UNIVERSITÀ DELLA CALABRIA

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Dipartimento di Farmacia e Scienze della Salute e della Nutrizione

Dottorato di Ricerca in

Medicina Traslazionale

CICLO

XXXIII

Progesterone Receptor B disrupts the metabolic reprogramming in breast cancer cell lines inducing death by blocking energy producing pathways, and p53 may be linked in this tumour suppressive-action.

Settore Scientifico Disciplinare

BIO/16

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ABSTRACT

Il carcinoma mammario (BC) rappresenta la più comune causa di morte per neoplasia nella donna. Nonostante i progressi fatti nella diagnosi precoce e nella terapia, esso resta una delle forme tumorali con prognosi infausta. Ciò può dipendere dalla sua notevole eterogeneità, infatti, il BC è classificato in diversi sottotipi in base alle caratteristiche istologiche, metaboliche, genetiche e molecolari. In particolare, le forme ormono-resistenti risultano le più aggressive, chemioresistenti e difficilmente curabili. Pertanto, è necessaria l'individuazione di nuove strategie terapeutiche.

Gli ormoni sessuali steroidei, tra cui l'estradiolo (E2) ed il progesterone (PRG) (Fuqua SAW et al., 2004), svolgono un ruolo importante nello sviluppo normale della ghiandola mammaria, e si ritiene che la progressione del cancro al seno sia influenzata da tali steroidi e dai loro recettori (Clarke CL et al., 1990, Dunnwald LK et al., 2007). Sebbene l'azione dei recettori per gli estrogeni nella carcinogenesi del seno, è stata oggetto di intensa indagine, non è ancora chiaro, anzi è controverso, il ruolo dei recettori per il PRG (PRs) (Horwitz KB 1987). Un decremento dei livelli di espressione dei PRs è generalmente associato con la progressione del carcinoma mammario. I PRs appartengono alla sottofamiglia dei recettori steroidei nucleari classici, e comprendono due isoforme, denominate PR-A e PR-B (Kastner P et al., 1990). Da modelli sperimentali emerge che i tumori al seno PR+ sono ben differenziati, a basso rischio di recidiva mentre tumori PR- sono metastatici e presentano un decorso più aggressivo dopo la perdita di PR-B rispetto ai tumori che lo esprimono. I dati dimostrano che i pazienti con tumori PR+ ma con prevalenza dell'isoforma A, hanno una probabilità di recidiva circa 3 volte maggiore rispetto ai pazienti con prevalenza di PR-B, e ciò risulta correlato alla resistenza al tamoxifene (TAM).

Nell'ultima decade sono stati identificati geni e pathways trasduzionali coinvolti nella resistenza endocrina e recentemente il metabolismo cellulare è considerato un innovativo target per le terapie anti-tumorali. È noto che molti segnali oncogenici alterano il metabolismo cellulare allo scopo di sostenere la crescita e la sopravvivenza della massa tumorale. La cellula tumorale esprime un metabolismo peculiare che è tuttora oggetto di indagine in quanto numerosi studi hanno anche evidenziato che le cellule tumorali possono vivere in un ampio spettro di stati bioenergetici che variano dalla predominanza del fenotipo glicolitico fino a quello parzialmente o totalmente fosforilativo. Il glucosio viene metabolizzato sia attraverso la glicolisi che lo Shunt dei Pentoso Fosfato (PPP), di tali vie la cellula cancerosa non metabolizza il glucosio solo per sostenere la richiesta di ATP o per incrementare la sintesi di acidi nucleici e di NADPH (necessario alla sintesi di acidi grassi ed alla rigenerazione del glutatione ridotto), utilizza anche direttamente gli intermedi di tali pathways metabolici.

In un nostro precedente studio, abbiamo dimostrato che il 17OH-progesterone (OHPg) tramite il suo recettore PR-B è in grado di attivare il gene PTEN. PR-B e PTEN cooperano nell'indurre la morte tramite autofagia nelle cellule di tumore mammario (De Amicis F et al., 2014).

Allo scopo di investigare altri tumour-suppressive pathways attraverso cui OHPg/PR-B svolge un'azione protettiva nel tumore al seno, abbiamo deciso, in questo progetto, di valutare un suo possibile ruolo nel metabolismo delle cellule tumorali ed un suo possibile link con un altro noto oncosoppressore, ossia il gene p53 che svolge anche un ruolo chiave nel metabolismo delle cellule tumorali. Ad oggi, nessun dato in letteratura ha riportato un ruolo di OHPg/PR-B sulla riprogrammazione metabolica di cellule di tumore mammario, nè una sua possibile cooperazione in tale azione con la p53.

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 Epidemiology

Breast cancer is the most frequently diagnosed cancer and leading cause of cancer death in women worldwide. Despite advances in the diagnosis and treatment of human malignancy, cancer remains among the leading causes of morbidity and mortality worldwide, with 7.5 million deaths attributed to this cancer in 2008. Breast cancer is now the most frequently diagnosed cancer and the leading global cause of cancer death in women, accounting for 23 % of cancer diagnoses (1.38 million women) and 14 % of cancer deaths (458,000 women) each year (Jemal A et al., 2011). Although breast cancer has a markedly higher incidence in developed countries, half of new breast cancer diagnoses and an estimated 60 % of breast cancer deaths, are now thought to occur in the developing world (Jemal A et al., 2011). 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 C, 2017). Due to the presence of these prevention programs and the efficient therapies, breast cancer mortality has decreased in developed countries, while it is increasing in underdeveloped countries (Harbeck N & Gnant M, 2017).

1.1.2 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 S et al., 2017). Other risk factors implicated in breast tumour are (Fig. 1):

- 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 S 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 an increased risk to develop breast tumour.
- Lifestyle. Alcohol consummation and diet style based only on sutured fat acid intake increase breast cancer risk. In particular, frequent alcohol consummation is associated with the highest level of circulating estrogen-related hormones by leading to ERα pathway activation (Sun YS et al., 2017).

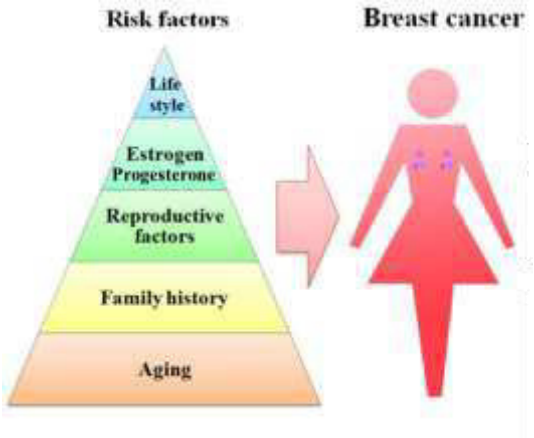


Figure 1 Schematic representations of breast cancer risk factors (Adapted from Sun YS et al., 2017).

1.1.3 Classification

Breast cancer is classified in (Fig. 2):

-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.

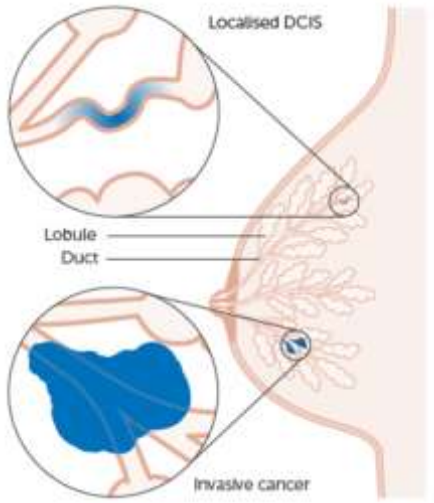


Figure 2 Breast cancer classification (Cancer Research UK).

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 T 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 auxiliary 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. 3) 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.).

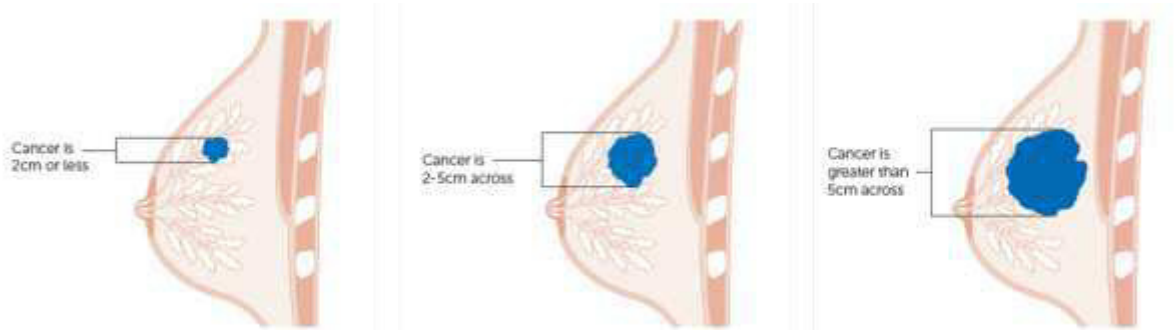


Figure 3 Breast cancer grading (Cancer Research UK).

1.1.4 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 whether 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 AG & Winer EP, 2019).

1.2 Steroid hormone receptors in breast cancer

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 C & O'Malley B, 2010).

The sex steroid hormones, Estrogen (E2) and Progesterone (Pg), play an important role in normal mammary gland development, and it is believed that breast cancer progression is influenced by theirself and their receptors (Fuqua SAW et al., 2004). The level of these steroid hormones receptors is a strong prognostic factor for patients with breast cancer and has been used in clinical management as an indicator of endocrine responsiveness (Fig. 4) (Toma S et al., 1985; Horwitz KB 1993). Approximately 75% of primary breast cancers express ER, and more than half of these cancers also express PR (McGuire WL 1978). Both ER and PR are prognostic factors, although both are weak and lose their prognostic value after long-term follow up (Bardou VJ et al., 1973).

Although the clinical significance of ER evaluation has been well established, the role of PR remains controversial, and whether PR assessment is necessary has been debated for years. (Hefti MM et al., 2013).

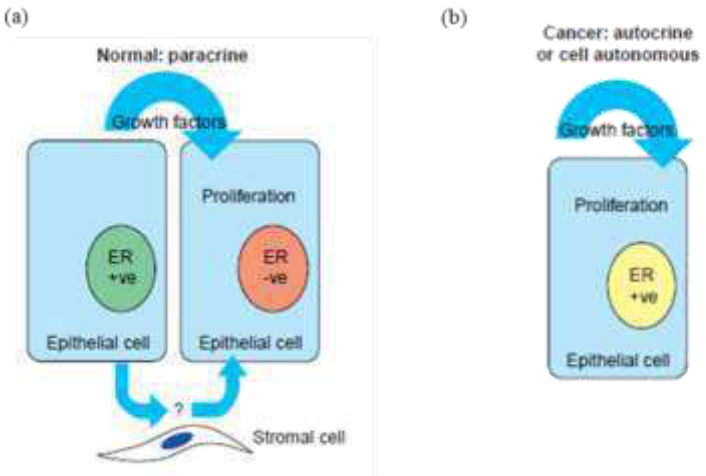


Figure 4 Estrogen receptor expression and cell proliferation in human breast tissues (Adapted from Clarke et al., 2004).

