
Monocarboxylate Transporters (MCTs) in breast cancer aggressiveness: therapeutic targeting and regulatory mechanisms
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Luísa Filipa Morais dos Santos
UMinho | 2017

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Tese de Doutoramento em Ciências da Saúde

Trabalho efetuado sob a orientação da
**Doutora Maria de Fátima Monginho Baltazar**
e da
**Doutora Céline Marques Pinheiro**

abril de 2017

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I hereby declare having conducted my thesis with integrity. I confirmed that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration.

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The work presented in this thesis was performed in the Life and Health Sciences Research Institute (ICVS), Minho University. Financial support was provided by grants from the Fundação para Ciência e Tecnologia (FCT) through individual fellowship (SFRH / BD / 87139 / 2012), by FEDER funds through the Operational Programme Competitiveness Factors - COMPETE and National Funds through FCT - Foundation for Science and Technology under the project POCI-01-0145-FEDER-007038; and by the project NORTE-01-0145-FEDER-000013, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF).

# ACKNOWLEDGEMENTS/AGRADECIMENTOS

## Acknowledgements/Agradecimentos

Gostaria de começar por agradecer à Professora Doutora Fátima Baltazar, minha orientadora, pela oportunidade que me deu em 2010 como bolseira de investigação. Durante estes seis anos foram muitas as vivências e por isso agradeço toda a confiança que depositou no meu trabalho, todos os conhecimentos que partilhou, as palavras amigas, a força que me deu, toda a disponibilidade, por toda a compreensão, simpatia e amizade… Muito Obrigada.

À Professora Doutora Céline Pinheiro, que na qualidade de orientadora desta tese, partilhou os seus ensinamentos e conselhos científicos. Um obrigado por todo o apoio e confiança que depositou em mim, pois mesmo longe pela distância sempre esteve presente e disponível para ajudar. Por todo o companheirismo e a amizade, um muito obrigado.

À Professora Doutora Cecília Leão, presidente da Escola de Medicina, e ao Professor Doutor Jorge Pedrosa, diretor do Instituto de Investigação em Ciência da Vida e da Saúde (ICVS), agradeço a oportunidade e pelas excelentes condições de trabalho que proporcionam a todos os seus colaboradores. Tem sido uma honra trabalhar no ICVS durante estes seis anos!

A todos os membros do domínio de investigação em Ciências Cirúrgicas, em especial ao coordenador Doutor Jorge Correia-Pinto. Obrigada pelo ambiente de trabalho que me proporcionou e por todos os momentos científicos.

A todos os meus colegas de laboratório, que foram sem dúvida um pilar importante nesta produção científica! Obrigada por todo o carinho, amizade, e conselhos … Em especial, à Céline, à Olga, à Vera, à Sara, à Sandra, à Marta VP, Marta Pojo, Marta Costa, Joana, Céline Gonçalves, Ana Xavier, Ana Oliveira, Bruno e Filipe.

Obrigada por todos os momentos de boa disposição quer no laboratório, quer fora dele, toda a paciência e partilha de conhecimentos.

Um agradecimento ainda mais especial às minhas queridas amigas Sara, Sandra e Ana Paula, pela vossa amizade, todo o apoio que me dão e por estarem sempre comigo, mesmo que longe!

A todos os meus amigos, um muito obrigado pela compreensão na falta de tempo para partilhar com eles e pelo apoio que sempre me deram!

Um especial agradecimento à minha família, em particular aos meus pais e à minha irmã Rita, porque sempre acreditaram em mim! Pela compreensão que sempre tiveram quando "trocava" o trabalho pela sua companhia, pelos sacrifícios que fizeram para poder chegar aqui, pela amizade, carinho, apoio e amor…. Muito Obrigada!

Por fim, ao Luís, amigo, namorado e futuro marido, pela compreensão e paciência, por esperares por mim depois de mais um dia longo de trabalho, pelos conselhos, por me ouvires… Por estares sempre ao meu lado em todos os bons e maus momentos e por nunca me deixares desistir!

*Para ti, estrelinha, que partiste cedo de mais e não me viste terminar aquilo de que tanto falavas… e que te levou…*

“Nada é difícil se for dividido em pequenas partes.”

Henry Ford

# ABSTRACT/RESUMO

**Abstract**

Breast cancer is a heterogeneous and multifaceted disease, being the most common cancer among women and the second leading cause of cancer-related death. Classified into distinct biological subtypes and with different prognostic implications, basal-like subtype is considered the most aggressive and, so far, with no targeted therapy available.

Referred as a hallmark of cancer, the "Warburg Effect" contributes to tumour malignancy mainly due to the high rates of lactate produced as a consequence of metabolic reprogramming. In this phenomenon, even in the presence of oxygen, cancer cells obtain energy mainly from glycolysis, increasing the levels of glucose uptake, which culminates in the production of high amounts of lactate that is transported across the plasma membrane through Monocarboxylate Transporters (MCTs). Several studies reported the upregulation of MCTs in different tumour types, including breast cancer, in which MCT1 overexpression was associated with basal-like breast carcinoma. However, the potential of MCTs as therapeutic targets and especially the regulation of these proteins in breast cancer have been little explored.

Thus, in this work, inhibition of MCT activity was accomplished using the MCT inhibitors α-cyano-4-hydroxycinnamate (CHC), quercetin and lonidamine, in a panel of breast cancer cell lines. The results evidenced that lactate transport inhibition decreased cell proliferation, migration and invasion, promoting cell death, mainly due to MCT1 inhibition, in the most sensitive cell lines. Additionally, downregulation of MCT1 and MCT4 by siRNA, in basal-like breast cancer cells, was performed in hypoxia and normoxia conditions, and showed that MCT knockdown decreased tumour cell aggressiveness, and, importantly, disrupted *in vivo* tumour formation and growth.

Furthermore, considering the enhancers of breast cancer progression, estrogen receptor (ER) signalling, Epithelial Mesenchymal Transition (EMT) and cancer microenvironment, we explored the effect of these players in the metabolic profile of breast cancer. We showed that low levels of glucose and exogenous lactate modulated

MCT expression, as well stimulation of a glycolytic profile by the EMT inducer TGF-β, in basal-like breast cancer cells. In the luminal breast cancer cells, stimulation of the ER pathway seems to not alter mRNA or protein levels of MCT1, MCT4, CD147, HKII and HIF-1α proteins, but, in contrast, increases lactate production.

This work provides novel evidence for the role and regulation of MCTs in breast cancer, pointing at these proteins as major players in breast cancer aggressiveness. Also, the results of MCT disruption *in vitro* and *in vivo*, support these proteins as targets in breast cancer therapy.

**Keywords:** Glycolytic phenotype; Monocarboxylate Transporters; Breast cancer; Targeting; Regulation.

**Resumo**

O cancro da mama é uma doença heterogénea e multifacetada, sendo o tipo de cancro mais frequente nas mulheres e a segunda causa de morte relacionada com esta doença. Classificada em diversos subtipos, com diferentes implicações de prognóstico, o subtipo basal é considerado o mais agressivo e, neste momento, sem terapias dirigidas disponíveis.

Referido como uma das características das células tumorais, o "efeito de Warburg" contribui para a malignidade dos tumores principalmente devido aos elevados níveis de lactato produzidos. Mesmo na presença de oxigénio, as células tumorais obtêm energia principalmente a partir da glicólise, elevando os níveis de glucose consumida e levando à produção exacerbada de lactato o qual é transportado através da membrana plasmática pelos Transportadores de Monocarboxilatos (MCTs). Vários estudos referem a expressão aumentada dos MCTs em diferentes tipos de tumores, incluindo o cancro da mama, no qual a sobre-expressão do MCT1 foi associada com o fenótipo mais agressivo (tipo basal). Contudo, e apesar dos vários estudos, o potencial dos MCTs como alvos terapêuticos e em especial a sua regulação no cancro da mama têm sido pouco explorados.

Assim, neste trabalho, foi efetuada a inibição dos MCTs, utilizando para isso os inibidores destes transportadores α-cyano-4-hydroxycinnamate (CHC), quercetina e lonidamina, em linhas celulares de carcinoma da mama. Os resultados obtidos evidenciaram que a inibição do transporte de lactato diminuiu a proliferação, migração e invasão celular, promovendo a morte celular, devido essencialmente à inibição do MCT1. Além disso, foi feita a inibição da expressão do MCT1 e MCT4 nas células de carcinoma da mama do tipo basal, utilizando siRNA, em condições de hipóxia e normóxia. Deste modo, mostrou-se que a inibição da expressão dos MCTs, diminuiu a agressividade tumoral, mostrando-se eficaz na disrupção da formação e crescimento tumoral *in vivo*.

Adicionalmente, uma vez que o MCT1 se encontra aumentado no carcinoma da mama, e tendo em conta os vários fatores que influenciam a progressão deste carcinoma, como sinalização do recetor de estrogénio (ER), a transição epitélio-mesenquimal (EMT) e o metabolismo glicolítico, foi explorada a influência destes fatores na regulação do perfil metabólico do carcinoma da mama. Os resultados mostraram que os baixos níveis de glucose e o lactato exógeno modularam a expressão dos MCTs, tal como a estimulação da EMT, nas células basais do carcinoma da mama. Nas células do tipo luminal MCF7, a estimulação da via ER parece não ter alterado os níveis de mRNA e de proteína dos MCT1, MCT4, CD147, HKII e HIF-1α, contudo, aumentou a produção de lactato.

Este trabalho apoia a utilização dos MCTs como alvos terapêuticos no carcinoma da mama, apresentando novas evidências sobre o seu papel e regulação. Os resultados da sua inibição *in vitro* e *in vivo*, demonstra também o seu papel como importantes intervenientes na agressividade do carcinoma da mama.

**Palavras chave:** Fenótipo glicolítico; Transportadores de Monocarboxilatos; Carcinoma da mama; Alvos terapêuticos; Regulação.

CONTENTS

# Contents

# ABBREVIATIONS

## Abbreviations

**AE** - anion exchanger

**AMP** - 5’ monophosphate

**AMPK** - 5’ monophosphate protein kinase

**ATM** - serine/threonine kinase gene

**ATP** - adenosine triphosphate

**BBB** - blood-brain barrier

**BRCA1** - breast cancer 1

**BRCA2** - breast cancer 2

**BSG** - basigin

**CAIX** - carbonic anhydrase IX

**CDH1** - E-cadherin gene

**CHC** - α-cyano-4-hydroxycinnamate

**CHEK2** - checkpoint kinase 2 gene

**CHO** - chinese hamster ovary

**CK5/6** - basal markers cytokeratin 5/6

**CPE** - choroid plexus epithelium

**CTLs** - cytotoxic T lymphocytes

**DBDS** - 4,4’-dibenzamidostilbene-2,2’-disulphonate

**DCIS** - ductal carcinoma *in situ*

**DIDS** - 4,4’-diisothiocyanostilbene-2,2’-disulphonate

**E2**- 17β-Estradiol

**ECM** - extracellular matrix

**EGFR** - epidermal growth factor receptor

**EMT**- epithelial-mesenchymal transition

**ER** - estrogen receptor

**ERK1/2** - extracellular signal-related kinase

**FDG** - 2-(18F)-fluoro-2-deoxy-D-glucose

**FGFR** - fibroblast growth factor receptor

**GLUT1** - glucose transporter 1

**GLUT4** - glucose transporter 4

**HER2** - human epidermal growth factor receptor 2

**HIF-1α** - hypoxia inducible factor 1 alpha

**HKII** - hexokinase II

**HRE** - hypoxia response element

**IBC** - inflammatory breast cancer

**IDC** - Invasive ductal carcinoma

**IGF-1** - insulin-like growth factor 1

**IGF1R** - insulin-like growth factor-1 receptor

**IL-8** – interleukine 8

**ILC** - invasive lobular carcinoma

**LAT1** – L-type amino acid transporter 1

**LCIS** - lobular carcinoma *in situ*

**LDH-A** - lactate dehydrogenase-A

**MCTs** - monocarboxylate transporters

**MMP** - matrix metalloproteinases

**NHE1** - $Na^+/H^+$ exchanger 1

**NPPB** - 5-nitro-2-(3-phenylpropylamino)-benzoate

**NSAIDs** - nonsteroidal anti-inflammatory drugs

**OXPHOS** - oxidative phosphorylation

**PALB2** - partner and localizer of BRCA2 gene

**p-CMBS** - p-chloromercuribenzenesulfonic acid

**PDK1** - pyruvate dehydrogenase kinase 1

**PDH** - pyruvate dehydrogenase

**PET** - positron emission tomography

**PFK-1** - phospho-fructokinase-1

**PGC-1α** - peroxisome proliferator-activated receptor-gamma co-activator 1 alpha

**pHe** - extracellular pH

**pHi** - intracellular pH

**PK** - pyruvate kinase

**PPARα** - peroxisome proliferator-activated receptor alpha

**PPARγ** - peroxisome proliferator-activated receptor gamma

**PPIs -** proton pump inhibitors

**PPP** - pentose phosphate pathway

**PR** - progesterone receptor

**PTEN** - phosphatase and tensin homolog gene

**ROS** - reactive species of oxygen

**RPE** - retinal pigment epithelium

**siRNA** - small interference RNA

**SLC16** - solute carrier family 16

**T3** - triiodothyronine

**T4** - thyroxine

**TCA** - tricarboxylic acid cycle

**TGF-β -** transforming growth factor beta

**TMDs** - alpha-helical transmembrane domains

**TNBC** - triple negative breast cancer

**TP53** - tumour protein p53 gene

**TSH** - thyroid stimulating hormone

**UTR** - untranslated region

**V-ATPase** - vacuolar-type $H^+$-ATPases

**VEGF** - vascular endothelial growth factor

# AIMS AND THESIS LAYOUT

## Aims

Metabolic reprograming was recently associated with cancer progression, being recognized as a hallmark of cancer. Importantly, the high amounts of lactate produced by this phenomenon are transported out of the cell by Monocarboxylate Transporters (MCTs), promoting acidification of the tumour microenvironment and enhancing tumour aggressiveness. Thus, several studies have reported upregulation of MCTs in several cancer types, including breast cancer, and, consequently, MCTs are considered as promising targets in cancer therapy.

Although the importance of MCTs as therapeutic targets in cancer has been widely discussed, their role in breast cancer is still poorly understood. Thus, the main aim of this thesis is to explore MCTs as therapeutic targets and understand their regulation in breast cancer.

To achieve the main goal, the work was subdivided into the following specific aims:

- To determine the effects of the inhibition of MCT activity in breast cancer cells, using *in vitro* models;
- To evaluate the impact of MCT downregulation in breast cancer, using *in vitro* and *in vivo* models;
- To identify putative MCT regulatory mechanisms in breast cancer cells, using *in vitro* models.

## Thesis layout

The present thesis is divided into four chapters:

**Chapter 1** contains a general introduction with a review of the literature, comprising a description of breast cancer, characterization of metabolism of normal and cancer cells, including glycolytic metabolism, and tumour microenvironment characterization, and finally a review about MCTs, the main molecules explored in this thesis.

**Chapter 2** is composed by two studies, published in international peer reviewed journals. In the first one, inhibition of MCT activity in breast cancer cells was evaluated using classical and non-classical MCT inhibitors, and, in the second one, the impact of downregulation of MCT expression on tumour aggressiveness was evaluated also in *in vitro* models, but, most importantly, in *in vivo* models (mouse xenografts).

**Chapter 3** is composed by unpublished results, exploring putative mechanisms of MCT regulation in breast cancer.

**Chapter 4** contains a general discussion of the results obtained in this work, and a general conclusion.

# CHAPTER 1: GENERAL INTRODUCTION

## 1.1. Breast cancer

The normal breast is composed by lobes and ducts. Each breast contains 15-20 lobes, and each lobe presents many smaller sections designated as lobules, which function is milk production, being all these structures linked by ducts (Figure 1). Breast tissue is also composed by blood vessels, lymph vessels, and surrounding stroma, which comprises more than 80% of the breast volume, providing nutrition and structural support to the normal epithelium [1].

Breast cancer is characterized by a molecular and clinical heterogeneity, and has been recognized as a multifaceted disease. This tumour type presents distinct biological subtypes, with a diverse range of clinical, pathological and molecular features, and different prognostic and therapeutic implications [2-4]. Breast cancer was still the most common diagnosed malignancy in 2016 and is the second cause of cancer-related death in woman [5].

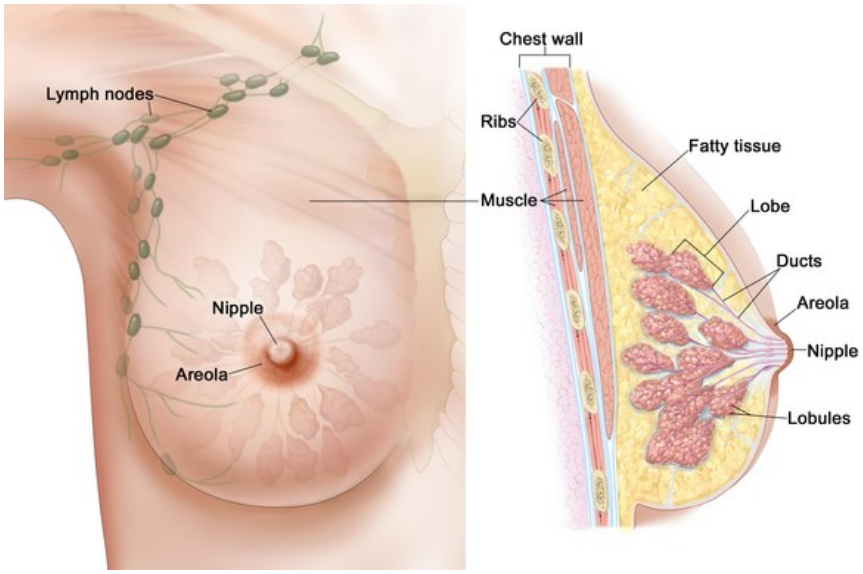


***Figure 1***- Anatomy of female breast [6].

Most of breast cancer types derive from epithelial cells, and so are classified as carcinomas, being adenocarcinomas, which start in the glandular tissue, a very common type. In fact, a single breast cancer can be a combination of different types of breast

cancer cells, with different origins, different grade of invasiveness or different molecular features [5, 7]. Although most of breast cancers derive from epithelial cells, there is evidence indicating that stroma plays an important role in cancer initiation and progression [1].

### 1.1.1. Breast cancer types and risk factors

Ductal carcinoma *in situ* (DCIS) originates from duct cells and is considered as non-invasive breast cancer, being cancer cells confined to the ducts, without invading the surrounding tissue. In contrast, invasive ductal carcinoma (IDC) has the same origin, but cancer cells are able to invade the surrounding breast tissues, like fat tissue (Figure 2). Invasive cancer cells are able to spread to other parts of the body through the lymphatic system, originating metastases. This is the most frequent type of breast cancer, and around 8 of 10 invasive breast cancers are IDC. Another type of breast cancer is the invasive lobular carcinoma (ILC), which starts in the lobules, and these cancer cells are also able to originate metastases (Figure 2). One in 10 cases of breast cancer is ILC, being its detection more difficult. Within the less common breast cancer types is the inflammatory breast cancer (IBC), an invasive type of breast cancer, with an incidence around 1-3% of all breast cancers. Other invasive cancer types like adenoid cystic carcinoma, low-grade adenosquamous carcinoma, medullary carcinoma, and papillary carcinoma are less frequent and, in general, have a better prognosis than IDC. Metaplastic carcinoma, micropapillary carcinoma, and mixed carcinoma are also less frequent but present worse prognosis than IDC [1, 5, 7-9].

It is known that 5-10% of breast cancer cases are hereditary, being in the majority of cases associated with *BRCA1* and *BRCA2* (Breast Cancer 1 and 2) gene mutations, and less commonly associated with mutations in *TP53* (Tumour Protein p53), *PTEN* (Phosphatase and Tensin Homolog), *CDH1* (E-cadherin), *ATM* (serine/threonine kinase), *CHEK2* (Checkpoint Kinase 2) or *PALB2* (Partner and Localizer of BRCA2) tumour suppression genes, among others [10]. Around 20-25% of familial breast cancer cases are caused by mutations in *BRCA1* or *BRCA2* genes, which increase the risk of breast and ovarian cancer, being *BRCA1* mutation associated with a risk two times higher than

*BRCA2* mutation [11]. Women who have these mutations tend to develop breast cancer at younger ages than women without these mutations, and, normally, breast cancer is found in both breasts [12].

Around 85% of breast cancers are diagnosed as sporadic cancers, occurring in woman with no familial history of breast cancer, which result from accumulation of several mutations in somatic genes, as a result of the aging process and lifestyle in general [10, 13, 14]. Many risk factors have been studied, and is known that about 40% of all breast cancer cases are linked to age, reproductive history, hormonal *status*, family history, lifestyle, and environmental factors [5, 14, 15].

Although the factors causing breast cancer can be useful to stratify patients and treatment options, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) *status* have demonstrated to be more useful to predict prognosis and treatment options [3, 4].

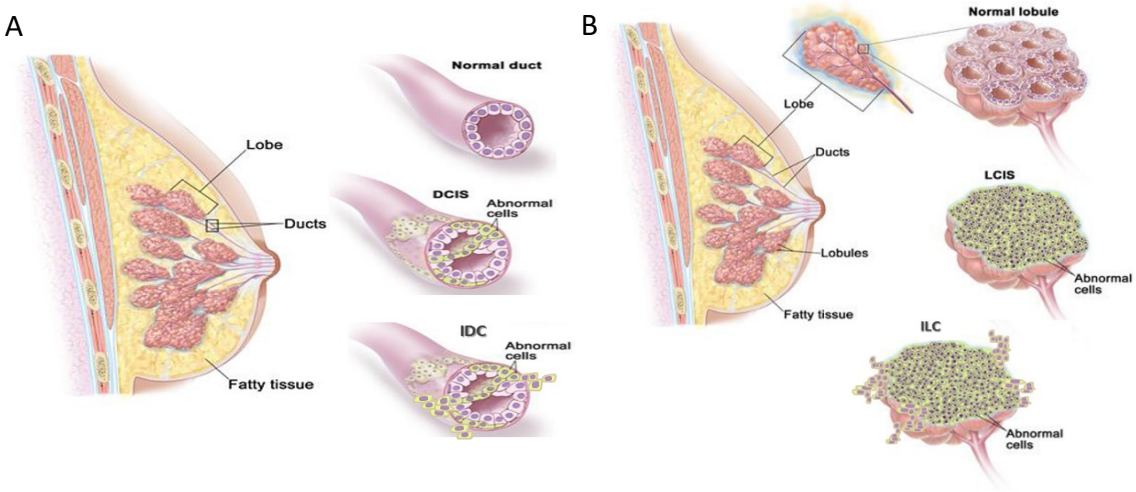


***Figure 2***- Stages of Ductal Carcinoma *in Situ* (DCIS) (A) and Lobular Carcinoma *in Situ* (LCIS) (B). IDC (Invasive Ductal Carcinoma); ILC (Invasive Lobular Carcinoma). Adapted from [16].

### 1.1.2. Molecular subtypes

Independently from the different types of breast cancer, based on the origin and organization of cancer cells under the microscope, breast cancer has been classified according to its gene profile into five major subtypes [17, 18]. The luminal subtype is one of the most common subtypes, being less invasive and presenting a more favourable

prognosis than the other subtypes. The luminal subtype is divided into luminal A (ER+ and/or PR+, HER2-) and luminal B (ER+ and/or PR+, HER2+). The HER2+ subtype is characterized by absence of ER and PR and presence of HER2, while triple negative breast cancer (TNBC) is characterized by absence of positivity for ER, PR and HER2. In fact, many authors refer basal-like subtype as TNBC, and many times these two subtypes are mentioned as being the same. However, basal-like subtype, besides not presenting ER, PR, and HER2 expression, is also characterized by presence of the basal markers cytokeratin 5/6 (CK5/6) and/or epidermal growth factor receptor (EGFR). It is reported that not all TNBC express basal markers, being positivity for basal markers generally associated with worse prognosis than TNBC itself [2, 12, 19]. These molecular signatures have been useful to indicate the prognosis and also to decide treatment options, being patients with luminal A subtype the ones who have the best prognosis and patients with basal-like the worst prognosis (Figure 3) [12, 17, 18, 20].

The fifth subtype is designated as normal-like, and presents, most of the times, normal cells associated with tumour cells [12, 17, 18, 20].

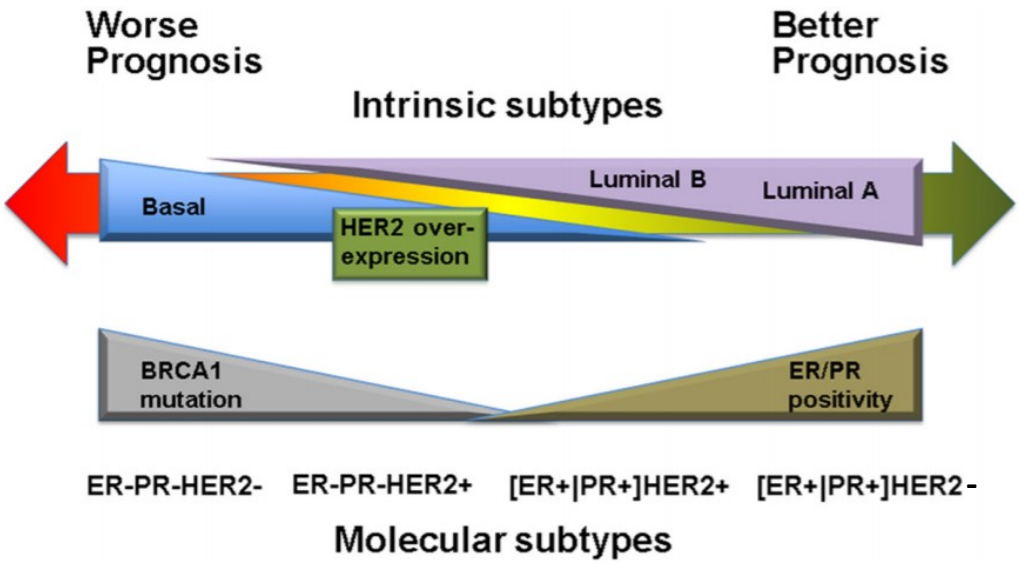


***Figure 3***- Patient outcome based in molecular breast cancer subtypes [12].

### 1.1.3. Breast cancer therapy

Currently, breast cancer patients can be separated according to treatment options, however, only patients with luminal and HER2+ subtypes can benefit from targeted therapy, while TNBC and basal-like only have chemotherapy and local treatments as options [14, 18, 21, 22]. Being TNBC responsible for 10-30% of all breast cancers, and associated with younger age, higher stage at diagnosis, and poor prognosis, the need for more specific therapies for this subtype is urgent [5, 19, 22].

Local treatments, like surgery and radiation therapy, could be good options for breast cancer at early stages. Surgery can include only removal of the part of the breast containing the tumour (breast-conserving surgery) or radical mastectomy, in which the entire breast is removed. Radiation therapy is frequently useful after breast-conserving surgery or mastectomy [7].

Systemic treatments are frequently used and can involve chemotherapy, hormone therapy and targeted therapy, being common the combination of these different types of treatment [7]. Chemotherapy can be administrated after surgery (adjuvant chemotherapy) or before surgery (neoadjuvant chemotherapy). Chemotherapy is especially used when breast cancers are very extensive and in advanced breast cancers, in which breast cancer has already metastasis. Currently, antracyclines (e.g. doxorubicin and epirubicin), taxanes (e.g. paclitaxel and docetaxel), the antimetabolite drug 5-fluorouracil, alkylating agents (e.g. cyclophosphamide), and platinum drugs (e.g. carboplatin), are often used in combination of 2 or 3 drugs, and in breast cancer cases with no indication for targeted therapies [23]. Tumours that present positivity for hormonal receptors, like ER and PR that are stimulated by estrogen or progesterone for breast cancer growth, benefit from targeted therapy like aromatase inhibitors, which block estrogen production, or tamoxifen and fulvestrant, which block ER signalling. In HER2 positive cancers, monoclonal antibodies that block this receptor, like trastuzumab and pertuzumab, as well as small molecule inhibitors, like lapatinib, are used [7, 24, 25].

## 1.2. Cell metabolism

Cell metabolism is a highly synchronized cellular process in which several metabolic pathways cooperate to convert nutrients into other molecules essential for specific cellular functions [26]. Differentiated tissues and quiescent cells obtain most of the adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS), which follows the oxidation of pyruvate into the tricarboxylic acid cycle (TCA), after the conversion of glucose into pyruvate during glycolysis [26, 27].

Glycolysis is a cascade of enzymatic reactions that converts one molecule of glucose in two molecules of pyruvate, which still contain most of the chemical potential energy of glucose [26, 28]. Glucose enters the cell by uptake through glucose transporters (GLUTs), and is then converted into glucose-6-phosphate by hexokinase (HK), following the conversion into pyruvate through different reactions, generating a net amount of 2 molecules of ATP per glucose. In the presence of oxygen (aerobic conditions), glycolysis is the first stage of complete degradation of glucose. After, oxidation of pyruvate into acetyl-CoA occurs followed by the TCA cycle in the mitochondria. In this situation, NADH, an OXPHOS fuel to maximize the production of ATP, is produced, with minimal production of lactate (Figure 4) [27, 29]. Exceptionally, in the absence of oxygen (anoxia) or under low oxygen levels (hypoxia), differentiated cells are able to reduce pyruvate into lactate through lactic acid fermentation, transferring electrons from NADH and regenerating $NAD^+$ essential for glycolysis to carry on [29]. In this case, the lactate produced needs to be transported out of the cells by monocarboxylate transporters (MCTs) [27, 30]. In both cases, there is production of protons, which must be exported out of the cell, leading to extracellular acidification [27].

