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Involvement of microRNAs in Androgen Receptor-dependent Breast Cancers

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1. Introduction

1.1 Breast cancer

1.1.1. Incidence and mortality

Breast cancer is the most common cause of cancer among women, accounting for over one-fifth of the estimated annual 4.7 million cancer diagnoses in females, and the second most common tumour, after lung cancer, in both sexes (1). There is at least a 10-fold variation in worldwide breast cancer incidence rates as a consequence of a range of socio-economically differences in the population prevalence of several factors such as reproduction, hormons and nutrition (2). The highest incidence rates occur in northern and western Europe, northern America, Australia and New Zealand, and in southern countries of South America. Also screening programmes, such as that introduced in several European countries in the late 1980s, influence trends in the incidence and mortality of breast cancer. In some countries mortality started decreasing before screening was introduced, but its decline also occurred in non-screened age groups, and in some countries without national screening programmes suggesting that the major factors of the observed tendencies vary among the countries, and may include earlier detection by screening, but also improvements in therapy (3). Mortality has increased from the 1950s until at least the 1980s in most European countries, particularly in East and South Europe and from the early 1990s there was an evident decline which has affected women aged less than 50 years. The pattern observed in the USA and Canada is broadly similar to that of Europe, with comparable increases in incidence in both white and black women (4). Similarly to Europe, a decline in mortality took place in 1980s, and from 1992 to 2000 breast cancer death rates among white women declined in 38 US states, whereas among blacks increases were observed in several states (5). Breast cancer incidence has a distinctive age-specific curve (Fig. 1): there is a rapid rate of increase before the menopause (age 40-50) that slows down after it probably due to diminishing levels of circulating oestrogens. Overall, breast cancer incidence rates increased during the most recent time period (2006-2010) among non-Hispanic white women aged 30 years to 49 years,

and African American women aged 60 years to 69 years, whereas rates decreased for Hispanic women aged 30 to 49, and 50 years to 59 years. Trends by ER (Estrogen Receptor) status were also estimated: overall there was a decrease in ER- tumors and an increase in ER+ tumors. During 2006 to 2010, the incidence rates of ER+ breast cancer increased in African American women with faster intensification among younger groups. Rates of ER+ breast cancer also grew among younger (30 years to 49 years) white women and Hispanic women aged 60 to 69 years, while significant decreases were observed for ER- breast cancer in most age/racial/ethnic groups (6).

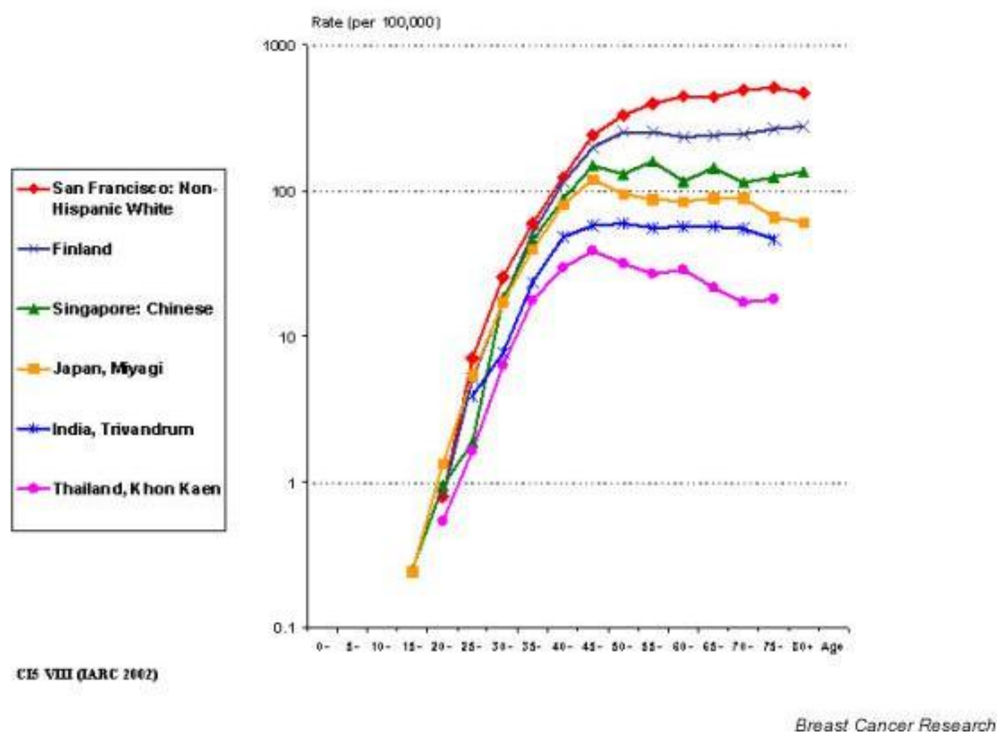


Fig.1: Age-specific breast cancer incidence rates in selected cancer registries

1.1.2. Risk factors

There are several risk factors linked to breast cancer: age, reproductive factors, personal or family history of breast disease, genetic pre-disposition, and environment. Age increases the risk of developing breast cancer in fact, in the United States, the probability of a woman developing breast cancer increases with age, from 0.5% up to 39 years, at 3.78% after 40 years (7). Initial DCIS or stage IIB, estrogen/progesterone receptor-negative, and age <50 years are all factors associated with higher recurrence and second primary rates. Women with a family history or greater breast density had

higher second primary rates, and women who received breast conserving surgery without radiation had higher recurrence rates. These evidences identify subgroups who may benefit from increased surveillance (8). Some proliferative breast lesions including usual ductal hyperplasia, intraductal papillomas, sclerosing adenosis and fibroadenomas confer a small increased risk of breast cancer development, while women with atypia have an approximately 4.3 fold greater risk of developing it, if compared to the general population (9). The risk increases in case of family history: women who have one, two or three affected first degree relatives have risk ratios of 1.80, 2.93 and 3.90 respectively, compared with women who have no affected relative (10). A genetic predisposition identified mutations in the genes BRCA1 and 2 is considered another predisposing factor to the onset of breast cancer as lifetime breast cancer risk ranges from 65% to 85% for BRCA1 mutation carriers and 45% to 85% for BRCA2 carriers. Other genes including homozygous ataxia-telangiectasia (ATM), CHEK2, BRIP1 and PALB2, confer a 20%-40% lifetime risk (11,12). Among the risks can be further listed endogenous hormone exposure, reproductive factors, and exogenous hormone exposure ones. The first ones can be associated with: an early menarche (13), the fact that nulliparous women are at an increased risk compared to parous women (14), breast feeding as a protective role (15), high endogenous sex hormone levels (16) and later onset at menopause (17). Evidences suggest also a relationship between the use of hormone replacement therapy (HRT) and breast cancer risk (18).

1.1.3. Molecular subtypes

Breast cancer studies has revealed substantial tumor heterogeneity consisting of different molecular subtypes, with distinct clinical and biological characteristics (19). In the recent years, molecular profiling of breast cancer by gene expression studies has provided important tool to discriminate different subtypes, each associated with clinical outcome and treatment response. Based on the molecular studies, breast carcinomas can be classified in main groups.

1.1.3.1 Luminal A and B subtypes

Luminal A subtype represents 50-60% of breast cancers and is the most common one. This subtype includes overexpression of genes related to cells of the luminal side of mammary ducts, estrogen receptor (ER) and ER transcription factors (20). The histological and IHC classification includes low histological grade, low Ki67 index, positive staining for ER, progesterone receptor (PgR) and luminal epithelial markers like cytokeratin (CK) 8 and 18. This subtype has the lowest rate of TP53 mutation. Luminal A is the subtype that shows the best prognosis with a 5-year survival rate of 90 % for patients with primary breast cancer (21), and generally is associated with less lymph nodes involvement, lower local and distant recurrent rates (22). Luminal B subtype varies from 10 to 30% and shares histological similarities with luminal A, only changing an higher Ki-67 index and a moderate histological grade (23). Currently, luminal B carcinomas are divided into luminal B/Her2-negative and luminal B/Her2-positive subgroups.

1.1.3.2 Her2-positive subtype

Her2-enriched subtype represents the 4-7% of breast carcinomas and is characterized by amplification, or overexpression of Her2 oncogene and genes located at the Her2 amplicon on chromosome 17q22.24. IHC and histological features include Her2 overexpression, negative staining for ER and PgR and high histological grade. It differs from luminal subtype because of the high incidence of advanced disease at the time of diagnosis, an increased risk of metastasis usually to brain, lung and liver, and a short overall survival (24).

1.1.3.3 Basal-like subtype

Basal-like subtype represents 10-20 % of breast cancers (21) and is characterized by overexpression of some genes associated to cells of the basal layer of mammary ducts, such as CK5/6, CK17, laminin, integrin b4, EGFR and fatty acid binding protein 7 (19,20). It usually shows a high Ki-67 index and high histological grade and IHC and it is positive for CK5/6 and CK17 staining. Although this subtype is characterized by the lack of expression of ER and PgR, as well as Her2 overexpression there are some differences between it and triple negative breast cancers (TNBCs). However, they largely overlap in 70-80 % of cases referring to morphology (ductal carcinoma of high

histological grade), association with BRCA1 mutations and lack of benefit from endocrine or Her2-based therapy.

1.1.3.4 Triple negative subtypes

Triple negative breast cancers (TNBCs) collect several subgroups of breast carcinomas characterized by different clinical, histological, immunoistochemical and pathological features. Their main feature is the lacking of ER, PgR and Her-2 expression, the reason why patients are insensitive to most currently ER-targeted, Her-2-based and hormonal therapies. This type of carcinoma affects 20-30% of all breast cancer patients and is more prevalent among young Latino, African and African-American women (25). More than 80% of BRCA-1 mutations are linked to triple negative groups and 20% of BRCA-1 and BRCA-2 mutations show deficiency in DNA repair mechanisms. TNBCs are highly malignant, poorly differentiated, more aggressive, and show a poor outcome (26). Furthermore, patients with TNBCs have twice as likely than other women to develop distant metastasis, showing a shorter survival. Recently, Lehmann *et al.* have analyzed gene expression profiles from breast cancer data sets and identified 587 TNBC cases. Cluster analysis identified 6 TNBC subtypes including basal-like 1 and basal-like 2 (BL1 and BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and a luminal androgen-receptor (LAR) subtype (27). The BL1 subtype is enriched in genes implicated in cell cycle, DNA replication, G2 cell-cycle pathway, RNA polymerase and G1 to S cell cycle (MYC, NRAS, PLK1, PRC1, TTK, CCNA2, AURKA, AURKB, CENPA, CENPF, BUB1, BIRC5). Furthermore, this subtype show a high Ki-67 mRNA expression and Ki-67 nuclear staining related to the high proliferative nature of the carcinoma. The BL2 subtype displays unique gene ontologies involving growth factor signalling (NGF, EGF, MET, Wnt/β-catenin and IGF1R pathways). The IM subtype is linked to immune cell processes, including immune cell signaling pathways (TH1/TH2, NK, B cell receptor signalling, T cell receptor signalling pathways), cytokine signaling (IL-12/17 and cytokine pathways) and immune signal transduction pathways (NFkB, TNF and JAK/STAT signalling). The M subtype is characterized by high expression of genes involved in cell motility and cell differentiation pathway (Wnt and TGF-β signalling). The MLS group is enriched in genes related to angiogenesis (TEK1, TIE1, EPAS1 and VEGFR2). In addition, MLS expresses low levels of proliferation genes in comparison to ML one. The LAR subtype is an ER-negative subgroup that show a high percentage of

genes linked to hormonally regulated pathways, such as steroid synthesis or androgen/estrogen metabolisms. It shows high androgen receptor (AR) mRNA expression, more than all other subtypes, and it is heavily positive for nuclear AR staining (27).

TNBC subtype	Cell line	Subtype correlation (*P* value)	Histology	Mutations[A]	Intrinsic subtype[B]	Basal subtype[C]
Basal-like						
BL1	HCC2157	0.66 (0.00)	DC	*BRCA1; STAT4; UTX*	Basal	Basal A
	HCC1599	0.62 (0.00)	DC	*BRCA2; TP53; CTNND1; TOP2B; CAMK1G*	Basal	Basal A
	HCC1937	0.28 (0.00)	DC	*BRCA1; TP53; MAPK13; MDC1*	HER2	Basal A
	HCC1143	0.26 (0.00)	IDC	*TP53*	Basal	Basal A
	HCC3153	0.24 (0.00)		*BRCA1*	Basal	Basal A
	MDA-MB-468	0.19 (0.06)	DC	*PTEN; RB1; SMAD4; TP53*	Basal	Basal A
	HCC38	0.19 (0.01)	DC	*CDKN2A; TP53*	Unclassified	Basal B
BL2	SUM149PT	0.30 (0.00)	INF	*BRCA1*	Unclassified	Basal B
	CAL-851	0.25 (0.00)	IGA	*RB1; TP53*	Basal	
	HCC70	0.24 (0.04)	DC	*PTEN; TP53*	Basal	Basal A
	HCC1806	0.22 (0.00)	ASCC	*CDKN2A; TP53; UTX*	Unclassified	Basal A
	HDQ-P1	0.18 (0.17)	IDC	*TP53*	Unclassified	
IM	HCC1187	0.22 (0.00)	DC	*TP53; CTNNA1; DDX18; HUWE1; NFKBIA*	Basal	Basal A
	DU4475	0.17 (0.00)	DC	*APC; BRAF; MAP2K4; RB1*	Unclassified	
Mesenchymal-like						
M	BT-549	0.21 (0.00)	IDC	*PTEN; RB1; TP53*	Unclassified	Basal B
	CAL-51	0.17 (0.00)	AC	*PIK3CA*	Unclassified	
	CAL-120	0.09 (0.00)	AC	*TP53*	Luminal B	
MSL	HS578T	0.29 (0.00)	CS	*CDKN2A; HRAS; TP53*	Unclassified	Basal B
	MDA-MB-157	0.25 (0.00)	MBC	*NF1; TP53*	Unclassified	Basal B
	SUM159PT	0.14 (0.00)	ANC	*PIK3CA; TP53 HRAS*	Unclassified	Basal B
	MDA-MB-436	0.13 (0.00)	IDC	*BRCA1; TP53*	Unclassified	Basal B
	MDA-MB-231	0.12 (0.00)	IDC	*BRAF; CDKN2A; KRAS; NF2; TP53; PDGFRA*	Unclassified	Basal B
LAR						
LAR	MDA-MB-453	0.53 (0.00)	AC	*PIK3CA; CDH1; PTEN*	Luminal A	Luminal
	SUM185PE	0.39 (0.00)	DC	*PIK3CA*	Luminal A	Luminal
	HCC2185	0.34 (0.00)		*PIK3CA*	Luminal A	Luminal
	CAL-148	0.32 (0.00)	AC	*PIK3CA; RB1; TP53; PTEN*	Luminal A	
	MFM-223	0.31 (0.00)	AC	*PIK3CA; TP53*	Luminal A/B	
Unclassified	HCC1395		DC	*ATR; BRCA2; CDKN2A; PTEN; FGFR1; PDGFRB; TP53*	Basal	
	BT20		IDC	*CDKN2A; PIK3CA; TP53*	HER2	Basal A
	SW527				Luminal B	

[A]Source: mutations taken from COSMIC database (www.sanger.ac.uk/genetics/CGP/cosmic/). [B]Molecular subtype determined by correlation with UNC/intrinsic breast centroids (29). [C]Basal subtype obtained from Neve RM et al. (32). AC, adenocarcinoma; ANC, anaplastic carcinoma; ASCC, acantholytic squamous cell carcinoma; C, carcinoma; CS, carcinosarcoma; DC, ductal carcinoma; IDC, invasive ductal carcinoma; IGA, invasive galactophoric adenocarcinoma; INF, inflammatory ductal carcinoma; MC, metaplastic carcinoma and MBC, medullary breast cancer.

Fig. 2 Subdivision of triple negative breast cancer cell lines into different subtypes (*Lehmann et al., J Clin Invest. 2011*)

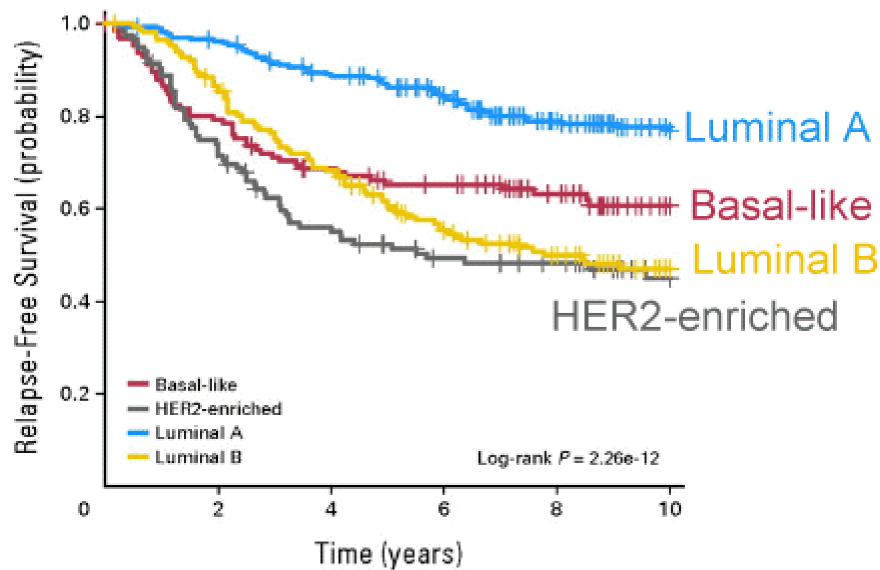


Fig. 3 Outcome of node negative women no adjuvant therapy based on genetic profile

1.1.4. Current treatment strategies

Being breast cancer a heterogeneous disease, characterized by several biological features, it is important to consider different clinical response patterns according to various treatment modalities and clinical outcomes. Today, biological characteristics such as tumor size, histological grade, patient's age, ER, PgR, Her2 status and lymph node involvement are not more sufficient to evaluate patient-tailored treatment strategies. Therefore, recent studies of global gene expression (GEP) have provided evidences to open a broad field in cancer research to study new potential targets, allowing to improve patient risk stratification and to ensure the highest chance of benefit and the least toxicity from treatments. In particular, adjuvant therapy, including chemotherapy, hormonal therapy, human epidermal growth factor receptor (HER2)–directed and radiation therapies, supports surgery in effecting cure of breast cancer.

