PRECLINICAL STUDY OF THE ANTITUMOR EFFECT OF MELATONIN IN DIFFERENT HEAD AND NECK CANCER MODELS: EVALUATION OF THE ROLE OF MITOCHONDRIA AND OXIDATIVE STRESS

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Memoria que presenta el graduado en Bioquímica

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como aspirante al grado de Doctor

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Dª. Germaine Escames Rosa, Catedrática de Fisiología de la Facultad de Medicina de la Universidad de Granada,

Certifica que Dª. Laura Martínez Ruiz, Graduada en Bioquímica, ha realizado bajo su dirección y en el Departamento de Fisiología e Instituto de Biotecnología de la Universidad de Granada, el trabajo titulado "Estudio preclínico del efecto antitumoral de la melatonina en diferentes modelos de cáncer de cabeza y cuello: evaluación del papel de la mitocondria y del estrés oxidativo" reuniendo las condiciones necesarias para optar al grado de Doctor.

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El interesado

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Laura Martínez Ruiz

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Martinez-Ruiz L, Florido J, Rodríguez-Santana C, López Rodríguez A, Guerra-Librero A, Fernández-Gil BI, García-Tárraga P, Garcia-Verdugo JM, Oppel F, Sudhoff H, Sánchez-Porras D, Ten-Steve A, Fernández-Martínez J, González-García P, Rusanova I, Acuña-Castroviejo D, Carriel VS, Escames G. Intratumoral injection of melatonin enhances tumor regression un cell line-derived and patient-derived xenografts of head and neck cancer increasing mitochondrial oxidative stress. Biomedicine and Pharmacotherapy, 2023. DOI: 10.1016/j.biopha.2023.115518.

Other publications:

Martinez-Ruiz L, López-Rodríguez A, Florido J, Rodríguez-Santana C, Rodríguez-Ferrer JM, Acuña-Castroviejo D, Escames G. Patient-derived tumor models in cancer research: evaluation of the oncostatic effects of melatonin. Biomedicine and Pharmacotherapy, 2023. DOI: 10.1016/j.biopha.2023.115581.

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Participation in research projects:

Name of the project: Estudio preclínico de diferentes formulaciones de melatonina para revertir la resistencia a fármacos asociada a la sobreexpresión de las bombas de flujo ATP-dependientes. **Reference:** PID2020-115112RB-I00. **Principal Investigator:** Germaine Escames. **Agency:** Ministerio de Ciencia e Innovación**. Date:** 01/09/2021 – 31/08/2024**. Founds:** 121.000 €

Name of the project: Nueva estrategia terapéutica para evitar la resistencia a la quimio y/o radioterapia asociada a la función

mitocondrial: evaluación de diferentes formulaciones de melatonina. **Reference:** P18-RT-3222**. Principal Investigator:** Germaine Escames. **Agency:** Consejería de economía, innovación y ciencia. Junta de Andalucía. **Date:** 01/01/2020 - 31/03/2023. **Founds:** 119.652 €

Name of the project: Estudio de los mecanismos de acción de la melatonina para reducir la quimiorresistencia: implicación de las proteínas ABC transporters. **Reference:** PPJIB2020-10**. Principal Investigator:** Laura Martínez Ruiz. **Agency:** University of Granada. **Date:** 01/01/2021 - 31/12/2021. **Founds:** 1.700 €

Name of the project: Conexión entre desincronización de los genes reloj y disfunción mitocondrial en la resistencia a la quimioterapia: evaluación de los efectos de la melatonina. **Reference:** SAF2017-85903-P**. Principal Investigator:** Germaine Escames. **Agency:** Ministerio de Economía y Hacienda**. Date:** 01/01/2018 - 30/06/2021**. Founds:** 108.900 €

Contributions to conferences related to this Doctoral Thesis:

Oral Communications

II congreso de Investigación PTS. Nueva estrategia terapéutica para evitar la resistencia a la quimioterapia asociada a la función mitocondrial. 2022. Granada (Spain). Alba López-Rodríguez; Alejandro Fábrega-Puentes; César Rodríguez-Santana; **Laura Martínez-Ruiz;** Javier Florido; Andrés Oliver-Ramirez; Germaine Escames.

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I congreso de Investigación PTS. Metabolic switch produced by melatonin at high concentrations induces apoptosis in cancer. 2019. Granada (Spain). Ana Guerra-Librero; Beatriz I. Fernández-Gil, Javier Florido, Ying-Qiang Shen; **Laura Martínez-Ruiz**; César Rodríguez-Santana; Jorge García-Checa; Darío Acuña-Castroviejo; Germaine Escames.

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IV Workshop de Jóvenes Biotecnólogos. Enhancement of radio- and chemotherapy by high melatonin concentration in head and neck cancer. 2017. Granada (Spain). Javier Florido; Beatriz I Fernández Gil; Ana Guerra-Librero; Ying-Qiang Shen; **Laura Martínez Ruiz**; Sergio García López; Ramy K Sayed; Miguel Mendívil Pérez; Viviana Soto-Mercado; Manuel González Díez; Darío Acuña Castroviejo; Germaine Escames.

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EACR Cancer Metabolism. Effect of melatonin in ABC transporter-mediated multidrug resistance associated with mitochondrial function. 2022. Bilbao (Spain). Alba López Rodríguez; César Rodríguez Santana; **Laura Martínez Ruiz;** Javier Florido, Germaine Escames.

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ABBREVIATIONS

Abbreviations

HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus
EMT	epithelial-to-mesenchymal transition
CSCs	cancer stem cells
TME	tumor microenvironment
MMPs	matrix metalloproteinases
EGFR	epidermal growth factor receptor
CDDP	cisplatin
IR	irradiation
aMT	melatonin
ROS	reactive oxygen species
AANAT	arylalkylamine-*N*-acetyltransferase
ASMT	acetylserotonin-*O*-methyltransferase
AFMK	N1-acetyl-N2-formyl-5-methoxykynurenine
AMK	N1-acetyl-5-methoxykinuramine
NO	nitric oxide
GSH	glutathione
GPx	glutathione peroxidase
SOD	superoxide dismutase
GR	glutathione peroxidase
HIF-1	hypoxia-inducible factor-1

RET	reverse electron transport
NK	natural killer
VEGF	vascular endothelial growth factor
EGF	epidermal growth factor
PDGF	platelet-derived growth factor
ECM	extracellular matrix
5-FU	5-fluorouracil
NSCLC	non-small cell lung cancer
PDX	patient-derived xenografts
PDO	patient-derived organoid
PDC	patient-derived cells
CRC	colorectal cancer
OSCC	oral squamous cell carcinoma
FBS	fetal bovine serum
DMSO	dimethyl sulfoxide
DMEM	Dulbecco´s Modified Eagles Medium
PG	propane-1,2-diol
DPBS	Dulbecco's phosphate-buffered saline
PNEU	PneumaCult-Ex Plus Medium
NSG	NOD SCID gamma
MRI	magnetic resonance imaging

HE	hematoxylin-eosin
PS	picrosirius
AB	alcian blue
EM	electron microscopy
TUNEL	deoxynucleotidyl transferase-mediated dUTP nick end labeling
PFA	paraformaldehyde
OCT	optimal cutting temperature
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium sulfate polyacrylamide gel electrophoresis
PVDF	polyvinylidene difluoride
DNP-hydrazone	2,4–dinitrophenylhydrazone
DNPH	2,4-dinitrophenylhydrazine
LPO	lipid peroxidation
MDA	malondialdehyde
4-HNE	4-hydroxy-2-nonenal
DCFH-DA	2',7'–dichlorofluorescin diacetate
SP1	sample 1
SP2	sample 2
SP3	sample 3
CAFs	cancer-associated fibroblast

“Nuestra recompensa se encuentra en el esfuerzo y no en el resultado;

un esfuerzo total es una victoria completa”

Mahatma Gandhi

RESUMEN

Resumen

El carcinoma de células escamosas de cabeza y cuello (HNSCC) es el sexto cáncer más común a nivel mundial, con casi 500.000 muertes cada año. El tratamiento actual del HNSCC es la combinación de cirugía, quimioterapia y radioterapia local. Sin embargo, estos tratamientos presentan importantes efectos secundarios y es frecuente el desarrollo de resistencia frente a los mismos, lo que conduce al fallo de las terapias y a la muerte del paciente.

Por tanto, es necesario el desarrollo de nuevas terapias con mayor eficacia y menores efectos adversos que los tratamientos ya existentes. En el desarrollo de terapias innovadoras destaca la melatonina, ya que carece de efectos secundarios y posee importantes efectos oncostáticos. Numerosos estudios han demostrado que la melatonina no solo potencia los efectos de los tratamientos existentes, sino que, además, reduce la toxicidad de estos. Esto se debe a que la melatonina tiene un efecto dual, disminuyendo el estrés oxidativo en las células sanas y aumentándolo en las células tumorales, donde, además, presenta efectos anti-proliferativos, pro-apoptóticos, anti-angiogénicos y anti-metastásicos. Sin embargo, a pesar de los numerosos estudios que demuestran los efectos anti-tumorales de la melatonina, existen resultados contradictorios

cuando se utiliza en modelos *in vivo*. Una posible causa podría ser que, para ejercer sus efectos oncostáticos, son necesarios niveles altos de melatonina en el tumor, siendo, por tanto, necesaria la búsqueda de una ruta de administración alternativa que permita aumentar la disponibilidad de la melatonina en el tumor y así establecer un tratamiento efectivo.

Por otro lado, un factor determinante en el fallo de los ensayos clínicos es el modelo preclínico empleado. Tradicionalmente, las líneas celulares ya establecidas han sido la herramienta más utilizada en la investigación contra el cáncer. Sin embargo, tras años en cultivo, la composición genética y el comportamiento de estas células se encuentra alterada y, como consecuencia, no son una representación real de los tumores de los pacientes. Para solventar este problema, en los últimos años se han desarrollado los modelos preclínicos derivados de pacientes, que proceden directamente de tumores de pacientes y, por tanto, mantienen la heterogeneidad y las características moleculares de los mismos. Estos modelos son más adecuados para estudiar la efectividad de los diferentes tratamientos y, por ello, en los últimos años ha aumentado significativamente su uso. Sin embargo, los estudios de

melatonina en modelos derivados de pacientes son prácticamente inexistentes.

Por tanto, nuestra hipótesis es que la melatonina, debido a sus propiedades oncostáticas demostradas *in vitro*, podría ser un agente prometedor como nuevo tratamiento frente al HNSCC o como terapia adyuvante de las terapias ya existentes. Sin embargo, es necesario el desarrollo de una nueva formulación o vía de administración que permita alcanzar altos niveles de melatonina en el tumor, necesarios para ejercer sus efectos oncostáticos. Estos estudios, además, deben desarrollarse no solo en los modelos preclínicos tradicionales sino también en los modelos derivados de pacientes, facilitando así la traslación a la clínica.

Para ello, el objetivo de este estudio ha sido analizar los efectos de diferentes formulaciones de melatonina *in vivo*, en diferentes modelos de ratones con xenoinjertos de células HNSCC. Analizamos los efectos de la melatonina en ratones con xenoinjertos de células Cal-27 y SCC-9, dos líneas celulares ya establecidas de HNSCC, y en ratones con xenoinjertos de tumores procedentes de pacientes. Estos ratones se trataron con diferentes formulaciones de melatonina, administrada intratumoralmente cada 24 horas durante 63 días. La melatonina fue

administrada sola o en combinación con cisplatino (CDDP), uno de los tratamientos actuales más empleados frente al HNSCC. Tras sacrificar los animales, se analizaron histológicamente los tumores para evaluar los efectos oncostáticos de la melatonina. También se analizaron la morfología y distribución de las mitocondrias, los niveles de ROS y la apoptosis. Además, se estudiaron los efectos de la melatonina en el proceso de migración, tanto en las líneas celulares ya establecidas como en células tumorales procedentes del tumor de un paciente.

Los resultados demostraron que la administración intratumoral de melatonina al 3% redujo el crecimiento tumoral en ratones con xenoinjertos de células Cal-27 y SCC-9, lo que se correlacionó con un aumento significativo de los niveles de melatonina en el tumor. El análisis histológico de los tumores tratados con melatonina confirmó una disminución de las áreas tumorales activas y mostró un aumento de la cápsula de colágeno que rodea al tumor, así como una diminución de las áreas adenoescamosas, lo cual se ha relacionado con un mejor pronóstico del cáncer. Además, la administración intratumoral de melatonina potenció los efectos del CDDP en cuanto a reducción del tumor. En concordancia con estos resultados, la melatonina aumentó los niveles de

LPO y de proteínas oxidadas, tanto sola como en combinación con CDDP, e incrementó significativamente la apoptosis en los tumores Cal-27. La melatonina al 3% también redujo los niveles de CD98, lo que sugiere un incremento de la diferenciación de las células tumorales.

Por otro lado, teniendo en cuenta que una de las principales dianas de la melatonina es la mitocondria, estudiamos la morfología y la distribución de las mitocondrias mediante microscopía electrónica. Las imágenes obtenidas mostraron que las mitocondrias de los tumores tratados eran más grandes y se encontraban en la periferia de las células y no alrededor del núcleo, lo que se ha relacionado con un mejor diagnóstico.

A continuación, se desarrollaron modelos celulares y de xenoinjertos derivados de pacientes a partir de biopsias de HNSCC. Los resultados demostraron que la melatonina redujo la proliferación celular y la formación de esferoides en el modelo celular y, además, disminuyó drásticamente el crecimiento tumoral en los ratones con tumores primarios, procedentes de pacientes en los 3 modelos analizados.

Finalmente, se demostró que la melatonina redujo la migración y las propiedades invasivas de las líneas celulares Cal-27 y SCC-9, además de alterar la expresión de los marcadores de transición epitelio-mesénquima en el modelo celular derivado de paciente.

Como conclusión, nuestro estudio ha elucidado el papel de la melatonina en la reducción del crecimiento tumoral, tanto sola como en combinación con CDDP, proponiendo la melatonina como una posible opción terapéutica frente al cáncer. Concretamente, la combinación de CDDP y melatonina podría ser un tratamiento prometedor en la terapia frente al HNSCC, abriendo la puerta a un futuro ensayo clínico con pacientes oncológicos para establecer un tratamiento con melatonina con aplicación clínica.

SUMMARY

Summary

Head and neck squamous cell carcinoma (HNSCC) is an aggressive malignancy, representing the sixth most common cancer worldwide and accounting for nearly 500,000 deaths every year. Current mainstay HNSCC treatment is the combination of surgery, cisplatin (CDDP)-based chemotherapy and concurrent locoregional radiotherapy. However, these treatments are associated with important related-toxicities and the development of resistance, leading to treatment failure and to the death of patients.

The poor outcomes for HNSCC demonstrate the need for novel therapies with less toxicity and more efficacy. Because of its oncostatic influence and lack of association with adverse effects, melatonin is of relevance to the development of innovative cancer treatments. Several studies have demonstrated that melatonin not only shows synergistic anticancer activity with other treatments, but also reduce the general toxicity arising from other treatments. This could be due to the "dual effect" of melatonin. On one hand, melatonin protects normal cells from a variety of insults due to its antioxidant effect in these cells, and on the other, melatonin suppresses tumor cell growth due to its pro-oxidant effect in these cells. Its oncostatic properties include pro-oxidant, anti-

proliferative, pro-apoptotic, anti-angiogenic and antimetastatic actions in tumor cells. Nevertheless, although a great deal of clinical evidence has confirmed the anticancer effects of melatonin, some conflicting results also exist when it is applied *in vivo*. One possible explanation is that high concentrations of melatonin within the tumor are necessary to exert its oncostatic effect, being therefore necessary to search for an alternative administration route to improve bioavailability and establish the optimal dosage for cancer treatment.

On the other hand, an important factor for failure in clinical trials is the inadequate biology of preclinical models. Traditionally, established human cancer cell lines have been the fundamental tool in cancer research. However, the genetic composition and behavior of established cancer cell lines has been altered after thousands of generations in culture, not faithfully representing real patient´s tumors. To solve this problem, the concept of patient-derived models emerged and gained extensive acceptance in cancer research. Since they proceed directly and recently from a patient´s tumor, these models might better retain the heterogeneity and molecular characteristics of patient tumors and be a better alternative to study cancer biology and drug sensitivity. Therefore,

the utilization of patient-derived models significantly increased in the recent 5 years. However, literature about the oncostatic effect of melatonin in patient-derived tumor models is scant.

Considering all above, our hypothesis is that melatonin, due to its oncostatic properties, is a promising agent in anticancer therapy, as new therapeutic approach and as a coadjutant therapy in HNSCC treatment. However, to establish a melatonin treatment with proper application in clinical practice, it is necessary to search for an alternative formulation or an alternative route of administration, which ensures a high bioavailability of melatonin in the tumor. These studies should be conducted not only in established HNSCC cell line models, but also in patient-derived models, which further resemble real tumors and ensure clinical translation.

Concerning this hypothesis, the general objective of this study was to analyze the effect of different formulations of melatonin *in vivo* in different HNSCC mice models. We analyzed the effect of melatonin in mice bearing established HNSCC Cal-27 and SCC-9 cell line xenografts or patient-derived tumor xenografts. Mice were treated with different formulations of melatonin, administered intratumorally each 24 hours for

63 days. Melatonin was administered alone or in combination with CDDP, one of the most common HNSCC treatment. After mice sacrifice, tumors were histologically analyzed to assess the oncostatic effects of melatonin. Mitochondria morphology and distribution, apoptosis and ROS levels were also measured in tumors. Finally, melatonin effects in migration process were also studied, both in established and patient-derived cells.

Results showed that intratumoral treatment with melatonin 3% reduced tumor growth in Cal-27 and SCC-9 xenografts, which correlated with a significant increase of melatonin levels in the tumors. The histology of tumors treated with intratumoral melatonin showed a decrease in tumor active areas, as well as an increase in collagen-rich capsule surrounding the tumors and a reduction of adenosquamous differentiation areas, which has been associated with a better cancer prognosis. Intratumoral injection of melatonin also potentiated the effects of CDDP reducing tumor growth. In line with these results, melatonin increased LPO levels and oxidized protein expression in Cal-27 tumors, alone or in combination with CDDP, and noticeably triggered ROS-dependent apoptosis, which resulted in an increase in the Bax/Bcl-2 ratio and TUNEL-positive nuclei. Moreover, melatonin 3% reduced CD98

levels compared to the control and CDDP groups, suggesting an increase in tumor cell differentiation.

On the other hand, considering that one of the main targets of melatonin is the mitochondria, we evaluated mitochondrial morphology and distribution by electron microscopy. According to previous *in vitro* results, in melatonin-treated tumors, mitochondria were larger and localized in the periphery of the cells and not in the perinucleus, which has been associated with a better cancer outcome.

Next, it was attempted to generate patient-derived cells (PDC) and patient-derived xenografts (PDX) from HNSCC tumor biopsies. Results demonstrated that melatonin reduced cell proliferation and spheroid formation in the PDC model. In addition, the intratumoral administration of melatonin 3% reduced tumor growth in the three PDXs analyzed.

Finally, it was demonstrated that melatonin impaired migration and invasive capacities of Cal-27 and SCC-9 cell lines, as well as altered epithelial to mesenchymal transition markers in the PDC model.

As a conclusion, our study elucidated the roles of intratumoral melatonin reducing tumor growth and synergizing CDDP, proposing

melatonin as a possible therapeutic option for cancer treatment. Specifically, the combined treatment with melatonin and CDDP may be a promising clinical approach to treat patients with HNSCC, encouraging a future clinical trial in cancer patients to establish a new melatonin treatment with proper clinical application.

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INTRODUCTION

1. Head and neck squamous cell carcinoma

Head and neck squamous cell carcinoma (HNSCC) is an aggressive heterogeneous group of malignancies arising from the epithelial lining of the oral and nasal cavity, pharynx, and larynx (Figure 1) (Cui et al., 2021; Shin et al., 2022). It represents the sixth most common human cancer worldwide (Cui et al., 2021; Guerra-Librero et al., 2021), accounting for over 900,000 new patients diagnosed and 470,000 deaths every year (Luo et al., 2022).

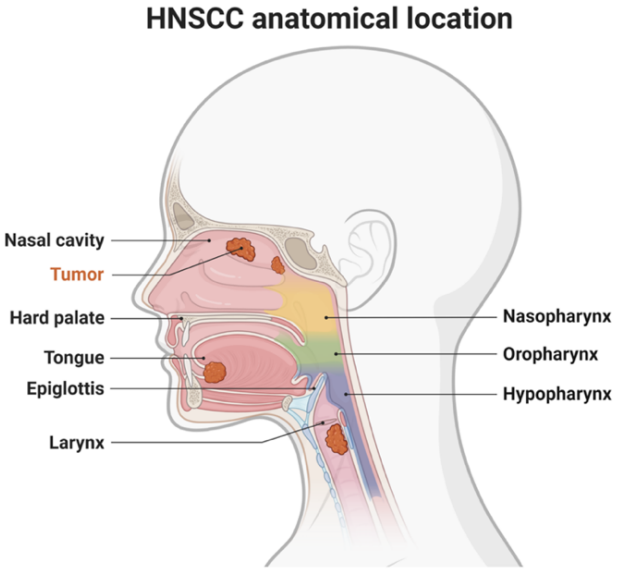

Figure 1. HNSCC anatomical location. Image created using Biorender.com.

The risk of HNSCC is strongly associated with tobacco use, alcohol consumption, and human papillomavirus (HPV) infection (Jin et al., 2022; Shin et al., 2022)**.** Concerning prognosis, generally HPV-positive HNSCC has a more favorable prognosis than those HPV-negative (Johnson et al., 2020) and, although HNSCC is highly curable at early stages, about 60% of HNSCC patients are diagnosed with the locoregionally advanced disease, which is associated with poor survival (Yao et al., 2021). Most of the causes of HNSCC high mortality rate are local recurrence and regional or distant metastasis (Cui et al., 2021; Luo et al., 2022).

1.1. HNSCC metastasis

As mentioned above, HNSCC is characterized by a high rate of recurrence and a high risk of lymph node metastasis (Y. Li et al., 2023; Sun et al., 2022). Particularly, this type of cancer frequently metastasizes to the cervical lymph nodes, which is considered a regional dissemination and occurs in as many 40% of cases (Figure 2) (Altuwaijri et al., 2021; Kisoda et al., 2022; Z. Zhang et al., 2012).

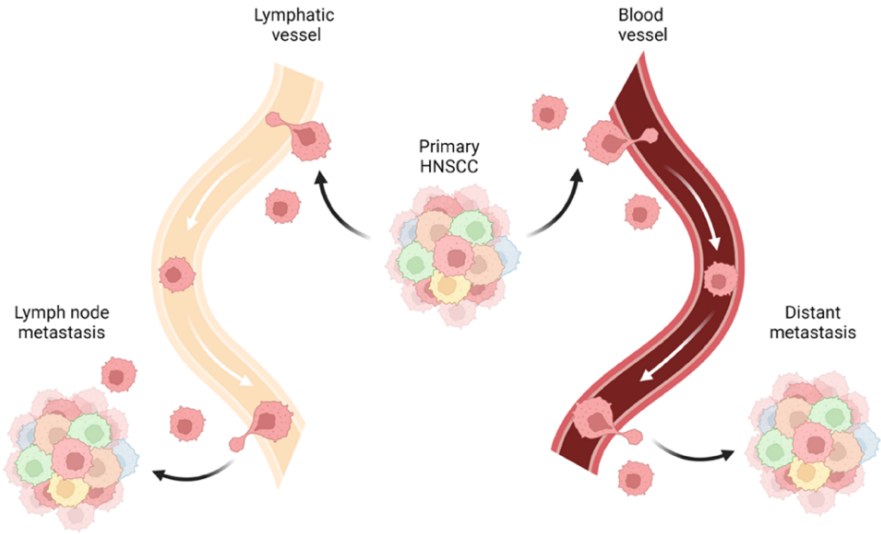

Figure 2. Schematic representation of loco-regional, and distant invasion in HNSCC. Image created using Biorender.com.

On the other hand, despite distant metastasis was once considered uncommon in HNSCC, the advance in the effective management of the primary tumor in HNSCC patients and the current longer survival, has uncovered a higher but variable risk prediction of distant metastasis (Bahig et al., 2022). The incidence of distant metastasis in HNSCC is lower than in other malignancies as melanoma, breast, or lung cancers, ranging from around 3–10% at initial clinical presentation with an additional 20–30% during disease (Vartanian et al., 2022). Nevertheless, autopsy studies point to an incidence of distant metastasis that is 3 to 4 times higher than reported in clinical series (Duprez et al., 2017).

