UNIVERSIDADE D
COIMBRA

Margarida Ribeiro da Silva

P-CADHERIN ROLE ON THE MITOCHONDRIAL BIOLOGY OF BREAST CANCER CELLS

Tese no âmbito do Mestrado em Bioquímica orientada pelo Doutor Ricardo Jorge Fernandes Marques e pelo Professor Doutor António Joaquim Matos Moreno e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologias da Universidade de Coimbra.

Setembro de 2019

Departamento de Ciências da Vida

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This work was performed at the Mitochondrial Toxicology and Experimental Therapeutics (MitoXT) group, at the Center for Neuroscience and Cell Biology of University of Coimbra, Portugal, under the supervision of Dr. Ricardo Marques (CNC, UC) and Dr. António J. Moreno (DCV, UC) and also of the group leader, Dr. Paulo J. Oliveira (CNC, UC).

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A vós.

Margarida

Abstract

Breast cancer is one of the most common neoplasms worldwide and remains the leading cause of cancer death in women in developed countries. P-cadherin, a cell adhesion glycoprotein, is overexpressed in 30% of breast cancer cases and was associated with poor prognosis and lower survival rates. P-cadherin regulates cell adhesion, migration and invasion of breast cancer cells and has been associated with glycolytic markers such as GLUT1, CAIX, MCT1 and CD147 in breast cancer tissue samples. As metabolic reprogramming is a hallmark of cancer, P-cadherin may be related to mitochondrial changes that justify this metabolic change. Thus, we intend to evaluate whether P-cadherin overexpression may influence mitochondrial biology of breast cancer cells.

This work focused on assessing how P-cadherin could modulate mitochondrial characteristics in two breast cancer cell lines: a luminal breast cancer model, MCF-7/AZ, by comparing its effects when overexpressed and a basal breast cancer line, BT20, in which a transient downregulation of P-cadherin was induced. These models served as a way to uncover P-cadherin biologic effects in mitochondria features and cellular metabolism.

The results indicate that P-cadherin may be involved in metabolic changes that dictate the primary form of ATP by breast cancer cells. This occurs possibly by stimulating glycolysis since there was a 25 % decrease in ATP levels when P-cadherin was overexpressed. However, no statistically significant differences were obtained that could correlate regulation of P-cadherin with oxidative phosphorylation changes, or alteration in mitochondrial membrane potential. Although P-cadherin does not appear to interfere with mitochondrial biogenesis processes, it may be involved in modelling mitochondrial dynamics, since a decrease of 39.61 % in MFN1 protein was observed in MCF-7/AZ cells overexpressing P-cadherin, while MFN1 increased following P-cadherin silencing in BT20 cells. These effects may eventually alter the mitochondrial biology of breast cancer cells. If these results are confirmed, it may support the described P-cadherin role in increased aggressiveness and metastatic capacity of cancer cells by modulating, at least partially, cancer cell metabolism.

Keywords: P-cadherin; Breast Cancer; Mitochondria; Metabolism.

Resumo

O cancro da mama é um dos neoplasmas mais comuns em todo o mundo e continua a ser a principal causa de morte por cancro em mulheres nos países desenvolvidos. A P-caderina, uma glicoproteína de adesão celular, está sobre expressa em 30% dos casos de cancro da mama e foi relacionada com pior prognóstico e menor taxas de sobrevivência. A P-caderina tem uma influência na adesão celular, migração e invasão das células de cancro da mama e foi associada com marcadores glicolíticos, como o GLUT1, CAIX, MCT1 e CD147 em amostras de tecido de cancro da mama. Como a reprogramação metabólica é um *hallmark* do cancro, a P-caderina poderá estar relacionada com alterações mitocondriais que justifiquem está mudança metabólica. Assim, pretendemos com este trabalho avaliar se a sobre expressão da P-caderina poderá influenciar a biologia mitocondrial das células de cancro da mama.

Este trabalho focou-se em avaliar como é que a P-caderina poderá modular características mitocondriais em duas linhas celulares de cancro da mama: um modelo de cancro da mama luminal, MCF-7/AZ, comparando os efeitos com a sobre expressão da P-caderina e uma linha de cancro da mama basal, BT20, na qual foi induzido o silenciamento transiente da P-caderina.

Os resultados indicam que a P-caderina poderá estar envolvida nas alterações metabólicas que ditam a principal forma de obtenção de ATP pelas células de cancro da mama. Isto ocorre possivelmente estimulando a glicólise, uma vez que houve uma diminuição de 25% nos níveis de ATP aquando da sobre expressão da P-caderina. No entanto, não foram encontradas evidências que relacionem o efeito da P-caderina com alterações na fosforilação oxidativa, ou potencial membranar mitocondrial. Embora a P-caderina não pareça interferir com os processos de biogénese mitocondrial, poderá está envolvida na modelação da dinâmica mitocondrial, uma vez que uma diminuição de 39,61 % na proteína MFN1 foi observada nas células MCF-7/AZ sobre expressando P-caderina, e um aumento da mesmo proteína após silenciamento da P-caderina nas células BT20. Estes efeitos poderão eventualmente alterar a biologia mitocondrial das células do cancro da mama. Se estes resultados forem confirmados, podem apoiar o papel descrito da P-caderina no aumento da agressividade e capacidade metastática de células cancerígenas modulando, pelo menos parcialmente, o metabolismo destas.

Palavras-chave: P-caderina; Cancro da Mama; Mitocôndria, Metabolismo.

Abbreviation List

2-DG	2-Deoxyglucose
Acetyl-CoA	Acetyl Coenzyme A
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
B2M	Beta-2-microglobulin
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2
CAIX	Carbonic anhydrase IX
CBD	Catenin-binding domain
CD49f	Integrin alpha 6
CDH3	P-cadherin gene
CLB	Catenin lysis buffer
CO_2	Carbon dioxide
CSC	Cancer stem cells
CYTB	Mitochondrial cytochrome b
DCIS	Ductal Carcinoma *In Situ*
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DRP1	Dynamin related protein 1
E-cadherin	Epithelial cadherin
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
FAK	Focal adhesion kinases
FBS	Fetal Bovine Serum
FIS1	Fission homolog protein 1
GLUT1	glucose transporter 1
HER2	Human epidermal growth factor receptor 2
HIF-1	Hypoxia-inducible factor 1
HKII	hexokinase II
IMM	Inner mitochondrial membrane
JMD	Juxtamembrane domain
KCl	Potassium chloride

KH_2PO_4	Monopotassium phosphate (Phosphate Buffer)
LCIS	Lobular Carcinoma *In Situ*
MCTs	Monocarboxylate transporters
MFF	Mitochondrial fission factor
MFN1	Mitofusin 1
MFN2	Mitofusin 2
MMP1	Matrix metalloproteinase 1
MMP2	Matrix metalloproteinase 2
mtDNA	Mitochondrial DNA
mtDNA	Mitochondrial DNA
Na_2HPO_4	Sodium phosphate dibasic
NaCl	Sodium chloride
NAD^+/NADH	Nicotinamide adenine dinucleotide
N-cadherin	Neuronal cadherin
NTC	No template control
OMM	Outer mitochondrial membrane
OPA1	Optic atrophy gene 1
PBS	Phosphate buffered saline
P-cadherin	Placental cadherin
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PR	Progesterone receptor
PVDF	Polyvinylidene difluoride
R-cadherin	Retinal cadherin
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
siRNA	Small interfering RNA
TCA	Tricarboxylic acid
TDLUs	Terminal duct lobular units
TEBs	Terminal end buds
TFAM	Mitochondrial transcription factor A
TMRM	Tetramethylrhodamine
TOM20	Translocase of outer mitochondrial membrane 20

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Chapter 1

INTRODUCTION

1.1. Breast Cancer

1.1.1. CANCER BIOLOGY

Cancer is a complex disease where normal cells acquire a neoplastic capacity that allows tumour development (Hanahan and Weinberg 2011). Cancer etiology involves multiple factors as mutations, that can be caused by hereditary gene alterations, lifestyle choices and environmental context or simply DNA replication errors (Paulmurugan 2012; Tomasetti, Li, and Vogelstein 2017).

During the process through which tumours develop, cancer cells acquire new characteristics and capabilities, termed cancer hallmarks. These hallmarks, that contribute to the pathological process, encompass the ability of tumour cells to maintain proliferative capacity, to be able to bypass growth suppressor signals and to enhance proliferative signaling. Immortal replicative capacity and resistance to cell death, as well as the induction of angiogenesis or the capacity for invasion and metastasis, are also crucial in the multistep process of neoplasm formation (Dai et al. 2016; Hanahan and Weinberg 2000). New emerging hallmarks justify the metabolic reprogramming of cells in order to sustain proliferation and cancer cells capacity to avoid immune destruction (Hanahan and Weinberg 2011a).

One of the most common neoplasms worldwide is breast cancer, which is the most common type of cancer in women (Makki 2015; Tao et al. 2015). Breast carcinomas are very heterogeneous, presenting various morphological features and types of classification. Due to breast cancer diversity of pathologic features and biological behaviors, carcinomas can develop in any of the cells of the mammary gland (Dai et al. 2016; Makki 2015).

The normal mature mammary gland is formed by a network of ducts and alveolar structures that join to form lobules (Shackleton et al. 2006), that are subdivided into lobuli and alveoli, and develop during puberty and result from duct branching and elongation. In the process terminal end buds (TEBs), at the tips of the ducts, and terminal duct lobular units (TDLUs) are formed (Sternlicht 2005; Sternlicht et al. 2006). TLDUs are involved in milk production during lactation and are the primary source of most cancers (Figueroa et al. 2014). The TLDUs comprise two distinct types of cells: basal (myoepithelial) cells and luminal epithelial cells, which are distinguished by immunohistochemistry (Paredes et al. 2002).

1.1.2. INCIDENCE OF BREAST CANCER

Breast cancer had the second-highest incidence in 2018, for both sexes, accounting for 11.6% of the cases (Bray et al. 2018). Among women, it was the most commonly diagnosed and the main cause of death by cancer, with metastatic spread taking a great part in this fatal outcome (Ismail-Khan and Bui 2010; Vieira et al. 2017).

It accounts for about 25% of all cancers in women and it is estimated that in 2018, worldwide, 2 088 849 new cases of breast cancer were identified and over 626 679 women died due to breast cancer (Figure 1) (F. Bray et al. 2018; Ghoncheh, Pournamdar, and Salehiniya 2016).

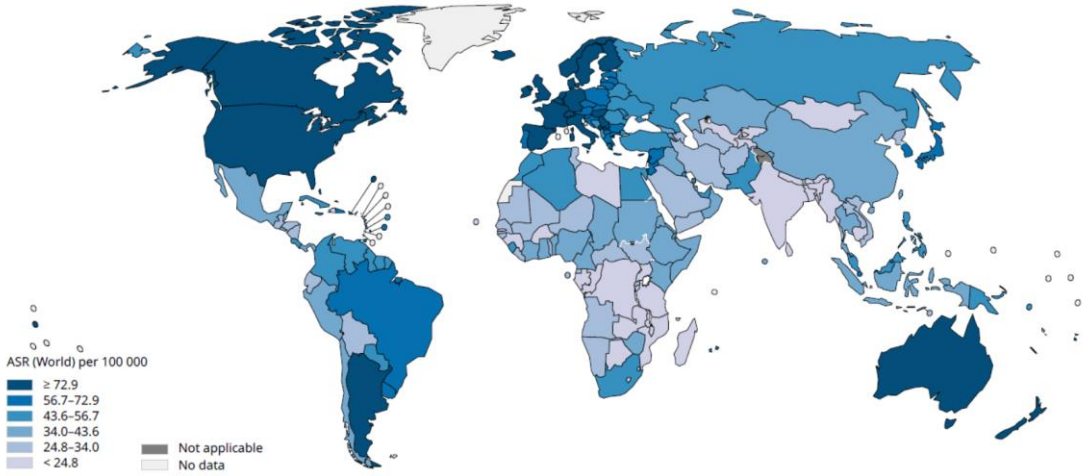

Figure 1 - Age standardized incidence rates of breast cancer, per 100 000 (world). Adapted from GLOBOCAN 2018

In Portugal, an estimated 6088 new cases were diagnosed, and 1570 deaths were associated with breast cancer in 2012, among women. This represents 30% of all cancer cases and 16% of all cancer-related deaths (Forjaz de Lacerda et al. 2018).

1.1.3. RISK FACTORS

There are several risk factors related to the onset and development of breast cancer. Intrinsic factors, such as family history and personal characteristics can increase the risk of the disease. In fact, the risk of breast cancer is about 2 times higher for women that had one first-degree female relative affected. Moreover, inherited rare mutations, such as *BRCA1* and *BRCA2* account for 5%-10% of all breast cancers cases (Tung et al. 2016).

Besides these, other intrinsic factors are higher breast tissue density, early menarche and beginning of menopause later on (Collaborative Group on Hormonal Factors in Breast Cancer 2016), but also high bone mineral density in postmenopausal women (Qu et al. 2013)

and naturally high endogenous hormone levels in premenopausal women (Endogenous Hormones and Breast Cancer Collaborative Group 2013).

Most extrinsic risk factors only account for low relative risk and can be divided in reproductive factors, obesity, physical activity, diet and environmental. Older age (> 35 years) at the first childbirth and lower number of children is associated with a higher risk of breast cancer (Albrektsen et al. 2005; Lambertini et al. 2016) while a longer breastfeeding time slightly reduces the risk of the disease (Faupel-Badger et al. 2013). There has also been found an association between breast cancer risk and fertility drugs (ovulation-stimulation drugs) (Sugawara and Nikaido 2014). Postmenopausal hormones have also been associated with a higher risk (Islami et al. 2015) since overweight postmenopausal women have a higher risk of contracting breast cancer, mainly due to higher estrogen levels released by fat tissue (Gunter et al. 2015; Picon-Ruiz et al. 2017). On the other hand, physical activity has a protective effect, reducing the risk of breast cancer by 10% to 20% (Pizot et al. 2016). Alcohol and tobacco are also risk factors for breast cancer (Gaudet et al. 2013; Liu, Nguyen, and Colditz 2015).

1.1.4. Breast Cancer Classification

Breast cancer is a very heterogeneous disease with grouping diverse subtypes and specific characteristics. There are also some methods of classification and treatments that can be adjusted to the different cases (Tao et al. 2015). There are both cases with good prognosis and slow growth as well as very aggressive cancers, whose classification is divided into up to twenty-one histological subtypes and four molecular subtypes (Dieci et al. 2014). Initially, the TNM system is determined using information on tumour size and how far it has spread (T), the extent of spread to the nearby lymph nodes (N) and metastatic spread (M). Then a stage of 0, I, II, III, or IV is assigned based on the overall extent of the carcinoma: *in situ*, local, regional or distant (Edge et al. 2010).

The histological classification of *in situ* carcinomas are subdivided in ductal carcinoma (DCIS), occurring in the epithelial cells of breast ducts, or lobular carcinoma (LCIS), growing in the lobules (Allred 2010; Eusebi et al. 1994). Regarding the main types of invasive carcinomas, histologically, these include infiltrating ductal, invasive lobular, ductal/lobular, mucinous (colloid), tubular, medullary and papillary. The most common type is the infiltrating ductal carcinoma which can further sub-classified as either well-differentiated - grade 1, moderately differentiated - grade 2, or poorly differentiated - grade 3 (Figure 2) (Malhotra et al. 2010).

