M 2013

U.PORTO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

# THE EFFECT OF DIETARY POLYPHENOLS ON GLUCOSE UPTAKE BY BREAST CANCER CELLS (MCF-7)

CLÁUDIA FILIPA MAIA AZEVEDO
DISSERTAÇÃO DE MESTRADO APRESENTADA
AO INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
DA UNIVERSIDADE DO PORTO EM
ONCOLOGIA- ESPECIALIZAÇÃO ONCOLOGIA MOLECULAR

**Cláudia Filipa Maia Azevedo**

# The effect of dietary polyphenols on glucose uptake by breast cancer cells (MCF-7)

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

**Orientador:** Prof. Dr. Fátima Martel

**Categoria:** Professora Associada

**Afiliação:** Faculdade de Medicina da Universidade do Porto

**Co-orientador:** Prof. Dr. Elisa Keating

**Categoria:** Professora Auxiliar Convidada

**Afiliação:** Faculdade de Medicina da Universidade do Porto

Ao longo da realização do trabalho experimental que esteve na base da dissertação do Mestrado em Oncologia tive várias ajudas e apoio de pessoas, às quais não posso deixar de expressar os meus sinceros agradecimentos.

Em primeiro lugar, à minha orientadora, Professora Doutora Fátima Martel, pela oportunidade e total disponibilidade oferecida, bem como por todos os conselhos e apoio científico que me transmitiu na discussão e orientação deste projeto.

À minha co-orientadora, Professora Doutora Elisa Keating pela possibilidade de desenvolver o presente trabalho relacionado com o seu tema de Pós-Doutoramento intitulado "Transmembrane transport in trophoblast and cancer cells: molecular similaritties and disparitties"; e pelo apoio e incentivo ao longo de todo o trabalho.

A todo o Departamento de Bioquímica da Faculdade de Medicina da Universidade do Porto agradeço o acolhimento, a ajuda e simpatia. Em especial, à Ana Branco e ao João Araújo agradeço a preciosa ajuda no desenvolvimento deste projeto.

Agradeço à Doutora Maria João Pinho, do Departamento de Farmacologia e Terapêutica da Faculdade de Medicina da Universidade do Porto, pela ajuda que gentilmente me deu na quantificação dos níveis de RNAm através de qRT-PCR.

À Professora Doutora Rita Negrão, à Raquel Costa e à Vera Machado pela ajuda e noções dadas aquando da realização do western-blot.

Ao Prof. Doutor João Tiago Guimarães, do Departamento de Patologia Clínica do Hospital de S. João, pela disponibilidade e prontidão, cuja colaboração foi sem dúvida uma mais-valia para a execução do projeto.

Ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto e à Diretora do Mestrado em questão, a Professora Doutora Berta Silva, por todo o apoio prestado durante o Mestrado.

E por último, mas não menos importante, um agradecimento carinhoso à minha família, aos meus amigos e aos escuteiros pelo apoio, motivação, disponibilidade e paciência que foram cruciais durante todo o meu percurso académico e na minha formação pessoal.

As células tumorais apresentam um metabolismo alterado, com um aumento da taxa de captação de glucose e produção de lactato relativamente ao metabolismo oxidativo (efeito de Warburg). Os polifenóis alimentares são conhecidos por possuírem efeitos quimiopreventivos e anticancerígenos. Recentemente, o nosso grupo verificou que os polifenóis quercetina (um flavanol) e a epigalocatequina-3-galato (um flavan-3-ol) inibem a captação de glucose em células de cancro da mama (células MCF-7). Por esta razão, decidiu-se investigar se outros polifenóis interferem também com a captação de glucose nestas células. Os polifenóis testados foram outros flavanóis (miricetina e kaempferol) e flavan-3-óis ((+) catequina e (-) epicatequina), uma flavona (crisina), uma isoflavona (genisteina), uma chalcona (xantohumol) e um stilbeno (resveratrol).

A captação de $^{3}$H-desoxi-D-glucose ($^{3}$H-DG) pelas células MCF-7 foi dependente do tempo e saturável ($K_m$=6.5±0.5 mM e $V_{máx}$=63.6±2.3 nmol/mg prot).

Agudamente (26 min), a miricetina (10-100 µM), a crisina (100 µM), a genisteína (10-100 µM), o resveratrol (10-100 µM), o kaempferol (10-100 µM) e o xantohumol (10-100 µM) inibiram a captação de $^{3}$H-DG. Por outro lado, a (+) catequina e a (-) epicatequina aumentaram-na ligeiramente (10-15%). Verificou-se que o kaempferol foi o inibidor mais potente da captação de $^{3}$H-DG nas células MCF-7, com um $IC_{50}$ de 4.0 (1.6-9.8) µM (o $IC_{50}$ da genisteína e do resveratrol foram 38.9 (15.2-99.4) µM e 67.2 (29.4-153.7) µM, respectivamente). O kaempferol (100 µM) comporta-se como um inibidor misto, uma vez que, aumenta o $K_m$ (para 15.6± 2.4 mM) e simultaneamente o $V_{máx}$ (para 106.9± 10.6 nmol/mg prot).

Cronicamente (24 h), o kaempferol foi também capaz de inibir a captação de $^{3}$H-DG ($IC_{50}$ de 13.6 (2.8-66.9) µM). Este flavonóide inibiu em cerca de 40% a transcrição do GLUT e, apesar de não significativo, este efeito foi acompanhado pela diminuição de produção proteica do GLUT1. Verificou-se ainda que a exposição das células ao kaempferol induziu um aumento dos níveis de lactato extracelular ao longo do tempo, o que sugere que este flavonóide inibe a recaptação de lactato através da inibição do MCT1.

Adicionalmente, o kaempferol revelou propriedades antiproliferativas e citotóxicas, uma vez que foi capaz de inibir o crescimento e reduzir a viabilidade celular. Estes efeitos do kaempferol foram mimetizados com uma concentração baixa de glucose no meio extracelular e foram revertidos com uma concentração alta de glicose no meio extracelular.

Em conclusão, o kaempferol (e, embora menos potentemente, alguns dos outros polifenóis) inibe a captação de glucose nas células MCF-7 de uma forma potente, aparentemente por bloqueio de membros da família GLUT de transportadores de glicose

(muito provavelmente o GLUT1). Este efeito parece contribuir para o efeito quimiopreventivo deste composto presente na dieta.

**Palavras-Chave** · Células do Cancro da Mama · Absorção de Glicose · Polifenóis · Kaempferol

## ABSTRACT

Cancer cells present an altered metabolism, with an increased rate of glucose uptake and lactate production instead of oxidative metabolism (the Warburg effect). Dietary polyphenols are known to possess cancer preventive and anticancer effects. Recently, our group verified that the polyphenols quercetin (a flavanol) and epigallocatechin-3-gallate (a flavan-3-ol) inhibited glucose uptake by the MCF-7 breast cancer cell line. So, we decided to investigate if other polyphenols could also interfere with glucose uptake by these cells. The polyphenols tested were other flavanols (myricetin and kaempferol) and flavan-3-ols ((+) catechin and (-) epicatechin), a flavone (chrysin) and an isoflavone (genistein), a chalchone (xanthohumol) and a stilbene (resveratrol).

Uptake of $^{3}$H-deoxy-D-glucose ($^{3}$H-DG) by MCF-7 cells was time-dependent and saturable ($K_m$=6.5±0.5 mM and $V_{máx}$=63.6±2.3 nmol/mg prot).

Acutely (26 min), myricetin (10-100 µM), chrysin (100 µM), genistein (10-100 µM), resveratrol (10-100 µM), kaempferol (10-100 µM) and xanthohumol (10-100 µM) inhibited $^{3}$H-DG uptake. By contrast, (+) catechin and (-) epicatechin slightly (by 10-15%) increased it. Kaempferol was found to be the most potent inhibitor of $^{3}$H-DG uptake by MCF-7 cells, with an $IC_{50}$ of 4.0 (1.6-9.8) µM (the $IC_{50}$ of genistein and resveratrol was 38.9 (15.2-99.4) µM and 67.2 (29.4-153.7) µM, respectively). Kaempferol (100 µM) behaved as a mixed-type inhibitor, since it simultaneously increased the $K_m$ (to 15.6±2.4 mM) and the $V_{máx}$ (to 106.9±10.6 nmol/mg prot).

Chronically (24h), kaempferol was also able to inhibit $^{3}$H-DG uptake ($IC_{50}$ of 13.6 (2.8-66.9) µM). This flavonoid was also found to inhibit by around 40% GLUT1 transcription and, although not statistically significant, this effect was accompanied by a decrease in GLUT1 protein production. We also verified that exposure of cells to kaempferol induced an increase in extracellular lactate levels over time which suggests that this flavanol inhibits lactate reuptake by inhibiting MCT1.

Additionally, kaempferol revealed antiproliferative and cytotoxic properties, because it inhibited cell growth and decreased cell viability. These effects of kaempferol were mimicked with low extracellular glucose conditions and reversed with high glucose extracellular conditions.

In conclusion, kaempferol (and, albeit less potently, some other polyphenols) potently inhibits glucose uptake by MCF7 cells, apparently by blocking members of the GLUT family (most probably GLUT1). This effect may contribute to the known chemopreventive effect of this dietary compound.



**Keywords** · Breast Cancer Cells · Glucose uptake · Polyphenols · Kaempferol

Azevedo C, Correia-Branco A, Araújo JR, Keating E, Martel F (2013) The polyphenol kaempferol potently inhibits glucose uptake in a human breast cancer cell line. XXII Porto Cancer Meeting & III Porto-Bordeaux Joint Meeting, Porto.

## LIST OF TABLES



## LIST OF FIGURES

| | |
|---|---|
| **$^{3}$H-DG** | $^{3}$H-deoxy-D-glucose |
| **ATP** | Adenosine-5'-triphosphate |
| **BrC** | Breast Cancer |
| **C** | Control |
| **cDNA** | Complementary DNA |
| **CYT B** | Cytochalasin B |
| **DCIS** | Ductal carcinoma *in situ* |
| **DEPC** | Diethylpyrocarbonate |
| **2-DG** | 2-Deoxy-D-glucose |
| **DMSO** | Dimethyl sulfoxide |
| **DNA** | Deoxyribonucleic acid |
| **DNAse** | Deoxyribonuclease |
| **DTT** | Dithiothreitol |
| **ECL** | Enhanced Chemiluminescence |
| **ETOH** | Ethanol |
| **GAPDH** | Glyceraldehyde-3-phosphate dehydrogenase |
| **GLUT** | Glucose transporter |
| **HMIT** | $H^{+}$ coupled myo-inositol transporter |
| **$IC_{50}$** | Concentration that originates 50% inhibition of $^{3}$H-DG transport |
| **IDC** | Invasive ductal carcinoma |
| **ILC** | Invasive lobular carcinoma. |
| **KF** | Kaempferol |
| **LCIS** | Lobular carcinoma *in situ* |
| **LDH** | Lactate dehydrogenase |
| **MCF-7** | Michigan Cancer Foundation – 7 |
| **MCT** | Monocarcarboxylate transporter |

| | |
|---|---|
| **MEM** | Minimum essential medium |
| **mRNA** | Messenger ribonucleic acid |
| **OXPHOS** | Oxidative phosphorylation |
| **PHZ** | Phloridzin |
| **qRT-PCR** | Quantitative reverse transcription-polymerase chain reaction |
| **RNA** | Ribonucleic acid |
| **ROS** | Reactive oxygen species |
| **SGLT** | $Na^+$-dependent glucose transporter |
| **SRB** | Sulforhodamine B |
| **TBST** | Tris buffered saline with Tween-20 |
| **TCA** | Trichloroacetic acid |

# CHAPTER I

# INTRODUCTION

# 1. BREAST CANCER

## 1.1 Characteristics and Epidemiology

Breast Cancer (BrC) is the most common malignancy in women and its incidence has been increasing over the years, especially in older women (*1-3*). It is the fifth most common cause of cancer death, lung cancer being on top of the list (*4*). In Portugal, 4 500 new cases are estimated to appear every year with a corresponding mortality of 1 500 (*2*). Fortunately, the death rate for breast cancer has been decreasing over the past decade, since there are faster and more efficient diagnostic methods and improved therapies. Also, divulgation about the symptoms and self-exams is as important as knowledge about risk factors, because it is estimated that about 90% of breast cancer could be treated and consequently curable if they are detected in an early stage (*2*).

Ninety five percent of breast cancers are carcinomas, since they arise from breast epithelial elements (*3, 5*). Normally, 85% of breast carcinomas originate in cells of ducts (ductal carcinoma) and 15% begin in the cells that line the lobules (lobular carcinoma). Non-carcinomatous breast cancers are rare and originate in the connective tissue of the breast (*1, 3*).

Cancer cells present some characteristics that distinguish them from normal cells, including loss of differentiation, uncontrolled growth, immortalization, loss of contact inhibition, increased invasive capacity, evasion from the host immune surveillance processes and the apoptotic signal restraints and induction of neo-angiogenesis (*6, 7*).

Cancer is a multistep process that includes initiation, proliferation and progression. Breast cancer carcinogenesis begins with an increase in the number of breast cells (hyperplasia), followed by the emergence of atypical breast cells (atypical hyperplasia), carcinoma *in situ* (noninvasive cancer) and finally, invasive cancer (Fig. 1) (*3*).

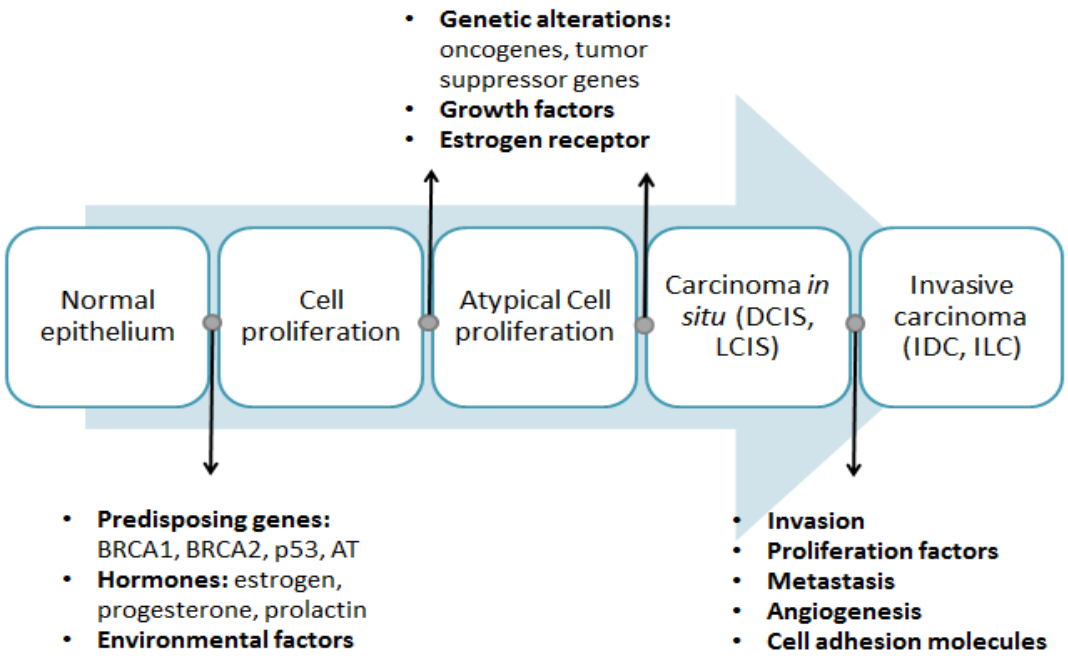



**Figure 1: Model of the multistep carcinogenesis in BrC.** DCIS: ductal carcinoma *in situ*; LCIS: lobular carcinoma *in situ*; IDC: invasive ductal carcinoma; ILC: invasive lobular carcinoma. Adapted from Bechmann *et al*. (1997).

