DEPARTAMENTO DE BIOLOGÍA CELULAR, FISIOLOGÍA E INMUNOLOGÍA

UNIVERSIDAD D CÓRDOBA

……

Identification of novel clinical and molecular factors for the diagnosis and aggressiveness of prostate cancer

Identificación de nuevos factores clínicos y moleculares para el diagnóstico y agresividad del cáncer de próstata

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ENRIQUE GÓMEZ GÓMEZ

CÓRDOBA, SEPTIEMBRE 2019

TITULO: *Identification of novel clinical and molecular factors for the diagnosis and aggressiveness of prostate cancer*

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Identification of novel clinical and molecular factors for the diagnosis and aggressiveness of prostate cancer

Identificación de nuevos factores clínicos y moleculares para el diagnóstico y agresividad del cáncer de próstata

……..

Memoria de Tesis Doctoral presentada por **Enrique Gómez Gómez**, Licenciado en Medicina, para optar al grado de **Doctor en Biomedicina**

Tesis Doctoral realizada bajo la dirección del Dr. **Raúl M. Luque Huertas** y de la Dra. **Julia Carrasco Valiente**.

En Córdoba, a septiembre de 2019

IMIBIC INSTITUTO MAIMÓNIDES DE INVESTIGACIÓN BIOMÉDICA DE CÓRDOBA
UNIVERSIDAD D CÓRDOBA
GECM Grupo de Endocrinología Celular y Molecular
DEPARTAMENTO DE BIOLOGÍA CELULAR, FISIOLOGÍA E INMUNOLOGÍA

El Dr. **Raúl Miguel Luque Huertas**, Profesor Titular del Departamento de Biología Celular Fisiología e Inmunología de la Universidad de Córdoba, y la Dra. **Julia Carrasco Valiente**, Facultativo Especialista del Área de Urología

INFORMAN

Que D. **Enrique Gómez Gómez**, Licenciado en Medicina, ha realizado bajo nuestra dirección el trabajo titulado "***Identification of novel clinical and molecular factors for the diagnosis and aggressiveness of prostate cancer***" reuniendo los méritos suficientes para optar al Grado de Doctor en Biomedicina.

Y para que conste, se firma la presente en Córdoba, a septiembre de 2019.

Fdo.: Dr. Raúl Miguel Luque Huertas

Fdo.: Dra. Julia Carrasco Valiente

TÍTULO DE LA TESIS: ***Identification of novel clinical and molecular factors for the diagnosis and aggressiveness of prostate cancer***

DOCTORANDO/A: Enrique Gómez Gómez

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES 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, en el periodo comprendido entre 2014 y 2019, el doctorando Enrique Gómez Gómez no solo ha superado con creces los objetivos planteados al comienzo de la misma, sino que ha desarrollado y validado técnicas experimentales de una gran utilidad para el grupo de investigación, que le han permitido obtener resultados muy relevantes en el campo del cáncer de próstata y obesidad y que quedan patentes en varias publicaciones. Concretamente, como fruto de su trabajo durante este periodo, ha publicado cuatro trabajos directamente relacionados con su Tesis Doctoral, en las revistas "*Journal of Clinical Medicine*", "*British Journal of Cancer*" y "*Journal of Cellular and Molecular Medicine"* todas ellas revistas de referencia dentro de nuestras áreas de investigación. Además, el trabajo realizado en este periodo ha dado lugar a otro artículo que está actualmente en segunda revisión en la revista "*BMJ Open*".

Por último, el doctorando ha presentado sus resultados en diferentes congresos de ámbito nacional e internacional.

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

Córdoba, septiembre de 2019

Firma del director

Fdo.: Raúl M. Luque Huertas

Esta Tesis Doctoral ha sido realizada en el Departamento de Biología Celular, Fisiología e Inmunología de la Universidad de Córdoba, bajo la dirección del Doctor Raúl M. Luque Huertas y Co-Dirección de la Doctora Julia Carrasco Valiente del Servicio de Urología del Hospital Universitario Reina Sofía de Córdoba. Sus estudios han sido realizados dentro de proyectos subvencionados por el Instituto de Salud Carlos III (FIS: PI13/00651, PI16/00264, DTS18/00131), fondos FEDER (CCB.030PM), Obra Social "la Caixa" (CaixaImpulse) y por la Junta de Andalucía (PI-0639-2012, PI-0541-2013, BIO-0139, CTS-1406). Durante el transcurso de la presente Tesis Doctoral se ha realizado una estancia de tres meses en dos periodos diferentes en el Departamento de Urologia de Londres (NHS) del University College London Hospitals (UCLH) e Imperial College London bajo la supervisión del Dr. Hashim Ahmed con el objeto de obtener la Mención Internacional en el Título de Doctor de la Universidad de Córdoba.

*"**El secreto de la felicidad no está en hacer siempre lo que se quiere sino en querer siempre lo que se hace"***
Y ADEMÁS SER AGRADECIDO".

(León Tolstói)

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Posteriormente, y como no, a mis dos directores de tesis **Raúl Luque Huertas** y **Julia Carrasco Valiente**, y aunque no aparezca como tal a **María José Requena**, porque ella es la que ha iniciado y mantenido todo en cada momento. A Raúl no solo le tengo que agradecer todo por dirigir esta tesis, si no por darme todas las oportunidades (Y MÁS) que han estado en su mano para mi formación y mi profesión, siendo además mi tutor durante dos años de aprendizaje máximo en la Beca Rio Hortega. En lo profesional queda patente, pero además creo que no es posible agradecer con palabras su trato en lo personal, siempre atento, dispuesto y disponible en los momentos duros,

de errores, de confusión que he tenido, así que GRACIAS. De María José y Julia es fácil describir porque han sido y son como Madres para mí, por supuesto lo son literalmente en lo profesional, pero también se han comportado así en lo personal, y no solo durante este periodo.

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Muchas gracias por todo!

List of publications

This Thesis is based on the research articles listed below, which will be referred in the text by their Roman numerals.

Article I: Gómez Gómez E, Salamanca Bustos JJ, Carrasco Valiente J et al. Observational study comparing the accuracy/variability between the ERPSC and the PCPT risk calculators for the prediction of significant prostate cancer in patients with PSA <10ng/ml [*BMJ Open* (Under review)].

Article II: Gómez-Gómez E, Carrasco-Valiente J, Campos-Hernández JP, et al. Clinical association of metabolic syndrome, C-reactive protein and testosterone levels with clinically significant prostate cancer. [*J Cell Mol Med*. 2019; 23(2):934-942. doi: 10.1111/jcmm.13994]

Article III: Frantzi M*, Gómez-Gómez E*, Blanca-Pedregosa A et al . CE-MS based urinary biomarkers to distinguish non-significant from significant prostate cancer. [*Br J Cancer*. 2019; 120(12):1120-1128. doi: 10.1038/s41416-019-0472-z].

Article IV: Gómez-Gómez E*, Jiménez-Vacas JM*, Carrasco-Valiente J, et al. Plasma ghrelin O-acyltransferase (GOAT) enzyme levels: A novel non-invasive diagnosis tool for patients with significant prostate cancer. [*J Cell Mol Med*. 2018; 22(11):5688-569. doi: 10.1111/jcmm.13845]

Article V:Gómez-Gómez E, Jiménez-Vacas JM, Pedraza-Arévalo S et al. Oncogenic role of secreted engrailed homeobox 2 (EN2) in prostate cancer [*J Clin Med* 2019; 8, 1400*;* doi:10.3390/jcm8091400]

*Equally contributors (First authors)

List of abbreviations

AA = Aminoacids

AACE = American Association of Clinical Endocrinologists

AMACR = Alpha-methylacyl-CoA racemase

APC = Adenomatous polyposis coli gene

AR = Androgen Receptor

AS = Active surveillance

AUC = Area Under the Curve

BMI = Body Mass Index

BRCA = Breast cancer gen

β2M = β-2-microglobulin

CE-MS = Capillary Electrophoresis-Mass Spectrometry

CDKN1A = Cyclin-dependent kinase inhibitor 1A

CK = Creatine kinase

CRP = C-Reactive Protein

CRPC= Castration Resistant Prostate Cancer

CSD = Cancer-specific death

CSS = Cancer Specific Survival

CTNNB1 = Beta catenin gene

DCE = Dynamic contrast-enhanced imaging

DDR = DNA damage repair

DLX1 = Distal-Less Homeobox 1

DRE = Digital Rectal Examination

DWI = Diffusion-weighted imaging

ECM = Extracellular Matrix

EGIR = European Group for the Study of Insulin Resistance

EN = Engrailed

ERG = Erythroblast transformation-specific (ETS) related gen

ERSPC = European Randomized Screening of Prostate Cancer

ETS = Erythroblast transformation-specific

ETV1/4 = ETS translocation variant

FCS = Foetal Calf Serum

FDA = U.S Food and Drug administration

FLI1 = Friend leukemia integration

FOXA1 = Forkhead Box A1

GC-MS = Gas chromatography mass spectrometry

GOAT = Ghrelin O-acyltransferase

GS = Gleason Score

GSTP1 = Glutathione S-Transferase Pi 1

HGF = Hepatocyte grow factor

hK2 = Human kallikrein 2

HOX = Homeobox genes

HPV-18 = Human papillomavirus -18

IDF = International Diabetes Federation

IDH1 = Isocitrate dehydrogenase 1

IL- 6 = Interleukin-6

iPSA = Intact PSA

LC-MS/MS = Liquid chromatography coupled to tandem-mass spectrometry

MAPK = Mitogen-Activated Protein Kinases

mCRPC = Metastatic Castration Resistant Prostate Cancer

MetS = Metabolic Syndrome

miRNAs = MicroRNAs

MMP = Matrix metalloproteinases

MpMRI = Multiparametric prostate magnetic resonance imaging

MUC3 = Mucin 3

NCOA2 = Nuclear receptor coactivator 2

NETs = Neuroendocrine tumors

NKX3-1 = Homeobox protein Nkx-3.1

NPV = Negative predictive value

NRIP1 = Nuclear receptor-interacting protein 1

PARP = Poly(ADP-Ribose) polymerase

PAX = Paired box genes

PCa = Prostate Cancer

PCA3 = Prostate cancer antigen 3

PCPT = Prostate Cancer Prevention Trial

PGA3 = Pepsinogen 3 preproprotein

PHI = The 'Prostate Health Index'

PI-RADS v2 = The Prostate Imaging Reporting and Data System version 2

PTEN = Phosphatase and tensin homolog

PI3K = phosphatidylinositol- 3-kinasa

PPV = Positive Predictive Value

RB = Retinoblastoma

RC = Risk Calculator

Sig PCa = Significant Prostate Cancer

siRNA = small interfering RNA

SPDEF = SAM Pointed Domain Containing ETS Transcription Factor

SPINK1 = Inhibitor serine peptidase Kazal type 1

SPOP = Speckle type BTB/POZ protein

TDRD1 = Tudor Domain Containing 1

TMPRSS2 = Transmembrane protease, serine 2

TNF = Tumor necrosis factor

TP53 = Tumor protein p53

TRUS = Trans-Rectal UltraSound

UGM = UDP-galactopyranose mutase

USPSTF = U.S. Preventive Services Task Force

VEGF = Vascular endothelial growth factor

WAT = White Adipose Tissue

WHO = World Health Organization

WT = Wild type

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0. Summary

Resumen

El cáncer de próstata (CaP) es la neoplasia sólida más frecuente en varones en países desarrollados. Desafortunadamente, la extraordinaria heterogeneidad y complejidad del CaP, unida a su variabilidad fenotípica/clínica, fuertemente influenciada por el ambiente endocrino-metabólico, hace difícil encontrar elementos clínico-moleculares comunes que faciliten estrategias diagnósticas, pronósticas y/o terapéuticas globales y efectivas.

Partiendo de esta base, la **hipótesis** de la presente Tesis Doctoral es que el estado metabólico y alguno de los factores asociados al mismo podrían estar relacionados con la presencia y agresividad del CaP, y que el estudio más profundo de algunas herramientas utilizadas habitualmente en la práctica clínica del CaP (calculadoras de riesgo o "Risk Calculators") y de algunas familias de proteínas poco estudiadas hasta la fecha en CaP podrían ser de gran utilidad para identificar nuevos métodos y/o biomarcadores de diagnóstico, pronóstico y/o tratamiento del CaP.

En primer lugar, estudiamos y comparamos la utilidad de herramientas predictoras de riesgo que se basan en calculadoras de riesgo (CRs) combinando diferentes variables clínicas, centrándonos en estudiar su efectividad y la variabilidad de su resultado en dos mediciones consecutivas del PSA. Específicamente, se usaron dos CRs: "Prostate Cancer Prevention Trial risk calculator 2.0 (PCPT-RC 2.0)" y "European Randomized study of Screening for Prostate Cancer risk calculator (ERSPC-RC)" seleccionando 510 pacientes para el análisis, observando una buena validación externa con ambas CRs. La comparación entre ambas CRs no reveló diferencias significativas. La adición de PSA libre mejoró la precisión del PCPT-CR [Área bajo la curva (AUC)= 0,65 vs. 0,73]. De acuerdo con los resultados de precisión, el análisis de la curva de decisión también fue similar entre las dos CRs, que mostraron un beneficio neto desde

un umbral de riesgo temprano, lo que significa que su implementación mejoraría la selección de pacientes para la biopsia de próstata, si bien, el ERSPC-CR tuvo mejor estabilidad para las variaciones intraindividuales de PSA.

En segundo lugar, se evaluó la asociación de Síndrome Metabólico (MetS), parámetros inflamatorios [(específicamente la proteína c-reactiva (PCR)] y niveles de testosterona con el resultado de la biopsia en una cohorte prospectiva de 524 pacientes con sospecha de Ca programados para una biopsia de próstata. Los resultados revelaron que no sólo la presencia de MetS, sino también un mayor número de criterios de MetS y un mayor nivel de PCR circulante, pero no de testosterona, se asociaron con un mayor riesgo de CaP significativo (CaP Sig; Gleason ≥7). Curiosamente, cuando analizamos cada criterio de MetS de forma independiente, encontramos que sólo dos criterios, una mayor circunferencia de la cintura y la presión arterial, se asociaron significativamente con un mayor riesgo de CaP Sig.

Para los análisis de nuevos biomarcadores, se realizó un estudio de un panel de marcadores peptídicos urinarios mediante CE-MS ("Capillary electrophoresis–mass spectrometry") utilizando una cohorte de 823 pacientes. El análisis en la cohorte de entrenamiento permitió la identificación de 19 biomarcadores peptídicos, de los cuales se pudieron obtener secuencias para 17. El panel de 19 péptidos seleccionados en la cohorte de entrenamiento (n= 543 pacientes) fue validado en un conjunto de validación independiente de 280 pacientes con un valor de AUC de 0,81 [Intervalo de confianza: 0,76-0,86]. Además, la comparación con el ERSPC-CR mostró que este panel claramente superó al nomograma para los diagnósticos de CaP Sig [modelo de 19 biomarcadores vs. ERSPC-CR; AUC= 0,82 vs 0,69, respectivamente (p= 0,02)], también en el análisis de la curva de decisión.

En relación con el estudio de biomarcadores aislados, la ghrelina O-acyltransferasa (GOAT) es una enzima clave que regula la actividad del sistema de la ghrelina, la cual se ha demostrado que está sobreexpresada en tejidos de la CaP (a nivel de ARNm y proteína) y sus niveles plasmáticos elevados en pacientes con CaP; sin embargo, su supuesta función como biomarcador no invasivo del CaP en pacientes de riesgo no ha sido estudiada previamente. Por tanto, se intentó evaluar el uso de la GOAT como biomarcador diagnóstico no-invasivo de CaP en un estudio caso-control de 312 pacientes divididos en tres grupos: Pacientes con CaP, pacientes con riesgo de CaP pero resultado negativo en la biopsia y controles sanos. Los niveles plasmáticos de GOAT fueron significativamente más altos en pacientes con CaP en comparación con pacientes sanos y pacientes en riesgo de CaP pero con resultado negativo en la biopsia. Además, estos niveles fueron aún más altos en pacientes diagnosticados con CaP Sig. De hecho, la selección de la subpoblación de pacientes con un PSA entre 3-20 ng/ml, reveló que la precisión de la GOAT para el diagnóstico de CaP Sig fue mejor que la del PSA en esta población (donde la precisión del PSA fue dramáticamente baja). El análisis de la asociación de los niveles de GOAT con características agresivas mostró una correlación entre los niveles de GOAT con el grado Gleason y una asociación con un mayor riesgo de ser diagnosticado con metástasis.

Por último, estudiamos más profundamente factores de transcripción que contienen homeodominio [genes *Engrailed (EN)* 1 y 2], que previamente habían sido evaluados en diferentes tumores, incluyendo CaP. Concretamente, se pretendió evaluar la utilidad de la EN2 como marcador no invasivo y estudiar, por primera vez, su papel oncogénico en CaP. En primer lugar, la *EN2* se sobreexpresó en las dos cohortes analizadas de tejidos de CaP en comparación con los tejidos control y en las líneas celulares tumorales en comparación con las células RWPE-1 de tipo normal, así como

en diversas cohortes de muestras externas disponibles (análisis "*in silico*"). De manera consistente, los niveles de EN2 en orina mostraron niveles más altos en pacientes con CaP, siendo el porcentaje de detección de EN2 en orina en pacientes con tumor del 75% vs. el 45% encontrado en los controles (p= 0,05). A continuación, se encontró que la proteína EN2 era secretada por líneas celulares de CaP, mientras que sus niveles se encontraban por debajo del límite de detección en las células RWPE-1 normales. Finalmente, se demostró que la EN2 aumenta el potencial tumorigénico en las células prostáticas, es decir, una mayor capacidad de proliferación, migración o secreción de PSA, mediante la modulación de ciertas vías de señalización claves en CaP. Curiosamente, nuestros resultados también muestran un aumento en la tasa de fosforilación del receptor de andrógenos (AR), sus variantes de splicing y de la ruta AKT después del tratamiento EN2 en LNCaP y/o 22Rv1.

Por todo ello, los resultados presentados en esta Tesis Doctoral constituyen una información novedosa y valiosa que respalda varias ideas: **1)** Existen herramientas gratuitas útiles para estimar el riesgo de CaP basado en variables clínicas habituales cuyo uso mejoraría la práctica clínica; **2)** El síndrome metabólico y parámetros relacionados con la inflamación (PCR) se encuentran asociados al diagnóstico de CaP clínicamente significativo y con su agresividad; **3)** El análisis de una combinación de marcadores peptídicos puede mejorar la capacidad de diagnóstico de CaP clínicamente significativo; **4)** la enzima GOAT puede ser un marcador útil para complementar la información del PSA, cuando este pierde más capacidad, y mejorar el diagnóstico de CaP significativo y su agresividad; **5)** Engrailed 2 podría utilizarse como biomarcador no invasivo para el diagnóstico del CaP, así como servir como una herramienta útil para el desarrollo de nuevos fármacos para esta patología.

Summary

Prostate cancer (PCa) is the most common solid neoplasm in men in developed countries. Unfortunately, the extraordinary heterogeneity and complexity of PCa, together with its phenotypic/clinical variability, strongly influenced by the endocrine-metabolic environment, makes it difficult to find common clinical-molecular elements that would facilitate global and effective diagnostic/prognostic/therapeutic strategies.

Based on this information, the **hypothesis** of this Doctoral Thesis is that the metabolic state and some of the factors associated to it could be associated to the presence and aggressiveness of CaP, and that a more profound study of some tools usually used in the clinical practice of PCa (Risk Calculators-RCs), as well as of some families of proteins poorly studied to date in PCa could be very useful for the identification of novel methods and/or biomarkers for the diagnosis, prognosis and/or treatment of PCa.

First, we studied and compared the usefulness of different risk predictor tools based on RCs combining different clinical variables, focusing on studying the effectiveness and the variability of their results within two consecutive PSA measurements. Specifically, two RCs were used: the "Prostate Cancer Prevention Trial risk calculator 2.0 (PCPT-RC 2.0)" and the "European Randomized study of Screening for Prostate Cancer risk calculator (ERSPC-RC)", selecting 510 patients for the analysis wherein both RCs showed good external validation. The comparison between the two RCs did not reveal any significant differences. The addition of free PSA improved the accuracy of the PCPT- RC (AUC= 0.65 vs. 0.73). According to the precision results, the decision curve analysis was also similar between the two RCs, which showed a net benefit from an early risk threshold, meaning that their implementation would improve

the selection of patients for prostate biopsy. However, the ERSPC-CR had better stability for intra-individual variations of PSA.

Secondly, we evaluated the association of Metabolic Syndrome (MetS), inflammatory parameters [specifically the C-Reactive Protein (CRP)] and testosterone levels with the biopsy result in a prospective cohort of 524 patients scheduled for a prostate biopsy with suspected PCa. Not only the presence of MetS, but also a greater number of MetS criteria and a higher level of circulating CRP, but not testosterone, were associated with an increased risk of significant PCa (Sig PCa; Gleason ≥7). Interestingly, when we analyzed each MetS criterion independently, we found that only two criteria, an increased waist circumference and blood pressure, were significantly associated with an increased risk of Sig PCa.

In the analysis of novel biomarkers, a study of a panel of urinary peptide markers by CE-MS ("Capillary electrophoresis–mass spectrometry") was carried out using a cohort of 823 patients. The analysis in the training cohort identified 19 peptide biomarkers, from which sequences for 17 could be obtained. The panel of 19 peptides selected in the training cohort (n=543 patients) was validated on an independent validation set of 280 patients with an AUC value of 0.81, ranged from 0.76 to 0.86. In addition, comparison with the ERSPC-RC showed that this panel clearly outperformed the nomogram for Sig PCa diagnoses [model with 19 biomarkers vs. ERSPC-RC; AUC = 0.82 vs 0.69, respectively (p= 0.02)], also in a decision curve analysis.

In relation to the study of isolated biomarkers, Ghrelin-O-acyltransferase (GOAT) is a key enzyme that regulates the activity of the ghrelin system and has been shown to be overexpressed in PCa tissues (at mRNA and protein levels) and its plasma levels elevated in PCa patients, but its potential role as a non-invasive biomarker of patients at-risk of PCa has not been studied previously. We evaluated GOAT as a

diagnostic marker of PCa in a case-control study of 312 patients divided into three groups: patients with PCa, patients at risk of PCa but with a negative result in the biopsy and healthy controls. Plasma levels of GOAT were significantly higher in patients with PCa compared to healthy patients and patients at risk of PCa but with negative biopsy result. In addition, these levels were even higher in patients diagnosed with Sig PCa. Moreover, the subpopulation of patients with a PSA between 3-20 ng/ml was selected for direct comparison with PSA, revealing that the accuracy of GOAT for the diagnosis of Sig PCa was significantly better than that of PSA in this population (where PSA accuracy was dramatically low). The analysis of the association of GOAT levels with aggressive features showed a correlation between Gleason grade and GOAT levels and also with an increased risk of being diagnosed with metastasis.

Finally, we studied transcription factors containing homeodomain [*Engrailed* (*EN*) genes 1 and 2], which have been previously evaluated in different tumors, including PCa. Specifically, we intended to evaluate the usefulness of EN2 as a non-invasive marker and study, for the first time, its oncogenic role. Firstly, EN2 was overexpressed in the two available PCa tissue cohorts compared to control tissues, in different tumor cell lines compared to the normal type RWPE-1 cells, as well as on available external cohorts of patients analyzed "*in silico*". Consistently, urine EN2 levels showed higher levels in patients with PCa, being the percentage of urine EN2 detection in patients with PCa of 75% vs. the 45% found in the controls (p= 0.05). EN2 protein was then found to be secreted by PCa cell lines, while its levels were below the limit of detection in normal RWPE-1 cells. In line with this, we found that EN2 increases the tumor potential in prostate cells, i.e. a greater capacity for proliferation, migration or PSA secretion, by modulating certain key signalling pathways. Interestingly, our results also show an increase in the phosphorylation rate of androgen

receptor (AR), AR splicing variants and AKT after EN2 treatment in LNCaP and/or 22Rv1.

Therefore, the results presented in this Doctoral Thesis constitute novel and valuable information that supports several ideas: **1)** The appropriate use of free tools to estimate the risk of PCa based on common clinical variables would improve the clinical practice; **2)** The MetS and parameters related to inflammation (CRP levels) are associated with the diagnosis of clinically Sig PCa and with its aggressiveness; **3)** The analysis of a combination of peptide markers can improve the ability to diagnose clinically Sig PCa; **4)** GOAT enzyme can be a useful marker to complement PSA information, when PSA loses more capacity, and to improve the diagnosis of Sig PCa and its aggressiveness; **5)** Engrailed 2 could be used as a non-invasive biomarker for the diagnosis of PCa, and could be a useful tool for the development of new drugs for this pathology.

I. Introduction

I-1. Prostate Cancer epidemiology

Prostate cancer (PCa) is diagnosed in approximately 1.3 million men by year all over the world (1); with a variable incidence that depends on the geographic area, being the most incident cancer type in the majority of countries (**Figure 1**). Currently, PCa is the most frequent cancer among European men, with a higher prevalence in the North and West of Europe, and an increasing trend in the East and South (2). The variation in PCa incidence is the result of the screening and early diagnosis programs (as we will further explain in subsequent sections). This high prevalence of PCa implicates more than 8 billion euros of cost in Europe, being the majority concentrated in the first year after diagnosis (3).

Patient survival has also been improved during the last decades. Specifically, 5-year survival rates evolved from 73.4% to 83.4% between 1999 and 2007 and are still improving. The explanation for this increase survival rates is not only an earlier diagnosis but also the advances in the treatments (4,5).

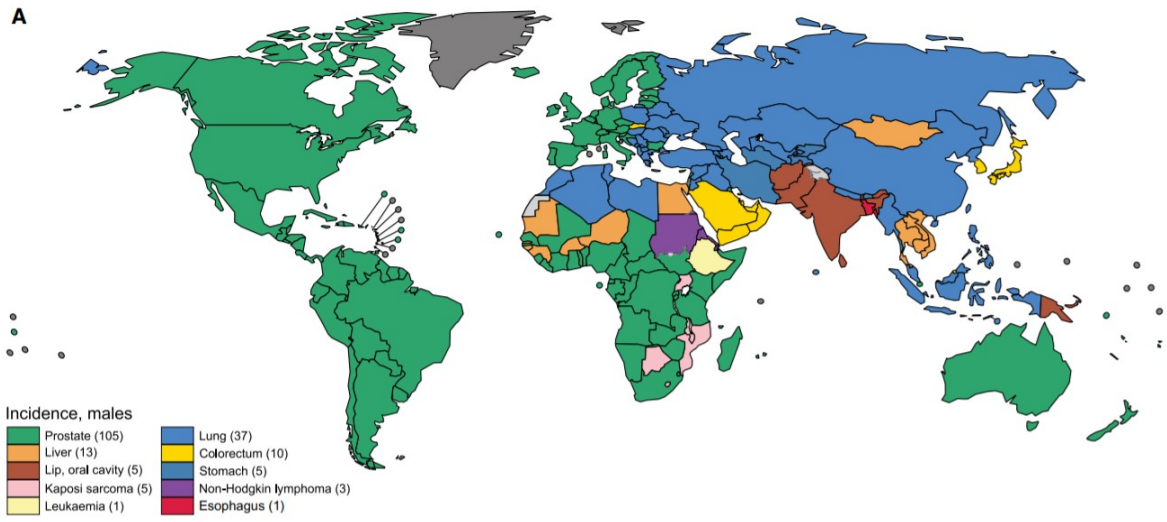

Figure 1. Global Maps Presenting the Most Common Type of Cancer Incidence in 2018 in Each Country Among Men.

I-2. Risk Factors

There are three main risk factors that have been clearly identified in the diagnosis of PCa: age, race and family history (hereditary factors). A recent systematic review, that covers the prevalence of incidental PCa in autopsies, showed an increase in the prevalence of PCa by decade of age [Odds Ratio (OR) = 1.7 95%; CI (1.6-1.8)], increasing from a 5% in patients younger than 30 years old to a 59% in patients older than 79 years old (6).

The race influence has been quite documented, showing a higher incidence and aggressiveness in Afro-American people, with also variations in the genomics of the tumors (7).

The hereditary influence is also one key risk factor as PCa is considered the cancer with highest proportion of family cases, ahead of breast and colorectal cancers. In fact, a Scandinavian study performed with twin brothers estimated that a 42% of global PCa risk can be due to hereditary factors (8,9). Furthermore, PCa risk duplicates with one first-degree relative affected, and increases by 5 to 11 times with two or more relatives, being this risk higher when a brother is affected compared to when the father is affected (10). However, this clear hereditary association has not been still explained by a specific group of genes (11). Carter *et al.* proposed the following definition for the hereditary PCa: Family history of PCa in three generations, and/or three first-degree relatives and/or two first-degree relatives if one was diagnosed below 55 years old (12).

Lynch *et al.* recently published a review about possible screening pathways in genetic predispose families revealing the important genetic heterogeneity of this tumor with a wide variety of suspicious alleles involved in the disease (13). However, consistent evidences have supported that some mutations, such as those affecting *BRCA*

gene and the variant G84 on *HOXB13* gene (rs138213197), are associated with an earlier development and more aggressive PCa (13). The IMPACT study showed that the positive predictive value (PPV) for a positive biopsy in patients with a *BRCA* mutation with a Prostate-specific antigen **(**PSA) >3 ng/mL was double than in the other populations, and that the diagnosis of PCa in patients younger than 50 years only occurred in the *BRCA* mutated patients (14,15).

In recent years, other risk factors of PCa clinical development, such as the diet, alcoholism, sexual behaviors and habits, metabolic status and a chronic inflammatory state, have been suggested (16–18). Interestingly, epidemiologic, molecular and clinical studies have shown evidence about the putative role of the metabolic syndrome in PCa, but further research is still necessary.

I-2.1 Metabolic Syndrome, inflammation and PCa

Metabolic Syndrome (MetS), previously named "X syndrome", was firstly described in 1987 as a group of atherosclerotic risk factors: insulin resistance, hypercholesterolemia and hypertension (19). Since then, different associations have assigned a variety of definitions for this syndrome [World Health Organization (WHO) – 1998, European Group for the Study of Insulin Resistance (EGIR) – 1999, American Association of Clinical Endocrinologists (AACE) – 2003, International Diabetes Federation (IDF) – 2005, etc.] (20). Among them, the most worldwide spread is the National Cholesterol Education Program-Adult Treatment Program III (NCEP- ATP III). For this consensus, a group of American experts agreed for a final report definition, for which is needed to meet at least three of the following criteria (21):

I. Abdominal circumference >102 cm (>40 in).

II. Serum HDL cholesterol <40 mg/dl (<1.0 mmol/l) or being actively treated for low HDL levels.

III. Serum triglycerides ≥150 mg/dl (≥1.7 mmol/l) or being actively treated for elevated triglycerides.

IV. Fasting glucose from 100 to 126 mg/dl or being actively treated for hyperglycemia.

V. Blood pressure ≥130/85 mmHg or being actively treated for hypertension.

The estimated prevalence according to "The National Health and Examination Study" (22) in America is approximately 34%, which has been corroborated in more recent series (23). In Spain, the estimated prevalence in population over 18 years old is also high [22.7% (21.7%-23.7%)] (24), but lower than in the American population.

From a clinical point of view, it should to be noted that the role of MetS and PCa association as a putative PCa risk factor have been proposed in some studies (25,26). Recently, Esposito *et al.* and Gacci *et al.* summarized the literature data in two meta-analyses. Specifically, these studies showed that the fact of being categorized as MetS/or not, was not associated with the diagnosis of PCa, but abdominal circumference and hypertension, as individual factors, were variables associated with the diagnosis of the disease (27). Meanwhile, Gacci *et al.* concluded that obesity was associated with worse oncologic outcomes in men with PCa, in particular with more aggressive tumor features (18). The explanation for this controversy could be found in the fact that the results of different studies varied pursuant to the cohort geographic localization, with heterogeneity in variables such as the prevalence of MetS, different MetS definitions, and the analytical methodologies and the effect of the different drugs intake in each cohort. Most studies have analyzed MetS as a dichotomous variable

(MetS; yes or no), with significant positive association in the European cohorts (25,26,28) and negative in the American cohorts (29,30).

The problem of analysing the MetS variable only with a dichotomous approach is that the independent effects and interactions between the different syndrome components are missed (30). In line with this, Bhindi *et al.* (31) studied the MetS with a quantitative approach considering each component as an independent risk factor. Interestingly, the final number of risk factors was positively correlated with the risk of being diagnosed with PCa.

The biological hypothesis of the association between MetS and PCa is mainly based on different factors/pathways (**Figure 2**): alterations in the insulin/IGF1 and adipokines system, dysregulation in hormones levels, and inflammation.

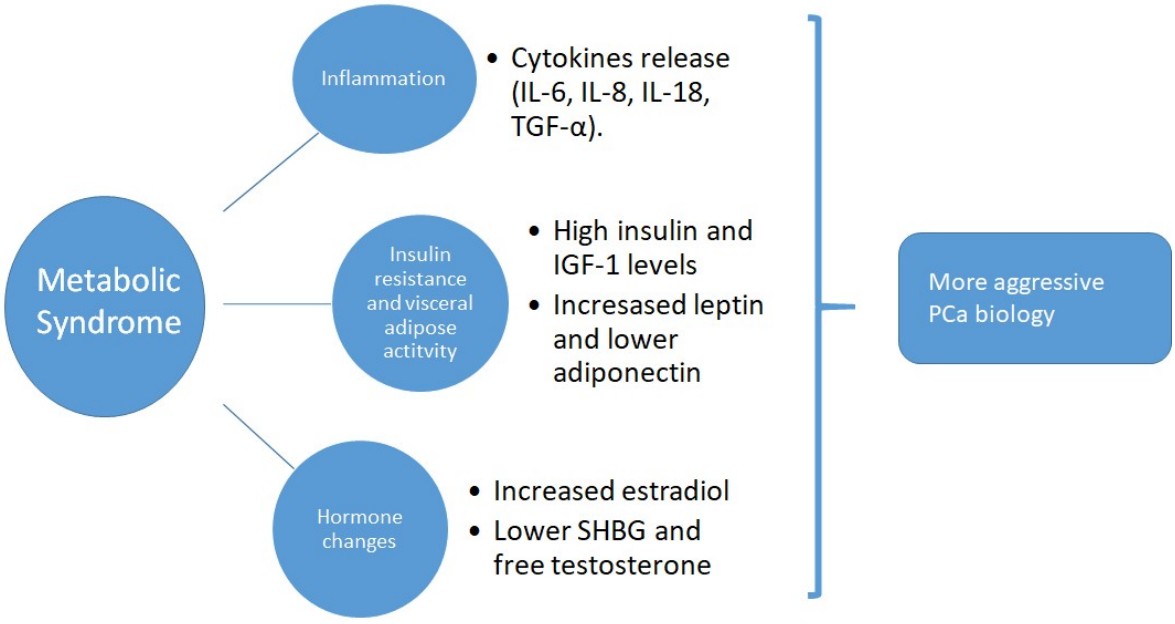

Figure 2. Biological hypothesis of the association between MetS and PCa aggressiveness (32).

Firstly, higher insulin and glucose levels and some insulin gene polymorphisms showed an increased risk of being diagnosed with PCa, with consistent literature supporting the influence of IGF-1 *in vitro* (e.g. increasing the proliferation and

migration rate of PCa cells) and *in vivo* (33–36). However, there are some data suggesting that long-term diabetes seems to protect from PCa. This could be explained by the fact that the progressive insulin resistance and the β-cells failure finally are not able to secrete insulin, consequently, conferring protection against PCa in long-term (36–38). In line with this, cohorts with long-term follow up have demonstrated how during the initial period of the disease, just soon after the diagnosis, there is no association. However, with the natural evolution of the disease, beyond 6 years, diabetes became a protective factor with a hazard ratio around 0.75 (39–41). Moreover, it should be taken into account the amount of evidence suggesting the possible antitumor effect of metformin in this type of cancer (42,43), which could be a potential explanation for this long-term protective effect and not in the initial phase.

Another pathway involved in the MetS-PCa association is hormone dysregulation. Specifically, MetS patients usually present higher levels of estradiol and lower levels of testosterone (44), and several studies hold up an association between lower testosterone levels and a higher risk of Significant Prostate Cancer (Sig PCa; Gleason ≥7) but not with a higher risk of any PCa (45). Even though, this association could be simplistic since blood levels of testosterone do not accurately reflect the androgenic function in the tissue. In fact, androgenic modulation of the polymorphisms and androgen receptor (AR) transcription factors, and also local androgen synthesis do not perfectly correlate with peripheral blood levels of androgens (46). This statement, together with the lack of consistent evidence about an increase in the risk of PCa due to androgen supplements, support the hypothesis of an AR saturation (47). According to that, the AR would only respond to a certain saturation threshold, with sensitive changes through low levels modifications but not with levels above the cut-off.

In addition, the role of obesity in PCa has been widely explored. The study of the relationship between the adiposity and PCa set up some evidences: saturated fats promote prostate carcinogenesis by an increase in IGF-1 signal, in estrogen receptor stress and by altering the immune system activation (48). In this relationship between obesity and PCa, it should be considered that most of the studies use as main variable the body mass index (BMI). Despite the fact that this variable is appropriate to measure the obesity, it has less accuracy than abdominal circumference, which has been better associated with the inflammatory status and cardiovascular risk (49). Moreover, there is some evidence supporting the abdominal circumference as a risk factor for PCa and for high-risk PCa, even after adjusting this parameter by BMI (50). In line with this, some studies only associate obesity with high-risk PCa and not with the low-risk disease (51). The negative correlation between BMI and PSA levels should be taken in consideration, inasmuch as obesity could mask the diagnosis and consequently driving to a diagnosis in a more advance disease with poorer prognosis. There are three main explanations for the relationship between obesity and PSA levels: first, the protein dilution, with a higher serum volume that dilute PSA circulating levels; second, the conversion of testosterone to estradiol by the aromatase; and, finally, the hypothalamic suppression with consequently lower levels of androgens and PSA (52,53).

Within the biologic explanation linking obesity and PCa, up to 50 factors released by the adipocytes known as adipokines (mainly secreted by the white adipose tissue or White Adipose Tissue (WAT)) could also play an important role. Some examples are cytokines (tumor necrosis factor –α (TNF–α), Interleukin-6 (IL- 6), etc.), angiogenic factors (vascular endothelial growth factor (VEGF), apelin, etc.) and others that are more specifically produced by adipocytes (leptin, adiponectin, resistin) which

promote proliferation, dedifferentiation and angiogenesis in PCa cell lines, conferring a worse prognosis (54,55).

Another key component of MetS is the hypertension, which has been independently associated with a higher risk of PCa. There is not wide biologic evidence supporting the association with this molecular pathway, but a possible biological explanation could be an increased sympathomimetic effect that would entail to an androgen guided cell-proliferation (30).

As a pathological syndrome, MetS induces a chronic inflammatory status which derives in multiple diseases. C-Reactive Protein (CRP) is an inflammatory marker whose levels have been shown to be more elevated in patients with different types of cancer compared to the healthy population (56). Very high CRP levels indicate an acute inflammation while levels lower than 10 mg/L are usually associated with a physiological condition of minor inflammation as a response to a metabolic stress situation such as obesity, high blood pressure, diabetes and similar (57).

To date, numerous studies have suggested the existence of a correlation between CRP levels and a worse prognosis in metastatic PCa (58,59), but there is only sparse data in localized disease, driving to inconclusive results (60–62). In line with this, a population-based study did not reach to associate higher levels of CRP with an increased risk of PCa (63), probably due to the methodology used with too high levels of CRP as reference (around 10 mg/L) in comparison with the studies in advanced disease that stablish lower cut-off levels. In fact, Lee *et al.* (64) found an association between CRP levels and PCa risk using a high-sensitivity CRP test, which is able to detect very low levels of CRP, suggesting that the analysis of a lower levels range of this protein might be able to demonstrate the association between CRP levels and PCa risk.

I-3. Pathophysiology of PCa

PCa evolves from benign tissue to malignant lesion by acquiring, over time, several genetic (DNA copy number variations, gene mutations, or chromosomal rearrangements) and intracellular signalling alterations (i.e. fosfatidilinositol- 3-kinasa (PI3K) and Mitogen-Activated Protein Kinases (MAPK) pathways), epigenetic changes (DNA methylation, histone modification or miRNA dysregulation) and other key molecular alterations (65). In early PCa, structural lesions (such as genomic rearrangements) are prevalent [i.e. gene fusions (erythroblast transformation-specific (ETS) related gen (ERG), ETS translocation variant (ETV1/4), and Friend leukemia integration (FLI1)], while point mutations occur less commonly compared to other solid tumors (i.e. colorectal cancer, melanoma) probably because PCa is not exposed to strong exogenous mutagens (65,66).

I-3.1. Early events in Prostate Cancer

It has been demonstrated that primary molecular complexity of PCa is high. In fact, it has been suggested that up to 74% of the primary PCa tumors falls into one of seven subtypes defined by specific gene fusions (ETS genes with AR-regulated genes) or mutations (e.g. speckle type BTB/POZ protein (SPOP), Forkhead Box A1 (FOXA1) and Isocitrate dehydrogenase 1 (IDH1)). In contrast, the remaining subset of PCa tumors (26%) are driven by unknown molecular alterations (65,67). Androgen signalling is the most relevant pathway in both primary and advanced PCa due to the fact that PCa tumor growth occurs in an androgen dependent manner (65,68). On one hand, in early stages of the disease, the *AR* gene or its protein products are not usually

altered (i.e. mutations in *AR*, amplifications of the gene or appearance of AR splicing variants, which are hallmarks of castration resistant PCa (69)), but many of its cofactors (i.e. nuclear receptor coactivator 2 (NCOA2), Nuclear receptor-interacting protein 1 (NRIP1)) and AR-regulated genes are involved in PCa development. On the other hand, androgens and AR are essential in Early Onset PCa-driven ETS fusion events (70,71). A path of progression due to genetic events was proposed by Bacca *et al.,* describing the deletion of Homeobox protein Nkx-3.1 (*NKX3-1)* or *FOXP1* and fusion of Transmembrane protease, serine 2 (*TMPRSS2)* and *ERG* genes (due to an increase in AR activity) as putative PCa promotors, modifying normal prostate epithelial differentiation and stimulating other oncogenic alterations (72). Subsequently, lesions or epigenetic silencing in tumor suppressors such as Phosphatase and tensin homolog (PTEN), Cyclin-dependent kinase inhibitor 1A (CDKN1A), retinoblastoma (RB) or tumor protein p53 (TP53) accumulate leading to increase cell growth, genomic instability and/or evasion of apoptosis (72). 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 to established clinical treatments.

I-3.2. Late events in Prostate Cancer: The development of Castration Resistant Prostate Cancer

Data from Robinson *et al.,* after the analysis of 150 metastatic Castration Resistant Prostate Cancer (mCRPC), revealed that the most frequent events of this stage of PCa (71.3%) were related with genes involved in the androgen signalling pathway (i.e. copy number alterations or mutations), being the *AR* as the most frequent mutated

gene (69). This type of modifications in *AR* have been associated with the development of androgen deprivation treatment resistance (73), although they have still not been widely implemented in daily clinical practice. Another alternative pathway of resistance is the presence of splicing variants which constitutively activate androgen receptor independently of ligand binding. These splicing variants are associated with resistance to androgen specific treatments (i.e. abiraterone and enzalutamide) (74), but not to chemotherapy, and still not approved direct treatment for these splicing variants modulation (75).