PR is an estrogen-regulated gene, and its synthesis in normal and cancer cells requires estrogen and ER. Therefore, it is not surprising that ER positive/PR-positive tumours are more common than ER-positive/PR-negative tumors. The etiology of ER-positive/PR-negative tumours is currently unclear. Some studies have shown that ER and PR status can change over the natural history of the disease or during treatment (Hull DF et al., 1983). For instance, sequential breast cancer biopsies have shown that ER levels are reduced slightly with intervening endocrine therapy, although complete loss is uncommon. In contrast, PR levels decrease more dramatically during tamoxifen therapy, with up to half of tumors completely losing PR expression when resistance develops (Gross GE et al., 1984) These ER-positive/PR-negative metastatic tumors then display a much more aggressive course after loss of PR compared with tumors retaining PR, and patients then have a worse overall survival, indicating a change in tumor cell–regulatory mechanisms (Gross GE et al., 1984; Balleine RL et al., 1999). Whether and how the loss of PR affects the poor clinical course of these tumors is at present unclear. Although Estrogen Receptor (ER) is an accepted predictor of response to endocrine therapy the role of Progesterone Receptor (PR) has been more controversial. Recent studies published on the largest retrospective analysis of early breast cancer treated with tamoxifen found that patients with ER+/PR+ tumors benefited much more from adjuvant tamoxifen therapy than those with ER+/PR- tumours (Bardou VJ et al., 2003*;* Rakha EA et al., 2007). Importantly, multivariate analyses, including lymph node involvement, tumour size, and age, show that PR status was independently associated with disease-free and overall survival. Progesterone Receptors belong to the subfamily of

classical nuclear steroid receptors; human PR proteins exist as two isoforms, termed PR-A and PR-B. Both PR-A and PR-B contain three functional domains including the N-terminus, a centrally located DNA binding domain (DBD), and C-terminal ligand binding domain (LBD) (Fig. 5). Three-dimensional structures of isolated DBD and LBDs have revealed common motifs for these regions. By comparison, little is known about the structure of the N-terminal domain. This is the least conserved region among family members with re-spect to both length and amino acid sequence. The N-domainis functionally important, as it is required for full transcriptional activity of steroid hormone receptors and for many cell- and target gene-specific responses. Other functional and structural determinants have been identified within these broader three domains. In addition to binding steroid hormone, the LBD contains determinants for dimerization (DI) in the absence of DNA, binding of heat shock proteins(hsps) and for nuclear localization sequence (NLS). The DBD contains a second NLS and dimerization domain that is dependent on DNA binding. Steroid receptors contain at least two transcription activation domains (AFs), AF-1 in the N-terminal domain and highly conserved AF-2 in the C-terminal LBD. These are autonomous transferable domains required for the DNA bound receptor to transmit a transcriptional activation response and they function as specific binding sites for coactivators. AF-2 located in the LBD is hormone-dependent and becomes activated as a result of the steroid hormone inducing a conformational change that creates a hydrophobic binding pocket for members of the p160 family of steroid receptor coactivators (SRCs). The coactivators that bind to and mediate the activity of AF-1 are yet not well defined.

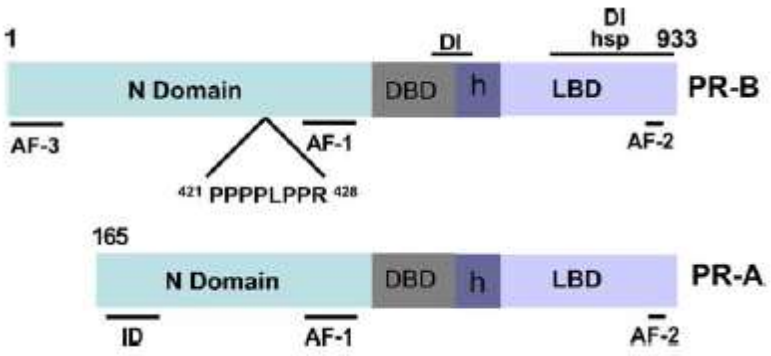


Figure 5 Structure of progesterone receptor PR

The human PR is expressed as two isoforms deriving from a single gene by alternate promoter usage. PR-A (94 kDa) differs from PR-B (120 kDa) by lacking 164 amino acids

(aa) at the N-terminus. Although the two forms of PR have similar steroid hormone and DNA binding activities, they have distinct transcriptional activities. PR-B in general, is a much stronger activator than PR-A. However, PRA can be a strong activator under specific cell and target gene contexts. The stronger activation potential of PR-B is due in part to the existence of a third activation domain (AF-3) within the first N-terminal 164 aa that is unique to PRB. Under certain cell and target promoter contexts PR-A is inactive as a transcription factor and can function as a ligand-dependent transdominant repressor of other steroid receptors including PR-B and the estrogen receptor (ER). PR-A can act in this repressor mode in response to the binding of either progestin agonists or antagonists. An inhibitory domain (ID) responsible for this transrepressor function has been mapped to the first 140 N-terminal (aa 165–305) amino acids of PR-A. Since the sequence within ID is present in both PR isoforms but is only active in the context of PR-A suggests the PR-B Specific N-terminal segment plays a role in suppressing the ID domain. Despite structural similarities, PR-A and PR-B regulate different subsets of genes and although PR-B is transcriptionally more active, there are genes, known to be involved in breast cancer progression, that are uniquely regulated by PR-A (Richer JK et al., 2002). *In vivo* the two PR isoforms are usually co-expressed at similar levels in normal cells, yet their ratio varies dramatically in different tissues, physiological states and disease (Boyd-Leinen PA et al., 1982; Kato J et al., 1993). With regard to the mammary gland, in transgenic mice, 3:1 over-expression of PR-A over PR-B results in extensive epithelial cell hyperplasia, excessive ductual branching, and disorganized basement membrane, all features associated with neoplasia. In contrast, over-expression of PR-B leads to premature ductual growth arrest and inadequate lobulo-alveolar differentiation (Shyamala G et al., 2000; Shyamala G et al., 1998). Moreover, the loss of coordinated PR-A and PR-B expression is an early event in carcinogenesis and is evident in pre-malignant lesions (Mote PA et al., 2002). A significant proportion of carcinomas expresses a predominance of PR-A isoform and this is associated with poor clinical outcomes: in endometrial cancer, PR-A isoform predominance is significantly more common in tumours of higher grade, indicating an association between PR-A isoform predominance and poor prognosis (Arnett-Mansfield RL et al., 2001). Although ER and PR are members of different steroid hormone receptor sub-families and recognize distinct hormone response elements, there is considerable biological evidence for cross-talk between the estrogen and progestin hormone receptor signalling pathways. In many cases, progestins suppress the stimulatory effects of estrogens in target cells, for

example estrogen increases the expression of both c-fos and PR mRNA in uterine cells, and progestins block this effect (Loose-Mitchell DS et al., 1988; Kirkland JL et al., 1992) which appear to be mediated via PR, but it is unclear if ER or some other component of the estrogen-ER signaling pathway is the target for repression. Also liganded PR can suppress estradiol-stimulated ER activity, with the magnitude of repression dependent on the PR isoform, progestin ligand, promoter, and cell type (Katzenellenbogen BS 2000).

1.3 Cancer cell metabolism

Cancer cells survival is strongly linked to a metabolic reprogramming. Disruption of tumour metabolism represents an elegant approach to induce cancer cell death. In fact, energy metabolism has been considered an innovative target in anticancer therapies, since metabolic changes are a common feature of cancerous tissues. However, it may help in understanding how, step by step, the metabolic pathways are arranged in comparison with normal metabolism to characterize a cancer metabolic phenotype.

The most characterizing metabolic phenotype observed in cancer cells is known as the Warburg Effect (Fig. 6), which involves the production of ATP through glycolysis instead of oxidative phosphorylation, even in presence of normal oxygen concentrations. However, metabolic reprogramming in tumours extends beyond the Warburg Effect. In fact, the classical theory on the metabolism of tumour cells (increased glycolytic activity and down-regulation of oxidative phosphorylation) is still under investigation as numerous studies have shown that cancer cells can live in a wide spectrum of bio-energy states ranging from the predominance of the glycolytic to the phosphorylating phenotype (Smolková K et al., 2011).

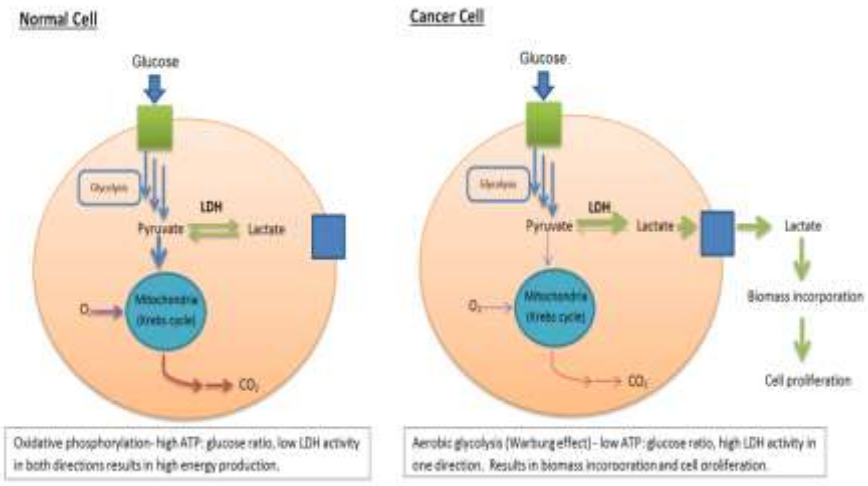


Figure 6 Warburg Effect

Estrogen and estrogen receptors (ERs) are well-known regulators of several aspects of metabolism, including glucose and lipid metabolism, and impaired estrogen signaling is associated with the development of metabolic diseases (Faulds Malin Hedengran, 2012).

In our previous study, we demonstrated that 17OH-progesterone (OHPg) via its PR-B receptor is able to activate the PTEN gene (De Amicis F et al., 2014). PR-B and PTEN cooperate by inducing death through autophagy and senescence in breast cancer cells (De Amicis F et al., 2016).

