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THE ROLE OF NUTRIENT TRANSPORT BY CANCER CELLS IN THE INTERACTION BETWEEN TYPE 2 DIABETES MELLITUS AND BREAST CANCER

CLÁUDIA RAQUEL SANTOS SILVA

TESE DE DOUTORAMENTO APRESENTADA
À FACULDADE DE MEDICINA DA UNIVERSIDADE DO PORTO EM
METABOLISMO - CLÍNICA E EXPERIMENTAÇÃO

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"The important thing is not to stop questioning. Curiosity has its own reason for existence. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of this mystery each day."

Albert Einstein

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RESUMO

Atualmente, o cancro da mama é a neoplasia maligna mais comum entre as mulheres a nível mundial, e cerca de 20-28% das mulheres que sofrem de cancro da mama têm também diagnóstico de diabetes mellitus tipo 2 (DM2). As alterações metabólicas associadas à DM2 têm sido associadas a uma maior incidência, uma progressão acelerada e aumento da agressividade do cancro de mama. No entanto, a relação entre a DM2 e o cancro de mama permanece em grande parte desconhecida. Nesse contexto, hipotetizamos que as características associadas à DM2 possam interferir com os transportadores de glicose e/ou glutamina e criar um ambiente metabólico favorável à iniciação, promoção e progressão do cancro de mama. Isto é de particular interesse no cancro de mama, dado que a dependência de glicose e/ou glutamina é particularmente aumentada em alguns subtipos deste tipo de cancro.

Portanto, o principal objetivo do trabalho incluído nesta tese foi investigar o efeito de características associadas à DM2 (hiperglicemia, hiperinsulinemia, hiperleptinemia, inflamação crónica de baixo grau e níveis aumentados de stresse oxidativo) na captação celular de glicose e glutamina, e se mudanças neste traço contribuem para o efeito negativo da DM2 na iniciação, promoção, progressão e resposta à terapia no cancro da mama.

Os resultados obtidos demonstraram que algumas características associadas à DM2 induziram um aumento na captação celular de glicose que foi sensível ao BAY-876 e mediada pelo transportador de glicose 1 (GLUT1), e um aumento na captação celular de glutamina, que foi maioritariamente dependente de Na^+, sensível ao GPNA e mediada pelo transportador de alanina, serina, cisteína 2 (ASCT2) em ambas as linhas celulares de cancro da mama (recetor de estrogénio positivo; ER^+ e triplo-negativo; TN) e ainda em células epiteliais não tumorais da mama. De todas as características associadas à DM2, o interferão-gama (IFN-γ) e a insulina apresentaram o efeito mais interessante. Notavelmente, o IFN-γ aumentou a captação de ^{3}H-GLN (em células transformadas com 7,12-dimethyl-benzanthracene (DMBA) e em células ER^+ e TN) e a captação de ^{3}H-DG (em células ER^+ e TN); curiosamente esse efeito ocorreu apenas nas células cancerígenas. A insulina estimulou a captação de ^{3}H-GLN (somente em células epiteliais não tumorais da mama) e a captação de ^{3}H-DG (em células ER^+, TN, células transformadas com DMBA e células epiteliais não tumorais da mama). De realçar que o efeito pró-proliferativo do IFN-γ em células ER^+ e TN está associado a um aumento da captação de ^{3}H-GLN sensível ao GPNA, bloqueado após silenciamento do transportador ASCT2 e mediado pela ativação das vias

de sinalização PI3K, STAT3 e STAT1. Por outro lado, o efeito pró-proliferativo da insulina foi dependente de um aumento na captação de ^{3}H-DG mediada pelo transportador GLUT1 em células ER^{+}, TN, células transformadas com DMBA e células epiteliais não tumorais da mama e mediada pelas vias de sinalização PI3K e mTOR, em células transformadas com DMBA e células não tumorais.

Globalmente, os nossos resultados fornecem evidências de que as características associadas à DM2, em particular a hiperinsulinemia e um estado inflamatório (IFN-γ), podem induzir alterações na captação de glicose mediada por GLUT1 e na captação de glutamina mediada por ASCT2, o que pode despoletar a iniciação, e/ou estimular a promoção e progressão do cancro de mama. Portanto, o transportador de glicose (GLUT1) e o transportador de glutamina (ASCT2) podem constituir um mecanismo pelo qual a DM2 participa na iniciação, promoção e progressão do cancro de mama, e um alvo para o tratamento do cancro de mama em pacientes com DM2.

ABSTRACT

Currently, breast cancer is the most common malignancy among women worldwide, and about 20-28% of women with breast cancer are also diagnosed with type 2 diabetes mellitus (T2DM). The metabolic alterations associated with T2DM have been linked to a higher incidence, accelerated progression and increase aggressiveness of breast cancer. However, the relationship between T2DM and breast cancer remains largely unknown. In this context, we hypothesized that T2DM-associated characteristics can interfere with glucose and/or glutamine cellular uptake, creating a favourable metabolic environment for the initiation, promotion, and progression of breast cancer. This is of special interest in breast cancer, as glucose and/or glutamine dependence is particularly evident in some breast cancer subtypes.

So, the main aim of the work included in this thesis was to investigate the effect of T2DM-associated characteristics (hyperglycemia, hyperinsulinemia, hyperleptinemia, and increased levels of inflammation and oxidative stress) upon glucose and glutamine cellular uptake, and if changes in this trait can contribute to the negative effect of T2DM in breast cancer initiation, promotion, progression, and response to therapy.

The results obtained demonstrated that some T2DM-associated characteristics induced an increase in glucose cellular uptake which was BAY-876-sensitive and GLUT1-mediated, while the increase in glutamine cellular uptake was mainly Na^+-dependent, GPNA-sensitive and ASCT2-mediated in both oestrogen receptor positive (ER^+) and triple-negative (TN) breast cancer cells and non-tumorigenic epithelial breast cells. Interferon gamma (IFN-γ) and insulin displayed the most interesting effect. Notably, IFN-γ increased ^{3}H-GLN uptake (in ER^+, TN and 7,12-dimethylbenz[a]anthracene (DMBA)-transformed cells) and ^{3}H-DG uptake (in ER^+ and TN cells) in a cancer cell-specific manner. Insulin stimulated ^{3}H-GLN uptake (exclusively in non-tumorigenic epithelial breast cells) and ^{3}H-DG uptake (in ER^+, TN, DMBA-transformed and non-tumorigenic epithelial breast cells). Of interest, the pro-proliferative effect of IFN-γ in ER^+ and TN cells was associated with an increase in ^{3}H-GLN uptake which was GPNA-sensitive, blocked by ASCT2 knockdown and mediated by activation of PI3K, STAT3 and STAT1. On the other hand, the pro-proliferative effect of insulin was dependent on an increase in GLUT1-mediated ^{3}H-DG uptake in ER^+, TN, DMBA-transformed cells and non-tumorigenic breast epithelial cells and mediated by PI3K and mTOR in DMBA-transformed and non-tumorigenic cells.

Overall, our data provide evidence that T2DM-associated characteristics, particularly hyperinsulinemia and IFN-γ, induce changes in GLUT1-mediated glucose and ASCT2-mediated glutamine uptake that can trigger to breast cancer initiation, promotion and progression. Therefore, the glucose transporter (GLUT1) and the glutamine transporter (ASCT2) may constitute a mechanism by which T2DM participates in breast cancer initiation, promotion and progression, and a target for treatment of breast cancer in T2DM patients.

LIST OF CONTENTS

LIST OF FIGURES

CHAPTER I

CHAPTER VI

LIST OF TABLES

CHAPTER VII

LIST OF ABBREVIATIONS

^{18}F-FDG	^{18}Fluoro-2-deoxy-D-glucose
^{18}F-fluciclovine	^{18}F-fluorocyclobutane-1-carboxylic acid
^{3}H-DG	^{3}H-deoxy-D-glucose
^{3}H-GLN	[2,3,4-^{3}H]-glutamine
acetyl-CoA	Acetyl coenzyme A
AGEs	Advanced glycation end products
AI	Artificial intelligence
ARNT	Aryl-hydrocarbon receptor nuclear translocator
ASCT	Alanine, serine, cysteine transporters
ATB$^{0,+}$	Basic amino acid transporter B(0^{+})
ATF4	Stress-responsive transcription factor
ATP	Adenosine triphosphate
BAY-876	[N4-[1-(4-cyanobenzyl)-5-methyl-3-(trifluoromethyl)-1H-pyrazol-4-yl]-7-fluoroquinoline-2,4-dicarboxamide]
DMBA	7,12-Dimethylbenz[a]anthracene
E_2	17β-oestradiol
E2F-3	E2F- transcription factor 3
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Oestrogen receptors
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
G6P	Glucose-6-phosphate
GDH	Glutamate dehydrogenase
GLS	Glutaminase
GLUD	Glutamate dehydrogenase
GLUL	Glutamine synthetase
GLUT	Glucose transporters
GOT	Glutamate–oxaloacetate transaminase

GPD	Glycerol-3-phosphate dehydrogenase
GPNA	L-g-glutamyl-p-nitroanilide
GPT	Glutamate–pyruvate transaminase
GSK-3β	Glycogen synthase kinase 3 beta
HER2	Human epidermal growth factor receptor 2
HIF-1α	Hypoxia-inducible factor 1-alpha
HK	Hexokinases
HS	Horse serum
HSP	Hexosamine synthesis pathway
IFN-γ	Interferon gamma
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IR	Insulin receptor
IRS	Insulin receptor substrate family
JAK	Janus kinase
LAT	L-type amino acid transporters
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinase
MeAIB	(Methylamino)isobutyric acid
MEK	Mitogen-activated protein kinase
miRNA-137	MicroRNA-137
mTOR	Mammalian target to rapamycin
mTORC1	Mammalian target to rapamycin complex 1
Na^+	Sodium
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NF-κB	Factor nuclear kappa B
Ob-R	Leptin receptor
OXPHOS	Oxidative phosphorylation
p53	Tumour protein 53

PAM50	Prediction Analysis of Microarray 50
PEP	Phosphoenolpyruvate
PET	Positron Emission Tomography
PGK	Phosphoglycerate kinase
PI3K	Phosphatidylinositol 3-kinase
PK	Pyruvate kinase
PKB	Protein kinase B
PPP	Pentose phosphate pathway
PR	Progesterone receptors
PSAT	Phosphoserine transaminase
RAGE	Receptor for advanced glycation end products
Rb	Retinoblastoma
rBAT	Neutral and basic amino acid transport protein
RNF5	Ring finger protein 5
ROS	Reactive oxygen species
SGLT	Sodium-dependent glucose co-transporters
SHBG	Sex hormone binding globulin
SHC	Adaptor protein
SNAT	Neutral amino acid transporters
STAT	Signal transducer and activator of transcription
T2DM	Type 2 diabetes mellitus
TBH	Tert-butylhydroperoxide
TCA	Tricarboxylic cycle
TN	Triple-negative
TNF-α	Tumour necrosis factor alpha
UICC	Union for International Cancer Control
VEGF-A	Vascular endothelial growth factor A
y^+LAT	y^+L amino acid transporter
α-KG	α-ketoglutarate

THESIS OUTLINE

This thesis is organized into **eight CHAPTERS.**

CHAPTER I provides a general introduction considering the current literature. **CHAPTER II** describes the project motivation and the specific aims**. CHAPTERS III**, **IV** and **V** are related to the experimental work and are structured according to the aims. **CHAPTER VI** provides a general discussion from work**. CHAPTER VII** presents the main conclusions and perspectives for future research. Lastly, **CHAPTER VIII** contains the list of cited references.

The information presented in the **CHAPTERS III, IV** and **V** is published in international peer review scientific journals, according to the following list:

CHAPTER III

Cláudia Silva, Nelson Andrade, João Tiago Guimarães, Emília Patrício, Fátima Martel. The *in vitro* effect of diabetes-associated markers insulin, leptin and oxidative stress on cellular characteristics promoting breast cancer progression is GLUT1-dependent. European Journal of Pharmacology, 2021; 898: 173980. doi:10.1016/j.ejphar.2021.173980

CHAPTER IV

Cláudia Silva, Nelson Andrade, Ilda Rodrigues, António Carlos Ferreira, Miguel Luz Soares, Fátima Martel. The pro-proliferative effect of interferon-γ in breast cancer cell lines is dependent stimulation of ASCT2-mediated glutamine cellular uptake. Life Sciences, 2021; 286: 120054. doi:10.1016/j.lfs.2021.120054

CHAPTER V

Cláudia Silva, Nelson Andrade, João Tiago Guimarães, Emília Cardoso, Catarina Meireles, Vanessa Pinto, Joana Paiva, Fátima Martel. The pro-proliferative effect of insulin in human breast epithelial DMBA-transformed and non-transformed cell lines is PI3K-, mTOR- and GLUT1-dependent. Cell Biochemistry & Function, 2022; 1-11. doi:10.1002/cbf.3681

CHAPTER I

INTRODUCTION

Introduction

Almost 463 million people live with diabetes globally nowadays and this condition is predicted to affect more than 700 million in 2045 [1]. Type 2 diabetes mellitus (T2DM), which accounts for almost 90% of diabetes cases [1, 2], constitutes one of the most common chronic diseases worldwide [3]. Breast cancer is the most frequent malignancy among women worldwide [4], and, in the last years, several reports have reinforced a link between breast cancer and T2DM [5-7] showing a 20-28% increased risk of breast cancer in women with T2DM [2, 7]. In line with this, several metabolic T2DM-related factors are known to influence tumour initiation, promotion, progression, and/or response to therapy [8-10]. However, the relationship between T2DM and breast cancer remains largely unknown.

1.1. Breast cancer

1.1.1. Epidemiology and risk factors

Breast cancer is the most frequently diagnosed cancer among women worldwide, and it is also the leading cause of cancer-related deaths in this gender. Although it can occur in both genders, its incidence is much higher in women and increases significantly with age [4].

In Portugal, according to the most recent data published by the World Health Organization, breast cancer was the most frequent cancer among women in 2020, with an estimated 7 041 new cases, representing 26.4% of all cancer cases in this gender [11]. In terms of mortality, breast cancer still has a high impact in Portugal, being the first cause of death by malignant diseases in woman [12], although a decrease in mortality rate has been noticed worldwide [4, 13]. Declines in breast cancer mortality could be further accelerated by expanding access to high quality prevention, early detection, and treatment services to all women [4, 13].

Breast cancer is a multifactorial disease and various factors contribute to its occurrence [14]. About 10% of all cases of breast cancer are related to genetic predisposition or family history, with variances by country and ethnicity [13, 15]. The most common cause of hereditary breast cancer is an inherited mutation in *BRCA1* or *BRCA2* genes, with an average cumulative lifetime risk of about 70% [13, 15]. Next generation sequencing has enabled panels of genes— beyond *BRCA1* and *BRCA2* — to be screened, including *ATM*, *CHEK2*, *PALB2*, *PTEN*, *STK11* and

TP53, to determine the inherited breast cancer risk [13, 16]. However, most cases of breast cancer occur sporadically [16]. A high proportion of breast cancer cases can be attributed to reproductive and hormonal risk factors (early age at menarche, later age at menopause, advanced age at first birth, fewer number of children, menopausal hormone therapy, less breastfeeding and oral contraceptives), lifestyle risk factors (obesity and overweight, physical inactivity, vitamin D deficiency, low-fibre diet, alcohol intake and smoking) and other risk factors (diabetes, air pollution, night work and radiation) [13-16].

1.1.2. Molecular and clinical classification

Breast cancer is a heterogeneous disorder composed by several histopathological and biological subtypes that have several molecular alterations, distinct clinical behaviours and different responses to therapy [13, 15, 17, 18]. Clinical stratification of breast cancers based on the expression of oestrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor 2 (HER2, also known as ERBB2), histological grade, type and size of tumour, and lymph node metastasis, influence the prognosis and have important implications in choosing therapy [16-18].

While histological stratification is still a common practice, the advances of gene expression profiling techniques allow immunohistochemistry (IHC) methods coupled with microarray technology to accomplish a more extensive breast cancer classification [15, 19, 20]. The best characterized gene expression classification system is the Prediction Analysis of Microarray 50 (PAM50) [21], which is a 50-gene classifier that groups breast tumours into five molecular or intrinsic subtypes, based on the expression pattern of certain genes (Figure 1). These subtypes are: (A) luminal A – this subtype accounts for about 40-70% of all breast cancers, is ER- and PR-positive, negative to HER2 amplification, has low proliferation rates (ki67<14%), tends to have good prognosis and its treatment typically involves hormonal therapy (despite some of these tumours are less responsive to chemotherapy, in which cases neoadjuvant or adjuvant chemotherapy is recommended); (B) luminal B – this subtype accounts for 10-20% of all breast cancers, is ER- and/or PR-positive, HER2-positive or negative, has high proliferation rates (ki67>14%), an intermediate prognosis, and similarly to luminal A subtype, its treatment involves hormonal therapy, but in some cases adjuvant chemotherapy is recommended; (C) HER2-enriched – this subtype makes up 10-15% of breast cancers, is ER- and PR- negative, positive to HER2 amplification, has a high grade, high ki67 index, an intermediate prognosis and is an

aggressive disease but responds well to targeted therapies aimed at the HER2 protein (such as transtuzumab, pertuzumab, lapatinib, capecitabine and ado-transtuzumab emtansine) and chemotherapy; (D) triple-negative (TN) – this subtype accounts for 10-20% of all breast cancers, is ER-, PR- and HER2-negative, has a high grade, high ki67 index, poor prognosis, is common in women with BRCA1 gene mutations and non-surgical treatment has been limited to conventional chemotherapy and radiotherapy, until the recent approval of the PARP inhibitor (Olaparib) for BRAC1 and BRAC2 mutation and; (E) normal-like – this subtype is ER- and/or PR-positive, HER2-negative, has low levels of Ki67 index and good prognosis, although it is still slightly worse than luminal A breast cancer [13, 15, 16, 18-20].

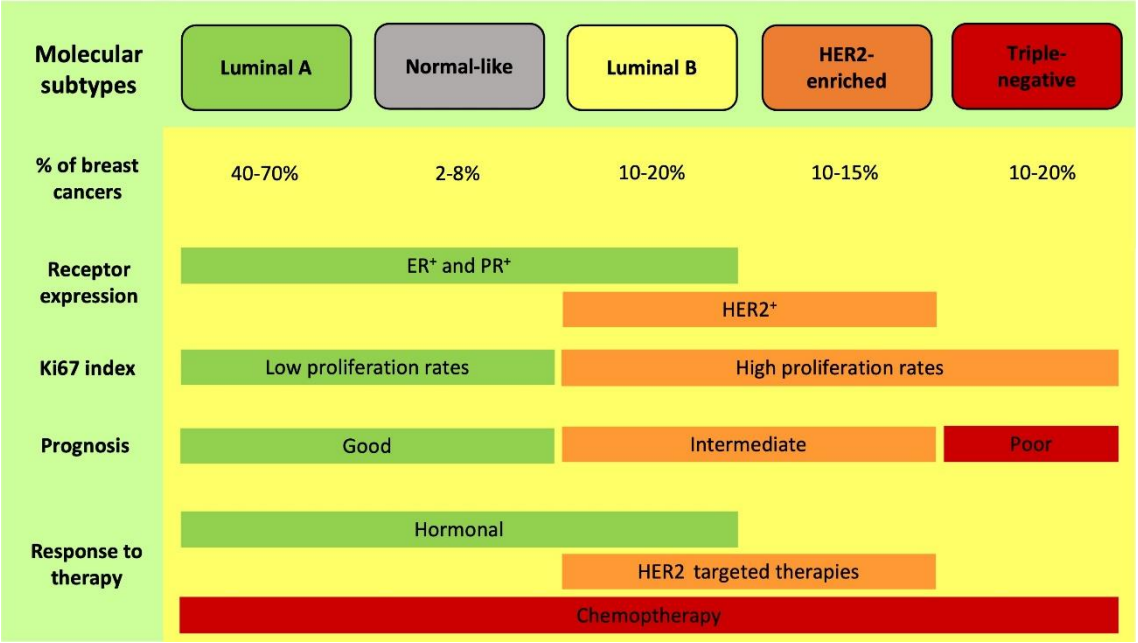

Figure 1. Molecular classification of breast cancer subtypes.

The molecular classification used in the clinic is based on histological grade and on immunohistochemical coupled with microarray technology for detection of ER, PR, HER2 and Ki67 and is used to stratify patients for therapy. This classification defines five molecular or intrinsic subtypes, each with distinct clinical outcomes and different approaches to therapy: luminal A, luminal B, HER2-enriched, triple-negative and normal-like. Abbreviations: ER: estrogen receptor; PR: progesterone receptor; HER2; human epidermal growth factor receptor 2.

Once breast cancer is diagnosed, clinical information of tumour according to the Union for International Cancer Control (UICC) is used to determine the stage of the disease, which will impact the treatment patients receive. The staging combines the clinical factors (referred above)

and stratifies the disease into one of 5 stages, from Stage 0 to Stage IV [15, 19]. This staging takes into account biological parameters that have predictive and prognostic value, and it provides more accurate prognostic information than the former staging systems [19].

1.2. Metabolic reprogramming in cancer

Cellular metabolism refers to the entire set of biochemical reactions that occur in a cell [22]. In order to sustain the increase in proliferation and rapid growth associated with tumorigenesis, cancer cells consume alternative nutrients and increase the production of macromolecules that contribute to cellular biomass and support generation of new cells [22]. As a result, cancer cells must undergo reprogramming of their metabolic pathways in order to obtain huge amounts of energy and materials [23]. These altered metabolic processes are recognized as an important hallmark of cancer, critical for successful neoplastic transformation and continued tumour cell growth [22, 24]. A classic example of a reprogrammed metabolic pathway in cancer cells is the "Warburg effect" or "aerobic glycolysis" [25]. An increase in glucose consumption by tumours in comparison to the non-proliferating normal tissue was first described more than a hundred years ago by Otto Warburg. He observed that tumour cells preferentially metabolize glucose to lactate via glycolysis rather than via oxidative phosphorylation (OXPHOS), even in the presence of oxygen, for adenosine triphosphate (ATP) production (Figure 2) [25]. This high-energy production pathway is essential to fulfil the energy demands and to maintain the increased demand for macromolecules (Figure 2) [26, 27].

While aerobic glycolysis had been regarded as the norm in cancer cells, some studies show that in many cancer cells, including breast cancer cells [28], mitochondria are functional and these cells still obtain ATP mainly through OXPHOS, through a "reverse Warburg effect" [29]. According to this model, a metabolic interplay between cancer cells and fibroblasts exists: stromal cells perform aerobic glycolysis and feed the cancer cells with the lactate they produce, which is then oxidized through tricarboxylic cycle (TCA) and OXPHOS. This interaction prevents the creation of an acidic condition in the tumour microenvironment, supporting and worsening the conditions of cancer (Figure 2) [29, 30]. This concept shows that the metabolic needs of tumour cells do not rely on a single metabolic strategy.

Besides glucose, tumour cells also rely on a wide variety of alternative fuels to supply various metabolic needs [31]. Another metabolite that has been identified as important for

tumour growth is glutamine, which is pivotal for biomass production, most notably as a nitrogen donor [24]. Indeed, in many cancer cells, glutamine demand dominates supply in periods of rapid growth [32, 33] and the survival of some types of cancer cells depends on glutamine, a phenomenon known as "glutamine addiction" (Figure 2) [32, 34, 35]. The dependence of tumour cells in alternative fuels may constitute tumour-specific metabolic vulnerabilities, and thus, may create additional therapeutic windows to eradicate tumour cells.

1.2.1. Glucose metabolism in cancer

Glucose is that the primary energy source of life on earth. It also serves as an important precursor for biomolecule synthesis and plays an important role in cell signalling [36]. Glucose metabolism is considered one of the most important aspects of cancer cell metabolism because it supplies intermediates and precursors for several other key metabolic pathways, including the generation of amino acids, nucleotides, and lipids [35, 37], as well as for generation of lactate to maintain the nicotinamide adenine dinucleotide (NAD^+)/reduced nicotinamide adenine dinucleotide (NADH) redox balance [23, 37]. This process, termed glycolysis [38-41], comprises several reversible enzymatic reactions and three irreversible reactions [38], where one molecule of glucose is converted into two molecules of pyruvate, being the energy released conserved in the form of two ATP and two reducing equivalent NADH [40, 42]. In well-oxygenated tissues, pyruvate enters the mitochondria and is completely oxidized to CO_2, a process that generates approximately 36 moles of ATP per mole of glucose. Under oxygen deprivation, pyruvate is converted to lactate, generating only two ATP molecules [43].

The first step of glycolysis is catalysed by hexokinases (HK) that phosphorylates glucose to produce glucose-6-phosphate (G6P) [38, 40]. This reaction is maybe the foremost important step in glucose metabolism, because it traps glucose [38, 40], which could preferably be exported by the glucose transporters, inside cell, as G6P. G6P is at the convergence point of not only glycolysis but also the pentose phosphate pathway (PPP), the hexosamine synthesis pathway (HSP) and glycogen synthesis [38, 41]. The PPP is composed of two branches: oxidative and non-oxidative [38, 41]. The oxidative branch generates two NADPH molecules, which is required to maintain intracellular redox homeostasis and fatty acid synthesis [38, 44]. The non-oxidative branch generates ribose-5-phosphate, which is used to produce new nucleotides for DNA and RNA synthesis [44]. Dihydroxyacetone phosphate, an intermediate metabolite of glycolysis, can be reduced to form glycerol-3-phosphate by the enzyme glycerol-3-phosphate

dehydrogenase (GPD), which can be used to provide glycerol for biosynthesis of membrane components, phospholipids, and triglycerols [40, 44]. In glycolysis, dihydroxyacetone phosphate is converted to 1,3-biphosphoglycerate, which is converted into 3-phosphoglycerate by phosphoglycerate kinase (PGK) in a reaction that generates ATP. 3-phosphoglycerate is used to generate phosphoenolpyruvate (PEP) in the subsequent reaction of glycolysis and participates in the serine biosynthesis pathway that branches from glycolysis to supply methyl groups to the one-carbon pathway, contributing to nucleotide and protein synthesis, methylation reactions, and NADPH production [23, 38]. In the last committed step of glycolysis, pyruvate kinase (PK) catalyses the conversion of PEP to pyruvate, generating ATP in this process [38, 45]. The final product of glycolysis, pyruvate, can be converted in aerobic settings into acetyl coenzyme A (acetyl-CoA) and thence enter the TCA cycle, whereas in anaerobic conditions, it can be converted into lactate by lactate dehydrogenase (LDH), a process named lactic fermentation (Figure 2) [37, 38].

In normal cells, glycolysis is coupled to OXPHOS to optimally synthesize intracellular ATP from glucose. However, cancer cells undergo a metabolic transformation, in which glycolysis is uncoupled from respiration and rewired to lactic fermentation. So, normal cells obtain ATP both from glycolysis and OXPHOS, while cancer cells mainly obtain ATP from glycolysis, even when there is sufficient oxygen [25, 43] This metabolic change is one of the first recognized biochemical hallmarks of cancer cells [22, 24], called the “Warburg effect” or “aerobic glycolysis” [25].

Although glycolysis yields a lower amount of ATP compared to mitochondrial OXPHOS, several benefits inherent to aerobic glycolysis may explain the preference of cancer cells for glycolysis instead of mitochondrial oxidation. One of those benefits is the fact that ATP production from glucose occurs up to 100 times faster with glycolysis than with OXPHOS [37, 45]. Moreover, the enhanced glycolytic rate in tumour cells, which is predominantly due to the overexpression or enhanced activity of key glycolytic enzymes, contributes to tumour progression [39], as it benefits fast proliferating of cells through the production of glycolytic intermediates needed for several metabolic pathways, including the generation of amino acids, nucleotides and lipids [45]. Another benefit relates to the high lactate levels that are released into the tumour environment, because lactate induces acidification of the tumour environment, favouring the development of a more aggressive and invasive phenotype [42].

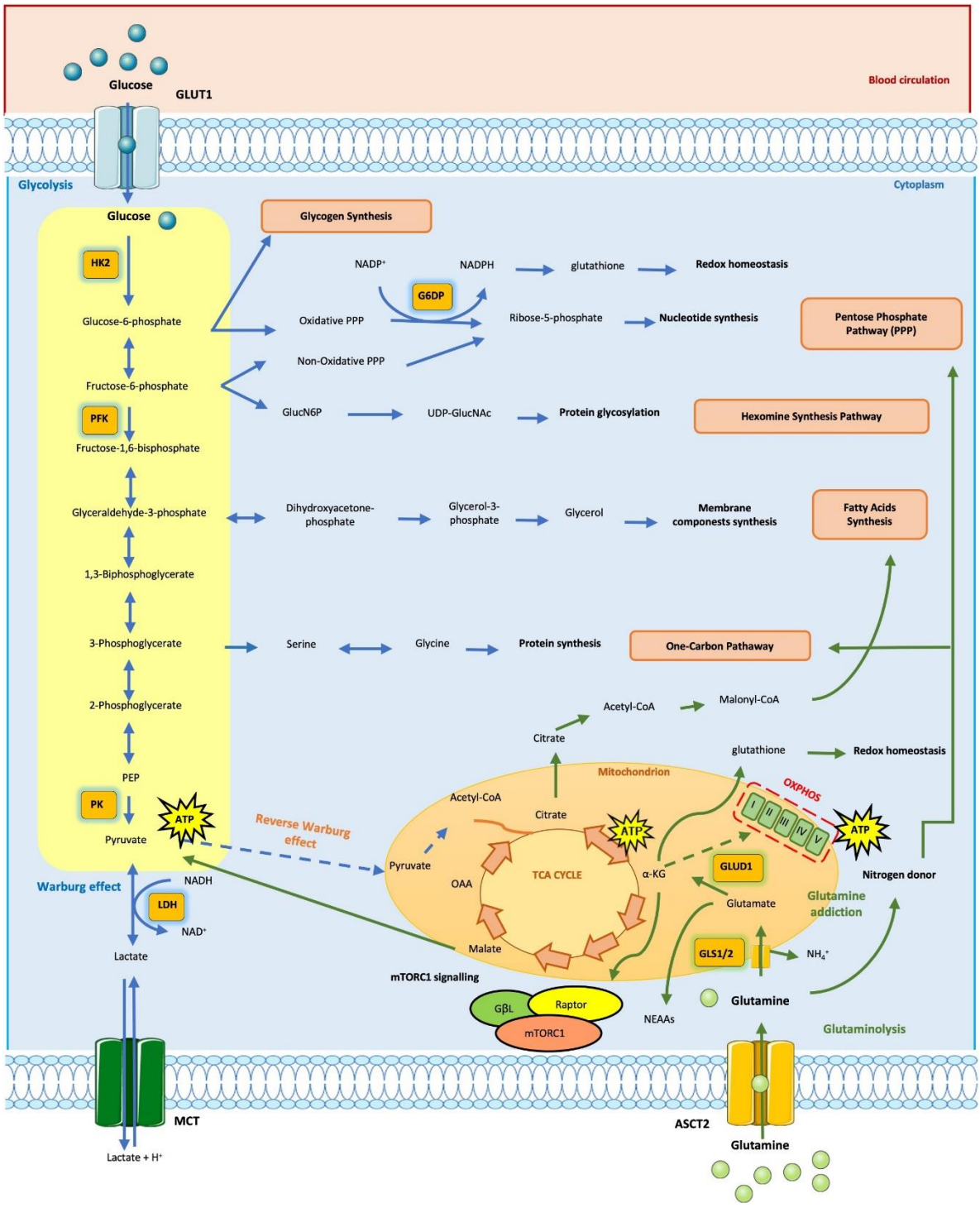

Figure 2. Overview of reprogramming glucose and glutamine metabolism in cancer cells.

Mitochondrial oxidation of glucose and glutamine through the TCA cycle and the OXPHOS is a highly efficient means of producing energy for differentiated cells. However, in case of malignant transformation, cells take up large amounts of glucose and glutamine, central biomolecules that provide cells with most of the energy and metabolites required for rapid growth and proliferation. Cancer cells upregulate an alternative pathway for glucose metabolism, called aerobic glycolysis. Although less efficient in generating ATP, aerobic glycolysis allows for more rapid metabolism of glucose and efficient regeneration of NAD^+, fundamental by ensuring a glycolytic flux, while preserving mitochondrial enzymatic activity for anabolic processes. Alternatively, glycolytic intermediates are diverted to provide substrates through glycogen synthesis, nucleotide synthesis, protein glycosylation, fatty acid synthesis and protein synthesis, cellular processes that are critical for highly proliferative cells. Pathways for the

metabolism of glutamine are also upregulated within the setting of increased proliferation. Glutamine is the primary source of nitrogen used for protein or nucleotide synthesis and supply the TCA cycle with carbon skeletons that maintain intermediates for synthesis of protein, nucleotide and fatty acids. In addition, glutamine also activates the mTORC1 pathway and can be used in the production of glutathione, to maintain tightly controlled redox balance. Blue arrows represent glucose metabolism and green arrows represent glutamine metabolism; dash arrows represent glucose and glutamine metabolism in differentiated cells. Abbreviations: GLUT1, facilitative glucose transporters 1; PPP, pentose phosphate pathway; NADPH, nicotinamide adenine dinucleotide phosphate; NAD, nicotinamide adenine dinucleotide; ATP, adenosine triphosphate; TCA cycle, tricarboxylic acid cycle; OXPHOS, oxidative phosphorylation; HK2, hexokinase 2; G6DP, glucose-6-phosphate dehydrogenase; PFK, phosphofructokinase; PK, pyruvate kinase; PEP, phosphoenolpyruvate; LDH, lactate dehydrogenase; MCT, monocarboxylate transporter; ASCT2, alanine, serine, cysteine transporter 2; acetyl-CoA, acetyl coenzyme A; α-KG, α-ketoglutarate; OAA, oxaloacetate; NEAA, non-essential amino acid; NH_4^+, ammonium; mTORC1, mammalian target to rapamycin complex 1.

Cancer cells show a remarkable plasticity in metabolic adaptation. There is no doubt that aerobic glycolysis appears to represent a selective advantage for tumour cells, as they become more resistant to apoptosis and acquire increased growth and invasive properties. Regardless of the mechanisms, glycolysis upregulation represents a clear advantage for cancers cells and at the same time a target for new anticancer therapies [42].

1.2.2. Glutamine metabolism in cancer

Although it is widely accepted that glucose is the dominant energy fuel for most cancers [46], another energy fuel of many cancer cells is glutamine [47], which is the amino acid most utilized by transformed cells [35].

Glutamine is a non-essential amino acid for normal cells, as it can be synthesized endogenously from the TCA cycle intermediate α-ketoglutarate (α-KG) by a process that involves glutamate dehydrogenase (GDH), which converts α-KG into glutamate, and glutamine synthetase (GLUL), which converts glutamate into glutamine using ammonia [48]. However, highly proliferative cancer cells, which use glutamine as an essential energy substrate, as well as for generation of nucleotides, lipids, and proteins [35, 49-51], consume glutamine at a rate exceeding that of glutamine biosynthesis [50].

The high demand of proliferating tumour cells for glutamine was first described in the 1950 by the physiologist Harry Eagle, who demonstrated that the optimal growth of cultured HeLa cells requires a 10- to 100-fold molar excess of glutamine in culture medium relative to other amino acids [52]. In later years, this was also observed in Ehrlich ascites carcinomas, hepatomas and carcinosarcomas [53, 54]. Indeed, several studies have shown that depletion of

glutamine from the tumour microenvironment is higher than in normal tissues [53, 55]. Accordingly, cancer cells deprived of glutamine die quickly [56], and increased glutamine metabolism in cancer was recently referred to as "glutamine addiction" [32, 34, 35].

Glutaminolysis is the process by which cells convert glutamine into TCA cycle metabolites through the activity of numerous enzymes [57]. During glutaminolysis, glutamine is converted into glutamate via glutaminase (GLS1 and GLS2), releasing ammonium ions [50, 57]. GLS1 is overexpressed in many cancers and is associated with a higher disease stage and poor prognosis [58]. Glutamate is subsequently converted into α-KG via two different pathways, one involving the activity of glutamate dehydrogenase (GLUD1 and GLUD2), which releases ammonium, and the other involving the activity of a group of transaminases, including glutamate–oxaloacetate transaminase (GOT), glutamate–pyruvate transaminase (GPT), and phosphoserine transaminase (PSAT), which transfer the amino nitrogen from glutamate to produce another amino acid (aspartate, alanine, serine, among others) and α-KG, without producing ammonia [50, 57, 59]. In the cytoplasm, α-KG participates in fatty acid biosynthesis and NAPDH generation while in mitochondria α-KG participates in the TCA cycle, supporting the OXPHOS pathway [50]. Alternatively, α-KG can escape the TCA cycle as malate being converted into pyruvate by malic enzyme in a process that leads to NADPH production, thus participating in the maintenance of redox homeostasis [51, 59, 60]. Pyruvate can, in turn, be transformed into lactate, restoring NAD^+ needed for anaerobic glycolysis and ATP production [51, 60] On the other hand, α-KG can converted to citrate by reductive carboxylation through the reverse TCA cycle, to support lipogenesis (Figure 2) [50, 61]. This is especially important in tumours to sustain their increased requirement for cell membranes and cell signalling [61]. Most of the carbon required for *de novo* fatty acid synthesis in normoxia conditions comes from glucose, which is converted to acetyl-CoA that condenses with oxaloacetate to produce citrate [57, 59]. However, under certain conditions, like hypoxia or when mitochondrial respiration is impaired, or during the formation of anchorage-independent tumour spheroids, cancer cells shift from glucose and glutamine oxidation to reductive carboxylation in order to maintain citrate levels [57, 61]. Thus, anaplerosis for energy production and fatty acid synthesis in cancer cells is extremely addicted to glutamine metabolism, especially under conditions of metabolic stress or oncogenic activation [59].

Glutamine has a versatile role in cell metabolism. First, glutamine participates as a source of nitrogen, which is required for the synthesis of nucleotides and non-essential amino acids [47, 49, 62], which are required for rapidly proliferating cancer cells [59]. A further role for

glutamine is that it provides a carbon source for TCA cycle and fatty acid synthesis via α-KG production [47, 49, 63, 64], and cancer cells require large amounts of lipids during cell division [59]. Alternatively, glutamine is involved in glutathione synthesis, which reinforces the antioxidant response and helps to maintain the redox balance, apoptosis and autophagy [47, 49, 65, 66]. Last, glutamine activates the mammalian target to rapamycin (mTOR) pathway by enhancing the influx of amino acids, including leucine, which maintains activation of the mammalian target to rapamycin complex 1 (mTORC1). This, in turn, regulates protein translation and prevents apoptosis in cancer cells (Figure 2) [47, 49, 67, 68].

Thus, glutamine metabolism is a central player in the regulation of uncontrolled tumour growth by modulating bioenergetic and redox homeostasis and by serving as a precursor for biomass synthesis [59].

1.2.3. Glucose transporters in cancer

Glucose, due to its hydrophilic nature, requires specific carrier proteins to cross the plasma membrane [69]. In mammals, two families of glucose transporters have been described: the secondary active Na^+-dependent glucose co-transporters (SGLT, gene family name solute carrier *SLC5A*) and the facilitative glucose transporters (GLUT, gene family name *SLC2A*) [27, 70, 71].

The SGLT family transfer glucose against its concentration gradient coupled with Na^+ transport down its concentration gradient, which is maintained by the Na^+/K^+ pump [27]. This family includes 12 different members in humans [72], among which SGLT1 and SGLT2 have been extensively characterized in the past several years [36]. SGLT1 is primarily expressed in intestine, while SGLT2 is highly expressed within the kidney cortex and plays a crucial role in renal reabsorption of glucose. The major substrates of SGLT1 are both glucose and galactose (1:1 ratio), whereas SGLT2 prefers glucose to galactose (2:1 ratio) [36]. Despite overexpression of the SGLT1 and SGLT2 transporters has been documented in several types of cancer, it has not been described in breast cancer [27, 71].

Human GLUTs are a major facilitator superfamily of solute carriers. This family is comprised of 14 transporter proteins categorized into three classes according to their sequence similarity, substrate specificity, physiological expression and function: Class I (GLUTs 1–4, 14), Class II (GLUTs 5, 7, 9, and 11), and Class III (GLUTs 6, 8, 10, 12, and 13/H^+-myso-inositol transporter (HMIT)) [36, 69, 70]. These proteins transport not only glucose but also fructose,

galactose, mannose, glucosamine, myoinositol, other hexoses and also some related compounds with variable affinities [69]. Among GLUTs, GLUT1, GLUT3, and GLUT4 have a higher affinity for glucose, allowing transport of glucose at a high rate under normal physiological conditions [27].

In order to achieve a glycolytic rate that is approximately 30-fold higher than normal, cancer cells take up glucose at an elevated rate [69]. This increased rate of cellular uptake of glucose is accomplished by overexpression of glucose transporters, which is observed in the generality of cancer cells [27], being often correlated with more aggressive and advanced-staged tumours [69].

1.2.3.1. Regulation of GLUT1 in breast cancer

The high rate of glucose uptake observed by cancer cells, is often associated with deregulated an increased expression of glucose carriers, particularly GLUT1 [27, 70].

GLUT1 is responsible for basal glucose uptake, and it is widely distributed in normal tissues [69, 73]. Other than that, GLUT1 is the predominant transporter overexpressed in many tumours, including hepatic, pancreatic, esophageal, brain, renal, lung, cutaneous, colorectal, endometrial, ovarian, cervical, head, neck and breast [73, 74] suggesting an important role of this transporter in the increased glucose uptake observed in cancer cells. Importantly, several studies have shown a close relationship between GLUT1 expression, tumour development, and poor prognosis in patients with breast cancer [75, 76]. In breast cancer, the extent of tumours positive for GLUT1 by immunohistochemistry varies from 42% to 91% [75, 77, 78]. Godoy et al. reported positive staining of GLUT1 in 91% of the invasive ductal carcinomas [78], while Younes et al. demonstrated an increased expression of GLUT1 only in 42% of breast tumours [75]. In untreated primary human breast cancers, 61% were GLUT1 positive [77]. Already, Brown and Wah showed that all the primary breast cancers and the lymph node metastases were GLUT1-positive, and that this transporter was expressed both on the cell membrane and in the cytoplasm of the tumour cells [79]. The localization of GLUT1 at the plasma membrane seems to be critical for breast cancer cell function [80]. Although it is not clear when does GLUT1 overexpression occurs during tumorigenesis, it appears that GLUT1 is particularly important for the initiation of breast tumorigenesis, affecting tumour formation and cell transformation, a fundamental part of the neoplastic process [80]. So, this gene has been proposed as oncogene [27].

GLUT1 is regulated through several molecular mechanisms. To date, in breast cancer cells, induction of GLUT1 expression by hypoxia-inducible factor 1-alpha (HIF-1α), by the c-MYC oncogene, as well as by the mutated gene tumour protein 53 (p53) [27, 34, 69, 81] and by the mutated Ras gene [69] has been exhaustively reported. Additionally, some hormones [82] and growth factors [83] seems also to interfere with GLUT1 expression in breast cancer cells. However, it is believed that additional molecular mechanisms can regulate GLUT1.

GLUT1 has been proposed as a potential marker of hypoxia [69]. The transcription factor HIF-1α, which is activated by hypoxia, is an important regulator of GLUT1 [81]. Increased gene expression of HIF-1α is known to be present in 56–76% of breast cancers [84], being its overexpression related to HER2 positivity [85] and TN breast cancer [86]. A complex of HIF-1α and aryl-hydrocarbon receptor nuclear translocator (ARNT) has been shown to bind to the GLUT1 promoter and upregulate its expression [84].

The transcription factor c-MYC is upregulated in many human cancers, linking altered cellular metabolism to tumorigenesis. In breast cancer, the expression of GLUT1 is also induced by c-MYC oncogene [81].

Concerning the tumour suppressor gene p53, it mutated in about 20%–30% of breast cancers and more often in ER^- breast cancer [81]. Apparently, in breast cancer cells, p53 regulatory functions depend on oxygen availability. Under normoxia conditions, the overexpression of the p53 correlates with a significant decrement in GLUT1; contrariwise, when oxygen availability decreases, p53 overexpression induces increased GLUT1 [87].

Although mutated forms of Ras are not associated with most breast cancers (5%), there is considerable evidence that Ras can promote breast cancer growth and development [88]. This appears to be associated with mutations in KRAS or BRAF, which binds Ras, upregulating the GLUT1 gene [69].

Additionally, some hormones, such as oestrogens, may provide another mechanism of GLUT1 upregulation. Indeed, MCF-7 cell line show increased proliferation when treated with 17β-oestradiol (E_2) [82, 89], and treatment with E_2 has been shown to stimulate glucose metabolism, by increasing the rate of glucose utilization via glycolysis and the TCA cycle [90].

Lastly, epidermal growth factor (EGF) is another regulator of GLUT1 in breast cancer. EGF actions are mediated by the EGF receptor (EGFR). Treatment of breast cancer cell lines with EGF promotes EGFR activation leading to a shift in glycolytic flux (increased lactate production) and this metabolic response seems to involve GLUT1 overexpression [83].

Increased GLUT1 expression may contribute to the increased uptake of ^{18}Fluoro-2-deoxy-D-glucose (^{18}F-FDG) by breast tumours observed by Positron Emission Tomography (PET) imaging. Among patients with tumours detectable by PET, it is evident that higher glucose uptake on scan represents more metabolically active and therefore more aggressive tumours [27, 73].

1.2.4. Glutamine transporters in cancer

Glutamine is one of the twenty different amino acids involved in protein synthesis in mammalian cells, but the biological functions of this particular amino acid go far beyond its role in protein synthesis, as shown in section 1.2.2. [48]. As glutamine is hydrophilic and water-soluble, it cannot diffuse into cells across the plasma membrane, and so its transmembrane transfer requires transporters [48, 91].

Amino acid transporters are involved in nutrient supply, cell signalling pathways, and cell homeostasis [49]. In normal cells, most amino acid transporters show a specific expression depending on tissue and stage of development [49, 51]. Cancer cells, on the other hand, tend to overexpress these transporters according to the specific tumour type [49, 92].

Based on their substrate specificity, regulatory properties and transport mechanism, amino acid transporters can be classified into distinct systems: system A, ASC, N and L [93, 94] and more recently, system x_c^-, system $B^{0,+}$ and system y^+L [49, 94].

On the other hand, based on their dependence on Na^+, amino acid transporters are divided into two categories: Na^+-dependent and Na^+-independent. Some of these transporters accept only neutral amino acids whereas others accept neutral as well as cationic amino acids, and yet others accept neutral, cationic, and anionic amino acids [47, 48, 93].

System ASC transports alanine, serine and cysteine along with other small neutral amino acids (valine, threonine and alanine) [95]. This system includes two Na^+-dependent antiporters, alanine, serine, cysteine transporters, ASCT1 (gene family name *SLC1A4*) and ASCT2 (gene family name *SLC1A5*) [92, 94]. As obligatory exchangers, they can coordinate either the influx and efflux of glutamine depending on the concentration gradients for the amino acid substrates across the plasma membrane [48, 92, 96]. Importantly, ASCT2 is the only member of this family that transports glutamine [94]. This family, and especially ASCT2, is frequently overexpressed in many cancer types, including breast cancer, colon cancer, lung cancer, prostate cancer, melanoma, neuroblastoma and glioblastoma [48].

System A preferentially transports alanine. It refers to a Na^+-dependent transport process selective for neutral amino acids, including alanine [48, 94, 97]. This system includes neutral amino acid transporter 1 (SNAT1, gene family name *SLC38A1*) and 2 (SNAT2, gene family name *SLC38A2*) that mediate the Na^+-dependent uniport of small neutral amino acids and are inhibited by (methylamino)isobutyric acid (MeAIB) [48]. SNAT1 appears to be upregulated and overexpressed in different breast cancer cell lines, particularly in ER^-, compared with normal breast tissues [98], while SNAT2 has been found to be upregulated in TN breast cancer [99].

System N preferentially transports amino acids with nitrogen in the side chain. It refers to a Na^+-dependent transport process selective for glutamine, asparagine, and histidine, all of which contain a nitrogen atom in the side chain [48, 94, 97]. This system includes SNAT3 (gene family name *SLC38A3*), SNAT5 (gene family name *SLC38A5*) and SNAT7 (gene family name SLC38A7) that mediate the uptake of glutamine, histidine and asparagine with a transport mode dependent on both Na^+ and H^+ [48].

System L preferentially transports leucine and others large neutral amino acids [94]. System L activity is mediated by four Na^+-independent transporters divided in two subgroups: the first includes light subunits of heterodimeric amino acid transporters, amongst which L-type amino acid transporter (LAT1 gene family name *SLC7A5*) and LAT2 (gene family name *SLC7A8*); the second subgroup includes cationic amino acid transporters: LAT3 (gene family name *SLC43A1*) and LAT4 (gene family name *SLC43A2*) [100]. LAT1 and LAT2 forms a heterodimer with the glycoprotein 4F2hc (also known as CD98), by the formation of a disulphide between two conserved cysteine residues [101]. Both are obligatory exchangers of almost all the amino acids, with a high preference towards histidine and with lower affinity to glutamine and threonine [92]. LAT3 and LAT4 are facilitated diffusers of neutral amino acids with a low affinity, and do not appear to require a binding partner. LAT3 and LAT4 deliver a straight range of neutral amino acids, including leucine, isoleucine, valine, phenylalanine and methionine, into cells [101]. Of the system L transporters, LAT1 is the one that is most overexpressed in multiple cancers [101] and its expression was shown to be essential for progression of various cancer [47, 102]. However, LAT2, LAT3 and LAT4 also show increased expression in many cancers and are critical for control of protein translation and cell growth through the mTORC1 pathway. Nevertheless, LAT3 and LAT4 show a more restricted expression in some cancers, for instance in breast cancer, comparatively to LAT1 or LAT2 [101, 103].

One of the recently described systems, xc^-, is represented only by the transporter xCT (gene family name *SCL7A11*), which functions as a Na^+-independent obligatory exchanger that imports extracellular cystine and induces the efflux of intracellular glutamate and plays a vital role in maintaining redox homeostasis [49]. High levels of xCT expression have been observed in several types of cancer [49]. In breast cancer, xCT seems to be important for the proliferation of TN breast cancer. Curiously, a subgroup of TN breast cancer require cysteine import via xCT, instead of glutamine [104].

As to system $B^{0,+}$, it includes the Na^+- and Cl-dependent neutral and basic amino acid transporter $B(0^+)$ ($ATB^{0,+}$) (gene family name *SLC6A14*), a transporter sensitive to membrane potential which has a broad affinity for all neutral and cationic amino acid substrates [49]. It is highly expressed in ER^+ breast cancer [105].

Finally, the recently described system y^+L includes the y^+L amino acid transporter 1 (y^+LAT1) (gene family name *SLC7A7*), which mediates the influx of dibasic and some neutral amino acids in a Na^+-independent manner [49]. Its overexpression has been reported in TN breast cancer [106] and $HER2^+$ breast cancer cells lines [107].

Finally, another SLC member is also involved in glutamine transport. This transporter, the neutral and basic amino acid transport protein (rBAT) (gene family name *SLC3A1*), is a Na^+-independent transporter of cystine and neutral and dibasic amino acids [49, 108], which has been reported to be associated to breast cancer tumorigenesis [108].

1.2.4.1. Regulation of ASCT2 in breast cancer

Glutamine is one of the key nutrients for rapidly proliferating tumour cells [109], and glutamine metabolism is closely associated to amino acid transporters [49]. ASCT2 is recognized as a primary transporter of glutamine [51], and is predominantly localized at the plasma membrane [110, 111]. ASCT2 is highly expressed in multiple cancers and is closely related to poor prognosis and malignant potential [51, 110, 111]. Intriguingly, ASCT2 expression is detected also in cancers deriving from human tissues in which normally the protein is not present [110]. So, this gene has been proposed as an oncogene [34].

ASCT2 is known to be upregulated in breast cancer [109, 112], and ASCT2 expression seems to vary depending on the molecular subtype [49, 109]. Indeed, a high ASCT2 expression was observed in highly-proliferative subtypes (HER2 and TN) [109, 112] and a lower expression in less aggressive tumours (luminal) [109]. This is in concordance with lower levels of glutamine

observed in TN and HER2$^+$ tumours compared with luminal A tumours, suggesting an increase in glutamine consumption in TN tumours [113, 114]. All these results support the conclusion that glutamine plays a role in breast cancer cell growth and proliferation, especially among the most aggressive types of breast cancer. So, understanding the regulation of ASCT2 in cancer cells may be important for to development of new targeted therapies. So far, mechanisms regulating ASCT2 by c-Myc, ring finger protein 5 (RNF5), retinoblastoma (Rb) and microRNA-137 (miRNA-137) have been confirmed [49, 51, 111]. However, other mechanisms are believed to regulate ASCT2.

Myc oncoproteins are particularly sensitive to glutamine deprivation [115]. c-Myc oncogene amplification is found in approximately 15% of breast cancers, particularly in basal-like type breast cancers [116]. In TN breast cancer, c-Myc is also associated with increased expression of and reliance on glutamine metabolism-related genes, including ASCT2 [109]. c-Myc binds to the promoter of ASCT2 and induces overexpression of ASCT2 and glutamine influx [117]. Furthermore, the stress-responsive transcription factor (ATF4) is also highly expressed in TN breast cancer cells. In these cells, ASCT2 is not only regulated by n-Myc but also by ATF4 [109]. Thus, it is probable that through ATF4 regulation of Myc, ASCT2 expression is modulated in breast cancer cells in response to amino acid concentrations and oestrogen receptor (ER)-stress conditions.

Another mechanism of ASCT2 regulation, associated with ER-stress conditions, involves the ubiquitin ligase RNF5. More specifically, chemotherapy-induced ER stress promotes the activation of RNF5, which stimulates the ubiquitination and degradation of ASCT2 in breast cancer cells, leading to decreased glutamine uptake. Thus, RNF5 seems to interfere with ASCT2 expression (which promotes breast tumorigenesis) [118].

Besides being regulated by oncogenes, ASCT2 expression is also regulated by the tumour suppressor gene Rb. More specifically, downregulation of Rb, which is normally observed in cancer, leads to overexpression of ASCT2 through the E2F- transcription factor 3 (E2F-3) [119].

Similarly, miRNA-137 was also shown to negatively regulate ASCT2 expression. The expression of miRNA-137 is frequently lost in many tumours, including breast cancer, resulting in increased ASCT2 expression. So, ASCT2 was identified as a critical downstream effector of miR-137, thus revealing a molecular link between DNA methylation, microRNA and tumour metabolism [120].