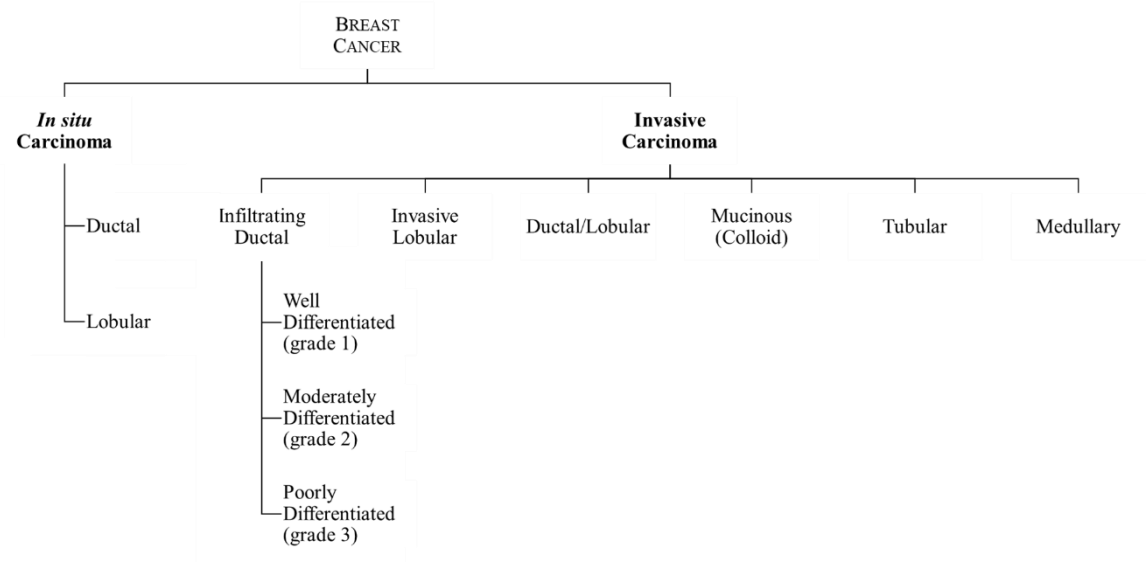

Figure 2 -Histological classification of breast cancer subtypes.

Molecularly, breast cancers can be classified accordingly to their expression levels of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). The presence or absence of these receptors leads to the distinction of four molecular subtypes: luminal A, luminal B, HER2-enriched and triple-negative (Figure 3) (Hon et al. 2016).

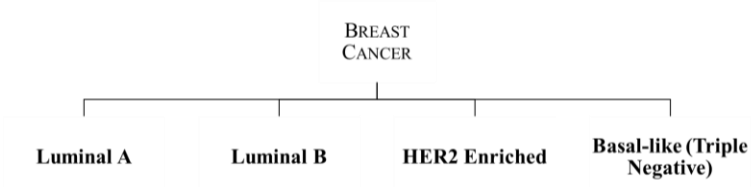

Figure 3 - Molecular classification of breast cancer.

Luminal A tumours are ER and/or PR positive and HER2 negative, being associated with the most favorable prognosis because they are endocrine-responsive. These cancers represent about 70% of the cases and are less aggressive with a slower development, which also facilitates treatment (Blows et al. 2010). Luminal B tumours are positive for all three receptors, also being endocrine-responsive. Those tumors account for around 12% of the cases and this subtype tends to be a higher grade and is associated with lower survival than luminal A (Haque et al. 2012). HER2-enriched tumours are only HER2 positive and more aggressive, that rapidly grow when compared to luminal A and B, representing only 5% of the cases (Romond et al. 2005). The triple-negative subtype lacks all three receptors and is

associated with a higher grade, is one of the deadlier, with fewer therapeutic options and shorter survival time. Triple-negative breast cancers represent about 12% of all cases and are twice as common in premenopausal African American women (Hon et al. 2016; Ismail-Khan and Bui 2010).

1.1.5. THERAPEUTIC APPROACHES

Invasive breast cancer is mostly treated through a combination of some type of surgery, which could be breast-conserving surgery or mastectomy, and other treatment, such as hormone therapy, radiation therapy, chemotherapy or targeted therapy (Bellavance and Kesmodel 2016; Y. Chen et al. 2016). For instance, hormone therapy also referred to as anti-estrogen therapy, is used to lower estrogen levels by interfering with estrogen receptors. One common drug used is Tamoxifen that can only be applied on hormone-responsive breast cancers (Burstein et al. 2014).

For patients with triple-negative breast cancer the only treatment is chemotherapy, individually or in combination with surgery (Hon et al. 2016). Adjuvant chemotherapies such as anthracyclines, platinum compounds and taxanes seem to be the most effective in treating triple-negative breast cancer (Wahba and El-Hadaad 2015). Although, treatment options available can be effective, cancer recurrence is still frequent especially in the first 5 years after treatment (EBCTCG 2005).

Sustaining proliferative signalling, escaping growth suppressors, promoting angiogenesis, resisting cell death, enabling replicative immortality and inducing invasion and metastases are the six hallmarks of cancer proposed in 2000 by Weinberg et al. Since then, two more additional hallmarks were found to be involved in cancer pathogenesis: evading immune and deregulating cellular metabolism (Hanahan and Weinberg 2011). The metabolic changes in cancer cells are driven by oncogene-directed metabolic reprogramming in order to maintain cell growth and proliferation (Ward and Thompson 2012).

1.1.6. CANCER STEM CELLS

The tumour microenvironment is constituted by a very diverse cell population, that changes as the primary tumour become invasive and metastatic (Figure 4) (Hanahan and Weinberg 2011a). The heterogeneity of cancer cells subpopulations, with varying degrees of differentiation, is reflected in a diversified histopathological classification, as is the case of breast cancer classification (Dieci et al. 2014; Hanahan and Weinberg 2011a).

Cancer stem cells (CSC), also known as tumour-initiating cells, are a subpopulation of those diverse association of cells types that are characterized by self-renewal and differentiation ability. Thus, these cells are able to initiate, propagate and hierarchically organize secondary tumours and promote relapses (Sousa et al. 2014; Vieira et al. 2017). CSC are hyphotisezed as being responsible for tumour initiation and propagation and, even though this cancer propagating cells don't have a determined origin, they are related with increased aggressiveness and higher metastatic lesions of tumours, which makes them resistant to most anti-cancer agents (Albergaria et al. 2011; Eyre et al. 2016).

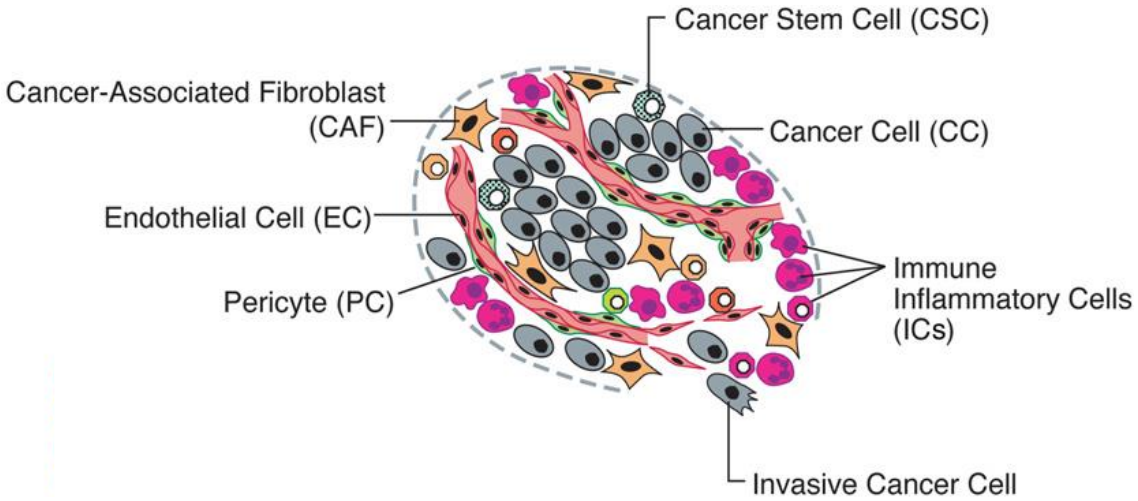

Figure 4 -The constitution of the tumour microenvironment, including cancer stem cells. (Hanahan and Weinberg, 2011)

Cancer stem cells can be derived from normal tissue stem cells that undergo oncogenic transformation, or from progenitor cells that are already oncogenic and assume a stem-like phenotype (Hanahan and Weinberg 2011). These cells present a modified tumour microenvironment, characterized by hypoxia, and altered metabolism, with low mitochondrial respiration and high rates of glycolysis (Cojoc et al. 2015; Pecqueur et al. 2013).

In breast tumours, CSC have also been detected. Markers of CSC, such as CD49f and CD44, can be found in basal-like (triple-negative) breast cancer, which is commonly heterogeneous and poorly differentiated. CD44-positive cancer cells with basal-like phenotype are commonly more motile and invasive, resulting in worse clinical outcome (Sorlie et al. 2001; Vieira et al. 2017).

Moreover, breast CSC present resistance to most anti-cancer agents, including chemotherapy and radiotherapy (Geng, Alexandrou, and Li 2014; Plaks, Kong, and Werb 2015). This is due to their dormancy, specific morphology, DNA damage repair capability and overall induction of antiapoptotic signaling, drug efflux pumps and detoxifying enzymes. The hypoxic niche where these cells reside can also provide additional protection to anti-cancer agents (Vinogradov and Wei 2012).

Taking into account all the evidence that relates CSC to the progression of tumours, metastasis and recurrence, is critical to understand the underlying mechanisms through how this CSC contribute to breast cancer aggressiveness and develop therapies targeted to eliminate them (Owens and Naylor 2013).

1.2. Cadherins

Cadherins are cell-to-cell adhesion molecules that form calcium-dependent adherents-type junctions (Takeichi 1995). Classical cadherins have a highly conserved cytoplasmatic domain and this cell adhesion molecules are crucial in intracellular signaling mechanisms that regulate cell polarity, differentiation, growth, and migration (Vleminckx and Kemler 1999). This family of glycoproteins is very important in morphogenesis, development and perpetuation of adult tissues (Paredes et al. 2005).

These adhesion molecules have three different domains: extracellular, transmembrane and cytoplasmic. The extracellular region leads to the formation of lateral dimmers that come together in a zipper-like structure with adjacent cells and determines the specificity of the molecule (George and Dwivedi 2004). The cytoplasmic region comprises the juxtamembrane domain (JMD), responsible for cell relocation, and the catenin-binding domain (CBD), necessary for cadherin function (Green et al. 2010; Van Roy and Berx 2008). Cytoplasmic catenins form a dynamic association between the cadherin tail and the actin cytoskeleton, in order to ease assembly into the junctional structure (Paredes et al 2007). This complex is a major regulatory mechanism that influences cell proliferation, differentiation, motility and survival, where the p120-catenin, β-catenin and α-catenin act as the main interaction partners (Figure 5) (Albergaria et al. 2011).

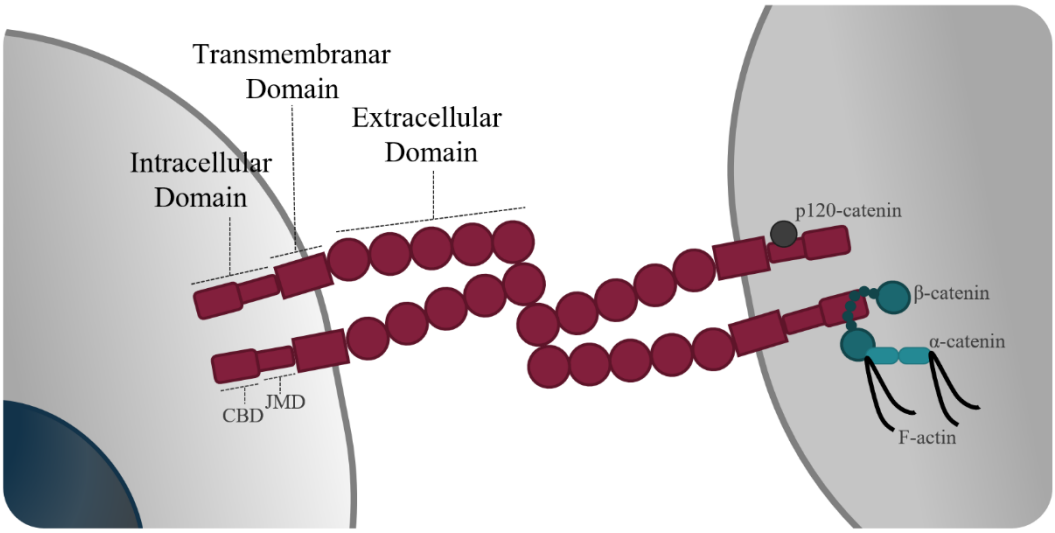

Figure 5 – Schematic representation of the structural composition of P-cadherin adhesive junction protein.

The distinct cadherins are denominated according to their distribution in the different tissues and include N-cadherin (neuronal), R-cadherin (retinal), E-cadherin (epithelial) and P-cadherin (placental) (Albergaria et al. 2011; Paredes et al. 2004). E-cadherin is the primarily expressed member of the classical cadherin family, being expressed in all mammalian epithelia (Van Roy and Berx 2008), and are of utmost importance in the maintenance of cell shape and polarity (Albergaria et al. 2011). In the normal adult mammary gland, E-cadherin is expressed in luminal epithelial cells and myoepithelial/basal cells (Paredes et al. 2007).

P-cadherin, also called placental cadherin, is encoded by the *CDH3* gene that maps to chromosome 16q22.1. It distinguishes itself from N- and E-cadherin in terms of molecular mass and immunological specificity but its structure is similar to classic cadherins (Paredes et al. 2007). P-cadherin is mostly expressed in basal cells (Paredes et al. 2002) and, even though it has a restricted expression, it is essential for the correct architecture of the mammary tissue and its inhibition has been associated with incorrect migration of myoepithelial cells during breast development (Albergaria et al. 2011).

1.2.1. CADHERINS ROLE IN CANCER

Dysfunction of cadherin levels has been associated with various disorders, including cancer. During the progression of epithelial tumours, the expression of E-cadherin decreased (Van Roy and Berx 2008). This loss of E-cadherin is linked with a higher expression of mesenchymal cadherins, called the cadherin switching. This switch is associated with more

proliferative and invasive cells in some tumours (Hazan et al. 2004; Wheelock et al. 2008). In fact, the transition from epithelial cells to mesenchymal cells (EMT – epithelial-mesenchymal transition), accompanying this cadherin switch, is a crucial step for metastatic spread of the primary tumour (Canel et al. 2013).

