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Targeting Anoikis-resistant P-cadherin-enriched Breast Cancer Cells by *in vitro* Metabolic Reprograming

Joana Sousa Pereira

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Dissertação de Candidatura ao grau de Mestre em Oncologia – Especialização em Oncologia Molecular submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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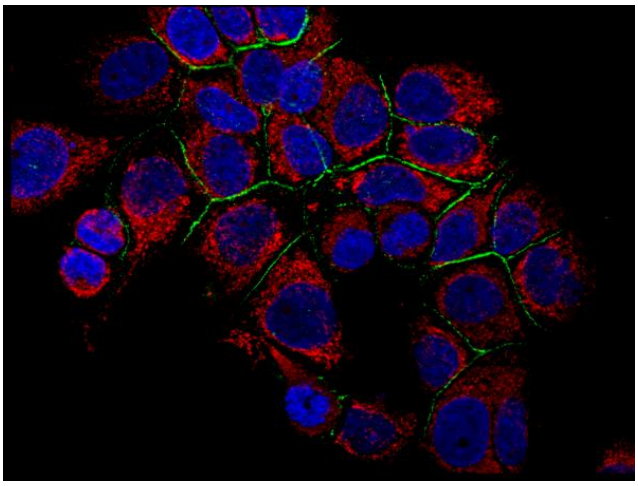

"*Success consists of going from failure to failure without loss of enthusiasm*."

Winston Churchill

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Chegando a esta fase, não podia de deixar de agradecer a quem me acompanhou e ajudou durante o percurso deste último ano. Desde já peço desculpa, se por alguma razão, me esquecer de mencionar alguém.

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ABBREVIATION LIST

ADP: Adenosine diphosphate

ALDH: Aldehyde dehydrogenase

BLBC: Basal-like breast cancer

CAIX: Carbonic anhydrase IX

CAT: Catalase

CCLE: Cancer cell line encyclopedia

CDH1: Cadherin 1 or E-cadherin gene

CDH3: Cadherin 3 or P-cadherin gene

CK: Cytokeratin

CoA: Coenzyme A

DAPI: 4,6-diamidine-2-phenylindolendihydrochrolide

DCA: Dichloroacetate

DCIS: Ductal carcinoma *in situ*

2-DG: 2-deoxyglucose

E-cadherin: Epithelial cadherin

ECM: Extracellular matrix

EMT: Epithelial mesenchymal transition

ER: Estrogen receptor

FACS: Fluorescence-activated cell sorting

GFPT: Glutamine-fructose-6-phosphate transaminase

GLUT: Glucose transporter

G6PD: Glucose-6-phosphate dehydrogenase

GPx: Glutathione peroxidase

HER2: Human epidermal growth factor receptor 2

hESC: Human embryonic stem cell

HSP70: 70 kilodalton heat shock protein

LDH: Lactate dehydrogenase

MCT: Monocarboxylate transporter

MFE: Mammosphere forming efficiency

mRNA: Messenger ribonucleic acid

N-cadherin: Neural cadherin

P-cadherin: Placental cadherin

PDC: Pyruvate dehydrogenase complex

PDH: Pyruvate dehydrogenase

PDK: Pyruvate dehydrogenase kinase

PDP: Pyruvate dehydrogenase phosphatase

PgR: Progesterone receptor

PKM2: Pyruvate kinase muscle enzyme 2

PPP: Pentose phosphate pathway

R-cadherin: Retinal cadherin

ROS: Reactive oxygen species

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

siRNA: Small interfering ribonucleic acid

SOD: Superoxide dismutase

TCA: Tricarboxylic acid

TCGA: The cancer genome atlas

TNBC: Triple-negative breast cancer

tPDH: Total pyruvate dehydrogenase

ABSTRACT

P-cadherin is a cell-cell adhesion molecule and an important mediator of the aggressive behavior and metastatic potential of breast cancer cells. This molecule is also a well-established indicator of poor patient prognosis in breast cancer. Importantly, P-cadherin expression promotes stem-like properties to breast cancer cells, such as tumorigenic capacity and *anoikis* resistance, being recognized as a breast cancer stem cell (BCSC) marker.

BCSCs are known to exhibit pro-glycolytic metabolic skills, allowing them to decrease oxidative stress, escape *anoikis*, survive in circulation and increase metastasis formation. Disturbing this survival skill by metabolic reprograming would target these properties and impact the efficacy of cancer treatment. Recently, we have demonstrated that P-cadherin aberrant expression is associated with hypoxic, glycolytic and acidosis markers in breast carcinomas, that hypoxia-inducible factor 1 alpha (HIF-1α) stabilization increases membrane P-cadherin expression and that P-cadherin enriched cell populations show increased glucose transporter 1 (GLUT1) and carbonic anhydrase IX (CAIX) expression, as well as high mammosphere forming efficiency. Moreover, preliminary data from the group points for the hypothesis that aberrant P-cadherin expression might have a role in cellular metabolic reprograming of BCSCs, acting as an antioxidant and enhancing cell survival in circulation by promoting *anoikis*-resistance.

Thus, the main aim of this work was to evaluate the role of P-cadherin in dichloroacetate (DCA) induced metabolic reprograming, a pyruvate dehydrogenase kinase (PDK) inhibitor, which promotes the shift from glycolysis to oxidative phosphorylation (OXPHOS). Using a panel of human breast cancer cell lines, we demonstrated that P-cadherin-enriched breast cancer cell lines are more sensitive to DCA. Interestingly, we also observed that P-cadherin expression modulates the levels of phosphor pyruvate dehydrogenase (pPDH), the inactive form of PDH. On the other hand, DCA also decreased the expression of P-cadherin, probably by its effect in pPDH. Interestingly, we demonstrate that treatment with DCA, decreases the survival of breast cancer cells, mainly in P-cadherin enriched breast cancer cells, being this effect more pronounced in anchorage-independent conditions. Finally, P-cadherin downregulation induces an increase of mitochondrial reactive oxygen species (ROS) production in triple-negative basal-like breast cancer (TN-BLBC) cells, probably being responsible for survival role attributed to P-cadherin in breast cancer cells.

Taking together, our results indicate that P-cadherin enrichment dictates the sensitivity of breast cancer cells to DCA-induced metabolic reprogramming, through its role in the modulation of pPDH expression in breast cancer cells. Thus, we suggest that P-cadherin

might be a valuable biomarker to predict the response to DCA treatment in breast cancer patients.

RESUMO

A caderina-P é uma molécula de adesão célula-célula e um importante mediador do comportamento agressivo e do potencial metastático das células de cancro da mama. Esta proteína é também um indicador de mau prognóstico destes tumores. A sua expressão promove propriedades estaminais, tais como capacidade tumorigénica e resistência a *anoikis*, sendo reconhecida como um marcador de células estaminais de cancro da mama.

As células estaminais de cancro da mama são conhecidas por apresentarem propriedades metabólicas que lhes permitem diminuir o stress oxidativo, escapar à *anoikis*, sobreviver em circulação e aumentar a formação de metástases. Assim, destabilizar estas capacidades de sobrevivência através da reprogramação metabólica pode aumentar a eficácia do tratamento do cancro. Recentemente, o nosso grupo demonstrou que a expressão aberrante de caderina-P está associada a marcadores de hipoxia, glicólise e de resistência a acidose, em carcinomas da mama. Mostramos ainda que a estabilização do HIF-1α aumenta a expressão membranar de caderina-P e que populações de células enriquecidas em caderina-P apresentam uma expressão aumentada de GLUT-1 e CAIX, bem como uma elevada capacidade de formação de mamosferas. Para além disto, resultados preliminares apontam para a hipótese de que esta molécula tem um papel na reprogramação metabólica das células estaminais do cancro da mama, atuando como um antioxidante e aumentando a sobrevivência celular em circulação através da promoção da resistência à *anoikis*.

Assim, o principal objetivo deste trabalho foi avaliar o papel da caderina-P na reprogramação metabólica induzida por dicloroacetato (DCA), um inibidor da piruvato desidrogenase cinase (PDK), que promove a alteração da glicólise para a fosforilação oxidativa. Os nossos resultados mostram, pela primeira vez, que as células enriquecidas em caderina-P são mais sensíveis ao DCA e que esta proteína é responsável pela modulação dos níveis de pPDH, a forma inativa da piruvato desidrogenase (PDH). Por outro lado, observamos também que o DCA diminui a expressão da caderina-P, provavelmente devido ao seu efeito na pPDH, e ainda que o tratamento com este composto diminui a sobrevivência das células preferencialmente enriquecidas em caderina-P, sendo este efeito mais pronunciado em condições independentes de ancoragem. Finalmente, o silenciamento da caderina-P induz um aumento da produção de espécies reativas de oxigénio (ROS) mitocondriais, sendo este provavelmente responsável pelo papel de sobrevivência atribuído à caderina-P em células de cancro da mama.

Em suma, este trabalho sugere que a expressão de caderina-P pode ser um biomarcador da sensibilidade à reprogramação metabólica induzida por DCA, em doentes com cancro da mama.

CHAPTER I

INTRODUCTION

1. CANCER METABOLISM

In the last decade, the interest in cancer metabolism has been highly increasing. Oncogenic alterations and the tumor microenvironment were found to contribute for the acquisition of distinct metabolic cell behaviors. With all the progress in this subject, the cellular reprograming of energy metabolism was recognized as a new hallmark of cancer cells [1].

1.1. WARBURG EFFECT

Glucose is the major macronutrient that allows energy generation for cellular processes through the oxidation of its carbon bonds [2]. Normal cells, in the presence of oxygen, rely mainly on mitochondrial oxidative phosphorylation (OXPHOS), in which glucose is metabolized into carbon dioxide (CO_2) and water by glycolytic pyruvate oxidation in the mitochondrial tricarboxylic acid (TCA) cycle [3]. In this process, oxygen is the final acceptor of electrons, flowing through the mitochondrial electron transport chain, and allowing the end of glucose oxidation and the generation of ATP (adenosine triphosphate) [4]. In anaerobic conditions, normal cells readdress pyruvate away from the mitochondria, producing considerable amounts of lactate and lower levels of energy [5].

However, it has been shown that, in a cancer context, cells produce considerable large amounts of lactate, even in the presence of oxygen, being their metabolism frequently referred as “aerobic glycolysis” or “Warburg effect”. This effect was described by Otto Warburg in the 1920s, where he hypothesized that cancer cells develop a defect in mitochondria leading to an impaired aerobic respiration and a subsequent reliance on glycolytic metabolism in order to provide energy [6, 7]. Nevertheless, successive work showed a normal mitochondrial function in most cancer cells, suggesting an alternative explanation for aerobic glycolysis in these cells [8].

Aerobic glycolysis is a less efficient process for ATP production in comparison to mitochondrial OXPHOS (2 ATP molecules instead of 36 by TCA cycle), which raises the question why do cancer cells perform this metabolic shift towards glycolysis. Several explanations fit perfectly to answer this question: 1) lactate production from glucose is faster than its complete oxidation in the mitochondria, so the fast production of energy can be rapidly tuned to support the demand for ATP synthesis by cancer cells; 2) the

Warburg effect functions as an adaptive mechanism to support the biosynthetic requirements of proliferative cells, in which increased glucose consumption is used as a carbon source for anabolic processes [9]; 3) the Warburg effect is also an advantage for tumor progression, since elevated glucose metabolism decreases the tumor microenvironment pH through lactate and protons secretion, allowing increased invasiveness of the surrounding areas by cancer cells [10, 11]; 4) increased glycolysis and OXPHOS impairment is also important in the modulation of reactive oxygen species (ROS), interfering directly in tumor cell's signaling. The homeostatic balance of ROS is essential for the appropriate functioning of normal cells. Excessive cellular ROS will damage cell membranes, nucleic acids, among others deleterious effects, but insufficient ROS will disrupt signaling processes that will benefit cell proliferation. Therefore, the Warburg effect causes alterations in mitochondrial redox potential and consequently changes in ROS production [2, 12].

In highly proliferative cells, such as cancer cells, ATP and NADH (reduced nicotinamide adenine dinucleotide) are not the only required products, and glycolysis and TCA do not function only for compensating cellular energetic demands. These pathways and their intermediate products are deviated to other molecular pathways, such as pentose phosphate pathway (PPP), hexosamine synthesis and serine/glycine synthesis pathways, in order to provide precursors for the synthesis of building blocks, such as lipids, proteins, DNA (deoxyribonucleic acid) and RNA (ribonucleic acid). Furthermore, PPP activation has been widely demonstrated in several types of cancer and associated with invasion, metastasis, angiogenesis, and resistance to chemo- and radiotherapy [13, 14]. The increase in flux through the PPP generates abundant reductive power in the form of NADPH (reduced nicotinamide adenine dinucleotide phosphate), which in turn allows increased ATP production and lipid synthesis, providing protection against oxidative damage (**Figure 1**). Like glucose, glutamine is also a substrate for tumor and proliferative cells, being an important mitochondrial substrate that is metabolized over glutaminolysis and involved in the protection of cells from oxidant injury, through glutathione and mitochondrial phosphate-activated glutaminase enzyme [15].

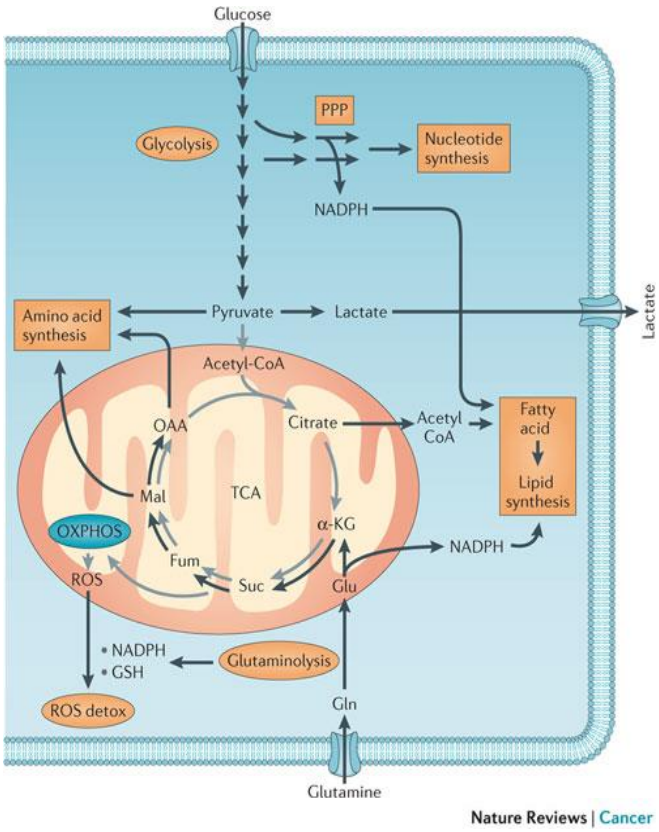

Figure 1. Proliferating cancer cells rely mainly on a glycolytic and glutamine-addicted profile (black arrows and orange boxes), instead of mitochondrial respiration (grey arrows), which is referred to as the Warburg effect and glutaminolysis. Cancer cells use glucose and glutamine as main sources of energy carbon precursors. The carbon flux through glycolysis and glutaminolysis is increased in cancer cells and allows the decrease of reactive oxygen species (ROS) levels, as well as the production of energy and precursor intermediates for feeding the pentose phosphate pathway (PPP) and a truncated tricarboxylic acid (TCA) cycle that further feed carbon intermediates into biosynthesis pathways, such as nucleotide, lipid and amino acid synthesis, that are used for making new cells. Adapted from Deblois G & Giguère V, Nature Reviews Cancer, 2012 [16].

