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ESCOLA SUPERIOR DE TECNOLOGIA DA SAÚDE DE LISBOA

MTOR Expression Regulation and New Anticancer Applications for Apigenin

Mestrando: Renata Wellenkamp de Carvalho Freire de Cervantes

Orientador: Prof. Doutora Ana Ramos [1,2]

1- Escola Superior de Tecnologias da Saúde de Lisboa (ESTeSL)

2- Centro de Investigação em Saúde e Tecnologia (H&TRC)

Mestrado em Tecnologias Clínico-Laboratoriais

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Júri:

Presidente- Doutora Edna Ribeiro [1,2]

Orientador- Doutora Ana Ramos [1,2]

Arguente- Doutora Vânia Gonçalves [3]

1-Escola Superior de Tecnologias da Saúde de Lisboa (ESTeSL)

2-Centro de Investigação em Saúde e Tecnologia (H&TRC)

3-Instituto Nacional de Saúde Doutor Ricardo Jorge

Mestrado em Tecnologias Clínico-Laboratoriais

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Resumo

A apigenina (4',5,7-tri-hidroxiflavona) é um dos flavonoides mais abundantes do mundo vegetal e um dos fenólicos mais pesquisados. Sabe-se que a apigenina reduz a proliferação celular nas células de cancro gástrico, mas resta saber se a apigenina reduz o mTOR nas células de cancro gástrico, se esta redução no mTOR é de alguma forma responsável pela supracitada diminuição da proliferação, e se a exposição à apigenina é total ou parcialmente responsável por esta diminuição. A via de sinalização do mTOR encontra-se hiperactivada em cerca de 70% dos cancros humanos, nomeadamente no cancro gástrico, assim como em doenças muito prevalentes na nossa população como a diabetes e as doenças cardiovasculares. mTOR é um centro celular que integra sinais intra e extracelulares em termos de energia, nutrientes, e disponibilidade hormonal, modulando as respostas moleculares para adquirir um estado homeostático através da regulação de processos anabólicos e catabólicos. Consequentemente, a desregulação da via mTOR tem sido associada a uma variedade de doenças humanas. Embora tenham surgido nos últimos anos grandes avanços relativamente aos reguladores e efeitos da via de sinalização do mTOR, pouco se sabe sobre a regulação da expressão genética do *mTOR*. Os dados atualmente disponíveis sobre as alterações de expressão de mTOR observadas em várias doenças, particularmente cancros humanos, são aqui apresentados, e o conhecimento atual sobre a regulação de mTOR a nível transcricional e translacional é apresentado de forma sistematizada e é descrito como diferentes miRNAs afetam a sinalização de mTOR em condições patológicas. Além disso, utilizando linhas celulares de cancrogástrico Gp202, foi possível caracterizar o potencial anti-tumorigénico da apigenina, descrevendo a sua influência na morfologia celular e nos mecanismos associados à morte celular. Este estudo ajudará na prossecução da investigação das propriedades anti-tumorais da apigenina em relação ao cancro gástrico, particularmente o seu possível efeito sobre o mTOR, bem como no desenvolvimento de novos inibidores do mTOR, uma vez que proporciona uma perspetiva exaustiva sobre a regulação da expressão do gene *mTOR*.

Palavras-Chave: Via de sinalização mTOR; Cancro Gástrico; Inibição mTOR; Diminuição da expressão proteica; Apigenina

Abstract

Apigenin (4',5,7-trihydroxyflavone) is one of the most abundant flavonoids in the plant world and one of the most researched phenolics. Apigenin is known to reduce cell proliferation in gastric cancer cells, but it remains to be seen whether apigenin reduces mTOR in gastric cancer cells, whether this reduction in mTOR is in any way responsible for the aforementioned decrease in proliferation, and whether exposure to apigenin is fully or partially responsible for this decrease. The mechanistic/mammalian target of rapamycin (mTOR) signaling pathway is hyperactivated in about 70% of human cancers, including gastric cancer, as well as in diseases that are very prevalent in our population such as diabetes and cardiovascular disease. mTOR is a central cellular hub that integrates intra- and extracellular signals in terms of energy, nutrient, and hormone availability, modulating the molecular responses to acquire a homeostatic state through the regulation of anabolic and catabolic processes. Accordingly, dysregulation of mTOR pathway has been implicated in a variety of human diseases. While major advances have emerged regarding the regulators and effects of mTOR signaling pathway, little is known about the regulation of *mTOR* gene expression. The currently available data regarding mTOR expression changes observed in several diseases, particularly human cancers, is presented here, and the current knowledge about mTOR regulation at the transcriptional and translational levels is systematized. It also demonstrated how different miRNAs affect mTOR signaling both in pathological conditions. Furthermore, utilizing Gp202 gastric carcinoma cell lines, it was possible to define apigenin's anti-tumor potential by establishing its influence on cell morphology and the mechanisms associated withcell death. This study, I believe, will assist in the pursuit of research into apigenin's anti-tumoralproperties in relation to gastric cancer, particularly its possible effect on mTOR, as well as in the development of new mTOR inhibitors since it provides an exhaustive perspective on the regulation of *mTOR* gene expression.

Key words: mTOR signaling pathway; Gastric Cancer; mTOR inhibition;
Decreased protein expression; Apigenin

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Muito Obrigada

“For a research worker, the unforgotten moments of his life are those rare ones which come after years of plodding work, when the veil over natures secret seems suddenly to lift & when what was dark & chaotic appears in a clear & beautiful light & pattern.”

Gerty Cori

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ABBREVIATIONS LIST

4E-BP1- Eukaryotic translation initiation factor 4E-binding protein 1
AKT- Protein kinase B
AMP- Adenosine monophosphate
ATP- Adenosine triphosphate
CDK- Cyclin-dependent kinase
CO2 – Carbon dioxide
DEPTOR- DEP Domain Containing MTOR Interacting Protein
DMSO – Dimethyl sulfoxide
DNA- Deoxyribonucleic acid
EFSA- European Food Safety Authority
EGFR- Epidermal growth factor receptor
EV- Extracellular vesicles
FBS – Fetal bovine serum
FMO- Flavin-containing monooxygenase
GSK3 - Glycogen synthase kinase 3
MCL- Mantle cell lymphoma
mRNA- Messenger RNA
mTOR - Mechanistic (previously known as "mammalian") Target of Rapamycin
mTORC – mTOR complex
NADPH- Nicotinamide adenine dinucleotide phosphate
NEC- Neuroendocrine carcinoma
NSCLC- Non-small cell lung cancer
p-4EBP1- Phosphorylated 4E-binding protein 1
p70S6K – Ribosomal protein S6 kinase beta-1
p-AKT- Phosphorylated AKT
PBS – Phosphate-buffered saline
PDGFR- Platelet derived growth factor receptor
PDK1 – 3-Phosphoinositide-dependent kinase 1
PI3K – Phosphoinositide 3-kinases
p-mTOR – Phosphorylated mTOR
PRA40- Proline-rich AKT1 substrate 1
PTEN – Phosphatase and tensin homolog
Raptor – Regulatory-associated protein of mTOR
Rheb – Ras Homolog, MTORC1 Binding
Rictor – Rapamycin-insensitive companion of mammalian target of rapamycin
TSC- Tuberous sclerosis complex
VEGFR- Vascular endothelial growth factor receptor
WHO- World Health Organization

Chapter One - Theoretical Context

1 Theoretical Context

1.1. Cancer and Its molecular mechanisms

According to the World Health Organization, “Cancer is a large group of diseases that can start in almost any organ or tissue of the body when abnormal cells grow uncontrollably, go beyond their usual boundaries to invade adjoining parts of the body and/or spread to other organs” (WHO). The latter process is called metastization and is a major cause of death from cancer (Seyfried, T. N., & Huysentruyt, L. C., 2013). While benign tumors may normally be surgically removed, malignant tumors are frequently resistant to such targeted therapy due to their spread to distant body regions (Cooper GM., 2000). Neoplasm and malignant tumor are othercommon names for cancer.

Chemical carcinogens, such as the products of tobacco pyrolysis; physical carcinogens, such as radiation; biological carcinogens, such as infectious agents (viruses), hormones, chronic inflammation, and oxidative stress are all factors that contribute to this form of illness (Santos & Teixeira, 2011). The process by which normal cells are transformed into cancer cells is divided into four phases: initiation, promotion, progression, and the potential for metastasis. (Figure 1.1)

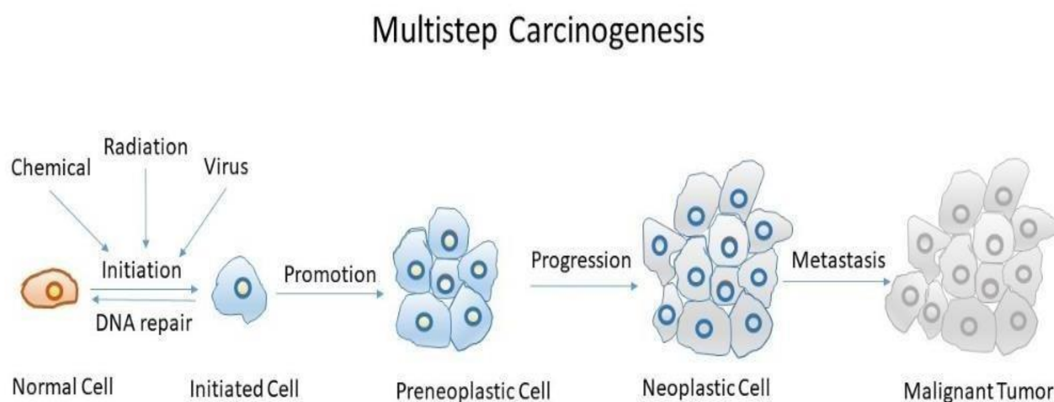

Figure 1.1 - Multistep process involved in carcinogenesis that transforms a normal cell into a malignant tumor. (Available at: https://med.libretexts.org/courses/american_public_university/apus%3a_an_introduction_to_nutrition_(byerley)/text/07%3a_nutrition_and_cancer/7.02%3a_carcinogenesis) accessed in November 2021.

The first stage of carcinogenesis consists of a mutation and/or genetic alterations in a single cell, i.e., it is the occurrence of an event that alters the cell genome through alterations in tumor suppressor genes and/or protooncogenes (Vineis, P., 2010). The next step in the multistep carcinogenesis model is the clonal expansion of the initiated cells (Vineis, P., 2010). The transformed cells require stimulation to proliferate at this point, and the way by which they promote neoplasm development divides the stimulation into three sorts (Cooper GM., 2000). The first type includes genotoxic chemicals, which induce direct DNA damage by forming DNA adducts. Second type are mitogens, which attach to receptors on cells and drive cell division, resulting in long-term hyperplasia. Finally, there are cytotoxic compounds that induce tissue damagethat leads to hyperplasia (Cohen & Ellwein, 1990). The accumulation of these changes (mutations), which cause a lack of physiological control over cell proliferation and a variety of biological functions, leads to malignant transformation(Stevens & Lowe, 2002). Finally, there is progression, which is a phase in which cell development becomes independent of the carcinogen or promoter, and there are already enough mutations to immortalize the cells. The final point of progression is the establishment of an invasive tumor. (Stevens & Lowe, 2002). DNA damage can be caused by both endogenous and exogenous agents, such as bile acids or reactive oxygen species, cigarette smoke, radiation, pollution, and many others (Chatterjee, N., & Walker, G. C., 2017).

DNA replicates normally in a healthy body, with no negative consequences for the organism. However, genetic material may occasionally be damaged as a result of a variety of external and internal stressors (Tiwari, V., & Wilson, D. M., 3rd, 2019). At the beginning of the twentieth century Theodor Boveri proposed that changes in a cell's genetic material may be the foundation of malignant transformation. Boveri's idea is based on the fact that cancer cells frequently exhibit chromatin abnormalities and aberrant mitotic figures (McKusick & Boveri, 1985). Over the following years other researchers converged on the same conclusion, stating that genotoxic chemical and physical agents could induce cancer and that certain forms of neoplasia would have hereditary transmission (Bishop, 1987). However, it was through the use of oncogenic retroviruses that the presence of genes capable of triggering malignant transformation was established for the first time in 1970 (Duesberg, P. H., & Vogt, P. K., 1970). These findings were confirmed when cell lines were transfected with tumoral DNA, resulting in malignant transformation (Bernstein & Weinberg, 1985). It is now known that cancer development involves multiple dysregulated processes leading to uncontrolled cell growth (Vogelstein et al., 2013). As cancerous cells progress through the tumor, they undergo a wide range of mutations in their characteristics. This is a multi-stage process that involves sequential mutations and/or epimutations, resulting in uncontrolled cell growth and hedonistic homeostasis regulation. Mutations that cause cancer affect the metabolism and behavior of

cells in a progressive manner. They change their proliferative control, give them unlimited life, change their communication with neighboring cells, and then provide them the ability to escape into the immune system. In summary, they are genetically and/or epigenetically tainted, but they can divide and multiply on their own (Hong S. N., 2018; Puneet, et al, 2018).

Throughout the cell cycle process, regulatory checkpoints regulate the compliance and adherence of the molecular and structural events that occur in accordance to the ideal cell cycle framework. (Elledge S. J., 1996; Barnum, K. J., & O'Connell, M. J., 2014). At the checkpoints, detected anomalies activate processes that lead to a variety of alternative outcomes, including a repair mechanism (DNA repair), cell arrest (senescence) pathways, necroptosis, and apoptosis (Barnum, K. J., & O'Connell, M. J., 2014). When a proliferating cell fails to activate any of these systems in unicellular organisms, the reproductive capability is reduced, but in multicellular species it leads to unregulated cell proliferation, which can lead to cancer (Kastan, M. B., & Bartek, J., 2004). The cell cycle is governed by two types of regulatory mechanisms. The first is extracellular and includes signaling pathways and intracellular processes that are controlled by checkpoints (Lukas, J., et al, 2004).

One important fact to be taken into consideration when talking about cells and cell signaling pathways is that they are not isolated from each other but are connected to form complex signalingnetworks. Various pathways govern cell proliferation, motility, and survival, and the changes that occur in cancer cells are the consequence of multiple modifications in the cell signaling machinery (Sever, R., & Brugge, J. S., 2015). (Figure 1.2)

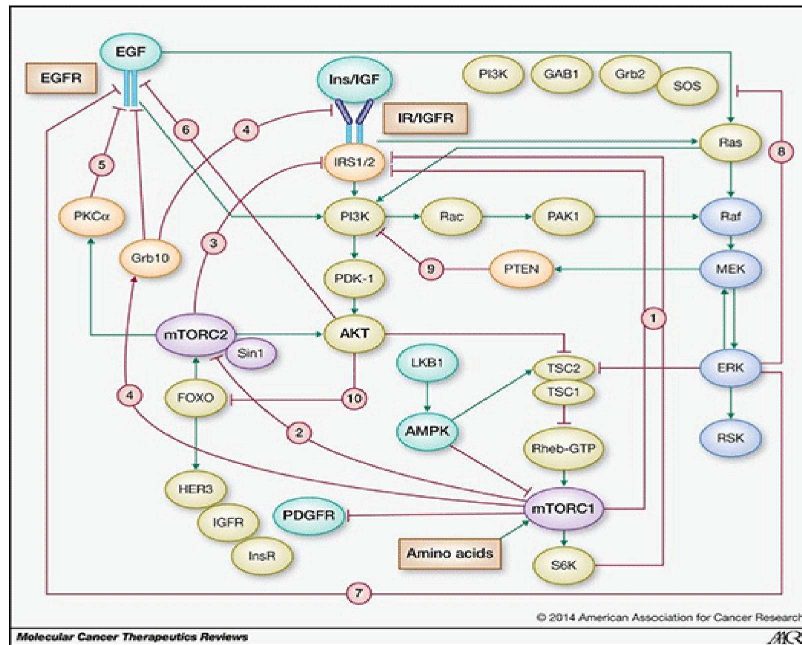

Figure 1.2 - PAM (pi3k/akt/mtor) pathway *(stimulatory connections in green; negative feedback loops in red) (from rozengurt e et al, 2014). (Available at: https://www.marycrowley.org/groundbreaking-research/cancer-pathways/); accessed in December 2021*

Since the mechanisms responsible for the formation of tumoral processes eventually come from changes in the regulation of cell cycle progression, the complexity of cell signaling networks has important implications for understanding the behavior of tumor cells, since all this knowledge is incredibly relevant in terms of therapeutic implications (Giancotti F. G., 2014).

The multi-stage and multi-hit models of carcinogenesis suggest that cancer is caused by a limited number of (two–seven) mutations (NORDLING C. O., 1953; Zhang, X., & Simon, R., 2005; Tomasetti, C., et al, 2015). Despite the availability of substantial genetic data (International Cancer Genome Consortium, 2010) and decades of research, the great majority of malignancies' particular mutations that cause carcinogenesis remain unknown.

The fact that no combination of mutations (hits) is responsible for all cases of cancer, even within a cancer subtype, is a possible explanation for the failure to discover these carcinogenic mutations. Carcinogenesis, on the other hand, is the outcome of one of many conceivable combinations of a limited number of hits. A realistic estimate of the number of such hits will aid in our understanding of how malignancies develop and the identification of the exact mutations responsible for individual cases of cancer (Dash, S., et al, 2019). Knowing the number of hits with some degree of certainty is required to identify particular combinations from an increasingly vast number of potential combinations. To do so, we must employ a mathematical model that helps to predict the number of hits without knowing or making assumptions about the extremely variable and difficult to estimate mutation rate. This is a serious drawback of existing models. (Anandakrishnan, R., et al, 2019).

1.2. THE mTOR SIGNALLING PATHWAY

It was during a journey to Rapa Nui (also known as Easter Island) in 1964 that Shegal and his colleagues discovered in bacterial *Streptomyces hygroscopicus* isolate from a soil sample, the component rapamycin, that have antifungal, immunosuppressive, and anti-tumor effects. (Eng, C. P., et al, 1984; Martel, R. R., et al, 1977). Several investigations of this new compound indicated that it inhibits TOR activity by building an inhibitory complex with its intracellular receptor, the FK506-binding protein, FKBP12, which binds a region in the C terminus of TOR proteins known as FRB (FKB12–rapamycin binding) (Chen et al. 1995; Choi et al. 1996; Chung, J., et al, 1992). Despite long-held beliefs, the entire mechanism of action of rapamycinremained mysterious until 1994, when biochemical investigations linked the mechanistic (previously known as "mammalian") Target of Rapamycin (mTOR) with the direct target of therapamycin-FKB12 complex in mammals (Kathryn G. Foster, et al 2010).

mTOR is a serine/threonine protein kinase that regulates cell growth and plays a major role in regulating protein synthesis. (Figure 1.3)

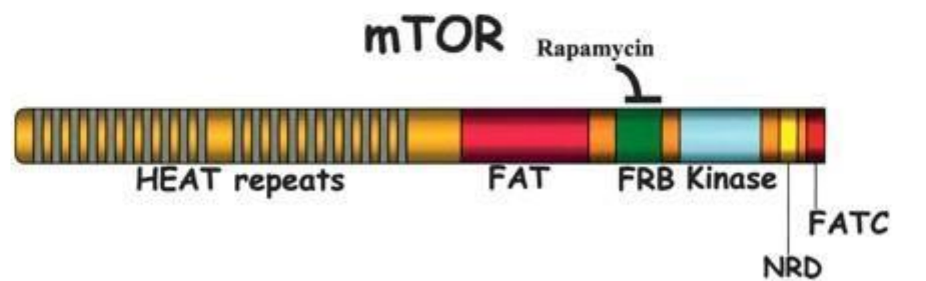

Figure 1.3 - The primary structure of mTOR and functional domains mTOR is a Ser/Thr kinase comprising of 2549 amino acids and contains several conserved functional domains. The N-terminus possesses 20 tandem HEAT repeats which serve as protein-protein interaction parts. The C-terminal domains contain a kinase domain which has sequence similarity with the catalytic domain of PI3K, a catalytic domain containing an FRB domain, a FAT domain and a FATC domain. A negative regulatory domain (NRD) is localized between the catalytic and FATC domains. *(Available at http://genesdev.cshlp.org/content/18/16/1926/f1.expansion.html; accessed in December 2021.*

The mTOR pathway is essential for cell survival, and mTOR dysregulation has major effects for the cell. This route controls critical cellular operations such as translation, transcription, protein stability, and cytoskeleton structure (Schenone, S., et al, 2011; Inoki,K., et al, 2005).

mTOR forms into two complexes, mTORC1 and mTORC2 (Laplante, M., & Sabatini, D. M., 2012). (Figure 1.4). MTORC1 is a heterotrimeric protein complex that includes a catalytic component, mTOR, as well as numerous additional proteins such as mLST8, DEPTOR, PRAS40, and a protein involved in cell control (RAPTOR) (Kim, D. H., et al,

2002; Hara, K., et al, 2002). This complex is rapamycin sensitive, and its function is connected to protein synthesis (Saxton, R. A., & Sabatini, D. M.; 2017). The mTORC2 complex is composed of the mTOR protein., Rictor, mLST8, and mSin1, which are involved inthe control of cytoskeleton activities such as stimulating actin fibers, paxillin, RhoA, RAC1, and protein kinase (PKC) (Pearce, L. R., et al, 2007; Yang, Q., et al, 2006; Sarbassov, D. D., et al, 2004). Except when treated for extended periods, mTORC2 complex is not susceptible to rapamycin (Schenone, S., et al, 2011; Jiang, B. H., & Liu, L. Z., 2008)

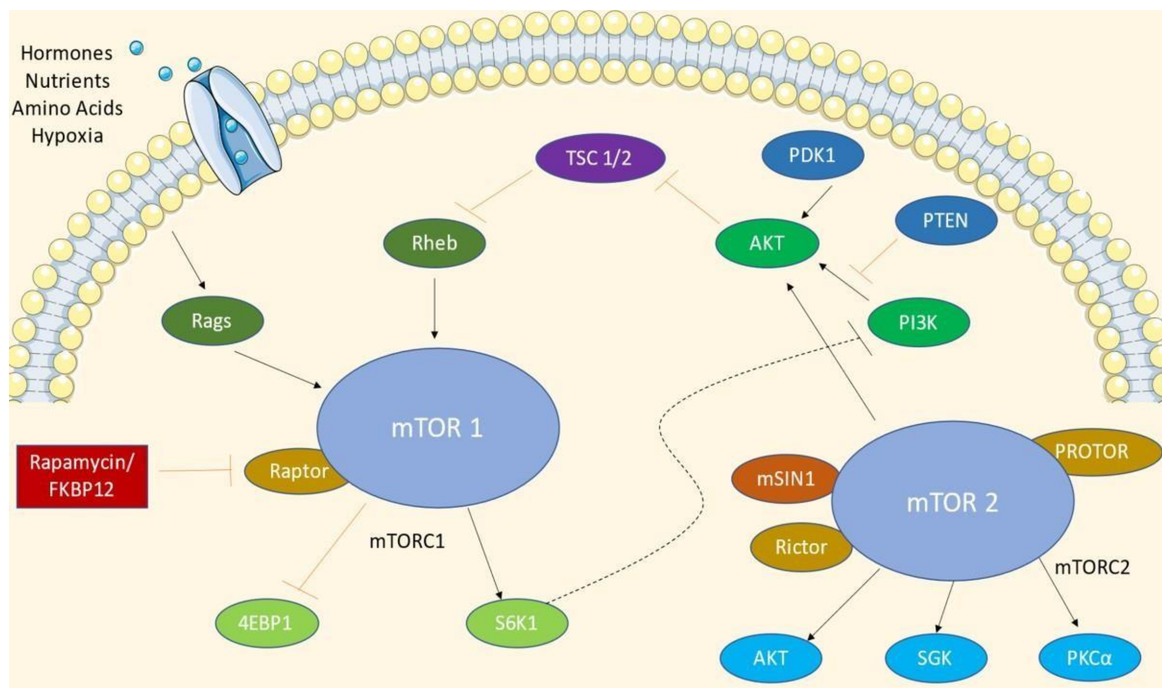

Figure 1.4 - Schematic representation of mTOR signaling pathway

Inactivation of tuberous sclerosis complex 1 (TSC1), or harmatin, and tuberous sclerosis complex 2 (TSC2), or tuberin, functions as a negative regulatory factor for mTOR, and may result in tuberous sclerosis complex syndrome. The TSC1-TSC2 heterodimer is a GTPase that is essential for mTORC1 activation. (Schenone, S., et al, 2011; Jiang, B. H., & Liu, L. Z., 2008) Inactivation of some tumor suppressor genes, such as PTEN, serine-threonine kinase 11 (STK11), or TP53, inhibits the TSC1-TSC2 complex, activating the mTOR pathway. The TSC1-TSC2 complex is inhibited through phosphorylation that can be caused by inactivation or mutation of PTEN and consequent increase in AKT, by inhibitionof AMP-activated protein kinase (AMPK) via STK11 and by increased mRNA levels causedby TP53 leading to mTOR activation (Schenone, S., et al, 2011; Feng, Z., et al, 2005; Zhang, Y., et al, 2017).