1.1.4.1 Traditional and new chemotherapy

Endocrine Therapy (ET) is the first choice treatment in the management of estrogen receptor alpha (ERα)-positive breast cancer and it is used pre-operatively (neoadjuvant), post-operatively (adjuvant), and in the metastatic/advanced disease setting (palliative treatment). Current ET is constituted by treatments which modulate or disrupt the process of estrogen production or the function or presence of the ER in breast cancer cells. In pre-menopausal women estrogen production is largely due to

ovarian follicles under the control of the anterior pituitary gland which produces luteinizing hormone (LH), and follicle stimulating hormone (FSH). In postmenopausal ones, estrogens are dependent on peripheral aromatisation, predominantly in the liver, adrenal glands, and adipose tissue. Then, estrogen exerts its effect binding to ER wich regulates target genes transcription.

1.1.4.2 Adjuvant cytotoxic chemotherapy and endocrine therapy in hormone receptor positive breast cancer

Currently, there are two major types of strategies available for hormone receptor positive breast cancers: cytotoxic chemotherapy and endocrine therapy. Several studies have shown the improvement of patients disease free survival (DFS) and overall survival (OS). Among several regimens, the most widely used is CMF (cyclophosphamide, methotrexate and fluorouracil). It was first introduced by Bonadonna *et al.*, (28), then several studies were carried out, including a meta-analysis showing a reduction of 6.2% in breast cancer related mortality after 10 years of follow up (29). Other important agents for adjuvant therapy are anthracyclines, among which the most used is Doxorubicin (Adriamycin) in stage II breast cancer patients. In particular, it has been reported it is effective in metastatic phase (30). Another important step in the chemotherapy has been the introduction of taxanes. Several clinical trials were performed, including a trial comparing sequential doxorubicin and docetaxel chemotherapy for lymph node positive breast cancer showing the superiority of doxorubicin in survival (31).

The hormone therapy is divided into two drugs categories: selective estrogen receptor modulators (SERMs) and aromatase inhibitors (AIs). SERMs bind to estrogen receptors in a competitive way and they interfere with DNA synthesis by recruiting co-repressors and inhibiting cell cycle progression. Among SERMs, Tamoxifen has recently been the gold standard for the treatment of ER positive breast cancer in both pre- and post-menopausal women. In 2011, the Early Breast Cancer Trialists' Collaborative Group reported that 5 years of tamoxifen treatment reduced recurrence rates and mortality (32), but it has been shown that only with ER levels of 10 fmol/mg of cytosol protein or above there was tamoxifen efficacy and benefit, representing a limit in the use of this kind of treatment. Furthermore, in the last years a series of causes of acquired resistance to tamoxifen have been identified: the reduction or loss of ER signalling, the

recent discovered of G protein-coupled estrogen receptor 1 (GPER or GPR30), changes in ER-related co-factors, overexpression of Epidermal Growth Factor Receptor (EGFR) remain a problem and contribute to the development of endocrine-resistance.

AIs act inhibiting the "aromatase" enzyme that converts circulating testosterone to estradiol (E2), and androstenedione to estrone, by aromatization. These pathways represents the main source of estrogen in post-menopausal women, for this reason AIs can act only if the primary source of estrogen is not available. The most common drugs of AI category are Anastazole, Letrozole and Exemestane, luteinizing-hormone-releasing hormone (LHRH) agonists depriving estrogens.

1.1.4.3 Adjuvant therapy in Her2 enriched breast cancer

Trastuzumab is the first monoclonal antibody developed to target HER2 transmembrane glycoprotein. It binds to the juxtamembrane domain of HER2 receptor leading to activation of p21 or p27 and transcription inhibition. It was approved in 1997 by the first US Food and Drug Administration (FDA) and has become the first-line treatment in any stage of HER2-enriched disease. However, resistance to Trastuzumab therapy still represents a pivotal issue in the treatment of HER2 overexpressing carcinoma, and this is mainly due to defective apoptosis pathways (33). TD-M1 (emtansine-trastuzumab conjugate) and Pertuzumab are newer agents targeting HER2 and are currently approved by the US FDA for the use in metastatic stage (34). In particular, the antibody-drug conjugate T-DM1 has cytotoxic activity due to the microtubule-inhibitory agent DM1, leading to an improvement in patient OS.

Lapatinib, another drug that binds to intracellular domain of HER2 protein, was the first small molecule developed to overcome Trastuzumab resistance. It has shown efficacy as a single agent or in combination with trastuzumab in metastatic setting. (35).

1.1.4.4 Adjuvant therapy for triple negative breast cancer

Since this tumor subtype lacks of effective molecular targets, chemotherapy remains the mainstay of adjuvant treatment. The platinum agents, Cisplatin and Carboplatin, have shown their DNA-damaging activity particularly in the triple-negative subgroup. Most part of basal-like subtypes associated to BRCA-1 mutations is sensitive to DNA-

damaging agents because of impaired repair by homologous recombination of double-strand breaks (DSB) induced by platinums, so these agents have become the best regimen in the metastatic stage (36). Anyway, standard treatments currently used in TNBC are the same as for hormone receptor positive carcinomas, whereas this group is sensitive to both anthracycline or taxane based regimen. Bevacizumab, an agent which targets the Vascular Endothelial Growth Factor (VEGF), is effective in TNBC, and clinical studies revealed the importance of angiogenesis and microenvironment in TNBC cells (37). TNBC is clearly a complex disease, and its impressive heterogeneity adds to the challenge of identifying targets and treatments. The focus will need to be on clinical trials of increasingly smaller subsets of TNBC patients, defined by molecular and genetic characteristics that classify a patient's tumor into a subtype (*i.e.*, receptor expression or mutation).

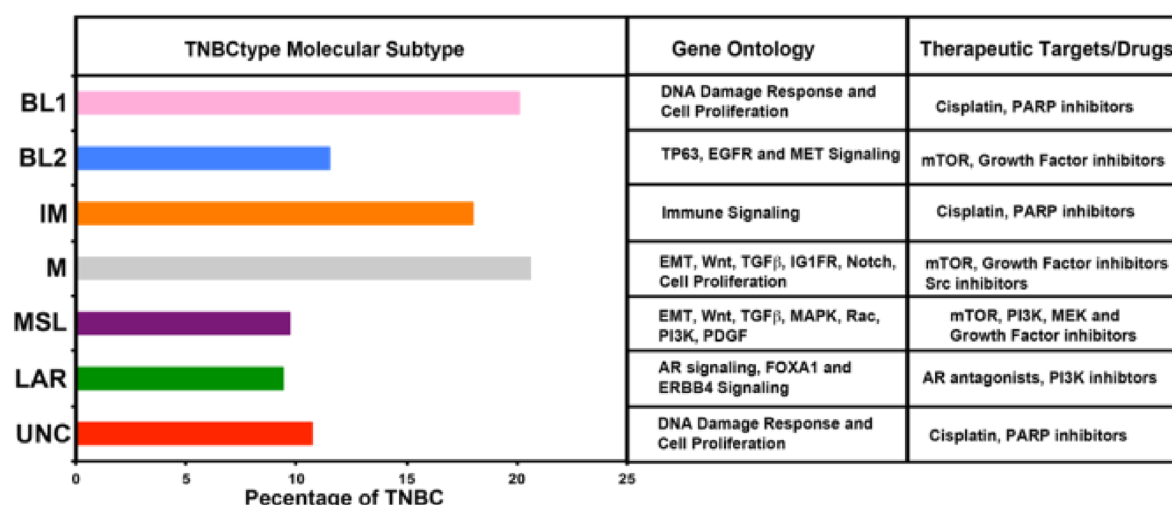


Fig. 4 Distribution of triple-negative breast cancer (TNBC) subtypes from The Cancer Genome Atlas with enriched gene ontology and potential therapeutic targets. BL1, basal-like subtype 1; BL2, basal-like subtype 2; IM immunomodulatory subtype; M, mesenchymal subtype; MSL, mesenchymal stem cell-like subtype; LAR, luminal subtype expressing androgen receptor; UNC, unclassified. (http://onlinelibrary.wiley.com/doi/10.1002/cncr.28914/full#cncr28914-fig-0001).

1.1.4.5 New promising targeted therapies

In recent years new strategies have been developed. Among new agents, Cyclin-dependent kinases 4 and 6 inhibitors are largely used, as Palbociclib, to target the Cyclin D-cyclin-dependent kinases 4/6-retinonoblastoma pathway that controls the cell cycle restriction point. mTOR pathway (mammalian target of rapamycin) is often activated in breast tumor and can represent a resistance mechanism to hormonal

therapies of which the most commonly used is Everolimus. New promising agents are Poly ADP-ribose polymerase (PARP) inhibitors, that block PARP enzymes activity, critical for appropriate recognition and repair of DNA breaks in cells with BRCA1/2 mutations. In this way, inhibition of PARP further compromises DNA repair leading to cell death. PARP inhibitors seem to be an attractive therapeutic options especially for BRCA-mutated cancers and TNBCs (38).

1.1.4.6 Androgen receptor antagonists

Recently, it has been shown that there is a subset of ER/PgR negative tumors expressing genes known to be direct targets of ER and responsive to estrogens, or usually expressed in ER positive breast tumors which are driven to proliferate by androgens (39). This has led to the development of non-steroidal anti-androgen Bicalutamide for the treatment of patients with subtype ER/PgR negative, but AR positive. In a study including 436 patients only 12% were positive for the expression of AR, and after Bicalutamide treatment for at least 12 weeks, only 4/21 patients had stable disease for more than 6 months, suggesting a probable efficacy in this patient group (40).

1.1.4.7 Metformin

Metformin is largely used in the treatment of diabetes mellitus type 2 reducing insulin resistance and diabetes-related mortality. Currently, there are wide pre-clinical studies and data showing the possible anticancer effect of Metformin in all breast cancer subtypes, as well as in cytotoxic therapy-resistant models. In a population-based study was observed the reduction of cancer risk after administration of Metformin to patients with diabetes mellitus type 2 (41, 42). The effects of Metformin were also investigated in TNBC cell lines. Liu *et al.* showed the anti-TNBC effects of Metformin both *in vitro* and *in vivo*, demonstrating the inhibition of cell proliferation, colony formation and apoptosis induction (43). Again, another study reported that Metformin can target JAK2/Stat3 signaling inhibiting proliferation and reducing P-Stat3 and P-mTOR levels in TNBC cell lines (44). On the basis of these and other studies, the anti-cancer activity of Metformin was hypothesized and should be explored as a therapeutic agent against TN aggressive subtype. There are currently several ongoing prospective clinical studies investigating the safety and efficacy of this agent in cancer patients.

1.2 Sex Hormone Receptors in Breast Cancer

Biological, epidemiological and clinical data strongly implicate the role of sex hormones, principally estrogens, in breast cancer yet their presence does not implicate the origin of malignant process. Despite the simple mechanism of ligand/receptor construct, the molecular machinery by which sex hormones regulate different processes in their target tissues are really more complex. The link between breast cancer and estrogens has always been recognized, but the real explanation for how these hormones exert their actions is perpetually under investigation. Protein receptors play a key role in hormonal actions including estrogen-stimulated growth of breast cancer cells. Sex hormone receptors belong to the steroid/thyroid superfamily of nuclear receptors and mediate the genomic action of estrogens by acting as ligand-dependent transcription factors. All receptors share three distinct but not autonomously functioning domains (Fig. 5). The first domain is NH_2 – terminal domain (NTD) that contains activating factor 1 (AF-1), the function of which is to mediate interaction between transcription factors and target-gene activation. The DNA-binding domain (DBD) contains two highly conserved zinc-finger regions necessary for high-affinity binding to estrogen response elements (EREs) in target genes and comodulating receptor dimerization with the ligand-binding domain (LBD). The third domain is the C-terminus that mediates ligand binding, receptor dimerization and nuclear translocation. Finally, AF-2 domain.

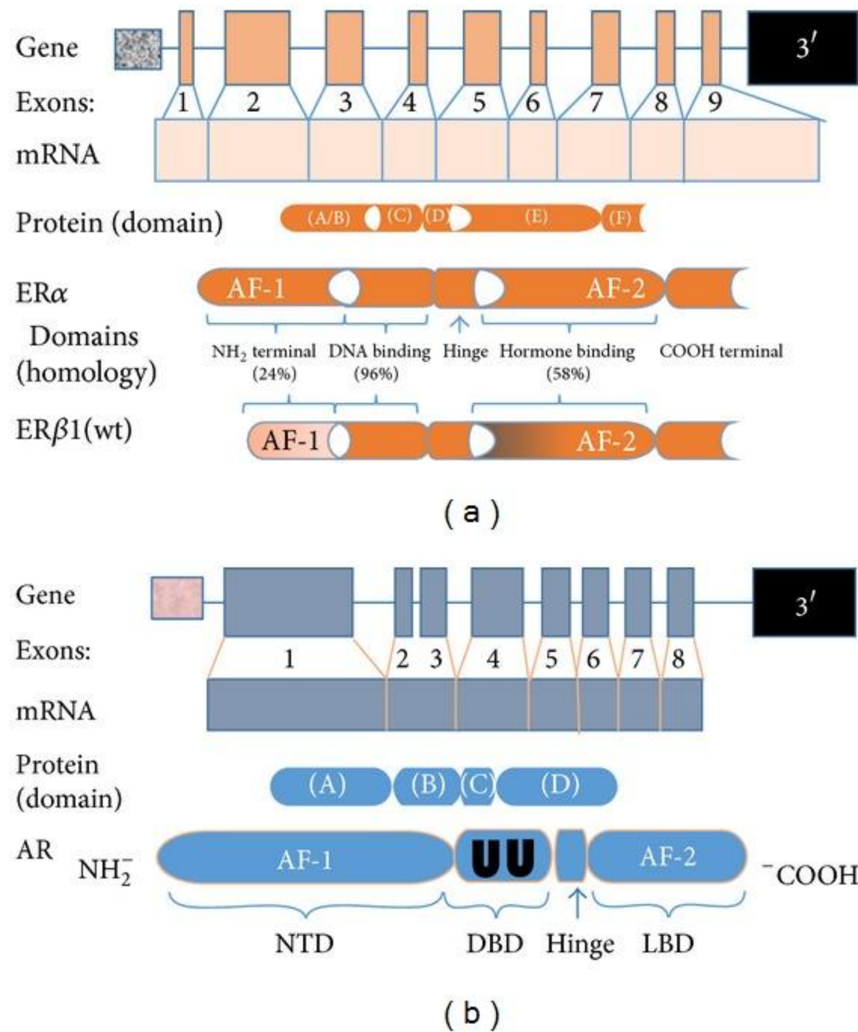


Fig. 5 (a) Estrogen receptor gene and protein (b) Androgen receptor gene and protein

1.2.1 Estrogen Receptor Signalling

Estrogens play a central role in reproduction and are regarded as the powerful female hormones involving reproductive capacity. Nowadays, estrogen is no longer viewed just as a female sex hormone but rather as a steroid hormone acting in both females and males. In addition to their central role in reproduction, estrogens also affect the cardiovascular, skeletal, immune and nervous systems and play a pivotal role in the initiation and progression of breast cancer and osteoporosis (45). All these functions are achieved both through the action of the endogenous estrogens: E1 (Estrone), E2 (Estradiol/17-beta Estradiol) and E3 (Estriol). Estrogen receptor has always been associated with breast cancer in two different ways: first, ER is the first element used for the assessment of endocrine therapy; then, it is a prognostic factor, as it is

associated with improved disease outcome. Molecular characterization of ER became possible when the ER gene was cloned by the Chambon Group (46), then, through mutagenesis studies, its structure was identified. Traditionally, estrogens act as nuclear transcription factors, interacting with coactivator or corepressor proteins, histone-modifying enzymes and proteins to modulate target genes through complex interaction. The pleiotropic and tissue-specific effects of estrogens are mediated by the differential expression of two distinct ER subtypes: ER-Alpha (ER-α) and and ER-Beta (ER-β), and their coregulators (47). The activities of a plethora of ER-interacting proteins converge to confer distinct functionalities on ERs, including the activation and repression of transcription, the integration of intracellular signaling pathways and the control of cell cycle progression. Both ERs are distributed widely in both genders. ER-α predominates in the uterus and mammary gland, whereas ER-β has significant roles in the central nervous, cardiovascular, and immune systems, urogenital tract, bone, kidney, and lungs. Typically, the majority of either ER-α or ER-β is found in the cytoplasm and nucleus. However, small amounts (2%) can associate with the cell membrane. The classical mechanism of steroid hormone action involves nuclear interactions of intracellular receptors, which are either cytoplasmic or nuclear. Binding of estrogens to ER releases the receptor from an inhibitory complex with HSPs (Heat Shock Proteins) and triggers conformational changes to allow ER-ligand binding to the palindromic ERE (Estrogen Response Element) located in the target gene promoters, stimulating gene transcription. Transcriptional activity requires the implication of AF1/AF2 domains. The transcriptional activity is also enriched by several regulatory cofactors including chromatin-remodeling complexes, coactivators and corepressors. Coactivators generally do not bind to the DNA but are recruited to the target gene promoters through protein-protein interactions with the ER. Some ER coactivators are members of the p160/SRC (Steroid Receptor Coactivator) family, as SRC1/NcoA1 (Nuclear Receptor Coactivator-1), NcoA2, NCOA3/AIB1/TRAM1/RAC3, CBPs (CREB-Binding Proteins) and p300 and the family of CITED (CBP/P300-Interacting Transactivator, With Glu/Asp-Rich Carboxy-Terminal Domain) proteins. Corepressors including NCoR (Nuclear Receptor Co-Repressor) and MTA1 (Metastasis Associated-1) protein have been implicated in the transcriptional silencing. The balance of receptors, coactivators and corepressors is a critical step in the classical pathway and it is crucial to promote signaling. A second classical mechanism of action involves protein-protein

interactions: in this pathway, ER-ligand complexes interact with transcription factors such as NF-KappaB (Nuclear Factor-KappaB), activator protein-1 and SP1 (Specific Protein-1) to influence gene transcription. There are different pools of ER in the cellular environment, including the plasma membrane, the mitochondria and the endoplasmic reticulum: in general, about 80% of ERs localize in the nucleus in the absence of estrogen, then ligand stimulation leads to nuclear accumulation and subsequently subcellular distribution of ER content varies from cell to cell. Over the years, studies have provided several critical aspects of estrogen signalling at the transcriptional level. In addition to traditional mechanism of action of ER, investigations have shown the existence of alternative rapid and nongenomic steroid actions. The nongenomic signalling includes the recruitment of second messengers such as NO (Nitric Oxide), RTKs (Receptor Tyrosine Kinases), GPCRs (G-protein–Coupled Receptors), and protein kinases including PI3K (PhosphatidylInosiol-3-Kinase), serine-threonine kinase Akt, MAPK (Mitogen-Activated Protein Kinase) family members and PKA and PKC (Protein kinases). The activation of GPR-30, a novel transmembrane intracellular estrogen-binding protein (48), and Akt pathway lead to antiapoptotic cascade. Activation of MAPK pathway drives to downstream cytoplasmic events or transcriptional events involving potentiation of AF1 activity: ER-ligand complex induces rapid phosphorylation of the adaptor proteins Src and SHC (SH2 Containing Protein), resulting in a SHC–GRB2 (Growth Factor Receptor Binding Protein-2)–SOS complex formation; this leads to the subsequent activation of Ras, Raf and MAPKs, including ERK-1/2 (Extracellular Signal Regulated Kinases), JNK (c-Jun N-terminal Kinase), and p38. They are then translocated to the nucleus and participate in gene transcription.

