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TITOLO TESI

"Novel insight into the protective role of androgen receptor in ER-positive breast cancer"

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ABSTRACT

Introduction: Androgen receptor (AR) role in breast cancer appears to be clinically relevant and disease context specific. In estrogen receptor (ER) α-positive primary breast cancers, AR positivity correlates with low tumor grade and a better clinical outcome. These clinical-pathological findings mirror androgen capability to counteract ERα-dependent proliferation in both normal and tumor mammary epithelium.

Tumor microenvironment is a key factor in cancer development and progression since the physical and hormonal paracrine exchanges with the epithelial compartment promote tumor cell proliferation and metastasis.

This research project reports additional molecular mechanisms in the dynamic interplay between AR and ER-α signaling pathway in breast cancer cells and showed AR expression and role in the stroma of ER-positive breast cancers.

Material and methods: MCF-7, T47D, SKBR3 breast cancer cells and cancer-associated fibroblats (CAFs) from biopsies of primary breast tumors (n=3); cell proliferation assay, transient transfection, quantitative Real Time PCR (qRT-PCR), western blotting (WB), immunoprecipitation assay (IP), chromatin immunoprecipitation assay (ChIP), RNA silencing, tunel assay, DNA affinity precipitation assay (DAPA), immunofluorescence analysis (IF), immunocytochemical staining (IHC), wound-healing scratch assay, F-actin staining assay, Boyden-chamber transmigration assay, matrigel-based invasion assay, cytokine array, zymography assay, mammosphere forming efficiency assay. Data were analyzed by ANOVA.

Results: This study demonstrated an androgen-dependent mechanism through which ligand-activated AR decreased estradiol-induced cyclin D1 protein, mRNA and gene promoter activity in MCF-7 cells. This mechanism involved the competition of AR and ERα for the steroid receptor coactivator AIB1, a limiting factor in the functional coupling of ERα with the cyclin D1 promoter. Indeed, AIB1 overexpression was able to reverse the down-regulatory effects exerted by AR. Co-immunoprecipitation studies showed that AIB1 preferentially interacted with ERα or AR in relation to their intracellular levels. In addition, ChIP analysis evidenced that androgen administration decreased E2-induced recruitment of AIB1 at the AP-1 site on the cyclin D1 promoter gene.

Moreover, this research project showed an increased expression of the pro-apoptotic protein BAD following androgen treatment while the levels of anti-apoptotic protein Bcl-2 as well as of the pro-apoptotic proteins BID and BAX remained unchanged. As consequence, the Bcl-2/BAD ratio was reduced, shifting the delicate balance between inhibitors and inducers of cell

death. Androgen stimulation increased also BAD levels into the nuclear compartment in ERα/AR-positive MCF-7 as well as in ERα negative/AR-positive SKBR3 cells. The androgen-regulated intracellular localization of BAD involved an AR/BAD physical interaction, suggesting a nuclear role for BAD upon androgen stimulation. Indeed, androgens induced both AR and BAD recruitment at the AP-1 and the ARE sites within the cyclin D1 promoter region, contributing to explain the anti-proliferative effect of androgens in breast cancer cells.

Finally, the study demonstrated AR expression and functionally activation upon androgen treatment in primary human breast CAFs. Androgen-activated AR affected CAFs secretory phenotype as evidenced by cytokine array performed on CAFs conditioned medium (CM). Co-culture experiments showed that CM from androgen-treated CAFs was less effective in stimulating MCF-7 and T47D cells motility and invasion compared to CM from untreated ones, indicating that androgens, via AR, may influence CAFs secretion of paracrine soluble factors involved in tumor cell motility and invasiveness sustainment.

Conclusions: Taken together all these data showed that ligand activated AR exerts a protective role in E2-dependent breast cancer development and progression by inhibiting CD1 expression through the squelching of the steroid receptor co-activator AIB1 and the overexpression of the pro-apoptotic protein BAD. Furthermore, the study highlighted the protective role of AR in the tumor microenvironment, since activated-stromal AR affects the paracrine factors secreted by CAFs reducing ER-positive breast cancer cell migration and invasiveness.

Thus, these findings reinforce the possibility to couple the androgen-based therapy with therapies targeting other important pathways in ERα-positive breast cancer patient treatment.

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1. INTRODUCTION

1.1 Breast cancer

Breast cancer originates from an increased and unregulated proliferation of breast tissue epithelial cells. These cells form ducts and lobules of mammary gland providing the main gland function, lactation.

1.1.1 The normal breast tissue

Normal breast tissue consists of two different compartments: the stroma, which provides the scaffold of the mammary gland and the epithelium which consists of several branching ductal-lobular systems forming a tree-like structure (Moffat & Going, 1996). Lobules produce milk and ducts connect in lobules to the nipple connection. Thus, this structure mirrors the main breast function, lactation (Winters, Martin, Murphy, & Shokar, 2017).

During a lifetime, breast tissue is involved in several morphological modifications, making it a dynamic structure. Indeed, mammary gland tissue is involved in recurring expansion and regression during the menstrual cycle, though the major changes occur in the pregnancy, lactation and involution. For this reason, the embryonic, pubertal, and reproductive phases are the three major development stages in normal breast tissue (Macias & Hinck, 2012).

Mammary gland development starts during embryogenesis. In post-natal development, the ducts spread into the fat pad until puberty (Arendt & Kuperwasser, 2015). In this stage, gland expansion is due to ductal growth and division forming terminal end buds which themself originate new ducts or alveolar buds. The type 1 lobules are composed by the alveolar buds clustering around the terminal duct. Mammary gland development is a long process that may stay incomplete in absence of pregnancy. In adult women, alveolar buds are called ductules and increase their number originating for the first the type 2 lobules and then the type 3 lobules (Fig. 1.1). This mechanism is parallel to the increase of lobules size and the decrease of each single structure (Russo & Russo, 2004). Type 4 lobules are present only during lactation.

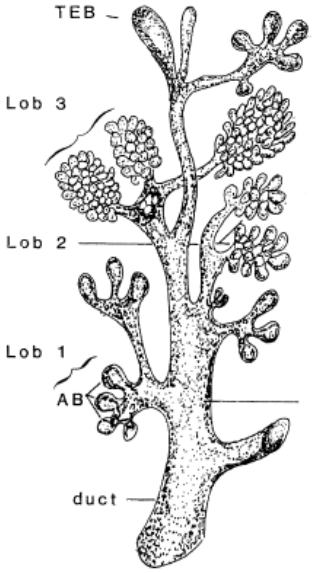

Figure 1.1 Diagrammatic representations of the lobular structures of the human breast (Adapted from Russo & Russo, 2004).

Type 1 lobules are less developed but they have the highest proliferation expression of both estrogen receptor-alpha (ERα) and progesterone receptor (PR). Moreover, type 1 lobules are present during the follicular phase and type 2 during the luteal phase of menstrual cycle, indicating that hormone levels may also influence mammary gland morphology (Arendt & Kuperwasser, 2015). Indeed, when sexual maturity is reached, a brief breast cell proliferation occurs during the menstrual cycle. In this cycle, there is a peak of estrogen during the follicular phase and of progesterone in the luteal phase, in which the mitosis in epithelium cell population is increased. Furthermore, during pregnancy, estrogen and progesterone promote milk-producing alveoli development and in post-menopause, when ovaries stop to produce both hormones, epithelial cells are substituted by stromal cell and adipose tissue (Hilton, Clarke, & Graham, 2018).

1.1.2 Mammary epithelial cell hierarchy

Inside normal breast epithelium different cell lineages that arise from multipotent stem cells. In a linear hierarchy, stem cells are at the apex. These cells divide symmetrically or asymmetrically to originate bipotent progenitors, which, in turn, yield to luminal and basal/myoepithelial cell populations (Arendt & Kuperwasser, 2015; Hilton et al., 2018).

Myoepithelial and luminal epithelial cells form the bilayered, tubular structure in which, during lactation, myoepithelial cells contract to allow milk secretion from alveolar luminal cells (Macias & Hinck, 2012).

By evaluating the expression of the cell-surface markers integrin alpha-6 (CD49f) and Epithelial cell adhesion molecule (EpCAM), within luminal breast cell are identified three different subpopulations:

- Luminal progenitor CD49f + and EpCAM +;
- Luminal mature CD49f- and EpCAM+;
- Basal CD49f+ and EpCAM-.

Luminal progenitor and mature cell subpopulations differ on steroid receptor expression: 28% of luminal progenitor cells express ERα and are negative to PR; 55% of luminal mature cells express ERα and 71% PR (Margan, Jitariu, Cimpean, Nica, & Raica, 2016).

1.1.3 Epidemiology

Breast tumour is the most prevalent malignancy in women and affects one in eight women during their lifetime. Along with lung and colon tumor, breast cancer is one of the most common cancers worldwide (Harbeck & Gnant, 2017). Currently, breast cancer prevention acts in two different ways: primary prevention, which includes chemoprevention or prophylactic surgery in women at high risk of developing breast cancer; secondary prevention that consists in the earlier detection and screening programs through mammography, clinical breast examination (CBE) and breast self-examination (BSE) (Coleman, 2017). Due to the presence of these prevention programs and the efficient therapies, breast cancer mortality has decreased in developed countries, while it is while it is increasing in underdeveloped countries (Harbeck & Gnant, 2017).

1.1.4 Risk factors

Non-genetic or genetic, non-modifiable or modifiable risk factors are involved in breast cancer formation. Germ-line genetic mutations in breast cancer susceptibility genes 1 and 2 (BRCA1 and BRCA2) lead to breast and ovarian cancer. When BRCA mutations occur, BRCA suppressor proteins are not able to inhibit the abnormal cell growth and proliferation, contributing to malignancy and cancer development (Winters et al., 2017). Other risk factors implicated in breast tumour are (Fig. 1.2):

- Age. The highest frequency of mammary tumour is at 40 years with a peak around 60 years, indicating increased incidence and mortality proportionally with age.
- Reproductive factors. Breast cancer is frequent when women experience an early menarche and/or a late menopause. Breast cancer risk is 20% higher if menarche occurs before age 11 and 12% higher if menopause occurs around ages 50-54, indicating ovarian hormones role in breast cancer promotion (Winters et al., 2017).

- Family history. Women with a first-degree relative affected by breast cancer have a highest risk to develop the diseases that may increase if the relatives are two or three.
- Estrogen and progesterone. Not only are the endogenous, but also exogenous estrogens and progesterone to be involved in breast carcinogenesis. Indeed, women that used oral contraceptives and the hormone replacement therapy have and increased risk to develop breast tumor.
- Lifestyle. Alcohol consummation and diet style based only on satured fat acid intake increase breast cancer risk. In particular, frequent alcohol consummation is asscociated with the highest level of circulating estrogen-related hormones by leading to ERα pathway activation (Sun et al., 2017).

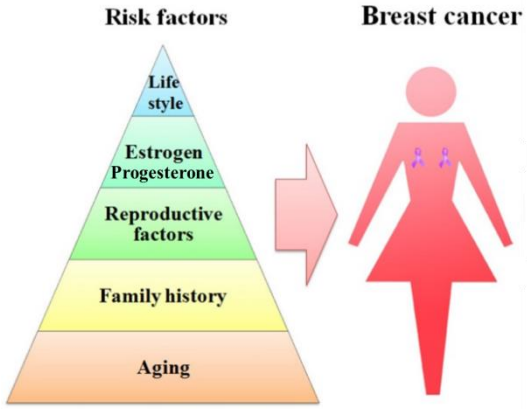

Figure 1.2 Schematic representations of breast cancer risk factors (Adapted from Sun et al., 2017).

1.1.5 Classification

Breast cancer is classified in (Fig. 1.3):

-Ductal carcinoma in situ (DCIS), a non-malignant and non-invasive proliferation of breast epithelial cells that are confined into the lobules and ducts;

-Invasive carcinoma, an abnormal proliferation of breast neoplastic cells that are infiltrating in the surrounding stroma.

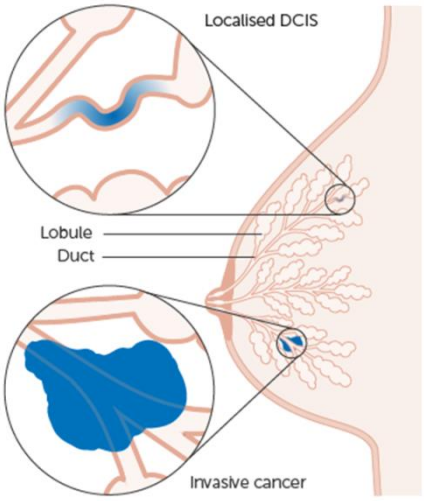

Figure 1.3 Breast cancer classification (Cancer Research UK).

Based on variations in estrogen receptor α (ERα), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) gene expression patterns, breast cancer is also classified in four molecular subtypes each with distinct clinical outcomes (Sorlie et al., 2001):

-luminal A (ERα+, PR+, HER2-);

-luminal B (ERα+, PR+, HER2+);

-HER2-enriched (HER2+);

-triple negative/basal like (ERα-, PR-, HER2-).

Moreover, breast cancer can be classified by evaluating the stage and the grade of the tumour. The stage indicates tumour size and the involvement of axillary nodes. The TNM is the most common staging system, where T indicates the size of the tumour, N describes whether there are any cancer cells in the lymph nodes, and M indicates whether the cancer has spread to a different part of the body. By the TNM system, breast cancer is staged I-IV. Stage I indicates that the tumour is 2 cm across or less, without lymph-nodes involvement; stage IV indicates a spreading tumour in another parts of the body, in particular brain, lung, liver and bone.

The grade is based on mitotic cells, nuclear shape and tubular differentiation. Breast cancer grades (Fig 1.4) are: grade 1, tumour cells are similar to the normal, grown slowly and aren't infiltrating; grade 2, an intermediate grade; grade 3, tumour cells are undifferentiated, grown faster and spread into the body ("Stages, types & grades of breast cancer | Cancer Research UK," n.d.).

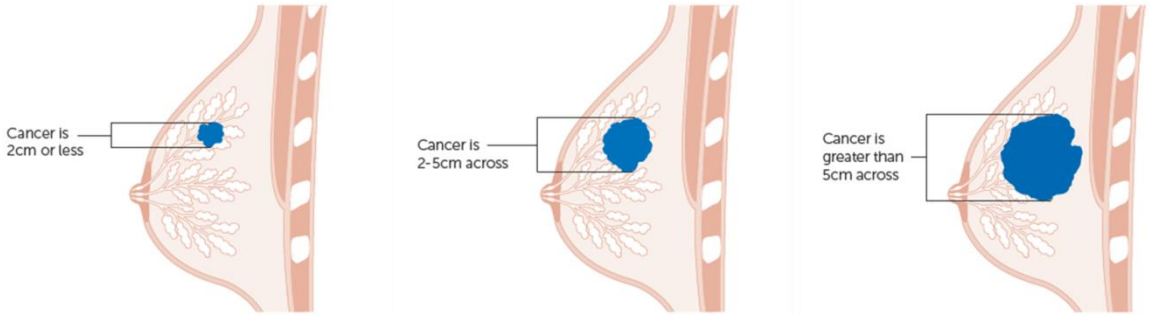

Figure 1.4 Breast cancer grading (Cancer Research UK).

1.1.6 Systemic therapy

- ERα+, PR+, HER2- subtype

Endocrine therapy aims to inhibit estrogen-dependent breast cancer cell growth. In pre-menopausal patients, tamoxifen is the main drug used in the standard endocrine therapy. It is an ERα+ selective ligand which competes with estrogen in ERα+ binding. The aromatase inhibitors anastrozole, exemestane and letrozole are used in post-menopausal patients to reduce the conversion of androgens in estrogens. Tumour stage and grade may indicate wheter to treat with chemotherapy and/or endocrine therapy. Adriamycin/cyclophosphamide, Adriamycin/cyclophosphamide/paclitaxeland Docetaxel/cyclophosphamide are examples of chemotherapy combinanations that are used in early breast cancer to prevent recurrences.

In metastatic ERα+, PR+, HER2- breast cancers, the early treatment is endocrine therapy in combination with cyclin-dependent kinase (CDK) 4/6 inhibitor like abemaciclib, palbociclib and ribociclib. Unfortunately, endocrine resistance often occurs and in this case the second option is the chemotherapy.

- Triple-negative subtype

Non-metastatic triple-negative breast tumors larger than 5 mm are treated with chemotherapy. Anthracylines and taxanes are the only approved by the Food and Drug Administration (FDA) in the treatment of this breast cancer subtype.

In metastatic triple-negative subtypes, chemotherapy is the only option for patients without BRCA1/2 mutation. Otherwise, when a fault in one of the BRCA genes is present, the inhibitors of poly [adenosine diphosphate-ribose] polymerase (PARP) enzymes are approved for use.

- HER2+

One of the greatest achievements in breast cancer treatment is the HER2-targeted therapy. Trastuzumab is a monoclonal antibody that targets the extracellular domain of HER2.

Moreover, the monoclonal antibody pertuzumab and tyrosine-kinase inhibitor neratinib reduce the risk of relapse in patient with high risk HER2+ breast cancer.

Taxanes in combination with trastuzumab and pertuzumab the first choice in metastatic HER2+ breast cancers; the second choice is the antibody-drug conjugate trastuzumab emtansine (Waks & Winer, 2019).

1.2 Steroid hormone receptors in breast cancer: the role of androgen receptor

Steroid hormones act by binding their own receptors that are ligand-activated transcription factors. These hormones not only regulate mammary gland growth but may favour breast carcinogenesis. Thus, cancer risk is linked to woman's reproductive history and lifetime hormonal exposure (Brisken & O'Malley, 2010).

1.2.1 Estrogen receptor

Steroid hormones ability to stimulate breast epithelium proliferation was experimentally demonstrated by implanting subcutaneously normal human breast tissue in an adult female athymic nude mouse. Estrogen and progesterone were administrated to mimic luteal and follicular phase of menstrual cycle. Estradiol increased cell proliferation during luteal phase, while progesterone co-administration with estrogen didn't influence it. Instead, long term treatment with estrogen and progesterone lead to an increased proliferation of normal mammary epithelium from post-menopausal women, highlighting the correlation with the increased breast cancer risk. During the premenopausal, normal estrogen receptor alpha (ER-α)-negative epithelial cells proliferate in response to steroid hormones. The growth is stimulated via paracrine signals by adjacent cells that express ESRA. The number of proliferating cells decreases with the age while the number of ER-α positive cells increases. The high expression of ER-α is a breast cancer risk factor. Indeed, ER-α expression is greater in normal cells near the tumour and in Caucasian women, in which breast cancer is more frequent. Moreover, proliferating tumour cells express ER-α, indicating that there is an autocrine mechanism to stimulate cell proliferation (Fig. 1.5) (Clarke, Anderson, & Howell, 2004).

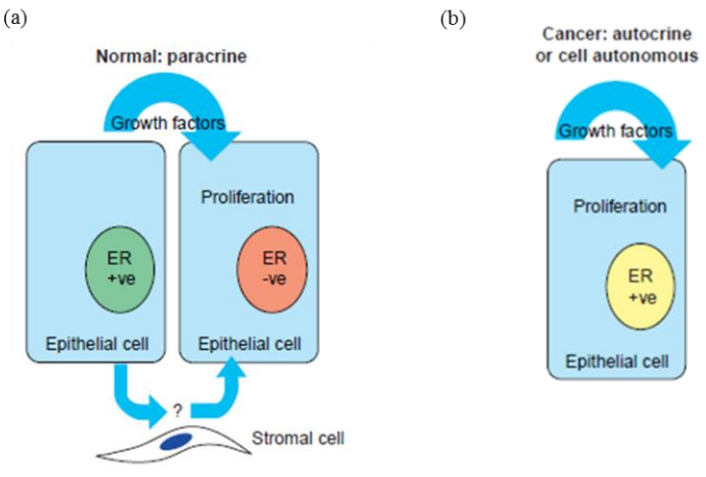

Figure 1.5 Estrogen receptor expression and cell proliferation in human breast tissues (Adapted from Clarke et al., 2004).

1.2.2 Progesterone receptor

Estradiol is responsible for ductal development and side branching while progesterone is necessary for alveolar growth of mammary gland. There are two different isoforms of progesterone receptor, each with a specific role: PR A is important for the reproductive capacity and PRB for the mammary gland development. PRA and PRB are both present in normal mammary gland epithelium, but during breast carcinogenesis, PRA is highest. Tumour cells express higher levels of PR than normal cells. Indeed, PR expression is stimulated by estrogens. In addition, PR stimulates cell proliferation through autocrine and or paracrine signals and its transcriptional activity. In breast cancer, PR is a good prognosis marker in breast cancer and an indicator of response to endocrine therapy (Clarke et al., 2004; Knutson & Lange, 2014).

1.2.3 Androgen Receptor

Despite estrogen and progesterone receptors are widely studied, only recently there is a growing interest to investigate the androgen receptor (AR) role in breast tumour carcinogenesis, prognosis and treatment. The AR is a transcription factor member of the nuclear receptor family, which includes ER and PR (Rahim & O'Regan, 2017). AR is the steroid receptor more expressed in luminal epithelial mammary cells and it is detectable approximately in ~90% of ERα+ and ~35-50% of triple negative breast tumours (Bleach & McIlroy, 2018).

- AR Structure

The *AR* gene located on the long arm of the chromosome Xq11-12 encodes for the receptor protein composed of 919-aminoacids and with three functional domains:

-The amino-terminal (NTD). It has the pivotal role in the transcriptional activity through the activation function domain-1 (AF-1), formed by the two transcription activation units Tau-1 and Tau-5.
-The DNA binding domain (DBD). Through two zinc-finger structures, it binds the androgen response element (ARE), two palindromic consensus sequence 5'-GGTACAnnnTGTTCT-3'.
-The carboxyl-terminal domains which is linked to the DBD by the hinge region. It has the ligand-binding domain (LBD) and activation function domain-2 (AF-2), important for the interactions with the ligand and the heat shock proteins.
The nuclear localization signal (NLS) links the DBD and hinge region and regulates the nuclear localization of AR (Giovannelli et al., 2018).

- Androgen metabolism

The common precursor of all steroid hormones is the cholesterol. The breast tissue doesn't express the enzymes involved in cholesterol metabolism into androgens. Androgens are produced both from ovaries and adrenal gland. Cholesterol is metabolized in androstenedione (4-dione) in ovaries and in the zona reticularis of adrenal gland (Fig. 1.6), in which it is also converted in dehydroepiandrosterone (DHEA) (McNamara & Sasano, 2015). However, dehydroepiandrosterone sulfate (DHEAS), is the major circulating sexual hormone precursor that is metabolized in androgens or estrogens in peripheral tissues, including breast tissue. Testosterone (T) and dihydrotestosterone (DHT) are produced in brain, bone and breast. In particular, T can be converted both in DHT by 5-α-reductase and in estradiol (E2) by aromatase. T and DHT are the only androgens able to bind the AR, but DHT is the most potent AR-ligand (Giovannelli et al., 2018; Rahim & O'Regan, 2017). Notably, DHT concentration is higher in breast tumour than in normal tissue, especially in DCIS breast cancer patients, indicating a possible intratumoural role of DHT (Takagi, Miki, Ishida, Sasano, & Suzuki, 2018).

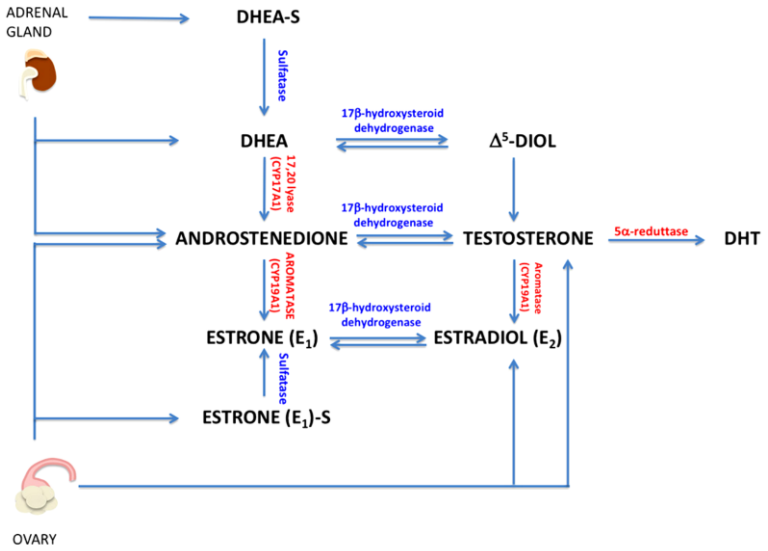

Figure 1.6 Androgens metabolism (Adapted from Giovannelli et al., 2018).