PI3K and PTEN are proteins that have been linked to reduced insulin sensitivity in malignant tumours. Mutations in the PTEN gene are observed in certain benign congenital diseases (e.g. Cowden, Bannayan Zonana) as well as in various tumours (e.g. breast, melanoma, prostate, kidney, ovarian, endometrial), emphasizing the involvement of PTEN and consequently PI3K in carcinogenesis (Zhang, Y., et al, 2017). AKT protein plays an important role in cell survival at multiple levels, including activation of mTOR, inhibition of GSK3 (glycogen synthase kinase 3), increased levels of β-catetin, and suppression of BAD protein, which is implicated in the apoptotic process (Zhang, Y.., et al, 2017), and is the main link between PI3K and the TSC complex upstream of mTORC1. AKT amplification is implicated in prostate, ovarian and breast cancers in humans, as well as in lymphomas in mice (Carnero, A., & Paramio, J. M., 2014). AKT activation is further responsible for changes in nuclear factor KB activity, hypoxia, and transcription factor alterations, all of which result in cell cycle modification and inhibition of apoptosis. AKT is typically activated in pancreatic tumours, serving as a biological marker of tumour aggressiveness (Marat, A. L., et al, 2017) (Figure 1.5).

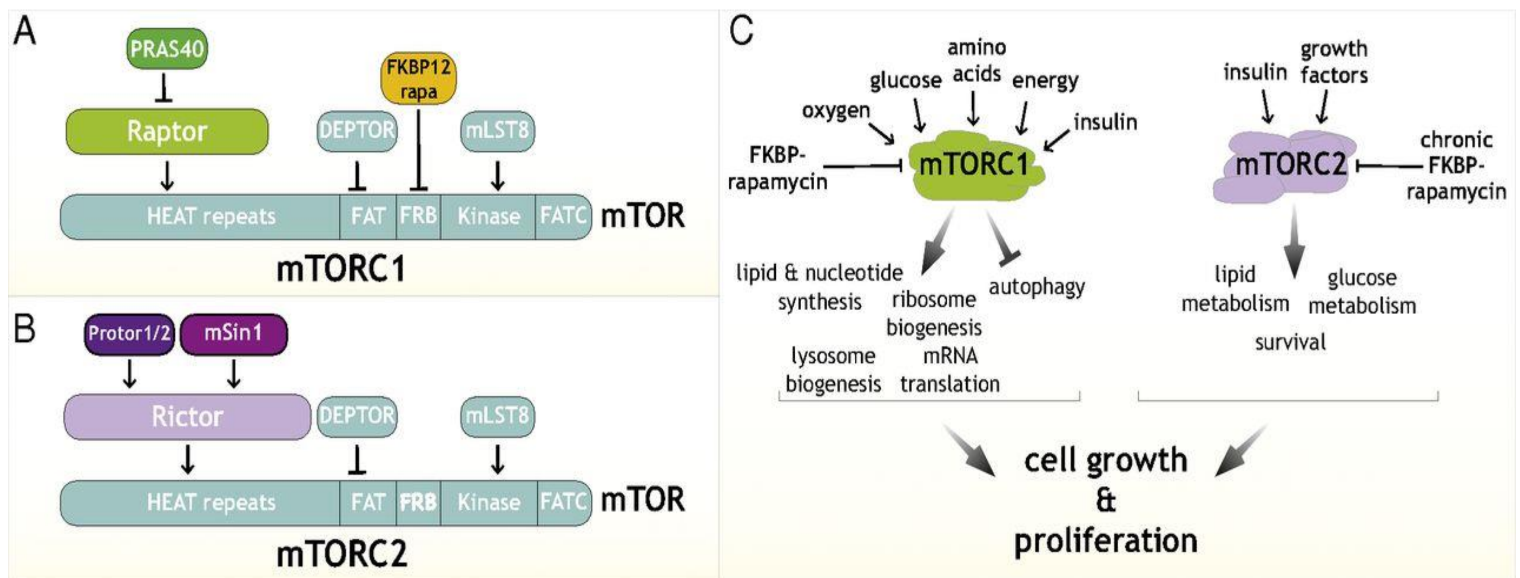

Figure 1.5 - mTOR complexes (A and B) stablished components of mtorc1 (A) and mtorc2 (B). (C) schematic showing the signals sensed by mtorc1 and mtorc2 and the processes they regulate to control growth (available at: https://www.pnas.org/content/114/45/11818); accessed in December 2021

1.3. THE mTOR PATHWAY IN CANCER

The mTOR signaling pathway is critical for cell growth and proliferation since it coordinates anabolic activities with oxygen, energy, and nutrition availability, as well as external signals. Cancer cells have the ability to sustain chronic proliferation in the absence of growth-promoting cues, which is a basic feature. This proliferative advantage is acquired, at least in part, by genetic processes that result in abnormal mTORC1 signaling activity (Cargnello, M., et al, 2015). Aberrant mTOR signaling caused by genetic changes at different stages of the signal cascade is frequent in several forms of cancer (Tian, T., Li, X., & Zhang, J., 2019). When activated mTOR signaling, stimulates cell proliferation and metabolism, which contribute to tumor initiation and progression. Furthermore, mTOR suppresses autophagy in a variety of ways. Changes in the PI3K/mTOR pathway are linked to cancer by intricate mechanisms such as amplification or hyperactivation of proto-oncogenes including *RAS, PI3K, AKT*, epidermal growth factor receptor (*EGFR*), *HEr2/neu*, *BCR-ABL*, or loss of function of tumor suppressor genes like *PTEN, TSC*, or *LKB 1.* (Shenone, S. et al, 2011). Several researchers have been focusing on the association between mTOR pathway and cancer, providing an overview of the protein levels of key pathway components in distinct neoplasia's. In addition, research in animal models have been performed to establish a relationship between the mTOR pathway and metabolism (Cornu, Albert, & Hall, 2012). From this studies there have been established a relation between mTOR pathway and: Lung cancer (Li L., et al, 2015; Karachaliou N., et al 2015; Alì G.,et al, 2011) Gastric cancer (Byeon S.J., et al, 2014; Bornschein J., et al 2015; LI M. et al 2012; Murayama T., et al 2009; Lohneis Philipp, et al 2014; Kasajima A. et al, 2011) Pancreatic cancer (Fujiwara M., et al, 2015; Han X., et al, 2013) Esophageal cancer (Chuang W. Y., et al, 2015; Wu N., et al, 2018); Breast cancer (Rojo F., et al, 2014; Beca F., et al, 2014; Mutee A., et al, 2009) Prostate and ovarian cancer (Kremer C. L., et al, 2006; Sutherland S. I., et al, 2014; Foster H., et al, 2010; Rogers-Broadway K. R., et al, 2019) Liver cancer (Yang Z., et al, 2011; Bennukul K., et al, 2014; Guerrero M., et al, 2019). As well as: Leukemia (Ulińska E., et al, 2016; Khanna A., et al, 2018), myeloma (Chen J., et al, 2018; Stockwin W., et al, 2016), gallbladder adenocarcinoma (Leal P., et al, 2013) and many others (Table S2.1).

1.4. THE mTOR PATHWAY AND GASTRIC CANCER

Gastric cancer (GC) is one of the most frequent cancers in the world, with about 600000 new cases annually in men and 300000 cases in women, and it is the fourth most prevalent causeof cancer death with an estimated 700000 deaths annually (Ferlay J., et al, 2010). Despite recent advances in treatment quality and alternatives, advanced gastric cancer remains one ofthe most difficult disease to cure, with a median overall survival (OS) of 10-12 months and a 5-year OS of 5-20 percent (Kahraman, S., & Yalcin, S. 2021), and surgery is still the only possibly curative treatment option. Even though a considerable number of GC patients have distant metastases at the time of diagnosis. The most prevalent location of metastases is peritoneal dissemination. Because positive peritoneal cytology (Cy1) is related with poor long-term prognosis, these individuals are classified as stage IV even if macroscopic carcinomatosisis absent (Bausys, A., et al, 2021). Patients with peritoneal metastases may benefit from cytoreductive surgery and intraperitoneal chemotherapy, but there is still an ambiguity when deciding on the best variant for the therapy (Abdel Mageed, H., et al, 2021). Despite substantial breakthroughs in Gastrointestinal (GI) cancer treatment techniques in recent decades, drug resistance via multiple mechanisms remains the most common reason of therapy failure in GI tumors (Lv, X., & Xu, G., 2021). Identifying the expression patterns of essential molecules involved in cell survival and the progression of gastric cancer may aid in the diagnosis and prevention of the disease. The solution might be found in the signaling cascades that govern cell survival and metabolism. Anomalies in each molecule involved in such cascades may result in increased viability and unrestricted multiplication of cancer cells. The PI3K axis has been demonstrated to be active in around one-third of human malignancies. Gastric cancer is one of the most commonly affected malignancies by this axis (Baghery Saghchy Khorasani, A., et al, 2021). Overactivated mTOR may potentially play a role in the regulation of GC cells' autonomous proliferation, cell growth, and differentiation (Tapia O., et al., 2014). Increased mTOR activation has been seen in 60–80 percent of stomach adenocarcinomas at various phases of the illness (Feng, Z., et al., 2005). Due to its potential in gastric cancer progression, the mTOR pathway has become the focus of development of new anticancer drugs. Several research have previously been undertaken with the goal of developing effective inhibitors for this pathway, which might lead to improved results in targeted therapy (Baghery Saghchy Khorasani, A., et al, 2021).

1.5. mTOR INHIBITORS

According to the National Cancer Institute an mTOR inhibitor is a substance that blocks a protein called mTOR, which helps control cell division. Blocking mTOR's action may keep cancer cells from growing and prevent the growth of new blood vessels that tumors need to grow. Some mTOR inhibitors are used to treat cancer. Rapamycin and its analogues, commonly known as rapalogs, are the most well-known mTOR inhibitors, and they have demonstrated some anti-tumor effects (Wang, X., & Sun, S. Y., 2009)

Rapamycin (sirolimus) is a well-known allosteric and PPI-targeting drug affecting the mTOR signaling pathway (Ballou & Lin, 2008) and was the first mTOR inhibitor to be discovered. It enters cells and forms an inhibitor complex with the intracellular receptor FKBP12, this complex binds to the C-terminus of TOR FRB proteins, where it has a lethal impact by blocking TOR signaling activities to downstream targets. (Oshiro, et al., 2004; Proud C. G., 2007)

Rapalogues were the first generation of mTOR inhibitors, which demonstrated to be useful in the treatment of select cancers but not in most solid tumors (Teng Q, et al, 2019). The mechanism by which they act is allosteric inhibition, resulting in mTORC1 inhibition and, in prolonged treatment, mTORC2 inactivation (Tian, T., et al, 2019). The Food and Drug Administration (FDA) authorized two water-soluble rapamycin derivatives, temsirolimus and everolimus, for the treatment of advanced renal cancer carcinoma (RCC) in 2007 and 2009, respectively. Everolimus was authorized by the FDA in 2011 for the treatment of individuals with progressing neuroendocrine tumors of pancreatic origin (PNET). Furthermore, temsirolimus was studied in several clinical studies for the treatment of advanced neuroendocrine carcinoma (NEC), advanced or recurrent endometrial cancer, and relapsed or refractory mantle cell lymphoma (MCL, approved in the European Union in 2009). Furthermore, a few studies with everolimus in patients with advanced gastric cancer, advanced non-small cell lung cancer (NSCLC), and advanced hepatocellular carcinoma were done. Ridaforolimus, a rapamycin analog, was also studied in clinical trials for advanced bone and soft-tissue sarcomas, as well as several other advanced solid tumors (Li, J., et al, 2014; Wander, S. A., et al, 2011). Nevertheless, rapalogs, have only shown minimal efficacy in big solid tumors in the clinic, and the reasons for rapalogs' limited clinical success have not been determined, butthey are likely related to the large number of mTORC1-regulated negative feedback loops that suppress upstream signaling systems such as receptor tyrosine kinase activation, PI3K-Akt signaling, and the Ras-ERK pathway and can be re-activated with rapamycin (Wander, S. A., et al, 2011).

Because the mTOR kinase domain is required for both rapamycin-sensitive and -insensitive activities, mTOR catalytic inhibitors have recently been created as a second generation of anti-mTOR therapies in which small compounds compete for the binding site (Zhang, Y. J., et al, 2011). Phosphorylation of mTOR does not occur in the absence of ATP, resulting in its deactivation and inhibition of mTORC1 and mTORC2 (Zhou, H. Y., & Huang, S. L., 2012). However, because mTOR and PI3K (phosphoinositide 3-kinase) are similar, these inhibitors may also inhibit PI3K, deregulating critical physiological functions while also causing adverse effects and significant toxicity (Qiu, H. Y., et al, 2021).

In light of the existing constraints, a third generation of mTOR inhibitors was developed based on the analysis of mTOR mutations that confer resistance to rapalogs or kinase inhibitors of mTOR (Rodrik-Outmezguine, V. S.,et al, 2016). To counteract these mutations, researchers created RapaLink-1, a molecule that consists of rapamycin connected to an mTOR kinase inhibitor and allows inhibition of the mutants (Rodrik-Outmezguine, V. S., et al, 2016). Studies have shown that RapaLink-1 had a better anti-cancer impact in various glioblastoma models than rapamycin or the mTOR kinase inhibitor sapanisertib (Fan, Q., et al, 2017; El Hage, A., & Dormond, O., 2021).

Novel mTOR inhibition techniques rely on direct mTOR kinase inhibitors that target both mTOR complexes at the same time (TORC1/2 inhibitors) (Guertin, D. A., & Sabatini, D. M., 2009). These TOR inhibitors with ATP-competitive catalytic sites selectively inhibit mTORC1 and mTORC2 kinase activity. TORC1/2 kinase inhibitors inhibited the mTORC1 downstream targets S6K and 4EBP1, as well as the mTORC2 signaling feedback loop, as revealed by suppression of AKTSer473 phosphorylation (Janes, M. R., 2010; Yu, K., 2010).

Aside from simultaneously inhibiting mTORC1/2, another interesting technique is to target the PI3K/AKT/mTOR signaling cascade at different levels. Dual PI3K/TORC kinase inhibitors targeting class I PI3K isoforms, mTORC1 and mTORC2, are now under preclinical and clinical research for this purpose (e.g., BEZ235 and XL765), which have the potential to outperform TORC1/2 kinase inhibitors by targeting PI3K in addition to TORC1/2 (Janes, M. R., 2010). Aside from innovative compounds possibly replacing rapalogs as mTOR inhibitors in the future, there is presently a window of opportunity to investigate the importance of other treatment methods (Wacheck V., 2010).

1.6. New therapeutic approaches in cancer

Tumors become more heterogeneous as they progress, resulting in a mixed population of cells with varying molecular characteristics and receptivity to therapy. This variability may be seen at both the geographical and temporal levels, and it is the driving force behind the formation of resistant phenotypes induced by a selection pressure during drug delivery (Dagogo-Jack, I., & Shaw, A. T., 2018). As a result, a thorough knowledge of these complicated events is critical for developing new, more accurate and efficient therapeutics. Several topics are now being investigated in the hunt for novel treatment techniques that may address existing difficulties associated with cancer medicines already accessible (Pucci, C., et al, 2019). One of the new approaches for cancer therapy focuses on targeted therapy that targets a specific region, such as tumor vasculature or intracellular organelles, while leaving the surrounding tissues unharmed, improving the treatment's specificity, lowering its downsides (Bazak, R., et al, 2015). Other of this approach focuses on nanomedicine which provides an option for biocompatible and biodegradable devices capable of delivering traditional chemotherapeutic medicines *in vivo*, boosting bioavailability and concentration surrounding tumor tissues and enhancing release profile and can be used for a variety of purposes spanning from diagnosticto therapy (Martinelli, C., et al, 2019). Extracellular vesicles (EVs), which are responsible for cancer formation, microenvironment change, and metastatic spread, have recently received alot of attention as effective drug delivery vehicles (Kumar, B., et al 2016). Another intriguing route is gene therapy and the production of apoptosis-inducing genes (Lebedeva, I. V. et al, 2003) and wild type tumor suppressors (Shanker, M., et al 2011), as well as targeted silencing oncogenes mediated by siRNAs, which is now being evaluated in several clinical studies throughout the world (Vaishnaw, A. K., et al, 2010).

Because of their anti-proliferative and pro-apoptotic qualities, natural antioxidants and several phytochemicals have lately been presented as anti-cancer adjuvant therapy (Chikara, S., et al 2018; Singh, S., et al, 2016; Singh K, et al, 2017). Thermal ablation of tumors and magnetic hyperthermia are expanding precision medicine potential by allowing treatment to be targeted in extremely small and precise locations, suggesting that they might be used in place of more intrusive procedures such as surgery (Brace C., 2011; Hervault, A., & Thanh, N. T., 2014)

Furthermore, emerging domains such as radiomics and pathomics are contributing to the creation of novel ways for gathering large quantities of data, developing new therapeutic strategies (Yu, K. H., et al, 2016), and accurately predicting responses, clinical outcomes, and cancer recurrence (Grove, O., 2015; Kong, J., 2013).

When combined, these tactics will be able to give the greatest personalized treatments for cancer patients, emphasizing the significance of integrating several disciplines to get the best results. In this review we will be focusing on the therapeutic potential of flavonoids which have gained a great deal of interest because of their wide range of pharmacological actions, including antioxidant, antimutagenic, antibacterial, antiangiogenic, anti-inflammatory, antiallergic, enzymatic activity modulators, and anti-cancer activity (Cushnie and Lamb, 2011; Kawai M., et al., 2007; García-Lafuente et al., 2009).

1.7. Natural antioxidants in cancer therapy

Every day, the human body is subjected to a variety of exogenous insults, including ultraviolet (UV) rays, air pollution, and tobacco smoke, which result in the production of reactive species, particularly oxidants and free radicals, which are responsible for the onset of many diseases, including cancer (Bhattacharyya, A., et al, 2014; Phaniendra, A., et al, 2015; Zegarska, B., et al, 2017). Diet is an exogenous agent, and some foods, such as processed meats, have been related to an increased risk of cancer, whilst other foods, such as fruits and vegetables, have been connected to a decreased risk of tumor development (Doll R et al. 1981; Santarelli, R. L., et al, 2008; Mentella, M. C., et al, 2019)

These molecules can be formed due to therapeutic medication administration, but they can also be made spontaneously within our cells and tissues by mitochondria and peroxisomes, as well as by macrophage metabolism during normal physiological aerobic activities. Damageto DNA (genetic changes, DNA double strand breaks, and chromosomal abnormalities (Cadet,J., et al, 1997) and other biomolecules (Gupta, R. K., et al, 2014) such as lipids (membrane peroxidation and necrosis (Gille, G., & Sigler, K., 1995) and proteins, can occur from oxidative stress and radical oxygen species (significantly altering the regulation of transcription factors and, as a consequence, essential metabolic pathways (Halliwell B., 2007).

Natural antioxidants such as vitamins, polyphenols, and plant-derived bioactive compounds have recently been investigated to introduce them as preventative agents and prospective therapeutic medications (Katz L, et al, 2016; Bernardini S, et al, 2018). These compounds have

anti-inflammatory and antioxidant capabilities and may be found in a variety of vegetables and spices (Iqbal J, et al, 2017). Vitamins, alkaloids, flavonoids, carotenoids, curcumin, berberine, quercetin, and many other compounds have been screened *in vitro* and tested *in vivo*, demonstrating significant anti-proliferative and pro-apoptotic properties, and have been introduced as complementary therapies for cancer (Chikara S, et al, 2018; Singh S, et al, 2016; González-Vallinas M, et al, 2013) Among these is apigenin, a low molecular weight anti-carcinogenic flavonoid which will be the focus of our work.

1.8. FLAVONOIDS

Flavonoids are phytochemicals that may be found in two forms: free aglycones and glycosidic conjugates. They are polyphenolic and have a phenyl benzopyrone structure (C6–C3–C6), which is categorized into flavones, flavanols, isoflavones, flavonols, flavanones, flavanonols, and chalcones based on the saturation level, C-ring substitution pattern, and central pyran ring opening (Hossain, M., et al 2016; Middleton et al., 2000; Ravishankar, D., et al 2013) (Figure 1.6). They are present in a group of substances found in plants and fruits that contribute to a well-balanced diet and positive health benefits. They have an influence on medicine because they serve as a scaffold for novel pharmaceuticals and have a wide variety of applications in the treatment of illnesses. Flavone derivatives stand out among the many forms of flavonoids because of their widespread presence in nature and well-established biological activity. The most common method for synthesizing halogenated flavones is to employ existing halogenated starting materials. Because it is more complex and entails some selectivity concerns, halogenation of the flavones core is less typical (Santos, R., et al 2020). Flavonoids have been identified as a rich source of chemicals with potential anti-cancer activities due to their diversity of structural patterns and the represent a potential route for anti-cancer medicines due to their ability to halt the cell cycle, induce apoptosis (Kuntz et al., 1999), impair mitotic spindle formation (Beutler et al., 1998), and inhibit angiogenesis (Mojzisa et al., 2008). Flavonoids have been shown to modulate several protein kinases (e.g. protein kinase-C, serine-tyrosine kinases) as well as epidermal growth factor receptors (EGFRs), platelet derived growth factor receptors (PDGFRs), vascular endothelial growth factor receptors (VEGFRs), and cyclin-dependent kinases (CDKs) (Singh and Agarwal, 2006). Flavonoids also exercise their chemopreventive action by inhibiting phase I metabolizing enzymes (e.g., cytochrome P450),

which metabolically activate a large variety of pro-carcinogens, causing carcinogenesis (Tsyrlov et al., 1994).

They also activate phase II metabolizing enzymes (such as GST, quinone reductase, and UDP-GT), which degrade carcinogens and remove them (Bu-Abbas et al., 1998). Flavonoids' chemopreventative effects are linked to their anti-cancer capabilities, which include the scavenging of reactive oxygen species (ROS) and growth-promoting oxidants, which are the primary catalysts for tumor formation. These findings underline flavonoids' potential as antiproliferative agents.

In thisreview will be given enfaces to a particular flavone, (4’, 5, 7-trihydroxyflavone) Apigenin, focusing on its health-promoting effects/therapeutic functions.

