FACULTAD DE MEDICINA
UNIVERSIDAD DE CANTABRIA

GRADO EN MEDICINA

TRABAJO FIN DE GRADO

La melatonina como inhibidor de la transición epitelio-mesénquima en el cáncer

Melatonin as inhibitor in the epithelial-mesenchymal transition in cancer

Autor/a: Olivia J. Cobos Torres

Director/es: D. Carlos M. Martínez Campa

Santander, Junio 2022

INDEX

ACKNOWLEDGMENTS

A mamá, a papá y a mi hermana, por su infinita paciencia y cariño y por ser mi ejemplo a seguir. Sin ellos estos seis años no hubieran sido posibles.

A mis cinco, que han hecho de esta ciudad un hogar y que sin duda son lo más bonito que me llevaré nunca.

A mi mejor amigo y compañero de vida, por su apoyo incondicional y por creer siempre ciegamente en mí.

Y por último, a mi tutor, D. Carlos Martínez Campa, por su tiempo, implicación y consejos.

ABBREVIATIONS

5-HTP: 5-Hydroxytryptophan
AANAT: Aralkylamine N-Acetyltransferase
APC: Adenomatous Polyposis Coli
CAF: Cancer Associated Fibroblast
CREB: cAMP Response Element Binding Protein
DLL4: Delta-Like Ligand 4
ECE1: Endothelin-Converting Enzyme 1
EMT: Epithelial-Mesenchymal Transition
FSP1: Fibroblastic Specific Protein 1
GATA3: GATA Binding Protein 3
GSK-3β: Glycogen Synthase Kinase 3 Beta
HIF-1α: Hypoxia Inducible Factor 1 Alpha
HIOMT: Hydroxyindole-O-Methyltransferase
hTERT: Human Telomerase Reverse Transcriptase
LAP: Latency Associated Peptide
LEF: Lymphoid Enhancement Factor
MET: Mesenchymal-Epithelial Transition
MMP: Matrix Metalloproteinases
NAS: N-Acetyl Serotonin
NF-κB: Nuclear Factor Kappa B
OCT4: Octamer-Binding Transcription Factor 4
PARP1: Poly ADP-Ribose Polymerase 1
PCBP1: Poly (rC)-Binding Protein
ROR: Retinoic acid-related Orphan Receptor
ROS: Reactive Oxygen Species
SCN: Suprachiasmatic Nucleus
TAM: Tumor Associated Macrophage
TGF-β: Transforming Growth Factor Beta
TNF-α: Tumor Necrosis Factor Alpha
TPA: 12-O-Tetradecanoylphorbol-13-Acetate
TPH: Trypthophan Hydroxylase
VEGF: Vascular Endothelial Growth Factor
ZEB1: Zinc Finger E-Box Binding Homeobox 1

ABSTRACT

Cancer is a worldwide problem that affects the whole population, as not only will one in three people suffer from the condition at least once in their lifetime, but also costs associated with the disease are extremely high. Throughout the years, many investigations have focused on finding a cure for different cancer types, targeting diverse stages of tumor development. One of them is the epithelial-mesenchymal transition (EMT), recognition of which helps reach that objective. This transition occurs during carcinogenesis and involves a shift from an epithelial cell to a mesenchymal cell, with the changes it entails in mobility, conformation, functionality and polarity. On the other hand, melatonin is an endogenous hormone mainly synthesized in the pineal gland in the brain. This hormone is a fundamental regulator of the sleep-wake cycle, as well as being anti-inflammatory and having multiple other functions. Not until recently, studies have also started to define melatonin as an anti-oncogenic substance. Therefore, both the EMT and melatonin play important roles regulating carcinogenesis, being the reason as to why their interplay seems to be of great importance. The multiple ways in which melatonin is capable of inhibiting the EMT, based on results obtained from observational and experimental studies, will be discussed in this systematic review.

RESUMEN

El cáncer es un problema mundial que afecta a toda la población, ya que no solo lo padecerá una de cada tres personas al menos una vez en su vida, sino que también los costes que la enfermedad supone son extremadamente altos. A lo largo de los años, numerosas investigaciones se han centrado en formas de combatir diferentes tipos de cáncer tratando la enfermedad a diferentes niveles en su progresión, y el descubrimiento de la transición epitelio-mesénquima (EMT) contribuye a alcanzar esa meta. Esta transición sucede durante la carcinogénesis y consiste en el paso de una célula epitelial hacia una célula mesenquimal, con los cambios que supone de movilidad, conformación, funcionalidad y polaridad. Por otro lado, la melatonina es una hormona endógena sintetizada principalmente en la glándula pineal en el cerebro. La función principal de la hormona es la sincronización de los ritmos circadianos (ciclo sueño-vigilia), además de ser antiinflamatoria y participar en muchos otros procesos. Recientemente también se le han otorgado multitud de funciones anti-oncogénicas. Por consiguiente, tanto la EMT, como su modulación por la melatonina, juegan un papel importante en la regulación de la carcinogénesis y es por ello por lo que la relación entre ambas resulta de gran importancia. Se expondrán las numerosas formas en las que la melatonina es capaz de inhibir la EMT, basándonos en resultados obtenidos a partir de estudios observacionales y experimentales.

KEYWORDS

Melatonin; EMT; Twist; Snail; ZEB; carcinogenesis; TGF-β; Wnt; β-catenin; Notch; Hedgehog; micro-RNAs

OBJECTIVES AND METHODOLOGY

Objectives

The aim of this review was to gather results of studies that associated melatonin with the epithelial-mesenchymal transition. Along the document, carcinogenesis will be defined as a three stepped theory. Moreover, the epithelial-mesenchymal transition will be dissected and the many properties of melatonin introduced. Lastly, we will establish the multiple ways in which melatonin inhibits the transition in a variety of cancer types. As a concluding section of this review, future perspectives as to what expect will be outlined.

Methodology

The methodology followed was an exhaustive and actualized review of studies published concerning melatonin as an anti-neoplastic hormone and an adjuvant in cancer treatment, in association with the epithelial-mesenchymal transition. Using the keywords previously mentioned, different databases, such as PubMed and Medline were used to research the latest studies. Actualized books and journals like Nature, The Cell and International Journal of Molecular Sciences were also consulted.

1. INTRODUCTION

Melatonin is a hormone synthesized at night by the pineal gland, which is below the corpus callosum and posterior to the third ventricle. As far as it is known, the pineal gland was first described with its current name in two texts written by Galen in the 2nd century (1). Although its existence was acknowledged thousands of years ago, it wasn't until the 20th century that its actual role was beginning to be understood. This understanding was initiated with Aaron B. Lerner who, searching for a cure for vitiligo, suspected something in the pineal gland was causing amphibians skin to change. Lerner and his colleagues were able to isolate a serotonin derivative (which was later named melatonin) from bovine pineal tissue (2).

Therefore, Descartes' early idea of the pineal gland being the "seat of the soul" (1), drastically changed over the years. It was now seen as an actual endocrine organ with its own function and secretion, melatonin being its main secreted product.

It has been discovered that, in primitive mammals, melatonin's main role lies within the circadian regulation of certain parameters such as temperature, sexual reproduction, feeding time, locomotor activity and endocrine functions. In humans, melatonin is mainly involved in the sleep-wake cycle, having the ability to restore disturbed sleep patterns when administered exogenously (3). Through a complex sequence of mechanisms, that will be further discussed in this document, when light hits the retina, melatonin ceases to be produced. On the contrary, higher concentrations of melatonin are produced at night, specifically between 12 and 3 a.m., with secretion starting around 8-10 p.m. After reaching its maximum peak of approximately 100-200 pg/ml, levels begin to descend to around 10-30 pg/ml (4). This circadian rhythm of production helps humans sleep when there is no light, endorsing a homogenous and higher quality rest.

However, as investigation has progressed over the years, more ways in which melatonin plays a role in our body have been discovered, including, among others, anti-inflammatory, anti-neoplastic, anti-oxidant and anti-aging properties. All these features make melatonin a molecule with great therapeutic potential.

Its anti-neoplastic role is the main objective of this systematic review. As we will see in further detail, many mechanisms have been proposed, including inhibition of angiogenesis, induction of apoptosis or telomerase inhibition. These pathways participate in all carcinogenic steps, having in common a phenomenon called the epithelial-mesenchymal transition (EMT). The EMT can be either involved in the deleterious action that carcinogenesis is or, on the other hand, it can be a mechanism participating in benign physiological processes.

Initially, it is of interest to clarify some concepts in order to understand what the EMT really is. An epithelial cell is a type of cell that is specialized and is found on epithelial tissue, which lines the outer surfaces of blood vessels and organs, as well as the inner surfaces of cavities inside the human body. Epithelial cells can be squamous, cuboidal or columnar and characteristically have apical-basal polarity. Mesenchymal cells, on the other hand, are unspecialized embryonic

cells that display a fusiform or stellate shape. They are part of the connective tissue and are known to have an anterior-posterior polarity (5).

Furthermore, it is known that both types of cells exhibit different biomarkers. Epithelial cells express E-cadherin, claudins, occludins and certain cytokeratins, while mesenchymal cells display N-cadherin and vimentin (6,7). Other factors characteristic of the transition state like Snail and Slug, will be further developed in its corresponding chapter.

Therefore, as the term itself implies, the EMT occurs when an epithelial cell is transformed into a mesenchymal one. However, the transition is not as straightforward and thus, it can be described as a spectrum, undergoing different stages and gradually losing cell-to-cell junctions as well as changing biomarkers along the way.

There are different types of EMT that have been classified as type one, two and three. The first one is involved in embryogenesis, being first mentioned in the late 20th century when describing embryonic development. Within embryogenesis, other EMT roles such as implantation and organ formation are also included. All three processes express N-cadherin and vimentin (8) and have therefore a mesenchymal phenotype. The loss of adhesion molecules in epithelial cells such as tight and adherens junctions, desmosomes and hemidesmosomes, leads to a higher migratory capability, essential for organ formation and development. This type of EMT generates the mesoderm and endoderm in embryonic gastrulation from the epiblast layer, as well as being involved in the neural crest migration. The endoderm will later on develop respiratory and gastrointestinal organs, while the mesoderm will form muscles, kidneys and red blood cells, (9). Mesenchymal cells can also undergo mesenchymal-epithelial transition (MET) in order to give rise to secondary epithelial cells. These secondary cells have been theorized to then participate in another EMT cycle, leading to more specialized cells such as astrocytes or chondrocytes (8).

Type two EMT participates in physiological wound healing, regeneration and fibrosis. When organ fibrosis is involved, an inflammatory response is activated, leading to a series of events including cellular migration, angiogenesis or extracellular matrix remodeling (10). Regarding the EMT, some epithelial cells undergo an intermediate transition into fibroblasts and later on myofibroblasts, which are essential in the physiological process of healing (8). As it occurs with embryo development, the gain in motility achieved with this transition will also be key for regeneration, as cells need to migrate to where the damage is. If myofibroblasts are not destroyed afterwards via apoptosis, they will continuously produce substances such as collagen, which will induce a pathological state of fibrosis, further damaging the organ concerned (11). Depending on the transcription factors implicated, this fibrosis generated by an EMT has been seen to occur in liver (from healthy hepatocytes to cirrhotic liver), kidneys, lungs and cardiac tissue (12).

The third and final type of EMT is the one involved in carcinogenesis. Carcinomas are a type of tumor derived from an uncontrollable epithelial cell division. Many mechanisms are involved in the development of a carcinoma, being EMT one of

them. It participates in its induction, progression (leading to *in situ* carcinoma and subsequently to invasive carcinoma) and metastasis (13). The acquired migratory phenotype that has already been stated is especially relevant in metastatic cancer, as it confers cells the ability to invade and disseminate towards healthy distant tissues. These cells experience later on a MET which would explain why secondary tumors are histologically similar to the primary one. Additionally, carcinogenic cells that have undergone an EMT acquire further malignant traits. These are, for instance, stemness features (giving them the ability to renovate by themselves), greater resistance to treatments (6), inhibition of its own senescence and metabolic alterations (14).

Therefore, melatonin's relationship with EMT lies on the fact that the hormone is capable of regulating different transcription factors, that will in turn overexpress certain genes or, on the contrary, inhibit others. This concept will be further on developed in this review, together with a much more detailed explanation of everything presented in this introduction.

2. CHAPTER ONE: STAGES IN CARCINOGENESIS

The set of processes and phenomena that lead to the generation and development of cancer is called carcinogenesis. The word "cancer" implicitly describes the action of an uncontrolled cell division that ultimately alters the structure and behavior of normal-functioning tissues that are nearby. Therefore, a carcinogenic cell, by definition, must have two properties. To begin with, it must have the ability to duplicate by itself, while bypassing suppressive mechanisms established by the organism. Secondly, it must also be able to infiltrate healthy tissues (15). In order for this to happen, a healthy normal cell must be transformed into a carcinogenic cell, and that's how the three-step theory was postulated. This theory supports that the cell must follow a series of phases, which have been denominated as "initiation, promotion and progression". As recently mentioned, the EMT plays a role in all three of them.

2.1 Initiation

The irreversible stage of initiation begins when disruption in the cells' DNA first start to appear. This could be secondary to mutations, deletions or translations, leading to chromosome instability (15). If this DNA alterations are not eliminated or repaired by physiological repair mechanisms, the cell can become quiescent forever or, in turn, proliferate inadequately. Mutations can occur spontaneously or be a consequence of the effect of various exogenous factors (carcinogens), such as radiation, tobacco, alcohol or some viruses, as illustrated in figure 1. The bond created between the carcinogen and the modified DNA nucleotide is called an adduct. This adduct, if capable of surpassing standard repair mechanisms, will be misread as a normal nucleotide and be therefore present in the next cell cycle. The more adducts, the greater probability of developing tumors in the organism (16).

Aside from intrinsic DNA alteration, epigenetic changes can also trigger the initiation phase. Promoter regions become methylated and, as consequence, a certain number of genes will be modified. These genes, that are also targeted by the carcinogens previously mentioned, can be either proto-oncogenes or tumor suppressor genes. The former will be activated, while the latter inactivated. Proto-oncogens like Ras or c-Myc normally regulate cell cycle progression, growth and development so when they are overexpressed, abnormal proliferation will take place. On the other hand, tumor suppressor genes (like p53, Rb, BRCA1/2, Bcl-2) are repressed, leading to higher mutational rates, uncontrolled growth and greater survival capacity. Specifically, p53 is a major suppressor gene, which participates in DNA reparation, as well as being a key component in the cell cycle regulatory machinery. Such is its importance, that more than half of all existing cancer cases have p53 mutations (17).

2.2 Promotion

If mutated cells enter the cell cycle and continuously divide, a large population of resistant cells will be created. They will possess certain advantages compared with the rest of the tissue (apoptosis evasion, higher proliferative rate or resistance to cytotoxicity) that will ensure the cell's survival when natural selection takes place. Contrariwise to the previous stage, promotion is reversible and the interval between these two first steps can be very prolonged (17).

This step also requires of certain inductors named "promoters", which will only act once the cell has been initiated in the previous step. Promoters normally have a non-mutagenic effect and will further on increase proliferation. Examples of these are sexual steroids (androgens and estrogens), prolactin, glucagon, UV light, phenobarbital or 12-O-Tetradecanoylphorbol-13-acetate (TPA). TPA is present in croton oil and its role as a promoter is carried out by activating protein kinase C, which consequently results in increased cell division (16).

2.3 Progression

If carcinogenic agents and promoters are found in constantly high concentrations, even more mutations will take place and the previously mentioned genes will be further on over-/underexpressed. This continuous accumulation of altered DNA creates more aggressive and malignant cells with higher resistance to treatment (15). Additionally, cells can acquire deleterious forms of apoptosis and inadequate growth control, as can be seen below in figure 1. These properties could explain the reason as to why when the sooner the cancer is diagnosed, the better prognosis the patient normally has.

Moreover, the microenvironment, and the cancer itself, will secrete different cytokines (such as proteases) and growth factors, while also recruit and activate macrophages and fibroblasts. The extracellular matrix will begin to form fibrose tissue and this, together with cellular necrosis achieved by macrophages, will lead to tissular damage, dysfunction and lastly, cellular death (18). One of the substances secreted by both the tumor and its surrounding cells is the vascular

endothelial growth factor (VEGF), that will trigger the creation of new blood vessels, allowing persistence of tumoral growth. In order for the continuously enlarging mass to survive, metabolic requirements are modified (such as increase in glucose and oxygen uptake), and these will only be satisfied with the newly created blood vessels. Furthermore, this increase in vessels allows carcinogenic cells to infiltrate the circulatory system, granting them the ability to invade different parts of the body and thus, initiating metastasis.

During this stage in carcinogenesis, cells will also become less differentiated, invading even further while avoiding the organism's attempt to suppress the tumor. This stem-like feature can be achieved through an EMT process and the transition will also confer higher motility to cancer cells, secondary to loss of cell-to-cell junctions. These changes promote once again the invasion of nearby healthy tissues, contributing to the metastatic process and ultimately organ failure (18).