1.2.2 Androgen Receptor

Androgens are commonly considered as male hormones, but they have been also detected at physiologically relevant levels in women (49).

These hormones play important biological roles in females. Different species of androgenic hormones are present in female circulation, including testosterone, DHEA and androstenedione (A4) produced by the ovaries and then, also with dehydroepiandrosterone-sulphate (DHEAS), secreted by the adrenal glands. Furthermore, testosterone, DHT and their metabolites are synthesised also in breast,

bone and brain tissues. Testosterone levels are variable during fertile period and gradually decline following menopause but in contrast, adrenal androgen production continues also after menopause (50). Testosterone has the potential to be metabolized within breast tissue to E2, the most potent natural ER-α ligand, or DHT, the most potent natural androgen receptor ligand, via the activity of aromatase and 5α-reductase enzymes, respectively, so this can influence the proliferative capacity of breast epithelial cells. Several studies have tried to analyze the possible correlation between circulating androgens and breast cancer growth and in general, high serum testosterone level has been associated with an increased risk in post-menopausal women; however, the relationship remains unclear and it becomes necessary to conduct additional studies. Androgen receptor (AR) is the intracellular receptor that mediates the biological effects of androgens and is a member of the family of steroid nuclear receptors: its structure is quite similar to that of ER (Fig. 6) and there are similarities between the AR and ER-α signalling pathway. AR protein levels are elevated (59–75% AR-positive epithelial cells) in ducts and lobules of the adult mammary gland and remain stable across the menstrual cycle and early stages of pregnancy, only showing a dramatic reduction during late stages of pregnancy and during lactation. In contrast, ER-α levels are lower (0–12.5% ER-α-positive epithelial cells) and change significantly during menstrual cycle and gestational stages (51). Similar patterns of AR and ER-α expression are present in the breast epithelia of women without breast cancer. AR has been detected in up to 85% of primary breast cancer and up to 75% of metastatic lesions (52), but this frequency appears to be variable among different breast cancer subtypes. In fact, AR seems to play different roles at different stages in various subtypes of the disease, so AR activity is becoming important in the evaluation of clinical practice. Most part of studies have demonstrated the possible inhibitory role of AR towards breast cancer cell lines (53,54), but some studies have found that androgens have a pro-proliferative activity (55,56). In prognostic studies about ER-α positive breast cancers, higher levels of AR confer a survival advantage, suggesting a possible role of AR as a tumor-suppressor in malignant breast epithelial cells: a probable explanation is that AR levels increase with the progression of ER-α positive malignancy in a homeostatic way to restore the balance of hormones activity normally involved in mammary epithelial cell proliferation. Furthermore, in ER-α positive breast cancer, AR has been found to

correlate with lower grade, reduced lymph node involvement and higher disease-free survival, proportionally to AR expression levels (57,58). In TNBCs, AR is expressed in 10-43% but its prognostic value in this subtype remains unclear, with some studies suggesting an increased associated mortality (59), some indicating no influence of AR expression (60). Anyway, McGhan *el.* found that AR expression correlates with higher tumor stage and an increase in lymph node metastasis (61). Hu *et al*. analyzed 211 TNBC cases and found that patients expressing AR had 83% of increase in overall mortality compared to AR-negative patients (59). Discrepancies in studies could be due to multiple factors, including the use of tissue microarrays *vs* whole-tissue sections, of different AR protein detection techniques, of several antibodies for AR immunodetection, measurement of AR mRNA trough different platform in trascriptome profiling studies and variable cut-off scores defining AR positivity. Furthermore, larger cohort numbers are necessary to determine a role for AR in specific patients tissues. TNBC expressing AR is called "*molecular apocrine*" , and it has been identified by Farmer *et al*. (62). This subtype has been studied *in vitro* using different breast cancer cell lines whose growth, demonstrated by AR silencing or treatment with Bicalutamide, is promoted by AR expression. Furthermore, Robinson *et al*. were able to show that, in the absence of ER-α, more than a half of AR binding events in the genome map in a similar pattern to that of ER-α in ER-positive cells, promoting the expression of ER-α target genes. In this subtype AR can, in some ways, mimick ER-α in a transcriptionally active manner through the pioneer factor FoxA1 and can stimulate an expression pattern more similar to that of ER-α in a ER-α positive cancer cell line than the profile reported for the AR in a prostate cancer cell line (63). Moreover, recent data show that over 90% of metastasis from luminal tumors retain FoxA1 expression (64) and the presence of AR and FoxA1 in metastatic lesions may mark a luminal to molecular apocrine transition and need an antiandrogenic rather than an antiestrogenic strategy. All these aspects suggest that in ER-α carcinomas AR can drive tumor progression and can represent a therapeutic target for this subtype of breast cancer.

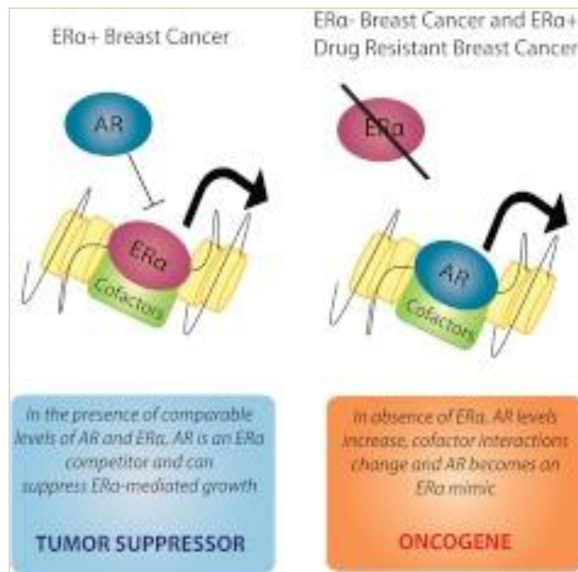


Fig. 6 Proposed dual role of AR in breast cancer (Hickey et al., Androgen Receptor in Breast Cancer, Mol Endocrinol. Aug 2012)

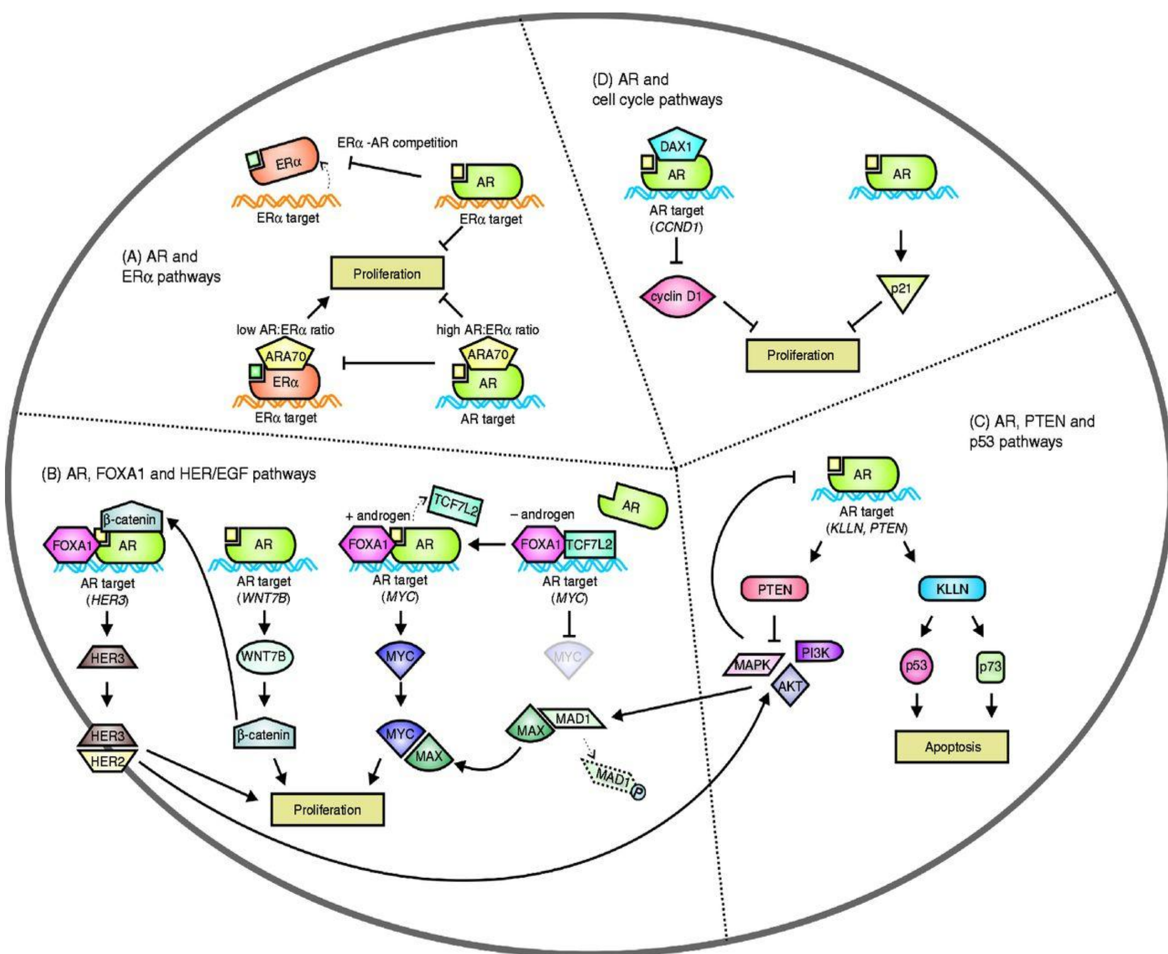


Fig. 7 Overview of pathways regulated by AR in breast cancer cells. (A) AR and ERα, (B) AR, FOXA1 and HER2, (C) AR, PTEN and p53 and (D) AR and cell cycle regulators (*McNamara K M et al. Endocr Relat Cancer 2014;21:T161-T181*)

1.3 Epigenetics and Cancer

Epigenetics is a term used to describe all heritable modifications in gene expression that are not caused by alterations in the DNA sequence. DNA methylation and histone modifications are the major epigenetic mechanisms, but there are also stable and long-term changes in the transcriptional machinery of a cell which are not necessary heritable. Epigenetic machinery drives critical processes such as X-chromosome inactivation, genomic imprinting, genomes reprogramming during differentiation and development, RNA interference connected to post-transcriptional gene silencing and genome imprinting. Deficiencies in this epigenetic mechanisms were found to be correlated to many human diseases including breast cancer.

1.3.1 DNA methylation

DNA methylation is the covalent post-replicative addition of a methyl group (-CH_3) onto the 5-carbon of the cytosine ring within CpG dinucleotides,catalyzed by DNA methyltransferases (DNMTs), which transfer the CH_3 –group from the methyl group donor S-adenosyl methionine. Usually gene promoters are rich in CpG dinucleotides, which are grouped to form a cluster called CpG islands. It is estimated that 60% of protein-coding mammalian genes hold CpG islands in the promoter region. In normal cells, CpG islands are usually unmethylated, while other single CpG dinucleotides throughout the genome are generally methylated. In cancer cells, on the contrary, CpG islands preceding tumor suppressor gene promoters are frequently hypermethylated, while promoter regions of oncogenes show a lower CpG methylation. Hypermethylation of gene promoters can lead to cell cycle regulating genes silencing, resulting in uncontrolled cell growth and tumorigenesis. Common genes found to be transcriptionally silenced via promoter hypermethylation include the cell cycle inhibitor Cyclin-dependent kinase inhibitor p16, the tumor suppressor gene p53, the DNA repair genes MLH1, MGMT and BRCA1 and the cell cycle regulator APC (65). Loss of imprinting and reactivation of transposable elements are the main mechanisms responsible of hypomethylation of CpG dinucleotides, and this can lead to chromosome instability as well (66).

1.3.2 DNA Methyltransferases

DNMTs hold in the C-terminal a catalytic domain that transfers methyl groups onto cytosine residues within DNA and they are the principal enzymes involved in hypermethylation of tumor suppressor genes. In mammals the DNMT protein family includes five members: Dnmt1, Dnmt2, Dnmt3a, Dnmt3b and Dnmt3b, but only three among them has catalytic methyltransferase activity (Dnmt1, Dnmt3a and Dnmt3b). Dnmt1 is classified as a *maintenance* methyltransferase because it shows a strong preference for hemimethylated over unmethylated DNA and its particular targeting of replication foci seems to allow copying of the parental DNA methylation pattern onto the newly synthetized DNA daughter strand (67). The Dnmt3 family has two catalytic members, Dnmt3a and Dnmt3b, which have methyltransferase activity towards unmethylated over hemi-methylated DNA, thus they are called *de novo* methyltransferases. Between the two remaining members of DNMT family, Dnmt2 and Dnmt3L, only Dnmt3L has shown to be able to stimulate *de novo* DNA methylation through the mediation of Dnmt3a (68).

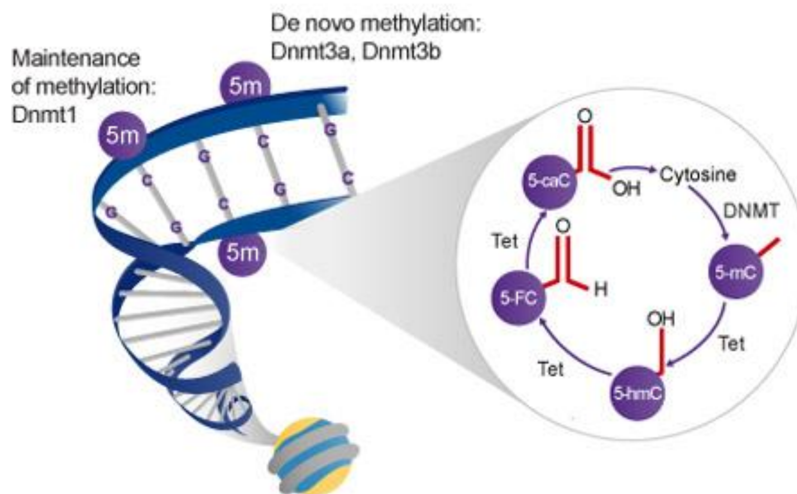


Fig. 8 Representation of the 3 main DNMT transferases

1.3.3 Histones modifications

Histone modifications are another fundamental process involved in epigenetic gene regulation. Histones are the major proteins that form chromatin, which can be classified into two forms: euchromatin, the accessible, less-dense and early-replicating, and heterochromatin the inaccessible, late replicating and gene-dense. Histones are

globular proteins carrying N-terminal tails that can undergo several chemical reactions by histone deacetylases enzymes (HDACs), histone acetyltransferases (HATs) and histone methyltransferases (HMTs), that cause the modification of eu- or heterocromatin. Histone changes can silence tumor suppressor genes despite the lower methylation of the gene's CpG island (an event that usually activates genes) (69). Gene transcription can be affected by acetylation, that modifies chromatin's structure that modulates the accessibility of transcription factors to their target DNA. So, it plays an important role in regulation of gene expression and in tumorigenesis.

1.4 microRNAs

MicroRNAs (miRNAs) are small non-coding RNAs of about 18-24 nucleotides (nt) of lenght, whose role in human carcinogenesis has been identified relatively recently. For many decades miRNAs have remained unknown to researchers until, in 1993, Victor Ambros discovered them in the nematode *C. Elegans*. In fact, during mutation sudies that determined changes in the timing of the development of the nematode, Ambros identified through genetic screening the gene *lin-4* encoding two small RNAs: one of about 22 nucleotides and the other of 61 nucleotides. It was observed that the shorter of them, interacting with the 3'untranslated region (3'UTR) of mRNA of lin-14, repressed its expression, thus reducing the amount of protein produced without changing the levels mRNA (70). The 22 nucleotide Lin-4 RNA was, by that time, recognized as an archetype of a large class of regulatory RNAs called "microRNAs" or "miRNAs". Subsequently, Reinhart *et al*. identified another gene coding for a second 22 nucleotides miRNA, let-7, involved in the transition from larval to adult in *C. elegans* (71). A year later over 100 miRNAs in both *D. melanogaster* and humans were identified (72, 73).

1.4.1 Nomenclature

Names are assigned to miRNAs, before the publication of their discovery, after they have been experimentally confirmed. The prefix "mir" is followed by a hyphen and a number that often indicates the order of identification (for example miR-123 was discovered and named before miR-456). The letter "r" of "mir" is written in lowercase

or uppercase, depending on whether we refer to the pre-miRNA or mature form, respectively. miRNAs that have nearly identical sequences except for one or two nucleotides, are annotated with the addition of a lower case letter after the number (*e.g.* miR-29a, miR-29b and miR-29c). Pre-miRNAs which are located in different regions in the genome but that generate a mature form identical to 100%, are indicated with an additional suffix in figures (*e.g.* the pre-miRNA hsa-mir-194-1 and hsa-miR-194-2 are located in different regions of the genome, but generate an identical mature miRNA: hsa-miR-194). In addition, a three-letter prefix identifies the species of origin (e.g. "hsa" for Homo sapiens, "mmu" for Mus musculus, "gga" for Gallus gallus and "rn" for Rattus norvegicus). Other prefixes that are used include "v" for a miRNA encoded by a viral genome, and "d" for a miRNA belonging to *D. melanogaster*. When two mature miRNAs are derived from opposite strands of the same pre-miR, these are marked with the suffix "-3p" or "-5p", which indicate respectively the filament "sense" and "antisense", previously indicated by the suffixes "s" and "as". Finally a miRNA is distinguished by its level of relative expression, if known, with an asterisk following the name: this indicates the miRNA resulting from double hairpin structure that is at higher levels in the cell (74).