The most common sites of distant metastases are the lung, bone and liver, accounting for approximately 70-85%, 15-39% and 10-30% of events, respectively (Chiesa-Estomba et al., 2021). However, it also can occur in the brain, the skin and in other unusual sites (LK Wong et al., 2021; Vartanian et al., 2022).

The incidence of distant metastasis is influenced by the location of the primary tumor, stage and certain biological characteristics, especially viral disease etiology since HPV positivity is associated with a higher risk of distant metastasis. Although rates of metastasis are equal for both types of tumors, HPV−negative HNSCC tumors tend to spread locally while HPV-positive tumors tend to spread to distant sites (Chiesa-Estomba et al., 2021; Duprez et al., 2017; Vartanian et al., 2022). Specifically, a recent meta-analysis on 5353 patients showed that (a) hypopharynx tumors, (b) staged 3-4 lesions, (c) positive lymph node more than 6 cm, (d) locoregional treatment failure and (e) poor differentiation significantly increase the risk of distant metastasis (Vartanian et al., 2022).

Concerning metastasis treatment, the management of distant metastatic disease can vary from palliative to curative intent in a few but highly selected patients. This is possible due to the advance in the

knowledge of primary tumor biology, disease dissemination and imaging techniques. However, most patients are generally considered incurable and, therefore, they are candidates for palliative treatment since no systemic therapy has curative potential in such a clinical scenario. Consequently, patients with metastasis have a dismal overall survival, around 10 months and a poor quality of life (Chiesa-Estomba et al., 2021; Ghosh et al., 2022; Vartanian et al., 2022).

At the cellular level, the development of distant metastasis in HNSCC is clearly a multifactorial phenomenon, with different processes involved on it. Two of the main characteristic of metastasis development are the initiation of epithelial-to-mesenchymal transition (EMT) and the tumor enrichment with cancer stem cells (CSCs) (Duprez et al., 2017; Renu et al., 2022).

1.1.1. Epithelial-to-mesenchymal transition (EMT)

EMT plays vital roles in tumor metastasis (Luo et al., 2022). Particularly, EMT is a biological process in which epithelial cells lose their epithelial characteristics and, instead, gain mesenchymal features. The

mesenchymal phenotype includes an enhancement of cell migration and the promotion of the dissemination, contributing EMT, therefore, to the metastasizing potential of dedifferentiated epithelial cancer cells (Figure 3) (Brylka & Böhrnsen, 2022; Kisoda et al., 2022; Law et al., 2021; Renu et al., 2022).

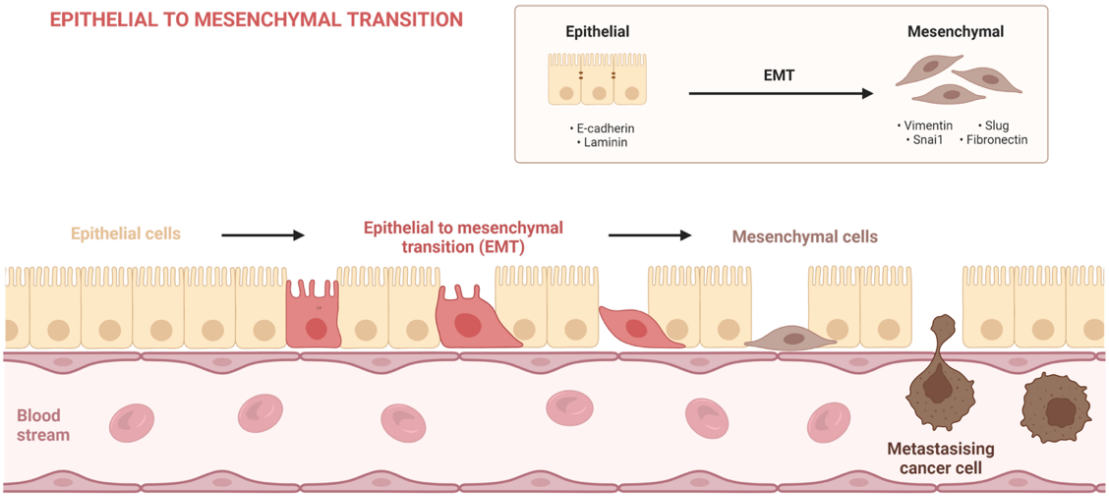

Figure 3. The overall EMT process. Image created using Biorender.com.

Because EMT is driven by growth factors and intracellular communication, the tumor microenvironment (TME) exerts a significant influence on tumor EMT. At first, cells of the TME (fibroblasts, stromal, endothelial and immune cells) try to contain tumor growth and

dissemination. However, the continuous cell–cell interactions and the stimulation of the TME often lead to a modulation of the TME to overcome the inhibitory signals, promoting the mesenchymal phenotype overexpression and therefore, the development of invasive characteristics (Brylka & Böhrnsen, 2022; Elmusrati et al., 2021). These invasive cancer cells enter the bloodstream and form the metastases via the bloodstream. Once the distinct site is reached, there is a development of the metastatic tumor via the growth of cancer cells (Renu et al., 2022).

At the molecular level, an EMT marker in HNSCC is the reduction of E-cadherin levels and the overexpression of vimentin (Maria de França et al., 2021) since vimentin expression is higher in cells with mesenchymal phenotype while the expression of E-cadherin is usually higher in epithelial cells (Brylka & Böhrnsen, 2022). Specifically, E-cadherin is a cadherin present in the epithelia which interacts with the cytoskeleton. This protein is engaged not only in cell adhesion and morphogenesis, but also in cellular signal transduction involving polarity, differentiation, growth, and cell migration; its loss or reduction has been associated with advanced stages of tumor, increased metastatic potential and lowered overall survival in a variety of epithelial neoplasms, including HNSCC

(Cavaliere et al., 2021). Meanwhile, vimentin is an intermediate filament protein that is ubiquitously expressed in normal mesenchymal cells to maintain the cellular architecture and tissue integrity and, also, participates in tumorigenesis, EMT and in the metastatic spread of cancer (P.-F. Liu et al., 2017).

Another process necessary for EMT is actin reorganization and invadopodia formation, mediated by protease matrix metalloproteinases (MMPs) (Renu et al., 2022). These enzymes support tumor cell invasion toward the basement membrane and stroma, the penetration of blood vessels, and metastasis by interacting with macromolecules on the basement membrane to degrade and stimulate extracellular matrix remodeling (Han et al., 2022)**.**

Consequently, E-cadherin and vimentin expression level, as well as MMPs activity are markers of EMT and, therefore, markers of HNSCC metastasis (Renu et al., 2022).

1.1.2. Cancer stem cells (CSCs)

Other hallmark of metastasis, as mentioned before, is tumor enrichment with CSCs. Emerging evidence indicates that a small population of cells in HNSCC patients, named CSCs, are involved in metastasis and tumor recurrence. These CSCs are a minor population of cells within tumor tissues with self-renewal potential and pluripotency, which means that they have the capacity to give rise to the heterogeneous lineages of cancer cells that comprise the tumor. In addition, CSCs have an enhanced invasion potential compared to non-CSCs. Hence, CSCs play vital roles in the processes of tumor initiation, progression and metastasis (Cirillo et al., 2021; Fitriana et al., 2021; Kisoda et al., 2022).

CSCs have been identified for different types of tumors, including HNSCC. In particular, CSCs of HNSCC present several surface markers such as CD44, CD98 and CD133, which high expression is usually considered an indicator of poor prognosis. Among them, CD44 is the most observed CSC marker in HNSCC, and which received a highest clinical attention (Cavaliere et al., 2021; Fitriana et al., 2021). CD44 is a cell-surface glycoprotein and a receptor for hyaluronic acid, involved in cell-cell

interaction, adhesion, migration, and metastasis of tumor cells. Similar functions present CD98, which constitute a transmembrane amino acid transporter, as well as CD133 (Peitzsch et al., 2019).

All the processes mentioned above are critical requirements for cell invasion and dissemination, conducting to metastasis. This phenomenon is responsible for the majority of HNSCC patient's death, leading to a five-year survival rate of less than 50%. Indeed, the survival rates have remained largely unchanged over the past 30 years, despite advances in the treatment of HNSCC (Yao et al., 2021).

1.2. HNSCC treatments

Regarding therapies, with the exception of early-stage HNSCC which are usually treated with surgery alone, the majority of HNSCC patients require multimodal intervention, such as surgery, radiotherapy, and/or chemoradiotherapy (Guerra & Devesa, 2021; Hardingham et al., 2023; Johnson et al., 2020).

However, considering than more than 80% of HNSCC tumors have an overexpression of the epidermal growth factor receptor (EGFR), the FDA

approved cetuximab, a monoclonal antibody against EGFR, for the treatment of HNSCC in 2006. This medication had modest success and it is only used in cisplatin (CDDP)-ineligible patients. Similarly, FDA has recently approved two immune checkpoint inhibitors (pembrolizumab and nivolumab) for the treatment of cisplatin-refractory recurrent or metastatic HNSCC (Hu et al., 2023).

Nevertheless, despite innovations and technological advances in cancer therapy over the past decades, current mainstay HNSCC treatment is the combination of CDDP-based chemotherapy and concurrent locoregional radiotherapy (Yao et al., 2021).

1.2.1. Radiotherapy

Radiotherapy is often used as a local cancer treatment when surgery resection is not possible. Indeed, this therapy is used as an adjunct or palliative treatment for many cancers, including HNSCC. In patients with this type of cancer, especially in those with an advanced disease, radiotherapy is often combined with chemotherapy, being this combination accepted as useful non-surgical treatment that preserves

organ function and adequately handles the tumors (Huang et al., 2023; Su et al., 2022).

Typically, radiotherapy is a treatment modality that uses irradiation (IR), which usually refers to high-energy photon radiation, such as X and gamma rays, and particle radiation. IR operate through direct or indirect actions. Directly, IR damages biomolecules, particularly DNA, which finally is disrupted, resulting in the termination of cell division and proliferation, and even in cell necrosis or apoptosis. IR indirect effects involve the injury of biomolecules by means of free radicals, largely by reactive oxygen species (ROS), which are mainly derived as the radiolysis product of water. ROS can damage biomolecules, producing single- or double-strand breaks of DNA and cross-linking of DNA–DNA or DNA–protein, resulting in cell death (Figure 4). All these processes undermine biomolecules and activate relevant signaling pathways to increase apoptosis of tumor cells (Fernandez-Gil et al., 2019; H. Wang et al., 2018).

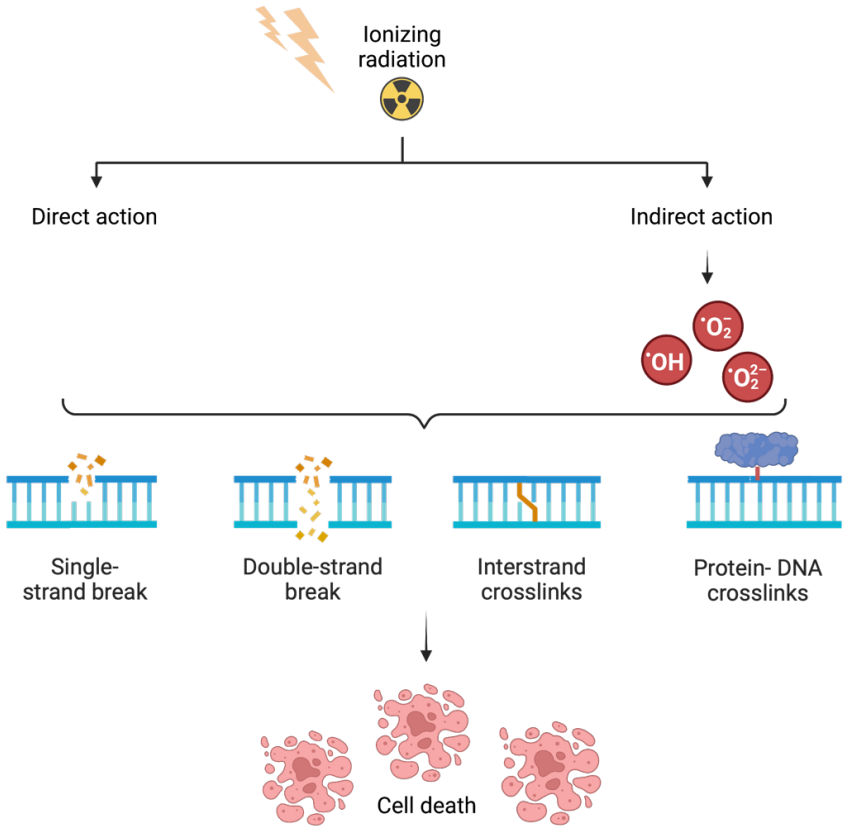

Figure 4. Mechanism of action of irradiation. Image created using Biorender.com.

Despite radiation therapy is an effective treatment for HNSCC, tumor cell resistance to this treatment is a major concern. One way to overcome this problem is to increase radiotherapy effectiveness by using radiosensitizers to enhance tumor cell radiosensitivity. Radiosensitizers are drugs that, when integrated with radiotherapy, increase the cell-killing effect of radiotherapy. However, only a few chemotherapy drugs

(such as CDDP and carboplatin) have had this effect so far (Fernandez-Gil et al., 2019; Maier et al., 2016; Su et al., 2022). Previous research showed that inducing chemotherapy with CDDP before radiotherapy improves overall survival in patients with advanced HNSCCs, which not only increases antitumor activity but also reduces the concentration of treatment administration (Su et al., 2022).

Other radiation-associated problem is acute and late toxicities in healthy organs. Typical acute toxicities include mucositis, dermatitis, dysphagia, decreased taste, and hoarseness. The late toxicities include radiation-induced osteonecrosis, xerostomia, subcutaneous fibroma, trismus, hypothyroidism, sensorineural hearing loss and throat stenosis (Okuda et al., 2022; Su et al., 2022). These side effects not only increase the likelihood of infection and bleeding but also reduce the patient´s quality of life (Su et al., 2022). However, advances in radiotherapy methods, such as intensity-modulated radiotherapy and fractionated radiotherapy, have led to dramatic reductions in radiation-associated adverse events. As a result, it has become possible to use chemotherapy in combination with radiotherapy while minimizing associated toxicities (Okuda et al., 2022).

1.2.2. Chemotherapy

As mentioned before, chemotherapy is frequently offered in combination with radiotherapy in those patients with advanced HNSCC (Amaral et al., 2022). This multidisciplinary approach is recommended to afford functional organ preservation and improved patient outcomes (Huang et al., 2023).

The most commonly used chemotherapy agents are platinum-based, being CDDP the treatment of choice for most HNSCC patients (Amaral et al., 2022; Fernandez-Gil et al., 2019). This chemotherapeutic medication is not only used in HNSCC treatment but is highly effective to treat other solid tissue cancers such as testicular, ovarian, lung and breast cancers (Sadiq et al., 2020; J. Zhang et al., 2020). Although it has several actions, CDDP's primary anti-tumor effect is through DNA damage in tumor cells preventing, on one hand, tumor cell duplication and inducing, on the other, the apoptosis of those cells (Kanno et al., 2021; Okuda et al., 2022; Sadiq et al., 2020).

Concerning the specific mechanism of action, once CDDP enters the cell, the molecule becomes activated by the displacement of chloride atoms by water molecules, resulting in the generation of a highly

electrophile molecule. Such an electrophile molecule can now reach easily with any nucleophile structure, resulting in the formation of interstrand and intrastrand crosslinks on nucleic acids that are resistant to DNA repair mechanisms. Consequently, cell proliferation process becomes impaired, which leads to a cascade of negative events culminate in mitochondria dysfunction, caspase activation, and ultimately apoptosis (Figure 5) (Romani, 2022; J. Zhang et al., 2020).

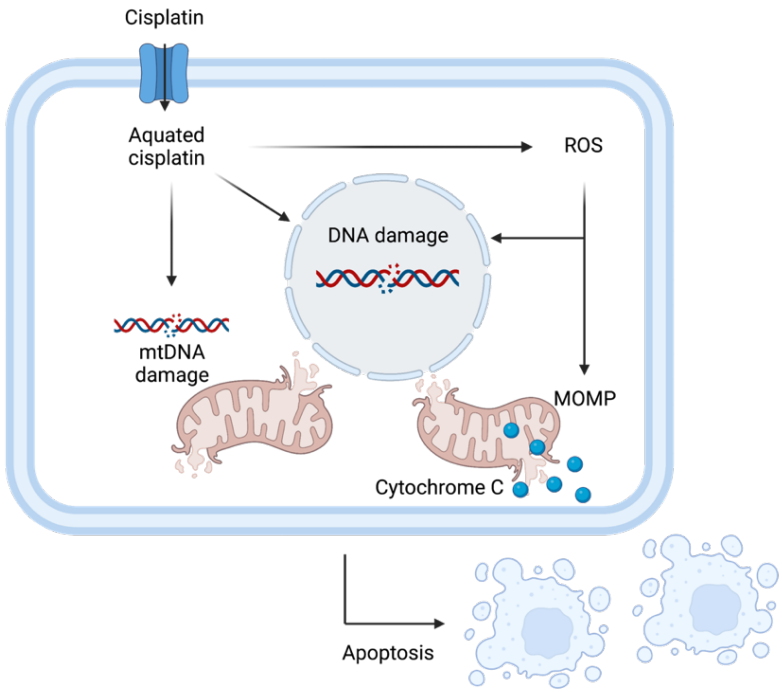

Figure 5. Cisplatin mechanism of action. Image created using Biorender.com.

Furthermore, CDDP contributes to cell apoptosis by increasing ROS formation. CDDP promotes ROS formation directly or indirectly through mitochondria-induced damage. Because mitochondria represent one of the main sources of ROS formation within the cell, the dysfunction in mitochondrial proteins, elicited by ROS directly but also via CDDP interaction, results in abnormal calcium uptake into the mitochondria, decreased mitochondrial membrane potential and ATP synthesis, and ultimately in the activation of the apoptotic pathway (Figure 5) (Romani, 2022; J. Zhang et al., 2020).

All these CDDP actions contribute to tumor cell death. However, healthy cells are also affected by CDDP treatment, initiating serious side effects that may cause physicians to stop the treatment (Sadiq et al., 2020). The main CDDP-related toxicities are (i) nephrotoxicity, primarily due to damage in kidneys proximal tubules since CDDP is excreted from this organ; (ii) myelosuppression, because of mutations in genes involved in hematopoiesis; (iii) nausea and vomiting, caused for the stimulation of the vomiting center directly or by others factors such as anxiety; and (iv) ototoxicity, due to CDDP-induced apoptosis of cochlear hair cells (Okuda et al., 2022).

Another important obstacle to effective cancer therapy is the CDDP resistance that tumor cells develop during chemotherapy. Clinically relevant levels of resistance emerge quickly during the treatment, which usually prevent achieving sufficient therapeutic effects and lead, finally, to treatment failure (Amaral et al., 2022; Okuda et al., 2022; Yamano et al., 2010).

In conclusion, treatment resistance, as well as side effects, remain critical drawbacks of existing treatments (Cui et al., 2021; Florido, Martinez-Ruiz, et al., 2022; Luo et al., 2022). These adverse events for survivors, coupled with the poor outcomes for HNSCC, demonstrate the need for novel therapies with less toxicity and more efficacy (Ghosh et al., 2022). In this regard, melatonin stands out. Because of its oncostatic influence and lack of association with adverse effects, melatonin is of particular relevance to the development of innovative cancer treatments (Florido, Martinez-Ruiz, et al., 2022). Several studies have demonstrated that melatonin not only shows synergistic anticancer activity with other treatments, but also has the ability to reduce the adverse effects or general toxicity arising from other treatments (Z. Liu et al., 2021; Sang et al., 2021; Y.-Q. Shen et al., 2018). These melatonin anti-tumor effects

have been demonstrated in different types of cancers (Minocha et al., 2022; Sánchez-Sánchez et al., 2022), including HNSCC (Shin et al., 2022).

2. Melatonin

Melatonin (N-acetyl-5-methoxy-tryptamine, aMT) (Figure 6) is a functionally pleiotropic indoleamine molecule, which was first discovered in the bovine pineal gland by Lerner in 1958 (LERNER et al., 1960). It was reported later that melatonin not only exists in animals, but also in other organism such as plants, clades of invertebrates and unicellular organisms including bacteria, suggesting the important biological role of this hormone (L. Wang et al., 2022; Zhou et al., 2021). Indeed, melatonin performs a wide spectrum of remarkable biological functions right from the regulation of biological rhythms to combat cancer (Minocha et al., 2022).

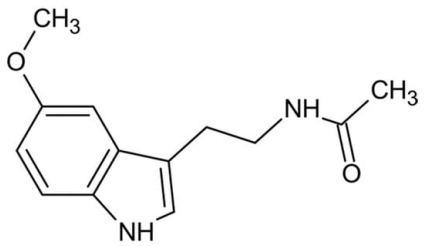

Figure 6. Chemical structure of melatonin. (Mannino et al., 2021)**.**

2.1. Melatonin synthesis and metabolism

Although at first it was thought that melatonin was only synthesized in the pineal gland, numerous investigations have confirmed its synthesis in other tissues (Minocha et al., 2022; Moslehi et al., 2022). Specifically, melatonin is synthesized in most organs and tissues and its concentration is one or two orders of magnitude higher than pineal melatonin (Acuña-Castroviejo et al., 2017).

Pineal melatonin production is under the control of the central pacemaker, located in the suprachiasmatic nucleus, that transforms the photoperiodic signal in an endocrine one, stimulating or inhibiting melatonin synthesis. Particularly, lacking light at night stimulates the release of noradrenaline from the sympathetic neurons projecting to the pineal gland, which results in the activation of the enzymes involved in melatonin synthesis and subsequently increases the level of melatonin produced by the pineal gland. The melatonin produced by the pineal gland is then released into the cerebrospinal fluid and blood vessels. Therefore, the level of melatonin in cerebrospinal fluid and blood reaches the maximal around 2:00 a.m. to 4:00 a.m. and decreases throughout the daytime (Acuña-Castroviejo et al., 2017; Rodríguez-Santana et al., 2023;

L. Wang et al., 2022). To note, the amphiphilic nature and the small size of melatonin facilitates its passage across cell membranes so it can access to other fluids and tissues as saliva, urine, preovulatory follicle, semen, amniotic fluid, and milk, as well as to different cellular compartments (Talib et al., 2021).

Contrary to pineal melatonin, extrapineal melatonin does not follow a circadian rhythm and is not released into the circulation, acting in an autocrine or paracrine manner (Acuña-Castroviejo et al., 2017).

Specifically, the biosynthesis of melatonin, pineal and extrapineal, occurs via the same enzymatic machinery. Cells firstly obtain tryptophan from circulation and convert it to serotonin. Serotonin is then converted to *N*-acetylserotonin by the action of arylalkylamine-*N*-acetyltransferase (AANAT) and finally to melatonin by acetylserotonin- *O*-methyltransferase (ASMT) (Figure 7), being these two enzymes the limiting steps for melatonin biosynthesis. Both enzymes are present in mitochondria, strengthening the idea that melatonin is produced in the mitochondria in perhaps all cells (Cucielo et al., 2022; L. Wang et al., 2022).

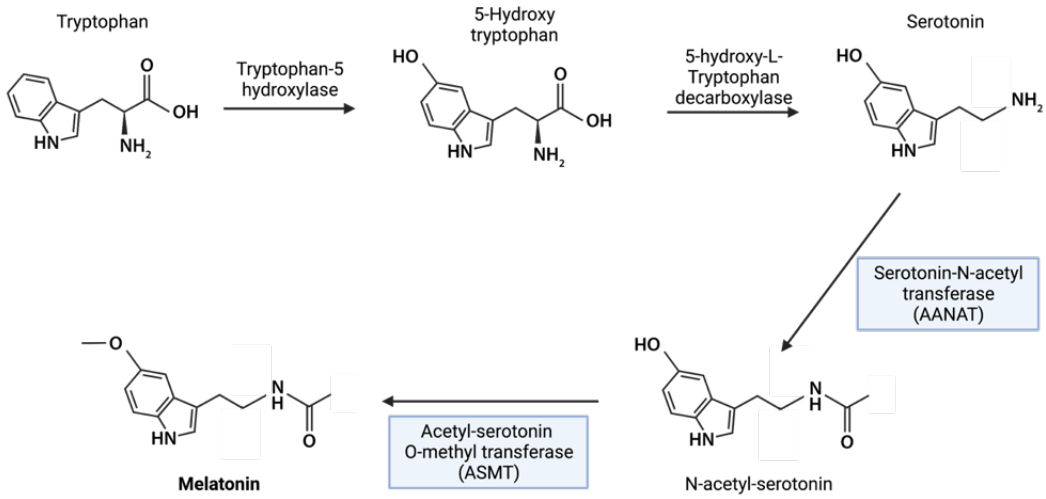

Figure 7. Biosynthesis of melatonin. Image created using Biorender.com.

