DEPARTAMENTO DE BIOLOGÍA
CELULAR, FISIOLOGÍA E INMUNOLOGÍA

Functional role and therapeutic potential of the somatostatin and ghrelin systems and their splicing variants in prostate cancer.

PAPEL FUNCIONAL Y POTENCIAL TERAPÉUTICO DE LOS SISTEMAS SOMATOSTATINA Y GHRELINA Y SUS VARIANTES DE SPLICING EN TUMORES DE PRÓSTATA.

Daniel Hormaechea Agulla

Córdoba 2017

TITULO: *Functional role and therapeutic potential of the somatostatin and ghrelin systems and their splicing variants in prostate cancer*

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Memoria de Tesis Doctoral presentada por **Daniel Hormaechea Agulla**, Licenciado en Biología, para optar al grado de **Doctor en Biomedicina**.

Los directores

Dr. Raúl M. Luque Huertas y Dr. Justo P. Castaño Fuentes
Profesores del Departamento de Biología Celular de la Universidad de Córdoba

En Córdoba, a 17 de abril de 2017

UNIVERSIDAD DE CÓRDOBA

IMIBIC

D. Raúl M. Luque Huertas y D. Justo Pastor Castaño Fuentes Profesores del Departamento de Biología Celular, Fisiología e Inmunología de la Universidad de Córdoba,

INFORMAN

Que D. Daniel Hormaechea Agulla, Licenciado en Biología, ha realizado bajo nuestra dirección el trabajo titulado "**Functional role and therapeutic potential of the somatostatin and ghrelin systems and their splicing variants in prostate cancer.**" y que bajo nuestro juicio reúne los méritos suficientes para optar al Grado de Doctor en Biomedicina.

Y para que conste, firmamos la presente en Córdoba, a 17 de abril de 2017.

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

TITULO DE LA TESIS: Functional role and therapeutic potential of the somatostatin and ghrelin systems and their splicing variants in prostate cancer.

DOCTORANDO: Daniel Hormaechea Agulla

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

(Se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

Durante el desarrollo de la presente Tesis Doctoral, el doctorando Daniel Hormaechea Agulla ha alcanzado e incluso superado los objetivos planteados al comienzo de la misma, al tiempo que ha desarrollado técnicas experimentales de gran utilidad para el grupo de investigación, que le han permitido obtener resultados muy relevantes en el campo del cáncer de próstata. Concretamente, como fruto de su trabajo ha publicado hasta la fecha un trabajo directamente relacionado con su tesis doctoral, en la revista *Cancer Letters*, de gran relevancia dentro de nuestra área de investigación. Además, el doctorando ha realizado una estancia en la universidad de Adelaida, Australia, donde amplió y perfeccionó diversos métodos de trabajo. Por último, el doctorando ha presentado sus resultados en diferentes congresos de ámbito nacional e internacional, de los que han derivado varios capítulos de libro.

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

Córdoba, 17 de abril de 2017

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

Esta Tesis Doctoral ha sido realizada en el Departamento de Biología Celular, Fisiología e Inmunología de la Universidad de Córdoba, bajo la dirección de los Dres. Raúl M. Luque Huertas y Justo P. Castaño Fuentes. Dicho proyecto fue subvencionado mediante los Proyectos PI13/00651, PI16/00264 (Proyectos de Investigación en Salud FIS, funded by Instituto de Salud Carlos III and co-funded by European Union (ERDF/ESF, "Investing in your future"), BFU2013-43282-R, BFU2016-80360-R (MINECO), BIO-0139, CTS-1406, (Junta de Andalucía) and CIBERobn. CIBER is an initiative of Instituto de Salud Carlos III, Ministerio de Sanidad, Servicios Sociales e Igualdad, Spain.

LIST OF ABBREVIATIONS

aa= Amino acids

ADT= Androgen deprivation therapy

AR= Androgen receptor

BPH= Benign Prostatic Hypertrophy

CK= Cytokeratin

CORT= Cortistatin

CRPC= Castration-Resistant Prostate cancer

DHT= Dihydrotestosterone

DRE= Digital Rectal Exam

ER= Estrogen Receptor

ERG=ETS-related gene

ETS=E26 transformation-specific or E-twenty-six

FFPE= Formalin-Fixed, Paraffin-Embedded

GHRL=Ghrelin

GHS-R = Ghrelin receptor

GOAT = Ghrelin O-acyltransferase

GR= Glucocorticoid Receptor

GS= Gleason Score

MBOAT = Membrane-bound O-acyltransferase

miRNA= micro-RNA

SNPs= Single Nucleotide Polymorphisms

SSAs= Somatostatin analogs

SST= Somatostatin

sst= Somatostatin receptors

PCa= Prostate Cancer

PCA3=Prostate Cancer Antigen 3

PIN= Prostatic Intraepithelial Neoplasia

PSA= Prostate-Specific Antigen

PR= Progesterone

PGR= Progesterone Receptor

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SUMMARY

1. SUMMARY

Cancer is one of the most serious and complex threats to the health of the population in the world. Specifically, prostate cancer is the second most common solid tumor in men, causing 300,000 deaths each year around the world. Since its appearance in the 1990s, the PSA ("Prostate Specific Antigen") test revolutionized the diagnosis of prostate cancer by increasing the number of patients diagnosed at an early-stage of the disease. However, due to the complex biology and heterogeneity of prostate cancer, most of the tumors detected by the PSA test show a low aggressiveness and the likelihood of the patient dying because of their development is also relatively low. Unfortunately, the treatment of "low-aggressive" tumors can produce more side effects than the tumor itself. In addition, because PSA can be elevated by benign causes (prostatitis, medication, etc.), it presents a considerable ratio of false positives. Although other biomarkers are currently being studied, none of them have shown enough clinical evidence to justify their use, and therefore, new biomarkers are required to complement the use of the PSA test.

Prostate cancer is dependent on androgen stimulation for its survival and cell proliferation. Two phases can be differentiated in the clinic of prostate cancer: a first phase in which patients are treated hormonally with anti-androgens or with LHRH analogs or antagonists, with the aim of blocking the production of androgens, and a second phase known as "Castration-Resistant Prostate Cancer" (CRPC), in which tumors become resistant to hormone therapy. Currently, it is well accepted that residual androgens remaining after castration and the androgen receptor play vital roles in the biology of the CRPC. In fact, the appearance of truncated forms of aberrant splicing from the canonical androgen receptor gene (i.e. AR-V7) is one of the most important phenomena of this phase, and currently, its presence is considered as a marker of prognosis and an indicator of resistance to hormone therapy.

Numerous neuropeptides, such as those belonging to the family of factors regulating the hypothalamic-pituitary axis, play multiple roles in various tissues under normal conditions and in pathological situations, such as cancer. A common feature of many of these altered endocrine-metabolic conditions is the atypical or ectopic presence, alteration or the total or partial loss of molecules that, like neuropeptides and their

receptors, can control key cellular functions. In fact, numerous studies indicate that the processes of alternative splicing, and in particular the appearance of forms of non-canonical or aberrant splicing, contribute to the development of tumor pathologies, and in many cases, are potential biomarkers of the disease, as it is the case of the androgen receptor variants in prostate cancer. In this context, the objective of this Doctoral Thesis has been to study the presence and functional role of two pleiotropic systems that control body homeostasis; specifically, the system formed by somatostatin (SST), cortistatin (CORT) and their receptors (sst1-5), including the sst5TMD4/5 splice variants, and the ghrelin system and their receptors (GHSR1a/b), including the splice variant In1-ghrelin, as well as the enzyme GOAT, in human samples of healthy prostate and prostate cancer. In addition, we evaluated the usefulness of some of these molecules as new biomarkers of diagnosis and/or prognosis of the disease, and their therapeutic potential in prostate cancer.

Initial evaluation of somatostatin and ghrelin systems expression using the quantitative real-time polymerase chain reaction (qPCR) demonstrated a profound dysregulation of some of the components of both systems in prostate cancer. Specifically, sst1 and sst5TMD4 receptors from somatostatin system, and GOAT enzyme and In1-ghrelin alternative splicing variant from ghrelin system, were found to be significantly overexpressed in prostatic tumor tissues compared to prostate healthy tissues. Overexpression of the sst1 receptor has been previously reported and has been related to the aggressiveness of the disease. On the contrary, our results are the first to demonstrate the overexpression of sst5TMD4, In1-ghrelin, and GOAT in prostate cancer.

The overexpression of the truncated sst5TMD4 receptor was especially marked in patients with metastases and with worse prognosis, and therefore could become a new biomarker of aggressiveness. This result was first found with the qPCR technique and was subsequently corroborated at the protein level in another cohort of patients using the immunohistochemistry technique (IHC). Next, the consequences of sst5TMD4 overexpression in prostate cancer were analyzed. For this, in vitro models (androgen-dependent and castration-resistant prostate cancer cell lines) and in vivo models (xenografts in immunodeficient mice) were used to demonstrate that sst5TMD4 overexpression clearly increased the malignancy of prostatic tumor cells (i.e. cell proliferation and cell migration), and that such effects were carried out through the modulation of various key cellular signaling pathways in the biology of prostate cancer

(i.e. MAPK / ERK, MAPK / JNK, MYC / MAX, WNT, RB and TGF-β), and dysregulation of the expression of oncogenes (i.e. CAV1, IL-6) and tumor suppressor genes (i.e. CDKN2A, APC, SFRP1, NRIP1, RARB, LOXL1). Finally, with the aim of demonstrating the therapeutic potential of sst5TMD4 in prostate cancer, endogenous expression of sst5TMD4 was silenced by the design, validation and use of a siRNA ("small interfering RNA") specific for the sst5TMD4 sequence. Silencing decreased cell proliferation and migration of these cells compared to control cells.

In the case of the enzyme GOAT, its expression was increased in prostate tumor samples compared to controls, where it was correlated with metabolic parameters of the patients, such as weight, BMI or presence of dyslipidemia. This result was corroborated at the protein level by IHQ using a GOAT-specific antibody. GOAT overexpression was also observed in prostate cancer-cell lines compared to normal prostate-like cell lines. In addition, our results demonstrated, for the first time, that prostate tumor cells are able to secrete GOAT, and that the treatment with different metabolic signals (i.e. insulin, IGF-1, ghrelin and/or In1-ghrelin peptides) regulate its expression at the level of the prostate. Interestingly, GOAT protein levels were increased in the plasma and urine of patients with prostate cancer compared to controls, and correlated with aggressiveness factors of the disease and with different metabolic parameters, such as the presence of diabetes and dyslipidemia. In addition, we demonstrated that plasma levels of GOAT could serve as a new non-invasive biomarker for the diagnosis of prostate cancer, especially in non-diabetic patients in whom the sensitivity and specificity of plasma levels of GOAT to detect the disease were particularly high.

Likewise, our results demonstrate that In1-ghrelin expression, but not native ghrelin, is significantly elevated in prostate cancer biopsies and prostate cancer cell lines, as well as in plasma and urine samples from patients with prostate cancer, in comparison with normal tissue samples or from healthy patients. Given that both In1-ghrelin and native ghrelin were expressed in healthy prostate tissue, we investigated the possible functional role that both molecules could have in prostate physiology. For this, cell cultures of healthy prostate cells treated with the acylated peptides of Inl-ghrelin or native ghrelin were performed. It was interesting to note that the treatment with In1-ghrelin, but not ghrelin, peptides increased PSA expression (mRNA) and secretion (protein). In addition, overexpression of In1-ghrelin, but not ghrelin, increased the proliferation of healthy prostate cells. Next, we analyzed the pathophysiological role that the

overexpression of In1-ghrelin could present in prostate cancer. We used different prostate cancer-cell lines that represent the broad spectrum of the disease. Both, treatment with In1-ghrelin peptides and transfection with an In1-ghrelin expression vector increased cell proliferation and migration of the different prostate cancer cell lines. Moreover, this result was corroborated in a preclinical model of in vivo xenograft in immunodefficient mice. We demonstrate that the pathophysiological effects of In1-ghrelin are likely mediated through the modulation of the ERK / kinase pathway activity, increased expression of certain oncogenes (i.e. CAV1, CAV2, IGFBP-5, LOXL1) and a decrease in certain tumor suppressor genes (i.e. APC, SFRP1, NRIP1). Finally, in order to demonstrate the therapeutic potential of In1-ghrelin in prostate cancer, In-1 ghrelin expression was silenced by the design, validation and use of two siRNAs ("small interfering RNA") specific for the In1-ghrelin sequence in several prostate cancer cell lines; such silencing decreased cell proliferation and PSA secretion from those cells in comparison to control cells.

Therefore, the results obtained in this Doctoral Thesis on the expression, regulation and the pathophysiological role of sst5TMD4 and In1-ghrelin variants and GOAT enzyme in the context of prostate cancer, clearly support the relevance of the somatostatin and ghrelin systems, especially their forms of alternative splicing, in the development and progression of this cancer type. Specifically, our findings demonstrate that the sst5TMD4 and In1-ghrelin splicing variants and the GOAT enzyme are overexpressed in prostate cancer where they induce important alterations related to tumor aggressiveness. Therefore, these molecules could serve as new diagnostic and/or prognostic biomarkers and could represent new therapeutic targets for the treatment of prostate cancer.

RESUMEN

1. RESUMEN

El cáncer es una de las amenazas más serias y complejas para la salud de la población mundial. En concreto, el cáncer de próstata es el segundo tipo de tumor sólido más frecuente entre los hombres, y causa 300.000 muertes al año en todo el mundo. Desde su aparición en la década de los 90, el test del PSA ("antígeno prostático específico") revolucionó el diagnóstico del cáncer de próstata, incrementado el número de pacientes diagnosticados en una fase temprana de la enfermedad. Sin embargo, debido a la compleja biología y heterogeneidad de los tumores prostáticos, la mayoría de los tumores detectados por el test del PSA presentan una baja agresividad, y la probabilidad de que el paciente muera debido a su desarrollo también es relativamente baja. Desafortunadamente, el tratamiento de tumores de "baja agresividad" puede producir por sí mismo más efectos secundarios que el propio tumor. Además, debido a que el PSA se puede elevar por causas benignas (prostatitis, toma de medicamentos, etc), presenta un elevada proporción de falsos positivos. Aunque se están estudiando en la actualidad otros biomarcadores, ninguno de ellos ha mostrado evidencia clínica suficiente como para justificar su uso y, por tanto, se requieren nuevos biomarcadores que complementen y/o mejoren el uso del test de PSA.

El cáncer de próstata depende de la estimulación androgénica para su supervivencia y proliferación celular. En la clínica del cáncer de próstata se pueden diferenciar dos fases: una primera fase en la cual los pacientes se tratan hormonalmente con el objetivo de bloquear la producción de andrógenos mediante anti-andrógenos o con análogos o antagonistas de LHRH, y una segunda fase conocida como "cáncer de próstata resistente a la castración" (en inglés CRPC "Castration-Resistant Prostate Cancer"), en la cual los tumores se vuelven resistentes a la terapia hormonal. Actualmente, se concede un papel vital a los andrógenos residuales tras la castración y al receptor de andrógenos en la biología del CRPC. De hecho, la aparición de formas truncadas de splicing aberrante del receptor de andrógenos (AR-V7) es uno de los fenómenos más importantes de esta fase, y su presencia se considera como un marcador de prognosis e indicador de resistencia a la terapia hormonal.

Numerosos neuropéptidos, como los pertenecientes a la familia de factores reguladores del eje hipotálamo-hipofisario, desempeñan multitud de funciones en diversos tejidos bajo condiciones normales o en situaciones patológicas, como el cáncer. Una característica común de muchas de estas situaciones endocrino-metabólicas es la presencia atípica o ectópica, la alteración o la pérdida total o parcial de moléculas que, como los neuropéptidos y sus receptores, pueden controlar funciones celulares clave. De hecho, numerosos estudios indican que los procesos de splicing alternativo, y en particular la aparición de formas de splicing no canónico o aberrante, contribuyen al desarrollo de las patologías tumorales, y en muchos casos son potenciales biomarcadores de la enfermedad, como es el caso de las variantes del receptor de andrógeno en cáncer de próstata. En este sentido, el **objetivo de la tesis doctoral** se ha centrado en estudiar la presencia y el papel funcional que ejercen dos sistemas pleiotrópicos de control del organismo; en concreto, el sistema formado por la somatostatina (SST), la cortistatina (CORT) y sus receptores (sst1-5), incluyendo las variantes de splicing sst5TMD4/5, y el sistema de la ghrelina y sus receptores (GHSR1a/b), que incluye la variante de splicing In1-ghrelina, y la enzima GOAT, en muestras humanas de próstata sana y de cáncer de próstata. Asimismo, se evaluó la utilidad de algunas de estas moléculas como nuevos biomarcadores de diagnosis y/o prognosis de la enfermedad, y su potencial terapéutico en cáncer de próstata.

El análisis inicial mediante la técnica de PCR cuantitativa en tiempo real (qPCR) de la expresión de los sistemas somatostatina y ghrelina demostró una profunda desregulación de algunos de los componentes de ambos sistemas en cáncer de próstata. Concretamente, se encontró que los receptores sst1 y sst5TMD4 del sistema somatostatina, y la enzima GOAT y la forma de splicing alternativo In1-ghrelina del sistema ghrelina, se encuentran significativamente sobreexpresados en tejidos tumorales prostáticos. La sobreexpresión del receptor sst1 se ha publicado previamente y se relaciona con la agresividad de la enfermedad. Por el contrario, nuestros resultados son los primeros que demuestran la sobreexpresión de sst5TMD4, In1-ghrelina y GOAT en cáncer de próstata.

La sobreexpresión del receptor truncado sst5TMD4 fue especialmente significativa en los pacientes con presencia de metástasis y con peor prognosis, pudiendo ser por ello un nuevo biomarcador de agresividad. Este resultado fue descubierto inicialmente con la técnica de qPCR y se corroboró después a nivel proteico en otra

cohorte de pacientes mediante la técnica de inmunohistoquimica (IHQ). Posteriormente, se analizaron las consecuencias que podría ejercer la sobreexpresión de sst5TMD4 en cáncer de próstata. Para ello, se usaron modelos *in vitro* (líneas celulares de cáncer de próstata andrógeno-dependientes y líneas resistentes a la castración) e *in vivo* (xenotransplantes en ratones inmunodeprimidos) para demostrar que la sobreexpresión de sst5TMD4 incrementa claramente la malignidad de las células prostáticas tumorales (ej. proliferación y migración celular) y que dichos efectos se llevaban a cabo a través de la modulación de diversas vías de señalización celular claves en la biología del cáncer de próstata (ej. MAPK/ERK, MAPK/JNK, MYC/MAX, WNT, RB y TGF-β), y de la desregulación de la expresión de oncogenes (ej. CAV1, IL-6) y genes supresores de tumores (ej. CDKN2A, APC, SFRP1, NRIP1, RARB, LOXL1). Finalmente, y con el objetivo de demostrar el potencial terapéutico de sst5TMD4 en cáncer de próstata, se silenció la expresión endógena de sst5TMD4 mediante el diseño, validación y utilización de un siRNA ("ARN pequeño de interferencia") específico para la secuencia de sst5TMD4. El silenciamiento disminuyó la proliferación y migración de las células en comparación con células control.

Por su parte, la expresión de la enzima GOAT se encontró incrementada en muestras tumorales prostáticas respecto a controles, y dicha expresión se correlacionó con parámetros metabólicos de los pacientes tales como el peso, su índice de masa corporal (IMC) o la presencia de dislipidemia. Este resultado se corroboró a nivel proteico, mediante IHQ, con un anticuerpo específico de GOAT. La sobreexpresión de GOAT se observó también al comparar líneas celulares de cáncer de próstata respecto a líneas celulares de próstata normal. Además, por primera vez se demostró que las células prostáticas tumorales son capaces de secretar enzima GOAT y que el tratamiento con diferentes señales metabólicas (ej. insulina, IGF-1, ghrelina y/o In1-ghrelina) regulan su expresión en la próstata. Mediante un ELISA específico se descubrió que los niveles de esta enzima están aumentados en el plasma y orina de pacientes con cáncer de próstata respecto a controles y que se correlacionan con factores de agresividad de la enfermedad, así como con diferentes parámetros metabólicos, tales como la presencia de diabetes y dislipidemia. Además, demostramos que los niveles plasmáticos de GOAT podrían servir como un nuevo biomarcador no invasivo para el diagnóstico del cáncer de próstata, especialmente en pacientes no diabéticos en los que la sensibilidad y especificidad de los niveles plasmáticos de GOAT para detectar la enfermedad son particularmente elevadas.

Asimismo, nuestros resultados demuestran que los niveles de In1-ghrelina, pero no los de ghrelina nativa, se encuentran significativamente elevados en muestras de tejido y en líneas celulares de cáncer de próstata, así como en muestras de plasma y orina de pacientes con cáncer de próstata en comparación con muestras de tejido normal o de pacientes sanos. Dado que tanto In1-ghrelina como ghrelina nativa se expresan en tejido sano prostático, investigamos el posible papel funcional que ambas moléculas podrían presentar en la regulación de la función prostática. Para ello se realizaron cultivos celulares de células prostáticas no tumorales, que se trataron con los péptidos acilados de In1-ghrelina o ghrelina nativa. Resultó interesante observar que el tratamiento con los péptidos de la In1-ghrelina, pero no con ghrelina aumenta la expresión (mRNA) y secreción (proteína) de PSA. Además, la sobreexpresión de In1-ghrelina, pero no de ghrelina, aumentó la proliferación de las células prostáticas sanas. Posteriormente analizamos el papel patofisiológico de la sobreexpresión de In1-ghrelina en cáncer de próstata. Para ello, utilizamos diferentes líneas celulares de cáncer de próstata que representan el amplio espectro de la enfermedad. Tanto el tratamiento con péptidos In1-ghrelina como la transfección con un vector de expresión de In1-ghrelina aumentó la proliferación y migración celular de las diferentes líneas celulares de cáncer de próstata. Más aún, este resultado se corroboró en un modelo preclínico *in vivo* de xenotransplante en ratones inmunodeprimidos. Se comprobó que los efectos patológicos de la In1-ghrelina se llevaban a cabo a través de la modulación de la actividad de la vía de las quinasas ERK, de un aumento de expresión de ciertos oncogenes (ej. CAV1, CAV2, IGFBP-5, LOXL1) y por una disminución de determinados genes supresores tumorales (ej. APC, SFRP1, NRIP1). Finalmente, y con el objetivo de demostrar el potencial terapéutico de In1-ghrelina en cáncer de próstata, se silenció la expresión endógena de In1-ghrelina mediante el diseño, validación y aplicación de dos siRNAs ("ARN pequeño de interferencia") específicos para la secuencia de In1-ghrelina en varias líneas celulares de cáncer de próstata; dicho silenciamiento disminuyó la proliferación celular y la secreción de PSA de esas células en comparación con células control.

Por todo ello, los resultados obtenidos en la presente Tesis Doctoral sobre la regulación de la expresión y el papel patofisiológico de las variantes de splicing sst5TMD4 e In1-ghrelina y de la enzima GOAT en el contexto del cáncer de próstata, apoyan claramente la relevancia de los sistemas somatostatina y ghrelina, especialmente de sus formas de splicing alternativo, en el desarrollo y progresión tumoral de este tipo

de cáncer. Específicamente, nuestros hallazgos demuestran que las variantes de splicing sst5TMD4 e In1-ghrelina y la enzima GOAT están sobreexpresadas en cáncer de próstata, donde inducen importantes alteraciones relacionadas con la agresividad tumoral. Por tanto, estas moléculas podrían servir como nuevos biomarcadores de diagnóstico y/o pronóstico y podrían representar nuevas dianas terapéuticas para el tratamiento del cáncer de próstata.

INTRODUCTION

2. INTRODUCTION

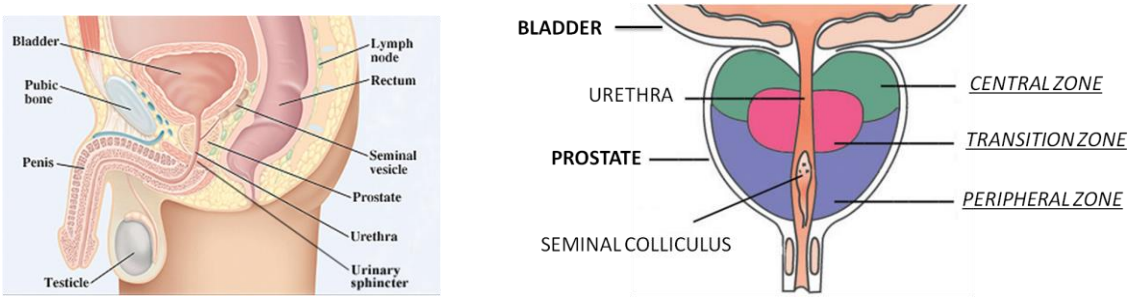
2.1. Prostate gland

The prostate is a gland about the size and shape of a walnut (20 g and 4 × 2.5 cm) which is part of the male reproductive system [1, 2]. Its physiological function is the production of a fluid that, along with sperm cells from the testicles and fluids from other glands, makes up semen [3]. The muscles of the prostate ensure that the semen is forcefully pressed into the urethra and then expelled outwards during ejaculation.

The prostate is located directly below the bladder and above the muscles of the pelvic floor. The rectum is behind the prostate, making it possible to feel the gland from the rectum using the finger. The ducts in the prostate gland flow into the urethra, which passes through the prostate.

The adult human prostate is organized in three zones: central zone, transition zone, and peripheral zone (70% of total volume), reflecting three distinct sets of ducts [4] (**Figure 1A**). Most prostatic tumors (70%) start from the peripheral zone, whereas the transition zone is the origin in 20% of total cases [5]. Prostatic ducts are lined by a pseudostratified epithelium that contains three major cell types: luminal secretory epithelial cells, basal epithelial cells, and rare neuroendocrine cells, which can be distinguished by their patterns of expression of specific differentiation markers [2, 6] (**Figure 1B**). These cells have an apical–basal polarity and secrete prostatic proteins and fluids from their apical surface into the prostatic lumen. Finally, the stromal layer is mainly formed by smooth muscle cells, and also contains fibroblastic, neuronal, lymphatic, and vascular cell types [2]. Reciprocal interactions between the epithelium and stroma are critical for maintaining prostate homeostasis and normal prostate function [7].

A.



B.

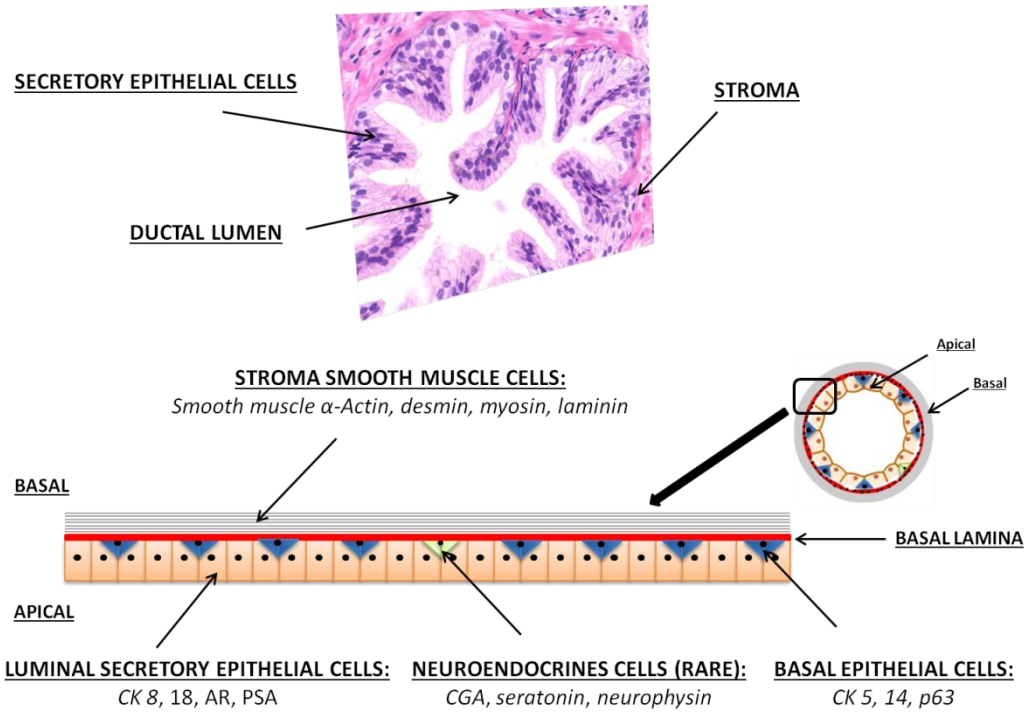


Figure 1: The human prostate gland. **A)** Sagittal view of the localization of prostate in the body, and different zones (peripheral, transition and central) inside the organ. **B)** Structure and cells of a prostatic duct. Each epithelial cell type (basal, luminal and neuroendocrine cells) harbors specific markers (indicated below each cell type) that allow its identification. Figure 1A is taken from https://www.fairview.org/healthlibrary/Article/87095 while Image 1B is taken from WebPatholohy website: http://webpathology.com/.

2.1.1. Physiological regulation of prostate by hormones and its dysregulation in prostate cancer

Prostate development and function is regulated by multiple hormones [2, 3, 8]. Specifically, different classes of steroids hormones (androgens, estrogens, progesterone

and glucocorticoids) initiate distinct actions via binding to their cognate nuclear receptor proteins [9]. However, clinical and laboratory data have shown clear evidence that androgens are the most important hormones regulating the prostate gland [2].

2.1.1.1. Androgens

Androgens synthesis and secretion are under the physiological regulation of the hypothalamic–pituitary–testicular axis, including the production of testosterone in testis (which represents 95% of total testosterone) [10, 11], and the activity of adrenal glands, which produce other androgens such as dehydroepiandrosterone sulfate and androstenedione [12] (**Figure 2**). Androgens exert their action through binding to a nuclear receptor called Androgen Receptor (AR), which controls the transcription of hundreds of genes involved in prostate development, growth, and function [13] (**Figure 2**). Specifically, during development, AR drives luminal epithelial proliferation and differentiation of the prostate [14]. Moreover, orchiectomy and/or chemical castration produces a total inhibition of mice prostate fetal development [15]. Several studies have also demonstrated that the Androgen/AR-axis is indispensable for prostate development [8, 16, 17] In fact, it has been shown that mice and human with dysfunctional AR expression do not develop a functional prostate [3, 16-18]. DHT (*5α-dihydrotestosterone*; the reduced metabolite of testosterone) is the main androgen involved in prostate function [19]. Intracellularly, testosterone is converted to DHT by the enzyme 5α-reductase; indeed, DHT has ~5-10 times higher affinity for the AR than testosterone [20]. In human, the deficiency of 5α-reductase enzyme produces a complete absence of prostate morphogenesis [21]. There are many genes whose expression is under the regulation of AR but KLK3 (kallikrein related peptidase 3, commonly known as PSA; prostatic specific antigen) is well-known for being one of the most important factors due to its critical function and clinical application. Specifically, PSA is involved in the liquefaction of seminal coagulum, presumably by the hydrolysis of semenogelin-1 and Galectin-3 [22-24]. Remarkably, PSA is almost exclusively expressed in the prostate epithelium (luminal cells) and clinically it is used as biomarker of PCa because its serum levels are generally higher in patients with PCa [25].

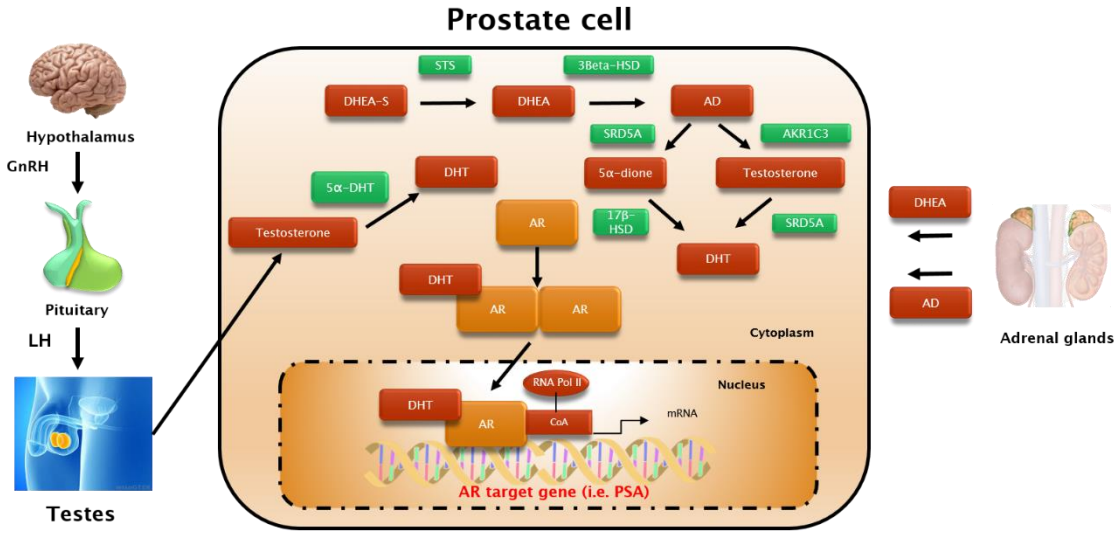


Figure 2: Representative scheme of the association between the hypothalamic-pituitary-testicular axis and adrenal gland with prostate cell intrinsic factors. The gonadotropin-releasing hormone (GnRH) is produced in the hypothalamus and stimulates the secretion of the luteinizing hormone (LH) from gonadotrope cells of the pituitary gland. Eventually, LH will reach the testes where it induces the secretion of testosterone from Leydig cells. When testosterone enters in the prostate, it is rapidly converted to dihydrotestosterone (DHT) by the 5α-reductase enzyme (5α-DHT) and binds to androgen receptor (AR). The AR-DHT complex enter in the nucleus and binds to AR-target gene domains in the DNA in order to regulate their expression. Other source of androgens that will be converted to DHT in prostate come from the adrenal glands which produce dehydroepiandrosterone sulfate (DHEA-S) and androstenedione (AD).

2.1.1.2. Estrogens

The majority of estrogens present in men are derived from the peripheric conversion, mainly in the adipose tissue, of androstenedione and testosterone to estrone and estradiol [26]; however, small amounts are also produced in the testes and directly secreted to blood [27]. Together with AR, ER (estrogen receptor) is also important to drive luminal epithelial proliferation and differentiation of fetal prostate [28]. Moreover, estrogens play a dual role in the regulation of adult prostate due to their antagonistic role, increasing or decreasing proliferation depending on the receptor to which it binds to [13]. Specifically, it has been shown that estrogens increase proliferation after binding ERα but decrease proliferation through ERβ [29]. In addition, there is a growing body of evidence supporting the notion that estrogens play a pathophysiological role in PCa mainly by the effects trigger binding to ERα, since this receptor is upregulated in PCa [30].

2.1.1.3. Progesterone

Progesterone (PR) is another steroid that controls prostate homeostasis [31]. PR exerts its effects through its receptor, PGR, whose two isoforms (PGR*A* and PGR*B*) are expressed in stromal cells] [31]. Several studies have shown that PR is able to decrease cell growth and invasion of normal prostate cells and PCa cell lines by binding to both PGR isoforms and through the modulation of different intracellular mechanisms [31, 32]. It has also been shown that PR protein levels are decreased in PCa stroma as compared with benign tissue, suggesting a putative role of this system in the homeostatic control of the prostate as well as in PCa progression [32].

2.1.1.4. Glucocorticoids

Glucocorticoids are produced in the adrenal gland and mediate systemic effects through binding to the Glucocorticoid Receptor (GR) [9]. GR is expressed in normal prostate, although its physiological role as regulator of prostatic biology is still unclear [33]. Mice lacking endogenous expression of GR in prostate do not show any significant change in cell growth or morphogenesis [34]. However, GR expression is decreased in early stages of human PCa with only 30% of primary prostatic tumors showing GR expression [35]. Moreover, some studies using *in vitro* models have shown a tumor-suppressor role of GR in PCa in terms of cell proliferation or migration [35]. Additionally, GR expression is upregulated in late phases of the disease (androgen ablation PCa/AR blockade), and, therefore, it has been suggested that GR overexpression in the prostate epithelia may be associated with the response to androgen deprivation therapy [36]. Recent studies have shown that AR and GR are capable of binding to the same sites on chromatin [36, 37] and that genes induced by GR and DHT are highly overlapping [9]. Furthermore, the treatment with AR antagonist increases GR expression in few days [36], a process called "*Steroid receptor switch*". This observation might be very important from a clinical point of view since hormone-refractory PCa patients are treated with chemotherapeutic agents (i.e. docetaxel) and with glucocorticoids to ease side-effects by chemotherapeutic agents [9]; therefore, the treatment with glucocorticoids could represent a tumor alternative pathway to bypass the androgen blockade [9].

2.2. Clinical significance

2.2.1. Prostatitis

Prostatitis is a common inflammation of the prostate that usually occurs in men under age 50 [38]. The cause can be due to an infection or other causes such as hormones dysregulations (i.e. estradiol), physical trauma, urine reflux or dietary habits (i.e. charred meat) [39]. Infiltration of immune cells (*T cells*, *B cells*, *macrophages* and *mast cells*, mainly) and production of cytokines (IL-6, IL-1 or TNF-α) are typical features of prostatitis [39].

Remarkably, some models reflect the possible relationship between inflammation and early PCa-onset in which the inflammation caused by different agents would produce a precursor lesion in the prostate called PIA (Proliferative Inflammatory Atrophy) that in many cases will be healed, but sometimes could be the precursor of other lesions named PIN (Prostatic Intraepithelial Neoplasia) and/or PCa [40-42]. In fact, morphological transitions between PIA, PIN, and PCa have been reported and some of the genetic alterations of prostate are shared with PIA and PIN lesions (i.e. loss of tumor suppressor genes such as *NKX3.1* and *p27*, promoter hypermethylation of *GSTP1*, etc.) [40, 41, 43].

2.2.2. Benign prostatic hyperplasia (BPH)

BPH is the most common urological disease in men after 50-year-old [44]. It courses as an enlargement of the prostate associated with symptoms like poor urine flow, intermittent stream or sensation of poor bladder emptying [44]. Age is the main risk factor for developing BPH since 50% of men over 75 ages have this disease [44, 45]. Androgen/AR signaling plays key roles in increasing cell proliferation in both stromal and epithelial cells, therefore boosting development of BPH [46]. Some patients present increased PSA levels in serum due to enlarged organ volume, inflammation and AR activity; however, BPH is not considered to be a premalignant lesion [47].

2.2.3 PIN

PIN is the most established precursor of PCa [48]. Indeed, both processes share genetic and molecular markers, with PIN representing an intermediate stage between benign epithelium and invasive malignant carcinoma [49]. As PCa, PIN is more common with advanced age with higher rates of occurrence in the peripheral zone of the prostate.

The presence of PIN lesions is associated with 20%-25% of PCa risk in subsequent repeat biopsies [49]. Based on how abnormal the patterns of cells look, PIN are classified as: 1) Low-grade PIN (LG-PIN; the patterns of prostate cells appear almost normal), and 2) High-grade PIN (HG-PIN; the patterns of cells look more abnormal).

Histologically, HG-PIN presents nuclear cell enlargement, hyperchromasia and prominent nucleoli [49].

2.2.4. Prostate cancer

2.2.4.1. Epidemiology

PCa is the second most common cancer in the male population and the fourth most common worldwide [50]. It is estimated that one of seven men will develop PCa in his time life [51]. In 2013, 1.4 million of new cases and 293,000 deaths were reported around the world, being the leading cause of cancer death for men in 24 countries [50]. The incidence is higher in developed countries and in Western population (North America and Europe) thanks to the PSA screening [50]. PCa is mainly diagnosed at old age (>60 years) and usually when the tumor is localized (81% of the cases; regional lymph nodes: 12%; distant: 4%) [51] (**Figure 3**). The 5-year relative survival is ≥ 99% when the tumor is localized or regional lymph nodes but only of 24% when the tumor is already distant at the diagnosis [51] (**Figure 3**).

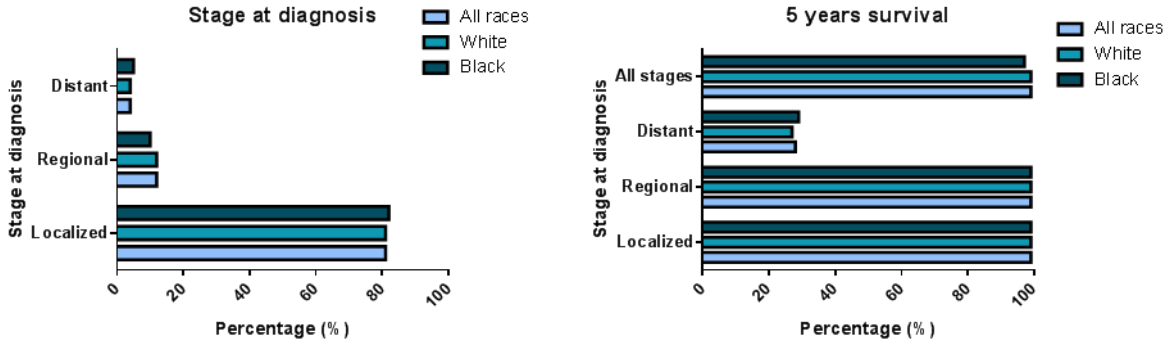


Figure 3: PCa stage at primary diagnosis and 5-year survival depending on stage at diagnosis in all races and white and black individuals in EEUU. Adapted from Siegel et al. [51].

2.2.4.2. Symptoms

Not everyone experiences symptoms of PCa, especially in early-stages of the disease. In many cases, the signs are first detected by a doctor during a routine check-up. Some patients will experience changes in urinary or sexual function that might indicate the presence of PCa. These symptoms include the need to urinate frequently (especially at night), difficulty starting urination or holding back urine, weak or interrupted flow of urine, difficulty in having an erection or painful ejaculation (http://www.cancer.net/cancer-types/prostate-cancer/symptoms-and-signs). However, these symptoms can also indicate the presence of other diseases or disorders (prostatitis or BPH). Lymph nodes (LNs) and bones represent the most common locations of metastatic disease so patients usually suffer of acute bone pain.

2.2.4.3. Risk factors

There are only three established risk factors for PCa: older age, ethnic origin (African-American men have the highest incidence rate) [52] and heredity [53, 54]. The risk for first-degree relatives is about two times higher than for men in the general population [53]. More specifically, it has been shown that this familial risk is four times higher for first-degree relatives of men with PCa diagnosed younger than 60 years than for the general population [54]. In line with this, only one definite PCa predisposition gene, the homeobox gene *HOXB13*, has been identified [55, 56]. More studies have shown that PCa patients with *BRCA2* mutations carriers present higher Gleason-score and worse prognosis than do non-*BRCA2* mutations carriers but the relationship between BRCA2 in PCa development is not clear [57]. External factors that, in some ways, may be also involved in the development of PCa are the type of diet, pattern of sexual behavior, alcohol consumption, exposure to ultraviolet radiation, chronic inflammation, infection for pathogens [58] and occupational exposure [59].

2.2.4.4. Classification

The classification of PCa is based on the TNM system (tumor–node–metastasis cancer staging system; The Union Internationale Contre le Cancer, 2010; http://www.uicc.org/resources/tnm), which is one of the most accepted cancer staging systems. The TNM system is based on the size and/or extent (reach) of the primary tumor (T), the amount of spread to nearby lymph nodes (N), and the presence of

metastasis (M) or secondary tumors formed by the spread of cancer cells to other parts of the body. A number is added to each letter to indicate the size and/or extent of the primary tumor and the degree of cancer spread (**Table 1**).

Table 1: TNM system classification of PCa tumors

PRIMARY TUMOR (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Clinically in-apparent tumor not palpable or visible by imaging
T1a	Tumor incidental histologic finding in ≤5% of tissue resected
T1b	Tumor incidental histologic finding in >5% of tissue resected
T1c	Tumor identified by needle biopsy (because of elevated prostate specific antigen [PSA] level)
T2	Tumor confined within prostate; tumors found in 1 or both lobes by needle biopsy but not palpable or reliably visible by imaging
T2a	Tumor involves one-half of 1 lobe or less
T2b	Tumor involves more than one-half of 1 lobe but not both lobes
T2c	Tumor involves both lobes
T3	Tumor extends through the prostatic capsule; invasion into the prostatic apex, or the prostatic capsule is classified not as T3 but as T2
T3a	Extracapsular extension (unilateral or bilateral)
T3b	Tumor invading seminal vesicle(s)
T4	Tumor fixed or invades adjacent structures other than seminal vesicles (e.g., bladder, levator muscles, and/or pelvic wall)
Pathologic (pT)*	
pT2	Organ confined
pT2a	Unilateral, involving one-half of 1 lobe or less
pT2b	Unilateral, involving more than one-half of 1 lobe but not both lobes
pT2c	Bilateral disease
pT3	Extraprostatic extensión
pT3a	Extraprostatic extension or microscopic invasion of the bladder neck
pT3b	Seminal vesicle invasión
pT4	Invasion of the bladder and rectum
REGIONAL LYMPH NODES (N)	
NX	Regional lymph nodes were not assessed
N0	No regional lymph node metastasis
N1	Metastasis in regional lymph node(s)
Pathologic	
pNX	Regional nodes not sampled
pN0	No positive regional nodes
pN1	Metastases in regional nodes(s)
DISTANT METASTASIS (M)*	

M0	No distant metastasis
M1	Distant metastasis
M1a	Nonregional lymph nodes(s)
M1b	Bone(s)
M1c	Other site(s) with or without bone disease

Pathologic evaluations of prostate cancer is based on Gleason grading [60] (**Figure 4**). This system has evolved since 1966 and, recently, it has been modified with a new system already accepted by the World Health Organization for the 2016 (WHO 2016) edition of Pathology and Genetics: Tumours of the Urinary System and Male Genital Organs [61]. The Gleason system is based on the glandular pattern of the tumor as identified at relatively low magnification. It comprises from 1 to 5, with 1 being the most differentiated and 5 being the least differentiated (**Figure 4**).

Gleason score of biopsy-detected PCa comprises the Gleason grade or the most extensive pattern (primary pattern), plus the second most common pattern (secondary pattern), if two are present. If one pattern is present, it needs to be doubled to yield the Gleason score. For three grades, the Gleason score comprises the most common grade plus the highest grade, irrespective of its extent [62]. When reporting prostatectomy specimens, the Gleason score is the sum of the most and second-most dominant (in terms of volume) Gleason grade. If only one grade is present, the primary grade is doubled. If a grade comprises < 5% of the cancer volume, it is not incorporated in the Gleason score (5% rule). The primary and secondary grades are reported in addition to the Gleason score. A global Gleason score is given for multiple tumours, but a separate tumour focus with a higher Gleason score should also be mentioned [62].

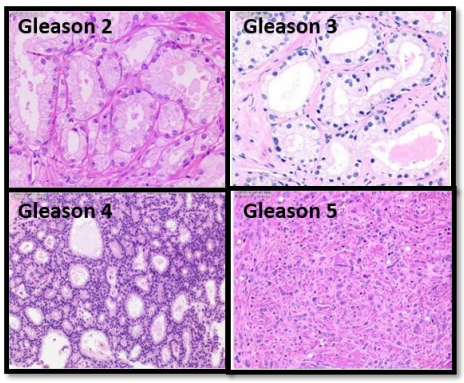


Figure 4: Representative images of Gleason score classification. **Gleason score 2**: The glands have abundant pale cytoplasm and prominent nucleoli; **Gleason score 3**: The glands have well-formed round, oval, or distorted lumens. There is nuclear enlargement and

hyperchromasia with prominent nucleoli. Basal cells are not seen; **Gleason score 4**: Glandular fusion is the hallmark feature of Gleason pattern 4. The image shows fused glands forming anastomosing irregular cords separated by moderate amount of stroma; **Gleason 5 score**: Solid sheet of relatively uniform neoplastic cells diagnostic of Gleason score 5+5=10. Images are taken from WebPatholohy website: http://webpathology.com/.

Due to its misleading clinical implications, Gleason score of 1 + 1 = 2 should not be rendered, regardless of the specimen type GS 2–4 should rarely be rendered in needle biopsies, if ever (5,6). Therefore, from a practical standpoint, Gleason pattern in contemporary practice starts at 3 and GS starts at 6 in prostate biopsy specimens and most transurethral resection of the prostate (TURP) and radical prostatectomy (RP). specimens [63, 64].

2.2.4.5. Types of prostate cancers

Most PCa tumors are adenocarcinomas (around 90%) with a typical luminal phenotype common to other epithelial cancers (i.e. breast and colon cancer) with loss of the basal layer [65]. Adenocarcinomas express AR and lack the expression of markers of basal cells such as CK5, CK14 and p63 [66]. In the past, it was accepted that luminal cells were the origin of PCa [67, 68], but nowadays that notion has been questioned since multipotent stem and progenitor cells within the basal epithelial are able to give rise to basal, luminal and neuroendocrine cells [67, 69]. In fact, with the appropriate oncogenic input (i.e. ERG, AKT and AR overexpression), normal basal cells, but not normal luminal cells, are able to produce adenocarcinomas [67], and even a mixture of adenocarcinoma and neuroendocrine PCa (by means of N-Myc overexpression and constitutive activation of AKT pathway) [70]. In addition, PCa origin is commonly multifocal, since about 80% of tumors contains >1 disease focus [71], and each of those may harbor different molecular alterations [72] which emphasize the intra-tumoral complexity and heterogeneity of PCa.

Rare types of PCa are ductal carcinoma, mucinous PCa, prostate sarcomas, signet ring cell PCa and neuroendocrine tumors (small- and large-cell PCa) [65]. The frequency of neuroendocrine PCa tumors (NEPC) is low (<2% of total PCa) [73] but normally behave very aggressively, since they cannot be detected by PSA screening (there is no expression of AR or PSA in neuroendocrine cells), and, therefore, many patients are

diagnosed in an advanced stage of the disease [74, 75]. Loss of RB1 and TP53 tumor suppressors and overexpression of MYCN and AURKA (Aurora Kinase A) commonly occurs in NEPC [70, 72], and, potentially, can be PCa clinical targets (i.e. AURKA inhibitors [70, 76]). Neuroendocrine cell differentiation is a process that helps the tumor to keep growing and bypass the castration-hormonal therapy [75].

2.2.4.6. Pathophysiology

PCa evolves from benign tissue to malignant lesion by acquiring, over time, several genetic alterations (DNA copy number variations, gene mutations, or chromosomal rearrangements), alterations in intracellular signaling (i.e Phosphoinositide 3-kinase pathway, MAPK kinase pathway), epigenetic changes (DNA methylation, histone modification or miRNA dysregulation) and other key molecular alterations (i.e. aberrant alternative splicing) [77]. While structural lesions (such as genomic rearrangements) are prevalent in PCa, point mutations occur less commonly compared to other solid tumors (i.e. colorectal cancer) probably because PCa is not exposed to strong exogenous mutagens [78, 79] (**Figure 5**). Somatic copy number alterations (SCNAs) are common in PCa, and imply the gain or loss of segments of genomic DNA, leading to amplification of oncogenes (i.e. AR, MYC) and/or deletion of tumor suppressor genes (i.e. TP53, PTEN, CDKN1A or APC) [78, 80]. Chromosomal rearrangements can also result in gene fusions, with aberrant function promoting oncogenesis (i.e. ETS gene fusion).

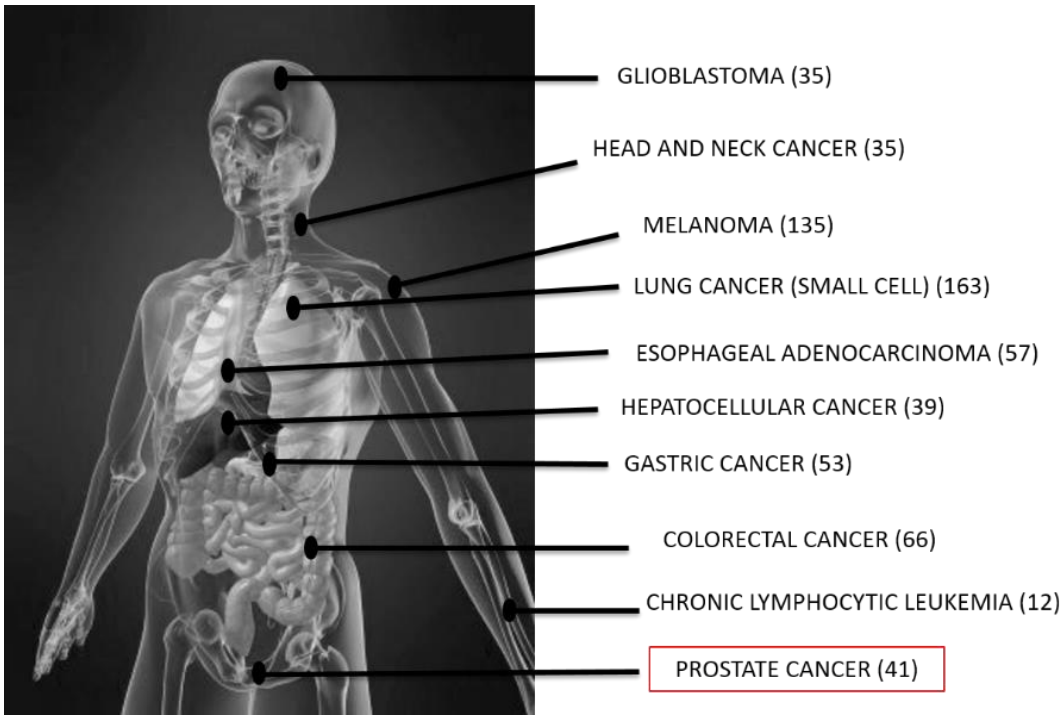


Figure 5: Number of somatic mutations (in parentheses) in representative human cancers, detected by genome wide sequencing studies. Adapted from Vogelstein et al. [79].