Finally, another regulatory mechanism of action of ASCT2 was also suggested. Tamoxifen and raloxifene, two selective ER modulators used in breast cancer therapy, seem to inhibit ASCT2-mediated glutamine uptake in MDA-MB-231 cells [121].

^{18}F-fluorocyclobutane-1-carboxylic acid (^{18}F-FACBC or ^{18}F-fluciclovine), a non-metabolized synthetic amino acid–based PET radiotracer transported via ASCT2, has shown promise in the imaging of breast and other cancers. ^{18}F-Fluciclovine activity seems to vary with histologic and immunohistochemical characteristics of malignant breast tumours and demonstrates a higher uptake in breast cancers with poor prognostic factors [122].

1.3. Breast cancer and type 2 diabetes mellitus

T2DM is one of the most common chronic diseases worldwide [3] and is characterized by insulin resistance and glucose intolerance [2]. It results from the body's inability to use insulin and is responsible for 90% of patients with diabetes around the world, being generally associated with obesity [2, 3].

The first clinical observations to suggest an association between diabetes and cancer date back to 1932 [123], but it was only recently that this evidence has received more attention. Indeed, in recent years, several studies have reinforced a link between several cancers and T2DM [5-7], including breast cancer, for which a 20-28% increased risk in women with T2DM was found [2, 7]. Interestingly, the link between T2DM and breast cancer appears to be most evident in post-menopausal women [2, 44] with an about 20–27% increased risk to develop breast cancer in these women [2, 6]. This suggests important interactions between key hormones and metabolic alterations in T2DM that predispose to the development of breast cancer [44]. Breast cancer in women with T2DM is often diagnosed at an advanced stage compared to women without T2DM [124]. Maybe for this, mortality after breast cancer diagnosis has been shown to be 50% higher in women with T2DM [2].

This suggests that the metabolic alterations that occur in T2DM provide a favourable metabolic environment for the development of breast cancer [44].

1.3.1. The molecular link

Several T2DM-related factors seems to influence tumour initiation (DNA damage from a carcinogen or reactive molecule), promotion (stimulation of initiated cells growth), and progression (more aggressive growth with angiogenesis and metastasis) and/or response to

therapy, and these have been implicated as key contributors to the complex effects of T2DM on cancer incidence and outcomes [8, 9]. It is thought that hyperglycemia and hyperinsulinemia act as triggers to cancerous phenotypes in T2DM. Additionally, altered adipokine levels (higher leptin and lower adiponectin levels) also seems to be implicated in cancer pathophysiology in T2DM patients. Moreover, the metabolic dysregulation observed in T2DM is responsible for a chronic low-grade proinflammatory condition (higher levels of interleukin-6 (IL-6), interleukin-1 beta (IL-1β), tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ)), leading to increased oxidative stress levels [2, 9, 44, 125, 126]. These metabolic changes subsequently lead to dysregulation of cell signalling pathways involved in cell proliferation, migration, angiogenesis, inflammation, invasion, and apoptosis (Figure 3) [2]. So, these T2DM-related factors could trigger and/or facilitate breast cancer initiation promotion and/or progression. Nonetheless, the mechanisms underlying the link between T2DM, and breast cancer have yet to be fully characterized.

1.3.1.1. Hyperglycemia

Hyperglycemia, resulting from an increase in systemic insulin resistance [2, 127], is the hallmark of T2DM and can have a profound effect on cancer cell biology (especially cancer initiation, proliferation, migration and invasiveness) [2, 44, 126, 127].

The long-term, poor glycaemic control observed in T2DM leads to a dysregulated metabolism responsible for increased circulating levels of insulin and production of a wide range of proinflammatory cytokines, such as IL-6 and TNF-α, but also, to increased production of reactive oxygen species (ROS) and platelet activation [2, 127, 128]. All these glucose-associated conditions can promote tumour progression [127].

Hyperglycemia also contributes to the formation of advanced glycation end products (AGEs) [2, 126, 127], which interact with its receptor for advanced glycation end products (RAGE), leading to ROS generation, activation of factor nuclear kappa B (NF-κB) and, finally, to cell damage [2, 127]. Upregulation of RAGE by AGEs promotes cell proliferation, migration and invasion, in TN breast cancer (Figure 3) [129]. Conversely, blockade of RAGE-mediated signalling inhibits breast tumour growth and metastasis [130].

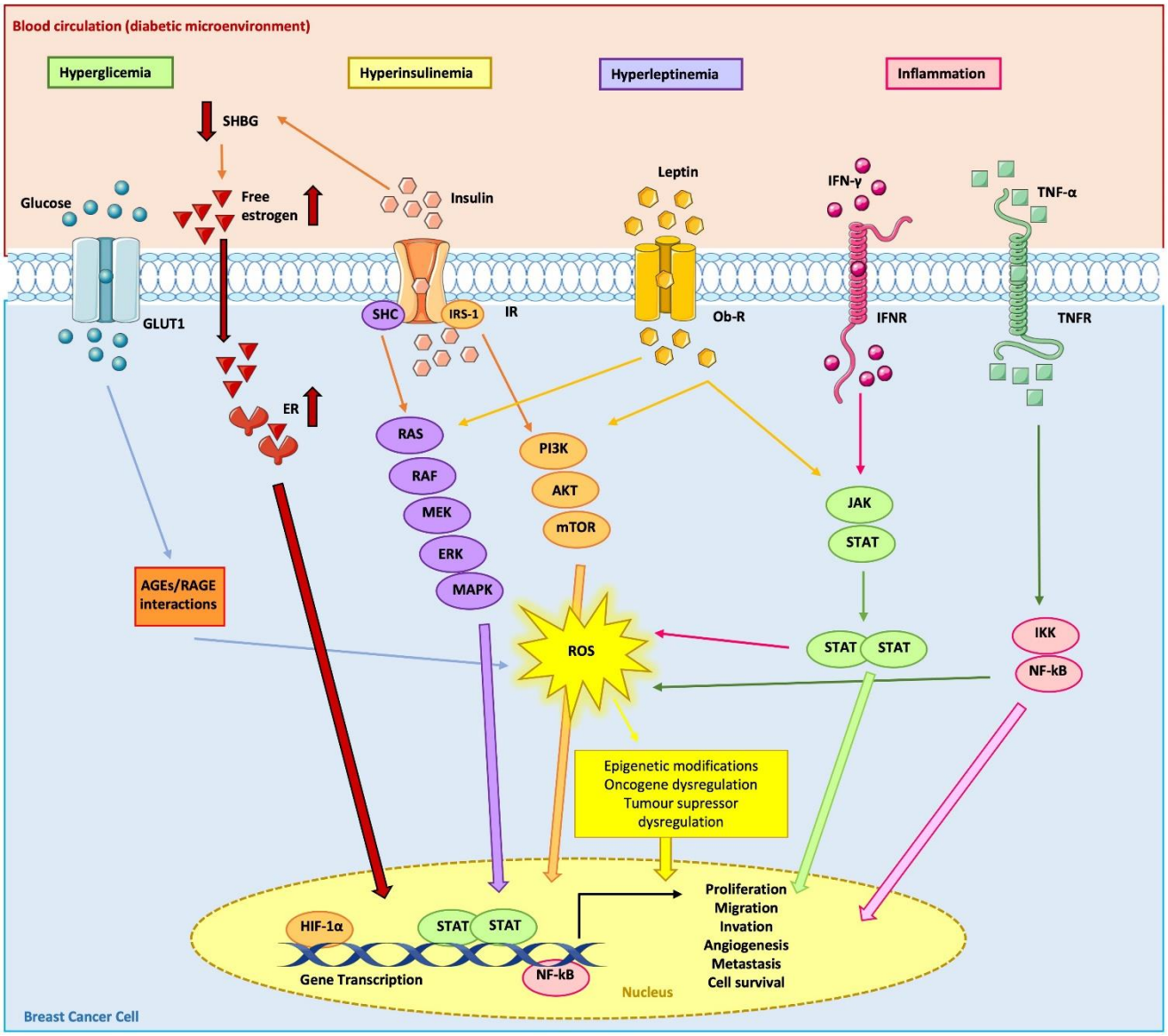

Figure 3. Impact of T2DM-related factors in breast cancer initiation, promotion, progression and/or response to therapy

T2DM causes insulin resistance (hyperglycemia and hyperinsulinemia), altered adipokine levels (hyperleptinemia), increased oxidative stress levels and a low-grade chronic inflammatory condition (higher levels of TNF-α and IFN-γ). These metabolic changes lead to dysregulation of cell signalling pathways involved in cell proliferation, migration, angiogenesis, invasion, metastasis, apoptosis and necrosis. Hyperglycemia increases the formation of AGEs, which, interacts with RANGE, leading to ROS production and cell damage. Furthermore, inflammation also stimulates ROS formation. Excessive oxidative stress causes epigenetic modifications, oncogene and tumour suppressor dysregulation and activation of transcription factors (NF-κB, STAT3 and HIF-1α). Hyperinsulinemia leads to activation of signalling pathways such as MAPK and PI3K pathways involved in carcinogenesis. Additionally, hyperinsulinemia leads to a decrease in SHBG levels, leading to an increase in free oestrogen concentrations and subsequent activation of ER leading to the enhancement of breast carcinogenesis. Similarly, hyperleptinemia stimulates activation of signalling pathways such as MAPK, PI3K and JAK/STAT stimulating breast cancer initiation, promotion and progression. So, all these T2DM-related factors can trigger and facilitate breast cancer transformation. Abbreviations: GLUT1, facilitative glucose transporters 1; AGE, advanced glycation end products; RAGE, receptor for advanced glycation end products; SHBG, sex hormone binding globulin; ER, oestrogen receptor; IR, insulin receptor; Ob-R, leptin receptor; IFN- γ; interferon gamma; TNF-α, tumour necrosis factor alpha; INFR, interferon receptor; TNFR, tumour necrosis factor receptor; ROS, reactive

oxygen species; SHC, adaptor protein; IRS-1, Insulin receptor substrate 1; RAF, proto-oncogene serine/threonine-protein kinase; MEK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; mTOR, mammalian target to rapamycin; JAK, janus kinas; STAT, signal transducer and activator of transcription; IKK, IκB kinase; NF-kB, factor nuclear kappa B; HIF1α, Hypoxia-inducible factor 1-alpha.

1.3.1.2. Hyperinsulinemia

Insulin is a polypeptide hormone produced by the pancreatic β-cells in response to an increase in blood glucose concentrations. The first step in activation of the insulin pathway, the binding of insulin to the tyrosine kinase insulin receptor (IR), stimulates glucose uptake in skeletal and cardiac muscle and adipose tissue [2, 131, 132]. IRs are present at the surface of most cells, although the levels are particularly high on targets for insulin action [133]. Breast tissue is no exception, as it is firmly established that IR is expressed in healthy breast tissue, as well as in breast cancer cells [2, 132, 133]. Furthermore, overexpression of IR is stimulated by insulin and leads to malignancy in breast epithelial cell lines [2, 132, 134] and high IR content correlates positively with tumour size and grade [132].

Several mechanisms contribute to the association between hyperinsulinemia caused by T2DM and breast cancer: activation of the insulin pathway, activation of the insulin-like growth factor (IGF)-1 pathway and altered regulation of endogenous sex hormones [132].

In the first mechanism, insulin binds to the IR, and once activated, the IR phosphorylates several intracellular proteins, including members of the insulin receptor substrate family (IRS) and adaptor protein (SHC) [2, 132, 133]. Binding of IRS to the IR leads to activation of the phosphatidylinositol 3-kinase (PI3K), which turns on the protein kinase B (PKB also known as Akt) pathway, and binding of SHC adaptor protein 1 to IR activates the mitogen-activated protein kinase (MAPK) pathway, through the activation of Ras/Raf/MEK/ERK cascade [2, 132, 133]. In general, the metabolic effects of insulin are mediated by the PI3K pathway, whereas the mitogenic effects of insulin involve the activation of Ras and the MAPK pathway [2, 133]. Both these pathways have important roles in tumorigenesis, such as increased cell survival, proliferation, motility, adhesion, angiogenesis and invasion (Figure 3) [2, 132].

Insulin is also capable of activating the IGF-1-signaling. IGF-1 and insulin, their receptors (IGF-R and IR, respectively) and their intracellular signalling pathways share similarities, particularly in hyperinsulinemic conditions [2, 127, 128, 132]. Of importance, in breast cancer, IGF-1 and IGF-R are considered stronger mitogenic, when compared to insulin/IR signalling [2, 127], because IGF-1 and IGF-R activation results in a stronger positive effect on breast cancer

cell proliferation and survival [127]. Moreover, the signalling pathways modulated by IGF-R are implicated in protection of breast cancer cells from apoptosis and development of drug resistance [2]. In addition, high circulating levels of IGF-1 and IGF-BP3 are associated with increased risk of premenopausal breast cancer [132]. So, the IGF system is also considered to be a key regulatory pathway in breast cancer [2, 132].

Lastly, in T2DM, high levels of insulin originate higher plasma levels of oestrogens and reduced plasma levels of sex hormone binding globulin (SHBG), that are strongly associated with breast cancer risk in postmenopausal women [2, 127, 132]. Thus, deregulation of sex hormones may be involved in the proliferation and survival of breast cells, inhibition of apoptosis and, possibly, in the enhancement of hormonal carcinogenesis (Figure 3) [2, 127]. Thus, deregulation of sex hormones can be an important link between T2DM and breast cancer [132].

1.3.1.3. Hyperleptinemia

Adipose tissue is an important organ for the production of adipokines, inflammatory cytokines and enzymes that are dysregulated in T2DM and potentially contribute to tumour growth and metastases [126]. The predominant cell population in the breast are the adipocytes [2]. Leptin is the one of several adipokines secreted by adipocytes [2, 126, 135, 136], and play a significant role in breast cancer risk and tumour progression [2].

The main function of leptin is the maintenance of energy homeostasis, participating in the anorexigenic pathway. In addition to this main function, it is known that leptin has effects on angiogenesis and on the proliferation of many cell types, including cells of breast tissue [136]. Enhanced expression of leptin and its transmembrane receptors (Ob-R) have been observed in malignant tissues of the breast and are associated with metastasis [137] and poor prognosis [126]. Additionally, an increase in the levels of leptin promotes breast tumour survival and growth [2]. Mechanistically, leptin activates important signalling pathways such as janus kinase (JAK)/signal transducer and activator of transcription (STAT), PI3K/Akt, and mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase 1/2 (ERK1/2) involved in cell proliferation, migration, angiogenesis, invasion and metastasis of breast cancer cells (Figure 3) [2, 126, 135]. These findings suggest that leptin might increase cancer risk.

1.3.1.4. Inflammation

It is currently well-accepted that T2DM promotes a state of chronic low-grade inflammation that leads the secretion of inflammatory cytokines (IL-6, IL-1β, TNF-α and IFN-γ), creating a microenvironment favourable to breast tumour development [9, 127, 138-140]. These cytokines have effects on cancer-related mechanisms such as cell survival, proliferation, angiogenesis and metastatic dissemination [141]. Indeed, the pathways of inflammation seem to promote activation of NF-κB, STAT3 and HIF-1α [2, 9, 139, 142]. These transcription factors coordinate the production of inflammatory mediators such as proinflammatory cytokines, which are directly involved in all stages of carcinogenesis (initiation, promotion, and progression) (Figure 3) [142]. NF-κB signalling plays an important role in the development and progression of breast cancer [139]. HIF-1α promotes the recruitment of immune cells and positively regulate the function of pro-tumorigenic inflammatory response cells, forming yet another molecular bridge between T2DM and breast cancer [9]. In addition, in postmenopausal women, greater levels of breast adipose tissue inflammation exist, which may contribute to the higher incidence of breast cancer in postmenopausal women [143]. Therefore, a chronic inflammatory state in T2DM patients may be one of the mechanisms associated with cancer initiation, promotion and progression [138, 139].

1.3.1.5. Oxidative stress

The metabolic abnormalities observed in T2DM, especially under conditions of poor metabolic control, not only cause a chronic pro-inflammatory condition, but also increase oxidative stress levels [2, 127, 128].

As already mentioned, high glucose levels in circulation contribute to the formation of AGEs, which promote the activation of NF-κB, leading to significant ROS formation [2, 127]. Under normal physiological conditions, these ROS are necessary for normal cell redox reactions, cellular function, and intracellular signalling; however, in high concentrations, ROS overload the antioxidant systems leading to an increase in oxidative stress, that can damage cell DNA by direct oxidation or by interfering with the mechanisms of DNA repair, thus increasing the risk of cancer (Figure 3) [2, 142].

Also, inflammation-induced ROS production has been linked to cancer initiation and progression [142]. Excessive ROS production render cancer cells resistant to apoptosis, disrupt cell anchorage sites and promote and sustain tumour angiogenesis [2]. Some markers of oxidative stress, including DNA adducts and lipid peroxidation products (malondialdehyde and 8-

isoprostanes) are frequently identified in breast cancer patients, suggesting that ROS are involved in the etiology and progression of breast cancer [144]. In addition, it has been reported, in breast cancer, that advanced stages of the disease are characterized by a more pronounced oxidative status than earlier stages, with a marked reduction of the antioxidant enzyme catalase activity and an enhanced lipid peroxidation together with higher nitric oxide levels [144].

Additionally, ROS have also been implicated in aberrant epigenetic modifications such as DNA hypermethylation and hypomethylation, resulting in an alteration in gene expression, with effects in malignant transformation and cancer progression (Figure 3) [2, 127]. Thus, excess ROS in T2DM patients seems to play also a role in cancer initiation, promotion and progression.

CHAPTER II

MOTIVATIONS AND AIMS

Motivations and Aims

The metabolic abnormalities associated with T2DM are linked to a higher incidence, accelerated progression and increased aggressiveness of breast cancer [2, 8, 9, 44]. So, it is likely that the metabolic environment occurring in T2DM can promote, directly or indirectly, breast tumour initiation, promotion, progression, and/or response to therapy [2, 8-10, 44, 126]. In this context, metabolic reprogramming, a recognized hallmark of cancer cells, has been attracted significant interest both from the perspective of understanding tumorigenesis and as a potential therapeutic target in breast cancer [145, 146]. Breast cancer cells rewire their cellular metabolism, upregulating expression of nutrient transporters in order to meet the increased energy demands [146]. In line with this, higher expression of the main glucose and glutamine transporters in cancer cells (GLUT1 and ASCT2, respectively) has been associated with cancer of higher grade, increased proliferation, malignant potential and poor prognosis [51, 75, 76, 111]. Therefore, these genes have been proposed as oncogenes and glucose and glutamine transporters are currently being investigated as potential therapeutic targets in cancer treatment [27, 34]. Basis on this, an effective GLUT1 and/or ASCT2 inhibition can be an alternative therapeutic strategy for reducing/preventing breast cancer. In recent years, several inhibitors of GLUT1 [27] and ASCT2 [51, 92] have emerged and showed antitumor effect. Among them, a promising compound, [N4-[1-(4-cyanobenzyl)-5-methyl-3-(trifluoromethyl)-1H-pyrazol-4-yl]-7-fluoroquinoline-2,4-dicarboxamide] (BAY-876) was identified as a new-generation selective inhibitor of GLUT1 [147]. On the other hand, L-g-glutamyl-p-nitroanilide (GPNA) features a glutamine structural analogy and effectively inhibits the Na^+-dependent amino acid transporter ASCT2 [49, 103, 111, 148].

Basis on this, the present work aims to investigate the effect of T2DM-associated characteristics (hyperglycemia, hyperinsulinemia, hyperleptinemia, and increased levels of inflammation and oxidative stress) upon glucose and glutamine transport, and whether changes in transport of these nutrients can contribute to the negative effect of T2DM in breast cancer initiation, promotion and progression, and response to therapy.

The specific aims of this study are detailed in the following chapters:

CHAPTER III - The effect of type 2 diabetes mellitus-associated characteristics upon glucose cellular uptake in breast cancer progression

This Chapter aims to study the *in vitro* effects of T2DM-associated markers on cellular characteristics promoting breast cancer initiation and/or progression, and to investigate if an effect on GLUT1-mediated glucose cellular uptake contributes to their effect in promoting initiation and/or progression of breast cancer cells. The specific aims are:

- To study the effect of T2DM-associated characteristics on cell proliferation, migration, growth, angiogenesis, viability and apoptosis in breast cancer and non-tumorigenic cell lines.
- To explore the effect of T2DM-associated characteristics on glucose cellular uptake and lactate production in breast cancer and non-tumorigenic cell lines.
- To investigate if T2DM-associated characteristics interfere with GLUT1-mediated glucose transport in breast cancer and non-tumorigenic cell lines.
- To evaluate the capacity of BAY-876 in reversing the effects of these compounds on glucose uptake, cell proliferation and viability in breast cancer and non-tumorigenic cell lines.

CHAPTER IV - The effect of type 2 diabetes mellitus-associated characteristics upon glutamine cellular uptake in breast cancer progression

This Chapter aims to explore if interference with ASCT2-mediated glutamine uptake by breast cancer cells, *in vitro*, contributes to the effect of T2DM-associated markers in promoting breast cancer initiation and/or progression. The specific aims are:

- To explore the effect of T2DM-associated characteristics on glutamine cellular uptake by breast cancer and non-tumorigenic cell lines.
- To examine the influence of GPNA, a pharmacologic inhibitor of the ASCT2 transporter, in preventing the stimulatory effects of these compounds on glutamine uptake.
- To investigate if T2DM-associated characteristics interfere with ASCT2 expression levels.
- To confirm the involvement of ASCT2 in the stimulatory effects of T2DM-associated characteristics, by performing targeted knockdown of ASCT2.
- To explore which signalling pathways mediate the effects of T2DM-associated characteristics on glutamine cellular uptake.

CHAPTER V – The effect of type 2 diabetes mellitus-associated characteristics upon glucose and glutamine cellular uptake in breast cancer initiation and promotion

This Chapter aims to induce and validate an *in vitro* model of breast cancer promotion and, thereafter, to investigate the influence of T2DM-associated markers in breast cancer initiation and or/promotion. The specific aims are:

- To induce carcinogenesis in a non-tumoral human breast epithelial cell line, by using the specific mammary chemical agent 7,12-Dimethylbenz[a]anthracene (DMBA), and to validate the model using, besides other method, artificial intelligence (AI) tools.
- To study the effect of T2DM-associated characteristics on cell proliferation, migration, growth, angiogenesis, viability, and apoptosis in DMBA-transformed and non-tumorigenic cell lines.
- To explore the effect of T2DM-associated markers on glucose and glutamine cellular uptake and lactate production in DMBA-transformed and non-tumorigenic cell lines.
- To investigate which signalling pathways mediate the effects of T2DM-associated characteristics.
- To evaluate the capacity of GLUT1 and ASCT2 pharmacologic inhibitors (BAY-876 and GPNA, respectively) in reversing the effects of these compounds.

CHAPTER III

THE EFFECT OF TYPE 2 DIABETES MELLITUS-ASSOCIATED CHARACTERISTICS UPON GLUCOSE CELLULAR UPTAKE DURING BREAST CANCER PROGRESSION

THE *IN VITRO* EFFECT OF DIABETES-ASSOCIATED MARKERS INSULIN, LEPTIN AND OXIDATIVE STRESS ON CELLULAR CHARACTERISTICS PROMOTING BREAST CANCER PROGRESSION IS GLUT1-DEPENDENT

Cláudia Silva, Nelson Andrade, João Tiago Guimarães, Emília Patrício, Fátima Martel.

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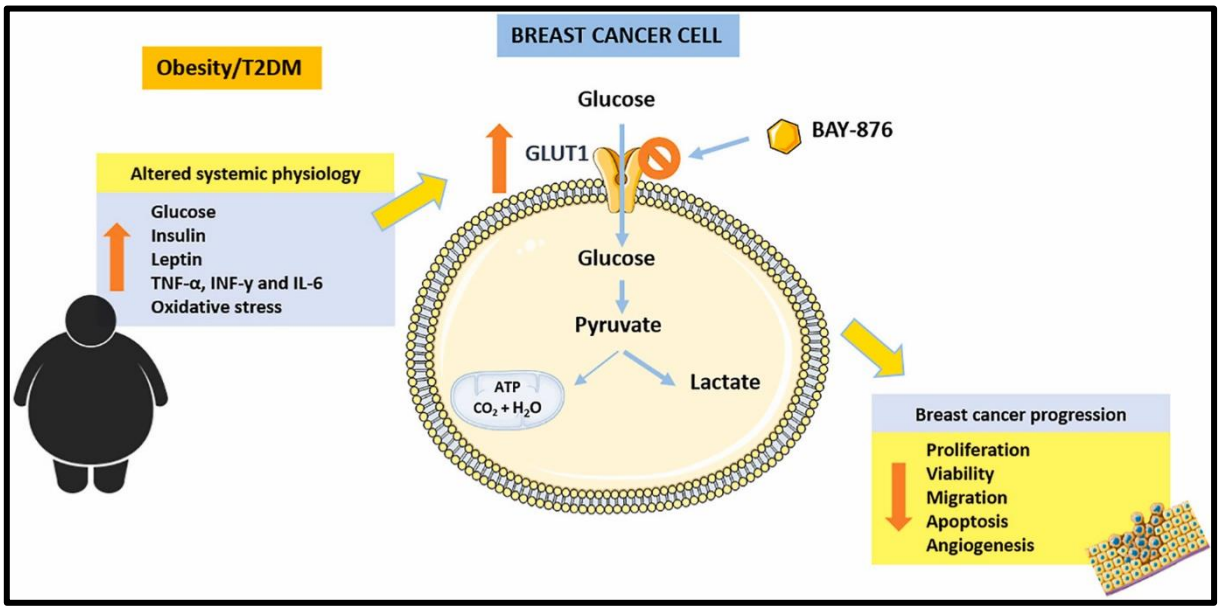
Graphical Abstract



Highlights

- Glucose uptake by breast cancer and non-cancer cell lines is mainly GLUT1-mediated
- Oxidative stress, insulin and leptin have breast cancer-promoting effects
- These markers increase GLUT1-mediated glucose uptake in breast cancer cells
- Oxidative stress and leptin increase GLUT1 mRNA levels in breast cancer cells
- These markers promote cancer cell growth and viability through GLUT1 stimulation

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The *in vitro* effect of the diabetes-associated markers insulin, leptin and oxidative stress on cellular characteristics promoting breast cancer progression is GLUT1-dependent

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ABSTRACT

Obesity and type 2 diabetes mellitus (T2DM) associate with increased incidence and mortality from many cancers, including breast cancer. The mechanisms involved in this relation remain poorly understood. Our study aimed to investigate the *in vitro* effect of high levels of glucose, insulin, leptin, TNF-α, INF-γ and oxidative stress (induced with tert-butylhydroperoxide (TBH)), which are associated with T2DM, upon glucose uptake by breast cancer (MCF-7 and MDA-MB-231) and non-cancer (MCF-12A) cells and to correlate this effect with their effects upon cellular characteristics associated with cancer progression (cell proliferation, viability, migration, angiogenesis and apoptosis).

^{3}H-DG uptake was markedly inhibited by a selective GLUT1 inhibitor (BAY-876) in all cell lines, proving that ^{3}H-DG uptake is mainly GLUT1-mediated. TBH (2.5 μM), insulin (50 nM), leptin (500 ng/ml) and INF-γ (100 ng/ml) stimulate GLUT1-mediated ^{3}H-DG (1 mM) uptake by both ER-positive and triple-negative breast cancer cell lines. TBH and leptin, but not insulin and INF-γ, increase GLUT1 mRNA levels. Insulin and leptin (in both ER-positive and triple-negative breast cancer cell lines) and TBH (in the triple-negative cell line) have a proproliferative effect and leptin possesses a cytoprotective effect in both breast cancer cell lines that can contribute to cancer progression. The effects of TBH, insulin, leptin and INF-γ upon breast cancer cell proliferation and viability are GLUT1-dependent.

In conclusion, T2DM-associated characteristics induce changes in GLUT1-mediated glucose uptake that can contribute to cancer progression. Moreover, we conclude that BAY-876 can be a strong candidate for development of a new effective anticancer agent against breast cancer.

1. Introduction

The current increase of obesity and type 2 diabetes (T2DM) on the human population is a headline concern worldwide, and both conditions are associated with an increased risk for the development of several types of cancer (Park et al., 2014; Pearson-Stuttard et al., 2018), including breast cancer (Lee et al., 2019). In 2018, breast cancer represented 11.6% of all cancers worldwide, and caused about 7% of cancer deaths. It constituted the second most frequent cancer and the second cause of cancer death, after lung cancer, in both sexes combined, and the most commonly diagnosed cancer and the leading cause of cancer death, in women (Bray et al., 2018). Breast cancer constitutes about 30% of all cancers attributable to high body-mass index and T2DM in women (Pearson-Stuttard et al., 2018). T2DM is not only a risk factor for breast cancer, but it is also associated with breast cancer progression and poor prognosis (Bray et al., 2018; Doerstling et al., 2017; Widschwendter et al., 2015).

A proposed biological mechanism underlying the link between T2DM, obesity and cancer relates to altered levels of T2DM-related factors, which influence tumour initiation, progression, and/or

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response to therapy (Doerstling et al., 2017). In particular, T2DM is associated with hyperinsulinemia, hyperglicemia, altered adipokines (higher leptin and lower adiponectin), chronic low-grade inflammation (higher interleukin-6 (IL-6), tumour necrosis factor α (TNF-α) and interferon- γ (INF- γ)) and increased oxidative stress levels (Doerstling et al., 2017; Micucci et al., 2016). These T2DM-related factors seem to drive tumour growth by engaging signaling pathways involved in cell proliferation, migration, angiogenesis, inflammation, invasion, and apoptosis (Doerstling et al., 2017), but the mechanisms underlying the link between T2DM and breast cancer have yet to be fully understood.

One of the cancer cell hallmarks corresponds to altered metabolic characteristics, which is known as metabolic reprogramming (Hanahan and Weinberg, 2011). One of the most well-known metabolic reprogramming features of cancer cells is the Warburg effect (aerobic glycolysis), which is characterized by increased glycolysis and lactate production regardless of oxygen availability in cancer cells. Aerobic glycolysis is often accompanied by increased glucose uptake (Cha et al., 2018; Hanahan and Weinberg, 2011; Vander Heiden, 2011). In line with this, increased expression of the main glucose transporter in cancer cells, the glucose transporter 1 (GLUT1) (also known as solute carrier family 2, facilitated glucose transporter member 1 (SLC2A1)), has been associated with cancers of higher grade, increased proliferation and malignant potential and consequently poor prognosis (Barbosa and Martel, 2020; Krzeslak et al., 2012; Martel et al., 2016). So, this gene have been proposed as oncogene (Martel et al., 2016). In this context, overexpression of GLUT1 in breast cancer is firmly established and GLUT1 inhibition appears to be a feasible cancer treatment (Adekola et al., 2012; Barbosa and Martel, 2020; Szablewski, 2013).

Very recently, a promising compound, [N4-[1-(4-cyanobenzyl)-5-methyl-3-(trifluoromethyl)-1H-pyrazol-4-yl]-7-fluoroquinoline-2,4-dicarboxamide] (BAY-876) was identified as a new-generation selective inhibitor of GLUT1. BAY-876 shows good metabolic stability *in vitro* and high oral bioavailability *in vivo* when used in nanomolar concentration (Siebeneicher et al., 2016). A recent work, studying the antitumor activity of BAY-876 in ovarian cancer cell lines and xenograft models, showed that BAY-876 dramatically inhibits tumorigenicity of both, cell lines and xenografts (Ma et al., 2018). The applicability of BAY-876 in breast cancer intervention remains, however, largely unknown.

So, in the present study, we aimed to investigate *in vitro* if the effect of T2DM-associated characteristics (high levels of glucose, insulin, leptin, inflammatory mediators and oxidative stress) on GLUT1-mediated glucose cellular uptake contributes to their effect on breast cancer progression. For this, we evaluated the capacity of BAY-876 in reversing the effects of these compounds on glucose uptake, cell proliferation and viability.

2. Materials and methods

2.1. Cells and cell culture

We used two breast cancer and one non-cancer cell line: MCF-7 (an estrogen receptor (ER)-positive human breast epithelial adenocarcinoma cell line; ATCC HTB-22; passage numbers 79-92), MDA-MB-231 (a triple negative human breast adenocarcinoma cell line; ATCC HTB-26; passage numbers 50-79) and MCF-12A (a non-tumorigenic human breast epithelial cell line; ATCC CRL-10782; passage numbers 30-53).

Cells were maintained in a humidified atmosphere of 5% CO_2-95% air and were grown in RPMI 1640 medium (catalogue #R6504, Sigma-Aldrich, St. Louis, MI, USA) supplemented with 2 mM L-glutamine, 10 mM sodium bicarbonate, 15% heat-inactivated FBS and 1% antibiotic/antimycotic (MCF-7 and MDA-MB-231 cell lines) or DMEM:Ham's F12 medium (1:1) (catalogue #FG4815, Biochrom, Berlin, Germany) supplemented with 20 ng/ml human epidermal growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml bovine insulin, 500 ng/ml hydrocortisone (all from Sigma-Aldrich, St. Louis, Missouri, USA), 5% heat-inactivated horse serum (Gibco, Life Technologies Corporation, CA, USA) and 1% antibiotic/antimytotic (MCF-12A cell line). Culture medium was renewed every 2–3 days, and the culture was split every 7 days.

For the determination of cell viability, proliferation, culture growth, migration, oxidative stress and angiogenesis, cells were seeded on 24-well culture dishes (2 cm^2; Ø 16 mm; TPP®, Trasadingen, Switzerland) and used at 80–90% confluence. For uptake experiments and for quantification of lactate production, cells were seeded similarly and used at 100% confluence. For apoptosis determination, cells were seeded on coverslips in 24-well plates and used at 50–60% confluence. For RNA extraction, cells were seeded on 21 cm^2 plates (21 cm^2; Ø 60 mm; Corning Costar, NY, USA) and used at 100% confluence.

2.2. Cell treatments

The concentrations of compounds to be tested were chosen based on literature and our own previous works (e.g. Andrade et al., 2018; Araujo et al., 2013; Silva et al., 2017; Thibault et al., 2007; Wolczyk et al., 2016).

To test the effects of glucose (Merck, Darmstadt, Germany), *tert*-butylhydroperoxide (TBH), insulin, leptin, TNF-α, INF-γ (all from Sigma-Aldrich, St. Louis, Missouri, USA) and/or BAY-876 (Tocris Bioscience, Bristol, United Kingdom) on cell proliferation, viability, apoptosis, migration, culture growth, angiogenesis, oxidative stress, RNA extraction, lactate production and glucose uptake, cells were exposed to these compounds for 24h in serum-free culture medium. To test the effect of these compounds on glucose cellular uptake, cells were exposed to these compounds for 24h in serum-free culture medium, and also during the 20-min pre-incubation and the 6-min incubation with ^{3}H-DG (see below).

Tested drugs were dissolved in serum-free culture medium (glucose), decane (TBH), HCl 0.01 M (insulin), 0.1% (w/v) BSA (leptin), 0.1% (w/v) phosphate buffered saline (PBS) (TNF-α and INF-γ) or DMSO 100 mM (BAY-876) (1% (v/v) final concentration), and controls were run in the presence of solvent.

2.3. Evaluation of oxidative stress

The formation of thiobarbituric acid-reactive substances (TBARS assay), which quantifies a lipid peroxidation biomarker -malondialdehyde- was used to determine oxidative stress levels. In brief, cells were exposed to TBH (2.5 μM) or vehicle for 24 h, and at the end of this period the reaction was started by addition of 50% (w/v) TCA to each sample, followed by a centrifugation for 2 min at 10620 g. Then, 1% 2-thiobarbituric acid was added to the supernatant and the reaction was carried out in a boiling water bath for 40 min. A pink-coloured complex was quantified spectrophotometrically at 535 nm using a microplate reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Results were normalized for total protein content (Bradford method).

2.4. Evaluation of culture growth

Cells were exposed to TBH (0.5–2.5 μM), glucose (15–30 mM), insulin (1–50 nM), leptin (10–500 ng/ml), TNF-α (1–100 ng/ml) or INF-γ (1–100 ng/ml) or vehicle for 24h. At the end of treatment, culture growth was determined by the sulforhodamine B (SRB) assay, which reports on intracellular protein content, as described (Silva et al., 2017).

2.5. Evaluation of cell proliferation

Cells were exposed to TBH (0.5–2.5 μM), glucose (15–30 mM), insulin (1–50 nM), leptin (10–500 ng/ml), TNF-α (1–100 ng/ml), INF-γ (1–100 ng/ml) and/or BAY-876 or vehicle for 24h, and cell proliferation rates were determined by a ^{3}H-thymidine incorporation assay, as described (Silva et al., 2017). DNA synthesis rate was evaluated by quantification of incorporation of ^{3}H-thymidine (mCi/mg total protein). Intracellular radioactivity was measured by liquid scintillation counting

(LKB Wallac 1209 Rackbeta, Turku, Finland). Results were normalized for total protein content (Bradford method).

2.6. Evaluation of cell migration

Cell migration rates were determined by a scratch injury assay. Briefly, cell monolayers were scratched with a 10 μl pipette tip and were afterwards treated for 24h with TBH (0.5–2.5 μM), glucose (15–30 mM), insulin (1–50 nM), leptin (10–500 ng/ml), TNF-α (1–100 ng/ml) or INF-γ (1–100 ng/ml) or vehicle. Images were obtained at 0 and 24h after the scratch, and quantification was performed using the ImageJ software (NIH, Bethesda, MD, USA).

2.7. Evaluation of cell viability

Cells were exposed to TBH (0.5–2.5 μM), glucose (15–30 mM), insulin (1–50 nM), leptin (10–500 ng/ml), TNF-α (1–100 ng/ml) INF-γ (1–100 ng/ml) and/or BAY-876 (500 nM) or vehicle for 24h. After this period, cellular leakage of lactate dehydrogenase (LDH) into the extracellular culture medium was determined, as described (Silva et al., 2017). LDH activity was expressed as the percentage of extracellular activity in relation to total cellular LDH activity.

2.8. Determination of apoptotic index

Cells were seeded on glass coverslips and were exposed to TBH (2.5 μM), insulin (50 nM), leptin (100 ng/ml), TNF-α (100 ng/ml) or INF-γ (100 ng/ml) or vehicle for 24h. Then, the apoptotic index was determined by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, by using the In Situ Cell Death Detection kit (Roche Diagnostics, Basel, Switzerland), as described (Silva et al., 2017). Immunofluorescence was visualized under a fluorescence microscope (Zeiss apopTome, Oberkochen, Germany). The apoptotic index was calculated as the percentage of apoptotic cells respective to total cell number.

2.9. Quantification of VEGF-A levels

Cells were exposed to TBH (2.5 μM), insulin (50 nM), leptin (500 ng/ml), TNF-α (100 ng/ml) or INF-γ (100 ng/ml) or vehicle for 24h. Then, VEGF-A levels were quantified using a human VEGF-A ELISA Kit (RAB0507; Sigma-Aldrich, St. Louis, Missouri, USA) according to the manufacturer's instructions. The optical density at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

2.10. Quantification of ^{3}H-deoxy-D-glucose (^{3}H-DG) cellular uptake

After exposure to TBH (0.5–2.5 μM), insulin (1–50 nM), leptin (10–500 ng/ml), TNF-α (1–100 ng/ml), INF-γ (1–100 ng/ml) and/or BAY-876 (10–500 nM) or vehicle for 24h, culture medium was first discarded and the cells were washed with 300 μl GF-HBS buffer (composition in mM: 20 HEPES, 5 KCl, 140 NaCl, 2.5 $MgCl_2$, 1 $CaCl_2$, pH 7.4) at 37 °C. Then, cell monolayers were pre-incubated for 20 min in GF-HBS buffer at 37 °C. Uptake was then initiated by the addition of 200 μl GF-HBS buffer at 37 °C containing ^{3}H-DG 10–20 nM (^{3}H-2-deoxy-D-glucose; specific activity 60 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO, USA). Incubation was stopped after 6 min by removing the incubation medium, placing the cells on ice, and rinsing them with 500 μl ice-cold GF-HBS buffer. Cells were then solubilized with 300 μl 0.1% (v/v) Triton X-100 (in 5 mM Tris-HCl, pH 7.4) and placed at 4 °C overnight. Intracellular radioactivity was measured by liquid scintillation counting (LKB Wallac 1209 Rackbeta, Turku, Finland). Results were normalized for total protein content (Bradford method).

2.11. Quantification of lactate release

After exposure to TBH (2.5 μM), insulin (50 nM), leptin (100 ng/ml), TNF-α (100 ng/ml) or INF-γ (100 ng/ml) or vehicle for 24h, extracellular lactate was measured with the lactate oxidase/peroxidase colorimetric assay, as described (Silva et al., 2019).

2.12. RT-qPCR

Total RNA was extracted from MCF-7, MDA-MB-231 and MCF-12A cells treated with TBH (2.5 μM), insulin (50 nM), leptin (100 ng/ml), TNF-α (100 ng/ml) or INF-γ (100 ng/ml) or vehicle for 24h and RT-qPCR was carried out as described (Silva et al., 2019). Cycling conditions for human glucose transporter 1 (SLC2A1; GLUT1) amplification were as follows: denaturation (95 °C for 5 min), amplification and quantification [95 °C for 10 s, annealing temperature (AT) for 10 s, and 65 °C for 10 s, with a single fluorescence measurement at the end of the 72 °C for 10 s segment] repeated 55 times, followed by a melting curve program [(AT + 10) °C for 15 s and 75 °C with a heating rate of 0.1 °C/s and continuous fluorescence measurement] and a cooling step to 37 °C for 30 s. The primer pair used for human GLUT1 amplification was 5′-GAT GAT GCG GGA GAA GAA GGT-3' (forward) and 5′-ACA GCG TTG ATG CCA GAC AG-3' (reverse). The amount of GLUT1 mRNA was normalized to the amount of mRNA of the housekeeping gene β-actin. Cycling conditions for human β-actin amplification were as follows: denaturation (95 °C for 5 min), amplification and quantification (95 °C for 10 s, AT for 10 s, and 65 °C for 10 s, with a single fluorescence measurement at the end of the 72 °C for 10 s segment) repeated 45 times, followed by a melting curve program [(AT + 10) °C for 15 s and 75 °C with a heating rate of 0.1 °C/s and continuous fluorescence measurement] and a cooling step to 37 °C for 30 s; the primer pair used for β-actin was: 5′-AGA GCC TCG CCT TTG CCG AT-3' (forward) and 5′-CCA TCA CGC CCT GGT GCC T-3' (reverse). Data were collected using the LightCycler 96 SW 1.1 analysis software (Roche, Mannheim, Germany), and results were analyzed by the comparative Ct (ΔΔCT) method (Schmittgen and Livak, 2008). β-actin mRNA expression levels were not affected by the treatment of the cells (data not shown).

2.13. Total protein determination

The protein content of cell monolayers was determined as described by Bradford (1976), using human serum albumin as standard.

2.14. Statistics

Data are expressed as means ± S.E.M. *n* indicates the number of replicates of at least 2 independent experiments. Statistical significance of the difference between two groups was evaluated by Student's *t*-test; statistical analysis of the difference between various groups was evaluated by the analysis of variance (two-way ANOVA) test, followed by the Newman-Keuls posthoc test. Analyses were done using the GraphPad Prism version 7.0 software (San Diego, CA, USA). $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. T2DM-associated characteristics induce changes in cell proliferation, migration, growth, angiogenesis, viability and apoptosis in breast cancer and non-cancer cell lines

Based on the hypothesis that T2DM-associated characteristics induce changes in glucose transport that can contribute to their negative effects on breast cancer progression, we studied the effect of a 24h-exposure to high levels of glucose (15–30 mM), insulin (1–50 nM), leptin (10–500 ng/ml), pro-inflammatory cytokines (TNF-α and INF-γ; 1–100 ng/ml) and high oxidative stress levels (by using the oxidative stress inducer

TBH (0.5–2.5 μM)) on cell proliferation (^{3}H-thymidine incorporation assay), culture growth (sulforhodamine B assay), viability (LDH leakage assay), migration (injury assay), apoptosis index (as assessed by the TUNEL assay) and angiogenesis (quantification of VEGF-A levels) in two breast cancer (MCF-7 and MDA-MB-231) cell lines (Figs. 1–3). Additionally, we also tested the effect of T2DM-associated characteristics in a breast non-tumorigenic cell line (MCF-12A).

TBH (2.5 μM) was able to induce oxidative stress, both in breast cancer (MCF-7 and MDA-MB-231) and non-tumorigenic (MCF-12A) cell lines, as assessed by an increase in lipid peroxidation levels (TBARS assay), although a more marked effect was observed in the non-cancer cell line (Fig. S1). TBH induced a concentration-dependent decrease in cell proliferation, culture growth and migration in MCF-7 cells, but opposite concentration-dependent effects on these parameters were observed in MDA-MB-231 cells (Figs. 1 and 2). In contrast, in both cell lines, TBH concentration-dependently decreased cell viability and also presented a pro-apoptotic and pro-angiogenic effect (Figs. 2 and 3). In the non-tumoral cell line, TBH was devoid of effect on cell proliferation, apoptosis and angiogenesis, but was cytotoxic and decreased culture growth and cell migration (Figs. 1–3).

High glucose (15–30 mM) caused no significant effect in culture growth, cell proliferation, cell viability and cell migration in both cancer and non-tumoral cell lines, with the exception of an increase in MCF-12A cell proliferation and in MDA-MB-231 cell migration (Figs. 1 and 2).

Insulin and leptin induced a concentration-dependent increase in proliferation and migratory capacity of the two breast cancer cell lines, although no consistent effect in culture growth was found (Figs. 1 and 2). Moreover, both compounds induced apoptosis in MCF-7 cells (Fig. 3). In contrast, their effects on breast cancer cell viability were distinct, as insulin and leptin induced a concentration-dependent decrease and increase in the viability, respectively (Fig. 2). Furthermore, insulin promoted angiogenesis in both cancer cell lines and leptin had a pro-angiogenic effect in MDA-MB-231 cells (Fig. 3). In contrast, in MCF-12A cells, insulin and leptin had no effect on cell proliferation, migration and angiogenesis, and reduced cell viability and culture growth; furthermore, leptin induced apoptosis (Figs. 1–3).

With regard to pro-inflammatory cytokines, TNF-α reduced cell proliferation rates and viability in all cell lines, but its effects on the other analyzed parameters were quite cell line-specific. In MCF-7 cells, it promoted culture growth, migration and apoptosis but had an anti-angiogenic effect; in MDA-MB-231 cells, it decreased culture growth, stimulated migration and angiogenesis and had no effect on apoptosis. Finally, in MCF12-A cells, TNF-α had no effect on culture growth, apoptosis and angiogenesis, causing only a decrease in migration rates (Figs. 1–3).

Finally, INF-γ induced a concentration-dependent decrease in cell proliferation in all three cell lines, but its effects on the remaining parameters were also distinct. In MCF-7 cells, an increase in culture growth, cell viability and apoptosis, and a decrease in cell migration and VEGF-A production was found (Figs. 1–3). In contrast, in MDA-MB-231

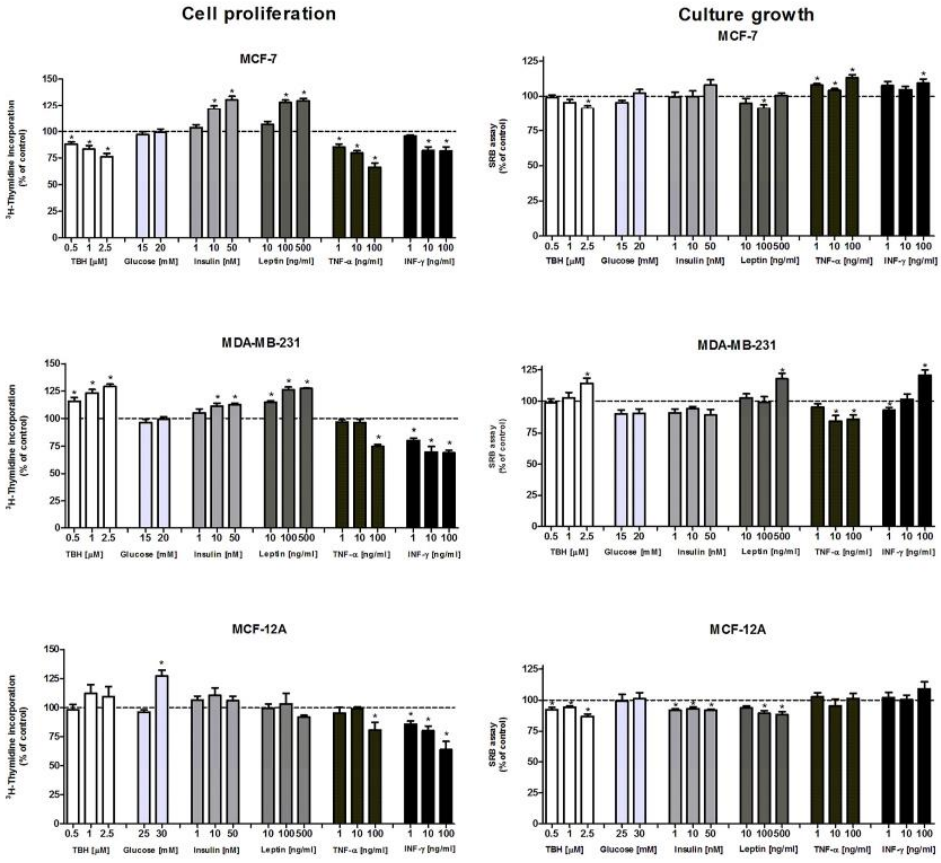

Fig. 1. Effects of T2DM-associated characteristics on cell proliferation and culture growth in breast cancer and non-tumoral cell lines.
Effects of TBH (0.5–2.5 μM), glucose (15–30 mM), insulin (1–50 nM), leptin (10–500 ng/ml), TNF-α (1–100 ng/ml) or INF-γ (1–100 ng/ml) (24h) on cell proliferation rates and culture growth of breast cancer (MCF-7 and MDA-MB-231)) and non-tumoral (MCF-12A) cell lines (n = 4–6). Data show arithmetic means ± S.E.M. *P < 0.05 vs control, by Student's *t*-test.

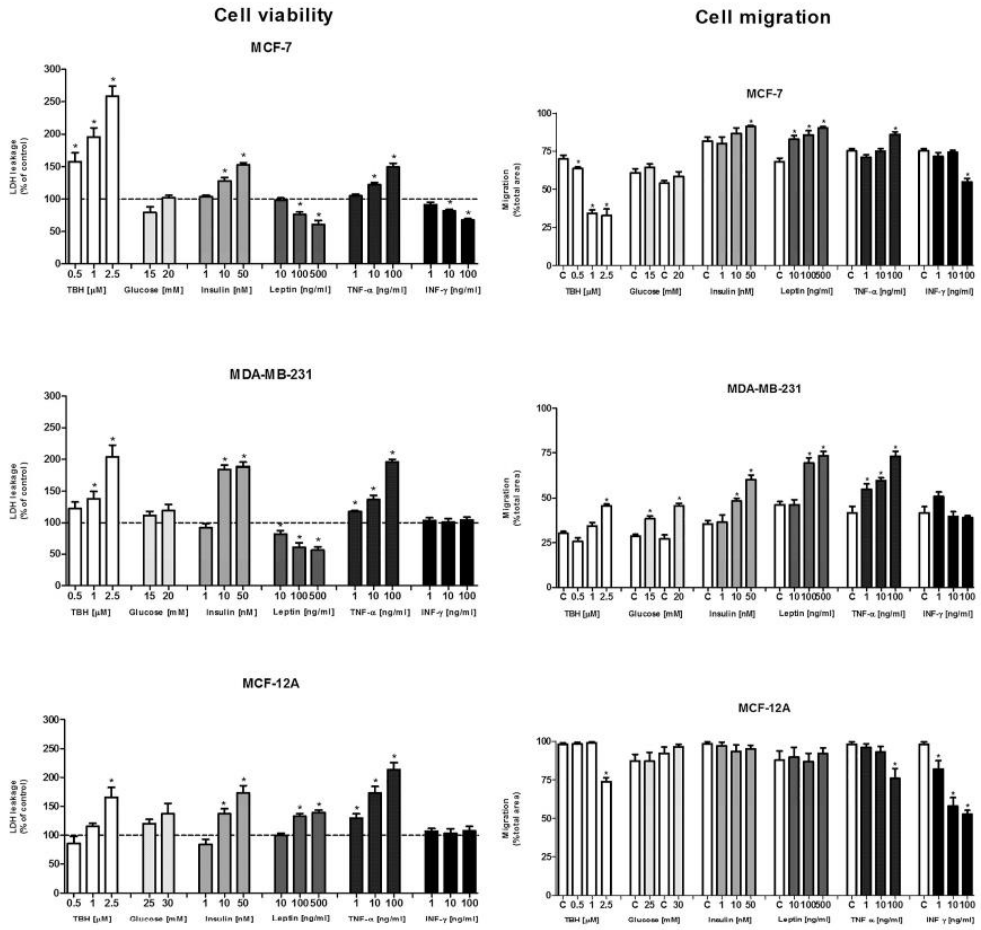

Fig. 2. Effects of T2DM-associated characteristics on cell viability and migration in breast cancer and non-tumoral cell lines.
Effects of TBH (0.5–2.5 μM), glucose (15–30 mM), insulin (1–50 nM), leptin (10–500 ng/ml), TNF-α (1–100 ng/ml) or INF-γ (1–100 ng/ml) (24h) on cell viability and cell migration of breast cancer (MCF-7 and MDA-MB-231) and non-tumoral (MCF-12A) cell lines (n = 4–6). Data show arithmetic means ± S.E.M. *P < 0.05 vs control, by Student's *t*-test.

cells, INF-γ stimulated culture growth and angiogenesis and did not affect viability, migration and apoptosis. Lastly, in MCF-12A cells, INF-γ had only pro-apoptotic, antiproliferative and anti-migratory effects (Figs. 1–3).

As a whole, these results show that, with the exception of glucose, T2DM are able to interfere with cellular characteristics important in the context of tumour progression in a cancer cell-specific way.

3.2. TBH, insulin, leptin and INF-γ increase cellular ^{3}H-DG uptake and lactate production in breast cancer cells

TBH, insulin, leptin, TNF-α and INF-γ were found to interfere with breast cancer cell proliferation, viability, apoptosis index, migration capacity and VEGF-A synthesis, and to differently interfere with these characteristics in a non-tumoral cell line. So, we selected these T2DM-associated markers for the next set of experiments.