1.2. OXIDATIVE STRESS IN CANCER

Mitochondria is the main intracellular source of ROS in most tissues, either in physiological and pathological conditions. ROS are a highly reactive group of oxygen-containing molecules, such as superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$), and singlet oxygen ($^1O_2$), being generated as metabolic by-products by biological systems [17]. At low or moderate concentrations, ROS function as signaling molecules, being implicated in different biological processes, such as cell adhesion, migration, proliferation, differentiation and survival [18]. An imbalance between the production of ROS and the ability of a biological system to detoxify these reactive products, leads to an excessive accumulation of ROS and, consequently, to cell and tissue damage, being this phenomenon known as oxidative stress [17]. This process can affect negatively several cellular structures, such as membranes, lipids, proteins, lipoproteins, as well as DNA [19]. Thereby, the maintenance of highly regulated mechanisms to control the levels of ROS is essential for normal homeostasis and proper response to environmental stimuli. In this context, cells display an antioxidant defensive system based mainly on enzymatic components, namely superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), in order to protect themselves from ROS-induced cellular damage [20].

Cells that undergo aerobic metabolism are subjected to some degree of oxidative stress [21]. However, undifferentiated cells such as stem cells, by residing in low oxygen tension compartments, maintain slow cycling proliferation, are quiescent, as well as can escape from oxidative stress damage associated with oxygenated tissues [22, 23]. Therefore, hypoxia induces a metabolic shift that diverge glucose metabolites to glycolysis, in order to maintain ATP production and prevent the increase of ROS concentration to a toxic level [24]. Accordingly, it has been described a difference in ROS levels between progenitor cells and their more mature progeny, which seems to be critical for maintaining stem cell function [25]. Thus, in the mammary gland, the luminal and the basal/myoepithelial cell layers, were found to present different ROS levels, being this difference attributed to variances in their mitochondrial content [26]. In this context, normal human basal mammary epithelial cells present low levels of ROS, which seems to be maintained by glutathione-dependent systems, while the matching purified luminal progenitor cells have higher levels of ROS, several glutathione-independent antioxidants and oxidative nucleotide damaging control proteins, and higher rate of oxygen consumption [27]. Furthermore, it is known that luminal progenitor cells are more resistant to glutathione depletion than basal cells, as well as to H_2O_2-induced oxidative stress and ionizing radiation [27]. Interestingly, mammary epithelial cells coordinate their responses to detachment through the increase of the SOD2 antioxidant system, decreasing the production of ROS from mitochondrial oxidation and, therefore, escaping extracellular matrix (ECM)-detachment cell death [28].

Similarly, in cancer cells, the Warburg effect causes alterations in mitochondrial redox potential, and, subsequently, changes the production of ROS, decreasing the concentration of ROS in these cells [12]. Furthermore, it has been widely described in human and murine models, that breast cancer cells presents decreased levels of ROS and high antioxidant defenses [26, 29], which besides being advantageous for the escape of these cells to oxidative stress induces cell death is also implicated in the therapy resistance [30], since ROS are critical mediators of ionizing radiation-induced cell death [31]. Accordingly, scarce ROS levels in breast cancer cells have been associated with diminished DNA damage in the presence of ionizing irradiation and with radio sensitization following the depletion of ROS scavengers [26].

1.3. METABOLIC REPROGRAMING IN CARCINOGENESIS

Normal cells evolve progressively to a neoplastic state through the acquisition of successive pathogenic mutations [1]. In a normal epithelium, short diffusion distance allows physiological levels of growth factors, substrates and metabolites. However, hyperproliferation carries cells away from the basement membrane, increasing the diffusion distance, resulting in regional hypoxia. In these conditions, the hypoxia-inducible factor 1 alpha (HIF-1α) induces the expression of products responsible for mediating changes in energy metabolism, pH regulation, angiogenesis, cell survival, cell invasion, as well as cell motility, by upregulating anaerobic glycolysis and increasing acidosis, to generate the required ATP [32, 33].

The metabolic plasticity of cancer cells is involved in cancer progression, drug resistance as well as in metastasis [34]. Thus, metabolic reprograming offers a wide range of potential targets to impair tumor initiation and progression, such as metabolic enzymes [35, 36]. Thereby, a metabolic targeting approach can prevent the nutrient supply for cancer cells and can also impair bioenergetics, in order to prevent an adaptive response to cell stress [36]. Currently, there are several anti-cancer strategies based on metabolic addiction of cancer cells, such as 2-deoxyglucose (2-DG), 3-bromopyruvate (3-BrP), and ionidamide, which target hexokinase (HK), as well as oxamate, that inhibits lactate dehydrogenase A (LDHA) [35], among others (**Figure 2**).

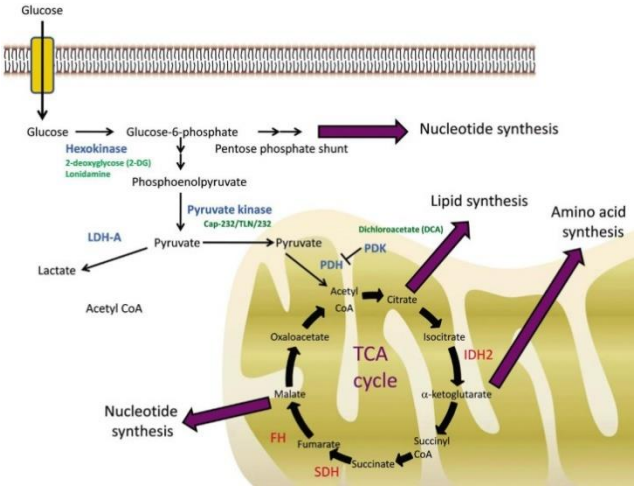

Figure 2. Targeting glucose metabolism in cancer cells. Key metabolic pathways and control points, which may serve as useful targets for cancer therapy, are shown schematically. Glucose enters in the cell by glucose transporters, being metabolized by glycolysis to pyruvate in the cytosol. Pyruvate is either converted to lactate through the action of lactate dehydrogenase-A (LDH-A), or imported into the mitochondrial matrix where it is converted to acetyl coenzyme A (CoA) via pyruvate dehydrogenase (PDH). Then acetyl CoA enter the tricarboxylic acid (TCA) cycle. In cancer cells, pyruvate often enters a truncated TCA cycle and its metabolites are diverted away from complete oxidation and into various biosynthetic pathways (purple arrows). The glycolytic intermediate glucose-6-phosphate can also be diverted into nucleotide synthesis pathways through the pentose phosphate shunt. Key enzymes which may be particularly promising targets for cancer therapy are shown in blue; drug inhibitors of these enzymes are shown in green. Pyruvate dehydrogenase kinase (PDK) suppresses activity of PDH and is itself inhibited by dichloroacetate (DCA). TCA enzymes which are known to be mutated in cancer are shown in red: IDH2 (isocitrate dehydrogenase 2), SDH (succinate dehydrogenase), and FH (fumarate hydratase). Adapted from Fogg *et al.*, 2011 [37].

A key branching point in the glycolytic pathway is the production of pyruvate [38]. Under normoxia, the pyruvate dehydrogenase (PDH) complex (PDC) governs the conversion of pyruvate into acetyl coenzyme A (CoA) through oxidative decarboxylation, controlling the flow of metabolites from glycolysis to the TCA cycle and, subsequently, the generation of ATP by mitochondria. This PDC activity is regulated by pyruvate dehydrogenase kinase (PDK), which phosphorylates and inactivates PDH [39]. Interestingly, it has been demonstrated that this cancer-specific metabolic remodeling can be reversed by dichloroacetate (DCA), a mitochondrial-targeting small molecule that inhibits PDK activity [38, 40, 41]. Thus, DCA can switch cancer cell metabolism from glycolysis to mitochondrial OXPHOS [38, 42] (**Figure 3**). Several studies have been demonstrating the potential role of DCA as an approach in cancer treatment, being already used in clinical trials [38, 43-45].

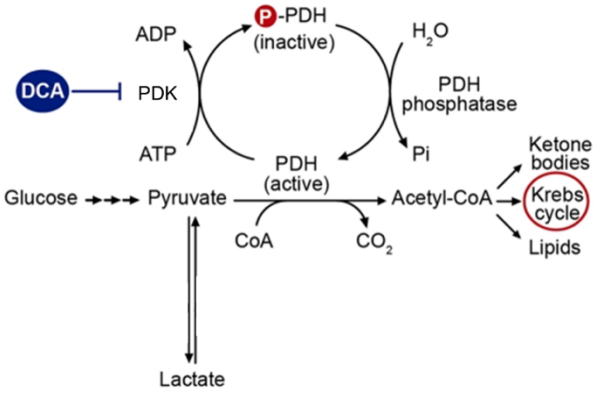

Figure 3. DCA inhibits the mitochondrial enzyme PDK, maintaining PDH in its active unphosphorylated state and facilitating the aerobic oxidation of glucose. PDH, located in the mitochondrial matrix, in its active unphosphorylated state mediates acetyl coenzyme-A formation from pyruvate, which feeds the electron transport chain responsible for ATP synthesis and oxygen consumption. Phosphorylation of PDH by PDK generates its inactive phosphorylated state. DCA-mediated inhibition of PDK renders most of PDH in the active form and then pyruvate metabolism switches towards glucose oxidation to CO_2 in the mitochondria. Adapted from Miquel E. *et al.*, 2012 [46].

1.4. METABOLIC ALTERATIONS IN BREAST CANCER

An increasing body of evidence have demonstrated that metabolic reprograming is fundamental for breast cancer initiation and progression [35]. In this context, Lu *et al.* proposed that metabolite alterations in several pathways, such as glycolysis, TCA and PPP, follows tumor progression in a mouse model of breast cancer [47]. Moreover, Shaw *et al.* demonstrated that there is a decrease in OXPHOS metabolism with breast cancer progression, with a decrease of cellular oxygen consumption rate (OCR) and an increased aggressiveness of breast cancer cells [48]. Also, comprehensive metabolic profiles identified metabolite deregulation in invasive breast carcinomas compared with

normal breast tissue, implicating changes in metabolic pathways during breast cancer progression [49]. Still, Budczies *et al.* identified key metabolic markers that separate cancer from normal tissue with high sensitivity and specificity [49].

Actually, analyzing the immunohistochemical expression of HIF-1α, glucose transporter 1 (GLUT1) and carbonic anhydrase IX (CAIX) in a histological model of breast cancer progression, Chen *et al.* demonstrated that there is as a powerful adaptive advantage associated to an aggressive phenotype in breast carcinomas [50].

A few years ago, gene expression analysis have improved the cellular and molecular understanding of breast cancer by identifying distinct molecular subtypes with different transcriptional signatures and clinical outcomes [51]. The basal-like breast cancer (BLBC) subtype has drawn the attention of the scientific community due to the lack of targets to therapy, since they are characterized by a triple negative phenotype, lacking the expression of hormone receptors (ER and PgR, estrogen and progesterone receptor, respectively), and human epidermal growth factor receptor 2 (HER2) [52]. Interestingly, this aggressive molecular subtype of breast carcinomas presents an increased response to hypoxia, as well as a predominant glycolytic metabolism. Several reports have demonstrated a differential expression of proteins induced by hypoxia and the development of a glycolytic/acid resistant phenotype in BLBC [53-55]. Kim *et al.* evaluated the metabolic phenotype of triple-negative breast cancer (TNBC), based on the immunohistochemical expression profiles of GLUT1 and CAIX, and observed a Warburg molecular phenotype in these tumors [56]. Accordingly, Doyen and co-workers also described a classical Warburg metabolism in TNBC, with high glucose uptake and increased lactate secretion, expressing glycolytic and hypoxic markers [57]. Still, several other authors showed that GLUT1, CAIX, monocarboxylate transporter 1 (MCT1) and cluster of differentiation (CD) 147 are differentially expressed in BLBC, as well as are associated to the absence of hormone receptors and expression of key basal markers, such as cytokeratin (CK) 5, EGFR (epidermal growth factor receptor), CK14 and vimentin. Moreover, these glycolytic markers were found to be associated with aggressive clinic-pathological characteristics in primary invasive breast carcinomas, such as high proliferation rates, high histological grade and poor patient's survival [53-55, 58, 59].

2. CANCER STEM CELLS

Over the years, increasingly evidence for the existence of cancer stem cells (CSCs), or tumor-initiating cells (TICs), has supported the implication of these cells in breast cancer development. Accordingly, there are some established markers to isolate these cancer cells that are highly tumorigenic, with high propensity to metastasize and resistant to therapeutic treatments [60].

2.1. DEFINITION AND CHARACTERIZATION

Tumor cells have high proliferative capacity, phenotypic plasticity and aberrant differentiation. Actually, stem cells and tumor cells share numerous properties and characteristics, namely self-renewal capacity, the reliance on similar signaling pathways, as well as biomarkers [61]. Stem cells are present in many different somatic tissues and are characterized by their self-renewal ability, the capacity to generate multiple cell lineages, as well as by the potential for sustained proliferation [62, 63]. These processes occur in a highly regulated manner, under the control of specific molecular machinery and influence of the surrounding microenvironment [61, 64].

The CSC hypothesis proposes that tumors have a hierarchical organization, being a small subpopulation of tumorigenic cells responsible for tumor formation and progression [65]. These cells with stem-like properties, or CSCs, are defined as a distinct population of cancer-initiating cells with the ability of self-renewal and to generate both further CSCs and more differentiated cancer cells [65]. In this way, CSCs are thought to originate the bulk of the primary tumor and to contribute to tumor heterogeneity [65]. Despite CSCs share numerous properties with normal stem cells, it is still not clear their origin [66, 67]. Until now, CSCs have been isolated from several human tumors, including leukemia [68], breast [63], melanoma [69] and colon [70], and the presence of these tumor cells subsets is strongly correlated with tumor recurrence and treatment failure [71]. Nevertheless, the isolation and characterization of these cells have been a major challenge in science [72].

2.2. BREAST CANCER STEM CELLS

In breast cancer, CSCs are designated breast cancer stem cells (BCSCs) and share important properties with mammary stem cells, such as the ability to proliferate and resist to radiation- and chemotherapy-induced cell death, allowing them to survive and to cause tumor recurrence [29, 73]. Although there is still not an universal criteria to characterize and identify BCSCs, several phenotypes and markers have been described to be able to identify and isolate these cells, such as $CD44^+$ $CD24^-$ phenotype or aldehyde dehydrogenase-1 (ALDH1) activity [60, 74-76]. The current gold standard method for assessing BCSCs activity is the ability of these cells to re-grow tumors in immuno-compromised mice, after being isolated by fluorescence activated cell sorting (FACS), using antibodies to specific cell surface markers or intracellular enzymes, such as ALDH (ADEFLUOR assay) [77]. Moreover, in non-adherent conditions, BCSCs, such as mammary stem and progenitor cells, are able to proliferate in an undifferentiated state, while differentiated cells die by *anoikis.* Thus*,* taking advantage of this *anoikis* resistance ability of BCSC, the Mammosphere Forming Efficiency (MFE) assay is also used to identify cancer cell populations enriched for stem-like properties [78].