Growth of adenocarcinomas is androgen-dependent, and therefore, endocrine-based therapies are targeted towards lowering serum androgens and/or inhibiting AR activity, normally known as androgen deprivation therapy (ADT) [81, 82] (**Figure 6**). Due to the PCa complexity, it is necessary to differentiate between the events that occur in an early phase of the disease (localized PCa), probably driving the development and progression of the disease, and those events that occur in a late phase, which takes place after the patient has gone through heavy ADT to control the progression of PCa [*CRPC* (Castration Resistant PCa) or *mCRPC* (metastatic CRPC)] [78, 80, 82-84] (**Figure 6**).F

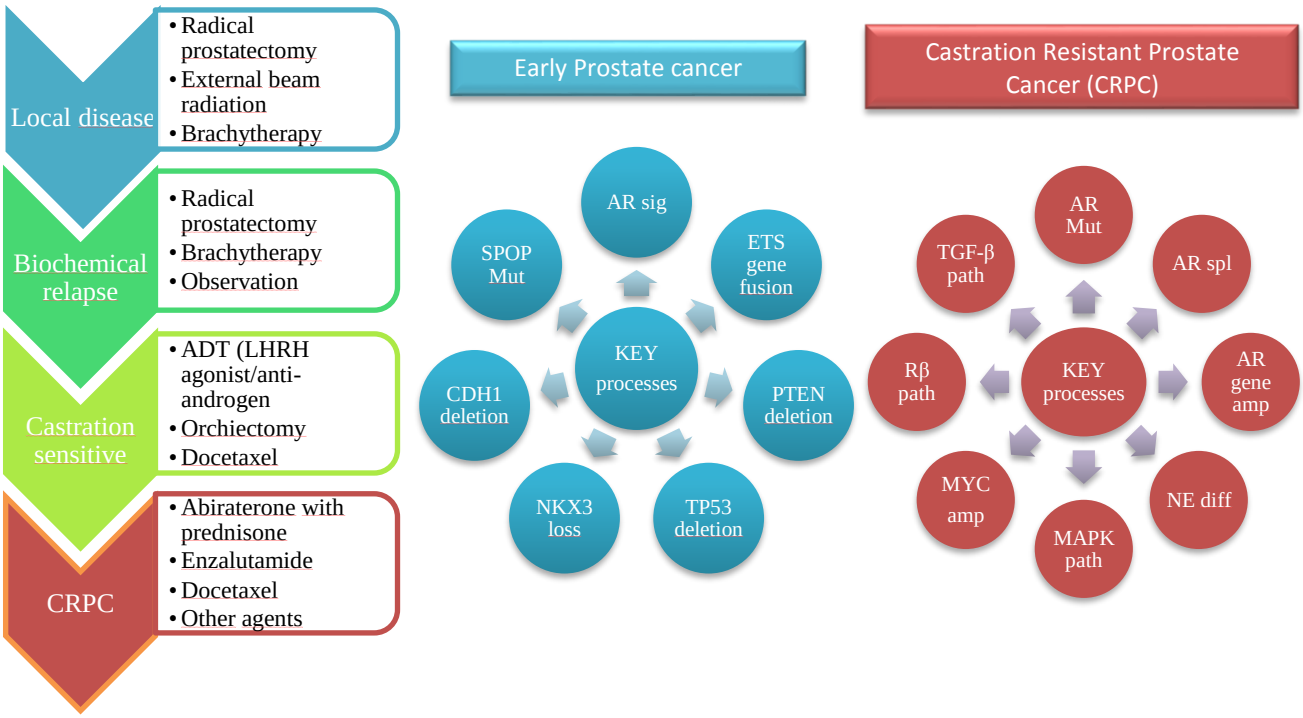


Figure 6: Clinical evolution of PCa with therapeutical options and molecular mechanism behind the pathology. AR sig= AR signaling; Mut= Mutation; AR spl: AR splicing (i.e. AR-V7); Amplif: Amplification; NE= Neuroendocrin; Path=pathway. Adapted from Prensner et al. [85] and Barbieri et al. [82]

2.2.4.6.1. Early events in PCa

The molecular complexity of primary PCa is remarkably high, and it has been proposed that 74% of the primary PCa tumors fall into one of seven subtypes defined by specific gene fusions (ETS genes with AR-regulated genes) or mutations (SPOP, FOXA1 and IDH1) [78]. In contrast, a significant subset of PCa tumors (26%) are driven by unknown molecular alterations [78]. Androgen signaling is the most relevant pathway in both primary and advanced PCa [78, 82, 83] and PCa tumor growth is androgen dependent. Although in early stages of the disease the AR gene or its protein product are not altered (mutations in AR, amplifications of the gene or appearance of AR splicing variants, which are hallmarks of castration resistant PCa), many of its cofactors (i.e. NCOA2, NRIP1) or AR-regulated genes are also involved in the appearance of PCa, and, in addition, androgens and AR are essential in Early Onset PCa (EO-PCa)-driven ETS fusion events [80, 84]. In line with this, Bacca et al. have proposed a ‘‘consensus path’’ of progression due to genetic events that could be similar to that in other solid tumors, like colorectal tumors [86]. Specifically, this path leading to PCa would begin with the deletion of NKX3-1 or FOXP1 and fusion of TMPRSS2 and ERG genes (due to an

increase in AR activity), modifying normal prostate epithelial differentiation [87] and promoting other oncogenic alterations [83]. Subsequently, lesions, or epigenetic silencing in tumor suppressors such as PTEN, CDKN1A, RB or TP53 accumulate [88, 89]; these alterations lead to increase cell growth, genomic instability and/or evasion of apoptosis [83]. Nevertheless, this vision may be oversimplified, since some events in PCa are exclusive (i.e. ETS fusion with presence of SPOP/CDH1 mutations) but others are common in the different subtypes. In sum, the complexity of primary PCa reflects, and probably underlies the wide range of patient response for established clinical treatments.

ETS gene fusions

The fusion between AR-regulated genes with members of the ETS family [78] is the most common alteration in primary PCa [90]. The prevalence of those rearrangements is 53% in primary PCa, being the fusion involving ERG the most common process (occurring in 46% of primary PCa) [78]. Up to 10 androgen-regulated genes have been found as 5' fusion partner of ETS gene [82, 90]. TMPRSS2 is the most common gene involve in this fusion, with the transcription factor ERG as 3' partner, resulting in activation of the TMPRSS2-ERG fusion oncogene [90]. Other members of the ETS family that serves as 3' partner included ETS variant 1, 4 and 5 (ETV1, ETV4, ETV5) [90].

In vivo models shows that the expression of ERG in mice prostate epithelial cells, at levels that are clinically relevant, is sufficient to cause the slow and partially penetrant emergence of age-related prostate tumors, a mechanism that involves the activation of Hippo pathway [91]. Consistent with this observation, *in vitro* experiments with forced ERG-overexpression in PCa cell lines increases pathophysiological features (i.e. cell migration) [92]. However, patients with ETS fusion tumors do not have a worse clinical outcome compared with other subtypes [93]. Most of the ETS positive subclasses harbor mutations or loss in PTEN but also in the tumor suppressor TP53 [78, 83]. However, other alterations as SPOP mutations, CDH1 deletions or SPINK1 overexpression occur exclusively in ETS-negative PCa showing again the molecular heterogeneity of PCa [78, 80, 83, 94].

PTEN

PTEN is as a tumor suppressor that negatively regulates intracellular levels of phosphatidylinositol-3, 4, 5-trisphosphate, and acts by negatively regulating AKT/PKB signaling pathway [95, 96]. It is mutated in a large number of cancers at high frequency; in fact, deletions in PTEN occur in 30-60% of primary PCa, with inactivating mutations in 5-10% of the cases [80]. Mostly, PTEN deletions occur in ERG-fusion cases [78, 80]. PTEN loss is often found in PIN lesions, which indicates that it is a common event in early PCa development; however, loss of PTEN is even more acute in advanced disease as it is shown in metastatic PCa [78, 94]. Moreover, a growing body of evidence shows that deletion of PTEN is associated with PCa aggressiveness [97, 98].

TP53

TP53 is the most commonly mutated gene in human cancer [99]. It is a tumor suppressor in many cancers, which induces cell growth arrest or apoptosis depending on the physiological context and cell type [99]. Several studies show deletions at the TP53 locus in approximately 25–40% of PCa samples [82], with point mutations in 5–40% of patients. Of note, about 25–30% of clinically localized PCa present alterations in TP53, and it is also found in PIN lesions, suggesting that these alterations are common in early events in the genomic history of the disease [82].

NKX3-1

NKX3-1 (NK3 Homeobox 1) is a transcription factor that acts as a negative regulator of epithelial cell growth in prostate tissue [100]. Moreover, it is crucial in the development of fetal prostate [101]. Deletion of NKX3-1 is a common event in early phases of PCa and cooperates with the activation of TMPRSS2: ERG [102]. Remarkably, *in vivo* models show that the loss of NKX3-1, in conjunction with the loss of PTEN, produces a constitutive activation of PTEN-AKT pathway disrupting normal cellular response to DNA damage and promoting PCa development [103].

SPOP mutations and CDH1 deletions

SPOP (E3 ubiquitin ligase adaptor speckle-type POZ protein) is one of the most frequently mutated genes in PCa (about 6-15% of localized PCa patients harbor point mutations in this gene) [78, 104]. PCa with mutant *SPOP* lacked ETS family gene rearrangements and showed a distinct pattern of genomic alterations [104]. PCa cells lines transfected with the most common SPOP mutant (the F133V variant) or with SPOP

siRNA showed increased invasion capacity compared to controls; however, cell proliferation and migration was not significantly altered [104, 105]. Moreover, SPOP can act as a tumor suppressor involved in AR signaling. Specifically, SPOP mutated protein cannot interact with SRC-3 protein or promote its ubiquitination and degradation (SRC-3 is a direct regulator of androgen receptor transcriptional activity, cell proliferation, and resistance to androgen deprivation therapy) [105]. Therefore, SPOP mutations abrogate its naive tumor suppressor role and control of SRC-3 in PCa.

CHD1 (Chromodomain-helicase-DNA-binding protein 1) is an enzyme that acts to remodel chromatin structure [106]. CDH1 is often loss in PCa (about 10-25% of cases) but exclusively in ETS negative tumors [78, 107]. Unfortunately, the role of CHD1 in PCa is still unclear; however, there is some evidence that CHD1 can act as a tumor suppressor, required for efficient recruitment of AR and for the regulation of expression of known AR-responsive tumor suppressor genes (i.e. NKX3-1 or FOXO1) [108].

AR signaling

AR undergoes several changes leading to enhanced activity in PCa, such as gene amplification, point mutations, and alterations in canonical splicing leading to constitutively active, aberrantly spliced variants, but most of these alterations take place in late stage metastatic CRPC [82]. Nevertheless, alterations in genes encoding proteins that regulate AR are common in early PCa; these include transcriptional co-activators, such as the nuclear receptor coactivator 2 gene (NCOA2), and E1A binding protein p300 gene (EP300), transcriptional co-repressors, like nuclear receptor repressor 2 gene (NCOR2), or interacting transcription factors nuclear receptor interacting-protein 1 (NRIP1-RIP140) [109], and chromatin regulatory elements [82]. FOXA1 (Forkhead Box A1), which is commonly overexpressed in PCa, binds to AR regulating its transcriptional activity and function [104]. Moreover, point activating mutations are found in this gene, and it has been demonstrated that FOXA1 mutants enhance cell growth and invasion in the presence of androgens [104].

Although AR does not seems to be structurally modified in primary PCa, its expression is higher in early onset PCa, an aggressive form of PCa diagnosed at age ≤55 years [84]. However, the most striking role of AR/androgens in early PCa comes from the evidence that this system is able to promote aberrant structural rearrangements (i.e. gene fusions), which are one of the first events in PCa [84]. *In vitro* experiments have

also demonstrated that the formation of ERG rearrangements with androgen-dependent genes can be induced by serum androgen levels in prostate epithelial cells [84, 110]. Therefore, it has been proposed that the increase in androgen levels promote structural rearrangements in the prostate (i.e. TMPRSS2: ERG), which initiate a cascade of genetic changes (i.e. PTEN or TP53 loss) driving progression and aggressiveness of human PCa.

2.2.4.6.2. Late events in PCa; the development of CRPC (castration resistant-PCa)

ADT by using anti-androgens and/or GnRH analogs is the gold standard of therapy for locally advanced PCa, metastatic PCa, and biochemically recurrent disease after the lack of success of localized treatments [111-113]. ADT is known to provide remission for a time (24-36 months) of the disease, accompanied with PSA decline [111]. However, the disease progresses despite continuous hormonal therapy and PSA levels increase again eventually (biochemical recurrence) [111]. This type of cancer is then known as castration-resistant PCa (CRPC) [113]. To date, there is no successful cure for metastatic castration-resistant PCa (mCRPC), which presents a mean survival time of only 16–18 months [111]. The molecular hallmarks of CRPC cancers are divided in **I)** AR-related pathways (**Table 2**) and, **II)** Non-AR-related pathways [76, 114].

I) AR-related pathways: To date, it is widely accepted that AR signaling plays a central role in this late phase of the disease, since the increase of PSA during ADT therapy reflects the re-activation of AR transcriptional activity [76, 111, 114]. Recently, the persistent significance of the AR axis in CRPC was pharmacologically validated by the clinical efficacy of androgen synthesis inhibitors (Abiraterone) and novel second-generation AR antagonist (Enzalutamide) [76, 111, 115-117]. In addition, other signaling pathways involved in AR function such as MAPK kinase, PIK3/AKT, Retinoblastoma (Rb), MYC/MAX or WNT pathways are dysregulated in CRPC, and contribute to the progression of the disease [82, 94, 114, 118] . The main mechanisms involved in the AR-axis maintenance in CRPC are summarized in **Table 2**.

Although not directly related with AR signaling, **neuroendocrine differentiation** is a crucial process driven by ADT therapy, and represents a critical step in the progression of PCa from adenocarcinoma to CRPC [70]. Neuroendocrine PCa cells only represent 2% of total cells of all primary PCa cells; however, ADT therapy produces an

expansion of this cell population, which does not express AR or PSA, and, therefore, is not a target for ADT therapy [70, 76]. Remarkably, it has been shown that some factors induced by castration (i.e. IL-6) enhance the neuroendocrine phenotype [119].

Table 2: Molecular mechanisms that promote persistent activation of AR in CRPC tumors.

Mechanisms that promote persistent AR axis activation in CRPC cells	Example
Persistence of intra-tumoral levels of androgens	Up-regulation and stimulation of enzymes involved in androgen synthesis (*AKR1C3*) [120]
AR overexpression	*AR* gene amplification, missense mutations in *AR* ligand-binding domain (LBD) [121, 122]
Expression of AR truncated variants	*AR-v7*, *AR-v4* [123-125]
Changes in the coregulatory components of the AR complex (coactivators and corepressors)	*SRC1*, *SRC2*, *SRC3*, *NRIP1 [109, 126]*
Activation of the AR complex via cross-talk with other signaling pathways	*PI3K/AKT*, *RAS/MAPK*, *MYC/MAX*, Retinoblastoma (*Rb*), *TGF-β*

II) Non-AR-related pathways. Some of the processes included in these pathways include: 1) Overexpression of anti-apoptotic proteins that circumvent the AR pathway (i.e. BLC-2[127]); 2) Tumor suppressor dysregulation (i.e. TP53 [128, 129]); 3) Alteration in miRNAs expression (i.e. MiR-21, MiR-27a, MiR-141, MiR-375 [130],[131]); 4) epigenetic changes (i.e. PTEN loss of expression due to CpG islands methylation); 5) alternative splicing [77, 132]; and, 6) gene fusions (i.e. ERG genes [10]).

2.2.4.7. Diagnosis

Most adenocarcinomas are diagnosed during PSA screening and/or digital rectal exam (DRE) [53]. Thanks to the PSA screening, almost 80% of PCa patients have tumors located in the prostate without metastasis at the time of the diagnosis [51]. If certain

symptoms or the results of PSA detection test are clearly positive, then a prostate biopsy is required. Accordingly, a core needle biopsy *by transrectal ultrasound (TRUS)-guided biopsy* is the main method used to diagnose PCa [53]. In biopsy specimens, adenocarcinoma diagnosis can be confirmed by the absence of immunostaining using p63 and cytokeratin 5/14 antibodies, both of which detect basal cells [133]. In addition, the sample will be evaluated for the presence and grade of the tumor (according to the Gleason system) and also diagnosed by the status of their primary tumors (T1-4, M0 and 1a-c, N0 or 1) [134].

Unlike most solid tumors, some biomarkers have been typically employed in PCa detection. First, PAP (prostatic acid phosphatase) was investigated as a serum biomarker of cancer in metastatic prostate patients [135]. In the 1980s, PAP was rapidly replaced by PSA, which has become one of the standard marker for the diagnosis of PCa in clinical routine. However, PSA value for the prognosis of PCa is controversial, and in many cases, leads to overdiagnosis and treatment of indolent cancer that produces more harm than good in the patient [136, 137]. Therefore, it is widely accepted that new PCa biomarkers should be identified to target unmet clinical needs in PCa management, including: 1) *risk indicators for disease with low PSA values (<10 ng/ml)*; 2) *prognostic markers to distinguish indolent from aggressive disease*; and 3) *biomarkers for metastatic cancer* [85]. Therefore, new genetics test have been devised in the last years that may fulfill the needs in PCa diagnosis (**Figure 7**).

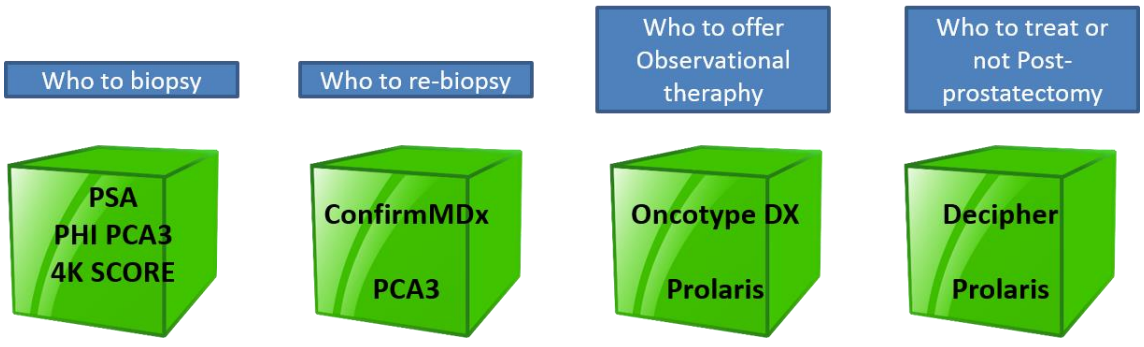


Figure 7: New tests for PCa clinical detection and management. After the identification of patients in whom a biopsy is required (based on results of specific tests such as PSA levels), new tests have been proposed, which are thought to fill out some of the current PCa clinical needs (represented as different buckets). The first bucket shows different promising tests, such as PHI, PCA3 and 4KSCORE, which might add specificity and sensitivity to PSA test in order to detect PCa patients. *Prostate Health Index (PHI)*, is a mathematical formula that combines total PSA, free PSA and proPSA [138], while the 4KSCORE incorporates a panel of four kallikrein protein biomarkers (total PSA, free PSA, intact PSA, and human kallikrein-related peptidase 2) and other clinical information in an

algorithm [139].. The second bucket shows new tests used to discern if the patient should undergo or not re-biopsy on suspicion of false negative in first biopsy. *ConfirmMDx* test is based on altered epigenetics factors that occur in the primary tissue (i.e. GSTP1, APC, RASSF1)[140]. PCA3 (Prostate cancer antigen 3) expression levels can be measured in urine with a commercial kit (Promega ®)[141]. The third bucket shows new tests that give information about if the patient should be treated or not when the tumor is localized and the Gleason score of the piece is low. *Oncotype DX* test measures 12 genes from Androgen receptor pathway (AZGP1, KLK2, SRDS12, FAM13C), cellular organization (FLNC, GSN, TPM2, GSTM2), proliferation (TPX2) and stromal response (BGN, SFRP4, COL1A1) [142]. Likewise, *Prolaris* test measures the expression of 46 genes (involved in cell cycle progression) that correlate with prostate tumor cell proliferation, to assess the aggressiveness of an individual patient's cancer [143]. Finally, the fourth bucket shows tests that inform which patient should be treated after prostatectomy. *Decipher* test measures the score obtained with the expression of 22 genes related with different cell processes (i.e. cell structure, adhesion, motility, or immune response), and provides information on tumors that will probably metastasize after radical prostatectomy. [144]

PSA and modifications

Kallikrein 3 (KLK3) is a gene that encodes PSA protein, a member of the tissue kallikrein family, which are serine proteases with diverse physiological functions [145]. A growing body of evidence suggests that many kallikreins are implicated in carcinogenesis and some have potential as novel biomarkers in cancer and other diseases [145]. PSA is expressed almost exclusively by the prostate and, therefore, its expression is tightly linked to the presence of prostatic cells [146]. Mature PSA is the result of proteolytic cleavages of two inactive precursor peptides, pre-proenzyme PSA (pre-proPSA) and proPSA [24], and it is secreted into semen in its final form. Under normal conditions, only low levels of PSA can be detected in blood, while it is highly secreted in urine, and the increase of serum PSA can represent the presence of PCa, because PSA is released into the blood as a consequence of disruption of normal prostate architecture [147]. As previously mentioned, the level of PSA is a continuous parameter for the presence of PCa: the higher the value, the more likely is the existence of the disease [53]. The exact cut-off level for what is considered to be a normal PSA value has not been universally and absolutely determined, but values of approximately <2-3 ng/ml are often used for younger men [53, 148], although low levels can underrate the presence of the disease [53] (**Table 3**). There is a high controversy with the specificity and sensitivity of PSA test (i.e. from 20 to 40% and 70 to 90%, respectively), depending on the PSA cutoff values used (for example, 2 vs. 3 ng/ml) with an area under the curve (ROC curve) of 0.55-0.70 for the ability of PSA to discern patients with PCa (1.0 is perfect discrimination and 0.5 is a coin toss) [149]. More importantly, the overdiagnosis, mainly by the detection

of harmless early-stage tumors with low Gleason score (indolent tumors), and consequently overtreatment, is accepted as the most important downside of PSA-test [150]. Therefore, there is a global controversy regarding whether PSA test should be used or not as a screening method for PCa. As an example, while the Prostate, Lung, Colorectal and Ovarian (PLC0) study performed in USA did not find any overall survival benefit using PSA-screening [151], the European Randomized Study of Screening for Prostate Cancer showed a 21% reduction of mortality based on PSA screening [152].

Table 3: Risk of PCa presence upon PSA blood levels.

PSA level, ng/ml	Risk of PCa%
0-0.5	6.6
0.6-1	10.1
1,1-2	17.0
2.1-3	23.9
3.1-4	26.9
PSA=prostate specific antigen, PCa=prostate cancer	

Modifications of PSA test have been reported and may improve PSA specificity (**Figure 8**). These modifications include PSA density, PSA density of the transition zone, age-specific reference ranges, and PSA molecular isoforms. One of the most promising markers is the use of the percentage of free PSA (% fPSA) to reduce the number of unnecessary biopsies and increase the rate of PC detected [153].

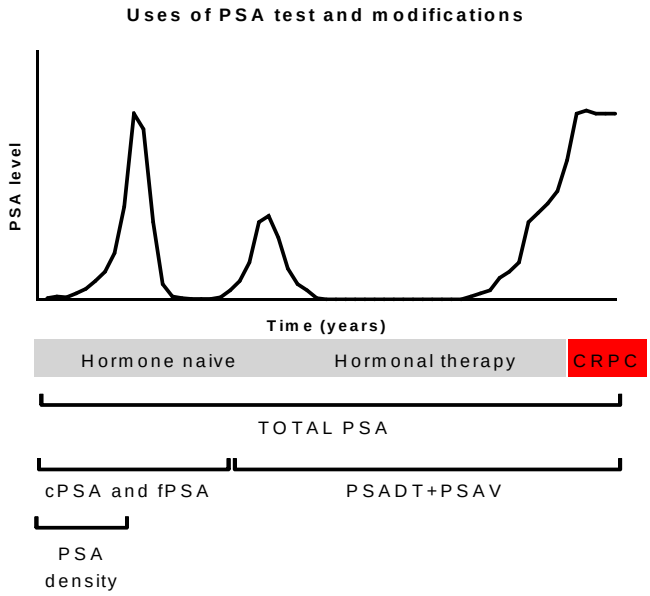


Figure 8: Use of PSA test and modifications through the different phases of the disease. cPSA: Complexed PSA; fPSA: free PSA; PSADT= PSA Doubling Time; PSAV=PSA velocity. **Modified from Prensner et al. [85]**

Prostate cancer associated 3 (PCA3)

PCA3 is a non-coding RNA highly overexpressed in prostate cancer [154]. In contrast to PSA, PCA3 is exclusively expressed by PCa cells [155]. Although its functional role is not clear, it seems to modulate and be regulated by androgen signaling [156]. PCA3 expression levels can be measured in urine with a commercial kit (Promega ®) [141]. The increased specificity of PCA3 is contrasted by a reduced sensitivity and, to date, the main application in clinical management of PCA3 could be the measurement of its levels in association with PSA levels, which might reduce the number of unnecessary biopsies after a negative biopsy in men with suspicion of PCa [157, 158].

Other promising biomarkers

In addition to PSA and PCA3, other biomarkers are currently being investigated [85]. As mentioned before, TMPRSS2-ERG fusion is one of the most common events in PCa [82]. However, TMPRSS2-ERG is absent in about 50% of PCa cases, and therefore, its use can be only suitable in multiplexed assays combined with other biomarkers (i.e. in conjunction with PCA3 in urine assays) [159]. Use of SNPs associated with PCa [160-162], metabolites [163], high-throughput approaches (RNA-Seq) [164], circulating tumor cells (CTCs) [165], exosomes [166] and miRNAs [167] are new avenues in PCa diagnosis and prognosis, although their use in clinical routine assays has not yet been well

developed. Interestingly, Taylor et al. [80] found that the level of DNA copy-number alterations can predict which group of patients will present biochemical recurrence while other groups with lesser DNA copy-number alterations may not even require surgery. Other independent study has corroborated this observation showing that the burden of DNA copy-number alteration correlates with higher Gleason score and PSA levels [78]. Another promising avenue is the use of miRNA in PCa diagnosis [167], since these molecules can be reproducibly extracted from a wide range of biological samples, accurately quantified by a variety of standard techniques, and are stable under different conditions (**Table 4** shows some of the most extensively investigated miRNAs in the serum of PCa patients) [167].

Table 4: Promising miRNA biomarkers in the detection of PCa.

miR	Trend	Putative targets	Pathway-Functional process
miR-141	Up	SHP	AR
miR-375	Up	MYC, CCND2	Proliferation
miR-221	Up	p27kip1	Cell cycle, AR
miR-181a	Down	LEF1, BCL2	EMT, apoptosis
miR-21	Up	p57kip2, PTEN	Proliferation

AR=androgen receptor; *EMT*=epithelial-mesenchymal transition

2.2.4.8. Treatment

The type of treatment that is specifically selected for each individual depends on several parameters such as the clinical stage, Gleason Grade, and patient preference [168] (**Table 5**).

Table 5: Treatment options for prostate cancer.

Stage (TNM Staging Criteria)	Standard Treatment Options
Stage I PCa (T1a, N0, M0, G1 [G1 = Well differentiated (slight anaplasia), (Gleason score of 2–4)])	Watchful waiting or active surveillance Radical prostatectomy External-beam radiation therapy (EBRT) Interstitial implantation of radioisotopes
Stage II PCa (T1b-d or T2, N0, M0, any Gleason)	Watchful waiting or active surveillance Radical prostatectomy EBRT with or without hormonal therapy Interstitial implantation of radioisotopes
Stage III PCa (T3, N0, M0, any Gleason)	EBRT with or without hormonal therapy Hormonal manipulations (orchiectomy or luteinizing hormone-releasing hormone [LH-RH] agonist) Radical prostatectomy with or without EBRT Watchful waiting or active surveillance
Stage IV PCa (T4, any N, M0-1, any Gleason)	Hormonal manipulations Bisphosphonates EBRT with or without hormonal therapy Palliative radiation therapy Palliative surgery with transurethral resection of the prostate (TURP) Watchful waiting or active surveillance
Recurrent PCa	Hormone therapy Chemotherapy for hormone-resistant prostate cancer Immunotherapy Radiopharmaceutical therapy/alpha emitter radiation
EBRT = external-beam radiation therapy; LH-RH = luteinizing hormone-releasing hormone; TURP = transurethral resection of the prostate; T = Primary tumor; N = Regional lymph nodes; M = Distant metastasis; G = Gleason score.	

http://www.cancer.gov/types/prostate/hp/prostate-treatment-pdq#section/_62.

2.3. Somatostatin system

Somatostatin (SST) is a neuropeptide expressed in the central nervous system and peripheral tissues [169] with an inhibitory functional role in the regulation of endocrine secretions in vertebrates [169] (**Figure 9**). Among others, SST inhibits the secretion of growth hormone (GH), thyroid-stimulating hormone (TSH), gastrointestinal (GI) hormones, pancreatic enzymes, and neuropeptides from different tissues, such as the pituitary, thyroid, stomach or pancreas [169, 170] (**Figure 9A**). SST acts through binding

to a family of G-coupled receptors encoded by 5 distinct, intronless genes called somatostatin receptors (ssts; sst1-5) (**Figure 9B**). The binding of SST to ssts triggers inhibitory cascades that negatively regulate cell proliferation and migration by the inhibition of downstream pathways, such as ERK and AKT [169]. SST is also able to inhibit cell proliferation of tumoral cells [171]. In line with this, synthetics SST analogs (SSAs; octreotide or pasireotide) with higher affinity for ssts and longer half-life than natural SST peptide, are a valuable clinical option in the treatment of different tumoral pathologies (i.e. pituitary and neuroendocrine tumors) and a new therapeutic avenue in other cancer types such as breast cancer or PCa [169, 172-175].

Somatostatin gene gives rise to a precursor called preprosomatostatin, which comprises about 120 aa [176]. After a process of proteolytic hydrolysis, two main products arise, SST-28 and SST-14, with 28 and 14aa, respectively. SST-14 and SST-28 bind to sst1-5 with low nanomolar affinity, with SST-14 having a slightly higher affinity for sst1-sst4, and SST-28 showing higher affinity for sst5 [177]. Although both peptides present similar physiological functions, SST-14 is predominantly produced in the central nervous system (CNS) but also in many peripheral organs, while SST-28 is mainly synthesized by mucosal epithelial cells along the gastrointestinal tract (GIT) [178].

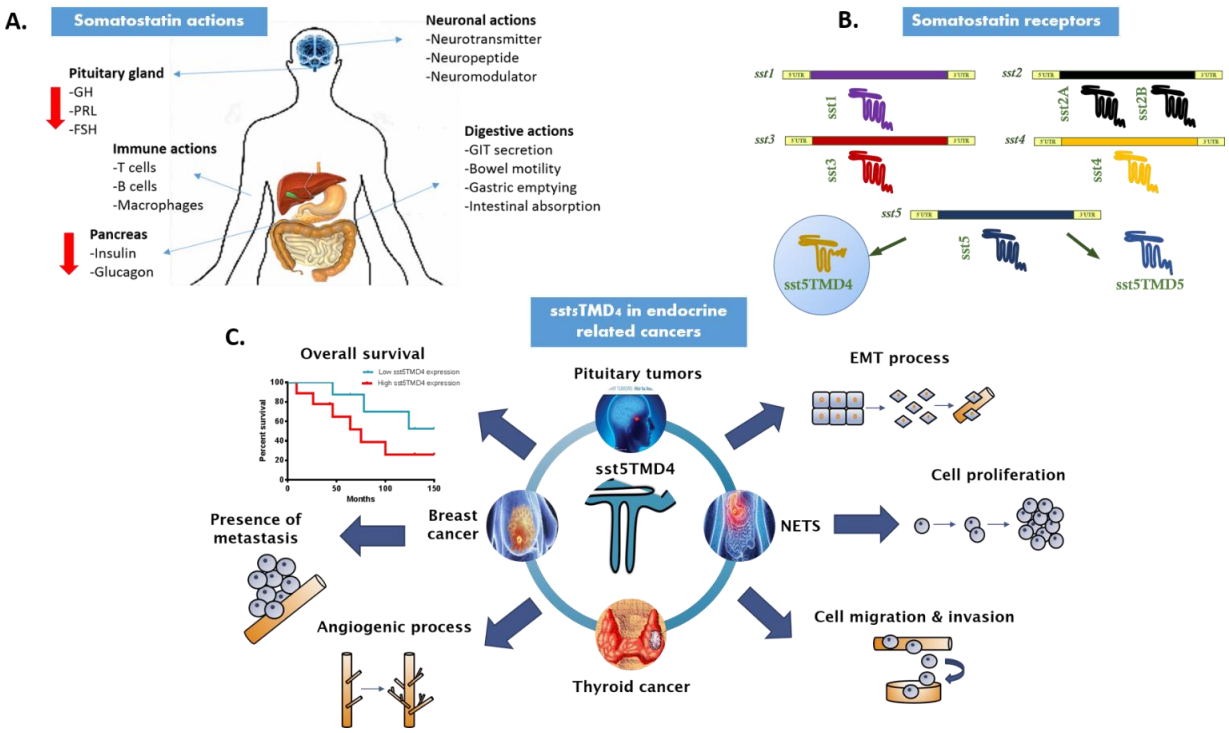


Figure 9: Somatostatin (SST) functional actions are mediate through binding to SST receptor subtypes (sst1-sst5). A. Multiple physiological actions of SST throughout the

body; **B**. sst receptors (sst1-5) are G-protein coupled receptor with seven transmembrane domains (TMD); **C**. Newly identified non-canonically spliced variants from the sst5 gene have recently been reported. Specifically, two variants with five and four TMDs (called sst5TMD5 and sst5TMD4, respectively) are generated together with the canonical, full-length sst5. sst5TMD4 spliced receptor has been associated with malignant features and aggressiveness in different types of endocrine-related tumors and cancers.

The complexity of the SST system increased twenty years after the discovery of SST, when a new SST-related peptide encoded by an independent gene was identified and named Cortistatin (CORT) [179]. Similarly to the SST gene, a larger precursor of 105 aa called precortistatin is cleaved to generates cortistatin-17 and cortistatin-29 in humans [180]. Interestingly, both peptides, SST and CORT, exhibit a strong structural, functional and pharmacological similarity, which may help to explain the ability of CORT to bind all canonical sst subtypes with comparable affinity to SST, and also the similar capacity of both peptides to regulate a number of common endocrine and non-endocrine relevant processes [179]. Nevertheless, it should also be emphasized that CORT actions do not always overlap with those of SST, and that CORT, but not SST, also binds and activates proadrenomedullin N-terminal peptide receptor (MrgX2) and shows binding affinity to ghrelin receptor (GHS-R1a) [181].

In addition, the existence of new variants of the sst5 receptor subtype derived from non-canonical splicing mechanisms has been recently described by our group in the human and other species [182] In particular, two additional truncated spliced variants of the human sst5 have been discovered, which display 4 and 5 transmembrane domains (TMDs) and were therefore named sst5TMD4 and sst5TMD5, respectively. Of special interest has been the study of the sst5TMD4 variant, which seems to play a relevant pathophysiological role, for it is barely expressed in normal tissues but markedly overexpressed in several endocrine-related tumors and cancers, including pituitary adenomas (PAs) [183, 184], breast cancer [185], poorly differentiated cancer and medullary thyroid carcinoma [186, 187], and neuroendocrine tumors NETs [188]. In these pathologies, sst5TMD4 presence correlates with clinical parameters of aggressiveness or bad prognosis, and promotes cell proliferation/migration/invasion and/or hormone secretion in primary cell cultures and/or model cell lines [183, 185, 187, 188] (**Figure 9C**). In addition, the presence of sst5TMD4 seems to impair the physiological anti-tumoral actions of SSAs in tumors. Specifically, studies from our group have proven that sst5TMD4 expression is negatively correlated with the ability of SSAs to reduce GH-

secretion in PAs [184], and inhibits the ability of sst2-transfected cells to respond to SST/SSAs [185].

2.3.1 Somatostatin system in PCa

ssts are expressed in PCa and PCa cell lines [189, 190]. sst1 has been shown to be overexpressed in PCa [191-193] and correlates with aggressive prostate cancer phenotype [192]. However, the expression of other ssts in normal vs. PCa tissues is more controversial. Specifically, sst2 has been reported to be downregulated [194] and upregulated [195] in primary PCa, and sst5 expression is expressed in a high proportion of PCa tissues, but is also present in normal prostate, and its levels do not seem to correspond to the grade of the disease [193, 196]. ssts are localized in secretory cells, basal cells, smooth muscle stromal cells and endothelial cells, being the sst1 the more frequently expressed in basal and endothelial cell compared with the other subtypes [197]. To date, the presence and localization of the truncated splicing variants sst5TMD4 and sst5TMD5 in PCa has not been explored.

The potential pharmacological use of SSAs in PCa has been evaluated in both, *in vitro* and *in vivo* studies. Specifically, *in vitro* treatment with SSAs targeting different ssts decreases the growth rate and increases cell cycle arrest through p21 in PCa cell lines [189]. Interestingly, the combined treatment of Lanreotide and Octreotide with Docetaxel enhances the induction of cell death in the PC-3 cell line [198, 199], a cell line with neuroendocrine differentiation features [200]. Thus, combining SSAs treatment with other therapeutic drugs might decrease tumor growth and provide a potential new avenue in PCa-treatment [172, 201], especially in those patients with neuroendocrine carcinoma or neuroendocrine differentiation after resistance to ADT therapy. Interestingly, it has been suggested that the imaging analysis of the presence of ssts on the cancer cell surface may provide a readily available, noninvasive means to distinguish adenocarcinoma from NEPC [202]. This distinction is important for therapeutic decision making and might open the door for developing novel radionuclide targets for the treatment of this aggressive subtype of PCa [202]. Indeed, a recent study focused on the combined used of sst scintigraphy technique (a type of radionuclide scan used to find carcinoid tumors using radioactive octreotide bound to ssts in the tumor) with docetaxel and octreotide treatment in CRPC patient with neuroendocrine differentiation showed a drastic size reduction of a lung metastasis with decreased in PSA level [174]. Moreover, SSAs reduces the plasmatic

levels of chromogranin A (CGA), a marker of neuroendocrine differentiation, in several trials [201, 203]. Somatostatin receptor based radio receptor therapy for treating advanced neuroendocrine tumors with somatostatin antagonist (vs. agonist) has been also shown to improve the diagnosis of PCa vs. other techniques [204].

SSAs may mediate their antitumor actions by decreasing the growth of tumors through two different mechanisms: 1) by directly targeting the membrane receptors (sst1-5) at the tumor surface; or 2) through an indirect inhibitory effect on secretory products, mainly exerted on growth-promoting factors such as growth hormone (GH) and insulin-like growth factor I (IGF-I). In fact, some studies have shown that the treatment with octreotide acetate significantly lowers IGF-1 levels and raises IGFBP-1 (its plasma binding protein) levels in patients with CRPC, with no effect in total PSA levels [203, 205]. Although the treatment with SSAs alone or in combinations have found some valuable clinical benefits (i.e. decrease in IGF-1 and CGA), other studies have not found improved overall survival, and the mechanistic reasons for those clinical failures are still completely unknown [205]. Therefore, although this system seems a plausible and valuable option in the treatment of PCa (especially in neuroendocrine tumors and CRPC patients with neuroendocrine differentiation), limited studies have been published focusing in the presence and functional role of many of the components of the SST-system in PCa. Accordingly, new studies focusing on the presence and regulation of the components of all the SST-system (including the splicing variants sst5TMD4 and sst5TMD5) in PCa are required.

2.4. Ghrelin system

Ghrelin is a hormone of 28-aa identified by reverse pharmacology as the endogenous ligand for the growth hormone secretagogue receptor (GHSR) [206]. Ghrelin was primarily found in the stomach, but is expressed in a wide range of tissues where it exerts multiple endocrine and paracrine actions [207] (**Figure 10**). Ghrelin requires to be acylated by the Ghrelin-o-acyl transferase (GOAT) enzyme [208] to exert most of its known functions through the canonical binding to GHSR1a, including stimulation of growth hormone release from the anterior pituitary, stimulation of appetite and gut motility, regulation of glucose homeostasis, and regulation of memory and of cardiovascular and immune systems [207, 209] (**Figure 10**).

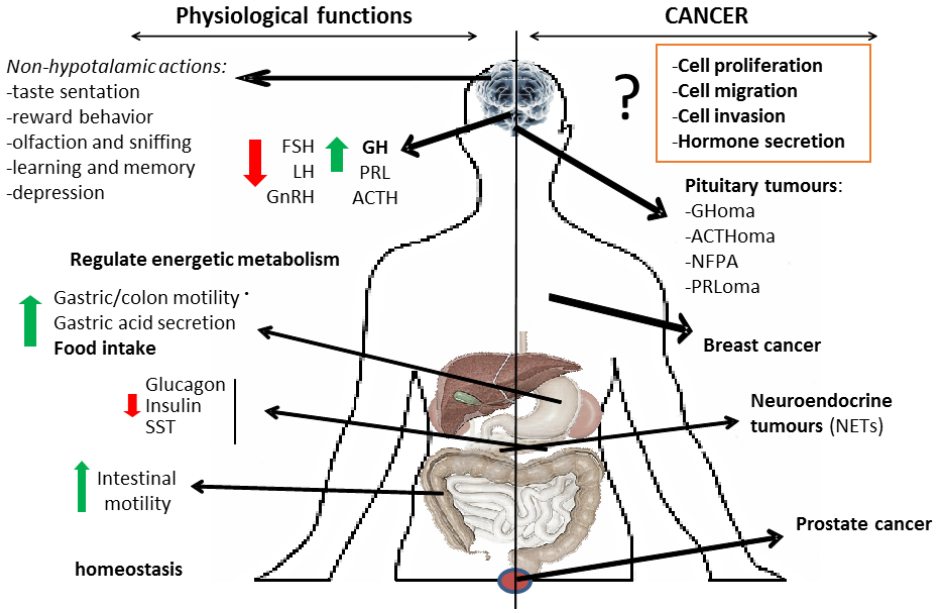


Figure 10: Functional and pathophysiological functions of ghrelin system through the body. The information of the image is taken from Gahete et al. [207].

On the other hand, although unacylated ghrelin (Des-acyl ghrelin) is the most common form of ghrelin in plasma, its functional roles are far from clear [210]. Interestingly, ghrelin and its receptor are expressed in a number of cancers and cancer cell lines and may play a role in processes associated with cancer progression, including cell proliferation, apoptosis, and cell invasion and migration; however, the precise roles of each component of the complex ghrelin-receptor system are still to be clearly defined (**Figure 10**) [207, 211, 212].

The mature ghrelin peptide results from proteolytic processing of a precursor peptide named preproghrelin [207, 213]. In humans, preproghrelin is encoded by a single-copy gene (GHRL) located on the 3p chromosome, which it is classically accepted to be composed of four coding exons (exons 1–4) [213] (**Figure 11A**). However, recent studies have shown the presence of upstream exons in the ghrelin gene (exon -1, exon 0, and extended exon 1) that can act as alternative sites for transcription initiation, and therefore, add complexity to the ghrelin gene regulation [214]. Indeed, some of these exons combine through alternative splicing processes to produce several mRNAs, which are further processed to generate functional peptides. In addition, a novel ghrelin variant generated by retention of intron 1 (In1), and consequently named In1-ghrelin, has been recently identified by our group [215] (**Figure 11B**). Interestingly, In1-ghrelin variant is upregulated in several endocrine-related cancers such as breast, pituitary and NETs [215-217]. In1-ghrelin variant shares the first 5-amino acids with native-ghrelin, which is the minimum sequence required for ghrelin acylation by GOAT and for binding and activation of GHSR1a [214] (**Figure 11B**); however, the amino acid sequence of In1-ghrelin is further modified by the retention of the intron 1. In line with this, GOAT expression has also been reported upregulated in some endocrine-related tumors [188, 215]. Remarkably, the expression of In1-ghrelin variant and GOAT, but not ghrelin, are positively correlated in breast cancer and neuroendocrine tumors [215, 217]. Based on these results (i.e. overexpression and correlations between In1-ghrelin, but not ghrelin, and GOAT enzyme) it has been suggested that In1-ghrelin variant could be the main substrate of GOAT in these endocrine-related tumors.

The originally orphan, growth hormone secretagogue receptor (GHSR) is encoded by a conserved single-copy gene located on chromosome 3 in humans [218] (**Figure 11C**). Human GHSR gene is composed of two exons, whose alternative splicing can generate two mRNA species, named GHSR1a and GHSR1b. GHSR1a mRNA, which includes exons 1 and 2, encodes a 366-amino acid G protein-coupled receptor (GPCR) with seven transmembrane domains (TMDs) (**Figure 11C**). In contrast, GHSR1b mRNA results from retention of the intron located between exons 1 and 2, which generates a 289-amino acid GPCR isoform with only five TMDs, and bearing a dissimilar 24-amino acid sequence at the C-terminal region compared with the GHSR1a sequence (**Figure 11C**). To date, the functional activity of truncated GHSR1b remains to be fully elucidated [207, 218, 219], while it is well-established that GHSR1a is the receptor responsible for

transducing the signal of acylated-ghrelin and the family of synthetic GH secretagogues. Nevertheless, there is emerging evidence that GHSR1b may exert pathologically-relevant actions, particularly by acting as a dominant-negative of its full-length partner GHSR1a [219-221].

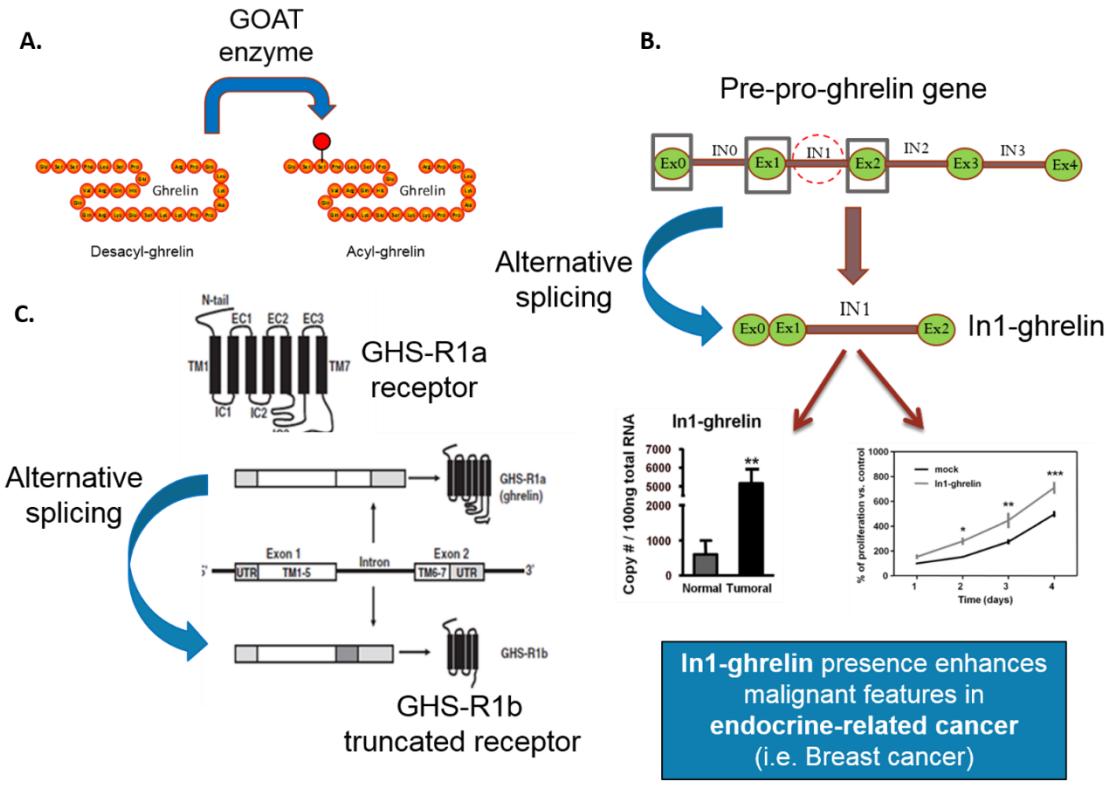


Figure 11: The ghrelin system. **A.** Ghrelin is found in plasma in two different forms: desacyl-ghrelin and acyl-ghrelin (active). GOAT enzyme is responsible for adding the acyl group in Serine 3 to ghrelin. Acyl-ghrelin is the only form able to bind to the growth hormone secretagogue receptor (GHS-R1a) and exert the majority of its known physiological and pathophysiological functions; **B.** Aberrant alternative splicing in the GHRL gene generates ghrelin and the spliced form In1-ghrelin variant which is commonly overexpressed and enhances malignant cancer features (i.e. cell proliferation, cell migration, hormonal secretion) in different cancer models; **C.** Alternative splicing in the GHSR gene generates the canonical GHSR1a but also an aberrant form called GHSR1b with pathophysiological features in endocrine tumors

2.4.1. Ghrelin system in PCa

Since 2002, several studies have reported the presence and investigated the potential pathophysiological role of ghrelin system in PCa [222-235]. These studies have shown that the majority of the components of the ghrelin system are locally expressed in the prostate gland. Particularly, ghrelin mRNA and protein expression has been reported in normal prostate, human benign hyperplasia, and prostate carcinoma [222] as well as in PCa cell lines [222, 223, 225]. Ghrelin peptide seems to be secreted from the prostate

[225], as it has been detected in the medium of the PC-3 cell line. In line with this, one study showed that "active" (acylated) ghrelin levels were higher in the blood of patients with PCa [228], but this point is controversial since other reports found no differences between patients with PCa and healthy individuals [226, 227]. In the prostate, ghrelin peptide is localized in the cytoplasm of glandular epithelial cells with lower staining intensity in the normal tissue compared with malignant epithelium [225].

There is much controversy on the putative physiological and pathological roles that ghrelin may play in the normal prostate and in PCa. Specifically, one group (led by Dr. L. Chopin) has reported that treatment with ghrelin peptide and/or overexpression of the canonical preproghrelin enhanced the growth rate and cell migration of PCa cell lines (PC-3 and LnCaP) [223, 225, 235], which could be mediated through the activation of key signaling pathways such as MAPK/ERK and/or PI3K/AKT/mTOR [225]. However, other studies from different and independent groups have shown opposite effects of ghrelin in terms of cell growth [222, 229, 231]. Interestingly, additional reports have also reported that other splicing variants generated from the ghrelin gene (i.e. exon 2- deleted preproghrelin, exon 3- deleted preproghrelin, in2c-variant) are also present in PCa, where they might be involved in controlling PCa malignancy features [225, 235, 236]. However, to date no studies have been published focusing in the presence and functional role of other components of the ghrelin-system in PCa, as is the case of the In1-ghrelin variant; therefore, new studies aimed at establishing the presence, regulation and functional role of some components of the ghrelin-system (including the splicing variant In1-ghrelin) in PCa are still required.

GOAT enzyme levels have been reported to be similarly abundant (both mRNA and protein) in normal and PCa tissues, although this study also demonstrated that its expression was significantly higher in PCa cell lines compared with normal prostate epithelial-derived cell lines [233]. This study also showed that acyl-ghrelin peptide treatment, but not desacyl-ghrelin, was specifically able to decrease GOAT expression in the normal-like RWPE1 cell line and in PC-3, but not in DU-145 or LnCaP, prostate cancer cell lines. Although this study shows for the first time that GOAT is expressed in PCa, many questions remains unanswered (i.e. the plasmatic expression of GOAT in patients with PCa or the regulation of this enzyme by different ghrelin-gene derived peptides or by other metabolic factors); Therefore, it is necessary to expand our knowledge of the presence and regulation of GOAT in PCa.

The presence of ghrelin receptors (GHSR1a and GHSR1b) in prostate cells has not been entirely defined, and the information published to date remains controversial [222, 223, 234]. Specifically, it has been reported that GHSR1a and GHSR1b expression was present in PCa cell lines (ALVA-41, LNCaP, DU145 and PC-3) [223], and that GHSR1a, but not GHSR1b, expression was also detected in normal prostate [223]. Conversely, another independent study showed that GHS-R1a and GHSR1b were not expressed in carcinomas, although GHS-R1b mRNA was present in 50% of hyperplasias [222]; moreover, in contrast to that previously reported, this study also showed that GHS-R1a and GHSR1b were expressed only in DU-145 cells. Recently, GHSR hypermethylation at the promoter and first exon was shown to be a common process in several cancers, including PCa, supporting the candidature of GHSR methylation as a highly accurate pan-cancer marker [234]. Therefore, as mentioned above, new studies focusing in the presence (in parallel), regulation and/or functional role of different components of the ghrelin-system (including the splicing variant In1-ghrelin, ghrelin, ghrelin-receptors and GOAT enzyme) using normal and PCa samples as well as normal and PCa cell lines in PCa are still required.

HYPOTHESIS AND OBJECTIVES

3. HYPOTHESIS AND OBJECTIVES

During the last years, our research group has focused much of its efforts in studying the role that two pleiotropic regulatory systems, those formed by somatostatin (SST)/cortistatin (CORT)/receptors (sst1-5 and sst5TMD4 and sst5TMD5 splicing variants), and by ghrelin/In1-ghrelin splicing variant/receptors (GHSR-1a and GHSR-1b) and GOAT enzyme, play on different pathological conditions, including several tumors and cancers. Although the presence of some receptors for SST/CORT and ghrelin in human PCa has been previously described, few studies have examined in detail the potential pathophysiological relevance of these regulatory systems. Based on this preliminary findings and our prior experience in other cancers, the initial **hypothesis of this PhD Thesis** was that the dysregulation of the SST/CORT and ghrelin systems, and, especially, of their splicing variants (sst5TMD4/5 and In1-ghrelin) and GOAT enzyme could directly and negatively influence PCa, and consequently, that their levels of expression in tumor tissues could provide useful information to improve diagnosis and prognosis of these tumors and/or to predict response to treatments. In addition, we thought that such molecules might be potential targets for the identification of new drugs (e.g. In1-ghrelin and sst5TMD4 receptor antagonists) and for the consequent development of future therapeutic strategies to treat these pathologies.

