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PROGRAMA DE DOCTORADO - BIOMEDICINA

Molecular, cellular and anatomopathological study of oral cavity tumors

Estudio molecular, celular y anatomopatológico de los tumores de la cavidad oral

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TITULO: *Molecular, cellular and anatomopathological study of oral cavity tumors*

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TÍTULO DE LA TESIS: Estudio molecular, celular y anatomopatológico de los tumores de cavidad oral

DOCTORANDO/A: Dª Alba Sanjuan Sanjuan

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

Durante el desarrollo de la presente Tesis Doctoral, en el periodo comprendido entre febrero 2017 y abril 2022, la doctoranda Alba Sanjuan Sanjuan ha superado los objetivos planteados al comienzo de esta. Durante los primeros 3 años del periodo, la doctoranda ha compaginado la clínica asistencial como residente de Cirugía Oral y Maxilofacial en el Hospital Reina Sofía (Córdoba) con el programa de Doctorado. Tras finalizar su residencia MIR, la doctoranda trasladó su formación profesional a Estados Unidos donde ha realizado dos años de Head and Neck Oncologic Surgery and Microvascular Reconstructive Surgery Fellowship en Jacksonville (Florida) y Detroit (Michigan). Durante esos dos años de alta demanda asistencial ha continuado con sus compromisos formativos, doctorales e investigadores ampliando sus conocimientos en investigación incluyendo un Experto Universitario en Metodología de la investigación. Así mismo, como fruto de su trabajo durante este periodo de doctorado ha publicado un artículo directamente relacionado con su Tesis Doctoral en una revista Q1 de referencia dentro del área de Oncología molecular. Así mismo, el trabajo realizado ha dado lugar a otro artículo científico relacionado con el análisis de la maquinaria de Splicing en el cáncer de cavidad oral que actualmente está en revisión. Adicionalmente, la doctoranda ha colaborado con diferentes investigadores de su ámbito de manera fructífera con un total de 19 artículos de autora o coautora y 3 capítulos de libro.

Por último, la doctoranda ha presentado los resultados de su Tesis en diferentes congresos de ámbito nacional e internacional incluyendo la obtención de un premio a la mejor comunicación poster oral en Sevilla 2019.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 22 de abril de 2022

Firma de los directores

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Dr. Raúl Miguel Luque Huertas, Catedrático del Departamento de Biología Celular, Fisiología e Inmunología de la Universidad de Córdoba y Dra. Susana Heredero Jung, Facultativo Especialista de Área en Cirugía Oral y Maxilofacial.

INFORMAN

Que Dª Alba Sanjuan Sanjuan, licenciada en Medicina, ha realizado bajo nuestra dirección el trabajo titulado "**Estudio molecular, celular y anatomopatológico de tumores de cavidad oral**" y que, según nuestro juicio, reúne los méritos suficientes para optar al Grado de Doctor en Biomedicina.

Y, para que conste, firmamos la presente en Córdoba, a 22 de abril de 2022

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No quiero comenzar sin el más importante de los agradecimientos que va dirigido a todos los pacientes que desgraciadamente sufren esta desafortunada y despiadada enfermedad. El cáncer de cavidad oral tiene un impacto inconmensurable y ellos continúan su lucha y depositan sus destinos en nuestras manos. La verdadera razón detrás de la investigación es siempre por y para ellos.

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Gracias a las personas más importantes de mi vida, mi familia. Mis padres, **Antonio y Ángela**, mi hermano **Marcos** y mi marido **Marek**. Ellos son mi apoyo más incondicional, mi colchón salvavidas y mi motor. Gracias por estar siempre a mi lado aun estando lejos. Os quiero.

¡Muchas gracias por todo!

List of Abbreviations

AC	Actinic Cheilitis
AJCC	American Joint Committee on Cancer
AS	Alternative Splicing
BMI	Body Mass Index
CNS	Central Nervous System
CNV	Copy Number Variations
CRP	C-reactive protein
DFS	Disease-Free Survival
DOI	Depth f Invasion
DLP	Dyslipidemia
DM	Diabetes Mellitus
EBV	Epstein–Barr virus
EMT	Epithelial-Mesenchymal Transition
ENE	Extranodular Extension
FBS	Fetal Bovine Serum
G	Histological Grade
GPCRs	G protein-Coupled Receptors
H&N	Head and Neck
HbA1	Glycosylated Hemoglobin
HBP	High Blood Pressure
HPV	Human papillomavirus
HNSCC	Head and Neck Squamous cell Carcinoma
IARC	International Agency for Research on Cancer
IHC	Immunohistochemistry
LRC	Locoregional Control
LVI	Lymphovascular Invasion
NCCN	National Comprehensive Cancer Network
NPC	Nasopharyngeal Carcinoma
OCC	Oral cavity cancer
OE	Oral Erythroplakia
OL	Oral leukoplakia
OLP	Oral Lichen Planus
OPMDs	Oral Potential malignant Disorders
OPSCC	Oropharyngeal Squamous cell carcinoma
OS	Overall Survival
OSCC	Oral Squamous Cell Carcinoma
OSMF	Oral Submucous fibrosis

PBS	Phosphate Buffered Saline
PCBP	Poly(rC)-binding protein (PCBP)
PCR	Polymerase Chain Reaction
pN	Pathological Cervical Staging or Cervical Metastasis.
PNI	Perineural Invasion
pT	Primary tumor Stage
PTI	Peritumoral Inflammation
qPCR	Quantitative Real-Time PCR
RBP	RNA-binding proteins
ROS	Reactive oxygen species
RR	Recurrence Rate
RT	Retrotranscription
RTOG	Radiation Therapy Oncology Group
SCC	Squamous Cell Carcinoma
SD	Standard Deviation
SEM	Standard Error of The Mean
SFs	Splicing Factors
SRIF	somatotropin release-inhibiting factor
SSAs	Somatostatin-Analogues
SST	Somatostatin
SSTs	Somatostatin-Receptors
TB	Tumor Budding
TNM	Staging system criteria T (tumor), N (nodal), M (metastasis).
UV	Ultraviolet
WPOI	Worse Pattern of Invasion

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Resumen

El carcinoma de células escamosas de cavidad oral continúa siendo una enfermedad agresiva y un desafío mundial con una tasa de supervivencia a los 5 años del 60%. Dependiendo de los factores de riesgo, la cirugía y la radioterapia adyuvante +/- quimioterapia siguen siendo la principal modalidad de tratamiento para la enfermedad local o avanzada. Por lo tanto, se necesitan con urgencia nuevos biomarcadores de diagnóstico, pronóstico y dianas terapéuticas para el cáncer de cavidad oral.

Es importante señalar que se ha comprobado que existe un componente de diferenciación neuroendocrina en algunos tumores que no se consideran clásicamente de origen neuroendocrino, incluido el carcinoma de células escamosas de pulmón y esófago, y más recientemente en la región de cabeza y cuello. En este contexto, la somatostatina (SST) es un neuropéptido inhibidor bien conocido que se produce en diferentes localizaciones a través del organismo, tanto centrales como sistémicas. Las acciones inhibitorias de SST están mediadas a través de los llamados receptores de SST (SSTs), que están ampliamente distribuidos en tejidos normales y tumorales, y regulan, entre otras actividades, la proliferación celular, la diferenciación y la angiogénesis en muchos tipos de tumores. Esta propiedad permite al sistema regulador de la SST ser muy útil clínicamente ya que es ampliamente utilizado en la obtención de imágenes tumorales [gammagrafía SST o gammagrafía con octreótido (un análogo sintético de SST que se une preferentemente al SST_2, receptor más ampliamente distribuido a través del organismo)]. Las células tumorales suelen expresar típicamente más de un SSTs, siendo el SST_2 el subtipo expresado con mayor frecuencia y, por tanto, el objetivo más importante del tratamiento. En consecuencia, los análogos sintéticos de SST (SSA), representan un objetivo terapéutico atractivo para tratar patologías tumorales positivas para SSTs ya que se ha demostrado que son capaces de controlar la hipersecreción hormonal y el crecimiento tumoral en algunos tipos de patologías tumorales.

Nuestra comprensión actual de la presencia de SST en carcinoma de células escamosas de cavidad oral es bastante escasa, y en algunos casos controvertida. Concretamente, estudios previos han demostrado que la expresión inmunohistoquímica relativa de algunos subtipos de SSTs está alterada en lesiones malignas en la laringe en comparación con tejido sano, así como en muestras tumorales del área de la cabeza y el cuello en comparación con muestras normales de mucosa orofaríngea (obtenidas durante la uvulopalatofaringoplastia) de otros pacientes. Sin embargo, hasta la fecha, no se han realizado análisis moleculares para analizar cuantitativamente, en paralelo, los niveles de expresión (número de copias) de todos los subtipos de SSTs en muestras de carcinoma oral en comparación con tejido sano (control; dentro de mismo paciente) mediante PCR cuantitativa. Hasta la fecha, tampoco se han estudiado los efectos directos de diferentes SSA en cultivos de células humanas primarias de carcinoma de cavidad oral. Por lo tanto, en base a la información mencionada anteriormente, consideramos que sería necesario interrogar los patrones de expresión de los receptores de la SST y su asociación con los datos clínicos, anatomopatológicos y de supervivencia del cáncer de cavidad oral.

Asimismo, la maquinaria molecular de splicing se ha comprobado que juega un papel fundamental para controlar las funciones celulares, y para regular los niveles de expresión génica y la especificidad tisular. Es más, la alteración de la maquinaria de *splicing* ha surgido recientemente como una característica importante del cáncer, con un gran potencial para servir como herramienta diagnóstica, pronóstica o terapéutica. Así, se ha comprobado que una ligera alteración en algunos de los componentes de la maquinaria (conocida como spliceosoma) puede afectar significativamente el patrón de expresión de multitud de genes claves y a la aparición de variantes de splicing oncogénicas. Concretamente, la desregulación de los factores de splicing que componen el spliceosoma lleva a una mala regulación del proceso de splicing y a la aparición aberrante de variantes que puede promover la iniciación del cáncer y afectar el fenotipo de las células cancerosas, incluida la proliferación, la apoptosis, la invasión y la metástasis de muchos tipos de cáncer.

En los últimos años, varios estudios se han centrado en el análisis y el impacto de algunos factores de splicing en el cáncer de cabeza y cuello. Sus resultados son muy variables, incluso contradictorios en cuanto al patrón de expresión de algunos factores de splicing en tejidos tumorales en comparación con tejido sano. Algunos de estos factores de splicing tienen un impacto en los tratamientos antineoplásicos al modular la apoptosis y permitir la sensibilización de las células cancerosas a los tratamientos terapéuticos. Estos factores pueden actuar como factores de supervivencia que disminuyen la apoptosis inducida por fármacos o, por el contrario, potencian los efectos pro-apoptóticos de los medicamentos quimioterapéuticos. Por todo ello, es necesario realizar un estudio que se centre en estudiar la alteración de la maquinaria del splicing en cáncer de cavidad oral ya que esto podría ayudar a identificar nuevos biomarcadores y dianas moleculares que ayuden a comprender mejor el comportamiento de estos tumores y al desarrollo de nuevas estrategias terapéuticas.

Teniendo en cuenta toda esta información, la **HIPÓTESIS INICIAL** de esta Tesis Doctoral es que la desregulación de los sistemas moleculares de la SST y de la maquinaria del splicing podría influir directamente la patofisiología de los tumores de cavidad oral y, en consecuencia, que sus niveles de expresión en los tejidos tumorales podrían proporcionar información útil para mejorar el diagnóstico y/o pronóstico de estos tumores, e identificar nuevas dianas terapéuticas para tratar a los pacientes con estas devastadoras patologías tumorales.

En base a esta hipótesis, el **OBJETIVO GENERAL** de esta Tesis Doctoral ha sido explorar la presencia, la posible desregulación y/o el papel funcional de algunos de los componentes de estos dos sistemas celulares clave involucrados en procesos críticos de la regulación celular [los receptores de somatostatina (SST), y por la maquinaria de splicing (componentes de spliceosoma y factores de splicing)] y que podrían estar asociados con el desarrollo, progresión y agresividad de los tumores de cavidad oral, con el objetivo final de descubrir nuevos biomarcadores y herramientas terapéuticas para

mejorar el diagnóstico, pronóstico, tratamiento y, por tanto, el manejo de los pacientes con estos tumores.

Para lograr este objetivo principal, hemos llevado a cabo diferentes **objetivos específicos**, tales como: 1) analizar cuantitativamente el perfil de expresión de los SSTs en una batería representativa de pacientes con carcinoma escamoso de cavidad oral clínicamente bien caracterizados en comparación con tejidos sanos adyacentes obtenidos dentro del mismo paciente; 2) evaluar la asociación in vivo entre la expresión de todos los SSTs en carcinoma de cavidad oral con parámetros de datos clínicos e histopatológicos relevantes; 3) explorar y comparar los efectos antitumorales directos de diferentes SSA (octreótido, lanreótido y pasireótido) en cultivos celulares primarios de carcinoma escamoso de cavidad oral; 4) caracterizar el patrón de expresión de elementos clave relacionados con el splicing (componentes del splicing y factores de splicing) en una batería representativa de pacientes con carcinoma escamoso de cavidad oral clínicamente bien caracterizados en comparación con tejidos sanos adyacentes obtenidos dentro del mismo paciente; 5) evaluar la asociación in vivo entre el patrón de expresión de elementos clave relacionados con el splicing (componentes del splicing y factores de splicing) en carcinoma escamoso de cavidad oral con parámetros de datos clínicos e histopatológicos relevantes; y 6) evaluar el potencial terapéutico de un inhibidor de la maquinaria de splicing (pladienolida B) en cultivos celulares primarios de carcinoma de cavidad oral.

Por tanto, la primera sección de esta Tesis Doctoral tenía como objetivo establecer los patrones de expresión de SST_{1-5} en tejidos tumorales de cavidad oral frente a tejidos sanos adyacentes obtenidos dentro del mismo paciente en una cohorte de pacientes bien caracterizada. Para llevar a cabo este primer objetivo, se realizó el análisis de la expresión de los receptores de SST por qPCR mediante un array basado en microfluídica en ambas muestras (tejido tumoral y sano). Los resultados de esta sección desvelaron un perfil de expresión diferente de los cinco subtipos de SSTs entre tejido tumoral y sano. Concretamente, la expresión de todos los receptores, excepto SST_1, se vio incrementada en tejidos tumorales en comparación con las muestras de control adyacentes sanas, siendo este aumento estadísticamente significativo para SST_2 y SST_3. Así, nuestros resultados apoyan y amplían los datos anteriores en otras patologías tumorales que indican que la expresión de algunos de los SSTs está aumentada en tejido tumoral versus tejido sano. Estos resultados han permitido aumentar el conocimiento del perfil de expresión de los receptores de SST en cáncer de cavidad oral. Nuestros resultados podrían considerarse clínicamente relevantes ya que la respuesta al tratamiento con SSA suele estar relacionada con la presencia de SST_2 en el tumor (especialmente de octreótido y lanreótido), por lo que SST_2 podría ser una importante diana terapéutica en el cáncer de cavidad oral a través del uso de diferentes SSAs (hipótesis que hemos explorado más adelante).

Otro objetivo dentro de la primera sección fue evaluar la supuesta asociación entre la expresión de los receptores SST_{1-5} y los datos de relevancia clínica e histopatológica en los pacientes con tumores de la cavidad oral (estadio, grado histológico, invasión tumoral, presencia de metástasis, recurrencia,

supervivencia global, etc.) y que son relevantes en el pronóstico del cáncer de células escamosas de cavidad oral. Los resultados revelaron que la expresión de SST_2 estaba relacionada con menor presencia de metástasis cervical, menor presencia de metástasis a distancia, así como una menor tasa de recurrencia. Además, los tumores con mayor expresión de SST_2 tenían asociación con factores histopatológicos de buen pronóstico, como mayor reacción inflamatoria peritumoral, menor profundidad de invasión y frentes de invasión más uniformes y menos infiltrativos. Estos resultados indican que el SST2 podría ser un marcador de pronóstico útil en los pacientes con tumores de la cavidad oral.

Además, esta sección también se centró en explorar por primera vez el efecto que tienen diferentes SSA (octreótido, lanreótido y pasireótido) en la tasa de proliferación celular en cultivos primarios de células tumorales de cáncer de cavidad oral procedentes de varios pacientes de la cohorte. Nuestros resultados demostraron que todos los SSAs (usados a una dosis de 10^{-7} M) fueron capaces de reducir significativamente la tasa de proliferación de cultivos de células tumorales primarias.

En conjunto, todos estos resultados señalan que los receptores de la somatostatina, especialmente SST_2, podrían tener un valor pronóstico en cáncer de cavidad oral, puesto que su alta expresión está relacionada con tumores de mejor comportamiento histopatológico y clínico. Pero, a su vez, también pueden tener un papel terapéutico en el cáncer de cavidad oral a la luz de los resultados en los cultivos celulares que muestran que el tratamiento con análogos de la somatostatina resulta en una reducción de la tasa de proliferación tumoral.

La segunda sección de esta Tesis Doctoral se centró en determinar la expresión y posible alteración de los componentes de la maquinaria de splicing en el cáncer de células escamosas de cavidad oral en comparación con muestras adyacentes-sanas. En primer lugar, nos propusimos caracterizar el patrón de expresión de un conjunto seleccionado de 59 componentes clave del spliceosoma y de factores de splicing en el tejido tumoral vs. las muestras de tejido sano. Nuestros resultados demuestran por primera vez que 12 de los 59 (20%) componentes de spliceosoma y factores de splicing analizados estaban desregulados en tejido tumoral en comparación con el tejido sano (SRSF4, SRSF5, SRSF9, SRSF10, NOVA1, ESRP1, ESRP2, RBM3, RBM10, TRA2A, TRA2B y TIA1). Algunos de estos elementos habían sido descritos previamente como alterados en cáncer oral, aunque no mediante la aproximación molecular establecida en este trabajo y sin usar los pacientes controles que se han incluido en este trabajo con muestras adyacentes de la cavidad oral procedentes del mismo paciente. En cambio, nuestros datos son los primeros en describir la alteración de diversos de los factores de splicing (ej. SRSF4, TRA2A y RBM10) en carcinoma de células escamosas de cavidad oral.

En este trabajo, también evaluamos las posibles asociaciones entre la expresión de los factores de splicing con datos clínicos e histopatológicos relevantes en esta patología tumoral. Nuestros resultados revelaron que la supervivencia se correlacionó positivamente con una mayor expresión de SRSF5, SRSF9, RBM3, TRA2A y TRA2B. Estos datos son novedosos, ya que hasta donde sabemos, somos los primeros en describir la relación entre la expresión de SRSF5, SRSF9, TRA2A, TRA2B, RBM3 y una

mejor supervivencia del cáncer oral. De ellos, TRA2B, SRSF9 y RBM3 también se asociaron con menos recurrencia o metástasis a distancia. La mayoría de los factores de splicing que mostraron una mejor supervivencia estaban relacionados con características clínicas e histopatológicas clave relacionadas con mejor pronóstico, como menor número de ganglios cervicales positivos (pN), menor número de ganglios con diseminación extracapsular y tumores con estadios más pequeños.

Asimismo, hemos observado a nivel histopatológico, que la expresión de TRA2B y TIA1 se asoció con factores de mejor pronóstico, como un menor grado de diferenciación (G), mayor reacción peritumoral inflamatoria o menor profundidad de invasión (DOI). Estos resultados apoyan estudios previos que demostraron que una mayor expresión de TIA1 se asocia con un mejor pronóstico. Sin embargo, hasta la fecha no existía información relacionada con el impacto de la expresión de TRA2B o TIA1 a nivel histopatológico en cáncer de cavidad oral. Así mismo, otro resultado novedoso que nuestro estudio desvela la relación de la expresión de NOVA1 y ESRP2 con factores clínicos e histopatológicos de buen pronóstico, como un frente de invasión tumoral expansivo y uniforme, mayor reacción peritumoral, menor estadio tumoral, menor número de ganglios cervicales positivos y menor tasa de recidiva regional. En conjunto, estos resultados demuestran una desregulación conjunta de diversos factores de splicing en cáncer de cavidad oral en comparación con las muestras de tejido sano adyacente, y que los niveles de expresión de algunos factores de splicing están asociados con características clínicas e histopatológicas clave de menor agresividad y, lo que es más importante, con la supervivencia general del paciente.

Por último, esta sección también se centró en explorar por primera vez el efecto que ejerce un inhibidor de la maquinaria de splicing (pladienolide B) en la tasa de proliferación celular de cultivos primarios de células humanas procedentes de tumores de la cavidad oral. Nuestros resultados demostraron que este inhibidor fue capaz de reducir significativamente la tasa de proliferación de cultivos de células tumorales primarias.

Como conclusión general, los estudios realizados en la presente Tesis nos permiten ampliar y avanzar en el conocimiento de las bases moleculares de la regulación fisiopatológica del cáncer de cavidad oral mediante el análisis de dos sistemas reguladores críticos: los receptores de somatostatina y la maquinaria de splicing. Específicamente, nuestros resultados demuestran que SST_2 y diferentes factores de splicing (SRSF4, SRSF5, SRSF9, NOVA1, ESRP1, ESRP2, RBM3, RBM10, TRA2A, TRA2B, TIA1 y SRSF10) representan puntos relevantes de regulación. Por lo tanto, estos factores de splicing podrían ser herramientas valiosas para desarrollar nuevos biomarcadores de diagnóstico y pronóstico y/o dianas terapéuticas para mejorar el manejo y la supervivencia de los pacientes con cáncer oral. Además, esta Tesis doctoral demuestra la eficacia de los SSA y el pladienolide-B como herramientas terapéuticas potencialmente útiles en carcinoma de células escamosas de cavidad oral.

Summary

Oral cavity squamous cell carcinoma (OSCC) continues to be an aggressive disease and a global challenge with a 5-year survival rate of 60%. Depending on the risk factors, surgery and adjuvant radiotherapy +/- chemotherapy continue to be the main treatment modality for local or advanced disease. Therefore, new diagnostic, prognostic biomarkers and therapeutic targets for oral cavity cancer are urgently needed.

It is important to point out that it has been confirmed that there is a neuroendocrine differentiation component in some tumors that are not classically considered to be of neuroendocrine origin, including squamous cell carcinoma of the lung and esophagus, and more recently in the head and neck region. In this context, somatostatin (SST) is a well-known inhibitory neuropeptide that is produced at different locations throughout the body, both central and systemic. The inhibitory actions of SST are mediated through the so-called SST receptors (SSTs), which are widely distributed in normal and tumor tissues, and regulate, among other activities, cell alteration, differentiation, and angiogenesis in many types of tumors. This property allows the SST regulatory system to be very useful clinically as it is widely used in tumor imaging [SST scintigraphy or octreotide scintigraphy (a synthetic analog of SST that preferentially binds to SST_2, the receptor most widely distributed across of the organism)]. Tumor cells usually express more than one SST, with SST_2 being the most frequently expressed subtype and, therefore, the most important target of treatment. Consequently, synthetic analogs of SST (SSA) represent an attractive therapeutic target to treat tumor pathologies positive for SSTs, since it has been shown that they are capable of controlling hormonal hypersecretion and tumor growth in some types of tumor pathologies.

Our current understanding of the presence of SST in squamous cell carcinoma of the oral cavity is quite scarce, and in some cases controversial. Specifically, previous studies have shown that the relative immunohistochemical expression of some SST subtypes is altered in malignant lesions in the larynx compared to healthy tissue, as well as in tumor samples from the head and neck area compared to normal samples from the larynx. oropharyngeal mucosa (obtained during uvulopalatopharyngoplasty) from other patients. However, to date, no molecular analysis has been performed to quantitatively analyze, in parallel, the expression levels (copy numbers) of all subtypes of SSTs in oral carcinoma samples compared to healthy tissue (control; within same patient) by quantitative PCR. To date, the direct effects of different SSAs on cultures of primary human oral cavity carcinoma cells have also not been studied. Therefore, based on the information mentioned above, we believe that it would be necessary to interrogate the expression patterns of SST receptors and their association with clinical, pathological and survival data of oral cavity cancer.

Likewise, the molecular machinery of splicing has been shown to play a fundamental role in controlling cellular functions, and in regulating gene expression levels and tissue specificity. Furthermore, the production of splicing machinery has recently emerged as an important feature of cancer, with great potential to serve as a diagnostic, prognostic, or therapeutic tool. Thus, it has been

proven that a slight alteration in some of the components of the machinery (known as a spliceosome) can significantly affect the expression pattern of many key genes and the appearance of oncogenic splicing variants. Specifically, deregulation of splicing factors that make up the spliceosome leads to dysregulation of the splicing process and aberrant appearance of variants that can promote cancer initiation and affect cancer cell phenotype, including wasting, apoptosis, invasion and metastasis of many types of cancer.

In recent years, several studies have focused on the analysis and impact of some splicing factors in head and neck cancer. Their results are highly variable, even contradictory in terms of the expression pattern of some splicing factors in tumor tissues compared to healthy tissue. Some of these splicing factors have an impact on antineoplastic treatments by modulating apoptosis and allowing cancer cells to be sensitized to therapeutic treatments. These factors can act as survival factors that decrease drug-induced apoptosis or, on the contrary, enhance the pro-apoptotic effects of chemotherapy drugs. For all these reasons, it is necessary to carry out a study that focuses on studying the alteration of the splicing machinery in oral cavity cancer, since this could help identify new biomarkers and molecular targets that help to better understand the behavior of these tumors and to development of new therapeutic strategies.

Taken all this information together, the **INITIAL HYPOTHESIS of this PhD Thesis** was that the dysregulation of the SST and splicing machinery systems could directly influence OSCC, and consequently, that their levels of expression in tumor tissues could provide useful information to improve diagnosis and/or prognosis of these tumors, and to identify novel therapeutic sources to treat patients with these devastating pathologies.

Based on this hypothesis, the **GENERAL AIM of this Doctoral Thesis** was to explore the presence, potential dysregulation, and/or functional role of components of two key cellular systems involved in critical regulatory processes [i.e., somatostatin receptors (SSTs), and by the splicing machinery (spliceosome components and splicing factors)] that could be associated with the development, progression, and aggressiveness of OSCC, with the ultimate goal of discovering novel biomarkers and therapeutic tools to improve the diagnosis, prognosis, treatment and, therefore, the management of the patients with these tumors.

To achieve this main aim, we have carried out different specific objectives such as: 1) to quantitatively analyze the expression profile of SSTs in a representative battery of clinically well-characterized OSCC tissues in comparison with adjacent healthy tissues obtained within the same patient; 2) to assess the putative *in vivo* association between the expression of all SSTs in the tumor of patients with OSCC and relevant clinical and histopathological data parameters; 3) to explore and

compare, side-by-side, the direct antitumor effects of different SSAs (octreotide, lanreotide, and pasireotide) in primary OSCC human cell cultures; 4) to characterize the expression pattern of key splicing-related elements (spliceosome components and splicing factors) in a representative battery of clinically well-characterized OSCC tissues in comparison with adjacent healthy tissues obtained within the same patient; 5) to assess the putative *in vivo* association between the expression pattern of key splicing-related elements (spliceosome components and splicing factors) in the tumor of patients with OSCC and relevant clinical and histopathological data parameters; and, 6) to assess the therapeutic potential of a splicing machinery inhibitor (pladienolide B) in primary OSCC human cell cultures.

Therefore, the first section of this Doctoral Thesis aimed to establish the expression patterns of SST_{1-5} in oral cavity tumor tissue versus adjacent healthy tissue obtained within the same patient in a well-characterized patient cohort. To carry out this first objective, the analysis of the expression of SST receptors was performed by qPCR using an array based on microfluidics in both samples (tumor and healthy tissue). The results of this section revealed a different expression profile of the five subtypes of SSTs between tumor and healthy tissue. Specifically, the expression of all receptors, except SST_1, was increased in tumor tissues compared to healthy adjacent control samples, this increase being statistically significant for SST_2 and SST_3. Thus, our results support and extend the previous data in other tumor pathologies that indicate that the expression of some of the SSTs is increased in tumor tissue versus healthy tissue. These results have allowed us to increase our knowledge of the expression profile of SST receptors in oral cavity cancer. Our results could be considered clinically relevant since the response to SSA treatment is usually related to the presence of SST_2 in the tumor (especially octreotide and lanreotide), so SST_2 could be an important therapeutic target in oral cavity cancer through the use of different SSAs (hypothesis that we have explored later).

Another objective within the first section was to evaluate the supposed association between the expression of the SST_{1-5} receptors and the data of clinical and histopathological relevance in patients with tumors of the oral cavity (stage, histological grade, tumor invasion, presence of metastases, recurrence, overall survival, etc.) which are relevant in the prognosis of OSCC. The results revealed that the expression of SST_2 was related to a lower presence of cervical metastases, a lower presence of distant metastases, as well as a lower rate of recurrence. In addition, tumors with higher expression of SST_2 were associated with histopathological factors of good prognosis, such as a greater peritumoral inflammatory reaction, less depth of invasion, and more uniform and less infiltrative invasion fronts. These results indicate that SST_2 could be a useful prognostic marker in patients with tumors of the oral cavity.

In addition, this section also focused on exploring for the first time the effect of different SSAs (octreotide, lanreotide, and pasireotide) on the rate of cell proliferation in primary cultures of oral cavity

cancer tumor cells from various patients in the cohort. Our results demonstrated that all SSAs (used at a dose of 10-7 M) were able to significantly reduce the rate of proliferation of primary tumor cell cultures. Taken together, all these results indicate that somatostatin receptors, especially SST2, could have a prognostic value in oral cavity cancer, since their high expression is related to tumors with better histopathological and clinical behavior. But, in turn, they may also have a therapeutic role in oral cavity cancer in light of the results in cell cultures that show that treatment with SSAs results in a reduction in the rate of tumor proliferation.

The second section of this Doctoral Thesis focused on determining the expression and possible alteration of the components of the splicing machinery in squamous cell cancer of the oral cavity in comparison with adjacent-healthy samples. First, we set out to characterize the expression pattern of a selected set of 59 key spliceosome components and splicing factors in tumor tissue vs. healthy tissue samples. Our results demonstrate for the first time that 12 of the 59 (20%) spliceosome components and splicing factors analyzed were deregulated in tumor tissue compared to healthy tissue (SRSF4, SRSF5, SRSF9, SRSF10, NOVA1, ESRP1, ESRP2, RBM3). , RBM10, TRA2A, TRA2B and TIA1). Some of these elements had previously been described as altered in oral cancer, although not by means of the molecular approach established in this work and without using the control patients that have been included in this work with adjacent samples of the oral cavity from the same patient. In contrast, our data are the first to describe the alteration of various splicing factors (eg. SRSF4, TRA2A and RBM10) in squamous cell carcinoma of the oral cavity.

In this work, we also evaluate the possible associations between the expression of splicing factors with relevant clinical and histopathological data in tumor pathology. Our results revealed that survival was positively correlated with increased expression of SRSF5, SRSF9, RBM3, TRA2A, and TRA2B. These data are novel, as to the best of our knowledge, we are the first to describe the relationship between the expression of SRSF5, SRSF9, TRA2A, TRA2B, RBM3 and better oral cancer survival. Of these, TRA2B, SRSF9, and RBM3 were also associated with less recurrence or distant metastasis. Most of the splicing factors that showed better survival were related to key clinical and histopathological features associated with better prognosis, such as fewer positive cervical nodes (pN), fewer nodes with extracapsular spread, and smaller tumor stages.

Likewise, we have observed at the histopathological level that the expression of TRA2B and TIA1 was associated with better prognostic factors, such as a lower degree of differentiation (G), greater peritumoral inflammatory reaction or less depth of invasion (DOI). These results support previous studies that showed that a higher expression of TIA1 is associated with a better prognosis. However, to date there was no information related to the impact of TRA2B or TIA1 expression at the histopathological level in oral cavity cancer. Likewise, another novel result that our study reveals is the

relationship between the expression of NOVA1 and ESRP2 with clinical and histopathological factors of good prognosis, such as an expansive and uniform tumor invasion front, greater peritumoral reaction, lower tumor stage, and fewer lymph nodes. positive cervical and lower rate of regional recurrence. Taken together, these results demonstrate a co-deregulation of various splicing factors in oral cavity cancer compared to adjacent healthy tissue samples, and that the expression levels of some splicing factors are associated with key clinical and histopathological features of lesser value. aggressiveness and, more importantly, with the overall survival of the patient.

Finally, this section also focused on exploring for the first time the effect of an inhibitor of the splicing machinery (pladienolide B) on the cell proliferation rate of primary cultures of human cells from oral cavity tumors. Our results demonstrated that this inhibitor was able to significantly reduce the proliferation rate of primary tumor cell cultures.

Taken together all the results of this Doctoral Thesis unveiled and expanded the knowledge of the molecular basis of the pathophysiological regulation of OSCC through the analysis of two critical regulatory systems: the somatostatin receptors and the splicing machinery. Specifically, our results demonstrate that SST_2 and different splicing factors (SRSF4, SRSF5, SRSF9, NOVA1, ESRP1, ESRP2, RBM3, RBM10, TRA2A, TRA2B, TIA1, and SRSF10), represent relevant points of regulation for OSCC. Therefore, these SFs could be valuable tools for developing novel diagnostic and prognostic biomarkers and/or therapeutic targets to improve the diagnosis, management, and survival of patients with OSCC. Moreover, this doctoral Thesis demonstrates the efficacy of SSAs and pladienolide-B as potential and useful therapeutic tools for human OSCC.

1. Introduction

Cancer represents one of the most challenging and complex health threats for the human population to date, despite the outstanding research and clinical efforts deployed over the last decades to fight this pathology[1–3]. Multiple studies suggest that human tumorigenesis is part of a multistep process. These sequential steps reflect genetic changes that cause progressive alteration of normal human cells into malignant cells requiring various rate-limiting steps, including genetic and epigenetic modifications[4]. Therefore, cancer is a highly heterogeneous and variable process, strongly influenced by changes in gene expression, but also by metabolic, nutritional, ambient, and lifestyle factors[5]. Despite this variability, most cancers share a group of common hallmarks, such as sustained proliferative signaling, evasion of growth suppressors, resistance to cell death, angiogenesis, activation of invasion and metastasis, or altered alternative splicing processes[6,7].

Among the different types of cancer, the work of this Thesis has been focused on **oral cavity cancer** (**OCC**), which are one of the most common malignancies in developing countries[2]. OCC arises from the oral cavity lining and are predominantly squamous cell carcinomas (SCC) arising from premalignant lesions through a multistep carcinogenesis process[8]. In this sense, neuroendocrine differentiation has been found in some tumors not considered to be of neuroendocrine origin, including SCC of the lung and esophagus[9], and recently, also found in larynx and head and neck SCC[10–12]. In this context, during the last years, our group has been interested in exploring the role of several endocrine systems, particularly the somatostatin (SST) system, in the development, progression and aggressiveness of different endocrine-related tumors, as well as in determining the suitability of certain members of this system as novel biomarkers for the diagnosis, prognosis and/or putative treatment of those endocrine-related tumors[13–21].

In spite that an easy physical examination (i.e. visual inspection and palpation) is often very revealing and allow an accurate impression of the extent of the disease, patients often present with advanced stage disease. Moreover, the underlying causes involved in the development of these tumors are still unknown and the tools available for predicting their prognosis, and, especially, for their treatment, are very limited and/or inefficient. Therefore, novel prognostic biomarkers and therapeutic approaches targets to combat this devastating pathology are urgently needed.

1.1. Oral Squamous Cell Carcinoma (OSCC)

1.1.1. Introduction to oral cavity cancer and epidemiology

Oral cavity squamous cell carcinoma (OSCC) consists of developing a soft tissue malignant neoplasm arising from the epithelium of the mucosa lining of the upper digestive tract, including the lip and the oral cavity. OSCC belongs to the Head and Neck (H&N) cancer classification, which combines

a heterogeneous group of tumor entities anatomically close to each other, but which differ in terms of etiology, histology, diagnostic, and treatment approaches. The head and neck cancer term is widely used to describe those tumors involving the oral cavity, the nasal cavity, paranasal sinuses, pharynx, larynx, and salivary glands[8]. Approximately 91% of all H&N cancer, including oral cavity cancer, are SCC. The other 9% are conformed by 2% of sarcomas and 7% of adenocarcinomas, melanomas, and other not well-specified tumors[22].

The incidence of neoplasms arising from the oral cavity is usually described in epidemiological studies together with the lip and pharynx[23]. The numbers of OCC are then sometimes unclear. According to recent statistics from the International Agency for Research on Cancer (IARC) (http://gco.iarc.fr/), OCC continues to be a worldwide challenge, with an incidence of new 377,646 cases in 2020, of whom almost 50% succumb to the disease. Furthermore, oral cancer will continue to increase with an estimated increase of 48.9% in 2040 with 562,484 cases (Figure I1).

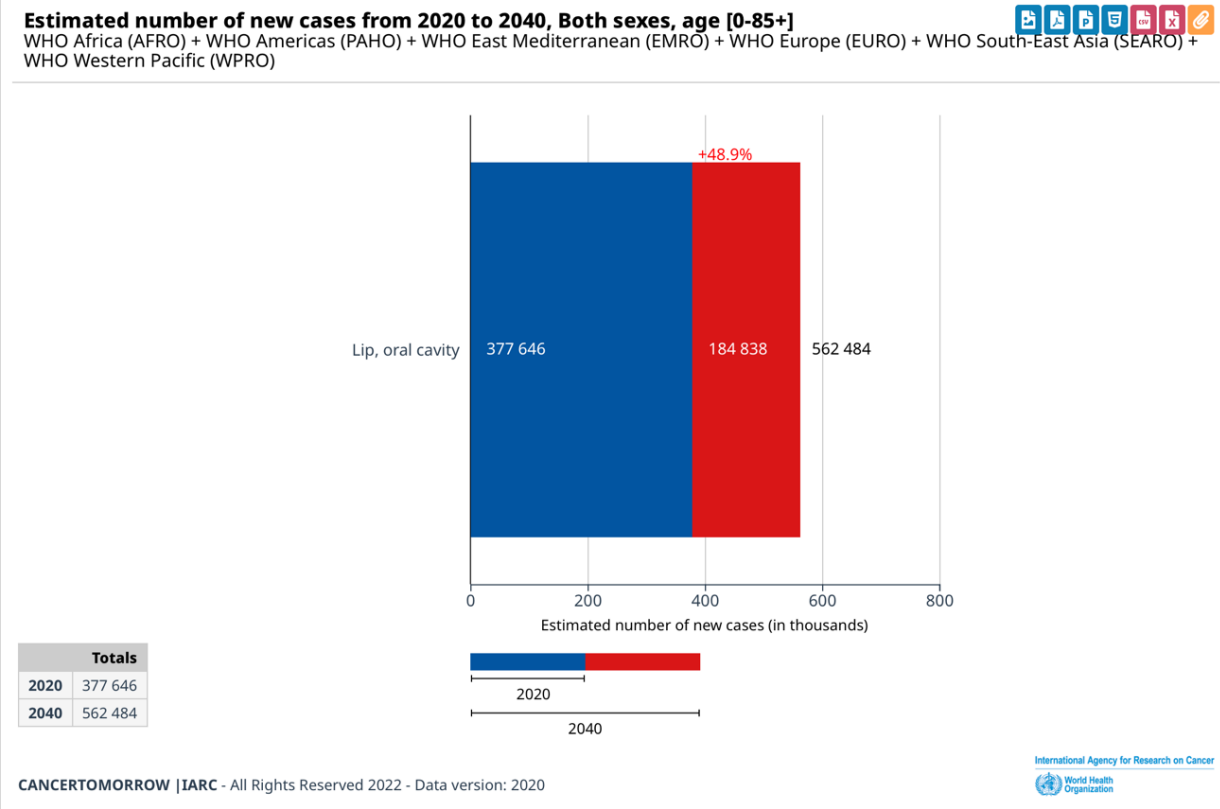


Figure I 1: Estimated number of new oral cavity cancer from 2020 to 2040. Data Source: GLOBOCAN 2020. International Agency for Research on Cancer (IARC) (http://gco.iarc.fr/), unadapted.

Oral cancer shows marked geographical differences, which are related to specific risk factors. Southern Asia dominates the gobal incidence of oral cancer, especially in South-Eastern and Central

Asia. According to Globocan 2020 for lip and oral cancer, Asia accounts the 65,8% of global incidence with 248,360 new cases, followed by Europe (mainly Central and Eastern Europe) with 17,3% (65,279 cases) (http://gco.iarc.fr/) (Figure I2).

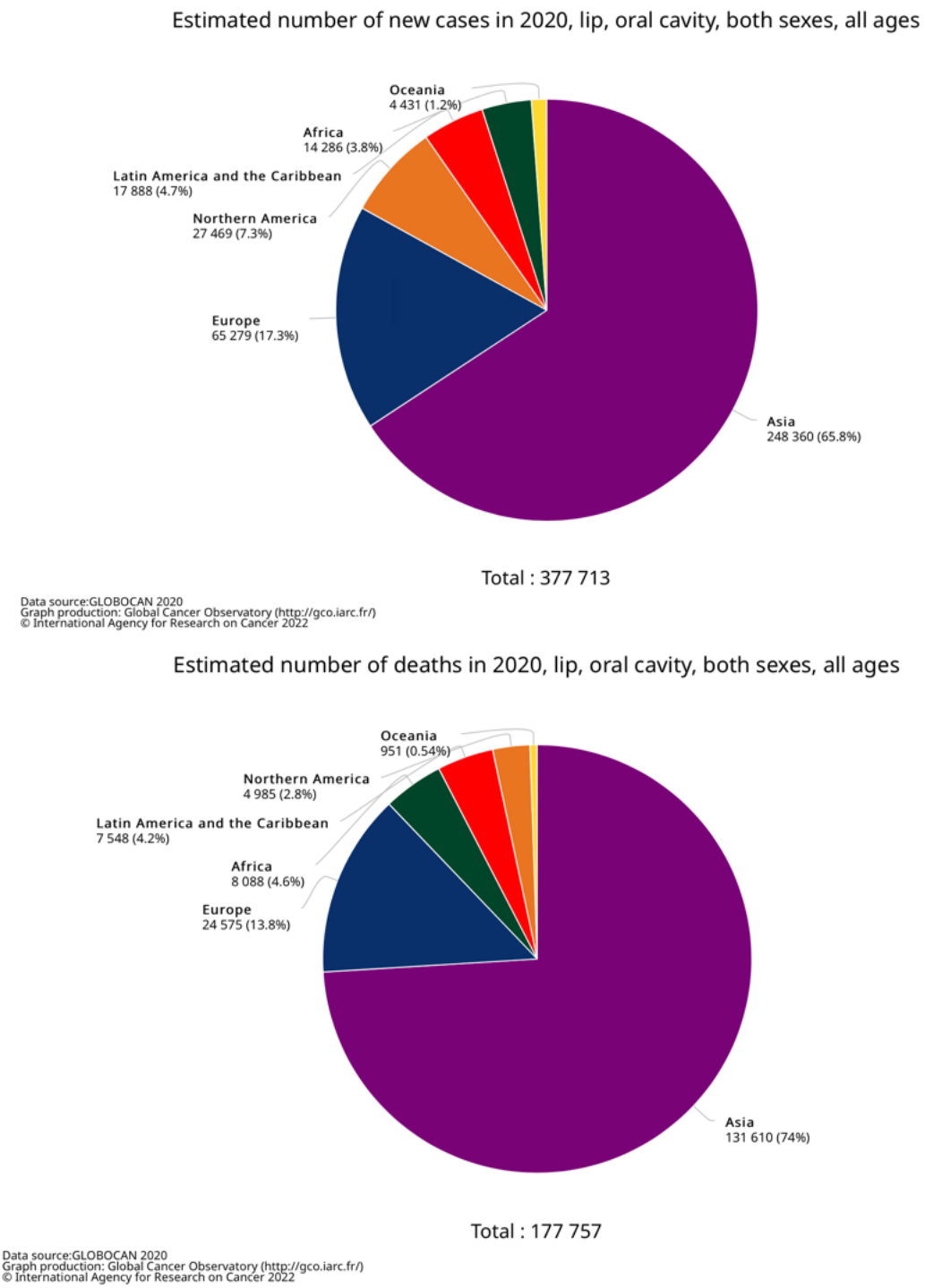
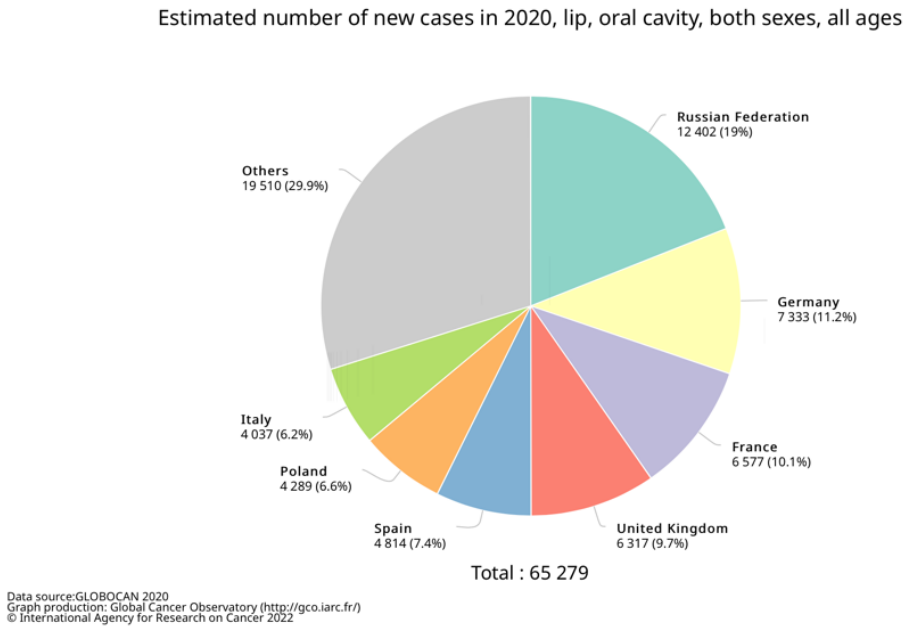


Figure I 2: Global distribution of estimated number of new cases (upper panel) and deaths (lower panel) of oral cavity cancer. Data Source: GLOBOCAN 2020. International Agency for Research on Cancer (IARC) (http://gco.iarc.fr/), unadapted.

For the most recent available data, the number of incident cases of lip and OCC for both genders and all ages in Spain was 4.814 number of new cases per year. This data positions Spain as the top fifth country in Europe with OSCC. Mortality accounts for 1.270 number of deaths for the same period, of which 791 were men and 479 were women (http://gco.iarc.fr/) (Figure I3).



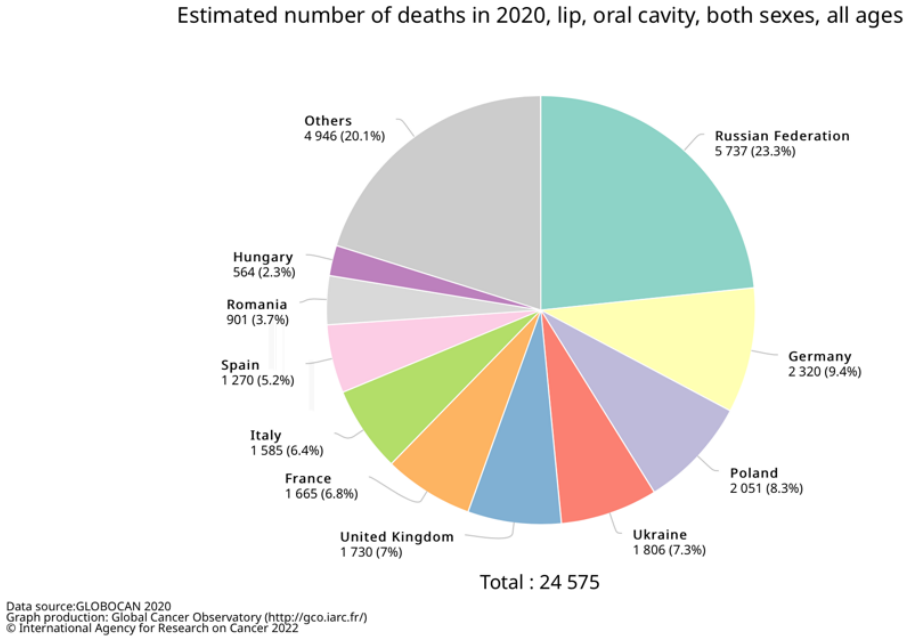


Figure I 3: Estimated number of new cases (upper panel) and deaths (lower panel) of oral cavity cancer. Data Source: GLOBOCAN 2020. International Agency for Research on Cancer (IARC) (http://gco.iarc.fr/), unadapted.

The worldwide number of deaths for OSCC in 2020 is 177,757 with 74% occurring in Asia vs. 13,8% in Europe. However, the European continent has the higher crude mortality rate with a 3.3 rate compared to 2.8 in Asia. This is due to the high crude mortality rate of the Central and Eastern Europe region (4.2). Spain in particular has a 2.7 crude mortality rate (http://gco.iarc.fr/) (Figure I4).

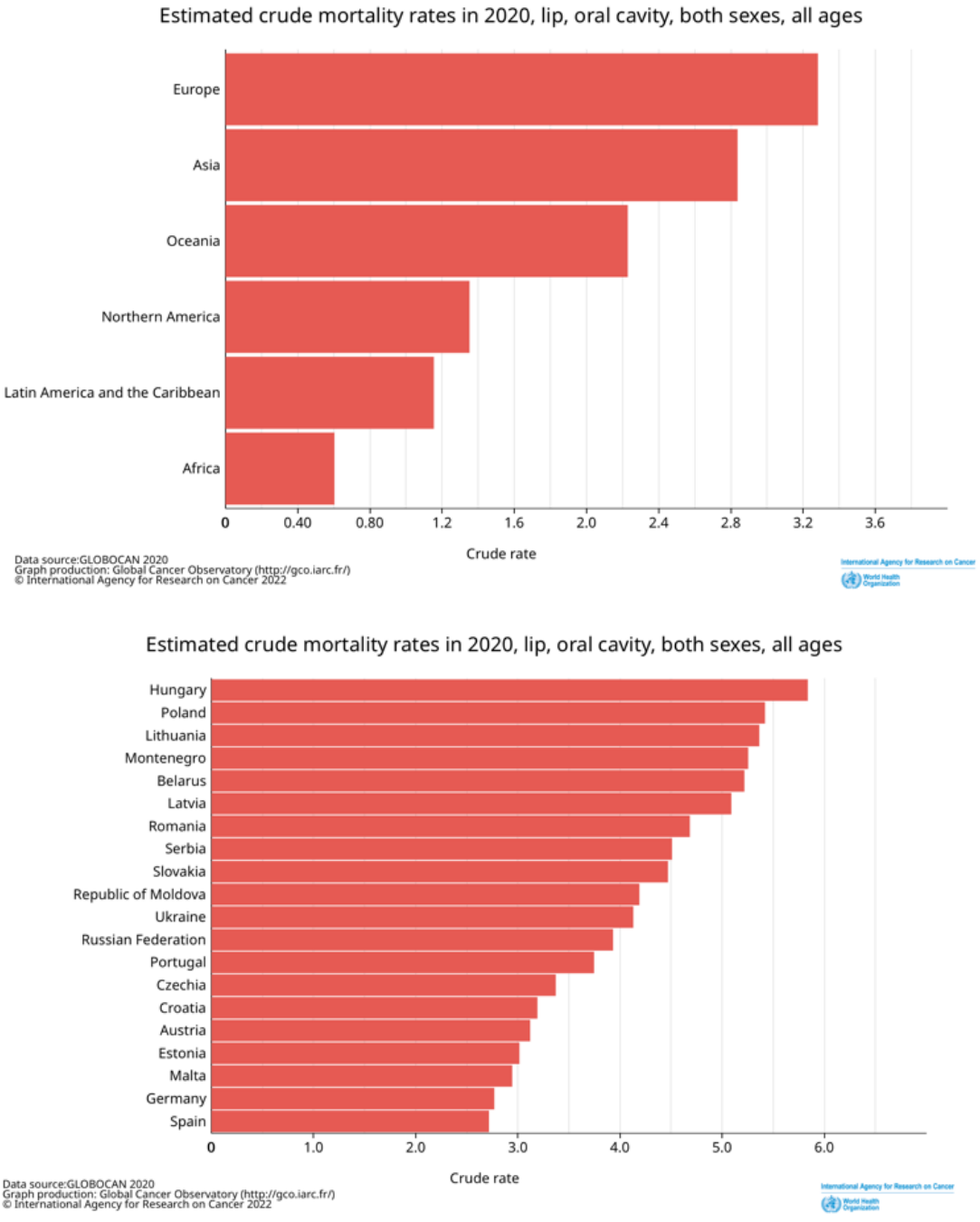


Figure I 4: Estimated global (upper panel) and European (lower panel) estimated crude mortality rate of oral cavity cancer in 2020. Data Source: GLOBOCAN 2020. International Agency for Research on Cancer (IARC) (http://gco.iarc.fr/), unadapted.