1.4.2 Biogenesis and degradation

Approximately 50% of miRNAs is embedded in introns of protein coding genes, or in non-coding RNA transcripts, thus identifying two different classes of transcription of miRNAs, "exonic" and "intronic", which require however similar biogenesis mechanisms. The transcription of a large number of miRNAs is then associated with the expression of host gene promoters, while other miRNAs are grouped into polycistronic transcripts, thereby allowing a coordinated expression (75). The miRNA is first transcribed in the nucleus by RNA polymerase II/III in a long precursor with a 5' CAP and a poly-A tail at the 3' (*pri*-miRNA) (76). The pri-miRNA is then transformed into an RNA of 70-100 nucleotides, with a hairpin structure (*pre*-miRNA), by a RNA-specific ribonuclease called Drosha, with the help of Dgcr8 (DiGeorge syndrome critical region gene 8, or Pasha) (77). Through Exportin 5 *pre*-miRNA is translocated into the cytoplasm, where a complex including Dicer (ribonuclease III) and TRBP (HIV-1 transactivating response RNA binding protein) cuts it in a duplex of 18-24 nucleotides, that interacts with an another large protein complex called RISC (RNA induced

silencing complex), comprising the Argonaute family proteins (Ago1-4 in humans). Once it is inside the complex, the two strands of the duplex miRNA: miRNA * are separated and followed by two different ways. The 3'-5' strand (miRNA *) is released into the cytoplasm where it is degraded, and the 5'-3'strand (mature miRNA) remains stably associated with RISC that mainly drives it, but not exclusively, to the 3' untranslated region (3'-UTR) of the target mRNA. It was recently shown that targeting can also occur in the 5' untranslated region (5'-UTR) and in the *open reading frame* (ORF) (78). Anyway, the decay process of miRNAs is still little known topic. Although initially miRNAs were considered very stable molecules, with a half-life of the order of days, recently "microRNases" involved in their turnover have been identified, as SDN in plants (small RNA degrading nucleases) and XRN-2 in animal cells (exoribonuclease 2). It seems that miRNAs, once completed their activity, or not having another target messenger, disassociate themselves from RISC complex and are led to degradation. It is emerging that a half-life of long duration is not considered as an invariant characteristic of miRNAs, but there are marked differences in their individual stability, furthermore the environment of the cell can influence the rate of turnover. Biochemical data support the hypothesis that the target mRNA can stabilize the complex miRNA-Ago, providing a possible mechanism that could control the duration of miRNA-life. Anyway, miRNAs turnover mechanisms are still largely unknown (79).

1.4.3 miRNAs function

miRNAs exert their regulation gene expression function through the control of the translation and the expression of their target mRNAs, and they are involved in a variety of biological processes (80,81). Following the association between miRNA-RISC complex and the target mRNA, the latter can undergo two different ways based on the complementarity degree of miRNA:mRNA. A perfect complementarity between base pairs leads to cleavage of the target (this occurs primarily in plants), while in the case of an imperfect annealing, silencing of the target mRNA will take place (predominant mechanism in nematodes and mammals), although in the latter case also a reduction of the target mRNA has been described (82). Recently, it was discovered that some microRNAs are able to up-regulate the translation of their specific targets. In fact, during cell cycle arrest, it seems that microRNAs, with the help of proteins such as AGO and FXR1, can activate the expression of a target gene recognizing target sites enriched

in AU elements (ARES) in the 3 '-UTR sequence (83). MiRNAs have their specificity and if we consider a number of different organisms, it is clear that miRNAs are differentially expressed during development stages, in different cell types and in various tissues (84). It has been estimated that approximately 9% of all mammal genes presents, in the 3'-UTR region, more than one target site for miRNAs and that 1314 of these genes are considered to be good candidates as target of miRNAs regulation, carrying more than two recognition sites. Despite this, it is not a rule that all predicted miRNA-mRNA pairs produce a biological effect, unless both RNAs are not expressed simultaneously in the same cell and in a sufficient concentration (85). Since only nucleotides from 2 to 8 of the 5'-UTR pair up perfectly with the 3'-UTR region of the mRNA target, identification of target mRNAs is quite complex (82, 86). Nucleotides not involved can pair with the sequence of the mRNA, but in most cases this is not necessary for the purposes of the intrinsic functionality of the molecule. Some miRNAs can indeed act as *small interfering* RNAs (siRNAs) by inducing cleavage of mRNA by forming a duplex miRNA: mRNA made possible by pairing between nucleotides in position 10 and 11, or it can happen that the extension of bases pairing between miRNA and mRNA can induce a balance between cleavage and degradation of the target (87). Studies carried out using bioinformatic algorithms applied to the probability of pairing between the first 2-8 bases of the sequence of mature miRNAs and the 3'-UTR of all human genes expressed, have highlighted that miRNAs are able to bind to more than a hundred target genes with different functions and that a single target gene can be regulated by different miRNAs (88,89). There are many algorithms developed in order to identify possible and probable target genes of a miRNA and, vice versa, miRNAs targeting a single gene. Among the most commonly used are cited DIANA-microT, TargetScan, Miranda and PicTar (85, 90, 91). Several papers, however, reported error rates associated with the analysis of these algorithms that suggest researchers to use more than one of these systems. In fact, as reported by Wei Wu *et al*. who examined all results obtained with these algorithms about a miRNA, more than 81% of miRNA:mRNA pairings are predicted by a single algorithm examined, while the false positive rate for each algorithm is around 20-30% (88). Literature also suggests to validate the predicted target, for example using luciferase assay (92). The exact mechanism of action of miRNAs is not yet completely undestood. Some studies suggest a repression of translation by association of the miRNA with the mRNA-ribosome

complex, before the process begins. Other studies support the hypothesis that this inhibitory action takes place once translation is started, following three possible modalities: impediment of elongation of the polypeptide due to a stacking of ribosomes along mRNA strand, direct action of the miRNA on the ribosome with induction of cleavage of two ribosomal subunits, instant degradation of the polypeptide during the elongation due to a protease activated by miRNA (93). MiRNAs can than participate actively in all molecular processes regulating them.

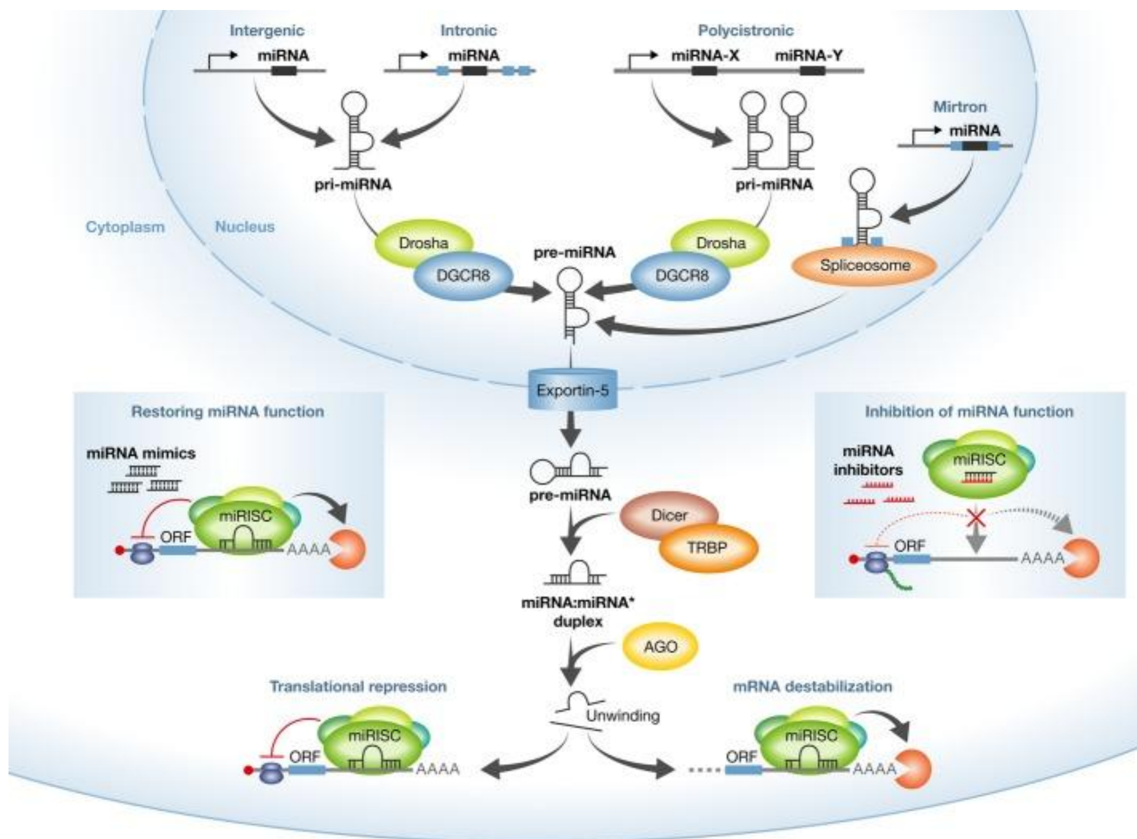


Fig. 9 Biogenesis of miRNAs and strategies employed to modulate miRNA activity: miRNA mimics and miRNA inhibitors

1.4.4 microRNAs and cancer

More than 50% of genes encoding miRNAs was found to be located in genomic regions associated with cancer (CARGs), about 19% within or adjacent to fragile sites of the genome (FRAs) and 43% was found in amplified genomic regions, or with frequent loss of heterozygosity (LOH) (94). It is therefore easy to understand the wide involvement

of miRNAs in carcinogenesis. Some miRNAs may have a dual function in cancer cells. Onco miRNAs and miRNA tumor suppressors may represent two different sides of the same gene, which can act in one way or another depending on tissue types and specific targets (95). Therefore, knowledge of expression profiles of miRNAs in various tumors could potentially have a significant diagnostic value, while the role of miRNAs as biomarkers for the prognosis of patients now seems to be evident (96). The study of these molecules has become of great interest to investigate rare and aggressive tumors, being very complicated to manage from the therapeutic point of view. The important role played by these small RNAs is confirmed by the evidence that they are often deregulated in tumor tissues (97). MiRNAs are frequently dysregulated in cancer patients. The group of Volinia has performed a detailed analysis of a large-scale expression profiling of miRNAs in 540 samples of 6 solid tumors (breast, colon, lung, pancreas, prostate and stomach). 137 different miRNAs, expressed in at least 90% of the samples analyzed, were considered. By comparison of all tumor tissues with all healthy tissues a change in expression of 31% of the total analyzed miRNAs has been revealed: 26 miRNAs were overexpressed and 17 under-expressed. In order to identify prognostic miRNAs, Volinia *et al*. have found a tissue-specific signature that can unequivocally be attributed to a particular tumor subtype. Among overexpressed miRNAs in all neoplasias. They found miR-21, miR-17-5p and miR-191, and in the same way 21 deregulated miRNAs have been identified, in at least three of the six solid tumors analyzed. Results have shown that prostate, colon, pancreas and stomach tumors have a very similar pattern, while lung and breast cancers show a quite different profile. A group of miRNAs that appears to be activated includes miR-210 and miR-213 that, with miR-155, represent the only overexpressed miRNAs in both breast and lung carcinomas, suggesting that in solid tumors the change in miRNAs expression seems to be unique, regardless of tissue's origin, and a common mechanism of involvement in carcinogenesis. In solid tumors, an increase of expression seems the most common event, while the loss of expression appears to be less frequent and more related to tissue specificity (98).

1.4.5 miRNAs in breast cancer

MiRNAs have been shown to play a critical role in the regulation of a wide range of pathological processes, including breast cancer. The first study describing genome profiling of miRNA in breast cancer identified 29 differentially expressed candidates, and 15 of them were used to distinguish between breast cancer and normal breast tissue (99). Furthermore, a correlation between some miRNAs and clinicopathological features, such as ER, PgR and Her2/neu status, tumor size or lymph node status, was found. In particular, the expression loss of members of the let-7 family was associated with clinical features, such as PgR status (let-7C), a positive lymph node status (let-7f-1, let-7a-3 and let-7a-2) or an high proliferation index (let-7c and let-7d) (100). Some miRNAs are classified as oncogenic because of their capacity to repress tumor suppressor genes, and numerous miRNAs have been demonstrated to be implicated in oncogenesis and/or metastasis process, that promote angiogenesis or induce epigenetic changes. For exemple, miR-10b is shown to be upregulated in breast cancer cells and correlated with increased cell migration and metastasis (101). Several studies have suggested that miR-155 is an oncogenic miR and its upregulation results in inhibition of tumor suppressor genes in breast cancer cells (102,103). Similarly, miR-21 upregulation was demonstrated in breast cancer cells and is considered as an oncogenic miRNA (104). Recently, miR-203 also has been shown to inhibit tumor suppressor protein SOCS3 expression in breast cancer cells (105). Other central miRs are represented by the miR-200 family which shows a role in cell stemness, cell proliferation, apoptosis also *in vivo* and it is implicated in the promotion of metastasis in breast cancer, reason why they are referred as MetastamiRs (106,107). Other central metastamirs include miR-520C, miR-373, miR-221, miR-222 and miR-9 (108). Furthermore, Zhao *et al*. reported that miR-221 and miR-222 overexpression contributes to tamoxifen resistance through negative regulation of ER-α, whereas knockdown of miR-221 and/or miR-222 restores ER-α expression and tamoxifen sensitivity (109), so they are part of that class of miRNAs implicated in drug resistance to chematerapeutic agents. Recently, miR-451 and miR-27 were shown to be involved in resistance of MCF-7 breast cancer cells to chemotherapeutic drug doxorubicin mediated by MDR-1 (110), and miR-328 was shown to regulate negatively the expression of the ATP binding cassette membrane transporter BCRP (111). Additionally, Kong *et al*. demonstrated that miR-155 contributes to chemoresistance in

breast cancer (112). Over the past decade, certain miRNAs have been reported as circulating in serum/plasma from cancer patients (113) and since then, several very interesting studies have revealed associations between circulating miRNAs and the presence of cancers, including breast cancer (114). Cuk *et al.* were able to associate for the first time circulating miR-127-3p, miR-376A and miR-652 to breast cancer, together with miR-148B, miR-376C, miR-409-3p and miR-801, finding them at significantly higher levels in the plasma of breast cancer patients when compared to healthy women (115), underling their potentially noninvasive biomarkers role. More recently, the group of Fabbri *et al.* has shown that exosomes released by lung cancer cells contain specific miRNAs, such as miR-21 and miR-29A, are able to bind and activate Toll-like receptors (TLR8 in humans and TLR7 in mice) in immune system cells surrounding the tumor tissue, and that are located in the interface between tumor tissue and healthy tissue. The activation of TLR by these exosomial miRNAs, triggers the pathway of NF-κB which leads to an increase in the production of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) by immune system cells, resulting in tumor growth and metastatic potential. This discovery has revealed a mechanism of action of miRNAs completely new, and the importance of exosomes as mediators of the cross-talk between tumor cells and the tumor microenvironment surrounding deserves to be mentioned (116). Furthermore, miRNAs have been shown to be able to trigger a prometastatic inflammatory response TLR-mediated (117). In breast cancer as well, some studies have been performed. For example, Melo *et al*. have found that exosomes derived from metastatic breast cancer cells showed higher enrichment in certain miRNAs when compared to exosomes derived from non metastatic breast cancer cells (118). Further investigation is certainly warranted to increase the understanding of circulating miRNAs as potential diagnostic, prognostic, and predictive biomarkers.

On the other side, some miRNAs are associated with tumor suppressive activity in breast cancer. So far, the most widely studied were miR-125A and miR-125B, associated with a reduction of the stress-induced RNA-binding protein HuR and therefore with a reduction in cell proliferation and migration and with increased apoptosis (119). Also miR-27B, miR-17-5p, miR-205, miR-206 and miR-145 are commonly considered tumor suppressive miRNAs in breast cancer, due to some evidences suggesting their role in the reduction of cell proliferation (120, 121, 122).

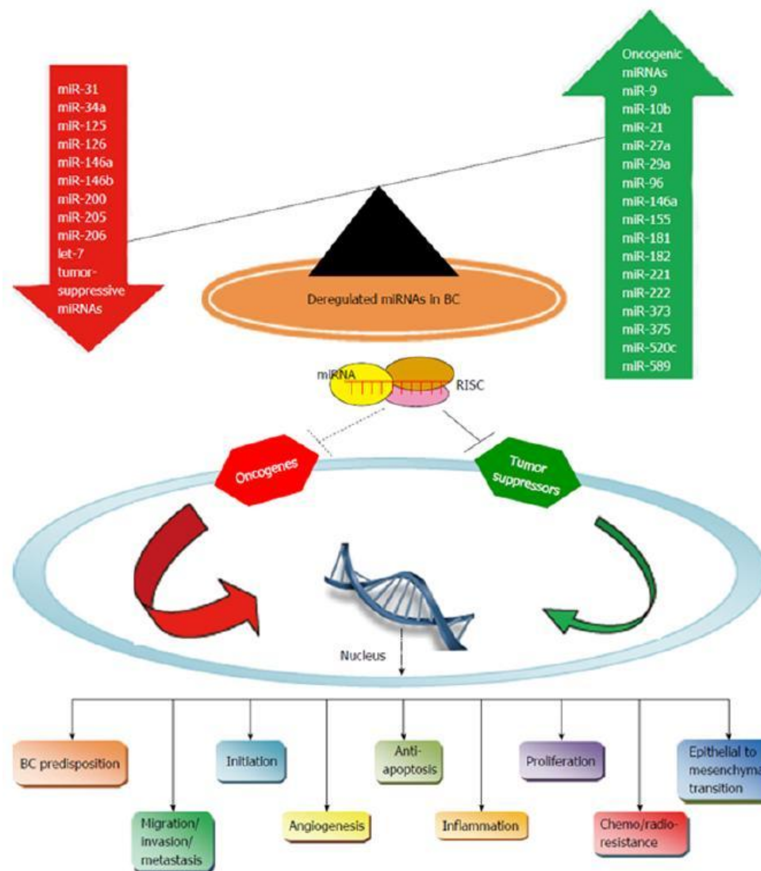


Fig. 10 Some major deregulated miRNAs in breast cancer

1.4.6 The miR-29 family and DNMTs

The family of miRs-29 (miR-29A, -29C and -29C) is downregulated in NSCLC (Non-small-cell lung carcinoma), as demonstrated by Fabbri *et al*. In *in vitro* experiments, the Authors have shown that miR-29s are able to target genes encoding DNMT3A and DNMT3B. They evaluate protein expression in 172 samples of healthy and tumor lung, and found a significant correlation between high expression of Dnmt3A and shorter survival, while this has not been observed with Dnmt3B. Expression level of mRNA has appeared inversely correlated with levels of miR-29s in NSCLC tissues. In addition, the analysis was extended to expression levels of mRNAs of two tumor suppressor genes, FHIT and WWOX, which are frequently silenced by methylation in promoter region. Transfection of miRNA-29A, -29B and -29C in lung adenocarcinoma cell lines led to a reduction of the expression of DNMT3A and 3B and induced demethylation of the promoter of FHIT and WWOX, resulting in their re-expression and inhibition of tumorigenicity *in vitro* and *in vivo*. This study confirms an important role of miRNAs as

regulators of epigenetic mechanisms involved in carcinogenesis (123). Also miR-148A and -148B have found to be involved in regulation of the coding sequence of Dnmt3B (124).