Based on this hypothesis, the **main objective of this thesis** was to determine the levels of expression and functional role of different components of the SST/CORT and ghrelin systems in PCa, especially those of the splicing variants sst5TMD4/5 and In1-ghrelin and GOAT enzyme, and to establish the possible association between their expression levels and: 1) the expression of other components of the SST/CORT and ghrelin systems (ligands, receptors and associated enzymes); 2) the expression of other regulatory systems and tumoral biomarkers well-known for its potential pathophysiological importance (e.g.: markers of proliferation, prognosis, etc.); and 3) the clinical information of the patients (e.g. levels of PSA, presence of metastasis, Gleason score, etc.).

Altogether, our ultimate aim was to establish the possible use of these splicing variants or other components of the SST/CORT and ghrelin systems, especially the GOAT-enzyme, as novel tumor biomarkers and promote their development as new diagnostic, prognostic and therapeutic targets.

To achieve this main objective, we proposed the following specific objectives:

1) To determine and analyze the expression levels of the aberrant splicing variants sst5TMD4, sst5TMD5 and In1-ghrelin, of GOAT-enzyme as well as others components of the SST/CORT and ghrelin systems and molecules of particular pathophysiological relevance (e.g. PSA, PCA3, etc.) in a wide, representative and clinically well-characterized set of human PCa samples, as well as in different human androgen-dependent and androgen-independent PCa cell lines (LnCAP, 22Rv1, PC-3, VCaP, Du145), compared to the expression levels of normal human prostate samples and the normal-like prostate cell line (RWPE-1), respectively.

2) To determine the association between the presence of the truncated receptors sst5TMD4/5, In1-ghrelin splicing variant and GOAT-enzyme and the clinical and pharmacological characteristics of patients with PCa, and to establish the possible quantitative associations between the presence of these splicing variants or GOAT-enzyme in PCa samples and the expression of other molecules of particular pathophysiological relevance in PCa analyzed in the objective 1.

3) To evaluate the functional response to different peptides of the SST and ghrelin systems (e.g. natural ligands, SSA and In1-ghrelin derived peptides) in primary human normal prostate cell cultures and in normal and PCa cell lines, and evaluate if this *in vitro* response on key functional processes associated with tumor progression and aggressiveness (e.g. cell proliferation, migration, etc.) is associated with the presence/absence of the aberrant splicing variants (sst5TMD4/5 and In1-ghrelin).

4) To explore the consequences of the overexpression/silencing of sst5TMD4/5, In1-ghrelin variant and/or GOAT-enzyme and other molecules from the SST and ghrelin systems on key functional processes associated with tumor progression and aggressiveness (e.g. cell proliferation, migration, PSA secretion, signaling pathways, etc.) in primary human normal prostate cell cultures, in normal and PCa cell lines, as well as on *in vivo* pre-clinical models of PCa (immunodeficient mice) that overexpressed/underexpressed sst5TMD4, sst5TMD5 or In1-ghrelin variant

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1. Patients and samples

All the studies involving human samples were approved by the Hospital Ethics Committee, conducted in accordance to the principles of the Declaration of Helsinki and written informed consent was obtained from all patients. Samples were obtained through the Andalusian Biobank (Servicio Andaluz de Salud). Different set of human samples obtained from PCa patients and control patients were collected. These samples included:

1) ***Formalin-fixed paraffin-embedded (FFPE) PCa tissues*** *(n=45)* obtained from radical prostatectomies of patient diagnosed with localized PCa (Gleason score 6-7 in final pathological evaluation) and their **non-tumoral adjacent tissues (N-TAR)** used as control. Available clinical parameters were collected from each patient (**Table 6**).

Table 6. Overall clinical and demographics data of patients from who formalin-fixed paraffin-embedded (FFPE) prostate pieces with low-intermediate grade tumors were obteined**.**

Parameter	Overall
Patients	n = 45
Age at diagnosis, Mean ± SD	62.27 ± 5.1
PSA level, ng/ml, Median (IQR)	4.8 (3.6-7.8)
Gleason score (%)	
6	12/45 (27%)
7	33/45 (73%)
EE, no. (%)	22/45 (48%)
PI, no. (%)	34/45 (75%)
VI, no. (%)	8/45(17%)
No.=number; SD=standard deviation; EE=extraprostatic extension; PI=perineural infiltration; VI=vascular invasion; FFPE= formalin fixed paraffin embedded; IQR=interquartile range	

2) ***Fresh prostate samples*** *(n=52)* obtained by core needle biopsies from patients diagnosed with palpable **high risk PCa** [PSA>20.0 and/or Gleason 8-10 and/or cT3, according to National Comprehensive Cancer Network (NCCN) guidelines classification] (**Table 7**). Histology of PCa was always confirmed by a specialized

anatomo-pathologist. Several techniques, such as computerized tomography (CT scan) and bone scan, were used to determine the presence of metastasis in these patients.

3) ***Normal prostate samples*** *(NPs, n=14*) from donor patients that underwent cystoprostatectomy due to bladder cancer. Histology of the normal prostate samples was always confirmed by a specialized anatomo-pathologist. Available clinical parameters were collected from each of these control patients (**Table 7**).

Table 7. Overall clinical and demographic data of patients from whom fresh PCa and normal prostate samples were obtained (by core needle biopsies or from cystoprostatectomy, respectively).

Parameter	Overall	Control	PCa	PCa No Met	PCa Met
Patients, no.	66	14	52	28	24
Age at diagnosis					
Median (IQR)	76 (67.5-81.25)	70 (62.2-80.7)	78 (69-81.7)	79 (69-83)	76 (69-80)
PSA level, ng/mL					
Median (IQR)			54.5 (37.2-212)	45 (22.5-61.5)	101.5 (51.2-771)
Gleason score					
7	-	-	18/52 (35%)	15/28 (53%)	3/24 (12%)
>7	-	-	34/52 (65%)	13/28 (47%)	21/24 (88%)
EE	-	-	17/52 (33%)	5/28 (18%)	13/24 (54%)
PI	-	-	27/52 (52%)	12/28 (43%)	15/24 (63%)

PCa=prostate cancer; No Met= no metastasis; Met= metastasis; nº= number; EE=extraprostatic extension; PI= perineural infiltration

4) ***Plasma and urine samples from patients with PCa*** (n=85) ***and healthy BMI-matched controls*** (n=28) for the evaluation of GOAT as new PCa biomarker (**Table 8**).

Table 8. Demographic and clinical characteristic of patients included in the study with plasma and urine samples for the evaluation of GOAT as PCa biomarker.

Parameter	Control (n=28)	PCa (n=85)	*p value*
Age, yr, mean (SD)	62.04 (9.6)	68.7 (8.3)	*0.0005*
Weight, kg, mean (SD)	84.56 (14.21)	81.5 (11.9)	0.27
Waist, cm, mean (SD)	105 (10.01)	105.5 (10.07)	0.83
BMI, mean (SD)	30.46 (4.4)	29.2 (3.8)	0.16
Ethnicity, (n)	Caucasian (27); Black (1)	Caucasian (84), Black (1)	-
Amylase	59 (52-75.3)	56 (46-75)	0.36
Albumin	4.16 (0.25)	4.20 (0.28)	0.54
Colesterol, mg/dL, mean (SD)	205.1 (34.69)	194.6 (38.93)	0.20
Triglycerides, mg/dL, median (IQR)	125.5 (93.25-178.5)	112 (90-151)	0.24
HDL, mg/dL, median (IQR)	45 (38-51)	45 (38.5-49.5)	0.84
Glycated hemogobin, (%)	5.6%	5.6%	0.4
Glucose, mg/dL, median (IQR)	109 (97.25-130.8)	102 (92-121.5)	0.15
Insulin, m U/L, median (IQR)	6.2 (3.3-10.7)	8.9 (5.5-11.4)	*0.045*
Diabetes, n (%)	9/28 (32.12%)	25/85 (29.4%)	0.81
Dyslipidemia, n (%)	5/28 (17.85%)	20/85 (23.95%)	0.60
Testosterone	5.3 (4.1-6.7)	4.8 (3.7-5.8)	0.16
Total PSA, ng/ml, median (IQR)	0,8 (0.4-1.1)	6.3 (3.8-9.5)	*<0.0001*
Free PSA, ng/mL, median (IQR)	-	0.81 (0.5-1.1)	-
Invasive tumors	-	14/85 (16%)	
Gleason score, n (%)			
6	-	41/85 (48%)	
7	-	38/85 (45%)	
8	-	5/85 (6%)	
9	-	1/85 (1%)	
cyfra_211	1,2 (1-1,80)	1,4 (1.1-2.1)	0.18
ca15_3	12 (7-21.7)	19 (14-24)	*0.046*
GOAT, ng/mL, median (IQR)	1.07 (0.9-3.2)	2.26 (1.3-6.1)	*0.0002*

PCa: prostate cancer; **Yr**= year; **SD**=standard desviation; **Kg**=kilogram; **cm**=centimeter. **BMI**=body mass index; **n**=number; **IQR**= interquartile range; Parameters with significant difference between the groups are indicated in *italic* letter.

5) ***Urine* samples *from patients with PCa*** (n=14) ***before and after*** prostate massage (**Table 9**).

Table 9. Demographic and clinical characteristics of patients included in the study for the analysis of urine GOAT levels before and after prostate massage

Parameter	**PCa**
Age, yr, mean (SD)	68.1 (7.1)
Weight, kg, mean (SD)	79.2 (9.9)
BMI, mean (SD)	28.8 (3.2)
Ethnicity, (n)	Caucasian (14)
Glycated hemoglobin, (%)	5.4 (5.2-5.8 %)
Glucose, mg/dl, mean (SD)	92.7 (8.4)
Insulin, m U/l, mean (SD)	8.6 (2.6)
Diabetes, n	0/14
Dyslipidemia, n	0/14
Testosterone ng/ml, mean (SD)	4.6 (2.2)
Total PSA, ng/ml, median (IQR)	5.9 (4.6-9.7)
Free PSA, ng/ml, mean (SD)	0.9 (0.3)
Invasive tumors, n (%)	2/14 (14%)
Gleason score, n (%)	
6	3/14 (21%)
7	9/14 (64%)
8	1/14 (7%)
9	1/14 (7%)
cyfra_21-1 U/ml, mean (SD)	1.6 (0.8)
ca_15-3 U/ml, mean (SD)	16.1 (6.9)
PCa: Prostate Cancer; **Yr**=Year; **SD**=Standard Deviation; **Kg**=Kilograms; **cm**=Centimeter; **BMI**=Body Mass Index; **n^{o}**=number; **IQR**=Interquartile Range.	

6) *Plasma* from another *cohort of* ***patients with PCa*** *(n=30)* ***and healthy donor patients*** *(n=20)* for detection of plasmatic In1-ghrelin and ghrelin levels (**Table 10**).

Table 10. Demographic and clinical characteristic of patients included in the study for the analysis of In1-ghrelin and ghrelin levels in the plasma.

Parameter	Control (n=20)	PCa (n=30)	*p value*
Age, yr, mean (SD)	63.8 (9.9)	74.8 (8.1)	*< 0.0001*
Weight, kg, mean (SD)	82.62 (12.8)	78.7 (12.3)	0.29
Waist, cm, mean (SD)	103.7 (10.7)	106 (10.9)	0.46
BMI, mean (SD)	29.8 (4.7)	28,8 (3.6)	0.54
Ethnicity, (n)	Caucasian, n=20	Caucasian, n=30	-
Amylase, median, (IQR)	60.5 (52.5-72.5)	54.5 (46.7-77.5)	0.26
Albumin, mean, (SD)	4.1 (0.23)	4.1 (0.33)	0.93
Colesterol, mg/dL, median, (IQR)	221 (184.3-244.8)	189.5 (163.5-206.5)	0.053
Triglycerides, mg/dL, mean (SD)	129.4 (39.2)	106.9 (41.5)	0.06
HDL, mg/dL, mean (SD)	45.8 (7.8)	48.2 (12.5)	0.44
Glycated hemogobin, (%)	5.5 (5.3-6.1)	5.7 (5.5-6.1)	0.59
Glucose, mg/dL, median (IQR)	107.5 (98.2-126.3)	97 (92-114)	0.06
Insulin, m U/L, mean (SD)	7.9 (6.9)	8.3 (3.1)	0.29
Testosterone	5.8 (1.6)	4.4 (2.1)	*0.01*
Total PSA, ng/ml, median (IQR)	0.85 (0.44-1.1)	25 (11.1-83.9)	*< 0.0001*
Free PSA, ng/mL, mean (SD)		0.74 (0.3)	-
Invasive tumors		14/30 (46,6%)	-
Gleason score, n (%)			
6		8/30 (26,6%)	-
7		9/30 (30%)	-
8		7/30 (23,3%)	-
9		6/30 (20%)	-
cyfra_211, media, (IQR)	1.2 (1-1.8)	1.8 (1.2-2.8)	*0.004*
ca15_3, median, (IQR)	14 (7-25.5)	23 (18.7-27)	*0.01*
Prepro-In1-ghrelin, pg/mL, median (IQR)	0 (0-0)	4.6 (0-18)	*0.03*

PCa: prostate cancer; **Yr**= year; **SD**=standard deviation; **Kg**=kilogram; **cm**=centimeter. **BMI**=body mass index; **n**=number; **IQR**= interquartile range; Parameters with significant difference between the groups are indicated in *italic* letter.

4.2. RNA, RT and qPCR assays

4.2.1. RNA isolation from FFPE samples

Total RNA from the FFPE samples was isolated using the RNeasy FFPE Kit (Qiagen, Limburg, Netherlands) according to the manufacturer's instructions.

Procedure:

- Place 4 FFPE cores (8µm sections) into a 2 ml microcentrifuge tube and close the lid.
- Add 1 ml limonene (xylene can also be used). Vortex vigorously for 10s, incubate at 56ºC for 5 min and centrifuge at full speed for 2 min. Repeat this step at least three times, until the paraffin is dissolved.
- Carefully remove the supernatant by pipetting without disturbing the pellet.
- Add 1 ml ethanol (96–100%) to the pellet, mix by vortexing, and centrifuge at full speed for 2 min.
- Carefully remove the supernatant by pipetting without disturbing the pellet. Carefully remove any residual ethanol.
- Keep the lid open, and incubate at room temperature (15–25°C) or at up to 37°C. Incubate for 10 min or until all residual ethanol has evaporated.
- Add 240µl Buffer PKD, and mix by vortexing.
- Add 10µl proteinase K. Mix gently by pipetting up and down.
- Incubate for 15 min at 56 ºC.
- Incubate for 15 min at 80 ºC.
- Place the sample on ice for 3 min and centrifuge for 15 min at full speed.
- Transfer the supernatant to a new microcentrifuge tube (not disturb the pellet).
- Mix 16µl DNase Booster Buffer and 10µl DNase I stock solution for sample. Mix by inverting the tube. Add 26µl of the mix to the sample.
-Incubate at room temperature for 15 min.
- Add 320µl Buffer RBC to break red cells and mix the lysate thoroughly.
- Add 720µl ethanol (100%) to the sample, and mix well by pipetting. Do not centrifuge.
-Follow the manufacturers' instructions for the next steps (column purification).

4.2.2. RNA isolation from fresh tissues (PCa samples by needle biopsies and normal prostate from cystoprostatectomy)

Total RNA from fresh prostate samples (PCa or normal prostate samples) was extracted using the AllPrep DNA/RNA/Protein Mini Kit followed by deoxyribonuclease treatment using RNase-Free DNase Set (Qiagen, Limburg, Netherlands).

Procedure:

Before starting:

*Kit DNase:

- Prepare DNase I stock solution by dissolving the lyophilized DNase I in 550µl RNase-free water.

*Kit extraction:

- Prepare a mix of β-mercapto + buffer RLT (10ul β-ME + 1ml RLT (this mix can be stored 1 month a RT)
- Add dithiothreitol (DTT) to buffer ALO (8mg DTT for each ml of buffer).
- Add ethanol (96-100%) to RPE, AW1 and AW2 buffers.
- Preheat EB buffer at 70ºC.

Protocol:

- Add suitable volume of RLT buffer + B-ME and homogenize (360-600ul if sample <20mg, or 600ul if sample >20mg)
- Centrifuge 3 min at full speed.
- Transfer to a DNA column (violet color)
- Centrifuge 30 sec at 10.000 rpm.
- Place column in a new 2ml tube.
- DNA is retained in the column while the liquid contains the RNA and proteins.
- Store column with DNA at 4ºC,
- Add ethanol 100% to the liquid in order to purify the RNA. Add 250ul if we started with 350ul, or 430ul if we used 600ul.
- Mix by pipetting, no vortex/centrifuge.
- Transfer up to 700ul to the RNA column (pink)
- Centrifuge 15 s at 10.000 rpm
- Transfer the liquid to a 2 ml tube and stored at 4ºC (Proteins).
- RNA is retained in the column.

RNA

- Add 350ul of RW1 buffer to the column.

- Centrifuge 15 sec at 10.000 rpm
- Discard the liquid
- Add 10ul DNase to 70ul RDD buffer, mix by inversion, spin.
- Add 80ul of this mix to the center of the column
- Incubate 15 min at RT
- Add 500ul of RPE buffer
- Centrifuge 15 sec at 10.000 rpm
- Discard the liquid
- Add 500ul RPE buffer
- Centrifuge 2 min at 10.000 rpm
- Place the column in a new 2-ml tube.
- Centrifuge 1 min at full speed.
- Place the column in a new 1.5-ml tube.
- Add 30-50ul of RNA-free water
- Centrifuge 1 min at 10.000 rpm
- Store RNA at -80ºC

Proteins

- Add 700ul of APP buffer to the liquid stored at 4ºC.
- Vortex and incubate 10 min at RT
- Centrifuge 10 min to full speed
- Discard supernatant with a pipette
- Add 500ul of 70% ethanol
- Centrifuge 1 min to full speed
- Discard supernatant with a pipette
- Incubate 5-10 min at RT in order to dry the protein-pellet.
- Add up to 100ul of ALO buffer and vortex
- Incubate 5 min at 95ºC
- Chill at RT
- Centrifuge 1min at full speed
- Transfer the supernatant into a new 1,5-ml tube.
- Store proteins at -20 ºC.

Genomic DNA

- Add 500ul of buffer AW1 to the column at 4 ºC

- Centrifuge 15 sec to 10.000 rpm
- Discard the liquid
- Add 500ul of AW2 buffer
- Centrifuge 2 min at full speed.
- Place column in a new 1.5-ml tube.
- Add 100ul of preheated-EB buffer
- Incubate 2 min at RT
- Centrifuge 1min at 10.000 rpm
- Store genomic DNA at 4ºC

4.2.3. RNA isolation from prostate cell lines

Total RNA from PCa and normal prostate cell lines was extracted with Trizol reagent (Thermo Scientific, Wilmington, NC, USA). Briefly, cells were seeded until confluence in 6 o 12-well-plates. Then, wells were washed two times with PBS buffer and, subsequently, 0.6ml Trizol was added and collected with lysed cells. RNA isolation was carried out by adding chloroform, centrifuging and collecting the aqueous phase. RNA was recovered and concentrated with 2-propanol precipitation and 70% ethanol washing steps. Finally, samples were dried and resuspended with 8µl of DEPC-treated water. Subsequently, samples were treated with 1µl (1 unit) of DNase (Promega, Barcelona, Spain) and incubated 30 min at 37°C, stopping the reaction by adding a Stop Solution and incubating at 65°C for 5 min.

4.2.4. Quantification of RNA concentration and Reverse-Transcription (RT)

Total RNA concentration and purity was assessed using Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, NC, USA), and subsequently retro-transcribed using random hexamer primers and the cDNA First Strand Synthesis kit (MRI Fermentas, Hanover, MD, USA).

Procedure:

- Add the following reagents in the order indicate below (RNA-free tubes).
- Template RNA: from 0.1 ng to 2.0 µg.
- 1 µl of Random Hexamer primer
- Water nuclease-free up to a volume of 12 µl.

- Mix gently, spin down and incubate at 65°C for 5 min.
- Cool on ice for 3 min.

Prepare a mix the following components in each sample:

- 5X Reaction buffer: 4µl
- RiboLock RNase Inhibitor (Enzime 1): 1µl.
- 10 mM dNTP Mix: 2µl
- RevertAid M-MuLV RT (200 U/µL; Enzime 2): 1µl.

Total volume of 20 µl. Then:

- Mix gently and centrifuge briefly.
- Incubate for 5 min at RT and then 60 min at 42 ºC.
- Finish the reaction by heating the sample 5 min at 70 ºC.

4.2.5. Conventional PCR and quantitative real-time PCR (qPCR) with SYBR green

Primers were designed using the bioinformatics tool Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) or Primer3 online tool (http://bioinfo.ut.ee/primer3-0.4.0/) with specific conditions to optimize the results in the downstream applications:

- Melting temperature of 60 ºC.
- Primer size: 20 pair of bases (range from 18-24)
- Primer % GC: Min: 40; Max: 60.
- Max. Self. Complementary: 4 (Ideally 0)
- Max. 3'Self. Complementary: No more than 1 (Ideally 0)
- Amplified sequence of 75 to 200pb
- Primers designed between different exons to prevent genomic amplification

Each set of primers was always evaluated for specificity by BLAST alignment (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

Conventional PCR has been routinely used along this work to validate qPCR primers, using the "DreamTaq DNA Polymerase" (Thermo Scientific, Wilmington, NC, USA) and the thermocycler "Supercycler Gradient Cycler" (Kyratec, Belgium).

In qPCR assays, cDNAs were amplified with the Brilliant III SYBR Green Master Mix (Stratagene, La Jolla, CA, USA) using the Stratagene Mx3000p system. qPCR of samples was always run in parallel with a standard curve to estimate the absolute mRNA copy number. Thermal profile consisted of an initial step at 95ºC for 3 min, followed by 40 cycles of denaturation (95ºC for 20 seconds), annealing (60ºC for 20 sec) and finally, a dissociation cycle to verify that only one product was amplified (by the melting curve). Expression levels were reported as absolute mRNA copy number/50ng of sample. For fresh samples a normalization factor was calculated with the expression levels of ACTB and GAPDH using GeNorm 3.3. For *in vitro* assays (normal prostates cell cultures and PCa cell lines) result were adjusted according to the value of ACTB.

4.3. sst5TMD4 and GOAT IHC analysis

sst5TMD4 immuno-histochemical staining was performed on 30 FFPE prostate samples with regions of normal prostate tissue and PCa tissue using a custom-made and previously validated polyclonal rabbit anti-human sst5TMD4 antibody (1:400) [188] (clone name:66498) using standard procedures [183, 185, 188]. In the case of GOAT, validation of IHC was implemented in formalin-fixed, FFPE prostate tissue samples obtained from radical prostatectomies, which have normal and tumoral regions from patients diagnosed with localized PCa (Gleason score 6-7 in final pathological evaluation). Finally, GOAT IHC staining was performed on biopsies pieces obtained from patients diagnosed with clinical high grade PCa (Gleason 7-10; n=16) and in normal-benign prostate samples. GOAT staining was analyzed using a commercially available human GOAT antibody that recognizes the middle region of the enzyme (AA 257-287; ABIN953340, Acris antibodies GmbH, Herford, Germany) using standard procedures (1:400).

Deparaffinized sections were incubated with sst5TMD4 and GOAT antibodies overnight at 37C, followed by incubation with the appropriate HRP-conjugated secondary antibody (Envision system, Dako, Barcelona Spain). Finally, sections were developed with 3,3´-diaminobenzidine (Envision system 2-Kit Solución DAB), contrasted with hematoxylin and mounted in an automatic mounter (Tissue-Tek® Film®, Sakura, Japon). A series of concentration-response tests were tested in order to determine the ideal concentration of antibody that need to be used to perform these analyses in prostate samples. For GOAT IHQ, pancreatic tissue was used as positive control. Two

independent pathologists performed the histopathological analysis of the tumors following a blinded protocol. In the analysis, +, ++, +++ stand for low, moderate, and high intensities of the tumoral region staining compared to the normal-adjacent region.

4.4. Analysis of Single Nucleotide Polymorphisms (SNPs) in the sst5 gene sequence

As previously mentioned, genomic DNA (gDNA) from tumoral (n=41) and control (normal prostates; n=9) samples were extracted using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Madrid, Spain) following manufacturer's instructions and subsequently quantified with Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, NC, USA). In order to identify SNPs that could be associated with the presence of sst5TMD4, specific primers to amplify the 5' and 3' ends of the cryptic intron spliced to generate the sst5TMD4 variant were designed and validated, and the resultant PCRs were further sequenced. Only variations located at the 3' ends side of the genomic sequence of the cryptic intron spliced to generate the sst5TMD4 were identified, where two previously reported SNPs were found among the samples analyzed. These SNPs were previously described in PubMed database as rs197055 [there are 3 different forms described for this SNP (A/C/T); however, in our studies we only found C and A variant] and rs12599155 [there are 2 different forms described (C and T); both of them were found in our samples].

4.5. Cultures: normal human prostate cells, normal-like prostate cell line and PCa cell lines.

4.5.1 Primary cell cultures from human prostate tissues

Normal prostate tissue was resected from donor patients after radical cytoprostatectomy surgery and transferred to the cell culture room in sterile cold (4ºC) culture medium (S-MEM, Gibco, Madrid, Spain) supplemented with 0.1%, BSA, 0.01% L-glutamine, 1% antibiotic-antimycotic solution, and 2.5% HEPES (Sigma-Aldrich, Madrid, Spain). Then, the tissue was washed three times in sterile culture medium and cut into small pieces. A portion of these pieces was rapidly frozen in liquid nitrogen ad stored at -80 ºC for RNA extraction. The remaining tissue was dispersed into single cells by enzymatic and mechanical disruption as previously reported [237] (see procedure

below). Dispersed cells were cultured onto tissue culture plates in PrEGM Prostate Epithelial Cell Growth Medium (Clonetics, cc-3165).

Procedure of enzymatic and mechanical disruption of prostate tissue

1. Transfer the prostatic tissue to a Petri dish (recommended size: 100 mm x 20 mm) with sterile culture medium.
2. Cut the tissue in small portions using sterile bladders and transfer to a sterile tube with PBS.
3. Centrifuge for 5 min at 1800 rpm. Aspire the supernatant and resuspend the cell pellet in 30 ml of PBS. Repeat this step one more time.
4. Discard the supernatant and resuspend the pellet in 20-30 ml of complete DMEM medium (supplemented with 10% Fetal Bovine Serum, 4 mM glutamine and 1% antibiotic-antimycotic solution). If the sample is collected in the morning, store at 4ºC until late afternoon.
5. Centrifuge for 5 min at 1800 rpm. Discard the supernatant.
6. Add the collagenase/dispase digestion medium (final concentration of both enzymes of 1mg/ml in complete DMEM medium). Incubate overnight at 37ºC in rotational movement.
7. Early in the morning, centrifuge for 5 min at 1800 rpm.
8. Discard the supernatant, wash cells in PBS buffer and centrifuge for 5 min at 1800 rpm. Discard the supernatant, resuspend the cell in 5 ml of Trypsin/EDTA solution and incubate for 5 min at 37ºC with occasional shaking.
9. Add 15 mL of DNase I solution (1 mg of recombinant DNase I in 50 mL of DMEM medium). Mix with Trypsin/EDTA solution. Centrifuge 5 min at 1800 rpm.
10. Discard the supernatant and resuspend in 10 mL of DMEM. Pass the cells through a sterile, siliconized glass pippete.
11. Pass the cells through a 100 µm filter. Centrifuge for 5 min at 1800 rpm.
12. Discard the supernatant and resupend the cells in 5 mL red blood cell lysis buffer. Incubate for 5 min on ice. Next, add 25 mL of PBS and centrifuge for 5 min at 1800 rpm.
13. Discard the supernatant and resupend the cell pellet in 10 mL of complete DMEM medium. Pass the solution through a 40 µm filter. Centrifuge for 5 min at 1800 rpm.
14. Discard the supernatant and resupend the cell pellet in 1-3 ml of complete PrEGM (PrEGM medium is supplemented with bovine pituitary extract, Insulin, Hydrocortisone, GA-1000, retinoic acid, transferrin, T3, Epinephrine and recombinant EGF).

15. Count the number of cells using the trypan blue method.

4.5.2. Cell lines

Normal-like prostate cell line (RWPE-1) as well as androgen dependent (22Rv1, LnCaP, VCaP) and independent (C4-2b, PC-3, DU-145) PCa cell lines were used in different assays. Each cell line was obtained from ATCC and cultured and maintained following manufacturers' instruction. All cell lines were validated by analysis of STRs (GenePrint® 10 System, Promega, Barcelona, Spain) and checked for mycoplasma contamination once a month by PCR as previously reported [238].

RWPE-1*:* Epithelial cells (markers cytokeratins 8 and 18) derived from the peripheral zone of a histologically normal adult human prostate from a 54-year old man, transfected with a single copy of the human papilloma virus 18 (HPV-18). This c ell line does not develop tumors in nude mice, and expresses AR and PSA (mRNA and protein levels) upon stimulation with DHT [239]. Doubling time: 58 h.

22Rv1: Human prostate carcinoma epithelial cell line derived from a xenograft that was serially propagated in mice after castration-induced regression and relapse of the parental. It develops tumors in nude mice, expresses AR (H874Y mutation) and PSA (both at RNA and protein levels) [240]. Cells express the following CKs: CK-8, CK-18. Other molecular markers: CD44, DD3 mRNA. Doubling time: 56 h.

LnCaP: Isolated from a needle aspiration biopsy of the left supraclavicular lymph node of a 50-year old man with metastatic-PCa. It develops tumors in nude mice and expresses high basal levels of PSA and AR (T877A mutation). Cells express the following CKs: CK-8, CK-18. Other molecular markers: DD3 mRNA. Constitutive activation of AKT pathway (inactivation of PTEN) [241]. Doubling time: 60 h.

VCaP: Cell line from a vertebral bone metastasis from a 59-year old patient with hormone-refractory PCa. It expresses high basal levels of AR (without mutations) and PSA (both at mRNA and protein levels), and develops tumors in nude mice. Cells express the following CKs: CK-8, CK-18. Other molecular markers: Rb, p53, DD3 mRNA. Double time: 5-6 days [242, 243].

PC-3: Cell line isolated from a bone metastasis of a grade IV prostatic adenocarcinoma from a 62-year old male. It develops tumors in nude mice and lacks expression of AR and PSA. Cells express the following CKs: CK-5, CK-8, CK-18. Other molecular markers: vimentin, NSE, BA-16, b-catenin, g-catenin, CBP. It lacks PTEN

expression. Doubling time: 33 h. Reported as cell line with neuroendocrine differentiation characteristics [200].

DU145: Derived from a metastatic brain lesion from a 69 years old Caucasian man. Develop tumors in nude mice. Does not express AR or PSA. Cells express the following CKs: CK-5, CK-8, CK-18. Other molecular markers: vimentin. Doubling time: 30 h [243].

4.6. Peptides

Human acylated ghrelin (SC1357, PolyPeptide Laboratories, Limhamn, Sweden), octreotide (GP-Pharm, Barcelona, Spain), IGF-1, insulin, paclitaxel and Ionomycin peptides (Sigma-Aldrich, Madrid, Spain) were purchased, while human acylated In1-ghrelin derived peptides (In1-19 and In1-40) were synthesized in collaboration with Ipsen Bioscience (Cambridge, MA, USA) and developed by CPC Scientific (Chinese Peptide Company, Hangzhou, China; see below). Pasireotide was generously provided by Herbert A. Schmid (Novartis Pharma AG, Basel, Switzerland).

4.7. Transfections (plasmid and siRNA)

4.7.1. Stable and transient transfection of ghrelin, In1-ghrelin, and sst5TMD4.

PC-3 and VCaP cell lines were stably transfected while DU145 was transiently transfected with pCDNA3.1 vector (Life Technologies, Madrid, Spain) empty (mock) or containing ghrelin, In1-ghrelin or sst5TMD4 transcripts and selected as previously reported [185]. Transfection was confirmed in all cases by qPCR and also by Western Blot (in the specific case of sst5TMD4).

Protocol

1. Seed the cells in a 6-well culture plate
2. When ~70% of confluence is reached, change the medium for 500µl of Opti-MEM.
3. Prepare 500 ng of each plasmid in a 1.5-ml Eppendorf.
4. Add 50 µl of Opti-MEM to each plasmid.
5. Prepare an additional mix of 50µl of optimum + 3µl of Lipofectamine-2000 (Gibco, Barcelona, Spain) for each transfection. Incubate the mix for 5 min at RT.

6. Add 50µl of the optimum-lipofectamine mix to the plasmid (with the 50µl of optimum) for an approximate total volume of 100µl.
7. Incubate for 30 min at RT.
8. Add to the seeded cells and incubate for 6-8 h at 37ºC.
9. Change the medium for normal growth medium (supplemented with 10% FBS). If a stable transfection is required, next day add genetic antibiotic (500µg/ml; Gibco, Barcelona, Spain), which selectively eliminates non-transfected cells.

4.7.2. Silencing of ghrelin, In1-ghrelin and sst5TMD4 by specific siRNA

For silencing assays, LnCaP (In1-ghrelin), PC-3 (sst5TMD4, In1-ghrelin) and DU-145 (sst5TMD4) cell lines were used. Cells were transfected with specific siRNAs designed and previously validated against In1-ghrelin (siRNA-1: 5'-GAGTCCTAAACAGACTGTT-3'; siRNA-2: 5'-CACUGUUUCUGGAAGGACATT-3') and sst5TMD4 (5'-CACAAAUCCUGGCAGGAGATT-3'), or scramble control (Catalog # 4390843, Invitrogen) using Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's instructions**.** After 48h, cells were collected for validation of the transfection (mRNA and/or Western Blot) and seeded for proliferation assays.

Protocol

1. Seed 200.000 cells in a 6-well plate (duplicate or triplicate for each transfection)
2. Incubate cells until ~70% of confluence is reached.
3. Transfect the cells with the desired siRNA concentration (25-100 nM).
4. Change the medium for 500µl of complete medium without antibiotics.
5. If the transfection is with 100 nM of siRNA, add 1 µl of siRNA (stock concentration of 50µM) to 50 µL of Opti-MEM medium in a 1.5 ml Eppendorf tube for each reaction.
6. Add 3,5µl of Lipofectamine RNAiMAX (Catalog # 13778-075, Thermo Scientific, Wilmington, NC, USA) to 50µl of optimum medium in another 1,5 ml Eppendorf tube for each reaction.
7. Add Lipofectamine mix to siRNA mix (dilution 1:1)
8. Incubate for 5 min at RT.
9. Add 100µl of this mix to the seeded cells.

10. Incubate for two days at 37ºC and 5% CO_2.

4.8. Functional assays

4.8.1. Measurements of proliferation rate

Cell proliferation was measured by Alamar Blue reagent (Biosource International, Camarillo, CA, USA), MTT tetrazolium salt colorimetric assay (Sigma-Aldrich, Madrid, Spain), and/or Tripan blue growth assay. Results were expressed as percentage referred to control (vehicle treated medium or mock transfected cells). All experiments were performed at least with three independent cell preparations.

Protocol for Alamar blue reagent

1. Seed 3000 cells per well for each treatment (triplicate or quadruplicate) with complete medium (10% of FBS). Incubate 1 day at 37ºC and 5% CO_2.
2. Change medium for complete growth medium without FBS in order to synchronize the cells. Incubate for 24 h.
3. Next day, dilute 10X Alamar blue stock with complete growth medium without FBS. Change the cell medium for the mix of Alamar blue 1x with complete growth medium without FBS for 3 h (100µl).
5. Read fluorescence at 560 nm (exciting) and 590 nm (reading) in a FlexStation system plate reader (Molecular Devices). This measurement will be the time 0h
6. Change medium for complete fresh medium with 10% FBS immediately after each measurement. In experiments using treatments, the medium will be changed immediately after each measurement for complete fresh medium alone with 10% FBS (control) or with different treatments at the selected concentration.
7. Repeat this process after 24 h, 48 h and 72 h.

Protocol for MTT assay

1. Seed 3000 cells per well for each treatment (triplicate or quadruplicate) with complete medium (10% of FBS). Incubate 1 day at 37ºC and 5% CO_2.
2. After the incubation, change medium for complete growth medium without FBS in order to synchronize the cells. Incubate for 24 h.

3. Next day, remove the cell medium and add 0.25% MTT reagent (Sigma Aldrich) resuspended in dPBS (Sigma Aldrich). Incubate for 3 h at 37ºC and 5% CO_2.

4. Discard the MTT solution from each well, and add acid-SDS solution (1g SDS, 10ml DMSO, 57.2µl glacial acetic acid) to lysate the cells.

5. Read absorbance at 570 nm in a FlexStation system plate reader (Molecular Devices).

6. Change medium for complete fresh medium with 10% FBS immediately after each measurement.

4.8.2. Measurements of cell migration

Cell migration was evaluated by Wound Healing assay as previously described [185]. Specifically, 500.000 cells were seeded in 6-well culture plates and incubate at 37ºC and 5% CO_2. When cells were completely confluents, a scratch was made with a sterile 200ul tip. Then, cells were incubated overnight at 37ºC and 5% CO_2 in complete medium without FBS in order to minimize cell proliferation effects on wound recovery. Next day, cell medium was replaced for medium with the specific treatment (or only with medium alone, used as control) and cells were incubating for 12h. At least, three independent and different pictures of the scratch were taken at 0 and 12h to monitor the wound healing. Wound healing was calculated as the area of a rectangle centered in the picture 1 h after the wound vs. the area of the rectangle just after doing the wound.

4.9. Hormonal measurements (ELISA and RIA techniques)

4.9.1. Measurement of PSA levels by ELISA

PSA (in human plasma or cell culture medium) was measured by a commercial ELISA kit (cat number: EIA-3719; Springfield, NJ, USA).

Protocol

1. Place all reagents and samples to RT

2. Format the required microplate wells.

3. Pipette 25 µL of standards, controls or samples into each well and incubate for 5 min at RT.

4. Add 100µL of PSA conjugate into each well, mix by moving the plate

5. Incubate 1h at RT with moderate shaking

6. Remove solution from the wells by aspirating the liquid or by decanting it.

7. Wash wells with distilled water (repeat this process five or six times)

10. Pipette 100µL of TMB-substrate solution into each well in darkness and cover the plate.

11. Incubate ~20 min at RT (18 °C - 25 °C) with moderate shaking. At this point, it is necessary to evaluate the changes in the color of the wells in order to stop the reaction before if this is necessary (to avoid saturation).

12. Add 100 µL/well of the stop solution (same order as substrate solution).

13. Read absorbance (OD) at 450 nm (blanking 630 nm).

4.9.2. Measurement of GOAT levels by ELISA

For the determination of GOAT protein level (serum, urine or cell culture medium), a commercial ELISA was used following the manufacturers' instructions (MyBioSource, San Diego, USA).

Protocol

1. Place all reagents, samples and standard at RT in order to pre-warm up all the reactive.
2. Calculate the wells required for standard curve (7 wells from 50 ng/ml to 0.78 ng/ml), blank and samples.
3. Add 100µl of standard or sample to each well. Protect from light. Incubate for 2h at 37 ºC in agitation.
4. Discard the liquid and add 100µl of the prepared Detection Reagent A. Incubate for 1h at 37 ºC.
5. Discard the liquid and wash for at least 3 times.
6. Add 100µl of the prepared Detection Reagent B. Incubate 30 min at 37ºC in agitation.
7. Discard the liquid and wash 5 times.
8. Add 90µl of the Substrate solution (in darkness). Protect from light. Incubate 10-20 min at 37 ºC in agitation.
9. Add 50µl of the Stop solution. Read at 450nm immediately.

4.9.3. Measurement of active ghrelin levels by ELISA

For the determination of active ghrelin (acylated at serine-3) protein level, a commercial ELISA kit was used following the manufacturers' instructions (Cat number: EZGRA-88K; Millipore, Madrid, Spain).

Protocol

1. Pre-warm all reagents to RT immediately before setting up the assay.
2. Calculate the wells required for the ELISA.
3. Wash each well with 300µL of diluted Wash Buffer (repeat this step 2 more times)
4. Add 20µL of the Matrix Solution to Blank, Standards and Quality Control (QC) wells
5. Add 30µL of the assay buffer to each of the Blank and sample wells
6. Add 10µL of the assay buffer to each of the Standard and QC wells.
7. Add in duplicate 20µL of Ghrelin Standards in the order of ascending concentrations to the appropriate wells.
8. Add in duplicate 20µL of QC1 and QC2 to the appropriate wells.
9. Add sequentially 20µL of the unknown samples in duplicate to the remaining wells.
10. Cover the plate with plate sealer and incubate at RT for 2 h with agitation
11. Remove plate sealer and decant solutions from the plate.
12. Wash wells 3 times with the diluted Wash Buffer (300 µL per well per wash).
13. Add 100 µL of the Enzyme Solution to each well. Cover plate with sealer and incubate with moderate shaking at RT for 30 min on the micro-titer plate shaker.
14. Remove sealer, decant solutions from the plate and tap plate to remove the residual fluid.
15. Wash wells 6 times with diluted Wash Buffer (300µL per well per wash). Decant and tap after each wash to remove residual buffer.
16. Add 100µL of Substrate solution to each well, cover plate with sealer and shake in the plate shaker for approximately 5-20 min.
17. Read absorbance at 450 nm (set 590 nm as control)

4.9.4. Measurement of active Prepro-In1-ghrelin levels by RIA

For the measurement of Prepro-In1-ghrelin levels, a competitive radioimmunoassay (RIA) was used following the manufacturers' instructions (cat

number: RK-032-42; Phoenix, Burlingame, CA, USA). Briefly, the assay is based upon the competition of 125I-peptide and prepro-In1-ghrelin peptide binding to the limited quantity of antibodies specific for prepro-In1-ghrelin peptide in each reaction mixture. As the quantity of sample in the reaction increases, the amount of 125I-peptide able to bind to the antibody is decreased. By measuring the amount of 125I-peptide bound as a function of the concentration of prepro-In1-ghrelin peptide (in standard reaction mixtures), it is possible to construct a "standard curve" from which the concentration of prepro-In1-ghrelin in the unknown sample can be determined.

Protocol

1. Dilute the RIA buffer with distilled water.
2. Reconstitute the standard peptide with 1ml of RIA buffer. Mix well and keep on ice.
3. Reconstitute the rabbit anti-peptide serum with 13ml of RIA buffer, mix well and keep on ice.
4. Reconstitute the positive Controls with 1ml of RIA buffer. Mix well and keep on ice.
5. Reconstitute samples with RIA buffer
6. Prepare dilutions of the standard as follows:

Tube Sample RIA Buffer Std. Conc. Stock Powder 1.0ml 12.8 µg/ml
0 10µl of Stock 990µl 128,000 pg/ml
A 10µl of 0 990µl 1,280 pg/ml
B 500µl of A 500µl 640 pg/ml
C 500µl of B 500µl 320 pg/ml
D 500µl of C 500µl 160 pg/ml
E 500µl of D 500µl 80 pg/ml
F 500µl of E 500µl 40 pg/ml
G 500µl of F 500µl 20 pg/ml
H 500µl of G 500µl 10 pg/ml

7. Set up initial RIA reactions in 12 x 75 mm polystyrene tubes.

a) Number tubes TC-1, TC-2, NSB-1, NSB-2, TB-1, TB-2 and #7 - #22 for the standards.

b) Number tubes #23, #24 for the positive controls.

c) Number tubes #25 up to #125 for the unknown samples.

d) Pipette 200µl of RIA buffer into each NSB tube.

e) Pipette 100µl of RIA buffer into each TB tube.

f) Pipette 100µl of standards H through A into duplicate tubes #7-#22.

g) Pipette 100µl of positive control in tubes #23 & #24.

h) Pipette 100µl of unknown sample into duplicate tubes: tube #25 and up.

i) Pipette 100µl of primary antibody (rabbit anti-peptide serum) into all tubes except TC and NSB tubes.

j) Vortex the contents of each tube.

k) Cover and incubate all tubes for 16-24 h at 4°C.

8. Reconstitute the 125I-peptide with 13ml of RIA buffer and mix well to make tracer solution.

9. Add 100µl of the tracer solution to each tube.

10. Vortex the contents in each tube.

11. Cover and incubate all tubes for 16-24 h at 4°C.

12. Reconstitute the Goat Anti-Rabbit IgG serum (GAR) with 13ml of RIA buffer

13. Reconstitute the Normal Rabbit Serum (NRS) with 13ml of RIA buffer.

14. Add 100µl of GAR to each tube except the TC tubes.

15. Add 100µl of NRS to each tube except the TC tubes.

16. Vortex the contents of each tube. Incubate all tubes at RT for 90 min.

17. Add 500µl of RIA buffer to each tube except the TC tubes and vortex.

18. Centrifuge all tubes (except the TC tubes) at 3,000 rpm for 20 min at 4°C.

19. Aspirate the supernatant (without touching the pellet) immediately following centrifugation.

20. Use a γ-counter to count the cpm of the pellet.

4.10. Mechanistic assays

4.10.1. Cancer pathway reporter assay

To evaluate the intracellular signaling pathways modulated by sst5TMD4 overexpression, a Cignal Finder 10-Pathway Reporter Array (Qiagen, Limburg, Netherlands) was initially used to simultaneously analyze ten relevant pathways that are critical regulators of cancer biology. Specifically, 40.000 sst5TMD4 stable-transfected PC-3 or mock cells were seeded in duplicates in a 96 well-array plate with Opti-MEM serum free medium and reverse transfected with the transcription factors-responsive

firefly luciferase construct specific for each pathway using Attractene Transfection Reagent (Qiagen), following the manufacturer's instructions. Cells were incubated for 24h at 37°C and 5% CO^2, under sterile conditions. Next day, medium was changed to complete growth medium (RPMI 1640 medium complemented with 10% fetal bovine serum, 2mM L-glutamine, and 0.2% of antibiotic) and cells were grown for an additional 24h. Subsequently, a Dual-Glo Luciferase assay (Promega, Barcelona, Spain) was performed under manufacturer's recommendations to monitor the activity of the different pathways. Relative firefly luciferase activity was measured and normalized to the constitutively expressed *Renilla* luciferase.

4.10.2. Measurement of free cytosolic calcium changes

Changes in free cytosolic calcium concentration ($[Ca^{2+}]_i$) in single cells were determined using fura-2AM dye (Molecular Probes, Eugene, OR).

Protocol

1. Seed 50,000 cells/coverslip (35-mm plates) in complete growth medium. Incubate for 24-48h until cells are 70% confluent.
2. Prepare medium with fura-2AM: 2 ml of phenol red-free DMEM containing 20 mM NaHCO3 (pH 7.4) + 2µl of pluronic acid + 5µl of FURA.
3. Remove the medium from the wells (red phenol interferes with the reaction), and add 1 ml of medium with FURA.
4. Incubate for 30 min at 37 ºC.
5. Remove the medium and add calcium medium.
6. Incubate for 15 min at RT.
7. Prepare treatments in this 15 min-incubation time.
8. Add 300µl of calcium medium. Set up the protocol in the computer.
9. Zoom with a 40X objective with Immersion Oil Type NF (Nikon, Tokyo, Japan) with the inverted microscope Eclipse TE2000-E (Nikon, Tokyo, Japan) coupled to a digital camera ORCA II BT (Hamamatsu Photonics, Hamamatsu, Japan).
10. Add 300µl of medium with the treatment.
11. Changes in free cytosolic calcium concentration ($[Ca^{2+}]_i$) are monitored over time after exciting the cells at 340nm (excitation peak of FURA binding to Ca^{2+})

and 380nm (excitation peak of FURA Ca^{2+}-free) and collecting the emission intensity at 510nm in the MetaFluor software.

12. Ionomycin (Sigma-Aldrich, Madrid, Spain) was always used as positive control.

4.10.2.1. Stable sst5TMD4-transfected PC-3 cells treated with SST analogs.

Mock- or sst5TMD4-stably-transfected PC-3 cells were grown onto glass cover slips and $[Ca^{2+}]_i$ was evaluated in response to SSA treatment (octreotide and pasireotide).

4.10.2.2. Normal human primary prostate cell cultures treated with ghrelin or In1-ghrelin.

$[Ca^{2+}]_i$ was evaluated also in normal human primary prostate cell cultures in response to ghrelin or In1-ghrelin peptides (In1-19 and In1-40).

4.10.3. RT2 Prostate Cancer PCR Array

Total RNA was extracted from 4 consecutive passages of mock, ghrelin, in1-ghrelin or sst5TMD4-stably-transfected PC-3 cells using the Absolutely RNA RT-PCR Miniprep Kit (Agilent, La Jolla, CA, USA). Total RNA quality was assessed using the Agilent's 2100 Bioanalyzer (Agilent technologies).

Protocol

1. Total RNA (0.75µg, obtained from the mixture of RNA from 4 consecutive passages) was incubated for 5 min at 42ºC with genomic DNA elimination mix (see mix below) and cooled on ice for 1 min.

Genomic DNA elimination mix

Component	Amount/volume for 1 reaction
RNA	0.75 µg
Buffer GE (genomic DNA elimination)	2 µl
RNase-free water	Variable µl
Total volume	10 µl

2. Next, first-strand cDNAs were synthesized using the RT^2 First Strand kit (catalog number 330401, Qiagen). The reaction mixtures (see below, total final reaction with the genomic DNA elimination mix= 20µl) were incubated at 42°C for 15 min,

followed by incubation at 95°C for 5 min and 91 µl of water was added and then cooled on ice.

Reverse transcription mix

Component	Volume for 1 reaction
-5X Buffer BC3	4 µl
-Control P2	1 µl
-RE3 Reverse Transcription Mix	2 µl
-RNase-free water	3 µl
Total volume	10 µl

3. RT^2 Prostate Cancer PCR array was used to simultaneously examine the mRNA levels of 84 genes associated with PCa development, including five "control genes" in a 96-well plate (catalog number 330231 PAHS-135ZA, Qiagen). A mix of 1350 µl of 2x RT^2 SYBR Green Mastermix, 102µl of cDNA and 1,248µl of RNase-free water (total volume=2700 µl) was prepared and 25µl of the mix was added into each well in a 96-well PCR-array plate (containing a panel of 84 genes).

4. Thermal profile consisted of an initial step at 95ºC for 10 min, followed by 40 cycles of denaturation (95ºC for 15 seconds), annealing (60ºC for 1 min) and finally, a dissociation cycle (melting curve) to verify that only one product was amplified.

5. Values from each gene were obtained for the threshold cycle (Ct) and normalized using the average of five housekeeping genes on the same array (HPRT1, B2M, RPLP0, GAPDH, and ACTB).

6. Relative amounts were calculated by the ΔΔCT method and further normalized to the values of their corresponding mock samples. The resulting values were reported as fold change; only genes showing a change of 2-fold or greater were considered for further validation.

7. The negative controls ensured a lack of DNA contamination and set the threshold for the absent/present calls.

8. The validation of the genes that showed some significant change in the array was carried out by qPCR with a different set of custom-designed primers (see below)

4.10.4. Western blotting

4.10.4.1. Validation of results from the cancer pathway reporter assay and RT^2 Prostate Cancer PCR Array by Western blotting

PC-3 cells overexpressing ghrelin, In1-ghrelin, sst5TMD4 and its respective control (mock-cells) were cultured (500,000 cells/experimental condition) in 6-well plates and incubated for 24h in complete growth medium (supplemented with 10% FBS). Then, medium was removed and cells were washed and lysed with SDS-DTT buffer as previously reported [185, 216]. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with 0.05% Tween-20 and incubated O/N at 4ºC with the primary antibodies for phospho-ERK1/2 (ref: 4370), phospho-cRAF (ref: 9421), phospho-AKT (ref: 9271), anti-DAXX (ref: 4533), anti-SFRP1 (ref:3534), anti-APC (ref:2504) and anti-B-tubulin (ref:2128) from Cell Signaling (Danvers, MA, USA), anti-ZNF185 (ref: ab83100), anti- IL-6 (ref:667) and anti-CDKN2A/p16INK4a (ref: ab81278) from Abcam (Cambridge, UK); anti-LOXL1 (ref: sc-166632), anti-NRIP1 (ref: sc-8997) and IGFBP5 (ref: sc-13093) from Santa Cruz (CA, USA); phospho-JNK from R&D system (ref: AF1205, Minneapolis, USA) and c-myc (ref: CSB-MA000041M0m. Wuhan, China). Secondary anti-rabbit (ref: 7074) and anti-mouse were purchased from Cell Signaling (Danvers, MA, USA). Proteins were developed using an enhanced chemiluminescence detection system (GE Healthcare, UK) with dyed molecular weight-markers. A densitometry analysis of the bands was carried out with Image J software.

4.10.4.2. Intracellular signaling pathways trigger after treatment with peptides from the ghrelin, somatostatin and insulin/IGF-1 systems in PCa cell lines.

LnCaP, 22Rv1 or PC-3 cells (250,000) were cultured in 6-well plates and incubated for 24 h in complete growth medium (supplemented with 10% FBS). Then, medium was removed and cells were starved overnight in HBSS medium (Gibco, Madrid, Spain). Next day, medium was replaced with FBS-free growth medium and treated with ghrelin and In1-ghrelin (In1-19/In1-40) peptides at 100nM concentration for 5-30 min, or IGF1 and insulin peptides at 100nM for 24 h. Medium without treatment was used as control vehicle. Then cells were washed and lysed with SDS-DTT buffer as previously reported [13, 33]. In the specific cases of cells treated with ghrelin and In1-ghrelin peptides, blots were incubated overnight at 4ºC with the primary antibodies for phospho-

ERK1/2, phospho-AKT, total ERK (ref: SC-154; Santa Cruz, CA, USA) and total AKT (ref: 9272S; Cell Signaling; Danvers, MA, USA).