- AR Mechanism of action

In absence of ligand, AR is in an inactivated state bound to the heat shock proteins (hsp) into the cytoplasm. Following androgens interaction, the AR dissociates from the hsp, is activated by phosphorylation, dimerizes and migrates into the nucleus where interacts with the ARE. By binding the ARE on the promoter region, the AR regulates the transcription of the target genes involved in cell division, proliferation, apoptosis and angiogenesis (Fig. 1.7). The AR may start the transcription by itself or may require the recruitment of others transcription factors or co-factors (Venema et al., 2019). Indeed, as nuclear receptor AR needs co-activators or co-repressor to control transcriptional activation. Different AR co-regulators are identified in prostate cancer cells such as BRCA1, Male germ cell-associated kinase (MAK), ARA70, SRC-1 (NCOA1), SRC-2 (NCOA2), and SRC-3 (NCOA3) (Bleach & McIlroy, 2018). In addition, growth factors, phosphorylation and co-activators may activate androgen receptor with a ligand-independent mechanism (Salvi, Bonafè, & Bravaccini, 2019). Furthermore, AR shows non-trascriptional/non-genomic actions: into the cytoplasm, AR may interact with phoshoinositide 3-kinase (PI3K), Src proteins and Ras GTPase; AR, moreover, may phosphorilate the mammalian target of rapamycin (mTOR), inactivate the forkhead box protein A1 (FOXOA1) or activate the protein kinase A (PKA) (Basile et al., 2017).

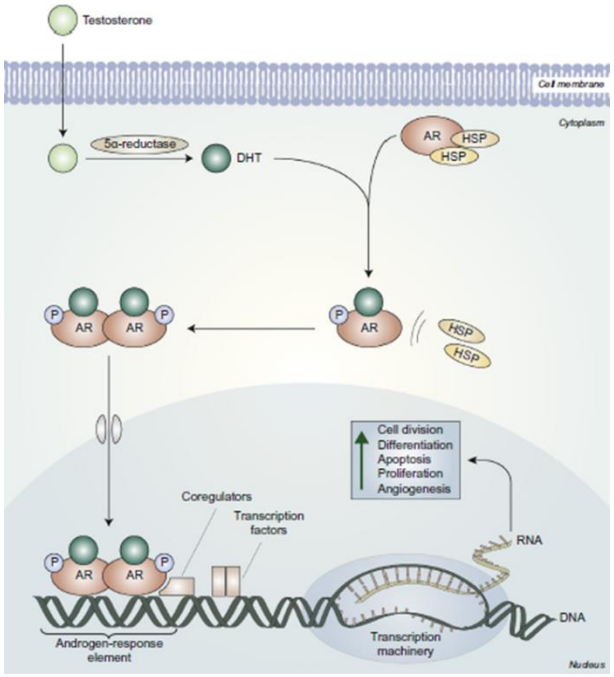

Figure 1.7 AR mechanism of action (Adapted from Venema et al., 2019).

- AR role in ERα+ positive breast tumour

AR action in breast cancer is strictly related to tumour subtype. Based on ERα and AR status, breast cancer it is also classified in three subtypes: basal positive (ERα+, AR+), basal negative (ERα-, AR-), and molecular apocrine (ERα-, AR+) (Bleach & McIlroy, 2018; Hickey, Robinson, Carroll, & Tilley, 2012).

Many evidence support androgen's ability to reduce tumour growth by exerting an anti-proliferative action in ERα+ breast cancers. *In vitro* studies showed that DHT and the synthetic AR-ligand mibolerone (Mb) inhibit E2-induced proliferation cells an effect reversed by the AR antagonist hydroxyflutamide or bicalutamide, highlighting the direct role of AR (Andò et al., 2002; Birrell et al., 1995; Cops et al., 2008). A mechanism to explain that was proposed in MCF-7 cells, in which androgen administration induced apoptosis and, moreover, arrested cell cycle progression by reducing cyclin D1 expression. Indeed, androgen stimulation promoted the AR binding on ARE on cyclin D1 promoter gene (CCND1), identified as an AR target gene, and the recruitment of the atypical orphan nuclear receptor DAX-1 and Histone Deacetylase 1 (Marilena Lanzino et al., 2010). The androgen-reduced cell proliferation is tightly related to activated-AR ability to counteract ERα genomic interaction. Several cross-talk mechanisms between the two receptors are reported in pre-clinical studies. A critical role in AR and ERα signalling activation involved the sharing of common co-regulators like ARA70. When AR: ER ratio was high, ARA70 promoted AR transactivation and ERα transcriptional activity was decreased, and vice versa, as showed in wild-type MCF-7 and AR overexpressed MCF7 and Hela cells (Marilena Lanzino et al.,

2005). In addition, in the human metastatic ductal epithelial ZR-75 breast cancer cells, that present equal levels of androgen and estrogen receptors, DHT treatment antagonized ERα target genes expression. This is due to the enrichment of AR-binding sites near ERα-target genes, suggesting AR has the ability to counteract ERα transcriptional activity in luminal breast cancer (Need et al., 2012). Interestingly, in a recent *in vivo* study, Z. Yu et al. showed the breast-selective activity of the synthetic AR ligand RAD140 and reduced tumour growth in AR/ERα+ breast cancer patient-derived xenograft models after treatment. This mechanism involved the down regulation of ESR1 and ERα+ target genes mRNA as well as of genes involved in cell growth promotion. Moreover, this effect is enhanced by the co-administration of RAD140 with the CDK4/6 inhibitor Palbociclib, approved in breast cancer treatment in combination with agents targeting the estrogen receptor, suggesting a possible use of RAD140 in breast cancer coadjuvant treatment (Yu et al., 2017).

Interestingly, the presence of a membrane androgen receptor was described even if the mechanism of action is still unknown. Kampaa et al. showed the presence of the membrane AR in T47D cells and in human breast cancer biopsies. In T47D cells, the treatment with the non-permeable testosterone conjugate with bovine serum albumin (T-BSA), induced apoptosis in a dose dependent manner by increasing the pro-apoptotic BAD and by reducing the anti-apoptotic Bcl2 protein levels. Moreover, T-BSA reversed the pro-apoptotic effects mediated by E2 (Kampaa et al., 2005).

Nevertheless, it must be reported that other studies showed a proliferative role of AR in ERα+ breast cancer cells. AR may promote MCF-7 breast cancer cells proliferation by increasing ERα recruitment on ERα-binding sites. This mechanism is consequent to an AR/ERα physical interaction, as proved by the highest AR nuclear localization following E2 stimulation in AR+/ERα+ MCF-7 and ZR-75-1 cells and not in AR+/ERα- MDA-MB-453 and MDA-MB-231 cells. In addition, the AR antagonist enzalutamide synergized with tamoxifen and fulvestrant to inhibit both basal and estrogen-dependent proliferation in MCF-7, ZR-75 and patient-derived xenograft BCK4 and PT12 cell lines and decreased E2 and DHT induced proliferation in PT12 primary tumours and metastases (D'Amato et al., 2016). Moreover, it was also described that androgen treatment may induce epithelial-to-mesenchymal transition, cell migration and metastasis in breast cancer cells. These mechanisms required the lysine-specific demethylase 1A (LSD1) or the recruitment of co-regulators NCOA1 involved in gene transcription (Azariadis et al., 2017; Feng et al., 2017). It is in accordance with RNA sequencing data from circulating tumour cells (CTCs) isolated from patients with ERα+ metastatic breast cancer, revealing AR expression and activation in bone metastasis. In particular, AR was overexpressed in breast CTCs derived from patients that received

aromatase inhibitor (AI) treatment for 725 days, whereas was negative in samples derived from patients that undergone treatment for 85 days suggesting a link between the reduced estrogen production and AR signalling (Aceto et al., 2018).

Moreover, Migliaccio et al. described in MCF-7 and LNCaP cells an association of AR with ER that did not involve a genomic interaction but was triggered by EGF-activated Src and consequently ER-AR-Src complex formation and cell proliferation stimulation (Migliaccio et al., 2006).

The dichotomous role of androgen receptor showed by these finding emphasize the importance of correlating AR expression and its role to hormonal milieu, experimental conditions and treatment.

The prognostic and predictive role of AR

AR is an emerging favourable prognostic marker in grade III, luminal A and ERα+ breast tumours during 5-10 years following diagnosis (Kensler, Poole, et al., 2019; Kraby et al., 2018).

A clinical meta-analysis carried out on AR protein and mRNA levels in 17,000 women with early-stage breast cancer, revealed that AR positivity improved disease-free survival (DFS) and over-all survival (OS) both in univariate (HR¼ 0.61; 95% CI, 0.52–0.72; OS: P < 0.001 and HR 0.62; 95% CI, 0.51–0.75; P < 0.001 respectively) and multivariate analysis (HR 0.46; 95% CI, 0.37–0.58; OS: P < 0.001 and HR 0.53; 95% CI, 0.38–0.73; P < 0.001 respectively). In addition, they analysed a subgroup to investigate the AR expression in the different breast cancer subtypes, AR presence was correlated with longer DFS and OS in univariate (HR 0.53; 95% CI, 0.44–0.63, P < 0.001 and HR¼0.59; 95% CI, 0.49– 0.72, P < 0.001, respectively) and multivariate analysis (HR 0.40; 95%CI, 0.31–0.52, P < 0.001 and HR¼0.37;95%CI 0.16–0.85, P ¼ 0.02, respectively) in ERα+ tumours. These data would confirm AR ability to antagonize ERα activity in relationship to their expression levels (Bozovic-Spasojevic et al., 2017). Recently, C.M. Venema et al. confirmed the favourable prognostic impact of AR in terms of DFS and OS in ERα+/HER2- tumour subtypes. This finding confirmed again that the AR may influence the breast cancer outcome in relationship to the receptor status and to the intrinsic molecular subtypes (Venema et al., 2019).

Furthermore, in a retrospective study of 159 patients with primary breast cancer, steroid receptor expression was analysed by immunohistochemistry. Overall survivor analyses (OS) performed in 89 primary tumours of luminal breast cancers patients showed a poor prognosis when the AR/PR and ER/PgR ratio were high (p=0.004 and p=0.21 respectively). Interestingly, in 24 patients androgen and estrogen receptor expression was detected both in

primary and metastatic sites. The increased AR/ER ratio was associated with better prognosis when it is present both in primary tumours and metastasis (p=0.011). However this study needs further investigations due the small case series (Bronte et al., 2019).

The analysis of AR expression in postmenopausal patients with ERα+ breast cancer recruited in the Breast International Group Trial 1-98 revealed that patients with AR+ early breast cancer showed smaller ($p<0.001$), lower-grade tumours ($p<0.001$) and often received conserving surgery ($p<0.001$). In these samples, AR expression was correlated to highest ER and PR levels whereas Ki67 didn't change. Nevertheless, AR expression wasn't associated to breast cancer-free interval (BCFI) and didn't affect the efficacy of letrozole or tamoxifen treatment (Kensler, Regan, et al., 2019).

Bronte et al. reported that AR expression didn't predict the efficacy of the aromatase inhibitors, used as first choice in ERα positive breast tumours, both in term of time to relapse (TTR) and progressive disease (PD) (Bronte et al., 2018).

In ZR-75-B and MCF-7 cells with low levels of Rho guanine nucleotide dissociation inhibitor (Rho GDI), which decrease is associated with tamoxifen resistance, AR stimulate EGFR activation that resulted in ERα phosphorylation and thus in tamoxifen-induced cell proliferation (Ciupek et al., 2015).

AR expression was associated with positive response to endocrine therapy and chemotherapy response in ERα + breast cancer (S. Park et al., 2012; Witzel et al., 2013) but other findings showed that AR positivity is associated with a less responsiveness to neo-adjuvant chemotherapy but better survival in ERα+ breast cancer (Okano et al., 2019).

These divergent results may be related to different criteria of analysis, experimental methods and samples number.

Androgens in breast cancer treatment

Since the 1940s, the AR agonist testosterone propionate and fluoxymesterone were used in the treatment of metastatic breast cancer and by 1988 the administration of the AR antagonist flutamide in patients was also described. Due to the local effects that leaded to virilisation, the metabolism into estrogens and the emergence of ERα modulator tamoxifen, the AR targeting wasn't a notable target in breast cancer treatment (Narayanan & Dalton, 2016; Venema et al., 2019).

Due to the 2-sided role in breast cancer, it is unsurprising that currently both agonist and antagonist of androgen receptor are under investigation in several clinical trials.

In post-menopausal women with early AR+/ERα+ breast cancer, the synthetic androgen receptor agonist (SARM) enobosarm is under investigation in the EMERALD study

(CRUK/15/075), on hold at the moment. The aim is to find the effects of the androgen treatment on breast cancer cell behaviour in the period that elapses by diagnosis to surgery. At the same time, the AR antagonist enzalutamide, approved for prostate cancer treatment, alone or in combination with the aromatase inhibitor exemestane approved for breast cancer treatment, is used in one cohort of post-menopausal patients with ERα+ breast tumour with size of at least 1 cm (NCT02676986). This study, still on-going, expects that in the week between the diagnosis and the surgery, enzalutamide can reduce the level and the activity of androgens that increased following AI administration.

The androgen agonist CR1447 (4-OH-testosterone) combines two mechanisms of action by interacting with the AR and inhibiting the aromatase enzyme. Thus, may have a higher activity than drugs with a single mechanism (NCT02067741) and it is under study in metastatic endocrine responsive and triple-negative/AR+ breast cancers. Moreover, another trial (NCT02910050) aims to evaluate the efficacy and the safety of the selective androgen receptor inhibitor bicalutamide and aromatase inhibitor in ER(+)/AR(+)/HER2(-) metastatic breast cancer patients who have disease progression after treatment of an aromatase inhibitor (Bleach & McIlroy, 2018).

1.3 Breast tumour microenvironment: the role of cancer-associated-fibroblasts

The tumour is already recognized as an organ and the stromal compartment is the scaffold for the tumour mass accordingly.

Through the identification of the hallmarks of cancer, Hanahan and Weinberg provided for the first time a complete view of tumour complexity. Indeed, tumour biology may be understood only by highlighting the role of the tumour microenvironment (TME) in tumorogenesis (Hanahan & Weinberg, 2011). This surrounding tissue is compounded by non-malignant cells that support tumour initiation, growth, progression and metastasis by producing and secreting growth factors, cytokines and by promoting tissue remodelling. Moreover, tumour stroma is involved in therapy resistance, thus there is a growing interest to target the breast tumour microenvironment in breast carcinoma treatment.

Different cells are contained in the TME (Fig. 1.8), including cancer stem cells (CSCs) that are source of differentiated cells by self-renew capability or by producing them; infiltrating immune cells like leukocytes, neutrophilis, mast cells, T-and B-lymphocytes and macrophages, named tumour-associated macrophages (TAMs); endothelial cells, necessary to supply oxygen and nutrients to epithelial tumour cells; pericytes that sustain the tumour endothelium; mesenchymal stromal cells, multipotent stromal cells, that may differentiate in

CAFs, TAMs, myeloid-derived suppressor cells (MDSC). Moreover, cells from tumour stroma can stimulate the surrounding cells with pro- and/or anti-tumorigenic properties or be stimulated by cancer cells to develop an aberrant tumour-associated phenotype (Eiro et al., 2019; Hanahan & Weinberg, 2011; Kalluri, 2016).

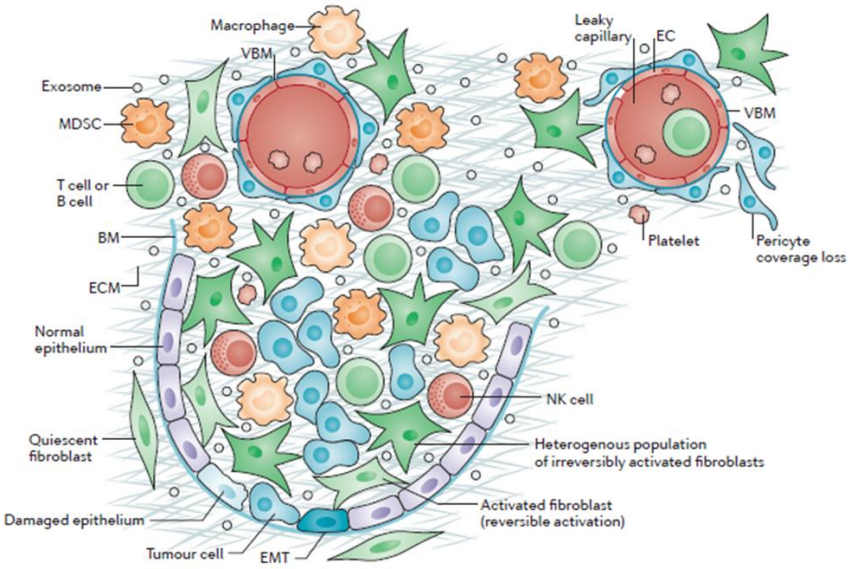

Figure 1.8 The tumour microenvironment (Adapted from Kalluri, 2016).

1.3.1 Cancer-associated fibroblasts

Fibroblasts were first described like cells producing collagen. In the normal tissue, fibroblasts are present in the interstitial space or near capillaries. These cells have a mesenchymal origin with spindle-shape morphology (Buchsbaum & Oh, 2016).

CAFs are the largest cell population inside breast stromal compartment. In normal mammary gland tissue, there are two different fibroblast populations with distinct gene expression profiles:

-Interlobular, $CD105^{low}/CD26^{high}$. They have an immune-related signature with interleukin 1 receptor type 1(IL1R1), interleukin 33 (IL33) and SCL39A8 expression;

-Intralobular, $CD105^{high}/CD26^{low}$. They are the main support to mammary gland by producing the extracellular matrix (ECM), they release ECM remodelling enzyme and growth factors, contributing to branching morphogenesis. In addition, $CD105^{high}/CD26^{low}$ fibroblasts share the expression profile of CAFs, thus by contributing to CAFs pool in breast TME (Houthuijzen & Jonkers, 2018).

From a hibernating, quiescent or resting state, fibroblasts are activated in the normal tissue during the wound-healing response. Because tumour is also defined as “wounds that do not heal”, activated fibroblasts are permanently present in cancer tissue. Once activated, they are

called myofibroblasts or cancer-associated fibroblasts (CAFs) and are identified by the positivity to different stromal activation markers:

-Alpha-smooth muscle actin (α-SMA), one of six actin isoforms. It is the main marker of myofibroblast differentiation;

-Fibroblast activation protein (FAP), type II integral membrane protein that belongs to the family of plasma membrane-bound serine proteinases. FAP is not expressed in normal tissues. However, it is not a specific marker to identify fibroblasts (Buchsbaum & Oh, 2016).

However, the origin of CAFs is still unclear and it might be different in relationship to tissue and tumour subtype. In breast cancer, CAFs may originate from resident fibroblast, epithelial or endothelial cells that undergo to epithelial or endothelial-to-mesenchymal transition and from mesenchymal stem cells (MSCs). Mammary gland is embedded in adipose tissue, rich of MSCs that are the main source of CAFs (Buchsbaum & Oh, 2016). By single-cell RNA sequencing of 768 transcriptomes of mesenchymal cell from the engineered MMT-PyMT mouse model of breast cancer, Bartoschek et al. identified four different CAFs subpopulations with specific origins, functions and histologically entities. The two main populations were vascular CAFs (vCAFs), originating from a perivascular localization, and matrix CAFs (mCAFs), arising from local fibroblasts. Interestingly, it was showed that the vCAFs and mCAFs gene signature was associated to increased risk to develop metastatic diseases in human cohorts (Bartoschek et al., 2018).

Fibroblasts contribute to tumourigenesis by producing ECM, releasing cytokines and chemokines, recruiting immune cells and by changing the normal tissue architecture through physical forces (Kalluri, 2016).

In particular, CAFs promote breast cancer cell growth by secreting fibroblast growth factors (FGFs), tumour growth factor (TGF-β), hepatocyte growth factor (HGF) and leptin. In addition, breast CAFs media is richer in 17beta-estradiol dehydrogenase (E2DH), enzyme involved in the conversion of estrone (E1) in estradiol (E2), than the media derived from normal fibroblasts, highlighting the direct role in promoting estrogen-dependent breast cancer cell growth.

Furthermore, CAFs stimulate cell local invasion by promoting epithelial-to-mesenchymal transition (EMT) in epithelial breast cancer cells and by affecting the surrounding extracellular matrix (ECM). Like reported by a recent study, ECM deposited by CAFs induces a mesenchymal morphology in premalignant breast cancer cells.

CAFs are also involved in breast cancer cell metastasis, moreover, by influencing the site of secondary tumours. Breast cancer cell growth and invasion are also promoted by CAFs through angiogenesis, lymphangiogenesis and the inhibition of cytotoxic immune cells.

Indeed, by releasing pro-inflammatory cytokines like interleukin-1 beta (IL-1β), interleukin-8 (IL-8), interleukin-10 (IL-10), tumour necrosis factor-alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1), interferon-β (IF-β) and stromal cell-derived factor-1(SDF-1) Breast CAFs create a perfect milieu to favourite chronic inflammation, a critical component of tumour progression (Buchsbaum & Oh, 2016). Many findings showed that fibroblasts contribute to metabolic reprogramming of the TME. Under aerobic condition, tumour cells convert glucose to lactate to produce energy with the "Warburg effect". Autophagy gives macromolecules required by tumour cells during metabolic stress like hypoxia and nutrient deprivation. Autophagy and angiogenesis are promoted by hypoxia-inducible transcription factor 1-alpha (HIF-α) which is increased in cancer tissue. In accordance with the "reverse Warburg effect", cancer cells induce the Warburg effect in tumour fibroblasts that provide lactate and pyruvate for oxidative mitochondrial metabolism in cancer cells.

CAFs have also a crucial role in breast cancer therapy resistance. Different mechanisms are involved, including CAFs ability to promote tamoxifen resistance in breast cancer cells by activating PI3K/AKT and MAPK/ERK pathways and by inducing ER-α phosphorylation at serine 118. Moreover, tamoxifen promotes breast cancer cell proliferation through GPER/EGFR/ERK pathway by activating GPER, CYP19A1 gene expression and estrogens synthesis in CAFs (Buchsbaum & Oh, 2016).

1.3.2 Steroid hormone nuclear receptors and breast CAFs

The abnormal activity of steroid nuclear receptors in breast CAFs contributes to tumour development. Indeed, cancer-associated-fibroblasts present a different gene signature of steroid hormones compared with fibroblasts isolated from normal breast tissues.

ER-α activity in breast CAFs is less pronounced and it may be related to the highest expression of the nuclear receptor in the tumour epithelial compartment. In contrast, estrogen receptor-beta (ER-β) is widely expressed in breast tumour fibroblasts. Androgen receptor is also present in breast CAFs but, as the other steroid receptor, its role is still unknown. Thus there is a growing interest to understand the steroid nuclear receptors role in hormone-dependent tumours including breast cancer (Cheng, Lee, Wahli, & Tan, 2019).

1.4 Hypotheses and aims

1.4.1 Hypotheses

Human breast cell proliferation is promoted by enhanced activity of the ER-α that regulates the transcription of target genes, which in turn regulate cell proliferation (Tyson et al., 2011).

Among these genes, cyclin D1 amplification plays a crucial role. Interestingly, within the androgen receptor protective role in ER-α positive breast cancer there is the AR ability to inhibits cyclin D1 gene transcription through the direct action at the promoter region (Marilena Lanzino et al., 2010). Here we hypothesized that AR may also regulate cyclin D1 expression by additional mechanism such as squelching of ER-α/AR shared co-activators or modulation of the mode of action of pro-apoptotic proteins which have been shown to be implicated in cell cycle regulation. In addition, due to the supporting role of stroma in tumour growth and progression, for the first time we investigated in primary breast cancer-associated-fibroblasts AR expression and activity that are still unknown.

1.4.2 Aims

1. To investigate if the AR/ER-α competition for the steroid receptor coactivator AIB1, important for ERα interaction on cyclin D1 promoter gene, might be an additional mechanism by which AR can modulate ERα genomic activity;
2. To evaluate if androgen treatment may modulate the expression, cellular distribution and function of BAD, a pro-apoptotic member of the Bcl-2 family proteins, whose expression is related to a significantly better disease free survival in (ER)α-positive human breast cancers.
3. To study the biological significance of androgens/AR signalling in breast tumour microenvironment and to investigate whether AR modulation in CAFs may affect their tumour promoting capabilities.

2. MATERIALS AND METHODS

2.1 Materials

Reagents and antibodies

Dihydrotestosterone (DHT), hydroxyflutamide (OH-Fl), bicalutamide (BIC) and estradiol (E2) were from Sigma Aldrich; Mibolerone (Mb) was from Perkin Elmer (Waltham, MA, USA); enzalutamide (ENZ) was a gift from Steve Lyons (Manchester Cancer Research Centre).