We used ^{3}H-DG to measure cellular glucose uptake. First, we compared ^{3}H-DG uptake (10 nM) in non-tumorigenic (MCF-12A) and breast cancer cell lines (MCF-7 and MDA-MB-231). ^{3}H-DG was taken up by the three cell lines, with a higher rate of uptake observed with the non-cancer cell line (Fig. S2). Uptake of ^{3}H-DG by the three cell lines was time-dependent and linear with time for up to 6 min of incubation (Fig. S2). On the basis of this information, subsequent experiments aimed at investigating the effect of T2DM-associated characteristics on ^{3}H-DG uptake were performed using a 6 min incubation time, for all cell lines.

Next, we exposed the non-cancer (MCF-12A) and the breast cancer cell lines (MCF-7 and MDA-MB-231) to TBH (0.5–2.5 μM), insulin (1–50 nM), leptin (10–500 ng/ml) TNF-α (1–100 ng/ml) or INF-γ (1–100 ng/ml) for 24h and their effect upon uptake of a low (10 nM) ^{3}H-DG concentration was quantified (Fig. 4). The effect of the highest concentration of each of these compounds on the uptake of a near physiological concentration of ^{3}H-DG (1 mM) (Fig. 4) and lactate production (Fig. 5) was also evaluated.

TBH concentration-dependently increased ^{3}H-DG (10 nM) uptake in all cell lines. This effect was also verified at a physiological ^{3}H-DG concentration (1 mM) (Fig. 4) and was associated with an increase in lactate production (Fig. 5).

In contrast, insulin, leptin and INF-γ possess distinct effects on ^{3}H-DG uptake by cancer and non-cancer cell lines. Insulin stimulated uptake of ^{3}H-DG (10 nM and 1 mM) and increased lactate production only in the cancer cell lines (Figs. 4 and 5). As to leptin, it increased uptake of ^{3}H-DG (10 nM and/or 1 mM) in the two breast cancer cell lines, associated with an increase in lactate production in MCF-7 cells. In contrast, ^{3}H-DG (10

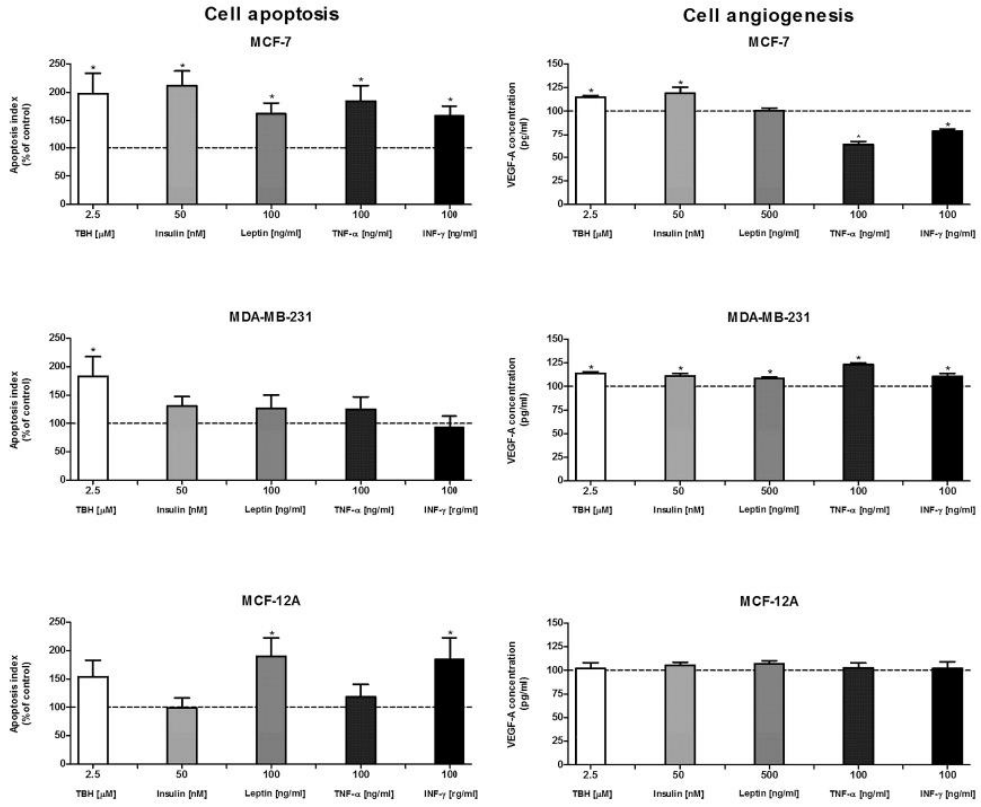

Fig. 3. Effects of obesity/T2DM-associated characteristics on apoptosis index and VEGF-A levels in breast cancer and non-tumoral cell lines.
Effects of TBH (2.5 μM), insulin (50 nM), leptin (100–500 ng/ml), TNF-α (100 ng/ml) or INF-γ (100 ng/ml) (24 h) on the apoptosis index and VEGF-A levels of breast cancer (MCF-7) and MDA-MB-231) and non-tumoral (MCF-12A) cell lines (n = 6). Data show arithmetic means ± S.E.M. *P < 0.05 vs control, by Student's *t*-test.

nM) uptake and lactate production were reduced by leptin in the non-cancer cell line. In relation to INF-γ, it increased uptake of ^{3}H-DG (10 nM and/or 1 mM) in the two breast cancer cell lines, associated with a parallel increase in lactate production, but decreased uptake of ^{3}H-DG and lactate production by the non-tumoral cell line (Figs. 4 and 5).

Finally, TNF-α was devoid of effect on uptake of ^{3}H-DG and lactate production in all cell lines (Figs. 4 and 5).

In summary, with the exception of TNF-α, the T2DM-associated features (TBH, insulin, leptin and INF-γ) were found to significantly increase ^{3}H-DG uptake and metabolism by breast cancer cells. Importantly, for insulin, leptin and INF-γ, this effect was cancer cell-specific.

3.3. ^{3}H-DG uptake in breast cancer and non-cancer cell lines is mainly GLUT1-mediated

The potent and selective GLUT1 transporter inhibitor BAY-876 (10–500 nM) concentration-dependently reduced ^{3}H-DG (10 nM) cellular uptake by both cancer (MCF-7 and MDA-MB-231) and non-cancer (MCF-12A) cell lines (Fig. 6). A similar inhibitory effect of BAY-876 (500 nM) was found in the two breast cancer cell lines (±75% inhibition) and it reduced ^{3}H-DG uptake by MCF-12A cells by 56%. So, GLUT1 plays an important role in the uptake of glucose by breast cancer and non-cancer cell lines.

3.4. TBH, insulin, leptin and INF-γ affect GLUT1-mediated cellular ^{3}H-DG uptake

We next decided to investigate if the T2DM-associated characteristics increase ^{3}H-DG cellular uptake by interfering with GLUT1. For this, we examined the influence of BAY-876 (500 nM) on the stimulatory effect of TBH (2.5 μM), insulin (50 nM), leptin (500 ng/ml) and INF-γ (100 ng/ml) upon the uptake of a low (10 nM) and high (1 mM) ^{3}H-DG concentration (Fig. 7).

Interestingly enough, the stimulatory effect of insulin, leptin and INF-γ on ^{3}H-DG (1 mM) uptake by the two cancer cell lines was abolished in the presence of BAY-876 (Fig. 7). This observation supports the conclusion that insulin, leptin and INF-γ interfere with GLUT1-mediated ^{3}H-DG uptake by breast cancer cell lines. In support of this conclusion, leptin appears to increase *GLUT1* mRNA levels in both MCF-7 and MDA-MB-231 cells (Fig. 8). As to insulin and INF-γ, they do not appear to increase GLUT1 transcription levels (Fig. 8). However, the observation that the stimulatory effect of insulin, leptin and INF-γ on ^{3}H-DG (10 nM) uptake did not disappear in the presence of BAY-876 suggests that these compounds probably affect not only GLUT1, but also a high-affinity glucose transporter which however, is not relevant in *in vivo* conditions.

In relation to TBH, the results obtained support the conclusion that it stimulates GLUT1 mediated ^{3}H-DG uptake in MDA-MB-231 and MCF-12A cell lines, because its stimulatory effect on ^{3}H-DG uptake was supressed in the presence of BAY-876 (Fig. 7). This effect of TBH is associated with an increase in *GLUT1* mRNA levels in MDA-MB-231 cells (Fig. 8). In contrast, in MCF-7 cells, the results obtained with BAY-876 indicate that TBH stimulates non-GLUT1-mediated ^{3}H-DG uptake (Fig. 7), although TBH was found to increase *GLUT1* mRNA levels (Fig. 8).

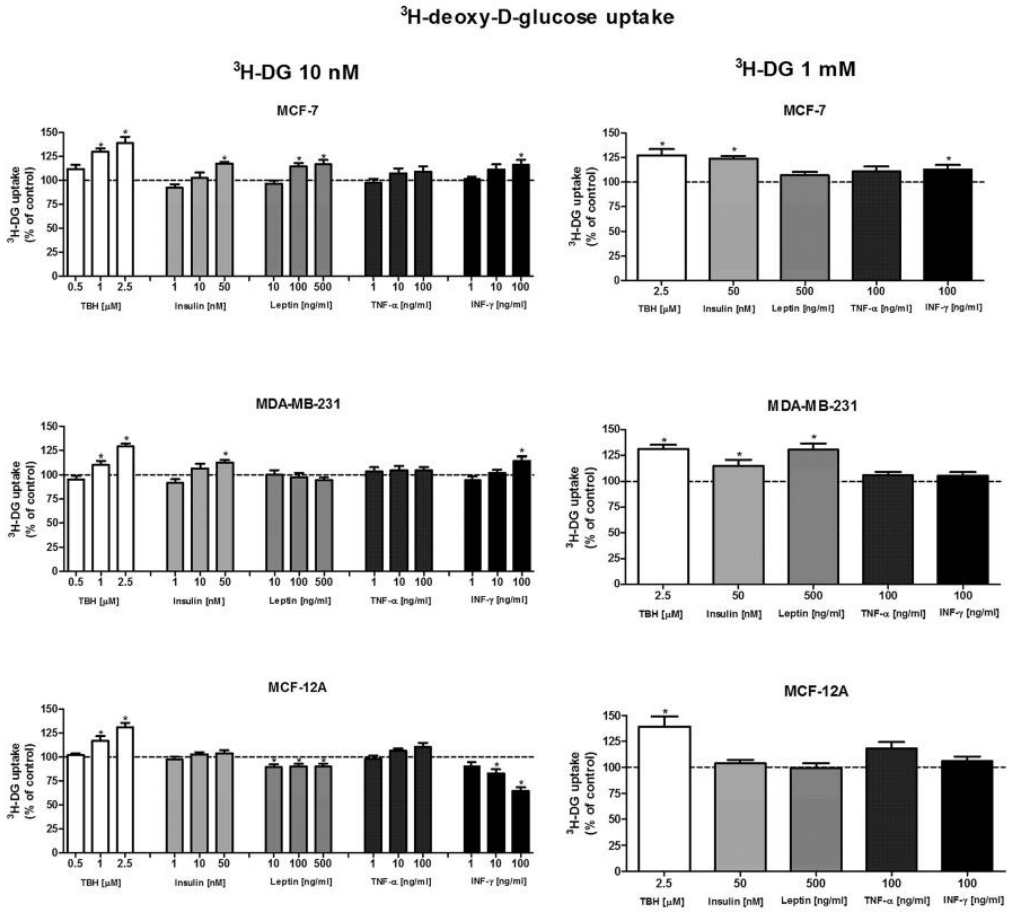

Fig. 4. Effects of T2DM-associated characteristics on the cellular uptake of a low and a high concentration of ^{3}H-DG by breast cancer and non-tumoral cell lines.

Effects of TBH (0.5–2.5 μM), insulin (1–50 nM), leptin (10–500 ng/ml), TNF-α (1–100 ng/ml) or INF-γ (1–100 ng/ml) (24h) upon uptake of ^{3}H-DG 10 nM and 1 mM (n = 8) by breast cancer (MCF-7 and MDA-MB-231) and non-tumoral (MCF-12A) cell lines (n = 8). Data show arithmetic means ± S.E.M. *P < 0.05 vs control, by Student's *t*-test.

3.5. The effect of TBH, insulin, leptin and INF-γ in cell proliferation and viability is dependent on interference with GLUT1-mediated glucose transport

Finally, the involvement of changes in GLUT1-mediated glucose uptake on the effect of TBH, insulin, leptin and INF-γ upon characteristics associated with cancer progression was evaluated. For this, the effect of BAY-876 upon the effect of these compounds on cell proliferation and viability was investigated (Fig. 9).

Alone, BAY-876 showed an antiproliferative effect in both cancer and non-cancer cell lines, being slightly more potent in the cancer cell lines. This effect was not associated with a decrease in cellular viability. On the contrary, the % of viable cells was increased in the presence of this GLUT1 inhibitor (Fig. 9).

In the presence of BAY-876, the effects of TBH, insulin, leptin and INF-γ upon cell proliferation and viability were completely abolished. This strongly suggests that interference with GLUT1-mediated glucose uptake is fundamental for the effect of these compounds upon proliferation and viability of breast cancer and non-cancer cell lines. The exception was the effect of leptin upon the proliferation of MDA-MB-231 cells (Fig. 9).

4. Discussion

The aim of this study was to investigate glucose cellular uptake as a molecular target linking T2DM-related factors (hyperglycemia, hyperinsulinemia, hyperleptinemia, increased levels of inflammation (TNF-α and INF-γ) or increased oxidative stress levels (Sanchez-Jimenez et al., 2019; Takatani-Nakase et al., 2014; Wei et al., 2017)) and breast cancer progression.

Firstly, we characterized the effect of T2DM-associated characteristics upon cellular features that contribute to breast cancer progression. Glucose caused only an increase in the migration capacity of MDA-MB-231 cells and an increase in proliferation of MCF-12A cells. So, glucose was excluded from the remaining experiments. Differently from our results, previous works reported that elevated glucose levels promote proliferation and migration (Hou et al., 2017; Takatani-Nakase et al., 2014; Wei et al., 2017) and inhibit apoptosis and necrosis (Baldari et al., 2017) of breast cancer cells, promoting tumour progression.

Insulin is a hormone well-known for its involvement in cell survival and proliferation, as well as for its mitogenic effect (Pollak, 2008), and it is firmly established that insulin receptors are frequently overexpressed in breast cancer cells (Rostoker et al., 2015; Wei et al., 2017). Our results show that insulin promotes proliferation, migration and VEGF-A

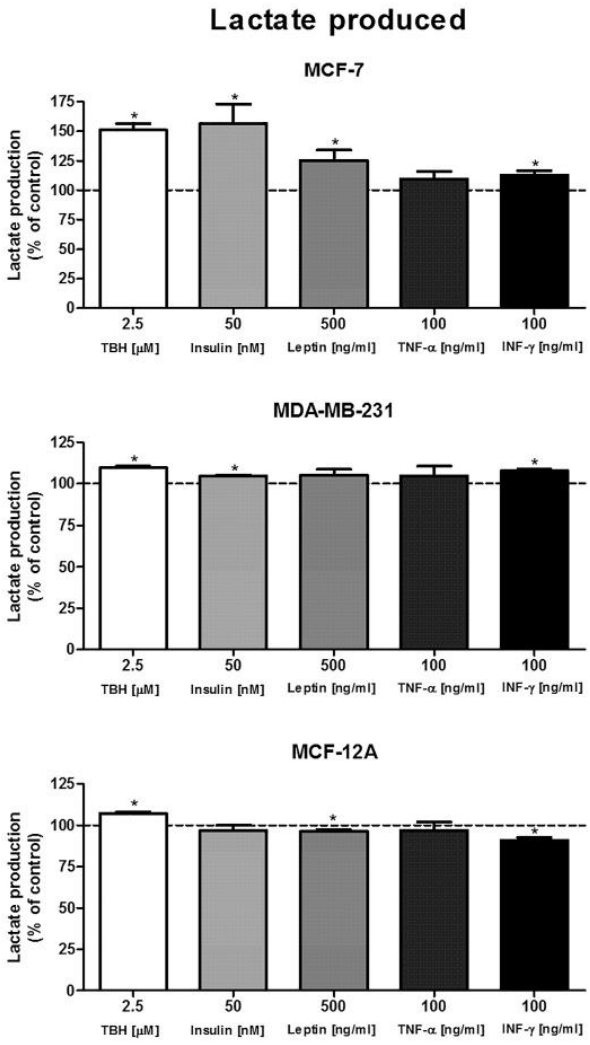

Fig. 5. Effects of T2DM-associated characteristics on lactate produced by breast cancer and non-tumoral cell lines.
Effects of TBH (5 µM), insulin (50 nM), leptin (500 ng/ml), TNF-α (100 ng/ml) or INF-γ (100 ng/ml) (24h) on lactate production (n = 4–6) by breast cancer (MCF-7 and MDA-MB-231) and non-tumoral (MCF-12A) cell lines (n = 4–6). Data show arithmetic means ± S.E.M. *P < 0.05 vs control, by Student's *t*-test.

production in both ER-positive and triple-negative breast cancer cell lines and additionally promotes apoptosis in the ER-positive breast cancer cell line. The pro-proliferative, pro-invasive (Wei et al., 2017), pro-angiogenic (Rose and Vona-Davis, 2012) and pro-apoptotic (Agrawal et al., 2017) effects of insulin in ER-positive cell lines was already observed by others. As to ER-negative breast cancer cells, in contrast to a previous work (Weichhaus et al., 2012), our results support the observation that insulin levels are epidemiologically linked to an increased risk of ER-negative breast cancer (Hirose et al., 2003).

Leptin stimulated proliferation, migration and the viability of both ER-positive and triple-negative breast cancer cell lines; moreover, it increased VEGF-A levels in the triple-negative cell line and promoted apoptosis in MCF-7 cells. In obese individuals, plasma leptin levels are risk factors for breast cancer (Okumura et al., 2002). The function of leptin appears to be complex, participating not only in the anorexigenic pathway, but also having effects in immune response, angiogenesis, and

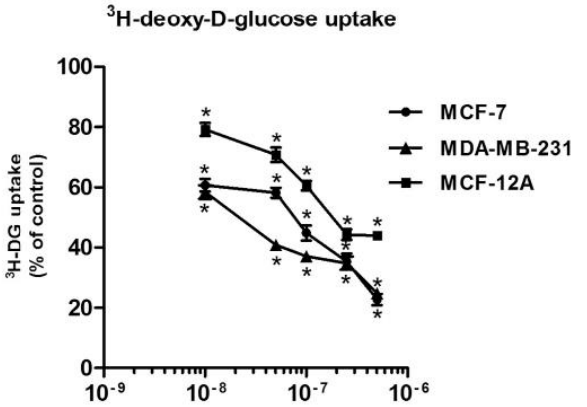

Fig. 6. Effect of BAY-876 on ^{3}H-DG uptake by breast cancer and non-tumoral cell lines.
Effect of BAY-876 (10–500 nM) for 24h on ^{3}H-DG 10 nM uptake by breast cancer (MCF-7 and MDA-MB-231) and non-tumoral (MCF-12A) cell lines (n = 8). Shown is arithmetic means ± S.E.M. *P < 0.05 vs control, by Student's *t*-test.

the proliferation of many different cell types, including breast tissue cells (Ando et al., 2019; Sanchez-Jimenez et al., 2019). So, leptin, like insulin, appears to be a growth factor for breast cancer cells (Sanchez-Jimenez et al., 2019). Indeed, a few studies showed that leptin stimulates breast cancer cell proliferation (Nkhata et al., 2009), by inducing alterations in cell cycle progression (Okumura et al., 2002), or by interfering with PI3K/AKT and MAPK signaling pathways (Nkhata et al., 2009; Sanchez-Jimenez et al., 2019), and promotes breast tumour angiogenesis through VEGF signaling (Gonzalez et al., 2006). In relation to apoptosis, our results agree with previous reports showing variable action of this hormone in breast cancer cell lines; either an anti-apoptotic effect (involving Bax or Bcl-2 proteins) (Nkhata et al., 2009; Perera et al., 2008), and a pro-apoptotic effect (caspase-mediated) were described (Naviglio et al., 2009).

In contrast, no effect on proliferation, migration and angiogenesis and decreased viability was found with both hormones, and additionally leptin was proapoptotic, in non-tumoral MCF-12A cells.

With respect to inflammatory cytokines, TNF-α presented an anti-proliferative and cytotoxic effect in all cell lines; in addition, this compound has a pro-migratory effect in cancer cell lines and an anti-migratory effect in non-cancer cell line, a pro-apoptotic effect in MCF-7 cell line and a proangiogenic effect in MDA-MB-231 cells and an anti-angiogenic effect in MCF-7 cells. As to INF-γ, this cytokine presented anti-proliferative effects in all cell lines, and increased viability, apoptosis and reduced migration and VEGF-A levels in MCF-7 cells, while a proangiogenic effect only was found in MDA-MB-231 cells, and, in MCF-12A cells, it had no effect on cell viability and angiogenesis, but stimulated apoptosis and had an antimigratory effect. Chronic inflammation induces an increase in TNF-α and INF-γ levels, thus creating a microenvironment favourable to tumour development in T2DM individuals (Sateesh et al., 2019). Some reports concluded that these compounds enhance breast cancer cell survival and proliferation, promote angiogenesis and metastatic dissemination (Barnes et al., 2003; Crespi et al., 2016; Mantovani et al., 2008; Pileczki et al., 2012; Pothiwala et al., 2009). On the contrary, a report concluded that TNF-α and INF-γ inhibited the growth rate and decreased the number of breast cancer cells (Wahyu Widowati et al., 2016), which is in line with our results. In the present work, TNF-α and INF-γ stimulated apoptosis of the MCF-7 cell line, but had no effect on MDA-MB-231 apoptosis rates. These observations are in line with previous reports of a proapoptotic effect of IFN-γ (Barnes et al., 2003; Ning et al., 2010; Zhang et al., 2003) involving protein 21 (p21) stimulation (Garcia-Tunon et al., 2007) and possibly mediated by tumour necrosis factor receptor 1 (TNFRI)

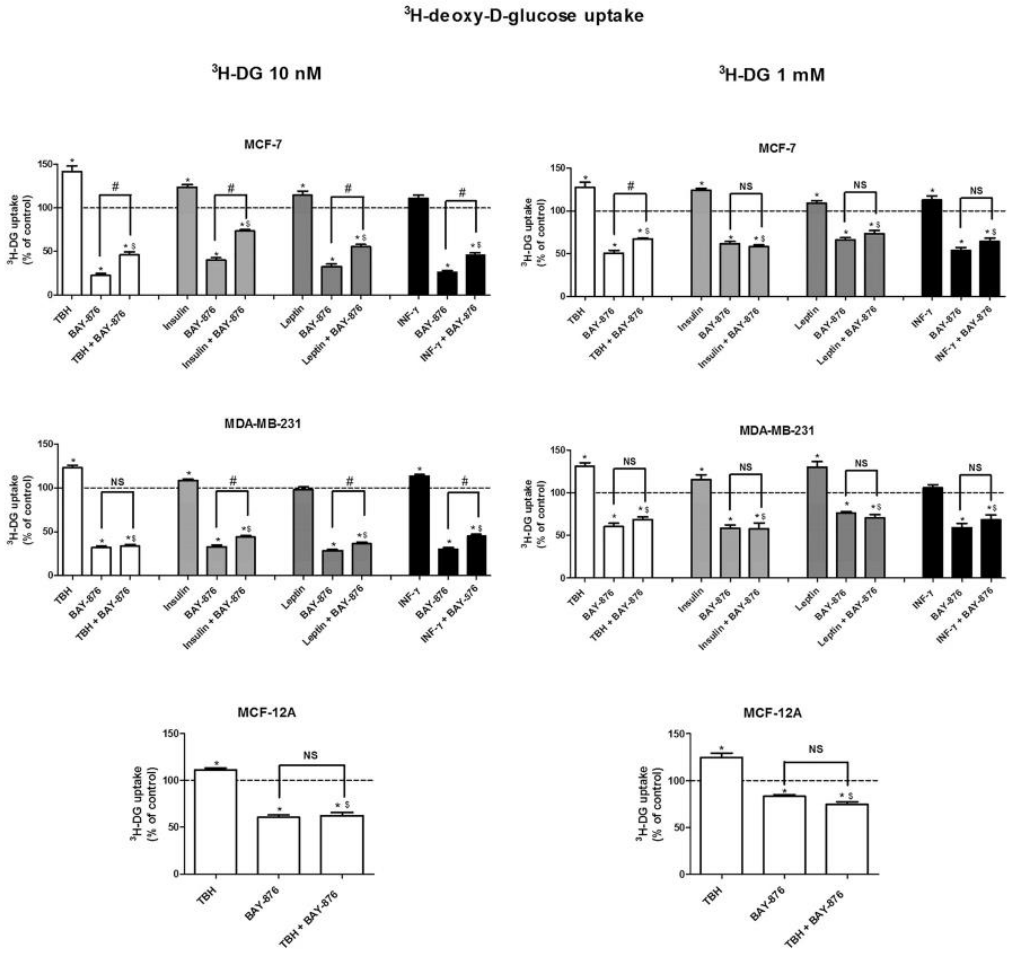

Fig. 7. The influence of BAY-876 on the stimulatory effect of T2DM-associated characteristics upon ^{3}H-DG uptake by breast cancer and non-tumoral cancer cell lines.

Effect of BAY-876 (500 μM) on the stimulatory effect of TBH (2.5 μM), insulin (50 nM), leptin (500 ng/ml) or INF-γ (100 ng/ml) (24h) upon the uptake of ^{3}H-DG 10 nM and 1 mM (n = 8) by breast cancer (MCF-7 and MDA-MB-231) and non-tumoral (MCF-12A) cell lines. Data show arithmetic means ± S.E.M. *P < 0.05 vs control; $ P < 0.05 vs TBH, insulin, leptin or INF-γ; # P < 0.05 vs BAY-876, by two way ANOVA with Newman-Keuls *post-hoc* test.

(Garcia-Tunon et al., 2007; Martinez-Reza et al., 2019). Although TNF-α is cytotoxic to certain tumour cell lines, it does not trigger apoptosis in normal cells (Battegay et al., 1995); this is in line with our results with MCF-12A cells showing no effect of TNF-α on apoptosis.

Finally, we showed that TBH presented anti-proliferative and anti-migratory effects in MCF-7 cells, and a pro-proliferative and pro-migratory in MDA-MB-231 cells, although it was cytotoxic, pro-apoptotic and pro-angiogenic in both cancer cell lines. In the non-cancer cell line, TBH was devoid of effect on proliferation, apoptosis and angiogenesis, but decreased cell and migration. Oxidative stress can be caused by several T2DM-associated mechanisms, including high levels of glucose, insulin, leptin, and chronic inflammation, overall contributing to a microenvironment favourable for the development and progression of T2DM-related cancers (Crespi et al., 2016). Moreover, it is known that tumour cells produce more reactive oxygen species than normal cells (Nourazarian et al., 2014). Reactive oxygen species have various biological effects and they have non-linear characteristics. In high oxidative stress environments, they may cause deleterious cellular effects (antiproliferative, pronecrotic and proapoptotic), while in low or medium oxidative stress environments, reactive oxygen species may induce DNA damage, inflammation and cell proliferation, eventually promoting carcinogenesis (Isnaini et al., 2018). So, the distinct effects of TBH in relation to the proliferation of the two breast cancer cells lines may be related to the inherent differences in oxidative stress/reactive oxygen species levels in MCF-7 (less aggressive and invasive potential) and MDA-MB-231 (more aggressive and invasive potential) cells. Namely, it is possible that, for the MCF-7 cell line, the concentration of TBH used is a very high dose, thus reducing proliferation, while in the more resistant cell line (MDA-MB-231), the concentration of TBH used is a low or medium dose, and so an increase in proliferation is observed. The increase in apoptosis caused by TBH in both breast cancer lines is probably mediated by the activation of the JNK and p53 (Hecht et al., 2016; Nourazarian et al., 2014; Sateesh et al., 2019). As to the pro-angiogenic effect of TBH, it agrees with the fact that many stimuli including hypoxia and oxidative stress can increase VEGF expression in cancer cells *in vitro* (Nourazarian et al., 2014).

As previously mentioned, GLUT1 is considered an oncogene (Barbosa and Martel, 2020; Martel et al., 2016) and GLUT1 is also the main glucose transporter in breast cancer cell lines (e.g., MCF-7 and MDA-MB-231) (Barbosa and Martel, 2020). Interestingly, intracellular

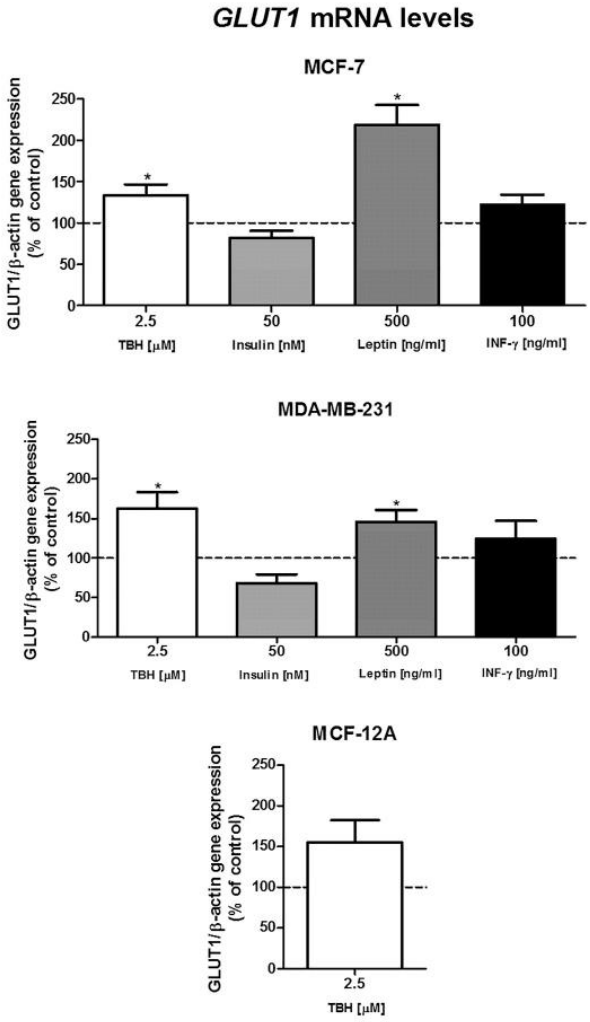

Fig. 8. Effects of T2DM-associated characteristics on *GLUT1* mRNA levels in breast non-tumoral and cancer cell lines.
Effects of TBH (2.5 μM), insulin (50 nM), leptin (500 ng/ml), TNF-α (100 ng/ml) or INF-γ (100 ng/ml) (24h) on *GLUT1* mRNA levels in breast cancer (MCF-7 and MDA-MB-231) and non-tumoral (MCF-12A) cell lines (n = 6). Data were normalized to the expression of β-actin. Data show arithmetic means ± S.E.M. *P < 0.05 vs control, by Student's *t*-test.

signaling pathways affected by T2DM-related markers (namely PI3K, AKT, mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK)) are known to cause changes in expression and/or activity of kinases and transcription factors that regulate GLUT1 expression, namely, HIF-1α, p53, and *c-myc* (Adekola et al., 2012; Barbosa and Martel, 2020; Martin and McGee, 2018). However, little is known about the effect of the metabolic changes found in T2DM on GLUT1. So, we decided to investigate the effect of T2DM-associated features in glucose (^{3}H-DG 10 nM and 1 mM) uptake and lactate production by the breast cancer and non-cancer cell lines.

TBH increased ^{3}H-DG uptake and lactate production in all cell lines. Insulin also increased ^{3}H-DG uptake and lactate production, but only in cancer cell lines, while leptin and INF-γ increased and decreased ^{3}H-DG uptake (and lactate production) in cancer and non-cancer cell lines, respectively. TNF-α showed no effect on ^{3}H-DG uptake and lactate production.

Importantly, in all cell lines, the effects of T2DM-associated characteristics on lactate production were consistent with their effects on ^{3}H-DG (10 nM) uptake. This shows that the effect on the uptake of a low ^{3}H-DG concentration (10 nM) is a very good indicator of glucose handling by the cells. Nevertheless, many of the effect of T2DM-associated markers were also observed at a ^{3}H-DG concentration (1 mM) comparable to the human physiological concentration (5 mM).

A recent study showed that BAY-876, a glycolysis-targeted anticancer agent, dramatically inhibited tumorigenicity of ovarian cancer cell line and xenografts, suppressing basal and stress-induced glycolysis in these cells (Ma et al., 2018). However, no information about BAY-876 has been described so far in breast cancer cell lines and so the applicability of this compound in breast cancer remains unknown (Siebeneicher et al., 2016). In the present work, we report for the first time the effect of the GLUT1 inhibitor BAY-876 on glucose uptake, proliferation and viability of breast cancer and non-cancer cell lines. By using BAY-876, we proved that ^{3}H-DG uptake by both cancer and non-cancer cell lines is mainly GLUT1-mediated and BAY-876-inhibited.

Finally, because an increase in glucose uptake is associated with a cancer phenotype (Coller, 2014; Oh et al., 2017), we tested the hypothesis that an increase in GLUT1-mediated uptake is involved in the cancer-promoting effect of T2DM-associated characteristics. For this, we first investigated if the stimulatory effects of T2DM-associated markers on ^{3}H-DG uptake (10 nM and 1 mM) is GLUT1-mediated, by testing their BAY-876-sensitivity and their effect on *GLUT1* mRNA levels. Then, we investigated if the effect of T2DM-associated markers on cancer-associated characteristics is GLUT1-dependent, by testing their BAY-876-sensitivity.

We conclude that insulin, leptin and INF-γ stimulate GLUT1-mediated uptake of ^{3}H-DG in both ER-positive and triple-negative breast cancer cell lines and that, while leptin increases GLUT1 transcription rates, insulin and INF-γ appear to interfere with GLUT1 at a posttranscriptional level. Moreover, we conclude that the effects of insulin, leptin and INF-γ upon breast cancer cell proliferation and viability are GLUT1-dependent, because they were abolished in the presence of the GLUT1 inhibitor. There was one exception, namely the effect of leptin on the viability of MDA-MB-231 cells. Nevertheless, the other results obtained with leptin (namely the observation that its stimulatory effect on ^{3}H-DG is GLUT1-mediated and that it increases *GLUT1* mRNA) led us to conclude that the effect of leptin on breast cancer-associated characteristics of the cell lines is also GLUT1-dependent.

Moreover, we also conclude that the effects TBH upon both ER-positive and triple-negative breast cancer cell proliferation and viability is GLUT1-dependent, because (1) TBH stimulated ^{3}H-DG uptake and *GLUT1* mRNA levels in both cell lines, (2) BAY-876 abolished its effect upon ^{3}H-DG uptake in MDA-MB-231 cells, and (3) BAY-876 abolished its effects upon breast cancer cell proliferation and viability, in both cell lines.

Although GLUT1 is the most expressed glucose transporter in breast cancer cells, various other members of the GLUT family were reported to be upregulated in breast cancer, namely GLUT3 (Kuo et al., 2019), GLUT4 (Garrido et al., 2015) and GLUT12 (Barbosa and Martel, 2020; Rogers et al., 2002). Our work, showing the involvement of GLUT1 in the effect of T2DM-associated markers on cancer progression-associated characteristics, shows the importance of GLUT1-mediated glucose uptake for cancer progression.

A point worth to discuss is the fact that insulin, leptin and INF-γ do not interfere with ^{3}H-DG uptake by non-cancer cells. So, their effect upon GLUT1 is cancer cell-specific. In contrast, TBH similarly interferes with ^{3}H-DG uptake by breast cancer and non-cancer cell lines. This suggests that hyperinsulinemia, hyperleptinemia and the proinflammatory environment found in T2DM favours breast cancer progression, while the increased oxidative stress levels associated with T2DM favours not only breast cancer progression but may also contribute to breast cancer initiation.

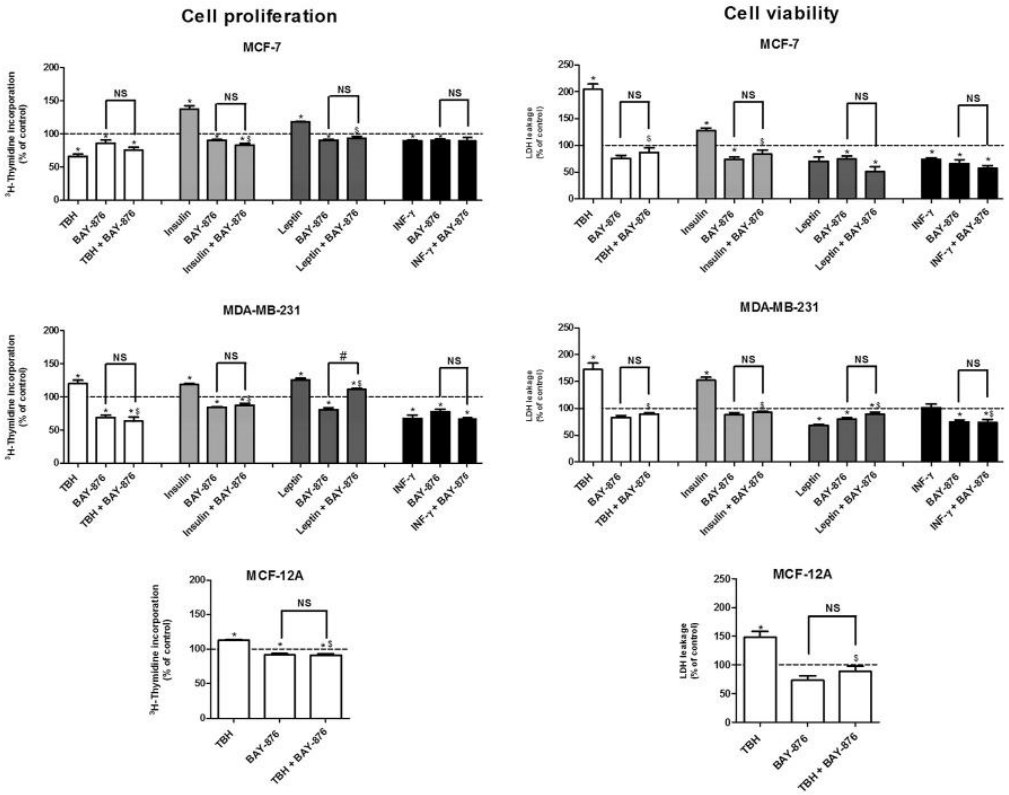

Fig. 9. The influence of BAY-876 on the effect of T2DM-associated characteristics upon the proliferation and viability of breast cancer and non-tumoral cancer cell lines.
Effect of BAY-876 (500 nM) on the effect of TBH (2.5 μM), insulin (50 nM), leptin (500 ng/ml) or INF-γ (100 ng/ml) (24h) on cell proliferation rates and viability (n = 6–8) of breast cancer (MCF-7 and MDA-MB-231) and non-tumoral (MCF-12A) cell lines. Data show arithmetic means ± S.E.M. *P < 0.05 vs control; $ P < 0.05 vs TBH, insulin, leptin or INF-γ; #P < 0.05 vs BAY-876, by two way ANOVA with Newman-Keuls post-hoc test.

5. Conclusions

We verified that TBH, insulin, leptin and INF-γ stimulate GLUT1-mediated uptake by both ER-positive and triple-negative breast cancer cells, and that their effects upon cell proliferation and viability are dependent on GLUT1 stimulation. Importantly, insulin and leptin (in both ER-positive and triple-negative breast cancer cell lines) and TBH (in the triple-negative cell line) have a proproliferative effect and leptin possesses a cytoprotective effect in both breast cancer cell lines that can contribute to cancer progression. Our results thus show that GLUT1 constitutes a molecular target for T2DM-associated characteristics and that an increase in GLUT1-mediated glucose transport can contribute to breast cancer progression in T2DM patients. Our results also indicate that the GLUT1 inhibitor BAY-876 is an effective inhibitor of proliferation and viability of both ER-positive and triple-negative breast cancer cells, thus constituting a promising therapeutic strategy for human breast cancer. Therefore, GLUT1 constitutes a mechanism by which T2DM participates in breast cancer progression, and a novel approach to treatment of breast cancer in T2DM patients. More investigation on this subject, namely involving evaluation of the efficacy of GLUT1 inhibition in breast cancer therapy in T2DM animal models, should be done.

CRediT authorship contribution statement

Cláudia Silva: Investigation, Writing – original draft. **Nelson Andrade:** Investigation. **João Tiago Guimarães:** Investigation. **Emília Patrício:** Investigation. **Fátima Martel:** Conceptualization, Formal analysis, Supervision, Visualization, Writing – review & editing, Project administration.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2021.173980.

References

Adekola, K., Rosen, S.T., Shanmugam, M., 2012. Glucose transporters in cancer metabolism. Curr. Opin. Oncol. 24, 650–654. https://doi.org/10.1097/CCO.0b013e328356da72.

Agrawal, S., Luc, M., Ziolkowski, P., Agrawal, A.K., Pielka, E., Walaszek, K., Zduniak, K., Wozniak, M., 2017. Insulin-induced enhancement of MCF-7 breast cancer cell

response to 5-fluorouracil and cyclophosphamide. Tumour Biol 39. https://doi.org/10.1177/1010428317702901, 1010428317702901.
Ando, S., Gelsomino, L., Panza, S., Giordano, C., Bonofiglio, D., Barone, I., Catalano, S., 2019. Obesity, leptin and breast cancer: epidemiological evidence and proposed mechanisms. Cancers 11, 62. https://doi.org/10.3390/cancers11010062.
Andrade, N., Silva, C., Martel, F., 2018. The effect of oxidative stress upon intestinal sugar transport: an in vitro study using human intestinal epithelial (Caco-2) cells. Toxicol. Res. 7, 1236–1246. https://doi.org/10.1039/c8tx00183a.
Araujo, J.R., Correia-Branco, A., Ramalho, C., Keating, E., Martel, F., 2013. Gestational diabetes mellitus decreases placental uptake of long-chain polyunsaturated fatty acids: involvement of long-chain acyl-CoA synthetase. J. Nutr. Biochem. 24, 1741–1750. https://doi.org/10.1016/j.jnutbio.2013.03.003.
Baldari, S., Garufi, A., Granato, M., Cuomo, L., Pistritto, G., Cirone, M., D'Orazi, G., 2017. Hyperglycemia triggers HIPK2 protein degradation. Oncotarget 8, 1190–1203. https://doi.org/10.18632/oncotarget.13595.
Barbosa, A.M., Martel, F., 2020. Targeting glucose transporters for breast cancer therapy: the effect of natural and synthetic sompounds. Cancers 12, 154. https://doi.org/10.3390/cancers12010154.
Barnes, B.J., Kellum, M.J., Pinder, K.E., Frisancho, J.A., Pitha, P.M., 2003. Interferon regulatory factor 5, a novel mediator of cell cycle arrest and cell death. Canc. Res. 63, 6424–6431.
Battegay, E.J., Raines, E.W., Colbert, T., Ross, R., 1995. TNF-alpha stimulation of fibroblast proliferation. Dependence on platelet-derived growth factor (PDGF) secretion and alteration of PDGF receptor expression. J. Immunol. 154, 6040–6047.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R.L., Torre, L.A., Jemal, A., 2018. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA A Cancer J. Clin. 68, 394–424. https://doi.org/10.3322/caac.21492.
Cha, Y.J., Kim, E.S., Koo, J.S., 2018. Amino acid transporters and glutamine metabolism in breast cancer. Int. J. Mol. Sci. 19, 907. https://doi.org/10.3390/ijms19030907.
Coller, H.A., 2014. Is cancer a metabolic disease? Am. J. Pathol. 184, 4–17. https://doi.org/10.1016/j.ajpath.2013.07.035.
Crespi, E., Bottai, G., Santarpia, L., 2016. Role of inflammation in obesity-related breast cancer. Curr. Opin. Pharmacol. 31, 114–122. https://doi.org/10.1016/j.coph.2016.11.004.
Doerstling, S.S., O'Flanagan, C.H., Hursting, S.D., 2017. Obesity and cancer metabolism: a perspective on interacting tumor-intrinsic and extrinsic factors. Front. Oncol. 7, 216. https://doi.org/10.3389/fonc.2017.00216.
Garcia-Tunon, I., Ricote, M., Ruiz, A.A., Fraile, B., Paniagua, R., Royuela, M., 2007. Influence of IFN-γ and its receptors in human breast cancer. BMC Canc. 7, 158. https://doi.org/10.1186/1471-2407-7-158.
Garrido, P., Osorio, F.G., Moran, J., Cabello, E., Alonso, A., Freije, J.M., Gonzalez, C., 2015. Loss of GLUT4 induces metabolic reprogramming and impairs viability of breast cancer cells. J. Cell. Physiol. 230, 191–198. https://doi.org/10.1002/jcp.24698.
Gonzalez, R.R., Cherfils, S., Escobar, M., Yoo, J.H., Carino, C., Styer, A.K., Sullivan, B.T., Sakamoto, H., Olawaiye, A., Serikawa, T., Lynch, M.P., Rueda, B.R., 2006. Leptin signaling promotes the growth of mammary tumors and increases the expression of vascular endothelial growth factor (VEGF) and its receptor type two (VEGF-R2). J. Biol. Chem. 281, 26320–26328. https://doi.org/10.1074/jbc.M601991200.
Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. Cell 144, 646–674. https://doi.org/10.1016/j.cell.2011.02.013.
Hecht, F., Pessoa, C.F., Gentile, L.B., Rosenthal, D., Carvalho, D.P., Fortunato, R.S., 2016. The role of oxidative stress on breast cancer development and therapy. Tumour Biol 37, 4281–4291. https://doi.org/10.1007/s13277-016-4873-9.
Hirose, K., Toyama, T., Iwata, H., Takezaki, T., Hamajima, N., Tajima, K., 2003. Insulin, insulin-like growth factor-I and breast cancer risk in Japanese women. Asian Pac. J. Cancer Prev. APJCP 4, 239–246.
Hou, Y., Zhou, M., Xie, J., Chao, P., Feng, Q., Wu, J., 2017. High glucose levels promote the proliferation of breast cancer cells through GTPases. Breast Cancer 9, 429–436. https://doi.org/10.2147/BCTT.S135665.
Isnaini, I., Permatasari, N., Mintaroem, K., Prihardina, B., Widodo, M.A., 2018. Oxidants-antioxidants profile in the breast cancer cell line MCF-7. Asian Pac. J. Cancer Prev. APJCP 19, 3175–3178. https://doi.org/10.31557/APJCP.2018.19.11.3175.
Krzeslak, A., Wojcik-Krowiranda, K., Forma, E., Jozwiak, P., Romanowicz, H., Bienkiewicz, A., Brys, M., 2012. Expression of GLUT1 and GLUT3 glucose transporters in endometrial and breast cancers. Pathol. Oncol. Res. 18, 721–728. https://doi.org/10.1007/s12253-012-9500-5.
Kuo, M.H., Chang, W.W., Yeh, B.W., Chu, Y.S., Lee, Y.C., Lee, H.T., 2019. Glucose transporter 3 is essential for the survival of breast cancer cells in the brain. Cells 8, 1568. https://doi.org/10.3390/cells8121568.
Lee, K., Kruper, L., Dieli-Conwright, C.M., Mortimer, J.E., 2019. The impact of obesity on breast cancer diagnosis and treatment. Curr. Oncol. Rep. 21, 41. https://doi.org/10.1007/s11912-019-0787-1.
Ma, Y., Wang, W., Idowu, M.O., Oh, U., Wang, X.Y., Temkin, S.M., Fang, X., 2018. Ovarian cancer relies on glucose transporter 1 to fuel glycolysis and growth: anti-tumor activity of BAY-876. Cancers 11, 33. https://doi.org/10.3390/cancers11010033.
Mantovani, A., Allavena, P., Sica, A., Balkwill, F., 2008. Cancer-related inflammation. Nature 454, 436–444. https://doi.org/10.1038/nature07205.
Martel, F., Guedes, M., Keating, E., 2016. Effect of polyphenols on glucose and lactate transport by breast cancer cells. Breast Canc. Res. Treat. 157, 1–11. https://doi.org/10.1007/s10549-016-3794-z.
Martin, S.D., McGee, S.L., 2018. Metabolic reprogramming in type 2 diabetes and the development of breast cancer. J. Endocrinol. 237, R35–R46. https://doi.org/10.1530/JOE-18-0037.
Martinez-Reza, I., Diaz, L., Barrera, D., Segovia-Mendoza, M., Pedraza-Sanchez, S., Soca-Chafre, G., Larrea, F., Garcia-Becerra, R., 2019. Calcitriol inhibits the proliferation of triple-negative breast cancer cells through a mechanism involving the proinflammatory cytokines IL-1beta and TNF-alpha. J. Immunol. Res. 2019, 6384278. https://doi.org/10.1155/2019/6384278.
Micucci, C., Valli, D., Matacchione, G., Catalano, A., 2016. Current perspectives between metabolic syndrome and cancer. Oncotarget 7, 38959–38972. https://doi.org/10.18632/oncotarget.8341.
Naviglio, S., Di Gesto, D., Romano, M., Sorrentino, A., Illiano, F., Sorvillo, L., Abbruzzese, A., Marra, M., Caraglia, M., Chiosi, E., Spina, A., Illiano, G., 2009. Leptin enhances growth inhibition by cAMP elevating agents through apoptosis of MDA-MB-231 breast cancer cells. Canc. Biol. Ther. 8, 1183–1190. https://doi.org/10.4161/cbt.8.12.8562.
Ning, Y., Riggins, R.B., Mulla, J.E., Chung, H., Zwart, A., Clarke, R., 2010. IFNgamma restores breast cancer sensitivity to fulvestrant by regulating STAT1, IFN regulatory factor 1, NF-κB, BCL2 family members, and signaling to caspase-dependent apoptosis. Mol. Canc. Therapeut. 9, 1274–1285. https://doi.org/10.1158/1535-7163.MCT-09-1169.
Nkhata, K.J., Ray, A., Schuster, T.F., Grossmann, M.E., Cleary, M.P., 2009. Effects of adiponectin and leptin co-treatment on human breast cancer cell growth. Oncol. Rep. 21, 1611–1619. https://doi.org/10.3892/or_00000395.
Nourazarian, A.R., Kangari, P., Salmaninejad, A., 2014. Roles of oxidative stress in the development and progression of breast cancer. Asian Pac. J. Cancer Prev. APJCP 15, 4745–4751. https://doi.org/10.7314/apjcp.2014.15.12.4745.
Oh, S., Kim, H., Nam, K., Shin, I., 2017. Glut1 promotes cell proliferation, migration and invasion by regulating epidermal growth factor receptor and integrin signaling in triple-negative breast cancer cells. BMB Rep 50, 132–137. https://doi.org/10.5483/bmbrep.2017.50.3.189.
Okumura, M., Yamamoto, M., Sakuma, H., Kojima, T., Maruyama, T., Jamali, M., Cooper, D.R., Yasuda, K., 2002. Leptin and high glucose stimulate cell proliferation in MCF-7 human breast cancer cells: reciprocal involvement of PKC-alpha and PPAR expression. Biochim. Biophys. Acta 1592, 107–116. https://doi.org/10.1016/s0167-4889(02)00276-8.
Park, J., Morley, T.S., Kim, M., Clegg, D.J., Scherer, P.E., 2014. Obesity and cancer–mechanisms underlying tumour progression and recurrence. Nat. Rev. Endocrinol. 10, 455–465. https://doi.org/10.1038/nrendo.2014.94.
Pearson-Stuttard, J., Zhou, B., Kontis, V., Bentham, J., Gunter, M.J., Ezzati, M., 2018. Worldwide burden of cancer attributable to diabetes and high body-mass index: a comparative risk assessment. Lancet Diabet. Endocrinol 6, 95–104. https://doi.org/10.1016/S2213-8587(17)30366-2.
Perera, C.N., Chin, H.G., Duru, N., Camarillo, I.G., 2008. Leptin-regulated gene expression in MCF-7 breast cancer cells: mechanistic insights into leptin-regulated mammary tumor growth and progression. J. Endocrinol. 199, 221–233. https://doi.org/10.1677/JOE-08-0215.
Pileczki, V., Braicu, C., Gherman, C.D., Berindan-Neagoe, I., 2012. TNF-alpha gene knockout in triple negative breast cancer cell line induces apoptosis. Int. J. Mol. Sci. 14, 411–420. https://doi.org/10.3390/ijms14010411.
Pollak, M., 2008. Insulin and insulin-like growth factor signalling in neoplasia. Nat. Rev. Canc. 8, 915–928. https://doi.org/10.1038/nrc2536.
Pothiwala, P., Jain, S.K., Yaturu, S., 2009. Metabolic syndrome and cancer. Metab. Syndr. Relat. Disord. 7, 279–288. https://doi.org/10.1089/met.2008.0065.
Rogers, S., Macheda, M.L., Docherty, S.E., Carty, M.D., Henderson, M.A., Soeller, W.C., Gibbs, E.M., James, D.E., Best, J.D., 2002. Identification of a novel glucose transporter-like protein-GLUT-12. Am. J. Physiol. Endocrinol. Metab. 282, E733–E738. https://doi.org/10.1152/ajpendo.2002.282.3.E733.
Rose, D.P., Vona-Davis, L., 2012. The cellular and molecular mechanisms by which insulin influences breast cancer risk and progression. Endocr. Relat. Canc. 19, R225–R241. https://doi.org/10.1530/ERC-12-0203.
Rostoker, R., Abelson, S., Bitton-Worms, K., Genkin, I., Ben-Shmuel, S., Dakwar, M., Orr, Z.S., Caspi, A., Tzukerman, M., LeRoith, D., 2015. Highly specific role of the insulin receptor in breast cancer progression. Endocr. Relat. Canc. 22, 145–157. https://doi.org/10.1530/ERC-14-0490.
Sanchez-Jimenez, F., Perez-Perez, A., de la Cruz-Merino, L., Sanchez-Margalet, V., 2019. Obesity and breast cancer: role of leptin. Front. Oncol. 9, 596. https://doi.org/10.3389/fonc.2019.00596.
Sateesh, R., Rao Bitla, A.R., Budugu, S.R., Mutheeswariah, Y., Narendra, H., Phaneedra, B.V., Lakshmi, A.Y., 2019. Oxidative stress in relation to obesity in breast cancer. Indian J. Canc. 56, 41–44. https://doi.org/10.4103/ijc.IJC_247_18.
Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C (T) method. Nat. Protoc. 3, 1101–1108. https://doi.org/10.1038/nprot.2008.73.
Siebeneicher, H., Cleve, A., Rehwinkel, H., Neuhaus, R., Heisler, I., Muller, T., Bauser, M., Buchmann, B., 2016. Identification and optimization of the first highly selective GLUT1 inhibitor BAY-876. ChemMedChem 11, 2261–2271. https://doi.org/10.1002/cmdc.201600276.
Silva, C., Correia-Branco, A., Andrade, N., Ferreira, A.C., Soares, M.L., Sonveaux, P., Stephenne, J., Martel, F., 2019. Selective pro-apoptotic and antimigratory effects of polyphenol complex catechin:lysine 1:2 in breast, pancreatic and colorectal cancer cell lines. Eur. J. Pharmacol. 859, 172533. https://doi.org/10.1016/j.ejphar.2019.172533.
Silva, C., Nunes, C., Correia-Branco, A., Araujo, J.R., Martel, F., 2017. Insulin exhibits an antiproliferative and hypertrophic effect in first trimester human extravillous trophoblasts. Reprod. Sci. 24, 582–594. https://doi.org/10.1177/1933719116667220.