4.11. Xenograft model

All experiments with mice were carried out following the European Regulations for Animal Care under the approval of the University of Cordoba Research Ethics Committee. Ten-week-old male athymic BALB/cAnNRj-Foxn1nu mice (Janvier Labs, Le Genest St Isle, France) were subcutaneously grafted in both flanks with $2x10^6$ of mock, ghrelin, In1-ghrelin or sst5TMD4-stably-transfected PC-3 cells (n=4-5 mice per condition) resuspended in 100µl of basement membrane extract (ref: 3432-005-001. Trevigen, Maryland, USA). Tumor growth was measured once per week during 3 months by using a digital caliper and calculating tumor volume. When the mouse was sacrificed, each tumor was dissected, fixed and sectioned for histopathological examination after hematoxylin–eosin staining and for necrosis and mitosis measurements. Another piece from the tumor was kept at -80ºC for later RNA extraction using Trizol reagent (Thermo Scientific, Wilmington, NC, USA).

4.12. Statistical analysis

Expression data obtained from fresh normal prostate samples and PCa biopsies (mRNA and protein) as well as plasma and urine samples from control and PCa patients (ELISA assays) were evaluated for heterogeneity of variance (if values had a Gaussian distribution) using the Kolmogorov–Smirnov test. Statistical differences were assessed by Mann–Whitney U test or by one-way or two-way ANOVA (comparison of tumor growth in xenografts) followed by Fisher's test. ROC curve was performed for evaluation of sensibility and specificity of GOAT, In1-ghrelin and sst5TMD4 in the different tissues and fluids analyzed [217]. Correlations between different molecules were studied using Spearman correlation test. Chi-Square test was used to compare the differences between the genomic frequencies of the different haplotypes of sst5 gene and the differences between groups of PCa patients with low/high sst5TMD4 and GOAT expression (samples were categorized in low and high sst5TMD4/GOAT levels according to median GOAT levels). P-values <0.05 were considered statistically significant. When p-values ranged

between <0.1 and >0.05, a trend for significance was indicated where appropriate. All statistical analyses were performed using the GraphPad Prism (La Jolla, CA, USA).

RESULTS

5. RESULTS

The results of this thesis have been subdivided in two main sections corresponding to the presence of different components of the SST-system (**section 5.1**) and Ghrelin-system (**section 5.2**) in human normal and PCa tissues and cell lines. Part of the results included in these two sections has been included in three independent manuscripts. The first one describing the presence and functional role of the truncated somatostatin receptor, sst5TMD4, in PCa (Title: "***The truncated somatostatin receptor, sst5TMD4, is overexpressed in prostate cancer, where it increases malignant features by altering key signaling pathways and tumor suppressors/oncogenes***"; under review). The second manuscript has been published in the journal "*Cancer Letters*" and it is focused on the identification of Ghrelin-O-acyl transferase (GOAT) enzyme as a potential non-invasive biomarker in PCa [Title: "***Ghrelin O-acyltransferase (GOAT) enzyme is overexpressed in prostate cancer wherein is associated with the metabolic status: potential value as non-invasive biomarker***"; Cancer Lett. 2016; 383(1):125-134]. The third manuscript is focused on the presence and functional role of the splicing variant In1-ghrelin in PCa (Title: "***In1-ghrelin splicing variant is overexpressed in prostate cancer wherein it increases aggressiveness features through regulation of key tumor suppressors/oncogenes***"; under review). It should also be mentioned that part of the results included in this thesis have been already patented [Patent-1: Title: "Ghrelin-O-acyl transferase (GOAT) and its uses" (registry number: PCT/ES2016/070844; Patent-2: Title: "Non-invasive diagnostic method of cancer" (registry number: P201631606)].

5.1. Analysis of the SST-system in PCa

5.1.1. Presence of different components of the SST-system in human normal and PCa tissues and cell lines

- **Analyses in fresh prostate tissues (normal and PCa samples)**: The expression of the different components of SST-system [ligands (SST and CORT) and receptors (sst1-sst5 and truncated sst5TMD4-5)] was evaluated in a battery of PCa biopsies from patients with high risk PCa (n=52) and in a set of normal prostate biopsies from healthy donor

patients (n=12) (**Table 7**). Although, SST and CORT peptides were expressed in normal (**Figure 12A**) and PCa samples (**Figure 12B**), CORT expression was higher than SST both in normal and PCa samples. sst2 was the canonical sst receptor expressed at highest levels in normal tissues, followed by sst5 and sst1 (**Figure 12A**), while sst1 was the most expressed sst in PCa (significantly overexpressed compared to normal tissues) followed by sst5 and sst2 (**Figure 12B**). The expression of sst3 and sst4 was not detectable in normal or PCa samples. When we analyzed the truncated sst5 (sst5TMD4 and sst5TMD5), only sst5TMD4 expression was significantly detected in prostate samples, and, remarkably, its expression was upregulated in PCa samples compared to normal prostate samples (**Figure 12A-B**). Interestingly, overexpression of sst1 in PCa samples compared with normal prostate samples, and its association with the aggressiveness of the disease has been already reported [192]. In contrast, it should be mentioned that this is the first study that demonstrates the presence and overexpression of sst5TMD4 in PCa compared with normal prostate tissues. Therefore, based on these results, we decided to further analyze the presence and functional role of this truncated sst5TMD4 in PCa (see below: section 4.1.2).

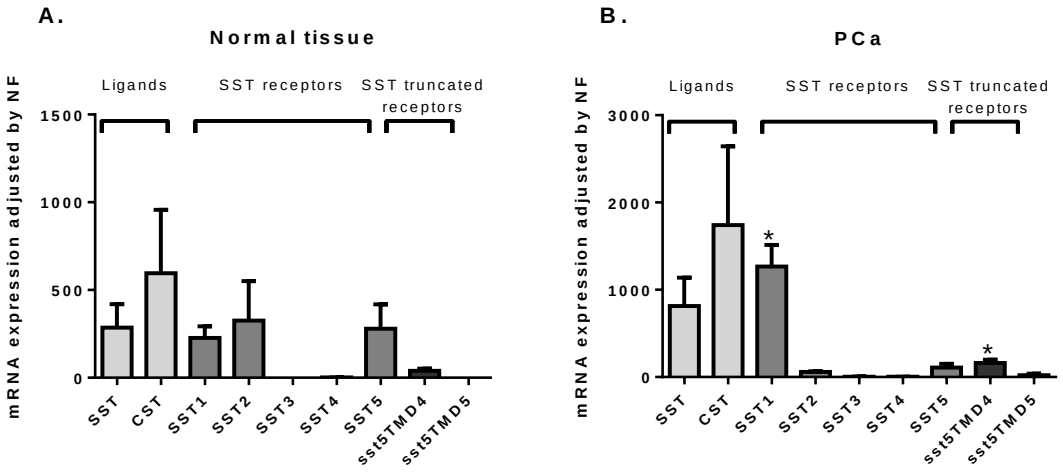


Figure 12: Expression of all the components of the SST-system in normal prostate and PCa samples. Expression levels of the different SST components in a battery of 52 fresh PCa samples compared to the expression found in 14 fresh normal prostates (Control). Absolute mRNA levels were determined by qPCR and adjusted by a normalization factor (NF) calculated from the expression levels of two housekeeping genes (ACTB and GAPDH). Data represent mean ± SEM. Asterisks (*p<0.05) indicate values that significantly differ between PCa samples and normal samples.

- **<u>Analyses in a normal prostate-like cell line and in different PCa cell lines</u>**: The expression of the SST-system was also analyzed in the normal-like prostate cell line (RWPE-1) and in different androgen-dependent (22Rv1, LnCaP, VCaP) and androgen-independent (PC-3, DU-145) cell lines, which represent several stages of the disease (**Figure 13**). While SST expression was barely detected in the different cell lines, CORT expression was detected in normal and PCa cell lines (**Figure 13**). Only sst5 and sst2 receptors were expressed in RWPE-1 (low expression of sst2) and PCa cell lines, with the exception of 22Rv1, which also expressed sst1. No expression was detected for sst5TMD4 and sst5TMD5 in RWPE-1, while sst5TMD4 (but not sst5TMD5) was expressed at low levels in all the PCa cell lines studied (**Figure 13**).

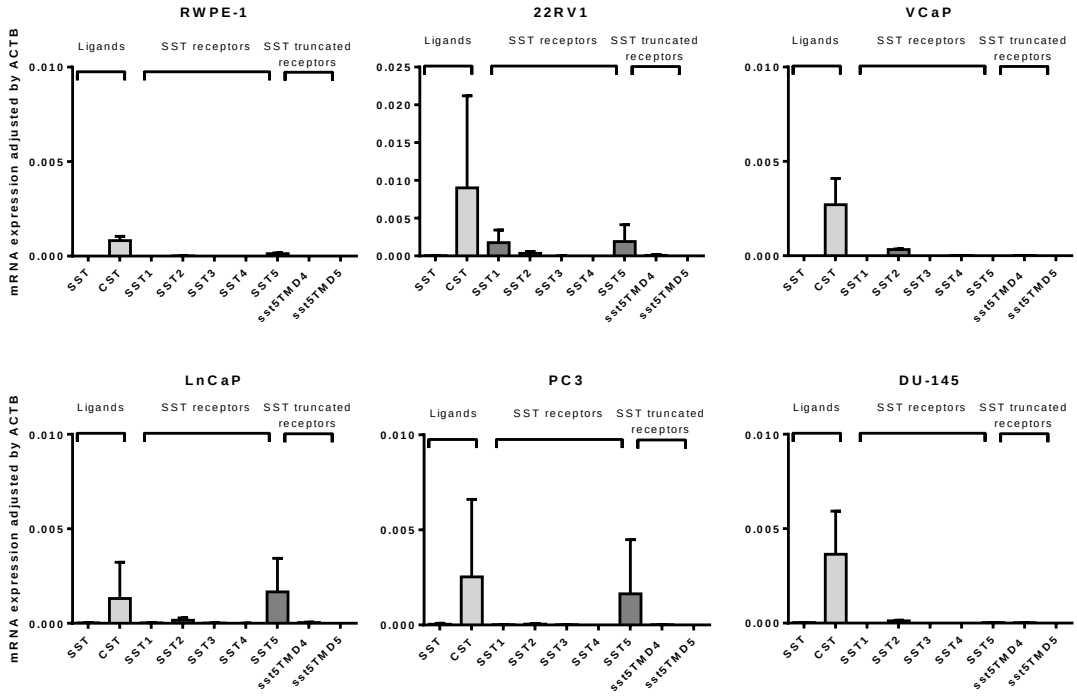


Figure 13: Expression of all the components of the SST-system in the normal-like (RWPE-1) and PCa cell lines (androgen dependent: 22Rv1, VCaP, LnCaP; androgen independent: PC-3, DU-145). Absolute mRNA levels from different passages (n≥3) were determined by qPCR and adjusted by ACTB. Data represents mean ± SEM.

5.1.2. Presence and functional role of sst5TMD4 in PCa

5.1.2.1. The truncated spliced receptor sst5TMD4 is overexpressed in PCa

sst5TMD4 expression was evaluated by qPCR technique in two independent cohorts of samples from PCa patients in different clinical stages [**Table-6** (FFPE samples) and **table-7** (fresh tissues; results previously indicated in Figure 12B)]. Specifically, sst5TMD4 expression was firstly measured in a cohort of 45 FFPE prostate samples from patients with low and intermediate grade tumours (Gleason Score 6-7) representing an early-stage of the disease (**Table 6**). Importantly, FFPE prostatic pieces harbor a tumoral region (PCa) and an adjacent free tumor region (N-TAR) defined by two expert anatomo-pathologists, which allows to compare sst5TMD4 expression in both regions from the same prostatic piece. These results revealed that sst5TMD4 expression was significantly higher in tumoral vs. N-TAR (p= 0.003; **Figure 14A**).

Next, expression of sst5TMD4, and of sst2 and sst5 (the two main pharmacological targets of SSA) was analyzed in a cohort of fresh PCa-biopsies collected from patients with high risk PCa [according to D' Amico classification (i.e. PSA > 20.0 and/or Gleason 8-10 and/or cT2c-T3); n=52)] representing a more aggressive and advanced form of the disease, and in 14 fresh normal prostate (NP)-tissues from patients that underwent cystoprostatectomy due to bladder cancer (absence of malignant glands was classified by two expert anatomo-pathologists) (**Table 7**). Consistent with the results obtained in FFPE samples, the overall expression of sst5TMD4 was higher in tumoral fresh-samples compared to NP-tissues (p=0.017; **Figure 14B**) while sst2 and sst5 expression was not significantly altered. Of note, sst5TMD4 expression was detected in more PCa-samples than sst2 or sst5 [88% (n=46/52) vs. 81% (n=42/52) or 77% (n=40/52), respectively], and its mRNA levels were significantly higher than those of sst2 or sst5 (**Figure 14C**). Interestingly, sst5TMD4 expression correlated with sst2 and sst5 expression levels in PCa but not in normal tissues (**Figure 14D**).

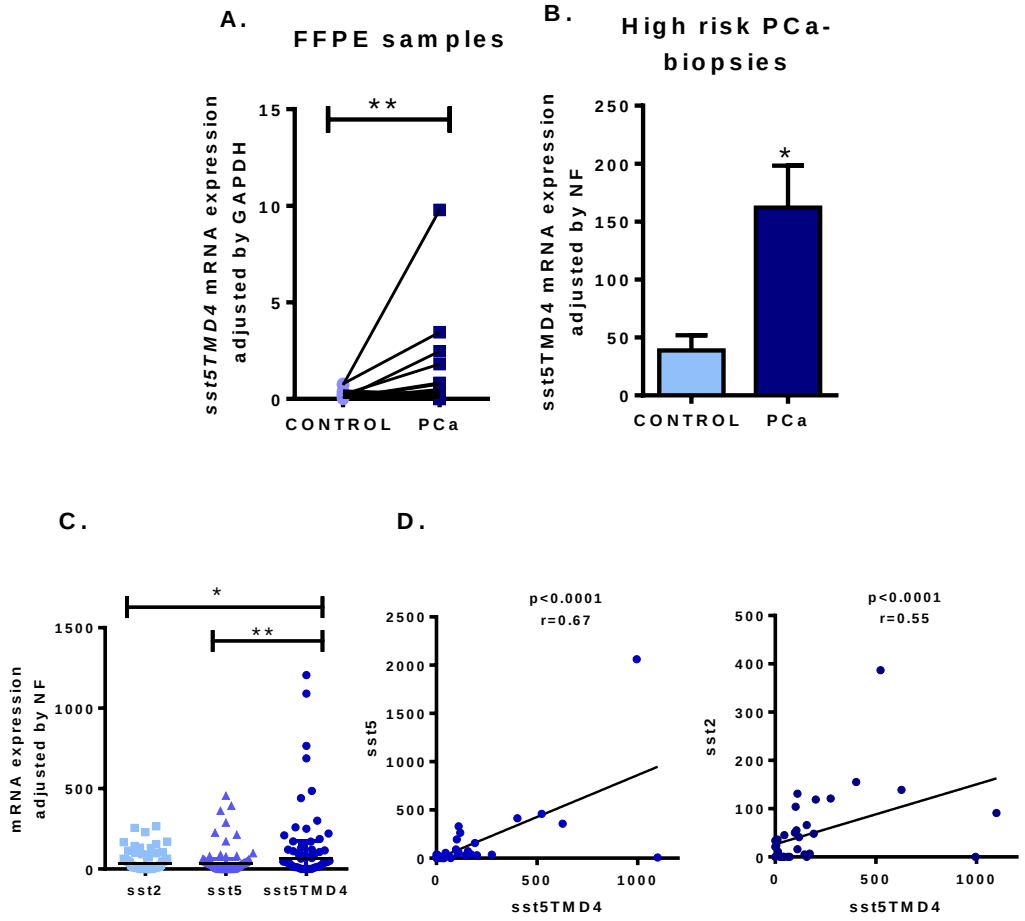


Figure 14: sst5TMD4 expression in two different cohorts of samples from patients with PCa. A. Paired analysis of sst5TMD4 expression in tumoral and non-tumoral adjacent regions (N-TAR) of formalin-fixed paraffin-embedded (FFPE) prostate pieces (n=45) from low/intermediate grade tumors (cohort-1). Absolute mRNA levels were determined by qPCR and adjusted by GAPDH (used as housekeeping gene); **B.** Expression levels of sst5TMD4 in a battery of 52 fresh PCa samples compared to the expression found in 14 fresh normal prostates (Control) (Cohort-2). Absolute mRNA levels were determined by qPCR and adjusted by a normalization factor (NF) calculated from the expression levels of two housekeeping genes (ACTB and GAPDH); **C.** Comparison of sst2, sst5 and sst5TMD4 expression in fresh PCa samples (n=52; cohort-2); **D.** Correlation of sst5TMD4 mRNA levels with sst5 and sst2 expression levels in fresh PCa samples (n=52; cohort-2). Asterisks (*p<0.05; **p<0.01) indicate values that significantly differ from the corresponding controls (A and B) or differences between the expression levels of sst5TMD4 and sst2 or sst5 (C).

5.1.2.2. sst5TMD4 levels (mRNA and protein) correlate with clinical aggressiveness features in PCa.

The mRNA levels of sst5TMD4, but not of sst2 or sst5 (**Figure 15A-C**), were elevated in patients with higher Gleason-score, a marker of PCa aggressiveness [244].

Furthermore, sst5TMD4 (but not sst2 or sst5) expression was significantly higher in metastatic vs. non-metastatic PCa or control samples (**Figure 15A-C**). Indeed, ROC-analysis indicated that sst5TMD4, but not sst2 or sst5, expression significantly discriminated between patients with and without metastasis (**Figure 15A-C**). When PCa patients were divided according to low or high sst5TMD4 expression (based on median expression), a greater proportion of patients with high sst5TMD4 expression presented metastasis, higher Gleason-score and extraprostatic extension compared with patients with low sst5TMD4 expression, while no such associations were observed for sst2 or sst5 expression (**Figure 15A-C**).

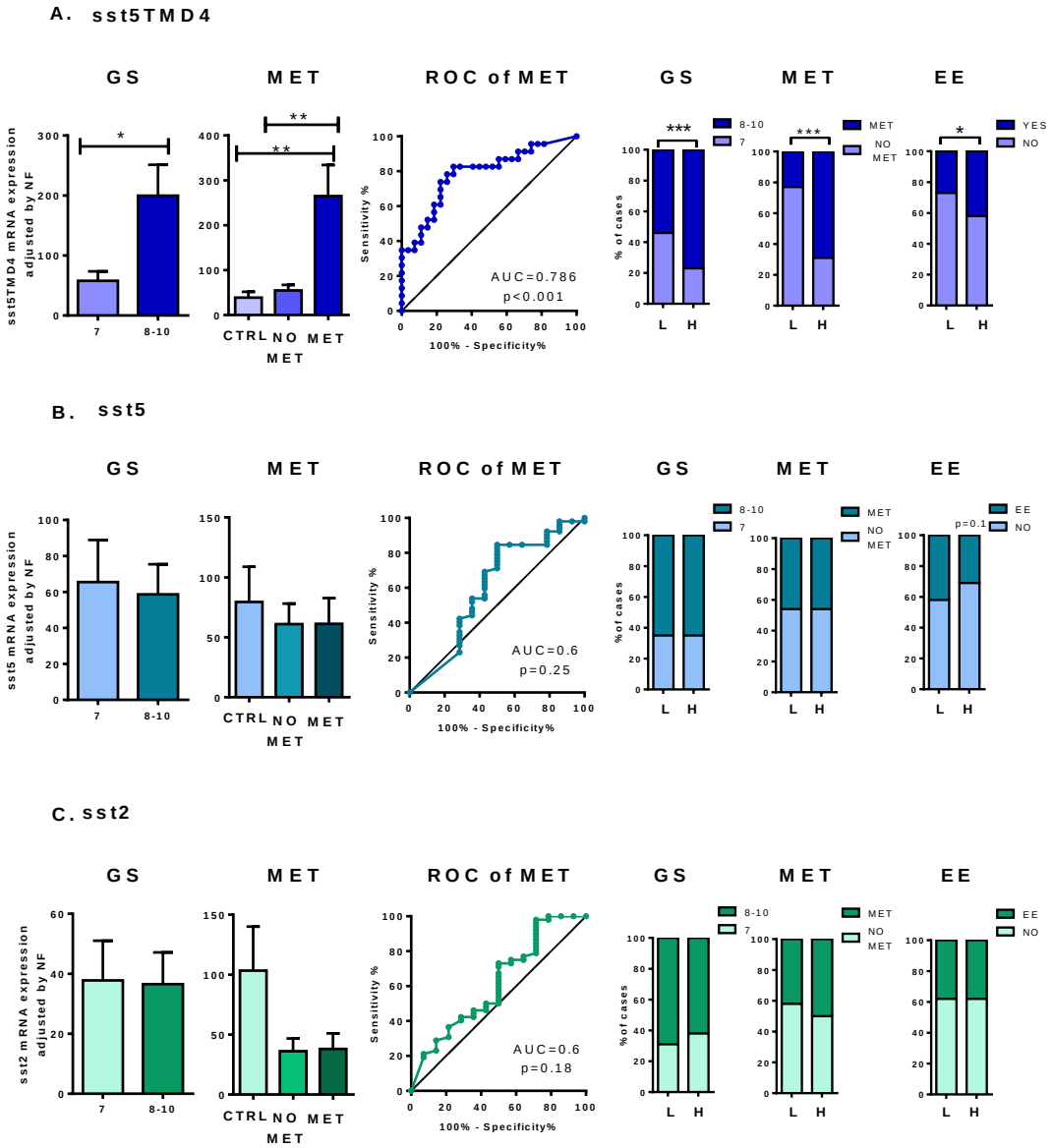


Figure 15: sst5TMD4 mRNA expression, but not sst5-sst2, is higher in patients with high Gleason score (GS) and metastasis (MET). Expression levels of sst5TMD4 (**A**), sst5 (**B**) and sst2 (**C**) in patients with intermediate (7) Gleason score (GS) compared with patients with high GS (8-10); Comparison of sst5TMD4 (**A**), sst5 (**B**) and sst2 (**C**)

expression levels found in fresh normal prostate samples (Control) and in PCa samples from patients with and without presence of metastasis (MET and NO MET, respectively); Receiver operating characteristic (ROC) curve analysis to determine the accuracy of sst5TMD4 (**A**), sst5 (**B**) and sst2 (**C**) expression as diagnostic test to discriminate between patients with presence of metastasis; Frequencies observed between two groups of PCa patients with low (L) or high (H) sst5TMD4 (**A**), sst5 (**B**) and sst2 (**C**) expression levels in terms of presence of metastasis, Gleason score level and presence of extraprostatic extension (EE). Asterisks (*p<0.05; *p<0.01; ***p<0.001) indicate values that significantly differ from the corresponding controls (GS of 7 vs. 8-10; MET samples vs. control or NO MET samples; samples from patients with low vs. high sst5TMD4 expression)**.**

Additionally, sst5TMD4 immuno-histochemical staining was performed on 12 FFPE-prostate pieces from radical prostatectomy (Gleason score 6-7) and in 18 FFPE-biopsies pieces obtained by needle biopsy (**Figure 16**). sst5TMD4 cell staining was predominantly granular and located at or close to the apical membrane (**Figure 16**). The analysis revealed that sst5TMD4 staining was always weaker in benign prostate-gland epithelium (BG; **Figure 16A**; staining of stromal regions was negligible) than in cancerous prostate-glands (BG vs. CG; **Figure. 16C**). Notably, although only one FFPE-prostate harboring a high-grade prostatic intraepithelial neoplasia (HG-PIN) lesion was available, sst5TMD4 staining was stronger in the pre-malignant lesion than the normal-adjacent region (**Figure 16B**), which suggests that sst5TMD4 overexpression could be associated with an early event in PCa. Consistent with sst5TMD4 mRNA results, sst5TMD4 staining was significantly stronger in samples from patients with higher Gleason-score pieces and in patients with metastasis (**Figure 16D**).

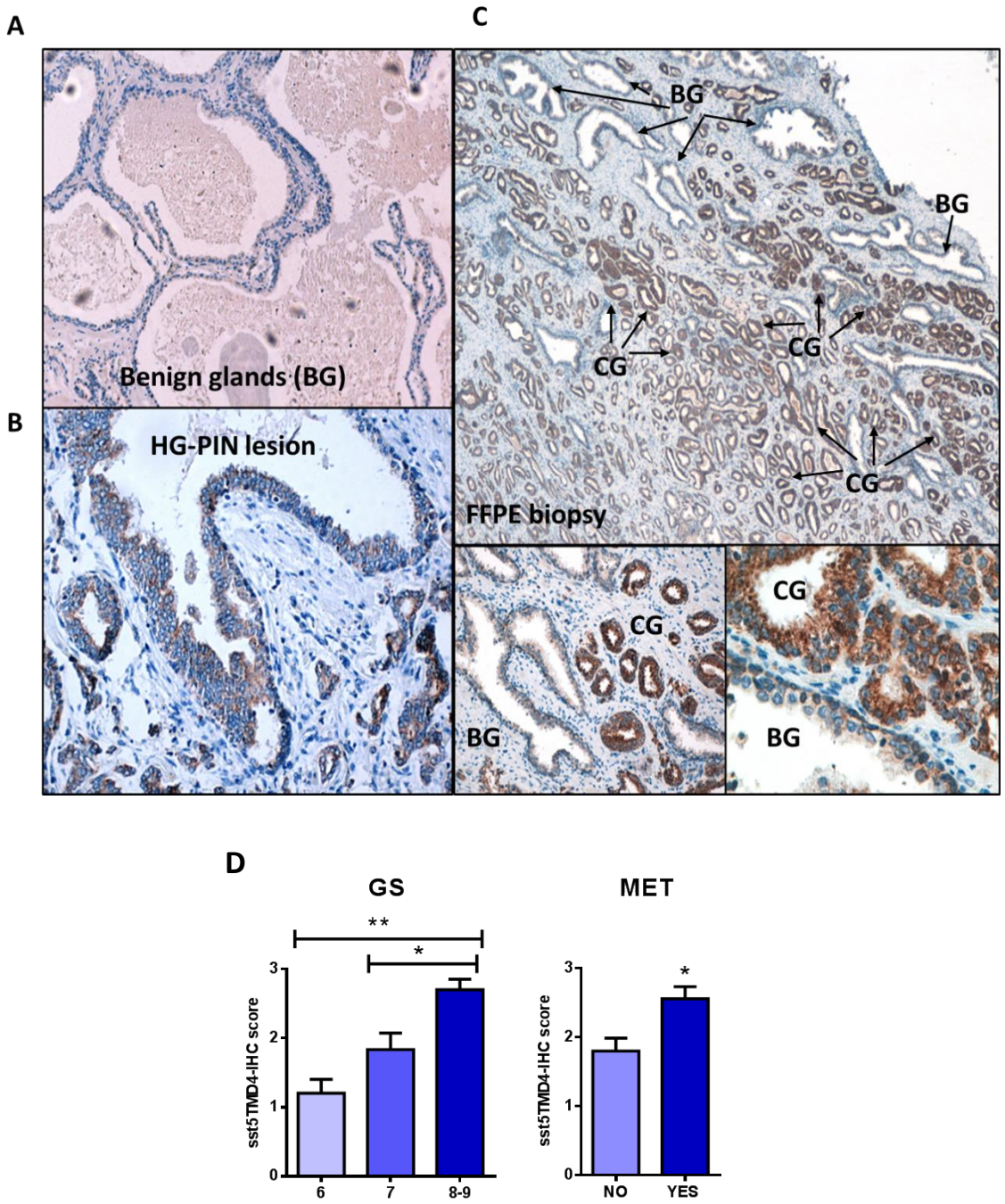
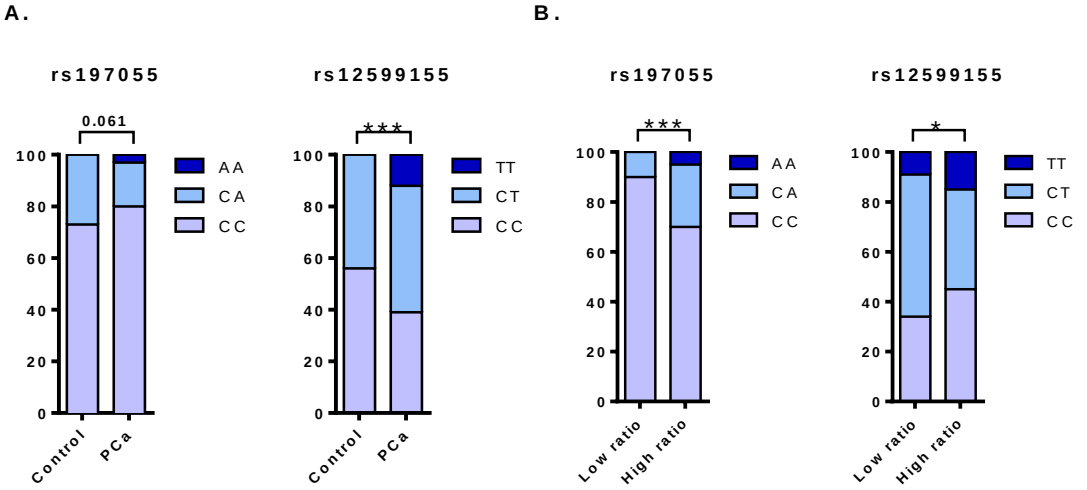


Figure 16: sst5TMD4 protein expression is higher in cancerous glands and correlates with higher Gleason score (GS) and presence of metastasis (MET). A-C. IHC analysis of sst5TMD4 in 12 FFPE prostate samples from patients with low-intermediate grade tumors and 18 prostate samples from high-risk PCa patients. Images are representative examples of sst5TMD4 staining in benign prostate glands (BG) (**A**) high-grade prostatic intraepithelial neoplasia (HG-PIN) (**B**) and prostates samples obtained from patients with PCa which include areas with normal-benign and PCa glands (BG and CG, respectively) (**C**) at 40X, 200X and 800X magnification. **D.** Correlation of sst5TMD4 IHC score (i.e. low, moderate, and high sst5TMD4 staining) with Gleason score (GS: 6, 7, 8-9 score; left-panel) and with presence of metastasis (MET: no or yes; right-panel). Data represent median (IQR) or mean ± SEM. Asterisks (*p<0.05; **p<0.01) indicate values that significantly differ from the corresponding controls (i.e. GS of 6 vs. 7 vs. 8-9; MET samples vs. NO MET samples).

5.1.2.3. sst5TMD4 is associated to two SNPs in PCa.

To analyze the putative implication of certain SNPs in sst5 splicing regulation, we sequenced the 5'- and 3'-ends of the cryptic intron spliced to generate sst5TMD4 and compared the frequencies of the observed SNPs (rs197055/rs12599155; **Figure 17**). We found significant differences in the frequencies observed for both SNPs between PCa vs. control-samples (**Figure 17A**), and also between PCa-samples with high and low sst5TMD4/sst5 mRNA ratio (**Figure 17B**). Indeed, two specific recessive homozygous haplotypes (AA for rs197055 and TT for rs12599155) were only found in PCa-samples, wherein they seem to be associated to a higher sst5TMD4/sst5 mRNA ratio (**Figure 17B**).

Figure 17: Analysis of SNPs in the sst5 gene sequence. Genomic DNA (gDNA) from PCa tissues (n=41) and normal prostate tissues (control; n=9) were used to identify SNPs that



could be associated with the presence of sst5TMD4. **A.** Differences in the frequencies observed for both SNPs between PCa vs. control-samples; **B.** Differences in the frequencies observed for both SNPs between PCa-samples with high and low sst5TMD4/sst5 mRNA ratio. Asterisks (*p<0.05; ***p<0.001) indicate values that significantly differ from the corresponding controls (control normal vs PCa samples; samples with low vs. high expression of sst5TMD4/sst5 ratio).

5.1.2.4. sst5TMD4 overexpression enhances pathophysiological features of PCa cell lines and induces larger tumors in nude-mice.

Since sst5TMD4 expression was clearly increased in PCa tissues compared with normal prostate tissues at the mRNA and protein level (Figures 14-16), we next sought to determine the functional effects of sst5TMD4 overexpression on PCa pathophysiological features (i.e. cell proliferation and cell migration) using *in vitro* cell

models (PCa cell lines stably transfected with sst5TMD4) and a preclinical *in vivo* model (xenografts).

5.1.2.4.1. Validation of sst5TMD4 overexpression in sst5TMD4-stably-transfected PCa cell lines.

As mentioned before, the expression level of sst5TMD4 was low in all prostate cell lines studied, including the VCaP, DU-145 and PC-3 (**Figure 18A**). Therefore, these cell lines represent adequate working models to analyze the effect of sst5TMD4 overexpression on PCa-associated pathophysiological processes. Successful overexpression of sst5TMD4 in transfected cells was confirmed by qPCR and Western Blot (**Figure 18B**).

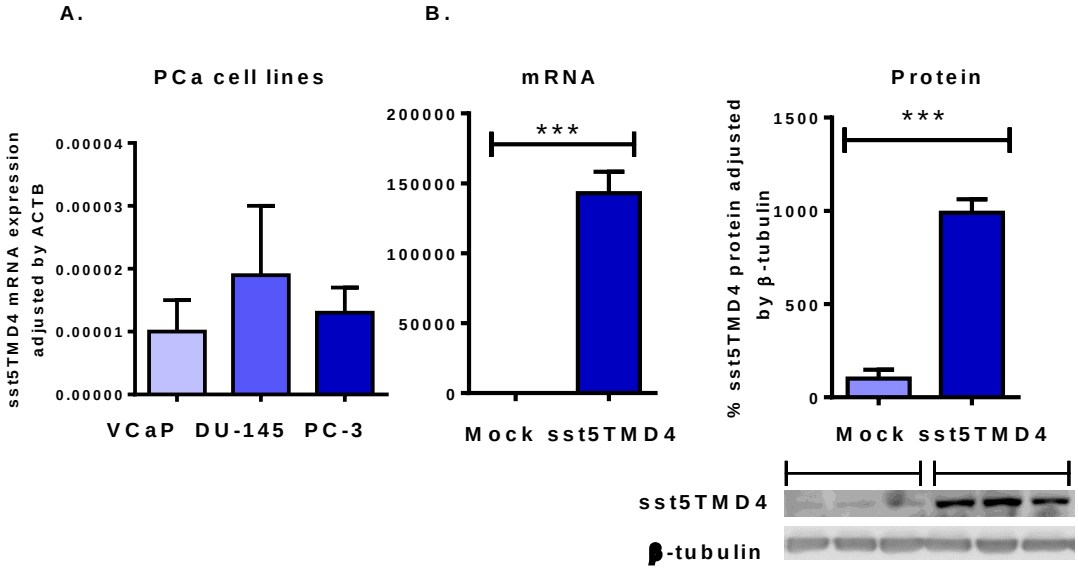


Figure 18: sst5TMD4 presence and validation of stable transfection of sst5TMD4 in PCa cell lines. **A.** sst5TMD4 mRNA levels in different PCa cell lines (VCaP, PC-3 and DU145). Absolute mRNA levels were determined by qPCR and adjusted by ACTB. **B**. Representative figures showing validation of sst5TMD4 overexpression in PC-3 cells (by qPCR and western-blot; one cell-passage with 3-technical replicates). Data represent mean ± SEM. Asterisks (***p<0.001) indicate values that significantly differ from the corresponding controls (mock-transfected cells).

5.1.2.4.2. sst5TMD4 overexpression enhances cell proliferation and migration of PCa cell lines.

sst5TMD4 stable overexpression increased cell proliferation in VCaP and PC-3 cell lines at 48h and/or 72h, and the results were further confirmed with a transient transfection in the DU145 cell line in which cell proliferation was increased at 24h and 48h (**Figure 19A**). Moreover, the pathophysiological role of sst5TMD4 in PCa was further analyzed by measuring the effect of sst5TMD4 overexpression on cell migration by wound-healing assay in the PC-3 cell line (**Figure 19B**). sst5TMD4 overexpression evoked a significant increase in the migration of PC-3 cells at 12h (**Figure 19B**).

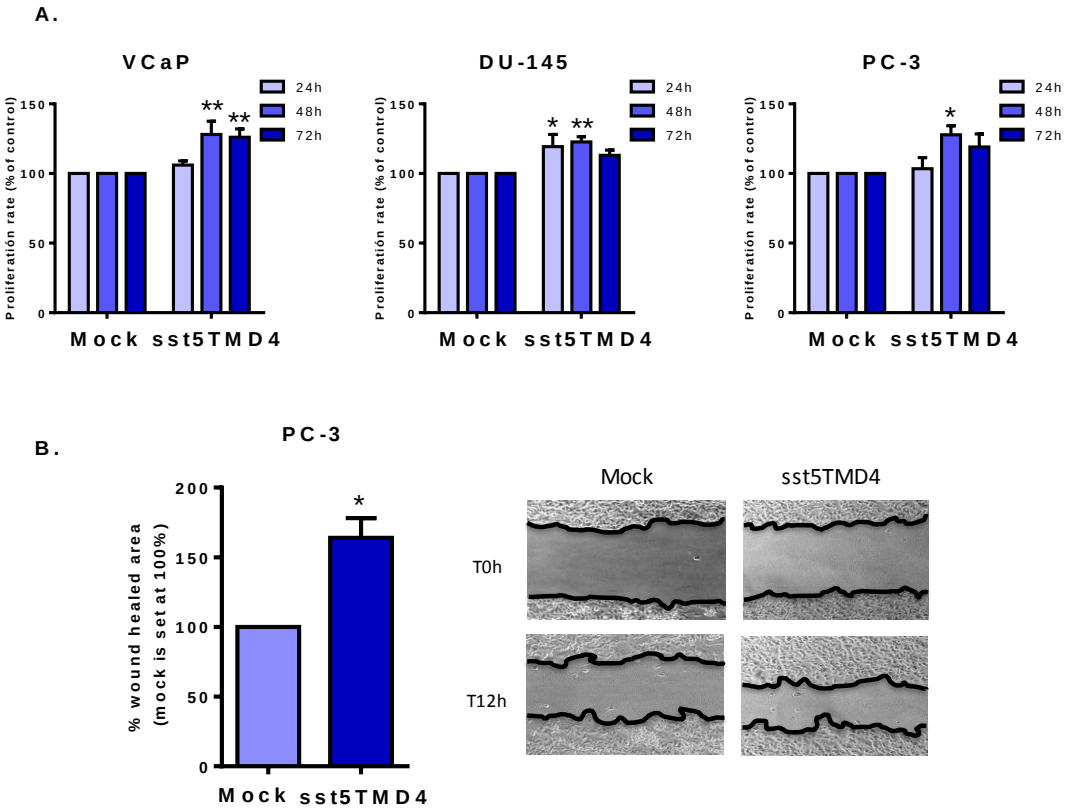


Figure 19: Functional effects of sst5TMD4 overexpression on pathophysiological parameters in PCa cell lines. A. Cell proliferation in sst5TMD4 stably-transfected-VCaP and PC-3, and in sst5TMD4 transiently-transfected DU145 cells, compared with their corresponding control (mock-transfected); **B**. Migration capacity level (Wound-healing) observed in sst5TMD4-stably-transfected PC-3 cells compared with control-cells (mock-transfected) (representative images showing the higher migration capacity of sst5TMD4-transfected cells 12h after the wound compared with mock-transfected cells are also included). All experiments were repeated at least three-times ($n \geq 3$). Data represent mean ± SEM. Asterisks (*$p<0.05$; **$p<0.01$) indicate values that significantly differ from the corresponding controls.

5.1.2.4.3. sst5TMD4-stably-transfected PC-3-cells induce larger tumors than control cells (mock cells) in a preclinical *in vivo* model.

The effect of the presence of sst5TMD4 on cell-proliferation was further evaluated on an *in vivo* model of subcutaneous xenograft induction in nude-mice (**Figure 20**). The overall size of the subcutaneous tumors induced by sst5TMD4-transfected PC-3-cells were significantly larger than those induced by mock-cells ($p<0.001$; **Figure 20A**). Although all tumors exhibited similar histotypes, tumors derived from sst5TMD4-transfected PC-3-cells showed a significantly higher necrosis, but not mitosis, grade (**Figure 20B**).

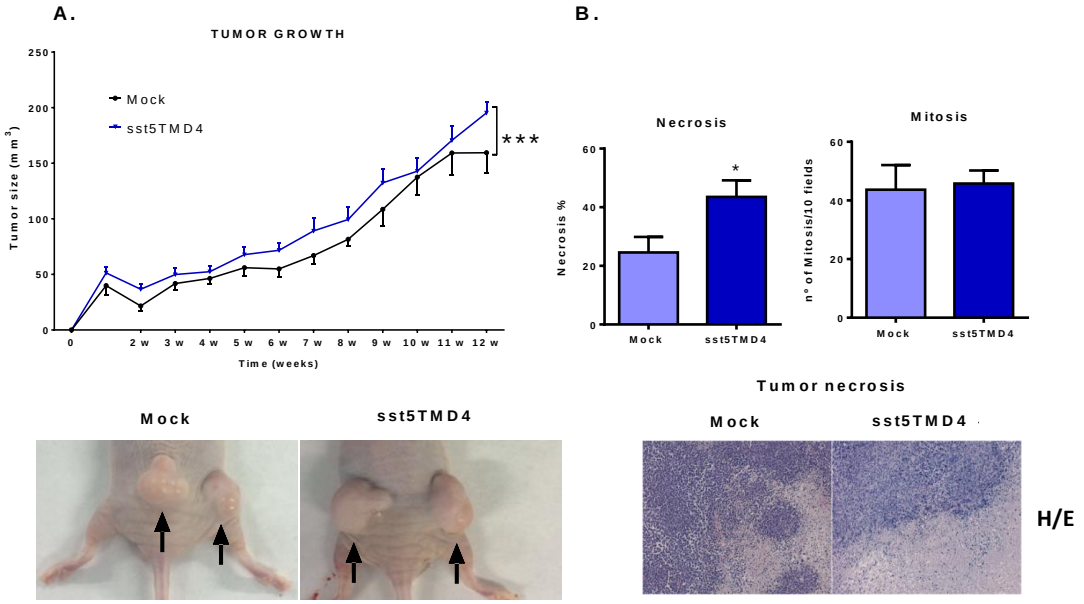


Figure 20: sst5TMD4 stable overexpression enhances tumor growth in preclinical models (NUDE mice). A. Growth-rate of subcutaneously inoculated mock- and sst5TMD4-transfected PC-3-derived tumors in nude-mice followed up to 12-weeks after inoculation. Below, representative images of mock and sst5TMD4-derived tumors after 12 weeks of growth. **B.** % of necrosis and number of mitosis (positive cells per 10 field) in mock- and sst5TMD4-transfected PC-3-derived tumors. Representative-images of hematoxylin–eosin (H/E) staining showing diffuse cell-proliferation and large areas of necrosis in sst5TMD4-trasfected PC-3-derived tumors compared with mock-derived tumors (with diffuse cell-proliferation and lower % of necrosis). Data represent mean ± SEM. Asterisks (*$p<0.05$; ***$p<0.001$) indicate values that significantly differ from the mock group.

5.1.2.5. sst5TMD4 silencing reverts pathophysiological features of PCa cell lines

Inasmuch as sst5TMD4 expression is upregulated in PCa and its overexpression (*in vitro* and *in vivo*) increases aggressiveness features of PCa cells, we next evaluated whether sst5TMD4 silencing could be a suitable clinical target in the disease (**Figure 21**). To this end, a specific siRNA against the specific region of sst5TMD4 was designed and

validated in our laboratory (successful silencing was validated by Western blot analysis; **Figure 21A**). Remarkably, sst5TMD4 silencing decreased proliferation rate (**Figure 21B**) and migration (**Figure 21C**) in PC-3 and DU145 cells

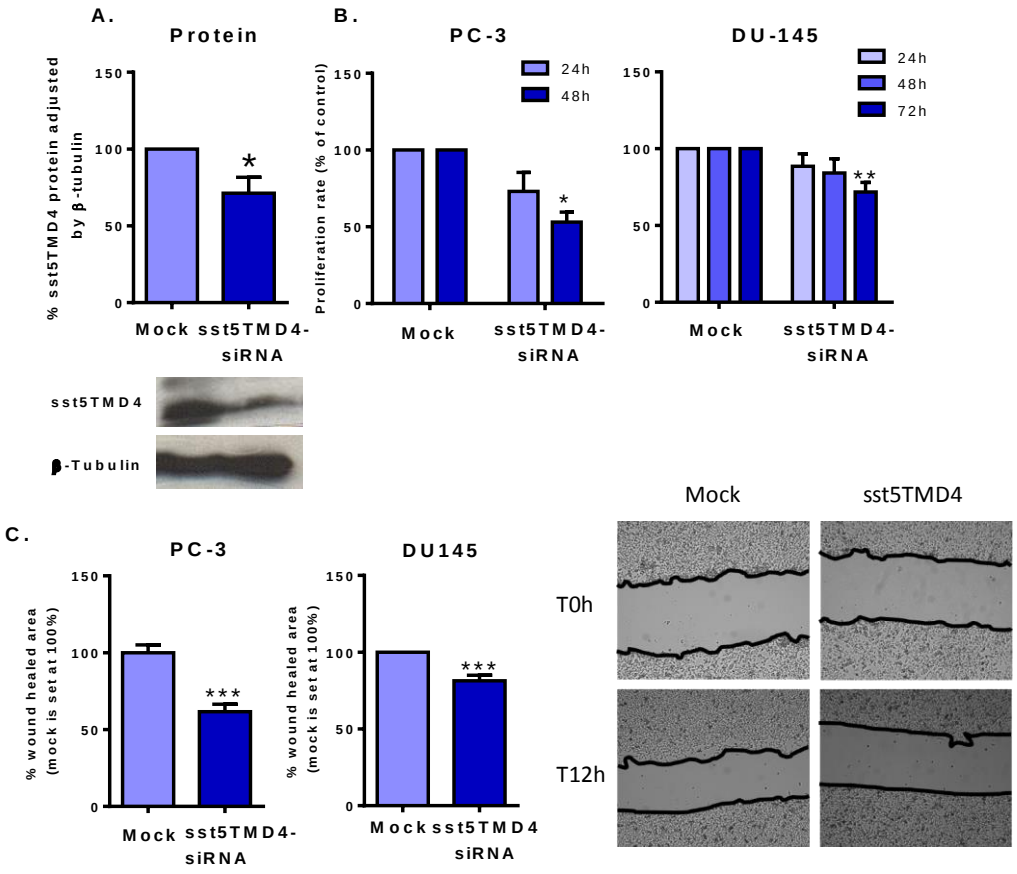


Figure 21: sst5TMD4 silencing decreases cell proliferation and migration of PCa cell lines. A. Validation by western-blot of sst5TMD4 silencing in PC-3 cells and representative-images showing this silencing (below). **B.** Proliferation of sst5TMD4-silenced PC-3 and DU145 cells compared with control scramble-transfected cells; **C.** Migration rates measured by the wound-healing technique of sst5TMD4-silenced PC-3 and DU145 cells compared with control scramble-transfected cells. Data represent mean ± SEM. Asterisks (*p<0.05; **p<0.01; ***p<0.001) indicate values that significantly differ from the mock groups.

5.1.2.6. sst5TMD4 overexpression modulates key cell signaling pathways and dysregulates the expression of oncogenes and tumor suppressor genes involved in PCa development and progression.

In order to determine the intracellular mechanisms involved in the pathophysiological processes triggered by sst5TMD4 overexpression in PCa-cells (i.e.

cell proliferation and cell migration), we next studied the consequences of sst5TMD4 overexpression on 10 key cancer-related pathways using a dual-luciferase reporter system (Cignal Finder Reporter Assay, Qiagen) and the RT_2 Prostate Cancer PCR Array comprising 84 genes involved in PCa development and progression.

5.1.2.6.1. Use of the Cignal Finder Reporter Assay revealed that sst5TMD4 overexpression enhances the activity of key cell signaling pathways. Validation of the results by qPCR and Western blot.

The differences in the relative expression of various key signaling pathway between sst5TMD4-stably-transfected and mock-transfected PC-3 cells are shown in **Figure 22A**. Specifically, the pathways more drastically activated in sst5TMD4-stably-transfected PC-3 cells compared with mock cells were Myc/Max (2.81-fold), MAPK/c-Jun (AP1; 2.61-fold), hypoxia (HIF1α; 2.51-fold), TGFβ (SMAD 2/3/4; 2.4-fold), pRB-E2F (E2F/DP1; 2.38-fold), Wnt (TCF/LEF; 2.05-fold) and MAPK/ERK kinase (ELK-1/SRF; 1.87-fold) with minor changes in the activation of Notch (RBP-Jk; 1.53-fold), p53 (1.23-fold) and NFKβ (1.08-fold). Remarkably, those pathways are usually dysregulated in PCa and are crucial in the pathophysiology of the disease [82].

Consistent with the increased activation of ELK-1/SRF and AP-1 transcription factors observed with the Cignal Finder Reporter Assay, several components from the MEK/ERK signaling pathway such as ERK (p=0.026) and JNK (p=0.042) exhibited increased basal phosphorylation (**Figure 22B**). Moreover, total protein level of the protooncogene c-myc, was clearly increased in sst5TMD4-stably-transfected PC-3 cells (p=0.0076, **Figure 22B**), which was in agreement with the activation of the transcription factor MYC/MAX (**Figure 22A**). Additionally, sst5TMD4-transfected PC-3-cells expressed higher TGF-β levels without changes in NOTCH (**Figure 22C**). In addition, due to the close relationship of PI3K/AKT pathways with tumoral pathologies, we also

analyzed the basal phosphorylation of AKT but did not find any alteration in the phosphorylation levels of this protein (data not shown).

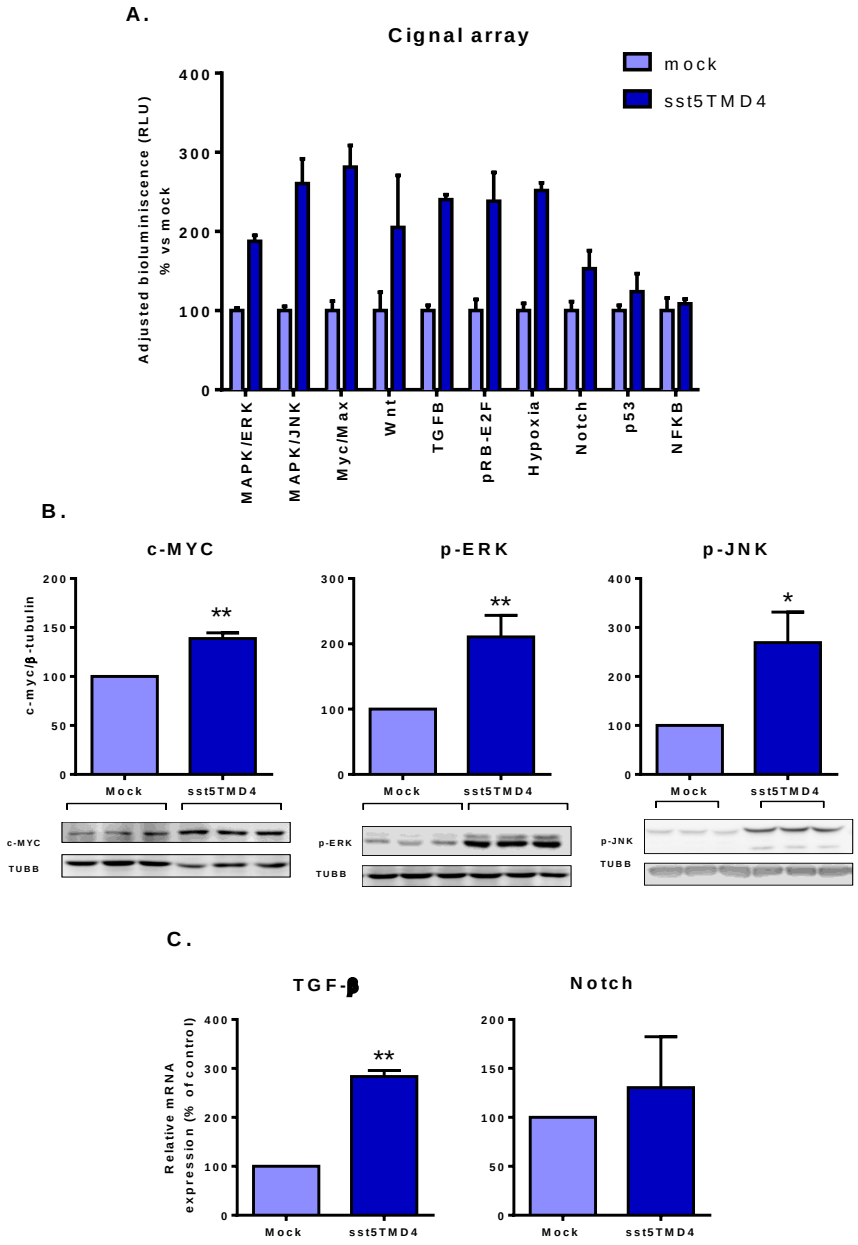


Figure 22: sst5TMD4 overexpression activates key intracellular signaling pathways in PCa cells. **A.** Results from the dual-luciferase Cignal Finder reporter assay of 10 cancer-related pathways. The ratio of bioluminisence (RLU) signal of sst5TMD4-stably-transfected PC-3 cells adjusted to control-mock cells (set at a 100%) is shown as increased fold change; **B.** Protein expression levels of total-myc, phospho-ERK1/2 and phospho-JNK (adjusted by β-tubulin levels) in sst5TMD4-stably-transfected PC-3 cells compared with control-mock PC-3 cells. A representative western blots image of all these proteins (one passage with tree technical replicates) are also shown; **C.** Expression of TGF-β and NOTCH in sst5TMD4-stably-transfected PC-3 cells. Absolute mRNA levels (% of variation compared to control-mock transfected-PC-3 cells) of TGF-β and NOTCH were measured in 4 consecutive passages (mock- and sst5TMD4-stably-transfected PC-3 cells). Absolute mRNA levels were determined by qPCR and adjusted by ACTB (used as

housekeeping gene). Values represent mean ± SEM. Asterisks (*p<0.05; **p<0.01) indicate values that significantly differ from the control.

5.1.2.6.2. Use of the RT_2 Prostate Cancer PCR Array revealed that sst5TMD4 overexpression alters the expression of key genes involved in PCa development and progression. Validation of the results by qPCR and Western blot.