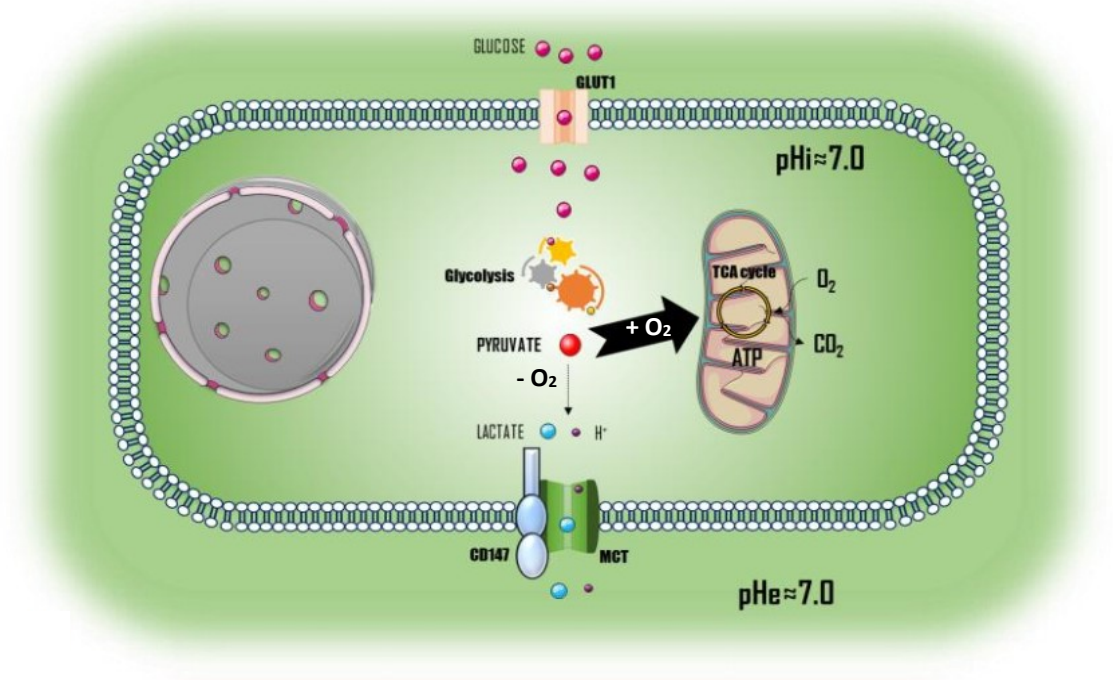


***Figure 4-*** Schematic representation of glucose metabolism in a normal cell. In the presence of oxygen ($+O_2$), non-proliferating tissues metabolize glucose into pyruvate, which is oxidized in mitochondria through OXPHOS. In the absence of oxygen ($-O_2$), glucose is metabolized into lactate. ATP (adenosine triphosphate); pHe (extracellular pH); pHi (intracellular pH); GLUT (glucose transporter); MCT (monocarboxylate transporter); TCA (tricarboxylic acid cycle). Adapted from [31].

The metabolic flux is controlled and regulated by several effectors, like hormones, small macromolecules and other extracellular factors, which maintain ATP levels almost constant, as well as glycolytic intermediates. For instance, 5' adenosine monophosphate protein kinase (AMPK) is a metabolic sensor that coordinates cellular uptake of glucose, β-oxidation of fatty acids and biogenesis of GLUT4 and mitochondria [32]. AMPK detects and reacts to fluctuations of 5' adenosine monophosphate (AMP) and, upon activation by AMP increase, inhibits anabolic pathways like fatty acid and protein synthesis, and activate catabolic pathways such as fatty acid oxidation, glucose transport and others [33, 34]. Also, ATP acts as a sensor, creating a negative feedback when oxygen is abundant, allowing mitochondria to oxidize pyruvate into bicarbonate ion ($HCO_3^-$) and then carbon dioxide and water [35].

HKII, phospho-fructokinase-1 (PFK-1), and pyruvate kinase (PK), crucial glycolytic enzymes, also suffer allosteric regulation by fluctuations of key metabolites, such as

lactate, which reflect the cellular balance between production and consumption of ATP [26, 36].

The levels of oxygen are also an important regulator of cellular and systemic responses, but these levels are also regulated by pathways that affect the expression and activity of several metabolic proteins [35, 37].

In contrast, metabolism of proliferating cells differs from quiescent and differentiated cells by showing high rates of glycolysis, lactate production, and biosynthesis of lipids and other macromolecules [27, 38]. Proliferating cells, like tumour cells, present an altered metabolism as a result of molecular changes during carcinogenesis, resulting in elevated dependency on glycolysis for energy production. This metabolic reprograming of cancer cells is nowadays defined as a new hallmark of cancer [39].

### 1.2.1. Cancer cell metabolism

Cancer is characterized by uncontrolled proliferation of malignant cells, in which genetic and epigenetic changes generate a new diversity of phenotypes making cancer cells morphologically and functionally different from each other [40, 41]. Metabolic reprograming, currently one of the ten hallmarks of cancer [39], is an adaptation of cancer cells to allow the elevated biosynthetic processes and energy production [29, 38]. In contrast to quiescent cells or differentiated tissues, tumour cells convert most of the incoming glucose into lactate (around 85%) even in presence of oxygen, by a phenomenon described by Otto Warburg, which is currently known as “Warburg effect” or aerobic glycolysis, while pyruvate metabolism through OXPHOS is less frequent (around 5%) (Figure 5) [27, 29, 42].

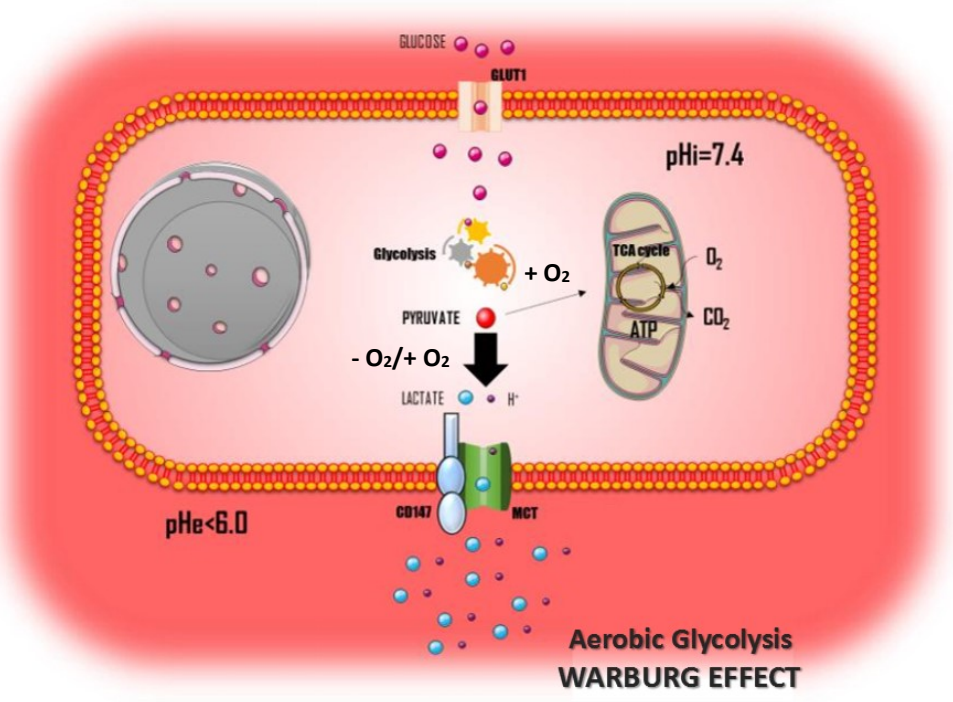


***Figure 5***- Schematic representation of metabolism in tumour cell. Glucose is metabolized to pyruvate, being then converted into lactate, even in presence of oxygen (+$O_2$), by a phenomenon denominated as aerobic glycolysis or Warburg Effect. ATP (adenosine triphosphate); pHe (extracellular pH); pHi (intracellular pH); GLUT (glucose transporter); MCT (monocarboxylate transporter); TCA (tricarboxylic acid cycle). Adapted from [31].

It is known that tumours contain aerobic (well oxygenated) and hypoxic (poorly oxygenated) regions, regulating, for that reason, the switch from oxidative to glycolytic metabolism [43]. Although most tumour cells are localized in hypoxic regions and thus depend on anaerobic glycolysis, some tumour cells remain glycolytic even when oxygen levels are restored, producing the majority of their ATP (60%) through this pathway, instead of OXPHOS [44, 45]. Although cancer cells use a less efficient pathway for ATP production, comparing to OXPHOS, this metabolic switch confers several advantages to cancer cells. Besides being a much faster pathway to produce ATP, it also generates carbon intermediates used in biosynthetic pathways, modulates reactive species of oxygen (ROS) and leads to evasion of cancer cells to the immune system [27, 29, 46, 47]. Additionally, upregulation of glycolysis leads to accumulation of pyruvate and production of lactate [46, 48], resulting in an acidic microenvironment. Multiple pH regulators cooperate for microenvironment acidification, like $Na^+/H^+$ exchanger 1

(NHE1) and its isoforms [49], vacuolar-type $H^+$-ATPases (V-ATPase) [50], MCTs [30], carbonic anhydrases (CAIX and CAXII) [51] and $HCO_3^-$ transporters and exchangers (NBCs and AEs) [52, 53]. This process confers advantages to cancer cells by killing normal cells [27, 54]. The escape to cell death is also favoured by the low extracellular pH, in combination with hypoxia, activating intracellular pathways like extracellular signal-related kinase (ERK1/2) [55]. By activation of ERK1/2, acidic pH also promotes cell proliferation, tumourigenesis, invasion, angiogenesis, cell differentiation and survival [56]. Moreover, activation of ERK1/2, acidification and hypoxia lead to release of proteases like cathepsin B [57], matrix metalloproteinases 2 and 9 (MMP2 and MMP9) [27, 58], promoting degradation of the extracellular matrix (ECM), leading to invasion and metastasis [27, 48]. Finally, low interstitial pH is also associated with upregulation of vascular endothelial growth factor (VEGF), supporting angiogenesis, which sustain tumour growth, invasion, and metastasis [59].

This hallmark is a characteristic of advanced cancers, and, as a result, the increase in glucose uptake by cancer cells is explored in clinics to diagnose tumours as well to monitor therapeutic response, using positron emission tomography (PET). The accumulation of 2-(18F)-fluoro-2-deoxy-D-glucose (FDG) in cancer cells is detected by PET, allowing the distinction of normal tissues from cancer cells, based on glucose uptake rates [46, 60].

### 1.2.2. Tumour microenvironment

The microenvironment of solid tumours, in which concentrations of several molecules, such as glucose, glutamine and/or oxygen, is heterogeneous, also contributes to selection of glycolytic phenotype. Different key physiological factors, like abnormal vascular structure, pH levels or metabolite transport are able to influence the characteristics of the tumour microenvironment [27, 59, 61, 62].

The carcinogenesis process and the early malignant phenotype are severely influenced by the microenvironment, occurring first of all in a avascular environment, being cells dependent on glucose and oxygen diffused through the blood vessels only

present in the stromal compartment (Figure 6) [27, 54]. After different stimuli, cells become hyperproliferative, and, as cells become even more distant from the blood vessels, pre-malignant lesions become hypoxic. This state promotes a metabolic switch to a hyperglycolytic phenotype, allowing cells to growth, and, consequently, the microenvironment becomes acidic and cells acquire an invasive phenotype (Figure 6). At this point, the acidic microenvironment is responsible for the selection of cells presenting an acid-resistant phenotype, a powerful proliferative advantage [54, 63, 64]. Several studies report induction of hyaluronan and its receptor CD44 by acidic microenvironment, promoting cancer cell invasion and metastasis [65, 66]. Also, the capacity of the acidic microenvironment to inhibit cytotoxic T lymphocytes (CTLs) and natural killer cells was reported, while inflammatory cells like macrophages are activated under this condition to participate in tumour progression [67]. This acid-resistant phenotype results from cellular adaptations mainly up-regulation of membrane pH regulators [27, 51], since prolonged exposure to low pH normally results in necrosis or apoptosis [68, 69].

Remarkably, being tumourigenesis a dynamic process with fluctuations of nutrient and oxygen levels, cancer cells are able to adapt their machinery to become, in a defined moment, normoxic cells, and, in another, change to hypoxic cells [70].

Tumour microenvironment is more than cancer cells; it also includes a mixed population of non-tumour cells, like hematopoietic and mesenchymal cells. In this context, lymphocytes, natural killer cells, macrophages, neutrophils and myeloid-derived cells, are also part of the tumour microenvironment, with interaction of T cells and macrophages with cancer cells through cytokines, promoting inflammatory responses [66, 71]. Additionally, fibroblasts, endothelial and stem cells are involved in tumour growth, angiogenesis and dissemination of cancer [65, 72, 73].

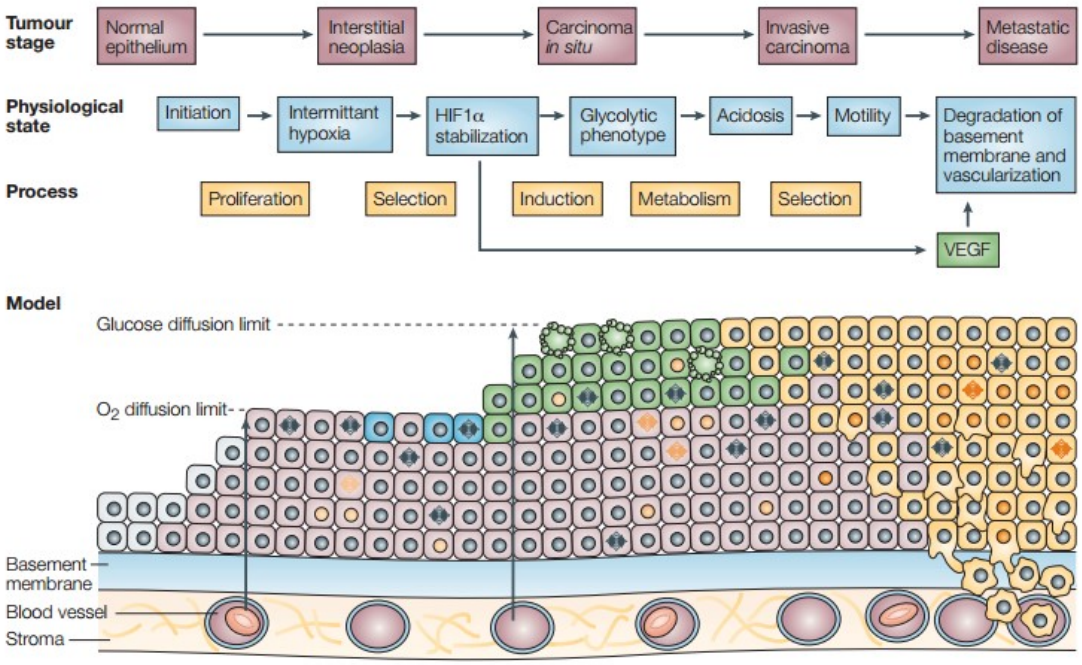


***Figure 6***- Schematic representation of carcinogenesis and its interaction with the microenvironment. In normal tissues, after intermittent hypoxia starts, the proliferation process initiates the hyperplasic stage to interstitial neoplasia, proceeding to carcinoma *in situ*, culminating with degradation of basement membrane, originating metastatic disease. Colour representation: grey - normal epithelial cells, pink - hyper-proliferative cells, blue - hypoxic cells, green - glycolytic cells and yellow - motile cells. HIF-1α (hypoxia inducible factor 1 alpha); VEGF (vascular endothelial growth factor) [27].

#### 1.2.2.1. **Hypoxia**

Hypoxia is a common feature of malignancy, particularly in solid tumours, supporting tumour invasiveness and metastasis, being associated with aggressiveness features. The hypoxia inducible factor 1 (HIF-1) is a key transcription factor involved in hypoxia response, being a heterodimer composed by a α-subunit (oxygen dependent) and β-subunit (oxygen independent). The α-subunit is degraded under higher oxygen conditions (>5% $O_2$), while in hypoxia conditions (<5% $O_2$) is stabilized and consequently translocated to the nucleus, forming a HIF-1α/β complex, which binds to target genes through hypoxia-response elements (HRE) [37, 74].

HIF-1α is responsible for regulating several pathways like angiogenesis, invasion, oxidative stress, treatment-resistance and metabolic switch to the Warburg effect [37].

The induction of several glycolytic-related proteins, such as glucose transporters GLUT1 and GLUT3, HKII, and lactate dehydrogenase-A (LDH-A), by HIF-1α has been described, supporting the dependency of cancer cells mainly on glycolysis [75, 76]. Also, HIF-1α-dependent upregulation of pyruvate dehydrogenase kinase 1 (PDK1), which inhibits the mitochondrial pyruvate dehydrogenase (PDH), preventing the conversion of pyruvate into acetyl-CoA, compromises oxygen consumption in OXPHOS (Figure 7), reinforcing the metabolic switch towards a hyperglycolytic phenotype [27, 77].

Additionally, HIF-1α induces the expression of proteins related to pH regulation (Figure 7), like CAIX, NHE1 and MCTs, which promote the efflux of protons and lactate [43], leading to acidification of the microenvironment. CAIX is a hypoxia marker and a prognostic indicator, which performs the reversible conversion of $CO_2$ to bicarbonate and a proton, contributing to acidification of tumour microenvironment and, consequently, to intracellular pH control [74, 78]. Induction of NHE1 by hypoxia was described in pulmonary myocytes [79, 80], pointing at this protein as an attractive target of HIF-1α in tumour cells. However, only limited data reports the upregulation of this pH regulator in tumour cells and hypoxic areas [63], being also suggested that NHE1 activity can be upregulated or downregulated according to cell type and oxygen availability [81].

Additionally, some studies report regulation of MCT1, MCT2 and MCT4 by hypoxia [82-84], however only MCT4 regulation was shown to be through activity on MCT4 promoter by HRE [74, 76, 84]. Up-regulation of MCT4 by HIF-1α contributes to the acid-resistant phenotype, facilitating the export of the newly formed lactate, allowing the continuous conversion of pyruvate into lactate and, therefore, stimulating aerobic glycolysis [28].

Furthermore, HIF-1α induces the expression of VEGF, allowing the recruitment of blood vessels, however, the structure of these vessels is disorganized, resulting in fluctuating areas of oxygen, which culminate in the upregulation of glycolysis [85-87]. Moreover, activation of HIF-1α is not only a response to low oxygen concentrations but its activation can be regulated by other factors, such as activation of oncogenes, including RAF and RAS, PI3K, AKT, mTOR, MYC and loss of tumour suppressor genes such as *TP53*, *PTEN* and *VHL* [74, 88].

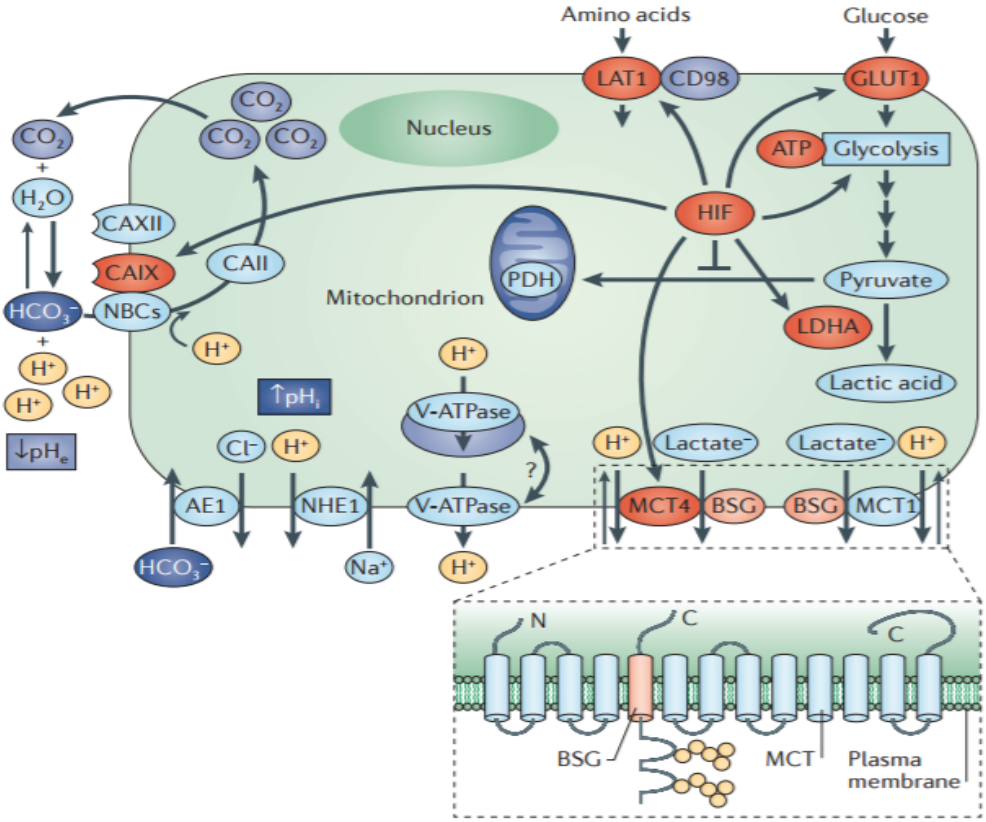


***Figure 7-*** Cellular model for the regulation of metabolic proteins and pH regulators by HIF-1α. To survive under acidic conditions, tumour cells express pH regulator proteins including NHE1 ($Na^+/H^+$ exchanger 1), CAIX, CAXII and CAII (carbonic anhydrases), $HCO_3^-$ transporters [$Na^+/HCO_3^-$ co-transporters (NBCs) and anion exchange protein 1 (AE1)], and MCT1 and MCT4 (monocarboxylate transporters). Metabolic fuel is provided to the cell by GLUT1 (glucose transporters) and LAT1 (L-type amino acid transporter 1). V-ATPase (vacuolar H+ -ATPases); PDH (pyruvate dehydrogenase); LDH-A (lactate dehydrogenase A); BSG (basigin); pHe (extracellular pH) [89].

### 1.2.2.2. **Lactate**

The glycolytic phenotype acquired by cancer cells during the carcinogenic process promotes acute and chronic acidification of the tumour microenvironment. This is one of the main causes of acidosis, due to production of lactate and carbonic acids, which maintain the extracellular compartment under acidic pH, combined with an alkaline intracellular pH (~7.2) [90, 91].

In the last years, lactate gained a relevant role in tumour progression, having been considered in several studies as a metabolic fuel, but also as a signalling molecule

in angiogenesis and evasion to immune surveillance [92]. In the metabolic symbiosis proposed by Sonveaux *et al.* [28], less oxygenated glycolytic cells produce lactate released by MCT4, while oxygenated oxidative cells consume the lactate produced, uptaken by MCT1. In this process, lactate has an important role in fuelling oxygenated tumour cells, while glucose can diffuse to the hypoxic regions where glycolytic cells use it to produce more lactate [28, 43].

Besides that, lactate have an important role in tumour progression by promoting escape to the immune system [93-95], cancer cell migration and invasion [58, 65], angiogenesis [96-98] and resistance to therapy [99, 100]. It is described that lactate is able to reduce the activation of dendritic cells, T cells and natural killer cells [93, 94]. Moreover, lactate stimulates tumour-associated macrophages (M2 macrophages), which play a role in immunosuppression and wound healing [101, 102]. Regarding migration and invasion, lactate is involved in production of hyaluronan and induction of its receptor CD44 [65], which is also responsible for cancer cell chemoresistance [99]. Activation of MMPs by lactate was also reported, leading to extracellular matrix degradation [58], promoting invasion and metastasis. Angiogenesis is also controlled by lactate, which promotes stabilization of HIF-1α, increasing VEGF and its receptor by tumour cells and endothelial cells [96]. Moreover, through crosstalk between tumour cells and endothelial cells, lactate induces interleukine-8 (IL-8) production by endothelial cells, resulting in new blood vessel formation [97]. It is reported stabilization of MYC by lactate, promoting VEGF, IL-8 and CD31 up-regulation, during prolonged hypoxia via ERK1/2 signalling pathway [98].

An association between lactate and patient poor prognosis, lower disease free and overall survival was also shown in different solid tumours, such as cervical cancer [103], head and neck [104], high grade gliomas [105] and non-small-cell lung cancer [106]. Moreover, the high clinical importance of lactate is supported by its association with incidence of metastasis [103, 104, 107], tumour recurrence and radioresistance [27].

#### 1.2.2.3. **Glucose**

Glucose plays a central role in cell metabolism and seems to be an excellent fuel and a versatile precursor, capable of providing a vast group of metabolic intermediates for biosynthetic reactions. This molecule has three most important fates: it may be stored in the form of glycogen; oxidized to pyruvate via glycolysis and subsequent OXPHOS to supply ATP and metabolic intermediates; or oxidized via the pentose phosphate pathway (PPP) to produce ribose 5-phosphate for nucleic acid synthesis and NADPH for reductive biosynthetic processes [108].

Nowadays, it is known that the high consumption of glucose by cancer cells is required for energy generation, culminating in lactate production even in presence of oxygen, by the Warburg effect [29, 47]. In fact, the augmented glycolytic activity of cancer cells has been explored for cancer diagnostic, being also useful to detect metastatic and recurrent lesions, and to identify high-grade disease through PET imaging [60].

The uptake of glucose is performed by the GLUTs, a family of transporters composed by 14 members. In cancer cells, up-regulation of GLUTs has been vastly described (reviewed in [109]), especially GLUT1, which is up-regulated by HIF-1α, being this transporter highly associated with FDG uptake in PET imaging [110].

In breast cancer, increase in GLUT1 expression and its association with glycolytic phenotype have been reported [111]. This study reported association of MCT1 and CD147 with GLUT1 and CAIX, supporting the involvement of this glucose transporter in the metabolic adaptation of breast cancer. Other studies have reported positivity for GLUT1 in breast cancers tissues, by immunohistochemistry [110, 112-115]. Additionally, it is known that estrogen and its receptor were able to increase GLUT1 levels in breast cancer cells [116]. Despite of GLUT1 being the most expressed glucose transporter in breast cancer, other glucose transporters like GLUT2 and GLUT3 have been detected in approximately 30% of invasive breast cancers, as well as GLUT4, but in fewer cases [110].

Although the uptake of glucose is a vital process to maintain glycolysis, it is known that limited glucose bioavailability is a key feature of the tumour

microenvironment, due to high glucose consumption and poor vascularization inside the tumour [117-119]. Characterization of glucose deprivation conditions in cervical cancer cell showed stabilization of MCT1-CD147 complex by a post-translational effect, inactivating endosome-dependent protein recycling [120].

#### 1.2.2.4. **Growth factors**

Growth factors and their receptors, receptor tyrosine kinases in particular, play an important role in cell growth promotion, cell proliferation, and tumorigenesis. The signalling pathways of growth factor receptors like HER2, insulin-like growth factor-1 receptor (IGF1R) and fibroblast growth factor receptor (FGFR) have been implicated in tumour growth, proliferation and therapy resistance in breast cancer [121, 122].

In breast cancer, an important independent pathway of tumour cell proliferation is HER2, a member of the EGFR family [123]. This receptor is overexpressed in approximated 15-20% of all breast cancer cases, mediating cell growth and survival through activation of its downstream mediators, like PI3K/AKT/mTOR and RAS/RAF/MEK/MAP kinases [124]. HER2 overexpression has been implicated in augmented glycolysis in breast cancer cells and in breast cancer patients [125-128].

IGF1R is a tyrosine kinase receptor, and, similar to HER2, mediates growth of breast cancer cells through PI3K and RAS/RAF/MEK/MAP kinases intracellular signalling [123], being also associated with increased glycolysis and proliferation [129].

FGFR is also a tyrosine kinase receptor, and signalling by its ligand FGF is implicated in several developmental processes including mesodermal patterning in the embryo and formation of numerous organ systems. Not surprisingly, its deregulation has serious implications in carcinogenesis [123].

#### 1.2.2.5. **Hormones**

Approximately 75% of breast cancers are positive for hormone receptors, namely ER and PR. This *status* has been helpful to predict response to targeted therapy in breast cancer patients [123]. Estrogen and progesterone regulate growth, differentiation and function of several tissues in the body, but are also associated with abnormal cell proliferation, resulting in tumourigenesis [130].

ER acts through 2 distinct, but interrelated, pathways, the nuclear and nonnuclear pathways. The nuclear pathway is activated by estrogen binding, dimerization of receptor and translocation to the nucleus, where, with interaction with co-regulator proteins, modulates gene transcription, being implicated in breast cancer pathogenesis. The nonnuclear pathway is mediated by estrogen and ER in the cytoplasm of cells, interacting with several signalling pathways, tyrosine kinases, and growth factor receptors, including HER2, IGF1R, and FGFR [123].

Studies correlating glycolysis and hormonal signalling are scarce, however, the association of MCT1 expression and absence of ER was described in breast cancer [131].

In other tissues, other hormones have been described as regulators of MCTs, like leptin in Caco2 cells (MCT1 up-regulation), thyroid-stimulating hormone (TSH) in rat thyroid cells (MCT1 up-regulation), noradrenaline in mouse neurons (MCT2 up-regulation), triiodothryonine (T3) in rat skeletal muscle (MCT4 up-regulation) and somatostatin in intestinal cells (MCT1 up-regulation) [132-136].

## 1.3. Monocarboxylate transporters (MCTs)

MCTs are essential molecules in the transport of endogenous monocarboxylates, such as pyruvate, lactate and ketone bodies (acetoacetate and β-hydroxy-butyrate), across the plasma membrane [137-140]. In order to maintain the high rates of glycolysis, the two molecules of lactate produced from each glucose molecule must be transported

out of the cancer cells [140], by these specific transporters. The efflux of lactate has an important role in most tumour cells, but also in high glycolytic tissues like blood red and white skeletal muscle [137]. In contrast, in tissues like brain, heart, red skeletal muscle, kidney and liver, lactate is the major gluconeogenic substrate, being its uptake of upmost importance [140]. MCTs are widely distributed throughout various mammalian tissues (Table 1) and cell types [139, 140].

### 1.3.1. MCT family

The Solute Carrier Family 16 (SLC16), also known as monocarboxylate transporter family, is composed by 14 members of related proteins, identified through sequence homology [137, 138, 141].

Structure prediction showed that MCT1-4, MCT7 and MCT8 are composed by 12 alpha-helical transmembrane domains (TMDs) (Figure 8) [140], being the N- and C-terminal located at the cytoplasm, while MCT6, MCT9-14 are composed by 10 TMDs [138, 139, 142]. Although TMDs are extremely conserved, N- and C-terminus and the large intracellular loop between TMDs 6 and 7, are regions in which some variations can occur [138, 139, 141, 142]. The variability is most frequent in transporters with 12 TMDs [138], being these variations related to substrate specificity, mainly regulated by C-terminal, or regulation of transport activity, correct structure maintenance or insertion at plasma membrane, mediated by the N-terminal domains [140]. It is also known that substrate affinity is affected by mutations in arginine ($Arg^{313}$) residue at TMD8, reducing, for instance, the affinity of MCT1 for lactate. Several designed drugs contain a carboxyl group incorporated in their chemical structure, making these compounds potential substrates for MCTs and MCTs as possible gateways for drug delivery [139, 140].

MCT1-4 were the first characterized members of the MCT family, facilitating the diffusion of monocarboxylates, like pyruvate, ketone bodies and lactate, being these substrates co-transported with protons in an equimolar manner through a symport mechanism [137, 139]. In opposition to MCT1, MCT2 and MCT4 (Table 1), MCT3 is described exclusively in the retinal pigment (RPE) and choroid plexus epithelia (CPE)

[137, 139, 141]. This transporter was previously found in chicken retina, presenting an affinity for lactate and pyruvate similar to MCT1 [137, 141].