The second pathway most altered in this pathology is PI3K, with up to 49% of somatic alterations, characteristically biallelic *PTEN* loss, which has been previously suggested to be associated with worse outcome in patients under abiraterone treatment (76). Next pathway altered is DNA damage repair (DDR), which is present in 23% of patients. Breast cancer 2 (*BRCA2)* is the most frequent altered gene, which opened a new druggable target as this patients are associated with higher sensitivity to Poly(ADP-Ribose) polymerase (PARP) inhibitors treatment such as olaparib (77). This pathway has also inheritance implications, since its analysis in patients with mCRPC has been proposed and currently evaluated in different clinical trials. Finally, the fourth most common alteration is Wnt pathway, with frequent mutations in Adenomatous polyposis coli gene (*APC)* and beta catenin gene (*CTNNB1)* (69).

I-4. Screening and diagnosis of PCa

PCa diagnosis has significantly evolved along decades, from only being reached after a symptomatic disease status with abnormal digital rectal examination (DRE) and a

biopsy, till today when most people are diagnosed guided by an unorganised screening or opportunistic setting.

Diagnosis evolution towards screening proposal was led by the PSA, discovered in the 80's (78) and supported by randomized studies such as the 'European Randomized Study of Screening for Prostate Cancer` (ERSPC) which shows a significant absolute and relative reduction in cancer-specific death (CSD) in the screened cohort, with better results with longer follow up. After 13 years of follow up, the number of people needed to screen was 781, the number of patients needed to be diagnosed with PCa was 27 in order to save a life, and a reduction in CSD of 27%, but with the cost of a PCa overdiagnosis (79). Before the publication of this trial update, results were more controversial. In this line, in 2013, a Cochrane review did not support the screening population, as the meta-analysis did not show significant reduction in mortality and the screening resulted in frequent and relevant adverse events from the subsequent prostate biopsies and also from the overdiagnosis and overtreatment(80). However, it specified that the benefit is reached after ten years of programmed screening so, in case of being carried out, it should be only offer to those people with a life expectancy of more than 15 years and anticipating those shown in the ERSPC trial by Schröder *et al* (79). The U.S. Preventive Services Task Force (USPSTF) made a recommendation against population screening program resulting in a decrease in PSA test demand. As a consequence, some studies have shown how, after 2012, the pattern of PCa diagnosis has changed towards a tendency in more locally advance and metastatic status (81).

Finally, the most recent recommendation is to offer PSA screening to well-informed patients with a life expectancy of more than 15 years after getting the following conclusions: '*PSA **screening** may reduce **prostate cancer** mortality risk, but*

is associated with false-positive results, biopsy complications, and overdiagnosis. Compared with conservative approaches, active treatments for screen-detected ***prostate cancer*** *have unclear effects on long-term survival but are associated with sexual and urinary difficulties'* (82).

With all this information, the different guidelines stablish their recommendations. The European Association of Urology guideline supports an individual approach according to the specific risk in well-informed patients with a life expectancy of more than 15 years and (83):

- Men with age >50 years old
- Men with age >45 years old and family history of PCa or Afro-Americans.
- Men with PSA levels of >1 ng/mL o >2 ng/mL at 40s and 60s years old, respectively, should be evaluated every 2 years, while those with lower levels could differ to an 8 years' period.

Moreover, new different screening schedules have been proposed by the Memorial Sloan Ketterin Cancer Center group (84), wherein it is emphasised the need of discussing pros and cons with the patient before starting the screening and also to have in mind an active surveillance management as tools to avoid overtreatment. The proposed schedule is shown in **Figure 3** (84). In line with this, and the favourable data from the ERSPC trial follow-up (79), a new recommendation has been made from the USPSTF with a more favourable approach to PSA screening (C recommendation) (85).

All these controversial data support the need to find new putative markers, predictive models and tools that improve the accuracy in order to better select which patients should undergo a prostate biopsy.

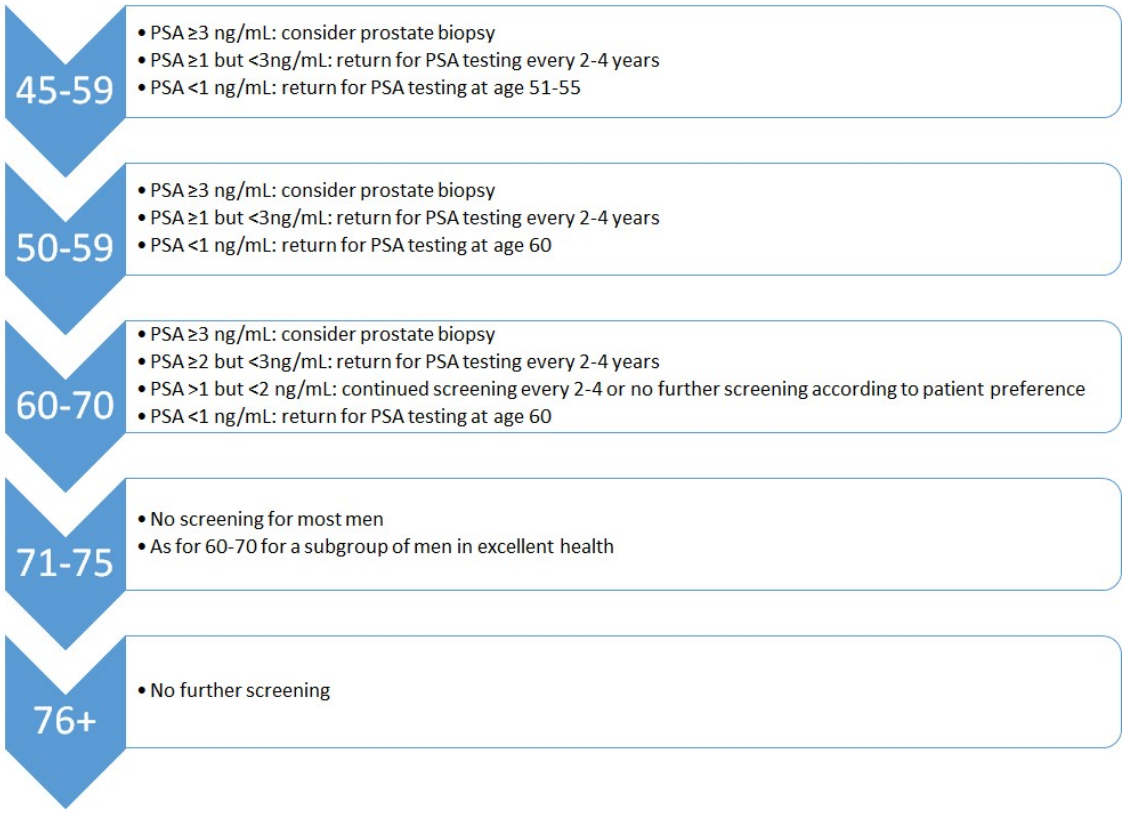

Figure 3. Memorial Sloan Kettering Cancer Center recommendations for Prostate Cancer Screening.

I-4.1. Tools used for diagnosis

PSA: It is a kallikrein-serine protease produced exclusively by epithelial prostatic cells [benign and tumor (although it has also been measured in saliva, breast and amniotic fluids) (86)]. The first description was on the 1980s by Papsidero and Stamey who found a correlation with PCa progression in 1987 (78). Most of the serum PSA is found as a complex with serum protease inhibitors α1-antiquimotripsine and α2-macroglobulin (PSA-α1-antiquimotripsine and PSA-α2-macroglobulin complexes). The rest of the PSA is inactive and is free in the peripheral blood (around 15%). In 1986, it was approved by Food and Drug administration (FDA) as a marker to cancer follow-up and, in 1994, as a PCa diagnostic biomarker for men older than 50s. Despite being very

sensitive, the main problem is its lack of specificity with a very high intra-individual variability, mainly affected by inflammation, prostate enlargement, and similar (87–90). Nowadays, its predictive ability is gradual with a non-clear cut-off value to indicate a prostate biopsy. There are some modifications and adjustments trying to improve its accuracy:

- Age adjusted PSA rising from a PSA of 2.5-3 ng/mL in men in their 50s to 4-5 ng/mL for men older than 70 years old.

- PSA density: It is a volume-adjusted PSA value. It has been shown as a useful tool to improve PSA specificity. It is not only possible to adjust by total prostate volume but also by specifically the transitional zone (91). Furthermore, this approach increases the accuracy to predict Sig PCa (92).

- PSA velocity and PSA double time: "PSA velocity" calculates the absolute increment in a period of time (usually one year), while the "PSA double time" is an exponential formula calculated with at least 2-3 prospective PSA values. Their value is limited because of a high variability and they have not shown to increase the accuracy of PSA alone (93).

- Free PSA: As previously explained, almost 15% of PSA circulate free in the serum. It has been shown to improve up to 15-20% the PSA specificity, with an inverse correlation with the risk of PCa. Usually, a higher free PSA level is associated with benign disease, decreasing from a 56% of tumor probability when the ratio free PSA/total PSA is <10% to only 8% when the ratio rises to more than 25% (94). However, it should not be forgotten that its levels are also influenced by prostate volume and the atmospheric temperature (95).

Digital rectal examination (DRE): It is considered the main complement to PSA levels when screening is carried out. An abnormal exploration should drive to prostate biopsy indication as up to 18% of PCa diagnoses are performed due to an abnormal DRE in patient with a low PSA. Despite being in correlation with Gleason Score (GS), its PPV drops to 5-30% when PSA levels are <2 ng/mL (96–98).

Risk calculators (RC): Based on these parameters and other clinical variables, such as age or family history, the clinician establishes the indication of prostate biopsy. To improve and facilitate this decision, there are some RCs which increase the accuracy of the diagnosis of Sig PCa compared to each of these variables independently. The two most important RCs are the Prostate Cancer Prevention Trial risk calculator 2.0 (PCPT-RC 2.0) (99) and the European Randomized study of Screening for Prostate Cancer risk calculator (ERSPC-RC) (100). Both RCs had an accuracy for the diagnose of Sig PCa higher than 0.75 in their original cohorts, but this accuracy decreased in the external validations (101).

- **PCPT-RC 2.0** is based on the following variables: PSA, age, family history, race, history of previous biopsy, and DRE. There is also a specific variable for patients with a PSA <10 ng/mL that include free PSA value.

It has been updated based on the first RC published in 2006 (102), over a more contemporaneous cohort of 1000 patients from the placebo branch of PCPT trial. This new modification and validation in a more contemporaneous cohort diagnosed by standard biopsies with at least 10 or more cores make it more reproducible. Furthermore, they also incorporated free PSA, improving significantly the prediction of

high-grade PCa compare to non-PCa, but not the prediction of any PCa (AUC 79.8% vs. 72.5%; p <0.05) based on a study over three different cohorts (99).

- **ERSPC-RC** offers up to 6 different variable combinations based on: PSA, DRE, history of previous biopsy, prostate volume calculated by trans-rectal ultrasound (TRUS) or DRE, and TRUS suspicious lesions.

In 2012, a revision trying to improve the RC accuracy with a prostate volume estimation by DRE was validated. This seems to improve the availability of the RC in the clinical practise, without the need of a more invasive technique (such as TRUS). In this research, they showed that DRE calculated volume infra-estimated the volume compared to TRUS, but that the categorization in three groups revealed a good correlation (25 cc, 40 cc and 60 cc) (100).

In recent years, these two RCs have been externally validated and direct comparisons have been performed. The two most recent evaluations were carried out by Poyet *et al.* (101) and by Foley *et al.* (103), who showed that ERSPC-RC 3/4 (without/with previous biopsy) + DRE outperformed PCPT-RC 2.0 in the ability to predict high grade PCa.

Recently, a new RC developed by the Prostate Biopsy Collaborative Group over a heterogeneous contemporary cohort has been proposed. It has been shown to outperformed PCPT–RC 2.0 for Sig PCa (Gleason ≥7) with an AUC difference of 0.03, and after stablishing a cut-off risk point of 10% the same prostate biopsy could be avoided but without missing any Sig PCa (104).

I-4.2. New Biomarkers for PCa

Numerous markers have been investigated, and are still currently under research, in order to improve the performance in the diagnosis of PCa. Some of these are briefly described below:

- PHI: The 'Prostate Health Index' is a mathematic formula that combines total PSA, free PSA, and (-2) proPSA. In 2012, this test received the FDA approval for its measures in blood of patients at risk of PCa (105). The marker, in the original prospective multicentre cohort, obtained an area under the curve (AUC) of 0.70, outperforming the PSA which only reached an AUC of 0.52 (106). In another European validation, the biomarker showed an AUC of 0.76 versus PSA density with an AUC of 0.61 (107). The PHI not only predicts PCa on the biopsy, but also is correlated with GS and associated with worse pathologic features in the prostatectomy specimens (108). It also showed prognostic ability in PCa patients already treated (105). Recent studies revealed that the cut-off point used to decide to undergo a biopsy should be adjusted and stablished depending on the prevalence of the disease and based on ethnicity (109).

- 4Kscore test: test that relies on the measurement of the combination of four prostate-specific kallikreins in the blood [total PSA, free PSA, PSA single chain intact PSA (iPSA), and human kallikrein 2 (hK2)] which has shown to improve the accuracy of PSA alone (110). However, its availability is limited (only specific laboratories around the world are able to carry out the analysis), mostly, due to the difficulty of measuring hK2 and iPSA. The panel includes not only the biomarkers but also other variables: age, DRE and information from previous biopsies. Its accuracy is higher for the prediction of high-grade PCa

(defined as GS ≥7) than for any PCa. The prospective validation published by Parekh *et al.* showed a reduction of unnecessary biopsies in up to 43% with a cut-off probability of 9%, although missing 10% of the diagnoses of high-grade PCa (111). Furthermore, 4Kscore test increased the prediction for Sig PCa in the prostatectomy specimens of a model based on clinical and pathological variables (age, DRE, PSA and biopsy result) from 0.81 to 0.84. This implementation would avoid surgery in 110 out of 334 patients (112). Further analysis in active surveillance proposes this test as a putative marker to better categorized the risk of reclassification, with a reduction of 27% in the number of biopsies only missing 6% of tumors with a GS ≥7 (113).

- *PCA3*: The expression of prostate cancer antigen 3 (114) is analyzed in urine, after a DRE, by the quantification of *PCA3* mRNA copies. The assay measures the concentration of *PCA3* and *PSA* RNA molecules and calculates the ratio of *PCA3* RNA molecules to *PSA* RNA molecules (PCA3 score). This test has the FDA approval for patients with previous negative biopsy. The cut-off points are not completely defined and vary between 23 and 35. Selecting the cut-off at 35 reached a sensitivity and specificity around 54-58% and 72-74%, respectively. It also correlates with the stage of the disease and predicts Sig PCa (115,116). In other settings, such as active surveillance, its role is not clearly established, showing worse results than others biomarkers previously described (117).

- TMPRSS2-ERG: The v-ets erythroblastosis virus E26 oncogene homolog 2 (ETS2) is a common event in PCa development with a high specificity for its diagnosis. Its measurement together with the *PCA3* in first pass urine improved the predictive ability of the ERSPC-RC from an AUC of 0.79 to 0.84 (115,118).

With this encouraging result, a commercial test combining both biomarkers with PSA in blood has been developed to predict the risk of aggressive PCa (119).

- SelectMDx: This is a new biomarker evaluated in urine after DRE which assesses urinary Homeobox C6 (*HOXC6)* and Distal-Less Homeobox 1 (*DLX1)* mRNA expression levels combined with traditional clinical risk factors (i.e., PSAD, DRE, PSA, age, history of prostate biopsy, and family history). This test is able to detect clinically significant PCa accurately, reducing the number of unnecessary prostate biopsies in a multicentre prospective study (120).

- Urinary exosomes: Exosomes are nano-vesicles excreted in urine from benign and malignant cells (114,121). They constitute a new non-invasive source of data from the tumor with potential diagnostic ability. It has been shown that there is no need of a DRE before urinary collection for their detection. Inside these vesicles, there are mRNAs, miRNAs, proteins, etc., that have been studied as putative markers by different groups (122). From all these studies, the one carried out by Donovan *et al.* (presented for the first time in ASCO 2015), showed how the analysis of three genes (*ERG*, *PCA3* and SAM Pointed Domain Containing ETS Transcription Factor (*SPDEF)*) could reach a negative predictive value (NPV) of 98.6% for high-grade PCa in those patients with a PSA between 2-10 ng/ml.

- Other markers based on RNA: Apart from those previously mentioned, inhibitor serine peptidase Kazal types 1 (SPINK1) and alpha-methylacyl-CoA racemase (AMACR) are also considered new biomarkers for PCa. The first one is an inhibitor serine peptidase Kazal type 1 whose urinary levels are associated with biochemical recurrence after radical prostatectomy in a specific negative *ETS*-gene population (123). The second one is an alpha-methylacyl-CoA

racemase which reach a sensibility and specificity over 70% for the diagnosis of PCa when its urinary levels are categorized by a specific cut-off point (124). Recently, a new 3 genes panel (*HOXC6*, Tudor Domain Containing 1 (*TDRD1)*, and *DLX1*) has outperformed *PCA3* in the diagnosis of Sig PCa, reaching an AUC of 0.8 when combined with the PSA (125). MicroRNAs (miRNAs) are small non-coding RNA that regulates gene expression in different biologic pathways. Up to date, some of them have been studied in blood and urine with a predictive and prognostic capacity, but the results are too preliminary and external validation are pending (115).

- Markers based on DNA: The analysis of DNA in urine has an advantage which is its relative stability and easy preservation (126). One of the most studied genes is the Glutathione S-Transferase Pi 1 *(GSTP1)*, whose promotor region is hypermethylated in almost 90% of prostate tumor cells. In a meta-analysis, which evaluated the marker in serum, plasma and urine over different populations, it was shown a high specificity of almost 90%, supporting this target gene as a very interesting marker (127). Its utility has been demonstrated when it is added to a predictive clinical model and the promotor of *APC* (128) increasing the AUC from 0.69 to 0.82 for the prediction of Sig PCa in prostatectomies specimen of patients with clinically low-risk disease, suggesting a relevant role in active surveillance (AS) management (129).

- New technologies in metabolomics and proteomics make them interesting options to perform massive analysis in urine and blood samples by liquid chromatography coupled to tandem-mass spectrometry, liquid chromatography–mass spectrometry (LC-MS/MS) (130–132) or gas chromatography (GC-MS) (133), among other techniques. In this sense, Jedinak *et al.,* found three proteins

(β-2-microglobulin (β2M), pepsinogen 3 preproprotein (PGA3), and mucin 3 (MUC3)), whose evaluation in urine could discriminate with a good accuracy between benign and tumor disease (AUC= 0.71) (134). Another protein explored was Engrailed-2 (EN-2) (Further information in Engrailed-2 and Prostate Cancer will be described below).

Within the most studied metabolites is sarcosine which was shown as a putative marker for early detection and aggressiveness prediction, although its usefulness was contradictory due to the negative results in the validations cohorts (135). Despite the metabolite profiles have not been explored as deep as genomic and proteomics, continuous work in normalization and technologic innovation is being carried out to cope with this gap in a promising field (122).

I-4.2.1. Proteomics/Peptidomics and Prostate Cancer

Proteins are the primary functional macromolecules of the cell. As cancer is a heterogeneous disease, its molecular features are not expected to be simple. A wide variety of proteins are dynamically up or down-regulated by cancer-related signalling, making the tumor tissue a tangle of proteins and secreted factors. In line with this, a study of cancer proteomics in a cell can include more than 1.5 million proteins (136,137). Proteomics is a discipline which has as main objective to improve disease management. Two of the main techniques implemented for peptide analysis are LC-MS and CE-MS (capillary electrophoresis–mass spectrometry). Nowadays, CE-MS seems to be more frequently used than LC-MS in clinical studies or in patient assessment, likely as a result of the increased reproducibility. However, a minor disadvantage of CE-MS may be the low loading capacity. This fact is of little relevance in profiling

studies for diagnostic purposes, since the analysis (typically not involving peptide sequencing) can be successfully performed with small quantities of starting protein material (138,139); that is the reason why CE-MS has been implemented in clinical peptidomics rather than proteomics (140,141).

Different approaches have been implemented to study protein as biomarkers of PCa in fluids (i.e. urine) (122). Most of them exploring only one protein or a specific panel of proteins such as UDP-galactopyranose mutase (UGM), Engrailed-2, Hepatocyte grow factor (HGF), IL-18BPa and others (142–144), but with the recent experimental approaches, a widely range of candidates can be explored.

CE-MS studies have previously demonstrated some urinary tumor specific peptides in an ample set of different tumors, most of them corresponding to fragments of collagen chains (145), possibly reflecting molecular changes in the extracellular matrix (ECM) organization and the altered activity of ECM-degrading proteases during tumor progression (146). A study from Theodorescu *et al.* showed a down-regulation of collagen fragments in PCa patients compared with negative biopsies. Collagen α-1 (III) and Collagen α-1 (I) are substrates of matrix metalloproteinases (MMP), a group of zinc finger endopeptidases with partially overlapping substrate specificity (147). Regulation of MMP activity has been found for different cancers (145,148,149). Considering the relation of these proteins with aggressiveness, studies evaluating these markers in clinically significant PCa versus non-significant PCa are awaiting.

I-4.2.2. Ghrelin O-acyltransferase and Prostate Cancer

Ghrelin system is a pleiotropic and complex system composed of several components, including ligands (e.g. native-ghrelin and In1-ghrelin variant) (150), and

receptors (GHSR1a and truncated GHSR1b) which are able to regulate multiple physiological processes (i.e. glucose/insulin-homeostasis, hormonal-release or cell-proliferation). Importantly, native-ghrelin and In1-ghrelin peptides share the initial 13 aminoacids (aa) and can be exclusively acylated (addition an octanoyl-group at the Ser-3) by the ghrelin O-acyltransferase (GOAT) enzyme, which is required for the binding and activation of the GHSR1a and, to exert the majority of their functions (151,152)**(Figure 4).**

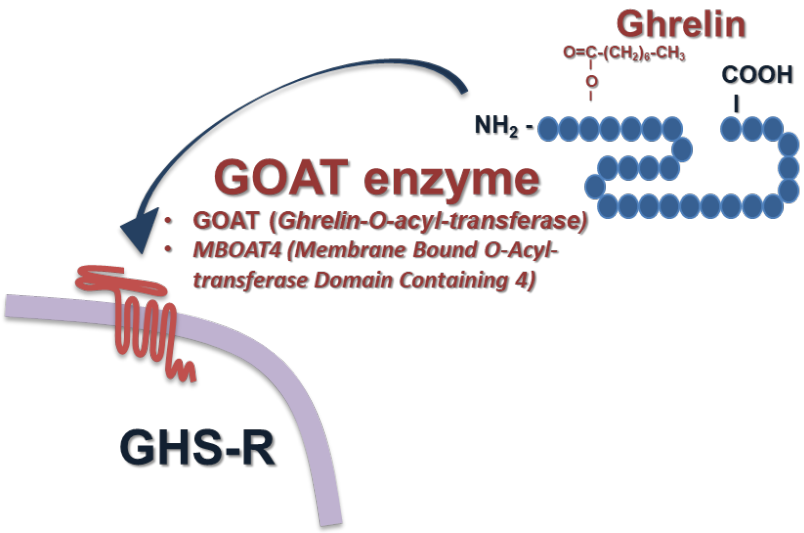

Figure 4. Ghrelin binds to its receptor but to do that, ghrelin needs to be modified with a unique octanoyl group by the GOAT enzyme.

GOAT enzyme is mainly expressed in stomach and pancreas but also in a large number of tissues (including the prostate) (153,154) and its levels are detectable and positively correlated with BMI in plasma (154). Interestingly, it has been demonstrated that GOAT is overexpressed in some tumor-tissues [i.e. breast, pituitary and neuroendocrine tumors (NETs)] compared with normal-tissues; however, to date, very limited data are available on the usefulness of GOAT as putative diagnostic marker in PCa (154,155).

The presence of GOAT in different types of human prostate samples (invasive and non-invasive) obtained from patients with and without PCa, and its relation to clinical and metabolic parameters of the patients (i.e. presence of metastasis, diabetes, dyslipidemia, etc.) has been recently evaluated. Interestingly, it has been reported that GOAT enzyme can also be directly secreted by PCa-cells and is consistently overexpressed in samples from PCa-patients (tissue/plasma/urine) where its expression seems to be conditioned by the metabolic-status. Furthermore, GOAT expression showed a high sensitivity/specificity in PCa discrimination, especially in non-diabetic patients, showing the possibility of considering this metabolic enzyme as a putative novel non-invasive PCa-biomarker alone or in combination with other biomarkers to provide a better PCa diagnosis (155).

I-4.2.3. Engrailed-2 and Prostate Cancer

Engrailed (*EN*) gene subfamily belongs to the Homeobox family which shares similar homeodomains. Specifically, homeobox genes include a hundred of members that encode different homeodomain proteins. Each homeodomain is constituted of three α-propellers, two of which have a *helix-turn-helix* conformation (the same as several transcription factors that bind DNA by interacting with the minor groove), while the other propeller ("recognition propeller") binds additionally with DNA bases directly by the mayor groove. Most of the homeodomains recognize a basal DNA element highly preserved which acts as promotor in multiples genes (TATA), being the T end in 5′ sense the key point for this recognition (so mutations in this base would not be able to bind the homeodomain). Homeobox gene family is subdivided in three main subfamilies: *HOX,* paried box *(PAX)* and *EN* genes.

HOX genes have been involved in several biological pathways that include cellular differentiation, homeostasis and functional maintenance of some adult organs and tissues (156). *In vivo* studies have shown the role of some genes of this family such as *HOXA9-11, HOXA13, HOXD13* and *HOXB13* in embryonic prostate development. Interestingly, the presence and role of some gene subtypes has been studied in prostate. Specifically, *HOXB13* has been found to be overexpressed in prostate tissue and androgen-dependent prostate cancer cell lines, but its expression is lower in androgen-independent ones (157,158). Its functional properties have been explored showing that it is able to promote cell-proliferation through developing an E2F pathway activation by p21 regulation (159). Moreover, *G84E* gene mutation in *HOXB13* is associated with an increased risk of hereditary PCa (160). Additionally, *HOXC* 4, 5, 6 and 8 are overexpressed in PCa cell lines and metastatic lymph nodes, with an association of HOXC8 with a loss of cell differentiation and higher GS (161,162). As previously described, HOXC6 is a promising non-invasive marker within a urinary panel of three compounds (HOXC6, TDRD1 and DLX1), showing an AUC of 0.77 which is better than previously well-known and stablished biomarkers such as the PCA3 or the PSA (0.68 and 0.72, respectively) (125).

PAX gene family has been less studied in PCa. Nevertheless, there are some data showing a possible functional role. Specifically, Gibson *et al.,* demonstrated that the inhibition of PAX expression results in alternate cell death pathways in PCa cells differing in p53 status (163).

Another subfamily comprises the *EN* genes. In vertebrates two variants have been described: Engrailed–1 (*EN1*) and Engrailed–2 (*EN2*), both showing similar properties. *EN1* is located in chromosome 2 (2q14.2) and *EN2* in chromosome 7 (7q36.3) (164). EN proteins have five different functional domains **(Figure 5).** The

homeodomain sequences confer the capacity of association with cytoplasmic vesicles and protein secretion, although the mechanism of secretion and internalization are not currently identified (165,166). A transcription and translation capacity have been attributed to these homeodomain sequences due to its affinity for binding directly to the translational eukaryotic factor 4E (167) in its N-terminal sequence. The main role of the EN proteins have been established as guidance for neural and embryonic axonal development (168). Meanwhile, its role in adults has been less explored until recently, when a high expression in EN-dependent neural cells development has been found (169), and also in different cancerous tissues such as glioblastoma, colorectal, ovary, breast or bladder tumors (170,171).

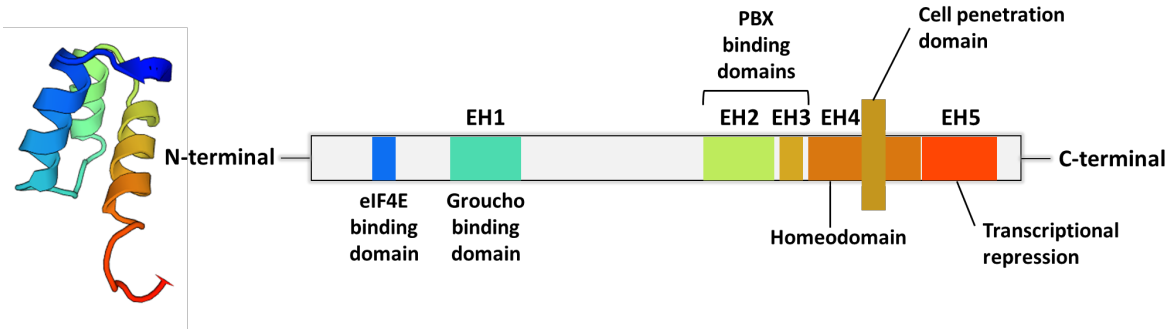

Figure 5. EN protein functional domains(168).

In 2008, the *EN2* expression in PCa cell lines was described for by Bose *et al.* (172), which demonstrated the influence of *EN2* expression in tumor survival, in the way that cell-proliferation significantly decreased when *EN2* expression was inhibited by "small interfering RNA" (siRNA). This work also demonstrated the link of *EN2* with *PAX2*, showing how its inhibition dysregulate *EN2* and vice versa. Later on, Morgan *et al.* bear out the overexpression of *EN2* in both androgen-dependent and androgen-independent cell lines, and in human tissue by quantitative analysis and

immunohistochemistry. Interestingly, it was demonstrated by immunohistochemistry that EN2 was expressed in the luminal area of the prostatic ducts and together with the information of secretion and internalization properties suggested a possible specific detection in urine (143). In fact, the expression of EN2 was initially evaluated in the first pass urine (5-10 mL) without prostate stimulation in a case-control study using as control group totally healthy people and patients with elevated PSA level but a negative prostate biopsy, as well as a custom-made antibody, not externally validated or commercialized. In this study, they showed with a stablished cut-off of 42.5 µg/L, that EN2 was overexpressed in PCa patients (66%) versus control (10-15%). These results allowed them to propose this protein as a putative urinary marker of PCa. However, even with a different average expression between groups, there were a huge absolute variability with ranges in both groups of 1.9-175 µg/L for control and 1.9-6510 µg/L in PCa patients (143). After that, the IMPACT cohort, which includes patients with high-risk of being diagnosed with PCa due to *BRCA1* and *BRCA2* mutations, was used to validate their results (14). They used the archived urine, and they found similar results with percentages of overexpression of 66% in PCa group versus 10% in the control group. Again, the level of expression was heterogeneous, and a significant number of patients did not undergo prostate biopsy; therefore, concluding that these results should be evaluated with caution. In a multivariate analysis, EN2 expression and not mutational status was a predictive factor of PCa (173).

Additionally, the association between EN2 levels and tumor volume and differentiation has been also studied. Specifically, Pandha *et al.* studied not only first pass but mid urine in patients before undergoing a prostatectomy demonstrating the existence of a positive correlation between expression levels of EN2 and tumor volume and stage, but not with tumor differentiation (GS) (174,175). In line with this, Marszall

et al. studied protein expression in urine with and without prostate stimulation using a commercial ELISA kit (EIAab Human Homeobox protein engrailed-2 ELISA Kit, Wuhan EIAAB Science Co., LTD, Whuan, China, catalogue number: E1851h). This study did not found difference in urine protein levels between patients with a without PCa without prostate stimulation, but this difference was found in urine collected after prostate stimulation [control average expression levels: 0.41 (0.00–1.93) ng/ml vs. tumors: 1.54 (0.00–7.25) ng/ml], with an association with a higher GS and tumor volume (T2c vs. T2a-b) (176).

I-4.2.4. Multiparametric prostate magnetic resonance imaging and Prostate Cancer Diagnoses

Multiparametric prostate magnetic resonance imaging (mpMRI) has an increasingly large role in the early detection and staging of PCa. Currently, mpMRI includes morphologic sequences (T1 and T2) that provide anatomical information about the gland, and diffusion-weighted imaging (DWI) and dynamic contrast-enhanced imaging (DCE) sequences that provide functional information that can help to classify the different findings of the morphologic sequences. The Prostate Imaging Reporting and Data System version 2 (PI-RADS v2) is a guideline for the interpretation of mpMRI results. It was published in 2015 as a method to decrease variability in the acquisition, interpretation and reporting of studies (177) (**Figure 6**).

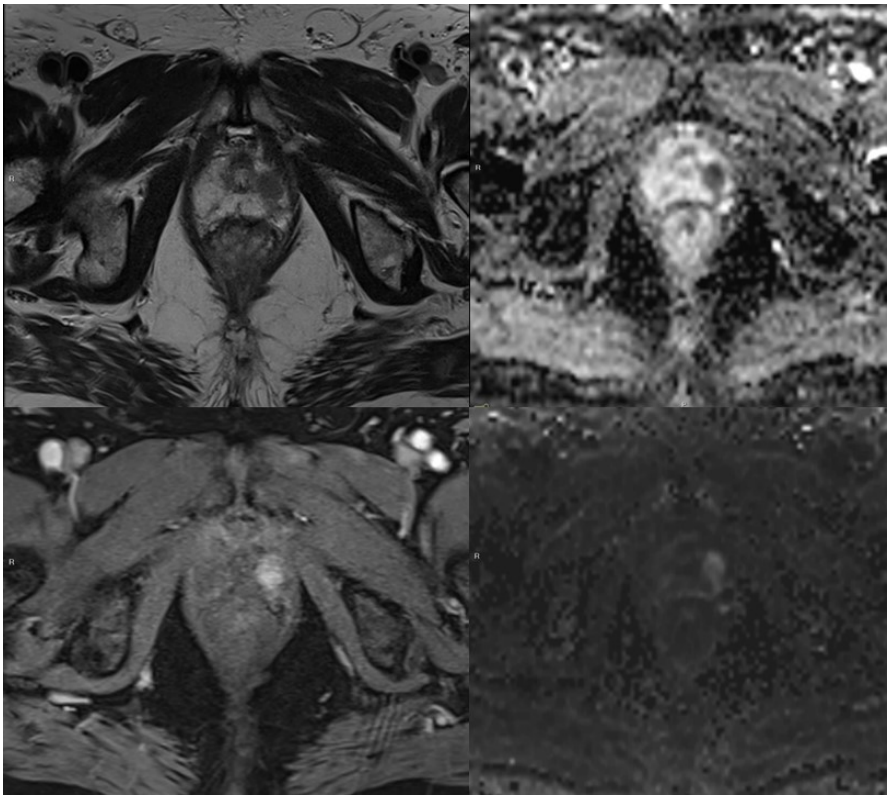

Figure 6. From up-down/left-right. T2 sequences, ADC value, contrasted enhanced T1 sequence and diffusion b-1400 value sequence showing a lesion PIRADS 4 in the left posterior peripheral zone of the apex.

Clinically, Sig PCa is defined in PI-RADS v2 based on the current uses and capabilities of mpMRI and MRI-targeted procedures: GS ≥7 (including 3+4 with prominent but not predominant Gleason 4 component), and/or volume ≥0.5 cm^3, and/or extra prostatic extension. PI-RADS v2 assessment uses a 5-point scale based on the likelihood (probability) that a combination of mpMRI findings on T2W, DWI, and DCE correlates with the presence of a clinically Sig PCa for each lesion in the prostate gland. The five PI-RADS v2 assessment categories are (**Figure 7**):

- PIRADS 1. Very low (clinically Sig PCa is highly unlikely to be present).
- PIRADS 2. Low (clinically Sig PCa is unlikely to be present).
- PIRADS 3. Intermediate (the presence of clinically Sig PCa is equivocal).
- PIRADS 4. High (clinically Sig PCa is likely to be present).

- PIRADS 5. Very high (clinically Sig PCa is highly likely to be present).

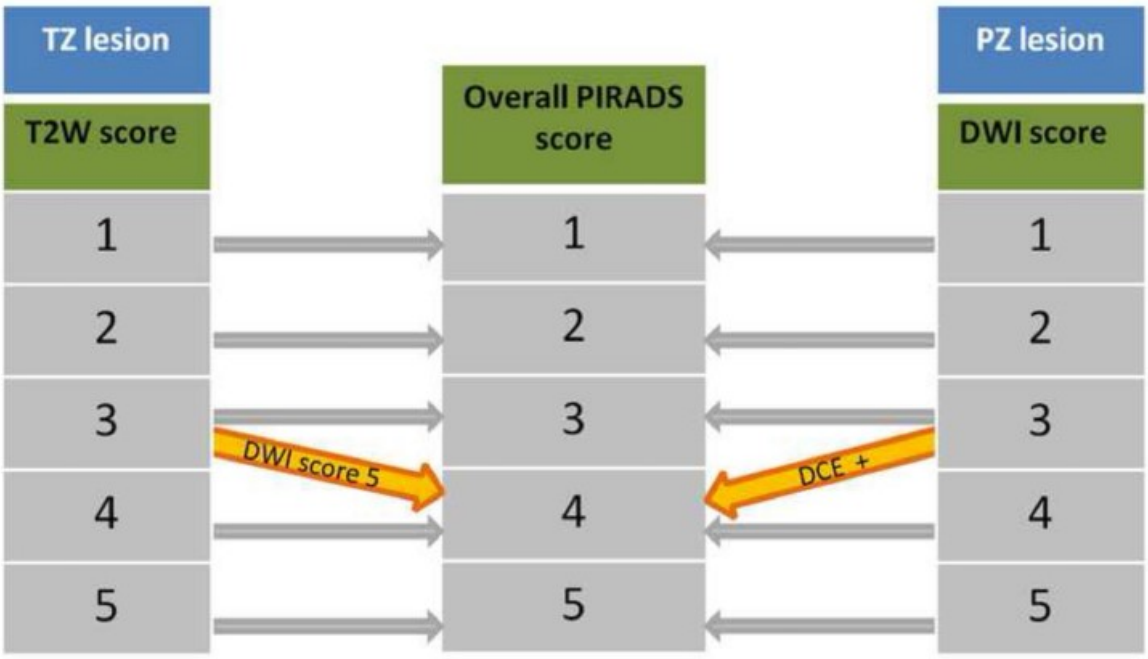

Figure 7. Stepwise approach to assign an overall PI-RADS score based on zonal location. TZ: transition zone. PZ: peripheral zone (178).

MpMRI accuracy for the detection of PCa varies between different clinical situations. When performed in the evaluation of patients with elevated PSA levels with previous negative prostate biopsy, mpMRI has been shown to identify clinically Sig PCa which would have been otherwise missed by routine systematic biopsy (179). Therefore, the evidence supports the role of mpMRI as cost-effective approach in patients with previous negative biopsy, while this is still less robust for biopsy naïve patients, justifying, to-date, the strong recommendation by the European guidelines only in patients with previous negative biopsy (83). However, recent studies mpMRI demonstrated promising results in both detection and exclusion of PCa, using an extensive prostate mapping biopsy as the referent or randomized strategy approach (180,181).

Given the current controversies regarding PCa screening, at least as important as the ability of mpMRI to detect clinically significant disease, is the potential for this screening strategy to reduce the over diagnosis of clinically insignificant PCa. This is based on its high NPV which, although it depends on the prevalence of Sig PCa in the cohort and definitions of positive mpMRI and Sig PCa, has been shown to be really high, reaching around a 88% [95% CI (77–99%)] (182).

Based on all this information, current EAU guidelines recommends to perform a mpMRI in patients at risk of PCa (183).

I-4.3. PCa pathology

Pathologic evaluations of PCa are based on Gleason grading. This system was created by Donald F. Gleason in 1966, and it has been modified since then, especially with a new system already accepted by the World Health Organization (WHO) for the 2016 edition of Pathology and Genetics: Tumors of the Urinary System and Male Genital Organs (184,185) (**Table 1**). The Gleason system is based on the glandular pattern of the tumor, identified at relatively low magnification, and ranges from 1 (most differentiated) to 5 (least differentiated).

GS of biopsy-detected PCa comprises the GS 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 GS. For three grades, the GS comprises the most common grade plus the highest grade, irrespective of its extent (83).

When reporting prostatectomy specimens, the GS is the sum of the most and second-most dominant (in terms of volume) GS. 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 GS (5% rule). Primary and secondary grades are reported in addition to the GS. A global GS is given for multiple tumors, but a separate tumor focus with a higher GS should also be mentioned.

Table 1 . Brief description of current pathology classification

New Group	Previous Gleason Score	Description
Grade Group 1	≤6	Only individual discrete well-formed glands
Grade Group 2	3+4 = 7	Predominantly well-formed glands with lesser component of poorly formed/fused/cribriform glands
Grade Group 3	4+3 = 7	Predominantly poorly formed/fused/cribriform glands with lesser component of well-formed glands
Grade Group 4	4+4; 3+5: 5+3 = 8	Only poorly formed/fused/cribriform glands or predominantly well-formed gland and lesser component lacking glands Predominantly lacking glands and lesser component of well-formed glands
Grade Group 5	9-10	Lacks glands formation

Due to its misleading clinical implications, GS 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 (186,187). Therefore, from practical standpoint, Gleason pattern in contemporary practice starts at 3 and GS starts at 6 in prostate biopsy specimens and most transurethral resection of prostate and radical prostatectomy specimens.

Most studies and the present doctoral thesis is based on ISUP 2005 GS classification (186). The recently accepted new ISUP 2014 Gleason grading (WHO 2016) (184) represents a compression of GS ≤6 to ISUP grade 1, and GS 9-10 to ISUP grade 5, whereas GS 7 is expanded to ISUP grade 2, i.e. 7 (3+4) and ISUP grade 3, i.e. 7 (4+3) showing a better prognosis correlation (188).

I-5. *In vitro* models for PCa research

In order to carry out translational research, it is essential the use of *in vitro* models in which tumor biology, markers expression and the functional role of different genes and proteins can be evaluated. Also, they are the first step to test potential novel treatments. In this line, multiple prostate cell lines have been developed and evolved during the last decades. They embrace from normal prostate to androgen-independent PCa cell lines, including different PCa stages and phenotypes. The first developed and still most used are LNCaP, PC-3, DU145 and 22Rv1 cell lines, and also normal prostate cell lines such as RWPE-1. Some of them, which have been used in this doctoral Thesis, are described below (for further information, the following references are recommended (189,190):

- **LNCaP:** cell type obtained from an aspirate of a lymph node metastasis in a Caucasian middle-aged patient (191). They express both androgen and estrogen receptors, PSA and hk2 in RNA and protein forms, but also Creatine kinase (CK)- 8 and 18, and "wild type" (WT) *TP53* gene (192). Characteristically, androgen

receptor has a T877A mutation that confers an increased response to steroids (193). Population doubling time is approximately 60 hours in a medium with foetal calf serum (FCS) concentrations of 2.5% to 10%. More than 60 cell line subtypes with specific characteristics have derived from the original (189). One of the main problems of this cell line is that cells adhere loosely to the substrate and are easily detached by tapping, shaking or pipetting. At high densities cells detach as sheets, making cell counts unreliable. Trypsinization of cells results in cell clumps, also making accurate cell counts difficult (194).