On the other hand, concerning melatonin degradation, this hormone is metabolized mainly by cytochrome P450 in the liver. It has been demonstrated that melatonin is metabolized to 6-hydroxymelatonin and N-acetylserotonin, which are subsequently converted to sulfate conjugates in human liver and excreted in the urine. However, a small portion of melatonin is also degraded by other tissues including skin and brain to form 6-hydroxymelatonin or N1-acetyl-N2-formyl-5-methoxykynurenine (AFMK). AFMK, together with N1-acetyl-5-methoxykinuramine (AMK) are two important melatonin metabolites that have excellent radical scavenging activity, as it will be explained later (Favero et al., 2017; Talib et al., 2021).

2.2. Effect in normal cells

Although the regulation of circadian rhythm is the most known consequence of melatonin release, it has been revealed that it exerts a wide range of biological effects, regulating various physiological processes (Moloudizargari et al., 2021; Moslehi et al., 2022; Y. Wang et al., 2022). Particularly, melatonin acts as an antioxidant, cytokine, biological response modifier and neuromodulator (Y. Wang et al., 2022).

Some of the biological effects carried out by melatonin are initiated following its coupling with its receptors, which exist both on the cellular membrane and in the nucleus (Figure 8), but other actions are receptor independent (Moloudizargari et al., 2021). Melatonin actions include its effect through specific MT1 and MT2 membrane receptors that mediated chronobiotic properties of the indoleamine; genomic effects, through nuclear receptors belonging to the ROR/RZR family of nuclear transcription factors, and regulatory effects through its binding to calmodulin and calreticulin, two proteins related to calcium homeostasis (Acuña-Castroviejo et al., 2017; Tarocco et al., 2019).

Among its functions, the most important one in healthy cells for this study is its role in antioxidant defense.

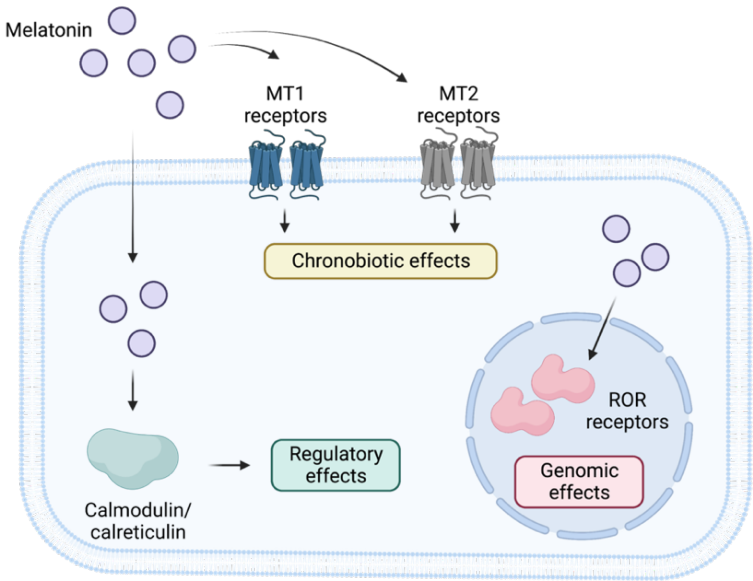

Figure 8. Melatonin receptors. Image created using Biorender.com.

2.2.1. Antioxidant properties

Melatonin has important antioxidant properties. This hormone, together with its metabolites, are potent free radical scavengers and broad-spectrum antioxidants with evolutionarily conserved properties (Florido, Rodriguez-Santana, et al., 2022).

Melatonin can participate in antioxidant defense direct or indirectly. Melatonin´s direct antioxidant effect is due to its direct radical scavenging activity since it can neutralize free radicals including ROS, nitric oxide (NO), and other products (Moloudizargari et al., 2021).

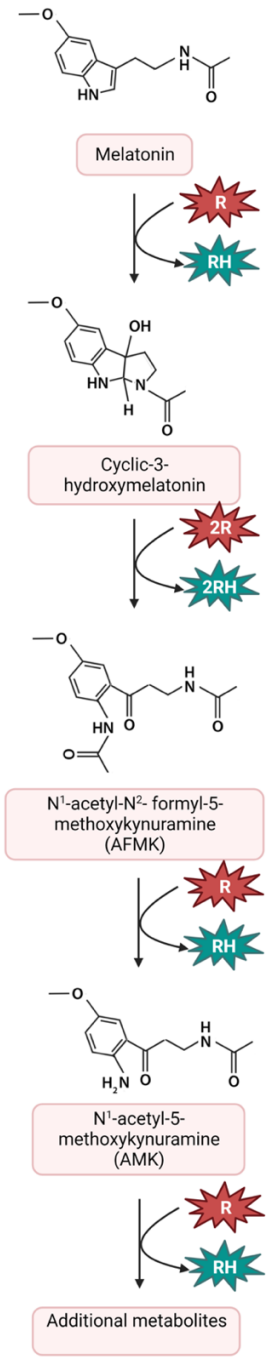

Figure 9. Scavenger cascade of melatonin and its main metabolites. Image created using Biorender.com.

Interestingly, this direct antioxidant effect includes melatonin's metabolites scavenging activity. Under the condition of severe oxidative stress, melatonin is found to be metabolized via enzymatic degradation or free radical interactive processes to produce many metabolites including AFMK and AMK, among others (Figure 9). All these metabolites can also act as antioxidants, being some of them even more potent than melatonin itself. Consequently, melatonin can generate a radical scavenger cascade with never ending action even with its degradation metabolites. Therefore, unlike other antioxidants, melatonin is very effective even in small doses since it can neutralize many radical products during this scavenger cascade (Talib et al., 2021).

On the other hand, indirect effects include the induction of antioxidant enzyme expression and activity (Moloudizargari et al., 2021). Melatonin can stimulate the expression and activity of antioxidant peptides and enzymes such as glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase and glutathione reductase (GR) (Woo et al., 2022).

In addition, melatonin plays an effective role in maintaining mitochondrial homeostasis, which protects against oxidative damage

(Florido, Rodriguez-Santana, et al., 2022). Particularly, melatonin increases the efficiency of the electron transport chain, thereby reducing electron leakage and decreasing the generation of free radicals (Y.-C. Li et al., 2021). Therefore, elevated release of melatonin can reduce oxidative stress and genomic instability markers in normal cells (Moslehi et al., 2022; Woo et al., 2022).

2.3. Effects in cancer cell

Regarding melatonin's effect in tumor cells, it has amply been reported that melatonin exhibits significant antitumor activity when used at high concentrations (Cucielo et al., 2022; Sánchez-Sánchez et al., 2022).

Melatonin oncostatic effect, which was first reported in the MCF-7 mammary adenocarcinoma line, was later reported in many experimental neoplasia models and human cancers (Kurhaluk & Tkachenko, 2022) including prostate, pancreas, lung and colorectal cancers (Cucielo et al., 2022). Its oncostatic effects include pro-oxidant, anti-proliferative, pro-apoptotic, anti-angiogenic and antimetastatic actions (Figure 10) (Cucielo et al., 2022; Minocha et al., 2022).

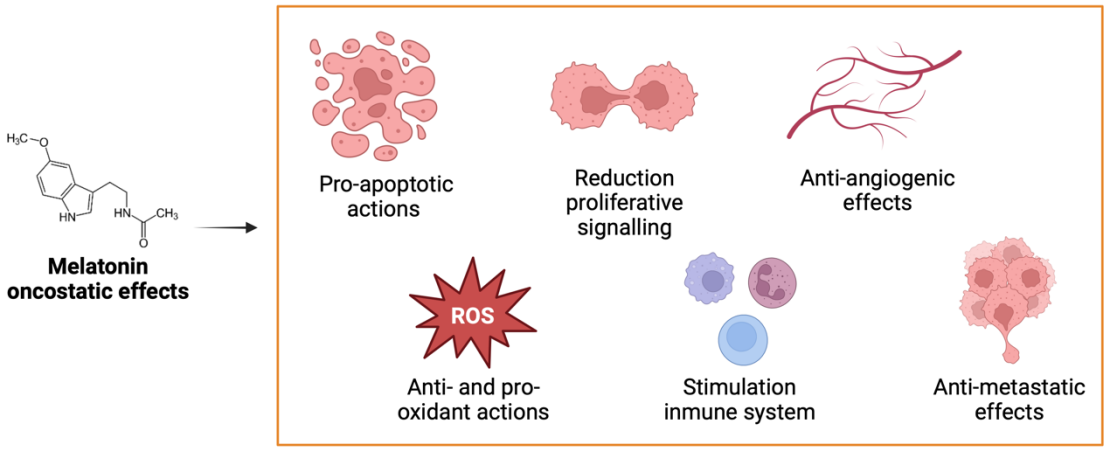

Figure 10. Melatonin oncostatic effects. Image created using Biorender.com.

2.3.1. Anti-proliferative

The tumor suppressive effects of melatonin have been partly attributed to the reduction of cancer promotion or progression, which are associated with antiproliferative activities (Bojková et al., 2018). Cancer cells are recognized by their ability to over-proliferate via modulation of protein expression and signaling pathways. The most critical and controlling pathways are hypoxia-inducible factor-1 (HIF-1), NF-κB, PI3K/Akt, insulin-like growth factor receptor, cyclin-dependent kinases and estrogen receptor signaling (Talib et al., 2021). As it is well known, melatonin is able to modulate the activation of a number of transcription factors and nuclear binding sites that are involved in these signaling

pathways and therefore, in carcinogenesis, inhibiting this process. Mainly, the antiproliferative properties of melatonin take place through cell cycle arrest (C.-J. Shen et al., 2016; C.-Y. Yang et al., 2017).

Moreover, melatonin's ability to decrease cancer cell proliferation has been ascribed to its differentiating properties in different cancer cell types. It has been documented that melatonin stimulates tumor cell differentiation in prostate, breast and gastric cancer, through different mechanism, resulting in a decrease of cancer cell growth. These studies suggest that melatonin acts as a differentiation inducer, reducing tumor cell proliferation (Bojková et al., 2018).

2.3.2. Pro-oxidant actions

Other of the main direct anticancer mechanisms of melatonin is its pro-oxidant action in tumor cells. Contrastively to its antioxidant effect in normal cells, several studies have demonstrated the prooxidant effect of melatonin in cancer cells (Woo et al., 2022). In this context, it has been widely demonstrated that high levels of melatonin are necessary to

induce ROS production in tumor cells (Florido, Martinez-Ruiz, et al., 2022; Florido, Rodriguez-Santana, et al., 2022).

Melatonin can induce oxidative stress in a receptor-dependent or -independent manner. In this regard, it has been suggested that melatonin's pro-oxidant activity involving receptors derives from binding to calmodulin, to which other enzymes associated with oxidative stress, also bind. One of these enzymes is phospholipase A2, which is involved in the release of important inflammatory mediators, such as prostaglandins, and in the production of ROS (Florido, Rodriguez-Santana, et al., 2022).

Regarding melatonin receptor-independent action, the indoleamine can induce oxidative stress directly by increasing ROS levels and indirectly by regulating the expression of various protein (Florido, Rodriguez-Santana, et al., 2022).

One of the main mechanisms by which melatonin indirectly increases ROS production is by the inhibition of tumor cell metabolic reprogramming. Through Warburg effect, tumor cells often exhibit a glycolysis upregulation and a decreased OXPHOS activity, which could reduce the production of H_2O_2 and O_2^- due to the reduced mitochondrial

activity. Therefore, the inhibition of metabolic reprogramming induced by melatonin results in an enhanced mitochondrial activity and thereby in an increased ROS production (Florido, Rodriguez-Santana, et al., 2022; Guerra-Librero et al., 2021).

In addition to the mechanism described above, we have previously demonstrated that melatonin may induce ROS production via reverse electron transport (RET). Complex I has been found to produce ROS in a forward or reverse direction, depending on the substrates used to feed the respiratory chain, suggesting that a change in cell metabolism induces RET. As previously explained, melatonin reverses metabolic reprogramming in some tumor cell, including HNSCC cell lines. This metabolism shift, together with other changes in mitochondrial function, such as an increase of succinate or an increase in the reduced form of CoQ10, explain the production of ROS via RET induced by melatonin (Florido, Martinez-Ruiz, et al., 2022; Florido, Rodriguez-Santana, et al., 2022).

Moreover, the pro-oxidant effects of melatonin can also be explained by the decrease in antioxidant capacity exhibited in the melatonin-treated cells since it decreases antioxidant enzymes such as

catalase, GPx and SOD in tumor cells (Florido, Rodriguez-Santana, et al., 2022).

Finally, melatonin regulates the expression of various proteins involved in metabolic pathways such as SIRT1 (Y. Cheng et al., 2013), SIRT3 (Chen et al., 2021) and AKT (Perdomo et al., 2020), all related with oxidative stress, leading to an increase in ROS production (Florido, Rodriguez-Santana, et al., 2022).

2.3.3. Pro-apoptotic actions

Closely related with melatonin's pro-oxidant actions is the apoptosis induction exerted by melatonin in tumor cells. Various groups have, for many years, reported that high concentrations of melatonin can promote ROS generation, leading to cell death in a variety of cancers. We have previously demonstrated a relationship between ROS levels and apoptosis, since the use of the antioxidant N-acetyl cysteine counteracts the effect of melatonin to induce apoptosis (Florido, Martinez-Ruiz, et al., 2022; Guerra-Librero et al., 2021; Ji et al., 2021), suggesting that melatonin induces apoptosis in cancer cells by increasing ROS production.

This is probably explained by the excessive levels of ROS induced by melatonin, which releases mitochondrial cytochrome C, leading to apoptosis in cancer cells (Florido, Rodriguez-Santana, et al., 2022; Woo et al., 2022).

Apoptosis, in general, consists of two intrinsic and extrinsic pathways. The intrinsic pathway is related to the release of cytochrome C from mitochondria and caspase recruitment while the external is independent of mitochondria and is initiated by the external stimuli (Moloudizargari et al., 2021). On this point, melatonin can induce both intrinsic and extrinsic pathways of apoptosis. At the intrinsic apoptotic pathway, melatonin activates caspases, increases cytosolic cytochrome C levels and modulates apoptosis protein-related expression. Specifically, melatonin increases Bax/Bcl-2 ratio and p53 expression, leading to programmed cell death. Extrinsically, melatonin augmented the expression of Fas and Fas-ligand. All these melatonin modulatory effects lead to tumor programmed cell death (Bojková et al., 2018; Sahar Baghal-Sadriforoush et al., 2022; Samanta, 2020; J. Zhang et al., 2020).

2.3.4. Immunomodulator effect

Alternatively, melatonin's immunomodulator effect also contributes to its oncostatic actions. Immune system within tumor has a pivotal role in invasion of cancer cells. In response to apoptosis or necrosis of cancer cells following treatments, several signaling pathways are activated after the interaction of apoptosis or necrosis products with immune system cells. Usually, macrophages digest apoptotic bodies and release tolerogenic cytokines which lead to the attenuation of immune system's activity against tumor cells. Moreover, some secreted cytokines from macrophages or lymphocytes trigger the development of new vessels through stimulation of angiogenesis factors. Therefore, modulation of immune system responses is an interesting strategy for suppressing tumor growth (Mortezaee et al., 2019). In this sense, melatonin exerts a tumor-suppressive capacity through the activation of anticancer immune cells, enhancing their viability and modulating cytokines release (Samec et al., 2021; Talib et al., 2021).

The fundamental physiological role of melatonin on the immunity has been well-described. Its immunomodulatory properties are mediated through the activated T-lymphocytes or enhanced immunity mediated by

CD8+ T cells. Melatonin has been shown to increase T-helper cell activity by releasing several specific cytokines (Bojková et al., 2018). In addition, it was reported that melatonin can stimulate natural killer (NK) cell production, as well as monocytes and leukocytes activity. The activation of these different immune cells increases immunosurveillance and leads to an augmented tumor cell death (Bojková et al., 2018; Talib et al., 2021).

On the other hand, while some investigators described melatonin as an immuno-stimulant, many other studies have also argued anti-inflammatory activities (Bojková et al., 2018). There is a strong relationship between chronic inflammation and tumor development. The outcomes of the inflammatory process might promote carcinogenesis via the formation of ROS and RNS, which result in DNA damage and cancer development. On this point, melatonin has shown an anti-inflammatory effect due to the suppression of NF-κB expression and the regulation of NLRP3 inflammasome, leading to a downregulation of inflammatory mediators (IL-6, IL-8, COX-2, and NO) (Talib et al., 2021).

2.3.5. Anti-angiogenic actions

Among the mechanisms involved in the anti-cancer effects of melatonin are antiangiogenic actions. New blood vessel formation, known as angiogenesis, is essential for tumor progression through the provision of nutrients and oxygen and removal of wastes. The inhibitory effects of melatonin, unlike other inhibitors of angiogenesis process, are exerted at different levels, by regulating different proteins involved in the formation of new vessels. However, vascular endothelial growth factor (VEGF) and HIF-1 seem like the main targets of melatonin (González et al., 2021; Moloudizargari et al., 2021; R. Wang et al., 2021).

MMPs and growth factors mainly including epidermal growth factor (EGF), VEGF and platelet-derived growth factor (PDGF) are key endogenous stimulators of angiogenesis. These growth factors have specific receptors, which are essential for its function and therefore, for angiogenesis process. In this regard, it has been demonstrated that melatonin can diminish the expression levels of these receptors, specifically VEGFR-2, the most effective receptor in angiogenesis induction. Melatonin also exerts its anti-angiogenic effects through

reducing VEGF mRNA levels in a dose-dependent manner (J. Cheng et al., 2018; Moloudizargari et al., 2021).

In addition, melatonin interferes with hypoxia-induced angiogenesis through diminishing HIF-1α, one of the two subunits of the HIF-1 heterodimer, and blocking the translocation of HIF-1 into the nucleus (Bojková et al., 2018; Moloudizargari et al., 2021; L. Yang et al., 2019).

These melatonin actions, together with its inhibitory effects on mediators for several signal transduction pathways, result in the inhibition of endothelial cell proliferation, survival, migration, invasion, cell permeability, budding, or tube formation, all contributing to melatonin angiostatic properties (González et al., 2021).

2.3.6. Anti-metastatic effect

Finally, melatonin exhibits various mechanisms to restrain metastasis, such as modulation of cell–cell and cell–matrix interaction, extracellular matrix remodeling by MMPs, readjustment of the cytoskeleton, EMT and angiogenesis (Talib et al., 2021).

On the one hand, melatonin modulates the cross talk between cells and the extracellular matrix (ECM) mainly via expression regulation of integrins and cadherins (Moloudizargari et al., 2021). As previously explained, different cadherins such as E-cadherin, are involved in early steps of metastasis, specifically in EMT (Moloudizargari et al., 2021). It has been found that melatonin control EMT through the upregulation of cell surface adhesion molecules, including E-cadherin in different cancer cell types, thereby decreasing the metastatic capacity of the tumor cells (Sánchez-Sánchez et al., 2022). Melatonin also decreases the expression of vimentin, which its upregulation has been frequently reported in invasive and poorly differentiated cancer cells (Targhazeh et al., 2022). Furthermore, melatonin modulates other EMT related protein expression including Snail, Slug, Twist, and Zeb (Moloudizargari et al., 2021; Targhazeh et al., 2022).

On the other hand, melatonin may exert its anti-metastatic effects by inhibiting the remodeling of ECM and the degradation of its main constituent including different types of collagens by MMPs. Specifically, melatonin decreases MMP9 levels, altering ECM remodeling and thereby interfering with tumor cell invasiveness (Moloudizargari et al., 2021).

All the melatonin effects explained above justify melatonin potential as an oncostatic agent in anticancer therapy. On the one hand, melatonin can suppress tumor development through different mechanism of action in tumor cells and, on the other, melatonin has the ability to protect normal cells from a variety of insults due to its antioxidant effect in these cells (Y.-Q. Shen et al., 2018; J. Zhang et al., 2020).

2.4. Clinical trials based on anticancer effects of melatonin

Melatonin interesting properties in both tumor and normal tissue suggest a remarkable effectiveness of melatonin treatment for clinical translation to patients with cancer (Moslehi et al., 2022).

One of the main considerations about the treatment in the field of oncology is the evaluation of the toxic effects and risk of new therapies directed against cancers (Talib et al., 2021). On this point, melatonin is a naturally produced hormone with an impressive long-term safety profile (Z. Liu et al., 2021). Indeed, various studies have demonstrated that melatonin exhibits oncostatic properties against different type of cancers with low or no toxicity even at high doses (Minocha et al., 2022). Specifically, some clinical trial studies have shown that patients with

cancer can tolerate some safe doses of melatonin like 5–20 mg/kg/day (Moslehi et al., 2022).

These clinical trial studies have been performed to evaluate the efficiency of melatonin as an anti-cancer agent (Moslehi et al., 2022). Almost all the clinical studies used melatonin in combination with other existing treatments or as protective therapy. Most of them evaluate melatonin effect in combination with other chemotherapeutic agents and not so often in combination with IR. Although emerging literature in the last few years has demonstrated that melatonin used alongside radiotherapy is able to augment the impact of ionizing radiation on tumors and can also prevent the latter's toxic effects on non-cancerous cells, the use of melatonin to enhance the effect of radiotherapy in human subjects has been poorly studied (Talib et al., 2021). Nevertheless, both clinical trials, combining melatonin with chemo- or radiotherapy, showed that application of melatonin led to positive impacts on the anticancer treatment (Talib et al., 2021; L. Wang et al., 2022). In general, melatonin administration enhances the efficiency of the therapies, alleviates treatments-related side effects and improves patient's life quality, including the reduction of the incidence of depressive symptoms and the

improvement of the quality of sleep of patient with cancer (Talib et al., 2021; L. Wang et al., 2022).

Some of the most important clinical trial regarding melatonin's oncostatic effects have been collected by Wang et al. (L. Wang et al., 2022) and will be summarized next. In a randomized, double-blind, clinical study, melatonin was co-administered to patients with HNSCC. Results showed that melatonin administration reduced mucositis, one of the main side effects of radiation in HNSCC patients, and ameliorated pain (Elsabagh et al., 2020; Lozano et al., 2021). In addition, combining melatonin with a CDDP-based standard treatment in this type of cancer reduced anemia, a common side effect of cisplatin (Lissoni et al., 2003). The effect of melatonin in ameliorating the side effects of chemotherapy was also investigated in patients with gastrointestinal cancer, showing that melatonin could maintain the body weight but failed to attenuate cachexia (Persson et al., 2005); and in metastatic colorectal patients, revealing that patients receiving a combined treatment with melatonin and subcutaneous IL-2 after a first-line therapy of 5-fluorouracil (5-FU) had a higher percentage of survival at 1 year compared to those who only received 5-FU treatment (Barni et al., 1995). Similarly, a randomized

clinical trial showed that metastatic breast cancer patients treated with both tamoxifen and melatonin had a higher relative response compared with those receiving tamoxifen alone (Lissoni et al., 1995).

The use of melatonin in non-small cell lung cancer (NSCLC) patients has been also investigated, showing that melatonin improved the quality of life but did not demonstrate any protective effects against chemotherapy-related side effects (Sookprasert et al., 2014). Besides, adjuvant melatonin following resection of NSCLC increased the 2-year disease-free survival in patients with late-stage disease but did not exhibit beneficial effects in quality of life, symptoms, or immune function (Seely et al., 2021).

Summarizing, although a great deal of clinical evidence has confirmed the anticancer effects of melatonin, some conflicting results also exist. Therefore, more *in vivo* studies and clinical trials are needed to establish its proper clinical application in cancer treatment (L. Wang et al., 2022).

3. Antitumor drug research models

Concerning research translation to clinical application, only ~ 5% of the drug candidates tested in clinical trials can be approved by the governmental drug administration. Although the therapeutic effect of each drug candidate is sufficiently confirmed in preclinical studies, limited efficacy accounted for more than 60% of failures during clinical trials in cancer therapy (Hou et al., 2022). An important factor for failure in clinical trials is the inadequate biology of preclinical models in which drugs are developed or tested, resulting in the inability to predict therapeutic efficacy in humans (Zeng et al., 2022). The existing heterogeneity between preclinical models and real patient tumors suggest that the evaluation model could be the obstacle for scientific research and anticancer drug development (Abdolahi et al., 2022; Hou et al., 2022).

3.1. Established cancer cell line-derived models

Traditionally, established human cancer cell lines have been the fundamental tool in cancer research and they have been widely applied, both *in vitro* and *in vivo*, for investigation of tumor biology and antitumor drugs testing. These models have been used to assess the effectiveness

of investigational anticancer compounds taking advantage of their ease of maintenance and propagation, relatively low cost, reproducibility and high-throughput evaluation. However, antitumor activity demonstrated with this approach is often not confirmed in clinical settings, mainly due to the low resemblance to human cancers *in vivo* (Genta et al., 2022).