Apart from geographical differences, OSCC shows a historically higher prevalence among men in their 6th-7th decade of life, being very rare in younger people[24]. According to IARC, the age-specific incidence of lip and oral cavity and pharynx, measured by Age-standardized rate (ASR), is 15.4 in men versus 3.6 in women. In Spain, the proportion fof OSCC in men vs. women had evolved since the 80´s when there was a very wide range of cases between genders. For instance, in a study performed by Capote et al. in 2020, they studied the evolution in the distribution of patients across the different decades according to sex. They observed a change in the sex ratio from 4.6:1 in the 1980s to 1.7:1 in 2000-2010[25]. The explanation for this rise in cases among women responds to the significantly increased proportion of women exposed to toxic habits like tobacco and alcohol[23,25].

However, studies of oral cancer among the non-smoking and non-alcohol consuming population show an increased proportion of OSCC among women in a bimodal distribution with increased rate at <50 years and at >70 years old[26]. Some authors studied the possible involvement of hormonal factors and the development of head and neck squamous cell carcinoma (HNSCC)[27–29]. They observed that estrogen and hormone-replacement-therapy (HRT) could protect against smoking-associated cancers, such as oral cancer. Estrogen postpones smoking-associated cancers by transitory maintenance of epithelial thickness and integrity in the upper aero-digestive tract[28], explaining why women with oral cancer have a higher mean age than men in their study. They also observed an increased risk of participants with age at menopause of <45 years compared to participants at menopause between 50-54 years[27]. Furthermore, estrogen deficiency is closely associated with insulin resistance (both in women and men) and elevated fasting glucose, a risk factor for the development of oral cancer in post-menopausal women, especially in the gingival location sub-group. The oral mucosa, especially the gingiva, is significantly affected by hormonal influences, seen in the development of atrophic, desquamative gingivitis in postmenopausal women with reduced estrogen levels [30]. However, to date, there is no study that shows gender has an impact on overall survival in OSCC[31,32].

During the last decades, especially the last 30 years, an increase in oral cancer cases among young patients, defined as <40- 45 years, has been described worldwide[32–37]. This new trend was first described for the oropharyngeal and tongue cancer subtypes[38,39]. The National Cancer Institute's Surveillance, Epidemiology, and End Results database provided evidence for an increase in the percentage of cases of SCC of the oral tongue in adults younger than 40 years, from 3% in 1973 to approximately 6% in 1993[36]. A study conducted at the University of Texas M. D. Anderson Cancer Center reported a dramatic increase in the number of cases of tongue cancer in young patients. They observed a change from less than 10% of all cases in 1963 to 15% to 25% of all cases in the mid-1990s[37]. Since then, this trend has continued[34]. Studies from Asia, the Middle East, and Africa have shown high percentages in the young population, particularly Nigeria and Pakistan, with percentages as high as 29% and 30%, respectively[40,41].

Most of the studies about OSCC in younger patients show gender predilection. Some have reported that females have a higher prevalence among the young patients developing oral cancer, even outnumbering men by 2:1[24,37,42,43]. In Europe, Scandinavia has shown a stable percentage of tongue cancer within younger age groups, but the trend revealed a persistent increase in females only[34,39]. However, this gender predilection between the older and younger patients with OSCC is not well accepted, and no consensus exists in the literature[44]. The overall pooled analysis for oral and oropharynx cancer proportions in young patients revealed significant heterogeneity across the studies[34].

Nevertheless, the importance of the OSCC trend among young patients relies upon the discussion that these patients have a worse prognosis than elderly patients. Several studies tried to demonstrate the worst outcome in this group, but addressing this issue is very difficult given the low incidence, making large samples very rare and agreement very difficult. Both conclusions have been established in the literature with articles showing a worse prognosis[43,45,46], equal survival rates[36,37,47,48], or better prognosis[49] in young patients[33]. Recently, a large multicentric study with 365 patients <45 years-old showed that younger patients share the same disease specific survival as the older population with similar rates of death from OSCC in multivariable models[50].

The hypothesis behind the possible reason for young patients' worse tumor behavior is the lack of predisposition to typical known risk factors like alcohol and tobacco. These two risk factors are dose and time-related[23,34]. The duration of the exposition in a patient younger than 40-45 years may not be enough for malignant transformation to occur[43], making the OSCC in young patients a completely different clinical entity characterized by different etiology and pathogenesis[34]. Besides, some authors found a low proportion of smokers and drinkers among the young population with OSCC[51]. Therefore, assuming a correlation between traditional risk factors and cancer in young subgroups may be unreasonable. However, it has also been described that the exposure of the epithelium to carcinogens at a young age may reduce the period of latency in carcinogenesis resulting in the early onset of SCC[52].

These differences regarding the exposure to typical risk factors raised concern to other possible etiological factors for H&N and oral cancer such as human papillomavirus (HPV)[53], genetic predisposition or susceptibility, and aberrant splicing dysregulation[54–57]. Despite the evidence regarding the different exposure to risk factors, there is no consensus on whether oral cancer in the young is distinct from that of older individuals, both theories proposed in the literature.

1.1.2. Etiology and Risk factors

Cancer is a complex process in which multiple components cooperate and are capable of causing a malignant transformation of a previous healthy tissue[54]. The considerable variation in worldwide incidence and mortality from the upper aero-digestive tract cancers is mainly attributed to the difference

in exposure to the major environmental and behavioral risk factors. The process of malignant transformation results from exposure that began years before the cancer development, which was probably shaped by broader socioeconomic factors[23].

The development of OSCC is strongly associated with epigenetic risk factors like environmental and lifestyle risk factors. The most critical lifestyle behavioral risk factors for oral cancer are tobacco usage, betel quid chewing, heavy alcohol consumption, and dietary micronutrient deficiency[58]. There are a significant number of environmental factors involved in oral cancer such as ultraviolet (UV) radiation, indoor and outdoor pollution, occupational exposures to radiation or chemical carcinogens, immunosuppression and, the recently very relevant effect of some "high-risk" genotypes of the HPV family, especially for the oropharyngeal and tongue cancer. The environmental insults presumably increase DNA damage, reduce murine double minute, increase p53 expression, and activate a cluster of genes associated with cell growth or cell death[59]. This process will be further explained in the molecular pathology section of the introduction.

1.1.2.1. Behavioral risk factors

a) Tobacco and alcohol consumption

Tobacco is considered the most important independent risk factor for the development of OSCC, as well as the etiology for other cancers such as lung, oral cavity, nasopharynx, oropharynx, hypopharynx, nasal cavity, accessory sinuses, larynx, esophagus, stomach, pancreas, colon, liver, kidney, ureter, urinary bladder, uterine cervix, and ovary (mucinous), as well as myeloid leukemia[60]. Tobacco could be used in different ways, such as smoked with cigarettes, cigars, beedi/bidi, reverse smoking, and smokeless tobacco products such as oral snuff or moist pouches[8].

Either smoked or smokeless, the oral cavity is the body´s first part exposed to tobacco products, which makes the oral mucosa susceptible to carcinogenic, microbial, immunologic, or clinical effects of its use. When tobacco is smoked, nicotine is primarily absorbed through the absorbent surface of the lungs and also by the oral and nasal mucosa[58]. On the contrary, smokeless tobacco (tobacco consumed without combustion) is placed inside the oral cavity in contact with the mucous membranes. The nicotine is absorbed to provide the desired effect[61]. This tobacco modality has become prevalent worldwide. While cigarette smoking has declined in the US in the previous decades, the use of smokeless tobacco has slightly increased from 2.7% in 2005 to 3.0% in 2010[62]. The proportion of smokeless tobacco in Spain remains very small.

Nicotine is not a carcinogen, but cigarettes and other combusted tobacco deliver a mixture of >7,000 chemicals, of which at least 70 are established carcinogens in smoked tobacco[63,64], and at least 16 in

unburned tobacco have been identified[65]. The most important carcinogens are the polycyclic aromatic hydrocarbons such as benzo[a]pyrene and aromatic amines, and the tobacco-specific nitrosamines, namely 4-(nitrosomethylamino)-1-(3-pyridyl)-1- butanone and N'-nitrosonornicotine[61,63,64]. This carcinogenic activity has been reported to be exerted through DNA adducts. Specifically, the drug-metabolizing enzymes, such as cytochromes P450 and glutathione-S-transferases, catalyze the detoxification of many cigarette smoke chemicals, forming reactive electrophilic intermediates that react with DNA and produce DNA addition products, known as DNA adducts. These are critical in the carcinogenic process because if they persist unrepaired, they can cause miscoding during DNA replication, resulting in a permanent change in the DNA sequence[63]. Minor DNA damage can result in mutations that can be part of the causal chain for malignant transformation, and sustained DNA damage can further perturb cell cycle control[8]. For example, suppose this mutation occurs in a critical gene, such as the *KRAS* oncogene or the *TP53* tumor suppressor gene. In that case, the result can be the loss of normal growth control processes and genomic instability[63].

The role of alcohol as an independent factor in oral carcinogenesis is still unclear. Alcohol is mainly formed by ethanol and water. The primary alcohol metabolizing enzymes are alcohol dehydrogenase, that oxidizes ethanol to acetaldehyde, and aldehyde dehydrogenase, which detoxifies acetaldehyde to acetate. Acetaldehyde is responsible for the oral carcinogenic effect of ethanol, owing to its multiple mutagenic effects on DNA[58]. Acetaldehyde interferes with the DNA synthesis and repair, induces sister chromatid exchanges and specific gene mutations, and inhibits the enzyme 6-methylguanitransferase which is responsible for repairing injuries caused by alkylating agents[61].

It is challenging to accurately calculate the influence of alcohol in the etiology of oral cancer, given that heavy drinkers also smoke heavily. However, it has been proven crucial only when considered in conjunction with tobacco[58,66]. Both have a strong dose-response relationship for oral and oropharyngeal cancer risk, increasing mortality[67]. It is well known that the addition of alcoholic drink intake to tobacco chewing/smoking increases the risk for oral cancer[58,68,69]. Alcohol increases the permeability of oral mucosa producing an alteration in morphology characterized by epithelial atrophy, which leads to easier penetration of carcinogens into the oral mucosa[61].

A study with data pooled from the International Head and Neck Cancer Epidemiology (INHANCE) Consortium showed that among smokers who never drank alcohol, there was a two-fold risk estimate for oral cavity and oropharyngeal cancer, which increased with frequency and duration of smoking. They also saw a similar two-fold risk of the oral cavity and oropharyngeal cancer for alcohol drinking among those who never smoked tobacco, but only in heavier alcohol drinkers (three or more drinks per day)[70]. The highest risks were observed in those who smoked tobacco and consumed alcohol heavily, with an increased five-fold risk[70].

b) Betel quid chewing

Betel quid chewing is a major worldwide risk factor for oral cancer. It is a mixture of areca nut, slaked lime (aqueous calcium hydroxide paste), with or without tobacco, condiments, and with or without sweeteners wrapped in a betel leaf [23]. It is estimated that 10-20% of the world´s population chew areca nut in some form, especially people from the South-eastern Asia[61], where the prevalence of betel quid usage among adults is dramatically high, between 25% and 50%, with peaks of 80–90% in some areas and among some rural ethnic groups[58]. Although very unpopular among the Spanish population, an increase in betel nut use in Italy has recently been described due to the immigrant population from countries where it is commonly used[65].

The primary purpose of betel quid chewing is to extract out the alkaloids, a natural cholinergic agonist similar to nicotine. Four alkaloids have been identified in the betel quid: arecoline, arecaidine, guvacoline, and guvacine. The sensation given by areca nut alkaloids is a stimulant action that increases the capacity to work with euphoric effects and heightened alertness. The Nitrosamine derivatives from each of the four major areca alkaloids are produced by endogenous nitrosation. Reactive oxygen species (ROS) are released in the oral cavity due to the auto-oxidation of polyphenols in the areca nut enhanced by the alkaline pH from slaked lime[71,72].

The damage caused to the oral cavity could have different presentation patterns like the local trauma and injury to the oral mucosa due to its abrasive nature or the malignant transformation of the epithelium to OSCC. Areca nut can disturb collagen homeostasis, cause crosslinks and accelerate the onset of oral submucous fibrosis (OSMF). OSMF is a collagen-related disorder in habitual chewers that can lead to injury-related chronic inflammation, oxidative stress, and cytokine production. Oxidative stress and subsequent ROS generation can induce cell proliferation, cell senescence, or apoptosis depending upon the level of ROS production. This process can lead to preneoplastic lesions in the oral cavity and subsequently to malignancy if chronically used[71].

c) Diet and nutrition

Dietary deficiencies, such as vitamin A and related carotenoids (especially beta-carotene), vitamin C, vitamin E, iron, selenium, folate, and other elements increase oral cancer risk[61,73]. Low consumption of fruits and vegetables and high consumption of red meat with high tobacco and alcohol lead to a 10- to over-20-fold excess risk of cancer of the oral cavity and pharynx[73]. The unfavorable role of the meat on cancers of the upper digestive tract has been attributed to its fatty acid composition and the presence of nitrites, N-nitroso compounds, heterocyclic amines, and polycyclic aromatic hydrocarbons[74].

A study in Italy showed the benefits of the Mediterranean diet as a protective factor against oral and oropharyngeal cancer. The protective role of those Mediterranean micronutrients includes the antioxidant effects, binding and diluting carcinogens in the digestive tract[74]. Antioxidants act by reducing free radical reactions that can cause DNA mutations and changes in lipid peroxidation of cellular membranes. Micronutrients also modulate carcinogen metabolism, work on cell differentiation maintenance, inhibit cell proliferation and oncogene expression, help immune function and inhibit the formation of endogenous carcinogens[8]. As Filomeno explains in his article, countries from the Mediterranean coast consume large amounts of olive oil, the primary source of monounsaturated fats. Olive oil has shown to have a favorable influence on oral cancer, possibly because of the antioxidant properties attributable to oleic acid itself and the presence of other nutrients, such as vitamin E and polyphenols[74–76].

1.1.2.2. Human papillomavirus

Some viruses favor the development of malignant tumors of the squamous epithelia. The prototypic viruses implicated in head and neck cancer development are different forms of human herpesvirus such as Epstein–Barr virus (EBV) and human papillomavirus (HPV)[61], being the first related to oral and oropharyngeal squamous cell carcinoma (OPSCC) and the latter with nasopharyngeal cancer[77].

Better known for cervical cancer, HPV is considered an independent factor for developing oropharyngeal cancer[78,79]. This sexually transmitted virus has more than 200 identified genotypes of which groups 1, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 are classified as oncogenic by IARC. HPV16 is the most common cause of HPV-related HNSCC with 80% prevalence, and HPV18 is the second with 3% prevalence[79]. HPV16 and HPV18 are the main high-risk oncogenic types for HNSCC, but others as HPV31, HPV33 and HPV35 are responsible for a subset of HPV-related HNSCC[51,61,68,79]. Risk factors in developing HPV infection include the lifetime number of oral sex partners as the most relevant factor, together with mouth kissing, vaginal and any sex, aged <18 years at the first time of oral sex, marijuana use, and history of cervical HPV infection[79,80].

How HPV goes from infection to cancer is still under study. However, it has been established that when the virus is not cleared in the mucosal lining of the tonsil and tonsillar crypts, it can lead to a precancerous stage or premalignant oropharyngeal disorders, which is usually missed, and with time turns into SCC[79]. The carcinogenesis process with HPV involves the expression of E6 and E7 proteins, which either inhibit or promote degradation of p53 and pRb and the release of the E2F transcription factor. This will result in the deregulation of the cell cycle and activation of viral replication within the host DNA. The degradation of pRb will produce the overexpression of p16, which is a Cyclin-dependent kinase inhibitor, allowing epithelial cells to escape from oncogene-induced senescence and activate survival signaling pathways[78,79,81]. The p16 is a hallmark of HPV-positive cancer, and it is used as a

biomarker. The detection of HPV can be determined by HPV DNA in situ hybridization or indirectly through p16 overexpression using immunohistochemistry[82].

Approximately 50% of patients with OPSCC are positive for HPV-16 DNA; however, contrary to this finding, oral SCCs are not typically associated with HPV presence, which could be because the epithelial tissue of the oral cavity differs from that in oropharynx structures. Only 20% of individuals with OCSCC are HPV-16 DNA positive[35].

The clinical trial study conducted by the Radiation Therapy Oncology Group (RTOG), RTOG 0129, confirmed that HPV-related tumors had shown a survival rate of 82.4% versus 57.1% for patients with HPV-negative tumors[82]. The changes in prognosis and the etiology shift from carcinogen-exposed to virally mediated cancer have led to establishing the ‘‘new’’ HPV-positive oropharyngeal cancer (HPV-OPSCC) patients´ category. These represent a unique population of HPV-related HNSCC patients who are typically younger, less likely to smoke and drink[35,51].

HPV-positive oropharyngeal (HPV-OPSCC) cancer has unique risk factors, demographic profile and prognosis, which may confer unique therapeutic considerations in the future. The change in etiology in HPV-OPSCC over the last 3-4 decades led to major changes in the 8th TNM classification of the American Joint Cancer Committee (AJCC), which established different classification systems for HPV-positive and HPV-negative oropharyngeal cancer[83].

1.1.2.3. Oral potentially malignant disorders

It is known that oral cancer may develop in areas with preexisting mucosal pathology. These lesions have been termed in the literature as “pre-cancer”, “precancerous state”, “premalignant lesions” or “intraepithelial neoplasia”[84]. Then, in 2005 the term “Oral potentially malignant disorders” (OPMDs) was established by the World Health Organization (WHO) during a workshop in London. They recommended this term when talking about oral mucosal disorders with an increased risk of cancer transformation. They established this terminology as it implies that not all lesions and conditions described under this term may transform to cancer; however, there is a family of morphological alterations amongst which some may have an increased potential for malignant transformation[85].

Oral PMDs are categorized into Oral leukoplakia (OL) (Figure I5), oral erythroplakia (OE) (Figure I5), oral submucous fibrosis (OSMF) (Figure I6), oral lichen planus (OLP) (Figure I6), and actinic cheilitis (AC) (Figure I7)[86]. Other PMDs are palatal keratosis associated with reverse smoking, discoid lupus erythematosus, dyskeratosis congenita, and epidermolysis bullosa[85,87].

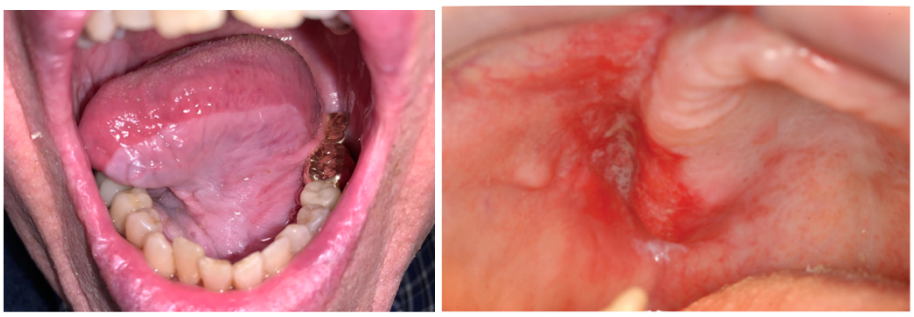


Figure I 5: Oral leukoplakia (OL) of the ventral tongue and floor of the mouth (left). Oral erythroplakia (OE) of the vestibular mucosa and alveolar ridge (right).

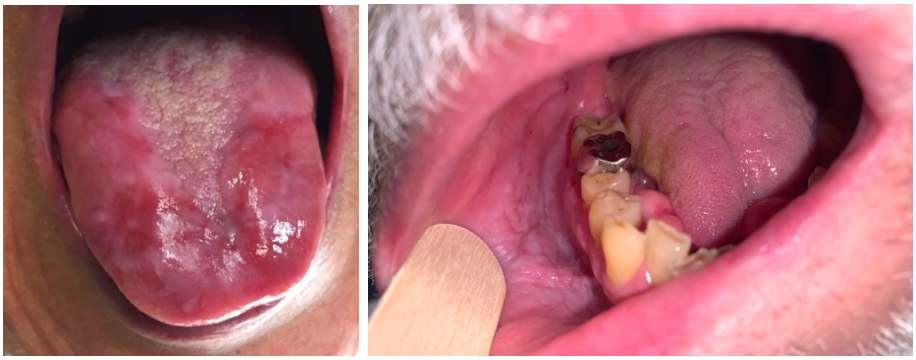


Figure I 6: Oral lichen planus (OLP) of the dorsal tongue (left). Oral submucous fibrosis (OSMF) of the right buccal mucosa (right).

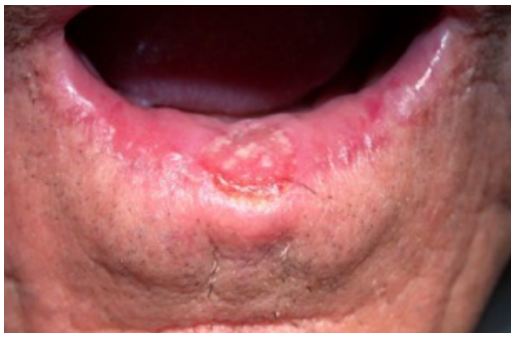


Figure I 7: Actinic cheilitis (AC) of the lower lip.

In 2018, Warnakulasuriya then proposed the inclusion of the term "Potentially Premalignant Oral Epithelial lesions." According to them, new evidence emerged that supports the inclusion of oral lichenoid lesions and graft vs. host disease as potentially malignant disorders[84]. Controversy continues whether oral lichen planus should be considered an OPMDs.

Similar to OSCC's global distribution, the OPMDs have a wide geographical variation due to differences in sociodemographic characteristics, the different types of used tobacco, and definition of

the disease[8,86,87]. A systematic review about OPMDs prevalence performed by Mello in 2018 found that Asia was affected the most by OPMD, followed by South America and the Caribbean. The pooled prevalence of OPMDs in Mello´s study was 4.47% (95%CI=2.43-7.08); but the prevalence of the different types of OPMDs varied: OSMF was 4.96% (95%CI=2.28-8.62); OL was 4.11% (95%CI=1.98-6.97); OE was 0.17% (95%CI=0.07-0.32), and AC was 2.08% (95%CI=0.94-3.67). Compared to the global prevalence, the overall prevalence in Europe was 3.07% (95%CI=1.64-4.93); and, concerning the subgroups, OL prevalence was 1.20% (95%CI=0.57-2.06), and AC was 15.32%[86].

Each one of the OPMDs has a different risk of malignant transformation. Warnakulasuriya *et al.* showed that the overall malignant transformation rate of leukoplakia was 3.5%. However, the rate varied in studies between 0.13% and 34%, with a mean of 14.9 %[72,87]. The most common site for OL is the buccal mucosa, but the site with a higher malignant transformation is the tongue, followed by a combination of tongue and floor of the mouth[72]. Erythroplakia is the OPMDs with the highest malignant transformation rates compared to the other oral lesions, ranging from 14% to 50%[86,88]. This highlights the importance of early detection and early treatment. Usually, erythroplakia shows at least some degree of dysplasia and even carcinoma *in situ* or invasive carcinoma[88]. For some authors, any red velvety lesions with or without white components in high-risk sites of the oral cavity should be considered, at the very least, carcinoma in situ or invasive carcinoma—unless proven otherwise[89]. AC is mainly caused by UV radiation and usually involves the lower lip with a high risk of SCC transformation; indeed, it is considered one of the main risk factors for lip cancer[87,90] and has demonstrated to have a greater risk for metastasis than SCC arising from other cutaneous parts[91].

Multiple studies are analyzing the cellular pathogenesis behind the malignant transformation of oral lesions. The most promising proposed some molecular tools to predict malignant transformation in head and neck carcinogenesis, such as the loss of heterozygosity (particularly in loci 3p and 9p), aneuploidy, and the biomarkers MMP9, survivin, cortactin, ki67, and cyclin D1[92].

1.1.2.4. Dental factors

Multiple dental factors could lead to OSCC, such as chronic oral mucosa trauma, poor oral hygiene, or periodontal disease. It is well known among oral and maxillofacial surgeons that chronic trauma of the oral mucosa is problematic, especially in patients that share other risk factors such as tobacco and alcohol. Chronic trauma results from a constant mechanical irritation to the mucosa epithelium. It can be caused by different sources such as teeth with sharp or rough surfaces, ill-lifting dentures or overextended flanges, and parafunctional habits[23]. An independently increased risk for oral cancer has been established with periodontal disease[93,94].

1.1.2.5. Genetic predisposition

Copper first described the genetic predisposition to develop head and neck cancer in 1995. He followed up relatives of former patients with HNSCC and found that 30% of them developed upper digestive tract cancers[54]. Garavello also confirmed a familiar risk of developing oral and pharyngeal cancer, independently of alcohol and tobacco, with a family history of oral and pharyngeal cancer and/or laryngeal cancer in first-degree relatives. The odds ratio with a first relative was 3.0 (95% CI, 1.9–4.9) for oral and pharyngeal cancer. This result increased to 7.1 (95% CI, 1.3–37.2) risk to develop oral and pharyngeal cancer and/or laryngeal cancer with 2 or more first-degree relatives affected[95]. Some studies have linked these family-related tumors to different gene polymorphisms in some carcinogen-metabolizing enzyme systems like the CYP1A1[96], ALDH2, or polymorphisms in genes involved in DNA repair maintenance like *XRCC1*[97].

1.1.2.6. Other

During the last years, various studies, meta-analyses, and systematic reviews have shown that patients with Diabetes Mellitus (DM) have a higher prevalence of oral cancer and OPMDs than the average population, which has been proved to be independent of geographical location[98,99]. The impact of obesity on OSCC survival is controversial in the literature with some papers describing obesity as an independent risk factor for OSCC patients[99–101], while others describe obesity as protective role[102,103].

Diabetes mellitus (DM) is one of the major comorbid conditions in the general population with a prevalence between 12-20% depending on the different ethnicities[104]. Van der Poll-Franse et al. studied the prevalence of DM in cancer patients, and analyzed if DM had an impact on stage at diagnosis, treatment, and prognosis. They found that younger patients with cancer had more prevalence of DM than general population of the same age. Cancer patients with DM experienced a significant increase in overall mortality (HR 5 1.44, 95% CI 1.40–1.49) as well as a worse prognosis compared to those without DM. Interestingly, they exposed that patients frequently were treated less aggressively due to the avoidance of diabetes related complications. They also speculated that diabetes could negatively influence the effect of cancer therapies[105]. The presence of diabetes has also been associated with increased cancer recurrence in a randomized colon cancer trial[106].

Patients with DM and oral cancer have an increased risk of oral cancer-related death of 2.9 times higher than the general population (95%CI=1.36- 3.22, p=0.001)[98]. Some *in vitro* and *in vivo* studies suggested that different peroxisome proliferator-activator receptors (PPAR) agonists, which are a DM treatment option, might suppress the growth of human OSCC cells, even if these cancer cells have no functional PPAR$_\gamma$[107]. Also, pioglitazone (a PPAR agonist) might potentially have a preventive effect on the development of oral cancer[108]. Hatton presented a phase II clinical trial with pioglitazone therapy

for dysplastic leukoplakia. This study showed that pioglitazone administered for three months at 45 mg/day elicited a response in 70% of subjects[109]. This theory was taken back by a Taiwan observational study conducted among humans with a diagnosis of DM. They found that Pioglitazone had a null association with oral cancer after multivariable adjustment, and that if only age and sex were considered for adjustment, a fake conclusion of a preventive role for pioglitazone might have been reached[110].

The relationship between DM and oral cancer is not yet well known, although it seems to be related to some biochemical and molecular factors. Hyper-insulin resistance means increased IGF1 receptor activation, which acts as an activator of proliferative and antiapoptotic factors, like Bcl-2, which is involved in cancer[98]. As a result of the proliferative action of hyperinsulinemia, an upregulation of the *CCND1* gene and its protein (cyclin D1) exists, which play a central role in the pathogenesis of oral cancer, not only increasing the proliferation but also favoring the migration capacity of malignant cells[92,98].

1.1.3. Molecular pathology in OSCC

Since completing the Cancer Genome Atlas in 2003, the mutational landscape of primary untreated Head and Neck cancer has been under the spotlight pending *in vitro* studies to understand the key regulatory pathways better, confirm malignant drivers, and discriminate potential therapeutic targets[111].

The new sequencing exome technologies improved the understanding of genomic regulation. In fact, since 2011, several studies have revealed specific alterations in elements of cell cycle control, squamous differentiation, mitogenic signaling, and epigenetic regulation that appear to drive the progression of HNSCC[8]. The molecular pathways that have been identified as contributors to the pathogenesis of HNSCC are[8,112,113]:

a) Alteration of the cell cycle control by tumor suppressor tumor protein p53 (tp53) mutation.
b) Alteration of the cell cycle control by the retinoblastoma tumor-suppressor gene (Rb) pathway and cyclin-dependent kinase inhibitor 2A (*CDKN2A*) mutations.
c) Alteration in cell growth and cell survival via the epidermal growth factor receptor (EGFR), Ras, and phosphatidylinositol-3-kinase (PI3K) pathways mutation.
d) Abnormal Squamous Differentiation through Notch Pathway mutation.

The tumor suppressor tp53 is involved in many cancer types, and its function is crucial in avoiding cancer development that it is sometimes referred to as “the guardian of the genome”. It was proven to be mutated in 47–69% of HNSCC[8,112,113] and its mutation is considered a prognostic factor in HNSCC. When tp53 is mutated, it is associated with decreased survival, higher resistance to chemotherapy, and higher locoregional recurrence after radiation therapy[114,115]. Despite this, the use of the tp53 as a

prognostic marker still brings controversy due to variability in testing techniques and inadequate sensitivity and specificity of various methodologies[8].

The other cell cycle regulator of importance in HNSCC is the Rb (retinoblastoma) pathway. It binds to E2F, a transcription factor, and arrests cell cycle progression. However, cyclin-dependent kinases CDK4 and CDK6 and the cyclin D1 (D1/CDK4/6 complex) can defeat this by phosphorylating Rb, which will in turn release E2F and allow the cell cycle to continue. This protein complex is inhibited by the tumor suppressor p16/INK4A (encoded by the *CDKN2a* gene), preventing Rb phosphorylation. The Rb pathway is commonly altered via loss of p16 or the upregulation of CCD1 (cyclin D1)[116]. When genetic alterations are combined with epigenetic silencing processes, such as methylation of p16-DNA, nearly 80% of HNSCC tumors exhibit loss of p16 function, which has been associated with decreased survival. Overexpression of cyclin D1 and loss of expression of $p16^{INK4a}$ have been identified as independent death predictors in tongue cancer[114] and can predict poor outcomes[8].

EGFR receptor has been found to be related to, and can be overexpressed in 90% of HNSCC, which made possible the development of multiple EGFR-targeting drugs that are very important for HNSCC, such as cetuximab. EGFR starts a signaling cascade that may lead to downstream signaling via the ras/raf/mapk pathway or the PIK3 pathway. Ras is a family of three genes, HRAS, KRAS, and NRAS. However, HRAS mutation is the one found in about 4-5% of cases of HNSCC[8,112]. PIK3 signaling is also an essential pathway for cell growth, differentiation, and cell migration. PIK3 mutations have been identified in more than 30% of HNSCC, making it a very common alteration. Besides, multiple mutations in the PIK3 pathway showed a relationship with advanced-stage tumors[8].

The use of the new sequencing technologies also emerged the importance of the NOTCH pathway in HNSCC. NOTCH consists of a family of four transmembrane receptors (NOTCH 1-4), and their signaling is responsible for various regulatory functions like cell control or differentiation. In HNSCC, it appears to have a tumor suppressor function. According to the Cancer Genome Atlas, the NOTCH1 mutations are confirmed to be present in about 19% of HNSCC cases [8,112].

1.1.4. Cellular pathology of OSCC

The evaluation of cellular and histopathological characteristics of a tumor is of significant importance for many reasons, but mainly to identify and validate ways to predict the tumor prognosis and, very importantly, to plan the treatment. Pathology parameters like depth of invasion (DOI) are critical for deciding whether a patient requires elective lymph node dissection or if a patient will require adjuvant therapy to avoid recurrence. Because of this, regular communication between the pathologist and the surgeon should exist, and the pathology report from the biopsy and the tumor resection specimen is vital in the cancer treatment process.

OSCC is triggered by a chain of events initiated or influenced by the risk factors described before in this thesis, maybe years before the diagnosis. This disease evolution is achieved in a stepwise manner that starts with microscopic alterations in the squamous epithelium such as hyperplasia, dysplasia, or carcinoma in-situ[8,117]. The epithelium of precursor lesions may be thick; however, in the oral cavity, it is often atrophic, which has an implication in the classification system that will further be described.

As previously mentioned in the OPMDs section, the principal cancer precursor lesions in the oral cavity are either white patches (OL), red patches (OE), or mixed red and white lesions. They have a higher risk of developing oral cancer[72,87], but not all of them will turn into dysplasia but will stay in the hyperplasia category. By definition, OPMDs lesions do not have invasion of the basal membrane[118]. Hyperplasia describes an increased number of cells in the spinous layer (acanthosis) or in the basal/parabasal cell layers. The main characteristic feature is that its architecture shows regular stratification without cellular atypia, which means variation in the size and shape of the keratinocytes[117,118].

1.1.4.1. Tumor histopathological factors

OSCC arises from keratinizing dysplasia and is graded into well differentiated (G1), moderately differentiated (G2), and poorly differentiated (G3) by the degree of the tumor squamous differentiation (G). Well differentiated carcinoma mimics the benign keratinizing epithelium with keratin pearls and intercellular bridges. On the other hand, poorly differentiated carcinoma shows anaplasia, abundant mitosis, and rare or no keratinization[118]. Some authors have stated that this classification lacks tumor discrimination with 90% of tumors of the oral and oropharyngeal region being classified as G2[119]. The correlation between G and survival has been described, but G alone is not recognized as a significant overall prognostic factor[119]. Nevertheless, G has shown to be of value as a prognostic factor in early-stage cancer[120].

By definition, SCC invades the basement membrane. This invasion can be classified as superficial or deep. The importance of this invasion is based on the increased risk for nodal metastasis as the tumor invades deeper. Different thresholds of the depth of invasion (DOI) warrant elective neck dissection in the literature, 2 to 4 mm being the traditional branch points[121–126]. The tumor's location also influences this decision. Some authors suggest a 2 mm cut-off point for the tongue, 3 mm for the floor of the mouth, and 4 mm for the retromolar and hard palate/alveolar ridge[124].

For decades, tumor thickness has been recognized as a prognosis factor of OSCC. A trend from describing tumor thickness to depth of invasion has been implemented in the latest TNM system[83]. DOI results from a perpendicular “plumb line” from the level of the basement membrane relative to the closest intact squamous mucosa to the deepest point of tumor invasion[77,127] (Figure I8). DOI helps differentiate between a thick, exophytic, but less invasive tumor and an ulcerated and deeply invasive

tumor with different prognosis[77,128]. In the latest 8th TNM edition of the American Joint Committee on Cancer (AJCC), DOI is divided into ≤5mm, >5 mm but ≤10 mm, and >10 mm and contributes to upgrading the T category (pT1,pT2,pT3,pT4)[77,83,126]. Some authors have failed to find a correlation between DOI and disease-free survival (DFS) or locoregional control (LRC) in the absence of other poor prognostic factors[129]. However, DOI continues to be an indicator of possible occult metastasis in OSCC and has significantly proved to be a major predictor of death in tongue SCC[128].

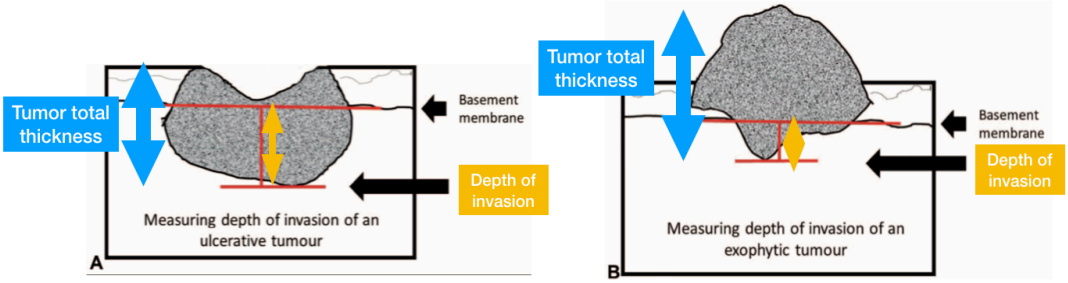


Figure I 8: Tumor thickness versus Depth of invasion (DOI). DOI helps differentiate between a thick, exophytic, but less invasive tumor and an ulcerated and deeply invasive tumor with different prognosis. Adapted figure from *Müller et al. 2019*[127].

Other histopathological relevant markers assessed in most pathological reports, besides tumor differentiation (G) and DOI, are growth pattern of tumor invasion, peri-tumoral inflammatory reaction (PTI)[127,130,131], presence of lymphovascular invasion (LVI), perineural invasion (PNI)[132], and tumor budding (TB)[133].

There are two types of clinical and histopathological growth patterns of tumor invasion front: exophytic and infiltrative. The infiltrative growth carries worse prognostic features and it is a useful predictor given that it is associated with increased likelihood of metastasis[118,134]. The infiltrative pattern can be classified as crumb (islet-infiltrating cells with wide fronts of invasion), trabes (thin infiltrating cords), or individual infiltrating cells. The trabes -"cord-like"-"strand-like" pattern is considered to be diffusely infiltrating SCC and shows the worse prognosis behavior and higher risk of metastasis[135].

Peritumoral inflammation (PTI) is the presence of inflammatory cells in the peritumoral microenvironment, and it exists commonly in many tumors, including the OSCC. Traditionally, PTI has been considered a defense mechanism against cancer progression and invasion[130,131,136,137]. It has been proposed that inflammatory activity, such as immunological response to the tumor, could be used as a prognostic factor. This theory is based on the belief that the lower the inflammatory infiltrate, the greater the risk of regional or distant metastasis[131,138], given that tumor progression is mediated mainly by the host's inability to mount a protective antitumor immune response. Jing study showed a 75% of cervical metastasis when low PTI compared to 37,5% and 29,4% in moderate and marked lymphocytic

peritumoral reaction[138]. However, the PTI´s role in the prognosis of OSCC is still controversial. Other studies suggest that peritumoral stromal inflammation is more likely to contribute to cancer development. Cancer cells can promote local migration of myelomonocytic cells, which, in turn, can express inflammatory mediators supporting pro-tumoral functions, such as cell proliferation, angiogenesis, stroma remodeling, and metastasis formation[139].

Affonso and Vieira have observed a correlation between a higher degree of malignancy (poorly differentiated tumors) and higher inflammatory intensity, suggesting an association between PTI and the degree of tumor differentiation. However, none of them could find a relationship with patient survival curves[131,136]. On the contrary, Campisi found that the presence of PTI acts as an independent predictive variable accounting for a better outcome without local recurrence. They saw that PTI-positive cases displayed a significantly lower risk of local recurrence and the highest mean lifetime[130].

Besides the local inflammation, a relationship between systemic inflammatory response (SIR) and cancer has also been studied. Some parameters used to measure systemic inflammation, such as C-reactive protein (CRP), albumin, white blood cell, neutrophil, lymphocyte, and platelet counts, have been reported to influence disease-related outcomes in some cancers[140,141]. In a study performed by Salas in unresectable HNSCC, CRP was found as the only significant factor in multivariate analysis that influenced response to chemo-radiation treatment. However, how this relationship works has not been yet clarified[141,142]. Further studies by Acharya and Khandavilli deepened into the relationship between CRP and OSCC. They found that a higher level of CRP is preoperatively present in larger size tumors (T3-T4). CRP level could be related to tumor cells releasing IL-6. The larger the tumor, the more cytokine release. Besides, they also observed a worse survival among patients with higher preoperative CRP and a positive relationship with clinical nodal status in OSCC[140,143].

Lymphovascular (LVI) and Perineural (PNI) invasion are also well-known histopathological tumor features that are considered as prognosis factors[118,144]. Both LVI and PNI definitions should be subclassified as either intratumoral or extratumoral and as focal or multifocal. To be considered PNI positive, the carcinoma should wrap around nerves while merely “bumping” into a nerve is not considered an invasion[77]. Both, PNI and LVI have been associated with increased risk of recurrence[119,145] and are indicators for the need of adjuvant radiation therapy[146].

Tumor Budding (TB) and Worst Pattern of Invasion (WPOI) are more newly described histopathological factors in OSCC. TB is defined as the presence of isolated undifferentiated malignant cells or small cell clusters (up to five cells) scattered in the stroma at varying distances from the invasive front. Its expression has been correlated with advanced tumor stage, larger tumor size, deeper invasion, an infiltrative pattern of invasion, nodal metastasis, distant metastasis, and the presence of extranodal extension (ENE), LVI, and PNI[133].

The WPOI has been included in the TNM system as a predictor outcome. Five different categories of WPOI are described (WPOI 1-5). However, the only WPOI level taken into consideration is whether or not WPOI-5 is present. The AJCC defines WPOI-5 as tumor dispersion of > 1 mm between tumor satellites. In the low-stage oral cavity cancer, tumors with >4 mm DOI and WPOI-5 positive have a higher risk for locoregional disease with a probability of developing locoregional recurrence of almost 42%[77].

1.1.4.2. Regional histopathological factors

The prognosis of OSCC is heavily impacted by the metastasis to the regional lymph nodes. The status of the cervical lymph nodes is the single most important prognostic factor affecting the patients´ survival[126,147,148]. In this context, the lymph nodes characteristics such as number, size, and laterality relative to the primary tumor location should be analyzed in patients with OSCC. These results help to determine the pathological cervical Staging (pN). These classifications will be further described in the 1.1.5 section.

Currently, the relevant histopathological results of the lymph node dissection include the number of pathological lymph nodes, the lymph nodes size, and the presence of Extranodal Extension (ENE). AJCC defines ENE+ as the extension of metastatic carcinoma through the fibrous capsule of a lymph node into the surrounding connective tissue, with or without the presence of a stromal reaction. A tumor that stretches the capsule without breaching it does not constitute ENE. AJCC also subcategorizes ENE as ENEma (macroscopic or gross ENE that is apparent to the pathologist's naked eye or extends > 2 mm beyond the nodal capsule under the microscope, or a soft tissue deposit that has completely destroyed nodal architecture), or ENEmi (microscopic ENE that is restricted to ≤ 2 mm from the nodal capsule)[77].

1.1.5. Diagnosis and Staging

Diagnosis of OSCC is made by physical examination, radiological studies, and confirmed by biopsy of the primary tumor. Different origins within the oral cavity carry different histopathologic behavior and prognosis. For this reason, the oral cavity subdivided into lip, tongue, gingiva, floor of the mouth, retromolar area, buccal mucosa, and hard palate[77] (figure I9).

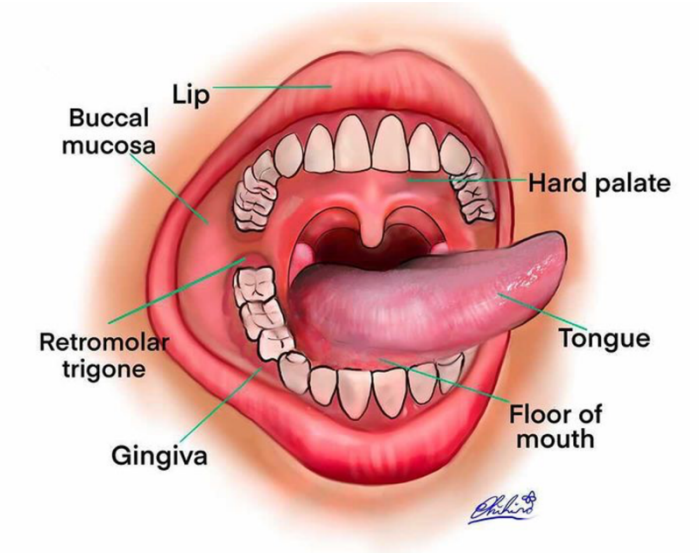


Figure I 9: Anatomical location of the different possible origins of squamous cell carcinoma within the oral cavity. Original picture.

Early detection in OSCC is critical for a good outcome and it mostly relies on physical exam. New trends for early detection with screening methods such as vital staining (toluidine blue), oral cytology using brush biopsy, and some light-based techniques (ViziLite® [Zila Pharmaceuticals, AZ, USA] and VELscope® [LED Dental Inc., BC, Canada) are emerging. However, there is no consensus regarding their use or literature showing improvement in early detection over physical exam[149].

A thourough physical examination, a biopsy of the primary tumor, and radiological studies such as computer tomography scan of the neck are necessary to determine the tumor Stage. A positron emission tomography (PET) scan can also be used to rule out or diagnose distant metastasis.

The AJCC TNM system is the used staging system which is based on three categories: T, N, and M. The histopathological analysis of the tumor specimen and neck dissection after surgery determine the Pathological Staging (pTNM) which is summarized in figure I10. Different combinations of these parameters establish the cancer Stages (I to IV) which are summarized in figure I11.

Tumor category (pT) refers to the tumor size (cm) and sub-classifies from pT1 to pT4. The pT includes DOI as an additional factor since the TNM 8th edition[77]. The Nodal involvement (pN) accounts for the metastatic disease in the cervical lymph nodes, which are sub-classified from pN1 to pN3b, as it is shown in figure I10. As previously mentioned, the cervical status possesses a tremendous prognosis value and correlates with a worse prognosis and a decreased overall survival rate[150]. pN accounts for the number and size of lymph nodes and their ipsilateral or bilateral location. In addition to this, extranodal extension (ENE), if present, modifies the pN category as shown in figure I10. M category corresponds to distant metastasis, which can be present in advanced disease[77,146].

Primary Tumor (pT) category	pT criteria
pTx	Primary tumor cannot be assessed
pTis	Carcinoma in situ
pT1	Tumor ≤ 2 cm and ≤ 5 mm depth of invasion (DOI)
pT2	Tumor ≤ 2 cm with DOI > 5 mm *or* tumor >2 cm and ≤ 4 cm with DOI ≤10 mm
pT3	Tumor > 2 cm and ≤ 4 cm with DOI >10 mm *or* tumor >4 cm with DOI ≤10 mm
pT4	Tumor >4cm with DOI >10 mm Or tumor invades adjacent structures

Pathological N (pN) category	pN criteria
pNx	Regional lymph nodes cannot be assessed
pN0	No regional lymph node metastasis
pN1	Metastasis in a single ipsilateral lymph node, 3 cm or smaller and ENE (-)
pN2a	Metastasis in a single ipsilateral lymph node, 3 cm or smaller and ENE (+) or larger than 3 cm but not larger than 6 cm and ENE (-)
pN2b	Metastasis in multiple ipsilateral lymph nodes, none larger than 6 cm in and ENE (-)
pN2c	Metastasis in bilateral or contralateral lymph nodes, none larger than 6 cm and ENE (-)
pN3a	Metastasis in a single lymph node larger than 6 cm and ENE (-)
pN3b	Metastasis in a single lymph node larger than 6 cm and ENE (+) or multiple ipsilateral, bilateral or contralateral lymph nodes with any ENE (+) or a single contralateral node of any size and ENE (+)

Metastasis (M) category	M criteria
M0	No distant metastasis
M1	Distant metastasis

Figure I 10: Pathological TNM classification of Oral squamous cell carcinoma. Adapted from *AJCC Cancer Staging Manual (8th edition).* Springer International Publishing: American Joint Commission on Cancer; 2017[77]. ENE, extracapsular extension; DOI, depth of invasion; pT, tumor size; pN, cervical metastasis.

Pathological Stage	Stage Criteria		
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T1, T2 T3	N1 N0, N1	M0 M0
Stage IVA	T1, T2 T3 T4	N2 N2 N0, N1, N2	M0 M0 M0
Stage IVB	Any T T4b	N3 Any N	M0 M0
Stage IVC	Any T	Any N	M1

Figure I 11: Tumor Stage classification of Oral squamous cell carcinoma. Adapted from *AJCC Cancer Staging Manual (8th edition).* Springer International Publishing: American Joint Commission on Cancer; 2017[77]ENE, extracapsular extension; DOI, depth of invasion; pT, tumor size; pN, cervical metastasis.

1.1.6. Treatment and outcomes

Treatment is based on the specific site of the disease, the stage, and the histopathologic risk factors. Single-modality therapy with surgery or radiotherapy can be selected for early-stage disease (Stage I and II). However, combined treatment modality with surgery and adjuvant radiotherapy/chemotherapy is the mainstream treatment modality for local or advanced Stages (Stage III and IV)[146]. Systemic

treatment must be individualized based on patient characteristics, and concurrent cisplatin with radiotherapy are preferred to fit patients with locally advanced disease. However, despite the therapeutic strategies and protocols, the OSCC 5-year survival rate is around 60%[151,152].

Surgical treatment of OSCC aims to remove the gross tumor and macroscopic oncological margins of at least 1 cm of healthy tissue with the objective of achieving free margins (>5mm)[150]. The cervical lymphadenectomy or neck dissection of the ipsilateral or bilateral sides will be performed depending on the presence of pathological lymph nodes by the time of the diagnosis, and depending on the initial tumor Stage, being recommended for patients with more than T2 to avoid risk of locoregional recurrence[123]. Selective neck dissection (levels I-III) is performed when there are no pathologic lymph nodes at the time of diagnosis, while a comprehensive neck dissection (levels I-V) is performed when the patient presents with clinically positive lymph nodes[150].

Adjuvant therapy with radiation is indicated depending on the presence of adverse prognostic risk factors such as multiple positive lymph nodes, PNI, LVI, pT3 and pT4 tumors, or positive lymph nodes in level IV. Patient with positive margins or ENE+ benefit from adjuvant concurrent chemotherapy, usually adding cisplatin to the adjuvant radiation therapy[150].