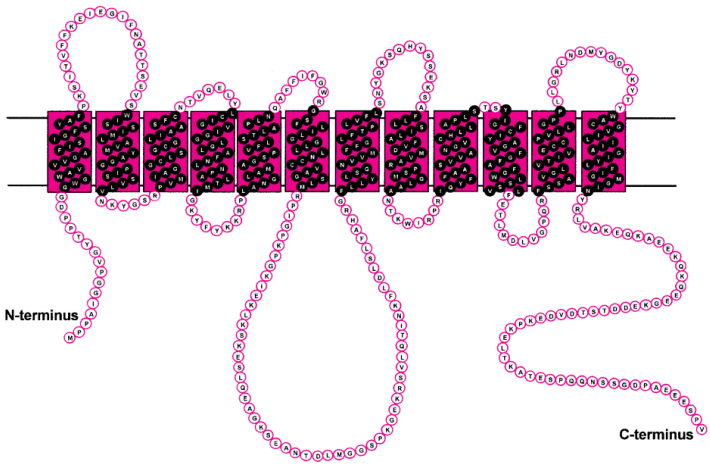


***Figure 8***- Predicted membrane topology of MCTs. MCT1 sequence is shown [140].

Characterization of MCT8 and MCT10 showed that pyruvate and lactate were not substrates for these transporters, and, in contrast, the transport performed by these two transporters is independent of proton or $Na^+$ coupling. MCT8 was described as a thyroid hormone transporter, which transports the thyroid hormones T3 and thyroxine (T4), while MCT10 transports aromatic aminoacids (Table 1). Both isoforms are characterized by a particular feature in the N-terminal of protein sequence, presenting a peptide sequence rich in proline, glutamic acid, serine and threonine (PEST motif), which is responsible for rapid protein degradation [138-140]. MCTs 5-7, 9, 11-14 were not yet characterized and their functional role and substrates remain unknown, being these called orphan transporters [138, 140, 141].

The main differences between MCT isoforms are related to substrate and inhibitor affinities, regulation of expression and tissue localization [30]. As MCT1, MCT2 and MCT4 are the most frequent isoforms associated with cancer [143], and the best characterized in human tissues, and they will be described in more detail.

**Table 1-** Monocarboxylate transporter (MCT) family members. Adapted from [142]**.**

| *Protein name* | *Main Substrates* | *Tissue distribution* | *Human gene name* | *Human gene locus* | *NCBI Reference Sequence* |
|---|---|---|---|---|---|
| ***MCT1*** | Lactate, pyruvate, ketone bodies | Ubiquitous | *SLC16A1* | 1p12 | NP_001159968.1 |
| ***MCT2*** | Lactate, pyruvate, ketone bodies | Kidney, brain | *SLC16A7* | 12q13 | NP_004722.2 |
| ***MCT3*** | Lactate | RPE, CPE | *SLC16A8* | 22q12.3-q13.2 | NP_037488.2 |
| ***MCT4*** | Lactate, pyruvate, ketone bodies | Skeletal muscle, chondrocytes, leucocytes, testis, lung, placenta, heart | *SLC16A3* | 17q25 | NP_001035887.1 |
| ***MCT5*** | O | Brain, muscle, liver, kidney, lung, ovary, placenta, heart | *SLC16A4* | 1p13.3 | NP_004687.1 |
| ***MCT6*** | O | Kidney, muscle, brain, heart, pancreas, prostate, lung, placenta | *SLC16A5* | 17q25.1 | NP_004686.1 |
| ***MCT7*** | O | Brain, pancreas, muscle | *SLC16A6* | 17q24.2 | NP_001167637.1 |
| ***MCT8*** | T3, T4 | Liver, heart, brain, thymus, intestine, ovary, prostate, pancreas, placenta | *SLC16A2* | Xq13.2 | NP_006508.1 |
| ***MCT9*** | O | Endometrium, testis, ovary, breast, brain, kidney, adrenal, RPE | *SLC16A9* | 10q21.2 | NP_919274.1 |
| ***MCT10 (TAT1)*** | Aromatic amino acids | Kidney, intestine, muscle, placenta, heart | *SLC16A10* | 6q21-q22 | NP_061063.2 |
| ***MCT11*** | O | Skin, lung, ovary, breast, pancreas, RPE, CPE | *SLC16A11* | 17p13.2 | NP_699188.1 |
| ***MCT12*** | O | Kidney | *SLC16A12* | 10q23.31 | NP_998771.3 |
| ***MCT13*** | O | Breast, bone marrow stem cells | *SLC16A13* | 17p13.1 | NP_963860.1 |
| ***MCT14*** | O | Brain, heart, ovary, breast, lung, pancreas, RPE, CPE | *SLC16A14* | 2q36.3 | NP_689740.2 |

O - Orphan Transporter; RPE- retinal pigment; CPE- choroid plexus epithelia

#### 1.3.1.1. **MCT1**

MCT1 was firstly identified in Chinese hamster ovary (CHO) cells and, subsequently, the human, rat and mouse homologue sequences were identified [137, 138], being this member the most well studied and functionally characterized [137].

The human MCT1 gene (*SLC16A1* - NP 001159968.1, NCBI reference sequence) is located in chromosome 1 (1p13.2-p12) and was firstl cloned in 1994, by Garcia and colleagues [144]. Structural organization and characterization of *SLC16A1* was achieved in 2002, showing 5 coding exons [145]. There is no evidence for alternative splicing in the 5' and 3' untranslated region (UTR) [140], and 6 transcripts were identified, resulting in 4 proteins with different sizes [137]. However, only the protein composed by 494 amino acids is the functional MCT1, resulting in a protein with molecular weight of ~54 kDa [139].

MCT1 presents an ubiquous distribution in human tissues, including blood-brain barrier (BBB), T-lymphocytes, spermatogenic cells, brain, apical membrane of RPE, inner ear, kidney, stomach, liver, gut epithelium, and others, but is highly expressed in heart and muscle [137].

MCT1 transport kinetics has been carefully studied using lactate, with this transporter presenting an intermediate affinity for the substrate, being involved in both uptake and efflux of monocarboxylates [138, 142]. The preference to transport a group of substrates with substitutions in C2 and C3 are clear, which include important metabolites such as L-lactate ($K_m$ 3-5mM), pyruvate ($K_m$ 0.7mM), acetoacetate ($K_m$ 4-6mM), and β-hydroxybutyrate ($K_m$ 10-12mM) [137, 142], although MCT1 also transports short chain (C2-C5) unbranched aliphatic monocarboxylates, such as acetate ($K_m$ 3.5mM) and propionate ($K_m$ 1.5mM) [30, 138, 142].

Transport of lactate is stereoselective, with $K_m$ for D-lactate 10 times higher than for L-lactate [146]. Other monocarboxylates like methanoate are poor substrates, while bicarbonate, dicarboxylates, tricarboxylates and sulphonates are not transported by MCT1 [141, 142]. It is known that mutations in specific amino acids can compromise the transport of the substrate. For instance, mutation of lysine 142 to glutamine in the loop between TMD 4 and 5 resulted in an increase in the $K_m$ for L-lactate from 5mM to 12mM

and a decreased stereoselectivity of the transporter, indicating the involvement of this residue in substrate recognition. In the same loop, a more drastic effect occurs with mutation of arginine 143 to glutamine, eliminating MCT1 transport activity [147].

Although MCT1 is needed to export or uptake monocarboxylates at the plasma membrane, its expression was also described at the mitochondria [148, 149] and peroxisomes [150], pointing at a participation of lactate oxidation in the maintenance of the organelles function.

#### 1.3.1.2. **MCT2**

The human MCT2 gene (*SLC16A7* - NP 004722.2, NCBI reference sequence) is located at the chromosome band 12q13, and has been cloned and sequenced in rat, mouse and human [140].

The expression of MCT2 is more restricted than MCT1, presenting high levels in testis [138, 141], followed by spleen, heart, kidney, pancreas, skeletal muscle, brain, and leukocytes with moderate to low expression [141]. MCT2 contains 484 amino acids, displays a molecular weight of ~52kDa and presents 60% of sequence identity with MCT1 [140], being alternative splicing described in rat and human [151, 152]. Similarly to MCT1, MCT2 catalyses the proton-linked transport of several monocarboxylates, with a higher affinity for pyruvate ($K_m$ 0.08mM) and L-lactate ($K_m$~1mM) [137, 139]. Moreover, MCT2 has a high affinity for ketone bodies and β-hydroxybutyrate [140, 141]. Due to the high affinity for monocarboxylates and its preferred localization in the most gluconeogenic tissues, this transporter is more adapted to perform the uptake of substrates [153].

#### 1.3.1.3. **MCT4**

MCT4 (*SLC16A3* - NP 001035887.1, NCBI reference sequence) was originally identified as MCT3 due to the high homology with chicken MCT3, being then renamed as MCT4 [30, 140]. *SLC16A3* gene encodes for MCT4 and is located at chromosome 17 (17q25.3), comprises 5 exons and 3 transcripts, being the protein product constituted by 465 residues, with a molecular weight of ~50kDa. The expression of this protein is higher in glycolytic tissues like white skeletal muscle fibres, astrocytes, white blood cells, chondrocytes and some mammalian cell lines [30, 140, 142], demonstrating its importance in the export of lactate [142]. MCT4 expression was also demonstrated in neonatal heart, which is more glycolytic than the adult heart, and in placenta, were lactate is transferred into the maternal circulation. Actually, the kinetic properties show a lower affinity for substrates than MCT1 and MCT2, presenting a $K_m$ of approximately 28mM for L-lactate and approximately 150mM for pyruvate [30, 142].

### 1.3.2. Regulation of MCT expression

Some studies have shown that MCT regulation can occur at both transcriptional and post-transcriptional levels, modifying protein expression, but also influencing transport activity, regulated by their chaperone proteins. Although little is known about this subject, these regulatory mechanisms appear to occur in a tissue-specific manner. Some studies reported MCT expression variation in different physiological and pathological conditions, especially MCT1 [138]. Besides that, alteration in MCT substrate concentrations and different signals from changes in cellular metabolism are able to alter MCT expression [30].

#### 1.3.2.1. **Transcriptional regulation**

The complex process of transcriptional regulation can be orchestrated by several molecules, from transcription factors, as the case of NFκB, to signalling molecules like lactate, both increasing MCT1 mRNA expression. For instance, in L6 cells cultured *in vitro* or in exercising muscle *in vivo*, an increase in MCT1 mRNA and protein levels was shown, due to the increase in lactate concentrations [138, 154]. Also, butyrate was able to stimulate MCT1 promoter activity, through NFκB [59, 154], and, in colonic epithelium, butyrate increased MCT1 mRNA and proteins levels in a concentration and time dependent manner [59, 138]. Moreover, butyrate enhanced the antitumour activity of 3-bromopyruvate through the increase of MCT4 expression in breast cancer cell lines [155]. The up-regulation of MCT1 through gene activation occurred also after chronic stimulation or exercise in rats and humans, while its down-regulation occurred as a consequence of denervation or spinal injury [30, 139, 142], being gene activation mediated by elevated calcium and AMP [30]. Moreover, during exercise, the peroxisome proliferator-activated receptor gamma and alpha (PPARγ and PPARα) and their co-activator 1 alpha (PGC-1α) induced MCT1 up-regulation, but the same was not observed for MCT2 and MCT4 [30].

Regulation of MCTs by hypoxia is described in several studies [28, 82-84, 156-161]. In heart muscle, after chronic hypoxia, an increase in MCT4 was observed [82], being also increased in bladder and breast cancer cells after hypoxia [158, 160]. Increase in MCT1 expression was reported in colorectal, breast and glioma cancer cells [84, 161], in neuronal, astrocytic and endothelial cells, mediated by HIF1-α [157]. Evidence showed the increase of MCT1 and MCT4 in adipocytes mediated by HIF-1α, and a decrease in MCT2 [83]. In opposition, a hypoxia-induced decrease in MCT1 and MCT4 in plantaris muscle was shown [82]. Nevertheless, there are controversial results on MCT regulation by hypoxia, once there is evidence that only MCT4 promoter is activated by HIF1-α through HRE [156].

Regulation of MCTs was also reported in different hormonal diseases, like diabetes, obesity and thyroid diseases, where a decrease in muscle MCT1 in obese rats was described [30].

In human breast cancer cells, MCT1 regulation by hypermethylation of CpG islands was also described in the promoter region [162]. Also, *c-MYC* and *n-MYC* oncogenes were described to regulate MCT1 gene expression, increasing MCT1 transcription [163], while INF-γ and TNF-α, pro-inflammatory cytokines, decrease its expression [164].

#### 1.3.2.2. **Post-transcriptional regulation**

Some studies have also demonstrated the regulation of MCTs at the post-transcriptional level, being most reports related to MCT1 and MCT2 [30]. An increase in MCT1 expression, without alteration in mRNA levels, was described in left ventricle hypertrophy [165], as well as during post-mitotic and G1 phases [30].

It was suggested that translational regulation can occur at 3'UTR of MCT1 mRNA, but not MTC2 nor MCT4 [140]. For instance, the initiation factor eIF4E, which is involved in translation, has its maximal peak of phosphorylation associated with maximal MCT1 expression [30].

Regulation of MCT1 by microRNAs was also reported, being miR-124 one of the most well-characterized, which decreases MCT1 protein expression in medulloblastoma, regulating MCT1 mRNA through binding to its 3' UTR [166].

Furthermore, hormonal regulation is also involved in post-transcriptional regulation of MCTs. For example, testosterone and TSH were described to increase MCT1 expression, without altering its mRNA levels [59, 135]. Additionally, a post-transcriptional mechanism was described in which MCT2 was increased in the brain by insulin and insulin growth factor 1 (IGF-1), mechanism that involves stimulation of the PI3K/AKT/mTOR pathway [30]. Moreover, noradrenaline induces MCT2 expression at the translational level, but not MCT1, in mouse neurons, with the requirement of a yet unknown transcriptional step [133]. Finally, there is no evidence that MCT1 regulation includs glycosylation [30].

#### 1.3.2.3. **Regulation of MCT activity**

Frequently, several membrane proteins are associated with glycosylated proteins to maintain their correct expression and function. Accordingly, several studies show that MCT1, MCT3 and MCT4 require the ancillary protein CD147 for membrane expression and activity, while MCT2 interacts with gp-70 [30, 167, 168].

CD147 (also known as basigin, EMMPRIN, OX-47 and HT7) and gp-70 (also known as embigin) are members of the immunoglobulin superfamily and are related to a small family of glycoproteins, which contain a single transmembrane domain containing a conserved glutamate residue, a short intracellular C-terminus, and a large glycosylated extracellular domain with two or three immunoglobulin domains depending on the splice variant [30, 169]. Moreover, studies have shown that, besides its role in MCT membrane location [170-172], CD147 is also important for MCT1, 3 and 4 activity [170, 173], but, on the other hand, MCT1 and 4 are also important regulators of CD147 [170]. Furthermore, studies indicate that covalent modification of CD147 results in inhibition of lactate transport, demonstrating the important role of CD147 in MCT function [167, 174].

CD147 was described as a tumour cell-derived MMP inducer, present in tumour cell surface, being involved in angiogenesis, multidrug resistance, activation and development of T cells, acting also as a receptor of cyclophilin A [175, 176]. Besides tumour cells, CD147 is also important in fetal, neuronal, lymphocyte and extracellular matrix development and tissue repair [177]. Thus, the contribution of MCTs is not restricted to their function as lactate transporters and pH regulators, but also play, indirectly, roles in angiogenesis, migration and invasion due to MCT/CD147 complex [142, 178]. Some studies reported plasma membrane expression of MCTs without CD147 expression, suggesting the existence of additional chaperones for their correct plasma membrane localization and activity, with studies identifying CD44 as another MCT chaperone [99, 179, 180].

### 1.3.3. Inhibitors of MCT activity

Several compounds have been described as classical inhibitors of MCTs, which inhibit lactate transport, and include inhibitors of three different categories. These are aromatic compounds, acting as competitor inhibitors, like 2-oxo-4-methylpentanoate and phenyl-pyruvate, and derivatives of α-cyanocinnamate, such as α-cyano-4-hydroxycinnamate (CHC) [181], ii) amphiphilic compounds, such as bioflavonoids (quercetin and phloretin), and anion exchanger (AE) inhibitors, such as 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) and niflumic acid [142], and iii) stilbene-derived compounds, as 4,4′-diisothiocyanostilbene-2,2′-disulphonate (DIDS) and 4,4′-dibenzamidostilbene-2,2′-disulphonate (DBDS), acting as irreversible inhibitors [182]. These inhibitors present a higher affinity for MCT1 and 2, being MCT2 more sensitive to inhibition by CHC, DBDS and DIDS than MCT1, but is insensitive to thiol reagents as p-chloromercuribenzenesulfonic acid (p-CMBS) [137, 138, 141, 142]. The specificity of this inhibitor is mediated by the ancillary protein CD147, but not by gp-70 [138, 141]. In opposition to MCT1, MCT3 is insensitive to inhibition by CHC, pCMBS and phloretin [137, 141]. In contrast, MCT4 exhibits a much lower affinity for a wider range of inhibitors than MCT1 [138, 142], but, exceptionally, MCT4 lactate transport is inhibited by a range of statins (drugs used to lower cholesterol levels) [183].

However, these compounds have been demonstrated to inhibit other transporters, like the mitochondrial pyruvate transporter (inhibited by CHC), or the chloride/bicarbonate exchanger AE1 (inhibited by DIDS and DBDS) [181, 184].

Other compounds have been studied as inhibitors of MCTs, like lonidamine, which is known to inhibit glycolysis in cancer cells. Initial studies referred lonidamine as inhibitor of hexokinase [185-187], but further studies showed the capacity to inhibit lactate transport mediated by MCT1 and 4 [186, 188, 189].

Also, the nonsteroidal anti-inflammatory drugs (NSAIDs), are transported by MCT1 and have been described as inhibitors of lactate transport [190]. Recently, 7-aminocarboxycourmarine family members have been identified as potent inhibitors of MCTs, demonstrating three times more activity than CHC [191, 192].

A new class of high-affinity and specific inhibitors has been designed by AstraZeneca to block MCT1 and 2. For instance, the compound AZD3965 is described as a MCT1 inhibitor and is currently being tested as anticancer agent in a phase I clinical trial for patients with prostate cancer, gastric cancer or diffuse large B cell lymphoma (ClinicalTrials.gov NCT01791595) [193]. Importantly, a related compound, AR-C155858, is a dual MCT1/2 inhibitor, being its activity modulated by the associated ancillary protein GP-70 [194, 195].

### 1.3.4. Role of MCTs in cancer

As previously described, the high levels of lactate produced by cancer cells is a common feature of tumours, being MCTs central molecules in the export of the accumulating lactate and pH regulation [27, 139]. Several studies demonstrate the up-regulation of MCT1 and MCT4 in tumours (Figure 9), like high-grade gliomas [196], colorectal carcinomas [197, 198], neuroblastomas [163], lung cancer [199, 200], cervical cancer [201], ovarian cancer [202, 203], breast carcinoma [131, 160, 162, 204], pancreatic cancer [205], melanomas [206], mesotheliomas [207, 208], bladder [209], kidney [210] and head and neck [211]. In gastrointestinal stromal tumours (GISTS), positive expression of MCT1 and downregulation of MCT4 was observed [212], while in another study only positive expression of MCT4 was reported [213]. MCT4 up-regulation was described in hepatocellular carcinoma [214]. In addition, up-regulation of MCT2 was reported in prostate [215], lung [199], pancreatic [216] and colorectal cancer [197, 198], as well in brain tumours [217]. In contrast, down-regulation of MCT1 expression was also described in liver [214], prostate tumours [218], as well as colorectal cancer (Figure 9) [219].

In breast cancer, a significant increase in MCT1 expression was described, in a subset of aggressive breast carcinomas (basal-like) compared with normal breast tissue. In this study, MCT1 and CD147 were associated with absence of ER and PR, high grade tumours, expression of cytokeratin 5 and 14, vimentin and Ki67, evidencing the role of

MCT1 in breast cancer aggressiveness [131]. Expression of MCT1 in breast cancer, especially in the aggressive basal-like subtype, was associated with GLUT1 and CAIX [204], which expression associated with shorter disease-free survival [161, 204].

In other solid tumours, association of MCT1 and 4 overexpression with negative prognostic markers have been frequently reported [31]. In cervical cancer, the association of lactate with poor prognosis, and a significant increase in MCTs from pre-invasive to invasive squamous lesions was observed [103, 201, 220]. Also, in neuroblastoma, MCT1 was highly associated with MYCN oncogene amplification, reinforcing the association with higher malignancy [163].

The available data supports that MCT expression is an adaptation to the hyperglycolytic phenotype, being upregulation of MCTs at the plasma membrane an advantage for tumour cells, allowing continuous export of lactate, and promoting tumour growth [143]. For this reason, these molecules are seen as promising targets in cancer therapy.

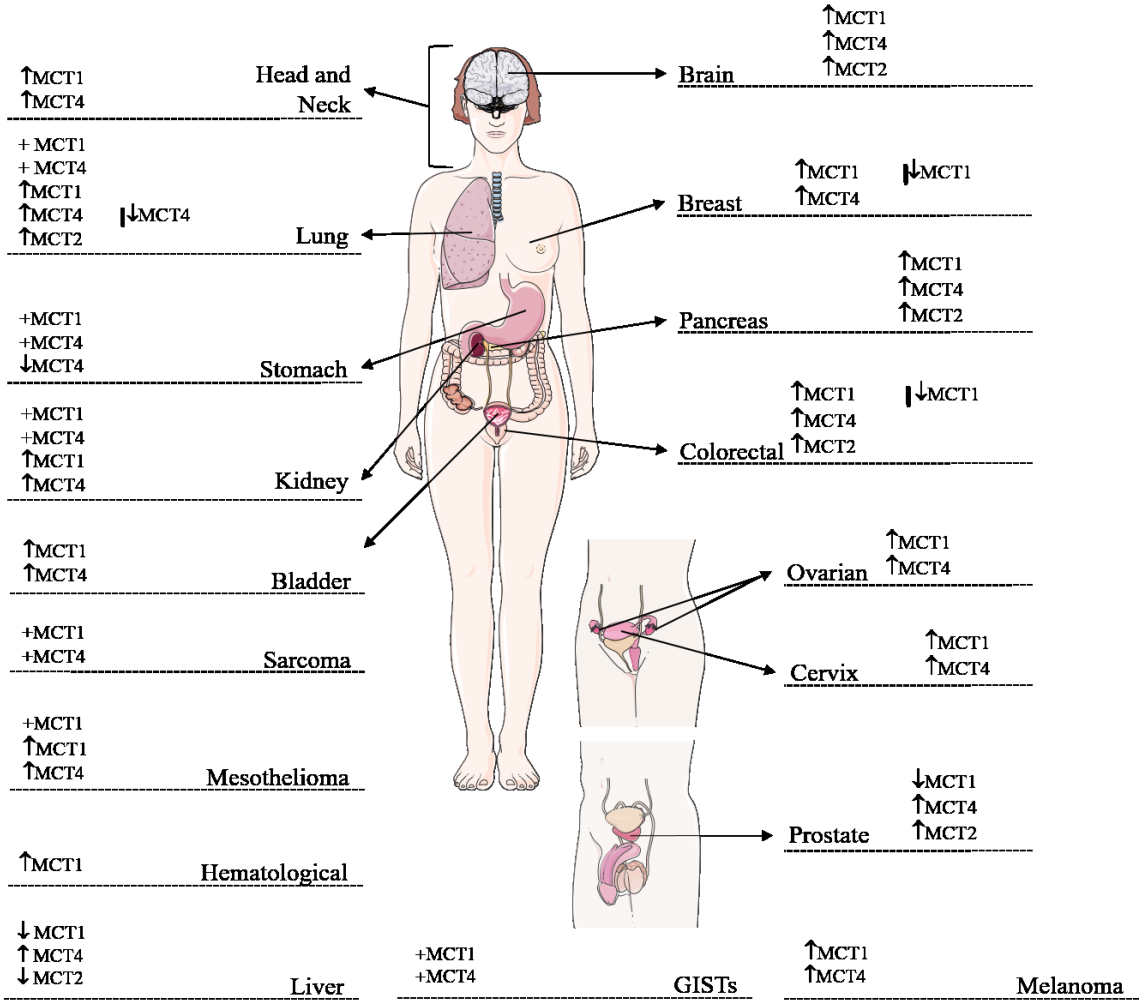


**Figure 9-** Overview of the MCT expression in different tumour types; **+** (high expression), ↑ (upregulation), ↓ (downregulation). Adapted from [31].

#### 1.3.4.1. **MCTs as therapeutic targets in cancer**

The increased interest of metabolic pathways in cancer therapy has motivated the development of several potential drugs, which are now in clinical trials [33, 90].

Being MCTs key molecules in the maintenance of the malignant phenotype of tumours, maintaining the hyperglycolytic and promoting the acidic-resistant phenotype, together with its upregulation in cancer, inhibition of MCTs emerges as a valuable

approach in cancer treatment (Figure 10) [143]. The adaptation of cancer cells to hypoxia can be compromised by inhibiting MCT1, disrupting the metabolic symbiosis between normoxic cells and hypoxic cells. While hypoxic cells are dependent on glucose and the Warburg effect to produce lactate, normoxic tumour cells are dependent on lactate exported by hypoxic cells. Thus, inhibition of MCT1 and glucose uptake is able to disturb this balance [28].

Although MCT inhibition will have a direct impact on pH regulation and cancer cell glycolytic rates, many other effects have been described as a result of this inhibition, like decreased cell viability [31, 143], increased cell death [28, 120, 155, 217], decreased tumour cell migration and invasion [120, 173, 178, 221] and also decreased angiogenesis [28, 97, 222].

*In vitro* inhibition of MCT1 induces a decrease in intracellular pH [28, 163, 223], enhancing cancer cell radiosensitivity [224] and improving the response to chemotherapy [225]. Also, CHC was used to inhibit MCT activity, showing a decrease of cell viability, migration and invasion of tumour cells [163, 226].

Further, small interference RNA (siRNA) has been used to silence MCT expression. Silencing of MCT1 and MCT4 was able to decrease cell migration and invasion [170, 178, 227], being supported by the interaction of MCT4 with beta 1-integrin [178]. *In vivo*, treatment with CHC decreased tumour growth [28, 31], sensitized cells to radiation [28] and decreased tumour invasion [228]. The combined silencing of MCT1 and MCT4, or CD147 demonstrated reduction of glycolysis and also in tumour growth [225, 229]. The specific inhibitor AZD3965 developed by AstraZeneca, was used *in vivo,* in a model of small lung cancer, and was shown to decrease tumour growth [230].

Being MCTs promising targets in cancer therapy, it is essential to understand the putative secondary effects in normal tissues. Considering the distribution of MCTs, specially MCT1, systemic delivery of inhibitors could affect mainly cardiac and skeletal muscle, being possible side effects muscle fatigue and intolerance to moderate-high physical exercise. In colonic epithelium, MCT1 is responsible for butyrate transport, and its inhibition can promote a decrease in cell proliferation and differentiation [59]. Although the secondary effects can be well tolerated by normal tissues [231], inhibition

of glycolysis as monotherapy has been disappointing, due to the high plasticity of tumour cells to reprogram their metabolism [33, 232]. However, combined therapy may bring promising results, as the one reported in lung cancer cells, in which inhibition of glycolysis by targeting basigin sensitizes cancer cells to phenformin [229]. Thus, there is a need to design new therapeutic strategies or combine this approach with current therapies to better improve cancer treatment.

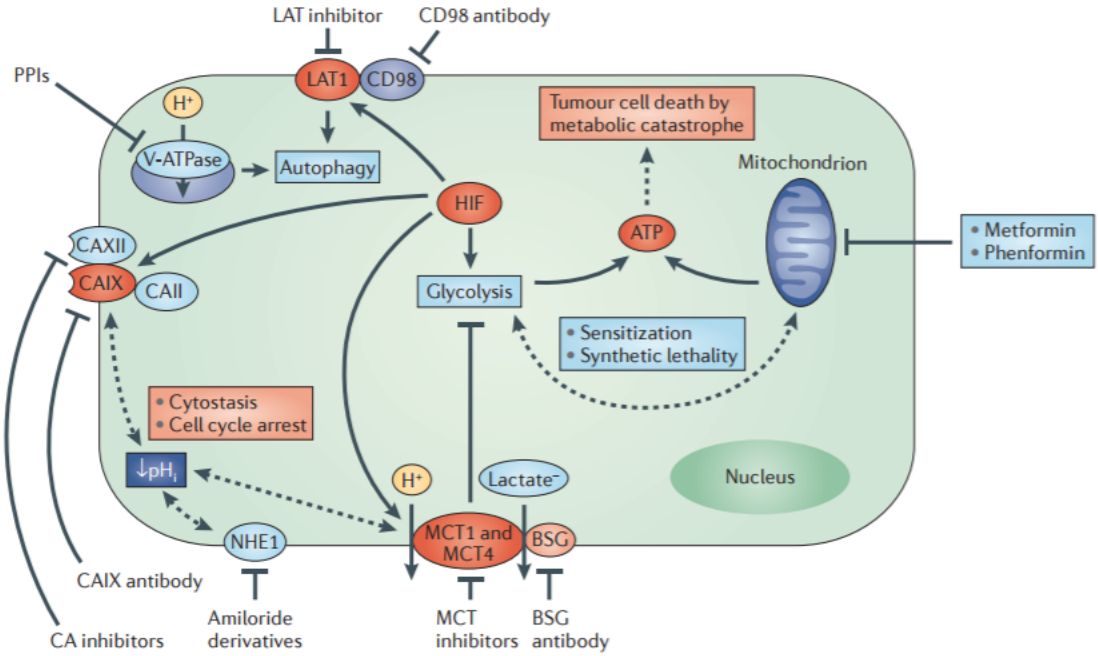


***Figure 10***- Potential targeting of metabolic pathways in cancer to achieve tumour cell killing. Potencial targets of inhibition are shown and interactions are represented in dashed lines. Metabolic stress caused by intracellular pH acidification, via inhibition of CAIX (carbonic anhydrase IX), MCTs (Monocarboxylate transporters) and NHE1 (Na+ /H+ exchanger 1), could be enhanced by metformin or phenformin to inhibit mitochondrial ATP production. PPIs (proton pump inhibitors); LAT1 (L-type amino acid transporter 1); BSG (basigin); HIF-1α (hypoxia-inducible factor) [89].

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# CHAPTER 2: MONOCARBOXYLATE TRANSPORTER TARGETING IN BREAST CANCER

The results presented in this chapter were published in the international peer reviewed journals:

**Morais-Santos, F.**, Miranda-Gonçalves, V., Pinheiro, S., Vieira, A. F., Paredes, J., Schmitt, F. C., Baltazar, F., and Pinheiro, C. *Differential sensitivities to lactate transport inhibitors of breast cancer cell lines*. Endocrine-Related Cancer, 2014. **21**(1): p. 27-38.