Szablewski, L., 2013. Expression of glucose transporters in cancers. Biochim. Biophys. Acta 1835, 164–169. https://doi.org/10.1016/j.bbcan.2012.12.004.

Takatani-Nakase, T., Matsui, C., Maeda, S., Kawahara, S., Takahashi, K., 2014. High glucose level promotes migration behavior of breast cancer cells through zinc and its transporters. PloS One 9, e90136. https://doi.org/10.1371/journal.pone.0090136.

Thibault, R., De Coppet, P., Daly, K., Bourreille, A., Cuff, M., Bonnet, C., Mosnier, J.F., Galmiche, J.P., Shirazi-Beechey, S., Segain, J.P., 2007. Down-regulation of the monocarboxylate transporter 1 is involved in butyrate deficiency during intestinal inflammation. Gastroenterology 133, 1916–1927. https://doi.org/10.1053/j.gastro.2007.08.041.

Vander Heiden, M.G., 2011. Targeting cancer metabolism: a therapeutic window opens. Nat. Rev. Drug Discov. 10, 671–684. https://doi.org/10.1038/nrd3504.

Widowati, Wahyu, Murti, Harry, Jasaputra, Diana Krisanti, Sumitro, Sutiman B., Aris Widodo, M., Fauziah, Nurul, Bachtiar, M.M.I., 2016. Selective cytotoxic potential of IFN-γ and TNF-α on breast cancer cell lines (T47D and MCF7). Asian J. Cell Biol. 11, 12. https://doi.org/10.3923/ajcb.2016.1.12.

Wei, M.L., Duan, P., Wang, Z.M., Ding, M., Tu, P., 2017. High glucose and high insulin conditions promote MCF7 cell proliferation and invasion by upregulating IRS1 and activating the Ras/Raf/ERK pathway. Mol. Med. Rep. 16, 6690–6696. https://doi.org/10.3892/mmr.2017.7420.

Weichhaus, M., Broom, J., Wahle, K., Bermano, G., 2012. A novel role for insulin resistance in the connection between obesity and postmenopausal breast cancer. Int. J. Oncol. 41, 745–752. https://doi.org/10.3892/ijo.2012.1480.

Widschwendter, P., Friedl, T.W., Schwentner, L., DeGregorio, N., Jaeger, B., Schramm, A., Bekes, I., Deniz, M., Lato, K., Weissenbacher, T., Kost, B., Andergassen, U., Jueckstock, J., Neugebauer, J., Trapp, E., Fasching, P.A., Beckmann, M.W., Schneeweiss, A., Schrader, I., Rack, B., Janni, W., Scholz, C., 2015. The influence of obesity on survival in early, high-risk breast cancer: results from the randomized SUCCESS A trial. Breast Cancer Res. 17, 129. https://doi.org/10.1186/s13058-015-0639-3.

Wolczyk, D., Zaremba-Czogalla, M., Hryniewicz-Jankowska, A., Tabola, R., Grabowski, K., Sikorski, A.F., Augoff, K., 2016. TNF-α promotes breast cancer cell migration and enhances the concentration of membrane-associated proteases in lipid rafts. Cell. Oncol. 39, 353–363. https://doi.org/10.1007/s13402-016-0280-x.

Zhang, M., Guo, R., Zhai, Y., Yang, D., 2003. LIGHT sensitizes IFNγ-mediated apoptosis of MDA-MB-231 breast cancer cells leading to down-regulation of anti-apoptosis Bcl-2 family members. Canc. Lett. 195, 201–210. https://doi.org/10.1016/s0304-3835(03)00148-4.

SUPPLEMENTARY DATA

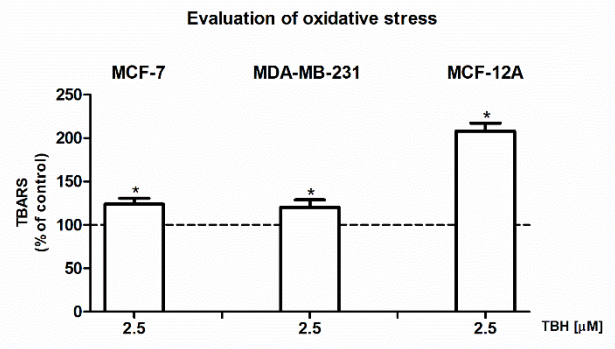


Fig. S1. Effects of TBH on lipid peroxidation levels in breast cancer and non-tumoral cell lines.

The effect of TBH (2.5 µM) for 24 h on lipid peroxidation levels (malondialdehyde) in breast cancer (MCF-7 and MDA-MB-231) and non-tumoral (MCF-12A) cell lines (n=8). Data show arithmetic means ± S.E.M. *P < 0.05 vs control, by Student's *t* test.

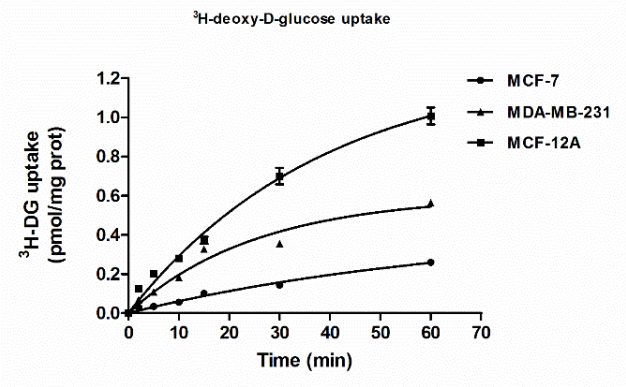
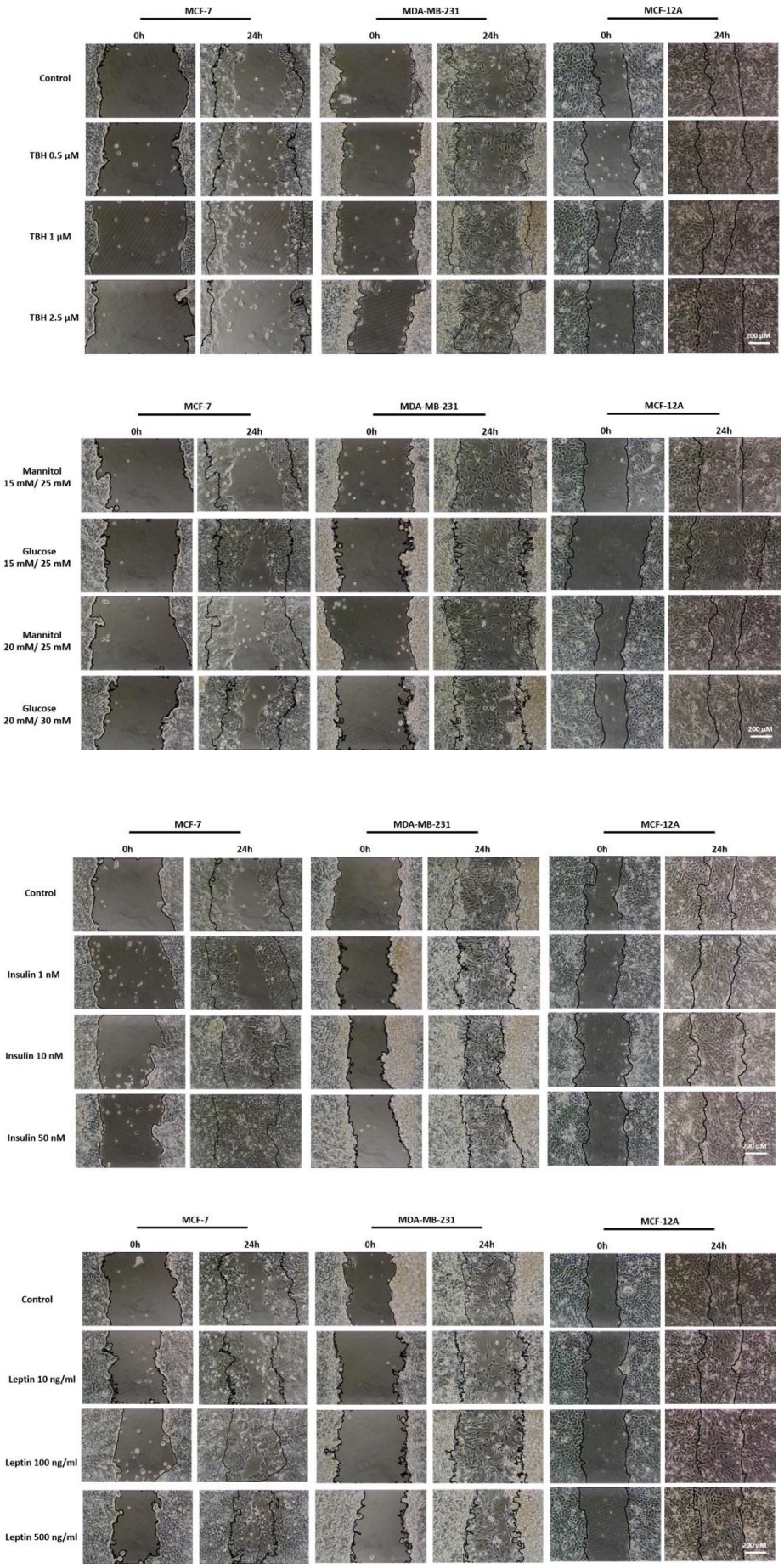


Fig. S2. Time-course of ³H-DG uptake in breast cancer and non-tumoral cell lines.

Time-course of ³H-DG uptake in breast cancer (MCF-7 and MDA-MB-231) and non-tumoral (MCF-12A) cell lines. Cells were incubated for various periods of time at 37°C with 10 nM ³H-DG (n=8). Shown is arithmetic means ± S.E.M.


MCF-7
MDA-MB-231
MCF-12A
0h
24h
Control
TBH 0.5 µM
TBH 1 µM
TBH 2.5 µM
200 µM
Mannitol 15 mM/ 25 mM
Glucose 15 mM/ 25 mM
Mannitol 20 mM/ 25 mM
Glucose 20 mM/ 30 mM
Insulin 1 nM
Insulin 10 nM
Insulin 50 nM
Leptin 10 ng/ml
Leptin 100 ng/ml
Leptin 500 ng/ml

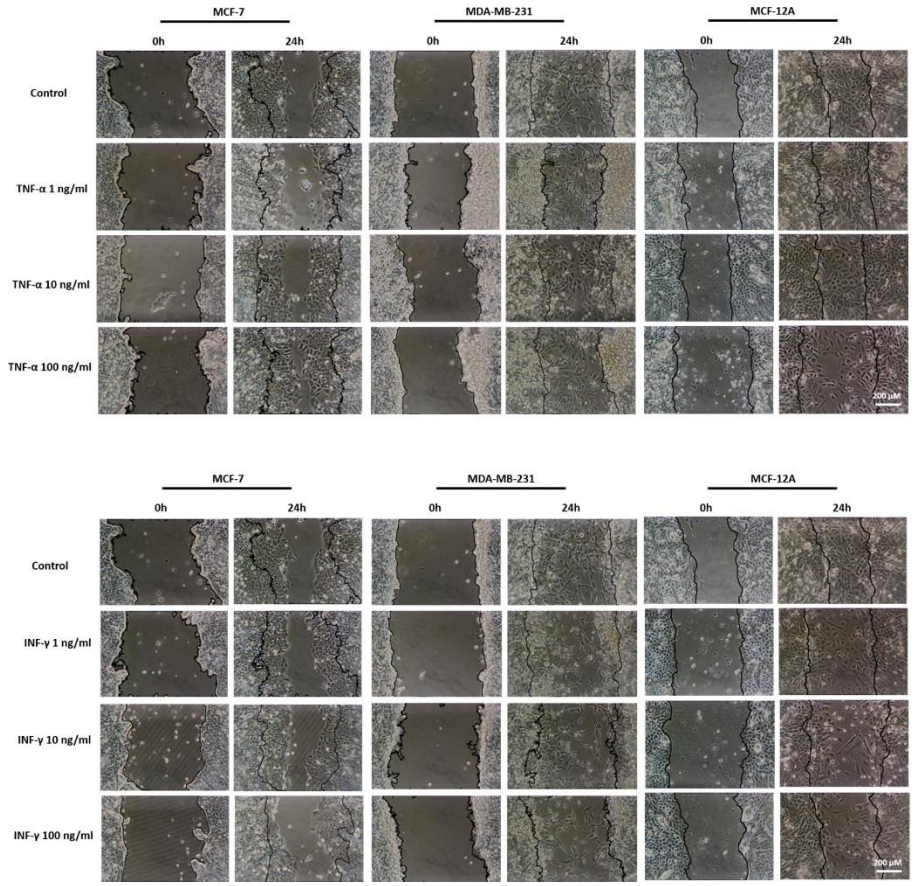


Fig. S3. Obesity/T2DM-associated characteristics affect migration of breast cancer and non-tumoral cell lines. Representative images of cell migration determined by the injury assay. Cell monolayers of breast non-tumoral (MCF-12A) and cancer (MCF-7 and MDA-MB-231) cell lines were scratched with a pipette tip and then treated for 24h with increasing concentrations of TBH (0.5-2.5 μM), glucose (15-30 mM), insulin (1-50 nM), leptin (10-500 ng/ml), TNF-α (1-100 ng/ml) or INF-γ (1-100 ng/ml) (n=4-6). Images were obtained at 0h and 24h after scratching. Scale bar: 200 μM.

CHAPTER IV

THE EFFECT OF TYPE 2 DIABETES MELLITUS-ASSOCIATED CHARACTERISTICS UPON GLUTAMINE CELLULAR UPTAKE DURING BREAST CANCER PROGRESSION

THE PRO-PROLIFERATIVE EFFECT OF INTERFERON-γ IN BREAST CANCER CELL LINES IS DEPENDENT STIMULATION OF ASCT2-MEDIATED GLUTAMINE CELLULAR UPTAKE

Cláudia Silva, Nelson Andrade, Ilda Rodrigues, António Carlos Ferreira, Miguel Luz Soares, Fátima Martel.

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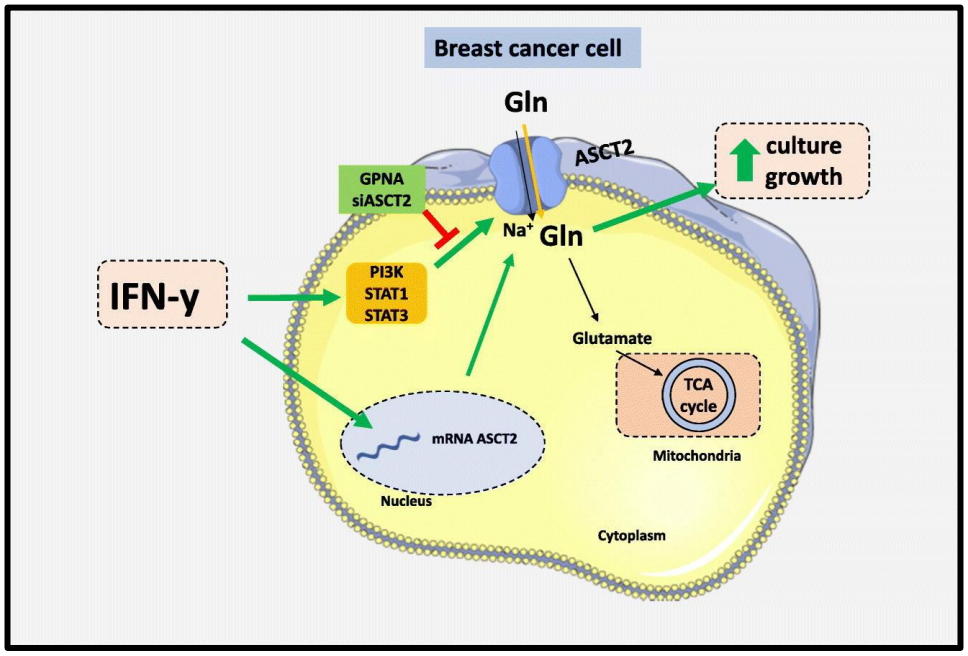
Graphical Abstract



Highlights

- Insulin increases ^{3}H-GLN uptake in a breast non-tumorigenic cell line
- IFN-γ increases ^{3}H-GLN uptake by breast cancer cell lines
- IFN-γ induced increase in ^{3}H-GLN uptake is GPNA- and ASCT2 knockdown-sensitive
- IFN-γ induced increase in ^{3}H-GLN uptake is mediated by PI3K-, STAT3- and STAT1
- The pro-proliferative effect of IFN-γ is dependent on an increase in ^{3}H-GLN uptake

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The pro-proliferative effect of interferon-γ in breast cancer cell lines is dependent on stimulation of ASCT2-mediated glutamine cellular uptake

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ABSTRACT

Aims: Type 2 diabetes mellitus (T2DM) is a risk factor for breast cancer initiation and progression. Glutamine (GLN) is a critical nutrient for cancer cells. The aim of this study was to investigate the effect of T2DM-associated compounds upon GLN uptake by breast cancer cells.
Main methods: The in vitro uptake of ^{3}H-GLN by breast cancer (MCF-7 and MDA-MB-231) and non-tumorigenic (MCF-12A) cell lines was measured.
Key findings: ^{3}H-GLN uptake in the three cell lines is mainly Na^{+}-dependent and sensitive to the ASCT2 inhibitor GPNA. IFN-γ increased total and Na^{+}-dependent ^{3}H-GLN uptake in the two breast cancer cell lines, and insulin increased total and Na^{+}-dependent ^{3}H-GLN uptake in the non-tumorigenic cell line. GPNA abolished the increase in ^{3}H-GLN uptake promoted by these T2DM-associated compounds. ASCT2 knockdown confirmed that the increase in ^{3}H-GLN uptake caused by IFN-γ (in breast cancer cells) and by insulin (in non-tumorigenic cells) is ASCT2-dependent. IFN-γ (in MDA-MB-231 cells) and insulin (in MCF-12A cells) increased ASCT2 transcript and protein levels. Importantly, the pro-proliferative effect of IFN-γ in breast cancer cell lines was associated with an increase in ^{3}H-GLN uptake which was GPNA-sensitive, blocked by ASCT2 knockdown and mediated by activation of the PI3K-, STAT3- and STAT1 intracellular signalling pathways.
Significance: IFN-γ and insulin possess pro-proliferative effects in breast cancer and non-cancer cell lines, respectively, which are dependent on an increase in ASCT2-mediated glutamine transport. Thus, an effective inhibition of ASCT2-mediated glutamine uptake may be a therapeutic strategy against human breast cancer in T2DM patients.

1. Introduction

Breast cancer is the most frequent cancer among women worldwide [1]. Two risk factors for breast cancer, frequently occurring simultaneously, are high body mass index and type 2 diabetes mellitus (T2DM) [2]. Obesity/T2DM at cancer diagnosis is also associated with a worst response to therapy and a poor prognosis, with an increased risk of local or distant recurrence and cancer-related death [3–5]. Multiple T2DM-associated factors are known to contribute to cancer initiation, progression and/or poor response to therapy, by directly or indirectly interfering with cell proliferation, migration, angiogenesis, inflammation, invasion, and apoptosis [4,6]. These include insulin resistance (hyperinsulinemia and hyperglycemia), altered adipokine levels (higher leptin and lower adiponectin), chronic low-grade inflammation (higher interleukin-6 (IL-6), tumor necrosis factor α (TNF-α) and interferon-γ (IFN-γ)) levels and increased oxidative stress levels [4,7,8]. Because the risk for breast cancer is higher and the efficacy of treatment is significantly lower in T2DM breast cancer patients, thus resulting in higher death ratios [9], a better understanding of the mechanisms involved in the interaction between T2DM and breast cancer is urgently needed.

Metabolic reprogramming, which was recently recognized as a hallmark of cancer cells, has attracted significant interest both from the perspective of understanding tumorigenesis and as a potential therapeutic target [10]. A century ago, Otto Warburg proposed what is now

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called the Warburg effect [11], which is characterized by increased glycolysis rates and lactate production by cancer cells regardless of oxygen availability [12,13]. Alongside with changes in glucose metabolism, cancer cells also show extensive metabolic flexibility at the amino acid level [14]. More specifically, in many cancer cells, glutamine demand dominates supply in periods of rapid growth [13,14] and the survival of some types of cancer cells thus depends on glutamine, a phenomenon known as "glutamine addiction" [13,15]. Glutamine supply to cancer cells involves several distinct transporters. Among these, the alanine-serine-cysteine transporter 2 (ASCT2; *SLC1A5* gene) is a primary transporter for glutamine frequently upregulated in multiple cancers [16,17], including breast cancer [18]. This transporter, which mediates Na^+-coupled influx of glutamine, preserves the biosynthetic pathways of rapidly growing cancer cells [18,19]. Higher expression of ASCT2 is correlated with poor prognosis of cancer patients [20], and this gene has been proposed as oncogene [15]. In line with this, ASCT2 inhibition or knockdown in various human cancer cell types markedly decreases intracellular glutamine levels and cell viability and proliferation [20–23]. So, ASCT2 has been attracting attention due to its role in supporting tumor growth and its potential as a therapeutic target in breast cancer, particularly in more aggressive types.

In this study, we aimed to investigate the effect of T2DM-associated characteristics (hyperinsulinemia, hyperleptinemia, and increased levels of inflammation and oxidative stress) upon glutamine uptake by breast cancer cells, in vitro, as a mechanism contributing to the negative effect of T2DM in breast cancer progression.

2. Materials and methods

2.1. Cell culture

We used two breast cancer cell lines (MCF-7 (an estrogen receptor (ER)-positive human breast epithelial adenocarcinoma cell line; ATCC HTB-22; passage numbers 79–94) and MDA-MB-231 (a triple negative human breast adenocarcinoma cell line; ATCC HTB-26; passage numbers 50–79)), and the non-tumorigenic MCF-12A epithelial cell line (ATCC CRL-10782; passage numbers 31–45).

Cells were maintained in a humidified atmosphere of 5% CO_2-95% air and were grown in RPMI 1640 (catalogue #R6504, Sigma-Aldrich, St. Louis, MI, USA) supplemented with 2 mM L-glutamine, 10 mM sodium bicarbonate, 15% heat-inactivated FBS and 1% antibiotic/antimycotic (MCF-7 and MDA-MB-231 cells) or DMEM:Ham's F12 medium (1:1) (catalogue #FG4815, Biochrom, Berlin, Germany) supplemented with 20 ng/ml human epidermal growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml bovine insulin, 500 ng/ml hydrocortisone (all from Sigma-Aldrich, St. Louis, Missouri, USA), 5% heat-inactivated horse serum (Gibco, Life Technologies Corporation, CA, USA) and 1% antibiotic/antimycotic (MCF-12A cells). Culture medium was renewed every 2–3 days, and the culture was split every 7 days.

For transport experiments, cells were seeded on 24-well culture dishes and used also at 100% confluence. For RNA and protein extraction, cells were seeded in 24-well (2 cm^2) and 6-well (9.6 cm^2) plates, respectively, and used at 100% confluence.

2.2. Cell treatments

The concentrations of compounds to be used were chosen based on our previous works [24,25] and are within the range of plasma levels found in T2DM patients [26–28].

To test the effects of *tert*-butylhydroperoxide (TBH) (0.5–2.5 μM), insulin (1–50 nM), leptin (10–500 ng/ml), TNF-α (1–100 ng/ml), IFN-γ (1–100 ng/ml), LY294002 (1 μM), tyrphostin AG490 (5 μM), fludarabine (1 μM) (all from Sigma-Aldrich, St. Louis, Missouri, USA) and/or GPNA (0.25–1 mM) (Santa Cruz Biotechnology, Dallas, Texas, USA), cells were exposed to these compounds for 24 h in serum-free culture medium.

Tested drugs were dissolved in decane (TBH), HCl 0.01 M (insulin), 0.1% BSA (w/v) in H_2O (leptin), 0.1% (w/v) phosphate buffered saline (PBS), (TNF-α, IFN-γ) DMSO (LY294002, tyrphostin AG490 and fludarabine) or H_2O (GPNA). The final concentration of the solvents in buffer and culture media was 1% (v/v). Controls for the drugs were run in the presence of the respective solvent.

2.3. Determination of ^{3}H-glutamine cellular uptake

After exposure to TBH, insulin, leptin, TNF-α, IFN-γ, LY294002, tyrphostin AG490, fludarabine and/or GPNA or vehicle for 24 h in serum-free culture medium, culture medium was discarded and the cells were washed with 300 μl GF-HBS buffer (composition, in mM: 20 HEPES, 5 KCl, 140 NaCl, 2.5 $MgCl_2$, 1 $CaCl_2$, pH 7.4) at 37 °C. Then, cell monolayers were pre-incubated for 20 min in GF-HBS buffer at 37 °C. Uptake was then initiated by the addition of 200 μl GF-HBS buffer at 37 °C containing ^{3}H-GLN ([2,3,4-^{3}H]-glutamine, specific activity 60 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO, USA) in GF-HBS buffer. Incubation was stopped after 6 min (except in time-course experiments) by removing the incubation medium, placing the cells on ice, and rinsing them with 500 μl ice-cold GF-HBS buffer. Cells were then solubilized with 300 μl 0.1% (v/v) Triton X-100 (in 5 mM Tris-HCl, pH 7.4) and placed at 4 °C overnight. Intracellular radioactivity was measured by liquid scintillation counting (LKB Wallac 1209 Rackbeta, Turku, Finland). Results were normalized for total protein content (Bradford method).

To test the Na^+-dependence of ^{3}H-GLN uptake, in some experiments the preincubation and incubation with ^{3}H-GLN was made in Na^+-free GF-HBS (NaCl being substituted by LiCl).

2.4. siRNA transfection

MCF-7, MDA-MB-231 and MCF-12A cells were plated and allowed to attach overnight. At 60% confluence, cells were transfected with 10 nM siRNA-ASCT2 (Ambion ID s12918) or siRNA-Selected Negative Control No. 2 (Ambion ID 4390846) in antibiotic-free growth medium using Lipofectamine 3000 and Opti-MEM Reduced Serum Medium (Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. Transfected cells were incubated at 37 °C and 5% CO_2 for 4-6 h after which the medium was replaced with fresh medium. After transfection (48 h), the cells were used for ^{3}H-GLN uptake and culture growth experiments. All transfections were performed in duplicate. The efficiency to siRNA-ASCT2 was confirmed by RT-qPCR.

2.5. RT-qPCR

Total RNA was extracted from MCF-7, MDA-MB-231 and MCF-12A cells treated for 24 h with TBH (2.5 μM), insulin (50 nM), leptin (100 ng/ml), TNF-α (100 ng/ml) or IFN-γ (100 ng/ml) or the respective vehicle, and RT-qPCR was carried out as described [29]. Cycling conditions for human alanine-serine-cysteine transporter 2 (*SLC1A5*; ASCT2) and L-type amino acid transporter 1 (*SLC7A5*; LAT1) amplification were as follows: denaturation (95 °C for 10 min), amplification and quantification [95 °C for 15 s, annealing temperature (AT) for 15 s, and 65 °C for 15 s, with a single fluorescence measurement at the end of the 72 °C for 15 s segment] repeated 40 times, followed by a melting curve program [(AT + 10)°C for 15 s and 75 °C with a heating rate of 0.1 °C/s and continuous fluorescence measurement] and a cooling step to 37 °C for 30 s. The primer pair used for human ASCT2 amplification was 5′-TGG TCT CCT GGA TCA TGT GG-3′ (forward) and 5′-TTT GCG GGT GAA GAG GAA GT-3′ (reverse). The primer pair used for human LAT1 amplification was 5′-GTG GAC TTC GGG AAC TAT CAC C-3′ (forward) and 5′-GAA CAG GGA CCC ATT GAC GG-3′ (reverse). Samples were analysed in duplicate and the levels of ASCT2 and LAT1 were normalized to the levels of the housekeeping gene, human β-actin. Cycling conditions for human β-actin amplification were as follows:

denaturation (95 °C for 5 min), amplification and quantification (95 °C for 10 s, AT for 10 s, and 65 °C for 10 s, with a single fluorescence measurement at the end of the 72 °C for 10 s segment) repeated 45 times, followed by a melting curve program [(AT + 10)°C for 15 s and 75 °C with a heating rate of 0.1 °C/s and continuous fluorescence measurement] and a cooling step to 37 °C for 30 s; the primer pair used for β-actin was: 5′-AGA GCC TCG CCT TTG CCG AT-3′ (forward) and 5′-CCA TCA CGC CCT GGT GCC T-3′ (reverse). Data were collected using the LightCycler 96 SW 1.1 analysis software (Roche, Mannheim, Germany), and results were analysed by the comparative Ct (ΔΔCT) method [30]. β-Actin mRNA expression levels were not affected by the treatment of the cells (data not shown).

2.6. Western blotting

MCF-7, MDA-MB-231 and MCF-12A cells treated for 24 h with insulin, TBH or TNF-α were lysed with RIPA Lysis Buffer (150 mM NaCl, 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH = 8.0) supplemented with 1:100 protease inhibitor cocktail and 1:100 phosphatase inhibitor cocktails 2 and 3 (both from Sigma-Aldrich, St. Louis, Missouri, USA). Protein concentration was determined using the BCA assay (Pierce). Proteins (20 μg) from each sample were subjected to SDS/PAGE on TGX Any-kD gels and transferred onto 0.2 μM nitrocellulose membranes using the Trans-Blot Turbo System (both from Bio-Rad, Berkeley, California, USA). Membranes were blocked with 4% BSA in TBS-Tween 0.1% and incubated overnight at 4 °C with primary antibody against ASCT2 (Cell Signalling #8057; 1:1000 dilution). For loading normalization, membranes were incubated with α-tubulin antibody (Sigma T5168; 1:10,000 dilution). Incubation with anti-rabbit and anti-mouse secondary antibodies (GE Healthcare NA934 or NA931; 1:10,000 dilution) was performed for 1 h at room temperature and chemiluminescent signal was detected on the Bio-Rad ChemiDoc™ system using Clarity Western ECL Substrate (Bio-Rad, Berkeley, California, USA).

2.7. Evaluation of culture growth

Cells were exposed to IFN-γ (1–100 ng/ml) and/or GPNA (1 mM) or vehicle for 24 h. At the end of treatment, culture growth was determined by the sulforhodamine B (SRB) assay, which reports on intracellular protein content, as described [24].

2.8. Total protein determination

The protein content of cell monolayers was determined as described by Bradford [31], using human serum albumin as standard.

2.9. Statistics

Data are expressed as arithmetic means ± S.E.M. or geometric means with 95% confidence intervals. *n* indicates the number of replicates of at least 2 independent experiments. Statistical significance of the difference between two groups was evaluated by Student's *t*-test; statistical analysis of the difference between various groups was evaluated by the analysis of variance (ANOVA) test, followed by the Newman-Keuls posthoc test. Analyses were done using the GraphPad Prism version 8.0 software (San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. ^{3}H-GLN uptake by breast cancer and non-tumorigenic cells is time- and Na^+-dependent

In the first series of experiments, the time-course and Na^+-dependence of ^{3}H-GLN uptake by non-tumorigenic (MCF-12A) and breast cancer (MCF-7 and MDA-MB-231) cell lines was determined. For this, the cell lines were incubated with ^{3}H-GLN (50 nM) for various periods of time, in the presence or absence of Na^+.

As shown in Fig. 1, all cell lines accumulate ^{3}H-GLN in a time-dependent way, and ^{3}H-GLN uptake is markedly Na^+-dependent in the three cell lines. Because ^{3}H-GLN uptake is linear for the first 5–6 min of incubation, subsequent experiments to characterize the uptake of ^{3}H-GLN were performed using a 6 min incubation time.

3.2. T2DM-associated compounds interfere with ^{3}H-GLN cellular uptake by breast cancer and non-tumorigenic cells

Based on the hypothesis that T2DM-associated conditions (hyperinsulinemia, hyperleptinemia, and increased levels of inflammation and oxidative stress) induce an increase in glutamine cellular uptake that contribute to the negative effects of T2DM in cancer progression, we investigated the effect of a 24 h-exposure to TBH (0.5–2.5 μM), insulin (1–50 nM), leptin (10–500 ng/ml) TNF-α (1–100 ng/ml) or IFN-γ (1–100 ng/ml) on the cellular uptake of ^{3}H-GLN (5 nM and 0.5 mM) by breast cancer (MCF-7 and MDA-MB-231) and non-tumorigenic (MCF-12A) cell lines.

As shown in Fig. 2, IFN-γ significantly stimulated ^{3}H-GLN (5 nM) total uptake by both breast cancer cell lines. In detail, total ^{3}H-GLN uptake increased by 20% and 40% in MCF-7 and MDA-MB-231 cells, respectively. This increase in total ^{3}H-GLN uptake was the result of an increase, in MCF-7 cells, of both Na^+-dependent and Na^+-independent ^{3}H-GLN uptake, whereas in MDA-MB-231, it was associated with an increase in Na^+-dependent ^{3}H-GLN uptake only. Interestingly, this stimulatory effect of IFN-γ on both breast cancer cell lines was similarly observed for a higher (0.5 mM) concentration of ^{3}H-GLN, equivalent to the human physiological concentrations (Fig. 2).

Moreover, the stimulatory effect of IFN-γ on ^{3}H-GLN uptake (5 nM and 0.5 mM) was not found in the non-tumorigenic cell line (MCF-12A) (Fig. 2); rather, in this cell line, an inhibitory effect of IFN-γ on ^{3}H-GLN uptake (5 nM only) was observed (Fig. 2). So, IFN-γ appears to have a cancer cell-specific positive effect on both total and Na^+-dependent glutamine uptake by both ER-positive and triple-negative breast cancer cells.

Besides IFN-γ, other T2DM-associated compounds were also found to induce changes in ^{3}H-GLN uptake. Total and Na^+-dependent ^{3}H-GLN uptake were enhanced by TBH in MDA-MB-231 cells, but a negative effect of TBH on total, Na^+-dependent and Na^+-independent ^{3}H-GLN uptake by MCF-7 cells was observed. In MCF-12A cells, TBH possessed distinct effects in relation to uptake of ^{3}H-GLN 5 nM and 0.5 mM: it decreased total and Na^+-dependent and increased Na^+-independent uptake of ^{3}H-GLN 5 nM, but an opposite effect on total and Na^+-dependent (increase) and Na^+-independent (decrease) uptake of a higher concentration of ^{3}H-GLN was observed (Fig. 2).

In relation to insulin, it was found to inhibit total, Na^+-dependent and Na^+-independent ^{3}H-GLN uptake by breast cancer cells, but to increase total and Na^+-dependent ^{3}H-GLN uptake by MCF-12A cells.

As to leptin, this compound decreased total and Na^+-dependent ^{3}H-GLN uptake in the three cell lines, and had distinct effects in Na^+-independent ^{3}H-GLN uptake, increasing it in MCF-7 and MCF-12A cell lines and decreasing it in the MDA-MB-231 cell line.

Finally, TNF-α was devoid of a significant effect on ^{3}H-GLN uptake by the breast cancer cell lines. In contrast, it shows opposite effects in relation to total and Na^+-dependent uptake of ^{3}H-GLN 5 nM (decrease) and 0.5 mM (increase), in non-tumorigenic (MCF-12A) cells (Fig. 2).

3.3. GPNA completely abolishes Na^+-dependent uptake of ^{3}H-GLN by breast cancer cells

We next tested the effect of different concentrations of GPNA (0.25–1 mM), a known ASCT2 inhibitor [32–34], on ^{3}H-GLN (5 nM) cellular uptake by the non-tumorigenic (MCF-12A) and the breast cancer

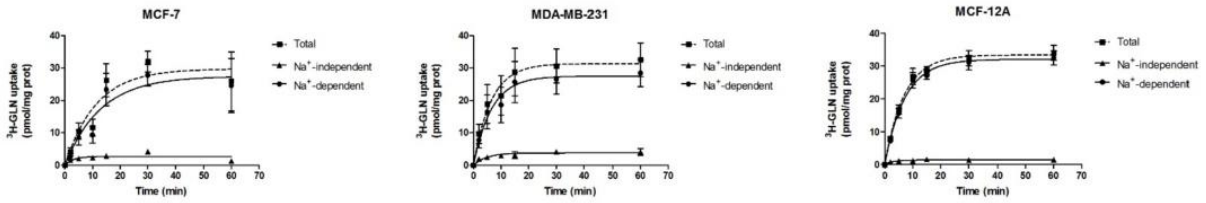

Fig. 1. Time-course and Na^+-dependence of ^{3}H-GLN uptake by breast cancer and non-tumorigenic cell lines.
Breast cancer (MCF-7 and MDA-MB-231) and non-tumorigenic (MCF-12A) cell lines were incubated for various periods of time at 37 °C with 50 nM ^{3}H-GLN (n = 4–6). Uptake was determined in the presence of Na^+ (total uptake) or absence of Na^+ (Na^+-independent uptake). Na^+-dependent uptake was calculated by subtracting Na^+-independent uptake from total uptake. Shown are arithmetic means ± S.E.M.

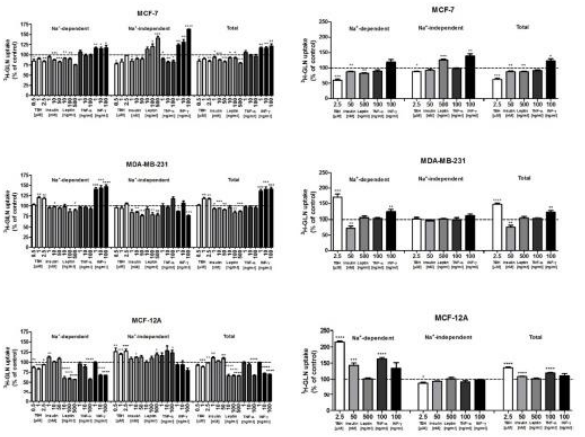

Fig. 2. Effects of T2DM-associated compounds on the cellular uptake of a low and a higher concentration of ^{3}H-GLN by breast cancer and non-tumorigenic cell lines.
Effects of TBH (0.5–2.5 μM), insulin (1–50 nM), leptin (10–500 ng/ml), TNF-α (1–100 ng/ml) or IFN-γ (1–100 ng/ml) for 24 h on Na^+-dependent, Na^+-independent and total uptake of ^{3}H-GLN 5 nM (left graphs) and 0.5 mM (right graphs) by breast cancer (MCF-7 and MDA-MB-231) and non-tumorigenic (MCF-12A) cell lines. Total and Na^+-independent uptake were determined in the presence or absence of Na^+, respectively. Na^+-dependent uptake was calculated by subtracting Na^+-independent uptake from total uptake (n = 4–6). Data show arithmetic means ± S.E.M. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$ versus control, by Student's *t*-test.

(MCF-7 and MDA-MB-231) cell lines.

As shown in Fig. 3, GPNA induced a concentration-dependent reduction in total ^{3}H-GLN uptake by the three cell lines. Although GPNA caused a maximal inhibition of total ^{3}H-GLN uptake similar in cancer and non-tumorigenic cell lines (about 70–75%), it was more potent (lower IC_{50}) in reducing total ^{3}H-GLN uptake by the cancer cell lines (Fig. 3). In relation to Na^+-dependent ^{3}H-GLN uptake, GPNA completely abolished it (±97% and ±99% inhibition), with a similar potency, in the two breast cancer cell lines (MCF-7 and MDA-MB-231 cells, respectively), but it was less potent and caused a lower inhibition (±75%) in MCF-12A cells (Fig. 3). Additionally, GPNA also caused a modest but significant decrease in Na^+-independent ^{3}H-GLN uptake in MCF-12A, MCF-7 and MDA-MB-231 cells (±30%, ±29% and ±18%, respectively) (Fig. 3).

3.4. GPNA prevents the stimulatory effects of T2DM-associated compounds on ^{3}H-GLN uptake by breast cancer and non-tumorigenic cells

The previous results showed that uptake of ^{3}H-GLN 5 nM and 0.5 mM (either Na^+-dependent, Na^+-independent or total uptake) is increased by leptin and IFN-γ in MCF-7 cells, by TBH and IFN-γ in MDA-MB-231 cells and by TBH, insulin, leptin and TNF-α in MCF-12A cells. So, we next decided to examine the influence of GPNA (1 mM) on the stimulatory effect of these compounds (24 h) upon the uptake of a low (5 nM) and a higher (0.5 mM) ^{3}H-GLN concentration.

Interestingly enough, in the three cell lines, GPNA inhibition was able to override the stimulatory effect of all these compounds on the uptake of both concentrations of ^{3}H-GLN (5 nM and 0.5 mM) (Fig. 4). This effect of GPNA was very pronounced in relation to uptake of the lower ^{3}H-GLN concentration, as the T2DM-associated compounds tested were completely devoid of effect in its presence. In contrast, in relation to uptake of the higher concentration of ^{3}H-GLN, the stimulatory effect of the T2DM-associated compounds was prevented, but the inhibitory effect of GPNA was reduced in the presence of the T2DM-associated compounds, in the two breast cancer cell lines, but not in the non-tumorigenic cell line (Fig. 4). Thus, the stimulatory effect of T2DM-associated compounds upon uptake of a low concentration of ^{3}H-GLN by the three cell lines is GPNA-sensitive. In contrast, the stimulatory effect of the T2DM-associated compounds upon uptake of a higher ^{3}H-GLN concentration is less affected by GPNA-induced inhibition in the cancer cell lines (Fig. 4).

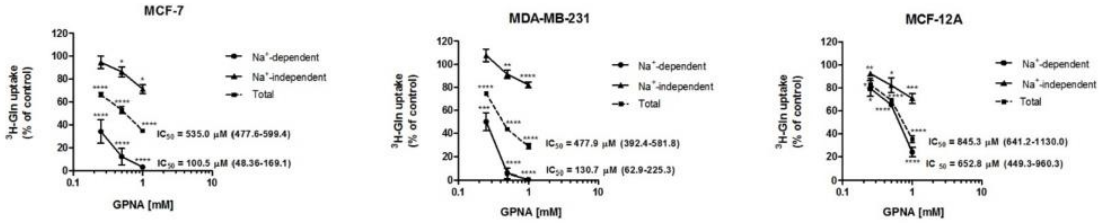

Fig. 3. Effect of GPNA on ^{3}H-GLN uptake by breast cancer and non-tumorigenic cell lines.
Effect of GPNA (0.25–1 mM) on ^{3}H-GLN (5 nM) uptake by breast cancer (MCF-7 and MDA-MB-231) and non-tumorigenic (MCF-12A) cell lines (n = 4). Uptake was determined in the presence of Na^+ (total uptake) or absence of Na^+ (Na^+-independent uptake). Na^+-dependent uptake was calculated by subtracting Na^+-independent uptake from total uptake. Shown is arithmetic means ± S.E.M. or geometric means with 95% confidence intervals. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$ versus control, by Student's *t*-test.

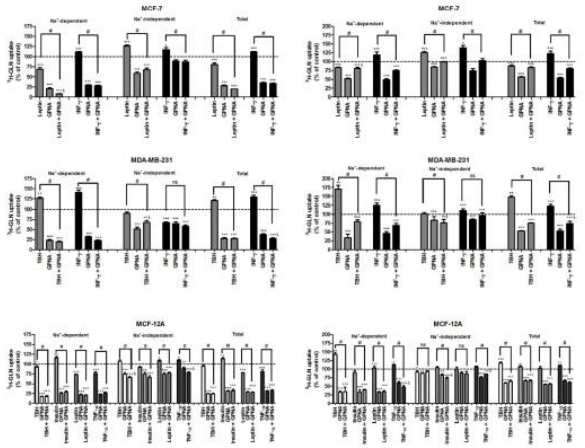

Fig. 4. The influence of GPNA on the stimulatory effect of T2DM-associated compounds upon the cellular uptake of a low and a higher concentration of ^{3}H-GLN by breast cancer and non-tumorigenic cell lines.
Influence of GPNA (1 mM) on the effect of TBH (2.5 μM), insulin (50 nM), leptin (500 ng/ml), TNF-α (100 ng/ml) or IFN-γ (100 ng/ml) for 24 h upon Na^+-dependent, Na^+-independent and total uptake of ^{3}H-GLN 5 nM (left graphs) and 0.5 mM (right graphs) by breast cancer (MCF-7 and MDA-MB-231) and non-tumorigenic (MCF-12A) cell lines. Uptake was determined in the presence of Na^+ (total uptake) or absence of Na^+ (Na^+-independent uptake). Na^+-dependent uptake was calculated by subtracting Na^+-independent uptake from total uptake. Data show arithmetic means ± S.E.M. (n = 4–6). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus control, $^{\$}P < 0.05$ versus GPNA, $^{\#}P < 0.05$ versus TBH, insulin, leptin, TNF-α or IFN-γ, ^{ns} not significant, by two way ANOVA with Newman-Keuls post-hoc test.

3.5. IFN-γ and insulin increase ASCT2 expression levels in breast cancer cells and non-tumorigenic cells, respectively

From the previous results, we concluded that the stimulatory effect of T2DM-associated compounds upon ^{3}H-GLN uptake is GPNA-sensitive and cell-specific. More specifically, in relation to the uptake of ^{3}H-GLN (5 nM): (1) in the ER-positive breast cancer cell line (MCF-7), IFN-γ stimulation of both Na^+-dependent and Na^+-independent uptake and leptin stimulation of Na^+-independent uptake is GPNA-sensitive; (2) in the triple-negative breast cancer cell line (MDA-MB-231), TBH and IFN-γ stimulation of Na^+-dependent uptake is GPNA-sensitive, and (3) in the non-tumorigenic cell line (MCF-12A), insulin stimulation of Na^+-dependent uptake and TBH, leptin and TNF-α stimulation of Na^+-independent uptake is GPNA-sensitive.

GPNA is broadly used as an ASCT2 inhibitor [33,35,36], but recent studies have demonstrated that it is also able to inhibit other Na^+-dependent and Na^+-independent glutamine transporters [32]. Namely, GPNA is also able to block the Na^+-coupled neutral amino acid transporter 1 (SNAT1) [37,38], SNAT2 [37,39], $ATB^{0,+}$ [36,40] and the Na^+-uncoupled transporters L-type amino acid transporter 1 (LAT1) [33,36,41,42] and LAT2 [33]. Hence, we decided to explore with more detail if ASCT2 is indeed involved in the GPNA-sensitive stimulatory effects of T2DM-associated compounds on ^{3}H-GLN uptake. Of the T2DM-associated compounds, we selected IFN-γ in breast cancer cells and insulin in non-tumorigenic breast epithelial cells for the next experiments, because IFN-γ and insulin stimulate Na^+-dependent ^{3}H-GLN uptake selectively in breast cancer and non-tumorigenic breast cells, respectively. So, for these two compounds, we next assessed their effects on ASCT2 mRNA and ASCT2 protein levels and after ASCT2 knockdown (Fig. 5).

In MCF-7 cells, IFN-γ has no effect on ASCT2 mRNA (Fig. 5A) and protein (Fig. 5B and C) levels. Therefore, the increase in Na^+-dependent ^{3}H-GLN uptake induced by IFN-γ does not result from an upregulation of the ASCT2 gene.

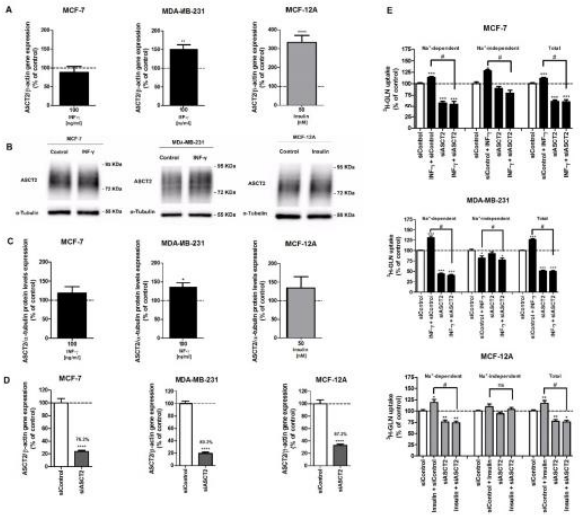

Fig. 5. Effects of T2DM-associated compounds on ASCT2 mRNA and protein levels and after ASCT2 knockdown in breast cancer and non-tumorigenic cell lines.
The effects of TBH (2.5 μM), insulin (50 nM), TNF-α (100 ng/ml) or IFN-γ (100 ng/ml) (24 h) on (A) *ASCT2* mRNA levels, assessed by RT-qPCR (n = 6) and on (B, C) ASCT2 protein levels, assessed by western blot (n = 6) in breast cancer (MCF-7 and MDA-MB-231) and non-tumorigenic (MCF-12A) cell lines. (D) The efficiency of ASCT2 knockdown (48 h) using siASCT2 (10 nM), was assessed by RT-qPCR in all cell lines. (E) Effects of insulin (50 nM) or IFN-γ (100 ng/ml) (24 h) after ASCT2 knockdown on Na^+-dependent, Na^+-independent and total uptake of ^{3}H-GLN 5 nM (n = 4) in breast cancer (MCF-7 and MDA-MB-231) and non-tumorigenic (MCF-12A) cell lines. In (A, E) data were normalized to the expression of β-actin. In (B, C) α-tubulin was used as loading control. (B) Representative blots are shown. (C) The graphs represent protein quantification of all blots. (E) uptake was determined in the presence of Na^+ (total uptake) or absence of Na^+ (Na^+-independent uptake). Na^+-dependent uptake was calculated by subtracting Na^+-independent uptake from total uptake. Data show arithmetic means ± S.E.M. (n = 4–6). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$ versus control, $^{\#}P < 0.05$ versus insulin or IFN-γ, ^{ns} not significant, by two way ANOVA with Newman-Keuls post-hoc test.

In MDA-MB-231 cells, the increase in Na^+-dependent ^{3}H-GLN uptake induced by IFN-γ was associated with an increase in ASCT2 mRNA (Fig. 5A) and protein (Fig. 5B and C) levels. Thus, we conclude that this compound induces an upregulation of the ASCT2 gene resulting in a stimulation of ASCT2-mediated ^{3}H-GLN uptake in this cell line.

Finally, in MCF-12A cells, insulin appears to stimulate Na^+-dependent ^{3}H-GLN uptake by increasing ASCT2 transcription rates, as this compound significantly increased ASCT2 mRNA levels (Fig. 5A) and tended to increase ASCT2 protein levels (Fig. 5B and C).

The effect of other T2DM-associated compounds on ASCT2 mRNA and protein levels and LAT1 mRNA levels was also assessed (Figs. S1 and S2). From these results, we could conclude that, in MCF-7 cells, the increase in Na^+-independent ^{3}H-GLN uptake caused by IFN-γ is not related to an increase of LAT1 mRNA levels and that, in contrast, leptin appears to stimulate LAT1-mediated Na^+-independent ^{3}H-GLN uptake by upregulating LAT1 gene transcription (Fig. S1). Moreover, in MDA-MB-231 cells, TBH had no effect on ASCT2 mRNA or protein levels (Fig. S2A–C). Therefore, the increase in Na^+-dependent ^{3}H-GLN uptake caused by this agent is not achieved by an increase in ASCT2 expression. Finally, in MCF-12A cells, TBH increased LAT1 mRNA levels (Fig. S1) but had no effect on ASCT2 mRNA or protein levels (Fig. S2A–C). Consequently, we conclude that TBH stimulates Na^+-independent ^{3}H-GLN uptake by increasing LAT1 transcription. In contrast, the increase of

Na^+-independent ^{3}H-GLN uptake induced by leptin and TNF-α is not associated with LAT1 upregulation (Fig. S1). Similarly, TNF-α had no effect on ASCT2 mRNA and protein levels (Fig. S2A–C).

3.6. The stimulatory effect of IFN-γ and insulin upon ^{3}H-GLN uptake by breast cancer cells and non-tumorigenic cells, respectively, requires ASCT2 expression

We next assessed the effects of IFN-γ and insulin after ASCT2 knockdown. For this, breast cancer (MCF-7 and MDA-MB-231) and non-tumorigenic (MCF-12A) cells were transfected with ASCT2-specific siRNA or a control-specific siRNA for 48 h. The efficiency of ASCT2 knockdown was confirmed by qRT-PCR. ASCT2 mRNA levels were markedly reduced by ASCT2-specific siRNA in MCF-7, MDA-MB-231 and MCF-12A cells, respectively (Fig. 5D).

Interestingly, the stimulatory effect of IFN-γ (in MCF-7 and MDA-MB-231) and insulin (in MCF-12A cells) on both total and Na^+-dependent ^{3}H-GLN uptake was abolished after transfection with ASCT2-targeting siRNA, as compared with control siRNA (Fig. 5E). This clearly indicates that IFN-γ (in breast cancer cells) and insulin (in non-tumorigenic cells) promote ASCT2-mediated ^{3}H-GLN uptake.

3.7. The stimulatory effect of IFN-γ upon ^{3}H-GLN uptake is PI3K-, STAT3- and STAT1-dependent in breast cancer cells

We next focused on IFN-γ, because this T2DM-associated compound possesses a very interesting effect, as it stimulates ^{3}H-GLN uptake (both total and Na^+-dependent; both at low (5 nM) and physiological (0.5 mM) levels) only in the breast cancer cell lines. We decided to investigate the intracellular signalling mechanisms by which IFN-γ stimulates ^{3}H-GLN uptake. For this, cells were treated with LY294002 (1 μM), tyrphostin AG490 (5 μM) or fludarabine (1 μM), which are specific inhibitors of phosphoinositide 3-kinase (PI3K), signal transducer and activator of transcription 3 (STAT3) and STAT1, respectively.

