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Effect of phytochemicals on nutrient uptake by pancreatic cancer cell lines

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

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“ ‘You could be a scientist’ (…) Somebody is believing in you, maybe that’s the inspiration.”

Katalin Karikó

Nobel Prize in Physiology or Medicine 2023

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A todos,
Muito obrigada!

Resumo

O cancro pancreático (PC) é atualmente uma das causas mais comuns de morte por cancro. Um dos *hallmarks* do cancro é a capacidade que as células tumorais adquirem para reprogramar o seu metabolismo, de modo a sustentar as suas necessidades energéticas. O efeito de Warburg é uma destas alterações, no qual as células tumorais promovem a glicólise com formação de lactato, mesmo em condições aeróbicas. Algumas células tumorais podem também tornar-se "viciadas em glutamina", aumentando a sua incorporação de glutamina e tornando este aminoácido indispensável ao seu funcionamento. Estas células podem também aproveitar o lactato libertado por outras células tumorais durante a glicólise, num processo denominado *shuttle* do lactato. Ao passar por esta alteração, as células tumorais podem utilizar o lactato, que seria de outra forma um resíduo metabólico, para produzir mais energia e prevenir a acidose láctica no microambiente tumoral. Como consequência destas alterações no metabolismo, alguns tipos de cancro apresentam sobre-expressão das proteínas de transporte desses nutrientes, incluindo o *GLUT1* (o principal transportador de glucose na maioria das células), o *ASCT2* (um transportador de glutamina dependente de sódio) e o *MCT1* (o principal importador de ácido láctico na maioria das células). Um tratamento alternativo para o cancro que tem ganho interesse nos últimos anos é o uso de compostos bioativos naturais. Trabalhos prévios do nosso grupo revelaram que alguns carotenoides e polifenóis têm atividade anti-tumoral e interferem com o transporte de nutrientes em algumas células tumorais. Neste trabalho, avaliamos o efeito de alguns carotenoides (astaxantina, β-caroteno, crocina e fucoxantina) e de alguns polifenóis (crisina, genisteína, kaempferol e quercetina) na incorporação de glucose (^{3}H-DG), ácido láctico (^{3}H-L) e glutamina (^{3}H-GLN) em duas linhas celulares de cancro pancreático, as células AsPC-1 e PANC-1. A crocina e a crisina foram os compostos com um efeito mais marcado, reduzindo a incorporação de nutrientes na linha AsPC-1. A crocina originou uma diminuição na incorporação de ^{3}H-DG e ^{3}H-L (10-20%) e a crisina originou uma diminuição na captação de ^{3}H-DG e ^{3}H-GLN (±20%). Exploramos depois mais profundamente os efeitos da crocina e da crisina (10 μM) nas células AsPC-1. A partir desses ensaios verificamos que: (a) a crocina e a crisina não alteram significativamente a expressão génica do *GLUT1*, *ASCT2* e *MCT1*; (b) a crocina e a crisina reduzem a viabilidade destas células, de uma forma dependente da concentração; (c) em combinação com inibidores das proteínas de transporte (BAY-876, NPPB e GPNA), a crocina e a crisina não têm um efeito citotóxico adicional. Concluindo, a crocina e a crisina promovem a diminuição da viabilidade nas células AsPC-1, possivelmente devido ao seu efeito inibitório na incorporação de glucose, ácido láctico e glutamina.

Palavras-chave: cancro pancreático, captação de nutrientes, glucose, ácido láctico, glutamina, *GLUT1*, *MCT1*, *ASCT2*, carotenoides, polifenóis, crocina, crisina

Abstract

Pancreatic cancer (PC) is currently one of the most common causes of cancer-related death. One of the hallmarks of cancer is the ability that cancer cells acquire to reprogram their metabolism in order to maintain the energetic requirements. The Warburg effect is one of these alterations, in which cancer cells promote glycolysis, even in aerobic conditions. Some cancer cells can also become “glutamine addicted”, increasing uptake of glutamine, which becomes indispensable for their function. Cells can also take advantage of the lactate released by other cancer cells during glycolysis, in a process called lactate shuttle. This alteration allows cancer cells to use lactate, otherwise a metabolic waste product, to produce more energy and to prevent lactic acidosis in the tumor microenvironment. Due to these changes in metabolism, some cancers present overexpression in nutrient transporter proteins, including *GLUT1* (the main glucose transporter in most cells), *ASCT2* (a sodium-dependent glutamine transporter) and *MCT1* (the main lactic acid importer in most cells). An alternative treatment for cancer that has increased in interesting in the latest years is the use of isolated natural bioactive compounds. Previous works from our group show that some carotenoids and some polyphenols have anticancer activities and interfere with nutrient transport on some cancer cells. In this work, we evaluated the effect of some carotenoids (astaxanthin, β-carotene, crocin and fucoxanthin) and some polyphenols (chrysin, genistein, kaempferol and quercetin) on glucose (^{3}H-DG), lactic acid (^{3}H-L) and glutamine (^{3}H-GLN) uptake by two PC cell lines, AsPC-1 and PANC-1 cell lines. We verified that crocin and chrysin were the compounds with highest effect on nutrient uptake, reducing nutrient uptake by AsPC-1 cells. Crocin promoted a decrease in both ^{3}H-DG and ^{3}H-L uptake (10-20%) and chrysin promoted a decrease in ^{3}H-DG and ^{3}H-GLN uptake (±20%). We then further explored the effect of crocin and chrysin (10 µM) on AsPC-1 cells. From these assays we concluded that: (a) crocin and chrysin do not significantly alter *GLUT1*, *ASCT2* and *MCT1* gene expression; (b) crocin and chrysin decrease cell viability, in a concentration-dependent manner; (c) in combination with nutrient transport protein inhibitors (BAY-876, NPPB or GPNA), crocin and chrysin do not have an increased cytotoxic effect. In conclusion, crocin and chrysin decrease AsPC-1 cell viability, most likely due to their inhibitory effect on glucose, lactic acid and glutamine uptake.

Key words: pancreatic cancer, nutrient uptake, glucose, lactic acid, glutamine, *GLUT1*, *MCT1*, *ASCT2*, carotenoids, polyphenols, crocin, chrysin

List of Abbreviations

#

^{3}H-DG	^{3}H-deoxy-D-glucose
^{3}H-GLN	^{3}H-glutamine
^{3}H-L	^{3}H-lactic acid

A

ASCT1	Alanine, serine, cysteine, threonine transporter 1
ASCT2	Alanine, serine, cysteine, threonine transporter 2
ALDH1A	Aldehyde dehydrogenase 1 family, member A1
Akt	Protein kinase B
ATCC	American Type Culture Collection
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine triphosphate

B

BMI	Body mass index
BRCA1	Breast cancer gene 1
BRCA2	Breast cancer gene 2
BTLA	B- and T-lymphocyte attenuator

C

CCL21	Chemokine (C-C motif) ligand 21
CCR7	C–C chemokine receptor 7
CDKN2A	Cyclin-dependent kinase inhibitor 2A
cDNA	Complementary DNA
COX2	Cyclooxygenase-2

D

DMEM	Dulbecco’s modified eagle medium
DMSO	Dimethyl sulfoxide

E

ECOG	Eastern Cooperative Oncology Group
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor

EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase

F

FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FOXM1	Forkhead box protein M1

G

GF-HBS	Glucose-free HEPES buffered saline
GLS	Glutaminase enzyme
GLUT	Sodium-independent glucose transporter
GPNA	L-γ-Glutamyl-p-nitroanilide

H

hENT1	Human Equilibrative Nucleoside Transporter 1
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HCV	Hepatitis C vírus
hnRNPA1	Heterogeneous nuclear ribonucleoprotein A1

I

IL	Interleukin

J

JNK	Jun N-terminal kinase

K

Ki	Inhibition index
KRAS	Kirsten rat sarcoma virus

L

LC3	Microtubule-associated protein 1 light chain 3
LDH	Lactate dehydrogenase enzyme

M

MAPK	Mitogen-activated protein kinases
MCT	Monocarboxylate transporter

miR	MicroRNA
MLH1	MutL homolog 1
MMP	Matrix metalloproteinases
MSH2	MutS homolog 2
MSH6	MutS homolog 6
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide

N

NADH/NAD^+	Nicotinamide adenine dinucleotide
NET	Neuroendocrine tumors
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NPPB	5-nitro-2-(3-phenylpropylamino)-benzoic acid

O

OXPHOS	Oxidative phosphorylation

P

PALB2	Partner and localizer of BRCA2
PARP	Poly-ADP ribose polymerase.
PC	Pancreatic cancer
PI3K	Phosphatidylinositol 3-kinase
PMS2	Post-meiotic segregation increased 2
PRSS1	Serine protease 1

Q

RT-qPCR	Quantitative reverse transcriptase polymerase chain reaction

R

RAGE	Receptor for Advanced Glycation Endproducts
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
ROS	Reactive oxygen species
RRM1	Ribonucleotide reductase M1 subunit
RRM2	Ribonucleotide reductase M2 subunit

S

SEM	Standard error of the mean

SGLT	Sodium-glucose cotransporter
SHH	Sonic hedgehog
SLC	Solute carrier family
SLC1A4	Solute carrier family 1 member 4
SLC1A5	Solute carrier family 1 member 5
SLC6A14	Solute carrier family 6 member 14
SLC16A1	Solute carrier family 16 member 1
SLC16A3	Solute carrier family 16 member 3
STAT3	Signal transducer and activator of transcription 3
STK11	Serine/threonine kinase 11

T

TCA	Tricarboxylic acid
TGF-β	Transforming growth factor-β
TNF-α	Tumour Necrosis Factor alpha
TRAIL	Tumor necrosis factor–related apoptosis-inducing ligand

U

USFDA	United States Food and Drug Administration
UV	Ultraviolet

V

VEGF	Vascular endothelial growth factor

W

WNT	Wingless-related integration site

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Chapter I – Introduction

1. Pancreatic cancer

The pancreas is a gland found between the spleen and duodenum, with exocrine and endocrine functions that play an essential role in nutrient digestion and metabolism and glucose homeostasis[1].

Pancreatic cancer (PC) can be defined as a group of malignancies, most of which classify as adenocarcinomas. Neuroendocrine tumors are less common, but usually have a better prognosis than exocrine tumors[2].

1.1. Epidemiology

PC is currently the 6th leading cause of death by cancer, worldwide, in both sexes, with approximately 467 409 deaths and the 12th most incident cancer with 510 992 new cases, in 2022 (Fig. 1)[3].

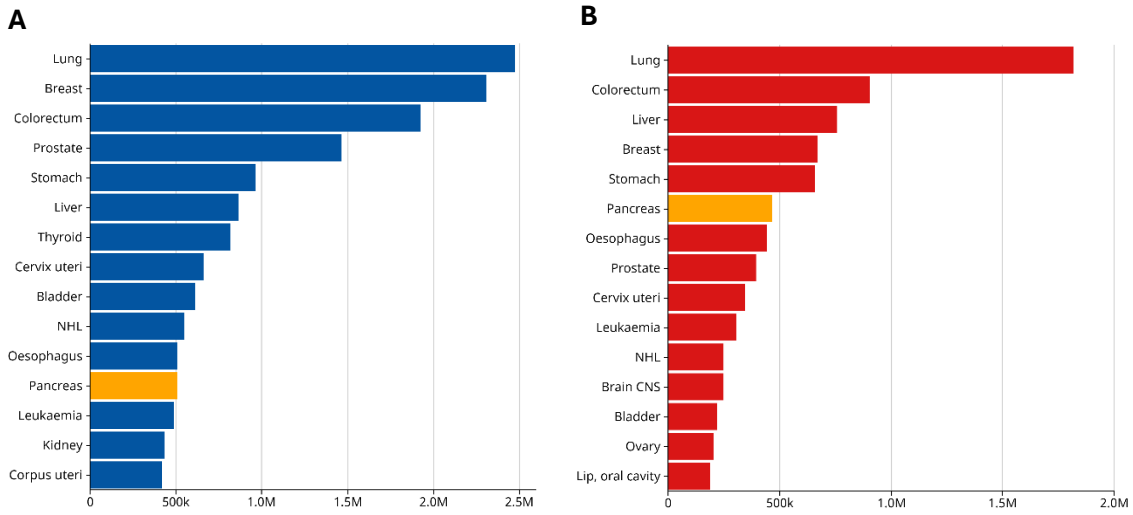

Figure 1: Bar chart representing the absolute numbers of incidence (A) and mortality (B) of the top 15 cancer sites, in 2022, for both sexes[3]. Pancreatic cancer is highlighted in both bar charts.

PC is currently one of the most lethal cancers with a 5-year survival rate of only 12% in the USA, in 2019[4]. This is mainly due to the absence of symptoms, which leads to a late diagnosis. PC incidence is expected to increase in the future due to the change in age distribution of the global population, with an increase in people with older age. Indeed, because PC rarely occurs in people before 40 years of age, population ageing is expected to lead to an increase in PC incidence[5].

The global burden of PC has been increasing rapidly throughout the last years, and this rise is expected to continue[1]. It is predicted that by 2025, PC might become the third

leading cause of death, after lung and colorectal cancer[6], surpassing esophagus, breast, gastric and liver cancers.

1.2. Etiology

PC is associated with many modifiable risk factors, although the mechanisms related to this risk increase are still relatively unknown. The strongest association with PC has been observed with chronic pancreatitis, smoking and alcohol consumption[7].

Chronic pancreatitis is an inflammatory disease that promotes the replacement of the pancreatic parenchyma by fibrous tissue, leading to a decrease in acinar and islet cells. The main factors that are known to promote this disease are smoking and alcohol drinking. It has been shown that patients with chronic pancreatitis have a significant increase in PC risk[7; 8].

Smoking has been shown to have the strongest association with PC development, with a relative risk of 2.0 to 4.0. Many studies show that smoking increases the risk of developing PC, when compared to non-smokers and to former smokers, and decreases the survival in PC patients[7; 8].

Furthermore, heavy alcohol drinking (4 or more drinks per day) has been shown to increase the risk of PC in some studies, but low and moderate alcohol drinking was not found to associate with PC. It was also found that this behavior increased PC risk in men, but showed no association in women[8]. Noteworthy, heavy alcohol drinking is related to the development of pancreatitis and diabetes mellitus, both of which are known to increase risk of PC[7].

In terms of diet, there have been many studies with inconsistent results about the effect of dietary habits on PC risk, but some studies suggest that a diet rich in carbohydrates, red meat and fats might contribute to a higher PC risk, while a diet rich in plant-based proteins, vitamins (including vitamin C, D and E), tea and coffee might have a protective effect[9].

Other modifiable risk factors include obesity, metabolic syndrome, periodontal disease, cholecystectomy, occupational exposure and infection[10].

Although lifestyle has a big impact on PC development, it is also dependent on non-modifiable factors like age, gender, race, and blood type[10].

Most of PC cancer cases occur in older people, with only approximately 10% of cases of PC diagnosed before 50 years of age[10]. This type of cancer is also more common in men than in women[11], although the incidence differences decrease with aging.

The incidence and mortality of PC has also shown differences between different ethnicities, being higher in African Americans, mainly due to being often diagnosed at advanced stages (due to socioeconomic status and increased risk behaviors, such as smoking, alcohol consumption and obesity) but also due to molecular differences between populations (higher frequency of CDKN2A and KRAS mutations). Asians have shown to be the individuals with a better prognosis and survival[10].
Blood group has also shown association with PC risk, being increased in A, B and AB blood types[12].

Although most PC are sporadic, around 10% of cases are hereditary syndromes or familiar PC cases[13]. Studies have shown that first-degree relatives of patients with PC have a 6.79-fold increase in PC risk[5].
Genes associated with PC susceptibility have also been identified, such as ATM, BRCA1, BRCA2, PALB2, STK11, CDKN2A, PRSS1 and mismatch repair genes (MLH1, MSH2, MSH6, PMS2)[5].

1.3. Symptoms and late diagnosis

PC is usually asymptomatic in early stages, although in some cases it manifests as vague and nonspecific symptoms (abdominal pain, nausea, vomiting, weight loss, jaundice, and others), which may mimic common conditions[1]. Only around 25% of PC patients show symptoms up to 6 months previous to diagnosis, while only 15% seek medical attention before this period[14].
Due to the mostly absent symptoms, this type of cancer is usually diagnosed in an advanced stage (45 to 55% of patients[15]) and has usually proliferated to other organs at the time of diagnosis[8].
Besides the absence of symptoms, PC is also difficult to diagnose in early stages due to unreliability of early tumor markers and localization of the pancreas (hidden in the retroperitoneum), which increases the difficulty in finding small resectable cancers with imaging techniques[16].

1.4. Treatment

Currently, surgery is the primary curative treatment for PC, although only 20% of PC patients present resectable disease[17]. Even though this is the only curative treatment, it presents a 5-year survival rate of 10-25% post-resection[1], high morbidity rates, and most PC patients have cancer recurrence even after tumor removal[17].

For patients with local disease, the treatment includes surgical resection, preoperative or postoperative chemotherapy, and possibly radiotherapy if necessary. For patients with borderline or locally advanced PC, treatment begins with chemotherapy, aiming to reduce the tumor size and allow surgical removal. For patients with metastatic disease, the only therapeutic options are chemotherapy and supportive care (Fig. 2)[1].