**Morais-Santos, F.**, Granja, S., Miranda-Gonçalves, V., Moreira, A. H., Queirós, S., Vilaça, J. L., Schmitt, F. C., Longatto-Filho, A. Paredes, J., Baltazar, F. and Pinheiro, C. *Targeting lactate transport suppresses in vivo breast tumour growth*. Oncotarget, 2015. **6**(22): p. 19177-89.

The results presented in this chapter were also presented in the poster format and published *in conference proceedings* in the following scientific meetings:

**Filipa Morais-Santos**, Céline Pinheiro, André Vieira, Joana Paredes, Fernando Schmitt, Fátima Baltazar (2013). *Monocarboxylate Transporters as therapeutic targets in breast cancer*. Advances in Molecular Oncology: Translating Molecular Biology into Cancer Treatment, São Paulo, Brazil, published in Clinics 68 (Suppl.9): S71.

**Filipa Morais-Santos**, Céline Pinheiro, André Vieira, Joana Paredes, Fernando Schmitt, Fátima Baltazar (2013). *Contribution of monocarboxylate transporters to the aggressive phenotype of breast cancer*. 11th International Congress on Targeted Anticancer Therapies 4 – 6 March 2013 Paris, France, published in Annals of Oncology 24 (Suppl.1).

## 2.1. Chapter Overview

Most of cancers, including breast cancer, increase the consumption of glucose to produce large amounts of lactate, even in presence of oxygen, by a phenomenon referred as Warburg effect [1-3]. In fact, based in the increase of glucose uptake, FdG-PET has been used as imaging technique for diagnosis and prognosis of breast cancer, namely to detect distant metastasis, as well as to follow therapy response [4]. In fact, many studies have demonstrated the importance of this phenotype in tumour progression and malignancy, due to the high levels of lactate transported out of tumour cells by MCTs [5-7]. Importantly, the association of lactate transport with increased cell proliferation, migration, invasion, angiogenesis, escape to cell death, as well suppression of anticancer immune response, has also been demonstrated [8].

Thus, in this chapter, two complementary studies, evaluating the effect of MCT inhibition in breast cancer, are presented.

In the first study, CHC, quercetin and lonidamine were used as MCTs inhibitors, and its effect was evaluated on cell viability, metabolism, proliferation, cell death, migration and invasion, in a panel of breast cancer cell lines. Importantly, in the most sensitive cell lines, a decrease in lactate production was shown, as well a decrease in cell proliferation, migration and invasion. The same effects were also achieved upon MCT1 downregulation, pointing at MCT1 as the main target.

Further, the second study showed the effect of targeting lactate transport, through MCT expression modulation, decreasing aggressiveness of breast cancer cells *in vitro* and, importantly, decreasing *in vivo* tumour growth.

The results of this chapter point at MCTs as promising targets in breast cancer therapy, demonstrating the importance of lactate in breast tumours.

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### 2.1.1. Differential sensitivities to lactate transport inhibitors of breast cancer cell lines

Research | F Morais-Santos et al. | Lactate transport inhibition in breast cancer cell lines | 21:1 | 27–38

# Differential sensitivities to lactate transport inhibitors of breast cancer cell lines

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## Abstract

The tumour microenvironment is known to be acidic due to high glycolytic rates of tumour cells. Monocarboxylate transporters (MCTs) play a role in extracellular acidification, which is widely known to be involved in tumour progression. Recently, we have described the upregulation of MCT1 in breast carcinomas and its association with poor prognostic variables. Thus, we aimed to evaluate the effect of lactate transport inhibition in human breast cancer cell lines. The effects of α-cyano-4-hydroxycinnamate, quercetin and lonidamine on cell viability, metabolism, proliferation, apoptosis, migration and invasion were assessed in a panel of different breast cancer cell lines. MCT1, MCT4 and CD147 were differently expressed among the breast cancer cell lines and, as expected, different sensitivities were observed for the three inhibitors. Interestingly, in the most sensitive cell lines, lactate transport inhibition induced a decrease in cell proliferation, migration and invasion, as well as an increase in cell death. Results were validated by silencing MCT1 expression using siRNA. The results obtained here support targeting of lactate transport as a strategy to treat breast cancer, with a special emphasis on the basal-like subtype, which so far does not have a specific molecular therapy.

**Key Words**
- breast
- molecular biology
- carcinoma

*Endocrine-Related Cancer* (2014) **21**, 27–38

## Introduction

Increased glucose uptake is a widely described phenomenon in cancer cells, being the rationale behind the whole-body non-invasive positron emission imaging technique, using $^{18}$F-fluorodeoxyglucose-positron emission tomography (FDG-PET). This technique is useful in the diagnosis and prognosis of breast cancer, especially regarding the detection of distant metastases, and recurrent disease as well as in monitoring response to therapy (Jadvar *et al.* 2009). This increased uptake of glucose, especially through glucose transporter 1 (GLUT1), is a consequence of an increased glycolytic metabolism that generates acids inside the cell. This fact leads to

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upregulation of some pH regulators, like carbonic anhydrase IX (CAIX) (Pouyssegur *et al.* 2006), to maintain the intracellular physiological pH, causing extracellular acidosis. The increased glycolytic metabolism ultimately leads to an increase in lactate release by cancer cells, also contributing to microenvironmental acidosis, as well as increased invasion (Stern *et al.* 2002) and suppression of anticancer immune response (Fischer *et al.* 2007). In this context, lactate has a central role in cancer aggressiveness and lactate transporters (monocarboxylate transporters (MCTs)) are currently seen as potential therapeutic targets in cancer treatment, with promising results obtained using *in vitro* and *in vivo* models (Mathupala *et al.* 2004, 2007, Colen *et al.* 2006, 2011, Fang *et al.* 2006, Sonveaux *et al.* 2008). There is an ongoing clinical trial using a specific MCT1/MCT2 inhibitor (Jones & Schulze 2012).

MCTs belong to a family of 14 members, with isoforms 1–4 being lactate proton symporters that exhibit different affinities for lactate (Halestrap & Meredith 2004). As the isoforms responsible for lactate efflux, MCT1 and MCT4 are probably the most promising in the cancer context and reports on MCT1 and MCT4 upregulation in a variety of tumours are becoming more frequent (Mathupala *et al.* 2004, Fang *et al.* 2006, Koukourakis *et al.* 2006, 2007, Pinheiro *et al.* 2008*a*,*b*, 2010, 2012). Importantly, we have described association of MCTs, especially MCT1, with poor prognostic variables (Pinheiro *et al.* 2008*b*, 2009, 2010, de Oliveira *et al.* 2012), reinforcing the potential of MCT1 as a cancer therapeutic target. Recently, our group described an increase in MCT1 expression in breast carcinomas, when compared with normal tissue. This enhanced MCT1 expression, as well as the expression of CD147, a MCT1/MCT4 chaperone (Kirk *et al.* 2000, Gallagher *et al.* 2007), was associated with basal-like-subtype tumours and other poor prognostic parameters (Pinheiro *et al.* 2010). Additionally, we found that MCT1, but not MCT4, was associated with GLUT1 and CAIX expressions, indicating a role of MCT1 in the hyperglycolytic and acid-resistant phenotype characteristic of less oxygenated (Pinheiro *et al.* 2011), instead of oxygenated cancer cells as pointed by others (Sonveaux *et al.* 2008).

Over recent years, different approaches have been used to inhibit lactate efflux from cancer cells, including MCT small-molecule inhibitors such as α-cyano-4-hydroxycinnamic acid (CHC; Colen *et al.* 2006, Sonveaux *et al.* 2008), lonidamine (Ben-Horin *et al.* 1995, Ben-Yoseph *et al.* 1998, Fang *et al.* 2006), flavonoids such as quercetin (Belt *et al.* 1979, Deuticke 1982, Wang & Morris 2007), and MCT siRNA (Mathupala *et al.* 2004, Fang *et al.* 2006, Sonveaux *et al.* 2008). The inhibition of MCTs has a direct effect on lactate transport, as well as on pH homeostasis, therefore having an important effect on cancer cell viability. In fact, studies on MCT inhibition are providing evidence for this strategy in the reduction of tumour malignancy, enhancement of radio-sensitivity and induction of cell death (Mathupala *et al.* 2007).

Despite the promising results with obtained for MCT inhibition in cancer, more efforts are needed to support inhibition of lactate transport and pH regulation as an alternative therapeutic strategy in cancer treatment. In this work, we were able to demonstrate the potential of MCT inhibitors in reducing breast cancer cell proliferation, migration and invasion as well as inducing cell death.

## Materials and methods

### Cell lines and culture conditions

The human breast cancer cell lines MDA-MB-468, MDA-MB-231, Hs578T, BT-20, MCF-7/AZ and SkBr3 were obtained from ATCC (Manassas, VA, USA) or from collections developed by Drs Elena Moisseva (Cancer Biomarkers and Prevention Group, Departments of Biochemistry and Cancer Studies, University of Leicester, UK), Marc Mareel (Laboratory of Experimental Cancerology, Ghent University Hospital, Belgium) and Eric Lam (Imperial College School of Medicine, Hammersmith Hospital, London, UK). The characteristics of each cell line are presented in Table 1.

All cell lines were routinely cultured in DMEM (Invitrogen), supplemented with 10% foetal bovine serum (Invitrogen) and 1% antibiotic solution (penicillin–streptomycin; Invitrogen), in a 37 °C humidified atmosphere with 5% $CO_2$.

### Drugs

Stock solutions of CHC, quercetin and lonidamine (Sigma–Aldrich) were prepared with DMSO (Sigma–Aldrich) and stored at −20 °C until use. Working solutions were freshly prepared in the culture medium without serum. DMSO concentration in the cell culture medium never exceeded 1%. All controls were performed using DMSO alone (vehicle).

### Downregulation of MCT1 expression

Silencing of MCT1 expression was achieved using siRNA (siRNA for MCT1, #4390824, Ambion, Foster City, CA, USA; scramble siRNA, #4390843 (Ambion)), using lipofectamine (RNAiMAX 13778-075, Invitrogen,

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**Table 1** Details of the origin, clinical, and pathological features of the tumours used to establish the breast cancer cell lines used in present study (Neve *et al.* 2006)

| Cell line | Origin | Age (years) | Pathology | Cancer subtype | ER status |
|---|---|---|---|---|---|
| MDA-MB-468 | Metastatic site (pleural effusion) | 51 | Adenocarcinoma | Basal A | – |
| MDA-MB-231 | Metastatic site (pleural effusion) | 51 | Adenocarcinoma | Basal B | – |
| Hs578T | Breast | 74 | Invasive ductal carcinoma | Basal B | – |
| BT20 | Breast | 74 | Invasive ductal carcinoma | Basal A | – |
| MCF-7/AZ | Metastatic site (pleural effusion) | 69 | Invasive ductal carcinoma | Luminal | + |
| SkBr3 | Metastatic site (pleural effusion) | 43 | Adenocarcinoma | Luminal ($Her2^+$) | – |

Carlsbad, CA, USA) as permeabilisation agent, according to the manufacturer's instructions.

### Evaluation of the metabolic behaviour of human breast cancer cell lines

**Glucose and lactate quantification** The metabolic behaviour of the different cell lines was determined by assessing extracellular amounts of glucose and lactate. Glucose and lactate were quantified using commercial kits (Roche, Basel, Switzerland and SpinReact, Sant Esteve de Bas, Girona, Spain, respectively), according to the manufacturer's instructions, as described previously (Miranda-Goncalves *et al.* 2013). Results are expressed as the total in micrograms for three independent experiments.

### Protein expression assessment

**Paraffin cytoblock and immunocytochemistry** Concentrated cell suspensions were used to prepare paraffin cytoblocks for immunocytochemistry of MCT1, MCT4 and CD147, as described previously (Miranda-Goncalves *et al.* 2013). See Table 2 for immunocytochemistry details. Negative controls were performed by using adequate serum controls for the primary antibodies (N1698 and N1699, Dako, Carpinteria, CA, USA). Cytoblock sections were counterstained with haematoxylin and permanently mounted. Cells were evaluated for positive expression, distinguishing cytoplasmic expression from membrane expression.

**Western blot** Western blot was performed as described previously (Miranda-Goncalves *et al.* 2013), using the same antibodies as for immunocytochemistry (anti-MCT1, 1:200; anti-MCT4, 1:500 and anti-CD147, 1:500). Goat anti-actin (1:500, sc-1616, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as loading control.

### Assessment of the effect of lactate transport inhibitors

**$IC_{50}$ determination** Cells were plated in 96-well plates and allowed to adhere overnight in a complete DMEM medium before incubation with a culture medium containing 3–15 mM of CHC, 10–100 μM of quercetin and 50–300 μM of

**Table 2** Details of the immunocytochemical procedure for MCT1, MCT4 and CD147

| Protein | Positive control | Antigen retrieval | Peroxidase inactivation | Detection system | Primary antibody: Company (reference) | Primary antibody: Dilution and incubation time |
|---|---|---|---|---|---|---|
| MCT1 | Colon carcinoma | Citrate buffer (10 mM, pH=6) 98 °C; 20 min | 0.3% $H_2O_2$ in methanol, 30 min | R.T.U. VECTASTAIN Elite ABC Kit (Vector Laboratories) | Santa Cruz Biotechnology (sc-365501) | 1:500, overnight, RT |
| MCT4 | Colon carcinoma | Citrate buffer (10 mM, pH=6) 98 °C; 20 min | 3% $H_2O_2$ in methanol, 10 min | Ultravision Detection System, Anti-polyvalent, HRP (Thermo Fisher Scientific) | Santa Cruz Biotechnology (sc-50329) | 1:500, 2 h, RT |
| CD147 | Colon carcinoma | EDTA (1 mM, pH=8) 98 °C; 15 min | 3% $H_2O_2$ in methanol, 10 min | R.T.U. VECTASTAIN Elite ABC Kit (Vector Laboratories) | Santa Cruz Biotechnology (sc-71038) | 1:400, overnight, RT |

Santa Cruz Biotechnology, Santa Cruz, CA, USA; Thermo Fisher Scientific, Fremont, CA, USA; Vector Laboratories, Burlingame, CA, USA.

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lonidamine. The effect of CHC on total biomass, measured by the Sulpho rhodamine B assay (TOX-6, Sigma–Aldrich), was evaluated after 24 h of treatment, while for quercetin and lonidamine the time of treatment was 48 h. $IC_{50}$ values were estimated with the GraphPad Prism 5 Software (GraphPad Software, Inc., La Jolla, CA, USA), applying a sigmoidal dose–response (variable slope) nonlinear regression, after logarithmic transformation.

**Metabolic profiling** After reaching confluence, cells of each cell line were incubated with each inhibitor at its $IC_{50}$ and aliquots of cell culture media were retrieved after 4, 8, 12 and 24 h, assuring confluences similar to the ones observed in the control. Glucose and lactate were quantified as described earlier.

**Cell proliferation assay** Proliferation of cells sensitive to treatment with inhibitors was assessed by bromodeoxyuridine incorporation, after treatment with CHC, quercetin and lonidamine at the respective $IC_{50}$ (24 h for CHC and 48 h for quercetin and lonidamine), as described previously (Miranda-Goncalves *et al.* 2013).

**Cell death assay** Cells were treated with CHC for 24 h, and quercetin and lonidamine for 48 h (at the appropriate $IC_{50}$) and cell death was assessed by simultaneous staining with FITC annexin-V and propidium iodide, as described previously (Miranda-Goncalves *et al.* 2013).

**Wound-healing assay** Cell migration was assessed by the wound-healing assay, as described previously

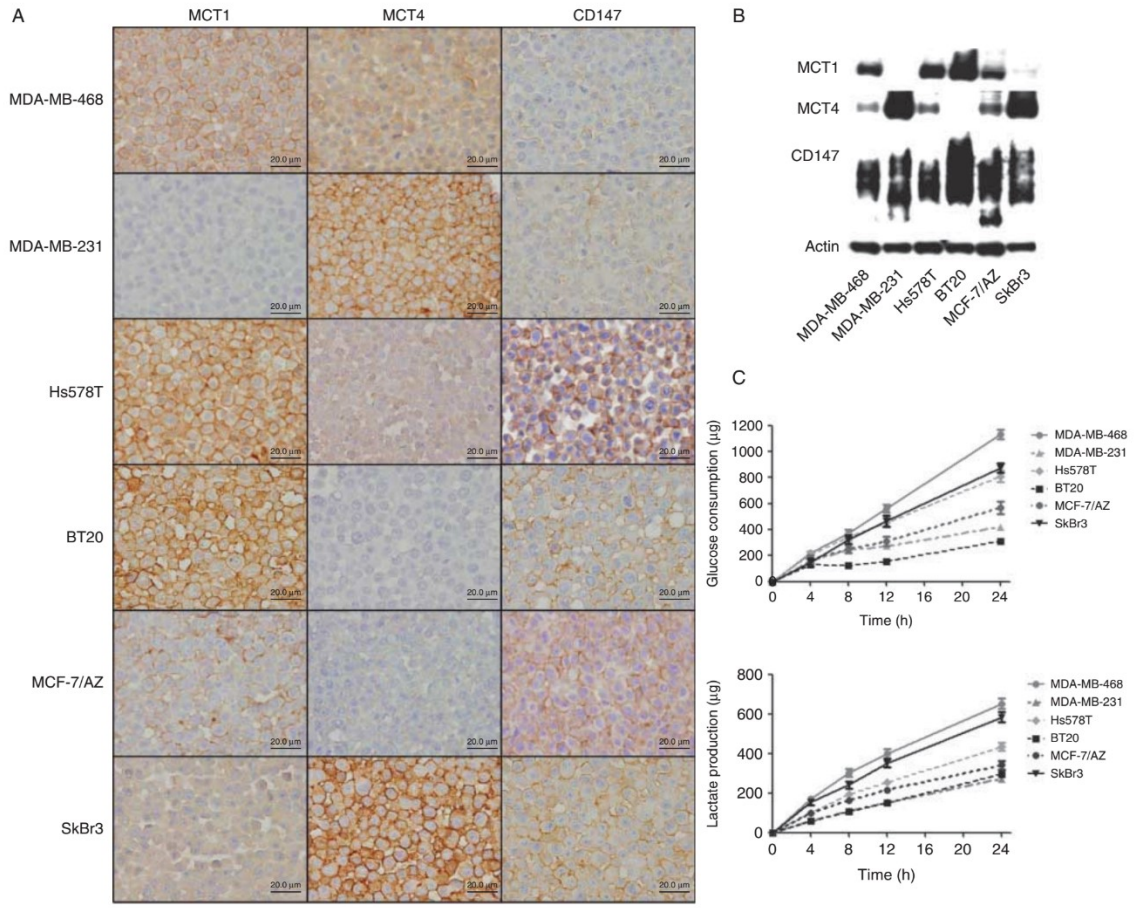


**Figure 1**

Characterisation of the metabolic profile of the breast cancer cell lines. Immunocytochemical expression of MCT1, MCT4 and CD147 in human breast carcinoma cell lines (scale bars 20 μm) (A). MCT1 (50 kDa), MCT4 (52 kDa) and CD147 (50–60 kDa for the highly glycosylated and 42 kDa for the low-glycosylated form) protein expression was detected by western blot in cell lysates (B). Extracellular amounts of glucose and lactate in the different human breast cancer cell lines, over time (4, 8, 12 and 24 h). Values are expressed as mean ± S.E.M. (C).

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(Miranda-Goncalves *et al.* 2013). Cells were exposed to half of the $IC_{50}$ for CHC (to avoid excessive proliferation inhibition and cell death) and of the $IC_{50}$ concentrations for quercetin and lonidamine. The 'wounded' areas were photographed at 0, 12, and 24 h.

**Invasion assay** Cell invasion assay was performed with the sensitive cell lines with invading capacity (MDA-MB-468, MDA-MB-231 and Hs578T), using 24-well BD Biocoat Matrigel Invasion Chambers (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions, as described previously (Miranda-Goncalves *et al.* 2013). Cells were seeded in a medium containing half of the $IC_{50}$ for CHC and of the $IC_{50}$ for quercetin and lonidamine, during 24 h.

### Statistical analysis

Data from three independent experiments, each one in triplicate, were stored in GraphPad Prism 5 Software. All conditions were examined for statistical significance using two-tailed Student's *t*-test for mean comparison, the threshold for significance being $P$ values $<0.05$.

## Results

### Cells with higher rates of glucose consumption produce more lactate

As depicted in Fig. 1A, membrane expression of MCT1 was only observed in MDA-MB-468, Hs578T, BT20 and, at a very low level, in MCF-7/AZ cell lines, while MCT4 was only clearly expressed at the plasma membrane of MDA-MB-231 and SkBr3 cells. CD147 was observed in the plasma membrane of all cell lines. MCT2 expression was not found in any of the cell lines analysed in this study (data not shown). Some intracellular expression was also observed for these markers in some cell lines. Western blot analysis confirmed the different levels of expression observed by immunocytochemistry (Fig. 1B).

The cell lines studied exhibited different levels of glycolytic metabolism (Fig. 1C). As expected, cells with higher rates of glucose consumption also produced more lactate; MDA-MB-468 and SkBr3 showed the highest glucose consumption rates, followed by Hs578T and MCF-7/AZ, while MDA-MB-231 and BT20 presented the lowest consumption rates. In accordance, MDA-MB-468, SkBr3 and Hs578T produced more lactate than MCF-7/AZ, BT20 and MDA-MB-231 in that order.

### Inhibition of lactate transport decreases glucose consumption and lactate production in most glycolytic cells

Breast cancer cell lines were treated once with the lactate transport inhibitors CHC, quercetin and lonidamine at increasing concentrations and $IC_{50}$ values were estimated by evaluating total cell biomass (Table 3). The $IC_{50}$ values show that MDA-MB-468, MDA-MB-231 and Hs578T cells were sensitive to CHC, quercetin, and lonidamine. MCF-7/AZ cells showed $IC_{50}$ values only for CHC and lonidamine, while sensitivity to quercetin was not sufficient to estimate the $IC_{50}$ within the range of concentrations used, and the same was observed for BT20 with all the inhibitors used. SkBr3 showed high $IC_{50}$ values for both quercetin and lonidamine.

To determine whether the induced-inhibitory effect in the different cell lines was due to metabolic disturbance, glucose consumption and lactate production were analysed (Fig. 2). Only MDA-MB-468 and Hs578T cells showed a significant decrease in both glucose consumption and lactate production, after treatment with any of the three inhibitors (Fig. 2A and B respectively). MCF-7/AZ also showed a significant decrease in glucose consumption and lactate production after CHC and lonidamine treatment, but not after quercetin treatment, which is in accordance

**Table 3** $IC_{50}$ values for CHC, quercetin and lonidamine for each cell line

| | CHC | | Quercetin | | Lonidamine | |
|---|---|---|---|---|---|---|
| Cell lines | $IC_{50}$ value calculated (mM) | $IC_{50}$ value used (mM) | $IC_{50}$ value calculated (μM) | $IC_{50}$ value used (μM) | $IC_{50}$ value calculated (μM) | $IC_{50}$ value used (μM) |
| MDA-MB-468 | 9.6 | 10 | 49.64 | 50 | 95.84 | 100 |
| MDA-MB-231 | 5.33 | 5 | 40.65 | 40 | 126.1 | 125 |
| Hs578T | 11.45 | 10 | 39.88 | 40 | 124.5 | 125 |
| BT20 | >15 | 10[a] | >100 | 50[a] | >300 | 125[a] |
| MCF-7/AZ | 9.44 | 10 | >100 | 50[a] | 123.6 | 125 |
| SkBr3 | 12.39 | 10 | 94.05 | 50[a] | 237.8 | 125[a] |

[a]These values do not correspond to the $IC_{50}$ value. For these cell lines, the highest $IC_{50}$ value obtained for the other cell lines was used.

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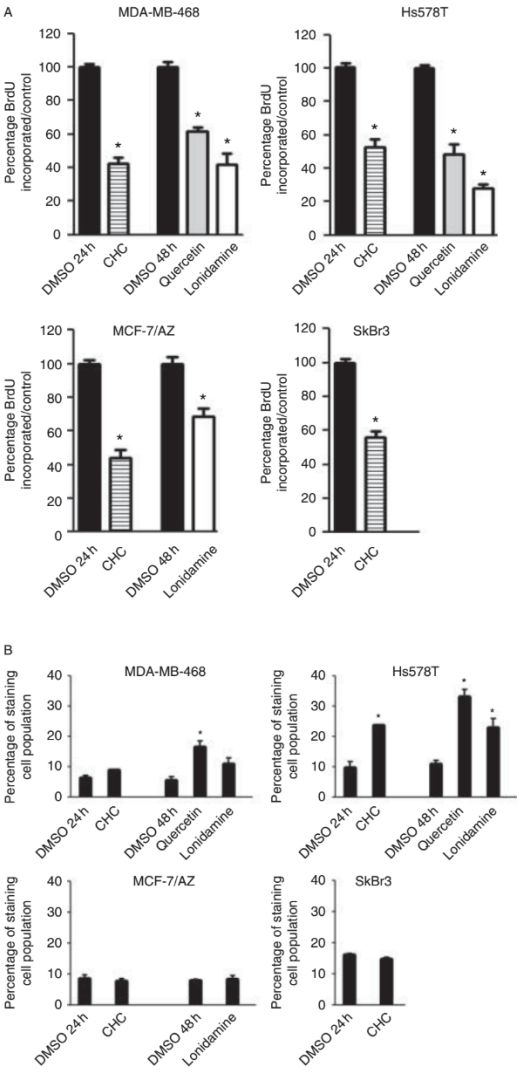


**Figure 3**
Effect of MCT inhibitors on cell proliferation and cell death. Cell lines were treated for 24 h with CHC ($IC_{50}/2$) and for 48 h with quercetin/lonidamine ($IC_{50}$). Cell proliferation (A) was evaluated by bromodeoxyuridine (BrdU) incorporation and cell death (B) by annexin-V/PI. Values are expressed as mean±S.E.M. with $*P<0.05$ compared with control (DMSO).

with the effect of quercetin on MCF-7/AZ biomass. In accordance with the higher $IC_{50}$ values determined for quercetin and lonidamine in the SkBr3 cell line, the glycolytic metabolism was only disturbed in this cell line after treatment with CHC. BT20 cells, with a very low sensitivity to the inhibitors, also showed a decrease in lactate production after CHC and quercetin treatment, but with no decrease in glucose consumption. Surprisingly, the MDA-MB-231 cell line, with high sensitivity to CHC and quercetin, showed no alterations at the metabolic level after treatment with either inhibitor. Metabolism assay confirmed that half of the $IC_{50}$ concentration of CHC was also able to significantly inhibit lactate efflux (Supplementary Figure 1A, see section on supplementary data given at the end of this article).

### Lactate transport inhibition reduces cancer cell aggressiveness

After assessing the sensitivity to the different inhibitors and the metabolic effects of this inhibition, the effect of the inhibitors was further evaluated for different tumour cell aggressiveness parameters in the most sensitive cells lines and whose metabolism was perturbed.

Figure 3A shows that all inhibitors induced a significant decrease in cell proliferation, which was more evident for lonidamine in MDA-MB-468 and Hs578T cells. CHC induced a prominent decrease in the proliferation of MCF-7/AZ and SkBr3 cells.

All inhibitors induced a significant increase in cell death in Hs578T cells, while only quercetin induced a significant increase in cell death in MDA-MB-468. Interestingly, for MCF-7/AZ and SkBr3 cell death was not affected by treatment with the inhibitors (Fig. 3B).

The influence of lactate transport inhibition on cell migration and invasion was also assessed. Importantly, the three inhibitors induced a significant decrease in cell migration (Fig. 4 and Supplementary Figure 2, see section on supplementary data given at the end of this article). Additionally, both quercetin and lonidamine induced a significant decrease in cell invasion for the two invading cell lines analysed (higher effect for lonidamine), while results on CHC were not consistent, as CHC induced a decrease in MDA-MB-468 invasion and had no effect on Hs578T (Fig. 5 and Supplementary Figure 3, see section on supplementary data given at the end of this article).

### Downregulation of MCT1 decreases lactate production and cell aggressiveness

In order to confirm that the previous results were a consequence of MCT1 activity inhibition, downregulation of MCT1 expression with siRNA was performed in Hs578T cells, which express MCT1 at the plasma membrane. An effective reduction in MCT1 expression was observed upon siMCT1 targeting (84.2%), as well as in CD147 expression (65.5%; Fig. 6A). MCT4 expression levels were not altered

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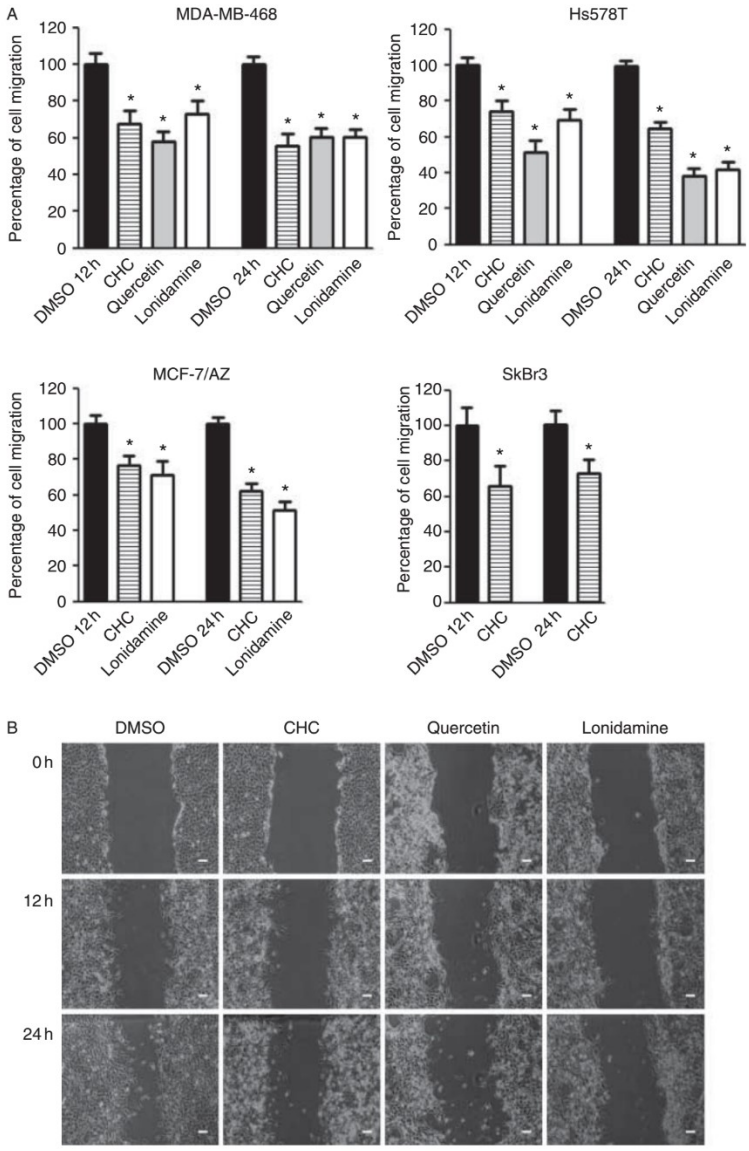


**Figure 4**

Effect of MCT inhibitors on cell migration. Cell migration was evaluated by the wound-healing assay in treated cells over 24 h with half the $IC_{50}$ for CHC and the $IC_{50}$ for quercetin and lonidamine. *$P<0.05$ compared with control (DMSO; A). Pictures represent Hs578T cell migration captured at 0, 12 and 24 h (scale bars 100 μm; B). Pictures of the remaining cell lines are available in Supplementary Figure 2.

by MCT1 downregulation. Similarly to MCT1 activity inhibition, MCT1 downregulation significantly decreased lactate production after 24 h of silencing; however, glucose levels were not affected (Fig. 6B and C). Importantly, there was a decrease in cell proliferation and migration capacity (Fig. 6D and E), similarly to the results obtained with inhibition of MCT1 activity.