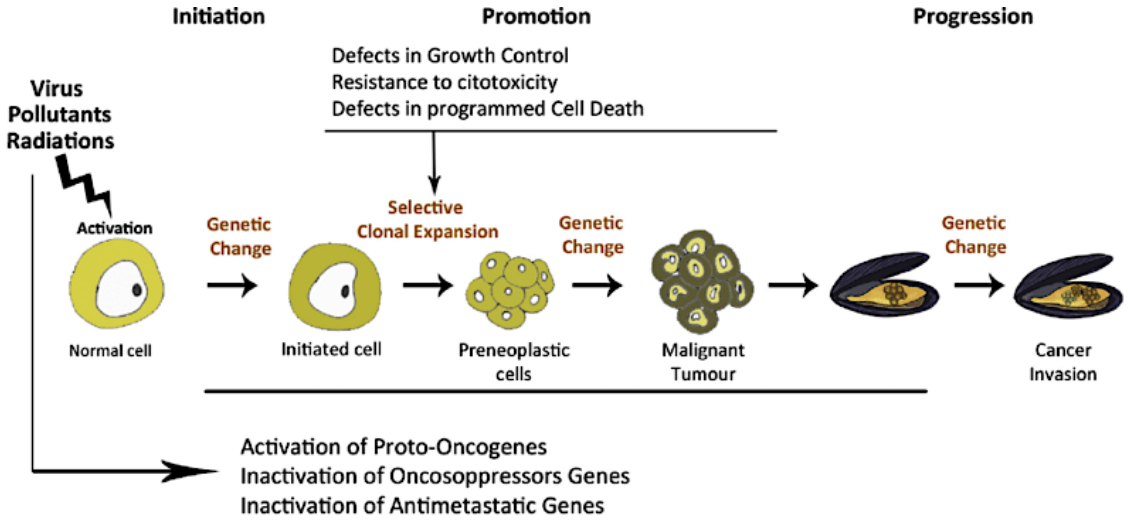

Figure 1*: *The three stages of carcinogenesis. Initiation, promotion and progression. *Several factors can be involved in activating the first step, such as viruses, pollutants or radiation. Genetic modification of normal cells (through the activation of oncogenes and inactivation of tumor suppressor genes) will initiate the cell, and selective clonal expansion will take place. This will ultimately lead to the formation of a malignant tumor, generating as a last step, a cancer capable of invading healthy tissues. Obtained from Carella F. et al. Journal of invertebrate pathology. 2015, 131. DOI: 10.1016/j.jip.2015.07.012.*(19)

3. CHAPTER TWO: EPITELIAL- MESENCHYMAL TRANSITION

The transition of an epithelial cell to a mesenchymal one will not only morphologically change the cell (from cuboidal to fusiform), but also lead to a series of functional changes that are the substrate of the roles EMT has on the organism. The cells will become more mobile, their polarity will become anterior-posterior, they will be resistant to apoptosis and more prone to disseminate (20).

The EMT occurs in different situations of an organism's development, these situations being three: embryogenesis, fibrosis and carcinogenesis.

Type one EMT is involved in implantation, embryo formation and posterior organogenesis. The trophoectodermal layer undergoes an EMT that helps with migration to the endometrium as well as helping with placenta implantation. It also participates in gastrulation, neural crest formation and more specific processes such as fusion of the palate and lip (21). Gastrulation will form two primordial layers called epiblast and hypoblast. The epiblast is the one with migratory capability, and will later on develop three other layers known as ectoderm, mesoderm and endoderm. The ectoderm is the most external layer and the neural crest (forming the peripheral nervous system, teeth and cartilage) and epithelial cells such as skin and hair will arise from it. The mesoderm is the medium layer and the excretory and circulatory system, together with muscle, will be formed once the embryo develops further. Lastly, the endoderm will end up forming the respiratory and gastrointestinal system. The relationship between EMT and gastrulation has been studied in several triploblastic animals (animals that have all three ectoderm, mesoderm and endoderm layers) like drosophila, sea urchin and zebrafish. Further *in vivo* studies are needed, but it is clear that EMT is a fundamental component of tissular organization during development (including cardiac valve formation), demonstrated with the loss of epithelial markers and the acquisition of mesenchymal ones (22).

Type two EMT occurs during tissue repair when a wound is formed. Cells change polarity and morphology, as well as acquiring greater mobility, all extremely necessary in order for the edges to close up. Not only has EMT been associated to skin wounds, but it also seems to have a role in organ regeneration when damage appears. This has been seen for example when the lacrimal gland, breast or cardiac tissue are injured (11). As mentioned previously, wound healing requires fibroblasts to turn into myofibroblasts, but if the latter perpetuates at the wound site, pathological fibrosis will begin to take place. If this fibrosis happens at the skin, hypertrophic scars will be formed. However, if it occurs internally, organs could become affected and functionality could be lost. EMT biomarkers such as increase in EMT-inductor Transforming Growth Factor β (TGF-β) has been observed at renal, hepatic, cardiac and pulmonary fibrosis (11), opening a new front for possible treatments of tissues that are difficult to repair.

The most studied type of EMT is type three as it is the one involved in carcinogenesis and is therefore the one with greater life repercussion. Understanding how EMT works at this stage is crucial for a better understanding of the process in order for newer treatments to be found. Unlike the other types, type three involves an EMT that is not controlled by the organism, as it leads to unwanted cell proliferation. In chapter one it was mentioned how carcinogenesis could be explained by the three-step theory: initiation, promotion and progression. EMT seems to have a greater impact in the tumor progression stage, as cancer cells lose adhesion with neighboring cells, thus allowing metastasis to occur (8). Furthermore, in order for metastatic cells to colonize healthy tissues, a MET must take place. It has been observed that the MET is necessary in the creation of a secondary tumor similar to the primary one, being not sufficient the initial EMT (14).

3.1 Biomarkers in the epithelial-mesenchymal transition

Epithelial cells lose a series of markers and gain others in order for the transition to a mesenchymal cell to take place. The better characterized epithelial markers described throughout many different studies are proteins like E-cadherin, Tight Junction Protein-1 (also known as zonula occludens or ZO-1), cytokeratin, laminin-1 and type IV α1-collagen which are extracellular proteins. Additionally, there are types of micro-RNAs related to epithelial cells, such as the mir-200 family. Micro-RNAs are single-stranded and non-coding RNAs involved in multiple gene expression and regulation. Along the transition process, cells will start losing these markers and depending on the amount they lose, the end product will be a purely mesenchymal phenotype or contrariwise, one that is in between the EMT spectrum. On the other hand, mesenchymal cells will express proteins like N-cadherin, osteoblast-cadherin and different types of integrins ($\alpha5\beta1$ and $\alpha V\beta6$). Vimentin, fibroblast specific protein 1 (FSP1) and β-catenin also appear as a more mesenchymal phenotype develops, together with extracellular molecules like α-1 I and III collagen, fibronectin and laminin-5. In regards with micro-RNAs, mir-10b and mir-21 are the ones acquired through the EMT. Moreover, certain transcription factors have also been described at the mesenchymal end of the transition, such as Snail (or Snail 1), Slug (or Snail 2), Twist, FOXC2, ZEB1 and Goosecoid (7).

Some of these changes are specific to the three stages previously mentioned. For type one EMT, most of the markers stated above will be present at some point of the transition. Cadherins are of great importance, as they allow tissues to be properly formed by acquiring adherens junctions. In embryogenesis, E-cadherin and ZO-1 will switch into N-cadherin. Epithelial cadherins provide cells with the tightness they need and are involved in the early stages of embryogenesis when the morula needs to be compacted. Contrarily, when embryogenesis develops, cell-to-cell adhesion is conferred by N-cadherin, which is useful in neural plate formation and cardiac muscle development. This change of cadherins, which is seen in all three types of EMTs, is promoted by transcription factor Snail, by repressing E-cadherin, together with an increase in vimentin and fibronectin. Cells present in the neural crest will integrate these changes, allowing them to migrate to other parts of the embryo, where, after a reverse transition (MET), the definitive tissues will develop (8). Additionally, $\alpha5\beta1$ integrin, which is fibronectin's receptor (7), will be present during gastrulation, where an extension function has been associated with it.

During type two EMT, tissue repair and fibrosis will take place, which will only be induced if inflammation, secondary to damage, is present. The molecules that are expressed during this process are mainly fibronectin, collagen and FSP1. FSP1 appears in fibroblasts induced via the EMT, being its presence demonstrated at renal fibrosis, liver fibrosis and pathological skin scars, together with higher Slug and Twist levels. FSP1 has also been observed during wound repair, as well as higher vimentin levels (both portraying a mesenchymal phenotype) and lower expression of type IV collagen. Furthermore, when analyzing wound sites, Slug appears to be overexpressed and if this is altered, wound healing seems to not be as efficient (11). Slug will decrease E-cadherin expression, allowing the necessary, yet not so strong, cell-to-cell adhesion.

The role that EMT plays in carcinogenesis has been demonstrated in multiple cancers, including breast, ovarian, lung, prostate and liver cancer. However, on the contrary to what it has being exposed above, the *in situ* ductal breast carcinoma has a total abolition of E-cadherin expression, while the invasive type still expresses this cadherin, being nevertheless, much more aggressive (23). It can be therefore interpreted, that the loss alone of just one epithelial marker (i.e. E-cadherin), might not confer the totality of the mesenchymal phenotype, nor the malignant properties associated with it. Certainly, in breast cancer positive for estrogen receptor ERα, metastasis was induced by Snail and Slug through repression of E-cadherin expression. Vice versa, Snail-induced EMT resulted in inhibition of ERα (24), proving the bidirectional relationship this hormonal cancer has with EMT. Additionally, elevated levels of Snail in breast cancer were associated with greater relapse, together with lower survival rates. Upregulation of vimentin was also demonstrated to decrease both E-cadherin and β1 integrin expression. Associated with this upregulation, an increase in invasiveness and motility was also observed, leading therefore to a worse cancer prognosis (23).

Moreover, overexpression of transcription factor ZEB1 in breast cancer stimulates higher dedifferentiation, which is the hallmark for many malignant tumors. Dedifferentiation causes cells that were previously differentiated to turn into stem-like cells. This has been seen to be associated with greater malignancy and higher therapeutic resistance (specially to chemotherapy), due to the fact that stem cells are able to renew by themselves and regenerate the neoplasia. Breast cancers resistant to certain treatments have stem-cell phenotypes, demonstrated by the use of specific markers such as $CD44^{+}/CD24^{-/low}$. Highly aggressive breast cancers have tested positive not only for these markers, but also for EMT markers like overexpression of Twist and vimentin and lower levels of mir-200 (20). In conclusion, there is a clear association between stem-like cells and EMT, opening up a possible new area of investigation for breast cancers that are therapeutically resistant.

As far as liver cancer is concerned, rising levels of Twist expression were seen to increase metastasis. In invasive colon cancer, Snail was also overexpressed. This last result was observed in lung cancer too (specifically, non-small cell cancer), together with an increase in the cytoskeletal marker vimentin. Both higher levels of vimentin and Snail in non-small cell lung cancer appeared in those that had a worse prognosis. Finally, in prostate carcinoma, E-cadherin's repression was affiliated with more malignant properties, as well as relapse after radiotherapy (23).

Therefore, all these results hint to the conclusion that EMT does indeed play a role in carcinogenesis, being able to confirm this through the biomarkers that have been associated to this transition. More profound studies of the pathways these markers are involved in will be necessary, since certain resistant cancers could be targeted with different and more efficient strategies.

3.2 Signaling pathways involved in the epithelial-mesenchymal transition

3.2.1 TGF-β signaling

For everything that has been exposed so far in this chapter to occur, something prior must take place. EMT must be stimulated and some molecules have already been identified to be responsible for it. One of them is the TGF-β. Tumors secrete many substances as regulators into their environment, and TGF-β is one of them. When the neoplasia is first starting to develop, TGF-β will actually limit the growth of it, yet when the cancer has progressed, the opposite effect will happen and proliferation and metastasis will be promoted (25). This last pro-oncogenic effect is carried out by several mechanisms, one of them being the induction of the EMT.

TGF-β has many signaling cascades, summarized in figure 2. The standard TGF-β activation pathway begins with the binding of TGF-β with TGFβ receptor 2 (TGFβR2) and receptor 1 or ALK5 (TGFβR1). This will lead to phosphorylation of proteins that act as secondary messengers known as Smad2/3, which will eventually be involved in EMT induction by binding to Smad4. Gene expression can also be induced through other pathways inside the Smad2/3 pathway. For example, as shown at number eight in figure 2, this can be achieved through the activation of the YAP/TAZ complex. YAP and TAZ are mechano-transducers that are also activated by extracellular stiffness. They can alter the matrix's properties that surround the tumor, conferring higher tumor progression and malignancy (26). Another alternative following the Smad2/3 phosphorylation, is the binding to the Poly(rC)-Binding Protein (or PCBP1) that triggers differential mRNA splicing. Additionally, as seen at number six, the ALK5-TGFβR2 association can lead to an alternative cascade of CDC42/Rac, Rho/ROCK, PI3K/Akt or even p38, all producing different effects through gene regulation.

It has been demonstrated that PI3K/Akt activation induces metastasis, and it is also responsible for treatment resistance in many different cancer types. These malignant traits are carried out through phosphorylation of the transcription factor Twist, which in turn promotes EMT. Twist has been linked to E-cadherin repression, increase in pro-oncogenic genes and worsening cancer prognosis (27). Other pathways that will be further on discussed, such as Wnt or NF-κB, also potentiate Twist expression, leading to all these EMT-associated events. In addition, it was identified how Twist activated mir-10b, which is one of the micro-RNAs that is acquired in the mesenchymal phenotype of EMT. Mir-10b is involved in tumor invasion, progression and metastasis, as seen in both *in vivo* and *in vitro* studies. Moreover, this micro-RNA inhibits gene HOXD10, causing an increase in RhoC (triggered by the binding of TGF-β to ALK5-TGFβR2) which has been seen to greatly induce metastasis (28). As a matter of fact, increase in mir-10b in breast cancer induced an EMT, while inhibition in mice of HOXD10 proved to significantly reduce lung metastasis (29). Furthermore, it has been discovered how the mir-200 family of micro-RNAs coexist in a feedback loop with transcription factors ZEB. This will result in an inhibition of ZEB factors when mir-200 are overexpressed, leading to an inhibition of the EMT (20).

Regarding the secondary messengers Smads, the pathways in which they are involved in tumor progression have also been linked to the EMT. Smads will repress E-cadherin expression via Snail, Twist and ZEB activation. When Smad4 is repressed in breast cancer cells, metastasis will be reduced, thus increasing survival rates. In addition, it has been observed how activation of Snail through Smad3/4 is involved in skin cancer and how inhibition of Smad2 promoted carcinogenesis even further (29).

TGF-β is normally synthesized as a precursor in the form of inactivated TGF-β associated with the Latency Associated Peptide (LAP). This complex can bind to different integrins. If bound to integrin αvβ6, as seen at number one, a traction force will detach TGF-β from LAP activating it, allowing TGF-β to follow any of the pathways already described. The increase in this integrin (which is in fact a mesenchymal marker for EMT) in breast cancer, has been associated with higher invasiveness and worse prognosis (29). On the other hand, visualized at number two, if both TGF-β and LAP bind to integrin αvβ8, certain proteases known as matrix metalloproteinases (MMPs) will cleave the union and set TGF-β free.

Lastly, endoglin is a glycoprotein that can form a receptor complex with ALK1 and TGFβR2, where TGF-β can bind to. Endoglin prevents degradation of VEGF, thus having a possible effect in angiogenesis. This ALK1 complex receptor, together with TGFβR2/ALK5-TGFβR2/ALK1 complex (shown at number five), can induce another Smad phosphorylation, known as Smad1/5/8. Smad1/5/8 will later on join to Smad4, once again promoting certain gene expression that will contribute to the EMT (25).

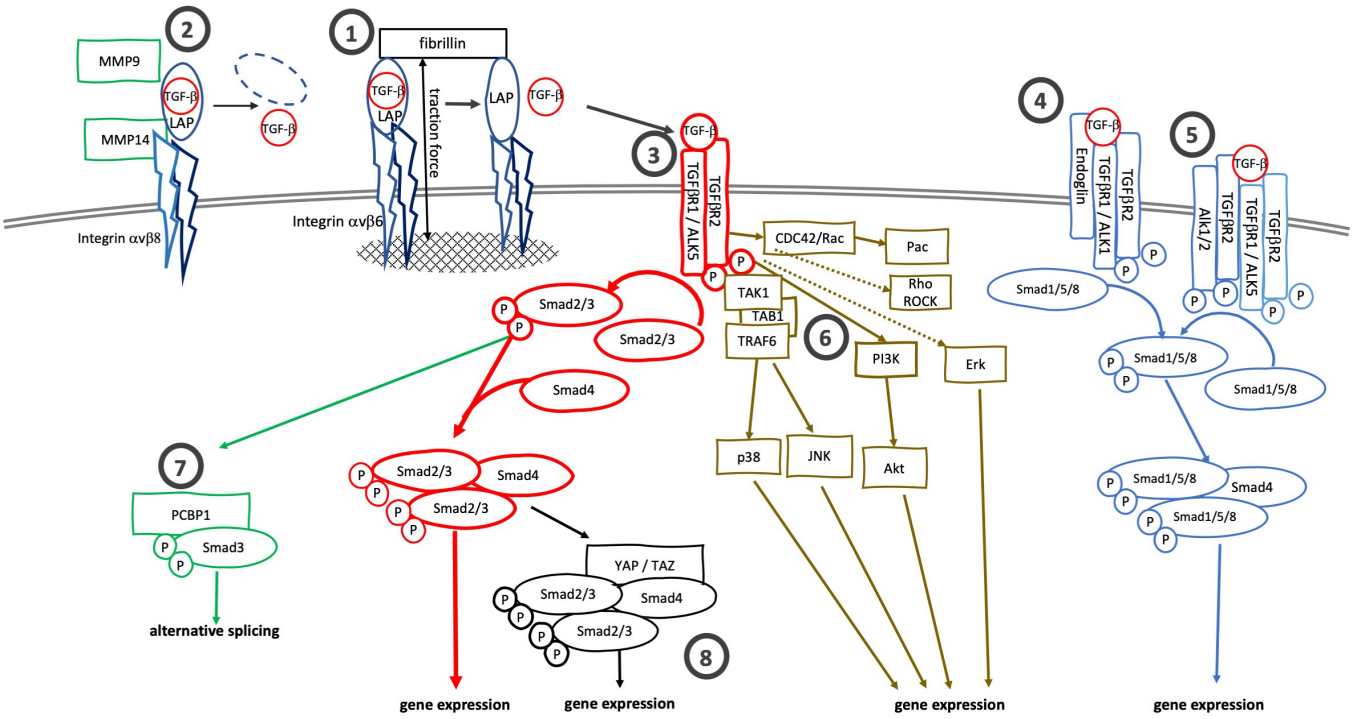

Figure 2: Different TGF-β signaling pathways. 1: *LAP-TGF-β complex binds to integrin αvβ6 and through a traction force, TGF-β is activated.* ***2:*** *Same complex can bind to integrin αvβ8, this time being TGF-β activated through proteolytic cleavage done by MMPs.* ***3***

(standard signaling cascade): *Activated TGF-β heterodimerizes TGF-β receptor 2 with TGF-β receptor 1 (ALK5) and activate Smad2/3 pathway.* ***6:*** *or either activate an alternate CDC42/Rac, Rho or PI3K/Akt pathway* ***4:*** *TGF-β can also bind to endoglin-TGFβR2-ALK1 leading to a Smad1/5/8 signaling.* ***5:*** *Same signaling can be triggered by binding of TGF-β to TGFβR2/ALK5- TGFβR2/ALK1 complex.* ***7:*** *Smad2/3 can bind to PCBP1 and induce an alternative splicing.* ***8:*** *Smad2/3 can also bind to YAP/TAZ complex. Obtained from: Stuelten et al. Frontiers in Cell and Developmental Biology.2021;9:764727.* (25).