The results, presented in Fig. 6, show that LY294002, tyrphostin AG490 or fludarabine treatment abolished the stimulatory effect of IFN-γ upon total and Na^+-dependent ^{3}H-GLN uptake by both breast cancer cell lines. These results support the conclusion that IFN-γ–induced stimulation of Na^+-dependent ^{3}H-GLN uptake in these cell lines is PI3K-, STAT3- and STAT1-dependent.

3.8. Inhibition/knockdown of ASCT2 abolishes the pro-proliferative effect of IFN-γ in breast cancer cells

We recently verified that IFN-γ stimulates MCF-7 and MDA-MB-231 cell proliferation/culture growth [43]. So, we decided to investigate if ASCT2 is involved in the pro-proliferative effect of IFN-γ in these cell lines. Interestingly enough, the stimulatory effect of IFN-γ on growth of these two cancer cell lines was abolished by GPNA (Fig. 7A) and ASCT2 knockdown (Fig. 7B). These observations strongly support the conclusion that the pro-proliferative effect of IFN-γ in these breast cancer cell lines is dependent on stimulation of ASCT2-mediated ^{3}H-GLN uptake.

4. Discussion

Many cancer cells require glutamine for survival and proliferation and thus exhibit a phenotype of "glutamine addiction" [13,15]; consequently, glutamine transport has been considered a target for cancer therapy. However, the approach is not straightforward due to the multitude of glutamine transporters in mammalian cells [36,44], and while glutamine has been shown to be critical in many cancer types, its importance for breast cancers is still not very well defined [45].

Plasma free glutamine levels are elevated in obesity [46], T2DM [46,47], and in breast cancer patients [47], suggesting that increased glutamine availability in T2DM or breast cancer state could be an important factor for these pathologies. So, we decided to investigate if

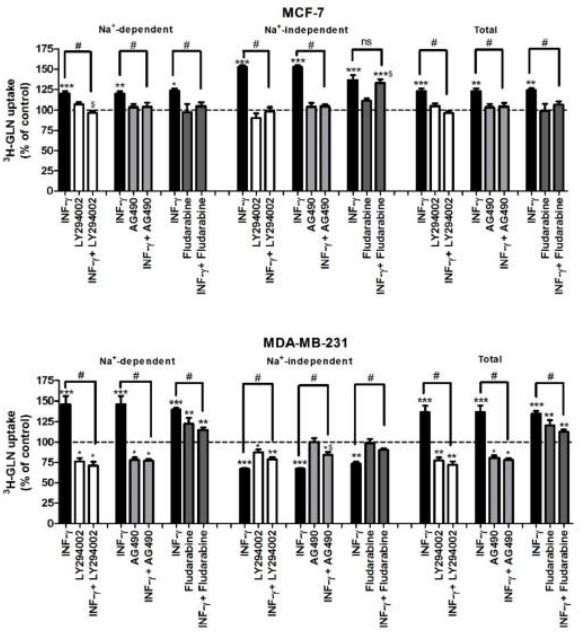

Fig. 6. The influence of specific inhibitors of intracellular signalling pathways (LY294002, tyrphostin AG490 and fludarabine) on the stimulatory effects of IFN-γ upon ^{3}H-GLN uptake by breast cancer cell lines.
Effect of IFN-γ (100 ng/ml) (24 h) in the presence or absence of LY294002 (1 μM), tyrphostin AG490 (5 μM) or fludarabine (1 μM) upon Na^+-dependent, Na^+-independent and total uptake of ^{3}H-GLN (5 nM) by breast cancer (MCF-7 and MDA-MB-231) cell lines. Uptake was determined in the presence of Na^+ (total uptake) or absence of Na^+ (Na^+-independent uptake). Na^+-dependent uptake was calculated by subtracting Na^+-independent uptake from total uptake. Data show arithmetic means ± S.E.M. (n = 4–6). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ versus control, $^{\$}P < 0.05$ versus LY294002, tyrphostin AG490 or fludarabine, $^{\#}P < 0.05$ versus IFN-γ, ^{ns} not significant, by two way ANOVA with Newman-Keuls post-hoc test.

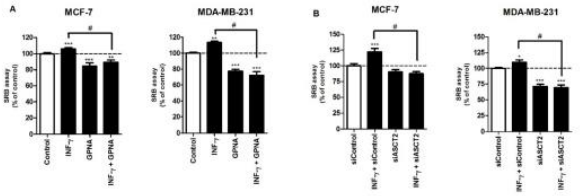

Fig. 7. The influence of GPNA or ASCT2 knockdown on the effect of IFN-γ upon growth of breast cancer cell lines.
(A) Effect of GPNA (1 mM) and (B) ASCT2 knockdown using siASCT2 (10 nM; 48 h) on the effect of IFN-γ (100 ng/ml) (24 h) on growth of breast cancer (MCF-7 and MDA-MB-231) cell lines (n = 8). Data show arithmetic means ± S.E.M. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs control; $^{\#}P < 0.05$ vs IFN-γ by two way ANOVA with Newman-Keuls post-hoc test.

T2DM-associated conditions (hyperinsulinemia, hyperleptinemia, and increased levels of inflammation and oxidative stress) induce an increase in glutamine transport that can contribute to the negative effect of T2DM in breast cancer progression.

We first verified that the three cell lines take up ^{3}H-GLN very efficiently and mainly by a Na^+-dependent mechanism. Because an increase in glutamine cellular uptake is expected to stimulate progression of breast cancer, we focused our analysis on T2DM-associated compounds with a stimulatory effect of upon Na^+-dependent ^{3}H-GLN (5 nM and/or

0.5 mM) uptake. Of the T2DM-associated conditions analysed, IFN-γ displayed the most interesting effect, as it induced a significant cancer cell-specific stimulatory effect on ^{3}H-GLN uptake (total and Na^+-sensitive) both at a low (5 nM) and a higher (physiological, 0.5 mM) concentration of ^{3}H-GLN. Nevertheless, other T2DM-associated compounds were also found to interfere with ^{3}H-GLN uptake. TBH increased total and Na^+-dependent ^{3}H-GLN uptake by MDA-MB-231 cells, but it reduced ^{3}H-GLN uptake by MCF-7 cells. Therefore, oxidative stress appears to have distinct effect in ^{3}H-GLN uptake by ER-positive (decrease) and triple-negative breast (increase) cancer cells. Insulin showed a differential response on ^{3}H-GLN uptake by breast cancer (reduction) and non-tumorigenic (increase) cells. Leptin reduced total and Na^+-dependent ^{3}H-GLN uptake by both breast cancer cell lines and, more pronouncedly, by non-tumorigenic cells. Moreover, Na^+-independent ^{3}H-GLN uptake was also stimulated by some of the T2DM-associated compounds. Namely, in MCF-7 cells, by IFN-γ and leptin, and in MCF-12A cells, by TBH, insulin, leptin and TNF-α.

With a few exceptions, the stimulatory effect of T2DM-associated compounds upon the uptake of 5 nM ^{3}H-GLN was also verified upon the uptake of a physiological level of ^{3}H-GLN (0.5 mM). So, analysis of the effect of these compounds on the latter is a very reliable indicator of their effect on glutamine uptake in vivo. The differences in the effect of T2DM-associated conditions on ^{3}H-GLN uptake at the two concentrations may reflect differential level-dependent recruitment of distinct glutamine transporters. In this context, it is known that ASCT2 exhibits a high affinity (Km = 0.02 mM) for glutamine, whereas e.g. LAT1 has a low affinity (Km = 1–2 mM) [48,49].

Although glutamine transporters are highly expressed by breast cancer and non-tumorigenic cell lines, distinct cell lines show different responses to glutamine. It is known that basal-like cells (MDA-MB-231) are very sensitive to glutamine availability, while luminal cells (MCF-7) are not, despite maintaining relatively high levels of glutamine transport [21]. Therefore, it is not surprising that some of the T2DM-associated compounds tested herein impact differently on ^{3}H-GLN uptake by different breast cancer cell types.

ASCT2, recognized as a primary glutamine transporter, is a Na^+-dependent neutral amino acid transporter. It mediates the exchange of amino acid substrates, which is crucial for the uptake of glutamine by rapidly growing tumor cells [35]. ASCT2 is overexpressed in different cancers and is closely related to poor prognosis [16,18], having thus being suggested as a target for specifically blocking cancer growth and development [50]. In recent years, several inhibitors of ASCT2 have emerged and showed a surprising antitumor effect [35,51]. Among them, GPNA features a glutamine structural analogy and inhibits the Na^+-dependent amino acid transporter ASCT2 [33,35,36]. Although more selective ASCT2 inhibitors exist [19], GPNA represents the more potent compound of a series of glutamine analogues synthesized by Esslinger et al. [34]. Based on this, we decided to use GPNA for ASCT2 inhibition.

We verified that GPNA caused a very marked inhibition in total ^{3}H-GLN uptake in both cancer and non-tumorigenic cells. Moreover, Na^+-dependent uptake by the two breast cancer cell lines, but not by the non-tumorigenic MCF-12A cell line, was completely abolished by GPNA. This suggests that Na^+-dependent ^{3}H-GLN uptake by breast cancer cells, but not by breast non-cancer cells, involves only GPNA-sensitive transporters and that, consequently, GPNA is a more effective inhibitor of glutamine uptake by breast cancer cells, when compared with non-tumorigenic cells. In this context, in a previous report, GPNA was also found to decrease ^{3}H-GLN uptake by MCF-7 and MDA-MB-231 cell lines, but to be devoid of effect on ^{3}H-GLN uptake by the non-tumorigenic MCF-10A cell line [21]. Additionally, we also verified that GPNA modestly inhibited Na^+-independent ^{3}H-GLN uptake in the three cell lines. An inhibitory effect of GPNA upon Na^+-independent ^{3}H-GLN uptake by MCF-7 cells was recently described, and it was concluded that this inhibitor hinders the activity of LAT1 and LAT2, which are expressed in this cell line [33].

Interestingly, in the three cell lines, GPNA was able to completely abolish the stimulatory effect of TBH, insulin, leptin, TNF-α or IFN-γ upon Na^+-dependent or Na^+-independent uptake of a low concentration of ^{3}H-GLN (5 nM). Moreover, GPNA abolished also the stimulatory effect of TBH, insulin and TNFα on Na^+-dependent ^{3}H-GLN (0.5 mM) uptake by the MCF-12A cells. However, it interfered less markedly with the stimulatory effect of T2DM-associated conditions on Na^+-dependent or -independent uptake in the two breast cancer cell lines. Therefore, stimulation of uptake of a low concentration of ^{3}H-GLN is GPNA-sensitive in both cancer and non-tumorigenic cell lines, while that of a higher concentration of ^{3}H-GLN is disrupted to a lesser extent by GPNA in breast cancer cell lines (but not in the non-tumorigenic cell line). These results support the hypothesis that low affinity glutamine transporter(s) less affected or even unaffected by GPNA participate in ^{3}H-GLN uptake in breast cancer cell lines only.

IFN-γ and insulin increased Na^+-dependent ^{3}H-GLN uptake selectively in breast cancer and non-tumorigenic breast cells, respectively. For this reason, we next investigated their effect on ASCT2 expression in these cell lines and concluded that the increase in Na^+-dependent ^{3}H-GLN uptake induced by IFN-γ results from an upregulation of ASCT2 transcription in MDA-MB-231 cells, but not in MCF-7 cells. Moreover, the stimulatory effect of insulin on ^{3}H-GLN uptake by MCF-12A cells is also associated with an increase in ASCT2 expression. The observation that the IFN-γ-induced increase in Na^+-dependent ^{3}H-GLN uptake in MCF-7 cells is not associated with a concomitant increase of ASCT2 mRNA or protein levels indicates that, in this cell line, this compound affects ASCT2 through a mechanism other than regulation of gene expression.

As previously reported, GPNA is broadly used as ASCT2 inhibitor, but recent studies have demonstrated that it is also able to inhibit other Na^+-dependent and -independent amino acid transporters [32]. Although Na^+-independent glutamine transporters are not the focus of this work, the co-expression of ASCT2 and LAT1 was described in other cell types [52,53], and GPNA was described to inhibit LAT1 in MCF-7 cells [33]. So, we decided to investigate the effect of T2DM-associated compounds that stimulated ^{3}H-GLN uptake on LAT1 transcription. We verified that leptin (in MCF-7 cells) and TBH (in MCF-12A cells) upregulated LAT1 transcription, whereas leptin, insulin and TNF-α (in MCF-12A cells) were devoid of an effect on LAT1 mRNA levels.

Because GPNA has a poor selectivity for the ASCT2 transporter, we further investigated the involvement of ASCT2 in the stimulatory effect of IFN-γ and insulin upon ^{3}H-GLN uptake by breast cancer and non-cancer cells, respectively, by performing targeted knockdown of ASCT2. Interestingly, the stimulatory effect on ^{3}H-GLN uptake of IFN-γ in both breast cancer cell lines and of insulin in the non-tumorigenic cell line were completely abolished after ASCT2 knockdown. These data strongly support the conclusion that IFN-γ (in MCF-7 cells and MDA-MB-231 cells) and insulin (in MCF-12A cells) increase ASCT2-mediated ^{3}H-GLN uptake.

The canonical signalling pathway mediating the effects of IFN-γ consists in the activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT), which promotes phosphorylation and activation of transcription factors such as STAT1 and STAT3. However, IFN-γ also activates non-canonical signalling pathways such as PI3K [54,55]. Deregulation of the JAK/STAT and PI3K pathways is often associated with increased tumorigenesis [54]. Interestingly, we concluded that the stimulatory effect of IFN-γ upon ^{3}H-GLN uptake involves activation of STAT1, STAT3 and PI3K signalling pathways in both cancer cell lines. These results thus support a relevant role of STAT1, STAT3 and PI3K in the increase of ^{3}H-GLN uptake and, therefore, in the promotion of breast cancer progression induced by IFN-γ.

In the final part of this work, we tested the hypothesis that interference with glutamine uptake by IFN-γ contributes to the negative effect of T2DM in breast cancer progression. We have recently verified that IFN-γ stimulates MCF-7 and MDA-MB-231 cell proliferation/culture growth [43]. As such, we investigated if the stimulatory effect of IFN-γ

upon breast cancer cell proliferation is ASCT2-mediated. Notably, we demonstrated that both pharmacological blockade and knockdown of ASCT2 abolished the pro-proliferative effect of IFN-γ in both ER-positive and triple-negative breast cancer cells. These results thus propose that ASCT2 mediates the negative effects of IFN-γ in breast cancer progression.

5. Conclusion

Our work shows that some T2DM-associated compounds induce changes in ASCT2-mediated glutamine transport that can contribute to breast cancer progression. Importantly, these effects are also evident in a breast non-tumorigenic cell line, suggesting that they may also contribute to breast cancer initiation in T2DM patients. Glutamine metabolism in the context of insulin resistance, altered adipokines, chronic low-grade inflammation and increased oxidative stress levels mechanisms is not yet fully understood. A better understanding of the mechanisms involved in the relationship between "glutamine addition" in breast cancer cells and T2DM is, therefore, warranted. Finally, our results suggest that an effective inhibition of glutamine uptake (as verified with GPNA and confirmed by ASCT2 knockdown) may be used as a therapeutic strategy against human breast cancer.

CRediT authorship contribution statement

Cláudia Silva: Investigation, Writing – original draft. **Nelson Andrade:** Investigation. **Ilda Rodrigues:** Investigation. **António Carlos Ferreira:** Investigation. **Miguel Luz Soares:** Investigation. **Fátima Martel:** Conceptualization, Formal analysis, Supervision, Visualization, Writing – review & editing, Project administration.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2021.120054.

References

[1] H. Sung, J. Ferlay, R.L. Siegel, Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, CA Cancer J. Clin. 71 (2021) 209–249, https://doi.org/10.3322/caac.21660.
[2] J. Pearson-Stuttard, B. Zhou, V. Kontis, et al., Worldwide burden of cancer attributable to diabetes and high body-mass index: a comparative risk assessment, Lancet Diabetes Endocrinol. 6 (2018) 95–104, https://doi.org/10.1016/S2213-8587(17)30366-2.
[3] F. Bray, J. Ferlay, I. Soerjomataram, Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, CA Cancer J. Clin. 68 (2018) 394–424, https://doi.org/10.3322/caac.21492.
[4] S.S. Doerstling, C.H. O'Flanagan, S.D. Hursting, Obesity and cancer metabolism: a perspective on interacting tumor-intrinsic and extrinsic factors, Front. Oncol. 7 (2017) 216, https://doi.org/10.3389/fonc.2017.00216.
[5] P. Widschwendter, T.W. Friedl, L. Schwentner, et al., The influence of obesity on survival in early, high-risk breast cancer: results from the randomized SUCCESS a trial, Breast Cancer Res. 17 (2015) 129, https://doi.org/10.1186/s13058-015-0639-3.
[6] S.M. Samuel, E. Varghese, S. Varghese, et al., Challenges and perspectives in the treatment of diabetes associated breast cancer, Cancer Treat. Rev. 70 (2018) 98–111, https://doi.org/10.1016/j.ctrv.2018.08.004.
[7] E.J. Gallagher, D. LeRoith, Obesity and diabetes: the increased risk of cancer and cancer-related mortality, Physiol. Rev. 95 (2015) 727–748, https://doi.org/10.1152/physrev.00030.2014.
[8] C. Micucci, D. Valli, G. Matacchione, et al., Current perspectives between metabolic syndrome and cancer, Oncotarget 7 (2016) 38959–38972, https://doi.org/10.18632/oncotarget.8341.
[9] K. Lee, L. Kruper, C.M. Dieli-Conwright, et al., The impact of obesity on breast cancer diagnosis and treatment, Curr. Oncol. Rep. 21 (2019) 41, https://doi.org/10.1007/s11912-019-0787-1.
[10] U.E. Martinez-Outschoorn, M. Peiris-Pages, R.G. Pestell, et al., Cancer metabolism: a therapeutic perspective, Nat. Rev. Clin. Oncol. 14 (2017) 113, https://doi.org/10.1038/nrclinonc.2017.1.
[11] O. Warburg, F. Wind, E. Negelein, The metabolism of tumors in the body, J. Gen. Physiol. 8 (1927) 519–530, https://doi.org/10.1085/jgp.8.6.519.
[12] E.M. Jaffee, C.V. Dang, D.B. Agus, et al., Future cancer research priorities in the USA: a lancet oncology commission, Lancet Oncol. 18 (2017) e653–e706, https://doi.org/10.1016/S1470-2045(17)30698-8.
[13] M.G. Vander Heiden, Targeting cancer metabolism: a therapeutic window opens, Nat. Rev. Drug Discov. 10 (2011) 671–684, https://doi.org/10.1038/nrd3504.
[14] A.J. Bott, S. Maimouni, W.X. Zong, The pleiotropic effects of glutamine metabolism in cancer, Cancers (Basel) 11 (2019), https://doi.org/10.3390/cancers11060770.
[15] Y. Kato, T. Maeda, A. Suzuki, et al., Cancer metabolism: new insights into classic characteristics, Jpn. Dent. Sci. Rev. 54 (2018) 8–21, https://doi.org/10.1016/j.jdsr.2017.08.003.
[16] A.D. Marshall, M. van Geldermalsen, N.J. Otte, et al., ASCT2 regulates glutamine uptake and cell growth in endometrial carcinoma, Oncogenesis 6 (2017), e367, https://doi.org/10.1038/oncsis.2017.70.
[17] E. Teixeira, C. Silva, F. Martel, The role of the glutamine transporter ASCT2 in antineoplastic therapy, Cancer Chemother. Pharmacol. 87 (2021) 447–464, https://doi.org/10.1007/s00280-020-04218-6.
[18] R. El Ansari, A. McIntyre, M.L. Craze, et al., Altered glutamine metabolism in breast cancer; subtype dependencies and alternative adaptations, Histopathology 72 (2018) 183–190, https://doi.org/10.1111/his.13334.
[19] C. Lopes, C. Pereira, R. Medeiros, ASCT2 and LAT1 contribution to the hallmarks of cancer: from a molecular perspective to clinical translation, Cancers (Basel) 13 (2021) 203, https://doi.org/10.3390/cancers13020203.
[20] Y. Hara, Y. Minami, S. Yoshimoto, et al., Anti-tumor effects of an antagonistic mAb against the ASCT2 amino acid transporter on KRAS-mutated human colorectal cancer cells, Cancer Med. 9 (2020) 302–312, https://doi.org/10.1002/cam4.2689.
[21] M. van Geldermalsen, Q. Wang, R. Nagarajah, et al., ASCT2/SLC1A5 controls glutamine uptake and tumour growth in triple-negative basal-like breast cancer, Oncogene 35 (2016) 3201–3208, https://doi.org/10.1038/onc.2015.381.
[22] Q. Wang, R.A. Hardie, A.J. Hoy, et al., Targeting ASCT2-mediated glutamine uptake blocks prostate cancer growth and tumour development, J. Pathol. 236 (2015) 278–289, https://doi.org/10.1002/path.4518.
[23] Q. Wang, K.A. Beaumont, N.J. Otte, et al., Targeting glutamine transport to suppress melanoma cell growth, Int. J. Cancer 135 (2014) 1060–1071, https://doi.org/10.1002/ijc.28749.
[24] C. Silva, C. Nunes, A. Correia-Branco, et al., Insulin exhibits an antiproliferative and hypertrophic effect in first trimester human extravillous trophoblasts, Reprod. Sci. 24 (4) (2017) 582–594, https://doi.org/10.1177/1933719116667220.
[25] N. Andrade, C. Silva, F. Martel, The effect of oxidative stress upon intestinal sugar transport: an in vitro study using human intestinal epithelial (Caco-2) cells, Toxicol. Res. 7 (2018) 1236–1246, https://doi.org/10.1039/c8tx00183a.
[26] A.K. Mishra, C.R. Parish, M.L. Wong, et al., Leptin signals via TGFB1 to promote metastatic potential and stemness in breast cancer, PLoS One 12 (2017), e0178454, https://doi.org/10.1371/journal.pone.0178454.
[27] X. Fang, R. Palanivel, X. Zhou, et al., Hyperglycemia- and hyperinsulinemia-induced alteration of adiponectin receptor expression and adiponectin effects in L6 myoblasts, J. Mol. Endocrinol. 35 (2005) 465–476, https://doi.org/10.1677/jme.1.01877.
[28] H. Alzamil, Elevated serum TNF-alpha is related to obesity in type 2 diabetes mellitus and is associated with glycemic control and insulin resistance, J. Obes. 2020 (2020) 5076858, https://doi.org/10.1155/2020/5076858.
[29] C. Silva, A. Correia-Branco, N. Andrade, et al., Selective pro-apoptotic and antimigratory effects of polyphenol complex catechin:lysine 1:2 in breast, pancreatic and colorectal cancer cell lines, Eur. J. Pharmacol. 859 (2019), 172533, https://doi.org/10.1016/j.ejphar.2019.172533.
[30] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative C(T) method, Nat. Protoc. 3 (2008) 1101–1108, https://doi.org/10.1038/nprot.2008.73.
[31] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[32] A. Corti, S. Dominici, S. Piaggi, et al., Gamma-glutamyltransferase enzyme activity of cancer cells modulates L-gamma-glutamyl-p-nitroanilide (GPNA) cytotoxicity, Sci. Rep. 9 (2019) 891, https://doi.org/10.1038/s41598-018-37385-x.

[33] M. Chiu, C. Sabino, G. Taurino, et al., GPNA inhibits the sodium-independent transport system L for neutral amino acids, Amino Acids 49 (2017) 1365–1372, https://doi.org/10.1007/s00726-017-2436-z.
[34] C.S. Esslinger, K.A. Cybulski, J.F. Rhoderick, Ngamma-aryl glutamine analogues as probes of the ASCT2 neutral amino acid transporter binding site, Bioorg. Med. Chem. 13 (2005) 1111–1118, https://doi.org/10.1016/j.bmc.2004.11.028.
[35] Y. Liu, T. Zhao, Z. Li, et al., The role of ASCT2 in cancer: a review, Eur. J. Pharmacol. 837 (2018) 81–87, https://doi.org/10.1016/j.ejphar.2018.07.007.
[36] Y.J. Cha, E.S. Kim, J.S. Koo, Amino acid transporters and glutamine metabolism in breast cancer, Int. J. Mol. Sci. 19 (2018), https://doi.org/10.3390/ijms19030907.
[37] A. Broer, F. Rahimi, S. Broer, Deletion of amino acid transporter ASCT2 (SLC1A5) reveals an essential role for transporters SNAT1 (SLC38A1) and SNAT2 (SLC38A2) to sustain glutaminolysis in cancer cells, J. Biol. Chem. 291 (2016) 13194–13205, https://doi.org/10.1074/jbc.M115.700534.
[38] K. Wang, F. Cao, W. Fang, et al., Activation of SNAT1/SLC38A1 in human breast cancer: correlation with p-akt overexpression, BMC Cancer 13 (2013) 343, https://doi.org/10.1186/1471-2407-13-343.
[39] A. Prat, C. Cruz, K.A. Hoadley, et al., Molecular features of the basal-like breast cancer subtype based on BRCA1 mutation status, Breast Cancer Res. Treat. 147 (2014) 185–191, https://doi.org/10.1007/s10549-014-3056-x.
[40] S. Karunakaran, S. Ramachandran, V. Coothankandaswamy, et al., SLC6A14 (ATB0,+) protein, a highly concentrative and broad specific amino acid transporter, is a novel and effective drug target for treatment of estrogen receptor-positive breast cancer, J. Biol. Chem. 286 (36) (2011) 31830–31838, https://doi.org/10.1074/jbc.M111.229518.
[41] P. Nicklin, P. Bergman, B. Zhang, et al., Bidirectional transport of amino acids regulates mTOR and autophagy, Cell 136 (2009) 521–534, https://doi.org/10.1016/j.cell.2008.11.044.
[42] Y. Cormerais, P.A. Massard, M. Vucetic, et al., The glutamine transporter ASCT2 (SLC1A5) promotes tumor growth independently of the amino acid transporter LAT1 (SLC7A5), J. Biol. Chem. 293 (2018) 2877–2887, https://doi.org/10.1074/jbc.RA117.001342.
[43] C. Silva, N. Andrade, J.T. Guimaraes, et al., The in vitro effect of the diabetes-associated markers insulin, leptin and oxidative stress on cellular characteristics promoting breast cancer progression is GLUT1-dependent, Eur. J. Pharmacol. 898 (2021), 173980, https://doi.org/10.1016/j.ejphar.2021.173980.
[44] Y.D. Bhutia, V. Ganapathy, Glutamine transporters in mammalian cells and their functions in physiology and cancer, Biochim. Biophys. Acta 2016 (1863) 2531–2539, https://doi.org/10.1016/j.bbamcr.2015.12.017.
[45] H.N. Kung, J.R. Marks, J.T. Chi, Glutamine synthetase is a genetic determinant of cell type-specific glutamine independence in breast epithelia, PLoS Genet. 7 (2011), e1002229, https://doi.org/10.1371/journal.pgen.1002229.
[46] Y. Zhou, L. Qiu, Q. Xiao, et al., Obesity and diabetes related plasma amino acid alterations, Clin. Biochem. 46 (2013) 1447–1452, https://doi.org/10.1016/j.clinbiochem.2013.05.045.
[47] X. Bi, C.J. Henry, Plasma-free amino acid profiles are predictors of cancer and diabetes development, Nutr. Diabetes 7 (2017), e249, https://doi.org/10.1038/nutd.2016.55.
[48] D. Fotiadis, Y. Kanai, M. Palacin, The SLC3 and SLC7 families of amino acid transporters, Mol. Asp. Med. 34 (2013) 139–158, https://doi.org/10.1016/j.mam.2012.10.007.
[49] N. Utsunomiya-Tate, H. Endou, Y. Kanai, Cloning and functional characterization of a system ASC-like Na+-dependent neutral amino acid transporter, J. Biol. Chem. 271 (25) (1996) 14883–14890, https://doi.org/10.1074/jbc.271.25.14883.
[50] S. Broer, Amino acid transporters as disease modifiers and drug targets, SLAS Discov. 23 (2018) 303–320, https://doi.org/10.1177/2472555218755629.
[51] C.T. Hensley, A.T. Wasti, R.J. DeBerardinis, Glutamine and cancer: cell biology, physiology, and clinical opportunities, J. Clin. Invest. 123 (2013) 3678–3684, https://doi.org/10.1172/JCI69600.
[52] T. Yazawa, K. Shimizu, K. Kaira, et al., Clinical significance of coexpression of L-type amino acid transporter 1 (LAT1) and ASC amino acid transporter 2 (ASCT2) in lung adenocarcinoma, Am. J. Transl. Res. 7 (2015) 1126–1139.
[53] H. Honjo, K. Kaira, T. Miyazaki, et al., Clinicopathological significance of LAT1 and ASCT2 in patients with surgically resected esophageal squamous cell carcinoma, J. Surg. Oncol. 113 (4) (2016) 381–389, https://doi.org/10.1002/jso.24160.
[54] F. Castro, A.P. Cardoso, R.M. Goncalves, et al., Interferon-gamma at the crossroads of tumor immune surveillance or evasion, Front. Immunol. 9 (2018) 847, https://doi.org/10.3389/fimmu.2018.00847.
[55] L. Martin-Hijano, B. Sainz Jr., The interactions between cancer stem cells and the innate interferon signaling pathway, Front. Immunol. 11 (2020) 526, https://doi.org/10.3389/fimmu.2020.00526.

SUPPLEMENTARY DATA

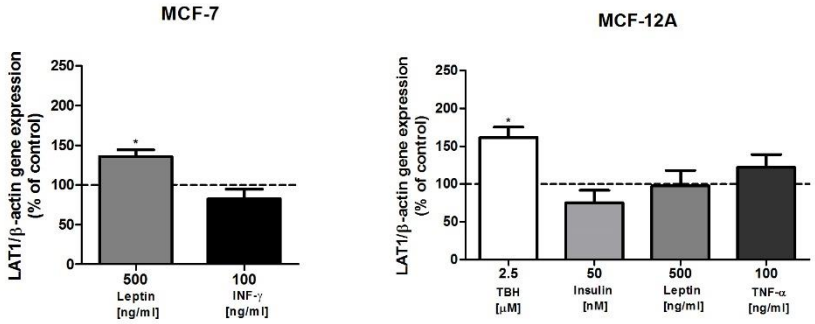

Fig. S1. Effects of T2DM-associated compounds on LAT1 mRNA levels in breast cancer and non-tumorigenic cell lines.

Effects of TBH (2.5 μM), insulin (50 nM), leptin (500 ng/ml), TNF-α (100 ng/ml) or IFN-γ (100 ng/ml) (24h) on LAT1 mRNA levels in breast cancer (MCF-7) and non-tumorigenic (MCF-12A) cell lines (n=6). Data were normalized to the expression of β-actin. Data show means ± S.E.M. *P < 0.05 versus control, by Student's *t* test.

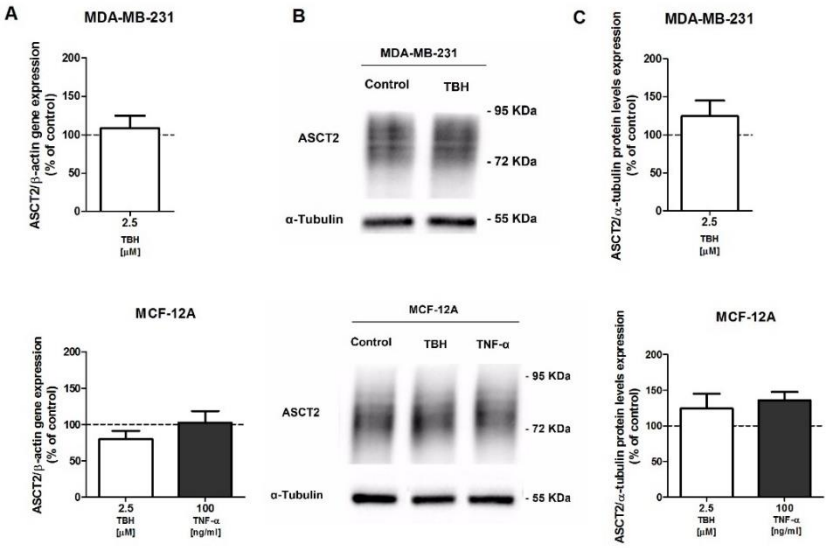

Fig. S2. Effects of T2DM-associated compounds on ASCT2 mRNA and protein levels in breast cancer and non-tumorigenic cell lines.

Effects of TBH (2.5 μM) and TBH (2.5 μM) and TNF (100 ng/ml) on **(A)** ASCT2 mRNA levels, assessed by RT-qPCR (n=6) and on **(B, C)** protein levels, assessed by western blot (n=6) in MDA-MB-231 and MCF-12A cell lines, respectively. In **(A)** data were normalized to the expression of β-actin. In **(B, C)** α-tubulin was used as loading control. (**B**) Representative blots are shown. (**C**) The graphs represent protein quantification of all blots. Data show means ± S.E.M. *P < 0.05 versus control, by Student's *t* test.

CHAPTER V

THE EFFECT OF TYPE 2 DIABETES MELLITUS-ASSOCIATED CHARACTERISTICS UPON GLUCOSE AND GLUTAMINE CELLULAR UPTAKE DURING BREAST CANCER INITIATION AND PROMOTION

THE PRO-PROLIFERATIVE EFFECT OF INSULIN IN HUMAN BREAST EPITHELIAL DMBA-TRANSFORMED AND NON-TRANSFORMED CELL LINES IS PI3K-, MTOR- AND GLUT1-DEPENDENT

Cláudia Silva, Nelson Andrade, João Tiago Guimarães, Emília Cardoso, Catarina Meireles, Vanessa Pinto, Joana Paiva, Fátima Martel

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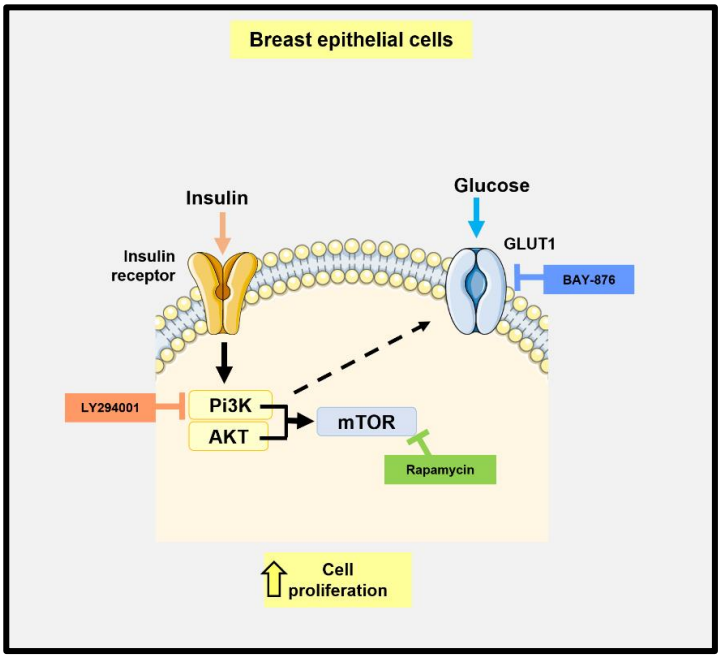
Graphical Abstract



Highlights

- Insulin stimulates proliferation of DMBA-treated and non-treated MCF-12A breast epithelial cell lines
- Insulin increases ^{3}H-DG uptake and lactate production in both cell lines
- The effect of insulin on cell proliferation and ^{3}H-DG uptake is mTOR and PI3K-dependent
- The effect of insulin on cell proliferation and ^{3}H-DG uptake is GLUT1 stimulation-dependent

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RESEARCH ARTICLE

CELL BIOCHEMISTRY & FUNCTION WILEY

The pro-proliferative effect of insulin in human breast epithelial DMBA-transformed and non-transformed cell lines is PI3K-, mTOR- and GLUT1-dependent

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Type 2 diabetes mellitus (T2DM) is linked to an increased risk of breast cancer. We aimed to investigate how T2DM-associated characteristics (high levels of glucose, insulin, leptin, inflammatory mediators and oxidative stress) influence breast cancer carcinogenesis, in DMBA-treated (MCF-12A$_{DMBA}$) and non-treated breast epithelial (MCF-12A) cell lines. Insulin (50 nM) promotes cell proliferation, ^{3}H-DG uptake and lactic acid production in both cell lines. The stimulatory effects of insulin upon cell proliferation and ^{3}H-DG uptake were hampered by rapamycin, LY294001 and BAY-876, in both cell lines. In conclusion, hyperinsulinemia, one important characteristic of T2DM, contributes to the initiation of breast cancer by a PI3K- and mTOR-dependent mechanism involving increased GLUT1-mediated glucose uptake.

Significance: The pro-proliferative effect of insulin in human breast epithelial DMBA-transformed and non-transformed cell lines is PI3K-, mTOR- and GLUT1-dependent.

KEYWORDS
breast cancer, DMBA, GLUT1, insulin, mTOR, PI3K

1 | INTRODUCTION

Type 2 diabetes mellitus (T2DM) has been linked to tumour initiation, accelerated progression and poor prognosis and response to treatment of several cancer types,[1,2] including breast cancer.[1] Currently, breast cancer is the most common malignancy among women worldwide and it is also the leading cause of cancer-related deaths in this gender,[3] and reports show a 20% to 28% increased risk of breast cancer in women with T2DM.[1,4]

T2DM is chronic metabolic disorder characterized by insulin resistance and glucose intolerance.[1] The insulin resistance-associated metabolic reprogramming leads to changes in circulation, namely hyperinsulinemia, altered adipokine levels (higher leptin and lower adiponectin levels), increased oxidative stress levels and a low-grade chronic inflammatory condition (higher levels of interleukin-6 [IL-6], tumour necrosis factor α [TNF-α] and interferon-γ [INF-γ]).[1,5,6] Tumour development is tightly controlled by complex intracellular signalling pathways that modulate their proliferation, survival, migration,

invasion and angiogenesis.[7] Interestingly, the above-mentioned T2DM-related factors interfere with these same signalling pathways.[5,7,8] Nonetheless, the mechanisms underlying the link between T2DM and breast cancer have yet to be fully characterized.

In order to meet the increased demands for rapid proliferation and cell survival, breast cancer cells reprogram their metabolic pathways.[1,9] Increased aerobic glycolysis irrespective of oxygen availability, also known as the Warburg effect, is a common metabolic adaption in cancer cells.[10] Additionally, many tumours show a higher demand of glutamine.[11] Interestingly, the diabetic environment induces a higher expression of the main glucose (the glucose transporter 1 [GLUT1]) and glutamine (alanine-serine-cysteine transporter 2 [ASCT2]) transporters in breast tissue[12,13] and it appears that GLUT1 is particularly important for the initiation of breast tumorigenesis.[14]

7,12-dimethylbenz[a]anthracene (DMBA) is a polycyclic aromatic hydrocarbon (PAH) model compound that has been extensively employed for studying breast carcinogenesis.[15] In a proliferating mammary epithelium, PAHs are metabolically activated to cause significant DNA damage, mutations, and malignant transformation of the subsequent cell population, thus initiating breast carcinogenesis.[16-18]

In this work, we investigated how T2DM-associated characteristics (high levels of glucose, insulin, leptin, inflammatory mediators and oxidative stress) can influence DMBA-induced breast carcinogenesis, by using a human breast epithelial cell line.

2 | MATERIALS AND METHODS

2.1 | Cell culture

The human breast epithelial cell line MCF-12A (obtained from American Type Culture Collection [ATCC CRL-10782, Rockville, MD], pass 50-62) was maintained in a humidified atmosphere of 5% CO_2-95% air and grown in DMEM:Ham's F12 medium (1:1) (catalogue #FG4815, Biochrom, Berlin, Germany) supplemented with 20 ng/mL human epidermal growth factor, 100 ng/mL cholera toxin, 0.01 mg/mL bovine insulin, 500 ng/mL hydrocortisone (all from Sigma-Aldrich, St. Louis, Missouri), 5% heat-inactivated horse serum (Gibco, Life Technologies Corporation, California) and 1% antibiotic/antimytotic. Culture medium was renewed every 2 to 3 days, and the culture was split every 7 days.

For the determination of cell viability, proliferation, migration and angiogenesis, cells were seeded on 24-well culture dishes (2 cm^2; Ø 16 mm; TPP, Trasadingen, Switzerland) and used at 80% to 90% confluence (4-day-old). For nutrient uptake experiments and for quantification of lactate production, cells were seeded similarly and used at 100% confluence (5-day-old). For apoptosis determination, cells were seeded on coverslips in 24-well plates and used at 50% to 60% confluence (3-day-old). For cell cycle analysis, cells were seeded on 21 cm^2 plates (21 cm^2; Ø 60 mm; Corning Costar, New York) and used at 100% confluence.

2.2 | DMBA-induced transformation of MCF-12A cells

The cells were treated with a mammary-specific carcinogen, DMBA (Sigma-Aldrich, St. Louis, Missouri). The MCF-12A cells (passage 50) were incubated with DMBA (2 μg/mL) for 24 hours followed by another 24 hours incubation with fresh DMBA in the culture medium. The surviving cells were washed with phosphate-buffered saline (PBS) and allowed to grow in fresh culture medium (without DMBA and with 5% FBS) until confluence, as described.[18] Cells grown in the same culture medium but treated with DMSO were used as control.

The transformation of MCF-12A$_{DMBA}$ cells was confirmed by analysis of the following parameters: rates of cell proliferation, colony formation and cell cycle analysis. In addition, both supernatants of the MCF-12A and MCF-12A$_{DMBA}$ cells were analysed using an optical sensing method: the intelligent Lab on Fibre (iLoF) tool[19-21] to investigate if there is a unique scattering signature of the carcinogen-induced process.

2.3 | Cell treatments

The concentrations of compounds to be tested were chosen based on the literature and our own previous works.[8,22-26]

To test the effects of glucose (30 mM) (Merck, Darmstadt, Germany), *tert*-butylhydroperoxide (TBH) (2.5 μM), insulin (50 nM), leptin (500 ng/mL), TNF-α (100 ng/mL), INF-γ (100 ng/mL), LY294002 (1 μM), rapamycin (100 nM) (all from Sigma-Aldrich, St. Louis, Missouri) and/or BAY-876 (Tocris Bioscience, Bristol, United Kingdom) on cell proliferation, viability, apoptosis, migration, angiogenesis, lactate production and glucose and glutamine uptake, cells were exposed to these compounds for 24 hours in serum-free culture medium.

Tested drugs were dissolved in serum-free culture medium (glucose), decane (TBH), HCl 0.01 M (insulin), 0.1% (w/v) BSA (leptin), 0.1% (w/v) PBS (TNF-α and INF-γ) or DMSO (BAY-876, LY294002 and rapamycin). The final concentration of solvents in buffer and culture media was 1% (v/v). Controls for the drugs were run in the presence of a solvent.

2.4 | Soft agar colony formation assay

Anchorage-independent growth ability was determined by soft agar colony formation assay. Briefly, we coated 6-well plates with 0.6% noble agar in complete media (1.5 mL/well) and allowed to solidify at room temperature for 30 minutes. Next, 15×10^3 cells were mixed with complete media containing 0.3% noble agar and added to the top of base agar and allowed to solidify for 30 minutes. Complete media (200 μL) was then added to each well to prevent drying, and the wells were incubated for 22 days at 37°C under 5% CO_2. Afterwards, colonies were stained with 0.01% crystal violet for 1 hour, and

the images captured with a microscope. Only colonies with diameter >50 μM were counted. The average number of colonies was quantified using the ImageJ software (NIH, Bethesda, Maryland).

2.5 | MCF-12A cells supernatants optical fingerprinting

The iLoF platform is a label-free, real-time and low-cost technology that agnostically analyses nanostructures in complex fluids through a highly focused laser beam, guided by a micro-lensed optical fibre, to obtain nanostructures' back-scattered signal signature.[19-21] This signal captures the specific combination of the Brownian motion of particles present in the sample under the influence of a harmonic electromagnetic potential generated by the electromagnetic field propagated by the polymeric micro-lensed fibre tip.[19,20,27] Through the application of artificial intelligence (AI) techniques to time and frequency-derived features extracted from the back-scattered signal, relevant information about the optical and biophysical properties of the bioparticles under analysis (refractive index, optical polarizability, size, shape) can be gathered in 'optical fingerprints' characterizing biosamples.

Back-scattered signals were collected from both DMBA-transformed ($MCF\text{-}12A_{DMBA}$) and non-tumoural (MCF-12A cells) supernatants to investigate if the difference in the light scattering signature of these two mediums was robust enough to translate the biochemical and cellular changes associated with the induced carcinogenesis. After the calculation of time and frequency-derived features from the back-scattered signals, an AI algorithm was trained to differentiate and identify the light scattering pattern associated with the DMBA-induced cells supernatant.

Three different technical replicates of each supernatant were measured and considered for analysis. Thus, the initial volume of each supernatant was divided into three different samples of 300 μL (totalling six different samples for the two supernatants). Then, a volume of 100 μL of each sample was pipetted onto a μ-dish 35 mm, glass bottom dish (ibidi GmbH, Gräfelfing, Germany), which was placed over the iLoF set-up for analysis. Back-scattered signals were acquired by micro-lensed polymeric probes when dipped in the samples, while laser light from a pigtailed 976 nm laser diode (S28-7602-500, Lumentum, California) was irradiated and focused by the microlenses at the tip of the optical fibres on the sample. The laser light was sinusoidally modulated with a frequency of 1 kHz and a sampling rate of 100 kHz. Samples were exposed to an average power of 13 mW during signal acquisition. The scattering radiation (acquired signal) was then collected by the same microlens used to focus the irradiation light, and guided to a photodetector (PDA36A2, Thorlabs, New Jersey) during 30 seconds. Then, the information was digitally converted and sent to a PC using a DAQ (NI USA-6361, X Series DAQ, National Instruments, Texas) at a sampling rate of 100 kHz. After each acquisition, the tip was displaced to a different position within the same sample and the process was repeated until a total of 10 signals of 30 seconds were acquired. Afterwards, the fibre tip was removed cleaned using a bleach solution (20%) to avoid cross contamination between samples. After acquisition, back-scattered signals were phase matched, filtered using a second-order Butterworth high-pass filter with a cut-off frequency of 500 Hz to remove noisy low-frequency components and split into portions of 10 seconds.[28] Then, a set of 98 time- and frequency-related features[19-21] was calculated for each 10 seconds epoch. Both signal processing and features calculation were run using a custom-built python script.

Before developing the AI algorithm to identify the scattering signature associated with the carcinogenesis process through the analysis of cells supernatant, the back-scattered derived features dataset was split into training and testing subsets.[29] The testing subset was composed of the features associated with the signals acquired from one of the three samples analysed per supernatant, which was randomly chosen from the three. The features associated with the remaining two samples for each supernatant were included in the training set.

The problem considered in this study included a binary classification. Back-scattered derived features were used to identify 'supernatant from DMBA-induced cells' from 'supernatant from non-treated cells'. The AI classification algorithm chosen for this problem was the random forests.[29] During the validation phase, the most suitable combination of values between the three parameters 'number of trees', 'number of predictors to sample' and 'minimum leaf size' was determined, based on the higher receiver operating characteristic (ROC) area under the curve (AUC) value attained using the 5-fold cross-validation method.[29] All the 98 features were used in the developed models. The performance of models was evaluated considering accuracy, sensitivity, specificity, ROC AUC and precision in the test set.

2.6 | Evaluation of cell proliferation

DNA synthesis rates were evaluated by quantification of incorporation of ^{3}H-thymidine (μCi/mg total protein), as described.[8,24] Intracellular radioactivity was measured by liquid scintillation counting (LKB Wallac 1209 Rackbeta, Turku, Finland). Results were normalized for total protein content (Bradford method).

2.7 | Evaluation of cell migration

Cell migration rates were determined by a scratch injury assay, as described.[8,24] Images were obtained at 0 and 24 hours after the scratch, and quantification was performed using the ImageJ software (NIH, Bethesda, Maryland).

2.8 | Evaluation of cell viability

Cell viability was evaluated by measuring the cellular leakage of lactate dehydrogenase (LDH) into the culture medium, as described.[8,24] LDH activity is expressed as the % of extracellular activity in relation to total cellular LDH activity.

2.9 | Quantification of VEGF-A levels

VEGF-A levels were quantified using a human VEGF-A ELISA Kit (RAB0507; Sigma-Aldrich, St. Louis, Missouri) according to the manufacturer's instructions. The optical density at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, Waltham, Massachusetts).

2.10 | Determination of apoptotic index

The apoptotic index was determined by the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay, by using the In Situ Cell Death Detection kit (Roche Diagnostics, Basel, Switzerland), as described.[8,24] Immunofluorescence was visualized under a fluorescence microscope (Zeiss apopTome, Oberkochen, Germany). The apoptotic index was calculated as the % of apoptotic cells in relation to total cell number.

2.11 | Cell cycle analysis

Changes in cell distribution across cell cycle stages were assessed by measurement of DNA content in the cells. For each sample, 1×10^6 cells were seeded on 21 cm^2 plates and were exposed to treatment for 24 hours. After the exposure, cells were centrifuged at 300 g for 5 minutes and fixed in 200 μL of 70% ethanol for 30 minutes at 4°C. Next, cells were washed in PBS with 2% BSA and re-suspended in 100 μL of propidium iodide (PI) solution (PI/Rnase, Immunostep) for 15 minutes at room temperature. Flow cytometry analysis was performed on a BD Accuri C6 flow cytometer (Becton, Dickinson and Company, Franklin Lakes, New Jersey). Results were analysed using FlowJo version 10.7.1 software (Ashland, Oregon). Results are expressed as % of total cells arrested in each phase in the cell cycle.

2.12 | Quantification of ^{3}H-deoxy-D-glucose and ^{3}H-glutamine cellular uptake

After exposure to TBH, glucose, insulin, leptin, TNF-α, INF-γ, LY294002, rapamycin and/or BAY-876 or vehicle for 24 hours, culture medium was discarded and the cells were washed with 300 μL GF-HBS buffer (composition in mM: 20 HEPES, 5 KCl, 140 NaCl, 2.5 $MgCl_2$, 1 $CaCl_2$, pH 7.4) at 37°C. Then, cell monolayers were pre-incubated for 20 minutes in GF-HBS buffer at 37°C. Uptake was then initiated by the addition of 200 μL GF-HBS buffer at 37°C containing ^{3}H-2-deoxy-*D*-glucose (^{3}H-DG) 10 nM (2-[1,2-^{3}H(N)-deoxy-D-glucose; specific activity 60 Ci/mmol) or ^{3}H-glutamine (^{3}H-GLN) 5 nM ([2,3,4-^{3}H]-glutamine; specific activity 60 Ci/mmol) (both from American Radiolabeled Chemicals, St. Louis, Missouri). Incubation was stopped after 6 minutes by removing the incubation medium, placing the cells on ice, and rinsing them with 500 μL ice-cold GF-HBS buffer. Cells were then solubilized with 300 μL 0.1% (v/v) Triton X-100 (in 5 mM Tris-HCl, pH 7.4) and placed at 4°C overnight. Intracellular radioactivity was measured by liquid scintillation counting (LKB Wallac 1209 Rackbeta, Turku, Finland). Results were normalized for total protein content (Bradford method).

2.13 | Quantification of lactate release

Extracellular lactate was measured with the lactate oxidase/peroxidase colorimetric assay, as described.[8,30]

2.14 | Total protein determination

The protein content of cell monolayers was determined as described,[31] using human serum albumin as standard.

2.15 | Statistics

Data are expressed as means ± SEM. *n* indicates the number of replicates of at least two independent experiments. Statistical significance of the difference between two groups was evaluated by Student's *t* test; statistical analysis of the difference between various groups was evaluated by the analysis of variance (two-way ANOVA) test, followed by the Newman-Keuls post hoc test. Analysis was done using the GraphPad Prism version 8.0 software (San Diego, California). $P < .05$ was considered to be statistically significant.

3 | RESULTS

3.1 | DMBA induces transformation of MCF-12A cells

Human breast epithelial (MCF-12A) cells were transformed using the mammary-specific carcinogen, DMBA. In order to confirm DMBA-induced carcinogenesis, colony formation ability (soft agar colony formation assay), rates of proliferation (^{3}H-thymidine incorporation assay) and cell cycle (flow cytometry analysis) were evaluated.

Anchorage-independent growth in the semisolid medium of soft agar is a strong indicator of a transformed phenotype.[32] As shown in Figure 1A,B, the resulting cell line MCF-12A$_{DMBA}$ originated an increased size and number of colonies compared with the non-tumoural MCF-12A cell line. Proliferation also has a decisive role in the dissemination of cancer cells,[9] and so we investigated the effect of DMBA on cell proliferation rates and cell cycle distribution. As shown in Figure 1C, the carcinogen-transformed cells showed a higher (±40%) proliferative potential as compared with the MCF-12A cells. Cell cycle analysis showed a significant decrease (±18%) in G2/M phase for MCF-12A$_{DMBA}$ cells as compared with the non-tumoural cell line (Figure 1D,E).