## Discussion

Basal-like tumours have an aggressive clinical behaviour (Sorlie *et al.* 2001, 2003, Sotiriou *et al.* 2003) and, in contrast to other molecular subtypes, do not have a specific molecular therapy (Matos *et al.* 2005, Paredes *et al.* 2007). This entails the search for new molecular

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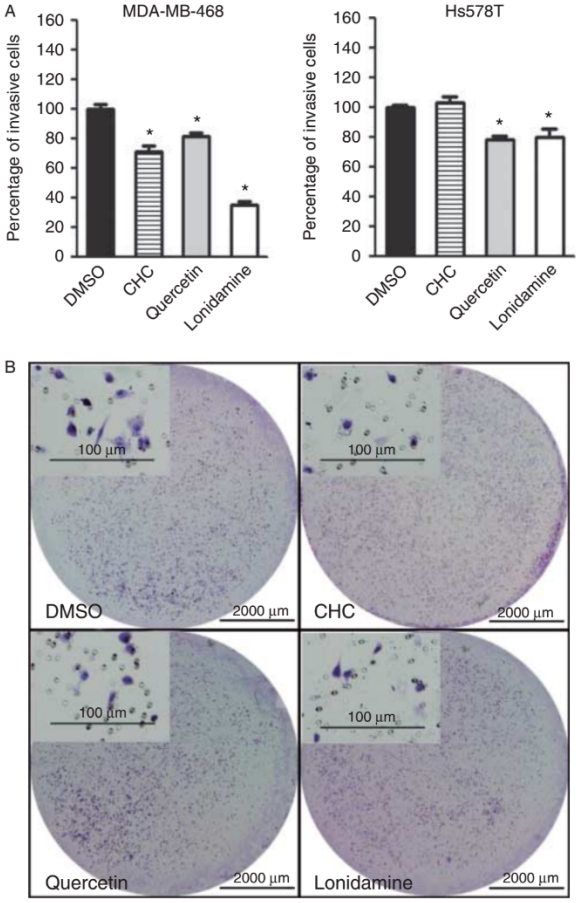


**Figure 5**
Effect of MCT inhibitors on cell invasion capacity. Cells were treated with half the $IC_{50}$ (CHC) or the $IC_{50}$ (quercetin/lonidamine) appropriate to that cell line and inhibitor combination, for 24 h, in matrigel invasion chambers. *$P<0.05$ compared with control (DMSO; A). Pictures represent Hs578T cell invasion at 24 h (scale bars 100 and 2000 µm; B). Pictures showing MDA-MB-468 are available in Supplementary Figure 3.

targets in this aggressive group of tumours, and, considering the increased expression of MCT1 in basal-like tumours (Pinheiro *et al.* 2010), as well as recent evidence showing MCTs to be effective anti-cancer targets (Mathupala *et al.* 2004, Colen *et al.* 2006, Fang *et al.* 2006, Sonveaux *et al.* 2008), MCT1 emerges as a promising therapeutic target that needs to be further explored in breast cancer.

In this work, we analysed MCT1 and MCT4 expression, as well as the expression of other relevant proteins in cancer metabolism, in a variety of human breast cancer cell lines. MCT1 was strongly expressed at plasma membrane in MDA-MB-468, Hs578T and BT20 (basal-like subtype cells), in accordance with our findings for human breast carcinoma samples (Pinheiro *et al.* 2010). In MDA-MB-231, also basal-like subtype cells, MCT1 was not detected, as described by others (Asada *et al.* 2003, Gallagher *et al.* 2007, Hussien & Brooks 2011), and the same was observed for the Her2-positive subtype cell line (SkBr3). In contrast, MCT4 was strongly expressed at the plasma membrane in MDA-MB-231, as described by others (Hussien & Brooks 2011), as well as in SkBr3. Therefore, it seems that the plasma expression of these two isoforms is mutually exclusive in breast cancer, suggesting different mechanisms of regulation. Importantly, we should not ignore the presence of intracellular expression of MCTs, especially MCT4. Actually, a recent study has shown mitochondrial expression of MCT2 and MCT4 in two breast cancer cell lines (MCF-7 and MDA-MB-231), indicating a role of MCTs in the mitochondria (Hussien & Brooks 2011). Additionally, two other studies have described a mitochondrial pyruvate carrier, which is a different protein from the MCTs (Bricker *et al.* 2012, Herzig *et al.* 2012). However, additional studies are required to elucidate if MCTs are working together, in parallel, or performing the transport of different substrates. Nevertheless, considering that CHC is incapable of crossing the plasma membrane, acting only outside the cell (Colen *et al.* 2006, 2011), when using this inhibitor, we believe that we are evaluating only the inhibition of MCT1 activity at the plasma membrane.

For comparison with CHC-induced effects and to validate our results concerning lactate transport inhibition in breast cancer cells, we used additional drugs described as lactate transport inhibitors, quercetin and lonidamine. Our results show that the human breast cancer cells studied have different responses to the inhibitors and that the underlying mechanisms seem to vary among them. In fact, the most sensitive cell line to both CHC and quercetin, MDA-MB-231, besides being negative for MCT1 and producing less lactate than the other lines, showed no alterations in glucose consumption or lactate production after treatment, indicating that other mechanisms, besides lactate transport inhibition, may account for the effects of CHC and quercetin on this cell line. In contrast, MDA-MB-468 and Hs578T, both basal-like-subtype cell lines with MCT1 positive plasma membrane expression and a more pronounced glycolytic phenotype, suffered a significant decrease in glucose consumption and lactate production, accompanied by a decrease in total biomass after exposure to the lactate transporter inhibitors. This was the expected result in a highly glycolytic cell line after MCT1 inhibition. The blockade of lactate efflux probably led to the accumulation of lactate in the cytoplasm, arresting glycolysis with

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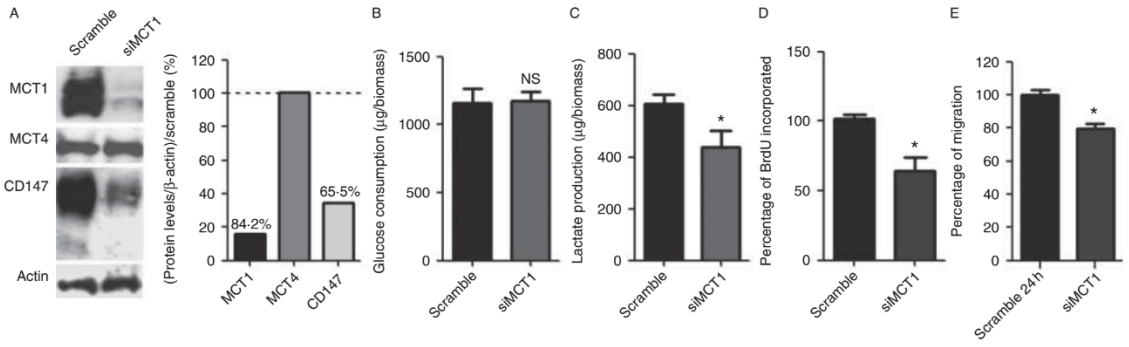


**Figure 6**

Downregulation of MCT1 in Hs578T. Cells were transfected with scramble or siMCT1, and expression of MCT1, MCT4 and CD147 was evaluated after 24 h (A). Effect of MCT1 downregulation on cell metabolism (B and C), proliferation (D) and migration (E). *$P<0.05$ siMCT1 cells compared with scramble; NS, not significant.

subsequent decrease in glucose uptake. Unexpectedly, SkBr3 was sensitive to the inhibitor-induced decrease in total biomass in a metabolic-dependent manner (although at a lower magnitude than MDA-MB-468 or Hs578T and not for the three inhibitors), albeit having undetectable MCT1 expression at the plasma membrane. MCT4 could be another CHC target; however, $K_i$ values for MCT4 are five to ten times higher than that for MCT1 (Halestrap 2012), ranging from 50 to 100 mM, concentrations not reached in the present assays. Additional targets, also affecting cancer cell metabolism, should be behind these effects. Intriguingly, BT20 basal-like-subtype cells, although highly positive for MCT1, were insensitive to treatment with all inhibitors. The reason for this insensitivity remains unclear. This may be due to the low proliferative rate of this cell line, accompanied by the low glycolytic metabolism. These cells could also rely on an alternative source of energy present in the culture medium that is also a substrate for MCT1, such as pyruvate. We evaluated extracellular lactate accumulation and these lactate concentrations could be a result of both lactate production and removal. However, in the presence of glucose, these cells preferred to consume glucose over lactate, some cell lines even being not able to consume lactate.

Although the effect on cell metabolism is extremely important for understanding the mechanisms of action of the different inhibitors, it was also crucial to unveil the contribution of MCT inhibition to other aggressiveness parameters, such as cell proliferation, death, migration and invasion. Importantly, we observed that the three inhibitors were able to inhibit proliferation, migration and invasion, as well as to induce cell death in breast cancer cells, this effect being more pronounced in MDA-MB-468 cells. As inhibition of MCTs will affect both lactate and proton efflux, intracellular accumulation of lactate will lead to glycolysis arrest and the consequent decrease in cell proliferation, as well as cell death induced by intracellular acidification. Also, the decreased migration and invasion capacity after lactate transport inhibition are in accordance with the contribution of both lactate and acidic microenvironment to the increased migration and invasiveness phenotypes of cancer cells (Walenta *et al.* 2002, Rofstad *et al.* 2006).

In general, we could observe that CHC has a broader effect on the behaviour of the breast cancer cells used in this study. Although used to target the same key metabolic proteins (MCTs), CHC, quercetin and lonidamine seem to act by different mechanisms, as a particular cell line could respond differently to each inhibitor. In fact, other metabolic targets have been described for these inhibitors that could mediate the breast cancer cell-altered phenotype associated with metabolic disturbance. Although being the most commonly used MCT1 inhibitor, CHC has also been described as a potent inhibitor of the mitochondrial pyruvate transporter (Halestrap & Denton 1974); however, permeability studies carried out by others show that CHC is not internalised by U-87MG glioma cells (Colen *et al.* 2006). Additionally, CHC has also been identified as an inhibitor of the anion exchanger 1 (Deuticke 1982), an important pH regulator that is responsible for $Cl^-/HCO_3^-$ membrane exchange (Kopito 1990). Different effects have been identified as mediating the antitumour activity of quercetin, including cell cycle arrest (Yang *et al.* 2006) and apoptosis (Granado-Serrano *et al.* 2006, Yang *et al.* 2006), as well as inhibition of the phosphatidylinositol 3-kinase/Akt pathway

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Received in final form 23 October 2013
Accepted 30 October 2013
Made available online as an Accepted Preprint
30 October 2013

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-13-0132



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### 2.1.2. Targeting lactate transport supresses in vivo breast tumour growth

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# Targeting lactate transport suppresses *in vivo* breast tumour growth

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**Keywords:** *monocarboxylate transporters, breast carcinoma, hypoxia, lactate, Warburg effect*

***Received:*** *January 21, 2015* ***Accepted:*** *May 04, 2015* ***Published:*** *May 14, 2015*

## ABSTRACT

**Background: Most cancers, including breast cancer, have high rates of glucose consumption, associated with lactate production, a process referred as "Warburg effect". Acidification of the tumour microenvironment by lactate extrusion, performed by lactate transporters (MCTs), is associated with higher cell proliferation, migration, invasion, angiogenesis and increased cell survival. Previously, we have described MCT1 up-regulation in breast carcinoma samples and demonstrated the importance of *in vitro* MCT inhibition. In this study, we performed siRNA knockdown of MCT1 and MCT4 in basal-like breast cancer cells in both normoxia and hypoxia conditions to validate the potential of lactate transport inhibition in breast cancer treatment.**

**Results: The effect of MCT knockdown was evaluated on lactate efflux, proliferation, cell biomass, migration and invasion and induction of tumour xenografts in nude mice. MCT knockdown led to a decrease in *in vitro* tumour cell aggressiveness, with decreased lactate transport, cell proliferation, migration and invasion and, importantly, to an inhibition of *in vivo* tumour formation and growth.**

**Conclusions: This work supports MCTs as promising targets in cancer therapy, demonstrates the contribution of MCTs to cancer cell aggressiveness and, more importantly, shows, for the first time, the disruption of *in vivo* breast tumour growth by targeting lactate transport.**

## BACKGROUND

Breast cancer is the most common cancer among women and the second leading cause of cancer-related mortality [1]. It is characterized by its clinical and molecular heterogeneity, as it is no longer seen as a single disease but rather as a multifaceted disease comprising distinct biological subtypes: luminal (A and B), HER2/neu+, normal breast-like, and basal-like [2, 3]. These molecular subtypes have important prognostic implications and different predictive values, being the basal-like subtype the most aggressive and with poorer prognosis, with no targeted therapy so far [4, 5]. Treatment of this breast cancer subtype with conventional chemotherapeutic

agents maintains the risk of breast cancer recurrence substantially high, around 30–40% [6], reinforcing the urgent need to look for new therapeutic targets.

It is known that most solid tumours, including breast cancer, have high rates of glucose consumption associated with lactate production, even in the presence of sufficient oxygen to sustain oxidative phosphorylation, by a process known as "Warburg effect" [7, 8]. The high glycolytic rates can provide several advantages to cancer cells, namely the acidification of tumour microenvironment by lactate extrusion, which is associated with higher cell proliferation, migration and invasion, angiogenesis and increased cell survival [9, 10]. To maintain the glycolytic flux, cancer cells up-regulate several proteins, including glycolytic enzymes and pH regulators, such as monocarboxylate transporters (MCTs) that will mediate the efflux of lactate [11].

The MCT family, Solute Carrier Family 16 (SLC16), comprises fourteen related proteins, being MCT1 and MCT4 responsible for the efflux of lactate coupled with a proton across the plasma membrane [12-15], contributing to the acidic tumour environment, which is adverse to non-tumour cells. These transporters require a protein chaperone, CD147, to be trafficked to plasma membrane and perform their activity [16].

The enhanced rates of glycolysis and glucose uptake in tumours are maintained by several adaptive mechanisms, including adaptation to hypoxia conditions [17-19], in which HIF-1α (Hypoxia inducible factor 1-α) is a key player by regulating several metabolism related proteins like the glucose transporter 1 (GLUT1), carbonic anhydrase 9 (CAIX) and MCTs [17, 20, 21]. In breast cancer, our group reported an association between MCT1 and both GLUT1 and CAIX expression, particularly in the basal-like subtype [22], which is associated with shorter disease-free survival. Also, MCT1 and CD147, alone or in co-expression, were also associated with estrogen receptor (ER) and progesterone receptor (PR) absence, high histological grade and proliferative index (Ki67), and presence of basal markers such as cytokeratin 5, 14 and vimentin [23], supporting the role of MCT1/CD147 in breast cancer aggressiveness as well as in the maintenance of the glycolytic phenotype. Consistently, several authors have demonstrated the importance of MCT inhibition in cancer, using both *in vitro* and *in vivo* models [for review see [24]]. Further, Morais-Santos *et al.* characterized the effect of targeting MCTs, especially MCT1, in a panel of breast cancer cell lines. Activity inhibition of MCTs with different inhibitors (quercetin, lonidamine and α-cyano-4-hydroxycinnamic acid (CHC)) decreased *in vitro* breast cancer cell aggressiveness, decreasing glucose consumption and lactate production, cell viability, proliferation, migration and invasion. Also, in Hs578T cells, which express MCT1 but lower MCT4, impairment of lactate transport led to increased cell death by apoptosis. Specific inhibition of MCT1 by siRNA in the same cells corroborated the results obtained by activity inhibition [25]. Also, results from the group in lung cancer and in glioblastoma models also anticipate the success of targeting MCTs [26, 27].

Thus, the promising results that have been emerging in the last years point at MCTs as new promising anticancer targets, however, more studies are needed to validate the potential of lactate transport inhibition in breast cancer treatment. In this study, we show that MCT1/4 knockdown reduced lactate transport, cell aggressiveness *in vitro* and, more importantly, inhibited tumour formation and reduced *in vivo* tumour growth.

## RESULTS

### Cancer cell metabolism is remodelled by hypoxia

A panel of three basal-like breast cancer cell lines, with different levels of MCT1/4 expression [25], was specifically used in this study to assess the importance of each MCT isoform in tumour progression.

MCT1, MCT4 and CD147 expression under normoxia and hypoxia conditions is shown in Figure 1A. In MDA-MB-468 cells, MCT1 and CD147 were found in the cytoplasm and plasma membrane, while MCT4 was found in the cytoplasm, being maintained in hypoxia (Figure 1A). MDA-MB-231 cells which do not express MCT1 [28], showed MCT4 and CD147 expression at both the cytoplasm and plasma membrane in normoxic and hypoxic conditions (Figure 1A). In BT20 cells, the reinforcement of MCT1 and CD147 plasma membrane expression after hypoxia is more evident, while MCT4, even after hypoxia, was not detected in this cell line (Figure 1A).

By Western blot analysis (Figure 1B), there was differential expression of MCTs and CD147 under hypoxia. In MDA-MD-468 cells, MCT1, MCT4 and CD147 protein expressions increased approximately 2-, 4- and 5-fold, respectively. Protein expression alteration in MDA-MB-231 cells was not so obvious, with only a 1.1- and 1.5-fold change in MCT4 and CD147, respectively. Finally, BT20 cells presented an increase in MCT1 of about 1.8-fold and an increase of CD147 of about 1.6-fold.

The glycolytic metabolism of the human breast cancer cell lines under hypoxia was evaluated by glucose and lactate quantification in the culture medium. All cell lines significantly increased the efflux of lactate, being accompanied by a significant increase in glucose uptake only in MDA-MB-468 cells (Figure 1C).

### Blocking lactate transport decreases the metabolic requirements to support cell aggressiveness

To better understand the role of each MCT isoform, a single transient knockdown for MCT1 (siMCT1), MCT4

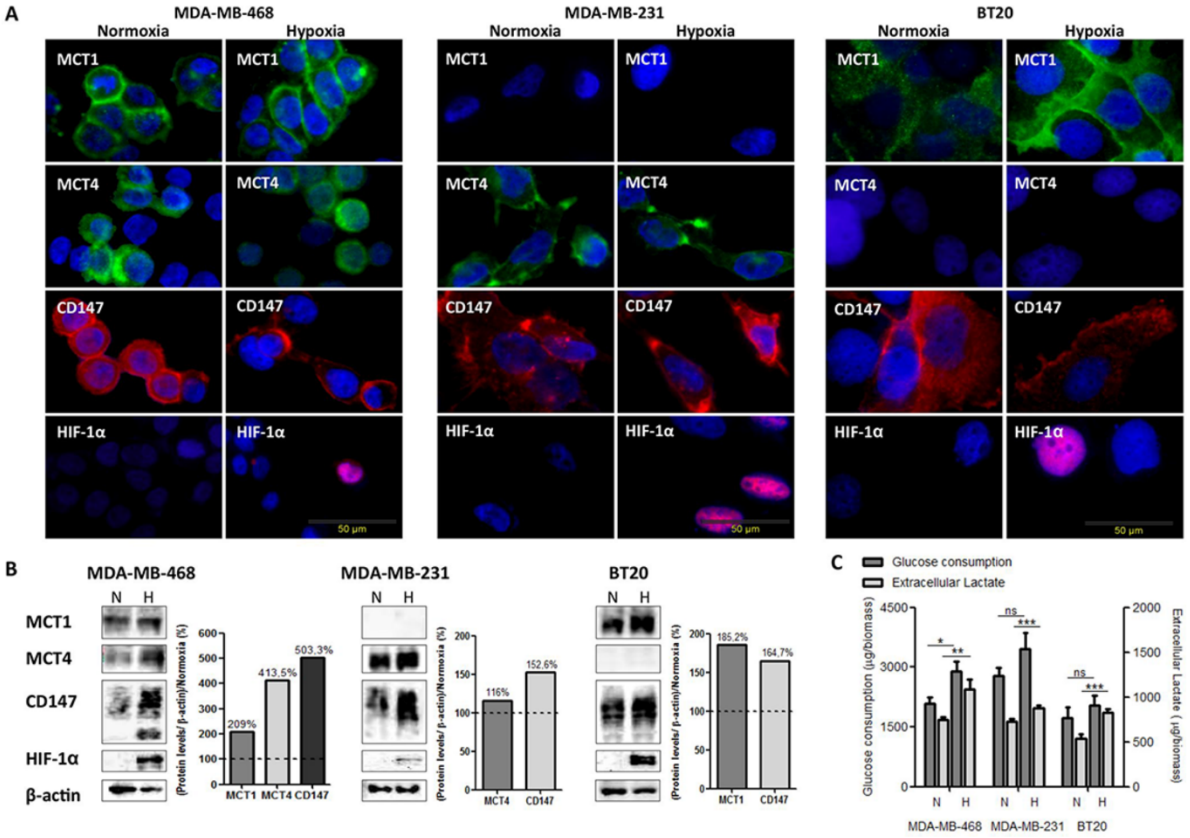


**Figure 1: The metabolic profile of breast cancer cell lines is modulated by hypoxia. A.** Immunofluorescence staining of breast cancer cell lines for MCT1 (green), MCT4 (green), CD147 (red) and HIF-1α (red) after 24 hours under normoxic (N) and hypoxic (H) conditions (magnification, x400; DAPI- blue nuclear staining). **B.** Western blot analysis of MCT1 (50kDa), MCT4 (52kDa), CD147 (31–65 kDa) protein expression under normoxic (N) or hypoxic (H) conditions. β-actin was used as loading control. The graphs represent protein quantification of each blot, compared with normoxia (dashed line). **C.** Glucose consumption and extracellular lactate production by breast cancer cell lines after 24 hours of normoxic (N) or hypoxic (H) conditions. Results are the mean of at least three independent experiments in triplicate ± SEM. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ normoxia *vs*. hypoxia.

(siMCT4) or double transient knockdown for MCT1 plus MCT4 (siMCT1/4) was performed, under normoxia and hypoxia conditions. As seen by Western blot (Figure 2A), MCT1 and MCT4 knockdown was almost complete in the three cell lines. Additionally, MCT knockdown was even more efficient in hypoxia (Supplementary Figure S1A). In MDA-MB-231 and BT20 cell lines, which only express one MCT isoform, MCT4 and MCT1 knockdown, respectively, was able to efficiently decrease the expression levels of CD147 (Figure 2A, Supplementary Figure S1A), while, in MDA-MB-468 cell line, only MCT1 or MCT1/4 knockdown was capable to reduce CD147 expression (Figure 2A and Supplementary Figure S1A).

In addition, in MDA-MB-468 cells, lactate release and cell proliferation were significantly decreased after MCT1 and double knockdown, in both normoxia and hypoxia, while MCT4 knockdown showed no effect (Figure 2B). Regarding MDA-MB-231 cells, MCT4 knockdown significantly reduced lactate secretion only in hypoxia conditions, while cell proliferation was significantly decreased under both normoxia and hypoxia (Figure 2C). In contrast, in BT20 cells, after MCT1 knockdown, lactate secretion and cell proliferation were only significantly decreased under hypoxic conditions (Figure 2D). The influence of MCT knockdown on cell biomass was also accessed and the results showed that only MCT1 knockdown induced a significant decrease on cell biomass, having a more profound effect under hypoxic conditions for both MDA-MB-468 and BT20 cells lines (around 30 and 40%, respectively) (Figure 2E).

To evaluate if the decrease of lactate-induced acidification of the extracellular media influenced other tumourigenic features, we also analysed cell migration and invasion. Thus, as observed in Figure 3A, the single knockdown of MCT1 or MCT4 and the double knockdown were able to significantly decrease cell migration in all cell lines (Figure 3A and Supplementary Figure S1B), in both normoxia and hypoxia. Regarding cell invasion, in

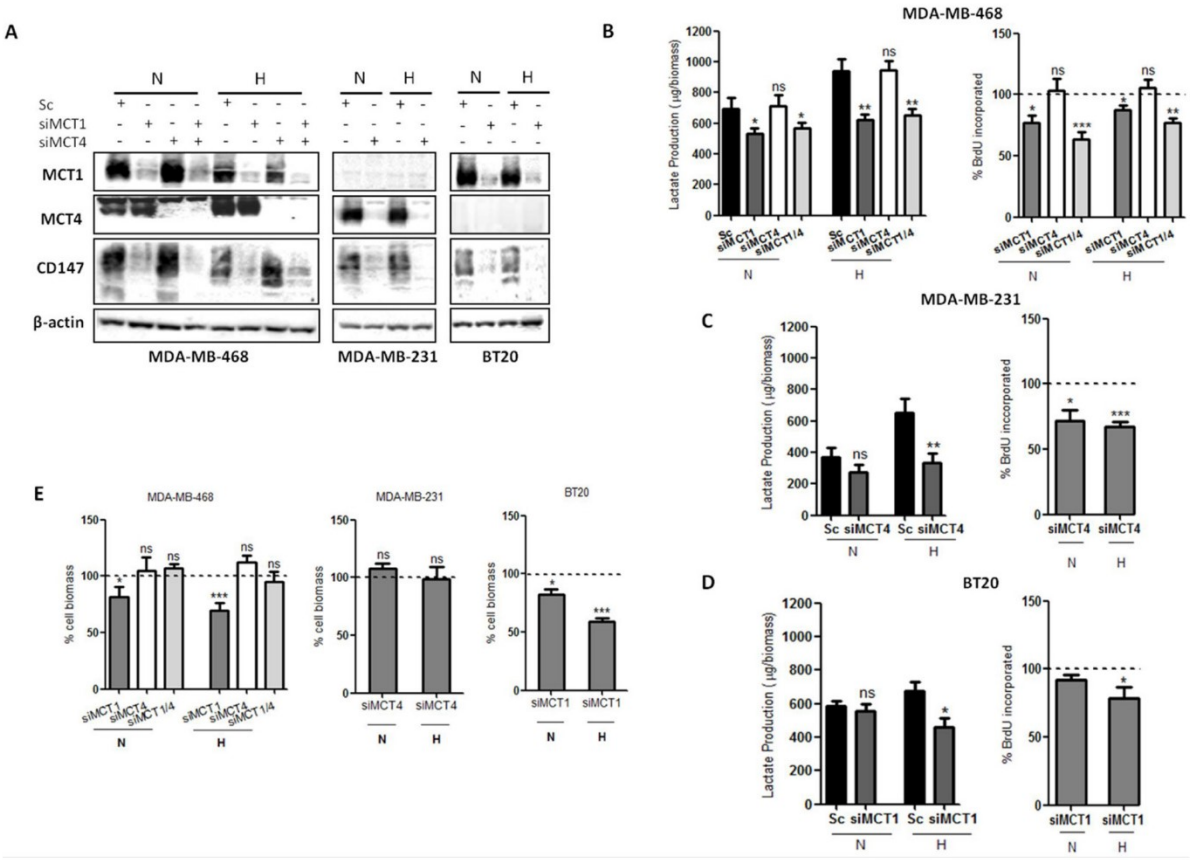


**Figure 2: MCT knockdown impairs glycolytic metabolism and cell proliferation. A.** Western blot results for MCT1, MCT4 and CD147 in silenced breast cancer cell lines under 24 hours of normoxia (N) or hypoxia (H). β-actin was used as loading control. **B,C,D.** Lactate production by silenced cells in normoxia (N) and hypoxia (H) compared to control (Sc - scramble) and percentage of cell proliferation by BrdU incorporation of silenced cells comparing to control (dashed line) in normoxia (N) and hypoxia (H). **E.** Percentage of cell biomass of silenced cells compared to respective control (dashed line). Results are the mean of at least three independent experiments in triplicate ± SEM. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

MDA-MB-468 cells, the percentage of invasive cells was significantly reduced in normoxia upon MCT1, MCT4 and MCT1/4 knockdown, while, in hypoxia, the inhibition of invasion caused by MCT knockdown was lost in siMCT1/4 (Figure 3B and Supplementary Figure S1C). MCT4 knockdown in MDA-MB-231 cells decreased cell invasion more effectively in normoxia, whereas MCT1 knockdown in BT20 cells only decreased significantly cell invasion in hypoxia (Figure 3B and Supplementary Figure S1C).

## MCT1/4 knockdown impairs tumour growth *in vivo*

To test the *in vivo* role of MCTs during cancer initiation, MDA-MB-468, MDA-MB-231 and BT20 cells with transient MCT knockdown were injected into the mammary fat pad of nude mice and tumour formation and growth was monitored. The duration of *in vitro* silencing in cultured cells in normoxic conditions showed that, in MDA-MB-468 cells, silencing of MCT1 and MCT4 was stable up to 10 days (Figure 4A, Supplementary Figure S2A). However, CD147 depletion previously observed after MCT1 and MCT1/4 silencing (Figure 2A) was not maintained after 10 days of silencing (Figure 4A and Supplementary Figure S2A). On the other hand, in MDA-MB-231 and BT20 cells, MCT4 and MCT1 knockdown remained at least until 14 days, as well as CD147 (Figure 4A). The efficiency of MCT4 knockdown in MDA-MB-231 cells and MCT1 knockdown in BT20 cells is shown in Supplementary Figure S2A. Additionally, along 10 days of silencing, there are significant differences in cell proliferation for siMDA-MB-231 and siBT20 cells, and no difference for siMDA-MB-468 cells, compared to controls (Supplementary Figure S2B). The silencing levels at the time of injection are shown by Western blot in each cell line panel (Figure 4B, 4C and 4D).

