DEPARTAMENTO DE BIOLOGÍA CELULAR, FISIOLOGÍA E INMUNOLOGÍA

Identification of novel biomarkers and therapeutic targets in the pathological interaction between the dysregulation of the endocrine-metabolic system and prostate cancer

Identificación de nuevos biomarcadores y dianas terapéuticas en la interacción patológica entre la desregulación del sistema endocrino-metabólico y el cáncer de próstata

Vicente Herrero Aguayo

Córdoba, Junio 2022

TITULO: *Identification of novel biomarkers and therapeutic targets in the pathological interaction between the dysregulation of the endocrine-metabolic system and prostate cancer*

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Identification of novel biomarkers and therapeutic targets in the pathological interaction between the dysregulation of the endocrine-metabolic system and prostate cancer

Memoria de Tesis Doctoral presentada por **Vicente Herrero Aguayo**, Graduado en Biología, para optar al grado de **Doctor en Biomedicina**

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En Córdoba, a 10 de Junio de 2022

UNIVERSIDAD D CÓRDOBA

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DEPARTAMENTO DE BIOLOGÍA CELULAR, FISIOLOGÍA E INMUNOLOGÍA

D. Raúl Miguel Luque Huertas y D. Manuel David Gahete Ortiz, Catedrático y Profesor Titular, respectivamente, del Departamento de Biología Celular Fisiología e Inmunología de la Universidad de Córdoba,

INFORMAN

Que D. Vicente Herrero Aguayo, Graduado en Biología, ha realizado bajo nuestra dirección el trabajo titulado "**Identification of novel biomarkers and therapeutic targets in the pathological interaction between the dysregulation of the endocrine-metabolic system and prostate cancer**" y que, bajo nuestro juicio, reúne los méritos suficientes para optar al Grado de Doctor en Biomedicina.

Y para que conste, firmamos la presente en Córdoba, a 10 de Junio de 2022.

Fdo.: Dr. Raúl Miguel Luque Huertas

Fdo.: Manuel David Gahete Ortiz

TÍTULO DE LA TESIS: Identificación de nuevos biomarcadores y dianas terapéuticas en la interacción patológica entre la desregulación del sistema endocrino-metabólico y el cáncer de próstata.

DOCTORANDO: Vicente Herrero Aguayo

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

Durante el desarrollo de la presente Tesis Doctoral, en el periodo comprendido entre septiembre de 2017 y Junio de 2022, el doctorando Vicente Herrero Aguayo no solo ha superado con creces los objetivos planteados al comienzo de la misma, sino que también 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 clínico y molecular de la obesidad y el cáncer de próstata, y que quedan patentes en diferentes publicaciones. Concretamente, como fruto de su trabajo durante este periodo, ha publicado 3 trabajos directamente relacionados con su Tesis Doctoral, en las revistas "Molecular Therapy Nucleic Acids" [Decil 1 (13/189) del área de "Medicine, Research & Experimental"; Factor de impacto: 8.886] y "Journal of Clinical Endocrinology and Metabolism" [Cuartil 1 (28/179) del área de "Endocrinology & Metabolism", Factor de impacto: 5.958], revistas de referencia dentro de nuestras áreas de investigación.

Por último, el doctorando ha presentado sus resultados en diferentes congresos de ámbito nacional e internacional, de los que han derivado varios capítulos de libro, y ha participado en el desarrollo de varias patentes.

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

Córdoba, 10 de Junio de 2022

Firma de los directores

Fdo.: Dr. Raúl Miguel Luque Huertas

Fdo.: Manuel David Gahete Ortiz

Esta Tesis Doctoral ha sido realizada en el Departamento de Biología Celular, Fisiología e Inmunología de la Universidad de Córdoba y en el Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), bajo la dirección de los Dres. Raúl Miguel Luque Huertas y Manuel David Gahete Ortiz. Dicho trabajo fue subvencionado mediante proyectos del Dicho trabajo fue subvencionado mediante proyectos/ayudas del MINECO (PID2019-105564RB-I00), Instituto de Salud Carlos III FIS (PI16/00264, PI17/02287, DTS18/00131, DTS20/00050), Junta de Andalucía (P20_00442; PEER-0048-2020, PI-0639-2012, PI-0541-2013, BIO-0139), Fundación La Caixa (CAIXAIMPULSE_003), Fundación para la Innovación y la Prospectiva en Salud en España (FIPSE-3188-2017), y del Centro de Investigación Biomédica en Red de la Fisiopatología de la Obesidad y Nutrición (CIBERobn). Durante el transcurso de la presente Tesis Doctoral se ha realizado una estancia de tres meses en el Departamento de Endocrinología de la Universidad de California en San Diego (UCSD) bajo la supervisión del Prof. Jerrold Olefsky, financiada por una ayuda de movilidad internacional para estudiantes de Formación de Profesorado Universitario (FPU) del Ministerio de Educación y Ciencia para la realización de estancias destinadas a la obtención de la Mención Internacional en el Título de Doctor de la Universidad de Córdoba, y de la cual ha derivado una publicación como coautor en la revista Diabetes (2022).

List of publications

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

Article I: Herrero-Aguayo V, Sáez-Martínez P, López-Cánovas JL, Prados-Carmona JJ, Alcántara-Laguna MD, López FL, Molina-Puerta MJ, Calañas-Continente A, Membrives A, Castilla J, Ruiz-Ravelo J, Alonso-Echague R, Yubero-Serrano EM, Castaño JP, Gahete MD, Gálvez-Moreno MA, Luque RM, Herrera-Martínez AD. Dysregulation of Components of the Inflammasome Machinery After Bariatric Surgery: Novel Targets for a Chronic Disease. J Clin Endocrinol Metab. 106(12):e4917-e4934. doi: 10.1210/clinem/dgab586. 2021.

[IF: 5.958, 28/179 (Q1) Endocrinology and Metabolism (JCI)]

Article II: Herrero-Aguayo V, Jiménez-Vacas JM, Sáez-Martínez P, Gómez-Gómez E, López-Cánovas JL, Garrido-Sánchez L, Herrera-Martínez AD, García-Bermejo L, Macías-González M, López-Miranda J, Castaño JP, Gahete MD, Luque RM. Influence of OB in the miRNome: miR-4454, a Key Regulator of Insulin Response Via Splicing Modulation in Prostate. J Clin Endocrinol Metab 106(2):e469-e484. doi: 10.1210/clinem/dgaa580. 2021.

[IF: 5.958, 28/179 (Q1) Endocrinology and Metabolism (JCI)]

Article III: Herrero-Aguayo V, Sáez-Martínez P, Jiménez-Vacas JM, Moreno-Montilla MT, Montero-Hidalgo AJ, Pérez-Gómez JM, López-Canovas JL, Porcel-Pastrana F, Carrasco-Valiente J, Anglada FJ, Gómez-Gómez E, Yubero-Serrano EM, Ibañez-Costa A, Herrera-Martínez AD, Sarmento-Cabral A, Gahete MD, Luque RM. Dysregulation of the miRNome unveils a crosstalk between OB and prostate cancer: miR-107 as a personalized diagnostic and therapeutic tool. Mol Ther Nucleic Acids 27:1164-1178. doi: 10.1016/j.omtn.2022.02.010. 2022

[IF: 8.886, 13/189 (D1) Medicine, Research & Experimental (JCI)]

List of abbreviations

ADT - Androgen deprivation therapy
AGO - Argonaute protein
AIM2 - Absent in melanoma 2
AR - Androgen receptor
ASC - Apoptosis-associated speck-like protein
ATP - Adenosine triphosphate
AUC - Area under curve
BMI - Body mass index
BPH - Benign prostatic hyperplasia
BS - Bariatric Surgery
CRP - C-reactive protein
CRPC - Castration-Resistant PCa
DAMPs - Damage-associated molecular patterns
DGCR8-DiGeorge syndrome critical region gene 8
DNA - Deoxyribonucleic acid
DRE - Digital rectal exam
FASN - Fatty-acid synthase
FDA - US Food and Drug administration
GO - Gene Ontology
GS - Gleason Score
GSDMD - Gasdermin D
HT - Hypertension
IL - Interleukin
IR - Insulin receptor
IR - Insulin resistance
KEGG - Kyoto Encyclopedia of Genes and Genomes
miRNAs - MicroRNAs
NEFAs - Non-esterified fatty acids
NLRC - NOD-like receptor CARD domain containing
NLRP - NOD-like receptor pyrin domain containing
NLRs - NOD-like receptors
NOD - Nucleotide binding oligomerization domain
NonSigPCa - Non-Significant Prostate Cancer
OB - Obesity
ODD - Odds Ratio
PAMPs - Pathogen-associated molecular patterns
PBMCs - Peripheral blood mononuclear cells
PCa - Prostate Cancer
PIN - Prostatic intraepithelial neoplasia
premiRNA - Precursor miRNA
PRRs - Pattern recognition receptors
PSA - Prostate-specific antigen
RISC - RNA-induced silencing complex
RNA - Ribonucleic acid
RNP - Ribonucleic protein
ROC - Receiver operating characteristic
ROS - Reactive oxygen species
RT-PCR - Real Time-Polymerase chain reaction
RYGB - Roux-en-Y gastric bypass
SAT - Subcutaneous adipose tissue
SG - Sleeve gastrectomy
SigPCa - Significant Prostate Cancer
T2DM - Type 2 Diabetes Mellitus
VAT - Visceral adipose tissue
WAT - White adipose tissue
XPO5 - Exportin 5

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Resumen

La **obesidad (OB)** es una enfermedad crónica metabólica con una incidencia creciente y una elevada morbilidad y mortalidad [1, 2]. La obesidad también se asocia a elevados costes económicos para los sistemas de salud y representa un problema de salud pública a nivel mundial [3, 4]. En concreto, en 2016, 650 millones de adultos eran obesos [5]; entre ellos, entre el 10% y el 30% presentaban trastornos metabólicos [6], entre los que se encuentran la diabetes tipo 2 (T2DM), la hipertensión (HT), las cardiopatías, el colesterol total y los triglicéridos elevados, los accidentes cerebrovasculares, la apnea obstructiva del sueño, la enfermedad del hígado graso no alcohólico y ciertos tipos de cáncer [7-9]. En este sentido, la cirugía bariátrica (BS) es un tratamiento bien reconocido para la OB que tiene resultados superiores para los pacientes que no han podido mantener la pérdida de peso por métodos no quirúrgicos [10]. En todo el mundo, los procedimientos bariátricos más realizados son el bypass gástrico en Y de Roux (RYGB) y la gastrectomía en manga (SG) por vía laparoscópica, donde ambos procedimientos abarcan casi el 80% de todas las operaciones bariátricas del mundo [11]. La BS induce y mantiene una pérdida de peso sustancial a través de diversos mecanismos, como la restricción calórica (debido a los cambios anatómicos inducidos por la cirugía en el tracto gastrointestinal), el aumento de la termogénesis inducida por las comidas, la modulación de los circuitos neuronales hipotalámicos (que regulan el equilibrio energético y/o la regulación del apetito), las modificaciones en las vías de señalización intestino-cerebro y los cambios en el gusto, las preferencias alimentarias y los patrones de conducta alimentaria [12-15]. Todos estos cambios se acompañan de una reducción de las complicaciones metabólicas, especialmente de la T2DM [16], y de una rápida alteración del estado inflamatorio, debida en parte a la modulación de la producción de citoquinas tras la BS, que puede ir acompañada también de una mejora de otras complicaciones metabólicas [17].

En este contexto, la inflamación es una respuesta inmunitaria protectora, en la que la función inmunitaria innata depende del reconocimiento de patrones moleculares asociados a patógenos, que proceden de patógenos invasores, y de patrones moleculares asociados al peligro, siendo inducidos de forma endógena por receptores de reconocimiento de patrones (PRRs) codificados en la línea germinal. La activación de los PRRs induce la producción de interferón-α, interferón-β y citoquinas proinflamatorias [18]. Estas respuestas están mediadas por la maquinaria del inflamasoma, un complejo multiproteico intracelular que desempeña un papel central en la inmunidad innata y es responsable de la activación de las respuestas inflamatorias. Este complejo está compuesto por algunas familias de PRRs, incluyendo el dominio de unión a nucleótidos, proteínas que contienen repeticiones ricas en leucina [receptores similares al dominio de unión a nucleótidos (NOD)], y por los receptores ausentes en el melanoma 2-like (AIM2- like) [19]. Los receptores tipo NOD (NLRs) y los receptores similares a AIM pueden oligomerizarse y actuar como andamios activadores de la caspasa-1, por ejemplo, activando la familia proinflamatoria de la interleucina (IL)-1 mediante la producción de IL-18 e IL-1β [20]. Sorprendentemente, la actividad de estos componentes del inflamasoma está regulada por diferentes proteínas reguladoras, vías metabólicas y un eje regulador mitocondrial [21]. En consecuencia, la activación de estos componentes del inflamasoma conduce a la secreción de diversas citoquinas inflamatorias y a la activación de receptores clave en las células inmunitarias, induciendo así la activación de cascadas inflamatorias, que pueden conducir en algunos casos a alteraciones del ciclo celular y a daños en el DNA [20, 21].

Diferentes componentes de la maquinaria del inflamasoma se han relacionado con diversos trastornos autoinflamatorios, autoinmunes y metabólicos, como la aterosclerosis, la T2DM y la OB [22]. Algunos estudios han descubierto que los

componentes del inflamasoma están desregulados en varios tejidos/células, incluidas las células mononucleares de sangre periférica (PBMC), y que estos cambios pueden estar asociados al desarrollo de la aterosclerosis, las enfermedades autoinmunes (por ejemplo, la esclerosis múltiple) y las neurodegenerativas (por ejemplo, el Alzheimer o el Parkinson) [20]. En la misma línea, se han descrito alteraciones en algunos componentes específicos del inflamasoma en la OB, pero sus funciones específicas aún no se conocen bien [23]. Sin embargo, hasta donde sabemos, aún no se han descrito completamente los cambios en los componentes del inflamasoma inducidos por la pérdida de peso aguda relacionada con el BS. Curiosamente, se ha informado de que las citocinas inducidas por el inflamasoma promueven las enfermedades inflamatorias relacionadas con la OB tras la estimulación de los metabolitos de la dieta alta en grasas y los cambios en el tejido adiposo que se producen durante el aumento de peso [24], lo que sugiere que la modulación del inflamasoma debido a la pérdida de peso puede estar asociada a una mejora de las comorbilidades relacionadas con la OB.

Como se ha mencionado anteriormente, la OB es la causa más común de resistencia a la insulina; sin embargo, la diabetes manifiesta no se desarrolla en la mayoría de los individuos obesos, ya que pueden preservar la normoglucemia mediante una mayor secreción de insulina, lo que da lugar a una hiperinsulinemia compensatoria [25]. Por el contrario, en los individuos susceptibles, la hiperinsulinemia compensatoria no se puede mantener, por lo que se desarrolla prediabetes y T2DM [6]. En este sentido, es fundamental comprender los mecanismos moleculares implicados en la aparición de la OB y sus comorbilidades asociadas. De hecho, se ha demostrado que el desarrollo y la progresión de la OB y otras comorbilidades asociadas, incluida la resistencia a la insulina [26], están estrechamente relacionados con la desregulación de factores metabólicos clave, especialmente los implicados en el control celular del balance

energético (por ejemplo, la insulina, la leptina, los transportadores de glucosa, etc.), que a su vez están controlados por mecanismos reguladores celulares específicos, como los microARN.

Los **microRNAs** (o **miRNAs**) son RNA cortos no codificantes (20-22 nucleótidos) que actúan a nivel post-transcripcional para regular la expresión génica [27]. Se están acumulando pruebas de que los miRNAs circulantes pueden actuar como factores endocrinos, ya que son liberados a la circulación por muchos tejidos [28]. De hecho, los miRNAs parecen servir como mensajeros endocrinos y paracrinos que facilitan la comunicación entre las células donantes y las dianas, ejerciendo así potencialmente importantes funciones en la integración de los órganos metabólicos [28]. Por estas razones, se ha postulado que los miRNAs circulantes podrían ejercer papeles cruciales en el desarrollo y la progresión de la OB y sus comorbilidades relacionadas. Estudios anteriores y dispersos han informado de que algunos miRNAs están desregulados en muestras de plasma de sujetos obesos, incluyendo miR-142-3p, miR-221 o miR-222 [29-34]. Sin embargo, estos resultados son inconsistentes entre los estudios y no pueden ser comparados debido a varios factores, incluyendo las diferentes características de la cohorte (obesos, obesos mórbidos, diabéticos), los diseños de los estudios (paralelo, transversal) o los métodos de detección (TaqMan, SYBR Green, micro o macro arrays, secuenciación de ARN pequeños o miRNA). Por lo tanto, el patrón completo de desregulación de los miRNAs en la condición de obesidad está aún por definir de forma fiable. De hecho, estudios anteriores han demostrado que las tecnologías de alto rendimiento pueden representar una de las herramientas más precisas y fiables para la cuantificación de miRNAs [35]. Lamentablemente, aunque varios de los estudios mencionados anteriormente han informado de la desregulación de

diferentes miRNAs en la condición de OB, hasta donde sabemos no hay estudios hasta la fecha que describan todo el miRNome utilizando arrays específicos de microRNAs.

Por último, como se ha mencionado anteriormente, la OB promueve la aparición de múltiples problemas/defectos patológicos, que provocan graves alteraciones de la homeostasis del organismo (por ejemplo, desregulación de las hormonas, factores de crecimiento e inflamatorios, etc.) que con frecuencia promueven o influyen en el desarrollo de las patologías graves, incluyendo diferentes tumores/cánceres relacionados con el sistema endocrino [36]. En este contexto, es importante mencionar que el **Cáncer de Próstata (PCa)** se ha convertido en el tipo de tumor más frecuente entre los hombres y representa un grave problema de salud en todo el mundo [36]. Una limitación clave en el manejo del PCa es que la prueba de cribado de referencia se basa en los niveles plasmáticos del antígeno prostático específico (PSA), un biomarcador que presenta profundos inconvenientes, especialmente en la llamada "zona gris" (definida como un rango de PSA de 3-10 ng/mL) [37]. De hecho, la prueba del PSA muestra una baja especificidad, ya que hay múltiples factores que pueden aumentar los niveles de PSA sin indicar necesariamente la presencia de un tumor, como la hiperplasia prostática benigna o las afecciones inflamatorias. Además, la prueba del PSA no es capaz de distinguir con precisión los tumores clínicamente relevantes de los casos indolentes [38]. Por estas razones, el análisis anatomopatológico de las biopsias de próstata, que representan una técnica altamente invasiva, sigue siendo necesario para diagnosticar adecuadamente el PCa en la actualidad. Por lo tanto, existe una importante necesidad clínica insatisfecha de identificar y validar nuevos biomarcadores diagnósticos de PCa no invasivos, fiables y específicos, idealmente desde un punto de vista personalizado y que muestren un potencial pronóstico y/o terapéutico.

En este sentido, los miRNAs son atractivos candidatos a biomarcadores ya que pueden ser extraídos de forma reproducible de una amplia gama de muestras biológicas y son generalmente estables y resistentes a diversas condiciones de almacenamiento [28, 39]. De hecho, estudios recientes han sugerido una relación entre los miRNAs circulantes y la presencia y el resultado del PCa [40-42]. En concreto, estudios recientes han identificado algunos miRNAs presentes de forma diferencial en muestras de plasma de pacientes con PCa; sin embargo, sólo algunos de ellos parecen derivar específicamente de los tejidos del PCa [43-45]. Lamentablemente, aunque varios estudios han identificado algunos miRNAs putativos específicos del PCa (por ejemplo, miR-141 [46], miR-375 [47] y miR-21 [48]), no hay consenso en la utilidad de los miRNAs plasmáticos como biomarcadores circulantes no invasivos del PCa. De hecho, no hay estudios que describan la desregulación de todo el miRNome en el PCa frente a los pacientes sanos. Por estas razones, este campo requiere una mayor investigación para determinar si un miRNA específico o una firma de miRNAs en plasma/suero podría asociarse con el riesgo de PCa y proporcionar un valor diagnóstico y pronóstico a través de una prueba rápida, fácil y no invasiva.

En base a toda la información mencionada anteriormente, en **esta Tesis Doctoral desplegamos un conjunto de enfoques clínicos y experimentales innovadores de uso común en nuestro equipo, incluyendo numerosas muestras clínicas y modelos celulares, para perseguir un triple OBJETIVO con la meta final de identificar nuevas y más personalizadas herramientas diagnósticas, pronósticas y terapéuticas para el manejo de la OB y el PCa.** Para ello, exploramos: 1) Los cambios en los componentes de la maquinaria del inflamasoma (es decir, componentes del inflamasoma y factores asociados a la inflamación) en pacientes obesos después de

la BS y su relación con los parámetros clínicos/bioquímicos al inicio y después de la BS; 2) El patrón de expresión diferencial de los miRNAs en la OB y su papel putativo en las comorbilidades relacionadas con la OB, como la resistencia a la insulina; y, 3) La desregulación del miRNome humano en pacientes con PCa, teniendo en cuenta la condición de OB de los pacientes (normopeso, sobrepeso u OB), y el potencial papel fisiopatológico y los mecanismos moleculares que subyacen al papel de miR-107 en el PCa.

