**DEPARTAMENTO DE BIOLOGÍA CELULAR, FISIOLOGÍA E INMUNOLOGÍA**


# Identification of novel molecular markers and contribution of splicing mechanisms in endocrine-related tumoral pathologies

## Identificación de nuevos marcadores moleculares y contribución de mecanismos de splicing en patologías tumorales endocrinas

**Sergio Pedraza Arévalo**

**Córdoba, 2019**

TITULO: *Identification of novel molecular markers and contribution of splicing mechanisms in endocrine-related tumoral pathologies*

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# Identification of novel molecular markers and contribution of splicing mechanisms in endocrine-related tumoral pathologies

## Identificación de nuevos marcadores moleculares y contribución de mecanismos de splicing en patologías tumorales endocrinas

Memoria de Tesis Doctoral presentada por **Sergio Pedraza Arévalo**, Licenciado en Biología, para optar al grado de **Doctor en Biomedicina**

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En Córdoba, a 13 de diciembre de 2019

DEPARTAMENTO DE BIOLOGÍA CELULAR, FISIOLOGÍA E INMUNOLOGÍA

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INFORMAN

Que D. Sergio Pedraza Arévalo, Licenciado en Biología, ha realizado bajo nuestra dirección el trabajo titulado "**Identification of novel molecular markers and contribution of splicing mechanisms in endocrine-related tumoral pathologies**" 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 13 de diciembre de 2019.

Fdo.: Dr. Justo P. Castaño Fuentes  Fdo.: Dr. Raúl M. Luque Huertas

**TÍTULO DE LA TESIS: Identification of novel molecular markers and contribution of splicing mechanisms in endocrine-related tumoral pathologies**

**DOCTORANDO: Sergio Pedraza Arévalo**

**INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS**

Durante el desarrollo de la presente Tesis Doctoral, realizada entre septiembre de 2015 y diciembre de 2019, el doctorando Sergio Pedraza Arévalo ha alcanzado sobradamente los objetivos planteados al comienzo de la misma, en la que ha podido desarrollar y validar técnicas experimentales novedosas que han sido de gran utilidad para el grupo de investigación y que le han permitido obtener resultados muy relevantes en el campo de los tumores relacionados con el sistema endocrino. De manera más específica, los resultados obtenidos durante el desarrollo de su trabajo le han posibilitado publicar un artículo directamente relacionado con su Tesis Doctoral en la revista "*Prostate*", de referencia internacional en el área de urología y nefropatías. Adicionalmente, se han producido dos trabajos más a partir de esta Tesis Doctoral, que se enviarán a publicar en un corto periodo de tiempo en revistas de prestigio de sus áreas correspondientes. Finalmente, el doctorando ha presentado los resultados de su Tesis en diferentes congresos y reuniones de ámbito nacional e internacional, así como participado en el desarrollo de varias patentes.

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

Córdoba, 13 de diciembre de 2019

Firma de los directores

Fdo.: Justo P. Castaño Fuentes

Fdo.: Raúl M. Luque Huertas

Esta Tesis Doctoral ha sido realizada en el Departamento de Biología Celular, Fisiología e Inmunología de la Universidad de Córdoba y en el Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), bajo la dirección de los Dres. Justo P. Castaño Fuentes y Raúl M. Luque Huertas. Dicho trabajo ha sido subvencionado mediante los proyectos del Instituto de Salud Carlos III FIS (PI16/00264) y del Ministerio de Economía (BFU2016-80360-R y BFU2013-43282-R) y con la financiación para el doctorando de una Ayuda para la Formación del Profesorado Universitario (FPU) del Ministerio de Educación, Cultura y Deporte (referencia FPU14/04290). Durante el transcurso de la presente Tesis Doctoral se ha realizado una estancia de tres meses en el Centro para la Investigación de la Diabetes de la Universidad Libre de Bruselas, bajo la supervisión del Dr. Decio L. Eizirik, financiada por una ayuda para Estancias Breves FPU del Ministerio Educación, Cultura y Deporte (EST17/00796), para la obtención de la Mención Internacional en el Título de Doctor de la Universidad de Córdoba.

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# RESUMEN

# Resumen

El cáncer es uno de los mayores retos que afronta la humanidad, pues comprende algunas de las patologías más graves que afectan a la salud a nivel mundial. Aunque se están realizado grandes esfuerzos y se han logrado avances significativos en investigación básica, clínica y traslacional durante las últimas décadas, el desarrollo de nuevas estrategias terapéuticas en oncología, más globales y a la vez precisas y eficientes, está limitado por la enorme heterogeneidad y complejidad de esta enfermedad. Para enfrentarse a dichas dificultades, Hanahan y Weinberg propusieron en el año 2000 y actualizaron en 2011 un grupo de alteraciones comunes compartidas por la mayoría de los tipos de cáncer a diferentes niveles, que se conocen como *hallmarks* o pilares del cáncer. Algunos de estos *hallmarks* están relacionados con la señalización hormonal, que se considera un componente importante en el control de la malignidad tumoral. En este contexto, la presente Tesis se ha enfocado fundamentalmente en el estudio de cánceres relacionados con el sistema endocrino, como el cáncer de próstata (CaP), fuertemente dependiente de la regulación hormonal, y distintos tipos de tumores neuroendocrinos (TNEs). El CaP es una de las patologías tumorales de mayor incidencia en hombres y una de las causas de muerte más comunes relacionadas con el cáncer en la población mundial. Por su parte, los TNEs son un grupo de neoplasias marcadamente heterogéneo, que surgen del sistema neuroendocrino difuso y se ha clasificado habitualmente según la localización del tumor. Concretamente, esta Tesis se enfoca en los tumores pancreáticos (PanNETs). Por último, analizaremos también un tipo tumoral estrechamente relacionado, los tumores neuroendocrinos hipofisarios (PitNETs).

Uno de los sistemas hormonales clásicamente relacionados con distintos tipos de tumores y que ha centrado el interés de nuestro grupo es el formado por la somatostatina y sus receptores ($SST_1$-$SST_5$). En particular, este sistema se ha relacionado con tumores neuroendocrinos (NET, PitNET), en los que los agonistas sintéticos de la somatostatina se emplean ampliamente para actuar sobre varios de sus receptores (ej. $SST_2$, $SST_5$) que sirven como efectivas dianas terapéuticas en esas patologías. En este contexto, destaca el reciente protagonismo del $SST_5$ como posible diana de tratamiento con nuevos análogos de SST, y por la existencia de variantes de splicing truncadas del mismo (ej. $SST_5$TMD4) relacionadas con agresividad tumoral en varios tipos de cáncer. Sin embargo, se conoce muy poco acerca de la biogénesis del $SST_5$ a partir de su gen *SSTR5*, así como del papel de otros receptores de somatostatina en patologías tumorales endocrinas.

El creciente descubrimiento de variantes de splicing anómalas que, como la mencionada $SST_5$TMD4, se sobreexpresan en distintos tipos de cáncer apoya la idea de que la alteración del proceso de splicing puede estar involucrada en el desarrollo y la agresividad tumoral, a través de la desregulación del perfil normal de splicing alternativo y de la generación de variantes oncogénicas. De hecho, en los últimos años, la alteración del proceso de splicing se empieza a considerar como un nuevo *hallmark* transversal del cáncer, pues afecta a todos los *hallmarks* ya descritos. No obstante, los datos disponibles sobre el splicing y su desregulación son aún escasos en muchas patologías tumorales, entre las que se incluyen los NETs.

Por todo lo expuesto, el **objetivo general** de esta Tesis ha sido determinar el papel que desempeñan los receptores de somatostatina y la maquinaria de splicing en distintos tipos de cánceres hormono-dependientes y tumores neuroendocrinos, así como los mecanismos de regulación subyacentes, con el fin último de descubrir nuevos biomarcadores y dianas farmacológicas con potencial para mejorar las aproximaciones diagnósticas y terapéuticas en esas patologías.

En este contexto, la **primera sección experimental** de esta Tesis se centró en el estudio del papel del $SST_1$ en CaP, explorando su presencia, alteración y posible papel funcional en esta patología. Los resultados mostraron una clara sobreexpresión de $SST_1$ en muestras de CaP con respecto a las de próstata normal. Además, en las muestras tumorales, la expresión de $SST_1$ se correlacionó con la del receptor de andrógenos (AR). Estudios *in vitro* con la línea celular de CaP 22Rv1 demostraron que el tratamiento con un agonista específico del $SST_1$, BIM-23926, disminuyó la proliferación celular y la secreción de PSA de estas células. Asimismo, el silenciamiento de la expresión del *SSTR1* incrementó, mientras que su sobreexpresión disminuyó, la proliferación de dicha línea celular. Mediante el uso de este agonista selectivo, estudiamos además las rutas de señalización implicadas en la función de $SST_1$. El tratamiento con BIM-23926 disminuyó la fosforilación de AKT tras 30 min, mientras que no se observaron cambios en la activación de otras proteínas importantes, como ERK, AR o JNK, ni tampoco en los niveles de $[Ca^{2+}]_i$, un segundo mensajero clásico en la señalización hormonal. Un tratamiento prolongado (24 h) con este agonista disminuyó la expresión de ARNm de *KLK3*, el gen que codifica el PSA, y *CCND3*, un importante regulador del ciclo celular, así como la expresión del propio *SSTR1*, lo que parece indicar una autorregulación a través de un bucle negativo. Ese tratamiento también provocó cambios en rutas de

señalización que se han relacionado con el AR; en concreto, la activación de $SST_1$ inhibió la expresión de varios oncogenes (como *ADAMTS1*, *VIPR*) y estimuló la del supresor tumoral *IGFBP5*. Finalmente, análisis *in silico* revelaron que la expresión de *SSTR1* podría estar regulada por varios miRNAs que correlacionan inversamente con este receptor en la base de datos The Cancer Genome Atlas (TCGA). Así, el tratamiento *in vitro* con uno de esos miRNAs, el miR-24, disminuyó los niveles proteicos y de ARNm de $SST_1$ en las células 22Rv1, observándose además una correlación de la expresión de ambos genes en la base de datos Memorial Sloan Kettering Cancer Center (MSKCC), que incluye muestras de metástasis.

La **segunda sección experimental** de esta Tesis se centró en el estudio de la regulación de la expresión del gen *SSTR5* en NETs, incluyendo PitNETs (especialmente somatotropinomas) y PanNETs. En primer lugar, aproximaciones *in silico* revelaron que existe un transcrito natural antisentido (TNA) que se superpone al gen *SSTR5* en el genoma, llamado *SSTR5-AS1*, y que hay cuatro islas CpG, regiones genómicas con una alta proporción de citosina-guanina, susceptibles de ser metiladas, entre esos dos genes. Aunque no se encontraron diferencias relevantes en la expresión de *SSTR5-AS1* en PitNETs respecto a hipófisis normal, se observó que el gen *SSTR5* sí se sobreexpresa en esa patología y, lo que es más interesante, que existe una marcada correlación directa entre la expresión de ambos genes (*SSTR5*/*SSTR5-AS1*) tanto en muestras de hipófisis normal como de somatotropinomas. Adicionalmente, descubrimos que la metilación de ADN en tres de las zonas CpG analizadas estaba alterada en somatotropinomas respecto a hipófisis normal. Es más, la metilación de una de dichas zonas, la que solapa con el centro del gran exón del gen *SSTR5*, incluyendo su zona de splicing alternativo, se correlacionó inversamente con la expresión tanto del receptor como de su antisentido en las muestras de somatotropinomas, pero no en las de hipófisis normal. En los PanNETs, observamos que el gen *SSTR5-AS1* se sobreexpresa en muestras tumorales respecto a su tejido adyacente no tumoral, mostrando esta expresión una correlación directa con la de *SSTR5* en ambos tejidos. Estos hallazgos nos impulsaron a realizar estudios *in vitro*, en la línea celular modelo de NETs BON-1, en las que observamos que el silenciamiento de *SSTR5-AS1* disminuía, a su vez, la expresión de *SSTR5*, que, de nuevo, mostraba una correlación directa con la de su TNA. Más aún, dicho silenciamiento aumentó la proliferación celular y la formación de colonias, apoyando la idea de que este antisentido podría contribuir a la agresividad de las células de NETs. El efecto inhibidor del

tratamiento con pasireotide, un análogo de somatostatina cuya diana predominante es el $SST_5$, en los parámetros celulares citados, también se vio alterado por el silenciamiento de *SSTR5-AS1*. De hecho, el silenciamiento del TNA disminuyó la activación de ERK y AKT y, llamativamente, potenció el efecto del tratamiento con pasireotide sobre la fosforilación de dichas proteínas, apoyando la probable relevancia de *SSTR5-AS1* en el control de la acción de $SST_5$.

En la **tercera sección experimental** de esta Tesis, nuestro objetivo fue estudiar la desregulación de la maquinaria de splicing y su posible papel funcional en PanNETs, buscando nuevos biomarcadores y/o dianas terapéuticas para esta patología. Primero, medimos la expresión de un grupo de 45 componentes de la maquinaria de splicing en muestras de PanNETs, comparadas con el tejido adyacente no tumoral, utilizando un array de PCR cuantitativa basado en microfluídica. Aproximadamente, el 50 % de los genes medidos, incluyendo algunos ARN nucleares pequeños que conforman el núcleo de la maquinaria, se observó que estaban sobreexpresados en las muestras tumorales, mientras que solo un factor se encontró infraexpresado. Un análisis de componentes principales y otros ensayos bioinformáticos seleccionaron cinco de los genes medidos como aquellos con las mejores características de agrupamiento para distinguir entre muestras tumorales y no tumorales; dichos genes fueron: *NOVA1*, *PRPF8*, *RAVER1*, *SRSF5* y *SNW1*. Además, observamos que los niveles de expresión de estos factores están asociados a importantes parámetros cínicos, como el índice Ki-67, necrosis, recidiva de la enfermedad, funcionalidad, pérdida de peso o invasión vascular. Uno de estos genes, *NOVA1*, mostró una curva ROC con un área mayor de 0,85 y su sobreexpresión en el tejido tumoral se confirmó a nivel proteico mediante inmunohistoquímica. Por ello, decidimos evaluar el posible papel funcional de este factor en las células de PanNETs. Así, descubrimos que la sobreexpresión de *NOVA1* aumentó la tasa de proliferación *in vitro* de dos líneas celulares modelo de PanNETs, BON-1 y QGP-1, y estimuló el crecimiento de tumores xenotrasplantados de células BON-1 en ratones. En cambio, el silenciamiento de *NOVA1* indujo en ambas líneas celulares una disminución de la proliferación, que estaba asociada a una reducción en la expresión de *CCND1* y un aumento de la de *CASP3*. Del mismo modo, el silenciamiento de *NOVA1* disminuyó la activación de ERK, PTEN y PDK1, sin alterar AKT, lo que sugiere que este factor puede actuar a través de acciones complejas y aparentemente contrapuestas. Curiosamente, el silenciamiento de *NOVA1* aumentó la fosforilación de p53 solo en las células QGP-1, en

las cuales, al mismo tiempo, disminuyó la expresión de la isoforma oncogénica *Δ133TP53*, sin alterar la canónica *TP53*. Estos resultados, junto con los expuestos sobre señalización, sugieren que *NOVA1* puede desempeñar un papel relevante en la regulación de la ruta de senescencia, involucrando a p53 y ERK, de manera célula-específica. Por otro lado, la disminución génica de *NOVA1* inhibió los niveles proteicos de ATRX y DAXX, así como la expresión de la isoforma truncada de *TERT*, lo que podría implicar a *NOVA1* en la regulación de la ruta de remodelación de la cromatina, particularmente relevante en PanNETs. Es más, en la línea celular QGP-1 el silenciamiento de este factor de splicing mejoró el efecto antiproliferativo de everolimus, un inhibidor de mTOR ampliamente usado en el tratamiento de PanNETs.

Por todo lo anterior, las principales conclusiones del trabajo presentado en esta Tesis son:

1. El gen *SSTR1* se sobreexpresa en CaP, donde podría ejercer relevantes acciones y estar regulado por miRNAs específicos. En concreto, $SST_1$ media la inhibición de la proliferación celular y la secreción de PSA en la línea celular de CaP 22Rv1, probablemente a través de rutas y mediadores relacionados con el AR, PI3K/AKT-CCND3.

2. La expresión de *SSTR5* en somatotropinomas y PanNETs puede estar controlada por mecanismos epigenéticos, que incluyen tanto metilación del ADN como procesos postranscripcionales, como la regulación mediada por un antisentido. En concreto, *SSTR5-AS1* podría contribuir a la regulación de características tumorales clave, tales como la proliferación, migración y formación de colonias, y en la respuesta al tratamiento con pasireotide, un análogo selectivo para $SST_5$.

3. Los componentes de la maquinaria de splicing están profundamente alterados, en general sobreexpresados, en PanNETs. Los niveles de algunos de ellos están asociados a importantes parámetros clínicos y permiten distinguir con alta eficiencia entre muestras tumorales y no tumorales. En especial, altos niveles del factor de splicing *NOVA1* provocan un aumento de la proliferación celular y la ruta de senescencia en modelos celulares de PanNETs, alterando rutas de señalización clave y comprometiendo la respuesta a everolimus.

Como **conclusión general**, los estudios presentados en esta Tesis permiten avanzar y profundizar en el conocimiento de las bases moleculares de la regulación

fisiopatológica de cánceres hormono-dependientes y tumores neuroendocrinos por dos receptores específicos de somatostatina y por la maquinaria de splicing. Concretamente, nuestros resultados demuestran que *SSTR1* en el caso de CaP, *SSTR5* en NETs y el factor de splicing *NOVA1* en PanNETs, constituyen puntos relevantes de regulación en estos tumores y como tales pueden servir como herramientas para el desarrollo de nuevos biomarcadores de diagnóstico y/o dianas terapéuticas para mejorar el futuro tratamiento de dichas patologías.

# SUMMARY

# Summary

Cancer represents one of the main challenges for the human being, in that it encompasses some of the most severe and health-threatening pathologies worldwide. Although great efforts are being implemented and significant advances are being reached in basic, translational and clinical research over the last decades, the development of novel and more global and useful therapeutic strategies in Oncology is hampered by the heterogeneity and complexity of this disease. In order to tackle these difficulties, Hanahan and Weinberg proposed in 2000 and updated in 2011 a group of common alterations shared by most cancer types, which were defined as the hallmarks of cancer. Some of those cancer hallmarks are related with hormonal signaling, which is considered an important element in the control of malignant features. In this context, this Thesis has been mainly focused in the study of diverse endocrine-related cancers, such as prostate cancer (PCa), which is strongly influenced by the hormonal milieu, and different types of neuroendocrine tumors (NETs). Indeed, PCa is one of the tumor pathologies with highest incidence in men and one of the most common causes of cancer-related deaths among worldwide population. On the other hand, NETs comprise a markedly heterogeneous group of neoplasia originated from the diffuse neuroendocrine system that have been typically classified by their location. Among them, this Thesis have been focused on pancreatic tumors (PanNETs). Finally, we will also analyze a tumor type closely related, as it is the case of pituitary neuroendocrine tumors (PitNETs).

One of the hormonal axes classically related to different types of tumors and that has represented the central interest of our group is the system comprised by somatostatin and its receptors ($SST_1$-$SST_5$). Particularly, this system has been classically linked to neuroendocrine tumors (NETs, PitNETs), wherein synthetic somatostatin agonists are widely used to act on several of its receptors (e.g. $SST_2$, $SST_5$), which represent useful therapeutic targets in these pathologies. In this context, it is remarkable the growing relevance of $SST_5$ as putative therapeutic target of novel somatostatin analogs, and due to the discovery of novel truncated splicing variants (e.g. $SST_5$TMD4), which are related with the aggressiveness of several cancer types. Nonetheless, very little is known about the biogenesis of $SST_5$ from its gene, *SSTR5,* and about the role of other SST receptors in endocrine-related tumors.

The growing identification of abnormal splicing variants that, similar to the above mentioned $SST_5TMD4$, are overexpressed in different cancer types reinforces the idea that the alteration of the splicing process may be involved in the development and aggressiveness of tumor pathologies, through the dysregulation of the normal alternative splicing pattern and the generation of aberrant isoforms with oncogenic potential. In fact, over the last years, the alteration of the splicing process is being considered as a novel and transversal cancer hallmark, in that it seems to be affecting to all the hallmarks previously described. However, the information regarding the splicing process and its dysregulation is still scarce in some tumor pathologies, including NETs.

Thus, for all the reasons indicated above, the **general aim** of this Thesis was to determine the role of somatostatin receptors and splicing machinery in different types of endocrine-related cancers and neuroendocrine tumors, as well as the underlying regulatory mechanisms, with the final purpose of discovering novel biomarkers and pharmacologic targets with potential to improve the diagnostic and therapeutic approaches in these pathologies.

In this context, the **first experimental section** of this Thesis was focused on the study of $SST_1$ in PCa, by exploring its presence, alteration and putative functional role in this disease. The results showed an evident overexpression of this receptor in PCa samples, compared to normal prostate samples. Additionally, in PCa samples, its expression was correlated with androgen receptor (AR) expression. *In vitro* studies with the PCa cell line 22Rv1 demonstrated that treatment with a specific agonist of $SST_1$, BIM-23926, decreased cell proliferation and PSA secretion of these cells. Likewise, the silencing of *SSTR1* expression increased, while its overexpression decreased, cell proliferation of this cell line. Through the treatment with the mentioned agonist, we next studied the signaling pathways implicated in the actions of $SST_1$. The treatment with BIM-23926 decreased the phosphorylation of AKT after 30 min, but no changes were observed in the activation of other important pathways, such as ERK, AR or JNK, or in the levels of $[Ca^{2+}]_i$, a classic second messenger associated to hormone signaling. A prolonged treatment (24 h) with this agonist decreased mRNA expression of *KLK3*, the gene encoding PSA, and *CCND3*, an important regulator of the cell cycle, as well as the proper *SSTR1* expression, which could mean a self-regulation of the receptor through a negative feedback. This treatment also changed AR-related signaling pathways; specifically, $SST_1$ inhibited the expression of several oncogenes (e.g. *ADAMTS1*, *VIPR*)

and increased the expression of the tumor suppressor *IGFBP5*. Finally, *in silico* analyses revealed that the expression of *SSTR1* may be regulated by several miRNAs, which were inversely correlated with *SSTR1* expression in The Cancer Genome Atlas (TCGA) database. The *in vitro* treatment with one of those miRNAs, miR-24, decreased the protein and mRNA expression of $SST_1$ in 22Rv1 cells, and a correlation between the expression of both genes was also observed in Memorial Sloan Kettering Cancer Center (MSKCC) database, which includes metastatic samples.

The **second experimental section** of this Thesis was aimed to study the regulation of the expression of *SSTR5* gene in NETs, including PitNETs (specially, somatotropinoma) and PanNETs. First, *in silico* approaches revealed that there is a natural antisense transcript (NAT) overlapping with the *SSTR5* gene in the genome, named *SSTR5-AS1*, and that there are four CpG islands, which are genomic regions with high proportion of cytosine-guanine that may be methylated, shared by these two genes. Although there were no changes in the expression of *SSTR5-AS1* in PitNETs compared to normal pituitary, *SSTR5* was overexpressed in this pathology and, more interestingly, there was a significant direct correlation between the expression of these two genes (*SSTR5*/*SSTR5-AS1*) in both normal pituitary and somatotropinoma samples. Furthermore, we found that the DNA methylation of three of the analyzed CpG islands was altered in somatotropinomas, compared to normal pituitary. Indeed, the methylation of the CpG island that overlaps with the center of the big *SSTR5* gene exon, which includes the region of alternative splicing, inversely correlated with the expression of the receptor and its NAT in somatotropinomas, but not in normal pituitary samples. In PanNETs, it was found that the *SSTR5-AS1* gene was overexpressed in tumor samples compared to non-tumor adjacent tissue, showing a direct correlation with the expression of the *SSTR5* in both tissues. These results led us to perform *in vitro* studies in the BON-1 NET cell line, wherein the silencing of *SSTR5-AS1* induced a decrease in *SSTR5* expression, which was again directly correlated with the expression of the NAT. Moreover, *SSTR5-AS1* silencing increased cell proliferation and colony formation, supporting a role of this antisense gene in the aggressiveness of NETs cells. The effect exerted by pasireotide, an analog of somatostatin that predominantly targets $SST_5$, in these cellular parameters was also altered after the silencing of *SSTR5-AS1*. In fact, NAT silencing decreased the activation of ERK and AKT proteins and, interestingly, enhanced

the effect of pasireotide on the phosphorylation of these two proteins, suggesting that the presence of *SSTR5-AS1* may be relevant in the action of $SST_5$.

In the **third experimental section** of this Thesis our aim was to study the dysregulation of the splicing machinery and its possible functional role in PanNETs, in order to identify novel biomarkers and/or therapeutic targets for this pathology. First, we measured the expression of 45 components of the splicing machinery in PanNETs samples, compared to non-tumor adjacent tissues, using a microfluidic qPCR array. Approximately, 50 % of the measured genes, including some small nuclear RNAs that comprise the core of the splicing machinery, were upregulated in tumor samples, while only one splicing factor was found downregulated. Principal component analysis and other bioinformatical tools served to select five of the measured genes (*NOVA1*, *PRPF8*, *RAVER1*, *SRSF5* and SNW1) as the best clustering features to distinguish between tumor and non-tumor samples. In addition, these factors were found to be associated to important clinical parameters, such as Ki-67 index, necrosis, disease relapse, functionality, weight loss and vascular invasion. One of these genes, *NOVA1*, exhibited an area under the ROC curve higher than 0.85 and its overexpression in tumor tissue was confirmed at protein levels through immunohistochemistry assays. Thus, we wanted to ascertain if this factor exerts a functional role in NETs cells. Interestingly, we discovered that the overexpression of *NOVA1* increased the proliferation rate of two PanNETs model cell lines, BON-1 and QGP-1, and the tumor growth of BON-1 xenografted tumors in mice. Moreover, silencing of this splicing factor decreased cell proliferation in those cell lines, which was associated to a decrease in *CCND1* and an increase in *CASP3* mRNA expression. In the same way, *NOVA1* silencing decreased the activation of ERK, PTEN and PDK1, without alteration of AKT phosphorylation, which suggests that this factor may act through complex and apparently opposed actions. Intriguingly, *NOVA1* silencing increased the phosphorylation of p53 only in QGP-1 cells, in which, at the same time, decreased the expression of *Δ133TP53* oncogenic isoform, without alteration of canonical *TP53*. These results, together with the previously mentioned in protein signaling, suggest a possible role of *NOVA1* in senescence pathway, involving p53 and ERK, in a cell-specific manner. On the other hand, genetic downregulation of *NOVA1* decreased ATRX and DAXX protein expression, as well as inhibited *TERT* truncated isoform, which suggests an implication of *NOVA1* in the regulation of the chromatin remodeling pathway, which is particularly important in PanNETs. Moreover, silencing of this splicing

factor in QGP-1 cell line improved the antiproliferative effect of everolimus, a mTOR inhibitor widely used in the treatment of PanNETs.

For all the above mentioned, the main conclusions of the work presented in this Thesis are:

1. The *SSTR1* gene is overexpressed in PCa, where it may be regulated by specific miRNAs and could have relevant functional implications. Specifically, $SST_1$ is directly related with the inhibition of cell proliferation and PSA secretion in 22Rv1 cell line, probably by the modulation of pathways and mediators linked to AR and PI3K/AKT-CCND3 pathways.

2. The expression of the *SSTR5* in somatotropinomas and PanNETs may be controlled by epigenetic mechanisms, including DNA methylation and post-transcriptional events, such as antisense-mediated regulation. In particular, *SSTR5-AS1* may be participating in the control of key tumor features, including proliferation, migration and colony formation, and in the effect of pasireotide treatment, a selective analog for $SST_5$.

3. The components of the splicing machinery are profoundly dysregulated, generally overexpressed in PanNETs. The levels of some of them are associated with important clinical parameters and could distinguish between tumor and non-tumor samples with a high efficiency. Specifically, the augmented level of the splicing factor *NOVA1* promotes an increase of cell proliferation and senescence pathway in PanNETs models, by altering key signaling pathways, and it is able to compromise the effectiveness of everolimus treatment.

As a **general conclusion**, the studies implemented in the present Thesis allow to expand and advance in the knowledge of the molecular basis of the pathophysiological regulation of endocrine-related cancers and neuroendocrine tumors by two specific somatostatin receptors and the splicing machinery. Specifically, our results demonstrate that *SSTR1* in the case of PCa, *SSTR5* in NETs and splicing factor *NOVA1* in PanNETs, represent relevant points of regulation for these tumors and, thus, they could be useful tools for the develop of novel diagnostic biomarkers and/or therapeutic targets to improve the future treatment of those pathologies.

# 1. INTRODUCTION

# 1. Introduction

Cancer represents one of the most challenging threats for human health, as it comprises some of the most severe and complex pathologies affecting the population around the globe [1]. For that reason, great efforts have been developed in the last decades in basic, translational and clinical research specifically aimed to fight cancer. However, the remarkable heterogeneity and complexity that characterize the diverse group of pathologies commonly referred to as cancer, hinder the discovery of novel, more precise and efficacious approaches to fight them, and thus cancer remains a real challenge for the biomedical research community [2]. It is well known that tumoral pathologies exhibit a wide number of intricate alterations, at different layers of complexity, from molecular to cellular and organismal levels; however, they all share also a discrete number of common features, which Hanahan and Weinberg systematized and defined as the *hallmarks of cancer*, in an attempt to provide a common conceptual framework to help improve the study of this disease by the scientific community [3, 4].

In addition to the key contribution of growth factors and immune/inflammatory systems mediators to the dysfunction of cancer cells, hormones of multiple kinds comprise a diverse critical regulatory component in cancer, wherein the endocrine molecular elements provide a selective, valuable window of intervention for diagnostics and therapy. In this context, among the different types of endocrine-related tumor pathologies [5, 6], the present Thesis will be focused on the study of some of the molecular underpinnings governing neuroendocrine tumors (NETs) a relevant hormone-dependent cancer, prostate cancer (PCa). This latter represents the second most common cancer among men worldwide, just after lung cancer, being their fifth leading cause of death by cancer [7, 8]. On the other hand, NETs are tumors that arise from neuroendocrine cells, which are distributed widely throughout the body. These tumors are characterized for their great heterogeneity and are classically classified by their location; in particular, we will focus on pituitary (PitNETs) and pancreatic NETs (PanNETs) [9, 10].

Neuropeptides and their receptors comprise multiple sets of interrelated, widely distributed regulatory molecular systems that, besides their primary physiologic regulatory roles, can be involved in the control of hormone-dependent tumors. Our group has been classically interested in studying the role of one of these systems, namely, that constituted by somatostatin and its receptors ($SST_1$-$SST_5$) [11], in the development and

progression of different endocrine-related tumors. Particularly, some of the elements of this system have provided highly valuable tools for the diagnosis and treatment of these tumors [12, 13]. In line with this, results from our team have unveiled the relevance of somatostatin receptor subtype 5 ($SST_5$) in PitNETs, where it may serve as a biomarker for treatment resistance [14]. In fact, we discovered two truncated, aberrantly spliced variants of $SST_5$ derived from the *SSTR5* gene, termed $SST_5$TMD4 and $SST_5$TMD5, which display specific, distinct features. In particular, $SST_5$TMD4 has been shown to be overexpressed in PitNETs, PanNETs, PCa and other tumoral pathologies, where its presence is related with oncogenesis and aggressiveness features [15-18]. Notwithstanding, the precise regulation and biogenesis of this receptor and the functional role of this and other SSTs are still very poorly known in several endocrine-related tumors. Accordingly, this will be one of the main subjects of this thesis.

In this scenario, and prompted by our discovery of these splicing variants, we came to realize, and decided to further explore, the emerging body evidence indicating that the alteration of the splicing process was not just an anecdotical observation in our setting, but, in fact, represents a frequent feature in many tumors and cancers, which has led some authors to propose that altered splicing should be considered as a novel cancer hallmark [19]. Indeed, its relevance is highly increasing in several tumoral pathologies as an underlying cause of tumoral heterogeneity and malignancy features, through the dysregulation of alternative splicing altering the normal profile of variants and generating aberrant isoforms [20-23]. However, unfortunately, the knowledge of alternative splicing and its (dys)regulation in many tumors and cancers, including NETs, is still very insufficient.

## 1.1 Cancer

Cancer is not considered nowadays as a single disease, but as a collection of diseases that can affect the whole body, in which cells start to proliferate uncontrolledly and spread into surrounding tissues and organs (National Cancer Institute, NCI, United States). This group of diseases is one of the major health problems for the human population worldwide. In fact, one out of each six deaths are currently caused by a tumoral pathology, which accounts for approximately 9.6 million deaths in 2017, being the second leading cause of death, just after cardiovascular diseases [1]. In terms of prevalence, 100 million people exhibited any type of cancer worldwide in 2017, more than the double in 1990, with percentages ranged between 0.4 and 5.5 % in each country (**Figure I1**).

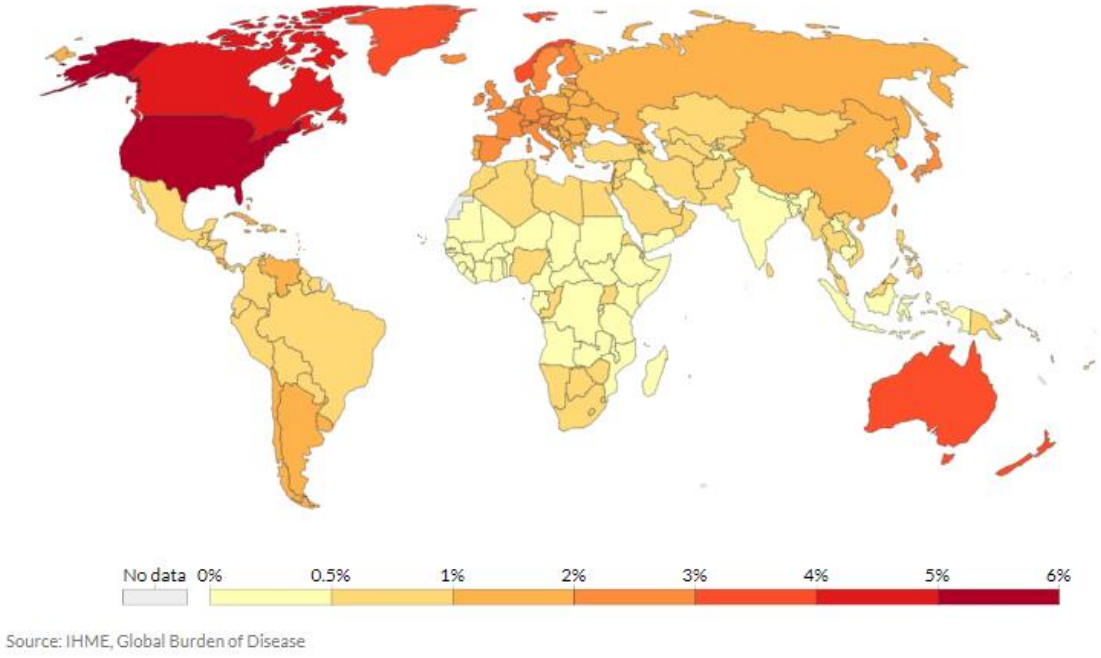


***Figure I1.*** *Percentage of population with any type of cancer by country.*
Portion of the population with any type of cancer measured as age-standardized percentage in 2017. Adapted from *GBD2016, Lancet 2017*, and Institute for Health Metrics and Evaluation.