To identify the downstream consequences of the changes in signaling-pathways observed using the dual-luciferase Cignal Finder reporter assay, we performed a qPCR-array comprising 84 genes involved in PCa (see **Table 11** at the end of the results section). The results showed a total of 10 genes whose expression was altered more than 2-fold in sst5TMD4-stably-transfected PC-3 cells compared with control-mock (**Figure 23A**; upregulated: GCA/CAV2/RASSF1; downregulated: SFRP1/NRIP1/LOXL1/ CDKN2A/RARB2/APC/IGFBP5). Further validation of these changes [i.e. genes found heavily altered (>2-fold) and some showing smaller changes (i.e. CAV1/IL-6/DAXX/ZNF185)] by qPCR using cDNA from several cell-passages and different primers sets (see **Table 12** at the end of the Results section) indicated that 5 genes were significantly upregulated (GCA/CAV1/CAV2/IL-6/DAXX) and 8 genes downregulated (SFRP/NRIP1/LOXL1/CDKN2A/RARB2/APC/IGFBP5/ZNF185) by sst5TMD4 overexpression (**Figure 23B**). Additionally, some of these changes were confirmed by Western-Blot (IL-6/SFRP1/NRIP1/LOXL1/CDKN2A/IGFBP5/ZNF185; **Figure 23C**). Interestingly, sst5TMD4-stably-transfected PC-3 cells-derived tumors (*in vivo* xenograft-model; previously presented in section 4.1.2.5.3 and figure 20) exhibited increased IL-6 and decreased SFRP1/IGFPB5 expression compared to mock-induced tumors (**Figure 23D**), which further validates the results observed previously in the PCR array.

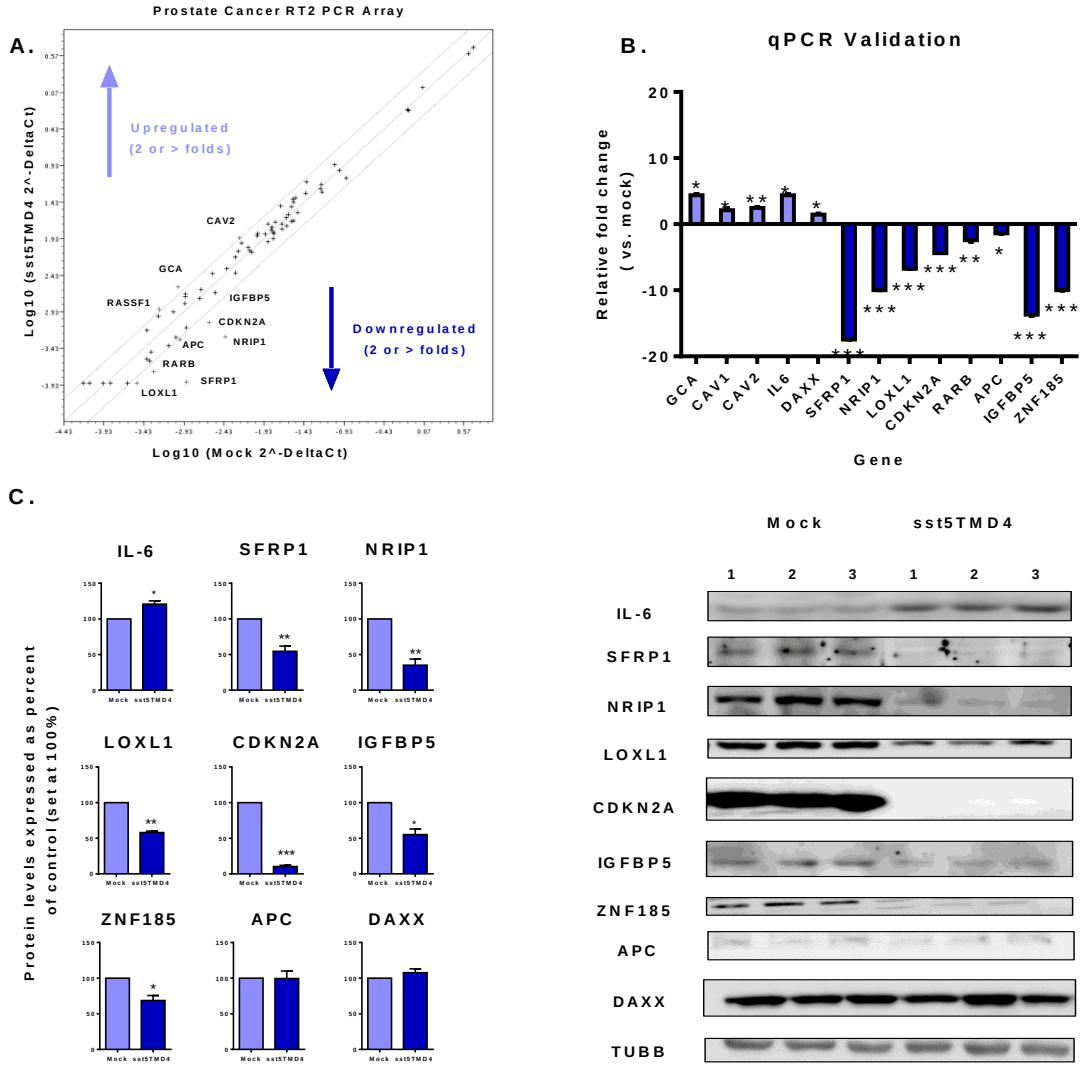
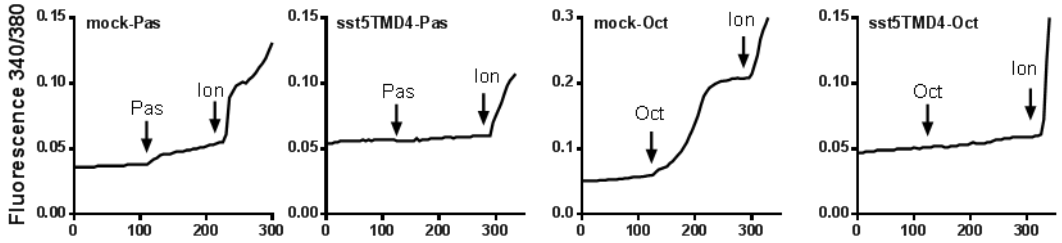


Figure 23: Mechanistic downstream consequences of sst5TMD4 overexpression in PCa-cells. A. Results from the RT_2 Prostate Cancer PCR Array showing changes in expression levels of some of the 84 genes involved in PCa in sst5TMD4-stably-transfected PC-3 cells compared with control-mock PC-3 cells; **B.** Validation by qPCR of genes dysregulated in the RT_2 Prostate Cancer PCR Array using different cell preparations and new sets of specific primers designed and validated by our group; **C.** Confirmation at the protein expression level (using Western Blot) of some of the changes observed in gene expression using the RT_2 Prostate Cancer PCR Array in sst5TMD4-stably-transfected PC-3 cells compared with control-mock PC-3 cells. Representative western blots image of all these proteins are also shown. Data are expressed as percent of control ("mock", set at 100%) within experiment; **D**. IL-6, SFRP1 and IGFBP5 mRNA expression levels in mock- and sst5TMD4-transfected PC-3-derived tumors (*in vivo* xenograft model). Results were normalized according to the value of ACTB (used as housekeeping gene). Asterisks (*p<0.05; **p<0.01; ***p<0.01) indicate values that significantly differ from the control.

5.1.2.7. PCa cell lines transfected with sst5TMD4 do not respond to SSAs in terms of changes in free-cytosolic Ca^{2+} concentration.

Evaluation of changes in free-cytosolic Ca^{2+} concentration (pathway tightly associated to SST-signaling) in PC-3 cells showed that whereas 21% and 28% of the cells not expressing sst5TMD4 (control-mock PC-3 cells) responded to SSAs (octreotide and pasireotide; **Figure 24A-B**), an evident blockade in this response was observed in sst5TMD4-overexpressing PC-3 cells (0% and 5% of the cells responded to octreotide and pasireotide, respectively). A similar response was found in the androgen-dependent LnCaP cells transiently-transfected with sst5TMD4, in that 16% and 14% of the control-mock cells responded to SSAs (**Figure 24C**), while sst5TMD4-overexpressing LnCaP cells barely responded to SSAs (4% and 2%, respectively).

A.



B.

Type of cell	SST analog	No. responsive cells	Total cells	(%) responsive cells	% Resp. Max.	Error	Time of max.response	Error
Mock	Pasireotide	32	114	28.1%	166.58	45.32	11.56	5.38
Mock	Octreotide	16	74	21.6%	150.67	19.83	8.44	2.18
sst5TMD4	Pasireotide	5	104	4.8%	185.30	18.72	8.00	0.68
sst5TMD4	Octreotide	0	74	0.0%	-	-	-	-

C.

Type of cell	SST analog	No. responsive cells	Total cells	(%) responsive cells	% Resp. Max.	Error	Time of max.response	Error
Mock	Pasireotide	10	73	14%	110.69	3.29	46.50	12.23
Mock	Octreotide	9	57	16%	147.03	23.87	38.33	17.78
sst5TMD4	Pasireotide	3	144	2%	110.79	7.01	8.33	2.72
sst5TMD4	Octreotide	5	132	4%	126.54	5.01	120.00	21.26

Figure 24: Effect of octreotide and pasireotide in kinetics of $[Ca^{2+}]_i$ in the presence and absence of sst5TMD4 in stably-transfected PC-3 cells and in transient transfected LnCaP cells (sst5TMD4 or mock-transfected, respectively; n≥3). A. Representative profiles of changes in $[Ca^{2+}]_i$ in mock or sst5TMD4-stably-transfected PC-3 cell cultures in response to octreotide (Oct), pasireotide (Pas) and Ionomycin (positive control)

administration (arrows) are shown; **B-C.** The number of responsive cells, total number of cells measured, percentage of responsive cells showing changes in $[Ca^{2+}]_i$ in response to octreotide and pasireotide, percentage of maximum response (±error) and time of maximal response (±error) to octreotide or pasireotide administration are indicated in stably-transfected PC-3 cells (**B**) and in transiently-transfected LnCaP cells (**C**).

5.2. Analysis of the ghrelin-system in PCa

5.1.1. Presence of different components of the ghrelin-system in human normal and PCa tissues and cell lines

- **Analyses in fresh prostate tissues (normal and PCa samples)**: The expression of the different components of ghrelin system [ligands (Ghrelin and In1-ghrelin), receptors (GHSR-1a and GHSR-1b) and GOAT enzyme] was measured in a battery of PCa biopsies from patients with high risk PCa (n=52) and in a set of normal prostate biopsies from healthy donor patients (n=12) (**Table 7**). Specifically, ghrelin, In1-ghrelin and GOAT were expressed both in normal and PCa samples (**Figure 25A-B**). Interestingly, In1-ghrelin and GOAT levels were upregulated in PCa compared with normal tissues (**Figure 25**A-B). In contrast, the expression of ghrelin receptors (GHSR-1a and GHSR-1b) was barely detected in both normal and tumor tissues (**Figure 25**). Based on these results, we decided to further analyze the presence and functional role of GOAT enzyme and In1-ghrelin variant in PCa (see below: sections 4.2.2 and 4.2.3).

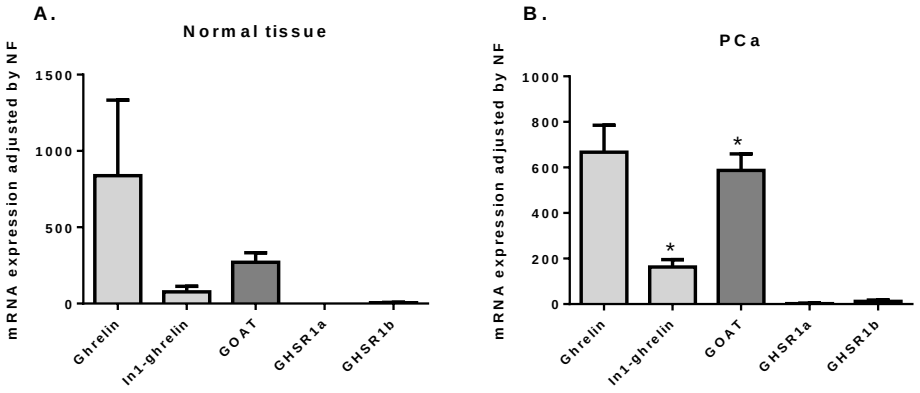


Figure 25: Expression of the components of the ghrelin-system in normal prostate and PCa samples. Expression levels of the different components of the ghrelin system in a battery of 52 fresh PCa samples compared to the expression found in 14 fresh normal prostates (Control). Absolute mRNA levels were determined by qPCR and adjusted by a normalization factor (NF) calculated from the expression levels of two housekeeping genes (ACTB and GAPDH). Data represent mean ± SEM. Asterisks (*$p<0.05$) indicate values that significantly differ between PCa samples and normal samples.

- **Analyses in a normal prostate-like cell line and in different PCa cell lines**: The expression of ghrelin-system was also analyzed in the normal-like prostate cell line RWPE-1, and in different androgen-dependent (22Rv1, LnCaP, VCaP) and androgen-

independent (PC-3, DU-145) PCa cell lines, which represent several stages of the disease (**Figure 26**). Our results revealed that GOAT and In1-ghrelin were the most expressed components of the ghrelin system in PCa cell lines (**Figure 26**). The expression of ghrelin was low or absent in the majority of the PCa cell lines analyzed. Moreover, the expression of the ghrelin receptors (GHSR1a and GHSR1b) was barely detected in both normal and PCa cell lines.

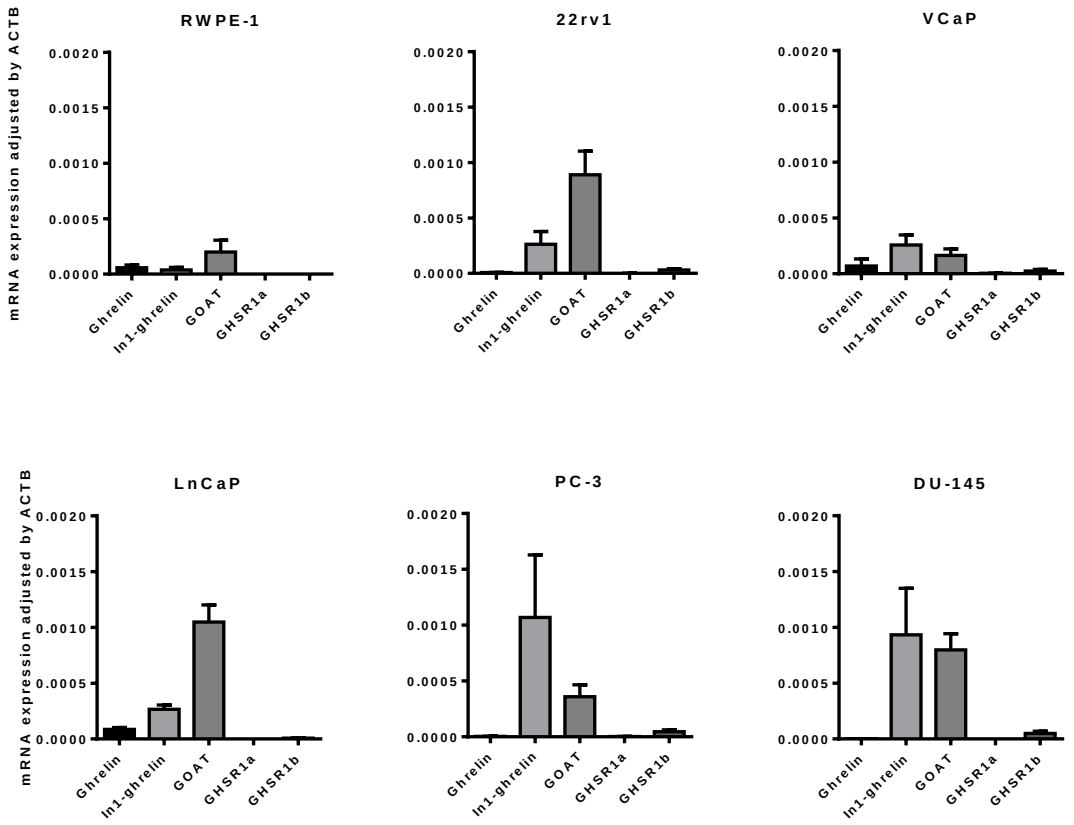


Figure 26: Expression of different components of the ghrelin system in normal-like (RWPE-1) and PCa cell lines (androgen dependent: 22Rv1, VCaP, LnCaP; androgen independent: PC-3, DU-145). Absolute mRNA levels from different passages (n≥3) were determined by qPCR and adjusted by ACTB. Data represents mean ± SEM.

5.2.2 Presence and functional role of GOAT in PCa

It should be mentioned that the results of this section have been published in the journal "*Cancer Letters*" [Title: "***Ghrelin O-acyltransferase (GOAT) enzyme is overexpressed in prostate cancer wherein it is associated with the metabolic status: potential value as non-invasive biomarker***"; Cancer Lett. 2016; 383(1):125-134]. A copy of this manuscript is included at the end of this thesis document.

5.2.2.1. GOAT enzyme is overexpressed in PCa tissues

Expression of GOAT enzyme was analyzed in a cohort of fresh PCa-biopsies collected from patients with high risk PCa [according to D' Amico classification (PSA > 20.0 and/or Gleason 8-10 and/or cT2c-T3); n=52)] and controls (n=12). Demographic and clinical parameters of the patients are detailed in **Table 6**. GOAT expression was increased in PCa tissues compared with normal tissues (p=0.047; **Figure 27A**). Remarkably, GOAT expression was able to discern between patients with presence of PCa and controls (ROC curve; p=0.028; **Figure 27B**).

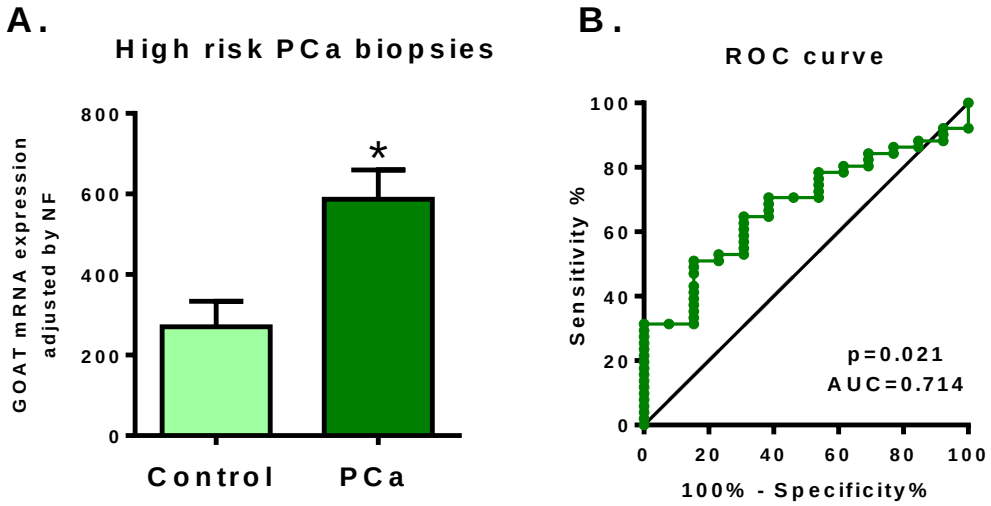


Figure 27: GOAT expression is upregulated in PCa tissues. A. Expression levels of GOAT in a battery of 52 fresh PCa samples compared to the expression found in 12 fresh normal prostates (Control). Absolute mRNA levels were determined by qPCR and adjusted by a normalization factor (NF) calculated from the expression levels of two housekeeping genes (ACTB and GAPDH). **B.** Receiver operating characteristic (ROC) curve analysis to determine the accuracy of GOAT expression as diagnostic test to discriminate between patients with presence of PCa. Data represents mean ± SEM. Asterisks (*p<0.05) indicate values that significantly differ from the control.

5.2.2.2. GOAT expression levels correlate with metabolic factors (BMI, body-weight and presence of dyslipidemia) in patients with PCa

We found that GOAT expression was positively correlated with body weight and BMI in PCa patients (**Figure 28A**) but not in controls (**Figure 28B**). In addition, GOAT expression tended to be higher in PCa patients with dyslipidemia (p=0.084; **Figure 28C**), and, a greater proportion of patients with high GOAT expression presented dyslipidemia compared with patients with low GOAT expression (**Figure 28D**). No correlations were found between GOAT expression and presence of metastasis, Gleason Score or circulating PSA (**Figure 28E**).

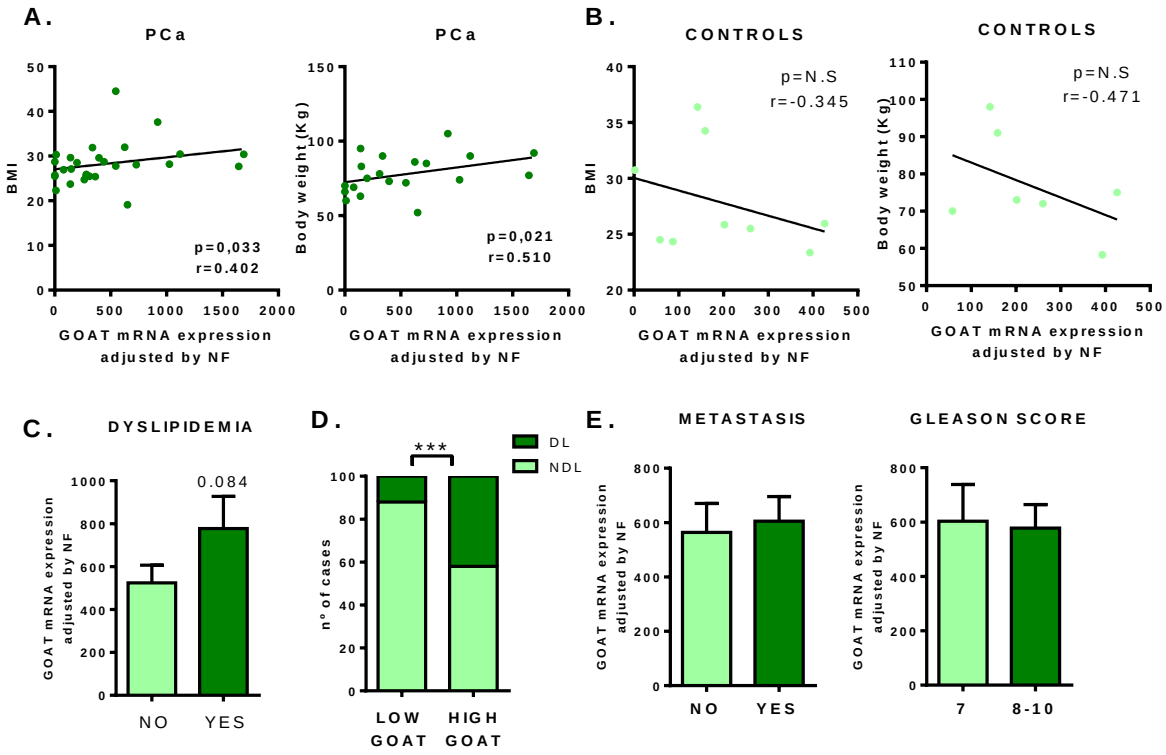


Figure 28: GOAT expression correlates with metabolic factors in patients with PCa. **A.** GOAT expression and BMI and body weight correlations in PCa patients; **B.** GOAT expression and BMI and body weight correlations in control patients; **C.** GOAT expression in patients without (NO) and with (YES) dyslipidemia; **D.** frequencies observed between two groups of PCa patients with low or high GOAT expression levels in terms of dyslipidemia; **E.** Comparison of GOAT expression in terms of presence of metastasis and Gleason Score. Graphs represent mean ± SEM. Asterisks (***p<0.001) indicate values that significantly differ from the corresponding controls. DL=dyslipidemia; NDL=non dyslipidemia.

5.2.2.3. GOAT protein levels are also upregulated in PCa tissues.

IHC for GOAT enzyme was also performed on FFPE-prostate samples harboring normal and tumoral regions, obtained from radical prostatectomies (n=4) of patients diagnosed with localized PCa (Gleason score 6-7), which revealed stronger GOAT staining in the tumoral vs. benign glands (**Figure-29A**). Interestingly, high-magnification images showed perineural-infiltration by tumoral prostate glands with high cytoplasmic (granular) and nuclear GOAT-staining, suggesting that GOAT overexpression could be associated with a malignant event in PCa. Due to the small set of FFPE samples available, we next implemented IHC analyses in a subset of samples (biopsies) from a cohort of clinically high-grade PCa samples already described above (n=16), which included PCa and normal-benign prostate regions. These analyses revealed that GOAT staining was very weak in normal-benign prostate samples (cytoplasmic-staining; **Figure-29B**) compared with the intense staining found in PCa samples [staining located at cytoplasmic (granular-staining) and nuclear level of the cancerous prostate-glands; **Figure-29B-C**). This staining in PCa samples seems to be specific for tumoral regions since the staining of the stromal cells within the tumor microenvironment was negative for GOAT (**Figure-29C**). Moreover, the comparison between the mRNA expression levels of GOAT in samples with moderate and high expression of GOAT by IHC suggests a relationship between mRNA and protein levels since samples with high GOAT by IHC presented higher (non-significant) levels of GOAT mRNA (data not shown). Interestingly, GOAT staining tended to be stronger in patients with higher Gleason-score (p=0.088; **Figure-29D**) and in patients with metastasis (p=0.079; **Figure-29E**).

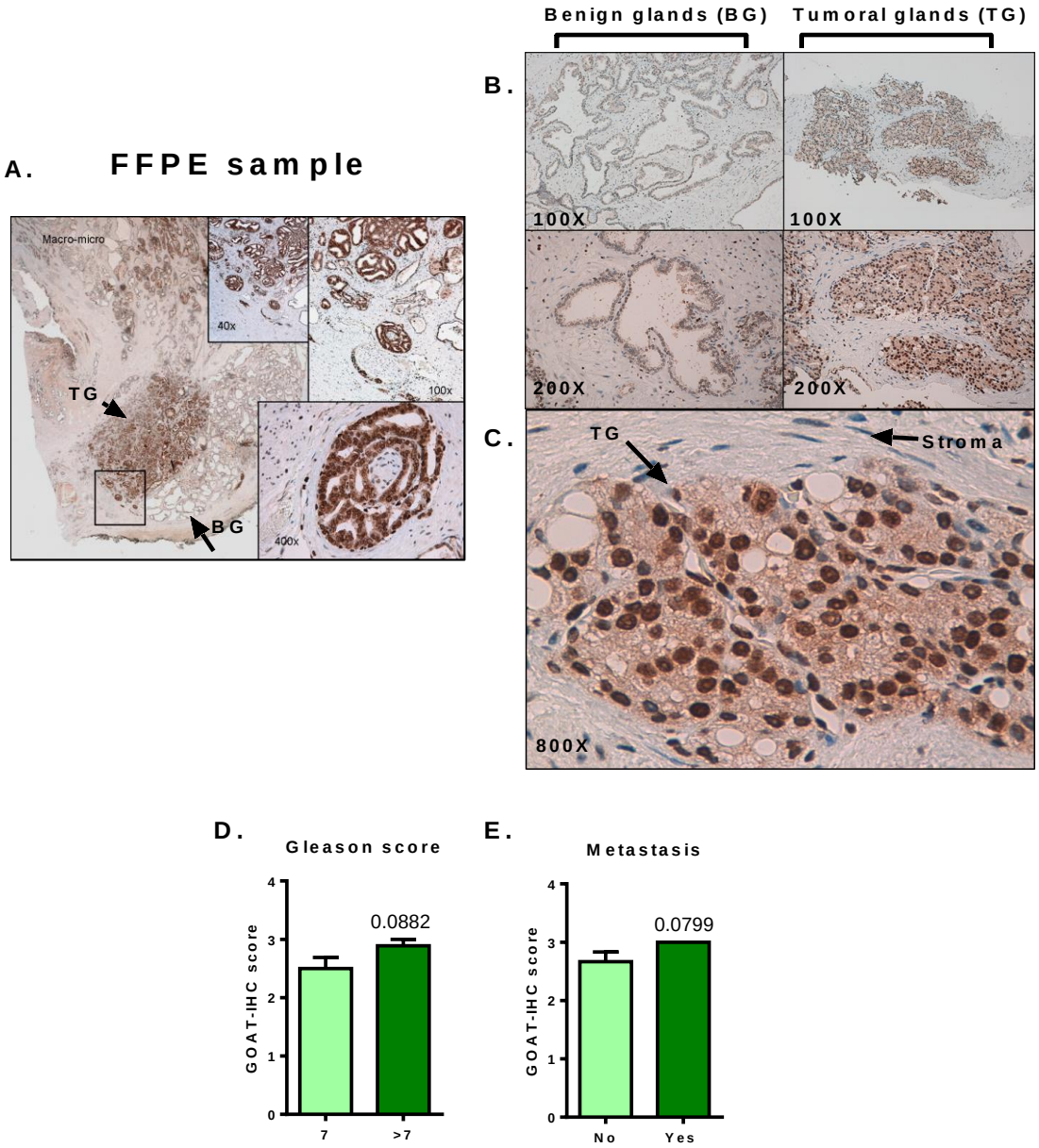


Figure 29: GOAT protein expression is increased in cancerous glands. A. IHC analysis of GOAT in a representative FFPE-PCa sample with benign and tumor glands at 40-400X magnification. The images show higher intensity of GOAT staining in tumor glands vs. benign glands, and intense GOAT staining in nerves infiltrated by tumor cells; **B-E.** GOAT IHC analysis in 16 biopsies from patients with high risk PCa; **B.** GOAT IHC staining in prostate biopsies with benign glands (BG; left-panel) or tumor glands (TG; right-panel) at 100-200X magnification; **C**. Cytoplasmic and nuclear GOAT staining in a tumoral gland at 800X magnification; **D.** Comparison of GOAT IHC score [low (1), moderate (2) or high (3) GOAT staining] with Gleason-score; **E.** Comparison of GOAT IHC score with presence of metastasis. In all cases, data represent mean ± SEM.

5.2.2.4. GOAT expression is higher in PCa cells vs. normal prostate cells and it is secreted and regulated by metabolic factors in normal and tumoral prostate cells.

GOAT expression was significantly higher in PCa cell lines (LnCaP, 22Rv1 and DU145) compared with the normal-like prostate cell line RWPE-1, and with human normal primary prostate cell cultures (**Figure 30A**). Remarkably, PCa cell lines were capable to secrete GOAT protein into the media, being this secretion higher in 22Rv1 vs. PC-3 cells (**Figure 30B**; 1.641 vs. 1.163 ng/ml, respectively; $p=0.005$, $n> 3$), which is in agreement with the higher GOAT mRNA levels observed in 22Rv1 vs. PC-3 (Figure 30A). To determine whether the expression of GOAT could be directly regulated by metabolic factors, normal human prostate cell cultures were treated with different metabolic factors [including its endogenous targets (acylated-ghrelin peptides: ghrelin and In1-ghrelin) and insulin and IGF1]. Particularly, In1-ghrelin peptide (In1-19), but not ghrelin, significantly increased GOAT expression in 22Rv1, but not in PC-3 cells (**Figure 30C**), and in normal prostate primary cell cultures (**Figure 30D**). Conversely, treatment with key metabolic factors such as insulin and IGF1, or their combination, significantly reduced GOAT expression in PC-3 cells ($p<0.001$; **Figure 30E**).

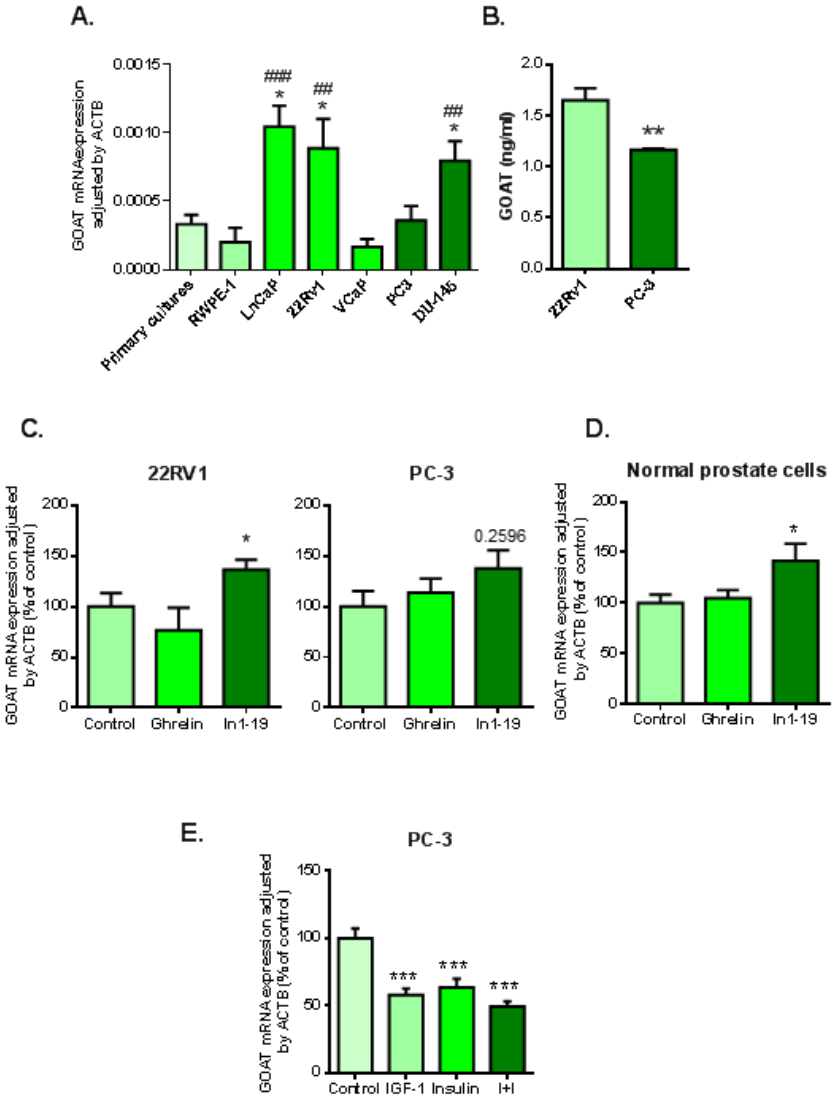


Figure 30: ***Regulation of GOAT expression by metabolic factors in normal prostate and PCa cell cultures*****. A.** GOAT expression in normal-like prostate cell line (RWPE-1) or prostate cancer cell lines (androgen-dependent: LnCaP, 22RV1 and VCaP, or androgen-independent: PC-3 and DU145) from different passages (n≥3). Asterisks (*p < 0.05) indicate values that significantly differ between normal RWPE-1 cells and PCa cell lines, while dashed (##p < 0.01; ### p < 0.001) indicate differences in GOAT expression between normal prostate primary cell cultures and PCa cell lines; **B.** Basal GOAT protein secretion measured by ELISA (after 24h-culture) in 22Rv1 and PC-3 cell lines (n=3). Asterisks **p<0.01) indicate values that significantly differ from 22Rv1 cells; **C-D.** Regulation of GOAT expression after treatment (10nM) with ghrelin or In1-ghrelin (In1-19) peptide in 22Rv1 or PC-3 PCa cell lines (C) and in normal prostate cell cultures (D) (24h, n≥3); **E.** Regulation of GOAT expression after treatment (10nM) with IGF-1, insulin or both (IGF-1+insulin; n≥3). In these expression experiments in response to treatments, asterisks (*p < 0.05; ***p<0.001) indicate values that significantly differ from controls. Data represent mean ± SEM. Absolute mRNA levels of GOAT were determined by qPCR and adjusted by the expression levels of a housekeeping gene (ACTB).

5.2.2.5. Plasma GOAT levels are elevated in patients with PCa in comparison with healthy control patients: association with pathophysiological parameters

Since GOAT is overexpressed in PCa tissues, secreted from PCa cell lines and can be detected in blood [245], we evaluated, for the first time, the presence of GOAT in plasma of PCa patients (n=85) and compared with the levels in healthy control patients (n=28) (Clinical/pathological features of this patient cohort are summarized in **Table 8**). Remarkably, plasmatic GOAT levels were significantly elevated in PCa patients compared with controls (**Figure 31A**). It should be noted that PCa-patients had similar body-weight and BMI but presented a slightly higher age than control-patients (~6 years; **Table 8**). Nevertheless, we implemented general linear models, which demonstrated that this difference was not responsible for the differences observed in plasmatic GOAT levels. Furthermore, when plasma GOAT levels were corrected by age, higher GOAT levels were clearly evident in PCa vs. control-patients (p-value=0.002**; Figure 31A).** Moreover, ROC-analysis showed the diagnostic potential for GOAT at the plasma level ($p<0.0001$; AUC=0.752; **Figure 31B**), wherein a value of 1.22ng/ml of GOAT plasma levels could discriminate between PCa vs. control-patients with a specificity of 67,8 % and a sensitivity of 81,1%.

Interestingly, plasmatic GOAT levels were associated with several pathophysiological factors. Specifically, GOAT levels were directly associated with total PSA levels and inversely correlated with free PSA (**Figure 31C),** and also associated positively with two cancer biomarkers (ca_15-3 and cyfra_21-1; **Figure 31D**). Moreover, a higher frequency of infiltrative tumors was found in patients with higher plasmatic GOAT levels (**Figure 31E**).

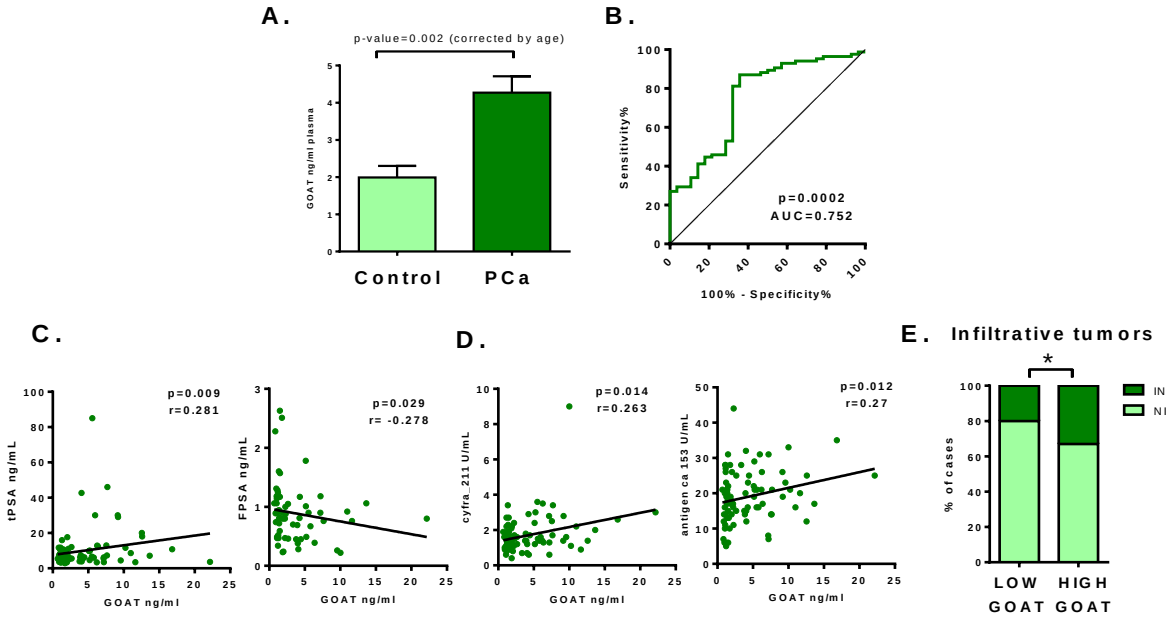


Figure 31: Analysis of the presence of GOAT in the plasma of patients with PCa and controls and association with pathophysiological parameters. A. GOAT plasmatic levels were determined by ELISA in a cohort of 85 PCa patients and 28 controls**; B.** Receiver operating characteristic (ROC) curve analysis to determine the accuracy of GOAT plasmatic levels as diagnostic test to discriminate between patients with PCa and controls**; C.** Correlation of plasma levels of GOAT and total PSA (tPSA) and free PSA (FPSA) levels in PCa patients; **D.** Correlation of plasma levels of GOAT with ca_15-3 and cyfra_21-1 tumor markers in PCa patients; **E.** Frequencies of infiltrative tumors observed in PCa patients with low vs. high GOAT plasmatic levels (according to median GOAT levels; IN=infiltrating tumors; NI=No infiltrating tumors). Values represent mean ± SEM. Asterisk (*p < 0.05) indicate values that significantly differ between groups.

5.2.2.6. Association with clinical-metabolic parameters of plasma GOAT levels with metabolic parameters in patients with PCa

We next analyzed the putative relationship between plasmatic levels of GOAT and several metabolic parameters (i.e. BMI, diabetes, dyslipidemia, etc.). Although we did not find correlations between GOAT plasma levels and BMI or body weight in control and PCa patients (**Figure 32A-B**), we observed that GOAT levels of control-patients (without PCa) were significantly higher in diabetic subjects compared with non-diabetic patients (**Figure 32C**), whereas no differences were found in plasmatic GOAT levels in control-patients with and without dyslipidemia (**Figure 32D**). Consistent with these results, plasmatic GOAT levels in control-patients were positively correlated with the levels of glycated hemoglobin (HbA1c; **Figure 32E**) and glucose (**Figure 32F**), two well-known clinical markers of diabetes. Accordingly, a significantly higher proportion of

patients with diabetes, but not with dyslipidemia, was found in control-patients with high plasmatic GOAT levels compared to those with low GOAT levels (**Figure 32G**). In contrast, plasmatic GOAT levels were similar in diabetic vs. non-diabetic patients with PCa (**Figure 32H**). A trend for elevation in plasmatic GOAT levels was found in patients with PCa that had dyslipidemia compared to those without dyslipidemia (p=0.06; **Figure-32I**). A positive correlation between plasmatic GOAT levels was also found with HbA1c, but not with glucose levels in PCa-patients (**Figure 32J** and **32K**, respectively). Indeed, a higher proportion of patients with diabetes as well as with dyslipidemia were found in PCa-patients having high plasmatic GOAT levels vs. PCa-patients with low GOAT levels (**Figure-32L**).

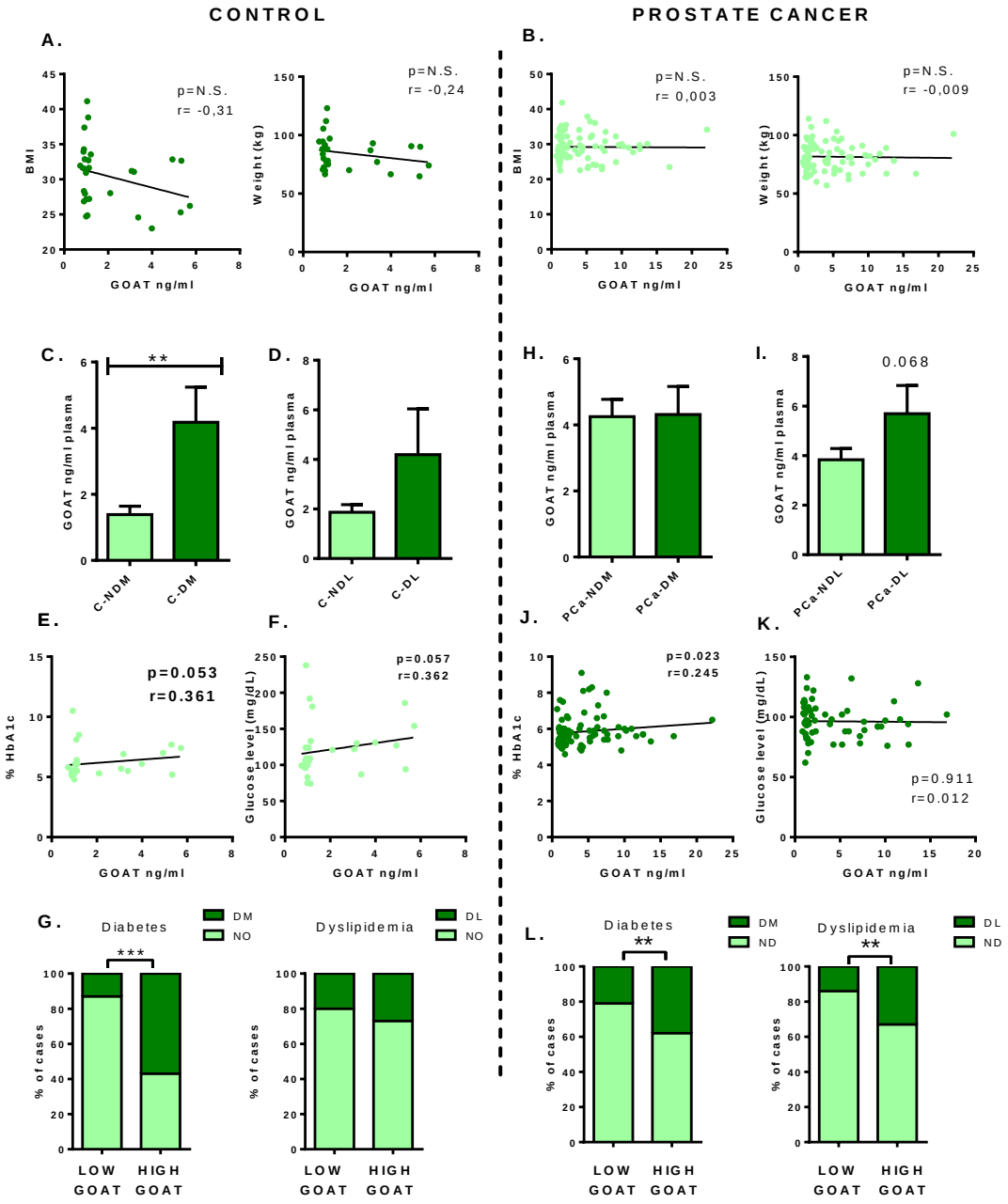


Figure 32: Association of GOAT levels with clinical-metabolic parameters in control and PCa patients. **A-B**. Correlations of plasma GOAT levels with metabolic factors (BMI and body weight) of control patients (A) and patients with PCa (B). **C-D.** Correlations of plasma GOAT levels with diabetes or dyslipidemia in controls patients (C-NDM: Controls without diabetes mellitus; C-DM: Controls with diabetes mellitus; C-NDL: Controls without dyslipidemia; C-DL: Controls with dyslipidemia). **E-F.** Correlations of plasma GOAT levels with HbA1c (E) and glucose (F) levels in control patients. **G.** Frequencies of diabetes and dyslipidemia observed in control patients with low vs. high GOAT plasmatic levels (Low GOAT: patients with low plasmatic GOAT levels; High GOAT: patients with high plasmatic GOAT levels). **H-I.** Correlations of plasma GOAT levels with diabetes or dyslipidemia in PCa patients (PCa-NDM: PCa patients without diabetes mellitus; PCa-DM: PCa patients with diabetes mellitus; PCa-NDL: PCa patients without dyslipidemia; PCa-DL: PCa patients with dyslipidemia). **J-K.** Correlations of plasma GOAT levels with HbA1c (J) and glucose (K) levels in PCa patients. **L.** Frequencies of diabetes and dyslipidemia observed in PCa patients with low vs. high GOAT plasmatic levels. Data represent mean ± SEM. Asterisks (**p < 0.01, ***p<0.001) indicate values that significantly differ between groups.

5.2.2.7. Plasma GOAT levels as non-invasive diagnostic biomarker of PCa in non-diabetic individuals

Based on the previous results, we next interrogated whether plasmatic GOAT levels could be a more specific PCa marker in non-diabetic patients. In fact, we found that plasmatic GOAT levels were higher in non-diabetic PCa-patients vs. control-patients ($p<0.001$; **Figure 33A**). Indeed, the area under curve was considerably improved when analyzing only non-diabetic PCa-patients [value of 0.854 (**Figure 33B**; only non-diabetic) vs. 0.752 (**Figure 31B**; diabetic+non-diabetic)]. In addition, the specificity/sensitivity to discriminate the presence of PCa was significantly higher when only the non-diabetic individuals were considered [a cut-off value of 1.142 ng/ml presented a specificity and sensitivity of 84.2% and 86.7% (**Figure 33A/B**) vs. 67.8% and 81.1% analyzing non-diabetic+diabetic patients (**Figure 31A/B**)]. Interestingly, plasmatic GOAT levels also positively correlated with total PSA, negatively with free PSA levels and positively with the cancer biomarker ca_15-3 (**Figure-33C**), and also exhibited a similar positive tendency with HbA1c ($p=0.080$; **Figure-33C**).

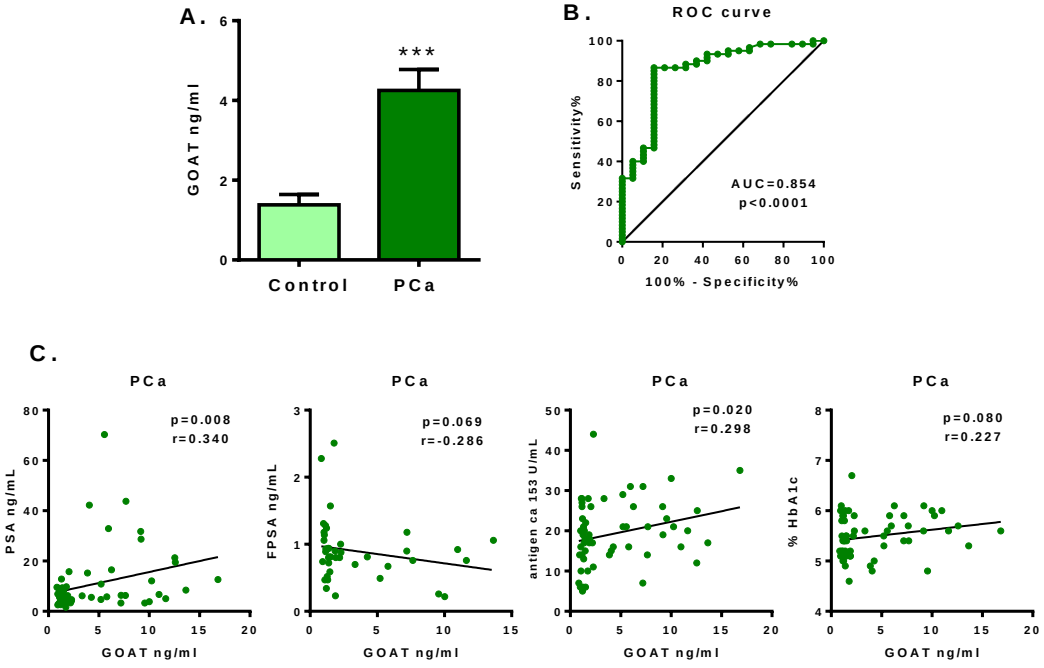


Figure 33: GOAT plasmatic levels in non-diabetic PCa patients compared to non-diabetic controls. A. GOAT plasmatic levels in non-diabetic PCa patients (n=60) compared to non-diabetic controls (n=19) were determined by ELISA. **B.** Receiver operating characteristic (ROC) curve analysis to determine the accuracy of GOAT plasmatic levels as diagnostic test to discriminate between PCa and controls patients within the population without diabetes. **C.** Correlations between plasma GOAT levels and total

PSA, free PSA, ca-153 and HbA1c in non-diabetic patients with PCa. Data represent mean ± SEM. Asterisks (***p<0.001) indicate values that significantly differ between groups.

5.2.2.8. Urine GOAT levels are elevated in patients with PCa in comparison to healthy-control patients: potential as non-invasive biomarker of PCa.

Urine GOAT levels were also significantly elevated in the same cohort of PCa-patients vs. control-patients (**Figure 34A**; **Table 8**). Actually, ROC-curve analysis showed that urine GOAT levels could discriminate between PCa vs. control-patients (**Figure 34B**; p<0.001, AUC= 0.716; a cut-off value of 1.061 ng/ml presented a sensitivity of 75% and specificity of 61%). Remarkably, this sensitivity/specificity was lower than that found with plasmatic GOAT levels, and the underlying reasons (which may involve sampling procedures, urine processing, etc.) are still not defined, and, therefore, warrant further investigation. Interestingly, no correlations were found between GOAT urine levels and metabolic parameters (BMI, body weight, diabetes or dyslipidemia) in control (**Figure 34C**) or PCa patients (**Figure 34D**), suggesting that GOAT urine levels are not relevantly influenced by these parameters Furthermore, urine GOAT secretion was increased after a prostate-massage in an additional cohort of PCa-patients (**Table 9**) compared to the same cohort of subjects before prostate-massage (**Figure 34E**).

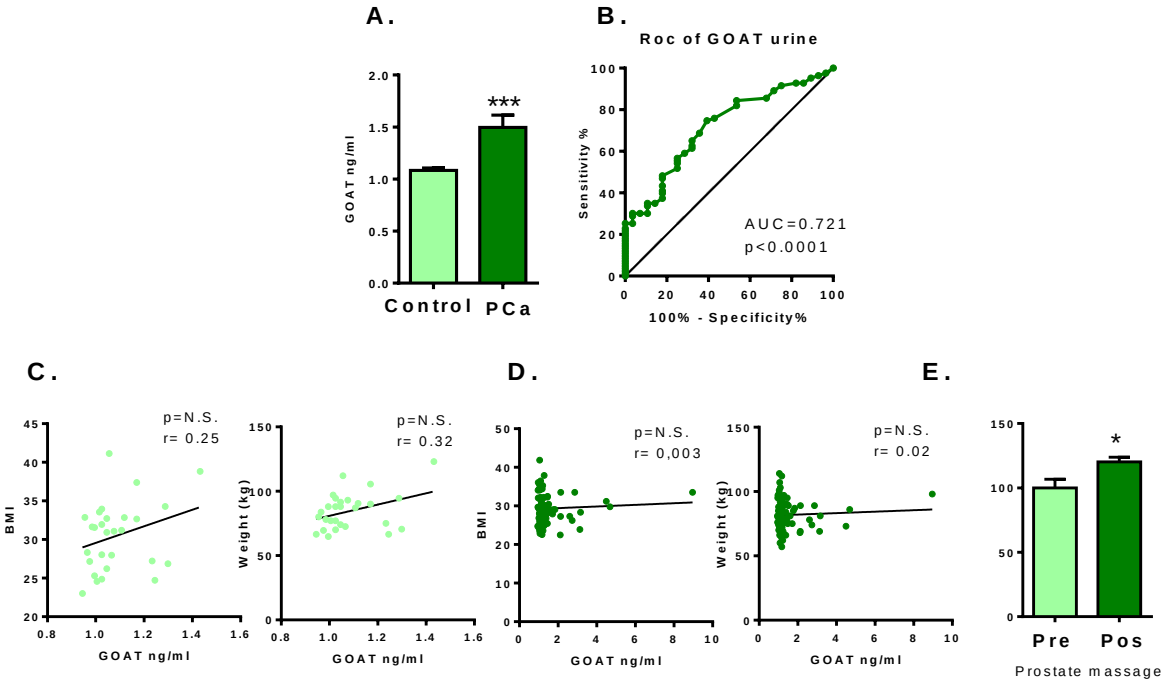


Figure 34: Evaluation of GOAT levels as a non-invasive PCa diagnostic marker in urine. A. GOAT urine levels in a population of 85 PCa patients compared to 28 normal controls determined by ELISA. **B.** ROC curve analysis to determine the accuracy of GOAT urine levels to discriminate between PCa and control patients; **C-D.** Correlations between urine GOAT levels and BMI and body weight in control patients (C) and PCa patients (D). **E.** Percentage of increase of urine GOAT secretion after prostate massage in PCa patients. Data represent Mean ±SEM. Asterisks (*$p < 0.05$; ***$p<0.001$) indicate values that significantly differ between groups.