La primera sección de esta Tesis Doctoral se centró en evaluar la expresión y los cambios en los elementos clave de la maquinaria del inflamasoma (es decir, los componentes del inflamasoma y los factores asociados a la inflamación) en pacientes con OB después de la BS [datos publicados en "Herrero-Aguayo V et al., J Clin Endocrinol Metab 106(12):e4917-e4934; 2021"]. En concreto, estos resultados demostraron una profunda desregulación de los componentes del inflamasoma tras la BS, en particular en los NLRs y en los reguladores del ciclo celular y del daño al ADN. Varios componentes se asociaron con comorbilidades metabólicas de base, incluyendo la T2DM (ligando de quimiocina con motivo C-C 2, receptor de quimiocina con motivo C-X-C 1, sirtuina 1), hipertensión (AIM2, ASC, receptor purinérgico P2RX7) y dislipidemia [ligando de quimiocina con motivo C-X-C 3 (CXCL3), dominio de pirina de la familia NLR (NLRP7)], y también mostraron cambios en su perfil molecular 6 meses después de la BS. Curiosamente, la huella de expresión génica de ciertos factores [dominio CARD de la familia NLR que contiene 4 (NLRC4), NLRP12, CXCL3), ligando de quimiocina con motivo C-C 8, receptor tipo Toll 4] diferenció con precisión las PBMCs pre y postoperatorias. La mayoría de los cambios fueron independientes de la técnica quirúrgica realizada. Además, descubrimos que el silenciamiento de

NLRC4/NLRP12 provocaba una alteración de la acumulación de lípidos, la tasa de apoptosis y la viabilidad celular en las células HepG2. Por lo tanto, todos estos resultados sugieren que ciertos componentes de la maquinaria del inflamasoma pueden desempeñar un papel importante como marcadores para predecir las comorbilidades relacionadas con la OB y representar objetivos putativos para revertir las complicaciones relacionadas con la OB. Además, este estudio ha revelado nuevas evidencias sobre el papel crítico de algunos NLRs (especialmente NLRC4) en los procesos fisiopatológicos, como la acumulación de lípidos, la viabilidad celular y la tasa de apoptosis.

Esta Tesis Doctoral también se centró en explorar el patrón de expresión diferencial de los microRNAs en la OB y su papel putativo en las comorbilidades relacionadas con la OB, como la resistencia a la insulina [datos publicados en "Herrero-Aguayo V et al., J Clin Endocrinol Metab 106(2):e469-e484; 2021"]. En concreto, encontramos que los niveles circulantes de miR-4454 eran más elevados en la OB, estaban asociados a parámetros metabólicos relevantes como la insulina o el índice HOMA, y eran modulados por intervenciones farmacológicas (metformina, estatinas) o médicas (BS). Además, los datos *in silico* e *in vitro* también sugirieron que la expresión de miR-4454 podría estar relacionada con la respuesta celular a la insulina en órganos relacionados con el sistema endocrino, como la próstata, ya que miR-4454 se asocia con una desregulación en elementos clave de la vía de señalización del receptor de insulina y con la inhibición de vías de señalización celulares clave como AKT y AMPK. Curiosamente, este estudio también mostró que las alteraciones celulares y moleculares encontradas en respuesta a la insulina desencadenadas por miR-4454 podrían estar asociadas a una desregulación en el proceso de splicing y a la alteración de isoformas de splicing metabólicamente relevantes, como las generadas a partir del gen del receptor de

insulina, que se han asociado al desarrollo de diferentes patologías, como la resistencia a la insulina, la T2DM, la OB, la aterosclerosis y el cáncer. Por lo tanto, los resultados de esta segunda sección de la Tesis Doctoral aportan nuevas y convincentes pruebas que apoyan la afirmación de que miR-4454 representa una prometedora herramienta diagnóstica, pronóstica y/o terapéutica, que merece la pena seguir explorando, en la OB y las comorbilidades asociadas, como la hiperinsulinemia y la T2DM.

Finalmente, en la última sección de esta Tesis Doctoral, analizamos la desregulación de todo el miRNome conocido en muestras de plasma humano de pacientes con PCa en comparación con las de voluntarios sanos con el fin de identificar nuevos y útiles biomarcadores de diagnóstico/pronóstico personalizado y potenciales herramientas terapéuticas en PCa [datos recientemente publicados en "Herrero-Aguayo V et al., Mol Ther Nucleic Acids 27:1164-1178; 2022"]. Nuestros resultados mostraron que los niveles plasmáticos de miR-107 podrían representar un útil biomarcador de diagnóstico personalizado del PCa, ya que sus niveles están aumentados en el plasma de los pacientes con PCa en comparación con los sujetos de control, utilizando dos cohortes independientes. Además, descubrimos que los niveles de miR-107 en plasma están asociados a parámetros oncogénicos clave como los niveles de PSA, el volumen tumoral, la testosterona o los niveles de CRP, lo que sugiere que los niveles elevados de miR-107 en plasma también podrían estar relacionados con la agresividad y la progresión del PCa. Además, los datos *in vitro* e *in silico* revelaron que miR-107 está implicado en la regulación del proceso de splicing y del metabolismo de los ácidos grasos, alterando la expresión del principal conductor FASN, lo que resulta en una reducción de las características de agresividad en las células independientes de los andrógenos. En particular, este estudio también aportó nuevas pruebas que demostraban que miR-107 podría representar un prometedor biomarcador personalizado para el PCa,

ya que los niveles de miR-107 eran más elevados en el plasma de pacientes obesos con PCa en comparación con los pacientes con PCa de peso normal. De hecho, miR-107 también mostró una fuerte capacidad discriminatoria entre el PCa Significativo (Gleason > 6) y el PCa No Significativo (Gleason < 6) en pacientes obesos, representando así no sólo un biomarcador de diagnóstico, sino también de pronóstico, ya que se ha informado de que la condición de obesidad representa un factor de riesgo para el desarrollo, la agresividad y la mortalidad del PCa. Por todo ello, estos resultados demuestran que miR-107 representa una prometedora herramienta diagnóstica, pronóstica y/o terapéutica, que merece la pena seguir explorando, en la asociación patológica entre PCa y OB.

En conjunto, los resultados globales de esta Tesis Doctoral demuestran que **la desregulación de ciertos componentes del miRNoma** (especialmente miR-4454 y miR-107) **y del sistema del inflamasoma** (especialmente algunos NLRs) **podría contribuir al desarrollo y la progresión del OB y el PCa, y podría estar estrechamente implicada en la asociación fisiopatológica entre ambas patologías, representando una fuente de dianas diagnósticas, pronósticas y terapéuticas novedosas y personalizadas que podrían utilizarse para mejorar el manejo y el tratamiento de los pacientes con estas devastadoras patologías.**

Summary

Obesity (**OB**) is a metabolic chronic disease with a growing incidence and elevated morbidities and mortality [1, 2]. OB is also associated with elevated economic costs for the health systems representing a worldwide public health problem [3, 4]. Specifically, in 2016, 650 million adults were obese [5]; among them, 10% to 30% presented with metabolic disorders [6] including type 2 diabetes (T2DM), hypertension (HT), heart disease, high total cholesterol and triglycerides, stroke, obstructive sleep apnea, nonalcoholic fatty liver disease, and certain types of cancer [7-9]. In this sense, **bariatric surgery** (**BS**) is a well-recognized treatment for OB that has superior outcomes for patients who have been unable to keep weight loss by nonsurgical methods [10]. Worldwide, the most commonly performed bariatric procedures are laparoscopic Roux-en-Y gastric bypass (RYGB) and sleeve gastrectomy (SG), wherein both procedures encompass nearly 80% of all bariatric operations worldwide [11]. BS induces and maintains substantial weight loss through a variety of mechanisms, including caloric restriction (due to anatomical surgical-induced changes of the gastrointestinal tract), increased meal-induced thermogenesis, modulation of hypothalamic neuronal circuits (that regulate energy balance and/or appetite regulation), modifications in gut-brain signaling pathways and changes in taste, food preferences, and eating behavior patterns [12-15]. All these changes are accompanied by reduction in metabolic complications, especially T2DM [16], and a rapid alteration of the inflammatory status, due in part to the modulation of cytokine production after BS, which may be also accompanied by improvement in other metabolic complications [17].

In this context, inflammation is a protective immune response, wherein innate immune function depends upon recognition of pathogen-associated molecular patterns, which are derived from invading pathogens, and danger-associated molecular patterns, being induced endogenously by germline-encoded pattern recognition receptors (PRRs).

The activation of PRRs induces production of interferon-α, interferon-β, and proinflammatory cytokines [18]. These responses are mediated by the **inflammasome machinery**, an intracellular multiprotein complex that plays a central role in innate immunity and is responsible for the activation of inflammatory responses. This complex is composed of some families of PRRs, including the nucleotide-binding domain, leucine-rich repeat containing proteins [nucleotide binding oligomerization (NOD) domain-like receptors (NLRs)], and by the absent in melanoma 2-like (AIM2- like) receptors [19]. NLRs and AIM-like receptors can oligomerize and act as caspase-1–activating scaffolds—for example, activating the proinflammatory interleukin (IL)-1 family through the production of IL-18 and IL-1β [20]. Remarkably, the activity of these inflammasome components is regulated by different regulatory proteins, metabolic pathways, and a regulatory mitochondrial hub [21]. Consequently, the activation of these inflammasome components leads to the secretion of diverse inflammatory cytokines and the activation of key receptors in immune cells, thus inducing the activation of inflammatory cascades, which can lead in some cases in cell-cycle alterations and DNA damage [20, 21].

Different components of the inflammasome machinery have been linked to a variety of autoinflammatory, autoimmune, and metabolic disorders including atherosclerosis, T2DM, and OB [22]. Some studies have found that inflammasome components are dysregulated in several tissues/cells including peripheral blood mononuclear cells (PBMCs), and that these changes may be associated with the development of atherosclerosis, autoimmune (e.g., multiple sclerosis), and neurodegenerative (e.g., Alzheimer´s or Parkinson´s) diseases [20]. In the same line, alterations in some specific inflammasome components have been described in OB, but their specific roles are still not well understood [23]. However, to the best of our

knowledge, changes in inflammasome components induced by acute weight loss related to BS have not been fully described yet. Interestingly, it has been reported that inflammasome-induced cytokines promote OB-related inflammatory diseases following stimulation by high-fat diet metabolites and adipose tissue changes that occur during weight gain [24], which suggests that inflammasome modulation due to weight loss may be associated to an improvement in OB-related comorbidities.

As mentioned above, OB is the most common cause of insulin resistance; however, overt diabetes does not develop in most obese individuals, as they can preserve normoglycemia through an enhanced insulin secretion, resulting in compensatory hyperinsulinemia [25]. Conversely, in susceptible individuals, compensatory hyperinsulinemia cannot be maintained, consequently developing prediabetes and T2DM [6]. In this sense, it is critical to understand the molecular mechanisms involved in the onset of OB and its associated comorbidities. In fact, it has been demonstrated that the development and progression of OB and other associated comorbidities, including insulin resistance [26], is closely linked to the dysregulation of key metabolic factors, especially those implicated in the cellular control of the energy balance (e.g. insulin, leptin, glucose transporters, etc.), which are, in turn, controlled by specific cellular regulatory mechanisms, such as microRNAs.

MicroRNAs (or **miRNAs**) are short non-coding RNAs (20-22 nucleotides) that act at post-transcriptional level to regulate gene expression [27]. Evidence is accumulating that circulating miRNAs can act as endocrine factors in that they are released to the circulation by many tissues [28]. Indeed, miRNAs seem to serve as endocrine and paracrine messengers that facilitate communication between donor and target cells, thereby potentially exerting important roles in metabolic organs crosstalk [28]. For these reasons, it has been postulated that circulating miRNAs could exert

crucial roles in the development and progression of OB and its related comorbidities. Previous, scattered studies have reported that some miRNAs are dysregulated in plasma samples of obese subjects, including miR-142-3p, miR-221 or miR-222 [29-34]. However, these results are inconsistent among studies and cannot be compared due to several factors, including different cohort characteristics (obese, morbid obese, diabetic), study designs (cross-sectional, transversal) or detection methods (TaqMan, SYBR Green, micro or macro arrays, small RNA or miRNA sequencing). Hence, the whole pattern of dysregulations of miRNAs in obese condition is still to be reliably defined. Indeed, it has been shown by previous studies that high-throughput technologies may represent one of the most precise and reliable tools for the quantification of miRNAs [35]. Unfortunately, although several of the previously mentioned studies have reported the dysregulation of different miRNAs in OB condition, to the best of our knowledge there are not studies to date describing the whole miRNome using microRNAs specific arrays.

Finally, as previously mentioned, OB promotes the appearance of multiple pathological problems/defects, which cause serious alterations of the homeostasis of the organism (e.g. dysregulation of hormones, growth and inflammatory factors, etc.) that frequently promote or influence the development of the severe pathologies including different endocrine-related tumors/cancers [36]. In this context, it is important to mention that **Prostate Cancer** (**PCa**) has emerged as the most frequent tumor type among men and represents a severe health problem worldwide [49]. A key limitation in PCa management is that the gold-standard screen test is based on the plasma levels of prostate-specific antigen (PSA), a biomarker that exhibits profound drawbacks, especially in the so-called “grey zone” (defined as a PSA range of 3-10 ng/mL) [37]. In fact, PSA test displays low specificity in that multiple factors can increase PSA levels

without necessarily indicating the presence of a tumor, such as benign prostatic hyperplasia or inflammatory conditions. In addition, PSA test is not able to accurately distinguish clinically relevant tumors from indolent cases [38]. For these reasons, the anatomo-pathological analysis of prostate biopsies, which represent a highly invasive technique, is still necessary to appropriately diagnose PCa nowadays. Therefore, there is an important unmet clinical need for the identification and validation of new, reliable and specific non-invasive PCa diagnostic biomarkers, ideally from a personalized point of view and showing prognostic and/or therapeutic potential.

In this sense, miRNAs are attractive biomarker candidates as they can be reproducibly extracted from a wide range of biologic samples and are generally stable and resistant to various storage conditions [28, 39]. Indeed, recent studies have suggested a relationship between circulating miRNAs and PCa presence and outcome [40-42]. Specifically, recent studies have identified some miRNAs differentially present in plasma samples from PCa patients; however, only some of them seem to be specifically derived from PCa tissues [43-45]. Unfortunately, although various studies have identified some putative PCa-specific miRNAs (e.g. miR-141 [46], miR-375 [47], and miR-21 [48]), there is not a consensus in the utility of plasma miRNAs as circulating non-invasive biomarkers for PCa. In fact, there are no studies describing the dysregulation of the whole miRNome in PCa vs. healthy patients. For these reasons, this field requires further investigation in order to ascertain whether a specific miRNA or a plasma/serum miRNA signature could be associated with PCa risk and provide diagnostic and prognostic value through a fast, easy, and non-invasive test.

Based on all the information mentioned above, in **this Doctoral Thesis we deploy a set of innovative clinical and experimental approaches commonly used in**

our team, including numerous clinical samples and cellular models, to pursue a triple OBJECTIVE with the ultimate goal of identifying new and more personalized diagnostic, prognostic, and therapeutic tools for the management of OB and PCa. To that end, we explored: **1)** Changes in components of the inflammasome machinery (i.e., inflammasome components and inflammatory-associated factors) in obese patients after BS and their relation with clinical/biochemical parameters at baseline and after bariatric surgery; **2)** The differential expression pattern of miRNAs in OB and their putative role in OB -related comorbidities such as insulin resistance; and, **3)** The dysregulation of the human miRNome in PCa patients, considering the OB condition of the patients (normoweight, overweight, or OB), and the potential pathophysiological role and the molecular mechanisms underlying the role of miR-107 in PCa.

The first section of this Doctoral Thesis was focused on evaluating the expression and changes in key elements of the inflammasome machinery (i.e., inflammasome components and inflammatory-associated factors) in OB patients after BS [data published in "Herrero-Aguayo V et al., J Clin Endocrinol Metab 106(12):e4917-e4934; 2021"] . Specifically, these results demonstrated a profound dysregulation of inflammasome components after BS, particularly in NLRs and cell-cycle and DNA damage regulators. Several components were associated with baseline metabolic comorbidities including T2DM (C-C motif chemokine ligand 2, C-X-C motif chemokine receptor 1, sirtuin 1), hypertension (AIM2, ASC, purinergic receptor P2RX7), and dyslipidemia [C-X-C motif chemokine ligand 3 (CXCL3), NLR family pyrin domain containing (NLRP7)], and also displayed changes in their molecular profile 6 months after BS. Interestingly, the gene expression fingerprint of certain

factors [NLR family CARD domain containing 4 (NLRC4), NLRP12, CXCL3), C-C motif chemokine ligand 8, toll-like receptor 4] accurately differentiated pre- and postoperative PBMCs. Most changes were independent of the performed surgical technique. Moreover, we found that the silencing of NLRC4/NLRP12 resulted in altered lipid accumulation, apoptosis rate, and cell viability in HepG2 cells. Therefore, all these results suggest that certain components of the inflammasome machinery may play important role as markers for predicting OB-related comorbidities and represent putative targets for reversing OB-related complications. Moreover, this study unveiled novel evidence for a critical role of some NLRs (especially NLRC4) in pathophysiological processes, such as lipid accumulation, cell viability, and apoptosis rate.

This Doctoral Thesis was also focused on exploring the differential expression pattern of microRNAs in OB and their putative role in OB-related comorbidities such as insulin resistance [data published in "Herrero-Aguayo V et al., J Clin Endocrinol Metab 106(2):e469-e484; 2021"]. Specifically, we found that the circulating levels of miR-4454 were higher in OB, associated with relevant metabolic parameters such as insulin or HOMA index, and modulated by pharmacological (metformin, statins) or medical (BS) interventions. In addition, *in silico* and *in vitro* data also suggested that expression of miR-4454 may be related to the cellular response to insulin in endocrine-related organs such as the prostate, inasmuch as miR-4454 is associated with a dysregulation in key elements of the insulin receptor signaling pathway and with the inhibition of key cellular signaling pathways such as AKT and AMPK. Interestingly, this study also showed that the cellular and molecular alterations found in response to insulin triggered by miR-4454 might be associated to a dysregulation in the splicing process and with the alteration of metabolically-relevant splicing isoforms, such as those generated from the

insulin receptor gene, which have been associated with the development of different pathologies, such as insulin resistance, T2DM, OB, atherosclerosis and cancer. Therefore, the results of this second section of the Doctoral Thesis provide new, compelling evidence supporting the contention that miR-4454 represents a promising diagnostic, prognostic and/or therapeutic tool, worth to be further explored, in OB and associated comorbidities, such as hyperinsulinemia and T2DM.

Finally, in the last section of this Doctoral Thesis, we analyzed the dysregulation of the whole known miRNome in human plasma samples from PCa patients compared to those from healthy volunteers in order to identify novel and useful personalized diagnostic/prognostic biomarkers and potential therapeutic tools in PCa [data recently published in "Herrero-Aguayo V et al., Mol Ther Nucleic Acids 27:1164-1178; 2022"]. Our results showed that the plasma levels of miR-107 might represent a useful personalized diagnostic biomarker of PCa since its levels are increased in plasma from PCa patients compared with control subjects using two independent cohorts. In addition, we found that plasma miR-107 levels are associated with key oncogenic parameters such as PSA levels, tumor volume, testosterone or CRP levels suggesting that high plasma miR-107 levels could also be related to PCa aggressiveness and progression. Moreover, *in vitro* and *in silico* data revealed that miR-107 is implicated in the regulation of the splicing process and the fatty acid metabolism, altering the expression of the main driver FASN, resulting in a reduction of aggressiveness features in androgen-independent cells. Notably, this study also showed novel evidence demonstrating that miR-107 could represent a promising personalized biomarker for PCa, in that miR-107 levels were higher in plasma from obese patients with PCa compared to normoweight PCa patients. Indeed, miR-107 also showed a strong discriminatory capacity between SigPCa and NonSigPCa in obese PCa patients, thus

representing not only a diagnostic but also a potential prognostic biomarker in that OB condition has been reported to represent a risk factor for PCa development, aggressiveness and mortality. Taken together, these results demonstrate that miR-107 represents a promising diagnostic, prognostic and/or therapeutic tool, worth to be further explored, in the pathological association between PCa and OB.

Altogether, the overall results of this Doctoral Thesis demonstrate that **the dysregulation of certain miRNAs** (especially miR-4454 and miR-107) **and key components of the inflammasome system** (especially NLRP12, NLRC4, TLR4, CCL8, and CXCL3) **could contribute to the development and progression of OB and PCa, and might be tightly implicated in the pathophysiological association between both pathologies, representing a source for novel and personalized diagnostic, prognostic and therapeutic targets that could be used to improve the management and treatment of patients with these devastating pathologies.**

Introduction

1. Metabolic diseases

1.1 Obesity

Obesity (OB) is an endocrine-metabolic disease tightly associated to multiple chronic diseases such as diabetes, cardiovascular disease, and cancer, which represent the main causes of death and disability in the Western world [50-54]. OB is defined as an excess of adipose tissue in the body. The most common approach to detect OB is the body mass index (BMI, weight in kg/height in m^2), wherein BMI > 30 indicates OB and BMI ranging 25-29.9 indicates overweight. The relationship between BMI and clinical outcomes has been thoroughly studied, and the fact that low BMI values associate with good health are nearly universally accepted. OB significantly raises the risk of T2DM, and both disorders are serious public health concerns worldwide, according to the World Health Organization's newest studies [WHO report 2020 and 2021]. Moreover, OB is considered as the second most frequent avoidable cause of cancer, and it is expected to replace smoking as the most common preventable cause of cancer [55]. The incidence of OB has tripled in the previous decades, with more than two-thirds (70.2%) of the adult population in the United States being overweight or obese, and almost half of adults (48.5%) having pre-diabetes or diabetes [54, 55].