Cadherin switch normally occurs during development and morphogenesis (Vleminckx and Kemler 1999). It is important to note that these alterations in the expression of epithelial and mesenchymal cadherins are not a requirement for the development or formation of metastases of all types of cancers. Moreover, cadherin switching may occur between different cadherins and differs greatly depending on the carcinoma (Wheelock et al. 2008). For instance, a switch from E-cadherin to N-cadherin is a marker of prostate cancer progression but an increase in P-cadherin expression may as well occur (Gravdal et al. 2007). In pancreatic cancer, there is also a decrease in E-cadherin levels (Hotz et al. 2007) and increased expression of N-cadherin (Shintani et al. 2006). A high expression of N-cadherin in breast cancer cells promotes invasiveness, motility and metastatic capacity of these cells (Hazan et al. 2000; Nieman et al. 1999). In high-grade breast carcinomas, negative for hormone receptors, P-cadherin expression is associated with lower E-cadherin expression (Palacios et al. 1995). In fact, P-cadherin expression is correlated with highly aggressive tumours and constitutes an indicator of poor patient prognosis (Peralta Soler et al. 1999).

1.2.2. P-CADHERIN AND BREAST CANCER

P-cadherin is overexpressed around 20% to 40% of breast cancer cases and was associated with poor prognostic for the patient, specifically short-term overall and disease-specific survival (Albergaria et al. 2011; Paredes et al. 2007). This adhesion protein is highly expressed in high histological grade, ERα-negative and proliferative invasive and aggressive breast tumours (Paredes et al. 2005a). It is one of the most important biomarkers for basal-like breast cancer and HER2-overexpressing breast carcinomas as well as noticeably expressed in inflammatory breast cancer (Albergaria et al. 2011; Sousa et al. 2014). P-cadherin has also been associated with alterations in breast cancer cells, affecting cell-cell adhesion and promoting cell migration and invasion. Moreover, this glycoprotein protein mediates cancer stem cell properties and suggested to be related to glycolytic as resistance to acidic features (Ribeiro and Paredes 2015).

Overexpression of P-cadherin is usually accompanied by a decreased in E-cadherin protein, which leads to tumour progression (Paredes et al. 2005a). This E- to P-cadherin switch may or may not involve EMT. EMT is a mechanism by which epithelial cells convert

to a mesenchymal morphology and this transition is usually necessary for the dissemination of cancer cells and development of metastasis (Ribeiro and Paredes 2015). However, in some breast cancer cells models, it may not even occur (Albergaria et al. 2011). For example, in the 4T1 cell model, that represents aggressive epithelial breast cancer, E-cadherin expression is maintained (Lou et al. 2008).

Disturbance of cell-cell adhesion is essential for the invasive behavior of tumours, one of the first steps in the metastatic process. In breast cancer, increased single cell motility and invasiveness are correlated with highly expressed P-cadherin. An explanation for this is that when E-cadherin is present, P-cadherin disrupts the interaction between E-cadherin and cytoplasmic catenins (p120ctn and βctn), promoting cancer cell invasion (Albergaria et al. 2011).

The inhibition of cell apoptosis, induction of tumour growth and cancer cell invasion promoted by P-cadherin is achieved through the activation of matrix metalloproteinases (MMPs), like MMP1 and MMP2 (Sousa et al. 2014). This MMPs participate in the degradation of the extracellular matrix and MMP2 releases P-cadherin extracellular domain, forming sPcad, a soluble form of P-cadherin, which itself induces a pro-invasive activity (Chakrabarty, Bernardes, and Fialho 2014).

ER-negative breast tumours have high expressing levels of P-cadherin and, Paredes *et al* (2004) established that the P-cadherin gene, *CDH3*, is an ER-repressed gene. This means that P-cadherin increase is related to or consequence of ER-signaling absence (Paredes et al. 2004). Furthermore, it has also been described that ER may, through a series of events, upregulate E-cadherin, an effect not observed in ER-negative tumours (Cowin, Rowlands, and Hatsell 2005). This demonstrates a connection between cadherins and other prognostic factors in breast cancer, suggesting that the poor prognosis of ER-negative breast carcinomas may be linked to the loss of E-cadherin together with P-cadherin overexpression.

1.2.3. P-CADHERIN AND CANCER STEM CELLS

P-cadherin expression is significantly associated with CD44 and CD49f expression in basal-like breast cancer carcinomas, potentiating the colonizing potential of CSC and contributing to increased metastatic capacity (Ribeiro and Paredes 2015; Vieira et al. 2017). This adhesion protein seems to regulate these CSC since its knockdown induces downregulation of CD44 and CD49f. Adding to that, the number of mammospheres formed decreased with lower P-cadherin expression (Vieira et al. 2012). It has also been described

that cancer cells that overexpress P-cadherin presented resistance to X-ray induced DNA damage, another shared property with CSC (Albergaria et al. 2011).

Moreover, a link between P-cadherin and an integrin signaling pathway has also been reported. Integrins facilitate ECM adhesion and the laminin receptor α6β4 integrin, which was found to be crucial in the progression of breast cancer, is regulated by P-cadherin. The fact that P-cadherin is involved in integrin signalling pathways means that, when activated, these pathways lead to activation of Sec and focal adhesion kinases (FAK). FAK is related to the maintenance of cancer stem cells, and their markers are decreased upon FAK inhibition. This explains, not only the invasive properties but also the stem cell phenotype induced by high levels of P-cadherin in breast cancer cells (Vieira et al. 2014).

Therefore, P-cadherin could be a CSC marker in human breast carcinomas and is an indicator of the development of metastases, being associated with the worst survival cases, particularly, triple-negative breast cancer subtype (Sousa et al 2014). The identification of P-cadherin has a breast CSC marker is regarded as an important target in the development of novel therapies against CSC (Cojoc et al. 2015).

1.3. Cancer Metabolism

Under normal conditions, the most efficient method of producing energy used by cells is through the oxidation pyruvate, end product of glycolysis, in the mitochondria through tricarboxylic acid (TCA) cycle and oxidative phosphorylation (Barbosa et al. 2012). In cancer, many cells go through a metabolic reprogramming from an oxidative phenotype to a more glycolytic one, even in oxygen-rich conditions (Ward and Thompson 2012). This switch, a core hallmark of cancer and termed the Warburg effect, occurs because cancer cells require ATP and intermediates for the synthesis of biomolecules, in order to maintain rapid cell division and viability (Feng et al. 2014; Pavlova and Thompson 2016).

The tumorigenesis process depends greatly on the catabolism of glucose, hence the higher consumption of glucose observed in various tumours (Pavlova and Thompson 2016). This higher increase of glucose intake is compensated by an increased expression of glucose transporters, such as glucose transporter 1 (GLUT1), that has high-affinity to glucose and regulates glucose uptake (Chen et al. 2010). Overexpression of GLUT1 has been associated with a higher expression of other molecular components of the glycolytic pathway, such as hexokinase II (HKII) (Xintaropoulou et al. 2018). HKII is an isoform of hexokinase, an

enzyme that catalyzes the first step of glycolysis (Glucose + ATP ⇄ Glucose-6-Phosphate + ADP), and that has been associated with high glycolytic tumours. When HKII binds to mitochondrial transmembrane channels, its ability to metabolize glucose is increased because the enzyme is less sensitive to negative feedback mechanisms and has preferential access to ATP generated by mitochondria (Mathupala, Ko, and Pedersen 2006; Pedersen et al. 2002). HK II has been shown to be overexpressed in various tumours such as breast cancer, ovarian cancer and pancreatic cancer (Chikamoto et al. 2017; Sousa et al. 2014).

During increased biosynthetic demand cells need precursor molecules and reducing equivalents to continue proliferating. To avoid downregulation of mechanisms of glycolysis, cancer cells convert pyruvate to lactate, producing NAD^+, instead of producing NADH in the conversion to Acetyl-CoA. Thus, cells avoid the negative feedback mechanism that result from the accumulation of NADH and maintain a lower ATP production, continuing to produce intermediates for biosynthesis (Namdari et al. 2011; Pavlova and Thompson 2016). The accumulation of lactate leads to an acidic intracellular environment, which means this molecule needs to be excreted through monocarboxylate transporters (MCTs). MCTs are involved in intracellular pH regulation and lead to extracellular acidification by the efflux of lactate (Chiche, Brahimi-Horn, and Pouysségur 2010; Feron 2009).

In breast cancer, cells start to proliferate away from the basal layer, resulting in regions defective on oxygen and nutrients (Chen et al. 2010). This hypoxic environment is known to induces a cellular response mediated by hypoxia-inducible factor 1 (HIF-1), that can induce GLUT1 expression, as well as HKII (Semenza 2010). Consequently, hypoxia can promote acidosis and enhance expression of transporters that regulate intracellular pH, such as carbonic anhydrase IX (CAIX) and monocarboxylate transporter 4 (MCT4) (Chiche, Brahimi-Horn, and Pouysségur 2010; Sousa et al. 2014).

1.3.1. MITOCHONDRIA IN CANCER

Mitochondria are essential organelles in cells and displaying also an important role in cancer cells. The sequence of metabolic changes through hypoxia, glycolysis and acidosis, provides cancer cells with an survival advantage, causing them to become more aggressive (Chen et al. 2010). ATP production levels in the mitochondria may decrease upon a metabolic switch due to not only bioenergetic modifications, but also alterations in the mitochondrial biogenesis and turnover that regulate mitochondrial mass, as well as changes in fission and fusion dynamics (Vyas, Zaganjor, and Haigis 2016).

1.3.1.1. Mitochondrial dynamics

Mitochondria are extremely dynamic organelles that form a well-designed and highly controlled network (Chen and Chan 2017). The morphology of mitochondria is determined by a balance of fusion, fission, biogenesis, degradation and mitophagy. This means that mitochondria can be arranged from highly interconnected networks to more fragmented ones. Alterations in these processes are associated with several pathologies and can be deregulated in cancer (Maycotte et al 2017; Senft and Ronai 2016; Vyas, Zaganjor, and Haigis 2016).

Fission is predominantly performed by dynamin related protein 1 (DRP1), which is recruited by fission homolog protein 1 (FIS1), mitochondrial fission factor (MFF) and other receptors (Chen and Chan 2017; Sebastián and Zorzano 2018). DRP1 leads to mitochondria constriction, causing fission (Figure 6) (Sheridan and Martin 2010). This process results in rounded, fragmented mitochondria, a phenotype that is associated with a lower ATP production derived from a reduced respiratory chain function (Senft and Ronai 2016; Vyas, Zaganjor, and Haigis 2016).

Mitofusins 1 and 2 (MFN1 and MFN2) in the outer mitochondrial membrane (OMM) and optic atrophy gene 1 (OPA1) in the inner mitochondrial membrane (IMM) mediate fusion and form elongated, interconnected mitochondria (Figure 6). This process is very important to maximize oxidative phosphorylation and crucial to dilute mitochondrial machinery errors, such as mutations in mitochondrial DNA (mtDNA) and oxidized lipids or proteins (Mishra and Chan 2016; Senft and Ronai 2016; Trotta and Chipuk 2017).

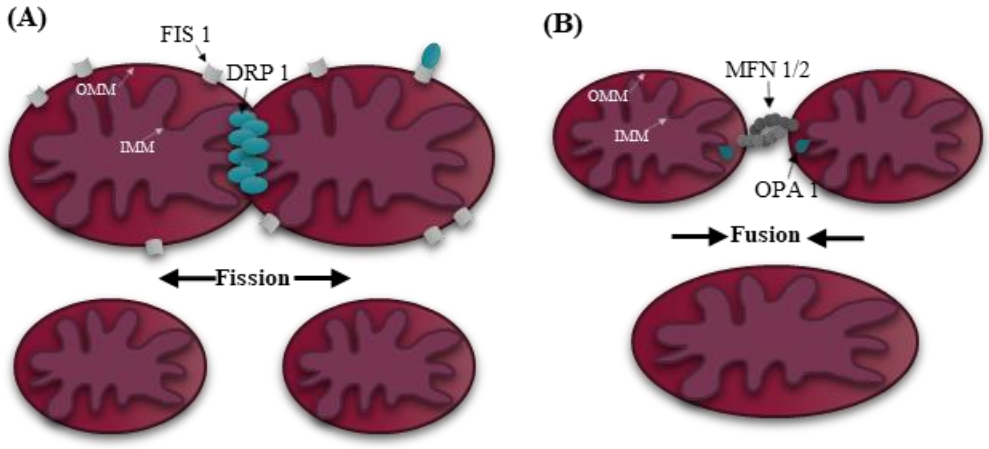

Figure 6 – Schematic representation of mitochondrial dynamics. (A) Mitochondrial fission is primarily driven by DRP1, which is recruited to mitochondria by FIS1. (B) MFN1 and MFN2 mediate outer mitochondrial membrane fusion together with OPA1 that drives inner mitochondrial membrane fusion.

In cancer cells, mitochondria often present decreased fusion and increased fission, with high levels of DRP1 and low expression of MFN1/2, resulting in more fragmented mitochondria (Cannino et al. 2018; Chen and Chan 2017; Vyas, Zaganjor, and Haigis 2016).

1.3.1.2. Mitochondrial biogenesis

Mitochondrial biogenesis is greatly influenced by the peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (PGC-1α), which is downregulated by HIF-1α in a predominantly glycolytic metabolism. PGC-1α also affects cellular metabolic pathways and influences mitochondria respiration (Lagory et al. 2015; Tan et al. 2016). PGC-1α works as a stress sensor in cancer cells and can be a promoter of carcinogenesis. In breast cancer, PGC-1α is known to promote anchorage-independent growth and enhance metabolic capacity (Fisher et al. 2011; Tan et al. 2016).

Mitochondrial transcription factor A (TFAM) is regulated by PGC-1α and is essential to maintain copy number, structure and efficient transcription of mtDNA (Gabrielson et al. 2014). TFAM binds to mtDNA creating U-turns and the number of TFAM that bind to a nucleoid will dictate the level of compaction of mtDNA (Figure 7) (Bonekamp and Larsson 2018; Kukat et al. 2015).

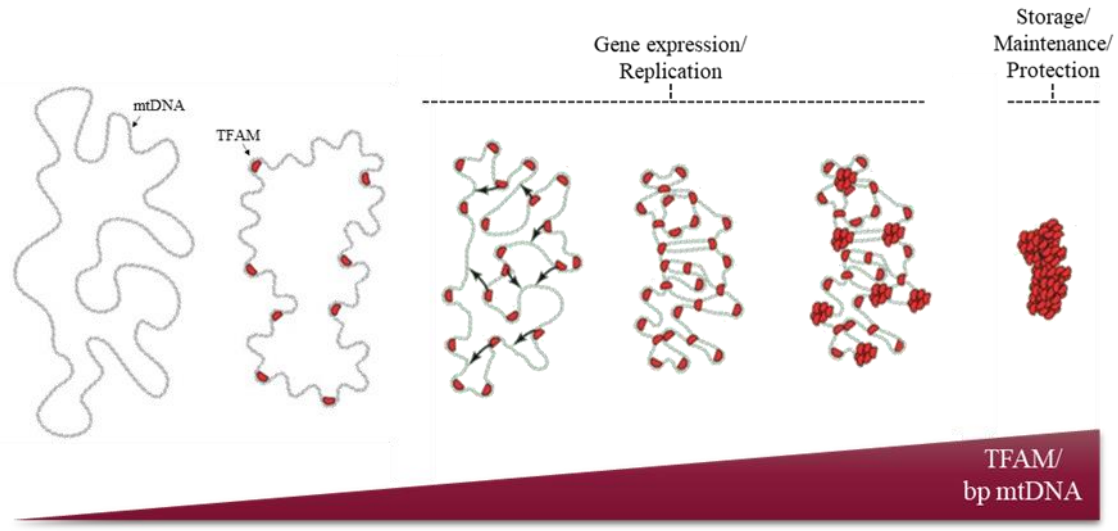

Figure 7 - Nucleoid compaction of mitochondrial DNA by TFAM. TFAM molecules bind to mtDNA and, through a combination of bending and cross-strand bindings compact mtDNA to form a tightly packaged mtDNA – nucleoid. Compaction increases as the ratio of TFAM per base pair of mtDNA grows. Adapted from Kukat et al. 2015.