The antibodies against AR (441), β-Actin (AC-15), BAD (C-7), BAX (B-9), Bcl-2 (C-2), BID (FL-195), Cyclin D1 (M-20), E-cadherin (G-10), ERα (F-10), GAPDH (FL-335), Lamin B (C-20), N-cadherin (H-63), Pan-Cytokeratin (C-11) and Vimentin (V9) were from Santa Cruz Biotechnology; the antibody against AR (D6F11), pAKT (Ser473) and AKT (#9272) were from Cell Signaling Technology; the antibody against α-SMA (1A4) was from Sigma Aldrich.

Cell cultures

Human breast cancer (MCF-7, T47D and SKBR3) from American Type Culture Collection-ATCC, were used. Media used to culture them are reported in Table 1. All cell lines were regularly tested for mycoplasma negativity (MycoAlert Mycoplasma Detection Assay).

CAFs isolation and culture

Primary Cancer Associated Fibroblasts were isolated from breast tumour biopsies derived from patients that were undergo surgery before to receive any pharmacological treatment and who signed informed consent in accordance with approved Human Subject's guidelines at Annunziata Hospital (Cosenza, Italy). After the transfer in our laboratory, the tumour biopsies were cut with scalpel into small pieces and then cultured at 37°C with 5% CO_2 air for about 3-4 weeks. During this period, we added fresh medium (reported in Table 1) and the CAFs had grown out from the pieces of tissue (Fig. 2.1). When the primary cells were confluent around the pieces, they were removed and the CAFs were washed with PBS trypsinized and cultured until 10-15 passages.

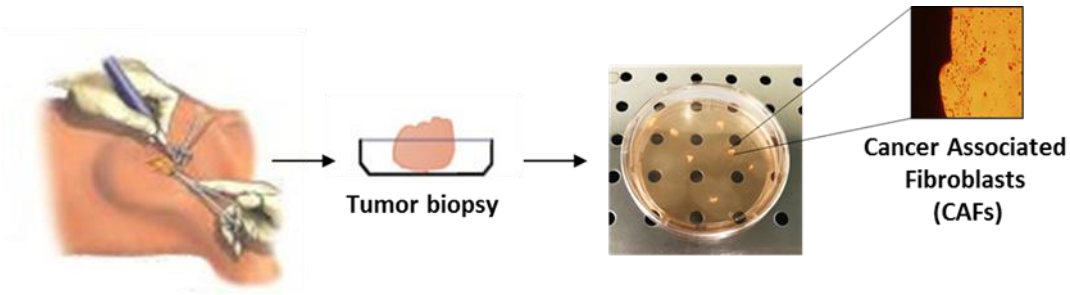

Figure 2.1 Breast CAFs isolation

CELL LINE	MEDIA	SUPPLEMENTS
MCF-7	DMEM (1X)	5% FBS, 1% Pen/Strep
T47D	RPMI 1640	10% FBS, 1% Pen/Strep, 1% L-Glutamine, 0,2 U/mL insulin
SKBR3	RPMI 1640 phenol red-free	10% FBS, 1% Pen/Strep, 1% L-Glutamine
HeLa	DMEM/F-12	5% FCS, 1% Pen/Strep, 1% L-Glutamine, 1% Eagle's nonessential amino acids
CAFs	50% MEDIUM 199 (1X) 50% F-12 Nut Mix (Ham) (1X)	15% FBS, 1% Pen/Strep

Table 1. Cell cultured media.

Before each experiment, breast cancer cells and CAFs were synchronized in phenol red-free serum free media (PRF-SFM) for 24h. All the experiments were performed in PRF-media containing 2.5 or 5% charcoal-treated (steroids depleted) Fetal Bovine Serum (PRF–CT). Cells were treated with 10^{-8} M E2, 10^{-7} M DHT, 10^{-8} M Mb, 10^{-6} M OHFl, 10^{-6} M BIC, 10^{-6} M ENZ and 10^{-8} M T-BSA.

- **Conditioned medium derived from CAFs**

CAFs were plated in full media until 70% of confluence, serum starved for 24h and then treated with vehicle, DHT or DHT+BIC. After 72h, the conditioned medium (CM) was collected, centrifuged and used to perform co-culture experiments.

2.2 Methods

- **Cell proliferation assays**

MCF-7 cells were seeded on six-well plates ($2x10^5$ cells/well) in 2.5% PRF–CT. After 24 h, cells were exposed for 3 days to and/or 10^{-8} M E2 and/or 10^{-6} M OHFl, or left untreated (-). CAFs were seeded on six-well plates (30000 cells/well) and were exposed to 10^{-8} M MIB or left untreated (-) for 3 and 6 days.

Cells were harvested by trypsin and drug effects on cell proliferation were measured by counting cells using a Burker's chamber; cell viability was determined by Trypan blue dye exclusion test.

- **Plasmids, transfections and luciferase reporter assays**

The following plasmids were used: Cyclin D1 promoter construct D1Δ-2966pXP2-Luc (a gift from Dr. A. Weisz, Università degli Studi di Salerno, Italy); wild-type AIB1 expression vector (a gift from Dr. B. O'Malley, Baylor College of Medicine, Houston TX USA); pcDNA3-AR (AR), encoding full-length androgen receptor, (a gift from Dr. M.J. McPhaul, UT-Southwestern Medical Center at Dallas TX, USA), the wild-type human ERα (HEGO) (a gift from Dr. P. Chambon, Université de Strasbourg, France).

Cells were transfected using Fugene 6 reagent (Roche Diagnostics) according to manufacturer's instructions. Renilla reniformis luciferase expression vector pRL-Tk (Promega) was used to assess transfection efficiency. Luciferase activity was measured with the Dual Luciferase kit (Promega).

- **Total RNA extraction, reverse transcription polymerase PCR and real-time RT-PCR assay**

Total RNA was extracted from MCF-7 cells and CAFs using TRIzol reagent and cDNA was synthesized by reverse transcription-polymerase chain reaction (PCR) method using a RETROscript kit. The expression of selected genes was quantified by real-time PCR using iCycler iQ Detection System (Bio-Rad, Hercules, CA). Five microliters of diluted (1:3) cDNA was analyzed using SYBR Green Universal PCR Master Mix, following the manufacturer's recommendations. The primers (Invitrogen) used are reported in Table 2.

GENE	FORWARD	REVERSE
Cyclin D1	5'-CCGTCCATGCGGAAGATC-3'	5'-AAACGTGGGTCTGGGCAA-3'
BAD	5'-GGAGGATGAGTGACGAGTTTGTG-3'	5'-GGGTGGAGTTTCGGGATGT-3'
FAP	5-AGAAAGCAGAACTGGATGG-3	5-ACACACTTCTTGCTTGGAGGAT-3
SDF1	5-TTACCCGCAAAAGACAAGT-3	5-AGGCAATCACAAAACCCAGT-3
CD147	5-GGGGCAGCGGTTGGAGGTTGT-3	5-GGGAGCCACGATGCCCAGGAAGG-3

Table 2. Primers sequences used for qRT-PCR

Each sample was normalized on the basis of its GAPDH or 18S ribosomal RNA content. The GAPDH or 18S quantification was performed using a TaqMan Ribosomal RNA Reagent kit (Applied Biosystems) following the method provided in the TaqMan Ribosomal RNA Control Reagent kit. The relative gene expression levels were normalized to a calibrator that was chosen to be the basal, untreated sample. Final results were expressed as n-fold

differences in gene expression relative to GAPDH or 18S ribosomal RNA and calibrator, calculated following the ΔΔThreshold cycle (Ct) method, as follows: n-fold=2- (ΔCt sample-ΔCt calibrator), where ΔCt values of the sample and calibrator were determined by subtracting the avarage Ct value of the GAPDH rRNA reference gene from the average Ct value of the different genes analysed. Assays were performed in triplicate.

- **Western Blotting**

Preparation of protein lysates

Total cell proteins were obtained from 70% confluent cells. Cells were washed three times with PBS, placed on ice and was added the lysis buffer (50mM Tris-HCl, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate) with the inhibitors of proteases activity (20 mg/mL aprotinin, 1% phenylmethylsulfonyl-fluoride, 0.1 mM sodium orthovanadate). After 5 minutes, cells were scrapered, transfer in an eppendorf tube, putted on ice, vortexed every 5 minutes for 30 minutes and centrifuged at 14 000 g for 10 minutes at 4°C. Supernatant was carefully pipetted into a new eppendorf tube. The cytoplasmic and nuclear proteins were obtained from 70% cultures. The cytoplasmic lysis buffer contained the following: 50mM HEPES pH 7.5, 150mM NaCl, 1% Triton X-100, 1.5mM MgCl2, EGTA 10mM pH 7.5, glycerol 10%, inhibitors (0.1mM Na3VO4, 1% PMSF, 20 mg/ml aprotinin). Following the collection of cytoplasmic proteins, the nuclei were lysed with the buffer containing 20mM HEPES pH 8, 0.1mM EDTA, 5mM MgCl2, 0.5M NaCl, 20% glycerol, 1% NP-40, inhibitors (as above).

Determination of lysates concentration

The protein concentration was measured by Bradford protein assay, which is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 (Bio-Rad) shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The first step was the preparation of a standard curve of absorbance using six solutions with increasing concentrations of BSA (1, 2.5, 5, 10, 15, 25 µg/µL). The concentration of sample was determined by adding 5 µL of each sample to 1 mL of reagent in a cuvette and measuring the absorbance at 595 nm.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were previously reduced with laemmlie buffer 6.8 pH (4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.004% bromophenol blue, 0.125% Tris-HCl) at 100°C for 5 minutes and then loaded onto 11% SDS-polyacrylamide gel with Thermo Scientific PageRuler Plus

Prestained Protein Ladder (Thermofisher). The electrophoresis was performed using 1X glycine buffer (1 M pH 7.5 Tris, SDS, glycine) at 90 Volt.

Transfer of protein

The gel were putted on a nitrocellulose membrane with 0.45 µm pore size and then inside two whatman paper filters and two sponges. The proteins were transferred onto nitrocellulose membrane using Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) with transfer buffer (1 M pH 7.5 Tris, SDS, glycine and methanol) at 90 Volts for 1 hour on ice.

Immunodetection

Membrane was blocked in 5% milk in Tris-buffered saline-Tween 1X (TBST: 0.1% Tween-20, 20 mM Tris, 150 mM NaCl, 5% BSA) shaking for 1 hour at room temperature, washed tre times with TBST and incubated overnight with the primary antibody at 4°C. Primary antibody dilutions were performed in 5% BSA in TBST 1X. Membrane was washed 3 times with TBST, incubated with the HRP conjugated secondary antibody for 1 hour at room temperature and then washed 3 times in TBST. At this point, proteins were detected using Clarity Western ECL Blotting Substrates (Bio-Rad) for 5 minutes. Images were captured using UltraCruz Autoradiography Film (Santa Cruz Biotecnology) or Odyssey Fc Imaging System (LI-COR Biosciences). The images were acquired by using Epson Perfection scanner (Epson, Japan) using Photoshop software (Adobe). The optical densities of the spots were analyzed by using ImageJ software (NIH; http://rsb.info.nih.gov/IJ) or Image studio software (LI-COR Biosciences).

- **Immunoprecipitation**

Total cell proteins were obtained from 70% confluent cells. Primary Ab weas incubated with protein A/G agarose (Santa Cruz Biotechnology) at 4ºC for 2h in PBS buffer. In control samples, the primary antibody was substituted with IgG. Then were added protein lysates and incubated at 4ºC over-night. The immunoprecitated proteins were washed three times with PBS buffer and separated on 11% polyacrylamide denaturing gel as previously described.

- **Chromatin Immunoprecipitation (ChIP) assay and PCR/real time PCR ChIP**

MCF-7 cells were grown in 15 cm dishes to 50-60% confluence, shifted to PRF for 24 h and then treated with 10^{-8} M E2 and/or 10^{-7} M DHT or left untreated in PRF-CT for 2h.

Thereafter, cells were washed twice with PBS and crosslinked with 1% formaldehyde at 37°C for 10 min. Next, cells were washed twice with PBS at 4°C, collected and resuspended in 200 µL of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl pH 8.1) and left on ice for 10 min. Then, cells were sonicated four times for 10 s at 30% of maximal power (Sonics,

Vibra Cell 500 W) and collected by centrifugation at 4°C for 10 min at 14,000 g. The supernatants were diluted in 1.3 ml of IP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl pH 8.1, 16.7 mM NaCl) followed by immunoclearing with 80µL of sonicated salmon sperm DNA/protein A agarose (UBI, DBA Srl) for 1 h at 4°C. Immuno-cleared chromatin was precipitated with anti-AIB1, anti-ERα, anti-AR or anti-BAD antibody. At this point, 60 µL salmon sperm DNA/protein A agarose were added and precipitation was further continued for 2 h at 4°C. After pelleting, precipitates were washed sequentially for 5 min with the following buffers: Wash A (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.1, 150 mM NaCl), Wash B (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.1, 500 mM NaCl), and Wash C (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris–HCl pH 8.1), and then twice with TE buffer (10 mM Tris, 1 mM EDTA). The immunocomplexes were eluted with elution buffer (1% SDS, 0.1 M NaHCO3). The eluates were reverse crosslinked by heating at 65°C and digested with proteinase K (0.5 mg/ml) at 45°C for 1 h. DNA was obtained by phenol/chloroform/isoamyl alcohol extraction. 2 µL of 10 mg/ml yeast tRNA (Sigma) were added to each sample and DNA was precipitated with 70% ethanol for 24 h at -20°C, and then washed with 95% ethanol and resuspended in 20 µL of TE buffer.

Immunoprecipitated DNA was analyzed in triplicates by real-time PCR by using 5 µl of the diluted (1:3) template DNA. The following primers (Invitrogen) spanning the AP-1 site of the Cyclin D1 promoter were used: forward 5'- CTTCGGTGGTCTTGTCCCA- 3' and reverse 5'- CTTCCCGTGCCGGCAATTTA- 3' Real-time PCR data were normalized with respect to unprocessed lysates (input DNA). Inputs DNA quantification was performed by using 5 µl of the diluted (1/50) template DNA. The relative antibody bound fractions were normalized to a calibrator that was chosen to be the basal, untreated sample. Final results were expressed as percent to the relative inputs.

Immunoprecipitated DNA was also analyzed by PCR by using 2 µL volume of each sample. The following specific primers pairs were used to amplify the ARE-containing cyclin D1 promoter fragment: 5'-TACCCCTTGGGCATTTGCAACGA-3' (forward); 5'-ACAGACGGCCAAAGAATCTCA-3' (reverse), and the AP-1-site containing cyclin D1 promoter fragment 5'-CTGCCTTCCTACCTTGACCA-3' (forward); 5'-TGAAGGGACGTCTACACCCC-3' (reverse). The amplification products were analyzed in a 2% agarose gel and visualized by ethidium bromide staining. The negative control was provided by PCR amplification without DNA sample.

The specificity of reactions was ensured using normal mouse IgG (Santa Cruz Biotechnology).

RNA silencing

AIB1 silencing experiments were performed using Stealth™ Select RNAi (Invitrogen Life Technologies) annealed duplexes. Non-specific (NS) siRNA was used as a control for non-sequence-specific effects. Cells were transfected with 100 pmol of siRNA AIB1 or NS siRNA, using Lipofectamine 2000 (Invitrogen Life Technologies), following manufacturer's instructions.

Tunel Assay

Apoptosis was determined by Dead End TM Fluorometricterminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) System (Promega) according to the manufacturer's instructions. After treatment the slides were immersed in 4% formaldehyde in PBS for 25 minutes at 4°C. The slides were washed twice in PBS for 5 minutes each time and cells were permeabilized by immersing slides in 0.2% Triton® X-100 in PBS for 5 minutes. After that, slides were washed twice in PBS for 5 minutes each time and then equilibrated with 100µl of equilibration buffer at room temperature for 5–10 minutes. Thus, 50µl of TdT reaction mix were added to the cells on an area no larger than 5 square centimetres without allow cells to dry completely. Slides were covered with plastic coverslips to ensure even distribution of the mix and incubated for 60 minutes at 37°C in a humidified chamber, by avoiding exposure to light from this step forward. The reaction was stopped by removing plastic coverslips and by immersing slides in 2X SSC for 15 minutes. Slides were washed three times in PBS for 5 minutes each time and mounting medium was added. To visualize all nuclei, Vectashield® with DAPI was used and the localized green fluorescence of apoptotic cells was detected by fluorescence microscopy.

DNA affinity precipitation assay

Nuclear extracts were obtained from cells stimulated with 10^{-7} M DHT for 2 h. 50 µg of nuclear proteins were mixed with 2 µg of specific biotinylated DNA probes (see below) in 400 µl of BufferD (20mMHEPES, pH7.9, 10% glycerol, 50mMKCl, 0.2mM EDTA, 1.5 mM MgCl2, 10 mM ZnCl2, 1 mM dithiothreitol, and 0.25% Triton X-100) and then incubated on ice for 45 minutes. After that, 50 µl of streptavidin-agarose beads (Invitrogen) were added, and the samples were agitated for 2 h at 4 °C. Next, the agarose beads-protein complexes were collected by brief centrifugation and washed twice in Buffer D. Proteins were uncoupled from DNA probes by the addition of 40 µl of SDS loading buffer and heating at 96 °C for 10 min. After removal of the beads, the supernatants were analyzed by WB for the presence of AR and BAD. The DNA motif probes were prepared by annealing a biotinylated sense oligonucleotide (for ARE, 5-Bio-GCTAAATTAGTTCTTGCAATTTAC - 3; for AP-1, 5-

Bio-AATGAGTCAGAATGGAGA-3) with nonbiotinylated antisense oligonucleotide (for ARE, 5-GTAAATTGCAAGAACTAATTTAGC; for AP-1, 5-GTGATCTCCCATTCTGACTCATT-3). Unlabeled probes were used as negative controls. As an additional control, a 10-fold excess of unlabeled probes was added to the nuclear lysates 30 min prior to the addition of the labeled probes to block specific probe-protein interactions.

- **Immunofluorescence assay**

Cells were platelet on 12-mm glass coverslips. After treatment cells were washed three times with PBS, fixed with 4% paraformaldehyde for 20 minutes, permeabilized with 0.2% Triton X-100 for 5 minutes, and incubated with 5% BSA to block non-specific for 1 hour at room temperature.. At the end of each step samples were washed three times with PBS. Blocked samples were incubated with primary anti-body BAD or α-SMA or AR at 4°C over-night and then with fluorescein-conjugated secondary at room temperature for 1 hour. DAPI (Sigma) staining was used for nuclei detection. Protein cellular localization was observed under a fluorescence microscope (Olympus BX51 fluorescence microscope; Olympus Italia S.R.L.). Cells were photographed at 100 magnification using ViewFinder Software, through an Olympus camera system dp50.

- **Immunocytochemical staining**

Cancer associated fibroblasts were grown on 12-mm coverslips. Cells were fixed with 2% paraformaldehyde for 30 minutes and then incubated with 3% H_2O in absolute methanol for 30 minutes to inhibit endogenous peroxidase activity and 15 % normal goat serum for 30 minutes to block non-specific binding of antibody. Anti-human Androgen Receptor (441) primary antibody or normal rabbit serum as control were added overnight at 4°C and goat anti rabbit antibody for 1h at room temperature was used as secondary antibody. Avidin-biotin horseradisch peroxidase complex (ABC complex/horseradish peroxidase complex) was applied for 30 minutes, and the chromogen 3–3- diaminobenzidine tetrachloride dihydrate was used as the detection system for 5 minutes. At the last, cells were visualized with 3,3'-Diaminobenzidine (DAB). Every step was followed by washing with TBS-T TBS-T (0.05 M Tris-HCl plus 0.15 M NaCl, pH 7.6, containing 0.05% Triton X-100).

- **Wound-healing scratch assay**

Cancer associated fibroblast and breast cancer cells were seeded on six-well plates. Monolayers were scratched with a pipette tip and cell debris were removed by aspirating the medium and by washing the cells twice with PBS. The resulting wound was monitored

during the time. After 24 and 48h of incubation, cells were fixed and stained with Coomassie brilliant blue (0,25 g Comassie Brillant Blue, 45 ml Methanol, 45 ml H_2O, 10 ml glacial acetic acid) and pictures were taken using phase-contrast microscopy at 10X magnification.

- **F-actin staining assay**

Alexa Fluor® 568 phalloidin (Life Technologies) was used to visualize F-actin in cell cultures. For adherent cells grown on glass coverslips, the staining procedure was performed by following manufacter's protocol. Cells were washed twice with prewarmed phosphate-buffered saline, pH 7.4 (PBS) and fixed the sample in 3.7% formaldehyde solution in PBS for 10 minutes at room temperature. After two or more washes with PBS, each coverslip was placed in a glass petri dish and incubated with a solution of acetone at =–20°C or 0.1% Triton X-100 in PBS for 3 to 5 minutes. Slides were washed two or more times with PBS and staining solution (5 µL methanolic stock solution into 200 µL PBS for each coverslip) was placed on the coverslip for 20 minutes at room temperature (generally, any temperature between 4°C and 37°C is suitable). To avoid evaporation, the coverslips were kept inside a covered container during the incubation. To reduce nonspecific background staining with these conjugates, 1% bovine serum albumin (BSA) was added to the staining solution. Slides were washed two or more times with PBS and cell nuclei were stained with DAPI. Pictures were obtained using an Olympus BX51 fluorescence microscope at 100 magnifications.

- **Boyden-chamber transmigration assay**

MCF-7 cells (60000 cells/chamber) were seeded into the top of 8 µm pore size 24-well transwell chambers in DCC 5%. CAFs-CM or CAFs seeded in the bottom were used as chemoattractant. After 24h, migrated cells were fixed with 4% paraformaldehyde, stained with DAPI and quantified by viewing five separate fields/membrane (10magnification) and expressed as mean numbers of migrated cells.

- **Matrigel-based invasion assay**

Invasion assay was performed using 8 µm pore size 24-well transwell chambers, pre-coated with Matrigel (0.4 µg/mL; BD Biosciences). MCF-7 cells (60000/chamber) were seeded with CAFs-CM into the top of chambers; regular full medium was used as chemoattractant. After 48h, invaded cells were quantified as reported for transmigration assay.

- **Cytokine array**

Conditioned media derived from CAFs were collected and clarified by centrifugation. The content of soluble factor was analysed by using Proteome Profiler Human XL cytokine arrays

according to the manufacturer's instructions (R&D Systems). 2.0 mL of Array Buffer 6 were pipetted into each well of the 4-Well Multi-dish. Each membrane was placed in a separate well and incubated for one hour on a rocking platform shaker. The samples were prepared by diluting the desired quantity to a final volume of 1.5 mL with Array Buffer 6. Array Buffer 6 was aspirated from the wells of the 4-Well Multi-dish and the prepared samples was added. The lid was placed on the 4-Well Multi-dish and incubated overnight at 2-8 °C on a rocking platform shaker. Each membrane was placed into individual plastic containers with 20 mL of 1X Wash Buffer. The 4-Well Multi-dish was rinsed with deionized or distilled water and dried thoroughly. Each membrane was washed for three times with 1X Wash Buffer for 10 minutes on a rocking platform shaker. For each array, 30 µL of Detection Antibody Cocktail were added to 1.5 mL of 1X Array Buffer 4/6. 1.5 mL of diluted Detection Antibody Cocktail was pipetted into the 4-Well Multi-dish. Each array was removed from its wash container, returned to the 4-Well Multi-dish containing the diluted Detection Antibody Cocktail and incubated for 1 hour on a rocking platform shaker. After washes into wash container, 2.0 mL of 1X Streptavidin-HRP were putted into each well of the 4-Well Multi-dish. The membrane were placed into the 4-Well Multi-dish containing the 1X Streptavidin-HRP and incubated for 30 minutes at room temperature on a rocking platform shaker. After washes into wash container, each membrane was placed on the bottom sheet of the plastic sheet protector with the identification number facing up. 1.0 mL of the prepared Chemi Reagent Mix was evenly placed onto each membrane and incubated for 1 minute. The membranes were placed with the identification numbers facing up in an autoradiography film cassette and 30 seconds, 1, 5 and 10 minutes exposure times were performed to detect signals. Spots density was measured using ImageJ software. Resulted values are the average from duplicate spots.

