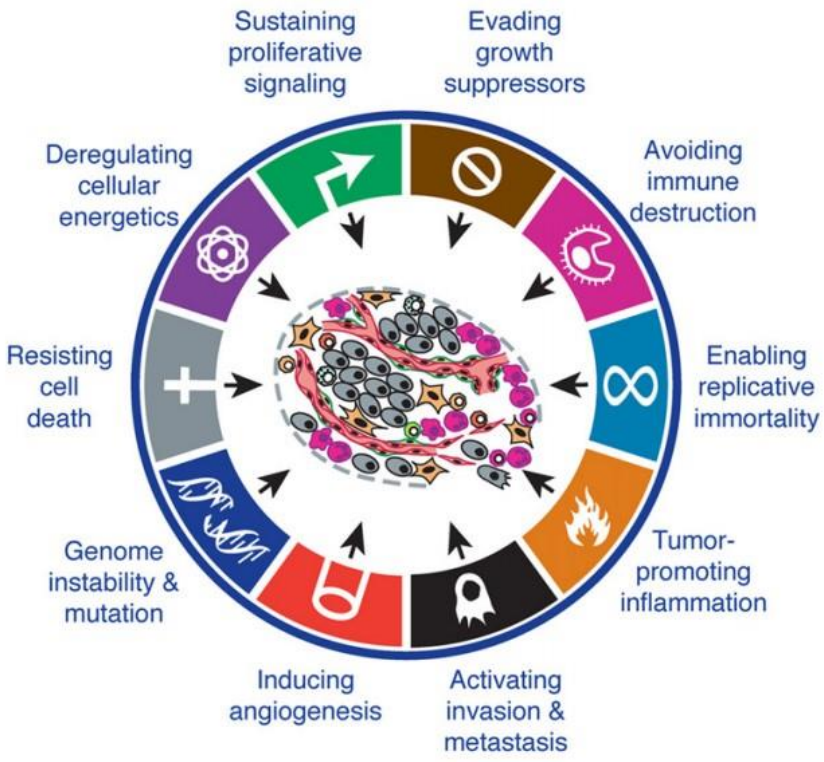


Figure 8. Hallmarks of cancer: the next generation. There are now 10 established hallmarks of cancer, including inflammation, metabolism and genomic instability (taken from 170).

5.1 Glucose metabolism

Under aerobic conditions, differentiated cells metabolise glucose to pyruvate via glycolysis in the cytosol and then the pyruvate enters the mitochondrial tricarboxylic acid (TCA) cycle for its complete oxidation. This reaction produces NADH (nicotinamide adenine dinclueotide NAD^+, reduced) which then fuels oxidative phosphorylation (OXPHOS) to

maximize the production of energy form ATP molecules. In the absence of oxygen, glycolysis is favoured and a small amount of pyruvate undergoes OXPHOS, whereas a high pyruvate quantity is converted in lactate in a process called fermentation. Otto Warburg first reported that in the presence of oxygen, proliferating cancer cells could reprogram their glucose metabolism, and thus their energy production, consuming glucose at a surprisingly high rate compare to normal cells by an increase of glycolysis and subsequent lactate release, in a state that has been termed “aerobic glycolysis”. This phenomenon is also known as the “Warburg effect” (figure 9) (173).

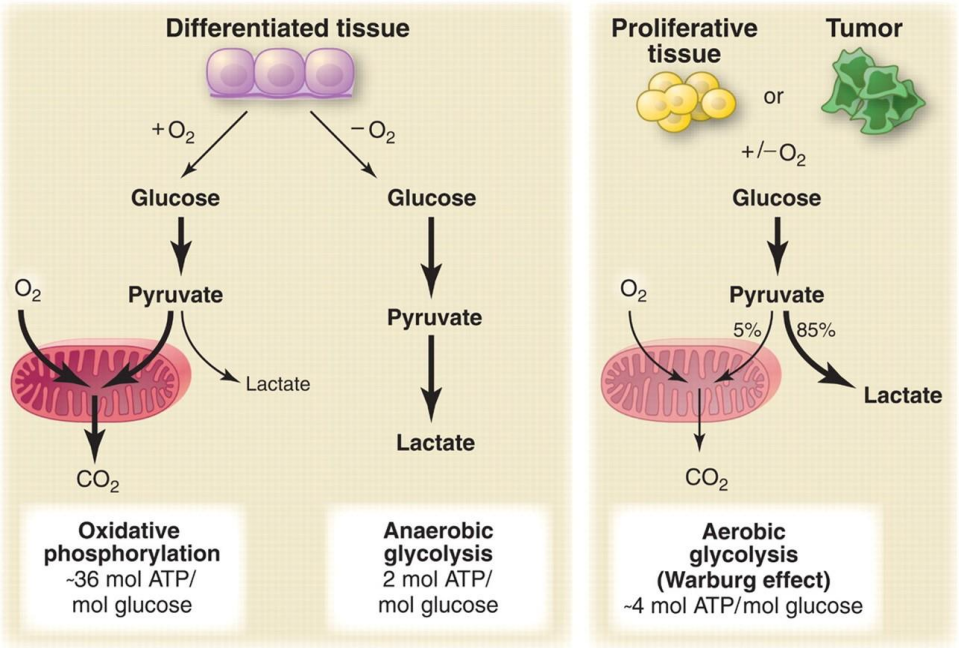


Figure 9. Comparison of glycolysis between a normal tissue and tumour/ proliferated tissue (taken from 174).

Warburg originally hypothesized that cancer cells developed a defect in mitochondria that led to an impairment in aerobic respiration and a subsequent reliance on glycolytic metabolism (173). However, subsequent works showed that mitochondrial function was not impaired in most cancer cells (175-177). The highly glycolytic rate provides several advantages for proliferating cells. First, an increase of glycolysis allows cells to use the most abundant extracellular nutrient, glucose, to produce ATP. Although the quantity of ATP produced by glycolysis is lower compared to that

obtained via OXPHOS, if the glycolytic flux is high enough, the percentage of cellular ATP produced by glycolysis can exceed that produced from aerobic respiration (173, 178). To allow an increase in glucose uptake, many cancer cells upregulate the glucose transporters, (GLUTs), which can contribute to a substantial increase in glucose import into the cytoplasm (179-181). Indeed, markedly increased uptake and utilisation of glucose have been documented in many human tumours using positron emission tomography (PET) with radiolabeled analogue of glucose (^{18}F-fluorodeoxyglucose, FDG). Furthermore, during cell growth and proliferation, cells need a large quantity of nucleotides, amino acids and lipids to create biomass. Glucose could be used to generate biomass as well as ATP. Degradation of this metabolite provides cells with intermediates needed for biosynthetic pathways, such as glycerol and citrate for lipids, non-essential amino acids for protein synthesis and through the oxidative pentose phosphate pathway, ribose sugars for nucleotides and NADPH (182). The switch from OXPHOS to glycolysis, with its concomitant accumulation of lactate produced and released in tumour microenvironment results in an increased acidity in the tumour microenvironment, which promotes tumour cells adaptation and contributes to the evolution of the tumour niche (182, 183). The availability of biosynthetic precursors is enhanced by regulation of the last rate-limiting step of glycolytic pathway, which is catalysed in normal cells by pyruvate kinase M1 (PKM1). PK catalyses the conversion of phosphoenolpyruvate (PEP) to pyruvate, with concomitant phosphorylation of ADP to ATP. It also exists an alternative splice form of PK, PKM2, which has reduced catalytic activity. This isoform is predominant in proliferating and cancer cells and can be allosterically activated by fructose-1-6-biphosphate (FBP) (184, 185). This activation can be countered by either phosphotyrosine binding to PKM2 or by phosphorylation of a specific tyrosine residue (Y105) of PKM2 by the activation of signalling pathways downstream of receptor tyrosine kinases. PKM2 can exist as both dimer and tetramer form (186, 187). The PKM2 dimer is less active compare to its tetrameric form in converting PEP to ATP and pyruvate (187, 188). While tetrameric PKM2 favours ATP production through TCA cycle, dimeric PKM2 plays a critical role in aerobic glycolysis (187). This reduced catalytic activity allows the reduction of the

glycolytic flux rate and causes the accumulation of intermediates that fuel several biosynthetic pathways (185). The dynamic equilibrium between the dimeric and the tetrameric form of PKM2 allows proliferating cells to regulate their need for anabolic and catabolic metabolism (187).

In addition, the PI3K/AKT/mTOR pathway has a crucial role in controlling glucose metabolism. The AKT signalling pathway promotes continued cell growth and coordinates the necessary metabolic change to support cell growth by increasing glucose uptake, glycolysis and ATP production. Activation of AKT signalling leads to the switch to glycolytic metabolism in cancer (189). AKT directly and/or indirectly regulates the transcription (190) and translation (191) of GLUT1. Furthermore mTOR, the downstream effector of PI3K/AKT, is at the crossroads of signalling pathway and is an hub for signals bringing the coordinated regulation of nutrient uptake, energy metabolism, cell growth, proliferation and cell survival (192, 193). Most importantly, mTOR is an upstream activator of hypoxia-inducible factor 1 α (HIF1-α) in cancer cells (194), which is a subunit of transcription factor that upregulates the expression of several genes involved in glycolysis metabolism (195). During normoxia, HIF1-α undergoes a degradation following posttranscriptional modifications. However, in several cancer cells HIF1-α is stable in presence of oxygen, and this is due to mutations which can involve HIF1-α itself or its regulators (196-198).

In addition to HIF1-α, other transcription factors are involved in the regulation of the glycolytic pathway, such as c-Myc and p53. Hexokinases (HK) are important enzyme that regulates the first step of glycolysis, the conversion of glucose in glucose 6-phosphate. HK2 is the isoform expressed specifically in skeletal muscle, adipocytes and in cancer cells (199). HK2 can be regulated by p53 as well as HIF1-α. The upstream regulatory elements of the HK2 gene contain response elements for protein kinase A, protein kinase C, HIF1-α and p53 (200, 201). In cancer cells, the HK2 gene is amplified, activated and induced by multiple signal cascades (202). Furthermore, HIF-1-α induces the expression of pyruvate dehydrogenase kinase 1 (PDK1) which phosphorylates and inhibit the pyruvate dehydrogenase complex (PDH) (203, 204). The inhibition of PDH impairs the entry of pyruvate into TCA cycle and promote the conversion of

pyruvate into lactate catalysed by the lactate dehydrogenase (LDH). LDH is upregulated in different types of tumour, probably due to an increased activity of HIF1-α and c-Myc, which regulate the expression of LDH (200). Lactate is transported out of the cancer cells across the plasma membrane by the monocarboxylate transporters (MCTs) family. MCT1 is utilised by oxidative tumours to upload exogenous lactate, produced by glycolytic cells in the tumour microenvironment, as energy source, which can be metabolised through OXPHOS (205). Conversely, MCT4 predominantly is used for lactate extrusion from glycolytic tumours (206). Overall, glucose uptake increase and lactate release, even in normoxic condition, are involved in cancer survival and proliferation via several mechanisms. Furthermore, the accumulation of lactate in cancer has been demonstrated to be of clinical relevance as prognostic markers (207).

5.2 Amino acids metabolism

Cancer cells have a continued and increased requirement for amino acids to meet their rapid proliferation. Amino acids can be divided into two classes: essential amino acids (isoleucine, leucine, methionine, valine, phenylalanine, tryptophan, hystidine, threonine and lysine) and non-essential amino acids (alanine, glutamate, glutamine, aspartate, asparagine and serine). Amino acids can be used as substrates for protein synthesis but also as source of energy (Figure 10).

Serine and glycine are linked to biosynthetic pathways and represent essential precursors for the synthesis of building blocks including protein and nucleic acids. Serine can be of external source (i.e. uptaken from the extracellular environment) or internal, when derived from glucose metabolism. Several studies suggested that cancer cells have an increased *de novo* serine synthesis via the phosphoglycerate dehydrogenase (PDGH) pathway. PHGDH oxidases around 10% of 3-phosphoglycerate produced during glycolysis by converting it to 3-phospho-hydroxypyruvate (209-211). This compound is then transaminated and dephosphorylated to serine. This compound is then transaminated and dephosphorylated to serine. Both *de novo* synthesised and imported serine can be further converted into glycine by the serine hydroxymethyl transferase (SHMT) enzyme, which is a direct transcriptional target of c-Myc (212).

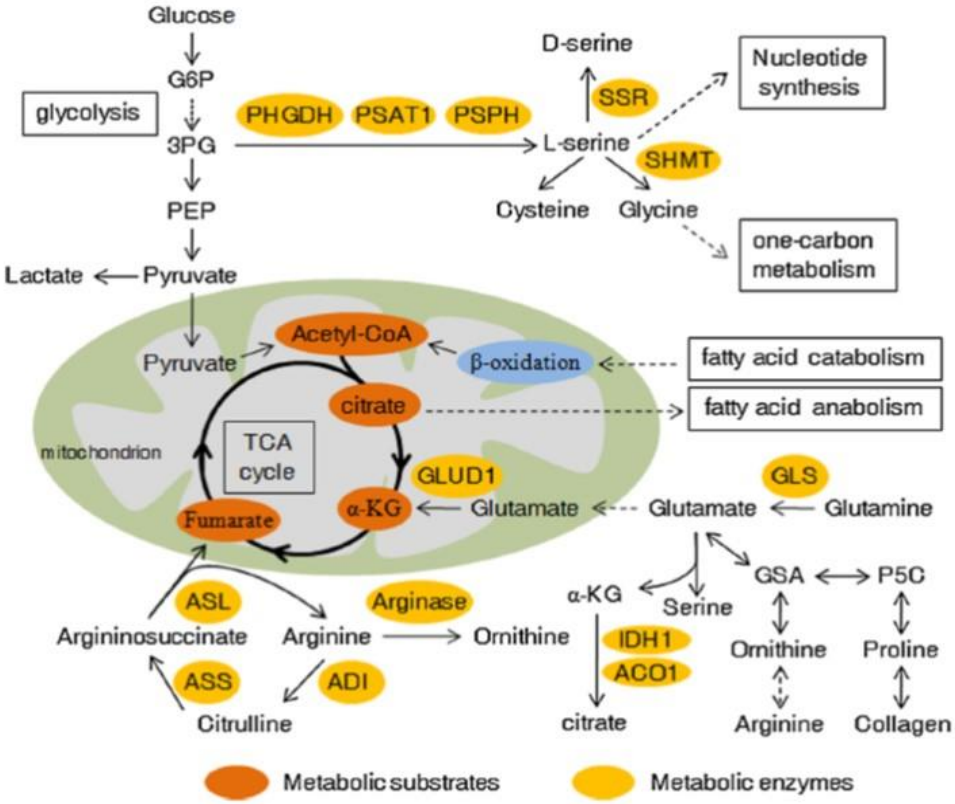


Figure 10. Amino acids metabolism in cancer cells and its crosstalk with other metabolism pathways. Amino acids synthesis, utilization, and involvement in other metabolism pathways are usually changed in cancer cells. Abbreviations: α-KG, α-ketoglutarate; GSA, glutamic semialdehyde; P5C, pyrroline-5-carboxylate; GLS, glutaminase; GLUD1, glutamate dehydrogenase 1; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase; ADI, arginine deaminize; IDH1, isocitrate dehydrogenase-1; ACO1, aconitase 1; SSR serine racemase. Dashed arrows represent indirect effects or serial reactions (taken from 208).

The glycine cleavage system represents a major metabolic pathway of the one-carbon metabolism that provides cofactors for purine and pyrimidine nucleotides biosynthesis essential for proliferating lymphocytes, cancer cells and foetal tissue (213, 214). PHGD is upregulated in highly metastatic breast cancers and therefore associated with poor prognosis (215). In addition to PHGDH, SHMT is also implicated in tumorigenesis. SHMT is targets of oncogene c-Myc, which is abnormally over-expressed in many tumours (216). Recent studies showed that exogenous glycine cannot be replaced by serine to support cancer cell proliferation. Indeed, cancer cells selectively consume exogenous serine, which is converted into intracellular glycine and one carbon units for building nucleotides (217). Moreover, the

uptake of exogenous glycine without the concomitant presence of serine in proliferative cells impaired the nucleotides synthesis (217). These data suggest that cancer cell proliferation is supported by serine rather than glycine consumption.

Another important amino acid involved in tumour progression is proline. Proline is a unique proteinogenic secondary amino acid which contributes to collagen formation, the most abundant protein in the body (218). Proline can be converted by reversible reaction to glutamate, in which α-pyrroline-5-carboxylate (P5C) and glutamic-γ-semi-aldehyde (GSA) are used as intermediates. Proline dehydrogenase (oxidase) (PRODH/POX) which catalyses the reaction from proline to P5C has a mitochondrial suppressor function and is induced by p53 and PPARγ and suppressed by c-myc and microRNA-23, a small non-coding RNA molecule with RNA silencing function that thus inhibits gene expression of mRNA target (219). Recent study indicated that proline metabolism deregulation is involved in cancer progression (220). Furthermore, GSA derived from glutamate or proline can be converted into ornithine, which is the precursor for arginine synthesis in the Urea cycle (218).

Arginine is an essential amino acid in several types of cancer and many tumour cells are sensitive to arginine deprivation when cultured *in vitro* (221). Arginine participates in many important metabolic pathways including biosynthesis of nitric oxide, nucleotides, proline and glutamate (222). Arginosuccinate, derived from the reaction catalysed by arginosuccinato synthase (ASS), is converted into L-arginine and fumarate. The latter links arginine metabolism to glucose-generated energy metabolism via the TCA cycle, being fumarate a TCA intermediate. A recent study revealed that some human cancers cells do not express ASS and they are unable to synthesize arginine *de novo* and therefore were susceptible to arginine deprivation therapy (223).

The most important amino acid for the survival and proliferation of human cancers is glutamine (224, 225), which is used for both energy generation and, as source of carbon and nitrogen, for biomass accumulation (226). Glutamine is imported into the cytoplasm via several membrane transporters (227) where it can be immediately metabolised or extruded out

of the cell by antiporters in exchange for other amino acids (228). In addition, glutamine-derived glutamate can exchange through the xCT (a heterodimer of two solute carrier transporters, SLC7A11 and SLC3A2) an antiporter for cysteine, which is quickly reduced to cysteine inside the cell (229). Glutamine catabolism begins with its conversion to glutamate, reaction catalysed by the glutaminase enzyme (GLS) (230). GLS expression and glutamine metabolism are activated by oncogenic transcription factor c-Myc in cancer cells (231, 232). In mitochondria, glutamate can then be converted through oxidative deamination to α-ketoglutarate (α-KG), which enters in TCA cycle to generate ATP. This reaction can be catalysed by either glutamate dehydrogenase (GDH), which is an ammonia-releasing process, or by several non-ammonia producing aminotransferases, which transfer glutamate-derived nitrogen to produce another amino acid and α-KG (233). In addition to energy and amino acids production, glutamine is involved in lipogenesis process. Glutamine metabolism can serve as an alternative source of carbons for fatty acids (FA) synthesis. Indeed, glutamine-derived α-KG can be reduced through reductive carboxylation to citrate, which is the precursor for fatty acid synthesis (234). It has been demonstrated that this reaction is important for cancer growth and involved in tumour progression (235-238). Many cancer cells utilise acetyl-CoA manly converted from glucose-derived pyruvate (239), but in glucose deprived-condition, citrate produced by glutamine-derived α-KG can be converted to acetyl-CoA by ATP citrate lyase (ACLY) enzyme (240), inducing glutamine dependent lipid synthesis. A recent study showed that the import of glucose-derived pyruvate into mitochondria by mitochondrial pyruvate carrier (MPC) suppressed GDH activity and glutamine-dependent acetyl-CoA formation. The MPC inhibition activated GDH and divert glutamine metabolism to generate both oxaloacetate and acetyl-CoA, indicating a compensatory mechanism that allow to cancer cells to generate lipid in glucose deprived-conditions (241). Furthermore, glutamine is involved directly in nucleotides biosynthesis. Indeed, glutamine-derived carbons are used for amino acids and lipids synthesis, glutamine-derived nitrogen contributes directly to *de novo* biosynthesis of purines and pyrimidines (242). In fact, synthesis of nucleotides from exogenous glutamine has been observed in human

primary lung cancer (243). Glutamine can also contribute to nucleotides biosynthesis trough other pathways. Aspartate derivate from glutamine via the TCA cycle and subsequent transamination serves as a crucial source of carbon for purines and pyrimidines synthesis (244, 245). Indeed, supplementation of aspartate can rescue cell cycle arrest caused by glutamine deprivation in cancer cells (246).

5.3 Lipid metabolism

In addition to glucose and amino acids metabolic reprogramming, also alterations in lipids and cholesterol metabolic pathways occur in different types of tumour (Figure 11) (247-249). Highly proliferative cancer cells show a strong lipids and cholesterol avidity and FA synthesis is frequently increased to satisfy the requirement of lipids for energy storage, synthesis of membranes and signalling molecules (250). Lipids as energy storage are utilised by different type of tumours. Normal and cancer store lipids in the form of lipid droplets (LDs) and LDs accumulation is higher in cancer than in normal cells (251-254). Indeed, high LDs are now considered as hallmark of cancer aggressiveness (253, 255-257). Citrate produced in the mitochondria is exported into the cytosol by the transport protein citrate carrier (CIC). CIC levels were found to be elevated in different human cancer cell lines and its activity was required for tumour proliferation *in vitro* and tumorigenesis *in vivo* (258). The first-rate limiting reaction of *de novo* lypogenesis is catalysed by ACLY enzyme, which links glucose and FA metabolism by converting citrate to oxaloacetate that can enter into TCA cycle and acetyl-CoA that is the precursor for FA synthesis in cytoplasm. It has been demonstrated that ACLY expression is increased in several types of cancer, including colorectal, breast, glioblastoma and ovarian cancer (260-262). Another rate-limiting enzyme involved in FA synthesis is acetyl-CoA carboxylase (ACC), which catalyse the carboxylation of acetyl-CoA to malonil-CoA. There are two isoforms of ACC, respectively ACC1 and ACC2. ACC1 is located in the cytosol and is highly expressed in lipogenic tissue, whereas ACC2 is embedded in the mitochondrial membranes and locates in oxidative tissues (263). ACC1 is responsible for the rate-limiting step of FA *de novo* synthesis and ACC2 seems to be involved in the regulation of FA oxidation (catabolism pathway).

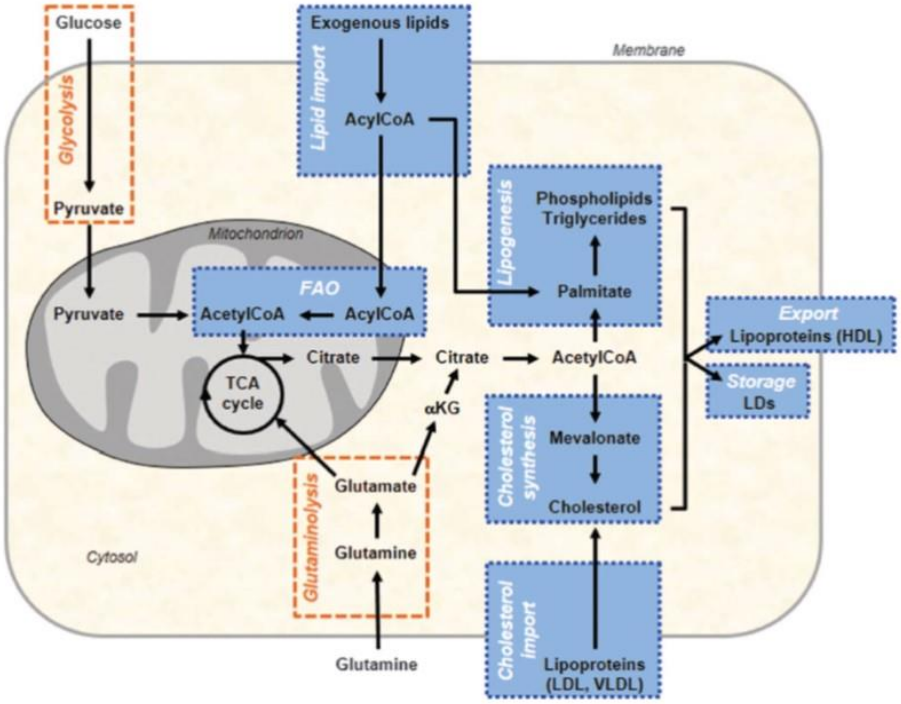


Figure 11. Lipid metabolic reprogramming in cancer. Lipid metabolic network (blue) includes import/export and catabolic pathways (fatty acid oxidation, FAO) as well as de novo synthesis pathways, such as lipids and cholesterol synthesis. Glucose- and/or glutamine-derived citrate, provided by the increased glycolysis and/or glutaminolysis (orange), are common precursors of lipogenesis and cholesterol synthesis. Cancer cells can also take up exogenous cholesterol, transported by LDL and very-low density lipoproteins (VLDL), to meet their cholesterol requirement. When cholesterol, PLs and TGs are in excess in tumours, they are exported into circulation as high-density lipoproteins (HDLs) or locally stored into LDs. Exogenous FAs taken up by cancer cells are broken down to produce energy through mitochondrial FAO process. Abbreviations: TCA cycle, tricarboxylic acid cycle; αKG, α-Ketoglutarate (taken from 259).

AMP-activated protein kinase (AMPK), a central energy sensor, is activated via phosphorylation by serine/threonine Kinase LKB1 (Liver Kinase B1), a known tumour suppressor (264) and strongly inhibits FA synthesis by phosphorylating and inactivating ACC1 (265). Another enzyme that was found deregulated in many types of tumour is fatty acid synthase (FANS), which catalyses the terminal step of *de novo* FA biogenesis and its activity provides a survival advantage in cancer cells (266).

Acetyl-CoA is a central node in carbon metabolism and is primarily generated in the mitochondria via glycolysis, lipids catabolism and amino acids metabolism (267). It represents an important carbon source and can be used for the synthesis of nucleotides, FA, cholesterol and glutamate, or

can undergo further oxidation via TCA cycle for ATP production. Many cancer cells are highly glycolytic and preferentially convert glucose-derived pyruvate in lactate. In this way, the majority of pyruvate does not enter in TCA cycle for the synthesis of citrate, which is transported to the cytoplasm for ACLY-mediated production of acetyl-CoA. A recent study showed that in highly glycolytic cancer cells, acetate is captured from the environment to be used as a carbon source for acetyl-CoA production (268). Acyl-CoA synthetase 2 (ACSS2) is a cytosolic enzyme that produces acetyl-CoA from acetate in a reaction that requires ATP. High expression of ACSS2 is associated with poor survival in triple negative breast cancers and the acetate uptake mediated by ACSS2 has been shown to support tumour cell growth and survival under nutrient-limiting conditions (268).

5.4 Therapies targeting tumour metabolic reprogramming

Altered tumour metabolism may be utilised as a target for cancer therapy. One of the metabolic changes exhibited by tumour cells is the increase of glucose uptake and subsequently a major glycolytic flux. Indeed, there are several drugs that have been designed to block or attenuate the glycolysis pathway hence inducing glycolysis-dependent cancer cells death. In addition to glycolytic rate-limiting enzymes, glucose transporters and other enzymes involved in other metabolic pathways have been selected as targets for drug screening (269). However, most of the promising drugs targeting glucose metabolism for anti-cancer therapy have only been tested in preclinical models, including the inhibitors of GLUT1 (WZB117) (152), HK2 (2-deoxy-glucose, methyljasmonate) (270) and LDHA (271). In addition, activators of metabolic enzymes can be used for potential treatment of cancer. Indeed, increased of PKM2 activity impairs tumour growth (272). Two small molecules, TEPP-46 and DASA-58, can specifically activate PKM2 in cancer cells, promoting the formation and stabilisation of tetrameric form of PKM2, which increase the glycolytic flux rate. It has been demonstrated that PKM2 activation alters metabolism in cultured cells and inhibits xenograft tumour growth (272). An increase of *de novo* FA synthesis for energy metabolism and membrane production can be advantageous for cancer cell survival and proliferation. Indeed, it has been demonstrated that hydroxycitrate treatment, an inhibitor of ACLY, is

able to reduce the stem like traits of cancer cell lines, a feature of aggressiveness in cancer progression (273). ACC1 and ACC2 inhibition has been utilised for the treatment of metabolic diseases such diabetes and dyslipidaemia and shows a potential as anticancer treatment (263). FANS inhibitors have also been identified, but none of them have been tested in the clinic (274). As far as amino acids metabolism is concerned, it has been identified one specific GLS inhibitor that blocks GLS and inhibits oncogenic transformation in preclinical models of cancer, without affecting normal cells (275). In addition to glutamine metabolic enzyme inhibition, the decrease of glutamine uptake can also impair cancer growth and tumour development (276). Furthermore, serine and glycine represent essential precursors for proteins synthesis, nucleic acids and lipids, especially through the participation of glycine in one-carbon metabolism. For such reason, several drugs targeting enzymes that catalyse the generation of tetrahydrofolate, involved in one-carbon metabolism, have been clinically approved in multiple cancers, such as methotrexate and Premetrexed (210).

6- Metabolic reprogramming and breast cancer

Metabolic reprogramming is a crucial event that occurs during tumorigenesis to support tumour growth, tissue remodelling and cancer metastasis. This switch is regulated by oncogenes and tumour suppressor genes and is influenced by tumour microenvironment. Breast tumours commonly develop a lipogenic phenotype and heavily rely on glucose and glutamine consumption for tumour growth. Metabolic profile analysis on biofluids, including serum and urine, derived from patients with breast cancer, have been performed to evaluate difference in metabolite profile in breast cancer patients compared to healthy women (277-281). These studies showed that the metabolic pattern of fluids derived from breast cancer patients is altered and identified three metabolites which were commonly reduced in breast cancer patients: glycine, choline and formate (277, 278). It has been suggested that the decrease in the levels of these metabolites may be attributed to their increased utilisation by cancer cells for anabolic reactions. In another study, glycine and choline consumption strongly correlated with proliferation rates of cancer cells, but not with

rapidly proliferating untransformed cells, suggesting that the glycine metabolism can be exploited as a potential therapeutic target (282). Moreover, recent studies reported that serine and formate metabolism are involved in breast cancer progression since they are involved in the production of NADPH. NADPH plays a critical role in maintaining cellular redox homeostasis through the regeneration of reduced glutathione, as well as in the synthesis of complex molecules such as nucleotides and lipids (283, 284). Furthermore, several reports have demonstrated the importance of *de novo* serine synthesis from glycine via the PHGDH pathway in breast cancer cells proliferation (285-287).