Each mitochondrion has multiple copies of mtDNA, a circular genome that encodes 13 proteins that are essential subunits of the OXPHOS complexes, but also 22 tRNAs and 2

rRNAs (Croteau, Stierum, and Bohr 1999; Malik and Rosa 2018). The number of copies of mtDNA is not fixed, unlike nuclear DNA, and the ratio of mtDNA copy number to nuclear DNA varies according to the needs of a tissue and may also be altered in disease situations (Dornfeld and Skildum 2018). Under conditions where mitochondrial biogenesis processes are altered, mitochondrial mass is also affected (Malik and Rosa 2018). An important marker of mitochondrial mass is the translocase of outer mitochondrial membrane 20 (TOM20) which is involved in protein import into the mitochondrial matrix (Fu et al. 2012).

1.3.2. METABOLISM AND CANCER STEM CELLS

Poorly differentiated tumours present an elevated glucose uptake and rely more on glycolysis. CSC also present an altered metabolic program characterized by decreased oxidative phosphorylation and ROS production (Feng et al. 2014; Krzeslak et al. 2012). These alterations occur in aerobic conditions, indicating that CSC are also affected by the Warburg effect. However, a hypoxic state has been shown to enhance this phenotype. The oxygen-depleted microenvironments found within a tumour may be essential to maintain the undifferentiated state of CSC since hypoxic niches within a tumour are enriched in CSC (Owens and Naylor 2013; Sousa et al. 2014). CSC also contain lower levels of reactive oxygen species (ROS), which could be a reason behind therapy resistance, because this allows these cells to reduce radiation-induced DNA damage due to enhanced ROS defense systems (Diehn et al. 2009).

Anchorage-independent growth is essential during cancer progression, meaning cancer cells have the ability to escape anoikis, a type of programmed cell death due to detachment from the extracellular matrix (ECM) or upon cell adhesion to an inappropriate matrix (Paoli, Giannoni, and Chiarugi 2013). This resistance to anoikis occurs as a result of the predominant glycolytic metabolism by cancer cells and leads to increased survival in circulation and metastasis propagation (Kim et al. 2012). This phenomenon may be exacerbated by CSC since these cells are related to more aggressive and metastatic cancers.

1.3.3. P-CADHERIN AND METABOLISM

Recently, Sousa *et al* (2014) demonstrated that up-regulation of P-cadherin leads to higher levels of GLUT1, which could lead to increased cell glucose uptake. This high expression of glucose transporters allows P-cadherin overexpressing cancer cells to have a higher glycolytic rate and may demonstrate that P-cadherin is implicated in a signaling pathway that interferes with the metabolic reprogramming of cells (Sousa et al. 2014).

Therefore, the altered metabolic state of cancer cells, which can be amplified by stem-like properties, is related to hypoxia adaptation and to P-cadherin overexpression. All these factors contribute to therapeutic resistance, which is supported by previous studies that show that breast cancer cells P-cadherin negative are more sensitive to cell death induced by radiation (Sousa et al. 2014). Monocarboxylate transporters (MCTs) were also found up-regulated in P-cadherin overexpressing cells. These transporters are involved in intracellular pH regulation and lead to extracellular acidification by the transport of lactate. This process is known to help the metastization process through the acidic degradation of the ECM (Pinheiro et al. 2011; Sousa et al. 2014).

1.4. AIMS OF THE STUDY

The main objective of this work was to understand if P-cadherin expression can modulate some of mitochondrial functions in breast cancer cells. Therefore, we intended to evaluate if P-cadherin levels could induce changes in mitochondrial morphology, by looking at the fusion and fission machinery, and biogenesis, as well as the effect on mitochondria membrane potential and cancer cells energetic state.

Chapter 2

MATERIALS AND METHODS

2.1. Reagents

Acrylamide/Bis Solution 40%, Blotting-Grade Blocker (non-fat dry milk), Laemmli buffer, Precision Plus Protein™ Standard Dual Color, Resolving Gel Buffer (1.5 M Tris-HCl), Stacking Gel Buffer (0.5 M Tris-HCl), Trans®-Blot Turbo™ RTA Transfer PVDF Kit (5x Transfer Buffer, Transfer Stacks and PVDF Membranes) and Clarity Western ECL Substrate were obtained from Bio-Rad (Hercules, California, USA). Protease Inhibitor Cocktail was acquired from Frilabo (Maia, Portugal), Ammonium Persulfate (APS) from Gerbu (Heidelberg, Germany) and CellTiter-Glo® Luminescent Cell Viability Assay Kit from Promega G7571 (Madison, Wisconsin, USA). From NzyTech (Lisbon, Portugal) were obtained Glycine, N,N,N′,N′-Tetramethylethylenediamine (TEMED), Sodium Dodecyl Sulphate (SDS) and Tris base. The reagents required for DNA extraction, DNase/RNase-free water and QIAamp® DNA Mini Kit, were obtained from Qiagen (Hilden, Germany), as well as Negative Control siRNA and Hs_CDH3_6 FlexiTube siRNA. From Promega (Wisconsin, USA), the CellTiter-Glo® Luminescent Cell Viability Assay was obtained. Adenosine 5'-triphosphate disodium salt, Bisbenzimide Hoechst 33342 trihydrochloride, Bovine Serum Albumin (BSA), Dimethyl sulfoxide (DMSO), Dithiothreitol (DTT), Methanol, Oligomycin, Potassium phosphate monobasic (KH2PO4), Propan-2-ol (Isopropanol), Sodium chloride (NaCl), Sodium phosphate dibasic (Na_2HPO_4), Tergitol™ solution (NP-40), Trixon X-100, Trypan Blue solution, Tween® 20 were received from Sigma-Aldrich (St. Louis, Missouri, USA). From Thermo Fisher Scientific (Waltham, Massachusetts, USA) were obtained the essential reagents for the cell culture such as 0.05% Trypsin-EDTA, Antibiotic-Antimycotic (10,000 units/mL penicillin, 10,000 µg/mL of streptomycin and 25 µg/mL amphotericin B), Dulbecco's modified Eagle's medium (DMEM), Fetal Bovine Serum, heat-inactivated (FBS), Ham's F-12 Nutrient Mix, Lipofectamine™ 2000 Transfection Reagent and Opti-MEM™, as well as the common reagents Pierce™ BCA Protein Assay Kit and Sodium chloride (NaCl).

2.2. Cell line and Culture Conditions

2.2.1. MCF7 CELL LINE

MCF-7 breast cancer cell line was introduced in 1973 by Dr. Herbert Soule, from a pleural effusion of a 69-year-old female with metastatic breast carcinoma (Soule et al. 1973). This cell line represents a classic breast cancer cell line that of a luminal-like breast carcinoma that is endocrine responsive. Consequently, these cells express estrogen, progesterone,

androgens and glucocorticoids receptors (Horwitz, Costlow, and McGuire 1975; Levenson and Jordan 1997).

MCF-7/AZ cell line was transfected with an expression vector with cDNA encoding full-length human P-cadherin, that co-expressed enhanced green fluorescent protein (EGFP), creating MCF-7/AZ.Pcad cell line (Joana Paredes et al. 2004). MCF-7/AZ as well as MCF-7/AZ.Pcad cell lines were provided by Dr Joana Paredes (I3S, Institute for Research and Innovation in Health, University of Porto). Thus, all experiments were performed on the MCF-7/AZ.Mock parental cell line, which normally has low expression levels of P-cadherin and the variant that overexpresses P-cadherin (MCF-7/AZ.Pcad).

Cells were grown in a mixture of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F-12 Nutrient Mix, in a ratio of 1:1, supplemented with 10% FBS heat-inactivated and 1% of antibiotic-antimycotic.

2.2.2. BT20 CELL LINE

BT20 cell line is derived from triple-negative breast cancer and was established in 1958 by Lasfargues and Ozzello. This cell line represents invasive and therapy-resistant tumours that are basal-like (Castles et al. 1993). These cells are negative for estrogen and progesterone receptors and previously observed to overexpress P-cadherin.

BT20 cell line was also kindly provided by Joana Paredes (I3S, Institute for Research and Innovation in Health, University of Porto). This cell line was grown in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% FBS heat-inactivated and 1% of antibiotic-antimycotic.

2.2.2.1. P-cadherin gene silencing in BT20 cells

Since BT20 cell line normally overexpresses P-cadherin, gene silencing expression was transiently induced by siRNA. BT20 cells were transfected with Hs_CDH3_6 siRNA to the target sequence AAGCCTCTTACCTGCCGTAAA, specific to P-cadherin gene and designated as BT20 siRNA CDH3. A scrambled siRNA sequence, Negative Control siRNA, with no homology to any mammalian gene, was used as control and designated BT20 siRNA scrambled (Sousa et al. 2014).

Cells were plated in a six-well plate with a cellular density of $3{,}5 \times 10^5$ cells/well to the confluence was around 60% to 70%. Gene silencing was performed 24 hours after cells were plated. Lipofectamine 2000 and siRNAs were diluted in Opti-MEM medium, in a ratio of 1:24 and 1:49, respectively, and were incubated for 5 minutes. The diluted siRNAs were

added to the diluted Lipofectamine 2000 and incubated for 20 minutes, in order to form the lipid complexes. 750 µL of serum-free DMEM medium was added to the cells and, to that, was added 250 µL of siRNA-lipofectamine, dropwise, to homogeneously distribute throughout the well.

After cells were incubated for four and a half hours, the medium was removed and added new complete DMEM media. After 48 hours of incubation, at 37˚C with 5% CO_2, assays were performed. CDH3 gene silencing was evaluated through western blotting.

2.2.3. Cell Culture

Both MCF-7/AZ and BT20 cell lines were kept in T25 or T75 flasks at 37ºC, 5% CO_2 and sub-cultured when they reached approximately 80-90% confluence. When doing this regular process, cells were rinsed with PBS (137 mM NaCl, 2,7 mM KCl, 4.3 mM Na_2HPO_4, 1,5 mM KH_2PO_4 and the pH adjusted to 7.2) and incubated with trypsin-EDTA, for 5 minutes at 37ºC. Afterward, the same volume of medium was added, cells centrifuged at 1200 rpm for 5 minutes and then resuspended and seeded.

2.3. Western Blotting Analysis

2.3.1. Total Protein extraction

BT20 cells were plated and transfected as described in 2.2.2.1, and were harvested after 48 hours of incubation. MCF-7/AZ.Mock and MCF-7/AZ.Pcad cells were plated in a six-well multiwell plate, in a cellular density of 2.5 x 10^5 cell/well, were allowed to adhere for 24 hours and, and after that time, assays were performed. To harvest the cells, they were washed twice with 2 mL of PBS and 200 µL of a mixture of catenin lysis buffer (CLB - 1% Triton X-100 and 10% of NP-40, in PBS) and proteases inhibitors (100:1) was added and let to incubate for 10 minutes, at 4ºC. Then, cells were scraped with cell scrappers and wells were collected two by two to a 1.5 mL eppendorf microcentrifuge tube . After resuspending the cells by up-and-down with a pipette, three 10 seconds vortexes were done and cell lysates were centrifuged at 14 000 rpm, for 10 minutes at 4ºC. The supernatant was recovered and stored at -80ºC until western blotting procedure was performed.

2.3.2. PROTEIN QUANTIFICATION

Protein was quantified by bicinchoninic acid method, using Pierce™ BCA Protein Assay Kit, according to the manufacturer's instructions. Protein lysates were diluted in a ratio of 1:10. A standard Bovine Serum Albumin curve was made, to which were compared the values of the samples, read at 560 nm in BioTek® Cytation3™ UV–vis multi-well plate imaging reader (BioTek, Winooski, Vermont, USA).

2.3.3. PROTEIN ELECTROPHORESIS AND TRANSFER

Protein (20 µg) was denatured at 95ºC, for 5 min, in 6x *Laemmli* sample loading buffer and DTT (9:1). Protein was then separated by 10-16% SDS-polyacrylamide gel, depending on the size of the protein of interest, in an electrophoresis system with running buffer (glycine 192 mM, tris base 25 mM, SDS 1% and the pH adjusted to 8.3) with a constant voltage of 150 V, at room temperature. To estimate protein molecular weight, a molecular weight standard was added to each gel.

Protein was then transferred to a PVDF membrane, either through wet-transfer during 90 minutes at a constant voltage of 250 mA, or through semi-dry-transfer using TransBlot® Turbo™ Transfer System, according to the manufactures instructions, during 10 minutes at 2.5 A, 25V. When using wet-transfer with transfer buffer (390 mM glycine, 480 mM tris base, 0.37 % SDS, 20 % methanol and the pH adjusted to 7.5), the transfer system was put in ice, to reduce the heat formed during transfer. After transfer, membranes were blocked in 5% non-fat milk in Tris-Buffered Saline Tween (TBS-T - 100 mM of tris base, 1.5 M of NaCl, 0.1% of Tween 20 ant the pH adjusted to 7.5) for 1 hour, at room temperature, with constant agitation. Membranes were then washed 3 times in TBS-T, for 5 minutes, and incubated overnight with the primary antibody against the protein of interest, at 4ºC with constant stirring (Table 1). After incubation, membranes were washed 3 times in TBS-T, the first wash for 10 minutes and the other two for 5 minutes, and incubated with the secondary antibody for 1 hour, at room temperature, under continuous agitation. Membranes were washed again and were incubated with ECL substrate, for 5 minutes at room temperature before reading fluorescence at the UVP Biospectrum 500 Imaging System (UVP LLC, Cambridge, UK) or VWR® Imager Chemi 5QE (Pennsylvania, EUA), through UV epi-ilumination (365 nm). Images were analysed using TotalLab TL120 1D v2009 (Nonlinear Dynamics Ltd., UK).

To normalise and assure equal protein loading in each well, membranes were incubated with anti-actin primary antibody for 1 hour, at room temperature, under constant agitation and the remaining procedure was the same as for the other antibodies.

Table 1 – List of primary and secondary antibodies used in western blotting protein analysis.