Despite it being derived from actual patients, cells derived from established cancer cell lines are much different from clinical cancer patient. This divergence depends on several factors (Genta et al., 2022; Hou et al., 2022).

Firstly, these tumor cells do not reflect the heterogeneity of real patient's tumors. The *in vitro* growth process results in the selection of clones with specific features promoting their survival, outliving other subpopulations (Genta et al., 2022). Furthermore, the addition of fetal calf serum to the culture medium may lead to cellular differentiation (Yin et al., 2021). Therefore, the progressive adaptation to culture conditions results in a loss of heterogeneity and differentiation (Genta et al., 2022).

Secondly, the genetic composition and behavior of established cancer cell lines has been altered after thousands of generations in culture (Hou

et al., 2022). Long-term *in vitro* cell culture showed alterations in tumor hallmarks caused by epigenetic and genetic changes (Abdolahi et al., 2022). In this context, to note, gene expression profiling has further demonstrated that cell lines obtained from diverse tumors resemble each other more than the corresponding clinical samples from which they were derived initially (Yin et al., 2021).

These cell lines even showed different phenotypes in different laboratories since they adapt to *in vitro* culture conditions, which, in turn, are frequently distinct between laboratories (Abdolahi et al., 2022). In addition, existing studies suggested that various classical cancer cell lines have been polluted or mixed by other cells, leading to inaccurate results (Hou et al., 2022).

Thirdly, using cancer cell lines it is impossible to replicate the microenvironment features, including molecules and tumor-stroma crosstalk, cell interactions, and the three-dimensional architecture of tumor niches (Miserocchi et al., 2022). The absence of a natural TME impairs the evaluation of drugs whose mechanism of action is based on cell-cell interactions or is related to angiogenesis (Genta et al., 2022).

As a result, to overcome these limitations, it is urgent to propose more accurate and effective evaluation models for predicting and evaluating the efficacy of drugs in cancer research (Hou et al., 2022; Yin et al., 2021).

In this context, the concept of patient-derived models emerged and gained extensive acceptance in cancer research, especially in translational medicine (Hou et al., 2022).

3.2. Patient-derived models

According to existing reports, there are three patient-derived models (Figure 11): i) patient-derived xenografts (PDX), established by transplanting fresh tumor tissue or cells from a patient into immunodeficient mice; ii) patient-derived organoid (PDO), formed from cells obtained by patient´s tumor digestion and transplanted to membrane extract with specific growth medium; and iii) patient-derived cells (PDC), which proceed from the digestion of a patient´s tumor (Hou et al., 2022).

Since they proceed directly and recently from a patient´s tumor, they encompass multiple factors such as histological structures,

malignant genotypes and phenotypes of real tumors. Therefore, these models might better retain the heterogeneity and molecular characteristics of patient tumors and be a better alternative to study cancer biology and drug sensitivity (Cromwell et al., 2022a; Lê et al., 2022a).

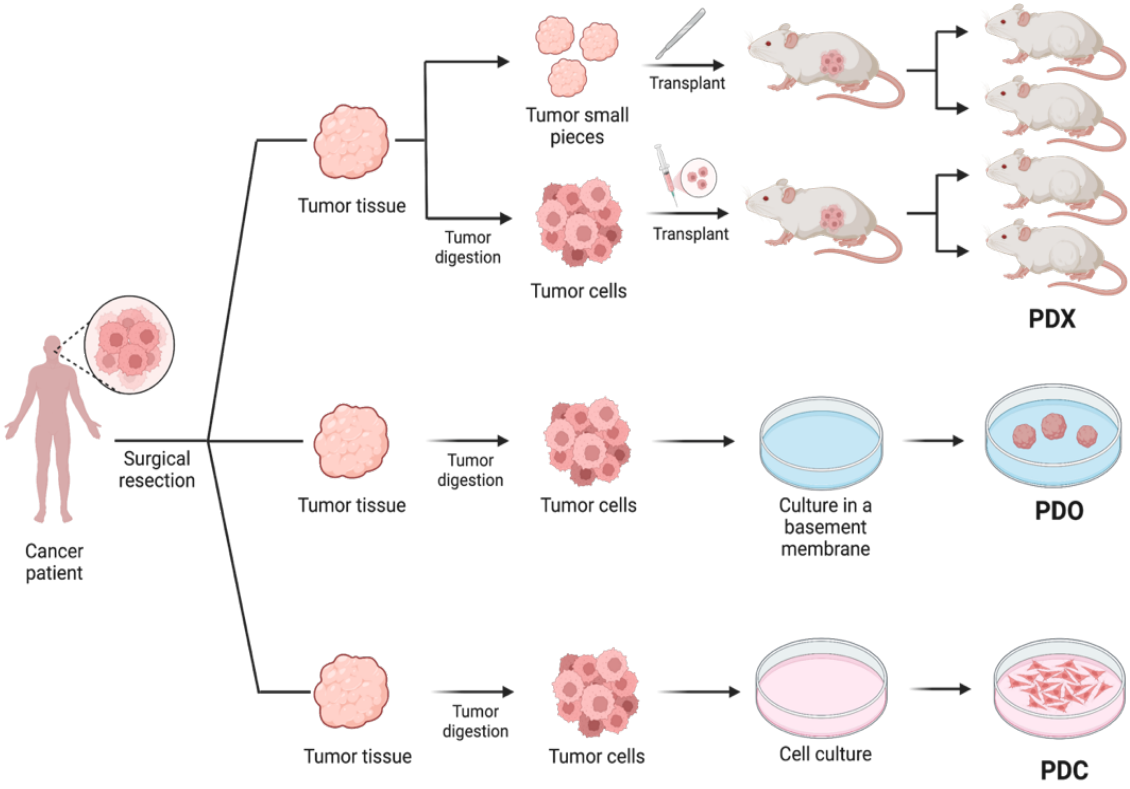

Figure 11. Process to obtain the different patient-derived models. Patient-derived xenografts (PDX); patient-derived organoids (PDO); patient-derived cells (PDC). Image created using Biorender.com.

While initially established decades ago, the utilization of these models significantly increased around the world especially in the recent 5 years. At present, patient-derived models are widely applied in various areas of medicine including fundamental research, drug development and clinical applications (Hou et al., 2022).

Fundamental research. Patient-derived models offer several advantages compared traditional cell line–derived models when it comes to fundamental research. In this sense, PDX could mimic TME found in actual patients. As a result, they provide a more accurate and reliable model for the evaluation of cancer biomarkers. Apart from PDX, PDO and PDC are also widely used in fundamental cancer research. Specifically, PDO models are valuable for investigating the mechanism of tumorigenesis and tumor immune evasion. One key advantage of PDO is that they can be genetically manipulated, making them a powerful tool for conducting studies in these areas (Hou et al., 2022; M. Li & Izpisua Belmonte, 2019; Maynard et al., 2020; Ullmann et al., 2020; Y. Wang et al., 2019).

Drug development. The three patient-derived models mentioned above are more appropriate models for drug development studies than

traditional cancer cell lines models. Among these models, the PDX model highlights as a remarkable option. It is considered a “clinical trial phase 0”, indicating that it serves as a pre-experiment or precursor of clinical trial phase II. PDX is also a reliable tool to identifying drug-sensitive markers and evaluating different drug combination strategies. Moreover, the PDC model, which is an excellent alternative to conventional cell lines, is extensively utilized in the process of discovering and developing anticancer drugs. It is particularly useful in the early stages of drug screening, contributing to more effective and promising drug candidates (Coussy et al., 2020; Hou et al., 2022; Kim et al., 2019; Meraz et al., 2019; Takahashi et al., 2019; Yoshimatsu et al., 2020).

Clinical application. The inherent variability among cancer patients results in diverse responses to the same treatment. Consequently, personalized therapeutic approaches, also known as personalized treatment, are essential in clinical practice (Bhimani et al., 2020). However, evaluating hundreds of drug candidates independently and determining the ideal strategy for each patient is impractical. Fortunately, patient-derived models have emerged as viable alternatives to identify the best therapeutic strategies. Directly derived from the

cancer patient, PDX may be the most homologous model of the human body which can be used to screen effective therapeutic strategy for a specific patient in the clinic. According to existing reports, PDX models have shown an overall predictive accuracy of 90%, indicating that they are highly suitable replacements for the actual patients themselves. These models provide a means to optimize and personalize treatment strategies, leading to better outcomes for individual cancer patients (Hou et al., 2022; F. Zhang et al., 2018).

In conclusion, the PDX, PDO, and PDC models are optimal humanized models currently in cancer research, which can successfully simulate the human body and clinical practice. The universalization of patient-derived models will support basic cancer research and provide additional scientific evidence for novel and effective drug development. For eventual clinical application, they will also yield more precise and reliable treatments (Hou et al., 2022).

3.2.1. Patient-derived xenograft

Among existing patient-derived models, PDX is the most reliable one, being of particular interest in translational medicine. As mentioned before, PDX are established by transplanting fresh tumor tissue or cells from a patient into immunodeficient mice (Genta et al., 2022; Zeng et al., 2022), being thereby developed from fresh human tumors without prior *in vitro* manipulation (Cybula & Bieniasz, 2022).

3.2.1.1. Advantages and limitations of PDX models

PDXs are characterized by the maintenance of the essential characteristics of the original patient's tumor, including histologic, biologic, and genetic features. In this case, tumor-specific characteristics are maintained through multiple mouse-to-mouse passages. These characteristics of PDX model justify them to be considered the most reliable reproduction of human cancers. For that reason, PDX models have been used to evaluate new therapeutic approaches and to perform pre-clinical drug testing (Cybula & Bieniasz, 2022).

Genomic and transcriptomic fidelity as well as preserving tumor heterogeneity are two of the key characteristics to predict responsiveness to antitumor compounds. In this sense, PDXs retain more than 80% of the genomic alterations harbored by the engrafted neoplastic tissue and encompass different populations of the original tumors (Genta et al., 2022), preserving the heterogeneity of human tumors (Cybula & Bieniasz, 2022). Other advantages of this animal model are that they maintain cell-to-cell and cell-to-matrix interactions, and therefore tumor microenvironment, better compared to other cancer models (Cybula & Bieniasz, 2022). In addition, PDX model maintain the ability to mimic a systemic response (Marshall et al., 2020).

However, PDX models also retain some important limitations, being the most important one the model establishment. The success rate of PDX establishment depends on multiple variables including the animal recipient. The probability of obtaining successful engraftment increases with the degree of immunosuppression in the animal host (Genta et al., 2022). Immunodeficient NOD/SCID or NOD/NSG mice are largely used to avoid tumor rejection since it lacks mature T and B cells and has deficient natural killer cell function (Lê et al., 2022a). However, PDXs generated in

immunodeficient mice cannot efficiently be used to study immunotherapies, since an intact immune system, and preferably one of human origin, is required for that purpose (Huo et al., 2020).

Cancer type is also important, being the most aggressive ones, which more frequently result in a successful engraftment. Moreover, the technique used to implant the tumor influence PDX establishment. Many techniques have been used to optimize engraftment, including orthotopic transplant or, in the case of hormone-dependent cancers, the addition of human hormones (Cybula & Bieniasz, 2022; Genta et al., 2022). Orthotopic grafting or injection into the circulation are associated with a higher success rate, up to 30–40%, than subcutaneous grafting (Lê et al., 2022a).

On the other hand, PDX modeling often requires large quantity of tumor tissue and tumor tissue material is usually scant, hindering PDX model establishment (Zhu et al., 2022). Most patients presenting with cancer disease often undergo a diagnostic needle biopsy rather than surgical resection and the biopsy material may be relatively scant. Nevertheless, when surgical resection is possible, tumor tissue obtained allowed PDX establishment (Kodack et al., 2017).

Finally, in addition to be technically cumbersome, PDX models are expensive and more time-consuming (Huo et al., 2020). PDX modeling requires 4‑8 months to determine the efficacy of treatment for a specific cancer, and the time lag between the transplantation of tumor tissue into mice and the initiation of treatment limits its extensive application (Zhu et al., 2022).

3.2.1.2. Applications of PDX model

This model is critical for improving our understanding of the genetic and molecular etiology of cancer disease, but especially for developing and validating effective therapies (Zeng et al., 2022). On this point, several studies have tested the response rate of different drugs to different cancers in PDX models, demonstrating that the response rate of PDX models to the drug is correlated with clinical outcomes. In fact, before a human clinical trial, PDX clinical trials are critical to evaluate anti-cancer therapies (Abdolahi et al., 2022). These types of studies, known as co-clinical trials, use laboratory data to guide clinical development or treatment strategies, with the final goal of identifying predictive

biomarkers and increasing the rate of success of experimental treatments (Genta et al., 2022).

Furthermore, application of PDX model could also lead to personalized medicine in oncology treatments. During the treatment of clinical tumors, the treatment plan applied is often non‑personalized and the individual differences between patients with the same tumor manifestations usually lead to treatment failure. However, the results of pre‑clinical experiments using the PDX model are often highly consistent with clinical facts and expectations, which is helpful for individualizing the treatment plan for each patient (Yin et al., 2021).

3.3. Melatonin and patient-derived models

Despite the utilization of patient-derived models significantly increased in the recent 5 years, literature about the oncostatic effect of melatonin in patient-derived tumor models is scant. Only 3 studies evaluate the role of melatonin in these models (Hasan et al., 2019; Sakatani et al., 2019; C.-Y. Yang et al., 2017).

In this regard, Sakatani et al. (Sakatani et al., 2019) established a PDO model of colorectal cancer (CRC) from cancer tissues collected from two patients undergoing surgery for CRC. After patient-derived organoids were obtained, melatonin treatment was assessed. For the treatment of patient-derived organoids, 2 mM melatonin was added to the culture medium and each organoid was cultured for 10 days. The growth of both patient-derived CRC organoids treated with 2 mM melatonin was significantly inhibited compared with the control, highlighting the growth inhibitory potential of melatonin.

On the other hand, melatonin oncostatic effect was evaluated in a PDC and PDX model of breast cancer by Hasan et al. (Hasan et al., 2019). Tumor cells were obtained from a mastectomy specimen of an African American woman and PDX model was established and propagated in SCID/Beige immunodeficient mice.

For *in vitro* experiments, cells obtained from the tumor (PDC model) were treated with melatonin at 10 µM for 36 hours, alone or in combination with tamoxifen, and with different melatonin-tamoxifen dug conjugates. In this case, some of the drug conjugates inhibited tumor cell viability and migration. Specifically, melatonin plus tamoxifen

demonstrated 80% inhibition of cell viability compared with vehicle whereas melatonin was without effect (Hasan et al., 2019).

Regarding *in vivo* experiments, two melatonin-tamoxifen conjugates were administered to mice by two routes of administration—subcutaneous and oral, being the subcutaneous administration of tamoxifen the group which served as the control group. This experiment was performed to analyze the protective effects of the conjugates. Results showed that one of the conjugates of melatonin and tamoxifen produce uterine protection and modulate estrogen/ER-dependent progesterone receptor mRNA expression in the mammary gland, suggesting that it has the potential to protect against the adverse effects on the uterus associated with chronic tamoxifen usage (Hasan et al., 2019).

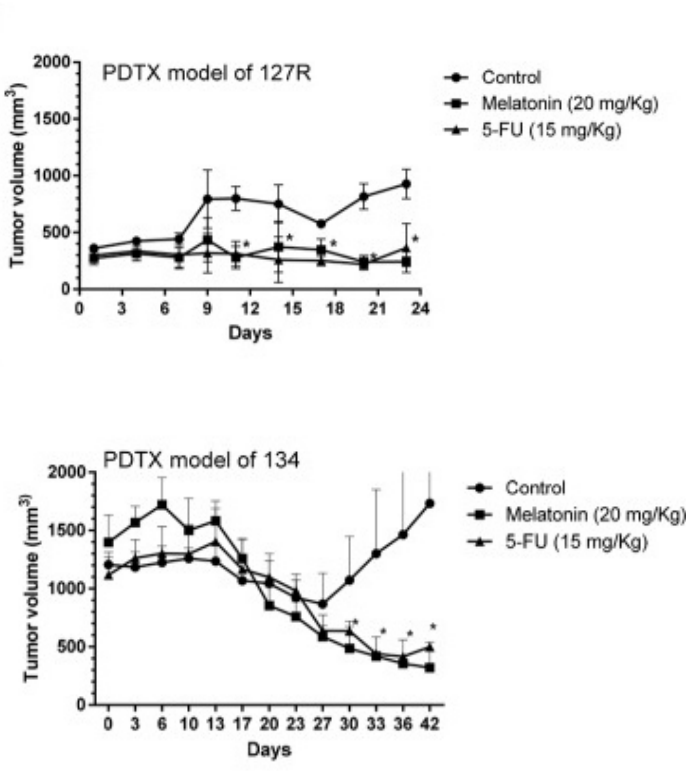

Figure 12. Changes in tumor volume in two different oral cancer PDX models. Tumors were treated for 24 and 42 days, respectively, with melatonin (20 mg/kg daily i.p.), 5-FU (15 mg/kg), and PBS (a vehicle control) (C.-Y. Yang et al., 2017).

The third study which study the effects of melatonin in a patient-derived model was performed by Yang et al. (C.-Y. Yang et al., 2017). They established oral cancer PDX models. Specifically, tumor specimens were obtained from oral squamous cell carcinoma (OSCC) patients with lymphatic metastases and were implanted subcutaneously into NSG mice. After serial transplantation, when tumors reached 500 mm^3, mice

were randomly divided into different experimental groups, receiving vehicle solution, melatonin (30 mg/kg/daily) or 5-FU; all trough intraperitoneal injection. Melatonin inhibited tumor growth in oral cancer PDX models (Figure 12) and induced oral cancer cells G0/G1 arrest. In addition, melatonin has low toxicity and has beneficial effects against cardiotoxicity.

Considering all explained above, to establish a melatonin treatment with proper application in HNSCC patient treatment, the evaluation of this formulation should be evaluated not only in established cancer cell line models but also in patient-derived models to ensure the translation to clinical application.

4. Background of the research group

As explained before, several groups have reported that high concentrations of melatonin can promote ROS generation in a variety of cancers, leading to tumor cell death (Junior et al., 2022; Y.-C. Li et al., 2021; Y. Wang et al., 2022). However, although a great deal of evidence has confirmed the anticancer effects of melatonin *in vitro*, some conflicting results exist when applied *in vivo*, indicating that the effects showed *in vitro* could not always be demonstrated *in vivo* (Y.-Q. Shen et al., 2018; L. Wang et al., 2022).

Specifically, in our Research Group, we perform a subcutaneous treatment with melatonin at 300 mg/kg day in mice with Cal-27 xenografts, an established cancer cell line of HNSCC. This treatment was performed alone or in combination with CDDP or IR, two of the most common HNSCC anti-cancer therapies (Yao et al., 2021). Results showed that subcutaneous administration of melatonin alone did not reduce tumor growth (Figure 13B), which was confirmed with the MRI of the control and treated tumors (Figure 13A). Surprisingly, although the combined treatment of subcutaneous melatonin and CDDP or IR significantly decreased tumor growth compared to the control group,

both CDDP and IR treatments alone resulted in similar reductions of tumor volume respect to the combined treatments (Figures 13D, F). This contrasted with the expectation that the combined treatments would be therapeutically more effective than treatment with CDDP or IR alone, based on previous *in vitro* results (Fernandez-Gil et al., 2019). To study the discrepancy between *in vitro* and *in vivo* results, melatonin levels in tumor were measured. As expected, the concentration of melatonin did not increase in tumors treated with melatonin alone or in combination with CDDP (Figure 13C, E). However, melatonin levels were slightly increased in tumors treated with melatonin and IR compared to the control group (Figure 13G). One possible explanation is the aggressiveness of IR treatment, which modify tumor microenvironment and could facilitate other drugs entry in tumors (Thompson & Maity, 2014; X. Zhang et al., 2023). For that reason, the combined treatment of melatonin and IR decreased tumor growth more significantly than the combined treatment with CDDP. These results suggest that high concentrations of melatonin within the tumor are essential for its oncostatic effect (Martinez-Ruiz et al., 2023; Y.-Q. Shen et al., 2018).

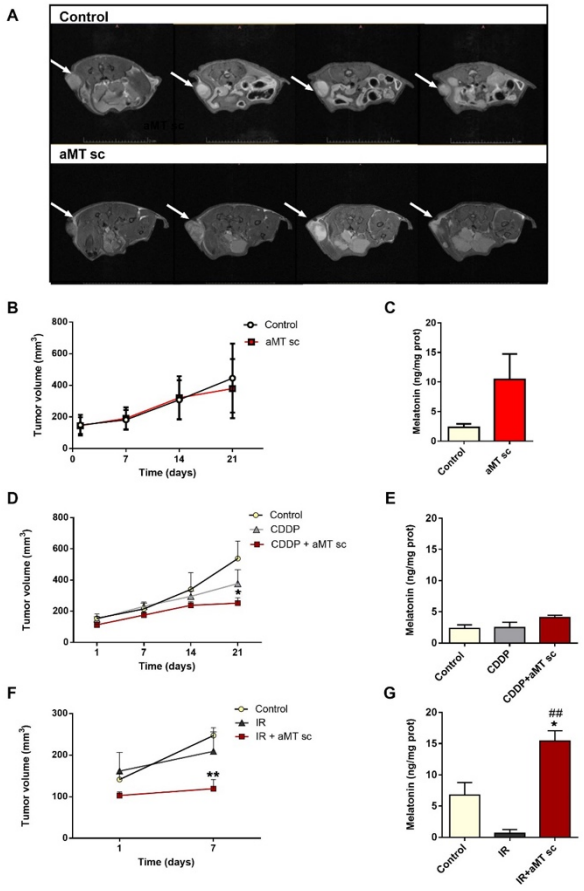

Figure 13. Subcutaneous melatonin treatment does not reduce tumor growth. (A) Representative axial T2-weigthed images obtained by MRI of mice bearing Cal-27 tumors treated with vehicle (control) or with melatonin subcutaneously (aMT sc) after 21 days of treatment. White arrows point tumor mass. (B, D, F) Tumor growth curves and (C, D, F) melatonin levels in tumors at the end of the treatments. For (B, C), treatments groups include control (mice treated with vehicle solution) and aMT sc (mice treated with melatonin at 300 mg/kg day subcutaneously); (D, E) experimental groups comprise control, mice treated with CDDP at 4 mg/kg day once per week (CDDP) and mice treated with CDDP plus melatonin (CDDP + aMT sc). Finally, (F, G) treatments groups involve control, mice receiving one doses of IR at 4 Gy (IR) and mice treated with the combination of IR and melatonin (IR + aMT sc). The data are presented as the means ± standard error of the mean (n=3-6 for each group, one-tailed unpaired t-test; *P < .05; **P < .01; ***P < .001 vs. control group and $^{\#\#}$ P < .01 vs. IR).

HYPOTHESIS AND AIMS

HNSCC is one of the most common malignancies and it is associated with a high mortality rate worldwide. Due to the heterogeneous biology of this type of cancer, treatment strategies are complex and most patients often require multimodal intervention such as surgery, radiotherapy, and/or chemoradiotherapy. However, the adverse side effects of radio- and chemotherapy and the high incidence of chemoresistance, which ultimately leads to treatment failure, limit the clinical usefulness of these therapies as anticancer treatments. Consequently, new HNSCC anticancer therapeutic strategies are required.

Melatonin has a dual effect in normal and tumor cells. Whereas in normal cells it is a potent free radical scavenger and antioxidant molecule, in tumor cells present oncostatic properties. Melatonin has anti-proliferative, pro-oxidant and pro-apoptotic, immunomulator, anti-angiogenic and anti-metastatic actions in different tumor cell types. Melatonin, therefore, can promote tumor cell death as well as protect in normal cells.

In this sense, melatonin oncostatic effect has been widely demonstrated in *in vitro* studies. However, when applied *in vivo*, some conflicting results exist since the results obtained *in vitro* could not always

be demonstrated *in vivo*. Specifically, in our Research Group we have evaluated a subcutaneous treatment with melatonin in mice with HNSCC xenografts, observing that, contrary to the results obtained *in vitro*, this melatonin treatment did not reduce tumor growth compared to the control group. It has also been demonstrated that the concentration of melatonin did not increase in tumors treated with subcutaneous melatonin respect to control tumors.

On the other hand, although established cancer cell lines have traditionally been the most commonly used models to study tumor biology and pharmacogenomics, these models have important limitations. In this regard, the use of patient-derived tumor tissues has transformed the field of drug research since they reflect the heterogeneity and retain the molecular characteristics of patient tumors, being thereby a better alternative to study cancer biology and drug sensitivity.