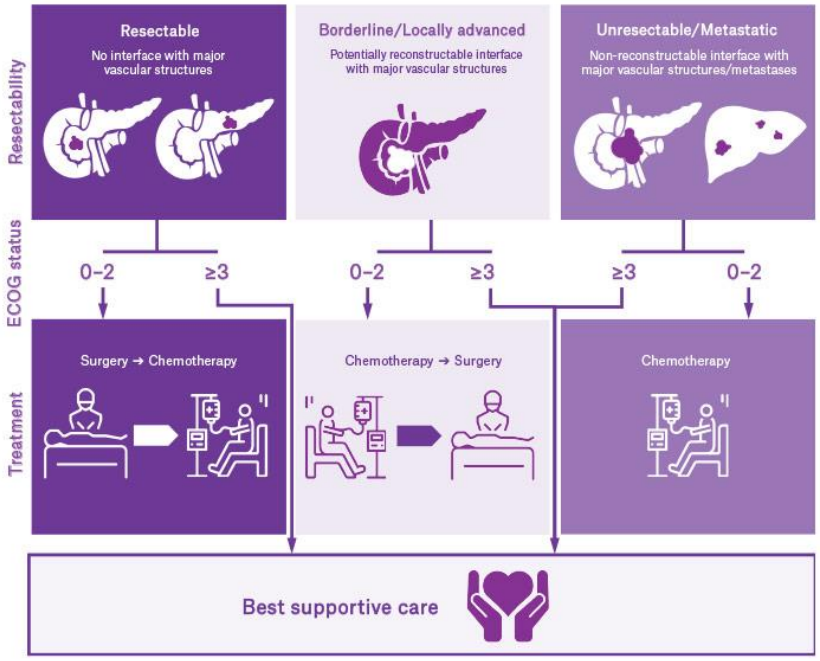

Figure 2: Treatment options for PC patients, depending on tumor resectability and ECOG (Eastern Cooperative Oncology Group) performance status of the patient[18].

There have been advances in the immunotherapy field, but it is not applicable to PC yet, due to the stromal barrier that blocks the infiltration of immune cells. Most clinical trials with immunotherapy in PC do not show promising results[19].

2. Hallmarks of cancer and cancer cell metabolism

The original hallmarks of cancer, published in 2000, consist in distinctive features that normal cells acquire allowing them to transform into cancer cells, and included the ability to resist cell death, induce angiogenesis, enabling replicative immortality, invasion and metastasis, growth suppressors evasion and sustained proliferative signaling. In 2011, the cancer hallmarks were updated ("The next generation") to include the ability to reprogram energy metabolism and evading immune destruction. In 2022, there was a latest update of these hallmarks ("New dimensions") which now include three new features: phenotypic plasticity and uninterrupted differentiation, epigenetic reprogramming and the microbiome[20; 21].

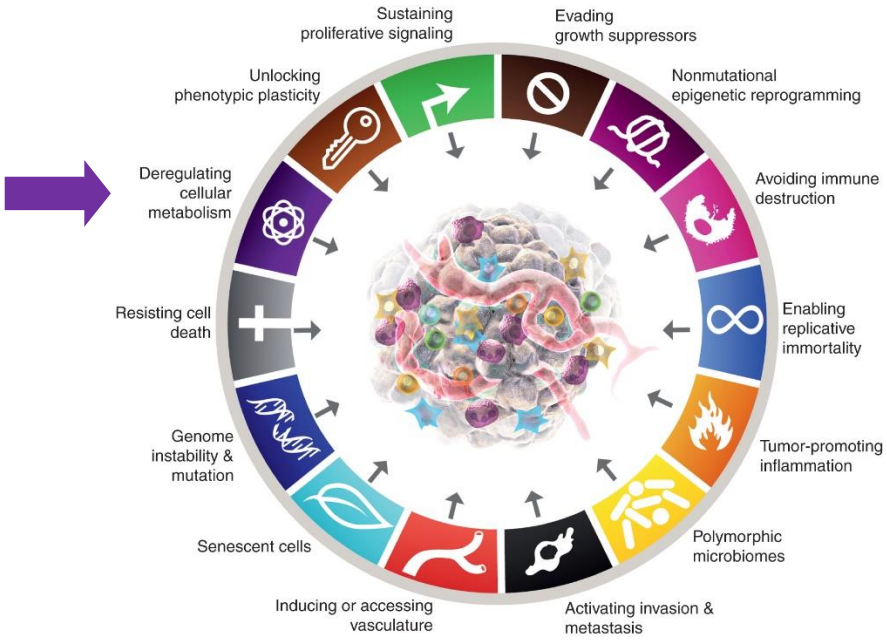

Figure 3: The hallmarks of cancer "New dimensions" (2022)[20].

As previously mentioned, one of the hallmarks of cancer included in 2011 consists in the deregulation of cellular metabolism, which is fundamental for the transformation of normal cells into cancer cells, due to the energetic requirements for increased proliferation[22].

2.1. Glucose metabolism

Glucose ($C_6H_{12}O_6$) is a sugar obtained from food and may enter the body in many different forms, such as galactose and fructose[23], and can also be released by the body through gluconeogenesis (production of glucose from non-carbohydrate components like glycerol, lactate and amino acids) or glycogenolysis (release of glucose by degradation of glycogen)[24]. This sugar is used as an essential energy source for aerobic and anaerobic

respiration and is stored in the liver and muscle tissues in the form of glycogen. Its blood concentration is mainly controlled by the pancreas, by secretion of the hormones insulin and glucagon, which promote the storage of glucose in tissues or the release of glucose to the blood, respectively[24].

In normal cells, energy can be produced from glucose in two ways: by fermentation or respiration. In both processes, glucose is converted into pyruvate by the glycolytic process, producing two ATP and NADH molecules in the process. Cells undergo fermentation mainly in anaerobic situations, like hypoxia. In this process, pyruvate is reduced into lactate, which is then secreted by the cells. This process results in the production of two ATP and two lactate molecules per molecule of glucose, and also regenerates the NAD^+ necessary for glycolysis. In respiration, pyruvate is transported into the mitochondria, where it is used in the oxidative phosphorylation (OXPHOS) process, producing higher amounts of energy (approximately 36 molecules of ATP per molecule of glucose)[25]. This process is the main source of energy production from glucose, accounting for 70% of the produced ATP[22].

2.1.1. Warburg Effect

In contrast to normal cells, cancer cells suffer a metabolic switch, known as the Warburg Effect, in which the cells use glycolysis with lactic acid production as the main energy production mechanism, even in the presence of oxygen (aerobic glycolysis)[22].

When compared to OXPHOS, glycolysis is an inefficient way to produce ATP, with lower ATP production per unit of glucose. However, glycolysis occurs 10 to 100 times faster than the complete OXPHOS process. With this, cells with a faster rate of ATP synthesis, but a lower yield may have a selective advantage when competing in the same environment[26].

It is also important to note that in aerobic glycolysis, most of the excess carbon from glucose is excreted as lactate. This can be quite beneficial for the cancer cells, since this process, as mentioned previously, not only regenerates the NAD^+ needed for glycolysis[26], but also leads to lactate accumulation in the tissue microenvironment, resulting in enhanced invasiveness[13; 27].

The remaining carbon excess can also be used for the *de novo* production of lipids, nucleotides and proteins[26].

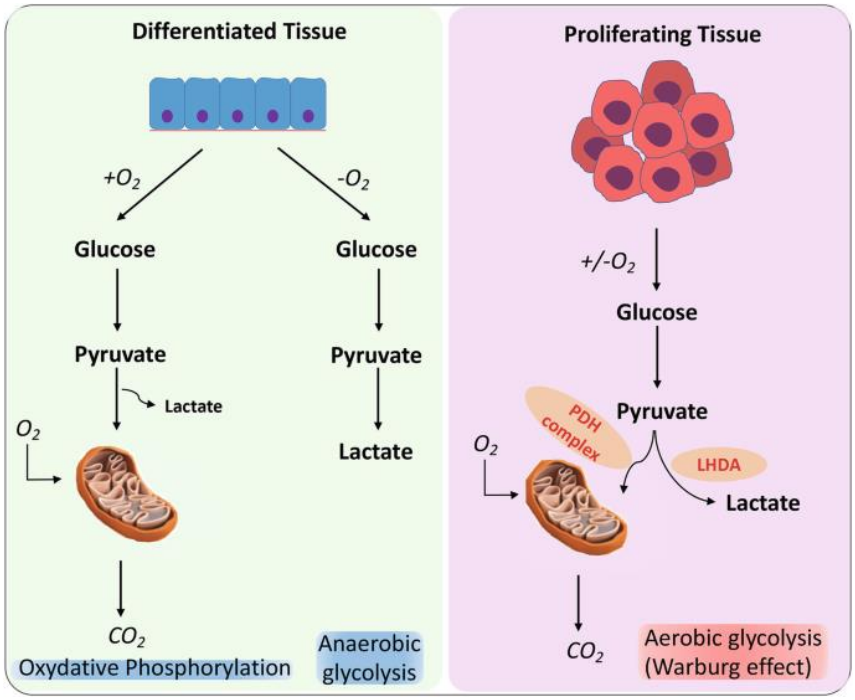

Figure 4: Warburg Effect and differences in the glucose usage between normal and cancer cells[28].

2.1.2. Glucose transport proteins

Glucose is a hydrophilic molecule, so it is not able to travel freely along the cellular membranes. Thus, transmembrane transporters are required for glucose cellular uptake[29]. These transporters are mainly divided into two categories: sodium-dependent (SGLT) and sodium-independent transporters (GLUT)[30]. SGLTs are able to transport glucose actively into the cells, against a concentration gradient, while GLUTs transfer glucose along a concentration gradient[29].

The GLUT family is composed for 14 members, but the most important and better-known glucose transporters are GLUT1-5[31].

Transporter	Location	Properties
(A)		
GLUT1	Almost universal	High capacity, relatively low K_m
GLUT2	Liver, β-cells, hypothalamus, basolateral membrane of small intestine	High capacity but low affinity (high K_m 15–20 mM); part of the glucose sensor in β-cells. Carrier for glucose and fructose in liver and intestine
GLUT3	Neurons, placenta, testes	Low K_m (1 mM) and high capacity
GLUT4	Skeletal and cardiac muscle, fat	Activated by insulin, K_m 5 mM
GLUT5	Mucosal surface in small intestine, sperm	Primarily fructose carrier in intestine

Figure 5: Main glucose transporters from the GLUT family, its location and properties[31].

GLUT1 is known to be the most widely expressed glucose transporter, maintaining the basal glucose transport in most cells. Its overexpression has been reported in many cancer cells, including breast, esophageal, lung, oral squamous cell carcinoma, rectal and pancreatic cancer, being associated with poor survival[32].

In PC, an overexpression of *GLUT1* was observed, but it depended on size, stage and cancer type. *GLUT2* is also overexpressed in malignant PC, while downregulated in neuroendocrine tumors (NETs). GLUT4 is also detected in PC, but it is underexpressed in these cells in comparison with non-PC cells[33].

2.2. Glutamine metabolism

Glutamine ($C_5H_{10}N_2O_3$) is the most abundant non-essential amino acid in our bloodstream, and even though glucose is the main fuel for most cells in the body, the rate of glutamine consumption is close to or higher than glucose[34]. Because of its high demand in the body, glutamine is known as a conditionally essential amino acid[35], meaning it is considered a non-essential amino acid, except when endogenous synthesis does not meet the metabolic needs of the body[36].

In normal cells, glutamine is converted to glutamate, by the enzyme glutaminase (GLS), and is then converted to α-ketoglutarate, by transamination, entering the tricarboxylic acid (TCA) cycle for energy production (Figure 6)[37]. Besides the production of ATP, glutamine is also used for nucleotide and amino acid synthesis, pH and redox balance, as well as signaling[38].

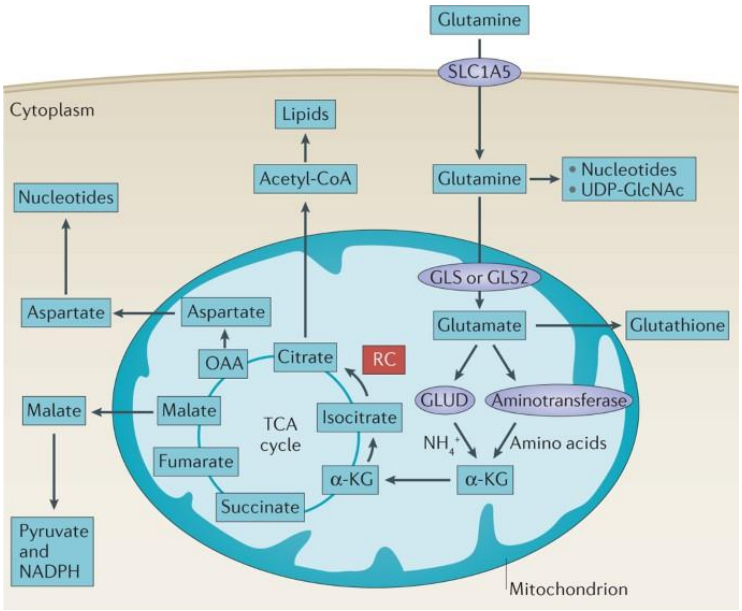

Figure 6: Glutamine metabolic fate in normal cells[37].

2.2.1. Glutamine addiction in cancer cells

Glutamine is a very important amino acid for cancer cells, being mainly used as a carbon and/or nitrogen source for biosynthetic reactions[35]. Some cancer cells are known to

increase their glutamine uptake and become dependent on this amino acid, which is also known as glutamine addiction[38].

Besides the increase in glutamine uptake, cancer cells are also able to synthesize glutamine from ammonia and glutamate, in nutrient deficiency situations[39].

In PC patients, particularly in patients who undergo chemotherapy, glutamine metabolism is very important since it can directly induce chemoresistance in tumor cells, leading to treatment failure[35].

2.2.2. Glutamine transport proteins

Glutamine is a hydrophilic molecule, and so, similarly to glucose, cannot diffuse into the cells[40]. Therefore, glutamine is transported into the cells through membrane transporters. In total, there are 14 glutamine transporters, belonging to four gene families: SLC1, SLC6, SLC7 and SLC38[35].

Gene name	Protein name	Amino acid substrates	Direction of glutamine flux
SLC1A5	ASCT2	Neutral	Influx/Efflux
SLC6A14	$ATB^{0,+}$	Neutral & Cationic	Influx
SLC6A19	B^0AT1	Neutral	Influx
SLC7A5	LAT1	Neutral	Influx/Efflux
SLC7A6	y^+LAT2	Neutral & Cationic	Influx
SLC7A7	y^+LAT1	Neutral & Cationic	Influx
SLC7A8	LAT2	Neutral	Efflux
SLC7A9	$b^{0,+}AT$	Neutral & Cationic	Efflux
SLC38A1	SNAT1 (SA2)	Neutral	Influx
SLC38A2	SNAT2 (SA1)	Neutral	Influx
SLC38A3	SNAT3 (SN1)	Neutral	Influx/Efflux
SLC38A5	SNAT5 (SN2)	Neutral	Influx/Efflux
SLC38A7	SNAT7	Neutral & Cationic & Anionic	Influx
SLC38A8	SNAT8	Neutral, Cationic & Anionic	Influx

Figure 7: Glutamine transporters in mammalian cells[40].

ASCT1 (SLC1A4) and ASCT2 (SLC1A5) are sodium-coupled transporters for neutral amino acids, selective for alanine, serine, cysteine, threonine, and glutamine. ASCT2 is one of the main glutamine transporters in cancer cells, being overexpressed in several cancers (breast, colon, lung, melanoma, neuroblastoma, glioblastoma and prostate cancer)[35; 40], including PC. It was also demonstrated that the glutamine transporter SLC6A14 ($ATB^{0,+}$) is overexpressed in PC as well[35].

2.3. Lactic acid/Lactate metabolism

Up to recently, lactate or lactic acid was manly known as a waste product of the glycolytic metabolism and its accumulation was often associated with inflammatory diseases and

cancer. Lactate was mainly associated with hypoxic conditions because in the absence of oxygen, the mitochondria can no longer produce energy, leaving fermentation as the only metabolic option for the cells[25].

However, more recently, it has been shown that, independently of oxygen availability, lactate is an inevitable product of glycolysis in many non-tumor cells and in a wide range of noncancerous diseases, such as pulmonary hypertension, pulmonary fibrosis, heart failure, atherosclerosis, and polycystic kidney disease[41].

Lactate can also be repurposed to produce more glucose, through the process of gluconeogenesis in the liver during high energy consumption times (e.g. intense physical activity)[41].

Recently, lactate has been recognized as an important part of glucose metabolism, and not just a waste product of this process. Not only is the concentration of lactate in circulation higher than any other substance used for energy production, but it has also been shown that lactate has direct function in organs, being crucial for the production of energy, acting as the main fuel for the TCA cycle[41].

2.3.1. Lactate shuttle in cancer cells

As previously mentioned, glycolysis is increased in cancer cells in aerobic conditions, leading to an increase in lactate production (Warburg effect). The fast production of lactate was the first molecular phenotype associated with cancer[25].

In the tumor microenvironment, lactate is manly produced and exported by hypoxic cancer cells, and can then absorbed by normoxic cancer cells, which convert this nutrient into pyruvate, through the lactate dehydrogenase B (LDH-B) enzyme, feeding the TCA cycle. This process is also known as the lactate shuttle (Figure 8). This not only increases the energy produced, but also protects the cancer cells, since the accumulation of lactate is toxic and can lead to lactic acidosis[41].

