ANA MARTA OLIVEIRA E SILVA

IMPACT OF ERUCIN IN KIDNEY CELLS: A MORPHOMETRIC ANALYSIS

Orientadora: Profª. Doutora Ana Sofia Fernandes

Dissertação de Mestrado Integrado em Ciências Farmacêuticas apresentada na Universidade Lusófona de Humanidades e Tecnologias/Escola de Ciências e Tecnologias da Saúde

Universidade Lusófona de Humanidades e Tecnologias

Escola de Ciências e tecnologias Saúde

Lisboa

2023

ANA MARTA OLIVEIRA E SILVA

Impact of Erucin in kidney cells: a morphometric analysis

Dissertação defendida em provas públicas na Universidade Lusófona de Humanidades e Tecnologias no dia 27-09-2023, perante o júri, nomeado pelo Despacho de Nomeação n. º350/2023 de 19 de setembro, com a seguinte composição:

Presidente: Prof. Doutor Luís Monteiro Rodrigues
Arguente: Profª Doutora Regina Menezes
Orientadora: Profª Doutora Ana Sofia Fernandes

Universidade Lusófona de Humanidades e Tecnologias

Escola de Ciências e tecnologias Saúde

Lisboa

2023

Resumo

O carcinoma renal representa mais de 80% dos casos de cancro renal, com aproximadamente 30% dos pacientes a desenvolver metástases após cirurgia.

Estudos recentes relataram uma associação inversa entre o consumo de frutas e vegetais com a redução da probabilidade de desenvolver Carcinoma de células renais.

Como a erucina, um composto comum em vegetais, demonstrou ser capaz de impactar a viabilidade de células cancerígenas, pretendemos avaliar se esta tem impacto a nível morfométrico em duas linhagens celulares distintas, em células não cancerígenas e células cancerígenas de Carcinoma de células renais, nomeadamente, Vero-E6 e 786-0.

Recorreu-se à análise morfométrica com o objetivo de identificar as principais anomalias celulares, por exemplo, formato, tamanho da célula, etc., que possam ser indicativos de alterações do citoesqueleto relacionadas com a capacidade migratória/invasiva das células.

Neste estudo, a erucina demonstrou ter a capacidade de modificar as características morfométricas das células renais. Estes resultados foram mais proeminentes na linha celular Carcinoma de células renais786-0 do que na linha celular Vero não cancerígena.

Embora os modelos celulares e animais sejam ferramentas importantes para decifrar os efeitos e mecanismos da quimio-prevenção, é igualmente fundamental direcionar esforços de pesquisa para estudos de intervenção em humanos a fim de determinar se os mesmos mecanismos se verificam .

Palavras-chave: Erucina, carcinoma renal, glucosinolatos, isotiocianato, morfometria, citosqueleto.

Abstract

Renal cell carcinoma represents over 80% of kidney cancer cases, with about 30% of the patient's developing metastasis after surgery.

Recent research has found ran inverse relationship between the consumption of fruit and vegetables and the risk of Renal cell carcinoma.

Because erucin, a common compound in vegetables, has been described to affect cancer cell viability, we aimed to evaluate if it has any morphometric impact on two distinct cells lines, the non-cancerous cells and cancerous Renal cell carcinoma cells, e.g., Vero-E6 and 786-0.

Morphometric analysis was used to identify key cellular anomalies, e.g., alterations in cell shape, size, etc, which may be indicative of cytoskeletal changes related to migratory/invasive capabilities.

In this study, ER was able to modify morphometric features of Renal cell carcinoma cells, that could be related to their ability to proliferate and form metastasis. These results were more prominent in the Renal cell carcinoma cell line 786-O than in the non-cancerous Vero-E6 cells.

Although cell and animal models are useful tools for studying the effects and mechanisms of chemoprevention, it's also critical to direct research efforts toward human intervention studies to conclude if the same mechanisms occur or whether food-derived compounds work in a completely different way in humans.

Keywords: Euricin, glucosinolates, isothiocyanate, RCC, glucosinolates, isothiocyanates, morphometric, cytoskeleton

List of abbreviations

AITC - Ally ITC

AR – Aspect ratio

AS-ITC - Serum albumin conjugate of ITC

BAP1 - Ubiquitin carboxyl-terminal hydrolase

BITC - Benzyl ITC

ccRCC - Clear cell RCC

CV - Cruciferous vegetables

CDCP1 - CUB-domain containing protein 1

CYS-ITC - cysteine conjugate of ITC

EMT - Epithelial-to-mesenchymal transition

ER – Erucin

ESP - Epithiospecifier protein

GI - Gastrointestinal

GLS - Glucosinolate

Glycys-ITC: glycine-cysteine conjugate of ITC

GPx - GSH peroxidase

GSH - Glutathione

GSH-ITC - Glutathione conjugate of ITC

GSTs - Glutathione S-transferases

HIF- β - Hypoxia-inducible factor β

HIF1-$\propto$ – Hypoxia-inducible factor 1-$\propto$

HIF2-$\propto$ – Hypoxia-inducible factor 2-$\propto$

I3C - Indol-3-carbinol

ITCs - Isothiocyanates

KDM5C - Lysine demethylase 5C

KDM6A - Lysine demethylase 6A

Kelch-like ECH-associated protein 1

MET - Mesenchymal epithelial transition

MRP-1 and MRP-2 - Multidrug resistance proteins 1 and 2

MYR - Myrosinase

Nac-ITC: N-acetylcysteine conjugate of ITC

PBRM1 - Protein polybromo-1

PDGF β -Platelet derived growth factor beta

PEITC - Phenylethyl ITC

PR-ITC: intracelular proteins conjugates of ITC

RET - Rearranged during transfection receptor

RCC- Renal cell carcinoma

ROS - Reactive oxygen species

SETD2 - Histone-lysine N-methyltransferase

SFN - Sulforaphane

SOD - Superoxide dismutase

TGF-$\propto$ - Transforming growth factor alpha

VEGF - Vascular endothelial growth factor

VHL - Von Hippel-Lindau

WHO - World Health Organization

Index

Resumo I
Abstract II
List of abbreviations ..... **III**
Figure Index..... **VII**
Table index ..... **IX**
1.Introduction..... **1**
2.Renal Cell Carcinoma..... **3**
2.1. Epidemiology.....**3**
2.2. Pathophysiology.....**5**
2.3. Can diet have an impact on renal cell carcinoma?.....**9**
3.Glucosinolates (GLS) **11**
3.1. What are they?.....**11**
3.2. Bioavailability **12**
3.3. Physiological effects.....**15**
4.Isothiocyanates.....**16**
4.1. What are they?.....**16**
4.2. Bioavailability **16**
5.Erucin.....**21**
5.1. What is Erucin (ER)? **21**
5.2. Bioavailability **22**
5.3. Bioactivity.....**24**
6. Objectives **27**
7. Methods **28**
7.1. Chemicals **28**
7.2. Cell lines **28**
7.3. Cells treatment.....**29**
7.4. Morphometric analysis **29**

7.5. Statistical Analysis 29
8. Results and discussion 30
9. Final considerations 37
10. Bibliography 39

Figure Index

Figure 1. Representation of the incidence of cancers through all ages, for both genders. Retrieved from Globocan NMSC, non-melanoma skin cancer, by WHO, 2021 (https://gco.iarc.fr/) 4

Figure 2. Representation of the incidence of cancers through all ages, for male gender. Retrieved from Globocan NMSC, non-melanoma skin cancer, by WHO, 2021 (https://gco.iarc.fr/) 4

Figure 3. Representation of the incidence of cancers through all ages, for female gender. Retrieved from Globocan NMSC, non-melanoma skin cancer, by WHO, 2021 (https://gco.iarc.fr/) 5

Figure 4. Representation of RCC stages. Retrieved from: PDQ Adult Treatment Editorial Board. Renal Cell Cancer Treatment (PDQ®): Health Professional Version. 2023 Feb 16. In: PDQ Cancer Information Summaries [Internet]. Bethesda (MD): National Cancer Ins 6

Figure 5. Representation of the consequences of VHL gene mutation. Retrieved from: "Focus on kidney cancer" by W.M.Linehan and B.Zbar, 2004, Cancer Cell (https://doi.org/10.1016/j.ccr.2004.09.006) 7

Figure 6. Molecular structure of GLS. Retrieved from: "Glucosinolate structures in evolution" by Niels Agerbirk ⇑, Carl Erik Olsen, 2012, Phytochemistry. (https://10.1016/j.phytochem.2012.02.005) 11

Figure 7. Detailed representation of the GLS breakdown. Retrieved from "Food as Pharma? The Case of Glucosinolates" by Edoardo Capuano, Matthijs Dekker, Ruud Verkerk and Teresa Oliviero, 2016, Current Pharmaceutical Design. (https:// 10.2174/1381612823666) 13

Figure 8. Representation of the structure of ITCs and several reactions an ITCs can undergo Adapted from "Are isothiocyanates potential anti-cancer drugs?" by Xiang WU, Qing-hua ZHOU, Ke XU, 2009. (https:// 10.2174/138161282366617012016 0832) 18

Figure 9. ITCs can increase the expression of phase II detoxifying and antioxidant enzymes, via Nrf2 activation (A), and thru the prevention the phosphorylation of Nf-kB and ultimately inhibit the transcription of pro-inflammatory genes (B). Retrieved from "The Nrf2 cell defence pathway: Keap1-dependent and -independent mechanisms of regulation" by Bryan h., Olayanju A., Goldring, C., Park B., 2012. (http:// 10.1016/j.bcp.2012.11.016) 19

Figure 10. The two pathways to obtain Erucin in vivo: 1) through the ingestion of rocket salad species and 2) through the reduction of SFN present in broccoli. Retrived from: "Biological Profile of Erucin: A New Promising Anticancer Agent from Cruciferous Vegetables", by Melchini M and Traka Maria, 2010. (http:// 10.3390/toxins2040593).21

Figure 11. Overview of the metabolic pathway of GLS and ITCs. Retrived from: "Isothiocyanates from Brassica Vegetables - Effects of Processing, Cooking, Mastication and Digestion" by Oliviero T., Verkerk R., and Dekker M. 22

Figure 12. Phase contrast photographs of the cell lines used in this work. Left: Vero cell line. Right: 786-O cell line. 28

Figure 13. Results obtained for three morphology parameters observed in the 786-0 cells (A) and Vero cells (B), namely cell area (A1, B1), perimeter (A2, B2), and aspect ratio (A3, B3).....33

Figure 14. Results obtained for two morphology parameters observed in the 786-0 cells (A) and Vero cells (B), namely circumference (A4, B4) and roundness (A5, B5).....34

Figure 15. Alterations in 786-0 cells morphology due to exposure to increasing ER concentrations. 34

Figure 16. Alterations in Vero cells morphology due to exposure to increasing ER concentrations. 35

Table index

Table 1. Biological Profile of Erucin: A New Promising Anticancer Agent from Cruciferous Vegetables. 24

Table 2. Summary of the five morphology parameters measured for Vero cell line after each of the incubation check time with four different ER concentrations. 31

Table 3. Summary of the five morphology parameters measured for 786-O cell line after each of the incubation check time with four different ER concentrations. 31

Table 4. The results obtained from Student's t test (two-sided) analysis for the data obtained for Vero cell line after each of the incubation check time with the different ER concentrations. 32

Table 5. The results obtained from Student's t test (two-sided) analysis for the data obtained for 786-O cell line after each of the incubation check time with the different ER concentrations. 32

1.Introduction

Renal cell carcinoma (RCC) represents the 10th most frequently diagnosed cancer in men and the 14th in women, accounting for 2.7% and 1.7% of all oncologic diagnosis, respectively. (WHO, 2021)

RCC represents over 80% of kidney cancer cases, with about 30% of the patients with RCC developing metastasis after surgery. According to World Health Organization (WHO), there are approximately 179 368 RCC related deaths per year, with RCC as the 16th most common cause of cancer worldwide. (WHO, 2021)

Although smoking, obesity, hypertension, and chronic renal disease are constant risk factors for RCC are, the origin of RCC is still fairly unclear. (Capitanio et al., 2018; Scelo & Larose, 2018)

The discovery of renal masses during abdominal imaging for nonspecific musculoskeletal or gastrointestinal problems has led to a rise in RCC incidence rates, especially first world countries. Although the majority of identified lesions are tiny tumors, a significant number of patients are diagnosed with locally advanced illness, with up to 17% of patients having distant metastases at the time of diagnosis. (Capitanio et al., 2018; Hsieh et al., 2018; Rossi, Klatte, Usher, & Grant, 2018)

Migration and adhesion of tumor cells are essential pre-requisites for the formation of metastases in malignant diseases. For a cancer cell to be able to disseminate and form metastases it requires cell abnormalities and also alterations induced within the proximal cells in a complex process that culminates with the entrance into the bloodstream or lymphatic circulation until it achieves a potential secondary tumor site, forming a metastasis. (Fares, Fares, Khachfe, Salhab, & Fares, 2020)

A recent meta-analysis of 13 prospective studies found that eating fruit and vegetables was related to a lower risk of RCC, and carotenoids found in fruits and vegetables may partly contribute to this protection. (Soundararajan & Kim, 2018)

Cruciferous vegetables (CV) are a term that includes cabbage, broccoli, cauliflower, brussels sprouts, and other vegetables with cross-shaped blooms. CV are high in sulfur-containing substances called as glucosinolates (GLS), precursors to products, such as isothiocyanates (ITCs) and indol-3-carbinol (I3C), that may help to minimize the risk of RCC. (J. Zhao & Zhao, 2013) ITCs have been reported to help prevent cancer by enhancing programmed cell death and autophagy, increasing the

transcription of tumor suppressor proteins, plus those previously silenced by epigenetic mechanisms and stimulating the ROS. (Higdon, Delage, Williams, & Dashwood, 2007)

One compound that has been identified as being capable to selectively impact cancer cell growth is 4-(methylthio) butyl isothiocyanate or erucin (ER). ER is extracted from rocket salads (from the family *Brassicaceae*) and extensively used in the Mediterranean diet and with increasing consumption globally, stimulated by the positive existing link that eating fresh raw food is good to facilitate the absorption of health-promoting phytochemicals.(Antonietta Melchini & Traka, 2014) ER has also been shown to react with H_2O_2 and alkyhydroperoxides to form water and an alcohol and thus to be a potent inducer of cellular antioxidants systems resulting in the reduction of ROS levels back to normal ones. (Antonietta Melchini & Traka, 2014)

It is increasingly important to understand the mechanisms by which the Mediterranean diet with its many dietary agents may prevent or retard the process of carcinogenesis, to successfully translate the laboratory findings to clinical approaches. (Antonietta Melchini & Traka, 2014) Lyons et al. defend that because cytoskeletal modifications are important for acquiring invasive capability, modest changes in cellular characteristics should express themselves in small but noticeable morphologic changes (e.g., shape). (Lyons et al., 2016) With this study we hope to demonstrate the relationship between the capacity of ER to decrease cell migration, with the altered cytoskeletal properties. (Lyons et al., 2016)

2.Renal Cell Carcinoma

2.1. Epidemiology

RCC ranks as the 16th most frequent cancer in the world (Fig. 1), and is the 10th most frequently diagnosed cancer in men (Fig. 2) and the 14th in women (Fig. 3), accounting for 2.7% and 1.7% of all new oncologic diagnosis worldwide, correspondingly. (WHO, 2021)

RCC represents over 80% of kidney cancer cases, and about 30% of the patients with RCC develop metastasis after surgery. Patients with RCC are at a higher risk of suffering some form of metastasis that can occur in one of three ways: (1) cancer cells can spread into the tissue around the tumour in the kidney; (2) cancerous cells can move into the lymph system and lastly (3) cancerous cells can enter the bloodstream and being deposited to another organ. (Kanwal, 2023)

RCC incidence rates have been rising, particularly in industrialized nations, due to an increase in the diagnosis of renal masses during abdominal imaging for nonspecific musculoskeletal or gastrointestinal symptoms. Although the majority of identified lesions are tiny tumors, a significant number of patients have locally progressed illness, with up to 17% of patients having distant metastases at the time of diagnosis. (Siegel & Miller, 2019)

Risk factors for RCC include hypertension, tobacco, obesity, and occupational exposure to known carcinogenetic chemicals such as trichloroethylene, which can lead to the development of RCC and increased mortality. Nonetheless, the etiology of RCC is largely unknown. (Capitanio et al., 2018; Capitanio & Montorsi, 2015; Rene, 2000)(Capitanio et al., 2018; Capitanio & Montorsi, 2015; Rene, 2000)