Not all BrC necessarily follow this progressive pattern and the same speed of progression, so some cancers may never progress beyond *in situ* disease (*3*), staying at stage 0 as shown in Table 1. Thus, BrC may be invasive, when they spread from the milk duct or lobule to other tissues in breast; or noninvasive (*in situ*), if remain confined to the ductal or lobular epithelium.

**Table 1:** Staging of Breast Cancer. Adapted from Kumar *et al*. (2010)

| Stage | T: Primary Cancer | N: Lymph Nodes | M: Distant Metastasis | 5-Year Survival (%) |
|---|---|---|---|---|
| **0** | DCIS or LCIS | No metastases | Absent | 92 |
| **I** | Invasive carcinoma ≤2 cm | No metastases | Absent | 87 |
| **II** | Invasive carcinoma >2 cm | No metastases | Absent | 75 |
| | Invasive carcinoma <5 cm | 1 to 3 positive | Absent | |
| **III** | Invasive carcinoma >5 cm | 1 to 3 positive | Absent | 46 |
| | Any size invasive carcinoma | ≥4 positive | Absent | |
| | Invasive carcinoma with skin or chest wall involvement or inflammatory carcinoma | 0 to >10 positive | Absent | |
| **IV** | Any size invasive carcinoma | Negative or positive lymph nodes | Present | 13 |

### 1.2 Risk Factors and Etiology

Breast carcinomas can be divided into sporadic and hereditary cases. The first ones correspond to the majority of all BrC cases and are associated with somatic genetic alterations, probably related to hormonal exposure, diet and lifestyle. Hereditary cases, on the other hand, are associated with germline mutations. The probability of a hereditary etiology increases with multiple affected first-degree relatives, when individuals are affected before menopause and/or have multiple cancers, or there are family members with other specific cancers. The inheritance of a susceptibility gene is the primary cause of approximately 12% of BrC (*3, 8*).

Women with an inherited BRCA1 or BRCA2 mutation have a greatly increased risk of developing BrC. Mutations in BRCA1 or BRCA2 are responsible for 52% and 32% of hereditary BrC cases, respectively. However, BRCA1 mutations also increase the susceptibility to ovarian cancer and the mutation in BRCA2 is frequently associated with male breast cancer. These two are the most common genes related to hereditary BrC, but other genes are also associated, such as ATM, CHEK2, RAD51, AR, DIRAS3, ERBB2, and TP53 (*9*).

Beyond this, BrC tumor type and degree of aggressiveness can be affected by age and gender, family and personal history, radiation, microenvironment and diet factors (*3*).

It is known that many aspects of women's diets and lifestyle have an effect on increasing or decreasing BrC risk. It is proved that obese women (especially after menopause) have an increased risk of BrC. Because our organism produces some estrogen in fat tissue, it is therefore more likely that obese women exhibit high levels of estrogen and, consequently, increased risk for breast cancer (*2*). Studies suggest that heavy cigarette smoking and high exposure to tobacco smoke are associated with an increase in BrC risk. On the other hand, physical activity can help to reduce it (*10*).

Diet has also an important role in promotion or inhibition of tumor progression. Factors like low dietary folate levels, high consumption of soy products, red meat or well-done meat and dairy products are under study for an association with an increase in BrC risk (*2, 3*). It is estimated that a 40% increase in BrC risk is associated with having 2 to 5 alcoholic drinks per day, although this effect can be counterbalanced with a high folic acid consumption (*11*). Some more dietary factors are known to decrease the risk, such as high consumption of fruits and vegetables, caffeine and large amounts of fiber (*10*).

In summary, ~20% of all cancers are caused by diet and around 30–40% can be prevented by it (*12-14*). So, it is important to analyze the effects of these dietary factors at a molecular level.

## 2. GLUCOSE UPTAKE IN BREAST CANCER CELLS

### 2.1 Glucose transporters

Glucose has several roles in our organism, since it is a precursor of glycoproteins, triglycerides and glycogen. Furthermore, and very importantly, glucose oxidation is a major source of metabolic energy in eukaryotic cells (*15, 16*).

Glucose can be synthetized within our body but it can also be obtained from the diet. Glucose obtained from diet is transferred from the lumen of the small intestine into the blood circulation, where it is transported together with glucose synthesized within the body. Since glucose (as the other sugars) is hydrophilic, it cannot enter the cell by simple diffusion and needs some specific transporters to cross the hydrophobic plasma membranes. In humans and other mammals, these transporters correspond to the Facilitative Glucose Transporters (GLUTs) and the Sodium-dependent Glucose Transporters (SGLTs) (*15*).

### 2.2 Facilitative Glucose Transporters (GLUTs)

The facilitative glucose transporters (GLUT) move sugars across cell membrane in favor of concentration gradient and without energy consumption (*17*).

The GLUT family of transporters is encoded by the SLC2A gene family that possesses 14 members. GLUTs are all intrinsic membrane proteins and they differ in their tissue distribution, having different affinities for sugars and responding to metabolic and hormonal regulation (*16, 18*).

Some models have been proposed indicating the orientation of GLUTs in the cell membrane. Macheda *et al.* (2005) suggest that the protein traverses the cell membrane 12 times, with the $NH_2^-$ and $COOH^-$ termini of the protein located on the cytoplasmic side of the membrane and a large intracellular loop between transmembrane domains (TM) 6 and 7 (Fig. 2) (*19*).

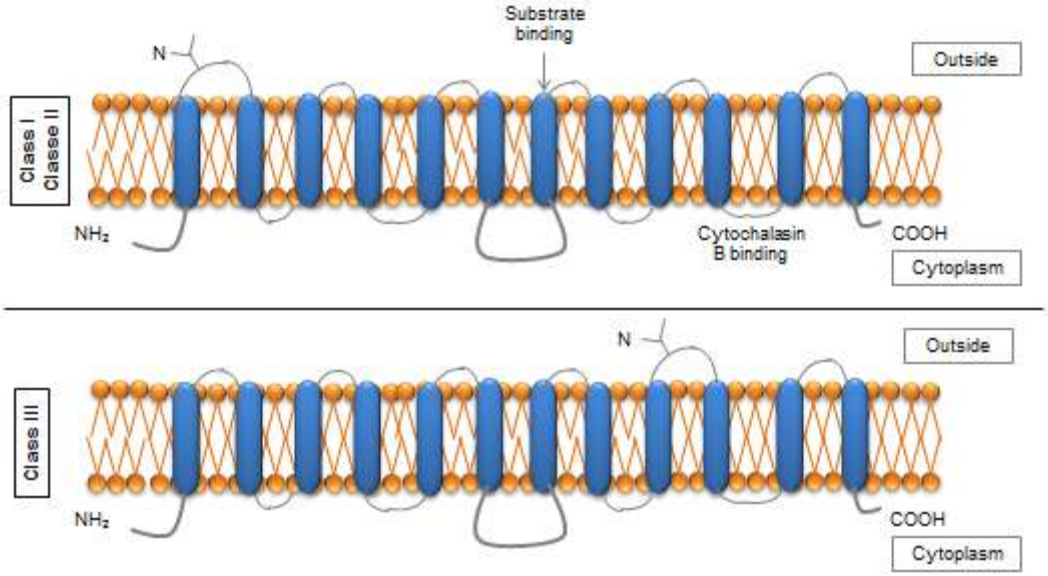



**Figure 2**: Model of the orientation of facilitative glucose transporter proteins in the cell membrane. Adapted from Macheda, M., *et al.*, (2005).

GLUT family members are divided into three classes according to the loop position and to their structural characteristics. We will next refer the GLUT members known to be present in MCF-7 cells (*16, 20*).

The class I and II have an extracellular loop between TMs 1 and 2 that contains an N-linked glycosylation site. Despite this similarity, these classes present several differences in terms of structure, function and tissue distribution (*15*).

**The class I** is constituted by GLUT1, GLUT2, GLUT3 and GLUT4.

The first member of this class, GLUT1, is normally found in brain, breast, erythrocytes, adipose tissue and it is believed that GLUT1 is responsible for basal glucose uptake in most tissues (*17*). This transporter is also present and even overexpressed in most cancers. It is highly expressed in cancers with higher grade and proliferative index and lower degree of differentiation. Thereby, it is associated with increased malignant potential, invasiveness and consequently, poor prognosis (*18*). Macheda *et al.* (2005) demonstrated that glycosylation of GLUT1 in between transmembrane domains 1 and 2, increases the efficiency of glucose transport. These same authors also show that hypoxia, estradiol and epidermal growth factor, which have an important role in BrC growth, can increase GLUT1 levels and glucose uptake in these cells (*19*).

GLUT2 is expressed in pancreatic β cells, liver and kidneys. It is thought that GLUT2 in β cells is important for the glucose-sensing mechanism of these cells and in bidirectional transport of glucose under hormonal control, in the liver. This glucose transporter is also characterized by its ability to transport fructose and is found on the surface of proximal renal tubules and enterocytes (*15, 20*).

GLUT3 is also present in most cancers but not so frequently as GLUT1. The affinity of GLUT3 to glucose is the highest of all GLUTs, and it is present in tissues with high necessity of fuel, such as brain (*15, 18, 20*).
GLUT4 is mostly found in heart, skeletal muscle and adipose tissue. In the presence of insulin, GLUT4 is translocated from vesicles of intracellular compartments to the cell membrane, resulting in 10-20 fold increase in glucose transport (*19, 21*).

GLUT5, GLUT7, GLUT9 and GLUT11 belong to **class II**.
GLUT5 only accepts fructose as a substrate, and it is responsible for fructose absorption in the small intestine. The presence of GLUT5 was observed in human BrC tissue, but not in normal breast tissue, indicating that fructose may play an important role in tumor growth (*19*). It is also present in small intestine, testes and kidneys.

**The class III** members lack the extracellular loop between TMs1 and 2 but contain a large extracellular loop and potential sites of N-linked glycosylation between TMs9 and 10. Apart from this, all members of this class (GLUT6, GLUT8, GLUT10, GLUT12 and HMIT) contain targeting amino acid sequences.
Similarly to GLUT4, GLUT12 is expressed in insulin-sensitive tissues, being translocated to the cell membrane in the presence of insulin. Studies suggest that GLUT12 was found in a human breast cancer cell line but is also expressed in the rat lactating mammary gland. This is considered a reactive gene since it is expressed in the embryo and down-regulated in adult tissues (*19*).

### 2.3 Sodium-dependent Transporters (SGLT)

This family of transporters transports glucose via a secondary active transport mechanism. The $Na^+$ electrochemical gradient provided by the $Na^+$ - $K^+$ ATPase pump is used to transport glucose into cells against its concentration gradient. There are 12 members of the human family, encoded by the SLC5A gene family (*16*).
SGLT1 moves glucose and also galactose (although with a much lower affinity) and is the only expressed in the small intestine. This member is also responsible for the recovery of any remaining glucose to avoid glucose loss in the urine (*15, 22*).

### 2.4 The Warburg effect

In normal cells, glucose is converted into pyruvate through glycolysis, and consequently into acetyl-CoA, which is used by the mitochondria to produce ATP. On the other hand, in poorly oxygenated cells or in cells that lack mitochondria, pyruvate is converted to lactate which is normally released from the cell. However, cancer cells present an altered metabolism, and therefore an increased lactate production even in the presence of oxygen. This is known as the Warburg effect. These cells present an increased rate of glucose uptake for ATP production, since the lack of oxygen supply contributes to an hypoxic microenvironment, which upregulates GLUT expression and lactate secretion (*16*).

The Warburg effect was observed for the first time by Otto Warburg in the first half of the $20^{th}$ century (*23*). This concept defines increased rates of glycolysis in the presence of adequate $O_2$ levels in cancer and non-transformed proliferating cells.

At the beginning this effect was thought to be caused by irreversible damage of oxidative phosphorylation (OXPHOS). However, this explanation was refutable after the observation of the Warburg effect in non-transformed proliferating cells, since these are not supposed to have irreversible damages on OXPHOS.

Cancer cells activate glycolysis in presence of $O_2$ to get a powerful growth advantage, faster ATP generation and to provide carbon skeletons for biosynthesis. Furthermore, some data suggest that the activation of glycolysis have an important role in the protection against cell death induced by ROS. Also, the persistent activation of glycolysis leads to environmental acidosis, which is toxic to normal cells and contributes to the tumor proliferation.

Most cancers have the following characteristics: overexpression of HIF-1, alkaline intracellular pH values, high generation of $O_2^{\bullet-}$ and $H_2O_2$ and alterations in OXPHOS. So, hypoxia and activation of HIF-1 play an important role in cancer cell metabolism, since they contribute the switch from OXPHOS to glycolysis (*24*).

Moreover, high levels of glucose transporters expression are associated with cancers of higher grade and proliferative index and in cancers of lower degree of differentiation. This is most probably related to the Warburg effect (*18, 25*).

With these evidences, some studies suggest that the upregulation of specific glucose transporters and the Warburg effect may be the key for the tumor surveillance and growth (*18*).

### 2.5 Glucose transport and BrC

Similarly to all cancer cells, BrC cells exhibit a high glycolytic activity compared with normal tissues. In BrC, glucose transport activity is strongly correlated with increased GLUT1 expression (*26*). GLUT1 is seen as a marker in early diagnosis as well as a new potencial therapeutic target. This transporter is a marker of hypoxia and glucose metabolism, and normally it is associated to a poor prognosis (*16*).

Nowadays, cancer therapy is based on two approaches: (1) conventional chemotherapy – an unspecific process that reaches all type of cells, eg. drugs that block nucleotide biosynthesis; and (2) targeted therapy – drugs designed to block specific components. Examples of the last one are: mTORC inhibitors (reduce glucose uptake), antisense oligonucleotides (downregulate the mRNA and protein expression of GLUT1), inhibitors of lactate dehydrogenase and monocarboxylate transporters, and anti-GLUT antibodies (induce growth arrest and apoptosis). The last one inhibits around 75% of breast cancer cell proliferation (*16, 17, 25*).

According to Aft *et al.* (2002), 2-deoxy-D-glucose (2-DG) strongly inhibits glucose metabolism and ATP production, causing growth inhibition and apoptosis. 2-DG is a structural analogue of glucose differing at the substitution of hydrogen for a hydroxyl group in the second carbon atom (Fig. 3). This modification causes an increased uptake, high intracellular levels of hexokinase or phosphorylating activity and low intracellular levels of phosphates, which lead to metabolic trapping (*27*). So, 2-DG is a promising agent to treat solid tumors, since it increases the efficiency of other therapies (*17*).