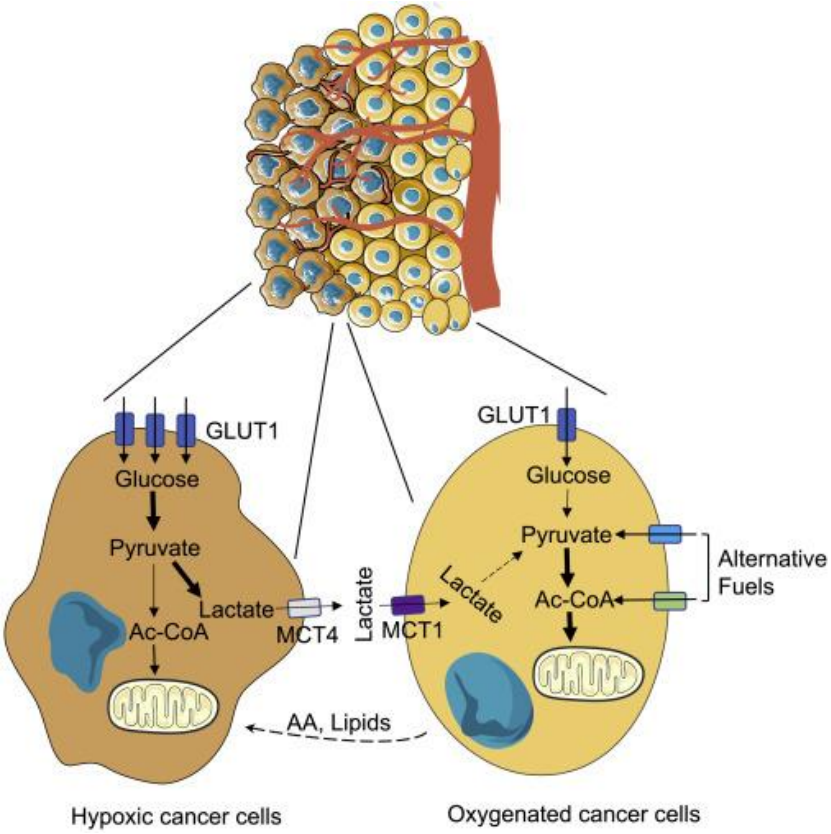

Figure 8: Metabolic Symbiosis between hypoxic and oxygenated cancer cells[42].

2.3.2. Lactate transporter proteins

Lactate is exported from and imported to the cells by using the monocarboxylate transporters (MCT) from the SLC16 family. In total, there have been 14 MCTs identified, with MCT1-4 being the most explored and characterized[43]. These transported allow the bidirectional transport of L-lactate, pyruvate and other monocarboxylates through the membranes[44].

MCT1 (SLC16A1) is found in most tissues[43], being upregulated in musculoskeletal and cardiac cells in response to the increased glycolysis in these cells[44].

MCT4 (SLC16A3) is the MCT with lowest affinity for lactate, being mainly used for the export of lactic acid from glycolytic cells[45].

In cancer cells, lactate produced by hypoxic cells is exported by the MCT4 transporter, and then incorporated into oxygenated cancer cells by the MCT1 transporter[41]. Therefore, tumor usually have overexpression of *MCT1* and *MCT4* transporters. Moreover, tumor cells may also overexpress *MCT2*, which normally is mainly expressed in the liver, where it promotes gluconeogenesis from lactate (Cori cycle), and in the kidney, for the excretion of lactic acid[45].

In PC, MCT1 and MCT4 have been correlated to cancer invasion, while MCT4 is also associated with cancer cell migration[46].

3. Dietary Bioactive Compounds

Currently, there is no agreement as to the definition of the term “bioactive compound”, being considered that it includes compounds that are able to interact with living tissues, with a wide range of possible effects[47]. Dietary bioactive compounds are mainly present in plant-based foods, including fruits, vegetables and whole grains[48].

More than 5000 dietary bioactive compounds have been isolated, including a wide variety of classes of compounds including alkaloids, nitrogen-containing compounds, organosulfur compounds, phenolics, phytosterols and carotenoids[49]. These have shown multiple health benefits (including autoimmune, cardiovascular, cerebrovascular, and neurodegenerative diseases, as well as metabolic syndrome and cancer[48]) possibly due to their antioxidant ability, allowing to balance the oxidative stress that promotes the development of many chronic diseases[50].

3.1. Carotenoids

Carotenoids are red, orange and yellow pigments, being one of the most common pigments in nature. They are produced by both photosynthetic and non-photosynthetic organisms, like plants, algae, fungi and bacteria. Although animals are not able to produce carotenoids (except spider mites, gall midges and pea aphids[51]), they obtain them by ingestion[52].

The most common carotenoids in human serum are α-carotene, β-carotene, β-cryptoxanthin, lycopene, lutein and zeaxanthin[53].

Despite the fact that carotenoids are very important for a well-balanced diet, they are not considered essential nutrients, since their absence is not directly correlated to a specific disease[54].

Their structure contains 15 conjugated double bonds, and they can be divided in two main classes: carotenes (pure hydrocarbons) and xanthophylls, which are composed by one or more oxygenated groups (epoxy, keto, carboxyl, hydroxyl and methoxy), being also known as oxygenated carotenoids[52].

In this project we are going to treat PC cells with a carotene – β-carotene – and three xanthophylls – astaxanthin, crocin and fucoxanthin.

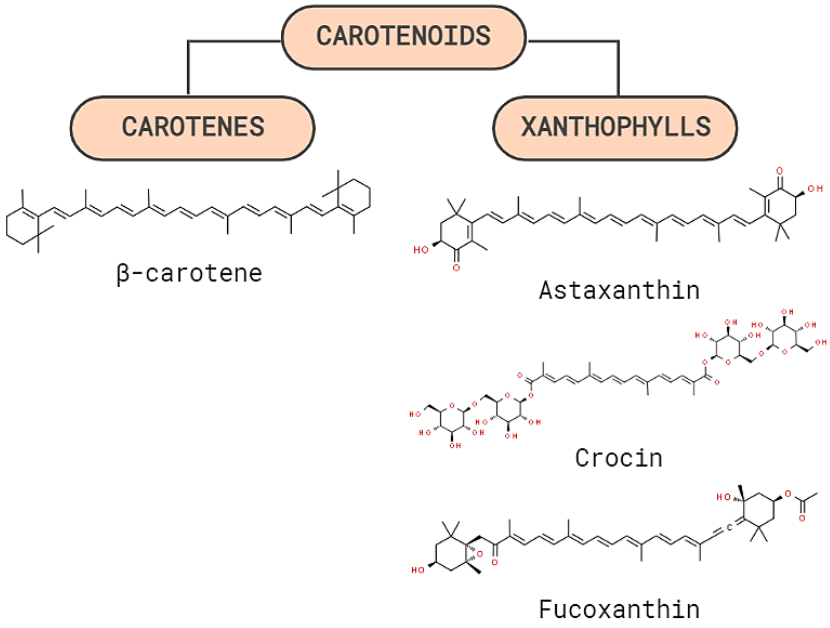

Figure 9: Representative image of the carotenoids involved in this project, with their respective chemical structure and classification (carotenes or xanthophylls). Figure created with Biorender. Chemical structures obtained from ChemSpider.

Due to their anti-oxidant activity, many carotenoids have shown important pharmacological and biological activities, such as anti-aging, osteo-protective, anti-diabetic, anti-inflammatory and anti-oxidant, cardio-protective, pro-vitamin and anticancer[52].

Most studies (epidemiological and experimental) suggest that carotenoids have a protective effect in various types of cancer. These compounds were shown to promote apoptosis, blockade of the cell cycle and metastasis, by modulation of oncogenic and anti-oncogenic proteins[51].

The main explored mechanisms of action of carotenoids are through PI3K/Akt pathway inhibition, WNT/B-catenin pathway modulation, suppression of metalloproteinases (MMPs) and suppression of TNF-α, NF-κB and VEGF[55].

3.1.1. Astaxanthin

Astaxanthin is an orange-red xanthophyll carotenoid with a structure alike β-carotene and lycopene, found in marine ecosystems, particularly in shrimp, salmon, lobster, and other, and gives them their pink or orange color by accumulation in body tissues[56]. Although these marine animals do not have the ability to synthesize astaxanthin, they obtain this

carotenoid from zooplankton (algae and microbes)[57]. The source with highest production of astaxanthin is the microalgae *Haematococcus pluvialis*[56].

This carotenoid was initially approved by the United States Food and Drug Administration (USFDA) in 1987, being used in aquaculture[58]. Currently, astaxanthin is also used in food, cosmetics and in the nutraceutical industry, showing to be safe for human use[57].

Astaxanthin has been shown to have antioxidant, anti-inflammatory, antiaging and anticancer effects[56; 57].

Astaxanthin is severely under researched in PC. To the best of my knowledge, the only evidence of its effect in PC showed that *in vitro* astaxanthin increases cell death in PC cells (Panc-1 and HTB-79) resistant to gemcitabine, by enhancing hENT1 and decreasing RRM1/2 expression, leading to an increase in sensitivity to gemcitabine. *In vivo*, the same study showed that astaxanthin induced apoptosis in a gemcitabine-resistant mouse model, in accordance with the previous *in vitro* results[59].

3.1.2. β-carotene

β-carotene is a carotenoid, particularly a terpenoid, with provitamin A properties. By oxidative cleavage, it is converted into 2 molecules of vitamin A. This vitamin is essential for various body functions, including normal development, vision and immune function[60].
This carotenoid is found in a wide variety of fruits and vegetables, giving them an orange-yellow color. The foods with highest β-carotene concentration are carrot juice, squash, spinach and orange sweet potatoes[61]. Most people consume a daily dose of β-carotene of approximately 1-2 mg, but this intake varies widely in the population[60].

β-carotene, like most carotenoids, has antioxidant properties which might contribute to a decrease in chronic disease risk, such as cancer, diabetes and cardiovascular diseases. This carotenoid is also detected in the skin, absorbing UV light and protecting against damages caused by photooxidation[62].

To the best of our knowledge, there are no published articles testing β-carotene in PC cells, but it was tested in hamster models for pancreatic carcinogenesis. It was found that β-carotene promoted a reduction in hyperplasia areas, carcinomas and total ductal lesions, at a concentration of 25 ppm, which corresponds to 0.3 mg per day[63]. Other researchers have found that β-carotene promoted a significant decrease in the number of

early ductal complexes and in the number of carcinomas, when the animals were supplemented with 56 mg/kg of β-carotene[64].

3.1.3. Crocin

Crocins are a family of carotenoids composed of mono- or di-glycosyl polyene esters of crocetin. They are mainly found in the stigmas of *Crocus sativus*, mainly known as saffron, being its main color pigment[65].

It has been used as a spice and food colorant and has been shown to be safe in pharmacological doses, causing no damage to organs[65].

Crocin has shown to have antioxidant, antidepressant, anti-ischemia and anticancer effects, as well as protective effects in the kidney, heart and skeletal muscle[66].

In PC BxPC-3 cells, crocin was shown to decrease cell viability and to induce apoptosis and cell cycle arrest[67]. In another study, it was shown to inhibit lipid peroxidation, decrease cell viability and induce apoptosis in BxPC-3 and Capan-2 cells[59]. Finally, *in vivo*, crocin promoted a 52% reduction in the pancreatic tumor volume in female athymic nude mice (NCR nu/nu)[68].

3.1.4. Fucoxanthin

Fucoxanthin is a marine xanthophyll carotenoid, present in macro and microalgae chloroplasts[69; 70]. This carotenoid is particularly abundant in brown seaweeds and is used in herbal medicines and dietary supplements, worldwide[69]. Fucoxanthin is one of the most abundant carotenoids, accounting for more than 10% of total carotenoid in nature[71].

In humans, fucoxanthin is metabolized mainly into fucoxanthinol, as this is the only metabolite found in plasma (although it might be converted into other metabolites inside organs and tissues)[70].

Fucoxanthin has shown anti-inflammatory, antioxidant and anticancer effects, and protection against cardiac, diabetic, hepatic, kidney, microbial, nervous system, obesity and respiratory diseases[69].

In PC, fucoxanthin promoted a decrease in cell viability, in combination with the chemotherapeutic drug gemcitabine, showing a synergetic effect in the MIA PaCa-2 cell line[72]. *In vivo*, this carotenoid decreased the pancreatic tumor size in C57BL/6J mice, mainly by the attenuation of BTLA and the CCL21/CCR7 axis[73]. Fucoxanthinol, the main fucoxanthin metabolite[70], induced apoptosis in PC cell lines KMPC44[74] and DLD-1 cells[75], promoted a decrease in cell growth in KMPC44 cells, suppressed the FAK/Paxillin,

PI3K/Akt and MAPK signaling pathways[74], while stimulating the growth of PANC-1 cells[75]. However, it is important to note that the cytotoxicity results from the study with PANC-1 cells are not fully trustworthy, since the MTT assay was used without accounting for the natural color of fucoxanthin, which might hinder the results of this colorimetric assay.

3.2. Polyphenols

Polyphenols are secondary metabolites produced by plants in response to stress and pathogens. These are mainly present in fruits, vegetables, cereals, coffee, tea, cocoa and others[76], with an average consumption of approximately 1g/day per person[77].

The structural characteristic of polyphenols is the presence of one or more phenolic units, which can be associated with several carbohydrates or organic acids. These compounds are mainly divided into two major categories: flavonoids (containing two phenol rings connected by an oxygenated heterocycle) and non-flavonoids (containing only one phenol ring). Flavonoids are then classified into the subclasses flavonols, flavones, flavanols, flavanones, isoflavanones and anthocyanidins, and the non-flavonoids into phenolic acids, lignans and stilbens, according to their molecular structure[76; 78].

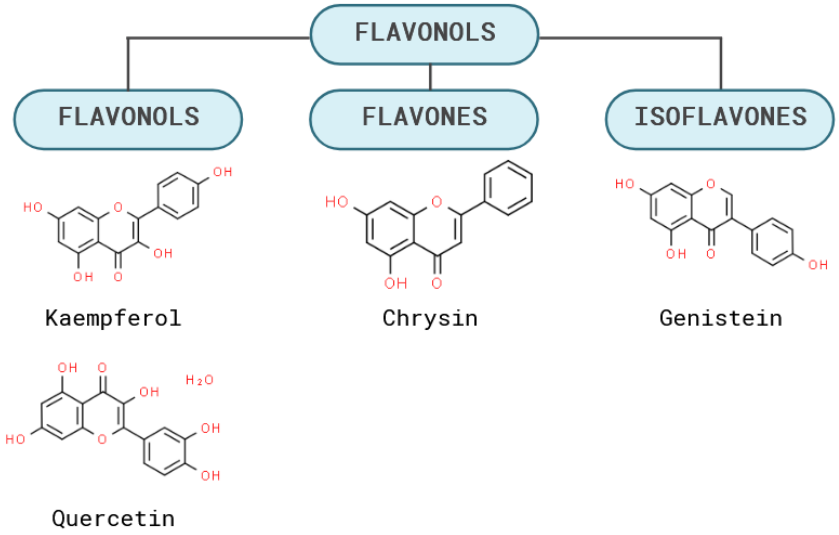

Figure 10: Representative image of the polyphenols involved in this study, with their respective chemical structure and classification (flavonols, flavones or isoflavones). Figure created with Biorender. Chemical structures obtained from ChemSpider.

In this project we are going to treat PC cells with the flavonoids chrysin (a flavone), genistein (an isoflavone), kaempferol and quercetin (two flavonols).

Polyphenols have been shown a protective effect against cardiovascular diseases, diabetes, lung damage, gastrointestinal problems, pancreatitis, neurodegenerative diseases and some cancers[79].

The main mechanism of polyphenols is the inhibition of reactive oxygen species generation and lipid peroxidation, leading to a reduction in oxidative stress. Polyphenols

also act against cancer through inhibition of NF-κB transcription factor (responsible for inflammation regulation and cell growth and survival), inhibition of pro-inflammatory enzymes (such as COX2, that increases cell proliferation and angiogenesis) and modulation of PI3K/Akt activity[55].

3.2.1. Chrysin

Chrysin (5,7-dihydroxyflavone) is a flavone mainly found in honey and propolis, but also in some fruits and plants used in traditional medicine[80].
In humans, its main metabolites are chrysin-7-glucuronide and chrysin-7-sulfate. Despite chrysin being a compound with low bioavailability, with a peak plasma concentration of 3-16 ng/mL, its metabolites, particularly chrysin-7-sulfate, can reach plasma concentrations of 450-4220 ng/mL[81].

Chrysin has shown various biologic effects, including antioxidant, anti-anxiety, antiviral, hepatoprotective, neuroprotective and anticancer[80].

In PC, chrysin was shown to decrease *in vitro* cell viability of various PC cell lines[82; 83], to increase cancer cell sensitivity to gemcitabine (both *in vitro* and *in vivo*)[83] and to induce apoptosis in the MIA PaCa-2 cell line[82]. *In vivo*, this polyphenol induced pancreatic tumor growth reduction in a mouse xenograft model[82].