Immunotherapy has been recently proposed as a treatment option for recurrent and metastatic HNSCC[146]. Specifically, the panel of experts in the National Comprehensive Cancer Network (NCCN) guidelines recommends immunotherapy (nivolumab and pembrolizumab) as category 1 preferred options for patients with recurrent or metastatic HNSCC who have progressed despite platinum-based chemotherapy[146]. These drugs target checkpoint inhibitors, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) or programmed cell death protein 1 (PD-1). This treatments act by blocking the immunosuppressive effect of these proteins, which are overexpressed in tumoral cells and tumor microenvironment, and by enhancing T-cell immunity. Multiple clinical trials are studying immunotherapy response in HNSCC compared to current guidelines with promising results[153,154].

The outcomes of OSCC are measured by 5-years overall survival (OS), Disease-specific survival (DSS), Disease-free survival (DFS), and recurrence. The disease can recur locally (Local recurrence, LR), regionally (Regional recurrence, RR), or disseminate to other regions as distant metastasis. The presence of regional metastasis at diagnosis is the most critical prognostic factor and predicts a worse survival. Besides this, different histopathological factors increase the risk of local or regional recurrence and distant metastasis, such as ENE+, DOI, close or positive margins, PNI, LVI, or G[77,144].

Based on the information previously described in this doctoral thesis, it is clear that all these data regarding the etiology and risk factors, the molecular and cellular pathology, and the diagnosis, staging, treatment and outcomes of the OSCC have significantly expanded our previous knowledge in the OSCC field. However, there are still many unknowns to be resolved regarding the molecular characterization

of these tumors, as well as many limitations in terms of the tools available for predicting their prognosis, and, especially, for their treatment. Therefore, **it is necessary to explore the presence, potential dysregulation, and functional role of novel components of critical molecular systems controlling the normal cellular physiology of oral cavity cells, which could serve to identify novel prognostic biomarkers and therapeutic targets to combat these devastating pathologies**. In this sense, the dysregulation of the **somatostatin system** and the **splicing process** have emerged as two potential regulatory events that are share by many cancer types (i.e. intracranial tumors, prostate and liver cancers, etc.)[14,155–162], but the available information in OSCC is very limited and fragmentary.

1.2. Somatostatin System

Somatostatin (SST) is a cyclic neuropeptide isolated from the ovine hypothalamus in 1973[163,164]. SST, also known as somatotropin release-inhibiting factor (SRIF) given its hypophysiotropic functions, is encoded by a single human gene located on chromosome 3q28, which is translated into pre-pro-SST, a 116-amino acid precursor protein that will be processed to produce the two biologically active isoforms: the tetradecapeptide SRIF-14 and the amino- terminally extended octacosapeptide SRIF-28[164].

This peptide is distributed along the central nervous system (CNS) and peripheral tissues (in most but not all organs), and its function depends on the location. Specifically, SST exerts a plethora of physiological actions (Figure I12) but most often performs inhibitory functions on a large number of hormones, including growth hormone, prolactin, thyrotropin, cholecystokinin, gastrin inhibitory peptide, gastrin, motilin, neurotensin, secretin, glucagon, insulin, and pancreatic polypeptide of the pancreatic islets of Langerhans [164–166]. In the CNS, SST is located in the anterior pituitary, the hypothalamus, limbic system, brain stem, and spinal cord[165]. It is believed to modulate, among other functions, cognition and locomotion via control of neurotransmission (Figure I12)[167].

Outside the CNS, SST is located in specific secretory cells of the gut and pancreas, salivary glands, in the renal systems of some species, etc.[165]. SST controls many functions, such as gastrointestinal motility, absorption and growth, inhibition of immunoglobulin synthesis, lymphocyte proliferation in lymphoid tissue[168], cell proliferation, differentiation, and angiogenesis, among others (Figure I12)[164,169,170].

SST isoforms acting as neurohormones, neurotransmitters, and/or neuromodulators are messenger molecules that elicit their function through G protein-coupled receptors (GPCRs), named SST receptors[171]. These receptors consist of a superfamily of signalling proteins that regulate many

biological processes and are well known for their role in cancer tumorigenesis, including the regulation of cell proliferation, invasion, metastasis, migration, adhesion, and angiogenesis (Figure I12)[164].

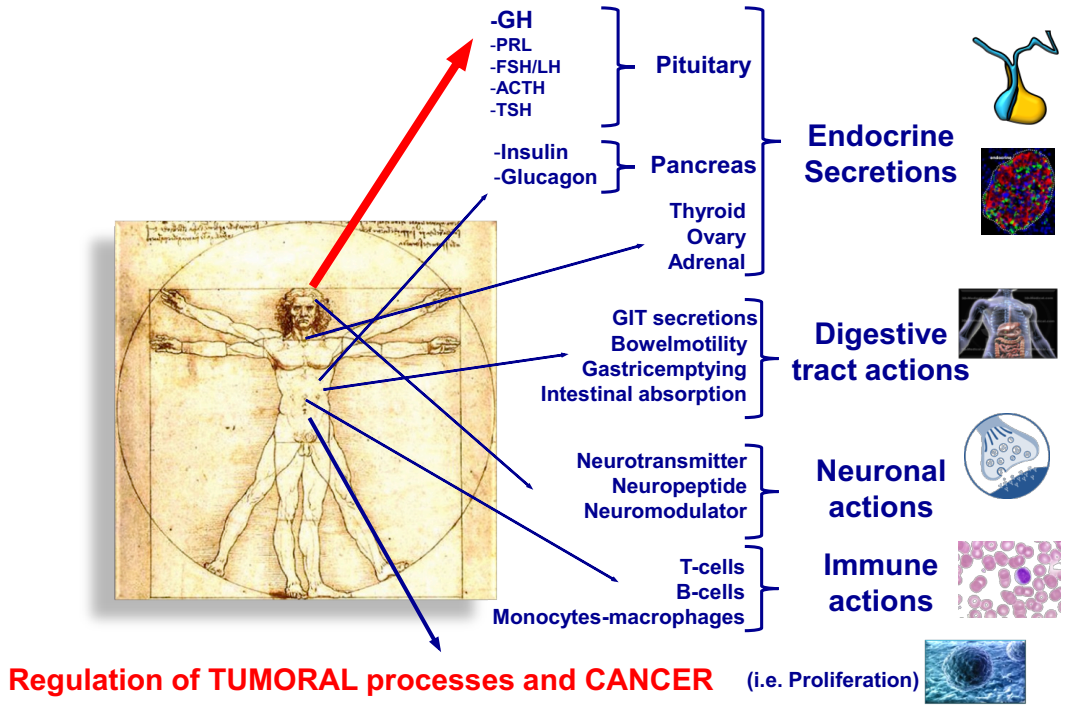


Figure I 12: Multiple physiological actions of SST have been described throughout the body which are mediate through binding to somatostaton receptor subtypes.

1.2.1. SST receptors

SST function is activated through their so-called SST receptors belonging to the family seven transmembrane GPCRs[164]. Specifically, distinct SSTRs (SST_{1-5}) have been cloned, encoded by five genes located at chromosomes 14q13, 17q24, 22q13.1, 20p11.2, and 16p13.3, respectively. Four of these genes lack introns. In addition, SST_2 and SST_5 are spliced to generate different variants named SST_{2A}, SST_{2B}, SST_5TMD4 and SST_5TMD5, and which differ in their C-terminal sequence[172,173].

Most of these receptors have been described that can deliver an antiproliferative signal, either by inhibiting mitogenesis or stimulating apoptosis. They modulate various intracellular signaling pathways such as adenylate cyclase, ion channels (K^+, Ca^{2+}), serine/ threonine, and tyrosine phosphatases, as well as phospholipase A2[164,174]. SST_1, SST_2, SST_4 and SST_5 induce G1 cell cycle growth arrest, while SST_3 is pro-apoptotic via the induction of p53 and BAX. SST_2, SST_3, and SST_5 are also antiangiogenic[169,175].

They all bind SST with higher affinity to SRIF -14 than SRIF-28 and show different affinity to SST analogs (or SSAs)[164]. The original classification of receptor subtypes was first based on their cloning discovery chronology. They were later subdivided into two major distinct subfamilies regarding their ability or inability to bind octapeptide SST analogs. One family comprises human SST_2, SST_3, and SST_5 receptors (initially referred to as SRIF 1 receptors), and the other is composed of SST_1 and SST_4 receptors (represented by SRIF 2 receptors)[164,175–177]. Compared to SST-14, analogs exhibit a low affinity for SST_1 and SST_4, whereas they bind SST_2 and SST_5 with a high affinity and bind SST_3 with moderate affinity[172].

Apart from the different SSAs affinity, these two families also share a structural resemblance. The amino acid sequences of human SST_1 and SST_4 receptors are 58% identical and 78% similar, while the identity of human SST_4 receptors with the remaining subtypes is around 40%. By contrast, human SST_5 receptors display higher amino acid sequence homology with SST_2 (52%) and SST_3 receptors (53%) than with SST_1 receptors (45%), consistent with the pharmacological properties ascribed to the respective receptor subtypes[164].

SSTs are widely distributed in normal and tumor tissues with distinct but overlapping expression patterns in human tissues[169]. They have shown to be present in several human cancer cells, including neuroendocrine, gastro-entero-pancreatic, brain, prostate, lung, and breast tumors. More than one subtype has been detected in each tumor histotype, being the SST_2 the most frequently expressed subtype and, thus, the most crucial therapy target[169,170,178–180]. Furthermore, SSTs also play a useful role in tumor imaging (SST-scintigraphy or octreotide scan)[181,182].

1.2.2. SST and oral cavity cancer

SST system (ligands and receptors) have been identified as tumor suppressor genes that exert potent antitumor and anti-secretory activities in several human cancers *in vitro* and *in vivo*. Interestingly, neuroendocrine differentiation has been found in some tumors not considered to be of neuroendocrine origin, including SCC of the lung and esophagus[9,183] and more recently, larynx[11,184–186], nasopharynx[187], and head and neck[12].

Previous studies have analyzed the SSTs profile in the HNSCC. Codon and Stafford studied the SSTs expression in benign, pre-malignant, and malignant lesions in the larynx and observed an increase in SST_5 and a loss of SST_2 in malignant samples. They also observed that SST_3 was scarce expressed in all samples[11,186]. On the other hand, Schartinger and coworkers studied SSTs expression in tumor samples of the head and neck area that included OSCC samples, and compared them to healthy oropharyngeal mucosa specimens (obtained during uvulopalatopharyngoplasty) from other patients.

They observed an increase in SST_1, SST_2, SST_4, and SST_5 with a low expression of SST_3[12]. In 2015, Misawa and coworkers published a paper about the methylation status of SST and SST_1 being correlated with reduced disease-free survival in patients with head and neck squamous cell carcinoma. Then, in 2018, they highlighted the potential role of SST as a biomarker for patients with laryngeal cancer with a low risk of relapse. They found that SST promoter was methylated in patients with laryngeal cancers with an OR of 0.080 (95% CI 0.018–0.349), while in the oral cavity, their results showed an OR of 1.7[184,185].

An upregulation of the SST_2 has also been recently associated with nasopharyngeal carcinoma (NPC) with improved survival in NPC patients with tumors positive for SST_2[187]. NPC is highly associated with Epstein-Barr virus (EBV) and this study proved that SST_2 is induced by EBV. Lechner and coworkers performed a whole-body ^{68}Ga-DOTATATE and ^{18}F-FDG PET/CT imaging on NPC patients and they found that SST_2 expression levels were also associated with *in vivo* uptake of ^{68}Ga-DOTA- peptides suggesting the potential use of this imaging to monitor SST_2 expression or as a target for SST_2 receptor-targeted radionuclide therapy (Lutetium-177, Ytrium-90). However, to data no study has evaluated the pattern of expression of SSTs in oral cavity SCC exclusively nor analyzes the relationship of SSTs expression with typical clinical and histopathologic prognostic factors of the OSCC.

1.2.3. Somatostatin Analogues

Somatostatin Analogues (SSAs) are synthetic compounds developed to treat several neoplasms in order to control abnormal hormonal secretion, reduce tumor volume, etc., including tumors secreting growth hormone and thyroid stimulating hormone[188,189]. The antiproliferative and antitumoral activity of SSAs can be direct or indirect. The direct function acts through activation of somatostatin receptors on tumor cells, leading to modulation of intracellular signaling transduction pathways[180]. The indirect function is independent of SST receptors and includes inhibiting angiogenesis, inhibiting growth factors and hormone synthesis, and immunomodulatory effects among others[190].

Natural SST peptides have poor clinical use because of their short half-life of approximately 2 min due to rapid proteolytic degradation in plasma[191]. Therefore, the pharmacokinetic profile of SSTs was improved with the development of synthetic SSAs with a shorter polypeptide chain. The first generation of short SSAs was octreotide (SMS- 201-995) and lanreotide (BIM-23014). These two SSAs are the current treatment for many tumors and are known for being preferential single receptor analogs, which means they mainly target a single SST receptor, mostly SST_2. Octreotide and lanreotide responses depend directly on the presence of the SST_2 receptor, which, if diminished, will result in no treatment response[191]. In this line, SST_2 analogues have shown to be effective at controlling for instance growth in neuroendocrine tumors[191]. However, some studies highlighted the incidence of resistant or poorly

responsive patients to these single-receptor analogs, which drifted into the search for a new treatment modality[180].

As previously explained, SST receptors have overlapping expression patterns in human tissues, share a structural resemblance, and undergo heterodimerization with each other and with other receptor families. This raised the necessity of developing a second generation of SSAs[180] based on the assumption that SSAs with multireceptor binding affinity would have a more potent antiproliferative effect[190]. Specifically, pasireotide, also known as SOM230, is a second-generation SSA metabolically stable with a plasma half-life in humans of approximately 12 hours. It binds with high affinity to $SST_{1,2,3 \text{ and } 5}$, which means all receptors but SST_4. Compared with octreotide, pasireotide has a 40-, 30-, and 5-fold higher binding affinity for SST_5, SST_1, and SST_3, respectively, and a lower affinity for SST_2[190]. Some studies using pasireotide have showed that it also works differently than octreotide and lanreotide, by producing rapid recycling of SST_2 to the plasma membrane after endocytosis and a rapid down-regulation of SST_3 after long-term exposure. Rapid down-regulation of GPCRs has been associated with the development of treatment tolerance. If SST_3 is rapidly down-regulated, it seems to be a less favorable pharmacological target for long-term administration of SSAs[191].

The only study testing SSAs in head and neck cancer is the one by Lechner et al. in nasopharyngeal cancer. They observed an upregulation of SST_2 and tested the impact of different SSAs (e.g. lanreotide and octreotide) on *in vitro* proliferation/survival of some NPC cell lines. Specifically, they found that lanreotide and octreotide did not affect SST_2 expression with no changes in cell death/apoptotic pathways. In contrast, 72-h treatment with PEN-221 (a *peptide ligand that is highly selective in targeting* SST_2 through the toxin DM1 ligation) led to significant downregulation of SST_2 expression, as well as upregulation of pathways related to apoptotic signaling and mitotic spindle formation dysregulation.

1.3. Splicing Process

1.3.1. Splicing process/Alternative splicing/ splicing factors

Eukaryotic genes are transcribed as pre-mRNAs which are composed of two distinct elements named exons and introns. The exons represent protein-coding expressed sequences scattered throughout the gene in between noncoding-protein intervening sequences, called introns. Both exons and introns are transcribed in the rising mRNA transcript and, co-transcriptionally, during messenger RNA (mRNA) maturation. Maturation consists of three steps that happen simultaneously: capping, addition of a poly-A tail, and **splicing**[192,193].

Splicing is responsible for efficiently removing the introns from the mRNA and joining the remaining exons together. The fact that most human genes contain multiple introns makes splicing a crucial step in gene expression[192,194]. The splicing process of a pre-mRNA is a complex mechanism in which many different elements are involved. Mistakes in defining introns and exons or their boundaries allow alternative patterns of intron removal, generating a diversity of transcripts[192]. In fact, the splicing process requires the formation of the splicing complexes or "**spliceosomes**" which are two ribonucleoproteic complexes: the major and minor spliceosomes, which act in different types of introns but share mechanisms of action[192,194,195] (figure I13). The biological significance of these spliceosome components relies on the detection of certain recognition sequences that are essential for the successful completion of the splicing process.

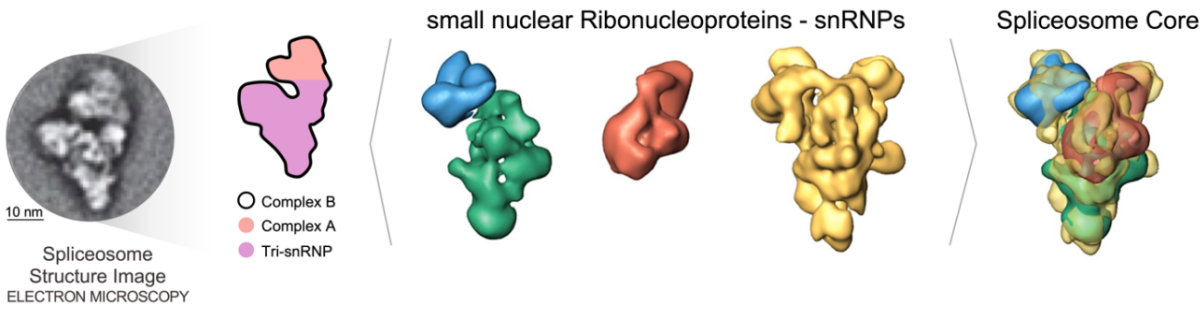


Figure I 13: Spliceosome structure. This caption displays an electron microscopy image (left) of negatively stained, affinity-purified human spliceosome core complex B[196]. Schematic representation of spliceosome core complex B (formed by Complex A and tri-snRNPs) together with 3D reconstruction of tri-snRNP complex (RNU4+RNU6+RNU5) and its folding organization[197].

The major spliceosome is the molecular machinery that catalyzes the splicing process of almost 99% of the rising mRNA. Its functional core comprises five small nuclear ribonucleoprotein particles (snRNPs): U1, U2, U4/U6, and U5. The minor spliceosome is composed of U11, U12, U4atac/U6atac, and U5. The spliceosomes are accompanied by more than 150 additional proteins called "*splicing factors and regulatory proteins*."[195]. The spliceosome catalyzes two sequential transesterification reactions, which involve a first nucleophilic attack at the 5′ splice site by the 2′ hydroxyl of conserved adenosine within the intron. This reaction generates a free 5′ exon and a cyclic (lariat) intermediate containing a 2′–5′ phosphodiester branch. Then, the free 5′ exon attacks at the 3′ splice site resulting in ligated exons and the lariat intron product (figure I14)[198].

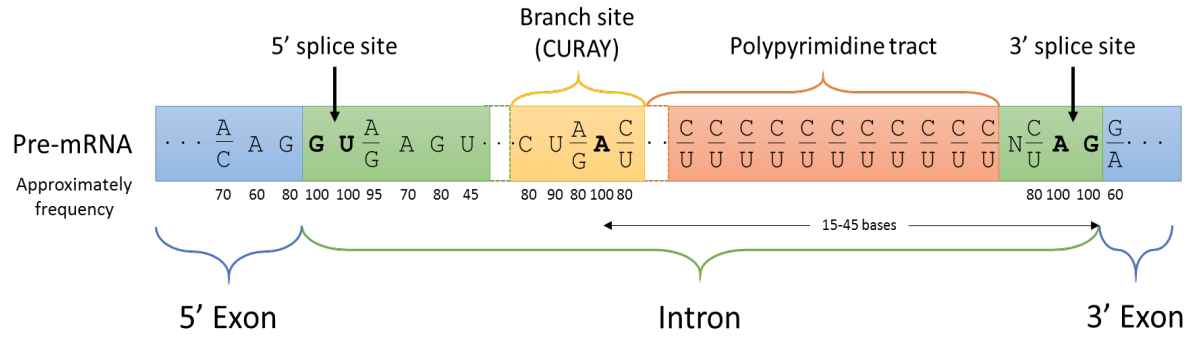


Figure I 14: Graphic scheme of splicing consensus sequences in the intron.

Alternative splicing (AS) is a post-transcriptional process. Different exons are included in mRNA after different pairs of splice sites and are selected in a pre-mRNA transcript resulting in different isoforms of protein and mRNA from one gene[55,195,199]. In fact, according to several studies, a high percentage of human genes (80-90%) are alternatively spliced[199], experiencing extensive opportunities for gene regulation and cell functioning, including cell growth, apoptosis, cell differentiation, and disease[195,200]. How AS happens is explained by four main processes: ‘‘cassette’’ exon skipping, alternative 5′ and 3′ splice site selection, alternative retained introns, and mutually exclusive exons[195].

The basic principle in splicing correctly recognizes introns and exons by the splicing machinery (spliceosome)[199]. The correct selection of splice sites in pre-mRNA is mediated partially by *cis*-acting RNA sequences that collectively comprise the ‘‘splicing code’’[195]. This code uses intronic dinucleotides GU and AG (for the major spliceosome) and GT–AG sites (for the minor spliceosome) at the 5′ and 3′ splice sites[57]. Nearly all introns belong to the U2-type, spliced by the major spliceosome, while the minor spliceosome is responsible for processing the rest of the introns or U12-type introns[195,201] Moreover, the spliceosome is a macromolecular complex whose functional core comprises several small nuclear ribonucleoproteins (snRNPs) subunits that regulate the splicing process. Its function is modulated by N300 splicing factors (SFs) that recognize specific sequences in exons and introns (figure I15)[202].

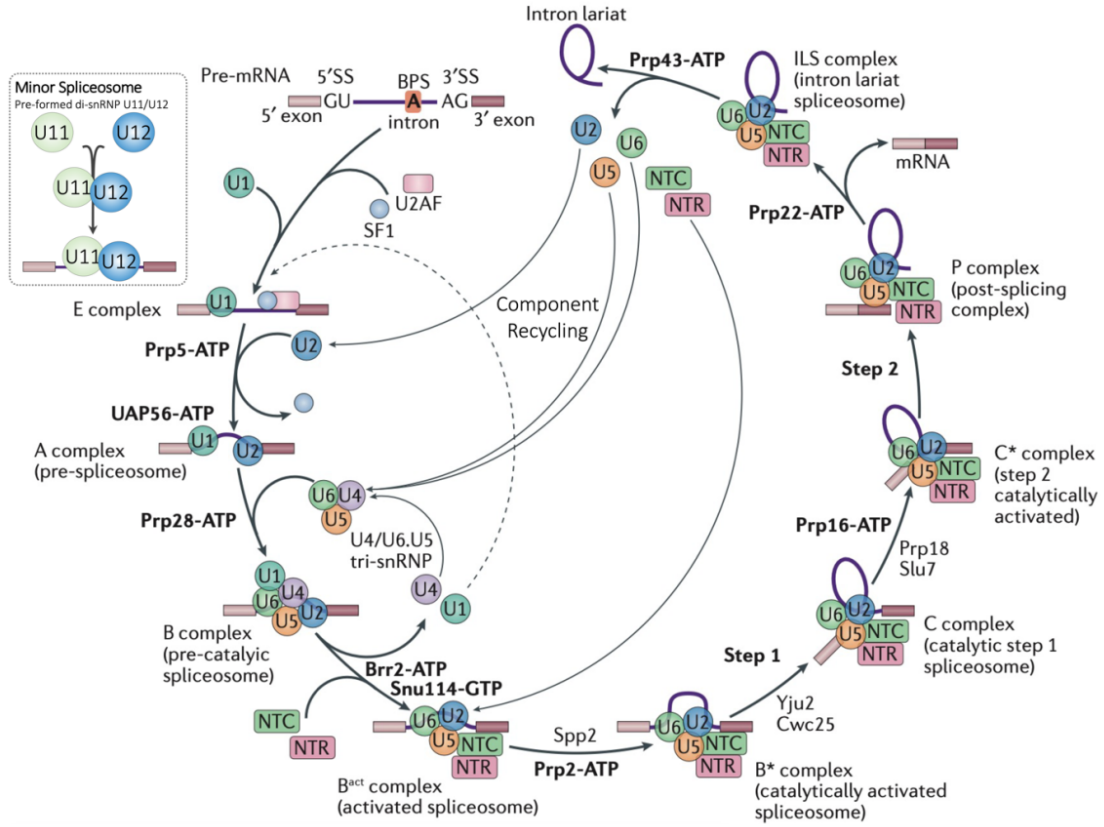


Figure I 15: Spliceosome cycle dynamics and catalytics reactions. Diagram showing the coordination and interactions of different spliceosome components in order to constitute the different complexes across the pre-mRNA splicing process. Several splicing factors play an important role in this process to finally produce a mature mRNA and intron lariat. All components are recycled at the end to process the next pre-mRNA. In the upper left box, a scheme is represented with the main difference between major and minor spliceosome, the pre-formed di-snRNP (RNU11+RNU12). Adapted from Shi 2017[203] **The RNUs nomenclature is replaced by Us in this figure (i.e., RNU1 is written as U1).*

The spliceosome must be guided to the correct splice sites. Which sequence of pre-mRNA is included or excised is decided by the RNA-binding proteins (RBP). These proteins, known as splicing factors (SF), bind to RNA with specificity to tissue and, therefore, control AS[204]. SFs are RNA-binding proteins that modulate the splicing process interacting with specific RNA sequences or motifs[205]. SFs are divided into four categories depending on the position and function of the *cis*-regulatory elements. They can act as enhancers or silencers. They are known as exonic splicing enhancers (ESEs) or silencers (ESSs) if, from an exon location, they function to promote or inhibit the inclusion of the exon they reside. They are known as intronic splicing enhancers (ISEs) or silencers (ISSs) if they promote or inhibit the use of adjacent splice sites or exons from an intron location[195,200]. They recruit trans-acting factors that activate or suppress splice site recognition or block the spliceosome components.

Several RBPs that function to control AS have been well studied. There are two major families of these trans-acting factors controlling splice site recognition and are known as the SR proteins and the heterogeneous ribonucleoproteins (hnRNPs)[195,205]. SRs are usually bound to ESEs and recruit the spliceosome components, while hnRNPs are linked to ISSs and ESSs, blocking the splicing process. Their activity is regulated by reversible phosphorylation, mediated by protein kinases like SRPK and CLK families, and kinases activated in different signaling pathways, such as MAPK, PI3K, AKT. Phosphorylation of SFs affects their binding to targeted transcripts, interactions with other proteins, and intracellular localization[206]. They both interact by competing with the binding sites or by altering the structure of the pre-mRNA, making the assembly of the spliceosome inaccessible[200,207]

Apart from the *trans*-acting factors, AS is also regulated at the tissue level, and tissue-specific RNA-binding splicing regulators cooperate to modulate protein-protein interaction networks. Among them, there are factors that involve the neural-specific factors NOVA[207] (NOVA1-2), PTBP2/nPTB/brPTB, and nSR100/SRRM4, as well as factors such as FOX1, FOX2 (also known as RBM9), RBM35a (also known as ESRP1), RBM35b (also known as ESRP2), MBNL, CELF, ETR, TIA, and STAR family proteins[195,200,207].

1.3.2. Splicing and cancer

Alternative splicing is an essential process for proteomic diversity, which is critical to controlling cellular and organ functions and regulating gene expression levels and tissue specificity[192,205]. AS has shown that it plays an essential part in defining tissue specificity[205]. According to Chen, each cell type has a unique repertoire of SR proteins and hnRNPs. A slight change in the architecture can significantly affect the pattern of AS, leading to alternative splicing misregulation. Because of this, if AS is disrupted, it can compromise the standard physiology of eukaryotic cells and promote cancer initiation by affecting proliferation, apoptosis, invasion, metastasis, etc. of many cancer types (Figure I16)[55,205].

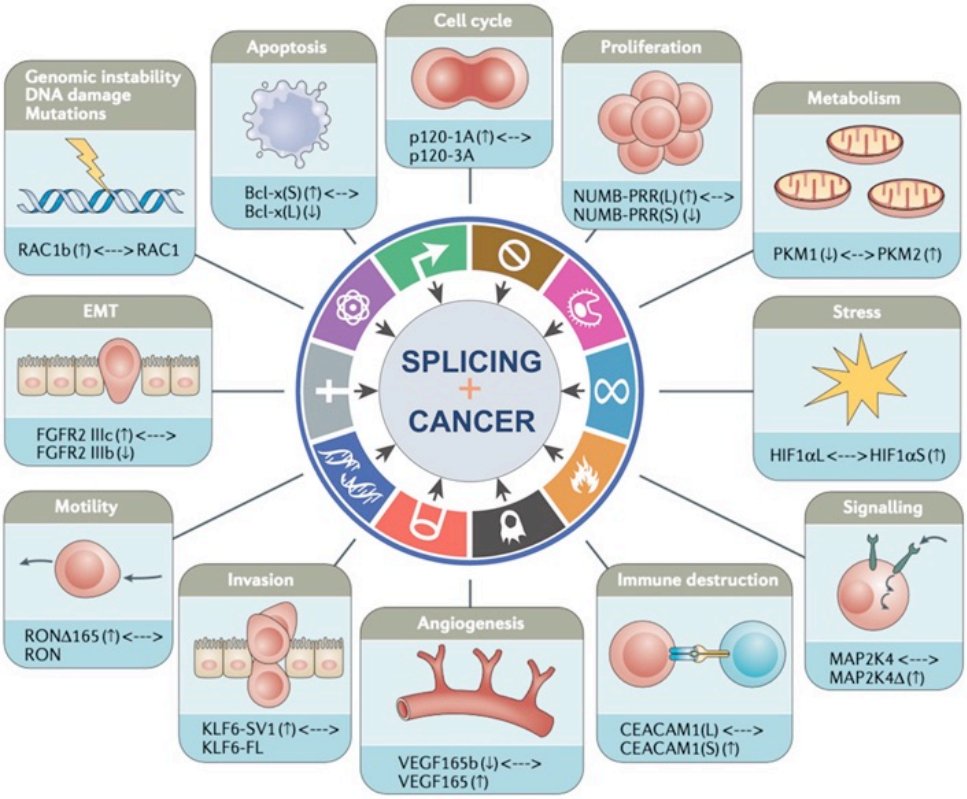


Figure I 16: Alternative splicing dysregulation in cancer. Different hallmarks of cancer are shown here together with alternative splicing dysregulation, which contributes to promoting different processes of tumorigenesis. Arrows up/down indicate a positive/negative association of the splicing variant with each tumorigenic process. Adapted from Bonnal et al.[208] and Hanahan et al.[209].

The alteration in splicing machinery can be caused by mutation of some components or alteration in SFs levels. Mutation in different SFs has been described, especially in hematopoietic cancer with mutation of SF3B1, SRSF2, and ZRSR2. RBM10 mutation has been described in solid tumors like lung adenocarcinoma, non-anaplastic thyroid cancer, colorectal or pancreatic carcinoma, and papillary mucinous neoplasms. SF3B1 has been related to breast tumors[210,211]. These mutations generally impair the recognition of regulatory sites, thereby affecting the splicing of multiple genes, including oncogenes and tumor suppressors[205,210,212].

Apart from these mutations, changes in the relative concentration of SFs can also trigger oncogenic processes[207,211]In solid tumors, SFs exhibit frequent changes at the copy number or expression levels but are rarely mutated[211]. Several potential prognostic biomarkers or therapeutic targets in HNSCC, such as SNPs, copy number variations (CNVs), methylation, lncRNAs, miRNAs, and mRNAs have been identified by high-throughput genomic and transcriptomic sequencing[56]. Systematic dysregulation of AS has recently emerged as an important cancer hallmark, with a great potential to serve as a source of novel diagnostic, prognostic or therapeutic tool. Therefore, in recent years, many studies have focused on the analysis and impact of SFs or AS events in OSCC or HNSCC[56,57,213–229].

An exhaustive bibliographic search revealed reported changes in the expression of approximately multiple spliceosome components and splicing factors in OSCC tissues (***Table I1***). Most of the changes reported relates to the upregulation or downregulation of certain spliceosomal elements, and often correlates with lower patient survival, tumor aggressiveness parameters and worse prognosis[56,218–222,230–240].

Table I 1: List of spliceosome components and splicing factors reported to be present in OSCC.

Splicing Factor	***Dysregulation*** *Normal tissue vs. tumor*	***Effects in OSCC/HNSCC OS or prognosis***	***References***
SRSF3	Upregulated	• Positive relationship between SRSF3 expression and tumor grading. • A significantly higher expression of the SR in patients with lymphatic metastasis	*Peiqi et al. 2016*
	Downregulated	• Better overall survival rates.	*Guo et al. 2019*
SRSF5	Upregulated	• Downregulation of SRSF5 in oral squamous cell lines retarded cell growth, cell cycle progression, and tumor growth.	*Yang et a. 2018*
SRSF9	Upregulated	• SRSF9 overexpression seemed to be a Hazard factor. No relationship with OS, DFS, Clinical Stage or Tumor grading.	*Liu et al. 2022*
	Unspecified	• Higher expression associated with poor prognosis	*Cao et al. 2020*
SRSF10	Upregulated	• Overexpression of SRSF10 was closely associated with poor survival.	*Yadav et al.2021*
hnRNP A1	Upregulated	• hnRNPA1 is required for the growth of OSCC cells. Overexpression of hnRNP A1 may be an early pathogenic event that could be used as a new biomarker for OSCC	*Yu et al. 2015*
hnRNP C	Unspecified	• Higher expression was correlated with poor outcomes	*Xing et al. 2019*
	Unspecified	• Higher expression associated with poor prognosis	*Cao et al. 2020*
hnRNP D	Upregulated	• Overexpression is associated with significantly reduced recurrence-free survival.	*Kumar et al. 2015*
hnRNP E2	Downregulated	• low-hnRNP E2 expression level was correlated with the histological grade of differentiation.	*Roychoudhury et al.2007*
hnRNP H1	Unspecified	• Higher expression was correlated with poor outcomes	*Xing et al. 2019*
hnRNP H2	Unspecified	• Higher expression was correlated with poor outcomes	*Xing et al. 2019*
hnRNP K	Upregulated	• High levels of hnRNP K were correlated with worse OS, DSS, and DFS and multiple clinicopathological factors with a poor prognosis like advanced tumor stage, positive node stage, advanced overall stages, extracapsular spread, and large tumor depths	*Matta et al. 2009, Wu et al. 2012*
	Unspecified	• Higher expression was correlated with poor outcomes	*Xing et al.*
	Upregulated	• Significant correlation between histological grades of differentiation and hnRNP K mRNA expression could not be predicted	*Roychoudhury et al.2007*
	Unspecified	• Higher expression associated with poor prognosis	*Cao et al. 2020*

hnRNP L	Upregulated	• Expression promotes the proliferation, invasion and metastasis of OSCC.	*Jia et al. 2016*
ESRP1 ***ESRP2***	Downregulated	• The expression levels of both ESRP1 and ESRP2 were low in normal epithelium but up-regulated in precancerous lesions and carcinoma *in situ*. Expression was maintained in advanced cancer cells but down- regulated in invasive fronts	*Ishii et al. 2014*
RBM3	Downregulated	• N/A	*Martinez et al. 2007*
NOVA1	Downregulated	• HNSCCa HPV-negative. The lower expression was an independent poor prognosis factor for OS and PFS and related to older age, advanced pT stage, and advanced pN.	*Kim et al. 2019*
	Upregulated	• HPV- positive oropharyngeal squamous cell carcinoma (SCC).	
TIA1A	Unspecified	• Higher expression associated with better prognosis	*Cao et al. 2020*
	Unspecified	• Higher expression was correlated with poor outcomes	*Xing et al. 2019*
TRA2B	Upregulated	• N/A	*Best et al. 2013*
CELF2	Unspecified	• Higher expression associated with better prognosis	*Cao et al. 2020*

SFs have an impact on anticancer drugs by modulating apoptosis and enabling sensitization of cancer cells to therapeutic treatments. They can act either as prosurvival factors that diminish drug-induced apoptosis or, oppositely, that potentiate pro-apoptotic effects of chemotherapeutics. The expression of SRSF1, for example, is downregulated after treatment with VX-680 in cervical cancer. The loss of SRSF1 expression sensitizes cancer cells to treatment induced apoptosis[206,241]. The hnRNP K splicing factor also interferes with treatment response in hepatocellular carcinoma by keeping high levels of genes that prevent the apoptosis in cells. 5´FU leads to downregulation of hnRNP K with the result of induction of apoptosis. Other splicing factors like SRSF2 have the opposite effect and potentiate pro-apoptotic effects of chemotherapeutics[206]. Furthermore, in some cancers the expression of some SFs is often indispensable for drug-induced apoptosis. This is the case of Cisplatin and SRSF4 in breast cancer, or PLX4720 (an inhibitor of mutated BRAF) in melanoma[242,243]. In head and neck cancer, SRSF3 has been shown that it can reduce the sensitivity of cancer cells to Paclitaxel (PTX) treatment. Specifically, PTX treatment significantly decreased SRSF3 expression and could inhibit cancer cell growth partially through the downregulation of SRSF3 expression[219]. Other SFs have also been associated to PTX such as TRA2A in breast cancer which promotes PTX resistance in breast cancer[219].

1.3.3. SR proteins & SR proteins in oral cavity cancer

SR proteins (serine/arginine-rich proteins) are a family of 12 members (SRSF1-12) that are nuclear factors involved in many steps of splicing regulation. They function as messenger-RNA-binding proteins and alternative SFs. SRs contain one or two RNA recognition motif(s) (RRM), and a protein-interaction arginine-serine rich (RS) domain[207,218]. As previously mentioned, SRs are most commonly

bound to ESEs mediated by the RS domain. However, they also cooperate with other positive regulatory factors to form larger splicing enhancing complexes by interacting with other RS domain-containing proteins, such as transformer 2 (TRA2), the SR-related nuclear matrix proteins SRm160 (also known as SRRM1), and SRm300 (also known as SRRM2)[207].

The main SR proteins that exhibit expression changes in tumors are SRSF1, SRSF2, SRSF3, SRSF5, SRSF6, and SRSF10[211]. In the last years, various studies have focused on the role of some of these SRs in OSCC. Specifically, SRSF1 (named SF2/ASF) is frequently upregulated in breast, lung, colon, and bladder tumors. It usually acts as a proto-oncogene that controls alternative splicing. It can associate with MYC[211,244], resulting in higher tumor grade and decreased survival in breast and lung cancer patients[245]. SRSF1 has also shown cisplatin and topotecan resistances when upregulated[211]. To date, it has not been established a relationship of SRSF1 to OSCC. Mutation in SRSF2 (alias SC35) is frequently observed in hematopoietic tumors and has also been described in ovary tumors[246], but up to now, it has no proven relationship to OSCC.

SRSF3 (alias SRp20) regulates the splicing process and involves multiple cellular processes, such as transcription termination. It usually acts as a proto-oncogene and is upregulated in oral tumors and lung, breast, ovarian, stomach, bladder, colon, and liver[211,218]. In a study by Peiqi and coworkers, higher levels of SRSF3, measured by immunohistochemistry, were observed in OSCC and moderate/severe dysplasia compared to normal tissues. They also reported a positive relationship of SRSF3 expression with high-grade cancer and lymphatic metastasis[218]. On the other hand, other studies have observed a lower expression of SRSF3 in head and neck cancer patients that were related to significantly better overall survival rates and disease-/progression-free survival rates. Based on these observations, SRSF3 was suggested as a valuable biomarker for the prediction of prognosis of patients with OSCC[219]. However, further studies are necessary to specifically explore the role of SRSF3 in OSCC due to the different controversial results of some of these studies.

SRSF5 (alias SRp40) upregulation has been associated with breast tumors with lymph node metastasis, oral tumors[217,220], breast, and lung cancer[211]. The relationship between SRSF5 and OSCC was studied by Yang et al., who focused their research on *in vivo* and *in vitro* SRSF5 expression. The expression levels of SRSF5 in human OSCC tissues were evaluated by immunohistochemistry. They found that OSCC tissues had significantly stronger staining of SRSF5 than normal control tissues suggesting that SRSF5 might have an oncogenic role. In fact, down-regulation of SRSF5 in oral squamous cell lines retarded cell growth, cell cycle progression, and tumor growth. Moreover, the expression of SRSF5 was positively correlated with SRSF3 in western blot assays, which confirmed that SRSF3 up-regulates the expression of SRSF5 by impairing its autoregulation in OSCC[220].

SRSF6 (named SRp55) is frequently up-regulated in breast, lung, pancreatic, colon cancers, and multiple skin subtypes of skin cancer. Its overexpression synergizes with MYC to promote the transformation of lung epithelial cell lines[211]; however, no relationship to OSCC has yet been defined. In addition, SRSF9 (named SRp30c) acts as an oncogene and it has been described as an unfavorable factor in cancers like bladder, cervical, and colorectal. Its effect on tumorigenesis has been related to its impact on diverse biological processes, such as tumor cell proliferation, apoptosis, migration, and invasion[235,247]. Liu et al. studied the expression of SRSF9 in pan-cancer samples. They observed an overexpression of SRSF9 in tumor samples compared to normal tissue in the majority of tumors, including the head and neck. Moreover, the overexpression of SRSF9 in head and neck seemed to be a hazard radio. However, no adverse prognostic role was found in head and neck, and no relationship with clinical stage or tumor grade was found. Significantly, Liu et al. also described that SRSF9 positively correlates with most immune checkpoints genes in most cancers which suggests that SRSF9 could be an immunotherapeutic and prognostic target[230].

SRSF10 (named SRrp40) regulates alternative splicing and is an atypical member of the SR protein family with a domain organization similar to SR proteins. SRSF10 plays an important role in the AS process by regulating the exon inclusion both positively and negatively, which depends on its binding at pre-mRNA relative to an alternative exon. It presence and potential role has been reported in colorectal cancer, cervical cancer and head and neck[231,248]. Moreover, Yadav et al. described the overexpression of SRSF10 in head and neck cancers, and a negative correlation of SRSF10 expression level with survival[231].

1.3.4. hnRNPs & hnRNPs in oral cavity cancer.

The hnRNP is a large and diverse family of RBPs involved in controlling splice site recognition, mRNA transport, and translation[211] There are thirty types of hnRNPs in human cells, (A–U), being the six core proteins known as A1, A2, B1, C1, B2, and C2, the most expressed[221]. As previously mentioned, hnRNP usually bound ISSs and ESSs, blocking the splicing process. They often work as antagonists to SR-protein-regulated alternative splicing events. Importantly, the upregulation and downregulation of some hnRNP proteins have been associated with cancer development. The most common types of hnRNP showing a relationship with cancer processes are the following: hnRNA A/B family and hnRNA K.

HnRNP A1, named PTB (polypyrimidine tract binding protein), is an hnRNP protein that regulates AS and translation. It can work either as an oncogene or as a tumor suppressor. It has proved to be overexpressed in Burkitt lymphoma, multiple myeloma, leukemia, lung, colorectal malignancies, and oral cancer[211,221]. Interestingly, HnRNP A1 upregulation in lung adenocarcinoma has been associated with increased tumor staging[211]. The role of hnRNP A1 in OSCC was studied by Yu and coworkers,

who found that hnRNP A1 was overexpressed in OSCC tissue, and it was also required for the growth of OSCC cells. The hnRNP A1 expression was independent of OSCC grade, suggesting that overexpression of hnRNP A1 may be an early pathogenic event that could be used as a new biomarker for OSCC[221].

HnRNP A2, B1 is a splicing regulator closely related to hnRNP A1, frequently overexpressed in lung, breast, colorectal, and brain tumors. Its high expression is correlated with microsatellite instability, increased tumor stage, and decreased overall survival[211]. The hnRNP B1 protein has been detected in OSCC and proposed as a useful marker for early detection of OSCC. However, no reports have described an association between the dysregulation of hnRNP A2/B1 and the pathophysiology of OSCC.

HnRNP C, heterogeneous nuclear ribonucleoprotein C1/C2, is a commonly expressed RNA-binding protein with cancer-promoting function. It has been associated with glioblastoma and breast cancer. In OSCC, hnRNP C overexpression has been described and associated with a worse poor survival[56,235]. Similarly, HnRNP D (an RBS protein) has been defined as an oncogene. This factor is associated with the dysregulation of many genes involved in the cell cycle, proliferation, and survival. For instance, it affects genes like c-myc, c-jun, c-fos, and tumor necrosis factor-α (TNF-α), which promote tumorigenesis. HnRNP D is another hnRNP that has been associated with OSCC[232]. Specifically, Kumar et al. reported overexpression of hnRNP D in human oral premalignant lesions by proteomic analysis with increased score in immunohistochemistry of hnRNP D protein among higher grade of dysplasia, tumor stage and tumor size. However, no relationship was found in nodal statues. They reported a significant reduced recurrence-free survival among patients with increased hnRNP D expression compared to those with low or no expression. Moreover, adiitional results showed that hnRNP D expression was a predictor for recurrent disease in patients with negative nodal status (pN-), but had no significant association with recurrence among patients with positive nodes (pN+)[232].

HnRNP E2 is one of the Poly(rC)-binding protein (PCBP) family of hnRNP. It mediates translation inhibition of the CCAAT/ enhancer-binding protein a (CEPBa) which inhibits granulocyte differentiation. Besides, along with hnRNP K, C, and E1, hnRNP E2 mediates translation of c-myc mRNA. The analysis of hnRNP E2 in oral cancer showed a downregulation of its mRNA expression that was correlated with histological grades of differentiation. The expression decreased in moderate to poorly differentiated SCC compared to well differentiated OSCC[233].

HnRNP K is a splicing factor that can act as a tumor suppressor and as an oncogene. Downregulation of hnRNP K has been observed to occur in AML and renal tumors, whether upregulation is present in breast, colorectal, and pancreatic[211]. HnRNP K up-regulates multiple downstream genes, including eIF4E and c-Myc, through transcriptional and post-transcriptional regulation, increasing cell

proliferation, anti-apoptosis, and metastasis[222,234]. Given its function, it has been proposed as a potential target for metastasis therapy. Moreover, the HnRNP K relationship with OSCC has been identified since an upregulation of hnRNP K mRNA in cancer compared with healthy oral tissue was observed[222,233,234]. Some studies have found overexpression of cytoplasm and nuclear hnRNP in OSCC and premalignant lesions[222,234]. Specifically, it was observed that nuclear hnRNP K expression increases from oral normal tissues to leukoplakia and frank malignancy which can serve as a diagnostic marker. Besides, high levels of hnRNP K were correlated with poor OS, DSS, and DFS of OSCC patients[234]. That overexpression was also related to multiple clinicopathological factors with a poor prognosis such us advanced tumor stage, positive node stage, advanced overall stages, extracapsular spread, and large tumor depths[234].

Finally, HnRNP L is a is a multifunctional splicing factor that is involved in nearly every step in mRNA expression and biogenesis[236,249]. It has been linked to several tumors including OSCC, esophagus, lung and breast. The expression of hnRNP L in OSCC has been described to be overexpressed in tumor samples compared to normal tissue [236,249]. Besides, its expression promotes the proliferation, invasion and metastasis of OSCC secondary to its impact on G2/M cell cycle progression and tumorigenesis *in vivo*[236]However, no study with histopathological or survival data have been published.

1.3.5. Other RNA-binding proteins in oral cavity cancer

- Epithelial-specific splicing factors ESRP1 and ESRP2: ESRP1 (named RBM35a) and ESRP2 (nameds RBM35b) belong to the RBM (RNA-binding motif) family of RNA- binding proteins. ESRP1, ESRP2, and the RNA-binding protein FOX2 homolog, RBFOX2, affect the splicing of genes involved in epithelial-mesenchymal transition (EMT), which is mainly characterized by changes in cell morphology, attenuation of cell-cell interaction, and loss of cell polarity, often leading to increased cell motility[237,250]. ESRP1and ESRP2 are tissue-specific splicing factors that contribute to the definition of the distinguishing characteristics of epithelial cells[207]. EMT is regulated by AS of some genes like CD44, which works in cell division, survival, and adhesion. In fact, the role of ESRPs in tumorigenesis is controversial because they have been classified as tumor suppressor factors and oncogenes. ESRP1/2 are often upregulated in the normal epithelium but downregulated in invasive fronts[211,237] In OSCC, the expression of ESRP1 and ESRP2 is down-regulated the normal epithelium but up-regulated in dysplasia, carcinoma *in situ,* and maintained in advanced cancer cells. However, ESRPs were down-regulated in invasive fronts that penetrated through the basement membrane into the stroma and those invading from cancer nests into stromal tissues. A knockdown of ESRP1 and ESRP2 showed increased cell motility in head and neck cancer cell lines that express these proteins through Rac1b and E-cadherin repression. All of this suggests that down-regulation of ESRP1 and ESRP2 is restricted to cells that acquire increased cell motility during cancer invasion[237].

- RBM proteins: Other types of RBM (RNA- binding motif proteins) are involved in cancer development. Specifically, RBM5 and RBM10 are two splicing factors that are homologs, and both share the capacity to regulate apoptosis by modifying Fas and BCl-x genes alternative splicing[251,252]. RBM5 and RBM10 are upregulated or downregulated in several solid tumors like breast, lung, prostate, and pancreas[211]. However, there are no studies about their relationship with oral cancer. Besides RBM5 and RBM10, there is also RBM9, also known as FOX2 which is another tissue-specific splicing factor that can influence many splicing processes, including those involved in epithelial-mesenchymal transition (EMT) in breast, pancreatic and colon tumors[207,211]. However, there are no studies hitherto that link its expression or any other RBM protein to oral carcinoma.

- TIA1: T-cell intracellular antigen-1 gene (*TIA1*) is another splicing factor that controls nuclear and cytoplasmic regulatory events including gene transcription, alternative pre-mRNA splicing and turnover, and translation of cellular mRNAs[253]. TIA1 has two variants *TIA1 variant 1* and *variant 2,* which encode two different isoforms, TIA1b and TIA1a, respectively[254]. TIA1 has proved to act as both, suppressor in gastric cancer[220,253], and oncogene in esophageal SCC[235,254]. Izquierdo et al. found that TIA1 expression was downregulated compared to normal tissue in a variety of tumors of epithelial origin such as, adrenal gland, lung, ovary, pancreas, parotid gland, skin, small intestine, stomach, thymus, and uterus. Besides, TIA1 down-regulation was associated with increased cell proliferation, tumor growth and invasion, suggesting TIA as a tumor suppressor[253]. On the other hand, Hamada et al. found by immunoreactivity that TIA1 was overexpressed in the cytoplasm and acted as an independent prognostic factor for worse overall survival[254]. TIA1 has been found to be expressed in OSCC[56,235]; however, the results show contradictory results with overexpression providing favorable or worse prognosis. No comparison data of levels of expression between tumor vs. healthy tissue were provided by these studies, and no association with histopathological data was analyzed.

- TRA2B: The *TRA2B* is a SR-like protein that acts as a splicing regulator in the cell nucleus, where it activates the inclusion of alternative exons. Overexpression of TRA2B has been found in lung, breast ovarian, cervical, prostate, colon, head and neck, and brain tumors. It has also described to be down-regulated in renal and thyroid cancer[211,240]. Its expression correlates with an aggressive phenotype, and a poorer prognosis has been associated with its expression in cervical cancer [211,240]. Although described as overexpressed in head and neck cancer in general, its impact on clinical, histopathological or survival data in HNSCC or OSCC has not been described yet.