Weight increase, and eventually OB, is thought to be the result of a long-term positive energy balance, although the pathophysiology of OB has been shown to be more complex [56]. Food intake and energy expenditure are conditioned by a complex combination of genetic, environmental, and psychological variables [57]. While the environment and socioeconomic situations influence behavior and cannot be targeted molecularly, the identification of genes and molecules that determine the susceptibility to OB can be molecularly addressed. According to studies on twins and families, the rate of BMI heritability is between 40-70% [58, 59]. More than 300 sites carrying

common variations in the general population have been found in large-scale genome-wide association studies, which indicate a significant link with OB features [57]. However, the effects of these loci on OB risk are minor, accounting for less than 5% of the variation in BMI [60, 61].

Whether epigenetic processes or interactions between genetic and environmental factors can explain the missing heritability is still being investigated. The identification of genes involved in body weight regulation that function in the central nervous system (Figure 1), such as leptin (Lep) and its receptor (Lepr), the melanocortin 4 receptor (Mc4r), and pro-opiomelanocortin (Pomc) are notable outputs of those research works [55]. Monogenic OB is caused by pathogenic mutations in human orthologous genes [62]. As a result, these were the first major breakthroughs in our knowledge of the systems that regulate appetite and fullness. Energy balance is controlled by complex interactions between the central nervous system, adipose tissue, and a plethora of other physiologic organs including the gut, liver, and pancreas (Figure 1).

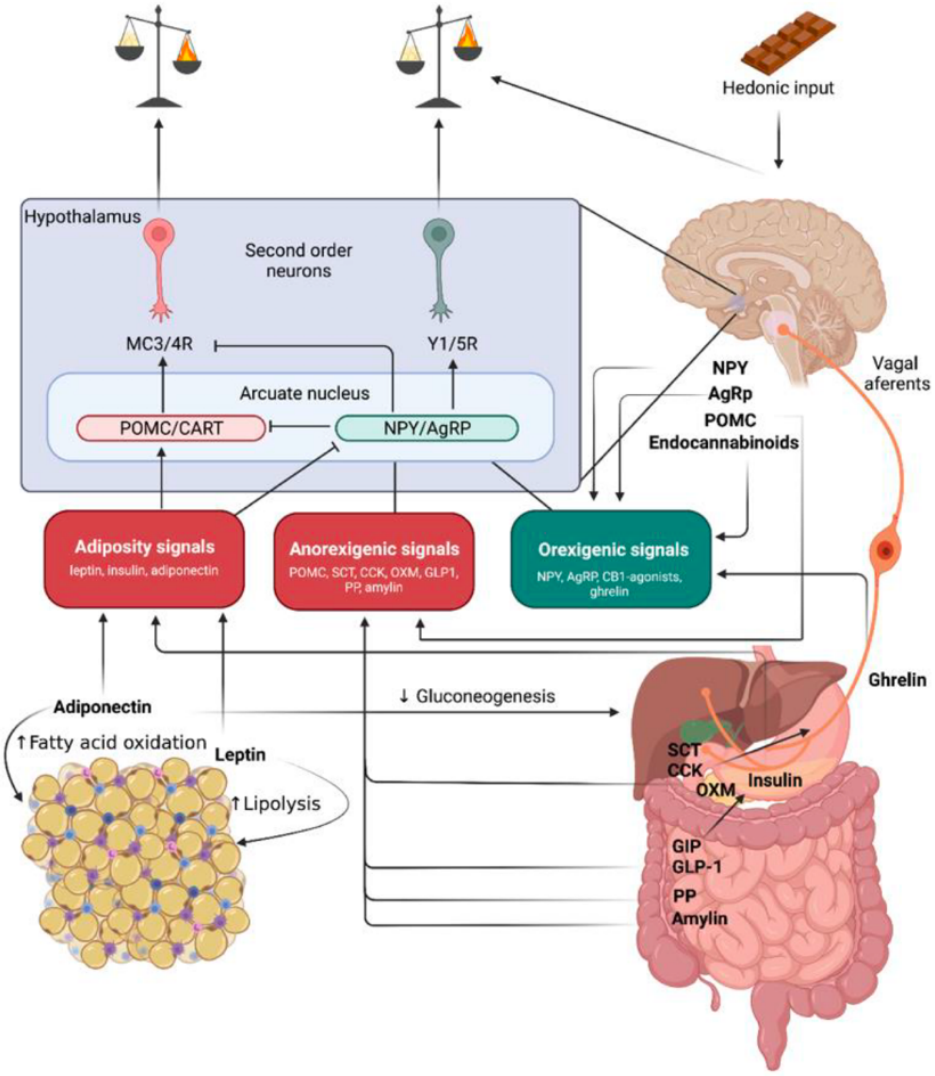

Figure 1. Energy balance signals integration. Source*: Gjermini et al., Biomolecules 2021.*

1.2 Insulin Resistance and Type 2 Diabetes

OB is the most important factor in the development of metabolic disorders, including insulin resistance and T2DM. By releasing non-esterified fatty acids (NEFAs) and glycerol, as well as hormones like leptin and adiponectin and proinflammatory cytokines [63, 64], the adipose tissue regulates metabolism. In OB, the production of many of these biomolecules is altered. Reduced phosphatidylinositol-3-OH kinase (PI3K) signaling in muscle and increased expression of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase in the liver are two mechanisms through which retinol-binding protein-4 (RBP4) induces insulin resistance (IR) [65]. Adiponectin, on the other hand, functions as an insulin sensitizer, promoting fatty acid oxidation through AMP-activated protein kinase (AMPK) and peroxisome proliferator activated receptor (PPAR) signaling [66, 67]. In addition to adipocyte-derived factors, increased release of tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1) and additional products of macrophages and other cells that populate adipose tissue might also have a role in the development of insulin resistance [63, 68]. TNF- α and IL-6 stimulate the c-Jun aminoterminal kinase (JNK) as well as the IB kinase (IKK-)/nuclear factor-B (NF-B) pathways through conventional receptor-mediated mechanisms, resulting in the overexpression of possible inflammatory mediators that can lead to insulin resistance. Insulin sensitivity also modulates β-cell function which is, in the majority of the cases, decreased in OB. Insulin-resistant individuals exhibit higher insulin response and lower hepatic insulin clearance than insulin-sensitive individuals, whether they are lean or obese. A feedback loop exists between insulin-sensitive organs and β-cells in healthy people, with β-cells increasing insulin supply in response to demand from the liver, muscles, and adipose tissue among other metabolic tissues [69]. In order to maintain glucose tolerance unchanged, changes

in insulin sensitivity must be matched by a proportionate yet opposite change in circulating insulin levels. Failure of this feedback loop results in a deviation from normal glucose tolerance and underlies the development of diabetes. The adaptive response to insulin resistance in a healthy β-cell comprises changes in both function and mass, and it is so efficient that normal glucose tolerance is maintained. However, when β-cell dysfunction is present, it leads to reduced glucose tolerance, impaired fasting glucose, and, in the worst-case scenario, T2DM.

One of the key variables contributing to the progression of T2DM is a persistent deterioration in β-cell function [70]. Postprandial and subsequent fasting glucose levels rise due to insufficient suppression of hepatic glucose synthesis and decreased efficiency of liver and muscle glucose uptake as a result of β-cell dysfunction and inadequate insulin secretion. Extremely high blood glucose levels commonly observed in diabetes may contribute to disease progression by having glucotoxic effects on the β-cell and negative effects on insulin sensitivity, both of which can be alleviated by reducing the glucose level therapeutically [71]. In healthy patients, however, boosting blood glucose levels for 20 hours has the exact opposite effect: it improves insulin sensitivity and improves β-cell function [72]. This suggests that a pre-existing, and perhaps genetically determined, risk is crucial for β-cell dysfunction to occur. This pre-existing problem eventually leads to a progressive impairment in insulin release and an increase in glucose levels, the latter of which aggravates the situation and contributes to β-cell failure. Elevated plasma NEFA concentrations are a second metabolic abnormality that may lead to progressive loss of β-cell function in a feed-forward way. Despite the fact that NEFAs are necessary for adequate insulin release, prolonged NEFA exposure *in vitro* and *in vivo* is linked to significant reductions in glucose-stimulated insulin secretion and reduced insulin biosynthesis [73, 74]. In humans,

increased NEFA levels caused by a lipid infusion contribute to the development of insulin resistance and also impede the expected compensatory β-cell response [75]. This dual effect makes them a good candidate to link insulin resistance and β-cell dysfunction in individuals with T2DM and those at risk of its development. This lipotoxic action can also work in tandem with glucose to cause even more harmful effects, a condition known as glucolipotoxicity.

It is important to mention that, from a molecular perspective, several mechanisms have been postulated to play a relevant role in the pathophysiological interplay between OB/insulin resistance/T2DM; however, the precise molecular mechanisms underlying the pathophysiological relationship between these pathological conditions are still to be fully elucidated.

1.3 Treatment

1.3.1 Pharmacological

Metformin is a first-line oral anti-diabetic drug commonly used to treat T2DM, while statins are used for the management of patients with hypercholesterolemia. Mechanistically, the direct effects of metformin and statins are mainly associated to AMPK activation and HMGCoA-reductase inhibition, respectively [76, 77]. However, several independent and/or additional mechanisms have been proposed to underlie the actions of metformin and/or statins, including TNFα inhibition [78], REDD1 upregulation [79] or inhibition of protein geranylgeranylation [80].

1.3.2 Bariatric Surgery

Bariatric surgery (BS) is a well-recognized treatment for OB that has superior outcomes for patients who have been unable to keep weight loss by non-surgical methods [10]. Worldwide, the most commonly performed bariatric procedures are

laparoscopic roux-en-Y gastric bypass (RYGB) and sleeve gastrectomy (SG), wherein both procedures encompass nearly 80% of all bariatric operations worldwide [11]. BS induces and maintains substantial weight loss through a variety of mechanisms, including caloric restriction (due to anatomical surgical-induced changes of the gastrointestinal tract), increased meal-induced thermogenesis, modulation of hypothalamic neuronal circuits (that regulate energy balance and/or appetite regulation), modifications in gut-brain signaling pathways and changes in taste, food preferences and eating behavior patterns [12-15]. All these changes are accompanied by reduction in metabolic complications, especially T2DM [16], and a rapid alteration of the inflammatory status, due in part to the modulation of cytokine production after BS, which might be accompanied by improvement in other metabolic complications [17].

2. Molecular alterations in Metabolic diseases

2.1 Inflammasome Regulation

Numerous research groups have shown that an alteration in the inflammatory status and, specifically, of some components of the inflammasome machinery could be associated with OB. Inflammasomes are multiprotein intracellular complexes that detect pathogenic microorganisms and sterile stressors, activate highly pro-inflammatory cytokines and are, in turn, responsible for innate immune system response [20]. These groups of intracellular multimeric protein complexes represent essential components of the host defense [81]. Inflammasomes activity depends upon the engagement of germline-encoded pattern-recognition receptors (PRRs), which activates innate immune system in response to harmful stimuli [19]. PRRs recognize the presence of unique microbial components [pathogen-associated molecular patterns (PAMPs), including bacterial flagellin] or damage-associated molecular patterns (DAMPs, such as uric acid

crystals), which are generated by endogenous stress. This recognition triggers downstream inflammatory pathways in response to infection and/or to repair damaged tissues [81]. Unfortunately, dysregulation of inflammasomes activation can exacerbate symptoms in infectious diseases and has been related to the development of autoimmune diseases, inflammatory disorders and even cancer [19, 81, 82].

Structurally, most inflammasomes are comprised by a PRR sensor protein [belonging to the nucleotide-binding domain (NBD): NLR; AIM2-like receptor: ALR; or the leucine-rich-repeat-(LRR)-containing families], an adaptor protein ASC (apoptosis-associated speck-like protein), and an inactive zymogen procaspase-1 [83, 84]. Depending on the PRR sensor protein, different inflammasome-cascades are activated (NLRP1, NLRP3, NLRP6, NLRP7, NLRP12 or NLRC4) (Figure 2) [85]. Once activated, sensor PRRs oligomerize through their central NACHT and HIN-200 domains (in the case of NLRs or ALRs, respectively), resulting in recruitment of ASC. Different studies suggest that ASC acts as a molecular platform for protein–protein interactions during inflammasome assembly. Specifically, the activation of NLRP3 and AIM2 inflammasomes precipitates ASC prion-like nucleation and, consequently, other ASC molecules are recruited into this structure, which provides a platform for caspase-1 activation and the subsequent cytokine processing [86, 87]. This activation cleavages procaspase-1 into active caspase-1 enzyme, which further cleaves proforms of several inflammatory cytokines including IL-1β and IL-18, into their active forms [85]. These released cytokines exert a wide variety of systemic functions (Figure 2). As such, IL-1β induces the expression of genes that control fever, pain threshold, vasodilatation and hypotension. The activation of IL-1β receptor also induces an endothelial cell response that enables the infiltration of immune cells to infected or damaged tissues [88]. IL-18 mediates adaptive immunity, and it is necessary for interferon-gamma (IFN-γ)

production. Active caspase-1 is also able to induce an inflammatory form of programmed cell death known as pyroptosis (Figure 2). Indeed, active caspase-1 activates gasdermin D (GSDMD) to form pores in the plasma membrane, inducing cell-pyroptosis [89, 90], which leads to pathological ion fluxes that produce cellular lysis and release of inflammatory intracellular contents [91]. In consequence, this process induces the exposition of intracellular pathogens and the generation of additional DAMPs [92, 93]. In addition, after inflammasome-induced cell death, ASC oligomers accumulate in the extracellular space, wherein the ingestion of ASC specks by macrophages results in lysosomal damage and additional IL-1β production, therefore, acting as a danger signal. Following this mechanism, inflammasome can induce a highly amplified systemic response [81]. Initially, pyroptosis was associated only with microbial infections, but recently it has been described in several inflammatory diseases and even in cancer, where it may affect all stages of carcinogenesis [94].

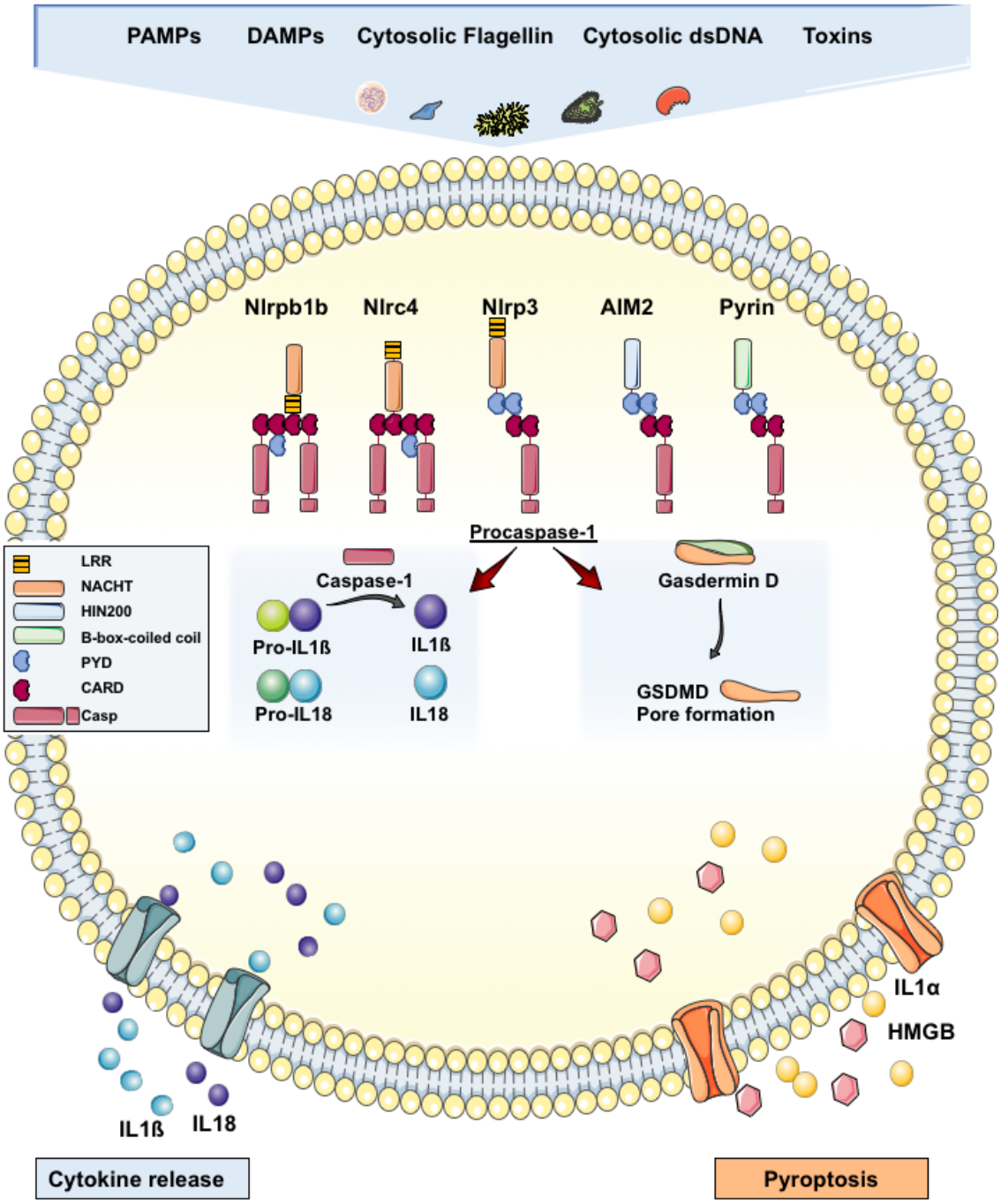

Figure 2. **General structure and activation of the inflammasome**. Source: *Herrera et al., Submitted in OB 2022.*

2.2 Role of Inflammasome in Metabolic diseases

Recent studies suggest a close relationship between the dysregulation of the inflammasome components and metabolic diseases. In particular, it has been described the upregulation of NLRP3 in subcutaneous adipose tissue (SAT) or visceral adipose tissue (VAT) from obese patients compared to lean controls [95-99], and its expression has been positively correlated with BMI, insulin resistance and negatively correlated to adiponectin levels [95-97]. NLRP3 inflammasome senses OB-associated DAMPs and is an important mechanism for insulin resistance [100-103]. Indeed, it has been suggested that NLRP3 limits insulin action, but the specific mechanism involved in this process is still unknown. Even more, an increased expression of NLRP3 has been associated with metabolic comorbidities, including dyslipidemia and T2DM [96, 97, 99]. In this context, VAT tissue from patients with metabolic comorbidities show increased expression of NLRP3 (and IL-1β) compared with patients without metabolic comorbidities [95].

Moreover, several OB-associated factors, such as increased levels of ceramides, reactive oxygen species (ROS), ATP and mitochondrial dysfunction, have been shown to activate the NLRP3 inflammasome in macrophages [83, 100, 103]. In agreement with these observations, the inhibition of NLRP3 inflammasome decreases OB-associated inflammation and improves insulin-sensitivity [95], while activation of NLRP3 inflammasome in combination with a Th1 shift in the T cell population in SAT increase the inflammatory processes in adipose tissue, which contributes to insulin resistance [104]. This study, which was performed in a small cohort of male subjects, revealed that an increased expression of caspase-1 and Th1 transcripts (CTBX21/CD3ε) was positively correlated with insulin resistance and impaired glucose metabolism. Specifically, the leakage of caspase-1 is correlated with decreased insulin resistance,

smaller adipocytes size and lower percentage of total fat mass [100]. However, although alterations in some specific inflammasome components have been described in OB, but their specific role is still not well understood, and changes in inflammasome components induced by weight loss related have not been fully described yet.

2.3 Splicing Process

Splicing is a sophisticated biological mechanism that processes immature or precursor RNA, eliminating the sequences that should not be included in the final RNA (introns) and joining the coding regions or exons, which lead to the generation of the mature RNA [105, 106]. However, the vast majority of genes (> 95%) are subjected to a highly regulated process of alterative splicing, rather than the simple cut-and-paste procedure known as canonical splicing [105, 107, 108]. This phenomenon allows for the formation of various sequence combinations by including and excluding certain sets of exons and introns, resulting in a variety of mature RNA transcripts from the same precursor, known as splicing variants, that may exhibit diverse or even opposite actions [109]. This is an important step in the cell that ensures proper gene expression control since it allows for a greater variety of transcripts from the same genes and increases the genome adaptability [108]. For all of these reasons, accurate splicing control is critical for the proper development and homeostasis of the cells and the organisms. In this context, the spliceosome, a ribonucleoproteic complex that recognizes certain RNA sequences to precisely identify and cleave introns before joining the neighboring exons, is responsible for splicing process and its meticulous regulation [110]. The major spliceosome (U2 spliceosome), which processes more than 99% of introns, and the minor spliceosome (U12 spliceosome), which functions on a small and particular collection of introns, are two distinct spliceosomes (Figure 3) that act individually in

mammals [111]. Based on the spliceosome processing, introns are classed as U2-type (or -dependent, GT-AT) or U12-type (or -dependent, AT-AC) [112]. Both spliceosomes present a core of small nuclear RNAs (snRNAs) called RNU1, RNU2, RNU4, RNU5, and RNU6 for the major spliceosome, and RNU11, RNU12, RNU4ATAC, and RNU6ATAC (RNU5 is present in both) for the minor spliceosome (Figure 3), which interact with other proteins to form small nuclear ribonucleoproteins (snRNP; U1-U6) that work together in a dynamic and coordinated manner to catalyze the splicing process [111, 112]. Furthermore, spliceosomes interact closely with splicing factors, a diverse group of over 300 molecules that complete the splicing machinery, assisting snRNPs in selecting and processing precise sequences, and participating dynamically in every step of the process [113, 114].