3.2.2. Genistein

Genistein (4', 5, 7-trihydroxyisoflavone) is an isoflavone, present in broccoli, cauliflower, clover sprouts, and mainly in soy-containing foods (soy powder, soy milk, tofu and soy beans and nuts). The foods with the highest content in genistein are fermented foods like miso and natto[84].
Genistein is extensively metabolized in humans, with a plasma level of total genistein in the micro molar range. The main metabolites of genistein are glucuronides (78%) and sulfates (20%), with main accumulation in the gastrointestinal tract and liver[85].

This compound, as much other natural bioactive compounds, has shown antioxidant, anti-inflammatory, antibacterial and antiviral, antidiabetic and anticancer properties[84].

In relation to PC, a few studies were previously conducted. Genistein was shown to decrease cell growth[86] and induce apoptosis, in various PC cell lines (PANC-1, Mia-PaCa2, AsPC-1, BxPC-3, PANC-28 and others)[87-91]. It was also shown to promote a decrease in colony formation[88; 89] and cell migration[86-88], as well as a decrease in NF-κB

DNA binding[90; 91], even though Mouria *et al.* observed that genistein had no effect on NF-κB activation in the Mia-PaCa2 cell line[92].

Treatment with genistein resulted in a decreased expression of miR-223 in gemcitabine-resistant AsPC-1 and BxPC-3 cells, reverting the epithelial-to-mesenchymal phenotype and improving chemotherapeutic drug sensitivity[86], as well as an increase in miR-34a in the AsPC-1 and Mia-PaCa2, which promoted cell growth suppression by downregulation of the Notch-1 signaling pathway[88]. This effect in Notch-1 was also reported by Wang *et al.* in the BxPC-3 cell line[89].

Additionally, genistein was found to induce some other effect in PC cell lines, such as downregulation of FOXM1[89], inhibition of the STAT3 pathway, as well as some targets of this pathway (survivin, cyclin D1 and ALDH1A)[87], reduction in MMPs[87; 89] and VEGF[89], and promoted mitochondrial apoptosis, mediated by ROS[87].

3.2.3. Kaempferol

Kaempferol (3,4',5,7-tetrahydroxyflavone) is a yellowish flavonoid found in many edible plants, such as tea, cabbage, broccoli, and kale, and also in plants used in traditional medicine[93; 94]. This compound is absorbed in the small intestine, suffering metabolic transformations in the liver. Its main metabolites are glucuronides and sulfoconjugates, mainly known as kaempferol glycosides[95].

Kaempferol and its metabolites have shown antioxidant, antidiabetic, anti-inflammatory, anti-microbial, neuro and cardio protective, and anticancer properties[94].

In relation to PC, kaempferol induced apoptosis in the MIA PaCa-2, PANC-1 and BxPC-3 PC cell lines[96-99], and inhibited cell growth and proliferation of the same cell lines and also of the SNU-123 cell line[97]. It was also shown to inhibit migration, invasion[97] and colony formation *in vitro* (PANC-1, MIA PaCa-2 and BxPC-3 cell lines) [98; 99]. The mechanism of action of kaempferol in PC is still unknown, but it has been shown to inhibit the Akt, ERK1/2 and Scr signaling pathways[97-99].

In vivo, kaempferol (100 mg/kg) decreased pancreatic tumor growth (including tumor weight and volume)[98].

3.3.4. Quercetin

Quercetin (3,3′,4′5,7-pentahydroxyflavone) is a flavonol naturally occurring in many fruits and vegetables, such as apples, cherries, onions, peppers, but also in other foods like wine and green tea, being mostly present as quercetin glycosides. The main source of quercetin in the human diet is onions, which contain quercetin-3,4'-diglucoside and

quercetin-4'-glucoside[100]. The average daily amount of quercetin ingested by humans is 25 mg, but this flavonol has a low bioavailability due to its limited water-solubility[101]. Quercetin is also used as a dietary supplement, mainly as quercetin aglycone with recommended daily doses of up to 1 g, but most commonly 500 mg[100].

Quercetin has shown antioxidant, anti-inflammatory, anticancer and gastroprotective effects, while also modulating the activity of various enzymes and promoting the decrease in apoptosis[102].

In PC, quercetin promoted a decrease in cell proliferation or growth in various PC cell lines[92; 103-111], in a concentration- and time-dependent manner[112-114]. *In vivo*, studies have also shown reduction in tumor cell proliferation and tumor growth[92; 107; 109; 112; 115]. However, some studies showed no effect of quercetin on PC cell proliferation (in MIA PaCa-2, CFPAC-1, Panc-1, and SNU-213 cells)[111; 116].

In terms of apoptosis, quercetin induced this process in PANC-1, PATU8988, BxPC-3 and MIA PaCa-2 cells, in various studies[104; 108; 112; 114; 115], while having no effect on PATU8988 cells[117]. *In vivo*, quercetin induced apoptosis in the pancreatic tumor tissue of mice[92; 112; 115]. Quercetin was also shown to promote an increase in activity of apoptosis-related proteins like caspase-3[92; 110] and 9, Bak[118] and PARP[92], while decreasing the activity of anti-apoptotic proteins like Bcl-XL[118].

As for cell cycle, quercetin was shown to arrest cell cycle on G2/M phase[115] (in BxPC-3 and MIA PaCa-2 cells) and on S-phase, while also promoting a decrease in cyclin D1 and an increase in p53 (in PANC-1 cells)[114].

Quercetin also promoted a decrease in cell migration and invasion in vitro[105; 106; 108; 112; 113; 115], and a reduction in metastasis development *in vivo*[112]. This effect might be related to the ability of quercetin to decrease MMPs expression (particularly MMP2 and MMP7), but also to an inhibitory effect on STAT3 pathway[105].

It is still unknown how quercetin acts in PC, but it has been shown that this flavonol acts as an antagonist of the sonic hedgehog (SHH) signaling and TGF-β/Smad signaling, leading to inhibition of the epithelial-mesenchymal transition (EMT), and to a decrease in cell proliferation, migration and invasion[112]. It has also been shown to activate TRAIL-induced apoptosis via JNK activation[117].

Additionally, it was demonstrated that quercetin promoted a decrease in IL-6, IL-8[119], RAGE[104] (associated with inflammation) and LC3-II protein expression[110] (associated with autophagy), and decreased cancer stem cell markers (particularly EpCAM, Nanog and Twist2)[107]. Quercetin also inhibited NF-κB[92; 115], and suppressed hnRNPA1[103] (associated

with tumor progression). Finally, one of quercetin metabolites – quercetin 3-O-glucoside – caused inhibition of the ERK signaling pathway[106] and EGFR signaling pathway[111].

Chapter II – Aims

Considering the importance of metabolic reprogramming in cancer cells and the burden of PC, the aim of this project was to determine the effect of various carotenoids and polyphenols upon nutrient uptake by PC cell lines.

Thus, our specific aims were to:

1. Determine the effect of the carotenoids astaxanthin, β-carotene, crocin and fucoxanthin on the uptake of glucose, lactic acid and glutamine, in two PC cell lines (AsPC-1 and PANC-1).

2. Determine the effect of the polyphenols chrysin, genistein, kaempferol and quercetin on the uptake of glucose, lactic acid and glutamine, in two PC cell lines (AsPC-1 and PANC-1).

3. Determine the effect of selected carotenoids/polyphenols on nutrient transporter gene expression (*GLUT1*, *ASCT2* and *MCT1*) by RT-qPCR.

4. Evaluate the cytotoxic effect of selected carotenoids/polyphenols using the MTT assay.

5. Investigate inhibition of nutrient uptake as a potential mechanism of action involved in the cytotoxic effect of the selected carotenoids/polyphenols, using nutrient transport protein inhibitors (BAY-876, GPNA and NPPB).

Chapter III – Material and Methods

1. Materials

^{3}H-2-deoxy-D-glucose (^{3}H-DG; specific activity 60 Ci/mmol); ^{3}H-lactic acid (specific activity 15 Ci/mmol); ^{3}H-L-glutamine (L-[2,3,4-^{3}H]; specific activity 60 Ci/mmol) (American Radiolabeled Chemicals Inc., St Louis, MO, USA). Chloroform; 2-propanol; D-glucose; potassium dihydrogen phosphate (KH_2PO_4); sodium bicarbonate ($NaHCO_3$); Triton X-100; (Merck, Darmstadt, Germany). Dipotassium phosphate (K_2HPO_4) (BDH Laboratory Supplies, Poole, UK). DMEM medium (PAN-Biotech, Aidenbach, Germany). L-γ-Glutamyl-p-nitroanilide hydrochloride (GPNA) (ChemCruz, Santa Cruz Biotechnology, Dallas, TX, USA). NYZol (NZYtech, Lisbon, Portugal). qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA). BAY 876 (Tocris Bioscience, Bristol, UK). KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB); antibiotic/antimycotic solution (100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B); astaxanthin; β-carotene; chrysin; crocin; diethyl pyrocarbonate (DPEC); dimethyl sulfoxide (DMSO); fetal bovine serum (FBS); fucoxanthin; genistein; kaempferol; L-glutamine; N-2 hydroxyethylpiperazine-N0-2-ethanesulfonic acid (HEPES); quercetin; RPMI 1640 medium; sodium pyruvate; trypsin–ethylenediaminetetraacetic acid (EDTA) solution (Sigma, St. Louis, MO, USA). DNAse I, RNAse H (Thermo Fisher, Waltham, MA, USA).

2. Cell culture

The AsPC-1 cell line is a PC cell line with high metastatic potential, initiated from cells derived from ascites of a 62-year-old woman with PC[120].

The PANC-1 cell line is a PC cell line with low metastatic rate, isolated from the pancreatic duct of a 56-year-old man with adenocarcinoma[121].

Both cell lines were obtained from American Type Culture Collection (ATCC) and used between passages 7-35 (AsPC-1) and 13-26 (PANC-1).

Cells were maintained in a humidified atmosphere of 95% air and 5% CO_2. Culture medium was changed every 2-3 days, and the culture split every 5-6 days in a 1:3 ratio (AsPC-1 cell line) or every 4-5 days in a 1:4 ratio (PANC-1 cell line).

AsPC-1 cells were grown in RPMI 1640 medium supplemented with 10 mM sodium bicarbonate, 2 mM L-glutamine, 0.01 mM sodium pyruvate, 25 mM HEPES, 10% heat-inactivated FBS and 1% antibiotic/antimycotic solution.

PANC-1 cells were grown in DMEM medium with 4.5 g/L glucose, supplemented with 2 mM L-glutamine, 10 mM sodium bicarbonate, 10% heat-inactivated FBS and 1% antibiotic/antimycotic solution.

For sub-culturing, cells were removed enzymatically (0.25% trypsin-EDTA, 3 min at 25°C, 5 min at 37°C for AsPC-1 and 2 min at 25°C, 2 min at 37°C for PANC-1) and sub-cultured in new plastic flasks (25 cm^2; TPP®, Trasadingen, Switzerland).

For nutrient uptake assays, cells were seeded on 24-well culture plates (2 cm^2; Ø 16 mm; TPP®) and used at 100% confluence (Fig. 11 and 12). For RNA extraction, cells were seeded on 21 cm^2 plates (TPP®) and were also used at 100% confluence. For the MTT assay, cells were seeded on 96-well culture plates (0.32 cm^2; Ø 6.4 mm; TPP®) and used at 90% confluence.

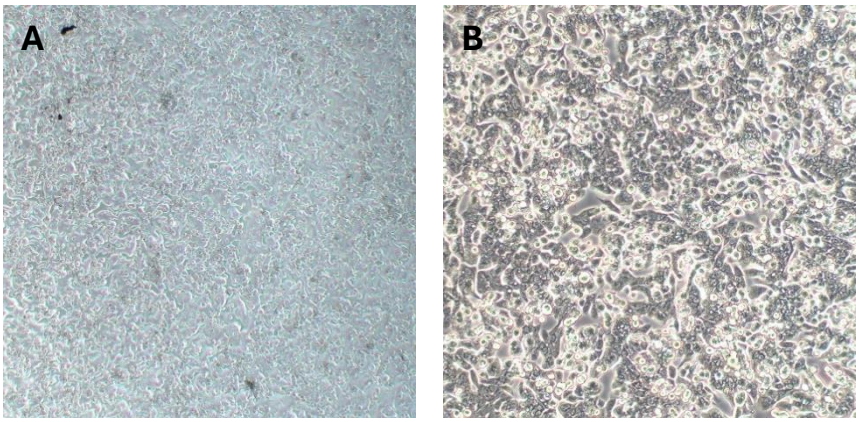

Figure 11: Representative microscope images of AsPC-1 flasks with 100% confluence, taken with 4x (A) and 10x (B) magnification.

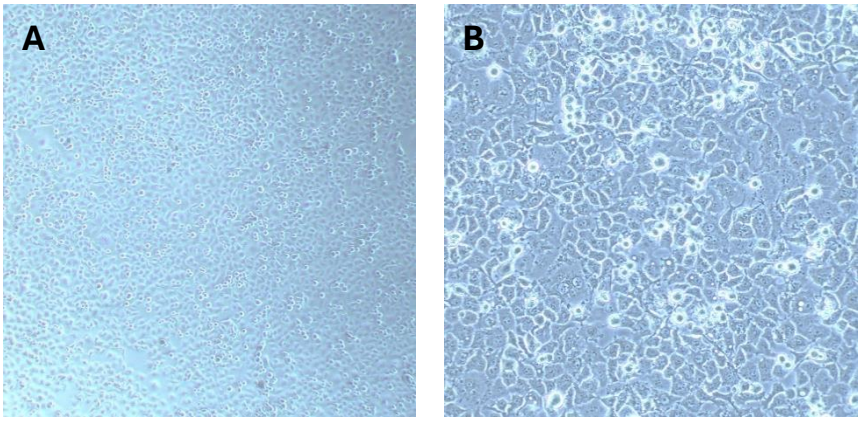

Figure 12: Representative microscope images of PANC-1 flasks with 100% confluence, taken with 4x (A) and 10x (B) magnification.

3. Cell treatments

In this study, we tested several carotenoids (astaxanthin, β-carotene, crocin and fucoxanthin) and polyphenols (chrysin, genistein, kaempferol and quercetin) in the following concentrations: 0.1, 1 and 10 µM. Cells were exposed to these compounds or solvents for 24h, in FBS-free culture medium.

Astaxanthin, β-carotene, chrysin, genistein, kaempferol and quercetin were dissolved in DMSO, crocin in H_2O and fucoxanthin in ethanol. The final concentration of these solvents in culture medium is 1% (v/v). Controls for these compounds contained the same amount (1% (v/v)) of the respective solvents.

4. Nutrient incorporation assays

After treatment of the cells (24h), the culture medium was discarded, and the cells were washed with 300 µL glucose-free HEPES buffered saline (GF-HBS; composition in mM: 140 NaCl, 5 KCl, 20 HEPES, 2.5 $MgCl_2$, 1 $CaCl_2$; pH 7.4) at 37 °C.

Then, cells were pre-incubated for 20 min with GF-HBS and the carotenoids/polyphenols in the concentrations previously mentioned, at 37°C.

After removal of the pre-incubation medium, the nutrient uptake begun with the addition of 200 µL GF-HBS buffer containing 10 nM ^{3}H-2-deoxy-D-glucose (^{3}H-DG), 5 nM ^{3}H-glutamine (^{3}H-GLN) or 10 nM ^{3}H-lactic acid (^{3}H-L). The incubation was stopped after 6 min (^{3}H-DG and ^{3}H-GLN) or 3 min (^{3}H-L) by removing the medium and washing the cells with 500 µL ice cold GF-HBS buffer. Cells were then solubilized with 300 µL 0.1% (v/v) Triton X-100 and left overnight at 4°C.

For ^{3}H-GLN uptake assay, besides the GF-HBS in the previous nutrient uptake assays, we also used GF-HBS with NaCl replaced by LiCl, in order to determine the sodium-independent component of ^{3}H-GLN uptake.

The ideal incubation periods with ^{3}H-DG and ^{3}H-GLN were previously determined for the AsPC-1 cell line by our group[122] – 6 min. The same incubation time was used with the PANC-1 cell line.

For ^{3}H-L incorporation assays, we initially determined the time-course of its uptake by the AsPC-1 cell line, by measuring the uptake at 1, 5, 10, 15, 30 and 60 min, in order to determine the ideal incubation time. From this experiment, we concluded that the ideal period of incubation of the cells with ^{3}H-L is 3 min. The same incubation time was used with the PANC-1 cell line.

When treated, cells were exposed to drugs not only for the 24h before the experiment, but also during the pre-incubation and incubation periods.

The intracellular radioactivity was determined by liquid scintillation counting (LKB Wallac 1209 Rackbeta, Torku, Finalnd), and the results normalized for total protein content.

5. RNA extraction and RT-qPCR

Total RNA was extracted from AsPC-1 cells after chronic treatment with crocin or chrysin (10 µM), using NZYol® isolation reagent, according to the manufacturer's instructions.

The extracted RNA was treated with DNAse I and the resulting DNA-free RNA was reverse transcribed using qScript cDNA SuperMix, according to manufacturer's instructions. The cDNA obtained from the previous reactions was then treated with RNase H, in order to degrade unreacted RNA.

For real-time qPCR (RT-qPCR) we used the transcription reaction mixture, and the PCR was carried out with a LightCycler® 96 (Roche, Nutley, NJ, USA). Ten µL reactions were set up in 96-well plates using *GLUT1*, *ASCT2*, *MCT1* or *β-actin* primers (Table 1), and 5 µL of KAPA SYBR® FAST qPCR Master Mix, according to the manufacturer's instructions. Cycling conditions are shown in Table 2. The fluorescence was measured in a single measurement at the end of the 72°C period.