3.2.2 Wnt signaling

Another EMT inducer is the protein Wnt. Wnt can follow a canonical pathway, in which β-catenin is involved in, and a non-canonical one, which is β-catenin independent. In the canonical pathway, Wnt will act as a ligand into specific receptors, which are known as Frizzled and low density lipoprotein-related protein (LRP5/6). When the binding of Wnt takes place, Dishevelled (sometimes shortened as Dsh or Dvl) will be phosphorylated, which will in turn inhibit the complex formed by the molecules Glycogen Synthase Kinase 3 beta (GSK-3β), Axin, Adenomatous Polyposis Coli (APC) and Casein Kinase 1 (CK1) as represented in figure 3. This complex is responsible for the ubiquitylation and degradation of β-catenin by the proteasome. Therefore, when the complex is inhibited, β-catenin will not be eliminated and its concentration will increase in the cytoplasm, leading to its translocation into the nucleus where it will exert different functions (30).

Once in the nucleus, β-catenin will interact with different transcription factors, being T-cell factor (TCF) and lymphoid enhancement factor (LEF) the ones most commonly identified with the induction of EMT. The Wnt/β-catenin pathway is therefore not only present in oncogenic development, but also in embryogenesis and fibrosis (31). Additionally, β-catenin can interact with the epithelial marker E-cadherin, creating strong cell-to-cell junctions. When E-cadherin is lost, the Wnt/β-catenin signaling will be affected and an increase of free β-catenin in the cytoplasm will take place (32). This will activate EMT once the catenin enters the nucleus. At the same time, secondary to an EMT effect, E-cadherin expression will be reduced, together with an increase in fibronectin, Slug and Twist (31).

β-catenin is also regulated by TGF-β, since the latter increases the former's intracellular availability by disassembling it from E-cadherin. This, as well as changing the epithelial conformity of the cell, allows β-catenin to enter the nucleus and exert its actions. Similarly, when TGF-β decreases, β-catenin will be degraded and the EMT will not take place (32). Another association of TGF-β with β-catenin has also been proposed, in the sense that LEF can be activated either by the binding of β-catenin itself, as mentioned above, or by binding of Smad proteins found in TGFβ-dependent pathways (31).

High concentrations of β-catenin inside the cell, especially high intranuclear levels, stimulate carcinogenesis and tumor progression. Abnormal and excessive activation of the Wnt/β-catenin pathway can be found in many different cancer types, such as colorectal, prostate or skin cancer (31). Indeed, most of the genes that will be activated secondary to this Wnt/β-catenin signaling are proto-oncogenes, such as cyclin-D1 or c-Myc (30). Mutations in β-catenin, APC or Axin

have also been found to promote malignant traits in several organs (32) (for example, APC mutations in familial adenomatous polyposis and colorectal cancer). Furthermore, excessive activation of this signaling leads to the induction of a mesenchymal phenotype by increasing Snail and Slug expression. It has been seen that when Slug is activated through the Wnt/β-catenin pathway with GSK-3β as a transducer, levels of the tumor suppressor gene BRCA1 will be lowered, augmenting the risk of developing aggressive breast cancer (31).

3.2.3 Notch signaling

Notch proteins are a family of transmembrane proteins that act as receptors and contain both extracellular and intracellular domains. When the ligands Jagged1 or Jagged2 bind to the receptor, the intracellular domain is cleaved by γ-secretase (24). The activated Notch will consequently act as a transcription factor inside the nucleus, where gene expression will be stimulated. Some of the genes that are affected are involved in carcinogenesis, being NF-κB, Akt or p21 some of them (31). NF-κB, or nuclear factor-kappa B, is a protein complex responsible for many different functions in the body, including inflammation and immunology. Excessive expression of this factor, however, has been correlated with tumor growth and progression. Akt was already mentioned in sections above, where a metastatic and angiogenic role was associated with it, possibly secondary to a Twist activation. Lastly, p21 is a kinase inhibitor and is therefore involved in the regulation of the cell cycle, meaning its mutation could lead to undesirable cell proliferation.

Additionally, Notch signaling is able to induce Snail and Slug expression, the latter being able to induce EMT by limiting E-cadherin and additionally activating β-catenin. This pathway also stimulates metastasis through EMT by reducing levels of mir-200, secondary to GATA binding protein 3 (GATA3) expression. On the contrary, it has been seen that when Notch is suppressed, invasiveness and progression are reduced in both lung and pancreatic cancer (31). In many different types of breast cancer, concentration of Notch's intracellular domain was seen to be elevated, while higher expression of Notch and Jag1 gene led to a poorer prognosis (24). All of these results set a new pharmacological front in where either Notch or Jagged inhibitors could possibly act as antineoplastic treatments. In this way, currently, several inhibitors of molecules involved in the Notch cascade are being developed, with further investigation still in need.

3.2.4 Hedgehog signaling

Mammals have three different types of Hedgehog proteins (Hh): Indian (IHH), Desert (DHH) and Sonic (SHH). When Hh proteins are not present, inhibition of Smoothened (Smo) by PTCH1 or PTCH2 receptor will take place, and therefore gene transcription will not occur. If on the other hand, Hh binds to PTCH receptor as seen in figure 3, Smo will interact with transcription factors Gli (Gli1 and Gli2 are activators, while Gli3 is an inhibitor). Later on, Gli factors will reach the nucleus and act upon certain genes that will be involved in apoptosis and the cell cycle (33).
The Hh signaling pathway has been mainly associated with embryo development, meaning alteration in any of the proteins involved could induce malformations or

abnormalities in the fetus. Cancer is also a secondary effect when the pathway malfunctions, as observed, among other tissues, in skin, blood, breasts and pancreas (33). Because of these findings, synthetic inhibitors of Smo and Gli are being developed, as an attempt to target and inhibit carcinogenesis. Molecularly, it was proven that when Smo was repressed, EMT in pancreatic cancer cells was inhibited. In addition, in neuroendocrine tumors, when Gli was present, E-cadherin was lost. Interrelation with the above pathways (TGF-β, Wnt and Notch) has also been observed, with Jagged2 and transcription factors FOX1/2 as mediators (30). Notch can also repress GSK-3β (14) and stabilize β-catenin's intracellular concentration, proving more ways in which the signaling pathways can be related.

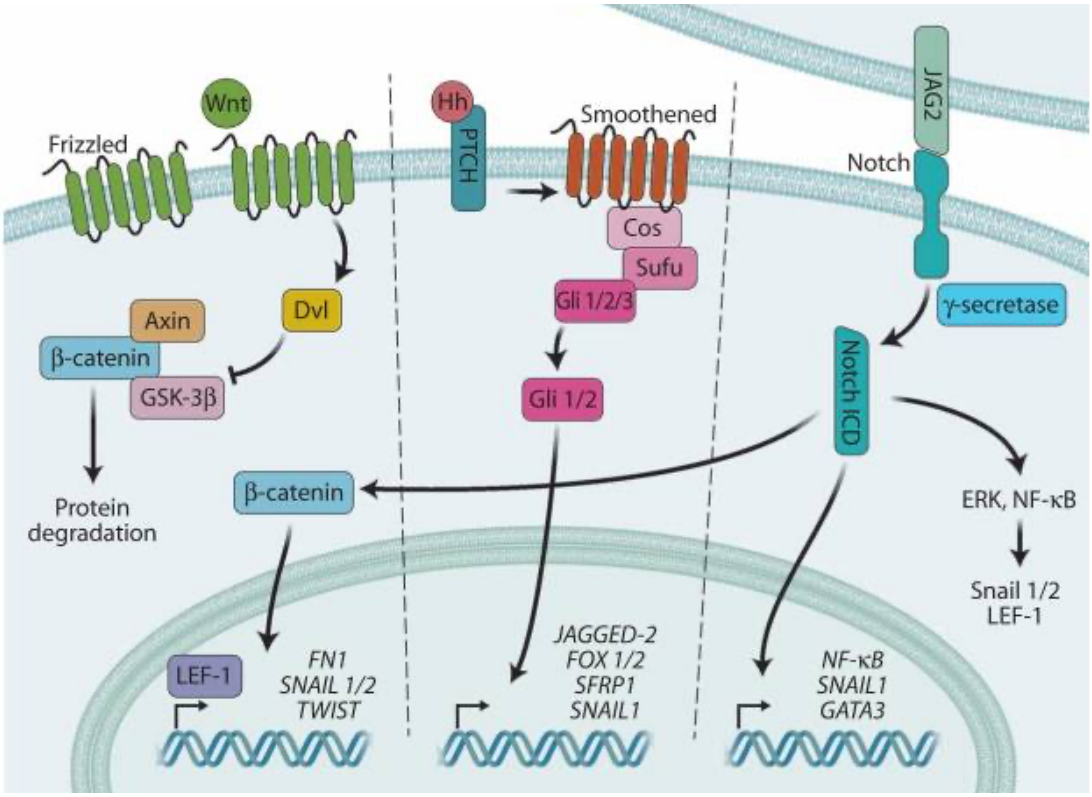

Figure 3: Wnt, Hedgehog and Notch signaling pathways. *Dvl: Dishevelled. LEF-1: lymphoid enhancement factor. FN1: fibronectin 1. SUFU: Suppressor of Fused (keeps Gli factors inactivated). Obtained from: Gonzalez DM et al. Sci Signal. 2014 Sep 23;7(344):re8. doi: 10.1126/scisignal.2005189.* (31).

In figure 3 it is summarized what it has already been stated about Wnt, Hh and Notch pathways. As it can be seen, all three of them, as well as the TGF-β pathway, can directly activate transcription factor Snail, which will promote EMT through different mechanisms. One of them, which has been previously mentioned, is the repression of E-cadherin. This is done through the inhibition of CDH1, which is a gene promoter of E-cadherin expression (24). Direct stimulation of mesenchymal genes can also be achieved through Snail activation, as well as inhibition of tight junction proteins and genes involved in the characteristic epithelial apical-basal polarity (14).

3.2.5 Hypoxia

Hypoxia is a common event in malignant neoplasms due to vessel compression (tumor mass effect), the increased oxygen demand by cells and the new vascularization created (24).

When oxygen is absent, certain enzymes (named PHD), that in aerobic conditions will trigger the degradation of hypoxia inducible factor 1 α (HIF-1α), are inhibited (31). Therefore, when hypoxia appears, HIFs will be activated and act as transcription factors. Indeed, HIF-1α is the one that has been most thoroughly studied, as many effects have been conferred to its transcriptional activity. Apoptosis, angiogenesis, therapeutical resistance, increase in cell stemness or metastasis are some of them (24). These are consequences secondary to EMT induction, through the expression of genes like TWIST or TGF-β. Moreover, HIF-1α stabilizes Snail and Slug and Notch's intracellular domain, as well as directly activating Notch and β-catenin (31).

HIF-1α has been proven in numerous studies to have an association with estrogen receptors in breast cancer. When this factor is present at high levels, a more aggressive ER+ cancer takes place, as proliferation, ER and VEGF are potentiated. If the malignancy continues to develop, HIF-1α induces a loss of ERα both *in vivo* and *in vitro* (24), which in turn could explain why some ER+ cancers eventually become resistant to hormonal therapy. Therefore, it could be theorized that with an HIF-1α inhibitor, ERα levels could stabilize throughout the carcinogenic process, thus augmenting the efficiency of hormonal treatment.

3.2.6 Non-transcriptional factors

Up until now it has been mentioned how some transcription factors, which are activated through different signaling pathways, are responsible for the promotion of the EMT. However, studies have also identified other ways in which this transition can be achieved. For example, components related with the extracellular matrix (ECM) can also be involved in signaling pathways that will eventually regulate specific genes. β1 integrin for instance is able to activate transition towards a mesenchymal cell through pathways where TGF-β acts upon (8). An already mentioned integrin, αvβ8, will also be responsible of activating TGF-β when found in its inactive state. Moreover, in figure 2 it was seen how another type of integrin (αvβ6) was also present in the TGF-β pathway and was actually correlated with higher invasiveness rates.

Collagen is another ECM substance playing an important role activating the transition. It can attach to either integrins or receptors known as DDR. Through these two types of mediators, cascades like NF-κB (which is part of the Notch pathway), GSK-3β (Wnt pathway) or p38 can be induced, where Snail, Slug and LEF-1 among others, will be expressed. Furthermore, it has been observed how when β1 integrin interacts extracellularly with type 1 collagen, E-cadherin is repressed and N-cadherin promoted. Deficiency of type 1 collagen has been seen to be specifically relevant in facial abnormalities during type 1 EMT, as well

as being a type 3 EMT inductor in different types of cancer (pancreas, breast and lung) (31).

Additionally, MMPs can be secreted during either type 2 or type 3 EMT. These proteases will degrade certain substances from the ECM, such as laminin-1 and type IV α1 collagen (8), that are indeed epithelial biomarkers, as well as cleaving cell-to-cell junctions.

Therefore, the EMT can, and certainly is, also induced through microenvironmental changes and not only through specific ligands binding to certain receptors. This suggests that a dynamic interaction between the environment and the cell has to exist if changes in the organism are desired.

4. CHAPTER THREE: MELATONIN

4.1 Structure

Melatonin is part of the acetamide group of compounds, which are the simplest form of amides that derive from acetic acid. It can also be called 5-methoxy-N-acetyltryptamine, hinting at its molecular structure that can be seen in figure 4. It is composed of an indole ring, an acyl-amino-ethyl chain in the 3rd position, (C=O, N-H, CH_2CH_3) and a methoxy group in the 5th (-O-CH_3).
Its structure is of great importance, as it confers high receptor affinity, ensuring that the principal molecule binding to the receptor is melatonin. These receptors in question are MT_1 and MT_2, which will be later described in the document.

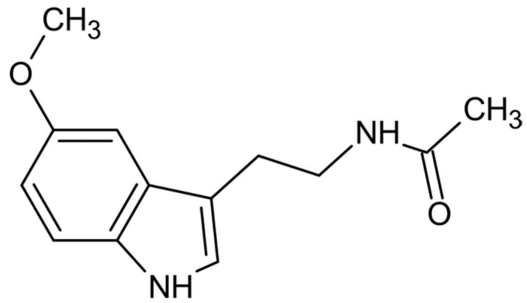

Figure 4: Melatonin structure. *It can be seen (from left to right): the methoxy group, the indole ring and the acyl-amino-ethyl side chain. Obtained from: Mannino, G. et al. Int. J. Mol. Sci. 2021, 22, 9996. DOI: 10.3390/ijms22189996.* (34).

4.2 Synthesis

The pineal gland synthesizes melatonin from the amino acid tryptophan following a series of enzymatic steps. As it is represented in figure 5, tryptophan is converted into 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase (TPH). Later on, 5-HTP is transformed into serotonin, by the enzyme L-tryptophan decarboxylase. The next step continues with the formation of N-acetyl serotonin (NAS) by aralkylamine N-acetyltransferase (AANAT), and finally NAS is transformed into melatonin by hydroxyindole-O-methyltransferase (HIOMT) (35).

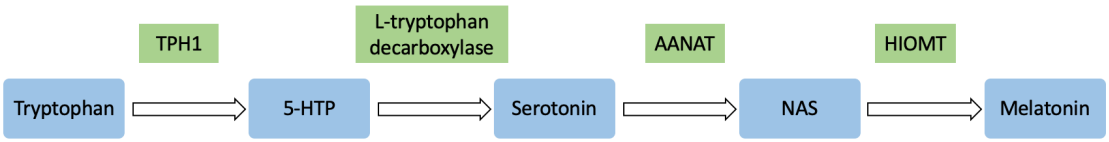

***Figure 5: The synthesis of melatonin**. The steps necessary for melatonin to be formed are represented in a schematic way. TPH1: tryptophan hydroxylase 1, 5-HTTP: 5-hydroxytryptophan, AANAT: aralkylamine N-acetyltransferase, NAS: N-acetyl serotonin, HIOMT: hydroxyindole O-methyltransferase.*

Throughout the years, it has been discovered that melatonin can also be produced elsewhere besides the pineal gland, in places such as the gastrointestinal tract, lymphocytes, bone marrow cells, retina, and skin. Not only does melatonin have an extra-pineal secretion, but it can also be found at far greater concentrations in some of these locations compared to those found in blood (35).