We know that breast cancer is a heterogeneous disease and breast tumours can be classified into several molecular subtypes with specific gene expression. In breast cancer, large difference in tissue metabolite profile have been observed between ER+ and ER- tumours, however these differences do not appear to further classify tumours into submolecular subtypes based on the gene expression profiles (288-290). The characterisation of glucose metabolism, glutamine consumption and glutamine dependence showed that only the ER- cancer cells lines were actually glutamine dependent, while ER+ cancer cell lines were mostly glycolytic (Figure 12) (229). This study suggests that the ER- breast cancer subset could benefit from GLS inhibitors or glutamine deprivation therapy, while the ER+ subset should be treated by glycolysis inhibition (229). However, further studies are needed to understand the metabolic alterations that characterise different types of breast cancer in order to identify possible prognostic and predictive metabolic markers and understand the subset that can benefit for the metabolic drugs currently used in the preclinical setting.

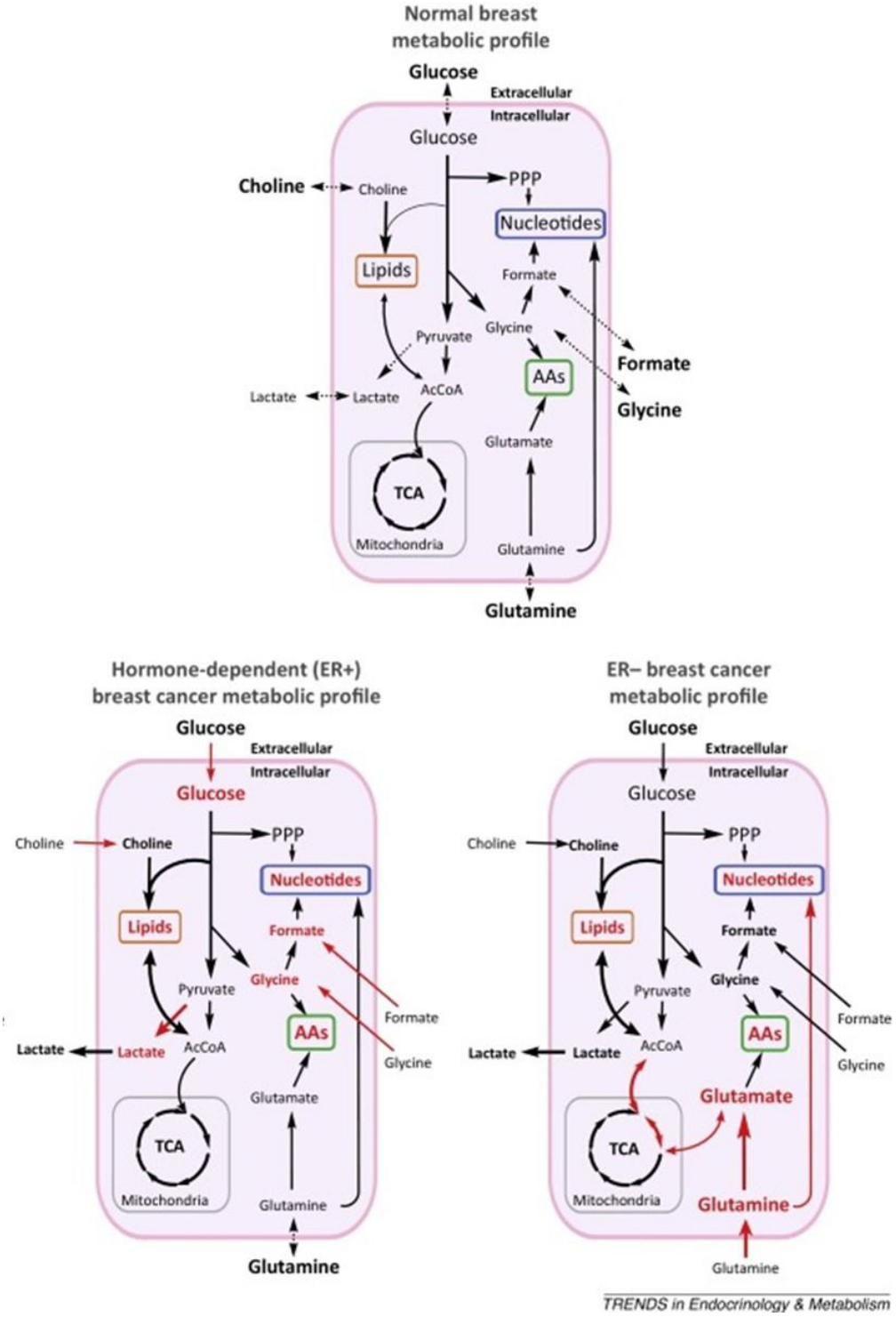


Figure 12. Metabolic alterations of breast cancer. Major metabolic changes in the development of oestrogen-dependent breast cancer promote the increased production of lipids used for the plasma membrane, nucleotides for DNA synthesis, and amino acids (AAs) for protein synthesis. This is supported through metabolic alterations, which include increased uptake of choline to synthesize lipids. Additionally, uptake of glycine and formate increases to support the production of nucleotides and AAs. ER+ breast cancers exhibit increased glucose consumption and aerobic glycolysis, increasing lactate production and pentose phosphate pathway (PPP) intermediates, which also fuel nucleotide production. By

contrast, ER- breast cancers exhibit a shift from glycolysis towards glutamine consumption to fuel the tricarboxylic acid (TCA) cycle as well as to donate nitrogen and carbon as precursors for proteinogenic AAs and nucleotides. Red indicates a significant increase in either metabolite or enzymatic pathway activity. Abbreviation: AcCoA, acetyl co-enzyme A (taken from 291).

6.1 Metabolism and therapy resistance in breast cancer

Emerging evidences show a relationship between deregulated cellular metabolism and cancer drug resistance, suggesting that interfering with these metabolic alterations can enhance the efficacy of common therapeutic agents or overcome resistance to chemotherapy or radiotherapy (292). It has been demonstrate that the development of endocrine therapy resistance in breast cancer cell lines, may be prevented or delayed by combining the AI letrozole with drugs targeting the PI3K/AKT/mTOR pathway, as described above (293). Recently, it has been shown that ER+ tamoxifen-resistant breast cancer cells are characterised by HIF-1α hyperactivation via modulation of AKT/mTOR, which results in enhanced aerobic glycolysis and a Warburg-like metabolism. Impairing glycolysis restored tamoxifen sensitivity in drug-resistant cells, suggesting that this metabolic reprogramming is not merely a consequence of signalling rewiring (294). However, a different study reported that ER+ tamoxifen-sensitive breast cancer cells showed a glycolytic phenotype when cultured alone. In presence of fibroblasts, these cells became resistant to tamoxifen and showed a metabolism relying on OXPHOS. Indeed, it has been proposed that while cancer associated fibroblasts are undergoing a Warburg-like metabolism, secreting lactate into the media, cancer cells are able to uptake such carbon source, undergoing OXPHOS, and this is sufficient to confer tamoxifen resistance (295). It seems possible to overcome tamoxifen-resistance by shifting breast cancer cells back to their glycolytic state. Another study showed that endocrine therapy may select a metabolic dormant cancer stem cell-like subpopulation, which is characterised by the loss of mitochondrial biogenesis. Importantly, the exit from this metabolic dormancy is orchestrated by interleukin 6 signalling that impacts on ER expression and promote the reacquisition of glycolytic and OXPHOS metabolic activity (296). These results highlight the importance of metabolic adaptability of cancer cells for endocrine therapy resistance and

suggest that targeting such metabolic reprogramming may improve the response to cancer therapeutics. Furthermore, the combination of chemotherapeutic drugs with metabolic inhibitors may represent a promising strategy to overcome drug resistance in cancer therapy.

6.2 Metabolic targeting in breast cancer

A potential therapeutic approach that can be exploited in ER+ breast cancer cells is based on interfering with altered metabolic pathways by metabolic poisons in combination with endocrine therapy. Recent studies have demonstrated that the anti-diabetic drug, Metformin, can exhibit direct antitumoral effects, or can indirectly decrease tumour proliferation by improving insulin sensitivity. Indeed, metformin inhibits the hepatic gluconeogenesis via AMPK activation, increases insulin sensitivity and glucose utilization by skeletal muscle and adipose tissue, resulting in reduced glucose and insulin levels in the blood stream (297). The decrease in insulin levels caused by metformin can reduce, in breast cancer cell lines, the activation of insulin pathways such as PI3K/Akt/mTOR and MEK/ERK1/2 leading to a decrease in tumour growth (298). Moreover, metformin is known to inhibit mitochondrial complex I *in vitro* (299) and it is thus possible that this targeting of the electron transport chain could inhibit tumour cell growth. This latter hypothesis has been questioned as cancer cells have the ability to survive on ATP produced exclusively by glycolysis. Furthermore, cancer cells have been shown to conduct glutamine-dependent reductive carboxylation to generate the TCA cycle intermediates required for cell proliferation when the electron transport chain is inhibited (300). Another candidate drug for the treatment of breast cancer is 2-deoxy-glucose (2-DG) which is a widely studied glucose analogue that acts as a competitive inhibitor of glucose metabolism. Upon transport into the cell, 2-DG is phosphorylated by HK; however, unlike glucose, it cannot be metabolized by phosphoglucose isomerase, and is unable to undergo further metabolic reactions. The increase of HK2 expression has been showed as a prognostic factor in breast cancer and is associated with high rate of proliferation in breast cancer cells (301). The combination of 2-DG with trastzumab treatment inhibits the survival of trastzumab-sensitive and -resistant breast cancers *in vitro* and *in vivo* models of HER2+ breast

cancers with more efficient inhibition of glycolysis via downregulation of heat shock factor1 and LDHA (302). In addition, LDHA inhibition has been showed to decrease HER2+ breast cancer cell proliferation under hypoxic conditions and interfere with tumorigenesis (176). Furthermore, the inhibition of LDHA by LDH inhibitor oxamate shows a synergistic inhibitory effect on taxol-resistant breast cancer cells by promoting apoptosis when combined with taxol (303).

In addition to the glycolytic pathway interference, glutamine transporters and glutaminolysis are currently investigated as potential pharmacological targets in cancer therapy. The amino acid transporter SLC6A14 is upregulated specifically in ER+ breast cancer cells and Blockade its targeting induces the impairment of cancer cell survival via mTOR activity inhibition and subsequent apoptosis and autophagy activation (304). Two novel GLS inhibitors have been showed to have antiproliferative activity in combination with chemotherapy in ER- breast cancer cells (305, 306). Concerning lipid metabolic reprogramming, different studies have shown that FASN inhibitor cerelenin acts synergistically with docetaxel in HER2+ cancer cells and docetaxel resistant breast cancer cells, indicating the role of FASN in breast cancer chemotherapy resistance (307). FASN blockade also induce synergistic chemosensitisation of breast cancer cells to other chemotherapy agents, such as paclitaxel, adriamycin, 5-fluorouracile and vinorlbine (307-309).

7- MicroRNAs

The microRNAs (or miRNAs) are a group of short non-coding RNAs (22-nucleotides) that mediate post-transcriptional gene silencing, thus controlling gene expression and assuming a key role in a variety of cellular process including proliferation, differentiation, apoptosis and metabolism (310-313). miRNAs bind to their target mRNAs and mediate their degradation with subsequent translation blockade (314). In humans, the majority of miRNAs are encoded by introns of non-coding or coding transcripts, but some miRNAs are encoded by exonic regions. Often, several miRNAs loci are in close proximity to each others, constituting a polycistronic transcription unit and the miRNAs within the same cluster are

generally co-transcribed, also if the individual miRNA can be additionally regulated at the post-transcriptional level (315). Generally, miRNAs that have identical sequence at nucleotides 2-8 of the mature miRNA are considered belonging to the same miRNAs “family”. Some miRNAs genes that reside in the introns of protein-coding gene share the promoter of the host gene, but it has been demonstrated that miRNA genes often have multiple transcription start sites (316) and that promoters of intronic miRNAs can be distinct form the promoters of their host genes (317). miRNAs are mainly transcribed by RNA polymerase II as a long primary transcript characterised by hairpin structure, called pri-miRNA. An individual pri-miRNA can either produce a single miRNA or contain clusters of two or more miRNAs that will be processed from a common transcript. In the nucleus, RNAse III Drosha processes this long pri-miRNA into 70-100 nucleotides pre-miRNA (318). This originated precursor molecule is exported by an Exportin-5 mediated mechanism to the cytoplasm (319), where it is additionally processed by RNAse Dicer III. Dicer is very large enzyme conserved among the species and containing different domains, including a double-strand RNA-binding domain (dsRBD), two RNAse III catalytic domains, one PAZ domain that binds the 3’-end of small RNAs, and other domains with ATPase and RNA-helicase activity (320). Dicer binds to the end of the pre-miRNA positions by its two catalytic domains and generate the mature 22-nucleotides double strand miRNA (320). This RNA molecule is named miRNA /RNA* and is constitute by the mature miRNA guide and the complementary passenger strand, the miRNA*. Many publications refer to the two strand pair as miR-3p/miR-5p, referring to the direction of the mature and functional miRNA. The so-called miRNA* was initially thought to be the strand subjected to degradation, instead more evidence suggest that it is not simply a non-functional product of miRNA biogenesis, but it can be selected as a functional strand and play significant biological roles (322). Dicer associates with transactivation-responsive RNA-binging protein (TRBP) which bind dsRNA (322). Although TRBP is not required for pre-miRNA processing, it enhances the fidelity of Dicer-mediated cleavage of pre-miRNAs and physically bridges Dicer with the Argonaute proteins (Ago1, Ago2, Ago3, Ago4) to participate in the assembly of miRNA induced silencing complex (miRISC) (322).

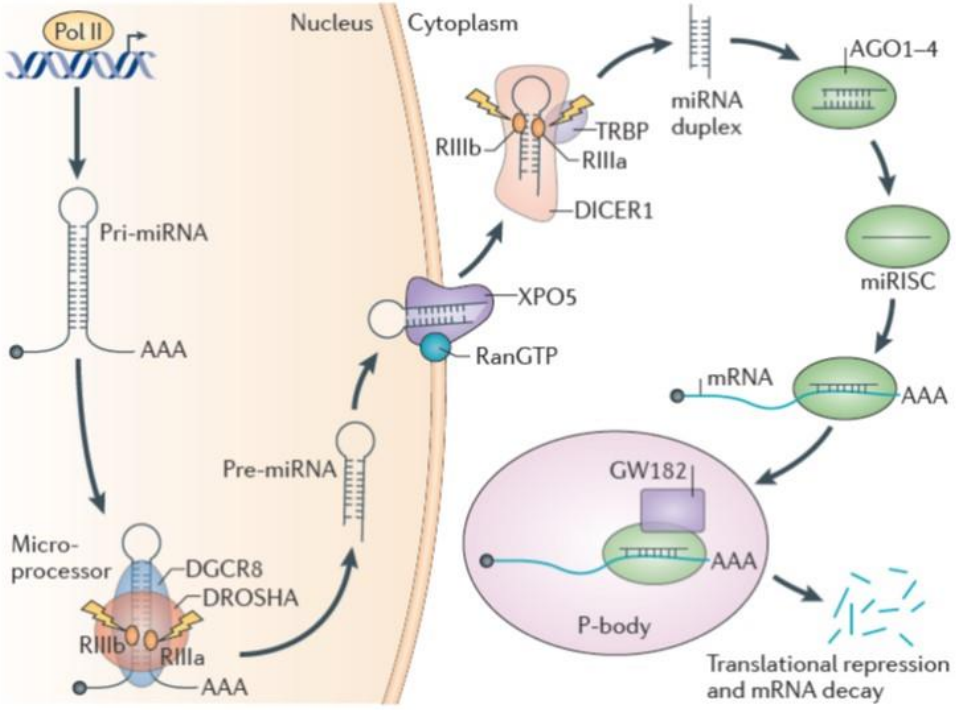


Figure 13. miRNA biogenesis pathway. miRNA genes are transcribed as primary miRNAs (pri-miRNAs) by RNA polymerase II (Pol II) in the nucleus. The long pri-miRNAs are cleaved by Microprocessor, which includes DROSHA and DiGeorge syndrome critical region 8 (DGCR8), to produce the 60–70-nucleotide precursor miRNAs (pre-miRNAs). The pre-miRNAs are then exported from the nucleus to the cytoplasm by exportin 5 (XPO5) and further processed by DICER1, a ribonuclease III (RIII) enzyme that produces the mature miRNAs. One strand of the mature miRNA (the guide strand) is loaded into the miRNA-induced silencing complex (miRISC), which contains DICER1 and Argonaute (AGO) proteins, directs the miRISC to target mRNAs by sequence complementary binding and mediates gene suppression by targeted mRNA degradation and translational repression in processing bodies (P-bodies). TRBP, transactivation-responsive RNA-binding protein (taken from (321).

As part of this complex, the mature miRNA is able to regulate gene expression at post-transcriptional level, binding for the most part through partial complementarity to target mRNAs, leading to mRNA degradation or translation inhibition (Figure 13). These two process can be carried out by any of Ago proteins (323). miRNAs can recognize their target mRNAs in the 3'-untranslated regions (UTRs), 5'UTR and the ORF (324-326). In addition to their gene expression silencing function, it has been demonstrated that miRNAs can upregulate translation upon growth arrest conditions (327). Considering the different mechanisms regulating the interaction between a miRNA and its target mRNA, it is not surprising that each miRNA has a

potential to target a large number of genes and thus the subsequent regulation can have different significance (328-331).

7.1 miRNAs and cancer

The first evidence that miRNAs could be involved in cancer initiation and progression derived from studies on chronic lymphocyte leukemia (CLL) (332). In particular, Croce's group reported that the locus including miR-15 and miR-16 on chromosome 13q14 is frequently deleted, resulting in the loss or reduced phenotypic expression of this miRNAs. Other studies have demonstrated that malignant tissues in human cancer patients exhibit distinctive miRNA expression signatures (333, 334) and distinct miRNAs are commonly up- or downregulated concurrently in distinct types of tumour and often associated with distinct cytogenetic abnormalities (334). For example, miR-17 and miR-21 were found upregulated in colon, lung, stomach and prostatic tumour, and mir-155 up-regulated in breast, lung and colon cancer (334). In contrast, miR-29 was down-regulated in CLL, acute myeloid leukaemia, lung and breast cancers (335-338). These miRNA expression patterns suggest that the regulation of these miRNAs is not a stochastic event, but the result of the genomic alterations leading to up-regulation or loss of function. Indeed, a considerable number of human miRNA genes are located at fragile sites or in genomic regions that are deleted, amplified or translocated in cancer (339). These genomic variations alter pri-miRNA transcription and miRNA expression, which leads to the aberrant expression of downstream target mRNAs. Thus, up-regulated miRNAs may act as oncogene and lost miRNAs as tumour suppressor and this deregulation can promote tumorigenesis and cancer progression (339, 340). In addition to structural genetic alteration, silencing of structurally normal miRNA genes by DNA promoter hypermethylation and/or histone acetylation has been described in solid and haematological tumours (341, 342). Saito and colleagues demonstrated that miR-127 is silenced by promoter DNA hypermethylation and down regulated in bladder cancer (341). Furthermore, deregulation of miRNAs expression can result from increased or decreased transcription from their respective genes by aberrant transcription factors activity. It has been showed that the miR-34 family transcription is induced directly by tumour suppressor p53 and high

expression of mir-34 correlate with high p53 levels in cancer cells (343, 344). Another study has demonstrated that c-Myc negatively regulates the transcription of miR-29 family members. In particular, c-Myc binds to conserved sequences of the miRNAs promoter leading to repression of miR-29 family members. This repression contributes to lymphomagenesis, in fact the restoration of the silenced miRNAs decreases the tumorigenic potential of the lymphoma cells (345). As a consequence of the different expression between normal and cancerous tissues, miRNAs have revealed a great potential as a new early diagnosis biomarkers. For example, over-expression of miR-205 and miR-21 in pancreatic ductal carcinoma has been reported to proceed phenotypic changes in the ducts, thus suggesting the possibility to use them for an early detection of this neoplasia (346). Furthermore, miRNAs are more stable than long mRNAs due to their small size, allowing a potential miRNA profiling and their possible use as a novel, minimally invasive biomarker. miRNAs can be reliably extracted and detected from frozen and paraffin-embedded tissues, blood (including plasma and serum) ((347), circulating exosomes (348) and from different biological fluids like urine (349) and saliva (350, 351). miRNAs have been shown to be able to predict also cancer prognosis. Several groups have successfully used miRNAs as prognostic markers to predict cancer outcome. For example, miR-155 overexpression and lethal-7 (let-7) miRNAs family downregulation are associated with poor prognosis in CLL and lung cancer (338), while in gastric cancer, a robust of 7 miRNAs signature can predict overall survival and relapse free survival (352). MicroRNAs signature can also be utilise to predict the response to specific therapies. For example, an increase of miR21 expression is sufficient to predict poor response to adjuvant therapy in addition to be an indicator of poor overall outcome in different types of tumour, including breast cancer (353, 354). Finally, miRNAs can also be used as potential target (336). The advantage of using miRNA approaches in therapy, both as targets and as therapeutic agents, is their ability to act simultaneously on multiple pathways such as proliferation, differentiation and survival. In summary, there are two main strategies to modulate the miRNAs expression in cancer. The direct strategy involves the use of oligonucleotides or viral constructs designed to block the expression of an oncogenic miRNA or to

reintroduce a miRNA that acts as a tumour suppressor. Conversely, the indirect strategy is based on the use of drugs that modulate the expression of miRNAs acting at the transcriptional level or during their maturation process (355). Although significant progresses have been made from the diagnostic and therapeutic point of view, there are still many obstacles prior to their clinical application.

7.2 miRNAs and breast cancer

Alterations in miRNA expression have been associated with tumorigenesis, metastasis and poor prognosis in human breast cancer. Different studies have demonstrated a significant deregulation in miRNAs expression in cancer versus normal breast tissue. Among these miRNAs, miR-10, miR-125, miR-145, miR-21 and miR-155 resulted deregulated in breast cancer, suggesting that they may potentially act as tumour suppressor genes or oncogenes (337, 356). In particular, miR-10, miR-21 and miR-155 are considered oncomiRs, because their upregulation inhibits tumour suppressing factors expression and promote cell proliferation, metastasis and angiogenesis or induce epigenetic changes, favouring tumour progression (140, 357-360). The increase of miR-10 correlates with increased cell migration and metastasis in breast cancer (361). The miR-155 exhibits its oncogenic ability by suppressing the expression of tumour suppressor genes, including protein Suppressor of cytokine expression 1 (SOCS1) both *in vitro* and *in vivo* (362). Similarly, miR-21 is upregulated in breast cancer and it is considered an oncomiR by inhibiting the expression of various tumour suppressor genes, such as tissue inhibitor metalloprotease (TIMP3) and PDCD4 (363, 364). In contrast, miRNAs that act as tumour suppressors can target mRNAs of various oncogenes and their regulation is critical in carcinogenesis (361). The first of these that has been identified is the let-7 family, which contains 12 members (365, 366). The oncogene RAS has been found to be a specific target of the let-7 family members and the restoration of let-7 expression reduces cell proliferation and mammosphere formation of breast cancer initiating cells and decrease metastasis *in vivo* (366). In many breast cancer cell lines and breast cancer patient samples, the level of miR-125a and miR-125b are often found to be downregulated. miR-125 directly target ETS, an

oncogenic transcription factor, and functions as a tumour suppressor miRNA (367). Another frequently downregulated tumour suppressor miRNA in breast cancer is miR-205, which is a negative regulator of epithelial to mesenchymal transition (EMT) and metastasis (337). Furthermore, a number of miRNAs are considered associated with molecular subtypes of breast cancer and individual miRNAs correlate with clinicopathological factors (368). Several studies have demonstrated a different miRNA profile in basal and luminal breast cancer subtypes (368-370). Specifically, let-7c, miR-10a and let-7f are associated with the luminal A subtype; whereas miR-18a, miR-135b, miR-93 and miR-155 are associated with the basal type. Additionally, mir-142-3p and miR-150 are associated with HER2 type (370), whereas miR-342 is predominantly expressed in ER+/HER2+ breast tumours (371).

miRNAs have also important roles in endocrine therapies response and some studies have attempted to identify miRNAs that contribute to the clinical benefits of hormonal therapies. MirR-342 influence ER expression and the response to tamoxifen (372, 373). It has been reported that miR-221/222 are upregulated in endocrine therapy resistant breast cancer (374). Recently, it has been demonstrated that miR-221/222 induce resistance to selective ER downregulators by β-catenin activation and subsequent repression of transforming growth factor-β- mediated growth inhibition (375). Furthermore, miR-221, miR-222 and mir181b directly target TIMP3 and can promote endocrine therapy resistance. Indeed, ER+ MCF7 breast cancer cells subjected to TIMP3 knockdown can survive and proliferate in the presence of tamoxifen (376). In addition, miR-30c has been identified as an independent predictor of tamoxifen response and it has been associated with increased progression-free survival in breast cancer patients (377). Recently, miR-301 expression has been found to be higher in tumour than normal tissues, and patients who suffered recurrence after tamoxifen treatment exhibit higher levels of miR-301 when compared to those who did not (378). miRNAs are also associated with resistance to AI (379). MiR-128a modulates the transforming growth factor-β-signalling and survival of letrozole resistant cell lines (380). A miRNA expression profiling before and after letrozole treatment in both preclinical and clinical settings revealed an increase in let-7f expression in the post-treatment

samples (381). Although aberrant miRNAs expression is important for breast tumorigenesis, it remains uncertain whether altered expression of miRNAs is the cause or the consequence of this pathological process. Understanding the molecular mechanism of action of miRNAs and the miRNAs profiling in breast cancer are essential area of research interest that will represent novel opportunities for the development of strategies for the diagnosis and treatment.

7.3 miR-155 and cancer

miR-155 is a typical multifunctional miRNA and its deregulation has been found associated with different types of cancer, cardiovascular diseases and viral infections. miR-155 was first described in 1989 and reported to be involved in the progression of lymphoma (382). It has more than 400 predicted gene targets (331)(11), including over 100 confirmed ones. miR-155 is over expressed in a number of neoplastic diseases and that it plays a significant role in the process of carcinogenesis, acting predominantly as an oncomiR (383). Several mechanisms have been suggested to explain this biological activity. For example, miR-155 was found to be one of the most potent miRNA suppressing apoptosis in human T cell leukemia Jurkat cells and in MDA-MB-453 breast cancer cells. In fact, over expression of miR-155 is followed by a substantial decrease of *tumour protein 53-induced nuclear protein 1* (TP53INP1), that is a nuclear protein able to induce cell cycle arrest and apoptosis through caspase-3 activation (373). This role in apoptosis could be responsible for the oncogenicity of miR-155 in several types of cancer. High levels of miR-155 have been found in different haematological cancers such as Hodgkin's and non-Hodgkin's lymphoma (384, 385), Burkitt's lymphoma (386) and CLL (387, 388). Furthermore, miR-155 gene was found to be over expressed in several solid tumours, such as thyroid carcinoma (389), breast cancer (334, 337, 390), colon cancer (334), cervical cancer (391), pancreatic ductal adenocarcinoma (PDAC) (392)(54), and lung cancer (393), where it is considered to be a marker of poor prognosis (338, 393).

In human, miR-155 is encoded by gene *MIR155HG*, also termed the B cell integration cluster (BIC locus), which is located on chromosome 21 (Figure 14) (394). Among its targets, there are Ras homolog family member A

(RhoA), forkhead box O3A (FOXO3a) and SOCS1 (395, 396). These genes in breast cancer can induce an increase in epithelial to mesenchymal cell transition, cell plasticity, cell survival, chemo-resistance and radio-resistance (395, 396). miR-155 deregulated targets have been associated to the development and progression of breast cancer, because of their involvement in cell growth and survival pathways (CCND1 and GAB3), cell migration and invasion (PAK2, RAB6A), apoptosis and proliferation (359, 397, 398).

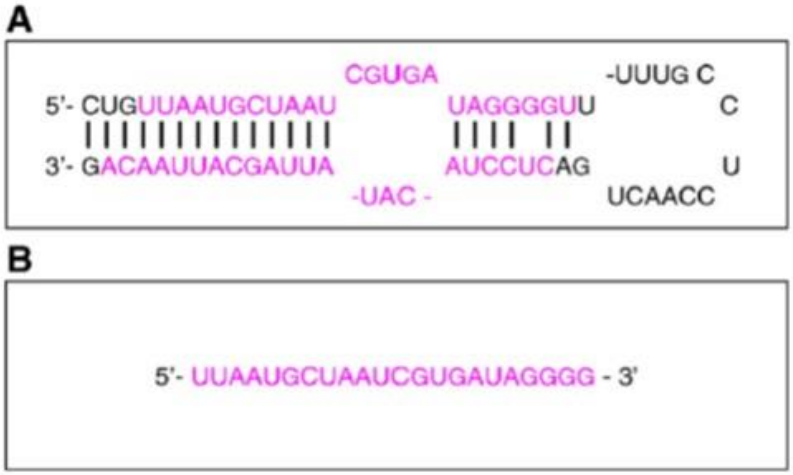


Figure 14. Maturation of miR-155. (A) PremiR-155 stem–loop structure. Nucleotide sequence of miR-155 RNA duplex is indicated in pink. (B) Mature single strand miR-155.

Furthermore, overexpression of miR-155 in breast cancer cells induces the activation of transcription factor STAT-3 through the Janus-activated kinase pathway and stimulation of breast cancer cells by the inflammatory cytokines, suggesting that mir-155 may serve as a bridge between inflammation and cancer. In addition, FOXO3a is a transcription factor which play a crucial role in apoptosis and cell growth by the regulation of a number of apoptosis/cell growth associated genes, and mir-155 can directly associate with FOXO3, blocking its transcription (395, 399). High miR-155 expression associated with low FOXO3a levels are present in recurrent breast tumours after radiotherapy or chemotherapy (400). In conclusion, different studies linked miR-155 expression to both invasiveness and recurrence of breast tumour and have demonstrated that the deregulated

expression of miR-155 and its target genes are of potential clinical prognostic value.

7.4 miR-23b and cancer

MiR-23b is a highly conserved miRNA and is transcribed as part of a cluster of miRNAs along with miR-27b and miR-24 (Figure 15) (401). MiR-23b gene is located on the long arm of chromosome 9 and its transcriptional process results in two mature miRNA transcripts, miR-23b-3p and the less studied miR-23b-5p.