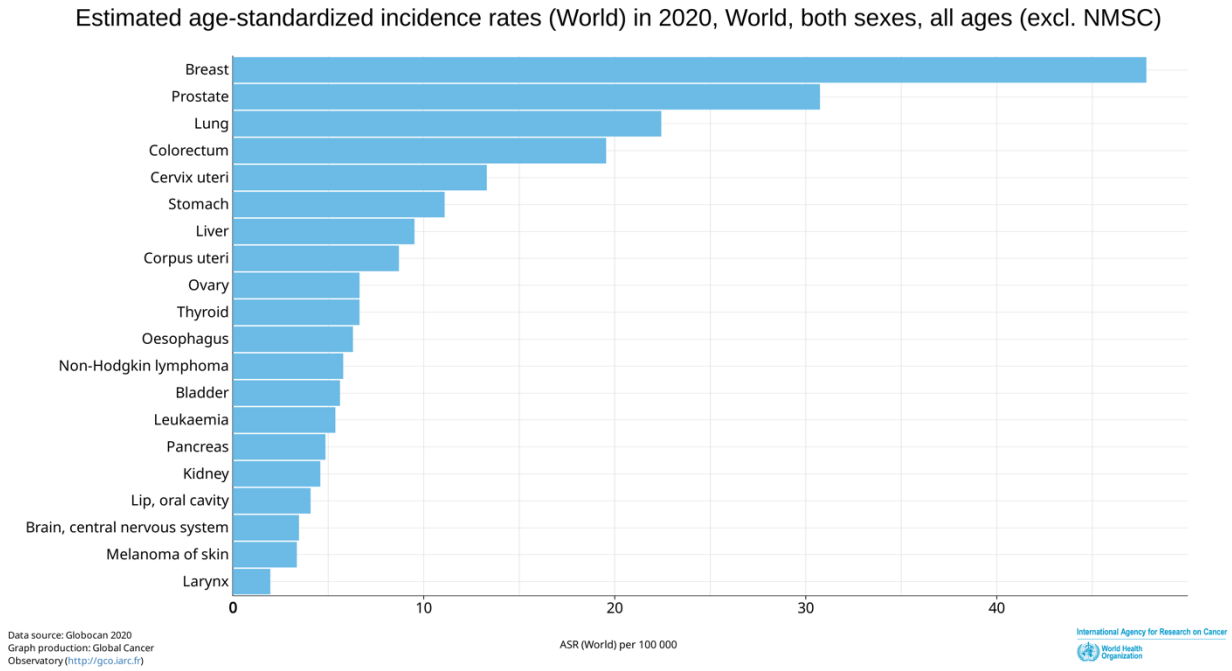

Figure 1. Representation of the incidence of cancers through all ages, for both genders. Retrieved from *Globocan NMSC, non-melanoma skin cancer, by WHO, 2021 (https://gco.iarc.fr/)*

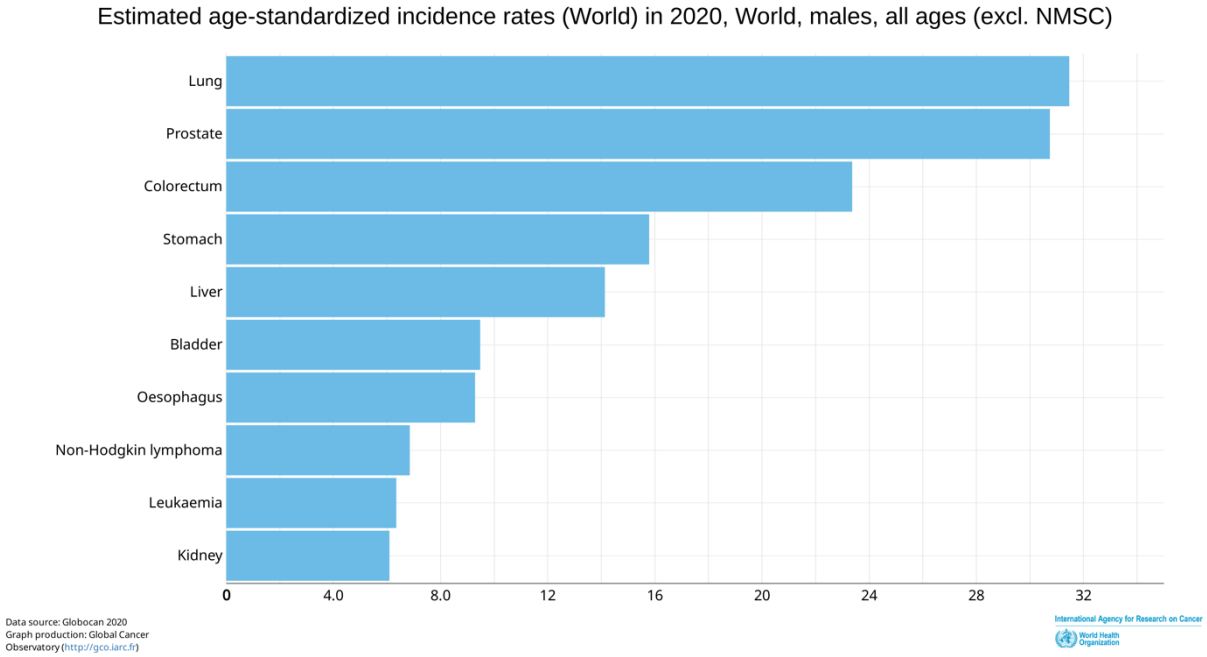

Figure 2. Representation of the incidence of cancers through all ages, for male gender. Retrieved from *Globocan NMSC, non-melanoma skin cancer, by WHO, 2021 (https://gco.iarc.fr/)*

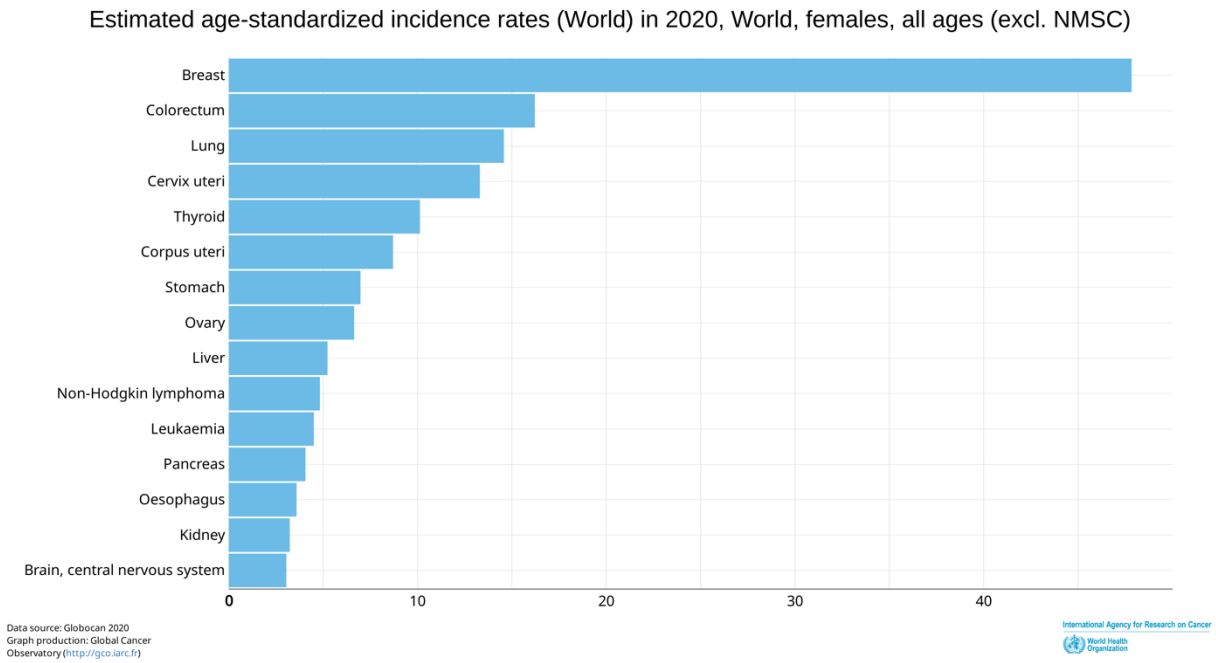

Figure 3. Representation of the incidence of cancers through all ages, for female gender. Retrieved from *Globocan NMSC, non-melanoma skin cancer, by WHO, 2021 (https://gco.iarc.fr/)*

2.2. Pathophysiology

RCC (Fig. 4) as previously mentioned is heterogeneous in its histology, origin of cell and driver mutations, being the most common subtypes of RCC the following: clear cell RCC (ccRCC), papillary RCC and chromophobe RCC. (Muglia & Prando, 2015; Srigley et al., 2013)

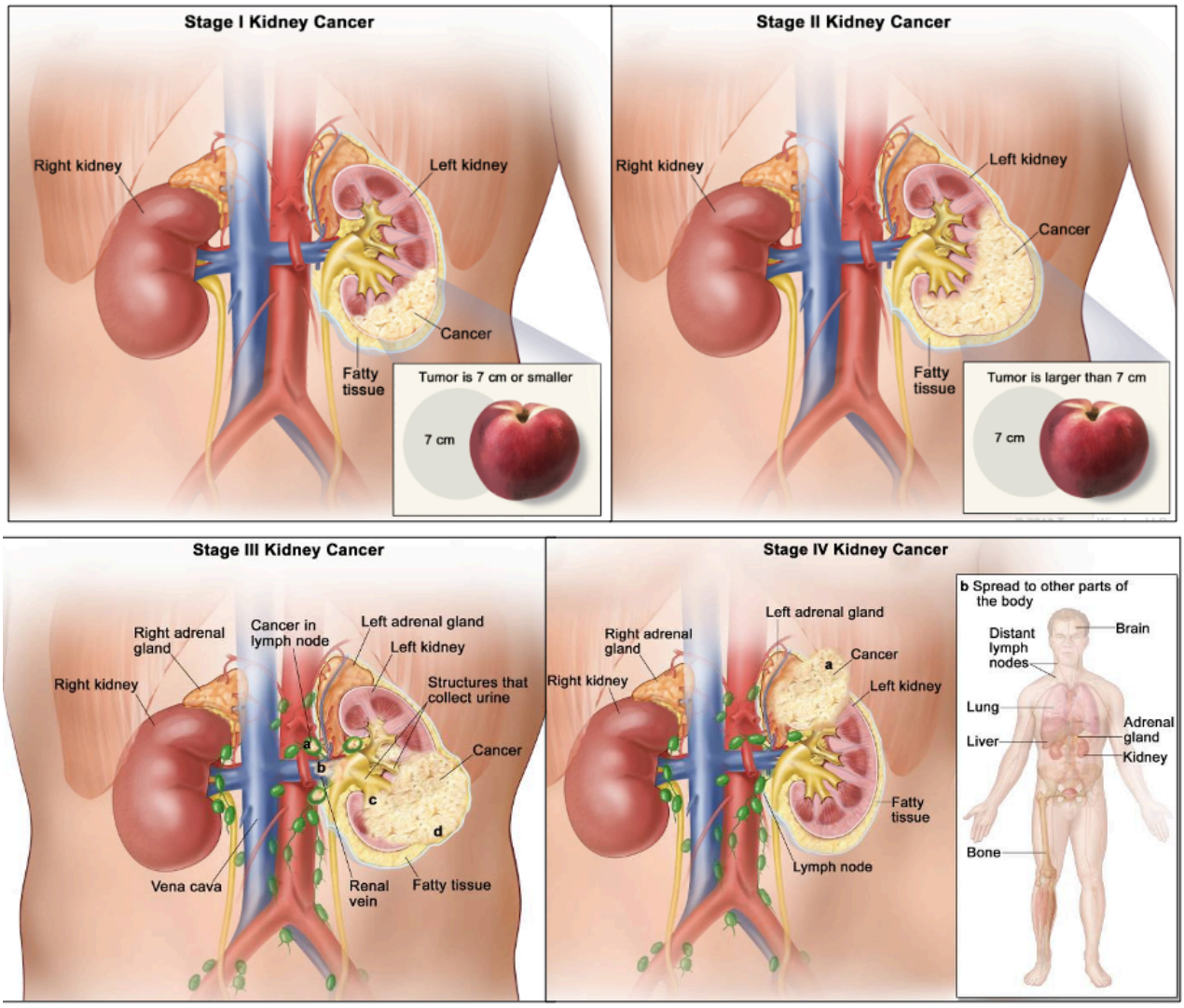

Figure 4. Representation of RCC stages. Retrieved from: *PDQ Adult Treatment Editorial Board. Renal Cell Cancer Treatment (PDQ®): Health Professional Version. 2023 Feb 16. In: PDQ Cancer Information Summaries [Internet]. Bethesda (MD): National Cancer Ins*

The most common pathophysiologic mechanism for the emergence of RCC, especially the ccRCC is the mutation of the Von Hippel-Lindau (VHL) gene on chromosome 3. The VHL gene (Fig. 5) is a tumor suppressor gene, therefore when it suffers mutation either by deletion due to cytogenetic abnormalities or due to somatic mutations, there is an increased probability to develop RCC. The VHL gene has information for a protein called pVHL, which acts as a tumor suppressor protein. This protein tends to form complexes with other proteins present in the cell, which ultimately results in the proteasomal degradation of several intracellular proteins. The pVHL also regulates the levels of several intracellular proteins, namely hypoxia-inducible factor 1-∝ and 2-∝ (HIF1-∝ and HIF2-∝). When these two proteins bind with each other, they act as transcriptions factors by biding to the DNA, promoting the transcription of several

growth factors, for example the vascular endothelial growth factor (VEGF), the platelet derived growth factor beta (PDGFB) and the transforming growth factor alpha (TGFA). (Curran & Kopp, 2021; Hsieh et al., 2018; Liao et al., 2022; Nabi, Kessler, Bernard, Flaig, & Lam, 2018)

When there's a normal concentration of oxygen in the cells, HIF1-∝ and HIF2-∝ are hydroxylated and attach with pVHL, consequential in the polyubiquitination of HIF-∝, making it a target for the pathway of proteasomal degradation. When there's a decrease of the usual concentration of oxygen, e.g., hypoxia, or the absence of the pVHL, the natural hydroxylation of HIF1-∝ and HIF2-∝ does not occur. Therefore, it is possible to verify an accumulation of HIF-∝, in the cell that dimerizes with the hypoxia-inducible factor β (HIF- β), forming the complex HIF-∝ - HIF-β. This complex then migrates to the nucleus where it acts up as a transcription factor, culminating in the increase of the mRNA levels and consequently the levels of VEGF, PDGF β and TGF-∝ and other controlling proteins, promoting the growth of the RCC (Figure 5). (Curran & Kopp, 2021; Hsieh et al., 2018; Liao et al., 2022; Nabi et al., 2018)

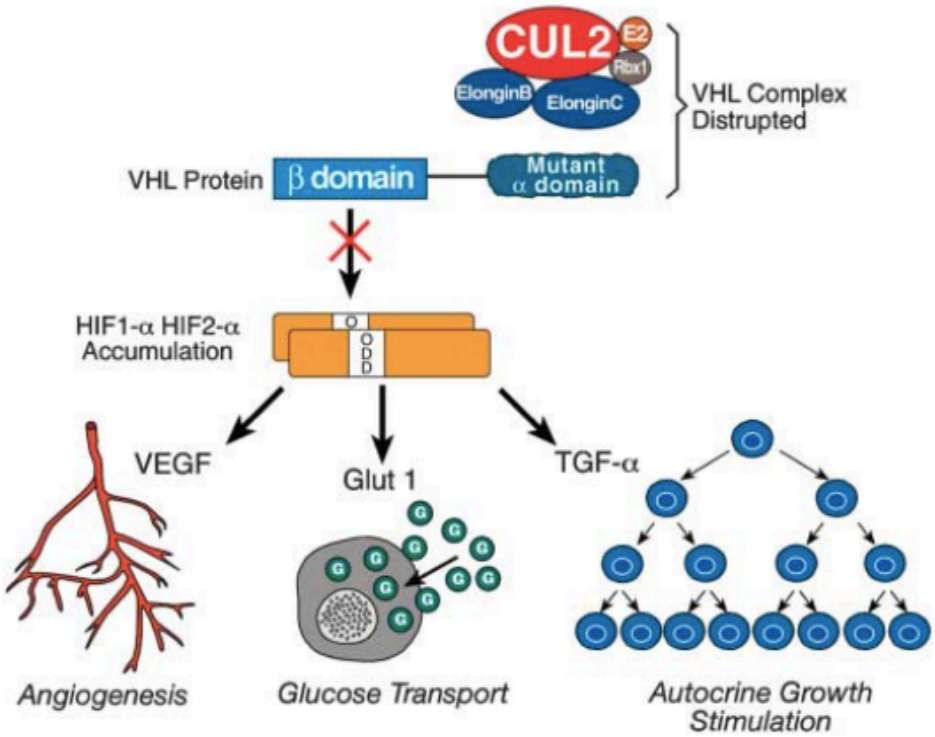

Figure 5. Representation of the consequences of VHL gene mutation. Retrieved from: "*Focus on kidney cancer" by W.M.Linehan and B.Zbar, 2004, Cancer Cell (https://doi.org/10.1016/j.ccr.2004.09.006)*

VHL protein promotes the production of genes involved in angiogenesis and invasion. Since in RCC this protein complex becomes defective (Fig.5) and fails to

perform its normal function, it leads to HIF-1α accumulation in cells and stimulates hypoxia-related genes such as VEGF and others which are themselves involved in angiogenesis and tumorigenesis, ultimately resulting in metastasis. (Jr, 2008)

The process of metastasis formation is complex and consists of the spreading of cancer cells from the primary or initial location of the disease to remote regions of the body. Nearly every part of the body can be reached due to the migration capability that cancer cells possess, which enables them to spread from the initial location through the bloodstream or the lymphatic vessels. There is little agreement on the biological processes that cause RCC metastasis. Grange et al., suggested that on a macromolecular level, tumor-derived microvesicles, that essentially split off from the primary tumor site, may help spread tumors via hematogenous routes. In fact, it has been found that these microvesicles appear to contain CD105-positive cells with a cancer stem cell profile as well as microRNas that promote angiogenesis.(Grange et al., 2011)

The liability of the immune system also plays a significant part in the formation of metastases, with several studies associating the development of metastases in various organs of the body with a decreased immune cells activity. (Finke et al., 2011)