2.3. BREAST CANCER STEM CELL'S METABOLISM

CSCs seem to adapt their metabolism to microenvironmental changes by conveniently shifting energy production between pathways or by acquiring intermediate metabolic phenotypes [79]. Therefore, emerging data have explored the metabolism of CSCs, revealing that these cells have a distinctive metabolic phenotype compared with the bulk of the tumor. However, there is still not a consensus about the metabolic behavior of BCSCs (**Table 1**).

Several authors claim that BCSCs have an increased glycolytic phenotype, which seems to be linked to a decrease in mitochondrial oxidative metabolism. In this context, Feng *et al.* demonstrated that mouse and human BCSCs present a more glycolytic phenotype compared with their differentiated progeny. These authors found that BCSCs present a higher ratio of lactate production to oxygen consumption, higher glucose consumption, as well as fewer and less active mitochondria than non-BCSCs [80]. Also, Ciavardelli *et al.* showed that BCSCs shift from OXPHOS to glycolysis, presenting increased expression of key enzymes of anaerobic metabolism, namely pyruvate kinase muscle isozyme 2 (PKM2) isoform, LDH and G6PD (glucose-6-phosphate dehydrogenase), as well as increased antioxidant defense systems [81]. Furthermore, Gammon *et al.* demonstrated that BCSCs, with epithelial-mesenchymal transition (EMT) characteristics, present high levels of HIF-1α, decreased mitochondrial mass and membrane potential,

consume less oxygen and present lower levels of ROS [82]. Still, Gordon *et al.* showed that BCSCs have the ability to adapt to microenvironment stress, such as starvation and hypoxia, by upregulating glucose transporters and switching to a more glycolytic phenotype to outcompete with their differentiated counterparts [83].

In contrast, other authors claim that BCSCs present increased OXPHOS characteristics and behavior. Vlashi *et al.* demonstrated that BCSCs rely mainly on mitochondrial oxidative metabolism, while the more differentiated progeny displays a more glycolytic phenotype [84]. Similarly, De Luca *et al.* also showed that BCSCs obtain energy mainly by OXPHOS and that mitochondrial biogenesis is required for anchorage-independent survival and propagation of stem-like cancer cells [85]. Also, Farnie *et al.* hypothesized that enhanced mitochondrial function could be partially responsible for chemo-resistance in BCSCs, since increased mitochondrial function confers a stem-like phenotype [86]. Accordingly, Lamb *et al.* claims that BCSCs present an increased mitochondrial mass and mitochondrial functional activity. They demonstrated that mammospheres are enriched for mitochondrial-related enzymes, as well as for proteins involved in mitochondrial biogenesis, proposing that increased mitochondrial biogenesis and decreased mitochondrial degradation are responsible for the accumulation of mitochondrial mass in BCSCs [87, 88]. Furthermore, the same authors also propose that mitochondrial mass could be a metabolic biomarker for anabolic BCSCs [89].

Table 1. Summary of described metabolic behavior of BCSC.

	BCSC isolation	Approach	Drug	Reference
Glycolytic	Sort $CD24^{-/low}$ cells within the sphere culture	Proteomic and targeted metabolomic analysis	2-DG	Ciavardelli *et al.*, 2014 [81]
	Sort $CD49f^{high}Epcam^{low}$, $CD49f^{low}Epcam^{high}$ cells by FACS	Transcriptome profiling using RNA-Sequencing	-	Feng *et al.*, 2014 [80]
	Sort $CD44^{high}ESA^{low}$ cells by FACS	Differences in the patterns of oxygen metabolism of sub-fractions of tumor cells	-	Gammon *et al.*, 2013 [82]
	From invasive carcinomas via FACS subpopulations expressing $CD49f^{high}CD24^{low}$, $CD49f^{high}CD24^{high}$, $CD49f^{low}CD24^{high}$ and $CD49f^{low}CD24l^{ow}$	Gene expression signatures of breast cancer stem and progenitor cells	-	Gordon *et al.*, 2015 [83]
OXPHOS	MFE	Quantitative proteomics analysis to identify mitochondrial therapeutic targets	-	Lamb *et al.*, 2014 [87]
	Sort BCSCs with low proteasome activity using FACS	Metabolic requirements of BCSCs and differentiated progeny	-	Vlashi *et al.*, 2014 [84]
	MFE with pre-treatment of monolayers with XCT790	Mitochondrial biogenesis as a target to impair CSCs propagation	XCT790	De Luca *et al.*, 2015 [85]
	MitoTracker Deep-Red staining to metabolically fractionate cells into mito-low and mito-high subpopulations by flow-cytometry	Therapeutic targeting of chemo-resistant CSCs	-	Farnie *et al.*, 2015 [86]
	MFE with previous fractionated GFP-high and GFP-low groups treated with MST-312 at day 0	hTERT-promoter-eGFP-reporter system to identify and purify a subpopulation of MCF-7 cells, with high hTERT transcriptional activity by FACS	MST-312, a telomerase inhibitor	Lamb *et al.*, 2015 [88]
	MFE of sorted MCF-7 cells for MitoTracker Deep-Red	Proteomic analysis of a humanized model of mouse mammary tumor virus	-	Lamb *et al.*, 2015 [89]
	MFE	Comparison between the proteome of MCF-7 cell monolayers and MCF-7-derived mammospheres using proteomic analysis	-	Lamb *et al.*, 2015 [90]
	MFE	Mitochondrial biogenesis as a selective target of CSCs	Antibiotics	Lamb *et al.*, 2015 [91]
	MFE with pre-treatment of monolayers with Atovaquone	Mitochondrial complex III and OXPHOS as a target to eradicate CSCs	Atovaquone	Fiorillo *et al.*, 2016 [92]
	MFE	Mitochondrial complex I as a target to kill BCSCs	Metformin	Hirsch *et al.*, 2012 [93]

Epcam: Epithelial cell adhesion molecule; ESA: epithelial surface antigen; GFP: green fluorescent protein; hTERT: Human telomerase reverse transcriptase.

3. P-CADHERIN: A CELL-CELL ADHESION MOLECULE

Classical cadherins are a family of molecules with important functions in cell-cell adhesion, tissue morphogenesis and cancer [94]. P-cadherin (placental cadherin) is one of the four classical cadherins (E-cadherin (epithelial), N-cadherin (neural) and R-cadherin (retinal)) [94], being the third to be identified and characterized [95].

3.1. STRUCTURE AND FUNCTION

CDH3, the gene encoding P-cadherin, share 66% of homology with the far more well characterized *CDH1* (the gene that encodes E-cadherin), being mapped in chromosome 16q22.1, a region that contains a cluster of several cadherin genes [96]. Specifically, *CDH3* gene is composed by 16 exons and exhibits a high degree of conservation in intron positions and a large intron after exon 2 [97].

P-cadherin has a molecular weight of 118 kDa and a similar molecular structure to that of classical cadherins [98]. The function and strength of P-cadherin-mediated adhesion depends on its dynamic association with catenins, through the cadherin-catenin complex [94, 98]. The cytoplasmic tail of P-cadherin contains two main domains: the catenin-binding domain (CBD), essential for cadherin function, and the juxtamembrane domain (JMD), which has been suggested to play a critical role in allowing cells to relocate [99] (**Figure 4**).

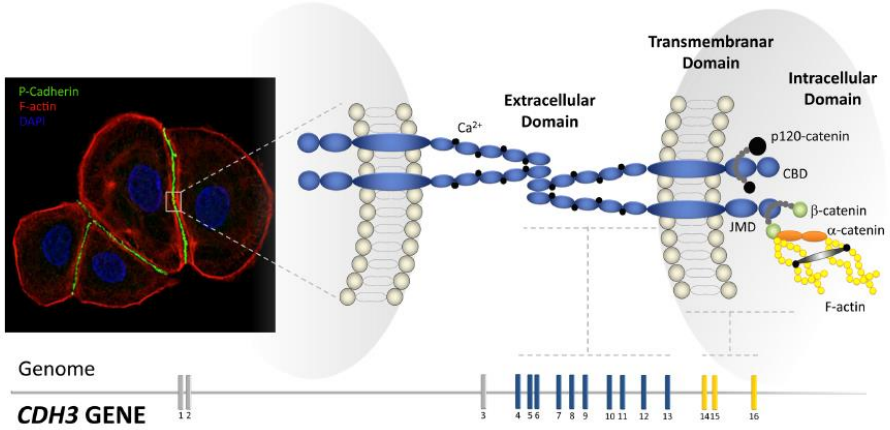

Figure 4. Schematic representation of the structure of the P-cadherin adhesive junction. Lateral clustering of P-cadherin molecules is required to form stable cell-to-cell contacts in BT20 breast cancer cells. In the intercellular space, P-cadherin extracellular domains interact with P-cadherin extracellular domains of adjacent cells to mediate cell-cell adhesion. Intra-cellular catenins bind to the cytoplasmic tail of P-cadherin. p120-ctn binds the cadherin tail at the juxtamembrane domain (JMD), whereas β-catenin binds to the distal catenin binding domain (CBD). α-catenin associates with β-catenin and is directly linked to the actin cytoskeleton. The lower panel represents the genomic structure of *CDH3*/P-cadherin gene, which is constituted by 16 exons: the extracellular part of P-cadherin is encoded by 10 exons (exons 4-13), whereas the transmembrane and intracellular domains are determined only by the information included in the last 3 exons (exons 14-16). Adapted from Albergaria *et al.*, 2011 [94].

3.2. ROLE IN STEMNESS AND IN CELL DIFFERENTIATION

P-cadherin has a crucial role in maintain the structural integrity of epithelial tissues. Moreover, it is accepted that this molecule participates in embryonic development and that it contributes to the biology of stem cells of the normal mammary gland and the hair follicle. Furthermore, this adhesion molecule is considered a biomarker for the isolation and characterization of stem cells, such as in human embryonic stem cell (hESC), as well as important mediator of stem cell activity through the modulation of signaling pathways [94, 100].

In the normal mammary gland, P-cadherin is restricted to the basal myoepithelial layer, contributing to the supra basal stem cell niche [101]. During normal breast development, P-cadherin has a critical role in the ductal mammary branching, being expressed by myoepithelial precursor cells, the cap cells, at the terminal end buds [102]. Moreover, P-cadherin function has been clarified by P-cadherin inactivation studies performed in mice. In this context, Radice *et al.* demonstrated that normal mammopoiesis is affect by P-cadherin deletion, since *CDH3*-null female mice, in the virgin state, present precocious mammary gland differentiation, breast hyperplasia, as well as dysplasia with age [103]. Several studies have elucidated that P-cadherin expression is crucial to the maintenance of normal breast epithelial architecture. Chanson *et al.*, by using an antibody that specifically antagonizes P-cadherin cell-cell interactions, demonstrated that the migration of mammary myoepithelial cells was compromised [104]. Furthermore, Nguyen-Ngoc *et al.* showed that P-cadherin loss causes precocious branching morphogenesis in matrigel, showing the importance of P-cadherin in the maintenance of normal breast epithelial architecture [105]. Taking together, these findings demonstrate that P-cadherin expression and signaling are essential for limiting the growth of the mature luminal epithelial cells, as well as for the maintenance of an undifferentiated state of the normal mammary gland, pointing to the role of P-cadherin as a putative stem cell marker.

3.3. P-CADHERIN IN BREAST CANCER

Due to their importance in normal development and tissue architecture, alterations in classical cadherins are implicated in disease [98]. Mutations in *CDH3* gene, resulting in abnormal P-cadherin expression, have been recognized as being responsible for congenital hypotrichosis with juvenile macular dystrophy, a rare autosomal recessive disorder characterized by short sparse scalp hair at birth and progressive macular retinal degeneration that leads to early blindness [106, 107]. Moreover, alterations in P-cadherin

expression have been widely associated with several solid tumors, including breast, prostate, colon, pancreatic and bladder cancer [98].

Particularly, in breast cancer, P-cadherin was found to be aberrantly expressed in 25% of ductal carcinomas *in situ* (DCIS), as well as in 20% to 40% of invasive breast carcinomas [98, 108-110]. P-cadherin is a marker of poor prognosis in breast cancer, associated with short-term overall and disease-free survival, as well as with distant and locoregional relapse-free interval [111-115]. P-cadherin is differentially expressed in poor prognosis BLBC [98]. Accordingly, its expression has been positively associated with poorly differentiated and high histological grade tumors, as well as with established markers of poor prognosis, such as ki-67, EGFR, CK 5/6 and CK14, and negatively associated with age at diagnosis, hormonal receptors (ER and PgR), and B-cell lymphoma 2 (Bcl-2) expression [82, 112-114]. Moreover, our group has demonstrated that P-cadherin expression shows higher sensitivity to distinguish the basal phenotype of breast carcinomas, being a reliable marker to be used in the daily practice of breast pathology laboratories for the identification these tumors [116].

We have also demonstrated that P-cadherin overexpression promotes cell motility, migration and invasion capacity and influences cell shape and cell polarity [117, 118]. Additionally, we observed that P-cadherin functional role is dependent on E-cadherin cellular context. Using *in vitro* and *in vivo* assays, as well as human primary breast cancer samples, we showed that the co-expression of E- and P-cadherin significantly enhanced tumor growth, is correlated with high histological grade, biologically aggressive behavior and with poor patient survival [119]. Taking together, targeting P-cadherin in breast cancer may be a good therapeutic approach, since normal associated counterparts exhibit low expression levels of this adhesion molecule [120].

3.4. P-CADHERIN AS A BREAST CANCER STEM CELL MARKER

Due to the high breast cancer heterogeneity, the definition of a single phenotype for BCSCs is a challenging task. The aggressiveness and the lack of target therapeutic approaches to BLBC has driven the attention to the need of better defining the CSC phenotype for this poor-prognosis breast carcinomas [121]. It has been reported that the luminal progenitor cell of the normal mammary gland hierarchy is the cell of origin for BLBC, since mutations in *BRCA1* (breast cancer susceptibility gene 1), a known suppressor of *CDH3* gene [122], was able to induce the formation of a breast carcinoma with basal phenotype [121].

Recently, our group has proposed P-cadherin as a BCSC marker and a valuable target to define the CSC phenotype and the cell of origin of BLBC [101]. We demonstrated that

P-cadherin expression is able to promote stem-like properties and is associated with the expression of CSC markers, such as CD44, CD49f and ALDH1 [101]. In addition, cell populations depleted for P-cadherin expression exhibited decreased *in vitro* self-renewal ability, lower capacity to grow colonies in 3D cultures and reduced tumorigenicity in nude mice [101]. Furthermore, P-cadherin expression is fundamental for the adhesion of cancer cells to ECM substrates, a critical step for metastatic dissemination. We demonstrated that its inhibition caused a significant decreased adhesion of breast cancer cells to the basement membrane substrate laminin and a major reduction in the expression of the laminin receptor α6β4 integrin [123]. The expression of this heterodimer is needed for the invasive capacity and increased MFE induced by P-cadherin expression, which might explain the stem cell and invasive properties induced by this protein in breast cancer cells [123].

BCSCs are able to survive and persist in the tumor, being responsible for recurrence of the disease [29, 73]. Remarkably, P-cadherin is considered a survival factor in breast cancer cells, since decreased P-cadherin expression increases breast cancer cell death in a caspase-dependent mechanism, as well as it promotes *anoikis* resistance, allowing cells to survive in anchorage-independent conditions [101, 119]. Still, this molecule confers resistance to radiation, since P-cadherin-enriched breast cancer cell population showed increased ability to survive in anchorage-independent conditions when irradiated, in comparison with P-cadherin depleted cells [124].