- PC-3: The origin of this cell line in a lumbar bone metastasis of a 62-year-old patient (195). Cells do not express AR and its grow is independent of androgen. PC-3 cells are 100% aneuploid, express CKs 5, 8 and 18, and contain a frameshift mutation in *TP53* that results in a premature stop codon (192). They need a medium with FCS concentrations of <1% to achieve a double time growth of 8.2 hours. There are some subtypes of this cell line (190), but not as much as of LNCaP.

- 22Rv1: It is a line originated from the primary CWR22R of a Gleason 9 PCa of a patient with bone metastasis. It was developed when to surmount overgrowth with mouse cells. Cells were grown on irradiated feeder cells to promote epithelial growth, trypsinized, stained for CD44 and sorted for a specific population of CD44 positive cells. Regrowth on feeder layers and sorting was repeated for an additional 2 times to derive the 22Rv1 line (196). Its cells express PSA (mRNA) and RA (protein), and also CK - 8 and 18 and have a p53 - Q331R mutation, and a H874Y mutation in the RA (192,197). 22Rv1 has a doubling time of 35 to 40 hours when grown on plastic.

- RWPE-1: This is a normal prostate cell line from the peripheral zone of the prostate of a 54-year-old man. The cells were immortalized with Human

papillomavirus -18 (HPV-18). A single cell clone from limiting dilution was selected to generate the RWPE-1 line (198). It stains positive for CKs 8 and 18, and negative for desmin and factor VIII, and cells respond to the synthetic androgen R1881 by increasing growth rate, PSA production and AR levels. Doubling time is 58 hours for RWPE-1(199).

II. Aims of the study

The **general aim of this study** was to further expand our cellular, molecular and clinically-relevant knowledge of PCa by proposing the following **main objectives**:

Objective 1: To further evaluate the effectiveness of different Risk Calculators in the prediction of PCa by analysing and comparing their accuracy and variability.

Objective 2: To explore the association of the metabolic and inflammatory status with the diagnosis of PCa.

Objective 3: To evaluate the diagnostic, prognostic and/or therapeutic value of molecular components of different key pathophysiologic regulatory systems in PCa using non-invasive fluids (blood and urine), tissue samples from PCa and healthy patients, and healthy and tumor prostate cells.

To achieve these main goals, we proposed the following **specific objectives** which have been associated to five scientific manuscripts directly derived from this Doctoral Thesis:

1) To perform a direct comparison of the accuracy and variability between the ERPSC and the PCPT Risk Calculators for the prediction of Sig PCa in patients with PSA <10ng/ml.

2) To examine the relationship and possible impact that inflammatory status, testosterone levels, and metabolic syndrome may have on PCa diagnosis.

3) To perform a proteomic analysis in order to determine the putative value of peptides in urine as diagnostic biomarkers for Sig PCa.

4) To evaluate the usefulness of GOAT enzyme as putative diagnosis marker for Sig PCa and its association with aggressive features.

5) To determine the usefulness of some components of the Engrailed gene subfamily as diagnostic and therapeutic biomarkers in PCa.

III. General results and discussion

This Ph.D. Thesis has been structured in different chapters corresponding to five independent scientific manuscripts, which were carried out to answer the previously proposed objectives.

3.1 Observational study comparing the accuracy/variability between the ERPSC and the PCPT risk calculators for the prediction of significant prostate cancer in patients with PSA <10ng/ml (Article I: *BMJ Open*: Under review)

Risk Calculators (RCs) are easy-to-use tools considering available clinical-variables that could help to select those patients with risk of PCa who should undergo a prostate-biopsy, avoiding unnecessary biopsies and over-treatment. Two of the most well-known RCs are the Prostate Cancer Prevention Trial risk calculator 2.0 (PCPT-RC 2.0) (99) and the European Randomized study of Screening for Prostate Cancer risk calculator (ERSPC-RC) (100).

However, two aspects that are less studied with the use of these RCs should be considered: **1)** the first one is the RC variability. It is an established fact that PSA has a really high intra-individual variability which could reach up to 10-20% (200), so it is always recommended to repeat the measure at least twice, between a not well determined period of time, before indicating a prostate biopsy (89,201). With this information, also the variability of these RCs and its clinical significance when they are used to select patients who should undergo a prostate biopsy should be studied; **2)** another aspect is the direct comparison in the most dubious population which comprises the patients with a PSA <10 ng/ml. In this population the better approach is to compare ERPSC–RC 3 or 4 + DRE versus PCPT v.2 + free PSA. This last variable has not been

included in most of the studies. This fact is really important because, as already published, its addition improves the accuracy for the diagnosis of Sig PCa (99).

For all the reason described above, we have performed a retrospective analysis in the ONCOVER cohort of patients. ONCOVER project (in the Urology branch) is a prospective design study in which urine and blood were collected from patients just before undergoing a prostate biopsy. A consecutive collection of more than 1,000 well clinically characterized biopsies were recruited (2013-2015).

To validate and directly compare ERSPC ¾ + DRE and the PCPT v2 + free PSA RCs, we focused on the patients who most benefit from these RCs, who are those with a PSA between 3-10 ng/ml. 510 patients of the ONCOVER cohort were selected for the analysis, and both RCs showed a good external validation. The comparison between both RCs revealed no significant differences, with an accuracy similar to that of Poyet *et al.* (101) for ESRPC and a better accuracy for PCPT + free PSA. As shown in Supplemental Figure 2 of Article I, the addition of free PSA improved the accuracy of the PCPT-RC [0.65 (0.59-0.71) PCPT1 v.2.0-RC vs. 0.73 (0.67- 0.79) PCPT1 v.2.0 + free PSA–RC; p= 0.02]. In agreement with the accuracy results, the decision curve analysis was also similar between both RCs, whom showed a net benefit from an early risk threshold, which means that their implementation would improve patient selection for prostate biopsy.

On the other hand, ERSPC-RC had better stability for intra-individual PSA variations than PCPT-RC 2.0. This could be simply explained by the fact that two values (PSA and free PSA) that suffer from this variability are used in the PCPT-RC 2.0 RC (202) while the use of an estimated volume in the ERSPC dilutes PSA variability. However, these results should be interpreted with caution, as volume estimation was performed by categorization of TRUS and not by DRE. It is true that this categorization

has been previously shown to be a good correlation (100). This likely depends on prostate volume (203), as well as low but certain inter-examiner variability (204), which could also increase ERPSC variability in an inter-clinician comparison.

Despite the similar decision curve, results from the sensitivity, specificity and ROC curve analysis show that the same risk threshold should not be used for both RC models. Both RCs are able to have similar performance, and the benefit of using any of both is similar in order to screen patients for a prostate biopsy, if the correct cut-off point is selected.

Altogether, our results showed that: 1) the use of both RCs (ERSPC and PCPT) could be a useful tool in the selection of patients who need prostate biopsy, and that both RCs showed similar accuracy for discrimination of Sig PCa; 2) ERSPC-RC showed higher stability than PCPT-RC for intra-individual PSA variations; 3) when comparing both RCs sensitivity and specificity, a higher rate of biopsies could be avoided with the ERSPC-RC vs. the PCPT-RC, but with a higher rate of Sig PCa missed. Thus, in those patients with a PSA between 3-10 ng/ml, these tools should be used in order to improve selection and specificity. The RCs specifically should be selected according to the variables available in the clinic. In addition, both RCs could also be used and the decision to undergo a biopsy be shared with the patient.

3.2. Clinical association of Metabolic Syndrome, C-Reactive Protein and testosterone levels with clinically significant prostate cancer (Article II: *J Cell Mol Med*. 2019 Feb;23(2):934-942. doi: 10.1111/jcmm.13994)

Nowadays, apart from the well-known PCa risk factors (age, race and family history), the contribution of lifestyle and environmental factors in the PCa has emerged as an interesting point of research. Recently, the influence that metabolic syndrome (MetS), hormonal alterations and inflammation might have on PCa risk has been a subject of controversial debate. In fact, MetS has been proposed as a promoter of numerous types of cancer, including PCa (18,27), although there are conflicting results depending on geographic distribution of the cohort and the studied endpoints. Furthermore, this association is mediated by inflammatory and hormonal factors that have also been associated with PCa (32), but clinical studies with common available markers evaluating their association are lacking.

In this study, we evaluated the association of MetS, inflammatory parameters (i.e. CRP) and testosterone levels with the result of the biopsy in a prospective cohort of 524 patients scheduled for prostate biopsy with suspected PCa. The presence of MetS was stablished according to the criteria of the National Cholesterol Education Program Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults, Adult Treatment Panel III criteria (ATP III), or being actively treated with specific drugs of each condition. The levels of hormones and proteins were evaluated in blood collected just before the prostate biopsy.

Our results revealed an association between some metabolic factors (especially obesity) with higher levels of CRP and testosterone. Not only the presence of MetS, but also a greater number of MetS criteria and a higher level of circulating CRP, but not of testosterone, were associated with a higher risk of Sig PCa. Interestingly, when we analyzed each MetS criterion independently, we found that only two criteria, a higher waist circumference and blood pressure (criteria I and V, respectively), were significantly associated with higher risk of Sig PCa. Furthermore, corroborating previous data (50), the association of waist circumference as a quantitative variable was maintained when also adjusting by the BMI. The association of these factors with Sig PCa was shown to be independent from clinical variables commonly used for its diagnosis, however, they do not seem to add significant value as predictive factors to improve the accuracy of a logistic model with clinical variables. Furthermore, CRP, but not the number of MetS factors, was correlated with GS on the biopsy.

An exploratory analysis of the association of drugs intake (i.e. statins and metformin) and the glycated haemoglobin (HbA1c) was further carried out. Specifically, no significant association between HbA1c levels or statin intake and the diagnoses of PCa or Sig PCa was observed in our cohort of patients. The analysis of metformin intake revealed a significant association with an increased risk of Sig PCa even when adjusting by glucose levels and HbA1c [OR= 2.74 (1.41-5.31); p <0.01]. However, these data should be interpreted with caution as only a limited number of patients were under metformin. Furthermore, the previous evidence showing the association of metformin with PCa clearly depends on the chronicity of the treatment which was not controlled in the present study (205).

The results provided by our study further reinforce the recent evidence supporting the association of MetS with Sig PCa (18), which consider obesity and hypertension as individual risk factors (27) and add further evidence of the increased risk of being diagnosed with Sig PCa with a higher number of MetS criteria (206). In addition, it shows for the first time an association between increased CRP levels, metabolic status and a higher risk of Sig PCa, as previous studies focused on the diagnosis failed to demonstrate this association probably due to the high cut-off levels used to investigate the CRP levels (63,64).

In summary, based on the results of this study, we can conclude that: 1) The presence of MetS and a greater number of MetS components was independently associated with an increased diagnosis of Sig PCa on biopsy, 2) CRP level was also independently associated with an increased risk of detecting Sig PCa and correlated with tumor aggressiveness at the time of prostate biopsy.

Altogether, based on the high incidence of MetS worldwide, especially in western countries, and considering the evident connection between some of the components of the MetS, the inflammatory status and the risk of PCa at the time of prostate biopsy, as well as of the association between inflammatory status with the aggressiveness of PCa found in our study, the results of the present study invites to suggest that interventional studies based on the control of MetS and inflammatory status in patients at risk of PCa might be a key point in the overall management of this disease. Therefore, future cellular/molecular/translational studies are crucial to understand the specific connections between individual MetS determinants and the pathophysiology of PCa.

3.3 CE-MS based urinary biomarkers to distinguish insignificant from significant prostate cancer (Article III: *Br J Cancer: 2019* Jun;120(12):1120-1128. doi: 10.1038/s41416-019-0472-z)

PCa is ranked as the second most frequently diagnosed cancer in men, and the most frequent non-skin cancer in developed countries. PCa diagnosis is currently mostly based on serum PSA testing, DRE and confirmed by a multi-core prostatic biopsy. Multiple factors not related to prostate malignancy may affect the level of blood PSA [inflammation, infection or presence of benign prostate hyperplasia]. Therefore, PSA lacks specificity particularly in the intermediate range, with only 22-27% of patients with PSA between 4–10 ng/ml to be positively confirmed with PCa after biopsy(207). Additionally, PSA screening and multicore biopsy have increased the detection rate of small, localized, well-differentiated PCa, resulting in over-diagnosis and over-treatment. For these and other many reasons, alternative diagnostic biomarkers, ideally non-invasive and specific, are necessary for the accurate detection of PCa.

In this sense, a wide variety of proteins are dynamically up or down-regulated by cancer-related signalling, making tumor tissue a tangle of proteins and secreted factors. Recent novel approaches have become available to find putative protein/peptides biomarkers for cancer diagnosis. This becomes more interesting in the case of PCa, wherein the current available biomarkers lack of enough accuracy, and wherein urine is an easy and non-invasive available sample. Specifically, few studies have implemented the capillary-electrophoresis coupled to mass spectrometry (CE-MS) technique to explore peptide biomarkers for the diagnoses of PCa (145,148). However, before the publication of this work, no studies had been implemented to determine a biomarker panel including MS-based biomarkers, which in combination to clinical

characteristics and the state-of-the art risk calculators can lead to improve non-invasive detection of Sig PCa.

For that purpose, a case-control study to detect urinary peptide markers by CE-MS [previously described (208)] was employed in a cohort of 823 patients. The cohort was divided in a training and set validation cohort according to the '2/3–1/3 rule'. Briefly, the samples were filtered by Centrisart ultracentrifugation filters (Sartorius, Göttingen, Germany) to retain proteins/polypeptides below 20 kDa that were subsequently desalted over PD-10 columns (GE Healthcare, Munich, Germany). The peptide extracts are lyophilized and resuspended in high-performance-liquid-chromatography-grade water. Mass spectral ion peaks representing identical molecules at different charge states are de-convoluted into single masses using Mosaiques Visu software (209). The peak list characterizes each peptide by its molecular mass, normalized migration time [min] and normalized signal intensity (210). Normalization of the CE-MS data was based on 29 collagen fragments that are generally not affected by disease and serve as internal standards (211).

The analysis in the training cohort enabled the identification of 19 peptides biomarkers, from which sequences could be obtained for 17, while two peptides could not be sequenced. The majority (14/17) were originated from various collagens. Peptide fragments originating from alpha-1 collagen of types (I), (XI), (XVII), (XXI) and alpha-2 type (I), (V), (IX), were most prominent and fragments of collagen type (VIII) chain were also identified. This most probably is depictive of the ECM rearrangements, resulting in proteolytic products, which are subsequently excreted in urine, so these collagen peptide fragments are oversecreted in Sig PCa cases. A unique motif (pGP) was present in most of the collagen sequences, which is a matrix-derived chemoattractant-derived from proteolytic cleavage of collagen by matrix

metalloproteinases. pGP after cleaved acts as a chemokine that binds to (C-X-C motif) receptors and is thus associated with neutrophil attraction in inflamed tissues (212). The other peptides were fragment of protein phosphatase 1 regulatory subunit 3A, which was identified with decreased abundance and fractalkine or (C-X3-C motif) ligand 1 and Semaphorin-7A, both upregulated in the group of patients with Sig PCa.

The panel of 19 peptides selected in the training cohort (n= 543 patients) were validated in an independent validation set of 280 patients with an AUC value of 0.81 ranged from 0.76 to 0.86 (95% CI) (p <0.0001). Furthermore, the comparison with the ERSPC-RC ¾ (n= 274, after excluding patients under 5-alpha-reductase inhibitors) showed that this panel clearly outperformed the nomogram for Sig PCa diagnoses [19-biomarker model vs ERSPC-RC; AUC= 0.82 (0.76-0.86) vs 0.69 (0.63-0.74), respectively (p= 0.02)], also in the decision curve analysis.

These results, although must be verified in a prospective trial to also assess the actual value in the context of patient management, are highly significant as discrimination between clinically significant and non-significant PCa is expected to have a positive impact on reducing over-treatment and the associated costs of unnecessary biopsies, improving patient compliance and alert the urologists to perform a more thorough examination in case of a positive result. In fact, the data presented in this study could demonstrate the utility of a multiple marker approach for improved non-invasive detection of Sig PCa. Taking into consideration the increased variability which is caused by the high intra-tumor heterogeneity, an intrinsic characteristic of cancer, a single biomarker is not expected to enable the discrimination of Sig PCa from non-significant with high accuracy. Therefore, a combination of biomarkers appears to be the currently best option to guide biopsies and active surveillance.

3.4 Plasma ghrelin O-acyltransferase (GOAT) enzyme levels: a novel non-invasive diagnosis tool for patients with significant prostate cancer (Article IV: *J Cell Mol Med*. 2018; Nov;22(11):5688-569. doi: 10.1111/jcmm.13845)

GOAT is a key enzyme regulating ghrelin system activity which has been shown to be overexpressed in PCa tissues (at the mRNA and protein level) (153–155) and plasma levels (155), but its putative role as non-invasive biomarker of PCa in patients with PSA levels ranging 3-20ng/ml (wherein precision of PSA is remarkably poor), and for the diagnosis of significant PCa (Sig PCa) has not been previously studied.

Therefore, in this study we aimed to evaluate the putative role of GOAT as diagnostic biomarker of PCa in a case-control study of 312 patients divided into three groups: PCa patients, patients at risk of PCa but negative result in the biopsy and healthy controls. GOAT plasma levels were evaluated by a commercial ELISA kit (MyBioSource, San Diego, USA) and the putative association with the PSA levels, and with aggressive features of PCa was explored. GOAT plasma levels were significantly higher in patients with PCa compared to healthy patients and patients at risk of PCa but negative result in the biopsy. Furthermore, these levels were even higher in patients diagnosed with Sig PCa (Gleason ≥7). The subpopulation of patients with a PSA between 3-20 ng/mL (which includes most of patients that usually undergo a prostate biopsy and wherein the PSA accuracy drops significantly) was selected to evaluate GOAT accuracy and compare it with accuracy of PSA. The analysis revealed that GOAT accuracy for the diagnosis of Sig PCa was better than PSA in this population (where PSA accuracy was dramatically low) [n= 77 Sig PCa patients; GOAT: AUC= 0.612 (0.531-0.693) vs. PSA: AUC= 0.494 (0.407-0.580); p= 0.035] and that its combination with other clinical variables (i.e. DRE, age and testosterone) further improved its predictive capacity and outperformed PSA for the diagnosis of Sig PCa

using the same model [GOAT: AUC= 0.720 (0.71-0.73) vs. PSA: AUC= 0.705 (0.695-0.716); p <0.001].

The analysis of the association of GOAT levels with an aggressive feature showed a correlation between GOAT levels with GS (R= 0.24; p= 0.001), and an association with a higher risk of being diagnosed with metastasis (OR= 1.01 95% CI (1.00-1.03); p= 0.03). Furthermore, an exploratory analysis of the patients initially treated with hormonotherapy (n= 19) indicated a tendency in the association of plasma GOAT levels with an earlier CRPC status [OR= 1.009: 95% CI (0.997-1.021); p= 0.145].

When viewed as a whole, the results of the present manuscript indicate that GOAT levels are markedly elevated in Sig PCa and are associated to aggressiveness features in PCa (i.e. GS and presence of metastasis), together with previous results of our group which showed a correlation of GOAT levels with In1-ghrelin variant levels (an alternative splicing variant of Ghrelin that confers aggressive features to PCa) in PCa (213), suggest that GOAT enzyme and In1-ghrelin variant could be functionally linked in PCa, where In1-ghrelin variant might be the primary target of GOAT, and that an autocrine/paracrine circuit involving these two components of the ghrelin system may possibly operate in PCa to increase aggressiveness features of PCa cells, setting the stage for future investigations.

Finally, and based on these results, it could be concluded that the measurement of plasma GOAT levels, in combination with PSA and/or an additional panel of clinical variables measured in PCa (i.e. age, DRE and testosterone levels), might be considered as a novel, complementary and non-invasive tool to provide a better diagnosis of PCa, especially for Sig PCa and for patients with grey-zone PSA levels, as well as a putative tool for the prediction of PCa aggressiveness.

3.5 Oncogenic role of secreted engrailed homeobox 2 (EN2) in prostate cancer (Article V: *J Clin Med* 2019;8, 1400*;* doi:10.3390/jcm8091400)

The homeodomain-containing transcription factors is a gene family that determines cell/tissue identity during normal embryonic development and which have been shown to be re-expressed by different tumor cell-types (214). Engrailed genes [Engrailed (*EN*) 1 and 2] are members of this family that mainly involve in neural development (168) but also have been shown to be expressed in different tumors, including PCa (170,171,174). Previous studies suggested that the engrailed variant 2 (EN2) which PCa-cells overexpress and secret, might serve as a potential diagnostic biomarker, however, its presence and functional role in PCa-cells is still controversial or unknown (174–176,215,216). In fact, the pathological role of EN1 has been poorly explored hitherto, and, consequently, it is not known if this factor could provide novel therapeutic targets for this highly incident and prevalent pathology.

Therefore, based on the information mentioned above, the objectives of this study were: 1) to analyze the utility of EN2 as a non-invasive diagnostic biomarker by measuring its expression and secretion levels in different, independent cohorts of samples from PCa patients and controls (prostate-tissues and urine); and 2) to investigate the oncogenic role of EN2 and its underlying molecular mechanisms as well as its putative value as a therapeutic target in PCa by using different prostate cell lines [normal (RWPE-1) and tumoral (LNCaP and PC3) cells] and diverse experimental approaches.

Firstly, EN2 was overexpressed in our two cohorts of PCa tissues compared to control and in tumor cell lines compared with normal-like RWPE-1 cells. This profile was corroborated *in silico* in two independent data sets (TCGA, MSKCC and

GRASSO). Consistently, urine EN2 levels showed higher levels and percentage of detection of EN2 in urine in patients with tumor (75%) vs controls (45%) (p= 0.05). Previous studies with this marker showed different results depending on the part of urine evaluated (143,173–175,217). Our data were in agreement with the results previously reported by Morgan *et al* (143), which also showed higher expression in mid-urine, not necessarily after prostate stimulation. Next, we confirmed EN2 overexpression in PCa cell lines (LNCaP and PC-3) vs. normal prostate cell line (RWPE-1) and, consistently, EN2 protein was found to be secreted from PCa cell lines (determined by ELISA in medium), while its levels were under the detection limit in the normal RWPE-1 cells. One of the most striking features of EN2 is that its protein does not seem to be localized in the nucleus of PCa cells but close to the luminal border of the cells, associated to secretory blebs (143). This is, indeed, consistent with the observation that cells from different established PCa cell lines can release EN2 protein to the medium [data presented herein and in Morgan *et al.*(143)], which, with previous studies suggesting its putative tumorigenic role (172), suggests that secreted EN2 could play a pathological role in PCa that remain poorly known.

In line with this, we have demonstrated that EN2 enhance tumorigenic potential in prostate cells, i.e. an increased capacity to proliferate, migrate or secrete PSA, by modulating certain signaling pathways. In particular, treatment with exogenous EN2 protein elicited an increase in the proliferation capacity of the PCa cell lines LNCaP and PC-3, an increase in the capacity to migrate of normal-like RWPE-1 and PC-3 PCa cells and an increase in PSA secretion from LNCaP cells, which are, all of them, parameters directly associated to the tumorigenic capacity of these cells (218). Interestingly, our results also show an increase in the phosphorylation rate of full-length AR in LNCaP and full-length and splicing variants of AR in 22Rv1, as well as an increase in

phosphorylation rate of AKT in LNCaP. Emerging evidence demonstrates a key role for the PI3K-AKT-mTOR signalling axis in the development and maintenance of CRPC stage (219). Moreover, preclinical studies have explained a direct connection between the PI3K-AKT-mTOR and AR signaling axes, revealing a dynamic interplay between these pathways during the development of androgen deprivation therapy ADT resistance (220), which suggest a putative role of EN2 by this interaction.

Next, a further exploration of dysregulated specific PCa genes was carried out. A PCa genes array analysis, with a 2-fold-change cut-off point, revealed that three genes were dysregulated after EN overexpression, showing an upregulation of *PSTGS1* and *EGR3*. Interestingly, these two genes are involved in the association between inflammation and cancer, mainly by upregulating cytokines such as IL6 and 8 (221,222). In contrast, *GSTP1* was downregulated, which has been shown to be hypermethylated in PCa and correlated with aggressive features (223).

When viewed together, our results provide compelling evidence to support the potential value of EN2 as a non-invasive diagnostic biomarker for PCa, and offer, as well, novel valuable information to consider its putative utility to develop new therapeutic tools in this pathology. In particular, we expanded and validated the higher expression of EN2 in PCa tissue vs. normal prostate, as well as its elevated levels in urine samples from PCa patients. In addition, we demonstrate herein, for the first time, that secreted EN2 protein could act as a tumorigenic factor in normal and tumoral prostate cells, by modulating key functional parameters and signaling pathways. Therefore, these data invite to explore further the identification and development of novel therapeutic targets related to *EN2* in this highly incident and prevalent pathology.

IV. General conclusions

The **main conclusions** associated to each objective/article included in the Thesis are described below:

Article I:

1) The use of both RCs (ERSPC and PCPT) could improve the selection of patients who need prostate biopsy since both RCs showed similar accuracy for discrimination of Sig PCa.

2) ERSPC-RC had better stability than PCPT-RC for intra-individual PSA variations.

3) When comparing both RCs and by using similar cut-off values for biopsy indication, a higher rate of biopsies could be avoided with the ERSPC-RC, but with a higher rate of Sig PCa missed.

General clinical conclusion: In those patients with a PSA between 3-10 ng/ml, these tools should be used in order to improve the selection of patients for prostate biopsy.

Article II:

4) There is an association between the presence of MetS, a greater number of MetS-components or CRP levels >2.5 mg/L with an increased Sig PCa diagnosis and/or with aggressive features, suggesting that MetS and/or CRP levels might influence PCa pathophysiology.

General clinical conclusion: Based on the high incidence of MetS worldwide, especially in western countries, and considering the evident connection between some of the components of the MetS and the risk of PCa at the time of prostate biopsy, as well as of the association between inflammatory status with the aggressiveness of PCa found in our study, the results of the present work invites to suggest that interventional

studies based on the control of MetS and inflammatory status in patients at risk of PCa might be a key point in the overall management of this disease.

Article III:

5) A 19-biomarker peptide panel approach is able to improve the non-invasive detection of Sig PCa, outperforming current gold standard tools.

General clinical conclusion: Effective discrimination, with a multi-peptide panel, between clinically significant and non-significant PCa is expected to have a positive impact on reducing unnecessary biopsies, improving patient compliance and also alert the urologists to perform a more thorough examination in case of a positive result. The results of this study, although highly significant, must be verified in a prospective trial to also assess the actual value in the context of patient management.

Article IV:

6) The measurement of plasma GOAT levels in addition to the PSA might represent a significantly better diagnostic marker than plasma PSA levels alone.

7) GOAT plasma levels show an association with aggressive features of the tumor**.**

General clinical conclusion: The measurement of plasma GOAT levels, in combination with PSA and/or an additional panel of clinical variables measured in PCa (i.e. age, DRE and testosterone levels), might be explored as a novel, complementary, non-invasive tool aiming to improve the diagnosis of PCa.

Article V:

8) There is higher expression of EN2 in PCa tissue vs. normal prostate, as well as its elevated levels in urine samples from PCa patients.

9) Secreted EN2 protein could act as a tumorigenic factor in normal and tumor prostate cells, by modulating key functional parameters and signaling pathways.

General clinical conclusion: We expanded and validated the higher expression of EN2 in PCa tissue vs. normal prostate, as well as its elevated levels in urine samples from PCa patients. In addition, we demonstrate, for the first time, that secreted EN2 protein could act as a tumorigenic factor in normal and tumor prostate cells, by modulating key functional parameters and signaling pathways. These data invite to further explore the identification and development of novel therapeutic targets related to *EN2* in this highly incident and prevalent pathology.

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VI. Articles

Article I

Observational study comparing the accuracy/variability between the ERPSC and the PCPT risk calculators for the prediction of significant prostate cancer in patients with PSA <10ng/ml

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Key Words: Significant prostate cancer; risk calculator variability; ERSPC; PCPT.

Tables and figures: 1 and 5

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ABSTRACT

Introduction: Risk Calculators (RCs) are easy-to-use tools considering available clinical-variables that could help to select those patients with risk of prostate-cancer (PCa) who should undergo a prostate-biopsy. **Objective:** To perform a comparison for the prediction of significant-PCa (SigPCa) between the European-Randomised Study of Screening for PCa (ERSPC) and the PCa Prevention-Trial (PCPT) RCs in patients with PSA between 3-10ng/ml through an evaluation of the accuracy/variability between two consecutive PSA-values. **Setting:** An observational study in a major university Hospital of the south of Spain. **Methods and participants:** An observational study was performed in patients who underwent a prostate-biopsy. SigPCa probabilities were calculated with the two PSA measures using ERSPC3/4+DRE and PCPTv2+free-PSA RCs. The prediction discrimination of SigPCa was determined by the area under the curve (AUC). Calibration, discrimination, and decision curve analysis were studied. The variability between both RCs-agreement was compared using Cohen's kappa coefficient. **Results:** 510 patients were analysed (87 diagnosed with SigPCa). The median PSA value were 5.3 and 5ng/ml for PSA1 and PSA2 respectively. Both RCs overestimated the risk in the case of high-risk probabilities. Discrimination ability for SigPCa was similar between models with an AUC=0.73(0.68-0.79) for ERSPC-RC vs. 0.73(0.67-0.79) for PCPT-RC. ERSPC-RC showed less variability than PCPT-RC, with a constant agreement (k=0.7-0.8) for usual range of clinical decision-making. Remarkably, a higher biopsies number would be avoided using the ERSPC-RC, but more SigPCa would be missed along all the risk probabilities. **Conclusions:** Both RCs had similar accuracy for the SigPCa discrimination. However, ERSPC-RC seems to be more stable for intra-individual PSA variations.

Strengths and limitations of this study

- This study highlights the need to spread the use of available free tools which would be useful in patient's selection for undergoing prostate biopsy.
- This study is the first to compare two available free risk calculators in patients with a PSA < 10ng/ml analyzing their variability between two consecutive different PSA levels.
- Although the clinical information of this study was extracted from a clinical practice cohort and with information that could be useful for urologists worldwide, this is a retrospective study and the use of TRUS biopsy for PCa diagnosis, even though is the standard in most populations, suffers from random error compared with template biopsy, which could have affected prediction results.

INTRODUCTION

Prostate cancer (PCa) is the second most frequently diagnosed malignancy in males worldwide, and the most frequent in developed countries(1). Its current standard of diagnosis is a prostate biopsy based on PSA levels and digital rectal examination (DRE). However, there are other available and complementary variables that could help to select those patients who should undergo a prostate biopsy (such as age, prostate volume, free PSA, family history, etc.), but these are not always used and/or well-integrated in daily clinical practice(2). In line with this, Risk Calculators (RCs) are easy-to-use tools that can help the clinicians to take advantage of all these available variables (3). The two main RCs are from the European Randomised Study for Screening of Prostate Cancer (ERSPC cohort; ERSPC-RC: http://www.prostatecancer-riskcalculator.com/seven-prostate-cancer-riskcalculators) and from the Prostate Cancer Prevention Trial (PCPT cohort; PCPT-RC: http://deb.uthscsa.edu/URORiskCalc/Pages/calcs.jsp). Both RCs have undergone some modifications, specifically the addition of estimated prostate volume in the ERSPC-RC (4,5). Furthermore, both RCs were originally developed from different patient cohorts and each RC uses different variables.

To date, limited external validations and comparisons have been performed by different groups (6–9). The two most important recent comparisons of the modified RCs were performed by Foley *et al* (6,7) and Poyet *et al* (9). Both found a better discriminatory ability for ERSPC-RC vs PCPTv.2-RC for the diagnoses of significant-PCa (Sig PCa) (AUC around 0.74 vs 0.69, respectively), but they also included patients with a high PSA of up to 50 ng/ml. Despite the possibility of using these RCs in patients with PSA levels up to 50ng/ml, it is clear that the advantages of using both RCs would probably increase in patients with a PSA under 10ng/ml, where the rate of positive biopsy for PCa clearly decrease, with an important number of unnecessary biopsies. Furthermore, in the case of the PCPTv.2-RC, the addition of the free PSA value in patients with a PSA under 10ng/ml seems to improve its accuracy (5), and, therefore, given its accessibility, this value should be included in the RC.

The intra-individual and inter-assay variability of PSA is already known (10–12) and, therefore, at least two measures are necessary before a prostate biopsy is indicated. In fact, it has been shown that approximately 25% of men with initial PSA levels between 4 and 10 ng/mL had normal PSA values upon repeat testing (13). In line with this, despite being primarily based on PSA level, the variability of the two RCs mentioned above has been poorly studied, and might have implications for patient management. Our group has recently evaluated this variability with the ERSPC-RC, which showed stable accuracy over a cohort of patients, but some changes with respect to an individual approach (14). To date, there is no study comparing the accuracy and variability of both RCs, the ERSPC + DRE vs. the PCPTv.2 + free PSA, for the prediction of Sig PCa. Therefore, the aim of this study was to perform a direct comparison between ERSPC + DRE and PCPTv.2 + free PSA RCs in patients with a PSA between 3-10ng/ml, evaluating the accuracy and variability of both methods in the prediction of Sig PCa.

MATERIALS AND METHODS

Study population and design

An observational retrospective study was performed in patients from ONCOVER cohort (1021 biopsies indicated by clinical practice wherein patients donated blood and urine before the biopsy). The study was carried out within the project approved by our Hospital Research Ethics Committee, and informed consent was obtained from all participants. Blood sample was obtained in the morning (between 8:00-10:00 am) after fasting overnight and then, the prostate biopsy was implemented according to clinical practice. The inclusion criteria for this study were: 1) PSA indication between 3-10 ng/ml; 2) Full clinical and laboratory data to fulfilled ERSPC-RC and PCPT-RC; 3) Age 55-80 years' old; 4) Two consecutives measurements of PSA levels within an interval of 12 weeks. Exclusion criteria included patients with a previously known PCa diagnosis or treatment that could modify PSA levels (Supplemental table 1).

Transrectal prostate biopsy was carried out under local anaesthesia by using a standard peri-prostatic block, a transrectal ultrasound transducer, and an 18G automated needle biopsy instrument. The prostatic volume was measured following the protocol used during transrectal ultrasound (TRUS), and usual recommendations were to take 12 cores in patients undergoing the first biopsy procedure, and a minimum of 16 biopsy cores for those who had a previous biopsy. Biopsy specimens were analysed by expert urologic pathologists according to the International Society of Urological Pathology (ISUP) 2005 modified criteria (15).

Main variables description

Demographic information and the medical history of each patient was obtained. PSA levels were measured twice within a period no longer than 12 weeks, as follows: 1) **PSA 1 and free PSA 1:** for biopsy indication; and, 2) **PSA 2 and free PSA 2:** before undergoing prostate biopsy. For both PSA measures were evaluated by Chemiluminescent Microparticle Immunoassays (ng/ml, by a CMIA;

Ref. 7k70; Abbott). The median and interquartile range of time between measurements was 6 (3-8) weeks.

Prostate volume: estimated by TRUS and categorized in three possible values, 25-40-60 ml, as recommended (4) (TRUS volume <30 = 25 mL, 30–50 = 40 mL, and ≥50 = 60 mL).

Significant/ high-grade (HG) prostate cancer (Sig PCa): PCa with a Gleason grade ≥ 7 on biopsy.

ERSPC-RC and PCPT-RC probabilities calculation

ERSPC: The formulas for the ERSPC-RC 3+DRE for patients at initial biopsy and the ERSPC-RC 4+DRE for patients at repeat biopsy were utilized in this study. These calculators use PSA, prostate volume, and DRE as variables, with, a negative prostate biopsy in ERSPC 4+ DRE in patients who had a previous biopsy. This provides a probability rating for any PCa or Sig PCa (Gleason ≥ 7).

ERSPC1/Sig PCa (1º Measure): Risk probability calculated by ERSPC-RC3 or 4 (if previous biopsy) ± DRE for any PCa using PSA 1/ Sig PCa – for HG PCa.

ERSPC2/Sig PCa (2º Measure): Risk probability calculated by ERSPC-RC 3 or 4 (if previous biopsy) ± DRE for any PCa using PSA 2/ Sig PCa – for HG PCa.

PCPT: The formulae for the PCPT-RC 2.0 + %free PSA was utilized in this study. This calculator uses race, age, PSA level, %free PSA level, family history of PCa, DRE and prior prostate biopsy. This gives a probability of negative biopsy, low grade PCa and Sig PCa (gleason ≥ 7).

PCPT1: Risk probabilities calculated by PCPT 2.0 + %free PSA using PSA 1 and free PSA 1.

PCPT2: Risk probabilities calculated by PCPT 2.0 + %free PSA using PSA 2 and free PSA 2.

The variability of PSA was calculated by the following formula: **| Measure 1– Measure2| / Measure 1**

Statistical analysis

A descriptive study was performed by calculating the median and interquartile ranges (IR) for the quantitative variables, and the absolute frequencies and percentages for the qualitative variables. A Student's T test for paired groups was used to compare the means of the quantitative variables (PSA 1 and 2).

The investigation of the comparative performance in the detection of Sig PCa of both RCs, ERSPC-RC and PCPT-RC, was performed, taking into account these four factors: discrimination capacity, calibration, clinical utility, and consistency against the observed variations in PSA levels for our dataset.

The discrimination ability of the models, i.e., their ability to separate those patients who had Sig PCa from those who do not, was assessed using the area under their Receiver Operator Characteristic (ROC) curve (AUC) (16), as measured in our sample. This is one of the most frequently used measurements of model discrimination, because of its independence of the selection of a specific decision threshold and its robustness against class imbalance. Confidence intervals for these AUCs were computed using bootstrapping. These AUCs were then compared to determine the relative performance of the models using DeLong tests (17). These tests were chosen because of their non-parametric nature, with few assumptions about the data, and their suitability for paired data, as both models were evaluated over the same dataset, properties which make this the most commonly used test to compare AUCs (18). For this comparison, we focused on the calculated risk score utilising the first measure of PSA (PSA 1; the value for the indication of the prostate biopsy).

The calibration of the calculators for our cohort was then investigated to determine the agreement between the frequency of the observed outcome (Sig PCa in our case) and the risks predicted by the model. Calibration plots were used for this purpose (19), enabling a visual evaluation of this agreement and the comparison between RCs.

To address the potential clinical utility of the models, we performed decision curve analysis on our data, as proposed by Vickers and Elkin (20). This method has the advantage of not requiring the

specification of the relative cost for false-positives and false-negatives, defining a net benefit as a function of the decision threshold at which one would consider obtaining a biopsy.

Finally, the stability of the predictions of both RCs, with regard to the observed intra-patient changes on PSA levels between measurements, was investigated using the Cohen's kappa (k) inter-rater agreement coefficient as a function of the decision threshold. This coefficient was selected due to its widespread use and robustness against random agreements, and thus, is a better measurement than naïve accuracy.

All the analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, Ill) and R version 3.2.3 (R Foundation for Statistical Computing, Vienna, Austria: URL https://www.R-project.org/). A <5% level of significance ($p<0.05$) was used to decide statistically significant differences.

Patient and public involvement

Participants and public were not involved in the development of research questions, study design or recruitment.

RESULTS

Cohort characteristics

In the present study, we analysed 510 patients who met the inclusion criteria previously described. Median age was 65 (60-70) years old, with a family history in 89 patients (17.5%) and a suspicious DRE in 82 patients (16.1%). The median PSA before prostate biopsy indication was 5.3 (4.3-6.9) ng/ml. 176 patients were diagnosed with PCa, 87 of those categorized as Sig PCa. Most patients (n=401; 78.6%) were biopsy-naïve and the median prostate volume was 35 (26-49) cc. Further cohort description according to Sig PCa status is shown in Table 1.

66 patients had a PSA 2 out of the range of 3-10ng/ml due to the variability (50 patients below 3ng/ml and 16 patients above 10ng/ml); thus, in this case, the % free PSA was not calculated, and the risk probability was calculated without the inclusion of this variable. The patients were maintained in the analysis as reflecting the variability of the PSA, and the application of the models in this real situations, although acknowledging that it could introduce a bias in terms of calibration and variability.

Direct comparison for Sig PCa prediction

Discrimination ability for Sig PCa was no different between the two models [ERSPC1-RC vs. PCPT1-RC: 0.73; 95% CI: (0.68-0.79) vs. 0.73; 95% CI: (0.67-0.79), respectively]. ROC curves are shown in Figure 1A. Similarly, no difference was found in the discrimination ability for any PCa. The comparison of the RC for both measures is described in Figure 1(B-D) with similar results but a tendency of better accuracy for PCPT2-RC vs ERSPC2-RC (p= 0.25). Supplemental Table 2 shows multiple comparisons by the DeLong test resulting in no differences between the two RCs for Sig PCa.

Both models tended to overestimate the risk for a high probability of Sig PCa, and slightly underestimate it for low risk patients, suggesting that the models would benefit from a recalibration for our population (Figure 2). None of the models predicted very high probabilities for most patients. The calibration curves for any PCa are shown in Supplemental Figure 1.

The decision curve analyses revealed that both RCs provided a clinical net benefit in the threshold probability range for Sig PCa (Figure 3). The net benefit was comparable between the two RCs for Sig PCa.

As shown in Supplemental Figure 2, the addition of free PSA clearly improved the accuracy of the PCPT-RC [0.65 (0.59-0.71) PCPT1 v.2.0-RC vs. 0.73 (0.67- 0.79) PCPT1 v.2.0 + free PSA –RC; p= 0.02].

Variability and clinical significance

PSA and free PSA change was significantly different between the two measures, but with low clinical variations [average PSA1 5.69 ng/ml vs. PSA2 5.39 ng/ml ($p < 0.05$) and average free PSA1 16.99% vs. free PSA2 18.03% ($p < 0.05$)]. Median variability of PSA was 14% (6-27%). Taking into account this variability of PSA, ERSPC proved to be more stable than PCPT. The k agreement between ERSPC1 and ERSPC2 was practically constant, 0.79±0.09 for the usual range of clinical decision (0-0.3). However, PCPT1 and PCPT2 showed wider variations, with a k agreement of approximately 0.55±0.32 in the same range, with a subsequent rapid decrease. The agreement between both models (ERSPC1 vs. PCPT1) proved to be worse for thresholds in this range, peaking 0.47 for a 17% risk, with an average 0.32±0.12 on the interval. The comparison between ERSPC2 and PCPT2 yielded similar results (Figure 4).

Direct comparison of sensitivity and specificity of both RCs along the different clinical risk thresholds showed that PCPT-RC has higher sensitivity and lower specificity than ERPSC-RC for a given threshold along the clinically useful region (Figure 5). The balance point is reached at a different risk threshold for each RC. The performances of both RCs at this point are comparable, as shown in Figure 5. Considering the superposition of their respective ROC curves to a good approximation (Figure 1), this means that a transformation of decision thresholds can make both models perform similarly.