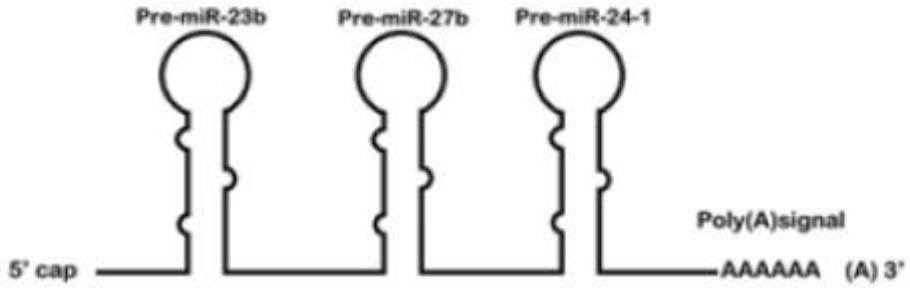


Figure 15. miR-23b~miR-27b~miR-24-1 cluster on chromosome 9.

MiR-23b is a classic example of pleiotropic modulator that influences and regulates a wide range of physiological cell functions, including proliferation, differentiation, motility and immune response. The dysregulation in miR-23b expression promotes the alteration of these cellular mechanisms and the development of diseases. In particular, it has been demonstrated that the miR-23b plays an important role in the tumorigenesis and progression of different types of cancer. It has been demonstrated that miR-23b functions as a tumour suppressor or as an oncogene in a cancer context-dependent manner (Figure 16) (402). MiR-23b has been found upregulated in different types of cancer, such as oral squamous and bladder tumours (403, 404). Similarly, it acts as oncomiR in renal cancer through inhibition of PTEN gene transcription, resulting in an hyperactivation of PI3K/AKT pathway (405). In contrast, in prostate cancer plays the role of suppressor, targeting the mitochondrial enzyme GLS (231). In hepatocellular carcinoma (HCC), miR-23b inhibits directly the transcription of uPA (Urokinase-Type Plasminogen Activator) and c-Met (or

HGFR, Hepatocyte Growth Factor Receptor) genes and the reduced expression of these two factors resulted in a decreased migration and proliferation of HCC cells (406). Similarly, miR-23b inhibits the formation of metastases of colon cancer by targeting FZD7 and MAP3K1 genes (407). Regarding the role of miR-23b in breast cancer, there are different studies that have shown opposing data on the effects of miR-23b in the processes of proliferation, migration and invasion of cancer cells. Wu *et al.* have reported that miR-23b is over-expressed in primary tumours and in the sera of these breast cancer bearing patients (408). Crucially, it has been demonstrated that miR-23b acts as a positive regulator of tumour growth in breast cancer by blocking the tumour suppressor Nischarin (NISCH).

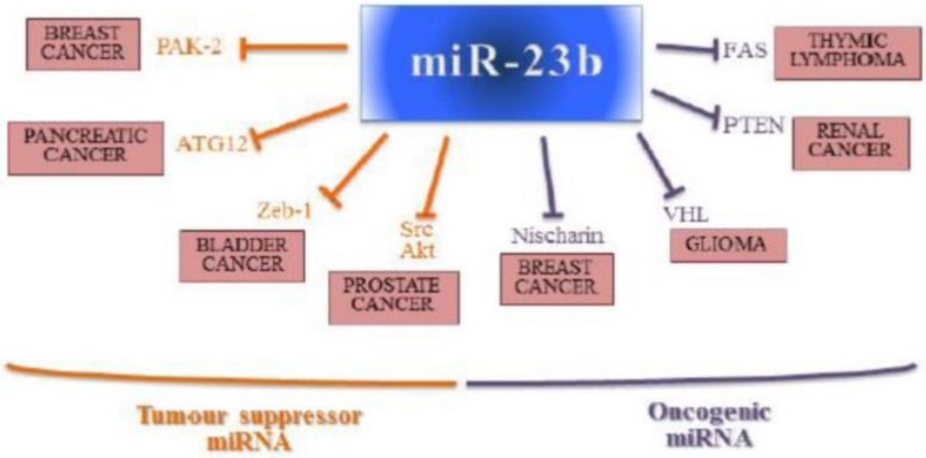


Figure 16. mir-23b as tumour suppressor or oncomir. Schematic representation of direct target in different types of tumour. Abbreviations: ATG-12, autophagy-related protein 12; VHL, Von Hippel-Lindau tumour suppressor; PTEN, phosphatase and tensin homolog (taken from 402).

NISCH is a binding partner for α5β1 integrin, interacts with members of the PAK (p21-Activated Kinase) family kinase and thereby regulates the metastatic behaviour of tumour cells (409, 410). Thus, miR-23b and Nischarin expression levels are inversely correlated in breast cancer: the up-regulation of the miR23b promotes proliferation and cell migration and associates with poor prognosis. Importantly, miR-23b knockdown reduces tumour growth and the formation of metastases *in vitro* and *in vivo* (411). Furthermore, the receptor HER2 and the growth factors EGF and TNF (Tumour Necrosis Factor alpha) promote the ectopic expression of miR-23b

via PI3K/AKT/NF-kB pathway, favouring its tumorigenic properties *in vitro* (411). Other studies have suggested the role of miR-23b as a tumour suppressor. In particular, it has been demonstrated that miR-23b has a role in cytoskeletal remodelling through the enhancement of cell-cell interaction, thus reducing cell motility and invasion during cancer progression (412). Moreover, miR-23b over expression or silencing significantly reduced or increased, respectively, cell invasion in breast cancer and its expression inversely correlates with breast cancer metastasis and tumour growth in *vivo* (413). An interesting study has been performed in radiotherapy resistant-derived pancreatic cancer cell lines, showing that the reduced levels of the miR-23b correlate with radioresistance and its overexpression sensitises the cells to radiation by inhibiting the radiation-induced autophagy (414).

7.5 miRNAs and tumour metabolism

In the last few years, different studies have demonstrated that a large number of miRNAs are involved in the regulation of cancer metabolism (Figure 17) (415). Several studies suggest that miRNAs regulate, in addition to glucose transporters family, several essential enzymes of glycolysis including HKs, glyceraldeid-3-phosphate dehydrogenase (GAPDH), and 6-phosphofructokinase (PFK1). It has been demonstrated that HK2, overexpressed in different types of cancer, can be regulated by miR-143. In particular, miR-143 downregulates HK2 and therefore inhibits glucose metabolism in head and neck squamous cell carcinoma (416), breast cancer (417), lung cancer (418) and colon cancer (419, 420). In addition, miR-155 represses miR-143 and promotes the transcription of HK2, inducing glycolytic phenotype in cancer cells (417). Similarly to glycolysis, the TCA cycle can be subjected to miRNAs control. For example, GLS is crucial in glutamine metabolism. Different studies suggest that p53, which is a direct target of miR-125, miR-30, miR-594, plays an essential role in sustain glutamine level by activating GLS2 (421). In addition, it has been demonstrated that c-Myc downregulates miR-23a and miR-23b in lymphoma and prostate cancer cells and leads to subsequent increase in GLS expression (422). Several miRNAs are involved in the control of lipid metabolism in cancer (423-426).

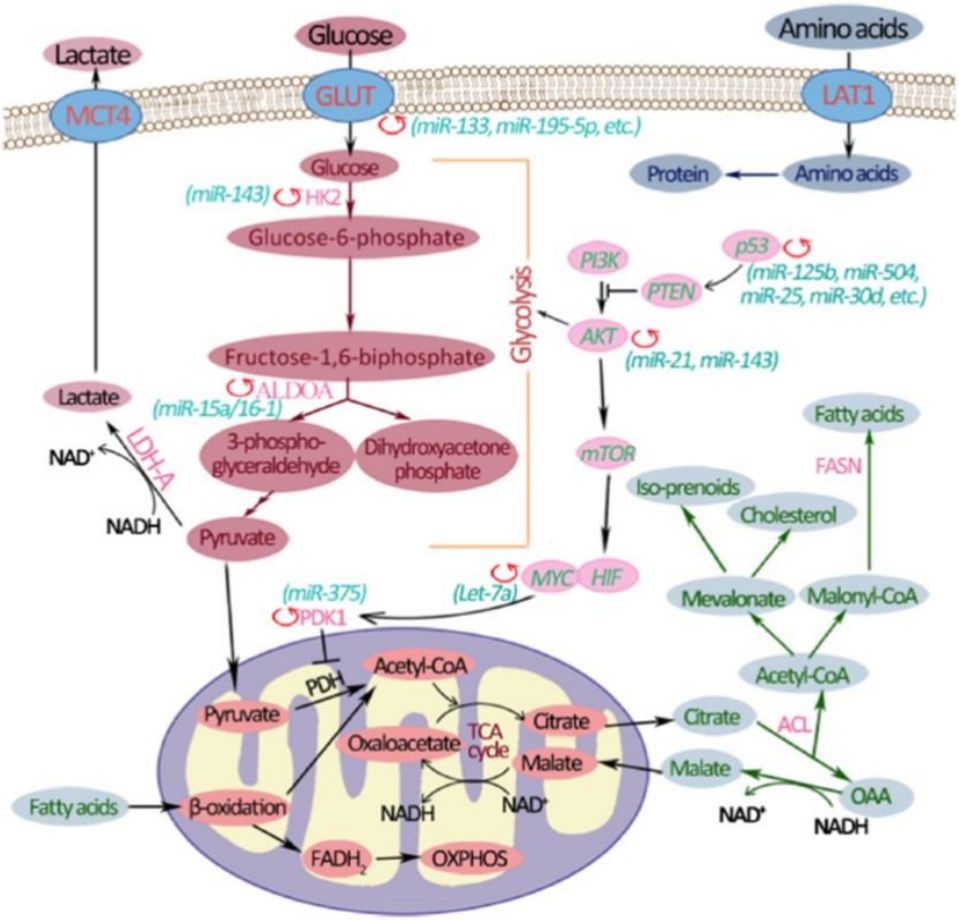


Figure 17. MicroRNAs regulate cell metabolism by targeting key metabolic enzymes and multiple oncogenic signalling pathways. miRNAs could regulate cell metabolism by modulating the expression of metabolic transporters (like GLUT) or enzymes (HK2, ALDOA and PDK1) and acting on p53, c-Myc and AKT/mTOR signalling pathways. The steps regulated by miRNAs are indicated by red circular arrows, and the related miRNAs are listed in the bracket. FASN, fatty acid synthase; GLUT, glucose transporter; HIF, hypoxia-inducible factor; LAT1, L-type amino acid transporter 1; LDH-A, lactate dehydrogenase isoform A; MCT, monocarboxylate transporter; PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase; PI3K, phosphatidylinositol 3-kinase (taken from 415).

In literature, it has been reported that miR-185 and miR-342 regulate lipids and cholesterol biosynthesis in prostate cancer cells by reducing the expression of transcription factor SREBP-1 and downregulating its target genes, including FASN and 3-hydroxy-3-methylglutarate CoA reductase (425). These data suggest that miRNAs are important in the control of cancer metabolic reprogramming by regulating the expression of genes involved in key metabolic pathways.

8- Autophagy

Autophagy, or cellular “self-eating”, is an evolutionary conserved process that occurs in all eukaryotic cells, from yeast to mammals (427, 428). It consist in a vesicular trafficking pathway where intracellular substrates, such as entire organelles, protein aggregates and specific proteins, are targeted for lysosomal degradation and recycling (429). During the autophagy, the portions of the cytoplasm and intracellular organelles are sequestered in double membrane bound structures called autophagosomes (figure 18). These autophagosomes then fuse with lysosomes to form autolysosomes, and the sequestered contents are degraded by lysosomal hydrolases and recycled. The degradation of intracellular aggregates and organelles is important to maintain cellular homeostasis. In addition to have a role in the turnover of protein and organelles, autophagy is involved in many physiological and pathophysiological processes (429-431). Low levels of basal autophagy prevent the gradual accumulation of damaged proteins and organelles in tissues that is toxic over time (429), and a strong activation of autophagy occurs when the cells are under stress conditions, such as nutrient deprivation and pathogen infections. Autophagy deficiency is thought to contribute to the pathogenicity in many diseases including neurodegenerative and liver disease, cancer and aging (432). Autophagy process is very complex, but in general, it can be divided into different phases: induction, vesicle nucleation, vesicle elongation and completion, docking and fusion, degradation and recycling. Up to 35 autophagy-related genes have been identified (ATGs). These genes, that compose the core machinery of autophagy, can be classified into several functional units: the Unc-52 like autophagy activating kinase 1 (ULK1) protein kinase complex, an initiating effector for the autophagic cascade; the VPS34-beclin 1 PI3K complex; two autophagy-specific ubiquitin-like (Ubl) conjugation systems; phopshatydilinositol-3-phosphate (PI3P) effector and the transmembrane recycling protein ATG9. Many ATG proteins contribute to the two Ubl conjugation reactions in which LC3 and ATG12 are the Ubl proteins (434).

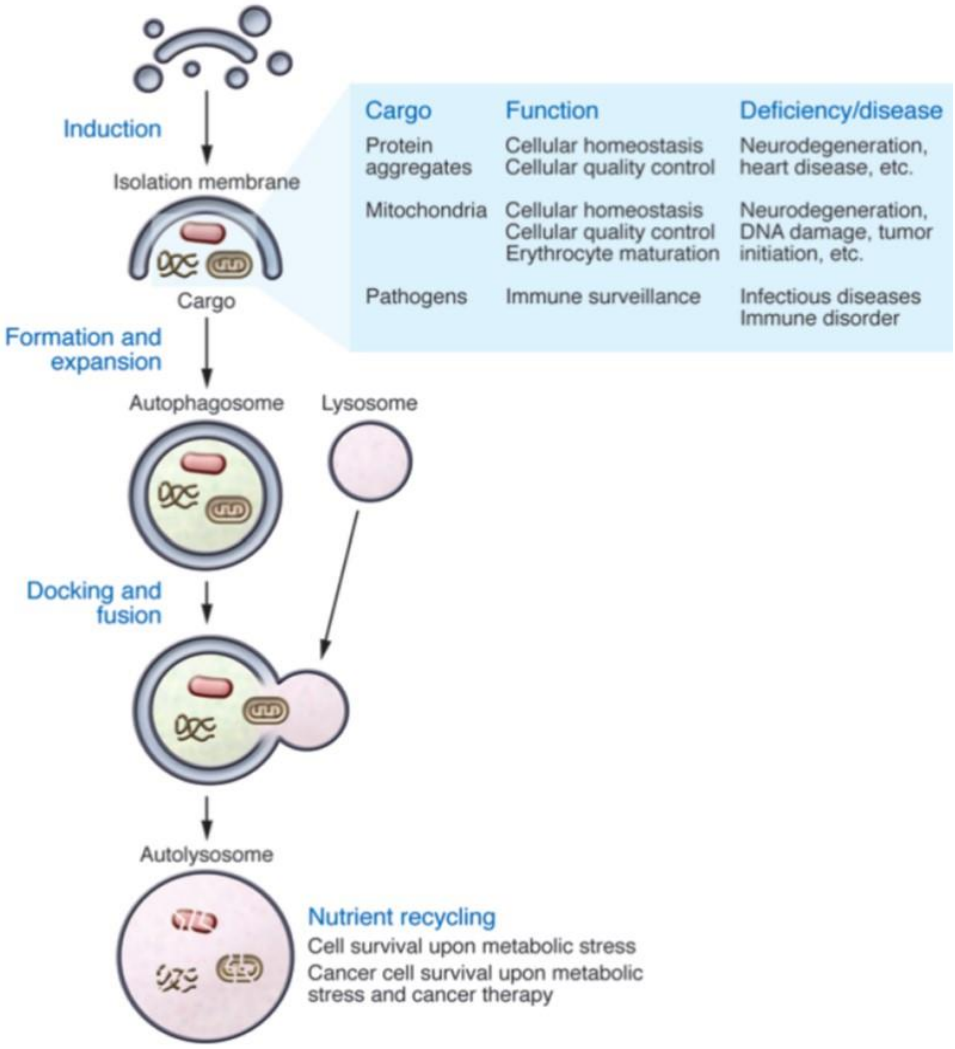


Figure 18. The autophagy pathway. Schematic of the intracellular membrane events involved in the autophagy and its biological functions (taken from 433).

Finally, LC3 is conjugated to phopshatidyl ethanolamine (PE), resulting in LC3-PE (known also LC3-II), which is required for autophagosome formation, cargo recognition and autophagic membrane tethering (435-437). Vesicles nucleation is the initial step in which protein and lipids are recruited for construction of the autophagosomal membranes. In mammalian cells, this process is initiated by activation of the class III PI3K/beclin1 complex. Numerous additional binding partners of this complex function as positive regulator, including BAX-interacting factor 1 (BIF-1), ATG14L and Ambra 1 (activating molecule in Beclin1 regulated autophagy protein 1) that promote the autophagy, or negative regulators

such as Rubicon and *B-cell lymphoma 2* (Bcl-2) that inhibit autophagic activation (438). Intracellular substrates, which must be degraded through autophagy, are targeted at least in part by poly-ubiquitination. It has been demonstrated that during autophagosome formation LC3-II act as a "receptor" at the growing phagophore membrane and interacts with "adaptor" molecules on the targets (e.g. protein aggregate) to promote their selective uptake into phagophore and degradation. The most characterised molecule in this regard is p62/SQMT1, a multifunctional adaptor molecule that promote turnover of poly-ubiquitinated protein aggregates (439). Other molecules, such as NRB1, function similarly to p62/SQMT1 in promoting turnover of poly-ubiquitinated proteins (440).

8.1 Autophagy and metabolism

Autophagy induction is important for normal cells to survive nutrients starvation, which is attributed to the recycling of intracellular component into metabolic pathways (430). The autophagy pathway and nutrient signalling intimately communicate between each other. A central node that coordinates this communication is the nutrient-sensing protein kinase complex mTOR complex 1 (mTORC1), which senses growth factors and amino acid levels in surrounding environment. This master regulator of cellular metabolism serves as an autophagy response switch by controlling the phosphorylation status, and therefore the autophagy activity, of the ULK1 complex. The main components of the ULK1 complex include the protein kinase ULK1 and its regulatory proteins ATG13 and FIP200 (437, 441, 442). Under nutrient-rich conditions, mTORC1 phosphorylates ATG13 and ULK1 to suppress the autophagy. When mTORC1 is inactivated by nutrient starvation or other stresses, the ULK1 complex becomes hypophosphorylated and active. There is an elegant feedback loop between mTORC1 activity and autophagy output. Upon nutrient starvation, mTORC1 is suppressed, leading to activation of ULK1-dependent autophagy. Downstream of autophagy, autophagic cargos are degraded into lysosomes to recycle building blocks, including amino acids, which reactivate mTORC1 and thereby attenuate ULK1-dependent autophagy (443, 444). Another important cellular energy sensor, AMPK, also plays a role in autophagy induction by phosphorylating TSC2 and the mTORC1

component Raptor, leading to inactivation of mTORC1 and subsequent activation of the ULK1 complex. Recently, AMPK has also been shown to directly interact with and phosphorylate ULK1 in a nutrient-dependent manner (445-449). One study suggests that AMPK-driven ULK1 phosphorylation is stimulated by glucose starvation, contributing to ULK1 activation (446). The mechanism of autophagy activation by nutrient deprivation is resumed in figure 19.

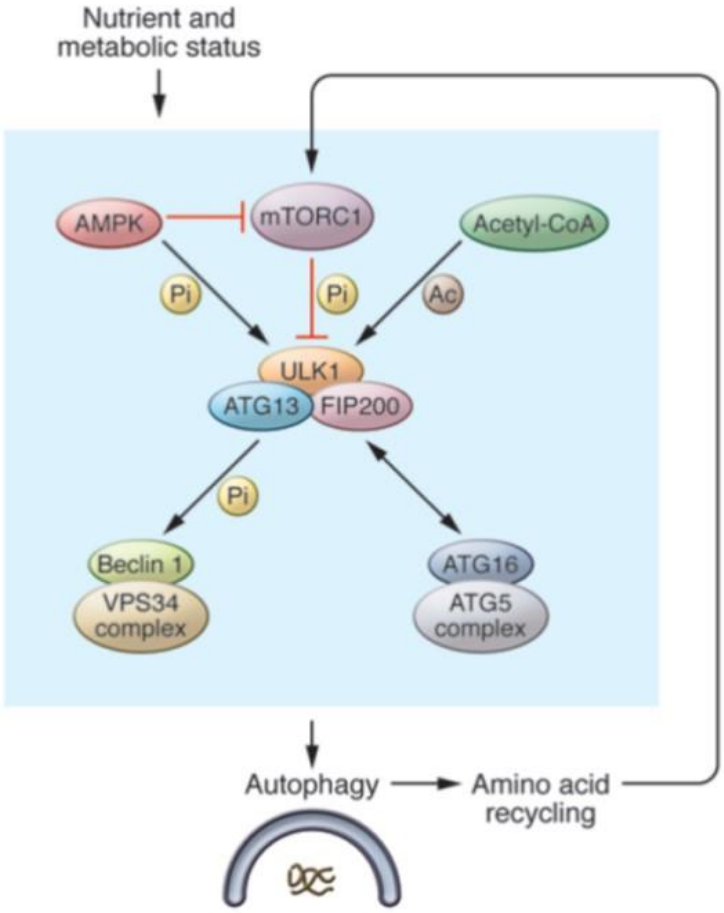


Figure 19. The autophagy pathway senses various nutrient signals via the ULK1 complex. Multiple nutrient-sensing mechanisms, including that modulated by mTORC1, AMPK, and acetyl-CoA, can directly interact with the ULK1 complex and thus regulate autophagy. Autophagy, by recycling amino acids to the cytoplasm, can reactivate mTORC1, and thus feedback suppress the autophagy function of the ULK1 complex. Pi, phosphorylation; Ac, acetylation; ATG16, ATG16L1. The dual-direction arrow indicates protein-protein interaction (taken from 433).

8.2 Autophagy and cancer

Autophagy has been reported to either inhibit or promote cancer cell proliferation, suggesting that the role of autophagy in cancer is controversial and context dependent (450). Autophagy was initially thought to be a tumour suppression mechanism. This concept derived from early reports that showed that the essential autophagy gene *ATG6/BECN1* was monoallelically lost in 40% to 75% of human prostate, breast, and ovarian cancers, indicating Beclin-1 as a tumour suppressor factor (451-453). Breast cancer cell lines frequently contain deletions of one allele of *BECN1*, which is necessary to induce autophagy in response to nitrogen deprivation (453). Introduction of *BECN1* into MCF7 breast cancer cells induced autophagy and inhibited tumorigenicity (453). Furthermore, levels of *BECN1* were significantly decreased in 18 out of 32 breast cancer samples, compared with normal epithelial cells from the breast (453). The contribution of the allelic deletion of *BECN1* to carcinogenesis has been demonstrated by two studies in which *BECN1+/–* mice showed an increased incidence of lung cancer, hepatocellular carcinoma, and lymphoma (454, 455). It has been hypothesised that the inhibition of autophagy could provide advantages for tumour development, because during tumorigenesis cancer cells could prefer to increase protein synthesis compared to their degradation in order to meet high growth and proliferation rate (456). Furthermore, it has been thought that autophagy decreases the mutation rate and suppresses oncogenesis by eliminating damaged organelles that produce genotoxic stresses such as free radicals (457). Therefore, blockage of autophagy could contribute to development of cancer not only by reducing the rate of protein degradation, but also by allowing genotoxic free radical to accumulate. Although autophagy is suppressed during the early stages of tumorigenesis, it seems to be upregulated during the later stages of tumour progression as a protective mechanism against stressful conditions (456, 458, 459). As the tumour grows, cancer cells that are located in the central areas of the tumour are poorly vascularized, so the induction of autophagy could allow them to survive in these low-nutrient and low oxygen conditions (456). Cancer cell lines of various origins, including colon cancer, breast cancer, melanoma,

hepatoma, and malignant glioma cells, undergo autophagy in response to nutrient deprivation (460-464).

Autophagy can promote the adaptation and survival under conditions that lead to cell death, such as anti-cancer therapy. For example, an increase of ATG12 expression confers radioresistance in pancreatic cancer cells, through an upregulation of autophagic processes (414). Studies in breast cancer cells showed that the induction of autophagy by anticancer therapies is usually prosurvival (465-467). It has been demonstrated that tamoxifen and fulvestrant induce autophagy in ER+ breast cancer cells (465, 468-472). Antioestrogen resistant cell lines exhibit increased basal autophagy when compared with their antioestrogen sensitive parental cells (469). Inhibiting autophagy via ATG5 silencing potentiates antioestrogen mediated cell death, indicating that antioestrogen-stimulated autophagy is a prosurvival event and a critical mechanism of endocrine therapy resistance (469). Analysis of publicly available human datasets indicates that ATG5, ATG7 and p62 are elevated in early recurring breast cancer when compared with breast cancers that show no recurrence (473). Furthermore, Cloroquine treatment, which inhibits autophagy by preventing degradation of autolysosome, in combination with antioestrogen therapies increases the sensitivity of resistant breast cancer cells to endocrine therapies (473). These results suggest that autophagy may be involved in cancer progression and in resistance to anticancer therapy, indicating that autophagy may become a therapeutic target to resensitise the cancer cells to the initial therapies.

Materials and Methods

Materials

- Unless specified, all reagents used for cells culture were purchased from Euroclone Group, Invitrogen and Sigma.
- Solutions and equipment for protein analysis were purchased from Biorad, except for PVDF membrane (Polyvinylidene fluoride), used for Western Blotting, which was provided from Millipore.
- Proteases and phosphatases inhibitors were from Sigma-Aldrich.
- Bradford reagent for protein dosage and all materials for SDS-PAGE were from Biorad.
- Chemiluminescence revelation kit was from GE Health Care.
- Matrigel was purchased from BD Biosciences.
- Transwells for invasion assays were from Costar (Euroclone Group). The Diff-Quick staining was purchased from BIOMAP SNC.
- The photographic plates were from Kodak.
- All radiolabelled molecules were purchased from PerkinElmer.
- Reagents for real-time PCR were from Qiagen or Applied Biosystem.

Drugs and Compounds

- E2: dissolved in 100% ethanol at 1 μM, stored at -20°C purchased from Sigma (E2758-1G).
- Androstenedione: dissolved in 100% ethanol at 10 μM stored at -20°C purchased from Sigma (46033)
- Letrozole: dissolved in 100% ethanol at 10 μM stored at -20°C purchased from Sigma (L6545)
- 4-OH tamoxifen: (hereafter simply tamoxifen) dissolved in 100% ethanol at 10 mM, stored at -20°C purchased from Sigma (H7904).
- ICI-182,780: (Fulvestrant) dissolved in DMSO at 10 mM, stored at -20°C purchased from Tocris Bioscience (1047).

- 2-DG: dissolved in Phosphate buffered saline (PBS) at 100 mg/ml stored at +4°C purchased form Sigma (D6134).
- Metformin: dissolved in PBS at 250 mM stored at -20°C purchased from Sigma (D150959).
- 3-Bromopyruvic acid: dissolved in PBS at 10 mg/ml stored at +4°C purchased from Santa Cruz Biotechnology (sc-260854B).
- Ilomastat: dissolved in DMSO at 50mM stored at- 20°C purchased from Chemicon International (CC1010).

Common use solution

- PBS (Phosphate buffered saline): 0.27 g/L di KH2PO4, 0.2 g/L KCl, 8.01 g/L NaCl , 1.78 g/L NaH2PO4 pH 7.4.
- Ripa lysis buffer: (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EGTA, 1mM sodium ortovanadate, 100 mM NaF).
- SDS−PAGE 4X Sample Buffer: 40% Glycerol, 240 mM Tris/HCl pH 6.8, 8% SDS, 0.04% bromophenol blue, 5% β-mercaptoethanol.
- SDS-PAGE 1X running buffer: 25 mM Tris, 192 mM glycin, 0.1% (W/V) SDS, pH 8.3.
- SDS-PAGE 1X blotting buffer: 25 mM Tris, 192 mM glycin, 10% methanol, pH 8.3.
- Blocking solution: non-fat dry milk 2 %, tween 0.05 % in PBS.
- Washing solution: tween 0.1 % in PBS (T-PBS).

Antibodies:

Antibody	Application	Dilution	Use	Source	Manufacturer
HK2	WB	1:1000	O/n 4°C	Rabbit	Cell Signalling
MCT1	WB	1:1000	O/n 4°C	Rabbit	Santa Cruz
MCT4	WB	1:1000	O/n 4°C	Rabbit	Santa Cruz
E-cadherin	WB	1:1000	O/n 4°C	Rabbit	Santa Cruz
Vimentin	WB	1:1000	O/n 4°C	Mouse	Santa Cruz
Rac1	WB	1:1000	O/n 4°C	Mouse	BD transduction lab.
RhoA	WB	1:1000	O/n 4°C	Mouse	Santa Cruz
SLC6A14	WB	1:1000	O/n 4°C	Rabbit	Abcam
p70S6K	WB	1:1000	O/n 4°C	Rabbit	Cell Signaling
p-p70S6K	WB	1:1000	O/n 4°C	Mouse	Cell Signaling
Beclin-1	WB	1:1000	O/n 4°C	Rabbit	Thermo Fischer
LC3 I/II	WB	1:1000	O/n 4°C	Rabbit	Thermo Fischer
AMPK	WB	1:500	O/n 4°C	Rabbit	Santa Cruz
P-AMPK	WB	1:500	O/n 4°C	Rabbit	Santa Cruz
Actin	WB	1:1000	O/n 4°C	Goat	Santa Cruz
Tubulin	WB	1:25000	O/n 4°C	Mouse	Sigma

Table 1. List of antibodies used in thesis. WB = Western blotting; O/n = overnight.

Cell lines

The ER+ wild-type (wt) MCF7 and ZR75-1 breast cancer cells were from American Type Culture Collection (ATCC) and are oestrogen dependent for survival and proliferation. Long-Term E2 Deprived (LTED) cell lines were obtained by culturing MCF7 cells in medium deprived of E2 for at least 20 weeks, to mimic the resistance to aromatase inhibitors. wt-MCF7 and wt-ZR75-1 cells were cultured in phenol red-free RPMI 1640 (Gibco, Life Technologies) supplemented with 10% Fetal Bovine Serum (FBS, Euroclone), 2 mM L-glutamine and 1 nmol/L E2 (both Sigma). LTED cells were cultured in steroid-depleted phenol red-free RPMI 1640 plus 10% dextran charcoal-stripped (DCC) FBS (Hyclone) and 2 mM L-glutamine (DCC medium). For clarity, MCF7-E2 are wt-MCF7 deprived of E2 for 72 h to mimic the acute-phase treatment with AI, whereas LTED cells mimic chronic treatment. MCF7 cells expressing human aromatase at clinical

relevant levels, known as MCF7-2A and MCF7-AROM1 were generated by stable transfection with a retroviral construct pBabe*AROM* expressing full-length human aromatase (*CYP19*) (474, 475). MCF7-2A were used as model of AI sensitivity and were maintained in RPMI 1640 (Euroclone) containing 10% FBS, 2 mM L-glutamine, and 1 mg/ml Geneticin/G418 (Invitrogen). For functional analysis, MCF7-2A were E2 deprived for 3 days by culturing in phenol red-free RPMI-1640 supplemented with 10% DCC. MCF7-TAM and MCF7-ICI cells were obtained by culturing MCF7 cells in DCC medium plus 1μM of 4-OH tamoxifen (MCF7-TAM) or 100 nM of fulvestrant (MCF7-ICI) for at least 12 weeks and represent cellular models of tamoxifen and fulvestrant resistance, respectively. Cells were amplified, stocked, and once thawed were kept in culture for a maximum of 4 months.