- **Zymography assay**

50 µL of CAFs-CM were precipitate with 200 µL of cold acetone at 20°C for 1h, after which the samples were centrifuged at 13000 g for 10 minutes. The supernatant was allowed and the pellet was suspended with 12 µL of solubilizing (TrisHcl 0,25M pH=6,8, SDS 10X, glicerolo, blu di bromofenolo). Samples were separates by electrophoresis onto 7,5% SDS-polyacrylamide of 0,75 mm containing 1% of porcin skin gelatin. Gel was washed with wash buffer (50 mM Tris-HCl, pH 7.5; 10 mM CaCl2; 2,5% Triton X-100) on rotation at 37°C for 30 minutes and then incubated with incubation buffer (50 mM Tris-HCl, pH 7.5; 10 mM CaCl2; 1% Triton X-100) on rotation at 37°C over-night. The Coumassie Brillant Blue solution (methanol, bidistilled water, glacial acetic acid, Coumassie Brillant Blue) was added for 10 minutes on a rocking platform shaker, wich was remuved by bleach solution (methanol,

bidistilled water, glacial acetic acid). The images were acquired by using an Epson Perfection scanner (Epson).

Mammosphere forming efficiency assay

Standard tissue culture plates were coated with poly (2- hydroxyethyl methacrylate) (pHEMA) as follows: dissolving 12 g pHEMA in 1 l of 95 % ethanol by stirring constantly on a heated plate. Once the solution was cooled, 1 ml/well was pipetted into a 6-well plate and placed in an oven at 40°C and left for 48 h, ensuring that the plates remained sterile.

1. Culture and detach cells at 70–80 % confluency according to standard protocols.
2. Centrifuge at 580 g for 2 min.
3. Remove supernatant and resuspend in 1–5 ml of ice cold PBS.
4. Use a 25 G needle to syringe the cell suspension three times, to ensure a single cell suspension has formed.
5. Use a haemocytometer to confirm a single cell suspension is present (if it is not a single cell suspension, syringe a further three times) and calculate the number of viable cells per ml using trypan blue.
6. Add 2 ml of mammosphere media (phenol red-free DMEM/F12 (Gibco) containing B27 supplement (no vitamin A; Invitrogen) and rEGF (20 ng/ml; Sigma)) or CAFs-CM to each well in a 6- well plate.
7. Plate 3000 cell/well in triplicate.
8. Incubate in a humidified atmosphere at 37°C and 5 % CO_2 for 5 days without moving or disturbing the plates and without replenishing the media.
9. After 5 days, count the number of mammospheres (at x40 magnification) which are greater than 50 μm diameter using a microscope fitted with a graticule.
10. Mammosphere forming efficiency (%) is calculated as follows:

(number of mammospheres per well=number of cells seeded per well)*100

Statistical analysis

Statistical analysis was performed using ANOVA followed by Newman-Keuls' testing to determine differences in means. All data are reported as the mean ± SD of three different experiments, each performed in triplicates. * $p \leq 0.05$ vs control.

3. AIB1 squelching as a mechanism contributing to the inhibition by androgens of estrogen-dependent cyclin D1 expression in breast cancer cell

3.1 Inhibition of estrogen-dependent proliferation by androgen receptor over-expression

We previously demonstrated that MCF-7 cells are androgen-responsive and that DHT treatment induces a transient increase in AR protein levels (Marilena Lanzino et al., 2005) similar to that seen in other cell types (Kemppainen, Lane, Sar, & Wilson, 1992; Yeap, Krueger, & Leedman, 1999).

Like the other steroid receptor, estrogen receptor transcriptional activity is regulated by co-activators. Cyclin D1 is a key regulator of estrogen-induced cell proliferation. In particular, the co-activator AIB1 is crucial in ER-α promoting cyclin D1 gene transcription (Planas-Silva, Shang, Donaher, Brown, & Weinberg, 2001).

Here we investigated the role of DHT-dependent signalling on the E2-induced proliferation of the ERα-positive MCF-7 cells and if a squelching of the AIB1 co-activator between AR and ER-α could exists. Consistent with previous reports (Andò et al., 2002; Marilena Lanzino et al., 2010), prolonged DHT administration resulted in a significant reduction of basal as well as E2-dependent MCF-7 cell proliferation (Fig. 3.1). To better investigate the role of androgen receptor, MCF-7 cells were transiently transfected with the pcDNA3-AR expressing the full length AR (MCF-7 cells/AR). The ectopic overexpression of AR "*per se*" reduced E2-dependent cell proliferation and further potentiates the inhibitory effects determined by DHT administration. Addition of the androgen antagonist hydroxyflutamide (OHFl) effectively reversed the inhibition of E2-induced cell growth exerted by DHT, suggesting that the effect was mediated by AR.

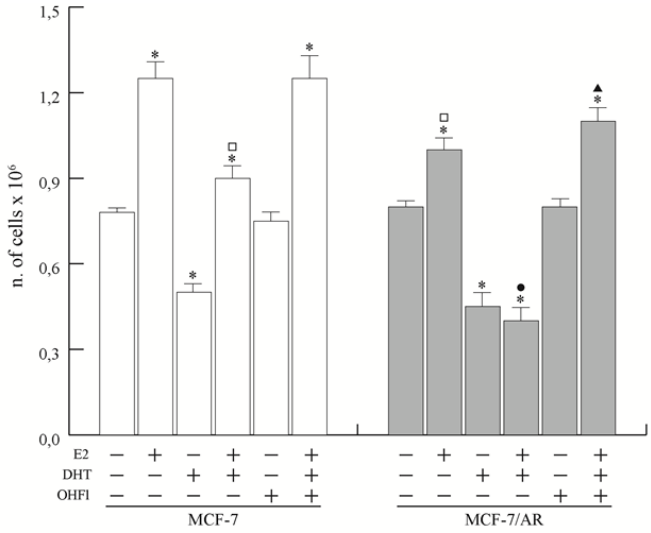

Figure 3.1 Over-expressed androgen receptor inhibits E2-dependent MCF-7 cells proliferation. MCF-7 cells and and MCF-7 transiently over-expressing AR (MCF-7/AR), were synchronized in PRF and treated with 10-7 M E2, and/or 10-7 M DHT, and/or 10-6 M OH-Fl in steroids depleted PRF-CT for 3 days. Data represent a mean±s.d. of three independent experiments, each in duplicate. *p ≤0.05 vs untreated MCF-7 cells; □p ≤0.05 vs. E2-treated MCF-7 cells; ●p ≤0.05 vs E2+DHT treated MCF-7 cells; ▲p ≤0.05 vs E2+DHT treated MCF-7/AR.

3.2 Inhibition of E2-induced cyclin D1 gene expression and promoter activity by androgen receptor over-expression

Since a key rate-limiting event in mitogenic estradiol signalling leading to S-phase entry is the induction of cyclin D1 (Musgrove, Lee, Buckley, & Sutherland, 1994) we investigated whether AR activation and/or its over-expression might modulate cyclin D1 expression.

To this aim, MCF-7 cells were transiently transfected with an empty vector or with a full length AR expression plasmid and left untreated or treated with E_2 and/or DHT for 48 hours. A significant reduction in the E_2-induced cyclin D1 protein expression levels was observed following DHT co-treatment in MCF-7 cells. Interestingly, AR overexpression *per se* determined a decrease of cyclin D1 protein content in response to E_2 stimulation, which was further reduced following DHT coadministration (Fig. 3.2A). A similar regulatory pattern was observed in terms of mRNA expression levels (Fig. 3.2B).

Next, we examined the possibility that AR activation by its own ligand and/or AR over-expression might negatively modulate the E_2/ERα induced cyclin D1 promoter transcriptional activity.

As shown in Fig. 3.2 C, in MCF-7 cells, a cyclin D1 promoter construct driving luciferase expression was induced by E_2 but significantly inhibited following DHT co-administration. Besides, the overexpression of AR resulted in the complete loss of the transcriptional signal induced by E_2 when compared with hormone stimulated activity in the absence of exogenous AR. Additionally, in these experimental conditions, a further decrease in E_2-dependent cyclin

D1 promoter activity was observed following DHT treatment. The androgen-dependent inhibition of E_2-activated signalling on cyclin D1 gene promoter was abrogated by the addition of the androgen antagonist OHFl, confirming the involvement of AR.

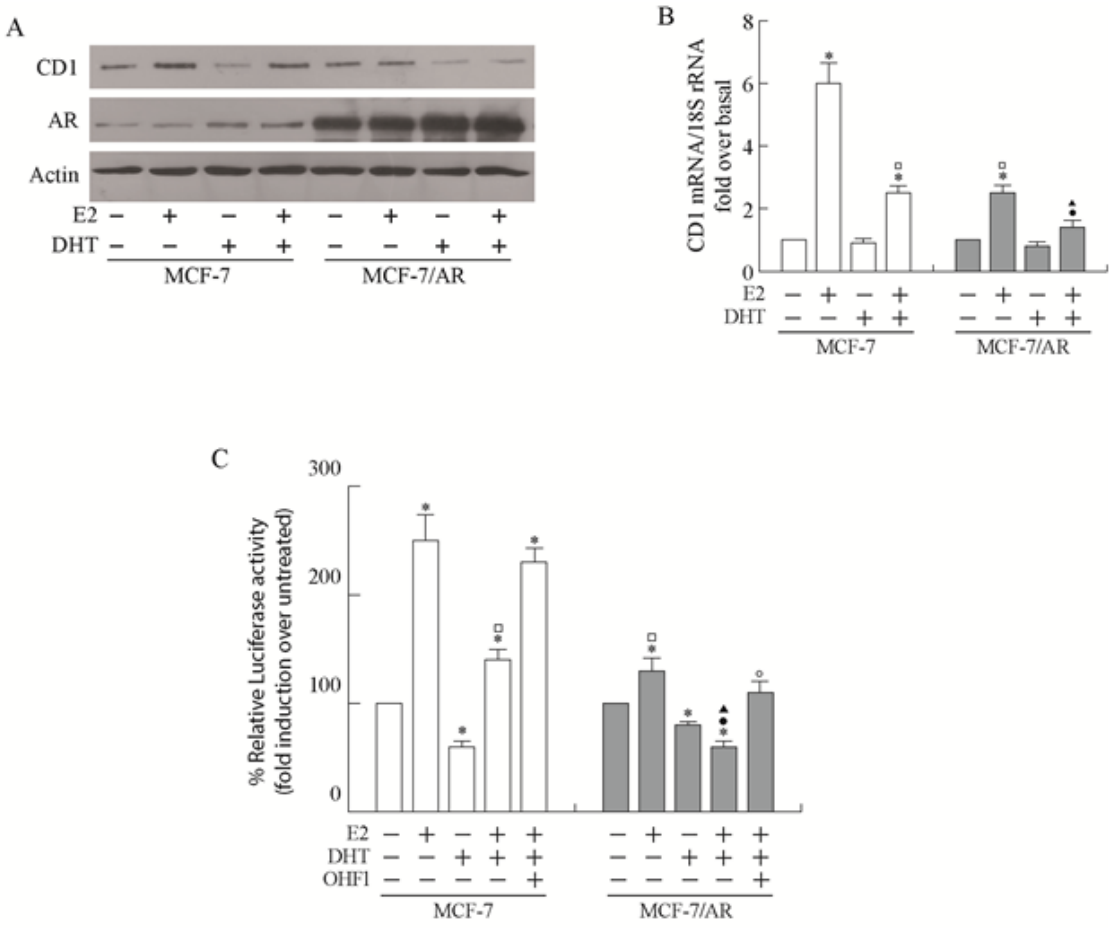

Figure 3.2 Estrogen induction of cyclin D1 expression and promoter activity is reduced by over-expression of androgen receptor. (A) Western blotting analysis of Cyclin D1 (CD1). MCF-7 and MCF-7/AR cells were treated as indicated. Actin was assessed as control of protein loading. (B) Quantitative Real Time RT–PCR from MCF-7 and MCF-7/AR cells treated as indicated. 18S rRNA was determined as control. Columns are the mean of three independent experiments each in triplicate; bars, SD; *p ≤0.05 vs untreated MCF-7 cells; □p ≤0.05 vs. E2-treated MCF-7 cells; ●p ≤0.05 vs E2+DHT treated MCF-7 cells. (C) MCF-7 and MCF-7/AR cells were transiently transfected with pCD1prom-Luc and treated as indicated. Columns are mean of three independent experiments and expressed as fold induction over untreated, which was assumed to be 100%; bars SD; *p ≤0.05 vs untreated MCF-7 cells; □p ≤0.05 vs. E2-treated MCF-7 cells; ●p ≤0.05 vs E2+DHT treated MCF-7 cells; ▲p ≤0.05 vs. E2-treated MCF-7/AR cells; ○p ≤0.05 vs. E2+DHT treated MCF-7/AR cells.

3.3 AIB1 overexpression rescues AR repression of estradiol-induced transcriptional activity of cyclin D1 promoter

The capacity of AR to compromise the transcriptional response dependent on a second receptor such as ERα, implies that shared components of the transcriptional machinery are involved (Marilena Lanzino et al., 2005; Torchia, Glass, & Rosenfeld, 1998). Therefore AR and ERα might use a common pool of co-factors present in limiting cellular concentrations. In this regard, we investigated the role of the steroid receptor coactivator AIB1 that is important in the functional coupling of ERα with the cyclin D1 promoter (Furth, Cabrera, Díaz-Cruz, Millman, & Nakles, 2011; Planas-Silva, Shang, Donaher, Brown, & Weinberg, 2001).

We first used an AIB1 siRNA approach, to selectively reduce AIB1 expression in MCF-7 cells. The AIB1 siRNA produced a >80% reduction in cellular AIB1 protein levels, which were still repressed after 72 hours (Fig. 3.3A). As shown in Fig 3.3B treatment with AIB1 siRNA completely negated the increase in cyclin D1 (CD1) protein expression induced by estradiol.

Interestingly, this pattern of cyclin D1 expression is similar to the one observed following AR over-expression (Fig. 3.3B), supporting the hypothesis that AIB1 is essential for E2-dependent cyclin D1 expression.

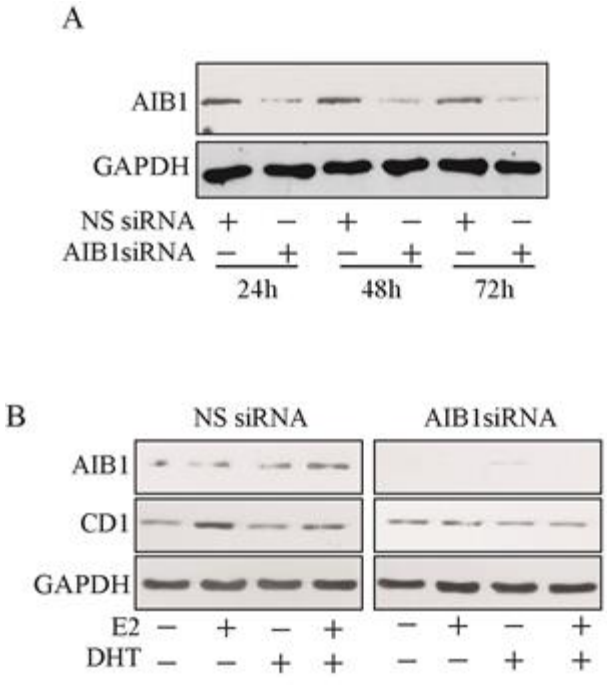

Figure 3.3 AIB1 is crucial for E2-induced Cyclin D1 (CD1) expression (A) Western blotting analysis of AIB1. MCF-7 cells were transfected with non-specific (NS) or targeted against AIB1 siRNA at different times, as indicated. GAPDH was used as loading control. (B) Western blotting analysis of AIB1 and CD1. MCF-7 cells

were transfected with non-specific (NS) or targeted against AIB1 siRNA and treated as indicated. GAPDH was used as loading control. Results are representative of three independent experiments.

Thus to assess whether AIB1 squelching might be involved in the transcriptional interference of AR on ERα transcriptional signal, we tested whether AIB1 overexpression could rescue AR repression of estradiol-induced transcriptional activity on cyclin D1 promoter.

As shown in Fig. 3.4A, progressively increasing amounts of ectopic AIB1 were able to restore the E2-dependent activation of cyclin D1 promoter activity, although in the presence of exogenous AR expression. Thus, AIB1 over-expression is able to completely abrogate the inhibitory effect induced by overexpressed AR and to re-establish E2-induced activity of cyclin D1 promoter in MCF-7 cells.

3.4 AIB1 interaction with either AR or ERα is related to the intracellular content of both steroid receptors

As AIB1 has been reported to be capable to bind and coactivate both ERα and AR (Nakles et al., 2011; Zhou et al., 2010) we investigated if AIB1 interaction with ERα and/or AR might be influenced by the intracellular levels of the two steroid receptors. To this aim, AIB1/ERα and/or AIB1/AR complex formation was analysed by co-immunoprecipitation assay in both MCF-7 and MCF-7/AR cells (Fig. 3.4B). Interestingly, in MCF-7 cells, which express high levels of endogenous ERα and low levels of AR (Marilena Lanzino et al., 2005), AIB1 co-immunoprecipitates predominantly with ERα in all the examined experimental conditions. On the contrary, following AR overexpression, a prevalent interaction of AIB1 with AR was observed. Specifically, in MCF-7/AR overexpressing cells we found a substantial decrease of AIB1/ERα complex and a concomitant increase of AIB1/AR interaction, compared to MCF-7 cells. As depicted in Fig. 3.4 C similar experimental conditions reproduced in Hela cells co-expressing ectopic AIB1, ERα and AR, determined analogous results. Indeed, to further prove whether AIB1 is capable to interact with AR and/or ERα in relationship to their intracellular levels, we next performed studies with HeLa cells, a well-known experimental model, which do not express AR or ERα (Kousteni et al., 2001; Smith, Oñate, Tsai, & O'Malley, 1996). In these experiments, coimmunoprecipitation assays were carried out in HeLa cells transiently cotransfected with both ERα and AR in a ratio of ERα /AR = 1:5 or at a ratio of ERα /AR = 5:1 in the presence of an excess of AIB1. Again, when AR content is higher than ERα (ratio ERα/AR = 1:5), AIB1 coimmunoprecipitates mainly with AR while, in the presence of an excess of ERα (ratio ERα /AR = 5:1), AIB1 primarily coimmunoprecipitates with ERα (Fig. 3.4 C).

It has been reported that AIB1 is required for ERα recruitment onto the estrogen responsive region of the cyclin D1 promoter (Planas-Silva et al., 2001; Sabbah, Courilleau, Mester, & Redeuilh, 1999). Thus, to highlight the biological implication of AR/ERα competition for a shared coactivator such as AIB1 in the regulation of cyclin D1 promoter, we evaluated its recruitment on cyclin D1 promoter by chromatin immunoprecipitation (ChIP) assay in MCF-7 and MCF-7/AR cells (Fig. 3.4D). Protein-chromatin complexes were immunoprecipitated using specific antibodies against AIB1 or ERα. The presence of the specific promoter sequence in the chromatin immunoprecipitates was analyzed by Real-time PCR using specific primers spanning the estrogen-responsive region of the cyclin D1 promoter that contains an AP-1 binding site.

As indicated in Fig 3.4D, in MCF-7 cells E2-induced recruitment of AIB1 on cyclin D1 proximal promoter was decreased by DHT co-administration. Alongside, in the same experimental conditions, also E2-dependent ERα binding to the AP-1 containing region was reduced (Fig 3.4E). Interestingly and consistent with the above reported data, AR overexpression greatly counteracts either the AIB1 or ERα occupancy of cyclin D1 promoter induced by E2.

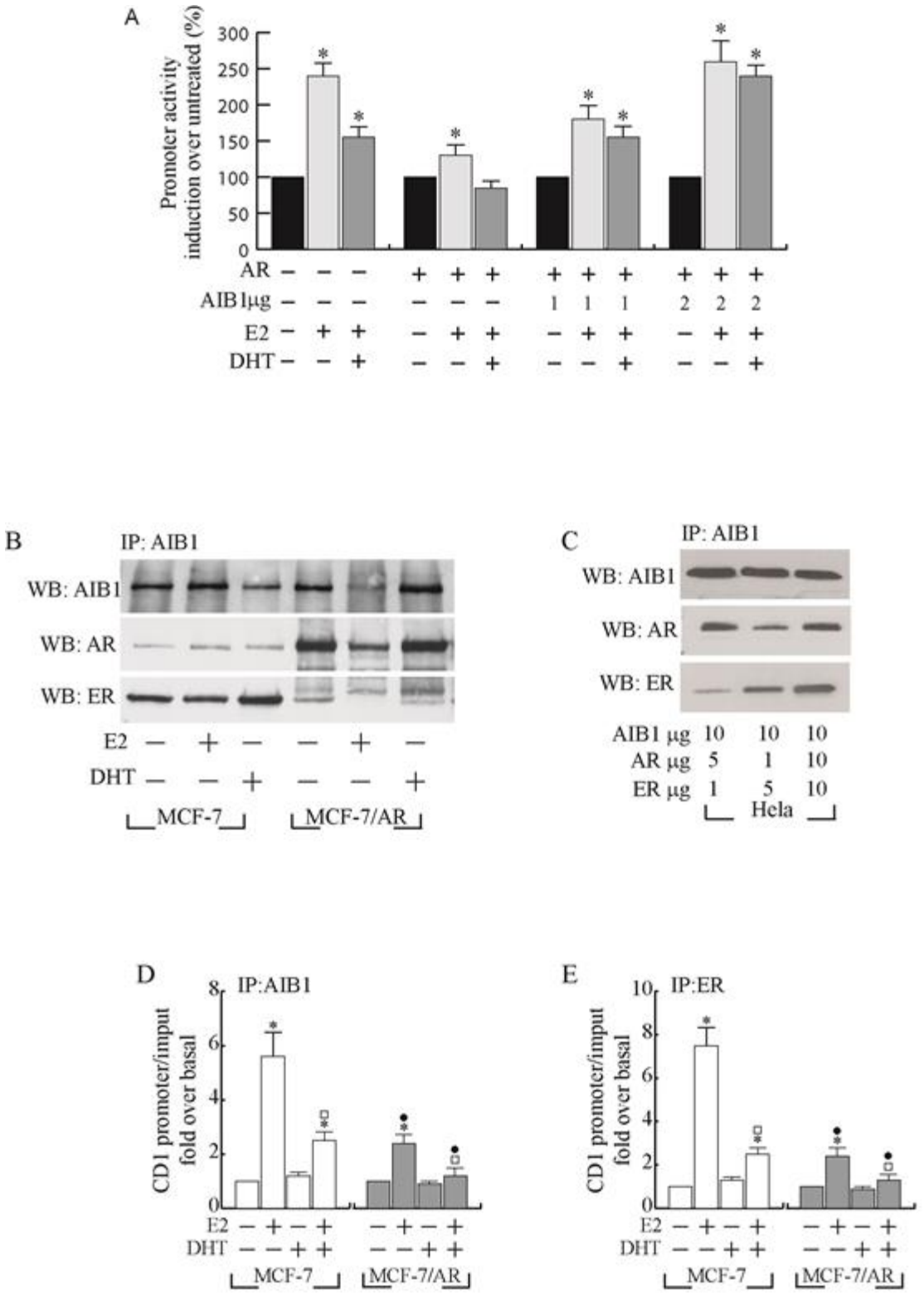

Figure 3.4 Over-expressed androgen receptor competes with ERα for AIB1 interaction. (A) MCF-7 cells, were transiently co-transfected with pCD1prom-Luc (0.25□g /well) and /or pcDNA3-AR (AR) and/or increasing amounts (given in □g/well) of full-length AIB1 expression plasmid (AIB1), and treated as indicated. Columns are mean of three independent experiments and expressed as fold induction over untreated, which was assumed to be 100%; bars SD; *p≤0.05. (B) Total cell extracts from MCF-7 and MCF-7/AR were immunoprecipitated (IP) with an anti-AIB1 antibody and immunoblotted (WB) to detect AIB1, ER and AR protein levels. Results are

representative of three independent experiments. (C) Total cell extracts from Hela cells transiently cotransfected with different amounts (given in □g/dish) of pcDNA3-AR (AR), Hego (ER) or full-length AIB1 expression plasmid as indicated were immunoprecipitated (IP) with an anti-AIB1 antibody and immunoblotted (WB) to detect AIB1, ER and AR protein levels. Results are representative of three independent experiments. (D and E) ChIP-qPCR performed on MCF-7 cells and MCF-7/AR cells using anti-AIB1 (D) or anti- ERα (E) antibodies, as indicated. IgG was used as control. Columns are the mean of three independent experiments. bars, SD; *p ≤0.05 vs untreated MCF-7 cells; □p ≤0.05 vs. E2-treated MCF-7 cells; ●p ≤0.05 vs E2+DHT treated MCF-7 cells.