Particularly in Spain, there is a 5-year prevalence of almost 800,000 patients, where more than 250,000 new cases were detected and more than 100,000 died in 2018 (source: Asociación Española Contra el Cáncer [AECC]). Actually, in 2030, cancer is predicted to become the leading cause of death, ahead of cardiovascular diseases, with a dramatic grow of mortality (source: American Society of Clinical Oncology, [24]). These data clearly reflect the importance of this problem in the society and the necessity of developing new approaches to fight it with higher effectiveness in the future.

The current increase in incidence of tumor pathologies has been related to several environmental factors, such as smoking or pollution, but also to an increased wealth, better medical services and associated extended life span, that also lead to an improved access to diagnostic techniques enabling to better detect these pathologies [25]. However, the associated increase in mortality implies the necessity of developing specific, sensitive and useful biomarkers for the diagnostic and prognostic of these pathologies, as well as new tools for the generation of therapeutic targets. For those reasons and in order to improve the study of cancer, the scientific community have adopted a group of common hallmarks for most types of cancers (**Figure I2**), which were defined and proposed by

Hanahan and Weinberg and include: sustained proliferative signaling, evasion of growth suppressors, avoiding of immune destruction, enabling replicative immortality, tumor-promoting inflammation, genome instability, deregulating cellular energetics, resistance to cell death, angiogenesis, and activation of invasion and metastasis [3, 4].

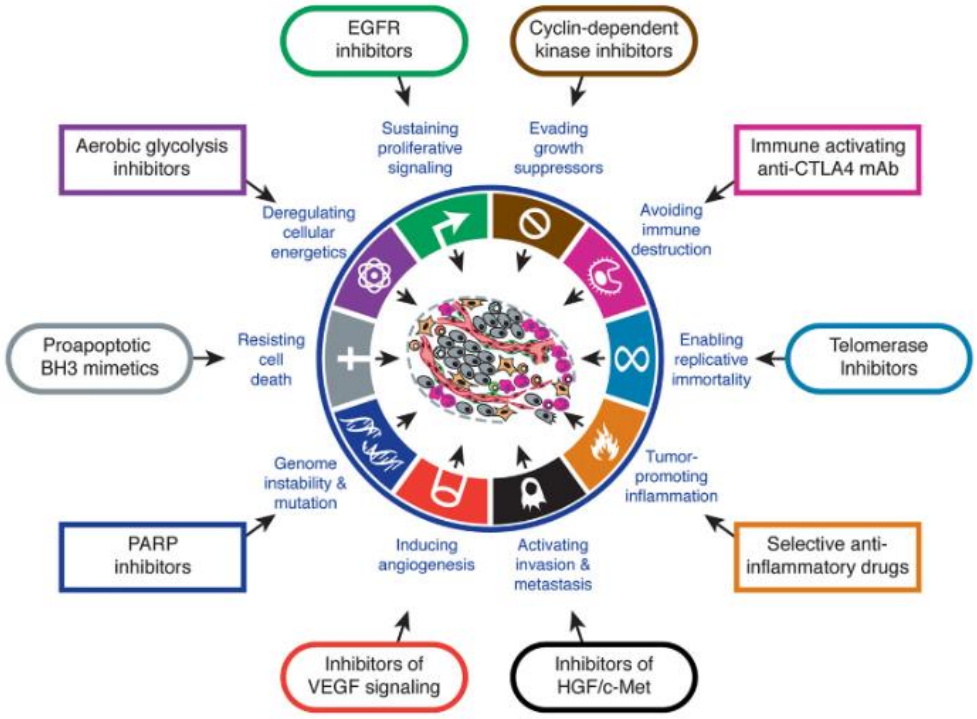


***Figure I2.*** *Hallmarks of cancer.*
Graphic representation of the update of hallmarks of cancer. Source: *Hallmarks of Cancer: The Next Generation, Hanahan and Weinberg, Cell 2011*.

One of the major problems that cancer research has to face is the remarkable heterogeneity and complexity that intrinsically characterize this group of pathologies. Indeed, even within a same "cancer type", individual tumors exhibit a wide number of intricate alterations at diverse levels, encompassing from molecular, cellular, genetic, epigenetic, and metabolic features, to clinical, anatomical, functional and pathological characteristics, which not only differ among the distinct types of cancer but, also, within the same type of cancer, among different patients, and even with a given tumor [2, 26]. As pointed out previously, this is due to the fact that cancer may arise from almost any cell type in the body, independently of its origin, localization or metabolic status, and thus give rise to a wide variety of malignancies with a variable etiology and pathology, which, in turn, evolve within a unique, specific microenvironment, an additional key factor to

take in account [27-30]. In any case, it is nowadays clear that oncogenesis and tumor progression is closely linked to the metabolic and endocrine alterations taking place in each patient, which is especially relevant in the so-called endocrine-related cancers [30-33].

## 1.2 Endocrine-related cancer

The terms endocrine-related cancer and hormone-dependent cancer have been classically used to refer to tumor pathologies that produce hormones, like pituitary and other neuroendocrine tumors (NETs), thyroid and adrenal cancers, etc., and also those that are strongly related to and responsive to endocrine signals, like peptide hormones and sex steroids, such as prostate, breast, ovary, endometrium, testis, etc. [34, 35]. Nevertheless, the definition of this term has been revised several times over the last years, since it has been observed that components of different additional hormonal systems are commonly dysregulated in tumor pathologies and exhibit tumorigenic potential, thus less classical hormone-sensitive cancers have been included in the endocrine-related cancer category, when they show certain sensitivity, at least at some stage, to hormonal systems. Therefore, the currently accepted meaning of endocrine-related cancers is ample and comprises those tumor pathologies that either develop in endocrine glands or in endocrine target tissues [5, 6]. In fact, the presence, importance and (dys)regulation of key endocrine signals, such as insulin or IGF-1 is a common feature in tumor pathologies [36, 37]; however, the knowledge about their role is still limited and there are multiple factors with unknown function in cancer. In this Thesis, we will focus on PCa and NETs, two types of endocrine-related cancers.

### 1.2.1 Prostate cancer

Prostate cancer (PCa) represents the third most common cancer when considering both sexes combined worldwide, and the second most common among men, just after lung cancer, and is the fifth leading cause of death by cancer in that group, according to GLOBOCAN 2018 and other studies (**Figure I3**). Almost 1.3 million new cases of PCa and 359,000 associated deaths worldwide were estimated for 2018 [7, 8]. In Spain, PCa is the most incident cancer and the third cause of death by tumor pathology in men population, with 31,728 new cases and 6,061 deaths in 2018 (source: AECC).

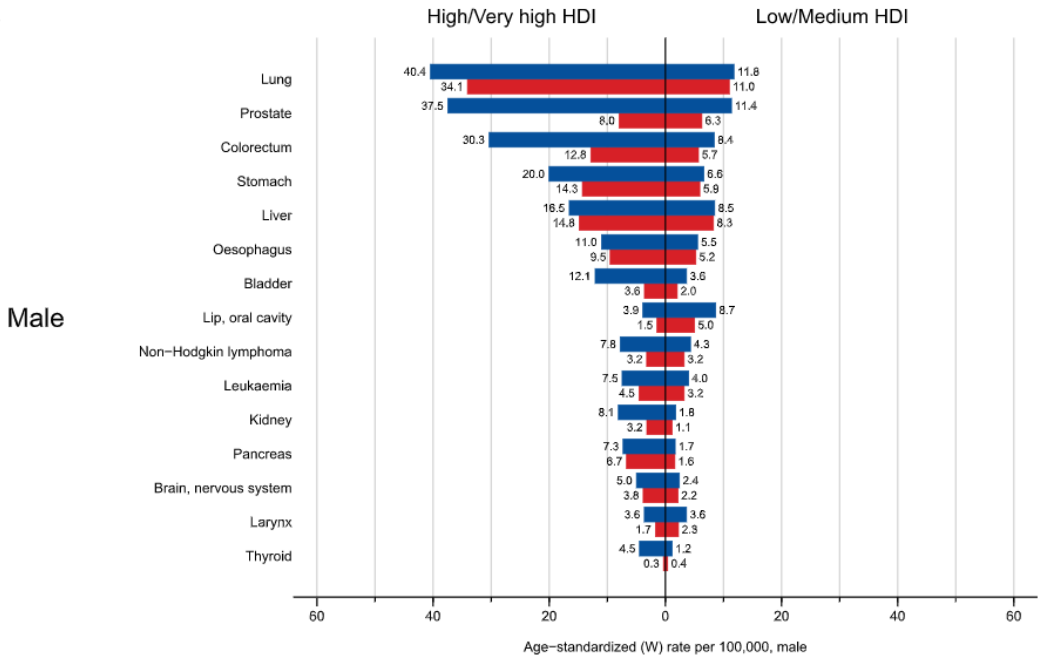


***Figure I3.*** *Cancer statistics of 2018 in male population.*
Incidence (blue) and mortality (red) rates of the 15 most common types of tumors in men population worldwide, in two groups of countries by Human Development Index (HDI), standardized by age. Source: GLOBOCAN 2018 study.

The relevance and severity of PCa has prompted a number of leading biomedical research groups worldwide to focus their work on the generation of novel knowledge to understand this disease. This has provided significant advances in diagnostic and therapeutic tools, which have enabled to achieve survival rates close to 100 % after 5 years when the tumor is localized [38-40]. Nonetheless, the available diagnostic tools are far from being as specific and sensitive as they should, and therapeutic strategies, including surgery, chemical castration, chemotherapy and androgen deprivation, have considerable associated side effects [41], which complicates options and choices for clinicians and patients. In addition, when PCa is detected at advances stages or the disease progresses, the prognosis prediction declines dramatically.

Currently, PCa is graded following the Gleason score, which measures the aggressiveness grade of the tumor based on its structure under the microscope and provides a valuable assessment of the disease [42]. On the other hand, the most commonly used biomarker for the detection and prediction of PCa, PSA, in spite of having provided

a helpful resource for general screening, is still very limited in the clinical practice [43]. PSA, or Prostate Specific Antigen, is a kallikrein serine-protease, expressed in the epithelial cells of the prostate gland, whose function consists in the dissolution of seminal clot. This biomarker is extensively used in the PCa diagnostic and it has a low cost [43]. However, PSA presents some problems in the diagnostic: it exhibits a high number of false positive and negative values and it does not segregate correctly between different aggressiveness groups [43-45]. This molecule is specific of the prostatic tissue but its secretion may be also increased in response to other pathologies, like benign prostatic hyperplasia, or activities, such as sex practice or bike cycling, which increase PSA blood levels and result in false positives [44]. On the other hand, there has been controversy in the last years about the cutoff of the PSA test, since it may exhibit false negatives when too high level is taken as reference [46]. Additionally, and although its levels are highly increased in case of metastasis, PSA it is not a good tumor progression marker, an information that is much needed to decide how to treat the patients [47]. For all these reasons, the biopsy represents the most extended and safe diagnostic practice to detect PCa. Therefore, there is a clear need to identify novel biomarkers that can help to avoid unnecessary biopsies, and to reduce their undesirable effects.

The therapeutic approaches to treat PCa are also still limited, particularly in advances states of the disease, despite the recent advances in the field [41]. In this context, it is worth noting that PCa has a marked endocrine nature, with its development and progress being closely influenced by sex steroid milieu, especially androgens [48, 49]. Moreover, other non-sex hormones, such as somatostatin, have also been related with normal prostate and PCa development [16, 50, 51]. However, the potential role of the somatostatin system and its underlying mechanisms in PCa are still poorly understood.

### 1.2.1 Neuroendocrine tumors

Neuroendocrine tumors (NETs) are a very heterogeneous group of neoplasms with rising incidence over the last years. This type of tumors arises from neuroendocrine cells, which share endocrine and nervous cell features, particularly the synthesis and secretion of hormones and neurotransmitters. Cells form the diffuse neuroendocrine system are distributed widely throughout the body, with higher presence in the gastrointestinal and respiratory tracts [9]. For this reason, NETs most commonly appear in respiratory (22-27 %) and gastroenteropancreatic tracts (62-67 %) (**Figure I4**) [52].

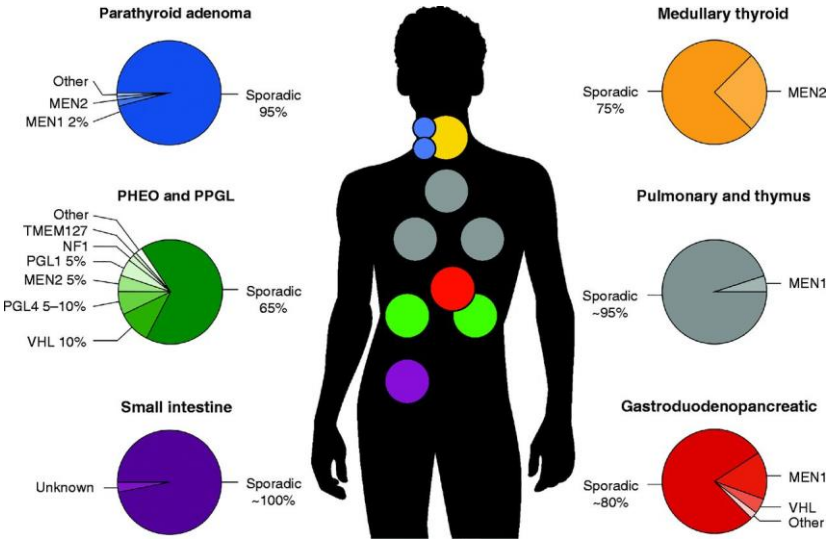


***Figure I4.*** *NETs main locations.*
Representation of the main localizations where NETs arise and the most common mutations; extracted from *Crona and Skogseid, EJE 2016.*

In spite of their high heterogeneity, these tumors were grouped together under the name *carcinoid*, coined by the pathologist Siegfried Obendorfer at the beginning of XX century, and they were classified, based the localization of the primary tumor [10, 53], into **foregut NETs**, tumors developed in the respiratory tract, thymus, stomach, duodenum and pancreas; **midgut NETs**, NETs from jejunum, ileum and appendix; and **hindgut NETs**, comprising tumors from large intestine, where more frequently appear in the rectum, and rarely, from presacral region. However, it was soon found that there are several types of NETs that are not included in those groups created in a first classification. Among them, NETs can be found in the thyroid [54], parathyroid, autonomic paraganglia or adrenal medulla [55, 56]. Moreover, in addition to these locations where NETs develop most frequently, these tumors can arise in, virtually, any type of tissue with neuroendocrine cells. Thus, NETs have been described in the literature in prostate [57], ureter [58], urinary bladder [59], ovary [60], cervix [61], breast [62], skin [63], testis [64], kidney [65], sublingual gland [66], gall-bladder [67] or sinonasal tract [68].

On the other hand, and in a different context, tumors derived from the anterior pituitary have been classically termed as pituitary adenomas, as they are considered as a benign pathology, because of their non-metastatic behavior. However, based on their frequent aggressiveness and associated morbimortality, the International Pituitary Pathology Club recently proposed a reclassification of this pathology and named it as pituitary neuroendocrine tumors or pitNETs [69].

In this Thesis, we will focus our studies on pancreatic NETs (PanNETs) and pituitary NETs (PitNETs).

PanNETs are one of the most common types and with a highest increase in the incidence in the last years (**Figure I5**) [70]. These tumors derive from hormone producing cells of the pancreas, which are grouped in the Langerhans islets [71], embedded in the exocrine part of the organ.

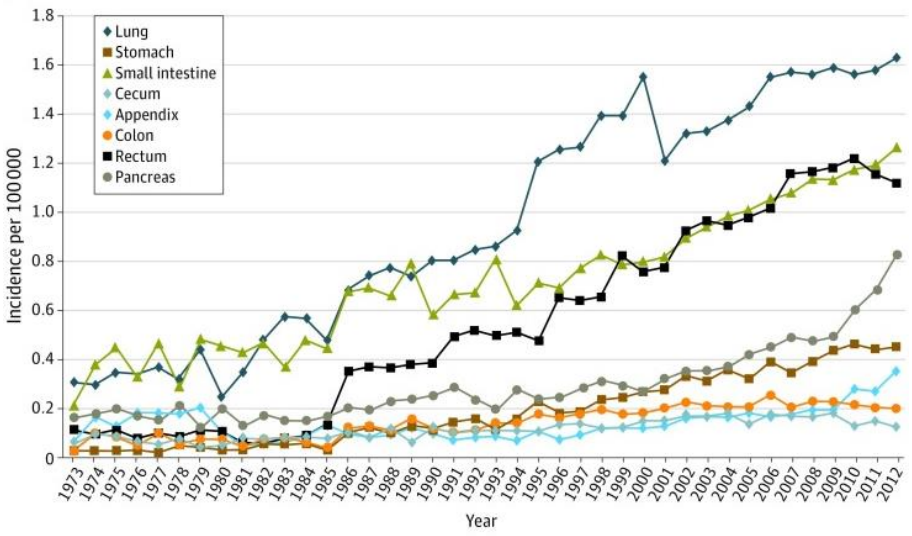


***Figure I5.*** *NETs incidence by location.*
Incidence of different types of NETs by location, showing their increase over the last decades and the recent rise in panNETs (grey circles). Source: *Dasari et al., JAMA Oncol. 2017*.

Due to the inaccuracy of the term carcinoid, which includes very diverse types of tumors with different prognosis and management, its use has been revised. Accordingly, the World Health Organization (WHO) and the European Neuroendocrine Tumors Society (ENETS) have proposed improved, novel classification systems for these tumors. In particular, gastroenteropancreatic NETs, based on the cellular differentiation and Ki-67 index, widely used in the study of these pathologies [72] are classified as follows:

1. Grade 1 neuroendocrine tumor: well differentiated cells and Ki-67 index lower than 3 %.
2. Grade 2 neuroendocrine tumor: well differentiated cells and Ki-67 index between 3 and 20 %.
3. Grade 3 neuroendocrine tumor: well differentiated cells and Ki-67 index higher than 20 %.

4. Grade 3 neuroendocrine carcinoma: poorly differentiated cell and Ki-67 index higher than 20 %.

Ki-67 is a heavy protein (395 kD, approximately) present in all the stages of the cell cycle, but not in quiescent cells in G0 stage. Accordingly, its immunodetection allows to identify and count cells with active cell cycle, providing a valuable index of tumor cell proliferation [73].

In addition to those classifications, PanNETs may be divided, following their capacity to produce and secrete hormones and/or amines, in functioning and non-functioning tumors. Functioning NETs are characterized by the production of one or more types of peptides and are typically linked to secondary syndromes due to the hypersecretion. They represent almost the half of PanNETs and are subdivided according to the peptide secreted, such as insulinoma, gastrinoma, glucagonoma, somatostatinoma and VIPoma. On the other hand, non-functioning PanNETs are non-hormone producing tumors and are commonly found in the head and neck of pancreas [74-76].

Over the last years, a number of genetic alterations that contribute to the tumorigenesis of PanNETs has been identified, particularly as mutations usually present in this kind of tumors, which may help in their characterization and study [77]. Mutually exclusive mutations in *ATRX* and *DAXX*, that lead to alternative lengthening of telomeres phenotype, have been found in a high percentage of panNETs [78], where they are related to the tumor development and progression [79]. In line with this, alterations in the telomerase gene (*TERT*) have also been related with length of telomeres and aggressiveness in cancer, where not only expression or mutations, but also alternative splicing has been linked to its activity [80, 81].

The diagnosis and clinical management of NETs is difficult due to their frequent lack of symptoms and the intrinsic high heterogeneity of this type of tumors (**Figure I6**). Until symptoms derived from hormonal hypersecretion or pain induced by mass effect appear, these tumors are very difficult to diagnose, being their finding often incidental, and thus, tumors are often detected when they have already acquired a high malignancy grade. The currently available biomarkers for the diagnosis and prognosis of these tumors are related with their secretion, such as chromogranin A, synaptophysin, serotonin or specific enolase [82, 83]. However, these biomarkers are not specific and powerful enough and the NETs heterogeneity hampers their suitability in several cases.

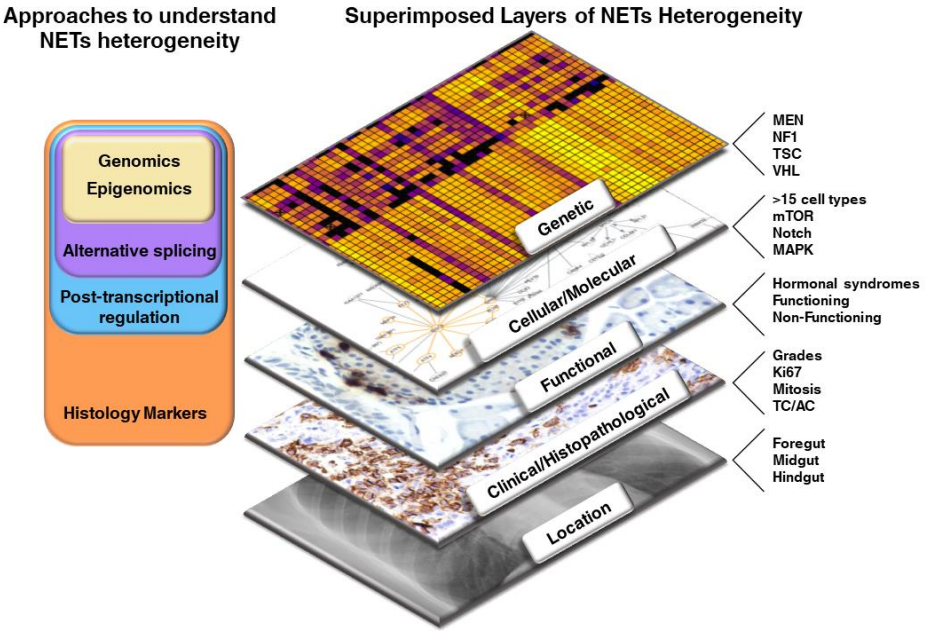


***Figure I6.*** *Levels of heterogeneity of NETs.*
Representation of the different layers of this heterogeneity interrelated and affecting each other. The left panel underscores some of the different approaches to address it. Source: Pedraza-Arévalo et al., Rev Endocr Metab Disord 2018.

Although heterogeneity also hinders the treatment of PanNETs, it may also provide a tool to personalize their medical and investigative approach [84]. Whenever feasible, surgery is the first therapeutic approach for PanNETs, whereas synthetic somatostatin analogs (SSAs) are the first line and most widely used pharmacological treatment [85-87]. Additionally, over the last years, novel treatments targeting specifically altered pathways have arisen, such as AKT/mTOR or tyrosine-kinase receptors, as is the case of everolimus and sunitinib, respectively [88, 89]. As well, greatly original therapeutic approaches have been developed, such as Peptide Receptor Radionuclide Therapy (PRRT), which allows the destruction of tumoral cells with radioactive isotopes [90, 91]. Nevertheless, the efficiency and suitability of these treatments is still insufficient and thus, it is clear that novel markers and therapeutic targets are necessary to improve the diagnostic and treatment of PanNETs.

On a different scenario, PitNETs have been classically considered a rare tumor pathology based on their low incidence, which, as is the case of other pathologies, may have been underestimated due to an impaired diagnosis. In fact, recent studies based on autopsy and imaging have revealed that these tumors are the most common intracranial neoplasms, with a prevalence between 10 and 22 % [92, 93]. As in the case of PanNETs,

their associated hypersecretion of pituitary hormones and the mass effect cause severe comorbidities, including growth alterations, hypogonadism, hypopituitarism, sexual dysfunction, infertility, and emotional disturbance, among others [94, 95]. Although these tumors rarely metastasize, they may invade and infiltrate local structures producing lesions and may cause complications derived from metabolic dysregulation, increasing their lethality [96].

Although the primary cause that initiates a PitNET is still unclear, it is widely accepted that these tumors arise from a monoclonal expansion of genetically altered cells, that are also potentiated by hypothalamic and/or peripheral factors [97, 98]. As in other tumors, there are different factors that increase cell proliferation which lead to tumor formation and growth, including genetic and epigenetic events, growth factors and even the microenvironment of the gland [98, 99], and research models have shown that disruption of cell cycle regulator is sufficient to promote pituitary tumorigenesis [100, 101]. However, as in the case of PanNETs, classic oncogenes and tumor suppressor genes are not frequently mutated in PitNETs [102]. In line with this, a growing set of PitNETs-specific disruptors has been described to be linked to their tumorigenesis, including *MEN1*, *AIP*, *GNAS* or WNT-catenin pathway components, the great majority of PitNETs being sporadic [98, 103].

PitNETs are primarily classified according to the hormonal hypersecretion that they show, namely growth hormone (GH), prolactin (PRL), follicle-stimulating (FSH)/ luteinizing hormone (LH), adrenocorticoropin (ACTH), and thyroid-stimulating hormone (TSH), with some tumors having plurihormonal nature, and other group do not show significant secretion and are termed non-functioning tumors [104, 105]. Particularly, GH-secreting tumors or somatotropinomas arise from GH-secreting cell or somatotropes, and cause gigantism or acromegaly, depending on the age of onset [106]. The excess of GH secretion causes growth acceleration if it starts before puberty ends, but in adults it causes extremity enlargement, facial and skeletal changes, and metabolic, gastrointestinal, cardiovascular and respiratory complications [107]. In the case of PitNETs, surgery is the first line treatment and the only curative approach to date; nevertheless, SSAs are frequently used either before surgery to control hormone secretion, or in persistent and recurrent disease in the case of GH-secreting tumors [108].

## 1.3 Somatostatin system

Somatostatin was originally isolated from ovine hypothalamus in 1973 and was called somatotropin release-inhibiting factor (SRIF), due to its capacity to inhibit GH secretion [109]. The human somatostatin gene (*SST*) encodes for a 116 aminoacidic precursor, named pre-pro-somatostatin, which is proteolytically processed to generate two different bioactive peptide isoforms: somatostatin-14, the most abundant, and somatostatin-28, which may be further cleaved to somatostatin-14 [110-112]. Additionally, differential processing of the same pre-pro-peptide can give rise to another non-cyclic 13-amino acids peptide, called neuronostatin [113].

Somatostatin exerts a wide number of pleiotropic actions throughout the body, especially in the central nervous system but also in peripheral tissues. The main functions of this peptide include modulation of neurotransmission, inhibition of pituitary hormone secretion, regulation of gastrointestinal tract including its endocrine and exocrine secretions, gut motility, blood flow, growth, pancreatic secretions, and, with rising evidence over the last years, regulation of the cell proliferation of normal and tumoral cells [12, 13, 114, 115].

In addition, cortistatin is another peptide with very similar sequence, structure and actions to somatostatin, likely due to their probable shared evolutionary origin from a common ancestral gene [116]. This peptide was discovered two decades after somatostatin, as a somatostatin-related peptide in the brain cortex, where it is expressed, thus its name. The cortistatin gene (*CORT*) also encodes for a pre-pro-peptide that is further processed to cortistatin-17 or cortistatin-29 definitive peptides [117, 118]. Although at first cortistatin was supposed to be a functional analog of SST, several studies have demonstrated that it carries out unique, even opposite, endocrine and non-endocrine actions than those from somatostatin, such as the modulation of sleep, neuronal activity and immune system regulation [118-121].

### 1.3.1 Somatostatin receptors

To exert their actions, both somatostatin and cortistatin bind to a family of 5 classic 7-transmembrane G protein-coupled receptors (GPCRs) class A, or rhodopsin-like, named $SST_1$ to $SST_5$, following the chronological order of their discovery and publication, which are encoded by 5 independent genes, *SSTR1-5* (**Figure I7**) [11]. Although there are no available crystal structures for any somatostatin receptor, it is

known that their sequences range in length is between 364 amino acids for $SST_5$ to 418 for $SST_3$. As in the case of other GPCRs, these receptors include a DRY motif that is involved in the coupling to G proteins. Studies of comparative genetics have shown that mammalian somatostatin receptors may have a common ancestral antecessor gene that was duplicated, resulting in two ancestral types of SSTs, that, later, give two groups of the current receptors: type 1, including $SST_2$, $SST_3$ and $SST_5$; and type 2, with $SST_1$ and $SST_4$ [11, 122, 123].

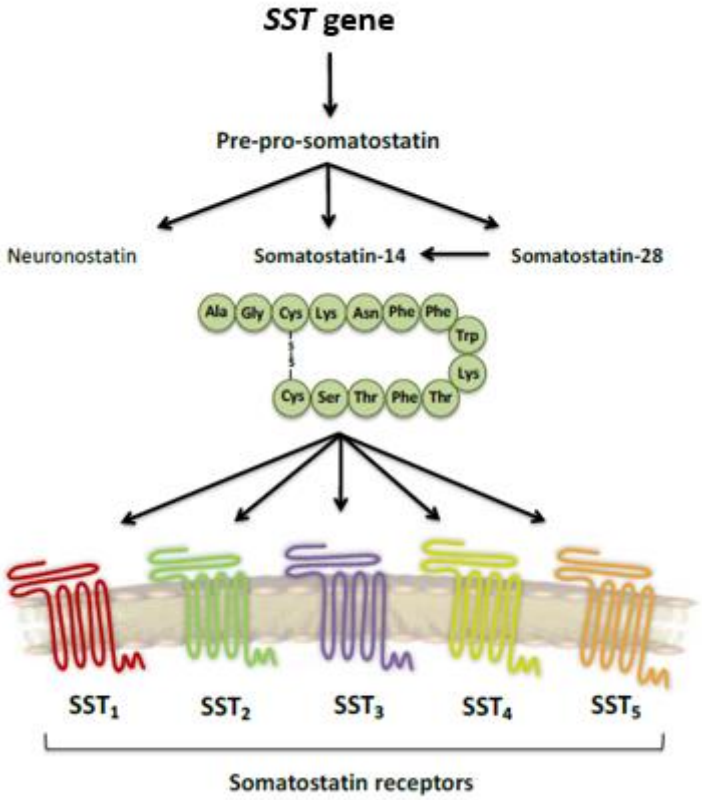


***Figure I7.*** *Components of the somatostatin system.*
Peptides derived from *SST* gene and binding receptors for SST peptides.

Since their discovery, evidence has accumulated demonstrating that this family of receptors is far more complex than originally envisioned, in that several of these receptors may be simultaneously present in the same cells and, additionally, are able to functionally and physically interact with each other or other GPCR, forming homo- or hetero-dimer complexes, which modulate the signaling pathways they activate and, thus, the actions that they can regulate [124].

Studies from our group have long been focused in the characterization of the somatostatin-$SST_{1\text{-}5}$ system in various physiological and pathological settings. In this

context, we discovered that the gene *SSTR5* not only encodes the canonical, full-length $SST_5$ but also generates distinct splicing variants, in several species (human, pig and rodents), which are functional despite being truncated, as they lack some transmembrane domains (TMD) [125-127]. In particular, the two human variants result from an alternative splicing event that eliminates a cryptic intron in the exon of *SSTR5* mRNA and have only 4 and 5 TMD, and therefore, we named them $SST_5$TMD4 and $SST_5$TMD5, respectively [125-127]. Additionally, the two truncated variants exhibit exclusive ligand-selective signaling properties, distinct distribution in normal tissues and different subcellular localization than the originally identified, long $SST_5$ isoform [125-127].

### 1.3.2 Somatostatin system and cancer

Somatostatin mostly exerts inhibitory functions in cells, particularly on hormonal secretion but also on cell proliferation and other fundamental processes. Somatostatin can exert these actions in both normal cells and tissues, as well as in tumoral cells, as it has been demonstrated in different tumor types [128]. In fact, it has been shown that somatostatin receptors are broadly and abundantly expressed in many cancers, especially in endocrine-related tumors, such as NETs or PCa. In general, $SST_2$ is the most expressed receptor in tumors, followed by $SST_5$, with a high tumor-specificity, $SST_1$, $SST_3$ and, finally, $SST_4$ [129, 130]. Further, the truncated $SST_5$ variant, mainly $SST_5$TMD4, has been also found to be highly expressed in several endocrine-related tumors, such as NETs [17, 18], thyroid cancer [131], breast cancer [132] or PCa [133], where it has been associated with tumorigenesis and malignancy features, likely by playing an inhibitory role over $SST_2$ and canonical $SST_5$, related to receptor interaction and intracellular retention [17, 126, 134]. This observation is particularly important in NETs and other endocrine-related cancers, given the key role of $SST_2$ and $SST_5$ in the tumor response to treatment with SSAs such as octreotide, lanreotide or pasireotide.

Indeed, most NETs, including PanNETs and PitNETs, express various SSTs at relatively high levels, which makes them responsive to somatostatin action, which decreases hormonal secretion and can also inhibit cell proliferation [135-137]. However, the clinical use of somatostatin is very limited due to its short half-life that reaches a maximum of three minutes [138]. For this reason, synthetic SSAs were developed with similar effects to those of natural somatostatin but with a longer half-life. The first generation of these compounds, octreotide and lanreotide, clinically used since 1988 and 2007, respectively, exhibit a preferential targeting for $SST_2$, with less affinity to $SST_5$

and, in the case of octreotide, for $SST_3$, and almost negligible binding for the other SSTs. These treatments have been widely used in the treatment of GH- and TSH-secreting PitNETs and also in PanNETs, to reduce hormonal secretion, control tumor volume and improve symptoms of the patients [86, 87, 139-142]. Nonetheless, a substantial proportion of patients are or become resistant to these treatments [135, 143]. For this reason, a second generation of SSAs was developed, with a multireceptor binding affinity, based on the idea that targeting more than one somatostatin receptor at the same time, as in the case of natural SST, could have more effectiveness in those poorly or non-responsive patients. From this group, the most widely used compound is pasireotide (or SOM230), approved in 2014, which exhibits a high affinity to $SST_5$, $SST_2$, $SST_3$ and $SST_1$, and accordingly referred to as a pan-SST agonist [144, 145]. However, data gathered in both experimental and clinical research has unveiled that the effect of these SSAs not only depends on their differential binding capacities to the various SSTs. On the contrary, it has been shown that, in the case of GH-secreting tumors, although the complete set of factors affecting SSA responsiveness is not fully defined, there are specific tumor features and molecular markers that relevantly influence the tumor response to SSAs, including granulation pattern, *AIP* and *GNAS* mutations, β-arresting, filamin A and e-cadherin expression, and, interestingly, *SSTR2*/*SSTR5* expression balance and the presence of $SST_5$ truncated variant $SST_5$TMD4 [11, 14]. Thus, it is important to understand the mechanisms underlying and governing the expression of the SSTR5 gene and its resultant receptor variants $SST_5$, $SST_5$TMD4 and $SST_5$TMD5, since it may affect NETs response to SSAs.