Methods

General culture conditions

Cell lines were grown under 5% CO2 at 37°C in their respective media. When passaging cells, growth medium was removed, washed with PBS and the cells incubated with a covering volume of trypsin. After the cells were detached, media was added to the cells to neutralize the trypsin and cells seeded into a new plate.

Frozen storage of cells

Cells were detached using trypsin, re-suspended in culture medium and pelleted by centrifugation at 1,000 g for 5 min. The cells were re-suspended in 1 ml of cell freezing medium (90% FBS and 10% DMSO) and then moved in specific freezing vials. Vials were then placed in polystyrene insulated boxes at -80°C for at least 48 h. After that, frozen vials were stored in liquid nitrogen.

Cell viability

Crystal violet survival assay. Crystal violet (CV) is a triphenylmethane dye (4-[(4-dimethylaminophenyl)-phenyl-methyl]-N,N-dimethyl-aniline) also known as Gentian violet (or hexamethyl pararosaniline chloride). Breast cancer cells were plated in 24-well culture dishes and treated as reported in

the Figures and described in the Results section. After the removal of the culture medium, the cells were fixed in a 4% formaldehyde solution at room temperature for 15 minutes and then incubate with CV solution for 10 minutes at 37°C. CV solution contains 0.5% CV in deionized water and 20% methanol. After incubation, CV was removed through aspiration and the cells were washed with PBS. Finally, CV uptaken by cells was solubilised with 2% SDS (Sodium dodecyl sulphate) solution through incubation in slow agitation for 20 minutes at 37°C. The solution containing CV solubilised was then collected and its absorbance was evaluated at a 595 nm wavelength. Absorbance is positively correlated to crystal violet amount bound to cells.

Protein manipulation

Protein extraction: cells were washed twice in PBS solution and then lysed with RIPA lysis buffer supplemented with proteases and phosphatases inhibitors. Protein lysates were collected, kept in ice and centrifuged at 6000 rpm for 10 minutes. After centrifugation, the supernatant was collected and total proteins were quantified with Bradford assay.

Protein quantification: protein quantification is evaluated with Coomassie Brilliant Blue (Bradford protein assay), which binds to basics and aromatics amino acidic residues (especially arginine) of the proteins, leading to maximum absorption at 595 nm wavelength. Thus, Coomassie Brilliant Blue intensity is positively correlated to protein concentration. To obtain the standard curve of reference, we used Bovine Serum Albumine (BSA), diluting BSA 2 mg/ml concentrated in deionized water and then obtaining rising BSA concentrations from 2 µg/mL to 15 µg/mL. Then Bradford reagent is prepared diluting 1/5 of starting solution with Coomassie Brilliant Blue in 4/5 of deionized water. To run the assay, 5 µL of each sample, opportunely diluted in 45 µL of water, were added to 950 µl of the working solution. After 5 minutes incubation, the absorbance of each sample is evaluated at a wavelength of 595 nm, subtracting the blank value. From the values obtained from the standard curve it is possible to create a curve of absorbance in function of its concentration, thus, interpolating absorbance values to the standard curve, it is possible to calculate the final protein concentration. Correlation between absorbance and concentration is

expressed by Lambert-Beer law: A=εdc, where ε represents the molar extinction coefficient, d the path length and c represents sample concentration. For each Western Blotting experiment from 20 to 50 µg of total proteins are loaded in each lane on a 4–20% pre-cast Mini-PROTEAN TGX Gel.

Polyacrylamide gel electrophoresis: it is a technique utilised for proteins separation based on their ability to move within an electric current, based on the length of their polypeptide chains or of their molecular weight.

SDS polyacrylamide gel electrophoresis (SDS- PAGE) samples are boiled for 5 minutes in a sample buffer containing SDS and β-mercaptoethanol, which leads to disulphuric bonds reduction and destabilization of eventual protein tertiary structure. In addition, sample buffer is supplemented with bromophenol blue, ionizing coloured-tracking solution for the electrophoretic run, and glycerol, which increases sample density and promotes its stratification at the bottom of the loading well.

Once the samples are loaded in the stacking gel, an electric field is applied across the gel, causing the negatively-charged proteins to migrate across the gel towards the positive electrode (anode). Stacking gel, characterised by very low acrylamide concentration (4%), is required to better stratify the samples before entering the separating gel. Proteins relative molecular mass is evaluated by comparison with protein ladder standard molecular weights, separated in the same gel. Running is carried on at 100V for almost 1 h.

Western blotting: Once the protein samples are run , in order to make the proteins accessible to antibody detection, they are moved from within the gel onto a membrane made of polyvinylidene difluoride (PVDF). The method for transferring the proteins is called electroblotting and uses an electric current to pull proteins from the gel into the PVDF membrane. The proteins embedded into the gel are transferred onto the membrane while maintaining the organization they had within the gel. Proteins transfer is carried out at 100V for 1 h and half. PVDF membrane must be previously activated through treatment with methanol for 10 seconds.

After electroblotting the PVDF membrane is incubated overnight in slow agitation at 4°C with specific primary antibodies in a blocking solution containing non-fat dry milk 2% and Tween 0.05%. After incubation, the membrane is washed three times with a washing solution containing PBS 1X and Tween 0.1% and, in order to reveal the specific protein, the membrane is incubated with horseradish peroxidase (HRP) conjugated secondary antibody for 1h at room temperature and then washed again for three times. In the chemiluminescence reaction horseradish peroxidase catalyses the oxidation of luminol into a reagent which emits light when it decays. Since the oxidation of luminol is catalysed by HRP, and the HRP is complexed with the protein of interest on the membrane, the amount and location of emission light is directly correlated with the location and amount of protein on the membrane. Chemiluminescent protein revelation is carried out with ECL Western Blotting reagents and developing of blots is carried out in the developing room placing imaging films on top of the membrane. Exposure is repeated, varying the time as needed for optimal detection.

In vitro Boyden motility and invasion assay

Transwell system is constituted by an upper chamber and a lower part (transwell) separated by 8 μm pore polyvinylpirrolidone-free polycarbonate filters (6.5 mm diameter). The day before the experiment is performed, the upper side of the porous polycarbonate filters was coated with 50 μg/cm^2 of Matrigel dissolved in sterile water and incubated over night at room temperature. The following day, the cells starved from 15-18 h were loaded into the upper compartment of the transwell (100.000 cells in 200 μl of starved medium) and placed into 24-well culture dishes containing 500 μl of complete growth medium. After 48 h of incubation at 37°C, non-invading cells were removed mechanically using cotton swabs from the upper chamber, and the micro porous membrane was stained with Diff-Quick solutions. For the migration assay, cells were starved from 15-18 h and were loaded in the upper compartment of the transwell, in the absence of Matrigel. The procedure is the same of the above described invasion test. Chemotaxis was evaluated by counting the cells migrated to the lower surface of the filters (six randomly chosen fields).

Three dimensional (3D) tumour spheroid invasion assay

96 well flat bottomed plate was coated with 50 µL/well of 1.5% of agarose to impair cell adhesion. For spheroid generation, 200 µL/well of MCF7-LTED suspension at optimized density (0.5×10^4 cells/mL) was distributed in agarose-coated 96-wells plate and centrifuged at 800 g for 3 minutes to facilitate cell aggregation. The plate was incubated for 4 days at 37°C, 5% CO_2. At day 4, when the spheroids were well formed, 100 µL medium was removed from wells and 100 µL Matrigel was gently added.

RhoA or Rac1 activity assay

RhoA-GTP and Rac1-GTP were analysed by pull-down assay from cell lysates. MCF7 and MCF7-LTED cells were directly lysed in RIPA buffer, the lysates were incubated with 10 µg Rhotekin-GST fusion protein (Becton Dickinson) or p21 activated kinase (PAK)-GST fusion protein, both absorbed on glutathione Sepharose beads for 1 h at 4°C. Immunoreactive RhoA or Rac1 were then quantified by western blot analysis. Lysates were normalised for RhoA or Rac1 content by immunoblot.

Gelatin zimography

Zimography was performed using cultured media collected in our experimental conditions. Aliquots of cultured media were electrophoresed on 8% SDS-PAGE co-polymerized with 0.13% type A gelatine under non-reducing conditions. Gels were washed twice in 2.5% Triton X-100 for 30 min and then incubated overnight at 37°C in 50 mM TRIS-HCl, pH 7.4, 200 mM NaCl and 5mM CaCl2 to allow MMPs activity. After incubation, the gels were stained for 90 minutes with a saturated Coomassie brilliant blue solution (methanol 40%, acetic acid 10%, distilled H_2O 50%) at room temperature and then destained by several washings in the same buffer without dye. After the wash in distilled water, the gels were scanned immediately with Quantity-One Image Analysis software (Bio-Rad). The bands containing gelatinase activity appeared transparent and were evident in the otherwise homogeneous blue gel.

FITC-collagen release assays

Cell suspension was copolymerized with non-labelled rat tail collagen I containing 2% FITC-labelled collagen monomers. Migration was allowed for 48 h, and solid-phase collagen containing the cells was pelleted, whereas FITC released into the supernatant was analysed by spectrofluorometry at 595 nm wavelength.

Gene expression and miRNA analysis

Total RNA was extracted from wt-MCF7 cells cultured in presence or absence of E2, and their LTED derivatives and subjected to miRNA and mRNA profiles analysis by Human miRNA and Gene Expression Microarrays (Agilent Technologies). Data were normalized and the differentially regulated genes (fold-change>1.5, adjusted p-value<0.05) were obtained using One-way ANOVA and Student-Newman-Keuls post hoc test. Gene expression data were then subjected to Gene Set Enrichment Analysis (GSEA, Broad Institute). The integrated analysis and reconstruction of post-transcriptional regulatory networks was performed using Magia 2.0 (476).

Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted from tissue culture cells, grown as a monolayer, using RNeasy Mini Kit (Qiagen). RNA concentration and quality of the samples were determined by measuring the UV absorbance at 260 nm and 280 nm on Nanodrop 1000 (Thermo Scientific) and 500 ng of total RNA were reverse transcribed to strands of cDNA using Quantitech Reverse Transcription Kit according to manufacturer's instructions (Qiagen). mRNA expression by qRT-PCR analysis was performed using QuantiFast SYBR Green (Qiagen) for GLUT1 (GLUT-1 Forward: 5'CGGGCCAAGAGTGTGCTAAA-3'; GLUT-1 Reverse: 5'-TAGCGATACCGGAGCCAATG-3'), Sso Advanced Universal SYBR Green Supermix (Bio-Rad) for Beclin-1 (qHsa CID0016032) and TNFAIP3 (qHsa CID0012648), and Taqman assay (Applied Biosystem) for HK2 (Hs0060686_m1) and SLC6A14 (Hs00924564_m1). Data were normalised on β-2 microglobulin for SYBR (Qiagen) or GAPDH (qHsa CED003874) for

SYBR (Bio-RAD), or GAPDH (Hs02758991_g1) and ACTB (4310881E) for TaqMan.

For miRNA analysis, total RNA, including small RNAs, was purified using miRNeasy kit (Qiagen). RNA concentration and the quality of the sample was evaluated using Nanodrop as described above, the reverse transcription reaction of 500 ng of total RNA was carried on using miScript II RT kit (Qiagen). The quantification of miRNAs expression level was assessed by qRT-PCR using miScript SYBR Green PCR kit and miScript Primer Assay Hs_miR_143_1, Hs_miR_155_2 and Hs_miR_23b_2 (Qiagen). Data were normalised on Hs_SNORD61_1 (Qiagen). All the amplifications were run on 7500 Fast Real-Time PCR System. Data were reported as relative quantity with respect to the calibrator sample using the 2-ΔΔCt method. For miR-155 analysis on patient-derived RNA, we used a ΔCt technique normalising on both miScript Primer Assay SNORD61 and RNU6-2 (Qiagen).

RNAi transfection

The day before the transfection 30 x 10^4 cells were plated in 6-well culture dishes in order to reach 70% of confluence the day after. Cells were transfected according to the protocol of mirVana miRNA Mimics RNAi Transfection (Invitrogen by Life Technologies) with 20 nmol/L anti-miR-155 (Ambion, MH12601), miR-23b-3p *mimic* (Ambion, MC10711) and miR-23b-3p *inhibitor* (Ambion, MH10711) or miRNA *mimic negative control#1* (Ambion, # 4464058), and anti-miRNA negative control (Ambion, AM17011) using Lipofectamine 2000 Reagent (Invitrogen). Functional analysis were performed after 48 h or 72h after transfection.

Glucose, lactate, glutamine and amino acids uptake

Breast cancer cells were plated in 6-well culture dishes and treated as reported in the Figures and described in the Results section. Radiolabeled nutrients uptake was evaluated in a buffered solution (140 mM NaCl, 20 mM Hepes/Na, 2.5 mM MgSO4, 1 mM CaCl2, and 5mM KCl, pH 7.4) containing 0.2 µCi/mL D-[U-^{3}H]-glucose, or 0.2 µCi/mL D-[U-^{14}C]-lactate, glutamine or L-[U-^{14}C]-amino acids mixture for 15 min at 37°C. Cells were subsequently washed with cold PBS and lysed with 0.1 M NaOH.

Incorporated radioactive nutrients derived signal was measured by liquid scintillation counting and normalised on protein content.

Detection of released CO_2 by radioactive glucose or lactate

Breast cancer cells were treated as indicated in the Results section and 0.2 µCi/mL [U-^{14}C]-glucose or U-^{14}C]-lactate was added for 15 minutes. Each dish had a taped piece of Whatman paper facing the inside of the dish soaked with 100 µL of phenyl-ethylamine-methanol (1:1) to trap the CO_2. Then 200 µL of 4 M H_2SO_4 were added to the cells. Acidification of the medium allow CO_2 release. Each plate was incubated for 37°C, 5% CO_2 for 1 h to permit $^{14}CO_2$ to be trapped into the Whatman paper. Finally, the Whatman paper was removed and transferred to scintillation vials for counting. Measuring CO_2 production is a read-out for OXPHOS evaluation. In fact, cells use glucose together with O_2 for producing energy, H_2O and CO_2, according to the reaction $C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O$ + energy. When a given radioactive substrate is administered to the cells and enters a catabolic reaction that leads to CO_2 production, radioactive CO_2 can be detected by a scintillation counter.

^{3}H thymidine incorporation assay

This assay allows measuring the proliferation rate of cells by ^{3}H-Thymidine incorporation into the new synthesised DNA. Cells were plated in 12-well culture dishes and incubated in culture medium with 1 µCi/ml of methyl-3H-Thymidine for 4 h leading to the incorporation of radioactive nucleotide into the new DNA synthesised during cell division. After incubation, the cells were washed twice with PBS and precipitated with 1 ml of 10% TCA (trichloracetic acid) for 15 minutes at room temperature. Cells were subsequently wash for three times with TCA solution and finally lysed with NaOH 1 M for 15 minutes at room temperature. Incorporated radioactive was assayed by liquid scintillation counting and normalised on protein content. Radioactivity is positively correlated to cell proliferation index.

In vivo experiments

Female Ncr Foxhead nude 6- to 8- week-old mice (Harlan) were kept under sterile conditions with free access to food and water. Mice were ovariectomised and then allowed to acclimatize for approximately 14 days.

MCF7-AROM1 xenografts were initiated by subcutaneous inoculation of 100 mL cell suspension containing 1 x 10^7 cells in Matrigel (BD Biosciences) into the right flank. Growth was maintained by androstenedione support through intradermal daily injection (100 µg/day). Tumours were grown to approximately 8-mm diameter and assigned to treatment groups. Mice continued to receive androstenedione support and were randomized to receive daily doses of vehicle (10% N-methyl-pyrollidone (NMP)/90% polyethylene glycol (PEG300) or letrozole (1 mg/kg in 150 mL of 10% NMP/90% PEG300). Letrozole was administered daily and mice were sacrificed after 21 days. All animal work was performed in collaboration with Dr Clare M. Isacke at Institute of Cancer Research (ICR, London), and carried out with UK Home Office approval.

Immunohistochemistry (IHC)

For GLUT1 IHC, antigen retrieval was carried out by microwave for 5 minutes at full power (900 W) in citrate buffer pH 6.0. Anti-GLUT1 antibody (Abcam) was applied at 1:3000 dilution. The stained slides were then scanned on a whole slide scanner (Nanozoomer 2.0-HT, Hamamatsu).

Statistical analysis

Statistical analysis of the data was performed using GraphPad Prism Software by Student's t-test or ANOVA as described in the figure legends and Results section. Differences were considered statistically significant when $p < 0.05$.

Results and Discussion

Despite the clinical benefit of endocrine therapy for the ER+ breast cancer subset, resistance to endocrine agents remains a problem, with a large proportion of women relapsing with endocrine resistant-disease. As extensively described in the Introduction section, several molecular mechanisms have been proposed to contribute to endocrine therapy resistance including hypersensitisation to oestrogen (477, 478) and ER activation via aberrant growth factor signalling (479). Different pharmacological compounds have been developed to target this altered signalling pathways in combination with endocrine therapy. However, given the adaptability of tumour cells, targeting a single growth factor or a downstream signalling hub likely leads to compensatory mechanism and many patients fail to benefit from these combined therapeutic approaches. It has been shown that targeting bioenergetic alterations sensitise breast cancer cells to chemotherapies (292, 480, 481) and to biological therapy, such as Herceptin (302).

The final goal of this study was to identify and characterise the metabolic phenotype of ER+ resistant breast cancer cells (compared to parental cells), with a particular focus on the metabolic reprogramming in response and adaptation to long-term oestrogen deprivation (LTED), a condition that mimics AI treatment. In addition, since this metabolic reprogramming could be involved in the resistance to endocrine therapy, understanding the metabolic reprogramming during LTED will help to identify potential metabolic-related predictive biomarkers of endocrine therapy response and/or potential therapeutic targets that can be further exploited for combinatorial treatment approaches.

Role of central carbon metabolism in response and adaptation to AI

The combination of letrozole and glycolysis inhibitors synergistically inhibits MCF7-2A cancer cell growth *in vitro*

As described in the Material and Methods section, MCF7-2A cell line is a cellular model sensitive to AI treatment. They stably express the aromatase enzyme and thus can convert the androgens into oestrogens. Therefore, when cultured in the presence of androstenedione (10 nM) they showed a dose-dependent decrease in cell survival in response to the AI letrozole (Figure 20A). Importantly, after 3 days of letrozole treatment, MCF7-2A cells showed a dose-dependent decrease in their glycolysis capacity, indicated by reduced radioactive [^{3}H] glucose uptake (Figure 20B). However, at this point time, MCF7-2A did not show a significant cell growth decrease, suggesting that the inhibition of glycolysis by letrozole precedes cell growth inhibition and indicates that glycolysis impairment is not merely a bystander effect of the cell growth inhibition. In fact, the inhibition of glucose uptake, index of glycolytic capacity, positively correlates with the inhibition of cell viability induced by letrozole, as shown by statistical correlation analysis (r = 0.92, P < 0.001, figure 20C), suggesting that combining letrozole with a glycolysis inhibitor may be more effective in decreasing cell survival than using AI alone. Indeed, the combination of letrozole treatment with glycolysis inhibitor 2-DG enhanced the effect of letrozole in inhibiting MCF7-2A cell survival, but had no effect on parental MCF7 cells, which have no endogenous aromatase expression (Figure 20D). It is important to note that the effect of 2-DG was synergistic with that of letrozole, as demonstrated by combination index analysis (Table 2). Comparable results were obtained when letrozole was combined with 3-bromopyruvate, another glycolytic inhibitor (Figure 20E), suggesting that AI sensitive breast cancer cells are dependent on glycolysis to survive and proliferate.

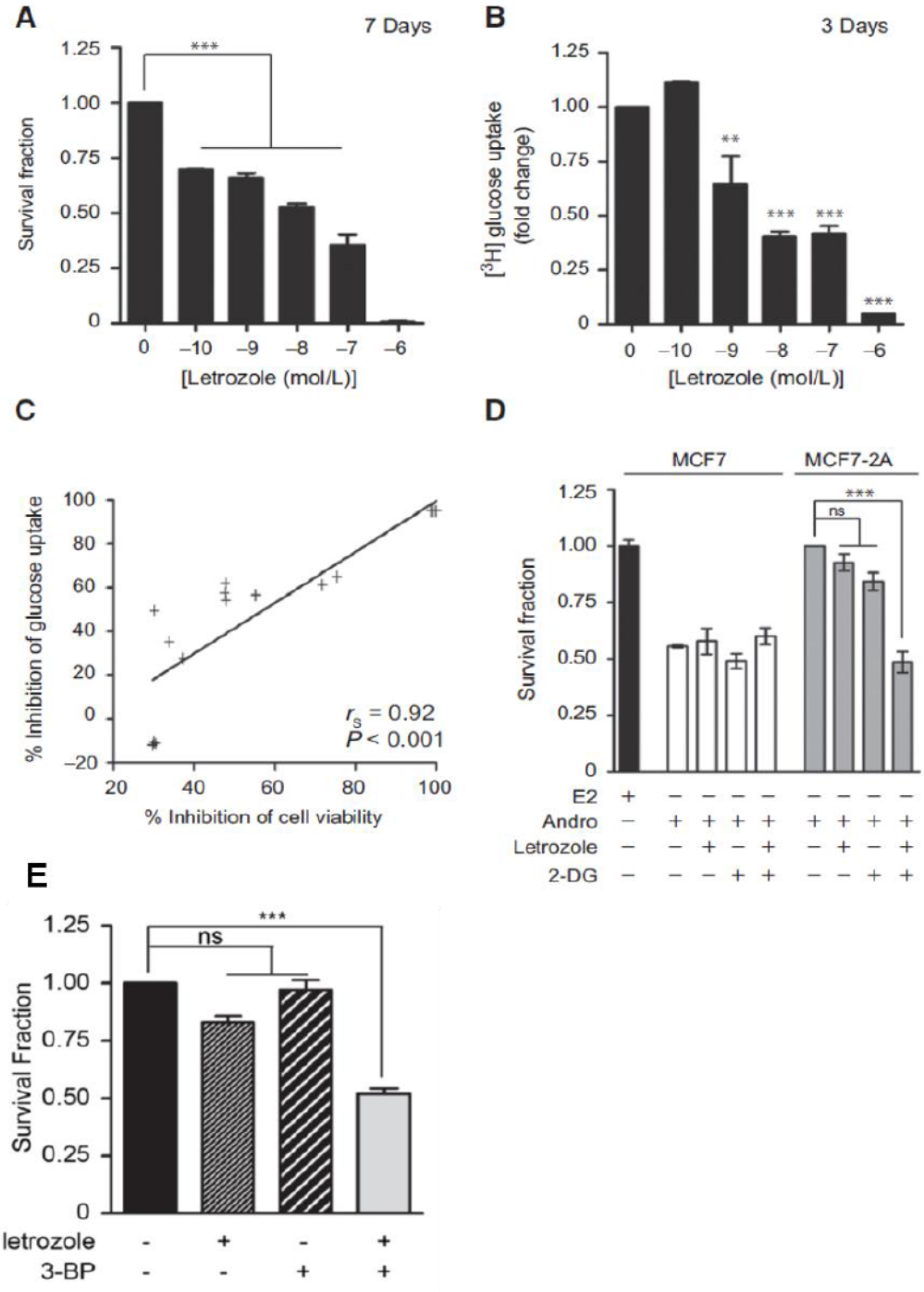


Figure 20. Letrozole impairs glycolysis in MCF7-2A cells and targeting glycolysis in combination with letrozole synergistically inhibits cancer cell growth. A, MCF7-2A were E2 deprived for 3 days with addition of 10 nmol/L androstenedione for the last 24 hours. Cells were then subjected to letrozole treatment with the indicated concentration. B, cells were treated as in A but glucose uptake was measured after 3 days of letrozole treatment. Data represent mean ± SEM, n = 3. 1 way ANOVA; Dunnett corrected; **, P < 0.01; ***, P < 0.001. C, correlation scatter plot of inhibition of glycolysis (glucose uptake) and inhibition of cell viability (r = 0. 92, P < 0.001). D, cells were treated as in A and after 24 hours received either letrozole, 2-DG (1 mg/mL), or the combination of both for further 72 hours. Parental MCF7 cells that have no endogenous aromatase expression responded to 1

nmol/L E2 but not to androstenedione (andro, 10 nmol/L), letrozole (10 nmol/L), or a combination of the two. E, cells were treated as in A and then subjected to letrozole treatment with or without 3-BP for further 72 hours. Data are presented as the ratio of cell survival inhibition measured compared with untreated cells. Data represent mean ± SEM, *n* = 3. 1 way ANOVA; Dunnett corrected; ***, *P* < 0.001; ns, not significant.

2-DG (mg/ml)	letrozole (nM)	Growth inhibition	CI	effect
0.2	10	51%	0.29	synergy
0.2	100	54%	0.27	synergy
0.5	10	53%	0.68	synergy
0.5	100	53%	0.69	synergy

Table 2. Combination Index (CI) analysis of MCF7-2A cells treated with letrozole and 2-deoxyglucose (2-DG). CI is a parameter obtained by Chou-Talalay method to analyse the drug combination effects (ref) and offers quantitative definition for additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1) in drug combinations.

Aerobic glycolysis is enhanced in an *in vitro* model of AI resistance

To investigate the role of the central carbon metabolism in the context of AI resistance, the expression of key molecular components of glycolytic pathway were analysed by Western Blot and qRT-PCR in MCF7-LTED, a cellular model of AIs resistance, compared to parental MCF7 cells. MCF7-LTED, independently to E2 stimulation, showed higher expression both at mRNA and protein level of the key glycolytic enzyme HK2 compared to parental MCF7 cells (Figure 21A and 21B). Furthermore, MCF7-LTED cells increased the expression of the glucose importer GLUT1 (Figure 21C) and of the lactate exporter MCT4 (Figure 21A). This increase in glycolysis-associated components correlated with an increase of glucose uptake (Figure 21D) accompanied by reduced glucose respiration, assessed by [^{14}C] CO_2 release (Figure 21E). Conversely, MCF7-LTED did not differ from parental MCF7 cells in lactate consumption, revealed by analysis of lactate upload (Figure 21F), but reduced the amount of lactate respired (Figure 21G). This data show that AI-resistant ER+ breast cancer cells have a

glycolytic phenotype associated to OXPHOS impairment with respect to parental cells.

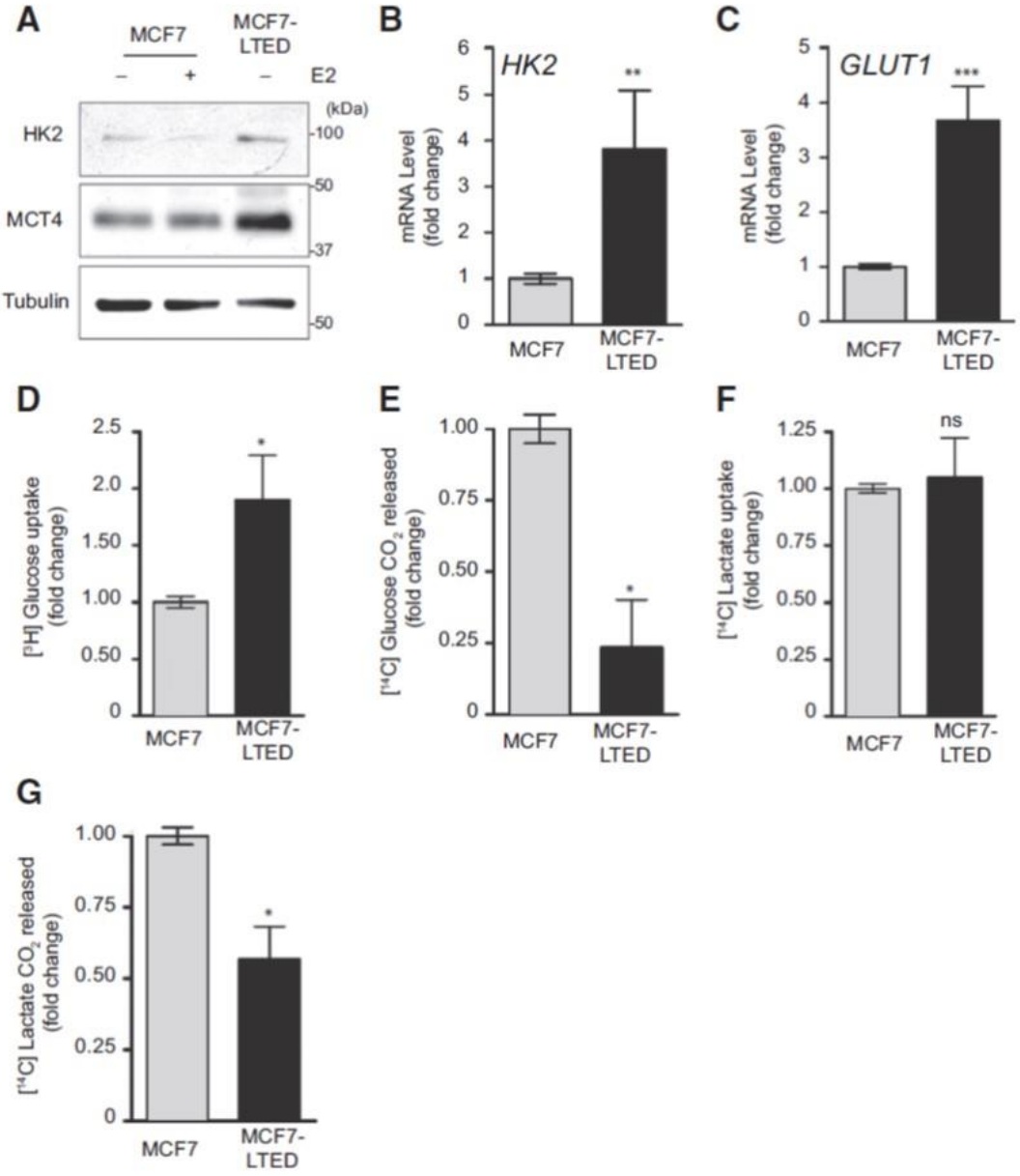


Figure 21. MCF7-LTED cells display higher aerobic glycolytic activity. A, MCF7-LTED cells were compared with wt-MCF7 in presence or absence of 1 nmol/L E2. Total protein lysates were subjected to Western blot analysis as indicated. B–G, MCF7-LTED cells were compared with wt-MCF7 and subjected to qRT-PCR (B and C) after 3-day culture or to radioactive assays (D–G) as described in Materials and Methods. Data represent mean ± SEM, $n = 3$. Student t test; *, $P < 0.05$, **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

MCF7-LTED cells display high metabolic plasticity following the metabolic targeting

To investigate whether targeting glycolysis could lead to decreased survival in MCF7-LTED cells, parental MCF7 cells and their LTED derivatives were exposed to the metabolic poisons, 2DG and Metformin, which inhibit glycolytic pathway and OXPHOS, respectively. Single drug treatment significantly impaired parental cell survival, both in presence and absence of E2 (Figure 22A). Conversely, no effects were observed in cell survival when MCF7-LTED were treated with metformin, and only minor survival fraction changes were noted when MCF7-LTED were treated with 2DG (Figure 22A). As expected, the combination of 2-DG treatment with Metformin dramatically impaired cell survival of both parental MCF7 and LTED cells (Figure 22A), suggesting that MCF7-LTED cells are capable of switching from glycolysis to OXPHOS metabolism when glycolysis is impaired. Indeed, in MCF7-LTED cells, 2-DG treatment decreased HK2 expression (Figure 22B) and glucose uptake (Figure 22C) while induced their ability to upload lactate (Figure 22D), which can be diverted to OXPHOS metabolism. We also analysed the effects of metabolic targeting in another breast cancer cell lines: ZR75-1 cells that have similar level of ER expression compared to MCF7, and their derived ZR75-1-LTED, which in contrast to MCF7-LTED show no ER expression (482). 2-DG and Metformin administration reduced the survival fraction of both parental and ZR75-1-LTED cells (Figure 22E). Crucially, combining metformin with 2-DG had an addictive effect. This suggests that ER expression is a prerequisite for the metabolic plasticity observed in MCF7-LTED. Indeed, following the treatment with the ER downregulator fulvestrant (ICI 182,780), parental MCF7 cells and their derivatives (LTED and MCF7-2A) showed impaired glucose uptake and enhanced OXPHOS (Figure 23).