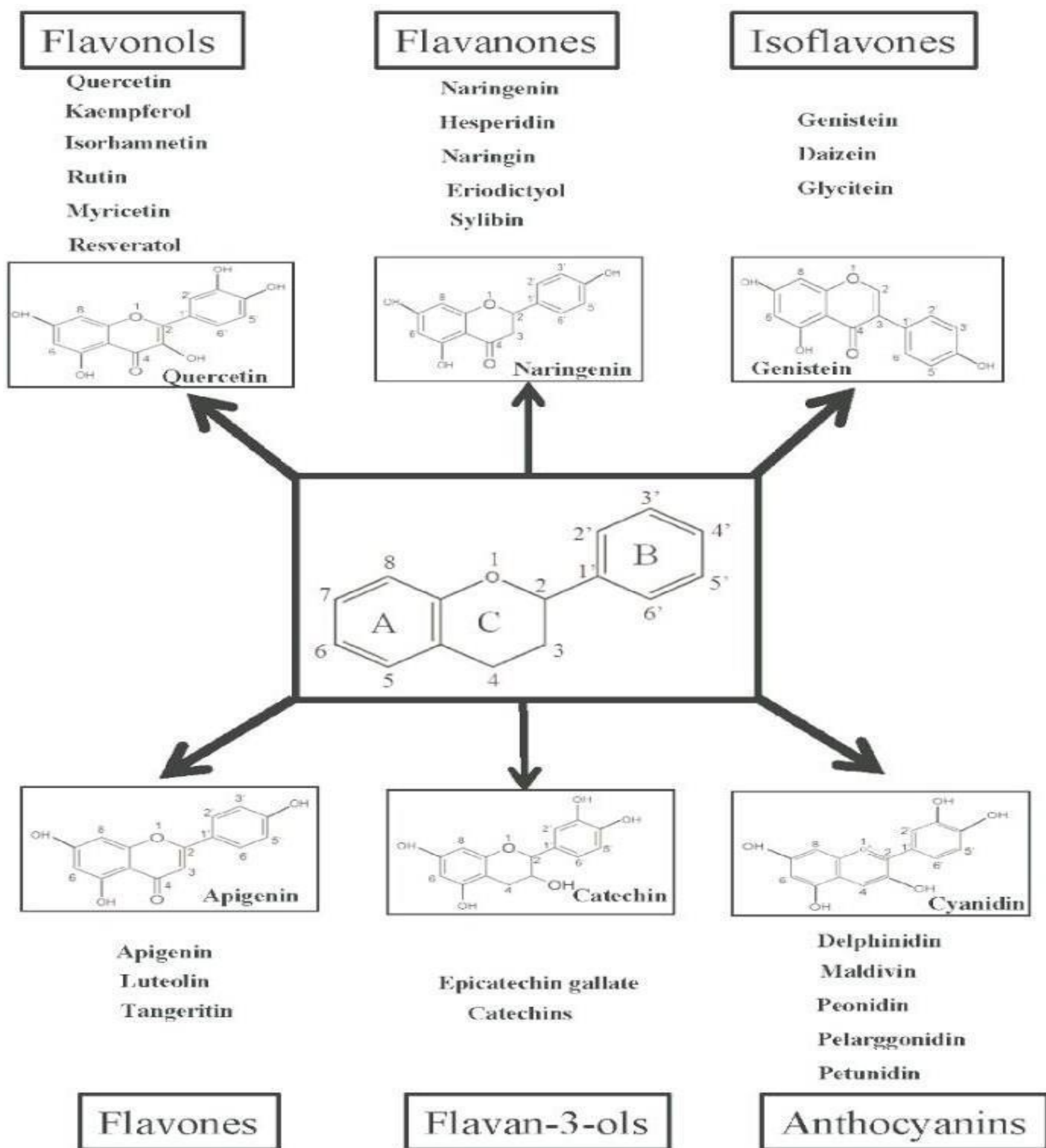

Figure 1.6 - *Classification and example of flavonoids and their chemical structures. Flavonoids are classified into six groups, including flavonol, flavanone, isoflavone, flavone, flavan-3-ols, and anthocyanin. Chemical structures of each of the six classes of flavonoids are shown as examples, including isorhamnetin for flavonol, naringin for flavanone, daizein for isoflavone, apigenin for flavone, catechin for flavov-3-ols, and cyanidin for anthocyanins. (Available at: https://www.researchgate.net/figure/classification-and-example-of-flavonoids-and-their-chemical-structures-flavonoids-are_fig3_301332394) accessed in November 2021*

1.9. APIGENIN

Among the several phenolic compounds, apigenin is one of the most well-known, with numerous nutritional and organoleptic properties that can contribute with its beneficial health effects (Hostetler, G. L., et al, 2017).

Apigenin (4,5,7-trihydroxyflavone) (Figure 1.7) is one of the most extensively distributed flavonoids in the plant world, as well as one of the most researched phenolics, and may be found in substantial amounts as glycosylated in vegetables (parsley, celery, onions), fruits (oranges), herbs (chamomile, thyme, oregano, basil), and plant-based drinks (tea, beer, and wine) (Hostetler GL et al, 2017). Increasing evidence demonstrates that apigenin metabolism is divided into two primary steps. In the presence of liver enzymes such as cytochrome P450, as well as the collaboration of nicotinamide adenine dinucleotide phosphate (NADPH) and flavin-containing monooxygenase (FMO), apigenin undergoes phase I metabolism (Cardona et al., 2013; Tang et al., 2017). In phase II metabolism, enteric and enterohepatic cycles contribute to the biotransformation of apigenin (Chen et al., 2007). Phase II metabolism requires glucuronidation and sulfation (Tang et al., 2017). Apigenin is bio-transformed into metabolites such as luteolin (Lut) and sulfated and glucuronidated conjugates throughout metabolism (Chen et al., 2003; Gradolatto et al., 2005).

It is well established that a high intake of flavonoids from vegetables and fruits is inversely related to cancer risk. The relationship between dietary flavonoids and their preventive effect as well as cancer risk reduction has been explored, among other things, in research on ovarian cancer, breast cancer, and the risk of neoplasm recurrence in individuals with resected colon cancer (Salehi, B., et al, 2019). Several of apigenin's biological properties have been detailed in a recent study, including cytostatic and cytotoxic activity against different cancer cells, anti-atherogenic and protective effects in hypertension, cardiac hypertrophy, and autoimmune myocarditis, among others (Zhou, X., et al, 2017). Because of apigenin's diverse pharmacological actions and relevance to human health, a thorough understanding of its mechanism of action is critical for potential therapeutic uses. When compared to other structurally similar flavonoids, it exhibits a low intrinsic toxicity on normal vs malignant cells (Ali, F., et al, 2017; Lotha, R. O. B. E. R. T., & Sivasubramanian, A. R. V. I. N. D. 2018). Apigenin promotes cell cycle arrest at many phases of proliferation, including G1/S and G2/M, via regulating the expression of certain CDKs and other genes (Takagaki, N., et al, 2005; Maggioni, D.,et al, 2013; Iizumi, Y., et al, 2013)), and is known to influence intrinsic apoptoticpathways by altering mitochondrial membrane potential and triggering the release of

cytochrome C in the cytoplasm, which results in the formation of APFA, activation of caspase 3, and activation of apoptosis (Seo, H. S., et al, 2014). Otherwise, regulate extrinsic apoptotic pathways by activating caspase-8.

Apigenin also induces apoptosis in cancer cells by altering the expression of Bcl-2, Bax, STAT-3, and Akt proteins (Seo, H. S., et al, 2012; Karmakar, S., et al, 2009), and activates anti-inflammatory pathways such as p38/MAPK and PI3K/Akt, as well as preventing IKB degradation and nuclear translocation of NF-B and decreasing COX-2 activity (Lee, J. H., et al, 2007; Huang, C. H., et al 2010).

Despite its relevance, there is a lack of information on the beneficial health potential of apigenin for humans, with respect to, inflammation or cognitive performance for instance, which is another noteworthy prospective use of this substance. This is most likely related to the fact that, despite its various beneficial benefits, apigenin has a relatively low water solubility (1.35 μg/mL) and high permeability (Zhang, J., et al 2012).

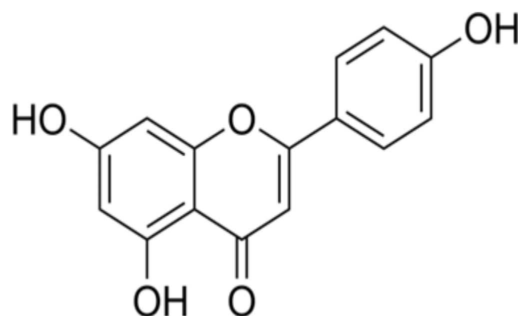

Figure 1.7 - Chemical structure of apigenin (available at: https://www.researchgate.net/figure/chemical-structure-of-apigenin_fig4_309626061) accessed in December 2021

1.10. Apigenin in cancer

In addition to anti-inflammatory and antioxidant properties, apigenin has been shown to have significant anti-cancer activity in various cancer cells, including breast cancer (Perrott et al., 2017), liver cancer (Qin Y et al., 2016), pancreatic cancer (Johnson and de Mejia, 2013), prostate cancer (Shukla et al., 2014), lung cancer (Pan, X et al., 2013), and colon cancer (Lee Y.et al., 2014).

Kashyap et al. (Kashyap, D., et al 2018) have reviewed apigenin's various therapeutic activities *in vitro* and *in vivo* systems. The many mechanisms behind apigenin's possible therapeutic impact were investigated, including cell cycle arrest, apoptosis, anti-inflammatory, and antioxidant function. They discovered an inverse relationship between flavonoid consumption and the incidence of all cancers, providing significant evidence of flavonoids' preventive function against lung cancer.

There have been studies that suggest apigenin might be used as a chemotherapeutic agent due to its low intrinsic toxicity and impressive effects on normal vs malignant cells when compared to other structurally similar flavonoids (Madunić, J., et al, 2018), Shukla and others (Shukla, S., et al, 2014) investigated the activity of apigenin in the treatment of prostate cancer. Apigenin induced a considerable decrease in the weight of the genitourinary apparatus, both dorsolateral and ventral prostate, according to their findings. They also show that apigenin can effectively decrease prostate cancer growth, at least in part by blocking the PI3K/Akt/FoxO signaling pathway (Shukla, S., et al, 2014; Salehi, B. et al, 2019).

So far, antitumor activities of apigenin like, encouraging apoptosis or autophagy, regulating cell cycle, preventing tumor cell migration and invasion, and triggering the patient's immune response have been reported within diverse types of tumors, *in vitro* and *in vivo* models (Table 1.1) (Ashrafizadeh, Milad et al. 2020)

Table 1.1 - Protective effects of apigenin on different human cancers and cell lines. (Tong, X., & Pelling, J. C., 2013) (Available at https://www.ncbi.nlm.nih.gov/pmc/articles/pmc3729595/) accessed in December 2021

Cancer types	**Apigenin's effects**
Breast cancer	Inhibition of proliferation, induction of apoptosis, suppression of cell invasion, anti-estrogenic activities
Prostate cancer	Induction of apoptosis and G_1 phase arrest of cell cycle, inhibition of cell growth, suppression of HIF-1α expression, inhibition of VEGF, inhibition of FAK/src
Colon cancer	Inhibition of cell growth, induction of G_2/M cell cycle arrest, increase in the stability of p53 protein, induction of apoptosis
Cervical cancer	Inhibition of cell growth through G_1 cell cycle arrest and apoptosis, suppression of motility and invasion
Skin cancer	Induction of autophagy, enhancement of apoptosis, inhibition of COX-2 expression, suppression of MMP-1 production
Lung cancer	Suppression of VEGF transcriptional activation, induction of apoptosis
Ovarian cancer	Inhibition of proliferation and VEGF expression, suppression of migration and invasion
Liver cancer	Inhibition of cell growth, induction of apoptosis, enhancement of radiation-induced cell death
Pancreatic cancer	Inhibition of Focal Adhesion kinase activation, suppression of HIF-1α, VEGF and Geminin expression, induction of G_2/M cell cycle arrest
Hematologic cancer	Inhibition of proliferation, induction of apoptosis, suppression of telomerase activity

1.11. Apigenin as a potencial mTOR inhibitor

Apigenin's structure was initially characterized in 1900, and it was first synthesized in 1939. (Li, B., et al, 1997). Apigenin originally came to the attention of scientists in the 1960s when itwas discovered to decrease histamine production from basophils and to have bronchial dilating effects in the lungs (Lefort, É. C., & Blay, J., 2013; Tong, X., & Pelling, J. C., 2013). Around 1980, the first report on apigenin's anticancer properties was published (Lefort, É. C., & Blay, J., 2013). In this day and age there is overwhelming evidence that plant-derived bioactive flavonoids have potent anticancer, cardiovascular disease, and age-related neurodegenerative illnesses therapeutic properties (Kashyap, D., et al, 2018). Several drug discovery studies have revealed that apigenin's medicinal potential is linked to its significant anti-inflammatory, anti-oxidant, and anti-tumor capabilities, (Kashyap, D., et al, 2018). When taken orally apigenin is systemically absorbed and recirculated via enterohepatic and local intestine routes. It has a bioavailability of around 30%, and it achieves peak circulation concentration (Cmax) after 0.5–2.5h (Tmax) of oral absorption, with an elimination half-life (T1/2) averaging 2.52±0.56h, (DeRango-Adem, E. F., & Blay, J., 2021).

It has been proven that apigenin has the ability to decrease cell proliferation, make cancer cells more susceptible to apoptosis, and prevent the formation of blood vessels to service the developing tumor. It also has effects that change the way cancer cells interact with their surroundings (Yan, X., et al, 2017). Apigenin has the ability to suppress cancer cell glucose absorption, extracellular matrix remodeling, cell adhesion molecules that engage in cancer growth, and chemokine signaling pathways that influence the route of metastasis into other regions (Lefort, É. C., & Blay, J., 2013). Lately apigenin has been implicated in numerous experimental and biological research as an anticancer agent. By regulating numerous signaling pathways, it causes cell growth inhibition and death in several types of tumors, including gastric cancer (Imran, M., et al, 2020). Currently apigenin is being investigated as a cancer chemopreventive drug and appears to offer protection against a wide range of malignancies, as described by many researchers as Patel, D., et al, 2007; Shukla et al., 2014 among others. Previous research has shown that apigenin can suppress cancer cell growth and proliferation, increase apoptotic cell death, cause cell cycle arrest, and alter mitochondrial membrane potential *in vitro* and *in vivo* (Hu, X. Y., et al 2015; Liao, Y., et al, 2014; Shukla, S.,et al, 2014; Park, S., et al, 2018). However, the specific mechanism behind apigenin's anti- tumor actions is yet unknown.

Autophagy is critical in the decomposition of unnecessary proteins and organelles and can be triggered by a variety of circumstances, including hypoxia, cellular stress, and nutritional deprivation (Singh, B. N., et al, 2012). Recent research suggests that targeted autophagy may be a viable cancer-fighting treatment method (Zhou, J., et al, 2016). In cancer cells, the mammalian target of rapamycin (mTOR) plays an important role in controlling the balance between cell proliferation and autophagy in response to cellular stress caused by chemotherapeutics (Chiarini, F., et al, 2015). Although previous research has shown that apigenin can promote autophagy and death in breast cancer cells and reduce cell proliferation in gastric cancer cells (Lefort, É. C., & Blay, J., 2013), it remains to be seen whether apigenin reduces mTOR in gastric cancer cells, whether this reduction in mTOR is in any way responsible for the aforementioned decrease in proliferation, and whether apigenin exposure is fully or partially responsible for this decrease. The mTOR signalling pathway is hyperactivated in around 70% of human cancers (He, H., et al, 2021), particularly gastric cancer (Byeon, S. J., et al, 2014; Bornschein, J., et al, 2015; Murayama, T., et al, 2009; Lohneis, P., et al, 2014; Li, M., et al, 2012; He, H., et al, 2021).

Previous studies have reported that apigenin could induce autophagy and apoptosis in breast cancer cells (Yang, J., et al 2019). However, the relationship between apoptosis and autophagy induced by apigenin remains unknown. Apigenin has been found to limit Akt function in several cell types by directly decreasing PI3K activity via the PI3K ATP-binding siteand then inhibiting Akt kinase activity (Tang, Q., et al, 2001). A study by Zhao et al. demonstrated that apigenin suppressed CK2 activity, reduced Cdc37 phosphorylation, disassociated the Hsp90/Cdc37/kinase client complex, and thereby triggered the degradationof several kinase clients, including Akt (Zhao, M., et al, 2011). More recently, Yang, et al demonstrated that the apigenin induced autophagy and apoptosis was via inhibiting PI3K/Akt/mTOR pathway (Yang, J., et al 2018). Also numerous studies *in vitro* as well as *in vivo* have been reporting apigenin to enhance apoptosis by inactivation of Akt (Budhraja, A., et al 2012; Cheong, J. W., et al, 2010). Apigenin has also been shown to inhibit ovarian tumormetastasis by down-regulating MMP-9, which is mediated by Akt signaling (He, J., et al,2012), to inhibit breast cancer metastasis by blocking the PI3K/Akt pathway (Lee, W. J., et al,2008), and to inhibit cancer angiogenesis by suppressing HIF-1 and VEGF expression, which is also related to Akt inhibition (Mirzoeva, S., et al, 2008; Liu, L. Z., et al, 2005). In comparison to the numerous publications in the literature establishing apigenin's inhibition of Akt activity, there are just a few papers demonstrating apigenin's inhibition of mTOR activity. Tong et al. recentlyrevealed that apigenin activated AMPK in human keratinocytes (Tong, X., et al 2012).Anotherresearch by Turktekin and colleagues found that apigenin suppressed mTOR expression in colon cancer cells (Turktekin, M., et al, 2011). Apigenin's capacity to inhibit both

the PI3K/Akt and the mTOR signaling pathways distinguishes it as a unique and different chemopreventive drug. Additionally, apigenin is linked with relatively minimal toxicity, making it more appealing for cancer chemoprevention (Tong, X., & Pelling, J. C., 2013).

Apigenin is known to inhibit cell proliferation in gastric cancer cells. In 2014, Chen, J., et al, have shown that apigenin can inhibit the growth of gastric carcinoma cells in dose- and time-dependent manner, and the inhibition is most effective on undifferentiated gastric carcinoma cells., Wu, K., et al stated that apigenin has the ability to inhibit the proliferation of human gastric cancer SGC-7901 cells, which is connected with its apoptosis-inducing activity (Wu, K.,et al, 2005), and another study conducted by, Kuo, C. H., et al, concluded that apigenin has the remarkable ability to inhibit Helicobacter pylori-induced atrophic gastritis and gastric cancer progression as well as possessing potent anti-gastric cancer activity (Kuo, C. H., et al, 2014). Still, it remains to be seen whether apigenin reduces mTOR in these cells, whether this inhibition of mTOR is in any way responsible for their decreased proliferation and survivor, and whether apigenin exposure is wholly or partly responsible for this decrease. When it comes to the flavone apigenin daily intake, relevant research is scarce. Nevertheless, a recent article reported on the average consumption of flavonoid compounds among adults in the European Union by country, region, and overall (Vogiatzoglou, A., et al, 2015), revealed that the average intake of apigenin per adult in Europe is 3 ± 1 mg/day using the food consumption data from the European Food Safety Authority (EFSA) and the FLAVIOLA Food Composition Database.

Apigenin is a naturally occurring low toxicity, nonmutagenic flavonoid that is widely distributed in food,and diets high in flavonoids has been linked to a variety of health benefits, for that reason, estimating apigenin consumption on a daily basis might aid in the right interpretation of the association between health outcomes and apigenin (Shukla et al., 2014). Based on the available literature, the beneficial effects of apigenin as a future anticancer modality are promising but they require further *in vitro* and *in vivo* studies to enable its translation from bench to bedside.

1.12. Objectives for the research

Main objective: The main objective is to perform an integrative analysis on mTOR expression regulation and the potential of apigenin as an mTOR inhibitor.

- Specific goals:
  - Systematize and critically analyse the processes of mTOR expression regulation.
  - Test the potential of apigenin in assay with Gp202 cell lines, assessing factors such as proliferation, and cell morphology.

CHAPTER TWO - SYSTEMATIC REVIEW OF MTOR

Systematize and critically analyze the processes of mTOR expression regulation. Submitted for publication

Regulation of mTOR expression in normal and pathological conditions

Renata Cervantes[1], Beatriz Simões[2], Tomás Justino[2], Ana Marques-Ramos[1,#]

[1]H&TRC-Health and Technology Research Centre, ESTeSL-Escola Superior de Tecnologia da saúde, Instituto Politécnico de Lisboa, Lisbon, Portugal

[2]ESTeSL, Escola Superior de Tecnologia da Saúde de Lisboa, Instituto Politécnico de Lisboa, Av. D. João II, Lote 4.69.01, 1990-096 Lisbon, Portugal

#corresponding author, A. Marques-Ramos ana.ramos@estesl.ipl.pt

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Abstract

The mechanistic/mammalian target of rapamycin (mTOR) is a central cellular hub that integrates intra- and extracellular signals in terms of energy, nutrient, and hormone availability, modulating the molecular responses to acquire a homeostatic state through the regulation of anabolic and catabolic processes. Accordingly, dysregulation of mTOR pathway has been implicated in a variety of human diseases. While major advances have emerged regarding the regulators and effects of mTOR signaling pathway, little is known about the regulation of mTOR gene expression. Here, we present the current available data regarding to the mTOR expression changes observed in several diseases, particularly in human cancers and systematize the current knowledge about the regulation of mTOR at the transcriptional and translational level. Furthermore, we demonstrate how different miRNAs affect mTOR signaling

both in normal and pathological conditions. We believe that our study will assist the development of new mTOR inhibitors as it gives an exhaustive perspective about the regulation of mTOR gene expression.

Introduction

The mechanistic target of rapamycin (mTOR) is a serine/threonine kinase that coordinates metabolism and growth of eukaryotic cells with external inputs such as nutrition and growth stimuli (Saxton RA, 2017). Over the last two decades, extensive research has demonstrated that mTOR is involved in key cellular processes, from protein synthesis to autophagy, and that hyperactivated mTOR signaling has been linked to cancer, diabetes, and the aging process (Laplante and Sabatini, 2012). It is a key component of two complexes, mTOR complex 1 (mTORC1) and mTORC2 that display several functions according to different downstream effectors (Laplante and Sabatini, 2012). By targeting 4EBPs and S6 kinases (S6Ks), mTORC1 induces protein synthesis, lipid and nucleotide biogenesis, and suppresses autophagy, lysosomal biogenesis, ultimately resulting in genome instability, cell survival, growth and proliferation (Laplante and Sabatini, 2012). mTORC2 targets several protein kinases, including Akt, by which it induces cell survival and proliferation (Oh and Jacinto, 2011). Dysregulation of mTOR is present in a myriad of diseases and it has been reported that mTOR hyperactivation occurs in more than 70% of human cancers (Oh, W. J., & Jacinto, E., 2011), one of which is gastric cancer (Jung, E. J., 2020). Although several inhibitors for this pathway have been created, such as rapamycin analogues (rapalogs) or ATP competitors, there are still significant limitations due to the lack of complete inhibition of the mTOR pathway and the co-inhibition of other target proteins that this form of inhibitor induces, which may have negative consequences for patient health and may translate into exacerbated side effects (Moschetta, Reale, Marasco, Vacca, & Carratú, 2014). To counteract the consequences of these mutations, a third generation of mTORi was created. These inhibitors have a rapamycin-FRB binding element connected to a TOR-KI (kinase incativator), allowing at least half of the ligand to bind when mutations prohibit theother half from binding. These mTORi can significantly suppress mTOR signaling. Nonetheless,these novel mTORi do not address the toxicity associated with off-targets. In recent years the investment in the development of biomarkers has been exponential, in this scope, several authors are seeing the expression of mTOR in different pathological conditions, such as Type 2 Diabetes Mellitus (Yang, L., et al, 2022; Tsai, K., et al, 2021), Alzheimer (Perluigi, M., et al,

2021), rheumatoid arthritis (Iwata, S., et al, 2021) and in particular in cancer (Kahraman, D. C., et al, 2019; Mossmann, D., et al, 2018). This review aims to shed light on the role of mTOR increased expression associated with a pathological condition.

Overview of regulation of mTOR signaling

The research of TOR began in the 1960s with a journey to Rapa Nui (also known as Easter Island), to discover natural compounds from plants and soil with potential medicinal use. There, a natural compound was identified and in 1972, Suren Sehgal isolated it from a bacteria called *Streptomyces hygroscopicus*, refined it, and reported it to have powerful anti-fungal action. He called it rapamycin in honor of its source and action (Singh, K., et al 1979). Rapamycin was first identified as an antifungal metabolite, but it was later proven to have immunosuppressive and anti-proliferative characteristics in mammalian cells, motivating researchers' interest in understanding how it operates (Li, J., et al, 2014). Several attempts were undertaken over the next two decades to study the cellular effects of this compound. In the 1990s large amounts of rapamycin were created and made available to the academic community as a result of a revived interest in rapamycin, which made it possible to discover the protein, called TOR (target of rapamycin) first in *Saccharomyces cerevisiae*, true the creation of a genetic screen to find resistant mutants based on its antifungal activity by Michael Hall and Joseph Heitman (Heitman et al., 1991). To fully appreciate the significance of Hall's discovery of yeast TOR, it was necessary to show that it was conserved in higher eukaryotes, as he projected. Snyder, Schreiber, Berlin, and Abraham found the human homolog of TOR to be the ortholog of yeast when they biochemically isolated it in 1994 (Saxton & Sabatini, 2017), which was then recognized as mTOR or mammalian TOR (now also referred to as mechanistic TOR). This serine/ threonine kinase belongs to the phosphatidylinositol 3-kinase-related kinase (PIKK) family, which includes several kinases involved in cell growth and cycle regulation, telomere length maintenance, DNA damage checkpoints, and recombination (Chrienova, Z., et al, 2021).

As previously stated, rapamycin inhibits cell proliferation, and once discovered, mTOR was linked to cell cycle progression by regulating proliferation. mTOR was later demonstrated to phosphorylate and block the CAP-dependent translation repressor

4EBP1 and phosphorylate and contribute to the activation of S6K1, both of which are key actors in translational regulation (Burnett, P. E. et al, 1998).