Gene expression is known to be regulated by a number of factors, among which extrinsic factors, such as epigenetic mechanisms, have gained great attention in recent years. Epigenetics involve the study of heritable changes in gene transcription through altering chromatin, without affecting the primary DNA sequence [146], and is emerging as a critical regulator of cell function, since its action controls multiple processes [147]. One of the main epigenetic modifications is DNA methylation, which is based on the addition of a methyl group to a cytosine preceding a guanine (CpG). CpG residues are enriched at CpG islands, regions of the genome that are frequently associated with promoter and enhancer function. Beside this, noncoding RNAs, may act as modular epigenetic regulators [148]. A particular type of noncoding RNAs, natural antisense transcripts (NATs), was described years ago [149], defined as transcripts derived from

the opposite strand to a protein-coding or sense gene, which can regulate the transcription of their corresponding sense genes, including at the chromatin level. The importance of NATs is arising as sequencing technologies improve, and, recent studies are deciphering the role of these NATs in some pathologies, including those of pituitary [150], where they show different roles, such as *AFAP1-AS1*, which seems to play a role PitNETs growth; or *C5orf66-AS1*, related to invasive PitNETs. Recently [151], the presence of a NAT for *SSTR5* was reported in laryngeal squamous cell carcinoma, where it may act as a tumor suppressor. Nevertheless, its role in PitNETs and PanNETs has not been explored yet.

In the case of PCa, its main hormonal regulation is exerted by androgens and other steroid hormones, which are tightly related with the appearance and progression of the disease [48, 152, 153]. However, prostate biology is also regulated by other neuroendocrine systems, in both normal and pathological circumstances [154, 155]. In particular, the components of the somatostatin axis are expressed in normal prostate, where they can regulate, as in other tissues, hormonal secretion and cell proliferation; interestingly, somatostatin receptors exhibit higher expression in hyperplasic and tumoral prostate [156-159]. This overexpression suggests a clinical opportunity for the treatment of PCa by targeting these receptors, whereas loss of their expression or presence of alternative splicing variants have been proposed as diagnostic and prognosis tools [16, 51, 160]. In this context, it is worth noting that SSAs have been employed in the treatment of PCa, where they exhibited limited effects as monotherapy but had more positive results when administered in combination with other PCa treatments, such as bromocriptine, triptorelin, dexamethasone or total androgen blockade; nevertheless, it seems clear that further studies with higher number of patients are needed in order to better understand the real benefits of SSAs treatment in PCa [161, 162].

Among the different SSTs, $SST_1$ (encoded by *SSTR1* gene) is not one of the best studied receptors, however, it has been shown to play important roles in some types of tumors, such as colon, breast, pancreas or lung cancer, where it has been related with malignancy features [163-166]. Interestingly, the *SSTR1* gene is also overexpressed in PCa and it has been related with aggressiveness features in this cancer [167, 168]. However, the knowledge about the role of $SST_1$ in PCa is still very limited, and its therapeutic potential remains unknown.

## 1.4 Splicing

The discovery of aberrantly spliced variants of $SST_5$ generated through a non-canonical splicing mechanism, coupled to their capacity to enhance aggressiveness in different types of tumors [16-18, 131], prompted us to study in more detail the process of splicing and to explore the growing evidence linking alterations in splicing with cancer and tumoral pathologies. Splicing is a process of pre-RNA maturation, by which introns are removed and exons are pasted together, resulting in mature RNA. This process is based fundamentally in two reactions of transesterification, whose targets are the phosphodiester bonds in the intron. The first of these reactions occurs in the so-called branch point, and the second in the binding with the previous exon (**Figure I8**) [169]. The process of splicing is carried out by the spliceosome, a macromolecular complex formed by five small nuclear ribonucleoproteins (snRNPs) that make up the core of the complex, and other associated proteins that facilitate the procedure [170]. Moreover, a set of approximately 300 auxiliary proteins act as splicing trans-regulator factors, participating in the regulation of the different steps of the process [171]. In the case of mammalians, there are two types of spliceosome: the first of them is the major spliceosome, composed by snRNPs U1, U2, U4, U5 and U6, which processes 99 % of the introns, named as U2-type (also U2-dependent, or GT-AT). The remaining 1 % of the introns (U12-type, U12-dependent, or AT-AC) are processed by the minor spliceosome, with a similar structure and analogous but distinct snRNPs, with the exception of U5, that is shared between both spliceosomes; the minor snRNPs are U11, U12, U4atac and U6atac [170, 172].

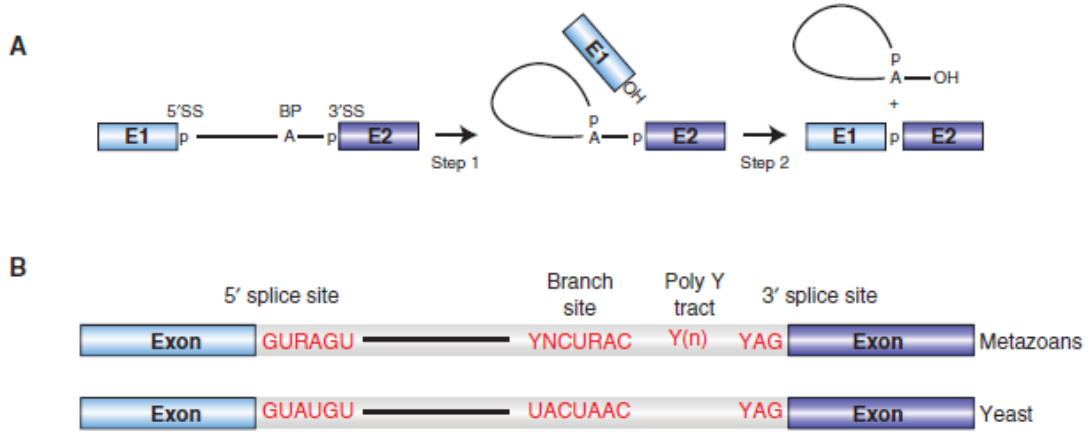


***Figure I8.*** *Splicing process.*
Simplified representation of the splicing process (A) and the conserved important sequences to carry out the procedure (B). Adapted from *Will and Lührmann, Cold Spring Harb Perspect Biol 2011*.

The splicing process, especially the pioneering studies, has been mainly investigated in laboratory research models that are easier to approach than mammals, like yeast, but it has been later shown that the key steps are very well conserved across species. Summarizing the classic explanation by Matera and Wang in 2014 [171] and other studies [21, 172] (**Figure I9**), U1 and U2 recognize and bind to 5' and 3' splice sites, respectively. Next, U2 recognizes sequences in the branch point and interacts with U1, forming the pre-spliceosome. Then, the preassembled U4-U5-U6 complex is recruited and several conformational changes take place to form a catalytically active complex, resulting in the U2/U6 structure that catalyzes splicing reaction. In addition, in this step U1 and U4 are released from the complex. At this moment, the first catalytic step is carried out, cutting the binding between the first exon and the intron-exon lariat intermediate. Finally, after some conformational changes, the second catalytic step leads to the separation of the intron and the second exon and the subsequent binding of both exons, leaving the post-spliceosomal complex with the intron lariat free. Finally, U2, U5 and U6 are released. All the described steps are firmly regulated by several spliceosome proteins, which ensure that the cuts and bindings are correct, make possible the sequence recognition, and put together and separate the other components.

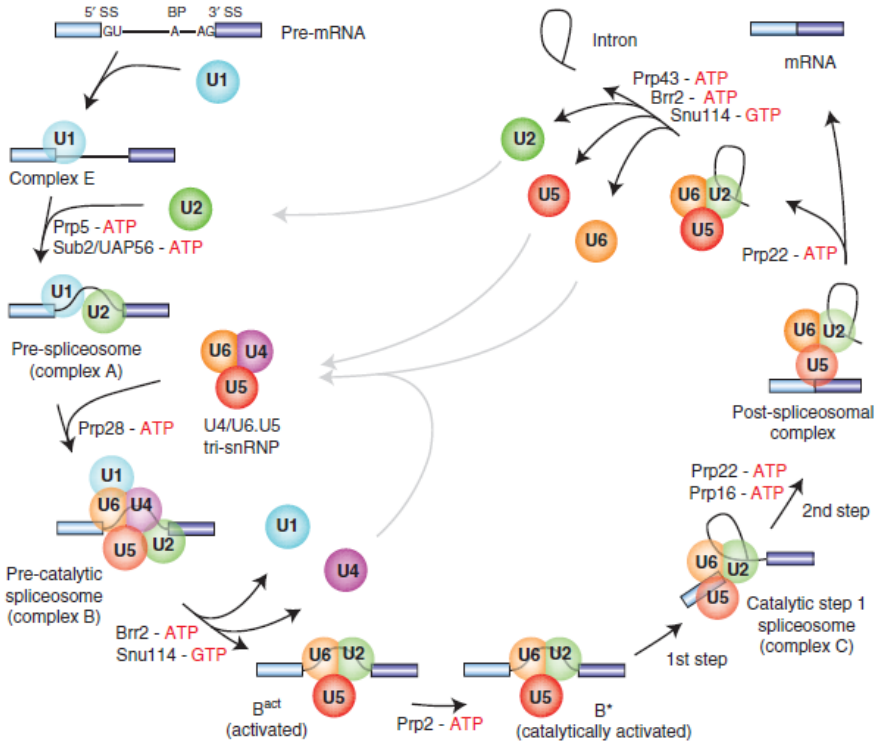


***Figure I9.*** *Spliceosome and splicing process.*
Schematic picture of the function of the spliceosome during the different steps of splicing process. Source: *Will and Lührmann, Cold Spring Harb Perspect Biol 2011*.

### 1.4.1 Alternative splicing

The basic splicing process, known as canonical or constitutive splicing, excises all introns and bind all exons from the pre-mRNA to generate mature RNA molecules. However, the vast majority of the genes (95 % approximately) undergo a more complex process known as alternative splicing, that comprises on or more of the following events, which alter the constitutive splicing process: 1) cassette exon skipping, an exon is excluded together with the two flanking introns; 2) alternative 5' splice site and 3) alternative 3' splice site, the exon is not fully included in the final RNA, but it is cut in a different site; 4) intron retention, there is no cutting of the intron, which is included in the mature RNA; and 5) mutually exclusive exons, two exons that cannot be included together, one of each is excluded in two different isoforms (**Figure I10**) [173-176]. As a result of this alternative splicing process, different mature RNA molecules, known as splicing variants or isoforms, are obtained from the same pre-mRNA, thereby conferring a great variety and depth to the genome, inasmuch as a single gene can give rise to several (even thousands) RNA variants, which, in turn, are translated into distinct proteins that may perform similar, different, or even opposite functions.

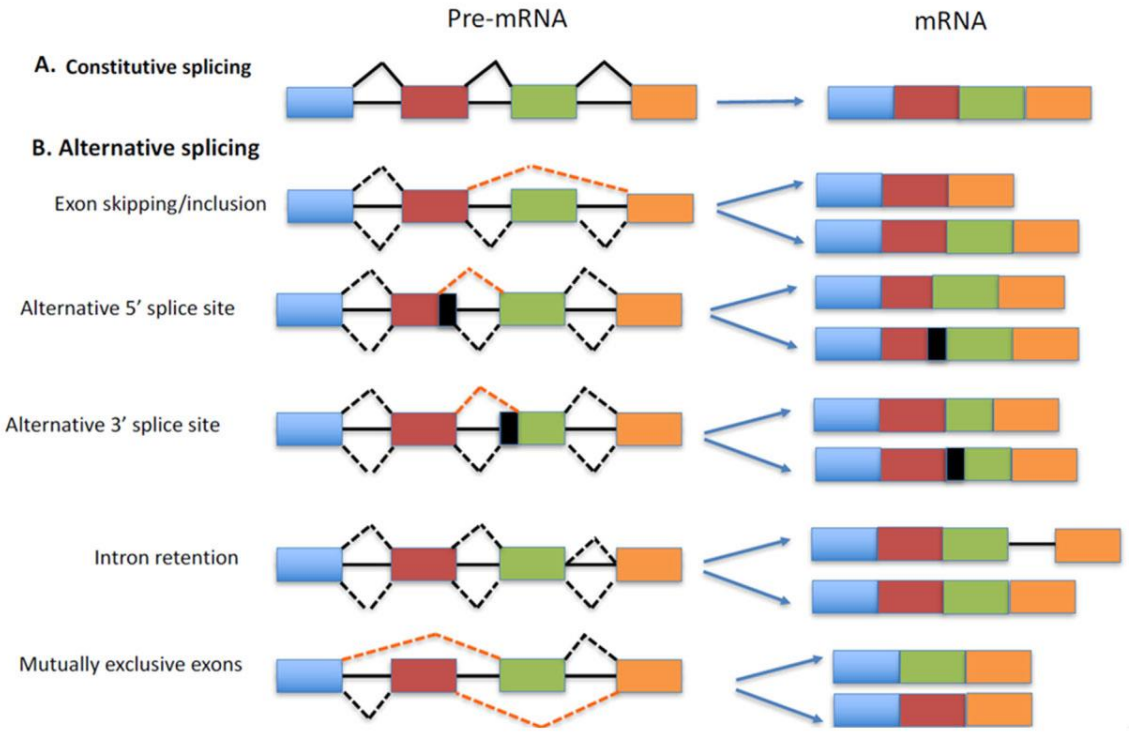


***Figure I10.*** *Alternative splicing.*
Graphic representation of the canonical splicing process (A) and the different types of alternative splicing that a molecule of RNA may suffer (B). Source: *Sen, Hepatoma Res 2018.*

Alternative splicing is tightly controlled by the action of cis- and trans-regulatory elements. The cis-regulatory elements consist in RNA sequences classified following their localization and function in the splicing process, as: ESE (exonic splicing enhancer), ISE (intronic splicing enhancer), ESS (exonic splicing silencer), and ISS (intronic splicing silencer) (**Figure I11**) [170]. On the other hand, the trans-regulatory elements are composed of proteins, the splicing factors, that regulate the process and (recognize?) the splice sites [170]. These elements are typically classified in two families, following the function they exert in the splicing process: serine-arginine proteins (SR-proteins) and heterogeneous nuclear ribonucleoproteins (hnRNPs) [177]. The first group, SR proteins, are usually enhancers of exon splicing, binding to ESE and recruiting the spliceosome components [178, 179], although some of them are also involved in other processes of the RNA biology, such as maturation, decay, transport or translation [180, 181]. Likewise, components of the hnRNPs family are also involved, besides the splicing process, in additional functions related to RNA, such as trafficking, stability and translation [182]. These hnRNPs proteins usually bind to splicing silencing sequences, which may be induced by competition against SR proteins for the binding sites or by altering the structure of the RNA, making specific zones inaccessible for the spliceosome [183, 184]. Moreover, there are many splicing factors, including *NOVA1*, that could act as inhibitors or as enhancers of the process, depending on their binding to silencing or enhancer elements. Thus, the function of these proteins will be determined by the nature of the sequence of interaction [171]. Therefore, the mature RNA variant generated from a given gene in a particular cellular environment represents the ultimate consequence from the dynamic interaction among splicing enhancers and silencers in order to define their precise assembling.

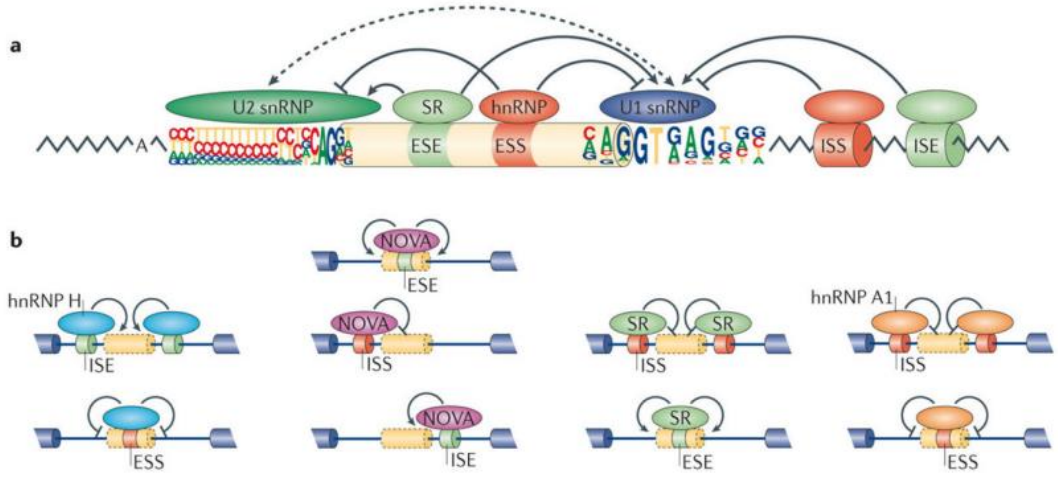


***Figure I11.*** *Regulation of the alternative splicing.*
This picture represents the regulatory actions of splicing factors over the alternative splicing process. A. Selection of the splicing site through cis sequences and splicing factors. B. Action of the splicing factors based on the context of the cis sequences they bind to. Source: *Matera and Wang, Nat Rev Mol Cell Biol 2014.*

## 1.4.2 Splicing and cancer

As a pivotal process for the precise and reliable transmission of information from DNA to RNA, the mechanism of splicing has to be under an exhaustive regulation. In fact, there is mounting evidence that the wrong functioning of alternative splicing, which can alter the normal proportion of the variants and the appearance of aberrant ones, can be the base of several pathologies [22, 185, 186]. This is particularly the case of tumor pathologies, which are so increasingly linked to the dysregulation of splicing landscape, that an altered splicing process is becoming recognized as one common hallmark for tumor development and progression [19, 187-189]. Is well known that tumor cells progress by developing mechanisms that allow them to adapt to the microenvironment; thus, alternative splicing can provide an increased genetic plasticity that would enable cancer cells to grow, become malignant, and take advantage against normal cells through the generation of oncogenic splicing profiles. Over the last years, many altered splicing profiles have been shown associated with tumoral pathologies in the literature [23, 190-193]. Specifically, a wide number of alternative splicing variants have been directly related with tumorigenesis, tumor progression and aggressiveness (**Figure I12**). One of the most representative examples of this is the case of androgen receptor variant 7 (*AR-V7*), characteristic in PCa [194]. This isoform results from an aberrant splicing that leads to the loss of exons 4-8 and the inclusion of the cryptic exon 3 in the mRNA of the *AR*

[195]. This mRNA is translated to an androgen receptor protein lacking its regulatory domain, which confers constitutive activity to the molecule, even in the absence of androgens, with the resulting increase in the growth of prostate tumors, which become resistant to treatments against these tumors, such as abiraterone or enzalutamide, and comprise the most aggressive PCa phenotype, known as castration resistant [196-198]. Another example is the alternative splicing of *TERT* mRNA. The alteration in its splicing leads to the appearance of an aberrant variant without the regulatory motif, that leads to a constitutive activity and an altered lengthening of the telomeres, accompanied by an increase in the aggressiveness features of non-small cell lung cancer [80, 199].

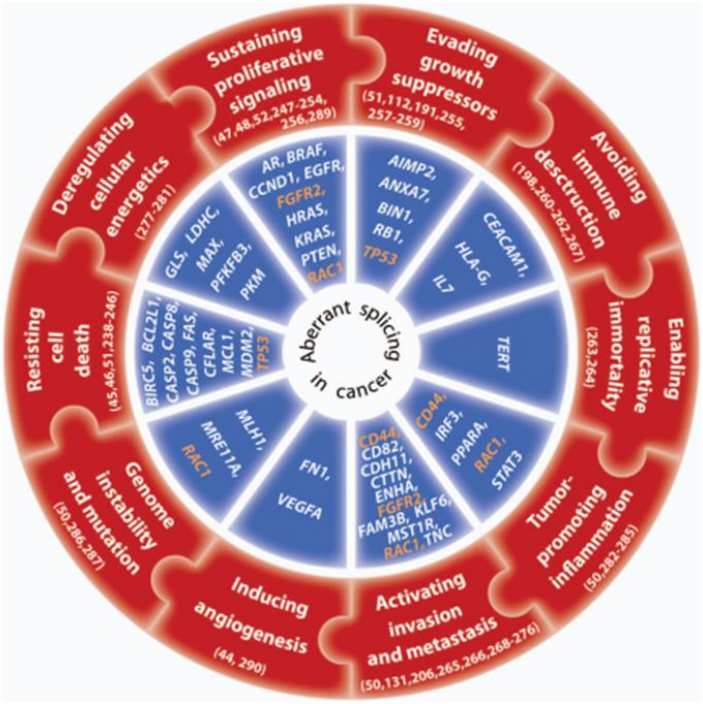


***Figure I12.*** *Alternative splicing and the hallmarks of cancer.*
Relationship between alternative splicing and the hallmarks of cancer, with several examples of dysregulated variants with each one of the hallmarks. Source: *Sveen et al., Oncogene 2015*.

The information regarding splicing and its alterations in NETs, and more specifically PanNETs and PitNETs is still limited, with only few examples of splicing dysregulation related with these tumors. Specifically, as described above, it has been shown that the truncated variant of the $SST_5$, $SST_5TMD4$, is overexpressed in NETs and related to aggressiveness features [17]. Additionally, our group also discovered in these tumors that a variant of the ghrelin hormone that retains the intron 1 (thus called In1-ghrelin) is overexpressed in NETs and other tumors and it related to higher aggressiveness [200, 201].

In this scenario, high efforts have been dedicated in recent years to elucidate the causes underlying the dysregulation of alternative splicing process in cancer. One of this causes is mutations in the regulatory sequences of splicing, such as the branch point, 3' and 5' splice sites or splicing factors binding motifs, that may hinder their recognition by adequate molecules. These mutations have been linked to several pathologies, including cystic fibrosis [202], and cancer [203, 204]. In addition, a most interesting potential cause for splicing dysregulation in pathologic conditions is the alteration of splicing machinery components, including their mutations, and altered expression levels or functionality (activity and/or subcellular location). Indeed, a growing list of studies are linking mutations in splicing machinery components and tumorigenesis, and, among those, it is of particular relevance the study of the *SF3B1* factor [205-207]. With the application of next-generation sequencing techniques, various mutations have been identified in this factor that may represent some of the most common splicing-related mutations in several types of cancer, as liver cancer [208, 209], and other tumor types, such as melanoma [210, 211], leukemia [212] or breast cancer [213]. Furthermore, recent publications show that components of the core of the spliceosome may be also mutated in cancer, as is the case of *RNU1*, mutated in several cancer types, where it is related with poor prognosis and could be a novel therapeutic target [214, 215].

In this context, the dysregulation of the expression of splicing machinery components has been widely studied in tumoral pathologies [216, 217]. In fact, splicing factors are starting to be considered as oncogenes and tumor suppressor genes [21]. There are many examples of SRs and hnRNPs altered in cancer. For instance, *SRSF1* has been shown to be overexpressed in colon and breast cancer, where it has a pro-tumorigenic role [218-220]; *SRSF6* acts as an oncogene in lung and colon cancer, where it is overexpressed [221]; *SRSF2* is also overexpressed in hepatocellular carcinoma, where it has been related to tumorigenesis and tumor development [222]. On the other hand, *PTBP1* (also known as hnRNP I) seems to act as a tumoral suppressor in colorectal cancer [223, 224], but it is overexpressed and promotes tumor growth and invasion in breast cancer [225], and causes gemcitabine resistance in pancreatic cancer [226]. Similarly, other splicing factors can also play distinct roles in different cancers, as occurs in the case of *NOVA1*, which has been related to tumor growth in non-small cell lung cancer through the alternative splicing of *TERT* [80], an event that can also be observed in other cancer cells [199], and has also been described as an oncogene in melanoma [227] and

osteosarcoma [228], whereas its downregulation is associated to poor prognosis and tumor progression in gastric cancer [229]. Thus, when viewed together, these results reveal that splicing alterations are strongly context-dependent and should not be regarded simplistically in the study of cancer, thus highlighting the importance of studying this process in each particular type of tumors. In this regard, there are very few studies about the dysregulation of splicing machinery in NETs, and have been mainly focused in lung high grade tumors [230, 231]. Recently, our group reported that the splicing machinery is profoundly altered in the most relevant types of PitNETs [232]. However, the splicing process and its potential alteration is poorly known in PanNETs and there are no studies addressing the general expression profile of the machinery implicated in this process.

# 2. OBJECTIVES

# 2. Objectives

The **general aim** of this Thesis was to determine the role performed by somatostatin receptors and the splicing machinery in different types of endocrine-related cancers and neuroendocrine tumors, as well as the regulation mechanisms underlying, with the final purpose of discover novel biomarkers and pharmacologic targets with potential to improve diagnostic and therapeutic approaches in those pathologies.

To fulfill this general aim, we proposed the following **specific objectives**:

**Objective 1**: To investigate the presence and possible dysregulation of somatostatin receptor subtype 1, *SSTR1*, in PCa, as well as to elucidate its functional role in this pathology in terms of tumorigenesis and malignancy features. Additionally, we will aim to look for important associations of this receptor with clinical parameters and factors that may be regulating its expression in this type of cancer, to have a bigger picture of the hormonal regulation of PCa.

**Objective 2**: To increase the poor understanding of the underlying mechanisms controlling the expression of the somatostatin receptor subtype 5, *SSTR5*, in NETs, specifically, those from pituitary and pancreas, besides to describe the functional implications of those mechanisms in the aggressiveness of these tumors, in order to better discern the real importance of this receptor.

**Objective 3**: To make a profile of the splicing machinery in panNETs and to evaluate the potential associations of the altered factors with relevant clinical parameters. From this point, we will aim to elucidate the functional role of the splicing machinery dysregulation in models of this neuroendocrine disease, regarding its relevant aggressiveness features.

# 3. MATERIALS AND METHODS

# 3. Materials and methods

## 3.1 Patients and samples

In this Thesis, samples from different cohorts of patients with endocrine-related tumoral pathologies have been included. All the studies presented herein were approved by the corresponding Hospital/University Ethics Committees and conducted in accordance with ethical standards of the Helsinki Declaration of the World Medical Association. Written informed consents from patients were obtained through the Andalusian Biobank (Servicio Andaluz de Salud). Patients were managed following current recommendations and guidelines. Data regarding physical examination, medical history, demography and laboratory work-up were obtained from routine visits using information available in clinical records.

First, fresh PCa samples (n = 52) were included, obtained by core needle biopsies, following NCCN guidelines [47]. To use as control, non-tumoral prostate samples were collected from patients after cystoprostatectomy due to bladder cancer but without PCa (n = 12). The appropriate classification of the samples as tumor or non-tumor was confirmed by expert pathologists and it is summarized in **Table 1**. Additionally, demographic and clinical parameters regarding tumor aggressiveness and metabolic status were collected. Briefly, included individuals exhibited a median of age of 76 years old at diagnosis. Regarding PCa cohort, all the samples had at least a Gleason score of 7, with a 65 % of higher grade. Additionally, a 33 % of the patients suffered extraprostatic extension and a 52 % perineural infiltration.

Regarding NETs, human PitNET samples were collected during transsphenoidal surgery from 27 acromegaly patients, and 11 normal pituitaries by autopsy from healthy donors. On the other hand, formalin fixed paraffin-embedded samples (FFPE, n = 20) were obtained from primary PanNETs; non-tumoral adjacent tissue, used as control, was extracted from the same piece and both tissues were separated by expert pathologists (patient features summarized in **Table 2**). The mean age of patients of PitNETs were 43-year old, while those with panNETs were 55-year old.

| Parameter | Overall | Control | PCa |
|---|---|---|---|
| **Patients, number.** | 64 | 12 | 52 |
| **Age; Median (IQR)** | 76 (67.5-81.25) | 70 (62.2-80.7) | 78 (69-81.7) |
| **PSA level, ng/ml; Median (IQR)** | | | 54.5 (37.2-212) |
| **Gleason score** | | | |
| **7** | - | - | 18/52 (35%) |
| **>7** | - | - | 34/52 (65%) |
| **Extraprostatic extension** | - | - | 17/52 (33%) |
| **Perineural infiltration** | - | - | 27/52 (52%) |

***Table 1.*** *Clinical and demographic parameters of patients with high risk of PCa.*

Overall clinical and demographic data of patients with high risk PCa in fresh normal prostates (control; obtained from cystoprostatectomy) and prostate cancer (PCa; obtained by core needle biopsies) samples.

| Parameter | PitNETs | PanNETs |
|---|---|---|
| **Number of samples** | 27 | 20 |
| **Age (years, mean ± SEM)** | 43 ± 11 | 55 ± 14 |
| **Body Mass Index (kg/m$^2$, mean ± SEM)** | 31.01 ± 6.83 | 28.00 ± 3.48 |
| **Gender (female, %)** | 63.2 | 57.1 |
| **Gender (male, %)** | 36.8 | 42.9 |
| **Smoking (%)** | N/A | 68.8 |
| **Family history of neoplasia (%)** | N/A | 12.5 |

***Table 2.*** *Summary of clinical parameters of the NETs patients.*

Overall clinical and demographic data of patients with these diseases that participated in the study.

## 3.2 Cell culture

In order to explore the functional aspects of the different molecules studied in this Thesis, we used several cell lines, as models for the pathologies of interest. Specifically, three PCa cell lines (22Rv1, DU145 and PC-3) and two panNETs cell lines (BON-1 and QGP-1) were used. PCa cell lines were previously validated by analysis of STRs (GenePrint® 10 System, Promega, Barcelona, Spain), while there is no STR profile for panNETs cell lines; all of them were checked for mycoplasma contamination by PCR, as

previously reported [233]. Cell lines were grown at 37 ºC, in a humidified atmosphere with 5.0 % of $CO_2$.

### 3.2.1 Cell lines

PCa cell lines were obtained from the American Type Culture Collection (ATCC). The 22Rv1 cell line was developed from a xenograft of CWR22R cells serially propagated in mice after castration-induced regression. The donor patient was diagnosed with primary prostatic carcinoma with Gleason score of 9 advanced to osseous metastasis. These cells are androgen-independent but sensitive to them, they express prostate specific antigen (PSA) and androgen receptor (AR) and their growth is weakly stimulated by dihydrotestosterone [234, 235]. The DU145 cell line was generated in 1975 from a PCa metastasis in the brain, and represented and important advance in PCa research, since previous cell lines came from mixtures of benign tumors and moderate adenocarcinoma. DU145 cells are androgen-independent and do not express PSA and AR [236, 237]. The PC-3 cell line was obtained from bone metastasis of a high grade PCa in 1979. These cells are similar to DU145, in that they are also resistant to androgen effects and do not express PSA and AR, but PC-3 cells are more aggressive than DU145 [236, 238].

On the other hand, in order to evaluate functional effects in NETs, we used the two most widely used model cell lines, BON-1 and QGP-1. BON-1 is a cell line derived from a metastasis in a peripancreatic lymph node of a non-functioning carcinoid tumor of the pancreas in 1986, and is considered as an aggressive model cell line. These cells secrete some markers that can be used to assess their behavior, such as serotonin (5-hidroxitriptamine or 5-HT), chromogranin A or neurotensin [239, 240]. The QGP-1 cell line was obtained from a primary pancreatic somatostatin-producing tumor, or somatostatinoma, that also secrete serotonin, in 1980. These cells exhibit a cluster and slow growth and a lower aggressiveness than BON-1 cells [241, 242].

DU145, PC3 and QGP-1 were cultured in RPMI 1640 (Lonza, Basel, Switzerland), while BON-1 cells were cultured in Dulbecco's Modified Eagles Medium complemented with F12 (DMEM-F12; Life Technologies, Barcelona, Spain), both supplemented with 10 % fetal bovine serum (FBS, Sigma-Aldrich, Madrid, Spain), 1 % glutamine (Sigma-Aldrich) and 0.2 % antibiotic (Gentamicin/Amphotericin B, Gibco, Thermo Fisher, Waltham, MA, USA).

### 3.2.2 Freezing/Thawing and maintenance of cells in culture

For freezing, aliquots of $10^6$ cells were resuspended in FBS with 5 % of dimethyl sulfoxide (DMSO) in cryotubes and maintained in a pre-warmed isopropanol bath that was then stored at -80ºC at least 24 h. After that, the cryotubes were cryopreserved under liquid nitrogen conditions.

For thawing, cryotubes containing $10^6$ cells were fast warmed in a water bath until ice disappeared, followed by resuspension of cells in pre-warmed complete medium. DMSO was removed by centrifugation and supernatant elimination. Then, cells were resuspended in the appropriate medium and seeded in 25 $cm^2$ flasks.

### 3.2.3 Reagents

$SST_1$ agonist BIM-23926 was provided by IPSEN (Milford, MA, USA). It was administered at 1 µM and 10 nM for proliferation and free cytosolic calcium concentration ($[Ca^{2+}]_i$) assays, and at 1 µM for the rest of the experiments. Pasireotide was provided by Novartis (Basel, Switzerland) and administered at 100 nM, as previously reported in other studies [243]. Everolimus was obtained from Sigma-Aldrich and used at 100 nM. Dilutions were made in corresponding media, depending on assay performed in each case.

### 3.2.4 Transfections

During this Thesis, several genetic alterations were performed through different transfection assays that are described as follow.

#### 3.2.4.1 Stable and transient transfections with plasmids and shRNA

Cell lines were transfected with commercial overexpression plasmids and shRNAs using Lipofectamine-2000 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions, as previously reported [16]. Briefly, between 100,000 – 200,000 cells, depending on cell line used, were seeded in 6-well plates and incubated for two days at 37 ºC and 5 % $CO_2$. Then, cells medium was retired and 500 µl of Opti-MEM (Thermo Fisher Scientific) were added. At the same time, 1 µg of each plasmid or shRNA of interest (containing *SSTR1* or *NOVA1* genes, or *SSTR5-AS1* silencing vector) or an empty vector, used as control and named as "mock" for overexpression and "scramble" for silencing, were mixed with 3 µl of Lipofectamine-2000 in 100 µl Opti-MEM and incubated for 30 min at room temperature. Next, those

transfection mixtures were added to the cells and incubated for 8 h. Finally, cell medium was replaced by 2 ml of complemented cell-specific medium. Success of transfections was validated by qPCR and/or western blot, comparing the expression of transcripts of interest with mock plasmid or scramble shRNA transfected cells. For subsequent studies, after transient transfection cells were incubated 48 h in order to allow plasmids to achieve the appropriate levels of gene expression.

For *SSTR1*-stably transfected cells, we treated them with geneticin in the medium, which selectively eliminates non-transfected cells. After two weeks of treatment, with successive plate changes, cells grew normally and experiments could start. In the case of *SSTR5-AS1* shRNA stable transfection, the protocol was similar, but the antibiotic used was puromycin.

#### 3.2.4.2 Transfections with siRNA and miRNA

Cell lines were transfected with specific siRNAs (targeting *SSTR1* and *NOVA1* genes; Ambion, Thermo Fisher Scientific and Origene, Rockville, MD, USA), which were validated in our laboratory. Specifically, cells were seeded in 6-well culture plates and transfected with *SSTR1* or *NOVA1* siRNAs and scramble siRNA, used as control, at 100 nM, using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen), following manufacturer instructions. Briefly, between 100,000 – 200,000 cells, depending on cell line used, were seeded in 6-well plates and incubated for two days. Then, cells medium was removed and replaced by 850 µl of 10 % FBS medium without antibody. At the same time 9 µl of lipofectamine were mixed with 300 µl of Opti-MEM and the appropriate amount of each siRNA and incubated for 5 min at room temperature. Finally, those transfection mixtures were added to the cells and incubated for 48 h, previous to subsequently studies, in order to allow siRNAs to achieve the appropriate inhibition of gene expression. Success of the silencing was validated by qPCR and/or western blot.