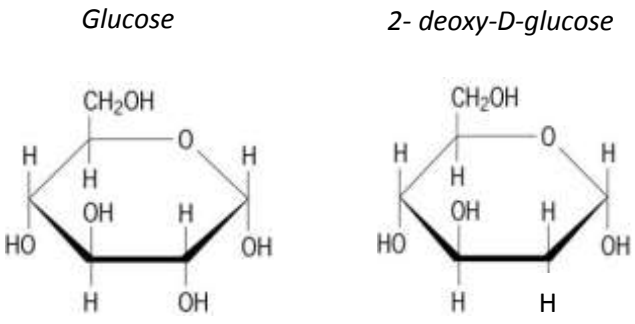



**Figure 3**: Structural comparison of glucose (left side) and 2-deoxy-D-glucose (right side).

## 3. POLYPHENOLS AS ANTICARCINOGENIC AGENTS

### 3.1 Polyphenol classes and anticarcinogenic effects

Several dietary compounds including vegetables, fruits and beverages are rich in polyphenols, which are secondary metabolites produced as a response to ultraviolet radiation, aggression by pathogens, disease, injury or stress (*28*). It is estimated that the total dietary intake is around 1g of polyphenols per day, wherein two-third are flavonoids and the remainder phenolic acids and other non-flavonoids (*29, 30*).

Polyphenols are constituted by at least one aromatic ring with one or more hydroxyl attached and can be classified into Flavonoids and Non Flavonoids according to their chemical structure and complexity (Fig. 4) (*31, 32*). Flavonoids are also classified according to their different characteristics: flavones are the most hydrophobic and isoflavones present structural similarities to estrogens.

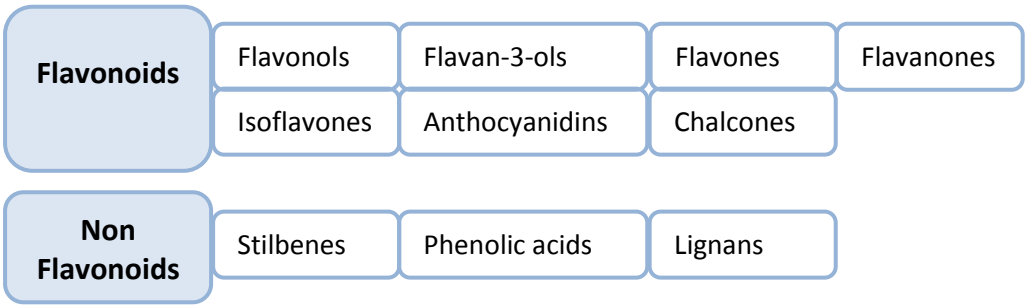



**Figure 4:** Schematic representation of the classes and subclasses of polyphenols.

Initially, the protective effect of dietary polyphenols was thought to be due to their antioxidant properties, which decreased free radicals levels within the body. Another antioxidant mechanism is the chelation of metals such as iron and copper ions, which prevent their participation in Fenton-type reactions and the generation of highly reactive hydroxyl radicals (*33*).

These are known to reduce the incidence of diseases, such as cardiovascular diseases, diabetes and cancer (*31*). Several epidemiological studies have found an association between certain foods rich in polyphenols, specially flavonoids, and a decrease in incidence of some types of cancer (renal cell carcinoma, breast, ovarian and colorectal) (*34*). Obviously, the health effects of polyphenols are influenced by the amount consumed and by their bioavailability (*28*).

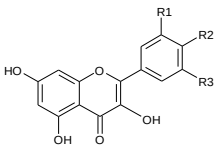
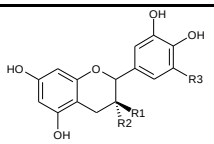
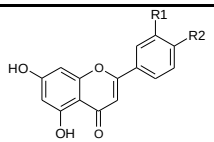
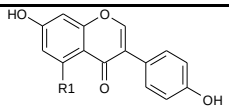
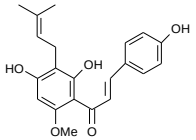
Certain dietary phenolic and their metabolites are able to modulate cell functions such as growth, proliferation, angiogenesis and apoptosis. The chemopreventive effect of some polyphenols appears to be related to the fact that they (1) inhibit carcinogen activation, catalyzed by cytochrome p450 enzymes (CYP450), (2) induce phase II enzymes, which

facilitate the elimination of certain carcinogens, (3) have antioxidant effect (*35, 36*). Beyond this, polyphenols can inhibit the metabolism of arachidonic acid, which is important to the production of many proinflammatory metabolites such as certain prostaglandins and leukotrienes (*37*). They are also the ability to interact with intracellular signaling cascades and with some pathways, such as NF-kB, activate protein-1 and mitogen-activated protein kinase (MAPK) (*31, 38*).

### 3.2 Flavonoids

Flavonoids are constituted by two aromatic rings connected by a heterocyclic ring: $C_6$-$C_3$-$C_6$ and usually they are conjugated to sugars (*39*). Depending on the classification method, the flavonoid group can be divided into several categories based on hydroxylation of the flavonoid nucleus as well as the linked sugar: flavonols, flavan-3-ols, flavones, isoflavones, flavanones, anthocyanidins and chalcones. In the present work, we studied the flavonoids present in Table 2 only. All these compounds were previously demonstrated to have an anticarcinogenic effect and they are present in high amounts in the human diet (their consumption varies between 26 mg and 1g per day) (*39*).

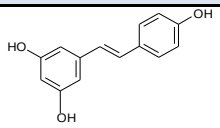
**Table 2:** Chemical structures of some selected flavonoids (*29*)

| Subclass of flavonoids | Molecular Structure | Functional groups and examples | Source |
|---|---|---|---|
| **Flavonols** |  | R1=H; R2=OH; R3=H: kaempferol<br>R1=OH; R2=OH; R3=OH: myricetin | Yellow onion, Curly kale, Leek, Cherry tomato, Broccoli, Blueberry, Black currant, Apricot, Apple, Beans, Black grape, Tomato, Black and Green tea infusion, Red wine |
| **Flavan-3-ols** |  <br>Monomeric | R1=H; R2=OH; R3=H: (+)catechin<br>R1=OH; R2=H; R3=H: (-)epicatechin | Chocolate, Beans, Apricot, Cherry, Grape, Peach, Blackberry, Apple, Black and Green tea, Red wine |
| **Flavones** |  | chrysin | Parsley, Celery, Pepper |
| **Isoflavones** |  | R2=OH: genistein | Soy flour, Soybeans, Miso, Tofu , Tempeh, Soy milk |
| **Chalcones** |  | xanthohumol | Beer |

### 3.3 Non Flavonoids

Non-flavonoids are structurally constituted by two aromatic rings connected by $C_6$-$C_2$-$C_6$. This group is divided into: phenolic acids, stilbenes and ligans. The main member of this class is resveratrol, a stilbene (Table 3), and its *trans* conformation has the ability to inhibit carcinogenesis (*31*).

**Table 3:** Chemical structure of the non-flavonoid resveratrol (*29*)

| Subclass of non-flavonoid | Molecular Structure | Functional groups and examples | Sources |
|---|---|---|---|
| Stilbenes |  | resveratrol | Red wine, peanuts |

## 4. AIM

Recently, our group verified that the polyphenols quercetin (a flavanol) and epigallocatechin gallate (a flavan-3-ol) inhibited glucose uptake by the breast cancer (MCF-7) cells (*21*).

So, in the present work, we decided to investigate if other polyphenolic compounds could also interfere with glucose uptake by MCF-7 cells and, if so, if this effect is involved in their anticarcinogenic activity.

We decided to test the effect of kaempferol (KF) and myricetin (two flavonols). Flavonols are the most abundant polyphenolics in food, KF being the main member of this class. According to Shiomi *et al.* (2013), myricetin can inhibit mammalian DNA polymerase, topoisomerase and human cancer cell proliferation (*40*). In addition, myricetin and KF can inhibit glucose efflux in human erythrocytes (*39*).

We also tested the effect of (+) catechin and (-) epicatechin (two flavan-3-ols). Studies show that (+) catechin inhibit HGF/Met signaling in immortalized and tumorigenic breast epithelial cells and suppress MCF-7 proliferation through TP53/caspase-mediated apoptosis; while (-) epicatechin have a potent and antiproliferative activity in human BrC cells (*41-43*).

A flavone member, chrysin, and an isoflavone, genistein, were also included in this study. Despite less common in fruits and vegetables, chrysin have also an important paper in cancer therapy. Several studies suggest that chrysin is able to inhibit aromatase in MCF-7, to induce apoptosis and to be an adjuvant in anti-cancer therapy (*44*). Li *et al.* (2013)

concluded that epigenetic processes are the most important mechanism involved in the inhibition of BrC by genistein (*45*). Isoflavones reduce the incidence of BrC, due to their competition with oestrogens, inhibiting tumor growth, which is dependent on oestrogens (*31*).

Xanthohumol, a member of the chalcones family was also studied. Monteiro *et al.* (2008) studied the effect of xanthohumol in BrC xenografts in nude mice and noticed that this compound has antiangiogenic, antiproliferative and anti-inflammatory effects (*46*).

Finally, a member of stilbenes, resveratrol, was also included. Su *et al.* (2013) shows the antioxidant and antiproliferative properties of resveratrol, and the fact that it reduces the viability, arresting the cell cycle in MCF-7 cells (*47*).

For this purpose, the acute and chronic exposure of the mentioned polyphenols on glucose uptake by BrC cells (MCF-7) was investigated. The cellular viability and proliferation as well as mRNA and protein expression were also evaluated.

# CHAPTER II

# MATERIALS AND METHODS

## 1. Materials

$^{3}$H-2-deoxy-D-glucose ($^{3}$H-DG; specific activity 50 Ci/mmol) (American Radiolabeled Chemicals Inc., St. Louis, MO, USA), $^{3}$H-thymidine (methyl-[$^{3}$H] thymidine; specific activity 79 Ci/mmol) (GE Healthcare GmbH, Freiburg, Germany). Dimethyl sulfoxide (DMSO), 2-deoxy-D-glucose, nicotinamide adenine dinucleotide (NADH), sulforhodamine B (SRB), cytochalasin B (from *Helminthosporum dematiodium)*, trichloroacetic acid (TCA), Tris, MEM M0643 (minimum essential medium), HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid), antibiotic/antimycotic solution (100 U/ml penicilin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericinB), phloridzin, cytochalasin B, catechin, epicatechin, xanthohumol, chrysin, resveratrol, myricetin, genistein, kaempferol, quercetin, triton X-100, trypsin-EDTA solution, sodium pyruvate (Sigma, St. Louis, MO, USA). Fetal bovine serum (Gibco, Life Technologies Corporation, CA, USA), Tripure® (Roche Diagnostics, Germany). D-glucose (Merck, Germany).

Drugs to be tested were dissolved in DMSO or ethanol. The final concentration of this solvent was 1% in HMBS buffer and 0.1% in culture medium. Controls for test compounds were run in the presence of the respective solvent.

## 2. Methods

### 2.1. MCF-7 cell culture

The MCF-7 cell line was obtained from the American Type Culture Collection (ATCC 37-HTB, Rockville, Md., USA) and was used between passage numbers 55 and 94. The cells were maintained in a humidified atmosphere of 5% $CO_2$-95% air and were grown in minimum essential medium (MEM) containing 5.56 mM glucose and supplemented with 10% fetal calf serum and 1% antibiotic/antimycotic solution. Culture medium was changed every 2 to 3 days and the culture was split every 7 days. For subculturing, the cells were removed enzymatically (0.25% trypsin-EDTA, 3 min, 37 ºC), split 1:6, and subcultured in plastic culture dishes (21 cm$^2$; ⌀, 60mm; TPP®, Trasadingen, Switzerland). For transport experiments, glucose metabolism assessment and for quantification of cell viability and growth, MCF-7 cells were seeded on 24-well plastic cell culture dishes (2cm$^2$; ⌀ 16mm; TPP®), and were used after 6-7 days in culture (90-100% confluence). For RNA and protein extraction, cells were seeded in plastic culture dishes (21 cm$^2$; ⌀, 60mm; TPP®) and were used 6-7 days after seeding. In all experiments, the medium was made free of fetal calf serum for 24 h before the experiments.

### 2.2. Transport studies

In the transport assays, $^{3}$H-DG was used as a substrate. DG is an unmetabolizable D-glucose analogue which is transported efficiently by GLUTs, but is poorly transported by SGLT1 (*48*).

The transport experiments were performed in HMBS buffer (composition, in mM: 140 mM NaCl, 5 mM KCl, 12.5 mM HEPES, 12.5 mM MES, 2 mM $MgCl_2$ and pH = 7.4). Initially the culture medium was aspirated and the cells (MCF-7) were washed with 0.3 ml HMBS buffer at 37ºC; then the cell monolayers were preincubated for 20 min in HMBS buffer at 37ºC. Uptake was then initiated by the addition of 0.2 ml buffer at 37ºC containing 20 nM $^{3}$H-DG (except in experiments for determination of kinetics of $^{3}$H-DG uptake). Incubation was stopped after 6 min (except in the time-course experiments, where incubation time was variable) by removing the incubation medium, placing the cells on ice and rinsing the cells with 0.5 ml ice-cold HMBS buffer. The cells were then solubilized with 0.3 ml 0.1 % (v/v) Triton X-100 (in 5 mM Tris-HCl, pH 7.4), and placed at room temperature overnight. Radioactivity in the cells was measured by liquid scintillation counting.

In experiments for determination of kinetics of $^{3}$H-DG uptake, cells were incubated for 6 min with increasing concentrations of $^{3}$H-DG: 0.02 µM, 1 µM, 10 µM, 100 µM, 300 µM, 1000 µM, 2500 µM, 5000 µM, 10 000 µM.

In experiments for determination of the chronic effect of compounds upon $^{3}$H-DG uptake, there was no preincubation period.

### 2.3 Treatment of MCF-7 cells

The concentration of compounds to be tested was chosen based on previous work of our group (*21, 49-51*).

In order to test the acute effect of compounds, cells were exposed to the compounds during both the 20-min preincubation and the 6-min incubation period with $^{3}$H-DG (20 nM) in HMBS buffer.

In order to test the chronic effect of compounds, cells were exposed to the compounds for 24h in serum-free culture medium followed by a 6-min incubation period in the presence of $^{3}$H-DG (20 nM) in HMBS buffer.

In some experiments, chronic treatment of the cells with kaempferol (100 µM; 24h) was performed in HMBS buffer with different concentrations of D-glucose (1 mM, 5.56 mM and 20 mM).

### 2.4 Protein determination

The protein content of cell monolayers was determined by Bradford method (*52*), using serum albumin as standard.

### 2.5 Evaluation of cellular integrity

*2.5.1 Determination of cellular viability (quantification of extracellular LDH activity)*

The effect of the polyphenols upon MCF-7 cellular viability was determined by using the lactate dehydrogenase (LDH) method. The intracellular enzyme LDH is released into the extracellular medium after cellular death. As described by Bergmeyer and Bernt (*53*), the activity of this enzyme is determined spectrophotometrically by measuring the decrease in absorbance of NADH during the reduction of pyruvate to lactate, according to the equation (1).

$$\text{Piruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{Lactate} + NAD^+ \qquad (1)$$

For this assay, cells were seeded on 24-well plates and submitted to acute or chronic treatment with polyphenols.