4. Unravelling the protective role of androgens/androgen receptor in breast cancer: when BAD goes good.

4.1 Androgens increase BAD expression in MCF-7 cells

Cancer cells acquire a highest viability by evading apoptosis that is well recognized as a "hallmark of cancer" (Hanahan & Weinberg, 2000). Therefore, the balance between the anti- and pro-apoptotic members within Bcl-2 family is a crucial role in cancer progression. In accordance with previous data showing synthetic AR agonist mibolerone (Mb) ability to inhibit human MCF-7 breast cancer cell line proliferation by inducing apoptosis (M. Lanzino et al., 2013), our first aim was to investigate the effect of androgens on pro/anti-apoptotic proteins expression following 1, 2 and 3 days of treatment. As showed in Fig. 4.1A, Mb administration caused, as expected a clearly evident AR up-regulation. Androgen treatment did not alter the expression of the anti-apoptotic Bcl-2 protein at any of the tested time point. As it regards pro-apoptotic protein content, cytosolic levels of BID and BAX remained unchanged too. Instead Mb administration induced a significant increase in the expression of the pro-apoptotic protein BAD that was evident after 24h exposure and persisted thereafter. As a consequence of the androgen-induced BAD expression, the Bcl-2/BAD ratio was reduced, shifting the delicate balance between inhibitors and inducers of cell death (Fig. 4.1B). QRT-PCR analysis confirmed the highest BAD expression upon androgen stimulation (Fig.4.1C), since Mb administration increased BAD mRNA levels in MCF-7 treated cells. In addition, also immunofluorescence analysis detected in MCF-7 cells cytoplasm and perinuclear region a clear BAD immunoreactivity, which was enhanced following Mb administration. Yet, more interesting was the observation that the fluorescence signal appeared to be markedly increased into the nuclear compartment, suggesting that androgen treatment caused the nuclear translocation of BAD (Fig. 4.1D), highlighting a potential new and "non-canonical" role for this pro-apoptotic factor. Thus, Mb administration increased not only AR, as expected, but also BAD mRNA and protein levels leading us to speculate a direct effect of AR on BAD expression. Thus, we focused our interest on the pro-apoptotic molecule Bcl-2 antagonist of cell death (BAD), which E2-induced phosphorylation/inactivation abrogates apoptosis in MCF-7 cells (R. I. Fernando & Wimalasena, 2004).

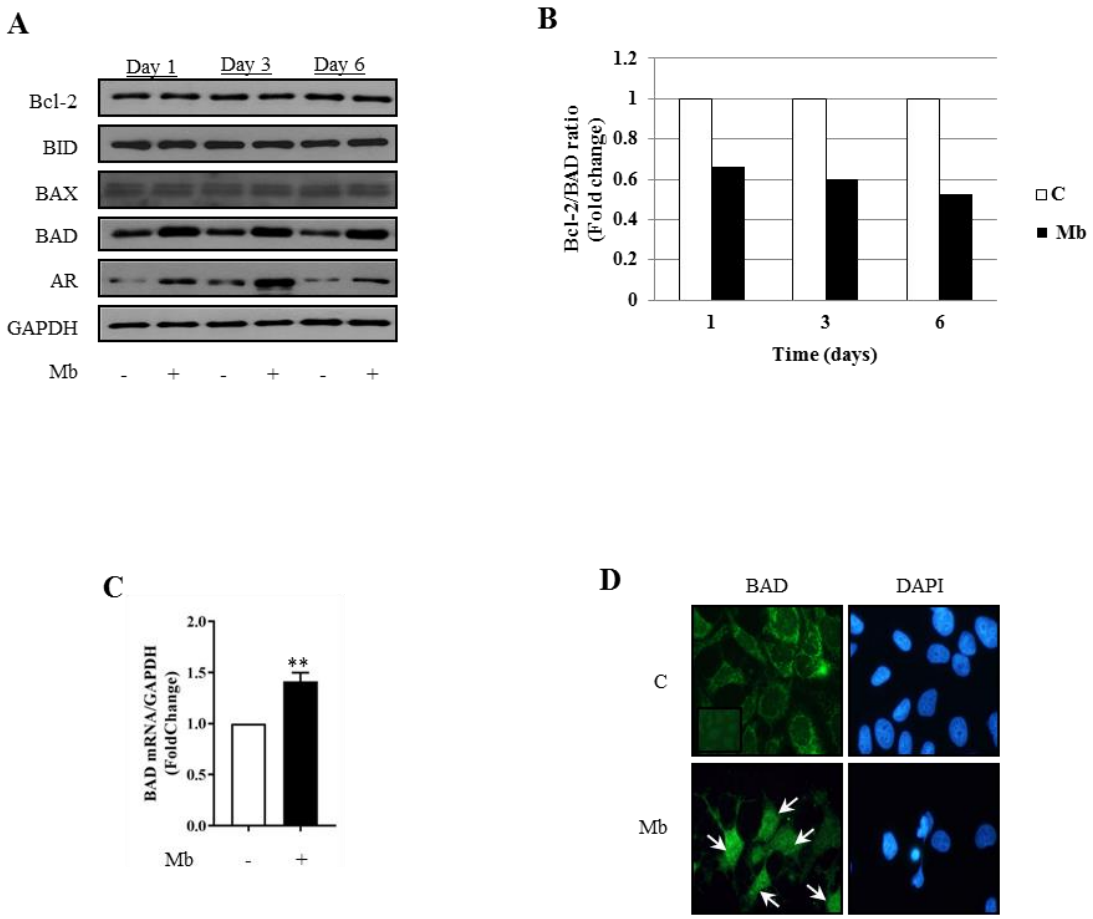

Figure 4.1 Androgens administration increases BAD expression in MCF-7 cells. (A) Western Blotting analysis of anti-apoptotic Bcl-2 and pro-apoptotic BID, BAX and BAD. MCF-7 breast cancer cells were treated with vehicle (-) or 10^{-8}M Mb for 1, 3 and 6 days. (B) Histogram represents the Bcl-2/BAD ratio. Band intensities were evaluated in terms of optical density (O.D.) arbitrary units and normalized to the relative GAPDH content. (C) Quantitative Real-Time RT-PCR from MCF-7 cells treated with vehicle (-) or 10^{-8}M Mb as indicated for 24h. GAPDH was determined as control. Data represent the mean ± S.D. of three separate experiments, each performed in triplicate. n.s. = non-significant; ** $p \leq 0.01$ vs control. (D) Immunofluorescence assay in MCF-7 cells treated with 10^{-8} M Mb for 24h. No immunoreactivity was detected when MCF-7 cells were incubated without the primary antibody (negative control: NC).

4.2 Ligand-activated AR induces BAD nuclear localization

To confirm BAD translocation in the nuclear compartment, western blotting assay was performed on the cytosolic and nuclear fractions of MCF-7 cells. In absence of Mb, BAD was mainly present in the cytoplasmic compartment, while upon Mb treatment, the nuclear abundance of BAD significantly increased. As expected, Mb treatment increased the cellular protein levels of its own receptor AR as well as its nuclear translocation. A similar pattern of BAD cellular levels and localization was also observed in the ERα negative/AR-positive SKBR3 breast cancer cells, even following administration of both Mb or the natural AR

ligand DHT (Fig. 4.2 A, B). These results highlight and strongly suggest a nuclear role for BAD upon androgen stimulation.

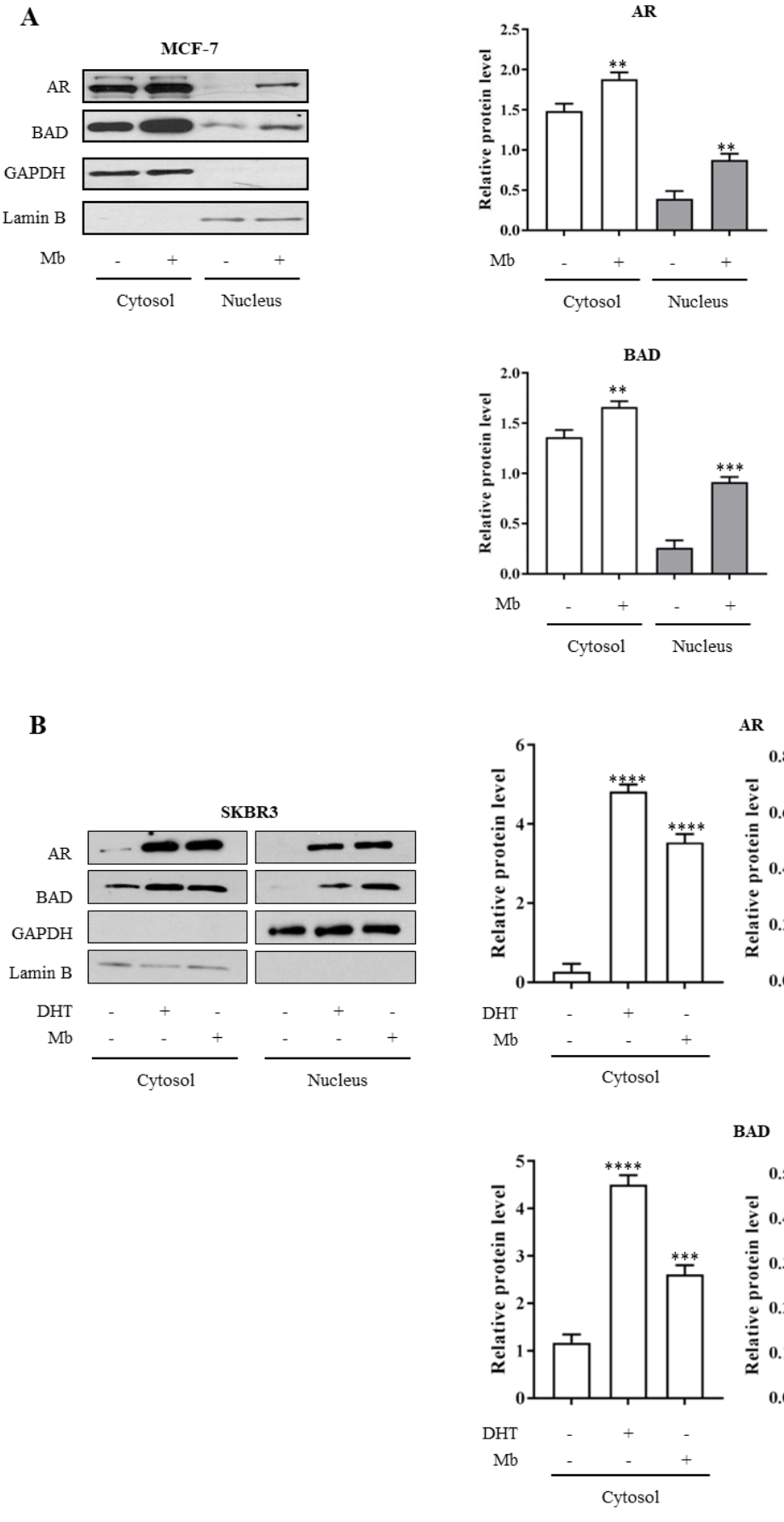

Figure 4.2 Androgens induce BAD nuclear localization. Western Blotting analysis of cytoplasmic and nuclear protein extracts from MCF-7 (A) and SKBR3 (B) breast cancer cells treated for 24h with vehicle (-) or 10^{-7}M DHT or 10^{-8}M Mb as indicated. LAMIN B and GAPDH were used as loading control. Histograms represent the mean±S.D. of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and normalized to the relative LAMIN B or GAPDH content. ** p≤0,01 and ***p≤0.001 vs untreated MCF-7 cells; *** p≤0,001 and ****p=0.0001 vs untreated SKBR3 cells;

4.3 Ligand-activated membrane AR doesn't influence BAD cellular localization in MCF-7 cells

Several observations indicated that, in addition to the classical intracellular androgen receptors, membrane androgen receptors (mAR) are also involved in the regulation of cell growth, motility and apoptosis in several tumors, including breast cancer and that these effect may occur independently of intracellular androgen receptors (Kampa et al., 2005; Pelekanou et al., 2010). Thus, to better understand if the membrane and/or the intracellular AR is able to modulate BAD expression and cellular localization, MCF-7 cells were treated with testosterone conjugated to bovine serum albumin (T-BSA), a compound unable to cross the plasma membrane. Unsurprisingly, T-BSA administration induced apoptosis as indicated by Tunnel Assay (Fig. 4.3A), showing a marked increase in the number of apoptotic nuclei following 6 days of treatment. In addition, western blotting was performed by using cytosolic and nuclear fractions of MCF-7 cells treated or not with T-BAS. As fig. 4.3B shows, Nevertheless, T-BSA administration didn't influence AR or BAD protein levels as well as their cellular localization, which was exclusively cytosolic. This evidence strongly suggests that BAD cellular levels and localization following androgen treatment just rely on activation of the classical intracellular AR.

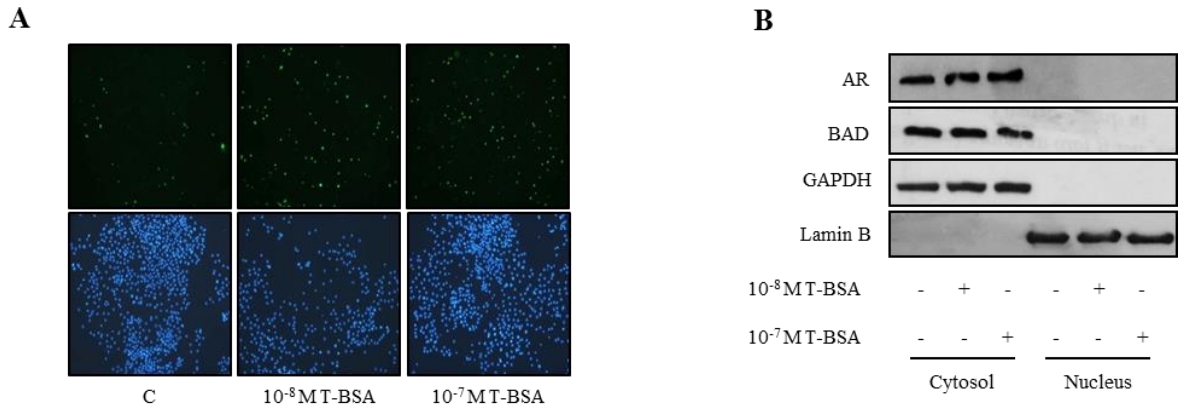

Figure 4.3 Long-term T-BSA administration induces apoptosis without influencing both AR and BAD expression and cellular localization in MCF-7 cells. (A) Cells treated with 10^{-8}M or 10^{-7}M T-BSA for 6 days were subjected to TUNEL nuclear staining and viewed by a fluorescent microscopy. DAPI staining for nuclei

detection. (B) Cytoplasmatic and nuclear protein extracts from MCF-7 cells treated with vehicle (-) or 10^{-8}M or 10^{-7}M T-BSA for 24h. LAMIN B and GAPDH were used as loading control.

4.4 DHT induces the formation of an AR/BAD complex in MCF-7 cells

To explore whether the androgen-regulated intracellular localization of BAD could implicate a physical interaction between AR and BAD, a co-immunoprecipitation assay was performed on cytosolic and nuclear fraction of MCF-7 cells left untreated or treated with DHT. As shown in Fig. 4.4 upon DHT treatment a clear increase of the AR/ BAD complex abundance could be observed into the nucleus. This result confirmed the possibility that AR regulates BAD nuclear translocation.

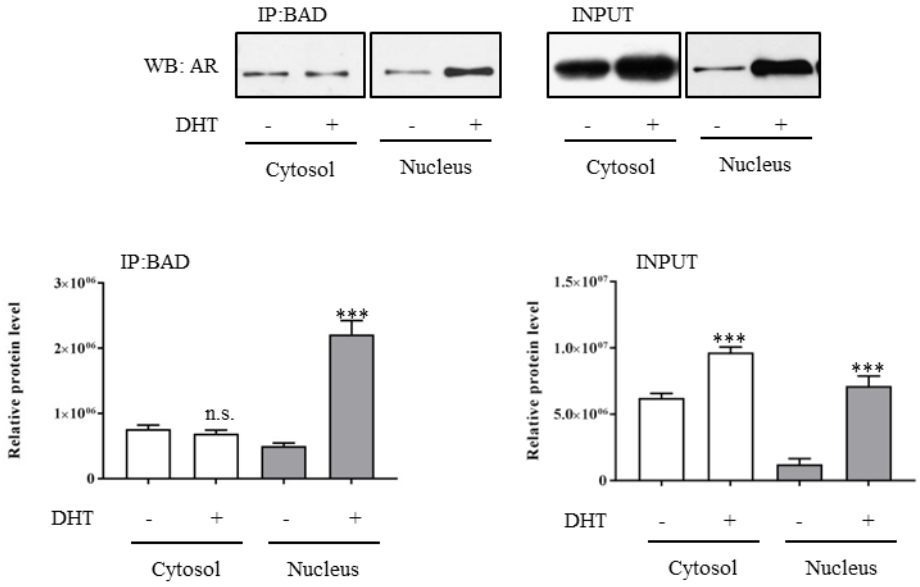

Figure 4.4 AR physically interacts with BAD in MCF-7 cells. 500 µg of either cytoplasmatic or nuclear lysates from MCF-7 cells treated with 10^{-7}M DHT for 24h were immunoprecipitated with anti-BAD Ab and immunoblotted to detect AR protein levels. Results were quantified by densitometric analysis and reported as optical density arbitrary units. n.s.= non-significant; *** $p \leq 0.001$ vs control.

4.5 Androgens induce BAD recruitment at AP-1 and ARE sites on the cyclin D1 promoter

The evidence of a nuclear AR/BAD interaction led us to investigate the biological significance of the role of AR/BAD complex within the nucleus. It has been previously demonstrated that nuclear BAD is able to influence breast cancer cell cycle progression by preventing cyclin D1 transcription, via a negative regulation of c-Jun at an AP-1 site within the cyclin D1 gene promoter (R. Fernando et al., 2007). In addition, cyclin D1 is a specific AR target gene whose repression contribute to explain the inhibitory role of androgens on breast cancer cell proliferation (M. Lanzino et al., 2010). Thus, we investigated whether a cooperation between AR and BAD in modulating cyclin D1 gene promoter might exist.

To this aim a double-stranded oligonucleotide containing the cyclin D1 promoter ARE- or AP-1-site were used in a DNA affinity precipitation assay (DAPA) to examine whether androgen treatment can influence BAD and/or AR protein accumulation at the AP-1 (CCND1-AP-1) and/or ARE (CCND1-ARE) consensus sequences. Figure 4.5A shows that, both AR and BAD were found associated with their own putative consensus oligonucleotide following DHT administration. Interestingly in these experimental conditions a clear association of AR to the AP-1 consensus site was also present. These results suggest that DHT administration induces a nuclear AR/BAD complex that may bind to the cyclin D1 promoter gene through the ARE and the AP-1 binding sites. This notion was further confirmed by ChIP assay experiments using anti-AR or anti-BAD antibodies, indicating that an AR/BAD complex do interacts with this region of the cyclin D1 gene as it exist in native chromatin. Indeed, both AR and BAD occupancy of either the ARE and AP-1 consensus sequences within the cyclin D1 promoter was induced in a ligand-dependent manner, being their recruitment enhanced by DHT administration (Fig. 4.5B).

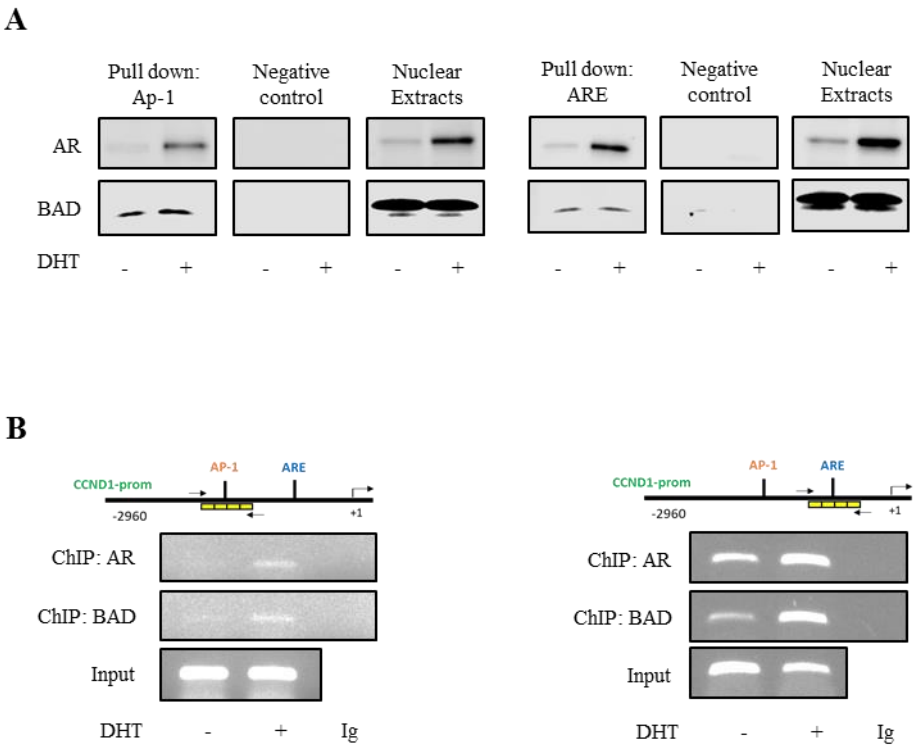

Figure 4.5 Ligand-activated AR and BAD bind to ARE and AP-1 sites on cyclin D1 promoter. (A) DAPA on nuclear extract from MCF-7 cells treated with 10^{-7}M DHT for 2 h. ARE or AP-1 biotinylated oligonucleotide were used. Unbound fraction, negative control (NC); nuclear extracts, positive control. (B) Chromatin from MCF-7 cells treated 10^{-7}M DHT for 2h was precipitated using anti-AR or anti-BAD antibodies. PCR was carried out using primers indicated by arrows and analysed by agarose gel electrophoresis. IgG control samples. DNA input, loading control.

4.6 AR and BAD expression is a predictive factor in ER-α positive breast cancer tumour

To better elucidate the role of AR and BAD co-operation, we investigated if our finding may have an impact on the outcome of ER-positive breast cancer patients that received endocrine treatment (Györffy et al., 2010). As Kaplan-Meier survival analysis shows (Fig. 4.6A and B), we found significantly increased overall survival (OS) and relapse-free survival (RFS) of individuals with elevated levels of AR or BAD. Taken together, these independent data sets showed that highest AR and BAD levels well correlate with patient responsiveness to tamoxifen therapy, suggesting to considerate the use of AR agonist in ER-positive breast cancers treatment.