4.3 Secretion and regulation

As it has been briefly summarized above, melatonin follows a circadian rhythm, meaning its levels rise at night-time and decrease with daylight in an approximate 24-hour cycle. This is all regulated with the help of the suprachiasmatic nucleus (SCN), the main circadian pacemaker that mammals have in their body. The SCN is located in the hypothalamus and is responsible for synchronizing all the peripheral circadian clocks the organism has in its cells. Both external and internal stimuli act upon the SCN in order to ensure the system is correctly adjusted. The main external stimulus is light, which excites the intrinsically photosensitive retinal ganglion cells acting through melanopsin. This generates an electrical signal that reaches the SCN through the retino-hypothalamic tract (36).

There is a particular sequence of structures, illustrated in figure 6, that connect the SCN with the pineal gland. Once the electrical impulse is generated in the retina and the SCN is reached, it propagates across the paraventricular hypothalamic nucleus. Then, it reaches the intermediolateral nucleus in the spinal cord and later on, the superior cervical ganglion. The latter, as a last step, grants the pineal gland its innervation, which is mainly sympathetic (37). Hence, melatonin is regulated by the production of noradrenaline, which will be inhibited when light is present (most effectively if it is blue light at 460–480 nm, since it is the frequency melanopsin is most sensitive to (38)).

On the contrary, noradrenaline is secreted from the superior cervical ganglion in the absence of light, acting on β-adrenergic receptors in the pineal gland. The activation of these sympathetic receptors increases cAMP levels, which in turn activate protein-kinase A, promoting on a genetic level the phosphorylation of cAMP response element binding protein (CREB). This CREB phosphorylation activates the transcription of the enzyme AANAT (39) and consequently, melatonin is produced.

Thus, it has been supported that AANAT is the rate-limiting enzyme in the synthesis route of melatonin (35) represented in the steps above in figure 5, meaning lower levels of AANAT will lead to lower melatonin concentrations. Others believe the enzyme HIOMT is as equally as important as AANAT (37).

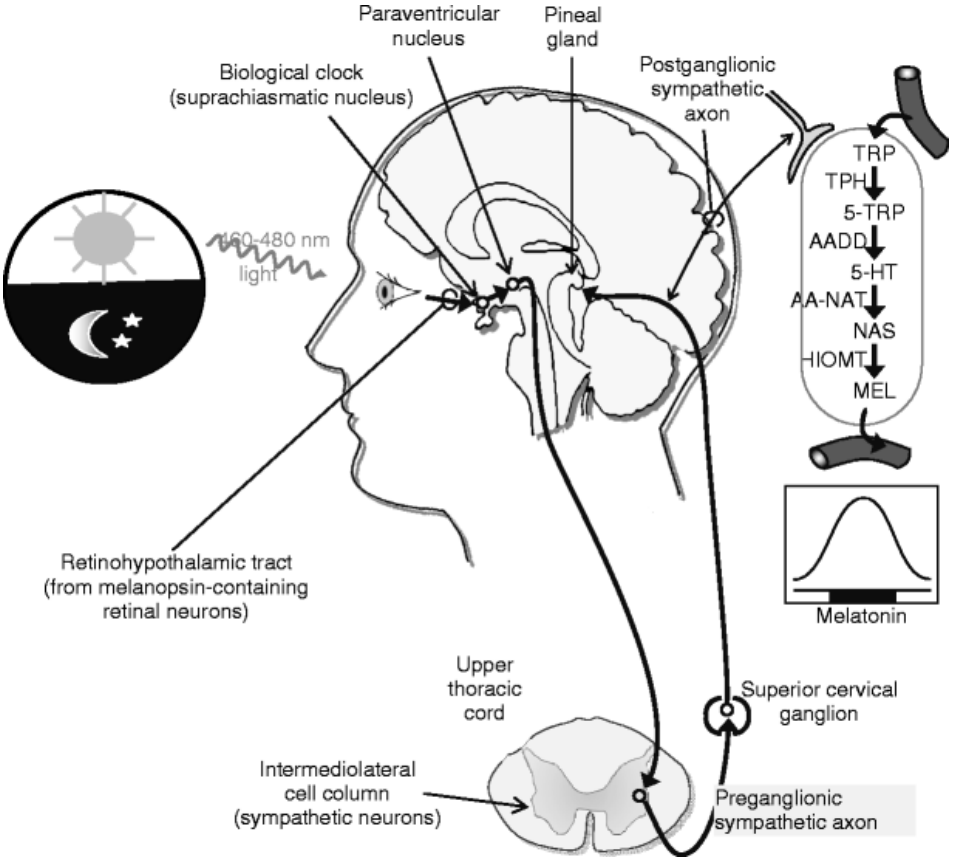

Figure 6: The influence of light/darkness in the synthesis of melatonin. *The pathway the electrical impulse has to travel in order to regulate melatonin's production is represented. In this case, the presence of light will inhibit its production. Obtained from: Korkmaz, A. et al. Rev Endocr Metab Disord 10, 261–270 (2009). DOI: 10.1007/s11154-009-9117-5.* (40).

4.4 Mechanism of action

There are two main ways in which melatonin can achieve its effects: by either binding and acting upon certain receptors, or by entering directly inside cells and as a result affecting intracellular proteins.

4.4.1 Receptor-related actions

If the first possibility takes place, melatonin acts upon two different G-protein-coupled receptors: MT_1 and MT_2. MT_1 is a type 1A receptor, encoded by a gene located at chromosome 4, while MT_2 is a type 1B receptor with its gene found in chromosome 11 (41). Both are cell-surface receptors and can be found mainly in the central nervous system (including retina and SCN), as well as also being present in many other peripheral locations (35).

When the activity of these receptors is triggered through the binding of melatonin, cAMP production, protein-kinase A activation and consequently also CREB phosphorylation are inhibited. On the other hand, ionic calcium levels are increased and protein-kinase C activated (41). The repercussion of it all depends on the type of cell melatonin is acting on.

Melatonin is a lipophilic molecule. Thus it is also able to interact with nuclear receptors aside from the ones present in the cell surface (MT_1 and MT_2). The main nuclear receptor described is the retinoic acid-related orphan receptor (ROR). ROR belongs to the NR1 nuclear receptor superfamily and is involved in many processes like cerebellar maturation, circadian regulation and bone formation (42).

4.4.2 Non-receptor related actions

As mentioned right above, the entrance of melatonin into different cells implies it can act directly towards certain molecules, and not only the ROR. One of the proteins it acts upon is calmodulin, a Ca^{2+} binding protein (41). This suggests melatonin is also involved in Ca^{2+} mediated processes, such as cytoskeletal modulation and cell physiology.

Actions inside mitochondria have also been described, fulfilling an important role as an anti-oxidant agent and as a protector for mutations in mitochondrial DNA (42).

4.5 General properties

Many properties have been attributed to melatonin throughout the years, such as being involved in the sleep-wake cycle, having anti-inflammatory and even possible anti-aging properties, being beneficial in cardiovascular or metabolic diseases and most importantly, having an anti-oncogenic action.

4.5.1 Circadian rhythm

The circadian rhythm is controlled by the SCN, being regulated with the presence or absence of light. In turn, melatonin is able to resynchronize the organism's 24-hour clock when it binds to MT_1 in the SCN. This chronobiological property is useful in many ways, being the regulation of the sleep-wake cycle one of them. This explains why totally blind individuals are more prone to suffer non-24-hour sleep-wake rhythm disorders, as exogenous light cannot be perceived by them (43). The non-24-hour sleep-wake rhythm disorder is characterized by the inability to synchronize in a 24-hour rhythm, meaning people will have inadequate sleep patterns with possible insomnia or hypersomnia, as well as altered hormone secretion levels which also follow a circadian rhythm (cortisol, TSH, etc.). People who suffer this type of disorder benefit from tasimelteon (41), which is an agonist of both MT_1 and MT_2 melatonin receptors. Additionally, those who suffer insomnia benefit from *Circadin* (prolonged-release melatonin, 2mg). The administration of *Circadin* is approved in individuals aged 55 or above, as melatonin production decreases with age (44). This gives sense into the fact that

elder people suffer most from insomnia, having trouble falling or maintaining sleep or even having non-restorative sleep. Thus, the fact that in both sleep disorders exogenous administration of melatonin is useful and seems to limit symptoms suggests how endogenous dysregulation of melatonin is the core of the problem.

Not only is it useful in sleep disorders, but also in circadian desynchronizations like those appearing when travelling long trans-meridian distances and experimenting jet lag. Jet lag disrupts the rhythmicity of internal clocks and can lead to poor quality sleep, inadequate eating patterns, fatigue and concentration and memory problems (45).

4.5.2 Anti-oxidant and anti-aging actions

Melatonin has been proven to be a potent endogenous anti-oxidative agent. Reactive oxygen species (ROS), such as free radicals like superoxides, peroxides and hydroxyls, are generated as byproducts in different metabolic pathways, mainly in the mitochondria. When cells are unable to eliminate them, they accumulate causing oxidative stress and as a result, several structures like membranes, lipids and even DNA can be damaged (42).

Levels of melatonin in mitochondria are very high compared to those in blood, suggesting the possibility that it is also synthesized and secreted inside, apart from using transporters (PEPT1 and PEPT2) to enter against gradient extracellularly. Melatonin's inhibition of ROS occurs due to scavenging actions, activation of antioxidant enzymes like quinone reductase 2 (QR2) or glutathione and inhibition of pro-oxidative enzymes like xanthine oxidase. It also works as a mitochondrial DNA protector, preventing detrimental alterations from occurring. ROS are also known to attack the electron transport chain (ETC) inside mitochondria, leading to the creation of even more ROS, starting a vicious cycle. Melatonin will guard the ETC from ROS with the mechanisms described above, as well as inhibiting the opening of the mitochondrial permeability transition pore (withholding cell death via apoptosis or necrosis) (44).

The anti-oxidant properties can be correlated to anti-aging properties, as all the damage ROS entails leads to cell dysfunction and consequently cell death, which are the underlying cause of aging. Furthermore, it is objectively seen how, with age, circadian rhythms are altered and melatonin concentration decreases (44), showing a two-way relationship between melatonin and aging.

4.5.3 Anti-inflammatory

Melatonin's anti-inflammatory actions have been successfully proven in animal models, showing how specific inflammatory cytokines are decreased (IL-1β, TNF-α and IL-6) whereas the anti-inflammatory ones are increased (IL-4 and IL-10) (44). However, further clinical trials are in need to be undergone, in order for results to be generalizable to every situation in humans.

By inhibiting p52 acetylation, melatonin also prevents the expression of iNOS and COX-2, two pro-inflammatory enzymes (46). This decreases leukotriene,

prostaglandin, thromboxane and prostacyclin concentration, all of which are molecules involved in inflammatory processes.

This property can be extended to many different mechanisms that have a substrate of inflammation. Therefore, melatonin acts in different areas such as cardiovascular system, central nervous system and respiratory tract, being useful in injuries or disorders caused by excessive inflammation. As a matter of fact, melatonin analogues have been used in more extensive ways than just for insomnia or sleep disorders. For instance, ramelteon and piromelatine are used for cardiovascular-associated problems, such as insulin resistance or high blood pressure (44).

4.5.4 Miscellaneous

There is sufficient evidence supporting that melatonin is involved in many other mechanisms, like acting as a protective agent against disruptors of the skin (47). It also has a role in metabolic pathways, being capable of properly managing diabetes or body weight, as well as reducing cardiovascular risks in the metabolic syndrome (44). It is also involved in fertility and reproduction, regulating gonadotropin secretion (LH, FSH) and puberty onset (39), being therefore beneficial in pathologies like polycystic ovary syndrome. Additionally, melatonin can have a positive effect when administered in depression (through MT_1 and MT_2 receptors and blockade of $5\text{-}HT_{2c}$ receptors) and in neurodegenerative conditions such as Parkinson or Alzheimer's disease. It is also able to maintain a correct muscle and bone function, preventing its loss in osteoporosis or other age-related afflictions (48).

4.6 General anti-neoplastic properties

Alluding chapter one, melatonin is positively involved in all three stages of carcinogenesis (initiation, promotion and progression). In addition, it participates in many other different pathways that inhibit tumor formation.

It was already mentioned how carcinogens like radiation (e.g., UV light) were responsible, through genomic instability, for the appearance of certain cancers such as melanomas. Melatonin's direct actions towards DNA, together with its anti-oxidant properties, make possible the inhibition of these pro-oncogenic effects. Hydroxyl groups generated with radiation are indeed the cause for most of the chromosomal instability generated, being melatonin capable of eliminating them. The previously mentioned increase in certain anti-oxidant enzymes by the indoleamine also contributes to the neutralization of these ROS. In addition, it has been repetitively demonstrated in animal studies that melatonin is capable of protecting the body from the adverse effects generated by radiation. The outcomes that can be observed in mice are increased survival rates, death prevention when lethal radioactive doses are administered and finally, avoidance of genomic alterations in red blood cells, as well as in bone marrow (49).

Furthermore, it has also been demonstrated that melatonin can activate DNA base-excision repair systems, which are the alterations that characteristically

occur with oxidative damage (49). A type of enzyme committed to DNA reparation is the poly ADP-ribose polymerase 1, or PARP1 for short, which is inhibited by melatonin. As contradictory as it might seem, the final outcome observed is still the inhibition of carcinogenesis, as PARP1-related pathways depend on the amount of existing DNA disruption. When the alteration is considerable, activation of PARP1 will be insufficient and so macrophages will eliminate the cell that carries damaged DNA. However, when cells are already carcinogenic, they use PARP1 to their advantage, as the reparation that this ribose confers them will make them thrive and survive with the mutated genome (50). Therefore, melatonin's inhibition of PARP1 seems only useful when cancer cells are present and not when cells are mildly mutated.

Another way in which the pineal hormone exerts its anti-neoplastic effects is by the inhibition of telomerase. Telomerase is an enzyme that elongates telomeres, a sequence that ends in TTAGGG in human chromosomes. The enzyme is highly activated in most cancer cells, conferring them the ability to divide indefinitely. Interestingly, melatonin has been seen to inhibit telomerase in various cancer types (51), illustrating once again another mechanism in which its anti-neoplastic actions are carried out.

Melatonin is also accountable for the induction of apoptosis, due to the fact that it interacts with mitochondria, which are organelles involved in caspase-dependent apoptotic pathways. The hormone specifically stimulates the expression of Bax, a pro-apoptotic protein that binds to Bcl-2 and abolishes the latter's anti-apoptotic effects. Additionally, cytochrome C and several caspases are also activated (48). Another way in which apoptosis is induced is by the activation of p53, a tumor suppressor factor involved in the cell cycle, in DNA reparation and in apoptosis (52).

The already mentioned data concerning the anti-inflammatory properties, and the fact that melatonin is secreted by lymphocytes, reveals melatonin's wide implication in immunology. Regarding inflammation, it alters the secretion of certain cytokines such as IL-2, IL-6, IFN-γ and TNF-α. Levels of IL-2 and IFN-γ are increased when melatonin is present. Both molecules activate NK cells, which results beneficial, as NK cells carry out a cytotoxic effect against cancer cells. On the contrary, melatonin inhibits IL-6 and TNF-α, which are pro-inflammatory cytokines (48). IL-6 is involved in a pathway that requires JAK-1 and STAT phosphorylation, which will consequently promote transduction of genes involved in proliferation and survival of cancer cells. TNF-α, when found at low concentrations, can have pro-oncogenic properties (53) which are counteracted by melatonin's effect.

Another important process that allows cancer cells to proliferate and to disseminate throughout the body is the growth of new blood vessels, a process known as angiogenesis. Many substances play a role in this process, being endothelin 1, with both direct and indirect impact on vessels, an important component. The indirect pathway involves activation of HIF-1α and the subsequent formation of VEGF. Melatonin downregulates the expression of VEGF and endothelin-converting enzyme 1 (ECE-1) (52), responsible for the creation of endothelin 1. As a result, angiogenesis is suppressed. Other important

molecules that contribute to cancer invasion are also downregulated with melatonin. These, as already mentioned, are Twist1, Survivin, Snail and Slug, which induce EMT and thus, cell migration. Twist1 promotes angiogenesis and metastasis, being regulated by the Akt signaling pathway which is also inhibited by melatonin. On the other hand, survivin carries out its pro-angiogenic effects by increasing VEGF secretion (54). In conclusion, melatonin inhibits angiogenesis by directly interacting with vessel-forming proteins, as well as suppressing molecules that induce EMT and trigger cell migration.

4.7 Antitumoral properties in hormonal tumors

Hormonal tumors are those that are heavily influenced by hormones, as they have specific receptors for them to bind to. Ovarian, breast, prostate or uterine cancers are some well-known hormone-dependent tumors. In order for these tumors to grow, they will need estradiol, progesterone or androgens.
Before, it was mentioned how melatonin is able to inhibit gonadotropins at a pituitary level, as well as estrogen synthesis at the gonads. FSH and LH will secrete estrogen and progesterone, so it is clear then how these effects could be broadened towards the understanding of the antitumoral properties in cancers that are estrogen-dependent.