The cycling program is followed by a melting curve program [(annealing temperature + 10°C) for 15s and 75°C with a heating rate of 0.1°C /s and continuous fluorescence measurement] and a cooling step to 40°C.

mRNA expression levels were analyzed using LightCycler analysis software (Roche, Germany). The amount of *GLUT1*, *ASCT2* and *MCT1* mRNA was normalized to the amount of mRNA of *β-actin* (housekeeping gene).

Table 1: Primer sequences and annealing temperature (AT) for real time RT-qPCR.

Target transporter	**Primer sequence (5'-3')**	AT (°C)
GLUT1	Forward: GAT GAT GCG GGA GAA GAA GGT Reverse: ACA GCG TTG ATG CCA GAC AG	60
ASCT2	Forward: TGG TCT CCT GGA TCA TGT GG Reverse: TTT GCG GGT GAA GAG GAA GT	65
MCT1	Forward: CAC CGT ACA GCA ACT ATA CG Reverse: CAA TGG TCG CCT CTT GTA GA	65
β-actin	Forward: AGA GCC TCG CCT TTG CCG AT Reverse: CCA TCA CGC CCT GGT GCC T	65

Table 2: Protocol time, temperature and cycle repeat for RT-qPCR.

PCR cycling step	Temperature (°C)	Protocol run time	Cycle repeat
Denaturation	95	5 min	1
Denaturation	95	10s	50
Annealing	AT (Table 1)	10s	
Extension	65 72	10s 10s	

6. Cell viability – MTT assay

MTT is a salt that, in the presence of metabolically active cells, is reduced into formazan crystals that present a purple color. Thus, we spectrophotometrically determined the quantity of formazan formed when cells were exposed to MTT, which is proportional to the number of cells with active mitochondria.

After AsPC-1 cell treatment (24h), 10 µL of MTT solution (5 mg/mL) were added to the culture medium and cells were incubated for 2h.

After the incubation period, the culture medium was removed, and cells were solubilized in 200 µL of DMSO.

To determine the absorbance values specific to formazan crystal, we measured the absorbance at 550 nm and subtracted it by the absorbance at 650 nm (corresponding to unspecific light absorption).

In this assay, the effect of nutrient transport protein inhibitors, namely BAY-876 (2.5 µM), GPNA (1 mM) and/or NPPB (0.5 mM), in combination with chrysin or crocin (10 µM), was also tested. In these experiments, these inhibitors were present for the period of AsPC-1 cell treatment (24h).

7. Protein determination

The protein content of the cell monolayers was determined as described[123], using human serum albumin as standard.

8. Calculation and Statistics

Results are expressed as arithmetic means with standard error of the mean (SEM). The value of n indicates the number of replicates of at least two different experiments. To determine the statistical difference between two groups, we used the Student's t test, considering the differences statistically significant when $p<0.05$. To determine the

difference between three or more groups, we used the one-way ANOVA test, followed by the Turkey's multiple comparisons test. Analyses were done using the GraphPad Prism version 8.0.1 software (San Diego, CA, USA).

8.1. Time-course analysis

For the ^{3}H-L uptake time-course analysis, we suited the following equation to the experimental data by a non-linear regression analysis.

$$A(t)=\frac{k_{in}}{k_{out}} \times (1 - e^{-k_{out} \times t})$$

In this equation, A(t) represents the accumulation of ^{3}H-L at the incubation time t, and k_{in} and k_{out} the rate constants of inward and outward transport, respectively.

Chapter IV – Results

1. Effect of carotenoids and polyphenols on nutrient uptake

1.1. Time-course of ^{3}H-L incorporation

Before investigating the effect of compounds on ^{3}H-L uptake, we determined the time-dependency of accumulation of this nutrient in AsPC-1 cells.

As shown in Figure 13, the incorporation of ^{3}H-L in cells is time-dependent and shows a linear relationship in the first 3 min of incubation. This information led us to conclude that a 3 min incubation time is the most appropriate for further experiments.

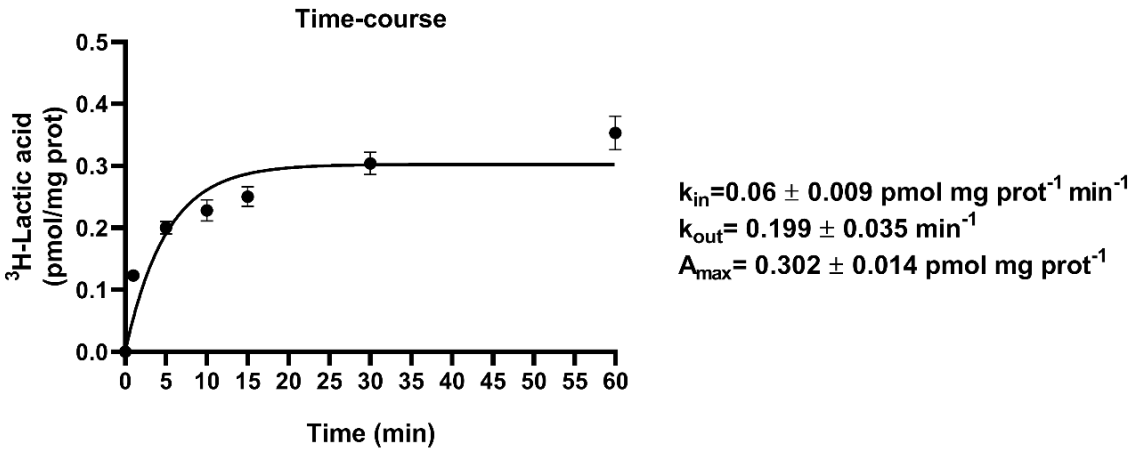

Figure 13: Time-course of ^{3}H-L uptake by AsPC-1 cells. Cells were incubated at 37°C with 10 nM ^{3}H-L for 1, 5, 10, 15, 30 and 60 min. Results are presented as pmol mg protein^{-1} with arithmetic means ± SEM (n=6-7). k_{in}: rate constant of inward transport; k_{out}: rate constant of outward transport; A_{max}: accumulation at steady state (t→∞).

Next, we tested the effect of carotenoids (astaxanthin, β-carotene, crocin and fucoxanthin) and polyphenols (chrysin, genistein, kaempferol and quercetin) in ^{3}H-L, ^{3}H-DG and ^{3}H-GLN uptake, using the AsPC-1 and PANC-1 cell lines.

1.2. Effect of carotenoids on ^{3}H-L, ^{3}H-DG and ^{3}H-GLN incorporation

To determine the effect of carotenoids on ^{3}H-L and ^{3}H-DG incorporation, AsPC-1 and PANC-1 cells were treated with astaxanthin, β-carotene, crocin and fucoxanthin (0.1-10 µM) for 24h, and then incubated with the radiolabeled nutrients for 3 min or 6 min, respectively.

As shown in Fig. 14, there was a decrease in ^{3}H-L uptake in AsPC-1 cells treated with crocin (1 µM; Fig. 14C) and fucoxanthin (0.1, 1 and 10 µM; Fig. 14D). Astaxanthin and β-carotene (Fig. 14A-B) had no significant effect in this cell line. Overall, fucoxanthin was the carotenoid with highest effect on ^{3}H-L uptake in the AsPC-1 cell line.
As for PANC-1 cells, astaxanthin (10 µM; Fig. 14A) decreased ^{3}H-L uptake, and no significant effect was observed with β-carotene, crocin and fucoxanthin (Fig. 14B-D).

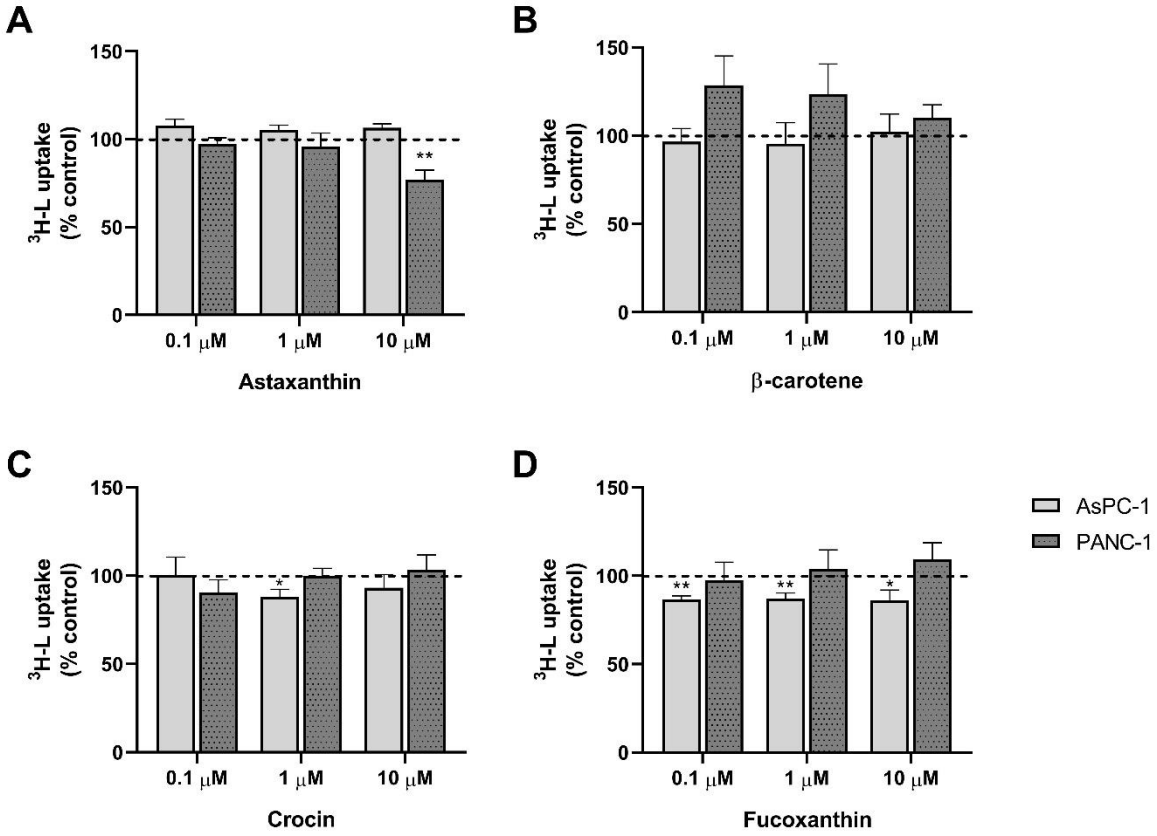

Figure 14: Effect of carotenoids on ^{3}H-L uptake by AsPC-1 and PANC-1 cells. After 24h exposure to the compounds (0.1, 1 and 10 µM) or solvent, cells were preincubated for 20 min, followed by an incubation for 3 min with 10 nM ^{3}H-L (n=6-8). Results are presented as pmol/mg of protein (% of control) with arithmetic means ± SEM. *Significantly different from control ($p<0.05$). **Significantly different from control ($p<0.01$).

As for the ^{3}H-DG uptake (Fig. 15), in the AsPC-1 cell line, crocin (0.1, 1 and 10 µM; Fig. 15C) promoted a decrease, while fucoxanthin (0.1 µM; Fig. 15D) increased the uptake of this nutrient. In contrast, astaxanthin and β-carotene (Fig. 15A-B) had no significant effect on the uptake of ^{3}H-DG uptake in this cell line. Altogether, crocin was the carotenoid with highest effect on ^{3}H-DG uptake, in the AsPC-1 cell line.

In the PANC-1, none of the tested carotenoids had a significant effect on ^{3}H-DG uptake.

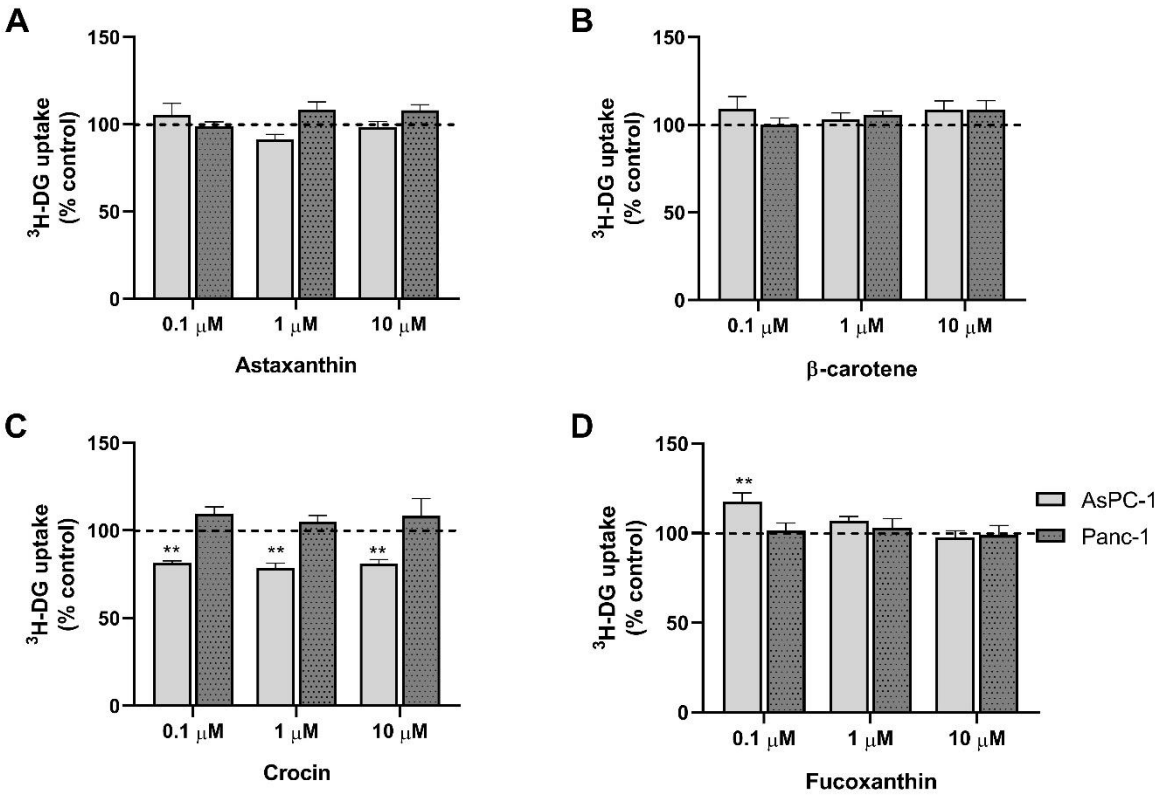

Figure 15: Effect of carotenoids on ^{3}H-DG uptake by AsPC-1 and PANC-1 cells. After 24h exposure to the compounds (0.1, 1 and 10 µM) or solvent, cells were preincubated for 20 min, followed by an incubation for 6 min with 10 nM ^{3}H-DG (n=6-8). Results are presented as pmol/mg of protein (% of control) with arithmetic means ± SEM. *Significantly different from control ($p<0.05$). **Significantly different from control ($p<0.01$).

To determine the effect of the carotenoids on ^{3}H-GLN uptake, both cell lines were incubated with the nutrient for 6 min, in the presence and absence of sodium.

In the AsPC-1 cell line (Fig. 16), of all the carotenoids tested, only β-carotene significantly decrease the total ^{3}H-GLN uptake by the cells (1 µM; Fig. 16B). As for the ^{3}H-GLN sodium-independent uptake, the carotenoids had no significant effect in this cell line (Fig. 16). Astaxanthin was the only carotenoid that promoted a significant decrease in the sodium-dependent uptake of ^{3}H-GLN (Fig. 16A).