DISCUSSION

Currently, considerable research is being carried out to find new diagnostic markers for Sig PCa, in order to reduce the number of biopsies and the over-diagnosis of insignificant PCa (21). These markers are based on body fluids (blood, urine) or image explorations (22,23). Some are recommended by guidelines such as the 4k score test, PCA3, and/or the Prostate Health Index (PHI) in body fluids (only PCA3 and PHI have been approved by the FDA) (24–26), or multiparametric magnetic resonance imaging (mpMRI), with recent evidence of its advantages in biopsy-naïve patients (27). However, costs and availability minimize their implementation worldwide, and, therefore, it is clear that additional and readily available tools, such as RCs, should be implemented in daily clinical practice. The two most used RCs are ERSPC-RC and PCPT-RC, which have been modified and adapted (4,5). Few external validations have been conducted, with varying results (7,9,28). Usually, external validations of RCs show worse performance than the original validations (8), a fact that is corroborated by our study. Therefore, based on all this information, evaluations, validations, and incorporation of RCs are needed (3).

The present study explores and compares for the first time, both the PCPT v2 + free PSA and the ERSPC + DRE, not only for accuracy but also for variability and clinical relevance. Our group previously explored the accuracy and variability of the ERSPC + DRE RC (14) but, in this study, we have specifically focused only on patients in the grey zone (PSA 3-10ng/ml) and compared the ERSPC + DRE RC vs. the PCPT v2 + free PSA, an analysis that has not been previously performed. This comparison showed that both RCs had similar accuracy for the discrimination of Sig PCa. However, ERSPC-RC had better calibration and stability for intra-individual PSA variations. Our methodology in calculating the volume is an estimation from the results of the TRUS measure, similar to Poyet *et al*, and following the recommendations of Roobol *et al.* (4). We have focused only on those patients with PSA between 3-10 ng/ml who require additional diagnostic information. The PCPT-RC option with free PSA, which increases the accuracy of discrimination between Sig PCa and no PCa (5), was calculated, as it is an easy-to-use and readily available tool for these patients.

As defined in the methodology, the first measure (ERSPC1 and PCPT1) was the focus of the direct comparison, as this was used as the indication for biopsy. The accuracy of both RCs was similar for Sig PCa in our study, showing an accuracy similar to other external validations such as Poyet *et al*. (9) and Foley *et al.* (7) for ESRPC (AUC= 0.73 and 0.74, respectively) and a better accuracy for PCPT1 v2.0 when adding free PSA (AUC= 0.70 and 0.69, respectively). Still, these results are far from ideal, and thus, additional data from imaging or fluid markers might be included to improve the accuracy of the RCs. In agreement with the accuracy results, the decision curve analysis was also similar between both RCs. In fact, both RCs showed a net benefit from an early risk threshold, which means that their implementation would be useful in the pathway of patient selection.

Studying the variability of the RCs improves our knowledge about their stability, which could translate into improved decision-making and selection of patients. Our PSA cohort showed a variability that was in the range of that previously shown in the literature (11,12,29). Our group and others (14,30) have demonstrated that a higher PSA variability is associated with a reduced risk of Sig PCa in a prostate biopsy, but it does not improve the accuracy of a RC. However, probability stability is important in order to trust RC probabilities at any point. Our study shows good agreement between the two ERSPC + DRE-RC probabilities, with good calibration and stability despite intra-individual PSA variations. PCPTv.2 + free PSA shows worse stability and higher variability, which could be explained simply by the fact that it uses two values (PSA and free PSA) that suffer from this variability (31), while the use of an estimated volume in the ERSPC dilutes the PSA variability. These results should be interpreted with caution, as volume estimation was performed by categorization of TRUS and not by DRE. It is true that this categorization has previously shown good correlation (4). This likely depends on prostate volume (32), as well as low but certain inter-examiner variability (33), which could also increase ERPSC variability in an inter-clinician comparison. It should also be taken into account that the clinical translation of this stability is not clear, firstly, because of the limitation of the use of a single estimated prostate volume and because the global accuracy of both RC are not significantly different, and seems to have a tendency to improve in the PCPT2 RC.

Calibration plots show that both models (PCPT-RC and ERSPC-RC) predict adequately only the actual risk of PCa and Sig PCa for low-risk patients, with a wider useful range in the case of PCa and a lower range in the case of Sig PCa. For higher risk patients, the calibration curves become irregular. This effect is accentuated for risks close to 1, as both models predict maximum risks of around 0.75 for Sig PCa. The models would benefit from recalibration for our population in the low-moderate risk region, considering that this is the region of greater interest for the model, as patients with a high predicted risk would probably undergo biopsy anyway. Nonetheless, despite not showing a good calibration in the usual range for clinical decision (0-0.3), visually ERSPC seems to be more consistent with a less fluctuating calibration in this range compared to PCPT, but at this point, this should be confirmed in future studies because no conclusion for direct comparison about calibration could be reached in the present study as quantitative analysis is outside the aim of this research. The comparison of coefficients between PCPT1 and PCPT2 and between PCPT2 and ERSPC2 showed that the differences between PCPT1 and PCPT2 were similar to those between ERSPC and PCPT models. As previously discussed ERSPC seems to be more insensitive and, therefore, robust to intra-individual variations of PSA compared to PCPT, while the predictive performance is similar and the clinical translation not clear yet.

Despite the similar decision curve, results from the sensitivity, specificity and ROC curve analysis show that the same risk threshold should not be used for both models. Both RCs are able to have similar performance, and the benefit of using any of them is similar in order to screen patients for a prostate biopsy, if the correct cut-off point is selected. It should be highlighted the importance of having an almost 100% negative predictive value, as the advantage of reducing unnecessary biopsies should not be at the cost of missing or delaying the diagnoses of a Sig PCa.

In clinical practice, the use of these RCs should be the first step in guiding the decision for further management of the patient. Patients with a confirmed, elevated PSA between 3-10ng/ml should be better stratified using other variables within a RC, as men with PSA levels >10ng/mL are likely to

proceed to biopsy regardless of other factors. Probably a specific cut-off point in the risk probability should not be used and take advantage of the known probabilities to discuss with the patient the biopsy indication as recommended by the PCPT-RC. In the situation in which the patient is in the low-risk group, according to both RCs (ERSPC and PCPT), the patient could continue with just follow-up. This fact has also been proposed by Alberts *et al.* (34) when applying new diagnostic markers, such as mpMRI. Specifically, they showed that following a negative recommendation from the ERSPC-RC would have avoided 62 (51%) of 122 mpMRIs and two (25%) of eight insignificant PCa diagnoses, missing three (10%) of 31 high-grade PCa. As the positive predictive value of these RCs is not as good as their negative predictive value, in case of discordance between both RCs or if there is an indication for a biopsy according to both RCs, other images or fluid biomarkers could increase the accuracy in order to potentially reduce the harm from unnecessary prostate biopsy and over-diagnosis (35). Specifically, Loeb *et al.* (36) has recently demonstrated that the incorporation of PHI into both RCs increases the accuracy of the diagnoses of Sig PCa. Another relevant point should be comment from the tendency of better predictive ability with the second evaluations of PSA, reinforcing the idea of the need of several PSA values to confirm the risk and discarded confounding factors. Furthermore, this analysis could suggest a tend to better discrimination ability of PCPT in the range of lower probabilities (when PSA is low), but further research would be needed to validate this affirmation. These risk calculators only show a static probability so other longitudinal variables and clinical judgment should be required for their application.

The present study has some limitations. First, despite the prospectively collected information, it is a retrospective study design. Second, prostate volume was an estimation and categorization from a TRUS calculation, and, therefore, it is not the actual approach for which the RC was developed. Third, the PSA values interval was not the same for all patients, which means the results should be interpreted with caution. Four, the use of TRUS biopsy for PCa diagnosis, although is the standard in most populations, suffers from random error compared with template biopsy (37), which could have

affected prediction results. However, the clinical information was extracted from a clinical practice cohort and with information that could be useful for urologists worldwide.

Altogether, our results showed that: 1) the use of both RCs (ERSPC and PCPT) could be an useful tool in the selection of patients who need prostate biopsy, and that both RCs showed similar accuracy for discrimination of Sig PCa; 2) ERSPC-RC showed higher stability than PCPT-RC for intra-individual PSA variations; 3) when comparing both RCs sensitivity and specificity, a higher rate of biopsies could be avoided with the ERSPC-RC vs. the PCPT-RC, but with a higher rate of Sig PCa missed. Thus, in those patients with a PSA between 3-10 ng/ml, these tools should be used in order to improve selection and specificity. The RCs specifically should be selected according to the variables available in the clinic. In addition, both RCs could also be used and the decision to undergo a biopsy be shared with the patient.

CONFLICTS OF INTEREST

Nothing to declare

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Footnotes

- **Contributors** E.G.G, R.M.L, MJ.R.T and J.C.V carried out the conception and design of the study; E.G.G, JJ.S.B, J.C.V, AM.B, J.V.R, I.M.B.A, J.M.L, JM.J.V contributed to the data acquisition; E.G.G, J.C.V, J.L.F.R, RM.L and MJ.R.T carried out the analysis and interpretation of data; E.G.G, J.C.V and RM.L drafted the manuscript; JJ.S.B, JL.F, AM.B, J.V.R, J.M.L, JM.J.V, and MJ.R.T carried out a critical revision of the manuscript for important intellectual content; E.G.G, and J.L.F.R performed the statistical analysis; RM.L, MJ.R.T and J.C.V supervised the work.
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- **Competing interests** None declared.
- **Ethics approval** This study was performed as part of the ONCOVER project. Ethical approval was obtained by the Reina Sofia Hospital Research Ethics Committee in accordance with the Declaration of Helsinki and informed consent was obtained from all participants for the project.
- **Provenance and peer review** Not commissioned; externally peer reviewed.
- **Data sharing statement** All data is shown within the manuscript
- **Patient consent for publication** Not required.

Figure legends

Figure 1: Receiver Operating Characteristic curves and Area Under the Curve values: **A**, for the ERSPC1 -RC (black) and PCPT1 -RC (grey) for Sig PCa; **B**, for the ERSPC1-RC and the ERSPC2-RC for positive biopsy; **C**, for the PCPT1-RC and the PCPT2-RC for positive biopsy; and **D**, for the ERSPC2-RC and the PCPT2-RC for Sig PCa.

Figure 2: Calibration plots for risk estimation, showing the agreement between predicted risk (horizontal axis) and the actual observed prevalence for people with that risk (vertical axis). The diagonal line shows the ideal behaviour of a perfectly calibrated RC, separating the upper left region where risks are underestimated from the lower right, where they are overestimated. **A**, Calibration plots for ERSPC1-RC Sig PCa risk estimation. **B**, Calibration plots for PCPT1-RCSig PCa risk estimation.

Figure 3: Results of the decision curve analysis. **A**, Net benefit for the prediction of Sig PCa on biopsy using the ERSPC1-RC (black line) and the PCPT1–RC (grey line) as a function of the risk threshold, compared to those benefits of the strategies of treating all patients (dashed line) and treating none (thin line). **B**, Plot demonstrating net reduction of interventions per 100 patients using the ERSPC-RC (black line) and the PCPT-RC (grey line).

Figure 4: Graphics showing Cohen's k coefficient, which evaluated the agreement between RCs, as a function of the decision threshold, with 1 being total agreement and 0 being the worst possible expected agreement between rates. **A**, Agreement between ERSPC1-RC and ERSPC2-RC for Sig PCa. **B**, Agreement between PCPT1-RC and PCPT2-RC for Sig PCa. **C**, Agreement between ERSPC1-RC and PCPT1-RC for Sig PCa.

Figure 5: Graphics showing sensitivities and specificities of both RCs along the clinically useful risk threshold. The ERSPC-RC (black line) and the PCPT-RC (grey line).

Table 1. Clinical and demographic characteristics of the cohort of patients categorized according to cancer status.

Variable	No Sig PCa n=423	Sig PCa n=87	All n=510
Age	64.0 (60.0-69.0)	68.0 (63.0-71.0)	65.0 (60.0-70.0)
Family History	81 (19.1)	8 (9.2)	89 (17.5)
Positive DRE	55 (13.0)	27 (31.0)	82 (16.1)
1 Serum PSA	5.3 (4.3-6.9)	5.8 (4.5-7.2)	5.3 (4.3-6.9)
1 free PSA %	16.2 (12.4-21.4)	12.5 (9-16.6)	15.9 (11.8-20.4)
2 Serum PSA	5.0 (3.7-6.6)	5.4 (4.1-6.7)	5.0 (3.8-6.6)
2 free PSA %	17.9 (13.9-23.4)	12.5 (9.1-16.3)	16.9 (12.8-22.1)
Prostate volume	38.0 (29.0-50.0)	26.0 (20.7-34.0)	35 (26-49)
First Biopsy	322 (76.1)	79 (90.8)	401 (78.6)
PCPT1 Sig PCa	0.08 (0.05-0.13)	0.16 (0.10-0.30)	0.09 (0.06-0.15)
ERSPC1 Sig PCa	0.05 (0.02-0.10)	0.12 (0.05-0.31)	0.05 (0.03-0.12)
PCPT2 Sig PCa	0.07 (0.04-0.11)	0.16 (0.08-0.27)	0.07 (0.05-0.13)
ERSPC2 Sig PCa	0.04 (0.02-0.08)	0.12 (0.05-0.30)	0.05 (0.02-0.11)
PCa	89 (21)	87 (100)	176 (34.5)

PCa= Prostate cancer; **Sig PCa=** significant PCa (Gleason ≥ 7 on biopsy); **No Sig PCa=** No cancer or non-significant PCa; **ERSPC1 / PCPT1 Sig PCa =** Probability of high grade PCa using the first measurement of serum PSA (at the time of biopsy indication by the urologist); **ERSPC2 / PCPT2 Sig PCa =** Probability high grade PCa using the second measurement of serum PSA (just before undergoing prostate biopsy). Median values (interquartile range) are expressed for quantitative variables, and absolute values (percentage) for qualitative variables.

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1

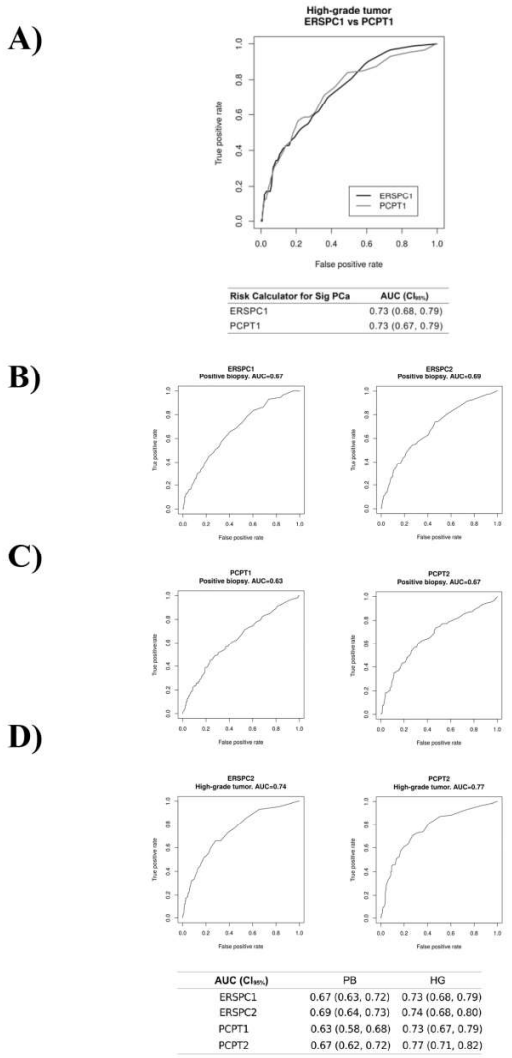
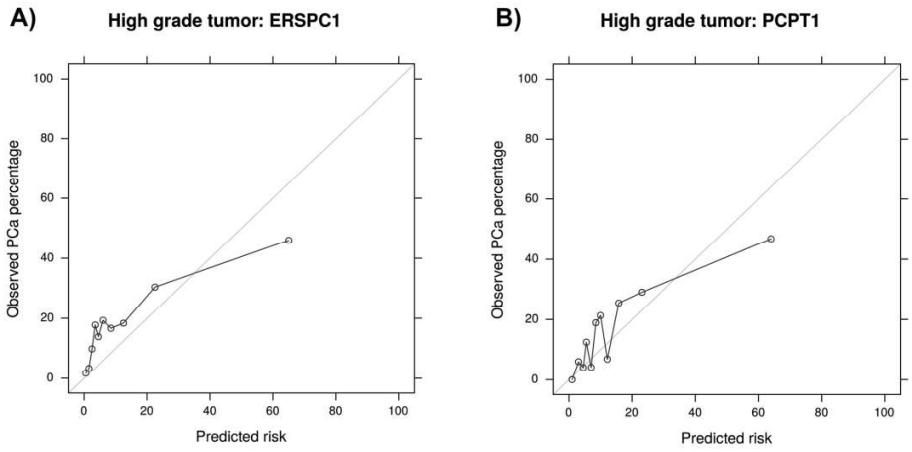


Figure 1: Receiver Operating Characteristic curves and Area Under the Curve values: A, for the ERSPC1 -RC (black) and PCPT1 -RC (grey) for Sig PCa; B, for the ERSPC1-RC and the ERSPC2-RC for positive biopsy; C, for the PCPT1-RC and the PCPT2-RC for positive biopsy; and D, for the ERSPC2-RC and the PCPT2-RC for Sig PCa.

145x244mm (300 x 300 DPI)

2



Calibration plots for risk estimation, showing the agreement between predicted risk (horizontal axis) and the actual observed prevalence for people with that risk (vertical axis). The diagonal line shows the ideal behaviour of a perfectly calibrated RC, separating the upper left region where risks are underestimated from the lower right, where they are overestimated. A, Calibration plots for ERSPC1-RC Sig PCa risk estimation. B, Calibration plots for PCPT1-RCSig PCa risk estimation.

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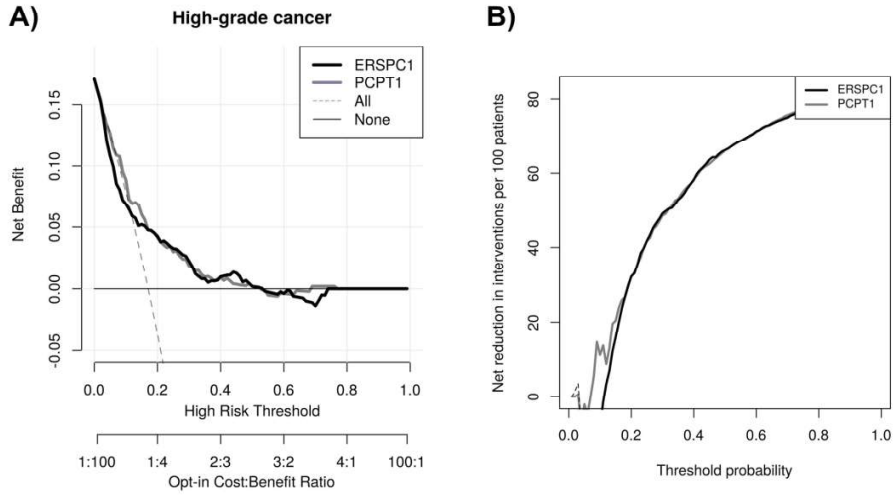
3



Results of the decision curve analysis. A, Net benefit for the prediction of Sig PCa on biopsy using the ERSPC1-RC (black line) and the PCPT1–RC (grey line) as a function of the risk threshold, compared to those benefits of the strategies of treating all patients (dashed line) and treating none (thin line). B, Plot demonstrating net reduction of interventions per 100 patients using the ERSPC-RC (black line) and the PCPT-RC (grey line).

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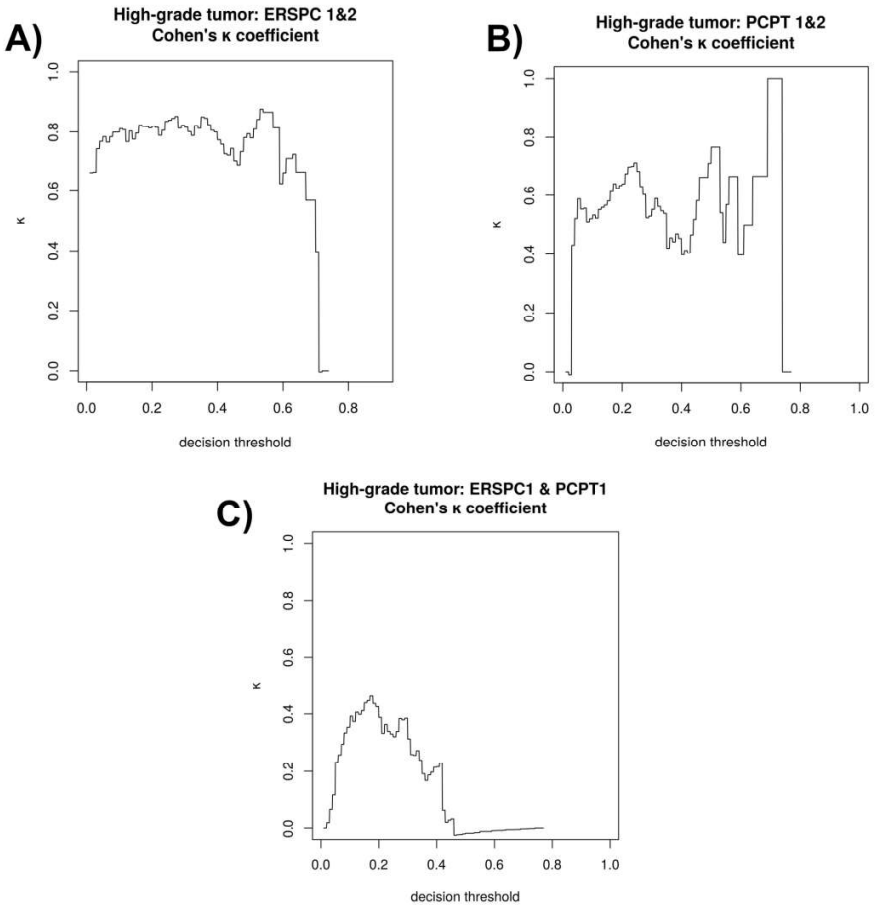
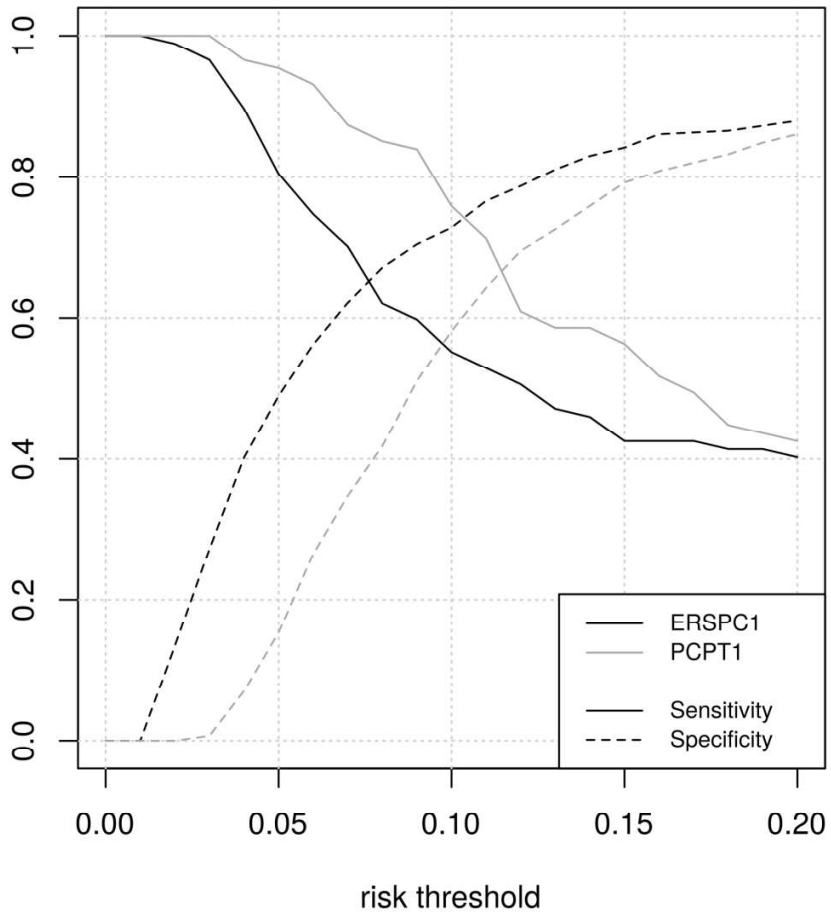
4



Graphics showing Cohen's k coefficient, which evaluated the agreement between RCs, as a function of the decision threshold, with 1 being total agreement and 0 being the worst possible expected agreement between rates. A, Agreement between ERSPC1-RC and ERSPC2-RC for Sig PCa. B, Agreement between PCPT1-RC and PCPT2-RC for Sig PCa. C, Agreement between ERSPC1-RC and PCPT1-RC for Sig PCa.

190x190mm (300 x 300 DPI)

5



Graphics showing sensitivities and specificities of both RCs along the clinically useful risk threshold. The ERSPC-RC (black line) and the PCPT-RC (grey line).

STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cross-sectional studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	3
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	5
Objectives	3	State specific objectives, including any prespecified hypotheses	6
Methods			
Study design	4	Present key elements of study design early in the paper	6
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	7
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants	7
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	8-10
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	7-8
Bias	9	Describe any efforts to address potential sources of bias	*8*
Study size	10	Explain how the study size was arrived at	7
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	7-8
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	8-10
		(*b*) Describe any methods used to examine subgroups and interactions	8-10
		(*c*) Explain how missing data were addressed	7
		(*d*) If applicable, describe analytical methods taking account of sampling strategy	8-10
		(*e*) Describe any sensitivity analyses	8-10
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	11
		(b) Give reasons for non-participation at each stage	11
		(c) Consider use of a flow diagram	Supplemental
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	11
		(b) Indicate number of participants with missing data for each variable of interest	11- Supplemental
Outcome data	15*	Report numbers of outcome events or summary measures	12
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	12
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	Supplemental
Discussion			
Key results	18	Summarise key results with reference to study objectives	14
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	17
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	16
Generalisability	21	Discuss the generalisability (external validity) of the study results	17
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	1

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

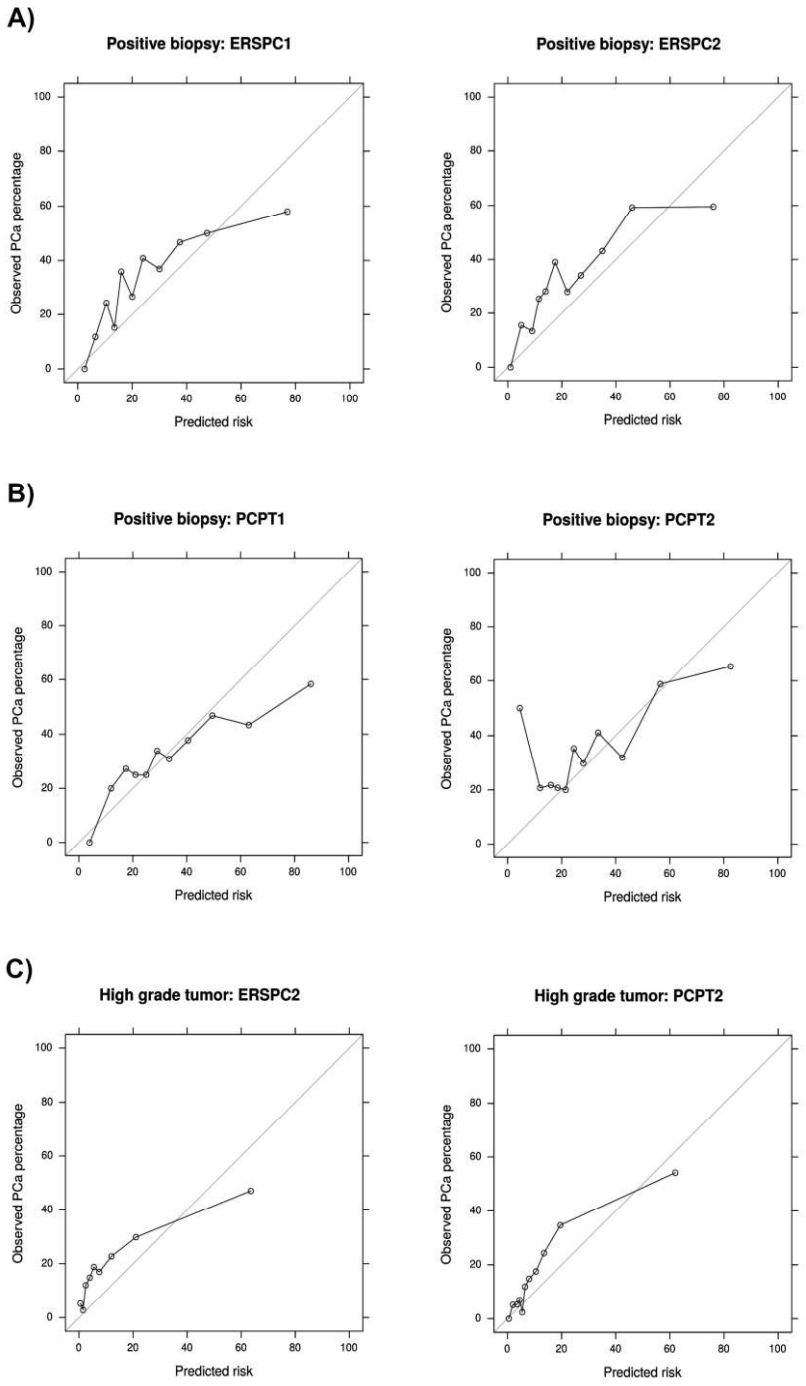
Supplemental Table 1. Patients excluded from the ONCOVER cohort for this study depends on the exclusion criteria.

Exclusion criteria	Number
Under active surveillance	25
2 consecutive PSA levels well recorded or affected	50
Prostate volume not well recorded	177
PSA of biopsy indication out of the range 3-10ng/ml , or Age out of the range 55-80	251
Total exclusions	511

Supplemental table 2: DeLong p values resulting from the pairwise comparison of the Area under the Receiver Operator Characteristic (ROC) curve (AUC) between Risk Calculators for significant Prostate cancer (Sig PCa) detection.

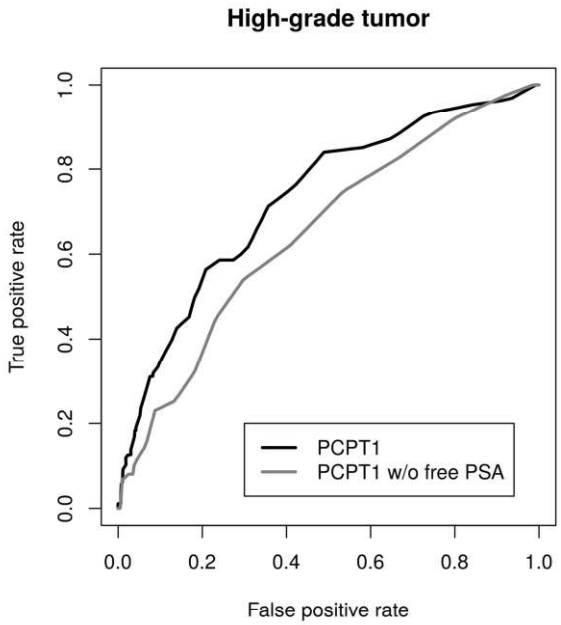
Sig PCa (p-value)	ERSPC1	ERSPC2	PCPT1	PCPT2
ERSPC1	X	0.51	0.95	0.19
ERSPC2	X	X	0.74	0.25
PCPT1	X	X	X	0.06

S1



Supplemental Figure 1: Calibration plots of the RCs in this cohort, demonstrating the agreement between predicted and observed probabilities: **A**, of a positive biopsy for the ERSPC1-RC and the ERSPC2-RC; **B**, of a positive biopsy for the PCPT1-RC and for the PCPT2-RC; and **C**, of a Sig PCa on the biopsy for the ERSPC2-RC and the PCPT2-RC.

S2



Risk Calculator for Sig PCa	AUC ($CI_{95\%}$)
PCPT1 without free PSA	0.65 (0.59, 0.71)
PCPT1 + free PSA	0.73 (0.67, 0.79)
DeLong p value: 0.02	

Supplemental Figure 2: Receiver Operating Characteristic curves and Area Under the Curve values for the PCPT1-RC without free PSA (black) and for the PCPT1-RC with free PSA (grey) to predict Sig PCa. P-value according to the DeLong test.

Article II

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ORIGINAL ARTICLE

WILEY

Clinical association of metabolic syndrome, C-reactive protein and testosterone levels with clinically significant prostate cancer

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Abstract

Recently, the influence that metabolic syndrome (MetS), hormonal alterations and inflammation might have on prostate cancer (PCa) risk has been a subject of controversial debate. Herein, we aimed to investigate the association between MetS-components, C-reactive protein (CRP) and testosterone levels, and the risk of clinically significant PCa (Sig-PCa) at the time of prostate biopsy. For that, men scheduled for transrectal ultrasound guided biopsy of the prostate were studied. Clinical, laboratory parameters and criteria for MetS characterization just before the biopsy were collected. A total of 524 patients were analysed, being 195 (37.2%) subsequently diagnosed with PCa and 240 (45.8%) meet the diagnostic criteria for MetS. Among patients with PCa, MetS-diagnosis was present in 94 (48.2%). Remarkably, a higher risk of Sig-PCa was associated to MetS, greater number of MetS-components and higher CRP levels (odds-ratio: 1.83, 1.30 and 2.00, respectively; $P < 0.05$). Moreover, higher circulating CRP levels were also associated with a more aggressive Gleason score in PCa patients. Altogether, our data reveal a clear association between the presence of MetS, a greater number of MetS-components or CRP levels >2.5 mg/L with an increased Sig-PCa diagnosis and/or with aggressive features, suggesting that MetS and/or CRP levels might influence PCa pathophysiology.

KEYWORDS
C-reactive protein, inflammation, metabolic syndrome, significant prostate cancer, testosterone

1 | INTRODUCTION

Prostate cancer (PCa) is the most common cancer among men in developed countries and a leading cause of mortality and morbidity globally.[1] The non-modifiable risk factors established for PCa are age, race and family history,[2] however, the contribution that lifestyle and environmental factors may have on PCa aetiology has been recently suggested, and certainly is still an active subject of debate.[3,4] In this sense, metabolic syndrome (MetS) is a widely prevalent disorder whose diagnosis consists on a combination of clinical and serological parameters including obesity (particularly abdominal adiposity), insulin resistance, elevated blood pressure,

elevated triglyceride levels and decreased levels of high density lipoproteins (HDL)-cholesterol.[5]

Several mechanisms have been proposed to explain the association between PCa and MetS including hormonal alterations (eg low circulating levels of testosterone), insulin resistance (eg high insulin and IGF-1 levels) and inflammation status (eg alterations in cytokines and C-reactive protein [CRP] levels, among others inflammatory-related molecules).[6] In this sense, we have recently uncovered the existence of a fine, germane crosstalk between the endocrine-metabolic status and the development and homoeostasis of the prostate gland, wherein key components of the insulin, IGF1 and adipokines axes, among other, could play a relevant pathophysiological role.[7,8] In addition, it has been suggested that low levels of testosterone could be linked with the presence of abdominal obesity, and this in turn, might cause an alteration in the metabolism of fatty acids promoting insulin resistance,[9] which might be associated to PCa risk[10,11]; however, the association between circulating testosterone levels, metabolic status and PCa progression/aggressiveness remains controversial.[12–15] Furthermore, circulating levels of CRP, one of the most useful markers to assess varying degrees of inflammation in disease states such as obesity, diabetes mellitus (DM), etc.,[16] have been found to be elevated in patients with different cancer types compared to healthy patients[17]; but the putative association between CRP levels, metabolic status, testosterone and PCa remains still unknown.[17]

Therefore, based on the information mentioned above, the aim of this study was to explore the associations and clinical consequences that the inflammatory status (using CRP levels), testosterone levels and MetS may have on the diagnosis and aggressiveness of PCa using a cohort of patients with and without MetS and/or PCa.

2 | PATIENTS AND METHODS

2.1 | Population

This is an observational study over an 18-month prospective cohort, in patients who underwent ultrasound guided prostate biopsy. The study was carried out within a project approved by our Hospital Research Ethics Committee, and informed consent was obtained from all participants. Specifically, blood sample was obtained in the morning (between 8:00 and 10:00 AM) after an overnight fasting and then, the prostate biopsy was implemented according to clinical practice. The inclusion criteria for this study was the indication of the biopsy by the clinician according to clinical practice. Recommendations to obtain a biopsy were the following: (a) in the case of non-previous biopsy, suspicious findings on digital rectal examination (DRE), PSA >10 ng/mL, or PSA 3-10 ng/mL if free PSA ratio was low (usually, <25-30%), and (b) in the case of patients with previous biopsies with persistently suspicious of PCa (ie elevated PSA, suspicious DRE, etc.). On the other hand, the exclusion criteria were: (a) wait circumference or other relevant clinical data not well-reported; (b) previously known PCa diagnosis, and (c) patients with acute infectious disease (not underwent prostate biopsy at this time).

2.2 | Clinical data

Demographics information and medical histories of each patient were obtained. Specifically, information of previous diagnoses of hypertension, DM and hypercholesterolaemia was collected, as well as family history of PCa and current usage of 5α-reductase inhibitors, metformin or statins. Moreover, each patient underwent a physical examination before the biopsy was carried out, including data of body weight (kg), height (cm) and waist circumference (cm). Specifically, the waist circumference was obtained by measuring the abdominal girth midway between the lowest rib margin and iliac crest while the patients were in a standing position.

As mentioned above, a blood sample (10 mL) was also collected after an overnight fasting period of ~8 hours. Levels of CRP (mg/L, by an Immunoturbimetric, High Sensitivity method; Ref. 6k26-30/41; Abbott), testosterone (ng/mL, by a Chemiluminescent Microparticle Immunoassay method [CMIA]; Ref. 7k73; Abbott), PSA (ng/mL, by a CMIA; Ref. 7k70; Abbott), HDL (mg/dL by an accelerator selective detergent method; Ref. 3k33-20; Abbott), triglycerides (mg/dL by a Glycerol Phosphate Oxidase method; Ref. 7D74-20; Abbott), glucose (mg/dL, by a Hexokinase/G-6-PDH method; Ref. 3L82-20 and 3L82-40) and glycated haemoglobin (HbA1c; %, by HPLC; Bio-Rad, Ref. 270-2000) were measured following the manufacturer's instructions.

MetS status of each patient was evaluated according to the National Cholesterol Education Program Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults, Adult Treatment Panel III criteria (ATP III).[18] For the diagnosis of MetS, at least three of the following criteria had to be met:

1. Waist circumference >102 cm (>40 inches).
2. HDL cholesterol levels <40 mg/dL (<1.0 mmol/L) or being actively treated for low HDL levels.
3. Serum triglycerides levels ≥150 mg/dL (≥1.7 mmol/L) or being actively treated for elevated triglycerides.
4. Fasting glucose levels ≥100 mg/dL (≥6.1 mmol/L) or being actively treated for hyperglycaemia.
5. Diagnosis of elevated blood pressure or being actively treated for hypertension.

2.3 | Prostate biopsy and pathologic analysis

Transrectal prostate biopsy was carried out under local anaesthesia using a standard peri-prostatic block, a transrectal ultrasound transducer, and an 18G automated needle biopsy instrument. Usual recommendations were to take 12 cores in patients undergoing the first biopsy procedure, and a minimum of 16 biopsy cores for those who had a previous biopsy. As recently reported,[19] all biopsy specimens were analysed by an expert urologic anatomo-pathologist according to ISUP 2005 modified criteria.[20]

2.4 | Statistical analysis

A descriptive study was performed by calculating the median and interquartile ranges for the quantitative variables and the absolute frequencies and percentages for the qualitative variables. The primary end-point of the study was the presence of a clinically Sig-PCa on biopsy. The tumours with a Gleason Score (GS) ≥7 were considered clinically Sig-PCa. The MetS variables were assessed in a dichotomous manner according to whether 3 or more of the ATP III diagnostic criteria were met, and quantitatively based on the absolute number of criteria met. The age, PSA levels and biopsy number were categorized (ie age [<60, 60-70 and >70 years], PSA [<3, 3-10, 10-20 and >20 ng/mL], and biopsies [1° or ≥2°]) to perform a multivariate analysis.

A Student's *t*-test was used for analysis of the quantitative data and a chi-squared test was used for the qualitative variables. A Pearson test was used to study the correlation between the quantitative variables. A receiver operating characteristic (ROC) curve analysis was performed to determine the best CRP levels cut-off for the diagnosis of Sig-PCa. Uni- and multivariate analyses were performed by logistic regression models to evaluate the association of the variables with PCa and Sig-PCa. ROC curve analysis was also performed to determine the predictive capability of the variables together in the total cohort and, a sub-analysis was also performed in patients with PSA <10 ng/mL. The De-long test was used to compare the area under the curve (AUC) values.

A <5% level of significance was used to decide statistically significant differences to make our conclusions comparable to those of the related research. All the analyses and graphics were performed with GraphPad prism 6, MedCalc statistical software and SPSS version 17.0.

3 | RESULTS

3.1 | Population description

Clinical data of 655 patients were selected to be included in this study; however, 131 patients were excluded based on the criteria mentioned above. Therefore, a total of 524 patients were finally included in the analysis. The demographic and clinical data from this cohort of patients according to the MetS status are shown in Table 1. Specifically, 240 of the patients (45.8%) satisfied the diagnostic criteria of MetS (n = 94 were diagnosed with PCa [39.2%] and, from those, n = 54 [57.4%] had Sig-PCa [GS ≥7]), while 284 of the patients had no MetS (n = 101 with PCa [35.6%] and, from those, n = 43 [42.5%] with GS ≥7) (Table 1). No statistical difference in family history of PCa, positive DRE or PSA levels were found between patients with or without MetS. However, patients with MetS were older, tended to have slightly higher prostate volume, had higher BMI, waist circumference, as well as elevated triglycerides, glucose and CRP levels but lower levels of HDL and testosterone (Table 1). A strong correlation between BMI and waist circumference was observed ($r = 0.87$; $P < 0.001$). Notably, the rate of Sig-PCa diagnoses was significantly higher in patients with MetS compared with patients without MetS ($P = 0.03$). Moreover, within the patients with MetS, waist circumference, glucose levels and hypertension were the most common criteria for the diagnosis of MetS (ie 205, 191 and 188 patients of 240, respectively; >75% of the patients with MetS; Table 1).

3.2 | Relationship between metabolic syndrome and circulating testosterone and CRP levels

Circulating levels of testosterone and CRP were analysed in the whole cohort of patients according to the individual diagnostic criteria of MetS (I, II, III, IV and V; Table 2). Interestingly, testosterone levels were significantly lower in patients that individually met each criterion of MetS compared to those that did not meet these criteria. In contrast, only patients that met the criterion I had higher CRP levels (Table 2).