After treatment, cellular leakage of the cytosolic enzyme lactate dehydrogenase (LDH) was determined as briefly described. 50 µl of the extracellular medium were added to 1.5 ml of phosphate / pyruvate solution (48 mM/ 0.6 mM respectively). Then, 25 µl of NADH solution (0.18 mM) was added, and the absorbance values were measured for 2 min at 340 nm. The reduction rate of absorbance was thus obtained and the enzyme activity calculated. Enzyme activity was expressed as the percentage of extracellular LDH activity in relation to the total cellular LDH activity. To determine total cellular LDH activity, control cells were solubilized with 0.5 mL of 0.1% (v/v) Triton X-100 (in 5 mM tris.HCl, pH 7.4) for 30 min at 37 ºC. This LDH activity is considered the total activity, since it represents 100% of cellular death.

*2.5.2 Determination of cell growth (sulforhodamine B assay)*

At the end of acute or chronic treatment with polyphenols, 62.5 µl of icecold 50 % (w/v) trichloroacetic acid (TCA) were added to HMBS buffer or serum free culture medium (500

µl) on each well to fix cells (1 h at 4°C in the dark). The plates were then washed five times with tap water to remove TCA. Plates were air-dried and then stained for 15 min with 0.4 % (w/v) SRB dissolved in 1 % (v/v) acetic acid. SRB was removed and cultures were rinsed four times with 1 % (v/v) acetic acid to remove residual dye. Plates were again air-dried and the bound dye was then solubilized with 375 µl of 10 mM Tris–NaOH solution (pH 10.5). The absorbance of each well was determined at 540 nm; samples were diluted in order to obtain absorbance values lower than 0.7.

### 2.6 Determination of cell proliferation rate

Cell proliferation rate was determined by the $^{3}$H-thymidine incorporation assay. Briefly, MCF-7 cells treated for 24 h with kaempferol (30 or 100 µM) or DMSO were incubated with $^{3}$H-thymidine 0.025 µCi/ml for 5 h, in serum freeculture medium. The excess of $^{3}$H-thymidine was removed with 300 µl of 10% trichloroacetic acid wash for 1 h at 4 ºC. After a 30 min drying period, 280 µl of NaOH 1 M were added, and the incorporated $^{3}$H-thymidine was measured by liquid scintillometry.

### 2.7 Assessment of glucose metabolism

Glucose metabolism was assessed by quantification of lactate in the extracellular medium of MCF-7 cells exposed for 4 h or 24 h to kaempferol 30 µM, 2-desoxy-D-glucose (DG) 2 mM or quercetin 100 µM (or the respective solvents) in serum free culture medium or in HMBS buffer with 5 mM D-glucose. Extracellular medium was collected and centrifuged at 8000 g for 10 min. Lactate concentration was then measured by the lactate oxidase/peroxidase colorimetric assay, as indicated by the manufacturer (Olympus Life and Material Science Europa GmbH, Hamburg, Germany), in the Department of Clinical Pathology of Hospital S. João.

### 2.8 RNA extraction and qRT-PCR

Total RNA was extracted from MCF-7 cells treated for 24 h with kaempferol 30 µM, using Tripure® isolation reagent, according to manufacturer's instructions (Roche Diagnostics, Germany). After removing the culture medium, Tripure® was added and the lysate was transferred to an eppendorf tube. Three layers were obtained after chloroform addition. The first layer, corresponding to RNA, was then isolated and treated with isopropanol for RNA precipitation and was washed with ethanol (75% ethanol in diethylpyrocarbonate

(DEPC)). Afterwards, 5 µg of RNA were used as template for complementary DNA production through the incubation with Mix dNTP's at 65ºC for 5 min and then 1 min at 4 ºC. Afterwards, 5 X First-Strand Buffer (250 mM Tris-HCl, pH 8.3 at room temperature; 375 mM KCl; 15 mM MgCl2), 0.1 M DTT and RNaseOUT™(40 U/µl) were added and incubated at 25 °C during 2 min. Reverse Transcriptase SuperScript II (200 U/µl) was added incubated at 25 °C during 10 min, followed by 50 min at 42°C and 70°C for 15 min. Samples were then incubated with 1 µl RNAse H for 20 min at 37 °C, to degrade unreacted RNA

The primer pair used for human GLUT1 amplification was 5'- GAT GAT GCG GGA GAA GAA GGT-3' (forward) and 5'- ACA GCG TTGA TGC CAG ACA G-3' (reverse). The amount of GLUT1 mRNA was normalized to the amount of mRNA of the housekeeping gene, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for which the primer pair was as follows: 5'- ATG GAG AAG GCT GGG GCT CAT-3' (forward) and 5'- GAC GAA GAT GGG GGC ATC AG-3' (reverse).

mRNA expression levels were analyzed using quantitative reverse-transcritpion PCR (qRT-PCR) and was carried out using a LightCycler (Roche, Nutley, NJ, USA). 20 µL reactions were set up in microcapillary tubes using 0.5 µM of each primer and 4 µL of SYBR Green master mix (LightCycler FastStart DNA Master$^{Plus}$ SYBR Green I Roche). Cycling conditions were as follows: denaturation (95°C for 5 min), amplification and quantification [95°C for 10 s, annealing temperature, and 65°C for 10 s, with a single fluorescence measurement at the end of the 72°C for 10 s segment] repeated 50 times, followed by a melting curve program [(annealing temperature +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 40°C for 30 s. Data were analyzed using LightCycler® 4.05 analysis software (Roche, Mannheim, Germany).

### 2.9 Protein extraction and Western- blotting

Proteins were extracted from MCF-7 cells treated for 24 h with kaempferol 30 µM, using RIPA isolation reagent, according to manufacturer's instructions (Roche Diagnostics, Germany). Protein concentration was quantified using the Pierce BCA Protein Assay Kit.

For Western blot, the samples (20 µg of protein) were resolved in 12% SDS-PAGE and electroblotted onto transfer membranes (Hybond$^{TM}$-C extra, Amersham Biosciences, England). Membranes were blocked during 1 h, in TBST (Tris buffered saline with Tween-20) containing 5% (w/v) nonfat dry milk, washed in TBST and incubated overnight with the

primary antibody, rabbit polyclonal anti-GLUT1 (Santa Cruz Biotechnology, Inc.) in a 1:100 dilution or with β-actin (ABCAM, Cambridge, UK) in a 1:3000 dilution. Then, after washing, membranes were incubated for 1 h with secondary anti-rabbit antibody (Santa Cruz Biotechnology, Heidelber, Germany) in a 1:2000 dilution. The membranes were again washed during 30 min with TBST and then incubated with ECL (Enhanced Chemiluminescence) reagent under a chemiluminescence detection system, the ChemiDoc XRS (Bio-Rad Laboratories). Band area intensity was quantified using the Quantity One software from Bio-Rad. β-Actin was used as loading control.

### 2.10 Calculation and statistics

For the analysis of the time-course of $^{3}$H-DG uptake, the parameters of equation (2) were fitted to the experimental data by a non-linear regression analysis, using a computer-assisted method (*54*).

$$A(t) = k_{in}/k_{out}(1-e^{-k}out^{\cdot t}) \qquad (2)$$

In equation (2), A(t) represents the accumulation of $^{3}$H-DG at time t, $k_{in}$ and $k_{out}$ the rate constants for inward and outward transport, respectively, and t the incubation time. $A_{max}$ is defined as the accumulation at steady state ($t \rightarrow \infty$).

For the analysis of the saturation curve, the parameters of the Michaelis-Menten equation were fitted to the experimental data by using a non-linear regression analysis, using a computer-assisted method (*54*).

For calculation of $IC_{50}$ values, the parameters of the Hill equation were fitted to the experimental data by using a non-linear regression analysis, using a computer-assisted method (*54*).

Arithmetic means are given with SEM and geometric means with 95% confidence intervals. n represents the number of replicates of at least two different experiments. Statistical significance of the difference between two groups was evaluated by the Student's t-test and statistical significance of the difference between three or more groups was evaluated by one-way ANOVA test, followed by Bonferroni test. Differences were considered to be significant whenever $P < 0.05$.

# CHAPTER III

# RESULTS

## 1. Characterization of $^{3}$H-DG uptake by MCF-7 cells

Initially, we characterized uptake of $^{3}$H-DG by MCF-7 cells and for that, the variation of $^{3}$H-DG uptake as a function of time (time-course) and substrate concentration (kinetics) was investigated.

### *1.1 Time-course of $^{3}$H-DG uptake*

For time-course studies, cells were preincubated for 20 min and then incubated with $^{3}$H-DG at 20 nM for various periods of time, (1, 5, 10, 15, 30 and 90 min), at 37 °C, in the absence or presence of cytochalasin B 50 µM (Cyt B; a GLUT inhibitor (*55*)) + phloridzin 500 µM (Phz; an SGLT inhibitor (*56*)).

As shown in figure 5, MCF-7 takes up $^{3}$H-DG in a time-dependent manner. Moreover, $^{3}$H-DG uptake is markedly reduced in the presence of Cyt B + Phz.

This analysis also showed that the uptake of $^{3}$H-DG in control conditions is linear for up to 6 min of incubation. Therefore, in all subsequent experiments, a 6-min incubation period with $^{3}$H-DG was used, thus ensuring that the remaining studies will focus on the uptake of $^{3}$H-DG.

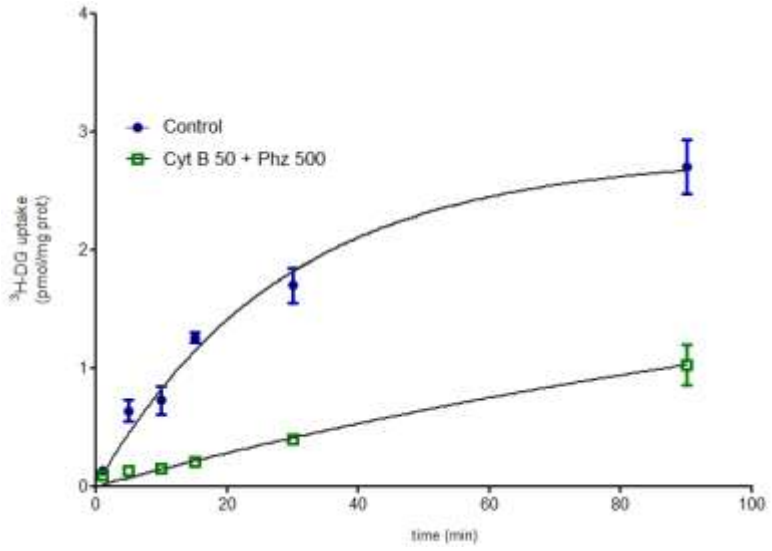



**Figure 5: Time-course of $^{3}$H-DG uptake by MCF-7 cells**. Cells were incubated at 37 °C and pH 7.4 with 20 nM $^{3}$H-DG for various periods of time, under control conditions or in the presence of phloridzin 500 µM + cytochalasin B 50 µM (Cyt B 50 + Phz 500). Shown are arithmetic means±SEM (n=4).

### *1.2 Kinetics*

To evaluate the kinetics of uptake of $^{3}$H-DG by MCF-7 cells, the cells were incubated with increasing concentrations of substrate (0.02-10 000 µM). Figure 6 shows that the uptake

of $^{3}$H-DG in these cells was saturable, and the $K_m$ and the $V_{máx}$ were found to be 6.5 ± 0.5 mM and 63.6 ± 2.3 µmol/mg prot, respectively.

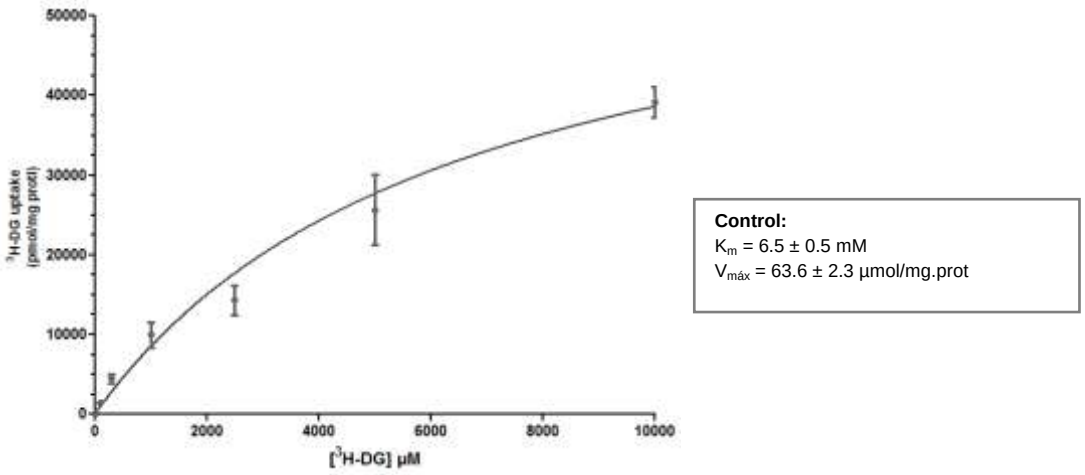



**Figure 6: Kinetics of $^{3}$H-DG uptake by MCF-7 cells.** MCF-7 cells were incubated at 37 ºC for 6 min in the presence of increasing concentrations of $^{3}$H-DG (0.2-10 000 µM). Shown are arithmetic means ± SEM (n = 3-14)

After this characterization, it was possible to proceed to the study of $^{3}$H-DG uptake modulation by polyphenols.

## 2. The acute effect of polyphenols upon $^{3}$H-DG uptake

### *2.1 Evaluation of $^{3}$H-DG transport inhibition*

In this series of experiments, we investigated the acute effect (26 min) of resveratrol, myricetin, genistein, kaempferol, (+) catechin, (-) epicatechin, chrysin and xanthohumol (10-100 µM) upon uptake of $^{3}$H-DG by MCF-7 cells.

As shown in Figure 7, four of the tested polyphenols (resveratrol, myricetin, genistein and kaempferol) inhibited $^{3}$H-DG uptake. By contrast, (+) catechin slightly (by 10-15%) increased it, and the others were devoid of significant effect.