Additionally, several molecular mediators of RCC metastasis have been discovered, such as CUB-domain containing protein (CDCP1) which has been suggested to promote metastasis in clear cell RCC with VHL alteration. CDCP1 is a Src family kinase substrate that has been shown to control anoikis resistance, migration, and matrix breakdown during tumor invasion. CDCP1 is regulated by a major transcription factor HIF-1, that controls the expression of dozens of genes involved in maintaining homeostasis, as concentration levels of oxygen change. (Uekita & Sakai, 2011) As CDCP1 is regulated by HIF-1, it leads to the activation of protein kinase C-d, which promotes cellular motility. (He, Briez, Bernard, Seuningen, & Leroy, 2009)

According to Ricketts, CJ. et al, most of clear cell RCC patients have chromothripsis of chromosome 3P, which results in the loss of 3p and as a result, in the inactivation of VHL protein, due to the impairment of its ubiquitin ligase function.(Ricketts et al., 2018; Sato et al., 2013)

Activated mammalian target of rapamycin (mTOR) pathways play an equally important part in tumorigenesis. mTOR pathways is made of two structurally and functionally different protein complexes, the mTOR complex 1 and the mTOR complex 2. mTOR pathways are involved in the regulation of cell viability, cell development, cell metabolism, protein synthesis, autophagy, and homeostasis. In other words, mTOR

pathways regulate many basic biological and physiological processes. In the case of the RCC that usually involves the loss or mutations of genes that themselves regulate mTOR it leads to aberrant mTOR signaling and the hyperactivation mTOR pathway, and finally to tumor initiation and progression. (Tian, Li, & Zhang, 2019)

The lack of p53 expression, a well-known tumor suppressor gene, and the mutant phosphatidylinositol 3-kinase (PI3K), which checks the availability of nutrients, mitogenic signals and cellular energy and oxygen levels, are two variables that may lead to mTOR pathway hyperactivation. (Gong, Caitano, Dizman, Govindarajan, & Pal, 2016)

There are other genes that contribute to cancer and RCC development as for example Ubiquitin carboxyl-terminal hydrolase (BAP1), protein polybromo-1 (PBRM1), histone-lysine N-methyltransferase (SETD2), lysine demethylase 5C (KDM5C) and lysine demethylase 6A (KDM6A). (Jr, 2008)

When metastasis in RCC occurs, it typically affects the pancreas, adrenal gland, bile, liver, lymph nodes, bones, brain, and gallbladder. Once RCC has spread, it is important to understand that different locations of metastasis may have different implications for prognosis. Patients with metastasized RCC have a survival rate up to eight months, with half succumbing within a year. Only about 10% of people with metastasis survive for more than five years. Up to date, cytokine immunotherapy was used as a first line medication. Nowadays, drugs that target VEGF and mTOR, bevacizumab and everolimus for example, have demonstrated higher efficacy and progression-free mortality. Targeted therapies, like tyrosine kinase inhibitors and some contemporary approaches include anti-mesenchymal epithelial transition (MET) and anti-rearranged during transfection receptor (RET.) (Gong et al., 2016)

2.3. Can diet have an impact on renal cell carcinoma?

Consumption of vegetables and fruits have always been thought to be beneficial to one’s health. Some vegetables and fruits were historically utilized as medication, but they are now thought to serve as a protective function in the genesis of different illnesses such as cancer and coronary heart diseases. (Verhoeven, Goldbohm, Poppel, & Verhagen, 1996)

Several studies refer to the important role, vegetable secreted compounds play in the inhibition of the enzymes involved in the carcinogen-activation, the activation of carcinogen-detoxifying enzymes, the induction of apoptosis and cell cycle progression.

(Chinni, Li, Upadhyay, Koppolu, & Sarkar, 2001; Cover et al., 1998; Kramer, 1997; Y. Zhang, 2004)

Zhang et al. discovered that the protective impact of vegetables was reduced after controlling the mass body index, suggesting that obesity may be an essential intermediary link between vegetables intake and the prevalence of RCC. Indeed, obesity has been identified as a risk factor for RCC by the European Association of Urology. (S. Zhang, Jia, Yan, & Yang, 2017)

Liao et al study found that vitamin C consumption is negatively related with the incidence of RCC. Vitamin C by being a ten-eleven translocation (TET) gene's coenzyme, helps to maintain the levels of 5-hydrxymethylcystosine (5hmc) which is linked to RCC tumorigenesis and RCC relapse. (Liao et al., 2022)

CV such as cabbage, broccoli, and cauliflower, are one type of vegetables that have been used for curative reasons since the prehistoric era and is nowadays thought to be cancer protective. (Verhoeven et al., 1996) CV have high concentrations of ITC, SFN and I3C, which several studies have found to have protective and anticancer properties, which will be detailed further in the next chapters. (Liao et al., 2022)

3.Glucosinolates (GLS)

3.1. What are they?

GLS are a big set of secondary biologically active metabolites with nourishing benefits, mostly found in a set of vegetables called cruciferous for their cross-shaped flowers, including cabbage, broccoli, cauliflower, brussels sprouts, and others CV. (J. Zhao & Zhao, 2013)

GLS belong to a natural group of organic compounds that contains sulfur and nitrogen, which derived from glucose and an amino acid. Each GLS (Fig.6) has a core carbon central atom that is connected to a thioglucose group by a sulfur atom; and bound to a sulfate group via a nitrogen atom. Furthermore, the core carbon is frequently attached to a changeable side group, accountable for the observed variation in biological activity. (Agerbirk & Olsen, 2012)

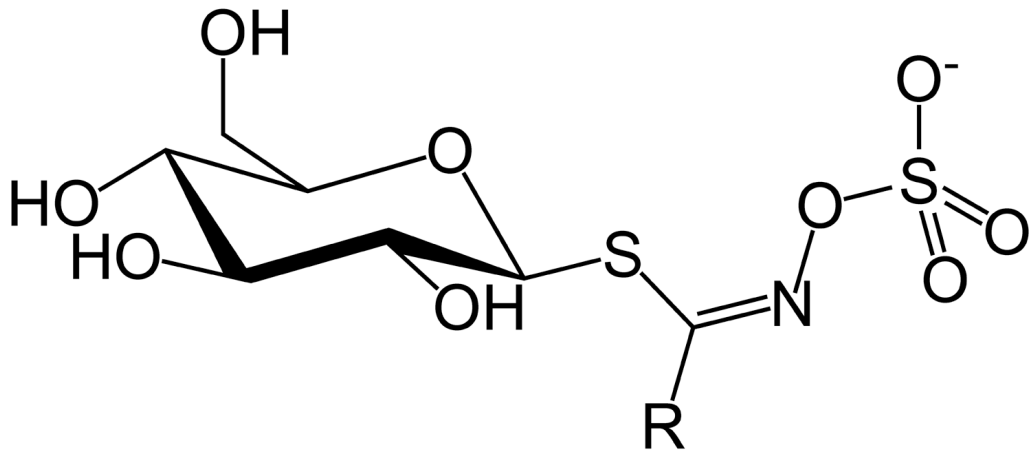

Figure 6. Molecular structure of GLS. Retrieved from: "*Glucosinolate structures in evolution" by Niels Agerbirk ⅄, Carl Erik Olsen, 2012, Phytochemistry. (https://10.1016/j.phytochem.2012.02.005)*

There are over 100 identified different GLS, grouped into four main groups: saturated aliphatic, unsaturated aliphatic, aromatic and indolyl.

Many of the physiological benefits proposed for the brassica vegetables in many researches, including in vitro, animal, human and epidemiological investigations are attributed to GLSs. (Capuano, Dekker, Verkerk, & Oliviero, 2017)

3.2. Bioavailability

The reactions needed to form each of the GLS products shown in figure 7, are complex and depend on the combination of several factors, e.g., the glucosinolate, temperature, pH and presence or absence of reducing agents such as ferrous ion. (Oliviero, Verkerk, & Dekker, 2018; Pdbiad, 2008)

In most cases in the presence of myrosinase (MYR), the glucosidic bond of GLS is hydrolyzed, forming an unstable aglycone while the glucose is released. (Fig.7) The aglycone fragments undergo a spontaneous reorganization (a Lossen chemical rearrangement) which ends in the elimination of the ion SO_4^{2-} resulting in the formation of metabolites, whose structures depend on the nature of the R chain of the GSL. While indolyl ITCs also eliminate SO_4^{2-} they undergo a different transformation process that results in thiocyanates. (Capuano et al., 2017)

All the aglycones, especially those formed under specific conditions such as the presence of reducing agents like ferrous ions and/or ascorbate and at lower pH values, eliminate SO_4^{2-} and H_2S, forming nitriles.

It has been demonstrated that the presence of epithiospecifier protein (ESP) promotes aglycone conversion to nitriles or epithionitriles. It is also known that the presence of ferrous ion as well as pH affects ESP action. Inactivation of ESP, for example, leads to increased ITC production under acidic (pH 4) or alkaline (pH 8) circumstances. If ESP is missing or inactive, nitrile production is preferred at low pH, whereas ITC creation rises at neutral or alkaline circumstances. (Oliviero et al., 2018)

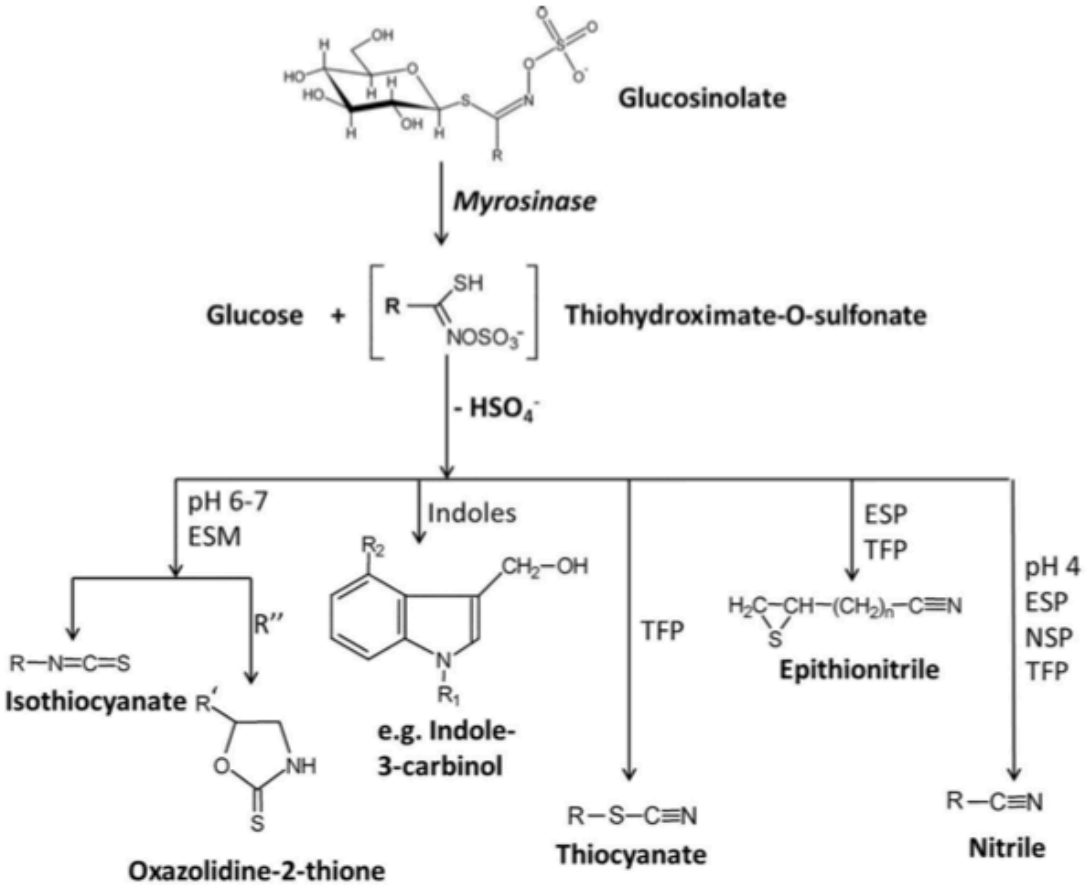

Figure 7. Detailed representation of the GLS breakdown. Retrieved from *"Food as Pharma? The Case of Glucosinolates" by Edoardo Capuano, Matthijs Dekker, Ruud Verkerk and Teresa Oliviero, 2016, Current Pharmaceutical Design. (https:// 10.2174/1381612823666)*

Rouzard et al. say that when large intact leaves, or florets of broccoli or cauliflower are eaten raw, both intact GLS and active MYR are ingested simultaneously, enabling the breakdown of GLS to occur within the alimentary tract and consequently increasing the conversion of GLS to ITCs and their bioavailability. (Johnson, 2003)

Cooking has been shown to promote the thermal breakdown and leaching of GLS and consequently decrease its concentration, while it also inhibits the activity of the enzyme MYR since high temperatures (above 80ºC) are known to cause the denaturation of proteins. According to Song L. and Thornalley P, during boiling of CVs the drastic losses of GSLs vary from 5% to 75% and consequently affects the concentration of secondary products. Newer studies, advice the choice of additional cooking processes such as steaming, microwaving or stir-frying, since the techniques do not induce such drastic losses of GSLs. (Song & Thornalley, 2007)

Due to the high importance of assuring the optimal characteristics and therefore beneficial effects for health, Dekker and co-workers have even developed a mathematical model describing these effects, allowing the possibility to quantify the losses of GLS and

its products, during storage, processing, and preparation of vegetables. (Dekker et al. 2000)

Understanding the absorption pathways of GLS its metabolites is crucial, but compared to other dietary bioactive chemicals, there is less evidence on how, where, and to what degree GLS and its hydrolysis products are freed, absorbed, distributed, metabolized, and excreted in humans. (Barba, 2016)

Once the GLS are consumed, some of them are absorbed immediately in the stomach, while the majority are absorbed in the small intestine, where, according to several studies, a tiny percentage of GLS can also be absorbed by the lining of the small intestine. In vivo, this absorption results in up to 5% of the ingested dosage being present in urine. The non-absorbed GLS compounds that may exist in the proximal intestine can still be hydrolyzed by the portion of MYR that remains in the plant consumed or by the bacterial microflora. (Barba, 2016)

Previous studies have found that the excreting of GLS metabolites fails to negligible levels when the numbers of colonic bacteria are reduced due to antibiotics. (Barba, 2016) Meaning that the bacterial microflora of the human colon also expresses some sort of MYR, activity that catalyzes the formation of a wide range of metabolites, depending on the pH and presence of cofactors. Getahun and Chung and Shapiro et al, found significant quantities of ITCs metabolites in urine of healthy human volunteers even after eating CV fully cooked and therefore with an inactivated myrosinase. (Barba, 2016)

By inoculation of previously germ-free rats with a strain of *Bacteroides Thetaiotaomicron* from human faeces capable of degrading GLS, it was found that it was possible to observe an increase in the excretion of GLS metabolites. A clear confirmation that bacteria with a MYR activity can breakdown GLS and lead to the formation of ITCs and presumably explain why even after eating cooked CV, the GLS metabolites like ITCs, can be found. (Barba, 2016)

Since the bioavailability of GLSs is intricately connected to their bioactive hydrolysis products, it will be further described in detail in the ITCs chapters.

3.3. Physiological effects

The high amount of GLS in CV, whether alone or in conjunction with other substances, is thought to be what distinguishes them from other vegetables in terms of chemoprevention. (Verhoeven et al., 1996) GLS and its breakdown products have been intensively studied for their potential health benefits.

Initially, research on GLS concentrated on the poisonous, anti-nutritive and goitrogenic qualities of the GLS compounds. GLS are precursors of other compounds, such as ITCs and I3C which have been shown in recent researches to have good health benefits against a variety of chronic diseases, including the risk of RCC. (Capuano et al., 2017; J. Zhao & Zhao, 2013)

Recent reports have identified GLS and their products as potent cancer-prevention agents in a variety of animal models, owing to their ability to inhibit metabolic phase I, primarily through the suppression of cytochrome P450 enzymes, which themselves metabolize and thus activate many carcinogenic agents. (Ahuja et al., 2010; Esteve, 2020)

In vitro studies, have shown that GLS and their metabolites can trigger phase II detoxification enzymes, as quinone reductase, glutathione-S-transferase and glucuronosyl transferases. (Rose, Ong, & Whiteman, 2005)

4.Isothiocyanates

4.1. What are they?

Isothiocyanates (ITCs) are known to be a set of hot and bitter substances, usually termed mustard oils and are probably the most thoroughly studied of the GLS products. (Fenwick, Griffiths, & Heaney, 1983)

ITCs are the hydrolysis products of GLS, usually found in CV such as broccoli, rocket salad, cauliflower, cabbage, and brussels sprouts. (Stewart & Mcdougall, 2012)

ITCs are also responsible for the plant sharp taste and ultimately its defense structure. The different species of CV generate ITCs in different proportions, and consequently different flavors. Overall ITCs are reactive compounds, mainly due to the electron-deficient central carbon atom that easily suffers a nucleophilic attack. (Stewart & Mcdougall, 2012)

4.2. Bioavailability

Salad species consumption has increased in the recent decade because to its health advantages, leading to a rise in more in-depth investigations of numerous vegetables and their key chemicals, including GLS and ITCs.