Considering all above, our hypothesis is that melatonin is a promising agent in anticancer therapy, as new therapeutic approach and as a coadjutant therapy in HNSCC treatment since it can enhance the effects of existing treatments and reduce the side effects of them. However, high

concentrations of melatonin in tumors are necessary to reach its oncostatic effects. In this regard, to establish a melatonin treatment with proper application in clinical practice, it is necessary to search for an alternative formulation or an alternative route of administration which ensures a high bioavailability of melatonin in the tumor. Moreover, this new formulation should be evaluated not only in established HNSCC cell line models, but also in patient-derived models, which further resemble real tumors and ensure clinical translation.

Concerning this hypothesis, the general objective of this study is to analyze the effect of different formulations of melatonin *in vivo* in HNSCC xenografts in order to increase melatonin levels in the tumor and therefore, reach the oncostatic effect of melatonin. For that purpose, the specific objectives are:

- To evaluate the effect of different formulations of melatonin in xenografts of Cal-27 and SCC-9 established cancer cell lines.
- To study if melatonin potentiates chemotherapy effects in mice with HNSCC xenografts.
- To investigate the effects of melatonin in HNSCC metastasis process.

- To analyze the effects of melatonin in HNSCC patient-derived tumor models.

MATERIALS AND METHODS

1. Cells and treatments

1.1. Established cancer cell lines

1.1.1. Cell culture

Two established head and neck squamous carcinoma cell lines were used in this study, Cal-27 (ATCC: CRL-2095) and SCC-9 (ATCC: CRL-1629). Both were obtained at the Cell Bank of the Scientific Instrumentation Centre in University of Granada from the American Type Culture Collection. The cells were cryopreserved in fetal bovine serum (FBS) (16000044, ThermoFisher Scientific, Madrid, Spain) at 5% of dimethyl sulfoxide (DMSO) (23489.297, VWR, Radnor, PA, USA).

Concerning cell culture, Cal-27 cell line was grown in Dulbecco's Modified Eagles Medium-GlutaMAX (DMEM, high glucose, GlutaMAX™ Supplement, 41965039, ThermoFisher). The medium was supplemented with 10% of FBS, 1 mM sodium pyruvate (S8636, Sigma Aldrich, Madrid, Spain) and 2% antibiotic/antimycotic solution (15240062, ThermoFisher Scientific). Meanwhile, SCC-9 cells were cultured in DMEM-F12 Nutrient Mixture Ham medium (11320033, ThermoFisher Scientific). In this case, the medium was supplemented with 10% FBS, 2% antibiotic/antimycotic

solution, 2 mM L-glutamine (25030081, ThermoFisher Scientific), 0.5 mM sodium pyruvate and 0.4 µg/mL hydrocortisone (H0888, Sigma Aldrich).

The cells were maintained in a humidified 37°C incubator with 5% CO_2. When they reached 60-70% of confluence, subculture was performed, changing the culture medium each 2-3 days if necessary. In addition, mycoplasma test using a PCR detection kit following the manufacturer's instructions (25235, LiliF Diagnostics, Burlington, MA, USA) was performed to confirm that cells were negative for mycoplasma.

1.1.2. Treatments

Melatonin (33457-24, Fagron Ibérica S.A.U., Terrasa, Spain) solution was prepared with 15% propane-1,2-diol (PG) (24414.296, VWR) in Dulbecco's phosphate-buffered saline (DPBS) (14190094, Life Technologies, Carlsbad, California, USA). This melatonin solution was filtered using a 0.2 µm pore filter (#PN 4612, Pall Corporation LifeSciences, California, USA) before cell treatment.

When cells reached 60-70% of confluence, FBS was removed from the culture medium. After 48 hours, cells were treated with melatonin at 500

and 1000 µM. Vehicle was added to control group. 24 hours later, the treatment was repeated and 48 hours after the first vehicle/melatonin treatment, the experiment was performed (Figure 14).

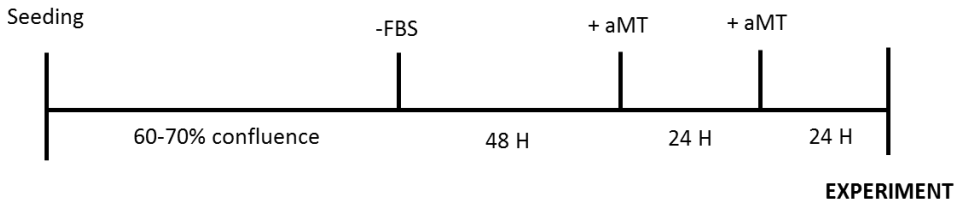

Figure 14. Melatonin treatment diagram in established cancer cell lines.

1.2. Patient-derived tumor cells

1.2.1. Tumor purification

A PDC model, obtained and characterized in Dr. Oppel laboratory (Oppel et al., 2019), was also used in the study. These cells proceed from a patient´s tumor tissue, which was collected from a surgery with informed consent of the patient, according to the declaration of Helsinki and as approved by the ethics committee of the Ruhr-University Bochum (AZ 2018-397). After collection, tumor tissue was processed as following.

Tumor tissue was minced into approximately 2-mm pieces and washed vigorously with DPBS to enhance the washing effect and reduce

the probability of contamination. Pieces obtained were digested using 2.5 mg/mL Collagenase NB4 standard (Nordmark Biochemicals, Uetersen, Germany) solution in DPBS + 3 mM $CaCl_2$ (433535, Carlo Erba, Sabadell, Spain) for 2 h at 37°C with gentle vortexing every 5–10 min. After that, the digestion mix was diluted with DPBS, pipetting up and down to further detach aggregates. Single cells and partially digested tumor pieces were then separated by filtration using a 40-µm cell strainer (SKU 43-50040-51, VWR) and placed in separate culture dishes. Finally, purified cells/cell aggregates were washed in DPBS and re-suspended in culture medium (Oppel et al., 2019).

1.2.2. Cell culture

Purified tumor cells and pieces were grown in PNEU- PneumaCult-Ex Plus Medium (PNEU), including 10 mL of the 50x PneumaCult-Ex Plus supplement (#05041, STEMCELL Technologies Inc., Vancouver, Canada), 1% 200 mM L-Glutamine, 1% 100x Penicillin/Streptomycin (PS-B, Capricorn Scientific, Ebsdorfergrund, Germany), 1% 250 µg/mL Amphotericin B solution (AMP-B, Capricorn Scientific) and 0.5 mL of a 96

µg/mL (0.2 mM) hydrocortisone solution. These fresh cultures were defined as passage 0 (Oppel et al., 2019).

As established cancer cell lines, the cells obtained from patient´s tumor tissue were maintained in a humidified 37°C incubator with 5% CO_2. These cells grow forming aggregates, which are referred as spheroids. Each 2 days, new medium was added to culture plates and cells were subcultured each 3-4 days. To split spheroids, these were disintegrated in a 37ºC water bath using accutase (ACC-1B, Capricorn Scientific) with gentle vortexing every 5–10 min.

1.2.3. Treatment

Melatonin solution was prepared as previously explained. Once spheroids were split, cells were cultured in PNEU and treated with melatonin at 500 or 1000 µM or vehicle solution each 24 hours for 2 days. Experiments about ROS production or apoptosis were performed after 48 hours of initial melatonin treatment (Figure 15).

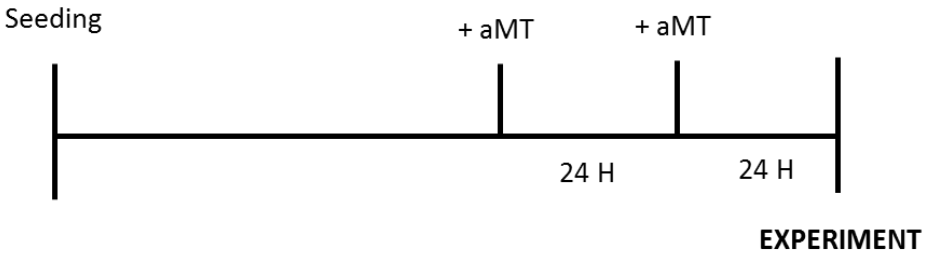

Figure 15. Melatonin treatment diagram in patient-derived cancer cells.

On the other hand, for proliferation analyses of patient-derived cancer cells, the treatment followed was different. In this case, spheroids formation should be assessed for a long period of time. Based on previous studies (Guerra-Librero et al., 2021), the treatment with melatonin at high concentrations each 24 hours results in tumor cell death, preventing experiment conduction after 5 days of treatment. For that reason, for proliferation analyses, cells were treated with melatonin at 500 µM each 72 hours for 21 days (Figure 16). Vehicle was added to the control group.

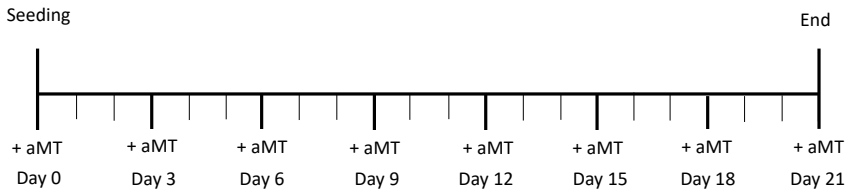

Figure 16. Melatonin treatment diagram in patient-derived cancer cells for proliferation analyses.

2. Animal models

All experiments were performed following a protocol approved by the Institutional Animal Care and Use Committee of the University of Granada (procedures 15/10/2020/119), developed in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (CETS # 123) and Spanish law (R.D. 53/2013).

2.1. Established cancer cell lines model

This study was conducted using 5- to 6-week-old athymic (nu/nu) nude mice (Janvier Labs, France) for established cancer cell lines xenografting. All mice were housed in appropriate sterile filter-capped cages and fed and given water ad libitum.

Traditionally, athymic nude mice have been used to model human tumor growth. The nude mutation prevents development of functional T cells and provided a model for engraftment of human cell lines derived from solid tumors (Shultz et al., 2014). On the other hand, the cells used

for that purpose were Cal-27 and SCC-9, two HNSCC lines derived from tongue tumors.

2.1.1. Xenografting in nude mice

For established cancer cell lines xenografting, first cell viability was assessed using the trypan blue exclusion test. Then, cell suspension was prepared with viable cells at 4 × 10^6 /0.2 mL concentration in DMEM (31053-044, ThermoFisher Scientific) and Matrigel Basement Membrane Matrix (354234, VWR) (1:1). This cell suspension was subcutaneously transplanted into the left flanks of the mice (Y.-Q. Shen et al., 2018).

Once xenografting was performed, mice body weights were measured using a digital balance, and tumor sizes were determined using a vernier caliper twice per week. Tumor volume was calculated with the following formula:

$$Tumor\ volume = \frac{(Width\ \times Length)^2}{2}$$

being length the longest dimension of tumors and width, the perpendicular dimension to length (Y.-Q. Shen et al., 2018).

2.1.2. Animal treatments

After xenografting, once tumor volume reached 100–200 mm^3, mice were randomly divided into different groups and drug treatment was initiated (Y.-Q. Shen et al., 2018).

First, an evaluation of the oncostatic effect of melatonin as an independent anti-cancer therapy was performed. In this study, mice were treated with vehicle solution or with melatonin at 0.3% or 3% each 24 hours for 63 days. Melatonin or vehicle solution was injected intratumorally to mice (60 μL of treatment solution/100 mm^3 tumor). Mice were sacrificed after 35 or 63 days of treatment (Figure 17).

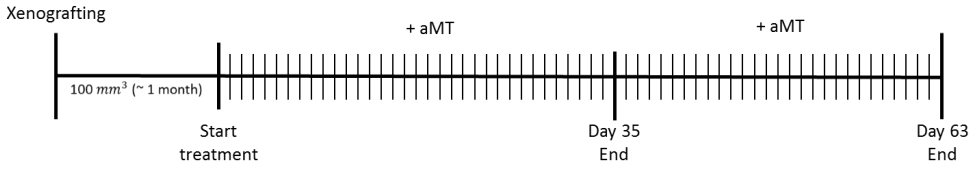

Figure 17. Melatonin treatment diagram in mice.

Next, the evaluation of melatonin as an adjuvant therapy was initiated. In this case, mice were treated with vehicle solution, CDDP and the combination of CDDP and melatonin. CDDP was injected intraperitoneally once per week at 4 mg/kg day (Y. Zhang et al., 2022). When it was combined with melatonin, melatonin was administered each

24 hours at 3% intratumorally, considering previous results obtained. In this case, mice were sacrificed after 35 or 49 days of treatment (Figure 18).

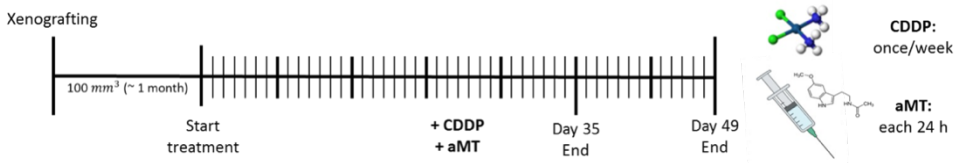

Figure 18. Combined CDDP and melatonin treatment diagram in mice.

To prepare treatment solutions, CDDP was dissolved in saline solution at 1.2 mg/mL. Melatonin was dissolved at 37.5% in PG and then diluted in saline solution. Both, melatonin and CDDP, were filtered using a 0.2 µm pore filter (#PN 4612, Pall Corporation LifeSciences, California, USA) before mice administration.

2.2. Patient-derived tumor model

NOD SCID gamma (NSG) mice were used for patient-derived tumor *in vivo* experiments. All mice were housed in appropriate sterile filter-capped cages and fed and given water ad libitum.

Traditionally, athymic nude mice have been used to model human tumor growth. However, the presence of an intact humoral adaptive immune system and an intact innate immune system, including high NK cell activity limits engraftment with most primary solid human tumors. In this context, a major leap forward in the engraftment of primary human cells, tissues, and tumors was the development of NSG immunodeficient mice. The *scid* mutation largely prevents the development of mature T and B lymphocytes of the adaptive immune system. Meanwhile, the NOD strain background confers intrinsic defects in innate immunity, including lowered NK cell activity, reduced levels of macrophage activation, abnormal dendritic cell development and function, and an absence of hemolytic complement. Finally, NSG mice also bear a targeted mutation in the IL2-receptor common gamma chain gene (Shultz et al., 2014). Therefore, NSG mice are a better alternative to perform PDX since the probability of obtaining a successful engraftment increases with the degree of immunosuppression in the animal host (Genta et al., 2022).

2.2.1. Tumor tissue purification

For PDX model, tumor tissues were obtained from medically indicated surgeries conducted at Klinikum Bielefeld with informed consent of the patients, according to the declaration of Helsinki and as approved by the ethics committee of the Ruhr-University Bochum (AZ 2018-397). Tumor pieces were digested using 5–10 mL of a 2.5 mg/mL Collagenase NB4 standard solution in DPBS + 3 mM $CaCl_2$ for 2 hours at 37°C. After digestion, purified cells/cell aggregates were washed in DPBS and frozen in 5% DMSO in FBS until xenografting (Oppel et al., 2019).

To note, for PDX, tumor cells were not cultured *in vitro*. The cells obtained from tumor tissues were frozen and then, directly transplanted into NSG mice.

2.2.2. Xenografting in NSG mice

For PDX, all cells derived from a tumor digestion were transplanted to one mouse. These cells were resuspended in DMEM and Matrigel Basement Membrane Matrix (1:1) in a total volume of 200 µL. The cell

suspension was subcutaneously transplanted into the left flanks of the mouse (Y.-Q. Shen et al., 2018).

In this case, to obtain a proper quantity of tumors to allow experiment conduction, several transplantations to different generation of NSG mice were required. For that purpose, once tumors reached an appropriate volume, approximately 800 mm^3, mouse was sacrificed, and tumor was extracted. This tumor was digested as previously explained and cells obtained were transplanted into 3 to 5 new mice. Once 8-10 tumors in mice were obtained, treatment was initiated.

2.2.3. Animal treatments

Treatment was initiated when tumor volume reached 100–200 mm^3. In this case, mice were randomly divided into two different groups, mice treated with vehicle solution or with melatonin. Regarding established cancer cell lines *in vivo* results, melatonin was injected at 3% intratumorally to mice each 24 hours and for 28 days. The treatment was performed only for 28 days since control tumors grew faster and mice bearing control tumors should be sacrificed at this point.

3. In vivo tests

3.1. Imaging techniques

3.1.1. Magnetic resonance imaging (MRI)

Various imaging modalities, including magnetic resonance imaging (MRI), have been widely used to monitor structural, functional, and molecular changes in cancer tissues both clinically and pre-clinically. Specifically, due to its non-invasive characteristics and high spatial resolution, MRI is one of the most powerful imaging tools available in diagnostic imaging (Haris et al., 2015) since it is highly sensitive to changes in the signal intensity of bone marrow and soft tissues (Azouz, 2002). Moreover, tumor progression as well as changes in tumor volume before and after treatment can be monitored by MRI (Y. Liu et al., 2018).

Procedure

The magnetic resonance experiments were carried out on a small animal Philips Achieva 3.0 TX (Amsterdam, The Netherlands) using an 8 Channel Sense Wrist Coil. Before imaging, mice were anesthetized with isoflurane at 3% and at 1-2% for maintenance during images acquisition. The animal was in prone position for a transverse acquisition covering the whole volume of the tumors. This planning was made from the sagittal

and coronal scout. Acquisition protocol consisted of a T2-weighted turbo spin-echo multi-slice sequence (T2W MS TSE) with a field of view (FOV) of 50x50x19 mm on the axial plane (27 slices), voxel size= 0.15x0.15x0.7 mm; TE=97 ms and TR=2800 (Sifre et al., 2022).

For images analysis, the total number of images acquired were considered, selecting for all the experimental groups the same image number to ensure a proper comparison.

3.1.2. Histological analyses

Histopathology is the study of the signs of the disease using the microscopic examination of a biopsy or surgical specimen that is processed and fixed. This imaging technique provides a comprehensive view of the disease and its effect on tissues since the preparation process preserves the underlying tissue architecture. As such, some disease characteristics, e.g., lymphocytic infiltration of cancer, may be deduced only from a histopathology image. Additionally, the diagnosis from a histopathology image is also possible in a considerable number of diseases, including almost all types of cancer (Gurcan et al., 2009).

Technically, for histological analyses, tumor sections are stained to visualize different components of the tissue. Specifically, in this study, tumor sections were stained with Hematoxylin-Eosin (HE), Picrosirius (PS), Alcian blue (AB) and Ki-67.

HE staining has been used by pathologists for over a hundred years. Hematoxylin stains cell nuclei blue, while Eosin stains cytoplasm and connective tissue pink. This staining method is used to perform a morphological evaluation of tumor tissues. Furthermore, division cells as well as apoptosis, necrosis or infiltrating cells could also be observed sometimes with this staining technique, permitting a proliferation evaluation of tumor cells (Gurcan et al., 2009).

PS stain is considered a highly specific and selective stain for collagen fibers due to its ability in differentiating between different types of collagen fibers in various pathological conditions. Therefore, this histochemical technique reveals the collagen capsule that surround tumors in most type of cancers (Manjunatha et al., 2015). Some studies mention that collagen fibers play an important role in ECM destruction and remodeling (Lattouf et al., 2014; Y. Li & Aparicio, 2013) since the

disruption of collagen capsules favors tumor invasiveness (Manjunatha et al., 2015).

On the other hand, AB was performed to analyze the glandular differentiation of tumor tissues. To note, HNSCC characterized by both squamous cell carcinoma and true adenocarcinoma. The two components occur in close proximity, but they tend to be distinct and separate (Rothenberg & Ellisen, 2012). Mucin production by the adenocarcinoma part is typically present, either intraluminal or intracellular (Rothenberg & Ellisen, 2012). A limited number of studies have investigated the impact of glandular differentiation on oncologic outcomes in different types of cancer, demonstrating that, in general, glandular differentiation was associated with an increased risk of developing high-grade carcinomas (Xu et al., 2017; Zhao et al., 2019).

Finally, proliferating neoplastic cells were identify by using antibodies against Ki-67 protein. The expression of Ki-67 is strongly associated with tumor cell proliferation and growth, and is widely used in routine pathological investigation as a proliferation marker (Koopman et al., 2018; LI et al., 2015). Contrary to Ki-67 analysis, the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)

assay is widely used as a measure of apoptotic cell death. This assay detects DNA breakage by labeling the free 3'-hydroxyl termini, detecting cells in apoptosis (Mirzayans & Murray, 2020).

Procedure

A. Tumor sample acquisition and preparation

For histological analyses, animals were anaesthetized by intraperitoneal injection of equitesin (1 mL/kg). After confirming complete anaesthetization through loss of all reflexes, animals were subjected to intracardiac perfusion with 3.7–4% neutral buffered paraformaldehyde (PFA, 9713.5000, VWR). Once perfused, tumors were carefully dissected, fixed in PFA for 24 hours and then, longitudinally sectioned for histological processing and analysis. Selected samples were dehydrated in ascending graded concentrations of ethanol, cleared in xylol and embedded in paraffin. Histological sections of 5 µm were dewaxed, hydrated and stained.

Sample preparation for TUNEL assay was different. In this case, after tumor fixation in PFA, tumors were dehydrated in 30% sucrose and embedded in optimal cutting temperature (OCT). OCT was used despite

paraffin since these embedded samples were later frozen and cut in a cryostat. Sections of 5 µm were used to perform immunofluorescence protocol.

B. Histochemical or immunohistochemical methods

As previously explained, HE staining was used for morphological evaluation of tumor progression. Tumor samples were incubated for 1 minute with hematoxylin followed by a 30 seconds incubation with eosin. The collagen network was assessed by using PS histochemical method, whereas proteoglycans and mucopolysaccharides (produced by adenosquamous cell clusters) were identified by using AB pH 2.5. Both methods consisted on a 30 minutes incubation with the corresponding reagents (García-García et al., 2021; Y.-Q. Shen et al., 2018).

On the other hand, proliferating neoplastic cells were identify by using antibodies against Ki-67 protein (MAD-000310QD, Vitro SA, Madrid, Spain). In this case, the immunohistochemistry method consisted on an overnight incubation at 4ºC with the corresponding antibody after an antigen retrieval with nitric citrate pH 6.

DeadEnd Fluorometric TUNEL system (G3250, Promega, Madison, WI, USA) was performed following the manufacturer's indications. Tumor sections were first permeabilized with Proteinase K at 20 µg/mL at room temperature for 10 minutes and then labeled with terminal deoxynucleotidyl transferase at 37°C for 1 hour. Finally, after stopping the labeling reaction with the appropriate buffer provided in the commercial kit, Hoechst (33342, Invitrogen, ThermoFisher Scientific) staining was carried out to stain cell nuclei.

Images were acquired with a fluorescence microscopy (Nikon Eclipse Ni-U microscope).

C. Images analyses

In order to determine tumor encapsulation quantitatively, the tumor/capsule ratio was calculated by using PS stained sections. In this sense, the whole tumor area including the PS positive capsule was determined and then the percentage of area occupied by the capsule was obtained. The same quantitative approach was applied to determine the percentage of AB positive areas occupied by mucopolysaccharides. These

quantitative approaches have been used in other studies (Carriel, Garzón, et al., 2017; Chato-Astrain et al., 2020).

The cell proliferation index was calculated in Ki-67 immunohistochemical stained sections following previous described protocols (Carriel, Scionti, et al., 2017). These protocols are based in selecting images of different parts of the tumors to count the number of cells positive for Ki-67. In this case, 5 images were randomly selected for each tumor and manual counting of Ki-67 positive cells was performed. The total number of cells of each section was also counted to calculate the proliferation index. The same quantitative approach was applied to determine the apoptotic cell percentage in TUNEL immunofluorescence sections.

All these images´ analyses were performed using Image J Software (Free Software Foundation Inc., Boston, MA, USA).

3.1.3. Electron microscopy analyses

The evaluation of tumors by transmission electron microscopy is often correlated with conventional histological and immunohistochemical studies (Eyden, 2002). In preclinical studies, transmission electron microscopy is routinely used to collect ultrastructural data from biological specimens at nanoscale resolution and to correlate structural images with biological functions (de Senneville et al., 2021). Understanding nanoscale structural changes can provide information about the physical state of cells or tissues. Specifically, in cancer research, the study of nanoscale structural alterations could reveal important information about the progress of carcinogenesis (Adhikari et al., 2020).