It is now known that mTOR regulates cell development and metabolism in response to environmental cues, ensuring that cells expand only under favourable conditions. When activated, promotes cell growth and proliferation by stimulating biosynthetic pathways suchas protein, lipid, and nucleotide production and inhibiting cellular catabolism via inhibition of the autophagy pathway (Rabanal-Ruiz & Korolchuk, 2018). Cell growth is more than just the formation of cell mass. It is a complicated process of balanced macromolecular synthesis that plays an important role in cell physiology and is regulated by signaling pathways, one of which is TOR kinase (Chrienova, Z., et al, 2021). According to its biochemical and genetic analysis, in eukaryotic cells, mTOR is found in two functionally different complexes mTORC1 and mTORC2. These two complexes phosphorylate distinct substrates and contribute to diverse physiological roles. They also have different sensitivity to rapamycin, While mTORC1 is susceptible to rapamycin, mTORC2 is resistantto acute rapamycin therapy, unless when chronic exposure to the chemical, where it has a deleterious impact on mTORC2 activity (Loewith, R., et al, 2002). mTORC1's main components are mTOR, mammalian lethal with sec-13 protein 8 (mLST8), and TOR regulatory associated protein (raptor). Additional components are DEP-domain containing mTOR interacting protein (DEPTOR) and Proline-rich Akt substrate 40kDa (PRAS40) (Li, J., et al, 2014). Experiments involving the rapamycin-induced suppression of mTORC1's signalingpathway have largely contributed to our understanding of its action. However, because mTORC2 is acutely resistant to rapamycin, our understanding of its role is restricted. The coreof the mTOR complex 2 (mTORC2) is composed of mTOR, Rictor (rapamycin-insensitive companion of mTOR), stress-activated protein kinase-interacting protein 1 (mSIN1), and mLST8. Additional regulatory components are, proctor 1/2 and DEPTOR (Laplante & Sabatini,2012, Loewith, R., et al, 2002, Chrienova, Z., et al, 2021). mTOR complex 1 (mTORC1) and mTORC2 integrate environmental, hormonal, and nutritional inputs from the intra and extracellular compartments. mTORC1 is activated in the lysosome by both amino acids and growth factors,whereas mTORC2 is mainly triggered by growth factors (Saxton, R. A., & Sabatini, D. M., 2017).

In the presence of growth factors, PI3K activates Akt, which inhibits TSC2, leading to the activation of Rheb, a key mTORC1 activator. This activation takes place at the lysosomal surface, where Rheb is found, and where mTORC1 is recruited when amino acids are available (Bond P., 2016). mTORC1 enhances protein synthesis, de novo lipogenesis, nucleotide

synthesis, glucose metabolism, ribosomal biogenesis, and cell cycle progression while negatively regulating endosomal biogenesis, autophagy, and proteasome assembly, all of which lead to cell survival and growth. Growth factors, subcellular location, GTPases, nutrients, and metabolites influence mTORC2 activity. This complex is in control of glucose homeostasis, ion transport, cell migration, and cytoskeleton rearrangement (Bond P., 2016).

mTORC1 is mainly activated by growth factors, such as insulin and insulin-like growth factor-1, through the conventional IRS-PI3K-AKT signaling pathway. Once this pathway is active, AKT phosphorylates and suppresses the tuberous sclerosis complex (TSC), a GTPase activating protein (GAP) that works on RHEB (Ras homolog enriched in brain). The insulin receptor substrates (IRS) proteins are phosphorylated when either receptor is activated. As a result, binding sites on these proteins are exposed, allowing them to interact with other proteins that have a Src Homology 2 (SH2) domain. One of the SH2 domain-containing proteins is phosphatidylinositol-3-kinase (PI3K). IRS stimulates PI3K by interacting with the kinase's SH2 domain (Myers, M. G., et al, 1992). Activated PI3K then phosphorylates inositol phospholipids embedded in the plasma membrane on a hydroxyl group at carbon 3. Phosphoinositides, such as phosphatidylinositol (3,4,5)-triphosphate (PIP3), are formed as a result. PIP3 binds to proteins with Pleckstrin homologies (PH) domains such as 3-phosphoinositide dependent protein kinase (PDK1) and Akt, causing them to be recruited to the plasma membrane (Bond P., 2016). Akt is thought to be a crucial upstream regulator of mTORC1, and it phosphorylates numerous additional proteins. Glycogen synthase kinase 3 (GSK3) (Cross, D. A., et al, 1995), proline-rich Akt substrate of 40 KDa (PRAS40) (Kovacina, K. S., et al, 2003), tuberous sclerosis complex 2 (TSC2) (Inoki, K., et al, 2005) and forkhead box class O (FoxO) proteins (Tran, H., et al, 2003) are the most well-studied Akt substrates. TSC2 and PRAS40 are both mTORC1 negative regulators. TSC2 binds to TSC1 and TBC1D7 to form the (TSC-TBC) complex and inhibits mTORC1 activity by means of its GTPase-activating protein (GAP) domain. Through the GTP-bound Rheb proteins (Rheb-GTP) mTORC1 is activated at the lysosomal membrane (Long, X., et al, 2005). The TSC-TBC complex can then regulate mTORC1 activity thanks to its GAP domain. Akt also helps mTORC1 by removing the PRAS40-induced inhibition.PRAS40 connects to the Raptor subunit of mTORC1, preventing it from engaging with substrates. Akt is also closely involved in inhibiting protein breakdown by influencing the activity of the FoxO family of proteins, which are protein breakdown regulators that modulate the ubiquitin-proteasome and autophagy-lysosomal proteolytic pathways (Sanchez, A. M., et al, 2014).

Another way of mTORC1 activation is through the presence of amino acids. When a cell is starvedof amino acids, mTOR may be located anywhere across the cytoplasm, but when amino acids

are added, mTOR quickly relocates to the cell's peri-nuclear area. Amino acids may boost mTORC1 activity by locating it to the lysosomal surface, where it may be activated by Rheb-GTP. Furthermore, amino acid intake stimulates mTORC1 via the branched chain amino acid/Rag pathway. The Ragulator-Rag complex was found to be responsible for mTORC1's lysosomal surface localization (Sancak, Y., et al, 2010). Rags, in turn, connect with the lysosomal membrane-anchored Ragulator protein complex. Rags' ability to interact with mTORC1 is determined by their guanine nucleotide binding status. RagA/B are coupled to GDP in an amino acid-depleted cell, while RagC/D are linked to GTP. The addition of amino acids causes a nucleotide exchange that favors RagA/GTP B's bound state over RagC/GDP bound state. The Ragulator, which is anchored in the lysosomal membrane, interacts with Rags, causing them to be localized to the membrane. Importantly, the Ragulator serves as a guanine nucleotide exchange factor (GEF) for RagA/B (Bar-Peled, L., et al, 2012), this activity is controlled by v-ATPase (Bar-Peled, L., et al, 2012). The GAP activity towards the Rags (GATOR1) complex is a GAP for RagA/B (Bar-Peled, L., et al, 2013). As a result, the Rags are deactivated, and mTORC1 is inhibited. GATOR1 activity is inhibited by another proteincomplex known as GATOR2 (Bar-Peled, L., et al, 2013). This inhibiting effect is mediated by Sestrin proteins in response to amino acids.

The control of mTORC2 activity is significantly less known than that of mTORC1. Nonetheless, growth factor-induced Akt Ser473 phosphorylation by mTORC2 is mediated through the phosphoinositide 3-kinase (PI3K) pathway. PI3K phosphorylates PI (4,5) P2 at the plasma membrane in response to growth factor stimulation, producing phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P3), which is counteracted by phosphatase and tension homolog (PTEN). Akt and its activating kinase PDK1 bind to PI(3,4,5)P3 selectively via their PH domains and are brought to the plasma membrane, where they are phosphorylated at Thr308 by PDK1. Recent studies also suggest that mTORC2 appears to be triggered by a variety of additional cues. PI3K inhibition decreases Akt phosphorylation at Thr308 and Ser473, the latter of which is an mTORC2 target site, according to several studies. The method by which mTORC2 phosphorylates Akt Ser473 is, however, a point of contention. It has been suggested that PI(3,4,5)P3 stimulates mTORC2 directly (Liu, P., et al, 2015). The PH domain of mSIN1 binds to the mTOR kinase domain and suppresses mTORC2 automatically. PI(3,4,5)P3 binds to the mSIN1 PH domain and recruits mTORC2 to the plasma membrane, but not the other PIPs. Furthermore, binding PI(3,4,5)P3 to the PH domain of mSIN1 relieves mTORC2 inhibition by mSIN1, which is consistent with a prior work that found PI(3,4,5)P3 directly enhances mTORC2 activity in vitro (Gan, X., et al, 2011). After PDK1 phosphorylates Akt Thr308, mTORC2 is localized and activated at the plasma membrane, and Akt phosphorylates Ser473. However, recent research employing the LocaTOR2 reporter called into question this concept, claiming

that mTORC2 is permanently present in the plasma membrane and is always active (Ebner, M., et al, 2017). As a result, Akt translocation to the plasma membrane is both essential and sufficient for mTORC2 phosphorylation. The activated growth factor PI(3,4,5)P3 may boost mTORC2 activity indirectly. Phosphorylation of mTOR Ser2481 is thought to be involved, although its role is unknown (Copp, J., et al, 2009; Soliman, G. A., et al, 2010). Another layer of growth factor-induced mTORC2 activation is supported by this positive feedback loop between Akt and mTORC2. In conjunction with the LocaTOR2 study, a model is suggested in which Akt Ser473 phosphorylation is mediated by both Akt translocation to the plasma membrane and Akt-induced increases in mTORC2 activity. However, whether phosphorylation of mSIN1 Thr86 is required for mTORC2 action remains unknown. The disparities in the results of numerous research on the issue clearly demonstrate that mTORC2 activity still need additional investigation.

When it comes to mTORC2 activation via subcellular localization, mTORC2 Is Active Mainly at Membrane Compartments, sitting at the crossroads of signaling pathways that govern metabolism and ion transport, through activation of the AGC-family kinases, the Akt, and the SGK1 proteins. However, how mTORC2 subpopulations are differentially regulated is poorly characterized. mTORC2 is mostly active in the plasma membrane, outer mitochondrial membrane, and endosomal vesicles, according to the LocaTOR2 reporter (Ebner, M., et al, 2017). mTORC2 has been linked to mitochondria and the ER in a variety of ways (Betz, C., & Hall, M. N., 2013) mTORC2 is also located in MAM (Mitochondria-associated membranes) and phosphorylates Akt, which phosphorylates MAM-associated proteins such the inositol 1,4,5-trisphosphate receptor and hexokinase 2 in response to growth factor stimulation (Betz, C., et al, 2013). These three mTORC2 subpopulations, however, appear to be distinct. MAM-localized mTORC2 is responsive to growth factors, whereas the LocaTOR2 reporter suggests that mTORC2 is active at mitochondria but not in the ER, and that mitochondria-localized mTORC2 is unaffected by PI3K and growth factors (Ebner, M., et al, 2017). It has been identified a connection of mTORC2 with actively translating ribosomes that is triggered by insulin-stimulated PI3K signaling and is independent of protein synthesis. Despite the possibility of ribosome-bound mTORC2 at MAM, the mechanism by which ribosome attachment activates mTORC2 is unclear. We now know a lot more about how mTORC1 perceives external nutrients and growth stimuli thanks to the discovery of the lysosome as a signaling hub. Surprisingly, perinuclear clustering of lysosomes was found to delay reactivation of not only mTORC1 but also mTORC2 and Akt upon serum replenishment (Jia, R., & Bonifacino, J. S., 2019). According to Rosner and his colleagues, mTORC2 translocate between the nucleus and the cytoplasm (Rosner, M., & Hengstschläger, M., 2008 and 2010). The disruption of mSIN1 and SGK1 localization to the nucleus and perinuclear compartment

by inhibiting c-PKC catalytic activity also inhibits mTORC2 phosphorylation of SGK1 Ser422 (Gleason, C. E., et al, 2019). Although the role of mTORC2 in the nucleus is undetermined, its subcellular distribution to the nucleus and perinuclear compartment may be important for its downstream effector SGK1 (Fu, W., & Hall, M. N., 2020). These data also show that there are many mTORC2 pools, each of which is controlled differently.

The importance of mTOR has lately been reinforced by the discovery of mTOR-associated proteins. mTOR creates diverse complexes with highly varied physiological roles when linked to different proteins. These results not only broaden the scope of mTOR's functions in cells but also hamper the regulatory network (Mishra, S., et al, 2021). As a result, it's more important than ever to fully comprehend the underlying molecular processes to better guide the development and application of anti-cancer therapies that target the mTOR signaling pathway (Figure 2.1).

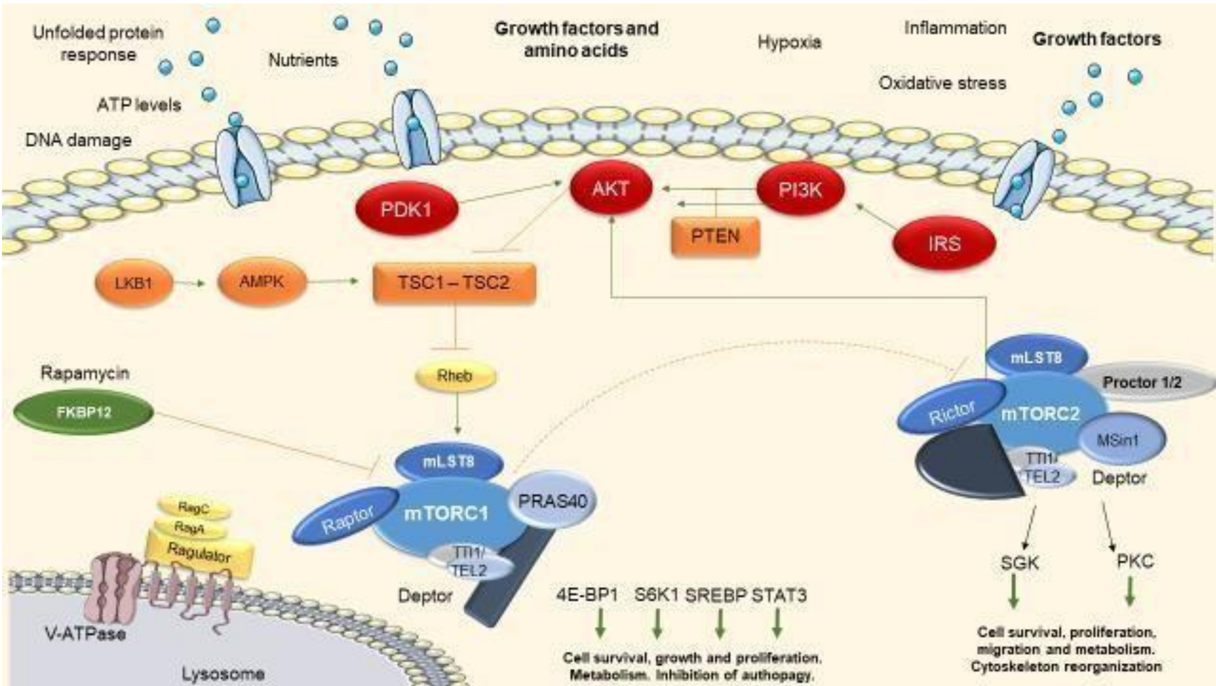


Figure 2.1 - mTOR signaling pathway

Methodology

The research question will be "Does cellular stress increase mTOR expression?", based on the PICO (population, intervention, control, and outcomes) methodology, where P is represented by Studies assessing changes in mTOr expression in situations of cellular stress; I: whether cellular stress condition induces alterationin mTOR expression; C: normal cellular conditions, and O: whether or not stress conditions increase mTOR expression.
The study reports on a search of available data published between the period of 1 January 2006 and 30 September 2020. The search terms aim to identify studies that characterize an increase in mTOR expression associated with stress and pathological conditions and Regulation of mTOR by microRNAs, with English as the chosen language. PubMed and Google Scholar were used as databases. Inclusion criteria were articles published between January 2006 and September 2020, which characterize mTOR expression associated with cellular stress and pathological conditions. Articles that didn't fulfil the inclusion criteria were not subjected to additional review, namely, review articles, non-English language articles, and articles that address the signaling pathway but not mTOR expression.

The selection of the articles was performed through Zotero in three rounds. The first round included a screening of all titles to exclude papers that were duplicated or unrelated to the topic. The second round consisted of an abstract screening. In the third round, considering the inclusion and exclusion criteria, the entire texts of all possibly relevant papers were evaluated. The following data was manually compiled: (1) Pathology, (2) Title, (3) The effect of mTOR, (4) Control Methods, (5) Analytical Methods, (6) Main Findings, and (7) References. We included 153 studies where 47 were studies regarding mTOR expression levels and disease and 106 regarding the Regulation of mTOR by microRNAs and observed an increased expression of mTOR in cancer pathologies as well as in other pathologies, especially in pathologies associated with the immune system and aging.

mTOR expression levels and disease

When we evaluated the expression of mTOR in the articles reviewed, results have shown an increase in the expression associated with various pathological conditions compared with a

normal condition. According to mounting data, mTOR appears to have a role in the etiology of Alzheimer's disease (AD). Furthermore, in AD, the levels of phosphorylated forms of mTOR are found to skyrocket. Liu, Y. C., et al (2017) were able to demonstrate, a considerable increase in the expression of mTOR and p-mTOR (at Ser2448) in the cortical tissues of Tg2576 mic. They also revealed that increased phosphorylation accompanies this increase in expression, which might indicate that in addition to increased expression, there is also increased activity of the mTOR complexes. Another interesting case is the study by Park, J. S., et al (2014) on lung tissues of pulmonary fibrosis patients where mTOR was shown to be highly expressed in the alveolar epithelial cells of individuals with pulmonary fibrosis, suggesting that mTOR may play a role in the etiology of idiopathic pulmonary fibrosis (IPF) by boosting alveolar epithelial cellproliferation. We also observed that increased expression of mTOR is related to several inflammatory skin pathologies, like psoriasis, allergic contact dermatitis, atopic dermatitis, andacne. When compared to healthy skin, mTOR gene expression was considerably enhanced inpsoriasis in bought lesional and non-lesional skin of psoriatic patients (Balato, A., et al, 2014; Monfrecola, G., et al, 2016). In most cases, expression was evaluated in terms of protein content, frequently by immunolabelling, to analyze differential protein expression, localization,and distribution at the tissue, cellular, and subcellular levels, however in some cases mRNA levels have also been assessed (Gödel, M., et al, 2011; Vilà, L., et al, 2012), in the latter situation, it was discovered that mRNA levels do not match to protein levels. This may be dueto post-transcriptional regulation.

When we analyzed the studies dealing with neoplasms, the relationship between the increased expression of mTOR and the pathology in question became evident, looking to gastric cancer studies, research by Byeon, S. J., et al. (2014), we found that the combination of p-mTOR and TSC1 status offered more robust survival information than either one alone. In addition, Bornschein, J., et al. (2015) found that the tumor core had greater mTOR and p-mTOR expression levels than the invasive front. Another study found that mTOR expression was present in 51.5 percent (17/33) of the samples, in contrast to low/absent expression in normal tissues (Li, M., et al, 2012). They also found a positive correlation between mTOR expression and tumor differentiation, lymph node metastasis, and clinical staging (Li, M., et al, 2012). There was no link found between gender, age, and invasive depth. Kasajima, A., et al. (2011)found that gastroenteropancreatic neuroendocrine tumors had high levels of mTOR, 4EBP1, p-4EBP1, p-S6K, and p-eIF4E. In foregut tumors, mTOR expression and activity were greater than in midgut tumors. When distant metastases were found in foregut tumors, mTOR expression was greater. Higher mTOR activity was linked to increased proliferative capability.

The findings ofstudies on esophageal cancer demonstrated that p-mTOR expression had no significant impact on patient survival (Chuang, W. Y., et al, 2015). However, one study in

advanced Esophageal Squamous Cell Carcinoma tumors found that PI3K/Akt/mTOR signaling hyperactivation was accompanied by overexpression of mTOR, proving that overexpression of mTOR is an independent adverse prognostic factor for overall survival (Wu, N., et al, 2018).

Overexpression of mTOR has also been documented in pancreatic cancer, with patients with high p-mTOR expression having a lower survival time (Han, X., et al, 2013). Almost all studies in breast cancer reveal p-mTOR overexpression (Rojo, F., et al, 2014; Beca, F.,et al, 2014; A Mutee, et al, 2009), as well as in cases of prostate cancer. Uliska, E., et al. (2016)discovered a link between mTOR expression and relapses in leukemia patients, and Guerrero,M., et al. (2019) also reported a link between mTOR expression and increased post-liver transplant tumorrecurrence rates in liver cancer patients. Most of the studies that reported cancer patients thathad a positive expression of mTOR, showed a trend towards worse prognosis and shorter overall survival.

Also noteworthy were the findings of Subbiah, V. et al. (2013), who found that overexpression of p-mTOR in desmoplastic small round cell tumors, Ewing's sarcoma, and Wilm's tumor resulted in constitutive activation of p70S6K, and of Lee, H. (2017), who found that expression of p-mTOR in cancer tissues was higher in adenocarcinoma than in other types of cancer, higher in metastatic cancer than in primary cancer; and also higher in the forefront of the infiltrating cancer cells. In solid tumors, mTOR activation has been linked to cancer cell invasion and migration.

Despite the importance of studying and understanding these processes, none of these studies attempted to verify the molecular mechanism that justifies increased expression. Interestingly, one of the articles showed, that Calcium/calmodulin-dependent protein kinase IV (CaMKIV) suppresses ubiquitin proteosomal degradation of mammalian target of rapamycin (mTOR) via inhibitory serine phosphorylation of GSK-3β and suppression of FBXW7 recruitment, as a result, both the macrophage and the kidney's autophagy are enhanced (Zhang Y et al., 2017).

Transcriptional regulation by mTOR

The process by which a cell manages the conversion of DNA to RNA, hence coordinating gene activity, is known as transcriptional regulation. It is a crucial biological mechanism that allows a cell to respond to a wide range of intra- and extracellular inputs. This highly dynamic process is made up of a series of biophysical events that are regulated by a large number of molecules that form larger networks and occur over a wide range of temporal and functional phases, from specific DNA-protein interactions to nucleoprotein complex recruitment and assembly. The key transcription levels include the recruitment and assembly of the whole transcription machinery, the initiation step, the pause release and elongation phases, as well as transcription termination (Lee, T. I., & Young, R. A., 2013). RNA polymerase II, general initiation transcription factors (TFIIA, -IIB, -IID, -IIE, -IIF, and -IIH), and the Mediator complex, (a multi- subunit compound that connects transcription factors bound at upstream regulatory elements and all the remaining apparatus at the promoter region), are all essential components of the basal transcription machinery (Schiano, C., et al, 2014).

Much is yet unknown about the multiplicity of pathways controlled at the transcriptional level by AMPK and mTOR, as well as all the transcription factors and other processes involved. Interestingly, both AMPK and mTOR have been demonstrated to; relocalize to the nucleus; be recruited to chromatin; modify histone marks; and phosphorylate numerous transcription factors directly or indirectly, in response to certain cellular cues (Bungard, D., et al., 2010; Audet-Walsh, E., et al., 2017, 2018; Khan, A. S., & Frigo, D. E., 2017; Giguère, V., 2020).

The AMPK and mTOR signaling pathways are emerging as key roles in the reproductive system and embryonic development, integrating several cellular functions (Audet-Walsh, E., et al, 2020). Because there is currently little study done in this area, it is a growing topic of interest. Yang and Malarkannan demonstrate the important function of both mTORC1 and mTORC2 in NK cell differentiation and maturation in a review focused on the role of mTOR transcriptional control of natural killer (NK) cell development. The authors begin by summarizing the relevance of mTOR activation by the IL-2/IL-15R cytokine receptors in committing lymphoid progenitors to the NK lineage in a comprehensive analysis. They then detail the different downstream transcriptional mechanisms triggered by mTORC1 or mTORC2 to promote early developmental stages and maturation of NK cells, a key component of the innate immune system, based on the most recent transcriptional profiling of NK cells performed by single-cell RNA-sequencing (Yang, C., & Malarkannan, S., 2020). Another study by Sukumaran et al. offered a broad perspective, emphasizing the importance of post-

translational modifications of transcription factors and histones, particularly direct phosphorylation, as well as epigenetic control via modulation of metabolites generated by intermediate metabolism. They also presented a computational analysis that revealed the identification of common transcription factors that regulate most of the genes in the AMPK and mTOR pathways, implying a coordinated transcriptional regulation mechanism to maintain abundance and stoichiometry in response to various environmental cues (Sukumaran, A., Choi, K., & Dasgupta, B., 2020). Multiple molecular pathways are clearly integrated by AMPK and mTOR signaling. Decoding their influence on the transcriptional regulatory network under physiological and pathological settings is a crucial future issue in the search for new biomarkers and therapeutic targets in this context.