Once ITCs are ingested or formed in the lumen of the gastrointestinal (GI) tract, they cross the GI epithelium, and the capillary endothelium cells by passive diffusion. Once absorbed, in the liver ITCs are rapidly conjugated to glutathione-by-glutathione S-transferases (GSTs), a class of phase II detoxification enzymes. The conjugated compound is then consecutively metabolized by the mercapturic acid pathway, meant to increase the solubility of ITCs, and encouraging a faster excretion in the urine.(Barba, 2016) Thus, ITCs absorption may be measured by quantifying the ITC conjugates discharged in urine, both as a proportion created by MYR contained in the vegetable and as a fraction formed by MYR-like activity of the intestinal bacteria.(Oliviero et al., 2018)

It has been disclosed that the overall average bioavailability of ITCs in raw and cooked cruciferous vegetables, are 61% and 10%, respectively.(Vermeulen, Berg, Freidig, Bladeren, & Vaes, 2006) Several studies have found that the concentration of ITCs conjugates excreted in urine and/or blood varies depending on the kind of broccoli consumed: (1) raw broccoli where MYR is still active; (2) cooked broccoli where MYR

is inert; and (3) crushed broccoli where ITCs are already produced prior to consumption. These results confirmed that ITCs excretion is increased when broccoli sprouts containing active MYR are consumed. (Clarke et al., 2011)

4.3. Bioactivity

In the beginning, ITCs were thought to have no health benefit effect since it is a GLS product, and some anti-nutritional or goitrogenic GLS were discovered in domesticated crops. Goitrogenic GLS, such as goitrin and organic nitriles, are known to block thyroid iodine absorption, enlarge the thyroid, and lower circulating thyroid hormone levels.. (Heaney & Fenwick, 1995) But according to Eun-ji Choi et al, no significant differences between the T3 and T4 concentrations were found, neither in the control nor the treatment groups. Thus more studies are needed to determine the effect of GLSs on thyroid hormones. (Choi, Zhang, & Kwon, 2014) Fortunately, recent studies have reported and demonstrated the therapeutic and prophylactic properties of these compounds.

ITCs have powerful antioxidant, anti-inflammatory, anti-microbial, neuroprotective, and cardioprotective properties. Various studies have found that ITCs have health advantages in conditions such as cancer, cardiovascular disease, and diabetes. The pharmacological benefits of ITCs are attributed to the electrophilic nature of the carbon residue (Fig. 8), which reacts with biological nucleophiles and nutrients such as amines, amino acids, proteins, thiols, and alcohols to produce a variety of compounds such as thioureas and dithiocarbamates. (Fimognari, Turrini, Ferruzzi, Lenzi, & Hrelia, 2012)

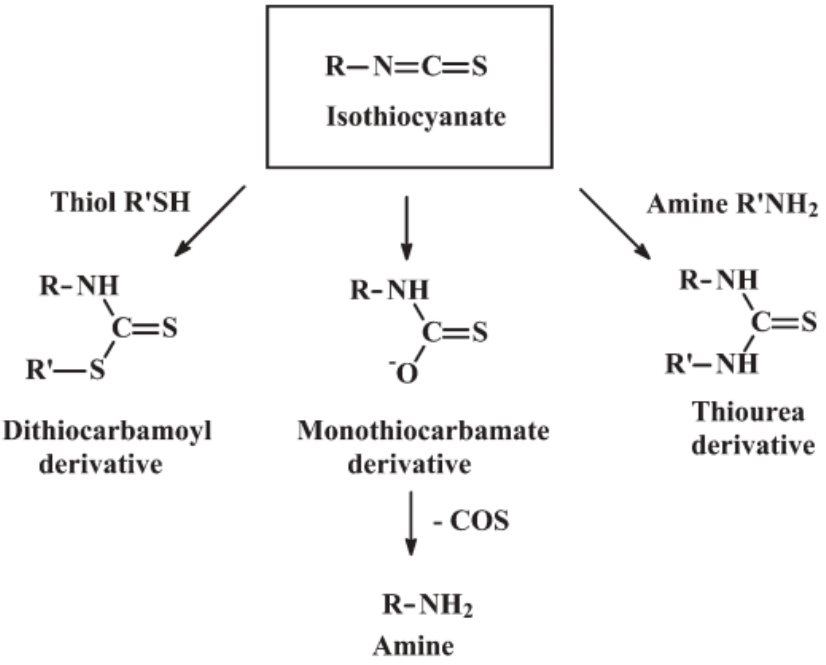

Figure 8. Representation of the structure of ITCs and several reactions an ITCs can undergo Adapted from *"Are isothiocyanates potential anti-cancer drugs?" by Xiang WU, Qing-hua ZHOU, Ke XU, 2009. (https:// 10.2174/138161282366617012016083 2)*

By far the most analyzed ITC is sulforaphane (SFN), followed by phenylethyl ITC (PEITC), benzyl ITC (BITC) and ultimately ally ITC (AITC). SFN is structurally related to ER, and several studies suggest that it can affect selectively cancer cell growth hence the reason for the ER to be the focus of this present work (Mastuo, Miyata, Yuno, Mukae, & Otsubo, 2020; Ullah, 2015)

A possible inhibitory activity of isothiocyanates against tumorigenesis appears to stem primarily from their ability to influence phase 1 and 2 biotransformation enzyme activities, thereby influencing several processes related to chemical carcinogenesis, such as carcinogen metabolism and DNA binding. (Higdon et al., 2007)

By far the most studied mechanism is Nrf2 mediated signaling pathway, with SFN being one of the strongest naturally occurring inducers of the Kelch-like ECH-associated protein 1 (Keap-1), which can up-regulate several protective genes, due to the activation of the nuclear translocation of Nrf2. The upregulated genes include most antioxidant and chemopreventive enzymes that protect against oxidative damage (Fig.9). (Bryan, Olayanju, Goldring, & Park, 2012)

Dong Z. et al discovered that SFN could significantly reduce nuclear translocation of the pro-inflammatory transcription factor nuclear factor (NF)-kB in the cells of the pancreas of a mouse by lowering the expression of NF-kB target genes that contain

information for pro-inflammatory mediators like tumor necrosis factor, interleukin-1, and interleukin-6. SFN, through inhibiting of the NF-kB pathway, also inhibits the inflammatory response, which includes the enzymes cyclooxygenase-2, prostaglandin E synthase, and inducible nitric oxide synthase. (Dong et al., 2016a)

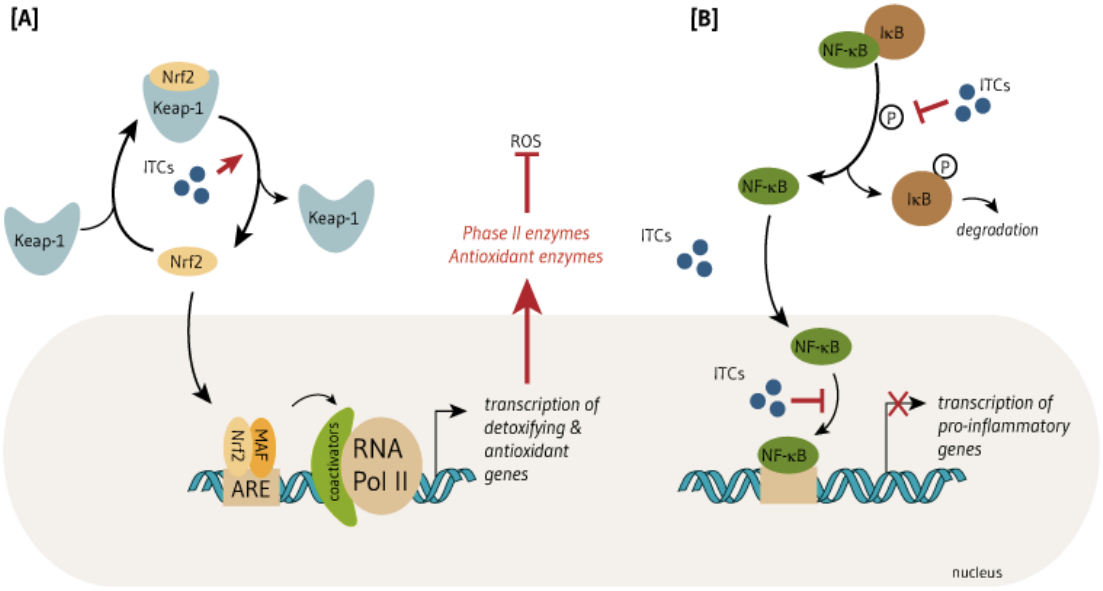

Figure 9. ITCs can increase the expression of phase II detoxifying and antioxidant enzymes, via Nrf2 activation (A), and thru the prevention the phosphorylation of Nf-kB and ultimately inhibit the transcription of pro-inflammatory genes (B). Retrieved from *"The Nrf2 cell defence pathway: Keap1-dependent and -independent mechanisms of regulation" by Bryan h., Olayanju A., Goldring, C., Park B., 2012. (http:// 10.1016/j.bcp.2012.11.016)*

ITCs have no selectivity towards normal or tumor cells. Therefore, in the case of Nrf2's hijacked by cancerous cells, a favorable redox balance ideal for the cells to acquire malignant properties, e.g, chemoresistance will be achieved. (Giudice & Montella, 2006; Shelton & Jaiswal, 2012) This behavior is called a hormetic dose response, a term that toxicologists usually use to refer to a "biphasic dose response to an environmental agent characterized by low dose stimulation and by high dose inhibitory or toxic effect."

According to Yongping et all., SF exhibited a hormetic dose response on cell growth, migration, and angiogenesis, which means that a lower concentration SF has a positive effect on cell growth, migration and angiogenesis be either of normal or carcinogenic cells, while in higher concentrations the opposite effect is observed. (Bao, Wang, Zhou, & Sun, 2014)

SFN was proven in multiple animal models to protect several tissues and organs by activating the Nrf2/ARE-dependent pathway (Esfandyari, Aleyasin, Noroozi, Sc, & Taheri, 2021) SFN, for example, decreased contrast agent-induced kidney injury in rats

by boosting Nrf2 nuclear translocation and raising HO^{-1} and NQO^{-1} expression. (Z. Zhao et al., 2016) SFN-mediated Nrf2 pathway activation also reduced oxidative damage-induced vascular endothelial cell injury in a rat model of type 2 diabetes mellitus.(Wang et al., 2014)

Pre-treatment with SFN restricted the decrease in glutathione (GSH) and the antioxidant enzymes, superoxide dismutase (SOD) and GSH peroxidase (GPx) in a rat model of hepatic ischemia reperfusion injury, where cellular damage was produced by the restoration of oxygen flow to a hypoxic liver. SFN also increased Nrf2, HO^{-1}, and NQO^{-1} expression while also decreasing ischemia death and apoptosis in liver cells. (Chi, Zhang, & Shen, 2015)

In a number of animal models, naturally occurring ITCs and their products have been demonstrated to suppress the development of chemically induced-malignancies of several organs including the lung, liver, esophagus, stomach, small intestine, colon, and breast. (Kumar, Tuli, Mittal, & Shandilya, 2015) Although observational studies show that increased intake of CV is associated with lower cancer risk in people, it is still difficult to tell whether such protective benefits are due to ITCs or other variables connected to the CV consumption. Clinical proof of ITCs' protective impact in humans is until today, still relatively limited. (Stewart & Mcdougall, 2012)

5.Erucin

5.1. What is Erucin (ER)?

ER (fig.10) was first isolated in the 1970s from seeds of *Eruca sativa* Mill. and is overall located in high levels in rocket salad species, but it is also found *in vivo* due to the decrease of the SFN, derived from broccoli. (Antonietta Melchini & Traka, 2014)

ER is a major component that has structural analogies with SFN. It is derived from glucoraphanin, a GLS present in CV and widely known for its chemopreventive properties. (Antonietta Melchini & Traka, 2014)

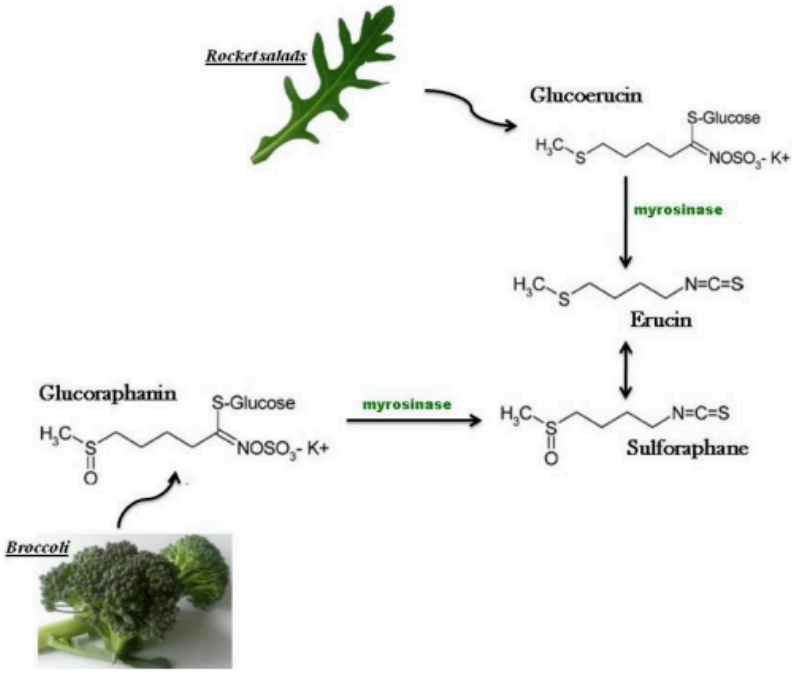

Figure 10. The two pathways to obtain Erucin in vivo: 1) through the ingestion of rocket salad species and 2) through the reduction of SFN present in broccoli. Retrived from: *"Biological Profile of Erucin: A New Promising Anticancer Agent from Cruciferous Vegetables", by Melchini M and Traka Maria, 2010. (http:// 10.3390/toxins2040593)*

5.2. Bioavailability

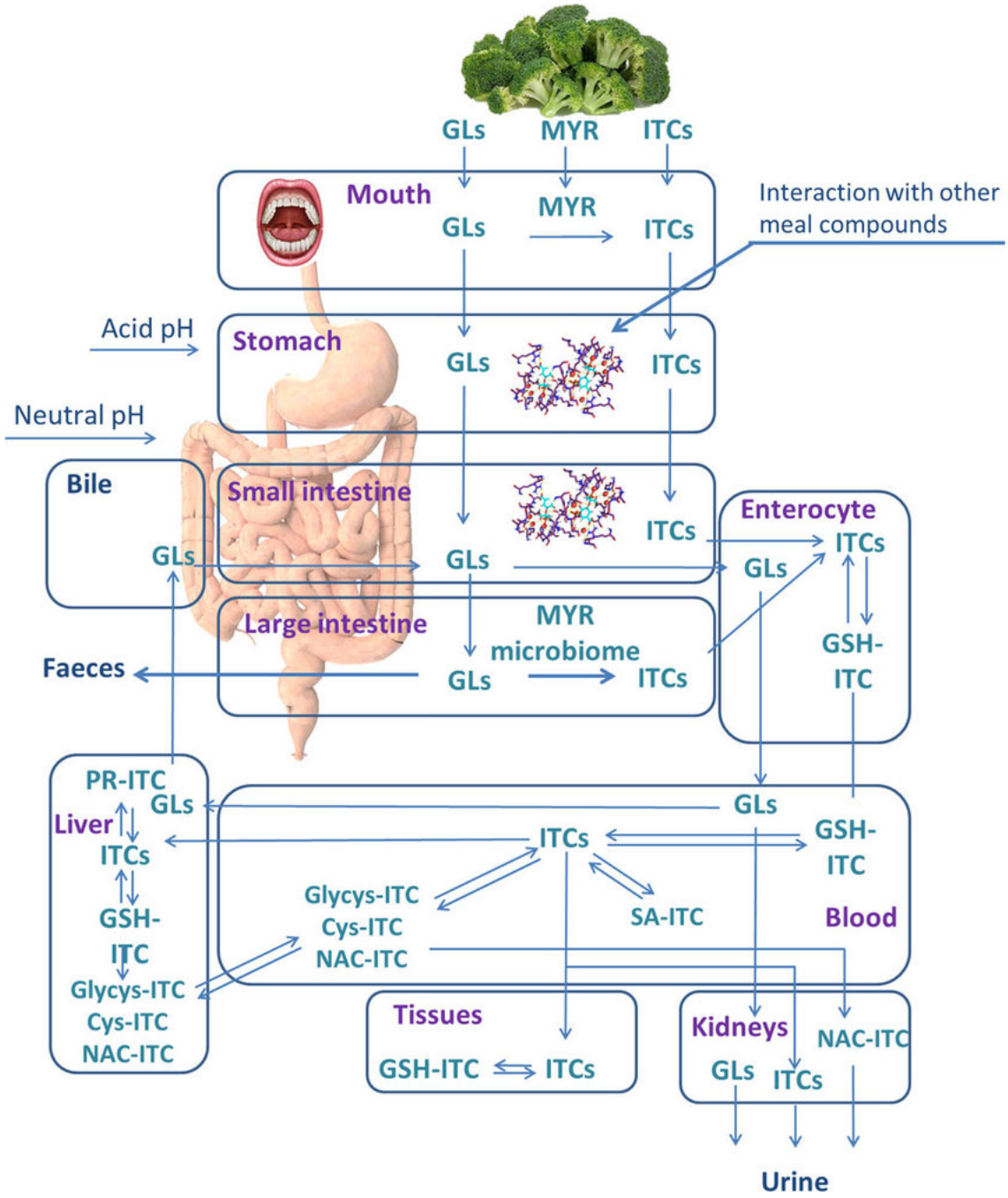

Figure 11. Overview of the metabolic pathway of GLS and ITCs. Retrived from: "*Isothiocyanates from Brassica Vegetables - Effects of Processing, Cooking, Mastication and Digestion" by Oliviero T., Verkerk R., and Dekker M.2018. (http:// 10.1002/mnfr.201701069)*

Legend: MYR: Myrosinase; GSH-ITC: Glutathione conjugate of ITC; PR-ITC: intracelular proteins conjugates of ITC; AS-ITC: Serum albumin conjugate of ITC; Nac-itc: N-acetylcysteine conjugate of ITC; Glycys-ITC: glycine-cysteine conjugate of ITC; CYS-ITC: cysteine conjugate of ITC.