In the *in vivo* model, MCT1 and MCT4 depletion resulted in a remarkable reduction of tumour growth for

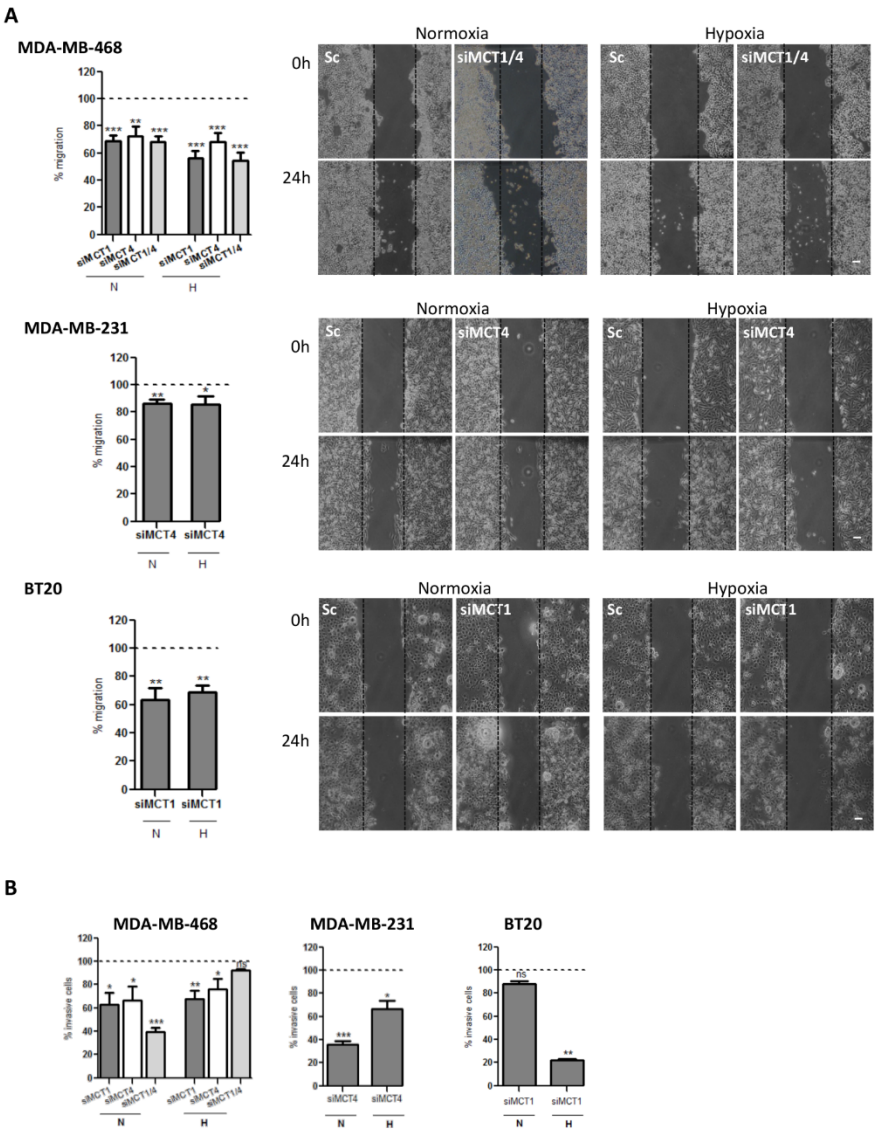


**Figure 3: MCT knockdown decreases *in vitro* cell migration and invasion. A.** Percentage of cell migration after 24 hours of normoxia (N) or hypoxia (H). Silenced cells were compared with the respective control (dashed line). Representative pictures of cell migration at 0 hours and 24 hours are shown (scale bar 100 μm). **B.** Percentage of invasive cells after 24 h of normoxia (N) or hypoxia (H). Silenced cells were compared with the respective control (Sc - scramble). Representative pictures of cell invasion at 24 hours of normoxia (left panel) or hypoxia (right panel) are shown. Results are the mean of at least three independent experiments in triplicate ± SEM. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

MDA-MB-231 and BT20 cells (Figure 4C and 4D). In particular, in MDA-MB-231 cells, at day 45 after injection, tumour volumes were 88 ± 23.4 $mm^3$ in the control group *versus* 20 ± 17.8 $mm^3$ in the MCT4 knockdown group. More importantly, in the MCT4 knockdown group, only 3 out of 8 injected animals developed tumours (Figure 4C). In BT20 cells, the depletion of MCT1 was also capable to significantly decrease tumour volume from 616 ± 162.4 $mm^3$ in the control group to 278.8 ± 37 $mm^3$ in the MCT1 knockdown group (Figure 4D). In this case, all the injected animals developed tumours. Finally, in MDA-MB-468 cells, the single or double MCT knockdown were not able to significantly reduce tumour volume or inhibit its formation (Figure 4B).

To investigate the effect of MCT knockdown in *in vivo* tumour growth inhibition, tumours were collected at the end of the experiment (day 45) and examined by immunohistochemistry for the expression of MCT1,

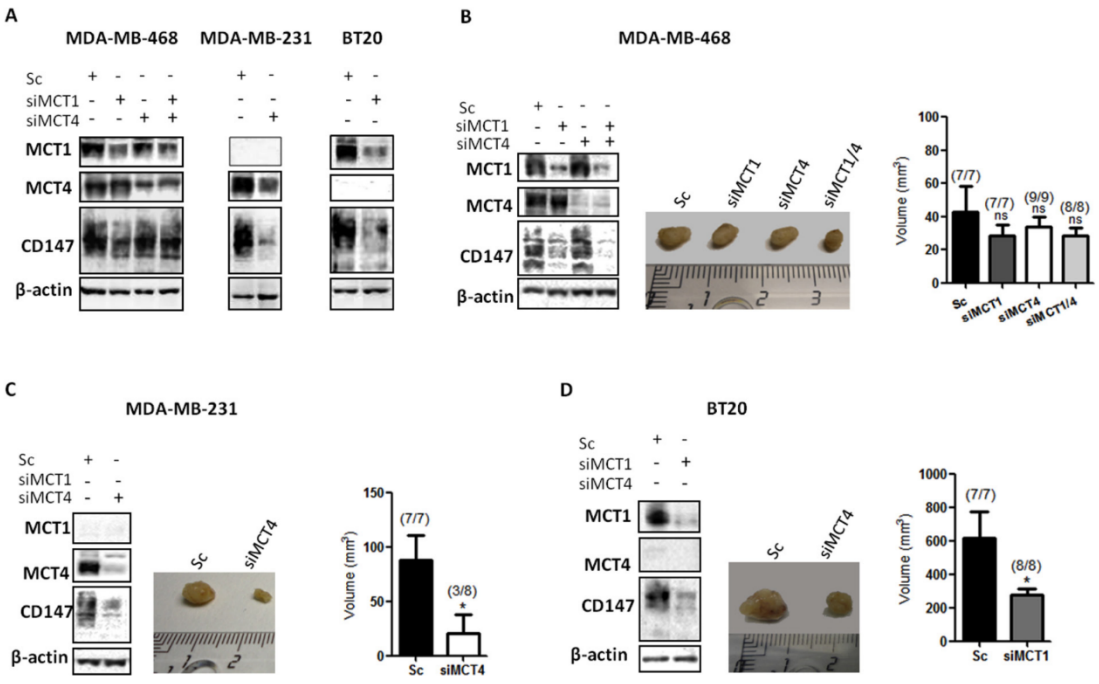


**Figure 4: Knockdown of MCT1 and MCT4 decreases tumour volume *in vivo*. A.** Western Blot results showing MCTs knockdown after 10 days in MDA-MB-468 cells and 14 days in MDA-MB-231 and BT20 cells. β-actin was used as loading control. **B,C,D.** MCT1, MCT4 and CD147 protein expression evaluated by Western Blot at the time of injection in the mammary fat pad of nude mice of the different cell lines. Representative picture of excised tumours after 45 days (middle panel) and the respective tumour volume ($mm^3$) in each animal group. The numbers in brackets indicate: number of animals with formed tumours/total number of animals in each group. The *in vivo* experiments were repeated twice. *: $p < 0.05$ siMCTs groups compared to scramble groups.

MCT4, CD147 and CAIX (Figure 5B, 5C), CD31 and Ki67 (Supplementary Figure S2D). H&E staining demonstrated that MDA-MB-468 silenced and control groups presented high levels of necrosis, when comparing to MDA-MB-231 or BT20 tumours (Figure 5A). Also, after 45 days, MCTs were re-expressed in cancer cells, however, in MDA-MB-468 tumours, the expression of MCT1 and CD147 was predominantly at the plasma membrane in siMCT1, siMCT4 (Supplementary Figure S2C) and siMCT1/4 groups (Figure 5B), while for MDA-MB-231 and BT20 tumours, MCT1, MCT4 and CD147 re-expression was limited to the cytoplasm (Figure 5B). MDA-MB-231 and BT20 tumours did not express MCT1 and MCT4, respectively (Figure 5B). Results also showed that the levels of proliferation (Ki67) and vessel density (CD31) were not altered between control and knockdown groups (Supplementary Figure S2D). Finally, CAIX expression, a cancer cell pH regulator, was more evident in MDA-MB-468 scramble and knockdown tumour groups (Figure 5B) than in MDA-MD-231 and BT20 tumours, which expression was in its majority in the control groups (Figure 5B) compared to the silenced groups.

## DISCUSSION

Glycolytic metabolism has recently been proposed as a fundamental mechanism in the metabolic reprogramming of cancer cells [10]. In fact, the large amounts of glucose consumed by tumour cells has been useful in the diagnosis of breast cancer using $^{18}$FDG PET-scanning, particularly in the detection of metastases of primary tumours, recurrent disease and to monitor therapy response [29, 30]. As a consequence, several proteins are differentially expressed to sustain the glycolytic phenotype of tumour cells, like some pH regulators such as CAIX, MCTs and other proteins of the glucose pathway [17, 23]. Being basal-like tumours a very aggressive group of breast tumours [3, 5], without specific molecular therapy [4] and with high MCT1 expression, and very low positivity in the normal breast tissue [23], this molecule is seen as a promising therapeutic target for this breast cancer subtype.

In this work, we exposed breast cancer cell lines to hypoxia to enhance glycolysis and impair oxidative phosphorylation, a common feature in tumours with disrupted vasculature [8, 31]. Our results show a shift from oxidative phosphorylation to a more glycolytic phenotype

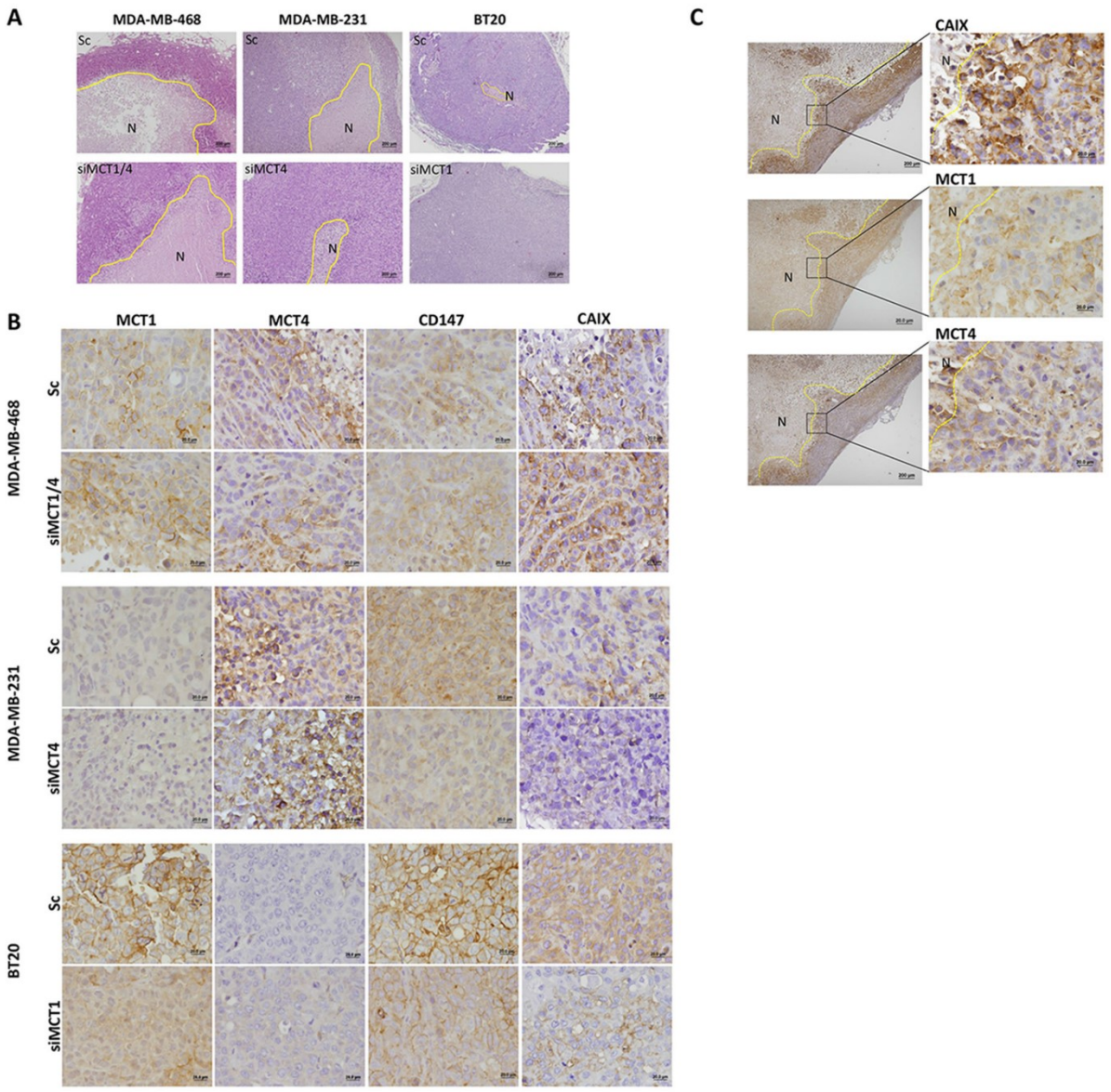


**Figure 5: MCT knockdown inhibits metabolism-related protein re-expression at the plasma membrane. A.** Hematoxylin and Eosin staining of representative tumours. **B.** Immunohistochemical expression of MCT1, MCT4, CD147 and CAIX in the excised tumours. **C.** CAIX, MCT1 and MCT4 expression in siMCT1/MCT4 MDA-MB-468 tumours in peri-necrotic areas. The areas surrounded by the yellow line represent necrosis (N).

in hypoxia, by increased lactate secretion, particularly in MDA-MB-468 and BT20 cells, as well as upregulation of the studied proteins, allowing cells to grow under intermittent hypoxia [9, 19] reinforcing the importance of glycolysis. Consequently, it is expected that hypoxic cells would be more dependent on MCT1/4 expression to export lactate, avoiding acid-induced necrosis and/or apoptosis. Thus, inhibition of MCTs by siRNA would be more effective in hypoxic conditions once cancer cells rely exclusively on glycolysis.

Our results show that MCT1 knockdown was more effective in hypoxia, with a higher decrease in lactate levels, cell biomass and cell invasion in BT20 cells. The drastic reduction observed in *in vivo* tumour growth corroborates these findings. Another study showed that treatment with metformin, which impairs oxidative phosphorylation forcing glycolysis, increase the response and the efficacy of MCT1 inhibitors [32]. Also, treatment of glycolytic cells, without MCT4, with a specific inhibitor of MCT1 showed a decrease in intracellular pH resulting in suppression

of tumour growth [33]. Results from our group also demonstrated that *in vitro* silencing of MCT1 decreased lactate efflux, migration and invasion in both glioma and breast cancer cells [25, 27], as it happened with MCT activity inhibition using classical MCTs inhibitors [25].

Furthermore, silencing of MCT4 in MDA-MB-231 cells, in which MCT1 is silenced by methylation [28], was also able to decrease lactate secretion and proliferation, but not cell biomass. However, hypoxic conditions seem to have no influence *in vitro* comparing to normoxia, probably because MDA-MB-231 cells present a more pronounced glycolytic phenotype, with high glycolytic rates even in the presence of oxygen (Warburg effect). As expected, migration and invasion were also affected, probably due to the interaction between MCT4 and β1-integrin at the leading edge of migrating cells, as reported by others in the same breast cancer cell line [34]. Also, the high reduction of tumour volume demonstrates the great potential of targeting MCT4 in tumours where MCT1 is absent. This was also corroborated by the observed inhibition tumour initiation in 5 of 8 animals, upon depletion of MCT4. Additionally, ectopic expression of MCT4 in transformed fibroblasts (poor tumourigenic cells) completely restored tumourigenicity, pointing at MCT4 as a pro-tumoural molecule [33].

The combined silencing of MCT1 and MCT4 in MDA-MB-468 cells was expected to also have some effect in tumour reduction since, *in vitro*, we demonstrated a decrease in lactate secretion. However, it fails to reduce cell biomass and invasion in hypoxia. Moreover, MCT1 knockdown had also no effect in reduction of tumour volume *in vivo*. As MCT4 expression was mostly cytoplasmic, the results obtained for MCT4 knockdown in these cells were expected. As shown in previous work [25], this cell line presents lower MCT1 and MCT4 expression than the other two cell lines, which probably means a lower dependence on MCTs than MDA-MB-231 and BT20 cells, not showing tumour growth inhibition.

Interestingly, contrary to MDA-MB-231 and BT20 cells, after 10 days of silencing, MDA-MB-468 cells re-expressed CD147. This protein play an essential role in MCT trafficking to the plasma membrane and activity of MCT1 and MCT4, but also in the regulation of matrix metalloproteinases production [35]. Although other authors attributed the major pro-tumoural role of CD147 by chaperoning MCTs, other CD147 pro-tumoural roles, like interaction with signalling integrins, CD98/LAT1 complex and promotion of metalloproteinases were also proposed [33]. Once CD147 was re-expressed within a few days after MCT knockdown, this protein may induce proteins with a pro-tumoural function, like metalloproteinases, failing in the reduction of tumour volume even with low MCT expression, however more studies are needed to support this hypothesis. A particular finding was the high levels of necrotic areas in MDA-MB-468 tumours compared to the other tumour xenografts. Although several studies have reported necrosis as an expected result from MCT inhibition [36-38] the marked levels of necrosis in the control group, excludes MCT knockdown as the cause for tumour necrosis. The proliferation curves along 10 days of silencing show that MDA-MB-468 silenced cells proliferate at the same rate as control cells, supporting the *in vivo* results for this cell line. Moreover, as re-expression of MCT1 and CD147 was at the plasma membrane in MDA-MB-468 tumours, this will probably prevent the decrease of tumour growth. In contrast, in tumours in which MCT expression is restricted to one MCT isoform (MDA-MB-231 and BT20 tumours), re-expression of MCTs and CD147 after 45 days was only cytoplasmic, suggesting a possible disruption in the trafficking to the plasma membrane.

CAIX plays a role in the maintenance of intracellular pH levels of glycolytic cancer cells [39]. This protein was previously associated with MCT1 in a subset of breast cancer basal-like tumours and was also correlated with a shorter disease-free survival [22], pointing at CAIX as a marker of tumour aggressiveness. Also, in another study using invasive breast carcinomas, it was shown that overexpression of CAIX was correlated with poor prognosis [40]. Our present results showed CAIX expression in all control groups, probably as an initial response to tumour hypoxia, with a particular strong expression in MDA-MB-468 tumours and, in this case, being also expressed in the knockdown groups, probably contributing to the aggressive behaviour of tumour. There was a clear CAIX staining in the peri-necrotic areas with correspondence to either MCT1 or MCT4 positivity, supporting the role of CAIX in the maintenance of intracellular pH in glycolytic cells expressing MCT1/4. In contrast, in tumours which volume was reduced after MCT knockdown, re-expression of CAIX was almost insignificant.

Importantly, the increasing interest in metabolic-related targets, like MCT1, is driving the development of new classes of specific and high-affinity inhibitors, including the MCT1 specific inhibitors developed by AstraZeneca. Currently, a Phase 1 clinical trial (NCT01791595) is recruiting patients with prostate cancer, gastric cancer or diffuse large B cell lymphoma, to evaluate the maximum dose, the potential side effects of the drug and the pharmacokinetic profile of AZD3965, a specific inhibitor for MCT1/MCT2, demonstrating the pharmacological interest in targeting MCTs in cancer therapy [41].

## MATERIALS AND METHODS

### Cell culture

The human breast cancer cell lines MDA-MB-468, MDA-MB-231 and BT20 were obtained from ATCC or from collections developed at Drs Elena Moisseva (Cancer Biomarkers and Prevention Group, Departments

of Biochemistry and Cancer Studies, University of Leicester, UK), Marc Mareel (Laboratory of Experimental Cancerology, Ghent University Hospital, Belgium) and Eric Lam (Imperial College School of Medicine, Hammersmith Hospital, London, UK).

All cell lines were routinely cultured in DMEM containing D-glucose (4,5 g/l), (Invitrogen), supplemented with 10% FBS (Invitrogen) and 1% penicillin–streptomycin (Invitrogen), in a 37°C humidified atmosphere with 5% $CO_2$. For experiments cells were cultivated in DMEM without FBS. Hypoxia was achieved using a modular incubator chamber (MIC-101 Billups-Rothenberg Inc.), with an atmosphere of 95% nitrogen and 5% $CO_2$, for 16 hours before starting the experiment, and maintained under hypoxia until the end of the experiment. Oxygen levels were monitored using an oxygen sensor (PAC 3500, Dräger) and never exceeded 1% at the end of the experiment.

### RNA interference and transfection

Silencing experiments were performed using 5 nM of Silencer Select Validated siRNAs from Ambion (MCT1 siRNA: s580 and MCT4 siRNA: s17417), as well as nontargeting control siRNA (Silencer Select Negative Control No.1 siRNA, 4390843, Ambion), using 1 µl/ml of Lipofectamine RNAiMAX (13778-075, Invitrogen), according to the manufacturer's instructions. Since total silencing was only observed after 4 days, being maintained for at least 2 additional days (confirmed by Western Blot), cells were plated at day 3 after silencing and, after overnight adherence, the experiments begin (t0) 4 days after silencing.

### Glucose and lactate measurement

The metabolic behaviour of the cell lines under the different treatment conditions was determined by analysing the extracellular amounts of glucose and lactate. For that, MDA-MB-468 ($6{,}8 \times 10^4$ cells/well), MDA-MB-231 ($3 \times 10^4$ cells/well) and BT20 ($4 \times 10^4$ cells/well) silenced and control cells were plated in 48-well plates and allowed to adhere overnight. Glucose and lactate quantifications were performed after 24 hours under normoxia or hypoxia conditions. Glucose and lactate were quantified using commercial kits (Roche and SpinReact, respectively), according to the manufacturer's instructions, as described previously [27]. Results are expressed as total µg of three independent experiments.

### Immunofluorescence

MDA-MB-468 ($9 \times 10^4$ cells/well), MDA-MB-231 ($4 \times 10^4$ cells/well) and BT20 ($6 \times 10^4$ cells/well) cells were plated on glass cover slips placed into 12-well plates and allowed to adhere overnight. Cells were then fixed after 24 hours under normoxic and hypoxic conditions. Briefly, cells were fixed with 4% paraformaldehyde during 15 minutes at room temperature and then washed 5 minutes with PBS 1x glicine 10 mM. After, cells were permeabilized with triton 0.1% diluted in PBS 1x for 4 minutes and then washed with PBS 1x (2 x 5 minutes). After blocking with bovine serum albumin 5% (BSA, Sigma Aldrich) for 30 minutes, cells were incubated with the respective primary antibodies: mouse anti-MCT1 (1:200, sc-365501, Santa Cruz Biotechnology), rabbit anti-MCT4 (1:500, sc-50329, Santa Cruz Biotechnology), mouse anti-CD147 (1:200, sc-71038, Santa Cruz Biotechnology) and mouse anti-HIF-1α (1:100, 610958, *BD Biosciences*) diluted in BSA 5%, overnight at room temperature. After washing with PBS 1x (2 x 5 minutes), cells were incubated with fluorochrome-conjugated anti-rabbit (1:500, A11008-AlexaFluor 488, Invitrogen Life Technologies) or anti-mouse (1:250, A11032- AlexaFluor 594, Invitrogen Life Technologies) secondary antibodies, diluted in BSA 5%, for 1 hour at room temperature. Finally, slides were washed with PBS 1x (2 x 5 minutes) and counter-stained with DAPI (Fluoroshield F6057, Sigma-Aldrich).

### Western blot

Cell lysis, protein sample preparation and Western blot were carried out as previously described [27]. Briefly, primary antibodies mouse anti-MCT1 (1:500, sc-365501, Santa Cruz Biotechnology), rabbit anti-MCT4 (1:500, sc-50329, Santa Cruz Biotechnology), mouse anti-CD147 (1:500, sc-71038, Santa Cruz Biotechnology), mouse anti-HIF-1α (1:500, 610958, *BD Biosciences*) and goat anti-actin (1:500, sc-1616, Santa Cruz Biotechnology) were used. Membranes were then incubated with the adequate secondary antibodies coupled to horseradish peroxidase (Santa Cruz Biotechnology) and bound antibodies were visualised by chemiluminescence (Supersignal West Femto kit, Pierce, Rockford, IL, USA). Protein quantification was performed using ImageJ Software (version 1.41).

### Cell biomass analysis

MDA-MB-468 ($1 \times 10^4$ cells/well), MDA-MB-231 ($6 \times 10^3$ cells/well) and BT20 ($7 \times 10^3$ cells/well) silenced and control cells were plated in 96-well plates and allowed to adhere overnight in complete DMEM medium. The effect of MCT knockdown on total biomass, measured by the Sulforhodamine B assay (TOX-6, Sigma-Aldrich), was evaluated after 24 hours of treatment (under normoxia and hypoxia), after a previous 24 hours period of hypoxic growth in the case of the hypoxia treatment condition. Viability curves were calculated with GraphPad Prism 5 software.

### Cell proliferation assay

MDA-MB-468 ($1 \times 10^4$ cells/well), MDA-MB-231 ($6 \times 10^3$ cells/well) and BT20 ($7 \times 10^3$ cells/well)

silenced and control cells were plated in 96-well plates and allowed to adhere overnight. Cell proliferation was assessed after 24 hours of normoxia and hypoxia. For the proliferation curves along 10 days, MDA-MB-468 (1000 cells/well), MDA-MB-231 (500 cells/well) and BT20 (500 cells/well) silenced and control cells were plated in 96-well plates and allowed to adhere overnight. Cell proliferation was assessed after 2, 4, 8 and 10 days. To determine the % of proliferation, cells were incubated with 20 μM bromodeoxyuridine (BrdU) for 6 hours before the end of each time point and BrdU incorporation was assessed according to manufacturer's protocol (BrdU, Cell Proliferation ELISA, Roche Diagnostics), as previously described [27].

### Migration assay

MDA-MB-468 ($6 \times 10^5$ cells/well), MDA-MB-231 ($2 \times 10^5$ cells/well) and BT20 ($3 \times 10^5$ cells/well) silenced and control cells were plated in 96-well plates and allowed to adhere overnight. At t0, silenced cell monolayers were washed and a "wound" was made by using a plastic pipette tip. The "wounded" areas were photographed by phase contrast microscopy at 0 and 24 hours. The migration distance was measured using the beWound - Cell Migration Tool (Version 1.5) (developed by A.H.J. Moreira, S. Queirós and J.L. Vilaça, Biomedical Engineering Solutions Research Group, Life and Health Sciences Research Institute- University of Minho; available at http://www.besurg.com/sites/default/files/beWoundApp.zip). beWound is an image processing tool to automate the measurement of cell migration rate in images from Wound-healing assay. This tool comprehends a three step approach: a) automatic image split into cell and "wounded" areas using an approach based on image appearance differences; b) extraction of detailed contours between compacted cell layer and "wounded" area; and, c) measurement of N user-defined lines (5 in current experiments), equally-spaced across the image and perpendicular to the "wound" main axis. This software allows to remove the user-dependency during measurements, while easing the analysis of large databases of images.

The migration distance relative to the control was calculated with the following formula at each time point: relative migration distance (%) = 100 (A–B)/a–b, where A/a is the width of cell wound before incubation, and B/b is the width of cell wound after incubation; A/B refers to the treated condition, a/b refers to the control condition.

### Invasion assay

Cell invasion assay was performed using 24 well BD Biocoat Matrigel Invasion Chambers, according to the manufacturer's instructions (354480, BD Biosciences) and as previously described [27]. MDA-MB-468 ($4 \times 10^4$ cells/well), MDA-MB-231 ($2{,}5 \times 10^4$ cells/well) and BT20 ($3 \times 10^4$ cells/well) silenced and control cells were plated into invasion chambers for 24 hours either in normoxia or hypoxia. Membranes were photographed in a stereomicroscope and invading cells were counted using the Image J software (version 1.41, NIH). Invasion was calculated as % of cell invasion normalised for the control condition.

### Mouse tumour models

Tumour induction was performed by orthotopic injection of $1 \times 10^6$ MDA-MB-468, MDA-MB-231 and BT20 cells silenced for MCTs, in the mammary fat pad of six to eight week-old female N:NIH(s)II: nu/nu nude mice. Tumour growth was monitored weekly using a calliper. At the end of the experiment (45 days), mice were sacrificed by cervical dislocation and the tumours removed, fixed in 10% buffered formalin, embedded in paraffin and sectioned for histological and immunohistochemical evaluations. Tumour volume was calculated according to the formula $V = (L \times W^2)/2$ (L= length, W= width). Animal experiments were carried out in accordance with the European Guidelines for the Care and Use of Laboratory Animals, Directive 2010/63/UE and the National Regulation published in 2013 *(Diário da República, 1.ª série- N.º 151-7 de agosto de 2013)*.

### Histology and Immunohistochemistry

A standard Haematoxylin and Eosin (H&E) staining was performed to assess the morphology of tumours. Immunohistochemistry was carried out as previously described [27]. The following primary antibodies were used: mouse anti-MCT1 (1:500, sc-365501, Santa Cruz Biotechnology), rabbit anti-MCT4 (1:500, sc-50329, Santa Cruz Biotechnology), mouse anti-CD147 (1:400, sc-71038, Santa Cruz Biotechnology), as previously described [25], rabbit anti-CAIX (1:2000, ab15086, Abcam) and mouse anti-Ki67 (1:200, AP10243CM, Gennova), during 2 hours at room temperature, using UltraVision Detection System Anti-polyvalent, HRP (Labvision Corporation). Goat anti-PECAM (CD31, 1:400, sc-1506, Santa Cruz Biotechnology) diluted in PBS, was incubated overnight at room temperature, for further incubation with biotinylated horse anti-goat (1:500, BA-9500, Vector Laboratories) secondary antibody, 1 hour at room temperature. In the remaining steps, the R.T.U. Vectastain Elite ABC Kit (Vector Laboratories) was used. Antigen retrieval to Ki67 and CD31 reactions were performed with citrate buffer, in the microwave for 15 minutes.

### Statistical analysis

Data from at least three independent experiments, each one in triplicate, was stored in GraphPad Prism 5 software. All conditions were examined for statistical significance using two-tailed Student's *t*-test for mean comparison, being the threshold for significance $p$ values $<0.05$.

## CONCLUSIONS

In summary, our results show that depletion of MCT1 and MCT4 in breast cancer decrease tumour cell aggressiveness *in vitro* and tumour formation and growth *in vivo*, pointing at MCTs as promising targets for cancer therapy. This work reinforces the previous *in vitro* results [25] and, more importantly, demonstrates for the first time disruption of *in vivo* breast tumour growth by targeting lactate transporters, emphasizing the importance of MCTs in breast tumour initiation and progression.