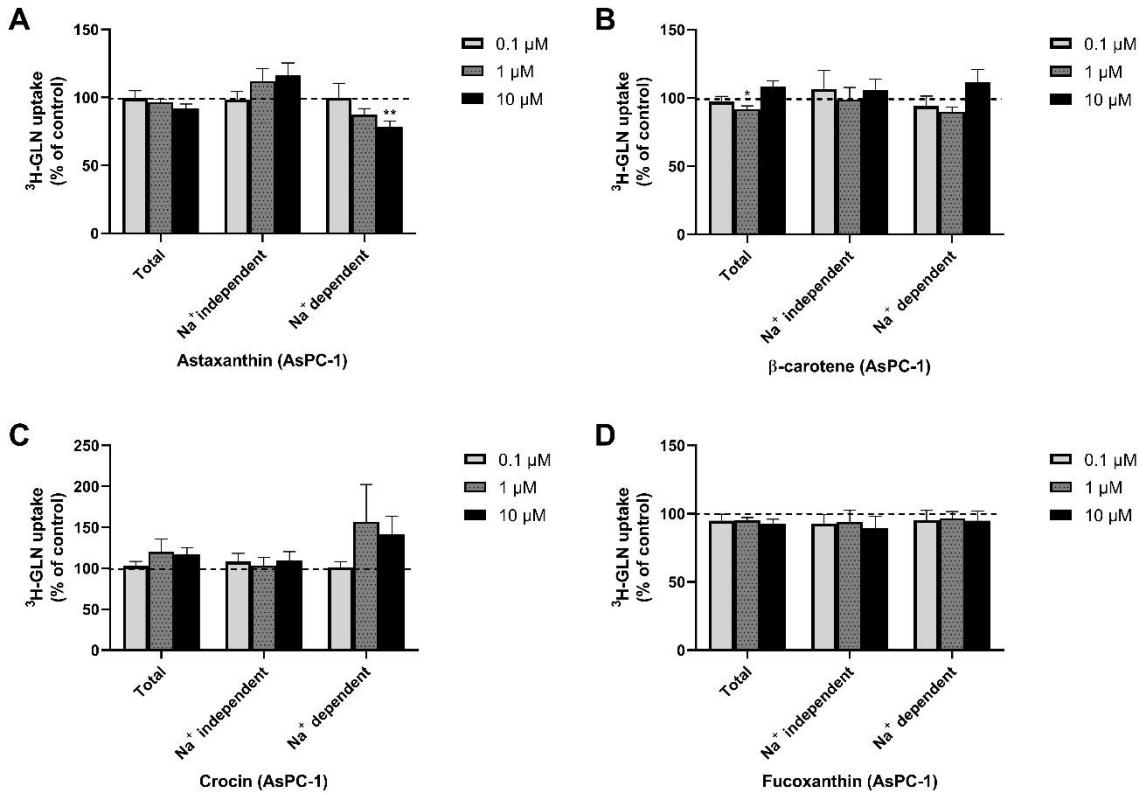

Figure 16: Effect of carotenoids on ^{3}H-GLN uptake (total, Na^{+}-independent and Na^{+}-dependent) by AsPC-1 cells. After 24h exposure to the compounds (0.1, 1 and 10 µM) or solvent, cells were preincubated for 20 min, followed by an incubation for 6 min with 5 nM ^{3}H-GLN (n=6). Results are presented as pmol/mg of protein (% of control) with arithmetic means ± SEM. *Significantly different from control ($p<0.05$). **Significantly different from control ($p<0.01$).

In the PANC-1 cell line (Fig. 17), astaxanthin, in all tested concentrations, had no overall effect on ^{3}H-GLN uptake (Fig. 17A). β-carotene promoted an increase in ^{3}H-GLN total uptake (1 µM), in ^{3}H-GLN sodium-independent uptake (10 µM) and in ^{3}H-GLN sodium-dependent uptake (1 µM) (Fig. 17B). Crocin promoted an increase in ^{3}H-GLN sodium-independent uptake (0.1 µM; Fig. 17C). Finally, fucoxanthin promoted a decrease in both total ^{3}H-GLN uptake (0.1 and 1 µM) and sodium-dependent ^{3}H-GLN uptake (0.1 and 1 µM) (Fig. 17D).

Overall, β-carotene and fucoxanthin were the carotenoids with highest effect on ^{3}H-GLN uptake in the PANC-1 cell line.

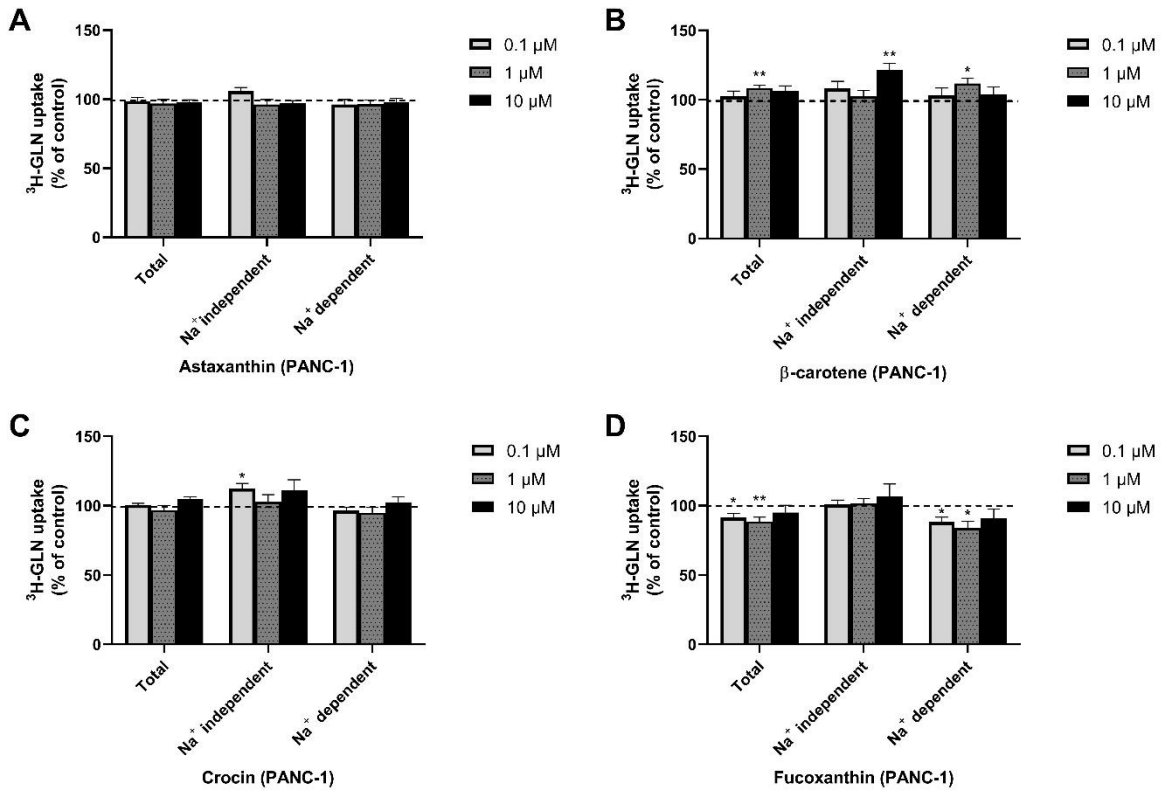

Figure 17: Effect of carotenoids on ^{3}H-GLN uptake (total, Na^{+} independent and Na^{+} dependent) by PANC-1 cells. After 24h exposure to the compounds (0.1, 1 and 10 µM) or solvent, cells were preincubated for 20 min, followed by an incubation for 6 min with 5 nM ^{3}H-GLN (n=6). Results are presented as pmol/mg of protein (% of control) with arithmetic means ± SEM. *Significantly different from control ($p<0.05$). **Significantly different from control ($p<0.01$).

1.3. Effect of polyphenols on ^{3}H-L, ^{3}H-DG and ^{3}H-GLN incorporation

Next, we determined the effect of polyphenols on ^{3}H-L, ^{3}H-DG and ^{3}H-GLN incorporation by AsPC-1 and PANC-1 cells. After a 24h treatment with chrysin, genistein, kaempferol and quercetin (0.1-10 µM), the cells were then incubated with the radiolabeled nutrients in the conditions previously described (3 min for ^{3}H-L and 6 min for ^{3}H-DG and ^{3}H-GLN).

Most polyphenols, particularly chrysin, genistein and kaempferol, had no significant effect on both AsPC-1 and PANC-1 ^{3}H-L uptake (Fig. 18A-C). The only polyphenol that promoted a significant increase in ^{3}H-L uptake was quercetin, in the lowest concentration tested (0.1 µM; Fig. 18D).

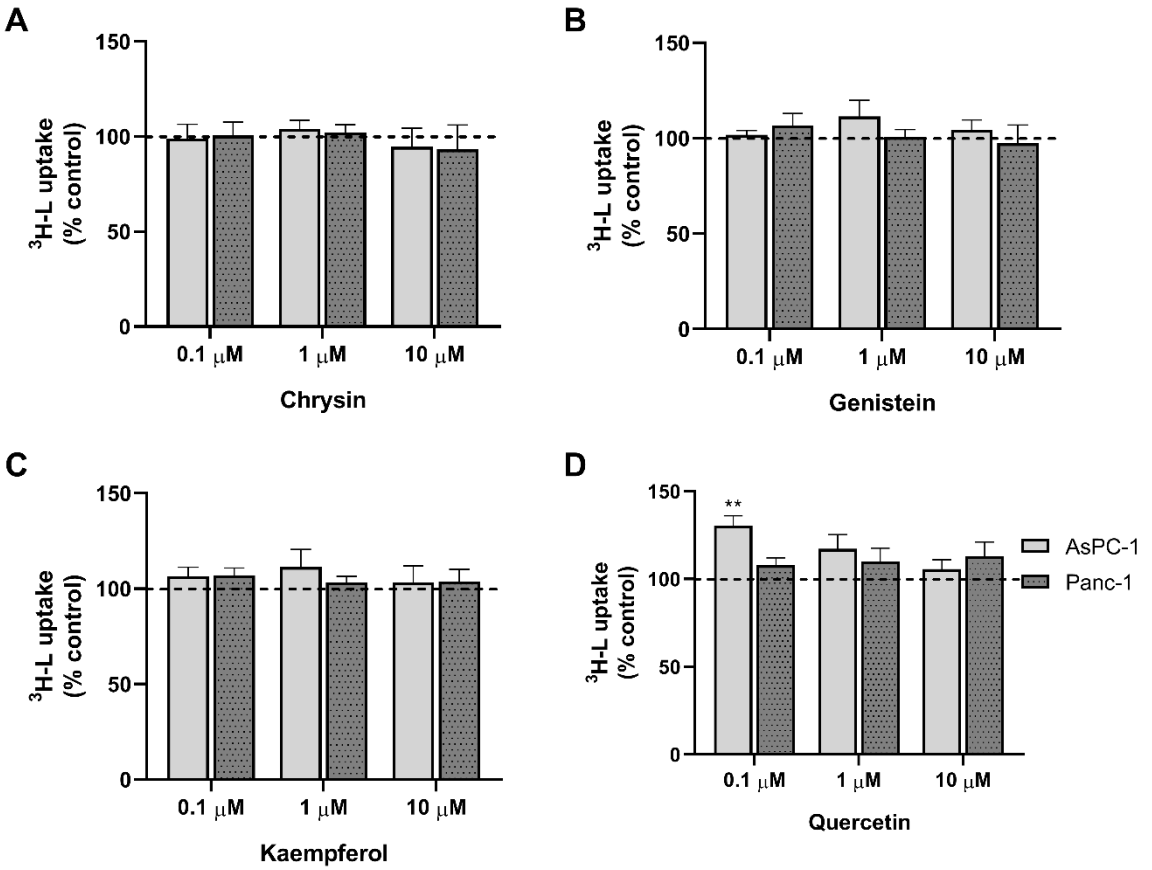

Figure 18: Effect of polyphenols on ^{3}H-L uptake by AsPC-1 and PANC-1 cells. After 24h exposure to the compounds (0.1, 1 and 10 µM) or solvent, cells were preincubated for 20 min, followed by an incubation for 3 min with 10 nM ^{3}H-L (n=6). Results are presented as pmol/mg of protein (% of control) with arithmetic means ± SEM. *Significantly different from control ($p<0.05$). **Significantly different from control ($p<0.01$).

As for ^{3}H-DG uptake, chrysin was the only polyphenol that had significant effects on the AsPC-1 cell line, by promoting a significant decrease in the incorporation of this nutrient (1 and 10 µM; Fig. 19A). Genistein, kaempferol and quercetin had no effect on ^{3}H-DG uptake on AsPC-1 cells (Fig. 19B-D).

In the PANC-1 cell line, genistein promoted a significant decrease in ^{3}H-DG uptake in the highest concentration tested (10 µM; Fig. 19B), while kaempferol (1 and 10 µM; Fig. 19C) and quercetin (1 µM; Fig. 19D) promoted a significant increase in the uptake of this nutrient. Chrysin had no significant effect on ^{3}H-DG uptake by this cell line (Fig. 19A).

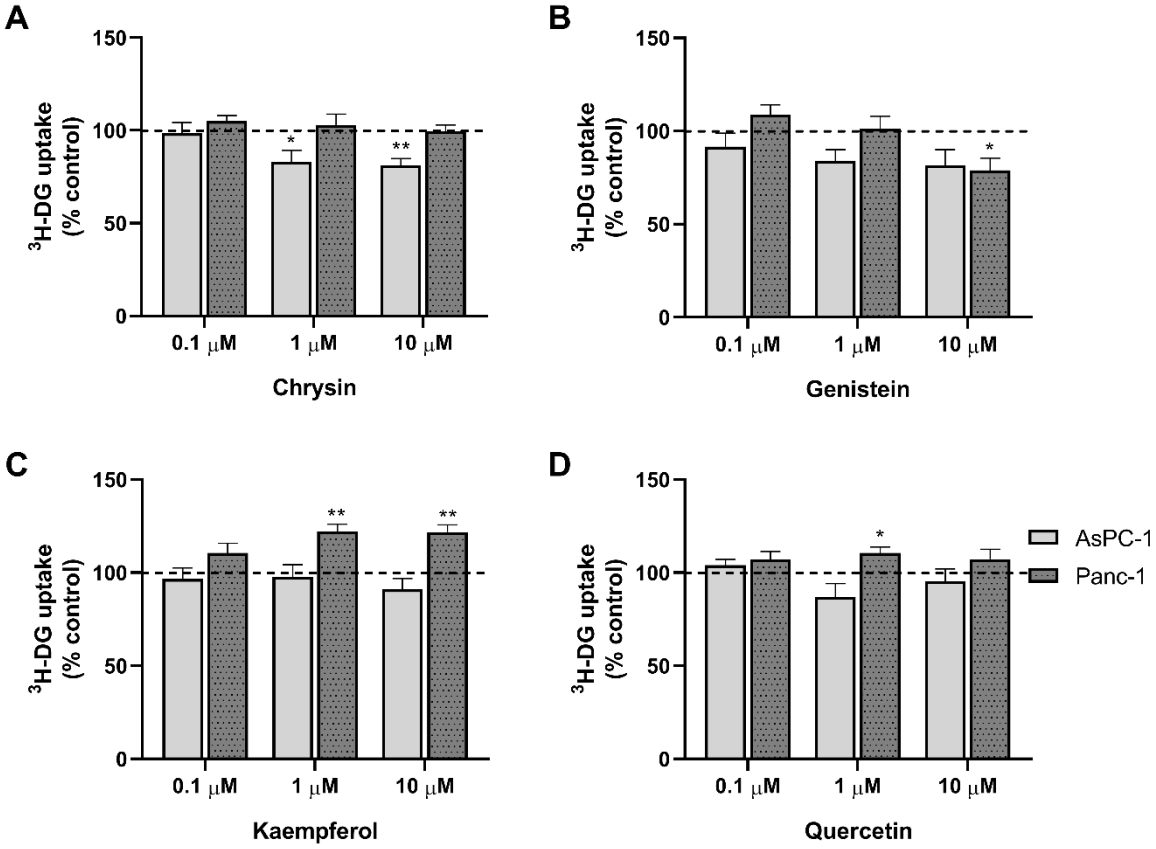

Figure 19: Effect of polyphenols on ^{3}H-DG uptake by AsPC-1 and PANC-1 cells. After 24h exposure to the compounds (0.1, 1 and 10 µM) or solvent, cells were preincubated for 20 min, followed by an incubation for 6 min with 10 nM ^{3}H-DG (n=6). Results are presented as pmol/mg of protein (% of control) with arithmetic means ± SEM. *Significantly different from control ($p<0.05$). **Significantly different from control ($p<0.01$).

In relation to ^{3}H-GLN uptake, most polyphenols showed no effects in the AsPC-1 cell line (Fig. 20). Particularly, genistein and kaempferol (Fig. 20B-C) had no effect on total, sodium-dependent and sodium-independent ^{3}H-GLN uptake. Chrysin also had no effect on ^{3}H-GLN total and sodium-independent uptake, but decreased sodium-dependent ^{3}H-GLN uptake in the highest concentration tested (10 µM; Fig. 20A). Quercetin was the polyphenol with the most marked effect on ^{3}H-GLN uptake, in the AsPC-1 cell line, by promoting a decrease in total and sodium-dependent ^{3}H-GLN uptake (0.1 µM), while increasing sodium independent uptake (0.1 and 1 µM) (Fig. 20D).