Additionally, the iLoF technique was capable of differentiating the supernatant of DMBA-transformed cells from the supernatant of cells not submitted to this carcinogen in a test set completely

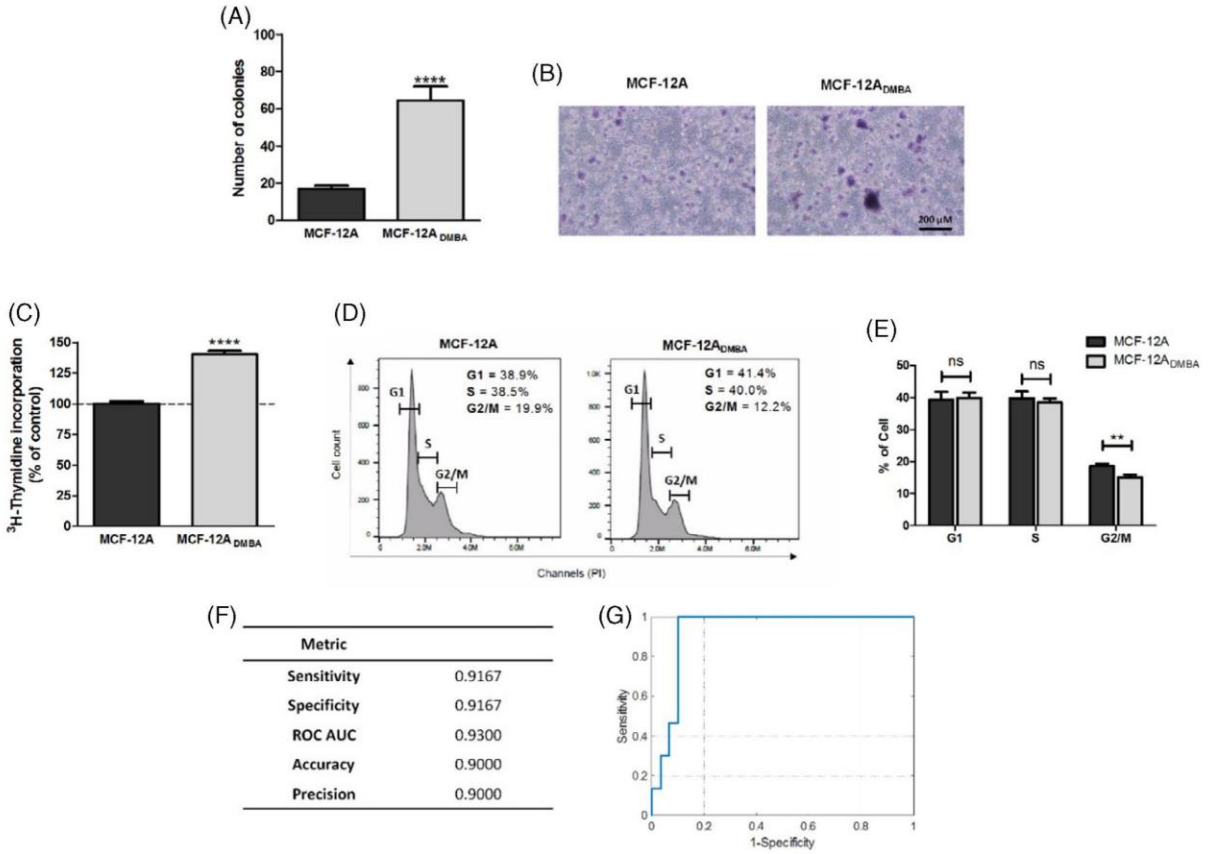

FIGURE 1 DMBA-induced transformation of MCF-12A cells results in enhanced anchorage-independent colony growth, increased cell proliferation, cell cycle changes and distinctive biophysical characteristics. The effect of DMBA-induced transformation (MCF-12A$_{DMBA}$) of the non-tumoural (MCF-12A) cell line on (A, B) soft agar colony formation rates (n = 6); (C) cell proliferation rates (n = 8); (D, E) cell cycle distribution (n = 9) and (F) iLoF technique performance metrics in the test set regarding the differentiation/identification of DMBA-induced cells supernatant from cells not submitted to DMBA, (G) ROC curve of the classification task in the test set. Data show arithmetic means ± SEM. **P* < .05; ***P* < .01; *****P* < .0001 vs control, by Student's *t* test. Scale bar: 200 µM

independent from the train set with a ROC AUC, accuracy, sensitivity and specificity above 90% (Figure 1F,G). These results, together with all cellular evidence of the carcinogenesis process associated with DMBA treatment of the cells, indicate that the biophysical characteristics of the supernatants are distinct enough to be used as a carcinogenesis optical fingerprint through the application of the iLoF technique.

In short, we proved that DMBA was able to induce transformation of the non-tumoural MCF-12A cell line.

3.2 | Insulin stimulates proliferation and induces changes in cell cycle in both DMBA-transformed (MCF-12A$_{DMBA}$) and non-cancer MCF-12A cells

To investigate the importance of T2DM-associated characteristics in breast cancer initiation, we characterized the effect of a 24 hours exposure to high levels to glucose (30 mM), insulin (50 nM), leptin (500 ng/mL), pro-inflammatory cytokines (TNF-α and INF-γ; 100 ng/mL) and high oxidative stress levels (by using the oxidative stress inducer TBH [2.5 µM]) on cell proliferation, viability, migration, apoptosis index and angiogenesis in DMBA-transformed (MCF-12A$_{DMBA}$) and non-tumoural (MCF-12A) cells.

As can be seen in Figure S1, TBH, glucose, leptin, TNF-α or INF-γ did not have a consistent effect on cellular features associated with cancer initiation. In contrast, insulin showed a pro-proliferative effect in both MCF12-A and MCF-12A$_{DMBA}$ cells, as cell proliferation rates increased by ±26% and ±38%, respectively (Figure 2A). Additionally, insulin increased the number of viable MCF12-A cells (Figure 2B) and increased (by ±26%) VEGF-A levels in MCF-12A$_{DMBA}$ cell culture (Figure 2D). Insulin did not affect migration and apoptosis rates in both normal breast epithelial and DMBA-transformed cells (Figure 2C,E,F).

We decided to focus on the pro-proliferative effect of insulin. To determine if insulin affects a specific phase of the cell cycle, we assessed its effect on the cell cycle profile in both MCF-12A$_{DMBA}$ and

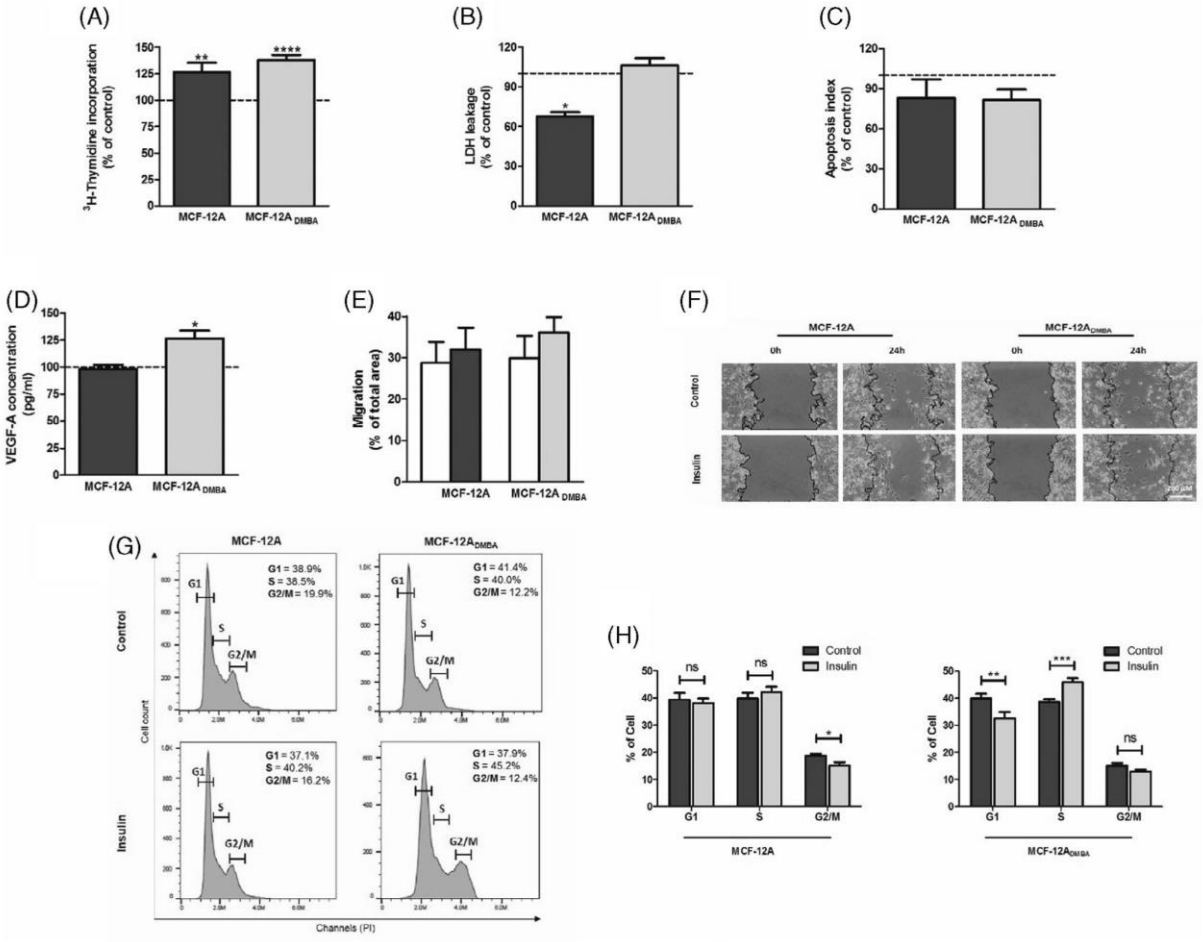

FIGURE 2 Insulin induces changes in cellular features in DMBA-transformed (MCF-12A$_{DMBA}$) and non-tumoural (MCF-12A) cell lines. Effect of insulin (50 nM) (24 hours) upon (A) cell proliferation rates (n = 6-9), (B) cell viability (n = 9), (C) apoptosis index (n = 6), (D) VEGF-A levels (n = 6), (E, F) cell migration (n = 6), and (G, H) cell cycle distribution (n = 9), in non-tumoural (MCF-12A) and DMBA-transformed (MCF-12A$_{DMBA}$) cell lines. Data show arithmetic means ± SEM. *P < .05; **P < .01; ****P < .0001; ns, not significant vs control, by Student's t test. Scale bar: 200 μM

MCF12-A cells. Our results show that insulin caused a significant decrease (±21%) in the number of cells arrested in G2/M in MCF-12A cells (Figure 2G,H). In contrast, in MCF-12A$_{DMBA}$ cells, insulin induced a significant decrease in the number of cells occupying the G1 phase (±18%) and a concomitant increase in the number of cells occupying the S phase (Figure 2G,H).

Overall, our data show that insulin induces changes in cellular features that can be relevant in the context of tumour initiation.

3.3 | Insulin modulates metabolic characteristics of DMBA-transformed (MCF-12A$_{DMBA}$) and non-cancer MCF-12A cells

Glucose and glutamine are important metabolic substrates of cancer cells. For this reason, glucose and glutamine uptake and lactate levels were quantified in DMBA-transformed MCF-12A and MCF-12A cells.

First, we compared ^{3}H-DG (10 nM) and ^{3}H-GLN (5 nM) uptake in non-tumorigenic (MCF-12A) and DMBA-transformed (MCF-12A$_{DMBA}$) cell lines. ^{3}H-DG and ^{3}H-GLN uptake by the both cell lines was time-dependent and linear with time for up to 5 to 6 minutes of incubation (Figure. S2). Based on this information, subsequent experiments to characterize the uptake of ^{3}H-DG and ^{3}H-GLN were performed using a 6 minutes incubation time. Moreover, ^{3}H-GLN uptake was markedly Na^+-dependent in both cell lines (Figure S2B,C).

Next, we evaluated the ability of insulin to interfere with ^{3}H-DG and ^{3}H-GLN uptake. As can be seen in Figure 3A, insulin promoted a significant increase in ^{3}H-DG uptake in both cell lines, in parallel with a significant increase in lactate production (Figure 3B). In relation to ^{3}H-GLN uptake, insulin stimulated both total and Na^+-dependent uptake in MCF-12A cells, but an opposite effect on total, Na^+-dependent and Na^+-independent ^{3}H-GLN uptake by MCF-12A$_{DMBA}$ cells was observed (Figure 3B).

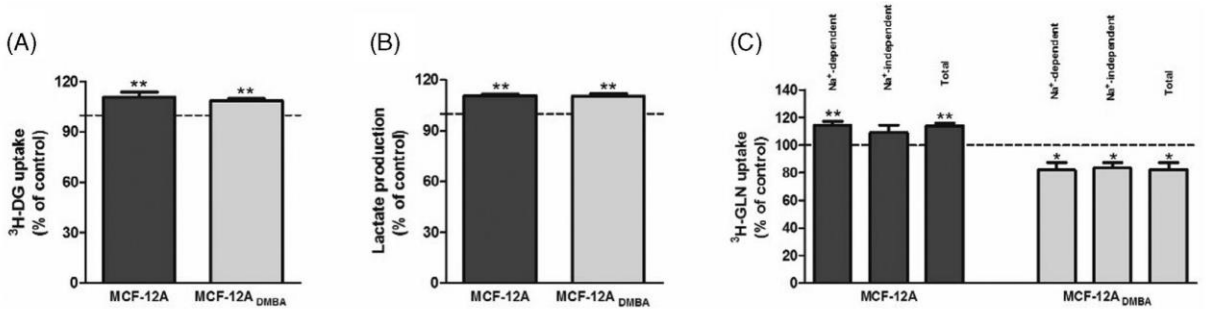

FIGURE 3 Insulin leads to changes on ^{3}H-DG and ^{3}H-GLN uptake and lactate production in DMBA-transformed (MCF-12A$_{DMBA}$) and non-tumoural (MCF-12A) cell lines. Effects of insulin (50 nM) (24 hours) upon (A) uptake of ^{3}H-DG 10 nM (n = 12), (B) lactate production (n = 4), and (C) uptake of ^{3}H-GLN 5 nM (n = 4) by non-tumoural (MCF-12A) and DMBA-transformed (MCF-12A$_{DMBA}$) cell lines. ^{3}H-GLN uptake was determined both in the presence of Na^+ (total uptake) and absence of Na^+ (Na^+-independent uptake). Na^+-dependent uptake was calculated by subtracting Na^+-independent uptake from total uptake. Data show arithmetic means ± SEM. *P < .05; *P < .01; vs control, by Student's t test

Taken together, these data suggest that glucose, but not glutamine, is likely to fuel the pro-proliferative effect of insulin in both non-tumoural and transformed breast cells.

The effect of other T2DM-associated characteristics on ^{3}H-DG and ^{3}H-GLN uptake and lactate production was also evaluated (Figure S3).

3.4 | The stimulatory effect of insulin upon ^{3}H-DG uptake is BAY-876-sensitive and PI3K- and mTOR-dependent in DMBA-transformed (MCF-12A$_{DMBA}$) and non-cancer MCF-12A cells

The previous results let us to hypothesize that the pro-proliferative effect of insulin in both MCF-12A$_{DMBA}$ and MCF-12A cells is related to a stimulatory effect on the facilitative glucose transporter 1 (GLUT1). This is because dysregulation of GLUT1 has been consistently observed in many different cancers, including breast cancer,[8,12,13] and its overexpression appears especially important for the initiation of breast tumorigenesis.[14] Accordingly, this gene was proposed as oncogene.[13,33] Very recently, a promising compound, [N4-[1-(4-cyanobenzyl)-5-methyl-3-(trifluoromethyl)-1H-pyrazol-4-yl]-7-fluoroquinoline-2,4-dicarboxamide] (BAY-876) was identified as a new-generation selective inhibitor of GLUT1.[34] Based on these facts, we decided to investigate if insulin interferes with GLUT1, by testing if the influence of BAY-876 (500 nM) on the stimulatory effect of insulin upon ^{3}H-DG uptake.

The results, presented in Figure 4A, show that BAY-876 alone caused a significant decrease (±50%) in ^{3}H-DG uptake in both cell lines. Interestingly, in the presence of BAY-876, the stimulatory effects of insulin upon ^{3}H-DG uptake were completely abolished in both cell lines (Figure 4A). Additionally, LY294002 (1 μM) and rapamycin (100 nM), which are specific inhibitors of the phosphoinositide 3-kinase (PI3K) and mammalian target of rapamycin (mTOR) intracellular signalling mechanisms, respectively, also abolished the stimulatory effect of insulin upon ^{3}H-DG uptake (Figure 5A). Alone, none of the inhibitors had significant effect on ^{3}H-DG uptake in both cell lines (Figure 5A). Taken together, these data strongly suggest that insulin stimulates GLUT1-mediated ^{3}H-DG uptake in both cell lines, and that this effect is dependent on PI3K- and mTOR-stimulation.

3.5 | The stimulatory effect of insulin upon cell proliferation is BAY-876-sensitive and PI3K- and mTOR-dependent in DMBA-transformed (MCF-12A$_{DMBA}$) and non-cancer MCF-12A cells

In the last series of experiments, we tested the influence of BAY-876, LY294002 and rapamycin upon the pro-proliferative effect of insulin in both cell lines.

BAY-876 alone caused a small but significant decrease in the proliferation rates in MCF-12A cells, but was devoid of effect in MCF-12A$_{DMBA}$ cells (Figure 4B). Interestingly, the pro-proliferative effect of insulin in both cell lines was completely abolished in the presence of BAY-876 (Figure 4B).

Finally, our results have shown that the pro-proliferative effect of insulin was prevented by simultaneous exposure to an inhibitor of PI3K (LY294002) or mTOR (rapamycin) (Figure 5B). As can also be seen in Figure 5B, LY294002 alone was able to reduce proliferation rates in both cells lines, and rapamycin also decreased cell proliferation in MCF-12A$_{DMBA}$ cells.

Taken together, these data strongly suggest that the pro-proliferative effect of insulin is dependent upon GLUT1, PI3K- and mTOR-stimulation, in both cell lines.

4 | DISCUSSION

The potential association between T2DM and breast cancer has been a subject of intense research, because several studies have shown increased risk of breast cancer development in women with T2DM.[1,4]

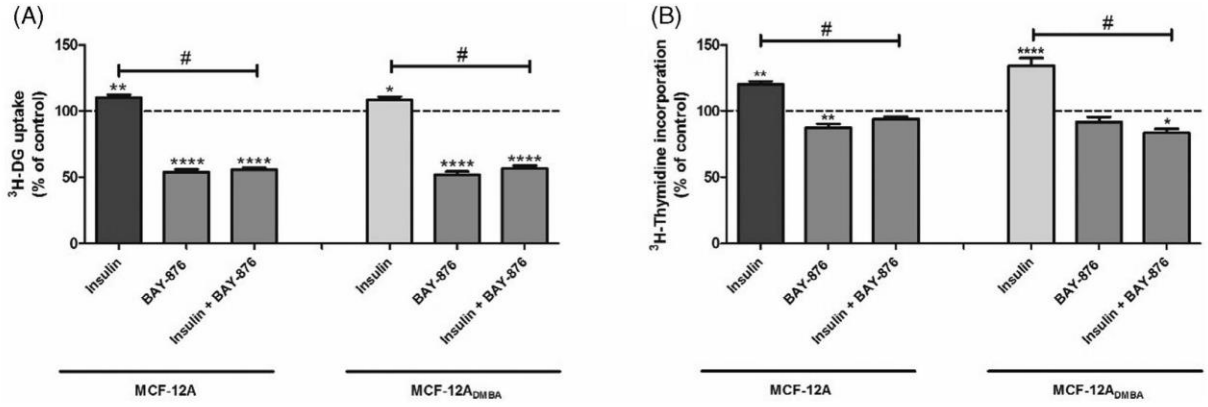

FIGURE 4 BAY-876 interferes with the stimulatory effects of insulin upon ^{3}H-DG uptake and proliferation of DMBA-transformed (MCF-12A$_{DMBA}$) and non-tumoural (MCF-12A) cell lines. Effect of insulin (50 nM) (24 hours) in the presence or absence BAY-876 (500 nM) upon (A) uptake of ^{3}H-DG 10 nM (n = 8), and (B) cell proliferation rates (n = 6) in DMBA-transformed (MCF-12A$_{DMBA}$) and non-tumoural (MCF-12A) cell lines. Data show arithmetic means ± SEM. *P < .05; **P < .01; ****P < .0001 vs control; $^{\#}P$ < .05 vs insulin, by two-way ANOVA with Newman-Keuls post hoc test

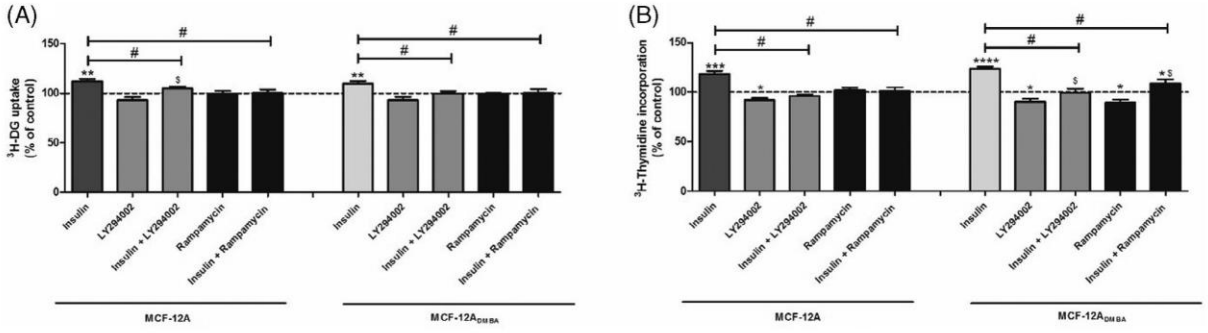

FIGURE 5 LY294002 and rapamycin interfere with the stimulatory effects of insulin upon ^{3}H-DG uptake and proliferation of DMBA-transformed (MCF-12A$_{DMBA}$) and non-tumoural (MCF-12A) cell lines. Effect of insulin (50 nM) (24 hours) in the presence or absence of LY294002 (1 μM) or rapamycin (100 nM) upon (A) uptake of ^{3}H-DG 10 nM (n = 8), and (B) cell proliferation rates (n = 9) in DMBA-transformed (MCF-12A$_{DMBA}$) and non-tumoural (MCF-12A) cell lines. Data show arithmetic means ± SEM. *P < .05; **P < .01; ***P < .001 vs control; $^{\$}P$ < .05 vs LY 294002 or rapamycin, $^{\#}P$ < .05 vs insulin, by two-way ANOVA with Newman-Keuls post hoc test

Thus, in the present study, we investigated the effect of T2DM-associated factors (hyperglycemia, hyperinsulinemia, hyperleptinemia, increased levels of inflammatory cytokines (TNF-α and INF-γ) and increased oxidative stress levels) upon cellular features that contribute to breast cancer initiation.

The use of a chemical carcinogen is an effective method to transform normal cells in order to study and compare the effects of a certain agent/compound on transformed cells.[18] In the present study, we used the chemical agent DMBA to induce carcinogenesis in a non-tumoural human breast epithelial cell line. Our results show that DMBA-transformed cells present several characteristics indicative of neoplastic transformation, namely an increase in cell proliferation rates and in the size and number of colonies and alterations in cell cycle distribution. These results prove that DMBA induced breast carcinogenesis in the non-tumoural MCF-12A cell line, confirming that this compound is an excellent model compound for inducing breast carcinogenesis.[16-18] In addition, in the present work, we use for the first time an AI-based technique—the iLoF method—to investigate if it is possible to generate an optical fingerprint specific to DMBA-induced cellular carcinogenesis. Through the optical fingerprinting of the biophysical properties of the supernatants of both DMBA-induced cells and the subpopulation of cells not submitted to this inducer, it was possible to distinguish/identify the subpopulation of cells not showing neoplastic-based features with a specificity and sensitivity above 90%, which were validated through cell proliferation assays and colony formation ability. Future work will involve the identification of the specific nanostructures present in cell supernatant, which contribute the most for the discriminatory nature of the scattering-based signal associated with the carcinogenesis process. Possible examples include specific extracellular vesicles (eg, exosomes)

produced by cells under neoplastic transformation, which significantly change the microscopic refractive index and optical polarizability of the supernatant, enhancing biophysical properties differences sensing between the two types of supernatants through scattering radiation using the iLoF method.

We then investigated the ability of T2DM-associated compounds to interfere with cell proliferation, migration, angiogenesis, viability, apoptosis, cell cycle distribution and glucose and glutamine uptake and lactate production in normal breast epithelial and DMBA-transformed cells. Of the tested compounds, insulin showed the more interesting results. Specifically, high levels of insulin present a pro-proliferative effect in MCF-12A$_{DMBA}$ and MCF12-A cell lines, accompanied by an increase in the number of MCF-12A viable cells. Curiously, a pro-angiogenic effect of insulin was observed in MCF-12A$_{DMBA}$ cells only, which suggests that the effect of insulin in regulating angiogenesis is specific to DMBA-transformed cells. The increased angiogenesis promoted by insulin seems to provide the vascular support that is essential for breast cancer initiation, observed in MCF-12A$_{DMBA}$ cells.[35] This effect of insulin agrees with a previous in vitro study from our group showing that high insulin concentrations promote cell proliferation and VEGF-A production in both ER-positive and triple-negative breast cancer cell lines.[8] So, insulin appears to stimulate angiogenesis associated with both cancer initiation and progression. Additionally, cell cycle analysis showed that insulin caused a slight but significant decrease in the G2/M phase population of non-tumoural cells, while promoting a decrease in the G1 phase cell cycle population accompanied by a significant increase in the number of cells occupying the S phase in DMBA-transformed cells. It is known that insulin upregulates the expression of cyclin D1 and E, which promote the entry of cells into the S phase in cancer cells.[36,37] Therefore, our data suggest that DMBA-transformed cells appear to acquire and share characteristics of breast cancer cells, having a similar response to high insulin levels.

Some evidence indicates that T2DM with its associated metabolic dysregulation, including hyperinsulinemia, increases the risk for a variety of cancers including breast cancer.[38,39] Hyperinsulinemia has a strong anabolic effect that leads to proliferative tissue abnormalities, stimulation of DNA synthesis, and cell proliferation.[40,41] It is thus not surprising that insulin may be an important molecular mediator of the T2DM-breast cancer connection.[38,41] For this reason, we decided to explore in more detail the impact of high levels of insulin on breast cancer initiation.

It is firmly established that metabolic reprogramming is fundamental for the development, rapid proliferation and survival of cancer cells.[9,12,42] The classical example of a reprogrammed metabolic pathway in cancer is the Warburg effect, characterized by increased glycolysis and lactate production in the presence of oxygen.[10] The hyperinsulinemia found in T2DM patients is a well-known activator of glycolysis.[12] In addition, plasma-free glutamine levels are elevated in both T2DM[43] and breast cancer[44] patients, suggesting that increased glutamine availability in T2DM could be important for breast cancer development. So, we decided to investigate the ability of insulin to change ^{3}H-DG and ^{3}H-GLN uptake and lactate production.

Interestingly, associated with increased proliferation, insulin also caused an increase in glucose uptake, accompanied by a significant increase in lactate production, both in normal breast epithelial and DMBA-transformed cells. Insulin may stimulate glucose uptake to support the increased ATP production needed for cell proliferation.[12,45] Curiously, the effect of insulin in ^{3}H-GLN uptake is distinct in normal breast epithelial and DMBA-transformed cells: total and Na^{+}-dependent ^{3}H-GLN uptake were enhanced in MCF-12A cells, but an opposite effect (decrease) on total, Na^{+}-dependent and Na^{+}-independent ^{3}H-GLN uptake by MCF-12A$_{DMBA}$ cells was observed. Unlike glucose, glutamine requirements are very heterogeneous among different cancer cell lines,[42] and it was previously reported that basal-like cells, but not luminal cell, are glutamine-sensitive.[46] Recent studies have demonstrated that the tissue of origin and the microenvironment can all impact cancer cell metabolism, including utilization of glutamine.[42] Interestingly, a recent study from our group showed that insulin reduces ^{3}H-GLN uptake by two breast cancer cell lines (MCF-7 and MDA-MB-231 cells) while increasing it in a non-cancer breast cell line (MCF-12A cells).[47] So, DMBA-transformed cells and the two breast cancer cell lines appear to show similar characteristics of ^{3}H-GLN uptake. Overall, the pro-proliferative effect of insulin in MCF-12A and MCF-12A$_{DMBA}$ is associated with a stimulation of ^{3}H-DG uptake, but these two cell lines have a differential response to insulin in relation to ^{3}H-GLN uptake. This suggests that glucose, but not glutamine, is essential for cell proliferation during breast cancer initiation.

A recent study by our group showed that the stimulatory effect of insulin upon cell proliferation and ^{3}H-DG uptake was abolished in the presence of the GLUT1 inhibitor BAY-876, both in ER-positive and triple-negative breast cancer cell lines.[8] BAY-876 is a recently promising compound identified as a new-generation selective inhibitor of GLUT1.[34] GLUT1 constitutes a principal mechanism for the increased proliferation and malignant potential of breast cancer cells,[8,12,13] and its overexpression appears especially important for the initiation of breast tumorigenesis,[14] as well as to maintain tumour growth.[12]

We thus decided to investigate if the stimulatory effect of insulin upon cell proliferation and ^{3}H-DG uptake is sensitive to BAY-876. Interestingly, BAY-876 was able to abolish both the increase in ^{3}H-DG uptake and the pro-proliferative effect of insulin in both MCF-12A and MCF-12A$_{DMBA}$ cell lines. These data thus strongly suggest that the pro-proliferative effect of insulin in both normal and DMBA-transformed breast epithelial cells is dependent on a stimulation of GLUT1-mediated glucose uptake.

Insulin binds to the insulin receptor and activates cell signalling pathways that are key regulators of cellular homeostasis. These signalling pathways are dysregulated in most aggressive cancers.[39] In the last set of experiments, we decided to further investigate the mechanisms involved in the effect of insulin upon ^{3}H-DG uptake and proliferation by investigating the involvement of mTOR and PI3K intracellular signalling pathways. By using specific inhibitors of mTOR and PI3K pathways, we could conclude that the stimulatory effect of insulin upon both proliferation and ^{3}H-DG uptake involves activation of mTOR and PI3K intracellular signalling pathways in both MCF-12A

and MCF-12A$_{DMBA}$ cell lines. These results indicate a relevant role of PI3K and mTOR in the pro-proliferative effect of insulin in breast cancer initiation.

In summary, our results suggest that insulin possess a pro-proliferative effect, which is dependent on an increase in GLUT1-mediated glucose uptake and mediated by the PI3K and mTOR signalling pathways, in both carcinogen-transformed and non-cancer cell lines. Our results thus indicate that insulin plays a central role that drives early breast cancer biology. In addition, the observation that inhibition of GLUT1, PI3K and mTOR could suppress the stimulatory effect of insulin on cell proliferation and glucose uptake in both normal breast epithelial and DMBA-transformed cells suggests that this approach could be effective for reducing/preventing breast cancer initiation in T2DM patients. Finally, the present work shows that insulin not only possesses a GLUT1-dependent pro-proliferative effect in both ER-positive and triple-negative breast cancer cell lines[8] but also promotes breast cancer initiation.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

Cláudia Silva: Investigation, Writing—Original Draft. Nelson Andrade: Investigation. João Tiago Guimarães: Investigation. Emília Cardoso: Investigation. Catarina Meireles: Investigation. Vanessa Pinto: Investigation. Joana Paiva: Investigation. Fátima Martel: Conceptualization, Formal analysis, Supervision, Visualization, Writing—review and editing, Project administration.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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REFERENCES

1. Samuel SM, Varghese E, Varghese S, Busselberg D. Challenges and perspectives in the treatment of diabetes associated breast cancer. *Cancer Treat Rev*. 2018;70:98-111.
2. Pearson-Stuttard J, Zhou B, Kontis V, Bentham J, Gunter MJ, Ezzati M. Worldwide burden of cancer attributable to diabetes and high body-mass index: a comparative risk assessment. *Lancet Diabetes Endocrinol*. 2018;6(2):95-104.
3. Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2021;71:209-249.
4. Larsson SC, Mantzoros CS, Wolk A. Diabetes mellitus and risk of breast cancer: a meta-analysis. *Int J Cancer*. 2007;121(4):856-862.
5. Doerstling SS, O'Flanagan CH, Hursting SD. Obesity and cancer metabolism: a perspective on interacting tumor-intrinsic and extrinsic factors. *Front Oncol*. 2017;7:216.
6. Vincent EE, Yaghootkar H. Using genetics to decipher the link between type 2 diabetes and cancer: shared aetiology or downstream consequence? *Diabetologia*. 2020;63(9):1706-1717.
7. Feng Y, Spezia M, Huang S, et al. Breast cancer development and progression: risk factors, cancer stem cells, signaling pathways, genomics, and molecular pathogenesis. *Genes Dis*. 2018;5(2):77-106.
8. Silva C, Andrade N, Guimaraes JT, Patricio E, Martel F. The in vitro effect of the diabetes-associated markers insulin, leptin and oxidative stress on cellular characteristics promoting breast cancer progression is GLUT1-dependent. *Eur J Pharmacol*. 2021;898:173980.
9. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144(5):646-674.
10. Warburg O, Wind F, Negelein E. The metabolism of tumors in the body. *J Gen Physiol*. 1927;8(6):519-530.
11. Vander Heiden MG. Targeting cancer metabolism: a therapeutic window opens. *Nat Rev Drug Discov*. 2011;10(9):671-684.
12. Martin SD, McGee SL. Metabolic reprogramming in type 2 diabetes and the development of breast cancer. *J Endocrinol*. 2018;237(2):35-46.
13. Barbosa AM, Martel F. Targeting glucose transporters for breast cancer therapy: the effect of natural and synthetic compounds. *Cancer*. 2020;12(1):154.
14. Wellberg EA, Johnson S, Finlay-Schultz J, et al. The glucose transporter GLUT1 is required for ErbB2-induced mammary tumorigenesis. *Breast Cancer Res*. 2016;18(1):131.
15. Wang H, Leung LK. The carotenoid lycopene differentially regulates phase I and II enzymes in dimethylbenz[a]anthracene-induced MCF-7 cells. *Nutrition*. 2010;26(11–12):1181-1187.
16. Calaf G, Russo J. Transformation of human breast epithelial cells by chemical carcinogens. *Carcinogenesis*. 1993;14(3):483-492.
17. Csiszar A, Balasubramanian P, Tarantini S, et al. Chemically induced carcinogenesis in rodent models of aging: assessing organismal resilience to genotoxic stressors in geroscience research. *GeroScience*. 2019;41(2):209-227.
18. Hussain-Hakimjee EA, Peng X, Mehta RR, Mehta RG. Growth inhibition of carcinogen-transformed MCF-12F breast epithelial cells and hormone-sensitive BT-474 breast cancer cells by 1alpha-hydroxyvitamin D5. *Carcinogenesis*. 2006;27(3):551-559.
19. Paiva JS, Jorge PAS, Ribeiro RSR, et al. Author correction: iLoF: an intelligent lab on fiber approach for human cancer single-cell type identification. *Sci Rep*. 2020;10(1):10775.
20. Paiva JS, Jorge PAS, Ribeiro RSR, Sampaio P, Rosa CC, Cunha JPS. Optical fiber-based sensing method for nanoparticle detection through supervised back-scattering analysis: a potential contributor for biomedicine. *Int J Nanomedicine*. 2019;14:2349-2369.
21. Paiva JS, Ribeiro RSR, Cunha JPS, Rosa CC, Jorge PAS. Single particle differentiation through 2D optical fiber trapping and Back-scattered signal statistical analysis: an exploratory approach. *Sensors*. 2018;18(3):710.
22. Andrade N, Silva C, Martel F. The effect of oxidative stress upon intestinal sugar transport: an in vitro study using human intestinal epithelial (Caco-2) cells. *Toxicol Res*. 2018;7(6):1236-1246.
23. Araujo JR, Correia-Branco A, Ramalho C, Keating E, Martel F. Gestational diabetes mellitus decreases placental uptake of long-chain

polyunsaturated fatty acids: involvement of long-chain acyl-CoA synthetase. *J Nutr Biochem*. 2013;24(10):1741-1750.
24. Silva C, Nunes C, Correia-Branco A, Araujo JR, Martel F. Insulin exhibits an antiproliferative and hypertrophic effect in first trimester human extravillous trophoblasts. *Reprod Sci*. 2017;24(4):582-594.
25. Wolczyk D, Zaremba-Czogalla M, Hryniewicz-Jankowska A, et al. TNF-alpha promotes breast cancer cell migration and enhances the concentration of membrane-associated proteases in lipid rafts. *Cell Oncol (Dordr)*. 2016;39(4):353-363.
26. Thibault R, De Coppet P, Daly K, et al. Down-regulation of the monocarboxylate transporter 1 is involved in butyrate deficiency during intestinal inflammation. *Gastroenterology*. 2007;133(6):1916-1927.
27. Novotny L, Hecht B. *Principles of Nano-Optics*. Cambridge: Cambridge University Press; 2006.
28. Eckardt RC. *Nonlinear Optics, Basics|χ(2)–Harmonic Generation*. Amesterdam: Elsevier; 2005.
29. Marques de Sá JP. *Pattern Recognition*. Berlin: Springer-Verlag; 2001.
30. Silva C, Correia-Branco A, Andrade N, et al. Selective pro-apoptotic and antimigratory effects of polyphenol complex catechin:lysine 1:2 in breast, pancreatic and colorectal cancer cell lines. *Eur J Pharmacol*. 2019;859:172533.
31. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;72:248-254.
32. Borowicz S, Van Scoyk M, Avasarala S, et al. The soft agar colony formation assay. *J Vis Exp*. 2014;92:51998.
33. Martel F, Guedes M, Keating E. Effect of polyphenols on glucose and lactate transport by breast cancer cells. *Breast Cancer Res Treat*. 2016;157(1):1-11.
34. Siebeneicher H, Cleve A, Rehwinkel H, et al. Identification and optimization of the first highly selective GLUT1 inhibitor BAY-876. *ChemMedChem*. 2016;11(20):2261-2271.
35. Rose DP, Vona-Davis L. The cellular and molecular mechanisms by which insulin influences breast cancer risk and progression. *Endocr Relat Cancer*. 2012;19(6):225-241.
36. Mawson A, Lai A, Carroll JS, Sergio CM, Mitchell CJ, Sarcevic B. Estrogen and insulin/IGF-1 cooperatively stimulate cell cycle progression in MCF-7 breast cancer cells through differential regulation of c-Myc and cyclin D1. *Mol Cell Endocrinol*. 2005;229(1–2):161-173.
37. Weichhaus M, Broom J, Wahle K, Bermano G. A novel role for insulin resistance in the connection between obesity and postmenopausal breast cancer. *Int J Oncol*. 2012;41(2):745-752.
38. Ferroni P, Riondino S, Buonomo O, Palmirotta R, Guadagni F, Roselli M. Type 2 diabetes and breast cancer: the interplay between impaired glucose metabolism and oxidant stress. *Oxidative Med Cell Longev*. 2015;2015:183928.
39. Yee LD, Mortimer JE, Natarajan R, Dietze EC, Seewaldt VL. Metabolic health, insulin, and breast cancer: why oncologists should care about insulin. *Front Endocrinol*. 2020;11:58.
40. Biello F, Platini F, D'Avanzo F, et al. Insulin/IGF Axis in breast cancer: clinical evidence and translational insights. *Biomolecules*. 2021;11(1):125.
41. Xia B, Hou L, Kang H, et al. NR2F2 plays a major role in insulin-induced epithelial-mesenchymal transition in breast cancer cells. *BMC Cancer*. 2020;20(1):626.
42. DeBerardinis RJ, Chandel NS. Fundamentals of cancer metabolism. *Sci Adv*. 2016;2(5):1600200.
43. Zhou Y, Qiu L, Xiao Q, et al. Obesity and diabetes related plasma amino acid alterations. *Clin Biochem*. 2013;46(15):1447-1452.
44. Bi X, Henry CJ. Plasma-free amino acid profiles are predictors of cancer and diabetes development. *Nutr Diabetes*. 2017;7(3):249.
45. Zhao K, Liu HY, Zhou MM, Zhao FQ, Liu JX. Insulin stimulates glucose uptake via a phosphatidylinositide 3-kinase-linked signaling pathway in bovine mammary epithelial cells. *J Dairy Sci*. 2014;97(6):3660-3665.
46. van Geldermalsen M, Wang Q, Nagarajah R, et al. ASCT2/SLC1A5 controls glutamine uptake and tumour growth in triple-negative basal-like breast cancer. *Oncogene*. 2016;35(24):3201-3208.
47. Silva C, Andrade N, Rodrigues I, Ferreira AC, Soares ML, Martel F. The pro-proliferative effect of interferon-gamma in breast cancer cell lines is dependent on stimulation of ASCT2-mediated glutamine cellular uptake. *Life Sci*. 2021;286:120054.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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SUPPLEMENTARY DATA

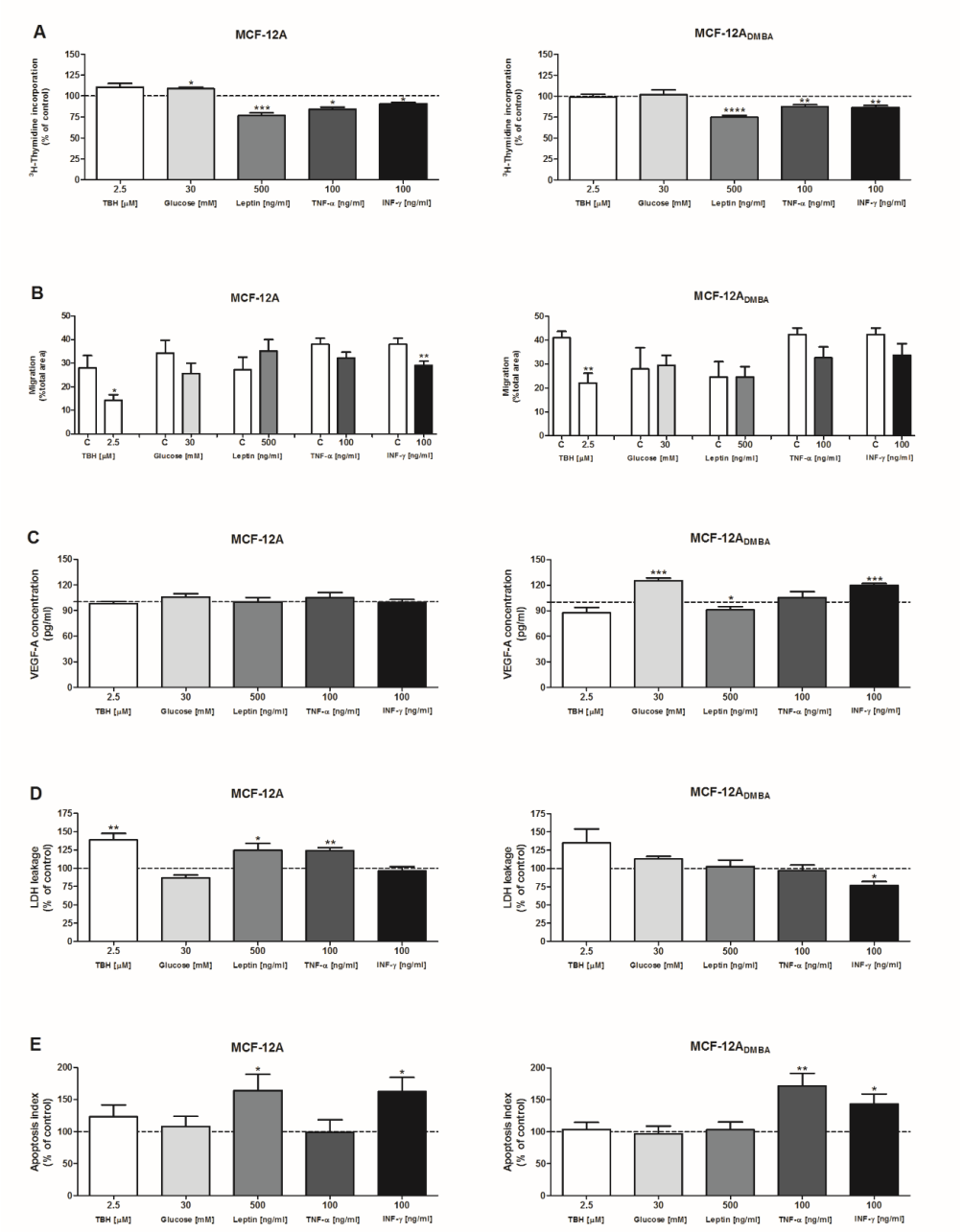

Figure S1. The effect of T2DM-associated characteristics on cell proliferation, migration, angiogenesis, viability and apoptosis in DMBA-transformed MFC12A (MCF-12A$_{DMBA}$) and non-tumoral (MFC12A) cell lines.

Effects of TBH (2.5 μM), glucose (30 mM) leptin (500 ng/ml), TNF-α (100 ng/ml) or INF-γ (100 ng/ml) (24h) on (A) cell proliferation rates (n=6-9), (B) cell migration (n=6-9), (C) VEGF-A levels (n=6), (D) cell viability (n=6-9) and (E) apoptosis index (n=6) in non-tumoral (MCF-12A) (*left graphs*) and DMBA-transformed MCF-12A cells (MCF-12A$_{DMBA}$) (*right graphs*). Data show arithmetic means ± S.E.M. *P < 0.05; **p<0.01; ***p<0.001 vs control, by Student's *t* test.

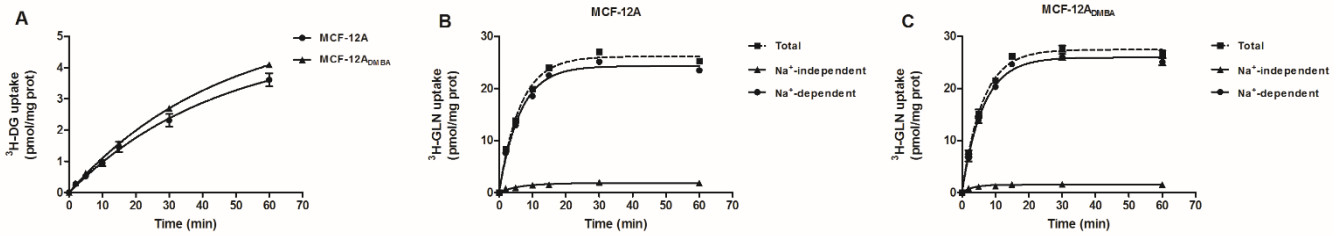

Figure S2. Time-course of ^{3}H-DG and ^{3}H-GLN uptake by DMBA-transformed MFC12A (MCF-12A$_{DMBA}$) and non-tumoral (MFC12A) cell lines.

DMBA-transformed (MCF-12A$_{DMBA}$) and non-tumoral (MCF-12A) cell lines were incubated for various periods of time at 37°C with (**A**) 10 nM ^{3}H-DG (n=8) or (**B**, **C**) 5 nM ^{3}H-GLN (n=4). ^{3}H-GLN uptake was determined both in the presence of Na^+ (total uptake) or absence of Na^+ (Na^+-independent uptake). Na^+-dependent uptake was calculated by subtracting Na^+-independent uptake from total uptake. Shown are arithmetic means ± S.E.M.

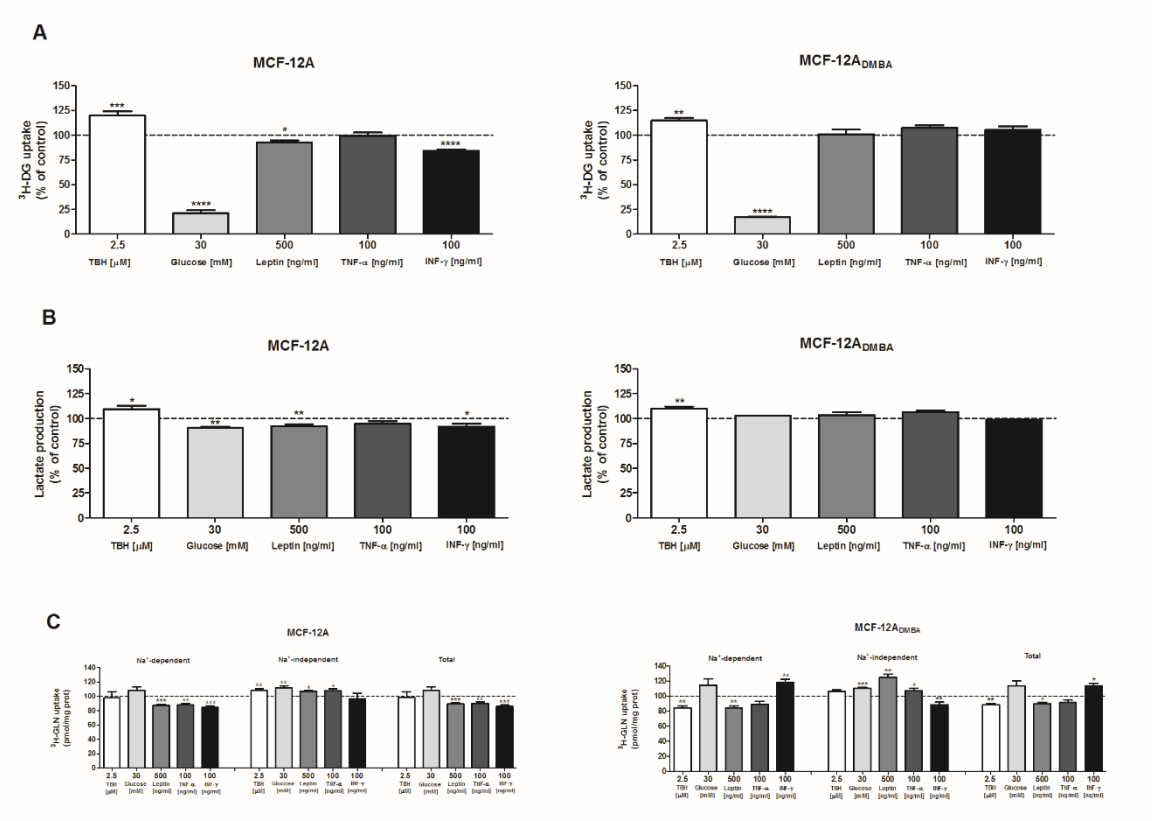

Figure S3. The effect of T2DM-associated characteristics on ^{3}H-DG and ^{3}H-GLN uptake and lactate production in DMBA-transformed MFC12A (MCF-12A$_{DMBA}$) and non-tumoral (MFC12A) cell lines.

Effect of TBH (2.5 μM), glucose (30 mM) leptin (500 ng/ml), TNF-α (100 ng/ml) or INF-γ (100 ng/ml) (24h) upon (A) uptake of ^{3}H-DG 10 nM (n=8-12) (B) lactate production (n=4) and on (C) uptake of ^{3}H-GLN 5 nM (n=4) in DMBA-transformed (MCF-12A$_{DMBA}$) (*right graphs*) and non-tumoral (MCF-12A) (*left graphs*) cell lines. ^{3}H-GLN uptake was determined in the presence of Na^+ (total uptake) or absence of Na^+ (Na^+-independent uptake). Na^+-dependent uptake was calculated by subtracting Na^+-independent uptake from total uptake. Data show arithmetic means ± S.E.M. *P < 0.05; **p<0.01; ***p<0.001; **** p<0.0001 vs control, by Student's *t* test.

CHAPTER VI

GENERAL DISCUSSION

GENERAL DISCUSSION

T2DM and breast cancer are diagnosed in the same individual more frequently than would be expected [2, 44, 127, 149]. During the last decades, many efforts were made to understand which factors contribute to the higher incidence of breast cancer seen among patients with T2DM compared to patients without T2DM. The metabolic alterations that occur in T2DM can probably explain this link [2, 8, 9, 44]. However, to date, the studies done haven't been sufficient to identify the mechanisms underlying the link between T2DM and breast cancer. It is therefore critical to discover new molecular targets that link T2DM and breast cancer and to develop new therapeutic approaches that prevent and control breast cancer initiation, promotion and progression in patients with T2DM. Cancer cells are able to utilize various forms of fuels such as glucose, amino acids or lactate to support proliferation [150]. Alterations in the hormonal and metabolic milieu associated with T2DM, namely insulin resistance (hyperinsulinemia and hyperglycemia), altered adipokine levels (higher leptin and lower adiponectin), chronic low-grade inflammation (higher TNF-α, IFN-γ, IL-6 and IL-1β levels) and increased oxidative stress levels, increase the availability of nutrients in the tumour microenvironment. By meeting the bioenergetics needs of tumour cells, these nutrients are essential to sustain rapid proliferation and unrestricted growth thus providing a favourable metabolic environment for the initiation, promotion and progression of breast cancer [2, 23, 44].

The present study focused in investigating the link between metabolic characteristics associated with T2DM and the cellular uptake of important nutrients for breast cancer cells. More specifically, we aimed to investigate the effect of T2DM-associated characteristics (hyperglycemia, hyperinsulinemia, hyperleptinemia, increased levels of inflammation and oxidative stress) in glucose and glutamine cellular uptake, and if changes in transport of these nutrients can contribute to the effect of T2DM in favouring cancer initiation, promotion, progression, and in negatively affecting response to therapy.

In addition, this work explored and compared the role of T2DM-associated characteristics in different breast cancer subtypes. The heterogeneity in breast cancer determines the course of disease, type of treatment and even patient survival [2]. Each subtype of breast cancer displays distinct features and metabolic alterations [146]. Some existing data suggests that the link between T2DM and breast cancer varies by intrinsic cancer subtype, but these data are yet limited [151]. Although there is some inconsistency in the literature, in T2DM women, the most

frequently found breast cancer subtype is TN breast cancer [151]. However, other tumours subtypes such as, ER^+, PR^+ and $HER2^+$ [152-154] are also frequently observed in T2DM women, compared to women without a history of T2DM. However, the biological mechanisms behind a potential increased risk of specific subtypes of breast cancer in women with T2DM are not well understood.

In our study, we decided to use the MCF-12A (ER^+, PR^+ and $HER2^-$) cell line, as a representative cell type of non-tumoral human breast epithelia, and two breast cancer cell lines, representatives of the luminal A and of the basal-like molecular subtypes: the MCF-7 (ER^+, PR^+ and $HER2^-$) and the MDA-MB-231 (TN; ER^-, PR^- and $HER2^-$) cell lines, respectively [155, 156]. MCF-7 and MDA-MB-231 cell lines represent a breast tumour cell in an early-stage and late-stage of progression, respectively [157-159]. So, we used these two cell lines with different metastatic potentials as models of breast cancer progression.