- NOVA: NOVA family is composed of two neuro-oncological ventral antigens that are described as neuron tissue-specific pre-mRNA splicing factors and are known as NOVA1 and NOVA2. NOVA1 is expressed in the central neuron system, and it is necessary for the development of the motor system and the survival of motor neurons[207,255]. According to some studies, genetic mutations and epigenetic

hypermethylation eliciting NOVA1 dysregulation appear to be rare in most cancers. Recent studies have shown that NOVA1 is enriched in normal fibroblasts and activated T cells and plays a role in various cancers like astrocytoma and oligodendroglioma, hepatocellular carcinoma, gastric cancer, and melanoma[239,255]. In a recent study, NOVA1 was proposed to be triggered by the inflammatory reaction to HPV infection in the oropharynx. They found NOVA1 over-expressed in tumor cells and microenvironment cells in human papillomavirus (HPV)-related oropharyngeal squamous cell carcinoma (SCC). NOVA1 overexpression was related to an inferior OS and PFS. However, in HPV-negative head and neck squamous cell carcinoma (HNSCC), NOVA1 was decreased or attenuated. The lower expression was also defined as an independent poor prognosis factor for OS and PFS and related to older age, advanced pT stage, and advanced pN[239].

Therefore, based on the described background and previous results, **it is necessary to interrogate the alteration of the components of the splicing machinery and its association with different cellular/molecular processes and clinical/histopathological characteristics in OSCC, which would lead to: 1) the discovery of novel molecular mechanisms to better understand the behavior of these tumors, and 2) uncover new biomarkers with diagnostic, prognostic, and therapeutic potential**. Moreover, based also in all the information mentioned above in other tumoral pathologies**, it is tempting to suggest that the strategy to target the spliceosome could be an innovative therapeutic approach in OSCC** but this avenue has not been tested so far.

2. Hypothesis and Objectives

During the last years, our team has focused much of its efforts in studying the role that two pleiotropic regulatory systems, those formed by **somatostatin** (ligands and receptors), and by the **splicing machinery** (spliceosome components and splicing factors), play on different pathological conditions, including several tumors and cancers. Although the presence of some components of these systems in human oral cavity squamous cell carcinoma (OSCC) has been previously described, few studies have examined in detail the potential pathophysiological relevance of these regulatory systems. Based on our prior experience in other cancers, the **INITIAL HYPOTHESIS of this PhD Thesis** was that the dysregulation of the SST and splicing machinery systems could directly influence OSCC, and consequently, that their levels of expression in tumor tissues could provide useful information to improve diagnosis and/or prognosis of these tumors, and to identify novel therapeutic sources to treat patients with these devastating pathologies.

Based on this hypothesis, the **GENERAL AIM of this Doctoral Thesis** was to explore the presence, potential dysregulation, and/or functional role of components of two key cellular systems involved in critical regulatory processes [i.e., somatostatin receptors (SSTs), and by the splicing machinery (spliceosome components and splicing factors)] that could be associated with the development, progression, and aggressiveness of OSCC, with the ultimate goal of discovering novel biomarkers and therapeutic tools to improve the diagnosis, prognosis, treatment and, therefore, the management of the patients with these tumors.

To achieve this main aim, we proposed the following **SPECIFIC OBJECTIVES** (**SO**):

SO1) To quantitatively analyze the expression profile of SSTs in a representative battery of clinically well-characterized OSCC tissues in comparison with adjacent healthy tissues obtained within the same patient in order to determine the utility of SSTs as putative diagnostic biomarkers.

SO2) To assess the putative *in vivo* association between the expression of all SSTs in the tumor of patients with OSCC and relevant clinical and histopathological data parameters (stage, histological grade, tumor invasion, presence of metastasis, recurrence, overall survival, etc.) in order to determine the utility of SSTs as putative prognostic biomarkers.

SO3) To explore and compare, side-by-side, the direct antitumor effects of different SSAs (octreotide, lanreotide, and pasireotide) in primary OSCC human cell cultures.

SO4) To characterize the expression pattern of key splicing-related elements (spliceosome components and splicing factors) in a representative battery of clinically well-characterized OSCC tissues in comparison with adjacent healthy tissues obtained within the same patient in order to

determine the utility of key dysregulated spliceosome-related elements as putative diagnostic biomarkers.

SO5) To assess the putative *in vivo* association between the expression pattern of key splicing-related elements (spliceosome components and splicing factors) in the tumor of patients with OSCC and relevant clinical and histopathological data parameters (stage, histological grade, tumor invasion, presence of metastasis, recurrence, overall survival, etc.) in order to determine the utility of key dysregulated spliceosome-related elements as putative prognostic biomarkers.

SO6) To assess the therapeutic potential of a splicing machinery inhibitor (pladienolide B) in primary OSCC human cell cultures.

3. Material and Methods

3.1. Study Cohort

The Ethics Committee of the Reina Sofia University Hospital (Cordoba, Spain) approved the study, which was conducted in accordance with the Declaration of Helsinki and national and international guidelines and approved by the Ethics Committee of the Reina Sofia University Hospital (Cordoba, Spain, Approval # 70180004). Written informed consent was obtained from all the patients.

A prospective observational case-control study was performed with 37 patients diagnosed with OSCC (lip, tongue, floor of the mouth, buccal mucosa, upper and lower gingiva, retromolar trigone, and hard palate). Patients were followed up for at least 24 months after surgery. Clinical variables were obtained from the clinical chart. Specifically, Body Mass Index (BMI), high blood pressure (HBP), diabetes mellitus (DM), dyslipidemia (DLP), smoking status, age, Stage (I/II/III/IV), histological grade (G1, G2, G3), tumor pT stage (pT1, pT2, pT3, pT4), cervical metastasis or pN (pN (pN0,pN1,pN2a,pN2b,pN2c,pN3), depth of invasion (DOI), perineural (PNI) or lymphovascular invasion (LVI), peritumoral inflammation (PTI) (absent, mild, moderate, severe), margin status (free >5 mm, close 1-5mm, positive <1mm), pattern of tumor invasion (infiltrative, exophytic), lymph nodes size and extranodular extension (ENE+) were recorded. Some variables like Stage, DOI, pT, pN and PTI were divided in subcategories (pNx4: pN0,pN1,pN2,pN3; DOIx3:<5 mm, 5-10mm, >10mm), or in dichotomous categories: margin statusx2: negative (>5 mm) /positive (<5mm); stagex2: I+II/III+IV; pTx2: pT1pT2/pT3+pT4; pNx2: pN0+pN1/pN2+pN3; pN-(pN0)/pN+(pN1,pN2,pN3); PTIx2: absent+mild/moderate+severe) to allow better analysis. Disease Overall Survival (OS) and OS rate at 24 months were calculated. OS was defined as the period between the diagnosis and death. Disease-Free Survival (DFS) was defined as the period between the primary surgery and the first recurrence, the last examination, or death. Three patients who died without recurrent disease before six months due to perioperative complications were classified as “lost data” for recurrence analysis. The recurrence date was established by the date of the recurrence biopsy result or the date of the imaging diagnosing the recurrence. The time to recurrence was defined as the time from the first surgery to confirmed recurrence. Overall Recurrence Rate (RR), Local Recurrence, Regional Recurrence, Local&Regional combined, and Distant Metastasis were calculated.

3.2. Sample collection

OSCC tissue samples (case) were obtained from the surgical specimen after resection. Healthy adjacent tissue samples (control) were obtained within the same patient from the buccal mucosa with a distance from the tumor greater than 2 cm. Then, both specimens were immediately deposited in cold culture medium, analyzed by an expert anatomo-pathologist and then, transported to the laboratory. The control sample and a fragment of the tumor tissue were frozen at -80 ºC for subsequent RNA isolation, retrotranscription, and expression analysis by quantitative real-time PCR (see below). When possible,

the remaining tumor tissue was used to perform cell cultures (see below). It should be mentioned that the tissue sample was consistently obtained safely and ethically and did not interfere with the pathologist's work in any case. A blood test was performed before surgery, in which we measured serum levels of insulin, C reactive protein (CRP), and glycosylated hemoglobin (HbA1).

3.3. RNA isolation and retrotranscription

Total RNA from all samples was extracted at the same time using the RNase-Free DNase Set (Qiagen, Limburg, Netherlands), according to manufacturer instructions, as previously reported[17,156,256]. The amount of RNA recovered, and its purity was determined using the Nanodrop One Spectrophotometer (Thermo Fisher Scientific, Madrid, Spain), and its quality was measured with the same device, using the Absorbance Ratio A260/280 and A260/230 and requiring a minimum of 1.8 in both. Retrotranscription of total RNA to cDNA was performed with the cDNA First Strand Synthesis kit (MRI Fermentas, Thermo Fisher Scientific), using random hexamers primers in a 20µl volume and following manufacturer's instructions, independently of the origin of the samples, as previously reported[257]. Briefly, 1 µg of total RNA from each sample was mixed with random hexamers and water, to match their concentrations, and incubated at 65 ºC for 5 min. Subsequently, appropriate buffers, dNTPs and reverse-transcriptase were added, and the mix incubated for 1 h at 42 ºC, stopping the reaction with an incubation of 5 min at 70 ºC.

3.4. Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was used to perform relative quantification of cDNA derived from retrotranscription of RNA from human samples. qPCR was performed using the Brilliant III SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) in the Stratagene Mx3000p system and specific (and validated) primers for each transcript of interest, as previously reported[156,258].

For each reaction, 10 µl of SYBER green, 8.4 µl of water, 0.3 µl of forward and reverse primers (10 µM), and 1µl of the sample (50 ng of cDNA) were used. The qPCR was made according to the following program: 1 cycle at 95°C for 3 min, 40 cycles of denaturing (95°C for 20 sec) and annealing/extension (61°C for 20 sec), and one last cycle where the final PCR products were subjected to graded temperature-dependent dissociation (55°C to 95°C increasing 0.5°C/30 sec) to verify that only one product was amplified. Specifically, human transcripts for SST receptors (SST_1, SST_2, SST_3, SST_4, SST_5) were used, as previously reported[259]. Additionally, human primers for major spliceosome components (n=10), minor spliceosome components (n=4), associated splicing factors (n=42) selected based on different criteria (key role in the splicing mechanism, alteration in other tumor pathologies, etc.) was

also used in a dynamic qPCR array (see below section "*Analysis of Splicing Machinery Components by a Customized qPCR Dynamic Array*"), as previously reported[156].

To control for variations in the amount of RNA used in the reverse transcription reaction and the efficiency of the reverse transcription reaction, the expression level (copy number) of each SST transcript was adjusted with a normalization factor (NF) calculated from Actin Beta, hypoxanthine-guanine phosphoribosyltransferase 1, and glyceraldehyde 3-phosphate dehydrogenase expression levels (used as housekeeping genes), as reported previously[156,259,260]. In this sense, samples were run, in the same plate, against a standard curve for each of the transcripts analyzed to estimate each transcript's absolute mRNA copy number and a No-RT sample as the negative control. Additionally, products were run on a 2% agarose gel and stained with RedSafe (iNtRON, ABC Scientific, Glendale) to confirm that only one band was amplified and no primer dimers were formed. An aliquot of the PCR products was then purified using the MinElute PCR Purification kit (Qiagen), and the purified PCR products were then sequenced to confirm target specificity.

3.5. Primary OSCC culture

OSCC tissues were placed after surgery in sterile cold PBS 1x (Omega Scientific, Tarzana EEUU) with 1% antibiotic-antimycotic solution and immediately dispersed into single cells under sterile conditions by enzymatic and mechanical disruption and cultured onto tissue culture plates in a serum-containing medium. Specifically, samples were minced into 1-2 mm^3 pieces with a sterile scalpel and washed twice with PBS 1x. Then, slices were incubated in a culture medium supplemented with Dispase (Invitrogen) and Collagenase I (Invitrogen) for 30-60 minutes, shaking at 37ºC (up to 2 hours). The dispersed cell suspension was centrifuged and washed twice. Cell incubation continued with 5ml of 0.05% Trypsin-EDTA (Sigma-Aldrich, Madrid, Spain) for 5 minutes at 37ºC, followed by incubation with 15 ml of DNase I (Promega, Madrid, Spain) for 5 minutes at 37ºC with siliconized pipette agitation every 5 minutes. Cells were filtered through a nylon gauze of 130-µm mesh (to avoid fibroblast contamination) and dissociated into individual cells by repeated smoothly tipped siliconized glass Pasteur pipette aspiration. Finally, the treatment of RBC lysis (BioLegend, London, UK) was performed to eliminate possible red cell contaminations. Cell number and viability (always higher than 95%) were determined by the trypan blue dye exclusion method (American Type Culture Collection, Manassas, VA) in a Neubauer Chamber. Cells suspension were seeded in RPMI 1640 (ThermoFisher Scientific) medium supplemented with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic, and 2mM L-glutamine in plates previously coated with poly-L-lysine to enhance cell adherence.

3.6. Cell Proliferation Assay

Primary OSCC cells were plated in 96-well plates at the density necessary to obtain a ~75% cell confluence in the control groups at the end of the experiment (10.000 cells/well). Twenty-four-hour later, serum-free medium was added for 24h. Then, cells were incubated for 3h in a 10% Alamar-Blue reagent/serum-free medium (Life Technologies, Thermo Fisher Scientific). Alamar-Blue reduction (basal cell viability) was determined in a FlexStation3 system (Molecular Devices, Sunnyvale, CA, USA) plate reader, exciting at 560 nm and reading at 590 nm. This reduction is proportional to the number of cells, so that the comparison between days is a relative reference of the cell proliferation. After this, different SSAs at 10^{-7} M [first-generation (Octreotide and Lanreotide; with high-affinity binding to SST_2 and SST_5) and second-generation (Pasireotide; a multireceptor-targeted SST with high affinity for SST_1, SST_2, SST_3, and SST_5), and vehicle-treated controls were added to wells (at least 4 wells/treatment) in 5% FBS medium for 24, 48 and 72 hours. 10^{-7} M was the dose previously reported to exert the most potent antitumor effect in different endocrine-related tumors[21,260]. Additionally, primary OSCC cells were treated with pladienolide-B at different doses (a splicing machinery inhibitor; 10^{-2}nM, 1nM and 100nM; Santa Cruz, Heidelberg, Germany), and vehicle-treated controls (at least 4 wells/treatment) in 5% FBS medium for 24, 48 and 72 hours. As previously reported[155], the Alamar-Blue reduction was measured every 24h. In this sense, medium was replaced by fresh 5 % FBS-medium immediately after each measurement (every 24 h), including treatment as appropriate. All assays were repeated a minimum of three times on independent days.

3.7. Analysis of Splicing Machinery Components by a Customized qPCR Dynamic Array

A Dynamic Array (Fluidigm, South San Francisco, CA, USA) was employed to simultaneously measure the expression levels of 59 genes in 37 oral cancer samples and normal healthy tissue. This custom array included components of the major spliceosome (n = 10) and minor (n = 4) spliceosome, associated SFs (n = 42), selected based on different criteria (key role in the splicing mechanism, alteration in other tumor pathologies, relationship with significant splicing variants in cancer, etc.), and the *ACTB*, *GAPDH* and *HPRT* as housekeeping genes. The array was prepared and measured following the manufacturer's instructions. Specifically, we performed a preamplification, exonuclease treatment, and the qPCR dynamic array following the manufacturer's instructions. Briefly, 12.5 ng of cDNA of each sample were pre-amplified using 1 µL of PreAmp Master Mix (Fluidigm) and 0.5 µL of a mix with all the primers together (500 nM) in a T100 Thermal-cycler (BioRad, Hercules, CA, USA) using the following program:

Initial denaturalization	95 ºC	2 min
14 cycles		
	95 ºC	15 s
	60 ºC	4 min

After preamplification, samples were treated with 2 µl of a 4 U/µl Exonuclease I (New England BioLabs, Ipswich, MA, USA) solution for 30 min at 37 ºC and 15 min at 80 ºC to remove the excess of primers. Then, samples were diluted with 18 µl of 1x TE Buffer at pH 8.3 (Thermo Scientific) in a 5-fold dilution. Next, 2.7 µl were mixed with 3 µl of EvaGreen Supermix (BioRad) and 0.3 µl of DNA Binding Dye Sample Loading Reagent 20X (Fluidigm); primers were diluted to 5 µM with 2X Assay Loading Reagent (Fluidigm).

Control line fluid was charged in the chip, and the Prime script program was run into the IFC controller HX. Finally, 5 µl of each primer and 5 µl of each sample were pipetted into their respective inlets on the chip, and the Load Mix script in the IFC controller software was run. After this program, the chip is put in the Biomark System following the manufacturer's protocol (Fluidigm). Data were processed with Real-Time PCR Analysis Software 3.0 (Fluidigm).

Primers used in this dynamic qPCR Array have been designed using the bioinformatics tool Primer Blast and Primer3 software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/ and http://bioinfo.ut.ee/primer3-0.4.0/), using as template the mRNA sequences from NCBI database. All the primers used in this Thesis are described in table M1, including the sequences, their application and the length of the amplicon in base pairs (bp). In order to standardize the methodology, basic requirements of the primers for qPCR were fixed in a Tm range of 59-61 ºC, and an amplified sequence of 80-200 pb. Additionally, in order to prevent genomic amplification, each primer, forward and reverse, was designed in different exons and, when possible, they were designed in the middle of an exon junction. Designed primers were synthesized by Integrated DNA technologies (Madrid, Spain). Subsequently, primers were validated by conventional PCR using cDNAs from human samples; PCR products were isolated with FavorPrep™ GEL/PCR Purification Kit (Favorgen, Vienna, Austria) and sequenced using sanger sequencing (STABVIDA, Portugal).

Table M 1: List of primers used in these studies. The name of the transcript, the application of the primers, the sequence of forward and reverse primer, and the size in base pairs are indicated.

Transcript	Primers application	Forward	Reverse	Size bp
ACTB	qPCR	ACTCTTCCAGCCTTCCTTCCT	CAGTGATCTCCTTCTGCATCCT	176
EIF4A3	qPCR	TGACCTCTACGACACACTGACC	AAGTTGGCTTCCCTCATTTTC	99
ESRP1	qPCR	TTTTGGGATCACTGCTGGGG	TGTCCCACCTTCTTGTTGGC	108
ESRP2	qPCR	AGAGCCCAGCAGTCAATTGTT	GTCTCACTGTCCACCACATCAG	96
GAPDH	qPCR	AATCCCATCACCATCTTCCA	AAATGAGCCCCAGCCTTC	122
HNRNPA1	qPCR	AAAGCCCTGTCAAAGCAAGA	AGTTGTCATTCCCACCGAAA	112
HNRNPA2B1	qPCR	CAGAGTTCTAGGAGTGGAAGAGGA	CCATTATAGCCATCCCCAAA	149
HNRNPA3	qPCR	ATGGGGCACACTCACAGATT	GCATCCACCTCTTCAACACA	102
HNRNPF	qPCR	AGTCCCACAGAACCGAGATG	CCAACCCTGAGAAGAACTGAAC	144
HNRNPG	qPCR	AGAGATTATGCACCACCACCA	CACGATCACGACCATATCCA	118
HNRNPK	qPCR	CTGGGGTGTCAGTTGTTGG	TGGTTTCAGTGTTAGGGAAGG	141
HPRT	qPCR	CTGAGGATTTGGAAAGGGTGT	TAATCCAGCAGGTCAGCAAAG	157
KHDRBS1	qPCR	GAGCGAGTGCTGATACCTGTC	CACCAGTCTCTTCCTGCAGTC	106
MAGOH	qPCR	GCCAACAACAGCAATTACAAGA	TTATTCTCTTCAGTTCCTCCATCAC	88
MBNL1	qPCR	TGACACCAATGACAACACAGTC	ATGTGCAGGGGGATGAAAG	94
MBNL2	qPCR	ACCACGCCTGTTATTGTTCC	TCCCTGCATACCTCCAGTTT	101
NOVA1	qPCR	TACCCAGGTACTACTGAGCGAG	CTGGTTCTGTCTTGGCCACAT	124
PRPF19	qPCR	CCAAGTTCCCAACCAAGTGT	GGCACAGTCTTCCCTCTCTTC	145
PRPF40A	qPCR	GCTCGGAAGATGAAACGAAA	TGTCCTCAAATGCTGGCTCT	130
PRPF8	qPCR	TGCCCACTACAACCGAGAA	AGGCCCGTCCTTCAGGTA	139
RAVER1	qPCR	GTAACCGCCGCAAGATACTG	CGAAGGCTGTCCCTTTGTATT	126
RBM10	qPCR	CAGCACTCCCTCAACATCCT	AGCACTTCTCTCGGCGTTT	127
RBM17	qPCR	CAAAGAGCCAAAGGACGAAA	TACATGCGGTGGAGTGTCC	107
RBM22	qPCR	CTCTGGGTTCCAACACCTACA	GGCACAGATTTTGCATTCCT	137
RBM25	qPCR	GCTAAATGCCCCCTCACAG	CTGGAAATCTGCGGAAAATG	86
RBM3	qPCR	AAGCTCTTCGTGGGAGGG	TTGACAACGACCACCTCAGA	98
RBM39	qPCR	AGTTGGATGGGATACCGAGA	TTGCCCTGAGCTGAATTTTT	102
RBM4	qPCR	GTCCCACCTGCACCAATAAG	CCGCTCCATGTGTACGAAG	104
RBM45	qPCR	CCCATCAAGGTTTTCATTGC	TTCCCGCAGATCTTCTTCTG	123
RBM5	qPCR	TCAGGCACCAGCAACTCTC	CGGTCTCGGTATTTCATCTCTC	124
RBM6	qPCR	CCAGGATGGAGAGAGCAAAA	CAGTAGTAAGGCGGACATAGGG	104
RNU1	qPCR	ATCACGAAGGTGGTTTTCC	GCAGTCGAGTTTCCCACA	114
RNU11	qPCR	AAGGGCTTCTGTCGTGAGTG	CCAGCTGCCCAAATACCA	108

RNU12	qPCR	ATAACGATTCGGGGTGACG	CAGGCATCCCGCAAAGTA	106
RNU2	qPCR	CTCGGCCTTTTGGCTAAGAT	TATTCCATCTCCCTGCTCCA	116
RNU4	qPCR	TCGTAGCCAATGAGGTCTATCC	AAAATTGCCAGTGCCGACTA	103
RNU4ATAC	qPCR	GTTGCGCTACTGTCCAATGA	CAAAAATTGCACCAAAATAA	85
RNU6	qPCR	CGCTTCGGCAGCACATATA	AAAATATGGAACGCTTCACGAA	101
RNU6ATAC	qPCR	TGAAAGGAGAGAAGGTTAGCACTC	CGATGGTTAGATGCCACGA	112
SF3B1	qPCR	CAGTTCCGTCTGTGTGTTCG	GCTGCCTTCTTGCCTTGA	101
SF3B2	qPCR	CTGCCAAACAGAAGCAAAAA	TGTGAGGGGACCTAAAACTTG	97
SFPQ	qPCR	TGGTAGGGGGTGAAAGTG	TTAAAAACAAGAAATGGGGAAATG	125
SND1	qPCR	ACTACGGCAACAGAGAGGTCC	GAAGGCATACTCCGTGGCT	101
SNRNP200/RNU5	qPCR	GGTGCTGTCCCTTGTTGG	CTTTCTTCGCTTGGCTCTTCT	103
SNW1	qPCR	ATGCGTGCCCAAGTAGAGAG	TCCCCATCCTCTTTTTCCA	134
SRPK1	qPCR	GAGCAAGAACATAACGGACCA	ACCCAACAAGCATTTCCAAG	134
SRRM1	qPCR	GTAGCCCAAGAAGACGCAAA	TGGTTCTGTGACGGGGAG	108
SRSF10	qPCR	CTACACTCGCCGTCCAAGAG	CCGTCCACAAATCCACTTTC	103
SRSF2	qPCR	TGTCCAAGAGGGAATCCAAA	GTTTACACTGCTTGCCGATACA	113
SRSF3	qPCR	TAACCCTAGATCTCGAAATGCATC	CATAGTAGCCAAAAGCCCGTT	117
SRSF4	qPCR	GGAACTGAAGTCAATGGGAGAA	CTTCGAGAGCGAGACCTTGA	110
SRSF5	qPCR	GCAAAAGGCACAGTAGGTCAA	TTTGCGACTACGGGAACG	92
SRSF6	qPCR	AGACCTCAAAAATGGGTACGG	CTTGCCGTTCAGCTCGTAA	82
SRSF9	qPCR	CCCTGCGTAAACTGGATGAC	AGCTGGTGCTTCTCTCAGGA	87
TCERG1	qPCR	GAGGAGCCCAAAGAAGAGGA	CACCAGTCCAAACGACACAC	112
TIA1	qPCR	TAAATCCCGTGCAACAGCAGA	TATGCAGGAACTTGCCAACCA	124
TRA2A	qPCR	TCAAAGGAGGCTATGGAAAGG	TGTGTGCGCTCTCTTGGTTA	90
TRA2B	qPCR	GATGATGCCAAGGAAGCTAAAG	AGGTAGGTCTCCCCATGTAAATTC	130
U2AF1	qPCR	GAAGTATGGGGAAGTAGAGGAGATG	TTCAAGTCAATCACAGCCTTTTC	120
U2AF2	qPCR	CTTTGACCAGAGGCGCTAAA	TACTGCATTGGGGTGATGTG	130

3.8. Statistical and bioinformatical analysis

For *in vitro* experiments, all data are expressed as mean ± SEM. Statistical analysis was done using SPSS (IBM, New York, NY, USA) and GraphPad Prism (La Jolla, CA, USA). Normality was assessed using Shapiro or Kolmogorov-Smirnov test and by visual inspection of the shape of histograms. We evaluate data heterogeneity of variance using the Kolmogorov-Smirnov test to compare the difference between the means of the gene's expression levels in tumor tissue and healthy tissues within the same

patient. Consequently, parametric (T-student) or nonparametric (Mann-Whitney U, Kruskal-Wallis) tests were implemented. One-Way ANOVA, Chi^2, or Fisher test analysis was performed to explore statistical differences between differences among two groups. *In vitro* cell proliferation experiments were assessed by multiple comparison tests (one-way ANOVA followed by Dunnet post hoc test) and performed in a minimum of three independent primary cultures from different patients (at least 4 replicates/treatment per experiment). As previously reported, to normalize values within each treatment and minimize intragroup variations in the different in vitro experiments (i.e., different ages of the tissue donor or metabolic environment), the values obtained were compared with vehicle-treated controls (set at 100%).

ROC curves were used to measure how well the expression of splicing machinery components could discriminate between different diagnostic groups. Statistical analysis of ROC curves was performed by calculating the Area Under the Curve (AUC) of each element and comparing them with the AUC of the reference line using the Student's t-test. Heatmaps and clustering analysis were performed using MetaboAnalyst 3.0. In this sense, the splicing machinery components that discriminate between oral cancer and healthy tissue were selected following two main criteria in all cases. First, the VIP score must be higher or equal to 1.5. This value is considered a significant threshold in this type of analysis. Second, and to screen the selected splicing machinery components by the first criteria, we chose only those with the best hierarchical clustering in the heatmaps (VIP Score >1.8). Moreover, PLS-DA analysis is a statistical method close to principal components analysis (PCA) that changes the maximum variance finding by a linear regression model in a different dimension showing the best elements to discriminate between different experimental groups. In this case, healthy and cancer tissue. The splicing statistical analyses from functional assays were assessed by paired parametric t-test or one-way ANOVA test followed by Dunnett's test for multiple comparisons. Data were expressed as mean ± SEM. Clinical correlations were evaluated by unpaired nonparametric Mann-Whitney test or the Spearman test.

Survival curves were calculated by Kaplan–Meier analysis, and the log-rank test was used to compare OS and Recurrence according to different variables. Parametric or nonparametric tests were used to analyze the relationship between risk factors, clinical and staging data, histopathological analysis, and SSTs or splicing factors expression levels. Pearson or Spearman correlation analyses were used to assessing the relationship between numerical variables.

P values ≤ 0.05 were considered statistically significant. A significant trend was indicated when *p*-values ranged between >0.05 and <0.1.

4. Results

4.1. Description of the cohort

Between December 2016 and April 2018, forty-one patients were operated in the Oral and Maxillofacial Surgery Department of the Reina Sofia University Hospital (Córdoba) for OSCC who met inclusion criteria and were selected for the study. Three OSCC samples were contaminated in the process. Of the rest, one sample was proven to be an outliner and excluded from the analysis. Therefore, we measured the epidemiological, staging, clinical, and histopathological data in 37 patients, and the expression levels of somatostatin receptors and key components of the splicing machinery in tumor (case) and healthy adjacent tissue samples (control) from these patients.

4.1.1. Epidemiological data and risk factors

The cohort of 37 patients comprised 19 men (51%) and 18 women (49%) with a mean age of 64 ± 2-years-old (26-86). Regarding the location of the lesion, 20 out of 37 patients (54%) had a tongue SCC, 6 patients (17%) had SCC in the floor of the mouth, 5 patients (13%) had an alveolar (superior, inferior) or hard palate SCC, 3 patients (8%) had buccal mucosa SCC, 1 patient had lip SCC (3%) and, in other 2 patients the cancer was at the retromolar trigone mucosa (5%). Location did not show any statistical gender predilection (p=0.10). However, women had more alveolar ridge SCC while men had more buccal mucosa than women (Figure R1).

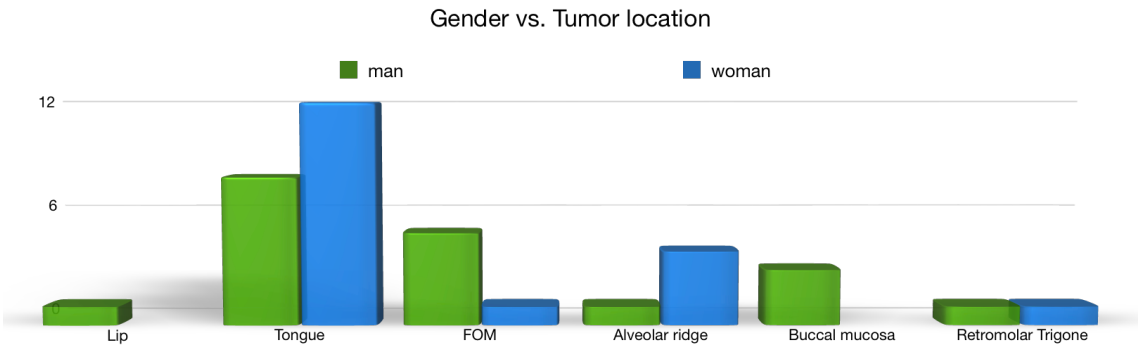


Figure R 1: Gender differences of tumor location. Women had more alveolar ridge SCC while men had more buccal mucosa. Abbreviation: FOM, Floor of the mouth.

Regarding the risk factors among the cohort, smoking and drinking habits were statistically predominant in men. While just 11% of women were ever smokers, men had a rate of 79%. (Figure R2). Women were more prone to have a diagnosis at an elder age (p=0.05). Neither BMI, HBP, DM, nor DLP showed gender predilection. Results from epidemiological data and the gender correlation are summarized in Table R1.

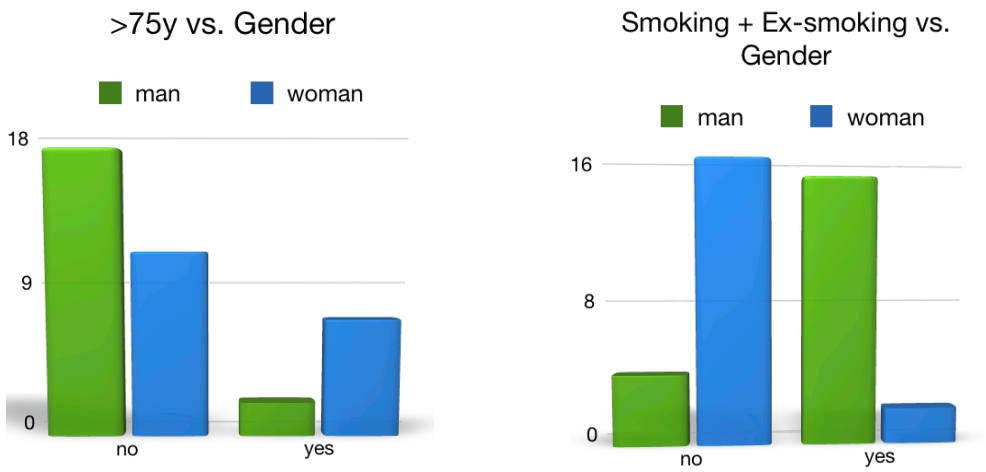


Figure R 2: Gender differences among risk factors. Gender distribution depending on age >75y (left) and smoking status (right). Women are elder when diagnosed and are usually non-smoker.

Table R 1: Epidemiological data, risk factors, and their gender correlation.

	Women	***Men***	***Total***	***Men vs. Women*** *p. value*	*Test*
	18 (49%)	19(51%)	37 (100%)		
<45y				p=0.28	Fisher
Yes	3/18 (17%)	1/19 (5%)	4 (11%)		
No	15/18 (83%)	18/19 (95%)	33 (89%)		
>75y				**p=0.05**	Fisher
Yes	7/18 (39%)	2/19 (10%)	9 (24%)		
No	11/18 (61%)	17/19 (90%)	28 (76%)		
Smoking	2/18 (11%)	10/19(53%)	12/37 (32%)	**p=0.01**	Fisher
Ex-Smoking	0/14 (0%)	5/7(71%)	5/21 (24%)	**p=0.001**	Fisher
Smoking +Ex-smoking	2/18 (11%)	15/19 (79%)	17/37 (46%)	**p=<0.001**	Fisher
BMI	29±6 (18-40)	27±4 (21-34)	28±5 (18-40)	p=0.10	Fisher
HBP	8/18 (44%)	8/19 (42%)	16/37 (43%)	p=1	Fisher
DM	4/18 (22%)	5/19 (26%)	9/37 (24%)	p=1	Fisher
DLP	5/18 (28%)	5/19 (26%)	10/37 (27%)	p=1	Fisher

Abbreviations: BMI, body mass index; DM, Diabetes Mellitus; DLP, dyslipidemia; HBP, high blood pressure.

4.1.2. Staging, clinical and histopathological data

Our cohort is comprised of 51% of patients with advanced Stages IV, 16% with Stage III, 27% Stage II, and 6% with Stage I. 35% of our patients belonged to pT4 tumors, 24% were pT3, 35% were pT2, and 6% were pT1. The cervical lymph node involvement was positive in 43% with pN1 in 11%, and with pN2 and pN3 both in 16% (Table R2). Neither pT, pN, stage data, nor any histopathological feature showed any gender predilection or statistical gender difference.

Table R 2: Clinical Staging data and their gender correlation.

	Women	***Men***	***Total***	***Men vs. Women***	
				p. value	*Test*
	18 (49%)	19(51%)	37 (100%)		
pT				p=0.68	X^2
pT1	1 (5%)	1 (6%)	2 (6%)		
pT2	5 (28%)	8 (42%)	13 (35%)		
pT3	4 (22%)	5 (26%)	9 (24%)		
pT4	8 (45%)	5 (26%)	13 (35%)		
pT combined				p=0.29	X^2
pT1+pT2	6 (33%)	9 (47%)	15 (40%)		
pT3+pT4	12 (67%)	10 (53%)	22 (60%)		
pN				p=0.53	X^2
pN0	9 (50%)	12 (63%)	21 (57%)		
pN1	3 (16%)	1 (5%)	4 (11%)		
pN2a	2 (11%)	0	2 (5%)		
pN2b	1 (6%)	1 (5%)	2 (5%)		
pN2c	1 (6%)	1 (5%)	2 (5%)		
pN3a	0	0	0		
pN3b	2 (11%)	4 (22%)	6 (17%)		
pN combined				p=0.43	X^2
pN0	9 (50%)	12 (63%)	21 (57%)		
pN1	3 (17%)	1 (5%)	4 (11%)		
pN2	4 (22%)	2 (10%)	6 (16%)		
pN3	2 (11%)	4 (22%)	6 (16%)		
pN combined x2				p=0.90	X^2
pN0/pN1	12 (67%)	13 (69%)	25 (68%)		
pN2/pN3	6 (33%)	6 (31%)	12 (32%)		
pN+ vs pN-				p= 0.41	X^2
pN-	9/18 (50%)	12/19 (63%)	21/37 (57%)		
pN+	9/18 (50%	7/19 (37%)	16/37 (43%)		
Stage				p=0.51	X^2
I	1 (6%)	1 (5%)	2 (6%)		
II	3 (17%)	7 (37%)	10 (27%)		
III	4 (22%)	2 (10%)	6 (16%)		
IV	10 (55%)	9 (48%)	19 (51%)		
Stage combined				p=0.19	X^2
I-II	4 (22%)	8 (42%)	12 (32%)		
III-IV	14 (78%)	11 (58%)	25 (68%)		

Abbreviations: pN, cervical metastasis (pN0/pN1/pN2a/pN2b/pN3); pNx4 (pN0/pN1/pN2/pN3); pNx2 (pN0+pN1/pN2+pN3), pN- (pN0) *vs*. pN+ (pN1, pN2, pN3); pT, tumor size (pT1,pT2,pT3,pT4); pTx2 (pT1+pT2/pT3+pT4); Stage (I/II/III/IV); Stage x2 (I+II/III+/IV).

Among the histopathological data reviewed (Table R3), the tumor´s differentiation grade showed equal G1 and G2 differentiation distribution while no G3 is present in any tumor sample. Most of the tumors had >5 mm of depth of invasion, and 60% of the tumors had moderate or intense peritumoral inflammation. Perineural invasion (PNI) and lymphovascular invasion (LVI) was present in 70% and 54% of the tumors, respectively. LVI had no gender predilection while PNI was present in 84% of men vs. 56% of women (p=0.05). Tumors with an infiltrative front of invasion were present in 81% of the patients while the other 19% showed an expansive front of invasion. Significantly, PTI showed a negative statistical correlation with pT, pN, Stage, PNI, and DOI (Table R4; Figure R3; Figure R4)

Table R 3: Histopathological data and their gender relationship.

	Women	***Men***	***Total***	***Men vs. Women*** *p. value*	*Test*
	18 (49%)	19(51%)	37 (100%)		
G				p=0.24	X^2
G1	11/18 (61%)	8/19 (42%)	19 (51%)		
G2	7/18 (39%)	11/19 (58%)	18 (49%)		
DOI x3				p=0.45	X^2
1- 5mm	3(17%)	1 (6%)	4 (11%)		
>5 - 10mm	6 (33%)	9 (47%)	15 (40%)		
>10 mm	9 (50%)	9 (47%)	18 (49%)		
PTI				p=0.74	X^2
Absence	1 (6%)	0 (0%)	1 (3%)		
Mild	7 (39%)	7 (37%)	14 (38%)		
Moderate	8 (44%)	9 (47%)	17 (46%)		
Intense	2 (11%)	3 (16%)	5 (13%)		
PTI x2				p=0.63	X^2
Absence/mild	8 (53%)	7 (35%)	15 (40%)		
Moderate/Intense	10 (47%)	13 (65%)	23 (60%)		
PNI				**p=0.05**	X^2
Yes	10 (56%)	16 (84%)	26 (70%)		
No	8 (44%)	3 (16%)	11 (30%)		
LVI				p=0.85	X^2
Yes	10 (56%)	10 (53%)	20 (54%)		
No	8 (44%)	9 (47%)	17 (46%)		
Invasion Front				p=0.73	X^2
Infiltrative	15 (83%)	15 (79%)	30 (81%)		
Expansive	3 (17%)	4 (21%)	7 (19%)		
Uniformity				p=0.73	X^2

Non-uniform	15 (83%)	15 (79%)	30 (81%)
Uniform	3 (17%)	4 (21%)	7 (19%)

Abbreviations: DOI, depth of invasion (1-5mm, 5-10mm,>10mm); G, grade; Invasion (expansive (+) *vs.* infiltrative (-)); LVI, lymphovascular invasion; PNI, perineural invasion; PTI, peritumoral inflammation (mild/moderate/severe) PTIx2 (absent+mild/ moderate + severe); Invasion front (poor defined tumor edges (-) *vs*. well defined edges (+))

Table R 4: Peritumoral inflammation relationship with other histopathological data.

	PTI	*Test*	***PTI x2***	*Test*
pT *(pT1, pT2, pT3, pT4)*	p=0.27 (-) R -0,50	X^2	**p=0.05 (-)** R -0,44	X^2
pT comb *(pT1+pT2, pT3+pT4)*	p=0.09 (-) R -0,40	X^2	**p=0.03 (-)** R -0,34	X^2
pN *(pN0, pN1, pN2a, pN2b, pN2c, pN3a, pN3b)*	p=0.45 R -0,29	X^2	**p=0.06 (-)** R -0,38	X^2
pN x4 *(pN0, pN1, pN2, pN3)*	p=0.33 R -0,27	X^2	p=0.09 R -0,35	X^2
pN x2 *(pN0/pN1, pN2/pN3*	p=0.09 R -0,31	X^2	**p=0.03 (-)** R -0,36	Fisher
pN-/pN+ *(pN0, pN+)*	p=0.20 R -0,20	X^2	p=0.08 (-) R -0,27	Fisher
Stage x4 *(I, II, III, IV)*	p=0.20 R -0,44	X^2	**p=0.02 (-)** R -0,41	X^2
Stage x2 *(I-II, III-IV)*	p=0.38 R -0,27	X^2	p=0.18 R -0,21	X^2
G *(G1, G2, G3)*	p=0.40 R -0,13	X^2	p=0.32 R -0,18	Fisher
DOI x3 *(1-5mm, >5-10mm, >10 mm)*	p=0.25 R -0,44	X^2	**p=0.02(-)** R -0,43	X^2
PNI *(Yes, No)*	p=0.34 R -0,27	X^2	**p=0.07 (-)** R -0,29	Fisher
LVI *(Yes, No)*	p=0.72 R -0,13	X^2	p=0.73 R- 0,09	Fisher
Front of invasion *(Expansive, Infiltrative)*	p=0.36 R -0,29	X^2	p=0.20 R -0,25	Fisher
Uniformity *(Uniform, Non-uniform)*	p=0.36 R -0,29	X^2	p=0.20 R -0,25	Fisher

Abbreviations: DOI, depth of invasion (1-5mm, 5-10mm,>10mm); DFS, disease Free Survival; G, grade (G1,G2,G3); Invasion [expansive (+) *vs.* infiltrative (-)]; LVI, lymphovascular invasion; OS, overall survival; pN, cervical metastasis (pN0/pN1/pN2a/pN2b/pN3); pNx4 (pN0/pN1/pN2/pN3);

pNx2 (pN0+pN1/pN2+pN3), pN- (pN0) *vs*. pN+ (pN1, pN2, pN3); perineural invasion; pT, tumor size (pT1,pT2,pT3,pT4); pTx2 (pT1+pT2/pT3+pT4); PTI, peritumoral inflammation (mild/moderate/severe) PTIx2 (absent+mild/ moderate + severe); RR recurrence rate; Stage (I/II/III/IV); Stage x2 (I+II/III+/IV); Invasion front [poor defined tumor edges (-) *vs*. well defined edges (+)]; (-), negative correlation; (+), positive correlation.

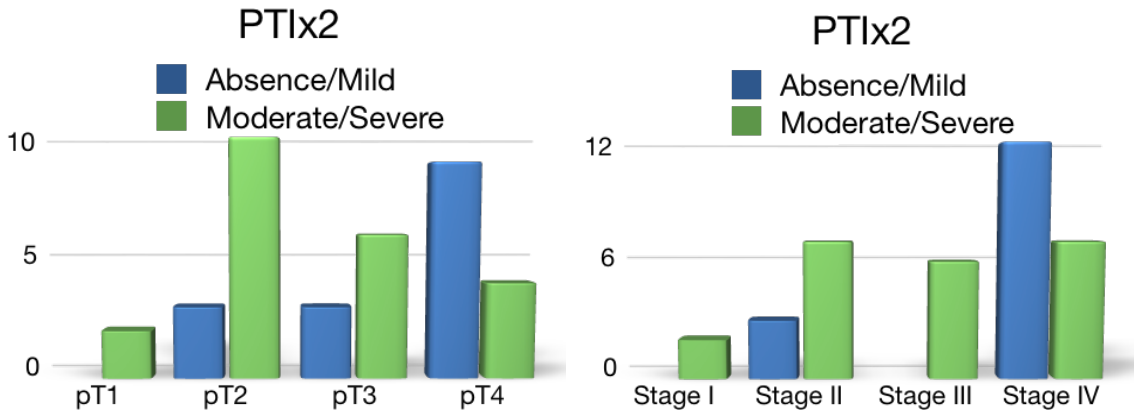


Figure R 3: Peritumoral inflammation (PTI, PTIx2) and its relationship with pT and Stage. Graphics of PTI distribution among pT and Stage showing that absence or mild PTI is related to higher Stage and bigger tumors (higher pT).

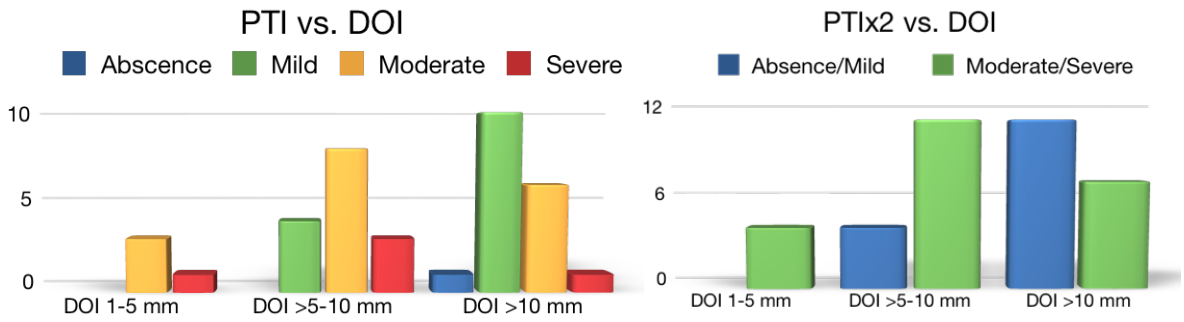


Figure R 4: Peritumoral inflammation (PTI and PTIx2) and its relationship with Depth of invasion (DOI). Graphics of PTI distribution among DOIx3 showing that absence or mild PTI is related to higher DOI.

4.2. Survival analysis

OS was calculated on all patients of the cohort. The follow up time of the study was 24 to 43 months depending on the time from surgery, but a minimum of 2 years was required for the survival and recurrence analysis. We analyzed OS at 2-years frame and overall disease survival. The disease OS was 70% and the OS at 2-years was 76%. OS rate was 33,6±2,4 months (range 2-43) while the OS rate

at 2-years follow up was 20.2±1.2 months (range 2-24). Neither of them showed gender statistical difference; however, a trend to worse prognosis in women could be observed in Kaplan Meier curves (Table R5; Figure R5).

Table R 5: Survival data and their gender correlations.

	General	*Men*	*Women*	*Men vs. Women*	
				p.value	*Test*
OS 2y (%)	28/37 (76%)	16/19 (83%)	12/18(66%)	p=0.28	Log-rank
OS at 2y (months)	20.2±1.2 (2-24)	20.6±2 (3-24)	19.7±1.6 (2-24)		
OS overall (%)	26/37 (70%)	15/19 (58%)	11/18 (42%)	p= 0.28	Log-rank
OS overall (months)	33,6±2,4 (2-43)	34,1±3,2	31,3±3,5		
DFS at 2y (%)	25/33 (76%)	13/17 (76%)	12/16(75%)	p=0.6	Fisher
DFS rate at 2y (months)	20.39 ±1.2 (2-24)	21,47±1.5 (2-24)	19,2±1.8 (6-24)		
RR at 2y (%)	9/34 (26%)	4/17 (24%)	5/17 (29%)	p=0.3	Log-rank
Local RR at 2y (%)	7/34 (21%)	3/17 (18%)	4/17 (23%)	p=0.5	Log-rank
Regional RR at 2y (%)	7/34 (21%)	3/17 (18%)	4/17 (23%)	p=0.6	Log-rank
Local&Regional RR at 2y(%)	5/34 (15%)	2/17 (12%)	3/17 (18%)	p=0.5	Log-rank
Distant metastasis at 2y (%)	3/34 (9%)	2/17(12%)	1/17 (6%)	p=0.5	Log-rank

Abbreviations: OS, overall survival; RR; recurrence rate; (-), negative correlation; (+), positive correlation

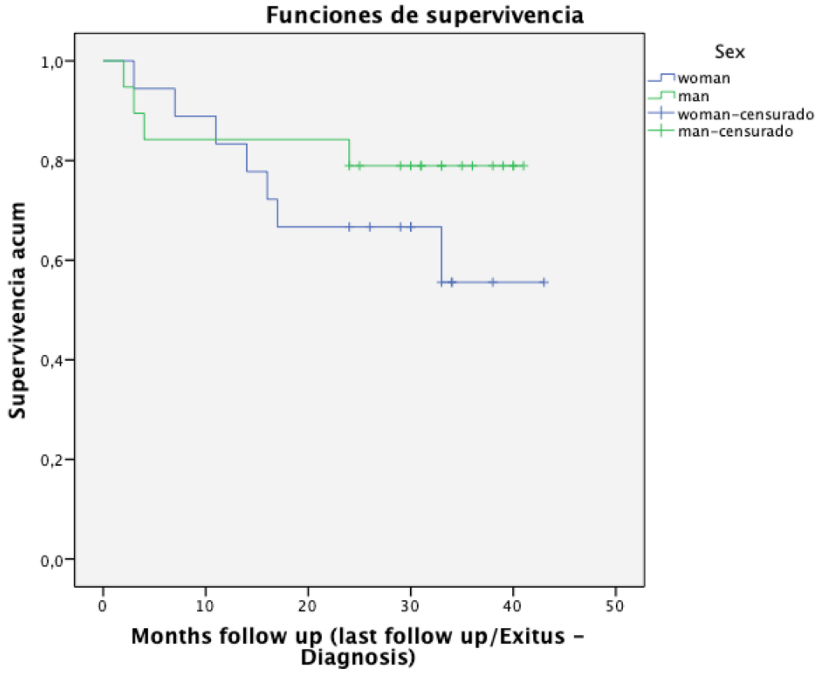


Figure R 5: Overall Survival Kaplan Meier curve. Overall Survival Kaplan Meier curve showing a trend to worse prognosis in women.

Among risk factors, age older than 75 years-old showed a statistical trend to worse OS (p=0.08, Log-rank test) with a statistical negative correlation. However, OS did not show any relationship with ever smoking or any other risk factor except for a significant statistical decrease among diabetic patients and patients with higher HbA1c levels (Figure R6; Table R6).