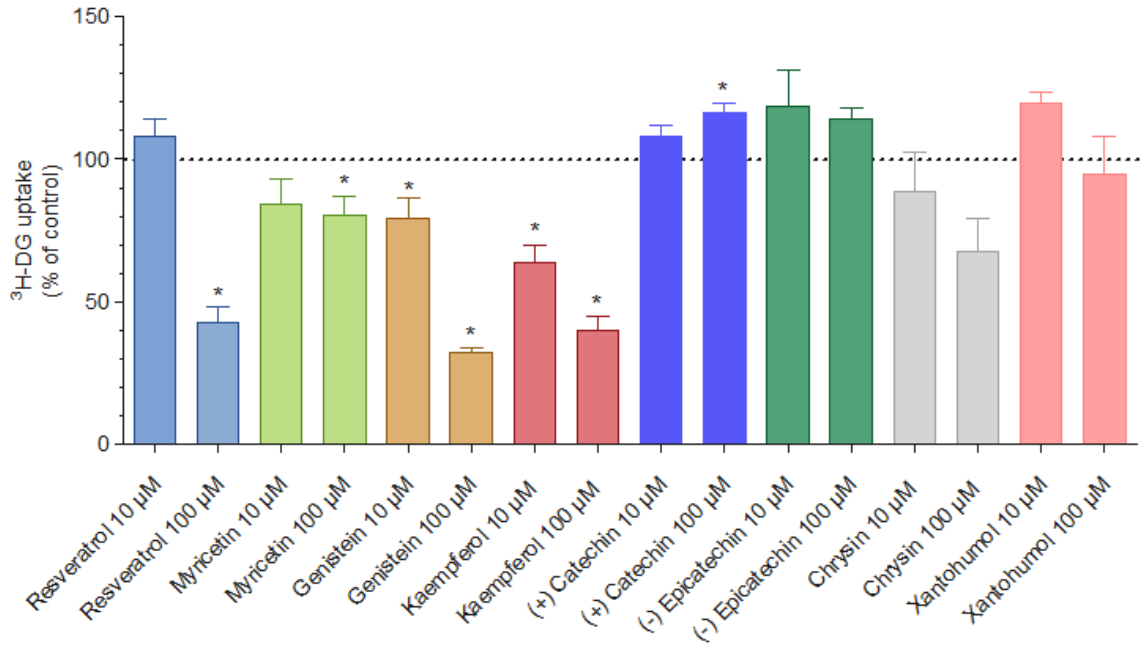



**Figure 7: The acute effect of several polyphenols on $^3$H-DG uptake.** Cells were preincubated for 20 min and then incubated for 6 min with 20 nM $^3$H-DG under control conditions (solvent) or in the presence of the polyphenols (10 and 100 µM). Polyphenols tested: flavanols (myricetin and kaempferol), flavan-3-ols ((+) catechin, (-) epicatechin), an isoflavone (genistein), a flavone (chrysin), a chalchone (xanthohumol) and a stilbene (resveratrol). Shown are arithmetic means±SEM. *Significantly different from control ($P < 0.05$).

The inhibitory effect of the polyphenols upon $^3$H-DG uptake was not related to a decrease in cell viability, as none of the polyphenols tested affected LDH leakage, indicating that these concentrations did not affect cell membrane integrity and thus cell viability (Fig. 8).

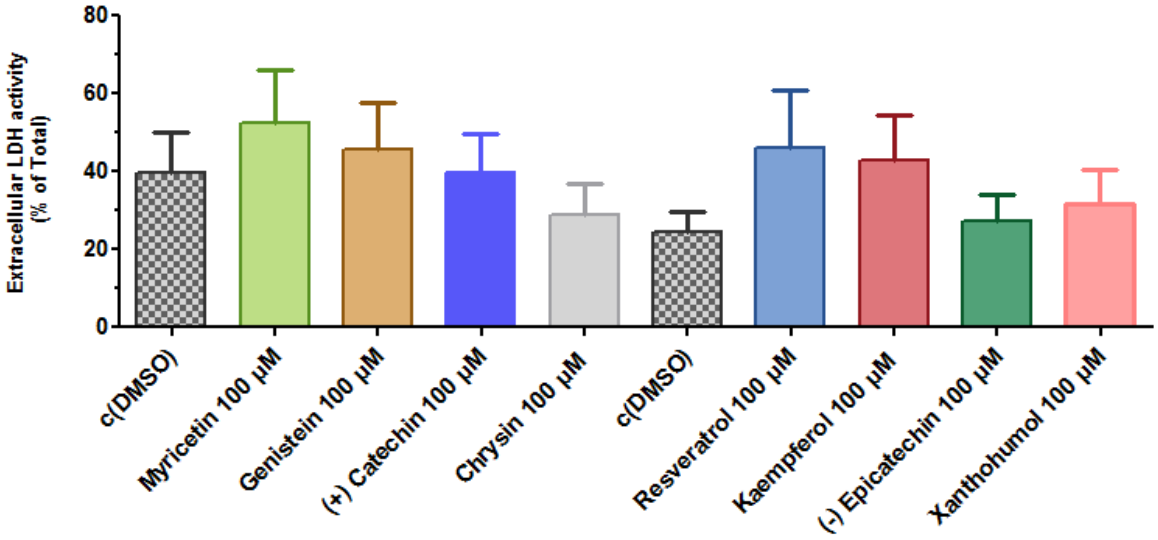



**Figure 8: The acute effect of studied polyphenols on cell viability.** After a 26 min exposure to polyphenols or to solvent (c(DMSO)), MCF-7 cellular viability was determined by quantification of extracellular LDH activity (n=12-17). Shown are arithmetic means ±SEM. *Significantly different from control ($P < 0.05$).

Resveratrol, genistein and kaempferol were found to cause the most marked inhibition of $^{3}$H-DG uptake (Fig. 8). Therefore, in the next series of experiments, we tested the effect of different concentrations of these compounds (1, 10, 30, 100, 185 or 300 µM) in order to calculate their $IC_{50}$.

Kaempferol was found to be the most potent inhibitor of $^{3}$H-DG uptake by MCF-7 cells, with an $IC_{50}$ of 4.0 (1.6-9.8) µM. The $IC_{50}$ of genistein and resveratrol was 38.9 (15.2-99.4) µM and 67.2 (29.4-153.7) µM, respectively (Fig. 9).

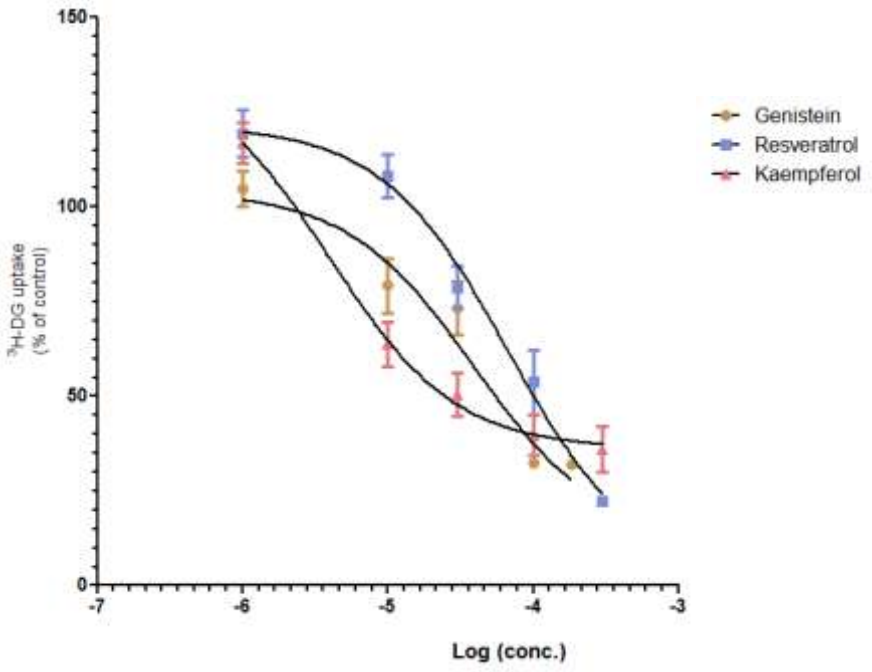



**Figure 9: Acute effect of different concentrations of kaempferol, resveratrol and genistein**. After a 26 min exposure to genistein, resveratrol and kaempferol, $IC_{50}$ was determined $IC_{50}$ results with 7<n<10. Shown are geometric means and the corresponding 95% confidence intervals

Again, the inhibitory effect of these compounds upon $^{3}$H-DG uptake is not related to a decrease in cell viability or growth (Fig. 10).

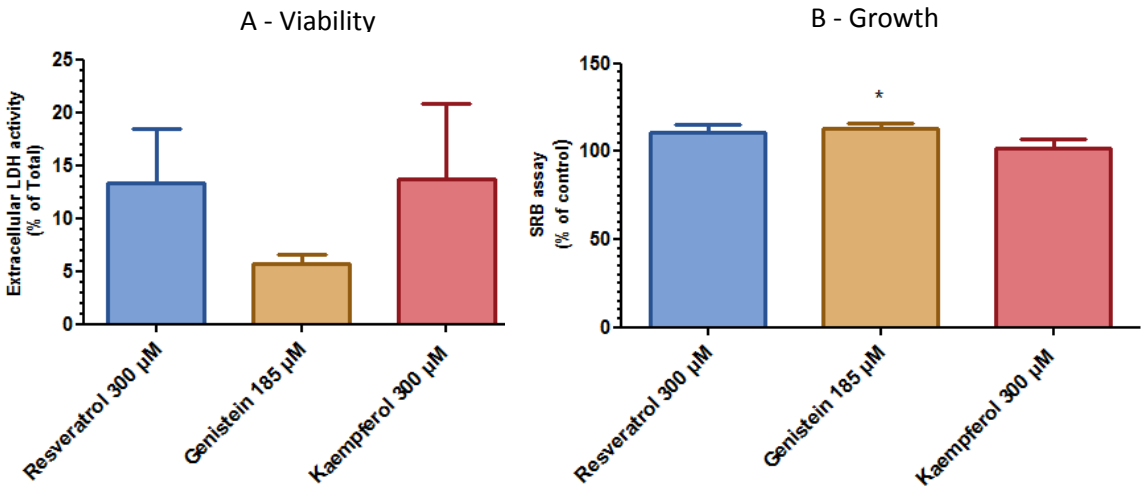



**Figure 10: Effect of resveratrol, genistein and kaempferol on cell viability (A) and cell growth (B).** After a 26 min exposure to polyphenols or to control (DMSO), MCF7 cellular viability was determined by quantification of extracellular LDH activity (n=6-8) and cellular growth was determined by the SRB assay (n=12-16). Shown are arithmetic means ±SEM. *Significantly different from control (P < 0.05).

### *2.2 Effect upon the kinetics of $^{3}$H-DG uptake*

We also evaluated the effect of the most potent of the tested polyphenols, kaempferol, upon the kinetics of $^{3}$H-DG uptake by MCF-7 cells. For this, cells were incubated with increasing concentrations of $^{3}$H-DG (0.02-10 000 µM) in the presence of kaempferol or the respective solvent. As can be seen in Table 4, KF 100 µM showed an unusual, mixed-type inhibitory effect, since it simultaneously increased the $K_m$ and the $V_{máx}$.

**Table 4:** Kinetic parameters of $^{3}$H-DG uptake by MCF-7 cells in the absence (DMSO) or presence of kaempferol 100 µM: Shown are arithmetic means ± SEM (n=3-14). * Significantly different from control (DMSO) (P<0.05)

| Compound | $K_m$ (mM) | $V_{máx}$ (µmol/mg prot) |
|---|---|---|
| **DMSO** | 6.47 ± 0.5 | 63.59 ± 2.3 |
| **KF100 µM** | 15.57 ± 2.4* | 106.93 ± 10.6* |

Considering all these results, we decided to further analyze the effect of KF. For this, we studied its chronic effect upon $^{3}$H-DG uptake, GLUT1 mRNA and protein expression, and upon cell viability and growth. Further, we investigated whether its anticarcinogenic (antiproliferative and cytotoxic) effect may be related to inhibition of glucose uptake.

## 3. The chronic effect of kaempferol

### *3.1 Modulation of $^{3}$H-DG uptake*

In a first series of experiments, we characterized the chronic (24 h) effect of kaempferol (0.3-30 µM) upon uptake of $^{3}$H-DG by MCF-7 cells.

This compound, when placed in contact with MCF-7 cells for 24h, caused a significant inhibition of $^{3}$H-DG uptake at 30 µM only (Fig. 11).

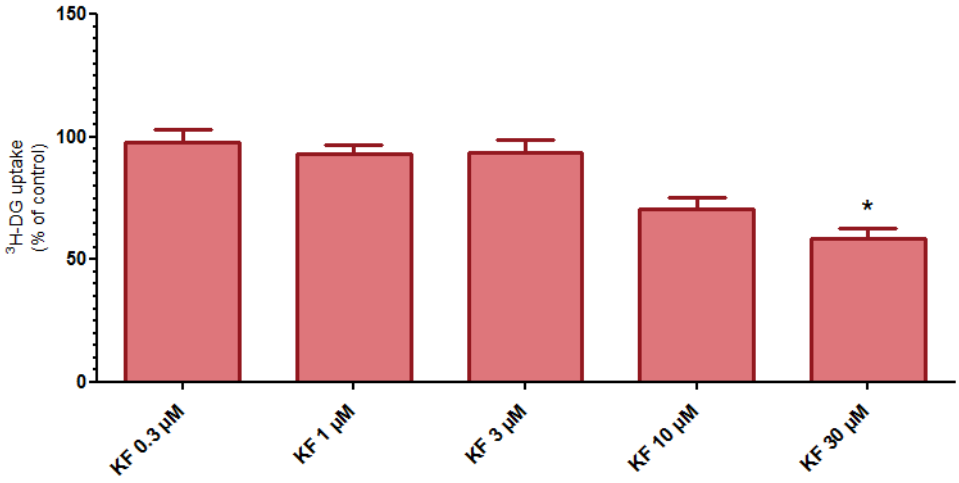



**Figure 11: Chronic effect of kaempferol upon $^{3}$H-DG uptake.** Cells were preincubated for 24 h and then incubated for 6 min with 20 nM $^{3}$H-DG under control conditions or in the presence of kaempferol (KF) at different concentrations: 0.3 µM, 1 µM, 3 µM, 10 µM and 30 µM. Shown are arithmetic means±SEM. *Significantly different from control ($P < 0.05$).

### *3.2 Effect upon lactate production*

Considering that these cells in culture perform mainly the Warburg effect, we investigated if the decrease in glucose cellular uptake in response to KF 30 µM exposure, as assessed by the decrease in $^{3}$H-DG uptake, would cause also a decrease in lactate production.

To clarify this, cells were incubated for 4 or 24h with KF 30 µM, QUE 100 µM or 2-DG 2 mM. After treatment, the extracellular medium was collected and lactate levels were quantified. QUE and 2-DG were used in order to compare their known effects with KF effect.

As shown in Figure 12, exposure of the cells to KF or QUE for 24 h caused a significant increase in the amount of extracellular lactate (especially evident for KF). On the contrary, 2-DG originated a strong decrease in the amount of lactate. When a shorter exposure to

these compounds was tested, KF and 2-DG were found to be devoid of effect; QUE, on the other hand, originated a decrease in the amount of lactate.