1.4.7 miRNAs and AR

Several studies have proposed a correlation between specific microRNAs and the expression of androgen receptor. Particularly, some androgenic-inducible microRNAs (miR-21, -101, -125b, -221, and -222) have been well-characterized in prostate cancer cells and found to be involved in the process of tumor progression associated with androgen (125). For instance, the onco-miR-21 has an androgen responsive element (ARE) within its promoter region and is directly up-regulated by androgens in prostate cancer cell lines. Again, 13 miRNAs (miR-135B, miR-185, miR-297, miR-299-3p, miR-34A, miR-34C, miR-371–3p, miR-421, miR-449A, miR-449B, miR-634, miR-654–5p, and miR-9) has been validated, able to regulate the 3'-UTR of AR and it has been demonstrated that some of them decreased androgen-induced proliferation of prostate cancer cells (126). Then other studies have been performed to investigate the role of microRNAs in AR pathway and, in breast cancer, the first evidence that highlights androgen-inducible miRs is reported to be miR-363 (127).

2. Aim of the thesis

Breast cancer is still a highly heterogeneous disease, characterized by differences both in biological features and in the response to the various existing therapies. Molecular biology and the latest discoveries in genetics have had a big impact on different aspects of prevention, prognosis and response to treatment. However, although in recent decades there have been improvements about hormone therapy, effective treatment options remain still limited mostly against TNBCs. In particular, the lack of expression of ESR1 in TNBCs is due in most cases to promoter hypermethylation. Promoter hypermethylation of tumor suppressor genes is one of the best known mechanisms involved in carcinogenesis. Consequently, the study of methylation has recently become a central theme in oncology research. Tumorigenesis is in fact a process characterized by an altered methylation status of key genes (tumor suppressor genes / oncogenes), as a result of increased expression of DNA methyltransferases (DNMTs). The genomic hypomethylation can lead to chromosomal instability and an increased mutation rate, thereby accelerating the progression of carcinogenesis. It was found that DNMT3B was overexpressed in 30% of breast cancers, and this appeared significantly related to the histological grade III, the negativity of ER and a marked expression of MK167 (128), suggesting that DNMT3B could represent a marker of aggressiveness and cancer proliferation, being the overexpression a cause of tumor growth rather than a consequence of it. Epigenetic factors and microRNAs are often, in cancer, deregulated in their activities. A close association between miRNAs and epigenetics is so established, as the same epigenetic mechanisms can sometimes be responsible for aberrations in the miRNoma (defined as the full spectrum of miRNAs of a specific genome). In fact, miRNAs are subject to the same rules of epigenetic regulation of any other coding gene and a specific group of miRNAs, called epi-miRNAs, can affect directly the effectors of epigenetic mechanism, such as DNMTs or HDACs (129). Furthermore, also ER has been demonstrated to be regulated by microRNAs, such as miR-206 that silences ER in *in vitro* studies (130). On the basis of these evidences and referring also to the study of Fabbri *et al*. (123), we have hypothesized that treatment with epi-miRNAs could lead to restoration of normal methylation pattern of ESR1 and then to its re-expression in TNBCs, finally re-establishing the

sensitivity to SERMs, particularly to Tamoxifen. Subsequently, being reported that 10-43% of TNBCs are positive for the expression of the androgen receptor (AR) (61) and that it is expressed up to 90% in primary tumors (52) and in 25% of metastases (40), we have speculated a possible involvement in the proliferation of TNBCs. There are experimental evidences which support this hypothesis, showing that AR represents a potential oncogene, especially in molecular apocrine subtype, in which AR seems to assume the role of the Er-α, where the latter is not expressed (63). So, we tested *in vitro* the modulation of AR operated by miRs, and in the same time we evaluated the effect of 3 drugs (Tamoxifen, Herceptin and Metformin), in order to consider a possible increase to their sensitivity, after AR silencing.

3. Materials and methods

3.1 Cell cultures

The human breast carcinoma cell line MDA-MB 231 and MDA-MB 453 were purchased from ATCC (American Type Culture Collection), and the cell line T-47D was gently purchased from Biochemistry, Biophysics and General Pathology Department of University of Naples. MDA-MB 231 and MDA-MB 453 were maintained in Leibovitz's L-15 medium (ATCC 30-2008, LGC *Standards*) supplemented with FBS (*Foetal Bovine Serum*) to a final concentration of 10%, according to the information sheet of the manufacturer. T-47D were maintained in RPMI-1640 medium (ATCC 30-2001, LGC *Standards*) supplemented with 10% FBS and 0,1 % Insulin Solution (*Sigma-Aldrich*). Penicillin-streptomycin (*PAA, Carlo Erba Reagents*) to a final concentration of 1% and MycoZap Prophylactic (*Lonza*) to a final concentration of 0,002% were added to all mediums. The cultures were maintained in an incubator Heraeus, in atmosphere composed of 95% air and 5% CO2. Every four days we proceeded to sub-cultivation of cell lines by using Trypsin-EDTA (PAA*, Carlo Erba Reagents*). Cell lines were tested every two months with MycoAlert ™ Mycoplasma Detection Kit (Lonza LT07-418, *Euroclone*) to check a possible contamination by mycoplasma.

3.2 Pre-miRNAs Transfection

Pre-miRNAs (pre-miRNA miR precursors, *Ambion*) and the corresponding negative control, SCR (Pre-miR miRNA precursor scrambled negative control # 1, *Ambion*) were used to transfect breast cancer cell lines, at a final concentration of 100 nM , with Lipofectamine 2000 (*Invitrogen*), in accordance with the instructions provided by the manufacturer. RNA and proteins were extracted from cell pellets collected at 24-48-72h after transfection, depending on the type of analysis.

3.3 Extraction of RNA and proteins

RNA was isolated with mirVana ™ miRNA Isolation Kit (*Ambion, Invitrogen*), following the protocol provided by the manufacturer. The quantification of extracted RNA was carried out using NanoDrop ND-1000 (*Thermo Fisher Scientific*). Total proteins were extracted, keeping samples on ice, with 1X RIPA lysis buffer (*Santa Cruz Biotechnology*) with the addition of 10 µl of PMSF, 10 µl of sodium orthovanadate and 15 µl of protease inhibitors, per ml of 1X RIPA lysis buffer, as recommended by the datasheet attached to the product. Then proteins were quantified following the protocol of the BCA Protein Assay (*Pierce, ThermoScientific*) and using a Multiscan EX microplate reader (*Thermo Labsystems*), with a wavelength filter of 490nm.

3.4 Protein expression analysis

Western blotting was used as a method of analysis to evaluate the expression of ER-α and AR. Proteins (20-50 µg) were denatured and separated by electrophoresis using a gel Criterion TGX Stain Free Gel Precast 4-20% (*Bio-Rad Laboratories*) and Laemmli Sample Buffer (*Bio-Rad*) with 5% of β-mercaptoethanol (*Carlo Erba Reagents*), in 1: 1 ratio with the sample. Electrophoretic run was performed at a constant voltage of 200V in a TRIS/Glycine/SDS 1X buffer (*Bio-Rad*). Proteins were then transferred on a PVDF membrane (Trans-Blot Transfer Turbo midi-format 0.2µm; *Bio-Rad Laboratories*) using the Trans Blot Turbo System (*Bio-Rad Laboratories*). The membrane was subsequently incubated for 2 hours at room temperature in a solution of Tween 20 (*Bio-Rad*) at 0.1% and 1X Dulbecco's Phosphate Buffered Saline (PBS; *Invitrogen*) supplemented with 5% milk powder (Blotting Grade Blocker Non Fat Dry Milk; *Bio-Rad*) in order to facilitate the saturation of non-specific binding sites. Primary antibodies and dilutions used are the following:

- Estrogen Receptor α (D6R2W *Cell Signaling*) 1:1000
- Androgen Receptor Antibody (#EPR1535(2) *Abcam*) 1:1000
- Vinculin clone FB11 (IgG1) monoclonal Ab (*Biohit*) 1:1000
- Beta Actin [AC-15] Antibody (HRP) (ab49900 *Abcam*) 1:50.000

Secondary antibodies and dilutions used are the following:

- Goat anti-rabbit IgG-HRP (*Santa Cruz*) 1:2000;
- Goat anti-mouse IgG-HRP (*Santa Cruz*) 1:2000;

- Precision Plus Protein Western C StrepTactin-HRP Conjugate (*Bio-Rad*) 1:10000;
- Biotinylated Protein Ladder (*Cell Signaling*) 1:1000

Blocking and immunological reactions were performed in accordance with the protocol Western Immunoblotting of Cell Signaling. The antigen-antibody reaction of chemiluminescence was performed using the SuperSignal West Femto (*Pierce Thermo Scientific*), the image was acquired using the Chemidoc (*Bio-Rad*) and analyzed using ImageJ Software.

3.5 miRNAs and mRNAs expression analysis

The analysis of the expression of miRs, for the evaluation of the efficiency of transfection, was performed using the TaqMan miRNA Assays (*Applied Biosystems, Lifetechnologies*). Briefly, the molecule of complementary DNA (cDNA) was synthesized using 10 ng of RNA as template, a Reverse Transcription gene-specific primer and TaqMan MicroRNA Reverse Transcription Kit (*Applied Biosystems, Lifetechnologies*). The expression of DNMT1, DNMT3A, DNMT3B and ESR1 was evaluated with the use of the TaqMan Gene Expression Assays (*Applied Biosystems, Lifetechnologies*). The cDNA was synthesized using 80ng of RNA as a template and the TaqMan RT PCR Kit (*Applied Biosystems, Lifetechnologies*). Quantitaive Real-Time (qRT-PCR) was performed with Applied Biosystems 7500 Real-Time PCR System (*Lifetechnologies*) using cDNA, TaqMan probes and TaqMan Universal PCR Master Mix (*Applied Biosystems, Lifetechnologies*). Experiments were conducted in triplicate and normalized to RNAU44 or GAPDH, used as internal controls (housekeeping genes). Relative expression levels were calculated using the method of comparative Ct (*ΔΔCt method*).

3.6 siRNA transfection

SMARTpool siGENOME AR siRNA (*Dharmacon*) and the corresponding negative control, siGENOME Non-Targeting siRNA #1 (*Dharmacon*), were used to perform AR silencing transfection, at a final concentration of 50 nM , with Lipofectamine RNAiMAX (*Invitrogen*), in accordance with the instructions provided by the manufacturer. RNA and proteins were extracted from cell pellets collected at 24-48-72-120-144h after transfection, depending on the type of analysis.

3.7 Drugs preparation

Tamoxifen ((Z)-1-(p-Dimethylaminoethoxyphenyl)-1,2-diphenyl-1-butene, trans-2-[4-(1,2-Diphenyl-1-butenyl)phenoxy]-N,N-dimethylethylamine) was purchased from *Sigma-Aldrich* in powder formulation and a working solution was prepared in Dimethyl sulfoxide (DMSO) (*CrioServ Bionichepharma*) to obtain a 100 mM concentration; tamoxifen was then tested at 0,5-1-5-10 μM and DMSO never exceeded 0,1%. Metformin (1,1-Dimethylbiguanide hydrochloride) was purchased from *Sigma-Aldrich* in powder formulation and a 100 mM working solution was prepared in fresh medium; metformin was then tested at concentrations of 5-10-20-40 mM. Herceptin was provided by the Pharmacy of our Institute at a concentration of 20 mg/ml, dissolved in sterile water and and 1 mM solution was used to perform experiments. After 24h post silencer-transfection, cells were trypsinized and seeded in 96-well plates. The day after cells were exposed to treatments at the different concentrations described above. The effect of Tamoxifen and Metformin was investigated at 24-48 and 72h, while that of Herceptin at 120-144 h.

3.8 CellTiter-Glo® Luminescent Cell Viability Assay

The CellTiter-Glo® Luminescent Cell Viability Assay (*Promega*) is a homogeneous method used to determine the number of viable cells in a culture, and it is based on the quantification of present ATP that indicates the existence of metabolically active cells. The procedure, according to the instructions provided by the datasheet, involves the adding of a single reagent (CellTiter-Glo ® Reagent) directly to cultured cells. This system "*add-mix-measure*" produces a cell lysis and the generation of a luminescent signal proportional to the ATP content, which, in turn, is directly proportional to the number of cells that are in culture.

3.9 Bromodeoxyuridine assay

After cells were seeded in 6-well plates and treated with Herceptin, they were incubated with a 60 μM Bromodeoxyuridine (BrdU) solution in 1 ml of medium. Cells were then centrifuged and fixed with 70 % of cold ethanol. The day after cells were washed in PBS 1X and incubated with first HCL 2M, then sodium tetraborate and Tween 20 0.5% + BSA 1%. Finally cells were incubated for 1 hour first with anti-BrdU antibody following a 1:50 diluition, secondly with a FITC-conjugated secondary

antibody diluted 1: 250 for 1 hour. After antibodies incubation, cells were washed with PBS 1X, stained with propidium iodide solution and incubated overnight at 4°C. The day after samples were analyzed by flow cytometry.

3.1 Data analysis

Data obtained from qRT-PCR were analyzed by Life TechnologiesTM 7500 Software v2.0.6 for 7500 Fast Real Time PCR System. Relative expression levels were calculated using the method of comparative Ct (ΔΔCt). The images of the Western Blot, acquired through Chemidoc, were analyzed using ImageJ Software. For viability assays in vitro survival curves were constructed using Excel software. The *Net Growth* was calculated as the ratio (OD treated - OD T_0 / OD untreated control - OD T_0) x 100. Viability experiments were conducted in octuplicate.

4. Results

4.1 Effect of miR-29A, -29B, -29C, -148A, -148B and -152 on the expression of DNMTs and ESR1

The silencing effect exerted on the DNMTs and ESR1 mRNAs by the miRs was assessed by qRT-PCR analysis performed on cDNA transcribed from RNA extracted from MDA-MB-231 cells transfected with miR-29A, miR-29B, miR-29C, miR-148A, miR-148B, and miR-152, or SCR, 72h post transfection. The analysis showed an effect of miR-29B and mir-148A on reducing the DNMT1 and DNMT3A expression (Fig. 11), and concomitantly slightly increasing the expression of ESR1, compared to SCR (Fig.12). No significant variations were observed for the other miRs on the expression of DNMTs and ESR1. 5-Azacitidine was used as an internal control, being a DNA demetylating agent able to inhibit the DNMTs.

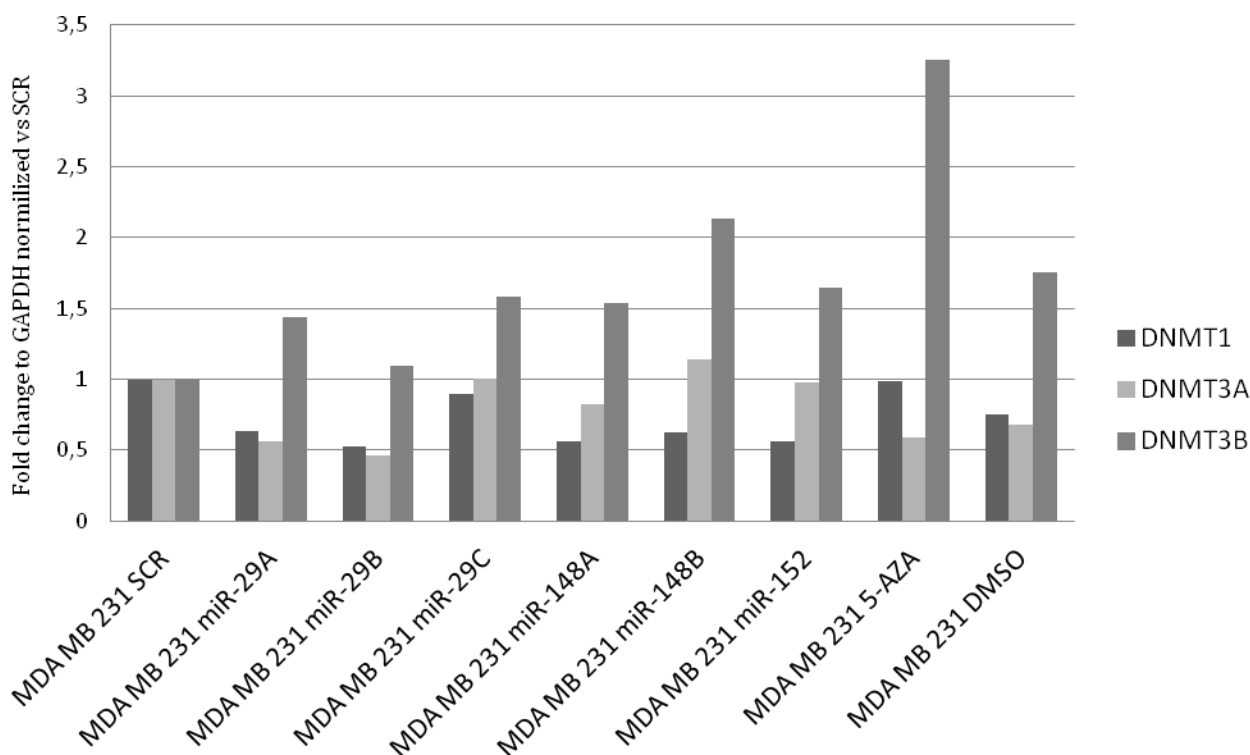


Fig. 11 DNMTs expression after transfection with miR 29A, 29B, 29C, 148A, 148B, 152 at 72h

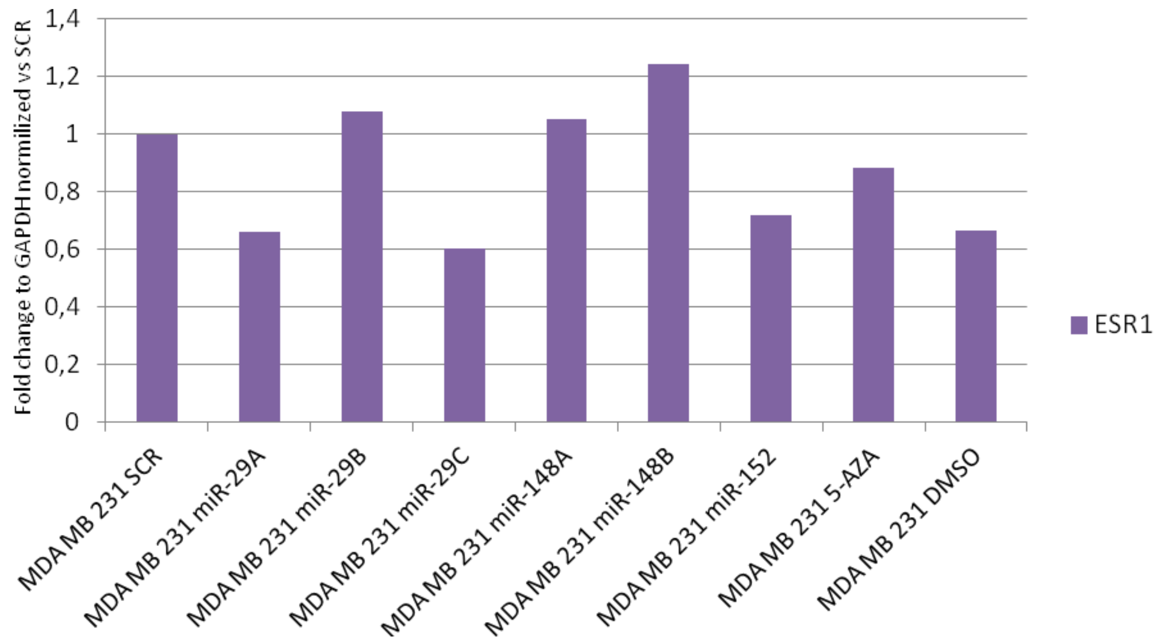


Fig. 12 ESR1 expression after transfection with miR 29A, 29B, 29C, 148A, 148B, 152 at 72h

4.2 Lack of ER-α re-expression after miR-29B, miR-148A and miR-191 transfection

A possible re-expression of estrogen receptor protein following the observed reduction of the expression of DNMT1 and DNMT3A mRNAs was evaluated by Western Blot analysis of protein extracts from MDA-MB 231 cells transfected with miR-29B, miR-148A, and miR-191, which was predicted to target DNMT3A, at 48h and 96h post transfection. No expression of estrogen receptor was observed, even in the samples treated with 2,5 µM 5-Azacitidine (Fig. 13).