## ACKNOWLEDGMENTS

We thank Drs Elena Moisseva (Cancer Biomarkers and Prevention Group, Departments of Biochemistry and Cancer Studies University of Leicester), Marc Mareel Laboratory of Experimental Cancerology, Ghent University Hospital, Belgium) and Eric Lam (Imperial College School of Medicine, Hammersmith Hospital, London, UK) for providing the breast cancer cell lines. We also thank Nuno Mendes, from IPATIMUP - Institute of Molecular Pathology and Immunology of University of Porto, Porto, Portugal, for the technical support during the *in vivo* assay, Magda Carlos and Sandra Costa, from Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus of Gualtar, Braga, Portugal, and ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal, for providing CD31 antibody and immunohistochemical protocol.

### Author's contribution

FM-S carried out the majority of the experiments, performed the statistical analysis and drafted the manuscript. SG and VM-G performed some experiments and helped in result analysis. AHJM, SQ and JLV developed the software for wound healing measurement and helped in figure construction. JP and FCS helped in the design of *in vitro* and *in vivo* experiments, interpretation of the results and manuscript revision. AL evaluated the immunohistochemical reactions in mouse xenograft samples. FB and CP conceived, and designed the study, coordinated the development of the tasks. All authors read and approved the final manuscript.

### Source of support

This work was supported by Fundação para a Ciência e a Tecnologia (FCT) (grant number PTDC/SAU-FCF/104347/2008), under the scope of "Programa Operacional Temático Factores de Competitividade" (COMPETE) of "Quadro Comunitário de Apoio III" and co-financed by the Fundo Europeu De Desenvolvimento Regional (FEDER). FM-S, VM-G and AHJM received doctoral fellowships from FCT (refs. SFRH/BD/87139/2012, SFRH/BD/ 51997/2012 and SFRH/BD/68270/2010, respectively). SG received a post-doctoral fellowship from UMINHO/BPD/18/2014 and CP from FCT (ref. SFRH/BPD/69479/2010).

## CONFLICTS OF INTEREST

The authors declare no conflicting interests.

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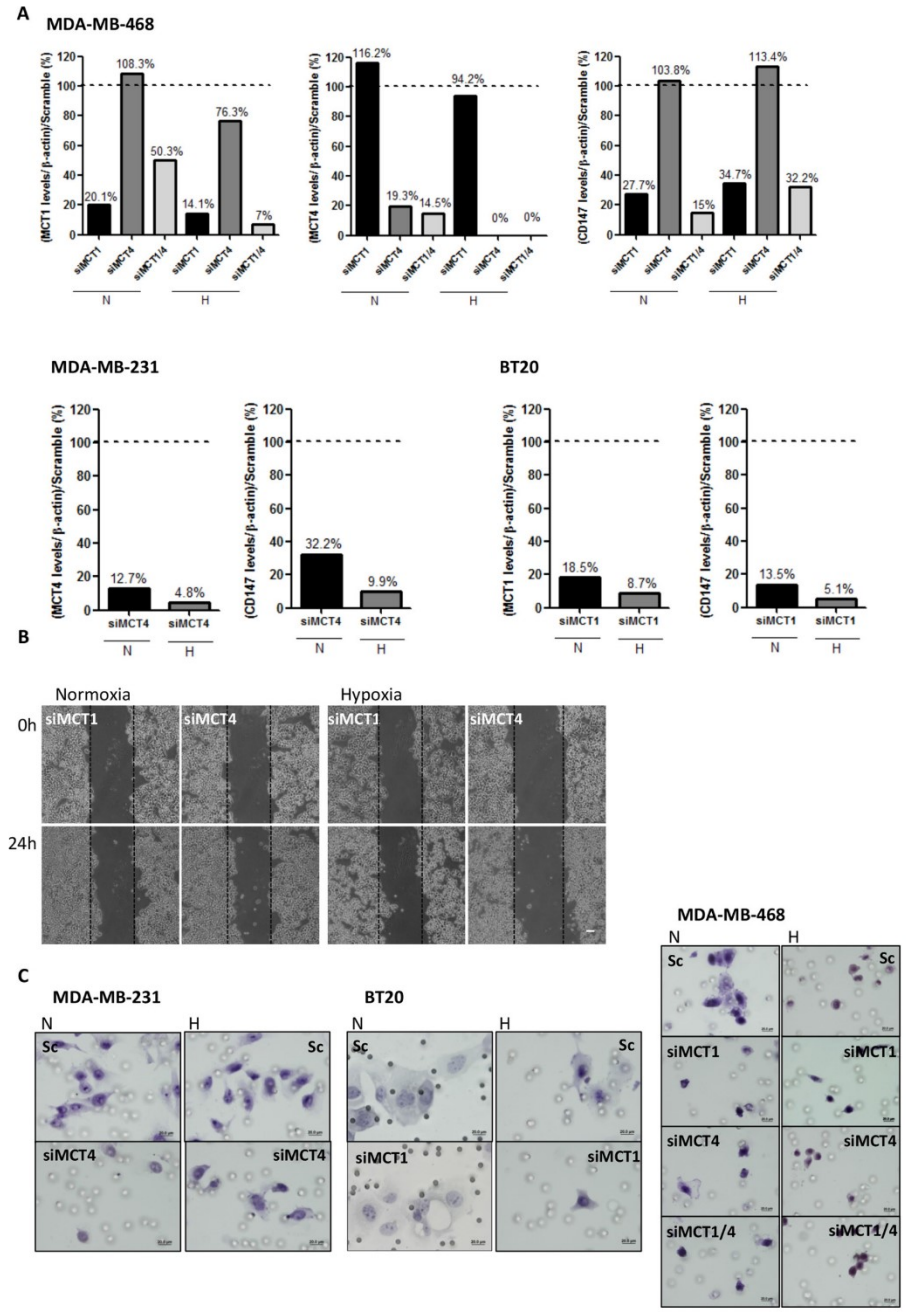
## SUPPLEMENTARY FIGURES




**Supplementary Figure S1: Protein levels of MCTs and CD147 and cell motility capacity. A.** MCT1, MCT4 and CD147 protein levels after MCT knockdown in normoxia (N) or hypoxia (N), measured after Western Blot analysis, compared to scramble (dashed line-100%). Quantification was performed using ImageJ. **B.** Representative pictures of MDA-MB-468 cell migration at 0 hours and 24 hours after MCT1 and MCT4 knockdown in normoxia or hypoxia (scale bar 100 μm). **C.** Representative pictures of MDA-MB-468, MDA-MB-231 and BT20 cell invasion at 24 hours in normoxia (N) or hypoxia (H) are shown. Silenced cells were compared to the respective control (Sc - scramble). Results are the mean of at least three independent experiments, each in triplicate.

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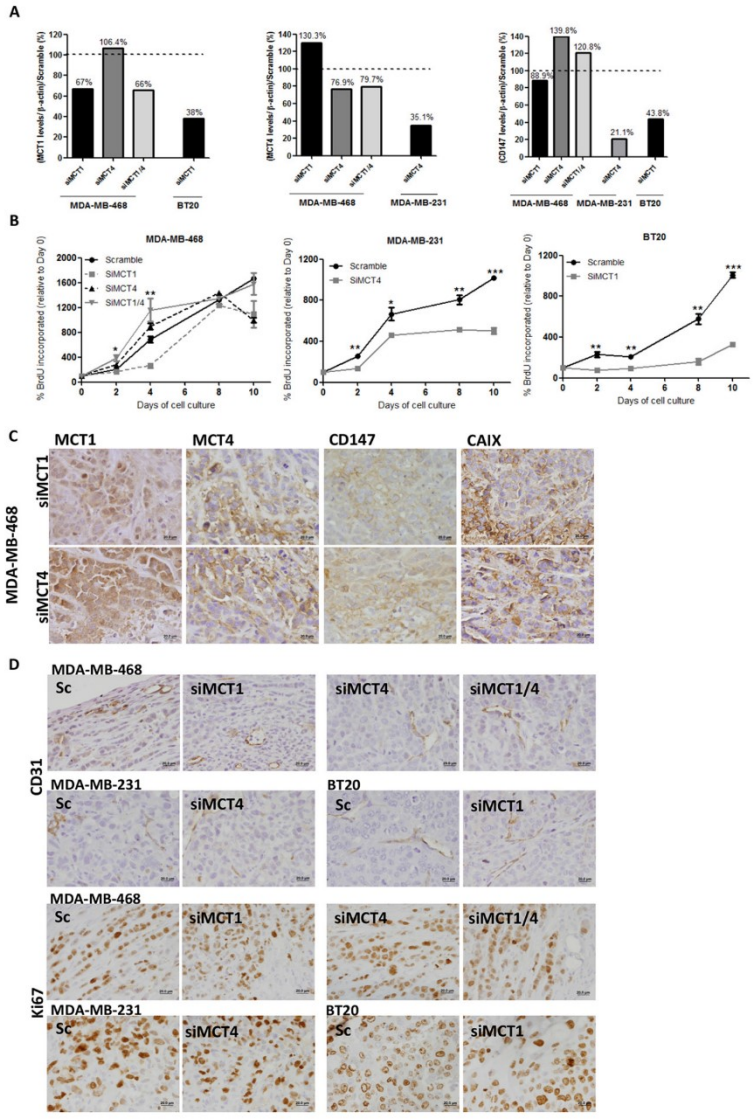


**Supplementary Figure S2: Protein levels of MCTs and CD147 after 10 and 14 days of silencing and *in vivo* protein expression. A.** MCT1, MCT4 and CD147 protein levels after MCT knockdown in normoxia, after 10 days of silencing in MDA-MB-468 cells or after 14 days of silencing in MDA-MB-231 and BT20 cells, measured after Western Blot analysis, compared to control (dashed line). Quantification was performed using ImageJ. **B.** Proliferation curves of BrdU incorporation along 10 days of cell culture. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$, siMCT1 (MDA-MB-468 and BT20) or siMCT4 (MDA-MB-231) compared with respective scramble. **C.** Immunohistochemical expression of MCT1, MCT4, CD147 and CAIX in excised MDA-MB-468 tumours after silencing of MCT1 and MCT4 (after 45 Days). **D.** Immunohistochemical expression of CD31 and Ki67 in excised tumours (after 45 Days).

# CHAPTER 3: MONOCARBOXYLATE TRANSPORTERS REGULATION IN BREAST CANCER

The results presented in this chapter are unpublished results, which are under preparation to be submitted for publication in an international journal:

**Morais-Santos, F.**, Granja, S., Pinheiro, C. and Baltazar, F., *Regulation of the metabolic profile of breast cancer cells by different microenvironmental modulators*. 2017

## 3.1. Chapter overview

Regulation of MCTs in cancer is poorly understood and studies about the regulatory mechanisms in breast cancer are scarce. However, regulation of MCTs by CD147 for its correct localization and function is a well-characterized mechanism [1-3]. More recently, another chaperone (CD44) emerged as an alternative protein involved in MCT plasma membrane localization [4]. Regulation of MCTs by hypoxia, has also been discussed but is not completely understood, especially in what concerns MCT1 [5-8]. However, a close association of MCT1 with hypoxia regions was shown in breast cancer samples, by association of MCT1, but not MCT4, with GLUT1 and CAIX [9]. Additionally, association of MCT1 expression with absence of both estrogen and progesterone receptors, expression of basal markers and high histological grade was shown [10], prompting us to pursue additional studies on its regulatory mechanisms.

Thus, in this chapter, several processes involved in enhanced breast cancer progression were considered, from epithelial mesenchymal transition, estrogen receptor signalling, to glycolytic metabolism. Breast cancer cells were stimulated with modulators linked to these pathways, like transforming growth factor β (TGF-β), 17β-Estradiol (E2), glucose, and lactate, being the metabolic behaviour of breast cancer cells evaluated, in order to unravel the role of these modulators in its regulation.

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### 3.1.1. Regulation of the metabolic profile of breast cancer cells by different microenvironmental modulators

*Manuscript under preparation*

# Regulation of the metabolic profile of breast cancer cells by different microenvironmental modulators

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*Manuscript under preparation*

## Abstract

Monocarboxylate transporters (MCTs) constitute one of the most important family of molecules involved in the maintenance of the glycolytic phenotype and intracellular pH homeostasis of cancer cells, which activity contributes to the tumour acidic microenvironment through lactate transport. In human breast cancer, MCT1 overexpression was associated with aggressiveness parameters, such as basal-like breast carcinoma, while *in vitro* and *in vivo* MCT inhibition caused a decrease in breast cancer cell aggressiveness and a reduction in tumour growth. However, the regulatory mechanisms involved with metabolic reprogramming, including MCT expression, in breast cancer remains poorly understood. Considering the several processes associated with enhanced breast cancer progression, like epithelial mesenchymal transition (EMT), estrogen receptor (ER) signalling and glycolytic metabolism, in the present study, we intend to unravel the influence of these modulators in the metabolic phenotype in breast cancer.

Three breast cancer cell lines and one normal epithelial cell line were stimulated with different concentrations of glucose, 17 β-estradiol, TGF-β and lactate, and the effects on cell metabolism and both mRNA and protein expression were evaluated.

The results demonstrate that low glucose concentrations are able to modulate MCT expression, mainly MCT4, promoting its increase in triple negative breast cancer (TNBC) cells. Exposure to 17 β-estradiol increased lactate production in MCF7 cells, but independently from MCT protein/mRNA levels, while TGF-β stimulation increased lactate export in these cells.

The results here presented indicate that the studied modulators participate in the modulation of the glycolytic metabolism of breast cancer cells, however the mechanisms underlying these effects must be further characterized.

**Keywords:** Monocarboxylate transporters; breast cancer; regulation; Warburg effect.

**Background**

According to data from the American Cancer Society, breast cancer is still the most common malignancy in 2016 and places second as the leading cause of cancer-related death in women [1].

Well defined molecular features allow to stratify breast cancer in different subtypes with particular phenotypes [2], which have important implications in prognostic and treatment [2, 3]. One of the most common subtypes of breast cancer is the luminal subtype- estrogen/progesterone receptor (ER/PR) positive. This subtype presents an epithelial phenotype, expressing several epithelial markers like E-cadherin, cytokeratin 8, 18, 19, claudins, occludins, and others [4], being less invasive and presenting a more favourable prognosis comparing to other subtypes [3, 5]. In contrast, triple negative subtype breast cancer - TNBC (negative for ER, PR and human epidermal growth factor receptor 2 receptor (HER2)) remains the most aggressive subtype and with poor prognosis [6, 7], reinforcing the urgent need for identification and validation of targeted therapies [7]. This subtype is frequently associated with mesenchymal markers, like vimentin and N-cadherin, which are associated with the invasive potential of these cells [4].

Several cancer hallmarks like sustaining proliferative signalling, evading growth supressors, escape to cell death, angiogenesis, inflammation, genome instability, and others, enable tumour growth and metastasis [8]. However, other signals from the microenvironment, including hormones, growth factors, glucose concentration, and others like levels of oxygen, also play a central role in the carcinogenic process [9, 10].

One of these hallmarks is the reprograming of energy metabolism, by which cancer cells consume high amounts of glucose and metabolize it into lactate, even in presence of oxygen. This phenomenon, known as “Warburg effect” or “aerobic glycolysis”, is characterized by faster production of ATP, compared to oxidative phosphorylation (OXPHOS). Further, this

metabolic phenotype provides other advantages to tumour cells, such as generation of fewer reactive species (ROS), synthesis of ATP independently of oxygen or production of precursors for the biosynthetic pathways, supporting cell proliferation [11]. Another advantage for the tumour cells is the production of large amounts of lactate, resulting in acute and chronic acidification of the tumour microenvironment, through the increase in extracellular lactate, which is associated with higher proliferation, survival, migration, invasion and angiogenesis [8, 12]. Lactate transport is mediated by monocarboxylate transporters (MCTs), a family of 14 members, but only MCT1 to MCT4 are responsible for the co-transport of protons and lactate through the plasma membrane [12-16]. MCTs present different affinities for their substrates; while MCT1 and MCT2 mostly perform the uptake of monocarboxylates, MCT4 is more adapted to transport these substrates out of the cells [13, 15, 16]. Several studies reported upregulation of these molecules in several tumour types [17-22], including in breast cancer [23]. Moreover, previous results from our group showed association of MCT1 expression with absence of ER and PR, expression of basal markers, high histological grade and increasing rates of proliferation, pointing at MCT1 and its chaperone (CD147) as relevant molecules in breast cancer aggressiveness [24]. Besides that, MCT inhibition was demonstrated as an effective strategy to decrease *in vitro* and *in vivo* tumour aggressiveness [25-28], decreasing tumour growth, lactate production, cell proliferation, migration and invasion in breast cancer [25], placing these molecules as promising targets in cancer therapy.

Concerning regulation of MCT expression, transcriptional and post-transcriptional mechanisms have been described in normal tissues [13, 14, 29]. In cancer, many studies report regulation of MCT expression by hypoxia [17, 25, 30, 31], although only *SLC16A3* (MCT4 encoding gene) promoter presents hypoxic response elements (HRE) in the promoter region [32-34]. High concentrations of monocarboxylic acids, like lactate and butyrate, have also been

reported to regulate MCT1 mRNA expression [35-38], and MCT4 protein expression in breast cancer [37]. Furthermore, hormonal regulation by testosterone [39] and thyroid stimulating hormone [40] has been described for MCT1, as well as regulation by insulin-like growth factor receptor type I which up-regulates MCT1 in normal tissues [41].

As the mechanisms underlying the modulation of glycolytic metabolism in cancer are still poorly understood, and considering the several processes involved in enhanced breast cancer progression, like epithelial mesenchymal transition (EMT), estrogen receptor (ER) signalling and metabolic intermediates in the present study, we intend to unravel the role of these modulators in the regulation of the metabolic phenotype in breast cancer.

**Methods**

***Cell culture***

The human breast cancer cell lines MCF7, Hs578t and MDA-MB-231 were obtained from ATCC and grown in DMEM containing 4.5g/L of D-glucose (Invitrogen), supplemented with 10% Fetal Bovine Serum - FBS (Invitrogen) and 1% penicillin-streptomycin (Invitrogen). The human non-tumourigenic epithelial cell line MCF10-2A was kindly provided by Professor Lígia Rodrigues (Centre of Biological Engineering, University of Minho, Braga, Portugal) and cultivated in DMEM/F12 (1:1), supplemented with 20ng/ml of epidermal growth factor (Peprotech), 100ng/ml cholera toxin (Sigma Aldrich), 0.01mg/ml insulin (Sigma Aldrich), 500ng/ml hydrocortisone (Sigma Aldrich), 5% FBS (Invitrogen) and 1% penicillin–streptomycin (Invitrogen). Cells were grown in a 37ºC humidified atmosphere with 5% $CO_2$.

For experiments with high glucose (HG - 25mM glucose), low glucose (LG - 5mM glucose) and low glucose plus lactate (LG+Lac - 5mM glucose + 10mM lactate) cells were plated in medium without FBS. For cell stimulation, recombinant Tumour Growth Factor-β2 (TGF-β2) (Peprotech) and 17 β-Estradiol (E2) (Sigma Aldrich) were added at a final concentration of 10ng/ml and 100nM, respectively. Before each

experiment, cells were glucose-starved for 40 minutes.

***Cell metabolism***

Stimulated and unstimulated cells were plated in 48-well plates at a density of 30.000 - 40.000 cells/well and allowed to adhere overnight. Extracellular lactate was quantified in the cell medium after 12 and 24 hours of cell stimulation, using a commercial kit (SpinReact) [28]. For each time point, the total protein (expressed as total biomass) was assessed by the sulforhodamine B assay (SRB, TOX-6, SigmaAldrich). Results are expressed as µg/total biomass of three independent experiments, in triplicate.

***Antibodies***

The following antibodies and dilutions were used: anti-MCT1 (1:500 dilution, sc-365501, Santa Cruz Biotechnology), anti-MCT4 (1:500 dilution, sc-50329, Santa Cruz Biotechnology), anti-CD147 (1:500 dilution, sc-71038, Santa Cruz Biotechnology), anti-hypoxia-inducible factor-1α (HIF-1α) (1:500 dilution, 610958, BD Biosciences), anti-hexokinase II (HKII) (1:4000. dilution, ab104836, Abcam), and anti-β-tubulin (1:3000 dilution, sc-23948, Santa Cruz Biotechnology).

***Western Blot***

After stimulation of human breast cancer and human non-tumourigenic epithelial cells for 24 hours, cells were lysed and the protein extracts collected for protein quantification using *DC* Protein Assay Kit (BioRad). Protein sample preparation and Western blot were carried out as previously described [28]. Incubation of primary antibodies was performed overnight at 4ºC, followed by incubation with the respective secondary antibody for 1 hour at room temperature. Bound antibodies were visualised by chemiluminescence (Supersignal West Femto kit, Pierce, Rockford, IL, USA). β-tubulin was used as loading control. Results are representative of three independent experiments.

***RNA extraction and cDNA synthesis***

RNA was extracted from cell lines using Trizol Reagent (15596-026, Invitrogen), according to manufacturer's instructions. The concentration and purity of RNA was evaluated by spectrophotometry (Nanodrop Technologies, Inc). RNA (1µg) was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems), as recommended by the manufacturer.

***Real-time quantitative PCR (RT-qPCR)***

RT-qPCR was performed using SsoFast EvaGreen Supermix (BioRad) according to the manufacturer's information. RT-qPCR was performed in a CFX96 detection system (BioRad) using the following optimized cycling conditions for β-actin, E-cadherin and Vimentin amplification: 30 seconds at 95 ºC for enzyme activation, 5 seconds at 95ºC for denaturation and 5 seconds at 58 ºC for annealing/extension, during 45 cycles. The melting curve was assessed at 65 ºC – 95 ºC, with increments of 0.5 ºC at each 5 seconds/step. For MCTs and CD147 amplification, the annealing temperature was modified to 53ºC and 56ºC, respectively, being the remaining conditions maintained. The following primers were used, at a 500 mM concentration:

| Gene | Forward sequence | Reverse sequence |
|---|---|---|
| E-cadherin | 5'-TGCCCAGAAAATGAAAAAGG-3' | 5'-GTGTATGTGGCAATGCGTTC-3' |
| Vimentin | 5'-GGGACCTCTACGAGGAGGAG-3' | 5'-AAGATTGCAGGGTGTTTTCG-3' |
| β-actin | 5'-GGACTTCGAGCAAGAGATGG-3' | 5'-AGCACTGTGTTGGCGTACAG-3' |
| MCT1 | 5'-TGACCATTGTGGAATGCTGT -3' | 5'- TTTCTGGTCCGGAGATTCTG -3' |
| MCT4 | 5'- TTCTTTGGCATCTCCTACGG-3' | 5'- TGGGCTTCTTCCTAATGCAG-3' |
| CD147 | 5'- GCCAGAAAACGGAGTTCAAG-3' | 5'- CCCTCGTTGATGTGTTCTGA -3' |

The expression levels were determined by ΔCq (quantification cycle) values using ΔΔCq method and normalized for β-actin results to calculate the relative gene expression. Results are presented as the mean ± SD of 1 independent experiment in duplicate.

***Statistical analysis***

GraphPad prism 5 software was used for statistical analysis, being all conditions examined for statistical significance using two-tailed Student's t-test for mean comparison, considering significant values for $p<0.05$.

**Results**

***Glucose limitation and exposure to exogenous lactate modulates lactate production in breast cells***

The high consumption of glucose by cancer cells is required for energy generation, culminating in lactate production even in presence of oxygen, by the Warburg effect [42, 43]. However glucose limitation is a feature of the tumour microenvironment, due to high glucose consumption and poor vascularization inside tumour [44-46]. Meanwhile, lactate gained a relevant role in tumour progression, having been considered in several studies as a metabolic fuel, but also as a signalling molecule in several processes [47].

To determine if different concentrations of glucose and exogenous lactate modify breast cancer cell metabolism, lactate production was measured in three breast cancer cell lines (MCF7, MDA-MB-231, Hs578t) and one normal breast epithelial cell line (MCF10-2A) (Fig. 1). In MCF10-2A and MCF7 cells, the metabolic profile with high and low glucose concentrations is very similar, with an increase in lactate secretion along time. However, after 12 hours with exogenous lactate, production of lactate by MCF10-2A normal cells stopped and, in contrast, increased in MCF7 cancer cells. In the TNBC cells MDA-MB-231 and Hs578t, lactate production was significantly higher in low glucose concentrations, along time. When

low glucose and exogenous lactate were combined, lactate production increased after 12 hours but decreased at 24 hours, comparing with LG condition, suggesting lactate consumption by these cancer cells.

***Estradiol exposure alters lactate production in ER positive cells***

Approximately 75% of breast cancers are positive for hormone receptors, namely estrogen and progesterone receptors being this phenotype helpful to predict response to targeted therapy in breast cancer patients [48]. However studies correlating glycolysis and hormonal signalling are scarce, the n of MCT1 expression and absence of ER was described in breast cancer [23]. To test if stimulation of ER was able to modify glycolytic metabolism, MCF7, MDA-MB-231 and MCF10-2A cells were stimulated with E2, and lactate production was evaluated (Fig. 2). The luminal breast cancer cell line MCF7, positive for estrogen receptor [49], increased lactate production after 12 hours of treatment, differently from the results obtained in the ER negative breast cancer

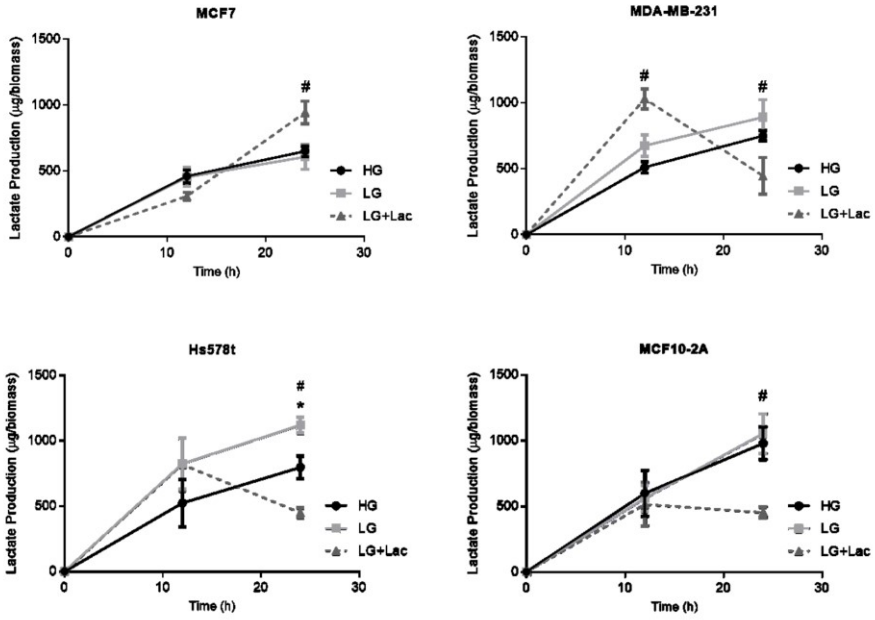


**Figure 1- Lactate production is affected by different glucose concentrations and addition of exogenous lactate.** Cells were cultivated in high glucose (HG), low glucose (LG) and low glucose+lactate (LG+Lac). Extracellular lactate was measured at 12h and 24h. Data shows means ± s.e.m. (n=3). * HG vs LG, $p \leq 0.05$ ; # LG vs LG+Lac, $p \leq 0.05$.

(MDA-MB-231) and normal epithelial (MCF10-2A) cells, in which no alteration was observed. However, after 24h, there were no significant differences in the presence of E2 in the 3 cell lines.

***Exogenous lactate promotes expression of the epithelial marker E-cadherin and decrease of mesenchymal marker Vimentin***

Several markers can be used to classify the EMT cell profile [4, 50], and, in the present study, this status was monitored by evaluation of RNA levels of E-cadherin (epithelial marker) and vimentin (mesenchymal marker) (Fig. 3). At standard conditions (HG), MCF7 cells expressed E-cadherin and very low levels of vimentin, consistent with its epithelial cell phenotype, as reported by others [9, 51]. In contrast, MDA-MB-231 and Hs578t expressed vimentin when grown in standard conditions (HG), but not E-cadherin, in line with the mesenchymal phenotype described for these cells [9, 51]. The normal epithelial cells, MCF10-2A, expressed low levels of both E-cadherin and vimentin at standard conditions (HG). Low concentrations of glucose (LG) did not affect the levels of E-cadherin or vimentin in the breast cancer cell lines MCF7 and HS578t. However, in MDA-MB-231 cells, after low glucose exposure, there was a decrease in vimentin, being E-cadherin expression unaltered.

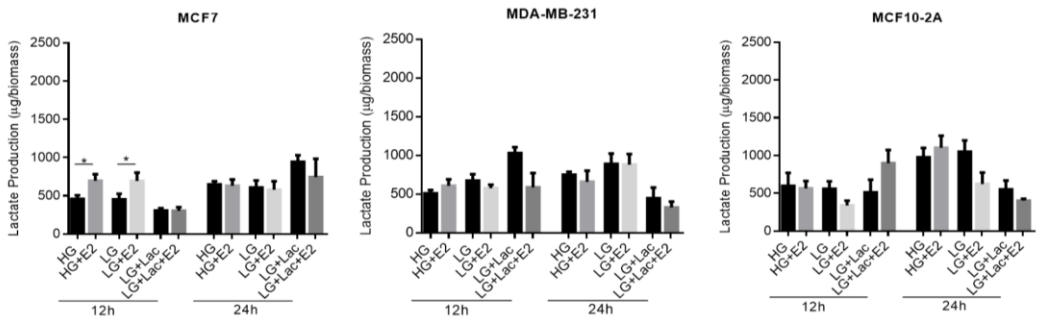


**Figure 2- Lactate produced by the luminal breast cancer cells MCF7 is affected by E2 treatment.** Cells were cultivated in high glucose (HG), low glucose (LG) and low glucose+lactate (LG+Lac) and stimulated with 100nM of 17 β-Estradiol (E2). Lactate was measured at indicated time points. Data show means ± s.e.m. (n=3).* $p \leq 0.05$.

After exposure to lactate (LG+Lac), there was an increase in E-cadherin expression in the breast cancer cell lines, with a more pronounced effect in MCF7 cells, reinforcing the epithelial phenotype of this cell line. In contrast, in mesenchymal cells MDA-MB-231 and Hs578t, lactate led to a decrease in vimentin expression.

Additionally, cells were stimulated with TGF-β, a potent EMT inducing factor [52], to promote this cellular transition. TGF-β stimulation decreased E-cadherin levels in MCF7 and MCF10-2A cells, and decreased E-cadherin and vimentin levels in MDA-MB-231 cells, compared to the respective controls. Particularly in Hs578t cells, TGF-β promoted an increase in vimentin and E-cadherin levels, although E-cadherin presented very low expression levels.