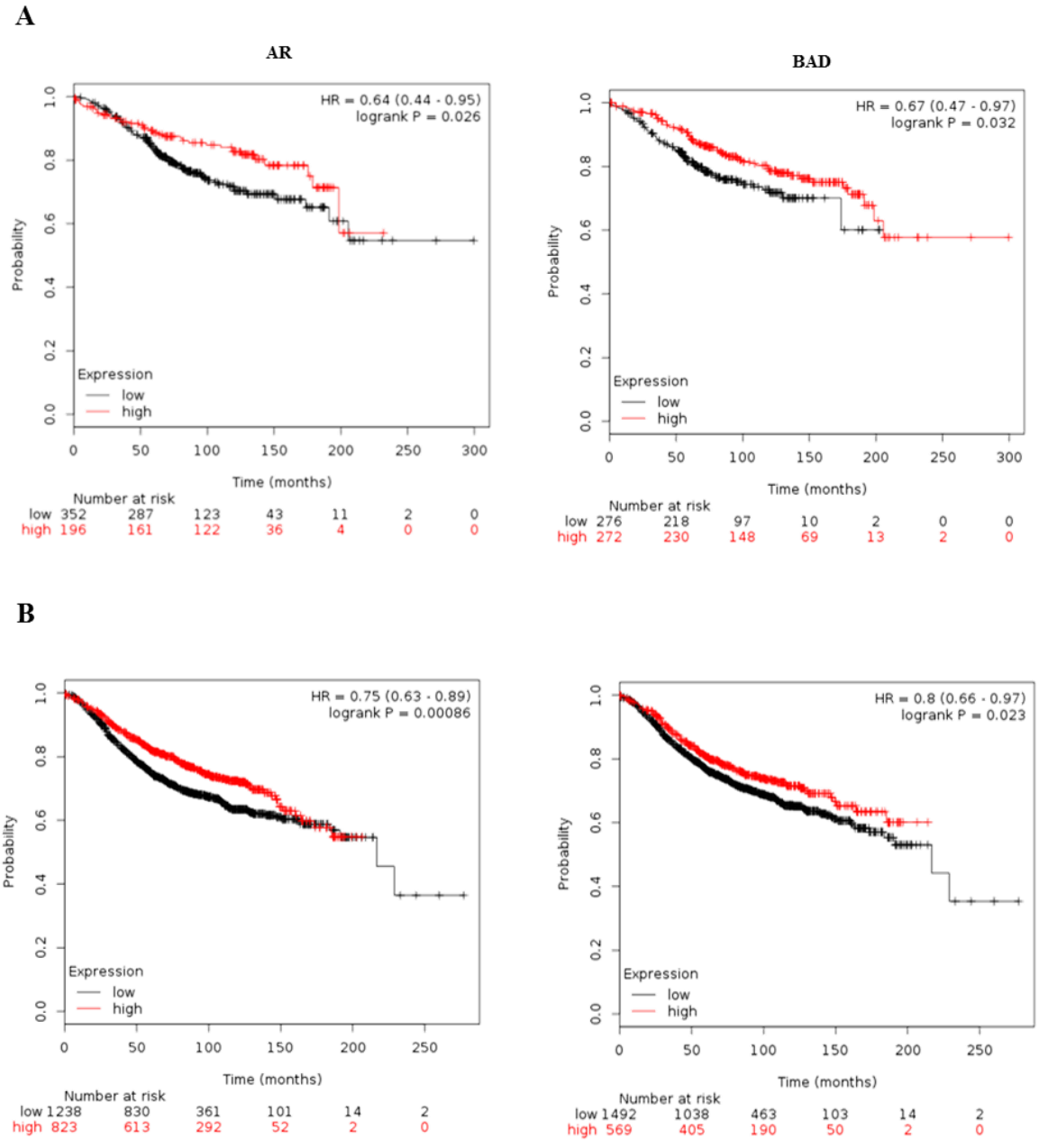

Fig. 4.6 AR and BAD are predictive of good response to endocrine therapy in ERα-positive breast cancer.

Kaplan-Meier plotter for overall (A) and relapse-free survival (B) for AR or BAD positive patients with ER-positive breast cancer receiving tamoxifen therapy.

5. Androgens affect breast cancer-associated fibroblasts (CAFs). A new role for breast stromal androgen receptor

To investigate androgen receptor (AR) expression in breast tumour stroma, cancer associated fibroblasts (CAFs) isolated from a total number of four patients with different pathologic features (Table 1) were used. In this study, the breast tumour fibroblasts used were named CAFs 1, 2, 3 and 4 (Table 1). Primary CAFs were characterized by evaluating the expression of the stromal activation markers α-Smooth Muscle Actin (α-SMA), Vimentin and Fibroblast Activation Protein (FAP) (Fig. 5.1).

TUMOR BIOPSY RECEPTOR PROFILE			
90% ER+	CAFs 1	50% ER+	CAFs 3
80% PR+		10% PR+	
3% Ki67		40% Ki67	
HER2 1+ (-)		HER2 1+ (-)	
60% ER+	CAFs 2	60% ER+	CAFs 4
70% PR+		70% PR+	
20% Ki67		20% Ki67	
HER2 2+		HER2 2+	

Table 1. Receptor profiles of breast tumours used to isolate CAFs.

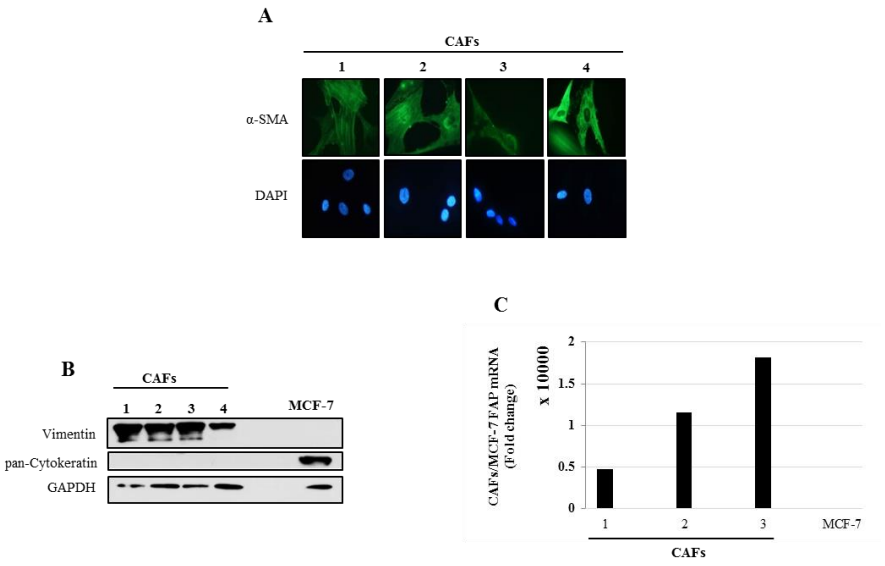

Figure 5.1 CAFs characterization. (A) Immunofluorescence assay were performed in breast CAFs to visualize α-SMA. No immunorectivity was detected when CAFs were incubated without the primary antibody (negative control; NC). (B) Western Blotting analysis of total protein fractions from CAFs and MCF-7 cells to investigate vimentin and pan-Cytokeratin expression. (C) mRNA extracted from CAFs and MCF-7 were analyzed with qRT-PCR to investigate FAP expression. GAPDH was used as housekeeping gene.

5.1 Primary human breast CAFs do express the androgen receptor

AR expression in primary human breast CAFs was investigated by performing immunoblotting analysis. As fig. 5.2A shows, all sets of CAFs expressed AR whose protein expression levels were up-regulated upon the stimulation with the natural ligand non-aromatizable dihydrotestosterone (DHT) for 24, 48 and 72h of treatment. Interestingly, AR expression was negatively regulated in presence of the AR antagonist enzalutamide (ENZ). Immunocytochemical staining and immunofluorescence analysis, following the treatment with the synthetic AR agonist mibolerone (Mb) and DHT respectively, confirmed increased AR expression highlighting a nuclear localization (Fig. 5.2 B and C), which suggested a genomic role of AR.

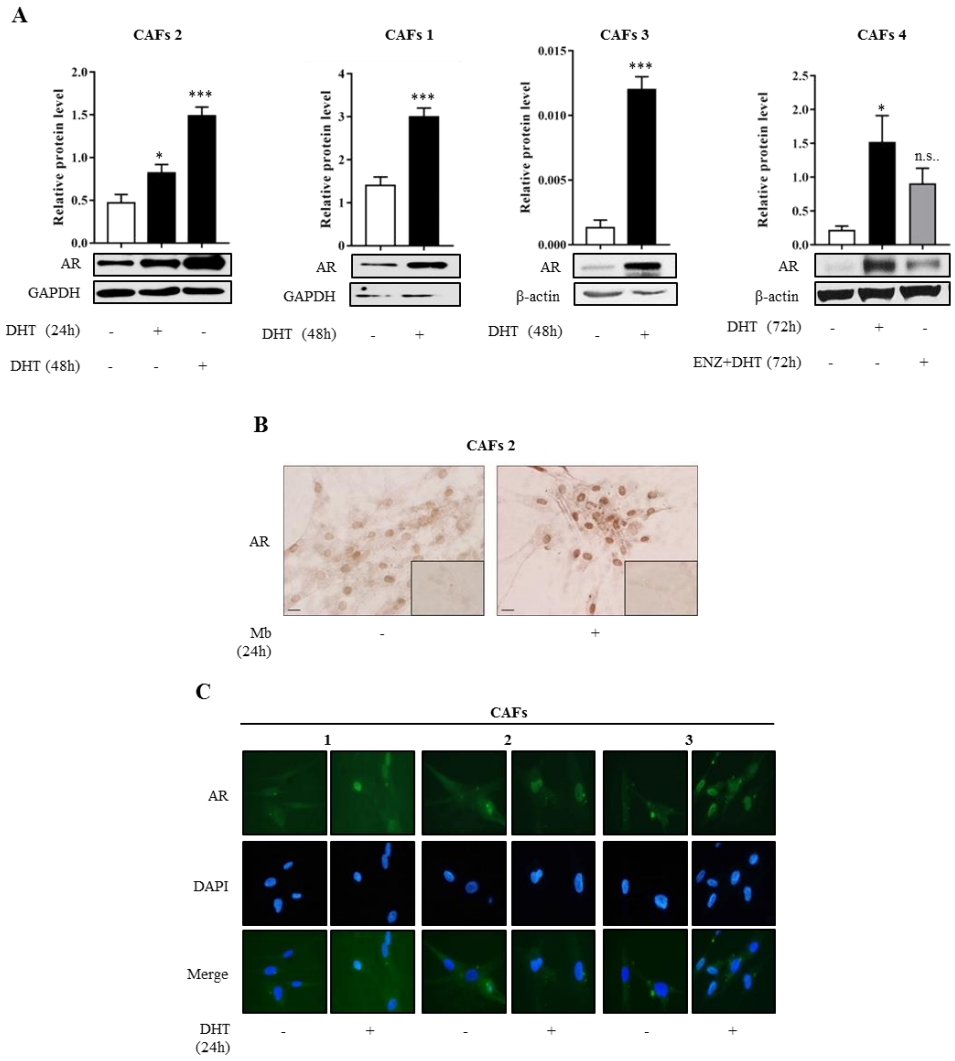

Figure 5.2 Androgens increase AR protein expression and nuclear localization. (A) Western Blotting analysis of total protein fractions from CAFs treated with vehicle (-), 10^{-7}M DHT or 10^{-7}M DHT+10^{-6}M ENZ for 24, 48 and 72h to evaluate AR expression. Histograms represent the mean±S.D. of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and normalized to the relative GAPDH or β-actin content. *p≤0.05 and ***p≤0.001 vs untreated CAFs; n.s. = non-significant (B)

Immunocytochemical staining and (C) Immunofluorescence assay were performed in breast CAFs untreated/ treated with AR agonist 10^{-8}M Mb or 10^{-7}M DHT to visualize AR expression and cellular localization. No immunorectivity was detected when CAFs were incubated without the primary antibody (negative control; NC).

5.2 Androgens have no effects on CAFs proliferation and motility

To better understand the role of activated AR in breast cancer stroma, we evaluated the effects on CAFs proliferation at first. As proliferation curve shows (Fig. 5.3), Mb did not influence CAFs proliferation until 6 days of treatment.

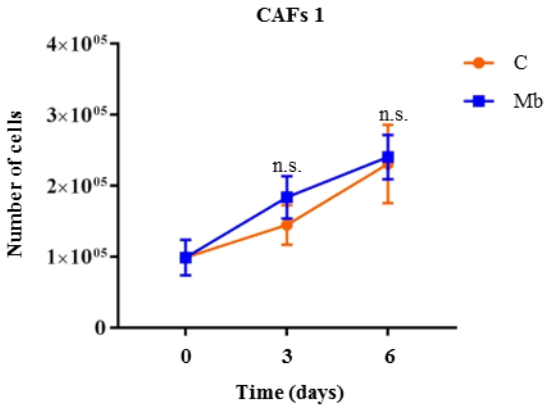

Figure 5.3 Androgen-activated AR does not influence CAFs proliferation. CAFs were platelet in presence or absence of 10^{-8}M Mb for 3 and 6 days. Cell counting by using a Burker's Chamber and cell viability by trypan blue dye exclusion were used to evaluate CAFs proliferation. n.s. = non-significant.

Thus, we investigated the effect on actin stress fibres formation which is a crucial event in cell motility (Pellegrin & Mellor, 2007). As F-actin staining shows (Fig. 5.4A), DHT did not influence actin contractility. This result was confirmed by wound-healing scratch assay showing that, either in presence or absence of DHT, CAFs migrated with the same speed to close the wound (Fig. 5.4B). These evidences suggested that androgens, via AR activation, do not affect fibroblast migratory capability.

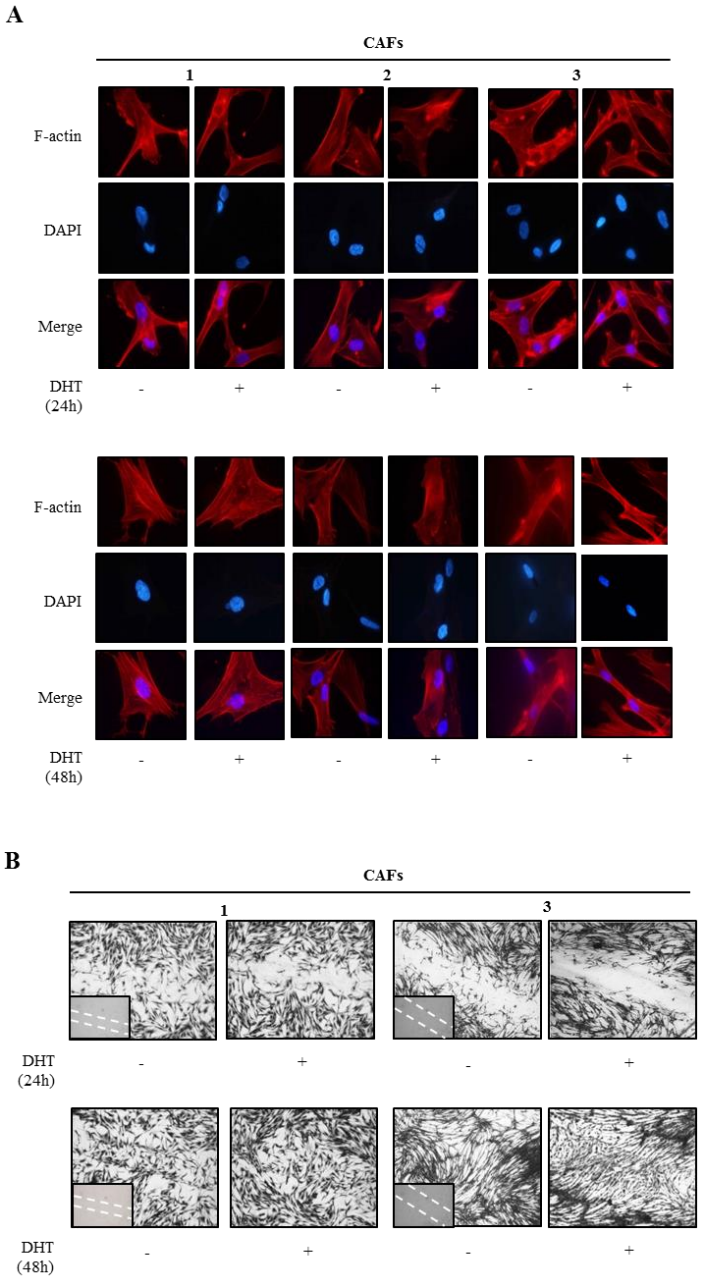

Figure 5.4 Androgen-activated AR does not influence CAFs migration. (A) CAFs treated in presence or absence of 10^{-7}M DHT for 24 and 48h were subjected to Phalloidin assay to visualize F-actin filaments. (B) Scratch assay on CAFs treated with vehicle (-) or 10^{-7}M DHT for 24 and 48h.

5.3 DHT treatment affects CAFs secretory phenotype

Cancer-associated fibroblasts have a crucial role in the paracrine cross-talk tumour-stroma by producing and secreting different cytokines. For this reason, we tested the effects of androgen administration on CAFs secretory phenotype by cytokines array. As Fig. 5.5A shows, DHT reduced the expression levels of several cytokines in CAFs-conditioned medium (CAFs-CM), some of which are critically involved in the regulation of cancer cell migration and invasive capabilities. In particular we focused our study on stromal cell-derived factor-1 (SDF-1), a chemokine that induces cell migration by specifically binding the C-X-C chemokine receptor

type 4 (CXCR4), and CD147, also named extracellular matrix metalloproteinase inducer (EMMPRIN) given that it is able to activate the matrix metalloproteases (MMPs). SDF-1 and CD147 reduced expression following DHT treatment was confirmed by Real-Time PCR (Fig. 5.5B). In uterine fibroblasts, the soluble CD147 ligand binds the receptor form on the cell surface, is internalized and, by activating the ERK1/2 signaling pathway, increases the expression of MMPs (Belton, Chen, Mesquita, & Nowak, 2008). Hence, zymography assay on CAFs-CM was performed to investigate MMP-2 and MMP-9 activity in presence or not of DHT. Androgen administration affected only the MMP-2 isoform by inhibiting its activity (Fig. 5.5C) and, thus, mirroring the reduced CD147 expression.

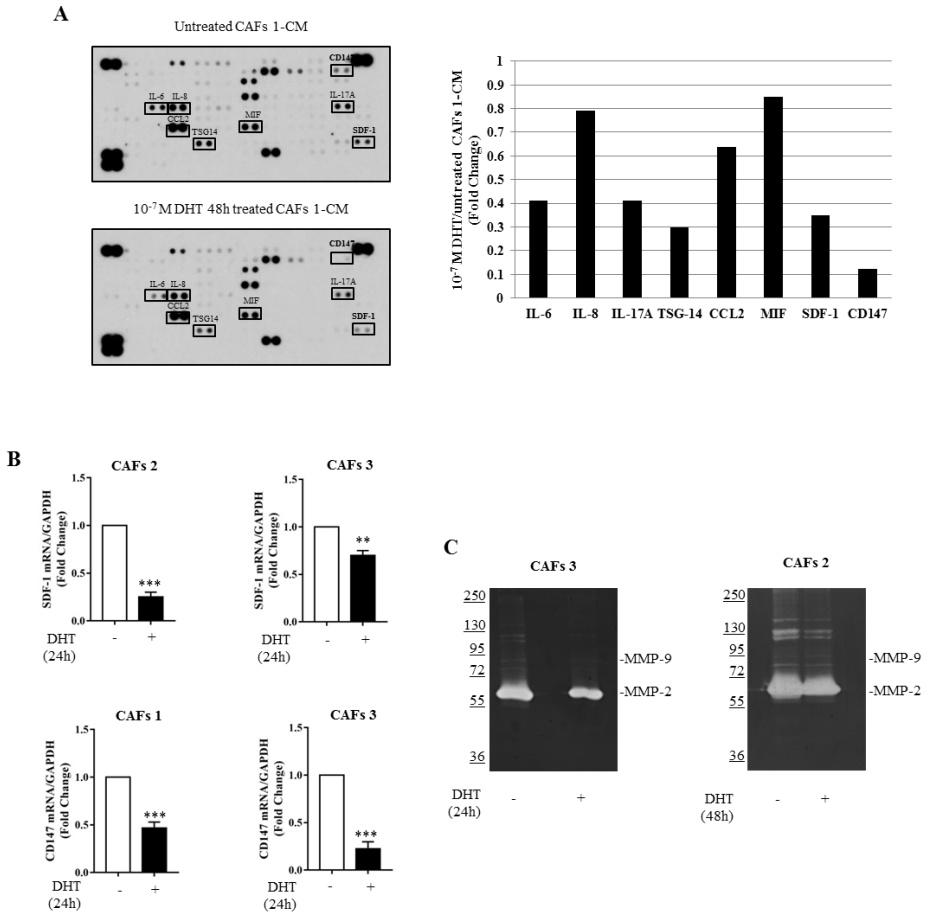

Figure 5.5 Activated AR reduces cytokines secretion from breast tumour fibroblasts. (A) Conditioned media from CAFs (CAFs-CM) untreated or treated with 10^{-7}M DHT were collected after 48h and screened for the expression of soluble secreted factors by cytokine array. (B) mRNA extracted from vehicle (-) or 10^{-7}M DHT-treated CAFs for 24h were analyzed with qRT-PCR to investigate SDF-1 and CD147 expression. GAPDH was used as housekeeping gene. Data represent the mean ± S.D. of three separate experiments, each performed in triplicate. **p≤0.01 and ***p≤0.001 vs untreated CAFs. (C) CM from CAFs treated or not with 10^{-7}M DHT for 24 and 48h were used to performed zymography assay.

5.4 DHT influences CAF-paracrine effects on breast cancer cell migration and invasiveness

Since DHT treatment, via androgen receptor, influences CAF's secretory phenotype, we inquired whether androgen-treated CAF-CM might influence ERα positive breast cancer cell motility by performing co-cultured experiments. As Fig. 5.6A shows, DHT treated CAFs-CM reduced MCF-7 cells migration. This data was confirmed by plating MCF-7 cells on the top and untreated/DHT treated CAFs on the bottom, to mimic *in vitro* carcinoma in situ (Fig. 5.6B). In addition, DHT treated CAF-CM reduced actin stress-fibres formation in MCF-7 cells.

In the same experimental condition, a similar result was also observed in T47D breast cancer cells. As wound-healing scratch assay shows, cancer cells migrated slowly to close the scratch in presence of DHT treated CAF-CM. Interestingly, the CM derived from CAFs treated with DHT in combination with the androgen receptor antagonist bicalutamide (BIC) reversed this effect (Fig. 5.6C).

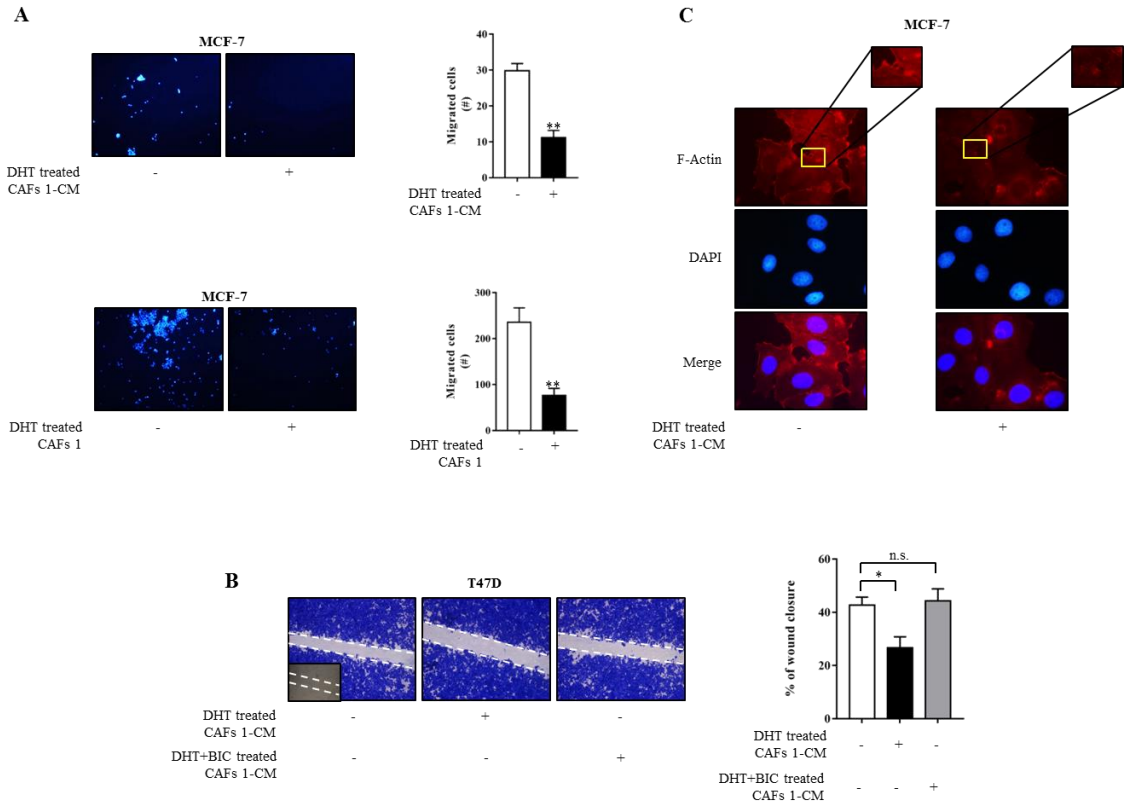

Figure 5.6 DHT treated CAFs-CM inhibits breast cancer cell motility. (A) MCF-7 breast cancer cell were incubated for 24h with conditioned media collected from CAFs (CAFs-CM) either untreated or treated with 10^{-7}M DHT (upper panel), or CAFs (lower panel) to perform Boyden-chamber transmigration assay. **p≤0.01 vs untreated CAFs-CM. (B) MCF-7 cells following 24h untreated/10^{-7}M DHT CAFs-CM exposure were stained to visualize actin filaments (F-actin). (C) Scratch assay was performed on T47D cells in presence of conditioned

media from untreated (CAFs-CM), 10^{-7}M DHT-treated (DHT-CAFs-CM) or 10^{-7}M DHT+10^{-6}M bicalutamide-treated (DHT/BIC-CAFs-CM) CAFs upon 24h of incubation. *p≤0.05 vs untreated CAFs; n.s. = non-significant

Next, we tested whether DHT may also affect CAFs-induced breast cancer cell invasive competence. As matrigel invasion assay shows (Fig. 5.7), MCF-7 cells incubated with DHT treated CAFs-CM exhibited lower invasiveness ability than the cells incubated with the untreated one.