4.7.1 Breast cancer

As of today, according to statistics from the Global Cancer Observatory, breast cancer is the most common type of cancer around the world. It represents around 11% of all cancer diagnosis, with more that 2 million cases, surpassing even lung neoplasms (55).

Not all breast cancers are positive for estrogen and/or progesterone receptors (ER and PR, respectively). Of all breast cancers, about 70% are positive for at least one hormone receptor (56) (also named luminal) and they rely on estrogen and/or progesterone for growth and survival. It is complicated to study in an isolated manner PR+ tumors, due to the fact that many of these receptors are expressed secondarily to ER responses. Therefore, concerning hormonal breast cancers, most studies in literature only investigate ER+ cancers or those positive for both ER and PR. The influence that melatonin has on these hormone-dependent tumors has been thoroughly investigated throughout the years in multiple different studies.

Melatonin carries out anti-tumor actions towards hormone-dependent cancers in several different ways. In MCF-7 cells, a human hormone-dependent breast cancer cell line (ER+ and PR+), cells would become arrested through the cell cycle in G_o/G_1 when melatonin was administered (57). This was, at least in part, accomplished through the activation exerted on p53, promoting apoptosis, as was previously mentioned in this review.

In addition, when the pineal hormone binds to receptor MT_1 in breast cancer cells, the binding of the estradiol-ERα complex to the estrogen response element (ERE) in DNA becomes blocked (58). This is believed to be the result of the

already described interaction between melatonin and calmodulin, since calmodulin binds to ERα and the hormone acts as a calmodulin antagonist. The interaction limits the otherwise stimulatory effect of estrogen in the hormone-dependent breast cancer. Therefore, it could be concluded that melatonin not only acts towards the epiphysis or gonads, but also directly towards cancer cells.

In another line of investigation, breast cancer risk was associated with presence of light during nighttime, since melatonin concentrations are lowered in this situation. At night, when melatonin levels are high, MT_1 receptors in cancer cells will be activated and all the anti-neoplastic mechanisms already described will be carried out. Therefore, cancer growth will follow a circadian-rhythm, growing at a lower rate at night (due to inhibition of estrogen synthesis and estrogen dependent pathways, among other mechanisms) and proliferating faster during the day. This pattern is reinforced when melatonin is administered exogenously right before nighttime. When the pineal gland is removed, as demonstrated in several animal studies, growth of the hormonal tumor will be consistent throughout the 24 hours of the day (59). Following this notion, investigators have researched if long night-shifts of work are related to increased breast cancer risk. The association has not been clearly identified in any clinical trial yet, although the evidence obtained throughout cohort, observational and animal studies is undeniable. The theorized mechanism that takes place seems to involve alteration in certain genes that are regulated by the circadian clock. Among the clock genes associated with breast cancer, CLOCK, CRY1/2 and PER3 (60) are stimulated by melatonin, as will be described in further sections.

4.7.2 Prostate cancer

Prostate cancer is one of the most common types of cancer among male population aged above 65, having a greater incidence in more developed countries. It is influenced by androgens such as dihydrotestosterone (61), the active form of testosterone.

Many studies have demonstrated the effect that melatonin has on prostate cancer. One mechanism described is that melatonin seems to confer the androgenic receptor (AR) found in cancer cells a different allocation (49). Normally, the AR is localized in the cytoplasm and will move into the nucleus when the androgen binds to it, in order to activate androgen-related genes. Melatonin prevents this translocation from happening and thus, causes androgen-dependent cancers to not grow or survive as they otherwise would with the correct androgenic supply.

Additionally, through melatonin's MT_1 receptors, several changes within the prostate cancer cell have been discovered. For example, pro-apoptotic factors like p38 and p53 are activated through phosphorylation and inhibition of negative regulators, respectively. Moreover, some specific micro-RNAs involved in epigenetic modulation are also upregulated (such as miRNA374b and miRNA3195). This effect decreases VEGF and HIF-1α levels, involved, as previously described, in angiogenesis. Furthermore, the binding of melatonin to MT_1 also inhibits the expression of the epidermal growth factor, necessary for tumor growth and proliferation (58).

In conclusion, numerous *in vivo* and *in vitro* studies prove how melatonin inhibits initiation of carcinogenesis, tumor proliferation and angiogenesis in prostate cancer.

4.7.3 Ovarian cancer

Ovarian cancer is the neoplasm with the worst prognosis when discussing cancers affecting the female reproductive system. It is known that this cancer is dependent on estrogen, progesterone and even testosterone. While breastfeeding and estrogen-based oral contraceptives confer protection against ovarian cancer, nulliparity, endometriosis, post-menopause status and increased age are risk factors (62).

Concerning ovarian cancer, results are not as consistent as in prostate or breast cancer, but they are, nevertheless, promising. Compared to same-aged females, those diagnosed with ovarian cancer are seen to have lower levels of melatonin (49).

Furthermore, studies conducted with rats which were administered ethanol (as a pro-carcinogenic agent) showed how melatonin was able to reduce ovarian cancer prevalence (63). The effect was accomplished through the induction of pro-apoptotic pathways (caspase-3 and p53) and reduction of survivin and Bcl-2 expression (64). The observed decrease of other mesenchymal markers will be discussed in its corresponding chapter, demonstrating even further the association between melatonin, ovarian cancer and EMT.

4.8 Melatonin as adjuvant in cancer treatments

Chemotherapy (CT) and radiotherapy (RT) are sometimes administered in cancer in hope of some kind of tumor regression. They can be used in combination with surgery or by their own and many different situations can take place. For example, some tumors might be unresectable, being these therapies the most convenient option. Alternatively, the tumor might be extremely large and CT could be advantageous in order for tumor size to be reduced before resection. Another indication for the use of adjuvant CT or RT would be if surgery is not capable of getting rid of the whole carcinogenic mass or if lymphadenectomy has been performed.
Hormonal therapies (HT) are also available and are clearly only effective if the cancer is hormone-dependent. Different receptors are targeted with these treatments, such as estrogen, progesterone or testosterone receptors.

The fact that some cancer lines are unable to retreat even after CT, RT or HT could imply that some kind of resistance has been developed. Here, melatonin can play a very important role, as it has been documented by several studies that the indoleamine is not only able to potentiate tumor regression, but also diminish side effects when associated to these therapies.

One of these mentioned studies demonstrated in MCF-7 breast cell lines, how, when tamoxifen (a selective estrogen-receptor modulator) was administered when circadian rhythms were altered, the tumor continued to progress. Circadian rhythms were disturbed by inducing light at night, thus decreasing melatonin concentration. The fact that the tumor showed no response, hints to the fact that when melatonin levels are low or absent, breast cancer is resistant to tamoxifen. Conversely, when these same cells were given melatonin in addition to tamoxifen, tumor growth not only stopped, but even clearly decreased (65). Furthermore, in this same study, in agreement with results obtained in previous investigations, it was seen that tumor onset sped up if light was present at a time when there should have been complete darkness (i.e., when melatonin secretion was inhibited) (49). It has also been described, in estrogen-dependent cancer cells, how melatonin is still capable of inducing apoptosis and inhibiting angiogenesis when concomitantly administered with tamoxifen (54).

Similarly, melatonin also seems to enhance tumor sensitivity towards GnRH analogues like triptorelin, used in prostate cancer to reduce testosterone levels. Therefore, melatonin could be efficient when prostate cancer becomes resistant to HT such as triptorelin. Moreover, results also imply that with melatonin's inclusion in the treatment, tumor growth is diminished, as well as toxicity created with this HT (58), such as restoration of thrombocytopenia to normal platelet levels.

Multiple researchers have proven the beneficial effect melatonin can have when combined to both CT and RT. The indoleamine has been combined with chemotherapeutic agents such as doxorubicin (an anthracycline antibiotic) in rats, where a higher survival rate and diminished tumor growth was observed. In addition, when treating cancer cells simultaneously with docetaxel and melatonin, inhibition of Bcl-2 and stimulation of Bax was observed, thus promoting apoptosis. Some other agents that have been investigated, where melatonin either acts as a synergist or limits its side effects, are cisplatin, etoposide, 5-FU, gemcitabine, valproic acid and sorafenib (58). However, despite the results of these studies being undeniable and very promising, little information is still yet known about the exact molecular effects melatonin has when integrated with CT. Oxidative stress, DNA alteration and creation of new blood vessels are typical adverse reactions seen after RT. It has previously been stated how melatonin is able to neutralize free radicals, protect DNA from possible damage and inhibit angiogenesis. Therefore, it could be said that melatonin neutralizes some side effects generated by RT. The underlying mechanisms are clear when *in vitro* studies have been carried out, whereas results are insufficient and even contradictory in clinical trials. Specifically in breast cancer cells that were radiated, melatonin decreased both activation and expression of enzymes like aromatase, 17 β-HSD1 and sulfatase (all involved in estrogen synthesis), suggesting its application as a tumor inhibitor in estrogen-dependent cancers. Other effects melatonin exerted associated to RT, that also reduced estrogen levels, were the increase in the rate of differentiation of fibroblasts to mature adipocytes and the inhibition of COX-mediated pathways. Additionally, expression of several angiogenic factors like Akt1, VEGF-A, TGFα and IGF-1 was also diminished (54).

In the MCF-7 cell line, melatonin increased p53 expression in those groups additionally undergoing RT, potentiating the apoptotic effect radiation can have on cancer cells. A higher number of them were also arrested in the cell cycle, preventing further tumor proliferation from occurring (58).

In conclusion, the effects that melatonin can have when combined with CT, RT or HT seem to be beneficial, as angiogenesis, tumor growth and side effects are reduced, while cell apoptosis and tumor sensitivity to treatments are potentiated. However, all of these results have been demonstrated in animal or *in vitro* studies, being the data gathered from human studies very scarce. Further investigation in this direction is essential, since results have the potential to search for and create new therapeutic targets.

5. CHAPTER FOUR: MELATONIN AND THE EPITHELIAL-MESENCHYMAL TRANSITION

Up until now, it has been discussed melatonin's oncostatic role and the implication of EMT within the human body. EMT is associated with carcinogenesis, as it has been consistently demonstrated in several works discussed in the different sections of this review. On the other hand, the pineal hormone is sometimes described as an anti-oncogenic substance. Therefore, it is not surprising that many researchers have tried finding an interconnection between the two of them. There is sufficient evidence correlating EMT with melatonin and their relationship will be exposed in this chapter.

5.1 Melatonin and EMT markers

Several studies using the MCF-7 cell line have shown that, when melatonin is administered, expression of E-cadherin increases, while N-cadherin and vimentin decrease (66). To what has been exposed so far, it is known that E-cadherin is associated with an epithelial phenotype and on the contrary, the latter with a mesenchymal phenotype. Regarding cancer, the mesenchymal phenotype will be more prone to invasiveness and will ultimately lead to a poorer prognosis. On the other hand, the epithelial phenotype is preferable, as adherens and tight cell junctions make cancer cell dissemination harder. Therefore, it seems melatonin reduces malignant traits such as metastasis in breast cancer cell lines. It has been suggested with plentiful data that the increase in E-cadherin and decrease in vimentin is secondary to a GSK-3β activation. When GSK-3β is activated, the degradation and thus, lack of translocation of β-catenin towards the nucleus will occur. In the nucleus, several EMT-associated transcription factors would otherwise be expressed, one of them being Snail, which is an E-cadherin repressor. Consequently, if β-catenin is eliminated by melatonin's presence like it is being proposed, Snail will not be activated and therefore E-cadherin expression will increase (66).

Stem-like cancer cells have a greater therapeutic resistance, as well as being more invasive and pro-metastatic than ordinary cancer cells. They have a mesenchymal phenotype and hence, can be transformed through an EMT.

Alluding what was previously stated, these cells express $CD44^{+}/CD24^{-/low}$, which is a marker for stem cells in breast cancer. Studies have shown that all of these $CD44^{+}/CD24^{-/low}$ cells also display octamer-binding transcription factor 4 (OCT4). OCT4 is a protein that preserves the stem cell's undifferentiated nature. It was seen how melatonin decreased OCT4 expression, being OCT4 associated to worse prognosis and higher resistance in several types of cancer (colorectal, prostate, glioma and lung cancer) (66). In addition, investigations regarding cancer stem cells in ovarian cancer have also shown similar results. Firstly, it was seen how the indoleamine was able to decrease Ki67 levels in ovarian cancer stem cells, as well as inducing apoptosis by increasing p53 (67). Overexpression of Ki67 would otherwise lead to higher cancer proliferation, invasion and metastasis. Hence, its inhibition sheds extra light on melatonin's possible anti-metastatic mechanisms. Moreover, when melatonin was administered in both ovarian cancer stem and non-stem cells, Snail, vimentin and ZEB1/2 decreased, while E-cadherin increased. Lastly, this same study reported inhibition of both MMP9 and MMP2. These metalloproteases cleave TGF-β from its inactive form, activating different possible pathways that will eventually induce EMT. It is then clear that melatonin does indeed play a role in not just ordinary cancer cell lines (from ovarian and breast cancer), but also in stem cells, which are those that are associated with the worst malignant traits (67).

Furthermore, in agreement with the results exposed above, in mice exposed to light at night and bearing MCF-7 xenografts, E-cadherin was repressed, while vimentin, Snail and β-catenin were induced. When endogenous melatonin was elevated, the opposite occurred (epithelial markers increased, while the mesenchymal ones decreased) (68). Therefore, all these results prove that *in vivo* data matches those of *in vitro* studies.

Aside from the MCF-7 breast cancer cell line, which is estrogen and progesterone dependent, a HER2+ cell line (named SKBR-3) has also been used as model. When a cancer is said to be HER2+, it implies that it tests positive for the human epidermal growth factor receptor 2. This type of breast cancer is associated with greater risk of metastasis and a more aggressive phenotype, compared to those which are HER2 negative. HER2 is involved in signaling cascades where ERK, MAPK and Rsk2 play an important role, and induction of EMT and metastasis are some outcomes their activation leads to. In addition, higher levels of Rsk2 are correlated with different neoplasms formation, as well as being present in about half of breast cancers. Moreover, when Rsk2 expression is suppressed in knockdown animals, metastasis decreases. Rsk2 phosphorylates Creb, a transcription factor which has been associated to malignant traits such as CT resistance and greater tumor promotion and progression. Other known pro-metastatic and EMT-related molecules that can be activated secondary to Creb phosphorylation are Stat-3 and Fascin. Mao et al. (68) demonstrated how exposure to light during nighttime completely suppressed melatonin production, which increased Erk, Rsk2, Creb, Stat-3 and Fascin expression. When melatonin was exogenously administered in SKBR-3 cells, or even when endogenous melatonin was present (when mice stayed in complete darkness) all these factors were inhibited. In MCF-7 xenografted mice, the same results were achieved. This anti-metastatic effect was achieved through MT_1 receptors, as MT_1 antagonists accomplished opposite results. In summary, the obtained results show that in

both HER2+ cell lines and in animals with HER2+ cancer xenografts, light exposure at night activates oncogenes, while presence of melatonin inhibits them.

Not only does melatonin act upon ER+/PR+ and HER2+ breast cancer cell lines, but it has also been recently demonstrated a role in the most aggressive subtype, in triple negative mammary cancer cell lines, such as CF41. CF41 cells are obtained from canines, and when Custódio et al. treated them in their study with melatonin and TGF-β silencing, E-cadherin and claudin-7 expression increased and N-cadherin and vimentin decreased. Additionally, in this same study, reduction of cell migration was also achieved (69).

Moreover, in recent studies, similar results have been obtained in carcinomas different than breast cancer. When melatonin was exogenously administered in endometrial adenocarcinoma cells, vimentin and Slug expression decreased, while E-cadherin and Numb increased. This last protein is involved in neurogenesis and has been associated with the inhibition of the Notch pathway. The outcome observed in the endometrial adenocarcinoma cells (diminished proliferation, invasion and migration) was achieved by melatonin through the suppression of the EMT via inhibition of 17β-estradiol (70). The same occurred in endometriotic endometrium when melatonin was administered. EMT markers and subsequently, invasion, proliferation and migration were reduced (71). This last study was also able to identify in endometriotic cells an increase in mesenchymal markers like Notch1, Slug, Snail and N-cadherin and a decrease in E-cadherin and Numb. Therefore, these results suggest that EMT could play an important role in the pathogenesis of endometriosis, establishing melatonin as a potential treatment. Another protein, other than Numb, that is heavily associated with the Notch pathway is Delta-like Ligand 4 (DLL4) present in ER+ breast cancers. In a study by Rajabi et al. it was seen how melatonin was able to diminish DLL4 expression in estrogen-dependent breast cancer cells, as well as being able to induce apoptosis (72). It was then proposed an alternative pathway in which melatonin is capable of inhibiting EMT, by avoiding DLL4 from interacting with Notch receptors and thus, limiting angiogenesis and cancer progression.

In another study investigating the relationship between lipopolysaccharides (LPS), prostate cancer and melatonin, it was observed how melatonin was able to inhibit migration and invasion of prostate cancer cells, regardless of whether they were induced by LPS or not. This was carried out by blocking EMT through different mechanisms: inhibition of inflammatory cytokines such as IL-6, inhibition of Stat-3 transcription factor (similarly to what was observed in HER2+ breast cancer cells) and suppression of Akt, GSK-3β and β-catenin pathways (73).

Many investigations regarding ovarian cancer and melatonin were already performed, however, in 2020, Bu et al. conducted a study where it was described for the first time the mechanism associating chronic restraint stress (CRS)-mediated metastasis of epithelial ovarian cancer and melatonin. Results showed how norepinephrine secretion, secondary to CRS, stimulated abdominal metastatic implantation of ovarian cancer, as well as increasing EMT markers such as Slug, Twist, Snail and β-catenin. In addition, melatonin was able to block the Akt/β-catenin/Slug signaling pathway and thus, decrease these

norepinephrine-promoted implantations (74), presenting itself as a possible therapeutic agent in ovarian cancer related to CRS.