In recent years, mounting evidence has also implicated p53 as a central player in the regulation of cellular energy metabolism (Vousden KH and Ryan KM, 2009; Liu J et al., 2015; Matoba S et al., 2006), alongside potential roles in the regulation of mitochondrial dynamics, beyond cell death (Wang W et al., 2010; Saleem A et al., 2015; McGowan EM et al., 2011). The p53 protein is a transcriptional factor that is stabilized within the nucleus upon DNA damage or oncogenic signaling. p53 regulates genes involved in cell cycle arrest, cell death (apoptosis), DNA repair, and senescence to prevent tumor development and growth (Levine AJ et al., 2009; Gnanapradeepan K et al., 2018; Vousden KH et al., 2002; Bieging KT et al., 2014; Khoo KH et al., 2014; Xie Y et al., 2016; Galluzzi L et al., 2015). p53 also localizes to the cytoplasm and the mitochondria, where it can regulate cytoplasmic cellular functions including apoptosis. Non-canonical functions of p53, including necrosis (Baumann, K, 2012), autophagy (Green DR and Kroemer G, 2009) and the less well-known functions of p53, necroptosis (Wu W et al., 2012) have all been shown to be p53-activated

specialized forms of mitochondria-programmed cell death processes (Gnanapradeepan K et al., 2018; Xie Y et al., 2016; Galluzzi L et al., 2015). Additional less characterized p53-associated processes that do not necessarily lead to cell death include autophagy, senescence, differentiation, and dormancy. Homeostasis regulation of the mitochondrial DNA involves both p53-nuclear transcriptional target genes, whose products translocate to the mitochondria or non-nuclear direct cytoplasmic effects of the p53 protein.

In order to investigate other tumor-suppressive pathways through which OHPg / PR-B signaling carries out a protective action in breast cancer, we decided in this project, to evaluate its possible action on the alteration of the metabolic reprogramming. Furthermore, we investigated a possible link with another known tumour suppressor, which performs a key role also in the cellular metabolism, the p53 gene (Kruiswijk F et al., 2015).

To date, no data in the literature have reported such an effect of the OHPg / PR-B signaling in breast cancer cells metabolism as well as a possible cooperation between OHPg / PR-B and p53 in interfering on the metabolic reprogramming.

1.4 Hypotheses and aims

1.4.1 Hypotheses

In our previous study, we demonstrated that 17OH-progesterone (OHPg) via its PR-B receptor is able to activate the PTEN gene (De Amicis F et al., 2014). PR-B and PTEN cooperateD in inducing death through autophagy and senescence in breast cancer cells (De Amicis F et al., 2016).

In order to investigate other tumor-suppressive pathways through which OHPg / PR-B signalling carries out a protective action in breast cancer, we decided in this project, to evaluate its possible action on the alteration of the metabolic reprogramming. Furthermore, we investigated a possible link with another known tumour suppressor, which performs a key role also in the cellular metabolism, the p53 gene. To date, no data in the literature have reported such an effect of the OHPg / PR-B signaling in breast cancer cells metabolism as well as a possible cooperation between OHPg / PR-B pathway and p53 in interfering on their metabolic reprogramming.

1.4.2 Aims

A) To evaluate the interference and role of OHPg/PR-B on the metabolic reprogramming of breast cancer cells, using two estrogen-responsive cell models such as MCF7 and T47D through the Seahorse analyzer. This method evaluates the rate of oxygen consumption in real time (OCR) and the rates of extracellular acidification (ECAR).

B) To compare the interference and role of OHPg/PR-B on the metabolic reprogramming of breast cancer cells, obtained by the seahorse analyser as well as considering the role of OHPg / PR-B signaling on the main kinases of various metabolic pathways, through Western Blotting analysis and enzymatic activities.

C) To investigate, in MCF7 cells, the possible cross-talk between the two PR-B tumor suppressors and the p53 gene.

D) To evaluate the possible role of this link in interfering with the metabolic reprogramming of MCF7 cells, thus expanding the understanding in the mechanisms underlying the control of progesterone and its receptor in malignant cell transformation processes.

E) To provide further clarification on the biology of breast cancer.

2 Materials and methods

Chemicals and Assay Kits 17-Hydroxyprogesterone (OHPg), aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMFS), sodium orthovanadate, Tris-Cl, MnCl2, NADP+, isocitrate and malate were from Sigma Chemical (Milan, Italy). Antibodies used were from Santa Cruz Biotechnology (Santa Cruz, CA). MitoProfile® Total OXPHOS WB Antibody Cocktail was from AbCam (Milan, Italy). Triglycerides content, lipase, glucose-6-phosphate dehydrogenase (G6PDH), Lactate Dehydrogenase (LDH) activities and glucose assays kits were from Inter-Medical (Biogemina Italia Srl, Catania, Italy). Molecular Probes ATP Determination Kit (A22066) was from Invitrogen (Milan, Italy).

Cell Cultures - Human breast cancer cells MCF7 and T47D (American Type Culture Collection-ATCC) have been stored according to supplier's instructions. Every 4 months, cells were authenticated by single tandem repeat analysis at our Sequencing Core; morphology, doubling times, and mycoplasma negativity were tested (MycoAlert; Lonza). MCF7 were maintained in DMEM/F-12 medium containing 5% FCS, 1% L-glutamine, 1% Eagle's nonessential amino acids, and 1 mg/ml penicillin/streptomycin in a 5% CO2 humidified atmosphere. T47D cells were routinely maintained in RPMI 1640 supplemented with 5% FCS (Sigma, Milan, Italy), 1 mg/ml penicillin/streptomycin (Sigma, Milan, Italy). For each experiment, cells cultured in phenol red and starved in serum-free medium for 24 hours (h), were treated with OHPg in medium containing 5 % charcoal-treated FCS to reduce the endogenous steroids concentration. The OHPg concentrations were chosen on the basis of our previous studies (De Amicis F et al., 2013). Furthermore, since in the major part of the experiments the 10 nM OHPg was the more efficacious in inducing an effect, we used this concentration in the Western blotting regarding the T47D.

Seahorse XF96 metabolic flux analysis (OCR and ECAR) - Real-time oxygen consumption rates (OCRs) and extracellular acidification rates (ECAR) rates were determined using the Seahorse Extracellular Flux (XFe96) analyzer (Seahorse Bioscience, USA). Briefly, 1×10^4 cells (MCF7 and T47D) per well were seeded into XFe96 well cell culture plates in presence of DMEM/F-12 and incubated for 24 h to allow cell attachment. After which, cells were starved in serum free media for 24 h and successively treated with 10 nM as well as 100 nM OHPg (these concentrations were chosen on the basis of our previously experiments) in medium containing 5 % charcoal-treated FCS. After 48h treatment, the cells were washed in pre-warmed XF assay media (or for OCR measurement, XF assay media supplemented with 10 mM glucose, 1 mM Pyruvate, 2 mM L-glutamine, and adjusted at 7.4 pH). Cells were then maintained in 175 µL/well of XF assay media at 37°C, in a non-CO2 incubator for 1 h. During the incubation time, we loaded 25 µL of 80 mM glucose, 9 µM oligomycin, and 1M 2-deoxyglucose (for ECAR measurement) or 10 µM oligomycin, 9 µM FCCP, 10 µM rotenone, 10 µM antimycin A (for OCR measurement), in XF assay media into the injection ports in the XFe96 sensor cartridge. Measurements were normalized by protein content (SRB assay).

Western blotting - Total protein extracts were obtained as previously described (Panza et al., 2017). Proteins were resolved on a 10 % sodium dodecyl sulfate–polyacrylamide gel,

transferred to a nitrocellulose membrane, probed overnight at 4 °C with the indicated antibodies β-actin was used as loading control.

Lipid-mediated transfection of siRNA duplexes - Cells were transfected with functionally verified siRNA directed against human PR-B, or against human p53 or with a control siRNA (Qiagen, Mi, Italy) that did not match with any human mRNA used as a control for non-sequence specific effects (NS siRNA). Cells were transfected using Lipofectamine 2000 reagent (Invitrogen, Paisley, UK) according to the manufacturer's instructions as previously reported (Panza S et al., 2017) and then treated as indicated.

Glucose assay Glucose oxidase catalyzes the oxidation of glucose to gluconic acid. The formed hydrogen peroxide is detected by a chromogenic oxygen acceptor, phenol, 4–aminophenazone in the presence of peroxidase. The intensity of the color formed is proportional to the glucose concentration in the sample (Santoro M et al., 2016). Data are presented as nM/μg protein.

Lactate Dehydrogenase (LDH) Assay LDH catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD^+. After the treatments, LDH assay was performed on cell lysates as previously described (Santoro M et al., 2016). Data are presented as absorbance change at 340 nm.

G6PDH assay activity The conversion of $NADP^+$ to NADPH, catalysed by G6PDH, was measured by the increase of absorbance at 340 nm as previously described (Santoro M et al., 2016). Data are presented as nM/min/μg protein.

ATP assay A bioluminescence assay for quantitative determination of ATP with recombinant firefly luciferase and its substrate D-luciferin (light emission at 560 nm at pH 7.8), was performed as previously described (Santoro M et al., 2016). Data are presented as nM/μg protein.

Isocitrate dehydrogenase 2 (ICDH2) activity assay ICDH activity was measured as previously reported (Gnoni GV and Paglialonga G, 2009). The reaction was performed on the cell lysate using a final 1 ml reaction volume containing 0.2 mg protein, 50 mM Tris-Cl pH 7.4, 5 mM MnCl2, 0.25 mM $NADP^+$, 0.25 mM isocitrate, at 37 °C. Reaction progress was monitored at 340 nm for 2 minutes (min); the NADPH production was calculated using an NADPH extinction coefficient of 6.26103 M-1 cm-1. Data are presented as nM/min/μg protein.

Malic enzyme (ME) activity assay ME activity was measured in the cell lysates as previously reported (Gnoni GV and Paglialonga G, 2009). The reaction was performed using

a final 1 ml reaction volume containing 0.3 mg protein, 50 mM Tris-HCl pH 7.4, 5 mM $MnCl_2$, 0.1 mM $NADP^+$, 5 mM malate, at room temperature. Reaction progress was monitored at 340 nm for 2 min; the NADPH production was calculated using an NADPH extinction coefficient of 6.26103 M-1 cm-1. Data are presented as nM/min/μg protein.

Triglycerides assay Triglycerides were measured in duplicate by a GPO-POD enzymatic colorimetric method according to manufacturer's instructions in cell lysates and as previously described (Santoro M et al., 2016). Data are presented as nM/μg protein.

Lipase activity assay Lipase activity was evaluated, by the method of Panteghini et al. (Panteghini M et al., 2001) based on the use of 1,2-o-dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin) ester as substrate, as previously described (Santoro M et al., 2016). Data are presented as nM/min/μg protein.

Acyl-CoA dehydrogenase activity assay (Fatty Acid Oxidation, FAO) Acyl-CoA dehydrogenases catalyze the initial step in each cycle of fatty acid β-oxidation in the mitochondria of cells. Assay was performed using a modification of a previously described method (Lehman TC et al., 1990). In brief, after protein lysis, 50 μg of proteins was added to the buffer containing 20 mm Mops, 0.5 mm EDTA and 100 lm FAD at pH 7.2. Reduction of FAD to FADH2 was read at 340 nm upon addition of octanoyl-CoA (100 μg M) every 20 s for 1.5 min. Data are expressed as nmol ⁄ min ⁄μg protein. The enzymatic activity was determined with three control media: one without octanoyl-CoA as substrate, another without the coenzyme (FAD) and the third without either substrate or coenzyme (data not shown).