Additionally, 22Rv1 cells were transfected with miRNA mimics of miR-24, miR-27, miR-383, miR-488 or with a negative control (20 nM; GenePharma, Shanghai, China) for 48 h, following a protocol similar to that applied for siRNAs. RNA was extracted using TRIzol reagent and 2 µl of extracted RNA were retrotranscribed with the Taqman microRNA Reverse transcriptase kit (Thermo Fisher Scientific) following the manufacturer's instructions. Taqman probes for hsa-miR-24-3p, hsa-miR-27b-3p, hsa-miR-383-5p, hsa-miR-488-3p, and RNU6 (used as housekeeping) were purchased

(Thermo Fisher Scientific). The validation of successful miRNA mimic transfection was determined by TaqMan Universal PCR Master Mix (Thermo Fisher Scientific) in the BioRad CFX PCR instrument (BioRad).

## 3.3 Functional assays

### 3.3.1 Proliferation and colony formation assays

We have used two different assays to measure cell proliferation. First, Alamar Blue fluorescent assay (Life Technologies, Thermo Fisher Scientific) was used to determine cell proliferation, as previously reported [244, 245]. Specifically, cells were seeded in 96-well plates at a density of 5,000 cells per well. After 24 h of starving, cell viability was analyzed at 0 h (basal), 24 h, 48 h and 72 h after transfection or treatment by measurement of fluorescent signal exciting at 560 nm and reading at 590 nm with Flex Station 3 device (Molecular Devices, Sunnyvale, CA, USA). More precisely, the day of the measurement, Alamar blue reduction was measured after cells were incubated for 3 h with 10 % Alamar blue/serum free medium. This reduction is proportional to the number of cells, so that the comparison between days is a relative reference of the cell proliferation. Medium was replaced by fresh 5 % FBS-medium immediately after each measurement (every 24 h), including treatment as appropriate. In all instances, cells were seeded per quadruplicate and all assays were repeated a minimum of three times.

Second, we used a variety of the colony formation assay, as it has been previously reported in the literature, to assess cell proliferation. This assay is based on the size that arising colonies reach, since this is a relative quantification of cell proliferation. Specifically, we seeded cell lines in a very low density (1,000 cells per well) in 6-well plates for 10 days. Treatment was added 24 h after seeding and refreshed it each 2-3 days. Next, cells were fixed and stained with a combination of violet crystal (0.5 %) and glutaraldehyde (6 %) for 30 min. Finally, cells were washed with tap water and pictures were taken for every well. Quantification of the area covered by cells was made using ImageJ software (National Institutes of Health).

Additionally, colony formation assay is based on the difficulties that cells find to grow up and proliferate when they are isolated, without other cells close to them. The genes that regulate this feature are expressed early when cells are seeded. For that reason, we treated cell lines during 24 h before the plating and seeded them at low density (1,000 cells per well) in 6-well plates for 10 days, without adding more treatments. The protocol

for staining and quantification is similar to that described above; however, in this case we did not quantify the area covered by the cells, but the number of colonies formed in each well.

### 3.3.2 Migration assay

Cell migration was evaluated by wound healing assay, based on the capacity of the cells to cover a wound made in a well with a high cell density, as previously reported [244]. Briefly, cells were seeded in 24-well plates and cultured them until they reached maximum confluence. Then, we made a wound in the middle on the well with a 200 µl sterile pipette tip. Cells were next incubated for 24 h in medium without FBS, in order to minimize cell proliferation effects on wound recovery, with the treatments as required. At least three random pictures were taken per well at 0 and 24 h after the wound was made. Wound healing was calculated as the uncovered area 24 h after the wound compared to the uncovered area just after wounding, which were all quantified with ImageJ software.

### 3.3.3 Xenograft model

Animal maintenance and experiments were carried out following the European Regulation for Animal Care and under the approval of the University of Córdoba Research Ethics Committee. Seven-week-old male athymic BALB/cAnNRj-Foxn1nu mice (Janvier Labs, Le Genest-Saint-Isle, France; n = 6 mice), were subcutaneously grafted in the flank with $3x10^6$ BON-1 cells transfected with mock and *NOVA1* plasmids in each flank, resuspended in 100 µl of basement membrane extract [16]. Tumor growth was monitored twice per week for 5 weeks, by using a digital caliper. After euthanasia of mice, each tumor was dissected, fixed, and sectioned for histopathologic examination after hematoxylin and eosin staining for the examination by expert pathologists. Additionally, a piece from each tumor was frozen for further RNA extraction with TRIzol reagent.

## 3.4 Molecular assays

### 3.4.1 Nucleic acids

#### 3.4.1.1 Genomic DNA and total RNA from fresh human samples

Genomic DNA (gDNA) and total RNA from human fresh samples were extracted using the “AllPrep DNA/RNA/Protein Mini Kit” (Qiagen, Madrid, Spain) following

manufacturer's instructions. Next, quantification of nucleic acids recovered was assessed with Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific), 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. Samples were homogenized with an IKA T25 Ultra-Turrax (Gemini BV laboratory, Apeldoorn, Netherlands) in the recommended RLT Buffer, allowing an accurate purification of gDNA and total RNA. Then, the homogenized samples were passed through two columns that retain firstly the gDNA and then total RNA, that were eluted with RNase- and DNase-free water, respectively.

#### 3.4.1.2 Total RNA from FFPE human samples

Regarding FFPE human samples, RNA was isolated using RNeasy FFPE Kit (Qiagen), according to the manufacturer's protocol. Briefly, FFPE sample slides were deparaffined with xylol and lysed with proteinase K, followed by heat treatment. Then, supernatant was treated with DNase and passed through a column that retain total RNA. Finally, RNA was eluted with RNase free water. The amount of RNA recovered and its quality was determined as described in the previous section.

#### 3.4.1.3 Total RNA from cell lines samples

Total RNA was extracted from different cell lines using TRIzol reagent (Sigma-Aldrich), according to manufacturer's protocol, as previously reported [244-246]. Briefly, cells were incubated until confluence in 6-well or 12-well plates until 70-80 % of confluence. Then, wells were rinsed with PBS and, subsequently, 1/0.6 ml TRIzol was added, collected and re-added several times, to lyse cells appropriately, and finally collected with lysed cells in 1.5 ml tubes. RNA isolation was carried out by adding chloroform and collecting the aqueous phase. RNA was precipitated, concentrated and washed with isopropanol and 70 % ethanol washing steps. Next, samples were dried and resuspended with 8 µl of DEPC-treated water. Subsequently, samples were treated with 1 U of DNase (Promega) and incubated for 30 min at 37 ºC, stopping the reaction by adding a Stop Solution and incubating at 65 ºC for 5 min. The amount of RNA recovered and its quality was determined as described above.

#### 3.4.1.4 Total RNA retrotranscription to cDNA

Retrotranscription of total RNA to cDNA was performed with the cDNA First Strand Synthesis kit (MRI Fermentas, Thermo Fisher Scientific), using random hexamers primers and following manufacturer's instructions, independently of the origin of the

samples. Specifically, 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.

#### 3.4.1.5 Conventional PCR

Conventional PCR has been used along this work with two aims: to study differential alternative splicing isoforms expression and to validate qPCR primers, both using PCR Master Mix (Thermo Fisher Scientific), that includes Taq DNA polymerase, reaction buffer, $MgCl_2$ and dNTPs. All conventional PCRs were carried out in a thermocycler T100 Thermal-cycler (BioRad). The thermal profile followed for conventional PCR was:

| | | |
|---|---|---|
| **Initial denaturalization** | 95 ºC | 30 s |
| **30-35 cycles** | | |
| | 95 ºC | 30 s |
| | 45-65 ºC | 30 s |
| | 72 ºC | 1 min/kb of amplicon |
| **Final extension** | 72 ºC | 5 min |
| **Hold** | 4-10 ºC | - |

Duration of the extension step depended on the length of each amplicon, where 1 min was needed per kb. Annealing temperature was adapted to each pair of primers, since we tried to design them to have around 60 ºC, but it was not always possible.

#### 3.4.1.6 Quantitative Real-Time PCR (qRT-PCR or qPCR)

Quantitative real-time PCR (qPCR) was used to perform relative quantification of cDNA derived from retrotranscription of RNA from human samples or cells lines. qPCR was performed using Brilliant III SYBR Green Master Mix in the Stratagene Mx3000p instrument (both from Agilent, La Jolla, CA, USA) as previously described [244-246]. Briefly, for each reaction, 10 µl of master mix, 0.3 µl of each primer, 8.4 µl of distilled $H_2O$ and 1 µl of cDNA (50 ng, when it was possible) in a 20 µl total volume were mixed. The thermal profile used for qPCR was:

| | | |
|---|---|---|
| **Initial denaturalization** | 95 ºC | 30 s |
| **40 cycles** | | |
| | 95 ºC | 20 s |
| | 60 ºC | 20 s |
| **Melting curve** | 0.5 ºC/30 s | |

Results were adjusted with a normalization factor, calculated from values of different combinations of *ACTB*, *GAPDH*, *HPRT* or *RNA18S1* housekeeping genes, depending on their suitability in each case, using Genorm Software, wherein the expression of the housekeeping genes did not differ between experimental groups.

#### 3.4.1.7 Quantitative PCR Dynamic Array

A Dynamic Array (Fluidigm, South San Francisco, CA, USA), based on microfluidic technique for gene expression analysis, was employed to measure the expression of a custom set of genes in 48 samples simultaneously. Specific primers for human transcripts of interest (**Table 3**) were designed in the same way than those for conventional PCR and qPCR, as explained in the next section. This custom array included components of the major spliceosome (n = 13), minor spliceosome (n = 4), associated splicing factors (n = 27) and the *ACTB*, *GAPDH* and *HPRT* as housekeeping genes. The array was prepared and measured following the manufacturer's instructions. Briefly, 12.5 ng of cDNA of each sample was preamplified using 1 µl of PreAmp Master Mix (Fluidigm) and 0.5 µl of a mix with all primers together (500 nM) in a T100 Thermal-cycler (BioRad), following the 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 TE Buffer at pH 8.3 (Thermo Scientific). 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 Prime script program was run into the IFC controller MX. 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).

#### 3.4.1.8 DNA methylation evaluation

The DNA methylation levels of CpG islands overlapping *SSTR5* and *SSTR5-AS1* genes were evaluated in the cohort of pitNETs samples and normal pituitary samples. Genomic DNA, extracted as described above, was used to this end, following a protocol previously reported by de la Rica and collaborators [247]. Specifically, we performed bisulfite conversion of the gDNA samples, converting unmethylated cytosines to uracil, while the methylated ones remain as cytosines. Methylation validation was performed by comparing enzyme restriction cuts, Hha I (GCGC) and Msp I (CCGG), on bisulfite transformed and not transformed samples. Once methylation was validated, we performed 8 PCR reactions per sample, one for each selected CpG zone (PCR1, **FigureM1**), with specific couples of primers that were modified with nucleotidic adaptors. Subsequently, we performed a second PCR (PCR2, **FigureM1**) pooling all the PCR1 products of each sample, using barcoded primers targeting adaptors of the PCR1, which allowed us to identify each sample in the following sequencing reaction. Finally, we pooled all the transcripts, making a single library with all the amplicons from all the samples, and performed a high throughput sequencing reaction (HTS).

#### 3.4.1.9 Gene expression profile microarray

Three independent passages from 22Rv1 cells stably-transfected with *SSTR1* in a pCDNA3.1 vector and the empty vector, as control, were used to measure a microarray Human Androgen Receptor Signaling Targets PCR Array PAHS-142Z (Qiagen). Expression profile array was measured using RT² qPCR SYBR Green ROX (Qiagen) in the Stratagene Mx3000p system. Total RNA of high quality was used, extracted using AllPrep DNA/RNA/Protein Mini Kit (Qiagen) and retrotranscribed using RT² First Strand Kit (Qiagen). Specific analysis of the results was performed with Data Analysis

Center (Qiagen, http://www.qiagen.com/shop/genes-and-pathways/data-analysis-center-overview-page/), following the manufacturer's instructions.

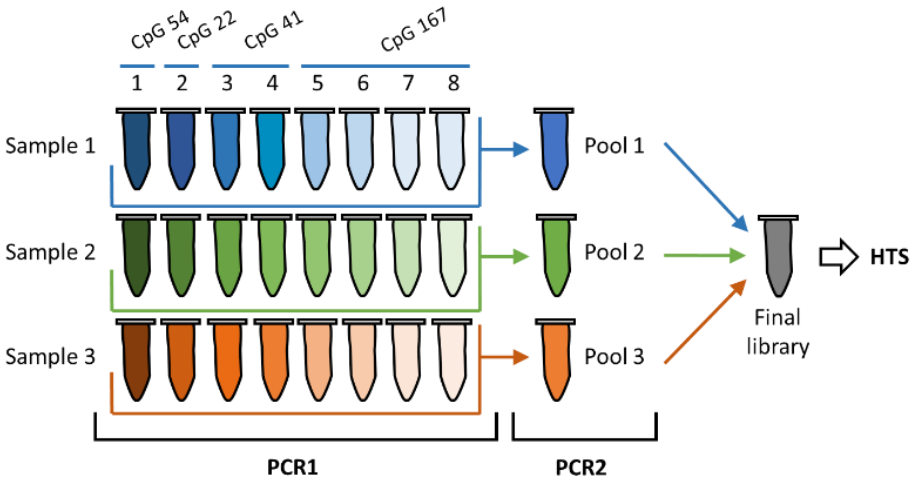


***Figure M1.*** *Workflow for DNA methylation assay of pituitary samples.*
PCR1 uses primers with a target-specific portion as well as part of the adaptors needed for HTS; PCR2, which is target independent, completes the adaptors and adds a barcode that allows sample pooling. After PCR1 all amplicons from the same sample can be pooled, reducing the scale of the sample-indexing PCR2; after indexing all samples can be pooled into a single tube and sequenced.

### 3.4.1.10 Primers design

Primers used during the present work for PCR and qPCR 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 3**, 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. Primers for alternative splicing analysis were less restrictive, since they amplified longer sequences. Designed primers were synthesized by Integrated DNA technologies (Madrid, Spain). Subsequently, primers were validated by conventional PCR using cDNAs from different cell lines as template; PCR products were isolated with FavorPrep™ GEL/PCR Purification Kit (Favorgen, Vienna, Austria) and

sequenced using the genomic services of the University Core Facilities, SCAI (Servicio Centralizado de Apoyo a la Investigación, University of Córdoba, Spain).

Finally, we also designed the primers for the PCR involving the DNA methylation assay (**Table 3B**). Those primers were designed using PyroMark software (Qiagen), applying special requirements: 300 bp amplicon; 58-60 ºC Tm; 22 nucleotides of length; including a high number of CG sites in the amplicon, but avoiding CG sites in the primer sequence, in order to include the maximum number of possible methylation but without affecting the primers efficiency; and including recognition sites for Hha I (GCGC) and Msp I (CCGG) restriction enzymes, to allow the methylation validation. Those primers were modified with target adaptors for the second PCR, as described in the original article. The primers for that second PCR were provided by the group that created the protocol and they targeted the adaptors of the first PCR primers and included barcodes to identify each sample.

**Table 3A**

| Transcript | Primers application | Forward | Reverse | Size (bp) |
|---|---|---|---|---|
| *RNA18S1* | qPCR | CCCATTCGAACGTCTGCCCTATC | TGCTGCCTTCCTTGGATGTGGTA | 136 |
| *ACTB* | qPCR | ACTCTTCCAGCCTTCCTTCCT | CAGTGATCTCCTTCTGCATCCT | 176 |
| *GAPDH* | qPCR | AATCCCATCACCATCTTCCA | AAATGAGCCCCAGCCTTC | 122 |
| *HPRT* | qPCR | CTGAGGATTTGGAAAGGGTGT | TAATCCAGCAGGTCAGCAAAG | 157 |
| *SSTR1* | qPCR | CACATTTCTCATGGGCTTCCT | ACAAACACCATCACCACCATC | 165 |
| *TP53* | qPCR | AAGGAAATTTGCGTGTGGAG | CCAGTGTGATGATGGTGAGG | 180 |
| *CCND3* | qPCR | GAGCTGCTGTGTTGCGAAG | TGCACGCACTGGAAGTAGGA | 143 |
| *KLK3* | qPCR | GTGCTTGTGGCCTCTCGT | CAGCAAGATCACGCTTTTGT | 108 |
| *ADAMTS1* | qPCR | CTCATCTGCCAAGCCAAAG | GCACACAGACAGAGGTGGAA | 100 |
| *IRS2* | qPCR | TTAGATGAGGCACCAACAAGG | AAGGCCAATGAAAACATCCA | 157 |
| *LIFR* | qPCR | CATCATCAGCGTAGTGGCTAAA | CCTTTCCCATCCCAACAAC | 116 |
| *NDRG1* | qPCR | ATTATTGGCATGGGAACAGG | GGGTTCACGTTGATAAGGACA | 101 |
| *IGFBP5* | qPCR | TGTGACCGCAAAGGATTCTAC | AAAGTCCCCGTCAACGTACTC | 129 |
| *SLC45A3* | qPCR | CCGGAGACACTATGATGAAGG | CAGAGAGAAGACCAGGGAGATG | 82 |
| *TSC22D3* | qPCR | TGATGTATGCTGTGAGAGAGGAG | ACGCTCTAGCTGGGAGTTCTT | 83 |
| *VIPR1* | qPCR | TGATCCCCCTGTTTGGAGT | CACCACAAAACCCTGGAAAG | 116 |
| *SSTR5* | qPCR | CTGGTGTTTGCGGGATGTT | GAAGCTCTGGCGGAAGTTGT | 183 |
| *SSTR5-AS1* | qPCR | AGCACAGGTGTTTCTGCTTCT | CCCTGCTCTGTCTTTCTCGT | 116 |
| *ATRX* | qPCR | TGTTTTCAGCCAGTCCCTCA | GCCACTTCCCCTCACCTTTA | 118 |
| *DAXX* | qPCR | AAGCCTCCTTGGATTCTGGT | CTGCTGCTGCTTCTTCCTCT | 237 |
| *MKI67* | qPCR | GACATCCGTATCCAGCTTCCT | GCCGTACAGGCTCATCAATAAC | 139 |
| *CCND1* | qPCR | CCTCGGTGTCCTACTTCAAAT | TCCTCCTCGCACTTCTGTTC | 108 |
| *CASP3* | qPCR | TTTTTCAGAGGGGATCGTTG | GTCTCAATGCCACAGTCCAGT | 97 |

| | | | | |
|---|---|---|---|---|
| *CELF1* | qPCR | AACAGAAGAGAATGGCCCAGC | TGCTGAAGGAGTGCTAAATACTG | 121 |
| *ESRP1* | qPCR | TTTTGGGATCACTGCTGGGG | TGTCCCACCTTCTTGTTGGC | 108 |
| *ESRP2* | qPCR | AGAGCCCAGCAGTCAATTGTT | GTCTCACTGTCCACCACATCAG | 96 |
| *KHDRBS1* | qPCR | GAGCGAGTGCTGATACCTGTC | CACCAGTCTCTTCCTGCAGTC | 106 |
| *MAGOH* | qPCR | GCCAACAACAGCAATTACAAGA | TTATTCTCTTCAGTTCCTCCATCAC | 88 |
| *NOVA1* | qPCR | TACCCAGGTACTACTGAGCGAG | CTGGTTCTGTCTTGGCCACAT | 124 |
| *PTBP1* | qPCR | TGGGTCGGTTCCTGCTATT | CAGATCCCCGCTTTGTAC | 111 |
| *RAVER1* | qPCR | GTAACCGCCGCAAGATACTG | CGAAGGCTGTCCCTTTGTATT | 126 |
| *RBM17* | qPCR | CAAAGAGCCAAAGGACGAAA | TACATGCGGTGGAGTGTCC | 107 |
| *RBM3* | qPCR | AAGCTCTTCGTGGGAGGG | TTGACAACGACCACCTCAGA | 98 |
| *RBM45* | qPCR | CCCATCAAGGTTTTCATTGC | TTCCCGCAGATCTTCTTCTG | 123 |
| *SFPQ* | qPCR | TGGTAGGGGGTGAAAGTG | TTAAAAACAAGAAATGGGGAAATG | 125 |
| *SND1* | qPCR | ACTACGGCAACAGAGAGGTCC | GAAGGCATACTCCGTGGCT | 101 |
| *SNW1* | qPCR | ATGCGTGCCCAAGTAGAGAG | TCCCCATCCTCTTTTTCCA | 134 |
| *SRRM1* | qPCR | GTAGCCCAAGAAGACGCAAA | TGGTTCTGTGACGGGGAG | 108 |
| *SRRM4* | qPCR | CCTTCACCACCTCCTCAC | TTCGGCACATTCCAGACA | 113 |
| *SRSF1* | qPCR | TGTCTCTGGACTGCCTCCA | TGCCATCTCGGTAAACATCA | 98 |
| *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 |
| *TIA1* | qPCR | TAAATCCCGTGCAACAGCAGA | TATGCAGGAACTTGCCAACCA | 124 |
| *TRA2A* | qPCR | TCAAAGGAGGCTATGGAAAGG | TGTGTGCGCTCTCTTGGTTA | 90 |
| *TRA2B* | qPCR | GATGATGCCAAGGAAGCTAAAG | AGGTAGGTCTCCCCATGTAAATTC | 130 |
| *PRPF40A* | qPCR | GCTCGGAAGATGAAACGAAA | TGTCCTCAAATGCTGGCTCT | 130 |
| *PRPF8* | qPCR | TGCCCACTACAACCGAGAA | AGGCCCGTCCTTCAGGTA | 139 |
| *RBM22* | qPCR | CTCTGGGTTCCAACACCTACA | GGCACAGATTTTGCATTCCT | 137 |
| *SNRNP70* | qPCR | TCTTCGTGGCGAGAGTGAAT | GCTTTCCTGACCGCTTACTG | 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 |
| *SF3B1 tv1* | qPCR | GCAGACCGGGAAGATGAATA | TTTTCCCTCCATCTGCAAAA | 88 |
| *SNRNP200* | qPCR | GGTGCTGTCCCTTGTTGG | CTTTCTTCGCTTGGCTCTTCT | 103 |
| *TCERG1* | qPCR | GAGGAGCCCAAAGAAGAGGA | CACCAGTCCAAACGACACAC | 112 |
| *U2AF1* | qPCR | GAAGTATGGGGAAGTAGAGGAGATG | TTCAAGTCAATCACAGCCTTTTC | 120 |
| *U2AF2* | qPCR | CTTTGACCAGAGGCGCTAAA | TACTGCATTGGGGTGATGTG | 130 |
| *Δ133TP53* | PCR | From Bernard et al., Oncogene 2013 | | |

| | | |
|---|---|---|
| *TERT* | PCR | From Mavrogiannou et al., Clin Chem 2007 |
| *TERT tv1* | PCR | |

| Table 3B | |
|---|---|
| **CpG Zone** | **DNA METHYLATION PRIMERS** |
| ZONE 1 Fw | ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTTGGGGGATGAAGAGT |
| ZONE 1 Rv | TGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAAACTCCCCAAACCCAACAAATAAA |
| ZONE 2 Fw | ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGGATGTTAGGGTATTTTGTGTTTT |
| ZONE 2 Rv | TGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCCCCAACAACCTACAAATATTC |
| ZONE 3 Fw | ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGGTTATTGTTAGTGGGATTAGG |
| ZONE 3 Rv | TGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACACAAAATAAAACCCCCAATAAAAAT |
| ZONE 4.1 Fw | ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTAGTAGTATTGTAGGGTAGGT |
| ZONE 4.1 Rv | TGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACATACAAACATTCCTTCCTCCTAAA |
| ZONE 4.2 Fw | ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGTATGTGTTGGTTTAGGGATTTATTA |
| ZONE 4.2 Rv | TGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACCAAAAAAAAACAACCCCAACATAT |
| ZONE 4.3 Fw | ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGAGTTTTTAGAAGGTTTTGTGTTTT |
| ZONE 4.3 Rv | TGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCTAACTTCAACCAACTCTATCC |

***Table 3.*** *Details of primers used for quantitative and traditional PCR (Table 3A), as well as methylation assays (Table 3B).*

## 3.4.2 Proteins

### 3.4.2.1 Western blot

Proteins from cell lines were extracted with SDS-DTT buffer, composed by 62.5 mM Tris-HCl, 2 % SDS, 20 % glycerol, 100 mM DTT and 0.005 % bromophenol blue. Cells were seeded in 6-well or 12-well plates and incubated at 37 ºC, 5 % $CO_2$ until 70-80 % of confluence. Then, proteins were extracted by using 200 µl pre-warmed SDS-DTT buffer and denaturalized by sonication during 10 s and boiling for 5 min at 95 ºC.

Proteins were separated by SDS-PAGE electrophoresis and transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5 % non-fat dry milk in Tris-buffered saline/0.05 % Tween-20 and incubated with the specific antibodies overnight at 4 ºC, followed by 1 h of incubation with the appropriate secondary antibodies (detailed antibodies are represented in **Table 4**, including the source, the reference, the dilution and the technique they were used for). Finally, proteins were detected using an enhanced chemiluminescence detection system (GE Healthcare, Madrid, Spain), following manufacturer's instructions, with dyed molecular weight markers. A densitometry analysis of the bands obtained was carried out

with ImageJ software. For normalization, we used a housekeeping protein, the Ponceau's red or total protein of the corresponding phosphorylated protein.

### 3.4.2.2 Immunohistochemistry

Immunohistochemical staining was performed to study the protein expression levels of NOVA1 in FFPE panNETs samples, using standard procedures [16, 154], including a heat-induced antigen retrieval step. Tissue sections were deparaffinized using xylol and a series of less concentrated ethanol solutions, and then treated with 10 mM sodium citrate pH 6 for 10 min in a microwave. Then, they were incubated with primary antibody overnight at 4 ºC, followed by incubation with the appropriate peroxidase-conjugated secondary antibody (antibody descriptions are included in **Table 4**). Finally, staining was developed with 3,39-diaminobenzidine (Envision system 2-Kit Solution DAB) and counterstained with hematoxylin. Negative controls of both primary and secondary antibodies were used in parallel, omitting them in separate samples. Particularly, NOVA1 protein expression was assessed in the total of 20 FFPE panNETs samples, including tumoral and non-tumoral adjacent tissue. An expert pathologist carried out a histopathologic analysis of the sections, following a blinded protocol, indicating +, ++ and +++ as low, moderate and high staining intensity of both tumoral and non-tumoral adjacent tissue.

| Antibody | Reference | Source | Technique | Dilution |
|---|---|---|---|---|
| Rabbit anti-human ERK1/2 | sc-154 | Santa Cruz Biotech | Western | 1:1000 |
| Rabbit anti-human p-ERK1/2 | #4370S | Cell Signaling | Western | 1:1000 |
| Rabbit anti-human AKT | #9272S | Cell Signaling | Western | 1:1000 |
| Rabbit anti-human p-AKT | #4060S | Cell Signaling | Western | 1:1000 |
| Rabbit Anti-human AR | ab133273 | Abcam | Western | 1:1000 |
| Rabbit Anti-human p-AR | ab71948 | Abcam | Western | 1:1000 |
| Rabbit Anti-human JNK | AF1387 | R&D systems | Western | 1:1000 |
| Rabbit Anti-human p-JNK | AF1205 | R&D systems | Western | 1:1000 |
| Rabbit Anti-human p-PDK1 | #3061 | Cell Signaling | Western | 1:1000 |
| Rabbit Anti-human p-PTEN | #9551 | Cell Signaling | Western | 1:1000 |
| Rabbit Anti-human p-p53 | #9284 | Cell Signaling | Western | 1:1000 |
| Rabbit Anti-human ATRX | HPA001906 | Sigma Aldrich | Western | 1:1000 |
| Rabbit Anti-human DAXX | HPA008736 | Sigma Aldrich | Western | 1:1000 |
| Goat Anti-human NOVA1 | #PA5-18895 | Thermo Fisher | Western/IHC | 1:1000/250 |
| Goat anti-rabbit IgG HRP-linked | #7074s | Cell Signaling | Western | 1:2000 |
| Rabbit anti-goat IgG HRP-linked | #31753 | Thermo Fisher | Western/IHC | 1:2000/500 |

***Table 4.*** *Details of antibodies used for the different protein experimental assays.*

#### 3.4.2.3 Evaluation of PSA secretion by ELISA

PSA secretion by 22Rv1 cell line was measured after treatment with the $SST_1$ agonist, BIM-23926. Briefly, cells were seeded in 12-well plates until 70-80 % of confluence. Then, they were FBS starved for 1 h and incubated with treatment (BIM-23926 at 1 µM or vehicle as control) for 24 h in absence of FBS. After that, media were collected and stored at -20 °C until PSA measurement, using commercial ELISA (#RAB0331, Sigma-Aldrich), following manufacturer's instructions. All the information about the assay can be accessed at the company website.

## 3.5 Bioinformatic analyses

### 3.5.1 *In silico* studies of *SSTR1* and possible regulatory miRNAs

Prediction of miRNAs that could regulate *SSTR1* expression was assessed *in silico* with three different software packages, freely available, including TargetScan, miRanda and DIANA. We followed several criteria in order to choose candidate miRNAs for further studies: I) predicted to bind the 3´UTR region in conserved sites among species; II) miRNA appears in, at least, two different software packages; III) number of poorly conserved sites among species; IV) good score in the different software packages (Total Context score and Aggregate PCT in Target Scan, mirSVR score and PhastCons score in Miranda, miTG score in Diana); and V) miRNA already published showing functional effect (http://www.mirbase.org/). In order to know and analyze the normalized expression levels of *SSTR1* and previously selected miRNAs in publicly available PCa cohorts, processed RNA-seq data from The Cancer Genome Atlas (TCGA, https://gdc-portal.nci.nih.gov/) and Memorial Sloan Kettering Cancer Center (MSKCC, https://www.mskcc.org/) were compiled. Using those data, we performed comparisons between PCa and control samples and correlations in tumoral samples with available clinical data.

### 3.5.2 Study of *SSTR5* gene context *in silico*

We performed an *in silico* study of the *SSTR5* gene through the USCS Genome Browser website (https://genome.ucsc.edu). We were looking for possible regulatory elements that may control *SSTR5* transcription and splicing, including NATs, CpG islands, histone markers and previous data that could help to further explore those findings, such as massive sequencing or probes in cell lines. The information obtained revealed the existence of an overlapping gene or NAT, *SSTR5-AS1*, which encodes a long

intergenic non-coding RNA. As well, there are 4 CpG-rich regions or CpG islands along both genes. Finally, there are several zones with histone markers, typically associated to promoter and/or enhancer sequences. Although all those findings could regulate *SSTR5* expression, we focused on NAT and CpG islands for further studies, as described above.

## 3.6 Statistical analysis

First, data were evaluated for parametric distribution with Kolmogorov–Smirnov test and were expressed as mean ± SEM (Standard Error of Mean). Statistical comparisons between groups were performed by unpaired parametric t-test and non-parametric Mann-Whitney U test, according to normality of included groups. Multiple comparisons of more than two groups were performed for analysis of variance (one-way ANOVA) followed by Dunnett's test. Pearson's or Spearman's bivariate correlations were performed for quantitative variables, in case they were parametric or not, respectively. The Receiver operating characteristic (ROC) curves were used to evaluate the suitability of genes to distinguish different groups of samples. Additionally, some analyses were performed in order to check the ability of several factors for distinguish between tumoral and non-tumoral samples. Random forest and simple logistic regression analyses were carried out with R language and followed by cross-validation in order to select a group of factors with a good ability to make clusters with samples. The heat map, principal component analysis and VIP score were performed through Metaboanalyst software (https://www.metaboanalyst.ca; McGill University, Montreal, Canada).

*In vitro* experiments were performed, at least, three times, as separate and independent experiments, carried out in different days and with different cell preparations. Statistical analyses were assessed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) and correlations were carried out using SPSS 22 (IBM SPSS Statistics Inc., Chicago, IL, USA). The p-values were two-sided and statistical significance was considered when $p < 0.05$, data is presented making specification for $p < 0.05$, $p < 0.01$ and $p < 0.001$.

# 4. RESULTS

# 4. Results

The aim of this Doctoral Thesis is to determine the role of somatostatin receptors and splicing machinery in different types of endocrine-related cancers and neuroendocrine tumors, and to explore their underlying regulatory mechanisms, with the final purpose of discovering novel biomarkers and pharmacologic targets. To better present how this global aim was pursued and for the sake of clarity, results have been subdivided in three experimental sections that will be presented separately.

## 4.1 Experimental section I: Somatostatin receptor subtype 1 ($SST_1$) as a biomarker and therapeutic target in prostate cancer

The somatostatin-$SST_{1-5}$ system comprises a complex and pleiotropic regulatory axis, which provides a relevant source of useful biomarkers and therapeutic targets for different pathologies, including endocrine-related tumors, as it has been shown for NETs and, especially, PitNETs [14, 248]. In this context, $SST_2$ and $SST_5$ have been the most valued targets, to which different SSAs were developed; however, in the case of PCa, the clinical findings regarding SSTs and the use of SSAs have been largely disappointing [249, 250]. Nevertheless, previous studies have demonstrated that other SSTs (e.g., $SST_3$) could also represent useful targets to develop therapeutic alternatives in some pathologies [251, 252]. In line with this, it has been shown that $SST_1$ could also be a relevant molecule in determining the response to SSAs [253]. Bearing this idea in mind, we decided to assess the expression levels of *SSTR1* in PCa samples and to investigate its regulation, functional implications, and potential utility as biomarker and/or therapeutic target in this pathology.

### 4.1.1 Overexpression of *SSTR1* in PCa and its association with relevant markers

As a first step, we evaluated, by qPCR, the expression levels of *SSTR1* in a cohort of fresh samples from patients with PCa (n = 52) and control samples without tumoral features (n = 12), whose clinical and demographic characteristics are summarized above (**Table 1**; Material and Methods). Interestingly, results from qPCR showed that $SST_1$ was expressed in a higher proportion of PCa samples than in control tissues (91 % in PCa vs. 75 % in non-tumoral samples), and also, that expression levels were markedly higher in PCa samples than in controls (**Figure R1**).

This initial analysis already suggested that $SST_1$ could be a valuable molecule in PCa, in that receptor overexpression would support its use as a putative treatment target. On the other hand, its potential as a biomarker in PCa required additional investigation.

Thus, we performed a ROC curve analysis showing that *SSTR1* expression was able to significantly (p = 0.048) discriminate tumoral from control samples (AUC = 0.68; **Figure R1**). These results pointed *SSTR1* as a possible biomarker for PCa, although additional analysis with more numerous patient cohorts would be needed to corroborate it.