Albeit there is an inadequate direct knowledge of the entire metabolism process of ER in humans, several studies have shown an *in vivo* interconversion of ER to SF, which associated with their structural resemblance, supports the hypothesis of a similar metabolic fate of the two components. (Antonietta Melchini & Traka, 2014)

Initially SF, similar to other ITCs, is exposed to enzymatic conjugation *in vivo* with the GSH that is catalyzed by glutathione transferases (GSTs). (Antonietta Melchini & Traka, 2014) The pace of the enzymatic interaction of ITCs with GSTs was shown to vary amongst structurally comparable molecules. SF has been demonstrated to be the weakest of the four GST isoenzymes among naturally occurring ITCs that only differ in the oxidation of the sulfur atom inserted into the carbon chain. However, ER looks to be the best, and hence its conjugation with GSH is predicted to be greater. (Oliviero et al., 2018; Wu, Zhou, & Xu, 2009)

Regarding the metabolization of ITCs (Fig.11), only when they reach the enterocyte occurs the first conjugation with GSH, followed by the main metabolization in the liver by a well-known route for the biotransformation of xenobiotic and endobiotic electrophilic compounds and their metabolites, the well-known mercapturic acid pathway. (Hanna & Anders, 2020)

Initially a spontaneous reaction between the -N=C= group of ITCs and the cysteine sulfhydryl group of GSH occurs, followed by the reaction with GST, culminating in the formation of the corresponding NAC-ITC conjugates that are themselves excreted in the urine. (Hanna & Anders, 2020; Y. Zhang, 2012)

According to the literature, mercapturic acids discharged in urine can be utilized to estimate ITC bioavailability since their excretion in urine indicates GLS ingestion, and hence the equivalent absorption of ITCs following CV consumption. (Hanna & Anders, 2020)

Vermeulen et al reported high excretion levels of ER and SF mercapturic acids, of approximability 94%, four hours after the consumption of rocket salads (Jiao, 1994) These findings also reported similar absorption (ER ka = 2.5 h^{-1}, SF ka = 2.0 h^{-1}) and excretion (ER ka = 0.24 h^{-1}, SF ka = 0.19 h^{-1}) rates after the ingestion of CV, showing similar *in vivo* kinetics for these structurally related ITCs. (Jiao, 1994)

Studies made by Kassahun, Kelem et al, assessed the reversibility of the oxidation-reduction biotransformation of the sulfur atom in ER and SF, showing that the oxidation of the sulfide in ER has a higher probably to happen than the reduction of the sulfoxide in SF. (Kassahun, Davis, Hu, Martin, & Baillie, 1997)

In humans, thiol-conjugates of SF are recognized to be the principal metabolic products generated by the mercapturic acid route. (Jiao, 1994) Because SF conjugation with thiols is a reversible process, multiple studies have proven that SF release in plasma and tissues under physiological circumstances is feasible and has been detected following CV ingestion. (Al et al., 2006; Conaway, Krzeminski, Amin, & Chung, 2001; Effery, 2007; Gasper et al., 2005)

5.3. Bioactivity

Among the several properties associated with ITCs, ER has demonstrated promising anticancer properties in various *in vitro* and *in vivo* studies, through similar processes as the ones already found for SFN. Research data indicate that ER may also exhibit possible protective properties against human cancer via a variety of pathways, which are outlined in table 1 below.(Antonietta Melchini & Traka, 2014)

Table 1. Biological Profile of Erucin: A New Promising Anticancer Agent from Cruciferous Vegetables.

Biological process	Molecular targets	Reference
Modification Phase I enzymes	CYP450 isoforms	Lamy, Schröder, Paulus, Brenk, & Stahl, 2008
Induction of phase II enzymes	Quinone reductase (QR) Glutatione transferase (GST)	Hanlon, Coldham, Sauer, & Ioannides, 2008; Jakubı, 2005; Unday, 2004; Y. Zhang, Talalay, Cho, & Posner, 1992
Up-regulation of Phase III detoxification system	Multidrug resistance proteins (MRP-1 and MRP-2)	Harris & Jeffery, 2008; Unday, 2004
Modulation of cell proliferation	Tumour suppressor proteins (p53, p21)	Lamy & Mersch-sundermann, 2009; A Melchini et al., 2009
	Cell cycle checkpoints	Fimognari, Michael, Iori, & Cantelli-forti, 2004;

		Jakubikova, Bao, & Sedlak, 2005; Unday, 2004
	Pro-apoptotic signals	Fimognari et al., 2004; Jakubikova et al., 2005; Lamy & Mersch-sundermann, 2009; Unday, 2004
	Androgen receptor (AR)	Kin & Sing, 2010
	Reactive oxygen species (ROS)	Doudican, Bowling, & Orlow, 2011

Zhang et al were the first to discover that ER is a major monofunctional inducer of phase II enzymes in several mouse tissues. Several follow-up studies have confirmed the Zhnag et al findings, both in animal and human cells. (Y. Zhang et al., 1992) Jakubíková et al studies showed that ER has a strong ability to induce phase II detoxification enzymes and induces a G2/M block in Caco-2 cells, stronger than that induced by SF. (A Melchini et al., 2009)

According to Melchini et al studies, ER can upregulate the proteins of p53 and p21 to hinder the proliferation of human lung cancer cells. According to Evelyn et al, ER has shown to induce both cell cycle arrest and apoptosis in human hepatoma HepG2 cells. (Lamy & Mersch-sundermann, 2009)

Because it may react with hydrogen peroxide and alkylhydroperoxides to generate water and alcohol, ER is considered to have direct antioxidant power. It also has indirect antioxidant ability, since it is a powerful inducer of cellular antioxidant systems such as thioredoxin reductase 1 (TrxR1), as Wang et al. demonstrated in human breast cancer MCF-7 cells.(Algimigli, 2005; Ang et al., 2005; Iori, Bernardi, Gueyrard, Rollin, & Palmieri, 1999)

Su-Hyeong et al have shown that ER was also effective in reducing the protein levels of androgen receptor on LNCaP human prostate cancer cells. (Kin & Sing, 2010)

Fimognari et al found that ER was able to cause cell cycle arrest, apoptosis and mitochondrial potential depolarization in human leukaemia cells and their multidrug resistance variants. (Fimognari et al., 2004) This study has shown that ER is able to induce a strong antiproliferative effect on human leukemia cells but has also shown no effect on non-transformed human T lymphocytes. A major advantage in comparison with SF, since

SF induced a strong antiproliferative effect on both transformed and non-transformed human T lymphocytes. (Dong et al., 2016a; Fimognari et al., 2004)

6. Objectives

A recent meta-analysis of 13 prospective studies found that eating fruits and vegetables was related to a lower risk of RCC, and carotenoids found in fruits and vegetables may play a role in this protection. (Soundararajan & Kim, 2018)

Since ER, a common compound found in CV, has been found to be able to selectively affect cancer cell growth, in the present work we intend to evaluate if ER has any morphometric effect on two cell lines, the Vero and 786-0. Cell lines represent the non-cancerous cells and cancerous RCC cells, respectively.

Morphometric analysis was used as a method to identify cellular anomalies, e.g., cell shape, size, etc, giving insights regarding cancer' expected behavior, since morphologic changes may be indicative of cytoskeletal changes related to the acquisition of invasive capacity.

Cells were analyzed regarding perimeter, area, roundness, circumference, and aspect ratio. Key characteristics that give us insight into the cell's cytoskeletal properties (Antonietta Melchini & Traka, 2014).

7. Methods

7.1. Chemicals

Cayman Chemical Company (Ann Arbor, MI, USA) supplied the ER ethanolic solution. Before each experiment, working solutions of ER at 2 mM in PBS with 2% ethanol were freshly made. As a vehicle control, the PBS solution containing 2% ethanol was used. The final quantity of ethanol in cell cultures was always 0,1% v/v.

Both Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were obtained from Biowest, USA. Both trypsin and penicillin/streptomycin solutions were purchased from Sigma-Aldrich, St Louis, MO, USA.

7.2. Cell lines

We used two kidney in vitro models (Fig.13). Vero cells are derived from the kidney of an African green monkey and are one of the mammalian continuous cell lines more commonly used as a model of non-cancer renal cells. As a model of renal clear cell carcinoma, we used 786-0 cells. Both cell lines were obtained from ATCC (Manassas, VA, USA). Both cell lines were cultured in DMEM, containing 10% FBS and 100 U ml^{-1} penicillin and 0.1mg mL^{-1} streptomycin. Cells were maintained at 37ºC, under a humidified air atmosphere containing 5% of CO_2 in air.

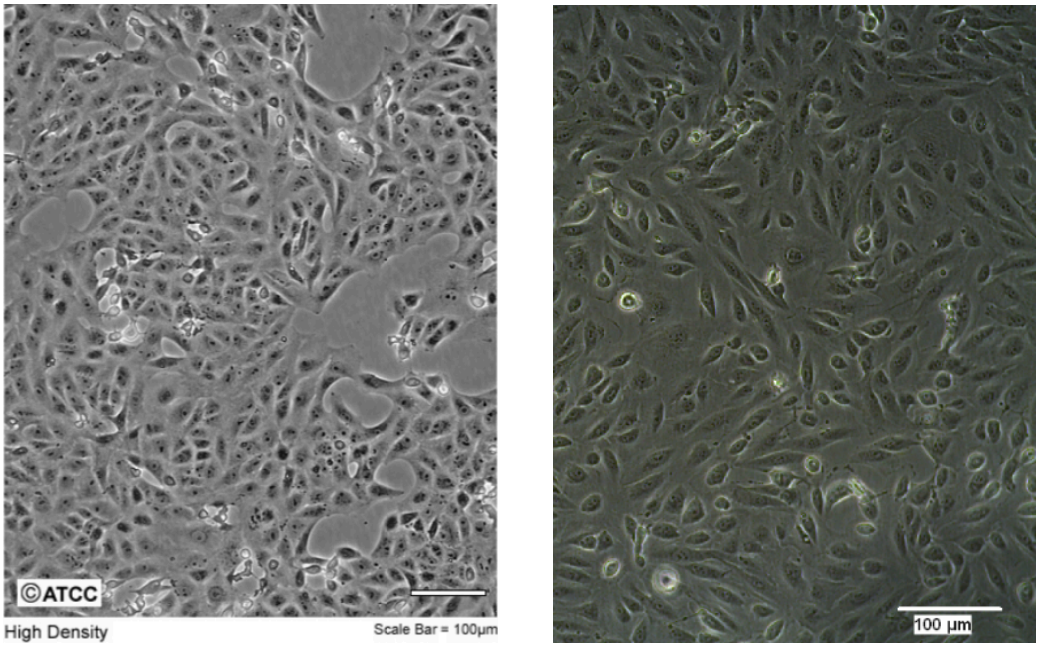

Figure 12. Phase contrast photographs of the cell lines used in this work. Left: Vero cell line. Right: 786-O cell line.

7.3. Cells treatment

Cells were seeded at a density of $2x10^5$ cells per well in 500 µL culture medium in 24-well plates and incubated for 24 h. Cells were then incubated with four different concentrations of ER (0 µM, 10 µM, 50 µM and 80 µM) and were checked at 6 different time points (t1=2h, t2=4h, t3=6h, t4=8h, t5=10h and t6=12h). Images were taken using an inverted phase contrast microscope (Olympus® CKX41) coupled to a digital camera (Olympus® SC20).

7.4. Morphometric analysis

Aspect ratio (AR), area, perimeter, circumference, and roundness were the parameters measured using a professional image processing software, Fiji® (Bethesda, MA, USA).

One by one, all images were processed to enhance contrast and brightness and ultimately to promote a more accurate measurement. One hundred cells were measured for each experimental condition.

7.5. Statistical Analysis

The median and standard variations of all the results obtained were calculated. The differences in mean values of the results observed in different conditions were evaluated by Student's t-test. The analyses were performed using the Microsoft excel software.

8. Results and discussion

CVs are receiving much attention due to its health-promoting properties and the promising results obtained in several clinical trials that show an inverse association between the intake of CVs and cancer cell proliferation risk. (Soundararajan & Kim, 2018) For example, Holloway et al., found that the treatment with SFN ultimately slowed down of the proliferation of brain cancer cells. (Holloway et al., 2017)

Consumption of cruciferous either fresh or raw has more beneficial effects than cooked or boiled, as bioavailability of ITCs in the former is higher, which leads to it being the main focusing on-going studies. (Soundararajan & Kim, 2018)

Although much study has concentrated on SFN, which is produced from broccoli, other ITCs such as ER seem promise. In vitro research suggests that ER can have a powerful antiproliferative effect on some human cancer cells. Hence it was the main focus of this study. (Antonietta Melchini & Traka, 2014)

On our study we carried out a morphometric analysis, using as parameters the AR, roundness, perimeter, area, and circumference. AR is frequently used to describe the elongation of the cell, with a higher AR meaning a more elongated cell morphology. Roundness helps to detect minute differences in cell contour and degree of curvature. Perimeter, area, and circumference give information on global cell morphology changes since malignant cells are distinguished by a big nucleus with an irregular size and shape. These morphological traits indicate metabolic changes, namely an increase in structures that allow cell cellular division and a decrease in structures associated with other metabolisms. (Lyons et al., 2016; Yu, Lim, Xiong, Tan, & Shim, 2013)

This work aimed thus to analyze morphological parameters in cancerous renal cells treated with ER. The results are summarized in Table 2 and 3.

Table 2. Summary of the five morphology parameters measured for 786-O cell line after each of the incubation check time with four different ER concentrations.

ER Concentration (µm)	Area		Perimeter		Circunference		AR		Roundness		Incubation check time (h)
	average value	standard deviation	average value	standard deviation	average value	standard deviation	average value	standard deviation	average value	standard deviation	
0	2751,690	1375,427	552,433	275,855	0,499	0,187	2,633	1,342	0,466	0,189	**2**
	2865,550	1427,143	312,244	67,468	0,454	0,106	3,346	2,450	0,380	0,104	**4**
	3019,340	1352,846	338,720	57,024	0,452	0,120	3,465	1,518	0,352	0,174	**6**
	2933,740	1395,132	343,528	61,907	0,399	0,138	3,653	2,654	0,338	0,262	**8**
	2007,667	677,198	251,406	68,236	0,447	0,145	3,237	0,877	0,420	0,204	**10**
	1790,500	983,927	237,378	96,944	0,498	0,172	3,904	1,705	0,457	0,230	**12**
10	2352,872	970,806	584,530	292,656	0,536	0,180	2,641	1,141	0,472	0,188	**2**
	2209,200	1461,795	250,000	96,842	0,362	0,157	2,659	1,624	0,525	0,276	**4**
	2645,800	979,145	281,187	58,189	0,327	0,191	3,474	1,812	0,399	0,260	**6**
	2343,300	1260,219	267,633	84,858	0,328	0,064	3,094	1,100	0,372	0,165	**8**
	1511,615	969,082	200,811	55,863	0,360	0,086	2,750	1,043	0,424	0,191	**10**
	1591,682	672,360	212,249	66,461	0,431	0,212	2,554	1,166	0,466	0,187	**12**
50	1699,613	1066,579	454,735	243,298	0,588	0,146	1,664	0,721	0,670	0,191	**2**
	1554,316	1461,795	250,000	96,842	0,554	0,157	1,945	1,624	0,535	0,276	**4**
	1110,585	848,458	189,930	53,918	0,583	0,127	2,659	0,712	0,577	0,188	**6**
	1209,200	750,408	183,748	58,402	0,584	0,138	2,203	0,830	0,587	0,187	**8**
	671,667	398,354	113,029	43,607	0,580	0,209	1,518	0,255	0,674	0,098	**10**
	564,714	261,438	113,252	31,700	0,552	0,138	1,478	0,300	0,701	0,130	**12**
80	1069,202	574,976	151,154	49,057	0,697	0,169	1,806	0,563	0,690	0,159	**2**
	1192,283	716,592	178,246	74,821	0,653	0,185	1,708	0,616	0,647	0,184	**4**
	775,733	555,568	114,202	35,776	0,701	0,108	1,472	0,356	0,711	0,143	**6**
	544,000	290,265	102,810	30,333	0,635	0,163	1,313	0,151	0,771	0,085	**8**
	504,083	189,980	100,580	28,933	0,683	0,170	1,345	0,149	0,773	0,090	**10**
	571,313	320,420	104,643	27,108	0,635	0,153	1,315	0,232	0,781	0,123	**12**

Table 3. Summary of the five morphology parameters measured for Vero cell line after each of the incubation check time with four different ER concentrations.