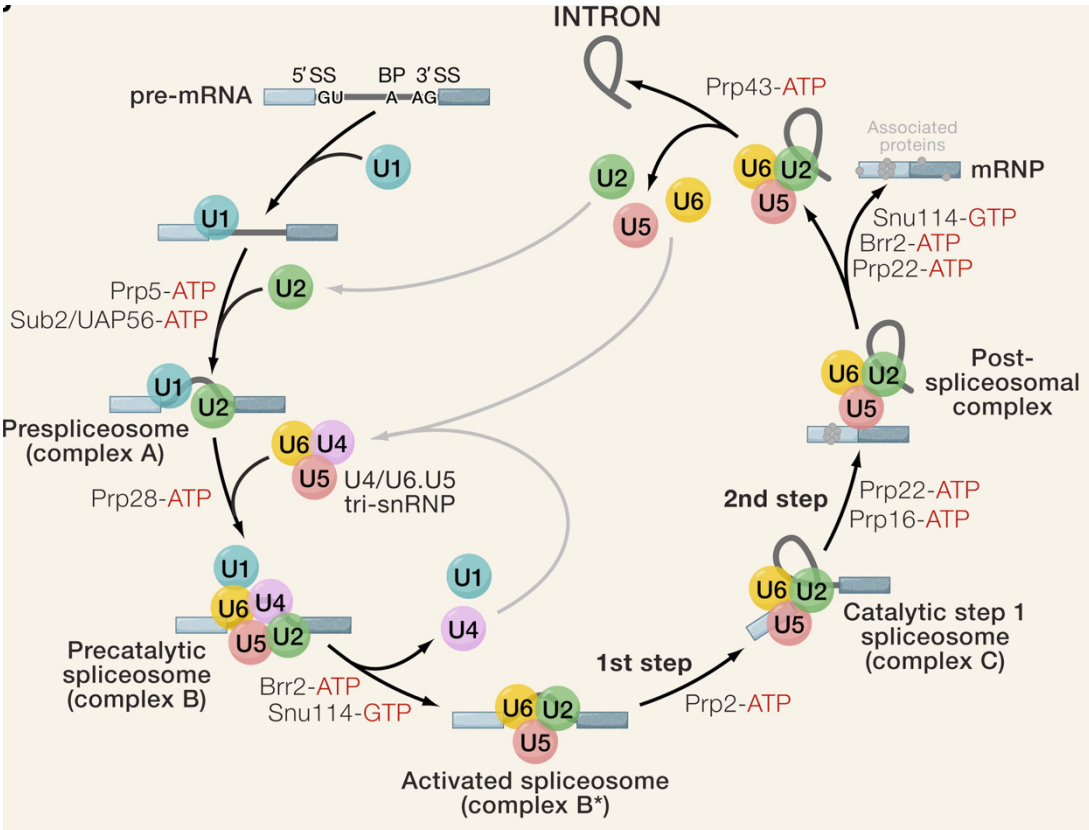

Figure 3. Schematic representation of pre-mRNA splicing by the Spliceosome. Source: *Wahl et al., Cell 2009*

2.4 Role of Splicing in Metabolic diseases

In this context, different changes in the expression level of splice variants and splicing factors may represent important drivers of metabolic diseases. For instance, the human insulin receptor (IR) presents two isoforms differing by the inclusion or not of exon 11 [115]. Insulin receptor type A (IR-A) lacking exon 11 is mostly expressed during prenatal life for growth and fetal development, whereas insulin receptor type B (IR-B) is primarily expressed in well-differentiated adult organs such as the liver for metabolic insulin action [116]. The binding affinity of IR-A and IR-B for insulin is similar, but they have distinct binding affinity for insulin-like growth factor 2 and proinsulin [117]. For that reason, insulin sensitivity might be associated with the alteration of insulin receptor isoform expression. In addition, splicing variants of other genes are also associated to metabolic diseases, including leptin receptor (LepR) or nuclear receptor corepressor (NCoR). Specifically, regarding the structural differences, the LepR isoforms are grouped into three classes: long, short, and soluble [118]. The long isoform is the full-length isoform, expressed in the hypothalamus and immune cells and participates in energy homeostasis and immunity [119]. The short isoforms can be expressed in various tissues and their main consequences are the internalization and degradation of leptin [120]. Lastly, the soluble isoform lacks the intracellular and cytoplasmic domains and is proposed to function as a carrier protein that regulates serum leptin concentration [118]. Similarly, NCoR exhibits different splice variants that exert opposed transcriptional effects [121]. The presence of exon 37, which encodes a third receptor interaction domain (RID), distinguishes between ω and δ splice variants. The expression of the first one predominates in preadipocytes and inhibits adipose differentiation. In contrast, NCoRδ predominates in mature adipocytes and triggers adipose differentiation [121]. However, although many studies have demonstrated the

role of several splice variants and splicing factors in the development and progression of different metabolic diseases, further investigation into the mechanism of splice variant production and their functional consequence is urgently needed.

2.5 miRNAs biology

It has been demonstrated that the development and progression of OB and other associated comorbidities, including insulin resistance [26], is closely linked to dysregulation of key metabolic factors, especially those implicated in the cellular control of the energy balance (e.g. insulin, leptin, glucose transporters, etc.), which are, in turn, controlled by specific cellular regulatory mechanism, such as microRNAs (miRNAs). miRNAs are short noncoding RNA molecules that act post-transcriptionally to regulate protein expression [27]. Frequently, miRNAs act to finely tune the levels of mRNA transcripts within the cells, but they can also function as repressors of protein production. Only a few hundred miRNAs are thought to regulate 30–80 % of the genes encoded in the human genome, with each miRNA targeting up to 100 genes and multiple miRNAs potentially acting on the same gene [122]. Within the 3' untranslated region of a specific mRNA transcript, a single miRNA can have several target sites, and bioinformatic predictions estimate that a single miRNA family can regulate up to 400 targets in human protein-coding genes [123].

Most of mature miRNA sequences are located within introns or exons of noncoding RNAs as well as introns of pre-mRNA [124, 125]. In the nucleus, pri-miRNAs are cleaved into stem-loop structures called precursor miRNAs (premiRNAs) by Drosha (a class 2 ribonuclease III enzyme enzyme; see below, Figure 4) [126]. After export of pre-miRNAs to the cytoplasm by Exportin 5 (XPO5) [127], they are cleaved near the loop into small double-stranded RNAs (dsRNAs) by Dicer [128]. The miRNA

duplex is subsequently loaded into an argonaute protein, which promotes the formation of the RNA-induced silencing complex (RISC), a ribonucleoprotein complex. In human, miRNA base pairing to its target is usually imperfect [129]. Base pairing guides mature miRNAs to the 3′ end of their target mRNA, causing mRNA instability and translational inhibition (Figure 4). Intronic miRNA expression is regulated by the same transcriptional factors as their host genes. Individual hairpin stability and processing may also be influenced by posttranscriptional regulatory pathways [124, 129].

The canonical miRNAs biogenesis pathway

Intergenic miRNAs with their own promoters are transcribed independently. Alternatively, some of them share a promoter with their host genes, while others are cotranscribed as a single primiRNA, comparable to polycistronic units [125]. The mature miRNA sequences are found in the introns or exons of noncoding RNAs, and many are derived from pre-mRNA introns (mirtron) [124]. The majority of miRNAs are transcribed by Pol II as large RNAs named pri-miRNAs, which contain at least one hairpin structure. Although all canonical pri-miRNAs contain a 5′ cap, they may lack a polyadenylation signal at the 3′ end [130]. Pre-miRNAs are formed in the nucleus when pri-miRNAs are cleaved into a 70-nucleotide stemloop structure [126]. This is performed by a microprocessor complex composed by Drosha (originally identified in Drosophila) and DiGeorge syndrome critical region gene 8 (DGCR8). Drosha is a member of RNase III enzymes, which are a type of double-stranded RNA-specific endoribonuclease. DGCR8 is a double-stranded RNA binding protein that functions as the noncatalytic subunit of the microprocessor complex [131]. The pre-miRNAs are subsequently exported to the cytoplasm by XPO5 and Ras-related nuclear protein (RAN), which is a small GTP binding protein [127]. PremiRNAs are cleaved near the

loop in the cytoplasm into small dsRNAs (one is the guide strand, the other is the passenger strand), which have two to three nucleotides overhangs on the 3′ end. Dicer, an RNase III enzyme, is involved in this cleavage, as well as TAR RNA binding protein (TRBP), a double-strand RNA binding protein [128](92). The miRNA duplex is loaded into AGO protein (using ATP-dependent chaperone proteins). The passenger strand of the miRNA duplex is evacuated once the AGO is returned to its normal conformation, resulting in single-stranded mature miRNA [130]. Following loading, AGO promotes the formation of a ribonucleoprotein complex known as RISC, which aids in the identification of the targeted mRNA (Figure 4). Base pairing directs mature miRNAs to their respective mRNA targets [129]. miRNAs bind to their mRNA targets by precise sequence complementarity in plants and extremely infrequently in animals, resulting in mRNA silencing [124]. In contrast, in humans precise pairing to the mRNA is not required. Trinucleotide repeat containing 6 (TNRC6) is an adapter protein which is recruited by AGO and interacts with the PABPC protein (poly(A)-binding) in the 3′ end of mRNA. It activates deadenylase complexes (particularly the CCR4–NOT [carbon catabolite repressor 4negative on TATA] complex). Deadenylases shorten the poly(A) tail of mRNA, causing mRNA instability by decapping and 5′3′ exonuclease activity. CCR4NOT and its recruitment of DEAD-box helicase 6 (DDX6), which binds the decapping complex and is reported to limit translation, also resulted in TNRC6 affecting translation efficiency [130]. Although translational repression keeps happening, its impact is minimal, and mRNA instability is the most common form of repression mediated by mammalian miRNAs [132].

The non-canonical miRNAs biogenesis pathway

Several alternative mechanisms for miRNA biogenesis have recently been discovered. Many groups of RNAs have structural and functional similarities to miRNAs, but they skip one or more steps in the canonical biogenesis route. Drosha and DGCR8 are required for the processing of canonical miRNAs, and noncanonical miRNAs can be produced in their absence [133]. In the synthesis of noncanonical miRNAs, there are many pathways, including Drosha-independent and Dicer-independent mechanisms. Certain debranched introns could behave as premiRNA hairpins in a well-known noncanonical pathway (mirtron). In the Drosha/DGCR8 independent mirtron pathway, introns are processed to lariat mirtrons by the spliceosome and subsequently debranched by the lariat debranching enzyme before folding into premiRNA hairpins (Figure 4). Immediately, XPO5 transports this pre-miRNA-like form to the cytoplasm, allowing the conventional pathways to continue [134].

Target recognition of the miRNA

Target recognition is primarily through base pairing between the miRNA seed (nucleotides 2–8) and sites within the 3′-untranslated region (3′-UTR) of the target mRNAs [135]. Targeting can also be facilitated by additional sequence elements, such as an unpaired Adenosine in the mRNA target sequence, corresponding to the nucleotide 1 in 5′ end of the mature miRNA [136]. The main repressive effects for each miRNA are mediated by these seven to eight nucleotide sites, which are identified by the most effective target prediction methods [137].

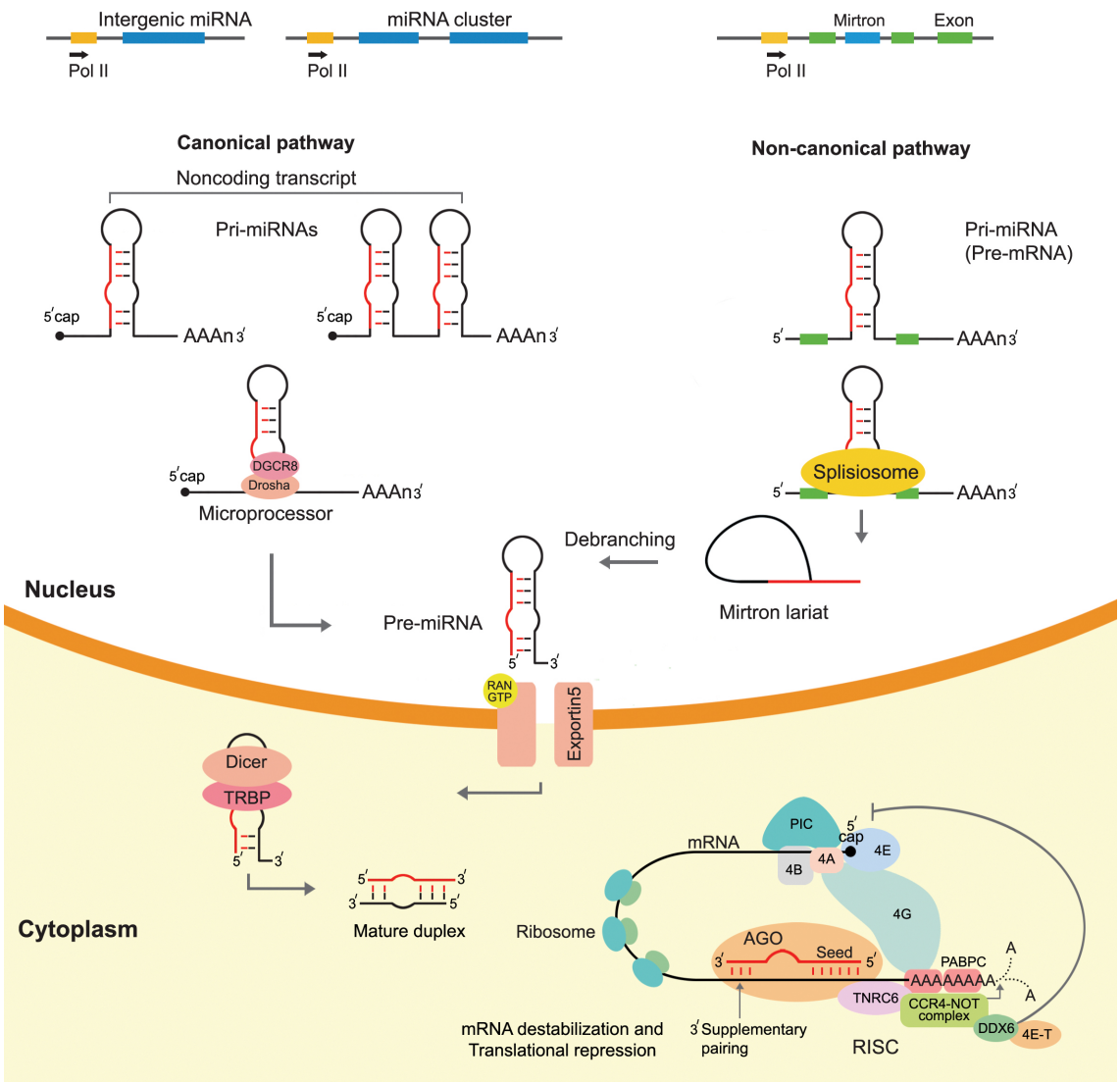

Figure 4. Overview of the miRNAs biogenesis: Canonical and non-canonical pathways. *Saliminejad et al., J Cell Physiol 2018.*

2.6 Role of miRNAs in Metabolic diseases

Evidence is accumulating that circulating miRNAs can act as endocrine factors in that they are released to the circulation by many tissues [28]. Indeed, miRNAs seem to serve as endocrine and paracrine messengers that facilitate communication between donor and target cells, thereby potentially exerting important roles in metabolic organs

crosstalk [28]. For these reasons, it has been postulated that circulating miRNAs could exert crucial roles in the development and progression of OB and its related comorbidities. Several studies have compared miRNA expression profiles in obese and lean white adipose tissue (WAT) from mice and humans. In fat, in cells from mice with diet-induced OB, which is phenotypically similar to OB in humans, 35 of the 574 detected miRNAs were differentially expressed [138]. Microarray screening of human WAT tissues has identified a number of miRNAs that are potentially dysregulated in patients with OB, both in those who also have T2DM and in those who do not have T2DM [139]. However, validation experiments, such as investigations of additional fat-cell models, studies with increased sample sizes and analysis with quantitative RT-PCR have confirmed a role for only a small number of miRNAs in OB [140, 141]. In addition, scattered studies have reported that some miRNAs are dysregulated in plasma samples of OB subjects, including miR-142-3p, miR-221 or miR-222 [29-34]. For example, using TaqMan technology, it was found in a cross-sectional study with 30 obese patients and 20 non-obese control participants that miR-17-5p and miR-132 were reduced in whole blood from obese patients compared with controls [33]; whereas other groups, using miRNA panels in a cross-sectional study with 20 obese patients and 20 control participants, found that miR-138, miR-376a and miR-503 were reduced and miR-15b increased in obese compared with controls [31]; and found that miR-222, miR-486, miR-146b, miR-146a, miR-20a, miR-15b and miR-26b′ were increased in obese compared with controls, using microRNA sequencing in a cross-sectional study with 100 obese patients and 146 normal control participants [28]. Notably, this seeming discrepancy does not exclude an important role for miRNAs in OB, as each miRNA has the potential to regulate the levels of numerous mRNAs. The lack of congruency between findings reported by different studies of miRNAs in OB is, however, difficult

to explain. Across these studies, only miR-221 was consistently found to be differentially expressed in human obese WAT compared with lean WAT; although it is important to note that this miRNA was found to be upregulated or downregulated depending on the study. Technical issues, such as differences in platforms and analysis methods, might, in part, explain the divergence of the reported results [142, 143]. The small sample sizes of the studied cohorts might also contribute to the observed discrepancies, as many miRNAs are not uniformly expressed across individuals. Unfortunately, although several of the previously mentioned studies have reported the dysregulation of different miRNAs in OB condition, to the best of our knowledge, there are no studies describing the whole miRNome using miRNAs-specific arrays.

3. Pathophysiological relationship between Obesity and Prostate Cancer

3.1 Prostate Cancer

Prostate cancer (PCa) is a disease characterized by uncontrolled growth and proliferation of prostate gland cells. The natural history of this tumor pathology implies the progression from benign tissue to prostatic intraepithelial neoplasia (PIN; which is considered a precursor of PCa [144]) and finally PCa, by acquiring numerous alterations at genomic, transcriptomic and epigenetic levels, in addition to other key molecular dysregulations [145]. The vast majority of PCa cases are adenocarcinomas (90%), mainly characterized by the expression of androgen receptor (AR) and the lack of basal-cell markers (e.g. CK5, CK14 and p63) [146]. The other PCa types are presented with very low frequency, including ductal carcinoma, mucinous PCa, prostate sarcomas, signet ring cell PCa and neuroendocrine tumors (NEPC), being all of them associated with poor prognosis [147, 148]. In addition to the histopathological classification, PCa

is also categorized based on the pharmacological response to androgen deprivation therapy (ADT): i) Hormone-sensitive PCa (tumors sensitive to ADT); and ii) Castration-Resistant PCa (CRPC) or hormone-refractory PCa (tumors insensitive to castration [149, 150]). Another factor that increases the complexity of this disease is the intra-tumor heterogeneity of PCa, reflected in its multifocal nature. Indeed, approximately 80% of PCa cases contain more than one focus [151], and each one of them may have different molecular alterations [152].

3.1.1 Epidemiology

Approximately 1.3 million men are diagnosed with PCa every year worldwide [153]. Specifically, PCa represents the cancer type with highest incidence among men in the vast majority of countries (Figure 5). Currently, it is the most frequent cancer type among European men, with a higher prevalence in the North and West of Europe, and an increasing trend in East and South Europe [154]. The variation in PCa incidence seems to be the result of specific screening and early diagnosis programs (further explained in Diagnosis section).

Fortunately, PCa patient survival has been improved during the last decades. The explanation for the increase in this survival rate seems to be the earlier diagnosis (due to screening programs) and the development of more effective treatments [155, 156].

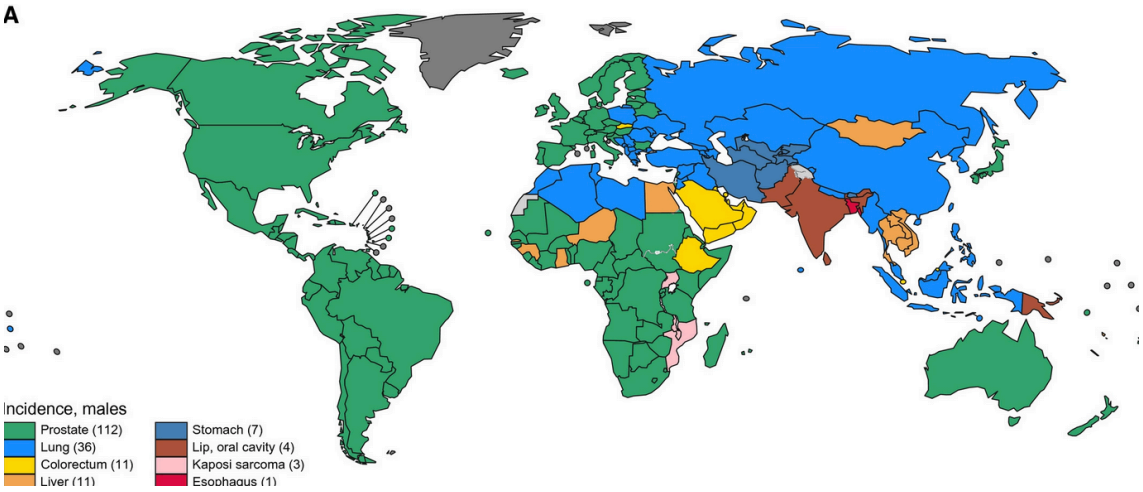

Figure 5. Global map presenting the most common types of cancer incidence in 2020 in each country among men. Source: *Globocan 2020*.