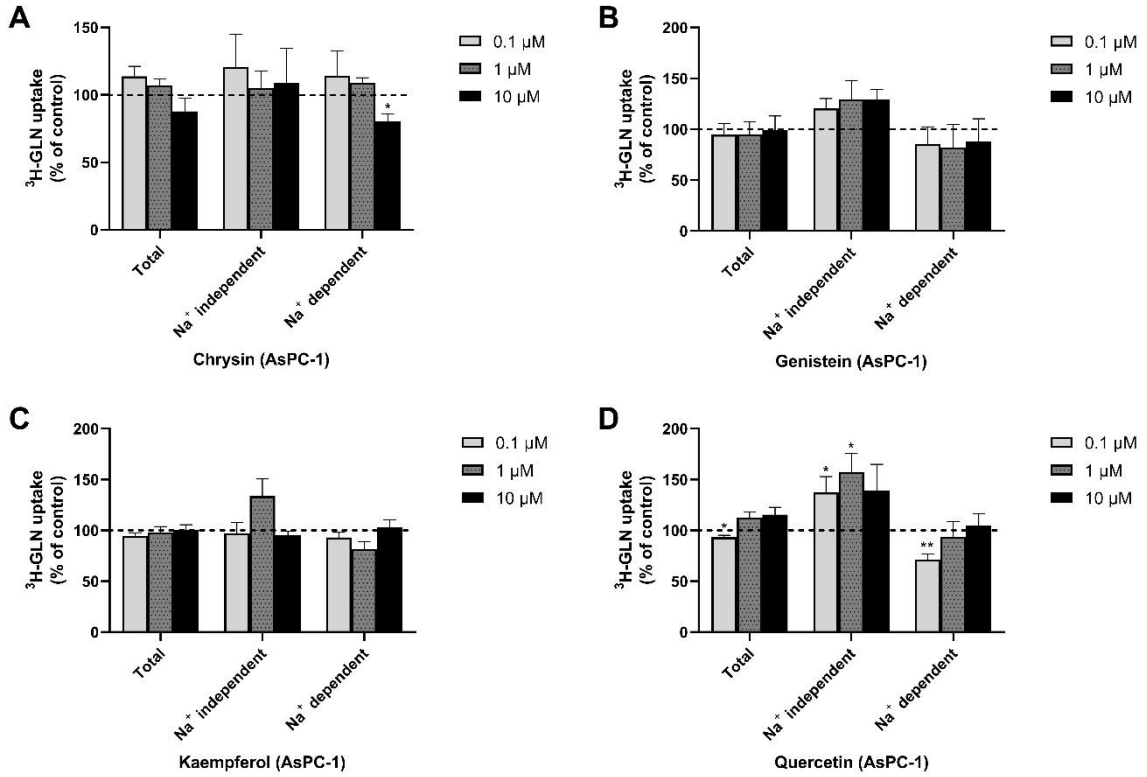

Figure 20: Effect of polyphenols on ^{3}H-GLN uptake (total, Na^{+} independent and Na^{+} dependent) by AsPC-1 cells. After 24h exposure to the compounds (0.1, 1 and 10 µM) or solvent, cells were preincubated for 20 min, followed by an incubation for 6 min with 5 nM ^{3}H-GLN (n=4-6). Results are presented as pmol/mg of protein (% of control) with arithmetic means ± SEM. *Significantly different from control ($p<0.05$). **Significantly different from control ($p<0.01$).

Finally, in the PANC-1 cell line, kaempferol promoted a decrease in ^{3}H-GLN total uptake (1 µM; Fig. 21C), while genistein promoted an increase in sodium-independent ^{3}H-GLN uptake (1 and 10 µM; Fig. 21B). Quercetin promoted a similar effect in both AsPC-1 and PANC-1 cell lines, promoting a decrease in total and sodium-dependent ^{3}H-GLN uptake, while increasing the sodium-independent uptake (Fig. 20D and 21D). Chrysin had no effect on ^{3}H-GLN uptake by this cell line (Fig. 21A).

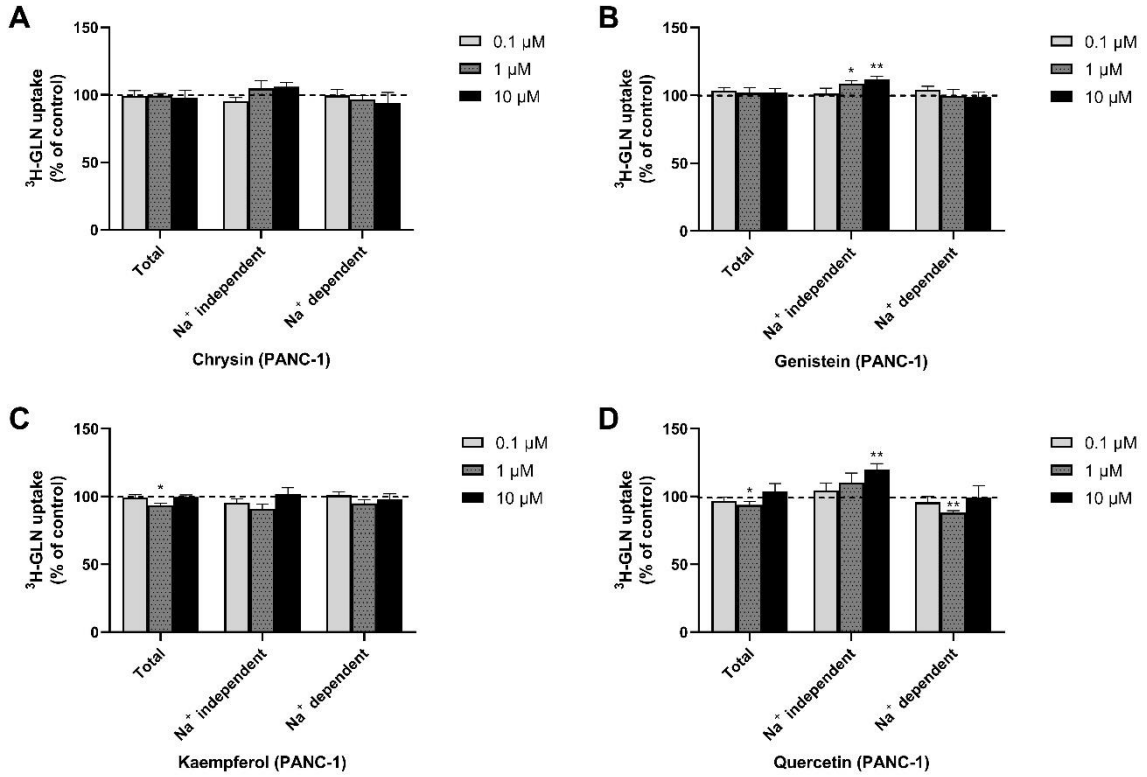

Figure 21: Effect of polyphenols on ^{3}H-GLN uptake (total, Na^{+} independent and Na^{+} dependent) by PANC-1 cells. After 24h exposure to the compounds (0.1, 1 and 10 µM) or solvent, cells were preincubated for 20 min, followed by an incubation for 6 min with 5 nM ^{3}H-GLN (n=6). Results are presented as pmol/mg of protein (% of control) with arithmetic means ± SEM. *Significantly different from control ($p<0.05$). **Significantly different from control ($p<0.01$).

Thus, from this set of experiments, we concluded that the polyphenols tested have essentially no effect on lactic acid cellular uptake, in both cell lines. We can also conclude that chrysin and quercetin were the polyphenols with highest effects on AsPC-1 cell line. Chrysin was the polyphenol with the lowest effect on PANC-1 cells. Quercetin was the only polyphenol that showed similar results in ^{3}H-GLN, in both cell lines.

2. Effect of crocin and chrysin on mRNA levels of nutrient transporters (*GLUT1*, *ASCT2* and *MCT1*)

Further assays were performed with the compounds (one carotenoid and one polyphenol) that presented the most interesting effects in the nutrient uptake assays, namely crocin and chrysin.

To investigate if crocin and chrysin interfere with the transcription rates of the nutrient transporter proteins *GLUT1*, *ASCT2* and *MCT1*, we determined their effects on the mRNA levels of these nutrient transporter proteins. For this, we isolated the mRNA from AsPC-1 cells treated with 10 µM of crocin and chrysin. Then, a qRT-PCR was performed with *GLUT1* and *ASCT2* as targets for the chrysin treated cells, and *GLUT1* and *MCT1* as targets for the crocin treated cells.

As observed in Fig. 22, there were no significant differences in nutrient transporter protein expression. Therefore, we determined that crocin and chrysin were not promoting a decrease in nutrient uptake by targeting the synthesis of the nutrient transporter proteins.

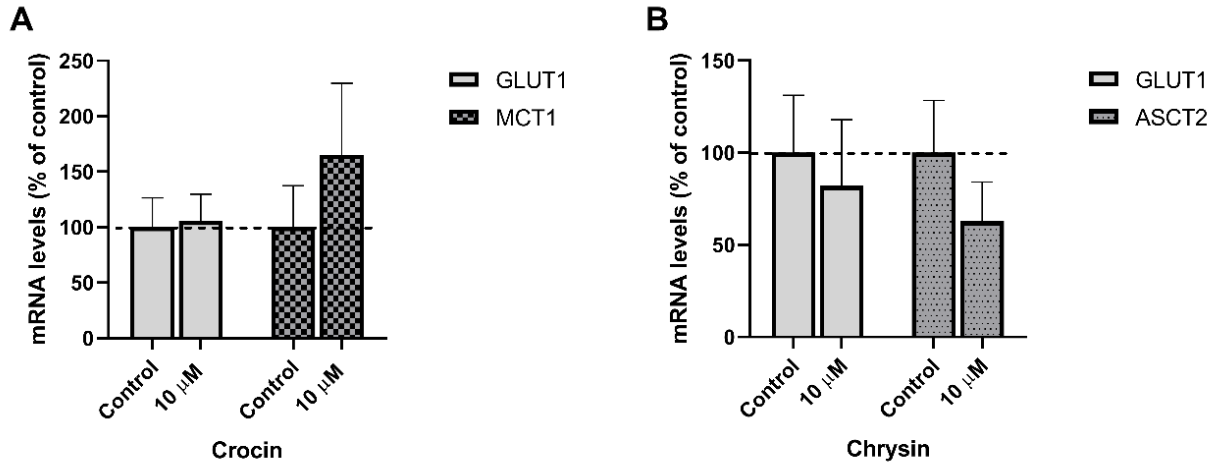

Figure 22: Effect of crocin (A) and chrysin (B) on *GLUT1*, *MCT1* and *ASCT2* mRNA levels in AsPC-1 cells. After a 24 h treatment with crocin 10 µM (A), chrysin 10 µM (B) or solvent (control), mRNA levels were quantified with RT-qPCR (n=4-6). Data were normalized to the expression of β-actin. Shown are arithmetic means ± SEM.

3. Effect of crocin and chrysin on cell viability

Next, the effect of different concentrations of crocin and chrysin (1, 10 and 20 µM) on AsPC-1 cell viability was determined. As seen in Fig. 23, both crocin and chrysin promoted a significant decrease on AsPC-1 cell viability in a concentration-dependent manner.

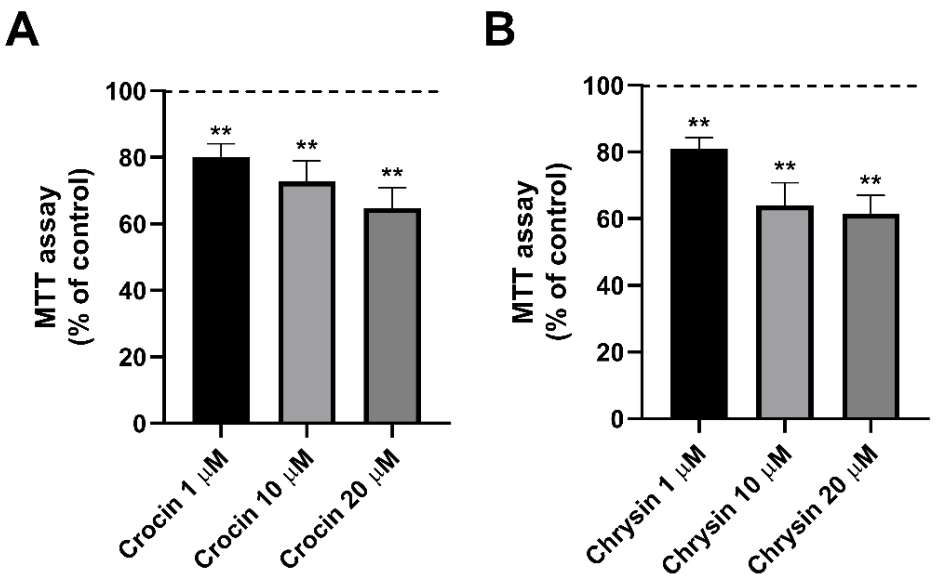

Figure 23: Effect of crocin and chrysin (1-20 µM) on cell viability. After a 24h exposure to crocin (A) or chrysin (B) 1, 10 and 20 µM, cell viability was assessed with the MTT assay (n=7-8). Arithmetic means ± SEM are shown. **Significantly different from control (P<0.01).

4. Influence of nutrient transport inhibitors (BAY-876, NPPB or GPNA) on the effect of chrysin or crocin on cell viability

In the final series of experiments, we wanted to verify if the cytotoxic effect of crocin and chrysin (10 µM) was dependent on nutrient uptake inhibition.

Confirming previous results, both crocin and chrysin at 10 µM promoted a decrease in cell viability, by the MTT assay (Fig 24).

As for the nutrient transporter inhibitors, Bay-876 and NPPB promoted a decrease in cell viability, but the effects of their combination with crocin (Bay-876 and NPPB) or chrysin (Bay-876) are not significantly different from the single treatments (Fig 24A-C). GPNA had no effect on AsPC-1 cells, either alone or in combination with chrysin (Fig 24D).

Since the combination of the nutrient transporter inhibitors and crocin/chrysin did not change their cytotoxic effect and therefore do not have an additive effect, it is suggested

that these compounds act by the same mechanism of inhibition, namely by inhibition of nutrient transport.

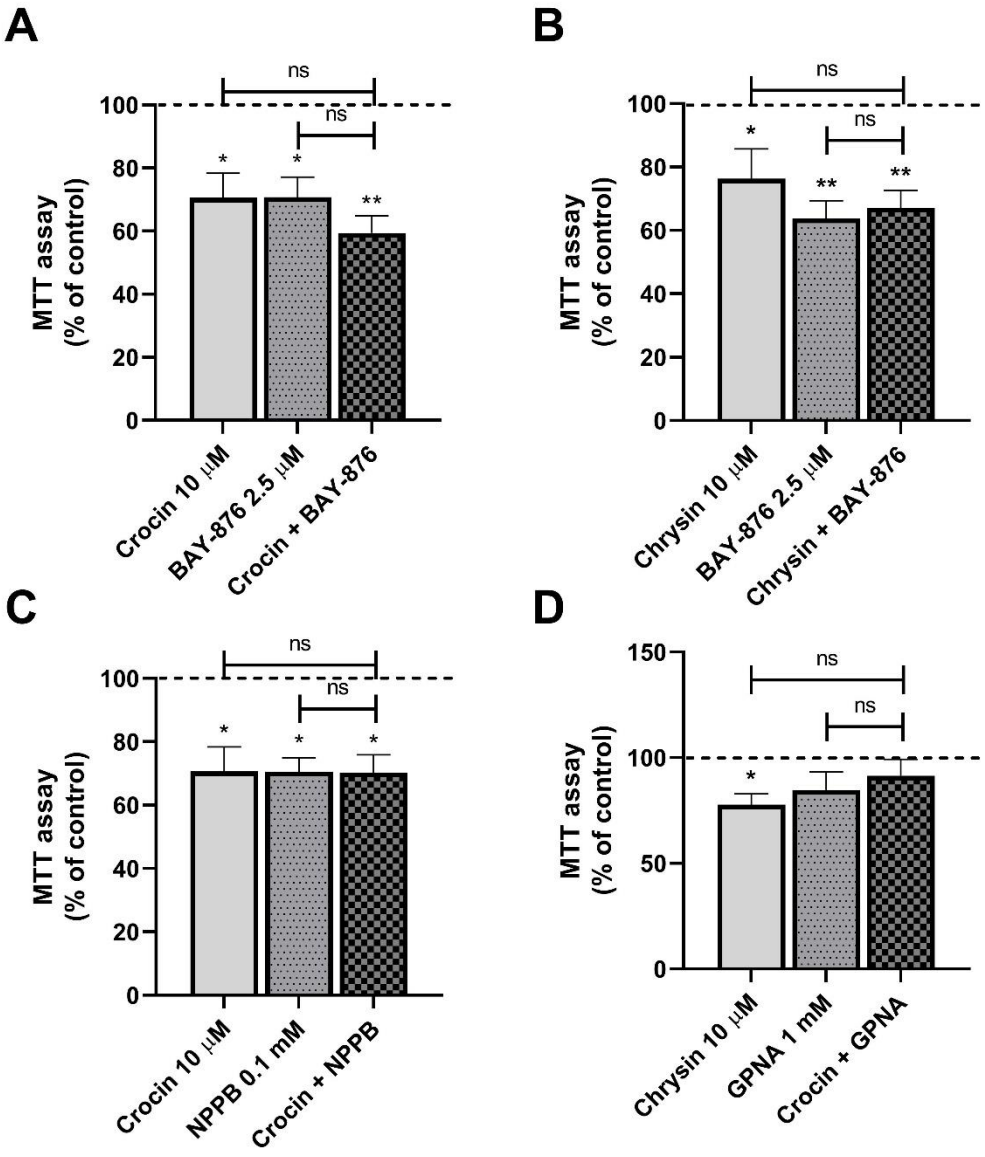

Figure 24: Effect of crocin or chrysin (10 µM) and nutrient transport inhibitors (2.5 µM BAY-876, 0.1 mM NPPB and 1 mM GPNA) on cell viability of AsPC-1 cells. After 24h exposure to crocin or chrysin 10 µM and nutrient transport inhibitors, cell viability was assessed with the MTT assay (n=6-12). Arithmetic means ± SEM are shown. *Significantly different from control (P<0.05) **Significantly different from control (P<0.01). ns not significantly different.

Chapter V – Discussion

Despite the advances in cancer treatment, PC is still one of the highest causes of cancer death worldwide, with a low 5-year survival rate of 9% reported in Europe and USA[3; 124].
The influence of diet in cancer has been a topic of interest in past years, and an alternative being explored is the use of isolated bioactive dietary compounds, such as carotenoids and polyphenols, in cancer treatment. The interest in these compounds has been increasing not only because of their properties but also because they are easily obtainable and have a lower toxicity, when compared to current anticancer therapies. Nevertheless, there is still a need to explore the underlying mechanisms of the anticancer effect of these compounds.