Primary Antibodies						
Name		MW (kD)	Dilution	Host species	Reference	Manufacturer
TOM20		20	1:1000	Rabbit	sc11415	Santa Cruz
TFAM (mtTFA)		25	1:1000	Goat	sc23588	Santa Cruz
PGC-1α		90	1:1000	Rabbit	sc13067	Santa Cruz
Opa-1		120	1:250	Mouse	sc393296	Santa Cruz
MFN-1		86	1:500	Rabbit	sc50330	Santa Cruz
DRP-1		80	1:500	Mouse	sc271583	Santa Cruz
Fis-1		17	1:250	Mouse	sc376447	Santa Cruz
OXPHOS cocktail	**ATP5A**	54	1:1000	Mouse	MS601	Abcam
	UQCRC2	48				
	SDHB	29				
	COX II	22				
	NDUFB8	18				
P-cadherin		120	1:1000	Mouse	6102228	BD Biosciences
Actin		43	1:5000	Mouse	MAB1501	Millipore

Secondary Antibodies				
Name	Dilution	Host species	Reference	Manufacturer
Anti-rabbit IgG, HRP-linked	1:5000	Goat	7074	Cell Signaling
Anti-mouse IgG, HRP-linked	1:5000	Horse	7076	Cell Signaling
Anti-goat IgG, HRP-linked	1:5000	Mouse	sc2354	Santa Cruz

2.4. Mitochondrial DNA Copy Number

2.4.1. GROWTH AND HARVESTING OF CELLS

Cell were grown, harvested and the DNA isolated according to QIAamp DNA Mini and Blood Mini Handbook (Qiagen 05/2016). BT20 cells and MCF-7/AZ cells were plated has described in section 2.2.2.1 and 2.3.1, respectively. 48 hours after plating for BT20 cells and after 24 hours for MCF-7/AZ cells, medium was aspirated, cells were washed with PBS

and 1 mL of trypsin-EDTA was added to each well and cells let to detach cell for 5 minutes, at 37ºC, 5 % CO_2. Wells were collected two by two in medium and centrifuged at 200 x g for 5 minutes. Supernatant was discarded, pellet was resuspended in 500 µL of PBS and centrifuged in 1.5 mL eppendorf microcentrifuge tube at 200 x g, for 3 minutes. The supernatant was discarded again, and the dry pellet was stored at -80ºC until total DNA extraction.

2.4.2. TOTAL DNA EXTRACTION

The dry pellet was resuspended in 200 µL of PBS and 20 µL of proteinase K was added. To lyse the cells, 200 µL of Buffer AL was added to each sample, that were then mixed by pulse-vortexing for 15 seconds and incubated at 56ºC, for 10 minutes. After a brief spin, 200 µL of ethanol 96% was added and mixed by pulse-vortexing, for 15 seconds. Another brief spin was performed, and 600 µL were applied to the QIAamp Mini spin column in a 2 mL collection tube. Column was centrifuged at 6000 x g, for 1 minute, the filtrate was discarded, and the column placed in a new 2 mL collection tube. 500 µL of Buffer AW1 was added to the column, centrifugation was performed at 6000 x g, for 1 minute, the filtrate was discarded, and the column placed in a new 2 mL collection tube. After adding 500 µL of Buffer AW2, columns were centrifuged at 20000 x g, for 3 minutes, the filtrate was discarded, and the column placed in a new 2 mL collection tube. Columns were centrifuged again at 20 000 x g, for 1 minute, and then placed in a new 1.5 mL microcentrifuge tube. Next, 200 µL of Buffer AE was added to the column and let to incubate at room temperature, for 5 minutes, and then centrifuged at 6000 x g, for 1 minute.

DNA concentration, yield and purity in the eluate were determined using Nanodrop 2000™ spectrophotometer (Thermo Fisher Scientific, USA), which was calibrated using Buffer AE. Samples were then sonicated for 10 minutes, using a Branson 5510® sonifier (Branson Sonic Power Company, Danbury, Connecticut, USA), and diluted in Buffer AE to a final concentration of 10 ng/µL.

2.4.3. QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION

mtDNA copy number determination was carried out by quantitative real time polymerase chain reaction (qRT-PCR) with Bio-Rad® CFX96TM Real-Time PCR System (Bio-Rad Laboratories, Hercules, California, USA). mtDNA copy number was quantified using CYTB gene and the nuclear-encoded B2M gene copy number was measured for normalization purposes (Table 2). Amplification of total cellular DNA was done using

SsoFast™ EvaGreen® supermix and 25 ng of DNA per well, with the primers being at a final concentration of 500 nM. For each primer was used a negative control without DNA, which was replaced by water (no template control - NTC). Efficiency curves were also made for each primer using a sample pool (one pool for each cell line) at 1:1, 1:10 and 1:100 dilutions. The annealing temperature used was 60°C.

To determine the mtDNA copy number, a ratio between copy number of the mitochondrial gene CYTB and the nuclear gene B2M was used. Data was analysed using Bio-Rad® CFX96 Manager software (version 3.1) (Bio-Rad Laboratories, Hercules, California, USA).

Table 2 – List of primers used in qRT-PCR

Gene	Designation	Forward Primer	Reverse Primer
B2M-DNA	Homo sapiens beta-2-microglobulin	GAATTCCAAATTCTGCTTGCTTGC	CCTCTAAGTTGCCAGCCCTC
h_CYTB	Mitochondrial cytochrome b	CCACCCCATCCAACATCTCC	GCGTCTGGTGAGTAGTGCAT

2.5. Fluorescence Microscopy

BT20 cells were plated and transfected in a six well multiwell plate, has described in 2.2.2.1. On the day before of the experiment, transfected BT20 cells, as well as MCF-7/AZ.Mock and MCF-7/AZ.Pcad cells, were plated in a chambered coverslip (ibidi GmbH, Germany), with a cellular density of 2 x 10^4 cells/well in 200 µL medium. On the day of the experiment, cells were washed with PBS and incubated with probes for 30 minutes at 37ºC, 5 % CO_2. Probes were prepared in the respective medium of each cell line and 200 µL were added to each well. The tetramethylrhodamine (TMRM) dye was added at 50 nM, which allows the study of mitochondrial membrane potential, and Hoechst 33342 at 1 µg/mL, to counterstain the nucleus. Images were then obtained with Zeiss Axiovert 200M inverted microscope (Zeiss, Germany), using a 40x air objective.

2.6. ATP levels measurements

BT20 cells were plated and transfected in a six well multiwell plate, has described in section 2.2.2.1. After transfection, cells were plated in a white 96 multiwell plates with clear bottom, with a cellular density of 2 x 10^4 cells/well in 150 µL of culture medium. MCF-7/AZ.Mock and MCF-7/AZ.Pcad were plated with a cellular density of 3 x 10^4 cells/well in

150 µL of culture medium. After 24 hours, inhibitors were added to the cells and incubated for 3 hours at 37ºC, 5% CO_2. The inhibitors oligomycin (2 µM), 2-Deoxyglucose (2-DG, 50 mM) were prepared in medium and added to the cells in a final volume of 100 µL. Afterwards, 50 µL of medium with inhibitors was removed and 50 µL of CellTiter-Glo® Luminescent Cell Viability Assay Kit was added to the cells. Luminescence was read at room temperature in BioTek® Cytation3™ UV–vis multi-well plate imaging reader (BioTek, Winooski, Vermont, USA) after 2 minutes of orbital shaking and 10 minutes of incubation to allow luminescent signal stabilization.

2.7. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7.04 (GraphPad Software, Inc.). To evaluate the effects P-cadherin expression, Shapiro-Wilk normality test was performed. If normality was verified, unpaired t-test was used. If not, nonparametric Mann-Whitney test was performed. To evaluate the effect on ATP levels with various inhibitors, an ordinary two-way ANOVA was performed. Data are presented as mean ± SEM. Statistically significance was considered if $p < 0.05$.

Chapter 3

RESULTS

3.1 P-cadherin expression in MCF-7/AZ and BT20 cells

In order to validate the differential expression of P-cadherin in the two cell models, in MCF-7/AZ and BT20, the protein levels were determined by western blotting technique. MCF-7/AZ cells, that normally have lower levels of P-cadherin, were transfected with an expression vector with cDNA encoding full-length P-cadherin to overexpress the protein (in MCF-7/AZ.Pcad) and kindly provided by Dr. Joana Paredes (I3S, Institute for Research and Innovation in Health, University of Porto). P-cadherin protein levels were increased by 65.69 % in MCF-7/AZ.Pcad cells (**p* = 0.0198), attesting that is overexpressed on this cell when compared to MCF-7/AZ.Mock cells (Figure 8A). BT20 cells present already a higher level of P-cadherin and so we transfected these cells with siRNAs in order induce a decrease of the protein levels. P-cadherin gene silencing were achieved successfully given the observed decrease of 90.38 % (***p* = 0.0085) in BT20 siRNA CDH3 cells, when compared to control cells, BT20 siRNA scrambled (Figure 8B).

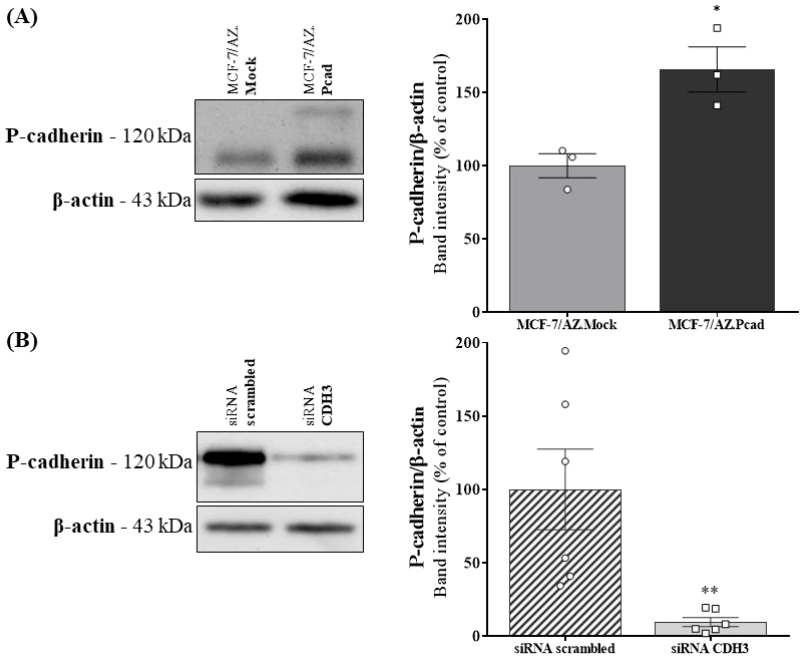

Figure 8 - Western blotting analysis of P-cadherin expression in MCF-7/AZ cell lines and in BT20 cell line.

(A) P-cadherin expression levels quantification in MCF-7/AZ.Mock and MCF-7/AZ.Pcad cell line of 6 independent experiments; a representative blot image is also presented. **(B)** P-cadherin expression levels quantification in siRNA scrambled and siRNA CDH3 BT20 cells, 48 hours post-transfection, from 6 independent experiments; a representative blot image is also presented. Results were normalized by β-actin and represented as percentage of the control. Data are expressed as mean ± SEM. Statistical analysis was calculated by using unpaired t-test.

3.2. Alteration of P-cadherin levels lead to changes in ATP production

To understand the main source of ATP production used by cells, oligomycin and 2-deoxy-D-glucose (2-DG) inhibitors were added to the cells. Oligomycin is an ATP synthase (Complex V) inhibitor and thus blocks OXPHOS (Hao et al. 2010). 2-DG inhibits glycolysis as it competes with glucose for hexokinase and inhibits glucose-6-phosphate formation. Instead, deoxyglucose-phosphate is produced, leading to hexokinase II inhibition and decreased ATP production via glycolysis (Aft, Zhang, and Gius 2002; Seo, Crochet, and Lee 2013).

When compared to control group MCF-7/AZ.Mock, P-cadherin overexpression in MCF-7/AZ.Pcad cells didn't show differences in total ATP production ($p = 0.9188$). Addition of oligomycin to both groups lead to a decrease in ATP production with P-cadherin overexpression (**$p = 0.0093$), which could indicate that ATP production was through mitochondria. However, these results must be taken in perspective given the surprising increase in ATP levels observed in MCF-7/AZ.Mock cells upon inhibition with oligomycin (**$p = 0.002$). This increase, compared to control cells without inhibitors, is quite unexpected because ATP synthase was inhibited, and the same did not happen in the MCF-7/AZ.Pcad cells with oligomycin. Even if the cells were compensating for ATP production with a higher glycolytic rate the levels should, in theory, not be higher than those obtained in control cells.

In MCF-7/AZ.Mock cells, in the case of 2-DG inhibition, compared to control without inhibitors, no significant differences were found in ATP levels ($p = 0.8270$). However, 2-DG led to a 25 % decrease in P-cadherin overexpression group was found comparatively to MCF-7/AZ.Mock cells, a result with plenty statistical significance (**$p = 0.0023$, Figure 9A). When comparing to control MCF-7/AZ.Pcad cells with no inhibitors, the cells showed a 14.63% decrease in ATP levels when inhibited with 2-DG ($p = 0.0854$). Although not statistically significant, this shows that P-cadherin-overexpressing cells are more susceptible to 2-DG inhibition. Taken together these results may indicate that MCF-7/AZ.Pcad cells are more dependent on glycolysis for ATP production than OXPHOS.

Silencing of P-cadherin (BT20 siRNA CDH3) in BT20 cells did not induce differences in total ATP levels ($p = 0.9725$) comparatively to control group BT20 siRNA scrambled, that present a higher expression of P-cadherin (Figure 9B). By comparing the two groups when treated with oligomycin, we observed a slight decrease in ATP levels in the siRNA CDH3 group, although not significative ($p = 0.1223$). When comparing BT20 siRNA CDH3 cells incubated with oligomycin to vehicle treated group (control), a nonsignificant trend was noticed ($p = 0.1302$). These results suggest that P-cadherin suppression may shift

cell to a more OXPHOS-dependent metabolism to obtain ATP. Analysis of BT20 siRNA scrambled cells did not present any differences on ATP levels with oligomycin, when comparing to the same cells without inhibitors ($p = 0.9659$).

From the evaluation of 2-DG inhibitory effect on ATP levels on BT20 siRNA scrambled comparatively to BT20 siRNA CDH3 cells no differences were found ($p > 0.9999$). This was surprising given the obtained result in the MCF7-7/AZ cells. However, both groups demonstrated a decrease in ATP levels with 2-DG relatively to their one control cells (vehicle treated), although without significance (Figure 9B). Therefore, this could mean that, independently of P-cadherin expression levels in BT20 cells, their ATP levels are still largely dependent on the glycolytic pathway. So. in BT20 cells, P-cadherin silencing seems to promote a more oxidative dependent metabolism, since the ATP levels decreased upon incubation with oligomycin, but it does not influence the glycolysis rate preponderance on these cells.

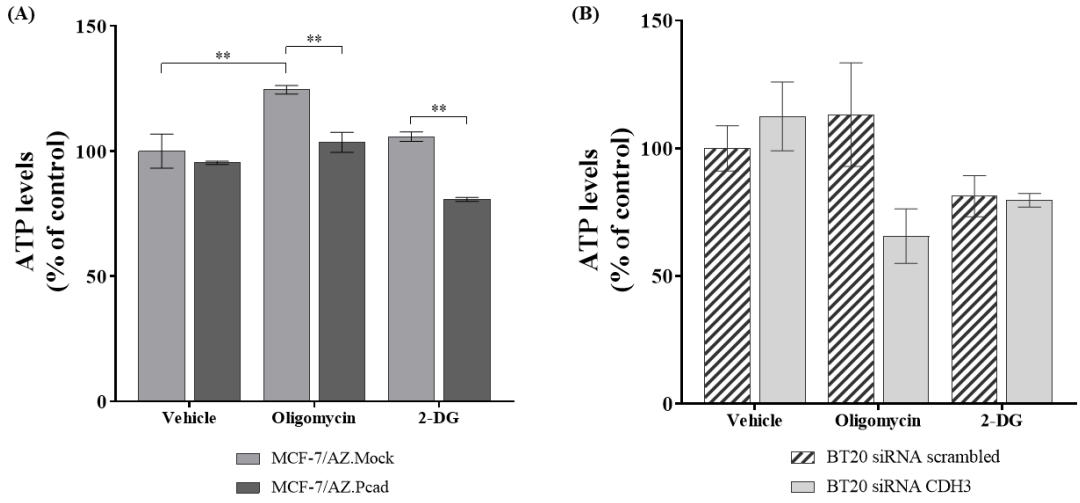

Figure 9 - ATP levels in MCF-7/AZ cells and BT20 cells.