Statistical Analysis - Data sets analyzed by Seahorse XF96 metabolic flux analysis (OCR and ECAR) was made using XFe96 software and GraphPad Prism software, using one-way ANOVA and Student's t-test calculations. All experiments were performed in quintuplicate, three times independently. The data obtained from triglycerides assay, lipase activity, G6PDH activity, and glucose as well as triglycerides quantification (six independent experiments using duplicate determinations), were presented as the mean ± SEM. The Western blot analyses were performed in at least four independent experiments. Each column of the Western blotting densitometric analysis, when we used the ab anti-OXPHOS represents the band intensities evaluated in terms of arbitrary densitometric units and were presented as the mean ± SEM. The differences in mean values were calculated using Analysis of Variance. The Wilcoxon test was used after analysis of variance as post hoc test. A value of $P \leq 0.05$ was considered as statistically significant. β-actin was used as a control

for equal loading and transfer. The numbers above blots represent the mean of the band optical density.

3 Results

3.1 The effects of OHPg on the MCF7 and T47D cells metabolic reprogramming were first evidenced by the Seahorse XF96 Analyzer (OCR and ECAR)

Cellular metabolism executed through a series of intracellular biochemical processes (glycolysis, Kreb Cycle, Electron transport and oxidative phosphorylation), results in the production of ATP, the release of heat and chemical byproducts (lactate and CO2) into the extracellular environment. Under typical *in vitro* cell culture conditions, the OCR is an indicator of mitochondrial respiration and of the ECAR is predominantly a measure of lactic acid formed during glycolytic energy metabolism. Measuring both OCR and ECAR simultaneously, may be indicative of the dynamic interplay between glycolysis and oxidative metabolism. To see metabolic adaptation dynamically and on live cells here, we evaluate the metabolic profile of MCF7 and T47D cells after 48 hours (h) treatment with OHPg (10 nM and 100 nM). In Fig.7 it was shown the metabolic profile of MCF7 cells based on OCR and ECAR (Panel A and F).

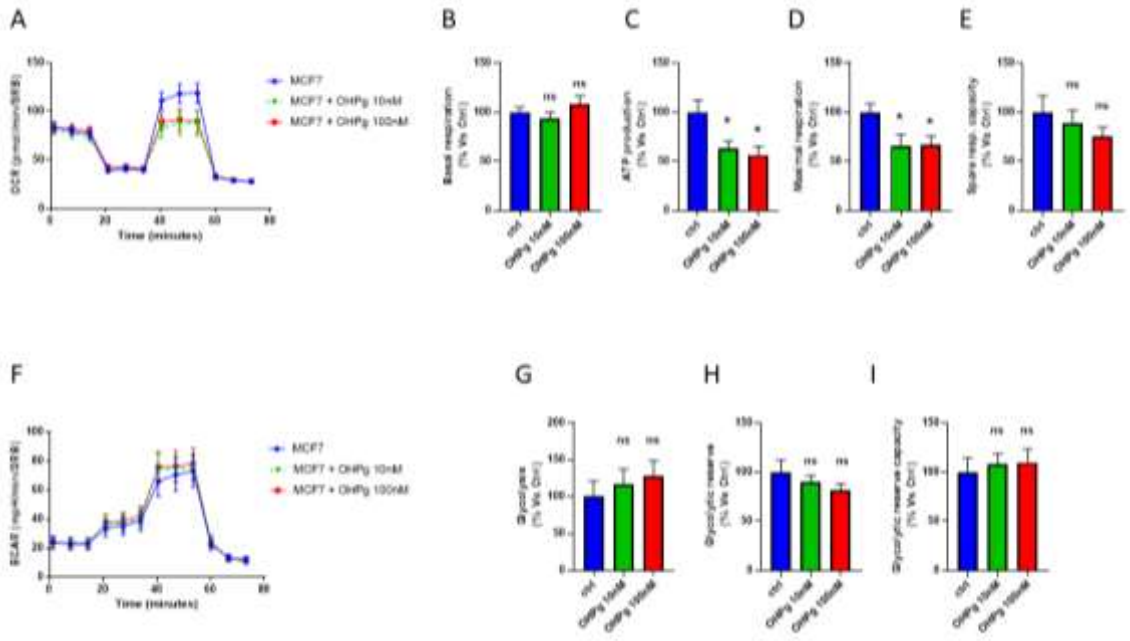


Figure 7: MCF7 cells showed a reduced mitochondrial ATP production upon OHPg. The Seahorse XF96 analyzer was employed to determine the mitochondrial function of MCF-7 cells, see Material and Methods. Data sets analyzed by Seahorse XF96 metabolic flux analysis (OCR and ECAR) was made using XFe96 software and GraphPad Prism software, using one-way ANOVA and Student's t-test calculations. All experiments were performed in quintuplicate, three times independently. A value of $P \leq 0.05$ was considered as statistically significant **A**: OCR; **B**: basal respiration; **C**: ATP production; **D**: maximal respiration; **E**: spare respiration capacity; **F**: ECAR; **G**: glycolysis; **H**: glycolytic reserve; **I**: glycolytic reserve capacity. * $p < 0.05$; ns = not significant.

From the results, the ATP and maximal respiration levels were significantly reduced after treatment with 10nM and 100nM OHPg (Panel C and D), while not significant changes were founded in basal respiration, spare respiratory capacity (Panel B and E) as well as in glycolysis, glycolytic reserve and glycolytic reserve capacity levels (Panel G, H and I). In Fig. 8 it can be observed the metabolic profile of T47D cells based on OCR and ECAR (Panel A and F).

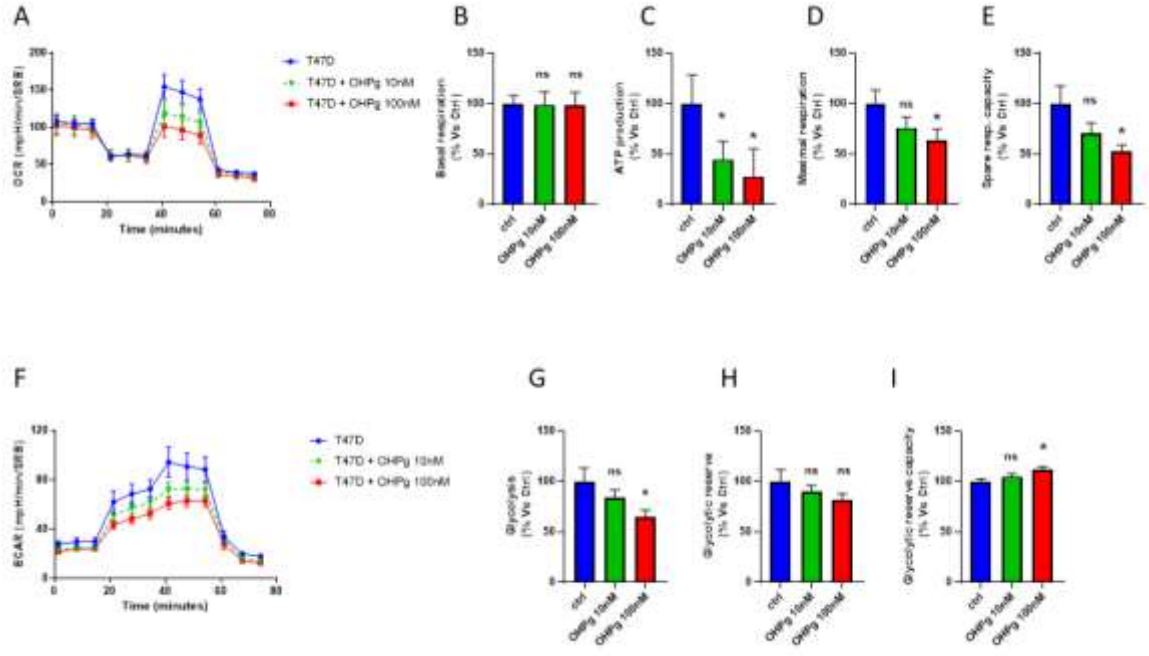


Figure 8: T47D cells showed a reduced mitochondrial ATP production and glycolysis upon OHPg. Seahorse XF96 analyzer was employed to determine OCR and ECAR in T47D cells, see Material and Methods. Data sets analyzed by Seahorse XF96 metabolic flux analysis was made using XFe96 software and GraphPad Prism software, using one-way ANOVA and Student's t-test calculations. All experiments were performed in quintuplicate, three times independently. A value of $P \leq 0.05$ was considered as statistically significant **A**: OCR; **B**: basal respiration; **C**: ATP production; **D**: maximal respiration; **E**: spare respiration capacity; **F**: ECAR; **G**: glycolysis; **H**: glycolytic reserve; **I**: glycolytic reserve capacity. * $p < 0.05$; ns = not significant.

From the results, the ATP levels were significantly reduced after treatment with 10 nM and 100 nM OHPg (Panel C). The maximal respiration and spare respiratory capacity levels were significantly reduced after treatment with OHPg 100nM (Panel D and E). After treatment with 100nM OHPg, glycolysis way was significantly reduced meanwhile glycolytic reserve capacity level were increased (Panel G and I respectively).

3.2 OHPg/PR-B regulated glycolysis and intracellular LDH activity in breast cancer cells

To better define how OHPg/PR-B could regulate the glycolytic pathway in our breast cancer cell lines, we determined the cellular glucose content, PFK1 expression as well as intracellular LDH activity after exposure to increasing OHPg concentrations for 24 h. To establish if OHPg acted through the PR-B, a set of cells were transfected with functionally verified siRNA directed against human PR-B in all the experiments performed (Fig. 9).

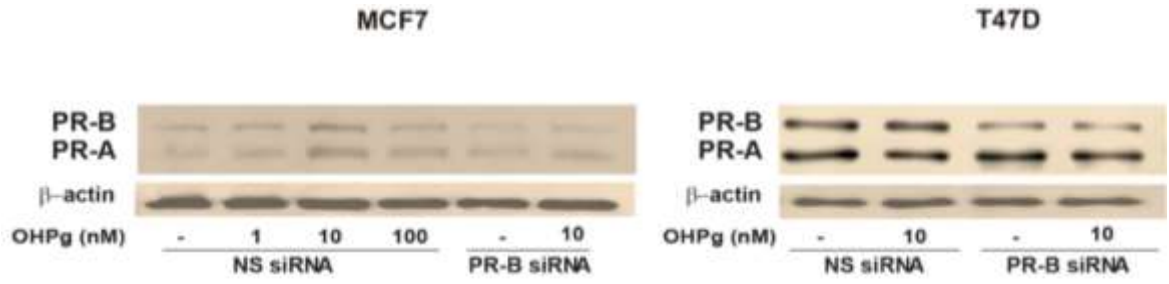


Figure 9: Immunoblot to verify that the used siRNA directed against human PR-B was really efficacy to silence the PR-B.

MCF7 and T47D cells transfected with a control siRNA and treated with the reported OHPg concentrations for 24h. In other sets of experiments MCF7 and T47D cells were transfected to verify the functionality of the siRNA directed against human PR-B without or with 10 nM OHPg. β actin was used as loading control. Autoradiograph shows the results of one representative experiment.

OHPg increased the glucose content with the respect to untreated cells, while PFK1 expression was drastically reduced. (Fig. 10A and B).