3.1.2 Risk factors

The development and progression of PCa have been associated with certain known risk factors that increase the probability of developing PCa, including aging, family history and race. Specifically, a systematic review by Bell et al. showed that the prevalence of PCa increases from 5% in patients younger than 30 years to 59% in patients older than 79 years [Odds Ratio (OR) = 1.7 by decade of age] [157]. In addition, familiar cases of PCa and the number of affected relatives have been associated with a higher PCa risk [158-160]. In fact, PCa is a tumor pathology with more family cases than breast and colorectal cancers, two malignancies with well recognized familial components [161]. According to an epidemiological study by Lichtenstein et al., approximately 40% of global PCa risk could be due to hereditary factors [162]. Specifically, 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 [163]. Therefore, hereditary PCa has been defined as "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" [161]. In this scenario, the extraordinary molecular heterogeneity of PCa hampers the finding of hereditary genetic alterations associated with PCa risk [164].

Finally, the influence of race on PCa risk has been broadly documented [165]. In particular, African American men have higher PCa risk and more aggressive tumors as compared to Caucasian men (7). In fact, it has been recently reported that, in USA, African Americans are more likely to suffer from PCa than European Americans (2-fold) and Asian Americans (4-fold) [166].

3.1.3 Diagnosis

PCa diagnosis has undergone a remarkable revolution since the establishment of the prostate specific antigen (PSA) as a non-invasive biomarker for PCa screening. PSA is a kallikrein-serine protease involved in the liquefaction of seminal coagulum [167, 168], which is mainly secreted by epithelial prostatic cells [although it has also been detected in nipple aspirate fluid, saliva, and amniotic fluid) [169]]. In 1994, PSA was approved by the Food and Drug Administration (FDA) as a PCa diagnostic biomarker for men older than 50 years. The diagnostic value of PSA was supported by the European Randomized Study of Screening for Prostate Cancer (ERSPC; published in 2014, after 13 years of follow-up), which showed a significant absolute and relative reduction in cancer-specific deaths in the screened cohort [170]. In fact, due to the PSA screening, the majority of PCa patients diagnosed nowadays present tumors with a less aggressive nature (e.g. located in the prostate, without metastasis at the time of the diagnosis) [166]. However, the main disadvantage associated with PSA screening is the overdiagnosis of PCa, with the consequent overtreatment [170]. In addition, other problem associated with PSA test is its low specificity, since plasma levels of PSA can

also be elevated in response to non-tumor conditions, such as benign prostate hyperplasia (BPH), prostatitis and sexual activity, among others [171, 172]. For that reason, nowadays there is not a clear cut-off value of PSA and prostate biopsy is usually required, especially when PSA ranges between 3-10 ng/mL (defined as the "PSA grey zone"). Therefore, as it has been mentioned above, the low specificity of PSA (especially in the grey zone) for detecting PCa cases makes the finding of additional non-invasive diagnostic biomarkers imperative.

Complementary, the digital rectal exam (DRE) is a clinical procedure by which the urologist is able to detect abnormalities in the prostate gland [173]. Specifically, PCa is usually linked to the presence of bumps on the normally smooth surface of the prostate. Previously to the PCa screening using PSA test, PCa was diagnosed after the appearance of symptoms by DRE and biopsy. Nowadays, DRE is considered the main complement for PSA levels to screen PCa. However, it should be noted that DRE diagnostic capability is dramatically reduced when PSA levels are <2 ng/mL [174-176].

Despite the establishments of the PSA test for PCa screening and even if its levels are elevated, a prostate biopsy is always needed to confirm PCa diagnosis. Nowadays, this technique consists on obtaining a core needle biopsy guided by transrectal ultrasound (TRUS). To interrogate the presence of prostate adenocarcinoma in the biopsy, p63 and cytokeratin 5/14 staining are analysed, which are absent in PCa cells [177]. Additionally, the grade of the tumors is evaluated by Gleason score (GS) and tumor–node–metastasis (TNM) systems [178]. Unfortunately, biopsies are associated with adverse effects such as bleeding, pain and infections [179] and, for that reason, the discovery of novel biomarkers with the ability to indicate when a biopsy should be obtained is urgently needed in order to avoid unnecessary biopsies.

GS is a pathological grade reported for the first time by Donald F. Gleason in 1966 [180]. Specifically, GS is based on the grade of histological dedifferentiation (glandular pattern) of the PCa tissue. The glandular pattern ranges from 1 (most differentiated) to 5 (least differentiated) [180]. GS is the sum of the glandular patterns of the most and second-most dominant (in terms of volume) PCa foci. If only one focus 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). Importantly, due to the misleading clinical implications of GS2-4, GS starts at 6 in prostate biopsy and radical prostatectomy specimens [181]. For that reason, PCa with GS6 is commonly known as **non-significant PCa** (NonSigPCa) while PCa with GS≥7 is known as clinically **significant PCa** (SigPCa). The last update of GS criteria was accepted in 2016 by the World Health Organization (WHO) [182, 183]. Briefly, the new International Society of Urological Pathology (ISUP) 2015 Gleason grading 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 [182-184] (Figure 6).

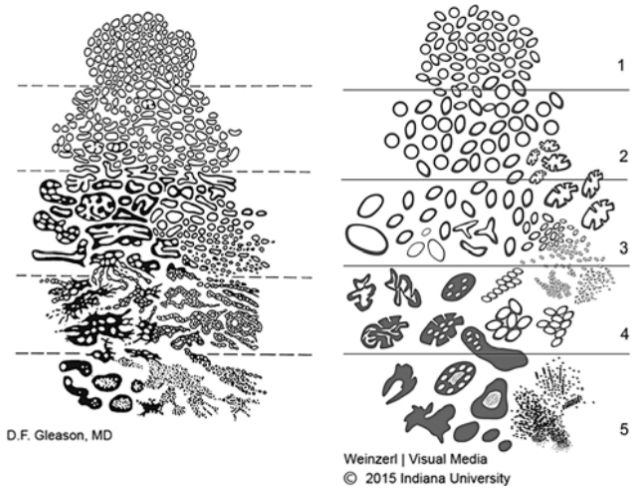

Figure 6. Comparison between the original (left panel) and the 2015 modified ISUP (right panel) Gleason Score schematic diagrams of PCa histologic patterns. Source: *Epstein et al., Am J Surg Pathol 2016*.

The diagnostic ability of PSA is especially questionable in the so-called **grey zone of PSA** (3-10 ng/mL), mainly due to the extremely low specificity of PSA in this range [37]. In addition, the prognostic value of PSA test is also very limited [185]. These drawbacks of the PSA test are associated with overdiagnosis and overtreatment of PCa patients, which could generate severe side effects in a significant percentage of patients [186, 187]. For that reason, the scientific community strive to identify novel PCa biomarkers that: i) could serve as risk indicators for disease with low PSA values (<10 ng/ml); ii) could act as prognostic markers to distinguish indolent from aggressive disease; iii) could predict PCa aggressiveness (prognostic biomarkers); and, iv) may be useful to diagnose metastatic cancer. In this sense, a broad number of novel biomarker panel tests as well as individual biomarkers have been already approved for the FDA or regulated by Clinical Laboratory Improvement Amendments (CLIA).

Specifically, serum based assays targeting kallikreins have been recently developed and proved their efficacy to diagnose PCa, including Prostate Health Index

(PHI) test and the four kallikrein (4K) score test. PHI is based on the levels of total-, free- and pro-PSA, while 4K score is an algorithm including free-, intact- and total PSA and kallikrein-like peptidase 2 (hK2) in addition to clinical data (i.e. Age, DRE and prior biopsy status) [188, 189]. Both tests show similar capacity for detecting PCa (PHI-AUC=0.69 vs 4K-AUC=0.70) [190]. Unfortunately, their specificity remains low (approximately 30%) [191].

On the other hand, the only FDA-approved urine-based test for reducing unnecessary biopsies is PCA3 test [38,39], which is based on the urine levels, after DRE stimulation, of the prostate cancer specific (PCA3) long non-coding RNA [192]. This test has shown high accuracy detecting PCa (sensitivity: 67%; specificity: 83%) [193]. In addition, Michigan Prostate (Mi-Prostate) score is based on the detection in urine of a gene fusion that is commonly present in PCa (*TMPRSS2-ERG*) combined with urine levels of PCA3, resulting in a AUC of 0.76 for detecting PCa and 0.78 for detecting high-grade PCa [194].

3.1.4 Treatment

The type of treatment for PCa patients is determined by a number of factors, including the clinical stage, Gleason Grade, and the patient preferences. The treatments strongly suggested by the European Association of Urology (EAU) Guidelines on Prostate Cancer 2020 according to the stage of the disease are summarized in Table 1.

Table 1. Therapeutic options for prostate cancer

Disease stage	Treatment
Low-risk localised disease **PSA<10 ng/mL and** **GS < 7 (ISUP 1) and cT1-2a**	- Active surveillance - LDR brachytherapy - IMRT
Intermediate-risk localised disease **PSA 10-20 ng/mL or** **GS 7 (ISUP 2/3) or cT2b**	- RP - LDR brachytherapy - EBRT plus ADT (4-6 months)
High-risk localised disease **PSA > 20 ng/mL or** **GS > 7 (ISUP 4/5) or cT2c**	- RP - EBRT plus ADT (2-3 years)
Locally advanced disease **Any PSA, any** **GS (any ISUP grade), cT3-4 or N+**	- RP - Adjuvant EBRT after RP - RT in combination with ADT (2-3 years)
Metastatic disease	- ADT plus docetaxel - ADT plus 2nd generation antiandrogens
Biochemical recurrence	- Salvage RT
Castration resistant disease	- Docetaxel - Abiraterone/Enzalutamide/Cabazitaxel - Radium 223 - Sipuleucel-T

LDR: Low dose brachytherapy; IMRT: Intensity-modulated radiotherapy; RP: Radical prostatectomy; EBRT: External beam radiation therapy; ADT: Androgen deprivation therapy.

3.2 Influence of OB in the development/progression of PCa

OB has been linked to an increased risk of malignancies such as colon, breast, endometrial, renal, gastric, esophageal, pancreatic, liver, and gall bladder in various studies [195, 196]. Several studies have shown the association of OB with the risk of PCa. Specifically, a prospective study of 3673 men in the United States showed that greater BMI) was an independent predictor of PCa (relative risk = 1.7 for BMI > 27.8 kg/m^2 compared with < 23.6 kg/m^2; p = 0.1). The percent change in BMI from baseline to age 50 was also positively associated with higher PCa risk (p = 0.01) [197]. Another prospective study from the United States found that BMI was slightly but positively linked with PCa, and that the link between OB and the risk of clinically relevant PCa was increased once well-differentiated, localized tumors were excluded [198]. However, a prospective study of 36,959 Swedish men found that the incidence of localized PCa was inversely related to BMI in middle-to-late adulthood (the rate ratio

for 35 kg/m^2 when compared to 22 kg/m^2 was 0.69 [95 % confidence interval (CI) 0.52–0.92)], but not in early adulthood. BMI in late adulthood was linked to a non-statistically significant increase in the risk of fatal PCa [rate ratio for every five-unit increase: 1.12 (0.88–1.43)], while BMI in early adulthood was linked to a lower risk of fatal PCa [rate ratio for every five-unit increase: 0.72 (0.51–1.01)] [199]. High BMI at a young age was inversely associated with overall risk of PCa (relative risk = 0.89, 95% CI 0.80–0.98, BMI 26 vs. 20–21.9, p = 0.01) and fatal and advanced disease, according to a prospective study of 141,896 men in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort [200]. A meta-analysis of 12 prospective studies of localized PCa (1,033,009 men, 19,130 cases) and 13 prospective studies of advanced PCa (1,080,790 men, 7067 cases) found an inverse linear relationship with BMI for localized PCa (p = 0.001, relative risk: 0.94 for every 5-kg/m2 increase) [201]. OB may thus have an opposite effect on the incidence of PCa risk in the early stages, depending on the type of PCa. Low testosterone levels in obese individuals could be one of the underlying causes of this inverse relationship between OB and localized PCa. Due to a decrease in luteinizing hormone (LH) pulse amplitude and serum LH levels, obese men have a decreased concentration of free testosterone [202]. The levels of total and free testosterone in the blood have been linked to an increased risk of low-grade PCa [203]. The link between testosterone, free testosterone, and the free-to-total testosterone ratio and PCa is, however, still controversial [204]. Furthermore, the effect of OB-induced systemic inflammation on the negative connection between BMI and localized PCa is uncertain. In this context, although OB has emerged as a high-risk factor associated with the development and aggressiveness of PCa, **the molecular, cellular, and endocrine-metabolic factors and/or mechanisms that underlie the pathophysiological association between OB and PCa are still poorly understood**.

3.2.1 Role of miRNAs in the pathophysiological relationship between OB and PCa

Recent studies have suggested a relationship between circulating miRNAs and PCa presence and outcome [40-42]. Specifically, recent studies have identified some miRNAs differentially present in plasma samples from PCa patients; however, only some of them seem to be specifically derived from PCa tissues [43-45]. Moreover, although various studies have identified some putative PCa-specific miRNAs (e.g. miR-141 [43-45], miR-375 [40, 47], and miR-21 [48]), unfortunately there is not a consensus in the utility of plasma miRNAs as circulating non-invasive biomarkers for PCa. In fact, to the best of our knowledge there are no studies describing the dysregulation of the whole miRNome in PCa vs. healthy patients. For these reasons, this field requires further investigation in order to ascertain whether a specific miRNA or a plasma/serum miRNA signature could be associated with PCa risk and provide diagnostic and prognostic value through a fast, easy, and non-invasive test.

In addition, it should be mentioned that the metabolic status of the patients has not been considered in the previous studies in order to define a diagnostic and prognostic model based on miRNAs expression profile. This suggests the necessity of considering the metabolic status of the patients, especially the OB condition, when developing and validating new biomarkers to improve the diagnostic and/or prognostic models in PCa. As mentioned above, only few recent studies have analyzed the role of miRNAs in the pathophysiological relationship between OB and PCa. In this scenario, it has been found that an increase in the expression levels of miR-301a due to hyperglycemia promotes cell proliferation by repressing p21 and Smad4 in PCa cells [205]. In addition, the level of miR-221-3p has been shown increased in bloodstream of PCa patients compared to healthy donors. This alteration might be associated to an

increase in the expression levels of miR-221-3p in WAT of mice inoculated with TRAMP-C1 PCa cells and fed with HFD [206].

Despite of all the information presented in the introduction of this Doctoral Thesis, and the advances acquired in the field over the last years, it is essential to precisely unveil the molecular, cellular and endocrine-metabolic events that underlie the development and progression of OB and PCa, as especially those involved in the pathophysiological association between both pathologies. In this context, some components of the inflammasome machinery as well as of the miRNome could represent important elements that might be useful as novel diagnostic, prognostic and therapeutic targets.

Aims of the study

The **GENERAL AIM of this study** was to further expand our cellular, molecular and clinical knowledge of two highly relevant pathologies, obesity and PCa, and further understand the pathophysiological association between them, by determining the pattern of dysregulation, and the associated underlying mechanisms, of key miRNAs and components of the inflammasome machinery in patients with obesity and/or PCa. The ultimate goal is to discover novel and personalized biomarkers and therapeutic tools to improve the diagnosis, treatment and management of these pathologies.

To achieve this main aim, we proposed the following **SPECIFIC OBJECTIVES** (SO), which have been devised to explore:

SO1) The presence and changes in components of the inflammasome machinery (i.e., inflammasome components and inflammatory-associated factors) in obese patients after bariatric surgery and their relation with clinical/biochemical parameters at baseline and after bariatric surgery;

SO2) The differential expression pattern of miRNAs in obese patients and their putative role in obesity-related comorbidities such as insulin resistance;

SO3) The dysregulation of the human miRNome in PCa patients, considering the obesity condition of the patients (normoweight, overweight, or OB).

SO4) The potential pathophysiological role and the underlying molecular mechanisms of miR-107 in PCa as well as their implication in the pathophysiological relationship between obesity and PCa.

Results and general discussion

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

3.1 Section I: Dysregulation of inflammasome components after bariatric surgery: novel targets for a chronic disease

Inflammasome has been linked to a variety of autoinflammatory, autoimmune and metabolic disorders including atherosclerosis, T2DM, and OB [23]. However, changes in inflammasome components induced by acute weight loss related to weight loss after bariatric surgery have not been fully described yet. In this context, it has been reported that inflammasome-induced cytokines promote OB-related inflammatory diseases following the stimulation by high-fat diet metabolites and adipose tissue changes that occur during weight gain [24], which suggests that inflammasome modulation due to weight loss may be associated to an improvement in OB-related comorbidities.

In the present study, **we have comprehensively evaluated the presence and changes in relevant components of the inflammasome machinery and associated elements after bariatric surgery** in a well-characterized cohort of obese patients, who underwent one of the two most common bariatric surgery procedures: RYGB and SG. Additionally, **their relationship with relevant baseline and follow-up anthropometric and biochemical characteristics was studied**.

To the best of our knowledge, this is the first report demonstrating that the expression of important inflammasome components is drastically dysregulated after bariatric surgery (especially NLRP12, NLRC4, TLR4, CCL8, and CXCL3). Our results

demonstrate that the expression of most NLRs is decreased 6 months after bariatric surgery, especially NLRP12. According to previous publications, NLRP12 seems to play an important role in reducing high-fat diet–induced OB by maintaining beneficial microbiota [207], and it also maintains colon microbiome diversity, which decreases colon inflammation and promotes specific microbes that reverse gut inflammation in chronic bowel inflammation diseases [208]. Recently, an antitumorigenic effect of NLRP12 has been also described by downregulating JNK activation in mouse hepatocytes [209]. JNK is activated by high-fat diet and OB, and its inhibition maintains insulin sensitivity [210]. NLRP12 is also associated with metabolic-induced cancer and chronic inflammatory diseases [211], suggesting that its role as target in the treatment of OB-induced comorbidities may be relevant and requires further attention. Importantly, our data demonstrate that NLRP12 was strongly decreased in PBMCs of patients after bariatric surgery, especially in women, and was significantly correlated with fat mass and abdominal perimeter after bariatric surgery, but not with the presence of T2DM before bariatric surgery. Thus, these data may be clinically relevant based on the critical metabolic role of NLRP12 as previously discussed.

Additionally, NLRC4 was strongly decreased after bariatric surgery in the PBMCs of our cohort of patients, and its expression was positively associated with fat mass and negatively associated with excess of BW loss. Regarding this, previous studies have reported direct actions of NLRC4 in adipocytes, where it can induce the activation of IL-1 signaling, promoting angiogenesis, and even breast cancer progression [212]. It is well known that bariatric surgery is associated with decreased risk of endocrine-related cancers, but the specific mechanisms are not well understood [213]. Therefore, based on the results of this study, we may speculate that one of these putative mechanisms may be related to the changes in this inflammasome component.

Another explanation for the observed changes in NLCR4 is gut microbiota. It is well-known that significant changes in the microbiota occur after bariatric surgery due to changes in the metabolism of bile acids, hormones, and gastric pH [214, 215]. Changes in the microbiota are related to the bariatric surgery procedure [216], in concordance with our results, since we observed significant differences in the inhibition of NLCR4 induced by RYGB and SG. However, further studies that include gut microbiota analysis should be performed.

The effect of bariatric surgery in liver disease is controversial. Some studies describe significant improvement in steatosis in nonalcoholic steatohepatitis, but changes in fibrosis are controversial, since both significant improvement and worsened condition have been described [217, 218]. Even more, liver disease is not a routine indication for bariatric surgery in patients with OB [219]. In this context, we evaluated the *in vitro* silencing of the expression of NLRC4 (and NLRP12) in liver cells, which resulted in increased lipid accumulation despite their association with decreased fat mass and excess body weight loss. These findings suggest that the modulation of inflammasome components may be cell dependent, as previously described [220], and the specific effects in liver cells should be evaluated separately. Related to this, NLRP3, NLRP6, and IL-18 can negatively modulate NAFLD progression in humans [221, 222] and represent important targets for this relevant OB-related comorbidity. Results about NLCR4 and NLRP12 are still needed.

TLR4 signaling pathway is recognized as a main trigger of OB-induced inflammatory response [223]. TLR4 is expressed not only in innate immune cells but also in other cell types, including adipocytes [223]. Additionally, it has been suggested that saturated fatty acids can also bind to TLR4 and activate TLR4-mediated signaling pathways [224], representing an additional link between inflammasome and OB [223].

Remarkably, this relation was observed in our study when post-surgery TLR4 expression levels were correlated with post-surgery BW. Moreover, correlation between TLR4, dyslipidemia, or lipid profile before or after bariatric surgery was observed. Importantly, TLR4 signaling is increased specially in nonalcoholic steatohepatitis compared to NAFLD [225]. In this sense, since we did not include patients with nonalcoholic steatohepatitis, this may explain the negative correlation between baseline TLR4 levels and liver enzymes serum values.

Previous studies have reported that CCL8 is increased in obese patients compared to lean subjects and in obese patients with insulin resistance compared to noninsulin resistant patients, and it is associated with other proinflammatory cytokines in adipose tissue [226, 227]. In concordance with this, CCL8 levels decreased 6 months after surgery and were correlated with body weight 6 months after surgery. Remarkably, CCL8 levels were not associated with the presence of dyslipidemia at diagnosis or its reversal. In this sense, further studies should be performed to confirm our results and to determine the clinical relevance of CCL8 in patients with metabolic syndrome.