ER Concentration (µm)	Area		Perimeter		Circunference		AR		Roundness		Incubation check time
	average value	standard deviation	average value	standard deviation	average value	standard deviation	average value	standard deviation	average value	standard deviation	
0	721,800	379,435	147,208	24,092	0,587	0,110	1,720	0,561	0,635	0,180	**2**
	983,500	436,995	145,654	33,456	0,656	0,110	1,698	0,451	0,743	0,198	**4**
	1713,220	781,391	162,246	40,474	0,586	0,100	1,677	0,429	0,769	0,167	**6**
	1619,000	872,255	161,027	42,647	0,661	0,109	1,794	0,538	0,671	0,173	**8**
	1348,600	565,603	144,658	30,361	0,566	0,058	1,664	0,160	0,661	0,092	**10**
	1358,600	570,559	145,936	31,322	0,574	0,062	1,757	0,301	0,621	0,125	**12**
10	868,545	329,435	146,387	15,481	0,574	0,158	1,600	0,713	0,698	0,196	**2**
	1132,538	436,995	145,696	25,397	0,695	0,090	1,555	0,235	0,705	0,185	**4**
	1180,000	1064,270	145,696	43,113	0,678	0,141	1,581	0,250	0,747	0,173	**6**
	994,412	146,680	164,502	18,227	0,671	0,154	1,605	0,702	0,628	0,201	**8**
	991,083	627,377	170,326	38,754	0,632	0,165	1,623	0,568	0,659	0,187	**10**
	900,000	375,345	130,995	33,524	0,662	0,138	1,589	0,628	0,637	0,179	**12**
50	1032,125	742,845	114,475	93,742	0,587	0,224	1,577	0,496	0,687	0,203	**2**
	1228,700	569,657	143,342	39,908	0,675	0,110	1,632	0,542	0,669	0,192	**4**
	1228,700	569,657	147,194	39,908	0,644	0,110	1,632	0,542	0,669	0,185	**6**
	1234,000	802,304	147,362	60,245	0,662	0,206	1,494	0,289	0,739	0,124	**8**
	1258,063	777,276	132,715	45,084	0,719	0,117	1,457	0,304	0,714	0,142	**10**
	900,000	375,345	136,924	33,524	0,719	0,138	1,457	0,628	0,737	1,138	**12**
80	805,500	355,550	117,612	19,964	0,594	0,121	1,592	0,255	0,738	0,125	**2**
	1032,636	412,956	132,466	47,654	0,574	0,149	1,440	0,437	0,641	0,134	**4**
	1076,636	544,575	136,886	30,006	0,694	0,059	1,408	0,297	0,734	0,126	**6**
	903,273	199,049	123,824	24,124	0,699	0,128	1,406	0,282	0,737	0,142	**8**
	740,222	243,493	134,939	39,161	0,707	0,207	1,331	0,487	0,765	0,187	**10**
	693,000	243,493	130,995	29,965	0,760	0,150	1,392	0,467	0,754	0,175	**12**

Table 4. The results obtained from Student's t test analysis for the data obtained for 786-O cell line after each of the incubation check time with the different ER concentrations.

ER Concentration (μm)	Area		Perimeter		Circunference		AR		Roundness		Incubation check time (h)
	t test	P value	t test	P value	t test	P value	t test	P value	t test	P value	
10	1,003	0,765	0,567	0,067	-0,030	0,085	0,763	0,930	-0,095	0,123	**2**
	2,180	1,070	0,873	0,098	0,655	0,073	2,340	1,094	1,340	1,094	**4**
	0,998	0,875	0,954	0,057	0,733	0,078	0,774	0,432	0,894	1,235	**6**
	1,100	0,768	0,988	0,063	0,754	0,079	1,745	0,876	0,789	1,576	**8**
	1,234	0,876	0,765	0,732	0,652	0,079	2,120	1,093	0,096	1,493	**10**
	0,667	0,723	0,654	0,053	0,641	0,072	3,450	2,345	0,099	1,345	**12**
50	2,897	0,052	2,230	0,056	-1,430	0,058	2,324	0,059	-1,400	0,059	**2**
	2,876	0,055	2,345	0,077	-1,497	0,063	2,653	0,056	-1,097	0,057	**4**
	3,342	0,058	2,560	0,059	-1,401	0,058	2,201	0,054	-1,123	0,058	**6**
	2,765	0,058	2,760	0,055	-1,487	0,059	2,342	0,059	-1,134	0,059	**8**
	2,875	0,054	2,987	0,055	-1,421	0,059	3,564	0,057	-2,420	0,057	**10**
	2,765	0,054	2,967	0,059	-1,489	0,062	4,123	0,058	-3,409	0,056	**12**
80	3,450	0,049*	3,230	0,053	-1,983	0,055	2,223	0,043*	-1,998	0,055	**2**
	2,983	0,047*	2,850	0,057	-1,897	0,043*	2,873	0,045*	-2,012	0,044*	**4**
	3,693	0,051	3,430	0,056	-2,101	0,038*	3,101	0,046*	-2,321	0,039*	**6**
	4,010	0,053	3,230	0,051	-1,887	0,047*	3,212	0,051	-3,231	0,049*	**8**
	3,123	0,048*	3,120	0,050*	-2,210	0,049*	3,124	0,049*	-3,123	0,049*	**10**
	2,853	0,049*	3,102	0,052	-1,889	0,052	3,122	0,052	-3,345	0,051	**12**

*; Statistically significant.

Table 5. The results obtained from Student's t test analysis for the data obtained for Vero cell line after each of the incubation check time with the different ER concentrations.

ER Concentration (μm)	Area		Perimeter		Circunference		AR		Roundness		Incubation check time (h)
	t test	P value	t test	P value	t test	P value	t test	P value	t test	P value	
10	0,500	0,765	0,975	0,765	0,543	0,585	0,985	1,200	0,875	0,983	**2**
	1,200	1,070	0,865	1,072	0,097	0,573	0,075	1,432	-0,954	1,234	**4**
	-1,124	0,875	0,771	0,775	1,123	0,059	0,081	1,432	-0,765	0,789	**6**
	-1,100	0,768	0,792	0,868	1,134	0,058	0,182	1,701	-0,675	0,653	**8**
	2,340	0,876	1,300	0,866	1,420	0,060	1,330	1,632	-0,343	0,568	**10**
	1,667	0,723	1,200	0,733	1,409	0,055	1,520	1,654	-0,410	0,543	**12**
50	-1,405	0,765	1,230	0,560	0,572	0,654	0,985	1,200	0,562	0,890	**2**
	-1,890	1,070	1,345	0,675	1,230	0,634	0,065	1,432	1,010	0,892	**4**
	1,124	0,875	1,560	0,580	1,789	0,053	0,071	1,432	1,432	0,992	**6**
	1,,34	0,768	1,760	0,580	0,456	0,910	0,192	1,701	-1,320	0,052	**8**
	0,981	0,876	1,987	0,540	-1,345	0,052	1,230	1,632	-1,430	0,051	**10**
	0,667	0,723	1,967	0,600	-1,450	0,056	1,420	1,654	-1,530	0,049	**12**
80	1,450	0,059	1,230	0,063	0,872	0,980	-0,998	0,055	0,983	0,055	**2**
	1,983	0,057	0,950	0,067	-1,340	0,430*	-1,210	0,054	-1,257	0,053	**4**
	1,693	0,051	1,130	0,066	0,730	0,560	-1,321	0,059	-0,990	0,048*	**6**
	1,010	0,052	1,230	0,061	0,892	0,570	-1,231	0,059	1,987	0,069	**8**
	1,123	0,058	1,120	0,060	1,234	0,450*	-1,123	0,059	1,893	0,069	**10**
	2,530	0,059	0,902	0,062	1,432	0,530	-1,345	0,061	1,889	0,055	**12**

*; Statistically significant.

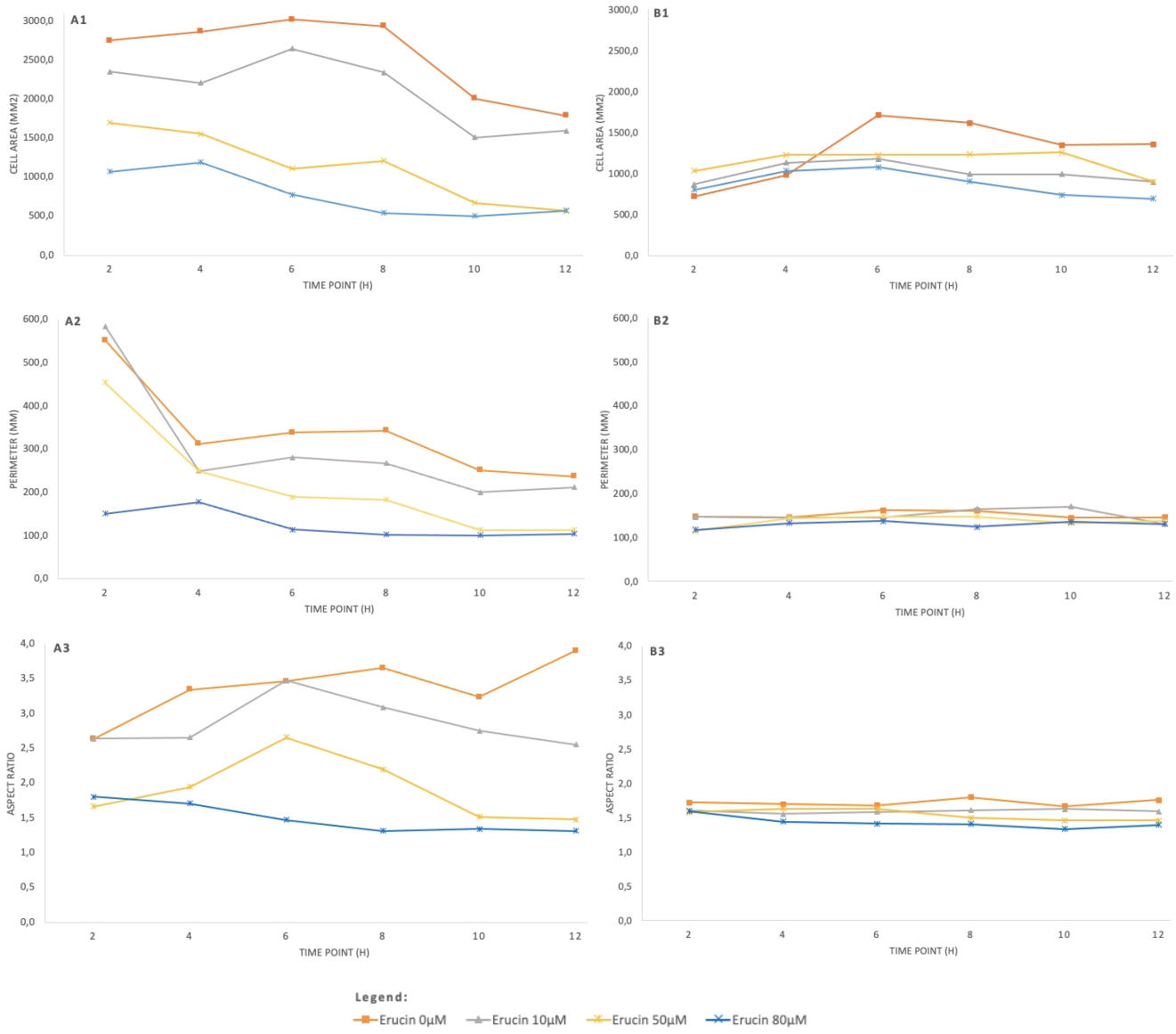

Figure 13. Results obtained for three morphology parameters observed in the 786-0 cells (A) and Vero cells (B), namely cell area (A1, B1), perimeter (A2, B2), and aspect ratio (A3, B3).

Comparing the results obtained in both cell lines, we can affirm that ER had a more prominent effect in the 786-O cell line. Regarding cell morphology, when higher concentrations of ER were administered, 50 and 80 µM, it is possible to verify an increased effect, with the values obtained for the area, perimeter, and AR decreasing (as shown on Figure 12 A1-A3) and the values of circumference and roundness increasing (as shown on Figure 13 A4-A5). These alterations in the 786-O cells morphology are easily verified in the images of the Figure 14. This same effect was observed throughout the time points. The student's test analysis indicated a significant impact for 786-O cell lines specially when the concentration of 80 µM was used (Table 4). The impact of ER on morphology was thus concentration- and time-dependent.

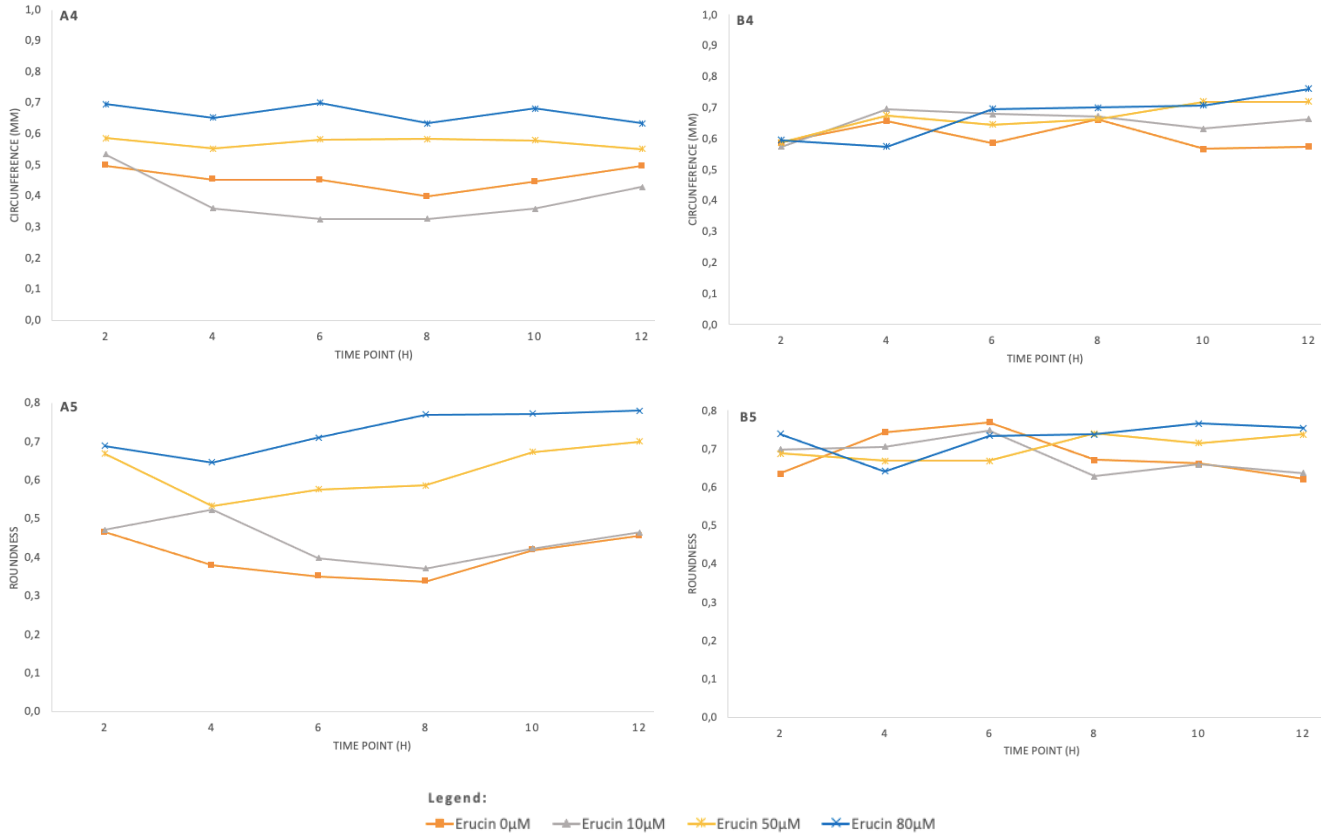

Figure 14. Results obtained for two morphology parameters observed in the 786-0 cells (A) and Vero cells (B), namely circumference (A4, B4) and roundness (A5, B5).

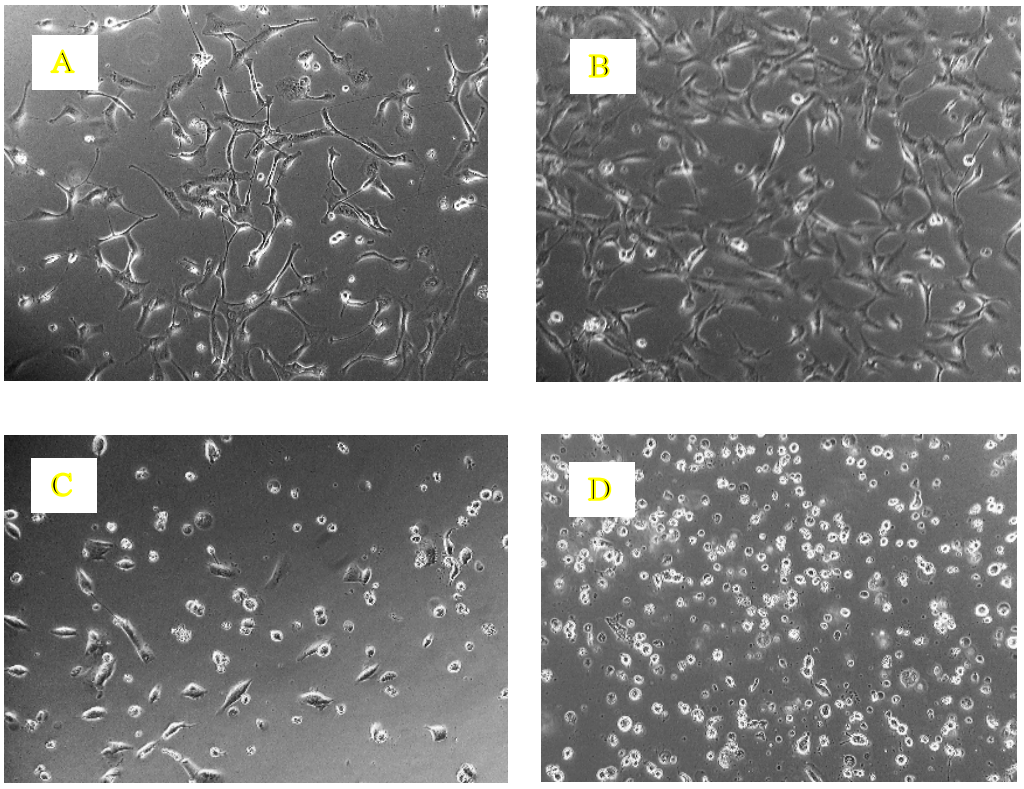

Figure 15. Alterations in 786-0 cells morphology due to exposure to increasing ER concentrations. (A)- 0 µM; (B) – 10 µM; (C)- 50 µM; (D) – 80 µM, after 12 h of incubation.