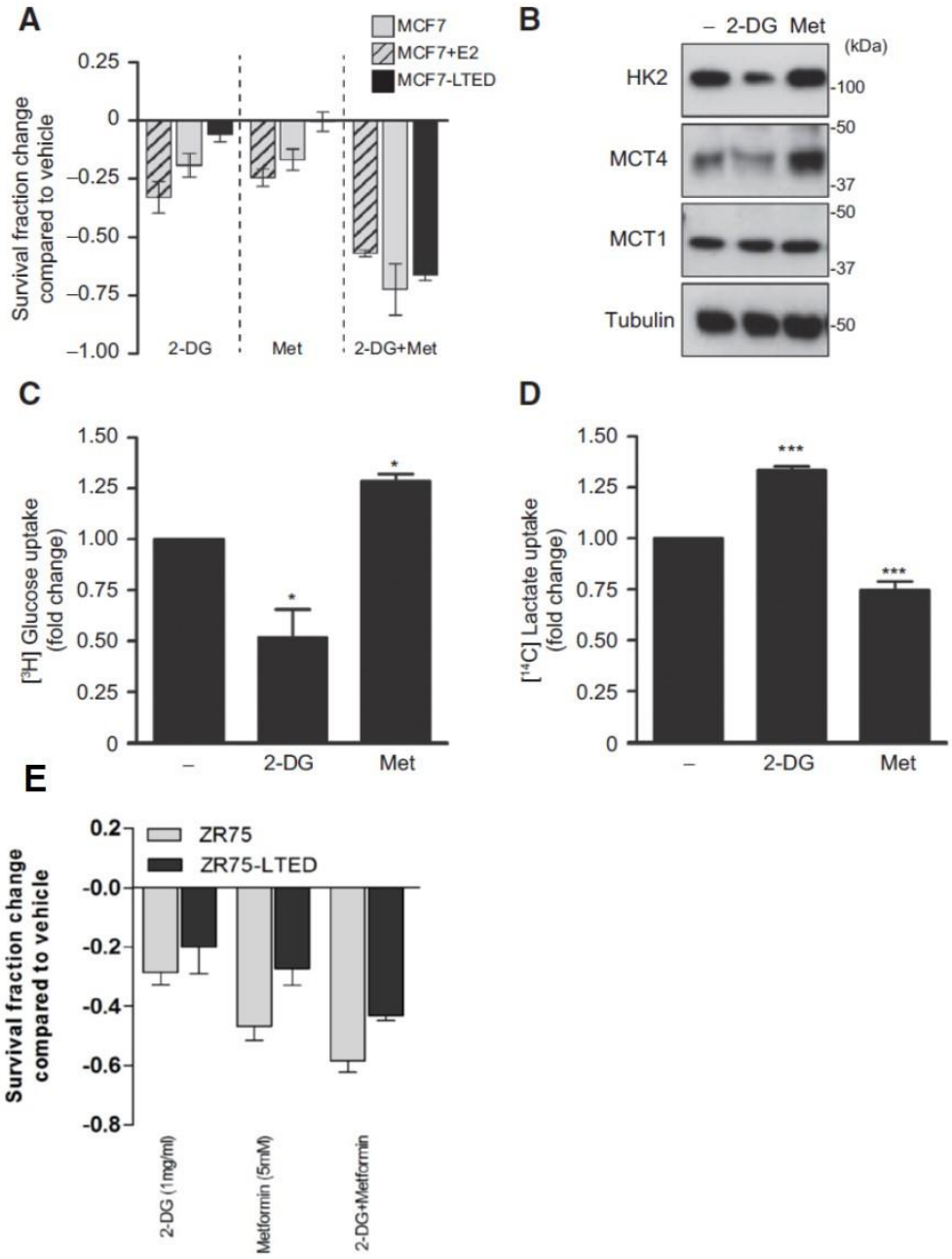


Figure 22 MCF7-LTED cells display metabolic plasticity and are insensitive to glycolysis targeting. A, MCF7-LTED cells were compared with wt-MCF7 in presence or absence of 1 nmol/L E2 and were subjected to 1 mg/mL 2-DG and 5 mmol/L metformin (Met) treatments. Data are presented as fold change of survival cell fraction compared with untreated cells. B–D, MCF7-LTED cells were treated with or without 2-DG or metformin and subjected to Western blotting (B), radioactive glucose uptake (C), or radioactive lactate uptake (D). 1 way ANOVA; Dunnett corrected; *, $P < 0.05$; ***, $P < 0.001$. E, ER- ZR75-1-LTED cells were compared to ER+ wt-ZR75 and were subjected to 2- DG and metformin treatments. Data are presented as fold change of survival cell fraction compared to untreated cells.

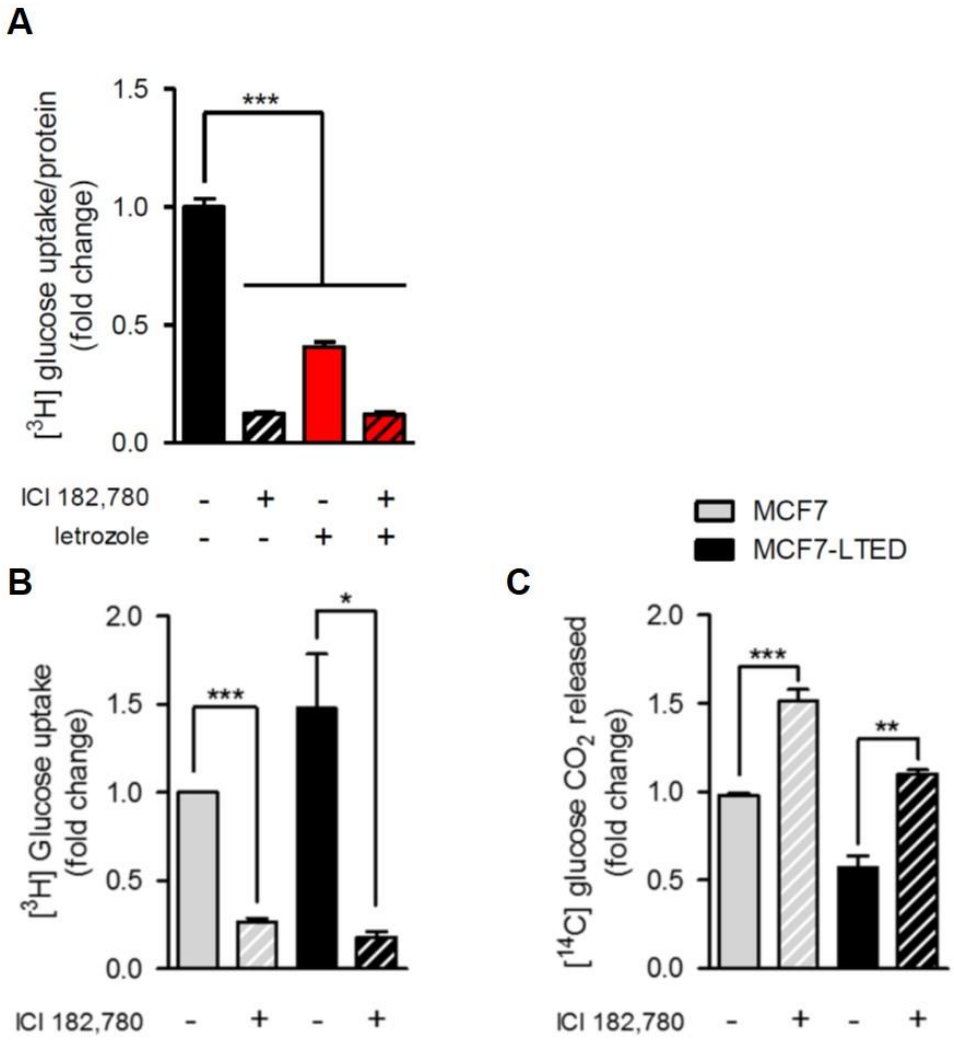


Figure 23. Fulvestrant (ICI 182,780) treatment impairs glucose uptake in MCF7-2A, wt-MCF7 and MCF7-LTED and increases CO2 production in wt-MCF7 and MCF7-LTED. A, MCF7-2A were E2 deprived for 3 days with addition of 10 nmol/L androstenedione for the last 24 hours. Then, cells were treated with 100 nM of ICI 182,780 in combination or not with letrozole for 72 hours and were subjected to radioactive assay. Data are presented as mean value ± SEM, *n* = 3. 1 way ANOVA; Bonferroni corrected; ***, *P*<0.001. B-C, MCF7-LTED were compared to wt-MCF7 and were subjected to 100 nM ICI 182,780 treatment for 72 hours. Glucose uptake (B) and CO2 production ©) were analysed by radioactive assay. Data are presented as mean value ± SEM, *n* = 3. Student *t* test *, *P* <0.05; **, *P* <0.01; ***, *P* <0.001.

In addition to metabolic plasticity, MCF7-LTED cells also display high motile plasticity

Since it is established that metabolic reprogramming and motile plasticity correlates with enhanced aggressiveness of cancer cells (170), we

analysed the motile and invasive behaviour of MCF7-LTED compared to parental MCF7 cells.

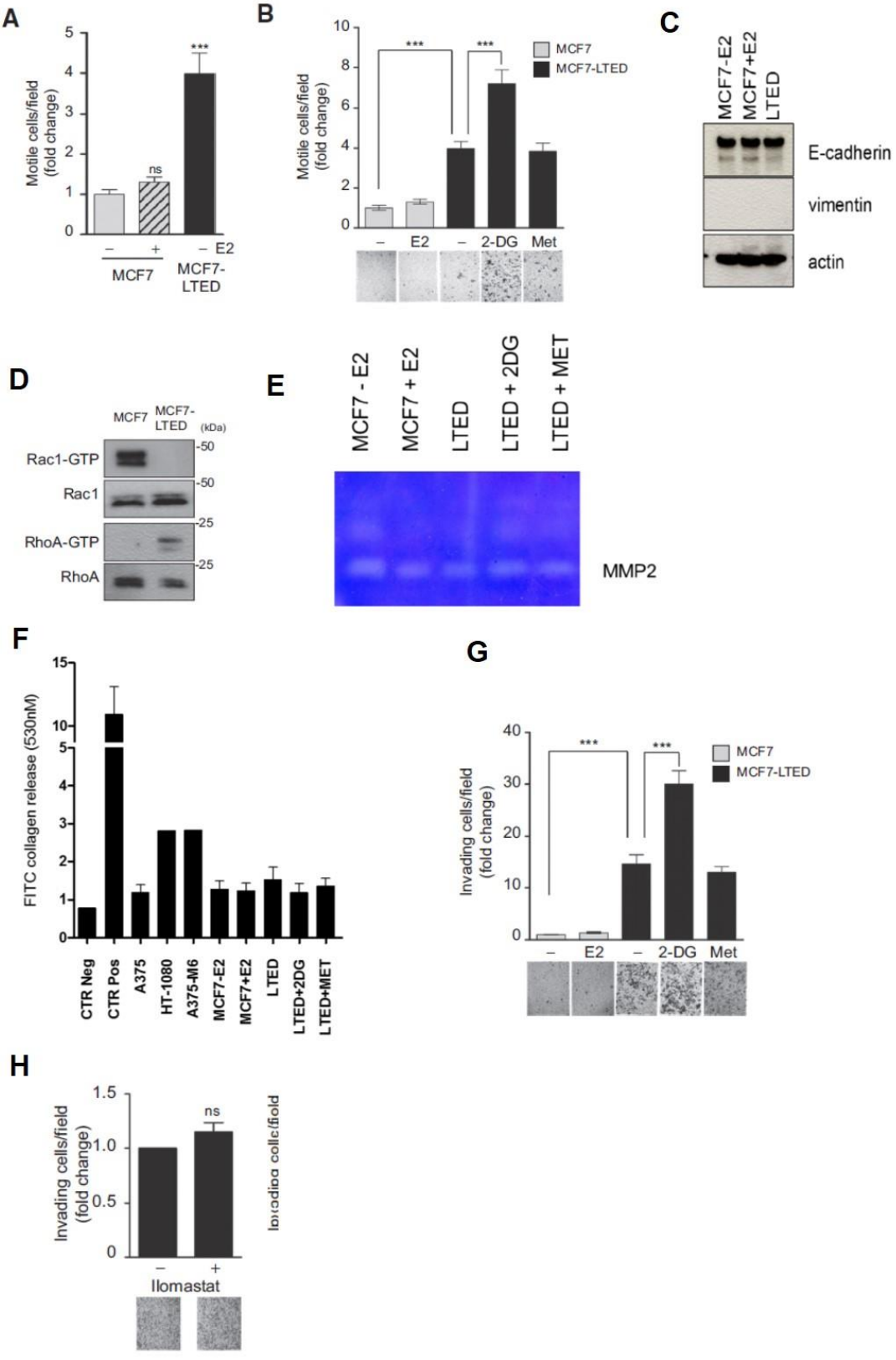


Figure 24. MCF7-LTED cells display high motile and invasive abilities and 2-DG administration enhances these features. A and B, MCF7-LTED cells were compared with wt-MCF7 in presence or absence of 1 nmol/L E2 and were subjected to 48-hours migration assay with or without drug treatments, as indicated; 1 mg/mL 2-DG and 5 mmol/L metformin (Met). 1 way ANOVA; Bonferroni corrected; ***, $P < 0.001$. C, MCF7- LTED cells were compared to wt-MCF7 in presence or absence of 1 nM E2. Total protein lysates were subjected to Western Blot to evaluate EMT markers. D, Rac1-GTP and RhoA-GTP were assayed as described in Materials and Methods. wt-MCF7 cells were compared to MCF7-LTED with or without metabolic target treatments and subjected to (E) gelatin zymography or (F) FITC-collagen release assays as described in Materials and Methods. F, A375 were used as negative control (i.e. amoeboid invasion) while HT1080 and A375-M6 used as positive cell model control (i.e. mesenchymal motility). CTR pos is collagenase. G, cells were treated as in B and subjected to invasion assay. H, MCF7- LTED cells were subjected to invasion assay in presence or absence of the MMP inhibitor ilomastat (50 mmol/L). Student t test; ns, not significant. Data represent mean values ± SEM, $n = 3$. 1 way ANOVA; Bonferroni corrected; **, $P < 0.01$; ***, $P < 0.001$.

To characterise whether metabolic plasticity was paralleled by motile plasticity, MCF7-LTED cells were first subjected to a Boyden assay, in the presence or absence of a Matrigel barrier that mimics the extracellular matrix. LTED cells showed increased migration, both in presence and in absence of E2 (Figure 24A and B) as well as increased invasion (Figure 24G) when compared with parental MCF7 cells. Epithelial-mesenchymal transition (EMT) marker levels, E-cadherin and vimentin, were unchanged in MCF7-LTED with respect to parental cells, suggesting that EMT is not directly involved in the enhanced motility (Figure 24C). Alternative to mesenchymal motility, cells can also adopt amoeboid motility allowing them to slide through the extracellular matrix (483). Amoeboid migration is characterised by MMP exclusion, inhibition of Rac1 and activation of RhoA (484). Accordingly, MCF7-LTED showed enhanced RhoA-GTP expression levels and decreased of Rac1-GTP (Figure 24D). In addition, gelatine zymography of parental and MCF7-LTED cells revealed no significance differences in metalloproteases (MMP) activity (Figure 24E) and fluorescence isothiocyanate (FITC)-collagen release assay showed no increase in collagen degradation (Figure 24F). Finally, treatment with MMP inhibitor Ilomastat did not reduce the invasive capacity of MCF7-LTED cells through Matrigel (Figure 24H).

Since 2-DG and metformin monotherapies had not substantial effect on MCF7-LTED cell survival, we investigated whether these treatments impeded their migratory capacity. Surprisingly, 2-DG treatment enhanced

MCF7-LTED cells motility (Figure 24B) and invasive abilities (Figure 24G), without affecting MMP activity (Figure 24E) and their collagen-degrading capacity (Figure 24F). These features were confirmed by three dimensional tumour spheroid assay. Indeed, MCF7-LTED spheroids embedded in Matrigel treated with 2-DG were more invasive that those generated from untreated MCF7-LTED cells, and the invading cells were characterised by single cell dispersal, resembling typical amoeboid migration (Figure 25).

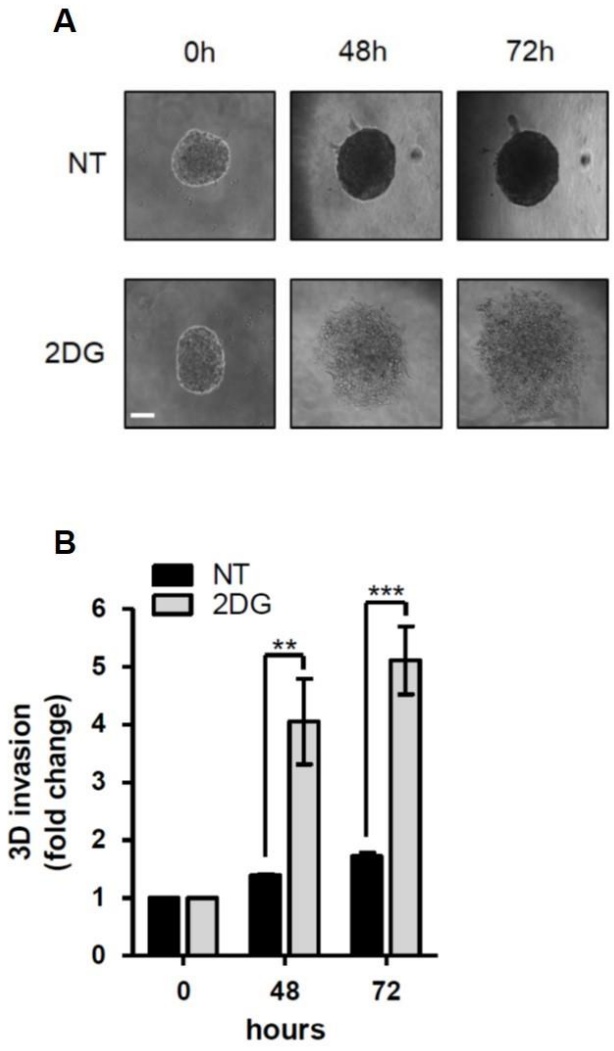


Figure 25. 2-DG increases MCF7-LTED tumor spheroid invasion in Matrigel. MCF7-LTED spheroids were generated as described in Materials and Methods and treated with or without 2-DG (1 mg/mL) and spheroid images were captured using an inverted microscope (Nikon Eclipse TS 100) equipped with a DS camera Nikon Digital Sight at 0, 48 and 72 hours. Scale bar, 20 μm (A). The invasion capacity was evaluated by measuring the invading area using ImageJ software (B). Data are presented as mean value ± SEM, $n = 3$. 2 way ANOVA; **, $P < 0.01$; ***, $P < 0.001$.

These data show that MCF7-LTED cells are characterised by amoeboid motility, in line with the absence of EMT markers expression and MMP activity.

ER-dependent miR-155 is responsible for metabolic and motile plasticity of MCF7-LTED cells

After the unexpected results obtained following the 2-DG treatment in the AI-resistant cell model, we investigated the molecular mechanism responsible for the metabolic and motile plasticity of MCF7-LTED cells. Deregulation of miRNA expression has been demonstrated in many types of cancer as they act as upstream regulators of mRNA expression of genes involved in carcinogenesis (485). In particular, miR-155 has been shown to be overexpressed in breast cancers and be modulated by oestrogen (29). In addition, miR-155 controls miR-143 expression, a miRNA known to target and decrease HK2 expression in MCF7 cells (417). Therefore, we hypothesised that miR-155 and miR-143 may be deregulated in MCF7-LTED cells and responsible for the increase of glycolytic metabolism noted in this cell model. qRT-PCR analysis showed that E2 induced a significant increase in miR-155 expression in parental MCF7 cells and that MCF7-LTED cells had higher (~3 fold) miR-155 expression when compared with their parental counterpart (Figure 26A). This increased miR-155 expression was paralleled by a significant reduction in miR-143 (Figure 26B), consistent with the increased HK2 expression both mRNA and protein levels (Figure 21A and B). E2-induced miR-155 expression was confirmed in MCF7-2A cells upon androstenedione administration (Figure 26C). Importantly, letrozole administration, which blocks the androgen to oestrogen conversion, restored basal miR-155 expression (Figure 26C). In addition, ER dependency was validate in both E2-treated MCF7 and MCF7-LTED cells by administration of ICI 182,780 (Figure 26D). Thus, the hypothesis was that enhanced miR-155 and HK2 expression in MCF7-LTED cells was related to their retention of a functional ER. This was further supported by the observation that miR-155 and HK2 expression were not significantly altered in ER- ZR75-1-LTED cells (Figure 26E and F). At this point, we targeted miR-155 to impair the metabolic properties of MCF7-LTED cells.

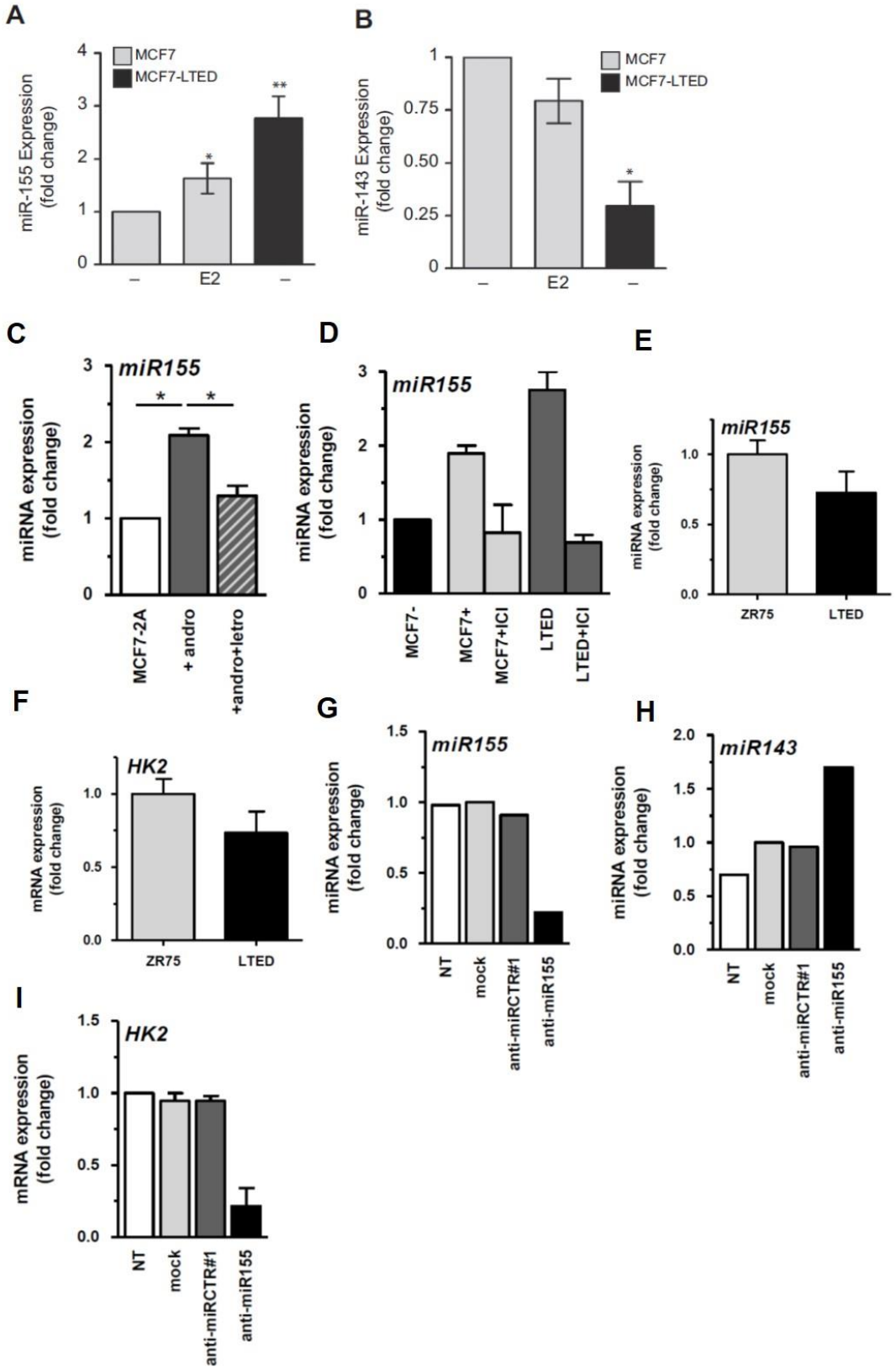


Figure 26. miR-155 is ER-dependent and control HK2 expression. A and B, MCF7-LTED cells were compared with wt-MCF7 in presence or absence of 1 nmol/L E2 and subjected to qRT-PCR. Data represent mean ± SEM, *n* = 3. 1 way ANOVA; Dunnett corrected; *, *P* <

0.05; **, $P < 0.01$.) MCF7-2A were E2 deprived for 3 days with addition of 10 nmol/L androstenedione for the last 24 hours with or without letrozole (100 nM) before qRT-PCR analysis. D, MCF7-LTED were subjected to the ER downregulator ICI 182,780 (ICI 1 μM) and compared to untreated MCF7- LTED and parental MCF7 cells. ZR75-LTED cells that do not retain ER expression were compared to wt-ZR75 cells and subjected to qRT-PCR to evaluate miR-155 (E) and HK2 (F) expression. MCF7-LTED cells were transfected with the indicated RNAi oligos and culture for further 72 hours before qRT-PCR analysis to evaluate miR-155 (G), miR-143 (H) and HK2 expression (I).

Anti-miR-155 caused a marked reduction of miR-155 and HK2 expression, and a corresponding increase of miR-143 expression, as expected (Figure 26G, H and I). Anti-miR-155 transfected MCF7-LTED cells showed a small change in cell survival when compared with anti-miR-scramble control. However, exposure of the anti-miR-155 transfected cells to metformin led to a marked reduction in cell survival (Figure 27A). In addition to its role in the control of glycolytic metabolism, miR-155 has been demonstrated to increase lymphoma cell motility (416). To evaluate whether miR-155 may also be responsible for 2-DG induced migration of MCF7-LTED cells, we compared the anti-miR-155 transfected cells to the anti-miR-scramble control cells. Notably, anti-miR-155 transfection reverted the invasive ability of MCF7-LTED cells when exposed to 2-DG (Figure 27B).

Glycolytic key players and miR-155 levels are decreased in AI-sensitive human breast cancer xenografts following the letrozole treatment

To determine the effects of letrozole treatment on the glycolytic key players, GLUT1 and HK2, and on miR-155 levels in breast cancer xenografts, MCF7 aromatase-transfected cell line, MCF7-AROM1, was injected subcutaneously into immunocompromised mice (see Materials and method). MCF7-AROM1 tumours are sensitive to letrozole administration. Indeed, letrozole-treated tumours were significantly smaller than those that were vehicle-treated (P=0.009, figure 28A). These tumours were subjected to IHC and/or proteins and RNA were extracted for Western Blot analyses and qRT-PCR analyses, respectively. Letrozole significantly reduced the expression levels of GLUT1, as shown by IHC (Figure 28B) and Western Blot analysis (Figure 28C), and that of HK2, both at protein (Figure 28C) and at mRNA levels (Figure 28D).