The MCF-12A cell line do not provides insight into the effects of a certain agent/compound on early-stages of malignant tissues, but is useful to understand its effects in normal tissue [160]. Thus, breast cancer initiation and promotion can be studied in this cell line, by using a specific breast carcinogen. In the present study, breast cancer initiation and promotion were induced in MCF-12A cells by using the mammary carcinogen DMBA. The assessment of *in vitro* transformation of human breast epithelial cells by chemical carcinogens requires the identification of malignant phenotypes - which are considered markers of cell transformation - such as enhanced growth, invasiveness, expression of clonal growth and anchorage-independent growth, which is a late marker and is correlated with tumorigenicity [22, 161, 162]. In the present study, we verified that DMBA-transformed MCF-12A cells presented several characteristics indicatives of neoplastic transformation, namely an increase in cell proliferation rates and in the size and number of colonies and alterations in cell cycle distribution. Additionally, we decided to use for the first time an AI-based technique – the iLoF method – to investigate if an optical fingerprint specific of DMBA-induced cellular carcinogenesis was generated. Interestingly, the iLoF technique was able to distinguish the supernatant of DMBA-transformed cells from the supernatant of cells not submitted to this carcinogen with a specificity and sensitivity above 90% **(Chapter V)**. Taken together, these results proved that DMBA induced breast carcinogenesis in the non-tumorigenic MCF-12A cell line, confirming this compound as an excellent model compound for inducing breast carcinogenesis [160, 161, 163].

In this study, we first investigated the importance of T2DM-associated characteristics upon cellular features that contribute to breast cancer initiation, promotion and progression. In this context, we analysed the effect of a 24h-exposure to high levels of glucose (15-30 mM), insulin (1-50 nM), leptin (10-500 ng/ml), pro-inflammatory cytokines (TNF-α and IFN-γ; 1-100 ng/ml) and high oxidative stress levels (by using the oxidative stress inducer tert-butylhydroperoxide (TBH); 0.5-2.5 μM) on cell proliferation, growth, migration, viability, apoptosis index and angiogenesis in breast cancer cells (MCF-7 and MDA-MB-231) and DMBA-transformed (MCF-12A$_{DMBA}$) and non-transformed breast epithelial (MCF-12A) cells. These characteristics of cancer cells are essential to sustain tumour growth and metastasis. Unexpectedly, of all tested compounds, glucose was the one that interfered the least with the cellular features mentioned. Glucose (15-30 mM) caused only an increase in MCF-12A cells proliferation rates, in VEGF-A production by MCF-12A$_{DMBA}$ cells and in the migration capacity of MDA-MB-231 cells **(Chapter III and Chapter V)**. We expected that the hyperglycemia that occurs in T2DM could have a more profound effect on breast cancer cell biology, by providing a greater glucose availability to drive cell proliferation. However, it is important to note that under control conditions, the *in vitro* experiments were conducted in media with non-physiological levels of glucose (11-17 mM, compared to 5.5 mM in plasma) [146]; therefore, an adaptation of cells to a high glucose environment may explain the lack of results.

Regarding insulin, this hormone (10-50 nM) was found to promote cell proliferation, migration, angiogenesis and viability in ER$^+$ and TN breast cancer cells and, additionally, it promoted apoptosis in the ER$^+$ cell line **(Chapter III)**. According to Gupta *et al.*, high insulin levels induce severe oxidative stress in MCF-7 cells, that result in cell death, whereas in MDA-MB-231 cells, which have a more aggressive phenotype, it induces proliferation [159]. In contrast, most previous *in vitro* studies indicated that insulin increases cell proliferation in ER$^+$ [133, 164, 165] but not in ER$^-$ [133, 165] breast cancer cells. Interestingly, MDA-MB-231 breast cancer cells have an elevated IR content as they carry an uncommon IR gene amplification [166], indicating that insulin may play a role in the metabolism of this cell line. Although there is some inconsistency in the literature, high insulin was reported to promote proliferation of MDA-MB-231 cells by alteration of glycogen synthase kinase 3 beta (GSK-3β), NF-κB, and histone H3 modifications, which may directly or indirectly modulate the expression of genes involved in proliferation [159]. DMBA-transformed cells were also analysed following insulin treatment. Similar to both breast cancer cell lines, insulin presented pro-proliferative and pro-angiogenic effects in MCF-12$_{DMBA}$ cells

(Chapter V) which suggests that the effect of insulin in regulating cell proliferation and angiogenesis is not specific to the breast tumour cells (both in early-stage and late-stage of progression), but also plays a central role in promotion of breast cancer. In addition, the increased angiogenesis induced by insulin seems to provide the vascular support that is essential for breast cancer promotion and progression [133]. Additionally, cell cycle analysis showed that insulin caused a decrease in the G1 phase population accompanied by a significant increase in the number of cells occupying the S phase in DMBA-transformed MCF-12 cells **(Chapter V)**. It is known that insulin upregulates the expression of cyclin D1 and E, which promote the entry of cells into the S phase in cancer cells [165, 167]. It is important to note that the MCF-12A cells used in the experiments to promote breast cancer (DMBA treatments), which were cultured in media with fetal bovine serum (FBS), presented a few different results in the cellular features in the presence of T2DM-associated markers, particularly insulin, when compared with cells that were cultured in media with horse serum (HS) (in the experiments to study breast cancer progression). More specifically, we verified some differences in the effect of insulin on cell proliferation, viability, ^{3}H-DG uptake and lactate production. In the non-tumorigenic cell line cultivated with HS, insulin increased cell viability **(Chapter III)**. Curiously, in the non-tumorigenic cell line cultivated with FBS, insulin reduced cell viability, enhanced proliferation and interfered with cell cycle progression **(Chapter III)**, indicating that insulin may also contribute to breast cancer initiation. It is well known that different sera are capable of distinctly affecting cell behaviours such as cell attachment, growth and proliferation [168]. Although some differences exist, our data suggest that DMBA-transformed cells appear to acquire characteristics of breast cancer cells, having a similar response to high insulin levels. So, insulin appears to stimulate proliferation associated with both cancer initiation, promotion and progression.

Concerning leptin, our data demonstrate that leptin (10-500 ng/ml) exerted distinct effects in the *in vitro* models of breast cancer progression (MCF-7 and MDA-MB-231), and promotion (MCF-12A$_{DMBA}$), as it increases cell proliferation, migration and viability in ER^{+} and TN cells, but decreases proliferation in DMBA-transformed breast epithelial cells; moreover, it presented a pro-angiogenic effect in the TN cell line, an anti-angiogenic effect in DMBA-transformed cells and promoted apoptosis in ER^{+} cells **(Chapter III and Chapter V)**. Leptin was previously reported to promote breast cancer cell proliferation, angiogenesis and metastasis and to inhibit apoptosis by regulating multiple intracellular signalling pathways including JAK/STAT3, MAPK/ERK, PI3K/AKT, PKC, JNK, and p38 [2]. Additionally, while high plasma leptin levels

increase the risk for breast cancer in postmenopausal women, lower leptin levels are related to higher breast cancer risk in premenopausal women [169], suggesting that leptin may increase or decrease risk of in breast cancer depending on menopausal status. However, most reports indicate that higher leptin serum levels are associated with advanced stage breast cancer [136, 170-173]. So, the role of leptin in breast cancer promotion has not been thoroughly defined, as there is no uniform conclusions in the literature about the effect of leptin on the risk and prognosis of breast cancer. Some studies comparing the influence of leptin in different breast cancer subtypes found significant differences in leptin and OB-R expression levels among breast cancer patients with different molecular subtypes ($HER2^+$, ER^+, and TN), with levels of leptin in TN > $HER2^+$ > ER^+ patients [174, 175]. Additionally, patients in the progressive disease group had high and low expression of leptin and OB-R, respectively [175]. Our results suggest that high leptin levels may aggravate breast tumour both in an early-stage and late-stage of progression (**Chapter III**). In contrast, no effect on proliferation, migration and angiogenesis, a decrease in cell viability and a pro-apoptotic effect was observed with this hormone in non-tumorigenic MCF-12A cells **(Chapter III)**. So, overall, our results suggest leptin appears to be involved in progression of breast tumorigenesis, but not in the initiation and promotion of breast tumorigenesis.

In this work, we studied two important pro-inflammatory cytokines involved in the inflammation process of T2DM, TNF-α and IFN-γ. TNF-α (1-100 ng/ml) presented an anti-proliferative, cytotoxic and pro-migratory effect in the two cellular models of cancer progression, an anti-migratory effect in the non-tumorigenic cell line, a pro-apoptotic effect in MCF-7 and MCF-$12A_{DMBA}$ cell lines, a pro-angiogenic effect in MDA-MB-231 cells and an anti-angiogenic effect in MCF-7 cells. As to IFN-γ (1-100 ng/ml), this cytokine presented an anti-proliferative effect in all cell lines (although it increased culture growth in ER^+ and TN breast cancer cells; see below), increased apoptosis and reduced migration in all cell lines with the exception of the TN breast cancer cell line, increased cell viability in ER^+ and DMBA-transformed MCF12A cells, and presented a pro-angiogenic effect in MDA-MB-231 and MCF-$12A_{DMBA}$ cells, but an anti-angiogenic effect in MCF-7 cells **(Chapter III and Chapter V)**. Some reports concluded that these compounds could create a local microenvironment conducive to enhanced breast cancer cell survival and proliferation, and to the promotion of angiogenesis and metastatic dissemination [141, 176-178], although the relationship may be influenced by the molecular subtype. For instance, Herrera *et al.* found that plasmatic TNF-α levels were high in patients with luminal tumours, but low in TN breast cancer [179]. On the contrary, other reports concluded that TNF-α and IFN-γ inhibited the

growth rate and decreased the number of ER^+ breast cancer cells [140, 177, 180, 181]. Some of these observations (the reduction in the growth rate) differs from our results. Unexpectedly, our results suggest that IFN-γ causes hypertrophy of ER^+ and TN breast cancer cells, since it reduces the synthesis of DNA (ie, an antiproliferative effect), while simultaneously increasing the cellular protein content (ie, culture growth). In relation to apoptosis, TNF-α and IFN-γ were reported to stimulate apoptosis in MCF-7 cells, but no effect on apoptosis was observed in MDA-MB-231 cells [140, 177, 181], thus corroborating our results and suggesting that the apoptosis mechanisms in different breast cancer subtypes respond differently to an inflammatory microenvironment.

ROS are known to have a controversial role during breast cancer initiation, promotion and progression [182], and our results are a proof of that. We showed that severe oxidative stress induced by TBH resulted in an anti-migratory effect in MCF-7, MCF-12A$_{DMBA}$ and MCF-12A cells and an anti-proliferative effect in MCF-7 cells, whereas in MDA-MB-231 cells, it presented pro-migratory and pro-proliferative effects, although a cytotoxic effect was found in all cell lines, except for the DMBA-transformed cells. Additionally, in both models of breast cancer progression, TBH was able to stimulate apoptosis and angiogenesis **(Chapter III and Chapter V)**. Oxidative stress appears to contribute to a microenvironment favourable for the development of T2DM-related cancers, as it has been widely implicated in the initiation and progression of breast cancer [159] and can be caused by several T2DM-associated mechanisms, including high levels of glucose, insulin, leptin, and chronic inflammation [176]. ROS present various biological effects, with non-linear characteristics. In high oxidative stress environments, they may cause deleterious cellular effects (anti-proliferative, pro-necrotic and pro-apoptotic), while in low or medium oxidative stress environments, ROS may induce DNA damage, inflammation and cell proliferation, eventually promoting carcinogenesis [183]. So, the distinct effects of TBH in relation to cell proliferation in the two progression models of breast cancer may be related to differences in ROS levels and ROS dependence in MCF-7 (less aggressive and invasive potential) and MDA-MB-231 (more aggressive and invasive potential) cells. In MCF-7 cells, high levels of ROS might result mainly in cell death, whereas in MDA-MB-231, it may stimulate proliferation. This can be explained if it is assumed that MDA-MB-231, but not MCF-7 cells, need high levels of ROS to maintain survival. Supporting our results, a recent study observed that increased ROS levels are essential for TN breast cancer cells survival, but not for survival of ER^+ breast cancer cells or non-tumorigenic breast cells [182]. Moreover, they identified the mitochondria (indeed one of the main sources of intracellular ROS) [184] as the main source of ROS in TN breast cancer cell lines

[182]. So, oxidative stress does not appear to trigger breast cancer initiation and promotion but seems to constrain early-stage of breast cancer while having as opposite effect in more advanced states.

Overall, our data show that several T2DM-associated characteristics induce alterations in cellular features that may eventually contribute to the initiation, promotion and progression of breast tumorigenesis.

Because glucose and glutamine are important metabolic substrates of cancer cells, we next evaluated the ability of T2DM-associated conditions to induce an increase in glucose (^{3}H-DG; 10 nM and/or 1 mM) and/or glutamine (^{3}H-GLN; 5 nM and/or 0.5 mM) uptake and/or lactate production, which can contribute to the stimulatory effect of T2DM in breast cancer initiation, promotion and/or progression. It is firmly established that metabolic reprogramming is fundamental for the development, rapid proliferation, invasion and survival of cancer cells [22, 23, 44, 146]. Metabolic reprogramming varies significantly between cancers based on genetics and environment, and may manifest through up or downregulation of specific metabolic pathways, through downstream effects of oncogenes [146]. The classical example of a reprogrammed metabolic pathway in cancer cells is the Warburg effect [25]. In addition, many cancer cells also display reliance on glutamine, termed glutamine addition [32, 34, 35] (see Introduction). However, little is known about the effect of the metabolic changes found in T2DM on glucose or glutamine metabolism in the context of breast cancer initiation, promotion or progression, and so we decided to investigate this point.

Of the T2DM-associated conditions analysed, IFN-γ and insulin displayed the most interesting effects. IFN-γ induced a cancer cell-specific stimulatory effect on ^{3}H-GLN cellular uptake (total and Na^{+}-sensitive) in cell models of either cancer promotion and progression (MCF-12A$_{DMBA}$, MCF-7 and MDA-MB-23 cells) and on ^{3}H-DG cellular uptake and lactate production in both cell models of breast cancer progression (MCF-7 and MDA-MB-231 cell lines), while reduced the ^{3}H-GLN and ^{3}H-DG cellular uptake in model of breast cancer initiation (MCF-12A). Similar to IFN-γ, insulin showed a differential effect in ^{3}H-GLN uptake by cell models of breast cancer promotion and progression (MCF-12A$_{DMBA}$ MCF-7 and MDA-MB-23 cells) (reduction) and initiation (MCF-12A) (increase). Regarding ^{3}H-DG uptake and lactate production, these were stimulated by insulin, in non-tumorigenic cells (MCF-12A cells cultivated with FBS) and in cell models of cancer promotion and progression (MCF-12A$_{DMBA}$ MCF-7 and MDA-MB-23 cells). Nevertheless, other T2DM-associated compounds were also found to interfere with ^{3}H-DG and/or ^{3}H-GLN cellular

uptake. Intriguingly, TBH increased total and Na^+-dependent ^{3}H-GLN uptake by MDA-MB-231 cells but reduced total and Na^+-dependent ^{3}H-GLN uptake by MCF-7, MCF-12A$_{DMBA}$ and MCF-12A cells; on the other hand, ^{3}H-DG uptake and lactate production were increased by TBH in all cell lines. As seen with cell proliferation, TBH presented distinct effects on ^{3}H-GLN uptake by the two models of breast cancer progression (MCF-7 and MDA-MB-231 cell lines). Increased ROS levels in TN breast cancer cells seem to be necessary for maintenance and survival of these cells, in comparison with ER^+ breast cancer and non-tumorigenic cell lines [182]. As to leptin, it reduced total and Na^+-dependent ^{3}H-GLN uptake by all cell models (breast cancer promotion and progression) and, more pronouncedly, by non-tumorigenic cells (initiation). In relation to ^{3}H-DG uptake, leptin stimulated ^{3}H-DG uptake and lactate production only in ER^+ cells and reduced these parameters in non-tumorigenic cells. TNF-α did not interfere with ^{3}H-GLN and ^{3}H-DG uptake and lactate production in cancer promotion and progression cell models, but reduced ^{3}H-GLN uptake in the non-tumorigenic cell line (Table 1) **(Chapter III, Chapter IV and Chapter V)**. Moreover, Na^+-independent ^{3}H-GLN uptake was also stimulated by some of the T2DM-associated compounds. Namely, in MCF-7 cells, by IFN-γ and leptin, in MCF-12A$_{DMBA}$ cells, by glucose and leptin and in MCF-12A cells, by glucose, TBH, insulin, leptin and TNF-α **(Chapter IV and Chapter V)**.

Importantly, in all cell lines, the effects of T2DM-associated characteristics on lactate production were consistent with their effects on ^{3}H-DG (10 nM) uptake. This shows that the effect on the uptake of a low ^{3}H-DG concentration (10 nM) is a very good indicator of glucose handling by the cells. Other interesting aspect is that, with a few exceptions, many of the effect of T2DM-associated markers were also observed at concentrations of ^{3}H-DG (1 mM) and ^{3}H-GLN (0.5 mM) comparable to the human physiological concentration (5.5 mM [146] and 0.5 mM [185], respectively) **(Chapter III and Chapter IV)**. So, analysis of the effect of these compounds on low ^{3}H-DG and ^{3}H-GLN concentrations is a very reliable indicator of their effect on glutamine or glucose uptake *in vivo*.

Lastly, the effect of glucose on ^{3}H-GLN and ^{3}H-DG uptake and lactate production was only evaluated in relation to the initiation and promotion of breast tumorigenesis, because, in ER^+ and TN breast cancer cells, cellular features associated with cancer progression such as cell proliferation, viability were not affected by glucose. In MCF-12A$_{DMBA}$ cells, glucose decreased ^{3}H-DG uptake, but did not interfere with lactate production and ^{3}H-GLN uptake (Table 1) **(Chapter III)**.

Altogether, these data suggest that T2DM-associated features, in particular hyperinsulinemia, hyperleptinemia, high inflammatory status (caused by IFN-γ) and oxidative

stress, can regulate nutrient transport to support development, rapid proliferation, invasion and survival of cancer cells. On the other hand, hyperglycemia do not seems to have a great impact upon glucose and glutamine transport in the context of cancer initiation, promotion and progression.

Table 1. Summary of the effect of T2DM-associated conditions upon ^{3}H-DG and ^{3}H-GLN uptake and lactate production in breast cancer initiation, promotion and progression cell models.

T2DM-associated characteristics	Cell type			
	MCF-7 (ER^{+}, PR^{+}, HER2)	**MDA-MB-231** (ER-, PR-, HER2)	**MCF-12A$_{DMBA}$** (ER^{+}, PR^{+}, HER2)	**MCF12A** (ER^{+}, PR^{+}, HER2)
Hyperglycemia				
Glucose uptake	x	x	Decreased	Decreased
Glutamine uptake	x	x	=	=
Lactate production	x	x	=	Decreased
Hyperinsulinemia				
Glucose uptake	Increased	Increased	Increased	Increased
Glutamine uptake	Decreased	Decreased	Decreased	Increased
Lactate production	Increased	Increased	Increased	Increased
Hyperleptinemia				
Glucose uptake	Increased	=	=	Decreased
Glutamine uptake	Decreased	Decreased	Decreased	Decreased
Lactate production	Increased	=	=	Decreased
Inflammation (TNF-α)				
Glucose uptake	=	=	=	=
Glutamine uptake	=	=	=	Decreased
Lactate production	=	=	=	=
Inflammation (IFN-γ)				
Glucose uptake	Increased	Increased	=	Decreased
Glutamine uptake	Increased	Increased	Increased	Decreased
Lactate production	Increased	Increased	=	Decreased
Oxidative stress				
Glucose uptake	Increased	Increased	Increased	Increased
Glutamine uptake	Decreased	Increased	Decreased	Decreased
Lactate production	Increased	Increased	Increased	Increased

Abbreviations: x, uptake not measured; =, uptake not altered.

An additional aspect to discuss is the fact that IFN-γ and insulin presented mostly cancer cell-specific effects. It is known that distinct cell lines show different responses to glucose and glutamine, which is in accordance with the presence of different metabolic profiles in different breast cancer subtypes [109, 146]. For instance, consistent with the Warburg effect, TN breast cancer cells present elevated glucose uptake and lactate secretion [146, 186], while ER^+ breast cancer cells seem to display the reverse Warburg effect [29, 146]. Additionally, it is known that basal-like cells (MDA-MB-231), but not luminal cells (MCF-7), are very sensitive to glutamine availability, despite maintaining relatively high levels of glutamine transport [109]. So, TN breast cancers upregulate aerobic glycolysis and display glutamine addiction, while ER^+ breast cancers present decreased glutamine catabolism and glycolytic flux. Recent studies have also demonstrated that the tissue of origin and the microenvironment can all impact cancer cell metabolism, including utilization of glucose and glutamine [109, 146]. For all these reasons, it is not surprising that some T2DM-associated compounds tested herein, present distinct responses in different breast cancer cell types and even in epithelial breast cells.

Next, we hypothesized that an increase in GLUT1-mediated and/or ASCT2-mediated uptake is involved in the cancer-promoting effect of T2DM-associated characteristics. As previously mentioned, GLUT1 and ASCT2 are the main glucose and glutamine transporters in cancer cells, respectively, being highly expressed in breast cancer cells, and their expression levels associate with increased proliferation, higher grade, malignant potential, and poor prognosis [51, 75, 76, 111]. Intriguingly, GLUT1 [146, 187] and ASCT2 [49, 109, 112] expression seems to vary depending on the breast cancer molecular subtype. GLUT1 overexpression appears to be especially important for the initiation of breast tumorigenesis [80], as well as to maintain tumour growth [44], while ASCT2 seems to support rapidly growing tumour cells [111], and its expression is detected also in cancers deriving from human tissues in which normally the protein is not present [110]. Therefore, these genes have been proposed as oncogenes [27, 34]. Targeting GLUT1 [27, 188, 189] and ASCT2 [109, 190-192] as a therapeutic strategy against cancer growth and development has been the focus of several recent studies.

In recent years, several inhibitors of GLUT1 [27] and ASCT2 [51, 92] have emerged and showed a antitumor effect. Among them, BAY-876 is a recently described promising compound identified as a new-generation selective GLUT1 inhibitor [147]. BAY-876 shows good metabolic stability *in vitro* when used in nanomolar concentrations [147]. The applicability of BAY-876 in

breast cancer intervention remains, however, almost unknown. On the other hand, GPNA features a glutamine structural analogy and inhibits the Na^+-dependent amino acid transporter ASCT2 [49, 103, 111, 148]. Although more selective ASCT2 inhibitors exist [92], GPNA represents the more effective compound of a series of glutamine analogues synthesized by Esslinger *et al.* [148] and has been widely used for this propose [102, 109, 193, 194]. We believed that effective GLUT1 and/or ASCT2 inhibition can be an alternative therapeutic strategy for reducing/preventing breast cancer initiation, promotion and progression in T2DM patients. Based on this, we decided to examine the influence of BAY-876 and GPNA on the stimulatory effects of T2DM-associated characteristics on ^{3}H-DG and ^{3}H-GLN uptake, respectively. Our findings revealed that the GLUT1 transporter inhibitor BAY-876 (10-500 nM) severely reduced ^{3}H-DG cellular uptake by both cancer cell lines (ER^+ and TN) (75% inhibition) whereas it showed a less marked effect (56% inhibition) in non-tumorigenic cells **(Chapter III).** Corroborating these results, GLUT1 mRNA levels were present in all cell lines, although its expression was 50% lower in non-tumorigenic cell lines compared with ER^+ or TN breast cancer [187]. It is possible that other glucose transporters (less responsive to inhibition with BAY-876) are also involved in glucose uptake by non-tumorigenic cells. Overall, we proved that ^{3}H-DG cellular uptake by breast cancer and non-tumorigenic cell lines is mainly GLUT1-mediated. It is important to note that, since its discovery in 2016 [147], preliminary anti-tumour activity of BAY-876 in breast cancer has been reported only in TN breast cancer cells. However, an heterogeneous response to GLUT1 pharmacologic inhibition was observed, as some TN cell lines (MDA-MB-231 and HCC1806 cells) were severely inhibited by BAY-876, whereas others (MDA-MB-436 and MDA-MB-468 cells) were more resistant to BAY-876 inhibition [189].

Concerning pharmacological inhibition of ASCT2, our results showed that GPNA (0.25-1 mM) inhibited Na^+-dependent ^{3}H-GLN uptake in both cancer (ER^+ and TN) and non-tumorigenic cells (97%, 99% and 75% inhibition, respectively). One interesting aspect observed in our work is that Na^+-dependent ^{3}H-GLN uptake by the two breast cancer progression cell lines, but not by the non-tumorigenic MCF-12A cell line, was completely abolished by GPNA **(Chapter IV)**. This means that Na^+-dependent ^{3}H-GLN uptake by ER^+ and TN breast cancer cells, but not by breast non-tumorigenic cells, involves only GPNA-sensitive transporters and that, consequently, GPNA is an effective inhibitor of glutamine uptake by breast cancer cells, when compared with non-tumorigenic cells. Similar results were found in a previous work, where GPNA was also found to decrease ^{3}H-GLN uptake by MCF-7 and MDA-MB-231 cell lines, but to be devoid of effect on the

non-tumorigenic MCF-10A cell line [109]. Curiously, we also verified that GPNA modestly inhibited Na^+-independent ^{3}H-GLN uptake in these three cell lines **(Chapter IV)**. An inhibitory effect of GPNA upon Na^+-independent ^{3}H-GLN uptake by MCF-7 cells was recently described, and it was concluded that this inhibitor hinders the activity of LAT1 and LAT2, which are expressed in this cell line [103].

In short, we were able to establish that, in both ER^+ and TN breast cancer cells and in non-tumorigenic breast epithelial cells, ^{3}H-DG uptake is mainly BAY-876-sensitive and ^{3}H-GLN uptake is mainly Na^+-dependent and GPNA-sensitive.

These previous results allowed us to speculate that the stimulatory effect of T2DM-associated characteristics upon ^{3}H-DG and ^{3}H-GLN uptake in MCF-7, MDA-MB-231, MCF-12A and MCF-12A$_{DMBA}$ cells, are related to a stimulatory effect upon GLUT1 and ASCT2 transporters, respectively. As previously reported, an increase in glucose and glutamine uptake is expected to promote progression of breast tumorigenesis, especially in TN breast cancer subtype [146]. Therefore, we focused our analysis on T2DM-associated compounds that increased uptake of ^{3}H-DG (10 nM and 1 mM) (TBH, insulin, leptin and IFN-γ in MCF-7 cells and MDA-MB-231 cells and TBH in MCF-12A cells) or ^{3}H-GLN (5 nM and 0.5 mM) (either Na^+-dependent, Na^+-independent or total uptake) (leptin and IFN-γ in MCF-7 cells, TBH and IFN-γ in MDA-MB-231 cells and TBH, insulin, leptin and TNF-α in MCF-12A cells). For this, we investigated if the T2DM-associated characteristics increased ^{3}H-DG and ^{3}H-GLN uptake by interfering with GLUT1 and ASCT2 by testing their BAY-876- and GPNA-sensitivity, respectively.

Interestingly enough, the stimulatory effect of TBH, insulin, leptin and IFN-γ on ^{3}H-DG uptake (10 nM and 1 mM) in both cancer cell lines (ER^+ and TN) and of TBH in the non-tumorigenic cell line was eradicated in the presence of the GLUT1 inhibitor (BAY-876; 500 nM) **(Chapter III).** This observation supports the conclusion that the increase in glucose cellular uptake induced by TBH, insulin, leptin and IFN-γ in both breast cancer cells (ER^+ and TN), and by TBH in normal breast epithelial cells is dependent on a stimulation of GLUT1-mediated glucose uptake. So, we next decided to explore with more detail the effect of these T2DM-associated compounds on GLUT1 transcription rates. The results obtained led us to conclude that leptin increased GLUT1 mRNA levels in both MCF-7 and MDA-MB-231 cells. As to insulin and IFN-γ, they do not appear to increase GLUT1 transcription rates. However, the observation that the stimulatory effect of insulin, leptin and IFN-γ on ^{3}H-DG (10 nM) uptake did not disappear in the presence of BAY-876 suggests that these compounds probably affect not only GLUT1, but also a

high-affinity glucose transporter which, however, is not relevant in *in vivo* conditions. In relation to TBH, the results support the conclusion that it stimulates GLUT1-mediated ^{3}H-DG uptake in MDA-MB-231 and MCF-12A cell lines by increasing GLUT1 transcription rates in MDA-MB-231 cells. In contrast, in MCF-7 cells, the results obtained with BAY-876 indicate that TBH stimulates non-GLUT1-mediated ^{3}H-DG uptake, although it was found to increase GLUT1 mRNA levels.

Given the importance of hyperactive glycolysis in support of the malignant features of cancer, we then assessed if the effect of T2DM-associated markers on proliferation and viability of breast cancer cells was GLUT1-dependent, by testing their BAY-876-sensitivity. Similarly to the effects on ^{3}H-DG uptake, the effects of TBH, insulin, leptin and IFN-γ upon breast cancer cell proliferation and viability were completely abolished in all cell lines by BAY-876, with one exception. The exception was the effect of leptin upon the proliferation of MDA-MB-231 cells **(Chapter III).** This strongly suggests that interference with GLUT1-mediated glucose uptake is fundamental for the effect of these compounds upon proliferation and viability of breast cancer and non-tumorigenic cell lines. It is worth to note that the applicability of BAY-876 in breast cancer remains largely unknown, and in the present work, we report for the first time the effect of BAY-876 on glucose uptake, proliferation and viability of breast cancer (ER^{+} and TN) and non-tumorigenic cell lines. Even though TN cells are considered much more dependent on glucose metabolism than ER^{+} cells [195] and GLUT1 inhibition showed a higher anti-proliferative effect on TN cells than on ER^{+} cells [196], in the present work ER^{+} and TN cells presented a similar response to BAY-876 in relation to both ^{3}H-DG uptake and proliferation, and a similar sensitivity of TBH, insulin, leptin and IFN-γ to inhibition with BAY-876. Additionally, one the main molecular pathways involved in the control of aerobic glycolysis is the PI3K/AKT/mTOR signalling pathway [197], and most T2DM-associated compounds act through this signalling pathway [2]. Mechanistically, PI3K signalling activates mTOR, which then activates the transcription factor HIF-1α. HIF-1α, in cooperation with other transcription factors such as Ras, Src, c-MYC and p53, regulates glycolysis and GLUT1 expression [197]. Thus, it is not surprising that the main T2DM-associated compounds may be important regulators of GLUT1.

Similarly, in the three cell lines, GPNA (1 mM) was able to completely abolish the stimulatory effect of TBH, insulin, leptin, TNF-α and IFN-γ upon Na^{+}-dependent or Na^{+}-independent uptake of ^{3}H-GLN (5 nM). Moreover, GPNA also abolished the stimulatory effect of TBH, insulin and TNF-α on Na^{+}-dependent ^{3}H-GLN (0.5 mM) uptake by MCF-12A cells. However, it interfered less markedly with the stimulatory effect of T2DM-associated conditions on Na^{+}-

dependent or -independent ^{3}H-GLN (0.5 mM) uptake in the ER^{+} and TN breast cancer cell lines. Thus, the stimulatory effect of T2DM-associated compounds upon uptake of ^{3}H-GLN (in a low concentration) was GPNA-sensitive in both cancer and non-tumorigenic cell lines, while uptake of a higher concentration of ^{3}H-GLN is disrupted to a lesser extent by GPNA in breast cancer cell lines (but not in the non-tumorigenic cell line) **(Chapter IV)**. These results support the hypothesis that low affinity glutamine transporter(s) less affected or even unaffected by GPNA participate in ^{3}H-GLN uptake exclusively in breast cancer lines.

Taken together, our results strongly indicate that the effect of T2DM-associated markers on cancer-associated characteristics appears not only to be GLUT1-dependent but also ASCT2-dependent. So, we decided to explore with more detail their effect on ASCT2 mRNA and protein levels. The observed increase in Na^{+}-dependent ^{3}H-GLN uptake induced by IFN-γ results from an upregulation of ASCT2 transcription and translation rates in MDA-MB-231 cells, but not in MCF-7 cells. Moreover, the stimulatory effect of insulin on ^{3}H-GLN uptake by MCF-12A cells is also associated with an increase in ASCT2 expression levels. In contrast, TBH increased Na^{+}-dependent ^{3}H-GLN uptake, although did not interfere with ASCT2 transcription and translation rates in MCF-7 and MCF-12A cells. Similarly, TNF-α promoted GPNA-sensitive uptake of ^{3}H-GLN, but did not affect ASCT2 transcription and translation rates **(Chapter IV)**. The observation that IFN-γ (in MCF-7 cells), TBH (in MCF-7 and MCF-12A cells) and TNF-α (in MCF-12A cells) induced an increase in Na^{+}-dependent ^{3}H-GLN uptake that is not associated with a concomitant increase of ASCT2 mRNA or protein levels suggest that, in these cell lines, these compounds affect ASCT2 by a mechanism other than regulation of gene expression. It should be noted that the activity of glucose or glutamine transporters depends not only on transcription or translation rates but is also dependent on membrane insertion of the transporters and on their intrinsic activity.

GPNA is broadly used as an ASCT2 inhibitor [49, 103, 111], but recent studies have demonstrated that it is also able to inhibit other Na^{+}-dependent and Na^{+}-independent glutamine transporters [190]. Namely, GPNA is also able to block the Na^{+}-coupled transporters SNAT1 [98, 191], SNAT2 [99, 191] and $ATB^{0,+}$ [49, 105] and the Na^{+}-uncoupled transporters LAT1 [49, 103, 198, 199] and LAT2 [103]. Although Na^{+}-independent glutamine transporters are not the focus of this work, the co-expression of ASCT2 and LAT1 was described in other cell types [200, 201], and GPNA was described to inhibit LAT1 in MCF-7 cells [103]. Hence, we also assessed the participation of LAT1 in the GPNA-sensitive stimulatory effects of T2DM-associated compounds on ^{3}H-GLN uptake. We observed that the increase in Na^{+}-independent ^{3}H-GLN uptake caused by

leptin (in MCF-7 cells) and TBH (in MCF-12A cells) is associated with an increase in LAT1 mRNA transcription rates, whereas IFN-γ (in MCF-7 cells) and insulin, leptin and TNF-α (in MCF-12A cells) were devoid of effect on LAT1 mRNA levels **(Chapter IV)**. Although a functional coupling between ASCT2 and LAT1 has been proposed in lung adenocarcinoma [200], esophageal squamous cell carcinoma [201] and even in breast cancer (ER^+), [103] our results do not suggest a reciprocal regulatory connection between these two transporters in the increased ^{3}H-GLN uptake caused by IFN-γ (in MCF-7 cells) and insulin, leptin and TNF-α (in MCF-12A cells).

Of the T2DM-associated compounds, IFN-γ and insulin stimulated Na^+-dependent ^{3}H-GLN uptake selectively in breast cancer and non-tumorigenic breast cells, respectively **(Chapter IV)**. For this reason, we focused our subsequent analysis in these two compounds. Because GPNA has a poor selectivity for ASCT2, we further investigated the involvement of ASCT2 in the stimulatory effect of IFN-γ and insulin upon ^{3}H-GLN uptake by breast cancer and non-tumorigenic cells, respectively, by performing targeted knockdown of ASCT2. Consistent with the results from pharmacological inhibition of ASCT2 with GPNA, the stimulatory effects of IFN-γ on ^{3}H-GLN uptake in both breast cancer cell lines and of insulin in the non-tumorigenic cell line were completely abolished after ASCT2 knockdown (Figure 4A and B) **(Chapter IV)**.

Our results also show that IFN-γ possesses a very interesting effect, as it stimulates ^{3}H-GLN uptake (both total and Na^+-dependent; both at low (5 nM) and physiological (0.5 mM) levels) only in the breast cancer cell lines **(Chapter IV).** So, we decided to explore the effect of this T2DM-associated marker in more detail. Glucose and glutamine transporters are known to be regulated by intracellular signalling mechanisms, and high levels of IFN-γ can affect several distinct intracellular signalling pathways [2, 133]. It is known that IFN-γ activates the JAK/STAT signalling pathway, which promotes phosphorylation and activation of transcription factors such as STAT1 and STAT3 [202, 203]. Moreover, IFN-γ also activates the PI3K signalling pathway [202, 203]. Deregulation of the JAK/STAT and PI3K pathways in cancer cells reprograms cellular metabolism by augmenting the activity of nutrient transporters, thereby supporting the anabolic demands of growing cells [202]. For instance, Avissar *et al.* showed that activation of the PI3K pathway induced ASCT2 expression and glutamine transport in human enterocytes [204]. For this reason, we decided to investigate the intracellular signalling mechanisms involved in the stimulatory effect of IFN-γ upon ^{3}H-GLN uptake in both ER^+ and TN breast cancer cells, by using specific inhibitors of these pathways. Interestingly, we concluded that the stimulatory effect of IFN-γ upon ^{3}H-GLN uptake involves activation of STAT1, STAT3 and PI3K intracellular signalling

pathways in both cancer cell lines (Figure 4A) **(Chapter IV)**. Overall, these results indicate a relevant role of STAT1, STAT3 and PI3K in the increase of ^{3}H-GLN uptake and, therefore, in the progression of breast cancer induced by IFN-γ.

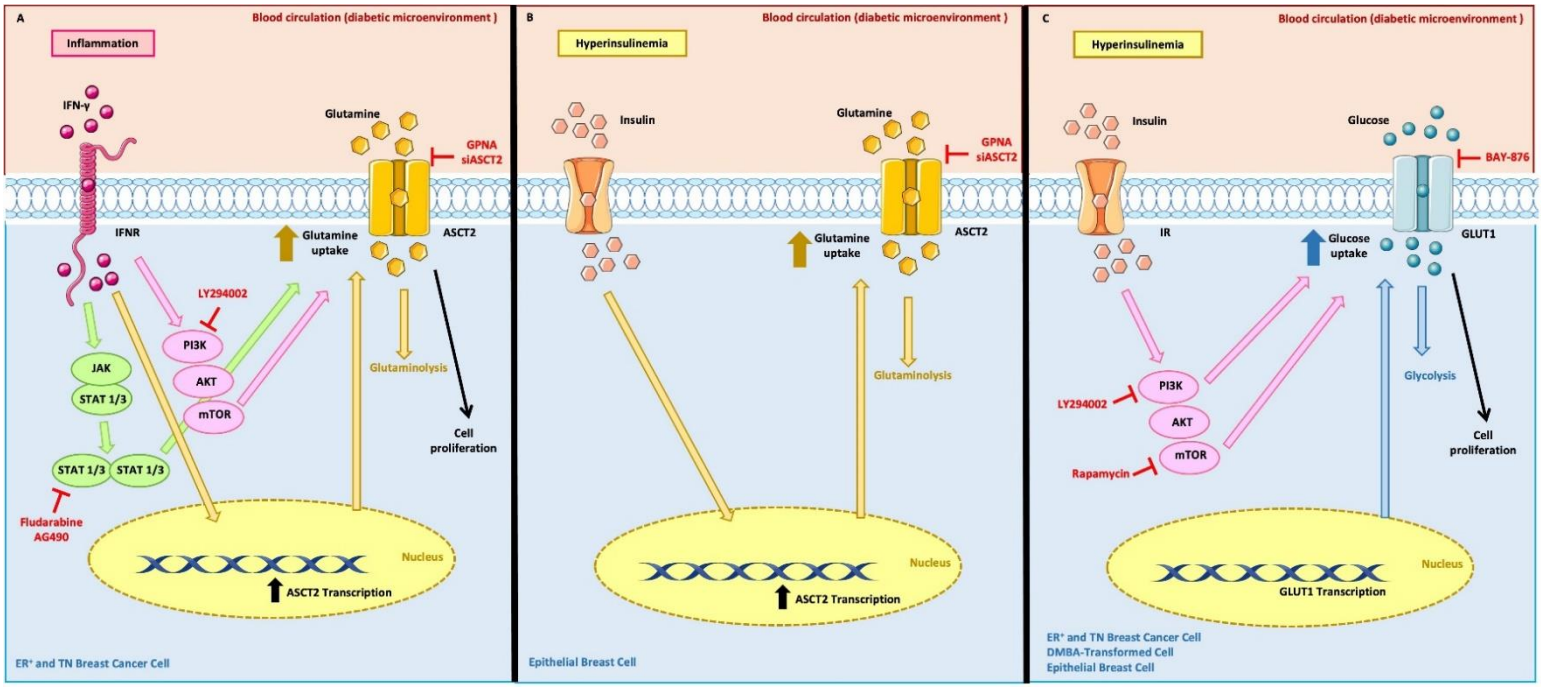

Figure 4. Schematic depiction of the effect of inflammation (IFN-γ) and hyperinsulinemia on glutamine and glucose cellular uptake in breast cancer cells, in DMBA-transformed cells and in breast epithelial cells.

(A) A high inflammatory status (IFN-γ) causes a pro-proliferative effect in ER^{+} and TN breast cancer cells, which is associated with an increase in glutamine uptake, and which is GPNA-sensitive, blocked after ASCT2 knockdown and mediated by activation of the PI3K-, STAT3- and STAT1 intracellular signalling pathways. (B) Hyperinsulinemia stimulates glutamine uptake in breast epithelial cells and which is GPNA-sensitive, blocked after ASCT2 knockdown. (C) Hyperinsulinemia stimulates glucose uptake and cell proliferation in ER^{+} and TN breast cancer cells, DMBA-transformed cells and breast epithelial cells, which is dependent on an increase in GLUT1-mediated glucose uptake and mediated by the PI3K and mTOR signalling pathways. Abbreviations: GLUT1, facilitative glucose transporters 1; ASCT2, alanine, serine, cysteine transporter 2; ER, estrogen receptor; TN; tripre-negative; IR, insulin receptor; IFN-γ; interferon gamma; INFR, interferon receptor; IRS-1, Insulin receptor substrate 1; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; mTOR, mammalian target to rapamycin; JAK, janus kinas; STAT, signal transducer and activator of transcription; HIF1α, Hypoxia-inducible factor 1-alpha; BAY-876, [N4-[1-(4-cyanobenzyl)-5-methyl-3-(trifluoromethyl)-1H-pyrazol-4-yl]-7-fluoroquinoline-2,4-dicarboxamide]; GPNA, L-g-glutamyl-p-nitroanilide.

From the previous results, we could conclude that the stimulatory effect of IFN-γ upon ^{3}H-GLN uptake: (1) is cancer cell specific; (2) is GPNA-sensitive; (3) is ASCT2-mediated; and (4) involves activation of STAT1-, STAT3- and PI3K signalling pathways **(Chapter IV).** Because IFN-γ promoted cell proliferation of ER^{+} and TN cells **(Chapter III),** we further investigated if the stimulatory effect of IFN-γ upon cell proliferation is ASCT2-mediated. Interestingly, both pharmacological blockade and knockdown of ASCT2 abolished the pro-proliferative effect of IFN-γ

in both ER^+ and TN breast cancer cells (Figure 4A) **(Chapter IV)**. These results thus strongly support the conclusion that ASCT2 is a mediator of the negative effects of IFN-γ in breast cancer progression.

Finally, of the tested T2DM-associated compounds, insulin showed the more interesting results, since its effects were quite similar in relation to breast tumour progression and promotion. More specifically, in MCF-7, MDA-MB-231 and MCF-$12A_{DMBA}$ cells, high levels of insulin presented pro-angiogenic effects. Curiously, these effects were cancer cell-specific. Moreover, in MCF-7, MDA-MB-23, MCF-$12A_{DMBA}$ and MCF12A cells (cultivated with FBS), insulin exhibited a pro-proliferative effect, increased ^{3}H-DG uptake and decreased ^{3}H-GLN uptake **(Chapter III, Chapter IV and Chapter V)**. These observations suggest that under hyperinsulinemic conditions, glucose, but not glutamine, is essential for cell proliferation during breast cancer promotion and progression and even cancer initiation. Numerous *in vitro* experiments consistently showed that insulin promotes growth of ER^+ human breast cancer cell lines [133], although the effects of insulin on TN human breast cancer cell lines are quite complex. Although TN and ER^+ cells have similar levels of IR, minimal or no stimulation of proliferation was observed with insulin in MDA-MB-231 cells [133, 205].

The PI3K/AKT/mTOR-signalling pathway is a well-established regulator of central glucose metabolism, aerobic glycolysis and proliferation [131, 133]. Dysregulation of the PI3K/AKT/mTOR-signalling pathway was shown to correlate with breast cancer disease progression [27, 34, 75]. So, we decided to investigate the intracellular signalling mechanisms involved in the stimulatory effect of insulin upon ^{3}H-DG uptake and proliferation in DMBA-transformed and non-transformed breast epithelial cells. Interestingly, we could conclude that the stimulatory effect of insulin upon both proliferation and ^{3}H-DG uptake involves activation of mTOR and PI3K intracellular signalling pathways in both MCF-$12A_{DMBA}$ and MCF12A cell lines (Figure 4C) **(Chapter V)**.

In addition, based on the fact that the pro-proliferative effect of insulin in ER^+ and TN breast cancer cells is dependent on GLUT1 stimulation **(Chapter III)** and GLUT1 overexpression appears especially important for the initiation of breast tumorigenesis [80], we investigated the influence of BAY-876 on the stimulatory effect of insulin upon cell proliferation and ^{3}H-DG uptake. Notably, we verified that in the presence of BAY-876 (500 nM), the effect of insulin upon cell proliferation and ^{3}H-DG uptake in MCF-$12A_{DMBA}$ and MCF-12A cells were completely abolished **(Chapter V).** Taken together, these data strongly suggest that insulin possesses a pro-proliferative

effect, which is dependent on an increase in GLUT1-mediated glucose uptake and mediated by the PI3K and mTOR signalling pathways, in both carcinogen-transformed and non-tumorigenic cell lines. Thus, we can conclude that insulin seems to promote cancer initiation of the breast epithelial cell and to stimulate promotion and progression of breast tumorigenesis by a GLUT1-dependent mechanism (Figure 4C).

In summary, several T2DM-associated characteristics, particularly hyperinsulinemia and a high inflammatory status (IFN-γ), interfere with GLUT1 and/or ASCT2 transporters to create a favourable metabolic environment for promotion and progression of breast tumorigenesis. Importantly, these effects are also evident in a breast non-tumorigenic cell line, suggesting that they may also contribute to breast cancer initiation in T2DM patients. Additionally, our results strongly suggest that targeting GLUT1 and ASCT2 can be an effective therapeutic strategy for reducing/preventing human breast cancer initiation, promotion and progression in T2DM patients.

CHAPTER VII

MAIN CONCLUSIONS AND FUTURE DIRECTIONS

MAIN CONCLUSIONS AND FUTURE DIRECTIONS

This study used a basic science approach to investigate the role of T2DM-associated characteristics (hyperglycemia, hyperinsulinemia, hyperleptinemia, increased levels of inflammation and oxidative stress) upon glucose (GLUT1) and glutamine (ASCT2) transport and the involvement of this effect on breast cancer initiation, promotion and progression, and to assess its potential as a therapeutic target.

The main conclusions of this work are:

- With the exception of glucose, all T2DM-associated characteristics (high levels of insulin, leptin, pro-inflammatory cytokines (TNF-α and IFN-γ) and oxidative stress (by using the oxidative stress inducer TBH)) induce changes in cellular features (proliferation, viability, growth, migration and angiogenesis) that can be relevant in the context of breast tumour promotion and progression, and even in cancer initiation.

- With the exception of glucose and TNF-α, all T2DM-associated features stimulate glucose and/or glutamine cellular uptake in the context of breast tumour initiation, promotion and/or progression.

- T2DM-associated characteristics present distinct effects in cellular features and glucose and glutamine uptake in different breast cancer subtypes.

- In both ER^+ and TN breast cancer cells and human epithelial breast cells, glucose cellular uptake is BAY-876-sensitive and glutamine cellular uptake is mainly Na^+-dependent and GPNA-sensitive.

- The increase in glucose cellular uptake induced by T2DM-associated characteristics is BAY-876-sensitive and GLUT1-mediated, while the increase in glutamine transport is principally GPNA-sensitive and ASCT2-mediated, in all cell lines.

- Of all the T2DM-associated characteristics analysed, insulin and IFN-γ display the most interesting effects, because their effect in cellular features relevant in the context of breast cancer, and glucose and/or glutamine uptake are: (1) mostly cancer cell-specific; (2) BAY-876-sensitive and GPNA-sensitive and (3) GLUT1-mediated and ASCT2-mediated, respectively.

- Insulin stimulates GLUT1-mediated uptake not only during breast cancer progression (in ER^+ and TN cells), but also during breast cancer promotion (DMBA-transformed cells) and initiation (non-transformed breast epithelial cells), and its stimulatory effect upon cell proliferation and viability is also dependent on GLUT1 stimulation. The effect of insulin upon both proliferation and glucose uptake in breast cancer promotion involves activation of mTOR and PI3K intracellular signalling pathways. Importantly, these effects are also evident in breast non-tumorigenic cell line, suggesting that they may also contribute to initiation of carcinogenesis in human epithelial breast cell. So, insulin may be an important molecular mediator of the T2DM-breast cancer connection.

- The pro-proliferative effect of the pro-inflammatory cytokine, IFN-γ, in ER^+ and TN breast cancer cells is associated with an increase in glutamine uptake, which is GPNA-sensitive, abolished after ASCT2 knockdown and mediated by activation of the PI3K-, STAT3- and STAT1 intracellular signalling pathways.

- GLUT1 and ASCT2 transporters appear to constitute a molecular target for T2DM-associated features, as an increase in GLUT1-mediated and ASCT2-mediated transport appear to contribute to breast cancer initiation, promotion and progression in T2DM patients.

- An effective inhibition of glucose and glutamine uptake may be used as a therapeutic strategy against breast tumours cell in early-stage or late-stage of progression, in the promotional stage of breast tumorigenesis and may even prevent initiation of carcinogenesis in human epithelial breast cell in T2DM patients.

We believe that this work contributes to a better knowledge on the impact of T2DM upon the initiation, promotion and progression of breast cancer. With this study, we tried to (1) further establish the relationship between T2DM and breast cancer initiation, promotion and progression; (2) understand and compare the role of T2DM-associated characteristics in different breast cancer subtypes; (3) understand how breast cancer cells utilize two of the main nutrients (glucose and glutamine) in the presence of T2DM-associated characteristics; and (4) propose personalized anti-cancer agents to improve treatment outcome and prognosis or even to block the initiation of tumorigenesis. Moreover, this work also suggest that some aspects should be investigated in more detail.

First, the relevance of our findings should be expanded to other proliferative breast cancer subtypes, namely luminal B and $HER2^+$ tumours. For instance, similar to TN, HER^+ breast cancers display altered glucose and glutamine metabolism [146], suggesting that these tumours may also be sensitive to GLUT1 and/or ASCT2 inhibition.

Next, it should be noted that our data answers some important questions, as to the efficacy and the optimal timing of GLUT1 or ASCT2 targeted therapies in T2DM patient; would they be best used in early breast cancer to slow or block development of disease, or in later-stage of disease, such as luminal and basal-like molecular subtypes of breast cancer. Our data show that *in vitro* GLUT1 and/or ASCT2 inhibition can be an effective therapeutic strategy for reducing/preventing breast cancer initiation, promotion and progression in the presence of some T2DM-associated compounds, but these results need to be validated *in vivo*. Future work must include analysis of GLUT1 and ASCT2 expression in the presence of T2DM and validation of the effectiveness of BAY-876 and GPNA *in vivo*, in order to reinforce the translation of our findings. The combination therapy of these two inhibitors should also be explored, as it could be relevant to achieve an optimal response, to increase the specificity of the treatment and, additionally, to reduce the development of drug resistance.

As with any therapeutic intervention, the potential to initiate adaptive pathways and resistance mechanisms in response to GLUT1 and/or ASCT2 inhibition should also be considered. Recent publications suggest that cancer cells may be able to adapt to long-term glucose or glutamine transporter inhibition by upregulating other transporters with similar substrate affinity. For instance, upregulation of SNAT1 and SNAT2 was reported in ASCT2-defecient cells [191]. Therefore, future work must understand what mechanisms enable this.

In this work we showed, for the first time, that an AI-based technique was able to distinguish/identify the supernatant of DMBA-transformed cells from that of non-tumorigenic cells. This discovery may lead to the identification of a new biomarker for the risk of breast cancer development in T2DM patients. So, future work should involve the identification of the specific nanostructures present in cell supernatant which contribute the most for the discriminatory nature of the scattering-based signal associated to the carcinogenesis process. Possible examples include specific extracellular vesicles (e.g., exosomes) produced by cells under neoplastic transformation.

Lastly, the biggest limitation of this study is that it draws conclusions only from cell lines, and it is known that cell lines are an imperfect model of human disease. Even within molecular subtypes, breast cancer is extremely heterogeneous, and no individual cell line and not even *in vivo* models can show all of this variation. Thus, gene expression analyses of tissue of the different molecular subtypes of breast cancer from patients with T2DM should be incorporated into this study in the future, in order to provide a better coverage of the intrinsic heterogeneity in samples from T2DM patients with breast cancer.

CHAPTER VIII

REFERENCES

REFERENCES

[1] P. Saeedi, I. Petersohn, P. Salpea, B. Malanda, S. Karuranga, N. Unwin, S. Colagiuri, L. Guariguata, A.A. Motala, K. Ogurtsova, J.E. Shaw, D. Bright, R. Williams, I.D.F.D.A. Committee, Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9(th) edition, Diabetes Res Clin Pract 157 (2019) 107843.

[2] S.M. Samuel, E. Varghese, S. Varghese, D. Busselberg, Challenges and perspectives in the treatment of diabetes associated breast cancer, Cancer Treat Rev 70 (2018) 98-111.

[3] Y. Zheng, S.H. Ley, F.B. Hu, Global aetiology and epidemiology of type 2 diabetes mellitus and its complications, Nature reviews. Endocrinology 14(2) (2018) 88-98.

[4] H. Sung, J. Ferlay, R.L. Siegel, M. Laversanne, I. Soerjomataram, A. Jemal, F. Bray, Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, CA: a cancer journal for clinicians (2021).

[5] P.J. Hardefeldt, S. Edirimanne, G.D. Eslick, Diabetes increases the risk of breast cancer: a meta-analysis, Endocrine-related cancer 19(6) (2012) 793-803.

[6] P. Boyle, M. Boniol, A. Koechlin, C. Robertson, F. Valentini, K. Coppens, L.L. Fairley, M. Boniol, T. Zheng, Y. Zhang, M. Pasterk, M. Smans, M.P. Curado, P. Mullie, S. Gandini, M. Bota, G.B. Bolli, J. Rosenstock, P. Autier, Diabetes and breast cancer risk: a meta-analysis, British journal of cancer 107(9) (2012) 1608-17.

[7] S.C. Larsson, C.S. Mantzoros, A. Wolk, Diabetes mellitus and risk of breast cancer: a meta-analysis, International journal of cancer 121(4) (2007) 856-62.