Regulation of mTOR translation

It is widely known that mTOR signalling operates and is necessary in a variety of physiological conditions associated with global protein synthesis reduction, such as in hypoxia and mitosis (Ramirez-Valle et al., 2010) and that the mTOR protein levels itself remain unchanged in those settings (Ramirez-Valle et al., 2010). Furthermore, it has been observed that some pathological conditions such as systemic lupus erythematosus are associated with a reduction in mTOR mRNA levels but an increase in mTOR protein levels (Vilà et al., 2012) (Table S 2.1). These data suggest that mTOR is subjected to regulation at the translational level. Indeed, our group demonstrated that mTOR is translated by an alternative and cap-independent mechanism that operates both in normal and stress conditions, allowing sustained mTOR protein levels regardless of the translational inhibitory cues (Marques-Ramos et al., 2017) (Figure 2.2). These findings might explain how mTOR is capable to be activated in a variety of physiological settings strongly associated with protein synthesis reduction. Furthermore, it gives a cue how mTOR evades the normal translational checkpoints and is over-expressed in a variety of diseases (Table S 2.1), as its translation is independent of cap and the initiation factors that are usually blocked by the control mechanisms of the cell (Marques-Ramos et al., 2017). Additionally, it opens a new avenue to counteract mTOR hyperactivation through reduction of mTOR expression, as our group is exploring.

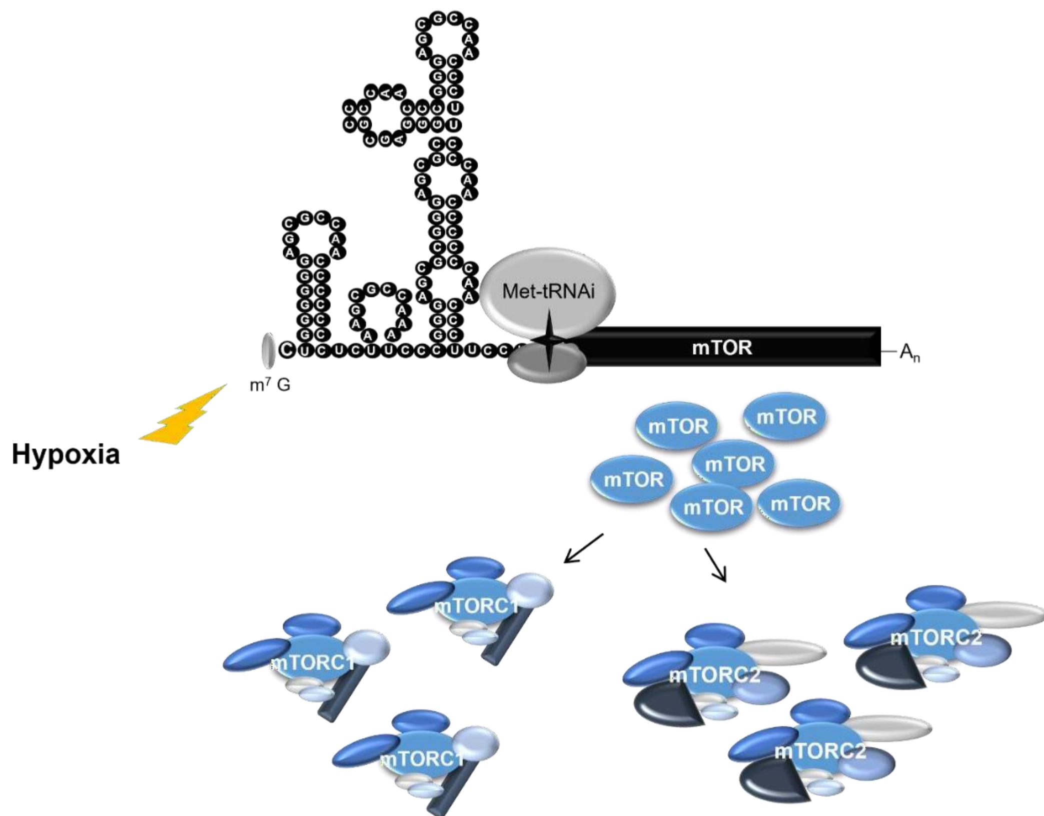


Figure 2.2 - mTOR is translated in a cap-independent manner (Marques-Ramos, A., et al 2017). Cap-independent translation ensures mTOR expression and function upon protein synthesis inhibition. *RNA (New York, N.Y.)*, *23*(11), 1712–1728. https://doi.org/10.1261/rna.063040.117

mTOR 5'UTR adopts a highly folded and evolutionary conserved structure that is capable to directly bind to the 40S ribosomal subunit in the absence of any initiation factor. This RNA scaffold assists cap-independent translation of mTOR, allowing sustained mTOR protein levels in translational inhibitory conditions (hypoxia). Cap-independent translation of mTOR occurs both in normal and stress conditions and is necessary for mTOR function.

Regulation of mTOR by microRNAs

MicroRNAs are highly conserved non-coding RNA molecules that play an important part in gene expression regulation. They are transcribed from DNA sequences by RNA polymerases II and III, which are then processed into precursors that undergo a sequence of cleavage events to create mature microRNA. (Macfarlane, L. A., & Murphy, P. R., 2010). miRNAs have been shown to interact with areas such as the 5′ UTR, coding sequences, and gene

promoters. miRNAs bind to target mRNAs through the 3’ untranslated region and causing mRNA degradation and translational inhibition. They can initiate translation or control transcription of their target genes. The interaction of miRNAs with their target mRNAs is influenced by a variety of factors, including miRNA sub-cellular localization, abundance, and the stability of miRNA-mRNA interactions. miRNAs can be released extracellularly and enter target cells through vesicles like exosomes or by binding to proteins such as Argonauts. Also, extracellular miRNAs act as chemical messengers, allowing cells to communicate with one another (O’Brien, J., et al, 2018).

miRNA dysregulation is a hallmark of cancer. Recent research has shown interactions between miRNAs and the mTOR pathway during cancer development (Figure 2.3). Such interactions appear to fine-tune many cellular processes and contribute qualitatively to cancer activity (Zhang, Y., et al, 2017). Most cancer types have interactions between miRNAs and mTOR signaling, according to current research (Zhang, Y., et al, 2017). Furthermore, they have been observed interacting in a variety of illnesses and physiological states. Integrating miRNAs with key protein components in the mTOR signaling pathway might also improve the specificity and sensitivity of existing treatments. Several clinical trials are now ongoing, and the power of integrating miRNAs with cancer signaling cascades to suit therapeutic needs holds a lot of promise (Zhang, Y., et al, 2017). A comprehensive list of miRNAs targeting mTOR pathway in several pathologies is listed in Table S 2.2.

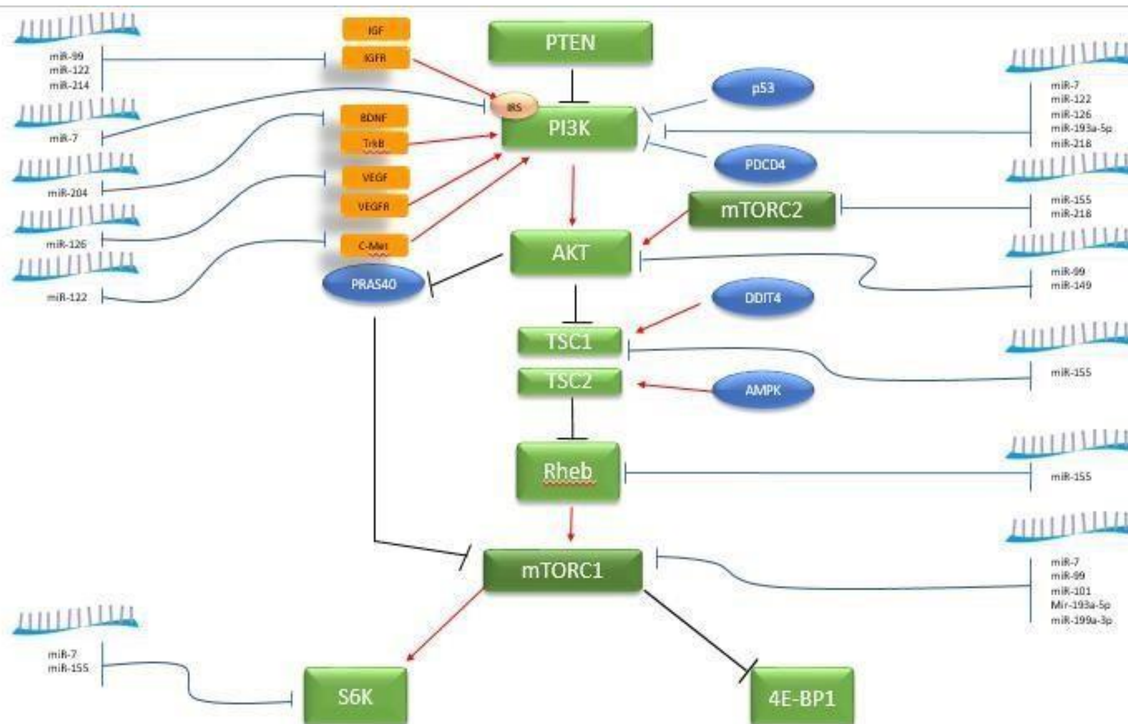


Figure 2.3 - Tumor-suppressive miRNAs that inhibit mTOR pathway

Conclusion

mTOR is a serine/threonine kinase that regulates eukaryotic cell metabolism and growth with external inputs such as nutrients and growth factors. (Saxton RA, 2017). In recent years, investment in biomarker research has been exponential; in this context, multiple authors have observed the expression of mTOR in pathologies, such as Type 2 Diabetes Mellitus. (Yang, L., et al, 2022; Tsai, K., et al, 2021), Alzheimer’s (Perluigi, M., et al, 2021), rheumatoid arthritis (Iwata, S., et al, 2021), and especially cancer (Kahraman, D. C., et al, 2019; Mossmann, D., et al, 2018). The identification of mTOR-associated proteins has increased the relevance of mTOR, which when coupled to different proteins, forms complexes with a wide range of physiological functions. These findings not only increase the range of mTOR actions in cells but also weaken the regulatory network. (Mishra, S., et al, 2021). In this review, we discuss the most recent data on mTOR expression variations reported in a variety of illnesses, including human cancers, and we systematize our current understanding of mTOR regulation at the transcriptional and translational levels.

It is well established that mTOR signaling functions and is essential under a range of physiological circumstances associated with reduced global protein synthesis, such as hypoxia and mitosis. (Ramirez-Valle et al., 2010) and that the mTOR protein levels themselves remain constant in those conditions (Ramirez-Valle et al., 2010). Furthermore, some clinical diseases, such as systemic lupus erythematosus, have been linked to a decrease in mTOR mRNA levels but an increase in mTOR protein levels (Vilà et al., 2012). The fact that mTOR translation is independent of cap and of initiation factors that are generally blocked by the cell's control mechanisms (Marques-Ramos et al., 2017) may explain how mTOR can be activated in a variety of physiological settings strongly associated with protein synthesis reduction, as well as how mTOR evades normal translational checkpoints and is over-expressed in a variety of diseases Furthermore, the list show how various miRNAs impact mTOR signaling in pathological situations. Recent studies has revealed interactions between miRNAs and the mTOR pathway during the development of cancer and it seems that these interactions appear to fine-tune numerous cellular processes and to contribute significantly to cancer activity. Furthermore, interactions between miRNAs and mTOR signaling have been discovered in most cancers as well as in a variety of diseases and physiological states. (Zhang,Y., et al, 2017). We hope that our research will contribute for the creation of novel mTOR inhibitors since it provides a comprehensive view of the control of mTOR gene expression.

Chapter Three - Apigenin assay

Test the potential of Apigenin in assay with Gp202 gastric cancer cell lines, assessing factors such as proliferation and cell morphology.

3.1. Relevance

Based on the information obtained in the course of the previously presented review, we found it relevant to evaluate the effect of apigenin on gastric cancer cells at different concentrations and exposure times, assessing morphology and determining cell proliferation. For this purpose, we used Gp 202 gastric tumor cell lines that overexpress mTOR (Gärtner, F., et al 1996) assessing factors such as proliferation and cell morphology.

3.2. Methodology

This task was designed and planned to have a fluid workflow with steps codependent on each other as shown in the schematic below (Figure 3.1)

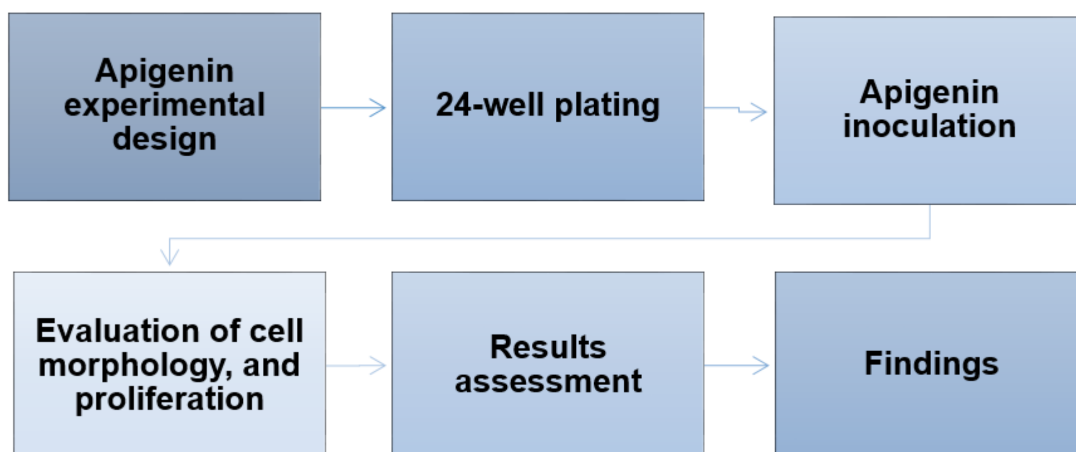


Figure 3.1 - Experimental workflow

3.2.1. Cell culture

Cell culture refers to the removal of cells from animals or plants and their subsequent growth under favorable artificial environmental conditions. For this project GP202 cells were used, a cell line established by i3s from diffuse primary gastric carcinoma of a 53-year-old Caucasian woman. These are adherent epithelial cells with a hexagonal or signet-shaped morphology (Figure 3.2), that were obtained via Ipatimup, ensuring sterility by microorganisms including Mycoplasma (Gärtner, F.,et al, 1996).

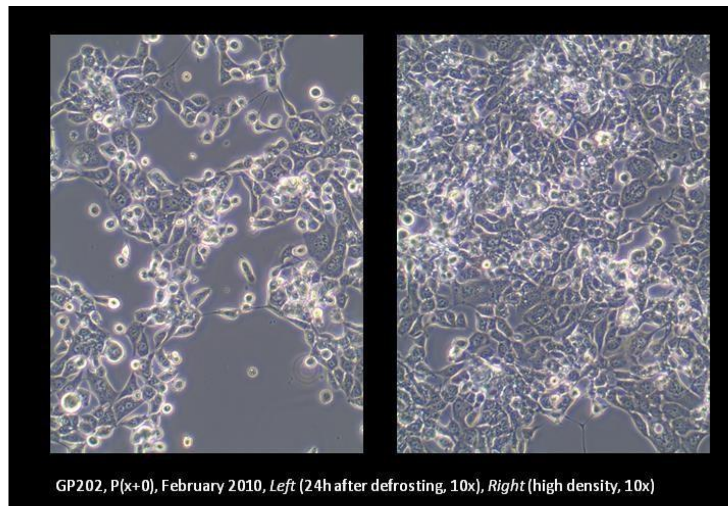


Figure 3.2 – GP202 cells 24h after defrosting (10x) - note the hexagonal morphology of the epithelial cells (source: https://www.ipatimup.pt/site/serviceview.aspx?title=gp202&serviceid=878); access December 2021.

Gp202 cells were cultivated in RPMI-1640 with l-glutamine (amino acid essential for cell proliferation) supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin and penicillin for inhibition of microbial agents. Prior to use, the cells were frozen (-80), T25 flasks (5 mLcapacity) were utilized for culture, and the cryotube containing the cells was put in a 37°C humid CO2 incubator to quickly thaw. Following this, cells were pipetted into flasks with culture medium under sterile conditions in a laminar flow chamber. The cells were kept in an incubator with 5% CO2, 95% oxygen at a temperature of 37°C for the course of the experiment. Culture media was replaced every 1-2 days, on average, in which the old medium was discarded and replaced with new medium (previously heated), using sterile 5ml graduated pipettes. Cells were then subcultured on alternate days depending on cell confluence. For this, the old culture medium was discarded and the cells were washed twice with pre-warmed calcium/magnesium-free PBS, then treated with 1 mL trypsin and incubated for about 3-5 minutes at 37°C or until their detachment from the culture flask wall was verified by microscopy. After the cells were detached from the plate by enzymatic digestion trypsin was inactivated by adding twice its volume (2 mL) of fresh culture medium. During the process of optimizing the protocol, we

discovered that increasing the washing with PBS from once to twice improve cell detachment from the flask wall. This is due to the fact that PBS helps to remove divalent cations from the adhesion of proteins such as cadherins, resulting in better flask detachment. Furthermore, we also found that changing the medium the day before transfer produced superior results, as it potentiated cell growth. To minimize the risk of microorganism contamination that could invalidate the results obtained, all manipulations were performed in a laminar flow chamber previously subjected to UV radiation for 30 minutes and all objects used for manipulation were previously cleaned with 70% alcohol.

3.2.2. Apigenin experimental design

To define the optimal doses and times of exposure to apigenin, the studies from Jiayu Chen et al, 2014 and Kun Wu et al, 2005 were considered, which refer to experimental tests carried out in HGC-27 cell line from metastatic lymph node of gastric cancer and SGC-7901cell line from human gastric cancer, which showed the best results in the following times and conditions. Based on these results we decided that in our assay cells would be exposed to 10µM and 20 µM of apigenin for 24h and 48h, in 24-well plates. (Figure 3.3).

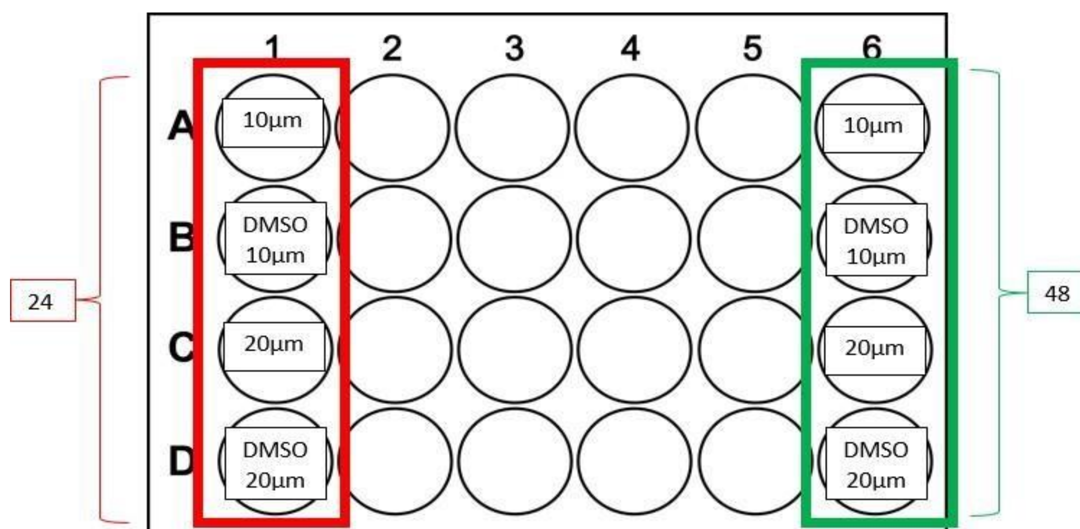


Figure 3.3 - Identification of plates for inoculation with apigenin: 10µM apigenin, 10µM DMSO control, 20µM apigenin, 20µM DMSO control, at 24h and 48h

3.2.3. 24-well plating

One day prior to apigenin treatment, cell were plated in 24-well. To achieve 80% of confluence at the moment of apigenin treatment, the optimal number of 120,000 cells in each well of a 24-well plate was established. This was done by pre-counting cells in a hemocytometer following

trypan blue staining (Figure 3.4). The trypan blue exclusion method is used for the determination of viable cells present in a cell suspension and is based on the principle that live cells have intact cell membranes and exclude dyes, such as trypan blue. For this procedure, the medium was removed from the culture flask when confluence was at 80% and cells were detached according to the previously described trypsinization procedure, then 100 µL of cells were withdrawn into a sterile eppendorf tube containing 100 µL of trypan blue and then we proceed to counting in a previously prepared hemocytometer cleaned with 70% alcohol after roughly 3 minutes of incubation with trypan blue (never exceeding 5 minutes to avoidincreasing cell death), brought to the microscope, where the mesh and quadrants were able to be seen.

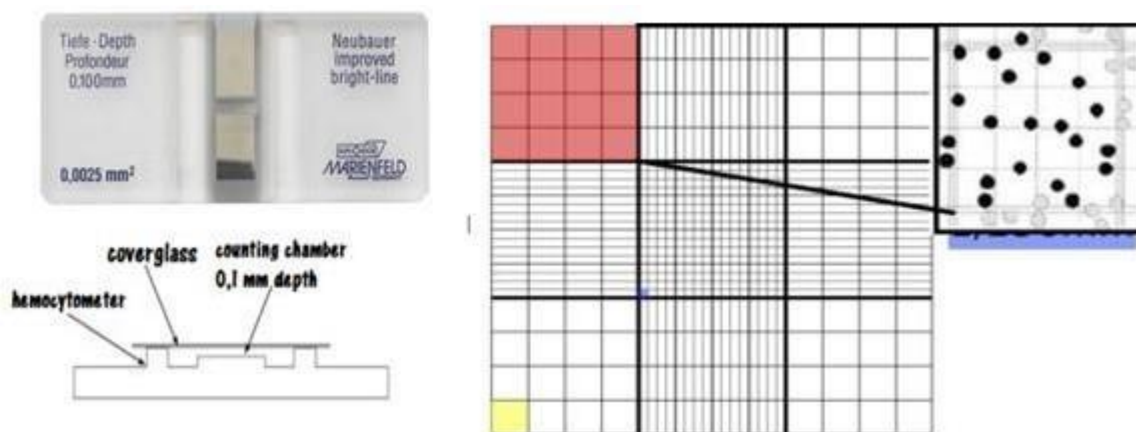


Figure 3.4 – Cell count scheme in a hemocytometer

The 4 peripheral quadrants were then counted as shown in figure 3.4 For a more reliable count it was imposed to count the cells that fall in the upper and left threshold, but not those in the right and lower threshold. From there, the average of viable cells is determined, and cells are calculated using the equation:

Cell count = average number of viable cells in the 4 grids x 10000 x dilution factor (dilution factor = 2 (100µL of cells + 100µL of trypan blue)

After estimating the number of cells in 1mL, the volume of cells to be pipetted from the flask into each well of the 24-well plate may be calculated so that each well has 120 000 cells in a volume of 500µL.

3.2.4. Apigenin inoculation

Apigenin (Sigma-Aldrich) was obtained freeze-dried, therefore it needed to be resuspended. Apigenin is soluble in ethanol, DMSO, and dimethyl formamide (DMF), and its solubility in these solvents is around 0.3, 15, and 25 mg/ml, respectively (Wang, M., et al, 2019). Since apigenin is only slightly soluble in water, for maximum solubility in aqueous buffers apigenin should be dissolved in DMF first, then diluted with the aqueous buffer of choice (Wang, M., et al, 2019). We decided to use DMSO as a solvent to make a stock solution. For each 15 mg of apigenin 1 ml DMSO was used as solvent to obtain a concentration of 15000µg/ml. The stock solution was stored at −20 °C.