Besides CCL8, it has been reported that CXCL3 is widely expressed in human adipocyte tissue, showing a higher expression level in preadipocytes compared with mature adipocytes, and it seems to regulate adipocyte differentiation possibly through the JNK pathway activation [228]. This fact may explain its correlation with baseline excess of body weight, but in our study, CXCL3 expression was significantly increased after bariatric surgery and negatively associated with baseline dyslipidemia and postsurgical HT. Additionally, a positive correlation between its postoperative expression levels and the initial excess of body weight was also observed. Therefore, although further studies would be required to determine the precise role of CXCL3, all

these findings may suggest the idea that this chemokine may have a protective role for metabolic comorbidities.

Furthermore, in our cohort, T2DM was associated with the expression of some inflammasome components including CCL2, CXCR1, and SIRT1, which may have important clinical implications. These findings, specifically related to CCL2 and CXCR1, were confirmed in a second cohort of patients. It has been described that CCL2 is expressed by insulin-producing cells, and it can produce insulitis and, consequently, islet destruction [229]. Additionally, CCL2 has been associated to diabetes induced kidney injury [230] and delayed wound healing [231], suggesting a role as putative target for preventing diabetes-related complications. Interestingly, the inhibition of CXCR1 has been suggested as a putative mechanism for reversing type 1 diabetes according to animal models [232, 233]. In this context, some CXCR1/2 inhibitors are currently being evaluated for treating inflammatory diseases including diabetes and several types of cancer [234, 235].

Remarkably, in our study, SIRT1 levels were also elevated in patients with T2DM, and its response to bariatric surgery seemed to be dependent on the presence of T2DM. In T2DM patients, the increase in the expression levels of SIRT1 induced after bariatric surgery may be associated, at least in part, to a downregulation in the expression of specific regulators, such as microRNA 448, which has been demonstrated by other groups [236]. Recently, another study revealed that kallistatin, whose circulating levels were increased in obese patients after RYGB, is implicated in the upregulation of SIRT1, triggering a downregulation of many inflammatory key genes [237]. These findings demonstrate that SIRT1 may have a relevant role in the regulation of many inflammatory components, especially under obese conditions. Importantly, previous publications suggest that some inflammasome components are differentially

expressed in patients with T2DM despite weight loss after a bypass surgery, suggesting a persistent inflammatory state in this population [238], which explains that some evaluated components remained unchanged after bariatric surgery in our cohort.

Previous publications suggest that AIM2 is associated with atherosclerosis and vascular disease [239, 240]. Additionally, mitogen-activated protein kinase 14 has been suggested as a cardiovascular prognostic marker [241]. Accordingly, in our cohort we observed increased levels of these components in patients with high blood pressure, suggesting a putative role of these inflammasome components in the reversion of cardiovascular events after weight loss induced by bariatric surgery.

Importantly, previous reports have described the link between NLRP3 and OB-associated comorbidities [23, 24]; even a significant reduction in NLRP3 expression levels in adipose tissue after bariatric surgery has been reported [241]. In our cohort, this finding was not confirmed, and, in contrast, a tendency to an increased expression of NLRP3 was observed; however, we could not evaluate this component in adipose tissue, which may explain the differences observed in both studies.

Additionally, circulating interleukins levels also reflected the mRNA expression of some inflammasome components, suggesting an appropriate correlation between molecular and circulating markers as has been previously described in humans [242, 243]. Importantly, the persistent levels suggest that despite weight loss 6 months after bariatric surgery, the OB-related inflammation state persists in these patients and that serum reductions would require increased body weight loss as has been previously observed in other studies [244].

Taken together, our results unveiled new conceptual and functional avenues in the OB/ bariatric surgery field, with potential clinical implications, by demonstrating for

the first time a clear dysregulation of key components of the inflammasome machinery (especially NLRP12, NLRC4, TLR4, CCL8, and CXCL3) in obese patients after bariatric surgery, which may be closely related to improvement and remission of OB-related comorbidities. Specifically, our data suggest that these specific components of the inflammasome machinery may play an important role as markers for predicting OB-related comorbidities and represent putative targets for reversing OB-related complications. Moreover, we unveil novel evidence for a critical role of some NLRs (especially NLRC4) in pathophysiological processes, such as lipid accumulation, cell viability, and apoptosis rate. Although these results should be confirmed in larger cohorts, including patients of different races and may also be reinforced, for instance, by the inclusion of gut microbiota analysis and by the evaluation of different metabolic tissues and further *in vitro* studies, our study provides solid, convincing evidence demonstrating that some components of the inflammasome machinery are associated and may play a critical pathophysiological role in the improvement and remission of OB-related comorbidities observed in the present study, offering a clinically relevant opportunity for novel targets that should be tested in humans.

These results have been published in "The journal of Clinical Endocrinology & Metabolism" (Article I of this Doctoral Thesis).

3.2 Section II: Influence of OB in the miRNome: miR-4454, a key regulator of insulin response via splicing modulation in prostate

Understanding the molecular mechanisms involved in the onset of OB is a critical objective for the scientific community, in that OB is a chronic metabolic disease of pandemic level associated with important comorbidities such as insulin resistance, T2DM, dyslipidemia, cardiovascular disease, etc., and with an increased risk of death [245]. Herein, **we explored the dysregulation of the whole miRNA landscape in human plasma samples**, discovering that miR-4454 levels are higher in OB, associated with relevant metabolic parameters such as insulin or HOMA index, and modulated by pharmacological (metformin, statins) or medical (bariatric surgery) interventions. In addition, our *in silico* and *in vitro* data also suggest that expression of miR-4454 may be related to the cellular response to insulin in endocrine-related organs such as the prostate [29, 246, 247], inasmuch as miR-4454 is associated with a dysregulation in key elements of the insulin receptor signaling pathway and with the inhibition of key cellular signaling pathways such as AKT and AMPK. Interestingly, we also show novel evidence demonstrating that the cellular and molecular alteration in response to insulin triggered by miR-4454 might be associated to a dysregulation in the splicing process and with the alteration of metabolically-relevant splicing isoforms, such as those generated from the insulin receptor gene, isoforms that have associated with the development of different pathologies, such as insulin resistance, T2DM, OB, atherosclerosis and cancer [117].

In particular, dysregulation of the expression pattern of miRNAs in OB was first explored by using an array based on Affymetrix technology. Results from the analysis of the whole miRNome demonstrated a significant dysregulation of the plasma levels of

26 miRNAs, wherein 7 of them exhibited capacity to discriminate between normoweight and control subjects. Among them, only the reduced levels of miR-21 have been also previously found in OB conditions in a cross-sectional study with 45 patients with T2DM and 42 control participants [248]. Importantly, it should be mentioned that only the dysregulation of miR-4454 could be corroborated by qPCR in two ampler sets of subjects (n=221 and n=20, respectively). Therefore, this is the first study demonstrating that the levels of miR-4454 are increased in plasma from obese patients compared with control subjects in three independent cohorts of subjects. Actually, the functional implications of this miRNA have only been explored to date in certain tumor pathologies such as bladder cancer [249] and metastatic melanoma [250, 251]. However, consistent with our study, alterations in the expression of miR-4454 have also been described in OB-related comorbidities including cardiovascular disease [252] and loss of β-cell function [253], but its dysregulation in OB and its putative pathophysiological role had not been reported until now.

Interestingly, plasma levels of miR-4454 were found to be associated with HOMA-IR, a crucial element in OB, used in many studies to define metabolically healthy OB [6, 254]. Intriguingly, circulating levels of miR-4454 were associated with insulin level but not with glucose level, suggesting a pathophysiological relationship between this miRNA and OB-associated comorbidities, such as hyperinsulinemia. This is further supported by the fact that the plasma levels of this miRNA were drastically lower in subjects treated with drugs commonly used to control certain OB-related complications, such as metformin or statins, in comparison with their control (non-treated) groups. In this sense, it should be mentioned that, nowadays, many studies have associated metabolic treatments with pathological alterations triggered by miRNAs [255-257]. Moreover, plasma levels of miR-4454 were also downregulated in patients

that underwent bariatric surgery, the most effective treatment for maintaining long-term weight loss in severe OB [10]. Altogether, these data demonstrate for the first time that miR-4454 is tightly modulated under obese conditions and that it may be implicated in the pathophysiology of OB or its associated comorbidities, such as hyperinsulinemia and insulin resistance. Indeed, although further studies would be necessary before reaching a precise and unequivocal conclusion in this regard, the data reported herein with miR-4454 together with previous observations indicating that specific miRNAs could be used as non-invasive diagnostic/prognostic biomarkers as well as therapeutic options in different endocrine-related pathologies [31, 258, 259], invite to suggest that miR-4454 could represent a promising and valuable tool in order to develop novel and predictive diagnostic/prognostic biomarkers and/or therapeutic tools for insulin resistance.

Interestingly, a screening of the expression levels in human tissues demonstrated that miR-4454 is highly expressed in liver and prostate, two endocrine organs strongly influenced by metabolic factors, including insulin, one of the hormones that exerts some of the most prominent physiological effects in both tissues [29][260]. In addition, the expression levels of miR-4454 in prostate and liver cells (RWPE-1 and HepG2, respectively) were altered when stimulated with a high dose of insulin, mimicking the hyperinsulinemia conditions observed in OB subjects. In striking contrast, expression levels of this miRNA were not altered in response to high dose of glucose, reinforcing the clinical association found herein, and indicating a positive correlation between miR-4454 and insulin, but not glucose, in human plasma samples, which further suggests that the expression of this miRNA may be regulated by insulin and linked to its levels.

In the same line, our *in silico* and *in vitro* studies demonstrated that miR-4454 is implicated in the cellular response to insulin in an organ or cell-type dependent manner.

Indeed, *in silico* GO analysis demonstrated that its target genes are involved, among others, in the insulin receptor signaling pathway. This result reinforces the hypothesis that miR-4454 is tightly associated with metabolic disorders such as OB and especially with the insulin metabolism. This may add some new clues to other studies demonstrating that the control of the metabolic homeostasis and, especially, the cellular response to insulin, is one of the pathways most regulated by miRNAs, including miR-7, miR-26a or miR-107 [261-263]. In particular, the *in vitro* overexpression of miR-4454 induced a significant dysregulation in key genes (insulin receptors and GLUT4) and pathways (Akt, AMPK and ERK) involved in insulin signaling in prostate, but not in liver, cells. These changes found in prostate (but not in liver cells) could be explained by the sophisticated molecular mechanism of regulation of miRNAs. Transcription of miRNA genes is regulated similarly to that of protein-coding genes, and is a major level of control responsible for tissue-specific or development-specific expression. In keeping with this, some miRNAs can exhibit different functions depending on the tissue in which they act due to the transcriptional regulatory network present in each cell type [30, 264]. As mentioned above, miR-4454 overexpression in prostate cells was associated to a decrease in the expression of *INSR* and *GLUT4*. GLUT4 is a glucose carrier entirely dependent upon insulin [265] and, although the role in the insulin-induced glucose uptake by adipose and muscle tissues and its regulation by several miRNAs have been characterized [259, 262], no previous studies have demonstrated this molecular association in the prostate gland.

Furthermore, a decrease in the phosphorylation levels of key insulin-related signaling pathway components was observed after the overexpression of miR-4454. In particular, AMPK regulates insulin homeostasis by reducing the phosphorylation of mTOR in the cytoplasm and also controls the glucose uptake by increasing expression

and translocation of GLUT4 [258]. This transcriptional regulation of GLUT4 by the activation of AMPK could also explain the reduction of the expression of this solute carrier in the cells overexpressing miR-4454. Moreover, the phosphorylation of AKT, the master downstream regulator in the insulin response, was also reduced. AKT activation also increases glycogen synthesis (through GSK-3 inhibition), protein synthesis (via mTOR signaling) and cell survival by the inhibition of pro-apoptotic factors, including Bad and FOXO transcription factors [266, 267]. Concerning ERK pathway, ERK1 deficient mice are protected against diet-induced OB and insulin resistance owing to decreased adipogenesis and elevated energy expenditure [268, 269]. Therefore, the overall dysregulation in these genes and signaling pathways might be associated with the pathophysiological alterations of some of the OB related comorbidities, such as hyperinsulinemia and hyperglycemia. Altogether, these results suggest that miR-4454 is modulated in response to insulin in prostate cells and that it can also regulate key genes and pathways involved in the cellular response to insulin, generating a regulatory feedback between insulin and miR-4454.

A particularly relevant finding of this study is that, while other miRNAs have been associated with the reduction of insulin receptor expression [270-272], the reduction of the expression of *INSR* triggered by miR-4454 is also associated herein with a dysregulation in the ratio between both *INSR* splicing isoforms (IRA and IRB). The differential physiological and pathological role of both isoforms is not completely known, but it has been demonstrated that their role are probably determined by the different binding affinity for insulin-like growth factors [272]. IRB is more abundant in adult tissues and it exerts mainly the metabolic actions of insulin, whereas IRA is mainly expressed in fetal and prenatal period and exerts mitogenic actions [272]. An

increase in the IRA/IRB ratio has been shown in conditions of insulin resistance in different insulin target tissues [273]. This capacity of miR-4454 to modulate the alternative splicing of the INSR gene is in complete agreement with the *in silico* prediction of the involvement of this miRNA with the modulation of RNA metabolism and splicing, including the regulation of the spliceosome, and could be of pathophysiological relevance, inasmuch as the control of the splicing process has been demonstrated to be tightly related to the insulin signaling pathway [117, 273, 274]. Of particular importance is the regulation of the ratio between both isoforms of the insulin receptor (IRA and IRB), which has been associated to different metabolic disorders such as T2DM [272] and also with several cancers types [275, 276].

Consistent with the previous idea, our *in vitro* studies revealed that overexpression of miR-4454 severely dysregulated several spliceosome components (*SNRNP200, RNU2* and *U2AF1*) and splicing factors (*CELF1, ESRP1, ESRP2, RBM8A, RBM45, SND1, SRRM4, SRSF9* and *SRSF10*) in prostate cells, wherein their expression levels correlated well with those of the total levels of IRS and with the expression of the splicing variant *IRB*, suggesting a causal association. Indeed, some of the spliceosome components and splicing factors found here to be controlled by miR-4454 have been implicated in different processes associated to the development or progression of OB and its related comorbidities, including cancer [277]. Namely, alterations in *ESRP1, SNRNP200* and *RNU2* could augment the risk of developing T2DM [278], dysregulation of the expression levels of *ESRP2, RBM45, SND1, SRSF10* and *U2AF1* have been observed in the liver of obese patients with steatosis [279], and SRSF10 has been described as a regulator of the production of lipin1a promoting adipocyte differentiation [280]. And more importantly, a previous study has proposed that the association of specific splicing factors such as CELF1 and SRp20 acts antagonistically

regulating INSR alternative splicing (causing exon skipping and increasing exon inclusion respectively) [281], which further support our results. Although more studies are necessary to better understand the cellular and molecular implication of miR-4454 as a regulator of the splicing process, it is clear that the dysregulation of the expression pattern of these splicing factors and spliceosome components trigger defects in the processing of splicing variants, including IR isoforms, and could, consequently, be involved in important metabolic disorders such as OB and T2DM [278, 279]. Therefore, these observations unveiled new conceptual and functional avenues, with potential therapeutic implications, which are worth exploring in future studies.

In conclusion, this study represents the first demonstration that the circulating levels of miR-4454 are increased in OB, associated with key clinical parameters (e.g. insulin levels, HOMA-IR), and modulated by OB-controlling interventions (metformin/statin treatment and bariatric surgery). Moreover, *in vitro* data revealed that miR-4454 is modulated by insulin and can impair the cellular response to insulin, in a cell type-dependent manner, through the modulation of the splicing process and important signaling pathways (Akt, AMPK and ERK). Altogether, our results provide new, compelling evidence supporting the contention that miR-4454 represents a promising diagnostic, prognostic and/or therapeutic tool, worth to be further explored, in OB and associated comorbidities, such as hyperinsulinemia and T2DM.

These results have been published in "The Journal of Clinical Endocrinology & Metabolism" (Article II of this Doctoral Thesis). .

3.3 Section III: Dysregulation of the miRNome unveils a crosstalk between OB and prostate cancer: miR-107 as a personalized diagnostic and therapeutic tool

Plasma PSA levels remain the current gold standard biomarker to diagnose PCa, which represents one of the tumors types with the highest incidence worldwide [282]. Unfortunately, PSA continues to show important limitations (especially in the range of 3–10 ng/mL, also named the "grey zone"), including compromised specificity, inasmuch as non-tumor conditions (e.g., infections, inflammation) can also increase PSA levels. Therefore, considerable research efforts have been focused on the identification of novel biomarkers that could complement or even replace plasma PSA in order to improve the diagnosis of PCa. In this context, microRNAs (miRNAs) have emerged as potential sources of non-invasive diagnostic biomarkers in several pathologies. Therefore, **this study was aimed at assessing for the first time the dysregulation of the whole plasma miRNome in PCa patients and its putative implication in PCa from a personalized perspective (i.e., OB condition)**.

In particular, our results from the analysis of the whole miRNome demonstrated a significant dysregulation of the plasmatic levels of 104 miRNAs, wherein 6 of them exhibited a great capacity to discriminate between PCa patients and healthy volunteers. The dysregulation of these miRNAs in PCa patients further supports previous data reporting the alteration of some of these miRNAs, including miR-107, in certain studies [41, 42, 283]. However, any of these studies have explored its putative role as diagnostic biomarkers as well as prognostic or therapeutic tools. Interestingly, the dysregulation of miR-107 and miR-191-5p was corroborated by qPCR in a second, ampler and independent cohort of subjects (n=295). The fact that the dysregulation of

other miRNAs was not corroborated may be explained by the fact that control patients from this ampler cohort are patients with suspect of PCa but negative results in the biopsy instead of healthy volunteers, as used in the first cohort. Nonetheless, we demonstrated that the diagnostic capacity of plasma miR-107 levels to discriminate between tumor and control patients significantly outperformed that of PSA levels. Most importantly, this improvement in the diagnostic ability of miR-107 persists when comparing only patients in the grey zone (wherein the capacity of PSA is extremely low). Remarkably, although miR-107 levels were higher in SigPCa patients compared to controls, these levels did not allow to discriminate between SigPCa and NonSigPCa patients; however, miR-107 levels correlated with relevant oncogenic parameters such as PSA levels [284], tumor volume [285], testosterone levels [286], and CRP [287] in SigPCa patients but not in NonSigPCa patients reinforcing also a putative prognostic capacity of plasma miR-107 levels.

Despite miR-107 plasma levels are increased in PCa patients, this miRNA exhibits an apparently controversial reduced expression in PCa tissue samples compared to their non-tumoral adjacent tissues as well as in all PCa cell lines analyzed compared to a non-tumor prostate cell line. These results are consistent with a previous study, which also suggests a tumor suppressor role of miR-107 in PCa cells [288]. However, it has not been demonstrated the reason why the levels of this miRNA increase in plasma samples from PCa patients while their levels are reduced in PCa cells. Here, we found that this discrepancy could be possibly explained by the higher capacity of PCa cells to secrete miR-107 to the extracellular medium compared to control prostate cells (possibly through a direct extracellular vesicle release and/or by a PCa-derived exosomal miRNA release), resulting in a lower level of miR-107 in PCa tissues compared with the plasma levels. These results are in line with other studies

demonstrating the same cellular phenomenon in the case of other miRNAs [289, 290]. Moreover, this is also consistent with the previously suggested tumor suppressor role of miR-107 in PCa [288] which was corroborated in the present study. Indeed, when miR-107 is overexpressed in androgen-independent PCa cells (i.e. DU145), several oncogenic features, including cell proliferation, migration and tumorosphere formation, were significantly reduced. However, it should be noted that the overexpression of miR-107 in an androgen-dependent PCa cell line (i.e. LNCaP), triggered an increase in cell proliferation and tumorosphere formation. These results obtained from androgen-dependent vs. androgen-independent PCa cells have not been reported before and suggest the idea that miR-107 could play a different endogenous role in PCa cells depending on the degree of tumor aggressiveness, maybe due to the sophisticated molecular mechanism of regulation of miRNAs in different cell types [27]. Therefore, although differential responses in androgen-dependent vs. androgen-independent PCa cells have been previously reported [291-293], this particular phenomenon observed in our study warrants further investigation in order to unveil its clinical implication.

Interestingly, more profound *in silico* (KEGG analysis) and *in vitro* studies demonstrated that miR-107 is implicated in the modulation of fatty acid metabolism, one of the main energy pathways used by tumor cells and whose dysregulation represent a hallmark of PCa [294]. In this sense, we observed several transcriptional dysregulations in genes implicated in this pathway in response to the overexpression of miR-107 in LNCaP and DU145 such as *ACADSB*, *ACADVL*, *CPT2*. Remarkably, only the expression level of *FASN*, the main driver of fatty acid metabolism [295], was decreased at mRNA level in both cancer cell lines. However, it should be noted that the protein levels of FASN were decreased in the androgen-independent PCa cell line DU145, while its levels were increased in LNCaP. Notably, these alterations could

explain, at least in part, the differential functional *in vitro* results previously discussed in DU145 vs. LNCaP cells, as FASN is a well-known oncogenic driver [296, 297]. Interestingly, the alteration in the expression of *FASN* (at mRNA and protein levels) observed in both PCa cell models in response to the overexpression of miR-107 was not found in the normal-like prostate cell model (PNT2). These results might suggest that the association of miR-107 with the cellular lipid metabolic process could be specific of PCa cells which reinforce the idea that this metabolic cellular process could be one of the main energy pathways used by prostate tumor cells to enhanced their agresiveness features.