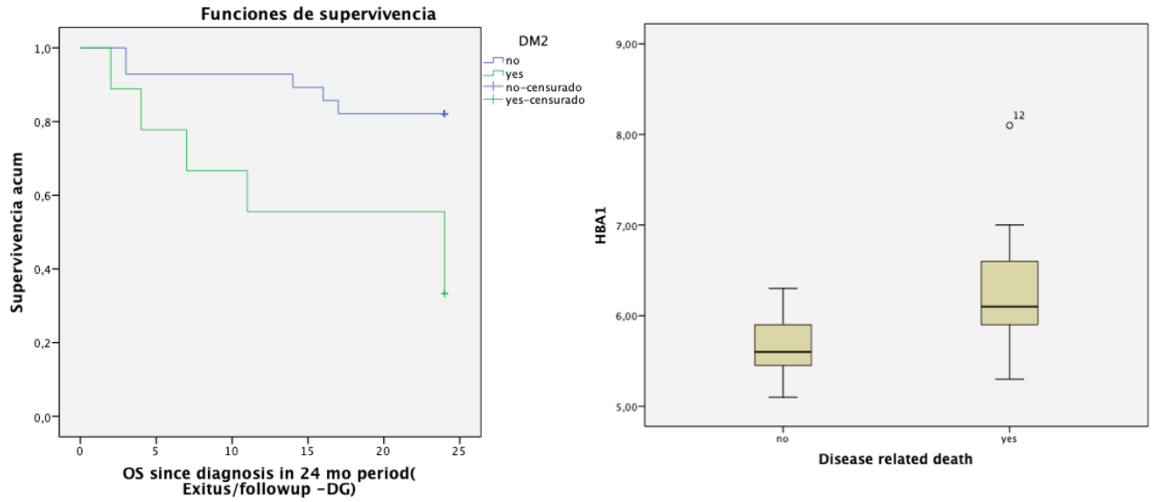


Figure R 6: DM and HbA1c relationship with survival analysis. 2-years OS Kaplan Meier curve showing a statistical relationship of worse survival in diabetic patients (left). HbA1c boxplot showing HbA1c is increased on patients with disease related death (right).

Table R 6: Risk factors and its relationship with OS and Recurrence analysis. Log-rank test and T-student.

	OS	***RR***	***Local RR***	***Regional RR***	***Local& Regional RR***	***Distant Metastasis***
	28/37 (76%)	9/34 (26.4%)	8/34 (23%)	7/34 (21%)	5/34(15%)	3/34 (9%)
>75y	p=0.08	**p 0,02 (+)**	**p<0.01 (+)**	**p=0.05 (+)**	**p<0.01 (+)**	p=0.78
No/Yes	R -0,32	R 0,40	R 0,52	R 0,35	R 0,50	R 0,04
<45y	p=0.43	p=0.9	p=0.40	p=0.66	p=0.46	p=0.57
No/Yes	R -0,15	R -0,01	R -0,18	R 0,04	R -0,15	R -0,11
Ever Smoking	p=0.25	p=0.11	p=0.06	p=0.31	p=0.21	p=0.66
No/Yes	R 0,12	R -0,26	R -0,30	R -0,15	R -0,20	R -0,06
Drinking	p=0.25	p=0.13	p=0.06	p=0.26	p=0.10	p=0.99
No/Yes	R 0,19	R -0,30	R – 0,37	R -0,22	R -0,30	R -0,01
DLP	p=0.85	p=0.72	p=0.39	p=0.39	p=0.11	p=0.14
Yes/No	R 0,01	R 0,04	R 0,14	R 0,14	R 0,27	R 0,25
HBP	p=0.77	p=0.63	p=0.94	p=0.91	p=0.39	p=0.35
No/Yes	R -0,02	R -0,06	R 0,04	R 0,04	R 0,18	R 0,18
DM	**p<0.01(-)**	**p<0.01(+)**	**p<0.001 (+)**	**p<0.01 (+)**	**p<0.001 (+)**	**p=0.04 (+)**
No/Yes	R -0,45	R 0,51	R 0,64	R 0,46	R 0,61	R 0,35

BMI *Cm2/kg*	p=0.88 R 0,04	p=0.64 R 0,08	p=0.16 R 0,30	p=0.87 R 0,01	p=0.18 R 0,25	p=0.27 R 0,14
CRP	p=0.80 R 0,08	p=0.60 R -0,15	p=0.88 R -0,08	p=0.97 R -0,03	p=0.58 R 0,05	p=0.74 R 0,04
HbA1c *</>6,5 continuos*	**p<0.01 (-) p=0,02 (-)** R -0,4	**p<0.01 (+) p<0,01 (+)** R 0,50	**p< 0.001 (+) p<0,001 (+)** R 0,65	**p= 0.05(+)** p= 0,13 R 0,27	**p= 0.01 (+) p= 0,01 (+)** R 0,44	p=0,57 p= 0,53 R 0,12
Insulin	p=0.64 R 0,14	p=0.78 R 0,06	p=0.35 R 0,19	p=0.54 R 0,12	p=0.15 R 0,28	p=0.19 R 0,20

Abbreviations: BMI, body mass index; CRP, C-reactive protein; DM, diabetes mellitus; DLP, dyslipidemia; HbA1c, hemoglobin A1c; HBP, high blood pressure;(-), OS, overall survival; RR; recurrence rate; negative correlation; (+), positive correlation

Regarding clinical data, OS was also statistically decreased with higher pT level by pT1/pT2/pT3/pT4 and pT combined, higher pN level, pN combined, and a higher Stage level (Table R7).

Table R 7: Clinical data vs. survival and recurrence data. Log-rank test

	OS	***RR***	***Local RR***	***Regional RR***	***Local& Regional RR***	***Distant Metastasis***
	28/37 (76%)	9/34 (27%)	8/34 (23%)	7/34 (21%)	5/34(15%)	3/34 (9%)
pT	**p<0,01 (-)** R -0,47	p=0.13 R 0,29	p=0.17 R 0,20	p=0.10 R 0,20	p=0.14 R 0,20	p=0.3
pT1	2/2:100%	1/2:50%	1/2:50%	1/2:50%	1/2:50%	0/3:0%
pT2	13/13:100%	1/13:8%	1/13:7%	0/13:0%	0/13:0%	0/13:0%
pT3	7/9: 78%	2/9: 22%	1/9:11%	2/9:22%	1/9:11%	2/9:22%
pT4	6/13:46%	5/10: 50%	4/10:40%	4/10:50%	3/10:30%	1/10:10%
pT x2	**p=0,01 (-)** R -0,41	p=0.078 R 0,26	p=0.21 R 0,16	p=0.057 R 0,20	p=0.17 R 0,20	p=0.1 R 0,19
pT1/pT2	15/15: 100%	2/15:13%	2/15:13%	1/15:7%	1/15:7%	0/15:0%
pT3/pT4	13/22: 59%	7/19:37%	5/19:26%	6/19:31%	4/19:21%	3/19:16%
pN	**p=0,01 (-)** R -0,42	**p=0.001 (+)** R 0,38	**p<0,01 (+)** R 0,25	**p=0.001 (+)** R 0,40	**p<0,01 (+)** R 0,28	**p<0.001 (+)** R 0,27
pN0	19/21:90%	3/20:15%	3/20:15%	2/20:4%	2/20:10%	0/20:0%
pN1	3/4:75%	1/4:25%	0/4:0%	1/4:25%	0/4:0%	0/4:0%
pN2a	1/2:50%	1/2:50%	1/2: 50%	0/2:0%	0/2:0%	0/2:0%
pN2b	2/2:100%	0/2:0%	0/2:0%	0/2:0%	0/2:0%	0/2:0%
pN2c	1/2:50%	0/1:0%	0/1:0%	0/1:0%	0/1:0%	0/1:0%
pN3a	0	0	0	0	0	0
pN3b	2/6:33%	4/5: 80%	3/5:60%	4/5:80%	3/5:60%	3/5:60%
pN x4	**p=0,01 (-)** R -0,43	**p=0.001 (+)** R 0,39	**p<0,01 (+)** R 0,26	**p≤0.001 (+)** R 0,40	**p=0.001 (+)** R 0,28	**p≤0.001 (+)** R 0,51

pN0	19/21:90%	3/20:15%	3/20:15%	2/20:10%	2/20:10%	0/22:0%
pN1	3/4:75%	1/4:25%	0/4:0%	1/4:25%	0/4:25%	0/4:0%
pN2	4/6:67%	1/5:20%	1/5:20%	0/5:0%	0/5:0%	0/6:0%
pN3	2/6:33%	4/5: 80%	3/5: 60%	4/5: 80%	3/5: 60%	3/5: 60%
pN x2	**p<0,01 (-)** R – 0,43	**p=0.01 (+)** R 0,34	**p=0.01 (+)** R 0,31	**p=0.02 (+)** R 0,31	**p=0.04 (+)** R 0,27	**p<0,01 (+)** R 0,52
pN0/pN1	22/25:88%	4/24:17%	3/24:12%	3/24:12%	2/24:8%	0/24: 0%
pN2/pN3	6/12:50%	5/10: 50%	4/10:40%	4/10:40%	3/10:30%	3/10:30%
pN-/pN+	**p=0,01 (-)** R -0,38	**p=0.03** R 0,31	p=0.16 R 0,16	**p=0.041** R 0,31	p=0.23 R 0,16	**p=0.02** R 0,48
pN-	19/21:90%	3/20:15%	3/20:15%	2/20:10%	2/20:10%	0/20:0%
pN+	9/16:56%	6/14:43%	4/14:29%	5/14:36%	3/14:21%	3/14:21%
Stage	**p<0,01 (-)** R -0,43	p=0.07 R 0,27	p=0.16 R 0,15	**p=0.04 (+)** R 0,30	p=0.10 R 0,18	p=0.25 R 0,30
I	2/2:100%	1/2:50%	1/2:50%	1/2:50%	1/2:50%	0/2:50%
II	10/10:100%	1/10:10%	1/10:10%	0/10:0%	0/10:0%	0/10:0%
III	6/6:100%	0/6:0%	0/6:0%	0/6:0%	0/6:0%	0/6:0%
IV	10/19:53%	7/16:44%	5/16:31%	6/16:38%	4/16:25%	3/16:19%
Stage x2	**p=0,05 (-)** R -0,42	p=0.27 R 0,16	p=0.49 R 0,07	p=0.16 R 0,22	p=0.36 R 0,13	p=0.17 R 0,23
I-II	12/12:100%	2/12:17%	2/12:17%	1/12: 8%	1/12: 8%	0/12:0%
III-IV	16/25:64%	7/22:32%	5/22:23%	6/22: 27%	4/22: 18%	3/22:14%

Abbreviations: OS, overall survival; pN, cervical metastasis (pN0/pN1/pN2a/pN2b/pN3); pNx4 (pN0/pN1/pN2/pN3); pNx2 (pN0+pN1/pN2+pN3), pN- (pN0) *vs*. pN+ (pN1, pN2, pN3); pT, tumor size (pT1,pT2,pT3,pT4); pTx2 (pT1+pT2/pT3+pT4); RR; recurrence rate; Stage (I/II/III/IV); Stage x2 (I+II/III+/IV); (-), negative correlation; (+), positive correlation.

Among histopathological factors, OS was negatively associated with an increased number of positive lymph nodes, an increased number of lymph nodes with ENE+, and a higher size of the positive lymph nodes. (Table R8; Figure R7). Higher PTI was significantly correlated with a better OS (Figure R8).

Table R 8: Lymph nodes data and its relationship with OS and Recurrence. T -student, U-Mann.

	K-S/Sph	***OS***	***RR***	***Local RR***	***Regional RR***	***Local& Regional RR***	***Distant Metastasis***	***DFS***
Nº pN lymph	p= 0,6	p= 0,12 R 0,01	p= 0,63 R -0,01	p= 0,24 R -0,22	p= 0,40 R -0,01	p= 0,30 R -0,20	**p=0,01 (+)** R 0,31	p=0,08 R -0,02
Nº pN +	p= <0,001	**p<0,01 (-)** R -0,47	**p= 0,04 (+)** R 0,38	p= 0,10 R 0,23	**p=0,01 (+)** R 0,47	p= 0,09 R 0,31	**p<0,01 (+)** R 0,49	**p<0,01 (-)** R -0,49
Nº pN ENE+	p= <0,001	**p<0,01 (-)** R -0,62	**p<0,01 (+)** R 0,57	**p= 0,01 (+)** R 0,49	**p<0,01 (+)** R 0,54	**p= 0,01 (+)** R 0,47	**p= 0,001 (+)** R 0,61	**p<0,001 (-)** R -0,72
Size pN+	p= 0,002	**p= 0,02 (-)** R -0,38	p= 0,09 R 0,31	p= 0,10 R 0,18	p= 0,07 R 0,10	p= 0,26 R 0,21	**p=0,02 (+)** R 0,42	**p=0,04 (-)** R -0,38

Abbreviations: ENE+, extranodular extension; OS, overall survival; pN+ (lymph node with metastasis); RR, recurrence rate; (-), negative correlation; (+), positive correlation.

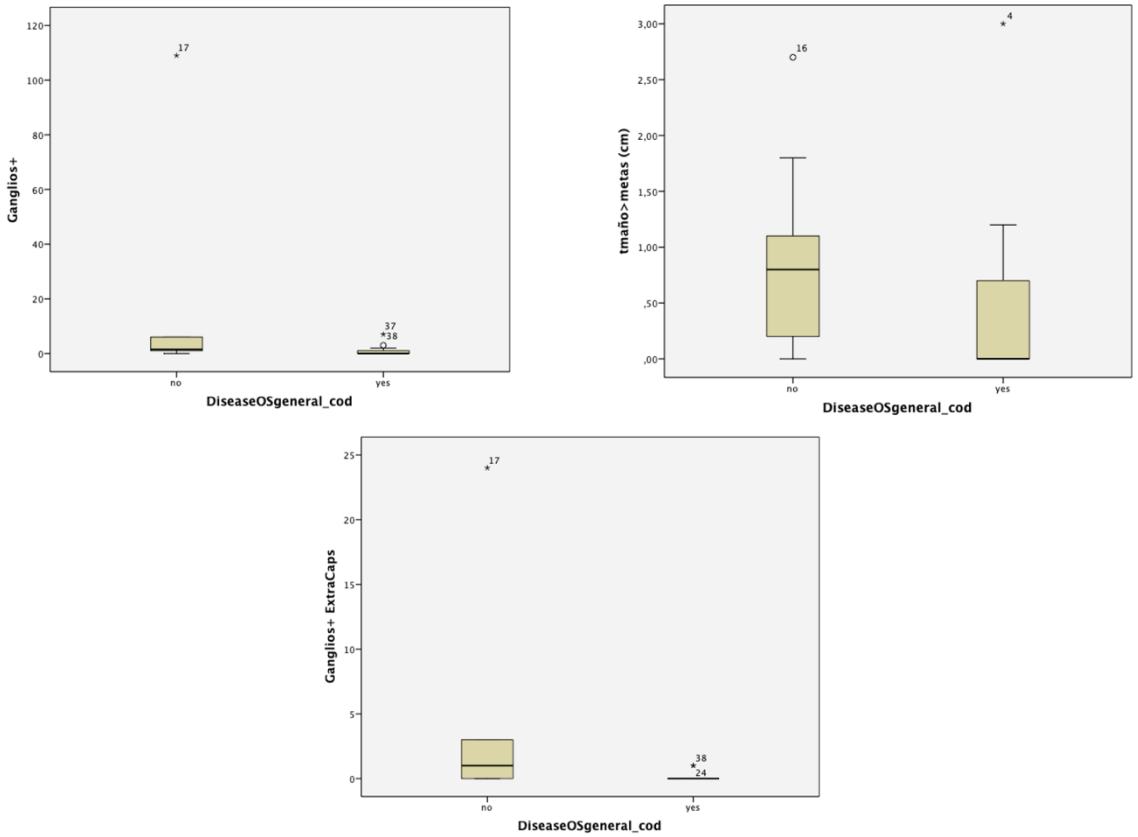


Figure R 7: Lymph node boxplots graphics and its relationship with OS data. OS was negatively associated with an increased number of positive lymph nodes, an increased number of lymph nodes with ENE+, and a higher size of the positive lymph nodes.

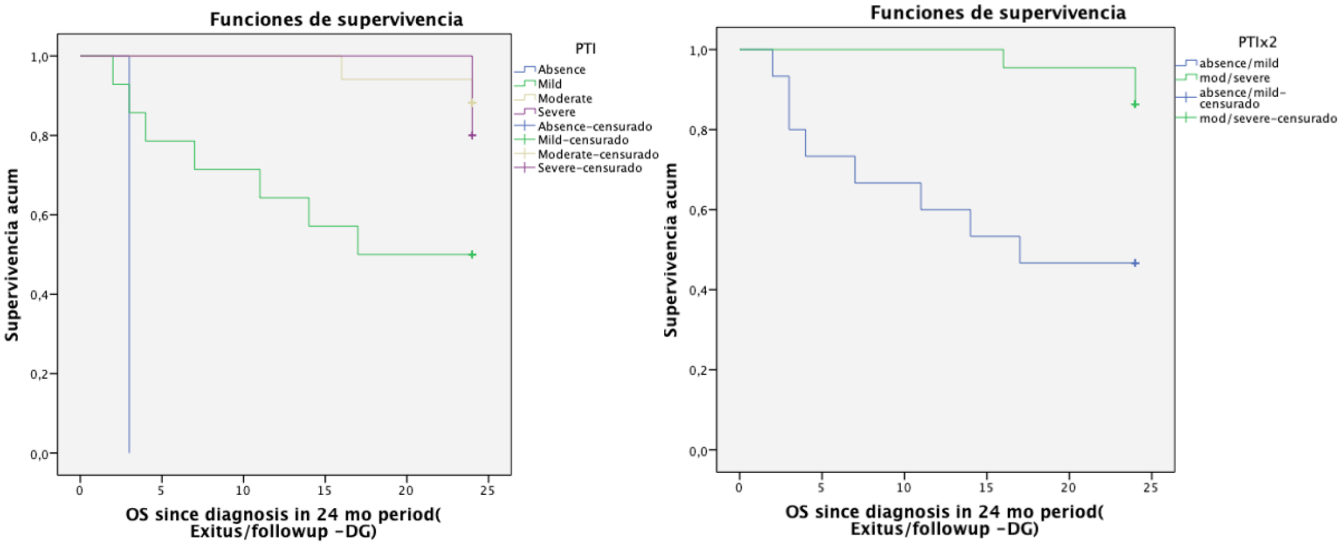


Figure R 8: Peritumoral inflammation reaction vs. Overall survival Kaplan Meier curve. 2-years OS Kaplan Meier curve showing a statistical relationship of worse survival on patients with absence or mild PTI.

A statistical trend for worse OS was found on tumor with non-uniform edges and infiltrative front of invasion. Margin status was also related to OS with a negative correlation with worse OS among patients with positive margins (Fig. R9). No other relationship was found between OS and G, DOI, LVI, or PNI (Table R9).

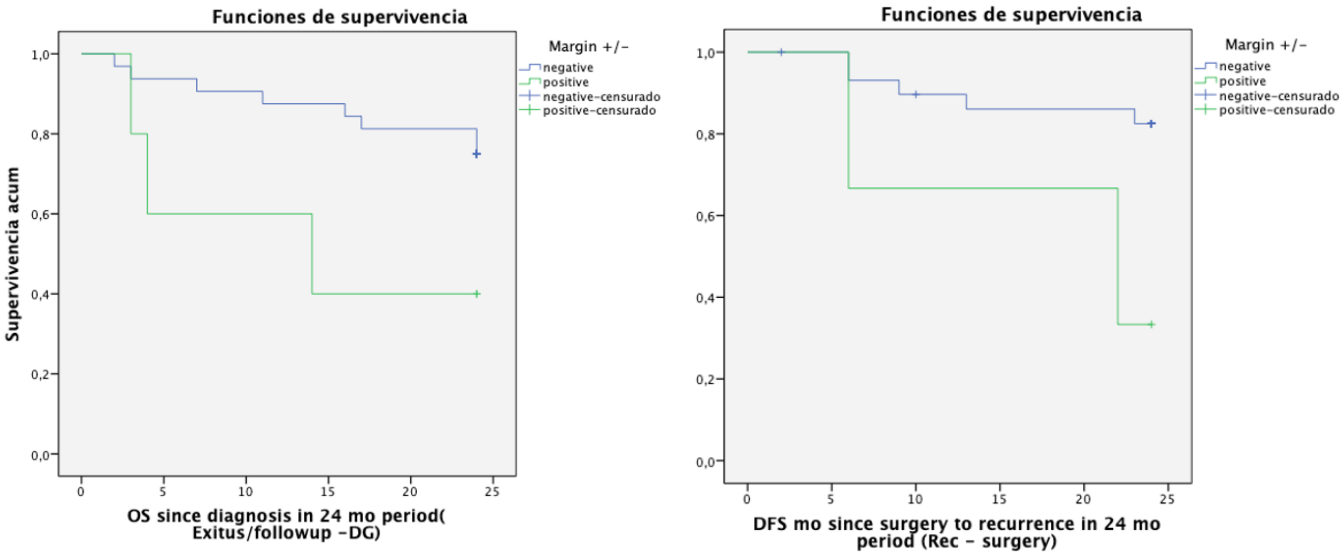


Figure R 9: Margin status vs. Overall survival Kaplan Meier curve and Disease-Free Survival. 2-years OS Kaplan Meier curve showing a statistical relationship of patients with positive margin with worse survival (left) and worse Disease-Free Survival (right).

Table R 9: Histopathological data and its relationship with OS and Recurrence analysis.

	OS	***RR***	***Local RR***	***Regional RR***	***Local& Regional RR***	***Distant Metastasis***
	28/37 (76%)	9/34 (26,4%)	8/34 (23%)	7/34 (21%)	5/43(15%)	3/34 (9%)
	p= 0.64	p=0.19	p=0.15	p=0.15	p=0.11	p=0,46
G	R -0,07	R 0,20	R 0,21	R 0,21	R 0,24	R 0,10
G1	15/19 (79%)	3/17:18%	2/17:12%	2/17:12%	1/17:6%	1/17:6%
G2	13/18 (72%)	6/17:35%	5/17:29%	5/17:29%	4/17:23%	2/17:12%
	p=0,10	p= 0.52	p=0.94	p=0.15	p=0.43	p=0,57
DOI	R -0,27	R 0,12	R -0,02	R 0,22	R 0,07	R 0,15
1- 5mm	4/4 (100%)	1/4:25%	1/4:25%	1/4:25%	1/4:25%	0/4:0%
>5 - 10 mm	14/15 (93%)	3/15:20%	3/15:20%	1/15:7%	3/15:20%	1/15:7%
> 10 mm	10/18 (56%)	5/15:33%	3/15:20%	5/15:33%	3/15:20%	2/15:13%
	p=<0,001	p= 0.15	p= 0.35	**p=0,02**	**p=0,05**	p=0,20
PTI	R 0,39	R -0,14	R 0,02	R -0,21	R -0,03	R -0,08
Absence	0/1:0%	0	0	0	0	0
Mild	7/14: 50%	5/12:42%	3/12:25%	5/12:42%	3/12:25%	2/12:17%
Moderate	16/17: 94%	2/17:12%	2/17:12%	0/17:0%	0/17:0%	0/17:0%
Severe	5/5: 100%	2/5:40%	2/5:40%	2/5:40%	2/5:40%	1/5:20%
	p=<0,001	p= 0.10	p= 0.47	**p=0,02**	p= 0.17	p=0,23
PTIx2	R 0,42	R -0,25	R -0,08	R -0,38	R -0,21	R -0,20
Absence/Mild	7/15 (47%)	5/12:42%	3/12:25%	5/12:42%	3/12:25%	2/12:17%

Moderate/Severe	21/22 (95%)	4/22:18%	4/22:18%	2/22:9%	2/22:9%	1/22:4%
	p=0,57	p=0.47	p=0.17	p=0.59	p=0.20	p=1
PNI	R 0,09	R -0,15	R – 0,27	R -0,11	R -0,24	R -0,01
Yes	9/11 (82%)	5/23:22%	3/23:13%	4/23:17%	2/23:9%	2/23:9%
No	19/26 (32%)	4/11:36%	4/11:36%	3/11:27%	3/11:27%	1/11:9%
	p=0,10	p=0.19	p=0.50	p=0.54	p=0.62	p=1
LVI	R -0,24	R 0,20	R 0,07	R 0,07	R – 0,08	R -0,10
Yes	13/20 (65%)	6/17:35%	4/17:23%	4/17:23%	2/17:12%	2/17:12%
No	15/17 (88%)	3/17:18%	3/17:18%	3/17:18%	3/17:18%	1/17:6%
	p=0,07	**p=0,08**	p=0,12	p=0,13	p=0,20	p=0,35
Front of invasion	R 0,31	R -0,30	R -0,25	R -0,25	R -0,21	R -0,15
Infiltrative	21/30 (70%)	9/27:33%	7/27:26%	7/27:26%	5/27:18%	3/27:11%
Expansive	7/7 (100%)	0/7:0%	0/7:0%	0/7:0%	0/7:0%	0/7:0%
	p=0,07	p=0,15	p=0,12	p=0,13	p=0,20	p=0,35
Uniformity	R 0,31	R -0,30	R -0,25	R -0,25	R -0,21	R -0,15
Uniform	21/30 (70%)	0/7:0%	0/7:0%	0/7:0%	0/7:0%	3/27:11%
Not uniform	7/7 (100%)	9/27:100%	7/27:26%	7/27:26%	5/27:18%	0/7:0%
Margin Status x2	**p= 0.05**	p=0.10	**p=0.03**	p=0.52	p=0.29	p=0.59
	R -0.26	R 0.35	R 0.36	R 0.21	R 0.22	R 0.03
Negative	24/32 (75%)	7/31 (23%)	5/31 (16%)	6/31 (19%)	4/31 (13%)	3/31 (9%)
Positive	2/5 (40%)	02/3 (67%)	2/3 (67%)	1/3 (33%)	1/3 (33%)	0/3 (0%)
	p=0.14	p=0.13	**p=0.07**	p=0.43	p=0.38	p=0.61
Margin Status x3	R -0.24	R 0.34	R 0.36	R 0.21	R 0.22	R 0.04
Free	5/6 (83%)	0/5 (0%)	0/5 /0%)	0/5 (0%)	0/5 (0%)	0/5 (0%
Close	19/26 (73%)	7/26 (27%)	5/26 (19%)	6/26 (23%)	4/26 (15%)	3/26 (11%)
Positive	2/5 (40%)	2/3 (67%)	2/3 (67%)	1/3 (33%)	1/3 (33%)	0/3 (0%)

Abbreviations: DOI, Depth Of Invasion (1-5mm, 5-10mm,>10mm); DFS, Disease-Free Survival; G, Grade; Invasion [expansive (+) *vs.* infiltrative (-)]; LVI, lymphovascular invasion; OS, overall survival; PNI, perineural invasion; PTI, peritumoral inflammation (mild/moderate/severe) PTIx2 (absent+mild/ moderate + severe); Invasion front [poor defined tumor edges (-) *vs*. well defined edges (+)]; RR, recurrence rate; (-), negative correlation; (+), positive correlation.

4.3. Recurrence and Disease-Free Survival analysis

4.3.1. Disease-free survival

Disease-free survival (DFS) at 24 months follow-up was present in 76% of our patients with a DFS length of 20,39 ±1,2 (2-24) months with no gender differences (Table R5). DFS did not show relationship with pT but did show a negative statistical correlation with pN and Stage (Table R10).

Table R 10: Disease-Free survival vs. clinical data. Disease-Free survival is expressed in months (mean) ± SEM.

	DFS *Yes*	*Test*	***DFS length*** *(months) Mean ± SEM*	*Test*
	25/33 (76%)		20,39 ±1,2 (2-24)	
pT	p=0.26 R -0,21	X^2	p=0.14 R -0,34	K-W
pT1	1/2(50%)		23,5±0,5	
pT2	12/13(92%)		23,8±0,15	
pT3	6/8(75%)		19±3,2	
pT4	6/10(60%)		16±2,6	
pT x2	p=0.24 R -0,23	X^2	p=0.13 R -0,33	U-Mann
pT1/pT2	13/15 (87%)		23,8±0,14	
pT3/pT4	12/18 (67%)		17,56±2	
pN	**p=0.06 (-)** R -0,35	X^2	**p=0.01 (-)** R -0,46	K-W
pN0	17/20(85%)		22,95±0,89	
pN1	3/4(75%)		20,50±3,5	
pN2a	1/1(100%)		24	
pN2b	2/2(100%)		24	
pN2c	1/1(100%)		24	
pN3a	0		0	
pN3b	1/5 (20%)		9,40±3,8	
pN x4	**p=0,014 (-)** R -0,35	X^2	**p=0.01 (-)** R -0,47	K-W
pN0	18/20(90%)		22,95±0,89	
pN1	3/4(75%)		20,50±3,5	
pN2	4/4(10%)		24±0,1	
pN3	1/5(20%)		9,40±3,8	
pN x2	p=0,1 R -0,29	X^2	**p=0.06 (-)** R -0,44	U-Mann
pN0/pN1	20/24(87,5%)		22,54±0,9	
pN2/pN3	5/9(56%)		15,89±3,2	
pN-/pN+	p=0.21 R -0,27	Fisher	**p=0,03** R -0,37	U-Mann
pN-	17/20 (68%)		23±0,9	
pN+	8/13 (32%)		16,5±2,4	
Stage	p=0,1 R -0,23	X^2	**p=0.06 (-)** R -0,35	K-W
I	1/2 (50%)		23,5±0,5	
II	9/10 (90%)		20,8±0,2	
III	6/6 (100%)		24±0,1	
IV	9/15 (60%)		17±2,3	
Stage x2	p=0,17 R -0,13	X^2	p=0.3 R -0,23	U-Mann
I-II	10/12 (92%)		23,75±0,17	
III-IV	15/21 (71%)		19,0±1,7	

Abbreviations: DFS, disease-free survival; OS, overall survival; pN, cervical metastasis (pN0/pN1/pN2a/pN2b/pN3); pNx4 (pN0/pN1/pN2/pN3); pNx2 (pN0+pN1/pN2+pN3), pN- (pN0) *vs*. pN+ (pN1, pN2, pN3); pT, tumor size (pT1,pT2,pT3,pT4); pTx2 (pT1+pT2/pT3+pT4); RR; recurrence rate; Stage (I/II/III/IV); Stage x2 (I+II/III+/IV); (-), negative correlation; (+), positive correlation.

Regarding histopathological factors, margin status had a statistical trend to worse DFS but no other differences were seen with G, DOI, LVI, PNI, PTI, tumor front of invasion or uniformity (Table R11). Significantly, DFS was decreased with a higher number of positive lymph nodes, higher number of lymph nodes with extracapsular involvement (ENE+) or lymph node size (Table R8).

Table R 11: Disease-Free survival vs. histopathological data. Disease-Free survival is expressed in months (mean) + SEM (Standard error of the mean.

	DFS *Yes*	*Test*	***DFS length*** *(months) mean±SEM*	*Test*
	25/33 (76%)		20,39 ±1,2 (2-24)	
G	p=0,11 R -0,30	X^2	p=0,07 R -0,24	U-Mann
G1	15/17:88%		22,29±1,16	
G2	10/16:62,5%		19,06±2,1	
DOI	p=0,18 R -0,18	X^2	p=0.26 R -0,21	K-W
1- 5mm	3/4:75%		23,7±0,2	
>5 - 10 mm	13/15:87%		22,8±0,9	
> 10 mm	9/14:64%		17,57±2,4	
PTI	p=0,06 R 0,17	X^2	p=0,12 R 0,19	K-W
Absence	0		0	
Mild	7/12:58%		16,75±2,6	
Moderate	15/16:94%		23,19±0,7	
Severe	3/5:60%		20,20±3,5	
PTIx2	p=0,1 (+) R 0,30	Fisher	p=0,05 R 0,29	U-Mann
Absence/Mild	7/12:58%		16,75±2,6	
Mod/Severe	18/21:86%		23,00±0,85	
PNI	p=0,7 R -0,05	X^2	p=0,87 R 0,10	U-Mann
Yes	17/22:77%		20,64±1,5	
No	8/11:73%		20,91±2	
LVI	p=0,4 R 0,15	X^2	p=0,31 R -0,23	U-Mann
Yes	11/16:69%		19,38±2	
No	14/17:82%		22±1,3	
Front of invasion	p=0,15 R 0,29	X^2	p=0,10 R 0,31	U-Mann
Infiltrative	18/26:69%		19,85±1,4	
Expansive	7/7:100%		24±0,1	
Uniformity	p=0,12 R 0,29	X^2	p=0,10 R 0,31	U-Mann
Not uniform	7/7:100%		19,85±1,4	
Uniform	18/26:69%		24±0,1	
Margin Status	**p=0.07** R -0.36	X^2	p=0.14 R -0.24	U-Mann
Negative	24/30 (80%)		20±1.2	
Positive	1/3 (33%)		17±5.6	
Margin Status	**p=0.07** R -0.36	X^2	p=0.14 R -0.24	U-Mann

Abbreviations: DFS, disease-free survival; DOI, Depth Of Invasion (>5mm, 5-10mm,>10mm); DFS, Disease-Free Survival; G, Grade; Invasion [expansive (+) *vs.* infiltrative (-)]; LVI, lymphovascular invasion; PNI, perineural invasion; PTI, peritumoral inflammation (mild/moderate/severe) PTIx2 (absent+mild/ moderate + severe); Invasion front [poor defined tumor edges (-) *vs*. well defined edges (+)]; (-), negative correlation; (+), positive correlation.

4.3.2. Recurrence rate

Recurrence rate (RR) was 26% (9/34) involving 24% of men and 29% of women with no statistical difference between gender. 7/34 patients (21%) had local recurrence, 7/34 patients (21%) had regional recurrence, 5/34 (15%) had both local&regional, and 3/34 patients (9%) had distant metastasis (Table R5).

The correlation of recurrence and risk factors showed that smoking, drinking and both smoking and drinking did not show a positive relationship with recurrence in our cohort sample. Patients older than 75years-old had higher rate of recurrence in general but also higher local, regional, and both local&regional recurrence. Among other risk factors, diabetes mellitus and higher HbA1c level were linked to a higher prevalence of recurrence (Table R6; Figure R10).

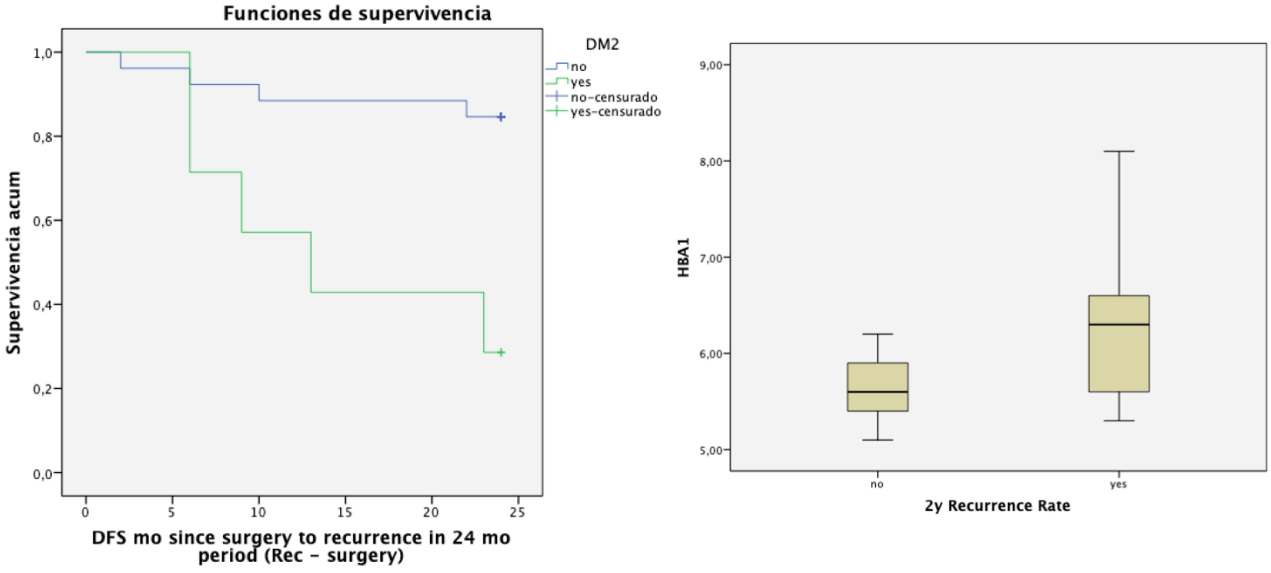


Figure R 10: Relationship of diabetes mellitus and HbA1c with DFS and 2-years general Recurrence Rate (RR). 2-years Kaplan Meier curve showing that diabetic patients have a log-rank relationship with higher Recurrence (left). HbA1c boxplot showing HbA1c is increased on patients with 2-years recurrence (right).

The correlation of RR and clinical data are briefed in Table R7. Results showed that pT classification did not show any relationship with any recurrence. However, recurrence has a statistically positive

correlation with a higher neck involvement (pN) related to a higher recurrence rate in general, local, regional, both local&regional, and distant metastasis. The pN classification pN0/pN1/pN2/pN3 and positive versus negative (pN-/pN+) also showed a correlation with recurrence rate in general, regional and distant metastasis. However, necks with cervical metastasis (pN+) had no relationship with the incidence of local recurrence. The higher number of positive lymph nodes and the ENE+ were strongly associated to recurrence rate, regional recurrence and distant metastasis. The size of the lymph nodes was also associated with distant metastasis (Table R8).

Among the histopathological factors included in the study, we found a correlation between a lower inflammatory peritumoral reaction and a higher regional recurrence rate and both local and regional (Figure R11). Positive margin status was correlated with increased local recurrence (Figure R12). However, G, DOI, PNI, LVI were not related to recurrence analysis (Table R9).

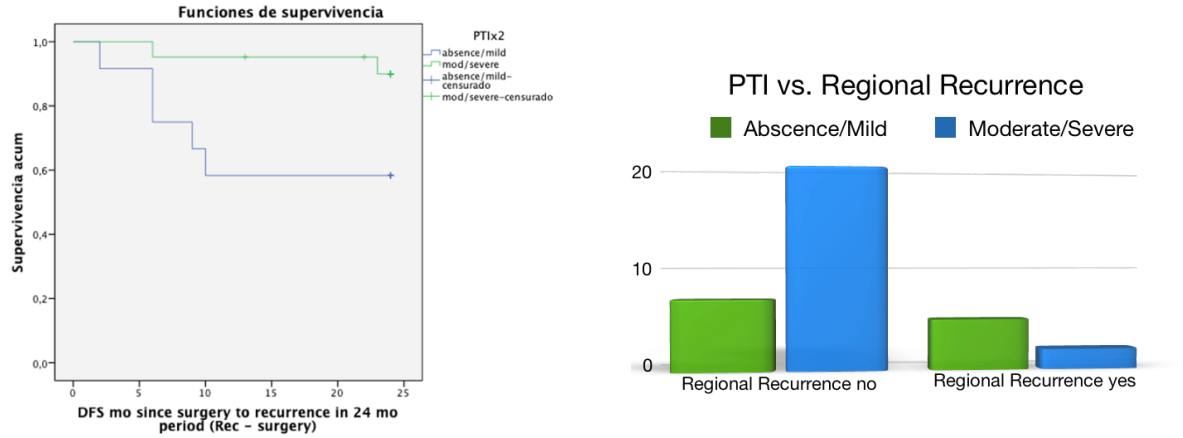


Figure R 11: Relationship of Peritumoral inflammation and Regional Recurrence Rate. 2-years Kaplan Meier curve showing that patients with lower PTI have a log-rank relationship with higher Regional RR (left). Graphics of PTI distribution among patients with 2-years Regional RR showing that patients with higher PTI have a statistical relationship with lower RR (right).

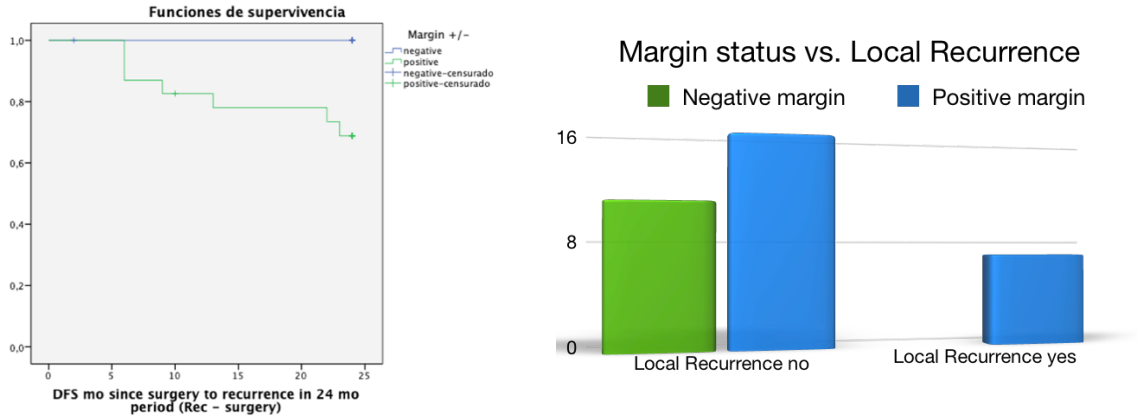


Figure R 12: Relationship of Margin status and Local Recurrence Rate. 2-years Kaplan Meier curve showing that patients with positive margins have a log-rank relationship with higher Local (left).

Graphics of PTI distribution among patients with 2-years Regional RR showing that patients with higher PTI have a statistical relationship with lower RR (right).

4.4. Expression of Somatostatin Receptors in OSCC vs. healthy oral cavity samples

A variable expression level for each of the five SST subtypes was found in OSCC (Figure R13). Specifically, the present work revealed that SST_1 is the dominant SST subtype expressed in healthy oral cavity tissues (mean ± SEM: 9408 ± 2737 mRNA copy number), followed by SST_2>SST_5>SST_4>SST_3 (5245 ± 999; 4432 ± 1437; 2454 ± 1312; 347 ± 157; respectively). In contrast, this profile was found to be altered in OSCC samples being SST_2 the dominant SST subtype expressed (mean ± SEM: 24,245 ± 5730 mRNA copy number), followed by SST_5>SST_4>SST_1>SST_3 (8698 ± 3561; 7295 ± 4381; 6318 ± 2648; 2171 ± 652, respectively). Thus, when we compared the expression levels between OSCC and healthy samples, we found that in general, the expression of all receptors, except SST_1, was increased in OSCC compared with healthy adjacent-control samples, being this increase statistically significant for SST_2 and SST_3 ($p<0.01$ *and* $p<0.001$, respectively). No sex differences were found in the expression of SSTs.

		Mean± S.E.M	Min	Max	Malignant vs Healthy p.value
SSTR1	· Healthy	9408 ± 2737	0,000	89100	
	· Tumor	6318 ± 2648	0,000	85600	p= 0,0607
SSTR2	· Healthy	5245 ± 999,5	0,000	17800	
	· Tumor	24245 ± 5730	0,000	147000	p=0,0024
SSTR3	· Healthy	346,9 ±156,9	0,000	3480	
	· Tumor	2171 ± 652,5	0,000	14100	p=0,0008
SSTR4	· Healthy	2454 ± 1312	0,000	44600	
	· Tumor	7295 ± 4381	0,000	129000	p=0,58
SSTR5	· Healthy	4432 ± 1437	0,000	36400	
	· Tumor	8698 ± 3561	0,000	111000	p=0,2442

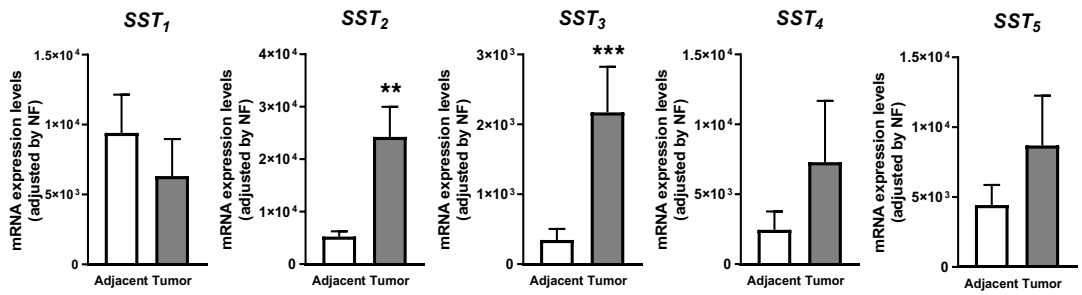


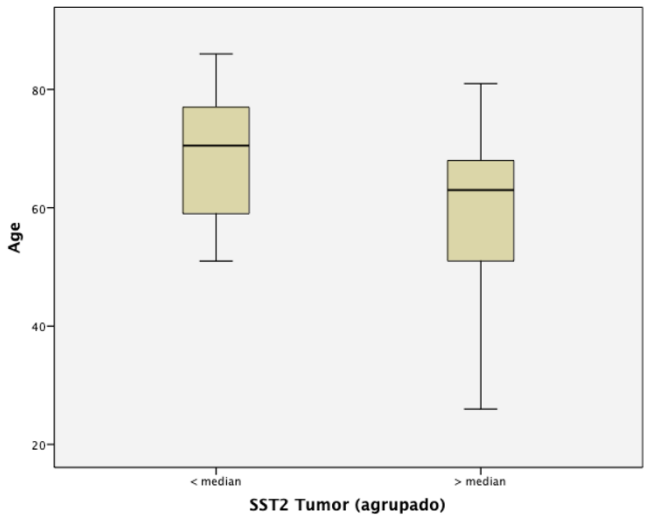
Figure R 13: mRNA expression levels of Somatostatin Receptors. mRNA expression levels of SSTs genes were measured by qPCR and adjusted by normalization factor. Values represent the mean ± SEM. Asterisk represents statistically significant differences (**, $p<0.01$; ***, $p<0.001$).

4.5. *In vivo* association between SST-Subtypes expression in OSCC with relevant risk factors, clinical and histopathological data

The analysis of the relationship of SSTs with the risk factors, clinical and histopathological factors was performed with univariant analysis comparing the receptor expression levels with parametric or non-parametric tests depending on the K-S test. To perform the relationship between the expression levels of SSTs in OSCC tissues and the different risk factors, clinical and histopathological data (including the Kaplan Meier curves), we represented the expression levels of SSTs as numerical or categorical [expression level higher (>) or lower (<) median values]. It should be noted that given the high number of analyses that were performed, and in order to simplify the representation of these associations, we decided to include only the "p" and corresponding "R" values of these analyses in the tables described below.

4.5.1. SSTs expression vs. risk factors

The analysis showed that there were no SSTs differences between men and female. Common OSCC risk factor, such as ever smoking and drinking, had very poor impact on SSTs expression. Age showed a negative correlation with SST_2 and SST_5. Besides, both are overexpressed in patients <45 years-old (Figure R14; Table R12). In general, all SSTs showed a negative correlation to Insulin, HbA1c and BMI (although this difference did not reach statistical significance in all the cases). Patients diagnosed with DM had less expression of SSTs. Regarding other risk factors such as HTN, we found that it had a significant negative correlation with SST_1, SST_2 and SST_3 expression. The expression of SST_2 and SST_3 had a negative correlation with CRP (Table R12-R13).



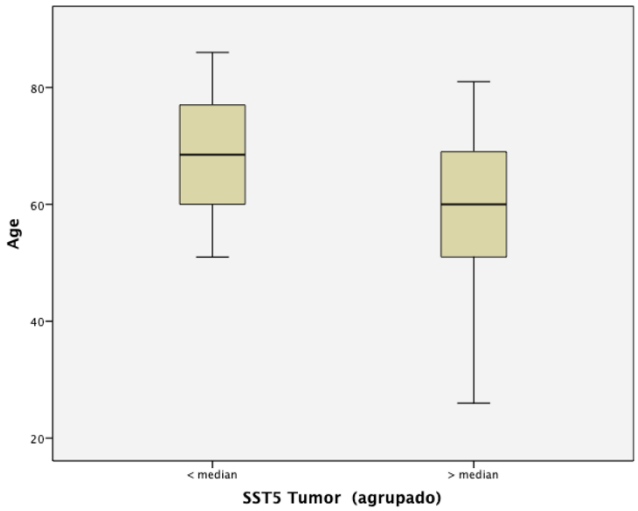


Figure R 14: Relationship between the expression levels of SST_2 and SST_5 with age. SST_2 and SST_5 expression has a negative correlation with age. SST_2 and SST_5 expression above median is present in younger patients.

Table R 12: SSTs Categorical expression and its relationship with risk factors. SSTs Categorical expression is expressed as >/< median. Chi2, Fisher or T-student tests are used to analyze the relationship between SSTs expression and risk factors. (-), negative correlation; (+), positive correlation.

	SST_1 *>/< median*	***SST_2*** *>/< median*	***SST_3*** *>/< median*	***SST_4*** *>/< median*	***SST_5*** *>/< median*	***Test***
Gender *Women/Men*	p=0.31 R -0,19	p=1 R 0.02	p=0.31 R -0.19	p=0.5 R -0.14	p=1 R 0.02	Fisher
Age *Continuous*	p=0.39 R -0,15	**p=0.02 (-)** R -0.39	p=0.33 R -0.16	p=0,38 R -0.15	**p=0.02 (-)** R -0.38	Fisher
>75y *No/**Yes***	p=0.71 R 0.08	p 0.12 R 0.31	p=1 R -0.04	p=1 R 0.05	p=0.12 R -0.31	Fisher
<45y *No/Yes*	p=0.33 R 0.19	**p=0.04 (+)** R 0.37	p=1 R 0.10	p=1 R 0.07	**p=0.04 (+)** R 0.37	Fisher
Ever Smoking *No/Yes*	p=0.18 R -0.25	p=0.74 R 0.08	p=1 R -0.02	p=0.3 R -0.21	p=1 R -0.02	Fisher
Drinking *No/Yes*	p=0.72 R 0.08	p=0.72 R 0.08	p=0.72 R 0.08	p=0.28 R 0.20	p=1 R -0.04	Fisher
DLP *No/Yes*	p=0.11 R -0.33	p=1 R -0.06	p=0.43 R -0.20	p=0.25 R -0.23	p=0.43 R -0.20	Fisher
HBP *No/Yes*	**p=0.04 (-)** R -0,38	**p=0.04 (-)** R -0.38	**p=0.04 (-)** R -0.38	p=0.29 R -0.23	p=0.73 R 0.08	Fisher
DM *No/Yes*	p=0.12 R – 0.31	p=0.12 R -0.31	p=0.44 R -0.17	p=0.34 R -0.21	p=1 R -0.04	Fisher
BMI *Cm2/kg*	p=0.25 R -0.14	p=0.06 R -0.29	p=0.17 R -0.28	**p=0.05 (-)** R -0.36	p=0.14 R -0.25	T-student
CRP	p=0.44 R -0.17	**p=0.01(-)** R -0.45	**p=0.05 (-)** R -0.32	p=0.11 R -0.25	p=0.98 R 0.07	T-student
HbA1c *(%)*	p=0.59 R -0.26	p=0.95 R -0.10	p=0.97 R -0.16	p=0.95 R -0.17	p=0.82 R -0.03	T-student
</>6,5	p =0.60	p= 0.60	p=0.50	p=0.39	p=0.65	Fisher
Insulin	p=0.44 R -0.20	p=0.14 R -0.23	p=0.12 R -0.22	p=0.19 R -0.23	p=0.40 R -0.14	T-student

Abbreviations: BMI, body mass index; CRP, C-reactive protein; DM, diabetes mellitus; DLP, dyslipidemia; HbA1c, hemoglobin A1c; HBP, high blood pressure;(-), negative correlation; (+), positive correlation.

Table R 13: SSTs Numerical expression and its relationship with risk factors. Spearman test and Nonparametric Kruskal-Wallis and U-Mann Whitney tests are used to analyze the relationship between SSTs expression and risk factors. (-), negative correlation; (+), positive correlation.