5.2.3. Presence and functional role of In1-ghrelin variants in PCa

5.2.3.1. In1-ghrelin, but not ghrelin, is overexpressed in PCa tissues and its levels are associated with GOAT-enzyme and PSA levels.

Expression of the In1-ghrelin variant and ghrelin was analyzed in a cohort of patients with high-risk PCa (n=52) and compared to normal prostate (NP) control samples (n=12) (**Figure 35**). Demographic and clinical characteristics of these cohorts of patients are included in **Table 6**. Specifically, ghrelin and In-ghrelin mRNA was detected in 83.3% and 66.6% of control patients and in 90% and 88.5% of PCa samples, respectively. Remarkably, In1-ghrelin, but not ghrelin mRNA was significantly overexpressed in PCa samples (**Figure 35A**). Indeed, ROC curve analysis demonstrated that In1-ghrelin, but not ghrelin expression could significantly discriminate between patients with or without PCa (**Figure 35B**). Moreover, In1-ghrelin, but not ghrelin expression was positively correlated with GOAT-enzyme expression (**Figure 35C**) and with PSA expression (**Figure 35D**). No further associations were found between In1-ghrelin or ghrelin and other pathological features, such as Gleason score or presence of perineural infiltration (data not shown). We also analyzed the expression levels of ghrelin receptor in this cohort of patients and found that GHSR1a was only detected in one tumor sample, while GHSR1b expression was detected in 6 control and 10 tumor samples, although its expression levels were very low (**Figure 35E**).

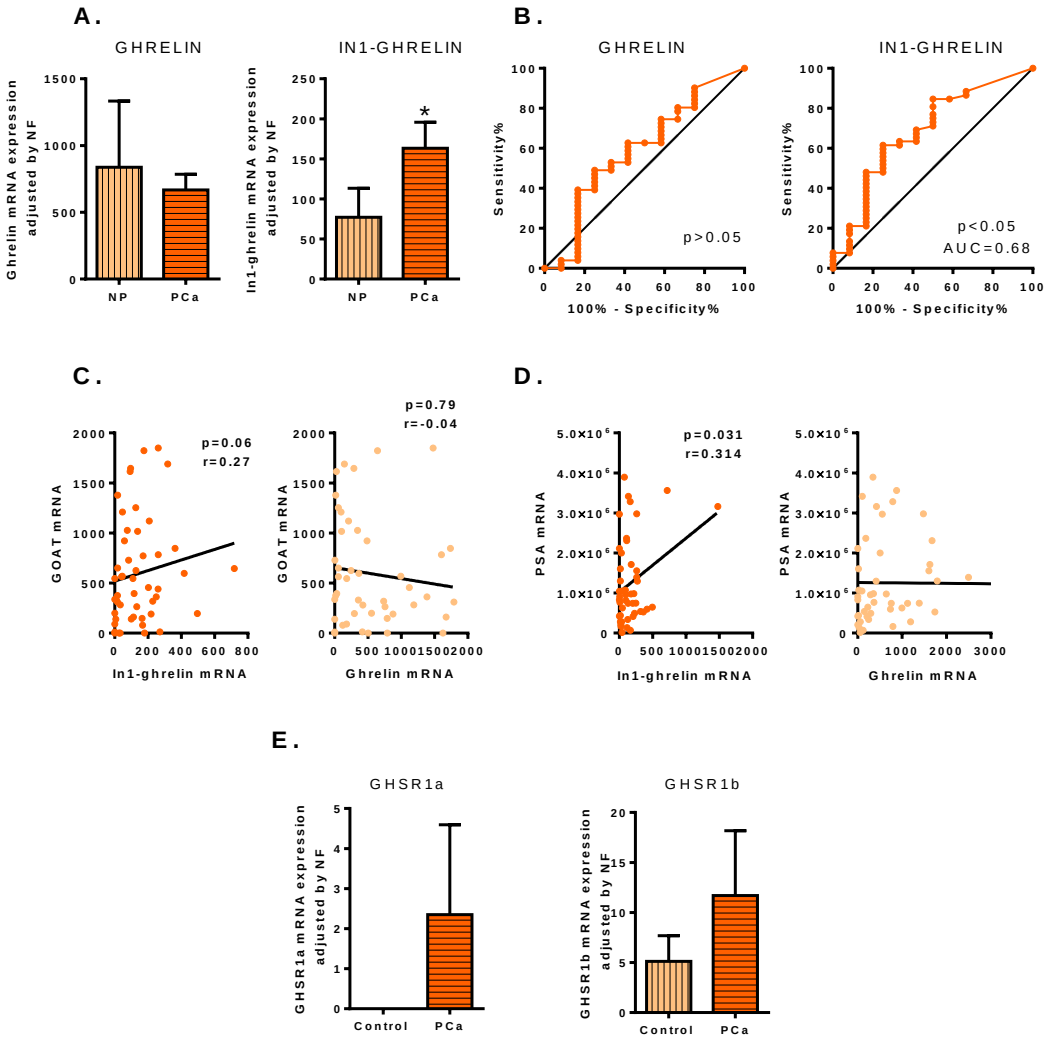


Figure 35: mRNA expression of ghrelin system in PCa. A. Ghrelin and In1-ghrelin expression in biopsies from patients with high-risk PCa (n=52) and normal prostates from patients that underwent cystoprostatectomy (n=12). Expression levels were determined by qPCR and adjusted by a normalization factor (NF) calculated from ACTB and GAPDH expression levels; **B.** ROC curve analysis to determine the accuracy of ghrelin or In1-ghrelin expression as diagnostic test to discriminate between high-risk PCa patients and controls using the same cohort; **C.** Correlations between ghrelin or In1-ghrelin expression with GOAT enzyme expression PCa samples (n=52); **D.** Correlations between ghrelin or In1-ghrelin with PSA in PCa samples; **E.** GHSR-1a and GHSR-1b mRNA levels in the same cohort of fresh samples. Values represent mean ± SEM. Asterisks (*p < 0.05) indicate values that significantly differ between groups.

5.2.3.2. In1-ghrelin, but not ghrelin expression is higher in castration-resistant PCa cells compared with androgen-dependent PCa cells and normal prostate cells.

Evaluation of mRNA expression of ghrelin-system in androgen-dependent and castration-resistant PCa cell-lines compared with the normal-like prostate cell line RWPE1 revealed that while native-ghrelin was expressed in normal RWPE1-cells and in LNCaP and VCaP PCa-cells (**Figure 36A**), its expression was almost undetectable in the other PCa cell-lines analyzed (**Figure 36A**). However, In1-ghrelin expression was commonly higher in all PCa cell-lines compared with the normal RWPE1-cells (**Figure 36B**), which is consistent with the data obtained from fresh PCa samples (**Figure 35A**). Moreover, In1-ghrelin expression was higher in castration-resistant cells (DU145/PC-3**)** compared to androgen-dependent cells (LNCaP/22Rv1/VCaP), suggesting that In1-ghrelin expression may vary through the different stages of PCa. Conversely, GHSR1a/b were barely expressed in PCa cell lines (**Figure 36C-D**), which is in contrast with previous studies showing the presence of GHSR1a in some of these PCa cell lines [223]. Therefore, in order to corroborate these data, we used different primers sets [designed from our group and validated in other samples, including a primer set used in the previously mentioned work [223], but again we did not find any evidence of detectable GHSR1a expression in PCa cell lines **(Figure 36E**).

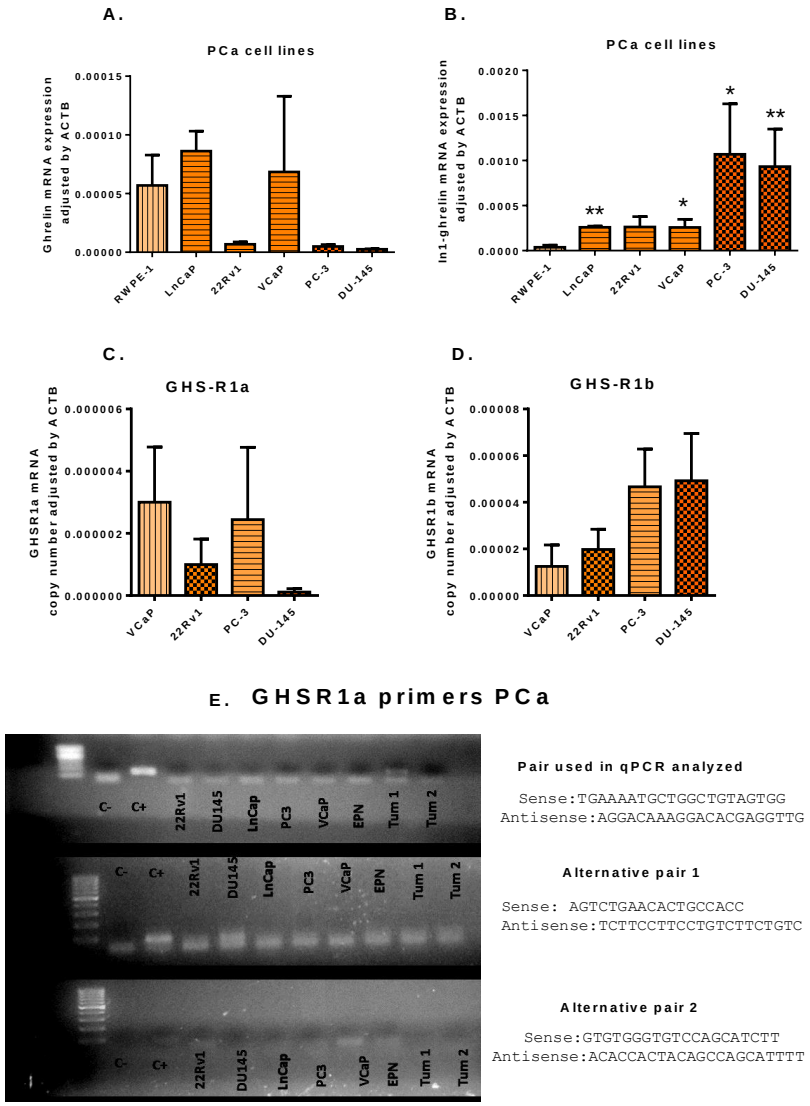


Figure 36: Study of the Ghrelin system expression in normal prostate and PCa cell lines. Ghrelin (A), In1-ghrelin (B) and GHSR1a (C) and GHSR1b (D) expression levels in normal-like prostate cell line (RWPE-1) and PCa cell lines (androgen-dependent: LnCaP, 22RV1 and VCaP or androgen-independent: PC-3 and DU145). Absolute mRNA levels from different passages (n≥3) were determined by qPCR and adjusted by ACTB. Data represents mean ± SEM. Asterisks (*p < 0.05; **p < 0.01) indicate values that significantly differ between a PCa cell line vs. the normal-like prostate cell line RWPE-1. **E.** Further analysis of GHSR1a expression on different PCa cell lines (22Rv1, DU-145, LnCaP, PC-3), normal-like prostate cell line (EPN) and PCa samples using several pairs of primers. As positive control, cDNA from a pituitary tumor with GHSR1a expression was used.

5.2.3.3. Plasma levels of In1-ghrelin, but not ghrelin, are higher in patients with PCa compared with healthy-control patients

We next studied the presence and regulation of circulating levels of native-ghrelin and In1-ghrelin in an independent patient's cohort. Demographic and clinical parameters of the patients are detailed in **Table 10**. Specifically, we found for the first time that

acylated In1-ghrelin was detected in plasma and its levels, but not those of native-ghrelin, are significantly higher in PCa-patients (n=30) compared to controls (n=20) (**Figure 37A**). Remarkably, ROC-curve analysis demonstrated that only acylated In1-ghrelin plasmatic levels could discriminate between patients with or without PCa (p=0.05; **Figure-37B**).

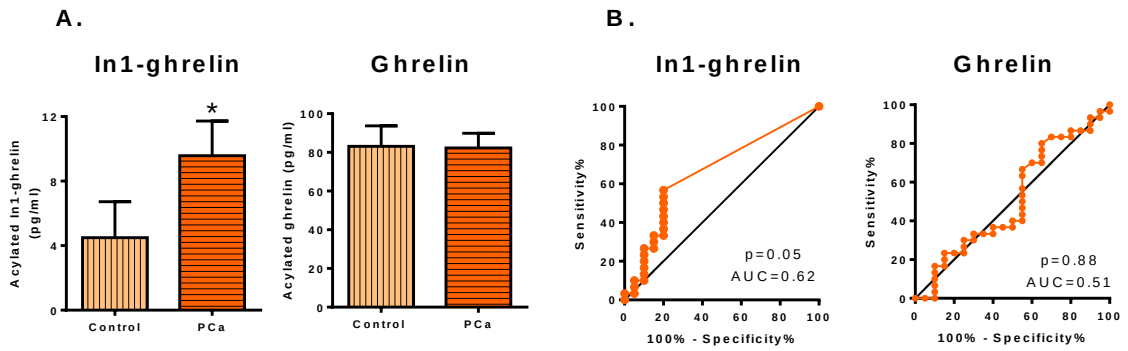


Figure 37: Ghrelin and In1-ghrelin levels in plasma. A. Circulating plasmatic levels of In1-ghrelin (by ELISA, n=30) and ghrelin (by RIA, n=20) in control-healthy patients compared with patients with PCa; **B.** ROC curve analysis to determine the accuracy of plasmatic acylated In1-ghrelin or acylated ghrelin levels as diagnostic test to discriminate between PCa patients and controls. Data represent mean ± SEM. Asterisks (*p<0.05) indicate values that significantly differ from controls patients.

5.2.3.4. Effects of In1-ghrelin and ghrelin treatment on normal prostate cell function.

To test the capacity of In1-ghrelin derived peptides (In1-19 and In1-40) and ghrelin to induce functional responses in normal prostate cells, we first measured the changes in $[Ca^{2+}]_i$ levels in single cells derived from primary normal prostate (NP) cell cultures (from men that underwent cystoprostatectomy for bladder cancer; **Figure 38**). Specifically, we found that ghrelin and In1-ghrelin could induce a stimulatory response in $[Ca^{2+}]_i$ in NP cell cultures; however, the percentage of responsive cells was different upon treatment (i.e. whereas 36.5% of NP-cells responded to In1-19 treatment, only 8.6% and 7.1% responded to ghrelin or to In1-40, respectively) (**Figure 38**). Moreover, In1-ghrelin peptides evoked a higher stimulatory response compared with ghrelin (i.e. an increase of 58% and 59% in response to In1-19 and In1-40 vs. an increase of 28% with ghrelin).

	Cells analyzed	% cell respond	% Max	Error	T resp	Error
Ghrelin	151	8.6	128.2	10.3	8.4	1.4
In1-19	252	36.5	159.8	9.4	11.8	1
In1-40	98	7.1	159.1	16.1	12.5	2

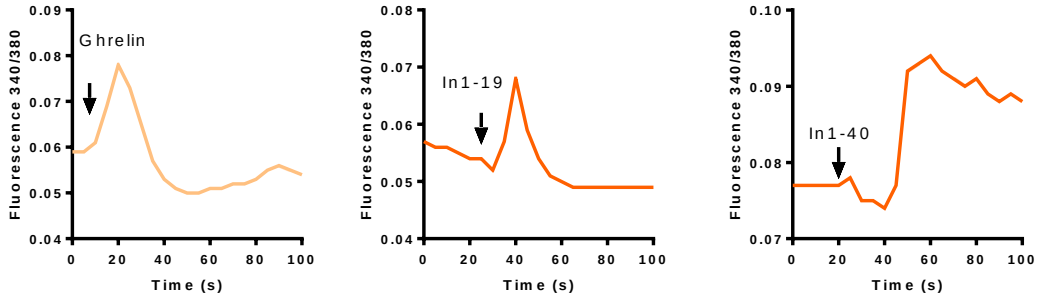


Figure 38: Effects of ghrelin and In1-ghrelin derived peptides (In1-19 and In1-40) on the levels of $[Ca^{2+}]_i$ in normal prostate cell cultures Total number of cells measured, percentage of responsive cells (% of cells resp.), percentage of maximum response (±error) and time of maximal response (±error) are indicated. Representative profiles of changes in $[Ca^{2+}]_i$ in response to ghrelin, In1-19 and In1-40 are also depicted. All treatments were used at 10nM (n≥3).

We next evaluated whether In1-ghrelin peptides or ghrelin could regulate other functional parameters such as PSA expression and secretion in primary NP cell cultures. Remarkably, In1-ghrelin treatment, but not native-ghrelin, increased PSA expression and secretion in NP-cell cultures at 4h and 24h, being In1-19 the most effective peptide (In1-40 only increased PSA expression/secretion at 4h with a non-significant increase in secretion) (**Figure 39A-B**).

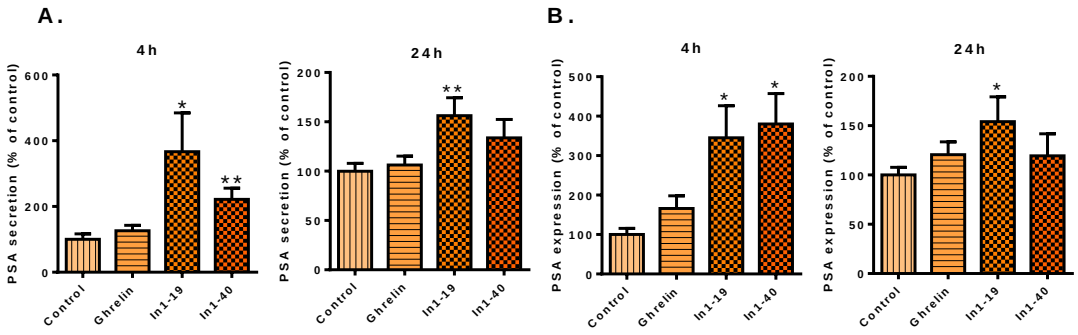


Figure 39: PSA secretion (A) and expression (B) after 4h or 24h of treatment with ghrelin or In1-ghrelin derived peptides (10nM). Data represent mean ± SEM. Asterisks (*p < 0.05; **p < 0.01) indicate values that significantly differ between normal prostate cell cultures treated with vehicle-control and ghrelin or In1-ghrelin derived peptides.

5.2.3.5. Effects of In1-ghrelin and ghrelin treatment or overexpression on cell viability/proliferation of normal primary prostate cell cultures and normal-like prostate cell line (RWPE-1)

Ghrelin or In1-ghrelin derived peptides treatment did not affect cell viability in primary NP-cell cultures or cell proliferation in normal RWPE1-cells (**Figure 40**). However, In1-ghrelin, but not native-ghrelin, overexpression increased the cell viability of primary NP-cell cultures after 48 of culture compared to mock (control)-transfected cell cultures (**Figure 40**).

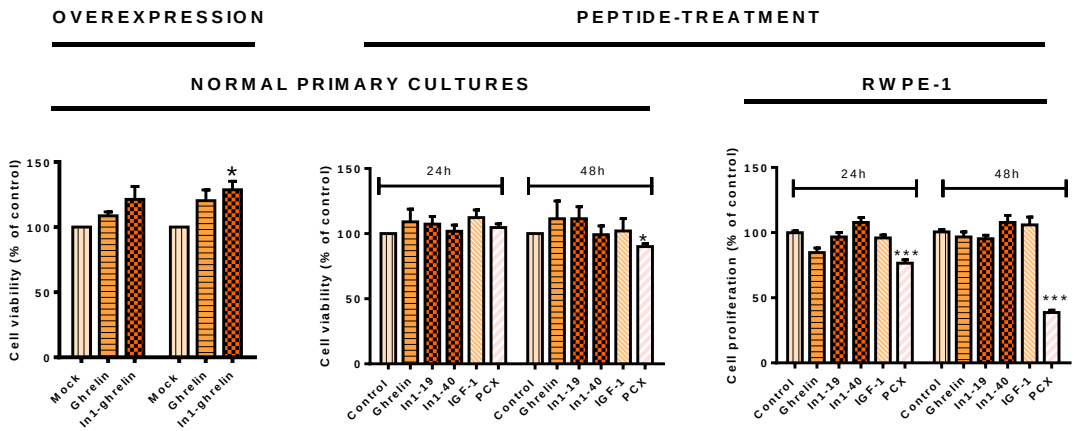


Figure 40: Cell viability of normal prostate cell cultures in response to ghrelin or In1-ghrelin treatment or overexpression. Cell viability of normal prostate cell cultures transfected with empty (mock), ghrelin or In1-ghrelin vectors and determined after 24-48h (left-panel; n≥3). Cell viability of normal prostate cell cultures (middle–panel) and cell proliferation of normal-like prostate RWPE-1 cell line (right-panel) after treatment with vehicle-control, ghrelin or In1-ghrelin derived peptides (In1-19 and In1-40) for 4-24h (n≥3). Asterisk represents significant differences (*p < 0.05; ***p < 0.001) between control and ghrelin or In1-ghrelin treatment or transfection. Values represent mean ± SEM.

5.2.3.6. Effects of In1-ghrelin and ghrelin treatment on pathophysiological features (cell proliferation and migration) of PCa cells

We next examined the effect of the treatment with ghrelin or In1-ghrelin peptides on cell proliferation of different PCa cell lines. Specifically, administration of native-ghrelin only increased cell-proliferation of 22Rv1 (at 24h) and PC-3 (24-48h) cell-lines, whereas it did not exert any significant effect in the rest of PCa cell-lines studied (i.e. LNCaP, VCaP or DU145; **Figure 41A-B**). However, In1-ghrelin peptides were able to increase cell-proliferation in several PCa cell-lines, both androgen-dependent

(22Rv1/VCaP/LNCaP) and -castration resistant cells (PC-3/DU145) (**Figure 41A-B**). Indeed, the effect of In1-ghrelin peptides treatment was more consistent and pronounced in castration-resistant PCa cell-lines (PC-3/DU145), wherein both peptides significantly increased cell-proliferation at 24-48h (**Figure 41A-B**). However, in androgen-dependent PCa cell-lines, both peptides increased proliferation at 24h in 22Rv1-cells, while only In1-40 increased proliferation at 48h in LNCaP/VCaP-cells (**Figure 41A-B**). In addition, we found that In1-ghrelin peptides, but not native-ghrelin, were able to increase migration of PC-3 cells (**Figure 41C**).

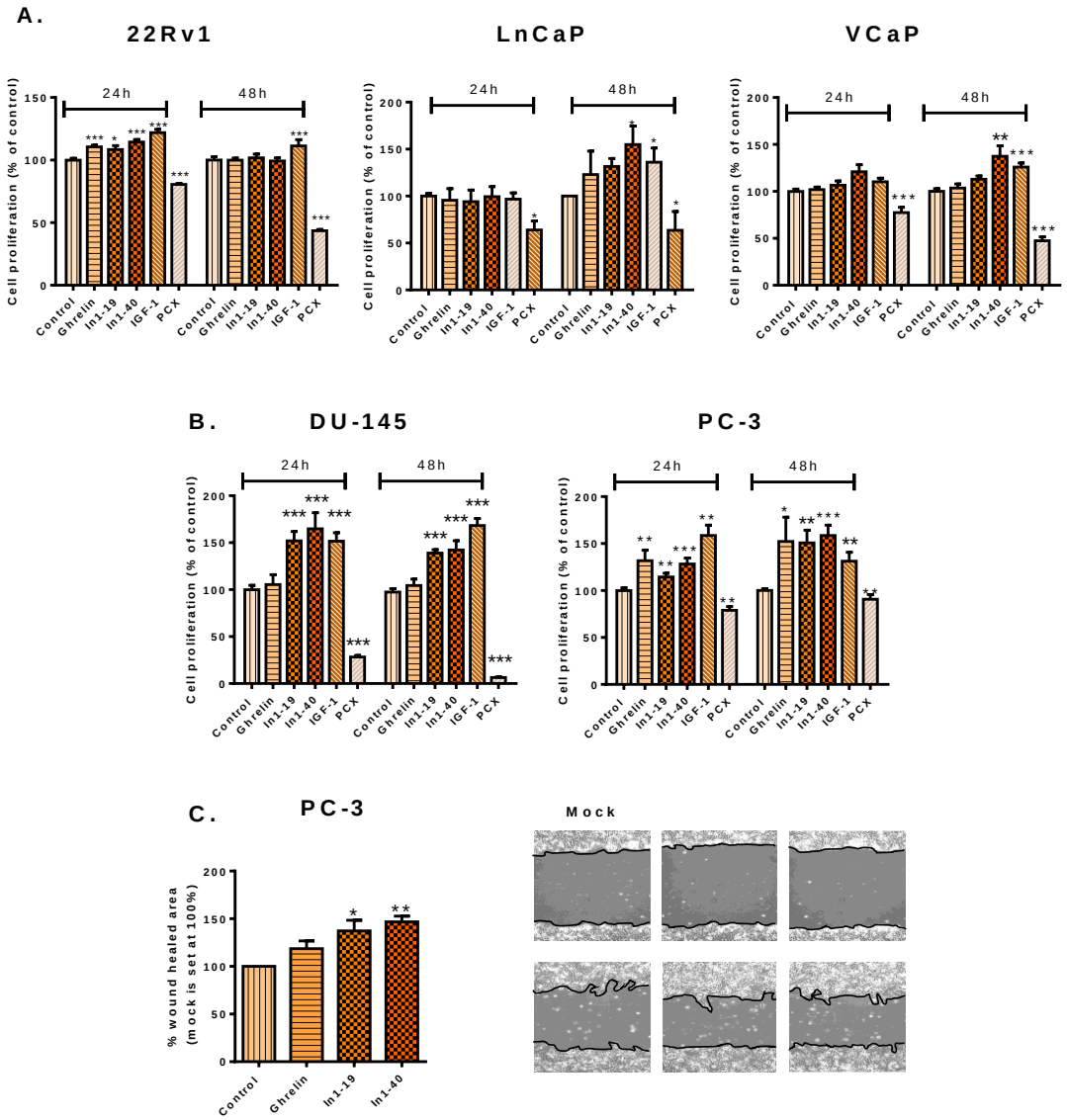


Figure 41: Effects of ghrelin and In1-ghrelin on PCa pathophysiological processes. Cell proliferation of androgen dependent (22Rv1, LnCaP and VCaP) (**A),** and androgen-independent (PC-3 and DU-145) (**B)** PCa cell lines cell lines after treatment with ghrelin or In1-ghrelin derived peptides (In1-19 and In1-40) for 4-24h (10nM; n≥3). Treatment with IGF-1 and paclitaxel (PCX) were used as positive and negative controls, respectively. Data represent mean ± SEM. Asterisks (*p < 0.05; **p < 0.01, ***p < 0.001) indicate differences

of a specific treatment with vehicle-treated controls within each time point. **C.** Migration capacity on PC-3 cells in response to ghrelin and In1-ghrelin peptides treatment determined by wound healing assay (12h; n≥3). Representative images showing the higher migration capacity of PC-3 cells after treatment with In1-ghrelin peptides are shown. Data represent mean ± SEM. Asterisks (*p < 0.05; **p < 0.01) indicate differences with vehicle-treated controls.

Finally, we demonstrated that In1-ghrelin peptides induced the phosphorylation on ERK1/2 in PC-3 and LNCaP cell-lines (**Figure 42**), with almost no detectable effect on AKT-phosphorylation

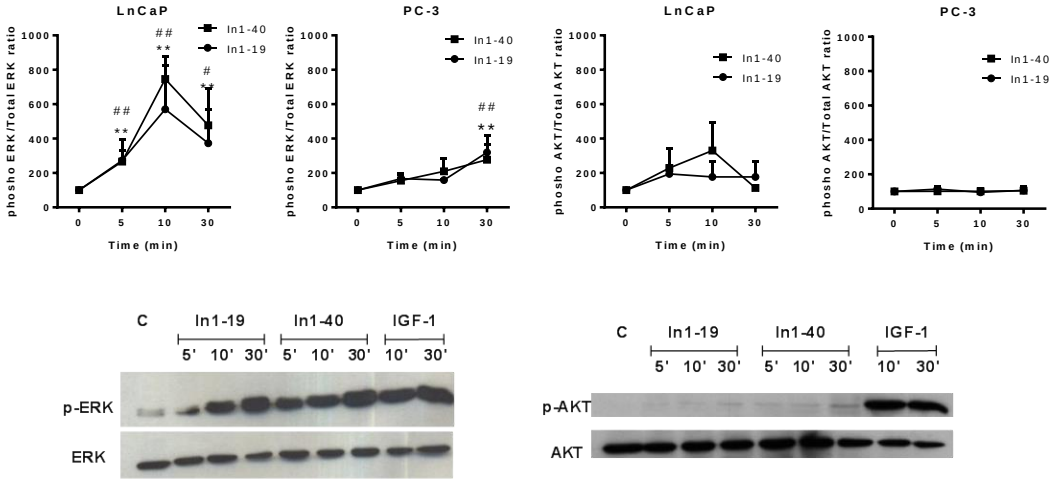


Figure 42: Phospho-ERK and phospho-AKT time-course activation after treatment with In1-ghrelin peptides (5-30 min) in LnCaP and PC-3 cell lines. Protein levels of phospho-ERK and phospho-AKT were adjusted by total ERK and AKT, respectively. Data represent mean ± SEM. Asterisks (**p < 0.01) indicate differences between In1-19 and vehicle-treated controls, and dashes (#< 0.05; ##p < 0.01) indicate differences between In1-40 and vehicle control treatment. Representative blots in LnCaP cell line are shown.

5.2.3.7. In1-ghrelin, but not ghrelin overexpression enhanced malignant features (cell proliferation and migration) of PCa cells

In order to examine the putative association between In1-ghrelin overexpression and malignant features in PCa cells, we selected one androgen-dependent (VCaP) and one independent (PC-3) cell line. First, we confirmed by qPCR the ghrelin or In1-ghrelin stable transfection in these cells (**Figure 43A**). Interestingly, we found that In1-ghrelin, but not ghrelin overexpression increased cell proliferation in VCaP and PC-3 cell lines at 48h (**Figure 43B**). Moreover, In1-ghrelin, but not ghrelin overexpression increased the migration of PC-3 cells after 12h by wound-healing assay (**Figure 43C**). Strikingly, the

overexpression of both ghrelin and In1-ghrelin variants increased the basal phosphorylation state of ERK, with no changes in Akt phosphorylation (**Figure 43D**).

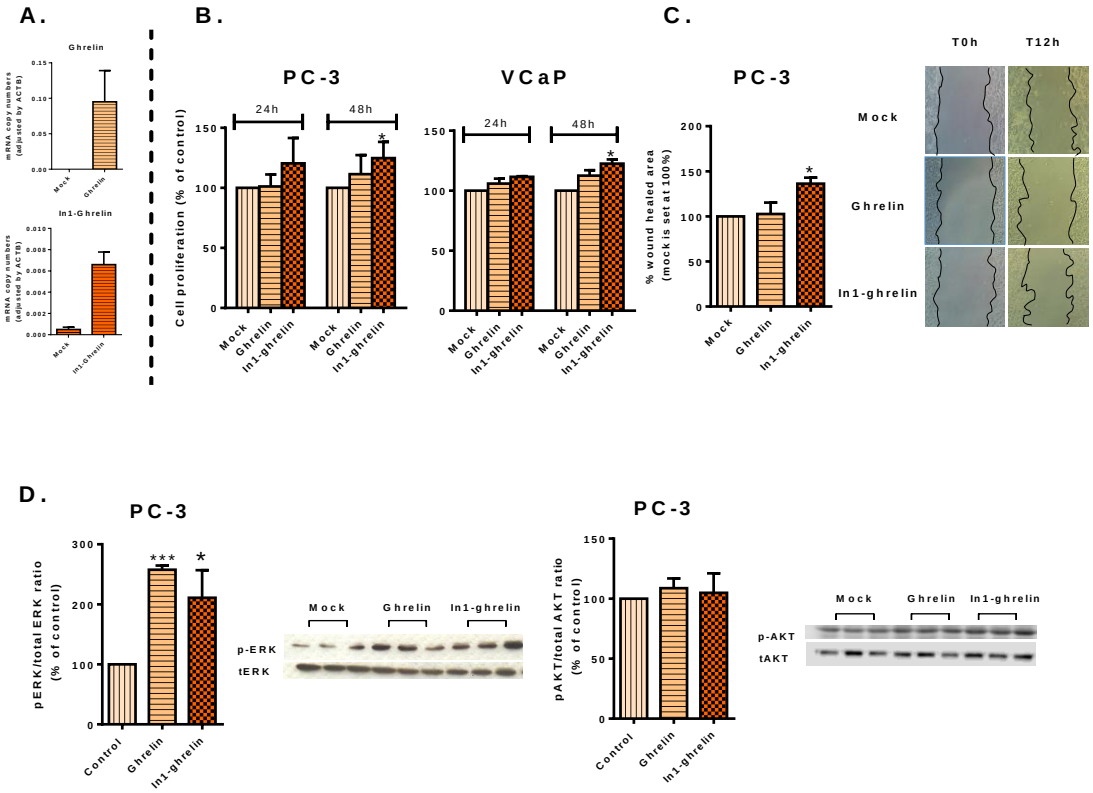


Figure 43: Effect of ghrelin and In1-ghrelin overexpression on PCa-cells pathophysiological processes. A. Validation of ghrelin and In1-ghrelin overexpression in PC-3-cells by qPCR technique; **B.** Cell proliferation of empty (mock), ghrelin and In1-ghrelin vectors stably transfected PC-3 and VCaP cell lines for 24-48h (n≥3); **C.** Cell migration of mock, ghrelin and In1-ghrelin stably-transfected PC-3 cell line after 12h by wound-healing assay (n≥3). Representative images showing the migration capacity of PC-3 cells transfected with mock, ghrelin and In1-ghelin are also indicated. **D.** phospho-ERK and phospho-AKT basal activation in PC-3 stably transfected cells (n≥3). Blots are representative of one cell passage with three technical replicates Data represent mean ± SEM (n≥3). Asterisks (*p<0.05; ***p<0.001) indicate values that significantly differ from the mock control cells.

5.2.3.8. In1-ghrelin overexpression enhanced the growth rate of PC-3-induced xenografted tumors

To further interrogate the pathophysiological effect of In1-ghrelin overexpression in PCa, an *in vivo* preclinical model was generated by subcutaneously inoculating ghrelin and In1-ghrelin stably-transfected or mock-control-transfected PC-3 cells in the flanks of immunodeficient mice and monitored the tumor growth for 12 weeks (**Figure 44**). Consistent with *in vitro* data, subcutaneous tumors induced by In1-ghrelin overexpression were larger than those induced with mock or ghrelin-transfected cells (p=0.0001 in both cases; **Figure 44A**). Moreover, histological-analysis revealed that tumors with stably-transfected ghrelin and In1-ghrelin cells showed a higher necrosis-grade (**Figure 44B**), without changes in percentage of mitotic cells (**Figure 44C**), compared to mock-induced tumors. Interestingly, tumors with stably-transfected ghrelin cells presented higher grade of inflammation than mock or In1-ghrelin derived tumors (**Figure 44D)**.

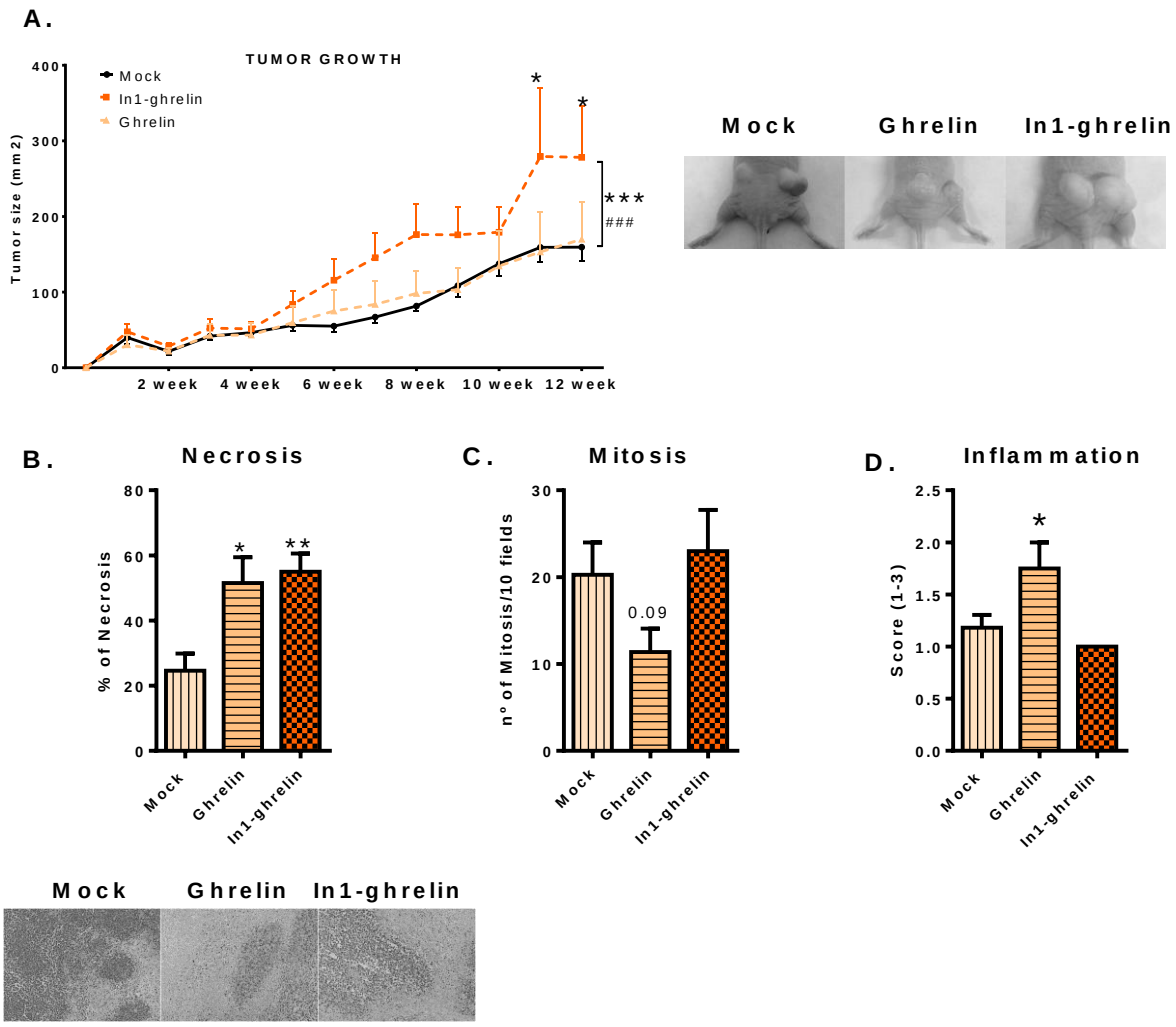


Figure 44: In1-ghrelin, but not ghrelin overexpression enhances tumor growth in NUDE mice. A. Growth rate of subcutaneously inoculated mock, ghrelin and In1-ghrelin-transfected PC-3-derived tumors

in nude mice (n=5) followed up to 12 weeks after inoculation. Statistical significance was evaluated by two-way ANOVA (***p<0.001 indicate differences between In1-ghrelin and mock, while #p<0.001 indicates differences between In1-ghrelin and ghrelin stably transfected cells). **B**. Percentage of necrosis in xenografted PC-3-derived tumors. Representative images of hematoxylin–eosin (H/E) staining are shown below. **C**. Number of mitosis/10 fields in xenografted PC-3-derived tumors. **D.** Grade of inflammation in xenografted PC-3-derived tumors measured as score 1-3. Data represent mean ± SEM (n≥3). Asterisks (**p<0.01; *p<0.05) indicate values that significantly differ from the mock control cells.

5.2.3.9. In1-ghrelin overexpression evoked a profound dysregulation of key genes involved in PCa development and progression.

In order to discover the molecular changes induced by In1-ghrelin overexpression in PCa, we performed a qPCR array comprising 84 key genes involved in PCa development and progression (For details of this array see **Table 11** at the end of the Results section). Specifically, we found 18 genes whose expression was altered more than 1.5-fold in In1-ghrelin stably-transfected PC-3-cells (13 upregulated genes: CAV1, CAV2, CDKN2A, DDX11, DLC1, FASN, GCA, IGFBP5, LOXL1, RASSF, SOX4, TFPI2 and USP5; and 5 downregulated genes: APC, GNRH1, RARB, SFRP1 and SHBG; **Figure-45A**), as well as 17 genes whose expression was altered in native-ghrelin stably-transfected PC-3-cells (7 upregulated: CASP3, CAV1, CAV2, CCND2, GCA, IL6 and VEGFA; and, 10 downregulated: CCNA1, CCND1, CDKN2A, DLC1, LOXL1, NRIP1, SFRP1, SOX4, TIMP2 and ZNF185; **Figure 45A**).

Next, we used qPCR analysis using cDNA from different passages of native-ghrelin and In1-ghrelin stably-transfected PC-3-cells and different sets of primers (for details of the specific primers see **Table 12** at the end of the Results section) to validate the results of the array. Thus, we observed that CAV1, CAV2, CDKN2A, IGFBP5 and LOXL1 were upregulated while APC, NRIP1 and SFRP1 were downregulated in In1-ghrelin stably-transfected PC-3-cells (**Figure 45B**). Similarly, we confirmed that IL6 was upregulated while CDKN2A, IGFBP5, LOXL1, NRIP1, SFRP1, SOX4 and ZNF185 were downregulated in native-ghrelin stably-transfected PC-3 (**Figure 45B**). Additionally, we confirmed some of these changes at the protein level [i.e. up-regulation of IGFBP5 and LOXL1 and downregulation of NRIP1 and SFRP1 in In1-ghrelin, and upregulation of IL6 and downregulation of CDKN2A, IGFBP5, LOXL1, NRIP1, SFRP1 and ZNF185 in native-ghrelin stably-transfected PC-3-cells (**Figure 45C**)]. Remarkably, some of the changes observed in the qPCR-array, real-time qPCR, and/or western-blot analyses, such as those observed for CAV1, LOXL1, IL-6 and SFRP1 were also further

validated in the xenografted-tumors (**Figure-45D**). Interestingly, as mentioned before, we also found a higher inflammatory cell-infiltration in the native-ghrelin, but not In1-ghrelin, stably-transfected PC-3-tumors (**Figure 44D**) which, together with the increase in IL-6 expression (**Figure 45B-D**), suggest a role of native-ghrelin in tumor inflammation.

Interestingly, some of these changes in mRNA and protein expression were similar in the In1-ghrelin and native-ghrelin stably-transfected PC-3-cells (e.g. SFRP1 and NRIP1 downregulation; **Figure 45B-D**); but, most noteworthy, some of the observed changes were regulated oppositely in both PCa cell-models (i.e. downregulation in native-ghrelin and up-regulation in In1-ghrelin stably-transfected PC-3-cells of LOXL1 and IGFBP5; **Figure 45B-D**). Therefore, these findings are reminiscent of the similar vs. disparate effects observed previously with native-ghrelin and In1-ghrelin in PCa-cells, respectively.

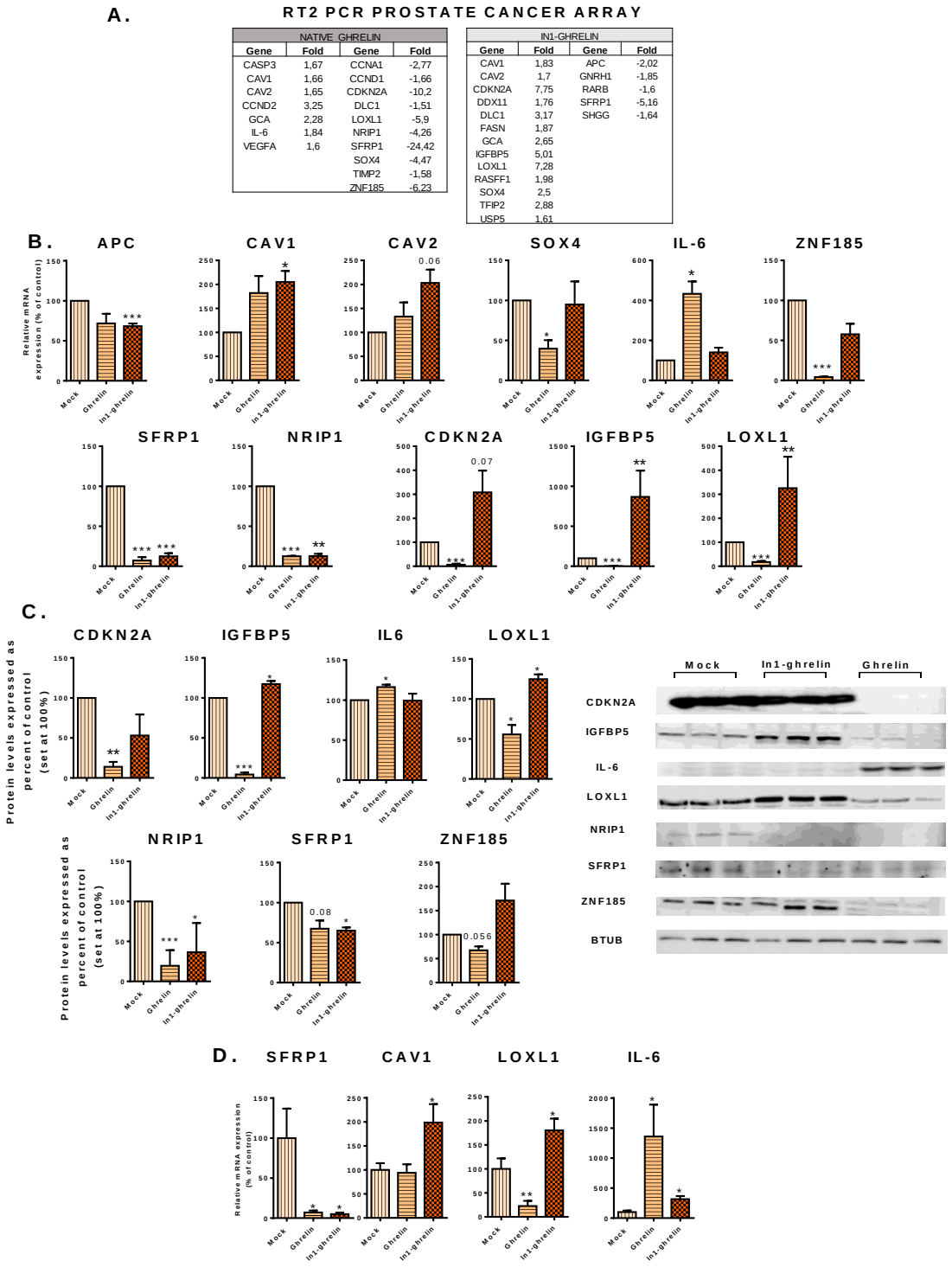


Figure 45: Gene and protein expression effects of ghrelin and In1-ghrelin overexpression in PC-3-cells and derived xenografted tumors. A. Results from the RT^2 Prostate-Cancer PCR-Array, which evaluates the expression of 84 genes involved in PCa development and progression performed in ghrelin and In1-ghrelin-stably transfected PC-3-cells compared with control-mock PC-3-cells. The tables indicate those genes whose expression change ≥ 1.5 folds; **B.** Validation by qPCR of genes dysregulated in the RT^2 Prostate-Cancer PCR Array using different cell preparations (n≥3) and new sets of primers. **C.** Validation by Western blot of the changes observed in the previous analysis. **D.** CAV1, LOXL1, SFRP1 and IL-6 mRNA expression levels in mock, ghrelin and In1-ghrelin transfected PC-3-

derived xenografted tumors. Results were normalized with ACTB. All preparations were repeated at least three times (n≥3). Values represent mean (±SEM). Asterisks (*p<0.05; **p<0.01; ***p<0.001) indicate values that significantly differ from the mock control.

It is also worth noting that In1-ghrelin stably-transfected PC-3-cells showed an overall increase in the expression of proangiogenic-factors (i.e. ANG1/ANG2/HIF1) as compared to mock- and native-ghrelin stably-transfected PC-3 cells (**Figure 46A**); being these differences only statistically significant for ANG1). Similar results were observed on the In1-ghrelin stably-transfected PC-3 derived xenografted-tumors (**Figure 46B**).

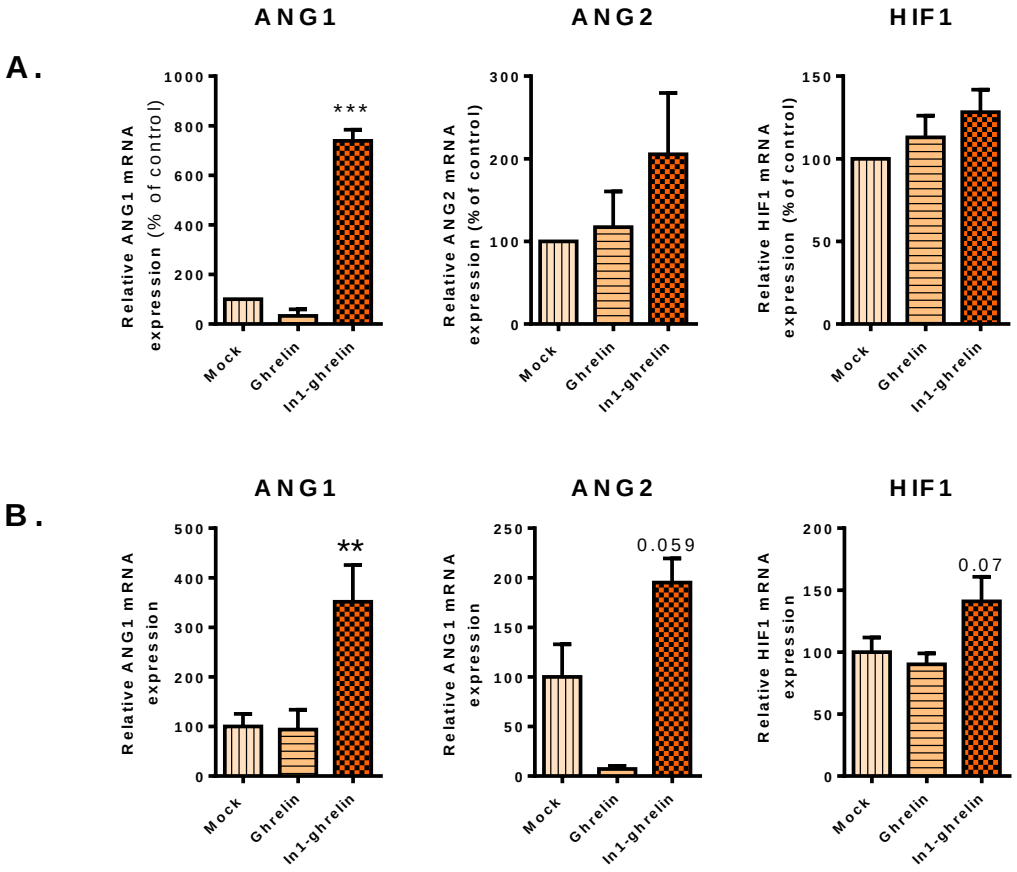


Figure 46: A. Expression of angiogenic factors in In1-ghrelin-stably transfected PC-3-cells and ghrelin-stably transfected PC-3 cells compared with control-mock PC-3-cells; **B.** Expression of angiogenic factors in xenografted tumors of stably transfected-PC-3 cells. Results were normalized with ACTB. All preparations were repeated at least three times (n≥3). Values represent mean (±SEM). Asterisks (*p<0.05; **p<0.01) indicate values that significantly differ from the mock control.

5.2.3.10. In1-ghrelin silencing decreased cell proliferation and PSA secretion

Downregulation of In1-ghrelin expression using two specific In1-ghrelin siRNAs (validation of both siRNAs is shown in **Figure-47A**), decreased cell-proliferation in PC-

3 and LnCaP cell lines at 24h and/or 48h [**Figure-47B**; siRNA-2 tended to decrease cell proliferation at 48h (p=0.06) but this difference did not reach statistical significance]. Moreover, In1-ghrelin silencing significantly decreased PSA secretion in LnCaP cell line using both siRNAs (**Figure-47C**).