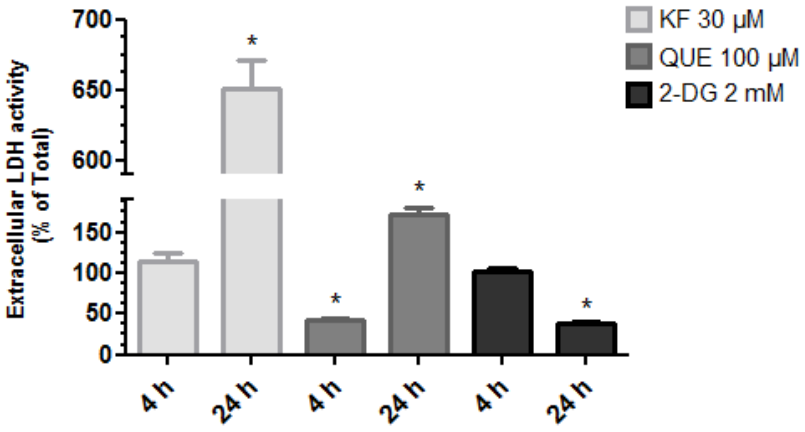



**Figure 12: Effect of kaempferol (KF), quercetin (QUE) and 2-deoxyglucose (2-DG) upon lactate production in MCF-7 cells**. Cells were treated at 37ºC for 4 or 24h with kaempferol 30 mM (n = 4-3), quercetin 100 mM (n = 3) or 2-DG 2 mM (n = 3-4). Shown are the arithmetic means ± SEM. * P <0.05 vs. Control (100%).

### *3.3 Modulation of GLUT1 mRNA expression*

Because KF inhibits $^{3}$H-DG transport into MCF-7 cells, we found important to investigate if this effect is related to modulation of expression of GLUT1, the main glucose transporter in these cells. So, we evaluated its effect upon GLUT1 transcription and translation, to realize if it interferes with mRNA synthesis and protein production.

For that, cells were treated for 24 h with KF 30 µM or with its solvent (DMSO). Subsequently, RNA was extracted and after cDNA synthesis, mRNA levels of GLUT1 were quantified by qPCR.

The results, shown in Figure 13, clearly show that the expression of GLUT1 mRNA is significantly reduced in response to kaempferol.

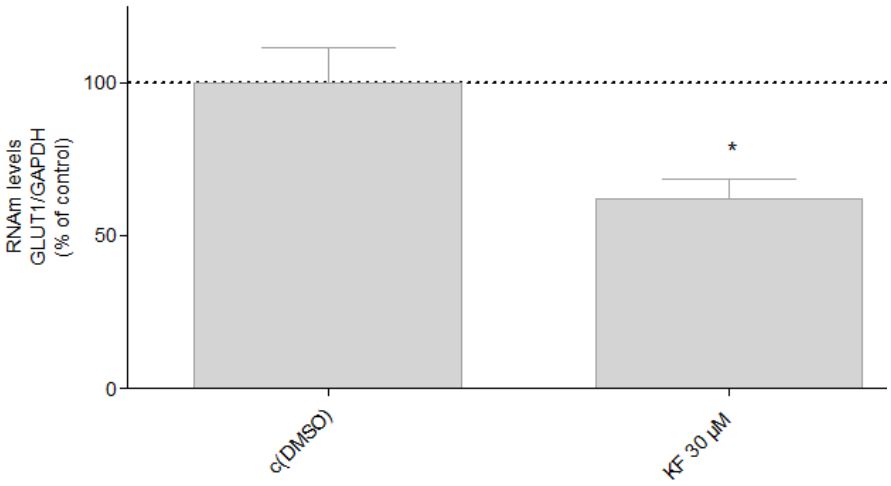



**Figure 13: Effect of kaempferol upon GLUT1 mRNA levels in MCF-7 cells**. Cells were treated at 37ºC for 24 h with kaempferol (KF) 30 µM (n = 6) or with its solvent (c(DMSO), dashed line, n = 6). Shown are arithmetic means ± SEM of GLUT1 expression relative to the internal control GAPDH. *Significantly different from control ($P < 0.05$).

### *3.4 Modulation of GLUT1 protein levels*

Next, the effect of KF (30 µM; 24h) upon protein levels was quantified by Western blotting. Through Figure 14, it is possible to observe that, although not statistically significant, there was a tendency for a decrease in GLUT1 protein levels in the presence of KF.

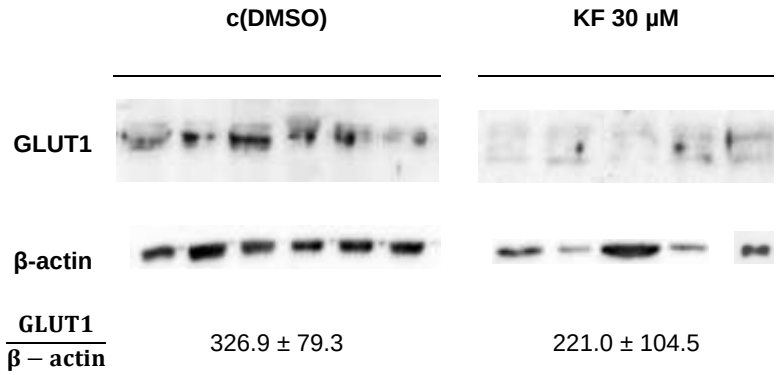



**Figure 14: Modulation of GLUT1 protein levels by kaempferol in MCF-7 cells.** Cells were treated at 37 ºC for 24 h with kaempferol (KF) 30 µM (n = 5) or with its solvent (c(DMSO), dashed line, n = 6). β-actin was used in order to normalize the results. Shown are arithmetic means ± SEM.

## 4. Antiproliferative and cytotoxic properties of kaempferol

In the next series of experiments, we aimed to investigate if KF presents an anticarcinogenic effect on MCF-7 cells and if this effect is related with inhibition of $^{3}$H-DG uptake.

### *4.1 Effect upon cell proliferation rate*

The rate of cell proliferation was evaluated by measuring the incorporation of $^{3}$H-thymidine by MCF-7 cells. As can be seen in Figure 15, KF 30 µM had no effect on MCF-7 proliferation rate, but a higher concentration (100 µM) markedly reduced it.

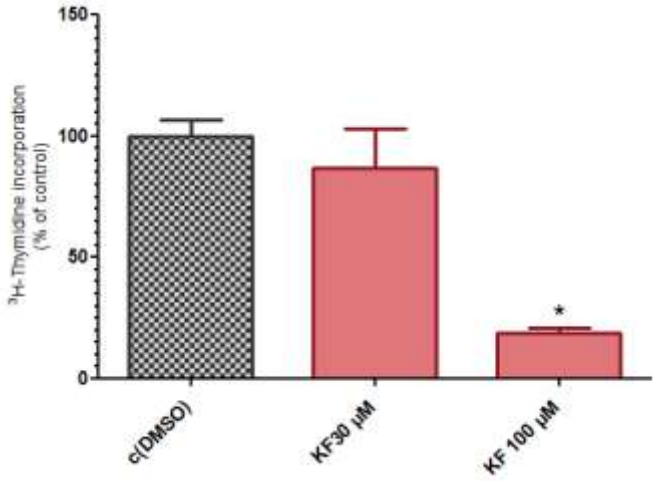



**Figure 15: Effect of kaempferol upon cell proliferation rate of MCF-7 cells**. Cells were cultured for 24h with DMSO (n = 9) or kaempferol (KF) 30-100 µM (n = 9). Shown are arithmetic means ± SEM. * P <0.05 vs. Control (100%).

### *4.2 Effect upon cell integrity*

As shown in Figure 16, KF 100 µM, but not KF 30 µM, caused a significant increase in LDH leakage (Fig. 16, A) and a significant decrease in cell growth (Fig.16, B).

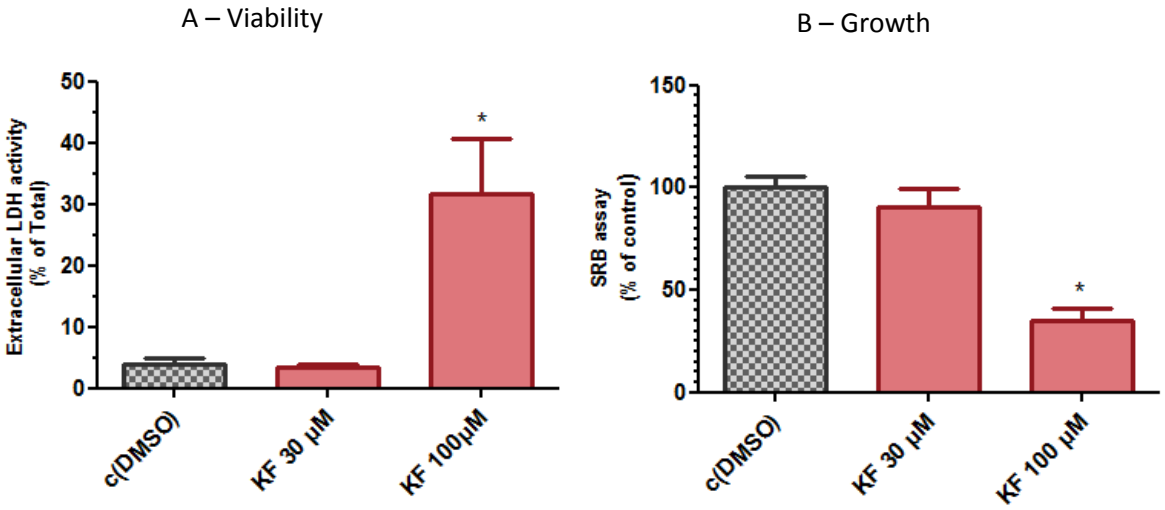



**Figure 16: Effect of kaempferol on viability (A) and growth (B).** After a 24h exposure to kaempferol (KF) at 30-100 µM or to its solvent (c(DMSO)), MCF-7 cellular viability was determined by quantification of extracellular LDH activity (n=12) and cellular growth was determined by the SRB assay (n=12). Shown are arithmetic means ±SEM. *Significantly different from control ($P < 0.05$).

## 5. Relationship between GLUT-mediated glucose transport and the antiproliferative and cytotoxic properties of kaempferol

In the last series of experiments, we investigated if inhibition of GLUT-mediated glucose transport is involved in the antiproliferative and cytotoxic effect of KF. For this, cells were exposed, during 24 h, to KF 100 µM or to its solvent (DMSO) in HMBS buffer with different glucose concentrations: 1 mM (Low Glucose – LG), 5.56 mM (Normal Glucose – NG) or 20 mM (High Glucose – HG).

### *5.1 Mimetization of kaempferol effect by Low Glucose-conditions*

Interestingly, low glucose conditions mimic the effect of KF upon cell viability (Fig. 17, A) and growth (Fig. 17, B). This result is in agreement with the hypothesis that the antiproliferative and cytotoxic effect of KF is dependent on inhibition of glucose entry into the cells.

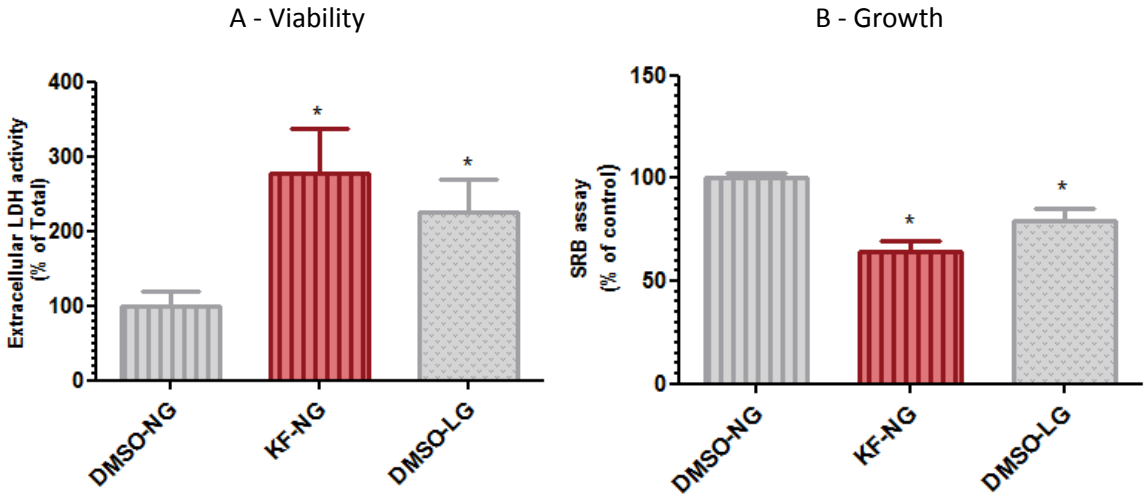



**Figure 17: Mimetization of the effect of kaempferol on cell viability (A) and cell growth (B) by low glucose-conditions.** Cells were exposed for 24 h to kaempferol (KF) at 100 µM or to its solvent (DMSO) in Normal Glucose-conditions (NG, 5.56 mM) or in low Glucose-conditions (LG, 1 mM). MCF-7 cellular viability was determined by quantification of extracellular LDH activity (n=10-11) and cellular growth was determined by quantification of whole cellular protein with SRB (n=12). Shown are arithmetic means ±SEM. *Significantly different from control (P < 0.05).

### *5.2 Reversal of the effect of kaempferol by High Glucose conditions*

A further confirmation of the conclusion that the cytotoxic effect of KF is dependent on inhibition of glucose entry into the cells is the observation that high glucose conditions were able to reverse the effect of KF in relation to cell viability (Fig. 18). However, no reversal could be found in relation to cell growth (results not shown).

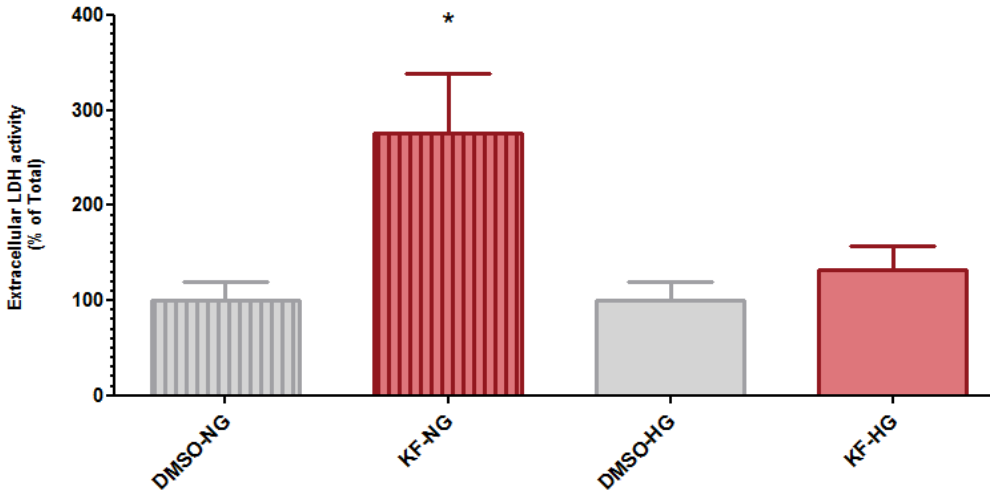



**Figure 18: Reversal of the effect of kaempferol on cell viability by high glucose-conditions.** Cells were exposed for 24 h to kaempferol (KF) at 100 µM or to its solvent (DMSO) in Normal Glucose-conditions (NG, 5.56 mM) or in High Glucose-conditions (HG, 20 mM). MCF-7 cellular viability was determined by quantification of extracellular LDH activity (n=10-12). Shown are arithmetic means ±SEM. *Significantly different from control (P < 0.05).