3.5. P-CADHERIN AND CANCER CELL METABOLISM

P-cadherin promotes stem-like properties to breast cancer cells and is recognized as a BCSC marker [101]. Although there is still no consensus, several authors claim that BCSCs exhibit pro-glycolytic metabolic skills, allowing them to decrease oxidative stress, being able to escape *anoikis*, survive in circulation and increase metastasis formation [125]. Interestingly, and in agreement with the reported glycolytic behavior of BLBC, we have recently showed that the expression of this basal epithelial marker P-cadherin associates with breast cancer cell populations harboring a glycolytic and acid-resistant phenotype, being significantly associated with the expression of HIF-1α, GLUT1, CAIX, MCT1 and CD147 in human breast carcinomas [125]. We also showed that P-cadherin expression is modulated by hypoxia in a time dependent manner through HIF-1α stabilization. Moreover, we observed that P-cadherin-enriched breast cancer cells exhibit increased GLUT1 and CAIX expression and that these cells comprise high MFE, suggesting that P-cadherin overexpressing BCSCs are more likely to exhibit increased glycolysis and to survive to metabolic-driven pH alterations [125]. Furthermore,

unpublished data from our group shows that P-cadherin silencing was able to decrease the extracellular acidification rate, as well as to modulate cellular ATP content of breast cancer cells. Still, we were also able to demonstrate that P-cadherin expression is associated with the production of low ROS levels, by inducing the upregulation of ROS scavenging systems, such as SOD1 and SOD2.

Taking together, we believe that this glycolytic and antioxidant role mediated by P-cadherin expression in breast cancer cells is likely to impact their ability to invade the surrounding tissue, to survive in circulation and to promote metastasis.

CHAPTER II

RATIONAL AND AIMS

P-cadherin expression promotes stem-like properties in breast cancer cells, such as tumorigenic capacity and *anoikis* resistance, being recognized as a BCSC marker [71]. BCSCs are known to exhibit pro-glycolytic metabolic skills, allowing them to decrease oxidative stress, being able to escape *anoikis*, survive in circulation and increase metastasis formation. Disturbing this survival skill by metabolic reprograming would target these properties and impact the efficacy of cancer treatment. Recently, we have demonstrated that P-cadherin-enriched populations are more likely to present a hypoxic, as well as glycolytic and acid-resistant phenotype [125]. Moreover, preliminary data from the group points for the hypothesis that aberrant P-cadherin expression might have a role in cellular metabolic reprograming of BCSCs, acting as an antioxidant and enhancing cell survival in circulation by promoting *anoikis*-resistance. Despite the recent implications of P-cadherin expression in metabolic behavior of breast cancer cells, nothing is known about the role of this basal epithelial marker in the sensitization of breast cancer cells to *anoikis* by metabolism reprograming.

Main Aim

The main aim of this work was to analyze the sensitivity of P-cadherin-enriched breast cancer cells to *anoikis* by *in vitro* metabolic reprograming using DCA.

Specific Aims

Using a panel of human breast cancer cell lines, the studies were performed in order to address the following aims:

TASK 1) To predict the association between *CDH3* expression and the expression of the DCA molecular targets in breast cancer, using bioinformatic predictive tools.

TASK 2) *In vitro* analysis of the P-cadherin role in metabolic reprograming induced by DCA in breast cancer cells, using two-dimensional (2D) monolayer, as well as anchorage-independent culture conditions.

TASK 3) Evaluation of the effect of P-cadherin expression in the modulation of oxidative stress in breast cancer cells.

CHAPTER III

MATERIALS AND METHODS

This chapter describes the materials and methods used for all the data presented in the results section.

MATERIALS

Cell Culture

Human breast cancer cell lines were obtained as follows: BT20, MDA-MB-468 and MCF-10A were acquired from American Type Culture Collection (Manassas, VA, USA), SUM149 was kindly provided by Dr. Stephen Ethier (University of Michigan, USA), and MCF-7/Az was kindly given by Prof. Marc Mareel (Ghent University, Belgium). MCF-7/Az cell line was retrovirally stable transduced to encode P-cadherin (MCF-7/Az.P-cadherin cell line), as described earlier by the group [126]. MCF-7/Az.Mock cell line, encoding only EGFP, was used as a control. Cells were routinely maintained at 37°C and 5% CO_2 in the following media (Invitrogen Ltd, UK): DMEM for BT20 and MDA-MB-468, and 50% DMEM/50% Ham-F12 for SUM149, MCF-10A and MCF-7/Az. In BT20, MDA-MB-468 and MCF-7/Az cell lines the media contained 10% heat-inactivated fetal bovine serum (FBS, Greiner bio-one, Belgium) and in SUM149 cell line, media was supplemented with 5% FBS, 5µg/ml of insulin and 1µg/ml of hydrocortisone (Sigma-Aldrich, USA). MCF-10A media was supplemented with 20ng/ml of epidermal growth factor (EGF, Sigma-Aldrich, USA), 0.5mg/ml of hydrocortisone, 100ng/ml of cholera toxin (Sigma-Aldrich, USA), 10µg/ml of insulin and 5% horse serum (Invitrogen). All media were supplemented with 100 IU/ml penicillin and 100 mg/ml streptomycin (Invitrogen Ltd, UK).

Primary Antibodies and Reagents

For Western blot, we used the following primary anti-human antibodies against: P-cadherin (clone 56, BD Transduction Biosciences, USA; diluted 1:500), phospho PDH (pPDH) at serine (Ser) residue 293 (ab177461, Abcam, UK; diluted 1:1000), total PDH (tPDH) (ab197956, Abcam, USA; diluted 1:3000) and 70 kDa heat shock protein (HSP70), as housekeeping (sc-7298, Santa Cruz; diluted 1:2000).

Presto blue reagent (Invitrogen, UK) was used to evaluate the viability of cells. Metabolic reprograming was induced by DCA (Sigma-Aldrich, USA), and MitoSOX™ Red reagent (ThermoFisher Scientific, UK) was used to measure mitochondrial ROS levels by immunofluorescence analysis.

METHODS

Bioinformatic analysis using public available gene expression databases of human breast cancer

To study the possible association between *PDK*, *PDHA1* and *CDH3* genes, we have used The Cancer Genome Atlas (TCGA), as well as the Cancer Cell Line Encyclopedia (CCLE) online databases, in breast cancer samples and cell lines, respectively.

Cell viability assay

Cells were plated in a 96-wells plate and treated with DCA in a daily basis. After 24h of treatment cells were washed with phosphate buffered saline (PBS) 1x and presto blue reagent was added at 1:20 diluted in culture medium. Cells were incubated at 37˚C, 5% CO_2 for 35 minutes and the fluorescence was read at 50% sensitivity top reading on the following wave-length (λ): $\lambda_{excitation}$ =560nm and $\lambda_{emission}$=590nm.

Protein extraction and western blot analysis

Protein lysates were prepared from cells using catenin lysis buffer [1% (v/v) Triton X-100 and 1% (v/v) NP-40 (Sigma-Aldrich, USA) in PBS] supplemented with 1:7 protease inhibitor cocktail (Roche Diagnostics GmbH, Germany, 11836170001) and with 1:100 phosphatase inhibitor cocktail 3 (Sigma-Aldrich, USA, P0044) for 10 min, at 4°C. Cell lysates were mixed with a vortex and centrifuged at 14000 rpm at 4°C, during 10 min. Supernatants were collected and protein concentration was determined using the Bradford assay (Bio-Rad Protein Assay kit, USA). Proteins were dissolved in sample buffer [Laemmli with 5% (v/v) 2-β-mercaptoethanol and 5% (v/v) bromophenol blue] and boiled for 10 min at 95°C. Samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were transferred into nitrocellulose membranes [Amersham Hybond enhanced chemiluminescence (ECL)]. For immunostaining, membranes were blocked for 1 hour with 5% (w/v) non-fat dry milk in PBS containing 0.5% (v/v) Tween20 and incubated overnight at 4°C with anti-P-cadherin, anti-pPDH, anti-tPDH and anti-HSP70. After washed with PBS-Tween20, membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse, or rabbit secondary antibodies (Santa Cruz Biotechnologies, USA) diluted 1:2000 for 1 hour. Proteins were then detected using ECL reagent (Amersham, USA) as a substrate. Quantity One software (Bio-Rad, USA) was used for quantification of the differences in protein expression comparing with HSP70 expression.

siRNA transfection

Gene silencing was performed with validated small interfering ribonucleic acids (siRNA), specific for *CDH3* (50nM, Hs_*CDH3*_6, Qiagen, USA). Transfections were carried out using Lipofectamine 2000 (Invitrogen, UK), according to manufacturer's recommended procedures. After incubation for 5 minutes, the siRNA and Lipofectamine 2000 solutions were mixed, incubated for additional 20 minutes and added to cell culture medium. A scrambled siRNA sequence, with no homology to any gene, was used as a negative control (Qiagen, USA).

Mammosphere Forming Efficiency (MFE) assay

After 24 hours of the siRNA transfection, cells were treated with DCA and incubated for 24 hours at 37°C, 5% (v/v) CO_2. After incubation, cells were enzymatically harvested and manually disaggregated to form a single-cell suspension and resuspended in cold PBS. Cells were plated at 500/cm^2 in non-adherent culture conditions, in 6-well plates coated with 1.2% poly(2-hydroxyethylmethacrylate)/95% ethanol (Sigma-Aldrich, USA) and allowed to grow for 5 days, in DMEM/F12 containing B27 supplement (Invitrogen, UK), 500ng/ml of hydrocortisone, 40ng/ml insulin, 20ng/ml EGF in a humidified incubator at 37°C and 5% (v/v) CO_2. MFE was calculated as the number of mammospheres (≥50μm) formed divided by the number of cells plated, being expressed as a percentage.

MitoSOX™ Red immunofluorescence assay

BT20 cells were cultured on glass coverslips and 24 hours later they were transfected with control and *CDH3* siRNA. Fresh media was added for 30 minutes before the experiment. Cells were washed with Hank's balanced salt solution (HBSS) and incubated with 1,5μM of MitoSOX™ Red for 45 minutes. After that, cells were washed with PBS, fixed with 4% paraformaldehyde (20 minutes) and washed twice with PBS, at room temperature. Each sample was mounted with Vectashield (Vector Laboratories, Inc, Burlingame, CA) containing 4,6-diamidine-2-phenylindolendihydrochrolide (DAPI) and visualized with Leica SP5 confocal microscope (Leica Microsystems GmbH, Germany).

Statistical analysis

Results are representative of three independent experiments. Quantifications are expressed as mean ± SEM (standard error of the mean) of the biological replicates considered. Statistical analyses were performed using Office Excel 2016 (Microsoft Corporation, Reading, UK). All statistical tests were two-sided and considered as significant when *P* value was lower than 0.05.

CHAPTER IV

RESULTS

I. BIOINFORMATICS ANALYSIS OF THE LINK BETWEEN P-CADHERIN EXPRESSION AND THE EXPRESSION OF DCA MOLECULAR TARGETS IN BREAST CANCER

Since PDK is a well-established target of DCA, the aim of this first part of the work was to search for evidences that would support and predict the link between the response of P-cadherin positive breast cancer cells to DCA treatment, through the association between DCA molecular targets (PDK and PDH) and *CDH3* expression. Therefore, in order to achieve this purpose, we examined online available gene expression databases.

CDH3 expression is correlated with *PDK1* and *PDHA1* expression in breast cancer

Using online TCGA database, we analyzed whether *CDH3* expression is correlated with the DCA molecular effectors, such as *PDK (1-4)* and *PDHA1* genes in breast cancer samples (**Figure 5**).

Interestingly, we observed that *CDH3* is positively correlated with *PDK1* (ρ=0.408) (**Figure 5A**) and *PDHA1* mRNA (messenger RNA) expression (ρ=0.335) (**Figure 5E**). However, no correlation was found between *CDH3* and the other *PDK* isoforms, namely *PDK2* (ρ=-0.235) (**Figure 5B**), *PDK3* (ρ=0.210) (**Figure 5C**) and *PDK4* (ρ=-0.083) (**Figure 5D**).

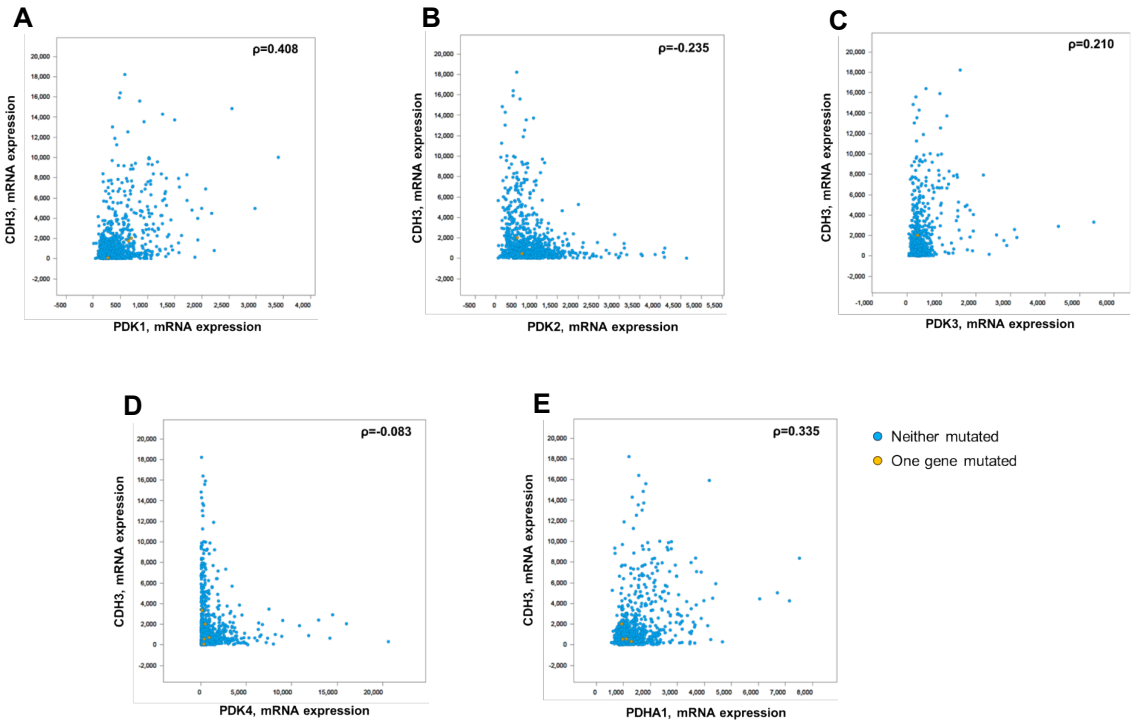

Figure 5. Correlation between *CDH3* and *PDK* isoforms and *PDHA1* genes in breast cancer. *CDH3* is positively correlated with **A)** *PDK1* (ρ=0.408) and **E)** *PDHA1* (ρ=0.335). However, no correlation was found between *CDH3* and **B)** *PDK2* (ρ=-0.235), **C)** *PDK3* (ρ=0.210) or **D)** *PDK4* (ρ=-0.083) in this type of cancer. Adapted from TCGA online gene database. (ρ: Pearson correlation coefficient)

II. *IN VITRO* ANALYSIS OF P-CADHERIN ROLE IN METABOLIC REPROGRAMING INDUCED BY DCA IN BREAST CANCER CELLS

The evidence found on the association between DCA molecular targets and *CDH3* expression using bioinformatic analysis, led us to go further on the role of this adhesion molecule in the DCA induced effects in breast cancer cells.