Finally, another cancer type that has been recently studied in association with melatonin is lung cancer. Data revealed that a higher Twist expression in lung cancer cells correlated with a higher CD133 expression, which is another biomarker for stem cells. Melatonin was able to reduce levels of both biomarkers (Twist and CD133) through the inhibition of p38/ERK and β-catenin pathways (75). The results obtained are consistent with those found in a more recent study, where high Twist levels were paralleled to a worse lung cancer stage and a lower MT_1 expression. Additionally, melatonin, by interacting with receptor MT_1, was able to limit Twist expression, which led to fewer liver metastasis *in vivo* (76). Therefore, these two studies showed evidence supporting melatonin's anti-metastatic effect by inhibiting EMT in lung cancer, which is one of the most prevalent types of cancer worldwide with a very high metastatic rate.
In conclusion, it can be said that the abundance of obtained data points towards potential mechanisms explaining melatonin's anti-oncogenic role. It could be either through direct inhibition of EMT-inducing pathways or through inhibition of other molecules which are involved in cancer progression and invasion. Either way, melatonin's effects arise in different types of cancer cell lines, including different breast cancer subtypes (ER+ and PR+, HER2+ and triple negative), ovarian, prostate, endometrial and lung cancer.

5.2 Melatonin and EMT in tumor microenvironment

Tumors are not just a solid mass with no interaction with its surroundings. In fact, tumors are in constant synergy with their microenvironment and will actually secrete multiple cytokines, as well as having a close relationship with other cells. Dendritic cells, lymphocytes, cancer associated fibroblasts (CAFs), tumor associated macrophages (TAMs) and endothelial cells are part of this microenvironment. Both immune and non-immune components will be decisive in the tumor's ability to grow and proliferate. Some are proto-oncogenic (TAMs, CAFs, endothelial cells, regulatory T cells), while others are anti-oncogenic (T helper cells: CD4+, cytotoxic T cells: CD8+, NK cells).

It has been suggested that melatonin inhibits transformation from conventional macrophages into TAMs (77). TAMs will release cytokines such as IL-10, IL-13, IL-4 and TGF-β. They will also induce secretion of free radicals and this, together with the interleukins, will facilitate angiogenesis and tumor proliferation.

The fibroblasts, or CAFs, on the other hand, will also produce a great amount of TGF-β, as well as VEGF, MMP2 and MMP9. CAFs have been associated to the most malignant types of cancer, especially in pancreatic cancer, and in less extent ovarian and breast cancer. The secretion of these cytokines by the fibroblasts will not only induce EMT, but also metastasis and angiogenesis. It has been demonstrated how melatonin is able to inhibit CAFs directly, as well as reducing the substances secreted by them and the unwanted interactions with other cells (77). TAMs, CAFs and regulatory T cells are also able to inhibit the activity of NK and CD8+ cells, which act as anti-tumor cells.

In addition, in MCF-7 breast cancer cells and in fibroblast-like cell lines, melatonin administration reduces expression of TNF-α, IL-1 and IL-11 (78). These results further on suggest the plausible relationship melatonin has with the microenvironment, specifically with the formation of fibrous tissue and the secretion of certain cytokines.

On the contrary, as previously stated, NK cells and CD8+ lymphocytes will secrete substances on a mission to suppress cancer cells. Secretion of IFN-γ and TNF-α help achieve this goal, and their release will be further reinforced by melatonin. These two molecules will ultimately activate caspases like caspase-3, leading to apoptosis of the cancer cells. In addition, melatonin is able to increase IL-2 levels, which strongly stimulate NK cells. When the pineal hormone is administered in already advanced cancers, rise in NK cell number can be observed, indicating the possible development of a heightened immune system. Furthermore, melatonin stimulates CD8+ cells, while also decreasing regulatory T lymphocytes (77).

These results show how melatonin is not only involved in reducing tumor growth itself, but also in regulating the many connections different types of cells have with the neoplasm. The hormone is able to potentiate anti-oncogenic mechanisms, while at the same time inhibit those that will promote cancer survival, angiogenesis and metastasis.

5.3 Melatonin, EMT and micro-RNAs

Micro-RNAs (or miRNAs) are single-stranded non-coding RNAs that regulate gene expression at a post-transcriptional level. There are a thousand types of miRNAs, and as discussed in previous chapters, some of them have been associated to each side of the EMT spectrum. The family of mir-200 are mostly found on the epithelial side of the transition and as figure 7 represents, they block ZEB1/2 expression. On the other hand, mir-10b and mir-21 are two subtypes that potentiate EMT, and are therefore commonly linked to mesenchymal cells. As can be seen on figure 7, there are several more that have been associated with the transition, each one with different targets, with an ultimate objective of either inhibiting or promoting an EMT.

Apart from associating miRNAs to specific EMT biomarkers, researchers have also hypothesized a relationship between miRNAs and different cancer types. Regarding breast cancer, Liu et al. came to the conclusion that FKBP3 and lnc010561 were downregulated by melatonin. FKBP3 codes for a binding protein important in immunoregulation, while lnc010561 is a transcript that does not encode a protein (long non-coding RNA). Both the binding protein and the long non-coding RNA are firmly related to and regulated by mir-30. When they are suppressed by melatonin, breast cancer proliferation and invasion are inhibited, while apoptosis is stimulated. Therefore, the study shows how the indoleamine is capable of downregulating certain genes related to EMT that will, at the end, suppress tumor progression in breast cancer cells. In figure 7, mir-30 is depicted as an inhibitor of the EMT factor Snail, thus being able to act as a tumor

suppressor. The micro-RNA has also been associated to inhibition of cell growth and metastasis, and consequently to a better prognosis too (80).

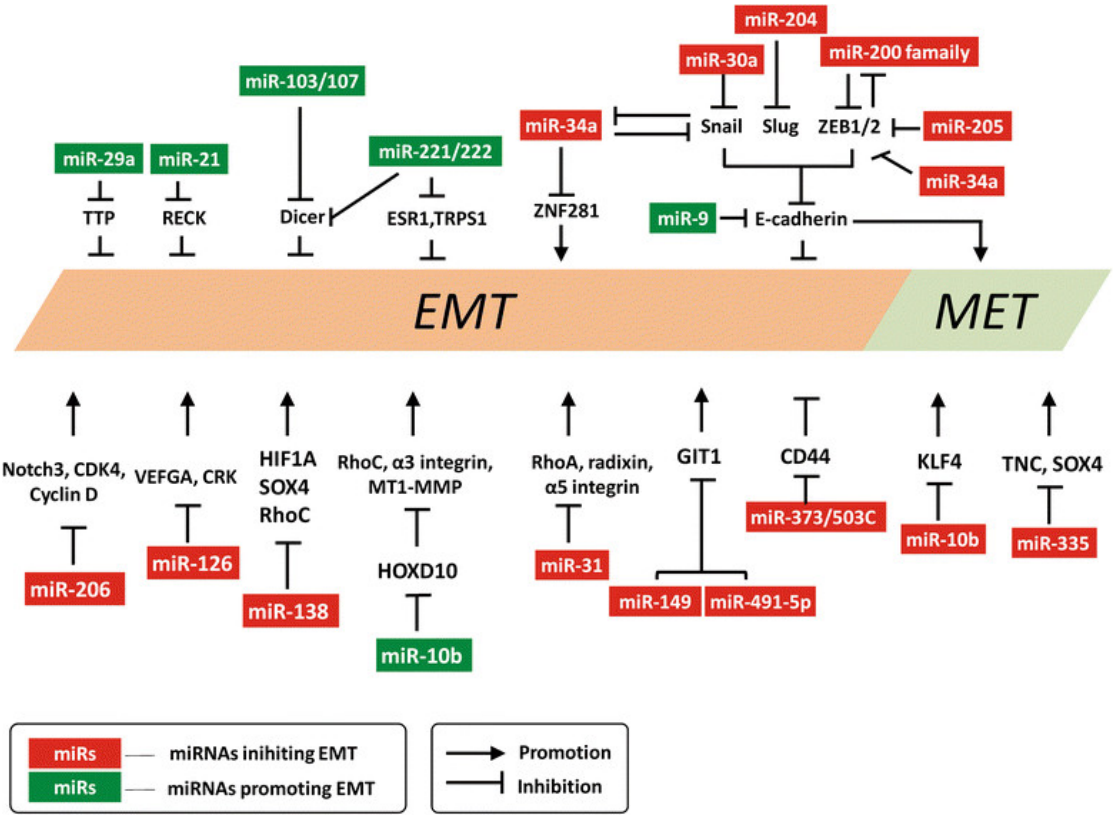

Figure 7: Micro-RNAs involved in the EMT and MET processes. *Some of the micro-RNAs that have been related to EMT and MET can be seen. In green, those that promote the transition and in red, those that inhibit it. EMT biomarkers that have already been described in this review, such as E-cadherin, Snail, Slug, ZEB1/2, VEFGA, cyclin D, Notch, MMPs are also illustrated. Obtained from: Chan et al., Lu-Hai. 2015. Journal of biomedical science. 22. 9. DOI: 10.1186/s12929-015-0113-7.*(79).

Similar results were obtained when studying triple negative breast cancer. Melatonin was able to inhibit breast cancer proliferation and metastasis by suppressing a different pathway: the lnc049808/mir-101/FUNDC1 axis. FUNDC1 was upregulated in breast cancer cells and its knockdown limited cancer progression. Like mir-30, mir-101 acted as a tumor suppressor and has previously also been associated to anti-oncogenic roles in many other cancer types (gastric, hepatocellular, osteosarcoma and cervical cancer) (81). Both studies proved that lnc010561/FKBP3 and lnc049808/FUNDC1, which are inhibited by melatonin, function as competing endogenous RNAs (ceRNAs) for mir-30 and mir-101, respectively. CeRNAs have recently been discovered as part of a network in which miRNA levels are regulated. Therefore, when melatonin inhibits these ceRNAs, miRNAs become available to undertake their anti-neoplastic roles. In conclusion, these results provide another reason as to why melatonin should be considered an alternative treatment for breast cancer.

The effect of melatonin has also been studied in another type of neoplasm: colorectal cancer. In 2019, Sakatani and colleagues demonstrated how sensitivity

towards 5-fluorouracil (5-FU) in resistant cells was regained after melatonin administration. It was seen how melatonin was able to stop cell growth, stimulate apoptosis and enhance the chemotherapy's cytotoxicity when administered in combination with 5-FU. Results also showed how expression of thymidylate synthase decreased, while expression of mir-215-5p was increased. The enzyme has been related to increased resistance to 5-FU in cancer, and has therefore also been associated to a worse prognosis for patients. Several micro-RNAs are known to decrease levels of thymidylate synthase, such as mir-203, mir-433 and mir-215-5p, being the latter the most significant one. This suggests melatonin's actions are achieved through the promotion of mir-215-5p expression, which blocks thymidylate synthase and in turn leads to a regained sensitivity to 5-FU (82).

NEDD9 is a molecule that promotes proliferation and metastasis in head and neck, breast and colorectal cancer. When mir-25-5p binds to the mRNA region of the NEDD9 molecule, its pro-oncogenic actions are inhibited. A recent study concluded that, when melatonin was administered in association with pterostilbene (commonly found in grapes and blueberries), overexpression of mir-25-5p was achieved. Parallel to this result, an increase in ROS, SOX10 and caspase-3-dependent apoptosis was also observed. SOX10 is known to suppress the Wnt/β-catenin pathway and thus, inhibit EMT from occurring. On the other hand, NEDD9 expression was reduced, as well as Ki67, which acts as a proliferation marker in tumors (83). In conclusion, results demonstrated how association of pterostilbene with melatonin synergistically potentiated the effect of the indoleamine. Both substances limited EMT in colorectal cancer cells by the overexpression of anti-neoplastic molecules (ROS, SOX10, caspase-3, mir-25-5p) and the inhibition of proto-oncogenic ones (NEDD9). A study conducted two years later showed similar results with different mir-RNAs. Here, it was seen how melatonin significantly reduced colony numbers in colorectal cancer cell lines (i.e., inhibiting proliferation and viability) and stimulated apoptosis by increasing levels of caspase-3. In mice bearing colorectal cancer xenografts, tumor growth and Ki67 expression were also inhibited after melatonin administration. The proposed mechanism was the upregulation of the mir-34a/449a cluster by the pineal hormone, which in turn was seen to negatively regulate Bcl-2 and Notch expression (84).

So far, we have discussed studies concerning micro-RNAs in breast and colorectal cancer, but some results have been found in other types of cancer, such as oral cancer. In one study, results concluded that in the presence of melatonin, mir-155 in oral cancer cells was reduced, while mir-21 was increased. Mir-155 has been associated to a worse prognosis in patients with oral carcinoma. In figure 7 it can be seen how mir-21 is in fact a micro-RNA that has been correlated to EMT promotion, meaning it also promotes carcinogenesis. These results seem contradictory with the vast amount of evidence that supports melatonin as an anti-neoplastic hormone. The researchers theorized a plausible reason could be the multiple binding sites mir-21 contains for different transcription factors (85). In another hand, Hsieh and colleagues demonstrated how melatonin was also able to induce apoptosis in vincristine-resistant oral cancer cells. Cells also experienced an increase in sensitivity to the chemotherapeutic agent, which is consistent with the multiple other studies that

have been reviewed so far in this document. These outcomes were achieved through the upregulation of mir-892a and mir-34b-5p among other molecules, both mediated, at least partially, by Akt and p38 activation (86).

Coincident data has been obtained in glioblastoma cells, where it is known that different micro-RNAs involved in carcinogenesis responded to melatonin. Ultimately, the hormone suppressed cell proliferation through the inhibition of HIF1-α, VEGF and MMP-9, all of which are EMT-promoting factors (87). Melatonin also suppressed VEGF and hence, angiogenesis and tumor proliferation, in osteosarcoma cells by inhibiting mir-424-5p (88). Another study centered around osteosarcoma cells found out that a type of long non-coding RNA, named lncRNA JPX, was not only upregulated in these cells, but also played fundamental roles in tumor growth and proliferation. It was seen that when osteosarcoma cells were treated with melatonin, lncRNA JPX expression was inhibited and, as a consequence, also metastasis and cell growth. Further investigation led to the conclusion that this inhibition was carried out through the suppression of the Wnt/β-catenin pathway, which, as stated earlier, is an EMT-inducing pathway (89).

Evidently, most recent studies are mainly focused on carcinomas, but interesting results were also obtained in radiation-induced lung injury. When a more action-prolonged form of melatonin (in a nanoparticle structure) was administered to lung cells damaged by radiation, caspase-3 levels were reduced. In addition, through the downregulation of mir-21 expression, decrease in inflammatory mediators (TNF-α and IL-6), TGF-β1 and Smad3 was also achieved (90). These results are concordant to those published in a study conducted in 2016 involving idiopathic lung fibrosis. It was reconfirmed how melatonin exerted a protective effect through the inhibition of two EMT-inducing pathways: Wnt pathway and Smad2/3 (TGF-β pathway) (91). Therefore, both studies illustrate different plausible underlying mechanisms in which melatonin can act as a type 2 EMT/fibrosis inhibitor. Additionally, melatonin's anti-inflammatory properties in lung fibrosis, whether this was idiopathic or secondary to radiation, were once again corroborated.

5.4 Melatonin, circadian rhythms and EMT

In the previous chapter dedicated to melatonin, it was mentioned how the hormone is regulated through circadian rhythms, meaning its concentration, and ergo effects, fluctuate throughout the 24 hours of the day. Not only the secretion of many hormones like melatonin, glucagon, insulin or cortisol is dictated by these intrinsic rhythms, but also the mechanistic actions of many cells in the immune system undergo circadian fluctuations. When this circadian rhythm is disrupted (for example during night shifts or when suffering jet lag after a long flight), the homeostasis in one's body becomes affected. These hormones will not reach the adequate levels required to exert their functions and thus, cells will not be as activated as they should, leading both to undesirable and detrimental outcomes. Evidence supports the debut of endocrinological diseases, such as type 2 diabetes and cardiovascular pathologies, in the context of circadian disruption. Not only are diseases more prone to appear, but also endocrine tumors have

demonstrated to be more prevalent in this scenario. To date, five of the ten cancers with highest worldwide incidence are endocrine (these are breast, prostate, cervix, uterus and thyroid) (55). As a matter of fact, the International Agency for Research on Cancer has classified night shift work, through disruption of the circadian rhythms, as a type 2A carcinogen.

Hadadi et al. reviewed the relationship between the circadian disruption (i.e., decreased levels of melatonin) and EMT-induced tumors. There are several clock genes which were already mentioned earlier in this document like CRY1, CRY2, NPAS2, PER3 and CLOCK that are related with greater breast cancer incidence (60). Others like PER2 and BMAL1 are associated to EMT transcription factors. The inhibition of the former in breast cancer cell lines results in EMT induction through Twist1, Snail and Slug, increasing stemness and invasiveness. The downregulation of the latter in colorectal cancer cells however, shifts the transition to the epithelial phenotype, resulting in more benign traits like higher treatment sensitivity and less tumor invasiveness (92). This same group found out that mice that had circadian rhythms disrupted, had much more incidence of mammary gland carcinoma and lung metastasis than those rodents that were exposed to equal times of light and darkness. In the cancer-affected mice, EMT genes like ZEB2, INHA (TGF-β gene) and CDH2 (N-cadherin gene) were overexpressed, while PER genes were downregulated.