Procedure

For electron microscopy (EM) analyses, animals were subjected to intracardiac perfusion with 2% PFA-2% glutaraldehyde (A17876, ThermoFisher Scientific) in 0.1 M phosphate buffer (pH 7.4, PB). After surgical resection, tumors were immediately fixed with the same fixative solution for 24 hours. Samples were then cut into 200-mm sections, washed with 0.1 M PB and kept in 0.1 M PB-0.05% sodium azide at 4°C until subsequent use (Ulloa-Navas et al., 2021). Sections were post-fixed

in 2% osmium tetroxide, dehydrated, and embedded in Durcupan resin (44611-44614, Sigma Aldrich). A diamond knife was used to cut semithin sections (1.5 mm), which were stained with 1% toluidine blue for light microscopy. Ultrathin sections (70–80 nm) were then cut, stained with lead citrate, and evaluated under an FEI Tecnai G2 Spirit transmission electron microscope (FEI Europe) using a digital camera (Morada Soft Imaging System; Olympus) (Cebrián-Silla et al., 2017).

3.2. Melatonin determination by HPLC

Melatonin quantification has been controversial for many years. However, HPLC is regarded as the most reliable method and it is successfully used for determining melatonin in different types of samples, including plasma, saliva, and tissue samples (Lin et al., 2012; Martins et al., 2017).

Procedure

Melatonin was extracted with trichloromethane and the organic phase was evaporated to dryness using SPD 2010 Speed Vac System (ThermoFisher Scientific). The samples were then analyzed by HPLC

(Shimadzu Europe GmbH, Duisburg, Germany) with a Waters Sunfire C18 column (150 × 4.5 mm, 5 µm). After stabilizing the column with the mobile phase (acetic acid 85 mM, EDTA 0.1 mM and acetonitrile 25%, pH 4.7), samples were injected into the HPLC system at 1 mL/minute flow rate. Melatonin fluorescence was measured using a Shimadzu RF-10A XL fluorescence detector (Shimadzu Europe GmbH) with 285-nm excitation and 345-nm emission wavelengths. Retention time was 8.9 minutes. A standard curve for melatonin was used to calculate the amount of melatonin in samples according to the peak area (Chahbouni et al., 2010; Y.-Q. Shen et al., 2018).

3.3. Western blot analysis

Western blotting is a well-known molecular biology method, that is commonly used to detect, identify and quantify specific proteins. In cancer research, is has been widely applied to detect key proteins for different purposes, from tumor evaluation to diagnostic or treatment selection applications.

Procedure

For western blot analysis, tumors were rapidly resected and frozen in liquid N_2 after animal sacrifice. After that, first, tumors were homogenized for protein extraction. For that, tumors were mechanically minced using appropriate blades and then, resuspended in a homogenization buffer containing Tris-HCl 100 mM pH 7.6 (1.319.491.211, Panreac, Castellar del Vallès, Spain), NaCl 1.5 M (131659, Panreac), 1% triton (7-878, Sigma Aldrich), DTT 50 mM (D0632, Sigma Aldrich), EDTA 0.5 M (ED4SS, Sigma) and a cocktail of protease inhibitors (78429, ThermoFisher Scientific) for its homogenization. Next, the homogenate samples were sonicated and subsequently centrifuged at 1000 G for 5 minutes at 4ºC. The supernatants obtained were used partly for protein quantification by Bradford assay and the rest for western blot sample preparation. Samples were prepared at a concentration of 40 µg protein/20 µL in sample buffer (Tris-HCl 0.5 M pH 6.8, 2% sodium dodecyl sulphate (SDS) (L3771 Sigma Aldrich), 10% 2-mercaptoethanol (ME00950250, Schalau), 20% glycerol and 0.025% bromophenol blue (B-8026, Sigma Aldrich)) and Tris 50 mM. To denature the proteins, samples were incubated at 99ºC for 10 minutes. Once the samples were prepared,

proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 15% acrylamide/bis-acrylamide gels (1610159, BioRad, Hercules, California, USA). Proteins were then wet transferred to a polyvinylidene difluoride (PVDF) membrane (IPVH00010, Sigma Aldrich). Once the proteins were transferred to the membranes, they were incubated with blocking buffer containing 5% milk or BSA in DPBS with 0.1% Tween 20. After that, they were incubated with the primary antibody (Table 1) diluted in blocking buffer overnight at 4°C. Following this incubation, the membranes were washed and incubated with the HRP-conjugated secondary antibodies at room temperature for 1 hour. Protein-antibody interaction was detected by chemiluminescence using the ECL™ Prime Western Blotting Detection Reagent (GERPN2232, GE Healthcare Life Sciences, Barcelona, Spain), analyzed with ChemiDoc MP Imaging System (BioRad) and quantified using ImageJ software. Protein band intensities were normalized to GAPDH. Data were expressed relative to control group.

Table 1. Antibodies used for WB analyses.

ANTIBODY	DILUTION	SOURCE	IDENTIFIER
Bax	1:200	Santa Cruz, Heidelberg, Germany	sc-7480
Bcl-2	1:100	Santa Cruz	sc-7382
Oxidized proteins	1:5000	Abcam, Cambridge, UK	ab178020
CD98	1:500	Abcam	ab108300
GAPDH	1:500	Santa Cruz	sc-166574
HRP Goat Anti-Mouse	1:1000	BD Pharmigen, San Jose, CA, USA	554002

3.3.1. Measurement of protein oxidation by western blot

A specific western blot protocol was followed for measuring oxidized proteins. These proteins were detected using Protein Carbonyl Assay Kit (ab178020, Abcam), which is designed for the measurement of protein carbonyl groups that are created by the oxidation of proteins. In the protein carbonyl assay protocol, carbonyl groups in protein side chains are derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reaction with 2,4-dinitrophenylhydrazine (DNPH) and finally, the DNP moieties are detected by western blotting. This kit has the advantage that

oxidized proteins are identified from a complex mixture, allowing the comparison of the oxidative status between samples.

Procedure

Samples were prepared according to manufacturer´s instruction with all the reagents provided in the commercial kit used. In this case, after minced mechanically, tumors were homogenized in DPBS. Protein extraction was achieved using an extraction buffer for 20 minutes on ice. Samples were subsequently centrifuged at 18000 G for 20 minutes at 4 ºC and the supernatants obtained were used, on one hand, for protein quantification by Bradford assay and, on the other, for western blotting sample preparation. Next, protein concentration was adjusted to 3 µg/µL to achieve derivatization reaction. As previously explained, proteins were derivatized by reaction with DNPH for 15 minutes. After neutralizing this reaction, samples were ready to be separated by SPS-PAGE. Following manufacturer's instructions, proteins were separated by SDS-PAGE in 15% acrylamide/bis-acrylamide gels and then, wet transferred to a PVDF membrane. Once in the membrane, they were incubated with blocking buffer containing 5% milk in DPBS with 0.1% Tween 20, followed by the incubation with the primary antibody (1:5000 in blocking buffer)

overnight at 4°C. Next day, the membranes were washed and incubated with the HRP-conjugated secondary antibody, which, in this case, is goat anti-rabbit IgG, at room temperature for 1 hour. Finally, protein-antibody interaction was detected by chemiluminescence and detected in ChemiDoc MP Imaging System, as previously explained. The intensity of the bands was normalized to GAPDH and data were expressed relative to the control group.

3.4. Measurement of lipid peroxidation (LPO)

Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals (Ito et al., 2019). It is usually used as an indicator of oxidative stress levels in cells and tissues since lipids are susceptible targets of oxidation because of their molecular structure abundant with reactive double bonds. Two of the most well studied markers of lipid peroxidation are malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE), which are generated via peroxidation of different fatty acids (Ho et al., 2013). Specifically, 4-HNE is produced as a major product of the peroxidative decomposition of polyunsaturated fatty acid and MDA, meanwhile, is in many instances the most abundant individual aldehyde

resulting from lipid peroxidation. Increased levels of both products are found in plasma and various organs under conditions of oxidative stress (Esterbauer & Cheeseman, 1990).

Procedure

For LPO evaluation, mice were sacrificed, and tumors, heart, lung and liver were resected and frozen in liquid N_2. In this case, the homogenization process was performed in Tris 20 M pH 7.4 and after a centrifugation at 2500 G for 10 minutes at 4°C, the supernatants obtained were used for LPO analysis. LPO was assessed using the Bioxytech LPO-568 assay kit (OxisResearch, Portland, OR, USA), which uses MDA and 4-HNE as markers. Samples were incubated with N-methyl-2-phenylindole and methasulfonic acid for 40 minutes at 45°C. N-methyl-2-phenylindole reacts with MDA and 4-HNE, yielding a stable chromophore with intense maximal absorbance at 586 nm. Protein concentration was also measured by Bradford assay to normalize results obtained.

Spectrophotometer measurements were performed in a Spectrophotometer FLX800 (Bio-Teck Instruments Inc., Winooski, VT, USA).

4. In vitro tests

4.1. Proliferation and spheroid formation assay

In suspension culture, cells tend to aggregate and go through the process of self-assembly. Therefore, cell aggregates of adjacent cells form a spheroid shape having wide utility in tumor and cancer research (Perche & Torchilin, 2012; Ryu et al., 2019).

The main feature of tumor-derived spheroids is the enrichment of CSCs or cells with stem cell–related characteristics due to their potential for self-renewal and differentiation. Hence, spheroids formation is considered a CSC proliferation marker. Consequently, the *in vitro* spheroid formation assay is a common assay used to measure the self-renewal and multipotent nature of the cancer stem cell subpopulations within a tumor or cancer cell line (Ishiguro et al., 2017; Oppel et al., 2019).

Procedure

For proliferation analysis, spheroids formed from patient-derived tumor cells, which were previously in culture, were dissociated with accutase at 37°C. Once a single cell suspension was obtained, one single cell was seeded in 100 µL of PNEU medium per well in a 96 well-plate (P-

96-450R-C, ThermoFisher Scientific) (Figure 19). These plates have round wells to facilitate single cell observation at the microscope. Just after seeding, each well was analyzed and observed at the microscope and those wells with no cells or more than one cell were eliminated from the study. Next, wells were divided into two groups, cells treated with vehicle solution and cells treated with melatonin at 500 µM. Cell division and growth, as well as spheroid formation, were monitored through the images obtained at day 0, 5, 14 and 21 with Olympus CKX41 Microscope (Olympus Deutschland GmbH, Hamburg, Germany). Diameter of spheroids and cell aggregates were later analyzed with ImageJ software.

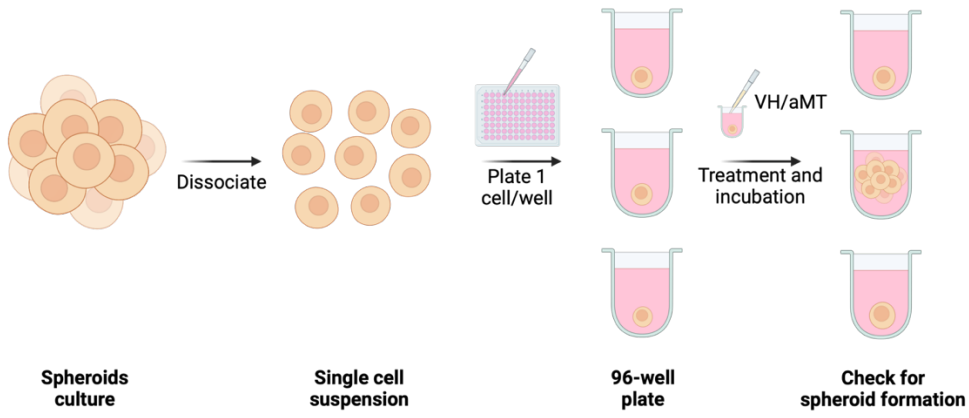

Figure 19. Overview of the spheroid formation assay protocol. Image created using Biorender.com

4.2. ROS production evaluation

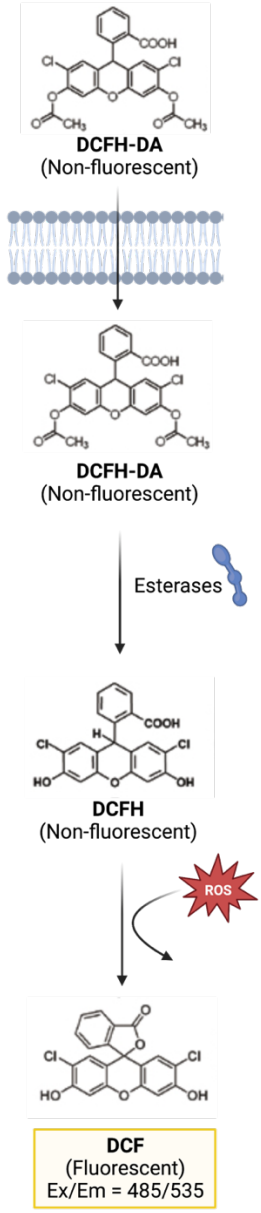

Figure 20. DCFH assay protocol. Image created using Biorender.com.

ROS levels were analyzed using the cell permeant reagent 2',7' – dichlorofluorescin diacetate (DCFH-DA), which is widely used to quantitatively assess reactive oxygen species in live cell samples (Fernandez-Gil et al., 2019; Florido, Martinez-Ruiz, et al., 2022; Guerra-Librero et al., 2021).

The DCFH assay protocol is based on the diffusion of DCFH-DA into the cell. It is then deacetylated by cellular esterases to a non-fluorescent compound (DCFH), which is later oxidized by ROS into 2', 7' – dichlorofluorescein (DCF). DCF is highly fluorescent and is detected by fluorescence spectroscopy with excitation / emission at 485 nm / 535 nm (Figure 20).

Procedure

Patient-derived tumor cells, dissociated from spheroids as previously explained for proliferation analysis and already treated, were seeded the afternoon before the experiment in dark 96-well plates (236105, ThermoFisher Scientific) at a density of 10,000 cells per well. Each group was performed in triplicate. On the day of the experiment, the culture medium was replaced with phenol red free medium and DCFH-DA

(D6883, Sigma) was added at 100 µM. Cells were then incubated for 30 minutes at 37°C. Subsequently, the cells were washed with DPBS and Krebs-Ringer bicarbonate buffer was added. Fluorescence was measured with a FLx800 plate fluorimeter for 45 minutes in 5-minute intervals at 485 nm excitation and 530 nm emission.

4.3. Western blot

Procedure

For western blot procedure, cell pellets were first recollected. Cells, already treated with melatonin or vehicle solution, were recollected using accutase for 5-10 minutes and centrifuged at 1000 rpm for 10 minutes. Cell pellets obtained were then resuspended and sonicated in 60 µL of homogenization buffer, composed of Tris-HCl pH 7.6 50 nM, DTT 7.7 mg/mL, cocktail of protease inhibitors and EDTA (78429, ThermoFisher Scientific) for protein extraction. They were subsequently centrifuged at a speed of 3,000 rpm for 20 minutes at 4°C and part of the supernatants obtained were used for protein quantification by Bradford assay and the rest for western blotting sample preparation. Samples were

prepared at a concentration of 40 µg protein/20 µL in sample buffer (the same composition for *in vivo* tests) and Tris 50 mM. Finally, proteins were separated by SPS-PAGE, transferred to PVDF membranes and incubated with the corresponding antibodies as previously explained in part 3.3. The primary and secondary antibodies used for *in vitro* western blotting analysis are summarized in the following table (Table 2). In this case, protein band intensities were normalized to GAPDH or β-actin.

Table 2. Antibodies used for *in vitro* WB.

ANTIBODY	DILUTION	SOURCE	IDENTIFIER
CD44	1:500	Abcam	ab157107
CD98	1:500	Abcam	ab108300
Caspase-9	1:500	Santa Cruz	sc-56076
GAPDH	1:500	Santa Cruz	sc-166574
β-actin	1:500	Santa Cruz	sc-47778
HRP Goat Anti-Mouse (anti-mouse)	1:1000	BD Pharmigen	554002
Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP (anti-rabbit)	1:2000	ThermoFisher Scientific	31460

4.4. Migration assays

The study of cell migration in cancer research is of particular interest as the main cause of death in cancer patients is related to metastatic

progression. To spread and disseminate throughout the body, cancer cells must migrate and invade through ECM, intravasate into blood circulation, attach to a distant site, and finally extravasate to form distant foci. In this context, various biological methods have been employed to study these events in detail. Specifically, the cell culture wound-closure and the transwell migration assays are widely used in the scientific community to evaluate tumor cell migration (Justus et al., 2014).

4.4.1. Transwell assay

The transwell migration assay is a commonly used test to study the migratory response of cells to different inducers or inhibitors. During this assay, endothelial cells are placed on the upper layer of a cell culture insert with permeable membrane and a solution containing a chemoattractant is placed below the cell permeable membrane. Following an incubation period, the cells that have migrated through the membrane are stained and counted (Justus et al., 2014).

Procedure

A Transwell assay was performed to determine the migration of Cal-27 and SCC-9 cells. Transwell chambers (3422, Corning, Kennebunk, USA) with a pore size of 8 µm were used for that assay. Cells were treated with vehicle, melatonin at 500 or 1000 µM as previously explained. The day before the experiment, 40,000 cells in 350 µL of DMEM with 0.5% FBS were seeded in the top chamber. In the lower chamber, 450 µL of DMEM with 2% FBS was added as chemoattractant, to induce cell migration (Figure 21). After the cells were incubated for 24 hours at 37°C in a 5% CO_2 atmosphere, cells on the top surface of the insert were removed with a cotton swab. Cells adhering to the lower surface, which were the cells that migrated, were fixed with 4% PFA, stained with DAPI and counted under a microscope Nikon Eclipse Ni-U microscope in nine fields.

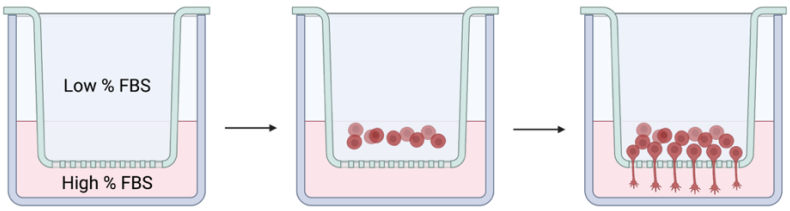

Figure 21. Transwell assay basis. Image created using Biorender.com.

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4.4.2. Wound healing assay

Wound healing assay is a simple, non-expensive, and highly reproducible method to study cancer cell migration *in vitro*. It is based on the observation that cells growing in a monolayer migrate to re-establish cell contacts after the development of an artificial wound. The conventional wound healing assay requires the formation of a cell monolayer that is scratched in order to create a wound. Cell migration is monitored as images are captured immediately after wound creation as well as at given time points during wound closure (Figure 22). Images are then compared in order to calculate cell migration (Freitas et al., 2021). Following the analysis of the wound closure over time when comparing to a control may reveal specific migration changes or an impaired migratory phenotype (Justus et al., 2014).

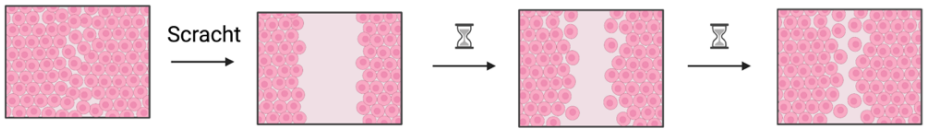

Figure 22. Wound healing assay basis. Image created using Biorender.com.

Procedure

Additionally, tumor cell invasion capabilities were evaluated by wound healing assay. For that assay, the cells were seeded and treated in a six-well plate (734-2323, VWR). After 48 hours of melatonin treatment and with a cell density of ~80%, linear scratches were created via scraping the cell monolayer with a 10 µL pipette tip, followed by washing with DPBS to remove the cell fragments and addition of fresh medium. Wound healing was observed for 48 hours. Images of the same wound area were observed under an inverted phase-contrast microscope and recorded at 0, 24 and 48 hours using a camera (OlympusBX61, Tokyo, Japan) after wound generation. ImageJ was employed to estimate the width of the wound. The migration rate was estimated through the percentage of scratch wound coverage, which was calculated as follows:

$$Scracth\ wound\ coverage\ (\%) = 100 - \left(\frac{Scratch\ wound\ distance\ at\ 24\ \ or\ 48\ hours}{Scratch\ wound\ distance\ at\ 0\ hours} \times 100\right)$$

5. Statistical analysis

All statistical analyses were performed using Prism 8 scientific software (GraphPad Software Inc.). Unpaired Student's *t* tests were used to compare differences between experimental groups and their respective controls. Data were expressed as the mean ± standard error of the mean of a minimum of three independent experiments. A $P < 0.05$ was considered statistically significant.

RESULTS

Part of the results shown in this section are included in the following article:

Martínez-Ruiz L, Florido J, Rodriguez-Santana C, López- Rodríguez A, Guerra-Librero A, Fernández-Gil BI, García-Tárraga P, Garcia-Verdugo JM, Oppel F, Sudhoff H, Sánchez-Porras D, Ten-Steve A, Fernández-Martínez J, González-García P, Rusanova I, Acuña-Castroviejo D, Carriel V, Escames G. Intratumoral injection of melatonin enhances tumor regression in cell line-derived and patient-derived xenografts of head and neck cancer by increasing mitochondrial oxidative stress.

Biomedicine & Pharmacotherapy, 2023

1. Intratumoral melatonin treatment drastically inhibits tumor growth in HNSCC xenografts

Although previous studies reported that melatonin leads to apoptosis in HNSCC cells in a dose dependent manner (Lang et al., 2021; SUNG et al., 2020), there are some conflicting results when melatonin it is applied *in vivo*, since the results obtained *in vitro* could not always be demonstrated *in vivo* (Y.-Q. Shen et al., 2018). These contradictory results could be due to melatonin should reach high concentrations in tumors to achieve its oncostatic effects. Specifically, Martinez-Ruiz et al. demonstrated that the subcutaneous administration of melatonin at 300 mg/kg day does not decrease tumor growth in Cal-27 xenografts, which was related to a low concentration of melatonin in treated tumors compared to control tumors (Martinez-Ruiz et al., 2023) .

To overcome the limitation of subcutaneous melatonin administration and to increase melatonin levels into the tumors, an intratumorally injectable of melatonin at 0.3 or 3% which is under patent (P201730598), was evaluated. To investigate the effect of this new formulation of melatonin in tumor growth *in vivo*, HNSCC cell line Cal-27

was injected into the flank of immunodeficient mice. Melatonin or vehicle solution was administered daily intratumorally for 63 days.

Results showed that intratumoral treatment with melatonin 3% reduced tumor growth compared to control group whereas melatonin 0.3% had no effect (Figure 23A). Particularly, the control group exhibited significant tumor growth from day 2 to day 41 with respect to the initial tumor volume (Figure 25A). However, melatonin 3% exhibited a decrease in tumor volume from day 34, which was significant on day 48, resulting finally in 60-100% regression of the tumor (Figure 23A and 25A). At the end of the treatment, melatonin 3%-treated tumors were smaller than 200 mm^3 (Figure 23A). This reduction of tumor volume correlated with a significant increase of melatonin levels in the tumors (Figure 23B). In contrast, control tumors and tumors treated with melatonin 0.3% exceeded 1000 mm^3 after 41 days (Figure 23A), the allowed volumes passed by Humane Endpoint Criteria, and the mice were sacrificed. These results were corroborated with MRI, which exposed the differences in control and treated tumors sizes, validating successful suppression of tumor growth with the intratumoral injections of melatonin 3% (Figure 23C).

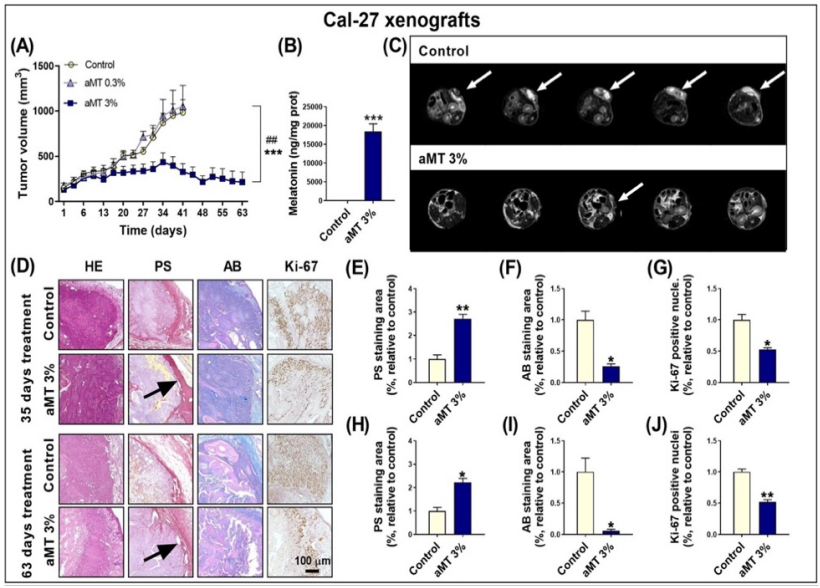

Figure 23. Intratumoral melatonin injection at 3% drastically inhibits tumor growth in Cal-27 xenografts after 63 days of treatment. (A) Tumor growth curve for mice bearing Cal-27 tumors receiving vehicle (control) or intratumoral melatonin injections (0.3 [aMT 0.3%] or 3% [aMT 3%]) every day for 63 days. Cal-27 control mice were sacrificed after 41 days of treatment because tumor volume exceeded 1000 mm^3. Data are presented as the means ± standard error of the mean (n=8-10 for each group). One-tailed unpaired t-test: ***P < .001 vs. control group and $^{\#\#}$P < .01 vs. melatonin 0.3% treated tumors. (B) Melatonin levels in tumors after 35 days of treatment. (C) Representative axial T2-weigthed images obtained by MRI of mice bearing Cal-27 tumors treated with vehicle (control; after 41 days) or melatonin 3% intratumorally (aMT 3%; after 63 days of treatment). White arrows indicate tumor mass. (D) Histological analysis of HE-stained tumors. Collagen capsule using PS staining (red). Mucin glandular structures are stained by AB (blue). Immunohistochemical identification of proliferating cells with Ki-67 antibody (brown). Scale bar = 100 µm. Asterisks mark central tumor cysts and black arrows indicate the capsule stained red by PS. (E, F, G, H, I, J. Quantitative histochemical analyses of PS, AB, and Ki-67 staining, showing an increase in the PS collagen capsule and a decrease in AB and Ki-67 staining after 3% melatonin treatment. Experimental groups include control tumors (mice treated with vehicle) and tumors treated with 3% melatonin intratumorally for 35 days (D, E, F, G) or 63 days (D, H, I, J). Data are presented as the means ± standard error of the mean (n=3 for each group). One-tailed unpaired t-test: *P < .05, **P < .01 vs. control tumors.