	SST_1 *Numerical*	***SST_2*** *Numerical*	***SST_3*** *Numerical*	***SST_4*** *Numerical*	***SST_5*** *Numerical*	***Test***
Gender	p=0.12	p=0.21	p=0.19	p=0.46	p=0.61	U-Mann
Women/Men	R -0.27	R -0.21	R -0.23	R -0.14	R -0.12	
Age	p=0.76	p=0.34	p=0.10	p=0,87	p=0,40	Spearman
Continuous	R -0.05	R -0.16	R -0.28	R -0.02	R -0.14	
>75y	p=0.44	p=0.56	p=0.38	p=0.43	p=0.40	U-Mann
*No/**Yes***	R 0.13	R -0.10	R – 0.16	R 0.09	R -0.15	
<45y	p=0.27	p=0.23	p=0.46	p=0.1	**p=0.07 (+)**	U-Mann
No/Yes	R 0.20	R 0.21	R 0.13	R 0.00	R 0.31	
Ever Smoking	p=0.08	p=0.59	p=0.83	p=0.23	p=0.61	U-Mann
No/Yes	R -0.31	R -0.09	R -0.04	R -0.23	R -0.09	
Drinking	p=0.93	p=0.58	p=0.58	p=0.37	p=0.76	U-Mann
No/Yes	R 0.16	R -0.09	R 0.10	R 0.17	R -0.05	
DLP	p=0.16	p=0.28	p=0.21	p=0.29	p=0.33	U-Mann
No/Yes	R -0.34	R -0.19	R -0.22	R -0.20	R -0.17	
HTN	**p=0.06 (-)**	**p=0.08 (-)**	**p=0.02 (-)**	p=0.38	p=0.40	U-Mann
No/Yes	R -0.33	R -0.29	R -0.40	R -0.17	R 0.15	
DM	p=0.09	**p=0.06 (-)**	**p=0.08 (-)**	p=0.34	p=0.81	U-Mann
No/Yes	R -0.29	R -0.32	R -0.31	R -0.19	R -0.04	
BMI	p=0.42	p=0.10	p=0.11	**p=0.04 (-)**	p=0.16	Spearman
Cm2/kg	R -0.26	R -0.13	R -0.30	R -0.39	R -0.25	
CRP	p=0.34	**p=0.01 (-)**	**p=0.07 (-)**	p=0.16	p=0.69	Spearman
	R -0.17	R -0.29	R -0.36	R -0.24	R 0.04	
***HbA1c** (%)*	p=0.25	p=0.63	p=0.23	p=0.67	p=0.69	Spearman
	R -0.21	R -0.09	R -0.22	R -0.08	R -0.07	
</>6,5	p=1	p=0.64	p=1	p=0.46	p=0.46	U-Mann
Insulin	p=0.26	p=0.20	p=0.21	p=0.19	p=0.42	Spearman
	R -0.20	R -0.11	R -0.28	R -0.30	R -0.11	

Abbreviations: BMI, body mass index; CRP, C-reactive protein; DM, diabetes mellitus; DLP, dyslipidemia; HbA1c, hemoglobin A1c; HBP, high blood pressure;(-), negative correlation; (+), positive correlation

4.5.2. SSTs expression vs. OS and Recurrence

Our results revealed that higher expression of *SST_2* (the dominant SST subtype expressed in OSCC samples) was related to a lower rate of regional recurrence and both local&regional recurrence (Table R14; Figure R15). We also found a trend for significant association between higher expression of *SST_2* and lower presence of distant metastasis. Moreover, we found that higher expression of *SST_5* showed a trend for a lower incidence of both local&regional recurrence (Table R14). No relationship was found between SSTs expression and OS.

Table R 14: *In vivo* association between SST-subtypes expression in OSCC with Overall Survival (OS), Recurrence Rate (RR), and Distant Metastasis. SSTs expression is expressed as categorical with ">/< median" analysis. *P-values* are calculated with Log-rank test for the analysis between SSTs >/< median analysis and OS, overall RR, Local RR, Regional RR, Local&Regional RR, and Distant Metastasis. U-Mann Whitney test is used for the analysis between SSTs Numerical analysis and OS, overall RR, Local RR, Regional RR, Local&Regional RR, and Distant Metastasis.

	OS	***RR***	***Local RR***	***Regional RR***	***Local& Regional RR***	***Distant Metastasis***
SST_1 >/< *median*	p=0.39 R -0.20	p=0.45 R 0.10	p=0.99 R -0.04	p=0.97 R -0.04	p=0.29 R -0.23	p=0.7 R -0.08
SST_2 >/< *median*	p=0.14 R 0.16	p=0.23 R -0.20	p=0.13 R -0.22	**p=0.04 (-)** R -0.37	**p=0.02 (-)** R – 0.43	**p=0.06 (-)** R -0.32
SST_3 >/< *median*	p=0.46 R -0.08	p=0.71 R 0.07	p=0.73 R -0.07	p=0.70 R 0.07	p=0.65 R- 0.08	p=0.53 R 0.10
SST_4 >/< *median*	p=0.78 R 0.05	p=0.53 R -0.09	p=0.42 R -0.13	p=0.41 R -0.13	p=0.27 R -0.18	p=0.14 R -0.26
SST_5 >/< *median*	p=0.38 R -0.20	p=0.38 R 0.01	p=0.48 R 0.01	p=0.47 R – 0.16	**p=0.05 (-)** R -0.38	p=0.76 R -0.06
SST_1 *Numerical*	p=0.95 R -0.17	p=0.92 R 0.13	p=0.70 R -0.02	p=0.41 R 0.03	p=0.41 R -0.15	p=0.12 R -0.06
SST_2 *Numerical*	p=0.77 R 0.16	p=0.68 R -0.23	p=0.96 R -0.21	**p=0.02** R -0.40	**p=0.02** R -0.41	p=0.58 R -0.07
SST_3 *Numerical*	p=0.27 R -0.01	p=0.44 R -0.08	p=0.44 R -0.21	p=0.54 R -0.08	p=0.54 R -0.23	p=0.66 R -0.06
SST_4 *Numerical*	p=0.42 R 0.01	p=0.96 R -0.07	p=0.78 R -0.09	p=0.76 R -0.11	p=0.76 R -0.15	p=0.66 R -0.07
SST_5 *Numerical*	p=0.07 R -0.17	p=0.22 R -0.08	p=0.10 R -0.25	p=0.29 R -0.18	**p=0.02** R -0.39	p=0.53 R -0.06

Abbreviations: OS, overall survival; RR, recurrence rate; (-), negative correlation; (+), positive correlation.

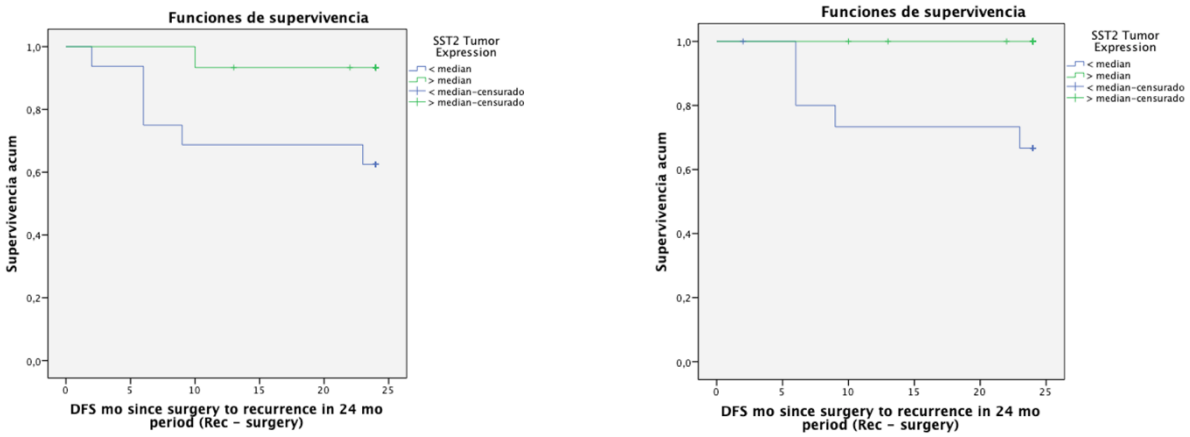


Figure R 15: Relationship between SST_2 expression and Regional RR and both Local&Regional RR. 2-years Kaplan Meier curve showing that patients with expression of SST_2 below the mean have a

log-rank relationship with higher Regional Recurrence (left) and both Local&Regional Recurrence (right).

4.5.3. SSTs expression vs. staging data and histopathological factors

The analysis showed that SST_2 expression was statistically increased in patients with less cervical nodal disease [pN (*p=0.02*), pNx4 (*p=0.02*), pNx2(*p=0.05*)]. Patients with pN- vs. pN+ showed higher SST_2 expression (Table R15)

Table R 15: *In vivo* relationship between SST-subtypes expression in OSCC and Staging data. SSTs Numerical expression is expressed as mean ± SEM in each category. Nonparametric Kruskal-Wallis and U-Mann Whitney tests are used to analyze the relationship between SSTs expression and Staging data.

	pT	***pTx2***	***pN***	***pNx4***	***pN x2***	***pN-/pN+***
SST_1 Numerical	*p=0.37* pT1:0 pT2:14515 ± 10312 pT3: 318394 ±312755 pT4:10161 ±6971	*p=0.34* pT1+pT2:12579 ±8985 pT3+pT4:133414 ±124958	*p=0.74* pN0: 133837 ±124939 pN1: 4819 ±3000 pN2a: 5003 ± 3128 pN2b: 68309 ± 67711 pN2c: 811 ± 811 pN3b: 2544 ±1910	*p=0.78* pN0:133837 ±124939 pN1: 4819 ±3000 pN2: 24709 ± pN3: 2544 ±1910	*p=0.92* pN0+pN1: 112334 ±104147 pN2+pN3:14633 ± 12181	*p=0.63* pN-: 133837 ±124939 pN+: 12016 ± 8921
SST_2 Numerical	*p=0.80* pT1: 23645±20298 pT2:25851±7180 pT3:321684±305878 pT4:24318 ±12041	*p=0.34* pT1+pT2: 25557±6501 pT3+pT4:143264 ±122292	***p=0.02, (-)*** pN0: 151455 ±121862 pN1: 12049 ±6652 pN2a: 36269 ±19590 pN2b: 41679 ±33279 pN2c: 3003 ±561 pN3b:1887 ±1074	***p=0.02, (-)*** pN0:151455 ±121862 pN1: 12049 ± 6652 pN2:26984±12566 pN3: 1887 ±1074	***p=0.05, (-)*** pN0+pN1:128221±101693 pN2+pN3:15576±7674	***p=0.03, (-)*** pN-:151455 ±121862 pN+:14636±5792
SST_3 Numerical	*p=0.41* pT1: 7051±7051 pT2: 1520615±151915 pT3:255331±251582 pT4:11187±9756	*p=0.19* pT1+pT2: 1318806±131685 pT3+pT4:108845±100565	*p=0.51* pN0:109093±100554 pN1:2189±1436 pN2a:2166±1935 pN2b:9877900±987692 pN2c: 0 pN3b:1648±1084	*p=0.13* pN0:109093±100554 pN1:2189±1436 pN2: 3293315±329228 pN3:1648 ± 1084	*p=0.97* pN0+pN1: 91275±8342 pN2+pN3: 1797125±179576	*p=0.94* pN-:109093±100554 pN+:1318475±1316879
SST_4 Numerical	*p=0.65* pT1:0 pT2:83614±75783 pT3:25890±25558 pT4: 11869±10693	*p=0.67* pT1+pT2:72466±65757 pT3+pT4:17477±11767	*p=0.33* pN0:67623±49981 pN1:1224±495 pN2a: 2012±2012 pN2b:35105±33658 pN2c:0 pN3b:989±989	*p=0.62* pN0: 67623±49981 pN1:1224±495 pN2:12372±11296 pN3:989±989	*p=0.87* pN0+pN1:56556±41789 pN2+pN3:7198±6180	*p=0.65* pN-:56556±41789 pN+:5605±4529
SST_5 Numerical	*p=0.24* pT1:0 pT2:5915±2586 pT3:174997±173940 pT4:17632±9363	*p=0.97* pT1+pT2:5126±2292 pT3+pT4:80597±69296	*p=0.87* pN0:793751±69346 pN1:11092±10581 pN2a:4833±1503 pN2b:7949±6748 pN2c:1575±1192 pN3b:5589±4638	*p=0.77* pN0:793751±69346 pN1:11092±10581 pN2:4786±2153 pN3:5589±4638	*p=0.58* pN0+pN1:67991±57803 pN2+pN3:5151±2278	*p=0.63* pN-: 793751±69346 pN+:6735±3100

Abbreviations: pN, cervical metastasis (pN0/pN1/pN2a/pN2b/pN3); pNx4 (pN0/pN1/pN2/pN3); pNx2 (pN0+pN1/pN2+pN3), pN- (pN0) *vs*. pN+ (pN1, pN2, pN3); pT, tumor size (pT1,pT2,pT3,pT4); pTx2 (pT1+pT2/pT3+pT4); Stage (I/II/III/IV); Stage x2 (I+II/III+/IV); (-), negative correlation; (+), positive correlation.

The negative relationship between SST_2 expression and pN was also observed with the categorical analysis where patients with higher SST_2 expression presented less cervical nodal disease [pN (p=0.04), pNx4 (p=0.05), pN-/pN+ (p=0.03); Table R16]. SST_2 had no relationship with pT; however, SST_3 expression above the median had a positive correlation with a higher pT and a higher Stage [pNx2 (p=0.03) and Stage×2 (p=0.04), respectively; Table R16].

Table R 16: *In vivo* relationship between SST-subtypes expression in OSCC and histopathological data. SSTs Categorical expression is expressed as >/< median. Chi2 or Fisher tests are used to analyze the relationship between SSTs expression and histopathological data.

	pT	***pTx2***	***pN***	***pNx4***	***pNx2***	***pN-/pN+***	***Stage***	***Stagex2***
SST_1 >/< *median*	p=0.46 R 0.13	p=0.5 R 0.14	p=0.41 R -0.01	p=0.30 R -0.01	p=0.54 R -0.04	p=0.44 R 0.08	p=0.37 R 0.17	p=0.17 R 0.22
SST_2 >/< *median*	p=0.68 R -0.15	p=0.20 R -0.19	**p=0.04 (-)** R -0.43	**p=0.05 (-)** R -0.43	p=0.08 R – 0.29	**p=0.03 (-)** R -0.38	p=0.42 R -0.19	p=0.11 R -0.26
SST_3 >/< *median*	p=0.13 R 0.33	**p=0.03 (+)** R 0.38	p=0.49 R 0.08	p=0.94 R 0.08	p=0.63 R= 0.08	p=0.62 R 0.08	p=0.16 R 0.33	**p=0.04 (+)** R 0.34
SST_4 >/< *median*	p=0.60 R 0.17	p=0.36 R 0.11	p=0.18 R 0.01	p=0.34 R 0.01	p=0.53 R -0.05	p=0.51 R 0.11	p=0.50 R 0.20	p=0.28 R 0.22
SST_5 >/< *median*	p=0.36 R 0.01	p=0.44 R -0.08	p=0.79 R 0.04	p=0.79 R 0.04	p=0.73 R 0.08	p=0.73 R 0.08	p=0.37 R=0.07	p=0.59 R -0.02
	G	**DOIx3**	**PTI**	**PTIx2**	**PNI**	**LVI**	**Invasion Front**	**Uniformity**
SST_1 >/< *median*	p=0.43 R 0.08	p=0.17 R 0.23	p=0.60 R 0.08	p=0.41 R 0.09	p=0.71 R 0.10	p=0.31 R 0.20	**p=0.08 (+)** R 0.31	**p=0.08 (+)** R 0.31
SST_2 >/< *median*	p=0.56 R -0.02	**p=0.04 (-)** R -0.21	**p=0.06 (+)** R 0.20	**p=0.08 (+)** R 0.32	p=0.57 R 0.01	p=0.60 R 0.02	**p=0.08 (+)** R 0.31	**p=0.08 (+)** R 0.31
SST_3 >/< *median*	p=0.56 R -0.02	p=0.52 R 0.17	p=0.37 R 0.13	p=0.58 R -0.02	p=0.10 R -0.27	p=0.31 R – 0.14	p=0.30 R 0.16	p=0.30 R 0.16
SST_4 >/< *median*	p=0.41 R -0.09	p=0.79 R 0.09	p=0.29 R 0.11	p=0.31 R 0.19	p=0.15 R -0.25	p=0.22 R -0.18	**p=0.02 (+)** R 0.40	**p=0.03 (+)** R 0.40
SST_5 >/< *median*	p=0.18 R -0.25	p=0.86 R -0.07	p=0.54 R 0.02	p=0.73 R 0.09	p=0.73 R 0.10	p=0.71 R 0.08	**p=0.01 (+)** R =0.46	**p=0.01 (+)** R =0.46

Abbreviations: DOI, Depth Of Invasion (>5mm, 5-10mm,>10mm); DFS, Disease Free Survival; G, Grade; Invasion [expansive (+) *vs.* infiltrative (-)]; LVI, lymphovascular invasion; OS, overall survival; pN, cervical metastasis (pN0/pN1/pN2a/pN2b/pN3); pNx4 (pN0/pN1/pN2/pN3); pNx2 (pN0+pN1/pN2+pN3), pN- (pN0) *vs*. pN+ (pN1, pN2, pN3); perineural invasion; pT, tumor size (pT1,pT2,pT3,pT4); pTx2 (pT1+pT2/pT3+pT4); PTI, peritumoral inflammation (mild/moderate/severe) PTIx2 (absent+mild/ moderate + severe); RR recurrence rate; Stage (I/II/III/IV); Stage x2 (I+II/III+/IV); Invasion front [poor defined tumor edges (-) *vs*. well defined edges (+)]; (-), negative correlation; (+), positive correlation.

Regarding histopathological factors, SST_5 expression was statistically increased in G1 tumors (*p=0.02*; Table R17), which are the well differentiated tumors. Moreover, $SST_{1,2,4,5}$ expression was statistically increased in OSCC that had an expansive front of tumor invasion compared to OSCC with an infiltrative front of tumor invasion (*p=0.01, p=0.03, p=0.05, p<0.01*, respectively; Table R17). Similarly, our results showed that $SST_{1,2,4,5}$ were overexpressed in OSCC with uniform tumor invasion edges compared to poorly defined ones (*p=0.08, p =0.08, p=0.02, p<0.01*; Table R16) (*p=0.01, p=0.03, p=0.05, p<0.01*, respectively; Table R17) (Figure R16).

Our data also revealed that patients with higher SST_2 expression more frequently presented tumor depth of invasion (DOI) of 5-10 mm compared to >10 mm and <5 mm (*p=0.04*; Table R16; Figure R17). SST_2 expression also showed a statistical tendency to be present on tumors which showed a higher peritumoral inflammation reaction [PTI (*p=0.06*) and PTIx2 (*p=0.08*); Table R16].

Table R 17: *In vivo* relationship between SST-subtypes expression in OSCC and histopathological factors. SSTs numerical expression is expressed as mean ± SD in each category. Spearman test and Nonparametric Kruskal-Wallis and U-Mann Whitney tests are used to analyze the relationship between SSTs expression and histopathological data.

	G	***DOI***	***DOIx3***	***PTI***	***PTIx2***
SST_1 *Numerical*	p=0.73 G1:143400±138998 G2:16222±9026	p=0.68 R -0.08	p=0.77 <5mm:0 5-10mm: 180295±166375 >10mm: 9534±5349	p=0.42 Mild: 2586±1178 Moderate:16856±156073 Severe: 8339±4533	p=0.90 Abs+mild: 8513±6027 Mod+sev:130371±118966
SST_2 *Numerical*	p=0.96 G1: 154884±135804 G2:27144±9505	p=0.42 R – 0.08	p=0.11 <5mm: 14620±9821 5-10mm: 190516±162368 >10mm:20776±9683	p=0.11 Mild: 10567±3660 Moderate:177377±152420 Severe:25336±14448	p=0.22 Abs+mild: 20281±10287 Mod+sev: 141177±116175
SST_3 *Numerical*	p=0.88 G1:114126±111900 G2:1170866±1161515	p=0.82 R -0.13	p=0.68 <5mm: 1525±3525 5-10mm: 1452796±1314132 >10mm: 9559±7310	p=0.14 Mild: 1133±452 Moderate: 1363582±1232491 Severe: 1702±1702	p=0.75 Abs+mild: 9511±8388 Mod+sev:1039325±940660
SST_4 *Numerical*	p=0.93 G1:55744±54997 G2:25478±13957	p=0.86 R -0.08	p=0.81 <5mm: 558±558 5-10mm:72668±65741 >10mm:21517±14620	p=0.18 Mild: 629±397 Moderate:19210±13131 Severe:198323±198093	p=0.39 Abs+mild: 9825±9203 Mod+sev: 61856±47519
SST_5 *Numerical*	**p=0.02 (-)** G1:86155±76907 G2:8097±6496	p=0.91 R -0.06	p=0.53 <5mm: 2890±2430 5-10mm:97734±92515 >10mm:13180±7217	p=0.44 Mild: 6133±3537 Moderate:91039±86784 Severe:8202±6380	p=0.80 Abs+mild :13630±8181 Mod+sev :71315±66089
	PNI	**LVI**	**Invasion front**	**Uniformity**	
SST_1 *Numerical*	p=0.81 Yes: 106694±100039 No:18963±13299	p=0.75 Yes:139111±131580 No:13367±8440	**p=0.01 (+)** Expansive: 448221±412110 Infiltrative: 5781±3024	**p=0.01 (+)** Uniform: 448221±412110 Non-Uniform: 5781±3024	
SST_2 *Numerical*	p=0.81 Yes: 117537±97878 No: 31020±10019	p=0.80 Yes: 150406±128653 No: 24433±7121	**p=0.03 (+)** Expansive: 446069±403333 Infiltrative: 19732±5736	**p=0.03 (+)** Uniform: 446069±403333 Non-uniform: 19732±5736	
SST_3 *Numerical*	p=0.15 Yes: 86476±80549 No:1979708±1975010	p=0.42 Yes: 113586±105890	p=0.17 Expansive: 3630478±3241581	p=0.17 Uniform: 3630478±3241581	

		No: 1237553±1234483	Infiltrative: 6073±4069	Non-uniform: 6073±4069
SST_4 ***Numerical***	p=0.15 Yes: 46034±39704 No: 28568±20697	p=0.31 Yes: 8520±6834 No: 79666±62136	**p=0.05 (+)** Expansive: 177277±163059 Infiltrative: 12858±8194	**p=0.05, (+)** Uniform: 177277±163059 Non-uniform: 12858±8194
SST_5 ***Numerical***	p=0.17 Yes: 66279±55467 No: 3146±575	p=0.63 Yes: 83845±72960 No: 5962±2429	**p<0.01 (+)** Expansive: 245340±28491 Infiltrative: 7462±40152	**p<0.01, (+)** Uniform: 245340±28491 Non-uniform: 7462±40152

Abbreviations: DOI, Depth Of Invasion (1-5mm, 5-10mm,>10mm); DFS, Disease Free Survival; G, Grade; Invasion [expansive (+) *vs.* infiltrative (-)]; LVI, lymphovascular invasion; PNI, perineural invasion; PTI, peritumoral inflammation (mild/moderate/severe) PTIx2 (absent+mild/ moderate + severe); Invasion front [poor defined tumor edges (-) *vs*. well defined edges (+)]; (-), negative correlation; (+), positive correlation.

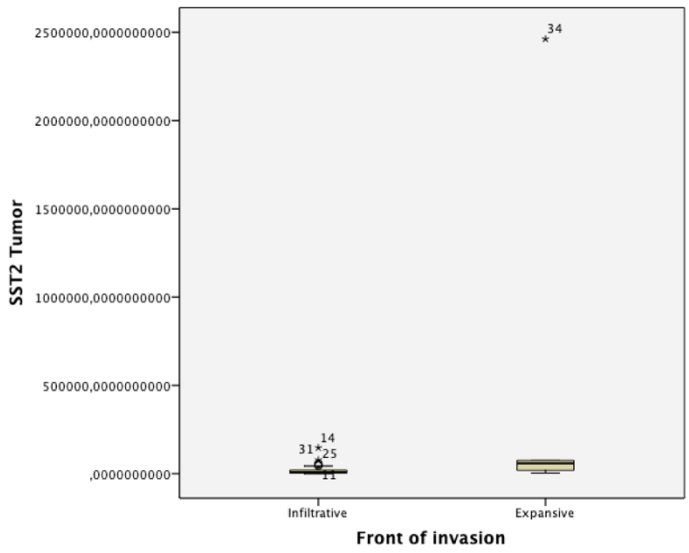


Figure R 16: Relationship between the expression levels of SST_2 and tumor invasion front. SST2 expression is > median on tumors with expansive front of invasion.

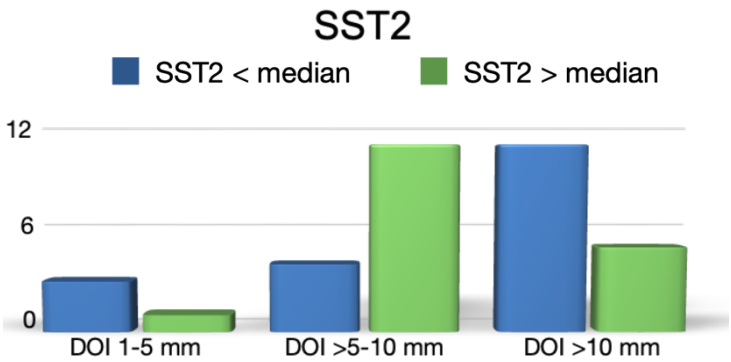


Figure R 17: Relationship between the expression levels of SST_2 and Depth of Invasion (DOI). *SST_2* higher expression is more frequently present with tumor depth of invasion (DOI) of 5-10 mm.

Finally, we found that the expression of SST_2 in OSCC had a negative correlation to the number of positive lymph nodes, the number of lymph nodes with ENE+, and their bigger size ($p<0.01$, $p=0.03$, and $p=0.05$, respectively; Table R18, Figure R18, Figure R19.

Table R 18: *In vivo* relationship between SST-subtypes expression in OSCC and lymph node pathological data. Spearman correlation test was used for the analysis between SSTs numerical expression and lymph node results.

	Nº + lymph	*Nº ENE+*	*Size (mm)*
SST1	*p=0.68*	*p=0.83*	*p=0.29*
Numerical	*R 0.07*	*R. 0.04*	*R 0.20*
SST2	**p<0.01 (-)**	**p=0.03 (-)**	**p=0.05 (-)**
Numerical	R -0.535	R -0.40	R -0.36
SST3	*p=0.63*	*p=0.96*	*p=0.62*
Numerical	*R 0.09*	*R -0.01*	*R 0.09*
SST4	*p=0.88*	*p=0.64*	*p=0.28*
Numerical	*R 0.02*	*R -0.08*	*R 0.20*
SST5	*p=0.94*	*p=0.84*	*p=0.89*
Numerical	*R 0.01*	*R 0.03*	*R 0.03*

Abbreviations: ENE+, extranodal extension; (-), negative correlation; (+), positive correlation.

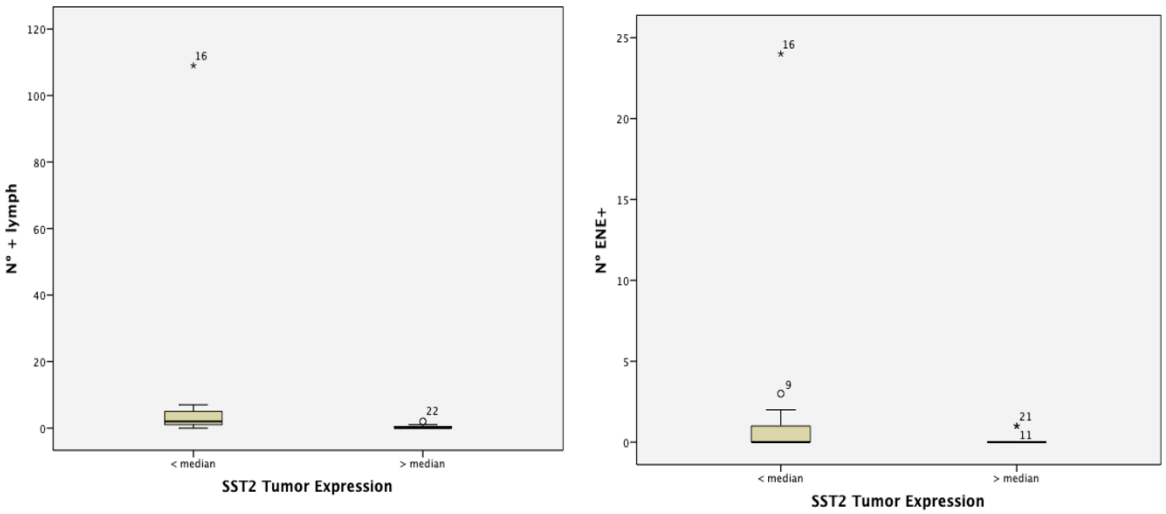


Figure R 18: Relationship of SST2 expression and number of positive lymph nodes (Nº + lymph) and number of lymph nodes with ENE+ (Nº ENE+). Patients with more Nº + lymph nodes (left) and more Nº lymph nodes with ENE+ (right) have < median SST2 expression. ENE+: Extranodal extension.

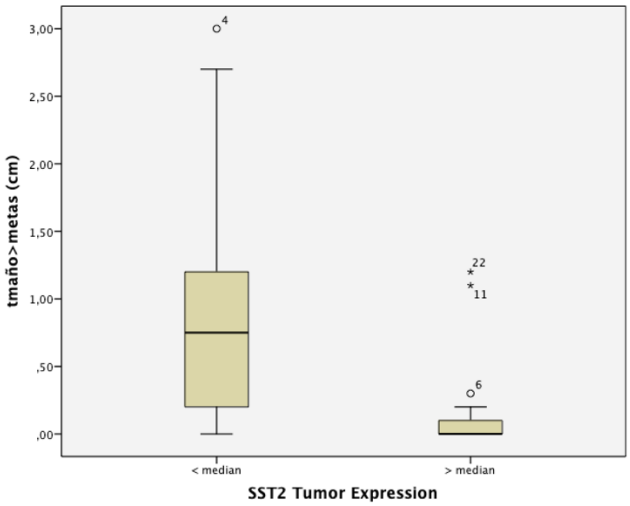


Figure R 19: Relationship of SST2 expression and size of positive lymph nodes. Bigger size lymph nodes have < median SST2 expression.

4.6. Antitumor Actions of First- and Second-Generation Somatostatin Analogues on Patient-Derived Primary Oral Squamous Carcinoma Cell Cultures

In the present study, we also explored the effect of different SSAs (Octreotide, Lanreotide, and Pasireotide) on the proliferation rate of patient-derived primary OSCC cell cultures. Remarkably, our results demonstrated that all SSAs tested (10^{-7} M) significantly reduce the proliferation rate of primary OSCC cell cultures OSCC (Figure R13). Specifically, all SSAs decreased proliferation rate at 24-, 48-, and 72-h of incubation (this inhibition was not statistically significant in the case of Octreotide at 24h and Lanreotide at 72-h; Figure R20).

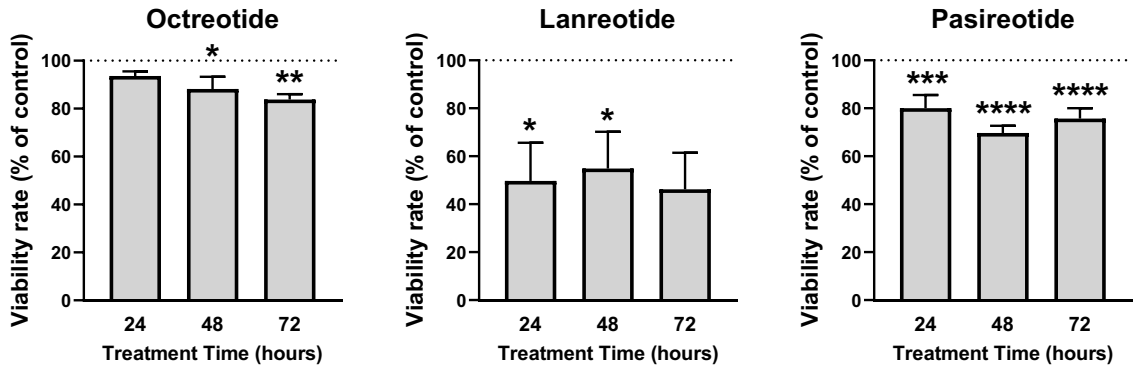


Figure R 20: Effect of different somatostatin analogues (Octreotide, Lanreotide and Pasireotide) on cell proliferation OSCC primary cell cultures. Proliferation rate (24- to 72- hour treatment) was measured by Alamar-blue reduction. Data are expressed as percent of vehicle-treated control (set at

100%). Values represent the mean ± SEM (n=3-4 tumors, 3-4 replicates/treatment). Asterisk represents statistically significant differences (*, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001)

4.7. Dysregulation of the expression of splicing machinery components in OSCC vs. healthy oral cavity samples

Another objective of this Doctoral Thesis was to determine the potential dysregulation of key components of the splicing machinery in OSCC as well as to explore the underlying relationship between this dysregulation with risk factors, clinical data and histopathological factors. To that end, the first step was to analyze the expression pattern of a selected set of key spliceosome components and splicing factors in the OSCC compared with non-tumoral adjacent tissue samples.

As expected, OSCC microfluidic array analysis of the spliceosomal landscape revealed a profound dysregulation of splicing machinery components (spliceosome and splicing factors), been significantly altered 12 of 59 (20 %) (Figure R21). Specifically, we found a clear downregulation of the splicing machinery components on the tumor sample with a p value <0.05 for *TRA2B, TIA1, SRSF4*, a p value <0.01 for *SRSF9, TRA2A*, a p value <0.001 for *ESRP1, NOVA1*), and a p value <0.0001 for *SRSF5, ESRP2, RBM10, RBM3*. In contrast, *SRSF10* was found to be upregulated in OSCC samples (p<0.05) (Figure R22).

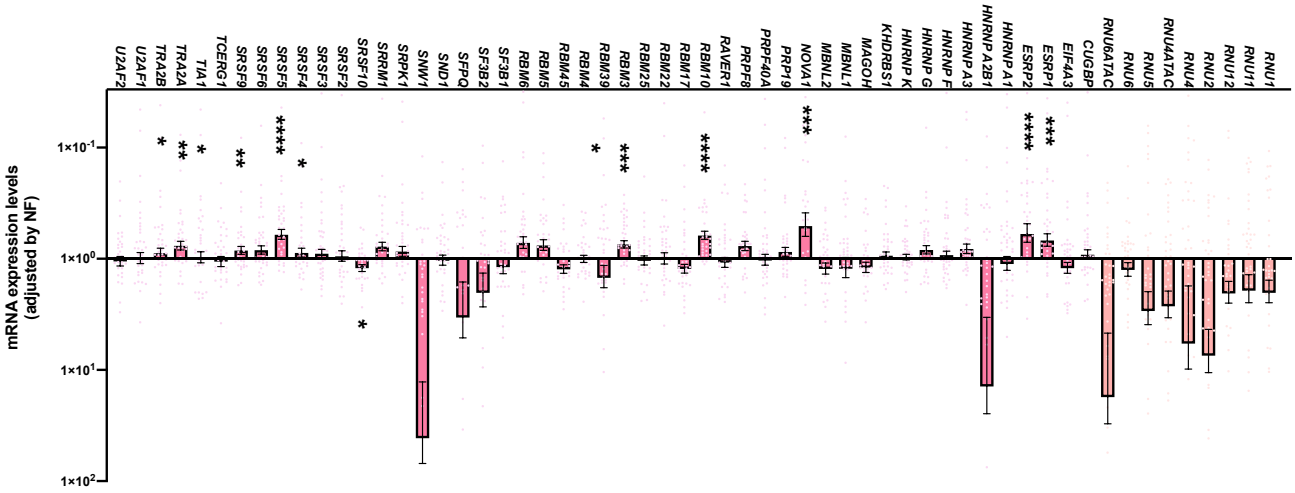


Figure R 21: Spliceosome components and splicing factors are dysregulated in OSCC. mRNA expression levels of splicing machinery components in OSCC samples compared with non-tumoral adjacent tissue. Data are represented by mRNA levels normalized by NF (Normalization factor; ACTB, HPRT and GAPDH) expression levels. (*, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001).

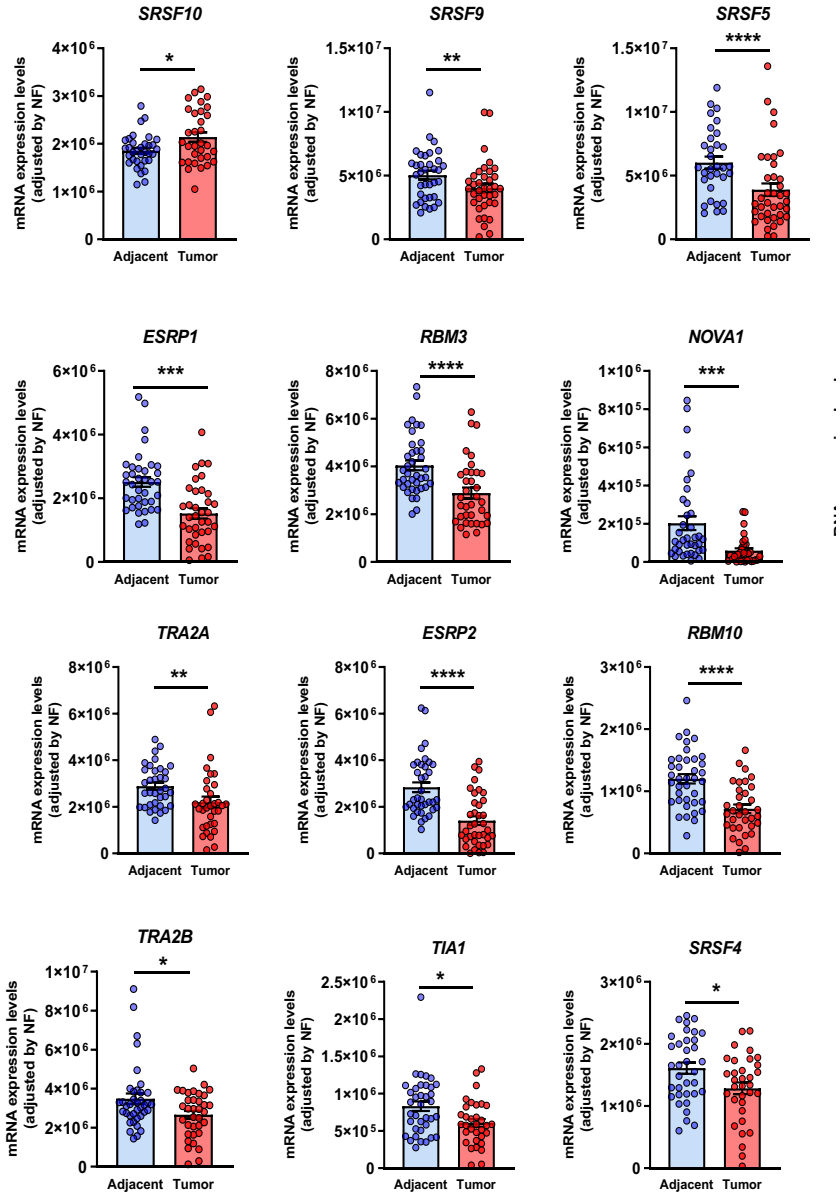


Figure R 22: Graphic description of mRNA expression levels of Dysregulated Spliceosome components in OSCC. mRNA expression levels of splicing machinery components in OSCC samples compared with non-tumoral adjacent tissue. Data are represented by mRNA levels normalized by ACTB expression levels. (*, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001).

Although non-supervised clustering bioinformatic approach (random-forest algorithm; Figure R23-A) with the expression levels of all the components of the splicing machinery analyzed did not reveal a clear clusterization or discrimination pattern between tumoral and controls tissues, a Sparse Partial Least Squares Discriminant Analysis (sPLS-DA; Figure R23-B) indicated a differential pattern of alteration between both samples types that was strong enough to clearly separate the tumor and non-

tumor groups. Moreover, a Variable Importance in Projection (VIP) Score of the PLS-DA analysis showed that the spliceosome components and splicing factors with a higher capacity of discrimination between the two samples groups were ESRP1, RBM10, ESRP2, RBM3 and NOVA1, being the most relevant genes for the classification model (VIP-Score > 1.8) (Figure R23-C).

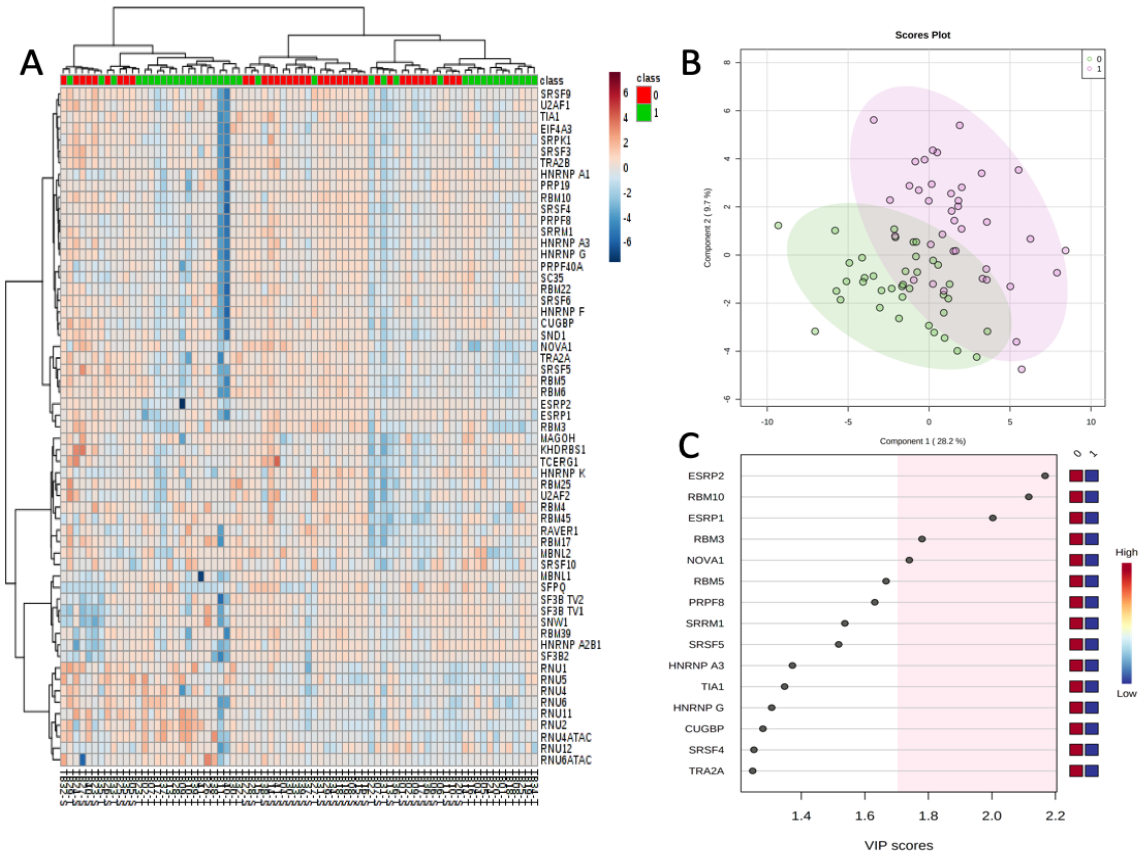


Figure R 23: Discriminatory value of splicing machinery components and splicing factors in OSCC. A) Unsupervised clustering analysis (Non-hierarchical heatmap) using the expression levels of the components of the splicing machinery in OSCC samples (1: green) compared with non-tumoral adjacent tissue (0: pink). Loading plot showing the capacity genes to discriminate between tumor vs. non-tumor adjacent tissues of the splicing machinery components analyzed in OSCC samples cohort. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study. **B)** Principal components analysis of the mRNA expression levels of the splicing machinery components in OSCC (1. green) and non-tumoral adjacent tissue (0: pink). **C)** VIP scores obtained from partial least squares discriminant analysis (PLS-DA) of all splicing machinery components studied.

For this reason, unsupervised hierarchical cluster analysis was repeated only with the top 5 genes of the VIP-Score identified with the higher capacity of discrimination between the tumor and non-tumor samples (ESRP1, RBM10, ESRP2, RBM3 and NOVA1) (Figure R24). These set of splicing machinery components allowed discriminating between the two sample types with higher capacity than the represented in Figure R24-A. Furthermore, a multiple receiver operating characteristic (ROC) curve analysis with the expression levels of these 5 components of the splicing machinery generated an area under the curve (AUC) of 0.876 95 % CI 0.742 – 0.961 (Figure R24-B), which demonstrate a potential capacity of discrimination of the selected components of the splicing machinery between tumor and non-tumor samples.

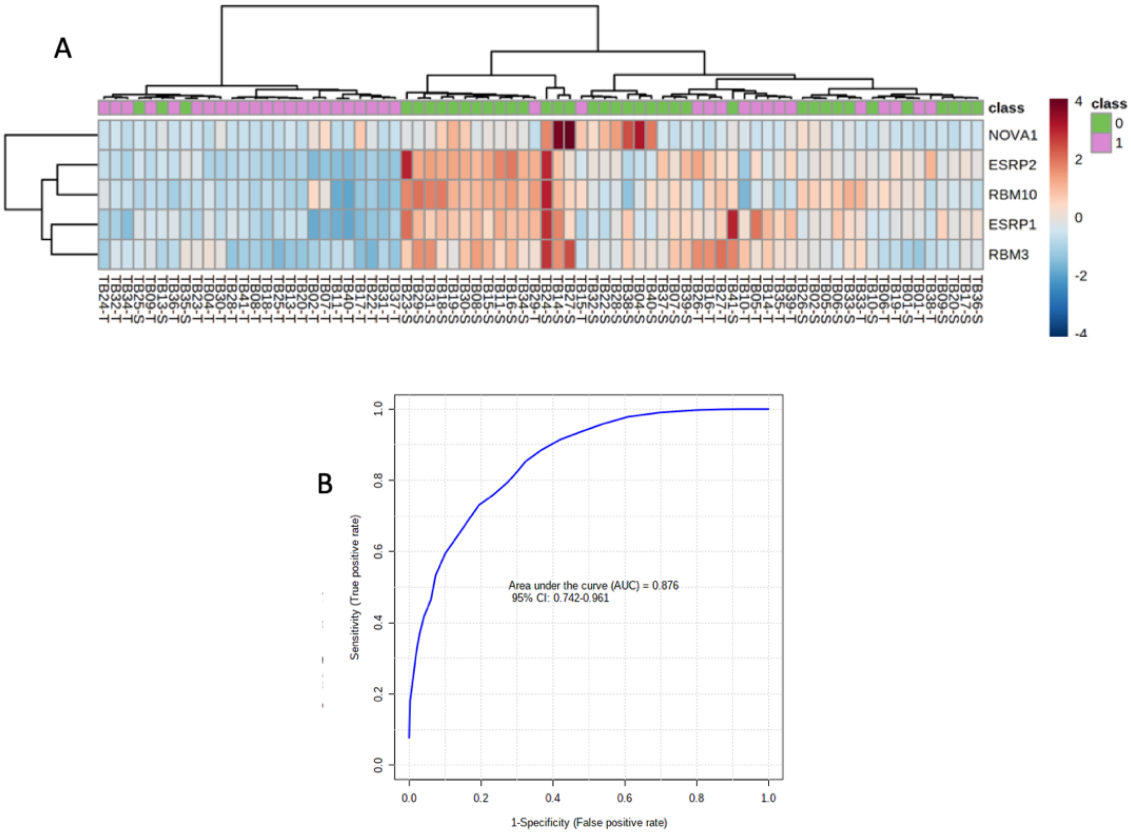


Figure R 24: Discriminatory value of top 5 genes of splicing machinery components and splicing factors in OSCC (ESRP1, RBM10, ESRP2, RBM3 and NOVA1). A) Unsupervised clustering analysis of mRNA expression levels of the 5 spliceosome components and splicing factors in OSCC samples (1; green) compared with non-tumoral adjacent tissue (0; red). **B)** Receiver operating characteristic (ROC) curve analysis with the expression levels of these 5 components of the splicing machinery.

Furthermore, individual ROC curve analysis with the 12 spliceosome components and splicing factors that were found to be significantly altered in OSCC samples compared with non-tumoral adjacent tissue (Figure R22) were also performed. As can be observed in Figure R25, AUC obtained ranged from 0.617 to 0.810. These data demonstrate that the expression of the spliceosomal components, and specially *SRSF10, SRSF9, SRSF5, TRA2A, ESRP2, RBM10, ESRP1, RBM3, NOVA1, TRA2B, TIA1, and SRSF4* are clearly dysregulated in OSCC samples and could serve as potential diagnostic biomarkers of OSCC. For this reason, we next studied the potential association between the dysregulation of the splicing machinery with risk factors, clinical, and histopathological characteristic of OSCC patients/samples was explored.

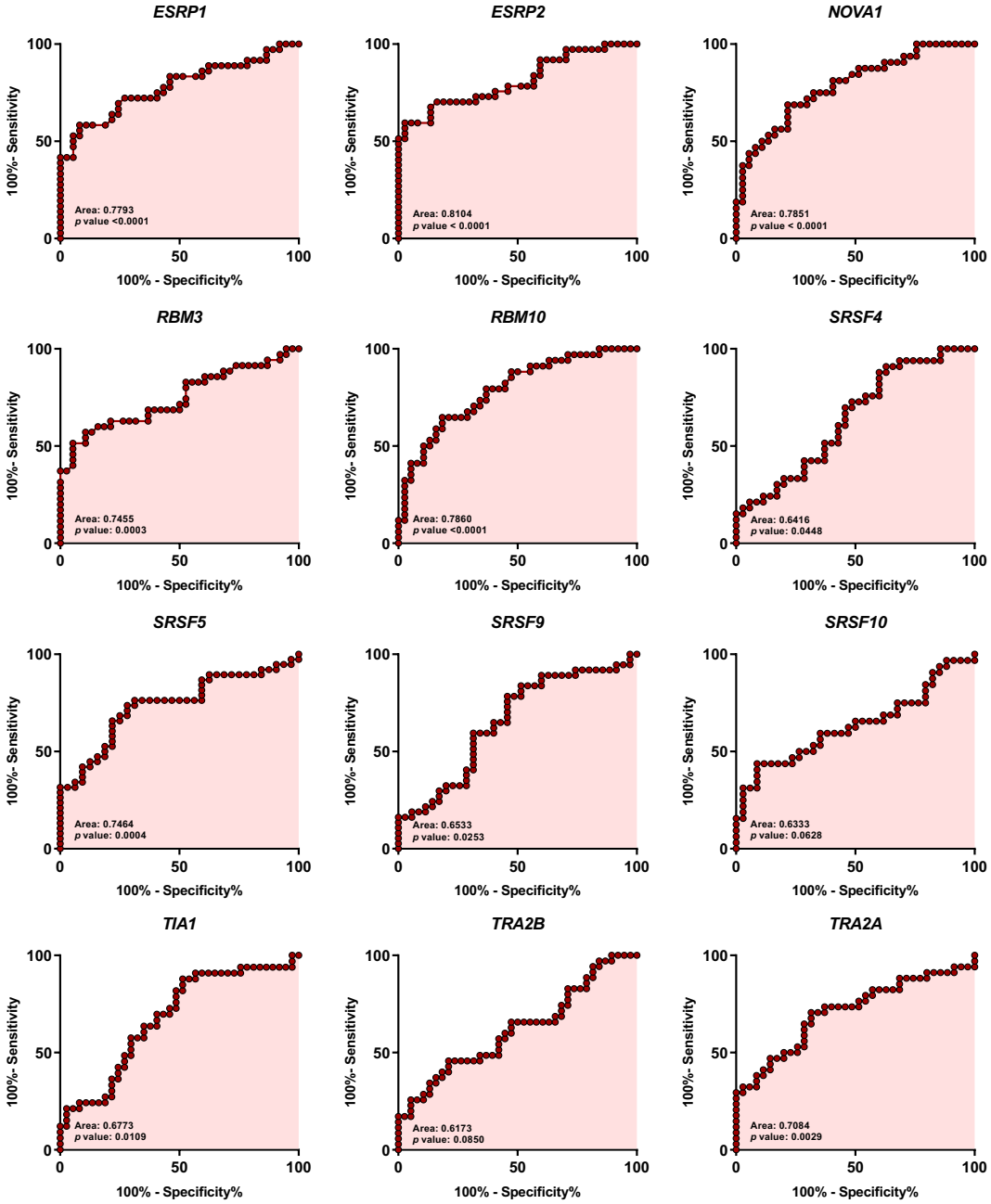


Figure R 25: ROC curve analysis of significantly expressed splicing machinery components in OSCC samples. ROC curve analysis of individual 12 spliceosome components and splicing factors

significantly altered in OSCC samples compared with non-tumoral adjacent tissue. AUC obtained ranged from 0.617 to 0.810.