As previously described, the selected carotenoids have shown anticancer effects in PC cells, by promoting an increase in cell death, inducing apoptosis and cell cycle arrest and even decreasing pancreatic tumor size in mice, although the numbers of studies regarding the effect of carotenoids on PC is relatively low.

To the best of our knowledge, this is the first study investigating the effects of the astaxanthin and crocin on nutrient uptake by cancer cells. Previous studies exploring the effects of these carotenoids on diabetes, insulin resistance and metabolic syndrome, found that astaxanthin promotes an increase in glucose uptake and consumption as well as in GLUT4 translocation in C2C12 myoblast cells and in human primary skeletal muscle cells[125]. In L6 myoblast cells, astaxanthin also promoted an increase in GLUT4 translocation to the cell membrane and increased glucose uptake[126]. *In vivo*, astaxanthin likewise promoted an increase in glucose incorporation into the skeletal muscle and increased oxidative phosphorylation in mice given a high fat diet[127].
In the present study using PC cells, we found that astaxanthin had no effect on ^{3}H-DG uptake by both cell lines. We also verified that astaxanthin had no effect on ^{3}H-L uptake by AsPC-1 cells, and on PANC-1 only promoted a decrease in ^{3}H-L uptake at the highest concentration tested (10 µM). On ^{3}H-GLN uptake, astaxanthin promoted a decrease in sodium-dependent ^{3}H-GLN uptake on AsPC-1 cells, also with the highest concentration tested, while having no effect on PANC-1 cells.

Regarding β-carotene, it was previously found that in the MDA-MB-231 breast cancer cell line this carotenoid promoted an increase in ^{3}H-DG uptake and a decrease in sodium-independent ^{3}H-GLN uptake, while having no effect on sodium-dependent ^{3}H-GLN uptake[128]. We found that, in both AsPC-1 and PANC-1 cells, β-carotene had no effect on

^{3}H-L and ^{3}H-DG uptake, but promoted a decrease in total ^{3}H-GLN uptake in AsPC-1 cells (at 1 µM) and an increase in total, sodium-dependent and sodium-independent ^{3}H-GLN uptake in PANC-1 cells (at 1 and 10 µM).

In a previous study in ovarian cancer cells, fucoxanthin has shown to decrease glucose uptake in OVCAR-3 and A2780 cells and also to inhibit glycolysis and the enzymes involved in this process[129].
In PC cell lines, we found that fucoxanthin promoted a decrease in ^{3}H-L uptake on AsPC-1 cells (and opposite of what was found in ovarian cancer cells[7]), and an increase in ^{3}H-DG uptake on AsPC-1 cells at the lowest concentration tested (0.1 µM). Fucoxanthin also promoted a decrease in total and in sodium-dependent ^{3}H-GLN uptake by PANC-1 cells (0.1 and 1 µM), while having no effect on AsPC-1 cells.

Normally, in cancer cells, an increase in glucose consumption and lactate production, because of aerobic glycolysis (the Warburg effect) exists[22]. The effect of fucoxanthin on AsPC-1 cells - increasing ^{3}H-DG uptake and decreasing ^{3}H-L uptake - can exacerbate the Warburg effect and therefore facilitate cancer cell growth. In detail, the increase in glucose uptake may result in a higher rate of aerobic glycolysis and thus ATP production, which will facilitate cancer cell proliferation. Besides, the reduction in lactate uptake by cancer cells can originate an increase in lactate concentrations around cancer cells, which have been linked to worse clinical outcomes in some cancers, due to the promotion of metastasis and angiogenesis, by restricting immune cell infiltration into the tumor and by promoting radio-resistance[130].

As for crocin, there was no available data on its effects on nutrient uptake by any cell type, but in our study, we found that it was the carotenoid with the highest inhibitory effect on nutrient uptake on AsPC-1 cells. Indeed, crocin promoted a decrease on ^{3}H-L uptake (at 1 µM) and on ^{3}H-DG uptake with all concentrations tested. Crocin had no effect on ^{3}H-GLN uptake by AsPC-1 cells, but increased the sodium-independent ^{3}H-GLN uptake on PANC-1 cells with the lowest concentration tested (0.1 µM).

The inhibition of glycolysis is a novel antineoplastic strategy being investigated, since cancer cells rely mostly on glycolysis for their energy requirements[131]. The inhibition of lactate uptake, usually by targeting *MCT1* and *MCT4*, has also shown promising effects in cancer therapy, by blocking the growth of glycolytic tumors[132]. With crocin, we found that it not only inhibits glucose uptake, but also inhibits lactic acid uptake, therefore inhibiting the

uptake of two important energy sources for cancer cells, showing that crocin has a strong anticancer potential against AsPC-1 cells.

In relation to the selected polyphenols, these have shown anticancer effects in PC cell lines, in agreement with what was previously described, which includes induction of a decrease in cell viability and of apoptosis[82; 86; 87; 96; 97; 104; 108]. *In vivo*, both chrysin and kaempferol promoted a decrease in pancreatic tumor growth[82; 98], while quercetin induced apoptosis in the tumor tissue[92; 112].

Their mechanisms of action in PC are still largely unknown, but genistein has reportedly inhibited the Notch-1 signaling pathway[88], causing a decrease in cell growth, as well as downregulation of FOXM1[89], which contributes to tumorigenesis and cancer progression[133]. It was also reported to inhibit the STAT3 pathway[87], which is known to regulate various processes like cell proliferation, migration, apoptosis, angiogenesis, metastasis and immune responses, being very important on the process of development and progression of various cancers[134]. As to kaempferol, it was shown to inhibit the EGFR signaling pathway[97], promoting an inhibition of cancer cell growth and migration. Finally, quercetin was also described to inhibit the STAT3 pathway[105], like genistein, and the SHH and TGF-β/Smad signaling pathways, resulting in the inhibition of the epithelial-mesenchymal transition[112]. Quercetin was also shown to promote apoptosis by JNK activation[117] and some of its metabolites appear to inhibit the ERK[106] and EGFR signaling pathways[111], like kaempferol.

As for nutrient uptake, our data shows that chrysin promoted a concentration-dependent decrease in ^{3}H-DG uptake by AsPC-1 cells, which is in agreement with previous studies showing that chrysin inhibited glucose and fructose uptake by other cancer cell types (Caco-2, HCC, MCF-7 and BeWo)[135-138], while inhibiting lactate production[136], thus apparently reversing the metabolic reprogramming of cancer cells. Our results also show that, in both PC cell lines, chrysin had no effect on ^{3}H-L uptake.

Prior research shows that chrysin led to an increase in the sodium-dependent ^{3}H-GLN uptake by the MCF-7 breast cancer cell line, while decreasing sodium-dependent ^{3}H-GLN uptake by another breast cancer cell line (MDA-MB-231)[139]. In our study, chrysin promoted a decrease in sodium-dependent ^{3}H-GLN uptake in the AsPC-1 cell line, while having no effect on PANC-1 cells.

Genistein had no significant effect on ^{3}H-L uptake on both cell lines but promoted a decrease in ^{3}H-DG in the PANC-1 cell line, with the highest tested concentration (10 μM), and an increase in sodium-independent ^{3}H-GLN uptake, also in the PANC-1 cell line.

Previous data show that genistein induces a decrease in glucose uptake in various cancer and non-cancer cell lines (HCC-LM3, Bel-7402, MCF-7, MDA-MB-231, A2780, CaOV3, ES2 and HL-60)[138; 140-143]. However, in low doses (1-13 µM) genistein has shown to promote an increase in glucose uptake and cell growth by breast cancer cell lines, while decreasing both glucose and glutamine uptake in higher doses (>20 µM)[144]. Moreover, in A431 and Colo205 cancer cells, genistein promoted an increase in glucose uptake, and in mice the *GLUT1* expression was significantly increased[145]. Treatment with genistein was also shown to decrease *GLUT1* expression in hepatocellular carcinoma[146] and in prostate cancer[147]. However, this decrease in prostate cancer cells was only observed in the first 6h of genistein exposure. After 24h and 48h exposure, *GLUT1* levels were increased. This study also showed that genistein promoted a decrease in GLUT4[147] protein levels.
So, our finding that genistein promotes a decrease in glucose uptake by PC cell lines agrees with most previous studies. However, we also verified that it increases glutamine uptake, which was not previously investigated.
To the best of our knowledge, there are no published studies regarding the effect of genistein on lactic acid uptake by cancer cells.

We found that kaempferol promotes an increase in ^{3}H-DG uptake by PANC-1 cells, which is an unexpected result given that in previous studies kaempferol was found to decrease glucose uptake[138; 148; 149], glucose consumption[148] and glycolysis[150] and to inhibit GLUT1 transporter[151] in some other cancer cell lines (eg. breast, esophagic, gastric and hepatocellular cancer cell lines).
We also found that kaempferol has no effect on ^{3}H-L uptake by both AsPC-1 and PANC-1 cell lines. The literature on this polyphenol indicates that it is able to decrease extracellular lactate levels in other cancer cell types [148-150] and also to decrease MCT1-mediated lactate uptake in a breast cancer cell line[138].
Kaempferol also promoted a decrease in total ^{3}H-GLN uptake by PANC-1 cells. A previous study on breast cancer cell lines showed an increase in ^{3}H-GLN uptake when cells were treated with kaempferol[139], which disagrees with the results we obtained in PC cells.
Overall, kaempferol had no effect on AsPC-1 nutrient uptake.

As for quercetin, we found that it increased ^{3}H-L uptake by AsPC-1 cells, at the lowest concentration tested (0.1 µM), while having no effect on PANC-1 cells. This result is very distinct from previous studies that reported an inhibition of MCT by quercetin in breast cancer cell lines[152], as well as a decrease in lactate production and consumption in breast cancer cells[153]. In insulinoma cells, quercetin was also found to inhibit the cellular uptake of L-lactate[154].

Quercetin also promoted an increase in ^{3}H-DG uptake on PANC-1 cells (at 1 µM) but had no effect on AsPC-1 cells. Prior research shows a very distinct effect of quercetin on glucose uptake (a decrease) by other cancer cell lines. Namely, in choriocarcinoma cells (BeWo), quercetin promoted a concentration-dependent decrease in ^{3}H-DG uptake, acting as a non-competitive inhibitor[155]. In uveal melanoma cells (92.1), quercetin also promoted a decrease in glucose uptake and suppressed the glycolytic rate[156]. The same was found in breast cancer cells (MCF-7 and MDA-MB-231), where quercetin reduced glucose uptake[153; 157], with a study finding a decrease in the level of *GLUT1* expression[157], while other finding an increase in *GLUT1* mRNA expression[153]. In hepatocarcinoma cell lines, quercetin was also found to decrease glucose uptake, while also increasing *GLUT1* expression[158]. This could be caused by the competitive inhibition of GLUT1, that can lead to GLUT recruitment from the cytoplasm to the membrane[158].

Finally, in relation to ^{3}H-GLN uptake, we found that in both cell lines, quercetin increased sodium-independent ^{3}H-GLN uptake while decreasing both total and sodium-dependent ^{3}H-GLN uptake. The main mechanism of uptake of glutamine in these cells was found to be sodium-dependent and mediated by the ASCT2 transporter[122], which is considered the most important glutamine transporter in cancer cells[159]. Interestingly, quercetin promoted the opposite effect on the sodium-independent ^{3}H-GLN uptake, which could mean that it is also acting on LAT1, which has been explored due to its potential in cancer and overexpression in many cancers[40]. Overall, total ^{3}H-GLN uptake was also decrease since the sodium-independent portion of the transport is very low compared to the sodium-dependent portion.

This possible effect of quercetin on LAT1 could be an interesting subject to study in the future. If this effect on LAT1 is caused by increased expression of this transporter, quercetin could be used in combination with nanoparticles with chemotherapeutic agents to better target PC cells, since LAT1 has been proven to enhance nanoparticles delivery[160]. However, LAT1 overexpression has also been associated with chemoresistance in some cancer[160], so it would need to be better studied in PC.

In a previous study, quercetin promoted an increase in sodium-dependent and total ^{3}H-GLN uptake by MCF-7 breast cancer cells[139], having an opposite effect than the one we found in this work. However, in MDA-MB-231, quercetin promoted a decrease in sodium-dependent ^{3}H-GLN uptake[139].

Overall, of all the polyphenols tested, chrysin was the one that showed a higher therapeutic potential by promoting a decrease in both ^{3}H-DG and in sodium-dependent ^{3}H-GLN uptake. As mentioned above, the inhibition of glucose uptake can be quite beneficial in cancer therapy, particularly by inhibiting the growth of glycolytic tumors[131].

Besides the inhibition of glucose uptake, chrysin also inhibits glutamine uptake, which is essential for “glutamine addicted” cancer cells. By inhibiting glutamine uptake and consumption by cancer cells, we can inhibit the growth and proliferation of cancer cells. This strategy for cancer treatment is being researched in the last years, with the development of many glutamine uptake inhibitors, but also inhibitors that target enzymes involved glutamine metabolism[159].
Altogether, in this study, we inferred that the AsPC-1 cell metabolism might be more sensitive to the carotenoids than the PANC-1 cells, in which the carotenoids have almost no effect on ^{3}H-DG and ^{3}H-L uptake. These results could be explained by the fact these cell lines are metabolically different, particularly when it comes to glycolysis, where AsPC-1 cells show a higher glycolytic rate than PANC-1[161].

Because, among all tested compounds, crocin and chrysin showed effects on nutrient uptake by AsPC-1 cells with the highest therapeutic potential, we decided to focus our study on further investigating their inhibitory effect of nutrient uptake by AsPC-1 cells, as well as on the possible relationship between this effect and their cytotoxic effect in these cells.

To determine if crocin and chrysin inhibited nutrient uptake by inhibiting their specific transporters gene expression, we performed a RT-qPCR targeting *GLUT1*, *ASCT2* and *MCT1*, which are the main transporters of glucose, glutamine and lactate, respectively. We found that crocin had no significant effect on *GLUT1* and *MCT1* gene expression, and that chrysin had no significant effect on *GLUT1* and *ASCT2* gene expression. This shows that these compounds do not affect nutrient transporter transcription rates.
This could mean that these compounds could target the nutrient transporter protein levels or the proteins directly, hindering their activity and therefore decreasing the amount of nutrients being transported into the cells. This was not further explored in this study but may be interesting to pursue in the future.

With the MTT assay, we determined that both crocin and chrysin promoted a concentration-dependent decrease in AsPC-1cell viability, which goes according to previous studies that obtained similar results in PC cell lines[67; 68; 80-83].

BAY-876 is a selective GLUT1 inhibitor with an IC_{50} value of 2 nM, but it also is able to inhibit GLUT2, GLUT3 and GLUT4 at higher concentrations[162]. NPPB (5-nitro-2-(3-phenylpropylaminobenzoate) is a MCT1 inhibitor with an inhibition index (K_i) of 9 µM, also inhibiting MCT4 at 240 µM[163]. GPNA (L-γ-glutamyl-p-nitroanilide) is a non-selective

competitive glutamine transport inhibitor and is commonly used as a ASCT2 inhibitor, although it also promotes inhibition of LAT1, LAT2, SNAT1 and SNAT2[164; 165].

To further investigate the anticancer effects of crocin and chrysin and if these are related to their effect on nutrient uptake, we assessed the cell viability when AsPC-1 cells were co-treated with BAY-876, NPPB and GPNA. We found that, by themselves, the inhibitors BAY-876 and NPPB promoted a decrease in AsPC-1 cell viability, while GPNA had no effect. This led us to believe that AsPC-1 cells might have a glutamine-independent metabolism, since their viability was unaffected by GPNA. This was previously observed in breast cancer cells, in which MCF-7 cells were found to be insensitive to ASCT2 inhibition with GPNA[166]. When BAY-876 was combined with chrysin or crocin, we found that this combination also promoted a significant decrease in cell viability, but it was not significantly different from the effect of the isolated treatments. The same was found when AsPC-1 cells were treated with NPPB and crocin. Because cells treated with crocin, chrysin or the inhibitors separately (except GPNA) caused a significant decrease in cell viability, that was not different when these compounds were combined, we believe that the cytotoxic effect of crocin and chrysin in the AsPC-1 cell line stems from the same mechanism of action of the inhibitors, i.e. by inhibiting glucose and lactic acid uptake.

Chapter VI – Conclusions

In conclusion, we were able to study the effect of various carotenoids and polyphenols on glucose, lactic acid and glutamine uptake by two PC cell lines, showing that these two PC cells lines have different metabolic sensibilities to the action of these compounds. We also showed that crocin and chrysin had a strong therapeutic potential by inhibiting nutrient incorporation by AsPC-1 cells. However, these compounds had no effect on nutrient transporter gene expression (*GLUT1*, *ASCT2* and *MCT1*). Crocin and chrysin were also found to promote a decrease in AsPC-1 cell viability, in a concentration-dependent manner, which appears to be related to their inhibitory effect on nutrient uptake.

Overall, this study contributes to the clarification of a possible mechanism of action involved in the anticancer properties of carotenoids and polyphenols, as well as of the potential of these compounds as therapeutic agents in PC.

The next logical steps would be to finish characterizing the mechanism of action by which crocin and chrysin inhibit nutrient uptake, but most importantly to determine if this effect is isolated to the AsPC-1 cell line, or if is also observed in other PC cell lines. We could also study a way to deliver these compounds into the pancreatic tumor tissue, since their bioavailability is quite low.

Chapter VII - References

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