[8] K.K. Collins, The diabetes-cancer link, Diabetes Spectr 27(4) (2014) 276-80.

[9] I.A. Durrani, A. Bhatti, P. John, The prognostic outcome of 'type 2 diabetes mellitus and breast cancer' association pivots on hypoxia-hyperglycemia axis, Cancer Cell Int 21(1) (2021) 351.

[10] S.S. Doerstling, C.H. O'Flanagan, S.D. Hursting, Obesity and Cancer Metabolism: A Perspective on Interacting Tumor-Intrinsic and Extrinsic Factors, Frontiers in oncology 7 (2017) 216.

[11] T.G.C. Observatory, Portugal Cancer Statistics, 2020. https://gco.iarc.fr/today/data/factsheets/populations/620-portugal-fact-sheets.pdf. (Accessed July 15th 2021).

[12] I.A. Gomes, C. Nunes, Analysis of the Breast Cancer Mortality Rate in Portugal Over a Decade: Spatiotemporal Clustering Analysis, Acta Med Port 33(5) (2020) 305-310.

[13] S. Loibl, P. Poortmans, M. Morrow, C. Denkert, G. Curigliano, Breast cancer, Lancet 397(10286) (2021) 1750-1769.

[14] Z. Momenimovahed, H. Salehiniya, Epidemiological characteristics of and risk factors for breast cancer in the world, Breast Cancer (Dove Med Press) 11 (2019) 151-164.

[15] Y. Feng, M. Spezia, S. Huang, C. Yuan, Z. Zeng, L. Zhang, X. Ji, W. Liu, B. Huang, W. Luo, B. Liu, Y. Lei, S. Du, A. Vuppalapati, H.H. Luu, R.C. Haydon, T.C. He, G. Ren, Breast cancer development and progression: Risk factors, cancer stem cells, signaling pathways, genomics, and molecular pathogenesis, Genes Dis 5(2) (2018) 77-106.

[16] N. Harbeck, F. Penault-Llorca, J. Cortes, M. Gnant, N. Houssami, P. Poortmans, K. Ruddy, J. Tsang, F. Cardoso, Breast cancer, Nat Rev Dis Primers 5(1) (2019) 66.

[17] F.K. Al-Thoubaity, Molecular classification of breast cancer: A retrospective cohort study, Ann Med Surg (Lond) 49 (2020) 44-48.

[18] S.K. Yeo, J.L. Guan, Breast Cancer: Multiple Subtypes within a Tumor?, Trends Cancer 3(11) (2017) 753-760.

[19] J.Y.S. Tsang, G.M. Tse, Molecular Classification of Breast Cancer, Adv Anat Pathol 27(1) (2020) 27-35.

[20] G.I. Uscanga-Perales, S.K. Santuario-Facio, C.N. Sanchez-Dominguez, S. Cardona-Huerta, G.E. Munoz-Maldonado, P. Ruiz-Flores, J.R. Barcenas-Walls, L.E. Osuna-Rosales, A. Rojas-Martinez, J.F. Gonzalez-Guerrero, J. Valero-Gomez, G.S. Gomez-Macias, A. Barbosa-Quintana, O. Barboza-Quintana, R. Garza-Guajardo, R. Ortiz-Lopez, Genetic alterations of triple negative breast cancer (TNBC) in women from Northeastern Mexico, Oncol Lett 17(3) (2019) 3581-3588.

[21] C.M. Perou, T. Sorlie, M.B. Eisen, M. van de Rijn, S.S. Jeffrey, C.A. Rees, J.R. Pollack, D.T. Ross, H. Johnsen, L.A. Akslen, O. Fluge, A. Pergamenschikov, C. Williams, S.X. Zhu, P.E. Lonning, A.L. Borresen-Dale, P.O. Brown, D. Botstein, Molecular portraits of human breast tumours, Nature 406(6797) (2000) 747-52.

[22] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, Cell 144(5) (2011) 646-74.

[23] R.J. DeBerardinis, N.S. Chandel, Fundamentals of cancer metabolism, Sci Adv 2(5) (2016) e1600200.

[24] N.N. Pavlova, C.B. Thompson, The Emerging Hallmarks of Cancer Metabolism, Cell metabolism 23(1) (2016) 27-47.

[25] O. Warburg, F. Wind, E. Negelein, The Metabolism of Tumors in the Body, The Journal of general physiology 8(6) (1927) 519-30.

[26] N. Aykin-Burns, I.M. Ahmad, Y. Zhu, L.W. Oberley, D.R. Spitz, Increased levels of superoxide and H2O2 mediate the differential susceptibility of cancer cells versus normal cells to glucose deprivation, Biochem J 418(1) (2009) 29-37.

[27] A.M. Barbosa, F. Martel, Targeting Glucose Transporters for Breast Cancer Therapy: The Effect of Natural and Synthetic Compounds, Cancers 12(1) (2020) 154

[28] K. Sun, S. Tang, Y. Hou, L. Xi, Y. Chen, J. Yin, M. Peng, M. Zhao, X. Cui, M. Liu, Oxidized ATM-mediated glycolysis enhancement in breast cancer-associated fibroblasts contributes to tumor invasion through lactate as metabolic coupling, EBioMedicine 41 (2019) 370-383.

[29] S. Pavlides, D. Whitaker-Menezes, R. Castello-Cros, N. Flomenberg, A.K. Witkiewicz, P.G. Frank, M.C. Casimiro, C. Wang, P. Fortina, S. Addya, R.G. Pestell, U.E. Martinez-Outschoorn, F. Sotgia, M.P. Lisanti, The reverse Warburg effect: aerobic glycolysis in cancer associated fibroblasts and the tumor stroma, Cell Cycle 8(23) (2009) 3984-4001.

[30] A.K. Witkiewicz, D. Whitaker-Menezes, A. Dasgupta, N.J. Philp, Z. Lin, R. Gandara, S. Sneddon, U.E. Martinez-Outschoorn, F. Sotgia, M.P. Lisanti, Using the "reverse Warburg effect" to identify high-risk breast cancer patients: stromal MCT4 predicts poor clinical outcome in triple-negative breast cancers, Cell Cycle 11(6) (2012) 1108-17.

[31] M.M. Keenan, J.T. Chi, Alternative fuels for cancer cells, Cancer J 21(2) (2015) 49-55.

[32] M.G. Vander Heiden, Targeting cancer metabolism: a therapeutic window opens, Nature reviews. Drug discovery 10(9) (2011) 671-84.

[33] A.J. Bott, S. Maimouni, W.X. Zong, The Pleiotropic Effects of Glutamine Metabolism in Cancer, Cancers 11(6) (2019) 770.

[34] Y. Kato, T. Maeda, A. Suzuki, Y. Baba, Cancer metabolism: New insights into classic characteristics, The Japanese dental science review 54(1) (2018) 8-21.

[35] C. Schiliro, B.L. Firestein, Mechanisms of Metabolic Reprogramming in Cancer Cells Supporting Enhanced Growth and Proliferation, Cells 10(5) (2021) 1056.

[36] D. Deng, N. Yan, GLUT, SGLT, and SWEET: Structural and mechanistic investigations of the glucose transporters, Protein Sci 25(3) (2016) 546-58.

[37] S. Cassim, M. Vucetic, M. Zdralevic, J. Pouyssegur, Warburg and Beyond: The Power of Mitochondrial Metabolism to Collaborate or Replace Fermentative Glycolysis in Cancer, Cancers 12(5) (2020) 1119.

[38] N. Hay, Reprogramming glucose metabolism in cancer: can it be exploited for cancer therapy?, Nature reviews. Cancer 16(10) (2016) 635-49.

[39] Z. Wu, J. Wu, Q. Zhao, S. Fu, J. Jin, Emerging roles of aerobic glycolysis in breast cancer, Clin Transl Oncol 22(5) (2020) 631-646.

[40] B. Kalyanaraman, Teaching the basics of cancer metabolism: Developing antitumor strategies by exploiting the differences between normal and cancer cell metabolism, Redox Biol 12 (2017) 833-842.

[41] J.H. Park, W.Y. Pyun, H.W. Park, Cancer Metabolism: Phenotype, Signaling and Therapeutic Targets, Cells 9(10) (2020) 2308.

[42] A. Annibaldi, C. Widmann, Glucose metabolism in cancer cells, Curr Opin Clin Nutr Metab Care 13(4) (2010) 466-70.

[43] R. Shiratori, K. Furuichi, M. Yamaguchi, N. Miyazaki, H. Aoki, H. Chibana, K. Ito, S. Aoki, Glycolytic suppression dramatically changes the intracellular metabolic profile of multiple cancer cell lines in a mitochondrial metabolism-dependent manner, Scientific reports 9(1) (2019) 18699.

[44] S.D. Martin, S.L. McGee, Metabolic reprogramming in type 2 diabetes and the development of breast cancer, The Journal of endocrinology 237(2) (2018) 35-46.

[45] S. Ganapathy-Kanniappan, J.F. Geschwind, Tumor glycolysis as a target for cancer therapy: progress and prospects, Mol Cancer 12 (2013) 152.

[46] J. Zheng, Energy metabolism of cancer: Glycolysis versus oxidative phosphorylation (Review), Oncol Lett 4(6) (2012) 1151-1157.

[47] R. El Ansari, A. McIntyre, M.L. Craze, I.O. Ellis, E.A. Rakha, A.R. Green, Altered glutamine metabolism in breast cancer; subtype dependencies and alternative adaptations, Histopathology 72(2) (2018) 183-190.

[48] Y.D. Bhutia, V. Ganapathy, Glutamine transporters in mammalian cells and their functions in physiology and cancer, Biochimica et biophysica acta 1863(10) (2016) 2531-9.

[49] Y.J. Cha, E.S. Kim, J.S. Koo, Amino Acid Transporters and Glutamine Metabolism in Breast Cancer, International journal of molecular sciences 19(3) (2018) 907.

[50] H.C. Yoo, Y.C. Yu, Y. Sung, J.M. Han, Glutamine reliance in cell metabolism, Exp Mol Med 52(9) (2020) 1496-1516.

[51] E. Teixeira, C. Silva, F. Martel, The role of the glutamine transporter ASCT2 in antineoplastic therapy, Cancer Chemother Pharmacol 87(4) (2021) 447-464.

[52] H. Eagle, The minimum vitamin requirements of the L and HeLa cells in tissue culture, the production of specific vitamin deficiencies, and their cure, J Exp Med 102(5) (1955) 595-600.

[53] J. Marquez, F. Sanchez-Jimenez, M.A. Medina, A.R. Quesada, I. Nunez de Castro, Nitrogen metabolism in tumor bearing mice, Arch Biochem Biophys 268(2) (1989) 667-75.

[54] L.A. Sauer, J.W. Stayman, 3rd, R.T. Dauchy, Amino acid, glucose, and lactic acid utilization in vivo by rat tumors, Cancer research 42(10) (1982) 4090-7.

[55] M.O. Yuneva, T.W. Fan, T.D. Allen, R.M. Higashi, D.V. Ferraris, T. Tsukamoto, J.M. Mates, F.J. Alonso, C. Wang, Y. Seo, X. Chen, J.M. Bishop, The metabolic profile of tumors depends on both the responsible genetic lesion and tissue type, Cell metabolism 15(2) (2012) 157-70.

[56] M. Yuneva, N. Zamboni, P. Oefner, R. Sachidanandam, Y. Lazebnik, Deficiency in glutamine but not glucose induces MYC-dependent apoptosis in human cells, J Cell Biol 178(1) (2007) 93-105.

[57] L. Yang, S. Venneti, D. Nagrath, Glutaminolysis: A Hallmark of Cancer Metabolism, Annu Rev Biomed Eng 19 (2017) 163-194.

[58] D. Yu, X. Shi, G. Meng, J. Chen, C. Yan, Y. Jiang, J. Wei, Y. Ding, Kidney-type glutaminase (GLS1) is a biomarker for pathologic diagnosis and prognosis of hepatocellular carcinoma, Oncotarget 6(10) (2015) 7619-31.

[59] Y.K. Choi, K.G. Park, Targeting Glutamine Metabolism for Cancer Treatment, Biomol Ther (Seoul) 26(1) (2018) 19-28.

[60] M. Scalise, L. Pochini, M. Galluccio, L. Console, C. Indiveri, Glutamine Transport and Mitochondrial Metabolism in Cancer Cell Growth, Frontiers in oncology 7 (2017) 306.

[61] E.R. Still, M.O. Yuneva, Correction: Hopefully devoted to Q: targeting glutamine addiction in cancer, British journal of cancer 120(9) (2019) 957.

[62] J.G. Cory, A.H. Cory, Critical roles of glutamine as nitrogen donors in purine and pyrimidine nucleotide synthesis: asparaginase treatment in childhood acute lymphoblastic leukemia, In Vivo 20(5) (2006) 587-9.

[63] R.J. DeBerardinis, A. Mancuso, E. Daikhin, I. Nissim, M. Yudkoff, S. Wehrli, C.B. Thompson, Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds

the requirement for protein and nucleotide synthesis, Proc Natl Acad Sci U S A 104(49) (2007) 19345-50.

[64] C.M. Metallo, P.A. Gameiro, E.L. Bell, K.R. Mattaini, J. Yang, K. Hiller, C.M. Jewell, Z.R. Johnson, D.J. Irvine, L. Guarente, J.K. Kelleher, M.G. Vander Heiden, O. Iliopoulos, G. Stephanopoulos, Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia, Nature 481(7381) (2011) 380-4.

[65] M. Yudkoff, D. Pleasure, L. Cregar, Z.P. Lin, I. Nissim, J. Stern, I. Nissim, Glutathione turnover in cultured astrocytes: studies with [15N]glutamate, J Neurochem 55(1) (1990) 137-45.

[66] A.E. Vaughn, M. Deshmukh, Glucose metabolism inhibits apoptosis in neurons and cancer cells by redox inactivation of cytochrome c, Nat Cell Biol 10(12) (2008) 1477-83.

[67] O. Yanagida, Y. Kanai, A. Chairoungdua, D.K. Kim, H. Segawa, T. Nii, S.H. Cha, H. Matsuo, J. Fukushima, Y. Fukasawa, Y. Tani, Y. Taketani, H. Uchino, J.Y. Kim, J. Inatomi, I. Okayasu, K. Miyamoto, E. Takeda, T. Goya, H. Endou, Human L-type amino acid transporter 1 (LAT1): characterization of function and expression in tumor cell lines, Biochimica et biophysica acta 1514(2) (2001) 291-302.

[68] R.V. Duran, W. Oppliger, A.M. Robitaille, L. Heiserich, R. Skendaj, E. Gottlieb, M.N. Hall, Glutaminolysis activates Rag-mTORC1 signaling, Mol Cell 47(3) (2012) 349-58.

[69] C.C. Barron, P.J. Bilan, T. Tsakiridis, E. Tsiani, Facilitative glucose transporters: Implications for cancer detection, prognosis and treatment, Metabolism 65(2) (2016) 124-39.

[70] P.B. Ancey, C. Contat, E. Meylan, Glucose transporters in cancer - from tumor cells to the tumor microenvironment, FEBS J 285(16) (2018) 2926-2943.

[71] H. Koepsell, The Na(+)-D-glucose cotransporters SGLT1 and SGLT2 are targets for the treatment of diabetes and cancer, Pharmacol Ther 170 (2017) 148-165.

[72] G. Gyimesi, J. Pujol-Gimenez, Y. Kanai, M.A. Hediger, Sodium-coupled glucose transport, the SLC5 family, and therapeutically relevant inhibitors: from molecular discovery to clinical application, Pflugers Arch 472(9) (2020) 1177-1206.

[73] L. Szablewski, Expression of glucose transporters in cancers, Biochimica et biophysica acta 1835(2) (2013) 164-9.

[74] M.L. Macheda, S. Rogers, J.D. Best, Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer, J Cell Physiol 202(3) (2005) 654-62.

[75] M. Younes, R.W. Brown, D.R. Mody, L. Fernandez, R. Laucirica, GLUT1 expression in human breast carcinoma: correlation with known prognostic markers, Anticancer research 15(6) (1995) 2895-8.

[76] C. Pinheiro, B. Sousa, A. Albergaria, J. Paredes, R. Dufloth, D. Vieira, F. Schmitt, F. Baltazar, GLUT1 and CAIX expression profiles in breast cancer correlate with adverse prognostic factors and MCT1 overexpression, Histol Histopathol 26(10) (2011) 1279-86.

[77] R.S. Brown, T.M. Goodman, K.R. Zasadny, J.K. Greenson, R.L. Wahl, Expression of hexokinase II and Glut-1 in untreated human breast cancer, Nucl Med Biol 29(4) (2002) 443-53.

[78] A. Godoy, V. Ulloa, F. Rodriguez, K. Reinicke, A.J. Yanez, L. Garcia Mde, R.A. Medina, M. Carrasco, S. Barberis, T. Castro, F. Martinez, X. Koch, J.C. Vera, M.T. Poblete, C.D. Figueroa, B. Peruzzo, F. Perez, F. Nualart, Differential subcellular distribution of glucose transporters GLUT1-6 and GLUT9 in human cancer: ultrastructural localization of GLUT1 and GLUT5 in breast tumor tissues, J Cell Physiol 207(3) (2006) 614-27.

[79] R.S. Brown, R.L. Wahl, Overexpression of Glut-1 glucose transporter in human breast cancer. An immunohistochemical study, Cancer 72(10) (1993) 2979-85.

[80] E.A. Wellberg, S. Johnson, J. Finlay-Schultz, A.S. Lewis, K.L. Terrell, C.A. Sartorius, E.D. Abel, W.J. Muller, S.M. Anderson, The glucose transporter GLUT1 is required for ErbB2-induced mammary tumorigenesis, Breast cancer research : BCR 18(1) (2016) 131.

[81] E.S.a.J.S. Koo, Glucose Metabolism and Glucose Transporters in Breast Cancer, Front Cell Dev Biol. 9 (2021) 728759.

[82] M.E. Lippman, G. Bolan, Oestrogen-responsive human breast cancer in long term tissue culture, Nature 256(5518) (1975) 592-3.

[83] K.H. Jung, E.J. Lee, J.W. Park, J.H. Lee, S.H. Moon, Y.S. Cho, K.H. Lee, EGF receptor stimulation shifts breast cancer cell glucose metabolism toward glycolytic flux through PI3 kinase signaling, PloS one 14(9) (2019) e0221294.

[84] A. Costa, D. Coradini, A. Carrassi, R. Erdas, A. Sardella, M.G. Daidone, Re: Levels of hypoxia-inducible factor-1alpha during breast carcinogenesis, J Natl Cancer Inst 93(15) (2001) 1175-7.

[85] A. Giatromanolaki, M.I. Koukourakis, C. Simopoulos, A. Polychronidis, K.C. Gatter, A.L. Harris, E. Sivridis, c-erbB-2 related aggressiveness in breast cancer is hypoxia inducible factor-1alpha dependent, Clinical cancer research : an official journal of the American Association for Cancer Research 10(23) (2004) 7972-7.

[86] M.S. Jin, H. Lee, I.A. Park, Y.R. Chung, S.A. Im, K.H. Lee, H.G. Moon, W. Han, K. Kim, T.Y. Kim, D.Y. Noh, H.S. Ryu, Overexpression of HIF1alpha and CAXI predicts poor outcome in early-stage triple negative breast cancer, Virchows Arch 469(2) (2016) 183-90.

[87] I. Hernandez-Resendiz, A. Roman-Rosales, E. Garcia-Villa, A. Lopez-Macay, E. Pineda, E. Saavedra, J.C. Gallardo-Perez, E. Alvarez-Rios, P. Gariglio, R. Moreno-Sanchez, S. Rodriguez-Enriquez, Dual regulation of energy metabolism by p53 in human cervix and breast cancer cells, Biochimica et biophysica acta 1853(12) (2015) 3266-78.

[88] L.B. Eckert, G.A. Repasky, A.S. Ulku, A. McFall, H. Zhou, C.I. Sartor, C.J. Der, Involvement of Ras activation in human breast cancer cell signaling, invasion, and anoikis, Cancer research 64(13) (2004) 4585-92.

[89] C. Nunes, C. Silva, A. Correia-Branco, F. Martel, Lack of effect of the procarcinogenic 17beta-estradiol on nutrient uptake by the MCF-7 breast cancer cell line, Biomed Pharmacother 90 (2017) 287-294.

[90] M. Neeman, H. Degani, Metabolic studies of estrogen- and tamoxifen-treated human breast cancer cells by nuclear magnetic resonance spectroscopy, Cancer research 49(3) (1989) 589-94.

[91] Y.D. Bhutia, E. Babu, S. Ramachandran, V. Ganapathy, Amino Acid transporters in cancer and their relevance to "glutamine addiction": novel targets for the design of a new class of anticancer drugs, Cancer research 75(9) (2015) 1782-8.

[92] C. Lopes, C. Pereira, R. Medeiros, ASCT2 and LAT1 Contribution to the Hallmarks of Cancer: From a Molecular Perspective to Clinical Translation, Cancers 13(2) (2021) 203.

[93] H.N. Christensen, Role of amino acid transport and countertransport in nutrition and metabolism, Physiological reviews 70(1) (1990) 43-77.

[94] B.P. Bode, Recent molecular advances in mammalian glutamine transport, J Nutr 131(9 Suppl) (2001) 2475S-85S; discussion 2486S-7S.

[95] H.N. Christensen, M. Liang, E.G. Archer, A distinct Na+-requiring transport system for alanine, serine, cysteine, and similar amino acids, The Journal of biological chemistry 242(22) (1967) 5237-46.

[96] N. Freidman, I. Chen, Q. Wu, C. Briot, J. Holst, J. Font, R. Vandenberg, R. Ryan, Amino Acid Transporters and Exchangers from the SLC1A Family: Structure, Mechanism and Roles in Physiology and Cancer, Neurochem Res 45(6) (2020) 1268-1286.

[97] M.S. Kilberg, M.E. Handlogten, H.N. Christensen, Characteristics of an amino acid transport system in rat liver for glutamine, asparagine, histidine, and closely related analogs, The Journal of biological chemistry 255(9) (1980) 4011-9.

[98] K. Wang, F. Cao, W. Fang, Y. Hu, Y. Chen, H. Ding, G. Yu, Activation of SNAT1/SLC38A1 in human breast cancer: correlation with p-Akt overexpression, BMC cancer 13 (2013) 343.

[99] A. Prat, C. Cruz, K.A. Hoadley, O. Diez, C.M. Perou, J. Balmana, Molecular features of the basal-like breast cancer subtype based on BRCA1 mutation status, Breast cancer research and treatment 147(1) (2014) 185-91.

[100] D. Fotiadis, Y. Kanai, M. Palacin, The SLC3 and SLC7 families of amino acid transporters, Mol Aspects Med 34(2-3) (2013) 139-58.

[101] Q. Wang, J. Holst, L-type amino acid transport and cancer: targeting the mTORC1 pathway to inhibit neoplasia, Am J Cancer Res 5(4) (2015) 1281-94.

[102] A.D. Marshall, M. van Geldermalsen, N.J. Otte, T. Lum, M. Vellozzi, A. Thoeng, A. Pang, R. Nagarajah, B. Zhang, Q. Wang, L. Anderson, J.E.J. Rasko, J. Holst, ASCT2 regulates glutamine uptake and cell growth in endometrial carcinoma, Oncogenesis 6(7) (2017) e367.

[103] M. Chiu, C. Sabino, G. Taurino, M.G. Bianchi, R. Andreoli, N. Giuliani, O. Bussolati, GPNA inhibits the sodium-independent transport system L for neutral amino acids, Amino acids 49(8) (2017) 1365-1372.

[104] L.A. Timmerman, T. Holton, M. Yuneva, R.J. Louie, M. Padro, A. Daemen, M. Hu, D.A. Chan, S.P. Ethier, L.J. van 't Veer, K. Polyak, F. McCormick, J.W. Gray, Glutamine sensitivity analysis identifies the xCT antiporter as a common triple-negative breast tumor therapeutic target, Cancer Cell 24(4) (2013) 450-65.

[105] S. Karunakaran, S. Ramachandran, V. Coothankandaswamy, S. Elangovan, E. Babu, S. Periyasamy-Thandavan, A. Gurav, J.P. Gnanaprakasam, N. Singh, P.V. Schoenlein, P.D. Prasad, M. Thangaraju, V. Ganapathy, SLC6A14 (ATB0,+) protein, a highly concentrative and broad specific amino acid transporter, is a novel and effective drug target for treatment of estrogen receptor-positive breast cancer, The Journal of biological chemistry 286(36) (2011) 31830-8.

[106] J.C. Leo, S.M. Wang, C.H. Guo, S.E. Aw, Y. Zhao, J.M. Li, K.M. Hui, V.C. Lin, Gene regulation profile reveals consistent anticancer properties of progesterone in hormone-independent breast cancer cells transfected with progesterone receptor, International journal of cancer 117(4) (2005) 561-8.

[107] R. Sweet, A. Paul, J. Zastre, Hypoxia induced upregulation and function of the thiamine transporter, SLC19A3 in a breast cancer cell line, Cancer biology & therapy 10(11) (2010) 1101-11.

[108] Y. Jiang, Y. Cao, Y. Wang, W. Li, X. Liu, Y. Lv, X. Li, J. Mi, Cysteine transporter SLC3A1 promotes breast cancer tumorigenesis, Theranostics 7(4) (2017) 1036-1046.

[109] M. van Geldermalsen, Q. Wang, R. Nagarajah, A.D. Marshall, A. Thoeng, D. Gao, W. Ritchie, Y. Feng, C.G. Bailey, N. Deng, K. Harvey, J.M. Beith, C.I. Selinger, S.A. O'Toole, J.E. Rasko, J. Holst, ASCT2/SLC1A5 controls glutamine uptake and tumour growth in triple-negative basal-like breast cancer, Oncogene 35(24) (2016) 3201-8.

[110] M. Scalise, L. Pochini, L. Console, M.A. Losso, C. Indiveri, The Human SLC1A5 (ASCT2) Amino Acid Transporter: From Function to Structure and Role in Cell Biology, Frontiers in cell and developmental biology 6 (2018) 96.

[111] Y. Liu, T. Zhao, Z. Li, L. Wang, S. Yuan, L. Sun, The role of ASCT2 in cancer: A review, European journal of pharmacology 837 (2018) 81-87.

[112] S. Kim, D.H. Kim, W.H. Jung, J.S. Koo, Expression of glutamine metabolism-related proteins according to molecular subtype of breast cancer, Endocrine-related cancer 20(3) (2013) 339-48.

[113] M.D. Cao, S. Lamichhane, S. Lundgren, A. Bofin, H. Fjosne, G.F. Giskeodegard, T.F. Bathen, Metabolic characterization of triple negative breast cancer, BMC cancer 14 (2014) 941.

[114] Y.M. Kanaan, B.P. Sampey, D. Beyene, A.K. Esnakula, T.J. Naab, L.J. Ricks-Santi, S. Dasi, A. Day, K.W. Blackman, W. Frederick, R.L. Copeland, Sr., E. Gabrielson, R.L. Dewitty, Jr., Metabolic profile of triple-negative breast cancer in African-American women reveals potential biomarkers of aggressive disease, Cancer Genomics Proteomics 11(6) (2014) 279-94.

[115] C.T. Hensley, A.T. Wasti, R.J. DeBerardinis, Glutamine and cancer: cell biology, physiology, and clinical opportunities, The Journal of clinical investigation 123(9) (2013) 3678-84.

[116] J. Xu, Y. Chen, O.I. Olopade, MYC and Breast Cancer, Genes Cancer 1(6) (2010) 629-40.

[117] D.R. Wise, R.J. DeBerardinis, A. Mancuso, N. Sayed, X.Y. Zhang, H.K. Pfeiffer, I. Nissim, E. Daikhin, M. Yudkoff, S.B. McMahon, C.B. Thompson, Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction, Proc Natl Acad Sci U S A 105(48) (2008) 18782-7.

[118] Y.J. Jeon, S. Khelifa, B. Ratnikov, D.A. Scott, Y. Feng, F. Parisi, C. Ruller, E. Lau, H. Kim, L.M. Brill, T. Jiang, D.L. Rimm, R.D. Cardiff, G.B. Mills, J.W. Smith, A.L. Osterman, Y. Kluger, Z.A.

Ronai, Regulation of glutamine carrier proteins by RNF5 determines breast cancer response to ER stress-inducing chemotherapies, Cancer Cell 27(3) (2015) 354-69.

[119] M.R. Reynolds, A.N. Lane, B. Robertson, S. Kemp, Y. Liu, B.G. Hill, D.C. Dean, B.F. Clem, Control of glutamine metabolism by the tumor suppressor Rb, Oncogene 33(5) (2014) 556-66.

[120] J. Dong, D. Xiao, Z. Zhao, P. Ren, C. Li, Y. Hu, J. Shi, H. Su, L. Wang, H. Liu, B. Li, P. Gao, G. Qing, Epigenetic silencing of microRNA-137 enhances ASCT2 expression and tumor glutamine metabolism, Oncogenesis 6(7) (2017) e356.

[121] V.K. Todorova, Y. Kaufmann, S. Luo, V.S. Klimberg, Tamoxifen and raloxifene suppress the proliferation of estrogen receptor-negative cells through inhibition of glutamine uptake, Cancer Chemother Pharmacol 67(2) (2011) 285-91.

[122] F.I. Tade, M.A. Cohen, T.M. Styblo, O.A. Odewole, A.I. Holbrook, M.S. Newell, B. Savir-Baruch, X. Li, M.M. Goodman, J.A. Nye, D.M. Schuster, Anti-3-18F-FACBC (18F-Fluciclovine) PET/CT of Breast Cancer: An Exploratory Study, J Nucl Med 57(9) (2016) 1357-63.

[123] M.H.C. Wilson E.B, Cancer and Tuberculosis with Some Comments on Cancer and Other Diseases, American Journal of Cancer Research 16 (1932) 227–250.

[124] H.K. Bronsveld, V. Jensen, P. Vahl, M.L. De Bruin, S. Cornelissen, J. Sanders, A. Auvinen, J. Haukka, M. Andersen, P. Vestergaard, M.K. Schmidt, Diabetes and Breast Cancer Subtypes, PloS one 12(1) (2017) e0170084.

[125] C. Silva, N. Andrade, J.T. Guimaraes, E. Patricio, F. Martel, The in vitro effect of the diabetes-associated markers insulin, leptin and oxidative stress on cellular characteristics promoting breast cancer progression is GLUT1-dependent, European journal of pharmacology 898 (2021) 173980.

[126] E.J. Gallagher, D. LeRoith, Obesity and Diabetes: The Increased Risk of Cancer and Cancer-Related Mortality, Physiological reviews 95(3) (2015) 727-48.

[127] P. Ferroni, S. Riondino, O. Buonomo, R. Palmirotta, F. Guadagni, M. Roselli, Type 2 Diabetes and Breast Cancer: The Interplay between Impaired Glucose Metabolism and Oxidant Stress, Oxid Med Cell Longev 2015 (2015) 183928.

[128] P. Vigneri, F. Frasca, L. Sciacca, G. Pandini, R. Vigneri, Diabetes and cancer, Endocrine-related cancer 16(4) (2009) 1103-23.

[129] H. Sharaf, S. Matou-Nasri, Q. Wang, Z. Rabhan, H. Al-Eidi, A. Al Abdulrahman, N. Ahmed, Advanced glycation endproducts increase proliferation, migration and invasion of the breast cancer cell line MDA-MB-231, Biochimica et biophysica acta 1852(3) (2015) 429-41.

[130] A.M. Korwar, H.S. Bhonsle, A.D. Chougale, S.S. Kote, K.R. Gawai, V.S. Ghole, C.B. Koppikar, M.J. Kulkarni, Analysis of AGE modified proteins and RAGE expression in HER2/neu negative invasive ductal carcinoma, Biochemical and biophysical research communications 419(3) (2012) 490-4.

[131] L.D. Yee, J.E. Mortimer, R. Natarajan, E.C. Dietze, V.L. Seewaldt, Metabolic Health, Insulin, and Breast Cancer: Why Oncologists Should Care About Insulin, Frontiers in endocrinology 11 (2020) 58.

[132] I. Wolf, S. Sadetzki, R. Catane, A. Karasik, B. Kaufman, Diabetes mellitus and breast cancer, The Lancet. Oncology 6(2) (2005) 103-11.

[133] D.P. Rose, L. Vona-Davis, The cellular and molecular mechanisms by which insulin influences breast cancer risk and progression, Endocrine-related cancer 19(6) (2012) R225-41.

[134] L. Frittitta, R. Vigneri, M.R. Stampfer, I.D. Goldfine, Insulin receptor overexpression in 184B5 human mammary epithelial cells induces a ligand-dependent transformed phenotype, J Cell Biochem 57(4) (1995) 666-9.

[135] D.V. Pham, P.H. Park, Tumor Metabolic Reprogramming by Adipokines as a Critical Driver of Obesity-Associated Cancer Progression, International journal of molecular sciences 22(3) (2021) 1444.

[136] F. Sanchez-Jimenez, A. Perez-Perez, L. de la Cruz-Merino, V. Sanchez-Margalet, Obesity and Breast Cancer: Role of Leptin, Frontiers in oncology 9 (2019) 596.

[137] S. Guo, M. Liu, G. Wang, M. Torroella-Kouri, R.R. Gonzalez-Perez, Oncogenic role and therapeutic target of leptin signaling in breast cancer and cancer stem cells, Biochimica et biophysica acta 1825(2) (2012) 207-22.

[138] J.A. Dias, G.N. Fredrikson, U. Ericson, B. Gullberg, B. Hedblad, G. Engstrom, S. Borgquist, J. Nilsson, E. Wirfalt, Low-Grade Inflammation, Oxidative Stress and Risk of Invasive Post-Menopausal Breast Cancer - A Nested Case-Control Study from the Malmo Diet and Cancer Cohort, PloS one 11(7) (2016) e0158959.

[139] K.H. Joung, J.W. Jeong, B.J. Ku, The association between type 2 diabetes mellitus and women cancer: the epidemiological evidences and putative mechanisms, Biomed Res Int 2015 (2015) 920618.

[140] Y. Ning, R.B. Riggins, J.E. Mulla, H. Chung, A. Zwart, R. Clarke, IFNgamma restores breast cancer sensitivity to fulvestrant by regulating STAT1, IFN regulatory factor 1, NF-kappaB, BCL2

family members, and signaling to caspase-dependent apoptosis, Molecular cancer therapeutics 9(5) (2010) 1274-85.

[141] A. Mantovani, P. Allavena, A. Sica, F. Balkwill, Cancer-related inflammation, Nature 454(7203) (2008) 436-44.

[142] A.B. Crujeiras, A. Diaz-Lagares, M.C. Carreira, M. Amil, F.F. Casanueva, Oxidative stress associated to dysfunctional adipose tissue: a potential link between obesity, type 2 diabetes mellitus and breast cancer, Free Radic Res 47(4) (2013) 243-56.

[143] T. Scully, A. Ettela, D. LeRoith, E.J. Gallagher, Obesity, Type 2 Diabetes, and Cancer Risk, Frontiers in oncology 10 (2020) 615375.

[144] C. Panis, V.J. Victorino, A.C. Herrera, L.F. Freitas, T. De Rossi, F.C. Campos, A.N. Simao, D.S. Barbosa, P. Pinge-Filho, R. Cecchini, A.L. Cecchini, Differential oxidative status and immune characterization of the early and advanced stages of human breast cancer, Breast cancer research and treatment 133(3) (2012) 881-8.

[145] U.E. Martinez-Outschoorn, M. Peiris-Pages, R.G. Pestell, F. Sotgia, M.P. Lisanti, Cancer metabolism: a therapeutic perspective, Nature reviews. Clinical oncology 14(2) (2017) 113.

[146] M.P. Ogrodzinski, J.J. Bernard, S.Y. Lunt, Deciphering metabolic rewiring in breast cancer subtypes, Transl Res 189 (2017) 105-122.

[147] H. Siebeneicher, A. Cleve, H. Rehwinkel, R. Neuhaus, I. Heisler, T. Muller, M. Bauser, B. Buchmann, Identification and Optimization of the First Highly Selective GLUT1 Inhibitor BAY-876, ChemMedChem 11(20) (2016) 2261-2271.

[148] C.S. Esslinger, K.A. Cybulski, J.F. Rhoderick, Ngamma-aryl glutamine analogues as probes of the ASCT2 neutral amino acid transporter binding site, Bioorganic & medicinal chemistry 13(4) (2005) 1111-8.

[149] W.H. Talib, A.I. Mahmod, S.F. Abuarab, E. Hasen, A.A. Munaim, S.K. Haif, A.M. Ayyash, S. Khater, I.H. Al-Yasari, L.T.A. Kury, Diabetes and Cancer: Metabolic Association, Therapeutic Challenges, and the Role of Natural Products, Molecules 26(8) (2021) 2179.

[150] E.M. McGowan, A. Simpson, J. McManaman, V. Boonyaratanakornkit, A.A. Hardikar, Hijacking of Endocrine and Metabolic Regulation in Cancer and Diabetes, Biomed Res Int 2015 (2015) 386203.

[151] H. Chen, L.S. Cook, M.C. Tang, D.A. Hill, C.L. Wiggins, C.I. Li, Relationship between Diabetes and Diabetes Medications and Risk of Different Molecular Subtypes of Breast Cancer, Cancer epidemiology, biomarkers & prevention : a publication of the American Association for

Cancer Research, cosponsored by the American Society of Preventive Oncology 28(11) (2019) 1802-1808.

[152] A. Crispo, L.S. Augustin, M. Grimaldi, F. Nocerino, A. Giudice, E. Cavalcanti, M. Di Bonito, G. Botti, M. De Laurentiis, M. Rinaldo, E. Esposito, G. Riccardi, A. Amore, M. Libra, G. Ciliberto, D.J. Jenkins, M. Montella, Risk Differences Between Prediabetes And Diabetes According To Breast Cancer Molecular Subtypes, J Cell Physiol 232(5) (2017) 1144-1150.

[153] M.L. Panno, M. Salerno, V. Pezzi, D. Sisci, M. Maggiolini, L. Mauro, E.G. Morrone, S. Ando, Effect of oestradiol and insulin on the proliferative pattern and on oestrogen and progesterone receptor contents in MCF-7 cells, J Cancer Res Clin Oncol 122(12) (1996) 745-9.

[154] G. Maskarinec, S. Jacobs, S.Y. Park, C.A. Haiman, V.W. Setiawan, L.R. Wilkens, L. Le Marchand, Type II Diabetes, Obesity, and Breast Cancer Risk: The Multiethnic Cohort, Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology 26(6) (2017) 854-861.

[155] K. Subik, J.F. Lee, L. Baxter, T. Strzepek, D. Costello, P. Crowley, L. Xing, M.C. Hung, T. Bonfiglio, D.G. Hicks, P. Tang, The Expression Patterns of ER, PR, HER2, CK5/6, EGFR, Ki-67 and AR by Immunohistochemical Analysis in Breast Cancer Cell Lines, Breast Cancer (Auckl) 4 (2010) 35-41.

[156] J. Dai, J. Jian, M. Bosland, K. Frenkel, G. Bernhardt, X. Huang, Roles of hormone replacement therapy and iron in proliferation of breast epithelial cells with different estrogen and progesterone receptor status, Breast 17(2) (2008) 172-9.

[157] F.G. Kern, S.W. McLeskey, L. Zhang, J. Kurebayashi, Y. Liu, I.Y. Ding, S. Kharbanda, D. Chen, D. Miller, K. Cullen, et al., Transfected MCF-7 cells as a model for breast-cancer progression, Breast cancer research and treatment 31(2-3) (1994) 153-65.

[158] R. Shrivastava, S. Sen, D. Banerji, A.K. Praharaj, G.S. Chopra, S.S. Gill, Assessment of non-invasive models for liver fibrosis in chronic hepatitis B virus related liver disease patients in resource limited settings, Indian J Pathol Microbiol 56(3) (2013) 196-9.

[159] C. Gupta, K. Tikoo, High glucose and insulin differentially modulates proliferation in MCF-7 and MDA-MB-231 cells, J Mol Endocrinol 51(1) (2013) 119-29.

[160] E.A. Hussain-Hakimjee, X. Peng, R.R. Mehta, R.G. Mehta, Growth inhibition of carcinogen-transformed MCF-12F breast epithelial cells and hormone-sensitive BT-474 breast cancer cells by 1alpha-hydroxyvitamin D5, Carcinogenesis 27(3) (2006) 551-9.

[161] G. Calaf, J. Russo, Transformation of human breast epithelial cells by chemical carcinogens, Carcinogenesis 14(3) (1993) 483-92.

[162] S. Borowicz, M. Van Scoyk, S. Avasarala, M.K. Karuppusamy Rathinam, J. Tauler, R.K. Bikkavilli, R.A. Winn, The soft agar colony formation assay, J Vis Exp (92) (2014) e51998.

[163] A. Csiszar, P. Balasubramanian, S. Tarantini, A. Yabluchanskiy, X.A. Zhang, Z. Springo, D. Benbrook, W.E. Sonntag, Z. Ungvari, Chemically induced carcinogenesis in rodent models of aging: assessing organismal resilience to genotoxic stressors in geroscience research, Geroscience 41(2) (2019) 209-227.

[164] M.L. Wei, P. Duan, Z.M. Wang, M. Ding, P. Tu, High glucose and high insulin conditions promote MCF7 cell proliferation and invasion by upregulating IRS1 and activating the Ras/Raf/ERK pathway, Molecular medicine reports 16(5) (2017) 6690-6696.

[165] M. Weichhaus, J. Broom, K. Wahle, G. Bermano, A novel role for insulin resistance in the connection between obesity and postmenopausal breast cancer, International journal of oncology 41(2) (2012) 745-52.

[166] V. Papa, G. Milazzo, I.D. Goldfine, F.M. Waldman, R. Vigneri, Sporadic amplification of the insulin receptor gene in human breast cancer, Journal of endocrinological investigation 20(9) (1997) 531-6.

[167] A. Mawson, A. Lai, J.S. Carroll, C.M. Sergio, C.J. Mitchell, B. Sarcevic, Estrogen and insulin/IGF-1 cooperatively stimulate cell cycle progression in MCF-7 breast cancer cells through differential regulation of c-Myc and cyclin D1, Mol Cell Endocrinol 229(1-2) (2005) 161-73.

[168] J. Franke, V. Abs, C. Zizzadoro, G. Abraham, Comparative study of the effects of fetal bovine serum versus horse serum on growth and differentiation of primary equine bronchial fibroblasts, BMC Vet Res 10 (2014) 119.

[169] H.R. Harris, S.S. Tworoger, S.E. Hankinson, B.A. Rosner, K.B. Michels, Plasma leptin levels and risk of breast cancer in premenopausal women, Cancer Prev Res (Phila) 4(9) (2011) 1449-56.

[170] F. Yi, S. Diao, X.L. Yuan, J.Y. Li, Association of plasma leptin levels and soluble leptin receptor with breast cancer, Zhonghua Yu Fang Yi Xue Za Zhi 52(3) (2018) 253-259.

[171] S. Ando, L. Gelsomino, S. Panza, C. Giordano, D. Bonofiglio, I. Barone, S. Catalano, Obesity, Leptin and Breast Cancer: Epidemiological Evidence and Proposed Mechanisms, Cancers 11(1) (2019) 62.

[172] K.J. Nkhata, A. Ray, T.F. Schuster, M.E. Grossmann, M.P. Cleary, Effects of adiponectin and leptin co-treatment on human breast cancer cell growth, Oncology reports 21(6) (2009) 1611-9.

[173] R.R. Gonzalez, S. Cherfils, M. Escobar, J.H. Yoo, C. Carino, A.K. Styer, B.T. Sullivan, H. Sakamoto, A. Olawaiye, T. Serikawa, M.P. Lynch, B.R. Rueda, Leptin signaling promotes the growth of mammary tumors and increases the expression of vascular endothelial growth factor (VEGF) and its receptor type two (VEGF-R2), The Journal of biological chemistry 281(36) (2006) 26320-8.

[174] X.L. Yuan, Z.P. Xu, C.R. Liu, L.P. Yan, P. Tao, P. Xiong, Q. Li, M. Zhou, H. Li, M. Zhao, J.Y. Li, Study of the association between polymorphism of persistent obesity, human leptin gene/leptin receptor gene and molecular subtypes of breast cancer, Zhonghua Yu Fang Yi Xue Za Zhi 51(6) (2017) 533-538.

[175] Y. Kong, Q. Dong, H. Ji, M. Sang, Y. Ding, M. Zhao, H. Yang, C. Geng, The Effect of the Leptin and Leptin Receptor Expression on the Efficacy of Neoadjuvant Chemotherapy in Breast Cancer, Med Sci Monit 25 (2019) 3005-3013.

[176] E. Crespi, G. Bottai, L. Santarpia, Role of inflammation in obesity-related breast cancer, Current opinion in pharmacology 31 (2016) 114-122.

[177] B.J. Barnes, M.J. Kellum, K.E. Pinder, J.A. Frisancho, P.M. Pitha, Interferon regulatory factor 5, a novel mediator of cell cycle arrest and cell death, Cancer research 63(19) (2003) 6424-31.

[178] V. Pileczki, C. Braicu, C.D. Gherman, I. Berindan-Neagoe, TNF-alpha gene knockout in triple negative breast cancer cell line induces apoptosis, International journal of molecular sciences 14(1) (2012) 411-20.

[179] A.C. Herrera, C. Panis, V.J. Victorino, F.C. Campos, A.N. Colado-Simao, A.L. Cecchini, R. Cecchini, Molecular subtype is determinant on inflammatory status and immunological profile from invasive breast cancer patients, Cancer Immunol Immunother 61(11) (2012) 2193-201.

[180] Wahyu Widowati, Harry Murti, Diana Krisanti Jasaputra, Sutiman B. Sumitro, M. Aris Widodo, Nurul Fauziah, M.M.I. Bachtiar, Selective Cytotoxic Potential of IFN-γ and TNF-α on Breast Cancer Cell Lines (T47D and MCF7), Asian Journal of Cell Biology 11(1) (2016) 12.

[181] M. Zhang, R. Guo, Y. Zhai, D. Yang, LIGHT sensitizes IFNgamma-mediated apoptosis of MDA-MB-231 breast cancer cells leading to down-regulation of anti-apoptosis Bcl-2 family members, Cancer letters 195(2) (2003) 201-10.

[182] F.L. Sarmiento-Salinas, A. Delgado-Magallon, J.B. Montes-Alvarado, D. Ramirez-Ramirez, J.C. Flores-Alonso, P. Cortes-Hernandez, J. Reyes-Leyva, I. Herrera-Camacho, M. Anaya-Ruiz, R. Pelayo, L. Millan-Perez-Pena, P. Maycotte, Breast Cancer Subtypes Present a Differential Production of Reactive Oxygen Species (ROS) and Susceptibility to Antioxidant Treatment, Frontiers in oncology 9 (2019) 480.

[183] I. Isnaini, N. Permatasari, K. Mintaroem, B. Prihardina, M.A. Widodo, Oxidants-Antioxidants Profile in the Breast Cancer Cell Line MCF-7, Asian Pacific journal of cancer prevention : APJCP 19(11) (2018) 3175-3178.

[184] S. Rodic, M.D. Vincent, Reactive oxygen species (ROS) are a key determinant of cancer's metabolic phenotype, International journal of cancer 142(3) (2018) 440-448.

[185] K. Wahi, J. Holst, ASCT2: a potential cancer drug target, Expert Opin Ther Targets 23(7) (2019) 555-558.

[186] S. Kim, D.H. Kim, W.H. Jung, J.S. Koo, Metabolic phenotypes in triple-negative breast cancer, Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine 34(3) (2013) 1699-712.

[187] I. Hamann, D. Krys, D. Glubrecht, V. Bouvet, A. Marshall, L. Vos, J.R. Mackey, M. Wuest, F. Wuest, Expression and function of hexose transporters GLUT1, GLUT2, and GLUT5 in breast cancer-effects of hypoxia, FASEB J 32(9) (2018) 5104-5118.

[188] Y. Ma, W. Wang, M.O. Idowu, U. Oh, X.Y. Wang, S.M. Temkin, X. Fang, Ovarian Cancer Relies on Glucose Transporter 1 to Fuel Glycolysis and Growth: Anti-Tumor Activity of BAY-876, Cancers 11(1) (2018) 33.

[189] Q. Wu, W. Ba-Alawi, G. Deblois, J. Cruickshank, S. Duan, E. Lima-Fernandes, J. Haight, S.A.M. Tonekaboni, A.M. Fortier, H. Kuasne, T.D. McKee, H. Mahmoud, M. Kushida, S. Cameron, N. Dogan-Artun, W. Chen, Y. Nie, L.X. Zhang, R.N. Vellanki, S. Zhou, P. Prinos, B.G. Wouters, P.B. Dirks, S.J. Done, M. Park, D.W. Cescon, B. Haibe-Kains, M. Lupien, C.H. Arrowsmith, GLUT1 inhibition blocks growth of RB1-positive triple negative breast cancer, Nat Commun 11(1) (2020) 4205.

[190] A. Corti, S. Dominici, S. Piaggi, E. Belcastro, M. Chiu, G. Taurino, S. Pacini, O. Bussolati, A. Pompella, gamma-Glutamyltransferase enzyme activity of cancer cells modulates L-gamma-glutamyl-p-nitroanilide (GPNA) cytotoxicity, Scientific reports 9(1) (2019) 891.

[191] A. Broer, F. Rahimi, S. Broer, Deletion of Amino Acid Transporter ASCT2 (SLC1A5) Reveals an Essential Role for Transporters SNAT1 (SLC38A1) and SNAT2 (SLC38A2) to Sustain Glutaminolysis in Cancer Cells, The Journal of biological chemistry 291(25) (2016) 13194-205.

[192] M.L. Schulte, A. Fu, P. Zhao, J. Li, L. Geng, S.T. Smith, J. Kondo, R.J. Coffey, M.O. Johnson, J.C. Rathmell, J.T. Sharick, M.C. Skala, J.A. Smith, J. Berlin, M.K. Washington, M.L. Nickels, H.C. Manning, Pharmacological blockade of ASCT2-dependent glutamine transport leads to antitumor efficacy in preclinical models, Nature medicine 24(2) (2018) 194-202.

[193] M. Hassanein, J. Qian, M.D. Hoeksema, J. Wang, M. Jacobovitz, X. Ji, F.T. Harris, B.K. Harris, K.L. Boyd, H. Chen, R. Eisenberg, P.P. Massion, Targeting SLC1a5-mediated glutamine dependence in non-small cell lung cancer, International journal of cancer 137(7) (2015) 1587-97.

[194] M. Bolzoni, M. Chiu, F. Accardi, R. Vescovini, I. Airoldi, P. Storti, K. Todoerti, L. Agnelli, G. Missale, R. Andreoli, M.G. Bianchi, M. Allegri, A. Barilli, F. Nicolini, A. Cavalli, F. Costa, V. Marchica, D. Toscani, C. Mancini, E. Martella, V. Dall'Asta, G. Donofrio, F. Aversa, O. Bussolati, N. Giuliani, Dependence on glutamine uptake and glutamine addiction characterize myeloma cells: a new attractive target, Blood 128(5) (2016) 667-79.

[195] I.F. Robey, A.D. Lien, S.J. Welsh, B.K. Baggett, R.J. Gillies, Hypoxia-inducible factor-1alpha and the glycolytic phenotype in tumors, Neoplasia 7(4) (2005) 324-30.

[196] L. Yang, J. Li, Y. Li, Y. Zhou, Z. Wang, D. Zhang, J. Liu, X. Zhang, Diclofenac impairs the proliferation and glucose metabolism of triple-negative breast cancer cells by targeting the c-Myc pathway, Exp Ther Med 21(6) (2021) 584.

[197] E. Shin, J.S. Koo, Glucose Metabolism and Glucose Transporters in Breast Cancer, Frontiers in cell and developmental biology 9 (2021) 728759.

[198] P. Nicklin, P. Bergman, B. Zhang, E. Triantafellow, H. Wang, B. Nyfeler, H. Yang, M. Hild, C. Kung, C. Wilson, V.E. Myer, J.P. MacKeigan, J.A. Porter, Y.K. Wang, L.C. Cantley, P.M. Finan, L.O. Murphy, Bidirectional transport of amino acids regulates mTOR and autophagy, Cell 136(3) (2009) 521-34.

[199] Y. Cormerais, P.A. Massard, M. Vucetic, S. Giuliano, E. Tambutte, J. Durivault, V. Vial, H. Endou, M.F. Wempe, S.K. Parks, J. Pouyssegur, The glutamine transporter ASCT2 (SLC1A5) promotes tumor growth independently of the amino acid transporter LAT1 (SLC7A5), The Journal of biological chemistry 293(8) (2018) 2877-2887.

[200] T. Yazawa, K. Shimizu, K. Kaira, T. Nagashima, Y. Ohtaki, J. Atsumi, K. Obayashi, S. Nagamori, Y. Kanai, T. Oyama, I. Takeyoshi, Clinical significance of coexpression of L-type amino acid transporter 1 (LAT1) and ASC amino acid transporter 2 (ASCT2) in lung adenocarcinoma, Am J Transl Res 7(6) (2015) 1126-39.

[201] H. Honjo, K. Kaira, T. Miyazaki, T. Yokobori, Y. Kanai, S. Nagamori, T. Oyama, T. Asao, H. Kuwano, Clinicopathological significance of LAT1 and ASCT2 in patients with surgically resected esophageal squamous cell carcinoma, Journal of surgical oncology 113(4) (2016) 381-9.

[202] F. Castro, A.P. Cardoso, R.M. Goncalves, K. Serre, M.J. Oliveira, Interferon-Gamma at the Crossroads of Tumor Immune Surveillance or Evasion, Front Immunol 9 (2018) 847.

[203] L. Martin-Hijano, B. Sainz, Jr., The Interactions Between Cancer Stem Cells and the Innate Interferon Signaling Pathway, Front Immunol 11 (2020) 526.

[204] N.E. Avissar, H.C. Sax, L. Toia, In human entrocytes, GLN transport and ASCT2 surface expression induced by short-term EGF are MAPK, PI3K, and Rho-dependent, Dig Dis Sci 53(8) (2008) 2113-25.

[205] A. Costantino, G. Milazzo, F. Giorgino, P. Russo, I.D. Goldfine, R. Vigneri, A. Belfiore, Insulin-resistant MDA-MB231 human breast cancer cells contain a tyrosine kinase inhibiting activity, Mol Endocrinol 7(12) (1993) 1667-76.