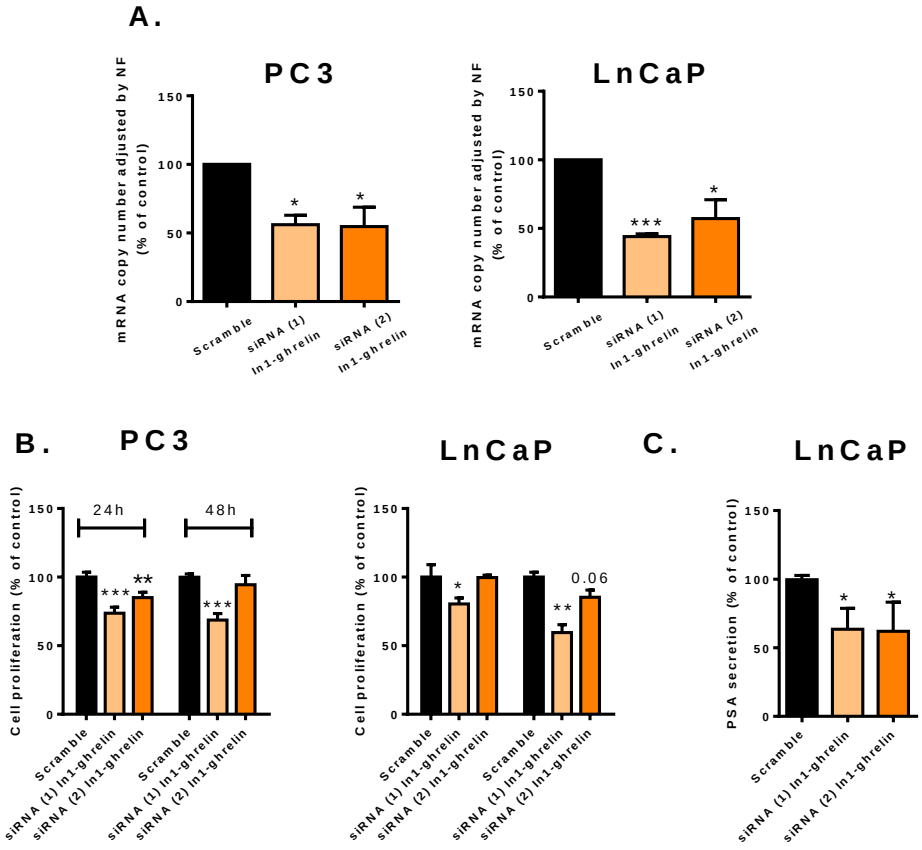


Figure 47: Effect of In1-ghrelin silencing on cell proliferation and PSA secretion. A. Validation by qPCR of In1-ghrelin silencing in PC-3 and LnCaP cells; expression levels were adjusted by a normalization factor (NF) calculated from ACTH and GAPDH expression levels. **B.** Proliferation rates of In1-ghrelin-silenced PC-3 and LnCaP cells compared with control scramble-transfected cells; **C.** PSA secretion of In1-ghrelin-silenced LNCaP cells compared with control scramble-transfected cells. All experiments were repeated at least three times (n≥3). Data represent mean ± SEM. Asterisks (*p<0.05; **p<0.01; ***p<0.001) indicate values that significantly differ from the scramble-control.

RESULTS: APPENDIX

Table 11. Prostate cancer finder RT2 Profiler PCR array data. In blue: gene expression increased ≥1,5**;** in red: gene expression decreased ≥1.5**;** in orange: gene expression increased 2 or more folds; in green: gene expression decreased 2 or more folds.

		2^(-Avg.(Delta(Ct))				Fold change		
Gene symbol	*Official full name*	Mock	In1-ghrelin	Ghrelin	sst5TMD4	In1-ghrelin	Ghrelin	sst5TMD4
ACACA	*Acetyl-CoA carboxylase alpha*	0.0220	0.0179	0.0196	0.0228	-1.23	-1.13	1.04
AKT1	*V-akt murine thymoma viral oncogene homolog 1*	0.0260	0.0324	0.0250	0.0323	1.24	-1.04	1.24
APC	*Adenomatous polyposis coli*	0.0011	0.0005	0.0009	0.0005	-2.02	-1.22	-2.19
AR	*Androgen receptor*	0.0000	0.0002	0.0001	0.0001	#	#	#
ARNTL	*Aryl hydrocarbon receptor nuclear translocator-like*	0.0040	0.0028	0.0054	0.0045	-1.45	1.34	1.13
BCL2	*B-cell CLL/lymphoma 2*	0.0012	0.0014	0.0012	0.0015	1.13	1	1.25
CAMKK1	*Calcium/calmodulin-dependent protein kinase kinase 1, alpha*	0.0009	0.0006	0.0007	0.0012	-1.48	-1.17	1.34
CAMSAP1	*Calmodulin regulated spectrin-associated protein 1*	0.0153	0.0148	0.0140	0.0144	-1.03	-1.09	-1.06
CASP3	*Caspase 3, apoptosis-related cysteine peptidase*	0.0191	0.0177	0.0318	0.0329	-1.08	1.67	1.73
CAV1	*Caveolin 1, caveolae protein, 22kDa*	0.0397	0.0728	0.0659	0.0706	1.83	1.66	1.78
CAV2	*Caveolin 2*	0.0058	0.0099	0.0096	0.0121	1.7	1.65	2.07
CCNA1	*Cyclin A1*	0.0001	0.0002	0.0001	0.0001	1.72	-2.77	1
CCND1	*Cyclin D1*	0.0273	0.0252	0.0165	0.0204	-1.08	-1.66	-1.34
CCND2	*Cyclin D2*	0.0004	0.0003	0.0013	0.0006	-1.33	3.25	1.61
CDH1	*Cadherin 1, type 1, E-cadherin (epithelial)*	0.0000	0.0002	0.0001	0.0001	#	#	#
CDKN2A	*Cyclin-dependent kinase inhibitor 2A*	0.0024	0.0186	0.0002	0.0008	7.75	-10.2	-2.91
CLN3	*Ceroid-lipofuscinosis, neuronal 3*	0.0083	0.0089	0.0073	0.0077	1.07	-1.13	-1.07
CREB1	*CAMP responsive element binding protein 1*	0.0096	0.0127	0.0134	0.0127	1.32	1.39	1.33
DAXX	*Death-domain associated protein*	0.0131	0.0186	0.0148	0.0185	1.42	1.13	1.41
DDX11	*DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 11*	0.0012	0.0022	0.0014	0.0019	1.76	1.12	1.54

DKK3	*Dickkopf homolog 3*	0.0000	0.0002	0.0001	0.0001	#	#	#
DLC1	*Deleted in liver cancer 1*	0.0001	0.0002	0.0001	0.0001	#	#	#
ECT2	*Epithelial cell transforming sequence 2 oncogene*	0.0269	0.0404	0.0346	0.0406	1.5	1.28	1.51
EDNRB	*Endothelin receptor type B*	0.0000	0.0002	0.0001	0.0001	#	#	#
EGFR	*Epidermal growth factor receptor*	0.0236	0.0198	0.0239	0.0253	-1.19	1.01	1.07
EGR3	*Early growth response 3*	0.0052	0.0049	0.0066	0.0040	-1.05	1.27	-1.29
ERG	*V-ets erythroblastosis virus E26 oncogene homolog (avian)*	0.0000	0.0002	0.0001	0.0001	#	#	#
ETV1	*Ets variant 1*	0.0131	0.0093	0.0103	0.0106	-1.41	-1.27	-1.23
FASN	*Fatty acid synthase*	0.0293	0.0529	0.0228	0.0426	1.81	-1.28	1.45
FOXO1	*Forkhead box O1*	0.0004	0.0003	0.0003	0.0002	-1.5	-1.48	-1.75
GCA	*Grancalcin*	0.0010	0.0026	0.0023	0.0025	2.65	2.28	2.57
GNRH1	*Gonadotropin-releasing hormone 1*	0.0013	0.0007	0.0013	0.0007	-1.85	1.04	-1.79
GPX3	*Glutathione peroxidase 3*	0.0000	0.0002	0.0001	0.0001	#	#	#
GSTP1	*Glutathione S-transferase pi 1*	0.7505	0.7320	0.8100	0.6625	-1.03	1.08	-1.13
HAL	*Histidine ammonia-lyase*	0.0000	0.0002	0.0001	0.0001	#	#	#
HMGCR	*3-hydroxy-3-methylglutaryl-CoA reductase*	0.0309	0.0278	0.0231	0.0266	-1.11	-1.34	-1.17
IGF1	*Insulin-like growth factor 1*	0.0000	0.0002	0.0001	0.0001	#	#	#
IGFBP5	*Insulin-like growth factor binding protein 5*	0.0001	0.0003	0.0001	0.0001	5.01	-1.1	-2.14
IL6	*Interleukin 6*	0.0005	0.0004	0.0008	0.0003	-1.19	1.84	-1.39
KLHL13	*Kelch-like 13*	0.0008	0.0007	0.0004	0.0004	-1.11	-1.84	-1.88
KLK3	*Kallikrein-related peptidase 3*	0.0000	0.0002	0.0001	0.0001	#	#	#
LGALS4	*Lectin, galactoside-binding, soluble, 4*	0.0001	0.0002	0.0002	0.0001	2.12	1.39	-1.19
LOXL1	*Lysyl oxidase-like 1*	0.0003	0.0022	0.0001	0.0001	7.28	-5.9	-2.93
MAPK1	*Mitogen-activated protein kinase 1*	0.0392	0.0396	0.0392	0.0486	1.01	-1	1.24
MAX	*MYC associated factor X*	0.0196	0.0195	0.0183	0.0188	-1	-1.07	1.04
MGMT	*O-6-methylguanine-DNA methyltransferase*	0.0146	0.0161	0.0147	0.0168	1.1	1.01	1.15

MKI67	*Antigen identified by monoclonal antibody Ki-67*	0.0619	0.0629	0.0546	0.0510	1.02	-1.13	-1.21
MSX1	*Msh homeobox 1*	0.0019	0.0025	0.0027	0.0024	1.31	1.4	1.25
MTO1	*Mitochondrial translation optimization 1 homolog*	0.0121	0.0110	0.0154	0.0135	-1.1	1.27	1.12
NDRG3	*NDRG family member 3*	0.0155	0.0138	0.0124	0.0137	-1.12	-1.25	-1.13
NFKB1	*Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1*	0.0077	0.0071	0.0078	0.0080	-1.08	1.01	1.04
NKX3-1	*NK3 homeobox 1*	0.0018	0.0017	0.0024	0.0018	-1.05	1.29	-1.02
NRIP1	*Nuclear receptor interacting protein 1*	0.0039	0.0031	0.0009	0.0005	-1.26	-4.26	-7.21
PDLIM4	*PDZ and LIM domain 4*	0.0062	0.0092	0.0073	0.0102	1.5	1.18	1.66
PDPK1	*3-phosphoinositide dependent protein kinase-1*	0.0154	0.0160	0.0159	0.0118	1.04	1.04	-1.3
PES1	*Pescadillo homolog 1*	0.0164	0.0187	0.0165	0.0197	1.14	1.01	1.21
PPP2R1B	*Protein phosphatase 2, regulatory subunit A, beta*	0.0145	0.0153	0.0143	0.0157	1.05	-1.01	1.08
PRKAB1	*Protein kinase, AMP-activated, beta 1 non-catalytic subunit*	0.0051	0.0048	0.0052	0.0066	-1.07	1.01	1.28
PTEN	*Phosphatase and tensin homolog*	0.0196	0.0115	0.0200	0.0160	-1.7	1.02	-1.22
PTGS1	*Prostaglandin-endoperoxide synthase 1*	0.0000	0.0002	0.0001	0.0001	#	#	#
PTGS2	*Prostaglandin-endoperoxide synthase 2*	0.0000	0.0002	0.0001	0.0001	#	#	#
RARB	*Retinoic acid receptor, beta*	0.0005	0.0003	0.0004	0.0002	-1.6	-1.35	-2.79
RASSF1	*Ras association (RalGDS/AF-6) domain family member 1*	0.0006	0.0011	0.0008	0.0013	1.98	1.43	2.2
RBM39	*RNA binding motif protein 39*	0.0610	0.0497	0.0574	0.0645	-1.23	-1.06	1.06
SCAF11	*SR-related CTD-associated factor 11*	0.0227	0.0182	0.0184	0.0175	-1.24	-1.23	-1.29
SEP7	*Septin 7*	0.1027	0.0842	0.0900	0.1013	-1.22	-1.14	-1.01
SFRP1	*Secreted frizzled-related protein 1*	0.0013	0.0002	0.0001	0.0001	-5.16	-24.42	-9.78
SHBG	*Sex hormone-binding globulin*	0.0004	0.0002	0.0003	0.0003	-1.64	-1.18	-1.53

SLC5A8	*Solute carrier family 5 (iodide transporter), member 8*	0.0000	0.0002	0.0001	0.0001	#	#	#
SOCS3	*Suppressor of cytokine signaling 3*	0.0006	0.0005	0.0006	0.0010	-1.11	1.08	1.79
SOX4	*SRY (sex determining region Y)-box 4*	0.0002	0.0006	0.0001	0.0001	2.5	-4.47	-1.84
SREBF1	*Sterol regulatory element binding transcription factor 1*	0.0056	0.0080	0.0040	0.0078	1.43	-1.41	1.4
STK11	*Serine/threonine kinase 11*	0.0026	0.0019	0.0032	0.0039	-1.4	1.2	1.47
SUPT7L	*Suppressor of Ty 7 (S. cerevisiae)-like*	0.0258	0.0194	0.0258	0.0201	-1.33	1	1.28
TFPI2	*Tissue factor pathway inhibitor 2*	0.0029	0.0083	0.0021	0.0021	2.88	-1.39	-1.37
TGFB1I1	*Transforming growth factor beta 1 induced transcript 1*	0.0012	0.0012	0.0012	0.0020	-1.05	1	1.67
TIMP2	*TIMP metallopeptidase inhibitor 2*	0.1255	0.0947	0.0794	0.0789	-1.33	-1.58	-1.59
TIMP3	*TIMP metallopeptidase inhibitor 3*	0.0001	0.0002	0.0001	0.0001	#	#	#
TMPRSS2	*Transmembrane protease, serine 2*	0.0000	0.0002	0.0001	0.0001	#	#	#
TNFRSF10D	*Tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain*	0.0075	0.0067	0.0071	0.0088	-1.12	-1.06	1.18
TP53	*Tumor protein p53*	0.0894	0.0890	0.0999	0.1212	-1	1.12	1.36
USP5	*Ubiquitin specific peptidase 5 (isopeptidase T)*	0.0099	0.0158	0.0099	0.0139	1.61	1.01	1.4
VEGFA	*Vascular endothelial growth factor A*	0.0273	0.0259	0.0438	0.0366	-1.05	1.6	1.34
ZNF185	*Zinc finger protein 185 (LIM domain)*	0.0009	0.0013	0.0001	0.0005	1.41	-6.23	-1.78

Table 12. Primers Sequences, product sizes obtained and GeneBank accession numbers of the primers designed and validated in our laboratory.

Template	GenBank Accession	Sense	Antisense	Product length (bp)
ACTB	NM_001101	ACTCTTCCAGCCTTCCTTCCT	CAGTGATCTCCTTCTGCATCCT	176
ANG1	NM_001146.3	GACAGATGTTGAGACCCAGGTA	TCTCTAGCTTGTAGGTGGATAATGAA	89
ANG2	NM_001147.2	GGATGGAGACAACGACAAATG	GGACCACATGCATCAAACC	78
APC	NM_000038	GAACCAAGGTGGAAATGGTG	AAAGCTGGATGAGGAGAGGAA	153
AR	NM_000044.3	GCAGGAAGCAGTATCCGAAG	GTTGTCAGAAATGGTCGAAGTG	112
CAV1	NM_001753	CGACCCTAAACACCTCAACG	CAGCAAGCGGTAAAACCAGT	148
CAV2	NM_001233	ACGACTCCTACAGCCACCAC	CAGCTTGAGATGCGAGTTGA	107
CDKN2A	NM_000077	ACCAGAGGCAGTAACCATGC	ACCTTCGGTGACTGATGATCTAA	121
DAXX	NM_001350	AAGCCTCCTTGGATTCTGGT	CTGCTGCTGCTTCTTCCTCT	237
GAPDH	NM_002046.5	AATCCCATCACCATCTTCCA	AATCCCATCACCATCTTCCA	122
GCA	NM_012198	ACAGCCAGTGCCAGAAACA	TCCACTTCACCATCCTGTCC	138
GHRL	NM_016362.3	CACCAGAGAGTCCAGCAGAGA	CCGGACTTCCAGTTCATC	215
GHSR-1a	NM_198407.2	TGAAAATGCTGGCTGTAGTGG	AGGACAAAGGACACGAGGTTG	168
GHSR-1b	NM_004122.2	GGACCAGAACCACAAGCAAA	AGAGAGAAGGGAGAAGGCACA	107
HIF1A	NM_001530.3	TTAGATTTTGGCAGCAACGAC	GGGTGAGGGGAGCATTACA	87
IGFBP5	NM_000599	TGTGACCGCAAAGGATTCTAC	AAAGTCCCCGTCAACGTACTC	129
IN1-GHRL	GU942497.1	TCTGGGCTTCAGTCTTCTCC	GCTTGGCTGGTGGCTTCTT	132
IL6	NM_000600	GGCAGAAAACAACCTGAACCT	CTCAAACTCCAAAAGACCAGTGA	115
KLK3	NM_001030047	GTGCTTGTGGCCTCTCGT	CAGCAAGATCACGCTTTTGT	108
LOXL1	NM_005576	CATTACCACAGCATGGACGA	GCCCTGGGTATGAGAGGTG	159
MBOAT4	NM_001100916.1	TTGCTCTTTTTCCCTGCTCTC	ACTGCCACGTTTAGGCATTCT	161
NRIP1	NM_003489	TCACAGGTCACAGCCAAAGA	GGGCGAGAAGCATTATTTCC	110
PCA3	NR_015342.2	CAGAGGGGAGATTTGTGTGG	TGTCATCTTGCTGTTTCTAGTGATG	172
RARB	NM_000965	TGCCTGGACATCCTGATTCT	GCATTGTGCATCTGAGTTCG	107
SFRP1	NM_003012	CATGCAGTTCTTCGGCTTCT	GTTGTCACAGGGAGGACACAC	145
SST1	NM_001049	CACATTTCTCATGGGCTTCCT	ACAAACACCATCACCACCATC	165
SST2	NM_001050	GGCATGTTTGACTTTGTGGTG	GTCTCATTCAGCCGGGATTT	185
SST5	NM_001053.3	CTGGTGTTTGCGGGATGTT	GAAGCTCTGGCGGAAGTTGT	183
sst5TMD4	DQ448304	TACCTGCAACCGTCTGCC	AGCCTGGGCCTTTCTCCT	98
TGF-β	NM_000660.5	CACGTGGAGCTGTACCAGAAA	CAACTCCGGTGACATCAAAAG	112
VEGFA	NM_001171623.1	TTAAACGAACGTACTTGCAGATG	GAGAGATCTGGTTCCCGAAA	93
ZNF185	NM_007150	CTGGCTACAAGATGACCACTGA	CCTCTGACCTCCGTTTCTGTT	144

DISCUSSION

6. DISCUSSION

6.1. The truncated somatostatin receptor, sst5TMD4, is overexpressed in prostate cancer, where it increases malignant features by altering key signaling pathways and tumor suppressors/oncogenes

Substantial progress in PCa medical treatment has been possible in recent years, owing to the emergence of novel therapies [246]. However, the available strategies are still clearly limited, emphasizing the critical need to identify new molecular biomarkers for PCa, to better diagnose, predict their prognosis and tumor behavior, and to provide tools to develop novel therapeutic strategies. SST/ssts are involved in body homeostasis regulation through inhibition of multiple cellular process, such as hormone secretion and cell proliferation, migration, and invasion [169]. In PCa, ssts are expressed and are capable of mediating such functions [189]; however, some initial, but limited, studies using SSAs reported no benefits in overall survival in PCa patients [205, 247], and the mechanistic reasons of this clinical failure are still unknown. Given that aberrant alternative splicing is one emerging cancer hallmark [132, 248], and that our group has demonstrated the presence and relevant pathological function of the spliced sst5TMD4 variant in other cancer types [183, 185, 187, 188, 249], we hypothesized that sst5TMD4 could be present and play a role in the development and progression of PCa or in the response to SSAs in PCa.

Our results demonstrated, for the first time, that **sst5TMD4**, but not sst2 or sst5 (the two main pharmacological targets of SSA), **is overexpressed (mRNA- and protein-level) in PCa samples** compared with non-tumoral adjacent regions (N-TAR) or normal prostates (NPs) tissues, and, importantly, that sst5TMD4 is strikingly **elevated in patients with higher Gleason-Score, and in those with metastasis**. Indeed, ROC curve analysis revealed that only sst5TMD4 expression (but not sst2 or sst5) could discriminate between patients that developed metastasis compared to those that did not develop them. These results suggest that **sst5TMD4 might represent a new biomarker of metastatic**

risk and exert a functional role in PCa pathology. These observations compare favorably with previous reports indicating that sst5TMD4 expression is consistently increased in several endocrine-related tumors compared with control-tissues, including neuroendocrine tumors (NETs) [188], breast [185] and pituitary [183] tumors. Indeed, sst5TMD4 association with poor prognosis markers and/or aggressiveness is a common observation in other tumors since sst5TMD4-expression was strongly correlated with lymph-node metastasis in breast cancers [185], and was also higher in invasive pituitary adenomas [183] and in lymph-node metastases of NETs compared with their corresponding primary tumors [188]. Accordingly, our present results further demonstrate that sst5TMD4 is the sst variant predominantly expressed in PCa-tissues (compared with sst2 and sst5), both in terms of number of PCa samples and expression levels (i.e. 2.2-fold higher). Thus, it is not unreasonable to suggest that **sst5TMD4 overexpression might be a common cellular/molecular signature across various endocrine-related tumors**.

Genomic approaches implemented herein demonstrated the **existence of two SST5 gene SNPs strongly associated to PCa-risk and sst5TMD4 expression**. Specifically, rs197055 AA and rs12599155 TT SNPs were only observed in PCa-samples (but not in NP-samples), wherein they correlated with higher sst5TMD4/sst5 ratio, suggesting a putative relationship of these SNPs with PCa pathogenesis. Additionally, we demonstrate, for the first time, that sst5TMD4-stably-transfected PC-3 cells and transiently-transfected LnCaP cells were not responsive to SSA-treatment (octreotide/pasireotide), while PC-3 and LnCaP cells without sst5TMD4 showed a clear change of $[Ca^{2+}]_i$ in response to these SSAs. This finding, together with the positive association found between expression levels of sst2/sst5/sst5TMD4 in PCa-tissues, might suggest a potential functional association between sst2/sst5/sst5TMD4 in PCa, and that the **high sst5TMD4-expression in PCa-cells could** help, in part, to **explain the inefficiency of SSA-therapy in the scarce and limited PCa clinical trials implemented hitherto**. Indeed, the downregulation of tumor suppressor pathways (CDKN2A, SFRP1/NRIP1/APC) elicited by sst5TMD4 overexpression (as will be discussed below) is coherent with the inhibitory role of sst5TMD4 on the negative signals normally initiated by SSAs through somatostatin receptors [250, 251]. Obviously, further work will be required to evaluate if the presence of sst5TMD4 might interfere with the effect of SSA

and/or other drugs currently used for the treatment of PCa (i.e. abiraterone or enzalutamide).

Based on these previous results, we further explored the functional role of sst5TMD4 in PCa-cell models. **sst5TMD4 overexpression evoked increased proliferation and migration in PCa-cells, whereas, in contrast, sst5TMD4 silencing decreased proliferation and migration in PC3-cells**. This demonstrates that this splice variant is functionally active in PCa-cells and that its presence is directly associated with aggressiveness features. In support of this notion, **sst5TMD4 overexpression promoted tumor growth in an *in vivo* preclinical-model**, wherein it increased tumor necrosis, likely due to higher tumor-volume. These results support and extend previous data showing that sst5TMD4 also influences aggressiveness features in other cancer cell models [i.e. breast (MCF7-cells); pancreas (BON1-cells) [185, 188]], suggesting that sst5TMD4 overexpression might be a common signature across different cancer types, which is directly associated to their aggressiveness features.

To investigate the signaling-pathways and mediators involved on sst5TMD4 actions in PCa cells, we used PC-3 cells as a suitable model. This demonstrated that **sst5TMD4 likely exerts its oncogenic functions through modulation of several pathways**, including an increased activation (basal-phosphorylation) of MAPK-signaling (ERK/JNK) pathways, which have been shown to cooperate in other tumoral pathologies to promote malignancy [252]. Additionally, this is consistent with previous reports showing that sst5TMD4 increases basal pERK/ERK-ratio in MCF7-cells [185]. Moreover, sst5TMD4 increased IL-6 expression, a positive regulator of ERK-activation [253] (**Figure 48**), while decreasing APC and SFRP1 expression, which are negative regulators of MAPK cascade [254, 255]. These results have special relevance because 25% of PCa cases have actionable lesions in MAPK pathway [256], and the biological consequences of MAPK substrate phosphorylation include increased proliferation, migration, differentiation, survival, angiogenesis, motility, and invasiveness [257].

sst5TMD4 overexpression also decreased the expression of some additional tumor suppressor candidates, including ZNF185, LOXL1 and NRIP1/RIP140 [109, 258, 259]. The decrease in NRIP1 expression is especially relevant because this transcriptional cofactor for nuclear receptors (i.e. androgen/estrogen/progesterone-receptors [109]) is a

negative regulator of androgen receptor expression in PCa [109], and, therefore, loss of NRIP1 expression induced by sst5TMD4 in PCa cells could increase the activity of androgen receptor in hormone-naïve but also in castration-resistant PCa, promoting the progression of the disease and reducing the response to androgen-deprivation therapies or AR-antagonists (i.e. enzalutamide). Moreover, NRIP1 is associated to WNT-pathway regulation, by increasing the APC-protein expression in colorectal cancer [260]. Indeed, sst5TMD4 overexpression decreased NRIP1 and APC and also SFRP1 (another candidate tumor-suppressor from WNT-pathway) expression, and was associated to the increased activation of TCF/LEF (a key WNT-pathway transcription factor), which altogether indicates that sst5TMD4 might affect the function of the WNT-pathway in PCa (**Figure 48**). Accordingly, sst5TMD4 enhanced the activation of c-MYC, a proto-oncogene associated to MEK/ERK [261] and WNT [262] pathways that control cell-cycle progression, cell-survival, and tumorigenesis, which is commonly amplified as an oncogene in PCa [80]. Finally, sst5TMD4 overexpression also enhanced the activation of RB pathway and decreased expression of CDKN2A (**Figure 48**), which is involved in the control of the RB pathway through the inhibition of cdk4/6, a kinase that ultimately controls the phosphorylation status of RB protein, a known tumor suppressor commonly lost in cancer [263]. Interestingly, sst5TMD4 overexpression has been associated to increased activity of c-Myc and JNK, two of the main negative regulators of CDKN2A [264], which could provide a plausible explanation for the drastic downregulation of CDKN2A gene in sst5TMD4-overexpressing cells. Consequently, when CDKN2A is down-regulated, cdk4/6 phosphorylates RB (an inactivating phosphorylation) leading to the activation of E2F-regulated genes involved in cell-cycle progression [263]. Therefore, since CDK4/6 inhibitors are being tested in PCa patients [76], this result should be considered to appropriately evaluate the efficiency of these drugs in patients with reduced CDKN2A and altered RB activity.

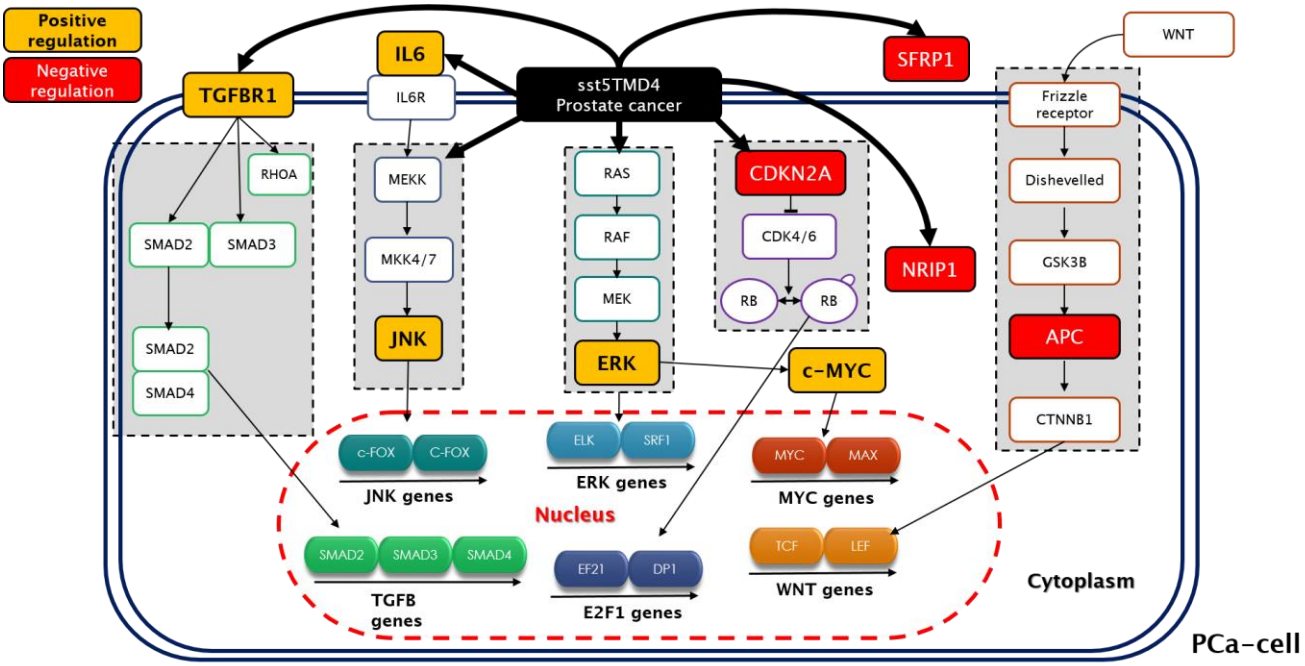


Figure 48: Working model summarizing the intracellular effects triggered by sst5TMD4 in human PCa cells. The data presented herein using several in vivo and in vitro PCa models suggest that the high expression of sst5TMD4 observed in human PCa, especially in metastatic PCa samples, might enhance the activation of MAPK (ERK/JNK), Myc/Max, WNT, TGF-β and retinoblastoma (RB) signaling pathways, which are known to be critical regulators of cancer biology. Specifically, sst5TMD4 might act at different regulatory levels by increasing the phosphorylation of proteins (i.e. MAPK cascade elements), modifying gene expression levels (i.e. APC, SFRP1, CDKN2A, NRIP1) and protein levels (i.e. MYC, SFRP1, CDKN2A, NRIP1) and/or by increasing the activation of transcription factors from several signaling pathways (i.e. TCF/LEF, E2F-DP1). Our data also indicate that the final impact of sst5TMD4 overexpression (and the regulation of the signaling pathways associated to sst5TMD4) in human PCa cells resulted in a significant promotion of aggressiveness features (i.e. increased proliferation, and migration) and also in the hampering of the normal response to SSAs in PCa cells.

Altogether, our findings demonstrate that the spliced variant sst5TMD4 is overexpressed in PCa, especially in metastatic-PCa, wherein it promotes *in vitro* and *in vivo* aggressiveness features by dysregulating key PCa signaling pathways and hampering the normal response to SSAs. Hence, these results suggest that **sst5TMD4 could represent a valuable tool to identify novel biomarkers to refine diagnosis and predict prognosis, and a promising player in the future development of therapeutic approaches involving SSAs** (alone or in combination with other therapeutic drugs) in PCa patients.

6.2. Ghrelin O-acyltransferase (GOAT) enzyme is overexpressed in prostate cancer wherein it is associated with the metabolic status: potential value as a non-invasive biomarker.

The introduction of PSA as a routine clinical marker led to an improved early detection of PCa and, thus, to an increase in PCa survival [50]. However, PSA-based screening for PCa has generated considerable debate because its diagnostic performance is relatively poor, considering that elevated PSA levels may be driven by benign conditions (i.e. prostatic hyperplasia or prostatitis) [85]. Prostate cancer antigen 3 (PCA3) has been proposed as the most prominent biomarker emerging as a non-PSA-based diagnostic test for PCa; however, unfortunately, PCA3 has also serious limitations (i.e. reduced sensitivity compared to PSA) [157]. Therefore, identification of novel, alternative, biomarkers for clinical diagnosis of PCa (ideally non-invasive) is urgently needed to improve and/or complement PSA/PCA3 screening.

Current evidence supports the necessity of implementing more personalized diagnosis and management strategies for cancer patients, in that inter-individual differences such as alterations in metabolic environment (i.e. diabetes and/or metabolic-syndrome) could be key to identify and deploy novel diagnostic biomarkers [85]. In this scenario, the enzyme GOAT might become of particular relevance in PCa [265, 266], for its critical role in whole body metabolism [267], and its wide bodily expression, which is finely regulated by changes in the metabolic environment in human tissues [215, 268], and is clearly dysregulated in different tumoral tissue types (i.e. breast cancer) compared with normal tissues [215-217]. In line with this, we demonstrate herein, for the first time, that **GOAT expression is markedly altered in PCa samples (at the mRNA and protein level) compared with control prostate samples (normal/benign tissues)**. Indeed, ROC curve analysis indicated that GOAT mRNA could predict PCa with a high sensitivity and specificity, suggesting that **GOAT levels might represent a novel, promising PCa biomarker**. GOAT protein expression showed a trend to be higher in PCa tissues with high Gleason-score and with metastasis, suggesting that **GOAT might be associated to malignant features in PCa**. These results partially differ from those reported in a previous study where GOAT mRNA expression was not altered in PCa

tissues vs. control samples [233]. However, it is possible that such differences may be related to the type of control sample used (i.e. healthy prostate-samples in our study vs. adjacent non-tumoral region of adenocarcinomas of prostate used in the previous study, which could be derived, at least in some cases, from prostates harboring high-grade multifocal PCa), or to the different primer set used to amplify GOAT in the qPCR, and/or to the precise characteristic of the primers (length, product-size, GC-content, etc.), which were not fully reported in that study. Nevertheless, our current observations are also partially consistent with this report [233] because both studies observed that **GOAT enzyme is significantly overexpressed in several PCa cell lines vs. normal prostate cells, supporting the idea that GOAT expression might be up-regulated in different stages of the PCa disease**. Moreover, our data compares favorably with previous reports indicating that GOAT is overexpressed in other tumoral tissues compared with normal tissues, including human neuroendocrine [217], pituitary [216], and breast [215] tumors, suggesting that **GOAT overexpression could be a common cellular signature across various endocrine-related tumors**.

Interestingly, our data revealed that **GOAT is secreted by PCa-cells**, and that its expression might be directly associated with its release, since GOAT secretion was significantly higher in the culture medium of 22Rv1 vs. PC3-cells, in a manner consistent with their expression levels (22Rv1>PC3). Indeed, IHC-analysis of GOAT in PCa samples revealed that **GOAT staining at the cytoplasmic level was granular, supporting the idea that this enzyme could be associated to a secretory mechanism in PCa cells**. Importantly, **GOAT expression was found to be specifically regulated by endocrine/metabolic factors (i.e. In1-ghrelin, but not ghrelin) in PCa-cells and in normal prostate cell cultures, suggesting that GOAT production could be functionally linked to In1-ghrelin at the prostate level**. In fact, this close relationship between In1-ghrelin and GOAT expression has been reported previously in other cancers [215, 217]. Hence, since In1-ghrelin is usually overexpressed in several endocrine-related tumors [215-217], it is conceivable that **In1-ghrelin, rather than ghrelin, could be the main substrate of GOAT** in these cancers, including **PCa**. Furthermore, other key metabolic factors (i.e. insulin/IGF-1) also modulated GOAT expression levels in PCa-cells, which might be associated with the positive correlation found in this study between GOAT expression levels and body weight and BMI in PCa patients, a similar association to that recently reported for circulating GOAT levels in normal weight, anorectic and

obese subjects [245]. Interestingly, **GOAT expression was higher in PCa-patients with dyslipidemia**, and a greater proportion of PCa-patients with dyslipidemia presented high GOAT mRNA levels compared with patients with low GOAT expression levels. Altogether, our results suggest the existence of a clear association between changes in GOAT expression levels and alterations in metabolic conditions in PCa-patients (i.e. body-weight, BMI and dyslipidemia), an idea that is in accordance with previous studies demonstrating the link between GOAT levels, lipid metabolism and whole body energy balance [269-271].

The results obtained hitherto prompted us to question whether GOAT levels could represent a suitable non-invasive biomarker for PCa under normal and/or altered metabolic conditions (e.g. diabetes or dyslipidemia). In fact, **plasmatic and urine GOAT levels were higher in PCa patients compared with healthy control individuals**. Moreover, **in PCa patients, plasma GOAT levels correlated positively with circulating total PSA (and negatively with free PSA) as well as with other cancer markers (ca_15-3/cyfra_21-1)** used to monitor other tumors (breast and lung cancer, respectively), and which have been proposed as possible PCa biomarkers in conjunction with PSA [272, 273]. Therefore, although we could not unequivocally establish a direct association between prostate and plasma/urine GOAT levels, due to the use of different patient's cohorts, the results presented herein indicate that **prostate GOAT tissue-expression (mRNA and protein) and plasma/urine GOAT protein levels are consistently and concomitantly elevated in PCa samples**. Indeed, ROC-curve analysis demonstrated that GOAT mRNA levels could predict PCa with a high sensitivity/specificity, and, most importantly, that plasma and urine GOAT levels also showed a good sensitivity (81.1% and 75%, respectively) and specificity (67.8% and 61%, respectively) for PCa detection. Altogether, our data indicate that **GOAT levels could represent a promising novel diagnostic biomarker (invasive and non-invasive) for PCa**.

There is increasing evidence that the alterations in the endocrine/metabolic milieu should be considered to appropriately stratify the patients, as well as to evaluate the diagnostic, prognostic and therapeutic potential of novel biomarkers in tumoral pathologies, including PCa. In this context, there is evidence that circulating GOAT levels depend on the metabolic environment (e.g. low levels in anorexic and high levels in

obese-patients [245]), and, most importantly, as shown here, that **GOAT expression is directly associated with dysregulations in metabolic-conditions (i.e. BMI/dyslipidemia) in PCa**, an observation that is in line with reports showing that altered endocrine/metabolic status (i.e. diabetes/metabolic-syndrome/BMI, etc.) is associated with the development/progression of several cancer-types [274], including PCa [275]. Accordingly, we hypothesized that plasmatic GOAT levels in PCa patients could also be linked to the metabolic status. Interestingly, we did find that **GOAT plasma levels were higher in individuals with alterations in glucose-metabolism (i.e. diabetes and/or dyslipidemia), and were positively correlated with the percentage of glycated-hemoglobin**, a gold standard for monitoring glycemic control [276], **in both control and PCa individuals**. Actually, irrespective of the presence of PCa, high plasma GOAT levels were associated with a higher proportion of diabetic and/or dyslipidemic patients, suggesting that dysregulated glucose-metabolism (diabetes) might be a confounding factor masking the potential of GOAT to discriminate between control and PCa patients when the whole population (non-diabetic plus diabetic patients) is considered. Therefore, we then sought to determine the potential of GOAT as a PCa biomarker after stratification of the patient cohort based on the presence of diabetes. Remarkably, **when only non-diabetic individuals are considered, we discovered that plasmatic GOAT levels are able to precisely discriminate the presence of PCa with high sensitivity and specificity**. These data clearly support the notion that **alterations in endocrine/metabolic milieu is a critical factor to ensure an adequate patient stratification and to assess the diagnostic, prognostic and therapeutic potential of novel biomarkers in cancer, including PCa**, as it is strongly suggested by the data presented herein for **plasma GOAT levels as a novel, suitable complementary PCa diagnostic test, especially for non-diabetic individuals**.

A final point of interest relates to the presence of GOAT in urine. After the discovery that prostate cells are able to secrete GOAT, being this secretion significantly higher in PCa cell-lines, we posited whether there is a potential utility of GOAT urine levels for the diagnosis of PCa, in that proteins in urine are mainly derived from epithelial cells in the urinary-tract [277, 278]. Indeed, changes in urine protein can reflect a dysfunction of cells within the urinary-tract better than those in blood samples, which are deeply influenced by homeostatic mechanisms [277, 278]. In support to this notion, our results demonstrate that **urine GOAT levels were also higher in PCa-patients**

compared with controls, showing a high specificity and sensitivity to diagnose PCa-patients. Thus, the data presented herein indicate that **GOAT might represent a novel non-invasive PCa biomarker in urine (wherein it is not severely influenced by the metabolic status)**.

In sum, to the best of our knowledge, the present data provides the first systematic analysis of the presence of the enzyme GOAT in different types of human prostate samples (invasive and non-invasive) obtained from patients with and without PCa, and in relation to the clinical and metabolic parameters of the patients (i.e. presence of metastasis, diabetes, dyslipidemia, etc.). Altogether, our results show, that **GOAT can be directly secreted by PCa cells and is consistently overexpressed in samples from PCa patients (tissue, plasma and urine), where its expression is drastically conditioned by the metabolic status**. Since GOAT expression showed a high sensitivity/specificity in PCa-detection, especially in non-diabetic patients, we propose that **GOAT might be considered as a novel non-invasive PCa biomarker alone or in combination with other biomarkers to provide a better PCa diagnosis**.

In fact, it should be mentioned that the results included in this section has been already patented for this purpose [Title of the patent: "*Ghrelin-O-acyl transferase (GOAT) and its uses*"; registry number: PCT/ES2016/070844] and also published in the journal "*Cancer Letters*" [Title: "*Ghrelin O-acyltransferase (GOAT) enzyme is overexpressed in prostate cancer wherein is associated with the metabolic status: potential value as non-invasive biomarker*"; Cancer Lett. 2016; 383(1):125-134]. A copy of this manuscript is included at the end of the Thesis document.

6.3. In1-ghrelin splicing variant is overexpressed in prostate cancer, wherein it increases aggressiveness features through regulation of key tumor suppressors/oncogenes

Previous studies have shown that ghrelin is expressed in both normal prostate (NP) and PCa tissues and cell lines with an increased staining of ghrelin peptide in malignant prostate epithelium compared with normal glandular tissue [225]. Interestingly, additional reports have shown that other ghrelin gene derived splicing variants are also present in PCa, where they could be involved in PCa malignancy [225, 235, 236]. Herein, we have expanded those results by demonstrating, for the first time, that **In1-ghrelin mRNA levels are overexpressed in a battery of PCa biopsies from patients diagnosed with high-risk PCa, compared to NP samples**, which is consistent with previous results indicating that **In1-ghrelin overexpression is a common hallmark shared by other endocrine-related tumors,** such as breast-cancer, pituitary tumors and NETs [215-217]. Nevertheless, although the expression of ghrelin was higher than that of In1-ghrelin in NPs, in our study cohort, ghrelin mRNA levels were not significantly elevated in PCa samples. Indeed, ROC-curve analysis revealed that only In1-ghrelin expression (but not ghrelin) could discriminate between patients with or without PCa, suggesting that In1-ghrelin merits further study as a potential novel biomarker in PCa. Interestingly, **In1-ghrelin, but not ghrelin levels positively correlated with GOAT-expression in PCa**, an association that has also been previously found in other endocrine-related tumors [215-217], and suggests that **In1-ghrelin may be the main ghrelin-gene variant functionally linked to GOAT in those tumors, which also reinforces the idea that an autocrine/paracrine-circuit involving these two components of the ghrelin-system may operate in PCa**. Indeed, this association might be particularly relevant in PCa pathology, as we have also demonstrated herein that GOAT is overexpressed in PCa patients, that it can be secreted by PCa cells, and its levels exhibit high specificity/sensitivity to predict PCa presence compared with other PCa biomarkers [279].

Remarkably, analysis carried out in an additional cohort of PCa patients and controls demonstrated that **acylated In1-ghrelin peptide could be detected in plasma, and that these levels, but not those of ghrelin are significantly higher in PCa patients than in**

healthy controls, which again would **suggest the possible utility of In1-ghrelin levels as a novel biomarker for PCa patients by using non-invasive (liquid) biopsies**. Moreover, consistent with the results found in PCa-biopsies, **In1-ghrelin mRNA expression was higher in PCa cell lines compared with the NP cell line**. Interestingly, we observed that **In1-ghrelin, but not ghrelin expression seemed to be higher in castration-resistant than in androgen-dependent cell lines, which would suggest that In1-ghrelin might play a role in the late PCa stages and/or in the progression of the disease**.

The fact that In1-ghrelin, and also ghrelin, were expressed at substantial levels in NP- and PCa-cells suggested that they could be exerting a functional role in the normal and pathological physiology of the prostate. Therefore, NP cell-cultures were used to test whether In1-ghrelin peptides and ghrelin can modulate signaling and functional parameters (such as PSA production and cell viability) in these cells. **In1-ghrelin peptides and ghrelin treatment evoked a signaling response in terms of changes in $[Ca^{2+}]_i$,** a key second messenger that has been previously linked to ghrelin system signaling [216, 280], and is functionally associated to PCa pathophysiology. Specifically, it has been shown that ion channel remodeling alters the nature of PCa cells Ca^{2+} influx, switching from an apoptotic to a pro-proliferative stage [281]. Nevertheless, it should be noted that the proportion of responsive cells and the magnitude of the stimulatory increase were consistently higher after In1-ghrelin than after ghrelin treatment in all experiments performed on primary NP cells. This observation suggests that In1-ghrelin variant could be playing a more pronounced function than ghrelin in prostate cells. Nonetheless, our results demonstrate a direct action of ghrelin-system splicing variants on NP-cells, which would imply the existence of receptors for In1-ghrelin, but also for ghrelin, in these cells. However, such putative receptors, likely, ought to be different from the classical GHSR1a, as we found that GHSR1a expression is virtually absent in the human prostate samples and PCa cell-lines analyzed herein. The existence of uncharacterized receptor(s) that mediate some of the biological effects of ghrelin, and likely of In1-ghrelin, has also been previously postulated in human prostate neoplasms and related cell-lines [222]. Interestingly, **In1-ghrelin, but not ghrelin overexpression clearly increased cell-viability, and In1-ghrelin treatment also increased PSA secretion and expression in NP cell-cultures**, which is consistent with data previously reported indicating that In1-ghrelin treatment enhances hormone-secretion in other cell-types (i.e. serotonin in NET

cells [217], GH in somatotropinoma cells, and ACTH in corticotropinoma cells [216]), and may suggest a **relevant role of In1-ghrelin in the malignization of NP cells**.

Ghrelin gene-derived variants, especially the In1-ghrelin variant, exerted a relevant effect on the pathophysiology of PCa cells. In particular, **In1-ghrelin treatment evoked an increase in proliferation on most of the PCa cell-lines tested, being its effect especially marked in castration-resistant cell-lines**. Similarly, **In1-ghrelin peptides significantly enhanced the migration-capacity of PC-3 cells**. The ability of In1-ghrelin peptides to increase cell-proliferation has been previously reported in human pituitary tumors (and in mouse AtT-20 cell-line) [216]. However, ghrelin administration only increased cell proliferation in LNCaP and PC-3 cell lines, which is in agreement with previous studies [225]. **These actions of In1-ghrelin peptides may be mediated, at least in part, through the activation of ERK signaling pathways**, as previously reported in other tumoral pathologies [216, 225]. Consistent with these data, **stable overexpression of In1-ghrelin, but not ghrelin enhanced cell proliferation, migration capacity and basal phosphorylation levels of PCa cells** (PC-3/VCaP cells). In line with this, In1-ghrelin overexpression has been shown to increase the cell proliferation of pituitary tumors [216], MDA-MB-231 breast cancer cell line [215] and BON-1 pancreatic cell line [217], and the migration capacity of NETs cell lines [217]. Moreover, to further explore this notion, we generated nude mice xenografted with In1-ghrelin or with ghrelin stably-transfected PC-3 cells, and found that **In1-ghrelin, but not ghrelin overexpression enhanced tumor growth in** this ***in vivo*** **preclinical model (i.e. larger tumors), wherein it increased tumor necrosis, likely due to higher tumor volume, supporting the idea that In1-ghrelin would increase the malignant features of PCa-cells**.

To gain further insight on the mechanisms underlying the actions of the ghrelin variants on PCa cells, we explored changes in the expression of a set of selected genes related to PCa. Specifically, **In1-ghrelin increased malignant features of PCa cells by altering the expression of key oncogenes, tumor-suppressor genes and genes associated to PCa pathophysiology** such as APC, CAV1, SFRP1, NRIP1, CDKN2A, IGFBP5 and LOXL1 [89, 282-286], which could help to explain the functional changes triggered by In1-ghrelin over-exposition (treatment and/or overexpression). Indeed, some changes were further confirmed at the protein-level (i.e. SFRP1, NRIP1, IGFBP5 and LOXL1) and in the In1-ghrelin stably-transfected PC-3-tumors, which showed an

increase in CAV1 and LOXL1, and a decrease in SFRP1 expression compared to mock cell-induced tumors. Intriguingly, ghrelin treatment and/or overexpression elicited the phosphorylation of ERK-pathway in PCa-cells and induced significant changes in the expression of certain oncogenes, tumor-suppressor genes and in genes associated to PCa-pathophysiology. Some of these changes were different (i.e. IL-6, SOX4 and ZNF185), common (i.e. decreased in SFRP1 and NRIP1) or opposite (i.e. LOXL1 and IGFBP5) to those observed by In1-ghrelin overexpression. However, our study demonstrates a marginal effect of ghrelin on the functional endpoints measured (*in vitro* proliferation and/or migration and *in vivo* proliferation and inflammation) compared to In1-ghrelin effects, suggesting that ghrelin could be playing a role on other endpoints not measured herein, and that **In1-ghrelin splice variant has a more relevant role than ghrelin in the aggressiveness of PCa-cells**. In line with this, the increase in LOXL1 and IGFBP5 expression observed in PCa-cells overexpressing In1-ghrelin could be pathophysiologically relevant and could be associated to the unique capacity of In1-ghrelin to enhance the malignancy features in PCa cells, since both factors have been shown to increase the aggressiveness of PCa cells [285-288]. Specifically, LOXL1 is able to enhance tumorigenesis and metastasis through active remodeling of tumor microenvironment [285, 287]. Moreover, IGFBP5 has been shown to play an important role in the castration phase of the disease, since upregulation in its expression accelerates progression to androgen-independence in PCa models [288], and enhances proliferation of PCa cells [286]. Accordingly, it is tempting to speculate that the upregulation of LOXL1 and IGFBP5 observed in PCa cells overexpressing In1-ghrelin might be associated to the increased aggressiveness features observed in PCa cells (i.e. *in vitro* and/or *in vivo* cell proliferation, migration, tumor growth and PSA secretion).

Finally, we further explored the utility of In1-ghrelin as a putative target to reduce PCa progression by analyzing the effect of In1-ghrelin silencing on PCa cell lines functional parameters. Remarkably, **In1-ghrelin silencing decreased cell proliferation of PC-3 and LNCaP cells, and PSA secretion from LNCaP cells, which, overall, suggest that In1-ghrelin could be considered as a novel target for the development of new and more specific therapies in PCa.**

Thus, when viewed together, our results indicate that **In1-ghrelin splicing variant is overexpressed in PCa, where it can regulate cell proliferation, migration,**

tumor growth and PSA secretion, through the modulation of the activation of certain signaling pathways (ERK-phosphorylation) and the expression of several oncogenes and tumor-suppressor genes, thereby suggesting a possible pathophysiological role for this splice variant in human PCa. The fact that the ghrelin system, particularly its In1-ghrelin variant, was strongly altered in PCa supports the idea that this system could contribute to PCa tumorigenesis, and **may provide novel tools to explore diagnostic/therapeutic targets in this pathology**.

In fact, it should be mentioned that the results included in this section, together with other results obtained in other endocrine-related tumoral pathologies, are part of a new patent recently presented by the research group [Title of the patent: "Non-invasive diagnostic method of cancer"; registry number: P201631606)].

CONCLUSIONS

7. CONCLUSIONS

1) The splice variant sst5TMD4 is overexpressed in prostate cancer, especially in patients with metastatic disease and high Gleason score, and promotes aggressiveness features (e.g. cell proliferation, migration) in *in vitro* and *in vivo* models, through the dysregulation of key prostate cancer signaling-pathways (e.g. ERK/JNK, MYC/MAX, WNT and RB), tumor suppressors (e.g. APC, SFRP1, CDKN2A, ZNF185) and oncogenes (e.g. CAV1, IL-6, DAXX).

2) The presence of sst5TMD4 hampers the normal response of prostate cancer cells to SSAs (octreotide and pasireotide), which might be one of the mechanism underlying the inefficiency of SSA therapy in the limited PCa clinical trials implemented hitherto.

3) Altogether, our results on sst5TMD4 suggest that it could represent a valuable tool to identify novel biomarkers to refine diagnosis and predict prognosis, and a promising player in the future development of therapeutic approaches involving SSAs in prostate cancer.

4) GOAT can be directly secreted by prostate cancer cells and is consistently and concomitantly elevated in samples from prostate cancer patients, including tumor tissue, plasma and non-invasive fluids (urine) compared with healthy-control patients. Elevated GOAT levels are associated to malignant features in prostate cancer and are severely influenced by the metabolic status (e.g. BMI, diabetes and dyslipidemia).

5) GOAT levels showed high sensitivity and specificity in prostate cancer detection, especially in non-diabetic patients. Thus, GOAT might represent a valuable novel biomarker for prostate cancer, alone or in combination with other biomarkers (e.g. PSA, PCA3), to improve prostate cancer diagnosis. Moreover, our data indicate that alterations in endocrine/metabolic milieu is a critical factor to ensure an adequate patient stratification and to assess the potential of novel biomarkers in prostate cancer.

6) The splice variant In1-ghrelin, but not ghrelin, mRNA levels are overexpressed, and positively correlated with GOAT expression, in prostate cancer, thus reinforcing the idea that an autocrine/paracrine-circuit involving these two components of the ghrelin-system may operate in prostate cancer.

7) In1-ghrelin could play a pathological role in late prostate cancer stages and/or in the progression of the disease, since its expression, but not that of ghrelin, is higher in castration-resistant than in androgen-dependent prostate cancer cell lines, and In1-ghrelin treatment or overexpression increases malignization of prostate cancer cells *in vitro* and *in vivo* (e.g. cell proliferation, migration, and/or PSA expression/secretion). These actions of In1-ghrelin are likely exerted by altering the expression of key oncogenes, tumor suppressor genes, and genes associated to prostate cancer pathophysiology (i.e. SFRP1, NRIP1, IGFBP5 and LOXL1).