Likewise, the *in silico* GO analysis revealed that miR-107 could be also associated with another relevant cellular process, the RNA-splicing, which has been recently reported by our laboratory to be tightly implicated in PCa aggressiveness [298]. In support of this idea, we found that several relevant splicing-related genes (such as *PTBP1*, *SRRM1*, *SRSF6*) were significantly altered in response to miR-107 overexpression in PCa cells. Although more studies are necessary to better understand the cellular and molecular implication of miR-107 in PCa, our results clearly suggest that the dysregulation of the expression of this miRNA triggers defects in the splicing process but especially in the fatty acid metabolism (possible due to the dysregulation of FASN), which are two relevant cellular/molecular processes that represent hallmarks of cancer [299, 300]. Therefore, these observations unveiled new conceptual and functional avenues, with potential therapeutic implications, which are worth to be explored in future studies.

Finally, our study also revealed that miR-107 is tightly associated with obese condition in patients, which suggests its putative role as a personalized diagnostic biomarker based on the obese status of the patients. In this sense, it should be

emphasized that PCa is strongly influenced by metabolic dysregulations including OB [301, 302]. Particularly, we observed that the diagnostic capacity of miR-107 significantly increased together with the increase of BMI, reaching its higher AUC value when comparing obese PCa patients vs. obese control patients. Furthermore, miR-107 significantly outperformed the diagnostic capacity of the gold standard PSA, especially when considering patients in the grey zone of PSA. Indeed, in the case of patients with OB, miR-107 did not only discriminate between tumor and non-tumor patients but also between NonSigPCa and SigPCa, which further reinforce its potential value as prognostic biomarker for this pathology.

In conclusion, this study represents the first demonstration that the plasma levels of miR-107 might represent a useful personalized diagnostic biomarker of PCa since its levels are increased in plasma from PCa patients compared with control subjects using two independent cohorts. In addition, we found that plasma miR-107 levels are associated with key oncogenic parameters such as PSA levels, tumor volume, testosterone or CRP levels suggesting that high plasma miR-107 levels could also be related to PCa aggressiveness and progression. Moreover, *in vitro* and *in silico* data revealed that miR-107 is implicated in the regulation of the splicing process and the fatty acid metabolism, altering the expression of the main driver FASN, resulting in a reduction of aggressiveness features in androgen-independent cells. Interestingly, this study also shows novel evidence demonstrating that miR-107 could represent a promising personalized biomarker for PCa, in that miR-107 levels were higher in plasma from obese patients with PCa compared to PCa patients with normoweight. Indeed, miR-107 also allowed a strong discriminatory capacity between SigPCa and NonSigPCa in obese PCa patients, thus representing not only a diagnostic but also a potential prognostic biomarker in that an OB condition has been reported to represent a

risk factor for PCa development, aggressiveness and mortality [303, 304]. Altogether, our results provide new, compelling evidence supporting the contention that miR-107 represents a promising diagnostic, prognostic and/or therapeutic tool, worth to be further explored, in the pathological association between PCa and OB.

These results have been published in "Molecular Therapy Nucleic Acids" (Article III of this Doctoral Thesis). .

General conclusions

The **MAIN CONCLUSIONS** associated to each section/article of this Doctoral Thesis are described below:

Article I:

1) Bariatric surgery induces a profound alteration in the gene expression pattern of key components of the inflammasome machinery in PBMCs of obese patients, which is associated to baseline metabolic comorbidities, including type 2 diabetes, and may be closely interconnected to the improvement and reversion of some obesity-related comorbidities after bariatric surgery.

2) Specific components of the inflammasome machinery (especially NLRP12, NLRC4, TLR4, CCL8, and CXCL3) may play an important role as markers for predicting obesity-related comorbidities, and might represent putative targets for reversing obesity-related complications.

Article II:

3) Obesity is associated to a drastic alteration of the plasmatic miRNA landscape, wherein miR-4454 levels are higher, associated with insulin-resistance (HOMA index and insulin levels) and modulated by obesity-controlling interventions [medical (metformin and statins treatment) and surgical (bariatric surgery)].

4) The expression of miR-4454 may be related to the cellular response to insulin in endocrine-related organs such as the prostate, inasmuch as miR-4454 is associated with a dysregulation of metabolically-relevant splicing isoforms (e.g. insulin receptor), and with the inhibition of key cellular signaling pathways (insulin, AKT AMPK and ERK),

5) miR-4454 might represent a promising and useful diagnostic, prognostic and/or therapeutic tool in obesity and associated comorbidities, such as hyperinsulinemia and T2DM.

Article III:

6) PCa is associated with a profound dysregulation of the whole miRNome, wherein plasmatic levels of 104 miRNAs were altered in PCa patients compared with control patients, representing a source of novel biomarkers.

7) miR-107 represents a novel and useful diagnostic and prognostic biomarker and a potential therapeutic tool for PCa, since its levels are increased in plasma from PCa patients compared with control subjects, and are associated with key oncogenic parameters (e.g. PSA levels, tumor volume, testosterone or CRP levels). In addition, miR-107 is implicated in the regulation of the splicing process and the fatty acid metabolism, altering the expression of the main driver FASN.

8) miR-107 levels also represents a novel and useful personalized diagnostic and prognostic biomarker in the pathophysiological association between obesity and PCa, since its levels are increased in plasma from obese with PCa compared to PCa patients with normoweight, and allowed a strong discriminatory capacity between SigPCa and NonSigPCa in obese patients.

Altogether, the overall results of this Doctoral Thesis demonstrate that **the dysregulation of certain miRNAs** (especially miR-4454 and miR-107) **and key components of the inflammasome system** (especially NLRP12, NLRC4, TLR4, CCL8, and CXCL3) **could contribute to the development and progression of OB and PCa, and might be tightly implicated in the pathophysiological association between both**

pathologies, representing a source for novel and personalized diagnostic, prognostic and therapeutic targets that could be used to improve the management and treatment of patients with these devastating pathologies.

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ARTICLE I

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Clinical Research Article

Clinical Research Article

Dysregulation of Components of the Inflammasome Machinery After Bariatric Surgery: Novel Targets for a Chronic Disease

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Abstract

Background: Obesity is a metabolic chronic disease with important associated morbidities and mortality. Bariatric surgery is the most effective treatment for maintaining long-term weight loss in severe obesity and, consequently, for decreasing obesity-related complications, including chronic inflammation.

Aim: To explore changes in components of the inflammasome machinery after bariatric surgery and their relation with clinical/biochemical parameters at baseline and 6 months after bariatric surgery.

Patients and methods: Twenty-two patients with morbid-obesity that underwent bariatric surgery (sleeve gastrectomy and Roux-en-Y gastric bypass) were included. Epidemiological/clinical/anthropometric/biochemical evaluation was performed at baseline and 6 months after bariatric surgery. Inflammasome components and inflammatory-associated factors [nucleotide-binding oligomerization domain-like

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receptors (NLRs), inflammasome activation components, cytokines and inflammation/apoptosis-related components, and cell-cycle and DNA-damage regulators) were evaluated in peripheral blood mononuclear cells (PBMCs) at baseline and 6 months after bariatric surgery. Clinical molecular correlations/associations were analyzed. Functional parameters (lipid accumulation/viability/apoptosis) were analyzed in response to specific inflammasome components silencing in liver HepG2 cells).

Results: A profound dysregulation of inflammasome components after bariatric surgery was found, especially in NLRs and cell-cycle and DNA damage regulators. Several components were associated with baseline metabolic comorbidities including type 2 diabetes (C-C motif chemokine ligand 2/C-X-C motif chemokine receptor 1/sirtuin 1), hypertension (absent in melanoma 2/ASC/purinergic receptor P2X 7), and dyslipidemia [C-X-C motif chemokine ligand 3 (CXCL3)/NLR family pyrin domain containing (NLRP) 7) and displayed changes in their molecular profile 6 months after bariatric surgery. The gene expression fingerprint of certain factors NLR family CARD domain containing 4 (NLRC4)/NLRP12/CXCL3)/C-C motif chemokine ligand 8/toll-like receptor 4) accurately differentiated pre- and postoperative PBMCs. Most changes were independent of the performed surgical technique. Silencing of NLRC4/NLRP12 resulted in altered lipid accumulation, apoptosis rate, and cell viability in HepG2 cells.

Conclusion: Bariatric surgery induces a profound alteration in the gene expression pattern of components of the inflammasome machinery in PBMCs. Expression and changes of certain inflammasome components are associated to baseline metabolic comorbidities, including type 2 diabetes, and may be related to the improvement and reversion of some obesity-related comorbidities after bariatric surgery.

Key Words: obesity, bariatric surgery, inflammasome, evolution, comorbidities

Obesity (OB) is a metabolic chronic disease with a growing incidence and elevated morbidities and mortality (1,2). It is associated with increased economic costs and represents a worldwide public health problem (3,4). Specifically, in 2016, 650 million adults were obese (5); among them, 10% to 30% presented with metabolic disorders (6), including type 2 diabetes (T2DM), hypertension (HT), heart disease, high total cholesterol and triglycerides, stroke, obstructive sleep apnea, nonalcoholic fatty liver disease, and certain types of cancer (7-9).

Bariatric surgery (BS) is a well-recognized treatment for OB that has superior outcomes for patients who have been unable to keep weight loss by nonsurgical methods (10). Worldwide, the most commonly performed bariatric procedures are laparoscopic Roux-en-Y gastric bypass (RYGB) and sleeve gastrectomy (SG), wherein both procedures encompass nearly 80% of all bariatric operations worldwide (11). BS induces and maintains substantial weight loss through a variety of mechanisms, including caloric restriction (due to anatomical surgical-induced changes of the gastrointestinal tract), increased meal-induced thermogenesis, modulation of hypothalamic neuronal circuits (that regulate energy balance and/or appetite regulation), modifications in gut-brain signaling pathways and changes in taste, food preferences, and eating behavior patterns (12-15). All these changes are accompanied by reduction in metabolic complications, specially T2DM (16), and a rapid alteration of the inflammatory status, due in part to the modulation of cytokine production after BS, which may be accompanied by improvement in other metabolic complications (17).

In this sense, inflammation is a protective immune response, wherein innate immune function depends upon recognition of pathogen-associated molecular patterns, which are derived from invading pathogens, and danger-associated molecular patterns, which are induced endogenously by germline-encoded pattern-recognition receptors (PRRs). The activation of PRRs induces production of interferon-α, interferon-β, and proinflammatory cytokines (18). These responses are mediated by the inflammasome machinery, a multiprotein intracellular complex that plays a central role in innate immunity and is responsible for the activation of inflammatory responses. This complex is composed of some families of PRRs, including the nucleotide-binding domain, leucine-rich repeat containing proteins [nucleotide-binding oligomerization (NOD) domain-like receptors (NLRs)] and by the absent in melanoma 2-like (AIM2-like) receptors (19). NLRs and AIM-like receptors can

oligomerize and act as caspase-1–activating scaffolds—for example, activating the proinflammatory interleukin (IL)-1 family through the production of IL-18 and IL-1β (20). Remarkably, the activity of these inflammasome components is regulated by different regulatory proteins, metabolic pathways, and a regulatory mitochondrial hub (21). Consequently, the activation of these inflammasome components leads to the secretion of diverse inflammatory cytokines and the activation of key receptors in immune cells, thus inducing the activation of inflammatory cascades, which can lead in some cases in cell-cycle alterations and DNA damage (20,21).

Different components of the inflammasome machinery have been linked to a variety of autoinflammatory, autoimmune, and metabolic disorders including atherosclerosis, T2DM, and OB (22). Some studies have found that inflammasome components are dysregulated in several tissues/cells including peripheral blood mononuclear cells (PBMCs) and that these changes may be associated with the development of atherosclerosis, autoimmune (eg, multiple sclerosis), and neurodegenerative (eg, Alzheimer´s or Parkinson´s) diseases (20). In the same line, alterations in some specific inflammasome components have been described in OB, but their specific role is still not well understood (23). However, changes in inflammasome components induced by acute weight loss related to BS have not been fully described yet. Interestingly, it has been reported that inflammasome-induced cytokines promote OB-related inflammatory diseases following stimulation by high-fat diet metabolites and adipose tissue changes that occur during weight gain (24), which suggests that inflammasome modulation due to weight loss may be associated to an improvement in OB-related comorbidities.

In this context, this study was aimed to evaluate for the first time the expression and changes in key elements of the inflammasome machinery (ie, inflammasome components and inflammatory-associated factors) in OB patients after BS. To that end, we used PBMCs, since gene expression patterns are commonly reflected in these cells and are closely related to the molecular profile of the disease (25). Specifically, we analyzed the gene expression levels of 4 groups of components of the inflammasome machinery: (1) NLRs, (2) regulators of inflammasome activation, (3) cytokines and inflammation/apoptosis-related components, and (4) cell-cycle and DNA damage regulators. Additionally, we aimed to explore the putative relations between gene expression levels of these components of the inflammasome machinery with different clinical and biochemical variables to determine potential mechanisms that may explain the reversion of metabolic and inflammatory comorbidities (associated with OB) after BS. Finally, we explored the role of key dysregulated inflammasome components on cell proliferation and apoptosis in liver cells to understand the potential underlying mechanisms that could explain the role of some key inflammasome components identified in this study in some OB-related comorbidities.

Material and Methods

Patients

This study was approved by the Ethics Committee of the Reina Sofia University Hospital (Cordoba, Spain), which was conducted in accordance with the Declaration of Helsinki and according to national and international guidelines. This is a prospective open-label study, wherein a written informed consent was signed by every individual before inclusion into the study. Twenty-two patients who underwent BS were included. Clinical records were used to collect full medical history of all patients (demographic and clinical characteristics of patients are summarized in Table 1). An additional cohort of 20 patients was evaluated to confirm the preliminary results, which clinical characteristics are summarized in Supplementary Table 2 (26). All patients were managed following available guidelines and recommendations. RYGB and SG bariatric procedures were performed in 15 and 7 patients, respectively. Clinical follow-up was performed by the same clinician in all cases at 6 months after BS. Body composition was evaluated using a multi-frequency bioimpedanciometer (TANITA MC-780MA), and waist circumference was measured at minimal expiration. Blood samples were obtained at baseline and 6 months after BS from all patients to obtain and analyze PBMCs.

Some formulas were used to evaluate body weight (BW) changes after BS as follows: body mass index (BMI) = weight (kg)/height (m^2); ideal weight for males = 25 × height (m^2); ideal weight for women = 24 × height (m^2); baseline excess of BW = baseline weight – ideal weight; expected BMI = (0.33 × baseline BMI) + 14; excess of BW lost = baseline excess of BW (kg)/overweight lost (kg); excess of BMI lost = [baseline BMI (kg/m^2) – current BMI]/(baseline BMI – 25).

Blood Sampling and Processing to Isolate PBMCs

Venous blood from all patients was collected in tubes containing EDTA at baseline and 6 months after BS. PBCMs were isolated as previously described (25,27).

RNA Extraction, Quantification, and Reverse Transcription

Total RNA from PBMCs was isolated using Direct-zol RNA kit (Zymo Research, Irvine, CA, USA) following

Table 1. Baseline and 6-month general characteristics of the evaluated patients

General characteristics	Total (n = 22)	Sleeve gastrectomy (n = 7)	Gastric bypass (n = 15)	*P*[a]
Sex				
Male	50 (11)	28.6% (2/7)	60% (9/15)	0.24
Female	50 (11)	71.4 (5/7)	40% (6/15)	
Age	46 ± 11.5	43.6 ± 12.5	47 ± 11.4	0.54
Type 2 diabetes	45.5 (10/22)	28.6% (2/7)	53.3% (8/15)	0.27
Hypertension	45.5 (10/22)	42.9% (3/7)	46.7% (7/15)	0.62
Dyslipidaemia	36.4 (8/22)	42.9% (3/7)	33.3% (5/15)	0.51
OSA	22.2 (4/18)	0% (0/7)	30.8% (4/13)	0.23
Baseline BMI, kg/m^2	45.4 ± 5.6	47.8 ± 3.5	44.3 ± 6.1	0.21
Baseline waist circumference, cm	137.6 ± 12.7	137.1 ± 10.5	137.9 ± 14.1	0.74
6-month BMI, kg/m^2	33.4 ± 4.4	34.2 ± 3.0	33.1 ± 4.9	0.49
6-month waist circumference, cm	115.7 ± 22.9	106.2 ± 8.9	119.8 ± 26.0	0.20
Baseline excess of body weight, kg	57 ± 16.5	59.9 ± 11.6	55.6 ± 18.5	0.49
Excess of body weight lost, %	26.1 ± 7.0	57.4 ± 13.4	25.1 ± 6.8	0.89
Excess of BMI lost, %	63.6 ± 18.2	59.1 ± 13.7	60.9 ± 17.4	0.95

Data given as % (n/total) or mean ± SD unless otherwise indicated.
Abbreviations: BMI, body mass index; OSA, obstructive sleep apnea.
[a]For the comparison between patients treated with sleeve gastrectomy with those treated with gastric bypass.

manufacturer's instructions and as previously described (25,27,28). The amount of RNA recovered was determined and its quality assessed by the NanoDrop2000 spectrophotometer (Thermo Fisher). Specifically, all the RNA samples passed the quality controls of 260/280 and 230/260 absorbance ratios between 1.8–2.0. As previously described (25,29,30), 1 µg of RNA was reverse transcribed to complimentary DNA using random hexamer primers with the First Strand Synthesis Kit (Thermo Fisher). RNA from the hepatic HepG2 cell model was isolated using TRI Reagent (Sigma-Aldrich, Madrid, Spain), followed by DNase treatment and as previously described (27,31,32).

Analysis of Components of the Inflammasome Machinery by Quantitative Polymerase Chain Reaction Dynamic Array Based on Microfluidic Technology

A 48.48 Dynamic Array based on microfluidic technology (Fluidigm, San Francisco, CA, USA) was developed and implemented to determine, simultaneously, the expression of 48 transcripts in 48 samples, following the same methods recently described (25,33). Specific primers for human transcripts of the inflammasome machinery including NLRs (n = 7), regulators of inflammasome activation (n = 15), cytokines and inflammation/apoptosis-related components (n = 18), and cell-cycle and DNA damage regulators (n = 5) were specifically designed and validated for this study [Supplementary Table 1 (26)]. In addition, 3 housekeeping genes were used [Supplementary Table 1 (26)]. The selection of this panel of genes was based on 2 main criteria: (1) the relevance of the given inflammasome components and other cell cycle regulators in the inflammatory and apoptotic process and (2) the demonstrated implication in the inflammatory response in metabolic disorders, especially in OB conditions. Details about the quantitative polymerase chain reaction (qPCR) dynamic array and primers design can be found in the supplemental information file (Supplemental Table 1) (26).

Preamplification, exonuclease treatment, and qPCR dynamic array based on microfluidic technology were implemented following manufacturer's instructions using the Biomark System and Real-Time PCR Analysis Software (Fluidigm), as previously described (34,35). Additional details are provided in Supplemental Table 1 (26). The expression level of each transcript was adjusted by a normalization factor obtained from the expression levels of 2 different housekeeping genes [beta actin (ACTB) and glyceraldehyde-3-phosphate dehydrogenase] using Genorm 3.3. This selection was based on the stability of the housekeeping genes analyzed among the experimental groups to be compared, wherein the expression of these 2 housekeeping genes was not significantly different among groups. Expression of the components of the inflammasome machinery in HepG2 cell lines was adjusted by *ACTB*, the most stable housekeeping gene in this model, as previously reported (32).

Quantitative Real-Time Polymerase Chain Reaction

Complimentary DNA was 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. Specifically, expression levels [absolute messenger RNA (mRNA) copy number/50 ng of sample] of C-X-C motif chemokine ligand 3 (CXCL3), C-C motif chemokine ligand 8 (CCL8), toll-like receptor 4 (TLR4), NLR family CARD domain containing 4 (NLRC4), NLRP12, C-C motif chemokine ligand 2 (CCL2), C-X-C motif chemokine receptor 1 (CXCR1), interleukin 6, and sirtuin 1 (SIRT1) were evaluated using the previously discussed validated primers. mRNA levels were normalized by hypoxanthine phosphoribosyl transferase, β-actin (BACT), and glyceraldehyde-3-phosphate dehydrogenase. All samples were run in the same plate against a standard curve to estimate mRNA copy number and a no-reverse transcribed sample as a negative control. Thermal profile consisted of an initial step at 95°C for 30 s, followed by 40 cycles of denaturation (95°C for 20 s) and annealing/elongation (60°C for 20 s), and, finally, a dissociation cycle (melting curve; 55°C-95°C, increasing 0.5°C/30 s) to verify that only 1 product was amplified.

Cell Lines

As previously described (32,36), liver-derived cell line HepG2 (ATCC, Manassas, VA, USA) was cultured in Minimum Essential Media (Thermo Fisher), with 10% fetal bovine serum (FBS; Sigma-Aldrich), 0.2% antibiotic-antifungal (Gentamicin/amphotericin-B; Thermo Fisher), and 0.5% sodium pyruvate cells were maintained at 37°C and 5% CO_2 under sterile conditions, validated by short-tandem repeat analysis (GenePrint 10 System, Promega, Barcelona, Spain) and tested for mycoplasma contamination (32,36).

Silencing Experiments by Specific siRNAs

Small interfering RNAs for NLRC4 (ID: s33830 catalog#4392420, Thermo Fisher) and NLRP12 (ID: s532697, catalog#4392420, Thermo Fisher) and a negative control (Scramble; Thermo Fisher) were used. For transfection, 150 000 HepG2 cells were seeded in 6-well plates, as previously described (32,36). Medium was replaced by antibiotic/antimycotic-free medium, and cells were transfected using Lipofectamine RNAiMAX reagent (Thermo Fisher). After 48 h, cells were detached and seeded to extract RNA and to implement functional assays.