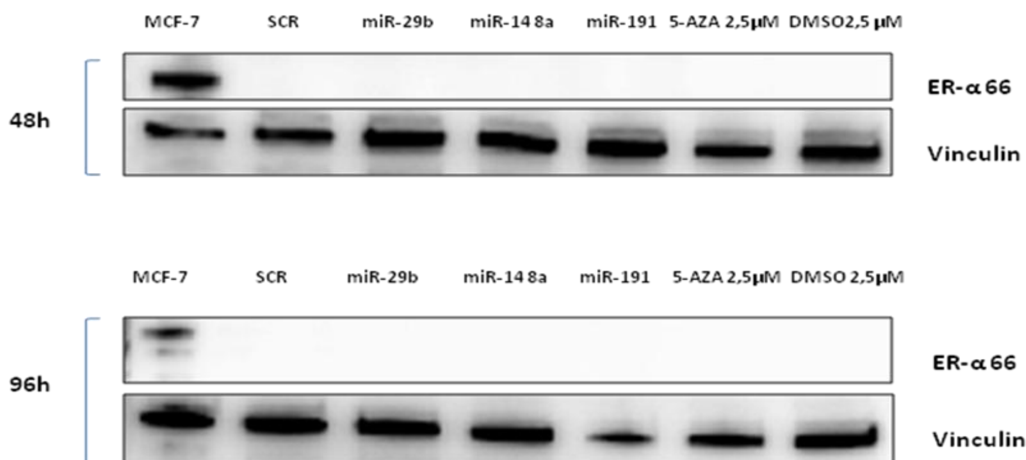


Fig. 13 Western blot after transfection of MDA-MB 231 with miR-29B, miR-148A and miR-191

4.3 TNBC cell lines AR/Her2 expression profiling

Not observing any results in the re-expression of the estrogen receptor, we then focused on the unclear role of AR. To test the expression of AR on several triple negative breast cancer cell lines a Western Blot was performed, using the prostate cancer cell line LNCap as positive control. AR expression was detected in the "molecular apocrine" subtype MDA-MB-453, and in the T-47D cell line (Fig.14-15). T-47D together with MDA-MB-453 were also tested for the expression of Her2 (Fig. 16).

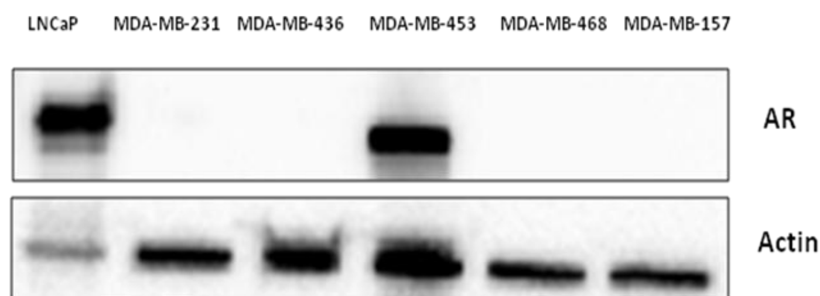


Fig. 14 Expression of AR on different TNBC cell lines by Western Blot

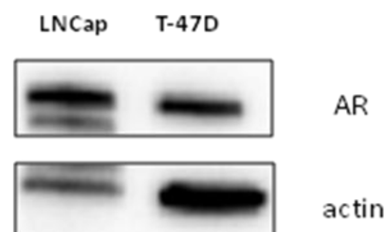


Fig. 15 Expression of AR in T-47D breast cancer cell line

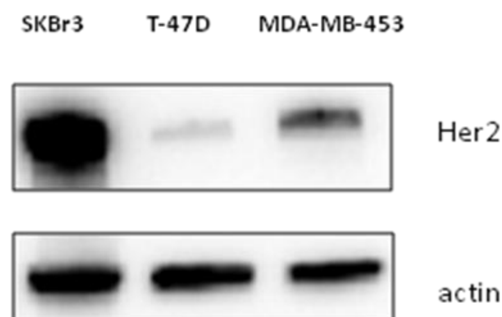


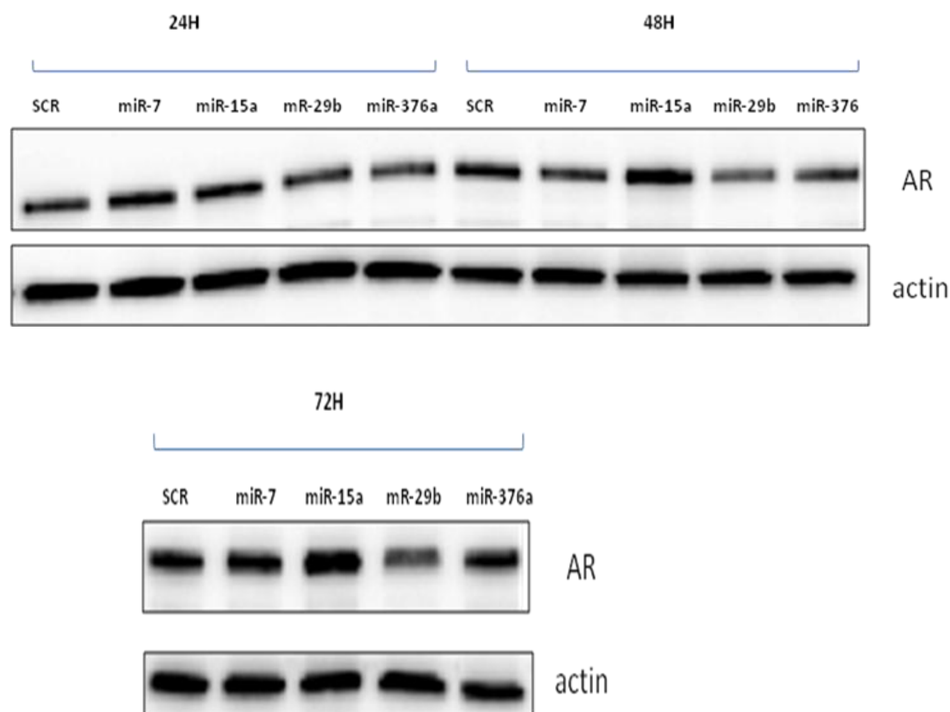
Fig. 16 Expression of Her2 in T-47D and MDA-MB-453 breast cancer cell lines compared to SKBr3 control

4.4 miRs which are predicted to target AR

After reviewing various databases for predictive binding site of microRNAs and target genes such as TargetScan (http://www.targetscan.org/), miRBase (http:// www.mirbase.org/), DIANA LAB (http:// diana.cslab.ece.ntua.gr/) and microRNA.org (http:// www.microrna.org/), we finalized the search to the choice of some miRs which showed stronger affinity for the 3'-UTR sequence of AR: miRs-7, -9, -15a, -16, -27a, -27b, -29a, -29b, -29c, -127-3p, -127-5p e -376.

4.5 AR modulation by miRs

A possible AR modulation worked by miRs-7, -9, -15a, -16, -27a, -27b, -29a, -29b, -29c, -127-3p, -127-5p and -376a, was evaluated by Western Blot analysis, performed on protein extracts from MDA-MB-453 cells transfected with the miRs mentioned above, or SCR at 24, 48 and 72h post transfection. Results showed a general tendency of all miRs tested, except miR-15a and miR-16, to down-regulate AR expression at 48h post transfection. Conversely, miR-15a and miR-16 showed an up-regulatory effect which remained constant for all the three analyzed times. The same costant effect, but in a down regulatory way, was instead explicated by miR-9 (Fig. 17-18-19).



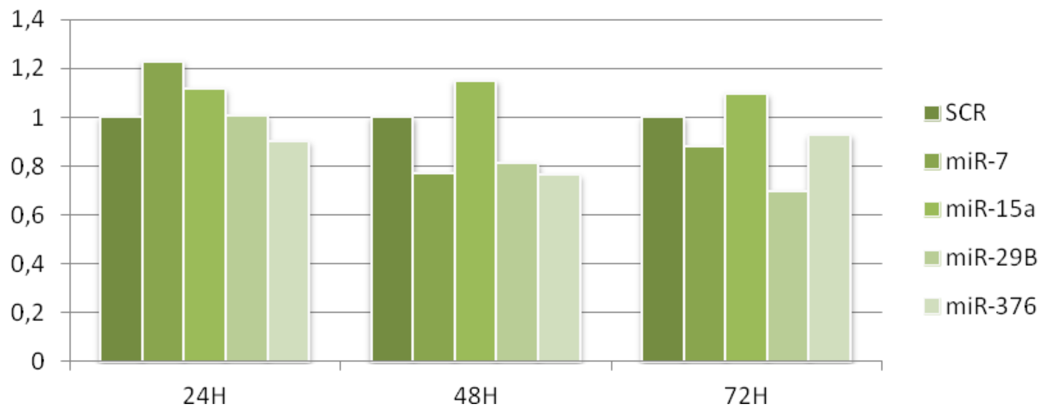
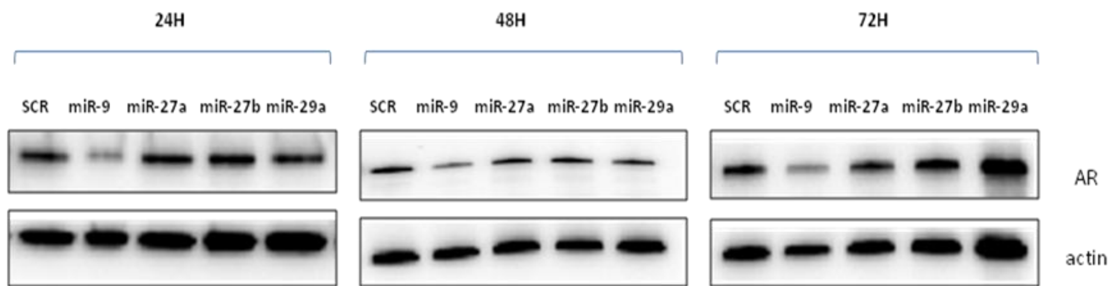


Fig. 17 Expression analysis of AR modulated by miRs-7, -15a, -29b, -376 on MDA-MB-453 breast cancer cell line



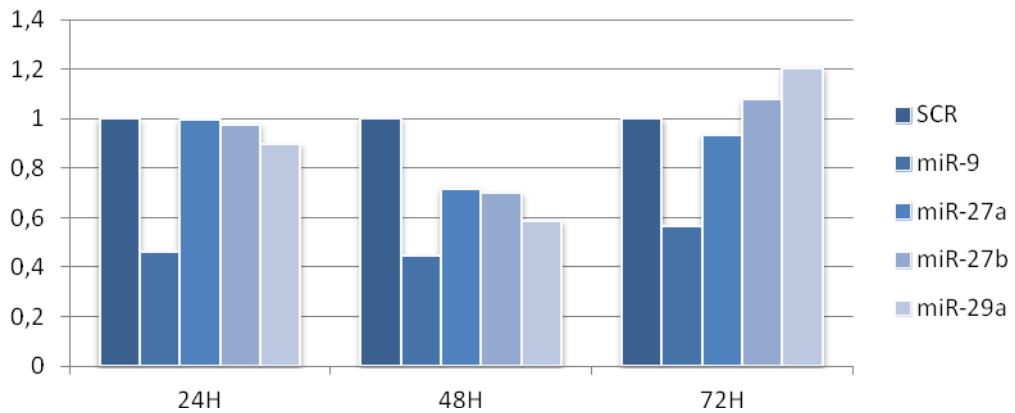


Fig. 18 Expression analysis of AR modulated by miRs-9, -27a, -27b, -29a on MDA-MB-453 breast cancer cell line

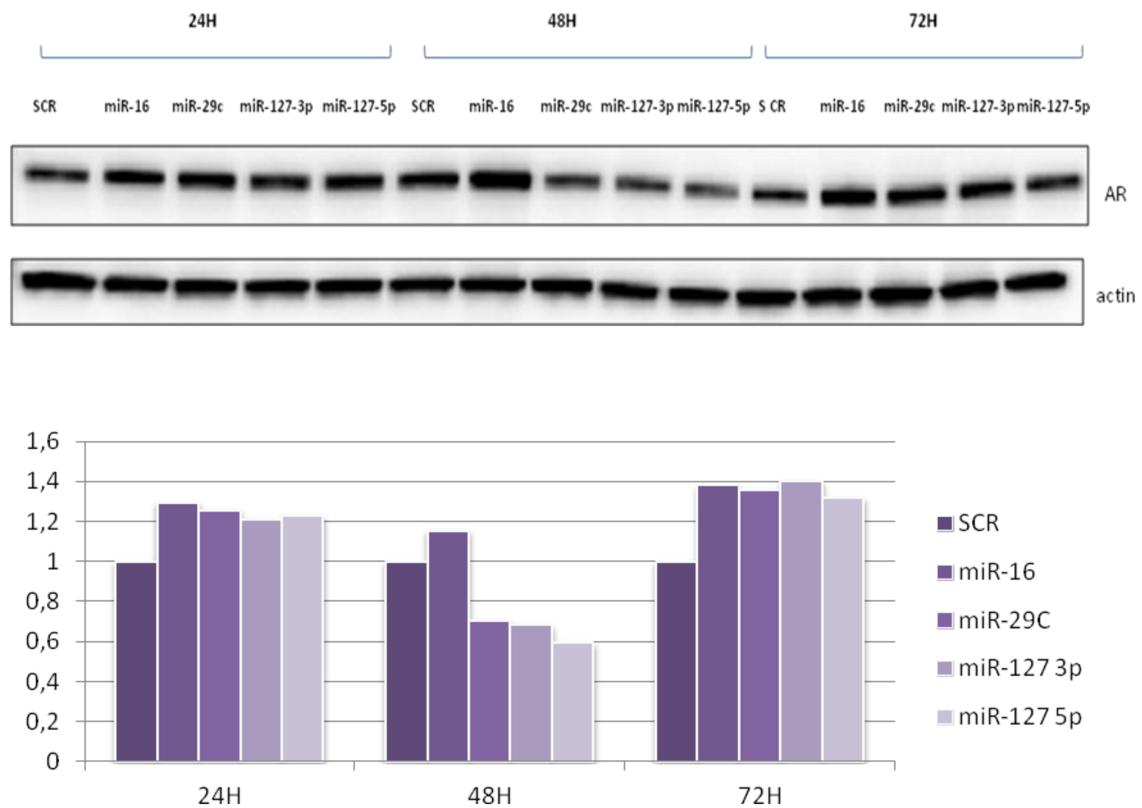


Fig. 19 Expression analysis of AR modulated by miRs-16, -29c, -127-3p, -127-5p on MDA-MB-453 breast cancer cell line

4.6 Silencing of AR is maintained up to 72 h post-transfection

A silencing kinetic study was performed in order to subsequently investigate the mechanism of some drugs. MDA-MB-453 were transfected with siRNAs at two different concentrations, 20nM and 50 nM, for 24, 48 and 72 hours. siRNA was effective in silencing AR from as early as 24h after transfection, and its effect is maintained up to 72h post-transfection (Fig. 20).

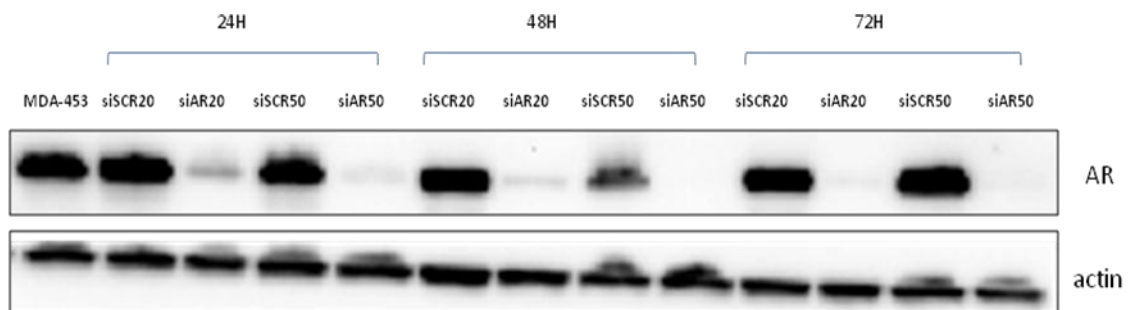


Fig. 20 AR silencing kinetic on MDA-MB-453 cell line

The maximum concentration of siRNA, 50 nM, was chosen and used to verify the effective silencing also on T-47D cell line (Fig. 21).