3.3 | Influence of MetS, CRP and testosterone levels in the diagnosis of PCa

We next analysed the influence of: (a) the MetS status; (b) each individual criterion of MetS; (c) the number of MetS criteria met and (d) circulating CRP or testosterone levels, on the diagnosis of PCa or Sig-PCa (GS ≥7) (Table 3). Specifically, we found that a greater number of MetS criteria tended to be associated with a higher risk of PCa ($P = 0.07$; being a higher blood pressure the only criteria significantly associated with the risk of PCa; Table 3). Interestingly, we found that the presence of MetS, a greater number of MetS criteria, and higher circulating CRP (but not testosterone) levels were significantly associated with a higher risk of Sig-PCa. Moreover, when we analysed each MetS criterion independently, we found that only criteria I (waist circumference) and V (elevated blood pressure) were associated with higher risk of PCa (although only a trend was found for Criteria I; $P = 0.07$; Table 3), as well as with higher risk of Sig-PCa (Table 3). However, no association was observed between criterion I or V and GS (data not shown). Furthermore, it should be mentioned that although a strong correlation between BMI and waist circumference was observed in our cohort, we did not found any association between BMI and the risk of PCa or Sig-PCa. On the basis of these results, we next analysed whether a greater number of MetS criteria or the circulating levels of CRP were associated to GS in PCa patients. Interestingly, our results revealed that only a higher circulating CRP levels, but not number of MetS, was positively correlated with a higher GS (GS = 6, GS = 7, GS >7; $P < 0.05$; Figure 1).

Further exploratory analyses were carried out to evaluate the association of drug intake or levels of HbA1c, with the diagnoses of both PCa and Sig-PCa. Specifically, no significant association between HbA1c levels or statin intake and the diagnoses of PCa or Sig-PCa was observed in our cohort of patients. However, the analysis of metformin intake revealed a significant association with an increased risk of Sig-PCa even when adjusting by glucose levels and HbA1c (odds ratio [OR]: 2.74 [1.41-5.31]; $P < 0.01$).

3.4 | MetS, CRP and testosterone levels as predictive factors of PCa

On the basis the previous results indicating the association between a higher risk of Sig-PCa with the diagnoses of MetS, a greater number of MetS criteria and higher circulating levels of CRP, we next implemented a multivariable analysis to determine the strength of the independent association of these factors with the risk of being diagnosed with a Sig-PCa. To that end, a ROC curve analysis was firstly performed to determine the best CRP cut-off levels for the diagnosis of Sig-PCa, which revealed that the best value was 2.5 mg/L for CRP (AUC 0.60; $P = 0.003$).

It should be mentioned that, as might be expected, a significant association was observed between the risk of detecting a higher rate of Sig-PCa in our cohort of patients and an older age (ie <60 vs 60-70, or vs >70 years old), an elevated PSA levels (ie <3 vs 3-10, vs 10-20, or vs >20 ng/mL) or, an abnormal DRE (Table 4). Conversely, this risk significantly decreased in those patients who had a larger prostatic volume and a previous negative biopsy. Therefore, based on these associations, and to accurately determine whether the presence of MetS, a greater number of MetS criteria, or circulating CRP levels might be used as predictive factors of Sig-PCa independently, we adjusted these three variables by age, family history, PSA, 5α reductase inhibitors intake, DRE, prostate volume and number of biopsies (Table 5). Remarkably, we found that the three variables analysed were significant associated with a higher risk of Sig-PCa as follow (Table 5): (a) Presence of MetS (OR: 1.83, 95% CI: 1.05-3.20, $P = 0.03$); (b) number of MetS criteria (OR: 1.30, 95% CI: 1.05-1.60, $P = 0.02$); and, (c) circulating CRP levels (>2.5 mg/L; OR: 2.00, 95% CI: 1.14-3.51, $P = 0.02$). In fact, ROC curve analyses confirmed that the presence of MetS, a greater number of MetS criteria, or circulating CRP levels might be used as additional diagnostic factors for Sig-PCa when are added to the common risk factors mentioned above (ie age, family history, PSA, 5α reductase inhibitors intake, DRE,

TABLE 1 Descriptive and comparative analysis of demographics and clinical variables according to the presence or not of MetS

Variable	MetS (n = 240)	No MetS (n = 284)	*P*-value	Total (n = 524)
Age; years old	66 (60-70)	64 (58-69)	0.01	65 (59-70)
Family History; yes	35 (14.6)	52 (18.3)	0.29	87 (16.6)
Positive DRE; yes	51 (21.3)	57 (20.1)	0.74	108 (20.6)
Serum PSA; ng/mL	5.6 (3.8-8.3)	5.8 (4.0-8.4)	0.43	5.7 (3.8-8.4)
5 alpha inhibitors	11 (4.6)	10 (3.5)	0.66	21 (4)
*Prostate volume; cm^3	39 (27-54)	34.2 (26-48)	0.06	35 (26-51)
BMI; kg/m^2	30.5 (28.2-33.3)	26.8 (25.0-29.0)	<0.01	28.4 (26.2-31.3)
Waist circumference; cm	109 (104-116)	99 (93.5-104.5)	<0.01	103 (97-111)
HDL; mg/dl	41 (35-46)	47 (42-55)	<0.01	44 (39-51)
Triglycerides; mg/dl	135 (95-176.8)	91 (74-115)	<0.01	106 (79-147)
Glucose; mg/dl	111 (100-129)	94 (87-101)	<0.01	100 (90-113.5)
Metformin; yes	57 (23.8)	9 (3.2)	<0.01	66 (12.6)
Statin: yes	124 (51.7)	44 (15.5)	<0.01	168 (32.1)
HbA1c; %	5.8 (5.5-6.2)	5.4 (5.1-5.6)	<0.01	5.5 (5.2-5.9)
CRP; mg/L	2.6 (1.4-4.8)	1.7 (0.9-4.1)	0.05	2.0 (1.1-4.4)
Testosterone; ng/mL	4.4 (3.5-5.7)	5.4 (4.4-6.7)	<0.01	5.04 (3.97-6.2)
MetS criteria				
Criteria I MetS	205 (85.4%)	86 (30.3%)	<0.01	291 (55.5)
Criteria II MetS	183 (76.3%)	81 (28.5%)	<0.01	264 (50.4)
Criteria III MetS	103 (42.9%)	26 (9.2%)	<0.01	129 (24.6)
Criteria IV MetS	191 (79.6%)	81 (28.5%)	<0.01	272 (51.9)
Criteria V MetS	188 (78.3%)	94 (33.1%)	<0.01	282 (53.8)
PCa; yes	94 (39.2%)	101 (35.6%)	0.42	195 (37.2)
Gleason Score ≥7; yes	54 (22.5%)	43 (15%)	0.03	97 (18.5)

BMI, body mass index; CRP, C-reactive protein; DRE, digital rectal examination; HbA1c, glycated haemoglobin; HDL, high density lipoprotein; PCa, prostate cancer; MetS, metabolic syndrome [Criteria: I. Waist circumference > 102 cm (> 40 in); II. HDL cholesterol levels <40 mg/dL (<1.0 mmol/L), or being actively treated for low HDL levels; III. Serum triglycerides levels ≥150 mg/dL (≥ 1.7 mmol/L), or being actively treated for elevated triglycerides; IV. Fasting glucose levels ≥100 mg/dL (≥ 5.55 mmol/L), or being actively treated for hyperglycaemia, and; V. Diagnosis of elevated blood pressure or being actively treated for hypertension].

Values are expressed in median and interquartile range for quantitative variables and in absolute number and percentage for qualitative variables. Statistical test: *t*-Student for quantitative variables and chi-squared for qualitative ones.

*n = 441 patients (No MetS = 236 and MetS = 205).

TABLE 2 Association between circulating C- reactive Protein and testosterone levels with each of the criterion (I, II, III, IV or V) of MetS

MetS criteria	C-reactive protein (mg/L)	*P*	Testosterone (ng/mL)	*P*
Criterion I				
Yes	2.7 (1.4-5.2)	<0.01	4.5 (3.6-5.8)	<0.01
No	1.5 (0.8-3.4)		5.5 (4.4-6.8)	
Criterion II				
Yes	2.4 (1.2-4.8)	0.40	4.6 (3.7-6.0)	<0.01
No	1.8 (1.0-4.0)		5.3 (4.2-6.5)	
Criterion III				
Yes	2.8 (1.5-4.9)	0.14	4.7 (3.6-6.0)	0.01
No	1.8 (1.1-4.1)		5.1 (4.1-6.3)	
Criterion IV				
Yes	2.1 (1.1-4.4)	0.65	4.5 (3.7-5.8)	<0.01
No	2.0 (1.1-4.4)		5.4 (4.2-6.5)	
Criterion V				
Yes	2.2 (1.2-4.7)	0.13	4.7 (3.9-6.0)	<0.01
No	1.9 (0.9-4.3)		5.3 (4.2-6.5)	

CRP, C-reactive protein; MetS, metabolic syndrome [Criteria: I. Waist circumference >102 cm (>40 in); II. HDL cholesterol levels <40 mg/dL (<1.0 mmol/L), or being actively treated for low HDL levels; III. Serum triglycerides levels ≥150 mg/dL (≥1.7 mmol/L), or being actively treated for elevated triglycerides; IV. Fasting glucose levels ≥100 mg/dL (≥6.1 mmol/L), or being actively treated for hyperglycaemia, and; V. Diagnosis of elevated blood pressure or being actively treated for hypertension].

Values express median and interquartile range. Statistical test *t*-Student.

TABLE 3 Univariate analysis showing the influence of MetS, circulating C-reactive protein or testosterone levels on the diagnosis of PCa, and clinically significant PCa (Gleason Score ≥7)

	PCa, n = 195			PCa, Gleason ≥7, n = 97		
Variable	OR	*P*	95% CI (OR)	OR	*P*	95% CI (OR)
MetS (yes)	1.17	0.39	0.82-1.66	1.62	0.03	1.04-2.54
No. of MetS criteria	1.13	0.07	0.99-1.29	1.23	0.02	1.04-1.45
MetS criteria						
Criterion I vs no MetS	1.39	0.07	0.97-1.98	1.71	0.02	1.08-2.72
Criterion II vs no MetS	0.96	0.83	0.67-1.37	1.06	0.78	0.68-1.65
Criterion III vs no MetS	1.05	0.84	0.69-1.58	1.23	0.41	0.75-2.02
Criterion IV vs no MetS	1.13	0.49	0.79-1.60	1.20	0.41	0.77-1.87
Criterion V vs no MetS	1.60	0.01	1.13-2.29	1.76	0.02	1.13-2.79
CRP (mg/L)	1.02	0.11	0.99-1.05	1.04	0.02	1.01-1.07
Testosterone (ng/mL)	0.93	0.15	0.85-1.02	0.96	0.48	0.85-1.08

CRP, C-reactive protein; OR, odds ratio; PCa, prostate cancer; MetS, metabolic syndrome [Criteria: I. Waist circumference >102 cm (>40 in); II. HDL cholesterol levels <40 mg/dL (<1.0 mmol/L), or being actively treated for low HDL levels; III. Serum triglycerides levels ≥150 mg/dL (≥1.7 mmol/L), or being actively treated for elevated triglycerides; IV. Fasting glucose levels ≥100 mg/dL (≥6.1 mmol/L), or being actively treated for hyperglycaemia, and; V. Diagnosis of elevated blood pressure or being actively treated for hypertension].

prostate volume and number of biopsies) with an AUC of 0.78 (0.72-0.84), 0.78 (0.73-0.84) and 0.77 (0.72-0.83) respectively (Figure 2A). It should be mentioned that the combination of these three clinical variables together did not significantly increase the predictive ability of the diagnosis of Sig-PCa (Figure 2A). However, a clear trend was found to diagnose Sig-PCa when adding the number of MetS, which might justify future evaluations in higher cohorts. Furthermore, in a sub-analysis in patients with a PSA<10 ng/mL, the AUC only showed a non-significant increase with the addition of the presence of MetS or the number of MetS criteria, but not with CRP levels (AUC of 0.76 vs 0.745) (Figure 2B).

4 | DISCUSSION

MetS and PCa are highly prevalent conditions worldwide. Current evidence suggests that MetS could play a role in the development and progression of several neoplasms, including PCa.[6,21] However, the specific components of MetS that may contribute to PCa risk and progression/aggressiveness in human remains controversial. In this sense, we have previously demonstrated the existence of a tight cross-talk between the metabolic status and the development and homoeostasis of the prostate gland, wherein key metabolic components (eg insulin, leptin, etc.) could play a relevant pathophysiological role at the prostate level.[7,8] Moreover, it has been shown that androgen-deprivation therapy in patients with PCa results in changes that overlap with MetS, including decreased insulin sensitivity, increased triglycerides and increased fat mass.[22] Despite the efforts and progresses made in recent years, it is imperative to determine the real impact of MetS, and/or of its individual components on PCa development, as well as the to determine the risk factors that comprise MetS in men with PCa to treat them accordingly.

In this study, we aimed at determining the potential associations and clinical consequences that MetS, each of the individual criterion of MetS, circulating testosterone levels and inflammatory status (using circulating CRP levels) may have on the risk and aggressiveness of PCa. As previously reported,[16,23,24] we observed an association between MetS and/or most of its individual criterion with lower circulating levels of testosterone and higher circulating levels of CRP. Furthermore, the analysis of the different clinical characteristics comparing patients with and without MetS revealed that patients with MetS had slightly higher prostate volume compared with patients without MetS, which is consistent with a recent report indicating an association of MetS parameters with benign prostatic

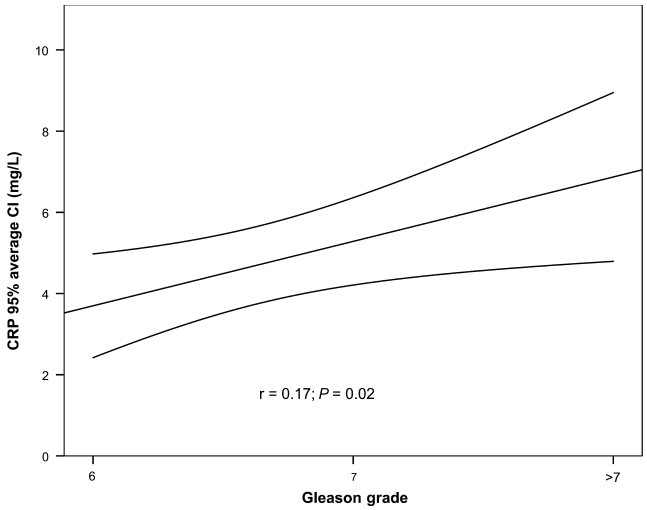


FIGURE 1 Correlation curve between circulating CRP levels and Gleason Score in patients with PCa

TABLE 4 Univariate analysis of common predictive factors of significant PCa on biopsy

	Sig-PCa (Gleason ≥7)		
Variables	**OR**	***P***	**95% CI (OR)**
Age 60-70 vs <60 (years old)	1.66	0.10	0.90-3.07
Age >70 vs <60 (years old)	5.35	<0.01	2.87-9.98
PSA 3-10 vs <3 (ng/mL)	2.67	0.07	0.93-7.66
PSA 10-20 vs <3 (ng/mL)	5.42	<0.01	1.70-17.34
PSA >20 vs <3 (ng/mL)	30.44	<0.01	8.73-106.11
DRE (suspicious)	3.70	<0.01	2.29-5.99
Prostate volume (cc)	0.98	0.02	0.97-0.99
Number of biopsy >1 (yes)	0.34	<0.01	0.18-0.66
Family history (yes)	0.74	0.35	0.39-1.39

DRE, digital rectal examination; PCa, prostate cancer; OR, odds ratio.
PSA - Adjusted by 5-α reductase inhibitors. [Prostate volume (N = 441 patients; PCa Gleason ≥7 = 79)].

enlargement in men surgically treated for this pathology.[25] These data might suggest that some component of the MetS could be connected with the prostatic growth and, therefore, given that the prevalence of MetS is increasing worldwide, the clinical control of MetS should be considered in patients at risk of PCa.

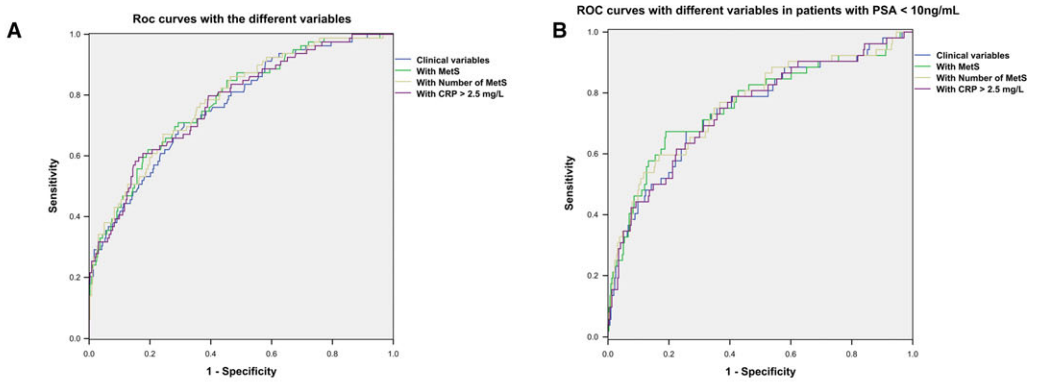
In line with this, the majority of the previous studies analysing the association between MetS and PCa have used the definition established by the NCEP ATP III,[21] which have often obtained inconsistent conclusions, probably due to the fact that the individual diagnostic criterion of MetS have not been consistently and uniformly examined in these studies.[21,26–39] In contrast, in this study, we have analysed the presence of MetS and of each MetS criterion independently using a significant cohort of patients (n = 524) with and without MetS, and with and without PCa (n = 240 with MetS [94 with and 146 without PCa] and n = 284 without MetS [101 with and 183 without PCa]). Remarkably, we found that the rate of Sig-PCa diagnoses was significantly higher in patients with MetS compared with patients without MetS (P = 0.03). Furthermore, our study indicated that the presence of MetS as well as a greater number of MetS criteria was significantly associated with a higher risk of Sig-PCa. In fact, multivariate analysis ROC curve analyses revealed that the presence of MetS and a greater number of MetS criteria could be used as diagnostic factors for Sig-PCa. Consistent with our study, Bhindi et al,[28] who previously investigated the criteria of MetS as quantitative variables, also observed that the greater the number of MetS criteria met, the greater the risk that patients had of harbouring a Sig-PCa. Interestingly, when we analysed each MetS criterion individually, we found that a higher waist circumference and elevated blood pressure (criteria I and V, respectively) were the only two factors significantly associated with an increased risk of PCa and of Sig-PCa in our cohort of patients, which is further supported by previous meta-analysis published on this specific topic.[40] In this sense, it should be mentioned that, although BMI has been commonly used to define obesity, BMI is probably less precise than the waist circumference which has been shown to have a stronger association with the inflammatory status and cardiovascular risk.[41] In fact, we found a strong correlation between both BMI and waist circumference in our cohort of patients; however, more individuals were considered as obese patients when waist circumference was used to categorize them vs BMI (ie 55% [waist circumference] vs 33% [BMI]). Furthermore, previous data have showed that waist circumference as a quantitative variable is associated with a higher risk of PCa or Sig-PCa after adjusting by BMI,[42] which is further validated in our cohort showing an OR: 1.07 (95% CI: 1.03-1.12, P = 0.002) for Sig-PCa.

Interestingly, since the use of metformin and statins and the risk of PCa is a controversial topic worldwide,[43–45] we also analysed this association in this study. Specifically, we did not observe an association between metformin or statins intake and the diagnosis of PCa in our cohort of patients; however, a clear association was found between metformin, but not statins, intake and the diagnose of Sig-PCa. Nevertheless, this observation should be taken with caution since, it was based on an exploratory analysis of drug intake and the presence of PCa at the time of prostate biopsy using a limited number of patients under metformin treatment and, without evaluating the period of time under the drug intake (which was not available in our cohort), being this latter parameter essential in this analysis since evidences have showed that only those patients with long-term consumption of metformin are the patients with less risk of any PCa.[44]

Since the available studies focusing on the association between circulating testosterone levels and the risk of developing PCa are in many instances controversial,[46,47] we next explored the association and independent predictive ability for Sig-PCa diagnoses of circulating testosterone levels among patient at risk of PCa and found no association between testosterone levels and an increased risk of PCa or Sig-PCa on the prostate biopsy in our cohort of patients. In contrast, we observed a clear association between elevated circulating CRP levels and a higher risk of Sig-PCa. Moreover, multivariate analysis showed that circulating CRP levels could be used as diagnostic factor for Sig-PCa. These observations are in part consistent with some, but not all[48–54] early reports showing that circulating CRP levels are associated with the prognosis of PCa (advanced and

TABLE 5 Multivariate analysis of the predictive ability of different variables (presence of MetS, number of MetS criteria or circulating CRP levels) to predict a higher risk of Sig-PCa adjusting by age, PSA, 5-α reductase inhibitors intake, DRE, prostate volume and number of biopsies

	Multivariate analysis of MetS			Multivariate analysis of number of MetS criteria			Multivariate analysis of CRP levels		
	OR	*P*-value	95% CI (OR)	OR	*P*-value	95% CI (OR)	OR	*P*-value	95% CI (OR)
Age 60-70 vs <60 (years old)	1.74	0.14	0.83-3.68	1.97	0.20	0.77-3.49	1.81	0.12	0.86-3.83
Age >70 vs <60 (years old)	4.78	<0.01	2.14-10.66	4.55	<0.01	2.04-10.18	5.04	<0.01	2.25-11.30
PSA 3-10 vs <3 (ng/mL)	2.64	0.09	0.85-8.19	2.66	0.09	0.85-8.28	2.42	0.12	0.79-7.44
PSA 10-20 vs <3 (ng/mL)	4.99	0.02	1.34-18.64	5.07	0.02	1.36-18.98	3.98	0.03	1.07-14.77
PSA>20 vs <3 (ng/mL)	19.69	<0.01	4.36-88.97	20.76	<0.01	4.57-94.28	13.94	<0.01	3.09-62.90
5-α reductase inhibitors intake	1.19	0.79	0.32-4.31	1.21	0.77	0.33-4.40	1.31	0.68	0.36-4.81
DRE (suspicious)	1.59	0.15	0.85-3.01	1.61	0.14	0.85-3.04	1.79	0.08	0.94-3.42
Prostate volume (cc)	0.98	<0.01	0.96-0.99	0.98	<0.01	0.96-0.99	0.98	<0.01	0.96-0.99
Number of biopsy >1 (yes)	0.32	<0.01	0.13-0.76	0.32	0.01	0.13-0.77	0.36	0.02	0.15-0.84
Family history (yes)	1.19	0.64	0.56-2.57	1.25	0.57	0.58-2.68	1.14	0.74	0.53-2.42
MetS (yes)	1.83	0.03	1.05-3.20						
No. of MetS criteria				1.30	0.02	1.05-1.60			
CRP >2.5 mg/L							2.00	0.02	1.14-3.51



Model	AUC	Sig Pca *P*	95%CI	*P*-value
Clinical variables	0.764	<0.001	0.706-0.822	
Clinical variables with MetS	0.779	<0.001	0.723-0.835	0.143
Clinical variables with number of MetS	0.782	<0.001	0.727-0.838	0.05
Clinical variables with CRP > 2.5mg/L	0.774	<0.001	0.716-0.831	0.395

Model in PSA < 10ng/mL	AUC	Sig Pca *P*	95%CI	*P*-value
Clinical variables	0.745	<0.001	0.667-0.823	
Clinical variables with MetS	0.76	<0.001	0.681-0.839	0.314
Clinical variables with number of MetS	0.76	<0.001	0.683-0.837	0.255
Clinical variables with CRP > 2.5mg/L	0.745	<0.001	0.668-0.822	0.989

FIGURE 2 ROC curves showing the predictive ability of different variables (Presence of MetS, number of MetS criteria or circulating CRP levels) to predict a higher risk of significant PCa (Sig-PCa) when are added to risk factors; age, family History, PSA, 5α reductase inhibitors intake, DRE, prostate volume and number of biopsies; (A) within the total cohort (n = 441 patients; PCa Gleason ≥7 = 79). (B) In patients with PSA <10 ng/mL (n = 368 patients; PCa Gleason ≥7 = 52) (for this analysis the PSA was not categorized and was evaluated as a continuous variable). *P*-value represents the comparison of each ROC curve with the basal ROC curve with the clinical variables alone

metastatic disease). Of note, our results also revealed that higher circulating CRP levels were associated with PCa aggressiveness since its circulating levels were positively associated with higher GS in our cohort of PCa patients. To the best of our knowledge, this is the first report showing that baseline circulating levels of CRP are associated with a higher risk of detecting PCa at the time of biopsy and demonstrating that circulating CRP levels could be used as a putative biomarker of PCa aggressiveness.

Finally, it should be mentioned that some observations reported in this study might have certain limitations and therefore, should be interpreted with some caution. First, although the use of TRUS biopsy for PCa diagnosis suffers from random error and false negative results in comparison with trans-perineal template biopsy, which might have affected the results of this study, it should be emphasized that TRUS biopsy is worldwide spread and the standard method in the current clinical practice.[55] Likewise, it would be

preferable to have compiled data of multiple CRP and testosterone levels from each patient over a larger time interval rather than a single value. Finally, the onset of MetS from diagnosis in each patient would ideally have been recorded as well to determine if the chronicity of the disease influences the degree of observed inflammation, and CRP levels. Nonetheless, based on the high incidence of MetS worldwide, especially in western countries, and considering the evident connection between some of the components of the MetS and the risk of PCa at the time of prostate biopsy, as well as of the association between inflammatory status with the aggressiveness of PCa found in our study, the results of the present work invites to suggest that interventional studies based on the control of MetS and inflammatory status in patients at risk of PCa might be a key point in the overall management of this disease. Therefore, future cellular/molecular/translational studies are crucial to understand the specific connections between individual MetS determinants and the pathophysiology of PCa.

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AUTHOR CONTRIBUTION

EG-G, JC-V, RML and MJR-T conceived and designed the project; EG-G, JC-V, JPC-H, AMB-P, JV-R, JR-G and MJR-T acquired data; EG-G, JC-V, AMB-P, JMJ-V and RML performed the analysis and interpretation of data; EG-G, JC-V and RML wrote the manuscript; JPC-H, AMB-P, JMJ-V, JV-R, JR-G and MJR-T revised the manuscript for important intellectual content; EG-G, JMJ-V performed the statistical analysis and imaging; RML and MJR-T obtained funding; RML supervised the work.

CONFLICTS OF INTEREST

Nothing to declare.

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Article III

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ARTICLE

Molecular Diagnostics

CE–MS-based urinary biomarkers to distinguish non-significant from significant prostate cancer

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BACKGROUND: Prostate cancer progresses slowly when present in low risk forms but can be lethal when it progresses to metastatic disease. A non-invasive test that can detect significant prostate cancer is needed to guide patient management.
METHODS: Capillary electrophoresis/mass spectrometry has been employed to identify urinary peptides that may accurately detect significant prostate cancer. Urine samples from 823 patients with PSA (<15 ng/ml) were collected prior to biopsy. A case–control comparison was performed in a training set of 543 patients ($n_{Sig} = 98$; $n_{non\text{-}Sig} = 445$) and a validation set of 280 patients ($n_{Sig} = 48$, $n_{non\text{-}Sig} = 232$). Totally, 19 significant peptides were subsequently combined by a support vector machine algorithm.
RESULTS: Independent validation of the 19-biomarker model in 280 patients resulted in a 90% sensitivity and 59% specificity, with an AUC of 0.81, outperforming PSA (AUC = 0.58) and the ERSPC-3/4 risk calculator (AUC = 0.69) in the validation set.
CONCLUSIONS: This multi-parametric model holds promise to improve the current diagnosis of significant prostate cancer. This test as a guide to biopsy could help to decrease the number of biopsies and guide intervention. Nevertheless, further prospective validation in an external clinical cohort is required to assess the exact performance characteristics.

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BACKGROUND

Prostate cancer (PCa) is ranked as the second most frequently diagnosed cancer in men,[1] and the most frequent non-skin cancer in developed countries.[2] Although PCa is diagnosed in 15–20% of men, the lifetime risk of death due to PCa is very low (3%),[3] mainly because low-risk forms progress slowly and the disease is well treatable in early stages. PCa diagnosis is currently mostly based on serum prostate-specific antigen (PSA) testing, digital rectal examination (DRE) and confirmed by a multi-core prostatic biopsy.[4] Multiple factors not related to prostate malignancy may affect the level of blood PSA [inflammation, infection or presence of benign prostate hyperplasia (BPH)]. Therefore, PSA lacks specificity particularly in the intermediate range, with only 22–27% of those patients with PSA between 4–10 ng/ml to be positively confirmed with PCa after biopsy.[5] In addition, PSA screening and multicore biopsy have increased the detection rate of small, localised, well-differentiated PCa,[6] resulting in over-diagnosis and over-treatment.[6–9] For those patients presenting with an indolent or clinically non-significant cancer (Gleason score (GS) < 7),[10] immediate treatment may not be beneficial and ideal management may be a conservative approach, such as active surveillance (AS).[11] Management of patients with non-significant PCa currently relies on repeated biopsies, series of PSA measurements and DRE, while the uncertainty to properly assess PCa imposes a significant social and economic burden on patients and health insurances because of the side effects and treatment costs.[12] For these reasons, better stratification of the risk for significant PCa (Sig PCa) appears beneficial to guide patient management.

Aimed at improving on the current discrimination of Sig PCa by non-invasive means, capillary-electrophoresis coupled to mass spectrometry (CE–MS) was employed to identify peptides specific for PCa in urine samples from patients with clinically significant and non-significant PCa. Urine was selected, as it presents several advantages over blood or tissue, among others: easy, non-invasive repeated sampling, effortless availability and high stability of the proteome. Although several candidate biomarkers have been described,[13–15] the currently available single biomarkers lack diagnostic accuracy for routine clinical application. At the same time, the high biological variability of PCa suggests that a combination of clearly defined, -omics derived biomarkers, rather than a single biomarker, may provide higher accuracy to detect cancer.[16–18] In this study, we aimed to establish a biomarker model to detect Sig PCa.

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METHODS

Study population and design

A case–control study was performed on patients who underwent a transrectal ultrasound (TRUS)-guided prostate biopsy from January 2013 to July 2015 in the Urology department, Reina Sofia Hospital, Cordoba, Spain, as part of the ONCOVER project. Ethical approval was obtained by the Reina Sofia Hospital Research Ethics Committee and informed consent was obtained from all participants for the project. ONCOVER cohort included patients who attended the urology clinic of Reina Sofia Hospital with a recommendation for a prostate biopsy according to clinical practice.[19] Patients provided a urine sample and underwent blood testing just before undergoing a prostate biopsy. Recommendations for biopsy indication were: suspicious findings on DRE, PSA > 10 ng/mL, or PSA 3–10 ng/mL if free PSA ratio was low (usually, <25–30%), and in patients with previous biopsies, a persistently suspicious indication of PCa (persistently elevated PSA, suspicious DRE, etc.). For transrectal prostate biopsy, 12 cores were obtained from patients undergoing the first biopsy procedure, and a minimum of 16 biopsy cores for those who had a previous biopsy. For this analysis, 823 PCa patients were included according to the following criteria: (a) PSA level <15 ng/mL on the day of the biopsy and (b) no previous diagnoses of PCa. For all 823 patients, complete records for all the main variables were available, including PSA, DRE, number of previous biopsies, 5-alpha-reductase inhibitor intake and pathology results. Information on prostate volume was additionally retrieved for 721 patients, based on the measurements that had been performed with TRUS during the biopsy. Because of missing data for 102 patients, and in order to avoid introducing any selection bias, for this analysis prostate volume was not included in the nomogram analysis, but only for comparison purposes (i.e., biomarkers compared to prostate volume). Patients treated with 5-alpha-reductase inhibitors for urinary symptoms were also included in the study, but excluded from the analysis for the comparison with PSA, as treatment with 5-alpha-reductase inhibitors is expected to affect PSA levels. All biopsy specimens were analysed by a urologic pathologist according to International Society of Urological Pathology 2005 modified criteria.[20] Clinical and laboratory data, including among others: age, PSA level (on the day of biopsy), the results of DRE, number of previous biopsies, prior treatment with 5-alpha-reductase inhibitors, prostate volume by TRUS, urinary creatinine and pathology results were collected and presented in the Supplementary Table 1. A score based on the risk calculator of the European Randomised Study of Screening for Prostate Cancer (ERSPC) was calculated (http://www.prostatecancer-riskcalculator.com/seven-prostate-cancer-risk calculators). The formulas that were utilised in this study, were ERSPC- 3, for those patients during initial biopsy, and ERSPC- 4, for patients during repeated biopsy. For the above estimates, the variables that are considered are PSA and DRE and the result of previous biopsy for those patients who underwent (biopsy before (ERSPC-4). GS was used in this study to discriminate Sig PCa (GS ≥ 7) from non-Sig PCa.

MS analysis

CE–MS analysis was performed for the 823 urine samples, following the previously established protocols for samples preparation and data acquisition, previously described in detail.[21] In brief, sample preparation was performed by diluting 700 µl urine aliquots from the urine collected from patients prior to the prostate biopsy, with two volumes (1.4 ml) alkaline buffer containing 2 M urea, 10 mM NH_4OH and 0.02% sodium dodecyl sulphate (pH 10.5). Subsequently, the samples were filtered by Centrisart ultracentrifugation filters (Sartorius, Göttingen, Germany) to retain proteins/polypeptides below 20 kDa and were subsequently desalted over PD-10 columns (GE Healthcare, Munich, Germany). The peptide extracts were lyophilised and resuspended in high-performance liquid-chromatography (LC) grade water. CE–MS analysis and data processing were performed according to ISO13485 standards yielding quality controlled urinary data sets.[21] Mass spectral ion peaks representing identical molecules at different charge states were deconvoluted into single masses using MosaiquesVisu software.[22,23] The peak list characterises each peptide by its molecular mass (kDa), normalised migration time (min) and normalised signal intensity (AU).[22,23] Normalisation of the CE–MS data were based on twenty nine collagen fragments that are generally not affected by disease and serve as internal standards.[24] After normalisation, all proteomics datasets were deposited, matched, and annotated in a Microsoft SQL database and used as input in the presented study. Transformation of the data (log-transformation) was performed prior to the statistical analysis, as previously described.[25]

Peptide sequencing and matching

Matching of the amino acid sequences with the CE–MS acquired ion peaks was based on mass correlation between CE–MS and LC-tandem MS analysis. The amino acid sequence was determined by MS/MS analysis using either a PACE CE or a Dionex Ultimate 3000 RSLS nanoflow system (Dionex, Camberly UK) coupled to an Orbitrap Velos instrument (Thermo Scientific), as previously described.[26] Protein matching and data analysis was based on Proteome Discoverer 1.2 (activation type: HCD; precursor mass tolerance: 5 ppm; fragment mass tolerance: 0.05 Da). No fixed modifications were selected, oxidation of methionine and proline were selected as variable modifications. The data were searched against the UniProt human database[27] without enzyme specificity. Further validation of the obtained peptide identifications is based on the assessment of the peptide charge at the working pH of 2.2 and the CE-migration time results.[28]

Statistical analysis

A case–control statistical comparison was performed to detect potentially Sig PCa biomarkers. The datasets were grouped into: (a) a case set of clinically Sig PCa ($n_{Sig} = 146$), including PCa patients with high-risk PCa (GS ≥ 7) and (b) a control set including clinically non-significant PCa (low-risk PCa; GS = 6) along with patients presenting with other aetiologies ($n = 677$). The groups were further divided into a discovery ($n_{Sig} = 98$ cases of Sig PCa; $n_{non\text{-}Sig} = 445$ controls) and validation set ($n_{Sig} = 48$ cases of Sig PCa, $n_{non\text{-}Sig} = 232$ controls), according to the '2/3–1/3 rule', as previously described.[16] Random sampling guarantees that each group/class is properly represented in all data subsets. Based on the literature,[29] this commonly used strategy of allocating two-third of cases for training is close to optimal for large sized datasets ($n \geq 100$) with strong signals (i.e., >85% full dataset accuracy).[29]

Further statistical analysis was performed to identify potential bias, considering the clinical data shown in Table 1. Mann–Whitney non-parametric test was used to investigate statistically significant differences between the two groups for continuous variables and chi-squared test for categorical variables, respectively. The urinary CE–MS profiles were compared for differences at the individual peptide excretion levels by applying the Wilcoxon rank sum test.[25] A frequency threshold of 70% in at least one of the two groups was applied. To increase the validity of the statistical approach, permutation analysis was performed by randomly excluding 30% of the samples and repeated five times. Statistical correction of the estimated p values for multivariate testing was performed based on the Benjamini–Hochberg method.[30] Only the peptides significant ($p < 0.05$) in all five permutation analyses were considered for further analysis.

Optimisation of the SVM-based biomarker model

The urinary peptide-based classifier was developed in the training set, using MosaCluster (version 1.7.0), a support vector machine

Table 1. Clinical and biochemical variables for the patients grouped into the discovery and validation set

Baseline characteristics	Discovery phase ($n = 543$)	Validation phase ($n = 280$)	p Value discovery vs. validation	Group 1: Non-significant PCa/Controls ($n = 677$)	Group 2: Significant PCa ($n = 146$)	p Value group 1 vs. group 2
Median age (IQR; y)	64.0 (11.0)	63.5 (12.0)	0.2947[a]	63.0 (11.5)	68.0 (10.3)	<0.0001[a]
PSA median (IQR; ng/ml)	5.4 (3.6)	5.0 (3.1)	0.2060 [a]	5.1 (3.3)	6.1 (4.1)	0.0013[a]
Digital rectal examination (Pos/Neg)	104/439	40/240	0.0576[b]	94/583	50/96	0.1453[b]
Previous biopsies (Y/N)	139/404	69/ 211	0.9895[b]	187/480	21/125	0.0007[b]
Median number of previous biopsies (IQR)	1 (1)	1 (0)	0.6366[a]	1 (1)	1 (0)	0.007[a]
Prostate volume (IQR; ml)	35.0 (25.0; $n = 481$)	36.0 (18.4; $n = 240$)	0.6701[a]	37.4 (23; $n = 594$)	28.0 (18; $n = 127$)	<0.0001[a]
PSA density (IQR; ng/ml^2)	0.14 (0.11; $n = 481$)	0.14 (0.10; $n = 240$)	0.3156[a]	0.20 (0.09; $n = 594$)	0.13 (0.15; $n = 127$)	<0.0001[a]
5α-reductase treatment (Y/N)	18/ 524	6/ 274	0.4640[b]	22/655	2/144	0.2219[b]
Median urinary creatinine (IQR; mmol/L)	7.6 (4.7)	8.3 (4.9)	0.0875[a]	7.9 (4.7)	7.8 (4.7)	0.5319[a]
Significant PCa	98 (18.0%)	48 (17.1%)	0.9345[b]			
Non-significant PCa	445 (82.0%)	232 (82.9%)	0.8532[b]			
Disease pathology—significant PCa						
Gleason 3 + 4/4 + 3	63 (64.3%)/20 (20.4%)	32 (66.6%)/9 (18.8%)	0.9290[b]/0.9945[b]			
Gleason 8	9 (9.2%)	5 (10.4%)	0.9459[b]			
Gleason≥9	6 (6.1%)	2 (4.2%)	0.9308[b]			
Disease pathology—non-significant PCa						
Gleason 6	99 (22.1%)	32 (13.8%)	0.0126[b]			
Benign—non-PCa aetiologies						
Benign prostatic hyperplasia; BPH	307 (69.1%)	175 (75.4%)	0.1033[b]			
Prostatic intraepithelial neoplasia; PIN	22 (5.0%)	12 (5.2%)	0.9425[b]			
Atypical small acinar proliferation; ASAP	17 (3.8%)	13 (5.6%)	0.3766[b]			

[a]Mann–Whitney test
[b]Chi-squared test
IQR interquartile range, *N* not received, *Neg* negative, *PCa* prostate cancer, *Pos* positive, *Y* received

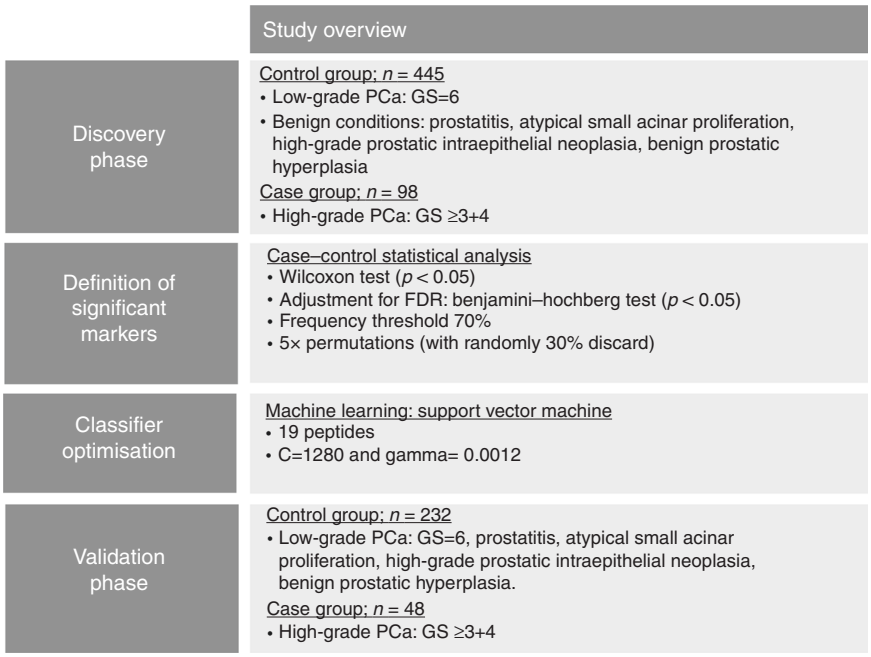


Fig. 1 Schematic representation of the study design and the analytical workflow for the development of urine CE–MS-based biomarker panel

(SVM)-based software. The classifier was optimised based on the shortlisted PCa specific biomarkers with each biomarker representing one dimension in the n-dimensional parameter space.[17] In additiony, the cut-off was established based on the discovery set. In the independent validation, the sensitivity and specificity estimates for the SVM-based peptide marker pattern were calculated based on the number of correctly classified samples. The receiver operating characteristic (ROC) plots and the respective confidence intervals (95% CI) were based on exact binomial calculations and were calculated in MedCalc 12.7.5.0 (Mariakerke, Belgium). Area under the curve (AUC) values were then compared using DeLong tests. Statistical comparisons of the classification scores in the validation cohort were performed by the Kruskal–Wallis rank sum test using MedCalc. To address the potential clinical utility of the models, we performed decision curve analysis, as proposed by Vickers and Elkin.[31] This method has the advantage of not requiring the specification of the relative cost for false-positives and false-negatives, defining a net benefit as a function of the decision threshold at which one would consider obtaining a biopsy. For the analysis MedCalc 12.7.5.0 (Mariakerke, Belgium) and R version 3.2.3 were used.