IIa) Analysis of the sensitivity of breast cancer cells to DCA-induced metabolic reprograming in 2D monolayer culture

In order to analyze the sensitivity of breast cancer cells to DCA, we treated different breast cancer cell lines with a range of DCA concentrations (0-100mM), during 24 or 48 hours. Using Presto Blue viability assay, we determined the half of maximal inhibitory concentration (IC50) of DCA, i.e. the concentration of DCA that induces 50% of cell death (DCA IC50): 98.6mM for MCF-7/Az, 94.6mM for BT20, 67.3mM for MDA-MB-468, 66.7mM for SUM149 and 47.4mM for MCF-10A (**Figure 6**).

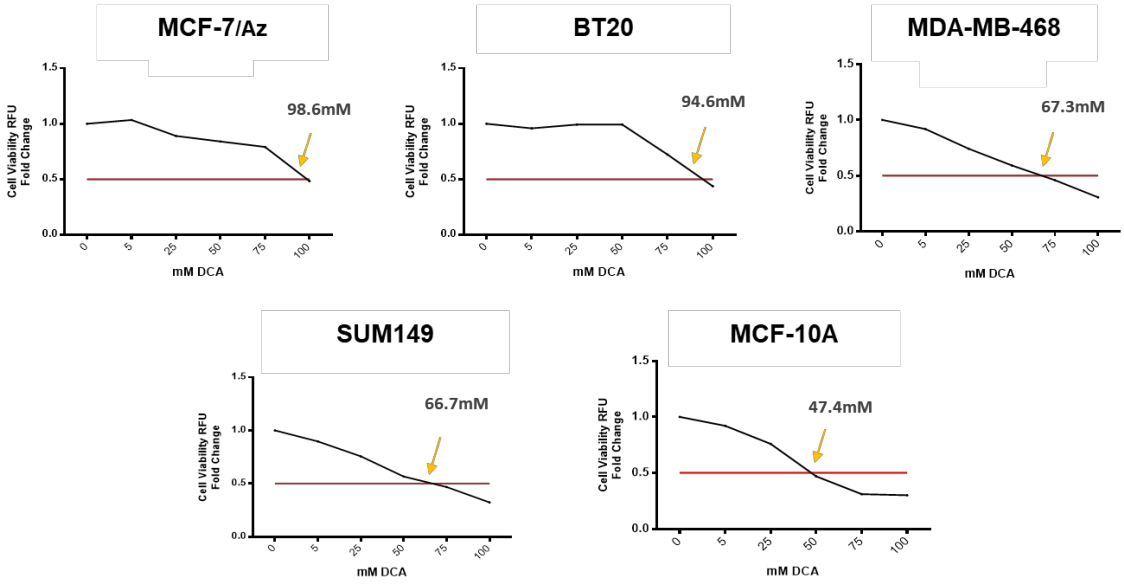

Figure 6. Breast cancer cell's viability in response to DCA. Breast cancer cells viability using presto blue fluorescence analysis in response to DCA was evaluated in a panel of breast cancer cell lines, including MCF-7/Az, BT20, MDA-MB-468 and SUM149, as well as in normal-like MCF10A cells. The concentration of DCA that induced 50% of cell death was 98.6mM for MCF-7/Az, 94.6mM for BT20, 67.3mM for MDA-MB-468, 66.7Mm for SUM149 and 47.4mM for MCF-10A. RFU: Relative Fluorescence Units.

We then went to evaluate the response of PDH phosphorylation at Ser 293 following DCA exposure, as a measurement of the inactive form of this protein, using western blot (**Figure 7**). We observed that 5mM of DCA was enough to induce a significant decrease in pPDH in BT20, SUM149, and MCF-10A cells. In MCF-7/Az breast cancer cells, we observed a decrease in pPDH expression with 25, 50 and 75mM of DCA. However, no alterations in pPDH levels were observed in MDA-MB-468 breast cancer cells, in any of the concentrations and time points used in this study.

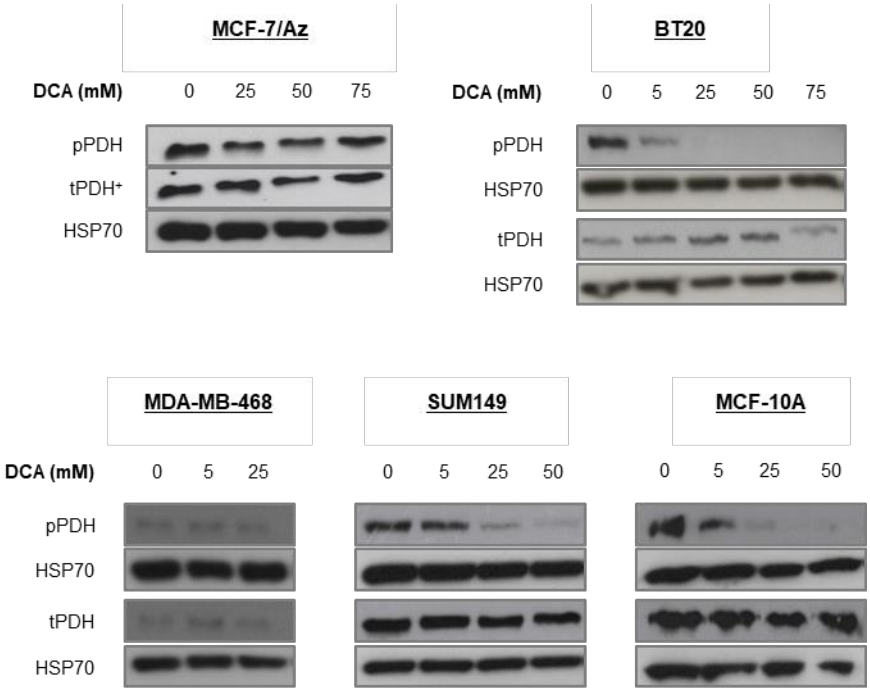

Figure 7. PDH phosphorylation status (Ser293) in response to DCA in breast cancer cell lines. A decrease in PDH phosphorylation levels using 5mM of DCA was observed in BT20, SUM149 and MCF-10A cells. In MCF-7/Az cells, higher concentrations of DCA (25, 50 and 75mM) were necessary to decrease pPDH in these cells. However, no alterations were observed in MDA-MB-468 breast cancer cells, in any of the concentrations and time points used in this study. $^{+}$Stripping from pPDH membrane.

P-cadherin is usually overexpressed in triple-negative basal-like breast cancer (TN-BLBC) and presents low expression levels in breast tumors with a luminal-like phenotype [71].

Comparing the levels of P-cadherin expression with the sensitivity to DCA in our panel of breast cancer cells, we were able to observe that luminal MCF-7/Az cells, with low levels of P-cadherin expression, presented a higher IC50 value and consequently less sensitivity to DCA, in comparison with P-cadherin-enriched TN-BLBC MDA-MB-468 and

SUM149 cells and normal-like MCF10A cells (**Figure 8**). However, BT20 cells presented both increased P-cadherin levels and increased IC50 (**Figure 8**).

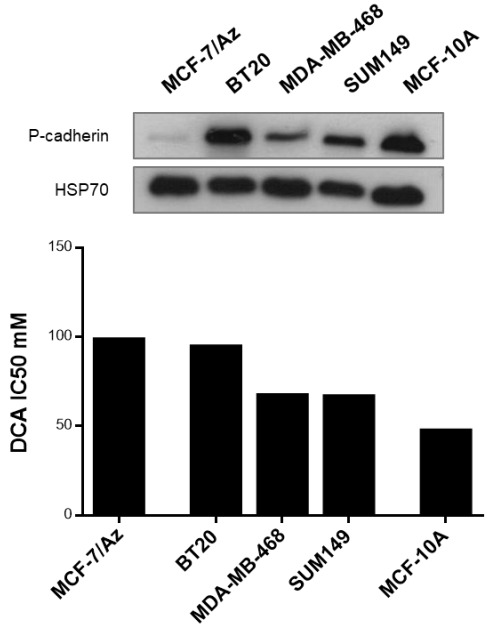

Figure 8. P-cadherin-enriched breast cancer cells have increased sensitivity to DCA. Using Presto Blue fluorescence analysis, we observed that TN-BLBC cells, namely MDA-MB-468 and SUM149, as well as MCF-10A normal-like breast cells, are more sensitive to DCA, in comparison with P-cadherin low expressing luminal breast cancer cells (MCF-7/Az). However, TN-BLBC BT20 cells present high P-cadherin levels and also lower sensitivity to DCA

These results suggest that breast cancer cells with higher P-cadherin expression present increased sensitivity to DCA treatment, while cells with low P-cadherin levels have decreased sensitivity to this metabolic modulator, except for BT20 cells.

IIb) P-cadherin expression Modulates pPDH levels in Breast Cancer Cells

In order to determine if P-cadherin was playing a role in DCA-induced signaling, we then went to evaluate the expression of pPDH in P-cadherin manipulated breast cancer cells (**Figure 9**). Thus, we used two different models: a MCF-7/Az luminal breast cancer cell model, where P-cadherin was constitutively overexpressed; and the BT20 TN-BLBC cell model, with P-cadherin-enriched breast cancer cells, where P-cadherin expression was silenced using a specific *CDH3* siRNA.

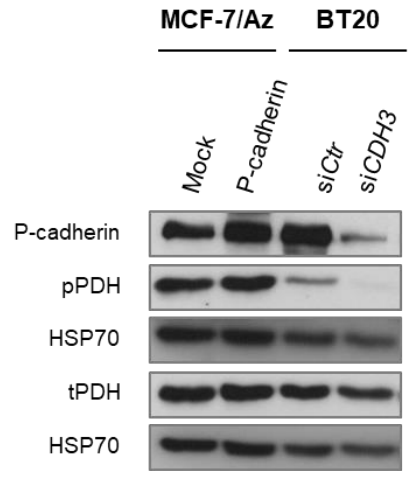

Figure 9. P-cadherin modulates pPDH expression in breast cancer cells. Western blot analysis showed an increase in pPDH expression levels when P-cadherin is overexpressed (MCF-7/Az.P-cadherin), as well as a decrease in pPDH expression upon *CDH3* downregulation in BT20 breast cancer cells.

Thereby, using western blot analysis, we observed that overexpression of P-cadherin (MCF-7/Az.P-cadherin) lead to an increase of pPDH expression, in comparison with control cells (MCF-7/Az.Mock) (**Figure 9**). Accordingly, *CDH3* downregulation in BT20 cells (BT20si*CDH3*) led to a decrease in the levels of pPDH expression, in comparison to BT20 cells transfected with the control siRNA (BT20si*Ctr*) (**Figure 9**). Thus, these results suggest that P-cadherin expression modulates pPDH expression in breast cancer cells.

IIc) DCA induces a decrease in P-cadherin expression in Breast Cancer Cells

Since the results above suggest that P-cadherin expression regulates pPDH levels, we then went to evaluate if there was a feedback loop and evaluate the effect of DCA on P-cadherin expression in breast cancer cells. Using western blot analysis, we observed that DCA treatment decreases the expression of P-cadherin in a dose-dependent manner in MCF-7/Az cells (**Figure 10A**). On the other hand, in TN-BLBC BT20 cells, 7.5m of DCA seems to decrease P-cadherin expression (**Figure 10B**).

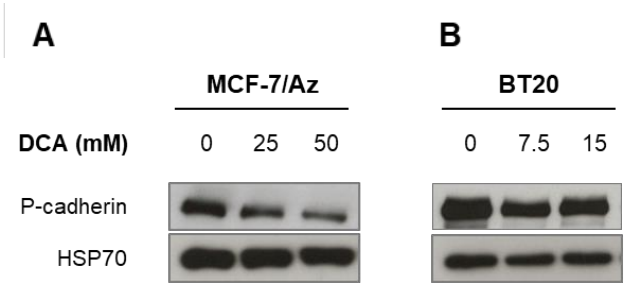

Figure 10. DCA decreases P-cadherin expression in breast cancer cells. A) Using western blot analysis, we observed a decrease in P-cadherin expression with DCA treatment in a dose-dependent manner in MCF-7/Az breast cancer cells. **B)** In TN-BLBC BT20 cells, 7.5mM of DCA induces a decrease in P-cadherin expression.

Taking together, these results suggest that, DCA probably induces specifically the apoptosis of P-cadherin-enriched cells, which lead to the downregulation of its expression.

IId) P-cadherin expression sensitizes Breast Cancer Cells to DCA-induced cell death

Since the results presented before suggests that P-cadherin-enriched breast cancer cells are more sensitive to DCA than the cells with lower P-cadherin expression, and that P-cadherin modulates the levels of pPDH in breast cancer cells, we next went to evaluate if P-cadherin expression, *per se*, was being responsible for the sensitivity of these cells to DCA in 2D monolayer culture conditions.

Thus, in order to assess the effect of DCA in MCF-7/Az breast cancer cells with different expression levels of P-cadherin, we treated MCF-7/Az.Mock and MCF-7/Az.P-cadherin cells with 25 and 50mM of DCA during 24 hours (**Figure 11**). We were able to observe that DCA induced a slight increase in cell death using 25mM and 50mM of this modulator, as previously presented above. Interestingly, we also observed that P-cadherin overexpressing cells presented a higher decrease in cell viability in comparison with the low P-cadherin expressing MCF-7/Az.Mock cells, when these cells were treated with 25mM of DCA during 24h (**Figure 11A**). Moreover, P-cadherin overexpressing cells present an increase of pPDH expression, either with and without DCA treatment (**Figure 11B**). We still observed that in control MCF-7/Az.Mock cells, DCA treatment induces a decrease in pPDH expression with both 25 and 50mM in comparison with MCF-7/Az.Mock non-treated cells (**Figure 11B**), consistent with the expected effect of DCA in pPDH levels.

In BT20 breast cancer model, *CDH3* downregulation induces a slight decrease in the viability of these cells, in comparison with BT20si*Ctr* with no treatment or upon 7.5 and 15mM of DCA during 24 hours (**Figure 11C**). However, there was no increased sensitivity of BT20si*Ctr* to DCA-induced cell death **(Figure 11C)**, as observed in MCF-7/Az model. Moreover, P-cadherin downregulation was accompanied by a decrease in pPDH expression, either with and without DCA treatment. Still, 7.5 and 15mM of DCA induced a decrease in pPDH expression when compared with the untreated cells (**Figure 11D**).