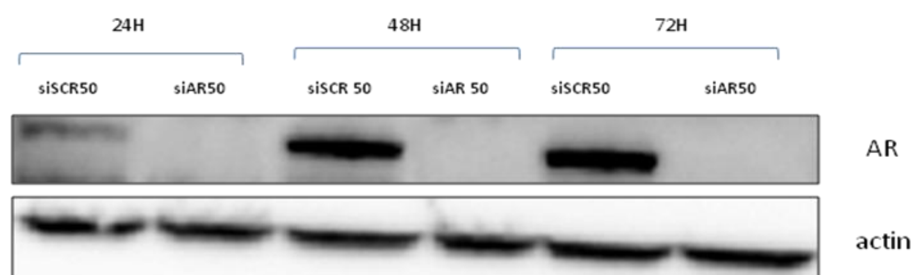


Fig. 21 AR silencing on T-47D cell line

4.7 Response to Tamoxifen, Metformin and Herceptin treatment

To evaluate changes in some drug sensitivity after AR silencing both MDA-MB 453 and T-47D cell lines were trated with Tamoxifen, or Metformin, or Herceptin, at different concentration in agreement with their respective pharmacokinetics, for 24-48-72-120-144h. About the treatment with Herceptin, T-47D cell line was chosen in function of its molecular profile (AR positive, Her2 negative) as an opposite model to MDA-MB 453 (AR positive, Her2 positive) and its effect was tested by a Bromodeoxyuridine flow cytometric assay. For Tamoxifen and Metformin a cell viability assay based on the ATP activity measurement was performed. No significant differences in Tamoxifen response in both cell lines were observed, except for treatment of MDA-MB-453 at 24h, where samples seemed more sensitive when silenced for AR protein (Fig. 22, 24). Metformin appeared to act more in samples silenced for AR compared to SCR, in both cell lines, , except in MDA-MB-453 at 48h (Fig. 26, 28). The silencing efficiency was confirmed by Western Blot analysis for each experiment (Fig. 23, 25, 27, 29).

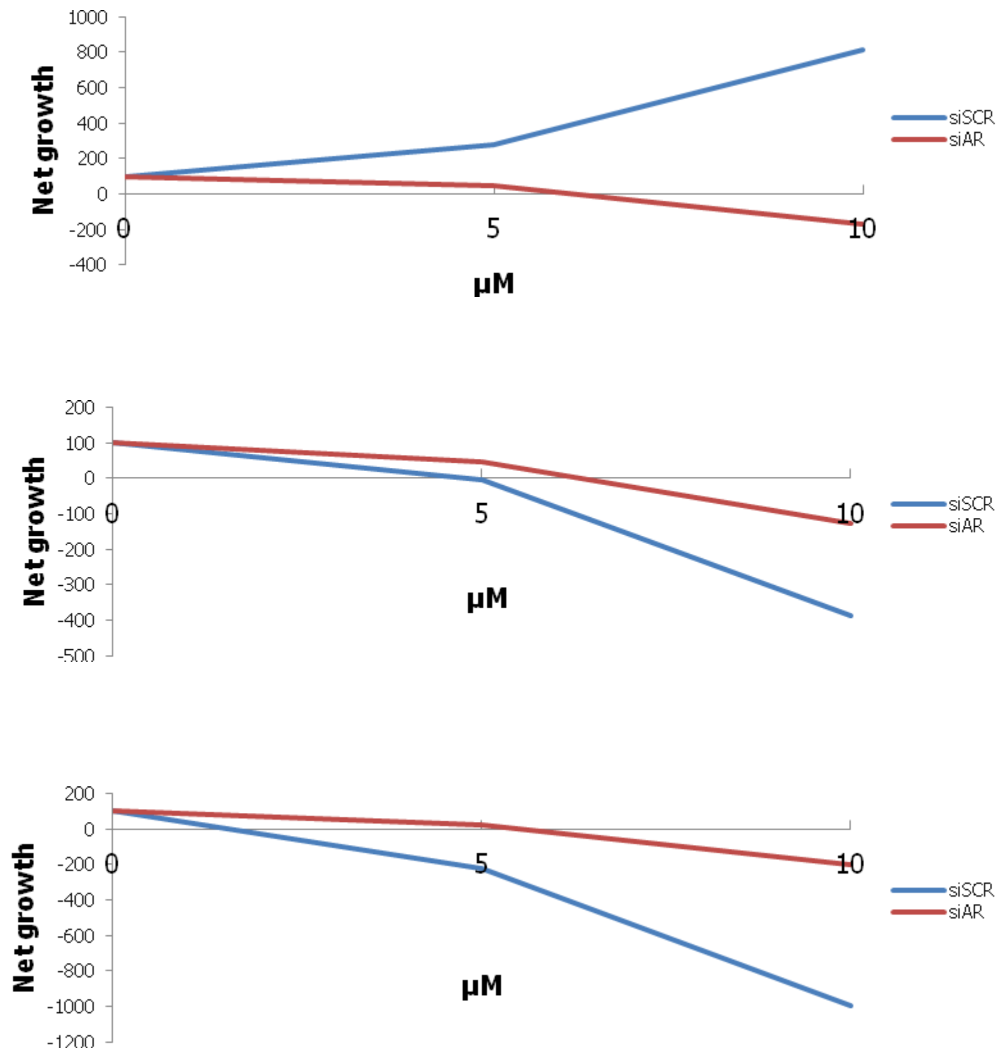


Fig. 22 Net growth analysis after Tamoxifen treatment 5-10 µM at 24-48-72h on MDA-MB-453 cells

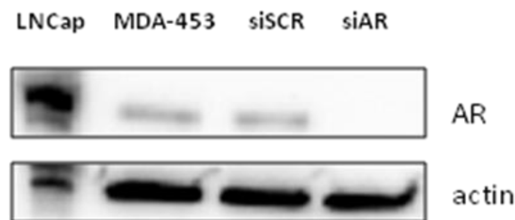


Fig. 23 Efficiency of silencing transfection on MDA-MB-453 cells

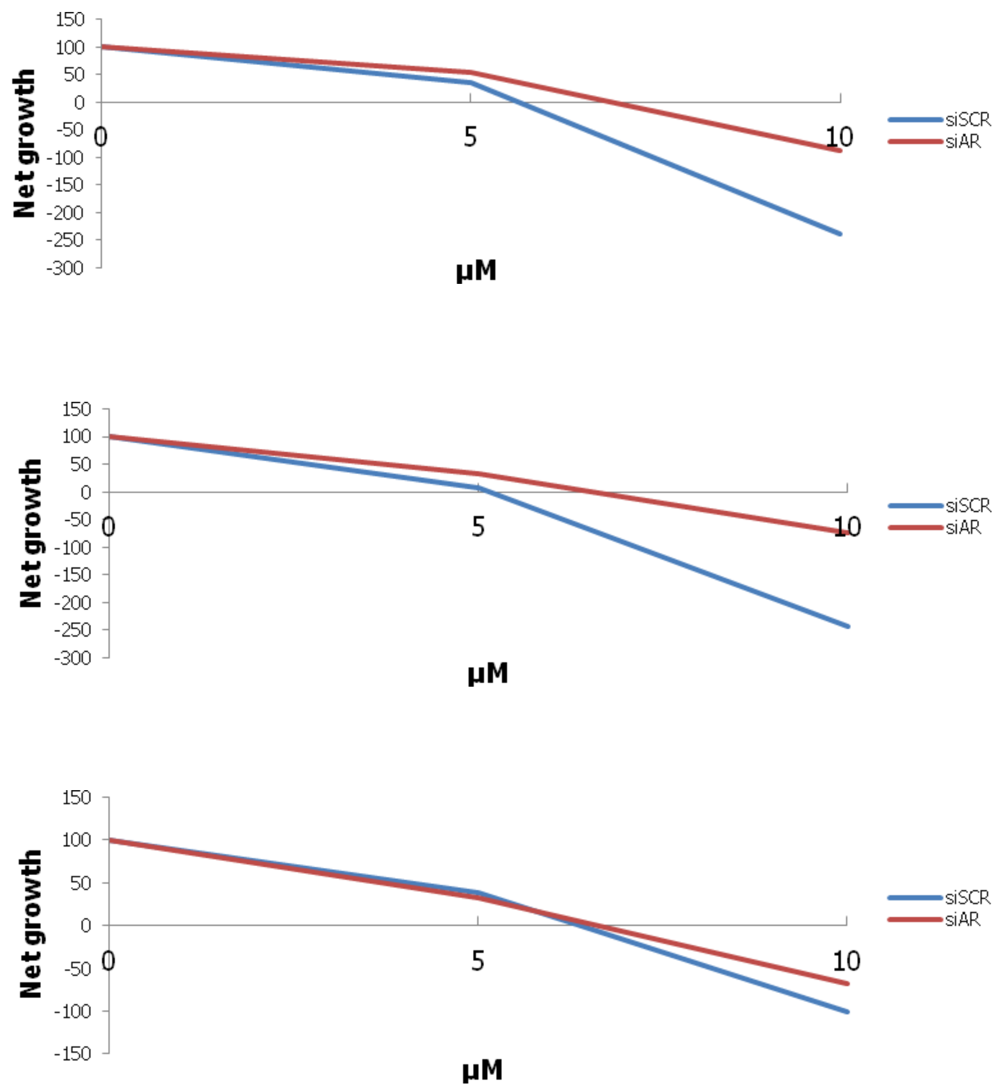


Fig. 24 Net growth analysis after Tamoxifen treatment 5-10 µM at 24-48-72h on T-47D cells

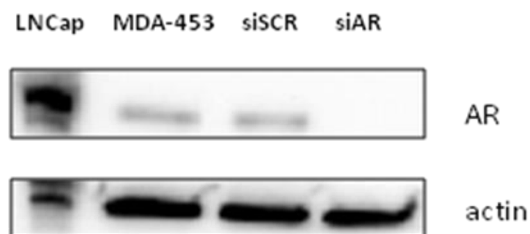


Fig. 25 Efficiency of silencing transfection on T-47D cells

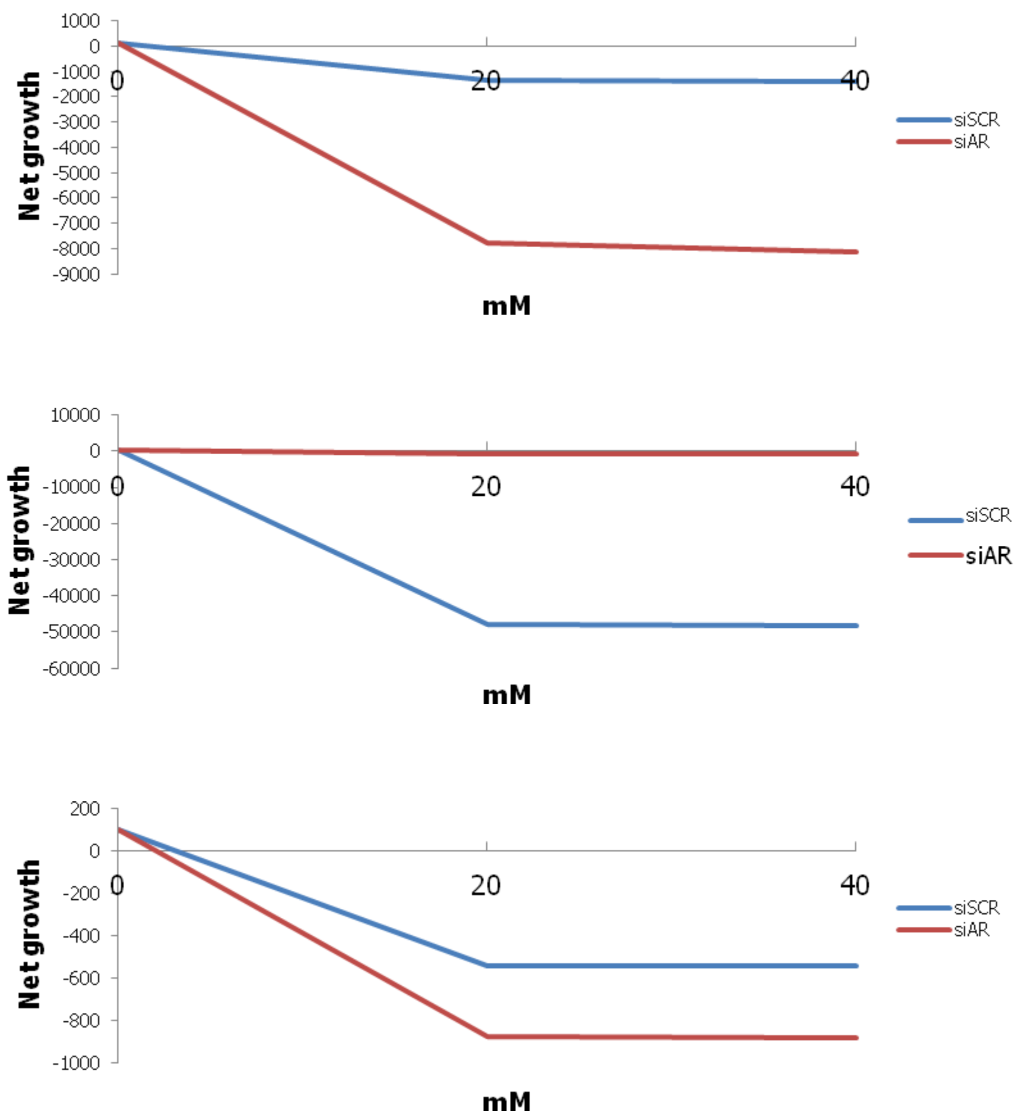


Fig. 26 Net growth analysis after Metformin treatment 20-40 mM at 24-48-72h on MDA-MB-453 cells

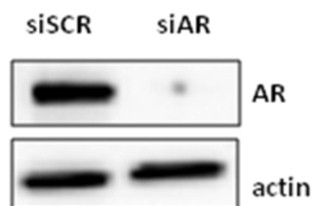


Fig. 27 Efficiency of silencing transfection on MDA-MB-453 cells

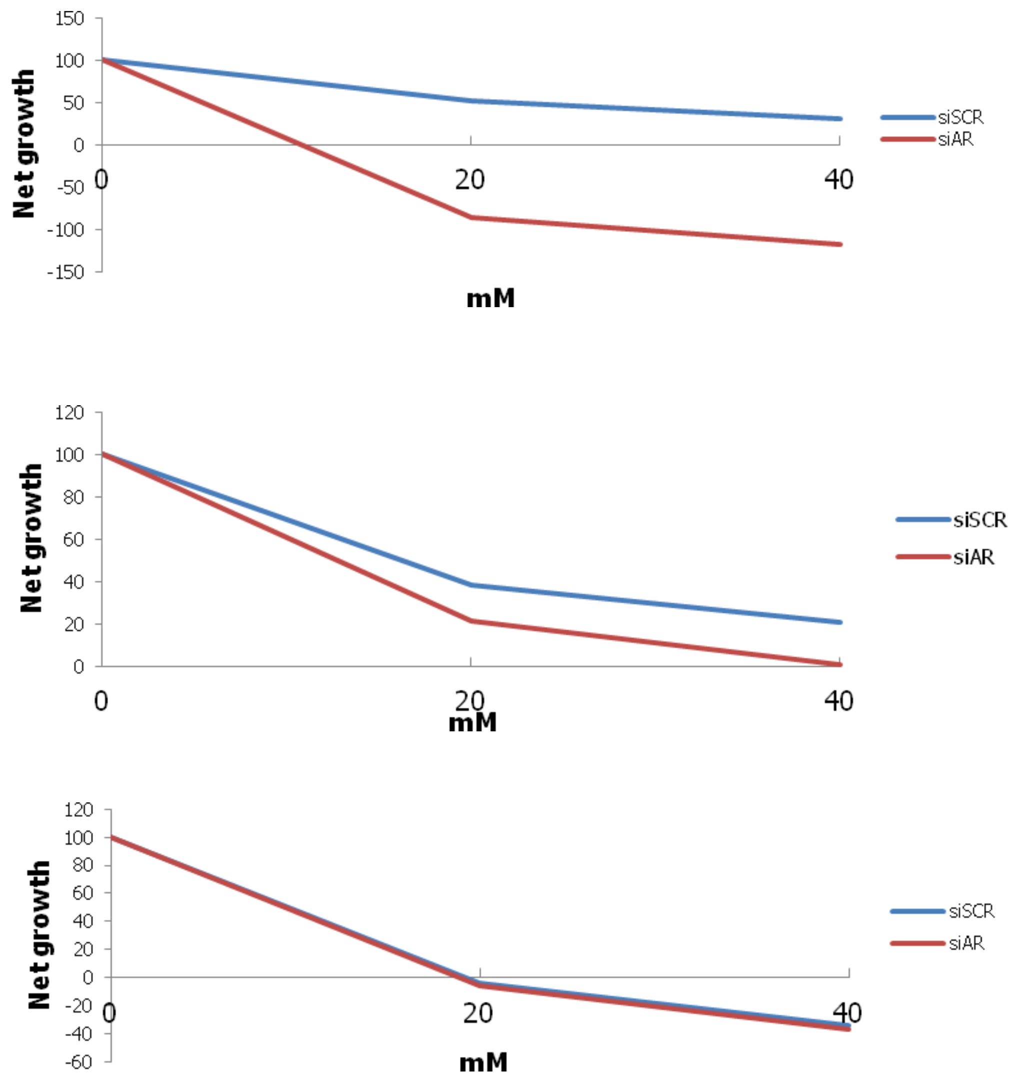


Fig. 28 Net growth analysis after Metformin treatment 20-40 mM at 24-48-72h on T-47D cells

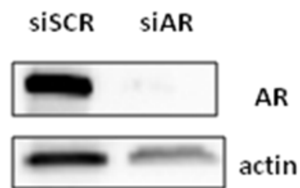


Fig. 29 Efficiency of silencing transfection on T-47D cells

Herceptin treatment in MDA-MB 453 cells silenced for AR showed a slight decrease in the percentage of proliferating cells, suggesting a response to the drug (Fig. 30, 31). No significant changes were observed in T-47D cells (Fig. 33, 34). The silencing efficiency was confirmed by Western Blot analysis for each experiment (32, 35).