Before inoculation with apigenin the cells were observed under an optic microscope to ensure that they were at 80% confluence. For the inoculation, apigenin was introduced by addition to the culture medium at concentrations of 10 µM and 20 µM. For the control group, DMSO was used to replace apigenin at the same concentrations (Table 3.1).

Table 3.1 - Scheme demonstrating the volume of apigenin and DMSO added to each well in the 24-well plate.

Condition	Apigenin 1	Control 1	Apigein 2	Control 2
24h	1 µl apigenin in 500µl culture medium	1 µl DMSO in 500µl culture medium	2 µl apigenin in 500µl culture medium	2 µl DMSO in 500µl culture medium
48h	1 µl apigenin in 500µl culture medium	1 µl DMSO in 500µl culture medium	2 µl apigenin in 500µl culture medium	2 µl DMSO in 500µl culture medium

The plates were incubated with 5% CO2 and 95% oxygen atmosphere for 24h and 48h respectively.

3.2.5. Cell morphology

The shape, structure, form, and size of cells are all described by cell morphology. Normal cells have uniform, ellipsoid forms, but cancer cells are typically irregular and contoured. Morphological traits play a vital part in the diagnosis of cancer (Senchukova, M. A., 2020). GP220 cells are adherent epithelial cells that usually develop in tiny clusters connected to the monolayer, with a subset of floating cells and present cells with small mucin vacuoles in the cytoplasm and cells with a classic hexagonal or signet-ring shape (Gartner, F., et al, 1996).

For the morphology evaluation the medium was discarded, and the cells were washed twice with 500 µl of PBS. Following this, the PBS was fully withdrawn (first with a 5mL pipette, then with a 1000 micropipette with blue and clear tips), then 500 µl of methylene blue was added and allowed to work for 60 seconds. After that, cells were rinsed again with 500 µl PBS. The plate was then brought to the microscope, where three representative fields from each well were photographed and the photographs were used to perform the morphological evaluation of eleven parameters as showed in table 3.2 based on a score (Table 3.3) from Carvalho et al, 2008. *The* following parameters were evaluated (Table 3.2)

Table 3.2 - Parameters employed in the morphological assessment

Atypical "naked" nuclei	Cells which are deprived of cytoplasm and have at least one abnormal nuclear feature
Abnormal mitoses	Presence of mythotic figures
Cohesive three-dimensional clusters	Groups of cells with nuclear overlapping (pseudostratification)
Excentric nuclei	Nucleus situated at one edge of the cell
High nucleus/cytoplasm ratio	The nucleus fills at least about 50% of the total cell size
Hyperchromasia	Chromatin darker than surrounding benign epithelium
Irregular nuclear membrane outlines	Presence of irregularities in the contour of the nuclear membrane
Poorly cohesive three-dimensional clusters	Groups in which cells contact each other only by small projections of the cytoplasm, resulting in "gaps" between cells.
Macronuclei	Nucleoli with a size of at least 2 µm
Signet ring cells	Cells with an intracytoplasmic vacuole that shifts the nucleus to the periphery and distorts the nuclear contour
Tumour diathesis	Presence of atypical cells in the background (acute inflammation and cell debris)

Table 3.3 – score used in the evaluation of cellular morphology

Score	**Visualisation**
0	Low visualisation
1	Moderate visualisation
2	High visualisation

- Low visualization (score 0): when between 0% and 20% of the cells of the observed field show the feature of the parameter under analysis

- Moderate visualization (score 1): when between 20% and 50% of the cells in the observed field show the feature of the parameter under analysis
- High visualization (score 2): when more than 50% of the cells of the observed field show the feature of the parameter under analysis

3.2.6. Cell proliferation and cell death

In the course of the morphological evaluation, we realized that through this first exercise we could make an estimate regarding proliferation and cell death. To do this we used cell counting tools from ImageJ that is a Java-based image processing program developed at the National Institutes of Health and the Laboratory for Optical and Computational Instrumentation (LOCI, University of Wisconsin).

3.3. RESULTS

3.3.1. Cell morphology

In the assays performed with apigenin concentration of 10 μM, all the morphological parameters defined on our score were able to be seen, except for abnormal mitoses, whereas on the assays performed with apigenin concentration of 20 μM results were similar, but there was also not possible to identify signet rings on our analyzed images (Figure 3.5). In boht cases the most prevalent parameters found were macronuclei, hyperchromasia and irregular nuclear membrane outlines as well as excentric nuclei. When analyzing the controls we were able to find that macronuclei and excentric nuclei were also prevalent, as well as tumor diathesis and poorly cohesive three-dimensional clusters witch were not prevalent parameters in the assays with apigenin. During the morphological analysis, it was possible to verify that the 10 μM concentration had better results regarding the observation of the morphological changes since the trials with the 20 μM had a very low concentration of cells, which made it more difficult to find morphological changes in large numbers. On the other hand, with a concentration of 20 μM it was much more noticeable the presence of atypical "naked" nuclei. We were also able to observe a general phenotypic alteration in the morphology of the cancer cells, in regards to its shape, decreased size, and cell fragmentation as well as a reduction of

the number of the cells. It was also evident, especially at 48 hours of incubation with apigenin, a fragmented aspect in the nuclei.

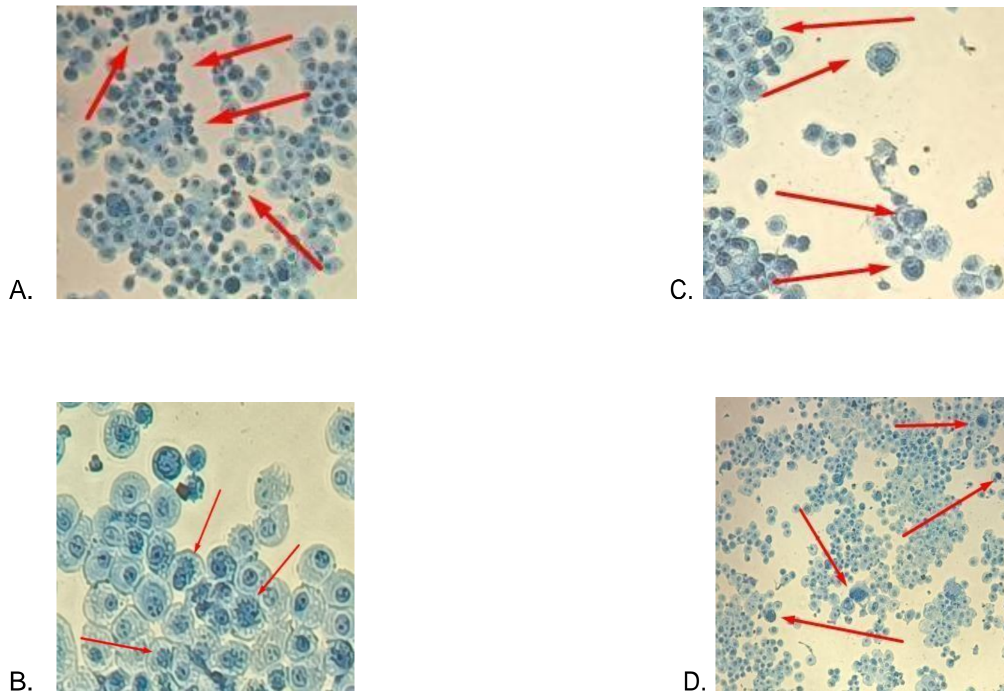
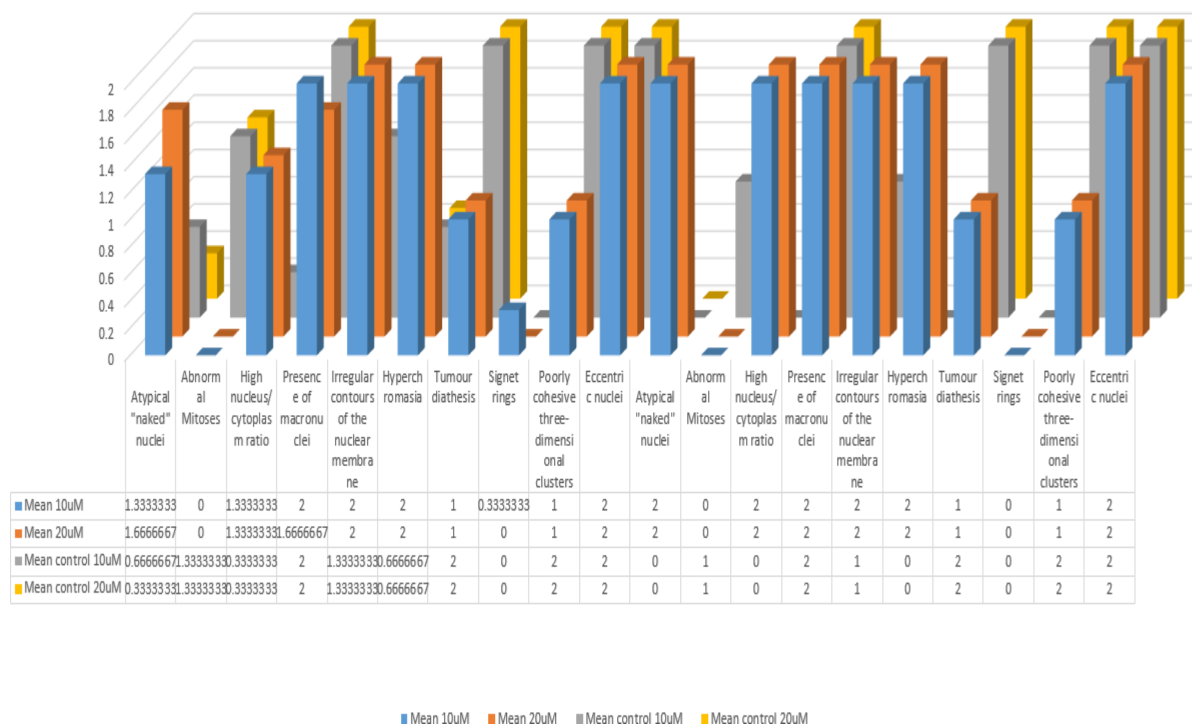


Figure 3.5 – A. Atypical "naked" nuclei, B. Irregular contours of the nuclear membrane, C. High nucleus/cytoplasm ratio and D. Hyperchromasia (10X)

As for the established intra-group score analysis, in both groups inoculated with apigenin (10 μM and 20 μM concentration), showed that score 2 which represents a high visualization of the parameters (when more than 50% of the cells of the observed field show the feature of the parameter under analysis)prevailed over score 1 representing a moderate visualization (when between 20% and 50% of the cells in the observed field show the feature of the parameter under analysis)and score zero which represents a low visualization (between 0% and 20% of the cells of the observed field show the feature of the parameter under analysis).

The inter-group analysis showed that score 2 was more frequent in the groups inoculated with apigenin regarding atypical "naked" nuclei (Figure 3.5 A), high nucleus/cytoplasm ratio(Figure 3.5 C), tumor diathesis, poorly cohesive three-dimensional clusters parameters which were less frequent in the control groups.



	Atypical "naked" nuclei	Abnormal Mitoses	High nucleus/ cytoplasm ratio	Presence of macronuclei	Irregular contours of the nuclear membrane	Hyperchromasia	Tumour diathesis	Signet rings	Poorly cohesive three-dimensional clusters	Eccentric nuclei
Mean 10uM	1.3333333	0	1.3333333	2	2	2	1	0.3333333	1	2
Mean 20uM	1.6666667	0	1.3333333	1.6666667	2	2	1	0	1	2
Mean control 10uM	0.6666667	1.3333333	0.3333333	2	1.3333333	0.6666667	2	0	2	2
Mean control 20uM	0.3333333	1.3333333	0.3333333	2	1.3333333	0.6666667	2	0	2	2

	Atypical "naked" nuclei	Abnormal Mitoses	High nucleus/ cytoplasm ratio	Presence of macronuclei	Irregular contours of the nuclear membrane	Hyperchromasia	Tumour diathesis	Signet rings	Poorly cohesive three-dimensional clusters	Eccentric nuclei
Mean 10uM	2	0	2	2	2	2	1	0	1	2
Mean 20uM	2	0	2	2	2	2	1	0	1	2
Mean control 10uM	0	1	0	2	1	0	2	0	2	2
Mean control 20uM	0	1	0	2	1	0	2	0	2	2

Figure 3.6 – Score Inter-group analysis (24h)

As for the total score (Figure 3.6), the groups inoculated with apigenin at both 24 and 48 hours obtained a total score of 13 while the control groups have a total score of 8 as presented in table 3.4. This was due to the fact that the cumulative value of the total score represents a higher prevalence of morphological changes in the analyzed images referring to the apigenin trials when compared to the controls.

Table 3.4 - Morphology score 24h (raw data for each experiment + mean, median and standard deviation for each condition)

	Morphology Score										
	atypical "naked" nuclei	Abnormal Mitoses	High nucleus/cytoplasm ratio	Presence of macronuclei	Irregular contours of the nuclear membrane	Hyperchromasia	Tumour diathesis	Signet rings	Poorly cohesive three-dimensional clusters	Eccentric nuclei	Total Score
10uM	2	0	2	2	2	2	1	0	1	2	13
10uM	0	0	0	2	2	2	1	1	1	2	10
10uM	2	0	2	2	2	2	1	0	1	2	13
mean 10uM	1.33	0	1.33	2	2	2	1	0.33	1	2	12
median 10uM	2	0	2	2	2	2	1	0	1	2	13
standard deviation	1.15	0	1.15	0	0	0	0	0.58	0	0	2.89
Control 10uM	0	1	0	2	1	0	2	0	2	2	8
Control 10uM	2	2	1	2	2	2	2	0	2	2	15
Control 10uM	0	1	0	2	1	0	2	0	2	2	8
mean C10uM	0.67	1.33	0.33	2	1.33	0.67	2	0	2	2	10.33
median controlo 10	0	1	0	2	1	0	2	0	2	2	8
standard deviation	1.15	0.58	0.58	0	0.58	1.15	0	0	0	0	4.04
20uM	2	0	2	2	2	2	1	0	1	2	13
20uM	1	0	0	1	2	2	1	0	1	2	9
20uM	2	0	2	2	2	2	1	0	1	2	13
mean 20uM	1.67	0	1.33	1.67	2	2	1	0	1	2	11.67
median 20uM	2	0	2	2	2	2	1	0	1	2	13
standard deviation	0.58	0	1.15	0.58	0	0	0	0	0	0	2.31
Control 20uM	0	1	0	2	1	0	2	0	2	2	8
Control 20uM	1	2	1	2	2	2	2	0	2	2	14
Control 20uM	0	1	0	2	1	0	2	0	2	2	8
mean C20uM	0.33	1.33	0.33	2	1.33	0.67	2	0	2	2	10
median control 20u	0	1	0	2	1	0	2	0	2	2	8
standard deviation	0.58	0.58	0.58	0	0.58	1.15	0	0	0	0	3.46

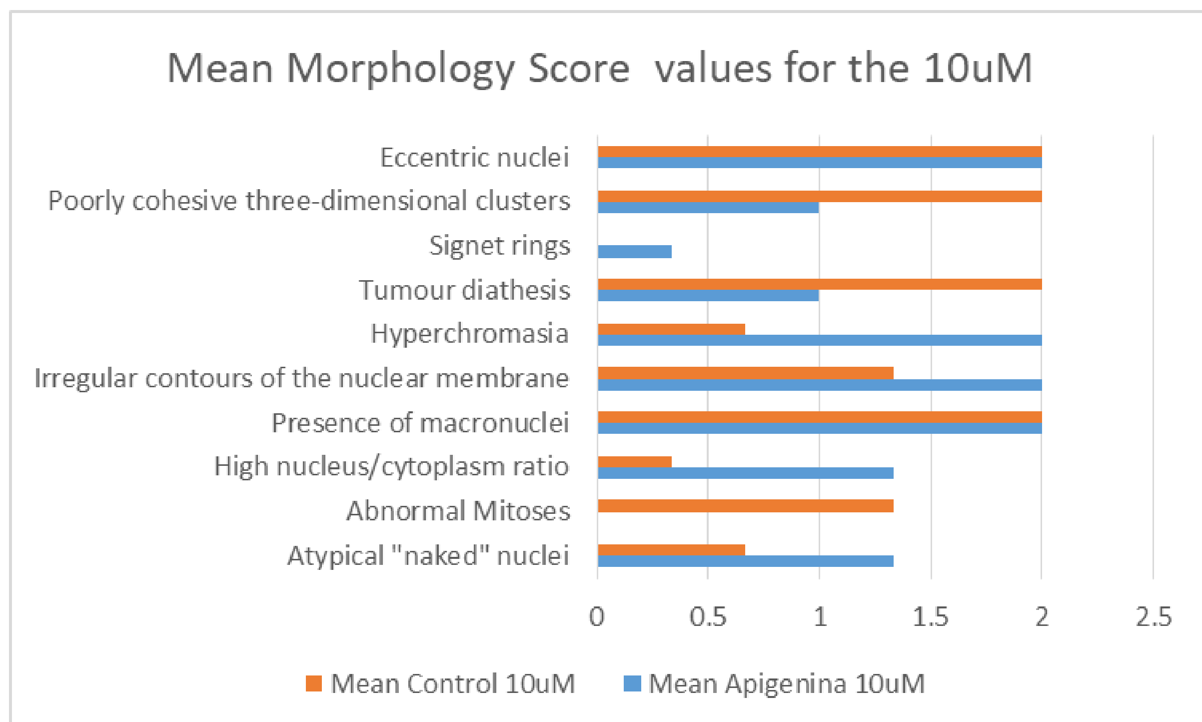


Figure 3.7 - Mean morphology score values for the 10 μM apigenin and 10 μM control assays at 24h

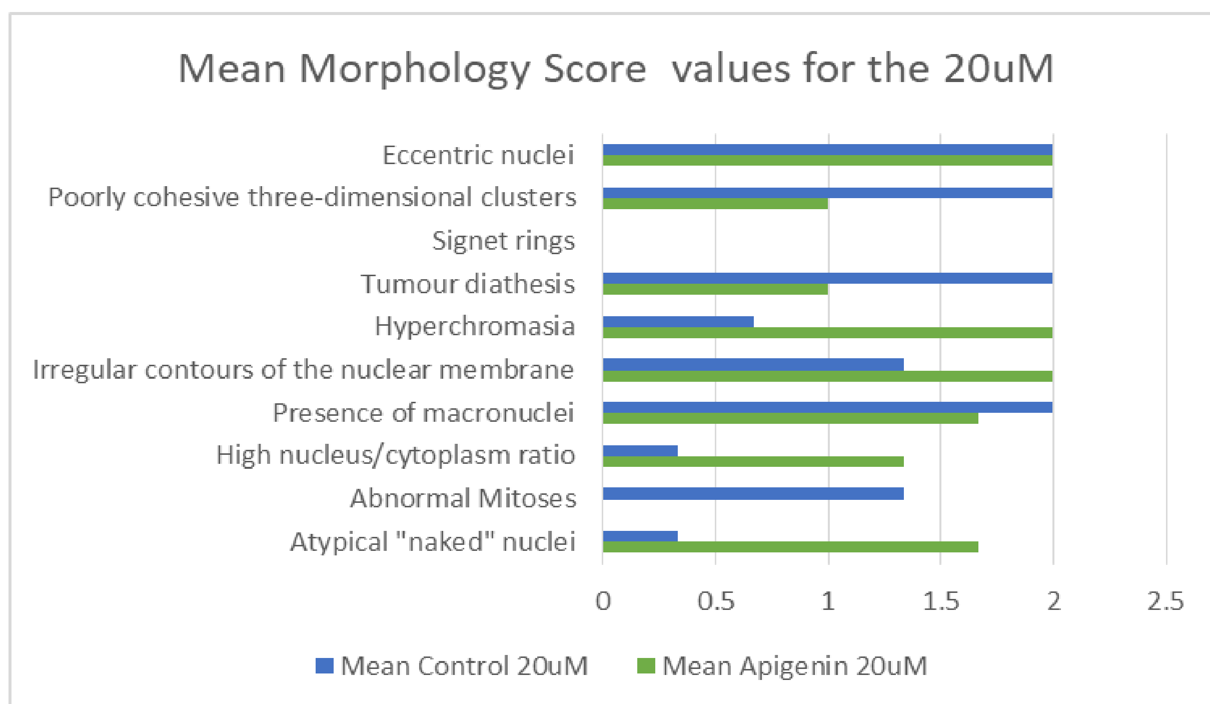


Figure 3.8 - Mean morphology score values for the 20 μM apigenin and 20 μM control assays at 24h

Gp202 cells were inoculated with apigenin at a concentration of 10 μM and 20 μM, for 24h and 48h, after which three representative fields from each well were analyzed and the mean valuewas used to extrapolate the percentage of population in each field as well as the divergence between each well (apigenin vs. control) at each exposure time (Figure 3.9). Each experimentwas performed in consecutive weeks one per week, all at the same conditions Overall it was possible to observe a decrease in cell proliferation compared to the control at both concentrations incubated for 24 hours, however, the cells incubated at 48 hours had very few cells which made difficult to make a more accurate assessment.

The results were very noticeable being for the 10 μM concentration, the mean quantity of cells , at 24h of 17.99% (range from 10.85% to 40.17%) and of 9.63% at 48h (range from 6.74% to 15.97%). As for the 20 μM assays the mean concentrations of cells were of 17.16% at 24h (range from 7.02% to 67.41%) and 6.82% at 48h (range from 3.05% to 14.43). Resulting in a difference in proliferation that ranges from 82,01% to 93,37% for 10 μM, and from 82,84% to 93,18% for 20 μM compared to the control condition (Table 3.5)

By observing the difference between the cell quantity of the inoculated cells in both incubation times, we thought that a concentration of 10 μM would have had a better outcome than the concentration of 20 μM regarding the availability of cells per well. However, the difference between the results for the concentrations of 10 μM and 20 μM at both exposure times was not statistically significant (p-value >0.05).

When comparing the results for 10 µM and 20 µM, the difference between cell quantity was of only 0.8% at 24h, and of 2.8% at 48h, which although represents a difference in the decrease of cell quantity, does not represent a statistically significant difference (p-value >0.05).

Then we compared the assays with 10 µM in both 24h and 48h which showed a difference of 8.36% regarding cell quantity, and the 20 µM also at 24h and 48h, and on this case the difference between cell count was of 10.34%. For the 10 µM concentration p-value was <0.05, as for the 20 µM the p-value was >0.05, showing that the longer exposure to apigenin had a bigger impact at a lower concentration clearly stating a time/dose relation.

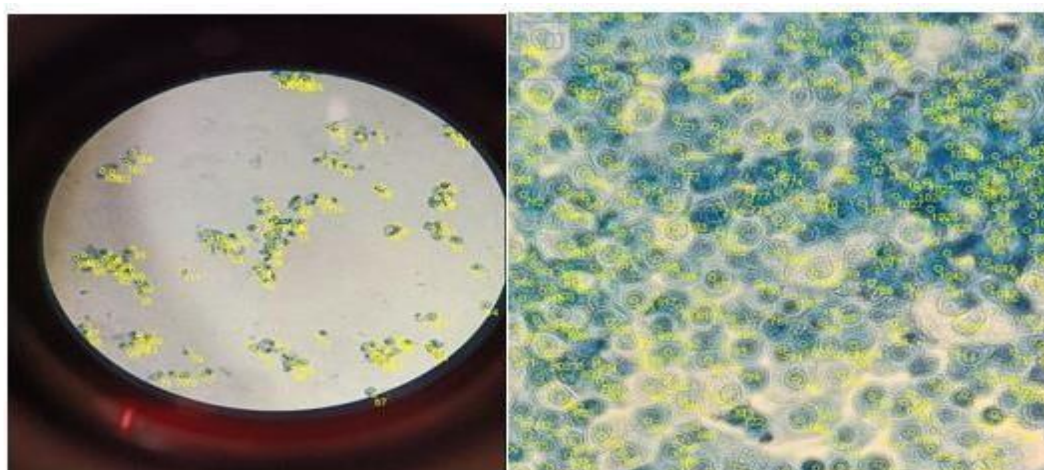


Figure 3.9 - Cell count - images extracted from ImageJ

Table 3.5 – Normalized cell count for each well at 24h and 48h for every trial.