# CHAPTER IV

# DISCUSSION

Breast cancer is one of the most devastating human diseases and some of the existent therapies are very toxic to normal cells. So, it is imperative to search for novel therapeutic compounds, especially natural compounds, since they are safe and have a favorable toxicity profile, which makes them suitable (*57, 58*).

Cancer cells present an altered metabolism, with an increased rate of glucose uptake and glycolysis in aerobic conditions instead of oxidative metabolism (the Warburg effect) (*25*). As a cell model of breast cancer, a human cell line derived from a malignant adenocarcinoma breast tissue, the MCF-7 cells, was used. As is already known, these cells are a prominent model system since they preserve several ideal characteristics particular to the mammary epithelium (*59, 60*).

This study was designed in order to characterize glucose transport in breast cancer cells, to provide information about the effect of polyphenols on glucose uptake, and to investigate inhibition of glucose uptake as being involved in the effect of these compounds on cell viability and growth.

For glucose transport experiments, 2-deoxy-D-glucose (2-DG) was used, since it is a glucose analog efficiently transported by GLUT family members but a very poor SGLT substrate (*48*) and cannot be metabolized by phosphoglucose isomerase (*61*).

According to the results obtained, $^{3}$H-DG uptake in MCF-7 cells was: (1) time-dependent; (2) saturable (with an $K_m$ of 6.5 ± 1.5 mM and a $V_{máx}$ of 63.6 ± 6.8 µmol/mg prot); (3) inhibited by cytochalasin B (CYT B), an inhibitor of glucose facilitative transporters (GLUTs) (*55*) and by phlorizin (PHZ), an inhibitor of sodium-dependent glucose transporters (SGLTs) (*56*). However, we speculate that SGLTs are not involved in this process. Previous studies from our group (*21*) have demonstrated that $^{3}$H-DG uptake by MCF-7 cells does not involve SGLT1, albeit being inhibited by PHZ. This conclusion was based on the fact that not only DG is a poor substrate of SGLT1, but also PHZ is a glucoside. So, the authors speculated that this flavonoid may be an unspecific competitive inhibitor of glucose transport and not a specific inhibitor of SGLT1 (*21*). These data suggest that, in these cells, glucose uptake is mediated by GLUTs. GLUT1 is likely to be the main mediator of glucose uptake by MCF-7 cells, since it is highly expressed in this cell line and it is known to be critical for glucose uptake in tumors (*18-20*).

Dietary polyphenols are known to possess cancer preventive and anticarcinogenic effects (*31*). These compounds are abundant in our diet, but their health effects are obviously influenced by the amount consumed and by their bioavailability. Recently, our group verified that the polyphenols quercetin (a flavanol) and epigallocatechin gallate (a flavan-3-ol) inhibited glucose uptake by MCF-7 cells (*21*). So, we decided to investigate if other polyphenols could also interfere with glucose uptake by these cells. Thus, in the second

part of this work, we studied the modulation of $^{3}$H-DG uptake by several polyphenols after an acute exposure: some flavanols (myricetin and kaempferol) and flavan-3-ols ((+) catechin and (-) epicatechin), a flavone (chrysin), an isoflavone (genistein), a chalchone (xanthohumol) and a stilbene (resveratrol). As mentioned in the *Introduction,* these polyphenols were chosen because several studies have shown their anticancer effects: genistein (*45*), kaempferol (*62*), resveratrol (*47*), xanthohumol (*46*), myricetin (*40*), (+) catechin and (-) epicatechin (*21*) and chrysin (*44*).

When MCF-7 cells were acutely treated (26 min) with myricetin, genistein, resveratrol, and kaempferol (KF) (10-100 µM), a concentration-dependent inhibition of $^{3}$H-DG uptake was observed. By contrast, (+) catechin slightly (by 10-15%) increased it. Through this it is possible to conclude that polyphenols belonging to different polyphenolic classes are able to impair glucose uptake and that their effects are strongly influenced by their chemical structure. This is evident in the disparate results of (+) catechin, (-) epicatechin and the epigallocatechin gallate, since they are at the same polyphenolic family and present different behaviors.

The cell integrity tests were important to prove that the above mentioned effects were not due to cellular death. So, by proving that the studied polyphenols don't interfere with viability and growth of MCF-7 cells (except genistein that seems to increase cell growth) we can conclude that their effect upon $^{3}$H-DG uptake is specific.

Determination of $IC_{50}$ for resveratrol, genistein and KF led us to conclude that KF was the most potent inhibitor of $^{3}$H-DG uptake by MCF7 cells, with an $IC_{50}$ of 4.0 (1.6-9.8) µM (the $IC_{50}$ of genistein and resveratrol was 38.9 (15.2-99.4) µM and 67.2 (29.4-153.7) µM, respectively). So, KF was chosen as the polyphenol to investigate in the remaining studies.

Flavonoids are classified according to their level of oxidation and to the pattern of substitution at the C ring. Individual compounds within a class differ in the arrangements of hydroxyl groups, which increase the water solubility (*29, 31*). KF (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) is a flavonol and normally is found in tea, broccoli, cabbage, kale, beans, endive, leek, tomato, strawberries and grapes (*63*). According to a Japanese study, the average daily intake (from japanese food) per capita of KF is 4.9 mg (*64*). However, in a Spanish study, it is estimated that human dietary intake may be approximately 10 mg/day (*63*). Normally, this compound is present at 26 ± 7 µg/d in urine. However these levels depend on the type of food consumed and on the amount of intake (*65*). KF and all the flavonoids are mostly found in food as *O*-glycosides and therefore these compounds need to be deglycosylated in order to be absorbed.

Some previous studies have found a link between the consumption of foods containing KF and a reduced risk of developing cancer. Some suggest that KF inhibits MCF-7 breast cancer cell proliferation through activation of the caspase cascade pathway (*66*); others suggest that KF is an antioxidant and can prevent lipid peroxidation, reducing ROS levels; that it is anti-inflammatory or that it inhibits angiogenesis *in vitro* and metastasis (*63*).

Analysis of the effect of KF upon the kinetic parameters of $^{3}$H-DG uptake showed a very unusual effect, since it simultaneously increases the $K_m$ and the $V_{máx}$ of $^{3}$H-DG uptake. So, KF seems to decrease the affinity while increasing the capacity of $^{3}$H-DG uptake. In other words, KF appears to inhibit uptake of a low concentration of $^{3}$H-DG while increasing uptake of a high concentration. Gonçalves *et al.* (2009) demonstrated the same effect with acetylsalicylic acid in modulation of butyrate transport in Caco-2 cells (*67*).

When MCF-7 cells were subjected to a chronic treatment (24h) with KF, it was verified that this flavonoid also inhibits $^{3}$H-DG uptake ($IC_{50}$ of 13.6 (2.8-66.9) µM), although less markedly than in the acute treatment. The chronic effect of KF was concordant with the study of Filomeni *et al.* (2010), that demonstrated a block in cellular uptake of glucose by this flavonoid in HeLa cells (*61*). However, this is the first work showing an inhibitory effect of KF upon glucose uptake in BrC cells.

We next decided to deepen knowledge on the KF effect at GLUT1 transcriptional and translational level. qRT-PCR results showed a significant decrease in the mRNA expression levels of GLUT1 after treatment with KF. A similar decrease in GLUT1 protein levels was observed in western-blot, although not statistically significant. The decreasing levels of mRNA and protein production are concordant with the inhibitory effect of KF on $^{3}$H-DG transport, suggesting that reduction of $^{3}$H-DG transport is due to downregulation of GLUT1 gene and protein expression.

Being glucose a main energy source for cancer cells, and so being necessary for their survival, glucose deprivation is expected to have a negative impact in cellular viability and growth. This fact was described by Munõz-Pinedo *et al.* (2003), who proved that the absence of glucose induces apoptosis in MCF-7 cells (*68*). Also, it is known that in human BrC, high GLUT1 expression tends to correlate with high proliferative activity and histological score (*69*).

So, in the last part of this work, we decided to investigate the effect of KF upon cell viability and growth and the relationship between inhibition of glucose uptake by KF and its effect upon cell viability and growth.

Chronic (24 h) treatment of the cells with KF (100 µM) caused a significant decrease in cell viability (as assessed by the quantification of extracellular lactate dehydrogenase activity) and growth (as assessed by the SRB assay and by quantification of $^{3}$H-thymidine

incorporation). So, KF presents antiproliferative and cytotoxic effects in MCF-7 cells. These results are in line with others studies, which demonstrated that KF is a compound which has effect in several areas, such as signal transduction, cell cycle, angiogenesis, metastasis and inflammation, and that it shows antiproliferative and cytotoxic effects in tumor cells. (*62*).

As already known, cancer cells produce lactate when they metabolize glucose, in order to produce their energy faster (*70*). So, we decided to analyze if inhibition of GLUT1 by KF would lead to a decrease in lactate production. Unexpectedly, exposure of cells for 24h to KF induced a very marked increase in the amount of extracellular lactate. In order to further investigate this finding, we also analyzed the effect of QUE and of 2-DG (since QUE is a known GLUT and MCT1 inhibitor and 2-DG is a GLUT inhibitor) and we tested a shorter exposure time. From the results obtained, we concluded that the increase in extracellular lactate concentration observed with KF (and, albeit less markedly, with QUE) treatment may be due to an inhibition of lactate reuptake, mediated by MCT1, in MCF7 cells (*70*). On the contrary, 2-DG, which is solely a GLUT1 inhibitor, caused a decrease in extracellular lactate.

Studies suggest that MCT1 (present in aerobic cells and responsible for the uptake of lactate) inhibition causes a switch from lactate-fuelled respiration to glycolysis. Aerobic tumor cells can now use glucose to fuel glycolysis and secrete lactate and hypoxic tumor cells, located far from blood vessels, die due to starved glucose and consequently energy failure (*70, 71*). In our study, cells don't have glucose due to GLUT1 block by KF and with the block of MCT1, they also don't have lactate, so these cells apparently don't have an energy source.

In the last part of this work, the relationship between inhibition of glucose uptake and the antiproliferative and cytotoxic effect of KF was investigated, by analyzing this effect of KF under several distinct concentrations of glucose. With these data, it was verified that low glucose mimics the effect of KF upon cell viability and growth, and that the effect of KF upon cell viability was reversed by high glucose. This observation is in agreement with Elstrom *et al.* (2004), who believe that inhibition of basal glucose transport may be a potential strategy for cancer treatment, because cancer cells are very sensitive to glucose concentration changes (*72*). In fact, glucose deprivation may induce kinases activation, changes in the redox state of the cell, or generation of free radicals and consequently induces cell death, making cells more fragile (*73*). On the other hand, elevated levels of glucose may promote increased glucose uptake (*74, 75*). These observations further validate GLUT1 as the main mediator of glucose transport in MCF-7 cells, since it is highly expressed in these cells and can be easily modulated by the presence of low or high

glucose (*69*). Taking all these into account, low glucose has a better treatment outcome comparing with high glucose.

So, in this work, we demonstrated that in MCF-7 cells KF impairs glucose utilization, which is associated with an anticarcinogenic effect. Some previous studies already showed a cytotoxic, antiproliferative and antitumoral effect of KF in ovarian (*76*), breast (*77*) and pancreatic cancer cells (*78*), but none related it with glucose utilization by cancer cells.

Summarizing, we suggest that KF directly inhibits GLUT1 and MCT1 and that cellular glucose and lactate deprivation induces several downstream effects such as cytotoxicity and proliferation arrest.

Taking all the results of this work into account, it is possible to create a model that proposes the effect of KF in MCF-7 cells (Fig. 19).

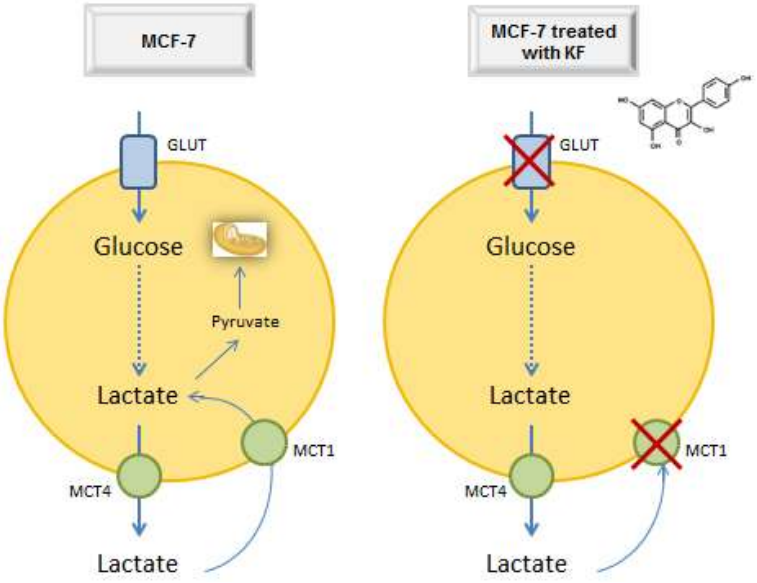



**Figure 19: Model of kaempferol-induced anticancer effects.** Kaempferol inhibits glucose and lactate uptake favoring the cytotoxic and antiproliferative effects.

# CHAPTER V

# CONCLUSIONS AND FUTURE PERSPECTIVES

Several polyphenols, belonging to different polyphenolic classes, impair glucose uptake by MCF-7 cells. The effect of these polyphenols upon glucose uptake does not appear to be related to their chemical structure.

As a whole, the data suggest that KF is the most potent inhibitor of glucose uptake, inhibiting GLUT1, which induces several downstream effects such as a decrease in glucose metabolism, cytotoxicity and proliferation arrest.

Overall, this study contributes to the elucidation of the mechanism involved in glucose transport in breast cancer cells and to a deeper knowledge of the health promoting effects of this flavonoid and its potential as therapeutic agent/adjuvant in breast cancer.

A link between cancer risk and the intake of some nutrients is well established. However, it is difficult to determine the effect of a single nutrient/compound in cancer prevention, because its effect may result from an interaction with other components present in food. So, in the future it is important to study KF interactions in the food matrix, its bioavailability and its effects at concentrations usually found in plasma.

Cancer cells are characterized by having a reverse pH gradient, since they present a higher internal pH (>7.4) and a lower external pH (6.7–7.1), which do not occur in normal cells. The internal pH, when high, is responsible for cell proliferation, evasion of apoptosis, metabolic adaptation and cell migration. On the other hand, a decreased external pH leads to the remodeling of extracellular matrix (ECM) and stimulates acid-activated proteases (*79*). In cells treated with KF, the extracellular pH is very low. So, from the results obtained we can raise the following questions: because MCT1 blockade leads to a decrease in $pH_{e,}$ can this contribute to tumor growth? There are some compensatory mechanisms associated to this effect? To answer them, more investigation is needed.

As it was demonstrate by Luo *et al.* (2012), KF nanoparticles achieve a strong and selective inhibition of ovarian cancer cell viability. Taking into account the nanotechnology evolution, it would be interesting also to analyze this option in BrC, since it seems to be a promising therapy (*76*).