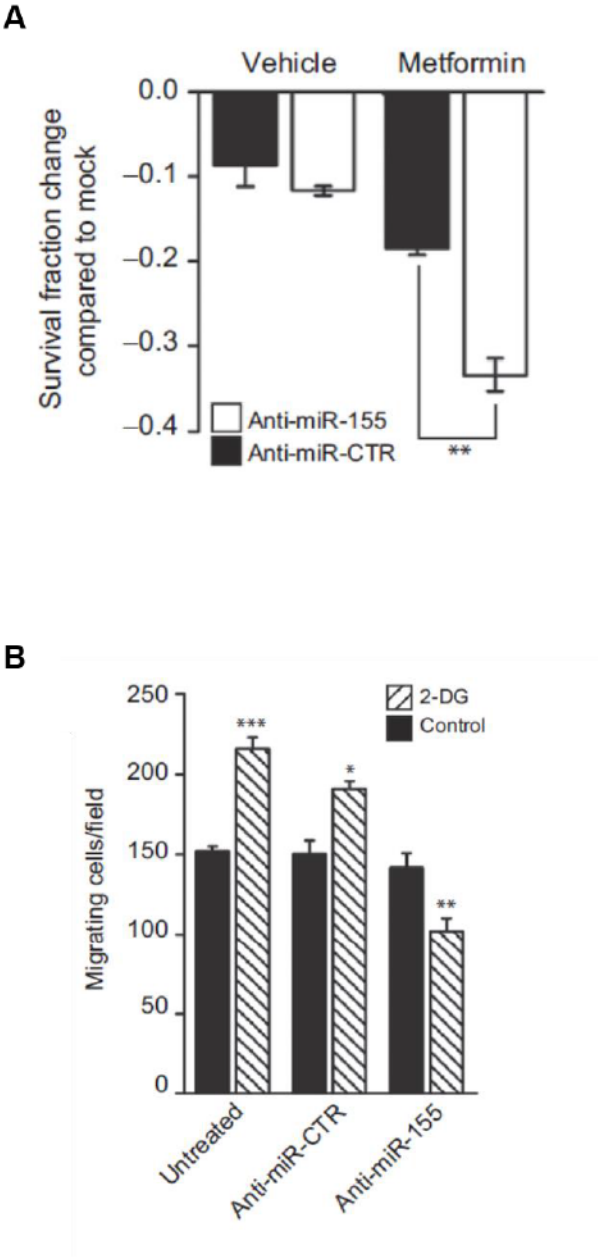


Figure 27. miR-155/miR-143 axis controls metabolic and motile plasticity of MCF7-LTED cells. A and B, MCF7- LTED cells were transfected with the indicated oligos and treated with 5 mmol/L metformin for further 72 hours before survival fraction calculation (A) or subjected to migration assays after further 48 hours of 2-DG treatment (B). Data represent mean ± SEM, $n = 3$. Student t test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Importantly, miR-155 levels were significantly reduced by letrozole administration (Figure 28E), reinforcing the *in vitro* results linking miR-155 expression and the glycolytic phenotype.

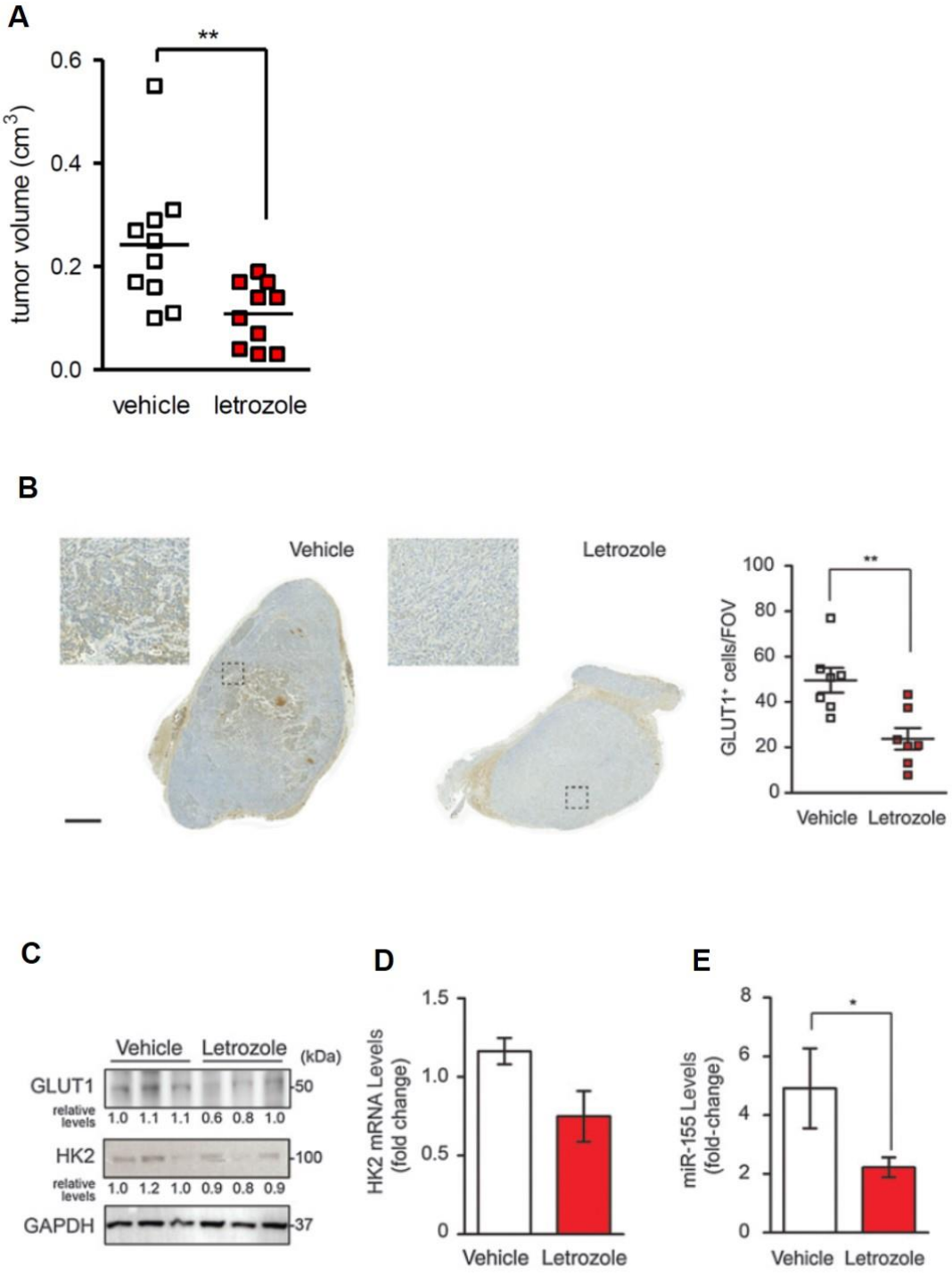


Figure 28. Letrozole administration decreases glycolytic key players and miR-155 levels in AI-sensitive human breast cancer xenografts. Mice under androstenedione support were treated daily with or without letrozole as described in material and methods (*n* = 10 per group). Tumour growth was assessed by calliper measurements of the two largest diameters. Volumes were calculated according to the formula: a × b2 × π/6, where a and b are orthogonal tumour diameters. (A) Tumour volume after 21 days treatment is shown. Data shown are from 10 mice per group ± SEM (Student *t* test, *P* = 0.009). B, representative IHC GLUT1 images of vehicle and letrozole-treated human MCF7-AROM1 xenografts (day

21 of treatment) and relative quantification per field of view (FOV). Data shown are from 7 mice per group ± SEM (Student t test, *P* = 0.004). Scale bar, 1 mm. C, total lysates extracted from MCF7-AROM1 tumours were subjected to Western blot analysis as indicated. Quantification shown below the blots was performed using ImageJ and normalized on GAPDH. Data represent mean ± SEM, *n* = 3. D and E, RNA was extracted and subjected to qRT-PCR for HK2 (D) and miR-155 (E). Data represent mean ± SEM, *n* = 4; Student *t* test: HK2, *P* = 0.084; miR-155, *P* = 0.048.

Glycolytic key players expression correlate with response to AI treatment *in vivo*

Next, we evaluated whether glycolytic players, such as GLUT1 and MCT4, which are differentially expressed in MCF7-LTED cells compared to parental cells, correlated with response to AI treatment. To obtain this information, we analysed publicly available gene expression data from biopsies of 52 ER+ breast cancer patients taken before and after 2 weeks of neoadjuvant letrozole treatment (486). The patients were subsequently divide into responders and non-responder defined by a more than 50% and less than 50% reduction, respectively, in tumour volume, following a further 3 months of letrozole treatment. Pairwise comparison shows a significant decrease in *GLUT1* expression after 2 weeks of letrozole treatment in the responder cohort (*P* = 0.008), but not in the non-responder cohort (Figure 29A). Conversely, *MCT4* expression increases in the non-responders cohort with a borderline statistical significance (*P* = 0.055, figure 29B), but not in the responder cohort. These findings were independently validated in gene expression data derived from 69 paired ER+ breast tumours biopsies taken pre- and post- 2- week neoadjuvant treatment with nonsteroidal AI anastrozole (487). The response to AI can be monitored by a Ki67 staining, a proliferation index that has been shown to predict poor long-term disease outcome (488). In particular, high levels of Ki67 after AI treatment correlate with poor prognosis. Pretreatment (i.e. samples before receiving anastrozole) *MCT4* expression showed a significant inverse correlation with pretreatment Ki67 levels (*r* = -0.28, *P* = 0.02; Figure 29C), suggesting that *MCT4* expression is not merely a surrogate marker of highly proliferating tumours.

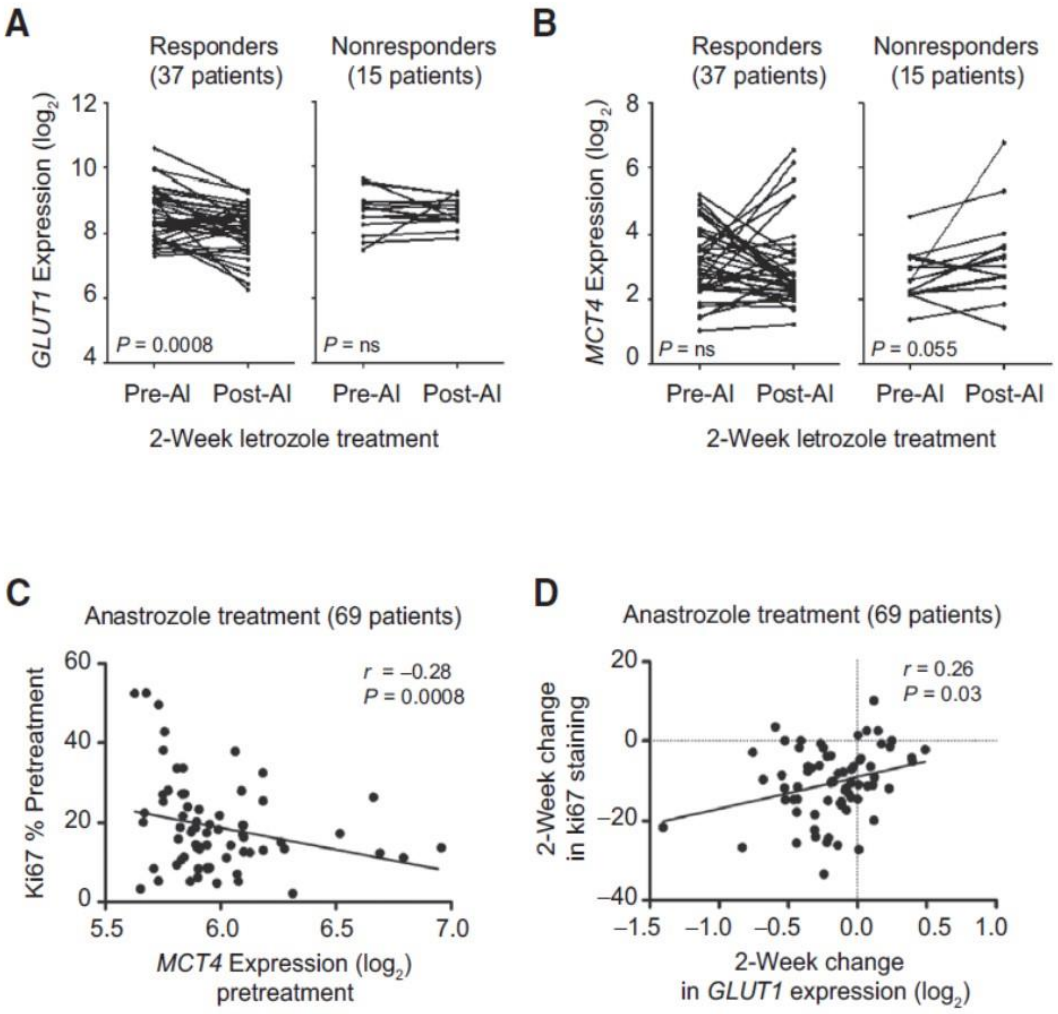


Figure 29. MCT4 and GLUT1 expression levels in the response to AI in clinical specimens. A–D, correlation of metabolic key players GLUT1 and MCT4 expression with response to AI. A and B, changes in 52 paired ER+ breast cancer samples pre- and post-2-week letrozole treatment. A, responder patients show a significant decrease in GLUT1. No significant change was observed in the non-responder group. B, conversely, MCT4 expression is increased in the non-responder group (Wilcoxon test). C and D, correlation of the MCT4 expression with Ki67 staining before receiving anastrozole treatment (C) or of the 2-week change in GLUT1 expression with the change in Ki67 staining between pre- and posttreatment biopsies (Spearman correlation; D). Paired pre- and posttreatment gene expression profiles and Ki67 IHC staining were available for 69 patients.

Furthermore, the change in *GLUT1* expression in the pre- and posttreatment samples positively correlated with the proportional 2-week change in Ki67 (r =0.26, P = 0.03; Figure 29D). In addition, we investigated whether the glycolytic component monitored in AI setting could also be responsible for patient stratification in breast cancers that had been treated with tamoxifen. Notably, the Kaplan-Meier analysis of publicly available

data revealed that patients characterised by higher levels of *HK2* (HR =1.72, *P* = 0.0015), *MCT4* (HR = 1.46, *P* = 0.019), or *GLUT1* (HR = 1.36, *P* = 0.062) showed poorer relapse-free survival when compared with lower expressing tumours (Figure 30A, B and C). Crucially, high miR-155-expressing tumours also showed a poorer prognosis in a cohort of patients that have been treated with tamoxifen (HR =3.62, *P* = 0.0048; Figure 30D), suggesting that miR-155 and glycolysis could also have a role in tamoxifen response.

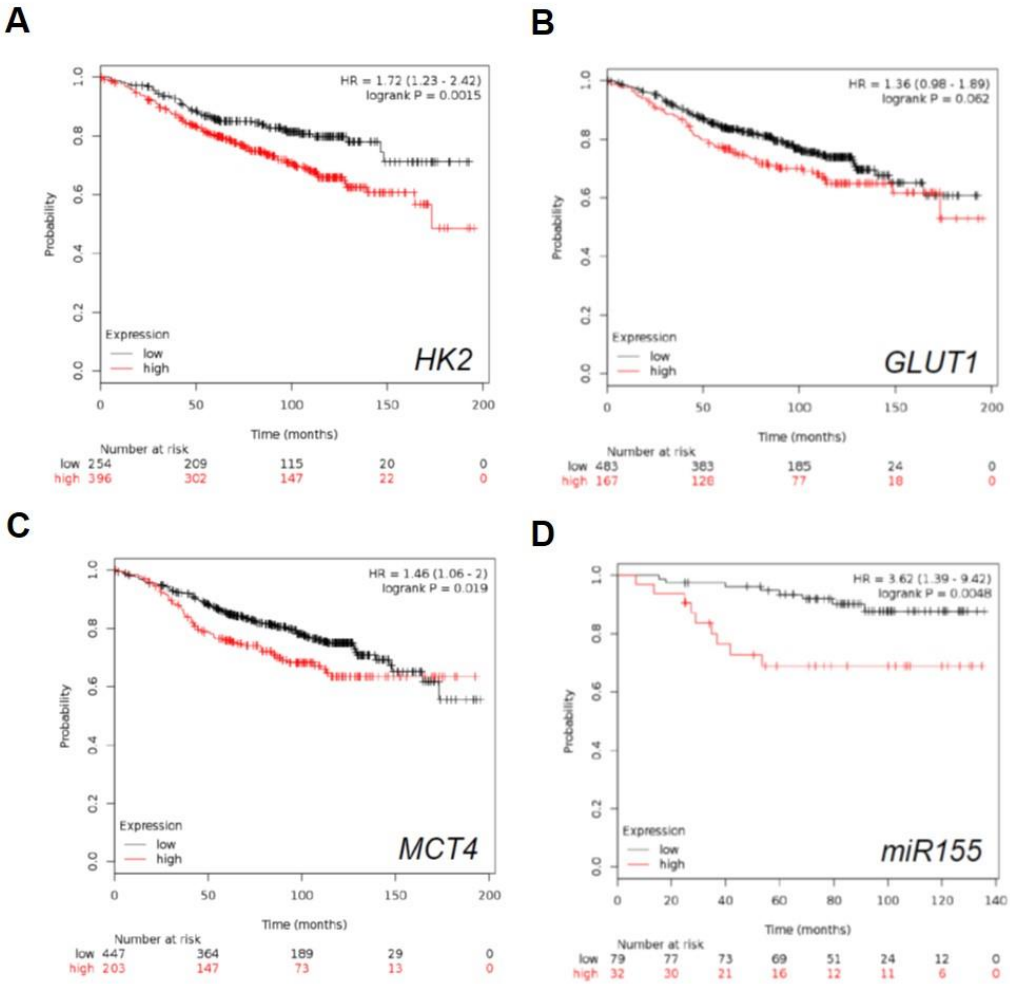


Figure 30. Tamoxifen-treated patients expressing high levels of HK2, GLUT1, MCT4 and miR155 show worst prognosis. Patients analysed were treated exclusively with tamoxifen and the best cut-off was chosen for the analysis. Relapse free survival data were retrieved using Km-plotter (489). Logrank p-value and hazard ratio (HR) with 95% confidence interval are shown.

These data support the idea that glycolytic metabolism plays an important role in the response and adaptation of breast cancer patients to AI treatment and potentially to other endocrine agents, such as tamoxifen.

miR-155 expression in ER+/HER- breast cancers identifies a subset of patients that do not respond to AI anastrozole

As our *in vitro* data suggested miR-155 as key regulator of the metabolic and motile reprogramming in AI-resistant cells, we wanted to validate whether this could be of clinical relevance. Therefore, we analysed by qRT-PCR the expression levels of miR-155 in 64 ER+ and HER2- breast cancer patients before undergoing anastrozole treatment (487). Ki67 levels were also monitored before and after 2 weeks of anastrozole treatment and used as an indicator of therapy response (482). Notably, pretreatment miR-155 levels positively correlate with the Ki67 levels posttreatment (r = 0.3593, P = 0.0035; Figure 31A) and with the proportional 2-week change in Ki67 (r = 0.2973, P = 0.0171; Figure 31B). Pretreatment miR-155 levels did not show a significant correlation with pretreatment Ki67 levels (r = 0.22, P = 0.08), thus excluding a possible association between miR-155 and proliferation. These results indicate that high miR-155 levels correlate with poor response to anastrozole therapy, reinforcing the idea that miR-155 plays a role in the response and adaptation to AI treatment in ER+ breast cancer.

Conclusion

These results show that ER-dependent miR-155 controls the metabolic plasticity of AI-resistant cells and allows them to shift *ad hoc* between oxidative phosphorylation and glycolysis. Clinical data confirm that an increased expression of key glycolytic enzymes and miR-155 correlates with poor prognosis in ER+ breast cancer patients. Crucially, targeting glycolysis in ER+ breast cancers may be of clinical benefit in combination with an AI in sensitive tumours, but could be detrimental once the tumour become resistant. However, targeting miR-155 could impair metabolic adaptation of AI-resistant tumours, therefore prolonging the efficacy of AI treatment.

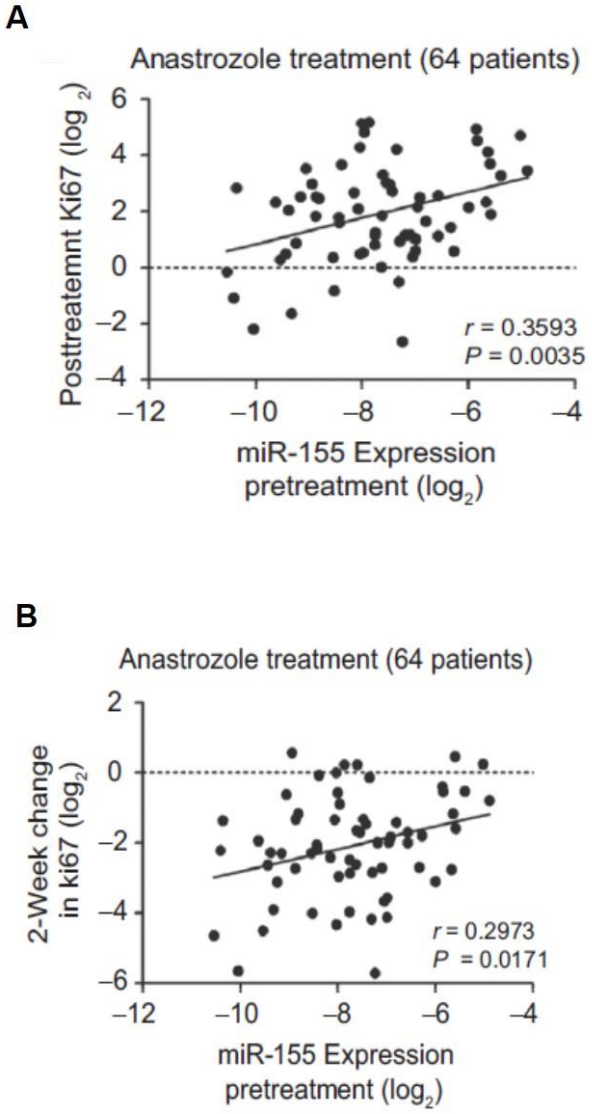


Figure 31. miR-155 expression levels in the response to AI in clinical specimens. correlation of the pretreatment miR-155 expression with the Ki67 staining after anastrozole treatment (E) or with the change in Ki67 staining between pre and posttreatment biopsies (Pearson correlation; F). RNA was available for 64 patients.

miR-23b-3p regulate amino acids metabolism and influences the response and adaptation to endocrine therapy of ER+ breast cancer

Since we have demonstrated that central carbon metabolic reprogramming is involved in response and adaptation to AI, we then asked whether the metabolic plasticity of resistant cells could also involve additional metabolic pathways and be responsible for the resistance to different endocrine agents. Therefore, we continued our studies in order to identify potential metabolic-related pathway alteration in addition to glucose metabolism reprogramming observed in AI-resistant cells.

Global gene expression and miRNAs analysis in parental MCF7 and MCF7-LTED cells show that miR-23/SLC6A14 node is deregulated in LTED cells and seems to have a role in endocrine therapy response.

To evaluate potential differences in metabolic-related pathway between AI-sensitive and -resistant cells, we analysed gene expression and miRNA profiles of MCF7-LTED cells compared to parental MCF7 cells. Total RNA was extracted from parental MCF7 cells, cultured in presence or absence of E2, and their LTED derivatives. mRNA and miRNA profiles were analysed on three biological replicates for each cell line using Human miRNA and Gene Expression Microarrays (Agilent Technologies). Statistical analysis based on 1 way ANOVA Benjamin-Hochberg corrected test (FDR<0.05, fold-change >2 and <-2) revealed 62 miRNAs and ~3,000 mRNAs differently regulated in MCF7-LTED versus MCF7+E2 cells, and 56 miRNAs and 2924 mRNAs differentially regulated between MCF7- LTED and MCF7-E2 cells (Figure 32A). Gene expression data were then subjected to Gene Set Enrichment Analysis (GSEA). It is interesting to note that GSEA confirms that the LTED model shares common features with other independent endocrine therapy resistant ER+ breast cancer models, with significant overlaps with the results of Craighton et al. (MSigDB M13661), highlighting that LTED cells are a good AI resistant cell model (Figure 32B). Furthermore, GSEA revealed that gene sets related to amino acid transporters (MSigDb M188; MSigDb M15239) were negatively

correlated with LTED cell profile, suggesting a role for amino acids metabolism in the response and resistance to AI (Figure 32C).

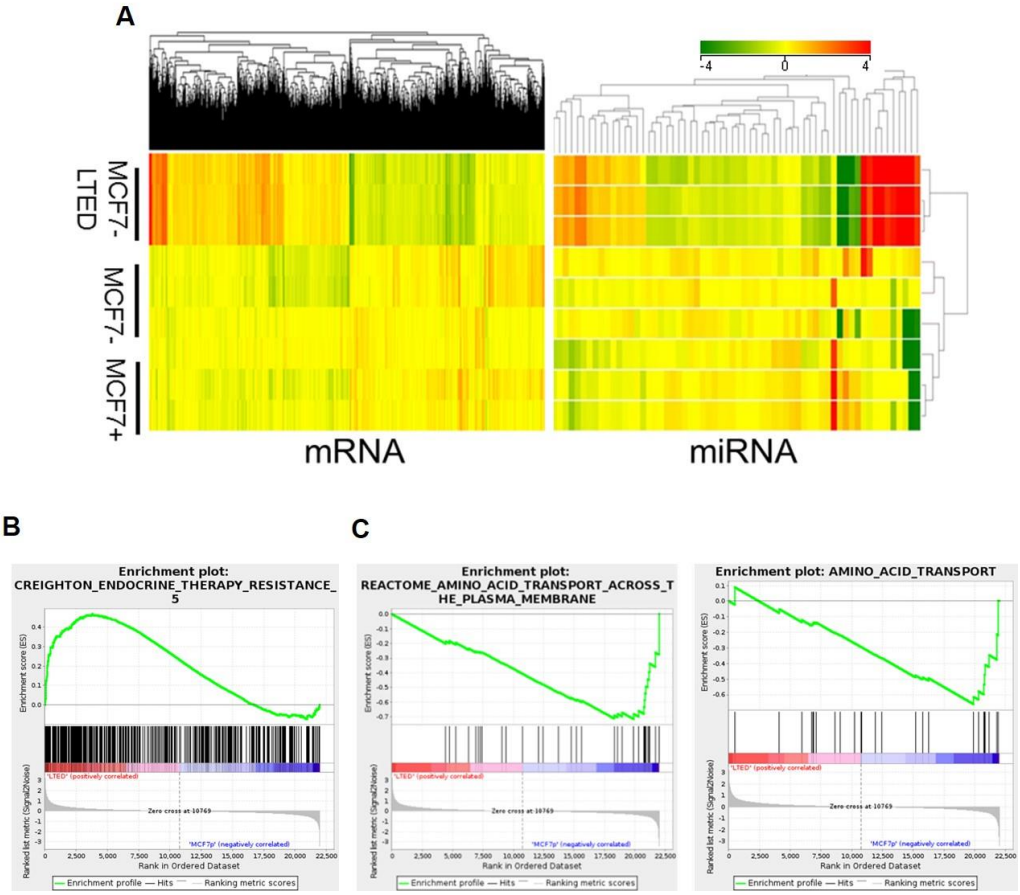
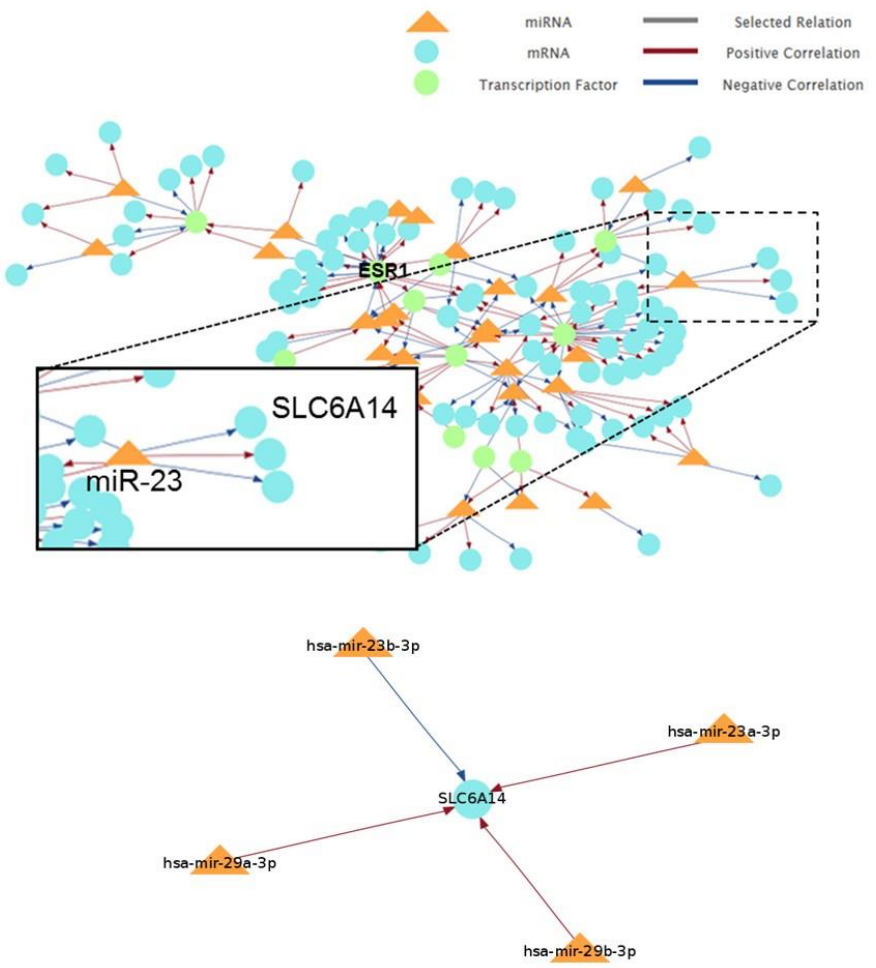


Figure 32. A Global gene expression and miRNA analysis in MCF7 and MCF7-LTED cells. A, Three biological replicates of MCF7 + or –E2 and MCF7-LTED cells were subjected to gene or miRNAs expression profiling using Agilent Technology. Statistical analysis performed based on 1 way ANOVA Benjamin-Hochberg corrected test (FDR < 0.05, fold-change > 2 and < -2) revealed 62 miRNAs and 3393 mRNAs differently deregulated in MCF7-LTED versus MCF7+E2 and 56 miRNAs and 2924 mRNAs differentially regulated in MCF7-LTED versus MCF7-E2 . B and C, GSEA confirms that genes associated to endocrine therapy resistance expressing in impendent ER+ breast cancer model positively correlated with LTED (B), and revealed that amino acid transporters are negatively correlated with LTED cell profile (C).

Integration analysis of miRNA and mRNA profiling using MAGIA 2.0 software (476) showed a key deregulated node controlling amino acids metabolism in MCF7-LTED cells between miR-23b-3p and amino acid transporter SLC6A14 (amino acid transporter Solute Carrier Family 6 Member 14), also known as $ATB^{0,+}$ (Figure 33A). The integrated analysis between miRNAs and mRNAs showed that there are other miRNAs that correlate with the expression levels of the SLC6A14 transporter, such as

miR-23a-3p, miR-29b-3p and miR-29b-5p. However, only miR-23b-3p showed an inverse correlation with SLC6A14 (Figure 33A).