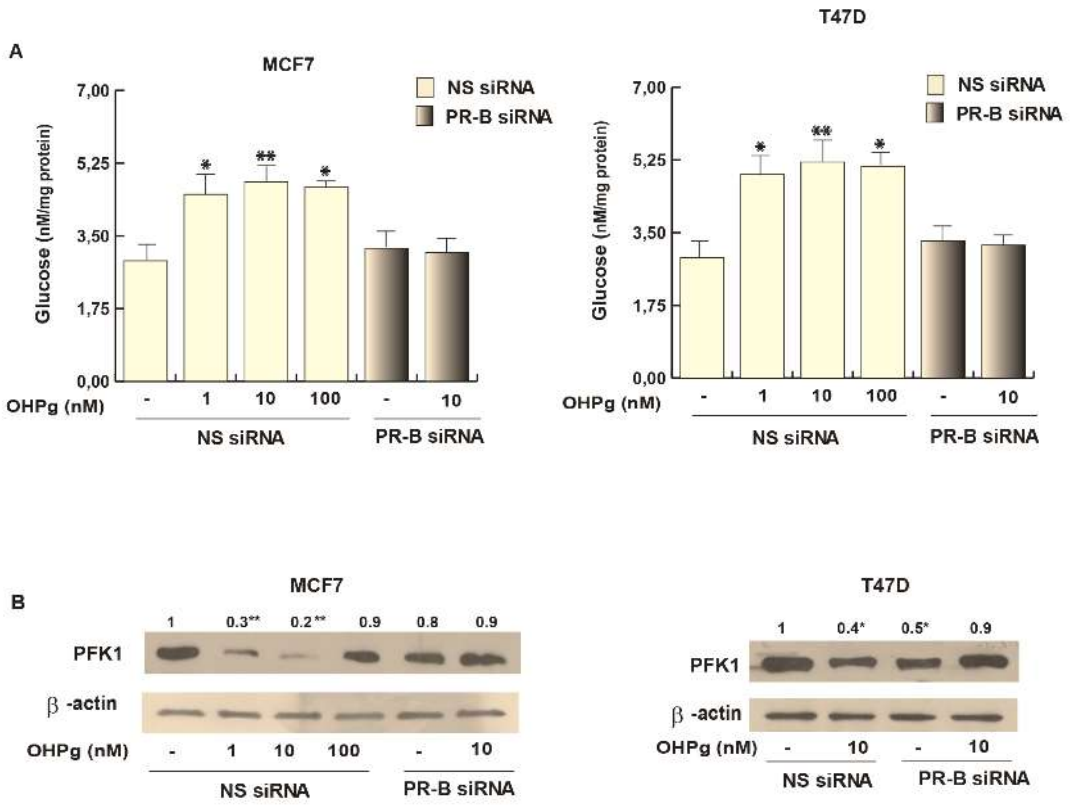


Figure 10 A and B: OHPg/PR-B regulate glycolysis in breast cancer cells.
A: Glucose assay was performed in MCF7 cells transfected with non-specific (NS)- or targeted against **A:** Glucose assay was performed in MCF7 and T47D cells transfected with a control siRNA and treated with the reported OHPg concentrations for 24h. In other sets of experiments MCF7 and T47D cells were transfected with functionally verified siRNA directed against human PR-B without or with 10 nM OHPg. Columns represent mean ± SEM of 6 independent experiments performed in duplicate. **P* < 0.05 versus (-); ***P* < 0.05 versus 10nM OHPg **B**: Immunoblot showing PFK1 expression. MCF7 and T47D cells were treated as in A. β actin was used as loading control. Autoradiograph shows the results of one representative experiment and the numbers on the top of the blot, are mean of four independent experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as fold over vehicle (-). **P* < 0.05;***P* < 0.05 versus (-).

The LDH enzyme catalyzes the reversible conversion of the final product of glycolysis, the pyruvate into lactate. This enzymatic activity, evaluated on cell lysate, increased in OHPg-treated cells (Fig. 10C). Similar results were obtained both on glucose content and on PFK1 expression as well as on the intracellular LDH activity in T47D cells. Although, the more efficacious concentration was the 10 nM OHPg as it concerns the glucose content and LDH activity, also the 1 nM and the 100 nM were significantly higher with respect to the control. The results also showed that the effect were mediated by PR-B.

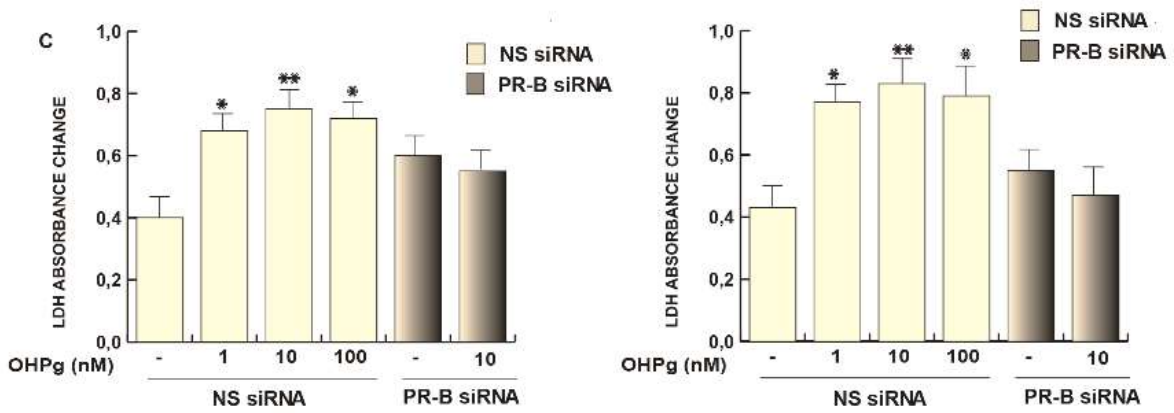


Figure 10 C: OHPg/PR-B regulated LDH activity in breast cancer cells.
C: LDH activity was performed in MCF7 and T47D cells treated as in A. Columns represent mean ± SEM of 6 independent experiments performed in duplicate. **P* < 0.05; ***P* < 0.05 versus (-).

3.3 OHP/PR-B effects on biosynthetic contributions in breast cancer cells

Glucose is also engaged through the pentose phosphate cycle (PPP), the main route in the cellular production of NADPH molecules. The PPP is upregulated in many types of tumors and the key rate limiting enzyme of the pathway is the G6PDH. Therefore, we tested the effect of OHPg/PR-B on the G6PDH activity in our cell models. OHPg treatment determined a significant reduction of the enzymatic activity at 10 and 100 nM OHPg (Fig. 11A) in both cell lines, addressing how the lowering of G6PDH in treated-cells also contributed to the decrease of glucose utilization.

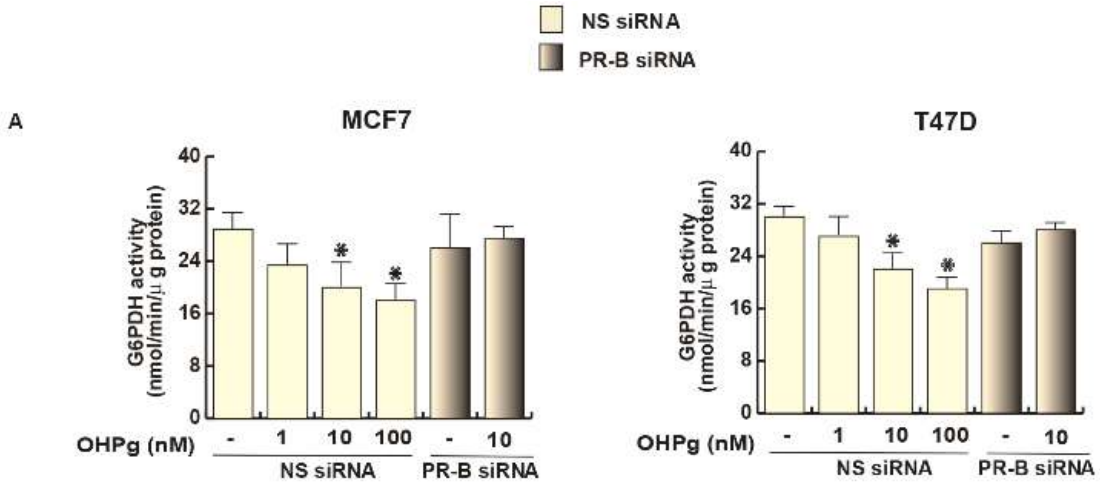


Figure 11 A: OHPg/PR-B affect biosynthetic contributions in breast cancer cells.

A: G6PDH activity was performed as reported in Materials and Methods. Columns represent mean ± SEM of 6 independent experiments performed in duplicate. *P < 0.05 versus (-).

To have more details of the OHPg/PR-B effect on biosynthetic state of breast cancer cells we investigated the activity of other two enzymes involved in the production of NADPH such as the ME and ICDH2. In MCF7 and T47D cells, (Fig. 11B, C), 10 nM OHPg were able to induce both enzymatic activities, in a significant manner. The results also showed that the effect were mediated by PR-B.

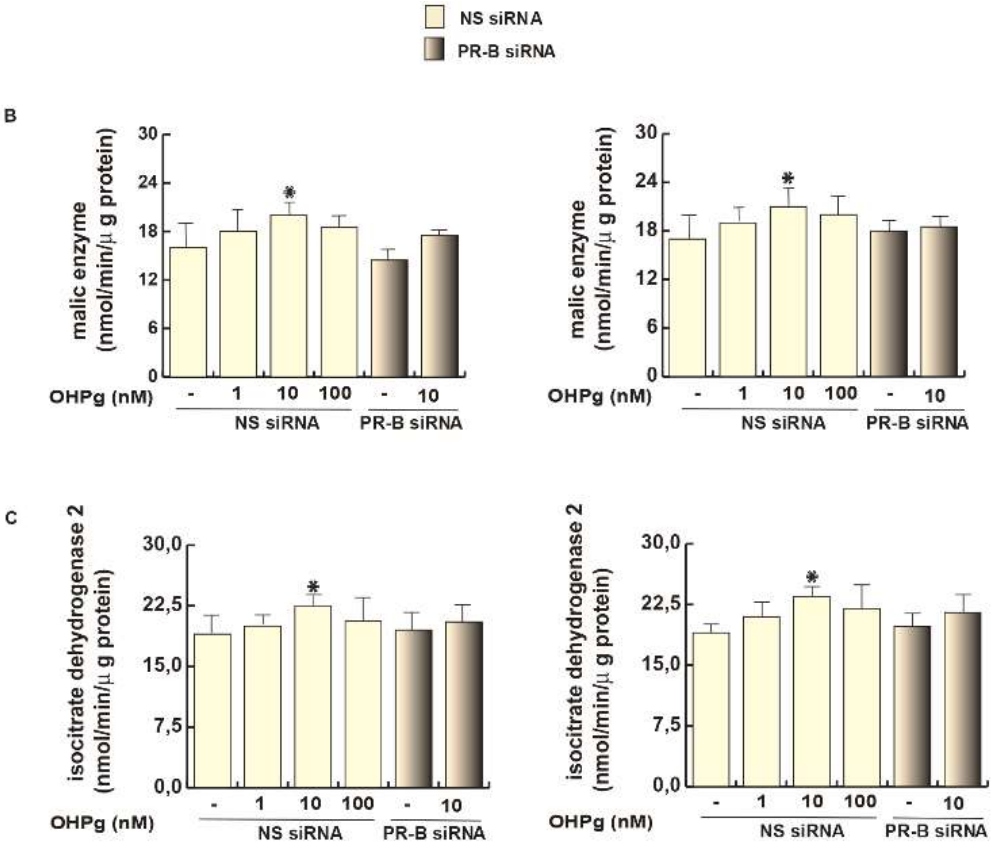


Figure 11 B and C: OHPg/PR-B affect biosynthetic contributions in breast cancer cells.
B: Malic enzyme assay **C:** Isocitrate dehydrogenase 2 activity were performed as reported in Materials and Methods. Columns represent mean ± SEM of 6 independent experiments performed in duplicate. *P < 0.05 versus (-).