For the Vero cell line, a clear effect of ER was observed at the highest concentration of 80 µM (Table 3). However, for the AR and roundness parameters at the concentration of 50 µM, it was possible to observe a difference, with a decrease of AR and an increase of roundness, meaning that the cells lost most their elongated cell morphology. Generally, for the Vero cell line we were able to observe a smaller decrease in area, perimeter, and AR while the circumference and roundness suffered an increase upon ER treatment. The student's test analysis indicated a weaker correlation Vero cell line (Table 5). Which allows to conclude that ER has a higher selectively for cancer cells, in agreement with other studies that found ER can induce a stronger antiproliferative effect on cancer human cells, than non-transformed cells. (Algimigli, 2005; Dong et al., 2016b; Jakubikova et al., 2005; Jakubı, 2005) Although these alterations in comparison with the 786-O cells, are not so strongly visible it is still possible to verify the impact of ER in the images of the Figure 15.

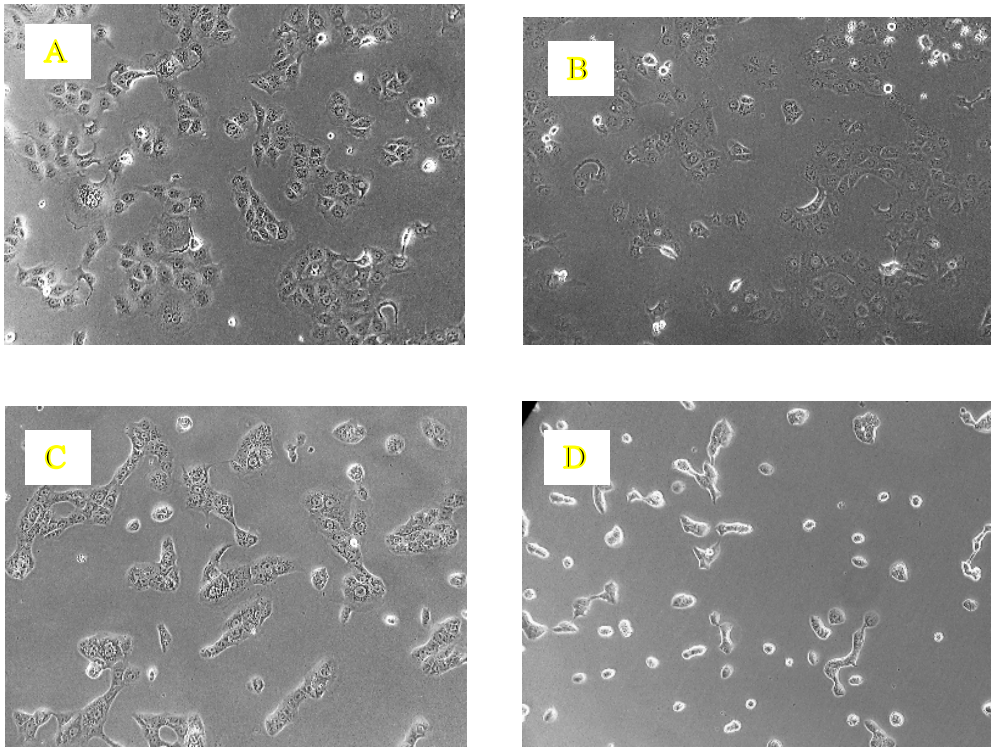

Figure 16. Alterations in Vero cells morphology due to exposure to increasing ER concentrations. (A)- 0 µM; (B) – 10 µM; (C)- 50 µM; (D) – 80 µM, after 12 h of incubation.

Our group found that ER has antiproliferative effects on the 786-O cell line and was able to lead to alterations on the cytoskeleton of the cell. These results are in agreement with other reports for similar ITCs compounds. For example, Zhou et al. findings indicate that SFN disrupts microtubules in human prostate cancer cells causing apoptosis, and Azarenko et al. studies suggest that ER inhibition of mitosis appears to be caused by suppression of spindle microtubule dynamics. (Azarenko, Jordan, & Wilson, 2014)

To evaluate biological activity in vitro and in vivo systems, it is critical to determine the levels of ITCs obtained in the systemic circulation following CV ingestion. Although there is currently no evidence for ER and its metabolites in humans, there is evidence that SF and its products circulate in human plasma at a maximum concentration of 2,2M 1.5 hours after intake of one serving of broccoli, with more than 40% being free SF. As a result, in order to attain the same benefits seen in the current study, a person would need to consume more than the standard diet provides. As a result, if pre-clinical investigations validate ER's relevance as a prospective addition to cancer treatment, ER would have to be administered as a medicine or nutraceutical rather than absorbed purely from diet. (Gasper et al., 2005)

It should also be mentioned that the concentrations used in this study were selected taking in consideration the cytotoxicity profile of ER studied by Guerreiro et al. At high concentrations, the cytotoxicity was greater in 786-O cancer cells than in non-tumor Vero-E6 cells. The concentration levels studied herein were not toxic, as evaluated by the MTT assay and confirmed using propidium iodide, a fluorescent intercalating agent that binds to DNA and allows the identification of dead cells.(Guerreiro et al., 2023)

9. Final considerations

Pharmaceutical development of innovative therapeutics for chronic illness has frequently resorted to the plant world to seek intriguing bioactive phytochemicals. Epidemiological data and its cell and animal models research appoint to CV as key sources of phytochemicals that are emerging as novel potential anticancer agents. (Antonietta Melchini & Traka, 2014)

In this present study, ER was able to modify morphometric features of RCC cells, that could be related with their ability to proliferate and form metastasis These results were more prominent in the RCC cell line 786-0 than the non-cancerous cell line Vero cells.

The degradation rate of GSL during food processing is still poorly understood, as it is a complex process due to the simultaneous generation of breakdown products and the high influence of the food processing method applied. (Simal-gandara, 2019) ITCs including ER and SFN are rapidly taken up into cells as GSH conjugates and due to their similarity in absorption and elimination rates, they show similar average bio availabilities.

Understanding the absorption pathways and metabolism of these compounds is critical. However, in comparison to what is known about many dietary bioactive chemicals, there is very scarce information on how, where, and what amount GLS and their hydrolysis products are freed, absorbed, transported, metabolized, and excreted in the human body. The majority of the available evidence comes from in vitro and animal investigations. (Simal-gandara, 2019)

Recent studies indicates that ITCs can exert their anticancer activity alone or in combination with chemotherapeutic microtubule-targeting drugs to synergistically enhancement of cancer cell death. (Cang, Ma, Chiao, & Liu, 2014; Cell, 2013)

In this work, we found that ER has led to notorious morphological alterations in both renal cell types studied. This new finding can help to explain the protective effect of CV against kidney cancer and should be further investigated.

Further structural and functional studies on the molecular interaction of ER with important signaling regulators of cancer cells should be conducted. (Soundararajan & Kim, 2018). It should also be further explored the cell viability when cells are treated with higher concentrations of ER.

Although cell and animal models are useful for studying the effects and mechanisms of chemoprevention, it is also critical to direct research efforts toward human

intervention studies to determine whether the same mechanisms exist in humans or whether food-derived compounds work in a completely different way. (Antonietta Melchini & Traka, 2014)

10. Bibliography

Agerbirk, N., & Olsen, C. E. (2012). Phytochemistry Glucosinolate structures in evolution. *Phytochemistry*, *77*, 16–45. https://doi.org/10.1016/j.phytochem.2012.02.005

Ahuja, I., Rohloff, J., Bones, A. M., Ahuja, I., Rohloff, J., Magnar, A., … B, A. M. (2010). Defence mechanisms of Brassicaceae : implications for plant-insect interactions and potential for integrated pest management . A review To cite this version : HAL Id : hal-00886509 Defence mechanisms of Brassicaceae : implications for plant-insect interac. *Agronomy for Sustainable Development*, *30*, 311–348.

Al, A. A., Mithen, R. F., Gasper, A. V, Shaw, P. N., Middleton, R. J., Ortori, C. A., & Barrett, D. A. (2006). Quantitative measurement of sulforaphane , iberin and their mercapturic acid pathway metabolites in human plasma and urine using liquid chromatography – tandem electrospray ionisation mass spectrometry. *Journal of Chromatography B*, *844*, 223–234. https://doi.org/10.1016/j.jchromb.2006.07.007

Algimigli, L. U. C. A. V. (2005). Direct Antioxidant Activity of Purified Glucoerucin , the Dietary Secondary Metabolite Contained in Rocket (Eruca sativa Mill .) Seeds and Sprouts. *Agricultural and Food Chemistry*, (53), 2475–2482.

Ang, W. E. I. W., Ang, S. H. W., Owie, A. F. O. H., Eckett, G. E. J. B., Ithen, R. I. M., & Ao, Y. O. B. (2005). Sulforaphane , Erucin , and Iberin Up-Regulate Thioredoxin Reductase 1 Expression in Human MCF-7 Cells. *Agricultural and Food Chemistry*, (53), 1417–1421.

Azarenko, O., Jordan, M. A., & Wilson, L. (2014). Erucin, the Major Isothiocyanate in Arugula (Eruca sativa), Inhibits Proliferation of MCF7 Tumor Cells by Suppressing Microtubule Dynamics. *PLOS One*, *9*(6), 1–12. https://doi.org/10.1371/journal.pone.0100599

Bao, Y., Wang, W., Zhou, Z., & Sun, C. (2014). Benefits and Risks of the Hormetic Effects of Dietary Isothiocyanates on Cancer Prevention. *PLOS One*, 1–19. https://doi.org/10.1371/journal.pone.0114764

Barba, F. J. (2016). Bioavailability of Glucosinolates and Their Breakdown Products : impact of Processing. *Frontiers in Nutrition*, *3*(August), 1–12. https://doi.org/10.3389/fnut.2016.00024

Bryan, H. K., Olayanju, A., Goldring, C. E., & Park, B. K. (2012). The Nrf2 cell

defence pathway : Keap1-dependent and -independent mechanisms of regulation. *Biochemical Pharmacology*. https://doi.org/10.1016/j.bcp.2012.11.016

Cang, S., Ma, Y., Chiao, J., & Liu, D. (2014). Phenethyl isothiocyanate and paclitaxel synergistically enhanced apoptosis and alpha-tubulin hyperacetylation in breast cancer cells. *Experimental Hematology & Oncology*, *3*(1), 1–6. https://doi.org/10.1186/2162-3619-3-5

Capitanio, U., Coleman, J., Gore, J. L., Sun, M., Wood, C., Russo, P., & Novara, G. (2018). Epidemiology of Renal Cell Carcinoma. *European Urology*, *75*(1), 74–84. https://doi.org/10.1016/j.eururo.2018.08.036

Capitanio, U., & Montorsi, F. (2015). Renal cancer. *The Lancet*, *6736*(15), 1–13. https://doi.org/10.1016/S0140-6736(15)00046-X

Capuano, E., Dekker, M., Verkerk, R., & Oliviero, T. (2017). Food as Pharma? The Case of Glucosinolates. *Current Pharmaceutical Design*, *23*, 2697–2721. https://doi.org/10.2174/1381612823666170120160832

Cell, T. (2013). Synergistic effect of paclitaxel and epigenetic agent phenethyl isothiocyanate on growth inhibition , cell cycle arrest and apoptosis in breast cancer cells Synergistic effect of paclitaxel and epigenetic agent phenethyl isothiocyanate on growth inhibitio. *Cancer Cell International*, *13*.

Chi, X., Zhang, R., & Shen, N. (2015). Sulforaphane reduces apoptosis and oncosis along with protecting liver injury-induced ischemic reperfusion by activating the Nrf2 / ARE pathway. *Hepatol Int*, *2*. https://doi.org/10.1007/s12072-014-9604-y

Chinni, S. R., Li, Y., Upadhyay, S., Koppolu, P. K., & Sarkar, F. H. (2001). Indole-3-carbinol (I3C) induced cell growth inhibition , G1 cell cycle arrest and apoptosis in prostate cancer cells. *Oncogene*, (November 2000), 2927–2936.

Choi, E., Zhang, P., & Kwon, H. (2014). Determination of Goitrogenic Metabolites in the Serum of Male Wistar Rat Fed Structurally Different Glucosinolates. *Toxicological Research*, *30*(2), 109–116.

Clarke, J. D., Hsu, A., Riedl, K., Bella, D., Schwartz, S. J., Stevens, J. F., & Ho, E. (2011). Bioavailability and inter-conversion of sulforaphane and erucin in human subjects consuming broccoli sprouts or broccoli supplement in a cross-over study design. *Pharmacological Research*, *64*(5), 456–463. https://doi.org/10.1016/j.phrs.2011.07.005

Conaway, C. C., Krzeminski, J., Amin, S., & Chung, F. (2001). Decomposition Rates of Isothiocyanate Conjugates Determine Their Activity as Inhibitors of Cytochrome

P450 Enzymes †. *Chem. Res. Toxicol*, *14*, 1170–1176.

Conaway, C. C., Wang, C., Pittman, B., Yang, Y., Schwartz, J. E., Tian, D., … Chung, F. (2005). Phenethyl Isothiocyanate and Sulforaphane and their N -Acetylcysteine Conjugates Inhibit Malignant Progression of Lung Adenomas Induced by Tobacco Carcinogens in A / J Mice. *American Association for Cancer Research*, (18), 8548–8558. https://doi.org/10.1158/0008-5472.CAN-05-0237

Cover, C. M., Hsieh, S. J., Tran, S. H., Hallden, G., Kim, G. S., Bjeldanes, L. F., & Firestone, G. L. (1998). Indole-3-carbinol Inhibits the Expression of Cyclin-dependent Kinase-6 and Induces a G 1 Cell Cycle Arrest of Human Breast Cancer Cells Independent of Estrogen Receptor Signaling *. *The Journal of Biological Chemistry*, *273*(7), 3838–3847.

Curran, C. S., & Kopp, J. B. (2021). PD-1 immunobiology in glomerulonephritis and renal cell carcinoma. *BMC Nephrology*, 1–20.

Dong, Z., Shang, H., Chen, Y. Q., Pan, L., Bhatia, M., & Sun, J. (2016a). by Modulating Nrf2-Mediated Oxidative Stress and NLRP3 Inflammatory Pathway. *Oxidative Medicine and Cellular Longevity*, 12. https://doi.org/10.1155/2016/7864150

Dong, Z., Shang, H., Chen, Y. Q., Pan, L., Bhatia, M., & Sun, J. (2016b). by Modulating Nrf2-Mediated Oxidative Stress and NLRP3 Inflammatory Pathway. *Oxidative Medicine and Cellular Longevity*, 1–12.

Doudican, N., Bowling, B., & Orlow, S. (2011). Enhancement of arsenic trioxide cytotoxicity by dietary isothiocyanates in human leukemic cells via a reactive oxygen species dependent mechanism. *Leuk Res.*, *34*(2), 1–15. https://doi.org/10.1016/j.leukres.2009.05.017.Enhancement

Effery, E. L. H. J. (2007). The Metabolic Fate of Purified Glucoraphanin in F344 Rats AND. *Agricultural and Food Chemistry*, *55*, 2861–2866.

Esfandyari, S., Aleyasin, A., Noroozi, Z., Sc, M., & Taheri, M. (2021). The Protective Effect of Sulforaphane against Oxidative Stress through Activation of NRF2 / ARE Pathway in Human Granulosa Cells. *Cell Journal*, (6), 692–700. https://doi.org/10.22074/cellj.2021.7393.This

Esteve, M. (2020). Mechanisms Underlying Biological Effects of Cruciferous Isothiocyanates / Indoles : A Focus on Metabolic Syndrome. *Frontiers in Nutrition*, *7*(September), 1–22. https://doi.org/10.3389/fnut.2020.00111

Fares, J., Fares, M. Y., Khachfe, H. H., Salhab, H. A., & Fares, Y. (2020). Molecular

principles of metastasis : a hallmark of cancer revisited. *Signal Transduction and Targeted Therapy*, (October 2019). https://doi.org/10.1038/s41392-020-0134-x

Fenwick, G. R., Griffiths, N. M., & Heaney, R. K. (1983). Bitterness in Brussels Sprouts (Brassica oleracea L . var . gernrnifera): The Role of Glucosinolates and Their Breakdown Products. *Sci.Foof Agric*, *34*, 73–80.

Fimognari, C., Michael, N., Iori, R., & Cantelli-forti, G. (2004). The new isothiocyanate 4- (methylthio) butylisothiocyanate selectively affects cell-cycle progression and apoptosis induction of human leukemia cells. *Investigational New Drugs*, (22), 119–129.