A



B

	LTED vs MCF7+E2	LTED vs MCF7-E2	MCF7+ vs MCF7-
miR23b-3p	+ 5,7	+ 4,06	-1,40
SLC6A14	-247,6	-248,8	-1,1

Figure 33. miR-23b-3p/SLC6A14 node is deregulated in AI-resistant MCF7-LTED cells. A, Integration analysis of mRNAs and miRNAs using Magia 2.0 software shows a key deregulated node between miR-23b-3p and amino acid transporter SLC6A14. Other miRNAs are found to be correlated to SLC6A14 expression, but only miR-23b-3p shows an inverse correlation with SLC6A14 expression. B, Gene expression data show an increased miR-23b-3p expression associated to low SLC6A14 in MCF7-LTED cells compared to

parental MCF7 in presence or absence of 1nmol/L E2. Normalized data were analysed by 1 way ANOVA Benjamin-Hochberg corrected test.

However, only miR-23b-3p showed an inverse correlation with SLC6A14 (Figure 33A). Indeed, gene expression analysis revealed that MCF7-LTED cells had a significantly lower amounts of the amino acid transporter SLC6A14 compared to MCF7+E2 (FC:-247.7) or MCF7-E2 (FC:-284.9) (Figure 33B) and that lower levels of SLC6A14 correlate with higher level of miR-23b-3p expression in MCF7-LTED compared to parental MCF7 cells in presence or absence of E2 (LTED vs MCF7+E2, FC:+5.7; LTED vs MCF7-E2, FC:+4.06; Figure 33B). Considering that miRNAs regulate gene expression through transcriptional inhibition of their target mRNAs, this inverse correlation between miR-23b-3p and SLC6A14 suggests that miR-23b-3p could directly regulate the SLC6A14 expression, and thus to have a role in the control of amino acids metabolism in MCF7-LTED cells.

AI-resistant MCF7-LTED cells have lower SLC6A14 expression when compared to parental cells

SLC6A14 is an amino acid transporter with unique characteristics. It is able to transport 18 of the 20 proteinogenic amino acids, except the non-essential and negatively charged glutamate and aspartate (304). This transporter is expressed at low levels in normal tissue, but its expression is up-regulated in different types of cancer, such as colon (490) and cervical (491) cancer. It has been demonstrated that SLC6A14 is upregulated also in breast cancer models, but only in the ER+ cell lines (492). SLC6A14 is able to transport large amount of leucine, an essential amino acid that is able to activate mTOR, glutamine, which provides carbon and nitrogen sources for tumour cells and arginine, an essential amino acid for those tumour cells that are lacking the arginine-synthesising enzyme (493, 494). These characteristics suggest that cancer cells increase SLC6A14 expression to meet the increasing demand for these amino acids due to their rapid growth. The treatment of ER+ breast cancer cells with the SLC6A14 blocker α-methyl-DL-tryptophan (α-MT) deprives the cells of glutamine, arginine and other essential amino acids, decreasing cell proliferation and causing apoptotic cell death (492). Considering the importance of SLC6A14 for the survival and proliferation of ER+ breast cancer cell lines, we hypothesised that the down regulation of SLC6A14 in

AI-resistant MCF7-LTED cells could confer advantages in term of survival and proliferation during the adaptation to oestrogen deprivation, making the resistant cells independent to external amino acids support for their growth.

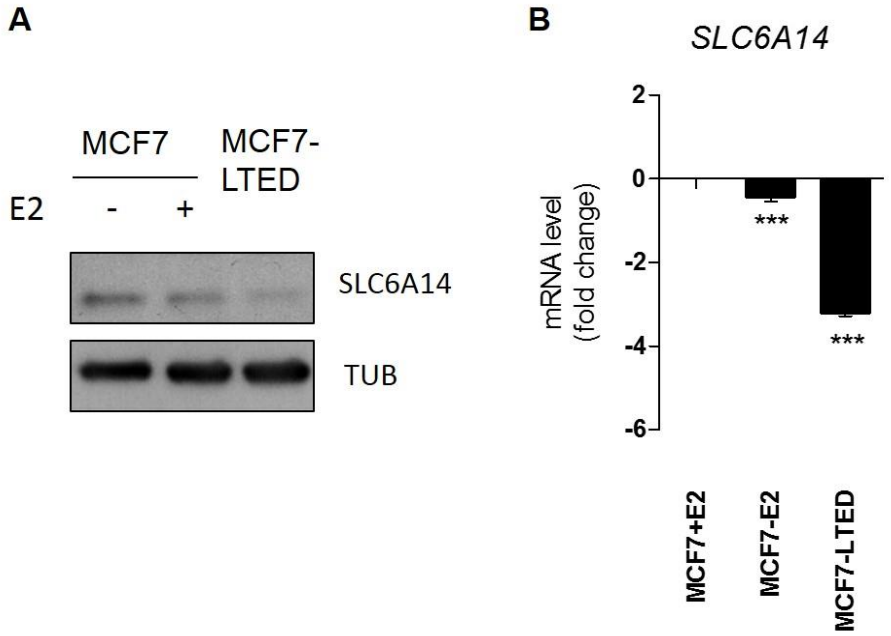


Figure 34. MCF7-LTED cells show an increase of SLC6A14 expression compared to parental MCF7 cells. A and B, MCF7-LTED cells were compared with wt-MCF7 in presence or absence of 1nmol/L E2. Total protein lysates or mRNAs were subjected to Western Blot analysis (A) and qRT-PCR analysis (B). Data represent mean ± SEM, *n* = 3 1 way ANOVA; Bonferroni corrected test*; ***, P*<0.001.

Gene expression data of SLC6A14 were confirmed by Western Blot and qRT-PCR in MCF7-LTED cells compared to parental MCF7 cells. The results showed an important downregulation in the expression of SLC6A14, both at protein (Figure 34A) and mRNA level (Figure 34B) in MCF7-LTED cells. The differences in SLCA614 expression revealed by Western Blot and qRT-PCR analyses are less than those obtained by Microarray analysis, but this could be caused by the different sensitivity of the two techniques utilised and/or the use of primers and probes that recognizes different regions of SLC6A14 transcript. Of note, the results showed also a weak reduction of SLC6A14 in MCF7-E2 compared to MCF7+E2, suggesting that SLC6A14 expression could be linked to E2 induced ER activation.

The increase of miR-23b-3p correlates with low SLC6A14 levels and decreases amino acids uptake in AI-resistant MCF7-LTED cells

The gene expression data of miR-23b-3p derived by microarray analysis (Figure 33B) were then confirmed by qRT-PCR analysis. MCF7-LTED showed an increased miR-23b-3p expression compared to parental MCF7 cells, in presence or absence of E2 (Figure 35A), which correlated with a downregulation of SLC6A14 expression (Figure 34A and B), suggesting that effect exerted by miR-23b-3p on the amino acid transporter SLC6A14 can be direct. To evaluate the role of SLC6A14 in amino acids metabolism, we analysed the capacity of uptaking exogenous amino acids of MCF7-LTED cells compared to parental MCF7 cells by administration of radioactive [^{14}C] amino acids pool and glutamine. Importantly, MCF7-LTED decreased the amino acids and glutamine consumption (Figure 35B and C), suggesting that this could be due to miR-23b-3p/SLC6A14 node deregulation in MCF7-LTED cells and have a role in the response and adaptation to oestrogen deprivation.

Low levels of SLC6A14 and high miR-23b-3p expression correlate with poor prognosis and lower survival in ER+ breast cancer patients

To investigate whether the deregulated expression of miR-23b-3p and amino acid transporter SLC6A14 could be of clinical relevance and used for patients stratification in ER+ breast cancer, we analysed the publicly available data of ER+ breast cancer patients to correlate the expression levels of miR-23b-3p and SLC6A14. Notably, Kaplan-Meier analysis revealed that ER+ breast cancer patients with low levels of SLC6A14 (n=909) showed poorer relapse-free survival (HR= 0.78, P=0.0051) compared to patients with high SLC6A14 expression (n= 893), who showed better prognosis (Figure 36A). Furthermore, in this cohort of patients the levels of SLC6A14 expression could discriminate the patients with good prognosis from those with bad prognosis following the endocrine therapy treatment. Indeed, patients with low levels of SLC6A14 (n=624) showed poorer prognosis (HR=0.77, P= 0.012) after endocrine treatment compared to patients with high SLC6A14 expression (n=601) (Figure 36B).

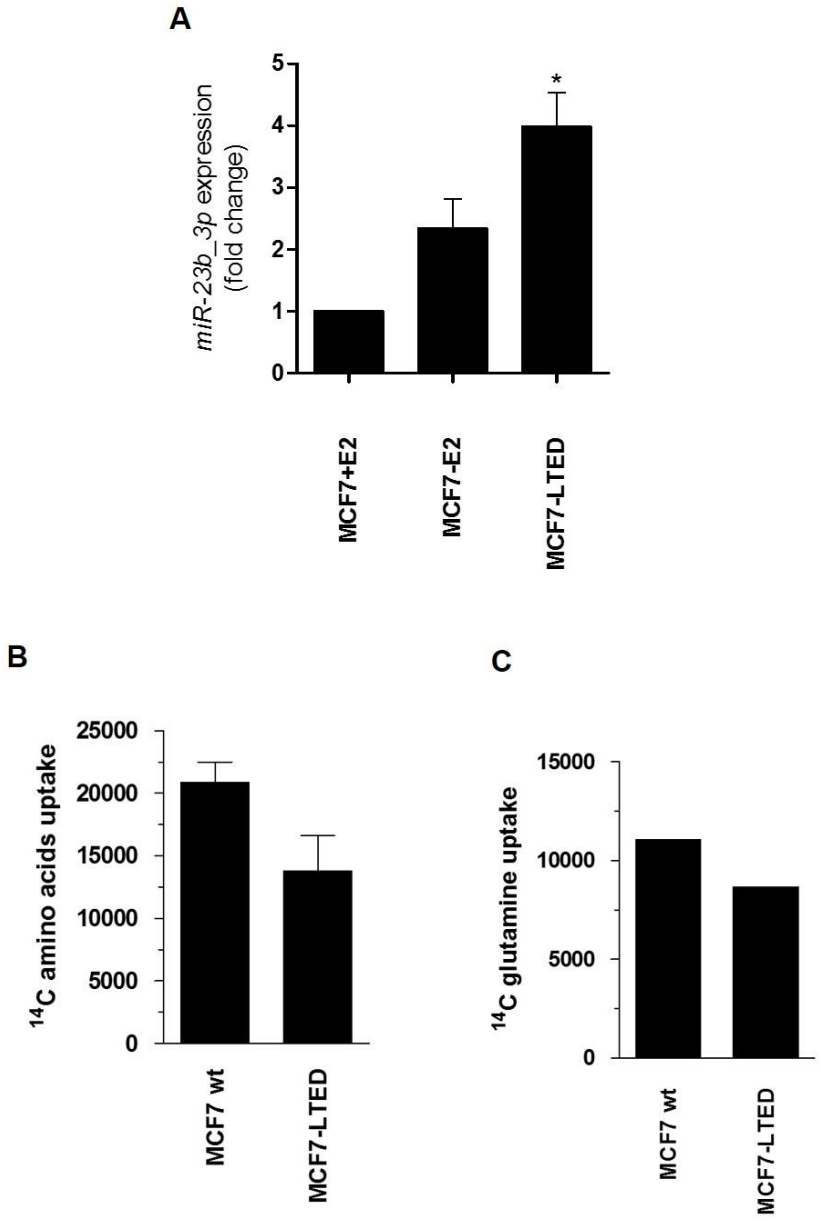


Figure 35. AI-resistant MCF7-LTED cells show an increase of miR23b-3p expression and a decreased amino acids uptake ability compared to parental MCF7 cells. A, MCF7-LTED were compared to wt-MCF7 cells in presence and absence 1nmol/L E2. Total miRNAs were extracted and subjected to qRT-PCR analysis. Data represent mean ± SEM, *n* = 3. 1 way ANOVA; Bonferroni corrected; *, *P* < 0.05. B and C, MCF7-LTED were compared with wt-MCF7 in presence of 1nmol/ E2 and subjected to radioactive amino acids pool uptake (B) and radioactive glutamine uptake (C) as described in Materials and Method.

In addition, analysis of publicly available TCGA ((*The Cancer Genome Atlas*) clinical data of 328 patients revealed that also miR-23b-3p could

have a prognostic value in ER+ breast cancer. Notably, high levels of miR-23b-3p in ER+ breast cancer patients correlated with lower survival (HR= 2.3, P= 0.018) compared to patients with low miR-23b-3p expression (Figure 36C). These results show that miR-23b-3p and SLC6A14 could be used respectively as prognostic and predictive markers, and suggest that the regulation of SLC6A14 expression by miR-23b-3p could be directly involved in the adaptation of ER+ breast cancer cells to oestrogen deprivation. To understand the role of miR23b-3p and SLC6A14 in the resistance to endocrine therapy, we further investigated the effect on survival and proliferation of ER+ breast cancer cells following interference with this pathway.

miR-23b-3p modulates SLC6A14 expression and influences the proliferation of MCF7-LTED cells in oestrogen-deprived conditions

To evaluate whether there is a direct link between miR-23b-3p and SLC6A14, we analysed the changes in SLC6A14 expression following RNA interfering approach against miR-23b-3p (see Material and methods). In particular, we overexpressed miR-23b-3p in parental MCF7 cells, where low miR-23b-3p levels are correlated to high SLC6A14 expression, by miR-23b-3p *mimic* transient transfection, a specific oligonucleotide that mimics the functions of miR-23b-3p. In contrast, in MCF7-LTED, where miR-23b-3p is expressed at higher levels and SLC6A14 is downregulated, we transfected a specific miR-23b-3p miRNA *inhibitor*, which inhibits miR-23b-3p expression and thus its functions. SLC6A14 expression was evaluated by Western Blot and qRT-PCR analyses. As expected, transfection with miR-23b-3p *mimic* increased miR-23b-3p expression in parental MCF7 cells compared to miRNA *mimic negative control#1*-transfected cells (Figure 37A) and induced a parallel decrease in SLC6A14 expression both at mRNA (Figure 37B) and at protein level (Figure 37C). In contrast, miR-23b-3p *inhibitor* decreased miR-23b-3p expression in MCF7-LTED cells compared to anti-miRNA *negative control#1*-tranfected cells (Figure 38A), with the subsequent increased in SLC6A14 expression both at mRNA (Figure 38B) and at protein (Figure 38C).

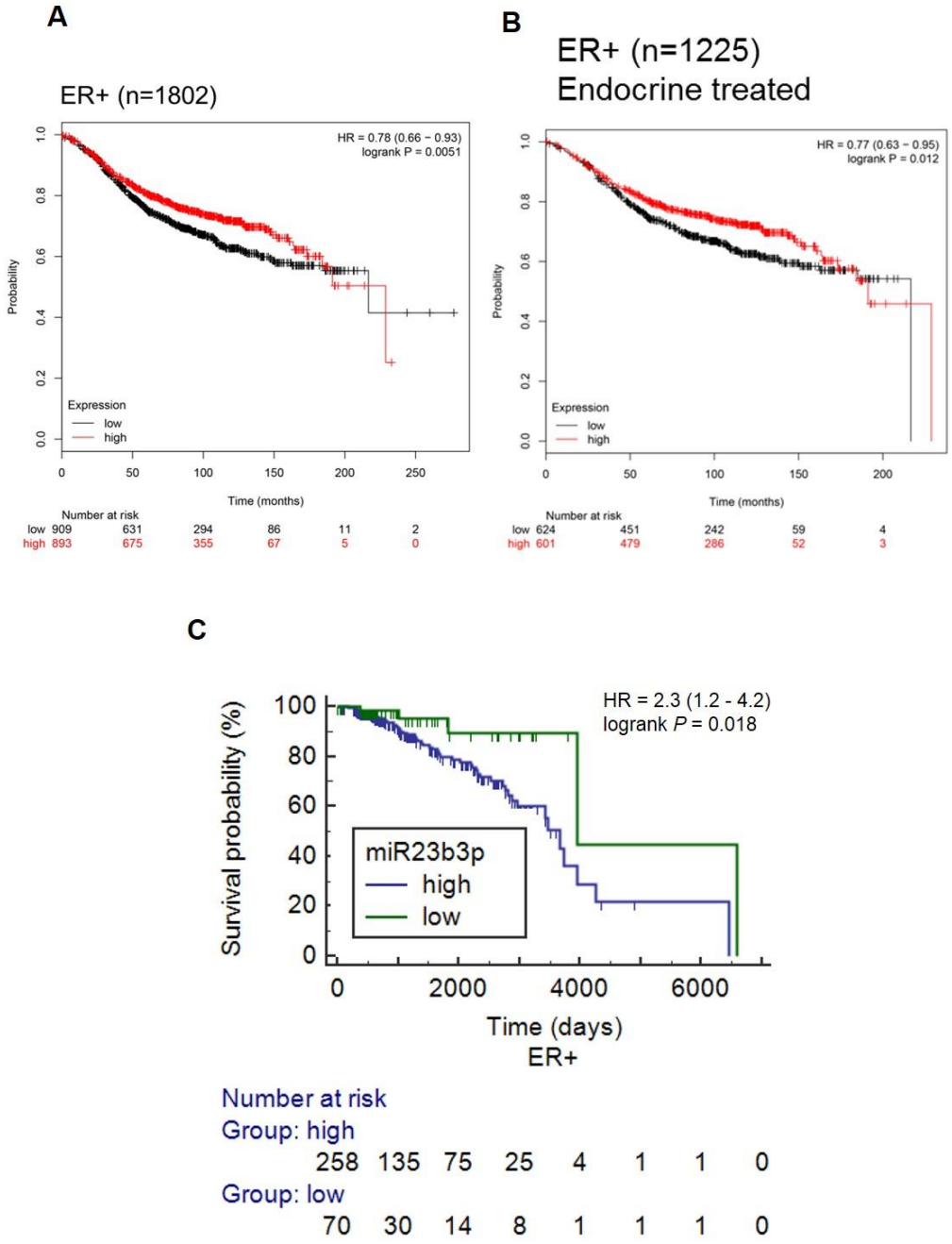


Figure 36. Low SLC6A14 levels and high miR-23b-3p expression correlate with poor prognosis and low survival in ER+ breast cancer patients. A and B, Kaplan-Meier analysis on SLC6A14 expression correlated with prognosis of ER+ breast cancer patients both untreated (A) and endocrine therapy treated (B). C, analysis of publicly available TCGA clinical data of 328 patients with ER+ breast cancer on miR-23b-3p expression in correlation with survival rate of ER+ breast cancer patients.

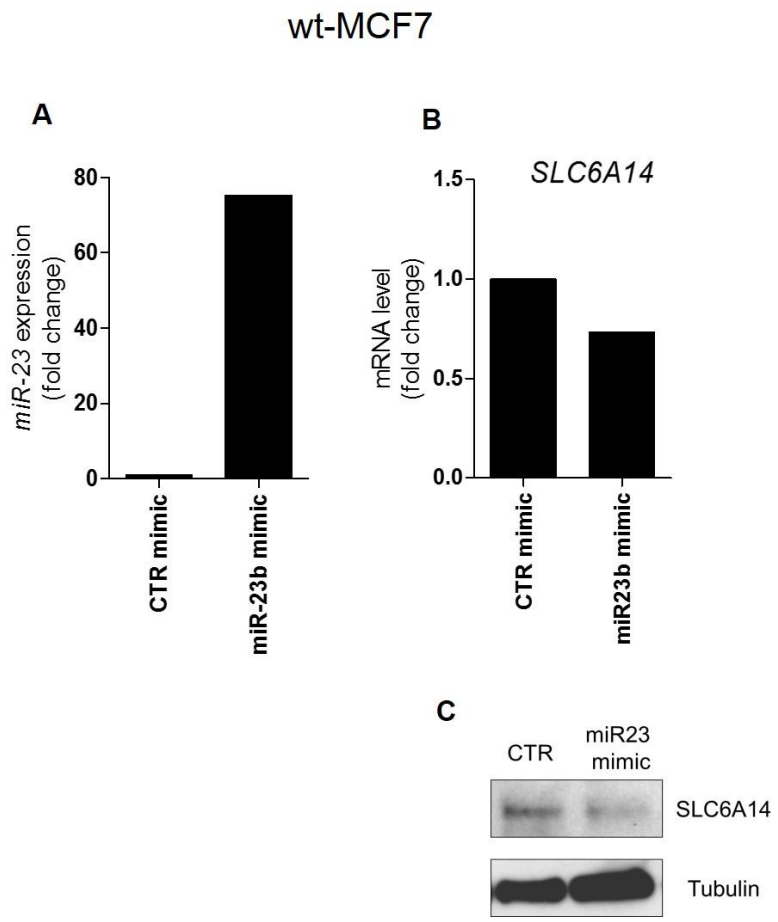


Figure 37. miR-23b-3p overexpression induces a decrease of amino acid transporter SLC6A14 expression in wt-MCF7 cells. A-C, Parental MCF7 cells were transfected with miR-23b-3p *mimic or* miRNA *mimic negative control#1,* as described in Materials and Methods. miR-23b-3p *mimic* transfected wt-MCF7 cells were compared to miRNA *mimic negative control#1* transfected cells. After 48 hours from transfection, total miRNAs were extracted and subjected to qRT-PCR analysis to evaluate miR-23b-3p expression (A). Total mRNA and protein content were subjected to qRT-PCR analysis (B) and Western Blot analysis (C) to evaluate SLC6A14 expression.

To investigate whether miR-23b-3p downregulation could impair the ability of miR-23b-3p *inhibitor*-transfected MCF7-LTED cells to adapt to oestrogen deprivation, we analysed the proliferation index of this cells in the absence of oestrogen by radioactive [^{3}H] thymidine incorporation analysis compared to MCF7-LTED cells transfected with miRNA *negative control#1*. Crucially, the cells transfected with miR-23b-3p *inhibitor* showed a reduced proliferation when compared to anti-miRNA *negative control#1*-tranfected MCF7-LTED cells (Figure 38D), suggesting that miR-23b-3p is involved in the proliferation and survival of ER+ breast cancer cells during oestrogen deprivation.

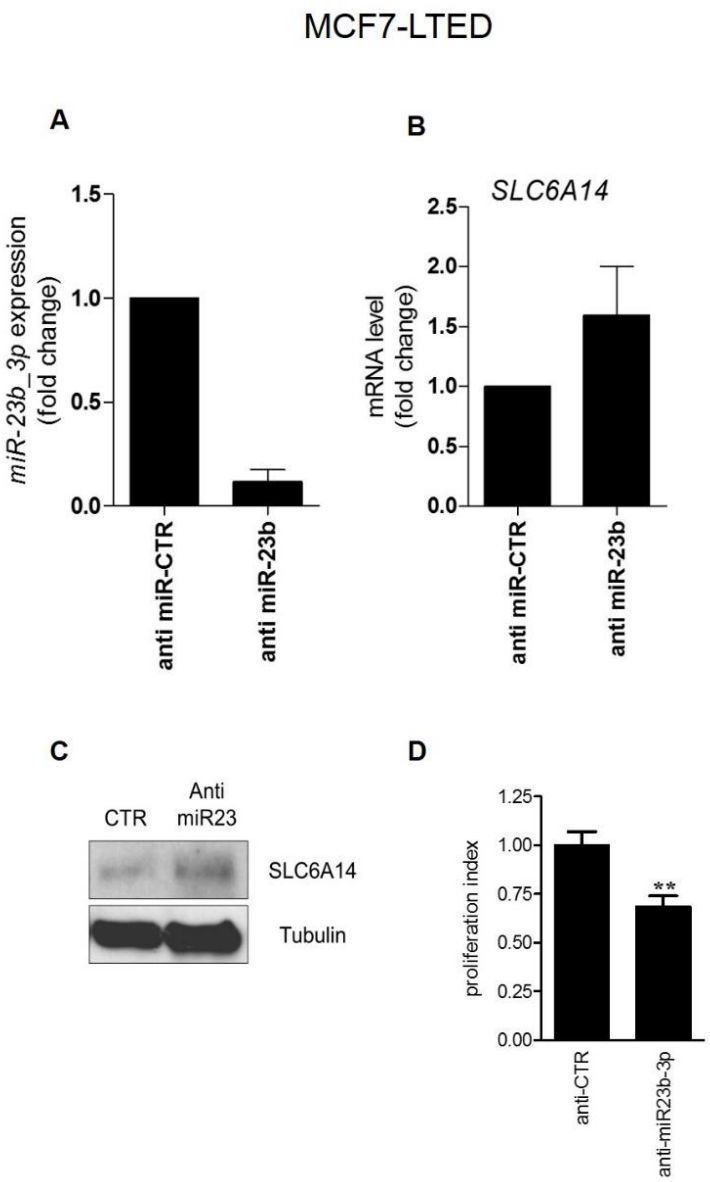


Figure 38. miR-23b-3p dowregulation induces an increased SLC6A14 expression and impairs proliferation of AI-resistant MCF7-LTED cells. A-D, MCF7-LTED cells were transfected with miR-23b-3p *inhibitor or* anti-miRNA *negative control#1,* as described in Materials and Methods. MCF7-LTED cells transfected with miR-23b-3p *inhibitor* were compared to anti-miRNA *negative control#1*-tranfected cells. After 48 hours from transfection, total miRNAs were extracted and subjected to qRT-PCR analysis to evaluate miR-23b-3p expression (A) and total mRNAs and protein lysates were subjected to qRT-PCR analysis (B) and Western Blot analysis (C) to evaluate SLC6A14 expression. D, proliferation index of MCF7-LTED in oestrogen-deprived condition by radioactive [^{3}H] thymidine incorporation. Data represent mean ± SEM, n = 3. Student t test; **, $P < 0.01$.

miR23b-3p/SLC6A14 node is involved in the response and resistance to tamoxifen and fulvestrant treatments.

To evaluate whether miR-23b-3p deregulation observed in AI-resistant MCF7-LTED cells was involved also in the resistance to other endocrine agents, we analysed miR23b-3p expression in tamoxifen (MCF7-TAM) and fulvestrant (MCF7-ICI) resistant cell lines derived by parental MCF7 cells (respectively, figure 39A and B).

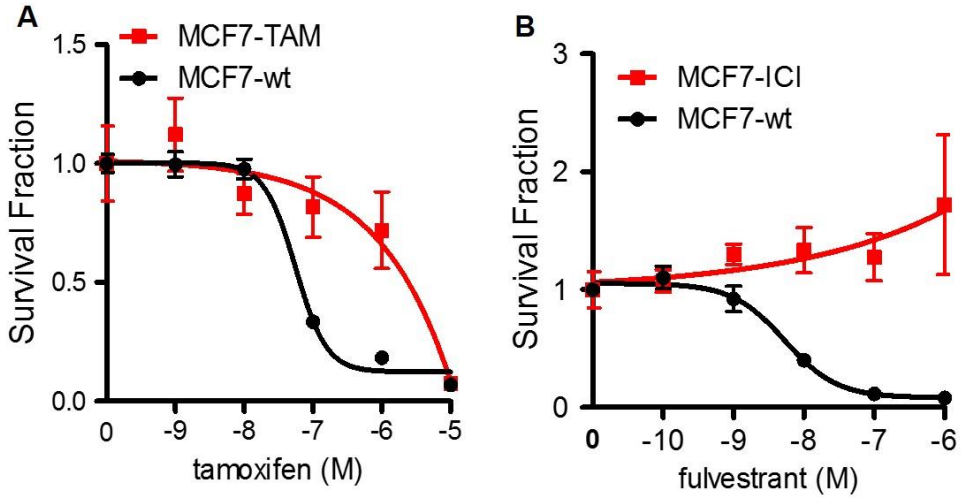


Figure 39. MCF7-TAM and MCF7-ICI cells are resistant to tamoxifen and fulvestrant treatments, respectively. A and B, wt-MCF7 cells were compared to MCF7-TAM or MCF7-ICI cells and treated with increasing doses of tamoxifen (A) or fulvestrant (B). Data are presented as fold change of survival fraction compared to untreated cells.

The results showed that MCF7-TAM and MCF7-ICI had an upregulation of miR-23b-3p levels (respectively 5 and 3 folds) compared to parental MCF7 cells (Figure 40A). Next, according to the hypothesis that SLC6A14 expression is regulated by miR-23b-3p, we investigated SLC6A14 expression in MCF7-TAM and MCF7-ICI cells and we found that the resistant cell lines showed a down regulation of SLC6A14 expression compared to parental MCF7 cells (Figure 40B). These results indicated that high miR-23b-3p levels correlated to low SLC6A14 expression in MCF7-TAM and MCF7-ICI cells, suggesting that miR-23b-3p can have a role not only in the AI response, but also in the resistance to tamoxifen and fulvestrant.

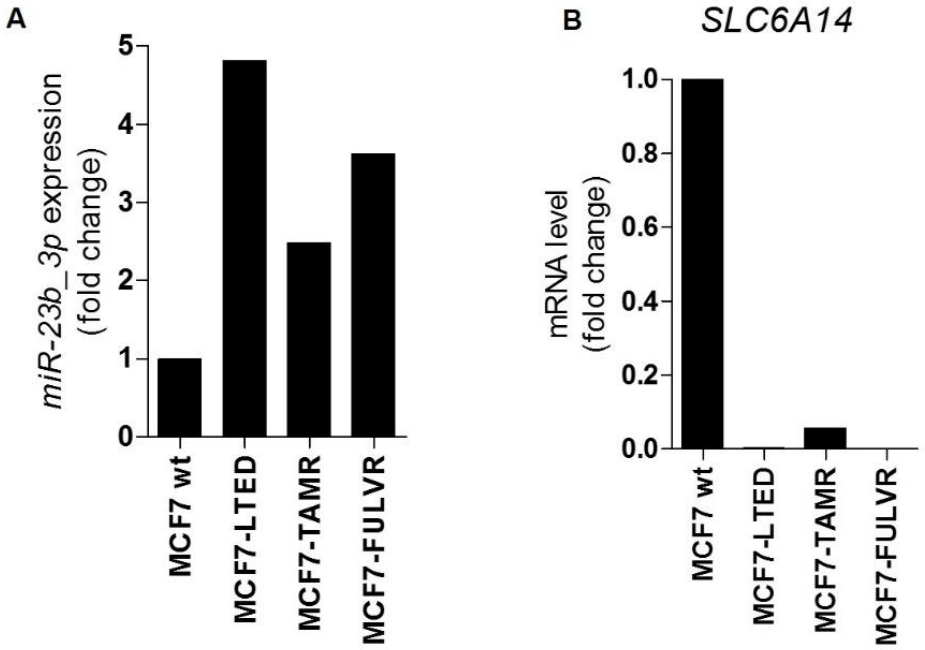


Figure 40. miR-23b-3p and amino acid transporter SLC6A14 are deregulated in endocrine therapy resistant cells compare to wt-MCF7 cells. A and B**,** wt-MCF7 were compared to endocrine therapy resistant MCF7-LTED, MCF7-TAM and MCF7-ICI cells derived by parental cells. Total miRNAs (A) and mRNAs (B) were extracted and subjected to qRT-PCR analysis to evaluate mR-23b-3p and SLC6A14 expression, respectively.