4.8. *In vivo* association between the dysregulation of the expression of splicing machinery components in OSCC with risk factors, clinical and pathological data

The analysis of the relationship between the expression of components of the splicing machinery and risk, clinical and histopathological factors of OSCC patients was performed with univariant analysis comparing the expression levels with parametric or non-parametric tests depending on the K-S test. To perform the relationship between the expression levels in OSCC tissues and the different variables, we represented the expression levels of mRNA as numerical or categorical [expression level higher (>) or lower (<) median values]. It should be noted that given the high number of analyses that were performed, and in order to simplify the representation of these associations, we also decided to include only the "p" and corresponding "R" values of these analyses in the tables described below.

4.8.1. Splicing dysregulation vs. risk factors

We found a positive correlation between *SRSF4 and SRSF9* expression and gender (men). (Figure R26). Age showed a correlation with *SRSF5, SRSF9, and TRA2A* expression, with higher mRNA levels among patients >75y (Table R19).

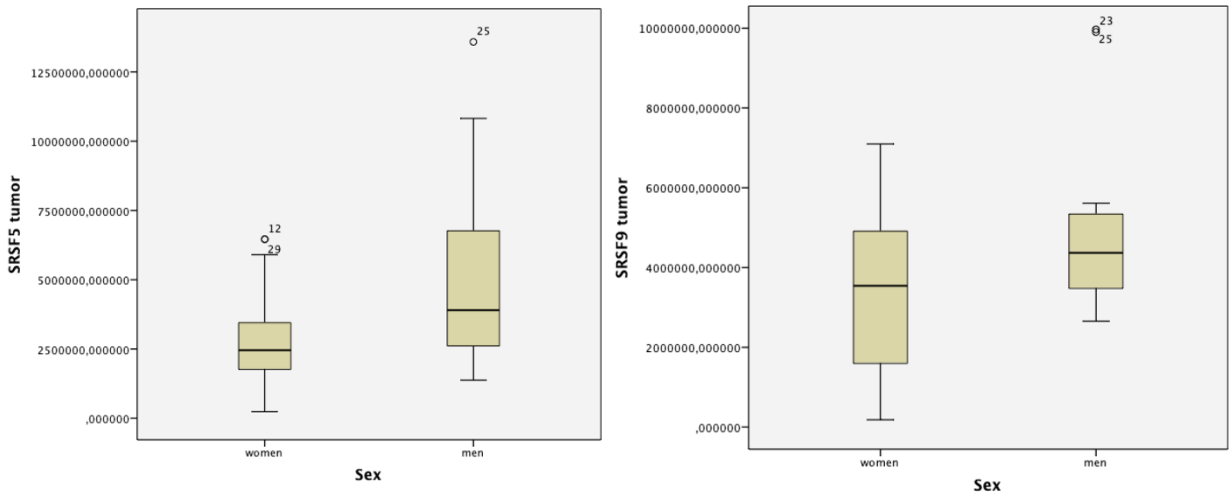


Figure R 26: Relationship of splicing factors SRSF5 and SRSF9 and gender. Boxplots showing that the expression of both, SRSF5 (left) and SRSF9 (right), is increased among men.

Table R 19: *In- vivo* splicing numerical expression and its relationship with risk factors. Spearman test and Nonparametric Kruskal-Wallis and U-Mann Whitney tests are used to analyze the relationship between Splicing factors expression and risk factors. (-), negative correlation; (+), positive correlation.

	SRSF4 ***Numerical***	***SRSF5*** ***Numerical***	***SRSF9*** ***Numerical***	***SRSF10*** ***Numerical***	***NOVA1*** ***Numerical***	***RBM3*** ***Numerical***	***RBM10*** ***Numerical***	*Test*
Gender	p=0.36	**p=0.03 (+)**	**p=0.04 (+)**	p=0.40	p=0.55	p=0.24	p=0.69	T-student
Women/Men	R 0.13	R 0,35	R 0.29	R 0.10	R 0.08	R 0.16	R 0.25	
Age	p=0.11	p=0.43	p=0.17	p=0.88	p=0.54	p=0.18	p=0.23	Pearson
Continuous	R -0.27	R -0.13	R -0.22	R 0.02	R 0.10	R 0.22	R -0.19	
>75y	p=0.22	**p=0.06 (+)**	p=0.01(+)	p=0.74	p=0.27	p=0.57	p=0.17	T-student
*No/**Yes***	R 0.20	R 0.35	R 0.45	R -0.01	R -0.01	R -0.13	R 0.20	
<45y	p=0.16	p=0.53	p=0.96	p=0.60	p=0.63	p=0.14	p=0.56	T-student
No/Yes	R 0.22	R -0.13	R -0.02	R -0.08	R 0.15	R -0.27	R 0.09	
Ever Smoking	p=0.41	p=0.26	p=0.54	p=0.77	p=0.23	p=0.70	p=0.64	T-student
No/Yes	R 0.27	R 0.17	R 0.17	R 0.24	R 0.16	R 0.16	R 0.28	
Drinking	p=0.46	p=0.32	p=0.18	**p=0.02 (+)**	p=0.56	**p=0.05 (+)**	p=0.22	T-student
No/Yes	R 0.18	R 0.13	R 0,30	R 0.37	R 0.12	R 0.25	R 0.25	
DLP	p=0.85	p=0.93	p=0.27	p=0.75	p=0.40	p=0.25	p=0.98	T-student
No/Yes	R -0.04	R 0.08	R 0.23	R 0.04	R -0.03	R 0.13	R 0.00	
HTN	**p=0.03 (-)**	**p=0.03 (-)**	p=0.10	**p=0.05 (-)**	p=0.65	**p=0.06 (-)**	**p=0.02 (-)**	T-student
No/Yes	R -0.43	R -0.33	R -0.31	R -0.37	R -0.20	R -0.31	R -0.41	
DM	p=0.43	p=0.54	p=0.94	p=0.33	p=0.39	p=0.99	p=0.94	T-student
No/Yes	R -0.12	R 0.07	R -0.07	R -0.22	R -0.10	R 0.05	R -0.06	
BMI	**p=0.03(-)**	p=0.72	p=0.32	p=0.33	**p=0.06 (-)**	p=0.13	p=0.75	Pearson
Cm2/kg	R -0.39	R -0.06	R -0.17	R -0.19	R -0.33	R -0.27	R -0.05	
CRP	p=0.19	p=0.17	p=0.33	p=0.43	p=0.54	p=0.18	p=0.97	Pearson
	R -0.24	R -0.23	R -0.17	R 0.15	R -0.11	R -0.24	R 0.00	
HbA1c *(%)*	p=0.95	p=0.92	p=0.70	p=0.73	p=0.72	p=0.77	p=0.60	Pearson
	R -0.01	R 0.01	R 0.07	R 0.06	R 0.06	R 0.05	R 0.09	
</>6,5	p=0.55	p=0.69	p=0.28	p=0.86	p=0.59	p=0.34	p=0.55	T-student
Insulin	p=0.12	p=0.31	p=0.57	p=0.77	p=0.29	**p=0.01 (-)**	p=0.45	Pearson
	R -0.28	R -0.17	R -0.09	R -0.05	R -0.18	R-0.48	R -0.13	
	ESRP1 ***Numerical***	***ESRP2*** ***Numerical***	***TRA2A*** ***Numerical***	***TRA2B*** ***Numerical***	***TIA1*** ***Numerical***			Test
Gender	p=0.39	p=0.27	p=0.10	p=0.08 (+)	p=0.26			T-student
Women/Men	R 0.20	R 0.28	R 0.25	R 0.26	R 0.13			
Age	p=0.27	p=0.98	p=0.39	p=0.85	p=0.41			Pearson
Continuous	R 0.18	R 0.04	R - 0.15	R - 0.03	R - 0.14			
>75y	p=0.48	p=0.40	**p=0.04 (+)**	p=0.28	p=0.24			T-student
*No/**Yes***	R -0.09	R 0.12	R 0.39	R 0.16	R 0.16			
<45y	p=0.30	p=0.24	p=0.53	p=0.62	p=0.70			T-student
No/Yes	R -0.17	R -0.19	R -0.06	R -0.11	R -0.03			
Ever Smoking	p=0.91	p=0.54	p=0.18	p=0.26	p=0.20			T-student
No/Yes	R 0.05	R 0.07	R 0.17	R 0.20	R 0.21			
Drinking	p=0.79	p=0.38	p=0.38	p=0.89	p=0.49			T-student
No/Yes	R 0.07	R 0.08	R 0.23	R 0.03	R -0.02			
DLP	p=0.41	p=0.33	p=0.50	p=0.67	p=0.64			T-student
No/Yes	R 0.14	R 0.16	R 0.24	R 0.09	R 0.12			
HTN	p=0.76	p=0.10	**p=0.09 (-)**	p=0.63	p=0.18			T-student
No/Yes	R -0.05	R -0.27	R -0.32	R -0.02	R -0.23			
DM	p=0.54	p=0.63	p=0.90	p=0.43	p=0.77			T-student
No/Yes	R 0.10	R 0.08	R -0.09	R 0.15	R 0.13			
BMI	p=0.37	p=0.33	p=0.91	p=0.33	p=0.76			Pearson
Cm2/kg	R -0.16	R -0.17	R -0.09	R -0.17	R -0.05			
CRP	p=0.82	p=0.26	p=0.87	p=0.51	p=0.49			Pearson
	R 0.04	R -0.20	R -0.02	R -0.11	R -0.12			
HbA1c *(%)*	p=0.27	p=0.91	p=0.94	p=0.63	p=0.62			Pearson
	R 0.20	R 0.01	R 0.01	R 0.08	R 0.09			
</>6,5	p=0.85	p=0.65	p=0.67	p=0.47	p=0.70			T-student
Insulin	p=0.82	p=0.51	p=0.99	p=0.31	p=0.39			Pearson
	R -0.04	R -0.11	R -0.02	R -0.18	R -0.15			

Abbreviations: BMI, body mass index; CRP, C-reactive protein; DM, diabetes mellitus; DLP, dyslipidemia; HbA1c, hemoglobin A1c; HBP, high blood pressure;(-), negative correlation; (+), positive correlation

Among the common OSCC risk factors, such as ever smoking and drinking, the later was present with higher expression of *SRSF10 and RBM3* (Table R19). Higher expression of *SRSF4 and NOVA1* was found among patients with lower BMI (Table R19). Higher expression of *RBM3* was present among patients with lower levels of insulin (Table R19). However, no correlation with CRP, DM or HbA1c was found. Regarding other risk factors such as HTN we found that it had a significant negative correlation with all splicing factors expression with a significant correlation or trend with *SRSF4, SRSF5, SRSF10, RBM3, RBM10, TRA2A* (Table R19).

4.8.2. Splicing dysregulation vs OS and Recurrence

Our results revealed that OS was positive correlated with higher expression of *SRSF5, SRSF9, RBM3, TRA2A, and TRA2B* (Table R20). Recurrence rate showed a positive statistical trend with lower expression of *TRA2B* (Table R20). We also found association between lower expression of *SRSF9* and local recurrence, and both local&regional recurrence (Table R20). Lower *NOVA1 and RBM3* expression was associated with distant metastasis. (Table R20; Figure R27).

Table R 20: *In vivo* association between splicing factors expression in OSCC with Overall Survival (OS), Recurrence Rate (RR), and Distant Metastasis. Splicing expression is expressed as categorical with ">/< median" analysis. *P-values* are calculated with Log-rank test for the analysis between >/< median analysis and OS, overall RR, Local RR, Regional RR, Local&Regional RR, and Distant Metastasis. U-Mann Whitney test is used for the analysis between Splicing Numerical analysis and OS, overall RR, Local RR, Regional RR, Local&Regional RR, and Distant Metastasis.

	OS	***RR***	***Local RR***	***Regional RR***	***Local& Regional RR***	***Distant Metastasis***	*Test*
SRSF4	p=0.35	p=0.91	p=0.61	p=0.42	p=0.20	p=0.90	T-student
Numerical	R 0.13	R 0.00	R -0.08	R -0.17	R -0.27	R -0.01	
SRSF5	**p=0.03 (+)**	p=0.47	p=0.65	p=0.40	p=0.44	p=0.76	T-student
Numerical	R 0.38	R -0.04	R -0.01	R -0.10	R -0.13	R 0.03	
SRSF9	**p=0.08 (+)**	p=0.32	p=0.35	p=0.25	p=0.18	p=0.93	T-student
Numerical	R 0.34	R -0.07	R -0.15	R -0.21	R -0.26	R 0.05	
SRSF10	p=0.13	p=0.63	p=0.94	p=0.23	p=0.34	p=0.35	T-student
Numerical	R 0.27	R -0.09	R -0.02	R -0.37	R -0.22	R -0.20	
NOVA1	p=0.57	p=0.96	p=0.35	p=0.14	p=0.13	p=0.28	T-student
Numerical	R 0.05	R -0.05	R -0.17	R -0.33	R -0.36	R -0,22	
RBM3	**p=0.09 (+)**	p=0.84	p=0.87	p=0.50	p=0.60	p=0.20	T-student
Numerical	R 0.30	R 0.01	R 0.05	R -0.10	R -0.09	R -0.22	
RBM10	p=0.67	p=0.72	p=0.32	p=0.51	p=0.24	p=0.96	T-student
Numerical	R 0.05	R -0.08	R -0.14	R -0.07	R -0.16	R -0.01	
ESRP1	p=0.14	p=0.63	p=0.90	p=0.49	p=0.44	p=0.70	T-student
Numerical	R 0.22	R -0.05	R 0.01	R -0.06	R -0.10	R 0.14	

ESRP2	p=0.14	p=0.44	p=0.43	p=0.85	p=0.54	p=0.27	T-student
Numerical	R 0.27	R -0.10	R -0.08	R -0.01	R -0.05	R 0.21	
TRA2A	**p=0.04 (+)**	p=0.69	p=0.80	p=0.40	p=0.40	p=0.73	T-student
Numerical	R 0.41	R -0.06	R -0.06	R -0.14	R -0.19	R -0.03	
TRA2B	**p=0.05 (+)**	p=0.34	p=0.37	p=0.15	p=0.40	p=0.64	T-student
Numerical	R 0,37	R -0.22	R -0.19	R -0.28	R -0.28	R -0.14	
TIA1	p=0.44	p=0.90	p=0.87	p=0.74	p=0.13	p=0.85	T-student
Numerical	R 0.06	R 0.17	R 0.16	R -0.04	R 0.05	R 0.00	
	OS	***RR***	***Local RR***	***Regional RR***	***Local& Regional RR***	***Distant Metastasis***	*Test*
SRSF4	p=0.82	p=0.33	p=0.79	p=0.85	p=0.41	p=0.93	Log-rank
>/< median	R -0.03	R 0.15	R 0.00	R -0.07	R -0.19	R 0.00	
SRSF5	p=0.30	p=0.99	p=0.92	p=0.77	p=0.51	p=0.66	Log-rank
>/< median	R 0.29	R 0.03	R 0.04	R -0.04	R -0.10	R 0.8	
SRSF9	**p=0.05 (+)**	p=0.11	**p=0.07 (-)**	p=0.14	**p=0.03 (-)**	p=0.98	Log-rank
>/< median	R 0.26	R -0.30	R -0.32	R -0.28	R -0.38	R -0.01	
SRSF10	p=0.17	p=0.94	p=0.83	p=0.24	p=0.44	p=0.11	Log-rank
>/< median	R 0.23	R 0.00	R 0.02	R -0.25	R -0.16	R -0.32	
NOVA1	p=0.35	p=0.46	p=0.46	**p=0.06 (-)**	p=0.10	**p=0.05 (-)**	Log-rank
>/< median	R 0.27	R -0.07	R -0.07	R -0.31	R -0.25	R -0.32	
RBM3	p=0.21	p=0.71	p=0.62	p=0.44	p=0.82	**p=0.05 (-)**	Log-rank
>/< median	R 0.35	R -0.01	R 0.14	R -0.12	R -0.01	R -0.33	
RBM10	p= 0.82	p=0.96	p=0.52	p=0.83	p=0.48	p=0.71	Log-rank
>/< median	R 0.01	R 0.01	R -0.12	R -0.03	R -0.12	R 0.07	
ESRP1	p=0.42	p=0.84	p=0.86	p=0.83	p=0.88	p=0.19	Log-rank
>/< median	R 0.14	R -0.03	R -0.03	R 0.04	R -0.02	R 0.24	
ESRP2	p=0.32	p=0.67	p=0.70	p=0.94	p=0.77	p=0.22	Log-rank
>/< median	R 0.28	R -0.05	R-0.04	R 0.03	R -0.03	R 0.23	
TRA2A	**p=0.02 (+)**	p=0.21	p=0.18	p=0.40	p=0.18	p=0.85	Log-rank
>/< median	R 0.43	R -0.23	R -0.23	R -0.17	R -0.25	R -0.03	
TRA2B	p=0.14	**p=0.07 (-)**	p=0.25	p=0.10	p=0.22	p=0.13	Log-rank
>/< median	R 0.40	R -0.27	R -0.14	R -0.26	R -0.18	R -0.25	
TIA1	p=0.58	p=0.17	p=0.10	p=0.65	p=0.34	p=0.93	Log-rank
>/< median	R 0.00	R 0.30	R 0.33	R 0.10	R -0.19	R 0.00	

Abbreviations: Overall survival (OS); recurrence rate (RR); (-), negative correlation; (+), positive correlation.

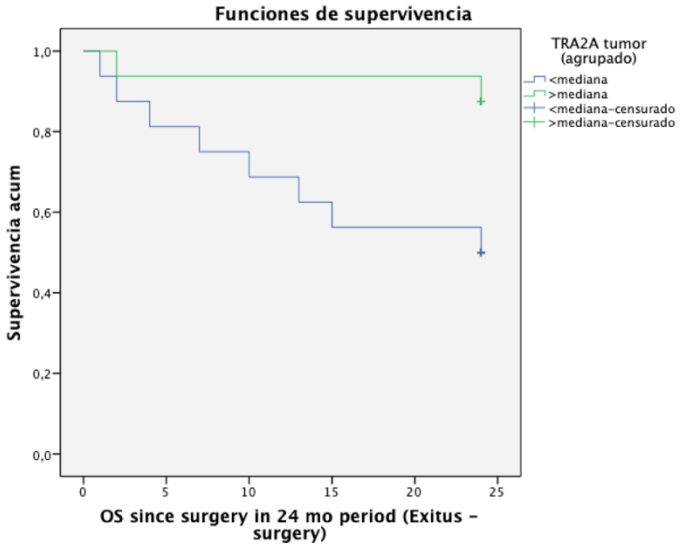


Figure R 27: Relationship between TRA2A expression and OS analysis. 2-years Kaplan Meier Survival curve showing that patients with expression of TRA2 above the median have a better survival.

4.8.3. Splicing dysregulation expression vs. Staging data and histopathological factors.

The Numerical analysis showed a trend for smaller tumors (smaller pT) when expression of *SRSF9 and TRA2A* was higher [pT (*p=0.08, p=0,08*); pTx2 (*0,08*); (Figure R28)]. This was also observed with the categorical analysis where also patients with higher expression of *TIA1* showed a trend for smaller pT [pT (*p=0.09*; pTx2 (*0,05*); Table R21].

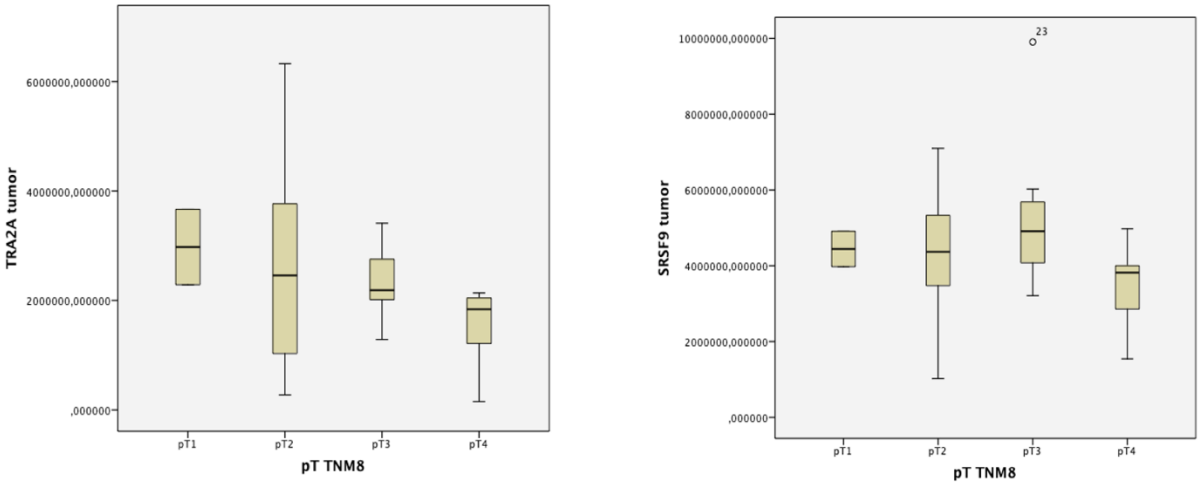


Figure R 28. Relationship between *SRSF9 and TRA2A* expression and pT. Boxplots showing that the expression of TRA2A (left) and SRSF9 (right) is increased in smaller tumors

Patients with higher expression of *TRA2A, TRA2B and TIA1* presented with less cervical nodal disease [pN-/pN+ (*p=0.07, p= 0,06, p=0,03*)], and less Stage [Stage x2 (*p=0.03, p= 0,09, p=0,05*); Figure R29, Figure R30, Figure R31; Table R21]. This was also observed with the categorical analysis where also patients with higher expression of *RBM3* had a trend with less cervical metastasis [pN-/pN+ (*p=0.07)*] and less Stage [Stage x2 (*p=0.09);* Table R21].

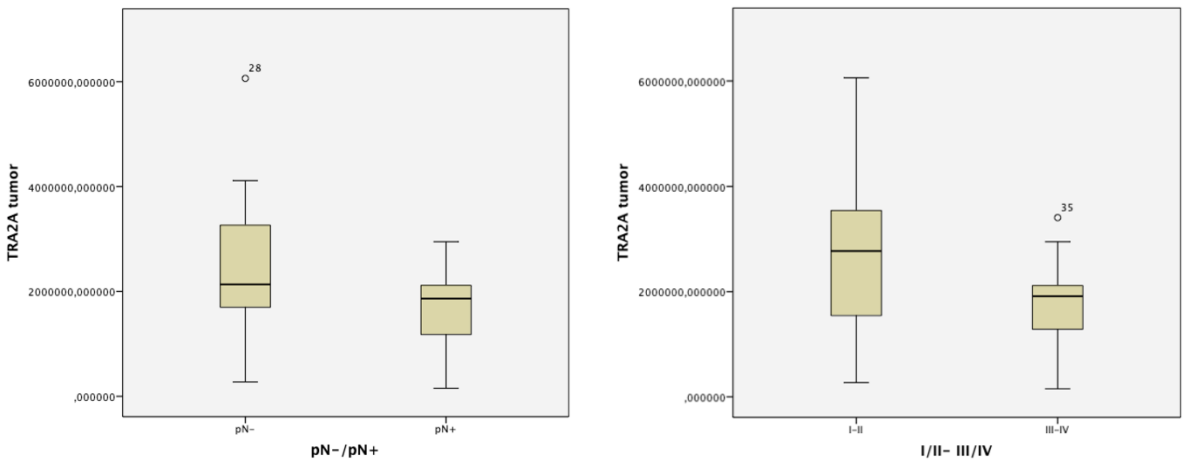


Figure R 29: Relationship between *TRA2A* expression and Staging data and histopathological factors. Boxplots showing that the expression of TRA2A is increased on patients with pN- (left) and lower Stages I-II (right).

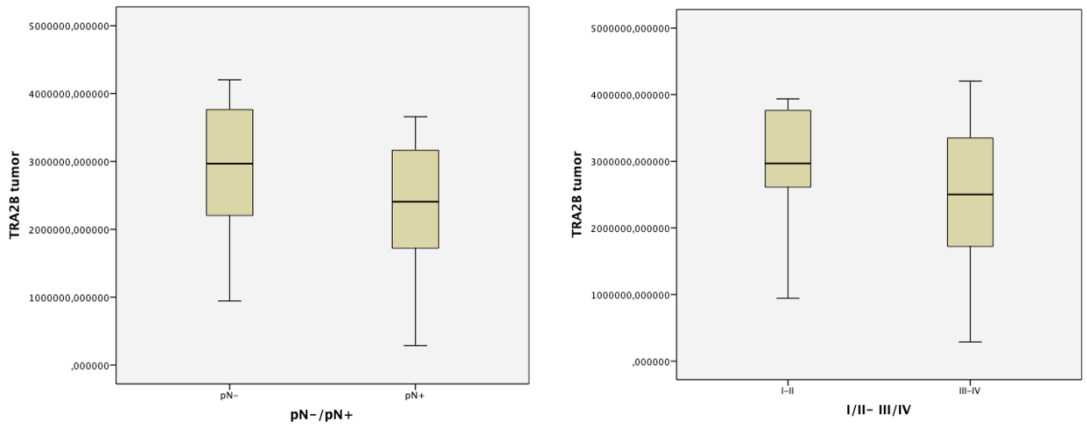


Figure R 30: Relationship between *TRA2B* expression and Staging data and histopathological factors. Boxplots showing that the expression of TRA2B is increased on patients with pN- (left) and lower Stages I-II (right).

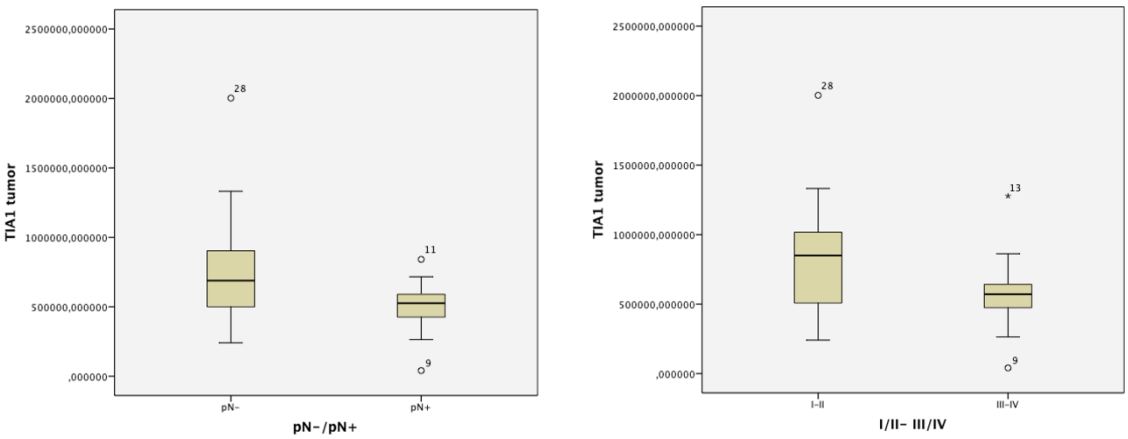


Figure R 31: Relationship between *TIA1* expression and Staging data and histopathological factors. Boxplots showing that the expression of TIA1 is increased on patients with pN- (left) and lower Stages I-II (right).

Table R 21: *In vivo* relationship between splicing expression in OSCC and staging data. Nonparametric Kruskal-Wallis and U-Mann Whitney tests are used to analyze the relationship between Splicing Numerical expression and Staging data. Chi2 or Fisher tests are used to analyze the relationship between Splicing Categorical expression and histopathological data.

	pT	*pTx2*	*pN*	*pNx4*	*pN x2*	*pN-/pN+*	*Stage*	*Stagex2*
SRSF4 Numerical	p=0.41 R 0.04	p=0.37 R 0.15	p=0.12 R -0.19	p=0.09 R -0.18	p=0.98 R -0.01	p=0.29 R -0.18	p=0.64 R 0.07	p=0.87 R 0.27
SRSF5 Numerical	p=0.13 R -0.37	**p=0.08 (-)** R -0.29	p=0.26 R -0.18	p=0.38 R -0.17	p=0.57 R -0.09	p=0.15 R -0.23	p=0.58 R -0.27	p=0.11 R -0.26
SRSF9 Numerical	**p=0.08 (-)** R -0.36	p=0.15 R -0.24	p=0.70 R -0.08	p=0.87 R -0.07	p=0.95 R -0.01	p=0.59 R -0.08	p=0.47 R -0.17	p=0.30 R -0.16
SRSF10 Numerical	p=0.23 R 0.09	p=0.49 R 0.12	p=0.11 R -0.02	p=0.33 R -0.01	p=0.44 R 0.14	p=0.80 R -0.04	p=0.13 R 0.16	p=0.80 R 0.04
NOVA1 Numerical	p=0.27 R -0.24	p=0.12 R -0.26	p=0.77 R -0.18	p=0.60 R -0.17	p=0.22 R -0.20	p=0.48 R -0.12	**p=0.08 (-)** R -0.25	p=0.18 R -0.22
RBM3 Numerical	p=0.88 R -0.07	p=0.90 R -0.02	p=0.13 R -0.16	p=0.12 R -0.16	p=0.88 R 0.02	p=0.18 R -0.23	p=0.42 R -0.05	p=0.51 R -0.11
RBM10 Numerical	p=0.98 R -0.02	p=0.77 R -0.04	p=0.76 R -0.12	p=0.91 R -0.11	p=0.51 R -0.11	p=0.50 R -0.11	p=0.14 R -0.01	p=0.54 R -0.10
ESRP1 Numerical	p=0.12 R -0.01	p=0.57 R 0.09	p=0.12 R -0.01	p=0.40 R 0.03	p=0.35 R 0.15	p=0.89 R -0.02	**p=0.02 (+)** R 0.11	p=0.46 R 0.12
ESRP2 Numerical	p=0.19 R -0.07	p=0.61 R 0.08	p=0.16 R -0.07	p=0.51 R -0.07	p=0.82 R 0.03	p=0.45 R -0.12	p=0.08 R 0.06	p=0.42 R 0.13
TRA2A Numerical	**p=0.08 (-)** R -0.40	p= 0.13 R -0.26	p=0.31 R -0.28	p=0.29 R -0.28	p=0.37 R -0.15	**p=0.07 (-)** R -0.31	p=0.40 R -0.35	**p=0.03 (-)** R -0.38
TRA2B Numerical	p=0.40 R -0.25	p=0.16 R -0.24	p=0.28 R -0.34	p=0.19 R -0.33	p=0.17 R -0.23	**p=0.06 (-)** R -0.33	p=0.71 R -0.26	**p=0.09 (-)** R -0.28
TIA1 Numerical	p=0.36 R -0.19	p=0.15 R -0.25	p=0.32 R -0.41	p=0.15 R -0.40	**p=0.04 (-)** R -0.36	**p=0.03 (-)** R -0.37	p=0.41 R -0.30	**p=0.05 (-)** R -0.33
	pT	***pTx2***	***pN***	***pNx4***	***pNx2***	***pN-/pN+***	***Stage***	***Stagex2***
SRSF4 >/< median	p=0.33 R 0.10	p=0.35 R 0.16	p=0.32 R -0.12	p=0.20 R -0.11	p=0.90 R 0.02	p=0.57 R -0.09	p=0.50 R 0.11	p=0.80 R 0.04
SRSF5 >/< median l	p=0.41 R -0.21	p=0.38 R -0.14	p=0.16 R -0.14	p=0.09 R -0.13	p=0.90 R -0.01	p=0.14 R -0.24	p=0.37 R -0.16	p=0.19 R -0.21
SRSF9 >/< median	p=0.37 R -0.23	p=0.31 R -0.16	p=0.89 R -0.14	p=0.65 R -0.14	p=0.71 R -0.06	p=0.31 R -0.16	p=0.89 R -0.11	p=0.48 R -0.11
SRSF10 >/< median	p=0.51 R 0.12	p=0.60 R 0.09	p=0.35 R -0.11	p=0.47 R -0.10	p=0.91 R -0.01	p=0.61 R-0.09	p=0.26 R 0.15	p=0.88 R 0.02
NOVA1 >/< median	p=0.35 R -0.27	p=0.12 R -0.25	p=0.26 R -0.31	p=0.30 R -0.30	**p=0.08 (-)** R -0.29	p=0.11 R -0.26	p=0.28 R -0.29	p=0.12 R -0.26
RBM3 >/< median	p=0.23 R -0.31	p=0.17 R -0.23	p=0.09 R -0.28	**p=0.08 (-)** R -0.27	p=0.59 R -0.09	**p=0.07 (-)** R -0.31	p=0.26 R -0.30	**p=0.09 (-)** R -0.29
RBM10 >/< median	p=0.97 R 0.03	p=1 R 0.00	p=0.69 R -0.06	p=0.97 R -0.05	p=0.71 R -0.06	p=0.73 R -0.05	p=0.82 R 0.07	p=1 R 0.00
ESRP1 >/< median	p=0.33 R 0.10	p=0.38 R 0.14	p=0.39 R 0.01	p=0.76 R 0.02	p=0.63 R 0.08	p=0.84 R -0.03	p=0.42 R 0.21	p=0.19 R 0.22
ESRP2 >/< median	p=0.42 R 0.05	p=0.31 R 0.16	p=0.12 R -0.09	p=0.20 R -0.08	p=0.71 R 0.06	p=0.31 R -0.16	p=0.89 R 0.11	p=0.48 R 0.11
TRA2A >/< median	**p=0.08 (-)** R -0.36	p=0.20 R -0.22	p=0.15 R -0.27	p=0.11 R -0.27	p=0.62 R -0.08	**p=0.05 (-)** R -0.33	p=0.13 R -0.27	**p=0.04 (-)** R -0.35
TRA2B >/< median	p=0.36 R -0.21	**p=0.09 (-)** R -0.29	p=0.17 R -0.34	**p=0.07 (-)** R -0.34	p=0.19 R -0.22	**p=0.07 (-)** R -0.31	p=0.36 R -0.26	**p=0.09 (-)** R -0.29
TIA1 >/< median	**p=0.09 (-)** R -0.22	**p=0.05 (-)** R -0.33	p=0.66 R -0.25	p=0.48 R -0.25	p=0.16 R -0.24	p=0.20 R -0.21	p=0.20 R -0.34	**p=0.05 (-)** R -0.34

Abbreviations: pN, cervical metastasis (pN0/pN1/pN2a/pN2b/pN3); pNx4 (pN0/pN1/pN2/pN3); pNx2 (pN0+pN1/pN2+pN3), pN- (pN0) *vs*. pN+ (pN1, pN2, pN3); pT, tumor size (pT1,pT2,pT3,pT4); pTx2 (pT1+pT2/pT3+pT4); Stage (I/II/III/IV); Stage x2 (I+II/III+/IV); (-), negative correlation; (+), positive correlation.

Regarding histopathological factors, the higher expression of *TRA2B and TIA* was related to better differentiated tumors with negative relationship with G *(p=0.01, p= 0,06*); Table R22 Figure R32). This was also observed with the categorical analysis where also patients with higher expression of *RBM10* were related to lower G grade (*p=0.01,*Table R22).

Table R 22: *In vivo* relationship between splicing expression in OSCC and histopathological data. Nonparametric Kruskal-Wallis and U-Mann Whitney tests are used to analyze the relationship between Numerical Splicing expression and histopathological data. Chi^2 or Fisher test were used to analyze the relationship between Categorical Splicing expression and histopathological data.

	G	***DOIx3***	***PTI***	***PTIx2***	***PNI***	***LVI***	***Invasion Front***	***Uniformity***
SRSF4 Numerical	p=0.21 R -0.21	p=0.80 R 0.06	p=0.27 R 0.23	p=0.10 R 0.28	p=0.84 R -0.03	p=0.91 R -0.01	p=0.48 R 0.12	p=0.48 R 0.12
SRSF5 Numerical	p=1 R 0.00	p=0.24 R -0.27	**p=0.06 (+)** R 0.39	**p=0.01 (+)** R 0.41	p=0.23 R -0.15	p=0.56 R -0.09	p=0.81 R 0.03	p=0.81 R 0.03
SRSF9 Numerical	p=0.96 R -0.01	p=0.21 R -0.26	**p=0.01 (+)** R 0.28	**p=0.01 (+)** R 0.40	p=0.36 R -0.19	p=0.40 R-0.14	p=0.30 R 0.17	p=0.30 R 0.17
SRSF10 Numerical	p=0.84 R -0.03	p=0.11 R 0.16	p=0.61 R 0.12	p=0.42 R 0.14	p=0.12 R 0.27	p=0.20 R 0.23	p=0.36 R 0.16	p=0.36 R 0.16
NOVA1 Numerical	p=0.24 R -0.19	p=0.39 R -0.14	p=0.11 R 0.21	p=0.16 R 0.24	p=0.88 R 0.02	p=0.76 R -0.05	**p=0.04 (+)** R 0.34	**p=0.04 (+)** R 0.34
RBM3 Numerical	p=0.83 R -0.03	p=0.75 R -0.12	p=0.46 R 0.11	p=0.46 R 0.12	p=0.59 R -0.09	p=0.12 R -0.26	p=0.13 R 0.26	p=0.13 R 0.26
RBM10 Numerical	p=0.17 R -0.23	p=0.59 R -0.15	p=0.31 R-0.02	p=0.64 R 0.07	p=0.27 R -0.18	p=0.26 R -0.18	p=0.65 R 0.07	p=0.65 R 0.07
ESRP1 Numerical	p=0.69 -0.06	p=0.82 R -0.04	p=0.56 R 0.04	p=0.73 R 0.05	p=0.69 R 0.06	p=0.57 R -0.09	p=0.12 R 0.26	p=0.12 R 0.26
ESRP2 Numerical	p=0.78 R -0.04	p=0.68 R -0.14	p=0.47 R 0.13	p=0.31 R 0.17	p=0.66 R-0.07	p=0.43 R -0.13	**p=0.06 (+)** R 0.30	**p=0.06 (+)** R 0.30
TRA2A Numerical	p=0.80 R 0.04	p=0.22 R -0.29	p=0.17 R 0.24	p=0.06 R 0.32	p=0.87 R 0.02	p=0.66 R 0.07	p=0.80 R -0.04	p=0.80 R -0.04
TRA2B Numerical	**p=0.01 (-)** R -0.43	p=0.24 R -0.29	**p=0.03 (+)** R 0.40	**p<0.01 (+)** R 0.46	p=0.49 R -0.11	p=0.62 R 0.08	p=0.25 R 0.19	p=0.25 R 0.19
TIA1 Numerical	**p=0.06 (-)** R -0.33	**p=0.04 (-)** R -0.44	**p=0.04 (+)** R 0.35	**p=0.01 (+)** R 0.45	p=0.27 R -0.19	p=0.91 R 0.01	p=0.68 R -0.07	p=0.68 R -0.07
	G	***DOIx3***	***PTI***	***PTIx2***	***PNI***	***LVI***	***Invasion Front***	***Uniformity***
SRSF4 >/< median	p=0.37 R -0.15	p=0.59 R 0.13	p=0.22 R 0.13	p=0.29 R 0.22	p=0.70 R 0.11	p=0.22 R 0.2	p=0.68 R -0.07	p=0.68 R -0.07
SRSF5 >/< median l	p=0.87 R -0.02	p=0.53 R -0.17	**p=0.08 (+)** R 0.36	**p=0.01 (+)** R 0.41	**p=0.09 (-)** R -0.28.	p=0.40 R -0.13	p=0.73 R 0.05	p=0.73 R 0.05
SRSF9 >/< median	p=0.73 R -0.05	p=0.57 R -0.12	**p<0.01 (+)** R 0.28	**p=0.02 (+)** R 0.40	p=0.71 R -0.06	p=0.73 R -0.05	p=0.67 R 0.07	p=0.67 R 0.07.
SRSF10 >/< median	p=0.83 R 0.03	**p=0.06 (+)** R 0.21	p=0.59 R 0.06	p=0.60 R 0.09	**p=0.03 (+)** R 0.37	p=0.21 R 0.22	p=0.68 R 0.07	p=0.68 R 0.07
NOVA1 >/< median	p=0.39 R -0.14	p=0.46 R -0.15	**p=0.04 (+)** R 0.36	**p=0.02 (+)** R 0.38	p=0.91 R -0.01	p=0.87 R -0.02	p=0.12 R 0.25	p=0.12 R 0.25
RBM3 >/< median	p=0.74 R 0.05	p=0.18 R 0.31	p=0.44 R 0.25	p=0. 13 R 0.25	p=0.32 R -0.16	p=0.31 R -0.17	p=0.28 R 0.18	p=0.28 R 0.18
RBM10 >/< median	**p=0.01(-)** R -0.39	p=0.54 R -0.16	p=0.28 R -0.03	p=0.73 R 0.05	p=0.13 R -0.24	p=0.50 R -0.11	p=0.37 R 0.14	p=0.37 R 0.14
ESRP1 >/< median	p=0.86 R -0.02	p=0.98 R -0.02	p=0.52 R -0.09	p=0.62 R -0.08	p=0.80 R 0.04	**p=0.06 (-)** R -0.31	**p=0.06 (+)** R 0.31	**p=0.06 (+)** R 0.31
ESRP2 >/< median	p=0.73 R-0.05	p=0.93 R -0.04	p=0.61 R 0.15	p=0.31 R 0.16	p=0.27 R -0.18	p=0.31 R -0.16	p=0.20 R 0.21	p=0.20 R 0.21

TRA2A	p=0.84	p=0.52	p=0.34	p=0.11	p=0.38	p=0.73	p=0.68	p=0.68
>/< median	R 0.03	R -0.19	R 0.22	R 0.27	R 0.15	R -0.09	R 0.07	R 0.07
TRA2B	**p<0.01(-)**	p=0.10	**p=0.04 (+)**	**p<0.01 (+)**	p=0.32	p=0.74	p=0.11	p=0.11
>/< median	R -0.53	R -0.35	R 0.47	R 0.48	R -0.16	R -0.05	R 0.27	R 0.27
TIA1	**p=0.02 (-)**	**p<0.01(-)**	p=0.20	**p=0.05 (+)**	p=0.10	p=0.86	p=0.68	p=0.68
>/< median	R -0.40	R -0.55	R 0.27	R 0.34	R -0.28	R -0.03	R -0.07	R -0.07

Abbreviations: G, grade; DOI, Depth of Invasion; DOIx3 (1-5mm, 5-10mm. >10mm); Invasion front [expansive (+) *vs.* infiltrative (-)]; PTI (mild, moderate, severe), PTIx2 (absent+mild/moderate+severe); PNI, perineural invasion; LVI, lymphovascular invasion; Uniformity [poor defined tumor edges (-) *vs*. well defined edges (+)]; (-), negative correlation; (+), positive correlation.

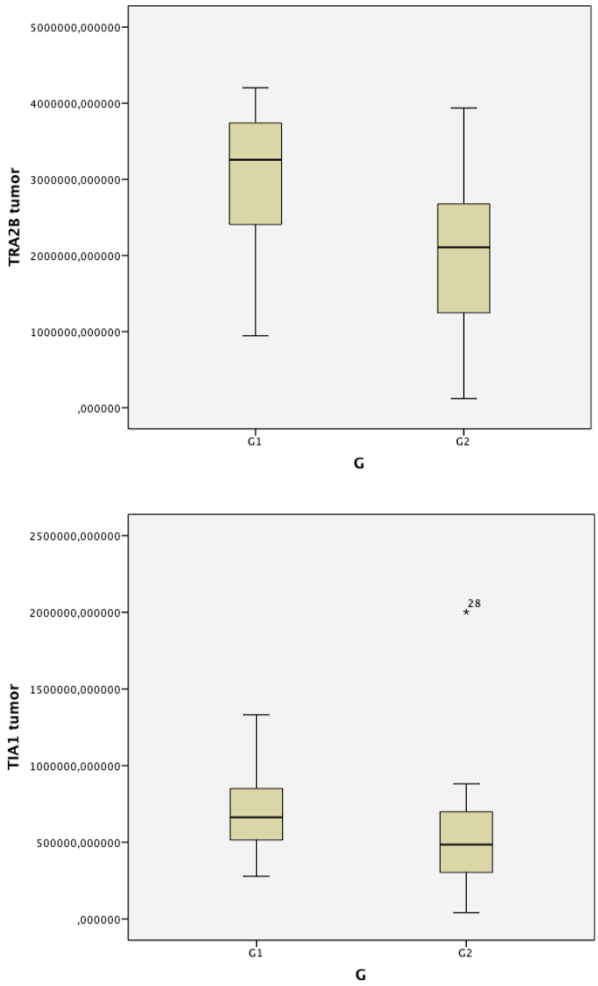


Figure R 32: *TRA2B and TIA1* expression according to Histological grade (G). Boxplots showing that the expression of TRA2B (upper panel) and TIA1 (lower panel) is increased on patients with well differentiated tumors (G1).

TIA1 expression was statistically increased in patients with smaller depth of invasion (DOI) [DOIx3 (*p=0.04, p<0.01*); Table R22]. Peritumoral inflammation showed a positive relationship with the expression of *SRSF5* [PTI (*p=0.06*); PTI x2 (*p=0.01*)], *SRSF9* [PTI (*p=0.01*); PTI x2 (*p=0.01*)], *TRA2B* [PTI (*p=0.03*); PTI x2 (*p< 0.01*)], and *TIA* [PTI (*p=0.04*); PTI x2 (*p=0.01*); Table R22; Figure R33]. This was also observed with the categorical analysis where also patients with higher expression of *NOVA1* were related to higher PTI [PTI (*p=0.04*); PTI x2 (*p=0.02*); Table R22].

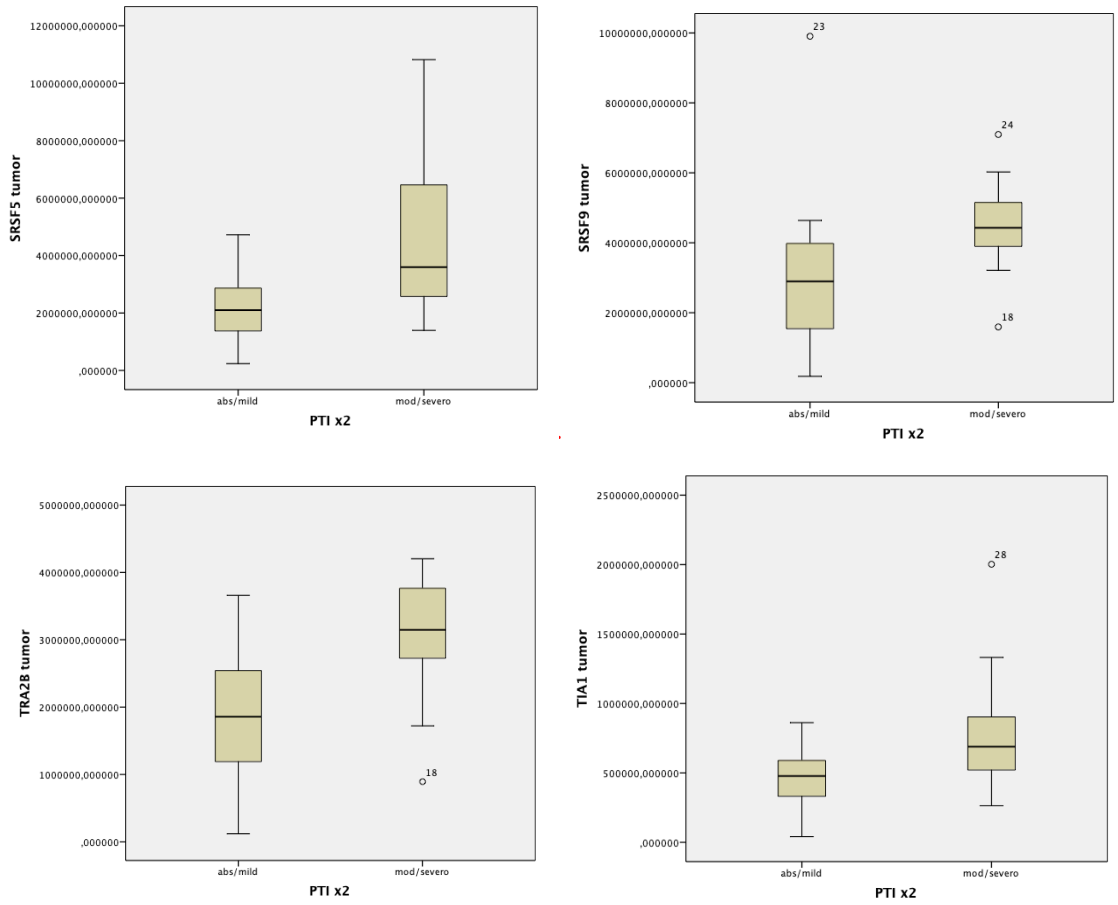


Figure R 33: *SRSF5, SRSF9, TRA2B and TIA1* expression according to Peritumoral Inflammation (PTI and PTI x2). PTI is expressed as PTI x2 (absence+mild/ moderate+severe).

Moreover, *NOVA1 and ESRP2* expression was statistically increased in OSCC that had an expansive front of tumor invasion compared to OSCC with an infiltrative front of tumor invasion (*p=0.04, p=0.06,* respectively; Table R22. Similarly, our results showed that *NOVA1 and ESRP2* were overexpressed in OSCC with uniform tumor invasion edges compared to poorly defined ones (*p=0.48, p =0.06*; Table R22).

Finally, we found that the expression of *SRSF5, TRA2A, TRA2b, and TIA1* in OSCC had a negative correlation to the number of positive lymph nodes, the number of lymph nodes with ENE+, and their bigger size (Table R23).

Table R 23: *In vivo* relationship between splicing expression in OSCC and lymph node pathological data. Spearman correlation test was used for the analysis between SSTs numerical expression and lymph node results.