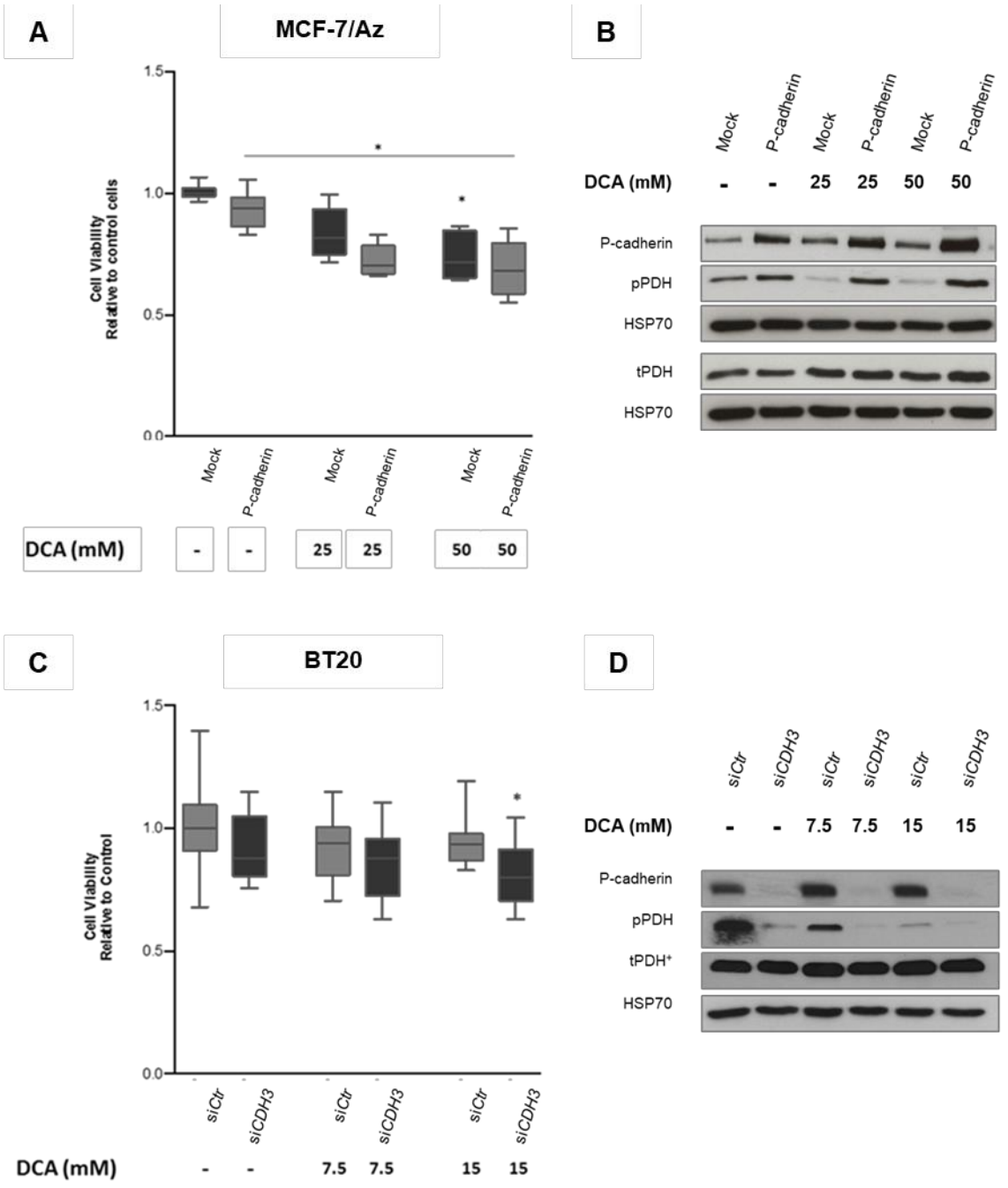

Figure 11. P-cadherin expression sensitizes MCF-7/Az breast cancer cells to DCA-induced cell death. In 2D monolayer cell culture conditions, **A)** P-cadherin overexpression in MCF-7/Az cells presents a higher decreased of cell viability in comparison to control cells (MCF-7/Az.Mock cells) when treated with 25mM of DCA. **B)** DCA treatment induces a decrease in pPDH expression with both 25 and 50mM in control cells, mainly in MCF-7/Az.mock cells. Moreover, P-cadherin overexpression leads to an increase in pPDH expression, either with and without DCA treatment. **C)** In TN-BLBC BT20 model, *CDH3* downregulation induces a slight decrease in the viability of these cells, in comparison with BT20si*Ctr* cells. **D)** With western blot analysis, we observed a decrease in pPDH expression with *CDH3* downregulation, with and without DCA treatment. [+]Stripping from pPDH membrane. n=3; Kruskal-Wallis test * p≤0.05. (* with no line: relative to control cells; * with line: association between line-linked conditions)

Since P-cadherin was described to be responsible for self-renewal of BCSCs [101], we also evaluated the effect of its expression in the survival of breast cancer cells to DCA-induced metabolic reprograming using an anchorage-independent culture system, which promotes the survival of *anoikis*-resistant breast cancer cells. Thus, using the both previously described breast cancer models, we treated cells with DCA during 24 hours and plated the cells in non-adherent conditions for 5 days, promoting the survival of *anoikis*-resistant breast cancer cells (**Figure 12**)**.**

In MCF-7/Az luminal model, we observed that P-cadherin overexpression induces an increase of self-renewal of these CSCs (**Figure 12A**). Interestingly, the treatment with 50mM of DCA induces a decrease in MFE, either in MCF-7/Az.Mock and MCF-7/Az.P-cadherin cells, being this effect higher in MCF-7/Az.P-cadherin BCSCs (**Figure 12A**). By western blot analysis, we observed an increase in pPDH expression in P-cadherin overexpressing cells, either with 25mM of DCA and without DCA treatment, in comparison with MCF-7/Az.Mock cells (**Figure 12B**).

In TN-BLBC BT20 model, *CDH3* downregulation induces a decrease in MFE of BT20 BCSCs, in comparison to control cells, either without or with 7.5mM of DCA during 24 hours (**Figure 12C**). Interestingly, when the cells were treated with 15mM of DCA, there was a high decrease in the number of mammospheres formed in the P-cadherin-enriched control cells, being this number similar to the one observed in cells with C*DH3* downregulation (**Figure 12C**). Also, western blot analysis demonstrates a decrease in pPDH expression with *CDH3* downregulation, with or without DCA, in comparison with control cells (**Figure 12D**).

Taking together, these results indicate that P-cadherin expression modulates the DCA-induced anoikis in BCSC.

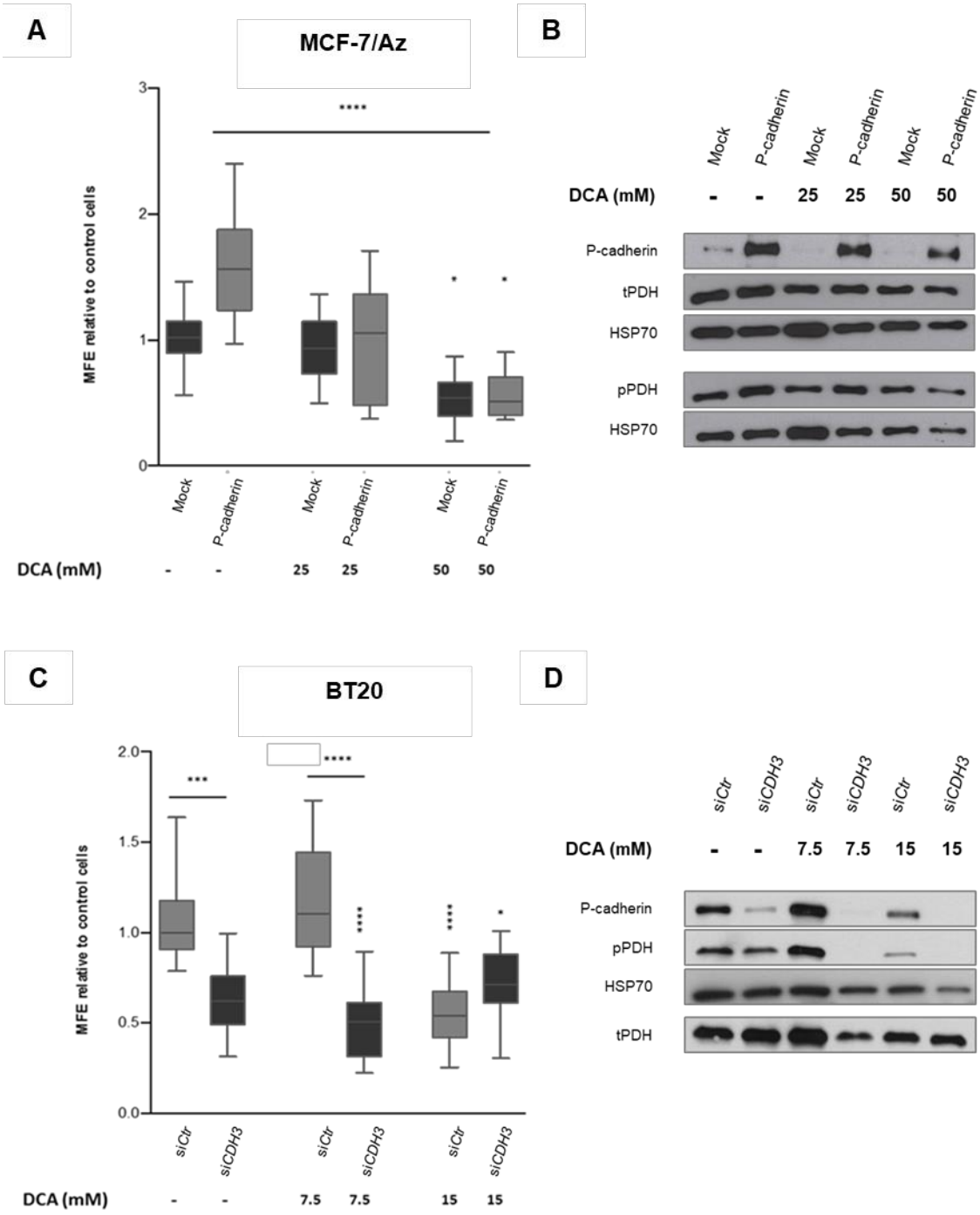

Figure 12. In anchorage-independent conditions, P-cadherin expression sensitizes BCSCs to DCA-induced cell death. A) P-cadherin overexpression induces an increase of self-renewal in MCF-7/Az BCSCs. Interestingly, 50mM of DCA induces a decrease in MFE, either in MCF-7/Az.Mock and in MCF-7/Az.P–cadherin cells, being the effect higher in BCSCs with increased P-cadherin expression. **B)** Western blot analysis showed an increase in pPDH expression in MCF-7/Az.P-cadherin cells, in comparison with MCF-7/Az.Mock breast cancer cells. **C)** The same effect was observed in TN-BLBC BT20 model, where a higher decrease in BT20si*Ctr* BCSC survival was observed upon 15mM of DCA, in comparison with BT20si*CDH3* BCSC. **D)** Western Blot analysis demonstrate a decrease of pPDH expression upon DCA treatment, in comparison with non-treated cells, as well as the decrease in pPDH upon *CDH3* downregulation, with or without DCA, in comparison with the control cells. n=3; Kruskal-Wallis test * p≤0.05; *** p≤ 0.001; **** p<0.0001. (* with no line: relative to control cells; * with line: association between line-linked conditions)

III. P-CADHERIN EXPRESSION MODULATES THE OXIDATIVE STRESS IN BREAST CANCER CELLS

Our group have described that P-cadherin is responsible for the glycolytic metabolism of BCSCs and mediates self-renewal with increased *anoikis*-resistance [125]. Moreover, it is accepted that this type of metabolism promotes lower levels of ROS, potentiating an undifferentiated phenotype and promoting a protection from *anoikis* [26]. Thus, in this part of the work, we went to evaluate if P-cadherin was also playing a role in the modulation of ROS. To achieve this goal, we used fluorescence-based ROS detection, using MitoSOX™ Red reagent, a fluorogenic dye that detects mitochondria derived superoxide anion in live cells.

We observed an alteration from a more pronounced peri-nuclear staining in BT20si*Ctr*, to a spread pattern of mitochondrial derived ROS staining in BT20si*CDH3* cells (**Figure 13A**). This shift was confirmed by the increase of the MitoSOxRed intensity in BT20si*CDH3* cells, in comparison with BT20si*Ctr* cells (**Figure 13B**), specifically measured in the membrane region between adjacent cells.

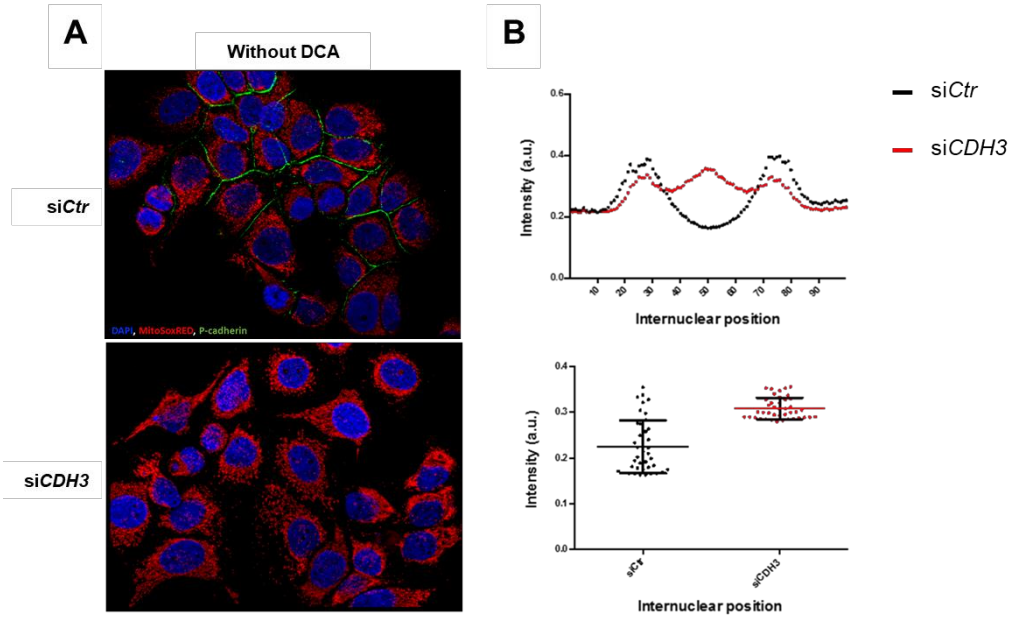

Figure 13. *CDH3* silenced TN-BLBC BT20 cells increases levels of mitochondrial derived ROS, namely superoxide anion. A) MitoSoxRed immunofluorescence of BT20 breast cancer cells with *CDH3* silencing demonstrates an increase and cytoplasmic spread of mitochondrial ROS, in comparison with the control cells. **B)** Internuclear fluorescence profiles of MitoSOX™ Red in TN-BLBC BT20 cells confirms the decrease in superoxide anion measured by MitoSOX™ Red immunofluorescence in comparison with the control cells. n=1. [immunofluorescence: DAPI (blue), MitoSoxRed (red), P-cadherin (green)].

Moreover, when cells were treated with DCA, we observed an increase of mitochondrial ROS, in comparison with the non-treated cells (**Figure 14**). Moreover, we were also able to observe the above described increase in mitochondrial ROS in *CDH3* silenced cells, whether treated or non-treated with DCA.