Fimognari, C., Turrini, E., Ferruzzi, L., Lenzi, M., & Hrelia, P. (2012). Mutation Research / Reviews in Mutation Research Natural isothiocyanates : Genotoxic potential versus chemoprevention. *Mutation Research-Reviews in Mutation Research*, *750*(2), 107–131. https://doi.org/10.1016/j.mrrev.2011.12.001

Finke, J., Ko, J., Rini, B., Rayman, P., Ireland, J., & Cohen, P. (2011). International Immunopharmacology MDSC as a mechanism of tumor escape from sunitinib mediated anti-angiogenic therapy ☆. *International Immunopharmacology*, *11*(7), 856–861. https://doi.org/10.1016/j.intimp.2011.01.030

Gasper, A. V, Al-janobi, A., Smith, J. A., Bacon, J. R., Fortun, P., Atherton, C., … Mithen, R. F. (2005). Glutathione S -transferase M1 polymorphism and metabolism of sulforaphane from standard and high-glucosinolate broccoli 1 – 3. *The American Journal of Clinical Nutrition*, *82*(6), 1283–1291. https://doi.org/10.1093/ajcn/82.6.1283

Giudice, A., & Montella, M. (2006). Activation of the Nrf2 – ARE signaling pathway : a promising strategy in cancer prevention. *Bioessays*, *28*, 169–181. https://doi.org/10.1002/bies.20359

Gong, J., Caitano, M., Dizman, N., Govindarajan, A., & Pal, S. K. (2016). ScienceDirect Metastasis in renal cell carcinoma : Biology and implications for therapy. *Asian Journal of Urology*, *3*(4), 286–292. https://doi.org/10.1016/j.ajur.2016.08.006

Grange, C., Tapparo, M., Collino, F., Vitillo, L., Damasco, C., Deregibus, M. C., … Camussi, G. (2011). Microvesicles Released from Human Renal Cancer Stem Cells Stimulate Angiogenesis and Formation of Lung Premetastatic Niche. *Cancer Research: Tumor and Stem Cell Biology*, *71*(15), 5346–5356. https://doi.org/10.1158/0008-5472.CAN-11-0241

Guerreiro, Í., Vidovic, B., Costa, J. G., Martins, M., Ferreira, S., Oliveira, N. G., … Fernandes, A. S. (2023). The Dietary Isothiocyanate Erucin Reduces Kidney Cell Motility by Disturbing Tubulin Polymerization. *Molecular Nutrition & Food Research*, *267*, *20058*, 1–10. https://doi.org/10.1002/mnfr.202200581

Hanlon, N., Coldham, N., Sauer, M. J., & Ioannides, C. (2008). Up-regulation of the CYP1 family in rat and human liver by the aliphatic isothiocyanates erucin and sulforaphane. *Toxicology*, *252*, 92–98. https://doi.org/10.1016/j.tox.2008.08.002

Hanna, P. E., & Anders, M. W. (2020). Critical Reviews in Toxicology The mercapturic acid pathway. *Critical Reviews in Toxicology*, *49*(10), 819–929. https://doi.org/10.1080/10408444.2019.1692191

Harris, K. E., & Jeffery, E. H. (2008). Sulforaphane and erucin increase MRP1 and MRP2 in human carcinoma cell lines. *Journal of Nutritional Biochemistry*, *19*, 246–254. https://doi.org/10.1016/j.jnutbio.2007.02.014

He, B., Briez, N., Bernard, D., Seuningen, I. Van, & Leroy, X. (2009). MUC1 , a New Hypoxia Inducible Factor Target Gene , Is an Actor in Clear Renal Cell Carcinoma Tumor Progression Se. *American Association for Cancer Research*, (14), 5707–5715. https://doi.org/10.1158/0008-5472.CAN-08-4905

Heaney, R. K., & Fenwick, G. R. (1995). Natural Toxins and Protective Factors in Brassica Species, Including Rapeseed. *NATURAL TOXINS*, *3*, 233–237.

Higdon, J. V, Delage, B., Williams, D. E., & Dashwood, R. H. (2007). Cruciferous vegetables and human cancer risk : epidemiologic evidence and mechanistic basis. *Pharmacol Res*, *55*(3), 224–236. https://doi.org/10.1016/j.phrs.2007.01.009

Holloway, P. M., Gillespie, S., Becker, F., Vital, S. A., Nguyen, V., Alexander, J. S., … Gavins, F. N. E. (2017). Sulforaphane Induces Neurovascular Protection Against a Systemic Inflammatory Challenge via Both Nrf2-Dependent and Independent Pathways. *Vascul Pharmacol*, *85*, 29–38. https://doi.org/10.1016/j.vph.2016.07.004.Sulforaphane

Hsieh, J. J., Purdue, M. P., Signoretti, S., Swanton, C., Schmidinger, M., Heng, D. Y., … Baker, T. (2018). Renal cell carcinoma. *Nat Rev Dis Primers*, *3*(17009), 1–42. https://doi.org/10.1038/nrdp.2017.9.Renal

Iori, R., Bernardi, R., Gueyrard, D., Rollin, P., & Palmieri, S. (1999). Formation of glucoraphanin by chemoselective oxidation of natural glucoerucin: a chemoenzymatic route to sulforaphane. *Bioorganic & Medicinal Chemistry Letters*, *9*, 1047–1048.

Jakubikova, J., Bao, Y., & Sedlak, J. A. N. (2005). Isothiocyanates Induce Cell Cycle Arrest , Apoptosis and Mitochondrial Potential Depolarization in HL-60 and Multidrug-resistant Cell Lines. *ANTICANCER RESEARCH*, *25*, 3375–3386.

Jakubı, J. (2005). Role of PI3K / Akt and MEK / ERK signaling pathways in sulforaphane- and erucin-induced phase II enzymes and MRP2 transcription , G 2 / M arrest and cell death in Caco-2 cells. *Biochemical Pharmacology*, *69*, 1543–1552. https://doi.org/10.1016/j.bcp.2005.03.015

Jiao, D. (1994). Identification and Quantification of the N-Acetylcysteine Conjugate of Allyl Isothiocyanate in Human Urine after Ingestion of Mustard1. *Cancer Epidemiology, Biomarkers & Prevention*, *3*(September), 487–492.

Johnson, I. T. (2003). Glucosinolates in the human diet . Bioavailability and implications for health. *Phytochemistry Reviews 1:*, *1*, 183–188.

Jr, W. G. K. (2008). The von Hippel – Lindau tumour suppressor protein : O 2 sensing and cancer. *Hypoxia and Metabolism*, *8*(November), 865–873. https://doi.org/10.1038/nrc2502

Kanwal, R. (2023). Advances in Cancer Biology - Metastasis Metastasis in renal cell carcinoma : Biology and treatment. *Advances in Cancer Biology - Metastasis*, *7*(August 2022), 100094. https://doi.org/10.1016/j.adcanc.2023.100094

Kassahun, K., Davis, M., Hu, P., Martin, B., & Baillie, T. (1997). Biotransformation of the Naturally Occurring Isothiocyanate Sulforaphane in the Rat : Identification of Phase I Metabolites and Glutathione Conjugates. *Chem. Res. Toxicol.*, (97), 1228–1233.

Kin, S.-H., & Sing, S. (2010). D,L-sulforaphane causes transcriptional repression of androgen receptor in human prostate cancer cells. *Mol Cancer Ther*, *8*(7), 1946–1954. https://doi.org/10.1158/1535-7163.MCT-09-0104.D

Kramer, H. (1997). Inhibition of Cytochromes P-450 and Induction of Glutathione S - Transferases by Sulforaphane in Primary Human and Rat Hepatocytes. *Cancer Research*, (57), 3649–3652.

Kumar, G., Tuli, H. S., Mittal, S., & Shandilya, J. K. (2015). Isothiocyanates : a class of bioactive metabolites with chemopreventive potential. *Tumor Biol.* https://doi.org/10.1007/s13277-015-3391-5

Lamy, E., & Mersch-sundermann, V. (2009). Research Article MTBITC Mediates Cell Cycle Arrest and Apoptosis Induction in Human HepG2 Cells Despite its Rapid Degradation Kinetics in the In Vitro Model. *Environmental and Molecular*

Mutagenesis, *50*(September 2008), 190–200. https://doi.org/10.1002/em

Lamy, E., Schröder, J., Paulus, S., Brenk, P., & Stahl, T. (2008). Antigenotoxic properties of Eruca sativa (rocket plant), erucin and erysolin in human hepatoma (HepG2) cells towards benzo (a) pyrene and their mode of action. *Food and Chemical Toxicology*, *46*, 2415–2421. https://doi.org/10.1016/j.fct.2008.03.022

Liao, Z., Fang, Z., Gou, S., Luo, Y., Liu, Y., He, Z., … Fu, Z. (2022). The role of diet in renal cell carcinoma incidence : an umbrella review of meta- analyses of observational studies. *BMC Medicine*, 1–26.

Lyons, S. M., Alizadeh, E., Mannheimer, J., Schuamberg, K., Castle, J., Schroder, B., … Prasad, A. (2016). Changes in cell shape are correlated with metastatic potential in murine and human osteosarcomas, 289–299. https://doi.org/10.1242/bio.013409

Mastuo, T., Miyata, Y., Yuno, T., Mukae, Y., & Otsubo, A. (2020). Molecular Mechanisms of the Anti-Cancer E ff ects of Isothiocyanates from Cruciferous Vegetables. *Molecules*, *25*(575), 1–22.

Melchini, A, Costa, C., Traka, M., Miceli, N., Mithen, R., Pasquale, R. De, … Martino, G. (2009). Erucin , a new promising cancer chemopreventive agent from rocket salads , shows anti-proliferative activity on human lung carcinoma A549 cells. *Food and Chemical Toxicology*, *47*(7), 1430–1436. https://doi.org/10.1016/j.fct.2009.03.024

Melchini, Antonietta, & Traka, M. H. (2014). Biological Profile of Erucin: A New Promising Anticancer Agent from Cruciferous Vegetables. *Experimental Hematology & Oncology*, *3*(5), 593–612. https://doi.org/10.3390/toxins2040593

Muglia, V. F., & Prando, A. (2015). Renal cell carcinoma : histological classification and correlation with imaging findings *. *Radiol Bras.*, *48*(3), 166–174.

Nabi, S., Kessler, E. R., Bernard, B., Flaig, T. W., & Lam, E. T. (2018). Renal cell carcinoma : a review of biology and pathophysiology [version 1 ; referees : 2 approved] Referee Status : *F1000Research*, *7*(0), 1–10. https://doi.org/10.12688/f1000research.13179.1

Oliviero, T., Verkerk, R., & Dekker, M. (2018). Isothiocyanates from Brassica Vegetables — Effects of Processing , Cooking , Mastication , and Digestion. *Molecular Nutrition & Food Research*, *1701069*, 1–11. https://doi.org/10.1002/mnfr.201701069

Pdbiad, C. (2008). Glucosinolates and their potential role in plant. *Period Biol*, *110*(4), 297–309.

Rene, Â. (2000). Risk factors for adult renal cell carcinoma : a systematic review and implications for prevention. *BJU International (2000), 86*(March), 20–27.

Ricketts, C. J., Cubas, A. A. De, Fan, H., Smith, C. C., Reznik, E., Bowlby, R., … Laura, S. (2018). The Cancer Genome Atlas Comprehensive Molecular Characterization of Renal Cell Carcinoma. *Cell Rep.*, *23*(1), 313–326. https://doi.org/10.1016/j.celrep.2018.03.075.The

Rose, P., Ong, C. N., & Whiteman, M. (2005). Protective effects of Asian green vegetables against oxidant induced cytotoxicity. *World Journal of Gastroenterology*, *11*(48), 7607–7614.

Rossi, S. H., Klatte, T., Usher, J., & Grant, S. (2018). Epidemiology and screening for renal cancer. *World Journal of Urology*, *36*(9), 1341–1353. https://doi.org/10.1007/s00345-018-2286-7

Sato, Y., Yoshizato, T., Shiraishi, Y., Maekawa, S., Okuno, Y., Kamura, T., … Miyano, S. (2013). Integrated molecular analysis of clear-cell renal cell carcinoma. *Nature Genetics*, (June). https://doi.org/10.1038/ng.2699

Scelo, G., & Larose, T. L. (2018). Epidemiology and Risk Factors for Kidney Cancer. *Clinical Oncology*, *36*(36), 3574–3582. https://doi.org/10.1200/JCO.2018.79.1905

Shelton, P., & Jaiswal, A. K. (2012). The transcription factor NF-E2-related Factor 2 (Nrf2): a protooncogene ? *The FASEB*, *2*, 1–10. https://doi.org/10.1096/fj.12-217257

Siegel, R. L., & Miller, K. D. (2019). Cancer Statistics , 2019, *69*(1), 7–34. https://doi.org/10.3322/caac.21551.

Simal-gandara, J. (2019). Glucosinolates : Molecular structure , breakdown , genetic , bioavailability , properties and healthy and adverse effects. *Advances in Food and Nutrition Research*, 1–46. https://doi.org/10.1016/bs.afnr.2019.02.008

Song, L., & Thornalley, P. J. (2007). Effect of storage , processing and cooking on glucosinolate content of Brassica vegetables. *Food and Chemical Toxicology*, *45*, 216–224. https://doi.org/10.1016/j.fct.2006.07.021

Soundararajan, P., & Kim, J. S. (2018). Anti-Carcinogenic Glucosinolates in Cruciferous Vegetables and Their Antagonistic Effects on Prevention of Cancers. *Molecules*, *23*(2983), 1–21. https://doi.org/10.3390/molecules23112983

Srigley, J. R., Delahunt, B., Eble, J. N., Egevad, L., Epstein, J. I., Grignon, D., … Tumor, R. (2013). The International Society of Urological Pathology (ISUP) Vancouver Classification of Renal Neoplasia. *Am J Surg Pathol*, *37*(10), 1469–

1489.

Stewart, D., & Mcdougall, G. (2012). The Brassicas – An Undervalued Nutritional and Health Beneficial Plant Family. *Food & Health Innovation*.

Tian, T., Li, X., & Zhang, J. (2019). mTOR Signaling in Cancer and mTOR Inhibitors in Solid Tumor Targeting Therapy. *International Journal of Molecular Sciences*, *20*(755), 1–34. https://doi.org/10.3390/ijms20030755

Uekita, T., & Sakai, R. (2011). Roles of CUB domain-containing protein 1 signaling in cancer invasion and metastasis. *Cancer Science*, *102*(11), 1943–1948. https://doi.org/10.1111/j.1349-7006.2011.02052.x

Ullah, M. F. (2015). Sulforaphane (SFN): An Isothiocyanate in a Cancer Chemoprevention Paradigm. *Medicines*, *2*, 141–156. https://doi.org/10.3390/medicines2030141

Unday, C. H. M. M. (2004). Induction of Phase II Detoxification Enzymes in Rats by Plant-Derived Isothiocyanates : Comparison of Allyl Isothiocyanate with Sulforaphane and Related Compounds. *Agricultural and Food Chemistry*, (52), 1867–1871.

Verhoeven, D. T. H., Goldbohm, R. A., Poppel, G. Van, & Verhagen, H. (1996). Epidemiological Studies on Brassica Vegetables and Cancer Risk. *Cancer Epidemiology, Biomarkers & Prevention*, *5*(September), 733–748.

Vermeulen, M., Berg, R., Freidig, A., Bladeren, P., & Vaes, W. (2006). Association between Consumption of Cruciferous Vegetables and Condiments and Excretion in Urine of Isothiocyanate Mercapturic Acids. *Agricultural and Food Chemistry*, (54), 5350–5358.

Wang, Y., Zhang, Z., Sun, W., Tan, Y., Liu, Y., Zheng, Y., … Sun, J. (2014). Sulforaphane Attenuation of Type 2 Diabetes-Induced Aortic Damage Was Associated with the Upregulation of Nrf2 Expression and Function. *Oxidative Medicine and Cellular Longevity*, 11.

WHO. (2021). GLOBOCAN 2021: estimated cancer incidence, mortality and prevalence worldwide in 2021. Retrieved from https://gco.iarc.fr/

Wu, X., Zhou, Q., & Xu, K. (2009). Are isothiocyanates potential anti-cancer drugs ? *Acta Pharmacologica Sinica*, *30*(5), 501–512. https://doi.org/10.1038/aps.2009.50

Yu, H., Lim, K. P., Xiong, S., Tan, L. P., & Shim, W. (2013). Functional Morphometric Analysis in Cellular Behaviors : Shape and Size Matter. *Adv. Healthcare Mater.*, 1–10. https://doi.org/10.1002/adhm.201300053

Zhang, S., Jia, Z., Yan, Z., & Yang, J. (2017). Consumption of fruits and vegetables and risk of renal cell carcinoma : a meta-analysis of observational studies. *Oncotarget*, *8*(17), 27892–27903.

Zhang, Y. (2004). Cancer-preventive isothiocyanates : measurement of human exposure and mechanism of action. *Mutation Research 555*, *555*, 173–190. https://doi.org/10.1016/j.mrfmmm.2004.04.017

Zhang, Y. (2012). The molecular basis that unifies the metabolism , cellular uptake and chemopreventive activities of dietary isothiocyanates. *Carcinogenesis*, *33*(1), 2–9. https://doi.org/10.1093/carcin/bgr255

Zhang, Y., Talalay, P., Cho, C.-G., & Posner, G. (1992). A major inducer of anticarcinogenic protective enzymes from broccoli: Isolation and elucidation of structure. *Proc.Natl. Acad.Sci.*, *89*(March), 2399–2403.

Zhao, J., & Zhao, L. (2013). Cruciferous Vegetables Intake Is Associated with Lower Risk of Renal Cell Carcinoma : Evidence from a Meta- Analysis of Observational Studies. *PLOS One*, *8*(10). https://doi.org/10.1371/journal.pone.0075732

Zhao, Z., Liao, G., Zhou, Q., Lv, D., Holthfer, H., & Zou, H. (2016). Sulforaphane Attenuates Contrast-Induced Nephropathy in Rats via Nrf2 / HO-1 Pathway. *Oxidative Medicine and Cellular Longevity*, 11–13.