RESULTS

Study cohort for patients with clinically significant and non-significant PCa

Proteomics profiling data were acquired from 823 patients suspicious for PCa. Out of those, 677 (82.3%) presented with non-significant PCa (GS = 6), benign or atypical conditions (control group) and 146 (17.7%) were included in the case group due to presence of Sig PCa. Men with Sig PCa were significantly older [median age = 68; interquartile range (IQR) = 10.3] compared to men from control group (median age = 63; IQR = 11.5; $p < 0.0001$). In addition, patients from the control group had significantly lower PSA levels (median = 5.1 ng/ml; IQR = 3.3) compared to those from case group [median = 6.1 ng/ml; IQR = 4.1; $p = 0.0013$]. Within the control group, 480 (70.9%) did not undergo any previous negative biopsy, while for patients with Sig PCa, the respective proportion was 85.6% ($n = 125$); ($p = 0.0007$). The clinical characteristics along with the sample distribution are presented in the Table 1.

Development of a biomarker model based on CE–MS urinary peptide profiling

For the identification of CE–MS specific biomarkers, a case–control comparison was performed in the discovery set of 543 patients, schematically depicted in Fig. 1. The comparison enabled the identification of 19 peptides displaying statistically significant differences in their distribution between patients with Sig PCa compared to the control group (Supplementary Table 2). The graphical depiction of the compiled urinary profiling signatures is comparatively presented in Fig. 2. Using the 19 statistically altered peptide markers an SVM machine learning algorithm was adopted and optimise to develop a classifier (Fig. 1).

Independent validation of the SVM-based biomarker model

Validation of the 19-biomarker model in the independent set ($n = 280$), in line with the recommendations for biomarker identification and reporting in clinical proteomics,[25] resulted in an overall AUC value of 0.81 ranged from 0.76 to 0.86 (95% CI: $p < 0.0001$). Fig. 3 presents the ROC curve, which at the pre-defined cut-off of −0.07 resulted in sensitivity levels of 90% (77–97; 95% CI) and specificity of 59% (52–65; 95% CI), respectively. Additional statistical analysis was performed, by application of a post hoc rank sum test to compare the scores between the case and control groups. As depicted in Fig. 4a, the classification of each group differs at the significance level of $p < 0.0001$. Moreover, as shown in Fig. 4b, there is a gradual increase in the 19-biomarker model score, as GS increases, while a significant difference is observed between the 19-biomarker model scores of GS 6 tumours and GS ≥ 7 ($p < 0.0001$).

Comparative analysis of the 19-biomarker model with clinical parameters

A direct comparison of the 19-biomarker model with PSA was performed in the validation set. Of note, out of 280 patients, 6 patients had received previous treatment with 5-alpha-reductase inhibitors, therefore for the comparative analysis only 274 patients were considered. As depicted in Fig. 5a, the multi-peptide model

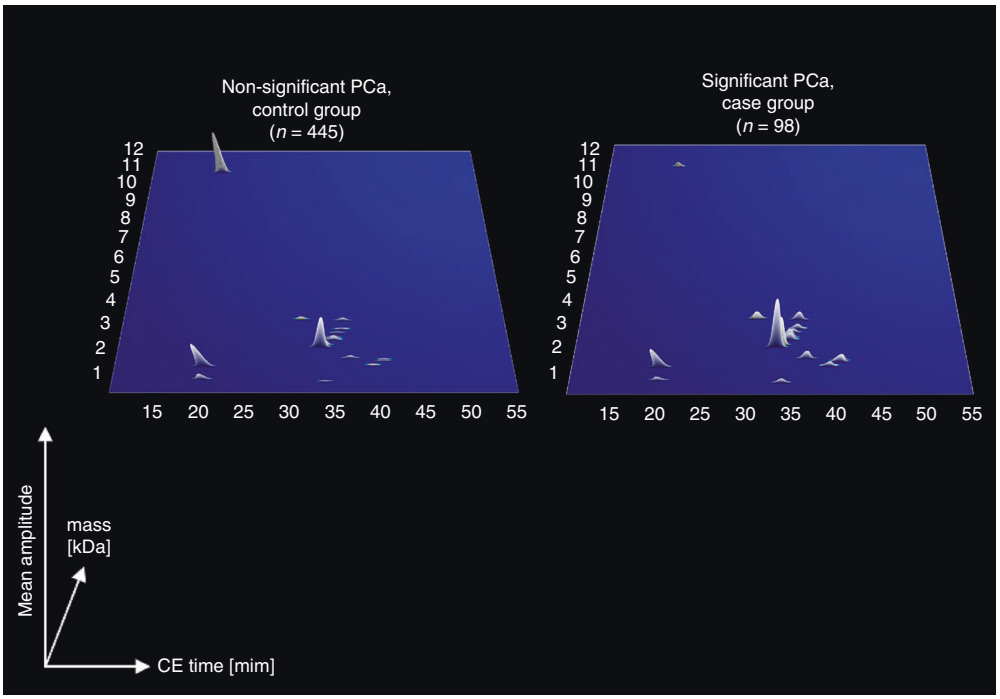
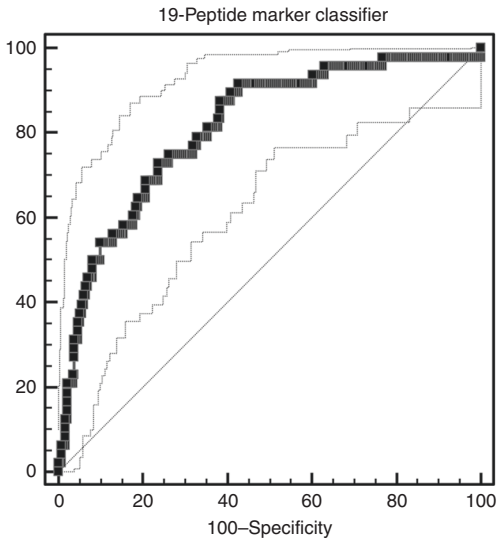


Fig. 2 Compiled average urinary profiling signatures of the patients with significant and non-significant PCa. The molecular mass (0.1–12 kDa) is shown on a logarithmic scale and is plotted against normalised migration time (15–55 min). Signal intensity is encoded by peak height and colour



Validation set	Performance
Sample size (*n*)	280
Case/control group (*n*)	48/232
Area under curve (AUC)	0.81
95% confidence interval	0.76–0.86
p value	<0.0001
Sensitivity (range) [%]	90 (77–97)
Specificity (range) [%]	59 (52–65)
Optimal cut-off	>−0.07

Fig. 3 Receiver operating characteristics (ROC) analysis performed in the independent validation cohort, displaying the performance of the 19-biomarker panel for discriminating the case group ($n_{Sig} = 48$) from the control group ($n_{non\text{-}Sig} = 232$). ROC characteristics, such as area under the curve (AUC), 95% confidence intervals (CI), and *p* value are provided for the classification of Sig PCa patients

significantly outperformed the PSA testing with the AUC values at 0.82 and 0.58, respectively ($p < 0.0001$). For those patients where clinical records on prostate volume were available ($n = 240$), an additional comparison between the 19-biomarker model and the prostate volume was performed, indicating a significantly better accuracy for the 19-biomarker model (AUC of 0.81) compared to prostate volume (AUC of 0.64; $p = 0.0103$). Moreover, logistic regression analysis was performed for the available clinical variables to assess the potential significant predictive value of each of those in the discrimination of Sig PCa. The included clinical parameters were: (a) the result of DRE, (b) presence of previous biopsy, (c) the number of previous biopsies, (d) prostate volume and (e) age. Based on the statistical comparison significant contribution to the outcome is revealed for age (odds ratio of 1.1, $p = 0.0366$), PSA (odds ratio of 1.2, $p = 0.0162$) and the 19-biomarker model (odds ratio of 2.2, $p < 0.0001$), while the presence and number of previous biopsies, prostate volume and the result of DRE were not significant predictors of Sig PCa. Combination of the significant variables (19-biomarker model, PSA and age) into a nomogram through the regression equation, resulted in an improved AUC value of 0.83, although not statistically significant ($p = 0.4344$) compared to the 19-biomarker model alone. In order

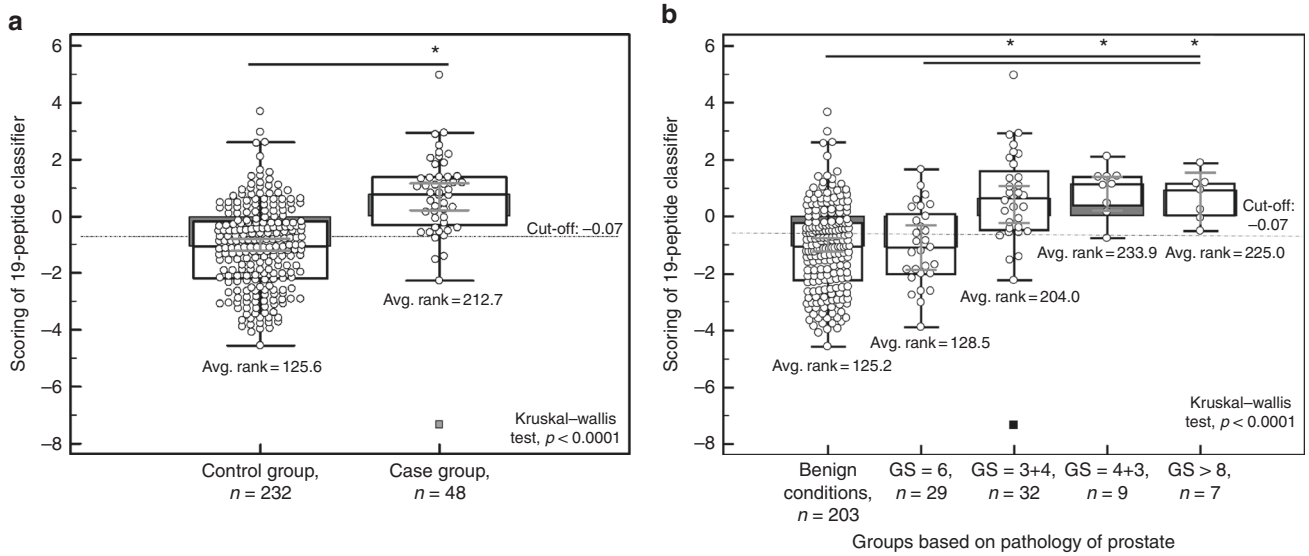
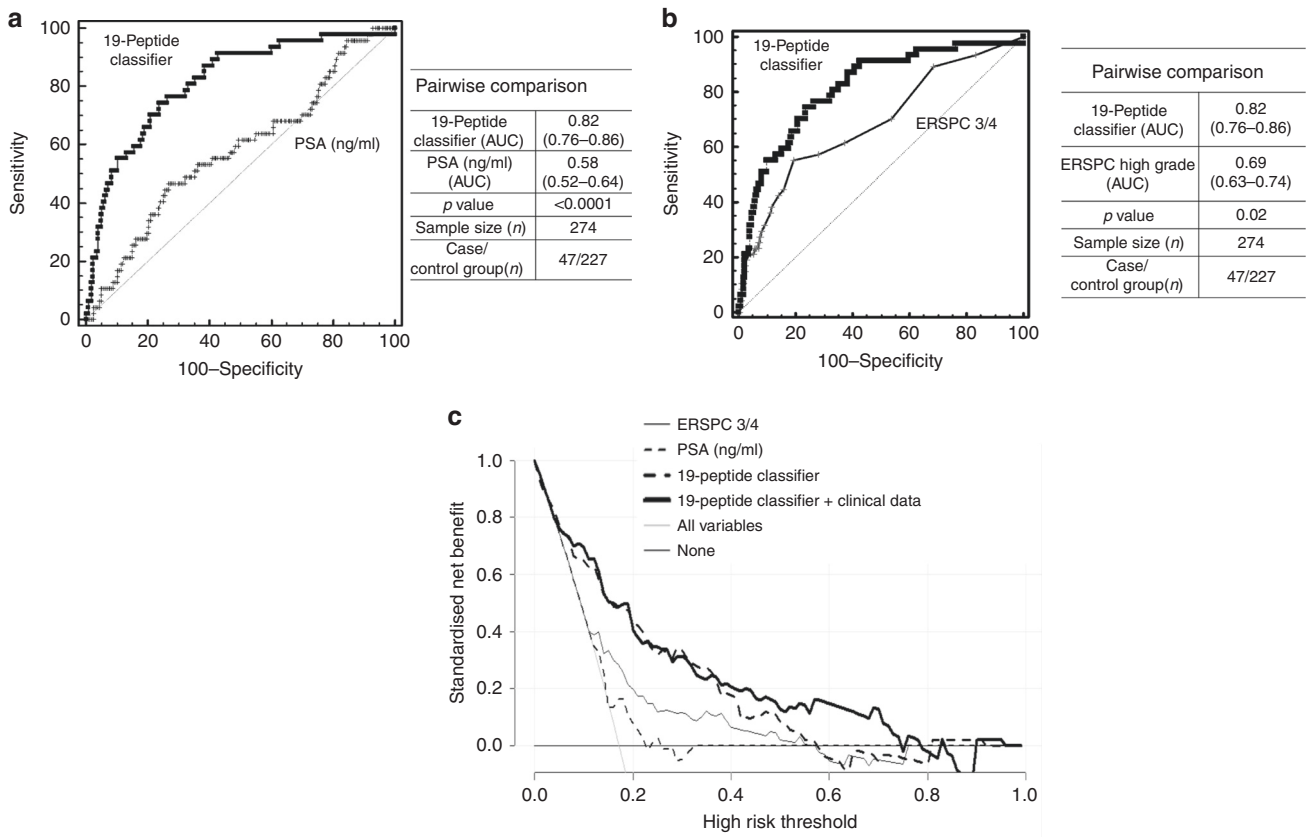


Fig. 4 **a** Classification scores, presented in Box-and-Whisker plots grouped according to the case group ($n_{Sig} = 48$) and control group ($n_{non\text{-}Sig} = 232$). **b** Classification scores displaying the level of discrimination across the different Gleason score. A post hoc rank-test was performed using Kruskal–Wallis test. *$p < 0.05$



Pairwise comparison	
19-Peptide classifier (AUC)	0.82 (0.76–0.86)
PSA (ng/ml) (AUC)	0.58 (0.52–0.64)
p value	<0.0001
Sample size (*n*)	274
Case/ control group(*n*)	47/227

Pairwise comparison	
19-Peptide classifier (AUC)	0.82 (0.76–0.86)
ERSPC high grade (AUC)	0.69 (0.63–0.74)
p value	0.02
Sample size (*n*)	274
Case/ control group(*n*)	47/227

Fig. 5 **a** Comparative analysis depicted by receiver operating characteristics (ROC) curves for the 19-biomarker panel and the PSA levels ($n = 274$). **b** Added value of the 19-biomarker panel over ERSPC -3/4 for high risk (Gleason ≥ 7) ($n = 274$; six patients from the validation set were excluded as previously treated with 5 alpha reductase inhibitors). **c** Results of the decision curve analysis. The net benefit for the prediction of Sig PCa on biopsy is shown, by using the different models as a function of the risk threshold, compared to the benefits of strategies for treating all patients (grey thin line) and treating none (grey thick line)

to investigate if the 19-peptide classifier can present an added value over the current state-of-the-art, the SVM-based score from the 19-biomarker model was further compared with the estimates of the ERSPC risk calculator for detecting high risk PCa (ERSPC—3/4), as presented in Fig. 5b. The 19-peptide classifier showed significantly better performance (AUC = 0.82; $p = 0.02$) compared to the ERSPC estimates (AUC = 0.69). To assess the clinical benefit of the 19-biomarker model, a decision curve

analysis was additionally performed. Based on the net plotting against the threshold probabilities for the comparisons between the 19-biomarker model alone and with clinical variables (PSA, age), PSA and ERSPC estimates, there is a clear benefit of the biomarker model, particularly in the lower range of the risk thresholds (Fig. 5c).

Sequencing of peptide biomarkers

Among the 19 peptide biomarkers, sequences could be obtained for 17, while 2 peptides could not be sequenced. The majority (14/17) were originated from various collagens. Peptide fragments originating from alpha-1 collagen of types (I), (XI), (XVII), (XXI) and alpha-2 type (I), (V), (IX), were most prominent and fragments of collagen type (VIII) chain were also identified. All the collagen peptide fragments are of increased abundance in the Sig PCa cases, apart from collagen alpha-1 (XVII) chain and collagen alpha-1(XXI), which are presented with decreased abundance. Interestingly, among the collagen fragments, a unique motif (pGP) is very prominent. The three remaining peptide markers were a fragment of protein phosphatase 1 regulatory subunit 3A, which was identified with decreased abundance and fractalkine or chemokine (C-X3-C motif) ligand 1 and Semaphorin-7A, both upregulated in the group of patients with Sig PCa.

DISCUSSION

Insignificant PCa are slowly progressing forms that may be better managed conservatively without immediate treatment. Nevertheless, as insignificant forms can progress to significant cancer, frequent monitoring is required to timely and accurately detect the progression. Currently, routine monitoring is based on either PSA, although associated low accuracy, or invasive biopsies.[32] More accurate non-invasive biomarkers are required to improve on the discrimination of Sig PCa. In this study, a biomarker model based on urinary peptides was established and validated in 823 patients suspicious for presence of PCa. This peptide panel enables the discrimination of non-significant PCa from clinically significant forms with high sensitivity and moderate specificity. The lower specificity is mostly attributed to the misclassification of clinically non-significant PCa (mainly GS of 6) as clinically significant forms. The clinical consequence of this observation can be weighted as acceptable, since patients with a positive score based on the 19-biomarker model would further undergo biopsy to rule out the presence of significant cancer.

The 19-biomarker model performs significantly better, when compared to PSA levels and also, when compared to the ERSPC risk calculator, demonstrating an added value of the biomarkers. Comparison with other clinical variables was also performed indicating a significant improvement of the 19-biomarker model, although particularly for prostate volume, missing data for 34 patients from the validation set do compromise the statistical power. An additional decision curve analysis was performed to assess the clinical benefit of the 19-biomarker model, in comparison with the current clinical standards, PSA and ERSPC calculator, demonstrating an improved net benefit of the 19-biomarker model, particularly in the low range of risk threshold.

Nowadays, several biomarkers have been tested in order to discriminate Sig PCa, such as 4K score test, PHI, PCA3, SelectMDx).[33] A direct comparison with those markers, was unfortunately not possible in the context of the presented study, as paired data were not available (as different cohorts and approximations were performed). However, the initial results shown in this study with an AUC higher than 0.80, is within the range of 0.74–0.90 which is shown by other biomarkers[34,35] and clearly justify implementation of this approach in a future investigative setting. In line with this and in order to facilitate comparisons, an additional prospective validation study design is planned, similar to other studies, such as the step approximation of the STHLM3 study, which was able to identify up to 21% of Sig PCa in patients with a PSA between 1 and 3 ng/ml.[35] In the prospective evaluation, inclusion of multiparametric magnetic resonance imaging is planned, as it has demonstrated an added value in the diagnostic approximation for Sig PCa with a high NPV, improving the detection of Sig PCa.[36,37]

Regarding the biomarker identity, sequences could be obtained from 17 of the 19 peptide markers, most of them derived from collagen origin and being in increased abundance. Collagen fragments represent the majority of urinary peptides, even in healthy individuals.[18] The increase in specific collagen fragments may depict extracellular matrix rearrangements, associated with tumour invasion and resulting in proteolytic products, which are subsequently excreted in urine. Previous studies,[18,38] reporting on CE–MS based biomarkers for detection of PCa (for discrimination of PCa patients from those without malignancy), also identified collagen fragments as being increased in abundance in cancer patients.[18,38] In the present study, a slightly different clinical design was followed, as the aim was to discriminate in patients that had PCa, those presenting with significant cancer from those with non-significant cancer. In the study by Theodorescu et al.,[18] four sequences out of twelve could be obtained, with one biomarker common in both studies: a fragment of Collagen alpha-1(I) chain. The other three biomarkers described by Theodorescu et al.[18] were not identified as significantly altered in this study, while an enrichment was observed for other sequences belonging to collagen alpha-1 and collagen alpha-2 chains, protein phosphatase 1 regulatory subunit 3A and fractalkine. The observed differences are attributed in part to the advancements of the technology enabling a better sequence coverage, but also the different clinical context, which in this study was the identification of differentially abundant cancer biomarkers between two cancer forms. A pGP motif was present in most of the collagen sequences. The pGP motif is a chemoattractant derived from proteolytic cleavage of collagen by matrix metalloproteinases. pGP motif binds to (C–X–C motif) receptors and is thus associated with neutrophil attraction in inflamed tissues.[39] In addition, protein phosphatase 1 regulatory subunit 3A, which is considered as a tumour suppressing molecule was identified with decreased abundance.[40] Overall, the observations at the urinary peptides of the patients with Sig PCa, depict features of cancer progression and tumour related inflammation.

The specific clinical impact of the non-invasive biomarker model would primarily be to guide patient management and reduce the number of invasive biopsies. As such, high sensitivity is required, for correct detection of significant PCa. In view of a positive test, the treating physician is alerted to perform a more thorough investigation, improving the overall accuracy in detection of Sig PCa. Lower specificity would result in more misclassifications of non-significant PCa as potentially significant, and as a consequence prostate biopsy to rule out significant cancer. Therefore, a false positive result will be clarified upon biopsy.

These encouraging results should be interpreted considering the limitations of the study: Firstly, although the use of TRUS biopsy for PCa diagnosis suffers from random error and false negative results in comparison with trans-perineal template biopsy,[37] which might have affected the results (underestimate the specificity and overestimate the sensitivity) of the present study, it should be noted that TRUS biopsy is the accepted standard method in the current clinical practice and mostly used in biomarkers studies. Secondly, comparison with prostate biopsy pathology and not prostatectomy specimens is a similar limitation, possibly affecting the results in the same way, but prostate biopsy is the first approximation to diagnose and to stablish the risk category of the patients, so that it might represent more clearly the clinical practice. Thirdly, urine was collected with no prostate stimulation which could diminish the

number of peptides specifically derived from the prostate secretion. Moreover, this study was performed retrospectively, however, on samples that were prospectively collected. Furthermore, the exact potential benefit for patients has to be assessed in a prospective trial. However, based on the data presented, implementation of this approach in an investigative setting appears highly justified.

The data presented in this study could demonstrate the utility of a multiple-marker approach for improved non-invasive detection of Sig PCa. Taking into consideration the increased variability which is caused by the high intra-tumour heterogeneity, an intrinsic characteristic of cancer, a single biomarker is not expected to enable the discrimination of Sig PCa from non-significant with high accuracy. Therefore, a combination of biomarkers appears to be the currently best option to guide biopsies and AS. Effective discrimination between clinically significant and non-significant PCa is expected to have a positive impact on reducing biopsies, improving patient compliance and also guide a more thorough examination in case of a positive result. The benefit for the management of patients under AS is also evident, as discrimination of the Sig PCa will result in improved guidance for initiation of definite treatment. Overall, improved non-invasive patient stratification is expected to present a positive impact on PCa patient management, by improving patient compliance and reducing over-treatment and the associated costs. The results of this study, although highly significant, will be assessed in a prospective trial to also determine the exact value in the context of patient management.

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AUTHOR CONTRIBUTIONS

M.F., E.G.G, H.M., and J.C.V. carried out the conception and design of the study; M.F., E. G.G., A.B.P., J.V.R. and J.C.V. contributed to the data acquisition; M.F., E.G.G., A.L., H.M. and J.C.V. carried out the analysis and interpretation of data; M.F. and E.G.G. drafted the paper; A.B.P., J.V.R., A.L., Z.C., A.S.M., R.M.L., M.J.R.T., H.M. and J.C.V. carried out a critical revision of the paper for important intellectual content; M.F. and A.L. performed the statistical analysis; R.M.L., M.J.R.T., H.M. and J.C.V. supervised the work.

ADDITIONAL INFORMATION

Supplementary information is available for this paper at https://doi.org/10.1038/s41416-019-0472-z.

Competing interests: H.M. is the founder and co-owner of Mosaiques Diagnostics. M.F. and A.L. are employed by Mosaiques Diagnostics.

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Ethics approval and consent to participate: This study was performed as part of the ONCOVER project. Ethical approval was obtained by the Reina Sofia Hospital Research Ethics Committee in accordance with the Declaration of Helsinki and informed consent was obtained from all participants for the project.

Data availability: All data generated or analysed during this study are included in this published article.

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Article IV

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ORIGINAL ARTICLE

WILEY

Plasma ghrelin O-acyltransferase (GOAT) enzyme levels: A novel non-invasive diagnosis tool for patients with significant prostate cancer

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Abstract

Early detection of PCa faces severe limitations as PSA displays poor-specificity/sensitivity. As we recently demonstrated that plasma ghrelin O-acyltransferase (GOAT)-enzyme is significantly elevated in PCa-patients compared with healthy-controls, using a limited patients-cohort, we aimed to further explore the potential of GOAT to improve PCa diagnosis using an ample patients-cohort (n = 312) and defining subgroups (i.e. significant PCa/metastatic patients, etc.) that could benefit from this biomarker. Plasma GOAT-levels were evaluated by ELISA in patients with (n = 183) and without (n = 129) PCa. Gleason Score ≥ 7 was considered clinically significant PCa. GOAT-levels were higher in PCa patients vs control patients, and in those with significant PCa vs non-significant PCa. GOAT-levels association with the diagnoses of significant PCa was independent from traditional clinical variables (i.e. PSA/age/DRE). Remarkably, GOAT outperformed PSA in patients with PSA-levels ranging 3-20 ng/mL for the significant PCa diagnosis [GOAT-AUC = 0.612 (0.531-0.693) vs PSA-AUC = 0.494 (0.407-0.580)]. A panel of key variables including GOAT/age/DRE/testosterone also outperformed the same panel but with PSA [AUC = 0.720 (0.710-0.730) vs

Enrique Gómez-Gómez and Juan M. Jiménez-Vacas Equally contribution.

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AUC = 0.705 (0.695-0.716), respectively]. Notably, GOAT-levels could also represent a novel predictive biomarker of aggressiveness, as its levels are positively associated with Gleason Score and the presence of metastasis at the time of diagnoses. Altogether, our data reveal that GOAT-levels can be used as a non-invasive biomarker for significant PCa diagnosis in patients at risk of PCa (with PSA: 3-20 ng/mL).

KEYWORDS
GOAT enzyme, non-invasive biomarker, significant prostate cancer

1 | INTRODUCTION

Prostate cancer (PCa) has emerged as the most frequent cancer type among men, with an estimation of 164 690 new cases in the United States for 2018 (10% of all new cancer cases).[1] The rate of diagnosis has increased since the 1990s with the introduction of the PSA test for early detection of PCa, and metastatic disease and specific mortality have been reduced in most western countries.[2] However, a key limitation in PCa management is that early PCa diagnosis is mainly based on the plasma levels of PSA, a biomarker that exhibits profound drawbacks. For instance, PSA test displays low specificity because of the fact that multiple factors can increase PSA levels, such as benign prostatic hyperplasia or inflammation conditions, and this test is not able to accurately distinguish clinically relevant tumours from indolent cases.[3] This leads to the overdiagnosis of PCa with many unnecessary biopsies and reduced patient quality of life (QoL), as well as to the overtreatment in a considerable number of patients.[4] Likewise, clinical management of aggressive PCa, that is metastatic and castration-resistant PCa (CRPC), also faces major limitations, including unresponsive patients and development of resistance to hormonal and chemical therapies.[5,6] Therefore, there is an important unmet clinical need for the identification and validation of new, reliable and specific biomarkers for early diagnosis of PCa, as well as for prediction of disease prognosis and treatment response, etc., which would improve patient survival and QoL and would reduce substantially the number of unnecessary biopsies in patients with suspect of PCa based on PSA test.

In line with this, and using a limited cohort of patients, we have recently demonstrated that ghrelin-O-acyltransferase (GOAT), a key enzyme regulating ghrelin system activity,[7-9] is overexpressed in PCa tissues (at the mRNA and protein level) and its plasma levels are elevated in PCa patients compared to healthy prostate tissues and to plasma from healthy controls, respectively.[10] Moreover, we observed that plasma GOAT levels could discriminate PCa, suggesting that GOAT might serve as a potential novel non-invasive biomarker of PCa.[10] However, in this previous pilot study, we could not establish whether plasma GOAT levels could be a significantly better diagnostic marker than PSA for the diagnosis of PCa, specially on those individuals with PSA levels ranging 3-20 ng/mL (wherein precision of PSA is remarkably poor), and for the diagnosis of significant PCa (Sig PCa). Accordingly, the aims of this study were (a) to valorize the utility of plasma GOAT enzyme levels alone, or in combination with other traditional clinical variables, as a tool for the detection of PCa, using a more representative, ample cohort of patients (n = 312) and by defining specific subgroups (e.g. Sig PCa vs non-Sig PCa) that could benefit from this biomarker; (b) to compare the utility of plasma GOAT vs PSA levels as diagnostic tools in this cohort of patients; and, (c) to determine the utility of plasma GOAT enzyme levels as a novel predictive biomarker of aggressiveness, by analysing its association with Gleason Score (GS), metastatic PCa and earlier CRPC status in the same cohort of patients.

2 | MATERIAL AND METHODS

This is a case–control study implemented with patients who donated blood under fasting conditions in the Reina Sofia University Hospital. The study was approved by the Hospital Ethic-Committee, and written informed consent from all patients was obtained. All samples were obtained through the Andalusian-Biobank (Servicio Andaluz de Salud, Spain).

2.1 | Patients and samples

Three cohorts of patients were included in the study:

1. Cohort 1: Healthy control population without suspected PCa (65 volunteers with a PSA < 2.5 ng/mL)
2. Cohort 2: Patients at risk of PCa (with suspected PCa) but with a negative biopsy result (64 patients scheduled for prostate biopsies according to clinical practise but with a negative result from the pathology analysis).
3. Cohort 3: Patients at risk of PCa (with suspected PCa) and with a positive biopsy result (183 patients scheduled for prostate biopsies according to clinical practise with a positive result of PCa from the pathology analysis).

Recommendation to undergo prostate biopsies within the population of patients included in this study was: (a) in the case of non-previous biopsies, suspicious findings on digital rectal examination (DRE), PSA > 10 ng/mL, or PSA 3-10 ng/mL if free PSA ratio was low (usually, <25%-30%), and; (b) in patients with previous biopsies (but with a negative result), a persistently suspected PCa. It should be noted that none of the patients was receiving any PCa-associated medical therapy or was subjected to surgery at the moment of the sample collection. Biopsy specimens were analysed by an uro-pathologist according to ISUP 2005 modified criteria.[11]

In order to determine and compare the levels of GOAT and PSA in plasma samples from all the patients included in this study (cohorts 1-3 mentioned above; a total of 312 samples), blood was collected early in the morning, after an overnight fast. Each blood sample was placed into a vacutainer tube containing sodium citrate, centrifuged 10 minutes at 1100 *g* (20°C) and subsequently plasma was aliquoted in tubes and kept at −80°C. Additionally, clinical, anthropometric and pathological features of all the patients were obtained and registered. In addition, testosterone levels were evaluated in patients at risk of PCa (cohorts 2 and 3).

2.2 | Determination of plasma GOAT, PSA and testosterone levels

For the determination of plasma GOAT levels, a commercial ELISA was used following the manufacture's instructions (MyBioSource, San Diego, USA), as previously reported.[10] GOAT ELISA kit exhibits a detection limit lower than 0.31 ng/mL and a detection range between 0.78 and 50 ng/mL. The intra- and interassay accuracy showed a CV lower than 10% and 12%, respectively. Samples were diluted 1:100 before performing the assay. Levels of PSA and testosterone were measured using technology of Chemiluminescent Microparticle Immunoassays (References 7k70 and 7k73, respectively; Abbott) following the manufacturer's instructions.

2.3 | Variables and statistical analysis

A descriptive study was performed by calculating the median and interquartile ranges for the quantitative variables and the absolute frequencies and percentages for the qualitative variables. One of the primary endpoints of the study was the presence of a clinically Sig PCa on biopsy. The tumours with a GS $\geq$ 7 were considered clinically Sig PCa. Student's *t* test was used for analysis of the quantitative data in case of two groups and ANOVA with Bonferroni's post hoc test in case of comparison between the three groups. A chi-square test was used for the qualitative variables. To study the correlation between GOAT levels and other clinical variables, a Pearson test was used. To address the diagnostic value of both PSA and GOAT measures, their associated ROC curves were built, showing the performance (specificity and sensitivity) for the different risk thresholds. The performance was then compared using DeLong tests over the respective areas under the curves (AUC). Then, the performance of multivariate models based on these measures, when complemented with additional clinical variables (age, DRE, BMI, testosterone, number of biopsies and family history) was investigated. These models were built using logistic regression, preceding the model construction with a feature selection step, using like-hood ratio test to discard variables that do not contribute to diagnostic performance. The performance of these models was then evaluated using 10-fold cross-validation, including the variable selection step to avoid selection bias. Similar to the case of univariate models, ROC curves and DeLong tests were used to compare the different models. An exploratory analysis for the association and prognosis value of GOAT was carried out. For this purpose, data from the follow-up and treatment with hormonotherapy according to clinical practise were also collected. A univariate Cox Regression analysis was carried out to explore the association of GOAT levels with the development of castration resistant disease (CRPC). A 5% level of significance (after adjusting for multiple comparisons, if specified) was used to decide statistically significant differences to make our conclusions

TABLE 1 Demographic/clinical data and anatomopathological characteristics of the three cohorts of patients included in this study

Variable	Healthy patients	Negative biopsy patients	PCa patients
Patients	65	64	183
Age			
Median (IQR)	51 (47-57)	64 (58-68)	67 (62-72)
PSA level (ng/mL)			
Median (IQR)	0.69 (0.46-1.03)	5.82 (4.42-6.88)	6.35 (4.15-12.53)
BMI			
Median (IQR)	29.07 (26.23-32.66)	28.23 (26.20-31.28)	28.44 (25.96-31.62)
>1 Biopsy		21 (32.8)	27 (14.8)
DRE (Abnormal)	-	8 (12.5)	69 (37.7)
Testosterone			
Median (IQR)	-	5.11 (3.99-6.48)	4.56 (3.69-5.84)
Family history		10 (15.6)	37 (20.2)
Gleason score			
<7	-	0	78 (42.6)
≥7	-	0	105 (57.4)
Metastasis (%)	-	0	7 (3.8)
Median (IQR) GOAT protein expression	231.68 (189.80-259.17)	242.42 (211.30-279.92)	263.51 (220.48-309.31)

PCa, Prostate Cancer; DRE, Digital Rectal Examination; BMI, Body Mass Index.
Values are expressed in Median (Interquartile range) for quantitative variables and absolute number (Percentage) for qualitative variables.

comparable to those of the related research. All the analyses and graphics were performed using GraphPad prism 6, SPSS version 17.0 and R version 3.2.3.

3 | RESULTS

3.1 | Descriptive characteristics of the cohort

A total of 312 patients were evaluated (65, 64 and 183 individuals from cohorts 1, 2 and 3, respectively). Clinical characteristics are depicted in Table 1 according to patient category. Patients with PCa (cohort 3) were older compared to patients with negative biopsy (cohort 2) and healthy patients (cohort 1) [67 (62-72) vs 64 (58-68) vs 51 (47-57), respectively; $P < 0.01$]. Patients with PCa had significantly higher plasma PSA levels compared to healthy patients [cohort 3 vs cohort 1; 6.35 (4.15-12.53) ng/mL vs 0.69 (0.46-1.03) ng/mL; $P < 0.05$], while a similar, albeit non-significant trend was found with the patients with negative biopsy [cohort 3 vs cohort 2; 6.35 (4.15-12.53) ng/mL vs 5.82 (4.42-6.88) ng/mL; $P = 0.11$]. No differences in BMI between groups of patients were found. The proportion of patients with previous biopsy and normal digital rectal examination (DRE) were significantly higher in cohort 2 (patients with negative biopsy) compared to the group of patients with PCa ($P < 0.01$). Testosterone levels were slightly lower in patients with PCa compared to patients with negative biopsy, but this difference did not reach statistical significance ($P = 0.09$). The percentage of

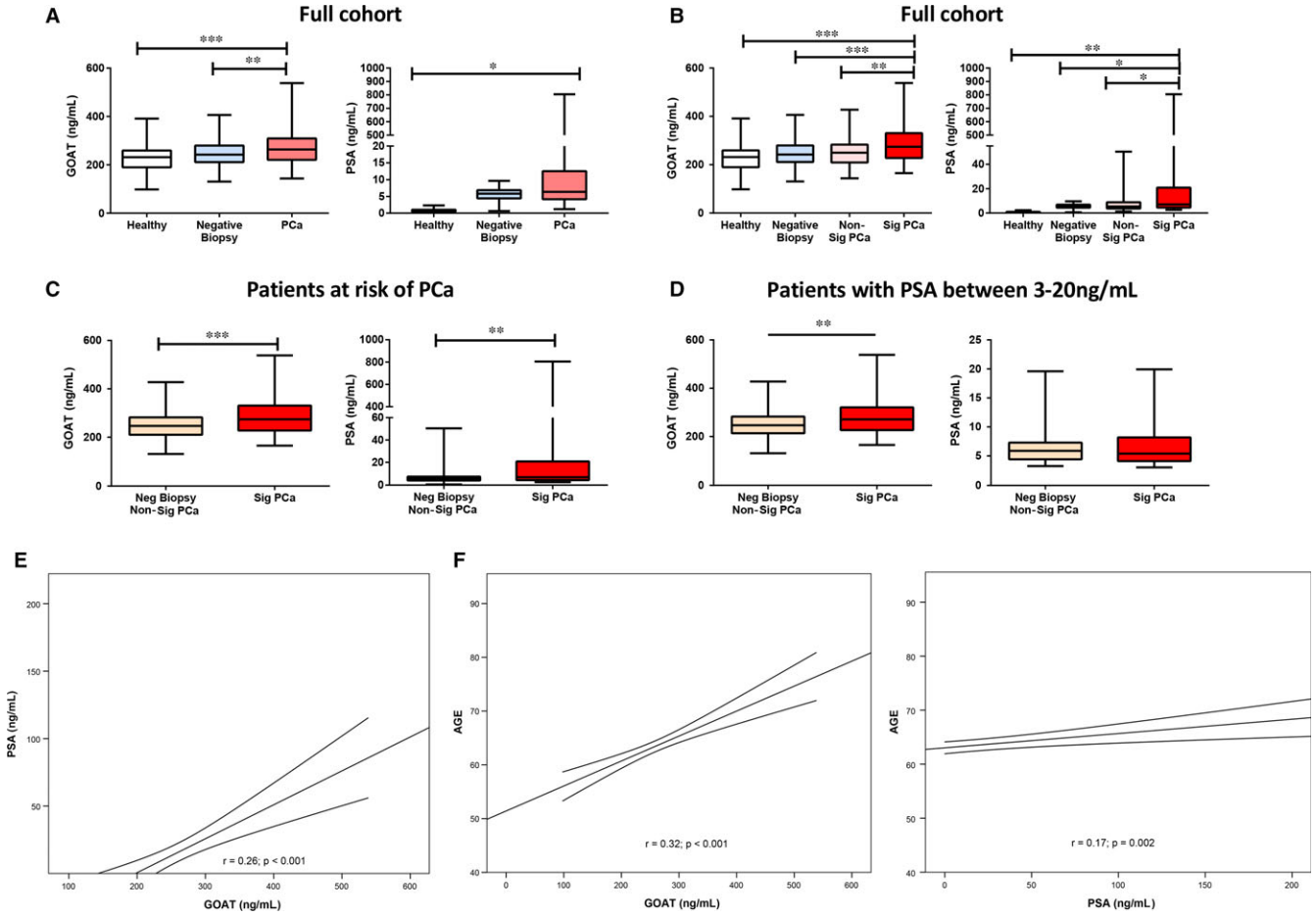


FIGURE 1 Plasma GOAT and PSA levels according to patient categorization. A, Comparison between plasma GOAT (left-graph) and PSA (right-graph) levels in healthy patients (n = 65), patients with suspected prostate cancer (PCa) but with a negative biopsy result (n = 64), and patients with confirmed PCa (n = 183). B, Comparison between plasma GOAT (left-graph) and PSA (right-graph) levels in healthy patients, patients with suspected PCa but with a negative biopsy result, and patients with PCa subclassified in non-significant PCa (non-Sig PCa; n = 78) and in Sig PCa (n = 105). C, Comparison between plasma GOAT (left-graph) and PSA (right-graph) levels in patients with Sig PCa (n = 105) compared to the combined group of patients with suspected PCa but with a negative biopsy together with patients with non-Sig PCa (n = 142). D, Plasma GOAT (left-graph) and PSA (right-graph) levels in patients with Sig PCa compared to the combined group of patients with suspected PCa but with a negative biopsy together with patients with non-Sig PCa, when considering only the patients with a PSA levels within the 3-20 ng/mL range (n = 77 and 125, respectively). In all cases, data represent mean ± SEM. Asterisks (*, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$) indicate values that significantly differ between groups. E, Correlations between GOAT levels and PSA levels in our cohort of patients. F, Correlations between GOAT (left-graph) or PSA (right-graph) levels and age in our cohort of patients. Coefficients of correlation were evaluated by Pearson's test. The graphics show the lineal adjusted method and mean confidence interval

TABLE 2 Multivariate analysis of the association of plasma GOAT levels with the diagnosis of prostate cancer (PCa) and Significant PCa (Sig PCa) adjusting with common clinical variables

	PCa (n = 183)			Sig PCa (GS ≥ 7; n = 105)		
Variable	OR	*P*	95% CI (OR)	OR	*P*	95% CI (OR)
PSA (ng/mL)	1.140	0.010	1.032-1.259	1.040	0.061	0.998-1.083
Age	1.043	0.078	0.995-1.094	1.070	0.003	1.024-1.119
DRE	2.573	0.031	1.090-6.074	4.177	0.000	2.118-8.235
Previous biopsy	0.333	0.004	0.156-0.710	0.495	0.084	0.223-1.100
Family history	1.479	0.360	0.640-3.417	1.104	0.800	0.513-2.376
GOAT (ng/mL)	1.006	0.049	1.000-1.012	1.007	0.005	1.002-1.012

GS, Gleason Score; DRE, Digital rectal examination; Previous Biopsy (Yes vs No); Family History (Yes vs No).

patients with family history did not differ between patients with PCa and with negative biopsy. Finally, 57% of the patients with PCa patients (cohort 3) had a GS of 7 or higher on the biopsy (Sig PCa; n = 105) and 4% (n = 7) presented metastasis at the diagnoses (Table 1).

3.2 | Capacity of plasma GOAT and PSA levels to predict the presence of PCa and Sig PCa

Plasma levels of GOAT were statistically higher in patients with PCa compared to patients with negative biopsy and healthy patients (Figure 1A, left panel). In contrast, PSA levels were higher in patients with PCa compared to healthy patients but not with patients at risk of PCa but with negative biopsy (Figure 1A, right panel). When patients with PCa were divided in two subgroups, with and without Sig PCa, we found that, although both plasma GOAT and PSA levels were significantly elevated in patients with Sig PCa (GS ≥ 7) compared to patients with non-Sig PCa (GS = 6), these differences were statistically more significant for GOAT vs PSA levels ($P = 0.002$ vs $P = 0.0145$; Figure 1B).