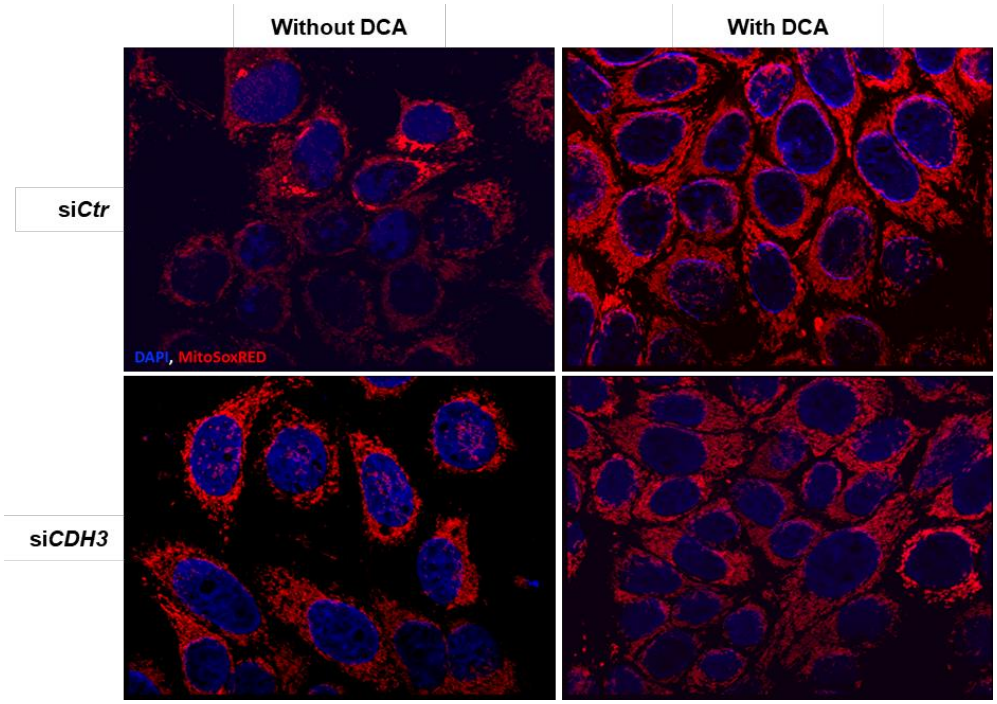

Figure 14. Mitochondrial ROS are increased upon *CDH3* downregulation and DCA treatment in TN-BLBC BT20 cells. DCA increases mitochondrial ROS in BT20 breast cancer cells. Moreover, our results show an increase in red fluorescence in *CDH3* downregulated cells, with or without DCA treatment. n=1 [immunofluorescence: DAPI (blue), MitoSoxRed (red)].

Although preliminary, our results show that P-cadherin silencing increases mitochondrial derived ROS production, suggesting that P-cadherin acts as a survival factor through the modulation of oxidative stress.

CHAPTER V

DISCUSSION

The results presented in this work provide novel insights about the role of P-cadherin in metabolic pathways, through the regulation of pPDH expression in breast cancer cells, as well as the possible clinical implications of P-cadherin expression in the response of breast cancer cells to DCA treatment.

Breast cancer is a heterogeneous disease with different biological behaviors, being divided into five distinct subtypes, according to different signatures and clinical outcomes, namely luminal A, luminal B, normal breast-like, HER-2 overexpressing and basal-like [51]. Over the past few years, our group has been describing several detailed functions of P-cadherin in breast cancer, a molecule which expression is differentially enriched in BLBC. These tumors are TNBC [127], associated with an aggressive behavior and worst patient prognosis [128, 129]. In this way, BLBC are characterized by the lack of ER and PgR, as well as the lack of HER-2 overexpression [130], which result in the resistance to anti-hormone therapies and HER-2 target therapies [131]. Histologically, BLBC are poorly differentiated carcinomas, present high nuclear and histological grade and frequently show medullary and metaplastic features, being the triple negative phenotype along with positivity for CK5 and/or EGFR the most suitable immunohistochemical criterion to identify these tumors [52, 132]. Interestingly, it has been reported that clinical features of breast tumors are reflections of specific glycometabolic pathways, attributing different metabolic behaviors to breast cancer molecular subtypes [133], which might contribute to a better orientation for clinical treatment [133]. Specifically, it has been described that BLBC present a stronger response to hypoxia, as well as a higher glycolytic metabolism than tumors with luminal characteristics, expressing hypoxic, glycolytic and acidosis markers, such as HIF-1α, GLUT1, CAIX, among others [55, 134]. Moreover, Dong *et al.* showed that the overexpression of PKM2, an important protein to control cell metabolism; glutamine-fructose-6-phosphate transaminase 1 (GFPT1), the first enzyme of hexosamine biosynthetic pathway; as well as G6PD, the first enzyme of PPP, were associated with poor prognosis of TNBC, as well as with worse response rate in neoadjuvant chemotherapy-treated breast cancer [133]. Interestingly, our group demonstrated that aberrant P-cadherin expression, a poor prognostic factor in breast cancer and a marker of BLBC, is associated with the hypoxic/glycolytic and acid resistant phenotype in breast cancer [125].

There are strong evidences that reversing the glycolytic metabolic behavior of cancer cells is a promising strategy that may offer a relative selectivity to target cancer cells [9]. Based on this concept, it has been described that DCA, by inhibiting PDK, decreases tumor growth in many cancer types, including breast cancer [39]. PDC is constituted by PDH and its regulatory proteins: PDK, which inhibits PDH by phosphorylation; and pyruvate dehydrogenase phosphatase (PDP), that activates PDH by dephosphorylation [135]. The phosphorylation of E1 subunit of PDH occurs at three specific serine residues, namely Ser 293 (site 1), Ser 300 (site 2) and Ser 232 (site 3) [136]. There are four PDK isoforms (1, 2, 3, 4), which differ in their intrinsic activity of PDH phosphorylation, tissue distribution, as well as in their sensitivity to the selective inhibitor drug, DCA [135]. Interestingly, each PDK isoforms is activated in response to different intracellular and extracellular conditions [137]. For instance, it has been demonstrated that liver-metastatic cells displayed increased HIF-1α, as well as increased PDK1, being this PDK isoform required for efficient liver metastasis in breast cancer patients [138]. Moreover, PDK2 is activated by acetyl-CoA and NADH, and inhibited by ADP (adenosine diphosphate); PDK3 is activated by ATP; and PDK4 is activated transcriptionally by hormonal signals, such as retinoic acid and glucocorticoids, being transcriptionally repressed by insulin [137].

There are only a few reports exploring the association of PDK isoforms with clinical and molecular features of breast cancer. Choi *et al.* studied the expression of several metabolism-related proteins such as GLUT-1, CAIX, PDK1, HIF-1α, among others, according to breast cancer molecular subtype, and found that PDK1 was not significant correlated with molecular subtypes of breast cancer [139].

In this work, we showed a positive correlation between P-cadherin and PDK1, as well as with PDHA1, in breast cancer samples database analysis. This finding is consistent with a CCLE analysis, showing increased expression of PDK1 in TNBC cell lines, specifically BT20 and MDA-MB-468 cells (data not shown).

It is described that, under hypoxia, HIF-1α activates PDK1 in order to direct pyruvate away from the mitochondria, to reduce mitochondrial oxygen consumption and decrease ROS production, helping cells to adapt to the hypoxic environment caused by oxygen deprivation [140]. In accordance, HIF-1α also induces an increase of membrane P-cadherin expression in breast cancer cells [125], as well as a reduction of ER, a known *CDH3* repressor [141].

Despite the fact that all four PDKs are able to phosphorylate PDH *in vitro*, PDK2 has the greatest activity to phosphorylate site 1, the most rapid and well-known mechanism for PDH inactivation, followed by PDK4, PDK1 and PDK3 [142]. Although PDK2 is ubiquitously expressed in different human tissues, while the other isoforms present tissue-specific distribution [42], this isoform is the most sensitive to pyruvate analog molecule, DCA [142]. Moreover, Sutendra *et al.* showed that DCA inhibit both PDK2 activity and HIF-1α in mammary carcinoma cells and that this isoform is associated with poor prognosis in breast cancer [143].

Through bioinformatic analysis (data not shown), we also observed an increased expression of *PDK1* isoform, and a slight increase of *PDK3* expression in ER negative breast cancer samples, in comparison with ER positive group. In contrast, *PDK2* and *PDK4* isoforms were increased in ER positive than in ER negative breast cancer. Still, we found that *PDHA1* presented an increased expression in ER negative breast cancer. We also observed a variability between the expression of the four *PDK* isoforms, as well as *PDHA1* gene in the breast cancer cell lines.

Despite the bioinformatic data presented and discussed in this work about PDK and PDH, nothing more is known about the association between P-cadherin and the expression of these key metabolic proteins in breast cancer.

P-CADHERIN-ENRICHED BREAST CANCER CELL LINES PRESENT INCREASED SENSITIVITY TO DCA

In the last decade, there is an increased recognition that metabolic reprograming is a result of an adaptation of cancer cells to environmental pressures. In this way, the upregulation of glycolysis and resistance to acid-induced apoptosis are cellular adaptations which support the proliferation and survival of cancer cells. Interestingly, it has been described that this cancer-specific metabolic remodeling can be reversed by DCA, in which by inhibiting PDK activity, leads to a reactivation of PDH, promoting the shift from glycolysis to oxidative metabolism [38, 40, 41]. Furthermore, it is described that DCA-induced metabolic reprograming occurs preferentially in glycolytic cancer cells, leading to a more oxidative phenotype and decreasing proliferation, while oxidative cells remain less sensitive to DCA treatment [43]. Currently, there are 7 clinical trials that studies the potential anticancer effect of DCA, including one specifically in breast cancer (clinicaltrials.gov). Despite that, the responses of cancer cells to DCA treatment have not been fully elucidated [38].

In this work, we demonstrate that TN-BLBC (MDA-MB-468 and SUM149) cells, as well as MCF-10A normal-like breast cells, are more sensitive to DCA, presenting lower DCA IC50 values, than luminal breast cancer cells. These results are in accordance with previous reports showing that TNBC cells are more glycolytic than luminal breast cancer cells [144], being more sensitive to DCA metabolic reprograming. Interestingly, since P-cadherin expression is enriched in TN-BLBC, our results also demonstrated that the cells with increased sensitivity to DCA were the ones presenting higher levels of P-cadherin expression. However, although BT20 breast cancer cells present high P-cadherin expression, these cells were the less sensitive to DCA induced cell death, in comparison with the others TN-BLBC cell lines used in this study. This result can be explained based on the preliminary and non-published observation from our group, where BT20 cells present a more oxidative behavior, similar to low P-cadherin expressing MCF-7 breast cancer cells, when compared with MDA-MB-468 and SUM149 breast cancer cell lines.
Moreover, in order to evaluate the efficacy of DCA in inhibiting PDK activity, we also evaluated the expression of the inactive form of PDH, pPDH, in our panel of breast cancer cell lines. Thereby, we demonstrated that 5mM of DCA is enough to induce a decrease in pPDH expression in BT20, SUM149, and MCF-10A cells. This is in accordance with the CCLE data, where we observed that BT20 breast cancer cell line present high levels of the most DCA sensitive isoform, *PDK2*. On the other hand, a higher DCA concentration is needed (25mM) in luminal MCF-7/Az breast cancer cells, in order to decrease pPDH levels, which is in line with their preferential oxidative behavior. Furthermore, MDA-MB-468 cells was the only breast cancer cell line with no alterations in pPDH levels induced by DCA, in accordance to their low PDK2 and high *PDHA1* expression levels from CCLE analysis.

P-CADHERIN EXPRESSION MODULATES THE EXPRESSION OF pPDH IN BREAST CANCER CELLS

BLBC present a glycolytic metabolism, expressing hypoxic and glycolytic markers [53, 145]. Also, the constitutive upregulation of glycolysis is likely to be a cellular adaption to hypoxic conditions in these tumors [35]. In this context, our group demonstrated, for the first time, the link between aberrant P-cadherin expression, a biomarker of BLBC, and the metabolic behavior of breast cancer cells [125]. In this report, P-cadherin expression is shown to be significantly associated with the expression of HIF-1α, GLUT1, CAIX, MCT1, as well as CD147 in human breast carcinomas [125]. Moreover, HIF-1α stabilization is accompanied by increased membrane P-cadherin expression, and that

the breast cancer cell fractions harboring high levels of P-cadherin are the same exhibiting a glycolytic and acid-resistant phenotype, presenting high amounts of GLUT1 and CAIX [125]. However, nothing more is known about the signaling between this adhesion molecule and the key metabolic proteins in breast cancer cells.

In this work, we observed that P-cadherin modulates the expression of the inactive form of PDH, pPDH. Specifically, we observed that overexpression of P-cadherin leads to an increase in expression of pPDH in these cells, either in 2D monolayers, as well as in anchorage-independent conditions. The increase of pPDH levels leads to a decrease in the flux of pyruvate into the TCA cycle, decreasing the respiration rate and possibly, a consequent increase of the glycolytic behavior of cells [39]. This result is not only in accordance with previous publications of our group showing that P-cadherin overexpression is associated with a glycolytic metabolism in breast cancer cells [125], but also with our unpublished data, where we observe that P-cadherin silencing decreases the extracellular acidification rate, a readout of a glycolytic impairment, in breast cancer cells. Moreover, we also observed a decrease in P-cadherin expression in MCF-7/Az cells induced by DCA, which might be due to the metabolic reprograming towards respiration, leading to a decrease in P-cadherin-enriched glycolytic cells. However, further studies are needed to evaluate if the alterations in PDH activity induces metabolic alterations that consequently decrease P-cadherin expression or whether it induces a direct effect in P-cadherin expression.

Furthermore, our group also demonstrate that breast cancer cells with increased P-cadherin expression, presenting a highly glycolytic behavior, is associated with increased cell motility, migration and invasion capacity [117, 118]. These cells present increased membrane transporters, namely CAIX and MCTs, in order to maintain pH homeostasis within the cells, inducing extracellular acidification. This acidification is known to facilitate *in vitro* cancer cell invasion and *in vivo* metastization [146].

Taking together, we demonstrate for the first time that P-cadherin expression modulates the levels of pPDH, which might be the mechanism responsible for the metabolic activity of this cell adhesion molecule in breast cancer cells. Still, since DCA induced a decrease in P-cadherin expression, we hypothesize that this metabolic modulator would also play a role in the impairment of aggressive properties mediated by P-cadherin expression in breast cancer cells.

P-CADHERIN EXPRESSION AS A VALUABLE BIOMARKER TO PREDICT THE RESPONSE TO DCA TREATMENT IN BREAST CANCER

Increasing body of evidences have been demonstrating that reversing the glycolytic metabolic behavior of cancer cells could be a promising strategy to target cancer cells in a selective way [9]. Accordingly, by inhibiting PDK, and consequently the activation of PDH, promoting OXPHOS in these cells, DCA decreases tumor growth in many cancer types, including breast cancer [39]. DCA-induced metabolic reprograming was already shown to eliminate preferentially BCSCs both *in vitro* and *in vivo* [80], suggesting that the decreased activity of PDH is responsible for the glycolytic behavior of BCSCs [80].

In this work, we demonstrate that P-cadherin expression modulates the sensitivity of BCSCs to DCA-induced cell death. We specifically showed that the silencing or the overexpression of P-cadherin, either decreases or increases, respectively, the sensitivity of BCSCs to DCA-induced breast cancer cells.