As previously recalled, IL-1β, TNF-α, IL-6 and TGF-β are all secreted by the tumor itself and its microenvironment, and ultimately all lead to EMT's induction, mostly through ZEB and Snail activation. Circadian dysregulation and low levels of melatonin will increase these pro-inflammatory cytokines (92) and consequently, increase the transcription factors responsible for the mesenchymal side of the transition in cells. The tumor microenvironment is further on altered by circadian dysregulation through the immune cells previously mentioned (NK cells, regulatory T cells, macrophages and fibroblasts), which are also controlled and regulated in an approximate 24-hour cycle.

Moreover, a study investigating the possible carcinogenic effects that night-shift work might have towards breast cancer, demonstrated how melatonin was able to reduce expression of MMP2 and MMP9, as well as decreasing activity of p38-related pathways (60). Inhibition of p38 is related to an increase in E-cadherin, as well as a decrease in Snail levels, secondary to gene expression becoming modified with TGF-β signaling cascades (29). The down-regulation of these factors adds to the anti-invasive and anti-proliferative properties of the pineal hormone.

In conclusion, when circadian rhythms do not run properly, concentration of different hormones like melatonin will be decreased. This has consequences on multiple levels, one being the augmented cancer risk that has been numerous times demonstrated in *in vivo* and *in vitro* studies. The risk rises secondary to the increase in EMT induction, by increasing pro-inflammatory cytokines, downregulating clock genes and altering immunological cells. Therefore, it can be postulated that repressors of these mechanisms could be a potential path for cancer treatment. In fact, monoclonal antibodies working as immunomodulators have been already tested for certain tumors. However, further investigation is

required in order for newer pathways to be targeted, such as the tumor microenvironment or clock-regulated genes.

5.5 Melatonin with other anti-neoplastic treatments

In the section “melatonin as adjuvant in cancer treatments” it was described how the hormone seemed to potentiate the anti-neoplastic effect of CT and RT, as well as decrease their toxicity when associated with them. Particularly, doxorubicin, which is an anthracycline class antibiotic, has been studied in association with melatonin. Menendez et al. demonstrated how melatonin counteracted doxorubicin’s side effects, while also enhancing its cytotoxic effects towards cancer cells (93). In this study, the chemotherapeutic agent clearly inhibited cell proliferation in MCF-7 cell lines (which are ER+ and PR+). Results also showed that when melatonin was associated with it, tumor proliferation decreased even further. However, in another cell line which was negative for hormonal receptors, melatonin did not potentiate doxorubicin’s anti-proliferative effect. It then seems, that melatonin’s synergism is only actually effective when hormone-dependent cancers are involved, having limited effect in those that are not regulated by hormones.

Moreover, in this same study, several genes related to cancer progression were inhibited even more when melatonin was introduced in the treatment, such as GATA3, c-Myc or Bcl-2. GATA3’s function lies on the ability to induce EMT through the inhibition of certain micro-RNAs. C-Myc is a widely known proto-oncogen involved in the expression of many genes that will trigger carcinogenesis. Lastly, Bcl-2 is an anti-apoptotic molecule which promotes persistence of cancer cells. On the other hand, Twist1 expression was increased by the anthracycline alone in the MCF-7 cells and, in the presence of melatonin, either alone or combined with doxorubicin, Twist1 was decreased. Twist is responsible for EMT induction through E-cadherin repression, overexpression of N-cadherin, vimentin and fibronectin, as well as MMP secretion and mir-10b activation. All of these outcomes will lead to higher cancer progression and metastasis, generating a cancer with a much worse prognosis. Moreover, Akt (participant in the PI3K/Akt pathway and Twist inducer) and VEGF were also downregulated by melatonin (93), meaning these proteins that are involved in angiogenesis were not expressed. Therefore, the several results obtained in Menendez et al.’s study adds to melatonin’s known anti-oncogenic role in hormone-dependent cancers, as some of the pathways responsible for promoting EMT were inhibited by the indoleamine.

Another previous study also supported these same results and emphasized the importance of adequate circadian rhythms. In it, it was demonstrated how with light exposure during night (i.e., melatonin secretion was suppressed) in mice with MCF-7 tumor xenografts, tumor latency was shortened, growth and metabolism increased and resistance to doxorubicin appeared. Contrariwise, addition of melatonin at night created opposite effects, including the reestablishment of sensitivity to doxorubicin (94). Therefore, this study managed to prove that when circadian rhythms are lost and doxorubicin resistance

emerges, administration of melatonin, and thus readjustment of the circadian rhythm, seems to have anti-oncogenic effects.

Besides doxorubicin, other compounds used in cancer treatment were tested in association with melatonin. Dabrafenib is a kinase-inhibitor type of agent, specifically a BRAF-V600E mutation inhibitor. This mutation is commonly found in melanomas and also recently discovered in a small percentage of anaplastic thyroid cancer. Thyroid cancer is a quite common type of cancer, generally with a good prognosis. The anaplastic subtype is the one exception, as it is highly aggressive and patients usually survive less than an additional year once they are diagnosed. Dabrafenib has been an effective treatment in initial stages, but unfortunately with time the thyroid cancer can develop resistance to it. Liao et al. (95) searched for an alternative with melatonin administration and results are encouraging. It was identified how viability of thyroid cells positive for BRAF-V600E was most inhibited when melatonin was associated with dabrafenib. This seemed to occur through an Akt-dependent pathway, as phosphorylated Akt was reduced with this combination of treatments. Additionally, the synergistic effect of both molecules increased the number of cells that became arrested in G_1 phase, limiting cyclin-D1 expression. Both melatonin and dabrafenib also decreased Bcl-2, while on the contrary, increased Bax and caspase-3 expression. All these events suggest a pro-apoptotic effect in thyroid cancer cells. Invasiveness and cell migration were also inhibited, through E-cadherin expression, together with N-cadherin and vimentin repression. Therefore, it was proven how melatonin in combination with dabrafenib, promoted an epithelial cell phenotype, while also limiting a mesenchymal type one. Finally, it was also demonstrated in the study how levels of human telomerase reverse transcriptase (hTERT) were reduced with the co-administration of both treatments. Back in the review it was stated how melatonin had an inhibitory effect with the telomerase enzyme, which, if active, confers cells uncontrolled proliferation. Hence, the newly detection of this ability to inhibit hTERT reinforces data from earlier studies. In thyroid cancer, expression of hTERT creates more aggressive cancers, as well as augmenting recurrence rates. All of these results enrich the possibilities of acquiring better survival rate responses in the anaplastic subtype, an objective that is truly necessary in this type of cancer.

Cisplatin is another common chemotherapeutic agent, which can be administered as treatment for advanced nasopharyngeal carcinoma. However, a non-negligible number of patients suffer from recurrence and metastasis due to developing resistance to cisplatin. Therefore, it was studied if melatonin helped regain sensitivity to CT in nasopharyngeal cancer cells. Results demonstrated that cells did indeed reverse their chemoresistance, as cell growth, migration and invasion was inhibited with melatonin's administration. Additionally, a correlation between resistant cells and higher levels of β-catenin in the nucleus was found. The underlying mechanism of melatonin's actions was the inhibition of the Wnt/β-catenin signaling pathway, as this study, together with several previous ones, reported that melatonin blocked β-catenin's translocation towards the nucleus. In conclusion, it was reported that melatonin, through the inhibition of the Wnt/β-catenin pathway, reduced chemoresistance to cisplatin in nasopharyngeal cancer cells (96). Therefore, with further investigation, combination of melatonin

and cisplatin could become in the future a plausible treatment for this head and neck type of cancer.

In an *in vivo* study, conducted to examine the effects achieved by the combination of melatonin and radiofrequency ablation (RFA) in lung cancer, it was observed how the hormone potentiated the anti-tumoral properties of the RFA. Melatonin was able to suppress tumor growth by stimulation of NK cell activity, which falls in line with results obtained commented on the section “melatonin and EMT in tumor microenvironment”. EMT-inducing pathways were also inhibited, such as Wnt, Hedgehog and NF-κB/Notch, as well as p53 being upregulated. All these results led to a decrease in tumor growth and malignancy, including in lung areas that had not been ablated. The study demonstrated how with the concomitant systemic administration of melatonin and local RFA, lung damage and cancer recurrence significantly diminished (97). These results offer a new approach in the simultaneous treatment and prevention of multiple lung tumors.

6. CONCLUSION AND PROSPECTS

Melatonin is an endogenous amide mainly secreted by the pineal gland. The hormone is an essential component in the sleep-wake cycle, following a circadian rhythm with maximum levels at night and minimum during daytime. Disruption of its approximate 24-hour cycle can lead to several disorders, such as jet lag, insomnia and even endocrine tumors. Over the years, additional actions, mostly carried out through MT_1 and MT_2 receptors, have been conferred to the hormone. These receptors are predominantly located in the central nervous system, but can also be found in adipocytes, skin, prostate, kidney, heart, arteries, breasts and ovaries. This broad distribution led researchers to wonder if melatonin could in fact have multiple other functions affecting different areas in the human body. Results show that the hormone does indeed possess other properties, such as anti-oxidative and anti-inflammatory ones, as well as being able to act upon carcinogenesis and neurological diseases. Melatonin has also been extensively associated with mitochondria, where it has the ability to neutralize free radicals, which are the core of many inflammatory diseases, including obesity and diabetes.

Despite having multiple functions, its anti-neoplastic role is by far the most promising one. An essential part of this role comes from the inhibition of the epithelial-mesenchymal transition. This transition changes immobile cells into those with greater migratory capability, generating cancers that will eventually be more aggressive, as they invade healthy organs with greater ease. The mesenchymal end of the transition is also associated with greater treatment resistance, meaning cancers that were previously sensitive to chemotherapy or radiotherapy will eventually stop responding to therapy. This is an immense obstacle many medical fields suffer as of today, as most cancer-related deaths are secondarily related to tumor resistance and hence, cancer relapse. For this reason, scientists are constantly searching for new treatments that could be useful in these situations. This is where melatonin must be considered. Studies have shown how melatonin is capable of not only reducing therapy toxicity, but also of augmenting their anti-oncogenic effect (in chemotherapy, radiotherapy,

hormonal therapy and even radioactive ablation). In combination with these therapies, melatonin reduces even further tumor growth, angiogenesis and relapse rates, as well as increasing apoptosis and tumor sensitivity. All these outcomes can be explained by melatonin's direct form of action, like its ability to inhibit angiogenic factors or reactive oxygen species, together with activation of apoptotic pathways and ability to lead cells into cell cycle arrest. Additionally, results can also be explained by the association melatonin has with the epithelial mesenchymal transition. Most markers related to epithelial cells are clearly potentiated with melatonin, while those related to the mesenchymal phenotype are inhibited by the pineal hormone. Not only are the biomarkers altered with melatonin administration, but also the outcomes the repression of mesenchymal cells entails. Tumor proliferation, invasion and metastasis are inhibited, ultimately leading to reduction of the cancerous mass.

Melatonin's inhibition of the epithelial-mesenchymal transition, that has been several times proven in both *in vivo* and *in vitro* studies, can be carried out through the tumor microenvironment. Here, expression of different immune and non-immune cells, with the common final attempt of suppressing carcinogenesis, will take place. In addition, melatonin has been demonstrated to be involved in several EMT-related signaling pathways, such as β-catenin, Notch, Hedgehog and TGF-β, as well as interacting with clock genes and stem cells. Most recent investigations focus on the relationship the hormone has with a type of non-coding RNA known as micro-RNAs. All studies seem to end up with the common outcome of inhibition of tumor progression. Results have been observed in breast, ovarian, prostate, osteosarcoma, thyroid and lung cancer, among others.

In conclusion, melatonin is an endogenous hormone with multiple anti-oncogenic properties that could benefit patients suffering cancer. Additionally, side effects appear to be rare and availability is wide with considerably low costs. Despite having all these advantages, melatonin is still scarcely used in clinical practice. Newer studies and clinical trials conducted in humans are still in need to be performed for the indoleamine to be widely accepted as a tumor suppressor. Implementation of circadian-friendly approaches not only in hospitals, but in everyday life, seems to be the next coherent step. Artificial lights in hospital beds should be limited, especially those with blue wavelengths, as they are the ones that most effectively inhibit melatonin's secretion. Cancer treatments could also be focused on following adequate circadian patterns, and administration of exogenous melatonin at the correct time, before and after chemotherapy or radiotherapy could also be implemented. Furthermore, in the long run, development of specific analogues or antagonists of certain components of pathways involved in the epithelial-mesenchymal transition might be essential to overcome cancer resistance.

7. BIBLIOGRAPHY

1. Laios K. The Pineal Gland and its earliest physiological description. HORMONES. 2017 Dec 26;13(3).
2. Reiter RJ, Tan DX, Galano A. Melatonin: Exceeding expectations. Vol. 29, Physiology. American Physiological Society; 2014. p. 325–33.
3. Zisapel N. New perspectives on the role of melatonin in human sleep, circadian rhythms and their regulation. British Journal of Pharmacology. 2018;175(16):3190–9.
4. Pozaa JJ, Pujolb M, Ortega-Albásc JJ, Romerod O. Melatonin in sleep disorders. Elsevier Sociedad Española de Neurología. 2018;34(5).
5. Young B, Geraldine O, Woodford P. Wheater's Functional Histology. 6th ed. Editors D, editor. Elsevier; 2014. 65–82 p.
6. Nieto MA, Yun-Ju Huang R, Jackson RA, Thiery JP. EMT: 2016. Cell. 2016;166(1):21–45.
7. Zeisberg M, Neilson E. Biomarkers for epithelial-mesenchymal transitions. The Journal of Clinical Investigation. 2009;119(6):1429–37.
8. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. The Journal of Clinical Investigation. 2009;119(6):1420–8.
9. Kiecker C, Bates T, Bell E. Molecular specification of germ layers in vertebrate embryos. Cellular and Molecular Life Sciences. 2015;73:923–47.
10. Eming S, Martin P, Tomic-Canic M. Wound repair and regeneration: Mechanisms, signaling, and translation. Science Transalational Medicine. 2014;6(265).
11. Stone R, Pastar I, Ojeh N, Chen V, Liu S, Garzon K, et al. Epithelial-Mesenchymal Transition in Tissue Repair and Fibrosis. Cell and Tissue Research. 2016;365(3):495–506.
12. Thiery JP, Acloque H, Yun-Ju Huang R, Nieto MA. Epithelial-Mesenchymal Transitions in Development and Disease. Cell. 2009;139(5):871–90.
13. Bakir B, Chiarella A, Pitarresi J, Rustgi A. EMT, MET, plasticity and tumor metastasis. Trends in Cell Biology. 2020;30(10):764–76.
14. Lu W, Kang Y. Epithelial-mesenchymal plasticity in cancer progression and metastasis. Developmental Cell. 2019;49(3):361–74.
15. Alberts B, Johnson A, Lewis J, Morgan D, Raff M, Roberts K, et al. Molecular Biology of the Cell. 6th ed. Lewis SG, Zayatz E, editors. Garland Science; 2015.
16. Holland-Frei J, Weston A, C.Harris C. Multistage Carcinogenesis . In: W Kufe D, E Pollock R, R Weichselbaum R, C Bast R, S Gansler T, F Holland J, et al., editors. Cancer Medicine. 6th ed. 2003.
17. Malarkey DE, Hoenerhoff M, Maronpot RR. Carcinogenesis: Mechanisms and Manifestations. In: Haschek and Rousseaux's Handbook of Toxicologic Pathology. Elsevier Inc.; 2013. p. 107–46.
18. Department of Nutritional Sciences and Toxicology University of California B. Chemical Carcinogenesis: Initiation, Promotion and Progression [Internet]. Berkley Rausser College ; Available from: https://nature.berkeley.edu/~dnomura/pdf/Lecture7Carcinogenesis.pdf
19. Carella F, Feist SW, Bignell JP, de Vico G. Comparative pathology in bivalves: Aetiological agents and disease processes. Journal of Invertebrate Pathology. 2015 Oct;131.