Crucially, the transfection of parental MCF7 cells with miR-23b-3p mimic conferred to sensitive MCF7 cells the ability to survive to tamoxifen and fulvestrant administration (when compared to miRNA *mimic negative control#1*-transfected cells) (Figure 41A and B), reinforcing the idea that miR-23b-3p overexpression could be involved in endocrine therapy resistance. Further studies are necessary to identify the role of miR-23b-3p in endocrine therapy resistance, in order to develop possible therapeutic approaches and confirm miR-23b-3p as predictive and prognostic marker.

Gene expression data reveals that autophagy-related markers are deregulated in MCF7-LTED cells compared to parental MCF7 cells

As our *in vitro* results suggested that AI-resistant MCF7-LTED cells are independent to exogenous amino acids for their growth and proliferation, we have hypothesised that they could be able to utilise endogenous proteins to sustain biosynthetic pathways. Nutrient deprivation is a potent

activator of autophagy pathway that it has been demonstrated to be able to either inhibit or promote cancer cells proliferation (450).

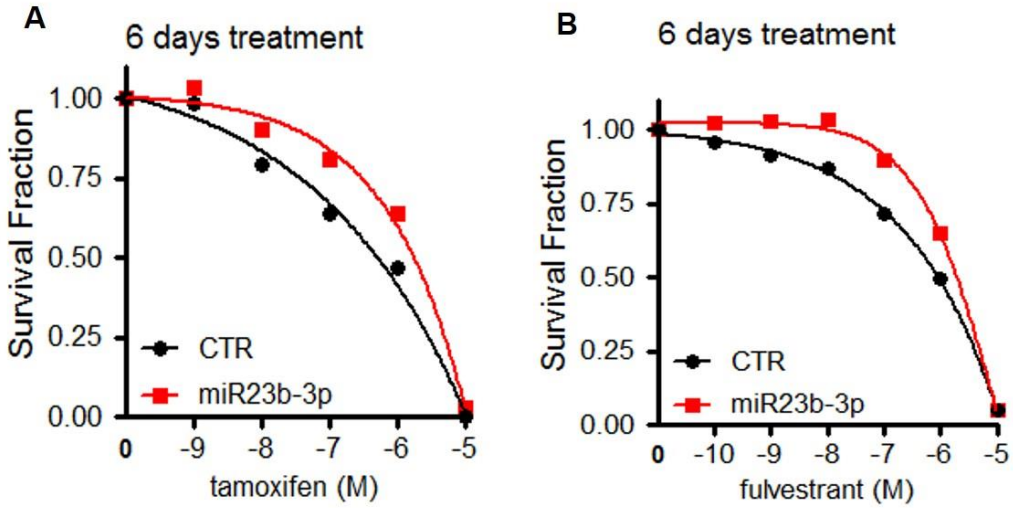


Figure 41. miR-23b-3p overexpression protects wt-MCF7 cells from tamoxifen and fulvestrant effect. A and B, wt-MCF7 cells were transfected with miR-23b-3p *mimic* or miRNA *mimic negative control#1.* miR-23b-3p *mimic* transfected MCF7 cells were compared with miRNA *mimic negative control#1* transfected cells and treated with increasing doses of tamoxifen (A) or fulvestrant (B). Data are presented as fold change of survival fraction compared to untreated cells.

In particular, low levels of exogenous amino acids inhibit mTOR sensor and activate autophagy via the activation of the class III PI3K/beclin1 complex (443). In addition, Beclin1 has a central role in the control of autophagy (438). Furthermore, SLC6A14 blockade induces amino acids deprivation, leads to inhibition of mTOR and activates autophagy in ER+ MCF7 cells (304). Importantly, our gene expression data on three biological replicates showed an increase of Beclin-1 expression in AI-resistant MCF7-LTED cells compared to parental MCF7 cells (Figure 42A). Another regulator of autophagic process is the *TNFα* (*tumour necrosis factor α*) *Induced Protein 3* (TNFAIP3 or A20) (495), which negatively regulates Beclin-1 expression by acting on Lys63 deubquitination (496). High levels of TNFAIP3 are associated with low levels of ubiquinated Lys63 and subsequently low activity of Beclin-1, resulting in an inhibition of the autophagic process (497). In addition, we found that TNFAIP3 is a putative target of miR-23b-3p by MIRGator database analysis, a platform that integrates publicly expression data of miRNA with those of mRNA and protein (498). In our

cell model, gene expression data showed that TNFAIP3 was downregulated in MCF7-LTED cells compared to parental MCF7 cells (Figure 42B), indicating that miR-23b-3p could indirectly regulate the expression of Beclin-1 by TNFAIP3 downregulation and subsequent activation of autophagy.

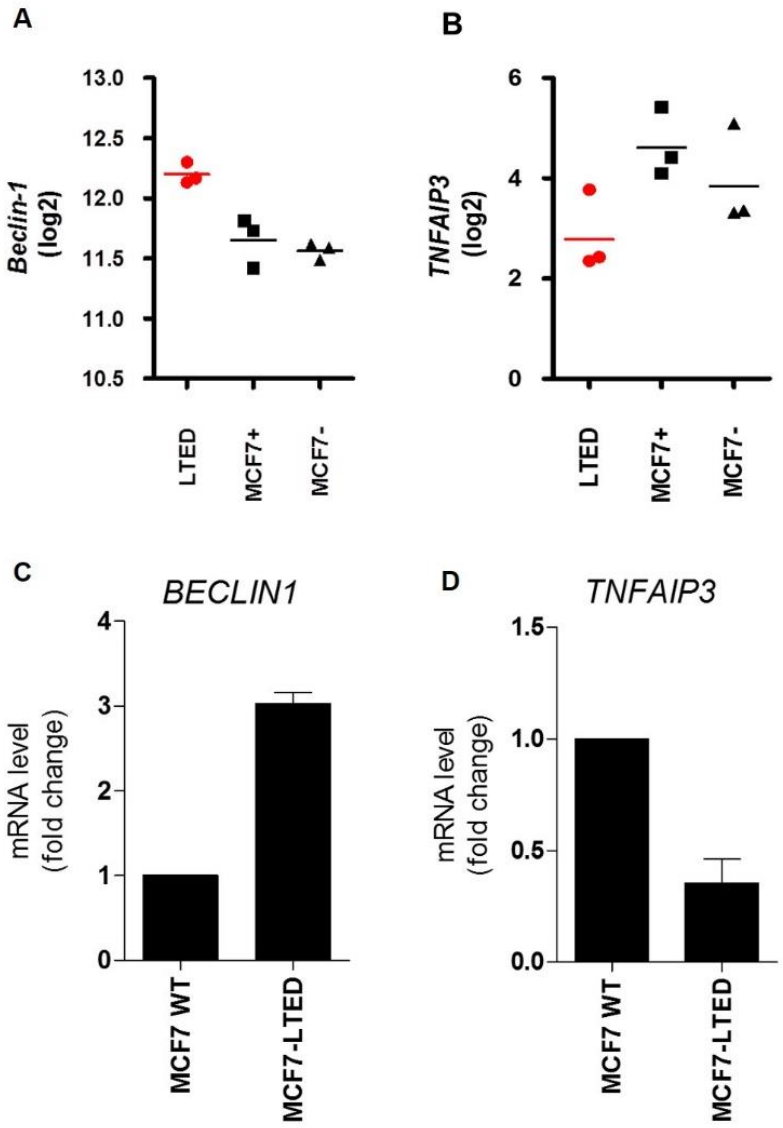


Figure 42. MCF7-LTED cells show an increase of Beclin1 expression and low TNFAIP3 levels compared to parental MCF7 cells. A and B, analysis of total gene expression data on Beclin1 (A) and TNFAIP3 (B) expression derived gene expression profile on three biological replicates of MCF7-LTED cells compared to MCF7 cells in presence or absence of 1nmol/L E2. Normalized data were analysed by 1 way ANOVA Benjamin-Hochberg corrected test. C and D, MCF7-LTED cells were compared to wt-MCF7 in presence of 1nmol/L E2. Total mRNAs were extracted and subjected to qRT-PCR for Beclin1 (C) and TNFAIP3 (D). Data represent mean ± SEM, *n* = 3.

This suggested that autophagy was activated in our cell model to promote the proliferation and survival of MCF7-LTED cells during oestrogen deprivation. We analysed Beclin-1 and TNFAIP3 expression by qRT-PCR in order to confirm the gene expression data. The results showed an increase of Beclin-1 expression (Figure 42C) associated with decreased TNFAIP3 expression (Figure 42D). Importantly, Kaplan-Meier analysis revealed that ER+ breast cancer patients characterised by low levels of TNFAIP3 showed poorer prognosis compared to patients with high TNFAIP3 expression. Statistical significance is achieved in the whole cohort of ER+ patients and in the subgroup that has been treated with endocrine therapy, (HR= 0.77, P= 0.0045, figure 43A, HR= 0.75, P= 0.041, figure 43B, respectively). These data suggest that autophagy activation is a pro-survival stimulus in AI-resistant cells and that the autophagy is involved in the adaptation of oestrogen deprivation, allowing MCF7-LTED cells to become independent to exogenous nutrients for survival and proliferation.

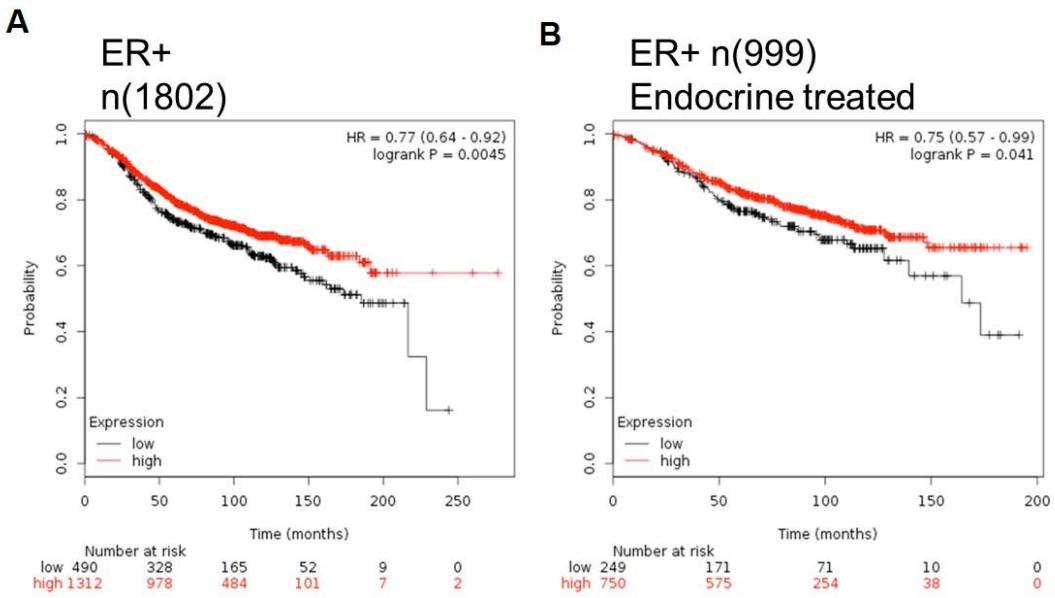


Figure 43. Low TNFAIP3 expression correlates with poor prognosis in ER+ breast cancer patients. A and B, Kaplan-Meier analysis of TNFAIP3 expression in correlation with prognosis in 1802 ER+ breast cancer patients (A) and in the subgroup of 999 patients treated with endocrine therapy (B).

AI-resistant MCF7-LTED cells show an autophagic phenotype compared to parental cells

Autophagy activation in AI-resistant MCF7-LTED cells compared to parental MCF7 cells was analysed by Western Blot. First, we evaluated the phosphorylation of ribosomal protein S6 kinase beta-1 (p70S6k) as a read-out of mTOR activation. mTOR phosphorylation and subsequent activation inhibits the recruitment of autophagy players and thus impairs autophagy. Crucially, p70S6K was dephosphorylated in MCF7-LTED cells compared to parental MCF7 cells (Figure 44A), indicating that mTOR was inactivated. In addition, MCF7-LTED cells showed increased Beclin-1 and LC3-II protein expression (Figure 44A). The presence of LC3-II is an indication of the autophagosome formation and activation of autophagic flux (499). Another cell energy sensor is AMPK, which is phosphorylated in nutrient deprived-condition and its activation in turn can inhibit mTOR activity (500).

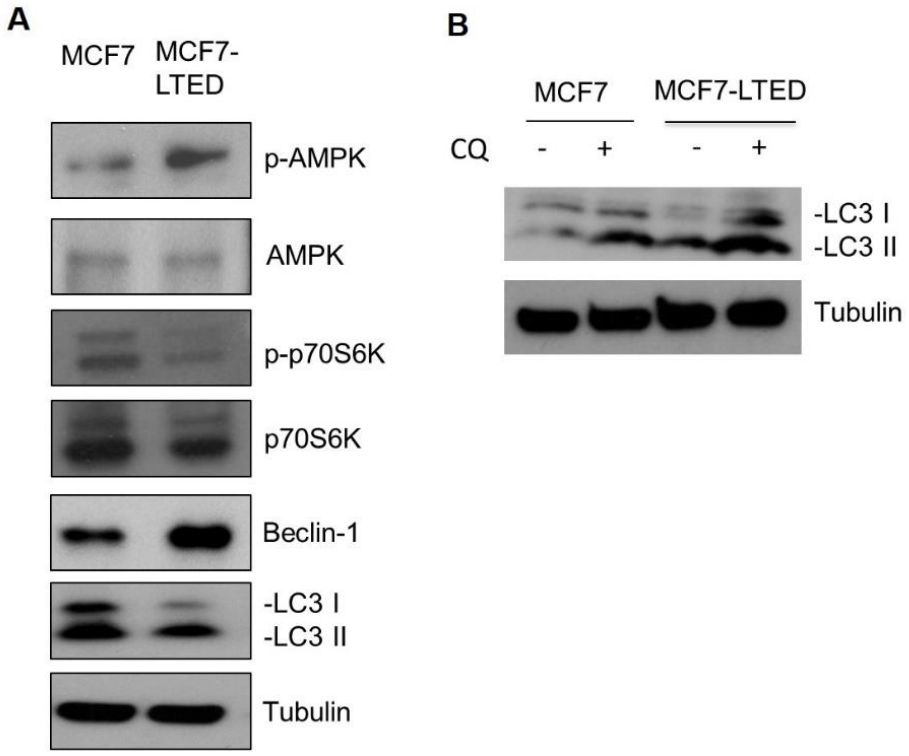


Figure 44. Analysis of the major autophagic markers in MCF7-LTED cells. A and B, MCF7-LTED cells were compared to wt-MCF7 cells in presence of 1nmol/L E2**.** Total protein content was subjected to Western Blot analysis in normal culture conditions (A) and with or without cloroquine (CQ) (B).

In our cell model, MCF7-LTED cells showed increased AMPK phosphorylation with subsequent inactivation of mTOR (analysed by the

phosphorylation of the downstream effector p70S6k) and increased Beclin-1 expression (Figure 44A). It has been reported that cloroquine (CQ) administration blocks autophagic flux by inhibition of lysosome degradation with subsequent LC3-II accumulation (499). Crucially, MCF7-LTED cells showed an increased LC3-II accumulation compared to parental MCF7 cells following CQ treatment (Figure 44B), indicating that AI-resistant cells could become independent to exogenous amino acids and activate autophagy for proliferation and survival. This metabolic reprogramming could be pivotal during oestrogen deprivation and represent another aspect of metabolic plasticity of MCF7-LTED cells.

Conclusion

MiR-23b-3p expression is increased in endocrine therapy resistant breast cancers and plays a role in the response to therapy. The acquisition of exogenous amino acids independence via SLC6A14 downregulation and subsequent activation of autophagy could be involved in therapy resistance of ER+ breast cancer. Further investigations are necessary to understand the role of miR-23b-3p in the control of autophagy and amino acids metabolic reprogramming in order to identify both potential metabolic related-biomarkers and therapeutic targets in endocrine therapy resistance.

Discussion

Approximately 70% of breast tumours are characterised by ER expression and are dependent on ER signalling for their growth and survival. Endocrine therapy is the standard of care for the treatment of this breast cancer subset and acts by targeting the ER pathway using different endocrine agents such as selective ER modulators, that compete with oestrogen to bind ER (e.g. tamoxifen), selective ER downregulators, that promote ER degradation (e.g. fulvestrant) or aromatase inhibitors (AI) that block oestrogen biosynthesis. In postmenopausal patients, AI have become the first-line treatment choice, showing higher efficacy than tamoxifen therapy. However, resistance to such agents remains a problem and many patients relapse with either *de-novo* or acquired resistance. Several mechanisms have been proposed to contribute to endocrine therapy

resistance, including hypersensitisation to oestrogen (488, 501) and activation via aberrant growth factor signalling (477). Recent studies have highlighted that combining endocrine therapy with HER2-targeting compounds (168, 478) or with inhibitors targeting downstream signalling effectors such as mTORC1, is superior to endocrine therapy alone (168). However, given the adaptability of tumour cells, targeting a single growth factor or a downstream signalling hub will likely lead to compensatory upregulation. Indeed, many patients fail to benefit from these combined therapeutic approaches and there remains an urgent need for more efficient therapeutic strategies.

Metabolic adaptation is essential for the cancer cells to satisfy the different energetic requirements that support a cancer cell from the initial proliferation, dissemination, therapy response and finally, the resistance. Several recent studies have reported that OXPHOS metabolism is associated with an aggressive phenotype of cancer cells (502) and characterise cancer cells that have developed resistance to different chemotherapeutic agents in various cancer models (503, 504). Conversely, reprogramming toward a hyperglycolytic metabolism has been associated with resistance to biologic agents such as Herceptin and Avastin in breast cancer (302, 505). To study the metabolic reprogramming that a cell undergoes in response and resistance to AI treatment, we have used an array of different *in vitro* and *in vivo* models. As such, we investigated primarily whether metabolic reprogramming could be responsible for breast cancer cell adaptation to LTED and whether metabolic targeting could be of any benefit in AI-sensitive and AI-resistant *in vitro* models. As breast cancer cell lines are characterised by low or no expression of endogenous aromatase, we have used cells transfected with the human aromatase gene as sensitivity model to AI (MCF7-2A). In addition, we have used ER+ cells adapted to LTED to study AI resistance, as lack of oestrogen in the medium mimics the hormone withdrawal that occurs during AI treatment (MCF7-LTED). However, such models have some limitations: MCF7 cells overexpressing aromatase do not account for the fact that androgen to oestrogen conversion predominantly occurs in the stromal cells of breast cancers, and LTED models cannot mimic the rewiring that cancer cells undergo when chronically treated with an AI. Therefore, to confirm the

clinical relevance of our *in vitro* findings, we have used tumour biopsies derived from patients enrolled in neoadjuvant trials of AI and a xenograft model that mimics letrozole clinical treatment. First, we have found that letrozole sensitivity of MCF7-2A is potentiated *in vitro* by concomitant antiglycolitic agents administration. In addition, we have observed reduced expression of the glycolytic-related components GLUT-1 and HK2 in a human xenograft model that is responsive to AI letrozole treatment . This indicates that blocking glycolysis in AI-sensitive breast tumour leads to therapeutic advantages in association with endocrine therapy. To identify then the metabolic pathways associated with AI resistance, we analysed the expression of key glycolytic components and performed tracking radioactive assay in ER+ breast cancer cell. The results showed that AI-resistant MCF7-LTED cells have a glycolytic phenotype compared to parental MCF7 cells. In addition, publicly available clinical data analysis show that ER+ breast cancer patients with poor prognosis are characterised by enhanced MCT4 and GLUT1 expression (i.e. hyperglycolytic phenotype) and therefore may have a potential value in predicting AI response. Taken together, these results highlight a “glycolysis dependency” of the MCF7-LTED cells and AI-sensitive models. Surprisingly, targeting glycolytic metabolism in AI-resistant MCF7-LTED cells did not affect cell survival. Indeed, these cells were capable of switching from glycolysis to OXPHOS metabolism and furthermore, 2-DG treatment enhanced their promigratory and proinvasive capabilities. This suggests that the acquisition of a hyperglycolytic phenotype correlates with aggressive clinical features of AI-resistant breast cancer. Indeed, ER+ luminal B tumours have poorer prognosis compared with luminal A with an increased risk of early relapse and resistance to endocrine therapy and chemotherapy (5) and it has been reported that luminal B breast cancers show a higher FDG-PET signal (i.e. higher glucose uptake) when compared with luminal A (506). Consequently, 2-DG used in combination with standard therapy may be of clinical benefit to the AI-sensitive tumours but detrimental to AI-resistant ones. Therefore, the metabolic plasticity of AI-resistant breast cancer could allow cancer cells adaptation to target treatments and be ultimately responsible for resistance and relapse.

Next, we evaluated the molecular components that could be responsible for the metabolic and motile reprogramming that MCF7 cells undergo under LTED conditions and after 2-DG treatment. Deregulation of miRNAs expression has been demonstrated involved in several types of cancer and the involvement of miRNAs has been previously described in breast cancer endocrine therapy response and resistance (379). As we have observed the metabolic plasticity exclusively in LTED cells that retain ER expression (MCF7), but not in those who lose it during LTED adaptation (ZR75-1), we focused our attention on those miRNAs regulated by ER signalling. Notably, miR-155 has been found to be associated with metastasis events and invasive properties of breast cancer (507) and E2 has been shown to upregulate miR-155 expression in MCF7 cells. Furthermore, it has been demonstrated that miR-155 regulate the expression of HK2 by miR-143 regulation (417) and increase the motility of lymphoma cells by impacting on RhoA activity (508). As miR-155 and miR-143 expression levels were found to be deregulated in MCF7-LTED cells, we hypothesised that miR-155 could be responsible for the altered metabolism observed in these cells and for the motile and invasive abilities displayed. Notably, impairing ER signalling by adding letrozole to androstenedione-treated MCF7-2A cells or by fulvestrant administration in MCF7-LTED cells, confirmed ER dependency of miR-155 expression. This was further confirmed in the xenograft-derived samples, where miR-155 expression was significantly decreased following ER signalling impairment, induced by in vivo letrozole administration. Finally, targeting miR-155 with anti-miR agents impaired 2-DG–induced motility of MCF7-LTED cells and potentiated the effect of metformin treatment, indicating that miR-155 targeting could have a potential therapeutic implication in AI-resistant tumours that retain ER expression. Crucially, miR-155 levels were significantly associated with response to AI therapy in ER+ breast cancers, identifying a subset of patients that could benefit from combinatorial approaches targeting miR-155 rather than AI monotherapy. Moreover, publically available data analysis showed that high miR-155 levels associated with poor prognosis on tamoxifen, suggesting that this miRNA could be involved in the resistance to additional endocrine agents. In conclusion, our results

highlight a potential diagnostic approach and therapeutic intervention based on miR-155 patient stratification in ER+ breast cancer.

Preliminary data that have been obtained in the last year of my PhD program suggest that in addition to AI-resistant cells the central carbon metabolic reprogramming, alteration of the amino acids metabolism is involved in the response and in the adaptation to oestrogen deprivation. Indeed, cancer cells can undergo different metabolic alterations to drive macromolecular biosynthesis for rapid cell growth and proliferation. Increased glutamine metabolism is an alternative energy source for cancer cells and is thought to be a central metabolic pathway cooperating with glycolysis by providing intermediates for amino acids (509, 510) and lipid synthesis necessary to sustain the higher proliferation rate (511). It has been reported that many types of tumour, including breast cancer, overexpress different amino acid transporters to support their rapid growth (512). First, we analysed the gene expression and miRNAs profiles of LTED and parental MCF7 cells (in presence or absence of E2) to identify possible deregulated metabolic pathways involved in the response to endocrine therapy. Notably, integration analysis of mRNA and miRNA profiling using MAGIA 2.0 software (476) revealed a key deregulated node controlling amino acids transport in MCF7-LTED cells composed of miR-23b-3p and the amino acid transporter SLC6A14. SLC6A14 is able to transport 18 of the 20 proteinogenic amino acids, excluding glutamate and aspartate. This transporter is expressed at low levels in normal tissue, but its expression is upregulated in different types of cancer, including ER+ breast cancer (304). By the upregulating this transporter, cancer cells can increase exogenous amino acids uptake to support their rapid growth and it has been demonstrated that selective blockade of SLC6A14 starves MCF7 cells of arginine, glutamine and essential amino acids, decreasing cell proliferation and causing apoptosis (304). Interestingly, AI-resistant MCF7-LTED cells show a decreased SLC6A14 expression compared to parental MCF7 cells, suggesting that this downregulation could confer a series of advantages to AI-resistant cells during the adaptation to oestrogen deprivation. Importantly, low levels of SLC6A14 correlated with increased miR-23b-3p expression in MCF7-LTED. This correlation between miR23b-3p and SLC6A14 was inverse in parental MCF7 cells, which showed high

SLC6A14 levels associated with low miR-23b-3p expression. Crucially, publicly available data analysis showed that low levels of SLC6A14 and high miR-23b-3p expression correlated with poor prognosis and low survival in ER+ breast cancer patients, highlighting that they could have a prognostic and predictive value, respectively. As expected, downregulation of SLC6A14 led to a decreased exogenous amino acids uptake in MCF7-LTED cells compared to parental MCF7 cells, suggesting that AI-resistant cells are independent of exogenous amino acids for proliferation and grow. In fact, gene expression data and Western Blot analysis revealed that decreased amino acids uptake is associated with the activation of autophagy in MCF7-LTED cells. The autophagy is a process that a given cell can activate to recycle organelles and proteins during nutrient starvation in order to obtain precursors for biosynthetic pathways (433, 513) Our data showed in MCF7-LTED cells an increase of autophagy, as demonstrated by mTOR inactivation and subsequent increased expression of the autophagic markers Beclin1 and LC3-II. This suggests that exogenous amino acids independence together with autophagy activation could represent a metabolic reprogramming that allows to AI-resistant cells to survive and proliferate during oestrogen deprivation. Furthermore, TNFAIP3 that controls Beclin-1 expression by deubiquitinization is dereg(259)ulated in MCF7-LTED and it is a putative miR-23b-3p target. Therefore, miR-23b-3p could regulate directly both amino acids uptake, autophagy and subsequent amino acids metabolism. Beclin-1 has a central role in the autophagic process and its overexpression is correlated with an increase of autophagic activation. Notably, Beclin-1 was overexpressed in MCF7-LTED cells compared to parental MCF7 cells. Next, we have observed that interfering with miR-23b-3p expression by transient transfection induced changes in SLC6A14 expression, indicating that a direct link between miR-23b-3p and amino acid transporter SLC6A14 might exist. Specifically, mimicking miR-23b-3p in parental MCF7 cells led to decreased SLC6A14 expression. However, following the increase of miR23b-3p, the levels of SLC6A14 were not completely abolished, as expected. This could be explained by the important role that SLC6A14 seems to play in the survival of parental MCF7 cells, and therefore other mechanisms may be involved in the maintenance of SLC6A14 expression

in MCF7 cells. In contrast, the inhibition of miR-23b-3p highly expressed in MCF7-LTED induced an increase in the SLC6A14 levels. Crucially, miR-23b-3p has a direct role in the favouring the survival in oestrogen deprived-conditions. In fact, following miR-23b-3p inhibition, AI-resistant MCF7-LTED cells decreased their proliferate capacity. In addition to AI-resistant cell model, we analysed two cell lines derived from parental cells that are resistant to tamoxifen and fulvestrant, to evaluate whether miR-23b-3p/SLC6A14 node deregulation may represent a common mechanism of resistance to endocrine therapy. The results showed that both resistant cancer cell lines had an increased miR-23b-3p expression associated with low SLC6A14 levels compared to parental cells. Crucially, mimicking miR-23b-3p in parental MCF7 cells increase the resistance to tamoxifen and fulvetsrant treatment, suggesting that miR-23b-3p could have a crucial role in the resistance to different endocrine agents. Further investigations are necessary to understand the role of amino acids metabolic reprogramming by deregulation of miR23b-3p/SLC6A14 node to identify possible therapeutic targets and/or potential prognostic and predictive biomarkers in ER+ breast cancer.

In addition to glucose and amino acids metabolic reprogramming, alterations in lipid-and-cholesterol associated pathways are also frequent in different types of tumour (259, 514). Recently, FASN expression has been recognized as an oncogene for its role in carcinogenesis and it has been reported to be upregulated in several cancers (250). We hypothesised that lipids metabolism could be reprogrammed in AI-resistant cell model. Preliminary data showed an increase in lipids biosynthesis from glucose and an increased lipid accumulation (lipid droplets) in MCF7-LTED cells when compared to parental MCF7 cells. It has been demonstrated that cancer cells have an increase in lipid droplets content compared to normal cells (255) and that high lipid accumulation is considered a hallmark of cancer aggressiveness. In AI-resistant cells, lipid droplets could be a source of energy in stress nutrient condition, but also represent an acetyl-CoA storage. In addition to its metabolic function, acetyl-CoA can function as acetyl-group donor for the acetylation of histones, an epigenetic event crucial in controlling cancer cells metabolism (515). Therefore, lipid metabolic reprogramming could confer an advantage to resistant cells in

terms of metabolic plasticity: depending on nutrient conditions, they could utilise lipid droplets as an acetyl-CoA storage to regulate the transcription of certain metabolic related-gene or as energy source instead of glucose, which we have shown to be fundamental in AI-resistant cells. High rate of *de novo* lipids biosynthesis could be necessary to maintain high lipid accumulation. Lipid metabolic alterations, together with glucose and amino acids metabolic reprogramming may be different aspects of a common phenotype, that is, high metabolic plasticity of AI-resistant cells needed for acquiring adaptive features that allow cell survival to oestrogen deprivation and to other stressful conditions thus promoting endocrine therapy resistance. Future aims of the current study are the identification of one or more molecular players involved in the described metabolic plasticity (e.g. miR-155 and miR-23-3p). On one hand, deregulated miRNAs could be used as potential clinical biomarkers, since there are still difficulties in targeting small RNAs (516); on the other hand an interesting area of research could be the investigation of potential targeting approaches of the pathways that are controlled by the deregulated miRNAs to be used as monotherapies or in association with current therapy.

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