(A) ATP levels in MCF-7/AZ.Mock and MCF-7/AZ.Pcad cells of 3 experiments; MCF-7/AZ.Mock cells with no inhibitors were considered as control. **(B)** ATP levels in BT20 transfected with siRNA scrambled or siRNA CDH3, 48 hours post-transfection, of 3 experiments; BT20 siRNA scrambled cells with no inhibitors were considered as control. Oligomycin (2µM) and 2-deoxy-D-glucose (2-DG, 50mM) inhibitors were incubate for 3 hours before measuring ATP levels using CellTiter-Glo® Luminescent Cell Viability Assay Kit. Data are expressed as mean ± SEM. *: $p<0.05$, **: $p<0.01$. Statistical analysis was calculated by using two-way ANOVA with Tukey's multiple comparisons test.

3.3. Changes in P-cadherin levels did not modify the amount of the components of the electron transport chain complexes

Given that different levels of P-cadherin expression in MCF-7/AZ.Pcad and BT20 siRNA CDH3 cells may be associated with metabolic changes, and so the OXPHOS machinery could be altered, we decided to analysis the expression of the electron transport chain complexes, to better understand the previous results. By western blotting technique, the protein expression levels for subunits of the electron transport chain complexes were evaluated in MCF-7/AZ and BT20 cells. To do so, NADH dehydrogenase (ubiquinone) 1 β subcomplex 8 (NDUFB8) from complex I, succinate dehydrogenase (ubiquinone) iron sulfur subunit (SDHB) from complex II, ubiquinol-cytochrome c core protein reductase II (UQCRC2) from complex III, cytochrome c oxidase subunit II (COX II) from complex IV and ATP synthase subunit α (ATP5A) from complex V were determined. In MCF-7/AZ cells, no significant differences were found in the expression levels of subunits of mitochondrial complexes I, III, IV nor V (Figure 10A). Unfortunately, we were not able to detect and quantify SDHB from complex II in MCF-7/AZ cells. In BT20 cells, P-cadherin silencing also did no induce a significant difference in the expression levels of OXPHOS subunits, despite all subunits were detected (Figure 10B).

Oppositely to what we were expecting, these results suggest that P-cadherin expression does not influence the mitochondrial complexes subunits expression on these cancer cells.

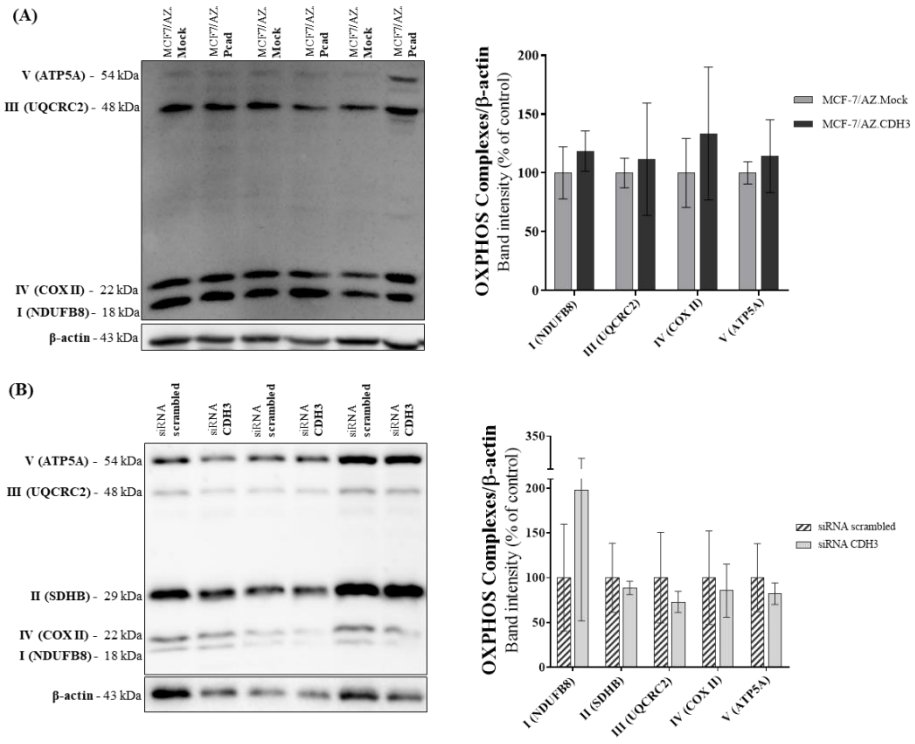

Figure 10 - Western blotting analysis of OXPHOS complexes expression in MCF-7/AZ and BT20 cell lines.

(A) OXPHOS levels protein quantification in MCF-7/AZ.Mock and MCF-7/AZ.Pcad cell line of 3 independent experiments; a representative blot image is also presented (n = 3); MCF-7/AZ.Mock cells were considered as control. **(B)** OXPHOS levels protein quantification in of siRNA scrambled and siRNA CDH3 BT20 cells, 48 hours post-transfection, from 5 independent experiments; a representative blot image is also presented (n = 3); BT20 siRNA scrambled cells were considered as control. OXPHOS complexes represented: CI (NDUFB8 - 18 kDa), CII (SDHB - 29 kDa), CIII (UQCRC2 - 48 kDa), CIV (COX II - 22 kDa) and CV (ATP5A - 54 kDa). Results were normalized by β-actin and represented as percentage of the control. Data are expressed as mean ± SEM. Statistical analysis was calculated by using unpaired t-test.

3.4. P-cadherin overexpression does not affect mitochondrial membrane potential

Possible changes in mitochondrial network and mitochondrial membrane potential were evaluated using TMRM probe. TMRM accumulates in active mitochondria depending on the mitochondrial membrane potential, hence the intensity of TMRM probe reflects the level of mitochondrial membrane potential. Using epifluorescence microscopy, no observable differences in mitochondrial network, that can be presented from filamentous tube-like structures to more fringed punctuated ones, were detected in MCF-7/AZ cells, as can be observed when comparing images of MCF-7/AZ.Mock cells (Figure 11A) with MCF-7/AZ.Pcad cells (Figure 11B). Regarding the mitochondrial membrane potential, P-cadherin expression levels did not alter this parameter but shows a tendency to decrease ($p = 0.0737$, Figure 11C). No differences in mitochondrial network were found either between control cells and BT20 siRNA CDH3 cells, with low P-cadherin levels (Figure 12A). Cells also maintained mitochondrial membrane potential after P-cadherin silencing, showing no significant differences ($p = 0.3420$, Figure 12B).

The absence of differences indicates that the P-cadherin protein, most likely, does not induce changes in the mitochondrial membrane potential of those cancer cells

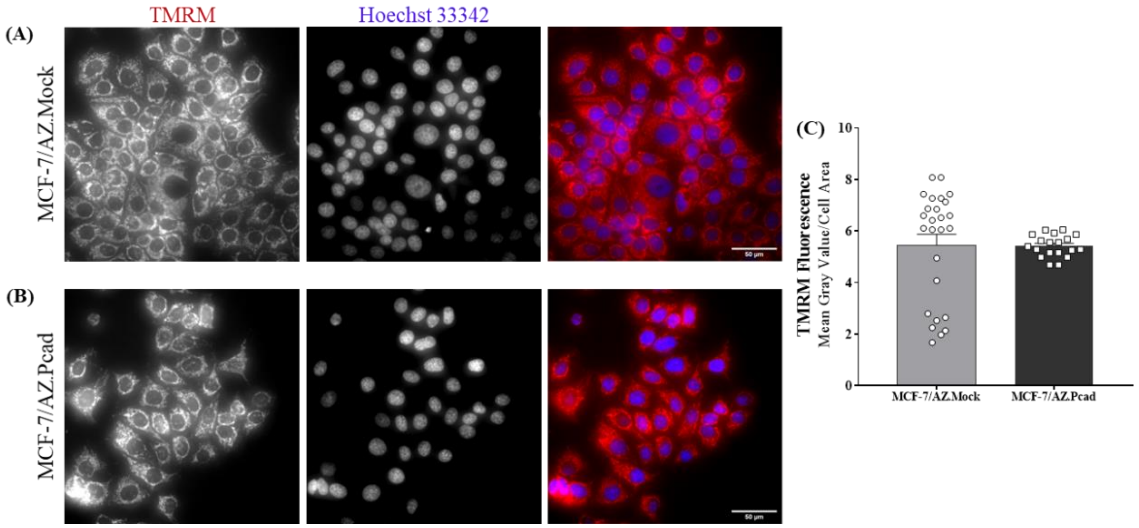

Figure 11 – Mitochondrial potential evaluation in MCF-7/AZ cells.

(A) Representative epifluorescence images of MCF-7/AZ.Mock and **(B)** MCF-7/AZ.Pcad cells, labelled with 50mM TMRM (red) and 1 µg/mL Hoechst 33342 (blue). Images were obtained using 40x objective. **(C)** Data are expressed as mean ± SEM of 3 independent experiments. Five to seven images were taken in each experiment. Average fluorescence of individual cells, mean gray value, was divided by the correspondent cell area (area of selection in square pixels), using ImageJ 1.52p software (USA). Statistical analysis was calculated using nonparametric Mann Whitney test.

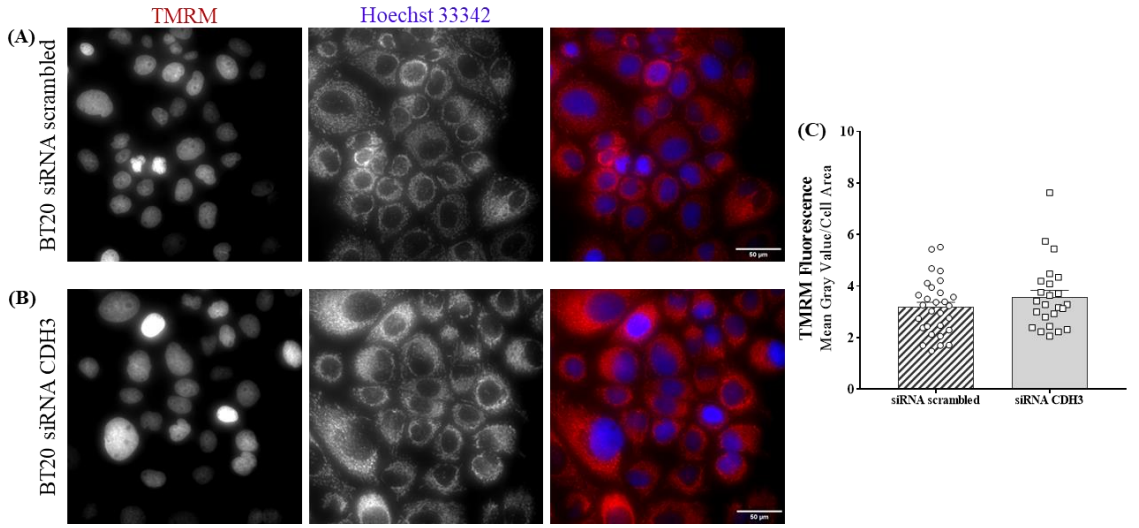

Figure 12 – Mitochondrial potential evaluation in BT20 cells, after P-cadherin silencing.

(A) Representative epifluorescence images of BT20 siRNA scrambled and **(B)** BT20 siRNA CDH3 cells, labelled with 50mM TMRM (red) and 1 µg/mL Hoechst 33342 (blue). Images were obtained using 40x objective. **(C)** Five to seven images were taken in each experiment, 72 hours after silencing. Average fluorescence of individual cells, mean gray value, was divided by the correspondent cell area (area of selection in square pixels), using ImageJ 1.52p software (USA). Statistical analysis was calculated using nonparametric Mann Whitney test.

3.5. Alterations in P-cadherin levels do not lead to mitochondrial remodelling

Taking in consideration that there may be metabolic changes associated with P-cadherin overexpression, we decided to evaluate the expression levels of mitochondrial biogenesis markers. These markers were analysed by western blotting and the mtDNA copy number was verified by qRT-PCR. P-cadherin overexpression, did not induced significant differences in TFAM expression levels (p = 0.8059, Figure 13A). In the case of PGC- 1α levels, there were also no differences with higher expression of P-cadherin (p = 0.2027, Figure 13B). Regarding mtDNA copy number, no differences were detected with higher levels of P-cadherin (p = 0.6782, Figure 13D). Mitochondrial content was also evaluated by assessing TOM20 protein expression in MCF-7/AZ.Pcad cells *versus* control MCF-7/AZ.Mock cells and no differences were found (p = 0.9113, Figure 13C). In BT20 cells, the levels of TFAM tended to decrease in cells where P-cadherin was silenced (Figure 14A), but without significant differences (p = 0.2855). PGC- 1α levels were also evaluated but without being observed differences in its expression with P-cadherin silencing (p = 0.7388, Figure 14B). mtDNA copy number was to evaluated by qRT-PCR (Figure 14C), and there were no statistical differences (p = 0.5747). These results appear to demonstrate that P-cadherin overexpression does not alter mitochondrial biology, in particular the mitochondrial biogenesis process.