8) Acylated In1-ghrelin peptide can be detected in plasma and its levels, but not those of ghrelin, are significantly higher in patients with prostate cancer than in healthy-controls, thus suggesting the possible utility of In1-ghrelin levels as a novel non-invasive diagnostic and prognosis biomarker for prostate cancer patients.

9) Taken together, our results reveal the existence of a marked, concomitant dysregulation of the expression of key components of the somatostatin and ghrelin systems, specially their splicing variants, in prostate cancer, wherein they might play a pivotal role in the regulation of pathophysiological processes associated with prostate cancer malignancy, and provide new clinical tools in the diagnosis, prognosis, and treatment of this tumoral pathology.

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Original Article

Ghrelin O-acyltransferase (GOAT) enzyme is overexpressed in prostate cancer, and its levels are associated with patient's metabolic status: Potential value as a non-invasive biomarker

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ABSTRACT

Ghrelin-O-acyltransferase (GOAT) is the key enzyme regulating ghrelin activity, and has been proposed as a potential therapeutic target for obesity/diabetes and as a biomarker in some endocrine-related cancers. However, GOAT presence and putative role in prostate-cancer (PCa) is largely unknown. Here, we demonstrate, for the first time, that GOAT is overexpressed (mRNA/protein-level) in prostatic tissues (n = 52) and plasma/urine-samples (n = 85) of PCa-patients, compared with matched controls [healthy prostate tissues (n = 12) and plasma/urine-samples from BMI-matched controls (n = 28), respectively]. Interestingly, GOAT levels in PCa-patients correlated with aggressiveness and metabolic conditions (i.e. diabetes). Actually, GOAT expression was regulated by metabolic inputs (i.e. In1-ghrelin, insulin/IGF-I) in cultured normal prostate cells and PCa-cell lines. Importantly, ROC-curve analysis unveiled a valuable diagnostic potential for GOAT to discriminate PCa at the tissue/plasma/urine-level with high sensitivity/specificity, particularly in non-diabetic individuals. Moreover, we discovered that GOAT is secreted by PCa-cells, and that its levels are higher in urine samples from a stimulated post-massage vs. pre-massage prostate-test. In conclusion, plasmatic GOAT levels exhibit high specificity/sensitivity to predict PCa-presence compared with other PCa-biomarkers, especially in non-diabetic individuals, suggesting that GOAT holds potential as a novel non-invasive PCa-biomarker.

Introduction

Prostate cancer (PCa) is the second most common cancer type in men [1]. PCa detection and management is mainly based in the use of circulating biomarkers. Specifically, prostate specific antigen (PSA) is the main biomarker used in clinical diagnosis of PCa; however, its use is controversial because, in many cases, leads to over-diagnosis and unnecessary treatment of indolent cancers [2,3]. Consequently, identification of novel, more specific and sensitive diagnostic/prognostic PCa biomarkers seem to be necessary [2].

There is now compelling evidence that severe metabolic alterations (i.e. metabolic-syndrome/diabetes/obesity) are tightly linked to increased risk of cancer [4–7], including PCa [8]. Actually, recent studies claim that presence of metabolic-syndrome should be

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Table 1
Demographic, clinical, anatomopathological characteristics and qPCR data from patients with high-risk PCa (needle biopsies) and normal prostate controls (Cystoprostatectomy).

	Overall	Control	PCa	p-value
Patients, n°	64	12	52	–
Age at diagnosis				
Median (IQR)	76 (68.2–81.7)	70 (61.5–81)	78 (69–81.7)	0.39
Weight				
Mean (SD)	77.5 (12.6)	76.9 (12.4)	77.7 (13)	0.87
BMI				
Mean (SD)	28.1 (4.7)	27.9 (4.5)	28.2 (4.9)	0.85
PSA level, ng/ml				
Median (IQR)	–	–	54.5 (37.2–212)	–
Dyslipidemia	17/64 (26%)	3/12 (25%)	14/52 (27%)	0.99
Diabetes	15/64 (23%)	3/12 (25%)	12/52 (23%)	0.99
Gleason score				
7	–	–	18/52 (35%)	–
>7	–	–	34/52 (65%)	–
Extraprostatic extension	–	–	17/52 (33%)	–
Perineural infiltration	–	–	27/52 (52%)	–
Metastasis	–	–	24/52 (46%)	–
Nº samples (%) in which GOAT was detected	60/64 (96%)	12/12 (100%)	48/52 (92%)	0.99

PCa = prostate cancer; n° = Number.

included in clinical assays for predicting PCa-risk, and diabetes should be one of the main parameters to be considered [9]. In line with this, several endocrine/metabolic factors known to be altered in diabetes/obesity, such as some components of the ghrelin-system, have also been associated to PCa [10,11]; however, their potential utility as biomarkers in PCa management has not been established hitherto.

The ghrelin system comprises a pleiotropic and complex network composed of several peptides, including native-ghrelin and In1-ghrelin [12], and receptors (GHSR1a/b), involved in the regulation of multiple patho-physiological processes (i.e. glucose/insulin-homeostasis, hormonal-release, cell-proliferation, etc.) [13]. Native-ghrelin and In1-ghrelin peptides share the initial 13 aminoacids (aa) sequence, which can be acylated (addition of an octanoyl-group at the Ser-3) by the ghrelin-O-acyl-transferase (GOAT) enzyme [12,14,15]. This acylation is required for native-ghrelin to bind and activate GHSR1a, and to exert thereby many of its functions [14,15]. Interestingly, several studies demonstrate that GOAT could play a key role in obesity/diabetes [16,17] [18–21], and may be relevant in coordinating the neuroendocrine response to metabolic stress, in that its expression can be finely regulated by the energy status (i.e. expression elevated in fasting and inhibited in obesity [22]), and by key metabolic factors (i.e. leptin increases [22] while insulin decreases GOAT expression [23]).

GOAT is mainly expressed in stomach and pancreas, but also in a number of additional tissues, including the prostate [24,25], and its levels are directly correlated with body mass index (BMI) in plasma [26]. Of note, our group and others have demonstrated that GOAT is overexpressed in some tumoral tissues, like breast [24], pituitary [12,27] and neuroendocrine tumors (NETs) [28], compared with normal tissues. However, very limited data is still available on GOAT in PCa [25]. Therefore, here we sought to analyze, for the first time: 1) the presence of GOAT in selected PCa-samples [invasive (biopsies; mRNA and protein levels) and non-invasive (protein levels

Table 2
Demographic and clinical characteristic of patients included in the study of plasmatic and urine levels of GOAT.

Parameter	Overall	Control (n = 28)	PCa (n = 85)	*p* value
Age, yr, mean (SD)	67.11 (9.1)	62.04 (9.6)	68.7 (8.3)	*0.0005*
Weight, kg, mean (SD)	82.31 (12.52)	84.56 (14.21)	81.5 (11.9)	0.27
BMI, mean (SD)	29.52 (4.01)	30.46 (4.4)	29.2 (3.8)	0.16
Ethnicity, (n°)	Caucasian (111), Black (2)	Caucasian (27), Black (1)	Caucasian (84), Black (1)	–
Glycated hemoglobin (%), median (IQR)	5.6 (5.3–6.1%)	5.6% (5.3–6.7%)	5.6% (5.2–6%)	0.4
Glucose, mg/dl, median (IQR)	105 (93.5–125.5)	109 (97.25–130.8)	102 (92–121.5)	0.15
Insulin, m U/l, median (IQR)	7.05 (4.8–11.3)	6.2 (3.3–10.7)	8.9 (5.5–11.4)	*0.045*
Diabetes, n° (%)	Non-diabetics (84), diabetics (34)	9/28 (32.12%)	25/85 (29.4%)	0.81
Dyslipidemia, n° (%)	Non-dislipidemic (88), dislipidemic (25)	5/28 (17.85%)	20/85 (23.95%)	0.60
Testosterone ng/ml	5.14 (3.72–5.93)	5.3 (4.1–6.7)	4.8 (3.7–5.8)	0.16
Total PSA, ng/ml, median (IQR)	5 (1.77–8.23)	0.8 (0.4–1.1)	6.3 (3.8–9.5)	*<0.0001*
Free PSA, ng/ml, median (IQR)	–	–	0,81 (0.5–1.1)	–
Invasive tumors	–	–	14/85 (16%)	
Gleason score, n° (%)	–			
6	–	–	41/85 (48%)	
7	–	–	38/85 (45%)	
8	–	–	5/85 (6%)	
9	–	–	1/85 (1%)	
cyfra_21-1 U/ml, mean (SD)	1.4 (1.1–1.9)	1.2 (1-1,80)	1,4 (1.1–2.1)	0.18
ca_15-3 U/ml, mean (SD)	17 (12–23)	12 (7–21.7)	19 (14–24)	*0.046*

PCa: prostate cancer; Yr = year; SD = standard deviation; Kg = kilograms; cm = centimeter; BMI = body mass index; n° = number; IQR = interquartile range.
Italic values represent significant difference in the parameter between PCa patients and control patients.

in plasma and urine)] compared with normal-control samples; 2) if GOAT expression/levels correlated with clinical/metabolic status (body-weight, BMI, diabetes, etc.) and with aggressiveness features (PSA levels, metastasis, etc.) of the patients; and 3) if GOAT is secreted from prostate cells, and whether its expression levels in prostate can be regulated by relevant metabolic factors (i.e. ghrelin, insulin, IGF-I) by using primary normal prostate cell cultures and/or PCa cell lines as model.

Materials and methods

Patients and samples

Three different sets of samples were included in the study. First, fresh PCa pieces (n = 52) obtained by core needle biopsies from patients diagnosed with clinical high-grade PCa (NCNN-guidelines) and samples from control patients without PCa (n = 12), who underwent cystoprostatectomy due to bladder-cancer, were collected. Experienced pathologists classified all these samples as normal/tumoral (Table 1). Second, plasma and urine samples from PCa patients (n = 85) and from healthy BMI-matched controls (n = 28) were collected (Table 2). Third, urine samples were collected before and after prostate massage in another cohort of PCa-patients (n = 14) (Table 3). In all cohorts, demographic/clinical parameters regarding tumor aggressiveness and metabolic status were collected. The study was approved by the Hospital/University Ethics-Committees and written informed consent from patients was obtained. All samples were obtained through the Andalusian-Biobank (Servicio Andaluz de Salud).

For more details of this and others materials/methods used (Reagents; Primary normal prostate cultures; GOAT IHC analysis; Normal-like prostate cell-line and prostate cancer cell-lines, Determination of GOAT expression in prostatic tissues by quantitative real-time RT-PCR (qPCR) and of plasma/urine levels by ELISA; Statistics), see Supplemental-Information [27–31].

Results

GOAT is overexpressed in PCa tissues in comparison to normal tissues wherein it is associated with metabolic-clinical parameters

qPCR analysis performed in PCa-tissues from patients diagnosed with clinical high-grade PCa revealed that GOAT was expressed in 92% (n = 48/52; Table 1) of the samples, and that its expression was markedly increased in PCa compared to non-tumor control tissues (p = 0.024; Fig. 1A). ROC analysis showed that GOAT expression significantly discriminated between PCa and controls subjects (AUC = 0.714; p = 0.021; a cut-off of 262 mRNA copies/50 ng cDNA presented 70.59% sensitivity and 66.67% specificity; Fig. 1B). The clinical/pathological features of this cohort of patients are included in Table 1. No correlation was found between GOAT mRNA levels and presence of metastasis, Gleason-score or circulating PSA levels (Supplemental Fig. S1A); in contrast, GOAT expression was directly correlated with some metabolic-clinical parameters, such as body-weight and BMI, in PCa-patients (Fig. 1C–D), but not in controls (Supplemental Fig. S1B). Moreover, GOAT expression tended to be higher in PCa patients with dyslipidemia (p = 0.084; Fig. 1E) and in fact, a significantly greater proportion of patients with high GOAT expression levels presented dyslipidemia (Fig. 1F).

Subsequently, GOAT-IHC was performed on FFPE-prostates harboring normal and tumoral regions obtained from radical prostatectomies (n = 4) of patients diagnosed with localized PCa (Gleason score 6–7), which revealed stronger GOAT staining in the tumoral vs. benign glands (Fig. 1G). Interestingly, high-magnification images showed perineural-infiltration by tumoral prostate glands with high cytoplasmic (granular) and nuclear GOAT-staining, suggesting that GOAT overexpression could be associated with a malignant event in PCa. Due to the small set of FFPE samples available, we next implemented IHC analyses in a subset of the biopsies from the cohort of clinical high-grade PCa samples described above (n = 16), which included normal-benign prostate regions. These analyses revealed that GOAT staining was very weak in normal-benign prostate samples (cytoplasmic-staining; Fig. 1H) compared with the intense staining found in PCa samples [staining located at cytoplasmic (granular-staining) and nuclear level of the cancerous prostate-glands; Fig. 1H–I]. This staining in PCa samples seems to be specific for tumoral regions since the staining of the stromal cells within the tumor microenvironment was negative for GOAT (Fig. 1I). Interestingly, GOAT staining appeared to be associated to GOAT mRNA levels and, tended to be stronger in patients with higher Gleason-score (p = 0.088; Fig. 1J) and in patients with metastasis (p = 0.079; Fig. 1K).

GOAT is expressed, secreted and regulated by metabolic factors in normal and tumoral prostate cells

GOAT mRNA expression was higher in PCa cell-lines (LnCaP/22Rv1/DU-145) compared with normal prostate cell line RWPE1 or normal prostate primary cells (Fig. 2A; n ≥ 3 from different cell preparations), supporting our previous data indicating that GOAT is overexpressed in PCa-tissues compared to normal-tissues (Fig. 1A). Moreover, PCa-cells (22Rv1 and PC3) were capable to secrete GOAT protein into the media (Fig. 2B), being this secretion higher in 22Rv1 vs. PC3-cells (1.641 vs. 1.163 ng/ml, respectively; p = 0.005; n > 3), which is in agreement with the higher GOAT mRNA levels observed in 22Rv1 vs. PC3 (Fig. 2A). Interestingly, treatment with In1-ghrelin (In1-19) [27], but not native-ghrelin significantly increased GOAT expression in 22Rv1, but not in PC3 cells (Fig. 2C-D; n = 3), and in normal prostate primary cell cultures (Fig. 2E; n = 3). Conversely, treatment with key metabolic factors such as insulin and IGF-I, or their combination, decreased GOAT expression in PCa-cells (p < 0.001; Fig. 2F; n ≥ 3).

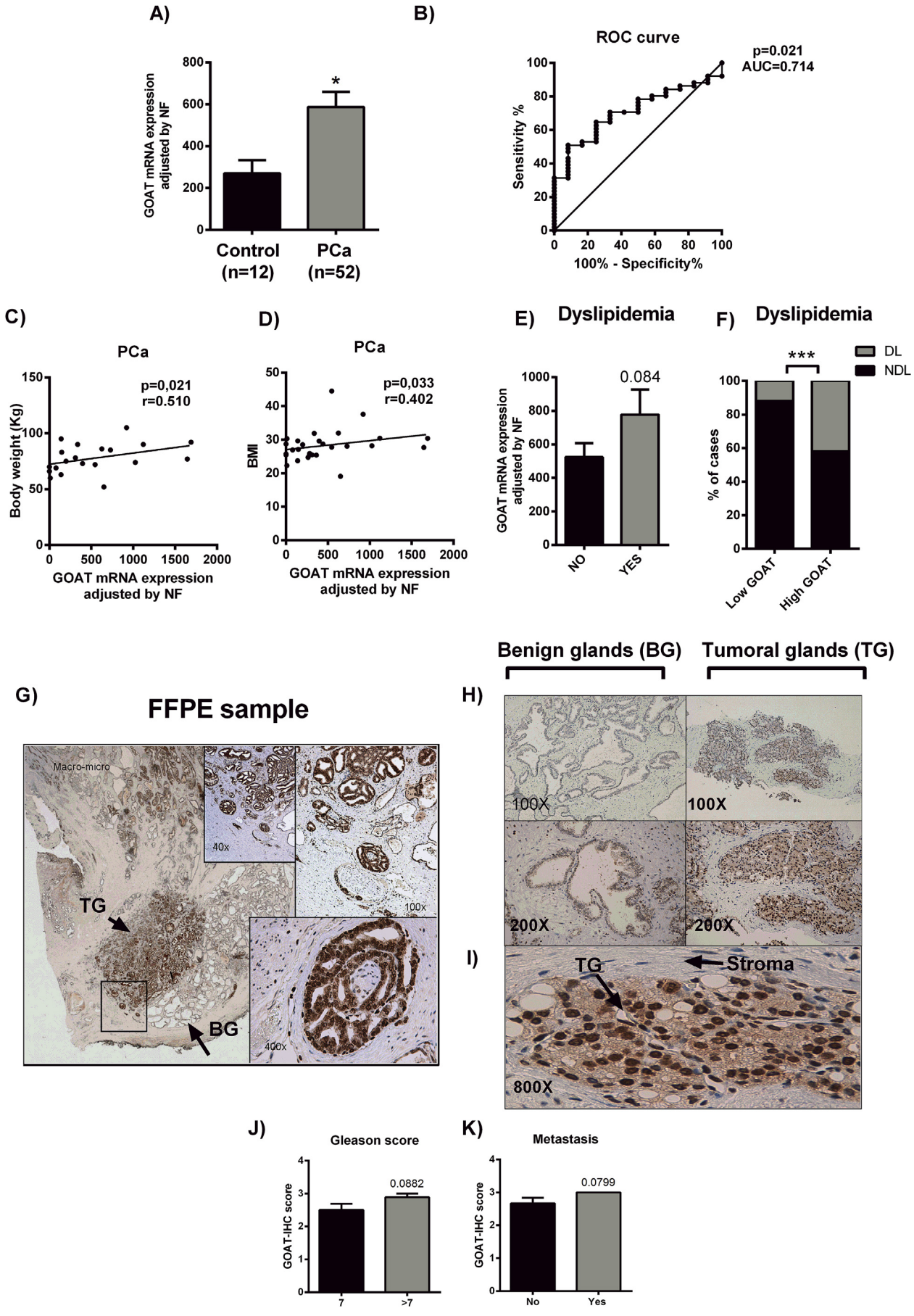
Plasma GOAT levels are elevated in patients with PCa in comparison to healthy controls: association with clinical-metabolic parameters

Plasmatic GOAT levels were elevated in PCa patients compared with controls (Fig. 3A). Clinical/pathological features of this patient cohort are summarized in Table 2. It should be noted that PCa-patients had similar body-weight and BMI but presented a slightly higher age than control-patients (~6 years; Table 2).

Table 3
Demographic and clinical characteristic of patients included in the study of urine GOAT levels after prostate massage.

Parameter	PCa
Age, yr, mean (SD)	68.1 (7.1)
Weight, kg, mean (SD)	79.2 (9.9)
BMI, mean (SD)	28.8 (3.2)
Ethnicity, (n°)	Caucasian (14)
Glycated hemoglobin, (%)	5,4 (5.2–5.8%)
Glucose, mg/dl, mean (SD)	92.7 (8.4)
Insulin, m U/l, mean (SD)	8,6 (2.6)
Diabetes, n°	0/14
Dyslipidemia, n°	0/14
Testosterone ng/ml, mean (SD)	4.6 (2.2)
Total PSA, ng/ml, median (IQR)	5.9 (4.6–9.7)
Free PSA, ng/ml, mean (SD)	0.9 (0.3)
Invasive tumors, n° (%)	2/14 (14%)
Gleason score, n° (%)	
6	3/14 (21%)
7	9/14 (64%)
8	1/14 (7%)
9	1/14 (7%)
cyfra_21-1 U/ml, mean (SD)	1.6 (0.8)
ca_15-3 U/ml, mean (SD)	16.1 (6.9)

PCa: prostate cancer; Yr = year; SD = standard deviation; Kg = kilograms; cm = centimeter; BMI = body mass index; n° = number; IQR = interquartile range.


A)
GOAT mRNA expression adjusted by NF
800
600
400
200
0
*
Control (n=12)
PCa (n=52)
B)
ROC curve
p=0.021
AUC=0.714
Sensitivity %
100
80
60
40
20
0
0 20 40 60 80 100
100% - Specificity%
C)
PCa
p=0,021
r=0.510
Body weight (Kg)
150
100
50
0
0 500 1000 1500 2000
GOAT mRNA expression adjusted by NF
D)
PCa
p=0,033
r=0.402
BMI
50
40
30
20
10
0
0 500 1000 1500 2000
GOAT mRNA expression adjusted by NF
E) Dyslipidemia
0.084
GOAT mRNA expression adjusted by NF
1000
800
600
400
200
0
NO
YES
F) Dyslipidemia
***
DL
NDL
% of cases
100
80
60
40
20
0
Low GOAT
High GOAT
G)
FFPE sample
Macro-micro
40x
100x
400x
TG
BG
H)
Benign glands (BG)
Tumoral glands (TG)
100X
100X
200X
200X
I)
TG
Stroma
800X
J)
Gleason score
0.0882
GOAT-IHC score
4
3
2
1
0
7
>7
K)
Metastasis
0.0799
GOAT-IHC score
4
3
2
1
0
No
Yes

Nevertheless, we implemented general linear models, which demonstrated that this difference was not responsible for the alterations in plasmatic GOAT levels observed. Furthermore, when plasma GOAT levels were corrected by age, higher GOAT levels were clearly evident in PCa vs. control-patients ($p = 0.002$). Moreover, ROC-analysis showed the diagnostic potential for GOAT at the plasma level ($p < 0.0001$; AUC = 0.752; Fig. 3B), wherein a value of 1.22 ng/ml of GOAT plasma levels could discriminate between PCa vs. control-patients with a specificity of 67,8% and a sensitivity of 81,1%.

Interestingly, plasmatic GOAT levels were associated with several pathophysiological factors. Specifically, GOAT levels were directly associated with total PSA levels and inversely correlated with free PSA (Fig. 3C), and also associated positively with two cancer biomarkers (ca_15-3 and cyfra_21-1; Fig. 3D). Moreover, a higher frequency of infiltrative tumors was found in patients with higher plasmatic GOAT levels (Fig. 3E).

We next analyzed the putative relationship between plasmatic levels of GOAT and several metabolic parameters (i.e. BMI, diabetes, dyslipidemia, etc.). Although we did not find correlations between GOAT plasma levels and BMI or weight in control and PCa patients (Supplemental Fig. S2A), we observed that GOAT levels of control-patients (without PCa) were significantly higher in diabetic subjects compared with non-diabetic patients (Fig. 3F), whereas no differences were found in plasmatic GOAT levels in control-patients with and without dyslipidemia (Fig. 3G). Consistent with these results, plasmatic GOAT levels in control-patients were positively correlated with the levels of glycated hemoglobin (HbA1c; Fig. 3H) and glucose (Fig. 3I), two well-known clinical markers of diabetes. Accordingly, a significantly higher proportion of patients with diabetes, but not with dyslipidemia, was found in control-patients with high plasmatic GOAT levels compared to those with low GOAT levels (Fig. 3J). In contrast, plasmatic GOAT levels were similar in diabetic vs. non-diabetic patients with PCa (Fig. 3K). A trend for elevation in plasmatic GOAT levels was found in patients with PCa that had dyslipidemia compared to those without dyslipidemia ($p = 0.06$; Fig. 3L). A positive correlation between plasmatic GOAT levels was also found with HbA1c, but not with glucose levels in PCa-patients (Fig. 3M and N, respectively). Indeed, a higher proportion of patients with diabetes as well as with dyslipidemia were found in PCa-patients having high plasmatic GOAT levels vs. PCa-patients with low GOAT levels (Fig. 3O).

Plasma GOAT levels as a non-invasive diagnostic marker of PCa in non-diabetic individuals

Based on the previous results, we next interrogated whether plasmatic GOAT levels could be a more specific PCa marker in non-diabetic patients. Plasmatic GOAT levels were higher in non-diabetic PCa-patients vs. control-patients ($p < 0.001$; Fig. 3P). Indeed, the area under curve was considerably improved when analyzing only non-diabetic PCa-patients [value of 0.854 (Fig. 3P/Q; only non-diabetic) vs. 0.752 (Fig. 3A/B; diabetic + non-diabetic)]. In addition, the specificity/sensitivity to discriminate the presence of PCa was significantly higher when only the non-diabetic individuals were considered [a cut-off value of 1.142 ng/ml presented a specificity and sensitivity of 84.2% and 86.7% (Fig. 3Q/P) vs. 67.8% and 81.1% analyzing non-diabetic + diabetic patients (Fig. 3A/B)]. Interestingly, plasmatic GOAT levels also positively correlated with total PSA (Fig. 3R), negatively with free PSA levels (Fig. 3S) and positively with the cancer biomarker ca_15-3 (Fig. 3T) and, also exhibited a similar positive tendency with HbA1c ($p = 0.080$; Fig. 3U).

Urine GOAT levels are elevated in patients with PCa in comparison to healthy-control patients: potential value as non-invasive biomarker

Urine GOAT levels were also significantly elevated in the same cohort of PCa-patients vs. control-patients (Fig. 4A; Table 2). Actually, ROC-curve analysis showed that urine GOAT levels could discriminate between PCa vs. control-patients (Fig. 4B; $p < 0.001$, AUC = 0,716; a cut-off value of 1.061 ng/ml presented a sensitivity of 75% and specificity of 61%). Remarkably, this sensitivity/specificity was lower than that found with plasmatic GOAT levels and, although the underlying reasons (likely, sampling procedures, urine processing, etc.) are still not defined, and thus further investigation is warranted. Interestingly, no correlations were found between GOAT urine levels and metabolic parameters (BMI, body weight, diabetes or dyslipidemia) in control or PCa patients (Supplemental Fig. S2B), suggesting that GOAT urine levels are not influenced by these parameters. Furthermore, urine GOAT secretion was increased after a prostate-massage in an additional cohort of PCa-patients (Table 3) compared to the same cohort of subjects before prostate-massage (Fig. 4C).

Discussion

The introduction of PSA as a routine clinical marker led to an improved early detection of PCa and, thus, to an increment in PCa survival [1]. However, PSA-based screening for PCa has generated considerable debate because its diagnostic performance is poor, considering that elevated PSA levels may be driven by benign conditions (i.e. prostatic hyperplasia or prostatitis) [2]. Prostate cancer antigen 3 (PCA3) has been proposed as the most prominent biomarker emerging as a non-PSA-based diagnostic test for PCa; however, unfortunately PCA3 has also serious limitations (i.e. reduced sensitivity than PSA) [32]. Therefore, identification of novel, alternative, biomarkers for clinical diagnosis of PCa (ideally non-invasive) is urgently needed to improve and/or complement PSA/PCA3 screening.

Current evidence supports the necessity of implementing more personalized diagnosis and management strategies for cancer patients, in that inter-individual differences such as alterations in metabolic environment (i.e. diabetes and/or metabolic-syndrome) could be key to identify and deploy novel diagnostic biomarkers [2]. In this scenario, the enzyme GOAT

Fig. 1. Analyses of the presence of GOAT in PCa tissues. **A**. GOAT mRNA expression was determined in a battery of 52 PCa samples compared to 12 normal prostates (Control). Absolute mRNA levels were determined by qPCR and adjusted by a normalization factor (NF) calculated from the expression levels of two housekeeping genes (ACTB and GAPDH); **B**. Receiver operating characteristic (ROC) curve analysis to determine the accuracy of GOAT expression as diagnostic test to discriminate between patients with PCa and controls; **C**. GOAT mRNA expression and body weight correlation in patients with PCa; **D**. GOAT mRNA expression and BMI correlation in patients with PCa; **E**. GOAT mRNA expression in patients with dyslipidemia; **F**. Frequencies of dyslipidemia observed in two groups of PCa patients with low or high GOAT expression levels (according to median GOAT levels) (DL = patients with dyslipidemia, NDL = Patients without dyslipidemia); **G**. IHC analysis of GOAT in a representative FFPE-PCa sample with benign and tumor glands at 40–400× magnification. The image shows higher intensity of GOAT staining in tumor glands vs benign glands, and intense GOAT staining in nerves infiltrated by tumor cells. **H**–**K**. GOAT IHC analysis in 16 biopsies from patients with high risk PCa; **H**. GOAT IHC staining in prostate biopsies with benign glands (BG; left-panel) or tumor glands (TG; right-panel) at 100–200× magnification; **I**. Cytoplasmic and nuclear GOAT staining in a tumoral gland at 800× magnification; **J**. Comparison of GOAT IHC score [low (1), moderate (2) or high (3) GOAT staining] with Gleason-score; **K**. Comparison of GOAT IHC score with presence of metastasis. In all cases, data represent mean ± SEM. Asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) indicate values that significantly differ from the corresponding controls.

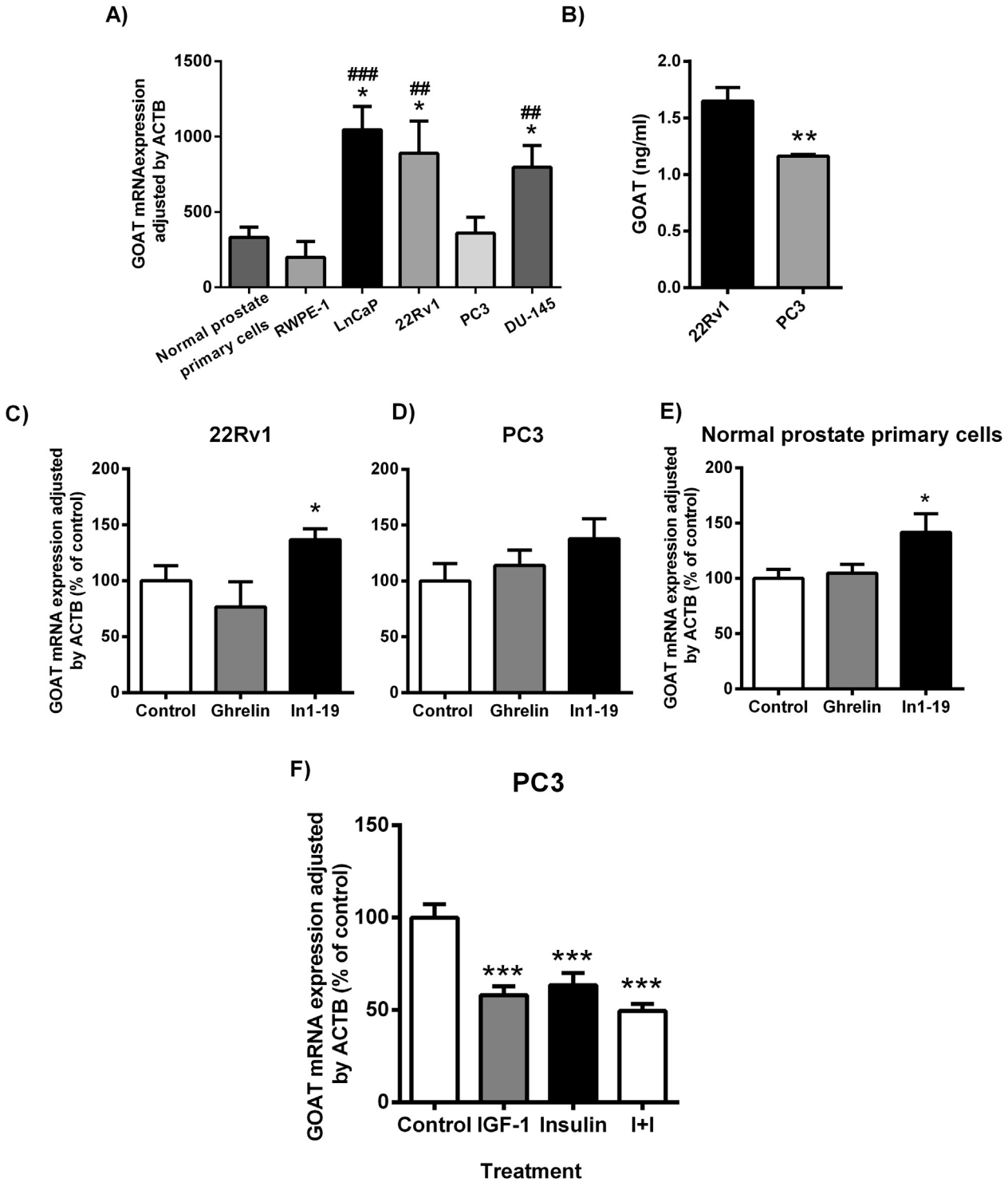


Fig. 2. Regulation of GOAT expression by metabolic factors. **A**. GOAT mRNA expression in normal prostate primary cell cultures, normal-like prostate cell line (RWPE-1) or PCa cell lines (androgen-dependent: LnCaP, 22Rv1 and VCaP or androgen-independent: PC3 and DU-145). Asterisks (*, $p < 0.05$) indicate differences in GOAT mRNA expression between RWPE-1 and PCa cell lines, while dashes (##, $p < 0.01$; ###, $p < 0.001$) indicate differences in GOAT mRNA expression between normal prostate primary cell cultures and PCa cell lines; **B**. GOAT protein secretion from 22Rv1 and PC3 cell lines (after 24 h-culture) determined by a specific ELISA kit ($n = 3$). Asterisks (**, $p < 0.01$) indicate values that significantly differ from 22Rv1; **C**. Regulation of GOAT mRNA expression after treatment (10 nM) with ghrelin or In1-ghrelin (In1-19) peptides in 22Rv1 ($n = 3$); **D**. Regulation of GOAT mRNA expression after treatment (10 nM) with ghrelin or In1-ghrelin (In1-19) peptides in PC3 ($n = 3$); **E**. Regulation of GOAT mRNA expression after treatment (10 nM) with ghrelin or In1-ghrelin (In1-19) peptides in normal prostate primary cell cultures, ($n = 3$); **F**. Regulation of GOAT mRNA expression after treatment (10 nM) with IGF-1, Insulin or both (IGF-1 + Insulin; $n \geq 3$). Absolute mRNA levels of GOAT were determined by qPCR and adjusted by the expression levels of a housekeeping gene (ACTB). In expression experiments in response to treatment, asterisks (*, $p < 0.05$; ***, $p < 0.001$) indicate values that significantly differ from controls. In all cases, data represent mean ± SEM of $n \geq 3$ independent experiments.

might become of particular relevance in PCa [14,15], for its critical role in whole body metabolism [18], and its wide bodily expression, which is finely regulated by changes in the metabolic environment in human-tissues [22,24], and is clearly dysregulated in different tumoral-tissue types (i.e. breast cancer) compared with normal-tissues [24,27,28]. In line with this, we demonstrate herein, for the first time, that GOAT expression is markedly altered in PCa-samples (mRNA/protein) compared with control prostate samples (normal/benign-tissues). Indeed, ROC curve analysis indicated that GOAT mRNA could predict PCa with a high sensitivity and specificity, suggesting that GOAT levels might represent a novel, promising PCa biomarker. GOAT protein expression showed a trend to be higher in PCa-tissues with high Gleason-score and with metastasis, suggesting that GOAT might be associated to malignant features in PCa. These results partially differ from those reported in a previous study where GOAT mRNA expression was not altered in PCa-tissues vs. control-samples [25]; however, it is possible that such differences may be related to the type of control sample used (i.e. healthy prostate-samples in our study vs. adjacent non-tumoral region of adenocarcinomas of prostate used in the previous study, which could be derived, at least in some cases, from prostates harboring high-grade multifocal PCa), or to the different primer set used to amplify GOAT in the qPCR, and/or to the precise characteristic of the primers (length/product-size/GC-content, etc.), which were not fully reported in that study. Nevertheless, our current observations are also partially consistent with this report [25] because both studies observed that GOAT-enzyme is significantly overexpressed in several PCa cell-lines vs. normal prostate cells, supporting the idea that GOAT expression might be up-regulated in different stages of the PCa-disease. Moreover, our data compares favorably with previous reports indicating that GOAT is overexpressed in other tumoral-tissues compared with normal-tissues, including human neuro-endocrine [28], pituitary [27] and breast [24] tumors, suggesting that GOAT overexpression could be a common cellular signature across various endocrine-related tumors.

Interestingly, our data shows that GOAT is secreted by PCa-cells, and that its expression might be directly associated with its release, since GOAT secretion was significantly higher in the culture medium of 22Rv1 vs. PC3-cells, in a manner consistent with their expression levels (22Rv1 > PC3). Indeed, IHC-analysis of GOAT in PCa-samples revealed that GOAT-staining at the cytoplasmic level was granular, supporting the idea that this enzyme could be associated to a secretory mechanism in PCa-cells. Importantly, GOAT expression was found to be specifically regulated by key endocrine/metabolic factors (i.e. In1-ghrelin, but not native-ghrelin) in PCa-cells and in normal prostate cell-cultures, suggesting that GOAT production could be functionally linked to In1-ghrelin at the prostate level. In fact, this close relationship between In1-ghrelin and GOAT expression has been reported previously in other cancers [24,28]. Hence, since In1-ghrelin is usually overexpressed in several endocrine-related tumors [24,27,28], it is conceivable that In1-ghrelin, rather than native-ghrelin, could be the main substrate of GOAT-enzyme in these cancers, including PCa. Furthermore, other key metabolic factors (i.e. insulin/IGF-1) also modulated GOAT expression levels in PCa-cells, which might be associated with the positive correlation found in this study between GOAT expression levels and body-weight/BMI in PCa patients, a similar association to that recently reported for circulating GOAT levels in normal weight, anorexic and obese subjects [26]. Interestingly, GOAT expression was higher in PCa-patients with dyslipidemia, and a greater proportion of PCa-patients with dyslipidemia presented high-GOAT mRNA levels compared with patients with low-GOAT expression levels. Altogether, our results suggest the existence of a clear association between changes in GOAT expression levels and alterations in metabolic conditions in PCa-patients (i.e. body-weight/BMI/dyslipidemia), an idea that is in accordance with previous studies demonstrating the link between GOAT levels, lipid-metabolism and whole body-energy balance [21,33,34].

The results obtained hitherto prompted us to question whether GOAT levels could represent a suitable non-invasive biomarker for PCa under normal and/or altered metabolic conditions (i.e. diabetes or dyslipidemia). In fact, plasmatic and urine GOAT levels were higher in PCa-patients compared with healthy-control individuals. Moreover, in PCa-patients, plasma GOAT levels correlated positively with circulating total-PSA (and negatively with free-PSA) as well as with other cancer markers (ca_15-3/cyfra_21-1) used to monitor other tumors (breast and lung cancer, respectively), and which have been proposed as possible PCa biomarkers in conjunction with PSA [35,36]. Therefore, although we could not unequivocally determine a direct association between prostate and plasma/urine GOAT levels, due to the use of different patient's cohorts, the results presented herein indicate that prostate GOAT tissue-expression (mRNA/protein) and plasma/urine GOAT protein levels are consistently and concomitantly elevated in PCa samples. Indeed, ROC-curve analysis demonstrated that GOAT mRNA levels could predict PCa with a high sensitivity/specificity and, most importantly, that plasma and urine GOAT levels also showed a good sensitivity (81.1% and 75%, respectively) and specificity (67.8% and 61%, respectively) for PCa-detection. Altogether, our data indicate that GOAT levels could represent a promising novel diagnostic biomarker (invasive and non-invasive) for PCa.

There is increasing evidence that the alterations in the endocrine/metabolic milieu should be considered to appropriately stratify the patients, as well as to evaluate the diagnostic, prognostic and therapeutic potential of novel biomarkers in tumoral pathologies, including PCa. In this context, there is evidence that circulating GOAT levels depend on the metabolic environment (i.e. low levels in anorexic and high levels in obese-patients [26]), and, most importantly, as shown here, that GOAT expression is directly associated with dysregulations in metabolic-conditions (i.e. BMI/dyslipidemia) in PCa, an observation that is in line with reports showing that altered endocrine/metabolic status (i.e. diabetes/metabolic-syndrome/BMI, etc.) is associated with the development/progression of several cancer-types [8], including PCa [9]. Accordingly, we hypothesized that plasmatic GOAT levels in PCa-patients would also be linked to the metabolic status. Interestingly, we did find that GOAT plasma levels were higher in individuals with alterations in glucose-metabolism (i.e. diabetes and/or dyslipidemia), and were positively correlated with the percentage of glycated-hemoglobin, a gold standard for monitoring glycemic control [37], in both control and PCa-individuals. Actually, irrespective of the presence of PCa, high plasma GOAT levels were associated with a higher proportion of diabetic and/or dyslipidemic patients, suggesting that dysregulated glucose-metabolism (diabetes) might be a confounding factor masking the potential of GOAT to discriminate between control and PCa-patients when the whole population (non-diabetic/diabetic-patients) is considered. Therefore, we then sought to determine the potential of GOAT as

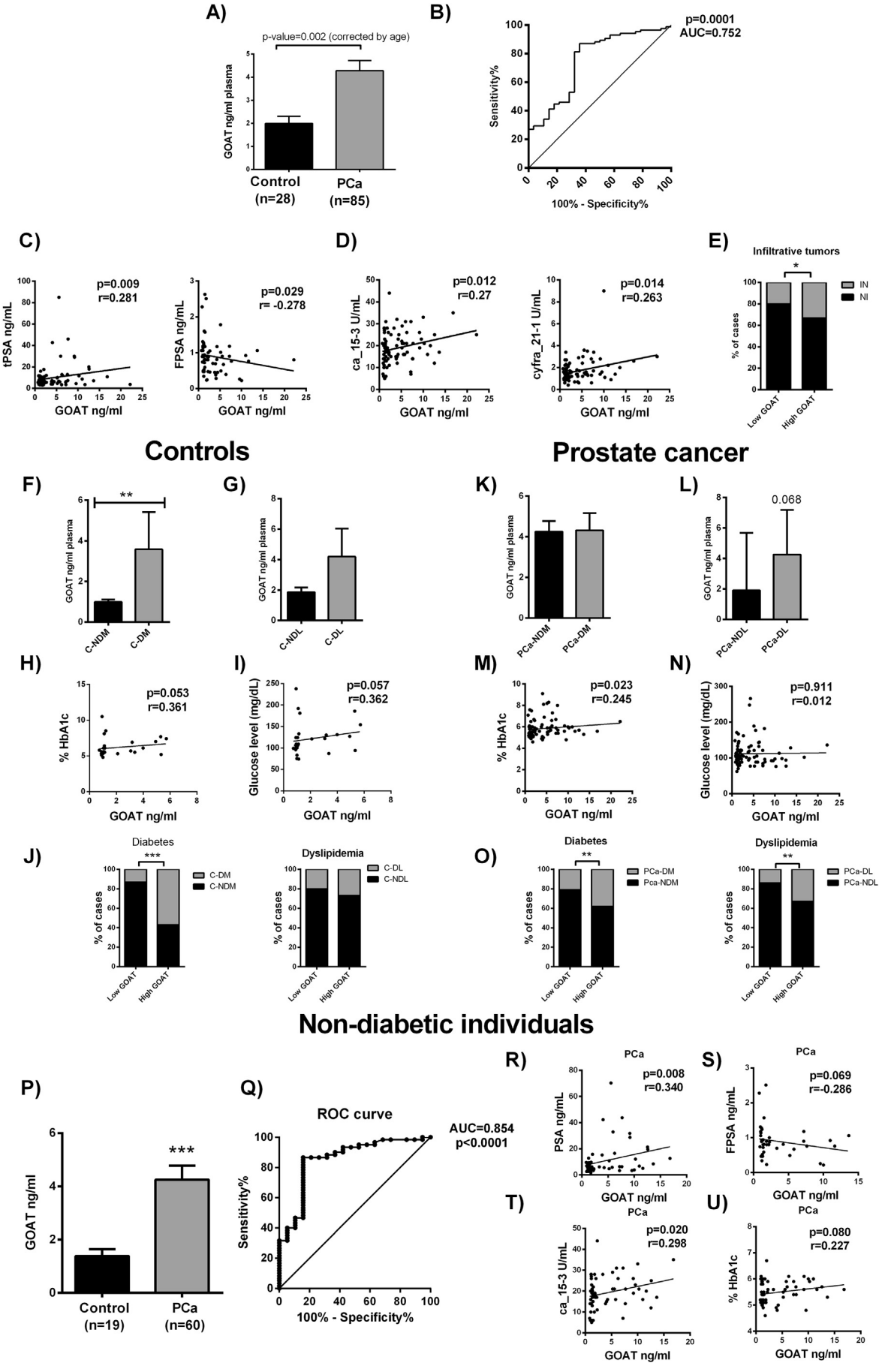

A)
p-value=0.002 (corrected by age)
GOAT ng/ml plasma
Control (n=28)
PCa (n=85)
B)
p=0.0001
AUC=0.752
Sensitivity%
100% - Specificity%
C)
p=0.009
r=0.281
tPSA ng/mL
GOAT ng/ml
p=0.029
r= -0.278
FPSA ng/mL
GOAT ng/ml
D)
p=0.012
r=0.27
ca_15-3 U/mL
GOAT ng/ml
p=0.014
r=0.263
cyfra_21-1 U/mL
GOAT ng/ml
E)
Infiltrative tumors
*
IN
NI
% of cases
Low GOAT
High GOAT
Controls
Prostate cancer
F)
**
GOAT ng/ml plasma
C-NDM
C-DM
G)
GOAT ng/ml plasma
C-NDL
C-DL
K)
GOAT ng/ml plasma
PCa-NDM
PCa-DM
L)
0.068
GOAT ng/ml plasma
PCa-NDL
PCa-DL
H)
p=0.053
r=0.361
% HbA1c
GOAT ng/ml
I)
p=0.057
r=0.362
Glucose level (mg/dL)
GOAT ng/ml
M)
p=0.023
r=0.245
% HbA1c
GOAT ng/ml
N)
p=0.911
r=0.012
Glucose level (mg/dL)
GOAT ng/ml
J)
Diabetes
***
C-DM
C-NDM
% of cases
Low GOAT
High GOAT
Dyslipidemia
C-DL
C-NDL
% of cases
Low GOAT
High GOAT
O)
Diabetes
**
PCa-DM
Pca-NDM
% of cases
Low GOAT
High GOAT
Dyslipidemia
**
PCa-DL
Pca-NDL
% of cases
Low GOAT
High GOAT
Non-diabetic individuals
P)
***
GOAT ng/ml
Control (n=19)
PCa (n=60)
Q)
ROC curve
AUC=0.854
p<0.0001
Sensitivity%
100% - Specificity%
R)
PCa
p=0.008
r=0.340
PSA ng/mL
GOAT ng/ml
S)
PCa
p=0.069
r=-0.286
FPSA ng/mL
GOAT ng/ml
T)
PCa
p=0.020
r=0.298
ca_15-3 U/mL
GOAT ng/ml
U)
PCa
p=0.080
r=0.227
% HbA1c
GOAT ng/ml

Fig. 3. Analysis of the presence of GOAT in the plasma of patients with PCa and controls. **A**. GOAT plasmatic levels were determined by ELISA in a cohort of 85 PCa patients and 28 controls; **B**. Receiver operating characteristic (ROC) curve analysis to determine the accuracy of GOAT plasmatic levels as diagnostic test to discriminate between patients with PCa and controls; **C**. Correlation of plasma levels of GOAT and total PSA (tPSA) and free PSA (FPSA) levels in PCa patients; **D**. Correlation of plasma levels of GOAT with ca_15-3 and cyfra_21-1 tumor markers in PCa patients; **E**. Frequencies of infiltrative tumors observed in PCa patients with low vs. high GOAT plasmatic levels (according to median GOAT levels; IN = infiltrating tumors; NI = No infiltrating tumors); **F**–**J**. Correlations of plasma GOAT levels with metabolic factors in controls; **K**–**O**. Correlations of plasma GOAT levels with metabolic factors in PCa patients (C-NDM: Controls without diabetes mellitus; C-DM: Controls with diabetes mellitus; PCa-NDM: PCa patients without diabetes mellitus; PCa-DM: PCa patients with diabetes mellitus; C-NDL: Controls without dyslipidemia; C-DL: Controls with dyslipidemia; PCa-NDL: PCa patients without dyslipidemia; PCa-DL: PCa patients with dyslipidemia; Low GOAT: patients with low plasmatic GOAT levels; High GOAT: patients with high plasmatic GOAT levels); **P**. GOAT plasmatic levels in non-diabetic PCa patients (n = 60) compared to non-diabetic (n = 19) controls measured by ELISA; **Q**. Receiver operating characteristic (ROC) curve analysis to determine the accuracy of plasma GOAT levels as diagnostic test to discriminate between PCa and controls patients within the population without diabetes; **R**. Correlations between plasma GOAT levels and total PSA in non-diabetic patients with PCa; **S**. Correlations between plasma GOAT levels and free PSA in non-diabetic patients with PCa; **T**. Correlations between plasma GOAT levels and ca_15-3 in non-diabetic patients with PCa; **U**. Correlations between plasma GOAT levels and HbA1c in non-diabetic patients with PCa. In all cases, data represent mean ± SEM. Asterisks (*, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$) indicate values that significantly differ between groups.

PCa-biomarker after stratification of the patient cohort based on the presence of diabetes. Remarkably, when only non-diabetic individuals are considered, we discovered that plasmatic GOAT levels are able to precisely discriminate the presence of PCa with high sensitivity and specificity. These data clearly support the notion that alterations in endocrine/metabolic milieu is a critical factor to ensure an adequate patient stratification and to assess the diagnostic, prognostic and therapeutic potential of novel biomarkers in cancer, including PCa, as it is strongly suggested by the data presented herein for plasma GOAT levels as a novel, suitable complementary PCa diagnostic test, especially for non-diabetic individuals.

A final point of interest relates to the presence of GOAT in urine. The discovery that prostate cells are able to secrete GOAT, being this secretion significantly higher in PCa cell-lines, we posited whether there is a potential utility of GOAT urine levels for the diagnosis of PCa, in that proteins in urine are mainly derived from epithelial cells in the urinary-tract [38,39]. Indeed, changes in urine protein can reflect a dysfunction of cells within the urinary-tract better than those in blood samples, which are deeply influenced by homeostatic mechanisms [38,39]. In support to this notion, our results demonstrate that urine GOAT levels were also higher in PCa-patients compared with controls, showing a high specificity and sensitivity to diagnose PCa-patients. Thus, the data presented herein indicate that GOAT might represent a novel non-invasive PCa-biomarker in urine (wherein it is not severely influenced by the metabolic status).

To the best of our knowledge, the present report provides the first systematic analysis of the presence of the enzyme GOAT in different types of human prostate samples (invasive and non-invasive) obtained from patients with and without PCa, and in relation to the clinical and metabolic parameters of the patients (i.e. presence of metastasis, diabetes, dyslipidemia, etc.). Altogether, our results show, for the first time, that GOAT-enzyme can be directly secreted by PCa-cells and is consistently overexpressed in samples from PCa-patients (tissue/plasma/urine), where its expression is drastically conditioned by the metabolic-status. Since GOAT expression showed a high sensitivity/specificity in PCa-detection, especially in non-diabetic patients, we propose that it might be considered as a novel non-invasive PCa-biomarker alone or in combination with other biomarkers to provide a better PCa-diagnosis.

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Statement of author contributions

R.M.L and D.H conceived and designed the project. D.H, E.G, A.I, J.C.V, E.R, F.L, S.P, J.V.R, M.D.G and R.M.L acquired the data. D.H, E.G, A.I, J.C.V, E.R, F.L, S.P, J.V.R, R.S, R.O, M.M.M, M.D.G, J.L.M, M.J.R, J.P.C and R.M.L performed the analysis and interpretation of data. D.H and R.M.L wrote the manuscript. E.G, A.I, S.P, M.D.G and J.P.C revised the manuscript for important intellectual content. D.H, E.G, A.I, M.D.G and R.M.L performed the statistical analysis. R.M.L, M.D.G

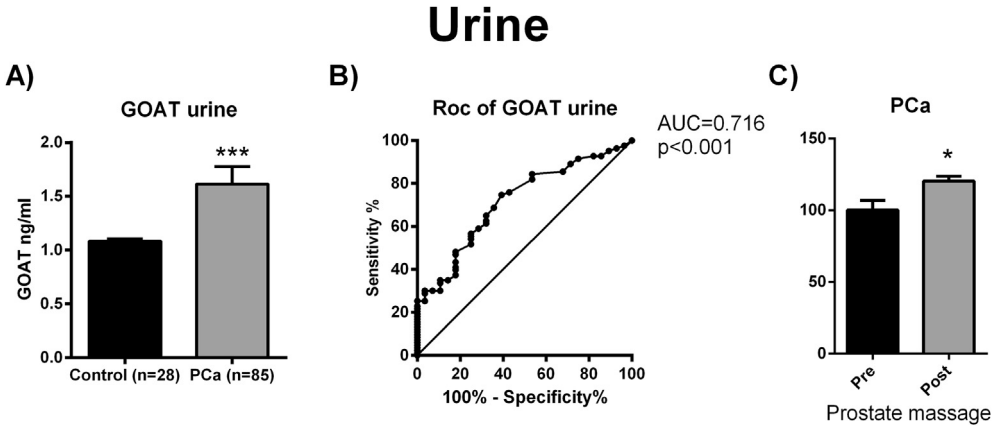

Fig. 4. Evaluation of GOAT levels as a non-invasive PCa diagnostic marker in urine. **A**. GOAT urine levels in a population of 85 PCa patients compared to 28 normal controls determined by ELISA; **B**. ROC curve analysis to determine the accuracy of GOAT urine levels to discriminate between PCa and control patients; **C**. Percentage of the increase of GOAT urine secretion after prostate massage in PCa patients (n = 14) from an additional cohort of patients. In all cases, data represent mean ± SEM. Asterisks (*$p < 0.05$; **$p < 0.01$, ***$p < 0,001$) indicate values that significantly differ between groups.

and J.P.C obtained funding's. F.L, E.R, R.S, R.O, M.M.M, J.L.M and M.J.R provided technical and material support. J.P.C and R.M.L supervised the work.

Disclosure statement

The authors have nothing to disclose.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.canlet.2016.09.022.

Conflict of interest statement

The authors have nothing to disclose.

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