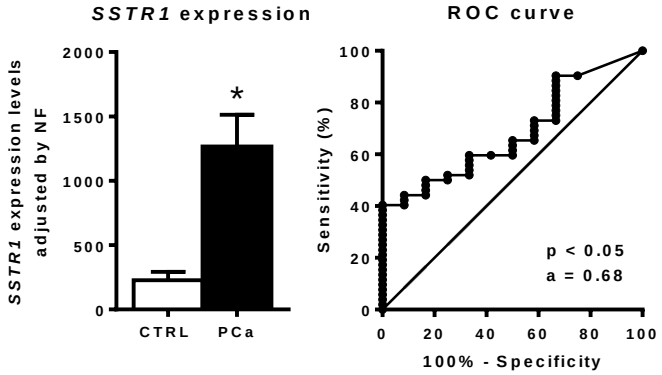


***Figure R1.*** *SSTR1 is overexpressed in PCa and can discriminate tumor vs. non-tumor tissue.* *Left panel:* Comparison of *SSTR1* expression in PCa biopsy samples compared to non-tumor control samples, adjusted by normalization factor (NF), calculated from the expression levels of three housekeeping genes (*ACTB*, *GAPDH* and *HPRT*). *Right panel:* Receiver operating characteristic (ROC) curve analysis, performed with the expression levels of *SSTR1* to determine the ability of its expression to discriminate between PCa patients and controls. Asterisk (*, $p < 0.05$) indicates values that significantly differ from controls. Data represent mean ± SEM.

To further examine the potential importance of $SST_1$ in PCa patients, we analyzed in more detail the clinical features recorded in the database of this patient cohort. Interestingly, this revealed that *SSTR1* expression correlated directly and significantly with *AR* expression in PCa patients, whereas, in clear contrast, mRNA levels of these two molecules did not correlate in control samples (**Figure R2**). This observation is important because *AR* is a highly relevant molecule in PCa, where it is overexpressed in pathological conditions, as it also occurs in our cohort (**Figure R2**).

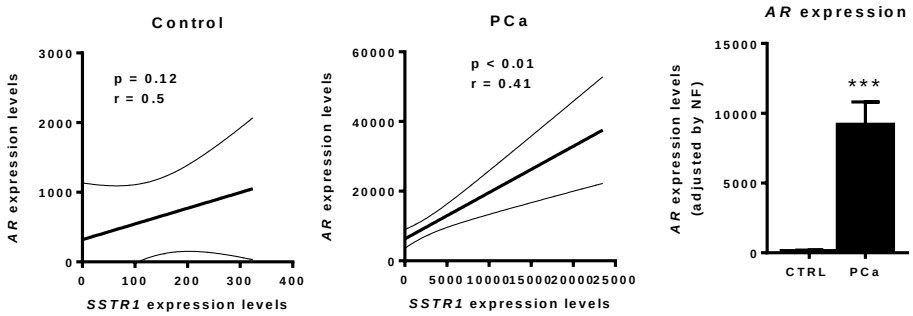


***Figure R2.*** SSTR1 *is associated with* AR *in PCa.*
Correlations between *SSTR1* and *AR* expression in tissues from control individuals (left panel) and PCa patients (middle panel). Comparison of *AR* mRNA levels between PCa and control samples (right panel). Asterisks (***, $p < 0.001$) indicate values that significantly differ between groups. Data represent mean ± SEM.

### 4.1.2 $SST_1$ exerts a functional role in PCa cell lines

The intense research activity in the field of PCa over the last decades has facilitated the development of a number of cell models amenable for experimental and preclinical studies. For that reason, and in order to select the best cell models to explore in detail the functional role of $SST_1$ and the regulation of the *SSTR1* gene in PCa, we first screened the expression of this receptor in a panel of PCa cell lines previously used in our laboratory (**Figure R3**).

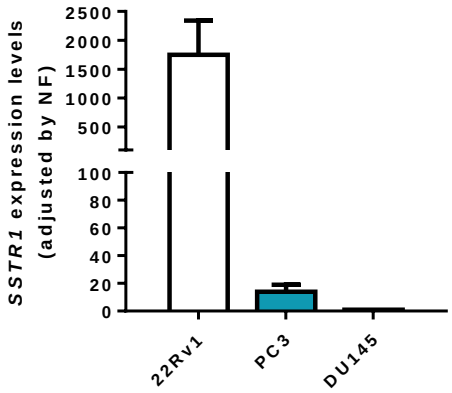


***Figure R3.*** SSTR1 *expression levels in various PCa model cell lines.*
Levels of *SSTR1* gene expression were measured by qPCR in three PCa model cell lines, including the androgen-sensitive cell line 22Rv1 and the androgen-independent cell lines PC3 and DU145. Data are expressed in normalized copy number and are mean ± SEM of 3 independent cell passages.

Results showed that the cell line with the highest expression was the androgen sensitive 22Rv1, with 1751 mRNA copies (adjusted by NF), which was considerably higher than PC3 and DU145 cell lines, that exhibited 14 and 1 mRNA copies (adjusted by NF), respectively. Additionally, the mRNA levels of *SSTR1* found in 22Rv1 cell lines

were closely similar to those found in fresh PCa samples. For those reasons, we selected 22Rv1 as the main model to further explore the functional role of *SSTR1* in PCa.

Having selected 22Rv1 as model cell line, our next aim was to assess the functional impact of $SST_1$ on key tumoral features. Specifically, we first measured the proliferation capacity of these cells in response to treatment with an $SST_1$ specific agonist, BIM-23926, at two different concentrations, 1 µM and 10 nM, during 72 h. Additionally, PSA secretion by this cell line was measured after 24 h of agonist treatment at 1 µM. Interestingly, treatment with 1 µM, but not 10 nM, of this $SST_1$ agonist significantly decreased cell proliferation at 48 h and 72 h, compared with their non-treated controls (**Figure R4A**). Moreover, this same concentration promoted a reduction in PSA secretion after 24 h (**Figure R4B**).

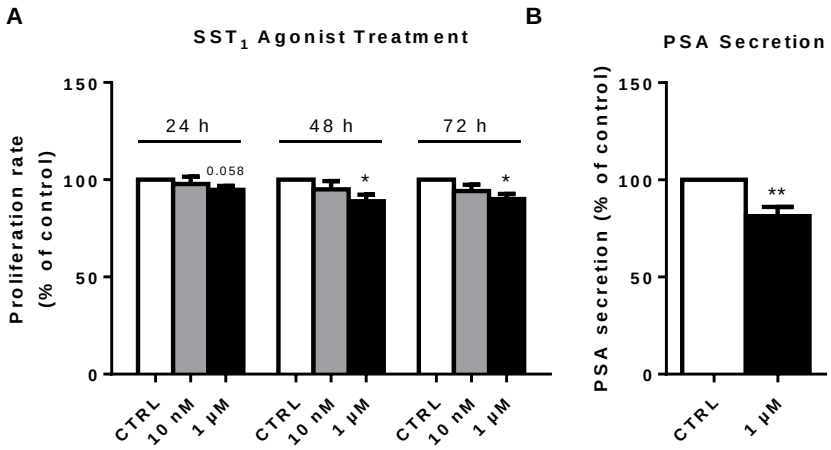


***Figure R4.*** *Treatment with the $SST_1$ agonist BIM-23926 inhibits cell proliferation and PSA secretion in 22Rv1 cells.*
**A**. Proliferation rate of 22Rv1 cells measured after 24, 48 and 72 h treatment with the $SST_1$ agonist BIM-23926, at two different concentrations, 10 nM and 1 µM, compared with the corresponding non-treated controls. **B**. PSA secretion from 22Rv1 cells after 24 h of treatment with 1 µM of the $SST_1$ agonist BIM-23926. Asterisks (*, $p < 0.05$; **, $p < 0.01$) indicate values that significantly differ from controls. In all cases, data are expressed as percentage of control and represent mean ± SEM of at least 3 independent experiments.

To further confirm and explore this initial evidence for a possible functional role of $SST_1$ in PCa cells, the expression of *SSTR1* gene was genetically modified in the 22Rv1 cell line. Surprisingly, stable overexpression of *SSTR1* with a specific pCDNA3.1 plasmid, without any additional exogenous treatment, cause a marked decrease in the proliferation rate of these cells over the 48 and 72 h after the seeding (**Figure R5A**), compared to cells transfected with a mock plasmid. In striking contrast, genetic silencing with a specific siRNA promoted a clear increase in cell proliferation at 24, 48 and 72 h (**Figure R5B**).

Both transfections were adequately validated as depicted in **Figure R5**. These results support and expand those obtained with the $SST_1$ agonist and provide further evidence that this receptor could have an antitumoral role in the PCa model cell line 22Rv1, which could potentially involve a relevant degree of constitutive activity for $SST_1$ in this cell line.

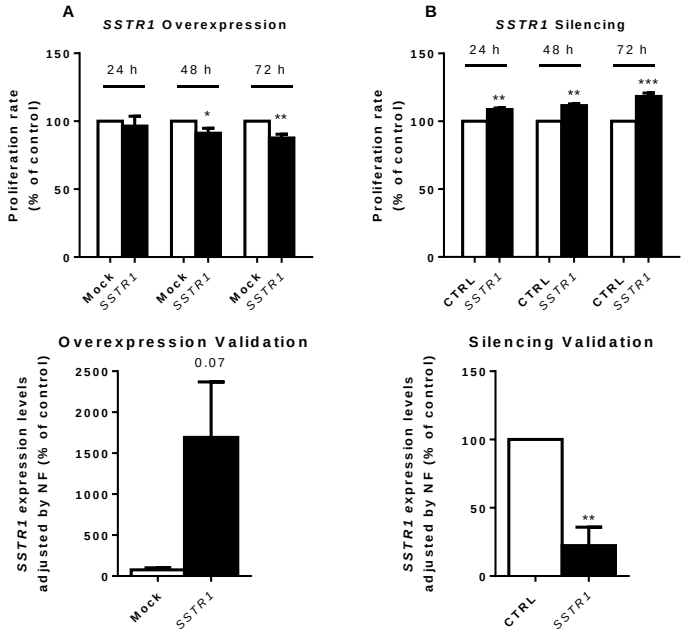


***Figure R5.*** *Altering* SSTR1 *expression modifies proliferation rate of 22Rv1 PCa cell line.*
Cell proliferation rate of 22Rv1 line after (**A**) *SSTR1* stable overexpression and (**B**) *SSTR1* silencing, compared with their respective controls, mock plasmid and scramble siRNA, at 24, 48 and 72 h, expressed as percentage of control. Transfection was validated in parallel and it is presented in the corresponding lower panel. Asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) indicate values that significantly differ from controls. In all cases, data represent mean ± SEM of $n \geq 3$ independent experiments.

### 4.1.3 $SST_1$ alters important signaling pathways in PCa cells

To better understand the mechanisms underlying the effects caused by ligand-induced $SST_1$ activation and *SSTR1* expression modifications in 22Rv1 cells, we next investigated the status of key signaling pathways for PCa. First, the concentration of free cytosolic calcium ($[Ca^{2+}]_i$), an important second messenger typically linked to SSTs inhibition of hormone release [254], was measured in response to treatment with the $SST_1$ agonist BIM-23926 at 1 µM and 10 nM in 22Rv1 cells. This revealed that only a low proportion of 22Rv1 cells responded to $SST_1$ agonist (**Figure R6**) and, moreover, that they showed a very limited response, thus suggesting that $SST_1$ signaling would not be primarily mediated through calcium as second messenger in PCa cells.

| $[Ca^{2+}]_i$ | *n* | PRC (%) | PMR (%) |
|---|---|---|---|
| **$SST_1$ Agonist 1 µM** | 0/4 | | |
| **$SST_1$ Agonist 10 nM** | 1/4 | 20 | 118 ± 5.4 |

***Figure R6.*** *Free cytosolic calcium concentration $[Ca^{2+}]_i$ kinetics in response to $SST_1$ agonist.*
Results from the $[Ca^{2+}]_i$ kinetics assay after treatment with $SST_1$ agonist at two different concentrations (1 µM and 10 nM); n, experiments with responsive cells/total experiments analyzed; PRC, proportion of responsive cells (considering the total number of cells analyzed in which a response was observed); PMR, percentage of maximum response.

We next focused on activation of key proteins from core cancer signaling pathways, such as AKT, ERK or JNK, and more specifically with PCa, like AR. To this end, we evaluated changes in their phosphorylation in response to 1 µM of the $SST_1$ agonist BIM-23926 at three different times, as determined by western blot (**Figure R7**).

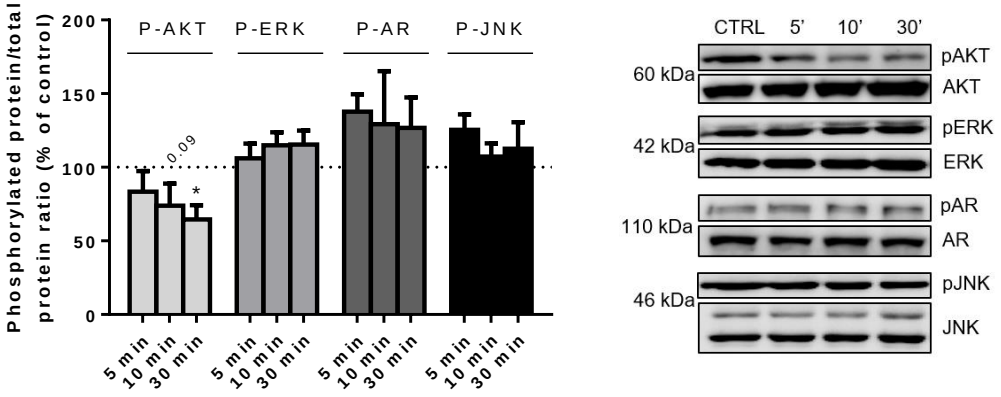


***Figure R7.*** *Downstream activation of signaling protein after $SST_1$ activation.*
Levels of phosphorylation of key proteins of core cancer signaling pathways, including AKT, ERK, AR and JNK, under BIM-23926 $SST_1$ agonist treatment (1 µM) at 5, 10 and 30 min, compared with non-treated control. Protein activation was normalized with the respective total protein in each case and expressed as percentage of control. Asterisks (*, $p < 0.05$) indicate values that significantly differ from controls. Data represent mean ± SEM of $n \geq 3$ independent experiments.

Results from western blot revealed that $SST_1$ agonist treatment decreased AKT phosphorylation at 30 min after treatment, whereas no similar changes were observed in the levels of phosphorylated ERK, AR or JNK. These results suggest that $SST_1$ operates through AKT, but not the other pathways assessed, to exert its functions in 22Rv1 cells.

We further evaluated the potential links between $SST_1$ activation and molecular targets typically altered in tumoral pathologies and, especially, in PCa, which could ultimately mediate the actions of this receptor, as, for example, transcription factors or cell cycle modulators. Specifically, we measured mRNA levels of *KLK3* (the gene than

encodes PSA protein), cyclin *CCND3*, the widely known *TP53*, and *SSTR1* itself, by qPCR, after 24 h of treatment with the $SST_1$ agonist BIM-23926 (**Figure R8**).

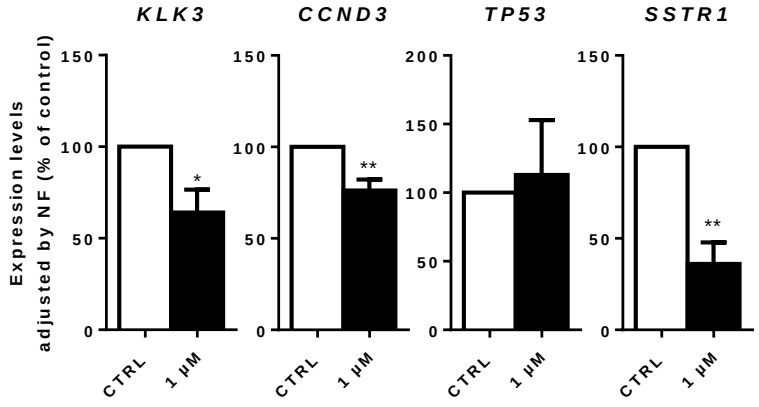


***Figure R8.*** *Levels of expression of key intracellular functional markers after $SST_1$ activation.* Levels of mRNA for *KLK3*, *CCND3*, *TP53* and *SSTR1* genes after 24 h of $SST_1$ agonist treatment at 1 µM, were measured by qPCR and compared with non-treated control. The mRNA levels were adjusted by normalization factor (NF) calculated from the expression levels of three housekeeping genes (*ACTB*, *GAPDH* and *HPRT*) and expressed as percentage of control. Asterisks (*, $p < 0.05$; **, $p < 0.01$) indicate values that significantly differ from controls. Data represent mean ± SEM of $n \geq 3$ independent experiments.

Interestingly, treatment with the $SST_1$ agonist BIM-23926 decreased expression of *KLK3* and *CCND3*, which agrees with and further support our previous finding, in that the inhibition of PSA secretion found earlier is accompanied by a downregulation of its coding gene, while the BIM-23926-induced decrease in proliferation is paralleled by a reduction in the expression of a key cyclin, *CCND3*, which regulates cell cycle. On the other hand, *TP53* expression was not altered by BIM-23926 treatment, suggesting that this receptor may not require this molecule to exert its actions. Of note, we also observed that treatment with the $SST_1$ agonist downregulated the expression of the *SSTR1* gene itself, implying that this receptor is self-regulated by a negative feedback when it is ligand-activated.

To explore in more detail the mechanisms underlying $SST_1$ effects and to focus our analysis more precisely in PCa, we carried out a PCR Array of Human Androgen Receptor Signaling Pathways, which is based on the measurement of mRNA levels for an ample but selected set of key genes involved in AR signaling and, thus, in PCa pathogenesis and aggressiveness. The array was measured in mRNA samples from 22Rv1 cells stably transfected with *SSTR1* plasmid, compared to those transfected with mock

plasmid. Results obtained showed that overexpression of *SSTR1* altered the expression of several genes, mainly related with tumoral progression (**Figure R9**).

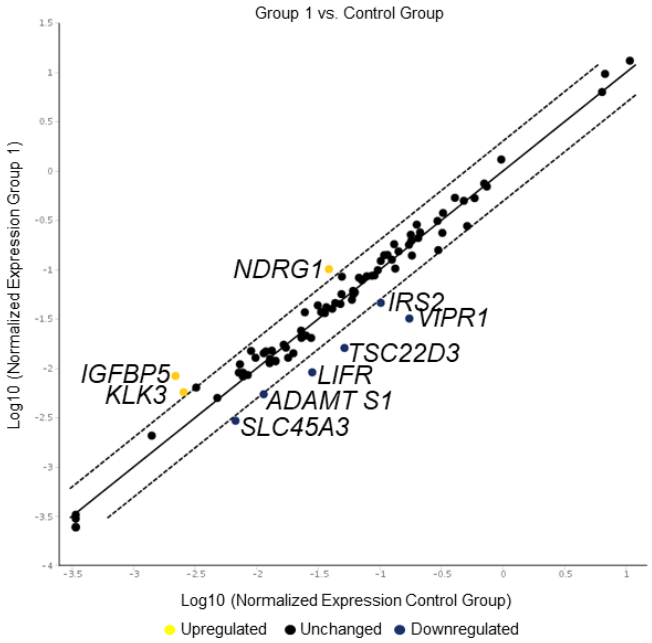


***Figure R9.*** *Effect of* SSTR1 *overexpression on AR-related Signaling Pathways in 22Rv1 cells.* A PCR Array of Human Androgen Receptor Signaling Pathways was measured in mRNA derived from 22Rv1 cells stably-transfected with *SSTR1* or mock plasmids. Scatter plot represents the changes observed between the respective mRNA levels when differences were over 2-fold. Upregulated genes are represented in yellow at the top of the image and downregulated genes at the bottom, in blue.

Specifically, we considered relevant those changes where genes exhibited differences higher than 2-fold. As shown in **Figure R9,** the genes overexpressed more than 2-fold between *SSTR1* and mock transfections were *IGFBP5*, *KLK3* and *NDRG1*, and are represented in yellow. In contrast, the downregulated genes included *ADAMTS1*, *IRS2*, *VIPR1*, *SLC45A3*, *LIFR* and *TSC22D*. However, since this PCR array did not enable to establish comparisons based on a statistical analysis, we decided to validate those results with a separate qPCR. This confirmed the upregulation of *IGFBP5* and the downregulation of *ADAMTS1*, *IRS2*, *VIPR1*, *SLC45A3* and *LIFR* (**Figure R10**).

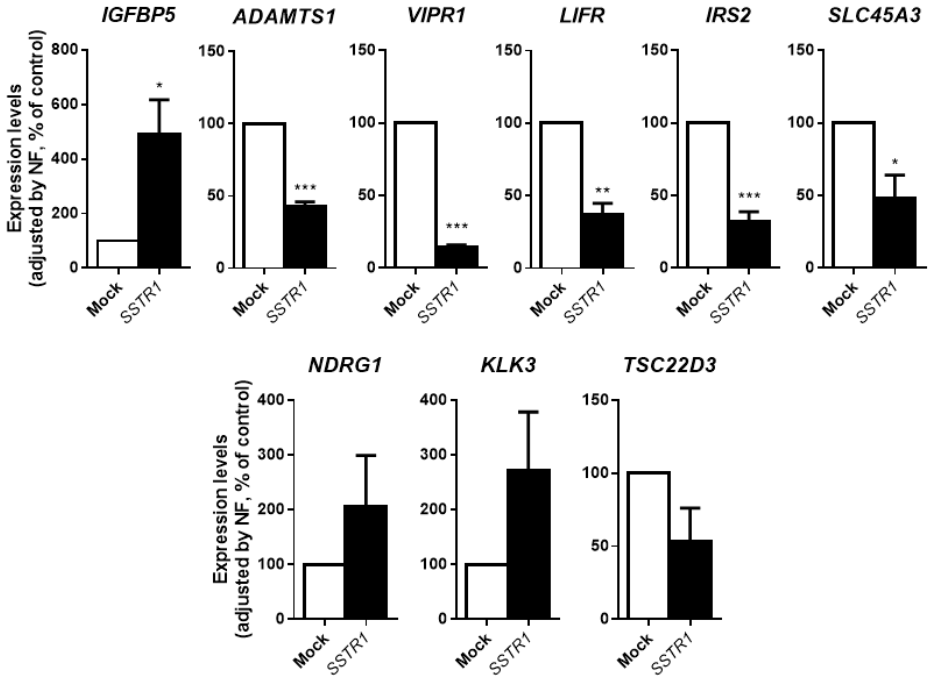


***Figure R10.*** *Validation by qPCR of changes found in the AR Signaling Pathways PCR Array.* Validation of alterations in genes found in the array was performed by qPCR. Absolute mRNA levels were adjusted by normalization factor (NF), calculated from the expression levels of three housekeeping genes (*ACTB, GAPDH* and *HPRT*). Asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) indicate values that significantly differ from controls. Data are presented as percentage of control and represent mean ± SEM of $n \geq 3$ independent experiments.

### 4.1.3 *SSTR1* expression in PCa can be regulated by microRNAs

In addition to examining the signaling pathways altered by *SSTR1* presence and those involved in mediating ligand-activated $SST_1$ actions, we interrogated the mechanisms that may be involved in regulating the expression of *SSTR1* itself in PCa, which may help to understand its overexpression in this pathology. To achieve this, we performed several *in silico* and *in vitro* assays aimed to identify possible miRNAs that may target *SSTR1* mRNA and regulate its expression. A first *in silico* approach revealed a number of miRNAs that could potentially recognize and bind the *SSTR1* mRNA, especially in the 3'UTR, with a high affinity (**Table 3**).

| | TARGETSCAN | | | | | | | | | | MIRANDA | | DIANA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Conserved sites | | | | Poorly conserved sites | | | | Total Cont. | Aggregate | mirSVR score | Phast score | miTG score |
| **miRNA vertebrates** | Total | 8mer | 7mer-m8 | 7mer-1A | Total | 8mer | 7mer-m8 | 7mer-1A | score | $P_{CT}$ | | | |
| miR-24/24ab/24-3p | 1 | 1 | 0 | 0 | 2 | 1 | 1 | 0 | -0.66 | 0.54 | -0.2766 | 0.5843 | 0.81 |
| miR-375 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | -0.1 | 0.38 | -0.1454 | 0.6823 | - |
| miR-27abc/27a-3p | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | -0.4 | < 0.1 | -1,147 | 0.6819 | 0.938 |
| miR-338/338-3p | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 0 | -0.2 | 0.22 | -0.4778 | 0.6259 | - |
| miR-383 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | -0.17 | 0.11 | -0.2704 | 0.5476 | - |
| miR-128/128ab | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | -0.14 | < 0.1 | -0.2057 | 0.6819 | - |
| miR-133abc | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | -0.2 | 0.19 | -0.3439 | 0.6259 | |
| **miRNA mammals** | | | | | | | | | | | | | |
| miR-653 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | -0.19 | < 0.1 | -0.7387 | 0.6823 | - |
| miR-326/330/330-5p | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | -0.12 | < 0.1 | -0.1452 | 0.6538 | - |
| miR-382 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | -0.11 | < 0.1 | -0.6519 | 0.6823 | 0.756 |
| miR-495/1192 | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 0 | -0.07 | < 0.1 | 0.5163 | 0.5476 | 0.911 |
| miR-488 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | -0.03 | < 0.1 | -0.1227 | 0.7118 | - |
| miR-335/335-5p | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | -0.02 | < 0.1 | -0.777 | 0.5595 | - |

***Table 5.*** *Prediction of potential miRNAs that could bind* SSTR1 *mRNA and regulate its expression.* Classification of miRNA that could regulate expression of *SSTR1*, based in three different software programs (packages): TargetScan, miRanda and DIANA. The miRNAs that were further *in silico* correlated with *SSTR1* mRNA expression are marked in gray. The upper situation in the table means a higher possibility of regulating *SSTR1* mRNA expression.

To reduce and refine the high number of hits obtained in this analysis, which could not be studied in full, we performed a further *in silico* screening of PCa samples data included in the publicly accessible database The Cancer Genome Atlas (TCGA). Quantitative correlations between the expression of *SSTR1* and that of the different miRNA revealed that only four of them (miR-24, miR-27b, miR-383, miR-488) were negatively and significantly correlated with *SSTR1* expression (**Figure R11**).

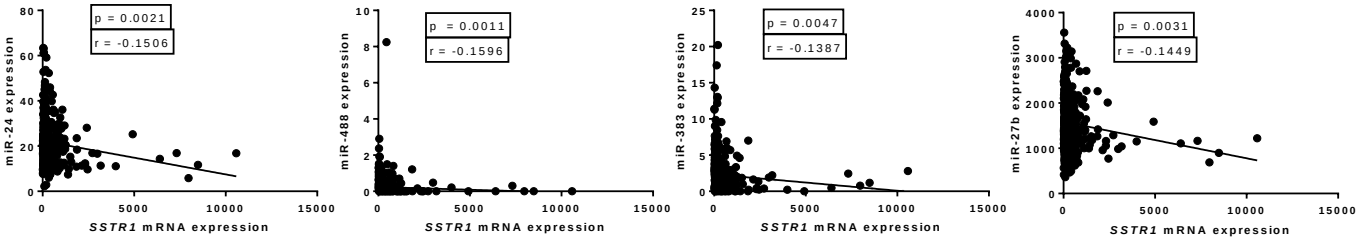


***Figure R11.*** *Correlations between miRNAs and* SSTR1 *expression levels in PCa public cohort.* Correlations between SSTR1 mRNA expression levels and expression of 4 different miRNAs (miR-24, miR-488, miR-383 and miR-27b) in PCa patients, from TCGA database.

When those miRNAs were transfected using mimics in 22Rv1 cells, only one of them, miR-24, was able to significantly decrease *SSTR1* expression, as it is shown in **Figure R12,** at both mRNA and protein levels.

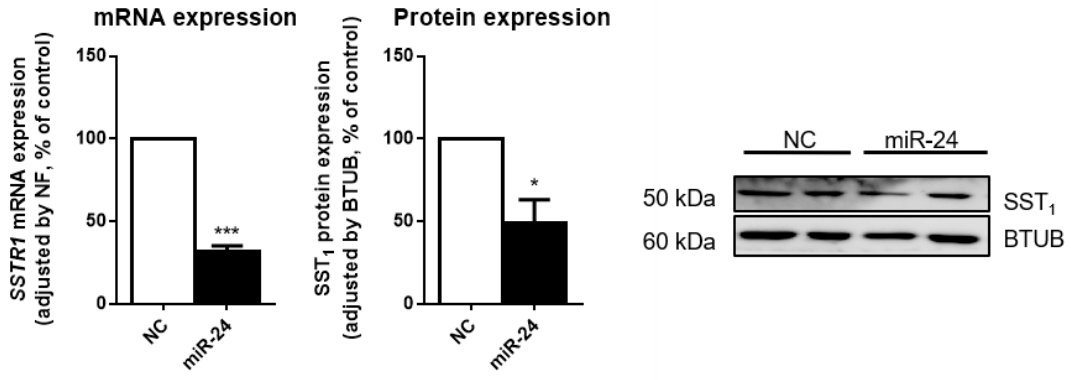


***Figure R12.*** *Changes in* SSTR1 *expression and $SST_1$ protein levels after miR-24 transfection.* The mRNA levels of *SSTR1* (left panel) and those of the $SST_1$ protein, (center and right panels), were reduced in 22Rv1 PCa cell line after transfection with miR-24, as compared with the corresponding negative controls (NC). Absolute mRNA levels were adjusted by normalization factor (NF), calculated from the expression levels of three housekeeping genes (*ACTB*, *GAPDH* and *HPRT*). Protein level was normalized by ß-tubulin (BTUB). Asterisks (*, $p < 0.05$; ***, $p < 0.001$) indicate values that significantly differ from controls. Data are expressed as percentage of control and represent mean ± SEM of $n \geq 3$ independent experiments.

An additional, more detailed analyses was then implemented *in silico* by using another PCa cohort available from the Memorial Sloan Kettering Cancer Center (MSKCC) dataset. Interestingly, we also found that miR-24 was clearly downregulated in PCa samples, compared with normal prostate (**Figure R13**). Moreover, the expression of this miRNA was lower in metastatic samples compared with primary tumor and normal prostate tissue. Of note, the pattern found in the expression of miR-24 was completely inverse to that from *SSTR1* expression, which was overexpressed in primary PCa samples and was expressed at even higher levels in metastatic tissue. These results agree with and support the data obtained from *in vitro* assays and further reinforce the idea that miR-24 can be a relevant player within the mechanisms regulating *SSTR1* expression in PCa.

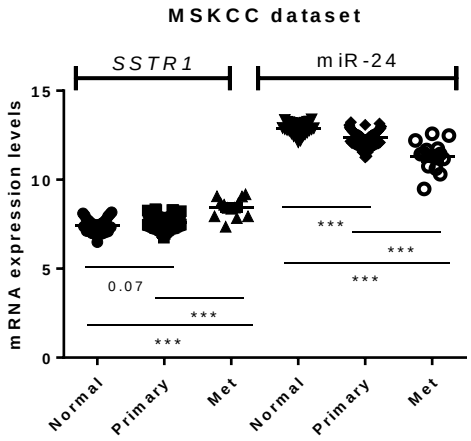


***Figure R13.*** *Expression of* SSTR1 *and miR-24 in a PCa cohort from the MSKCC dataset.* Data on the expression of *SSTR1* (left) and miR-24 (right) in normal prostate, primary PCa and metastatic tissue were obtained from the MSKCC dataset and statistical differences were assessed. Asterisks (***, $p < 0.001$) indicate values that significantly differ between the indicated groups.

## 4.2 Experimental section II: Epigenetic and post-transcriptional regulation of somatostatin receptor subtype 5 ($SST_5$) in NETs

The pathophysiological relevance and clinical utility of the somatostatin-SSTs system is most prominent in NETs and, particularly, in PitNETs [14, 248]. Among the five SSTs, the main receptor in this context is undoubtedly $SST_2$, both because it is, overall, the SST with wider and higher expression in most NETs and also, for this same reason, because the most used and effective SSAs primarily target $SST_2$ [11]. However, over the last years $SST_5$ is emerging as a very attractive receptor in this field, in that it is also widely expressed in these tumors and, besides binding the first generation SSAs, octreotide and lanreotide, is the primary target for new generation SSA pasireotide. Nevertheless, the precise functioning and regulation of $SST_5$ is less well known than that of $SST_2$. Thus, whereas there is ample evidence that $SST_5$ primarily acts as an inhibitory receptor, other data suggest that it may play a more complex role [11]. In line with this, it has been described that $SST_5$ can be a biomarker in PitNETs, where $SST_2/SST_5$ balance and the presence of $SST_5$ truncated variant, $SST_5$TMD4, have been proposed as markers of SSAs responsiveness in somatotropinomas [11].

Yet, in spite of the growing interest in $SST_5$, the available knowledge on the mechanisms underlying the biogenesis of this receptor and the regulation of its expression is still limited. Interestingly, a recent report suggests that *SSTR5* expression may be

regulated through DNA methylation and by a NAT, called *SSTR5-AS1*, in laryngeal squamous cell carcinoma [151]; however, there is still no information about this processes in NETs. Accordingly, in this experimental section we aimed to improve our understanding of the mechanisms controlling *SSTR5* gene expression and $SST_5$ biogenesis in PanNETs and PitNETs.

### 4.2.1 DNA methylation and natural antisense transcript (NAT) regulates *SSTR5* transcription in somatotropinomas

As an initial approach, we performed an *in silico* study of the structure of the *SSTR5* gene (**Figure R14**). The information obtained from the UCSC Genome Browser (version GRCh37/hg19) revealed the existence of an overlapping gene in humans, *SSTR5-AS1*, which encodes a long intergenic non-coding RNA, that could regulate *SSTR5* expression, as it has been shown for other NAT. Moreover, there are four CpG-rich regions or CpG islands, named heretofore as Zones 1-4, which are susceptible zones of methylation, along both genes, which could also regulate their expression.

Those CpG islands are located in sites of interest, for they could be important in the control of the expression of these genes. Thus, Zone 1 overlaps with the last exon of the NAT and Zone 2 falls on the big intron of NAT, two intergenic zones that have been related to expression regulation previously. Zone 3 coincides with the first exon of the *SSTR5* gene, partially overlapping with its promoter, and also with another part of the larger intron of the *SSTR5-AS1*. Finally, Zone 4 was the largest region identified ans was subdivided into three subzones for the purpose of the study: Zone 4.1 overlaps with the start of the NAT, possibly with its promoter, and the intron of *SSTR5*; Zone 4.2 falls in the exon of *SSTR5* and coincides with the CDS of the canonical $SST_5$; Zone 4.3 overlaps with the center of the big exon of *SSTR5* gene, including its zone of alternative splicing, and the zone immediately previous to the *SSTR5-AS1* gene.

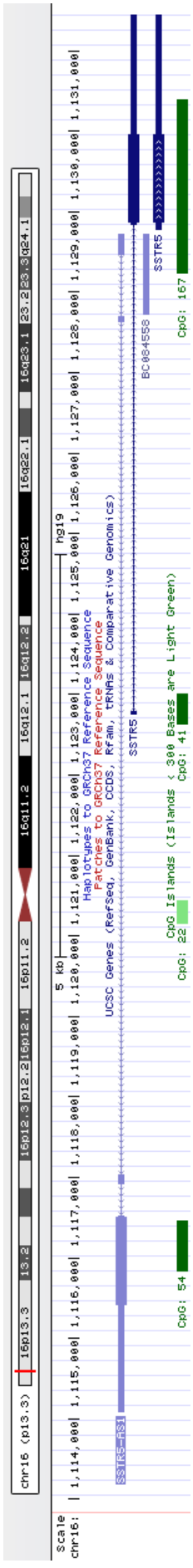


***Figure R14.*** *USCS Genome Browser representation* SSTR5 *locus.*
The picture represents the genomic situation of *SSTR5* gene and its NAT, *SSTR5-AS1*. Green boxes represent CpG islands, renamed as Zones in this study.