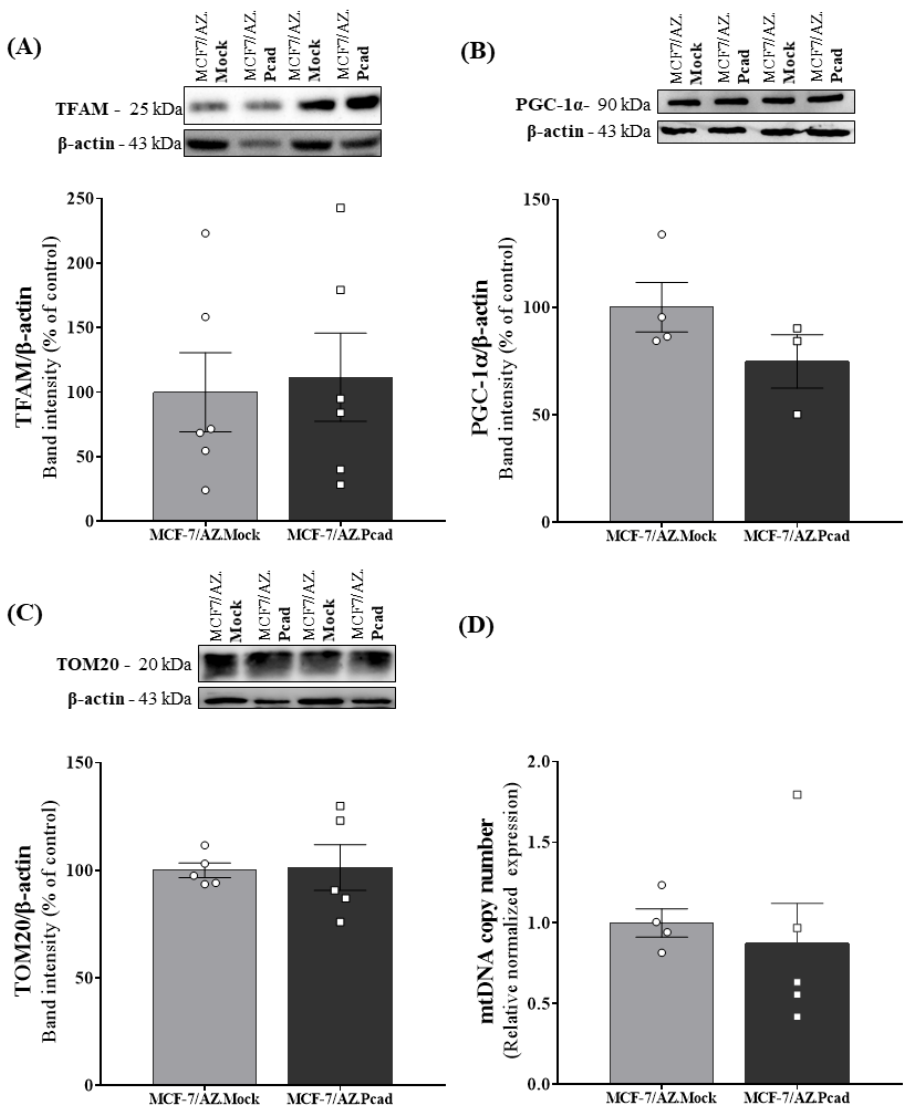

Figure 13 - Western blotting analysis of protein expression of proteins related to mitochondrial biogenesis and mtDNA copy number in MCF-7/AZ cells.

(A) TFAM expression levels quantification in MCF-7/AZ.Mock and MCF-7/AZ.Pcad cell line of 6 independent experiments; a representative blot image is also presented (n = 2). **(B)** PGC-1α expression levels quantification in MCF-7/AZ.Mock and MCF-7/AZ.Pcad cell line of 4 independent experiments; a representative blot image is also presented (n = 2). **(C)** TOM20 expression levels quantification in MCF-7/AZ.Mock and MCF-7/AZ.Pcad cell line of 5 independent experiments; a representative blot image is also presented (n = 2). **(D)** mtDNA copy number after P-cadherin overexpression. mtDNA copy number was measured by quantifying CYTB expression, normalized to B2M nuclear gene expression, relative to control. MCF-7/AZ.Mock cells were considered as control. Results were normalized by β-actin and represented as percentage of the control. Data are expressed as mean ± SEM. Statistical analysis was calculated by using unpaired t-test.

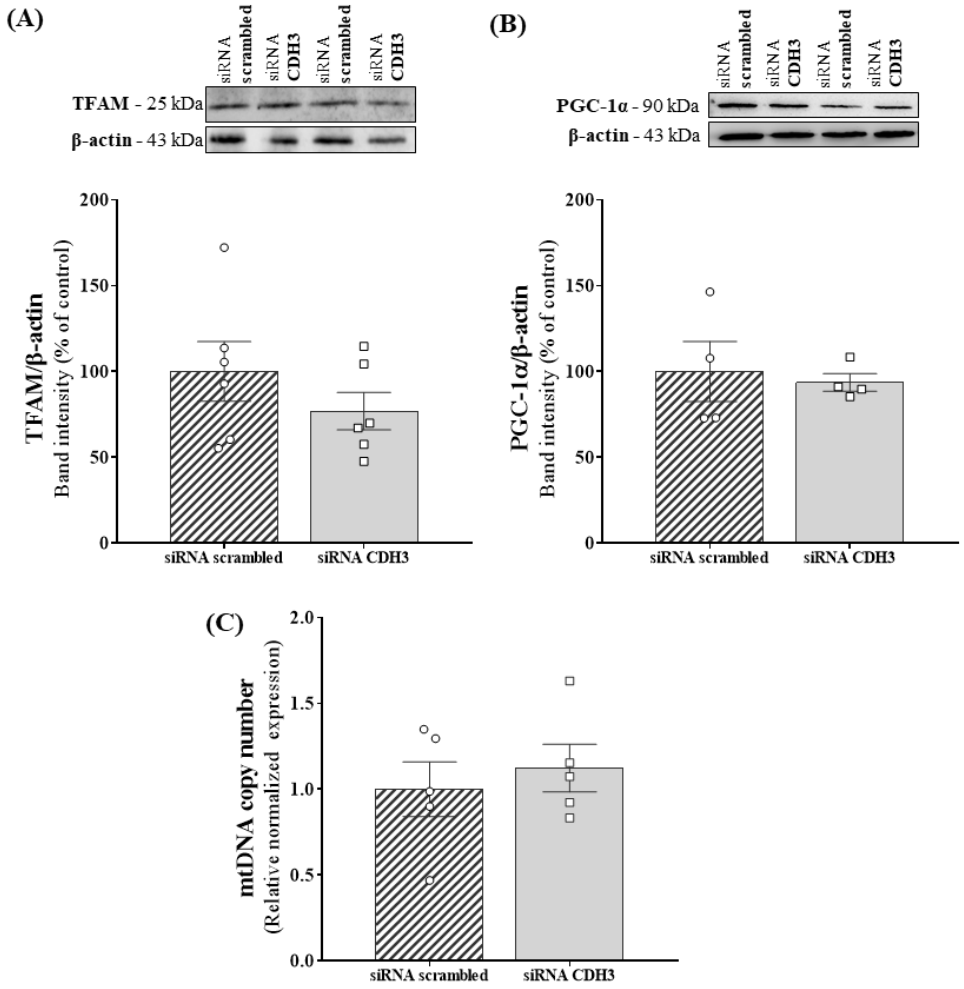

Figure 14 - Western blotting analysis of protein expression of proteins related to mitochondrial biogenesis and mtDNA copy number in BT20 cells.

(A) TFAM expression levels quantification in BT20 siRNA scrambled and BT20 siRNA CDH3 cell line, 48 hours post transfection, of 6 independent experiments; a representative blot image is also presented (n = 2). **(B)** PGC-1α expression levels quantification in BT20 siRNA scrambled and BT20 siRNA CDH3 cell line, 48 hours post transfection, of 4 independent experiments; a representative blot image is also presented (n = 2). **(C)** mtDNA copy number after P-cadherin silencing. mtDNA copy number was measured by quantifying CYTB expression, normalized to B2M nuclear gene expression, relative to control. BT20 siRNA scrambled cells were considered as control. Results were normalized by β-actin and represented as percentage of the control. Data are expressed as mean ± SEM. Statistical analysis was calculated by using unpaired t-test.

3.6. Higher levels of P-cadherin may lead to a more fragmented mitochondrial network

Expression levels of proteins related with mitochondrial dynamics namely fusion and fission events were determined by western blotting technique. In MCF-7/AZ cells, when higher levels of P-cadherin are present, fusion related protein mitofusin 1 (MFN1) levels showed a tendency to decrease of 39.61 % (Figure 15A), although with no statistical differences (p = 0.2000). On the other hand, mitochondrial fission-related protein, dynamin related protein 1 (DRP1) (Figure 15C), did not present statistical differences (p = 0.5912). Correspondingly, mitochondrial fission 1 protein (FIS1) levels (Figure 15B), also showed no differences (p = 0.2513). In BT20 cells, MFN1 levels tended to increase after P-cadherin silencing (p = 0.23605, Figure 16A), although no differences were detected in another fusion related protein, optic atrophy gene 1 (OPA1) (p = 0.6738, Figure 16B). Just as in MCF-7/AZ cells, in BT20 cells fission related protein DRP1 levels showed no differences with altered P-cadherin levels (p = 0.6456, Figure 16C). These results, although not statistically significant, may indicate that when P-cadherin is present at high levels, mitochondrial fusion processes are less active, even though there are no differences in proteins related to fission processes. This imbalance in mitochondrial dynamics, may lead to a more fragmented mitochondrial network. Therefore, can somehow support the differences observed in the ATP measurements, at least, in the MCF-7/AZ cells

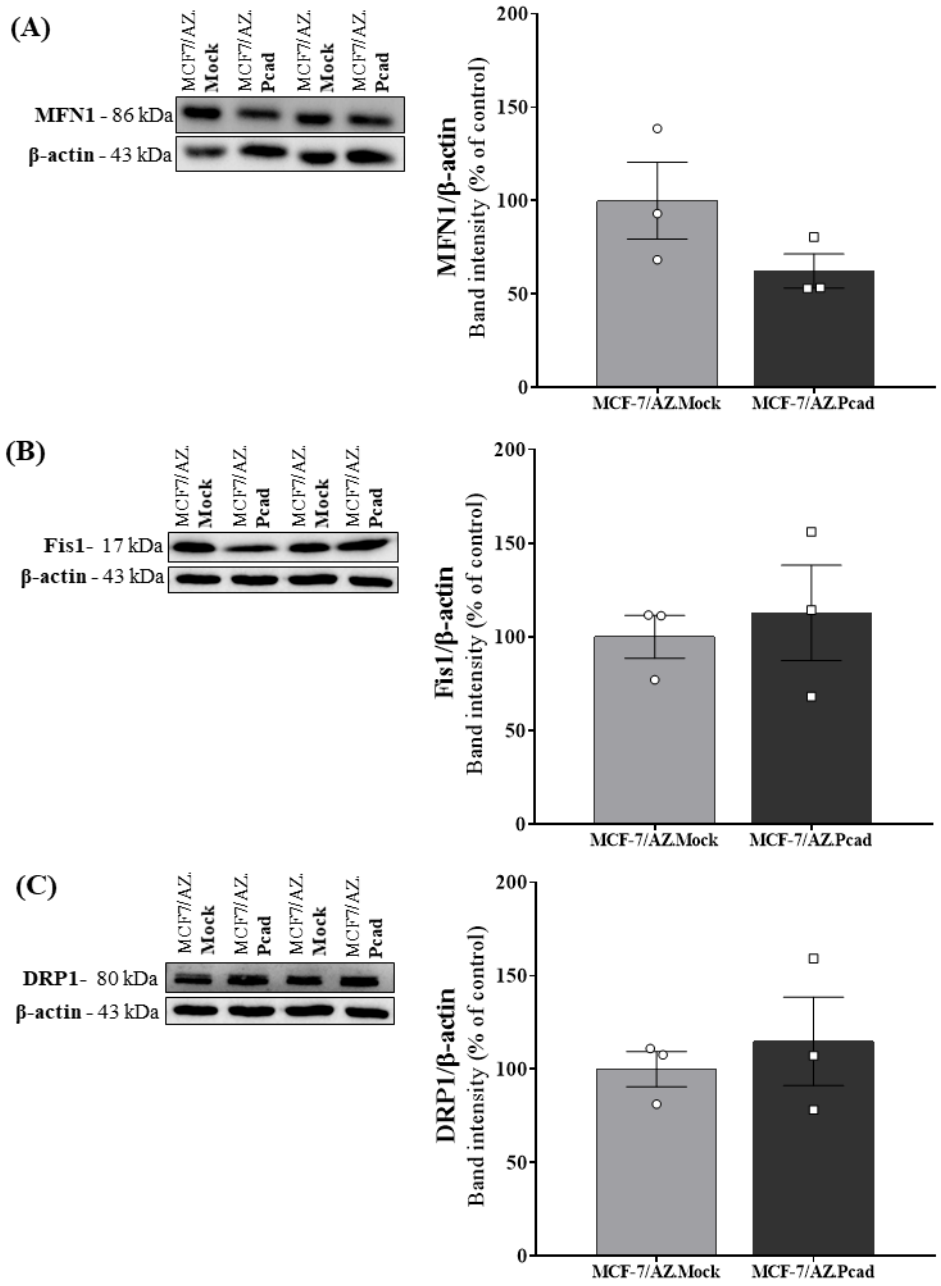

Figure 15 - Western blotting analysis of protein expression of proteins related to mitochondrial dynamics in MCF-7/AZ cells.

(A) MFN1 expression levels quantification in MCF-7/AZ.Mock and MCF-7/AZ.Pcad cell line of 3 independent experiments; a representative blot image is also presented (n = 2). **(B)** Fis1 expression levels quantification in MCF-7/AZ.Mock and MCF-7/AZ.Pcad cell line of 3 independent experiments; a representative blot image is also presented (n = 2). **(C)** DRP1 expression levels quantification in MCF-7/AZ.Mock and MCF-7/AZ.Pcad cell line of 3 independent experiments; a representative blot image is also presented (n = 2). MCF-7/AZ.Mock cells were considered as control. Results were normalized by β-actin and represented as percentage of the control. Data are expressed as mean ± SEM. Statistical analysis was calculated by using unpaired t-test.

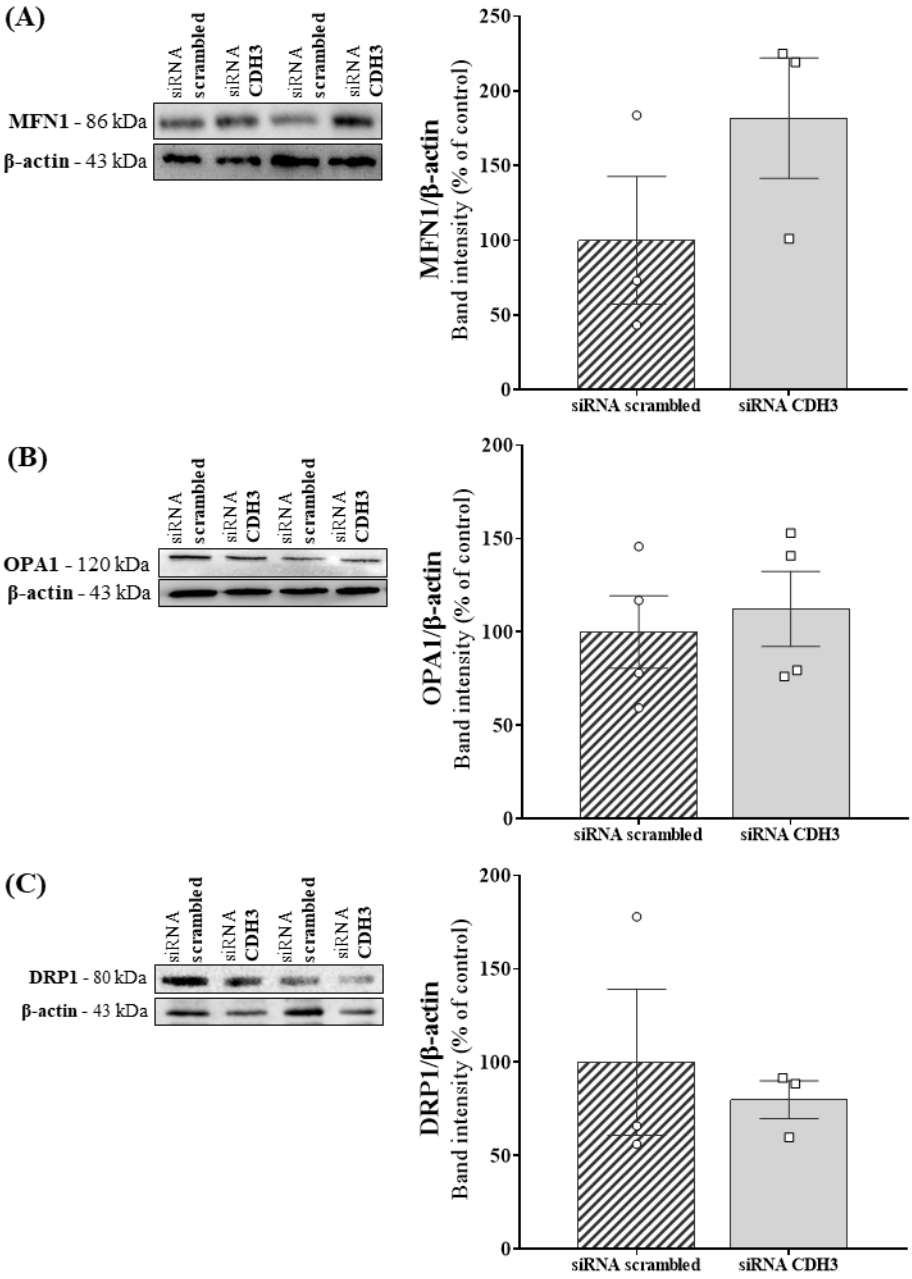

Figure 16 - Western blotting analysis of protein expression of proteins related to mitochondrial dynamics in BT20 cells.