3.4 OHPg/PR-B modulate bioenergetic requirements in breast cancer cells

Due to rapid proliferation and accelerated activities, tumors are constantly in high demand of ATP (Liem MP et al., 2014). Our data showed that in response to OHPg stimulation, the ATP content is reduced in both cell lines at all the concentrations used and PR-B siRNA reversed the effect (Fig. 12A).

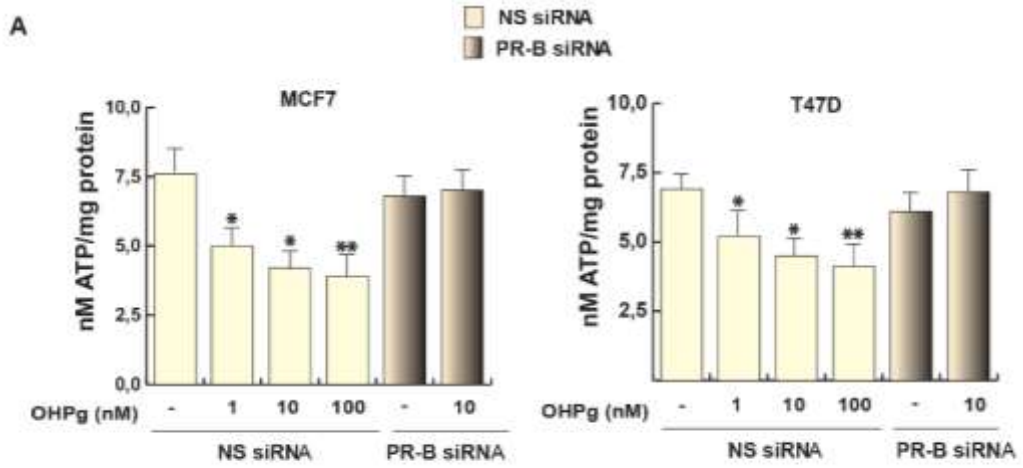


Figure 12 A: OHPg/PR-B modulate bioenergetic requirements in breast cancer cells
A: ATP assays in MCF7 and T47D were performed as above mentioned. Columns represent mean ± SEM of 6 independent experiments performed in duplicate. **P* < 0.05; ***P* < 0.05 versus (-).

Next we focused on the mitochondrial oxidative phosphorylation (OXPHOS) components. In MCF7 cells, the expression levels of CII, CV and CIII were reduced by OHPg/PR-B (Fig. 12B), while CIV in a less extent. As it concerns CI expression, it was induced by 10 nM OHPg via PR-B and barely reduced by 1 and 100 nM OHPg. Surprisingly, PR-B siRNA further reduced the basal protein content, particularly for CI, CII, CIV and CV complexes. Altogether, these results corroborate the decreased ATP cellular content as obtained by the Seahorse method, after OHPg stimulus.

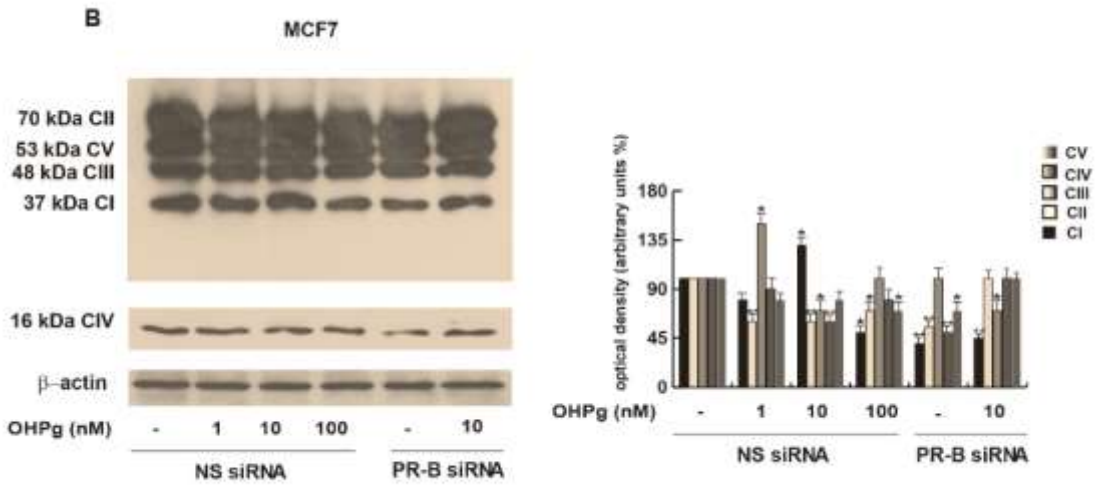


Figure 12 B: OHPg/PR-B modulate bioenergetic requirements in breast cancer cells
B: Immunoblot showing OXPHOS in MCF7 treated as above reported. β actin was used as loading control. Autoradiograph shows the results of one representative experiment of four independent experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as fold over vehicle (-). **P* < 0.05; ***P* < 0.05 versus (-).

3.5 OHPg/PR-B effects on the replenishment of the tricarboxylic acid cycle (TCA)

Pyruvate can be either reduced to lactate and shuttled out of the cell or transported to the mitochondria to be oxidized by the pyruvate dehydrogenase (PDH), which is regulated and blocked by the pyruvate dehydrogenase kinase (PDK), limiting the pyruvate conversion into Acetyl-CoA (Kyle SM and Brian NF, 2015). As shown in Fig. 13A, the PDH expression is significantly reduced after 10 nM OHPg treatment in both cell lines. PR-B-siRNA partially counteracted the OHPg effect. OHPg/PR-B did not influence PDK content in both cell lines cells.

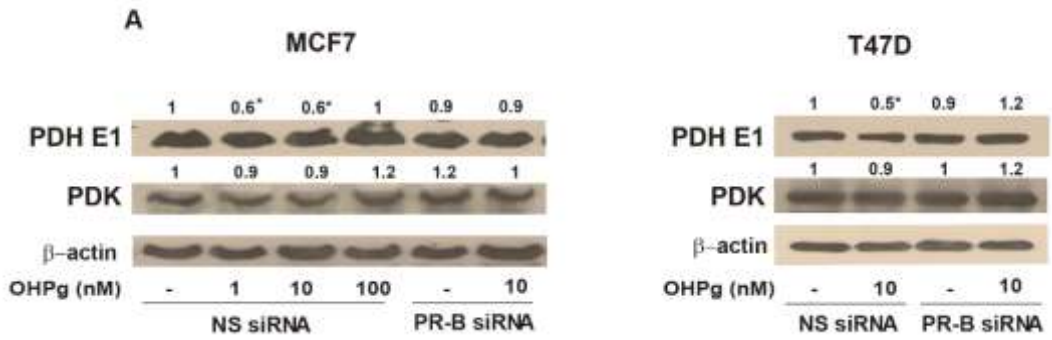


Figure 13 A: OHPg/PR-B effects on the replenishment of the TCA cycle

A: Immunoblot showing PDH E1 and PDK. **A** was performed as reported in Materials and Methods. β actin was used as loading control. Autoradiographs show the results of one representative experiment and the numbers on the top of the blot, are mean of four independent experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as fold over vehicle (-). *P < 0.05; **P < 0.05 versus (-).

The replenishment of the TCA cycle intermediates may also occur at level of α-ketoglutarate dehydrogenase (KGDH) complex which would catalyze alpha-KG from different sources. From our data, given the scarce pyruvate produced due to reduced glycolysis process, limited entrance and then poor conversion into Acetyl-CoA, we examined a possible alteration of the KGDH expression. As observed in Fig. 13B, OHPg induced the expression of the KGDH. Of consequence the OHPg-action may refuel the TCA cycle at level of the KGDH, thus inducing a 'truncated' TCA cycle in breast cancer cells. PR-B-siRNA counteracted OHPg effect.

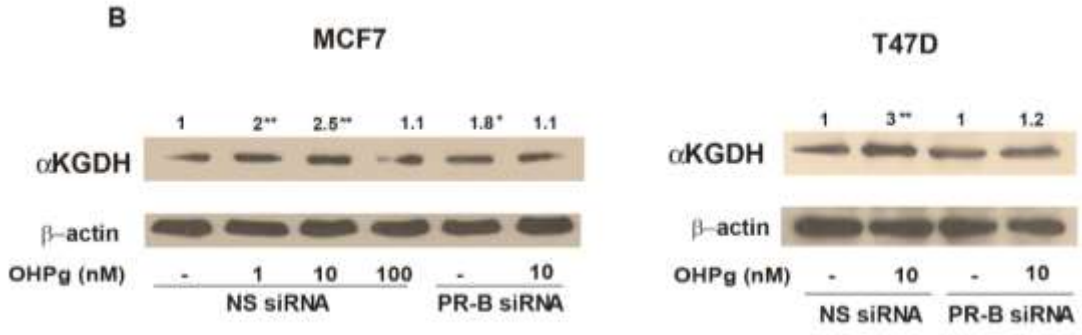


Figure 13 B: OHPg/PR-B effects on the replenishment of the TCA cycle
B: Immunoblot showing αKGDH. **B** was performed as reported in Materials and Methods. β actin was used as loading control. Autoradiographs show the results of one representative experiment and the numbers on the top of the blot, are mean of four independent experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as fold over vehicle (-). *P < 0.05; **P < 0.05 versus (-).

3.6 OHPg/PR-B action on de novo fatty acid synthesis

An increased use of a 'truncated' TCA cycle, includes diversion of citrate to cytosolic export for lipid synthesis. Cancer cells frequently upregulate *de novo* fatty acid synthesis to satisfy their energy demands and this process has been linked to a poor prognosis in breast cancer (Zhang F and Du G, 2012). It consists of a multiple step process involving enzymes such as ATP citrate lyase (ACLY), Acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS). Interestingly, we observed that the ACLY content increased after 1 and 10 nM OHPg treatment via PR-B in MCF-7 (Fig. 14). Conversely, both ACC and FAS expression

decreased. Interestingly, it appears that the absence of the PR-B per se significantly blocked the ACC. Similar results were observed in T47D cells. As it concerns ACLY and FAS, the PR-B-siRNA counteracted OHPg effect.