In the first experimental assay, we evaluated the RNA expression levels of the two genes of interest, *SSTR5* and *SSTR5-AS1*, in a cohort of 11 normal pituitary (NP) samples and 27 samples of somatotropinoma tumors causing acromegaly (**Figure R15**).

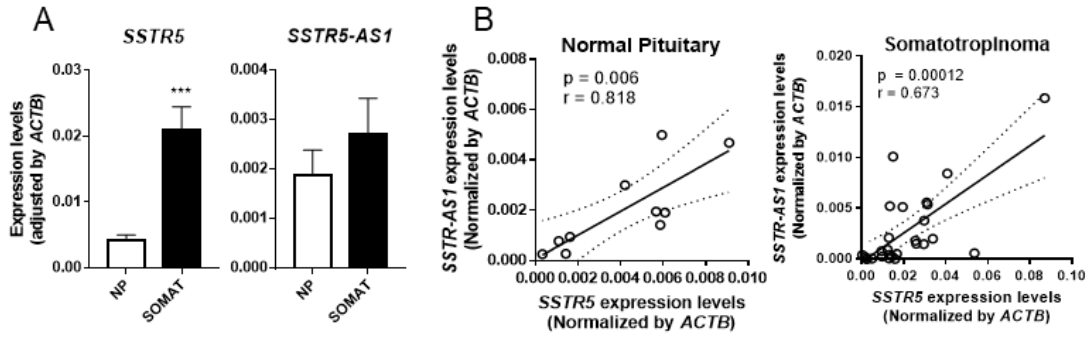


***Figure R15****. Relationship between expression levels of* SSTR5-AS1 *and* SSTR5 *in somatotropinomas.*
Expression levels of *SSTR5* and *SSTR5-AS1* (A) and correlations between (B) them in somatotropinomas (SOMAT) and NP, normalized by *ACTB* and measured by qPCR. Asterisks (***, $p < 0.001$) indicate values that significantly differ from control. Data represent mean ± SEM.

Interestingly, *SSTR5* was clearly overexpressed in somatotropinoma samples compared to normal pituitary tissue, whereas expression levels of *SSTR5-AS1* gene showed a similar trend but did not exhibit a significant change (**Figure R15A**). On the other hand, it is worth noticing that the expression of both genes showed a clear direct correlation in both NP and somatotropinoma samples (**Figure R15B**), which could suggest a putative functional association between these two genes.

As a next step, we measured the methylation levels of these four zones in the same cohorts of somatotropinoma and NP samples. The results showed that three of the zones examined exhibit significantly distinct levels in somatotropinoma and NP samples (**Figure R16**).

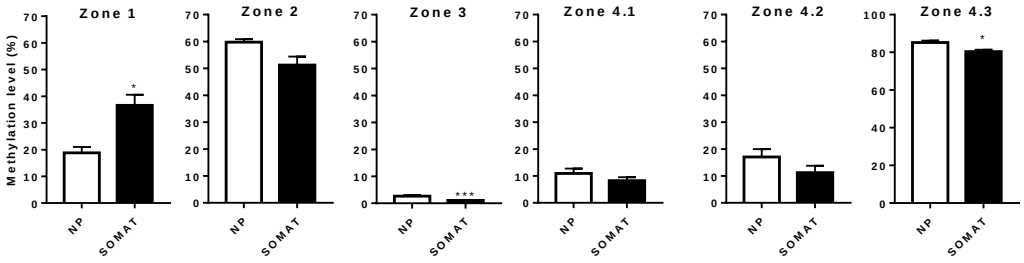


***Figure R16****. Methylation levels of CpG islands from* SSTR5 *context.*
Comparison of methylation levels between somatotropinoma (SOMAT) and normal pituitary (NP) samples, expressed as percentage, of the CpG islands, renamed as zones, around *SSTR5* and its NAT genomic context. Asterisks (*, $p < 0.05$; ***, $p < 0.001$) indicate values that significantly differ from control. Data represent mean ± SEM.

Specifically, Zone 1 was more intensely methylated in somatotropinomas (almost double) than in NP. In contrast, Zone 3, which exhibited very low levels of methylation in all the samples, exhibited a lower degree of methylation in somatotropinoma than in NP samples. In Zones 4.1 and 4.2, methylation levels were low, but no significant differences were observed; whereas, in Zone 4.3 methylation levels were lower in somatotropinomas than in NP, but displayed very high levels in both cases. Similarly, Zone 2 showed high methylation levels, although no significant differences were observed between groups.

Next, we compared the expression of *SSTR5* and *SSTR5-AS1* genes with the methylation levels of the CpG islands overlapping them in the genome. Remarkably, we found that the expression of both genes was tightly and inversely correlated with methylation levels of Zone 4.3 (**Figure R17**) in somatotropinoma but not in NP samples, whereas they did not show any similar significant correlation with methylation levels of any of the other zones examined.

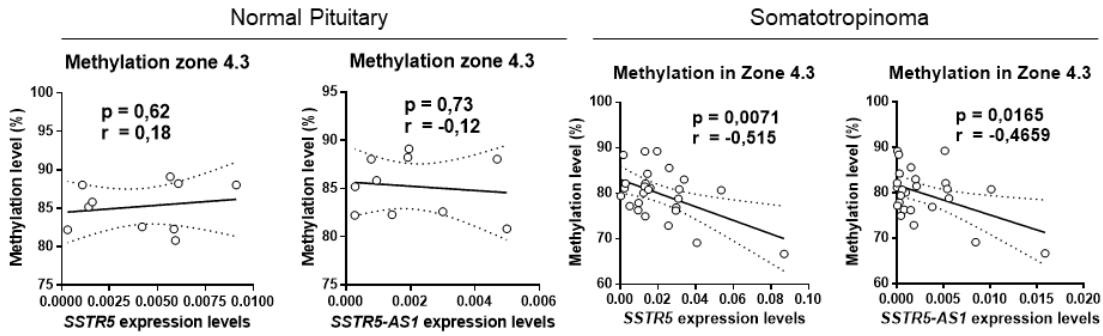


***Figure R17.*** *Correlations between gene expression levels of SSTR5 and SSTR5-AS1 and the DNA methylation levels*
Correlations between percentage of DNA methylation levels in Zone 4.3 and RNA expression levels of *SSTR5* and *SSTR5-AS1* in NP (left) and somatotropinoma (right) samples.

These results reveal that DNA methylation in Zone 4.3, which overlaps with the area of the big exon of *SSTR5* where non-canonical alternative splicing would take place and with the zone immediately previous to *SSTR5-AS1* gene, is related with the expression of these two genes, in a manner that might be related with the pathological context, as it was present in somatotropinomas but did not occur in healthy, normal pituitary.

### 4.2.2 Relationship between *SSTR5* and its NAT is also present in panNETs

In order to investigate whether the relationship between *SSTR5* and its NAT *SSTR5-AS1* is also present in other tumors where the somatostatin-SST system is important, we extended our study to PanNETs. To this end, we measured the expression of both genes in a cohort of 15 panNETs, comparing tumoral tissue with their paired non-tumoral adjacent tissue (NTAT), used as reference. Results from this analysis revealed that, while *SSTR5* expression did not differ between both regions, the levels of *SSTR5-AS1* mRNA were significantly higher in tumoral samples (**Figure R18A**). On the other hand, the expression levels of these genes were directly and strongly associated in both tumoral and non-tumoral tissue, reinforcing the idea of a functional link between them. Unfortunately, the methylation levels of these samples could not be measured due to the poor quality of the DNA from formalin fixed paraffin-embedded samples.

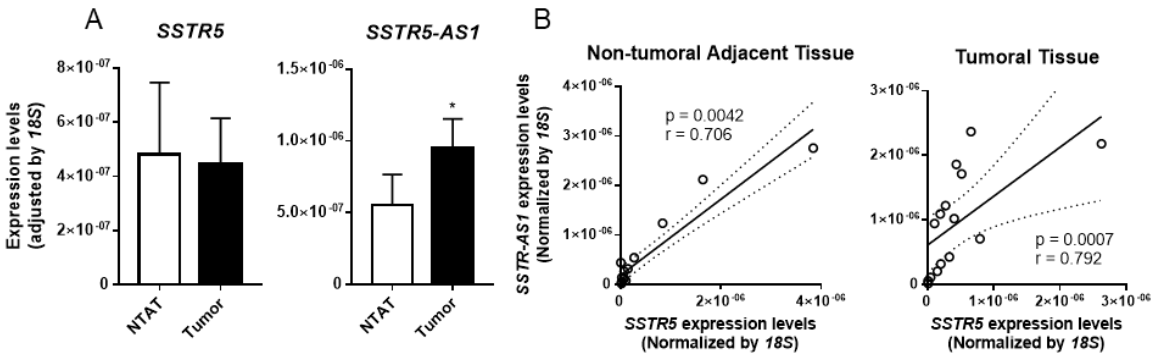


***Figure R18.*** *Relationship between the expression levels of SSTR5 and SSTR5-AS1 in PanNETs.* Expression levels of *SSTR5* and *SSTR5-AS1* (A) and correlations between (B) them in tumoral and paired non-tumoral adjacent tissue from 15 PanNET samples, measured by qPCR and normalized by *RNA18S1*. Asterisks (*, $p < 0.05$; ***, $p < 0.001$) indicate values that significantly differ from control. Data represent mean ± SEM.

### 4.2.3 *SSTR5-AS1* silencing decreases *SSTR5* expression and alters pasireotide effects

In an attempt to better understand the potential functional roles of *SSTR5-AS1* in NETs, we performed a stable silencing of *SSTR5-AS1* using a specific shRNA, which could help to decipher the possible link between this NAT and the *SSTR5* gene. For these and the ensuing assays, the PanNET model cell line BON-1 was used, also due to the lack of suitable human cell models for PitNETs. After silencing, cells were treated with pasireotide, a second generation SSA with high affinity for $SST_5$, in order to test if *SSTR5-AS1* may impact in the cell response to this treatment. Interestingly, the first observation was that *SSTR5-AS1* silencing by 30 %, concomitantly decreased *SSTR5* expression in BON-1 cells (**Figure R19A**).

Treatment with pasireotide (100 nM; 24 h) increased the expression levels of both *SSTR5* and *SSTR5-AS1*, suggesting the existence of a positive feedback regulatory mechanism linking $SST_5$ activation and the expression of this receptor, which may also involve NAT. Intriguingly, whereas silencing of *SSTR5-AS1* abrogated the stimulatory effect of pasireotide on the expression of this NAT, the same did not occur with *SSTR5*, in that pasireotide also tended to elevate *SSTR5* expression under NAT silencing. Moreover, mRNA levels of *SSTR5* and its NAT again correlated directly in *SSTR5-AS1* silenced and control conditions, showing mixed in **Figure R19B**.

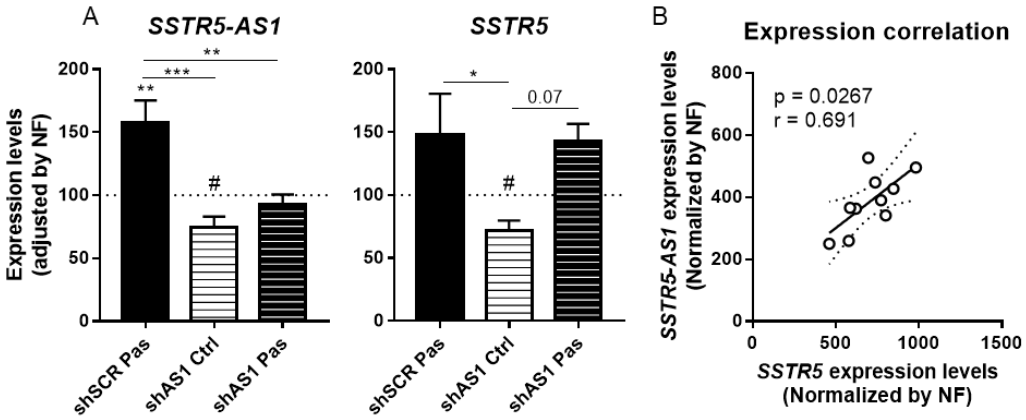


***Figure R19.*** *Silencing of* SSTR5-AS1 *decreases* SSTR5 *expression in BON-1 cells.*
Expression levels of *SSTR5-AS1* and *SSTR5* (A) and correlations between them (B) were evaluated in the BON-1 cell line after *SSTR5-AS1* silencing (striped bars) and 24 h treatment with pasireotide 100 nM (black), and were adjusted by normalization factor (NF) with *ACTB*, *GAPDH* and *HPRT* housekeeping genes. Asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) indicate values that significantly differ between groups under one-way ANOVA analysis; # symbol indicates values that significantly differ from control under t test. In all cases, data represent mean ± SEM of $n \geq 3$ independent experiments.

### 4.2.3 Decrease in *SSTR5-AS1* expression promotes aggressiveness features *in vitro*

To further examine the functional role of *SSTR5-AS1*, we next tested whether the presence of this NAT influences tumor aggressiveness features in the BON-1 cells *in vitro* model. Specifically, we measured proliferation, colony formation and migration of these cells under *SSTR5-AS1* silencing and pasireotide treatment. This approach first showed that NAT silencing significantly increased cell proliferation under basal culture conditions. In contrast, the effect of pasireotide in proliferation was not evident, since its

treatment did not induce any significant change under control conditions or after NAT silencing (**Figure R20A**).

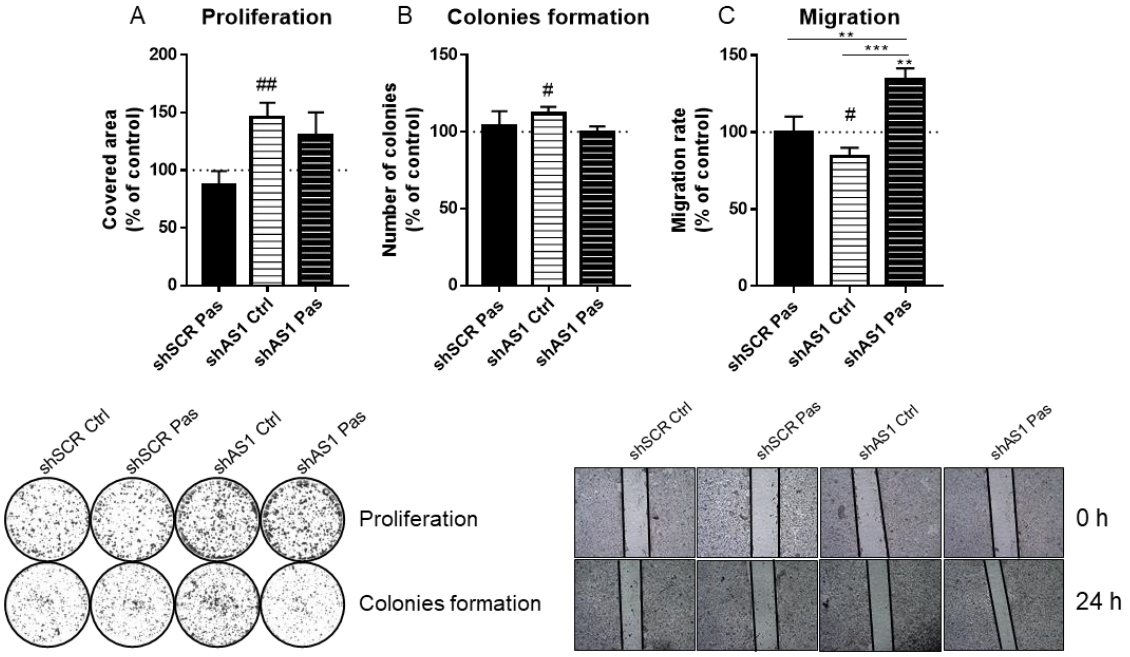


***Figure R20.*** *Alteration of aggressive features after* SSTR5-AS1 *silencing in BON-1.*
**A.** Proliferative rate of BON-1 cell line after 10 days of silencing (striped bars) and/or pasireotide treatment (black), represented as the area covered in the well. **B.** Capacity to form colonies under *SSTR5-AS1* silencing (striped bars) and/or 24 h of pre-treatment with pasireotide (black), measured by number of colonies after 10 days of incubation. **C.** Migration rate under *SSTR5-AS1* silencing (striped bars) and/or pasireotide treatment (black), after 24 h of the wound, represented by healed area. Asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) indicate values that significantly differ between groups under one-way ANOVA analysis; # symbols indicate values that significantly differ from control under t test. In all cases, data are presented as percentage of control and represent mean ± SEM of $n \geq 3$ independent experiments.

Interestingly, colony formation was also elevated after *SSTR5-AS1* silencing, as compared to its scramble control, further supporting a role of this NAT in enhancing malignancy features of these NET cells. Conversely, no changes were observed with pasireotide treatment (**Figure R20B**). In contrast with the above, *SSTR5-AS1* silencing did not increase but decreased cell migration, compared to scramble shRNA, thus suggesting a disconnection between the actions of *SSTR5-AS1* on the distinct functional cell features. Of note, while pasireotide, as in the previous parameter measured did not affect migration under control conditions (scramble shRNA), but surprisingly increased migration when *SSTR5-AS1* was silenced (**Figure R20C**). These observations highlight the relevance of the consequences that alteration of the NAT may affect the function of *SSTR5* gene and the response of $SST_5$ to drug treatment.

In line with this, we finally evaluated the impact of *SSTR5-AS1* on the activation of key proteins within typical signaling pathways regulated by $SST_5$. Thus, activation of AKT and ERK were assessed after *SSTR5-AS1* silencing and 10 min of pasireotide treatment. Results obtained showed that NAT silencing decreased both AKT and ERK activation, compared to scramble shRNA (**Figure R21**). Interestingly, pasireotide treatment did not alter these proteins under control conditions (scramble shRNA), and was similarly unable to modify their decreased levels after *SSTR5-AS1* silencing.

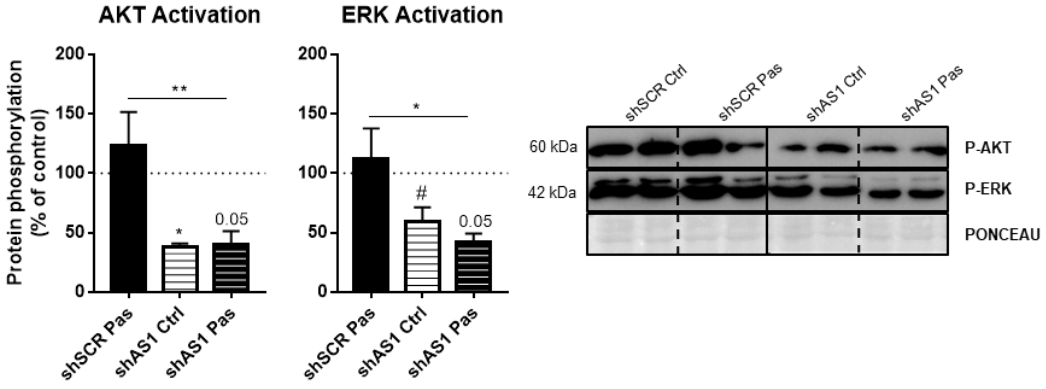


***Figure R21.*** *Silencing of* SSTR5-AS1 *alters key $SST_5$-related signaling pathways in BON-1 cells.* Protein phosphorylation of AKT and ERK in BON-1 cell line after *SSTR5-AS1* silencing (striped bars) and 10 min of pasireotide treatment (black). This activation was measured by western blot and normalized with Ponceau. Asterisks (*, $p < 0.05$; **, $p < 0.01$) indicate values that significantly differ between groups one-way ANOVA analysis; # symbol indicates values that significantly differ from control under t test. In all cases, data represent mean ± SEM of $n \geq 3$ independent experiments.

## 4.3 Experimental section III: The splicing machinery is dysregulated in pancreatic neuroendocrine tumors: role of *NOVA1* overexpression in enhancing tumor aggressiveness and malignancy

It is now widely accepted that tumor cells frequently express splice variants of proteins that carry out different, or even opposite, functions than the canonical ones. In this context, our group discovered the truncated variant of $SST_5$, $SST_5$TMD4 [17], and showed its presence and pathological implications in PitNETs and PanNETs [17, 18]. Likewise, we identified a novel variant of the ghrelin gene, named In1-ghrelin, and also found its association with aggressiveness in PitNETs [255] and PanNETs [201]. These findings, coupled to the increasing evidence linking alteration of the splicing process with cancer prompted us to explore this issue in more detail in PanNETs. Accordingly, in the present section of the Thesis we aimed at obtaining novel information on the molecular

profile of the splicing machinery in PanNETs and to examine the functional role of specific components of this machinery that could be dysregulated in NETs, with the ultimate goal of finding novel, promising biomarkers and/or therapeutic targets to improve diagnosis and treatment of this pathology.

### 4.3.1 Splicing machinery is dysregulated in panNETs in association with clinical features

To assess the expression profile of a selected set of components of the splicing machinery, we employed a qPCR Array based on microfluidics, and measured their mRNA levels in a cohort of 20 primary tumors from patient with panNETs, comparing the tumor tissue with the non-tumoral adjacent tissue, used as reference/control. As described in Materials and Methods, the set of components measured included all major spliceosome (n = 13), and minor spliceosome (n = 4) elements, and a group of associated splicing factors (n = 27) that were selected based on the literature. This approach revealed, for the first time, that seven components of major spliceosome, two of the minor spliceosome, and ten splicing factors were upregulated, whereas only one splicing factor, *ESRP2*, was downregulated, in tumoral samples compared to control tissue (**Figure R22**). The changes observed include both small nuclear RNAs (snRNAs), which comprise the core of the spliceosome, and associated proteins of this core and auxiliary splicing factors, and, altogether, account for nearly half of the components measured, thus highlighting that the splicing machinery is severely altered in panNETs.

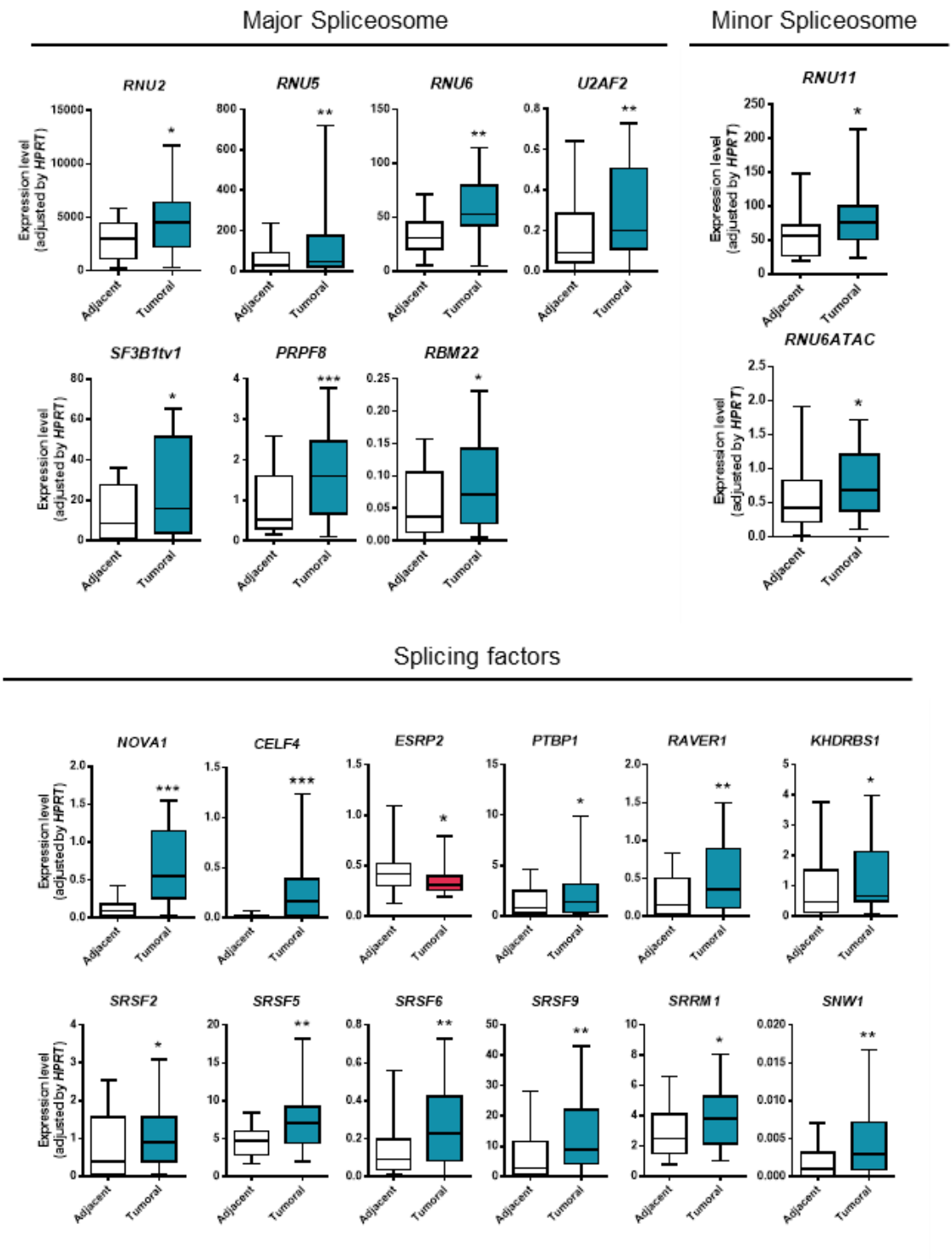


***Figure R22.*** *The pattern of expression of the splicing machinery components is severely dysregulated in PanNETs.*
Expression levels of key components of the splicing machinery in PanNETs **Tumoral** samples as compared to their paired non-tumoral **Adjacent** tissue used as reference/control. The RNA levels were determined by qPCR and adjusted by *HPRT* housekeeping gene. Asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) indicate values that significantly differ from control. Data represent mean ± SEM of n = 20 independent samples.

Interestingly, the potential relationship between the factors whose expression was found here to be altered is supported by the information already available in the literature, as it was shown by a STRING analysis (**Figure R23A**). This observation suggests that the components of this machinery may be altered together in response to shared mechanisms and with a common aim of appropriately regulating the alternative splicing process.

Given the high number of altered components, we sought to classify them attending to their features as possible biomarkers, by using a systematic statistical approach. To this end, we performed a Principal Component Analysis (**Figure R23B**), and a random forest and simple logistic regression analyses, which indicated the molecules that combinedly presented with highest changes and better clustering features. Specifically, this approach identified five genes that stand out among all those measured: *NOVA1*, *PRPF8*, *RAVER1*, *SRSF5* and *SNW1*. Of note, these five splicing machinery components were found to correlate significantly and positively with each other in our cohort (**Figure R23C**), further reinforcing the contention, already suggested by the STRING analysis, that a functional link may be in place among them and that a complex, interrelated functional network may likely exist within the splicing machinery. A more detailed analysis of these five factors revealed that they are, overall, highly overexpressed in tumoral tissue with respect to the non-tumoral adjacent reference tissue, and, interestingly, that the increased gene expression level was consistently observed in the vast majority of samples analyzed (**Figure R23D**).

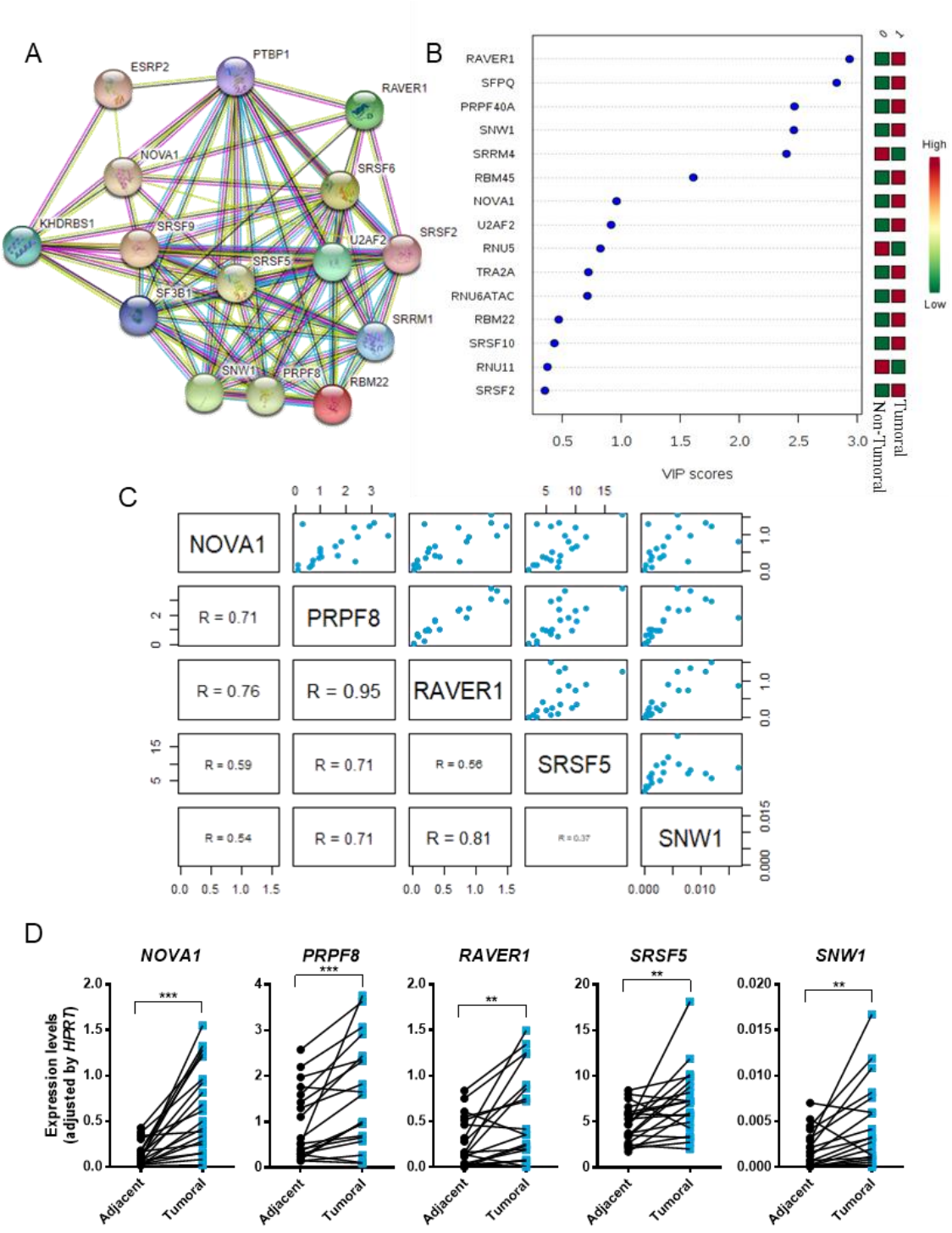


***Figure R23.*** *Classification, selection and interrelationship of splicing machinery components altered in PanNETs.*

**A.** STRING analysis of relationships among altered components based on the information available in the literature. **B.** Principal component analysis of all the altered components identified. **C.** Correlations between *NOVA1*, *PRPF8*, *RAVER1*, *SRSF5* and *SNW1* splicing machinery components. The upper panel represents the scatter plot of the correlations and the lower panel represents the R of each correlation, the higher the size the better the correlation obtained. **D.** Paired comparison of these five components between tumoral and adjacent tissue. Asterisks (**, $p < 0.01$; ***, $p < 0.001$) indicate values that significantly differ from control.

Having selected a limited number of elements, we next examined in further detail the potential association of the levels of expression of these molecules with relevant clinical features recorded in the patient database. This approach revealed that four out of the five altered splicing machinery components selected displayed significant correlations with key parameters related to tumor functionality and patient prognosis, which may provide additional information on the importance of these alterations (**Figure R24**).

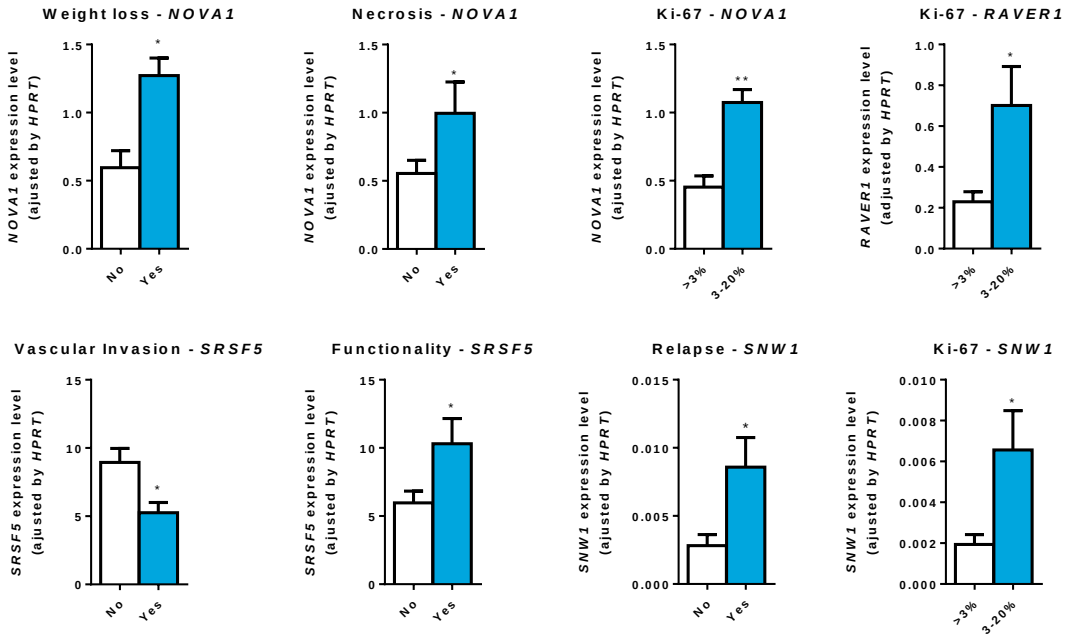


***Figure R24.*** *Associations between expression levels of the splicing machinery components in tumoral tissue and relevant clinical parameters.*
Associations between clinically relevant parameters of patient and tumors and the expression levels of the altered splicing machinery components selected on the basis of their higher changes in tumoral samples. Asterisks (*, $p < 0.05$; **, $p < 0.01$) indicate values that significantly differ from control. Data represent mean ± SEM of n = 20 independent samples.

Specifically, high levels of *NOVA1* gene expression showed a strong association with several parameters, namely weight loss, necrosis of the primary tumor and Ki-67 index, this latter being typically linked to the proliferative status of the tumoral cells. Interestingly, this Ki-67 index was also associated with expression levels *RAVER1* and *SNW1*, suggesting a notable relationship between cell proliferation and altered splicing. Moreover, a high expression of *SNW1* was related also to relapse of the disease. Finally, a higher expression of *SRSF5* was linked to a higher presence of functionality but lower vascular invasion.