P-cadherin promotes stem-like properties to breast cancer cells and is recognized as a BCSC marker, being associated with the expression of CSC markers, such as CD44, CD49f and ALDH1 [101]. Although the reports about the metabolic behavior of cancer stem cells are increasing exponentially, there is still no consensus about their metabolic behavior. Some authors claim that normal and cancer cells with stem like properties use preferentially glycolysis over OXPHOS as their main source of energy [147, 148]. Specifically, there are reports showing that BCSCs present a glycolytic behavior, with higher ratio of lactate production to oxygen consumption, higher glucose consumption, as well as fewer and less active mitochondria than non-BCSCs [80, 82, 83]. Moreover, the use of metabolic modulators as a search for therapeutic applications against BCSC are being widely used also to demonstrate the glycolytic behavior of these cells [81]. For instance, Ciavardelli *et al.* demonstrated that treatment with 2-DG, an inhibitor of glycolysis, inhibits BCSCs proliferation, presenting a synergistic effect in BCSC death when in combination with doxorubicin [81].

In contrast, other authors claim that BCSCs present an increased OXPHOS behavior, with increased mitochondrial mass and mitochondrial functional activity [84-89]. Furthermore, Hirsch *et al.* described that the combination of metformin, a mitochondrial inhibitor of complex I, and doxorubicin kills BCSCs, as well as non-stem cancer cells in culture, reduces tumor mass and prolongs remission in a more effective manner than each drug alone [93]. Also, targeting BCSCs with atovaquone, a selective inhibitor of OXPHOS, induces apoptosis in these cancer cells population [92].

In this work, we demonstrate that, in 2D monolayer culture conditions, MCF-7/Az.P-cadherin breast cancer cells present higher levels of cell death when treated with two different concentrations of DCA, in comparison with the control MCF-7/Az.Mock cells. Moreover, in BT20 breast cancer model, *CDH3* downregulation presents increased resistance to DCA induced cell death. Interestingly, these results were more pronounced when the cells were cultured in anchorage-independent conditions, in both breast cancer models. In anchorage-independent conditions, we observed an increase of self-renewal in P-cadherin-overexpressing cells, MCF-7/Az.P-cadherin and BT20si*Ctr*, in comparison with the respective low P-cadherin-expressing counterparts, MCF-7/Az.Mock and BT20si*CDH3* cells. These results are in accordance with described P-cadherin's *anoikis*-promoting role previously reported by our group, where we demonstrate that P-cadherin-enriched breast cancer cell populations have increased ability to survive in *anoikis* promoting conditions [101]. Interestingly, we observed a decrease in MFE, when MCF-7/Az cells were treated with 50mM of DCA, being this effect higher in P-cadherin-enriched MCF-7/Az cells than in MCF-7/Az.Mock cells. The same results were observed in BT20 model, where 15mM of DCA induced a higher decrease in the number of mammospheres formed in BT20si*Ctr* cells, with high P-cadherin levels, in comparison with P-cadherin-silenced BT20 cells. Taking together, we demonstrate that the silencing of P-cadherin expression decreases the sensitivity of BCSCs to DCA-induced cell death, which suggests that P-cadherin enrichment dictates sensitivity of BCSCs to DCA-induced metabolic reprograming.

The effect of P-cadherin in the sensitization of DCA-induced breast cancer cell death being potentiated in anchorage-independent conditions can be explained by PDK modulation, as well as to the alterations in metabolic properties induced by these conditions. It is reported that untransformed human mammary cells are able to upregulate the expression of PDK upon matrix detachment [28]. Thus, cell detachment reprograms cells to rely on glycolysis by attenuating the flux of glycolysis-metabolites into mitochondrial oxidation. Importantly, altered glucose metabolism is a crucial factor in the modulation of *anoikis* sensitivity, in which decreased glucose oxidation confers *anoikis* resistance. Therefore, due to the Warburg effect, cancer cells have survival advantage in anchorage-independent conditions [28]. Thus, metabolic reprograming induced by DCA can restore cancer cells sensitivity to *anoikis* in matrix detachment conditions, impairing the metastatic potential of breast cancer cells in circulation.

The breast cancer heterogeneity is, among other factors, reflected in the metabolic behavior of cell population and their plasticity. However, there are reports describing that DCA-induced metabolic reprograming occurs preferentially in glycolytic cancer cells [43].

Accordingly, our results reinforce the role of P-cadherin as an inducer of glycolytic behavior of breast cancer cells, since P-cadherin-enriched cell populations are more sensitive to DCA. Furthermore, the enhanced effect of DCA observed in anchorage independent conditions, where BCSCs are selected, suggests that these BCSCs present preferentially a glycolytic behavior.

Thus, since our results shows that P-cadherin expression modulates PDH activity in breast cancer cells and, consequently, the metabolic behavior of cells, we hypothesize that P-cadherin overexpressing breast carcinomas, with stem-like properties and increased glycolytic behavior, would present better response to DCA induced metabolic reprograming and cell death. For this reason, we also hypothesized that P-cadherin expression in breast cancer might be a valuable biomarker to predict the response to DCA treatment in this type of cancer.

CDH3 DOWNREGULATION INCREASES MITOCHONDRIAL ROS IN TN-BLBC BT20 CELLS

ROS include several oxygen-containing molecules, with inherent chemical properties that confer reactivity to different biological targets [149]. Interestingly, in the last two decades, it has been described that ROS have double-faced role, since it drives proliferation, as well as apoptosis in cancer cells [150]. At early stages of cancer development, the antioxidant activity decreases and ROS promotes cancer initiation through the induction of DNA damage and genomic instability. On the other hand, at late stages, tumor cells increase their intracellular antioxidant systems, such as NADPH and glutathione, escaping to oxidative stress-induced apoptosis [151]. Therefore, in order to an antioxidant strategy be effective in cancer treatment, it must target ROS when they promote proliferation rather than apoptosis, i.e. early stage of the disease.

In addition to being highly proliferative, cancer cells are resistant to *anoikis*, a type of apoptotic cell death caused by the detachment of cells from the ECM. [28]. With the metabolic alterations caused by the Warburg effect, cancer cells have the advantage to avoid the production of excess ROS and overcome *anoikis*. Moreover, increased aerobic glycolysis also increase the glucose flux into the PPP, which is a major pathway to produce NADPH, a cellular antioxidant that protects cells against the oxidative stress. Taking together, by enabling cancer cells to avoid the overproduction of ROS and increasing their antioxidant systems, the Warburg effect maintains the redox

homeostasis, as well as it promotes resistance to oxidative stress induced *anoikis* and, subsequently, metastatic spread of cancer cells [152, 153].

In this work, we demonstrate an increase in mitochondrial derived ROS levels upon *CDH3* downregulation in TN-BLBC BT20 cells. This finding is in accordance with preliminary data from our group, showing that P-cadherin expression modulates oxidative stress in breast cancer cells, namely ROS levels, through the regulation of SOD1 and SOD2 expression and activity. Moreover, these results are also in accordance with the low levels of ROS and antioxidant systems described in BCSCs, where P-cadherin is known to play a role in the maintenance of their self-renewal [81, 82, 101]. Thus, some authors claim that normal and cancer cells with stem-like properties exhibit increased adaptation to oxidative stress, with enhanced antioxidant protective systems and low levels of ROS [147, 148]. It is known that breast cancer cells, including BCSCs, present increased antioxidant defenses and low levels of ROS, allowing them to escape to oxidative stress induced *anoikis* [47, 80]. Diehn *et al*. observed that normal mammary epithelial stem cells, as well as BCSCs, present lower levels of ROS when compared with their corresponding mature progeny cells and non-tumorigenic cells, respectively [26].

Some chemotherapeutic agents, as well as radiotherapy, induces cancer cell death, in part, by the production of free radicals [154]. In this context, Phillips *et al.* showed that BCSCs are a radio-resistant subpopulation of breast cancer cells, which increase in number after short courses of fractionated irradiation [29]. Thus, the rapid repopulation of cancer cells observed during gaps in radiotherapy might be due to the presence of CSCs, which could be responsible for recurrence, metastasis and therapeutic resistance [29]. In this way, it is possible that the therapeutic resistance presented by these cells might be mediated by their increased capacity to overcome intracellular oxidative stress levels [155].

In this work, we were also able to observe that BT20si*Ctr* cells present a pronounced peri-nuclear staining, while BT20si*CDH3* cells present a more spread pattern of mitochondrial ROS staining. Since it is known that P-cadherin is involved in the maintenance of stem-like properties in BCSCs, as well as it is associated with the expression of CSC markers, such as CD44, CD49f and ALDH1 [101], this result is in line with reports suggesting that peri-nuclear mitochondrial arrangement is an indicator of stemness [156-158].

Finally, our findings also suggest that P-cadherin acts as survival factor [119], due to its role in the modulation of oxidative stress. Since ROS are generated mainly as a

byproduct of OXPHOS, through the incomplete reduction of oxygen, and DCA promotes the entrance of pyruvate into the TCA and subsequently its mitochondrial oxidation [40], this drug promotes the production of mitochondrial derived ROS. The production of these ROS can ultimately trigger caspase activation and apoptosis through the release of cytochrome c and other pro-apoptotic proteins [159]. Accordingly, Sutendra *et al.* demonstrated that DCA decrease mitochondrial membrane potential and increase mitochondrial ROS, PDH activity, as well as mitochondrial respiration in mammary carcinoma cells [143]. In this work, we observed an increase in mitochondrial derived ROS upon DCA treatment in comparison with non-treated cells, whether with and without *CDH3* downregulation. Since the immunofluorescence analysis is not a quantitative technique, these results need to be further quantified and validated.

CHAPTER VI

CONCLUSIONS

The results presented and discussed in this work allowed us to conclude that:

1. **Breast cancer cell lines enriched for P-cadherin are more sensitive to DCA-induced metabolic reprograming and cell death than the ones with low P-cadherin expression levels, probably due to their increased glycolytic behavior.** Using Presto Blue fluorescence analysis, we were able to demonstrate that P-cadherin-enriched breast cancer cell lines present a lower DCA IC50 value in comparison with the ones expressing low levels of this adhesion molecule.

2. **P-cadherin expression modulates the expression of pPDH in breast cancer cells.** Using Western blot analysis, we showed that P-cadherin expression increases the levels of pPDH, the inactive form of PDH, and, in contrast, P-cadherin downregulation decreases pPDH expression, either with and without DCA treatment.

3. **P-cadherin expression sensitizes breast cancer cells to DCA-induced BCSC *anoikis*.** Through the MFE assay in anchorage independent conditions, we observed that breast cancer cells with increased P-cadherin expression have a higher response to DCA-induced cell death than the ones with low P-cadherin expression. Therefore, we hypothesized that P-cadherin expression might be a valuable biomarker to predict the response to DCA treatment in breast cancer patients.

4. **P-cadherin downregulation induces an increase of mitochondrial derived ROS production in TN-BLBC BT20 cells.** Therefore, we hypothesized that P-cadherin is a survival factor in breast cancer cells probably due to its role in the regulation of oxidative stress in these cells.

CHAPTER VII

PROPOSED MODEL

P-cadherin-enriched breast cancer cells are more likely to present a glycolytic and acid resistant phenotype [125], being enriched in glycolytic transporters, such as GLUT1, allowing the entrance of glucose into the cells, which is then converted into lactate, and transported for the extracellular medium by MCT1 transporter [53]. In order to maintain the intracellular pH, CAIX is upregulated as well in these cells, contributing for cancer cell survival [54]. Moreover, preliminary data from our group suggest that P-cadherin expression plays a role in the modulation of oxidative stress, by increasing the expression and activity of scavenging systems, such as SOD1 and SOD2, respectively, decreasing the levels of ROS in BCSCs (**Figure 15**).

In this work, we demonstrate for the first time that P-cadherin has a role in the modulation of PDH levels in BCSCs, by increasing the inactive form of this protein, pPDH, contributing for the glycolytic behavior, as well as for the survival of these cells. Thereby, and since DCA-induced metabolic reprograming occurs preferentially in glycolytic cancer cells [43], the treatment of BCSCs with DCA, an inhibitor of PDK, activates PDH and reverses the glycolytic phenotype of these cells, by increasing their mitochondrial function and, consequently, the production of mitochondrial ROS, decreasing the survival of BCSCs (**Figure 15**).

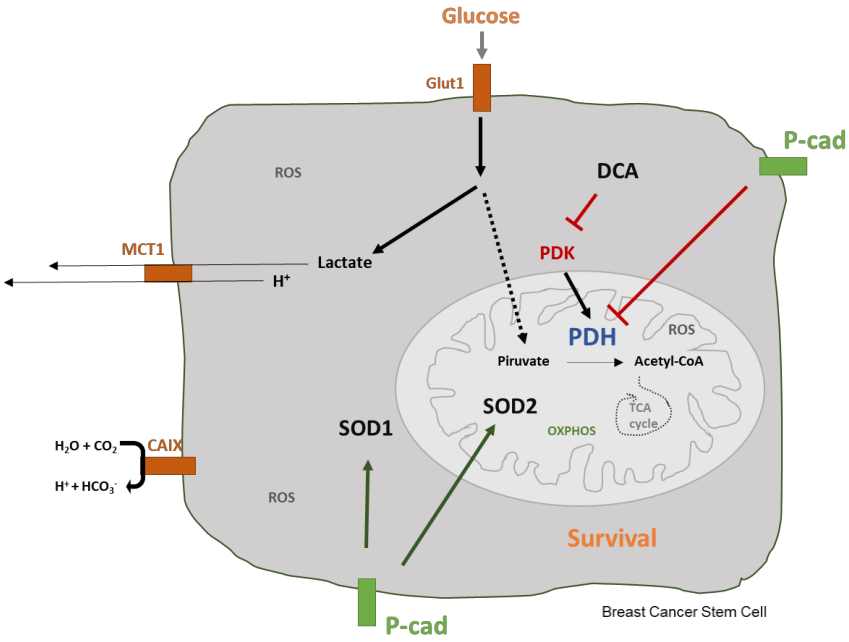

Figure 15. Schematic representation of the proposed model of this work. P-cadherin has a role in the modulation of PDH levels, by increasing the inactive form of this protein, contributing for the glycolytic behavior, as well as for the survival of BCSCs. When these cells are treated with DCA, PDK is inhibited, activating PDH and cells rely on oxidative phosphorylation, increasing the production of ROS and decreasing the survival of BCSCs.

CHAPTER VIII

FUTURE PERSPECTIVES

In the last few years, several oncogenic effects have been attributed to P-cadherin expression in a breast cancer context. Importantly, this molecule plays a role as a metabolic modulator, a new emerging hallmark of cancer. This work reinforces the role of P-cadherin as a metabolic modulator, as well as describes for the first time this molecule as a biomarker of the response of DCA-induced metabolic reprograming, through its ability to modulate PDK/PDH signaling. Although some data needs to be further validated, this work raises many interesting questions and challenges that remain to be elucidated. Thus, additional questions should be addressed in future studies:

1. Understand the combinatory effect of DCA-induced metabolic reprograming with oxidative stress induced by a chemotherapeutic agent widely used for breast cancer treatment, such as doxorubicin.

2. Evaluate the role of P-cadherin as a biomarker of resistance to oxidative stress induced by chemo- and radio-therapy in breast cancer cells.

3. Validate the *in vitro* findings described in this work, using *in vivo* models.

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