Additionally, plasma GOAT and PSA levels were also found to be higher in patients with Sig PCa compared to the combined group of patients at risk of PCa but with a negative biopsy together with patients with non-Sig PCa (Figure 1C), being these differences again statistically more significant for GOAT vs PSA levels. Importantly, when the patients with a PSA range between 3 and 20 ng/mL (the most ambiguous region of PSA levels, which leads to a high false-positive rate and, therefore, to a high number of unnecessary prostate biopsies) were analysed in more detail, we found that plasma GOAT, but not PSA, levels were significantly higher in patients with Sig PCa compared to the combined group of patients with negative biopsy and with non-Sig PCa (Figure 1D).

Interestingly, plasma GOAT levels positively correlated with plasma PSA levels (Figure 1E), but not with testosterone levels ($r = -0.044$; $P = 0.49$; data not shown), in this cohort of patients, which is consistent with our previous study using a different cohort of patients.[10] Moreover, a positive correlation was found between plasma GOAT or PSA levels with age (Figure 1F).

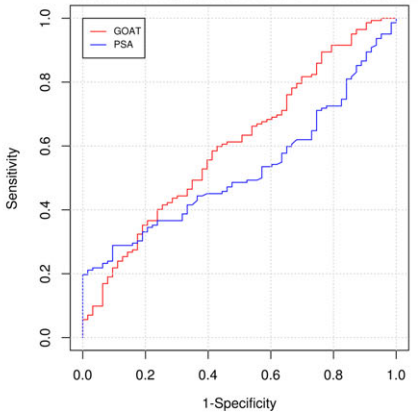
3.3 | Comparison of the predictive ability of GOAT and PSA to detect PCa and Sig PCa in the PSA grey zone

We next applied a multivariate analysis to evaluate the association of plasma GOAT levels with the diagnosis of PCa and Sig PCa adjusting with usual clinical variables analysed in PCa patients (PSA, age, DRE, etc.; Table 2). This revealed that GOAT levels are independent of these variables used in clinical practice, with the strongest association for DRE in the Sig PCa [OR = 4.18 (2.12-8.24)].

To explore the potential capacity of prediction of plasma GOAT levels compared to PSA levels, patients from cohorts 2 and 3 with a PSA range between 3 and 20 ng/mL were analysed. This analysis revealed that GOAT was a better biomarker than PSA for the diagnoses of PCa [n = 140 PCa patients; GOAT levels: AUC = 0.595 (0.509-0.681) vs PSA levels: AUC = 0.513 (0.432-0.594); Figure 2A]. This difference between both biomarkers was particularly significant for the diagnosis of Sig PCa, wherein the AUC improved for GOAT levels and worsened for PSA levels, [n = 77 Sig PCa patients; GOAT: AUC = 0.612 (0.531-0.693) vs PSA: AUC = 0.494 (0.407-0.580); $P = 0.035$; Figure 2B].

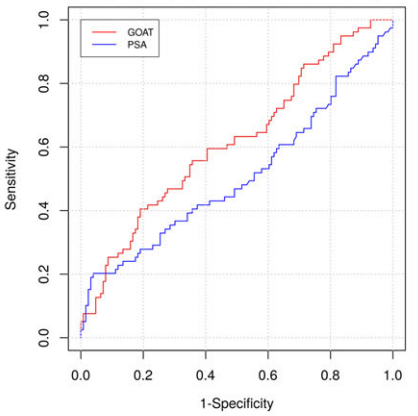
FIGURE 2 Capacity of plasma GOAT and PSA levels to predict the presence of prostate cancer (PCa) and significant (Sig) PCa. A-D. Graphics showing the receiver operating characteristic (ROC) curves analyses of the capacity of GOAT (red line) and PSA (blue line) to diagnose: A, PCa in patients with PSA ranging 3-20 ng/mL; B, Sig PCa in patients with PSA ranging 3-20 ng/mL; C, PCa in patients with PSA ranging 3-10 ng/mL; and D, Sig PCa in patients with PSA ranging 3-10 ng/mL. E-F, Graphics showing the ROC curve analysis of the capacity of models combining age, DRE and testosterone with GOAT levels (red line) or PSA (blue line) to predict the presence of Sig PCa in patients ranging 3-20 ng/mL PSA levels (E), or in patients ranging 3-10 ng/mL PSA levels (F). AUC and CI of each ROC curve are depicted in the tables below. These analyses were performed using patients with suspected PCa (cohorts 2 and 3)

A



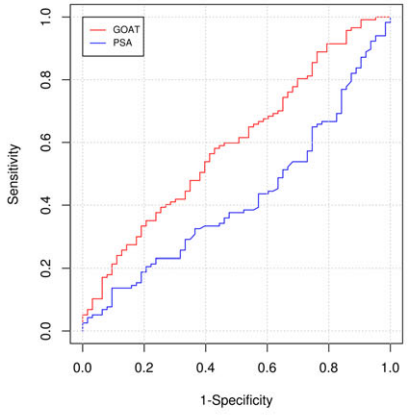
Variable	AUC for PCa	95%CI
GOAT	0.595	0.509 - 0.681
PSA	0.513	0.432 - 0.594

B



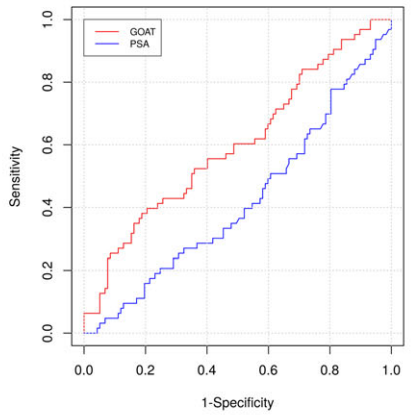
Variable	AUC for Sig PCa	95%CI
GOAT	0.612	0.531 - 0.693
PSA	0.494	0.407 - 0.580

C



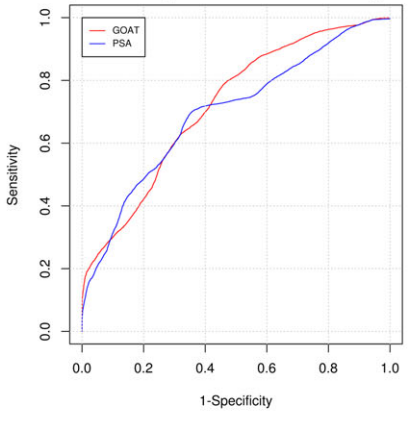
Variable	AUC for PCa	95%CI
GOAT	0.586	0.497 - 0.674
PSA	0.417	0.330 - 0.504

D



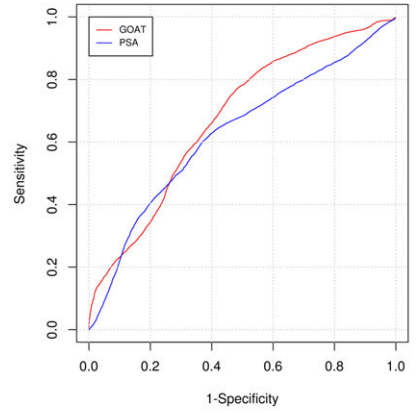
Variable	AUC for Sig PCa	95%CI
GOAT	0.595	0.506 - 0.684
PSA	0.416	0.328 - 0.505

E



Variable	AUC for Sig PCa	95%CI
Model with GOAT	0.720	0.710 - 0.730
Model with PSA	0.705	0.695 - 0.716

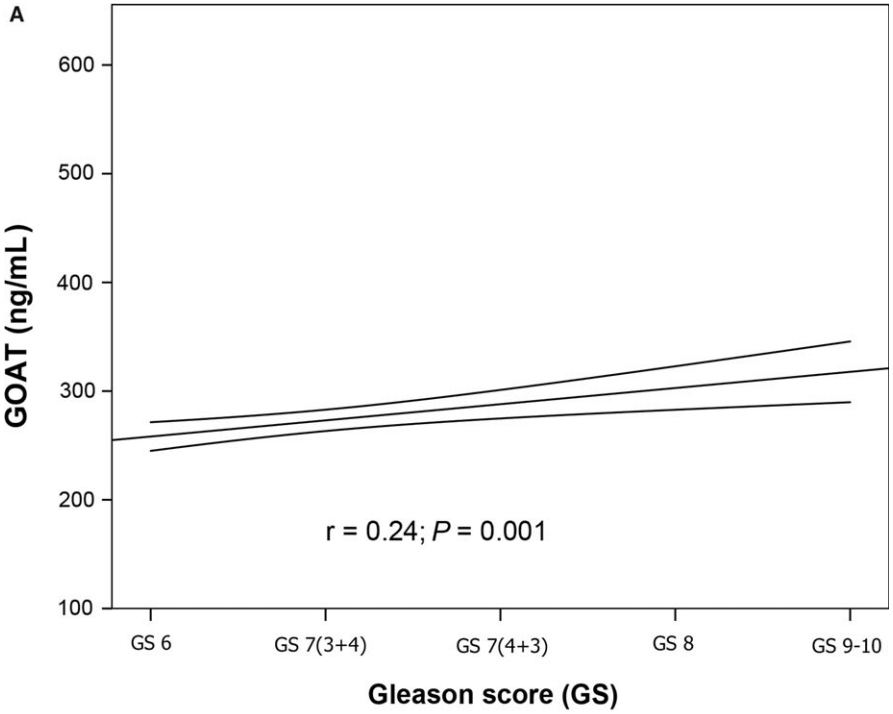
F



Variable	AUC for Sig PCa	95%CI
Model with GOAT	0.680	0.669 - 0.692
Model with PSA	0.636	0.623 - 0.648

This analysis was also applied to assess the predictive capacity of plasma GOAT levels, compared to PSA levels, in patients with a more restricted range of PSA, of 3-10 ng/mL, the so-called PSA grey zone (Figure 2C,D). The results clearly indicated that GOAT levels are a significantly better indicator than those of PSA to predict PCa in these patients [n = 117 PCa patients; GOAT levels: AUC = 0.586 (0.497-0.674) vs PSA levels: AUC = 0.417 (0.330-0.504), $P < 0.01$), Figure 2C]. Likewise, as illustrated in Figure 2D, the same was true for the population with Sig PCa, where GOAT levels significantly outperformed the predictive potential of PSA

A



B

Variable	Metastasis OR	p	95%CI
GOAT	1.01	0.03	1.00-1.02

C

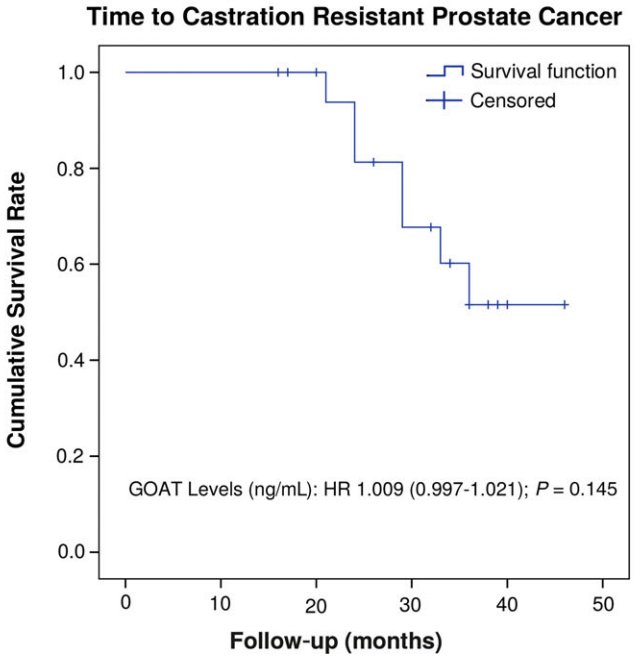


FIGURE 3 Association of plasma GOAT levels with aggressive features of prostate cancer (PCa) patients. A, Correlation between plasma GOAT levels and PCa Gleason Score. Coefficient of correlation was evaluated by Pearson's test. The graphic shows the lineal adjusted method and mean confidence interval. B, Association (odds ratio, OR) between plasma GOAT levels and the presence of metastasis at diagnosis evaluated by computerized tomography and bone scan. C, Representation of progression-free survival curve from 19 patients treated with hormonotherapy. Results of univariate Cox regression analysis analysing the association of GOAT levels and the time to the event are depicted

levels in this group of patients [n = 63 Sig PCa patients; GOAT levels: AUC = 0.595(0.506-0.684) vs PSA levels: AUC = 0.416 (0.328-0.505); $P < 0.01$].

Based on the previous results, a multivariate model based on GOAT or PSA levels complemented with an additional panel of clinical variables analysed in PCa (i.e. age, DRE and testosterone levels) was implemented to determine whether this combination could improve the accuracy of detection of PCa in patients with PSA levels between 3-20 ng/mL (Figure 2E) and 3-10 ng/mL (Figure 2F). This analysis revealed that the combination of this panel of clinical variables with plasma GOAT levels is significantly more efficient in detecting Sig PCa than when combined to plasma PSA levels [$P < 0.001$ in both cases; Figures 2E,F].

3.4 | Association of plasma GOAT levels with aggressiveness features of PCa patients

Association between aggressiveness features of the cohort of patients with PCa revealed that plasma GOAT levels showed a significant correlation with GS ($r = 0.24$; $P = 0.001$; Figure 3A). Remarkably, high GOAT levels were associated with the presence of metastasis at the time of diagnosis, as evaluated by computerized tomography and bone scan ($P = 0.03$; Figure 3B). Furthermore, an exploratory analysis in the patients initially treated with hormonotherapy (n = 19) and a median follow-up according to clinical practise of 35 months (26.75-39) indicated a tendency in the association of plasma GOAT levels with an earlier castration-resistant prostate cancer (CRPC) status (OR = 1.009: 95% CI (0.997-1.021); $P = 0.145$; Figure 3C).

4 | DISCUSSION

PCa is a major health problem and a leading cause of mortality and morbidity globally.[1] PSA has been used as the gold standard biomarker for the diagnosis of PCa since the 1990s, although its use remains controversial because of its lack of specificity. Specifically, although the proportion of men with metastatic PCa at the time of diagnosis have decreased dramatically with the introduction of PSA as a screening test, more men are being diagnosed with PCa, with the majority having early stage, clinically indolent disease, the majority of which may never have led to harm.[12] In addition, many men with benign conditions such as inflammation or hyperplasia are also being diagnosed and biopsied based on the results of the PSA test.[3] Moreover, it has been proposed that treatment of indolent cancer may cause a patient more harm than good as biopsies and PCa treatments have been associated with psychological distress, loss of bodily function, pain, suffering for patients and with a decrease in the patient QoL.[13] Consequently, these data have led to widespread criticism that PCa is now an "overdiagnosed" and "overtreated" cancer based on the PSA test. Therefore, there is an urgent need for the identification of new diagnostic and prognostic biomarkers for PCa, especially for Sig PCa, in order to improve the clinical management of PCa and to reduce the elevated number of biopsies and the overdiagnosis of non-significant PCa.[4]

In this context, there have been numerous efforts to improve the performance of the PSA test based on PSA derivatives (ie, PSA "density," PSA velocity and doubling time, free PSA, etc.); however, measurement of these derivatives has modestly improved care in that they are largely hindered by the same issues confounding PSA itself.[14] Additionally, other non-invasive biomarkers to diagnose PCa have been proposed [i.e. prostate cancer antigen 3 (PCA3), the gene fusion product TMPRSS2-ERG, the 4k score test, the Prostate Health Index (PHI) in body fluids, multiparametric magnetic resonance imaging (mpMRI), etc.],[15-20] but many of these tests are currently adjunctive to PSA, and head-to-head studies to determine whether these tests perform well in the absence of PSA screening are lacking. Moreover, PSA remains an inexpensive test and, thus, costs and availability of these alternative tests minimize their implementation worldwide. Therefore, additional accessible biomarkers should be implemented in daily clinical practice, especially those with a prognostic and predictive value of Sig PCa at the point of screening, which is the current greatest unmet clinical need, as this may reduce unnecessary interventions.

In line with this, our group and others have recently demonstrated that GOAT enzyme is overexpressed (at the mRNA and/or protein level) in PCa tissues and PCa cell lines compared to healthy prostate tissues and normal cell lines,[10,21] and, most importantly, we also reported that GOAT is oversecreted in PCa cells compared to normal prostate cells.[10] In fact, this initial, pilot study from our group revealed that plasma GOAT levels could discriminate between PCa and healthy subjects, suggesting that this enzyme might be used as a potential novel non-invasive biomarker of PCa.[10] However, this previous study was implemented with a limited cohort of patients and we could not establish therein whether plasma GOAT levels could be a better diagnostic marker than PSA for the diagnosis of PCa, specially on those individuals with PSA levels ranging between 3 and 20 ng/mL, the most ambiguous region wherein precision of PSA is remarkably poor, as well as for the diagnosis of Sig PCa. Consequently, the present study is the first to demonstrate that GOAT could be a significantly better diagnostic marker than PSA, exhibiting higher AUC, on those individuals with PSA levels ranging 3-20 ng/mL and especially for the diagnosis of Sig PCa. In this scenario, it is worth noting that the overexpression of GOAT has been demonstrated at tissue level in other endocrine tumours,[22] but, to the best of our knowledge, this is the first study evaluating GOAT plasma level and to analyse its putative utility as biomarker for cancer diagnosis. Therefore, although the role of GOAT as possible biomarker in other endocrine tumours cannot be completely ruled out and that its specificity for PCa needs to be further explored, this study strongly suggests that GOAT levels might represent a novel, valuable biomarker for Sig PCa.

We further explored the potential predictive capacity of plasma GOAT levels compared to PSA levels and found that plasma GOAT levels show a significant better AUC than plasma PSA levels in patients with PCa, but specially in patients with a PSA < 20 ng/mL

(wherein the capacity of PSA is significantly worse) or most importantly, with a PSA < 10 ng/mL (known as the PSA grey zone), having an independent association with the clinical variables commonly used in clinical practice. Furthermore, a multivariate model based on GOAT or PSA levels complemented with an additional panel of clinical variables measured in PCa such as age, DRE and testosterone levels demonstrated that GOAT levels could be efficiently complemented with these clinical parameters to significantly increase its accuracy for the prediction of Sig PCa, which altogether reinforce the idea that GOAT enzyme might represent a promising biomarker, complementing PSA determination for the diagnosis of Sig PCa.

Based on the clear association found between plasma GOAT, but not PSA, levels with Sig PCa, we hypothesized that plasma GOAT levels in PCa patients might be linked to the aggressiveness of PCa. Remarkably, our results indicate that plasma GOAT levels could represent a novel predictive biomarker of aggressiveness, as we found that its levels are positively associated with GS (i.e., higher GOAT levels in patients with GS $\geq$ 7) as well as with the presence of metastasis at the time of diagnoses. Moreover, plasma GOAT levels tended to be associated with an earlier diagnosis of CRPC, which might also indicate that this enzyme may serve to develop future therapeutic target for PCa. In line with this, we have recently demonstrated that GOAT enzyme is positively correlated in PCa with the levels of the In1-ghrelin splicing variant, but not with those of native-ghrelin, wherein the presence of In1-ghrelin variant drastically increased the aggressiveness features of PCa, acting as a true oncogene in this pathology.[23] In fact, this previous study demonstrated that In1-ghrelin silencing diminished the aggressiveness of PCa cells (e.g. proliferation capacity) suggesting that In1-ghrelin could be considered as a novel target for the development of new and more specific therapies in PCa. When viewed as a whole, the results of the present manuscript indicating that GOAT levels are markedly elevated in Sig PCa and are associated to aggressiveness features in PCa (i.e. GS and presence of metastasis), together with the previous results showing a strong correlation of GOAT levels with In1-ghrelin variant levels in PCa,[23] invite to suggest that GOAT enzyme and In1-ghrelin variants could be functionally linked in PCa, where In1-ghrelin variant might be the primary target of GOAT, and that an autocrine/paracrine circuit involving these two components of the ghrelin system may possibly operate in PCa to increase the aggressiveness features of PCa cells, which set the stage for future investigations.

In sum, the present report provides the first comparative analysis to determine the potential utility of plasma levels of GOAT, in combination with other traditional clinical variables (i.e. age, DRE and/or testosterone), as diagnostic tools for the detection of PCa, using an ample cohort of patients (n = 312) and defining clinically relevant subgroups (e.g. Sig PCa vs non-Sig PCa). Our results show, for the first time, that the measurement of plasma GOAT levels might represent a significantly better diagnostic marker than plasma PSA levels, exhibiting higher AUC, particularly on those individuals with PSA levels ranging 3-10 ng/mL (the PSA grey-zone) or 3-20 ng/mL. Moreover, as plasma GOAT levels showed a significant better AUC than plasma PSA levels for the detection of Sig PCa and its levels were associated with aggressiveness features of PCa, we propose that the measurement of plasma GOAT levels, in combination with PSA and/or an additional panel of clinical variables measured in PCa (i.e. age, DRE and testosterone levels), might be considered as a novel, complementary, non-invasive tool to provide a better diagnosis of PCa, especially for Sig PCa and for patients with grey-zone PSA levels, as well as a putative tool for the prediction of PCa aggressiveness.

AUTHOR CONTRIBUTIONS

E. Gómez-Gómez, JM Jimenez-Vacas and RM. Luque conceived and designed the project; E. Gómez-Gómez, JM. Jiménez-Vacas, J. Carrasco-Valiente, AM. Blanca-Pedregosa, J. Valero-Rosa, T. González-Serrano, M.D. Gahete, MJ. Requena-Tapia, and RM. Luque acquired data; E. Gómez-Gómez, JM. Jiménez-Vacas, V. Herrero-Aguayo, J. Carrasco-Valiente, A. León-González, JL. Fernández-Rueda, MD. Gahete, MJ. Requena-Tapia, JP. Castaño, and RM. Luque performed the analysis and interpretation of data; E. Gómez-Gómez, JM. Jimenez-Vacas and RM. Luque wrote the manuscript; J. Carrasco-Valiente, V. Herrero-Aguayo, AM. Blanca Pedregosa, AJ. León-González, J. Valero Rosa, JL. Fernández-Rueda, T. González-Serrano, J. López-Miranda, MD. Gahete, JP. Castaño and MJ. Requena-Tapia revised the manuscript for important intellectual content; E. Gómez-Gómez, JM. Jimenez-Vacas, JL. Fernández-Rueda and RM. Luque performed the statistical analysis; RM. Luque and MJ. Requena-Tapia obtained funding; RM. Luque supervised the work.

RM. Luque is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

CONFLICTS OF INTEREST

The authors confirm that there are no conflicts of interest and have nothing to disclose.

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Article V

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Oncogenic Role of Secreted Engrailed Homeobox 2 (EN2) in Prostate Cancer

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Abstract: Engrailed variant-2 (EN2) has been suggested as a potential diagnostic biomarker; however, its presence and functional role in prostate cancer (PCa) cells is still controversial or unknown. Here, we analyzed 1) the expression/secretion profile of EN2 in five independent samples cohorts from PCa patients and controls (prostate tissues and/or urine) to determine its utility as a PCa biomarker; and 2) the functional role of EN2 in normal (RWPE1) and tumor (LNCaP/22Rv1/PC3) prostate cells to explore its potential value as therapeutic target. EN2 was overexpressed in our two cohorts of PCa tissues compared to control and in tumor cell lines compared with normal-like prostate cells. This profile was corroborated in silico in three independent data sets [The Cancer Genome Atlas(TCGA)/Memorial Sloan Kettering Cancer Center (MSKCC)/Grasso]. Consistently, urine EN2 levels were elevated and enabled discrimination between PCa and control patients. EN2 treatment increased cell proliferation in LNCaP/22Rv1/PC3 cells, migration in RWPE1/PC3 cells, and PSA secretion in LNCaP cells. These effects were associated, at least in the androgen-sensitive LNCaP cells, with increased AKT and androgen-receptor phosphorylation levels and with modulation of key cancer-associated genes. Consistently, EN2 treatment also regulated androgen-receptor activity (full-length and splicing variants) in androgen-sensitive 22Rv1 cells. Altogether, this study demonstrates the potential utility of EN2 as a non-invasive diagnostic biomarker for PCa and provides novel and valuable information to further investigate its putative utility to develop new therapeutic tools in PCa.

Keywords: engrailed homeobox variants; prostate cancer; aggressiveness; biomarker

1. Introduction

Prostate cancer (PCa) is diagnosed in approximately 899,000 men per year worldwide [1] and is the most frequent non-skin cancer in developed countries among men [2]. Since the 1990s, with the introduction of the prostate specific antigen (PSA) test for the detection of PCa, the possibility of early diagnosis has been improved and, consequently, metastatic disease and specific mortality rates have been reduced in most Western countries [3]. However, the management of patients with PCa still faces several limitations. Firstly, the PSA test displays low specificity due to the influence

of multiple factors that increase PSA levels, such as benign prostatic hyperplasia or prostatitis [4], and it is not able to accurately distinguish clinically-relevant tumors from indolent cases. Similarly, various PSA-related derivatives, such as PSA velocity, PSA density, and free-to-total PSA ratio, have only provided limited improvements in terms of specificity [5]. Thus, there is no consensus-based recommendation with regard to population screening based on PSA measurement due to the proven risk of over-diagnosis and over-treatment in a considerable number of patients [6]. Secondly, progression of PCa is tremendously complex and its treatment is severely hampered by the lack of satisfactory therapeutic alternatives. Indeed, a significant number of patients are resistant or develop resistance to hormonal castration, the first-line medical therapy in this cancer type, and their disease progresses towards a castration-resistant state (CRPC), wherein the therapeutic alternatives are limited and, in many cases, insufficient [7,8]. Therefore, it seems essential to validate alternative diagnostic biomarkers to complement the PSA test, and to identify novel molecular targets in order to develop additional and more effective therapeutic tools.

In this scenario, the homeodomain-containing transcription factors comprise a gene family that controls cell and tissue identity during normal embryonic development, and have been shown to be strikingly re-expressed by different tumor cell types [9], wherein they could provide novel diagnostic biomarkers or therapeutic targets. Together with *HOX* and *PAX*, *engrailed-homeobox* (*EN*) genes are key members of the homeobox family. *EN* genes were originally characterized in *Drosophila melanogaster* and, later on, in different vertebrates species [10,11]. In human, two *EN* genes (*EN1* and *EN2*) located on chromosome 2 (2q14.2) and 7 (7q36.3), respectively, have been discovered, which slightly differ in function [12]. EN proteins are transcription factors capable to modulate multiple processes at different stages of development, involving transcriptional and translational regulation [9], but also present an unconventional ability to be secreted from producing cells, and to be internalized by others [13,14]. The main role of these proteins during embryonic development is to regulate neural development and embryonic axonal guidance [15]. However, they have been also shown to be expressed in different tumor pathologies, such as leukemia, glioblastoma, colon, ovarian, breast, bladder, and PCa [16–18]. In the particular case of PCa, EN2 has been found to be over-expressed in human PCa cells compared to normal prostate epithelial cells or stroma cells [19,20], suggesting its putative utility as a PCa biomarker. In fact, some studies have shown that EN2 can be detected in urine from PCa patients, wherein it could serve as a non-invasive diagnostic biomarker [20–24]. However, the accuracy of EN2 as diagnostic biomarker and the methodological procedure for EN2 assessment in urine samples are still a matter of debate, inasmuch as the sensitivity and specificity of this biomarker is considerably variable among studies and the values fluctuate depending on the existence of previous prostate massage [20–24]. Moreover, little is known about the potential tumorigenic role of EN2 in PCa since only a single study has shown that its silencing could be associated to a decrease in PCa cell proliferation [19]. Strikingly, EN2 protein does not seem to be localized in the nuclei of PCa cells but, rather, close to the luminal border of the cells, associated to secretory blebs [20]. Accordingly, it has been reported that different established PCa cell lines can release EN2 protein to the media, thereby suggesting that secreted EN2 could play a pathological role in PCa [20]. However, this pathological role has been poorly explored hitherto, and, consequently, it is not known if EN2 could provide novel therapeutic targets for this highly incident and prevalent pathology.

Therefore, based on the information mentioned above, the objectives of this study were: (1) To analyze the utility of EN2 as a non-invasive diagnostic biomarker by measuring its expression and secretion levels in different, independent cohorts of samples from PCa patients and controls (prostate tissues and urine); and (2) to investigate the oncogenic role of EN2 and its underlying molecular mechanisms as well as its putative value as a therapeutic target in PCa by using different prostate cell lines (normal (RWPE-1) and tumor (LNCaP, 22Rv1 and PC3) cells) and diverse experimental approaches.

2. Material and Methods

2.1. Patients and Samples

This study was approved by the Ethics Committee of the IMIBIC/Reina Sofia University Hospital (Córdoba, Spain), performed according to the Declaration of Helsinki, and patients were treated following national and international clinical practice guidelines. A written informed consent was required before collection of samples, which were managed by the Andalusian Health System Biobank (Córdoba, Spain). The study of tissue samples included: (1) Formalin-fixed paraffin-embedded (FFPE) PCa tissues ($n = 33$) obtained from radical prostatectomies of patients diagnosed with clinically localized low-intermediate grade PCa (Table 1), which presented tumor and adjacent non-tumor control tissues, and; (2) fresh biopsy samples ($n = 23$) from patients with locally-advanced PCa (palpable in digital rectal examination (DRE)) and fresh non-tumor prostate samples (NPs, $n = 7$) derived from patients that underwent cystoprostatectomy due to bladder cancer (Table 2). All diagnoses (tumor and non-tumor cases) were confirmed by specialist uropathologists. Evaluations of prostatectomies and biopsies were performed following the 2010 and modified 2005 ISUP criteria, respectively [25,26]. Urine samples were collected between 8:00 and 10:00 after, at least, eight hours of fasting in 1.5 mL aliquots and stored at −80 °C for subsequent analyses. Urine samples were obtained from: (1) Patients with PCa confirmed by positive biopsy ($n = 24$), and (2) control individuals, which included, first, subjects with no suspicious urologic symptoms, low PSA (<2.5 ng/mL) and normal DRE who voluntarily participated in this study ($n = 10$), and, second, patients with suspect of PCa but negative results on the systematic trans-rectal ultrasound-guided biopsy, which showed PSA < 10 ng/mL ($n = 10$; Table 3). It should be mentioned that urine levels of EN2 after DRE were also analyzed in the cohort of patients with PCa ($n = 24$).

Table 1. Overall clinical and demographic data and expression levels measured in formalin-fixed paraffin-embedded (FFPE) prostate pieces from patients with clinically-localized prostate cancer (PCa).

Variable	Overall
Number of patients	33
Age at diagnosis	
Median (IQR)	62 (58–66)
BMI	
Median (IQR)	27.7 (25.8–31.3)
PSA level, ng/mL	
Median (IQR)	6 (4.4–9.5)
Gleason score in prostatectomy specimen (%)	
6	9 (27.3)
7	23 (69.7)
8	1 (3)
EE, *n*° (%)	21 (63.6)
PI, *n*° (%)	28 (84.8)
VI, *n*° (%)	8 (24.2)
Relative *EN2* mRNA expression in FFPE piece [a]	
Tumor tissue	
Median (IQR)	0.173 (0.002–1.473)
Non-tumor adjacent tissue	
Median (IQR)	0.008 (0.000–0.477)
Ratio tumor/non-tumor tissue	
Median (IQR)	3.451 (1.260–12.212)

Table 1. *Cont.*

Variable	Overall
Relative *EN1* mRNA expression in FFPE piece *	
Tumor tissue	
Median (IQR)	0.723 (0.209–2.828)
Non-tumor adjacent tissue	
Median (IQR)	0.421 (0.149–1.145)
Ratio tumor/non-tumor tissue	
Median (IQR)	1.197 (0.325–3.249)

EE = extraprostatic extension; PI = perineural invasion; VI = vascular invasion; FFPE = formalin-fixed paraffin-embedded; IQR = interquartile range. a FFPE prostate piece with delimited tumor tissue and non-tumor adjacent tissue. * EN1 (n = 18) and EN2 expression (Ct) was calculated by qPCR, adjusted with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and analyzed by Delta (Ct) method.

Table 2. Overall clinical and demographic data of fresh samples from patients with normal prostates and PCa samples.

Variable	Overall	Control	PCa
Patients	30	7	23
Age at diagnosis			
Median (IQR)	73 (64–79)	67 (59–79)	76 (67.0–80.0)
PSA level, ng/mL			
Median (IQR)	-	-	40 (22–70)
Dyslipidemia (%)	7 (23.3)	2 (28.6)	5 (21.7)
Diabetes (%)	8 (26.7)	2 (28.6)	6 (26.1)
* BMI			
Median (IQR)	27.19 (25.2–29.83)	25.87 (24.50–34.24)	27.44 (25.46–29.65)
Gleason score			
=7	-	-	8 (34.8)
>7	-	-	15 (65.2)
EE (%)	-	-	6 (26.1)
PI (%)	-	-	14 (60.9)
[#] Metastasis (%)	-	-	13 (56.5)
N° samples (%) in whichEN2 was detected	25 (83.3)	3 (42.9)	22 (95.7)
[a] Median(IQR) *EN2* mRNAexpression	445 (8–2265)	0 (0–9)	874 (173–2650)

PCa = prostate cancer; EE = extraprostatic extension; PI = perineural invasion. [#] Metastasis (N or M stage) [a] *EN2* expression (Ct) was calculated by qPCR, adjusted by normalization factor (Beta-actin (ACTB) and GAPDH) and analyzed with copy numbers using a standard curve. * BMI; *n* = 22 (missing data).

Table 3. Demographic and clinical characteristic of patients included in the study of urine EN2 levels.

Variable	Control (*n* = 10)	Negative Biopsy (*n* = 10)	PCa (*n* = 24)	*p*-Value
Age, Years				
Median (IQR)	56 (52–61)	56 (53–59)	68 (59–71)	<0.01
Waist circumference, cm				
Median (IQR)	107 (99.5–111.8)	103 (98.3–109)	104.5 (100–111.5)	0.88
BMI				
Median (IQR)	32.4 (27.5–33.1)	30.1 (27.33–31.97)	29 (26.67–30.48)	0.15
PSA, ng/mL				
Median (IQR)	0.87 (0.6–1.6)	3.6 (3.0–4.0)	5.7 (4.6–9.7)	<0.01
DRE, abnormal (%)	0 (0)	0 (0)	9 (37.5)	<0.01
Prostate Vol, cc				
Median (IQR)	-	37 (23.1–45.8)	37 (25.59–52.5)	0.55
1° Biopsy (%)	-	9 (90)	17 (70.8)	
Gleason grade (%)				
=6			6 (25)	

Table 3. *Cont.*

Variable	Control (n = 10)	Negative Biopsy (n = 10)	PCa (n = 24)	p-Value
≥7			18 (75)	
Nº Pathologic cores				
Median (IQR)			2 (1–4)	
EN2 Urine				
Median (IQR)	0 (0–0.21)	0.02 (0.00–0.30)	0.19 (0.01–0.43)	0.05

PCa = prostate cancer; Yrs = year; cm = centimeters; BMI = body mass index; IQR = interquartile range; PSA = Prostate specific antigen; DRE = digital rectal examination; Vol = volume. Statistical analysis: Non-parametric test for independent groups comparing non-tumor (control + negative biopsy) versus PCa patients.

2.2. Datasets Analysis

Processed freely available RNA-seq data from The Cancer Genome Atlas (TCGA, https://gdc-portal.nci.nih.gov/) and the Memorial Sloan Kettering Cancer Center (MSKCC, https://www.mskcc.org/) regarding Prostate Cancer Adenocarcinoma (PRAD) were compiled and used for subsequent analysis. In addition, available PCa Grasso cohort data from Gen expression Omnibus (GSE35988) were also used for the analysis. Furthermore, free available cell lines data from Cancer Cell Line Encyclopedia (https://portals.broadinstitute.org/ccle) was also used.

2.3. EN2 Protein, Reagents, and Cell Lines

Recombinant protein of EN2 was purchased from Origene (TP311220; Origene, Rockville, MD, USA) and IGF1 and Paclitaxel from Life Technologies (Madrid, Spain).

Normal-like prostate cell line (RWPE-1) and PCa cell lines (LNCaP, 22Rv1 and PC3) were obtained from ATCC, validated by analysis of Short Tandem Repeats (STRs) (GenePrint® 10 System, Promega, Barcelona, Spain), and checked for mycoplasma contamination by PCR, as previously reported [27]. RWPE-1 cells were cultured in Keratinocyte-serum free medium (SFM)(Gibco, Waltham, MA, USA), while LNCaP, 22Rv1 and PC3 cells were maintained in Roswell Park Memorial Institute medium (RPMI) 1640 (Lonza, Basel, Switzerland), supplemented with 10% Fetal Bovine Serum (FBS), 1% glutamine, and 0.2% antibiotic, as previously reported [27,28]. All cell lines were grown at 37 °C, in a humidified atmosphere with 5.0% CO_2.

2.4. RNA Isolation, Reverse-Transcription, and Real-Time Quantitative PCR (qPCR)

Total RNA from FFPE samples was isolated using the RNeasy FFPE Kit (Qiagen, Limburg, Netherlands) following the manufacturer's instructions. The set of fresh samples was extracted using the AllPrep DNA/RNA/Protein Mini Kit followed by deoxyribonuclease treatment using RNase-Free DNase Set (Qiagen, Limburg, Netherlands). Total RNA was also isolated from cell lines using TRIzol Reagent (Life Technologies, Barcelona, Spain) following the manufacturer's protocol and subsequently treated with DNase (Promega, Barcelona, Spain). Quantification of the recovered RNA was assessed using NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, NC, USA). Briefly, one microgram of total RNA was retro-transcribed to cDNA with the First Strand Synthesis kit using random hexamer primers (Thermo Scientific, Madrid, Spain). cDNAs were amplified with the Brilliant III SYBR Green Master Mix (Stratagene, La Jolla, CA, USA) using the Stratagene Mx3000p system and specific primers for each transcript of interest. Expression levels (absolute mRNA copy number/50 ng of sample) of *EN1* (sense: GCAACCCGGCTATCCTACTT; antisense: CGATCCGAATAACGTGTGC) and *EN2* (sense: GAACCCGAACAAAGAGGACA; antisense: ACCTGTTGGTCTGGAACTCG) were measured using primers designed with Primer3 software and methods previously reported [29,30]. Normalization of all genes was done according to *GAPDH* expression levels or to a normalization factor, obtained by the expression levels of two control genes (*GAPDH*, sense: AATCCCATCACCATCTTCCA and antisense: AAATGAGCCCCAGCCTTC; and *ACTB*, sense: ACTCTTCCAGCCTTCCTTCCT and antisense: CAGTGATCTCCTTCTGCATCCT).

2.5. Measurements of Cell Proliferation

Cell proliferation of RWPE-1, LNCaP, 22Rv1, and PC3 cell lines in response to EN2 treatment was determined using the Alamar blue fluorescent assay (Life Technologies, Madrid, Spain), as previously described [27,31]. Different concentrations of EN2 peptide (10^{-6} to 10^{-9} M) were initially tested in RWPE-1 and PC3 cell lines (Figure S1). Based on these results, the dose of 10^{-7} M was selected for further experiments in all the prostate cell lines included in subsequent experiments (i.e., RWPE-1, LNCaP, 22Rv1, and PC3 cells), as we found that this was the only dose that increase proliferation rate in PC-3 (but not RWPE-1) cells. Moreover, the increase in proliferation rate in response to 10^{-7} M of EN2 was corroborated in 22Rv1 cells (IGF1 and Paclitaxel were used as control of proliferation enhancement and inhibition, respectively). Briefly, cells were seeded in 96-well plates at a density of 3000 to 5000 cells per well and subsequently serum-starved for 24 h. Then, after 3 h of incubation with 10% Alamar blue serum-free medium, basal proliferation rate was obtained by measuring the fluorescent signal of reduced Alamar, exciting at 560 nm and reading at 590 nm using the FlexStation III system (Molecular Devices, Sunnyvale, CA, USA). Subsequently, medium was replaced by fresh medium containing 5% FBS and the treatments to be tested immediately after each measurement and proliferation rate was determined after 24 h incubation. Results were expressed as percentage referred to control (vehicle-treated). In all cases, cells were seeded per quadruplicate and all experiments were performed a minimum of three times.

2.6. Measurements of Migration Capacity

Cell migration was evaluated in RWPE-1 and PC3 cells by wound-healing technique as previously reported [27,28]. Briefly, 300,000 cells were cultured in 12-well plates and then a wound was made using a 200 µL sterile pipette tip on confluence conditions. Then, the wells were rinsed using PBS and subsequently incubated for 24 h in serum free medium. Wound healing was evaluated as the area of a rectangle centered in the picture 24 h after the wound vs. the area of the rectangle just after the wound was performed. At least three experiments were performed in independent days.

2.7. Measurement of Free Cytosolic Calcium Concentration ($[Ca^{2+}]_i$)

Cells were plated on coverslips at a density of 100,000 cells per well and changes in $[Ca^{2+}]_i$ in RWPE-1, LNCaP, and PC3 cell lines after treatment with EN2 protein were tracked in single cells using fura-2/AM (Molecular Probes, Eugene, OR, USA) as described previously [27,32].

2.8. Microarray of Gene Expression Profile

Microarray experiment was carried out using the Human Androgen Receptor Signaling Targets PCR Array PAHS-142Z (Qiagen, Limburg, Netherlands). Three independent passages of LNCaP cells treated for 24 h with EN2 protein mixed in one pool, and the respective vehicle-treated controls were used. Retrotranscription was performed using RT^2 First Strand Kit (Qiagen, Limburg, Netherlands) and expression was measured using RT^2 qPCR SYBR Green ROX (Qiagen, Limburg, Netherlands) in Stratagene Mx3000p system. Results were analyzed with Data Analysis Center (Qiagen, http://www.qiagen.com/shop/genes-and-pathways/data-analysis-center-overview-page/), following the manufacturer's instructions.

2.9. Western Blot

RWPE-1, LNCaP, and PC3 cells were treated with EN2 peptide for 8 min for the evaluation of AKT, ERK, and Androgen Receptor (AR) phosphorylation levels by western blot using standard procedures, as previously reported [27,28,30]. Furthermore, AR and AR splice variants (SVs) phosphorylation levels by western blot were also evaluated in 22Rv1 cells. Briefly, proteins were extracted from cells seeded in 12-well plates using pre-warmed Sodium dodecyl sulfate- Dithiothreitol (SDS-DTT) buffer (62.5 mM Tris-HCl, 2% SDS, 20% glicerol, 100 mM DTT and 0.005% bromophenol blue), followed by sonication

during 10 s and boiling for 5 min at 95 °C. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline/0.05% Tween 20 and incubated with the specific primary antibodies [p-AKT (Ser47; Ref. CS9271S), Akt (Ref. CS9272), p-ERK1/2 (Ref. CS4370), ERK1/2 (Ref. CS154) from Cell Signaling (Danvers, MA, USA); p-Androgen Receptor (p-AR; Ser210; Ref. AB71948) and AR (Ref. AB133273) for both full-length and SVs ARs, from Abcam (Cambridge, UK)], tubulin beta (TUBB) (Ref. #2128S, Cell-Signaling), and the appropriate secondary antibodies (Cell Signaling)]. Proteins were detected using an enhanced chemiluminescence detection system (GE Healthcare, Madrid, Spain) with dyed molecular weight markers. A densitometry analysis of the bands obtained was carried out with ImageJ software, using total protein as normalizing factor of correspondent phosphorylated protein or TUBB as normalizing factor of total proteins.