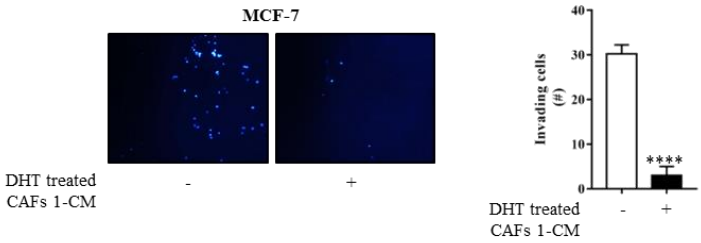

Figure 5.7 DHT treated CAFs-CM inhibits breast cancer cell invasive capabilities. Matrigel invasion assay was used to evaluate the invasive ability of MCF-7 breast cancer cells in presence of conditioned media from CAFs untreated (CAFs-CM) or treated with 10^{-7}M DHT (DHT-CAFs-CM) for 48h of co-cultured. ****p≤0.0001 vs untreated CAFs-CM.

5.5 DHT influences CAF-paracrine effects on breast cancer cell EMT

The epithelial-to-mesenchymal transition (EMT) is a biological process with a crucial role in tumour cell invasion, metastasis and chemoresistance promotion.

We investigated the effects of untreated/DHT treated CAFs-CM both on epithelial and mesenchymal markers. In MCF-7 cells, CM from DHT-treated CAFs increased the expression of E-cadherin, the loss of which enhances tumour cell spreading. Interestingly, the highest protein levels of the epithelial marker E-cadherin were parallel to a reduced expression of the mesenchymal marker N-cadherin in T47D cells (Fig. 5.8A). These evidence suggest that DHT-reduced CAFs secretory phenotype influences the EMT in breast cancer cells. For this reason, we investigated the Akt signalling pathway, whose activation by phosphorylation is largely recognized as an EMT inducer in tumour cells. As fig. 5.8B shows, the incubation of T47D cells with DHT treated CAFs-CM resulted in reduced phosphorylation of AKT compared to cells co-cultured with untreated CAFs-CM.

Future studies will be focused to better investigate these effects.

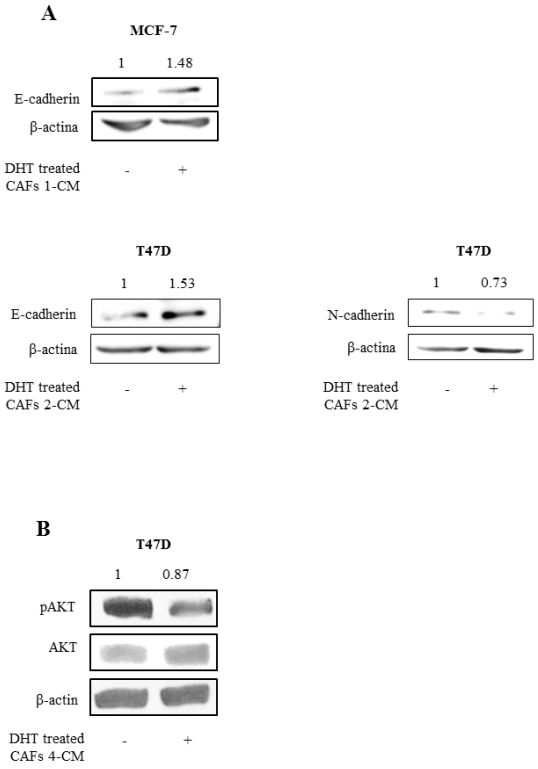

Figure 5.8 DHT treated CAFs-CM may reduce EMT in breast cancer cells. (A) Total protein fractions derived from MCF-7 and T47D cells, incubated for 24h with untreated/10^{-7}M DHT CAFs-CM, were used to evaluate the expression of EpCAM, E-cadherin, N-cadherin and AKT/pAKT (B). β-actin was used as loading control.

5.6 DHT-reduced CAFs secretory phenotype inhibits breast cancer stem cell formation

Tumour cells that undergo the epithelial-to-mesenchymal transition may acquire stem cell traits (Mani et al., 2008). Furthermore, our data from cytokine array showed in DHT treated CAFs-CM a reduced secretion of IL-8 and CCL-2, which are key regulators in cancer stem cells (CSCs) activity (Singh, Simões, Clarke, & Bundred, 2013; Tsuyada et al., 2012). Thus, we performed mammosphere forming efficiency assay (MFE) to evaluate the effects of untreated or DHT treated CAFs-CM on T47D cells stemness activity. We showed that CM derived from untreated CAFs increased mammosphere formation compared with T47D cells cultured in presence of mammosphere media. In addition, in presence of conditioned media derived from CAFs treated with DHT, mammosphere formation is significantly reduced (Fig. 5.9).

Taken together, these data demonstrate that androgens, by affecting the paracrine factors secreted by CAFs, inhibit cell spreading and can be useful in preventing tumour relapses.

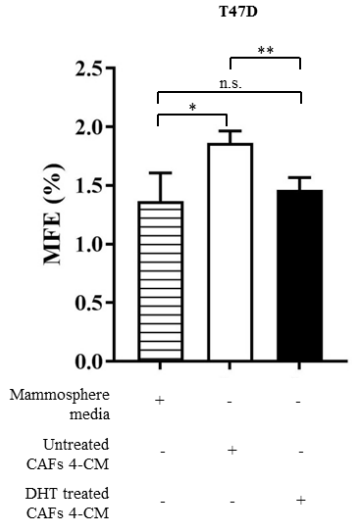

Figure 5.9 DHT treated CAFs-CM reduces mammospheres formation in breast cancer cells. Conditioned media from untreated (CAFs-CM) and 10^{-7}M DHT-treated (DHT-CAFs-CM) were used to evaluate mammosphere formation in T47D cells. The values represent the means ± SD of three different experiments each performed in triplicate. n.s. = non-significant; *p=≤0.05; **p=≤0.01.

6. Discussion

Androgen receptor (AR) is expressed in 70% of breast tumour (Peter et al., 2011). Even though AR expression in normal and neoplastic breast tissue has been known for a while (Allegra et al., 1979) only in the last years there is a growing interest to investigate the role of androgens/AR in breast cancer growth, progression, prognosis and treatment. AR role in breast tumour is strictly related to tumour subtype (Nieto, Rider, & Cramer, 2014). Recent clinical and experimental evidence suggests that AR signalling pathway has mainly inhibitor effects on normal mammary epithelial cells and plays a protective role in breast carcinogenesis in estrogen receptor-α (ER-α) positive breast cancer (Labrie et al., 2003; Yeh et al., 2003).

Estrogens have a central role in the proliferation and the differentiation of normal mammary epithelial cells as well as the development and progression of breast cancer (Feigelson & Henderson, 1996; Frech et al., 2005; Korach, 1994). Indeed, human breast tumourigenesis is promoted by enhanced activity of the ER-α that regulates the transcription of target genes, which in turn direct cellular proliferation (Tyson et al., 2011). Among these genes, cyclin D1 plays a pivotal role, as highlighted by several lines of evidences. In cyclin D1 knockout mice, mammary gland development is profoundly impaired and more evident during pregnancy when ovarian steroids fail to induce their massive proliferative changes (Fantl, Stamp, Andrews, Rosewell, & Dickson, 1995; Sicinski et al., 1995). Cyclin D1 over-expression has been reported in about 50% of invasive breast cancer (Tobin & Bergh, 2012) and strongly correlates with ER levels (Hui et al., 1996; JARES et al., 1997; Yang et al., 2006). The mechanism by which estrogens regulate cyclin D1 levels in hormone-responsive breast cancer cells is mainly transcriptional. Although no estrogen-responsive element- (ERE)–related sequence has been identified in the cyclin D1 promoter, several potential estrogen responsive sites have been mapped in the cyclin D1 proximal promoter (Altucci et al., 1996; Castro-Rivera, Samudio, & Safe, 2001; Liu et al., 2002; K. J. Park, Krishnan, O'Malley, Yamamoto, & Gaynor, 2005; Sabbah et al., 1999).

In this scenario emerges an aspect in the protective role of androgen receptor in ER-α positive breast cancer based on androgens ability to inhibit cyclin D1 expression through a ligand-dependent mechanism involving AR binding on androgen-responsive-elements (ARE) on cyclin D1 promoter gene (CCND1-ARE) (Marilena Lanzino et al., 2010).

Here we demonstrated that AR can reduce the E_2-induced cyclin D1 transcription gene by squelching the co-activator AIB1 and by increasing the pro-apoptotic protein BAD expression levels.

It is recognized that ERα-mediated transcription is a highly complex process involving a multitude of co-regulatory factors and cross-talk among distinct signalling path-ways (Hall, Couse, & Korach, 2001; Marilena Lanzino et al., 2005). A number of non-mutually exclusive mechanisms by which the action of steroid receptors might be competitive do exist, including homo- and heterodimers formation, structural analogy of activating ligands, binding to shared DNA response elements or sequestration of transcriptional co-regulators present in limiting cellular concentrations (Carroll et al., 2005; Marilena Lanzino et al., 2005; Migliaccio et al., 2005; Peters et al., 2009).

Our data shows that the AR interferes with E_2/ER-α dependent transcriptional induction of cyclin D1 by sharing the steroid receptor coactivator AIB1, whose abnormal expression is associated with malignancies in estrogen target tissues, such as ovarian and breast cancer (Anzick et al., 1997; H. J. List, Reiter, Singh, Wellstein, & Riegel, 2001). Very recently, it has been proposed that, in ERα-positive/HER2-negative invasive breast carcinoma, AIB1 could serve as a new putative prognostic biomarker, with its expression (high AIB1 vs low AIB1) being associated to breast cancer mortality (Narbe et al., 2019). More, interestingly, AIB1 has a unique role in regulating estrogen-dependent signalling as it is essential for ERα transcriptional activity (Shao et al., 2004; Tikkanen et al., 2000). This peculiarity of AIB1 serves as a mechanism by which it influences the growth of hormone-dependent breast cancer as suggested by the observation that depletion of AIB1 affects estrogen-dependent cell proliferation and survival in ER-positive MCF-7 human breast cancer cells, causing a reduction of MCF-7 xenografts growth in mice (Karmakar, Foster, & Smith, 2009; Heinz Joachim List et al., 2001). Specifically, in MCF-7 cells, AIB1 represents a rate-limiting factor for estrogen-dependent growth (Heinz Joachim List et al., 2001) since its cellular levels influence the ability of ERα to interact with the cyclin D1 promoter in an estrogen-dependent manner (Planas-Silva et al., 2001).

Our data establish that, in MCF-7 cells, exogenously expressed AIB1 reverses the AR repression of E2-dependent transcriptional activity of cyclin D1 promoter suggesting that the transcriptional interference between AR and ERα on cyclin D1 promoter might actually involve competition for limiting amounts of AIB1 in the cell. In our experimental models, the ability of AIB1 to modulate AR/ERα interplay is dependent on the steroid receptor cellular content since in MCF-7 cells expressing high levels of endogenous ERα and low levels of AR, AIB1 interacts predominantly with ERα. In contrast, AR overexpression induces a dominant interaction of AIB1 with AR. This co-activator squelching between the two steroid receptors impacts on ERα-driven transcription of growth regulatory genes. In MCF-7 cells, specific AR ligand activation is able to determine a significant decrease in the estrogen-

induced recruitment of AIB1 onto the AP-1 site containing region of the cyclin D1 promoter. Consistent with the notion that AIB1 is fundamental for ERα recruitment within the estrogen responsive sequence of the cyclin D1 promoter (Planas-Silva et al., 2001), a similar reduction in ERα occupancy of the AP-1 site containing region was also evidenced. Our results well correlate with previous findings showing that loss of AIB1 affects ERα-mediated signalling by both directly inhibiting transcriptional initiation and blocking ERα turnover, which may further compromise transcriptional regulation by the receptor (Shao et al., 2004). In addition, we showed that specific AIB1 knock-down completely abrogated E2 effect on cyclin D1 expression.

The shift from a benign to a malignant tumour is related to disease acquisition of different traits, presented by Hanah and Weinberg as "hallmarks of cancer" (Hanahan & Weinberg, 2000, 2011). These include tumour cells ability to elude the apoptosis. Bcl-2 family proteins, which includes members with pro- or anti-apoptotic activity, has a crucial role in apoptosis regulation. Many studies are focused on apoptotic regulators and cancer malignancy and progression. In particular, is emerging that the role of the pro-apoptotic molecule Bcl-2 antagonist of cell death (BAD) is not only restricted to the canonical cell death promotion. Low levels of BAD are reported in cytoplasmic and nuclear compartments in breast cancer tissues compared to normal ones (Cekanova et al., 2015). In primary breast cancer, BAD expression is correlated with highest survival, is a good prognostic marker and sensitizes cells to chemotherapy (Craik et al., 2010). Furthermore, It has been reported that BAD over-expression in MCF-7 breast cancer cells inhibits G1 to phase S cell cycle transition, cell growth and cyclin D1 expression (R. Fernando et al., 2007).

Our data indicate that activated AR promotes BAD recruitment on cyclin D1 promoter gene highlighting a cooperative role of the two factors in inhibiting basal and E2-dependent breast cancer cell proliferation. In line with previously evidences proving androgens ability to reduce MCF-7 cell proliferation by inducing apoptosis (M. Lanzino et al., 2013), here we show that, within a subset of Bcl-2 family members, androgens via AR, influence only BAD protein levels. The increased BAD expression may help the shift of the balance between anti- and pro-apoptic factors in favour of the latter. More interestingly, AR activation increases BAD nuclear localization highlighting, upon androgen administration, a nuclear role for this protein. Remarkably, this mechanism of action is not limited to a specific cell subtype since BAD nuclear translocation was also present in AR positive/ER-α negative breast cancer SKBR3 cells. Regulation of BAD cellular compartmentalization is exclusively affected by the intracellular AR and is closely linked to a AR/BAD physical interaction into the nuclear compartment. Herein, both BAD and AR occupy the AP-1 site containing region of the cyclin

D1 promoter which is important for the previously demonstrated ability of BAD to prevent cyclin D1 transcription (R. Fernando et al., 2007) and to antagonize the mitogenic effects exerted by estradiol via induction of cyclin D1 (R. Fernando et al., 2004). Therefore our study define a novel cooperative mechanism by which androgens, in addition to the above described squelching of AR/ER shared coactivators and to their ability to directly reduce the transcription of the cyclin D1 gene (Lanzino M et al 2010), also modulate the expression, cellular distribution and function of BAD, forcing its ability to act as a cell cycle inhibitor. In this scenario, a particular relevance is deserved by data from Kaplan-Meier survival analysis showing that in breast cancer patients, highest AR and BAD levels well correlate with patient responsiveness to tamoxifen therapy, suggesting the possibility to considerate the use of AR agonist in ER-positive breast cancers.

The protective role exerted by activated AR in ER-positive breast cancers is not restricted to the cancer epithelial compartment only. At the end of our studies, we presented the ability of breast stromal AR to affect the paracrine signal between breast stromal and tumour compartment.

Tumour tissue is embedded in the surrounding microenvironment (TME) that actively sustains cancer initiation, progression and therapy response. The stroma regulates cell epithelial function through physical and hormonal paracrine exchanges, thus offering a favourable environment for proliferation and metastasis. Cancer-associated fibroblasts (CAFs) are the main cell population inside cancer stroma and profoundly affect the tumour microenvironment. Hence there is a growing interest to better understand the gene networks and pathways mediated by CAFs to target the microenvironment and improve cancer patient survival. In this context, while nuclear receptors in tumour cells have been extensively investigated, their implications in cancer microenvironment are quite underappreciates. The AR has been found to be expressed in the stroma of both normal and tumour mammary tissue (Knower et al., 2013; Li et al., 2010) but its role in stromal-epithelial interactions is still completely unknown.

Herein we demonstrate, in primary human cancer associated fibroblasts (CAFs), the expression of AR, its activation and nuclear translocation following androgen administration, feature of its transcriptional activity. Despite CAFs proliferation and motility are not influenced upon androgen stimulation, AR activation influences their secretory phenotype. Indeed, tumour fibroblasts drive cancer cell proliferation and metastatic spread by producing and releasing hormones, growth factors, cytokines and enzymes involved in extracellular matrix degradation and remodelling (Hanahan & Coussens, 2012; Kalluri, 2016; Orimo et al., 2005). Through these mechanisms, CAFs also contribute to epithelial-to-mesenchymal

transition of breast cancer cells (Soon et al., 2013). Our findings evidence a reduced expression of many cytokines and in particular of SDF-1, a molecule that promotes cell migration by specifically binding the CXCR4 receptor, and of CD147, also called Extracellular Matrix Metalloproteinase Inducer (EMMPRIN), a protein able to activate matrix metalloproteases. In line with this evidence, a reduced activity of metalloproteases-2 (MMP-2) is reported. These alterations in CAFs' secretory phenotype affect their tumor-promoting ability on ER-α positive breast cancer cells. Indeed, conditioned media derived from CAFs treated with androgens significantly reduces the migratory and invasive competence of breast cancer cells. This effect occurs with concomitant changes in the expression of epithelial/mesenchymal markers, such as increase of the epithelial one E-cadherin and decrease of N-cadherin, underlining a protective adjustment of the functional interaction between tumour associated fibroblast and cancer cells. Remarkably, conditioned media derived from CAFs treated with DHT also reduces spheres-forming ability of breast cancer cells which is consistent with the notion that EMT program is not only the biological process that drives to carcinoma cell invasion and metastasis dissemination, but is also linked to cell entrance in cancer stem cell (CSC) state, contributing to cancer recurrence and therapy resistance (Shibue & Weinberg, 2017).

In conclusion, we demonstrated that the physical squelching of the AR/ER shared coactivator AIB1 and the AR-induced recruitment of BAD on site preferentially targeted by estrogens may represent at least two of the several potential mechanisms through which AR might negatively modulate ERα-dependent signalling and inhibit breast cancer cells proliferation. We also proved a novel role of stromal AR which, by inhibiting the expression of different cytokines secreted by CAFs, may reduce cancer progression and metastatic dissemination.

Taken together, our studies underline, once more, the protective role of AR in ER-positive breast cancer cells, the existence in breast cancer cells of a dynamic interplay between AR and ER-α signalling pathway, related to hormonal milieu, but also highlight, for the very first time in our knowledge, a remarkable activity of the AR in breast CAFs supporting the idea of coupling androgen-based therapy with therapies targeting other important pathways for the treatment of ERα-positive breast cancer patients.

References

- *Aceto N, Bardia A, Wittner BS, Donaldson MC, O'Keefe R, Engstrom A, Bersani F, Zheng Y, Comaills V, Niederhoffer K, Zhu H, Mackenzie O, Shioda T, Sgroi D, Kapur R, Ting DT, Moy B, Ramaswamy S, Toner M, Haber DA, and Maheswaran S "AR Expression in Breast Cancer CTCs Associates with Bone Metastases". Mol Cancer Res 2018, 16(4)).*
- *Allegra JC, Lippman ME, Thompson EB, Simon R, Barlock A, Green L, Huff KK, Do HM, Aitken SC "Distribution, frequency, and quantitative analysis of estrogen, progesterone, androgen, and glucocorticoid receptors in human breast cancer". Cancer research 1979, 39: 1447-1454.*
- *Altucci L, Addeo R, Cicatiello L, Dauvois S, Parker MG, Truss M, Beato M, Sica V, Bresciani F, Weisz A. "17beta-estradiol induces cyclin D1 gene transcription, p36D1-p34cdk4 complex activation and p105Rb phosphorylation during mitogenic stimulation of G(1)-arrested human breast cancer cells". Oncogene 1996, 12(11):2315–24.*
- *Ando S, De Amicis F, Rago V, Carpino A, Maggiolini M, Panno ML, et al. "Breast cancer: from estrogen to androgen receptor". Molecular and cellular endocrinology 2002,193(1-2):121-8.*
- *Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan XY, Sauter G, Kallioniemi OP, Trent JM, Meltzer PS "AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer". Science. 1997, 277(5328):965–8.*
- *Arendt LM, Kuperwasser C. "Form and function: how estrogen and progesterone regulate the mammary epithelial hierarchy". J Mammary Gland Biol Neoplasia. 2015, 20(1–2):9–25.*
- *Azariadis K, Kiagiadakia F, Pelekanoua V, Bempia V, Alexakisa K, Kampaa M, Tsapisa A, Castanasa E, Notasa G "Androgen Triggers the Pro-Migratory CXCL12/CXCR4 Axis in AR-Positive Breast Cancer Cell Lines: Underlying Mechanism and Possible Implications for the Use of Aromatase Inhibitors in Breast Cancer". Cell Physiol Biochem 2017, 44:66-84.*
- *Bartoschek M, Oskolkov N, Bocci M, Lövrot J, Larsson C, Sommarin M, Madsen CD, Lindgren D, Pekar G, Karlsson G, Ringnér M, Bergh J, Björklund A & Pietras K "Spatially and functionally distinct subclasses of breast cancer-associated fibroblasts revealed by single cell RNA sequencing". Nature Communications 2018, 9:5150.*
- *Basile D, Cinausero M, Iacono D, Pelizzari G, Bonotto M, Vitale MG, Gerratana L, Puglisi F "Androgen receptor in estrogen receptor positive breast cancer: Beyond expression". Cancer Treatment Reviews 61 2017, 15–2.*
- *Belton RJ, Jr., Chen L, Mesquita FS, and Nowak RA "Basigin-2 Is a Cell Surface Receptor for Soluble Basigin Ligand". THE JOURNAL OF BIOLOGICAL CHEMISTRY 2008, VOL. 283, NO. 26, pp. 17805–17814.*
- *Birrell SN, Bentel JM, Hickey TE, Ricciardelli C, Weger MA, Horsfall DJ, Tilley WD. "Androgens Induce Divergent Proliferative Responses in Human Breast Cancer Cell Lines". J. Steroid Biochem. Malec. Bioi. 1995, Vol. 52, No.5, pp. 459-467.*
- *Bleach R and Mcllroy M "The divergent function of androgen receptor in breast cancer; analysis of steroid mediators and tumour intracrinology". Frontiers in Endocrinology 2018, 9:594.*
- *Bozovic-Spasojevic I, Zardavas D, Brohee S, Ameye L, Fumagalli D, Ades F, de Azambuja E, Bareche Y, Piccart M, Paesmans M, and Sotiriou C "The Prognostic Role of Androgen Receptor in Patients with Early-Stage Breast Cancer: A Meta-analysis of Clinical and Gene Expression Data". Clin Cancer Res 2017, 23(11).*
- *Brisken C and O'Malley B "Hormone Action in the Mammary Gland". Cold Spring Harb Perspect Biol 2010, 2:a003178.*
- *Bronte G, Rocca A, Ravaioli S , Scarpi E , Bonafè E, Puccetti E, Maltoni R, Andreis D, Martinelli G, and Bravaccini S "Evaluation of Androgen Receptor in Relation to Estrogen Receptor (AR/ER) and Progesterone Receptor (AR/PgR): A New Must in Breast Cancer?". Hindawi Journal of Oncology 2019, Article ID 1393505.*
- *Bronte G, Rocca A, Ravaioli S, Puccetti M, Tumedei MM, Scarpi E, Andreis D, Maltoni R, Sarti S, Cecconetto L, Fedeli A, Pietri E, De Simone V, Asioli S, Amadori D and Bravaccini S "Androgen receptor in advanced breast cancer: is it useful to predict the efficacy of anti-estrogen therapy?". BMC Cancer 2018, 18:348.*
- *Buchsbaum RJ and Oh SY "Breast Cancer-Associated Fibroblasts: Where We Are and Where We Need to Go". Cancers 2016, 8, 19.*
- *Cancer Research UK https://www.cancerresearchuk.org/about-cancer/breast-cancer/stages-types-grades 2019.*
- *Carroll JS, Liu XS, Brodsky AS, Li W, Meyer CA, Szary AJ, Eeckhoute J, Shao W, Hestermann EV, Geistlinger TR, et al. "Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1". Cell. 2005, 122(1):33–43.*