Knowledge from future experiments, together with data obtained from “in vivo” studies, might help to sustain future nutritional recommendations in order to improve health.

# CHAPTER VI

# REFERENCES

1. National Institutes of Health, (2011) Breast Cancer, In *A.D.A.M. Medical Encyclopedia*, PubMed Health.
2. Portal de Oncologia Português, Cancro da Mama, Retrieved 17.09.2012, from http://www.pop.eu.com/portal/publico-geral/tipos-de-cancro/cancro-da-mama/o-cancro-da-mama.html.
3. Kumar V. *et al.,* (2010) The Breast in "Robbins and Cotran Pathologic Basis of Disease", *Elsevier*, 8th Edition.
4. Hutchinson L, (2010) Breast Cancer: Challenges, controversies, breakthroughs, *Nature Reviews Clinical Oncology 7*, 669-670.
5. Richie R. and Saxanson J, (2003) Breast Cancer: A Review of the Literature, *Journal of Insurance Medicine 35*, 85-101.
6. Hanahan D and Weinberg R, (2000) The Hallmarks of Cancer, *Cell 100*, 57-70.
7. Beckmann M, *et al.* (1997) Multistep carcinogenesis of breast cancer and tumour heterogeneity *Journal of Molecular Medicine 75*, 429-439.
8. DeVita *et al.* (2008) Cancer of the Breast in "DeVita, Hellman & Rosenberg's Cancer: Principles & Practice of Oncology", *Lippincott Williams & Wilkins*, 8th edition
9. San Diego State University, "Every Woman Counts, Resources for Health Professionals." Retrieved 8.10.2012, from http://qap.sdsu.edu/education/bcrl/Bcrl_riskfactors/bcrl_riskfactors_index.html.
10. Warren B and Devine C, (2004) Understanding Breast Cancer Risk and Risk Factors Associated With Diet and Lifestyle, *Sprecher Institute for comparative Cancer Research 49*.
11. Seitz H *et al.* (2012) Epidemiology and Pathophysiology of Alcohol and Breast Cancer: Update 2012, *Alcohol and Alcoholism 47*, 204-212.
12. Key T, *et al.* (2004) Diet, nutrition and the prevention of cancer, *Public Health Nutrition 7*, 187-200.
13. Donaldson M, (2004) Nutrition and cancer: A review of the evidence for an anti-cancer diet, *Nutrition Journal 3:19*.
14. Ledesma N, Women's Health Matters - Nutrition & Breast Cancer, In *UCSF Medical Center*.
15. Wood I, Trayhurn P, (2003) Glucose transporters (GLUT and SGLT): expand families of sugar transport proteins, *British Journal of Nutrition 89*, 3-9.
16. Szablewski L, (2012) Expression of glucose transporters in cancers, *Biochimica et Biophysica Acta*, 1835(2): 164–169.
17. Calvo M *et al.* (2010) Potential role of sugar transporters in Cancer and their relationship with anticancer therapy, *International Journal of Endocrinology*, 1-14.
18. Krzeslak A, *et al.* (2012) Expression of GLUT1 and GLUT3 Glucose Trnasporters in Endometrial and Breast Cancers, *Pathology Oncology Research, 18*, 721-728.
19. Macheda M, *et al.* (2005) Molecular and cellular regulation of glucose transporters (GLUT) proteins in cancer, *Journal of Cellular Physiology 202*, 654-662.
20. Medina R and Owen G, (2002) Glucose transporters: expression, regulation and cancer, *Biological Research 35 (1)*, 9-26.
21. Moreira L, *et al.* (2013) Quercetin and Epigallocatechin gallate seriously impair glucose uptake and metabolism by MCF7 cells and compromise both cell survival and proliferation, *Experimental Cell Research,* 319:1784-1795.
22. Janku F, *et al.* (2013) A kinase-independent biological activity for insulin growth factor-1 receptor (IGF-1R): implications for inhibition of the IGF-1R signal, *Oncotarget 4*, 463-473.
23. Modica-Napolitano J, *et al.* (2007) Mitochondria and Human Cancer in Current Molecular Medicine", Bentham Science Publishers Ltd, 121-131.
24. López-Lázaro M, (2008) The warburg effect: why and how do cancer cells activate glycolysis in the presence of oxygen?, *Anti-Cancer Agents in Medicinal Chemistry 8*, 205-312.

25. Cairns R, *et al.* (2011) Regulation of cancer cell metabolism *Nature Reviews| Cancer 11*, 85-95.
26. Li J, *et al.* (2008) Significant increase of glucose transport activity in breast cancer, *Chinese Journal of Pathology 37 (2)*, 103-108.
27. Aft R, *et al.* (2002) Evaluation of 2-deoxy-D-glucose as a chemotherapeutic agent: mechanism of cell death, *British Journal of Cancer 87*, 805-812.
28. Manach C, *et al.* (2004) Polyphenols: food sources and bioavailability *The American Journal of Clinical Nutrition 79*, 727-747.
29. Negrão and Faria (2009) Natural Polyphenols as Anti-Oxidant, Anti-Inflammatory and Anti-Angiogenic Agents in the Metabolic Syndrome, in "Oxidative Stress, Inflammation and Angiogenesis in the Metabolic Syndrome", ed. Soares R and Costa C, Springer, pp. 147-152.
30. Scalbert A, *et al.* (2005) Dietary polyphenols and the prevention of diseases, *Critical Reviews in Food Science and Nutrition 45(4)*, 287-306.
31. Crozier A, *et al.* (2009) Dietary phenolics: chemistry, bioavailability and effects on health, *The Royal Society of Chemistry 26*, 1001-1043.
32. Ross J and Kasum C, (2002) Dietary Flavonoids: Bioavailability, Metabolic Effects, and Safety, *Annual Review of Nutrition 22(1)*, 19-34.
33. Frei B. and Higdon J, (2003) Antioxidant activity of tea polyphenols in vivo: evidence from animal studies, *Journal of Nutrition 133*, 32-75-3284.
34. Jing L, *et al.* (2010) Phytochemicals, antioxidant properties and anticancer investigations of the different parts of several gingers species (Boesenbergia rotunda, Boesenbergia pulchella var attenuata and Boesenbergia armeniaca), *Journal of Medicinal Plants Research 4*, 27-32.
35. Xu C, *et al.* (2005) Induction of phase I, II and III drug metabolism/transport by xenobiotics, *Archives of Pharmacal Research 28*, 249-268.
36. Rushmore T and Kong A, (2002) Pharmacogenomics, regulation and signaling pathways of phase I and II drug metabolizing enzymes, *Current Drug Metabolism 3*, 481-490.
37. Lambert J, *et al.* (2005) Inhibition of carcinogenesis by polyphenols: evidence from laboratory investigations, *American Journal of Clinical Nutrition 81*.
38. Nijveldt R, *et al.* (2001) Flavonoids: a review of probable mechanisms of action and potential applications, *American Journal of Clinical Nutrition 74*, 418-425.
39. Martin H, *et al.* (2003) The inhibitory effects of flavonoids and antiestrogens on the Glut1 transporter in human erythrocytes, *Chemico-Biological Interactions 146*, 225-235.
40. Shiomi K, *et al.* (2013) Inhibitory effects of myricetin on mammalian DNA polymerase, topoisomerase and human cancer cell proliferation, *Food Chemistry 139*, 910-918.
41. Bigelow R and Cardelli J, (2006) The green tea catechins, (-)Epigallocatechin-3-gallate (EGCG) and (-)Epicatechin-3-gallate (ECG), inhibit HGF/Met signaling in immortalized and tumorigenic breast epithelial cells, *Oncogene 25*, 1922-1930.
42. Alshatwi A, (2010) Catechin hydrate suppresses MCF-7 proliferation through TP53/Caspase-mediated apoptosis, *Journal of Experimental & Clinical Cancer Research 29*, 1-9.
43. Nagarajan S, *et al.* (2008) Biocatalytically Oligomerized Epicatechin with Potent and Specific Anti-proliferative Activity for Human Breast Cancer Cells, *Molecules 13*, 2704-2716.
44. Stapel J, *et al.* (2013) Polyphenol compounds with anti-carcinogenic qualities: Effects of quercetin (flavonol), chrysin (flavon), kaempferol (flavanol), naringenin (flavanon) and hesperidin (flavanoid) on in vitro breast cancer, *Journal of Medicinal Plants Research 7*, 2187-2196.
45. Li Y, *et al.* (2013) Epigenetic Regulation of Multiple Tumor-Related Genes Leads to Suppression of Breast Tumorigenesis by Dietary Genistein, *PLOS | one 8*, 1-12.

46. Monteiro R, *et al.* (2008) Xanthohumol Inhibits Inflammatory Factor Production and Angiogenesis in Breast Cancer Xenografts, *Journal of Cellular Biochemistry*, 1-9.
47. Su D., *et al.* (2013) Comparision of Piceid and Resveratrol in Antioxidation and Antiproliferation Activities In Vitro, *PLOS | one 8*, 1-13.
48. Wright E, *et al.* (2003) Intestinal absorption in health and disease-sugars, *Best Practice & Research Clinical Gastroenterology 17*, 943-956.
49. Araújo J, *et al.* (2008) Modulation of Glucose Uptake in a Human Choriocarcinoma Cell Line (BeWo) by Dietary Bioactive Compounds and Drugs of Abuse, *Journal of Biochemistry 144*, 177-186.
50. Keating E, *et al.* (2007) Acute and chronic effects of some dietary bioactive compounds on folic acid uptake and on the expression of folic acid transporters by the human trophoblast cell line BeWo, *Journal of Nutritional Biochemistry 19*, 91-100.
51. Gonçalves P, *et al.* (2011) In Vitro Studies on the Inhibition of Colon Cancer by Butyrate and Polyphenolic Compounds, *Nutrition and Cancer*, 1-13.
52. Bradford M, (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal Biochemisty 72*, 248-254.
53. Bergmeyer H and Bernt E, (1974) Lactate dehydrogenase, *Methods in Enzymatic Analysis Academic Press, New York*.
54. Muzyka A, *et al.* (2013) GraphPad Prism from Windows. Version 5.03.
55. Klip A and Paquet M, (1990) Glucose Transport and Glucose Transporters in Muscle and Their Metabolic Regulation, *Diabetes Care 13*, 228-243.
56. Loike J, *et al.* (1996) Sodium-glucose cotransporters display sodium- and phlorizin-dependent water permeability., *The American Journal of Physiology 271*, 1774-1779.
57. Harun F, *et al.* (2012) Autophagic Cell Death Is Induced by Acetone and Ethyl Acetate Extracts fromEupatorium odoratum In Vitro: Effects on MCF-7 and Vero Cell Lines, *The Scientific World Journal*, 1-9.
58. Lazzeroni M, *et al.* (2011) The science behind vitamins and natural compounds for breast cancer prevention. Getting the most prevention out of it., *The Breast 3*, 36-41.
59. Cell Biolabs, Inc. Creating Solutions for Life Science Research, (2011) MCF-7/ GFP Cell Line, AKR-221
60. Yang H, *et al.* (2006) Growth inhibition and induction of apoptosis in MCF-7 breast cancer cells by *Antrodia camphorata*, *Cancer Letters 231*, 215-227.
61. Filomeni G, *et al.* (2010) Carcinoma cells activate AMP-activated protein kinase-dependent autophagy as survival response to kaempferol-mediated energetic impairment, *Autophagy 6*, 202-216.
62. Chen A and Chen Y, (2013) A review of the dietary flavonoid, kaempferol on human health and cancer chemoprevention, *Food Chemistry 138*, 2099-2107.
63. Calderón-Montaño J, *et al*. (2011) A Review on the dietary Flavonoid Kaempferol, *Mini-Reviews in Medical Chemistry 11*, 298-344.
64. Kimira M, *et al.* (1998) Japanese Intake of Flavonoids and Isoflavonoids from Foods Mitsuru, *Journal of Epidemiology 8*, 168-175.
65. Vries J, *et al.* (1998) Plasma concentrations and urinary excretion of the antioxidant flavonols quercetin and kaempferol as biomarkers for dietary intake, *American Society for Clinical Nutrition 68*, 60-65.
66. Diantini A, *et al.* (2012) Kaempferol-3-O-rhamnoside isolated from the leaves of Schima wallichii Korth. inhibits MCF-7 breast cancer cell proliferation through activation of the caspase cascade pathway, *Oncology Letters 3*, 1069-1072.
67. Gonçalves P, *et al.* (2009) Modulation of butyrate transport in Caco-2 cells, *Naunyn-Schmiedeberg's Archives of Pharmacology*, 379:325-336.

68. Younes M, *et al.* (1995) GLUT1 expression in human breast carcinoma: correlation with known prognostic markers, *Anticancer Research 15*, 2895-2898.
69. Munõz-Pinedo C, *et al.* (2003) Inhibition of Glucose Metabolism Sensitizes Tumor Cells to Death Receptor-triggered Apoptosis through Enhancement of Death-inducing Signaling Complex Formation and Apical Procaspase-8 Processing, *The Journal of Biological Chemistry 278*, 12759-12768.
70. Feron O, (2009) Pyruvate into lactate and back: From the Warburg effect to symbiotic energy fuel exchange in cancer cells, *Radiotherapy and Oncology 92*, 329-333.
71. Semenza G, (2008) Tumor metabolism: cancer cells give and take lactate, *The Journal of Clinical Investigation 118(12)*, 3835-3837.
72. Elstrom R, *et al.* (2004) Akt Stimulates Aerobic Glycolysis in Cancer Cells, *Cancer Research 64*, 3892-3899.
73. Graham N, *et al.* (2012) Glucose deprivation activates a metabolic and signaling amplification loop leading to cell death, *Molecular Systems Biology 8*, 1-16.
74. Heiden M, (2011) Targeting cancer metabolism: a therapeutic window opens, *Nature Reviews| Drug Discovery 10*, 671-684.
75. Alberghina L, *et al.* (2012) Cancer cell growth and survival as a system-level property sustained by enhanced glycolysis and mitochondrial metabolic remodeling, *Frontiers in Physiology 3*.
76. Luo H, *et al.* (2012) Kaempferol nanoparticles achieve strong and selective inhibition of ovarian cancer cell viability, *International Journal of Nanomedicine 7*, 3951-3959.
77. Kim B, *et al.* (2008) Sustained ERK activation is involved in the kaempferol-induced apoptosis of breast cancer cells and is more evident under 3-D culture condition, *Cancer Biology & Therapy 7*, 1080-1089.
78. Zhang Y, *et al.* (2008) *Ginkgo biloba* Extract Kaempferol inhibits cell proliferation and induces apoptosis in pancreatic cancer cells, *Journal of Surgical Research 148*, 17-23.
79. Webb B, *et al.* (2011) Dysregulated pH: a perfect storm for cancer progression, *Nature Reviews | Cancer 11*, 671-677

*Tenho a certeza de que hoje somos senhores do nosso destino, que a tarefa que temos perante nós não está acima das nossas forças; que as dores e dificuldades não estão para lá das nossas capacidades de resistência física. Enquanto tivermos fé na nossa própria causa e uma indomitável vontade de ganhar, a vitória não nos será negada.*

Winston Churchill