	Nº + lymph	*Nº ENE+*	*Size(mm)*
SRSF4 Numerical	p=0.10 R – 0.32	p=0.45 R -0.15	p=0.19 R -0.25
SRSF5 Numerical	**p=0.03 (-)** R -0.38	**p=0.03 (-)** R -0.38	**p=0.07 (-)** R -0.32
SRSF9 Numerical	p=0.12 R -0.28	p=0.77 R -0.05	p=0.29 R 0.19
SRSF10 Numerical	p=0.75 R -0.06	p=0.58 R 0.11	p=0.91 R 0.02
NOVA1 Numerical	p=0.17 R -0.25	p=0.32 R -0.18	p=0.45 R -0.14
RBM3 Numerical	p=0.29 R -0.20	p=0.54 R -0.12	p=0.54 R -0.12
RBM10 Numerical	p=0.17 R -0.25	p=0.74 R -0.06	p=0.77 R -0.05
ESRP1 Numerical	p=0.55 R -0.11	p=0.76 R 0.05	p=0.65 R 0.08
ESRP2 Numerical	p=0.18 R -0.24	p=0.50 R -0.12	p=0.69 R -0.07
TRA2A Numerical	**p=0.01 (-)** R -0.48	p=0.22 R -0.24	**p=0.04 (-)** R -0.39
TRA2B Numerical	**p<0.01 (-)** R -0.55	**p=0.04 (-)** R -0.38	p=0.01 (-) R -0.44
TIA1 Numerical	**p<0.01 (-)** R -0.57	**p=0.09 (-)** R -0.33	**p=0.01 (-)** R -0.47

Abbreviations: ENE+, extranodal extension; (-), negative correlation; (+), positive correlation.

4.9. Antitumor Actions of an inhibitor of the splicing machinery (pladienolide-B) on Patient-Derived Primary Oral Squamous Carcinoma Cell Cultures

Based on the previous results indicating that the expression of key spliceosomal components is consistently dysregulated in OSCC samples, and that a clear relationship is found between some of these components with important clinical, histopathological, and survival data, we next explored whether the inhibition of the activity of the splicing machinery might influence the pathophysiology of the OSCC cells. To that end, we performed a pharmacological experimental approach by blocking the activity of SF3B1 (a central and core component of the splicing machinery), using a specific inhibitor (pladienolide-B). First, we performed dose-response experiment using three different concentrations of pladienolide-B in one primary

OSCC cell culture at different incubation times (Figure R34-A). We found that the 100 nM dose was the most effective concentration for reducing cell proliferation rate at 24-, 48- and 72-h of incubation (Figure R34-A).

Then, we used pladienolide-B (100 nM) in different OSCC cell culture specimens and demonstrated that the inhibition of the activity of the splicing machinery was able to significantly decrease the proliferation rate in a time-dependently manner in OSCC primary culture (Figure R34-B).

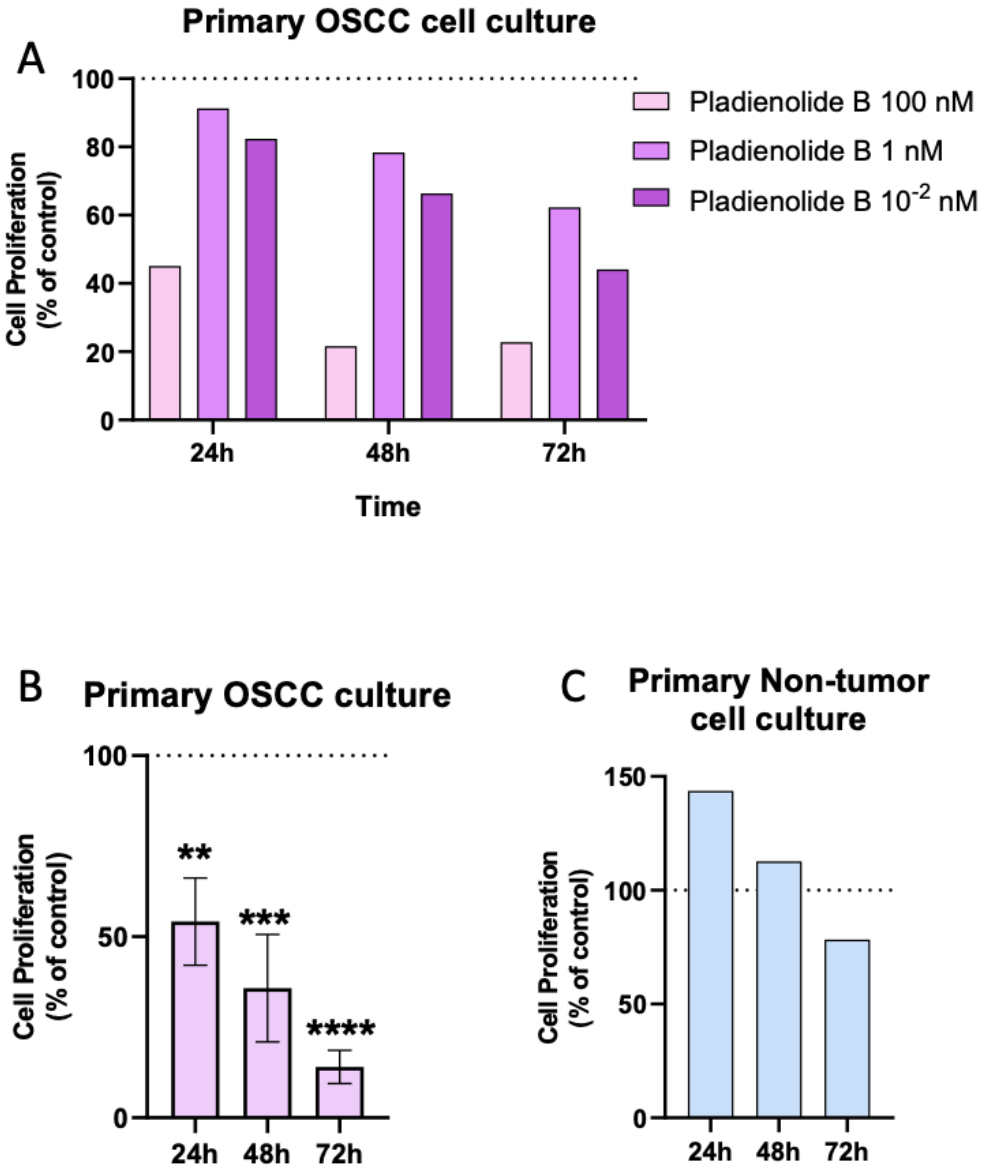


Figure R 34: Pharmacological inhibition of splicing machinery with Pladienolide B *in vitro* decreases proliferation rates in OSCC cells. A) Proliferation rate in response to different doses of Pladienolide B in primary OSCC cell culture compared to vehicle-treated control cells (Control set at 100%) (n=1). **B)** Proliferation rate in response to Pladienolide B administration in primary OSCC cell cultures (n=3) and **(C)** primary Non-tumor cell culture (n=3). Control set as 100%, represented as a dotted line. Asterisks indicate significant differences (**p<0.01; ***p<0.001; ****p<0.0001)

5. Discussion

5.1. General considerations

Oral squamous cell carcinoma (OSCC) continues to be an aggressive disease with an increasing incidence that constitutes a worldwide challenge[1]. Despite all efforts, the overall OSCC 5-year survival rate is only 60%[151,152]. The high incidence, frequent occult onset, low survival rate, and the limited and inefficient treatments, clearly emphasize the necessity of identifying novel biomarkers for these tumors. These potential biomarkers would help refine OSCC diagnosis, improve the prediction of their prognosis and tumor behavior, and provide novel tools to develop efficient therapeutic targets. In this scenario, rising evidence from our and other groups in the last decades have documented that the somatostatin system and the spliceosome and associated proteins are often altered in disease states, including different cancer types, which augments pathobiological versatility (e.g. through generation of distinct/novel alternative splicing variants)[156,159,161,259,261,262].

However, to the best of our knowledge, no studies had reported hitherto a comprehensive analysis to ascertain whether the somatostatin system (especially somatostatin receptors) and the splicing machinery is altered in OSCC tissues as compared with adjacent healthy tissues obtained within the same patient, and whether this potential alteration might be associated to key clinical and pathological parameters in these patients. Therefore, based on our prior experience in other cancer types, in this doctoral thesis we aimed to determine for the first time the expression profile of the somatostatin receptors, and of a representative set of spliceosome components and splicing factors, and their relationship with key clinical features of OSCC samples/patients, because we considered that it might provide useful information to improve diagnosis and/or prognosis of these tumors, and to identify novel therapeutic sources to treat patients with these devastating tumoral pathologies.

5.2. OSCC Epidemiology and Risk factors

The presented study depicts a population of 51% men and 49% women diagnosed with OSCC with a mean age of 62±10y and 65±16y, respectively. Traditionally, OSCC shows a higher prevalence among men in their 6th-7th decade of life[83] However, over the last decades, a significant change in OSCC gender predilection has been noted with a progressively narrowed gender gap between males and females[25], consistent with our cohort population with almost equal gender distribution and age. Our study also shows similar results to the literature, with a higher proportion of women of extreme ages (<45 and >75)[26].

In our cohort, cancer has a higher prevalence in some locations. The tongue and floor of the mouth (FOM) were the most common locations with 20 and 8 patients, respectively, followed by alveolar ridge/hard palate SCC with 5 patients. This data is consistent with the literature with a superior proportion of SCC in the tongue and FOM[32,263]. The location distribution has no statistical gender predilection (p= 0,10), but women had more alveolar ridge SCC while men had more buccal mucosa

than women. This data agrees with previous studies that described an increased proportion of gingivobuccal cancer in women with 70 or more years of age[26,264]. The explanation for this could be in the hormonal effect on the oral mucosa, especially the gingiva. Atrophic and desquamative gingivitis is more common in postmenopausal women with reduced estrogen levels[30] which could increase the risk of alveolar ridge mucosa cancer in elder women[26,264].

The relationship between the tumor location and the OS is controversial. Previous studies have failed to show OS differences among oral cavity cancer sites[32,265]. Bell and coworkers studied the impact of tumor location on overall survival and found that subsite alone is not associated to significant difference in OS. However, the risk for nodal metastasis in OSCC increases according to the location of the primary tumor from the lip to the oropharynx, and the presence of clinically positive lymph nodes is the single most important clinical predictor in determining survival[263].

Among risk factors contributing to OSCC in Europe, tobacco consumption[61] continues to be the most significant, and it is known to be more predominant among men. In our cohort, tobacco consumption difference between gender was significantly different ($p<0.001$). Just 11% of women were smokers or ex-smokers, while 78% of men had a history of tobacco use which is consistent with the data of the literature[25]. No other risk factors were statistically relevant between gender in our study, with a similar distribution of variables such as BMI, HBP, DM, DLP.

5.3. OSCC overall survival compared to risk factors, staging, and histopathological data

OSCC is a challenging disease due to its nature, complicated approach, and patient comorbidities. The OSCC outcome depends on many factors, especially on the stage of the disease at initial diagnosis and more specifically, the degree of cervical node involvement[263]. All efforts in oral cancer management aim to avoid a late diagnosis and ensure appropriate early treatment. However, early diagnosis is still not always achieved, and patients normally present with advanced disease that is in many cases notoriously difficult to treat even with all the available means.

The 5-year and 10-year survival rates are the most commonly used landmarks for analyzing cancer OS. OSCC 5-year overall survival has been described to be not higher than 60%[151,152,263]. In our cohort, OS is measured at 2 years to allow all patients to have the same follow-up, given that the last included patient was in April 2018. Our patients' OS at 2 years was 76% with a rate of 20,4±1,2 months (range 2-24 months). Patients continue to be followed to achieve the 5-year survival rate.

The relationship between age and OSCC survival have been controversial for decades, with the theory that younger patients have worse survival than the elderly population[43,45,46]. The appropriate analysis of this relationship has been complicated given the low rate of OSCC among young patients.

However, the recent multicentric study by Oh and coworkers that included 365 patients younger than 45years-old showed that younger patients share the same disease-specific survival that the older population with similar rates of death from OSCC in multivariable models[50]. Our results showed no statistical relationship between OS and age but did show a statistical trend with worse OS for patients >75 years old (p=0.08). This probably could be related to age-related associated coexisting comorbidities or tolerance to treatment secondary effects.

Among risk factors and patients' comorbidities contributing to OSCC in our cohort, diabetes mellitus showed a significant relationship with poor OS. In contrast, the common risk factors for OSCC did not impact the overall survival of our patients. In this sense, the association between diabetes and survival has been widely reported in several cancer pathologies[266–268]. Diabetes is an important comorbid condition in the general population, with a prevalence between 12-20%, depending on the different ethnicities[104]. DM was prevalent in 26% of our patients without gender differences and carried a significant negative correlation with overall survival (p<0,01) and a positive correlation with recurrence rate (p<0,01). Moreover, the HbA1c % also showed a statistically negative correlation with OS (p<0,01). The relationship mechanism between diabetes mellitus and OSCC is not yet well known; however, DM patients have a higher prevalence of oral cancer and oral potential malignant disorders with an increased risk of oral cancer-related death 2.9 times higher than the general population[98]. Van der Poll-Franse and coworkers studied the prevalence of diabetes in cancer patients and analyzed if impacted stage at diagnosis, treatment, and prognosis. They found that younger patients with cancer had more prevalence of diabetes than the general population of the same age. Cancer patients with DM experienced a significant increase in overall mortality (HR 5 1.44, 95% CI 1.40–1.49) and a worse prognosis than those without diabetes[105]. Moreover, Van der Poll-Franse et al. exposed that diabetic patients were frequently treated less aggressively to avoid diabetes-related complications. They also speculated that diabetes could negatively influence the effectiveness of cancer therapies[105]. Over the last decade our group has demonstrated the tight relationship between dysregulation of metabolic conditions and the development, progression and aggressiveness of different cancer types [269–274]. In this line, the results of our thesis show a correlation between DM and OSCC survival and recurrence. This highlights the importance of considering other comorbidities and risk factors in OSCC population, especially factors associated to metabolic conditions.

Historically, women have a lower proportion of OSCC. Given that low proportion, an accurate comparative survival analysis between gender is complicated. No study has proved a relationship between OS and sex to date. Yet, some authors have a still unproven belief that women with OSCC carry a worse prognosis. Our results show a trend with 66% 2 years OS in women compared to 83% in men. Several studies have aimed to discover different prognosis factors between genders. Specifically, some studies have found that history of smoking was an independent prognosis factor in oral cancer,

corresponding to a worse prognosis among men[31]. However, despite smoking history proving to be a worse prognosis factor in men, women who do usually not smoke have no improved survival. Our results also showed no gender preference among established overall survival risk factors such as advanced stage, pN, or pT grading. Additionally, there are no differences in histopathological characteristics among genders. The lack of statistical differences in any clinical or histopathological factors might be related to our small sample of patients; however, our data is consistent with what has been described in the literature[95].

Our cohort of patients presented with advanced tumors (stages III or IV) in 66% of the cases. The tumor size was pT4 in 34% and pT3 tumors in 24%. It should be mentioned that the inclusion criteria of this study included all tumors with enough volume to allow sample removal from the specimen without interfering with the pathology analysis. Therefore, this limitation significantly affected the number of early-stage tumors included, which significantly unrepresented pT1 patients (2 patients). As a result, the OS and recurrence analysis among those early-stage tumors may not be as well depicted in our cohort as in the normal population. The pT grading (pT1,pT2,pT3,pT4) did show an obvious impact on OS with a decreased survival in advanced pT3+pT4 (59%) compared to pT1+pT2 (100%). This data is consistent with the literature that shows that increased tumor size endorses a worse prognosis[77,83,119,275].

Almost 20 to 30% of OSCC patients usually present with positive cervical lymph nodes by the time of diagnosis[32,263]. The results of the cervical lymph node involvement of our patient cohort showed that the neck was positive in 42% of our population (16 patients). Among those, we find pN1 in 4 patients (10%), and pN2 and pN3 in 6 patients (16% and 16%). The higher presence of positive neck (pN+) in our study than in the general population may be related to the lower depiction of pT1 or early stage among our patients. As previously discussed, cervical nodal metastasis in OSCC is the most significant clinical predictor of DFS and OS[77,119,263,275]. Similar to those studies, our results showed that higher pN grading correlates with a poorer OS. Besides the grading, the numerical analysis of the number of positive lymph nodes, the number of lymph nodes with ENE+ present, and the larger size of the lymph nodes are also significantly related to a worse DFS and OS. ENE+ was present in 26% of our node-positive patients and was the feature that showed the highest negative correlation with OS. This statement is also consistent in the literature[275].

The OS and histopathological features analysis showed that higher PTI was significantly correlated with a better OS ($p<0.001$). Patients with mild or absent PTI had 47% OS compared to 95% OS among patients with moderate or severe PTI. When the relationship of PTI with other histopathological factors was analyzed, PTI had a significant negative relationship to pT, pN, stage, and DOI. These results are consistent with those studies that have observed worse cancer-specific and OS rates with scarce or absent lymphocytic infiltration[276]. Our data agree with the studies that consider PTI a defense

mechanism against cancer progression and invasion[130,131,136–138]. Besides the local inflammation, systemic inflammatory response, measured by preoperative CRP levels in OSCC, has been described to be present in larger tumors and associated with worse OS and nodal metastasis[140,143]. However, we did not find similar results in our cohort which could be related to the size of our cohort.

We classified the margin status as margin status x2 [negative (>1-5 mm) or positive (<1mm)], and as margin status x3 [positive (<1mm), close (1-5mm), or free (>5 mm)]. Positive margins were correlated with a worse OS (p=0.05). A significant reduction in OS was observed when smaller margins were obtained. Patients with negative margins had 75% of OS compared to the 40% of those with positive margins. In this context, margin status influence on OS and DFS is controversial in the literature. Although some studies have not found a relationship between positive margins and outcomes[276], most consider margin status a significant predictor of OS and recurrent disease[119,265,277,278]. Moreover, margin status is one of the criteria for adding adjuvant therapy consisting of radiation vs. chemoradiation, especially for larger tumors. Several authors have studied the impact of radiation on positive margins, with results showing no apparent impact on survival or decreased local recurrence[119]. A recent paper investigated the effect of radiation therapy on early OSCC with positive margins and suggested that the addition of radiation therapy did not improve the patient´s OS[278]. Therefore, if radiation does not increase the OS of early cancers with positive margins, this may open the door for other possible adjuvant therapy options not as aggressive as chemotherapy.

Although not statistically significant, higher DOI correlated with lower OS in our study. Tumors with depth <5 mm had 100% OS, while it was 93% for tumors >5 to 10 mm, and 56% for tumors >10 mm. This finding is consistent with the studies that describe DOI as an indicator of possible occult metastasis in OSCC, which has proved to be a significant predictor of death in these patients[128]. In this sense, we consider that the statistical relationship would be improved with a more substantial number of patients in our study.

The presence of non-uniform edges and infiltrative front of invasion was correlated with a worse OS with a statistical trend (p=0.07). This relationship is consistent with the literature results that show diffusely infiltrating SCC has a worse prognosis[135,276]. The relationship between PNI with poor OS has been described by several studies[145]. However, others have failed to find that association[265,276]. Similar to those, our results showed no relationship between PNI or LVI with OS.

5.4. OSCC disease-free survival and recurrence data compared to risk factors, staging, and histopathological data

Recurrence in OSCC is a significant predictor factor of patient prognosis. The presence of tumor relapse has been linked to an increased mortality rate of up to 92%. The recurrence rate (RR) of OSCC has been described to vary between 7 to 47%[265,277]. Local, regional, or combined recurrence are the most

common types in OSCC[123,276]. In our sample, the general RR was 26% (9/34 patients), with similar results for local (21%) and regional recurrence (21%). Out of these, both local&regional recurrence was present at the same time in five patients (15%). The tumor relapse usually occurs during the first two years after the first treatment, with some studies describing up to more than 94% of the patients with relapses before that[265,279,280]. 66% of the events happened within the first year after the surgery in our cohort. The importance of the timing of the recurrence has been addressed in many studies, and recurrences during the first 12 months are considered early recurrences which carry a worse OS[265,279].

Given the significant association between recurrence and mortality rate, it is evident that one of the keys for treating patients with OSCC relies on figuring out which patient has a higher probability of recurrence and how to address this. Many clinical and histopathological factors have been associated with a higher recurrence rate in OSCC[119]. In our cohort of patients, the correlation between recurrence and risk factors showed that smoking and drinking had no positive relationship with recurrence. Similar to these results, the smoking status by the time of surgery did not seem to affect the probability of tumor recurrence in the literature[276]. This can be related to patients' tobacco cessation after the first diagnosis. Among other risk factors, diabetes mellitus and higher HbA1c levels were linked to a higher prevalence of recurrence in our study. These results are consistent with a randomized trial that found a significantly increased overall mortality and cancer recurrence rate in patients with colon cancer and diabetes mellitus[106].

Many predictive models of potential histopathological factors with a higher likelihood of recurrence have been described in HNSCC[137]. Several clinical and histopathological factors have been related to a higher risk for locoregional recurrence, such as advanced stage at diagnosis, higher G, ENE+, positive PNI or LVI, higher DOI, positive margin status, an invasive pattern of invasion, and higher lymph node ratio[137,145,265,276,280]. Our results showed no relationship between pT grading and recurrence analysis. This is not consistent with the literature where higher pT has been broadly understood as a risk factor for increased locoregional recurrence[77,83,265]. We believe that this might be primarily due to the underrepresented pT1 in the cohort, and the fact that one pT1 patient did have a recurrence. On the contrary, our results showed a positive correlation between recurrence analysis and the presence of cervical nodal disease (pN). The cervical nodal disease is associated with higher recurrence rate in general, local, regional, both locoregional, and distant metastasis. The pN classification pN0/pN1/pN2/pN3 and positive versus negative (pN-/pN+) also correlated with recurrence rate in general, regional, and distant metastasis. The higher number of positive lymph nodes and their extracapsular involvement (ENE+) were strongly associated with recurrence rate, regional and distant metastasis. The size of the lymph nodes was also associated with distant metastasis. These results are widely consistent with the literature reporting that cervical nodal metastasis in OSCC is the most

significant clinical predictor for DFS[263,275]. This also shows that our sample depicts the usual recurrence behavior of squamous cell carcinoma[77,83,265].

As previously stated, margin status is an accepted prognostic factor in OSCC. However, controversy exists regarding its impact on OS and recurrence. Many studies have failed to prove the relationship of involved margins with locoregional failure[281], while others described an increased local recurrence in tumors with closer margins[119,282]. Our results showed that patients with positive margins had a high local recurrence rate (p=0.03). Among patients with negative margins, patients with free margins had no local recurrence, while those with close margins had a 19% of local recurrence. This was still significantly lower than the 67% local recurrence that patients with positive margins had (p=0.07). No relationship between regional or distant recurrence and margins status was found. Our results then agree with studies favoring increased recurrence rates with positive margins[119]. We believe that the heterogeneity of the margin definition and the different margin sampling techniques with or without considering frozen sections might play a significant role in analyzing the effect of margin status in OSCC survival and recurrence analysis.

Finally, the lower peritumoral inflammatory reaction was associated with a higher regional recurrence rate (p=0.02) and both local&regional (p=0,05). These results agree with the associating the lymphocytic reaction with a good prognosis[276]. Other histopathological factors such as G, DOI, PNI, and LVI were not related to tumor relapse in our sample. Although G is not recognized as a significant overall prognostic factor in the literature[119], the other mentioned factors have been associated with poor outcomes[128,132,144]. We believe DOI does not reach significance due to the limited number of pT1 (<5mm DOI) patients present in the study based on the limiting size selection criteria, together with the fact that one of those pT1 patients did have a recurrence.

5.5. SST system in OSCC

As previously mentioned, to the best of our knowledge, no studies had reported hitherto a comprehensive analysis to ascertain whether the somatostatin system (especially SSTs) is altered in OSCC tissues as compared with adjacent healthy tissues obtained within the same patient, and whether this potential alteration might be associated to key clinical and pathological parameters in these patients. Therefore, the following section of this Doctoral Thesis was aimed to analyzed the expression profile of the somatostatin receptors in patients with OSCCC and their association with key clinical features in order to find novel diagnosis and/or prognosis biomarkers, and to identify novel therapeutic sources to treat patients with OSCC.

5.5.1. *SST$_s$* are overexpressed in OSCC samples compared to control tissues

* Context for studying the SST system in OSCC: Oral cancers are among the most common malignant tumors worldwide, significantly reducing patients' quality of life[152]. Importantly, although OSCC is

considered a disease of old age, a recent clinical scenario witnessed its increasing incidence among young individuals[33]. In fact, according to recent statistics from the International Agency for Research on Cancer (http://gco.iarc.fr/), the number of OSCC cases that are newly diagnosed each year is very worrisome and will continue to increase. Therefore, this high incidence, together with the hidden onset and low survival rate, clearly emphasizes the necessity of identifying novel biomarkers for these tumors. These potential biomarkers would help refine OSCC diagnosis, improve prediction of prognosis and tumor behavior, and provide tools to develop novel therapeutic targets. In this context, neuroendocrine differentiation has been found in some tumors not considered to be of neuroendocrine origin, including SCC of the lung and esophagus[9,183] and more recently, larynx[11,184–186], nasopharynx [187], and head and neck[12]. In this regard, SST is a well-known inhibitory neuropeptide that is produced in different central and systemic locations[163,169]. As previously mentioned, SST inhibitory actions are mediated through their so-called SSTs, which are widely distributed in normal and tumor tissues, and regulate, among other activities, cell proliferation, differentiation, and angiogenesis in many tumor types[164]. In this sense, tumors cells typically express more than one SST-subtype, being the most frequently expressed SST2 subtype, and thus the most important target[169,170,178–180]. Consequently, synthetic SST analogues (SSAs) represent an attractive therapeutic target to treat the SST-positive tumor pathologies controlling hormone hypersecretion and tumor growth[283,284]. However, our current understanding of the presence of SSTs on OSCC is quite scarce and unclear, and to the best of our knowledge, no molecular analyses have been performed to analyze quantitatively, in a side-by-side manner, the expression levels (copy number) of all SSTs subtypes in OSCC samples, compared to healthy tissue (control; within the same patient) using quantitative PCR. Moreover, to date, the direct effects of SSAs on primary OSCC human cell cultures have not been tested.

Therefore, based on the information mentioned above, the objectives of this section of the doctoral thesis were: (1) to quantitatively analyze the expression profile of SSTs in OSCC vs. adjacent healthy tissues obtained within the same patient in a well-characterized cohort of patients; (2) to assess the putative in vivo association between the expression of all SSTs in the tumor and relevant clinical/histopathological data parameters (stage, histological grade, tumor invasion, presence of metastasis, recurrence, overall survival, etc.); and (3) to explore and compare, side-by-side, the direct antitumor effects of different SSAs (octreotide, lanreotide, and pasireotide) in primary OSCC human cell cultures.

First, we investigated the expression pattern of all SSTs in parallel using a quantitative PCR method in a group of samples derived from patients with OSCC (n=37; tumor *vs*. adjacent non-tumor tissues and evaluated their potential relationship with key clinical and pathological parameters). To the best of our knowledge, this was the first time that the expression of SST in OSCC has been thoroughly and quantitatively (mRNA copy number) analyzed in a relatively large series of samples. In the present

series, we observed a differential SST expression pattern in OSCC tissues (SST_2>>SST_5>SST_4 >SST_1>SST_3) compared to their corresponding adjacent non-tumor tissues (SST_1>SST_2>SST_5>SST_4>SST_3). Moreover, we demonstrated an overall increase in the expression of $SST_{2,3,4,5}$ in OSCC samples, compared to control tissues, being this overexpression statistically significant for SST_2 and SST_3 levels. This might be considered an important clinical finding, as the responsiveness of SSAs is critically dependent on the presence of SSTs, and because the treatment with available SSAs (e.g., first generation compounds, octreotide and lanreotide, which preferential bind to SST_2) has become the mainstay of medical therapy for tumor control in neuroendocrine disorders expressing SSTs, such as pituitary and gastroenteropancreatic neuroendocrine tumors[283,284]. In this sense, and as previously mentioned, during the last decade, neuroendocrine differentiation has been found in some tumors not considered to be of neuroendocrine origin, including SCC of the lung, esophagus, larynx, head, and neck[9,11,12,183–187] suggesting that the use of neuropeptides analogues (e.g., SSAs) could be used as a potential therapeutic avenue for OSCC.

Our observations compare favorably with previous reports indicating that the expression of different SST-subtypes, including SST_2 and SST_3, is consistently increased in other tumors compared with normal tissues, including human prostate[262,285], pituitary[256,258,261], neuroendocrine tumors[170,260] and brain tumors[157] among others. In this context, previously scarce studies analyzed SSTs expression in head and neck tumors using semi-quantitative immune-histochemical staining. They indicated head and neck squamous cell carcinoma specimens from different locations express different SST subtypes. Specifically, Lechner and coworkers found an upregulation of SST_2 in nasopharyngeal cancer[187]. In laryngeal cancer, Codon and Strafford found a downregulation of SST_2 in pre-malignant and malignant laryngeal samples compared to healthy tissue[11,186] On the contrary, Schartinger and coworkers studied head and neck cancer samples that included mainly OSCC and found overexpression of SST_1, SST_2, SST_4, and SST_5 with a low expression of SST_3 in cancer samples compared to healthy tissue from other patients´ oropharyngeal mucosa[12]. Our cohort is similar to Schartinger´s study and shares similar results.

Therefore, based on our results and the previous studies, it seems reasonable to suggest that overexpression of SSTs might be a typical cellular/molecular signature across various tumor types, including OSCC. This overexpression may be related to the antiproliferative signal, either by inhibiting mitogenesis or stimulating apoptosis that SSTs are known to carry out[286]. Moreover, these results might suggest that SSAs may have a therapeutic role in these tumors, a hypothesis that has been tested later on in this Doctoral Thesis.

5.5.2. *SST$_s$* are associated with relevant clinical and pathological data of OSCC patients with better prognosis

Another relevant finding of our study was that the expression levels of different SSTs, especially *SST$_2$* (the dominant SST subtype expressed in OSCC samples), were associated with risk factors, clinical and histopathological malignant features.

Firstly, we found a correlation between higher *SST$_2$* expression and less regional metastasis. Moreover, *SST$_2$* also had a negative correlation to the number of positive lymph nodes, the number of lymph nodes with ENE+, and their size. These are well-known risk factors in OSCC for regional recurrence and distant metastasis[126,263,275]. Additionally, we observed that the expression of *SST$_2$* and *SST$_5$* was related to a lower rate of regional and locoregional recurrence. On the contrary, the expression of *SST$_3$* (the SST subtype with lower levels in OSCC samples) was positively correlated with a higher pT and a higher Stage. Therefore, these results show that the main clinical correlations were associated with *SST$_2$* expression in OSCC. In fact, SST$_2$ acts as a dominant receptor in these tumors which it seems to be associated with decreased malignancy features (better outcomes, better DFS, less nodal and distant metastasis), suggesting a potential predictive value as a metastatic and recurrence biomarker.

Regarding risk factors, we observed a negative correlation between SST$_{2,5}$ and age, being both receptors overexpressed in patients <45 years old. In our cohort, age showed a statistical trend with worse survival for elderly patients and a higher recurrence rate. As previously stated, the higher *SST$_2$* and *SST$_5$* expression was related to a lower recurrence rate. Interestingly, the literature has no consensus regarding the impact of age on OSCC, with mixed results[33,36,37,43,45–50]. However, what is well known is that young patients with OSCC have a lack of long-term exposure to known OSCC risk factors, which makes young patients with OSCC a possible different entity[34]. Therefore, these results open the discussion about SSTs overexpression behaving as a potential protective biomarker in these patients.

Prognostic factors of oral cavity cancer are well known and under continuous review, as discussed in the introduction. DOI was recently introduced as an additional factor more accurately depicting pT in the most recent 8th edition of the AJCC TNM Staging Manual[83]. Tumor pT was classified according to the size but upgraded if a higher depth of invasion was found. Our results showed a negative correlation between SST$_2$ and DOI. We observed a higher expression of *SST$_2$* (*p=0.04*) in tumors with DOI 5-10 mm (15/37) compared to >10 mm (18/37) or <5mm (4/37). We believe tumors with less depth of invasion (< 5 mm DOI) have less SSTs expression. However, our results may not have achieved that due to the small number of pT1 (<5mm DOI) analyzed in the study based on the limiting selection criteria.

Besides DOI, recent papers have also focused on the impact of tumor budding and the pattern of invasion in recurrence rate[133,287]. We have found that SSTs are related to histopathological factors

present in tumors with a less aggressive histopathological behavior, such as an expansive front of invasion vs. infiltrative front of invasive, lower histopathological grade (G1), or uniform edges compared to poorly defined ones. These histopathological factors have been related to less risk for locoregional recurrence[137,145,265,276,280].

Moreover, a higher *SST₂* expression showed a statistical tendency to be present on tumors with higher PTI. The peritumoral inflammation has been considered a defense mechanism against cancer progression and invasion[130,131,136–138]. It has been proposed that inflammatory activity, such as an immunological response to the tumor, could be used as a prognostic factor since the lower the inflammatory infiltrate, the greater the risk of regional or distant metastasis[131,138]. However, the PTI's role in the prognosis of OSCC is still very controversial. Other studies suggest that peritumoral stromal inflammation is more likely to contribute to cancer development[139]. In our cohort, higher PTI was associated with better OS, lower recurrence rate and negatively correlated with histopathological factors such as pT, pN, Stage, and DOI. Besides the local inflammation, a relationship between systemic inflammatory response (SIR) and cancer has also been studied. In OSCC, higher levels of CRP were associated with larger tumors, positive nodal status, and worse survival[140,143]. Although CRP was not significantly correlated to OS in our cohort, the SSTs analysis showed a statistically negative correlation between the expression of SST_2 and SST_3 and CRP levels which also favors the relationship of overexpression of SST_2 and SST_3 with good prognosis features.

5.5.3 OSCC cells are responsive *in vitro to SSA*

All the data mentioned so far support the idea that the expression of *SST₂* is associated with relevant clinical and pathological data featuring OSCC patients with better prognosis behavior. This statement, together with the continuous rise of OSCC cases, the bad outcomes despite all therapeutic efforts, and the diverse heterogeneity of recurrence predictors, provides a scientific rationale to propose testing novel therapeutic strategies, such as a randomized controlled trial of the effect of an SSA (especially those that preferentially bind to *SST₂*) in the oral squamous cell carcinoma.

The present study indicates that OSCC tissues express high levels of different SST-subtypes (especially *SST₂*), which might be considered an important clinical finding, as the responsiveness of SSAs is critically dependent on the presence of SSTs, and because the treatment with available SSAs (e.g., first-generation compounds, octreotide and lanreotide, which preferential binding to *SST₂*) has become the mainstay of medical therapy for tumor control in neuroendocrine disorders expressing SSTs [such as pituitary and gastroenteropancreatic neuroendocrine tumors[260,283,288].

SSAs can bind any SSTs, but their therapeutic actions are assumed to be mainly mediated through SST_2 and SST_5 activation[179]. Some studies have observed a significant downregulation of SST_2 expression on *in vitro* nasopharyngeal cancer cell lines after 72-h treatment with the SSA PEN-221[187].

However, the same study did not observe the same results with the treatment of lanreotide and octreotide, which did not affect SST_2 expression with no changes in cell death/apoptotic pathways[187]. Our results demonstrated that all SSAs tested (10^{-7} M) significantly reduced the proliferation rate of primary OSCC cell cultures. Therefore, to the best of our knowledge, our results are the first to demonstrate that OSCC cells are responsive *in vitro* to the first and second generation of SSAs (octreotide, lanreotide, and pasireotide). All SSAs decreased proliferation rate at 24-, 48-, and 72-h of incubation, with the most significant effect for pasireotide. As described before, single receptor analogs (octreotide and lanreotide) target a single SST receptor, mostly SST_2. However, pasireotide has a higher binding affinity for SST_5, SST_1, and SST_3, respectively, and a lower one for SST_2. Our results showed that all SSTs but SST_1 were overexpressed in tumor samples with a significant difference for SST_2 and SST_3, and a statistical trend for SST_4 and SST_5. This is relevant due to pasireotide´s effect decreasing the proliferation rate of OSCC cells *in vitro*, which highlights not just SST_2 as a therapeutic molecular target in OSCC, but also SST_3 and SST_5. Thus, it seems plausible that additional factors, besides the simple abundance of a given SST, critically influence the SSA response in OSCC cells.

In summary, in the section of this doctoral thesis we have assessed the expression levels of all five SST subtypes in OSCC by qPCR, and it is the first series to compare the expression levels of each receptor between OSCC and control (adjacent non-tumor) tissues. Additionally, although the role of SSTs as possible prognostic biomarkers and therapeutic targets in OSCC needs to be further explored, this study strongly suggests that: (1) expression levels of SST_2 could be related with less rate of regional recurrence, both local and regional and less incidence of distant metastasis, suggesting that the assessment of SST expression profiles by qPCR may represent an effective screening tool to predict prognosis of OSCC; and, (2) SSAs exert antitumoral effects on OSCC cells, opening new avenues to explore their potential as novel targeting therapy for patients with OSCC.

5.6. Spliceosome and associated proteins in OSCC

* Context for studying the splicing machinery in OSCC: rising evidence in the last two decades has documented that the spliceosome and associated proteins are often altered in different cancer types, which augments pathobiological versatility through generation of distinct/novel alternative splicing variants[156,161,208,289–292]. In fact, it has been reported that multiple spliceosome components and splicing factors seem to be altered in OSCC, and these changes often correlates with lower patient survival, tumor aggressiveness parameters and worse prognosis[56,218,220–222,230–240,293]. However, to the best of our knowledge, no studies had reported hitherto a comprehensive analysis to ascertain whether the splicing machinery is altered in OSCC tissues as compared with adjacent healthy tissues obtained within the same patient. Thus, we aimed to determine for the first time the expression profile of a representative set of spliceosome components and splicing factors and their relationship with key clinical and

pathological features of OSCC samples/patients, as well as to determine the therapeutic potential of an inhibitor of the activity of the splicing machinery using primary OSCC cell cultures.

5.6.1. Splicing machinery is dysregulated in OSCC samples compared to control tissues

As mentioned before, OSCC is one of the cancer types with a poor prognosis with overall survival below 60% at 5y, which shows that the diagnostic and therapeutic strategies are ineffective; therefore, it is highly necessary the identification of novel molecular elements that could be used as biomarkers to refine OSCC diagnosis, improve prediction of prognosis and tumor behavior, and provide tools to develop novel therapeutic targets. In this sense, the splicing process has emerged as a novel source for identifying new biomarkers for the diagnosis and prognosis of numerous types of cancers, including OSCC. However, these studies have not comprehensively explored the global dysregulations of spliceosomal components and splicing factors in OSCC.

Therefore, based on the information mentioned above, the objectives of this section of the doctoral thesis were: 1) to characterize the expression pattern of key splicing-related elements (spliceosome components and splicing factors) in a representative battery of clinically well-characterized OSCC tissues in comparison with adjacent healthy tissues obtained within the same patient in order to determine the utility of key dysregulated spliceosome-related elements as putative diagnostic biomarkers; 2) to assess the putative *in vivo* association between the expression pattern of key splicing-related elements (spliceosome components and splicing factors) in the tumor of patients with OSCC and relevant clinical and histopathological data parameters (stage, histological grade, tumor invasion, presence of metastasis, recurrence, overall survival, etc.) in order to determine the utility of key dysregulated spliceosome-related elements as putative prognostic biomarkers, and; 3) to assess the therapeutic potential of a splicing machinery inhibitor (pladienolide B) in primary OSCC human cell cultures.

First, the results obtained in this study demonstrate for the first time a drastic dysregulation of the expression profile of the splicing machinery in a well-characterized cohort of OSCC compared with healthy-adjacent tissues, where a representative set of SFs was markedly altered [12 out of 59 components (20 %)]. Specifically, we found a downregulation of SRSF4, SRSF5, SRSF9, NOVA1, ESRP1, ESRP2, RBM3, RBM10, TRA2A, TRA2B and TIA1, and an upregulation of SRSF10. Indeed, although bioinformatics analyses did not defined an expression-based molecular fingerprint of the alteration of all the components of the splicing machinery analyzed that was able to perfectly discriminate between OSCC vs. control tissues, a variable importance in projection (VIP) score of the PLS-DA using the SFs with a higher capacity of discrimination between the two samples groups (ESRP1, ESRP2, RBM3, RBM10, and NOVA1) revealed the existence of a differential pattern of alteration between OSCC and control samples. Moreover, individual ROC curve analysis of each component of the splicing machinery found to be altered in OSCC (SRSF4, SRSF5, SRSF9, NOVA1,

ESRP1, ESRP2, RBM3, RBM10, TRA2A, TRA2B and TIA1) revealed that all these SFs could serve as potential diagnostic biomarkers of OSCC (AUC obtained ranged from 0.617 to 0.810). Interestingly, the potential diagnostic capacity clearly improved when the ROC curve analysis was performed with the 5 SFs with a higher capacity of discrimination (ESRP1, ESRP2, RBM3, RBM10, and NOVA1; AUC of 0.88) which reinforced the potential capacity of discrimination of the selected SFs between OSCC and non-tumor samples. Therefore, all these data together demonstrate that OSCC curse with a global splicing dysregulation in humans.

In this context, the expression levels of SFs in OSCC samples vary in the literature. Some SFs have been described to be upregulated or downregulated, or even oppositely altered, in OSCC samples. Other studies describe the expression of some SFs in OSCC but do not compare their expression with healthy samples. For example, different authors have described that SRSF3 can be downregulated or upregulated in OSCC samples[218,293] For example, different authors have described that SRSF3 can be downregulated or upregulated in OSCC samples (Peiqui, Guo). At the same time, the level of expression of other SFs such as hnRNP C, hnRNP H1, hnRNP H2, hnRNP K, TIA1, or CELF is not specified whether it is downregulated or upregulated[56,235]. In our study, we found an overall downregulation of the majority of SFs altered on tumor samples (11 out of 12: SRSF4, SRSF5, SRSF9, NOVA1, ESRP1, ESRP2, RBM3, RBM10, TRA2A, TRA2B and TIA1). These results were consistent with prior studies that also found down-regulation of ESRP1 and ESRP2[237], NOVA1[239], and RBM3[238] in OSCC tissues, but are in contrast to other studies indicating that SRSF5 and SRSF9 are upregulated in these tumoral samples. To the best of our knowledge, this is the first study to describe the downregulation of SRSF4, SRSF9, RBM10, TRA2A, TRA2B, and TIA1 in OSCC as compared with healthy-adjacent tissues. Moreover, we found that SRSF10 expression was upregulated in our cohort of OSCC samples which is consistent with a previous study[231]. Our results are in accordance with previous results demonstrating that the splicing machinery, especially many of the SFs found to be altered in OSCC samples, is drastically dysregulated in different diseases, including tumoral pathologies (i.e. tumors of the prostate, brain, pituitary, etc.)[156,158,161,294,295].

Therefore, based on our results and the previous studies, it seems reasonable to suggest that the dysregulation of the splicing machinery, especially of key SFs, might be a typical cellular/molecular signature across various tumor types, including OSCC, thus opening the possibility of identifying novel diagnostic and prognostic biomarkers that would help to manage this devastating pathology, and to provide novel tools to develop efficient therapeutic targets (i.e. inhibitor of the splicing machinery: see section 5.6.3).

5.6.2. Splicing factors are associated with relevant clinical and pathological data featuring OSCC patients with better prognosis

All the data mentioned in the previous section might suggest that the alteration of different SFs could be associated with relevant clinical and pathological data in OSCC patients. In fact, this Thesis provides novel data demonstrating that the expression levels of some of the SFs identified to be altered in OSCC might be associated with key clinical and histopathological features of less aggressiveness and, most importantly, with overall patient survival, suggesting a potential utility of the expression of some SFs as prognostic biomarkers in OSCC.

Specifically, our results revealed that OS was positively correlated with higher expression of *SRSF5, SRSF9, RBM3, TRA2A, and TRA2B*. Interestingly, the expression of all of these SFs was downregulated in tumor samples compared with healthy-adjacent tissues, and their expression in the OSCC tissue was associated with better OS. To the best of our knowledge, this is the first study demonstrating the relationship between these SFs with oral cancer better survival. Notably, the levels of TRA2B, SRSF9, and RBM3 were also associated with less recurrence or distant metastasis, suggesting that these SFs might have pathophysiological relevant in this tumoral pathology suggesting a causal link between dysregulation of these SFs and OSCC aggressiveness.

Importantly, most of these SFs that were associated with improved OS and less recurrence or distant metastasis were observed to be also related to key clinical and histopathological features related to better outcomes, such as less cervical nodal disease (pN). pN is a significant predictor factor of patient prognosis, increasing mortality rate if present[77,119,263,275]. Moreover, we found that patients with higher expression of RBM3, TRA2A, TRA2B, and TIA1 had less cervical nodal disease and tumors with smaller stages. This study also revealed that the expression of SRSF5, TRA2A, TRA2B, and TIA1 in OSCC was negatively correlated with the number of positive lymph nodes, the number of lymph nodes with ENE+, and their bigger size. Therefore, these results might be clinically relevant because the identification of potential biomarker(s) showing a correlation with clinical or histopathological features associated with a higher likelihood of cervical metastasis and with the outcome of aggressiveness of the OSCC is crucial for the possible development of new molecular profiles and for the management of patients diagnosed with this terrible tumoral pathology. In support of the clinical and pathophysiological relevance of the alteration of key SFs in cancer is a recent analysis in 33 cancer types (through The Cancer Genome Atlas database) revealing that putative cancer driver mutations occur in 119 genes encoding critical SFs[296]. Definitely, further studies have to be performed in order to identify the potential mechanisms of actions and signaling pathways underlying this link between the dysregulation of these SFs and the oncome of critical clinical or histopathological features in OSCC patients.

Furthermore, TRA2B and TIA expression levels were also associated with other key histopathological factors related to better outcomes, such as lower grade of differentiation, higher PTI,

or less DOI. These results are in accordance with a previous study indicating that higher expression of TIA was associated with better prognosis[235]. However, although TRA2B expression has been previously described to be altered in head and neck cancers, no information related to its impact on OSCC had been previously reported. Consequently, this is the first study to describe a more detailed knowledge of the histopathological relationship between TRA2B and TIA and OSCC patients.

Although other splicing factors, such as NOVA1 and ESRP2, did not reach significance in overall survival analysis in our cohort, they did show a relationship with other relevant clinical and histopathological data. Specifically, their expression was downregulated in tumor samples, and it was positively associated with histopathological factors of good prognosis, such as an expansive and uniform front of tumor invasion compared to infiltrative and poorly defined. NOVA1 expression was also related to higher tumor PTI, lower stage, less cervical metastasis, and lower regional recurrence rate. In this sense, both NOVA1 and ESRP2 downregulation has also been previously associated with OSCC[237,239]. In fact, our results support previous studies indicating that low ESRP2 expression is found in normal epithelium, upregulated in precancerous lesions and carcinoma *in situ*, but downregulated in invasive fronts[237]. Moreover, NOVA1 lower expression has also been described as an independent poor prognosis factor for OS and recurrence in OSCC HPV-negative[239], consistent with our results.

Importantly, SFs are considered molecular tools for chemotherapy response, acting as either pro-survival factors that diminish drug-induced apoptosis or, oppositely, that potentiate pro-apoptotic effects of chemotherapeutics[206]. The specific influence of individual SFs on the efficacy of chemotherapy drugs used in head and neck cancer has only been studied in the case of SRSF3, which showed it reduces the sensitivity of cancer cells to Paclitaxel (PTX) treatment. Other SFs have also been associated with PTX efficacy, such as TRA2A promoting resistance to PTX in breast cancer[219]. In this context, our results describe for the first time the association between TRA2A and OSCC, among other SFs, and their relationship might unveil the role of these newly described SFs as therapeutic targets in OSCC. In line with this, several reports have indicated that cancer cells are particularly vulnerable to splicing alterations, and that these changes might relevant from a therapeutic point of view since the transcriptomic landscape of cancer cells makes them particularly vulnerable to the pharmacological inhibition of the splicing[208,291]. In fact, modulators of the activity of the splicing machinery are currently being used in clinical trials offering a novel approach to treat different cancer types. Therefore, based on these evidences and on the results of this Doctoral Thesis, we wondered whether the inhibition of the activity of the splicing machinery might reduce the aggressiveness features in OSCC cells (see next section).

5.6.3 OSCC cells are responsive *in* vitro to an inhibitor of the activity of the splicing machinery

The present study indicates that the expression levels of several key SFs are altered in OSCC compared with adjacent-healthy tissues of the oral cavity suggesting a potential role of this splicing dysregulation in the pathophysiology of OSCC. In fact, nowadays it is well-recognized that the splicing process and its regulation are highly relevant for understanding every hallmark of cancer, to the point that splicing alterations constitute another cancer hallmark[7,297,298]. In this context, our study also provides an initial, unprecedented proof-of-concept on the suitability of splicing dysregulation as a novel potential target for OCSS treatment by demonstrating that the pharmacological disruption of the splicing process with a specific drug may have antitumor effects in these tumors. Specifically, we tested the direct *in vitro* effect of pladienolide-B in primary OSCC cell cultures. These results demonstrate for the first time that treatment with pladienolide-B significantly inhibited cell proliferation in OSCC cells, which compares well with recent data from our group demonstrating that pladienolide-B reduced proliferation rate in prostate, pituitary, liver, pancreas, and brain tumors[156,159–161].

All the data presented so far provide original, compelling evidence that the expression of several SFs is significantly altered in OSCC compared with healthy-adjacent oral cavity tissues, and that the alterations in the levels of some of these SFs are functionally linked to critical pathophysiological features in OSCC, which further reinforce the potential clinical and pathophysiological importance of the dysregulation of the splicing machinery in cancer. Moreover, our data highly the inhibition of the splicing machinery as a putative and efficient pharmacological target in OSCC, offering a clinically relevant opportunity worth to be explored in humans.

6. Conclusions

The main conclusions of the work presented in this Thesis are:

1. The expression levels of different somatostatin receptors are significantly altered in OSCC compared to adjacent healthy control tissues. Specifically, our findings highlight the potential role of SST_2 as a good prognostic biomarker for OSCC.

2. Different SSAs (octreotide, lanreotide, and pasireotide) can exert antitumoral effects (reduction in cell-proliferation) on OSCC cells, opening new avenues to explore their potential as targeted therapy for OSCC.

3. The components of the splicing machinery are profoundly dysregulated, generally downregulated, in OSCC compared to adjacent healthy tissues obtained within the same patient. The levels of some splicing factors are associated with significant clinical and histopathological parameters, reinforcing the potential clinical and pathophysiological importance of the dysregulation of the splicing machinery in cancer.

4. The inhibition of the splicing machinery (with pladienolide-B) could be an efficient pharmacological approach in OSCC, offering a clinically relevant opportunity worth to be explored in humans.

GLOBAL COROLLARY

As a **general conclusion**, the studies implemented in the present Thesis allow us to expand and advance the knowledge of the molecular basis of the pathophysiological regulation of OSCC through the analysis of two critical regulatory systems: the somatostatin receptors and the splicing machinery. Specifically, our results demonstrate that *SST_2* and different splicing factors (SRSF4, SRSF5, SRSF9, NOVA1, ESRP1, ESRP2, RBM3, RBM10, TRA2A, TRA2B, TIA1, and SRSF10), represent relevant points of regulation for OSCC. Therefore, these SFs could be valuable tools for developing novel diagnostic and prognostic biomarkers and/or therapeutic targets to improve the diagnosis, management, and survival of patients with OSCC. Moreover, this doctoral Thesis demonstrates the efficacy of SSAs and pladienolide-B as potential and useful therapeutic tools for human OSCC.

7. References

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