### 4.3.2 The splicing factor *NOVA1* as a putative biomarker for panNETs

The next step in our study was to assess the potential value of the five selected splicing machinery components as biomarkers for PanNETs, by testing their ability to accurately discriminate between tumor/non-tumor tissue and separately cluster these tissue samples. To this end, we made ROC curves for each component (**Figure R25**).

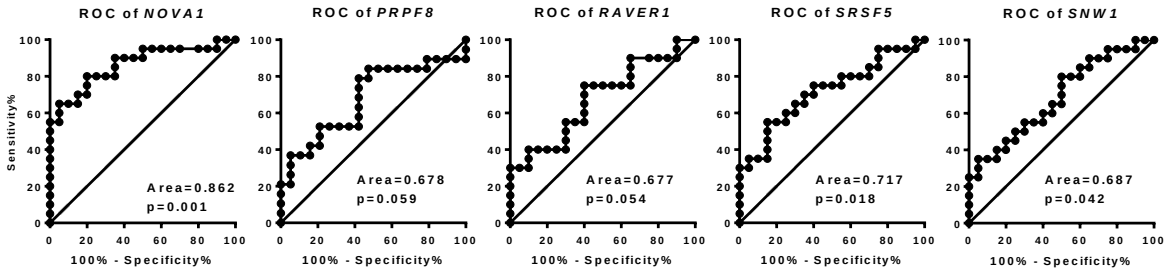


***Figure R25.*** *ROC curves of the five selected splicing machinery components.*
Receiver operating characteristic (ROC) curve analysis was developed to determine the accuracy of *NOVA1*, *PRPF8*, *RAVER1*, *SRSF5* and *SNW1* expression to discriminate between tumoral and non-tumoral samples. Data represent n = 20 independent samples.

These analyses showed that *NOVA1* expression exhibited the highest area under the curve (AUC), above 0.86, whereas the other four factors yielded AUC ranging between 0.65-0.75. These results indicate that *NOVA1* would be the best candidate for biomarker in PanNETs from those we have considered in this study, which prompted us to study this factor in more detail.

Accordingly, we first tested whether the overexpression of *NOVA1* observed in our cohort at the mRNA level could be validated at the protein level. To this end, we applied an immunohistochemical analysis by expert pathologists, who confirmed the higher presence on NOVA1 protein in tumor tissue, compared with non-tumoral adjacent tissue (**Figure R26**). As can be observed in the representative picture shown in this figure, the staining of NOVA1 protein is clearly more prominent in the tumoral gland than in the non-tumoral adjacent tissue (NTAT), particularly in the endocrine tissue of the normal pancreas (Langerhans islets). This is a key observation, inasmuch as Langerhans islets provide the most appropriate control tissue for PanNETs, since the rest of the normal pancreas is predominantly comprised by exocrine tissue. Actually, this is one of the main limitations in the use of reference tissue in the study of PanNETs, and the results from immunohistochemistry help to overcome it.

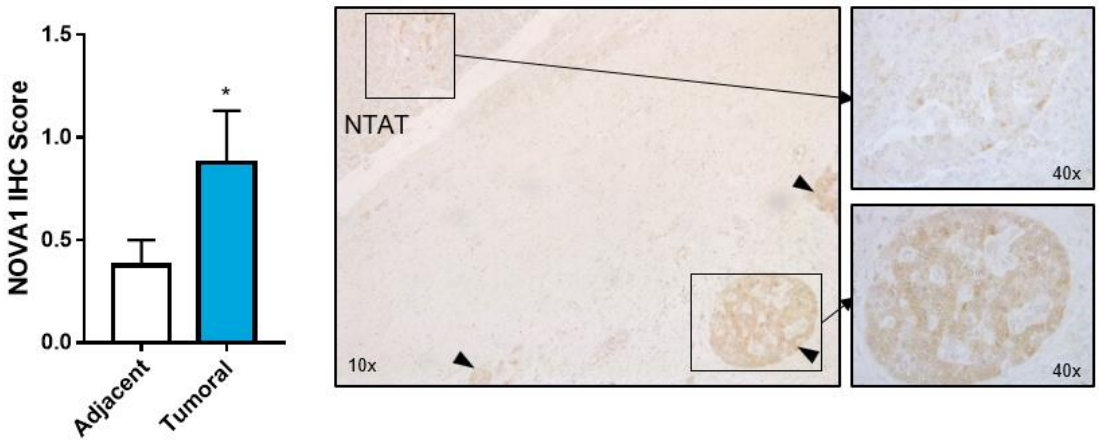


***Figure R26.*** *Immunohistochemical analysis of NOVA1 protein in PanNETs.*
Immunohistochemistry of NOVA1 protein was carried out in tissue section from PanNETs, and staining in tumoral versus non-tumoral adjacent tissue was evaluated and scored by expert pathologists (**Left**). **Right***:* A representative picture of a NOVA1 immunohistochemistry showing tumor and non-tumoral adjacent tissue (NTAT). Arrow heads point tumoral glands. Asterisks (*, $p < 0.05$) indicate values that significantly differ. Data represent mean ± SEM of n = 20 independent samples.

### 4.3.3 Overexpression of *NOVA1* increases cell proliferation and tumor growth

After uncovering the overexpression and accompanying biomarker potential of the splicing factor NOVA1 in PanNETs, we next aimed to explore the possible functional role and mechanisms of action of this factor in PanNETs cells. To this end, we first tested the expression of *NOVA1* in two PanNETs model cell lines, QGP-1 and BON-1. We observed that both cell lines exhibited appreciable mRNA levels of *NOVA1*, which were high enough to perform silencing assays to examine the effect of *NOVA1* loss, but sufficiently moderate to also allow overexpression studies of this factor (**Figure R27A**). Since we had observed that *NOVA1* was overexpressed in PanNETs, we initially overexpressed it in the two cell lines, and assessed functional features that could inform about tumor cell aggressiveness. Interestingly, in line with the previous results, *NOVA1* overexpression increased cell proliferation in both cell lines, as measured by Alamar Blue assay, at different time points (24, 48, 72 h) after transfection (**Figure R27B**). Overexpression was adequately validated (**Figure R27C**).

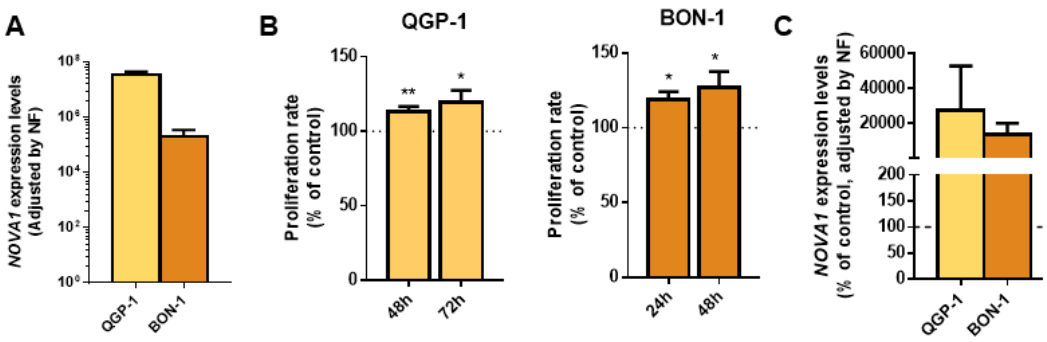


***Figure R27.*** NOVA1 *overexpression augments proliferation in PanNET cell lines QGP-1 and BON-1*.
**A**. *NOVA1* expression levels in QGP-1 and BON-1 PanNETs cell lines as measured by qPCR. **B**. Proliferation rate of QGP-1 (light) and BON-1 (dark) at 48 and 72 h or 24 and 48 h, respectively, after *NOVA1* overexpression compared with mock plasmid transfection, used as control (100 %), marked as tick line. **C**. Validation of *NOVA1* overexpression by plasmid transfection in QGP-1 and BON-1 cell lines. Asterisks (*, $p < 0.05$; **, $p < 0.01$) indicate values that significantly differ from control. Data are expressed as percentage of control and represent mean ± SEM of $n \geq 3$ independent experiments.

To test whether the functional ability of NOVA1 to enhance cell proliferation *in vitro* could also occur in a more clinically relevant experimental setting, we developed a preclinical model of PanNET xenograft tumors in mice. To this end, we selected BON-1 rather than QGP-1 cells, because the first exhibit a more aggressive phenotype (they derive from a metastasis), and also, because there is far more reported experience using BON-1 xenografted tumors in the literature. Thus, BON-1 cells transfected with *NOVA1*- or a mock-plasmid were transfected xenografted in nude mice. As shown in **Figure R28**, BON-1 cells overexpressing *NOVA1* exhibited a higher growth rate over time than mock-control cells, producing larger tumors at the end of the experimental period.

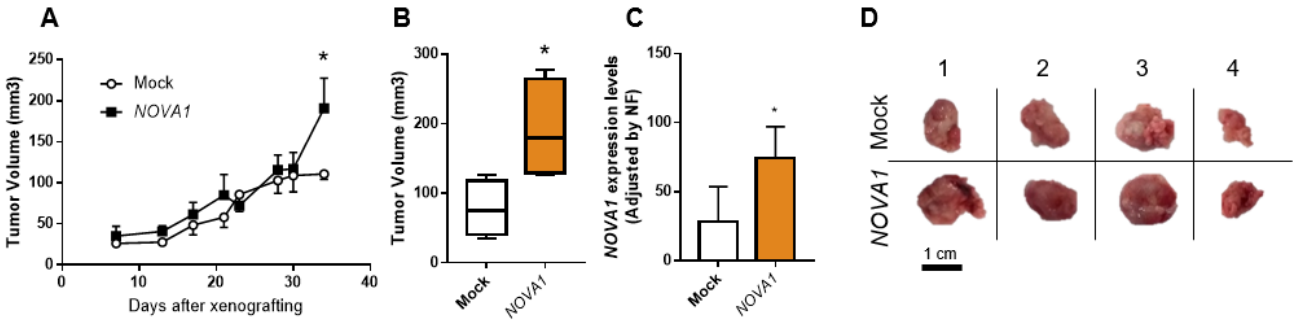


***Figure R28.*** NOVA1 *overexpression in BON-1 cells increases tumor growth in xenografted mice.*
**A.** BON-1 xenografted tumor growth in nude mice with *NOVA1* overexpression compared to mock. **B.** Comparison of tumor size at time of euthanasia; tumor volume is expressed as $mm^3$. **C.** Validation of *NOVA1* overexpression in tumor xenografts after euthanasia, represented as mRNA levels adjusted by normalization factor (NF; with *ACTB*, *GAPDH* and *HPRT* housekeeping genes). **D.** Picture of paired xenografted tumors with mock and *NOVA1* overexpression. Asterisks (*, $p < 0.05$) indicate values that significantly differ from control. Data represent mean ± SEM of $n \geq 3$ independent experiments.

Taken together, these and the previous results strongly support the idea that the splicing factor NOVA1 is directly related with cell proliferation in PanNETs and that increased levels of this factor may contribute to tumor aggressiveness.

### 4.3.4 *NOVA1* as putative therapeutic target in panNETs

Having demonstrated that *NOVA1* is overexpressed in PanNETs and has the functional capacity to enhance PanNET cell proliferation *in vitro* and *in vivo*, our next aim was focused on the study of its potential as a therapeutic target in these tumors. Given that this gene was overexpressed in the PanNETs samples, our experimental approach was to silence *NOVA1* expression in QGP-1 and BON-1 cell lines using a specific siRNA (**Figure R29**). Remarkably, this revealed that *NOVA1* silencing (validated both at mRNA and protein levels, **Figs R29B and C, respectively)** consistently decreased proliferation rate in both cell lines, compared to scramble siRNA, used as control (**Figure R29A**).

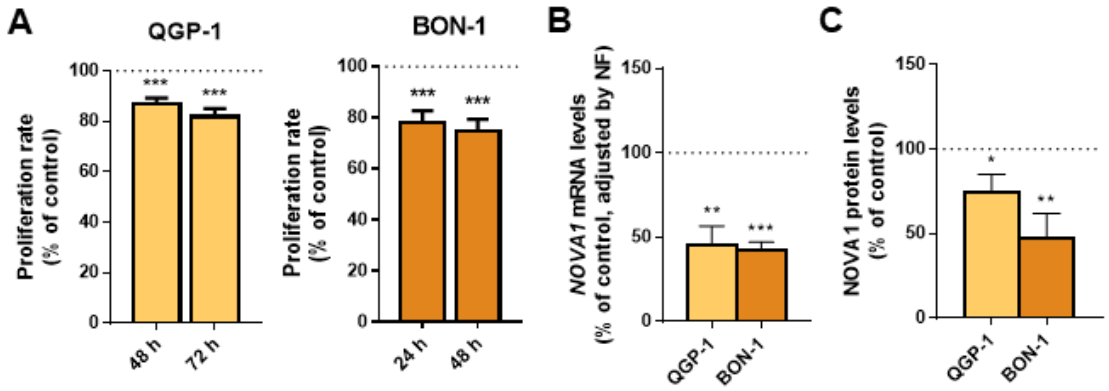


***Figure R29.*** NOVA1 *silencing decreases cell proliferation in QGP-1 and BON-1 cell lines.* **A.** Proliferation rate of QGP-1 (light; left) and BON-1 (dark; right) at 48 and 72 h or 24 and 48 h, respectively, after *NOVA1* silencing, compared with scramble siRNA used as control. **B.** Validation of *NOVA1* silencing by qPCR and **C** by western blot in both cell lines. Absolute mRNA levels were determined by qPCR and adjusted by normalization factor with *ACTB*, *GAPDH* and *HPRT* housekeeping genes and protein by Ponceau. Data are represented as percentage of control (100 %; marked as a tick line). Asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) indicate values that significantly differ from control. In all cases, data represent mean ± SEM of $n \geq 3$ independent experiments.

To investigate the mechanisms underlying the cellular alterations caused by changes in *NOVA1* gene expression, we assessed the mRNA levels of markers commonly related to key cell functions in cancer. In keeping with the results presented hitherto, *NOVA1* silencing decreased, in both cell lines, the expression of *CCND1*, a well-known regulator of cell cycle tightly linked to cell proliferation. Likewise, *NOVA1* silencing

increased the expression of *CASP3*, a key positive regulator for apoptosis. In contrast, no changes were found in *MKI67*, the gene encoding the protein measured for Ki-67 index (**Figure R30**).

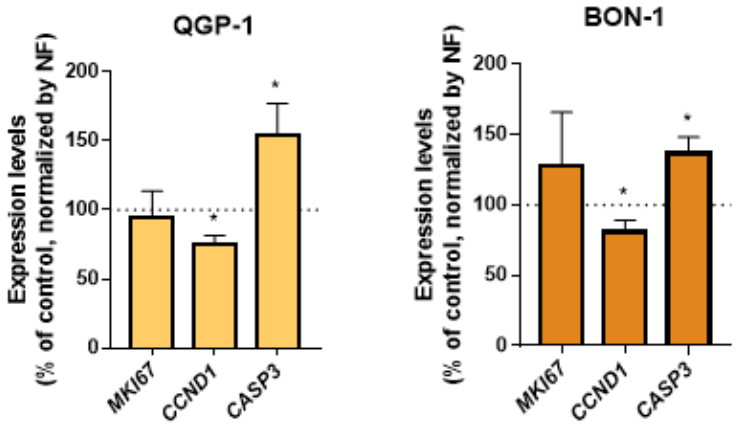


***Figure R30.*** *Expression levels of three key cell markers MKI67, CCND1 and CASP3 under* NOVA1 *silencing in two PanNETs cell lines.*
Expression levels of *MKI67*, *CCND1* and *CASP3* in QGP-1 (light; left) and BON-1 (dark; right) cell lines were measured under *NOVA1* silencing, compared with scramble siRNA. Absolute mRNA levels were determined by qPCR and adjusted by normalization factor with *ACTB*, *GAPDH* and *HPRT* housekeeping genes. Data are represented as percentage of control (100 %, marked as a tick line). Asterisks (*, $p < 0.05$) indicate values that significantly differ from control. Data represent mean ± SEM of $n \geq 3$ independent experiments.

We next interrogated how *NOVA1* silencing would affect activation of major signaling pathways typically involved in PanNETs. This showed that *NOVA1* silencing decreased ERK activation, which suggest that MAPK pathways may mediate *NOVA1* actions on cell proliferation. On the other hand, *NOVA1* silencing decreased phosphorylation of both PTEN and PDK1 proteins, two pivotal mediators that play opposite roles in the activation of PI3K/AKT pathway, while AKT activation was not altered. These results suggest a complex role for *NOVA1*, whose expression may lead to seemingly opposite effects is some signaling pathways. In this sense, whereas both cell types showed a similar response in most cases, *NOVA1* silencing increased the phosphorylation of p53 in QGP-1 but did not alter it in BON-1 (**Figure R31**).

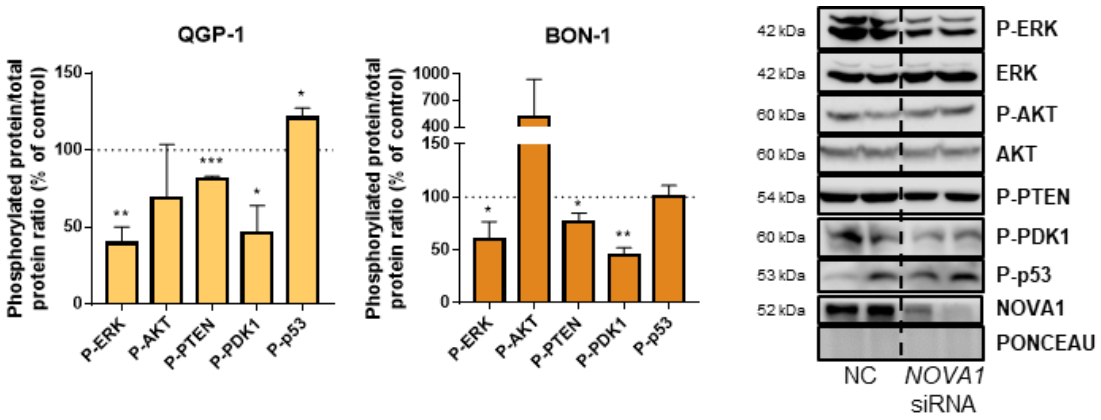


***Figure R31.*** *Effects of* NOVA1 *silencing in the phosphorylation of key signaling proteins.* Protein phosphorylation of ERK, AKT, PTEN, PDK-1 and p53 in QGP-1 (light; left) and BON-1 (dark; right) cell lines after *NOVA1* silencing, compared with scramble siRNA. This activation was measured by western blot and normalized with total protein or with Ponceau. Data are represented as percentage of control that was marked as a tick line. Asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) indicate values that significantly differ from control. Data represent mean ± SEM of $n \geq 3$ independent experiments.

The pivotal role of p53 in cancer and its distinct response to *NOVA1* silencing in the two cell types prompted us to explore this molecule in more detail. In particular, we evaluated the expression of two different isoforms of *TP53*, the gene encoding p53 protein, in order to study if *NOVA1* silencing exerts any effect on *TP53* transcription, since previous results revealed controversial effects in these cell lines. Interestingly, we observed that this silencing clearly decreased the expression of *Δ133TP53* isoform without altering that of the canonical *TP53* in QGP-1, whereas, in contrast, both variants were significantly decreased in BON-1 (**Figure R32**). These results fit nicely with the changes in protein activation shown above and can be functionally relevant, since Δ133p53 protein isoform has been linked to canonical p53 inhibition [256], which happened in QGP-1 but no in BON-1 cell line.

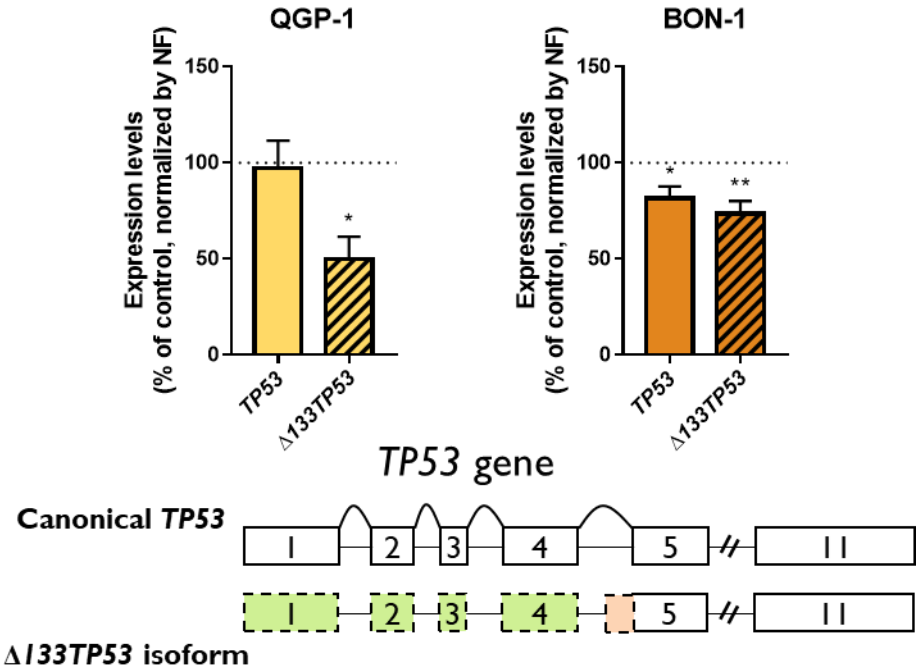


***Figure R32.*** NOVA1 *may regulate the transcription of* TP53 *gene in PanNETs cells.*
*TP53* (open bars) and *Δ133TP53* (striped bars) mRNA expression levels in QGP-1 (light; left) and BON-1 (dark; right) under *NOVA1* downregulation, compared with scramble. The lower panel shows a schematic representation of the two *TP53* isoforms evaluated in this study. Green boxes represent exon skipped and orange alternative exon start in *Δ133TP53* isoform. Data are represented as percentage of control (100 %; marked as a tick line). Asterisks (*, $p < 0.05$; **, $p < 0.01$) indicate values that significantly differ from control. Data represent mean ± SEM of $n \geq 3$ independent experiments.

### 4.3.5 Chromatin remodeling pathway is altered under *NOVA1* downregulation

Chromatin remodeling is a universally relevant process in normal and tumoral cells. However, this pathway has been shown to play a particularly relevant role in PanNETs, where mutations and/or dysregulation of some of its components have been identified as informative biomarkers in panNETs, especially ATRX, DAXX and the related telomerase (encoded by *TERT* gene). Therefore, we studied how alterations in *NOVA1* may cause changes in these biomarkers. In an initial approach, we found that both *ATRX* and *DAXX* were overexpressed in our cohort of tumors, compared to non-tumoral adjacent tissue (**Figure R33A**). In line with this, silencing of *NOVA1* led to a decrease in ATRX and DAXX protein levels in both cell lines (**Figure R33B**).

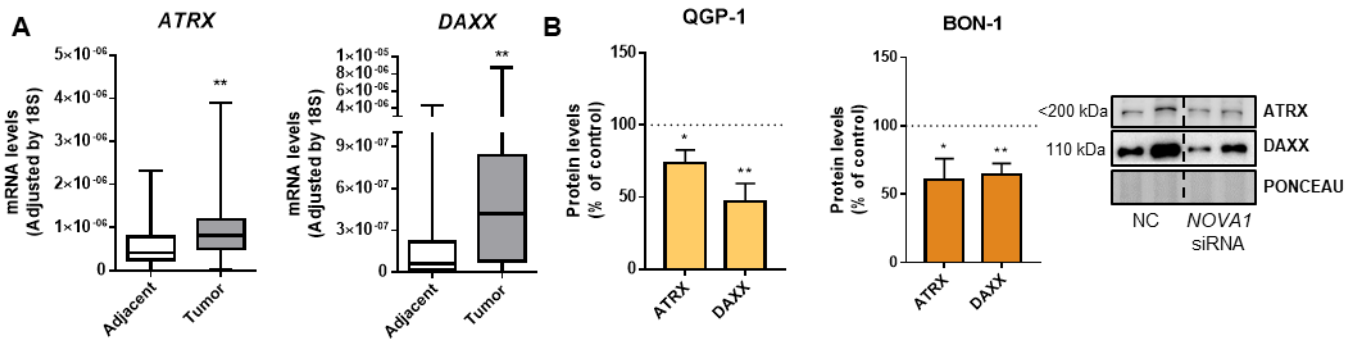


***Figure R33.*** NOVA1 *silencing alters expression of* ATRX *and* DAXX *genes in PanNETs cells.* **A.** *ATRX* and *DAXX* mRNA levels were measured in our cohort of PanNETs samples compared to non-tumoral adjacent tissue, used as control. Absolute mRNA levels were determined by qPCR and adjusted by *RNA18S1* housekeeping gene. **B.** ATRX and DAXX protein levels after *NOVA1* silencing. Protein levels were measured by western blot, normalized with Ponceau and represented as percentage of control. Asterisks (*, $p < 0.05$; **, $p < 0.01$) indicate values that significantly differ from control. In all cases, data represent mean ± SEM of $n \geq 3$ independent samples or experiments.

These results were somewhat unexpected and their functional implications are difficult to interpret, since most of the information related to these genes and their prognostic importance has been related to their mutations rather than their expression levels. Nevertheless, although further studies will be required to clarify these findings, they seem to be of relevance in that both *ATRX*, *DAXX* are considered as tumor suppressor genes, and therefore, the changes observed directly link *NOVA1* to this important pathway in PanNETs. Moreover, in support of this notion, we observed that *NOVA1* silencing decreased the expression of the oncogenic splicing variant of *TERT* gene (named as tv1), without altering the expression of the canonical one (**Figure R34**).

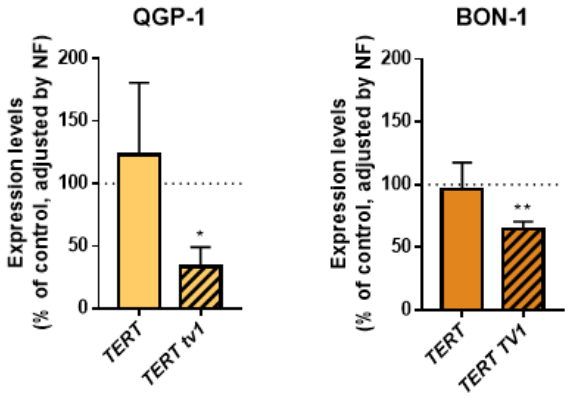


***Figure R34.*** NOVA1 *silencing differentially regulates the splicing of the* TERT *gene.* Expression levels of *TERT* (open bars) and *TERT tv1* (striped bars) under *NOVA1* silencing, compared to scramble control, in QGP-1 (light; left) and BON-1 (dark; right). Absolute mRNA levels were determined by qPCR and adjusted by normalization factor with *ACTB*, *GAPDH* and *HPRT* housekeeping genes. Asterisks (*, $p < 0.05$; **, $p < 0.01$) indicate values that significantly differ from control. Data are presented as percentage of control and represent mean ± SEM of $n \geq 3$ independent samples or experiments.

### 4.3.6 Expression of *NOVA1* can influence treatment effectiveness in panNETs

The associations found earlier between dysregulated *NOVA1* expression and key clinical parameters in our cohort of panNETs, coupled to the functional findings described hitherto, invited to explore whether alteration of *NOVA1* could influence the responsiveness of PanNETs to the main medical treatments available for this disease. To test this notion, *NOVA1*-silenced cells were treated with everolimus, lanreotide, octreotide and sunitinib, four currently used clinical treatments of panNETs, as compared with scramble-silenced cells. This revealed that *NOVA1* downregulation significantly improved the antiproliferative effect of everolimus in QGP-1 cells at 72 h of treatment, whereas no additive effect was observed in BON-1 cell line (**Figure R35**).

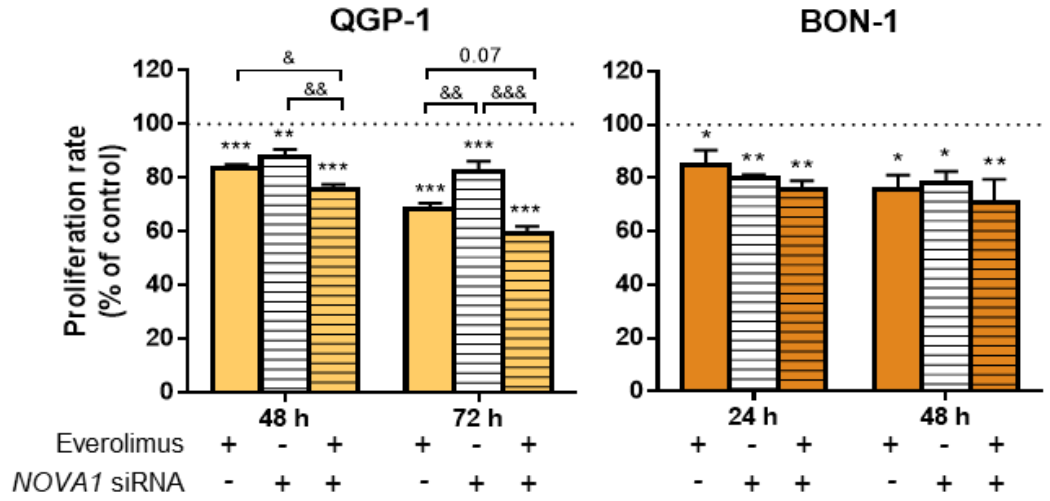


***Figure R35.*** NOVA1 *silencing enhances everolimus antiproliferative effect in PanNETs cells.* Proliferation rate of QGP-1 (light; left) and BON-1 (dark, right) cell lines after *NOVA1* silencing (striped bars) plus treatment with everolimus, compared with the non-treated scramble siRNA (open bars), used as control. Asterisks and & symbols (*/&, $p < 0.05$; **/&&, $p < 0.01$; ***/&&&, $p < 0.001$) indicate significant differences against the control or between groups, respectively. Data are presented as percentage of control and represent mean ± SEM of $n \geq 3$ independent experiments.

In contrast, the other treatments tested in this experimental approach did not change their effects after *NOVA1* silencing in these cells (**Figure R36**).

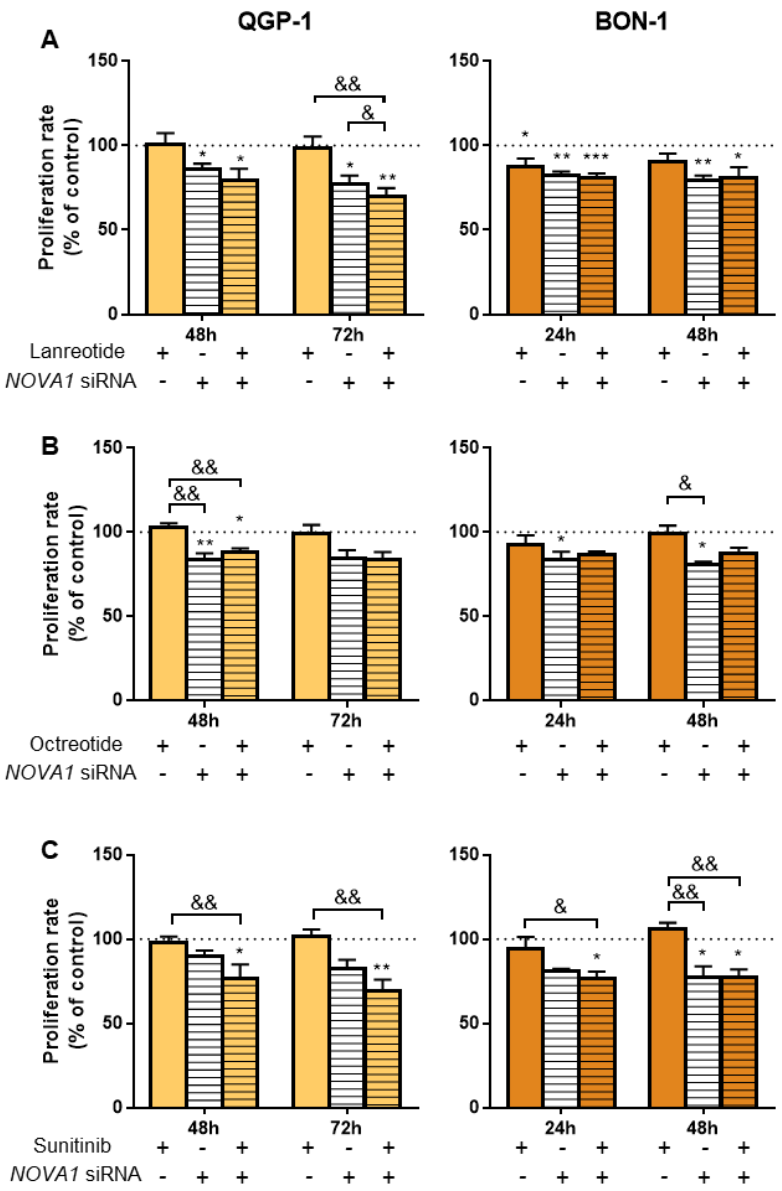


***Figure R36.*** *Effect of* NOVA1 *silencing on the effect of different PanNETs treatments.*
Proliferation rate with combination of *NOVA1* silencing and PanNETs classical treatments: lanreotide (**A**), octreotide (**B**) and sunitinib (**C**), in QGP-1 (grey, left) and BON-1 (black, right). Asterisks and & symbols (*/&, $p < 0.05$; **/&&, $p < 0.01$; ***, $p < 0.001$) indicate significant differences against the control or between groups, respectively. Data are presented as percentage of control and represent mean ± SEM of $n \geq 3$ independent experiments.

These results indicate that *NOVA1* expression may influence selectively the effectiveness of some current treatments, as it is the case of everolimus, but not others on QGP-1 and BON-1 cells, at least at the times and doses tested. Nevertheless, this type of interaction is not simple, seems to be dependent on the cell type considered and may be related to the specific pathways linked to this splicing factor and the action of everolimus.

# 5. DISCUSSION

# 5. Discussion

Cancer and tumor pathologies represent one of the main problems for human health worldwide, as they comprise the second leading cause of death in industrialized countries. Despite the efforts of the scientific community and the resources invested in studying these pathologies to improve our knowledge and seek novel clinical approaches to combat them, their remarkable heterogeneity and complexity, derived from both genetic and environmental factors, hinder the finding of solutions, and pose a big challenge to the society [1]. Tumor heterogeneity pervade all levels of tumor biology, from clinical to molecular, and impacts all cancers, being also present among patients with the same type of cancer and even within a single tumor of a given patient [2, 26]. To address this challenge, the scientific community adopted a common theoretical framework, proposed by Hanahan and Weinberg [refs], in the form of a group of common hallmarks that characterize virtually all type of cancers, and comprise a set of shared features that allow to study all cancers from a complex but common point of view (Figure I2) [3, 4]. Of note, a relevant proportion of these hallmarks are tightly related to metabolic-endocrine axes, as it has long been known that dysregulations in this homeostatic systems can severely impact, at various points, in the appearance, development and malignancy of the tumors [34]. Those effects are particularly important in the so-called hormone-dependent or endocrine-related tumors, in which tumor genesis and evolution is profoundly conditioned by metabolic-endocrine dysregulations [6]. Within this group, the present Thesis is focused in the study of prostate cancer (PCa) and neuroendocrine tumors (NETs). The first, PCa, is second most common cancer among men worldwide, after lung cancer, being their fifth leading cause of death by cancer [7, 8]. The second, NETs, are often dismissed due to their underestimated frequency, but represent a major challenge due to their great heterogeneity, even within a same pathology [10]. In this case, we focused on PitNETs and, particularly, on PanNETs.