2.10. Determination of PSA and EN2 Levels by ELISA

PSA secretion was measured after EN2 treatment (10^{-7} M concentration) in the LNCaP cell line using a specific commercially available ELISA kit (DRG Diagnostics, Marburg, Germany). Briefly, cells were seeded in 12-well plates at 70% confluence in serum-starved medium and 24 h later media were collected and stored at −20 °C until measurement. Results are expressed as the percentage of PSA secretion vs. vehicle-treated cells. Three independent experiments were performed on separate days, in which cells were plated per triplicate. In addition, EN2 levels were determined in medium from RWPE-1, LNCaP, and PC3, as well as in urine from PCa patients and control individuals using a commercially available ELISA kit (Wuhan EIAAB Science Co., Wuhan, China) following the manufacturer's instructions. All the information regarding specificity, detectability, and reproducibility for each of the assays can be accessed at the website of the company.

2.11. Statistical Analysis

Descriptive results were expressed as mean ± standard error of the mean (SEM) or median and interquartile range in case of quantitative data, and in absolute value and percentage in case of qualitative variable. Paired or unpaired (parametric or non-parametric) tests were performed to determine significant differences between two groups. The receiver operating characteristic (ROC) curve was performed for evaluation of the accuracy of EN2 as a diagnostic marker in the different tissues and fluids analyzed. p-values ≤ 0.05 were considered statistically significant and a trend for significance was indicated when p-values ranged between <0.1 and >0.05. Statistical analyses were performed using SPSS 17.0 (IBM SPSS Statistics Inc., Chicago, IL, USA) and GraphPad 7.0 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. EN2 Is Overexpressed in Tissue and Urine Samples from PCa Patients Compared with Controls

Analysis of *EN1* and *EN2* mRNA levels in FFPE prostate pieces from patients with low-intermediate grade PCa revealed that *EN2* expression was significantly higher in tumor vs. non-tumor adjacent tissue, specifically in 24 out of 33 samples (72.73%; Figure 1a), whereas *EN1* mRNA levels did not differ (Figure S2a,b). *EN2* overexpression was confirmed in an independent cohort of fresh PCa biopsies from high-risk/locally-advanced PCa patients ($n = 23$) vs. fresh normal prostate tissues ($n = 7$) (Figure 1b, left-panel). Of note, ROC analysis showed that *EN2* mRNA levels clearly discriminated between PCa and control subjects (AUC = 0.96; $p < 0.001$; Figure 1b, right-panel). These results were further corroborated by analyzing public databases obtained from The Cancer Genome Atlas (TCGA; $n = 52$ tumor and 52 non-tumor adjacent; Figure 1c) data portal, wherein 41 out of 52 samples (78.85%) showed a clear overexpression of EN2, and from the Memorial Sloan Kettering Cancer Center (MSKCC; $n = 179$) dataset (Figure 1d) which showed higher levels of *EN2* mRNA in PCa samples compared to controls. Although no other relevant clinical associations with Gleason score or other tumor-related

pathologic parameters were found in these cohorts (data not shown), in silico analysis using the Grasso cohort indicated that EN2 expression tends to be overexpressed in CRPC samples (Figure S2c).

In line with the previous data, mean urine EN2 protein levels were clearly elevated in PCa patients compared with healthy controls (Figure 1e, left-panel). Specifically, EN2 was detected in urine samples from 18 out of 24 (75%) of the patients with PCa vs. only in 45% of controls. In this sense, ROC curve analysis suggested the potential of urine EN2 levels to discriminate between PCa patients and controls [AUC = 0.66 (0.50–0.83); $p = 0.06$] (Figure 1e, right-panel).

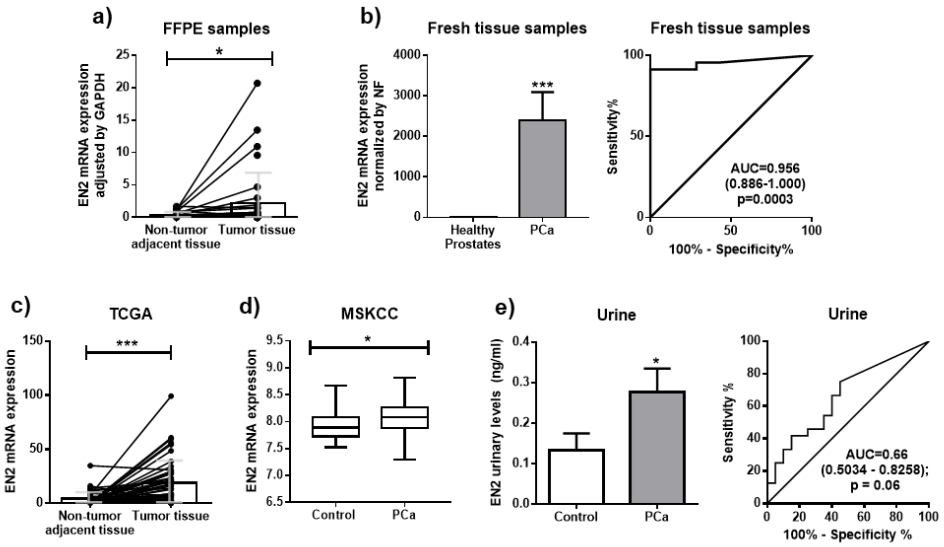

Figure 1. *EN2* is overexpressed in prostate tissue and urine from PCa patients. (**a**) Paired analysis of EN2 mRNA expression levels in PCa tissue and matched adjacent non-tumor tissue from 33 Formalin-fixed paraffin-embedded (FFPE) prostatectomy samples. Absolute mRNA levels were determined by qPCR and adjusted by *GAPDH* housekeeping gene. (**b**) mRNA expression levels of *EN2* in a battery of 23 PCa samples and compared to the expression levels found in seven normal prostates. Absolute mRNA levels were determined by qPCR and adjusted by normalization factor (NF). Receiver operating characteristic (ROC) curve analysis to determine the accuracy of EN2 to discriminate between tumor and healthy tissue. (**c**) Analysis of *EN2* mRNA expression levels in 52 PCa samples and 52 non-tumor adjacent samples from TGCA data set. (**d**) Analysis of *EN2* mRNA expression levels in 29 non-tumor tissue and 150 PCa tissues from the MSKCC data set. (**e**) Evaluation of EN2 levels as a non-invasive PCa diagnostic marker. EN2 urinary levels in 24 PCa patients (filled bars) compared to 20 controls (healthy and negative biopsy patients; open bars), determined by ELISA assay, without prostate massage (left panel). ROC curve analysis to determine the accuracy of EN2 to discriminate between tumor and healthy patients (right panel). Data represent mean ± SEM. * $p \leq 0.05$, *** $p < 0.001$ indicate values that significantly differ between groups.

3.2. EN2 Is Overexpressed, Secreted, and Modulates Aggressiveness Features in PCa Cells

Analysis of the expression of *EN2* in different prostate cell lines (Figure 2a) revealed that the mRNA of this variant was virtually absent in the normal-like prostate cell line RWPE-1, while it was clearly over-expressed in the two PCa cell lines analyzed, LNCaP and PC3. Available online data from the Cancer Cell Line Encyclopedia further confirmed this overexpression in PCa cell lines compared to normal prostate cells (Figure S3). Consistently, EN2 protein was found to be secreted from PCa cell lines (determined by ELISA in medium), while its levels were under the detection limit in the normal RWPE-1 cells (Figure 2b). In support of this latter observation, our data also suggest that the EN2 present in the urine might be mainly derived from prostate cells because urine EN2 levels were clearly increased after DRE in the same cohort of PCa patients previously described in Table 3 ($n = 24$; Figure S4).

Treatment with EN2 protein significantly increased cell proliferation rates in all PCa cell lines analyzed (LNCaP, 22Rv1 and PC3) but not in the normal RWPE-1 cells, compared to vehicle-treated controls (Figure 2c). In contrast, treatment with EN2 protein increased migration rate in both RWPE-1 and PC3 cell lines (Figure 2d). Interestingly, PSA secretion was also augmented after 24 h of treatment with EN2 protein in LNCaP cells (Figure 2e).

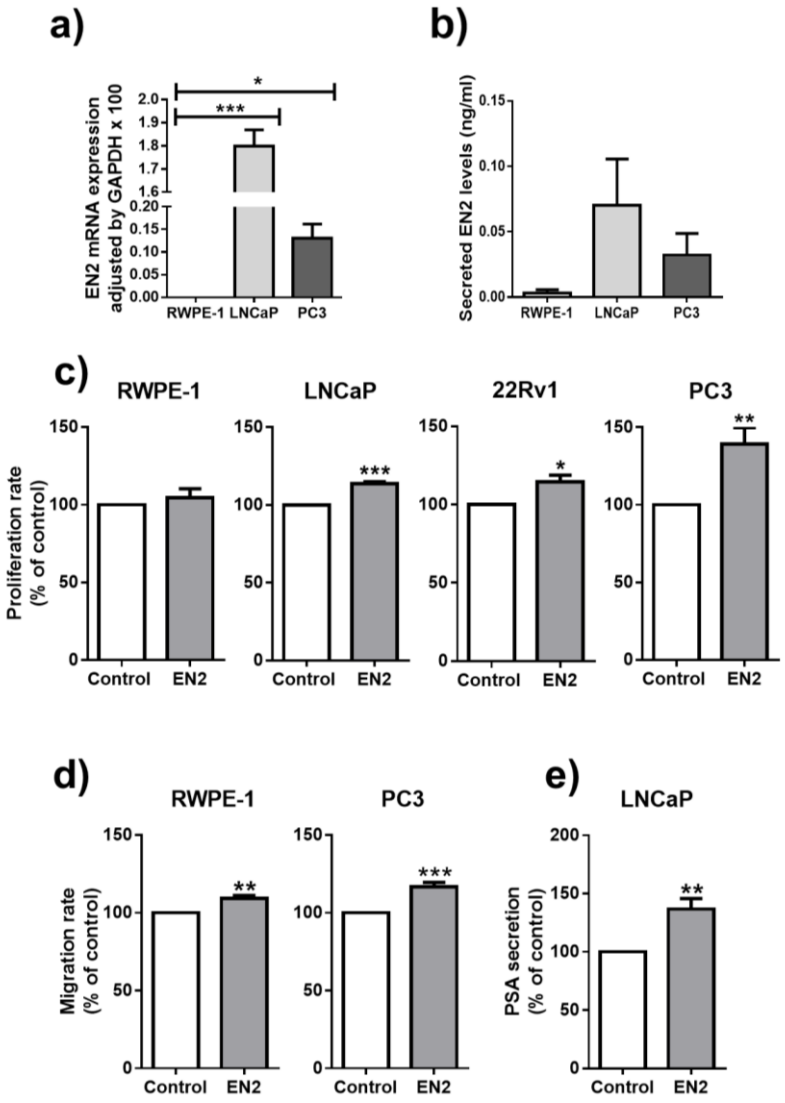

Figure 2. *EN2* expression and its functional role in prostate-derived cell lines. (**a**) *EN2* mRNA expression levels in normal-like prostate cell line, RWPE-1, and PCa cell lines, LNCaP and PC3, determined by qPCR and adjusted by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. (**b**) EN2 secretion levels from RWPE-1, LNCaP, and PC3 cell lines, determined by ELISA. (**c**) Effect of 24 h treatment with EN2 protein on cell proliferation rate in (from left to right) RWPE-1, LNCaP, 22Rv1, and PC3 cell lines compared to vehicle-treated controls. (**d**) Effect of 24 h treatment with EN2 protein on cell migration rate in RWPE-1 and PC3 cell lines, compared to vehicle-treated control. (**e**) PSA secretion from LNCaP cell line treated with EN2 protein compared with vehicle-treated controls (after 24 h culture) determined by a specific ELISA kit. Data represent mean ± SEM and they are expressed as percentage of vehicle-treated controls (set at 100%) within experiment. * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate significant differences compared to control.

3.3. EN2 Modulates Key Signaling Pathways and Molecular Targets in PCa Cells

In order to unveil the molecular mechanisms underlying the pro-tumorigenic actions of *EN2* in PCa, we first determined the capacity of EN2 protein to modulate free cytosolic calcium concentration ($[Ca^{2+}]_i$). Our results revealed that treatment with EN2 protein did not alter $[Ca^{2+}]_i$ kinetics in RWPE-1, LNCaP, or PC3 cells (Figure S5), while ionomycin elicited the appropriate response in all cell lines, indicating that EN2 does not alter this signaling pathway. In marked contrast, western blot analysis revealed that treatment with EN2 protein (10^{-7} M; 8 min) increased the phosphorylation of AKT and AR, but not ERK protein in LnCaP cells (Figure 3 and Figure S6). Furthermore, AR signaling modulation by EN2 was also corroborated in the androgen sensitive 22Rv1 cells, wherein treatment with EN2 increased the phosphorylation of full-length (Figure 3 and Figure S6) but also the splicing variants of AR (Figure S7), while it did not alter total AR levels. On the other hand, no changes in the phosphorylation levels of these proteins were found in response to EN2 treatment in normal-like RPWE-1 or androgen-insensitive PC3 cells (Figure 3 and Figure S6).

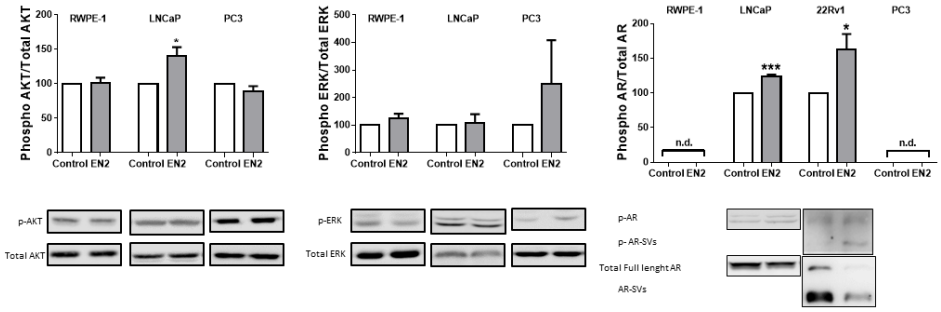

Figure 3. Downstream consequences of EN2 treatment in RWPE-1, LNCaP, 22Rv1, and PC3 cells. Phosphorylation of key signaling pathways (AKT, ERK, and AR; from left to right) after EN2 treatment during 8 min, compared with non-treated control (full western blot images in Figure S6). n.d means non-detectable levels. AR-SVs = AR splice variants. Data represent mean ± SEM and they are expressed as percentage of the ratio (set at 100%). * $p \leq 0.05$, *** $p < 0.001$ indicate significant differences compared to control.

In addition, to explore a putative association between EN2 and key factors in PCa, we measured, in LNCaP cells treated with EN2, a PCR Array of human androgen receptor signaling pathways, which allows the measurement of the mRNA levels of a wide number of key genes involved in pathways related with AR. This array revealed that several genes, mainly related with tumor progression, were altered when cells were treated with EN2 compared with vehicle-treated control cells (Figure 4a). Specifically, when considering significant differences in genes with a fold change higher than 2, the array revealed the upregulation of *EGR3* and *PTGS1* and the downregulation of *GSTP1* in response to EN2 protein treatment (Figure 4b).

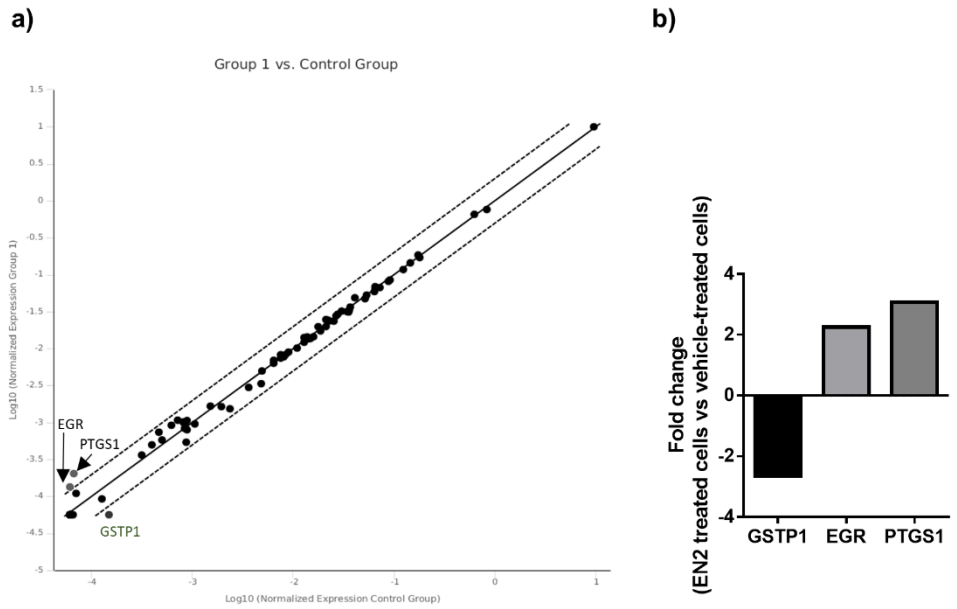

Figure 4. PCR array of human AR signaling pathway. (**a**) Representation of differences (two-fold change) between control and EN2-treated LNCaP cells using scatter plot. Upregulated genes are at the top of the image and downregulated genes at the bottom. (**b**) Representation of $\log_2$ fold changes in significantly-altered genes [Glutathione S-transferase P 1 (*GSTP1*), Early growth response 3 (*EGR3*), and Prostaglandin-Endoperoxide Synthase 1 (*PTGS1*)] between control and EN2-treated LNCaP cells.

4. Discussion

The high incidence and prevalence of PCa represent a major health problem worldwide [1], whose management is also hampered by the limited availability of appropriate diagnostic, prognostic, and therapeutic tools. In particular, PCa screening based on the current gold-standard PSA and the DRE remains controversial, mainly due to the high rate of over-diagnosis and unnecessary prostate biopsies [33]. In addition, although the molecular characterization of this type of cancer has provided new predictive and prognostic markers, as well as novel therapeutic targets [7,34], their universality and applicability are still a matter of debate. For these reasons, it seems crucial to identify new molecular biomarkers which would help to refine the diagnosis, to improve the prediction of the prognosis and behavior of the tumor, and to provide tools to develop novel therapeutic approaches.

In this scenario, earlier studies suggested that the gene family of homeodomain transcription factors might play a relevant role in the pathophysiology of PCa and, hence, that they could provide novel tools for the diagnosis, prognosis, and/or treatment of this pathology [21,35–37]. Specifically, previous reports showed that *EN2* is overexpressed in tumor prostate tissues and PCa cell lines compared with normal prostate tissue and normal-like cell lines [19,20]. In the present study, we have confirmed and expanded these findings by corroborating in independent and ample cohorts of patients that *EN2* is overexpressed in PCa samples with different grades of differentiation and aggressiveness, in comparison with normal prostate tissues but also with their respective adjacent non-tumor tissue. It is methodologically worth noting that the differences could be observed in both FFPE and frozen samples. Moreover, these results have been further substantiated by analyzing public databases from The Cancer Genome Atlas data portal and from the MSKCC dataset, which indicate that *EN2* is overexpressed in different cohorts of PCa patients and; therefore, suggest the universality of this biomarker.

Most relevant from the diagnostic point of view is the fact that EN2 can be found in urine, the less invasive liquid biopsy, wherein its levels have been shown to be elevated in PCa patients compared to controls [20–24]. In particular, Morgan et al. demonstrated that patients with PCa had 10-fold increased urine EN2 levels compared to controls, showing a specificity of 88% to diagnose PCa [20], which suggested the putative utility of urine EN2 levels as a novel non-invasive PCa biomarker. Indeed, the

same group validated these results by using patients with high risk of PCa included in the IMPACT cohort [21], and later correlated the urinary levels of EN2 with tumor stage and volume in patients treated with radical prostatectomy (first-pass and midstream urine samples were evaluated) [22,23]. However, a subsequent study by Marszałł et al. did not find different urinary levels of EN2 between patients with and without PCa when using complete urine samples, although they found higher EN2 urinary levels in urine after prostate massage [24]. Consequently, in that the sensitivity and specificity of this biomarker is considerably variable among studies and the values can fluctuate depending on the existence of previous prostate massage, the appropriateness and accuracy of EN2 as a PCa diagnostic biomarker, as well as the methodological procedure for EN2 assessment in urine samples, are still a matter of debate [20–24]. In this scenario, our results are in accordance with Morgan et al., showing higher levels and discriminatory capacity of EN2 in urine from patients with PCa versus controls, using an independent cohort of patients. Nevertheless, it should be taken into account as a limitation that the use of TRUS biopsy for PCa diagnosis, despite being the current standard in most populations, suffers from some false negative results and random error compared with template biopsy. In this series of studies, the authors recommended measuring the first part of the urine, but they have also found association with PCa volume with EN2 mid-urine levels. In contrast, we have observed similar differences by measuring EN2 levels in whole urine, which may suggest that EN2 urinary levels could represent a valuable PCa diagnostic tool without the necessesity of prostate stimulation if whole urine is used. However, this hypothesis should be further validated in subsequent studies using different, ampler cohorts of patients. It is also important to note that, as Marszałł et al. [24], we have analyzed EN2 urinary levels with a commercial ELISA kit and, maybe, our results could be improved with better EN2 detection systems. Indeed, Morgan et al., who first described the possible utility of this marker, used an non-commercial ELISA but their method has not been validated by other groups [20].

Interestingly, earlier studies also suggested that EN2 could play a tumorigenic role in PCa in that its silencing is associated to a decrease in PCa cell proliferation [19]. One of the most striking features of EN2 is that its protein does not seem to be localized in the nucleus of PCa cells but, rather, close to the luminal border of the cells, associated to secretory blebs [20]. This is, indeed, consistent with the observation that cells from different established PCa cell lines can release EN2 protein to the medium (data presented herein and in Morgan et al. [20]), and that urine EN2 levels increase after DRE in PCa patients (data presented herein and in Marszałł et al. [24]). Altogether, these results suggest that secreted EN2 could play a pathological role in PCa that remains poorly known. These reasons prompted us to explore herein the putative tumorigenic role of secreted EN2 protein in normal and tumor prostate cells, inasmuch as this information could pave the way towards the identification and development of novel therapeutic avenues in PCa. This approach led us to demonstrate, for the first time, that secreted EN2 protein can act on normal and tumor prostate cells by modulating certain signaling pathways and cancer-associated genes, which ultimately results in an enhance tumorigenic potential in these cells (i.e., an increased capacity to proliferate, migrate, or secrete PSA). In particular, treatment with exogenous EN2 protein elicited an increase in the proliferation capacity of the PCa cell lines LNCaP and PC3, an increase in the capacity to migrate of normal-like RWPE-1 and PC3 PCa cells and an increase in PSA secretion from LNCaP cells, which are, all of them, parameters directly associated to the tumorigenic capacity of these cells [38]. Interestingly, our results also show that EN2 treatment evoked a modest but significant increase in the phosphorylation rate of full-length AR in LNCaP and full-length and SVs AR in 22Rv1, as well as an increase in phosphorylation rate of AKT in LNCaP. In this sense, the PI3K/AKT signaling pathway is a frequently dysregulated pathway in cancer [39], and specifically in PCa [40], wherein signaling cross-talk and functional synergism between PI3K/AKT and AR pathways have been previously reported [41]. Furthermore, the association between the dysregulation of full-length and SVs AR signaling and the process of promoting oncogenesis of all stages of PCa has been also widely demonstrated [42]. In support of these findings, our present results indicate that this tumorigenic capacity could likely be associated to the modulation of the expression of certain cancer-associated genes, in that the PCR-based array implemented herein to analyze the

response of LNCaP cells to EN2 treatment revealed a relevant modulation of a discrete number of genes, including the upregulation of *PSTGS1* and *EGR3* and the downregulation of *GSTP1*. It is worth noting that both upregulated genes have been clearly shown to be involved in the association between inflammation and cancer [43,44]. In particular, EGR3 has been previously reported to be overexpressed in PCa cells and to upregulate inflammatory cytokines such as IL6 and IL8, which play an important role in PCa and contribute to disease progression and to the onset of castration resistance [43,45]. We also found that EN2 treatment elicited a significant down-regulation of *GSTP1*, which is known to be hypermethylated in PCa and to be correlated with the aggressiveness of the disease [46]. Nevertheless, since we observed that EN2 treatment had also the capability of modulating some functional parameters in RWPE-1 and PC3 cells, wherein it did not alter the phosphorylation levels of AKT, we could conclude that additional pathways activated by exogenous EN2 may exist, which may help to explain the tumorigenic role exhibited in these cell lines.

When viewed together, our results provide compelling evidence to support the potential value of EN2 as a non-invasive diagnostic biomarker for PCa, and offer, as well, novel valuable information to consider its putative utility to develop new therapeutic tools in this pathology. In particular, we expanded and validated the higher expression of *EN2* in PCa tissue vs. normal prostate, as well as its elevated levels in urine samples from PCa patients. In addition, we demonstrate herein, for the first time, that secreted EN2 protein could act as a tumorigenic factor in normal and tumor prostate cells, by modulating key functional parameters and signaling pathways. Therefore, these data invite to explore further the identification and development of novel therapeutic targets related to *EN2* in this high incidence and prevalent pathology.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/8/9/1400/s1. Figure S1: Dose response analysis of EN 2 treatment in RWPE 1 and PC 3 cell proliferation. Figure S2: Expression of EN1 in PCa and EN2 in CRPC PCa, Figure S3: Free available expression levels of EN2 in normal and tumor prostate cell lines, from Cancer Cell Line Encyclopedia (https://portals.broadinstitute.org/ccle), Figure S4: Urinary levels of EN2 before and after digital rectal examination in the cohort of patients with PCa (n = 24), Figure S5: Representative profiles of [Ca+2]i in RWPE-1, LNCaP and PC3 cell lines (n = 2) in response to EN2 protein, Figure S6: Western blot images: full-length gels and blots, Figure S7: Phosphorylation of AR splice variants after EN 2 treatment in 22Rv1 cells .

Author Contributions: Conceived and designed the project: G.-G.E., G.M.D. and L.R.M. Acquired data: G.-G.E., J.-V.J.M., P.-A.S., L.-L.F., H.-A.V., H.-A.D., S.-S.R., G.-S.T., C.-V.J. Performed the analysis and interpretation of data: G.-G.E., J.-V.J.M., P.-A.S., L.-L.F., H.-A.V., H.-A.D., V.-R.J., I.-C.A.,L.-G.A.J., S.-S.R., G.-S.T., C.J.P., C.-V.J., G.M.D. and L.R.M. Wrote the manuscript: G.-G.E., J.-V.J.M., G.M.D. and L.R.M. Revised the manuscript for important intellectual content: P.-A.S., H.-A.V., H.-A.D., V.-R.J., I.-C.A., L.-G.A.J., S.-S.R., G.-S.T., R.-T.M.J., C.J.P. and C.-V.J. Performed the statistical analysis: G.-G.E., J.-V.J.M. and P.-A.S. Obtained funding: R.-T.M.J. and L.R.M. Supervised the work: G.M.D. and L.R.M.

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Conflicts of Interest: The authors declare no conflict of interest

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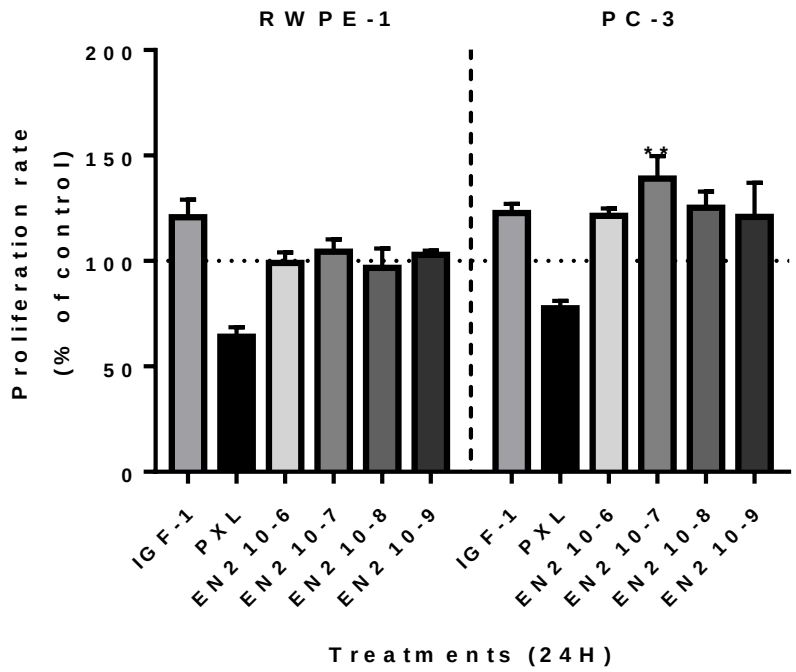
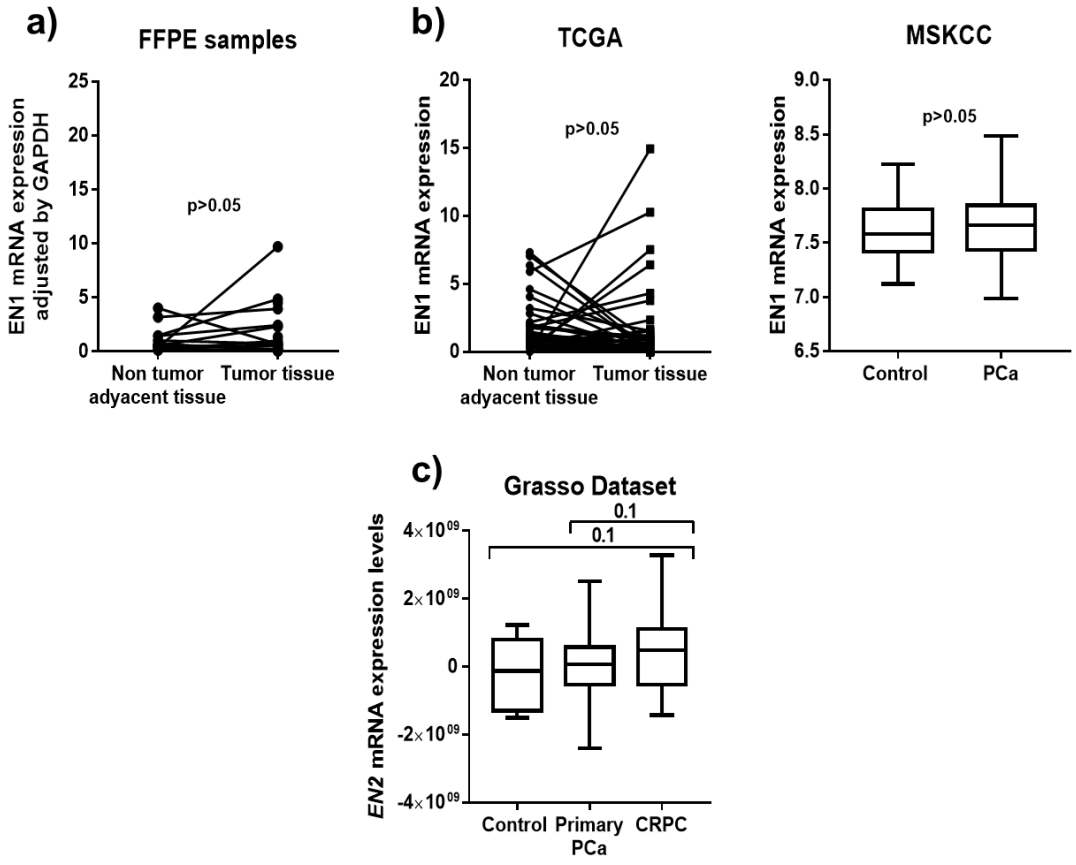
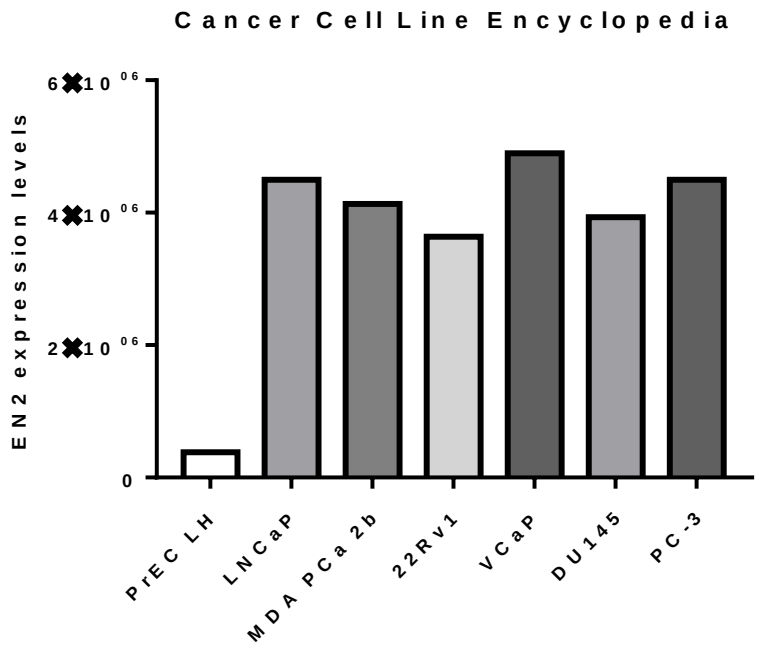


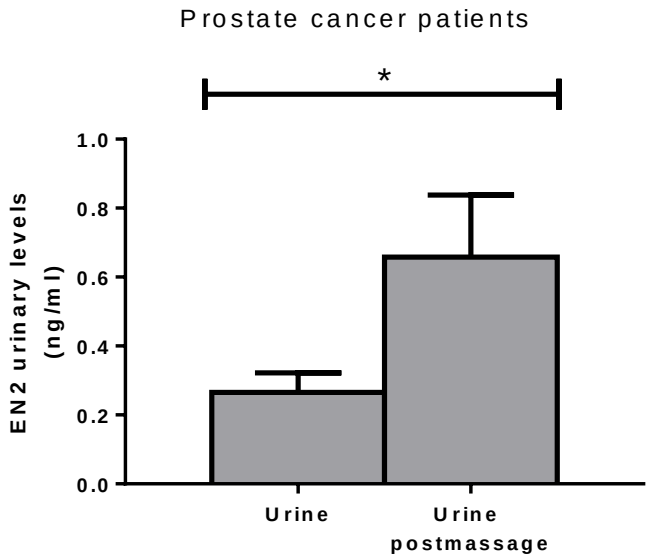
Supplemental figure 1. Dose-response analysis of EN2 treatment in RWPE-1 and PC3 cell proliferation. Proliferation rate in response to EN2 treatment at 10-6 to 10-9 M at 24 h. Data represent mean ± SEM. *, $p \leq 0.05$; **, $p < 0.01$, indicate values that significantly differ from the reference. PXL means paclitaxel



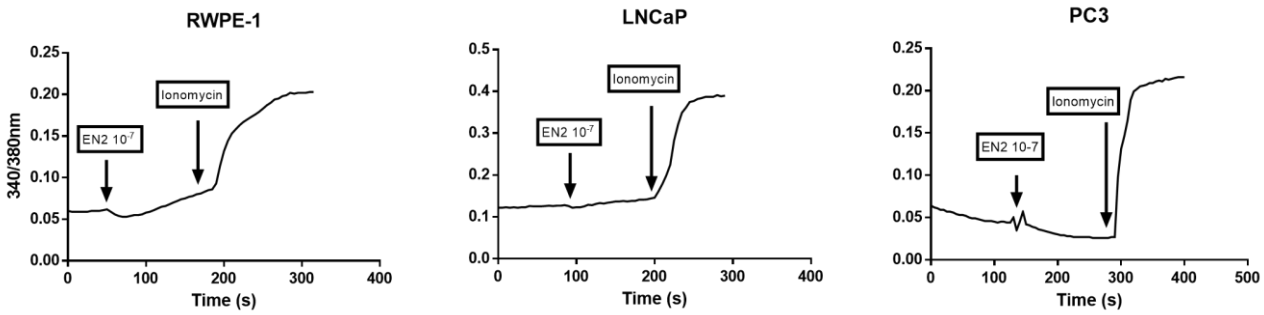
Supplemental Figure 2. Expression of *EN1* in PCa and EN2 in CRPC PCa. **A**. Paired analysis of *EN1* mRNA expression levels in PCa tissue and matched with adjacent non-tumor tissue from 18 FFPE prostatectomy samples. Absolute mRNA levels were determined by qPCR and adjusted by GAPDH housekeeping gene **B.** Analysis of *EN1* mRNA expression levels in 52 PCa tissue and 52 non-tumor adjacent tissue from TGCA data set (left) and analysis of *EN1* mRNA expression levels in 29 non-tumor tissue and 150 PCa tissue from the MSKCC dataset (right). **C.** Analysis of EN2 mRNA expression levels in 12 Control, 49 primary PCa and 27 CRPC samples from the Grasso cohort dataset Data represent mean ± SEM.



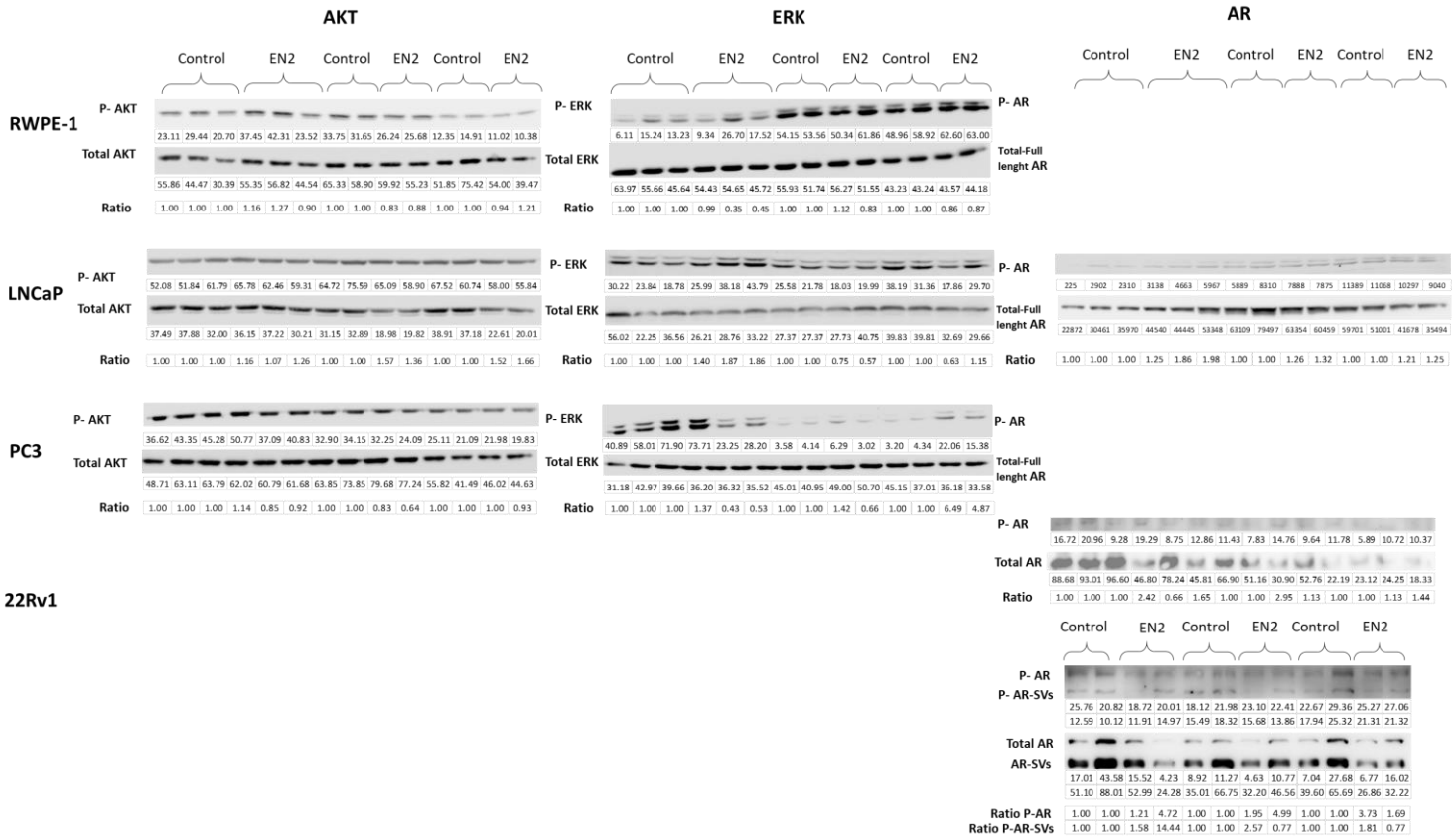
Supplemental figure 3. Free available expression levels of *EN2* in normal and tumoral prostate cell lines, from Cancer Cell Line Encyclopedia (https://portals.broadinstitute.org/ccle).



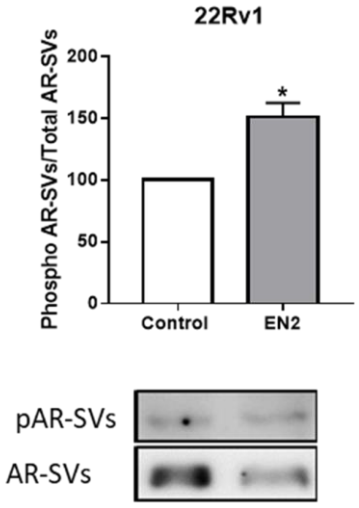
Supplemental figure 4. Urinary levels of EN2 before and after digital rectal examination in the cohort of patients with PCa (n= 24). Data represent mean ± SEM. *, $p \leq 0.05$.



Supplemental figure 5. Representative profiles of $[Ca^{+2}]_i$ in RWPE-1, LNCaP and PC3 cell lines (n=2) in response to EN2 protein. No changes was observed in response to EN2, while ionomycin elicited the expected response.



Supplemental figure 6. Western blot images: full-length gels and blots.
Phosphorylation of key signalling pathways (AKT, ERK and AR; from left to right) after EN2 treatment during 8 min, compared with non-treated control. Total protein was used as normalizing factor of the respective phosphorylated protein. AR-SVs; AR splice variants. It should to be noted that protein ratios of EN2 treated cells has been compared against protein ratios of control cells (which has been set at 100 %), therefore minimizing the variability of the data. Arbitrary units of densitometry analyses as well as final data (expressed as ratio of control cells) are shown under each blot. * Second western-blot showing not only full-length but also AR-SVs.



Supplemental figure 7. Phosphorylation of AR splice variants after EN2 treatment in 22Rv1 cells. A. Phosphorylation after EN2 treatment during 8 min, compared with non-treated control (full western blot images in supplemental figure 6). AR-SVs= AR splice variants. Data represent mean ± SEM and they are expressed as percentage of the ratio (set at 100%). *, $p \leq 0.05$; indicate significant differences compared to control.