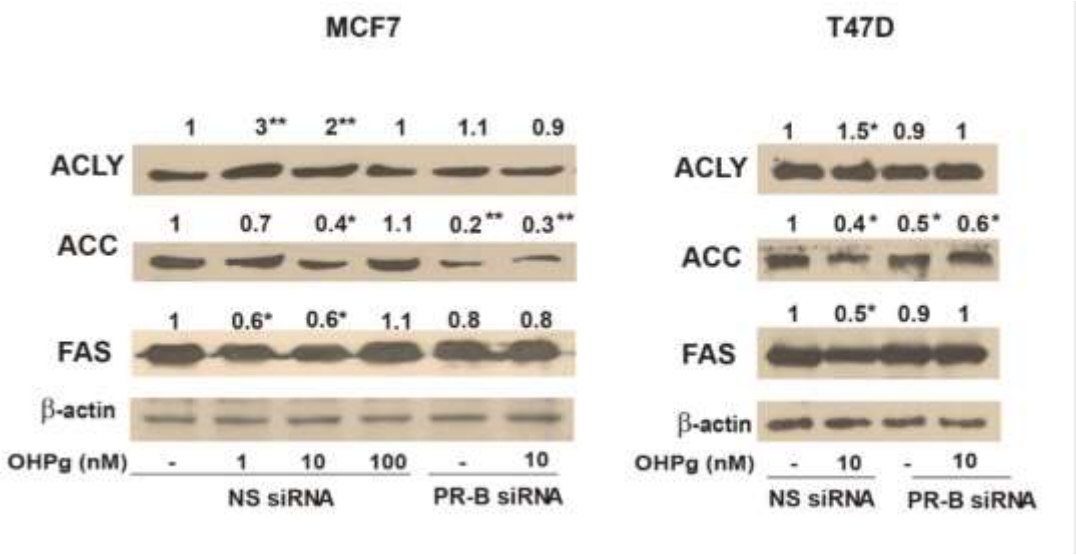


Figure 14: OHPg effects on *de novo* fatty acid synthesis in breast cancer cells
Immunoblot showing ACLY, ACC and FAS. The cells were treated as reported in Materials and Methods. Immunoblots were performed as reported in Materials and Methods. β actin was used as loading control. Autoradiographs show the results of one representative experiment and the numbers on the top of the blot, are mean of four independent experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as fold over vehicle (-). *$P < 0.05$; **$P < 0.05$ versus (-).

3.7 OHPg/PR-B promoted a lipid lowering effect in breast cancer cells

Similar to glucose, fatty acid metabolism supports both the biosynthetic and the bioenergetics requirements for cell proliferation and survival. OHPg-treated MCF7 as well as T47D cells through PR-B reduced the triglycerides content compared to untreated cells (Fig. 15A).

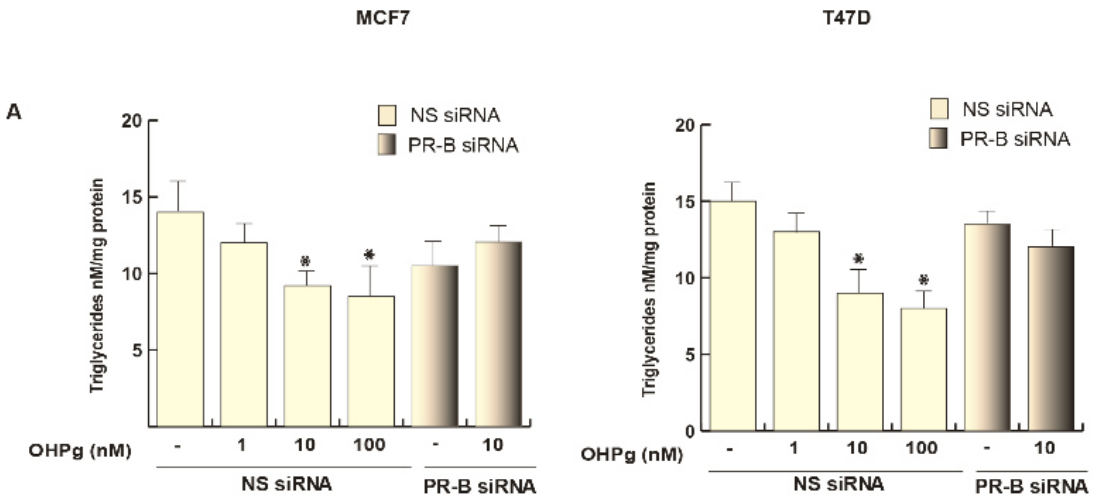


Figure 15 A: OHPg/PR-B effects on lipid metabolism in MCF7 and T47D cells
A: Triglycerides assays was performed in Materials and Methods. Columns represent mean ± SEM of 6 independent experiments performed in duplicate. **P* < 0.05 versus (-).

Alongside, the lipase activity and concomitantly FAO, both fatty acid catabolic processes, increased at all the OHPg concentrations used (Fig. 15B, C). The PR-B-siRNA counteracted at least in part the OHPg effect

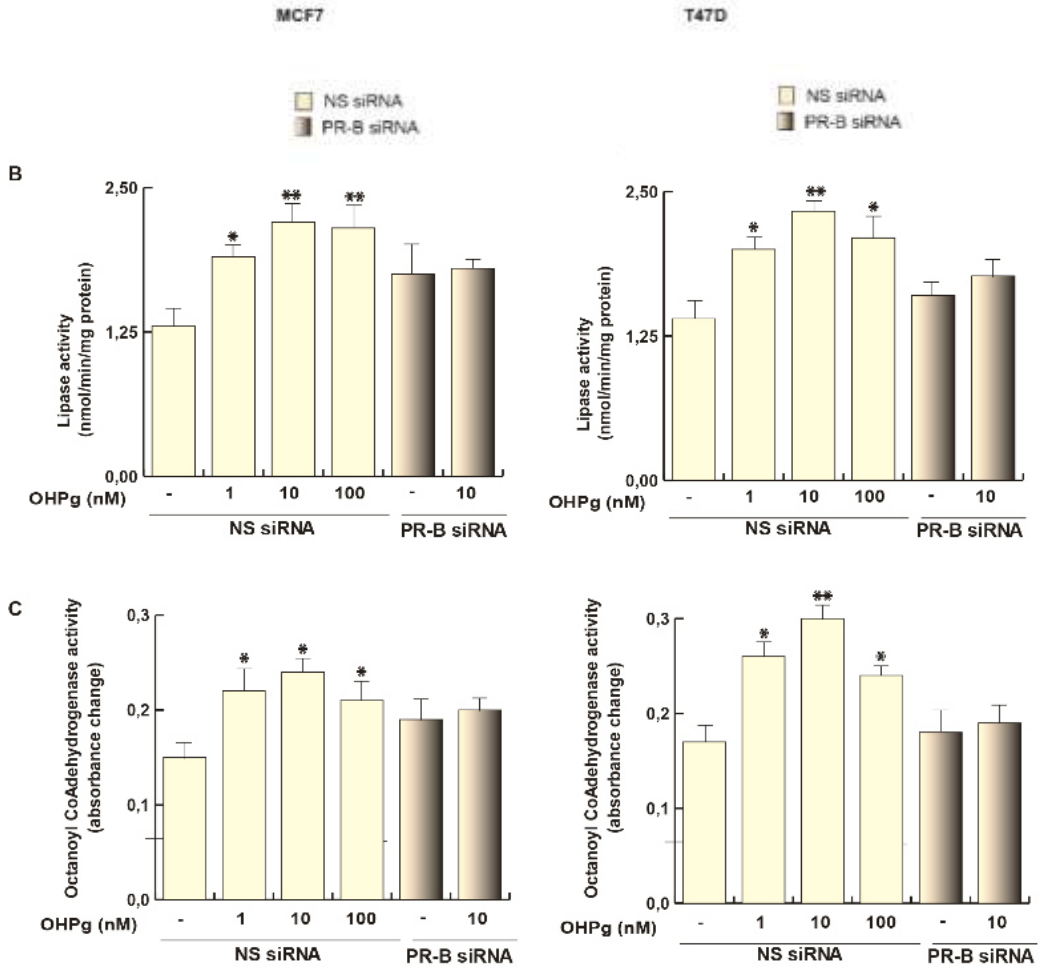


Figure 15 B and C: OHPg/PR-B effects on lipid metabolism in MCF7 and T47D cells
B: Lipase activity assays **C:** Acyl-CoA dehydrogenase activity assays and cell treatments were performed in Materials and Methods. Columns represent mean ± SEM of 6 independent experiments performed in duplicate. **P* < 0.05; ***P* < 0.05 versus (-).

3.8 OHPg/PR-B altered the metabolic reprogramming through a mechanism which may involve the p53 in MCF7 breast cancer cells

Our previous study indicated that OHPg/PR-B up-regulated the expression of the tumor suppressor gene phosphatase and tensin analog (PTEN), which is required both for the negative modulation of phosphatidylinositol 3-kinase/Akt-dependent cell proliferation (De Amicis F et al., 2014; Amorim MA et al., 2010) and another finding demonstrated a relationship with p53 for the regulation of cell survival (Sung-Po Hsu and Wen-Sen L, 2011) p53 has been shown to influence metabolic pathways such as glycolysis, oxidative phosphorylation, lipolysis, lipogenesis, β-oxidation (Zhang XD et al., 2010).

Thus, we hypothesized that PR-B and p53 may converge in a tumor suppressor activity by disorganizing the metabolic reprogramming in breast cancer cells. OHPg treated cells showed an increase of p53 levels both in the nucleus and in the cytosol (Fig. 16A), PR-B siRNA reduced this effect (Fig. 16B).