Over the last years, our group has studied several endocrine axes in the quest for novel biomarkers and therapeutic targets for endocrine-related tumors. In this context, some components of the somatostatin-SST system have been shown to be linked to development, progression and aggressiveness of these tumors, and have been proposed as biomarkers and/or therapeutic targets [252]. In fact, our group described the relative abundance of $SST_5$ in pitNETs as a biomarker for SSA treatment resistance [14]. Moreover, we discovered two aberrantly-spliced truncated variants of $SST_5$, $SST_5$TMD4

and $SST_5$TMD5, that are present in different endocrine-related tumors, including PitNETs [18, 257], PanNETs [17], PCa [16] and other tumoral pathologies [15, 131, 132], where, especially $SST_5$TMD4, has been related with oncogenesis and aggressiveness features. However, the knowledge on the regulation and biogenesis of this receptor, and on the functional role of other SSTs, are still poorly known in endocrine-related tumors.

The discovery of the existence and pathophysiological relevance of the splice variants of $SST_5$ (and those of ghrelin) pointed to the splicing process, an in particular to its underlying machinery, as an opportunity to better understand these pathologies, and also, as a source of potential biomarkers and therapeutic targets. Actually, alteration of the splicing process is increasingly considered as a novel cancer hallmark, as its relevance is emerging in tumor pathologies, as a cause of malignancy and, also, heterogeneity. However, little is known on the implications of splicing dysregulation in endocrine related tumors, particularly NETs.

For all these reasons, the main aim of this Thesis was to determine the role of SSTs and splicing machinery in different types of endocrine-related cancers and NETs, as well as the underlying regulatory mechanisms, in order to find novel biomarkers and pharmacologic targets with potential to improve the diagnostic and therapeutic approaches in these pathologies.

## 5.1 Novel biomarker and therapeutic target in prostate cancer: the role of *SSTR1*

PCa represents, as pointed out earlier, the third most common cancer with both sexes combined worldwide, and the second most common in men, just after lung cancer, being the fifth leading cause of death by cancer in that group [8]. For 2018, it was estimated that there were almost 1.3 million new cases of PCa and 359,000 associated deaths around the world [7]. In this sense, and despite the intrinsic difficulties of studying tumoral pathologies, over the last years research in this field has helped to increase the therapeutic options to treat PCa, which currently include surgery, chemotherapy, radiotherapy and chemical castration or androgen-deprivation [41]. In addition, valuable biomarkers, such as prostate specific antigen (PSA), have allowed to increase the proportion of patient survival with localized tumor, which now approaches 100 % [38]. Nevertheless, current clinical strategies entail important side effects and there is still a lack of tools to guide the selection of the correct treatment, which, altogether, complicate

the work of clinicians and the life of patients [45, 258]. Therefore, it is necessary to deepen our knowledge of PCa, in order to find novel biomarkers to improve diagnosis and prognosis, and to develop new therapeutic targets for the management of this disease.

In this scenario, it is worth noting that PCa has a relevant endocrine facet, as it is strongly influenced by androgens [48, 49] and other steroid hormones [153] in its genesis and progression, as well as by other neuroendocrine systems that are involved in both, normal prostate function and tumor development [155]. In line with this, it has been documented that the main components of the somatostatin-SST system are present in normal and tumoral prostate, where they can regulate different cell functions, including proliferation, as it happens in other tissues [157]. In fact, this neuroendocrine system is known to play a primary regulatory role in a number of endocrine-related tumors, especially PitNETs and PanNETs, where it represents a significant source of biomarkers and therapeutic targets [17, 18, 257]. In particular, $SST_2$ and $SST_5$ are valuable therapeutic targets for the use of SSAs in the treatment of NETs [86, 139, 140, 259]. Nevertheless, in spite of the promising initial experimental studies [260], results from clinical trials have been disappointing in other cancer types, such as lung, breast or non-endocrine gastrointestinal cancers [161]. In the specific case of PCa, early efforts were made to test the clinical use of $SST_2$- and $SST_5$-targeted SSAs, but the results were not positive in relation to relapse of the disease, or even negative, with worsening of tumor development [249, 261]. Nonetheless, as mentioned above, these first generation SSAs are mainly targeted to $SST_2$ and $SST_5$, with very low, almost ineffective binding to other SSTs, whereas different studies have revealed that other SSTs may play also a relevant role in diverse tumoral pathologies [251, 252]. In particular, some studies have shown that $SST_1$ may significantly modulate the response to SSAs by inhibiting cell proliferation through the interaction with tyrosine phosphatases [253]. Accordingly, we decided to explore the role of $SST_1$ in PCa, evaluating its presence, potential functional role and clinical relevance.

Our initial approach revealed that *SSTR1* mRNA is present in a high proportion of samples of PCa, where this receptor is overexpressed as compared to normal prostate in this cohort. This finding compares favorably with previous reports [167], where $SST_1$ appeared overexpressed in PCa and was regarded as a prominent candidates to provide information about the prognosis of this pathology. However, the mechanisms regulating *SSTR1* expression were poorly known. Interestingly, in this Thesis we have discovered

that the expression of *SSTR1* in PCa could be regulated by mechanisms involving non-coding RNAs. Specifically, we identified four miRNAs (miR-24, miR-488, miR-383 and miR-27b) that were likely related to the *SSTR1* gene, in that they exhibited a high probability to bind the mRNA of this receptor and, more importantly, their expression was inversely correlated with that of the *SSTR1* gene in the TCGA public cohort of PCa samples. Further studies led us to demonstrate that one of them, miR-24, was able to directly regulate the mRNA levels of *SSTR1* in the 22Rv1 PCa cell line. Moreover, expression of this miRNA is clearly and inversely correlated with *SSTR1* expression in the MSKCC PCa database, where, notably, miR-24 expression was progressively reduced while that of *SSTR1* concomitantly increased from normal prostate to PCa and, finally, to metastatic samples. Taken together, these results reinforced the notion that *SSTR1* may be involved in PCa physiopathology, possibly under the regulation of miR-24, and thus, that it deserved to be studied further as a possible biomarker and/or therapeutic target for this disease.

Indeed, our subsequent work clearly demonstrated that $SST_1$ exerts a profound antiproliferative effect in PCa cells, consistent with results reported for other tumoral pathologies, including glioma or pancreatic cancer [262, 263]. To generate these data, we performed proliferation assays in response to treatment with a selective $SST_1$ agonist, BIM-23926, and also after genetically altering *SSTR1* expression in 22Rv1 PCa cells. This revealed not only that ligand-induced $SST_1$ activation is able to decrease PCa proliferation, which is a significant observation, but also, that the levels of expression of this receptor clearly influence the proliferation rate of PCa cells as well, which suggests that $SST_1$ may sustain a certain degree of autonomous, constitutive inhibitory activity. Notwithstanding this, it remains to be established whether these situations also operate *in vivo*, in prostate tumor cells.

To explore the mechanisms underlying these effects, we assessed the status of different signaling pathways in response to $SST_1$ agonist treatment or after altering *SSTR1* expression in 22Rv1 PCa cells. The first approach showed that treatment with the $SST_1$ agonist did not alter $[Ca^{2+}]_i$ kinetics, implying that the actions mediated by this receptor may be independent of this second messenger. Likewise, western blot assays indicated that ERK or JNK activation was not altered either. In striking contrast, BIM-23926 treatment clearly decreased phosphorylation of AKT, a classic, well-known effector involved in activation of cell proliferation [264]. These observations suggest that $SST_1$

activation leads to a decrease in cell proliferation through an inhibition of the PI3K pathway, which has been previously linked to other components of the somatostatin-SST system [244, 265]. Similarly, activation of $SST_1$ with the selective agonist inhibited the expression of *CCND3*, the gene that encodes cyclin D3, an important regulator in the progression of the cell cycle through G1 phase, which has been previously associated to promotion of cell proliferation in cancer [266, 267], and which, interestingly, can be also regulated by the activation of AKT [268]. Consequently, our results invite to propose that activation of $SST_1$ by the BIM-23926 agonist in 22Rv1 PCa cells reduces cell proliferation though a mechanism that would involve the inhibition of the PI3K/AKT/CCND3 pathway. On the other hand, our findings may also imply that the effects caused by $SST_1$ activation would not involve the promotion of apoptosis of 22Rv1 cells through altered p53, since expression of *TP53* was unchanged, although, further studies would be necessary to unequivocally support this conclusion.

In addition to its direct role in regulating PCa cell proliferation, our study reveals that $SST_1$ may play relevant functions in PCa by interacting with the AR. Indeed, mRNA levels of *SSTR1* were tightly and directly associated with those of *AR* in our cohort of PCa samples, whereas such correlation was not found in non-tumoral samples. This observation highlights the interesting features of *SSTR1* as a target in PCa, since AR is one of the key molecules in the pathophysiology of this cancer. To examine this notion in further detail, we analyzed the involvement of AR and its associated pathways in the action of $SST_1$ in the 22Rv1 PCa cell line, using, as model, the overexpression of $SST_1$ in these cells. Consistent with our predictions, the increase of *SSTR1* expression induced important alterations in a number of molecules involved in AR function and signaling. These changes included upregulation of *IGFBP5* gene expression and downregulation of *ADAMTS1*, *IRS2*, *VIPR1*, *SLC45A3* and *LIFR* expression. The gene *IGFBP5* codes for insulin-like growth factor binding protein 5, an interesting molecule that plays a relevant role in the regulation of cell growth, differentiation and behavior, by binding and regulating IGF-1, and acting as a tumor suppressor in different cancers, such as melanoma or ovarian cancer [269, 270]. Accordingly, our results, coupled to the previous findings from other groups, further reinforce the idea that $SST_1$ may play a valuable role in PCa by activating tumor suppression systems.

In support of this contention, we found that the genes that were downregulated after *SSTR1* overexpression have been linked to tumor progression in the literature. Thus,

it has been shown that *ADAMTS1* is overexpressed in tumor tissues in relation with the activity of TGF-β [271] or even metastasis of breast cancer, where it may serve as a biomarker of prognosis [272]. *IRS2* is a very well-known oncogene that encodes for insulin receptor substrate 2, which participates in the transduction of insulin and IGF-1 signaling; this gene has been related to the inhibition of apoptosis in non-small cell lung cancer [273, 274]. Also, *VIPR1*, the receptor of the vasoactive intestinal peptide (VIP), is overexpressed in some types of tumors, where it can play antiapoptotic, proangiogenic and pro-proliferative functions [275, 276]. The potential relevance of this last gene is reinforced by the evidence gathered for other growth factors, such as growth hormone releasing hormone [277, 278] of gastrin-releasing peptide, which have been proposed and tested as therapeutic targets in PCa, where they act as tumor inducers [279]. Similarly, *SLC45A3*, also known as prostein, a molecule implicated in several cell processes including lipid metabolism, has previously been reported to be abundantly present in PCa tissue, where it has been used as biomarker due to its high expression [280, 281]. Finally, the *LIFR* gene regulates various cellular processes, including proliferation, differentiation and survival, and has been proposed as a negative prognosis biomarker in melanoma, as well as linked to treatment resistance in breast cancer, where it was explored as therapeutic target [282-284]. Altogether, these results provide compelling evidence to support an antitumoral role for $SST_1$ in PCa.

Intriguingly, despite the strong connection between *SSTR1* overexpression and AR-related signaling, we observed that activation of $SST_1$ with BIM-23926 did not alter AR protein phosphorylation, suggesting that the relationship AR-$SST_1$ may not involve a direct activation of AR itself but could require additional mechanisms to establish the observed functional link. In relation with this, a 24 h treatment with the $SST_1$ agonist inhibited PSA secretion in 22Rv1 cells, suggesting that the functional role of this receptor in PCa can involve a reduction of PSA over-secretion [285], which is closely associated to AR activity in these tumors [286]. Clearly, the precise nature of the interrelation between $SST_1$ and AR signaling warrants further investigation.

When viewed together, the results from the first part of this Thesis revealed that *SSTR1* is overexpressed in PCa with respect to non-tumoral prostate samples and that its expression may be regulated by specific miRNAs, particularly miR-24, which could play a relevant role in this pathology. In addition, we demonstrate that $SST_1$ is able to inhibit two key features of PCa cells, proliferation and PSA secretion, in the 22Rv1 cells model,

likely by modulating a signaling pathway integrating PI3K/AKT-CCND3 and also, through alteration of AR signaling. Therefore, our results demonstrate that $SST_1$ may represent in the future a novel biomarker and therapeutic target in the fight against PCa.

## 5.2 Regulation of *SSTR5* expression by epigenetic and post-transcriptional mechanisms in neuroendocrine tumors

There is now ample evidence that the somatostatin-SST system plays a key pathophysiological role in various tumors, particularly in NETs, where detection of specific SSTs and use of synthetic SSAs provide valuable diagnostic and therapeutic tools [11]. Actually, SSAs are currently employed to control tumor growth and/or associated symptoms in somatotropinomas (PitNETs) and in PanNETs, when surgery, the first line treatment and only curative approach to date, is not fully effective or cannot be applied [86, 139, 287, 288]. The basis for SSA action in these tumors is the abundant expression SSTs, and in particular $SST_2$, the primary molecular target of first generation SSAs, lanreotide and octreotide [11]. Unfortunately, an appreciable proportion of patients are unresponsive to SSA or develop resistance during treatment [11, 143, 289]. However, NETs also express high levels of other SSTs, especially $SST_5$, which would enable the use of alternative pharmacological approaches to treat these tumors. Indeed, although $SST_5$ also binds with high affinity first generation SSAs, it is even a better target for the second generation SSA pasireotide [290]. In fact, this novel SSA is being used (or tested) already for the treatment of different types of NETs [291-294], which supports the present and future potential of $SST_5$ as a target in these diseases.

On the other hand, the biology of $SST_5$ seems to differ substantially from that of $SST_2$ or the other SSTs, and is still far from being fully understood [11, 295]. Thus, for example, high $SST_5$ expression, in relation to that of $SST_2$, has been linked to SSAs resistance in acromegaly, instead of being associated to a good response [14]. Likewise, human *SSTR5* is the only gene of the SSTR family that, despite lacking typical introns in its coding sequence, can give rise to aberrant splice variants, e.g. $SST_5$TMD4, which are overexpressed in NETs and have been linked to oncogenic processes and SSA resistance [11, 14, 17]. These and other reasons support the importance to advancing in our understanding of the mechanisms regulating the expression of *SSTR5* and the biogenesis of $SST_5$ and to identify putative factors controlling its functioning in NETs.

In this scenario, we initially applied an *in silico* analysis of the *SSTR5* gene region that revealed the existence of a natural antisense transcript (NAT) overlapping in the genome with *SSTR5* gene, which had already been named, accordingly, *SSTR5-AS1*, but whose role or regulation had not been still reported at that time. Furthermore, a closer analysis revealed that, distributed along the loci of these two genes, there are four CpG islands susceptible of being methylated. We then decided to analyze in detail these two original features of *SSTR5* in NETs. Specifically, presence and relative abundance of *SSTR5-AS1* with respect to *SSTR5* was examined in somatotropinomas and PanNETs, whereas methylation levels of the different islands were measured in the cohort of somatotropinomas. Results from this latter approach revealed, for the first time, that some of these CpG islands were differentially methylated in somatotropinoma samples, compared with normal pituitary (NP). Specifically, the CpG island overlapping the last exon of the NAT gene *SSTR5-AS1* was more methylated in somatotropinomas than in NP, whereas the one overlapping the first exon of *SSTR5* and its putative promoter was hypomethylated in somatotropinomas compared to NP, and the most distant zone, overlapping the area where alternative splicing is presumed to occur, in the middle of the large exon of *SSTR5* and previous to the NAT, was significantly less methylated in somatotropinomas than in NP. The potential importance of these observations resides in the widely accepted role of DNA methylation in the control of gene expression, as well as its relation with other actions regulating DNA biology, including splicing [296-298]. In fact, methylation levels of this later area, referred to as Zone 4.3 in our study, was tightly associated with *SSTR5* and *SSTR5-AS1* expression in somatotropinomas, where lower levels of methylation were linked to higher expression of these genes, but not in NP samples. These findings clearly suggest that methylation of this CpG island can be related to the expression of these two genes in a pathologically relevant context, which is in agreement with results from a recent study where this *SSTR5* context was examined in laryngeal carcinoma [151]. The lack of association between methylation Zone 4.3 and *SSTR5*/*SSTR5-AS1* expression in NP is intriguing, and could suggest a differential regulatory role of this interaction in normal somatotropes, or perhaps a distinct contribution of the heterogeneous cell population comprising healthy pituitary tissue, as compared to the monoclonal population of tumoral somatotropes found in GH-secreting tumors. Nonetheless, the present findings provide original evidence that methylation of intragenic CpG island in the *SSTR5* gene can influence the expression of this gene and its NAT, thereby providing a novel avenue to further explore and understand the regulation

of *SSTR5* expression, not only in tumor somatotropes, but also in normal pituitary cells, as well as in other tumor cell types.

There is increasing interest in NATs given their ability to regulate the expression of their sense genes [149]. Consequently, we analyzed the expression of *SSTR5-AS1* and its relationship with that of *SSTR5* on the same cohort of somatotropinoma samples as well as in an additional set of PanNETs. Interestingly, *SSTR5-AS1* expression in PanNETs was higher in tumor tissue as compared to the non-tumoral adjacent tissue. In contrast, no such differences were found in somatotropinomas compared to NP. However, in both PitNETs and PanNETs, as well as in their respective control tissues, we discovered an interesting common behavior: there was a marked, direct association between the expression of *SSTR5-AS1* and that of *SSTR5*. These results are in agreement with the findings reported in laryngeal carcinoma [151], and support a close relationship between the control of both genes, which may involve a regulation by common factors, but also a direct interaction of the two genes during their expression. This latter mechanism is likely to be in place, in that our results proved that silencing of *SSTR5-AS1* with a specific shRNA caused a marked decrease in *SSTR5* expression *in vitro* in BON-1 cells.

We next sought to further understand the precise functional role of *SSTR5-AS1* gene in NETs, by evaluating different mechanistic endpoints on the PanNETs BON-1 cell model after silencing this NAT. This approach revealed that *SSTR5-AS1* silencing had a profound functional impact, as increased cell proliferation and colony formation in BON-1 cells. The reason for these actions may relate to the inhibition of *SSTR5* expression mentioned above, since this receptor can exert antitumoral functions and has been shown to have ligand-independent constitutive activity [11, 254, 299]. In contrast, *SSTR5-AS1* silencing caused a decrease in cell migration, which would apparently imply that this NAT, either directly or through $SST_5$ could contribute to sustain the migratory capacity of BON-1 cells under basal culture conditions. These observations unveil an apparent disconnection between two typical tumoral features, in that a reduction in the expression of this NAT would concomitantly increase proliferation but increase migration. Obviously, it would be of interest to explore whether these actions caused by the partial loss of *SSTR5-AS1* bear similar consequences *in vivo*, particularly in tumors. Nonetheless, these seemingly contradictory actions (given the antitumoral role of *SSTR5)*, may be mediated through a distinct ability of *SSTR5-AS1* to influence downstream signaling, as its silencing decreased activation of AKT and ERK, two key players in pathways

controlling a wide number of cell functions and with a complex cross-talking regulatory network. Typically, AKT and ERK inhibition are related with antitumoral actions [300, 301], which would be in keeping with the downregulation of migration observed after *SSTR5-AS1* silencing. In fact, these pathways have been previously related with *SSTR5* in the literature [11]. However, these reductions would not similarly fit with the increased proliferation and colony forming assays, thus suggesting that additional mechanisms must be in place underlying these actions and therefore, that further studies are necessary to fully understand the mechanisms mediating *SSTR5-AS1* function.

A final set of studies was aimed to ascertain whether *SSTR5-AS1* may influence the response of BON-1 cells to the SSA pasireotide, which preferentially targets $SST_5$. Interestingly, this revealed, for the first time, that pasireotide treatment increases *SSTR5* expression in PanNET cells, similar to that previously reported by our group in tumor pituitary cells [302]. But, most importantly, pasireotide also increased *SSTR5-AS1* expression, which could imply that the positive feedback between the activation of $SST_5$ and the expression of this receptor may involve or, at least be related to, that of the NAT itself. In fact, the presence of *SSTR5-AS1* shRNA impeded pasireotide to increase NAT expression, whereas it did not seem to fully abrogate its ability to upregulate *SSTR5* expression. On the other hand, in keeping with our previous findings in PanNET cell lines [243], the functional and signaling actions of pasireotide in BON-1 cells were somewhat limited, as it did not alter most of the parameters measured, nor was able to overcome the reduction in AKT and ERK activation caused by *SSTR5-AS1* silencing. Oddly enough, under this silencing pasireotide stimulated cell migration as indicated by the wound healing assay. These results confirm the unexpected limited ability of pasireotide to influence key functional parameters in PanNETs bearing $SST_5$ and, at the same time, unveil an association between $SST_5$ activation, expression of *SSTR5* and its NAT, *SSTR5-AS1*, and the actions of pasireotide on a key feature in cancer cells, migration, which would therefore warrant further investigations in PanNETs cells.

In sum, our study uncovers two novel mechanisms that can contribute to the regulation of *SSRT5* expression in cells from PanNETs and somatotropinomas, namely, epigenetic modulation by differential methylation of intragenic regions, and post-transcriptional events mediated by a natural antisense of *SSTR5*, *SSTR5-AS1*. The results presented herein reveal that methylation of specific *SSTR5* gene CpG regions can be the associated to the upregulation of both *SSTR5* and *SSTR5-AS1* expression. Similarly,

*SSTR5-AS1* may influence *SSTR5* and *SSTR5-AS1* expression as well, but in addition can influence thereby NET cell aggressiveness features, including proliferation, migration and colony formation, and can be involved in the limited response of PanNET cells to pasireotide. The precise contribution of these new regulatory mechanisms of $SST_5$ biology to the clinical behavior and pharmacological response of pituitary and pancreatic NETs as well as other tumors awaits future elucidation.

## 5.3 The splicing machinery is dysregulated in pancreatic neuroendocrine tumors: role of *NOVA1* overexpression in enhancing tumor aggressiveness and malignancy

Alteration of alternative splicing is increasingly regarded as a novel, transversal cancer hallmark, as it pervades all the individual hallmarks defined previously by disrupting the normal pattern of splicing and generating new, aberrant protein isoforms [19, 187, 303]. Thus, splicing dysregulation has been associated to many types of tumors [188], but also to other diseases, including major endocrine pathologies [304]. In line with this, there is also mounting evidence that defects in splicing also affect endocrine-related tumors, such as PitNETs [232] or lung NETs [305]. As explained in detail earlier, our original discoveries of novel isoforms of $SST_5$ [248] and ghrelin [200] and their contribution to tumor aggressiveness in NETs [17, 201] led us to hypothesize that the splicing machinery, as the core engine generating splice variants, could be involved in these events. However, although some studies had shown alterations in isolated splicing factors in NETs [306], a complete description of the splicing machinery had not been reported hitherto. Hence, our team decided to systematically characterize the pattern of expression of a representative set of components of the splicing machinery, including the core of the spliceosome and a group of selected splicing factors. This approach has recently widened our knowledge of the dysregulation of this machinery in PitNETs [232] as well as in prostate cancer [307]. Accordingly, in this section of the Thesis we aimed to obtain, for the first time, a similar panoramic view of the pattern of expression of the splicing machinery in PanNETs and to study its potential dysregulation.

Comparison of the expression profile of the splicing machinery between tumor tissue and non-tumor adjacent tissue (used as a reference), revealed that mRNA levels of nearly 50 % of the genes measured was upregulated, whereas only one component, *ESRP2*, was downregulated in tumor samples. These results indicate that the expression of the splicing machinery, a core macromolecular complex that plays a central functional

role in the cell, is profoundly altered in the tumor tissue, thereby disclosing a previously unrecognized dysregulation that most likely would entail patho-functional consequences in tumor cells. In close agreement with these findings, application of a similar experimental approach in the four major classes of PitNETs (non-functioning tumors, somatotropinomas, corticotropinomas and prolactinomas) [232], as well as in prostate cancer [307], has recently evidenced comparable levels of alteration (around 50 %) in the expression of the components of the splicing machinery. Therefore, dysregulation of this machinery seems to be a conserved feature across different types of endocrine-related tumors, which is in line with the growing list of studies linking defects in splicing factors and spliceosome components as a source of tumor development [205, 214, 222, 308]. Altogether, this information provides both new avenues for oncological research and a plethora of novel points of intervention to identify novel biomarkers and treatment targets. However, it seems mandatory to select among those altered factors the ones with most probable functional relevance and potential clinical value.

In line with this idea, we applied a detailed bioinformatic and statistical analysis of our results, which enabled to identify five genes, *NOVA1*, *PRPF8*, *RAVER1*, *SRSF5* and *SNW1*, that stand out over the rest, both because of their overexpression in virtually every single paired sample, and for their clustering ability to separate tumoral from non-tumoral samples. In keeping with the latter, generation of ROC curves showed that, despite the very limited number of samples employed, these factors were able to significantly discriminate tumoral from adjacent tissues, thus inviting to explore their potential as putative biomarkers in the future. Moreover, the possible relevance of changes in these factors in NETs was further supported by the observation that their increased levels were associated with important clinical parameters, such as vascular invasion, disease relapse or Ki-67 index, a widely used score for tumor cell proliferation with prognostic and clinical value. Among these five factors, *NOVA1* was selected for further studies based, initially, on its best fitted ROC curve (AUC > 0.86), but subsequently on a specific immunohistochemical analysis. This approach served not only to confirm at the protein level the mRNA overexpression observed in the tumor tissue, but also illustrated the intense confinement of NOVA1 immunostaining on the neuroendocrine tumor cells, as compared to the low levels present in normal endocrine cells of the non-tumoral surrounding tissue, further pointing at this gene as a putative novel biomarker for PanNETs. Moreover, the close association of *NOVA1* expression

levels with Ki-67 index and tumor necrosis strongly suggested that this splicing factor could be functionally linked to key tumor cell features such as cell proliferation and death [309, 310]. These data, coupled to the rising evidence indicating that NOVA1 plays relevant, even oncogenic roles in different tumors [80, 199, 227] led us to focus our efforts in ascertaining the functional and mechanistic underpinnings of this factor through *in vitro* and *in vivo* studies.

Initial *in vitro* studies using two PanNET model cell lines, BON-1 and QGP-1 cells, proved that the predicted association of NOVA1 upregulation in NETs with cell proliferation is likely to have a direct functional basis. Thus, *NOVA1* overexpression by plasmid transfection in two cell lines with quite distinct intrinsic expression levels of the factor caused a similar, significant increase in the basal proliferation rate of the two cell types in culture. Furthermore, a preclinical model based on an immunodeficient mice with xenografted tumors indicated that BON-1 cells overexpressing *NOVA1* also display a higher proliferation rate than mock-transfected cells *in vivo*, thereby producing larger tumors. On the contrary, *NOVA1* silencing in these cells decreased their proliferation rate, which is also in line with that found in other tumors, like astrocytoma, melanoma, non-small cell lung cancer and osteosarcoma [80, 227, 228, 311], where the signaling pathways mediating NOVA1 actions may differ depending on the type of cancer studied. In PanNETs, our results reveal that *NOVA1* silencing increases ERK phosphorylation and *CCND1* mRNA levels, suggesting that it increases cell proliferation through activation of the MAPK pathway, and the subsequent involvement of CCND1, which are known to interact in pediatric brain tumors [312] . However, the increase in *CASP3* expression suggests that an involvement of cell apoptosis should not be discarded in this context. In addition, the lack of changes in *MKI67* expression after altering *NOVA1* expression does not seem to agree with the association observed in tumors between *NOVA1* mRNA levels and Ki67 index in NETs, which invites to speculate an indirect relationship of these two molecular markers. Nevertheless, by and large, the present data provide original, compelling evidence that NOVA1 is a plausible enhancer of cell proliferation in PanNETs that deserve further study.

Inasmuch as NOVA1 is a pre-mRNA binding factor known for its role in splicing, we sought to ascertain possible splicing-related mechanisms that may underlie its functional effects on NET cells. To this end, we tested whether *NOVA1* silencing could alter the splicing of the telomerase gene, *TERT*, in BON-1 and QGP-1 cells, which,

indeed, was the case, as it decreased *TERT* transcript variant 1 (tv1) without altering the total expression of the gene. These results demonstrate that *NOVA1* silencing alters the selection of the *TERT* variants during splicing process, favoring the decrease of the truncated variant, which is known to exert a constitutive action that increases the length of telomeres and enhances tumor cell aggressiveness features in non-small cell lung cancer [80]. Actually, in support of our present findings, in that study *NOVA1* also increases tumor growth and promotes survival advantage of tumor cells by favoring the generation of a high proportion of *TERT* truncated variant during the splicing process [80]. Intriguingly, and possibly in relation to the above, *NOVA1* silencing in the NET cell lines also decreased mRNA and protein levels of *ATRX* and *DAXX*, two genes related to chromatin remodeling and lengthening of telomeres [313] that are considered tumor suppressors, as their mutations/loss are linked to NET aggressiveness and bad disease prognosis [78, 314]. Unfortunately, available knowledge on the meaning and regulation of *ATRX* and *DAXX* expression in NETs is not as advanced as that on their mutations, which prevent us to understand the significance of their overexpression in the tumor tissue of our NET cohort and of their decrease after *NOVA1* silencing. This notwithstanding, our results certainly suggest that NOVA1 may be involved in the regulation of the expression of these factors and, together with telomerase results, link NOVA1 with the chromatin remodeling and lengthening of telomeres pathways, which are known to play a central role in NETs.

To gain further insight into the signaling pathways mediating NOVA1 function, we next focused on those linked to cell proliferation and PanNETs oncogenesis. This revealed that activation of PTEN and PDK1, two key components of the PI3K/AKT pathway, essential in PanNETs, was inhibited under *NOVA1* silencing. This finding is apparently contradictory in that these proteins are functional antagonist in the activation of this pathway, where PTEN is a key inhibitor and PDK1 an important activator, closely related to AKT protein [315, 316]. In fact, AKT activation itself was not altered after *NOVA1* silencing, suggesting that, to exert its actions, NOVA1 may distinctly regulate specific components of this complex signaling network, which may even play opposite roles. Of note, PTEN inhibition has been shown to increase cell senescence, without changes in AKT, through direct interaction with the mTOR-p53 pathway [317]. Interestingly, we found that *NOVA1* silencing promoted p53 activation in QGP-1 but not in BON-1, a difference that may be related to the *CDKN2A* inactivating mutation found

in BON-1 [239], which would impede p53 activation, as it is its main driver in the context of senescence. In line with the above, and supporting the idea that *NOVA1* silencing can activate senescence, we observed that in QGP-1 cells this silencing also downregulated selectively the *Δ133TP53* isoform, without altering full *TP53* gene expression, a relevant finding because truncated *Δ133TP53* acts as a direct inhibitor of full-length, canonical p53, especially in senescence context. As expected, in BON-1 cells responded differently in this context, where *NOVA1* silencing inhibited the expression of both *Δ133TP53* and *TP53*. Taken together, these results suggest that the favorable actions of *NOVA1* silencing in NET cells could be exerted by increasing cell senescence, through the activation of PTEN/p53 pathway and the accompanying biasing of *TP53* transcription against the truncated *Δ133TP53* isoform. It remains to be elucidated whether NOVA1, through its pre-mRNA binding capacity, can interact directly with *TP53* transcripts to modulate their processing balance.

The modulation of *NOVA1* may also entail clinically relevant implications, in that *NOVA1* silencing increased the antiproliferative effect of everolimus, an mTOR inhibitor a widely used in NETs, in QGP-1 cells, whereas no such additive effect was found in BON-1 cells. These differential, cell line-dependent results might be in consonance with our previous findings and support a role of *NOVA1* on senescence, given that, in parallel and for the same reason exposed above, the additive effect was observed in QGP-1 cells but no in BON-1 cells. Although, our present findings also suggest that the action of NOVA1 in MAPK pathway may not be independent of mTOR, because its silencing in BON-1 cells has an effect in the activation of ERK, in spite of it not being additive to everolimus action. These complex differences between cell lines could be attributable to distinct mutations in specific components of the AKT/mTOR pathway that are differentially present in one cell line and not in the other one. This is, for example, the case of *TSC2*, one of the most important inhibitors of mTOR [318], that is mutated in BON-1 but not in QGP-1 [239], a divergence that could distinctly influence the effect of everolimus and its combined action with *NOVA1* silencing. Thus, because the set of specific mutations substantially differs in each PanNET patient and may even evolve over time in a given tumor [319-321] our results suggest that further research on *NOVA1* may guide to identify novel relevant targets with therapeutic potential in a personalized manner.

When viewed together, our results reveal, for the first time, that the splicing machinery is profoundly altered in PanNETs, most of its components being upregulated. Expression of some components is associated with clinical parameters and can efficiently discriminate between tumoral and non-tumoral samples. Importantly, *in vitro* and *in vivo* studies revealed that the factor *NOVA1* can modulate proliferation and senescence in PanNETs cell lines, where it alters key signaling pathways and splicing mechanisms, and may alter the response to everolimus. These data support the splicing factor NOVA1 as a promising candidate to develop novel biomarkers and therapeutic target in PanNETs.

# 6. CONCLUSIONS

# 6. Conclusions

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

1. The *SSTR1* gene is overexpressed in PCa, where it may be regulated by specific miRNAs and could have relevant functional implications. Specifically, $SST_1$ is directly related with the inhibition of cell proliferation and PSA secretion in 22Rv1 cell line, probably by the modulation of pathways and mediators linked to AR and PI3K/AKT-CCND3 pathways.

2. The expression of the *SSTR5* in somatotropinomas and PanNETs may be controlled by epigenetic mechanisms, including DNA methylation and post-transcriptional events, such as antisense-mediated regulation. In particular, *SSTR5-AS1* may be participating in the control of key tumor features, including proliferation, migration and colony formation, and in the effect of pasireotide treatment, a selective analog for $SST_5$.

3. The components of the splicing machinery are profoundy dysregulated in PanNETs, generally overexpressed. The levels of some of them are associated with important clinical parameters and could distinguish between tumor and non-tumor samples with a high efficiency. Specifically, the augmented level of the splicing factor *NOVA1* promotes the increase of cell proliferation and senescence pathway in PanNETs models, by altering key signaling pathways, and it is able to compromise the effectiveness of everolimus treatment.

**GLOBAL COROLLARY**

As a **general conclusion**, the studies implemented in the present Thesis allow to expand and advance in the knowledge of the molecular basis of the pathophysiological regulation of endocrine-related cancers and neuroendocrine tumors by two specific somatostatin receptors and splicing machinery. Specifically, our results demonstrate that *SSTR1* in the case of PCa, *SSTR5* in NETs and splicing factor *NOVA1* in PanNETs, represent relevant points of regulation for these tumors and, thus, they could be useful tools for the develop of novel diagnostic biomarkers and/or therapeutic targets to improve the future treatment of those pathologies.

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