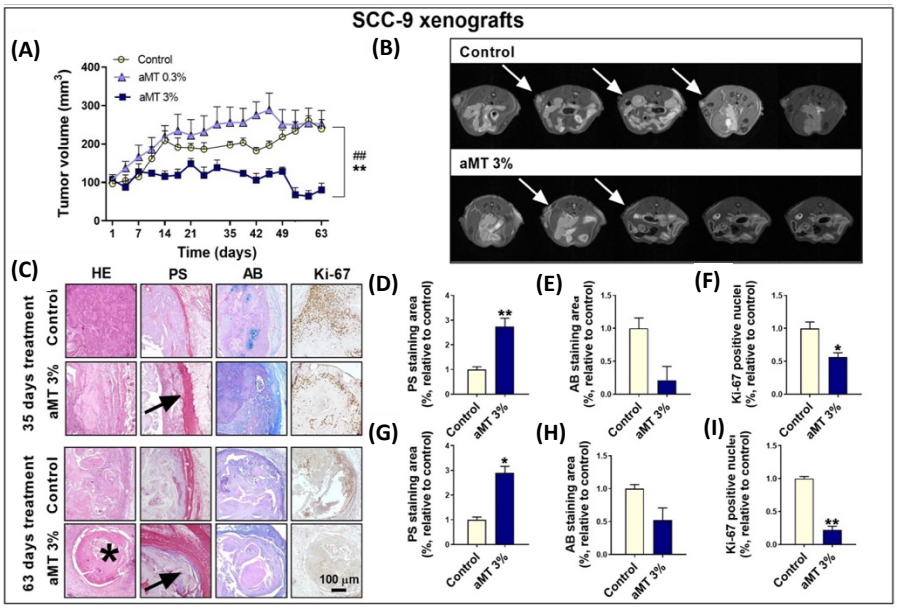

Figure 24. Intratumoral melatonin injection at 3% disminishes tumor growth in SCC-9 xenografts after 63 days of treatment. (A) Tumor growth curve for mice bearing SCC-9 tumors receiving vehicle (control) or intratumoral melatonin injections (0.3 [aMT 0.3%] or 3% [aMT 3%]) every day for 63 days. Data are presented as the means ± standard error of the mean (n=8-10 for each group). One-tailed unpaired t-test: **P < .01 vs. control group and ##P < .01 vs. melatonin 0.3% treated tumors. (B) Representative axial T2-weigthed images obtained by MRI of mice bearing SCC-9 tumors treated with vehicle (control) or melatonin 3% intratumorally (aMT 3%) after 63 days of treatment. White arrows indicate tumor mass. (C) Histological analysis of HE-stained tumors. Collagen capsule using PS staining (red). Mucin glandular structures are stained by AB (blue). Immunohistochemical identification of proliferating cells with Ki-67 antibody (brown). Scale bar = 100 µm. Asterisks mark central tumor cysts and black arrows indicate the capsule stained red by PS. (D, E, F, G, H, I) Quantitative histochemical analyses of PS, AB, and Ki-67 staining, showing an increase in the PS collagen capsule and a decrease in Ki-67 staining after 3% melatonin treatment. Experimental groups include control tumors (mice treated with vehicle) and tumors treated with 3% melatonin intratumorally for 35 days (C, D, E, F) or 63 days (C, G, H, I). Data are presented as the means ± standard error of the mean (n=3 for each group). One-tailed unpaired y-test: *P<.05, **P<.01 vs, control tumors.

Considering the great potential of intratumoral melatonin treatment, its effect was also studied in SCC-9 xenografts. The results were similar to those obtained in Cal-27 derived tumors. Melatonin 3% decreased tumor growth compared to control group whereas melatonin 0.3% had no effect (Figures 24A and 25C). Again, these results were corroborated with images obtained with MRI (Figure 24B). Of interest, the melatonin effects in SCC-9 xenografts were less significant than in Cal-27, which could be due to its lower proliferation rate (Tabosa et al., 2022).

To further evaluate the effect of intratumoral melatonin treatment, histological analyses of Cal-27 and SCC-9 derived tumors were performed after 35 and 63 days of treatment, which showed some differences among experimental groups as compared to their respective controls. In all cases, histology confirmed the development of an epidermoid carcinoma with AB positive adenosquamous differentiation clusters (Figure 23D and 24C). The histology of tumors treated with intratumoral melatonin showed a decrease in tumor active areas in both cell line xenografts, which was more evident after 63 days (Figure 23D and 24C). In addition, the formation of a collagen-rich capsule was noted in melatonin-treated tumors after 35 and 63 days of treatment (Figure 23D,

E, H and 24C, D, G). Interestingly, the effect of the treatment on tumor defined PS positive connective capsule was more evident after 35 days (Figure 23D, E, and 24C, D). This effect of melatonin was in accordance with previous studies, which demonstrated that the administration of subcutaneous melatonin in mice promotes collagen capsule formation, although it has no effect in tumor growth (Martinez-Ruiz et al., 2023). The formation of a capsule was a good signal because it could make the dissemination of tumor cells difficult (Ghirardi et al., 2022; Zou et al., 2022).

Furthermore, intratumoral melatonin resulted in a significant reduction in the adenosquamous differentiation areas in Cal-27 tumors (Figure 23D, F, I), which has been associated with a better cancer prognosis (Xu et al., 2017; Zhao et al., 2019).

The effectiveness of the intratumoral treatment was demonstrated through the quantitative evaluation of Ki-67, which revealed a significant reduction in cell proliferation in the active areas of treated tumors in a time-dependent manner (Figure 23D, G, J and 24C, F, I). Interestingly, after melatonin treatment, the active areas remained at subcapsular tumor zones (Figure 23D and 24C).

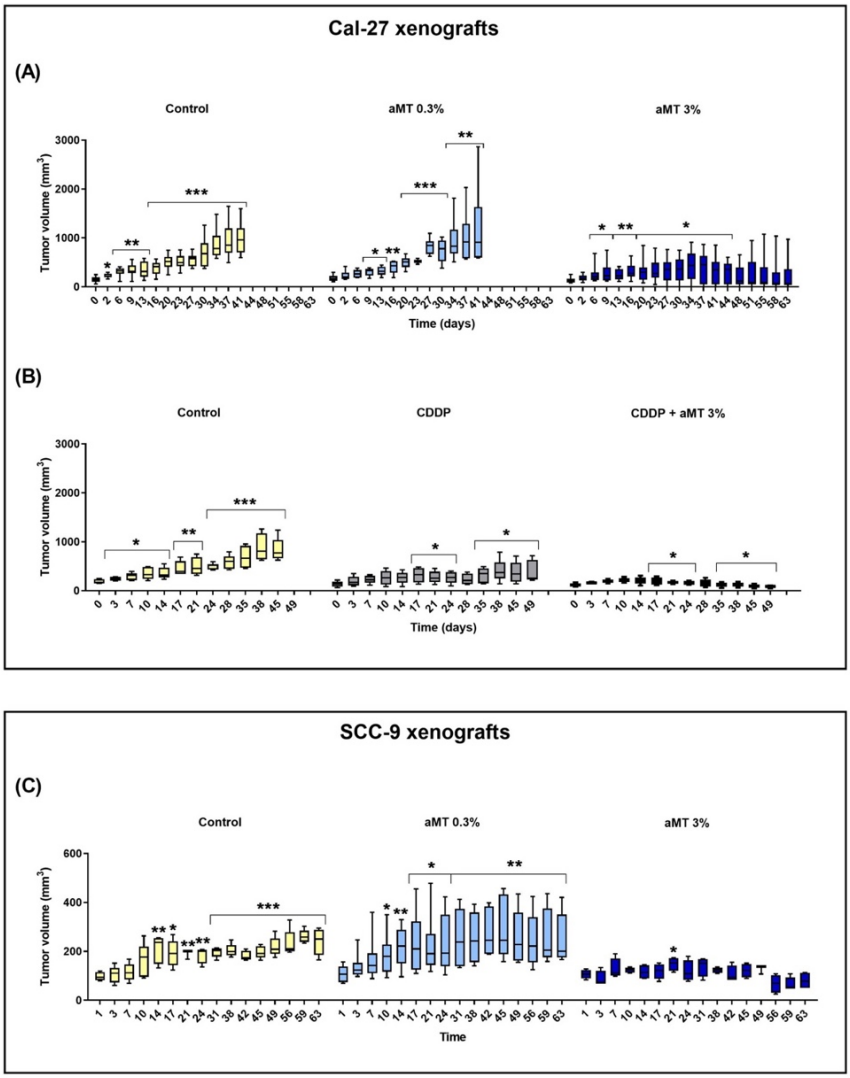

Figure 25. Intratumoral melatonin injection at 3% suppresses tumor growth and synergizes CDDP in HNSCC xenografts. (A, B) Tumor growth curve for mice bearing Cal-27 or (C) SCC-9 tumors. (A, C) Experimental groups comprise control (tumors treated with vehicle) and tumors treated with intratumoral melatonin injections (0.3 [aMT 0.3%] or 3% [aMT 3%]) every day for 63 days. (B) Experimental groups include tumors treated with vehicle (control), cisplatin at 4 mg/kg day once per week (CDDP), and the combination of CDDP plus intratumoral melatonin at 3% (CDDP + aMT 3%). Data are presented as the means ± standard error of the mean (n=4-10 for each group); One-tailed unpaired t-test: *P < .05, **P < .01, ***P < .001 vs. initial tumor volume of each group.

All of these findings demonstrated that the oncostatic effect of melatonin depended on reaching a high concentration in the tumors (Y.-Q. Shen et al., 2018).

2. Intratumoral melatonin treatment potentiates CDDP effects to decrease HNSCC tumor growth

Next, we examined the capacity of the intratumoral injection of melatonin to potentiate the cytotoxic effects of CDDP in Cal-27 xenografts. Melatonin 3% was administered intratumorally every 24 hours and CDDP intraperitoneally once per week for 49 days. Intratumoral melatonin potentiated the effects of CDDP on tumor growth (Figures 26A and 25B). Tumors treated with intratumoral melatonin plus CDDP began to show a decrease in volume from day 14 and had a significant decrease after 35 days of treatment and for the follow-up period (Figure 25B). In contrast, after 35 days, tumors treated with CDDP alone continued to grow (Figures 26A, B and 25B). Strikingly, there were no tumors in mice that received the combined treatment after 49 days, as shown on MRI (Figure 26C). Tumors hardly disappeared after 49 days

of combined treatment, which highlights the great clinical potential of melatonin treatment.

The combined treatment of intratumoral injection of melatonin and intraperitoneal administration of CDDP were also evaluated histologically after 35 days (Figure 26D). In line with above results, histology revealed a reduction of the tumor development with a significant increase of the collagen-rich capsule in the combined treatment-group (Figure 26D, E). CDDP, as well as the melatonin plus CDDP group, resulted in a significant reduction in the adenosquamous areas (Figure 26D, F).

These data indicate that intratumoral administration of melatonin potentiates the cytotoxic effect of CDDP, especially to decrease tumor growth and increase the collagen capsule, indicating that it could be a proper clinical approach to improve the effectiveness of existing anti-cancer therapies.

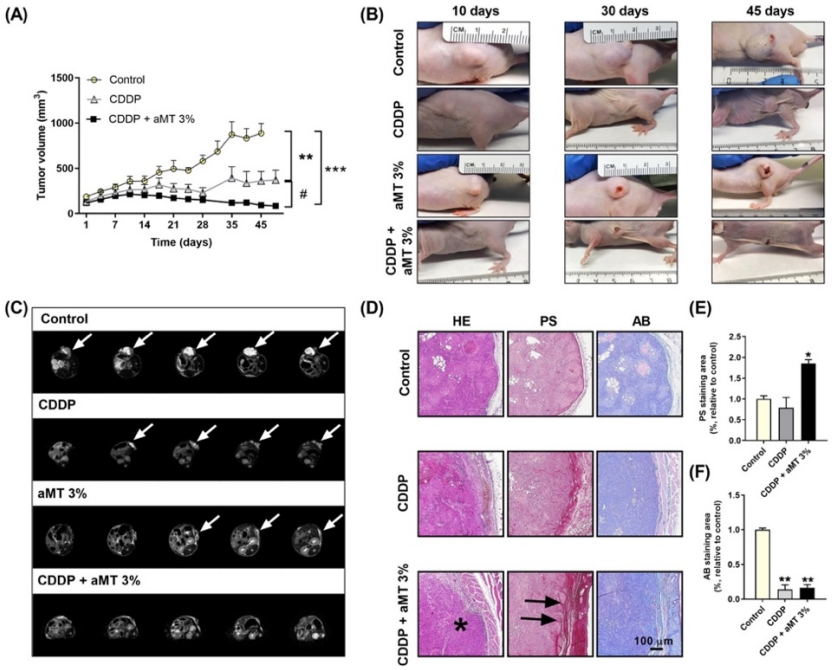

Figure 26. Cal-27 tumors disappear after 49 days of treatment with intratumoral melatonin and CDDP. (A) Tumor growth curve for mice bearing Cal-27 tumors treated with vehicle (control), cisplatin at 4 mg/kg day once per week (CDDP), and the combination of CDDP plus intratumoral melatonin at 3% (CDDP + aMT 3%). Data are presented as the means ± standard error of the mean (n=5-8 for each group). One-tailed unpaired t-test: **P < .01, ***P < .001 vs. control group and $^{\#}$P < .05 vs. CDDP treated tumors. (B) Representative images acquired after 10, 30, and 45 days of treatment. (C) Representative axial T2-weigthed images obtained by MRI of mice bearing Cal-27 tumors treated with vehicle (control), CDDP, intratumoral injections of melatonin at 3%, and the combination of CDDP + aMT 3%. White arrows indicate tumor mass. (D) Histological analysis of HE-stained tumors. Collagen capsule using PS staining (red). Mucin glandular structures are stained by AB (blue). Scale bar = 100 µm. Asterisks mark central tumor cysts, and black arrows indicate the capsule stained red by PS. (E, F) Quantitative histochemical analyses of PS and AB staining, showing an increase in PS collagen capsule and a decrease in AB staining area after 35 days of treatment with the combination of CDDP plus melatonin at 3%. Experimental groups include control tumors (mice treated with vehicle) and tumors treated with CDDP or the combined treatment of CDDP + aMT 3% for 35 days (D, E, F). Data are presented as the means ± standard error of the mean (n=3 for each group). One-tailed unpaired t-test: *P < .05, **P < .01 vs. control tumors.

3. Melatonin stimulates ROS production and apoptosis in Cal-27 xenografts

Given our previous study in which melatonin induced ROS complex I RET under anaerobic metabolism and led to apoptosis from excessive ROS production in HNSCC cells (Florido, Martinez-Ruiz, et al., 2022), we investigated the contribution of intracellular ROS to the potentiation of CDDP-induced cell death. Next analyses of melatonin mechanism of action were performed after 35 days of treatment. For this purpose, we analyzed oxidative stress levels. Melatonin increased LPO levels and oxidized protein expression in Cal-27 tumors (Figure 27A, B). Interestingly, treatment with melatonin plus CDDP enhanced these pro-oxidant effects and noticeably triggered ROS-dependent apoptosis, which resulted in an increase in the Bax/Bcl-2 ratio and TUNEL-positive nuclei compared to the CDDP group (Figure 27C, D, E).

Finally, considering that an increase in ROS is related to cell differentiation (Mendivil-Perez et al., 2017), we analyzed CD98 expression, a cancer stem cell marker in HNSCC (Martens-de Kemp et al., 2013). Melatonin 3% reduced CD98 levels compared to the control and

CDDP groups (Figure 27F), suggesting an increase in tumor cell differentiation.

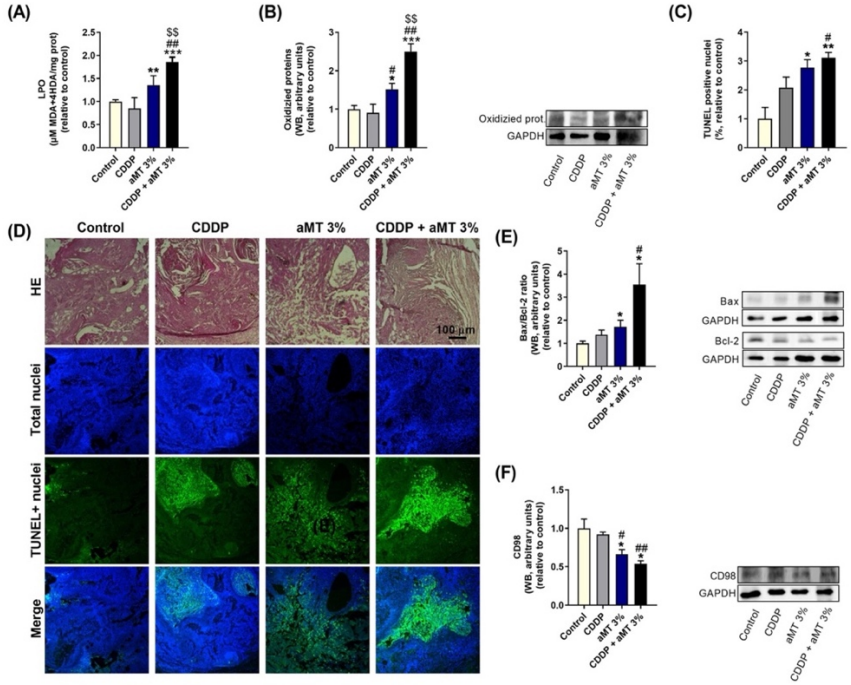

Figure 27. Intratumoral melatonin at 3% increases oxidative stress and apoptosis in Cal-27 xenografts tumors. (A, B) Study of oxidative stress levels and (C-E) apoptosis in Cal-27 tumors after 35 days of treatment. (A) LPO evaluation by spectrophotometric techniques. (B) Western blot (WB) analysis of oxidized proteins. (C) Percentage of apoptotic cells designated as the apoptotic index and analyzed by TUNEL assay. (D) TUNEL+ nuclei (apoptotic nuclei, green), DAPI stained nuclei (total nuclei, blue), and HE staining of the corresponding zone of the analyzed tumor. Scale bar = 100 µm. (E) Analysis of Bax/Bcl-2 expression by WB. (F) CD98 expression measured by WB. Experimental groups include control (mice treated with vehicle) and tumors from mice treated with CDDP at 4 mg/kg day once per week (CDDP), melatonin at 3% intratumorally (aMT 3%), or with the combined treatment of CDDP + aMT 3% for 35 days. Data are presented as the means ± standard error of the mean (n=4-9 for each group). One-tailed unpaired t-test: *P < .05, **P < .01, ***P < .001 vs. control tumors; $^{\#}$ P < .05, ## P < .01 vs. CDDP and $^{\$\$}$ P < .01 vs. aMT 3%.

Overall, these findings confirm that melatonin, at high concentrations, induces ROS production which promotes tumor cell apoptosis, also *in vivo*, which could explain the reduction of tumor growth observed macroscopically with the intratumoral melatonin treatment.

4. Intratumoral melatonin promotes changes to mitochondrial morphology in HNSCC xenografts

Considering that one of the main targets of melatonin is the mitochondria, we evaluated mitochondrial morphology and distribution by EM in Cal-27 xenograft tumors. EM images showed clear changes between control and treated tumors (Figure 28).

Mitochondria in the control and CDDP group had a spheroid and ovoid morphology with poorly developed cristae. Interestingly, in control tumors, mitochondria presented perinuclear localization (Figure 28). The redistribution of mitochondria from the periphery of the cell to the perinuclear region has been associated with hypoxic conditions and, therefore, with tumor progression and treatment resistance (Thomas et al., 2017; Ye et al., 2011).

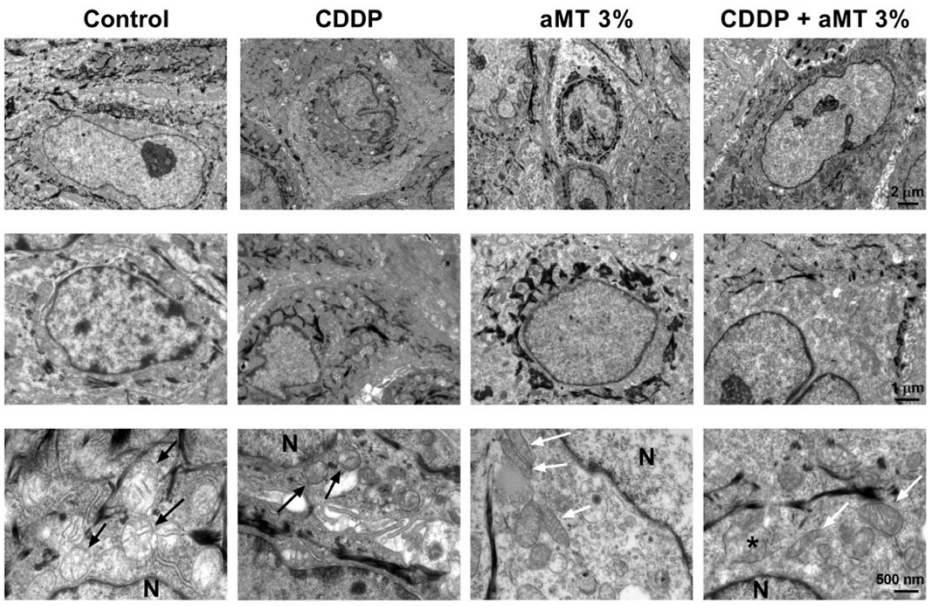

Figure 28. Melatonin-induced changes in mitochondrial morphology and distribution in Cal-27 xenografts tumors. Alterations in mitochondrial morphology analyzed by EM in Cal-27 xenografts. In control and CDDP tumors, the typical morphology of the Cal-27 mitochondria was observed as a round shape and few mitochondrial cristae (black arrows). Mitochondria are localized in the perinuclear region. In melatonin-treated tumors, mitochondria are elongated, with an increased number of cristae (white arrows) and following a homogenous distribution among the cellular cytoplasm. Some disruption in the mitochondrial membrane is appreciated in the melatonin-treated groups (black asterisk). Scale bar = 2 µm, 1 µm and 500 nm.

However, intratumoral melatonin treatment resulted in changes in the mitochondrial structure, morphology, and pattern of cytoplasm localization. Mitochondria were larger with well-defined cristae compared to the control and CDDP groups (Figure 28), which is indicative of a more developed organelle. In these tumors, mitochondria were

localized in the periphery of the cells and not in the perinucleus. However, most of these elongated mitochondria had disruptions in their mitochondrial membrane, which could indicate that high doses of melatonin damaged the mitochondria (Figure 28). These results were consistent with previous *in vitro* results (Guerra-Librero et al., 2021).