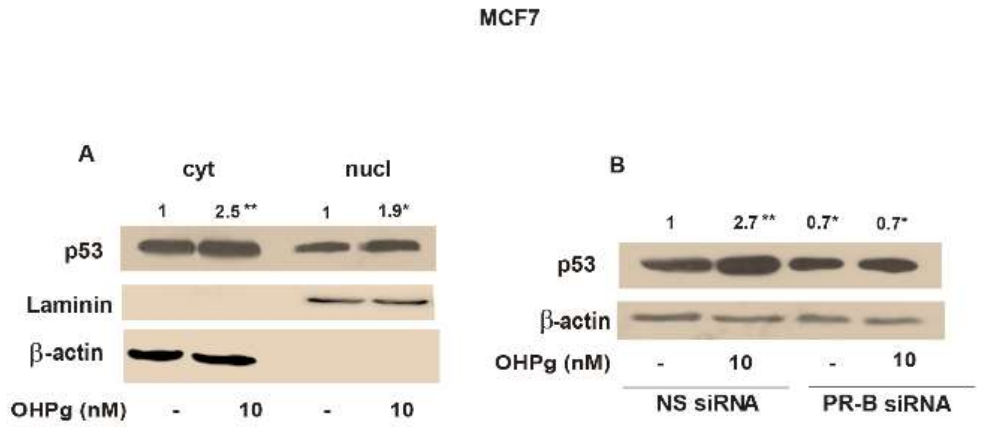
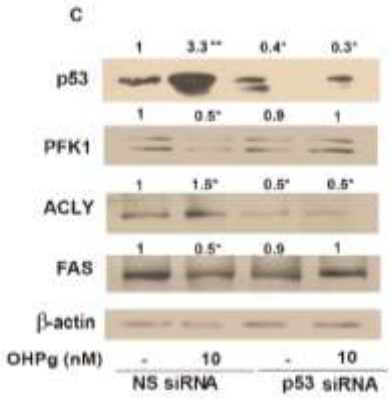


Figure 16 A and B: OHPg/PR-B influence the metabolic reprogramming through p53 in MCF7 breast cancer cells

A: Immunoblot showing p53. Cytosolic and nuclear cells lysates were obtained from MCF7 cells treated with vehicle (-) or 10nM OHPg for 24h. **B:** Immunoblot showing p53. MCF7 transfected with NS- or targeted against PR-B siRNA treated with vehicle (-) or 10nM OHPg for 24h. β actin was used as loading control. Autoradiograph shows the results of one representative experiment and the numbers on the top of the blot, are mean of three independent experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as fold over vehicle (-). $P < 0.05$ versus (-); $^{**}P < 0.05$ versus 10nM OHPg.

Next we performed p53 silencing studies to evaluate the effects upon OHPg treatment on key metabolic phases. p53 specific siRNA reversed the OHPg-induced effects observed on PFK1, ACLY, FAS, (Fig. 16C) LDH and G6PDH activities (Fig. 16D, E). These data strongly suggest a cooperation between PR-B and p53 in disrupting breast cancer cell metabolic reprogramming.



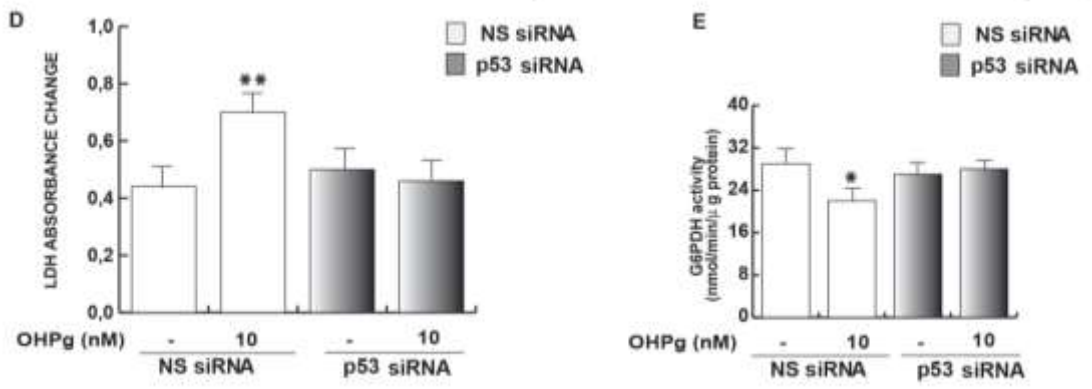


Figure 16 C, D and E: OHPg/PR-B influence the metabolic reprogramming through p53 in MCF7 breast cancer cells

C: Immunoblot showing p53, PFK1, ACLY, FAS. MCF-7 transfected with NS- or targeted against p53 siRNA treated with vehicle (-) or 10nM OHPg for 24h. β actin was used as loading control. **D**: LDH activity was performed in MCF7 cells transfected with non-specific (NS) - or targeted against P53 siRNA treated with vehicle (-) or 10 nM OHPg concentration for 24h. Columns represent mean ± SEM of 3 independent experiments performed in duplicate. $**P < 0.05$ versus control. **E:** G6PDH activity was performed in MCF7 cells as indicated. Columns represent mean ± SEM of 3 independent experiments performed in duplicate. $*P < 0.05$ versus control.

4 Discussion

Cancer metabolic programs need of energy and metabolites to support large-scale biosynthesis and other major processes of tumorigenesis. Cancers are extremely heterogeneous diseases with individual metabolic features. The literature has indicated that the Warburg phenotype is not exclusive and that a decrease of mitochondrial function is not a general feature of cancer cells (Jose C et al., 2011; Valcarcel-Jimenez L et al., 2017). Although, aerobic glycolysis is often found in malignant tumors, OXPHOS still contributes to energy production in cancers. The contribution ratio of glycolysis versus OXPHOS for the total ATP yield varies in different cancers (Slavov N et al., 2014). Therefore, carefulness and a multifaceted approach are important to define the metabolic phenotype of a cancer.

Herein, we evidenced various features of tumor metabolism influenced by OHPg/PR-B in human breast cancer cells, unveiling a novel protective role for OHPg/PR-B in human breast cancer. Interestingly, this new action may involve p53 addressing to a crosstalk between the two onco-suppressor pathways.

In this finding, in MCF7 and T47D cells, first we studied the metabolic phenotype by the Seahorse XF96 Analyzer, measuring both OCR and ECAR simultaneously, since this may be indicative of the dynamic interplay between glycolysis and oxidative metabolism. From our data it emerges that glycolysis way as well as the ATP and maximal respiration levels were significantly reduced after treatment with OHPg. To better characterize the metabolism of our cell lines, we analyzed different key factors which regulate both glucose metabolism and ATP production.

By evaluating glucose content and its metabolism in MCF7 and T47D cells upon increasing OHPg treatments, we observed that glucose content increased significantly, whereas the PFK1 expression was eminently decreased, while concomitantly the intracellular LDH activity increased. LDH is a long-lived protein that appears to be degraded mainly or exclusively by autophagy. Our data and this observation well fit with our recent findings demonstrating that OHPg *via* PR-B/Bcl-2 axis drives an impaired autophagy that facilitates

the irreversible growth arrest determining breast cancer cell fate (De Amicis F et al., 2014; 2016). Lactate synthesis can exert a metabolic control over glycolysis, through the inhibition of PFK1 enzyme, a rate limiting step of the glycolytic pathway (Leite TC et al., 2011). Recently, Mulukutla and co-authors showed lactate inhibitory effect on PFK1, which in turn results in slowing down the glycolytic flux (Mulukutla BC et al., 2014). It is known that the inhibition of PFK1 determines the accumulation of fructose-6-phosphate, then isomerized to glucose-6-phosphate fueling also the PPP. This pathway which is the primary cellular source of NADPH, is crucial to sustain antioxidant and anabolic function in many cancer cells (DeBerardinis Ralph J et al., 2016). Our data showed that OHPg/PR-B was able to lower the G6PDH activity thus affecting the PPP rate. The low glycolytic flux as well as the blockage of the PPP may explain the higher glucose levels as we found during OHPg treatment, which thus is accumulated instead of being used. Another confirmation came from our recent study, where we demonstrated that OHPg/PR-B induced the autophagy process through PTEN, which causes the inhibition of the PI3K/AKT signal (De Amicis F et al., 2014) and AKT is a well-known stimulator of glycolysis, therefore the downregulation of its signaling cascade can also explain the low glycolytic flux as it emerged from this finding.

Other than the PPP, also the ME and ICDH2 cytosolic enzymes are involved in the production of NADPH (Ren JG et al., 2014), and interestingly both enzymatic activities increased after OHPg stimulation in our cell models. Pyruvate can be either reduced to lactate and shuttled out of the cell or transported to the mitochondria to be oxidized by the pyruvate dehydrogenase (PDH), which is regulated and blocked by the pyruvate dehydrogenase kinase (PDK), limiting the pyruvate conversion into Acetyl-CoA (Kyle SM and Brian NF, 2015).

PDH and KGDH complexes regulate the large majority of carbon flow into the TCA cycle, derived from pyruvate and other sources respectively (Mullen AR et al., 2014). Our data showed that the refueling of TCA cycle may occur prevalently through the KGDH, since its expression increased, while that of PDH was reduced by OHPg. The heightened need in cancer cells for biosynthetic intermediates may also result in the increased use of a 'truncated' TCA cycle. Unfortunately, the use of a truncated TCA cycle presents the risk of altering the mitochondrial integrity and this is in agreement with the altered expression of the OXPHOS components as we observed.

Accordingly, our results showed a strong reduction of the ATP content. As it is known, the oxidation of NADH mainly occurs via mitochondrial OXPHOS, where electron transport is

coupled along 4 enzyme complexes (CI–CIV), and ATP is synthesized at CV level (Reinecke F et al., 2009). PR-B appears involved on the stability of all five complex. Altogether, these data indicate an internal electron chain transport deregulation consistent with the low amount of ATP produced.

A continuous use of the 'truncated' TCA cycle, includes the diversion of citrate in the cytosol to be used for fatty acid *de novo* synthesis (Lussey-Lepoutre C et al., 2015). In fact, citrate may be cleaved by ACLY into oxaloacetic acid (OAA) and Acetyl-CoA. The latter, may be used for lipid synthesis, a multiple step process involving enzymes such as ACC and FAS. ACC is also very important for tumorigenesis as inhibition of ACC stops cancer growth in prostate cancer cells (Shurbaji MS et al., 1996). FAS elevation is observed in breast, prostate and other types of cancer (Menendez JA et al., 2007). OHPg increased ACLY expression, whereas ACC and FAS levels were reduced, implying a switch towards the OAA cytosolic formation. This in turn through ME and ICDH2, which activities were increased by OHPg, sustained pyruvate and thus lactate production. These data are also in agreement with the increased intracellular LDH activity as we found. Furthermore, OHPg induced a general lipid lowering effect since *de novo* fatty acid synthesis and triglyceride levels decreased, while concomitantly the lipase activity and the FAO increased. Fatty acids are an important energy source that can produce more ATP than carbohydrates when required. These observations and our data suggest that OHPg/PR-B may induce the lipid degradation in breast cancer cells to recover energy, failing however in their intent.

Interestingly, metabolic stress also triggers primarily an adaptive (rather than pro-death processes) p53 response (Mulukutla BC et al., 2014; Mihaylova MM and Shaw RJ, 2011). p53 supports the expression of PTEN, which inhibits the PI3K pathway, thereby suppressing glycolysis (Stambolic V et al., 2001). From our data, OHPg/PR-B treatment induced p53 expression suggesting a molecular link between PR-B and p53 in the disruption of breast cancer cells metabolic reprogramming. In the main biochemical ways tested, p53 silencing greatly counteracted OHPg effects, suggesting that both PR-B and p53 converge in this tumor suppressive action.

Collectively, our findings indicated for the first time that activation of the PR-B by its own natural ligand influences several metabolic pathways in breast cancer cells, blocking both glucose, oxidative and lipid metabolism. Therefore, we discovered another distinctive protective action of OHPg / PRB against breast cancer. Intriguingly, it appears that the

OHPg/PR-B effects in this context are mediated by p53 tumor suppressive pathway, although their molecular link needs to be better investigated.

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