	Cells % at 24h				Cells % at 48h			
	Apigenin 10	Control 10	Apigenin 20	control 20	Apigenin 10	control 10	Apigenin 20	control 20
1st test run	13.43	100.00	8.20	100.00	10.27	100.00	4.88	100.00
	14.31	100.00	7.42	100.00	15.97	100.00	9.31	100.00
	21.36	100.00	7.02	100.00	9.19	100.00	6.35	100.00
2nd test run	19.18	100.00	27.07	100.00	7.98	100.00	14.43	100.00
	15.74	100.00	10.77	100.00	9.72	100.00	6.35	100.00
	40.17	100.00	67.41	100.00	6.74	100.00	3.05	100.00
3rd test run	10.85	100.00	8.77	100.00	4.95	100.00	5.69	100.00
	15.91	100.00	8.92	100.00	14.62	100.00	6.10	100.00
	11.00	100.00	8.90	100.00	7.25	100.00	5.18	100.00
mean	17.99	100.00	17.16	100.00	9.63	100.00	6.82	100.00
median	15.74	100.00	8.90	100.00	9.19	100.00	6.10	100.00
standard deviation	9.0020124	0	19.83869	0	3.611585	0	3.298015	0

Table 3.6 - Differential cell count % in each assay for both concentrations at 24h and 48h (cells/plate).

24 h						
	Apigenin 1	Control 1	reduction	Apigenin 2	Control 2	reduction
1st experiment	243.33	1563.33	-84.43%	131.67	1748	-92.47%
2nd experiment	283	1174	-75.89%	393.67	1105.67	-64.39%
3rd experiment	143.33	1114	-87.13%	98.33	1110.33	-91.14%

48h						
	Apigenin 1	Control 1	reduction	Apigenin 2	Control 2	reduction
1st experiment	180.67	1567.67	-88.47%	130.67	2016.33	-93.52%
2nd experiment	165.33	2084.67	-92.07%	170	2109.67	-91.94%
3rd experiment	133	1594.33	-91.66%	89	1582.67	-94.38%

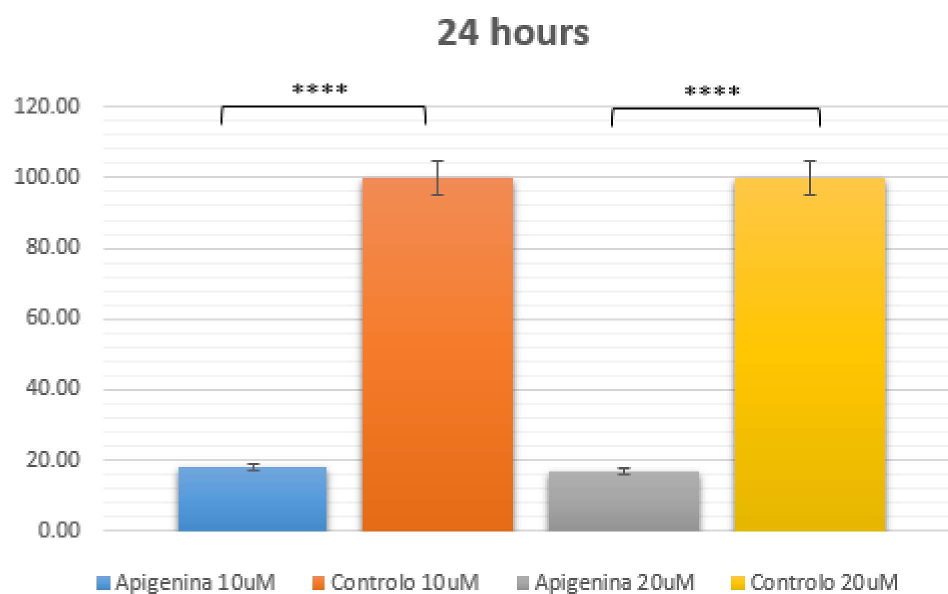


Figure 3.10 - Diagram representing cell proliferation for apigenin normalized to control conditions for both 10μM and 20μM concentrations at 24h (p-value < 0.001). Data is presented as mean and error bars represent standard deviation of the mean cell proliferation

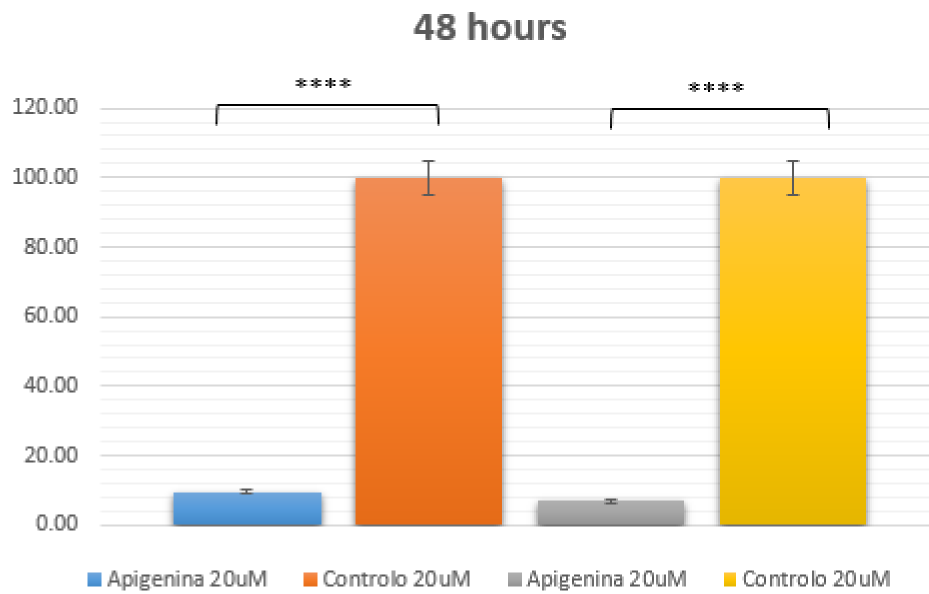


Figure 3.11 - Diagram representing cell proliferation for apigenin normalized to control conditions for both 10μM and 20μM concentrations at 48h (p-value < 0.001). Data is presented as mean and error bars represent standard deviation of the mean cell proliferation

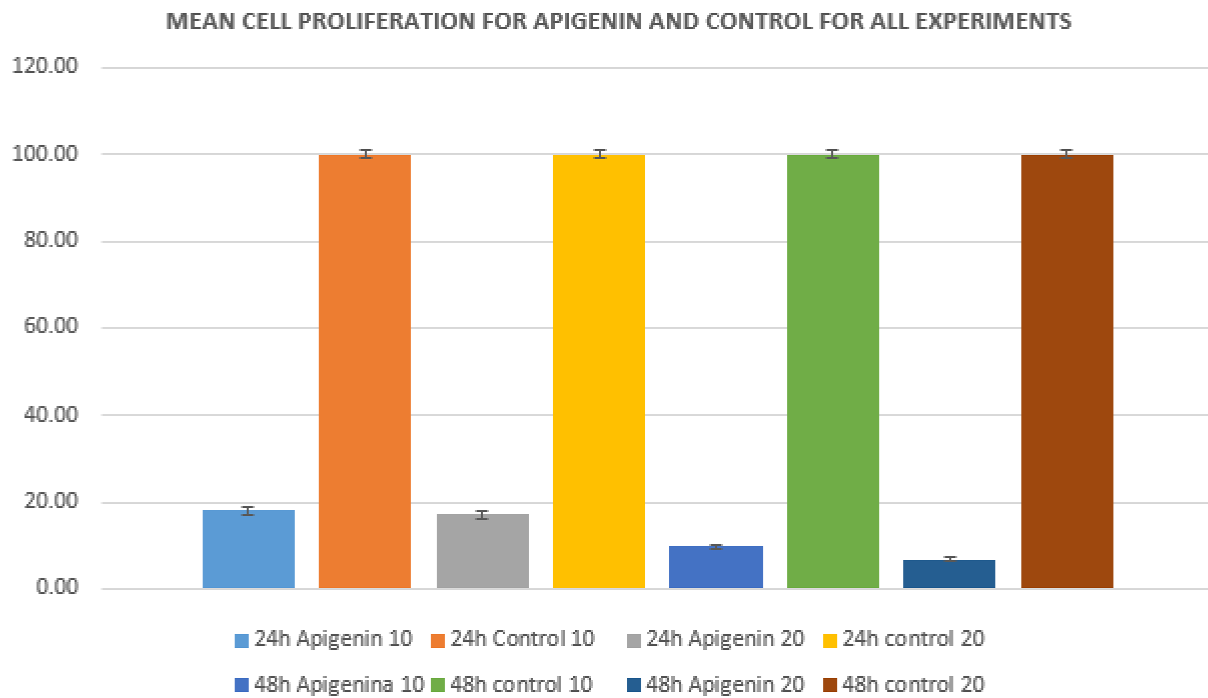


Figure 3.12 - diagram representing cell proliferation for apigenin normalized to control conditions at both times and concentrations (p-value > 0.05/T-test). Data is presented as mean and error bars represent standard deviation of the mean cell proliferation on each test.

Cell apoptosis is an intentionally designed cell death mechanism that keeps the internal environment steady. It comprises the activation, expression, and control of multiple genes. Abnormal cell apoptosis is a significant etiology for most malignant tumors, and discovering effective and low toxicant agents to induce tumor cell apoptosis has been a long-term goal in cancer therapy, but no medicine has yet to achieve a true cure for gastric carcinoma (Elmore S. (2007).

Apigenin has been shown in recent studies to have antibacterial, anticancer, antiparasite, and antioxidant properties. Apigenin was also shown to have anticancer properties in several studies and it may inhibit tumor cell proliferation by causing cell apoptosis. The effect and mechanism of apigenin on gastric cancer and normal cells, on the other hand, had not been yet described (Chen. J., et al, 2014).

In this study, we were able to see in the assays performed with apigenin for both concentrations of 10 μM and 20 μM that the more prevalent morphological alterations observed were macronuclei, hyperchromasia, irregular nuclear membrane outlines, and eccentric nuclei followed by atypical "naked" nuclei, high nucleus/cytoplasm ratio (Figure 3.5). We were also able to observe a phenotypic alteration in the morphology of the cancer cell when inoculated with apigenin, changing its shape, decreasing in size, and presenting cellular and nuclear fragmentation, indicating visual evidence of cell death, which can be understood as a mechanism of aggregation of the cellular components in the apoptotic process (Kroemer, G., 2007; Hail, N., 2006)., This observations are in line with the expected results when compared with other studies performed with apigenin on gastric tumor cells.

In a study from 2015, Wu and colleagues reported morphological changes such as disintegrated nuclear membranes, condensation of chromatin and broken nuclei found by fluorescent staining, when assessing SGC-7901 cells gastric carcinoma cells inoculated with apigenin, they were also able to identify a typical apoptosis peak through flow cytometry analyses after the cells were treated with apigenin for 48 h. In another study from Chen et al, 2014, is was demonstrated that apigenin could reduce mitochondrial membrane potential of gastric carcinoma cells which was concluded through the results regarding morphology that showed typical apoptotic morphological changes of the cell nucleus.

The results reported in these studies are in line with the results obtained in our trial, which brings us closer to a positive conclusion regarding the impact of apigenin on gastric cancer cell morphology and possibly in apoptosis.

Other research has already shown that apigenin can promote autophagy and death in breast cancer cells (Yang, J., et al 2019). In the study from Chen et al, 2014 it was demonstrated that apigenin is able to induce effects on viability and proliferation in gastric carcinoma cell lines HGC-27 and SGC-7901 with results of 0.04% and 2.14% respectively in increased apoptosis and altered mRNA levels of Bax, Bcl-2 and caspase-3 after treatment with apigenin 10 µg / ml. These results are in line with the results obtained in our study.

Also in the study from Wu et al, 2015, in which SGC-7901 cells were inoculated with apigenin at 20, 40 and 80 µmol/L over a periods of 24 and 48h, they were able to observe an increase in apoptosis at 48h of exposure of 5.76%, 19.17% and 29.30% respectively.

Recent studies have also shown that apigenin exhibits anti-proliferation effects on several forms of cancer cells such as prostate cancer cells breast cancer cells, leukemia cells, and colon cancer cells (Shukla, S., et al, 2014; Tong, X., & Pelling, J.C., 2013) and enhances gap junctional intracellular communication changes in human liver cells and induces morphological changes in some cells (Wu et al, 2015).

In our study, we also investigated the proliferation activity changes of gastric cells after apigenin inoculation. The data showed that apigenin could inhibit the proliferation activity of gastric cancer cell lines GP-202. The proliferation inhibition rate was dose and time-dependent,and the influence was more effective in the 10 µM dose at 48h cells than the 20 µM 48h cells or both concentrations at 24h (Table 3.6).

When compared to other studies that tested apigenin in Gastric cancer cells, our results are very promising since they revealed the same course of action from apigenin regarding proliferation and the conditions associated with time-dose response. In Wu et al, 2015, SGC-7901 cells inoculated with apigenin, they observed on day 4 at the concentration of 80 µM an inhibition rate (IR) of 90%. The growth IRs at concentrations of 20, 40, and 80 µM were 38%, 71%, and 99% respectively on day 7. Chen et al, 2014 also stated that the gastric cancer cell proliferation ability was inhibited by different concentrations of apigenin after 24, 48, or 72 h treatment, and the inhibition effect was time and dose-dependent.

However, Apigenin's mechanism of action on gastric cancer and normal cells had not been yet established and the link between apigenin-induced apoptosis and autophagy is still uncertain and it would be interesting to do a different approach on this research testing again, using different concentrations of apigenin and performing additional analysis such as nuclear morphology, flow cytometry or evaluate cyclin levels to assess apoptosis and cell cycle changes. It would also be interesting to seeif apigenin is able to improve the level of apoptosis precursor protein Bax, and downregulate anti-apoptotic protein Bcl-2, leading to the decrease of the mitochondrial membrane potential,

activating the caspase- 3 cascade and resulting in apoptosis . This was assessed in previous research and it would be interesting to see if we can replicate the results.

CONCLUSION

The main objective of this work was the deepening of knowledge and skills of independent work, the elaboration of a well-grounded systematic review relevant to the scientific community, and the development of the initial phase of the practical work following the knowledge acquired from the research conducted.

This work is structured to first define the key concepts, providing the reader with a solid theoretical basis for understanding the results and discussion; secondly, we present the article already submitted for publication resulting from the systematic review; then, we present the practical component with the assays performed and the results obtained. In the discussion, the theoretical framework, the literature review, and the results of the trials are related, analyzed, and discussed; finally, this conclusion is based on the evidence gathered throughout the work.

In recent years, investment in biomarker research has been exponential; in this context, multiple authors have observed the expression of mTOR in pathologies, such as Type 2 Diabetes Mellitus. (Yang, L., et al, 2022; Tsai, K., et al, 2021), Alzheimer's (Perluigi, M., et al, 2021), rheumatoid arthritis (Iwata, S., et al, 2021), and especially cancer (Kahraman, D. C., et al, 2019; Mossmann, D., et al, 2018). Apart from the possibility of novel chemicals replacing rapalogs as mTOR inhibitors in the future, there is now a window of opportunity to examine the significance of different therapeutic approaches (Wacheck V., 2010).

Vitamins, polyphenols, and plant-derived bioactive chemicals are examples of natural antioxidants that have lately been explored for use as prophylactic agents and potential therapeutic drugs (Katz L, et al, 2016; Bernardini S, et al, 2018). In this context, flavonoids have been shown to modulate several protein kinases (e.g. protein kinase-C, serine-tyrosine kinases) as well as epidermal growth factor receptors (EGFRs), platelet derived growth factor receptors (PDGFRs), vascular endothelial growth factor receptors (VEGFRs), and cyclin-dependent kinases (CDKs) (Singh and Agarwal, 2006).

Apigenin is one of the most widely distributed flavonoids in plants, as well as one of the most researched phenolics. Because of its diverse pharmacological actions and relevance to human health, a thorough understanding of its mechanism of action is critical for potential therapeutic uses. When compared to other structurally similar flavonoids, it exhibits a low intrinsic toxicity on normal vs malignant cells (Ali, F., et al, 2017)., promotes cell cycle arrest at many phases

of proliferation, including G1/S and G2/M, via regulating the expression of certain CDKs and other genes (Takagaki, N., et al, 2005; Maggioni, D., et al, 2013), and is known to influence intrinsic apoptotic pathways by altering mitochondrial membrane potential and triggering the release of cytochrome C in the cytoplasm, which results in the formation of apoptotic peptidase activating factor, activation of caspase 3, and activation of apoptosis (Seo, H. S., et al, 2014).
The systematic review discusses the most recent data on mTOR expression variationsreported in a variety of illnesses, including human cancers, and systematized our current understanding of mTOR regulation at the transcriptional and translational levels. It waspossible to establish that the identification of mTOR-associated proteins has increased the relevance of mTOR, which when coupled to different proteins, forms complexes with a wide range of physiological functions. These findings not only increase the range of mTOR actionsin cells but also weaken the regulatory network. (Mishra, S., et al, 2021), that the mTOR signaling is essential under a range of physiological circumstances associated with reduced global protein synthesis, such as hypoxia and mitosis. (Ramirez-Valle et al., 2010). mTOR protein levels themselves remain constant in those conditions (Ramirez-Valle et al., 2010). The fact that mTOR translation is independent of cap and of initiation factors that are generally blocked by the cell's control mechanisms (Marques-Ramos et al., 2017) may explainhow mTOR can be activated in a variety of physiological settings strongly associated with protein synthesis reduction, as well as how mTOR evades normal translational checkpoints and is over-expressed in a variety of diseases. We were also able to show how various miRNAs impact mTOR signaling in pathological situations through recent studies that revealed interactions between miRNAs and the mTOR pathway during the development of cancer which appear to fine-tune numerous cellular processes and to contribute significantly to cancer activity. Furthermore, interactions between miRNAs and mTOR signaling have beendiscovered in most cancers as well as in a variety of diseases and physiological states. (Zhang,Y., et al, 2017).
As for the experimental part of the work, it is based on the fact that numerous *in vitro* and*in vivo* research have indicated that apigenin increases apoptosis by inactivating Akt (Budhraja,A., et al 2012; Cheong, J. W., et al, 2010). Apigenin has also been shown to inhibit ovarian tumor metastasis by down-regulating MMP-9, which is mediated by Akt signaling (He,J., et al,2012), to inhibit breast cancer metastasis by blocking the PI3K/Akt pathway (Lee, W.J., et al, 2008), and to inhibit cancer angiogenesis by suppressing HIF-1 and VEGF expression, both ofwhich are related to Akt inhibition (Mirzoeva, S., et al, 2008; Liu, L. Z., et al, 2005). However, in comparison to the numerous articles revealing apigenin's suppression of Akt activity, there are just a few papers demonstrating apigenin's inhibition of mTOR activity. True the experiments preformed, it was possible to confirm that apigenin clearly suppresses cell growth in gastric carcinoma GP202 cell lines. The results obtained corroborate earlier findings

that apigenin has anti-cancer potential with a significant impact on cell proliferation as well as in cell morphology which showed phenotypical alterations, such as changes in cell and nucleus format and the presence of atypical characteristics associated with the apoptotic process. It was possible to conclude that incubation of cells with 10 μM of apigenin for 48h had a better outcome, which indicates a time-dose relation regarding apigenin action. Due to the fact that at the 20 μM assays the quantity of cells per well was too low , it became harder to observe many morphological changes, however, the 20 μM also presented good results when compared to control in both 24h and 48h period.
It will be interesting to continue this project by performing additional experimental steps, such as a fluorescent microscopy examination to further investigate the morphological alterations in the nucleus, flow cytometry investigation of apigenin-induced apoptosis and cell cycle alterations and to analyse Bcl-2, Bax and caspase-3 mRNA expression through quantitative real- time fluorescence polymerase chain reaction (Q-RT-PCR) method.
Additionally, it would also be interesting to preform Western Blot of the proteins involved in mTOR signalling pathway, like mTOR, p-4EBP1 (as a target of the mTORC1 complex) and p-AKT (as a target of the mTORC2 complex) to assess the effects of apigenin against mTOR, and mRNA quantification to confirm that only translation is being altered and that there is no interference of the mRNA in either its production or its stability, which should help to construct a more solid conclusion of the action of apigenin in cancer, specifically whether it is related to the signaling pathway in gastric cancer cell lines.
Although the future is unknown, we can say that significant progress is being made toward identifying more targeted types of cancer therapy, trying new approaches, and choosing to combine previously established therapies with new discoveries. Apigenin is a promising cancer inhibitor with low toxicity and no mutagenic effect that might unlock new possibilities for cancer treatment in humans and allow to reduce the negative impact of current treatments as well as their limitations, in addition to significantly impacting the mortality rates associated with neoplasia and having a direct impact on cancer patients' quality of life. If we can establish the relationship between apigenin's effect on gastric cancer cells and the mTOR signaling pathway, we may be close to taking a major step forward in cancer therapeutics.

These are the reasons why I feel that research is a source of optimism for the future.

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I – APPENDIX

Table S 2.1 - Characteristics of studies included in the systematic review for mtor expression in association with condition

Condition	mTOR expression	Ref.
Brain		
Alzheimer	β-amyloid increased the expression of mTOR and p-mTOR (at Ser2448)	Liu, Y. C., et al (2017)
Vascular dementia	mTOR protein levels were decreased at 21- and 28-days after chronic cerebral hypoperfusion (CCH) in the hippocampal CA1 region. Levels of p-mTOR protein were significantly and transiently increased at 7 days after permanent bilateral common carotid arteries occlusion surgery.	Park, J. A., & Lee, C. H. (2017)
Lung		
Idiopathic pulmonary fibrosis	The expression of mTOR correlated with the fibrosis score and lung function decline.	Park, J. S., et al (2014)
Skin		
Dermatitis	mTOR expression was significantly increased in psoriasis, allergic contact dermatitis and atopic dermatitis.	Balato, A., et al (2014)
Acne	mTOR gene expression was increased in the skin of acne patients (either in involved or non-involved skin)	Monfrecola, G., et al (2016)
Pathological scar	Increased mTOR expression in pathological scar fibroblasts.	Tang, Z. M., et al (2017)
Gastrointestinal Tract		
Diabetic nephropathy	Elevated mTOR mRNA levels	Gödel, M., et al (2011)
Lupus Erythematosus	Increase in mTOR protein levels in liver samples from a murine model of systemic lupus erythematosus, despite a reduction in mRNA levels.	Vilà, L., et al (2012)
Blood		
Blood	mTOR expression in peripheral blood of patients with osteoarthritic vary from high to low, in which high levels are associated with increased incidence of synovitis.	Tchetina, E. V., et al (2013)
	In cord blood cells, the presence of IL2, IL7 and IL15 altogether resulted in an increase of mTOR expression at day 14, and a decrease at day 21. The reduction of mTOR expression was observed when cells were treated with IL2 or IL15 alone but not IL7.	Mohammadian, A., et al (2017)

Cancer	mTOR expression	Ref.
Lung	Meta-analysis demonstrated that there is no association between mTOR and p-mTOR expression and the prognosis of non-small cell lung cancer (NSCLC).	Li, L., et al (2015)
	In EGFR-mutant NSCLC samples mTOR expression was: low or intermediate in 62.5% of the cases and high 37.5%. The group with high mTOR and BIM expression had shorter overall and progression-free survival to erlotinib.	Karachaliou, N., et al (2015)
	Expression of p-Akt and p-mTOR was higher in typical carcinoid tumor (TC) and atypical carcinoid tumor (AC) than large-cell neuroendocrine carcinoma (LCNEC) and small-cell lung carcinoma (SCLC). Furthermore, an association between p-mTOR expression and tumor size was observed in SCLCs and LCNECs.	Alì, G., et al (2011)
GASTRIC	High p-mTOR expression associated with adverse clinicopathologic parameters, namely poorer survival. Combination of p-mTOR and TSC1 status provided more strong survival information than each alone.	Byeon, S. J., et al (2014)
	Higher expression of mTOR and p-mTOR in the tumor center compared to the invasive front.	Bornschein, J., et al (2015)
	Immunohistochemistry of paraffin-embedded sections from gastric cancer cases reveled that mTOR expression was present in 51.5% (17/33) of the samples, in opposition to the low/absent expression in normal tissues. A positive correlation was observed between mTOR expression and tumor differentiation, lymph node metastasis and clinical staging. No correlation was observed with gender, age and invasive depth.	Li, M., et al (2012)
	Cytoplasmic p-mTOR expression was associated with tumor progression and poor survival in opposition to nuclear p-mTOR expression.	Murayama, T., et al (2019)
	Immunohistochemistry of gastric neuroendocrine tumours demonstrated p-mTOR and p4E-BP1 expression in 88.57% of cases.	Lohneis, P., et al (2014)
	Gastroenteropancreatic neuroendocrine tumours presented high levels of mTOR, 4EBP1, p-4EBP1, p-S6K and p-eIF4E. Both expression and activity of mTOR were higher in foregut than in midgut tumours. In foregut tumours, expression of mTOR was higher when distant metastases were present. Activity of mTOR was correlated with higher proliferative capacity.	Kasajima, A., et al (2011)