5. Intratumoral melatonin injection reduces tumor growth in patient-derived tumors

Importantly for possible clinical applications, next, we examined the oncostatic effect of melatonin in primary tumors derived from patients, which closer resemble real tumors, more than established cancer cell lines (Cromwell et al., 2022b; Lê et al., 2022b)**.**

First, it was attempted to generate PDC from tumor biopsies. A finished culture is one in which the cancer cells are free of stromal fibroblasts; can be cryopreserved, thawed, and re-grown; and share the same driver mutation(s) as the initial biopsy specimen. One of the main reasons for cell-line failure was the outgrowth of stromal cells, primarily stromal fibroblasts (Lê et al., 2022a). The PDC used for this study was obtained from a human biopsy of head and neck cancer tissue, which was

processed and characterized by Oppel et al. (Oppel et al., 2019), and is referred to here as sample 1 (SP1). As previously reported by these authors, primary HNSCC cells require specific culture medium and conditions to avoid cancer-associated fibroblasts (CAFs) overgrowth. It has been demonstrated that PNEU-medium provides a selection advantage to epithelial tumor cells and facilitates their proliferation so that they are not overgrown by CAFs, supporting the self-renewal of individual HNSCC cell clones (Oppel et al., 2019). Consequently, in this study, after patient-derived tumor tissue digestion, the obtained SP1 cells were grown in PNEU-medium (Figure 29A). After serial passages, a culture free of CAFs was obtained and these SP1 cells were used for subsequent experiments. First, proliferation rate was analyzed in this PDC model. SP1 cells grow forming cell aggregates, which culminate in spheroid formation. These spheroids are aggregates of single cells which self-assembly (Ryu et al., 2019). Melatonin at 500 µM decreased SP1 division rate, reducing the percentage of dividing cells from day 10 and to day 21 (Figure 29B), as well as the diameter of the cells aggregates formed in a time-dependent manner (Figure 29C, D). Interestingly, spheroids formation, which is a CSC proliferation marker (Ishiguro et al., 2017;

Oppel et al., 2019), was completely abolished with melatonin 500 µM (Figure 29C, E).

Next, as the direct anticancer effects of melatonin correlated with increased ROS levels and apoptosis in established HNSCC cell lines (Guerra-Librero et al., 2021), ROS and apoptosis levels were also studied in this primary culture. Consistent with previous findings (Fernandez-Gil et al., 2019; Florido, Martinez-Ruiz, et al., 2022; Guerra-Librero et al., 2021), melatonin at 1000 µM increased ROS production and caspase-9 expression in SP1 primary culture (Figure 29F-H).

Moreover, an *in vivo* study with PDX was performed from human biopsies of head and neck cancer tissues. In this case, after the digestion of tumor tissue, the obtained cells were not cultured *in vitro*, and they were directly transplanted into NSG mice. When tumors reached an appropriate volume, approximately 800 mm^3, the mice were sacrificed and tumors extracted, digested and transplanted again into 3-5 new mice to obtain a proper quantity of tumors to allow experimentation (Figure 30A). Different generations of tumors in NSG mice were required.

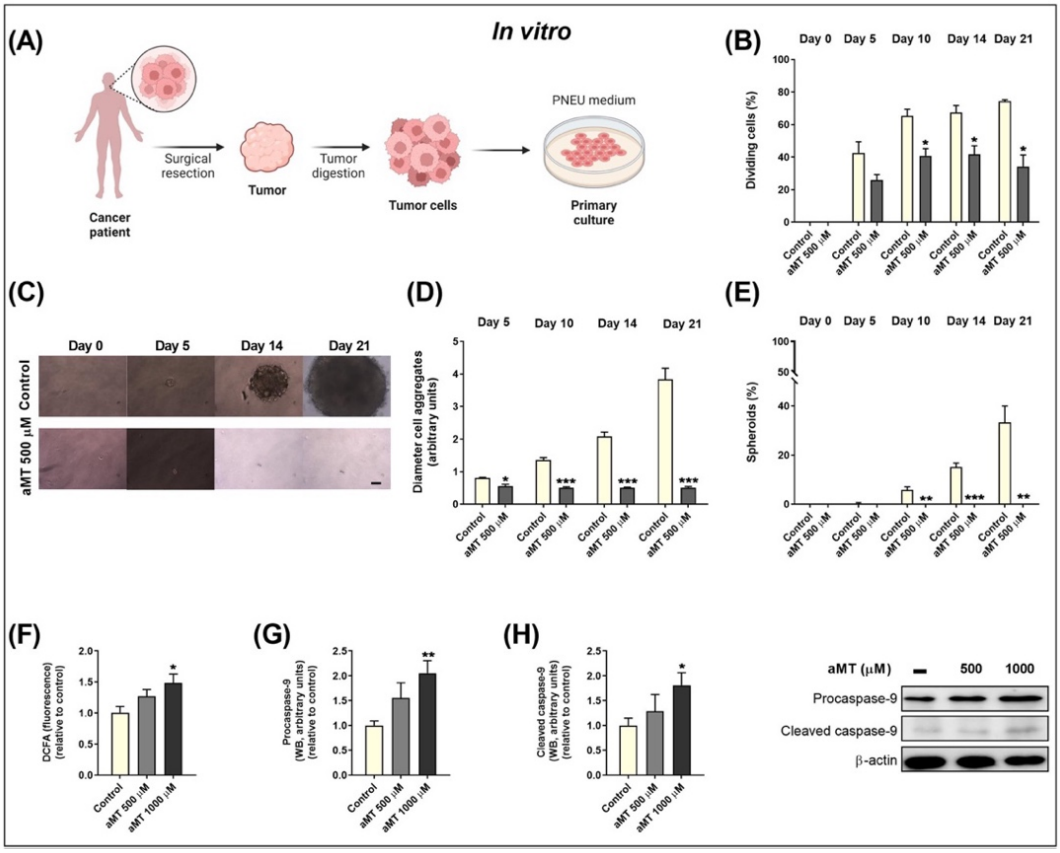

Figure 29. Melatonin inhibits proliferation and increases apoptosis in patient-derived tumor cells. (A) Scheme of the procedure followed to obtain a PDC (B, C, D, E) In vitro proliferation analysis of SP1 cells. (B) Percentage of SP1 cells in division; (C) Representative pictures of SP1 cells; (D) Diameter of the cell aggregates formed; (E) Percentage of spheroids generated in control cells (treated with vehicle) and cells treated with melatonin at 500 µM (aMT 500 µM) after 5, 10, 14, or 21 days of treatment. Cells were treated with vehicle or melatonin at 500 µM every 72 hours to avoid cell death induced by melatonin. Data are presented as the means ± standard error of the mean (n=3 for each group). One-tailed unpaired t-test: *P < .05, **P < .01, ***P < .001 vs. control of the respective day. (F, G, H) Evaluation of ROS production and apoptosis in SP1 cells. (F) Measurements of intracellular ROS levels by fluorimetry after staining with the DCFA fluorescent probe. (G, H) Procaspase and cleaved caspase-9 analysis by Western blot. Experimental groups include control cells (treated with vehicle) and cells treated with melatonin at 500 or 1000 µM for 48 hours.

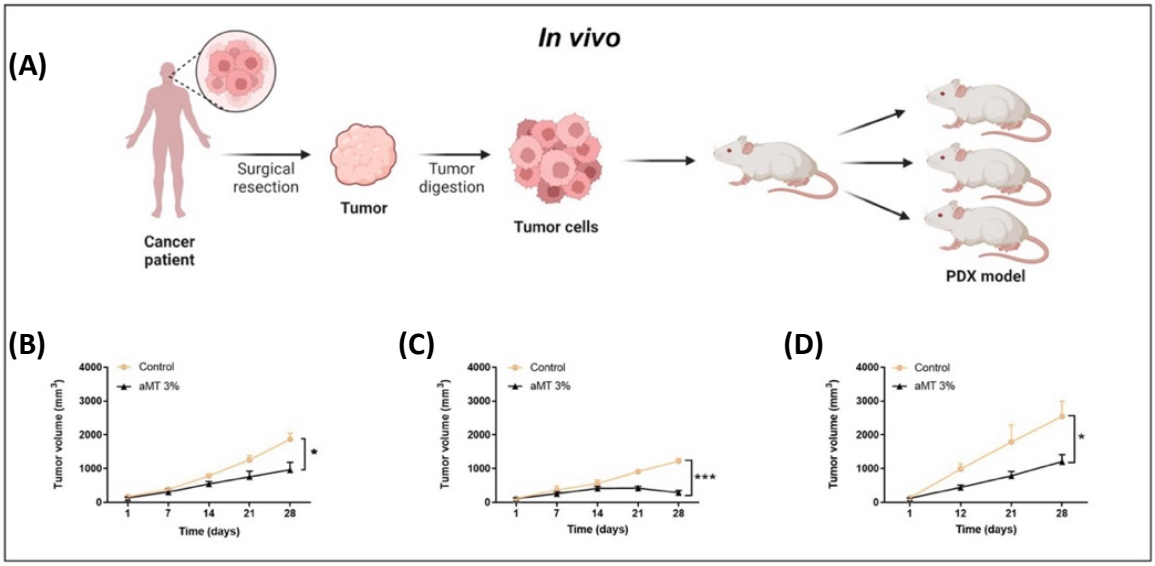

Figure 30. Melatonin suppresses tumor growth in three different HNSCC PDXs. (A-D) *In vivo* analysis of melatonin's oncostatic effect in three different PDXs, demonstrating that intratumoral administration of melatonin at 3% diminishes tumor growth in PDXs. (A) Scheme of the procedure followed for PDXs. (B, C, D) Tumor growth curves of mice bearing (B) SP1, (C) SP2, and (D) SP3 xenografts treated with vehicle solution (control) or the intratumoral administration of melatonin at 3% (aMT 3%). Data are presented as the means ± standard error of the mean (n=4 for each group). One-tailed unpaired t-test: *P < .05; **P < .01, ***P < .001 vs. control group.

Three different tumors derived from human biopsies were used in this study: SP1, SP2 and SP3. When tumors reached 100-200 mm^3, treatment was initiated. Melatonin 3% was administered intratumorally to mice each 24 hours for 28 days. As shown in Figure 30, the intratumoral administration of melatonin 3% reduced tumor growth compared to control group in the three primary tumors evaluated (Figure 30B-D).

Melatonin treatment was especially effective in SP2 primary tumors because, in this PDX model, melatonin not only exhibited oncostatic effect, but also antitumoral. SP2 tumors practically disappeared after 28 days of melatonin treatment (Figure 30C), which is more effective than the 63 days in established Cal-27 xenografts. These data further support the great clinical potential of our melatonin treatment.

6. Melatonin reduces cell migration *in vitro*

Finally, considering HNSCC frequently metastasizes to cervical lymph nodes (Kisoda et al., 2022; Y. Li et al., 2023), and high levels of ROS enable metastatic progression (Chang & Pauklin, 2021), the effect of melatonin on cell migration was explored in both established and patient-derived cells. Wound-healing assay showed that the migration capacities of both Cal-27 and SCC-9 cells were repressed by melatonin in a dose and time dependent manner (Figure 31A, C). As observed in the pictures obtained under the microscope, Cal-27 control cells practically covered all the scratch wound generated after 48 hours, while the coverage of the scratch wound was slower in the cells treated with melatonin at 500 and

1000 µM (Figure 31B). In SCC-9 treated cells, the distance of the scratch generated was even bigger after 48 hours compared to the initial distance, resulting in negative values for scratch wound coverage rate (Figure 31C, D). This could be due to SCC-9 low proliferation rate. These results were corroborated with Transwell system. Melatonin impaired invasive capacities of both Cal-27 and SCC-9 cells, reducing the number of migrated cells after 48 hours, as clearly shown in the microscope images (Figure 31E-H).

Moreover, the expression of two EMT markers such as vimentin and E-cadherin (Maria de França et al., 2021) were determined in SP1 primary cells, which represents a better model for metastasis evaluation (Hou et al., 2022). Melatonin drastically inhibited vimentin levels in SP1 cells at 500 and 1000 µM but increased E-cadherin at the highest dose (Figure 31I, J). Overall, these data demonstrate melatonin´s ability to reduce tumor cell migration and invasion in both established and primary cells.

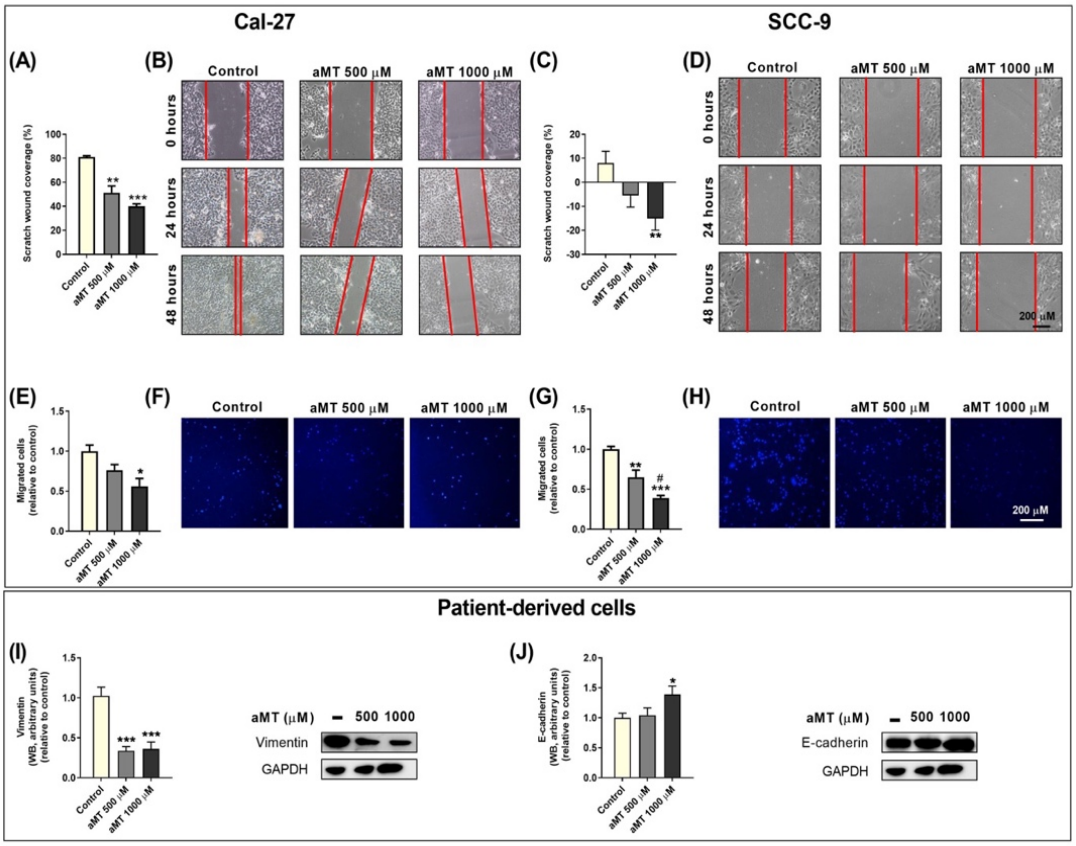

Figure 31. Melatonin impairs migration and invasive capacities in Cal-27 and SCC-9 cells and inhibits metastasis markers in SP1 cells. (A-D) Cell migration analysis of Cal-27 (A, B) and SCC-9 (C, D) cells by wound healing assay. (A, C) Quantitative analysis of scratch wound coverage and (B, D) representative images of scratched and recovering wound areas (marked by red lines) on confluence monolayers of cancer cells 0, 24, and 48 hours after wound generation. (E-H) Study of invasive capacities of Cal-27 (E, F) and SCC-9 (G, H) cells using the Transwell system. (E, G) Quantitative analysis and (F, H) representative images of migrated cells after 48 hours of melatonin treatment. (I, J) Analysis of EMT markers expression in SP1 primary cells. (I) Vimentin and (J) E-cadherin analysis by Western blot. Experimental groups include control (cells treated with vehicle) and cells treated with melatonin at 500 or 1000 μM (aMT 500 and 1000 μM) for 48 hours. Data are presented as the means ± standard error of the mean (n=3-5 for each group). One-tailed unpaired t-test: *P < .05, **P < .01, ***P < .001 vs. control; # P < .05 vs. aMT 500 μM.

DISCUSSION

Despite numerous studies demonstrating the anticancer effects of melatonin (Kurhaluk & Tkachenko, 2022; Sánchez-Sánchez et al., 2022), contradictory results have been reported when it is applied *in vivo* (Y.-Q. Shen et al., 2018). Different melatonin treatments have been evaluated with different and controversial results (Schernhammer et al., 2012; Seely et al., 2021; Sookprasert et al., 2014). However, to the best of our knowledge, this is the first study proposing intratumoral administration of melatonin for the treatment of HNSCC, providing evidence that melatonin can exert its oncostatic effect only by reaching high concentrations in the tumors.

We previously reported that melatonin administered subcutaneously did not significantly reduced tumor growth alone or in combination with IR or CDDP. The reduction of tumor volumes obtained with the combined treatment of subcutaneous melatonin plus CDDP or IR was similar to those obtained with IR or CDDP treatments alone (Martinez-Ruiz et al., 2023). These data were not in line with other studies where melatonin was proposed as an adjuvant therapy to chemo- and radiotherapy, providing synergistic antitumoral outcomes and relieving drug resistance (Berk et al., 2007; Lissoni et al., 1996; Z. Liu et al., 2021;

Sakatani et al., 2019; M. Zhang et al., 2021). Melatonin has been reported to sensitize cancer cells to chemotherapy via promoting apoptosis (Alonso-González et al., 2020; M. Zhang et al., 2021) and autophagy in cancer cells (Zhao et al., 2019). Similarly, the combination of melatonin with radiotherapy could also enhance cancer cell death through different pathways (González-González et al., 2019).

The apparent discrepancies between our results and other studies were due to the melatonin levels reaching tumors. Melatonin administered subcutaneously did not increase melatonin concentration in tumors compared to control mice. These data suggest that melatonin can exert an oncostatic effect only at high concentrations in tumors (Martinez-Ruiz et al., 2023).

Next, given that only at high concentrations melatonin exhibits oncostatic properties and that intratumoral administration was recently developed in immunotherapy (Alvarez et al., 2021), we decided to evaluate intratumoral injection of melatonin to increase its bioavailability in the tumor. Intratumoral injection was extremely efficacious, thereby establishing the proof of concept for intratumoral melatonin administration. Surprisingly, the treatment with melatonin 3%

administered intratumorally drastically inhibited tumor growth in Cal-27 and SCC-9 xenografts, remaining very small pieces of tumor after 63 days of treatment. In addition, the histological evaluation of treated tumors revealed a significant increase of collagen capsule that surrounds the tumors, and a reduction of the adenosquamous differentiation areas in Cal-27 tumors. Tumor capsule disruption has been broadly associated with a higher recurrence rate since it selectively favors proliferation, invasion and dissemination of the overlying tumor stem cells (Abdulaziz et al., 2015; Ghirardi et al., 2022; Song et al., 2014). On the other hand, the reduction of adenosquamous areas have been related to a better cancer outcome (Ghirardi et al., 2022; Xu et al., 2017; Zhao et al., 2019). In particular, other authours have demonstrated that glandular differentiation is significantly associated with higher recurrence and progression rate in patients (Zhao et al., 2019). Accordingly, both parameters, the strengthen of tumor capsule and the reduction of adenosquamous areas are good prognostic markers.

To further investigate a feasible clinical treatment, a combined treatment of intratumoral melatonin and CDDP was assessed. We do not exclude the possibility of combining melatonin with other agents, though

we selected intraperitoneal CDDP. Results showed that melatonin synergized the effects of cisplatin, stimulating apoptosis, increasing oxidative stress and reducing CSCs markers.

It is well known that melatonin is capable of increasing apoptosis rates and promoting mitochondrial damage in tumor cells, mainly by stimulating ROS production and proapoptotic activity in different cancer cell lines (Cucielo et al., 2022; Florido, Martinez-Ruiz, et al., 2022; Guerra-Librero et al., 2021). Congruently, our findings demonstrated that melatonin, also *in vivo*, stimulated apoptosis and increased oxidative stress levels in Cal-27 xenografts.

The increase in CDDP cytotoxicity is partly due to enhanced mitochondrial function. EM images evidence that melatonin induced mitochondrial elongation and facilitated the formation of cristae in HNSCC xenografts. The cristae shape has been demonstrated to regulate the stability and assembly of respiratory chain supercomplexes, which affects respiratory efficiency, increasing ROS levels (Guerra-Librero et al., 2021). Moreover, mitochondrial elongation correlates with a clear metabolic shift from glycolysis to oxidative phosphorylation that, in turn, induces ROS-dependent mitochondrial uncoupling. These findings are in

accordance with our previous results demonstrating that melatonin induces ROS complex I RET and leads to apoptosis from excessive ROS production, but only at high doses (Florido, Martinez-Ruiz, et al., 2022).

Additionally, melatonin treatment decreased CD98 expression, a CSC marker. CSCs are gaining clinical interest in recent years due to increasing evidence that CSC populations within a tumor have to be eliminated to increase cancer cure rates. In HNSCC, some CSC markers as CD98 were described as indicators of tumor cell populations with enhanced tumorigenic potential and reduced sensitivity to chemo- or radiotherapy (Martens-de Kemp et al., 2013; Peitzsch et al., 2019). Accordingly, the reduction of CD98 expression with intratumoral melatonin treatment should be a signal of a better cancer outcome.

Thus, our results indicate that the combined treatment of melatonin and CDDP could offer some advantages in terms of the clinical translation of this therapeutic approach. Therefore, all things considered, our study opens the way to other intratumoral therapy combinations that may include melatonin, even immunotherapy.

On the other hand, we must consider the genetic and epigenetic differences between the cell lines and original tumors, which make it difficult to evaluate how much of the original tumor biology is retained in established cell line models that have been maintained long-term *in vitro* (Cromwell et al., 2022a; Kodack et al., 2017; Lê et al., 2022a). In this sense, patient-derived tumor models have transformed the field of drug researching (Huo et al., 2020). However, culturing cancer cells from solid tumors has generally not been rapid or readily feasible since biopsy material is usually scant and it is difficult to eliminate CAFs (Kodack et al., 2017). Nevertheless, in this study a PDC model was successfully established. In this case, cells from patient´s tumor were culture in PNEU-medium, which provides a selection advantage to epithelial tumor cells, avoiding CAFs overgrowth (Oppel et al., 2019). As expected, melatonin at high doses decreased proliferation rate and increased ROS levels and apoptosis in this PDC. Interestingly, melatonin completely abolished spheroid formation, which are considered a CSC marker (Ishiguro et al., 2017; Oppel et al., 2019). These results appear to be congruent with other studies showing that melatonin suppress CSC-like phenotypes in other

types of cancer (M.-T. Liu & J. Reiter, 2017; Phiboonchaiyanan et al., 2021).

Moreover, considering that PDX represent a promising pre-clinical model which may be used to predict drug-responses (Huo et al., 2020), melatonin oncostatic effect was evaluated in three different HNSCC PDXs. Intratumoral melatonin also reduced tumor growth in three different PDXs, which revealed the potential of intratumoral melatonin injections. These results agree with other studies which demonstrate the oncostatic effect of melatonin in other PDX models from colon and oral squamous cell carcinoma (Sakatani et al., 2019; C.-Y. Yang et al., 2017). However, these studies are scare and more investigation about primary tumors and melatonin should be assessed.

Finally, the complex role of ROS in mediating tumor progression remains controversial. Several studies have suggested that increasing production of mitochondrial ROS enhances the metastatic capacity of tumor cells (Chang & Pauklin, 2021). It has also been demonstrated that melatonin induces an increase of mitochondrial ROS levels and a loss of stemness in HNSCC (Shin et al., 2022). This is in line with our findings that melatonin increased ROS levels and suppressed the migration and

invasion of HNSCC cells *in vitro* and regulated EMT-related markers in a PDC model. Therefore, the reduction of invasive capacities of HNSCC cells exerted by melatonin could be due to a reduced CSC population within these tumors. However, more experiments are indispensable to understand the mechanism of action of melatonin in metastasis.

Summarizing, our study elucidated the roles of intratumoral melatonin reducing tumor growth and synergizing CDDP. Considering our results and that melatonin is well acknowledged as a drug with no significant toxicity both in physiological and pharmacological concentrations (Kennaway, 2019; Priprem et al., 2017), we propose melatonin as a possible therapeutic option for cancer treatment. Specifically, the combined treatment with melatonin and CDDP may be a promising clinical approach to treat patients with HNSCC, encouraging a future clinical trial in cancer patients to establish a new melatonin treatment with proper clinical application.

CONCLUSIONS

First: Intratumoral melatonin treatment drastically inhibits tumor growth in HNSCC xenografts.

Second: Intratumoral melatonin treatment potentiates CDDP effects to decrease HNSCC tumor growth.

Third: Melatonin stimulates ROS production and apoptosis in Cal-27 xenografts.

Fourth: Intratumoral melatonin promotes changes to mitochondrial morphology in HNSCC xenografts.

Fifth: Intratumoral melatonin injection reduces tumor growth in patient-derived tumors.

Sixth: Melatonin reduces cell migration and EMT marker expression *in vitro.*

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