20. Dave B, Mittal V, M Tan N, C Chang J. Epithelial–mesenchymal transition, cancer stem cells and treatment resistance. BioMed Central [Internet]. 2012 [cited 2021 Dec 24];14(202). Available from: http://breast-cancer-research.com/content/14/1/202
21. Gonzalez DM, Medici D. Signaling mechanisms of the epithelial-mesenchymal transition. Science Signaling. 2014 Sep 23;7(344).
22. Nakaya Y, Sheng G. Epithelial to mesenchymal transition during gastrulation: An embryological view. Development, Growth & Differentiation. 2008 Oct 15;50(9).
23. Ribatti D, Tamma R, Annese T. Epithelial-Mesenchymal Transition in Cancer: A Historical Overview. Translational Oncology. 2020 Jun;13(6).
24. de Francesco E, Maggiolini M, Musti A. Crosstalk between Notch, HIF-1α and GPER in Breast Cancer EMT. International Journal of Molecular Sciences. 2018 Jul 10;19(7).
25. Stuelten CH, Zhang YE. Transforming Growth Factor-β: An Agent of Change in the Tumor Microenvironment. Vol. 9, Frontiers in Cell and Developmental Biology. Frontiers Media S.A.; 2021.
26. Dupont S, Morsut L, Aragona M, Enzo E, Giulitti S, Cordenonsi M, et al. Role of YAP/TAZ in mechanotransduction. Nature. 2011 Jun 8;474(7350):179–84.
27. Tang H, Massi D, Hemmings BA, Mandalà M, Hu Z, Wicki A, et al. AKT-ions with a TWIST between EMT and MET [Internet]. Available from: www.impactjournals.com/oncotarget
28. Ma L, Teruya-Feldstein J, Weinberg RA. Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. Nature. 2007 Oct 11;449(7163):682–8.
29. Hao Y, Baker D, ten Dijke P. TGF-β-Mediated Epithelial-Mesenchymal Transition and Cancer Metastasis. International Journal of Molecular Sciences. 2019 Jun 5;20(11).
30. Shang S, Hua F, Hu ZW. The regulation of β-catenin activity and function in cancer: therapeutic opportunities. Oncotarget. 2017 May 16;8(20).
31. Gonzalez DM, Medici D. Signaling mechanisms of the epithelial-mesenchymal transition. Science Signaling. 2014 Sep 23;7(344).
32. Tian X, Liu Z, Niu B, Zhang J, Tan TK, Lee SR, et al. E-Cadherin/β-Catenin Complex and the Epithelial Barrier. Journal of Biomedicine and Biotechnology. 2011;2011.
33. Jamieson C, Martinelli G, Papayannidis C, E. Cortes J. Hedgehog Pathway Inhibitors: A New Therapeutic Class for the Treatment of Acute Myeloid Leukemia. Blood Cancer Discovery . 2020 Sep;1(2).
34. Mannino G, Pernici C, Serio G, Gentile C, Bertea CM. Melatonin and Phytomelatonin: Chemistry, Biosynthesis, Metabolism, Distribution and Bioactivity in Plants and Animals—An Overview. International Journal of Molecular Sciences. 2021 Sep 16;22(18).
35. Tordjman S, Chokron S, Delorme R, Charrier A, Bellissant E, Jaafari N, et al. Melatonin: Pharmacology, Functions and Therapeutic Benefits. Current Neuropharmacology. 2017 Feb 28;15(3).
36. García-Porrero JA, Hurlé González JM. Human Neuroanatomy. Madrid, Spain: Editorial Médica Panamericana; 2015. 369–71 p.
37. Borjigin J, Zhang L, Calinescu A. Circadian Regulation of Pineal Gland Rhythmicity. Molecular Cell Endocrinology. 2011;349(1):13–9.

38. Bonmati-Carrion M, Arguelles-Prieto R, Martinez-Madrid M, Reiter R, Hardeland R, Rol M, et al. Protecting the Melatonin Rhythm through Circadian Healthy Light Exposure. International Journal of Molecular Sciences. 2014;15(12):23448–500.
39. Melmed S, Auchus RJ, Goldfine AB, Koenig RJ, Rosen CJ. William's Textbook of Endocrinology. 14th ed. Philadelphia: Elsevier; 2020. 124–126 p.
40. Korkmaz A, Topal T, Tan DX, Reiter RJ. Role of melatonin in metabolic regulation. Reviews in Endocrine and Metabolic Disorders. 2009 Dec 13;10(4).
41. Emet M, Ozcan H, Ozel L, Yayla M, Halici Z, Hacimuftuoglu A. A Review of Melatonin, Its Receptors and Drugs. The Eurasian Journal of Medicine. 2016;48(2):135–41.
42. Cipolla-Neto J, do Amaral FG. Melatonin as a Hormone: New Physiological and Clinical Insights. Vol. 39, Endocrine Reviews. 2018. 990–1028 p.
43. Uchiyama M, Lockley S. Non-24-Hour Sleep-Wake Rhythm Disorder in Sighted and Blind Patients. Sleep Medicine Clinics. 2015;10(4):495–516.
44. Chitimus D, Popescu M, Voiculescu S, Panaitescu A, Pavel B, Zagrean L, et al. Melatonin's Impact on Antioxidative and Anti-Inflammatory Reprogramming in Homeostasis and Disease. Biomolecules. 2020;10(9):1211.
45. Herxheimer A. Jet Lag. BMJ Clinical Evidence. 2014;2014.
46. Deng W, Tang S, Tseng H, Wu K. Melatonin suppresses macrophage cyclooxygenase-2 and inducible nitric oxide synthase expression by inhibiting p52 acetylation and binding. Blood. 2006;108(2):518–24.
47. Slominski A, Hardeland R, Zmijewski M, Slominski R, Reiter R, Paus R. Melatonin: A Cutaneous Perspective on Its Production, Metabolism, and Functions. Journal of Investigative Dermatology. 2018;138(3):490–9.
48. Ferlazzo N, Andolina G, Cannata A, Costanzo MG, Rizzo V, Currò M, et al. Is Melatonin the Cornucopia of the 21st Century? Antioxidants. 2020 Nov 5;9(11).
49. Reiter RJ, Rosales-Corral SA, Tan DX, Acuna-Castroviejo D, Qin L, Yang SF, et al. Melatonin, a full service anti-cancer agent: Inhibition of initiation, progression and metastasis. Vol. 18, International Journal of Molecular Sciences. 2017.
50. Wang L, Liang C, Li F, Guan D, Wu X, Fu X, et al. PARP1 in Carcinomas and PARP1 Inhibitors as Antineoplastic Drugs. International Journal of Molecular Sciences. 2017 Oct 8;18(10).
51. Talib W. Melatonin and Cancer Hallmarks. Molecules. 2018 Feb 26;23(3).
52. Srinivasan V, Spence DW, Pandi-Perumal SR, Trakht I, Cardinali DP. Therapeutic Actions of Melatonin in Cancer: Possible Mechanisms. Integrative Cancer Therapies. 2008 Sep 1;7(3).
53. Montfort A, Colacios C, Levade T, Andrieu-Abadie N, Meyer N, Ségui B. The TNF Paradox in Cancer Progression and Immunotherapy. Frontiers in Immunology. 2019 Jul 31;10.
54. González A, Alonso-González C, González-González A, Menéndez-Menéndez J, Cos S, Martínez-Campa C. Melatonin as an Adjuvant to Antiangiogenic Cancer Treatments. 2021; Available from: https://doi.org/10.3390/cancers

55. World Health Organization- International Agency for Research on Cancer. Global Cancer Observatory [Internet]. 2020. Available from: https://gco.iarc.fr/today/online-analysis-table?v=2020&mode=cancer&mode_population=continents&population=900&populations=900&key=asr&sex=0&cancer=39&type=0&statistic=5&prevalence=0&population_group=0&ages_group%5B%5D=0&ages_group%5B%5D=17&group_cancer=1&include_nmsc=1&include_nmsc_other=1
56. American Cancer Society. Breast Cancer Hormone Receptor Status [Internet]. Available from: https://www.cancer.org/cancer/breast-cancer/understanding-a-breast-cancer-diagnosis/breast-cancer-hormone-receptor-status.html
57. Cos S, Mediavilla MD, Fernández R, González-Lamuño D, Sánchez-Barceló EJ. Does melatonin induce apoptosis in MCF-7 human breast cancer cells in vitro? Journal of Pineal Research. 2002 Mar;32(2).
58. Menéndez-Menéndez J, Martínez-Campa C. Melatonin: An anti-tumor agent in hormone-dependent cancers. Vol. 2018, International Journal of Endocrinology. Hindawi Limited; 2018.
59. Blask DE, Dauchy RT, Sauer LA. Putting Cancer to Sleep at Night. Vol. 27, Endocrine. 2005.
60. Gehlert S, Clanton M. Shift Work and Breast Cancer. International Journal of Environmental Research and Public Health. 2020 Dec 20;17(24).
61. Daniyal M, Siddiqui ZA, Akram M, Asif HM, Sultana S, Khan A. Epidemiology, etiology, diagnosis and treatment of prostate cancer. Vol. 15, Asian Pacific Journal of Cancer Prevention. Asian Pacific Organization for Cancer Prevention; 2014. p. 9575–8.
62. Roett MA, Evans P. Ovarian Cancer: An Overview [Internet]. Vol. 80. 2009. Available from: www.aafp.org/afp.
63. Chuffa LGA, Fioruci-Fontanelli BA, Mendes LO, Fávaro WJ, Pinheiro PFF, Martinez M, et al. Characterization of Chemically Induced Ovarian Carcinomas in an Ethanol-Preferring Rat Model: Influence of Long-Term Melatonin Treatment. PLoS ONE. 2013 Dec 18;8(12).
64. Chuffa LGA, Alves MS, Martinez M, Camargo ICC, Pinheiro PFF, Domeniconi RF, et al. Apoptosis is triggered by melatonin in an in vivo model of ovarian carcinoma. Endocrine-Related Cancer. 2016 Feb;23(2).
65. Dauchy RT, Xiang S, Mao L, Brimer S, Wren MA, Yuan L, et al. Circadian and Melatonin Disruption by Exposure to Light at Night Drives Intrinsic Resistance to Tamoxifen Therapy in Breast Cancer. Cancer Research. 2014 Aug 1;74(15).
66. do Nascimento Gonçalves N, Colombo J, Lopes JR, Gelaleti GB, Moschetta MG, Sonehara NM, et al. Effect of melatonin in epithelial mesenchymal transition markers and invasive properties of breast cancer stem cells of canine and human cell lines. PLoS ONE. 2016 Mar 1;11(3).
67. Akbarzadeh M, Movassaghpour AA, Ghanbari H, Kheirandish M, Fathi Maroufi N, Rahbarghazi R, et al. The potential therapeutic effect of melatonin on human ovarian cancer by inhibition of invasion and migration of cancer stem cells. Scientific Reports. 2017 Dec 1;7(1).
68. Mao L, Summers W, Xiang S, Yuan L, Dauchy RT, Reynolds A, et al. Melatonin represses metastasis in Her2-postive human breast cancer cells by suppressing RSK2 expression. Molecular Cancer Research. 2016 Nov 1;14(11):1159–69.

69. Custódio PR, Colombo J, Ventura F v., Castro TB, Zuccari DAPC. Melatonin Treatment Combined with TGF-β Silencing Inhibits Epithelial-Mesenchymal Transition in CF41 Canine Mammary Cancer Cell Line. Anti-Cancer Agents in Medicinal Chemistry. 2020 Jul 24;20(8).
70. Zhang H, Qi S, Liu Z, Li C, Li M, Zhao X. Melatonin inhibits 17β-estradiol-induced epithelial-mesenchymal transition in endometrial adenocarcinoma cells via upregulating Numb expression. Gynecologic and Obstetric Investigation. 2022 Feb 7;
71. Qi S, Yan L, Liu Z, Mu Y lan, Li M, Zhao X, et al. Melatonin inhibits 17β-estradiol-induced migration, invasion and epithelial-mesenchymal transition in normal and endometriotic endometrial epithelial cells. Reproductive Biology and Endocrinology. 2018 Dec 23;16(1).
72. Rajabi A, Saber A, Pourmahdi M, Emami A, Ravanbakhsh R, Khodavirdipour A, et al. Anti-Cancer Effect of Melatonin via Downregulation of Delta-like Ligand 4 in Estrogen-Responsive Breast Cancer Cells. Recent Patents on Anti-Cancer Drug Discovery. 2020 Dec 29;15(4).
73. Tian QX, Zhang ZH, Ye QL, Xu S, Hong Q, Xing WY, et al. Melatonin Inhibits Migration and Invasion in LPS-Stimulated and -Unstimulated Prostate Cancer Cells Through Blocking Multiple EMT-Relative Pathways. Journal of Inflammation Research. 2021 May;Volume 14.
74. Bu S, Wang Q, Sun J, Li X, Gu T, Lai D. Melatonin suppresses chronic restraint stress-mediated metastasis of epithelial ovarian cancer via NE/AKT/β-catenin/SLUG axis. Cell Death & Disease. 2020 Aug 18;11(8).
75. Yang YC, Chiou PC, Chen PC, Liu PY, Huang WC, Chao CC, et al. Melatonin reduces lung cancer stemness through inhibiting of PLC, ERK, p38, β-catenin, and Twist pathways. Environmental Toxicology. 2019 Feb;34(2).
76. Chao CC, Chen PC, Chiou PC, Hsu CJ, Liu PI, Yang YC, et al. Melatonin suppresses lung cancer metastasis by inhibition of epithelial–mesenchymal transition through targeting to Twist. Clinical Science. 2019 Mar 15;133(5).
77. Mu Q, Najafi M. Modulation of the tumor microenvironment (TME) by melatonin. Vol. 907, European Journal of Pharmacology. Elsevier B.V.; 2021.
78. Alvarez-García V, González A, Alonso-González C, Martínez-Campa C, Cos S. Melatonin interferes in the desmoplastic reaction in breast cancer by regulating cytokine production. Journal of Pineal Research. 2012 Apr;52(3).
79. Chan SH, Wang LH. Regulation of cancer metastasis by microRNAs. Journal of Biomedical Science. 2015 Dec 23;22(1).
80. Liu P, Xie X, Yang A, Kong Y, Allen-Gipson D, Tian Z, et al. Melatonin Regulates Breast Cancer Progression by the lnc010561/miR-30/FKBP3 Axis. Molecular Therapy - Nucleic Acids. 2020 Mar;19.
81. Yang A, Peng F, Zhu L, Li X, Ou S, Huang Z, et al. Melatonin inhibits triple-negative breast cancer progression through the Lnc049808-FUNDC1 pathway. Cell Death & Disease. 2021 Aug 16;12(8).
82. Sakatani A, Sonohara F, Goel A. Melatonin-mediated downregulation of thymidylate synthase as a novel mechanism for overcoming 5-fluorouracil associated chemoresistance in colorectal cancer cells. Carcinogenesis. 2019;40(3).

83. Jung JH, Shin EA, Kim JH, Sim DY, Lee H, Park JE, et al. NEDD9 Inhibition by miR-25-5p Activation Is Critically Involved in Co-Treatment of Melatonin- and Pterostilbene-Induced Apoptosis in Colorectal Cancer Cells. Cancers (Basel). 2019 Oct 29;11(11).
84. Ji G, Zhou W, Li X, Du J, Li X, Hao H. Melatonin inhibits proliferation and viability and promotes apoptosis in colorectal cancer cells via upregulation of the microRNA-34a/449a cluster. Molecular Medicine Reports. 2021 Jan 5;23(3).
85. Hunsaker M, Barba G, Kingsley K, Howard KM. Differential MicroRNA Expression of miR-21 and miR-155 within Oral Cancer Extracellular Vesicles in Response to Melatonin. Dentistry Journal. 2019 May 1;7(2).
86. Hsieh MJ, Lin CW, Su SC, Reiter RJ, Chen AWG, Chen MK, et al. Effects of miR-34b/miR-892a Upregulation and Inhibition of ABCB1/ABCB4 on Melatonin-Induced Apoptosis in VCR-Resistant Oral Cancer Cells. Molecular Therapy - Nucleic Acids. 2020 Mar;19.
87. Doğanlar O, Doğanlar ZB, Delen E, Doğan A. The role of melatonin in angio-miR-associated inhibition of tumorigenesis and invasion in human glioblastoma tumour spheroids. Tissue and Cell. 2021 Dec;73.
88. Vimalraj S, Saravanan S, Raghunandhakumar S, Anuradha D. Melatonin regulates tumor angiogenesis via miR-424-5p/VEGFA signaling pathway in osteosarcoma. Life Sciences. 2020 Sep;256.
89. Li Y, Zou J, Li B, Du J. Anticancer effects of melatonin via regulating lncRNA JPX-Wnt/β-catenin signalling pathway in human osteosarcoma cells. Journal of Cellular and Molecular Medicine. 2021 Oct 21;25(20).
90. Wang S, Li J, He Y, Ran Y, Lu B, Gao J, et al. Protective effect of melatonin entrapped PLGA nanoparticles on radiation-induced lung injury through the miR-21/TGF-β1/Smad3 pathway. International Journal of Pharmaceutics. 2021 Jun;602.
91. Yu N, Sun YT, Su XM, He M, Dai B, Kang J. Melatonin attenuates TGFβ1-induced epithelial-mesenchymal transition in lung alveolar epithelial cells. Molecular Medicine Reports. 2016 Dec;14(6).
92. Hadadi E, Acloque H. Role of circadian rhythm disorders on EMT and tumour–immune interactions in endocrine-related cancers. Endocrine-Related Cancer. 2021 Feb;28(2).
93. Menéndez-Menéndez J, Hermida-Prado F, Granda-Díaz R, González A, García-Pedrero JM, Del-Río-Ibisate N, et al. Deciphering the molecular basis of melatonin protective effects on breast cells treated with doxorubicin: Twist1 a transcription factor involved in emt and metastasis, a novel target of melatonin. Cancers (Basel). 2019 Jul 1;11(7).
94. Xiang S, Dauchy RT, Hauch A, Mao L, Yuan L, Wren MA, et al. Doxorubicin resistance in breast cancer is driven by light at night-induced disruption of the circadian melatonin signal. Journal of Pineal Research. 2015 Aug;59(1).
95. Liao Y, Gao Y, Chang A, Li Z, Wang H, Cao J, et al. Melatonin synergizes BRAF-targeting agent dabrafenib for the treatment of anaplastic thyroid cancer by inhibiting AKT/hTERT signalling. Journal of Cellular and Molecular Medicine. 2020 Oct 1;24(20):12119–30.
96. Zhang J, Xie T, Zhong X, Jiang HL, Li R, Wang BY, et al. Melatonin reverses nasopharyngeal carcinoma cisplatin chemoresistance by inhibiting the Wnt/β-catenin signaling pathway. Aging. 2020 Mar 23;12(6).

97. Li M, Hao B, Zhang M, Reiter RJ, Lin S, Zheng T, et al. Melatonin enhances radiofrequency-induced NK antitumor immunity, causing cancer metabolism reprogramming and inhibition of multiple pulmonary tumor development. Signal Transduct Target Ther. 2021;6(1).