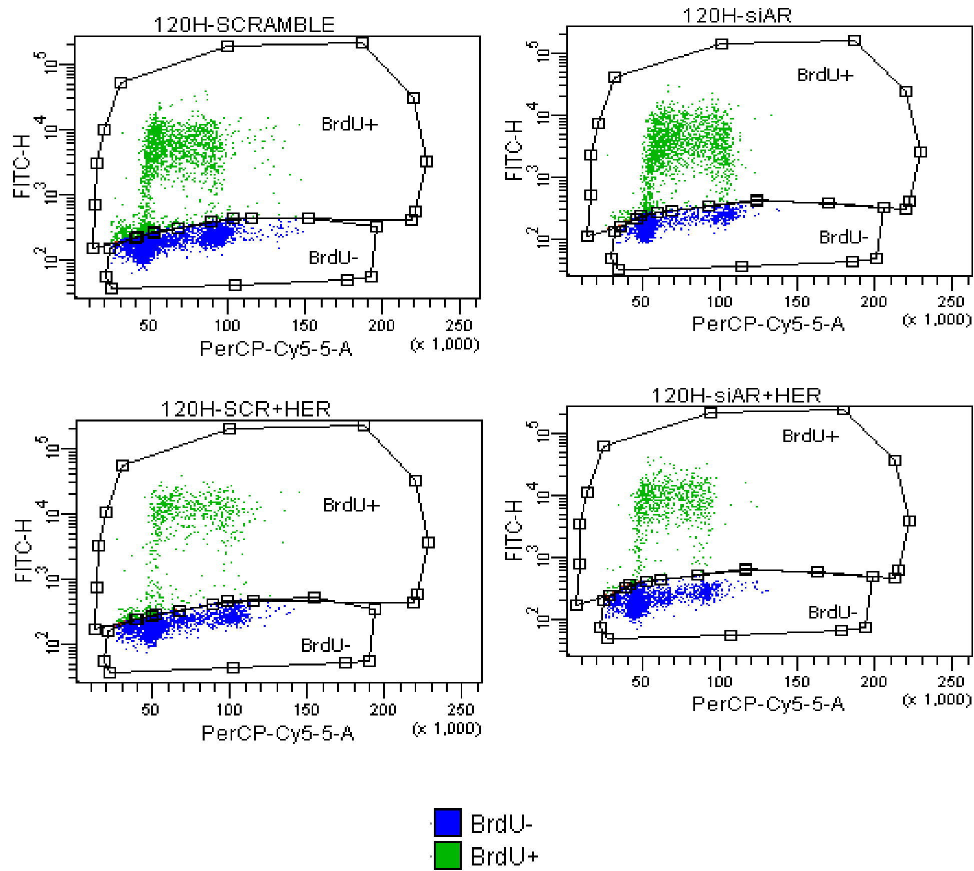


Fig. 30 Flow cytometric analysis of MDA-MB 453 cells after treatment with Herceptin 1 mM for 120h

	Scr	Scr+Her	siAR	siAR+Her
% BrdU-	82.0	85.4	71.8	83.9
% BrdU+	18.7	14.4	27.9	15.8

Tab. 1 Percentage of incorporating BrdU and non-proliferating MDA-MB-453 cells at 120 hours

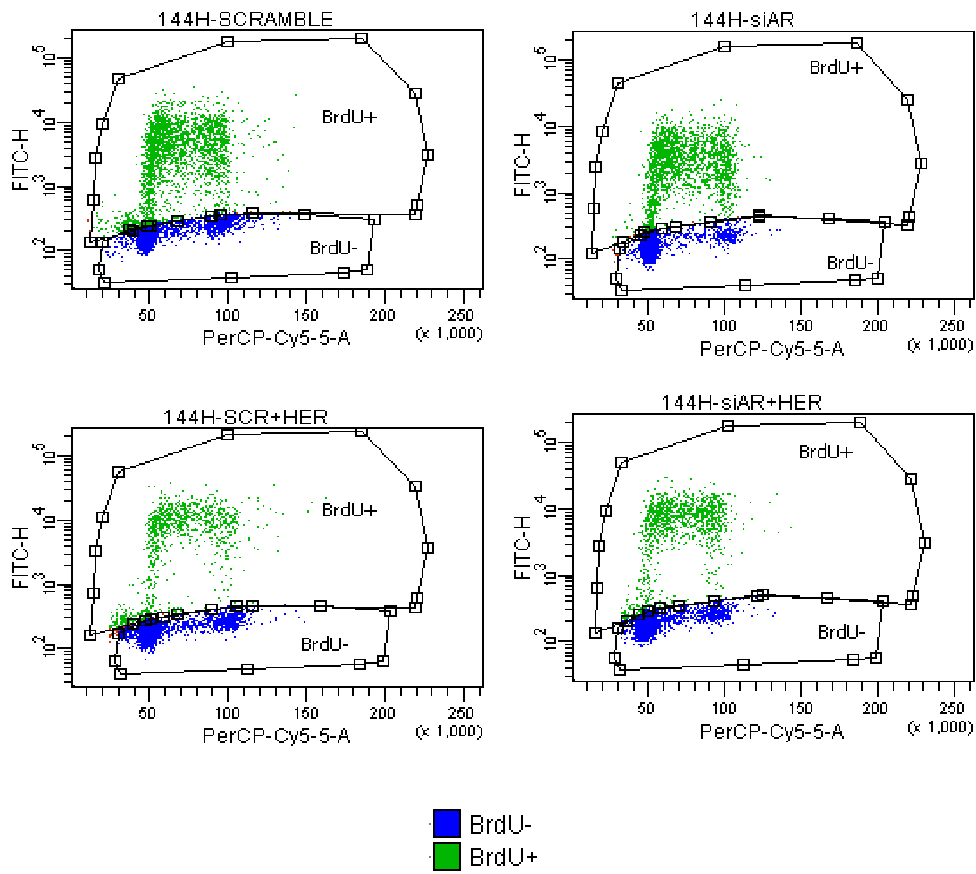


Fig. 31 Flow cytometric analysis of MDA-MB 453 cells after treatment with Herceptin 1 mM for 144h

	Scr	Scr+Her	siAR	siAR+Her
% BrdU-	74.2	80.4	69.7	81.2
% BrdU+	26.4	18.2	30.1	18.9

Tab. 2 Percentage of incorporating BrdU and non-proliferating MDA-MB-453 cells at 144 hours

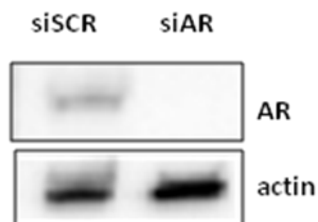


Fig. 32 Efficiency of silencing transfection on MDA-MB-453 cells

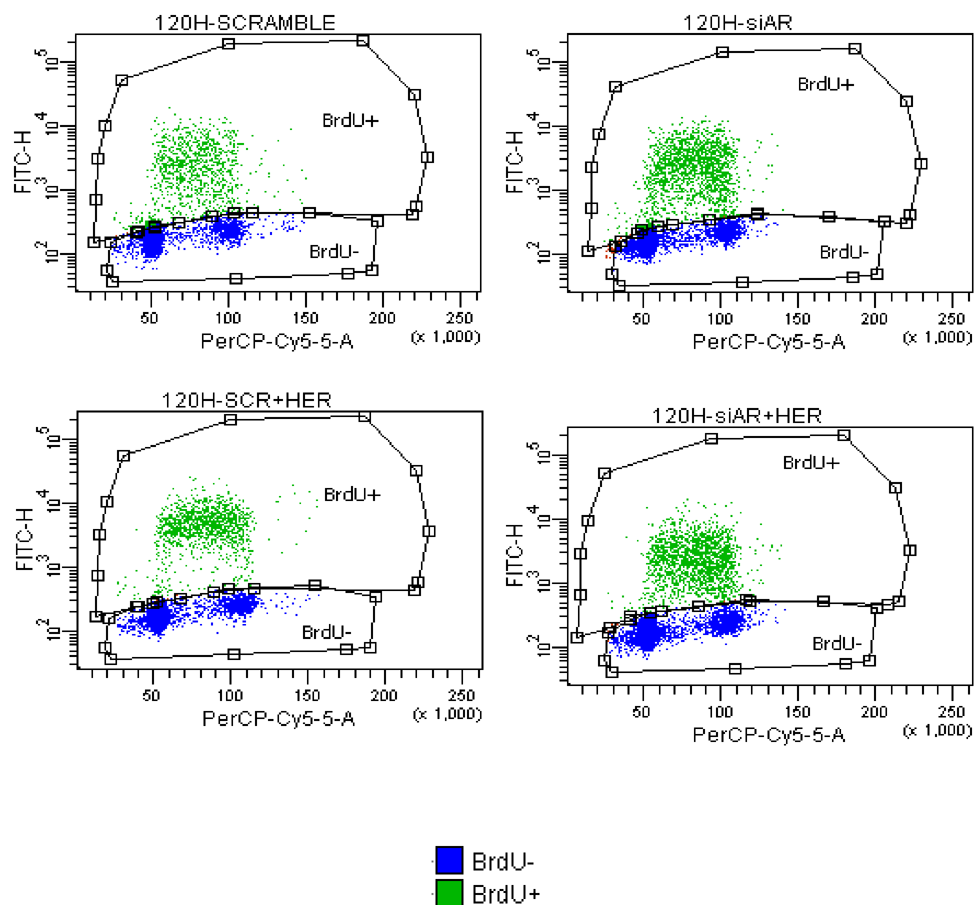


Fig. 33 Flow cytometric analysis of T-47D cells after treatment with Herceptin 1 mM for 120h

	Scr	Scr+Her	siAR	siAR+Her
% BrdU-	83.4	83.9	80.4	80.5
% BrdU+	16.9	16.0	19.2	19.3

Tab.3 Percentage of incorporating BrdU and non-proliferating T-47D cells at 120 hours

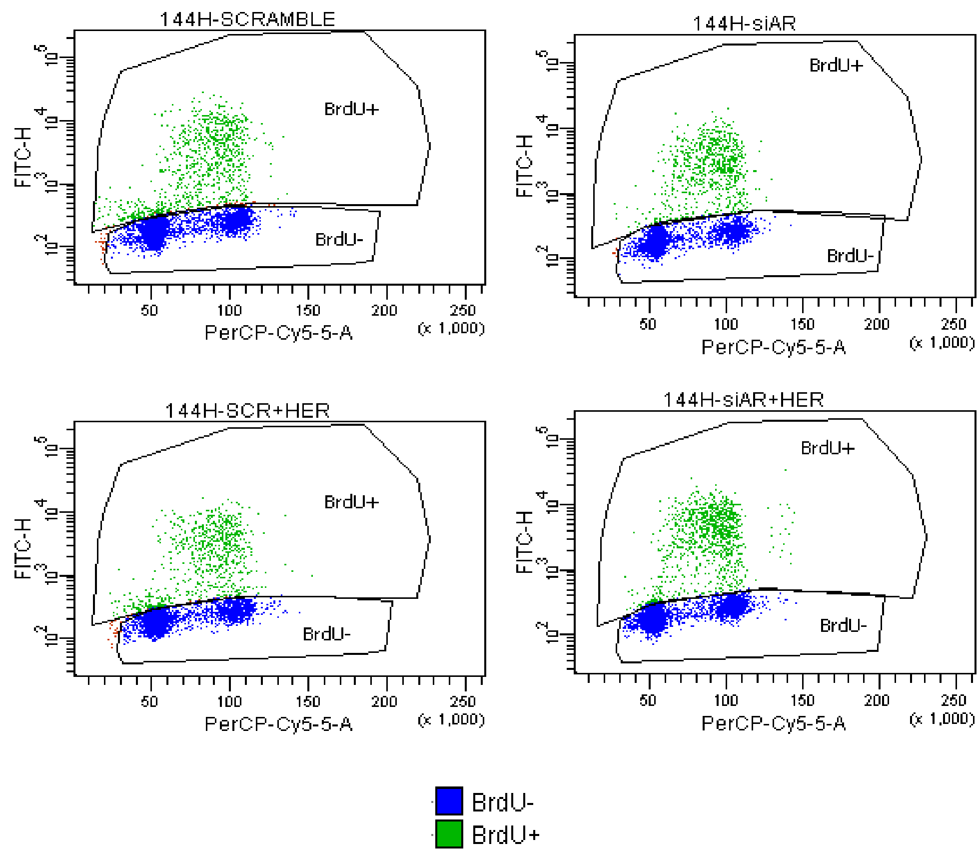


Fig. 34 Flow cytometric analysis of T-47D cells after treatment with Herceptin 1 mM for 144h

	Scr	Scr+Her	siAR	siAR+Her
% BrdU-	86.5	88.0	86.0	87.6
% BrdU+	12.5	11.3	13.6	12.6

Tab. 4 Percentage of incorporating BrdU and non-proliferating T-47D cells at 144 hours

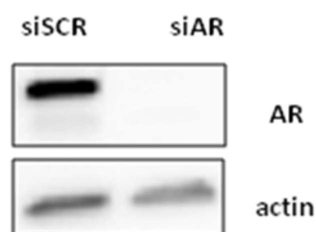


Fig. 35 Efficiency of silencing transfection on T-47D cells

5. Discussion

Breast cancer is a highly heterogeneous disease, characterized by differences both in biological aspects, both in the response to the various existing therapies and, although in recent decades progresses in hormone therapy have been made, effective therapeutic options against TNBCs are still limited. They represent a very aggressive tumor subtype, characterized by a reduced overall survival and a diminished risk of relapses (131). In particular, the lack of expression of ESR1 is due in the most of cases to hypermethylation in CpG islands of the promoter resulting in gene silencing, and an altered methylation status of genes is a consequence of the increased expression of DNMTs. Furthermore, in breast cancers negative for the expression of ER-α, AR is expressed in 10-43% and some studies suggested an increased associated mortality, other studies showed that AR can drive tumor progression and might represent a therapeutic target for this subtype of breast cancer (62, 64). MiRNAs are non-coding RNA that carry out regulation of genes by controlling translation and expression of their target mRNAs, and they are involved in a variety of biological processes (80, 81, 82, 83). A strong correlation between epigenetic and microRNA was observed, particularly miRs 29A, 29B and 29C showed complementary sequence with 3'-UTR region of DNMTs 3A and 3B, restoring the normal patterns of DNA methylation and inducing the re-expression of the hypermethylated gene (123). The identification of tumor-specific miRNAs and their molecular targets represent a key step to characterize the role of miRNAs in human tumorigenesis and might be important for the identification of new therapeutic targets. The understanding of molecular mechanisms associated with genes deregulation by miRNAs in human cancer, still remains a difficult task to investigate. In fact, although bioinformatics programs can help in the prediction of hypothetical target genes, *in vitro* experimental procedures are needed for the validation of molecular targets. Currently many target genes of miRNAs have been identified in human cancers, but despite encouraging experimental evidences linking miRNAs to cancer biology, little is known about cellular and molecular connections in which miRNAs are involved. In fact, the understanding is greatly complicated by the ability of miRNAs to target multiple molecular pathways, sometimes linked to each other, sometimes with opposite functions. Furthermore, miRs can behave as onco-miRNAs or miRNA-tumor

suppressors depending on tissue types and specific targets, representing two different sides of the same gene (95). All this aspects give rise to a complex regulatory network where biological effects and features of a single miRNA not always allow a linear explanation. Recently, the potential role of miRNAs as molecules able to restore drugs sensitivity when used in combination with pharmaceutical agents has become an exciting field to be investigated. One of the advantages of using miRs as therapeutic agents is represented by the fact that they are molecules physiologically present in the cells and so associated with a hypothetical lower risk of toxicity. Furthermore, they are able to induce a phenotype at low concentrations since they silence multiple genes simultaneously limiting their amount used to obtain a biological effect. Both these features make miRNAs excellent "drugs", as demonstrated by their introduction in experimental phase II clinical trials for the treatment of viral hepatitis type C (132). Data presented in this thesis had the initial aim to investigate the possible role of DNMTs silencing operated by the miRs-29 family, and other selected miRs to confer again a normal methylation pattern of ESR1, leading to a re-expression of it in TNBCs and thereby restoring a supposed sensitivity to Tamoxifen. For this purpose a series of experiments were conducted to analyze the possible role of miRs-29 family, identified as first possible candidates and predicted in targeting DNMTs, and of miR-148A, miR-148B, miR-152 and miR-191. The selected model for the *in vitro* studies was the TNBC cell line, MDA-MB-231. At 72h post transfection, miRs-148A and miR-29B were found to be involved in the reduction of the expression of DNMT1 and DNMT3A and in a concomitantly slight increase of ESR1 expression compared to the negative control. MiR-148B was seen to decrease the expression of DNMT1 correlated to an increase in the expression of ESR1, compared to negative control. These data were not confirmed at protein level, as no protein expression of estrogen receptor was observed after transfection of cells with miRNAs precursors. Consequently the results obtained and on the basis of data in the literature, our focus has shifted on Androgen Receptor with the purpose to assess the effect of some predicted targeting miRs (miRs-7, -9, -15A, -16, -27A, -27B, -29A, -29B, -29C, -127-3p, -127-5p and -376) on its expression. Transfection of cells with precursors of selected miRNAs has led to down-regulation of Androgen Receptor at protein level at 48h post transfection by all miRs considered, except by miR-15A and miR-16 which were observed up-regulating the receptor at every time considered.

Finally, assays were conducted to study Tamoxifen, Herceptin and Metformin in order to estimate a possible increase of drugs sensitivity after AR silencing in MDA-MB 453 and T-47D cell lines. In Tamoxifen treatments, cells seemed more sensitive when silenced for AR protein only in MDA-MB-453 at 24h post-treatment, might be due to activation of molecular pathways in the absence of expression of estrogen receptor. Studies on Metformin have confirmed our hypothesis of an increase of drug sensitivity due to AR silencing in both cell lines. Analysis of Herceptin assays showed how MDA-MB 453 samples silenced for AR have a slight decrease in the percentage of proliferating cells, demonstrating a possible increase in the response to treatment. These preliminary data provide the basis for further study of the modulation of the expression of AR by microRNAs, and how this mechanism can have an impact on cancer treatment. Not less important, it will be interesting to understand the molecular mechanisms underlying these interactions. Our results, although preliminary, might provide further evidence for a possible future use in clinical field of these small molecules.

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