Bladder	mTOR protein levels were elevated in tumours of urothelial patients that did not respond to neoadjuvant chemotherapy and decreased in complete responders.	Winters, B. R., et al (2018)
Pancreatic	High expression of PTEN seemed to prolong survival duration. Patients with positive p-mTOR expression seemed to have shorter survival duration.	Han, X., et al (2013)
Esophageal	p-mTOR immunostaining ranged from 1 to 165, with a median of 80. An H-score of 80 or more was considered high p-mTOR expression (n = 39), whereas an H-score of 79 or lower was considered low expression (n = 36). Expression of p-mTOR had no significant influence on patient survival. High p-mTOR expression was strongly associated with high tumor grade (grade 3; P = 0.0014).	Chuang, W. Y., et al (2015)
	In advanced Esophageal Squamous Cell Carcinoma tumors PI3K/Akt/mTOR signaling hyperactivation is accompanied with overexpression of mTOR. High expression of mTOR and other mTOR signaling components were closely related to the presence of lymph nodes metastases and advanced TNM stage. Overexpression of mTOR was proved to be an independent adverse prognostic factor for overall survival.	Wu, N., et al (2018)
Breast Cancer	Overexpression levels of pmTOR was detected in 100% of true interval cancers, but only in 25 (31.6%) of screen-detected cancers (P < 0.001).	Rojo, F., et al (2014)
	Luminal breast cancer (smaller, lower grade tumors) - 43.8% positive for pmTOR	Beca, F., et al (2014)
	A total of 104 (47.7%) tumors were positively stained for p-mTOR. The protein expression could be detected in the cytoplasm (38.1%, 83/218), the nuclear (7.8%, 17/218) and perinuclear (1.8%, 4/218) areas respectively. p-mTOR was more frequent in patients with lymph node metastasis Patients with PIK3CA mutations or p-mTOR expression had significantly shorter overall survival	Wang, J., et al (2017)
	A significantly higher number of breast cancer tissues were found to express the mTOR protein.	A Mutee, et al (2009)
Prostate	Expression of PTEN decreased and mTOR signaling pathway markers increased in PIN and in cancer as compared to normal cells in most	Kremer, C. L., et al (2006)

	samples. Overexpression of 4EBP1 and p-4E-BP1 was observed in PIN and cancer.	
	p-mTOR expression was found to increase across the progression model with mean staining in non-neoplastic samples of 40 compared to 98 in PIA, 107 in HGPIN, and 136 in cancer (P<0.001), but without significant increase between HGPIN and PIA. Correlation of high p-mTOR expression with outcome in PCa showed a trend towards worse prognosis, but this was not statistically significant.	Sutherland, S. I., et al (2014)
Leukemia	mTOR expression was found to be more frequent at ALL relapses than at the first diagnosis of the disease (7/10 vs. 8/31).	Ulińska, E., et al (2016)
	Significant high level of mTOR expression was seen in non-responders compared to responders of ALL (14.2 vs. 2.4, p < 0.001). It was also observed that non-responder group of both B-ALL and T-ALL had significant higher expression than responders (10.3 vs. 2.6, p < 0.01 and 19.0 vs. 2.1, p < 0.001).	Khanna, A., et al (2018)
Myeloma	Downregulation of mTOR was associated with hypermethylation of its promoter following treatment with curcumin, which may occur through regulating the expression of DNMT3.	Chen, J., et al (2019)
	On univariate analysis, high mTOR and p-mTOR were associated with male gender (75% versus 30.4%, p=0.04).	Stockwin, W., et al (2016)
Ovarian	Quantitative RT-PCR revealed that DEPTOR, RICTOR, RAPTOR, and mTOR are differentially expressed in paclitaxel-sensitive and -resistant (TaxR) ovarian cancer cell lines (n=3). There was an up-regulation of DEPTOR (2.4-fold), RICTOR (1.5-fold) and mTOR (1.2-fold) in PEO1TaxR ovarian cancer cells when compared with paclitaxel-sensitive PEO1 cells. A down-regulation (0.6- fold) was detected for RAPTOR (Figure 1A). With regards to SKOV-3, a significant up-regulation of DEPTOR (1.8- fold) and down-regulation of RAPTOR (0.6-fold), RICTOR (0.7-fold) and mTOR (0.6-fold) was found in SKOV-3TaxR cells when compared with parental SKOV-3 cells	Foster, H., et al (2010)
	Upregulation of DEPTOR constitutes a prognostic marker in ovarian cancer and is observed in response to mTOR pathway inhibition.	Rogers-Broadway, K. R., et al (2019)
Liver	Hepatocellular carcinoma HepG2 cells - over-expression of mTOR	Yang, Z., et al (2014)
	mTOR pathway is over-expressed in patients with multinodular HCC and is it associated with increased post-LT tumour recurrence rates.	Guerrero, M., e al. (2019)

Sacral chordoma	Expression of mTOR showed mainly in the cytoplasm of tumor cells. The positive expression of mTOR in the sacral chordoma was 62.5 % (25/40), significantly higher than that in the normal tissues (P = 0.030). The overall mean expression scores for PTEN and mTOR staining in sacral chordoma were 1.23 and 3.62, respectively. Positive expression of mTOR appears to correlate with negative expression of PTEN in sacral chordoma tissues (P = 0.021).	Chen, K., et al. (2014)
Brain	Astrocytoma (grade III) - Mtor 70% Glioblastoma (grade IV) - Mtor 81.8% Oligodendroglioma (grade III) - Mtor 20%	Annovazzi, L., et al. (2009)
	atypical primary meningiomas (grades I, II and III) - Cytoplasmic p-mTOR immuno-expression was seen in 39/48 (81%) atypical meningiomas	Barresi, V., et al. (2019)
Laryngeal carcinoma	The expression of mTOR in LSCCs ranged from 0.0% (in two cases) to 80.2%. The mean mTOR expression in patients with and without recurrent LSCC was 27.1% ± 25.2% and 14.2% ± 16.5%, respectively. The locoregional recurrence rate was significantly higher among LSCC patients whose mTOR expression was >35.3% (Fisher's exact test, p = 0.003). The DFS was also significantly shorter in cases of LSCC whose mTOR expression was >35.3% (log-rank test, p = 0.013).	Marioni, G., et al. (2012)
Testicular seminoma	There is an interaction between mTOR signalling pathways and testicular germ cell seminoma.	Yaba, A., et al. (2016)
Colorectal adenomas	Nuclear P38 correlated to low-grade dysplasia (Kendall P<0.01/tau=-0.254) and to decreased adenoma size (P<0.01/tau=-0.267). Nuclear P38 also correlated to cytoplasmic or membrane mTOR (P<0.01/tau=-0.223 and P<0.01/tau=-0.340) and to cytoplasmic CD133 (P<0.01/0.293). An inverse relationship was observed to Ki67 (P<0.00/ tau=-0.110).	Hanna, J. W., et al. (2014)
Gallbladder Adenocarcinoma	Immunostaining for phospho-mTOR was positive in 82 of 128 tumors (64.1%) and in 24% of chronic cholecystitis cases (16% nonmetaplasia and 32% with metaplasia) (P, .001). Survival analysis indicated that a high phospho-mTOR immunohistochemical expression was associated with poorer prognosis in patients with advanced GBC (P ¼ .02).	Leal, P. et al. (2013)

JEG-3 and BeWo human placental choriocarcinoma cell lines	RT-PCR analysis revealed that BeWo and JEG-3 cells express mTOR, 4EBP and the S6 kinase isoforms ·1, ·2, ß1 and ß2 at mRNA level. PCR analyses for the above genes produced PCR products of 537 bp for mTOR, 299 bp for 4EBP, and 585 bp, 480 bp, 468 bp, 336 bp for the ·1, ·2, ß1 and ß2 S6K isoforms	Mparmpakas, D., et al. (2010)
Desmoplastic Small Round Cell Tumor (EWS/WT1), Ewing's Sarcoma (EWS/FLI1) and Wilms' Tumor (WT1)	Overexpression of p-mTOR in desmoplastic small round cell tumors, Ewing's sarcoma and Wilm's tumor, which resulted in constitutive activation of p-p70S6K.	Subbiah, V. et al. (2013)
Phosphorylated mTOR Expression Profiles in Human Normal and Carcinoma Tissues	Expression of p-mTOR in adult and fetal normal tissues: intestinal crypt, intrahepatic bile ductile, pancreatic duct, distal nephron of the kidney, umbrella cell of urothelium, mesothelial cell, and choroid plexus in normal tissues. Expression of p-mTOR in cancer tissues: higher in adenocarcinoma than in other types of cancers; in metastatic cancer than in primary cancer; and in the forefront of the infiltrating cancer cells. mTOR activation was associated with cancer cell invasion and migration in solid tumors.	Lee H. (2017)

II– APPENDIX

Table S 2.2 - Characteristics of studies included in the systematic review for miRNA associated with effect on mTOR in association with pathology

Pathology	miRNA	Effect on mTOR	Biological effects	Ref.
Acute Kidney Injury	miR-199a-3p	Decreased expression and direct target	–	Yang, A., et al 2019
Acute lymphoblastic leukaemias	miR-99a e miR-100	Decreased expression and direct link	Inhibition of proliferation and increase of apoptosis	Li, X. J., et al 2013
acute respiratory distress syndrome human	miR-7-5p	Decreased expression and direct link	–	Qin, K., et al 2016
Adrenocortical tumours	miR-99a and miR-100	Decreased expression and direct link	–	Doghma, M., et al 2010
Aging	miR-496	Decreased expression and direct link	–	Rubie, C., et al, 2016
	miR-99b-5p	No effect on MTOR expression or mTOR protein but is a direct target of miR-99b-5p	Decreased protein synthesis	Zacharewicz, E., et al, 2020
Alcoholic liver disease	miR-155	Decreased expression and direct link	–	Babuta, M., et al, 2019
Anaplasic thyroid cancer	miR-99a	Decreased expression and direct link	Decreased viability; increased apoptosis; decreased proliferation	Huang, H. G., et al, 2015
Benign prostatic hyperplasia	miR-96-5p	Decrease of mRNA	–	Zhang, N., et al, 2018

Bladder cancer	miR-100	Decreased expression and direct link	Inhibits cell proliferation and motility ; Cell cycle arrest ; Inhibits tumorigenesis	Xu, C., et al 2013
	miR-99a-5p	Decreased expression and direct link	Decreased cell proliferation and cell cycle	Liu, Y, et al, 2019
	miR-99a-5p	Decreased expression	Increased effects of BITC	Lin, J. F., et al 2019
Breast cancer	miR-96	Decreased expression and direct link	_	Razaviyan, J., et al, 2018
	miR-100	Decreased expression and direct link	Overexpression of miR-100 increased the effect of paclitaxel on cell cycle arrest, multinucleation and apoptosis.	Zhang, B., et al, 2016
	miR-99a	Decreased expression (protein and mRNA) and direct binding	Suppression of self-renewal and sphere formation. Decreased tumourigenecity in vivo. Decreased invasion and migration.	Yang, Z., et al, 2014
	miR-99a	Decreased expression and direct link	_	Song, Y., et al 2014
	miR-99a	Decreased expression and direct link	Decreased proliferation and increased apoptosis	Hu, Y., et al, 2014
Brown adipocyte differentiation	miR-199a-3p	Decreased expression (mRNA and protein level) and direct binding	_	Gao, Y., et al, 2018
Carcinoma hepatocelular	miR-100	Decreased expression and direct link	Increased autophagy ; Increased apoptosis	Ge, Y. Y., et al, 2014

	miR-199a	Decreased expression and direct target	Reduced cell proliferation, invasion and migration ; AMSC-Exo-199a significantly sensitized HCC cells to doxorubicin	Lou, G., et al, 2020
	miR-199a-3p	Decreased expression and direct link	Increased apoptosis ; Increased sensitivity to dexorubicin ; Decreased invasion ; Cell cycle inhibition	Fornari, F., et al, 2010
	miR-497 ; miR-99a	Decreased expression and direct link	Inhibits proliferation;	Cheng, H., et al, 2017
Cardiac hypertrophy	miR-99a	Decreased expression and direct link (non verified)	Proliferation: Decreased; Apoptosis: Decreased	Li, Q., et al, 2016
cardiovascular disease endothelial and vascular smooth muscle cells	miR-100	Decreased expression and direct link (non verified)	Inhibition of neovascularization on vitro and in vivo. No change in viability.	Grundmann, S., et al, 2011
Cervical cancer	miR-634	Decreased expression and direct link	Decreased migration and invasion. Decreased proliferation.	Cong J., et al, 2016
	miR-99a ; miR -99b	Decreased expression and direct link	Decreased proliferation and invasion	Wang, L., et al, 2014
Cholangocarcinoma	MiR-199a-3p	Decreased expression and direct link	Increased sensitivity to cisplatin	Li, Q., et al, 2017
Chondrosarcoma	miR-100	Decreased expression and direct link	Increased sensitivity to cisplatin	Zhu, Z., et al, 2014
Chronic cerebral hypoperfusion	miR-96	Decreased expression and direct link	_	Liu, P., et al, 2018

Colorectal cancer	miR-99b-5p	Decreased expression and direct link	Decreased migration, did not affect proliferation	Li, W., et al, 2015.
	miR-100	Decreased expression and direct link	Invasion: decreased Migration: decreased MMP: decreased activity	Jiang, Y., et al, 2017
	miR-144	Decreased expression and direct link (Reduction of miR-144 expression by Anti-miR-144 transfection induced an elevation in mTOR mRNA expression)	Increased sensitivity to rapamycin; Decreased proliferation	Iwaya, T., et al, 2012
	miR-338-3p	Decreased expression and direct link	Increased resistance to 5-fluoracil	Han, J., et al, 2017
c-Src-mediated tumor growth	miR-99a	Decreased expression and direct link	Decrease in tumour growth	Oneyama, C., et al, 2011
Dermal wound healing	miR-99	Decreased expression and direct link (non verified)	_	Jin, Y., et al, 2013
Diabetes	miR-99a	Decreased expression and direct link	Decreased insulin-induced glucose consumption and lactate production	Li, W., et al, 2013
Endometrial cancer	miR-199a-3p	Decreased mTOR protein levels and direct binding (miR-199A-3p transfection did not result in a significant difference in mTOR mRNA expression levels (data not shown), but a significant reduction in mTOR protein expression was observed)	Decreases proliferation	Wu, D., et al, 2013
Esophageal carcinoma	mR-99a/100	Decreased expression and direct link	Decreased proliferation, increased apoptosis	Sun, J., et al, 2013

	miR-100	Decreased expression and direct link	Invasion and migration: decreased. No changes in apoptosis and proliferation in ESCC.	Zhang, N., et al, 2014
	miR-100	Decreased expression and direct target	Hsa_circ_0006168 can "absorb" miR-100 and increase mTOR expression to facilitate ESCC proliferation, migration and invasion. (RNAcirc by "absorbing" miRNA-100 consequently leads to increased expression of mTOR and thus increase proliferation, migration and invasion)	Shi, Y., et al, 2019
Estrogen mediation	miR-199a-3p	Decreased expression (mRNA and protein level) and direct binding	Increased autophagy	Fu, J, et al, 2018
Fibrosarcoma	miR-520c ; miR-373	Blocking of mRNA translation	_	Liu, P. and Wilson, M.J., 2012
Gastric cancer	miR-101-2	Decreased expression and direct link	Decreased cell viability ,colony formation ,migration and cell invasion and increased cell death.	Riquelme, I., et al, 2016
	miR-199a-3p	Decreased expression and direct link	Decreases proliferation	Peng, W., et al, 2013
	miR-224	Increased expression and direct binding (mTOR mRNA expressions were higher by miR-224 mimics)	Promotes proliferation, migration and invasion ; decreases apoptosis	Zhang, Y., et al, 2016

Glioblastoma	miR-548x ; miR-4698	Decreased expression and direct target	Decreased viability; decreased proliferation; decreased cell growth. No effect on apoptosis.	kalhori, M.R., et al, 2020
	miR-579	Decreased expression and direct target	–	Kalhori, M. R., et al, 2019
Glioma	miR-1229-3p	Decreased expression and direct link	Increased apoptosis; decreased proliferation; decreased invasion; decreased migration.	Cao, Q., et al, 2019
	miR-128	Decreased expression and direct link a mRNA	–	Chen, P. H., et al 2016
	miR-193a-5p	Decreased expression and direct link	Decreases TMZ-induced autophagy which consequently makes cells sensitive to TMZ cytotoxicity	Jiang, C., et al 2018
	miR-199a-3p	Decreased expression and direct link	Decreases cell proliferation but has no effect on invasiveness or apoptosis	Shen, L., et al, 2015
	miR-15a/16	Decreased expression and direct link	In CD8 cells deficiency of this miRNA led to increased activation, proliferation and cytotoxicity	Yang, J., et al, 2017
HCV	miR-99a	Decreased expression and direct target	Decreased viral replication;	Lee, E. B., et al, 2020
Head and neck carcinoma	miR- 27a*	Decreased expression and direct target	Decreased cellular vibility	Wu, X., et al 2013
	miR-99 e 100	Decreased expression and direct link (non verified)	Decreased proliferation; increased apoptosis.	Chen, Z., et al, 2012
	miR-27a	Decreased expression and direct link	Decreased viability. Increased apoptosis.	Wu, X., et al, 2013

Hepatic fibrosis	miR-101		Inhibition of proliferation; decreased survival;	Lei, Y., et al, 2019
Hepatocellular Carcinoma	MiR-758-3p	Decreased expression and direct link	Decreased migration and invasion. Decreased proliferation.	Jiang, D., et al, 2017
Hepatocellular Carcinoma	miR-99a	Decreased expression and direct link	Decreased proliferation. Does not influence apoptosis or metastasis	Li, D., et al, 2011
Hypertrophic heart disease	miR-96	Decreased expression and direct link	Increased hypertrophy	Sun, X., & Zhang, C., 2015
Influenza A infection	miR-101	Decreased expression and direct link	Decreased viral protein replication and translation	Sharma, S., et al, 2020
Intracerebral haemorrhage	miR-144	Decreased expression and direct link	Decreased autophagy and increased inflammatory response	Wang, Z., et al, 2017
	miR-144	Decreased expression and direct link (ref. Work)	Increased autophagic activity and inflammation	Yu, A., et al, 2017
LPS-induced endothelial cell inflammation	miR-99a	Decreased expression and direct link (non verified)	Decrease in inflammation factors	Bao, M. H., et al, 2016
Lung Adenocarcinoma	miR-33a-5p	Decreased expression and direct link	Inhibits proliferation ; increases sensitivity to celastrol	Li, Y. J., et al, 2018
	miR-99a	Decreased expression and direct link	Decreases proliferation and increases apoptosis	Gu, W., et al, 2013
Lung carcinoma	miR-497-5p	Decreased expression (protein mTOR) and direct target	Increased sensitivity to cisplatin	Hou, Z., et al, 2021
	miR-100-5p	Decreased expression and direct link	Increased sensitivity to cisplatin	Qin, X., et al, 2017

	miR-193a-5p and -3p	Decreased expression (protein and mRNA) and direct binding	Decreased proliferation; decreased invasion; decreased migration. Reversal of epithelial-mesenchymal conversion. Decreased metastases (in vivo)	Yu, T., et al, 2015
Lupus	miR-183	Decreased expression and direct link	Increased survival of sick mice.	Li, X., et al, 2019
Myoblast differentiation	miR 199a-3p	Decreased expression and direct link	With miR-100 decrease - Proliferation: increases Migration: increases Invasion: increases	Jia, L., et al, 2013
Nasopharyngeal carcinoma	miR-3188	Decreased expression and direct link	Decreases G1/S transition in cell cycle and proliferation; Increases sensitization to 5-FU	Zhao, M., et al, 2016
	MiR-646	Decreased expression and direct target	_	Song, Y. L., et al, 2019
	miR-99a	Decreased expression and direct target	Decreased proliferation	Wu, S. H., et al, 2019
Neuropathic pain	miR-183	Decreased expression and direct link a mRNA	_	Xie, X., et al, 2017
non-small cell lung cancer (NSCLC)	miR-99a	Decreased expression and direct link	Increased sensitivity to radiation, increasing apoptosis and decreasing proliferation.	Yin, H., et al, 2018
Obesity and type II diabetes	miR-100	Decreased expression and direct link	Reduced differentiation of adipocytes. No change in viability.	Pek, S. L. T., et al, 2016

Oral lichen planus	miR-199	Decreased expression and direct link	_	Wang, L., et al, 2019
Osteoarthritis	miR-100-5p	Decreased expression and direct link	_	Wu, J., et al, 2019
Osteosarcoma	miR-101	Decreased expression and direct link	Inhibition of proliferation ; Increased apoptosis	Lin, S., et al, 2014
	miR-199a-3p	Decreased expression and direct link (non verified)	Decreased migration and proliferation.	Duan, Z., et al, 2011
Ovarian cancer	miR-100	Decreased mTOR protein levels	Proliferation: Inhibit ; Apoptosis : Increases ; Cell cycle: stops ; Increased sensitivity to cisplatin	Guo, P., et al, 2016
	miR-1271	Decreased expression and direct target	With miR-100 decrease - Proliferation: increases Migration: increases Invasion: increases	Chen, T., et al, 2019
Pancreatic cancer	miR-99a	Decreased expression (protein and mRNA) and direct binding	With miRNA inhibition: increased proliferation; increased migration; increased invasion.	Li, D., et al, 2014
	miR-99b	Decreased expression and direct link	Decreased proliferation and radiation resistance	Wei, F. et al, 2013
Prostate cancer	miR-1271-5p	Decreased expression and direct target	Decreased proliferation; decreased invasion; decreased migration.	Shi, J., et al, 2020
	miR-144	Decreased expression and direct link	_	Liu, J., et al, 2016

	miR-495	Decreased expression and direct link	Decreases proliferation, migration and invasion	Li, J. Z., et al, 2016
	miR-96	Decreased expression (protein and mRNA) and direct binding	COMPLEX - Increased miR-96 may increase autophagy, but its ectopic increase, above a certain value may decrease autophagy	Ma, Y., et al, 2014
Pulmonary hypertension	miR-100	Decreased expression and direct link	Suppressed proliferation	Wang, A. P., et al, 2015
Renal carcinoma	miR-99a	Decreased expression and direct link	Decreased migration, invasion and proliferation	Cui, L., et al, 2012
	miR-144	Decreased expression and direct link	Decreased proliferation	Xiang, C., et al, 2016
Rheumatoid arthritis	miR-7	Decreased expression	_	Tang, X., et al, 2019
	miR-498	Decreased expression (mRNA and protein level) and direct binding	_	Li, G., et al, 2019
Salivary adenoid carcinoma	miR-144-3p	Decreased expression and direct link	Inhibition of proliferation and induction of apoptosis	Huo, F., et al, 2016
SMN deficiency	miR-183	Decreased expression and direct link	_	Kye, M. J., et al, 2014
Spinal cord injury	miR-99b-5p	Decreased expression and direct link	Inhibition of miRNA led to decreased apoptosis and increased proliferation	Cao, F., et al, 2017
	miR-421-3p	Decreased expression and direct link	Increased autophagy	Wang, J., et al, 2020

	miR-199a-3p	Decreased expression and direct link (non verified)	–	Liu, G., et al, 2012
T-cell differentiation	miR-99a ; miR-150	Decreased expression and direct link	Stimulates differentiation of Trag. cells	Warth, S. C., et al, 2015
Tumours of the oral cavity	miR-99a	Decreased expression and direct link	Decreased proliferation and increased apoptosis	Yan, B., et al, 2012
Type 2 diabetes mellitus (T2DM)	miR-99a	Decreased expression and direct link	Decreased insulin-induced proliferation and migration	Zhang, Z. W., et al, 2017
Vascular disease	miR-761	Decreased protein expression and direct binding (does not degrade mRNA)	–	Cho, J. R., et al, 2015
Vascular endotelial cells	miR-101	Decreased expression and direct link	Decreased vascular proliferation	Chen, K., et al, 2012
well-differentiated hepatocyte-derived carcinoma and poorly differentiated primary gastric mucinous adenocarcinoma cell lines	miR-129-3p	Decreased expression and direct target	Increased autophagy	**Sun, W., et al, 2019**.