~ *Castro-Rivera E, Samudio I, Safe S. "Estrogen regulation of cyclin D1 gene expression in ZR-75 breast cancer cells involves multiple enhancer elements". J Biol Chem. 2001, 276(33):30853–61.*
~ *Cekanova M, Fernando RI, Siriwardhana N, Sukhthankar M, de la Parra C, Woraratphoka J, Malone C, Ström A, Baek SJ, Wade PA, Saxton AM, Donnell RM, Pestell RG, Dharmawardhane S, and Wimalasena J "BCL-2 family protein, BAD is down-regulated in breast cancer and inhibits cancer cell invasion". Exp Cell Res. 2015, 331(1): 1–10.*
~ *Cheng HS, Lee JXT, Wahli W, Tan NS "Exploiting vulnerabilities of cancer by targeting nuclear receptors of stromal cells in tumor microenvironment". Molecular Cancer 2019, 51.*
~ *Ciupek A, Rechoum Y, Gu G, Gelsomino L, Beyer AR, Brusco L, Covington KR, Tsimelzon A, Fuqua SAW "Androgen receptor promotes tamoxifen agonist activity by activation of EGFR in ERa-positive breast cancer". Breast Cancer Res Treat 2015, 154:225–237.*
~ *Clarke RB, Anderson E and Howell A "Steroid receptors in human breast cancer". TRENDS in Endocrinology and Metabolism 2004, Vol.15 No.7.*
~ *Coleman C"Early Detection and Screening for Breast Cancer". Seminars in Oncology Nursing 2017, 33(2), 141–155.*
~ *Cops EJ, Bianco-Miottoa T, Moorea NL, Clarke CL, Birrell SN , Butlera LM, Tilleya WD "Antiproliferative actions of the synthetic androgen, mibolerone, in breast cancer cells are mediated by both androgen and progesterone receptors". Journal of Steroid Biochemistry & Molecular Biology 110 2008, 236–243.*
~ *Craik AC, Veldhoen RA, Czernick M, Buckland TW, Kyselytzia K, Ghosh S, et al. "The BH3-only protein Bad confers breast cancer taxane sensitivity through a nonapoptotic mechanism". Oncogene 2010, 29:5381–91.*
~ *D'Amato NC, Gordon MA, Babbs B, Spoelstra NS, Carson Butterfield KT, Torkko KC, Phan VT, Barton VN, Rogers TJ, Sartorius CA, Elias A, Gertz J, Jacobsen BM, and Richer JK "Cooperative Dynamics of AR and ER Activity in Breast Cancer". Mol Cancer Res 2016, 14(11).*
~ *Eiro N, Gonzalez LO, Fraile M, Cid S, Schneider J, Vizoso FJ, "Breast Cancer Tumour Stroma: Cellular Components, Phenotypic Heterogeneity, Intercellular Communication, Prognostic Implications and Therapeutic Opportunities". Cancers 2019, 11, 664.*
~ *Fantl V, Stamp G, Andrews A, Rosewell I, Dickson C. "Mice lacking cyclin D1 are small and show defects in eye and mammary gland development". Genes Dev. 1995, 9(19):2364–72.*
~ *Feigelson HS, Henderson BE "Estrogens and breast cancer carcinogenesis". Carcinogenesis 1996, 17(11):2279–84.*
~ *Feng J, Li L, Zhang N, Liu J, Zhang L, Gao H, Wang G, Li Y, Zhang Y, Li X, Liu D, Lu J and Huang B "Androgen and AR contribute to breast cancer development and metastasis: an insight of mechanisms". Oncogene 2017, 36, 2775–2790.*
~ *Fernando R, Foster JS, Bible A, Ström A, Pestell RG, Rao M, Saxton A, Baek SJ, Yamaguchi K, Donnell R, Cekanova M, Wimalasena J "Breast Cancer Cell Proliferation Is Inhibited by BAD REGULATION OF CYCLIN D1". THE JOURNAL OF BIOLOGICAL CHEMISTRY 2007, VOL. 282, NO. 39, pp. 28864–28873.*
~ *Fernando RI and Wimalasena J "Estradiol Abrogates Apoptosis in Breast Cancer Cells through Inactivation of BAD: Ras-dependent Nongenomic Pathways Requiring Signaling through ERK and Akt". Molecular Biology of the Cell 2004, Vol. 15, 3266–3284.*
~ *Frech MS, Halama ED, Tilli MT, Singh B, Gunther EJ, Chodosh LA, Flaws JA, Furth PA. "Deregulated estrogen receptor alpha expression in mammary epithelial cells of transgenic mice results in the development of ductal carcinoma in situ". Cancer Res. 2005, 65(3):681–5.*
~ *Furth PA, Cabrera MC, Diaz-Cruz ES, Millman S, Nakles RE. "Assessing estrogen signaling aberrations in breast cancer risk using genetically engineered mouse models". Annals of the New York Academy of Sciences. 2011, 1229:147-55.*
~ *Giovannelli P, Di Donato M, Galasso G, Di Zazzo E, Bilancio A, and Migliaccio A "The Androgen Receptor in Breast Cancer". Frontiers in Endocrinology 2019, 9: 492.*
~ *Gyorffy B, Lanczky A, Eklund AC, Denkert C, Budczies J, Li Q, Szallasi Z. "An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1809 patients". Breast Cancer Res Treatment 2010; 123(3):725-31.*
~ *Hall JM, Couse JF, Korach KS. "The multifaceted mechanisms of estradiol and estrogen receptor signalling". J Biol Chem. 2001, 276(40):36869–72.*
~ *Hanahan D and Weinberg RA "Hallmarks of Cancer: The Next Generation". Cell 2011, 144.*
~ *Hanahan D and Weinberg RA "The Hallmarks of Cancer" Cell 2000, Vol. 100, 57–70.*
~ *Hanahan D, Coussens LM "Accessories to the crime: functions of cells recruited to the tumor microenvironment". Cancer Cell. 2012, 21(3):309-22.*
~ *Harbeck N, Gnant M "Breast cancer". Lancet 2017, 389: 1134–50.*

~ *Hickey TE, Robinson JLL, Carroll JS, and Tilley WD "Minireview: the androgen receptor in breast tissues: growth inhibitor, tumour suppressor, oncogene?". Mol Endocrinol 2012, 26(8):1252-1267.*
~ *Hilton N, Clarke CL, Graham JD "Estrogen and progesterone signalling in the normal breast and its implications for cancer development". Molecular and Cellular Endocrinology 466 2018, 2e14.*
~ *Houthuijzen JM & Jonkers J "Cancer-associated fibroblasts as key regulators of the breast cancer tumour microenvironment". Cancer and Metastasis Reviews 2018, 37:577–597.*
~ *Hui R, Cornish AL, McClelland RA, Robertson JF, Blamey RW, Musgrove EA, Nicholson RI, Sutherland RL. "Cyclin D1 and estrogen receptor messenger RNA levels are positively correlated in primary breast cancer". Clin Cancer Res. 1996, 2(6):923–8.*
~ *Jares P, Rey MJ, Fernandez PL, Campo E, Nadal A, Munoz M, Mallofre C, Muntane J, Nayach I, Estape J, et al. "Cyclin D1 and retinoblastoma gene expression in human breast carcinoma: correlation with tumour proliferation and oestrogen receptor status" J Pathol. 1997, 182(2):160–6.*
~ *Kalluri R "The biology and function of fibroblasts in cancer". Nat Rev Cancer 2016, 23; 16 (9):582-98.*
~ *Kalluri R and Zeisberg M "Fibroblasts in cancer". Nat Rev Cancer 2016, 23;16(9):582-98.*
~ *Kampaa M, Niflia AP, Charalampopoulosb I, Alexakia VI, Theodoropoulosc PA, Stathopoulosd EN, Gravanisb A, Castanasa E "Opposing effects of estradiol- and testosterone-membrane binding sites on T47D breast cancer cell apoptosis". Experimental Cell Research 2005, 41– 51.*
~ *Karmakar S, Foster EA, Smith CL. "Unique roles of p160 coactivators for regulation of breast cancer cell proliferation and estrogen receptor-alpha transcriptional activity". Endocrinology 2009, 150(4):1588–96.*
~ *Kemppainen JA, Lane MV, Sar M, Wilson EM. "Androgen receptor phosphorylation, turnover, nuclear transport, and transcriptional activation. Specificity for steroids and antihormones". The Journal of biological chemistry 1992, 267(2):968-74.*
~ *Kensler KH, Poole EM, Heng YJ, Collins LC, Glass B, Beck AH, Hazra A, Rosner BA, Eliassen AH, Hankinson SE, Winer EP, Brown M, Tamimi RM "Androgen Receptor Expression and Breast Cancer Survival: Results From the Nurses' Health Studies". JNCI J Natl Cancer Inst 2019, 111(7): djy173.*
~ *Kensler KH, Regan MM, Heng YJ, Baker GM, Pyle ME, Schnitt SJ, Hazra A, Kammler R, Thürlimann B, Colleoni M, Viale G, Brown M and Tamimi RM "Prognostic and predictive value of androgen receptor expression in postmenopausal women with estrogen receptor-positive breast cancer: results from the Breast International Group Trial 1–98". Breast Cancer Research 2019, 21:30.*
~ *Knower KC, Chand AL, Eriksson N, Takagi K, Miki Y, Sasano H, Visvader JE, Lindeman GJ, Funder JW, Fuller PJ, Simpson ER, Tilley WD, Leedman PJ, Graham J, Muscat GE, Clarke CL, Clyne CD "Distinct nuclear receptor expression in stroma adjacent to breast tumors". Breast Cancer Res Treat 2013, 142(1):211-23.*
~ *Knutson TP, Lange CA "Tracking progesterone receptor-mediated actions in breast cancer". Pharmacology & Therapeutics 142 2014, 114–125.*
~ *Korach KS. "Insights from the study of animals lacking functional estrogen receptor". Science. 1994, 266(5190):1524–7.*
~ *Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, et al. "Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity". Cell. 2001, 104(5):719-30.*
~ *Kraby MR, Valla M, Opdahl S, Haugen OA, Sawicka JE, Engstrøm MJ, Bofin AM "The prognostic value of androgen receptors in breast cancer subtypes". Breast Cancer Research and Treatment 2018, 172:283–296.*
~ *Labrie F, Luu-The V, Labrie C, Belanger A, Simard J, Lin SX, Pelletier G "Endocrine and intracrine sources of androgens in women: inhibition of breast cancer and other roles of androgens and their precursor dehydroepiandrosterone". Endocr. Rev. 2003, 24:152-182.*
~ *Lanzino M, De Amicis F, McPhaul MJ, Marsico S, Panno ML, Andò S "Endogenous coactivator ARA70 interacts with estrogen receptor alpha (ERalpha) and modulates the functional ERalpha/androgen receptor interplay in MCF-7 cells". J Biol Chem. 2005, 27; 280 (21):20421-30.*
~ *Lanzino M, De Amicis F, McPhaul MJ, Marsico S, Panno ML, Ando S. "Endogenous coactivator ARA70 interacts with estrogen receptor alpha (ERalpha) and modulates the functional ERalpha/androgen receptor interplay in MCF-7 cells". The Journal of biological chemistry 2005, 280(21):20421-30.*
~ *Lanzino M, Maris P, Sirianni R, Barone I, Casaburi I, Chimento A, Giordano C, Morelli C, Sisci D, Rizza P, Bonofiglio D, Catalano S, Andò S "DAX-1, as an androgen-target gene, inhibits aromatase expression: a novel mechanism blocking estrogen-dependent breast cancer cell proliferation". Cell Death and Disease 2013, 4 e724.*
~ *Lanzino M, Sisci D, Morelli C, Garofalo C, Catalano S, Casaburi I, Capparelli C, Giordano C, Giordano F, Maggiolini M, Andò S. "Inhibition of cyclin D1 expression by androgen receptor in breast*

cancer cells--identification of a novel androgen response element". Nucleic Acids Res. 2010, 38(16):5351-65.

~ *Li S, Han B, Liu G, Li S, Ouellet J, Labrie F, Pelletier G "Immunocytochemical localization of sex steroid hormone receptors in normal human mammary gland". J Histochem Cytochem. 2010, 58(6):509-15.*

~ *List HJ, Lauritsen KJ, Reiter R, Powers C, Wellstein A, Riegel AT. "Ribozyme targeting demonstrates that the nuclear receptor coactivator AIB1 is a ratelimiting factor for estrogen-dependent growth of human MCF-7 breast cancer cells". J Biol Chem. 2001, 276(26):23763–8.*

~ *List HJ, Reiter R, Singh B, Wellstein A, Riegel AT. "Expression of the nuclear coactivator AIB1 in normal and malignant breast tissue". Breast Cancer Res Treat. 2001, 68(1):21–8.*

~ *Liu MM, Albanese C, Anderson CM, Hilty K, Webb P, Uht RM, Price RH Jr, Pestell RG, Kushner PJ. "Opposing action of estrogen receptors alpha and beta on cyclin D1 gene expression". J Biol Chem. 2002, 277(27):24353–60.*

~ *Macias H and Hinck L "Mammary gland development". WIREs Dev Biol 2012, 1:533–557.*

~ *Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, Campbell LL, Polyak K, Brisken C, Yang J, Weinberg RA "The epithelial-mesenchymal transition generates cells with properties of stem cells". Cell. 2008, 133(4):704-15.*

~ *Margan MM, Jitariu AA, Cimpean AM, Nica C, and Raica M "Molecular Portrait of the Normal Human Breast Tissue and Its Influence on Breast Carcinogenesis". J Breast Cancer 2016, 19(2):99-111.*

~ *McNamara KM, Sasano H "The intracrinology of breast cancer". Journal of Steroid Biochemistry & Molecular Biology 2015, 172-178.*

~ *Migliaccio A, Castoria G, Di Domenico M, Ciociola A, Lombardi M, De Falco A, Nanayakkara M, Bottero D, De Stasio R, Varricchio L, Auricchio F. "Crosstalk between EGFR and extranuclear steroid receptors". Ann N Y Acad Sci. 2006, 1089:194-200.*

~ *Migliaccio A, Di Domenico M, Castoria G, Nanayakkara M, Lombardi M, de Falco A, Bilancio A, Varricchio L, Ciociola A, Auricchio F "Steroid receptor regulation of epidermal growth factor signaling through Src in breast and prostate cancer cells: steroid antagonist action". Cancer Res. 2005, 65(22):10585–93.*

~ *Moffat DF, & Going JJ "Three dimensional anatomy of complete duct systems in human breast: pathological and developmental implications". J Clin Pathol 1996, 49(1), 48-52.*

~ *Musgrove EA, Lee CS, Buckley MF, Sutherland RL. "Cyclin D1 induction in breast cancer cells shortens G1 and is sufficient for cells arrested in G1 to complete the cell cycle". Proceedings of the National Academy of Sciences of the United States of America 1994, 91(17):8022-6.*

~ *Nakles RE, Shiffert MT, Diaz-Cruz ES, Cabrera MC, Alotaiby M, Miermont AM, et al. "Altered AIB1 or AIB1Delta3 expression impacts ERalpha effects on mammary gland stromal and epithelial content". Mol Endocrinol. 2011, 25(4):549-63.*

~ *Narayanan R and Dalton JT "Androgen Receptor: A Complex Therapeutic Target for Breast Cancer". Cancers 2016, 8, 108.*

~ *Narbe U, Sjostrom M, Forsare C, Bendahl PO, Alkner S, Leeb-Lundberg LMF, Lovgren K, Ryden L, Ingvar C, Ferno M. "The estrogen receptor coactivator AIB1 is a new putative prognostic biomarker in ER-positive/HER2-negative invasive lobular carcinoma of the breast". Breast Cancer Res Treat. 2019 Vol. 175, No. 2, p. 305-316.*

~ *Need EF, Selth LA, Harris TJ, Birrell SN, Tilley WD, and Buchanan G "Research Resource: Interplay between the Genomic and Transcriptional Networks of Androgen Receptor and Estrogen Receptor in Luminal Breast Cancer Cells". Mol Endocrinol 2012, 26(11):1941–1952.*

~ *Nieto CM, Rider LC, Cramer SD "Influence of stromal-epithelial interactions on androgen action". Endocr Relat Cancer 2014, 21(4): T147-60.*

~ *Okano M, Oshi M, Butash AL, Asaoka M, Katsuta E, Peng X, Qi Q, Yan L and Takabe K "Estrogen Receptor Positive Breast Cancer with High Expression of Androgen Receptor has Less Cytolytic Activity and Worse Response to Neoadjuvant Chemotherapy but Better Survival". Int. J. Mol. Sci. 2019, 20, 2655.*

~ *Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA "Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion". Cell 2005, 121(3):335-48.*

~ *Park KJ, Krishnan V, O'Malley BW, Yamamoto Y, Gaynor RB. "Formation of an IKKalpha-dependent transcription complex is required for estrogen receptor-mediated gene activation". Mol Cell. 2005, 18(1):71–82.*

~ *Park S, Park HS, Koo JS, Yang WI, Kim SI, Park BW "Higher expression of androgen receptor is a significant predictor for better endocrine-responsiveness in estrogen receptor-positive breast cancers". Breast Cancer Res Treat 2012, 133:311–320.*

~ *Pelekanou V, Notas G, Sanidas E, Tsapis A, Castanas E, Kampa M "Testosterone membrane-initiated action in breast cancer cells: Interaction with the androgen signalling pathway and EPOR". Molecular Oncology 2010, 4(2):135-49.*
~ *Pellegrin S and Mellor H "Actin stress fibres". Journal of Cell Science 2007, 120, 3491-3499.*
~ *Peter MB, Shaaban AM, Rajan SS, Maraqa L, Horgan K, Speirs V "Investigating and critically appraising the expression and potential role of androgen receptor in breast carcinoma". Horm Mol Biol Clin Investig 2011, 7(1):273-8.*
~ *Peters AA, Buchanan G, Ricciardelli C, Bianco-Miotto T, Centenera MM, Harris JM, Jindal S, Segara D, Jia L, Moore NL, et al. "Androgen receptor inhibits estrogen receptor-alpha activity and is prognostic in breast cancer". Cancer Res. 2009, 69(15):6131–40.*
~ *Planas-Silva MD, Shang Y, Donaher JL, Brown M, Weinberg RA. "AIB1 enhances estrogen-dependent induction of cyclin D1 expression". Cancer research. 2001, 61(10):3858-62.*
~ *Rahim B and O'Regan R "AR Signaling in Breast Cancer". Cancers 2017, 9, 21.*
~ *Russo J, Russo IH "Development of the human breast". Maturitas 49 2004, 2–15.*
~ *Sabbah M, Courilleau D, Mester J, Redeuilh G. "Estrogen induction of the cyclin D1 promoter: involvement of a cAMP response-like element". Proc Natl Acad Sci U S A. 1999, 96(20):11217–22.*
~ *Salvi S, Bonafè M, Bravaccini S "Androgen recaptor in breast cancer: A wolf in sheep's clothing? A lesson from prostate cancer". Seminars in Cancer Biology 2019, https://doi.org/10.1016/j.semcancer.2019.04.002.*
~ *Shao W, Keeton EK, McDonnell DP, Brown M "Coactivator AIB1 links estrogen receptor transcriptional activity and stability". Proc Natl Acad Sci USA. 2004, 101(32):11599–604.*
~ *Shibue T and Weinberg RA "EMT, CSCs, and drug resistance: the mechanistic link and clinical implications". Nat Rev Clin Oncol 2017, 14(10):611-629.*
~ *Sicinski P, Donaher JL, Parker SB, Li T, Fazeli A, Gardner H, Haslam SZ, Bronson RT, Elledge SJ, Weinberg RA. "Cyclin D1 provides a link between development and oncogenesis in the retina and breast". Cell. 1995, 82(4):621–30.*
~ *Singh JK, Simões BM, Clarke RB & Bundred NJ "Targeting IL-8 signalling to inhibit breast cancer stem cell activity". Expert Opin. Ther. Targets 2013, 17(11).*
~ *Smith CL, Onate SA, Tsai MJ, O'Malley BW "CREB binding protein acts synergistically with steroid receptor coactivator-1 to enhance steroid receptor-dependent transcription". Proceedings of the National Academy of Sciences of the United States of America 1996, 93(17):8884-8.*
~ *Soon PS, Kim E, Pon CK, Gill AJ, Moore K, Spillane AJ, Benn DE, Baxter RC. "Breast cancer-associated fibroblasts induce epithelial-to-mesenchymal transition in breast cancer cells". Endocr Relat Cancer 2013, 20(1):1-12.*
~ *Sørlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Lønning PE, Børresen-Dale AL. "Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications". Proc Natl Acad Sci USA 2001, 98: 10869–74 .*
~ *Sun YS, Zhao Z, Yang ZN, Xu F, Lu HJ, Zhu ZY, Shi W, Jiang J, Yao PP, Zhu HP "Risk Factors and Preventions of Breast Cancer". Int. J. Biol. Sci. 2017, 13(11): 1387-1397.*
~ *Takagi K, Miki Y, Ishida T, Sasano H, Suzuki T "The interplay of endocrine therapy, steroid pathways and therapeutic resistance: Importance of androgen in breast carcinoma". Molecular and Cellular Endocrinology 2018, 466:31-37.*
~ *Tikkanen MK, Carter DJ, Harris AM, Le HM, Azorsa DO, Meltzer PS, Murdoch FE. "Endogenously expressed estrogen receptor and coactivator AIB1 interact in MCF-7 human breast cancer cells". Proc Natl Acad Sci U S A. 2000, 97(23):12536–40.*
~ *Tobin NP, Bergh J "Analysis of Cyclin D1 in breast Cancer: a call to arms" Curr Breast Cancer Rep. 2012, 4(3):171–3.*
~ *Torchia J, Glass C, Rosenfeld MG "Co-activators and co-repressors in the integration of transcriptional responses". Curr Opin Cell Biol. 1998, 10(3):373-83.*
~ *Tsuyada A1, Chow A, Wu J, Somlo G, Chu P, Loera S, Luu T, Li AX, Wu X, Ye W, Chen S, Zhou W, Yu Y, Wang YZ, Ren X, Li H, Scherle P, Kuroki Y, Wang SE "CCL2 Mediates Cross-talk between Cancer Cells and Stromal Fibroblasts That Regulates Breast Cancer Stem Cells". Cancer Res 2012, 72(119).*
~ *Tyson JJ, Baumann WT, Chen C, Verdugo A, Tavassoly I, Wang Y, Weiner LM, Clarke R. "Dynamic modelling of oestrogen signalling and cell fate in breast cancer cells". Nat Rev Cancer 2011, 11(7):523–32.*
~ *Venema CM, Bense RD, Steenbruggen TG, Nienhuis HH, Qiu SQ, van Kruchten M, Brown M, Tamimi RM, Hospers GAP, Schröder CP, Fehrmann RSN,. de Vries EGE "Consideration of breast cancer subtype in targeting the androgen receptor". Pharmacology & Therapeutics 200 2019, 135–147.*
~ *Waks AG, Winer EP "Breast Cancer Treatment". JAMA 2019, Volume 321, Number 3.*

~ *Winters S, Martin C, Murphy D, Shokar NK "Breast Cancer Epidemiology, Prevention, and Screening". Progress in Molecular Biology and Translational Science 2017, 151:1-32.*
~ *Witzel I, Graeser M, Karn T, Schmidt M, Wirtz R, Schütze D, Rausch A, Jänicke F, Milde-Langosch K, Müller V "Androgen receptor expression is a predictive marker in chemotherapy-treated patients with endocrine receptor-positive primary breast cancers". J Cancer Res Clin Oncol 2013, 139:809–816.*
~ *Yang C, Trent S, Ionescu-Tiba V, Lan L, Shioda T, Sgroi D, Schmidt EV "Identification of cyclin D1- and estrogen-regulated genes contributing to breast carcinogenesis and progression". Cancer Res. 2006, 66(24):11649–58.*
~ *Yeap BB, Krueger RG, Leedman PJ. "Differential posttranscriptional regulation of androgen receptor gene expression by androgen in prostate and breast cancer cells". Endocrinology 1999, 140(7):3282-91.*
~ *Yeh S, Hu YC, Wang PH, Xie C, Xu Q, Tsai MY, Dong Z, Wang RS, Lee TH, Chang C "Abnormal mammary gland development and growth retardation in female mice and MCF7 breast cancer cells lacking androgen receptor". J. Exp. Med. 2003, 198:1899-1908.*
~ *Yu Z, He S, Wang D, Patel HK, Miller CP, Brown JL, Hattersley G, Saeh JC "Selective Androgen Receptor Modulator RAD140 Inhibits the Growth of Androgen/Estrogen Receptor–Positive Breast Cancer Models with a Distinct Mechanism of Action". Clin Cancer Res 2017, 23(24).*
~ *Zhou XE, Suino-Powell KM, Li J, He Y, Mackeigan JP, Melcher K, et al. "Identification of SRC3/AIB1 as a preferred coactivator for hormone-activated androgen receptor". The Journal of biological chemistry 2010, 285(12):9161-71.*