(A) MFN1 expression levels quantification in BT20 siRNA scrambled and BT20 siRNA CDH3 cell line, 48 hours post transfection, of 3 independent experiments; a representative blot image is also presented (n = 2). **(B)** OPA1 expression levels quantification in BT20 siRNA scrambled and BT20 siRNA CDH3 cell line, 48 hours post transfection, of 4 independent experiments; a representative blot image is also presented (n = 2). **(C)** DRP1 expression levels quantification in BT20 siRNA scrambled and BT20 siRNA CDH3 cell line, 48 hours post transfection, of 3 independent experiments; a representative blot image is also presented (n = 2). BT20 siRNA scrambled cells were considered as control. Results were normalized by β-actin and represented as percentage of the control. Data are expressed as mean ± SEM. Statistical analysis was calculated by using unpaired t-test.

Chapter 4

DISCUSSION

Breast cancer is one of the most common cancers worldwide, being the most common carcinoma in women. It can develop from any cell type of the mammary gland and can develop slowly and with good prognosis, as well as it can be a very aggressive and difficult tumour to treat. Thus, given tumours high cell heterogeneity composition, plasticity and mutation capacity, there is a need find novel approaches to improve patients survival (Albergaria et al. 2011; Haque et al. 2012; Varum et al. 2011). Therefore, it is imperative to find molecular markers that may be a therapeutic target for the treatment of these mammary carcinomas. P-cadherin overexpression has been associated with more aggressive tumours and higher metastasis and invasion capacity (Paredes et al. 2005). This adhesion protein has been detected at high abnormal levels in breast cancer cells, such as triple negative breast cancers that are the most aggressive and difficult type of tumours to treat. Tumours have regions in which glycolysis is predominant, usually associated with hypoxia niches, displaying the Warburg effect and characteristics associated with cancer stem cells (Cannino et al. 2018; C. L. Chen et al. 2010; Sousa et al. 2014). P-cadherin overexpression has been associated with glycolytic markers, such as GLUT1 and CAIX, and has the ability to model its expression (Sousa et al. 2014). In cells representative of basal tumours, P-cadherin silencing was shown to induce a reduction in expression of these markers, indicating the involvement of this adhesion protein in the metabolic reprogramming of cancer cells (Sousa et al. 2014). These markers are related to tumour changes that make it more acquire a more aggressive phenotype, which are related to a glycolytic phenotype (Cannino et al. 2018; C. L. Chen et al. 2010). As mitochondria is a fundamental organelle in cellular metabolism, this study aimed to evaluate changes in mitochondrial biology of breast cancer cells with different levels of P-cadherin.

In order to uncover the effects of P-Cadherin, we used the luminal-like and endocrine responsive breast MCF-7/AZ cell line, previously stable transfected with the gene, to obtain a low and high P-cadherin expression lines, respectively, MCF-7/AZ.Mock and MCF-7/AZ.Pcad cell line (Figure 8A) (Ribeiro et al. 2010). It was essential to evaluate the effects of P-cadherin overexpression on a responsive hormone cell line, commonly used as a more initial stage of breast cancer, and to understand if P-cadherin expression could potentiate the aggressiveness of these cells and alter their metabolic features (Joana Paredes et al. 2004). On the other hand, the BT20 cell line has high levels of P-cadherin and represents triple-negative basal-like tumours, being ideal for evaluating the effects of P-cadherin silencing (siRNA CDH3) in an aggressive model of cancer cells (Figure 8B) (Ribeiro et al. 2010).

The metabolic phenotype of breast cancer cells was evaluated in order to assess the main source of ATP production (Figure 9). MCF-7/AZ.Pcad cells, with high levels of P-

cadherin, apparently had a more glycolytic metabolism, seen by a decrease in ATP production due to the inhibition of 2-DG. This is in accordance to the described in the literature. In the case of oligomycin inhibition, it was also possible to verify differences when overexpressing P-cadherin in MCF-7/AZ cells. However, this is only true because there is an increase in ATP levels in MCF-7/AZ.Mock cells incubated with oligomycin, when compared to those same cells incubated with the vehicle. This increase was quite unexpected because, since ATP synthase is inhibited by oligomycin, there should be no increase in ATP levels.

In the case of BT20 cells, when P-cadherin was silenced, the greatest change in ATP levels was observed with oligomycin inhibition. These results seem to show that, with low levels of P-cadherin, BT20 siRNA CDH3 basal tumour cells showed a preferentially more oxidative metabolism. Regarding 2-DG inhibition, both BT20 siRNA scrambled and BT20 siRNA CDH3 cell show similar levels of ATP and are equally affected by blocking ATP production through the glycolytic pathway.

Taken these results together, we found that high levels of P-cadherin in MCF-7/AZ cells, seems to have an exacerbated dependence on glycolytic metabolism to obtain ATP. On the other hand, in BT20 cells, P-cadherin levels does not seem to influence glycolysis, but its downregulation may turn these cells towards a more OXPHOS metabolism. Changes in the metabolic behaviour may indicate a possible role of P-cadherin in the metabolic shift of breast cancer cells.

Metabolic reprogramming to a more glycolytic metabolism is essential to sustain the anabolic needs of growing and proliferating cancer cells (Ward and Thompson 2012). To understand whether this change led to a change in OXPHOS complexes, protein levels of oxidative chain subunits were measured (Figure 10). In MCF-7/AZ cells, there was no decrease in protein expression of the OXPHOS complexes to accompany glycolytic phenotype in cells with higher P-cadherin expression. In the case of BT20 cells, as there was no clear P-cadherin influence on the preferred source of ATP production, we were no able either to find differences in the expression levels of the oxidative chain complexes.

However, it is important to note that in both experiments, to identify the main source used for ATP production and to check the expression levels of OXPHOS complexes, only 3 experiments were performed, except for the measurement of BT20 cell complex levels, in that 5 experiments were performed. So, we can only take these data as preliminary results and more experiments to draw more sustained conclusions. Nonetheless, these results are in line with recent findings that state that the metabolic switch of cancer cells is not due to dysfunctional mitochondria, but rather to a calculated alteration of cancer cells to keep up with anabolic cell needs (Ward and Thompson 2012). Nevertheless, these results do not show

the functioning of the chain complexes. For this, the activity of each mitochondrial complex should be analysed as well as measuring oxygen consumption rate in both cell line models in future work is highly need. Tis way, it cannot be verified whether in fact overexpression of P-cadherin does effectively alter the capacity of the mitochondrial oxidative chain.

Along with OXPHOS complexes, a very important point for checking the mitochondrial function of P-cadherin effect on cancer cells is the measurement of membrane potential. The mitochondrial membrane potential is generated and maintained by complexes I, II and IV of the electron transport chain, by pumping protons to the mitochondrial intermembrane space. The membrane potential is essential for ATP synthesis through oxidative phosphorylation and levels are kept stable in normal conditions (Twig, Las, and Shirihai 2013; Zorova et al. 2018). Using a potential-dependent fluorescent dye, tetramethylrhodamine methyl ester (TMRM) (Scaduto and Grotyohann 1999), no statistical differences in mitochondrial membrane potential were detected, although there was a slight tendency to decrease when P-cadherin was overexpressed in MCF-7/AZ cells comparatively to control (Figure 11). No changes in membrane potential were detected either in BT20 cells (Figure 12). The above-mentioned results indicate that P-cadherin does not influence mitochondrial oxidative capacity. These results are consistent with previous studies showing that while cancer cells produce much of their ATP through glycolysis, mitochondria remain functional, maintaining their membrane potential (Maldonado et al. 2010).

Noticeably relevant for assessing changes in mitochondrial biology is the understanding how P-cadherin potentially influences mitochondrial biogenesis. To this end, protein levels of PGC-1α, essential for coordination of mitochondrial metabolism and biogenesis, TFAM expression levels, regulated by PGC-1α, and TOM20 protein levels, which is a marker of mitochondrial mass (Gabrielson et al. 2014; Lagory et al. 2015; Vega-Naredo et al. 2014) were measured. In MCF-7/AZ cells and BT20 cells, P-cadherin expression did not promote significant differences in PGC-1α neither TFAM expression levels (Figure 13 and Figure 14). So, P-cadherin does not appear to alter mitochondrial biogenesis. Mitochondrial mass marker TOM20 marker also didn't show changes in expression levels in MCF-7/AZ.Pcad cells (Figure 13), demonstrating that mitochondrial mass remains at constant levels even with P-cadherin overexpression. Unfortunately, and after many attempts of reaching a promising result, we could not analyse the protein expression levels corresponding to TOM20 in BT20 cells, and, consequently, we could not assess the mitochondrial mass alterations in this experimental condition. Since mtDNA replication and condensation is controlled by TFAM, we have evaluated mtDNA copy number. In both MCF-7/AZ and BT20 cells, there were no differences in mtDNA copy number with P-cadherin

overexpression. In fact, a decrease in mtDNA copy number has already been found in several cases of breast cancer compared to healthy tissue. This decrease in mtDNA content has already been associated with increased aggressiveness, invasion, progression and metastatic capacity of cancer cells (Fan et al. 2009; Mambo et al. 2005; Reznik et al. 2016). Although we did not find any differences in mtDNA copy number upon P-cadherin overexpression, there may be some relationship between this cell adhesion protein and the aggressiveness of tumours in which it is overexpressed.

Also, particularly important in mitochondrial biological processes is mitochondrial dynamics. Mitochondrial network organization and alteration allows cells to adapt to stress conditions (Senft and Ronai 2016). For this we evaluated the levels of proteins involved in the processes of mitochondrial fusion and fission. In the two cell lines with P-cadherin overexpression, MCF-7/AZ.Pcad and BT20 siRNA scrambled, MFN1 protein levels tended to decrease (Figure 15 and Figure 16). Regarding OPA1 expression in BT20 cell line, no significant differences were found with P-cadherin overexpression. These results seem to indicate that there is a tendency for mitochondrial fusion processes to decrease when P-cadherin is overexpressed. In the case of mitochondrial fission-related proteins, no significant differences were found in their expression levels upon P-cadherin overexpression. Still, it is possible to detect a slight upward trend in DRP1 and FIS1 for MCF-7/AZ.Pcad (Figure 15), and increased DRP1 in the case of scrambled BT20 siRNA scrambled (Figure 16). These preliminary results, although not significant, are consistent with what is found in more aggressive cancer cells and support, even if partially, our data from ATP measurements. In these cells, the mitochondrial network tends to be more fragmented, with a fusion decrease and increased fission, characterized by a decrease in MFN1 expression and an increase in DRP1 levels. (Cannino et al. 2018; H. Chen and Chan 2017; Senft and Ronai 2016; Vyas, Zaganjor, and Haigis 2016). This pattern of increase in fission and decrease in fusion is verified with P-cadherin overexpression when compared to cells with low levels of P-cadherin. Thus, P-cadherin may be involved in some way, directly or indirectly, in mitochondrial dynamics processes.

Chapter 5

CONCLUSION AND FUTURE PERSPECTIVES

It has been found that P-cadherin may be involved in the metabolic switch to a more glycolytic phenotype of breast cancer cells, which may be related with its aggressiveness. More specifically, P-cadherin appears to turn MCF-7/AZ cells more dependent on glycolysis to obtain ATP, while and oppositely to is later results no differences were observed in BT20 cells. However, silencing of this adhesion protein influences oxidative phosphorylation as this BT20 cell line seems to have a tendency rely more on mitochondria. There was no evidence of the role of P-cadherin in modelling the expression of oxidative chain subunits or in altering mitochondrial membrane potential, and so it was not possible to confirm if and how P-cadherin loss can eventually affect mitochondrial oxidative capacity. Nonetheless, future trials to prove these results are needed. It would be very important to evaluate the activity of the complexes, as well as only their expression levels. Together, and in order to better understand whether P-cadherin has an influence on mitochondrial capacity, it would also be advantageous to measure mitochondrial respiration. By measuring the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) we could obtain data that would allow us to better understand P-cadherin effect on mitochondrial function.

Considering the expression of mitochondrial biogenesis markers, no differences were detected, showing that P-cadherin does not appear to influence these processes. Similarly, no changes in mtDNA copy number were detected, and there was a slight downward trend for P-cadherin overexpression. These results may show a relationship between aggressiveness of tumours with high P-cadherin expression and aggressiveness of tumours with fewer copies of mtDNA.

A potential influence of P-cadherin on mitochondrial fusion and fission processes was found. Strong trends were found to decrease fusion processes upon P-cadherin overexpression in both MCF-7/AZ and BT20 cells. Although fluorescence microscopy has not detected any visible differences in mitochondrial network architecture, it can be directly or indirectly altered by P-cadherin overexpression. Given the small number of experiments for each protein, more experiments would be important to increase the statistical robustness. In addition, it is also crucial to evaluate the expression of other mitochondrial dynamics-related proteins, such as MFN 2, in order to have a more robust characterization of mitochondrial dynamics.

In the future it would also be very important to repeat all these experiments under more physiological conditions, the next step being to use 3 dimensional models, using mammospheres. The principle behind the formation of mammospheres is that, since cells are plated under non-adherent conditions, only stem cells are able to survive and multiply to form mammospheres. (Albergaria et al. 2011; Shaw et al. 2012; Vieira et al. 2012). These stem

cells, CSCs, which are part of the heterogeneous tumour cell population, are the most aggressive because they survive treatments and promote breast tumour metastases and relapse. In addition, CSCs were related to P-cadherin overexpression, which further underlines the importance of conducting this more physiological study.

Thus, P-cadherin may be influencing processes related to biology and mitochondrial function, but we cannot definitively conclude this influence only with these results. Future studies that may demonstrate the mechanism of action of P-cadherin on metabolic and mitochondrial changes in breast cancer cells are needed to confirm whether this cell adhesion protein is indeed capable of altering mitochondrial biology and cells metabolism to favour the ability invasive and metastatic cell populations of breast cancer. If confirmed, P-cadherin could be looked as an interestingly target with high therapeutic potential due to its association with aggressive tumours and influence in cancer cell metabolism remodelling.

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