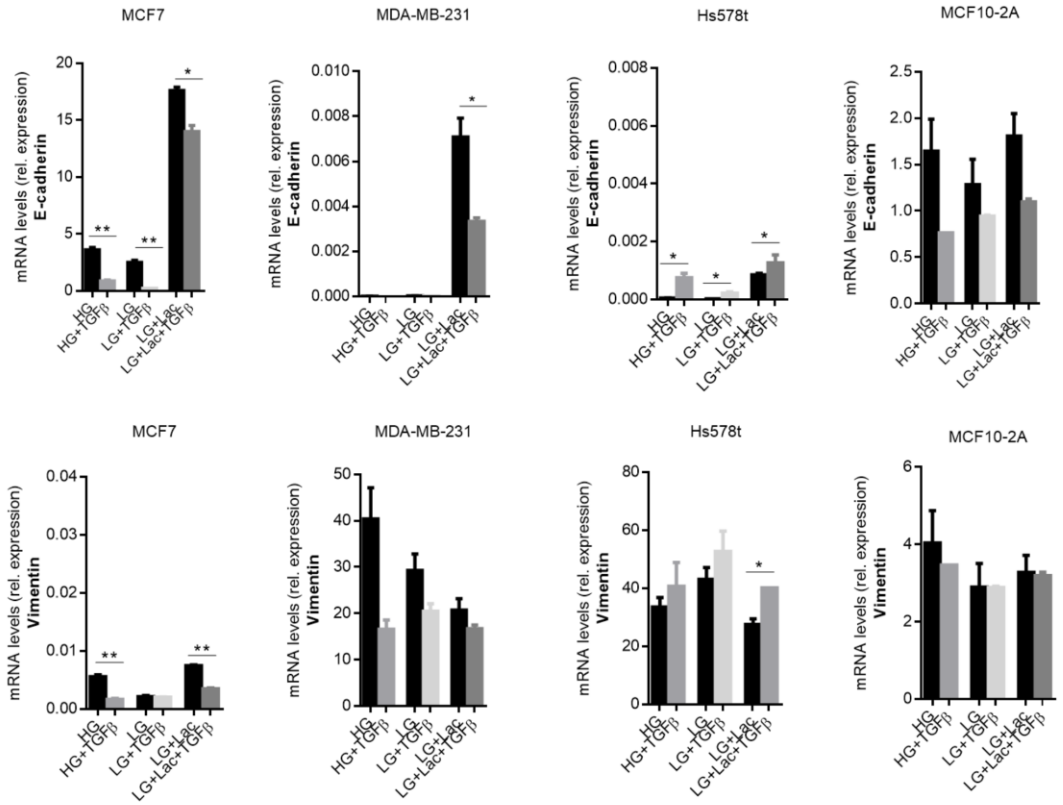


**Figure 3- TGF-β and lactate stimulation modifies the pattern of epithelial-mesenchymal markers.** Cells were stimulated with 10ng/ml of TGF-β at 12h. Cells were lysed for RNA preparation followed by reverse transcription, being cDNA amplified by RT-qPCR using specific primers. All conditions were normalized for β-actin mRNA levels * $p \leq 0.05$; ** $p \leq 0.01$.

***TGF-β increases lactate production in epithelial cells***

To understand the impact of TGF-β in lactate secretion by cancer and normal cells, lactate production was measured at 12 and 24 hours after TGF-β stimulation (Fig. 4). Treatment with TGF-β in MCF7 cells promoted an increase in lactate production in high and low concentrations of glucose, at 24 hours. It should be noted that, among the four cells lines used, this is the only epithelial cells that can undergo EMT. In Hs578t cells, lactate production decreased in the presence of TGF-β combined with low concentrations of glucose at 12 and 24 hours. In MDA-MB-231 cells and in the normal epithelial cells MCF0-2A, TGF-β did not affect lactate production.

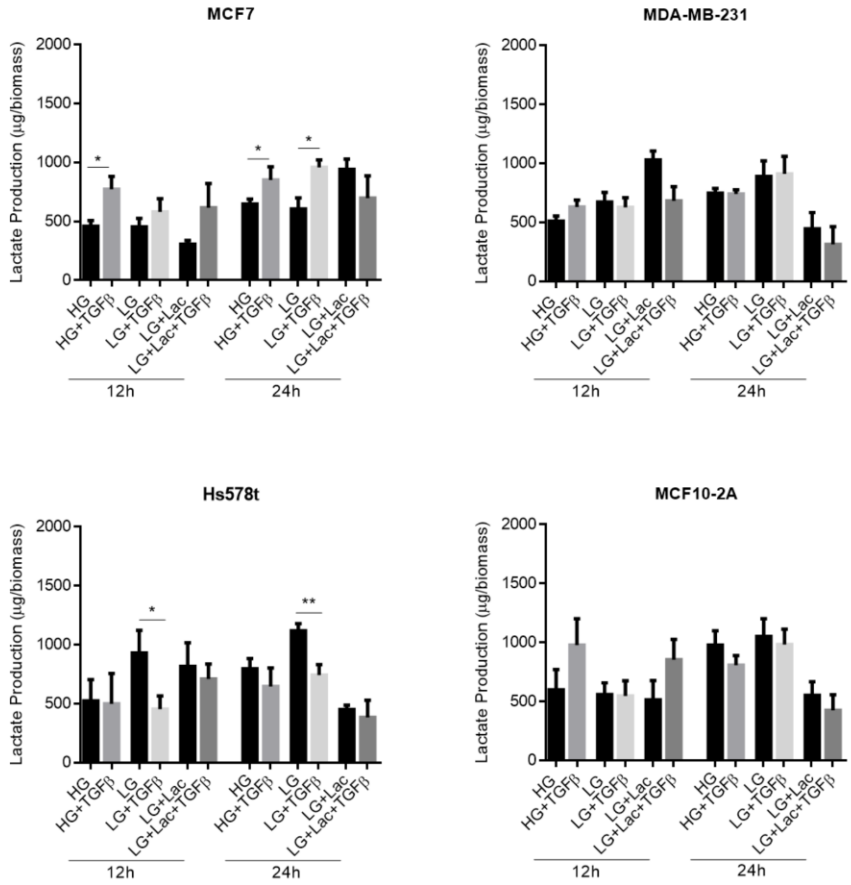


**Figure 4- TGF-β modulates lactate secretion of MCF7 luminal breast cancer cells and Hs578t triple negative breast cancer cells in an opposite way.** Cells were cultivated in high glucose (HG), low glucose (LG) and low glucose+lactate (LG+Lac) and stimulated with 10ng/ml of TGF-β. Lactate was measured at indicated time points. Data show means ± s.e.m. (n=3).* $p \leq 0.05$; **$p \leq 0.01$.

***Expression of glycolytic proteins in cancer cells is modulated by glucose concentrations***

To evaluate if different concentrations of glucose modulate glycolytic protein expression, cells were exposed to different glucose concentrations in culture media, being protein analysed by Western blot (Fig. 5). In MCF7 cells, low glucose increased the expression of several proteins like MCT1, MCT4 and HKII, compared with high glucose conditions. In combination with exogenous lactate, this effect was partially reverted, being protein expression similar to standard conditions (HG). In contrast, the mesenchymal cells MDA-MB-231 and Hs578t showed increased expression of MCT4 and HIF-1α under low glucose conditions, but not MCT1, which appeared to decrease in Hs578t cells. In MCF10-2A cells, variation of glucose concentrations did not seem to influence the levels of the studied proteins. TGF-β treatment decreased MCT1 and CD147 in Hs578t cells. Also, E2 stimulation did not induce protein expression alterations in MCF7 and MCF10-2A cells.

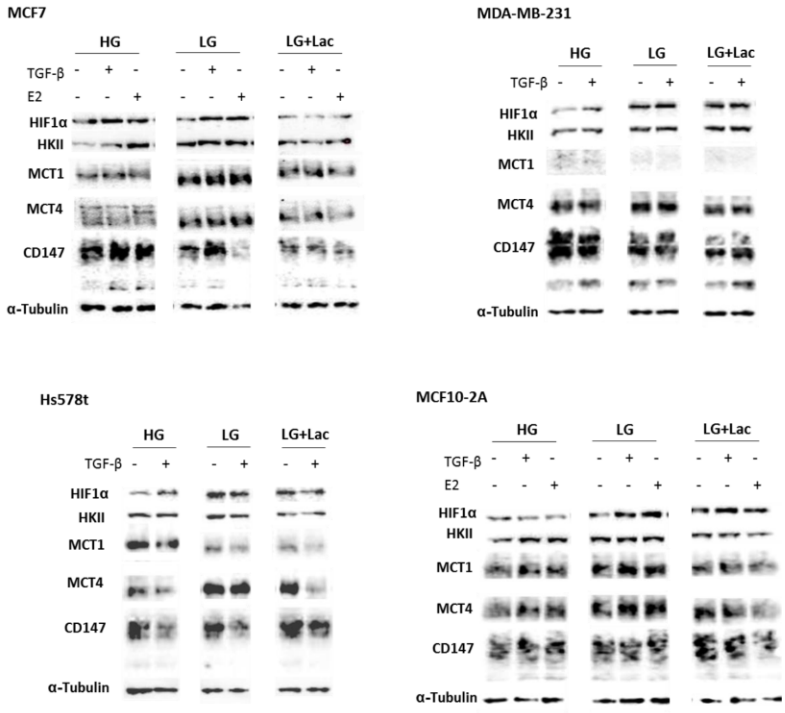


**Figure 5- Metabolism-related protein expression pattern is altered by glucose concentration.** Cells were treated with 100nM of E2 or 10ng/ml of TGF-β during 24 hours. Cells were lysed and protein extracts were analysed by Western blot. β-tubulin was used as loading control.

***mRNA modulation by TGF-β and glucose***

To investigate if the alterations in MCT1, MCT4 and CD147 levels were consequence of altered gene expression, mRNA levels by RT-qPCR were assessed after glucose, TGF-β and/or E2 stimulation (Fig. 6). Regarding mRNA level regulation by glucose, in general, there was a slight decrease in MCTs and CD147 mRNA levels from HG to LG concentrations, with the exception of MCT4 and CD147 mRNA in Hs578t, which increased from HG to LG.

In the normal epithelial cells MCF10-2A and in TNBC cells MDA-MB-231 and Hs578t, TGF-β promoted a similar effect, decreasing the mRNA levels of MCT1, MCT4 and CD147. In MCF7 cells, TGF-β decreased MCT1, CD147 and MCT4 only when combined with lactate. Stimulation with E2 did not alter mRNA levels in MCF7 and MCF10-2A cells, presenting similar levels to the control, with the exception of LG+Lac+E2 stimulation, which decreased MCT1 mRNA levels in MCF7 cells.

**Discussion**

Recent studies have demonstrated the importance of the Warburg effect in cancer, being a feature of aggressiveness, which culminates in the production of large amounts of lactate [14]. Although hypoxia is the main inducer of the glycolytic phenotype in cancer cells, glycolysis is elevated even in tumour cells that are in normoxic conditions. Indeed, many other factors like oncogenes and signalling pathways are involved in the glycolytic switch, which is actually acquired very early in carcinogenesis, even before tumours experience hypoxia [43].

Regarding the large amounts of lactate produced from increased glycolytic activity (5-10 mM intratumoural levels) [53, 54], MCTs have an important role, by transporting lactate across the plasma membrane of cancer cells [14]. In fact, several studies report the adaptive features of cancer cells during the glycolytic switch [11, 12], but less is known about regulation of MCTs by the different microenvironment *stimuli* during breast

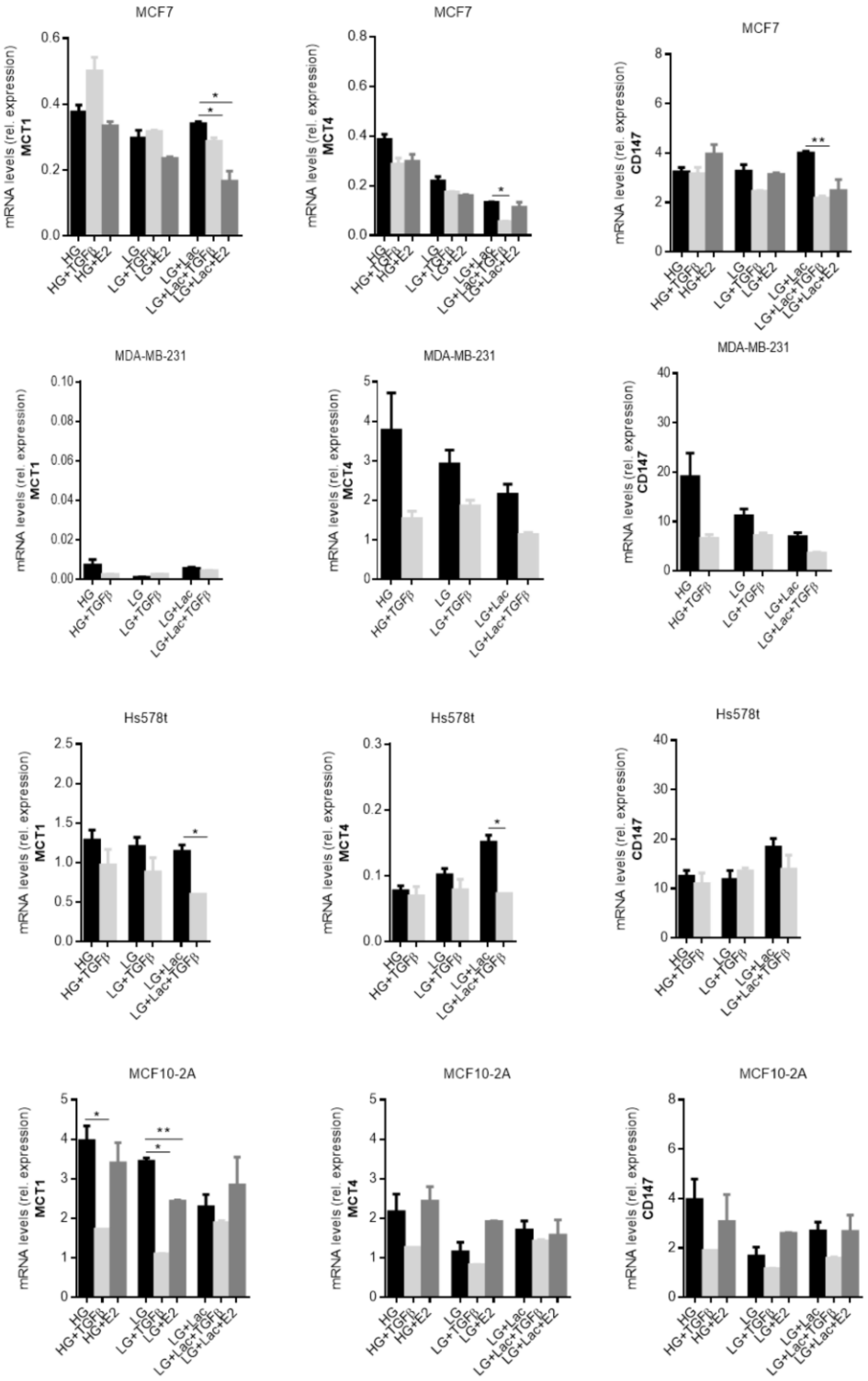


**Figure 6- TGF-β stimulation promoted alteration in mRNA levels of the proteins.** Cells were stimulated with 100nM of E2 or 10ng/ml of TGF-β during 12 hours. Cells were lysed for RNA preparation followed by reverse transcription, being cDNA amplified by RT-qPCR using specific primers * $p \leq 0.05$; ** $p \leq 0.01$.

cancer carcinogenesis. The uptake of glucose, is a crucial process to maintain glycolysis, however, its levels are frequently lower in the tumour microenvironment than in normal conditions, a consequence of high glucose consumption and poor vascularization of solid tumours [45, 46].

Thus, to understand the influence of glucose levels in MCT regulation, we cultured breast cancer cells in low glucose concentrations (LG, 5mM) *vs* high glucose concentrations (HG, 25mM). In the most aggressive cancer cells, MDA-MB-231 and Hs578t, low glucose levels promoted an increase in lactate production, which was accompanied by an increase in MCT expression, with particular emphasis on MCT4. According to the affinity for lactate, MCT1 and MCT2 were described as being primarily involved in lactate uptake, while MCT4 is more adapted for lactate export [13, 14], justifying the increase in MCT4 protein expression under these conditions. However, the levels of mRNA for MCT4 and its chaperone CD147, only accompanied this increase in protein for Hs578t cells but not MDA-MB-231 cells. This could be explained by the known translational or post-translational regulation of the expression of these proteins [14]. In normal and luminal cancer cells, MCF10-2A and MCF7 respectively, exposure to low glucose did not alter de levels of lactate production, however there was increased expression of MCT1 and MCT4 in MCF7 cells at the protein level, but again this change was not accompanied by the corresponding mRNA levels.

Lactate, is an important molecule in tumour microenvironment capable of modulating several processes including immunosurveillance, invasion, migration, angiogenesis, and others, and also modulate the activity of different cells, like endothelial cells and macrophages [32, 55]. Furthermore, lactate was able to regulate the metabolism of neighbour cells, being lactate transported out of glycolytic cells used by oxidative cells [47, 56], or by endothelial cells which perform the uptake of lactate through MCT1, promoting activation of the angiogenic process [57], or even in tumour fibroblasts in which uptake of lactate contributes to the tumour homeostasis

[58]. Being lactate levels in the tumours usually higher (5mM-10Mm) than glucose concentrations [53], the effect of 10 mM of lactate combined with low glucose was tested in the present study. Up to 12 hours of incubation, normal and TNBC cells continue to produce lactate, and, after this time until 24 hours, lactate starts to decrease, probably due to lactate consumption, which could be explained by an increase of MCT2 expression, for example.

Around 70% of breast cancer cases present overexpression of ERα, which in an initial phase most of breast cancer patients respond to anti-estrogen therapy [48, 59]. These intracellular receptors and steroid hormones, like 17-estradiol, play an important role in development and progression of breast cancer, by activation of nuclear and non-nuclear pathways promoting abnormal cell proliferation [48, 60]. Thus, we investigated the influence of ER pathway activation on MCT regulation, using MCF7 cells. In fact, treatment with 17 β-estradiol increased lactate production at 12 hours, but did not modify the protein expression or mRNA levels of MCTs. Since previous results from our group showed correlation between MCT1 and absence of ER expression [23], it was expected that ER pathway activation was not associated with MCT regulation. Nevertheless, it would be important to verify the activation of ER pathway by exposure of breast cancer cells to the synthetic 17 β-estradiol. However, in cells that not express ER (MCF10-2A and MDA-MB-231), lactate production was not affected after 17 β-estradiol treatment.

Another fundamental feature in the steps of invasion and metastasis, is the epithelial-mesenchymal transition (EMT), which is a highly regulated process associated with altered expression of several genes, downregulating many epithelial markers, linked with static and polarized cells, and upregulating mesenchymal markers that are linked with invasive and motile cells [4, 52]. In this way, we tested if MCT activity and expression could be affected by EMT, induced by TGF-β treatment in normal and breast cancer cells. We show that in luminal MCF7 cells treatment with TGF-β increase

the export of lactate, but these alterations were not accompanied by changes in glycolic phenotype-related molecules (protein and mRNA levels), as corroborated by Liu *et al.* in a recent study [61]. Additionally, the increase in lactate export was associated with a decrease in E-cadherin levels, supporting the association with EMT, as supported by others [9, 61]. Concerning MCF10-2A and MDA-MB-231 cells, TGF-β did not modulate lactate export neither protein/mRNA levels of the glycolytic-phenotype proteins. However, in Hs578t cells that express mesenchymal markers, TGF-β decreased lactate production being this accompanied by a decrease in MCT1 and CD147.

Interestingly, exposure to exogenous lactate promoted increase in E-cadherin mRNA expression in the breast cancer cell lines, being this expression reverted by TGF-β. We show that variation in the levels of glucose did not modulate mRNA levels of epithelial or mesenchymal markers, but in contrast exogenous lactate induced expression of E-cadherin and a decrease of vimentin mRNA levels (Fig.3), like if reversion of the transition occurs. This phenomenon has no implications in lactate production or implication in protein and mRNA levels.

**Conclusions**

We have shown that low glucose concentrations have an important effect in the modulation of MCT expression, mainly MCT4, and consequently increases the export of lactate in the most aggressive breast cancer cells. It was often difficult to make the correspondence between changes in protein levels and mRNA levels, which could be explained by some type of translational or posttranslational regulation, warranting further studies to investigate this.

Further, exposure to exogenous lactate in TNBC cells, promoted a change of metabolic phenotype, consequence of the uptake of lactate. This alteration could be also related to the increase in E-cadherin, making breast cancer cells less aggressive. 17 β-estradiol increased lactate production in MCF7 cells, but independently of MCT protein/mRNA levels alteration.

As described by others, EMT stimulation was associated with increase in lactate export in MCF7 cells, but curiously, some type of mesenchymal phenotype reversion was observed in Hs578t cells, with a decrease in lactate production and consequently in MCT1 and CD147 protein levels.

These results highlight some mechanisms that modulate glycolytic metabolism, which must be further explored to better understand the Warburg effect in breast cancer but also to identify novel targets to improve breast cancer therapy.

**Funding**

This study was supported by Projecto Estratégico- LA 26 – 2013-2014 (PEstC/SAL/LA0026/2013) and ON.2 SR&TD Integrated Program (NORTE-07-0124-FEDER- 000017)" co-funded by Programa Operacional Regional do Norte (ON.2- O Novo Norte), Quadro de Referência Estratégico Nacional (QREN), through Fundo Europeu de Desenvolvimento Regional (FEDER). FM-S received a fellowship from Fundação para Ciência e Tecnologia (FCT) REF. SFRH/BD/87139/2012.

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# CHAPTER 4: GENERAL DISCUSSION

## 4.1. General Discussion

The major findings of this PhD thesis will be presented and discussed in this chapter, as well as how these results contribute to the exploitation of Monocarboxylate Transporters as a valuable targets in breast cancer therapy, especially in the basal like subtype, which remains with no specific molecular targeted therapy.

### 4.1.1. Monocarboxylate transporters inhibition in breast cancer

Glycolytic metabolism has gained a novel interest in the reprogramming of cancer cells, being recently proposed as a new hallmark of cancer [1]. As a consequent of increased glucose consumption and to maintain the homeostasis of cancer cells, several proteins, like glycolytic enzymes, glucose transporters and pH regulators, as CAIX, NHEI and MCTs are differentially expressed [2-4]. In fact, previous results from our group and others demonstrate altered expression of MCTs in several cancer types, including breast cancer, of which the upregulation of MCT1 and its association with GLUT1 and CAIX in aggressive breast cancer subtype were reported [5]. Moreover, the association of MCT1 and CD147 with absence of estrogen and progesterone receptors, high histological grade and high proliferative capacity of breast cancer cells [6], point at MCT1/CD147 complex as a central player in breast cancer aggressiveness, contributing to the maintenance of the glycolytic phenotype of these cells. Considered by other authors as players in tumour cell viability and aggressiveness, some studies showed the importance of MCTs inhibition *in vitro* and *in vivo* in several tumours types [7-12]. However, the role of MCTs, and in particularly MCT1, needed to be further studied to support their exploitation as therapeutic targets in breast cancer.

Importantly, in this thesis, the effect of MCT inhibition, using classical MCT inhibitors such as CHC and quercetin, and non-classical inhibitors like lonidamine, has been characterized. The expression of high levels of MCT1 in breast cancer cells supports the findings in breast carcinoma samples [5, 6] and also highlights the importance of MCT1 in this type of cancer. The *in vitro* results demonstrated a decrease of glucose consumption and lactate production in the glycolytic cell lines MDA-MB-468 and

Hs578T, after inhibition of MCTs with the three different inhibitors (Chapter 2). Also, the inhibitors were able to decrease cancer cell malignant features like proliferation, migration and invasion, and induce cell death. Although the most affected cells presented both MCT isoforms (MCT1 and MCT4), the observed effects were most evident for MCT1 inhibition. This might be explained by the fact that CHC $k_i$ values for MCT4 are five to ten times higher than for MCT1 [13], requiring the use of much higher concentrations compared with the ones used in this study. Thus, to validate the results obtained with MCT1inhibition, its expression was downregulated using siRNA, and the effects on decreasing cell metabolism, proliferation and migration were similar to the obtained with CHC, corroborating the previous results.

However, in order to achieved more clear results on the inhibition of MCTs, and supported by the success of similar approaches in other cancer types [8, 9], in the second study, we performed the *in vitro* and *in vivo* inhibition of MCTs in breast cancer, using the siRNA approach, showing for the first time inhibition of tumour formation and reduction of tumour growth (Chapter 2). In this study, four cell lines were exposed to hypoxia, in order to enhance the dependency on glycolysis [14-16]. Thus, disruption of oxidative phosphorylation increased dependency of hypoxic cells on MCT1/4 for lactate transport. Consequently, inhibition of MCTs in hypoxic cells proved to be more effective than normoxic cells. Also, MCT knockdown in cells that express only one MCT isoform, as knockdown of MCT1 in BT20 cells, was more effective in inhibit lactate transport, decreasing cell biomass and invasion, and, importantly, induced a drastic reduction of *in vivo* tumour growth. These findings corroborate the previous *in vitro* results from our group [17], while others achieved similar *in vivo* results in cells without MCT4, decreasing intracellular pH and supressing tumour growth in colon cancer [18]. Although inhibition of MCT4 in MDA-MB-231 cells in hypoxia was less effective than in normoxia, the reduction of tumour growth and inhibition of tumour initiation exceeded the expected outcomes, based on the *in vitro* results. The efficacy of MCT inhibition in both studies supports the efficiency of targeting lactate transport to overcome breast cancer aggressiveness, especially in basal-like subtype. Importantly, the increasing interest in targeting MCTs in cancer led pharmaceutical companies to developed specific inhibitors of MCT1, one of which is currently under phase I clinical trials in patients with prostate and gastric cancer, and diffuse large B cell lymphoma [19].

Although MCTs have emerged as new candidates for cancer therapy, it is reasonable to anticipate adverse effects and toxicity in normal tissues. Being MCT1 expressed at almost all organs, systemic delivery of MCT inhibitors will not be exempt of adverse reactions. Indeed, side effects upon MCT1 inhibition using lonidamine were reported, including muscle fatigue, myalgia, asthenia, testicular pain, and gastro intestinal discomfort, however with no serious organ toxicity [20, 21], supporting the use of MCT inhibitors in cancer therapy.

### 4.1.2. Microenvironmental modulators of the monocarboxylate transporters

Concerning MCT regulation, different mechanisms have been reported in normal [13, 22-26] but also in cancer [20, 27-31] tissues. Several studies have reported upregulation of MCT1 mRNA and protein in muscle during exercise [13, 22, 23], regulation by butyrate in colonic epithelium [24] or even the regulation of MCTs expression associated with hormonal disorders, like diabetes, obesity and thyroid diseases [13]. Additionally, regulation of MCT1 was described by thyroid hormones or MCT2 by insulin pathways [13, 20, 32] and noradrenaline [33]. Also, the regulation of MCTs by hypoxia in heart muscle, astrocytic and endothelial cells has been discussed [25, 26].

In cancer, the regulation of MCT1/4 by their protein chaperone CD147 has been well studied, being this protein crucial for MCT1 and MCT4 trafficking to the plasma membrane [34-38]. In fact, the contribution of MCTs to the malignant phenotype is not only related to lactate transporter and acidification, but also with MCT close association with CD147, which has important roles in angiogenesis, migration and invasion [39, 40]. In opposition, MCT regulation by hypoxia has been a controversial topic, especially in what concerns MCT1 [16, 41-43], as only *SLC16A3* (MCT4) promoter presents HREs [44]. Additionally, cooperation of HIF1α with c-myc was described as regulator of aerobic glycolysis, being *SLC16A1* expression activated by this proto-oncogene [28, 30, 31].

With the results obtained in this thesis, we expect to have contributed to understand the mechanisms of regulation in breast carcinogenesis. In fact, upregulation of MCT1 has been reported by our group in breast cancer [6], results which were

corroborated in breast cancer cell lines (Chapter 2). Regulation of MCT expression by hypoxia was also reported in Chapter 2, where an increase of MCT1, MCT4 and also CD147 in breast cancer cells exposed to hypoxia was demonstrated, showing a shift from oxidative phosphorylation to a glycolytic phenotype [45].

Moreover, in Chapter 3, possible regulators of the metabolic phenotype were studied, considering the several players of enhanced breast cancer progression. Limitation of glucose inside tumours can be a consequence of the Warburg effect, due to the high levels of glucose consumption, but also caused by poor vascularization in tumours [46, 47]. We showed that low glucose levels were able to increase MCT expression, particularly MCT4, although not accompanied by mRNA upregulation. It is known that lactate levels are higher in the tumour microenvironment [48], and so we also tested the influence of lactate in breast cancer cell metabolism. Indeed, the metabolic phenotype of TNBC cells changed, evidencing consumption of this metabolite. As previously reported by our group, an association between MCT1 expression and absence of ER expression was verified [6], and, knowing that around 70% of breast cancer cases present overexpression of ER, playing an important role in breast cancer development and progression [49, 50], we also evaluated the role of this player in metabolic regulation. In fact, stimulation of ER increased lactate production in luminal cells but did not modify the expression patterns of glycolytic-related proteins, as expected. Finally, and considering the main process of invasion and metastization of tumours - the EMT process [51], increased lactate production was associated with a decrease of E-cadherin in cells that expressed epithelial markers. However, no alterations in protein or mRNA levels were observed in the glycolytic markers.

New clues concerning regulation of MCTs and maintenance of the glycolytic phenotype have been proposed in this thesis. However, further studies are needed to better explore the translational and post-translational MCT regulation, especially considering the low number of experiments in this specific study.

## 4.2. Conclusions and future perspectives

To summarize, the results presented in this thesis support the exploitation of MCTs as potential targets, especially MCT1, which are upregulated in breast cancer. Additionally, *in vitro* and *in vivo* studies reinforced the role of MCTs and lactate in the aggressive behaviour of breast cancer cells. Also, this thesis highlights the importance of the glycolytic metabolism for the maintenance of tumour growth, supported by *in vivo* results. Concerning regulation of the metabolic phenotype, the present work demonstrates modulation of MCT expression and lactate production by different glucose concentrations, increase in lactate production by 17 β-estradiol stimulation and decrease of MCT expression and lactate production by TGF-β, in specific breast cancer cell lines.

However, considering that the main goal of MCTs targeting in cancer is clinical application, more studies are necessary to investigate the efficacy of MCT inhibitors. Concerning MCT regulation, many other pathways related to MCT activity should be explored in breast cancer, namely gene regulation by methylation and mutations, interacting proteins, miRNAs, hypoxia, EMT, AKT pathway, c-myc, and other mechanisms by which MCTs may be controlled.

Whilst many other studies will complement the knowledge on MCTs in cancer, this thesis contributed with important findings in the field of glycolytic metabolism in breast cancer.

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