Lipid Accumulation Determination in HepG2 Cells

As previously described (27), HepG2 cells were treated with oleic acid (OA) to assess the effect of the silencing of selected NLRs on lipid accumulation, since these cells are widely used as normal-like liver model (27). In brief, cells were seeded, and once 80% of cell confluence was reached, cells were cultured in FBS-free medium and 0.5% free fatty acid bovine serum albumin for 1 h. Then, HepG2 cells were treated with 500 mM OA in FBS-free medium and 2% bovine serum albumin for 24 h. After the incubation time, medium was removed, and treated cells were washed with phosphate-buffered saline (PBS) and fixed with 10% paraformaldehyde for 15 min. Control cells were treated with OA-free medium. After removing paraformaldehyde, cells were washed and incubated with 60% isopropanol. Then, Oil Red O working solution was added for 10 min in agitation and washed. Optical density was measured at a wavelength of 520 nm in the FlexStation 3 system (Molecular Devices, Sunnyvale, CA, USA).

Cell Viability

Cell viability was measured by methylthiazolyldiphenyl-tetrazolium bromide assay (Sigma-Aldrich) at 24, 48, and 72 h, as previously described (32). Briefly, 10 000 cells/well were plated in 96-well plates, and on the day of measurement, 100 µL of methylthiazolyldiphenyl-tetrazolium bromide diluted in Dulbecco's PBS (Sigma-Aldrich) was added to the cells and then incubated 3 h at 37°C. Subsequently, cells were detached with lysis buffer (10% sodium dodecyl sulfate, 0.56% glacial acetic acid in dimethylsulfoxide) and absorbance measured using the FlexStation system plate reader at 570 nm. In all instances, cells were plated per quadruplicate. Results are expressed as percentage *vs* control cell.

Apoptosis

To evaluate the apoptotic rate, 4′,6-diamidino-2-phenylindole (DAPI) staining method was used, as previously described (32). Briefly, 150 000 cells/well were plated and cultured in 6-well plates. Transfected cells were incubated for 24 h. The cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature and then washed twice with PBS. DAPI solution was added at room temperature for 10 min and washed twice with PBS. The detection was performed with fluorescence inverted microscope (Zeiss Axioskop 2; Zeiss, Thornwood, NY) equipped with a charged-coupled device digital camera for image capture and processing with Axiovision (Zeiss) software. Images were captured using appropriate fluorescence filters for DAPI. Magnification shown is ×400 using ×40 oil immersion objective. Four fields of view were randomly taken to be photographed.

Statistical Analysis

Between-group comparisons were analyzed by the Mann-Whitney U test (nonparametric data) or the Kruskal-Wallis test (nonparametric data when we compared more than 2 groups). Paired analysis was performed by Student *t* (parametric data) or Wilcoxon test (nonparametric data). Chi-squared test was used to compare categorical data. Statistical analyses were performed using SPSS statistical software version 20, and GraphPad Prism version 6. Data are expressed as mean ± standard error of the mean and percentages. *P*-values < 0.05 were considered statistically significant. Heatmaps and clustering analysis were performed using MetaboAnalyst 4.0 (37). In this sense, the inflammasome machinery components that discriminate between baseline and 6 months after BS were selected following 2 main criteria. First, the Variable Importance in Projection score must be higher or equal than 1.5, this value being considered as a significant value in this type of analysis. Second, we chose only components that were sufficient to get the best hierarchical clustering in the heatmaps.

Results

Patient Population and Clinical Evolution After BS

A total of 22 patients who underwent BS were included in the study. Patients were treated according to the available clinical guidelines (38,39). SG and RYGB bariatric procedures were performed in 7 and 15 patients, respectively. Baseline characteristics of both group of patients were comparable, as well as the effect of both types of surgeries on BW and BMI after 6 months (Table 1). However, at baseline, excess BW in men was higher than in women (38.43 ± 14.83 *vs* 27.32 ± 8.36; $P < 0.05$). Patients with baseline HT showed higher BW before surgery (62.22 ± 16.73 *vs* 50.65 ± 14.51; $P = 0.05$). After BS, when the body composition measured by bioimpedanciometry was evaluated, both surgical techniques decreased fat mass and water $P < 0.05$—especially RYGB ($P < 0.001$), but RYGB did not decrease lean mass ($P > 0.05$) (Table 2). However, lean mass decreased in patients who underwent SG, which is consistent with previous reports (40-42), likely due to the fact that gastric pouch in these patients is reduced in comparison with RYGB. Consequently, the meal volume is significantly reduced during the first months after surgery; in particular, the tolerance of specific protein-enriched oral supplements is worsened. Interestingly, serum aspartate aminotransferase, alanine aminotransferase, and total cholesterol values significantly decreased after SG but not after RYGB. Importantly, decreased serum albumin and transferrin levels occurred after 6 months in both groups, but prealbumin levels significantly decreased only after SG ($P < 0.05$). Testosterone levels in males seemed to increase in both groups, although this change was statistically significant only after RYGB ($P < 0.05$). C-reactive protein significantly decreased only after RYGB, but insulin and C-peptide decreased in both groups ($P < 0.05$). Since C-reactive protein is an unspecific marker of inflammation, this fact is probably related with baseline weight and the lack of sufficient weight loss after 6 months in both techniques (after 6 months most patients were still obese). All anthropometric and biochemical comparisons between both groups at baseline and after 6 months of follow-up are depicted in Table 2.

Different Components of the Inflammasome Machinery Are Dysregulated After BS

NLRs; regulators of inflammasome activation, cytokines, inflammation/apoptosis-related components; and cell-cycle and DNA damage regulators were evaluated in PBMCs from patients at baseline and 6 months after BS. In general, the expression of a high proportion of the components analyzed in PBMCs decreased after BS [Supplemental Figure 1 (26)]. Among the NLR family, gene expression of NLRP7, NLRP12, NLRC4, and BIRC1 significantly decreased 6 months after BS (Fig. 1A). All the evaluated NLRs are depicted in Supplemental Figure 2A (26). Although this overall effect was similar after both surgical techniques, a more profound decrease in the expression levels of NLRP12 and NLRC4 were observed after SG and RYGB, respectively [Supplemental Figure 2B (26)].

In the case of the regulators of inflammasome activation and response [all the evaluated components are depicted in Supplemental Figure 3A (26)], we found that the expression of AIM2, caspase 1, c-Jun N-terminal kinase (JNK) isoform 2, P2X purinoceptor 7, and TLR4 significantly decreased after BS (Fig. 1B). However, when both surgical techniques were compared, TLR4 significantly decreased after RYGB and not after SG [Supplemental Figure 3B (26)].

When cytokines and inflammation/apoptosis-related components were evaluated, the gene expression of CCL8, IL-6R, nuclear factor kappa B, and transforming growth factor beta decreased after BS (Fig. 1C), while other components were not significantly altered after surgery [Supplemental Figure 4A (26)]. Among them, the decrease of CCL8 was more evident in patients who underwent SG compared to those who underwent RYGB [Supplemental Figure 4B (26)]. On the other hand, we found that CXCL3 and IL-6 significantly increased after BS (Fig. 1C), wherein the same pattern was observed after both types of surgery, especially the increase in the expression of CXCL3 after RYGB [Supplemental Figure 4B (26)].

Table 2. Biochemical evaluation of the evaluated patients at baseline and 6 months after bariatric surgery

General characteristics	Total (22)					Sleeve gastrectomy (14)					Gastric bypass (15)				
	Baseline		6 months			Baseline		6 months			Baseline		6 months		
	Mean	SD	Mean	SD	*P*[a]	Mean	SD	Mean	SD	*P*[b]	Mean	SD	Mean	SD	*P*[c]
Baseline weight (kg)	123.7	21.4	91.1	15.3	**0.000**	124.7	23.8	93.1	17.6	**0.018**	121.7	16.4	86.7	8.1	**0.001**
Fat mass (%)	49.3	4.6	30.9	9.8	**0.000**	48.8	5.2	31.8	8.3	**0.018**	50.4	2.9	28.9	13.0	**0.001**
Fat mass (kg)	61.0	12.0	28.2	10.5	**0.000**	61.0	13.9	29.7	10.1	**0.028**	61.0	5.8	25.1	11.2	**0.001**
Lean mass (%)	51.8	2.7	62.2	9.0	0.109	51.7	3.8	62.9	9.5	0.310	51.9	0	59.8	9.3	0.180
Lean mass (kg)	61.0	14.5	60.1	13.9	0.313	61.4	16.5	61.9	15.3	**0.028**	60.2	10.1	56.6	10.5	0.572
Water (%)	38.5	3.5	49.9	8.2	**0.001**	38.5	3.3	49.1	6.5	0.109	38.5	4.0	51.7	11.2	**0.018**
Water (kg)	50.4	14.5	45.3	10.4	**0.003**	51.5	14.2	45.5	11.0	**0.018**	47.5	17.9	44.8	9.9	**0.012**
Glucose	90.9	15.0	84.5	26.8	**0.030**	90.6	16.3	89.9	30.9	0.352	91.6	12.7	72.7	7.1	0.300
Uric acid	10.9	13.9	8.6	15.5	**0.006**	13.0	16.5	5.3	1.3	1.000	6.5	1.0	15.5	27.4	**0.004**
Magnesium	2.1	0.2	2.1	0.2	0.908	2.1	0.2	2.1	0.2	0.051	2.1	0.1	2.1	0.2	0.751
AST	31.3	13.7	23.8	10.4	**0.011**	31.1	12.1	25.8	9.5	**0.018**	31.7	17.7	19.6	11.7	0.078
ALT	37.2	21.0	24.9	15.8	**0.007**	34.8	17.9	28.8	17.0	**0.018**	42.3	27.3	16.6	8.7	0.158
GGT	42.8	39.4	26.9	18.9	**0.005**	47.5	46.7	30.1	21.2	0.499	32.6	13.1	20.1	11.2	0.075
AP	73.5	21.4	81.1	29.3	0.118	72.7	15.9	82.7	27.6	0.063	75.1	31.7	77.7	34.8	0.140
Total cholesterol	176.9	32.0	182.0	41.2	0.848	178.6	36.7	170.7	31.0	**0.046**	173.1	20.3	206.3	52.1	0.233
HDL	40.5	13.6	54.2	12.4	**0.001**	42.3	15.0	55.5	12.9	**0.046**	37.0	10.7	51.6	12.0	**0.008**
LDL	104.2	27.9	111.2	41.1	0.478	107.4	30.4	99.4	31.2	**0.018**	97.8	23.2	133.1	50.3	0.328
TG	144.8	50.4	99.7	21.2	**0.000**	130.5	47.8	96.5	21.7	**0.018**	175.4	44.3	106.6	19.6	**0.004**
Albumin	4.6	0.2	4.3	0.3	**0.000**	4.6	0.3	4.4	0.3	**0.043**	4.5	0.2	4.2	0.1	**0.003**
Prealbumin	23.4	4.7	21.5	3.8	**0.046**	23.0	5.5	21.9	4.5	**0.028**	24.1	2.5	20.6	1.6	0.329
CRP	10.0	6.0	3.6	3.4	**0.001**	8.6	6.3	1.9	2.4	0.752	12.7	4.7	6.3	3.2	**0.008**
HbA1c	5.9	0.8	5.7	1.3	**0.022**	6.0	0.9	5.9	1.5	**0.042**	5.7	0.5	5.3	0.5	0.154
LH (U/L)	5.2	4.9	4.6	1.9	0.099	4.0	1.7	4.4	1.8	0.500	7.9	8.5	5.0	2.2	0.139
FSH (U/L)	7.3	7.3	5.9	1.7	0.569	4.9	2.1	6.1	1.8	0.225	12.4	11.8	5.3	1.6	0.082
Testosterone (ng/dL)	3.3	0.9	501.8	319.8	**0.005**	3.3	0.9	452.0	342.4	0.180	2.9	1.5	701.0	19.8	**0.012**
Estradiol (pg/mL)	91.8	33.5	170.4	100.2	0.225	100.3	50.1	106.7	42.5	0.180	83.3	9.0	266.0	77.8	1.000
Progesterone	4.0	5.4	2.2	4.1	0.833	7.5	6.0	3.7	6.0	0.593	0.5	0.2	0.7	0.3	0.414
DHEAS (mcg/dL)	166.0	164.1	169.3	154.5	0.177	170.2	197.4	158.4	160.7	0.753	158.3	85.0	193.0	151.7	**0.033**
Insulin (mU/L)	19.3	19.5	5.9	2.4	**0.000**	**20.6**	**23.2**	**6.0**	**2.7**	**0.018**	16.5	7.4	5.5	1.9	**0.002**
TSH (mU/L)	2.2	1.6	4.3	9.9	0.654	2.4	1.9	5.2	12.4	0.091	1.9	0.8	2.7	1.3	0.087
Androstenedione (ng/mL)	2.6	1.3	2.3	1.3	0.135	2.6	1.6	2.3	1.4	0.600	2.5	0.7	2.3	1.1	0.177
C-peptide (ng/mL)	9.0	28.2	1.3	0.5	**0.001**	11.7	34.2	1.3	0.5	**0.018**	3.3	1.3	1.2	0.6	**0.019**
AMH (ng/mL)	2.8	1.7	1.5	0.6	0.109	3.2	2.6	1.4	0.7	0.109	2.4	1.3	1.8		0.180
Folic acid (ng/mL)	15.0	7.1	12.0	7.1	0.149	15.3	7.8	14.2	7.6	**0.043**	14.4	6.1	7.3	1.8	0.683
Vitamin B12 (pg/mL)	456.3	287.5	372.8	150.5	0.422	373.3	78.2	365.6	141.9	1.000	495.12	81.8	387.2	177.4	0.235
Iron (mcg/dL)	72.9	20.4	87.2	25.0	**0.020**	74.7	19.6	86.9	25.0	0.128	69.0	23.1	87.9	26.8	0.084
Ferritin (ng/mL)	171.8	200.2	148.3	139.0	0.794	187.4	237.0	140.1	150.0	0.499	138.5	85.6	165.9	121.1	0.510
Transferrin (ng/mL)	269.5	39.5	225.9	60.4	**0.001**	273.6	43.4	233.2	69.3	**0.028**	260.6	30.5	210.1	33.8	**0.026**
Urine calcium/creatinine (mg/mg)	0.1	0.1	0.1	0.1	**0.002**	0.1	0.0	0.1	0.1	**0.027**	0.1	0.1	0.2	0.1	**0.045**

Significant differences are highlighted in bold.

Abbreviations: 25-OH vitamin D, 25-hydroxy vitamin D; ALT, alanine aminotransferase; AMH, anti-Müllerian hormone; AP, alkaline phosphatase; AST, aspartate aminotransferase; CRP, c-reactive protein; DHEAS, dehydroepiandrosterone sulfate; FSH, follicle stimulating hormone; GGT, gamma-glutamyltransferase; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LH, luteinizing hormone; PTH, parathyroid hormone; TG, triglycerides; TSH, thyroid-stimulating hormone.

[a]*P* refers to the comparison between baseline characteristics and the 6-month follow-up in the whole cohort.

[b]*P* refers to the comparison between baseline characteristics and the 6-month follow-up after sleeve gastrectomy.

[c]*P* refers to the comparison between baseline characteristics and the 6-month follow-up after gastric bypass.

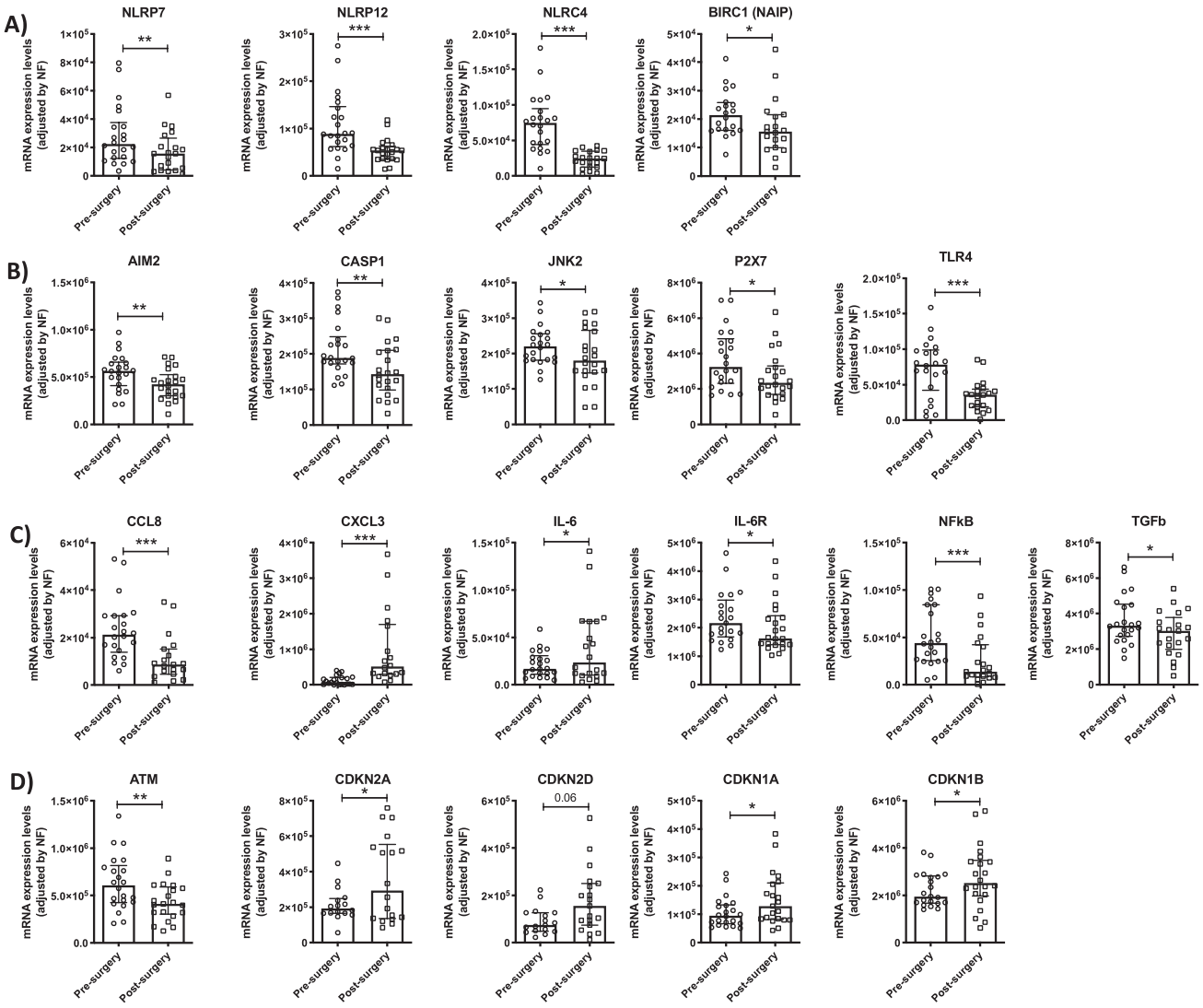

Figure 1. Significant changes in inflammasome components before, and 6-month after bariatric surgery. (A) Nucleotide-binding oligomerization domain-like receptors. (B) Regulators of inflammasome activation. (C) Cytokines and inflammation/apoptosis-related components. (D) Cell cycle and DNA damage regulators. Data represent the median ± interquartile range. Asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) indicate significant changes in the messenger RNA expression by paired analysis between the clinical variables.

Finally, cell-cycle and DNA damage regulators were mostly upregulated 6 months after BS (cyclin dependent kinase inhibitors (CDKNs) 1A, 1B, and 2A), while only the expression level of ATM decreased (Fig. 1D). The expression of CDKN2D was not significantly increased 6 months after BS but a trend for significance was observed ($P = 0.06$; Fig. 1D). When we analyzed the gene expression of these components in response to both surgical techniques, we found virtually the same changes, but none of them reached statistical significance [Supplemental Figure 5B (26)]. The absolute change in the percentage of the median for all the evaluated inflammasome components according to the type of surgery is presented in Supplemental Table 3 (26).

Key Components of the Inflammasome Machinery Are Associated With Critical OB-Related Metabolic Comorbidities at Baseline and 6 Months After BS

When the personal medical history of the patients was evaluated, we observed that PBMCs from patients with baseline T2DM exhibited increased levels of CCL2, CXCR1 and SIRT1 compared to nondiabetic patient PBMCs ($P < 0.05$) (Fig. 2A). Six months after BS, patients with T2DM significantly lost more BW and decreased their BMI compared to nondiabetic patients ($P < 0.05$) (Fig. 2B). Interestingly, patients with T2DM presented higher expression levels of CCL2, CXCR1, IL-6, and SIRT1 at 6 months after BS ($P < 0.05$) (Fig. 2C).

To validate these results, an additional cohort of 20 patients was evaluated using qPCR. Specifically, 10 patients who underwent SG and RYGB were included [specific characteristics of this cohort are depicted in Supplemental Table 2 (26)]. The evaluated genes were selected based on the previously described results. Patients with T2DM presented increased mRNA levels of CXCL3 and tended to express increased mRNA levels of CCL2 and CXCR1 (Fig. 3A). Six months after surgery, the mRNA expression levels of the evaluated genes were similar in both groups as previously observed (Fig. 3B). In this validation cohort, mRNA expression of the evaluated genes was similar in patients who underwent to RYGB or SG [Supplemental Figure 6 (26)].

In the same line, we observed that PBMCs from patients with baseline HT exhibited elevated gene expression levels of AIM2, ASC, mitogen-activated protein kinase 14, and P2X purinoceptor 7 ($P < 0.05$) (Fig. 4A). Six months after BS, PBMCs of patients with baseline HT exhibited a significant decrease in the expression of CXCL3 and an increase in the expression of NLRP1

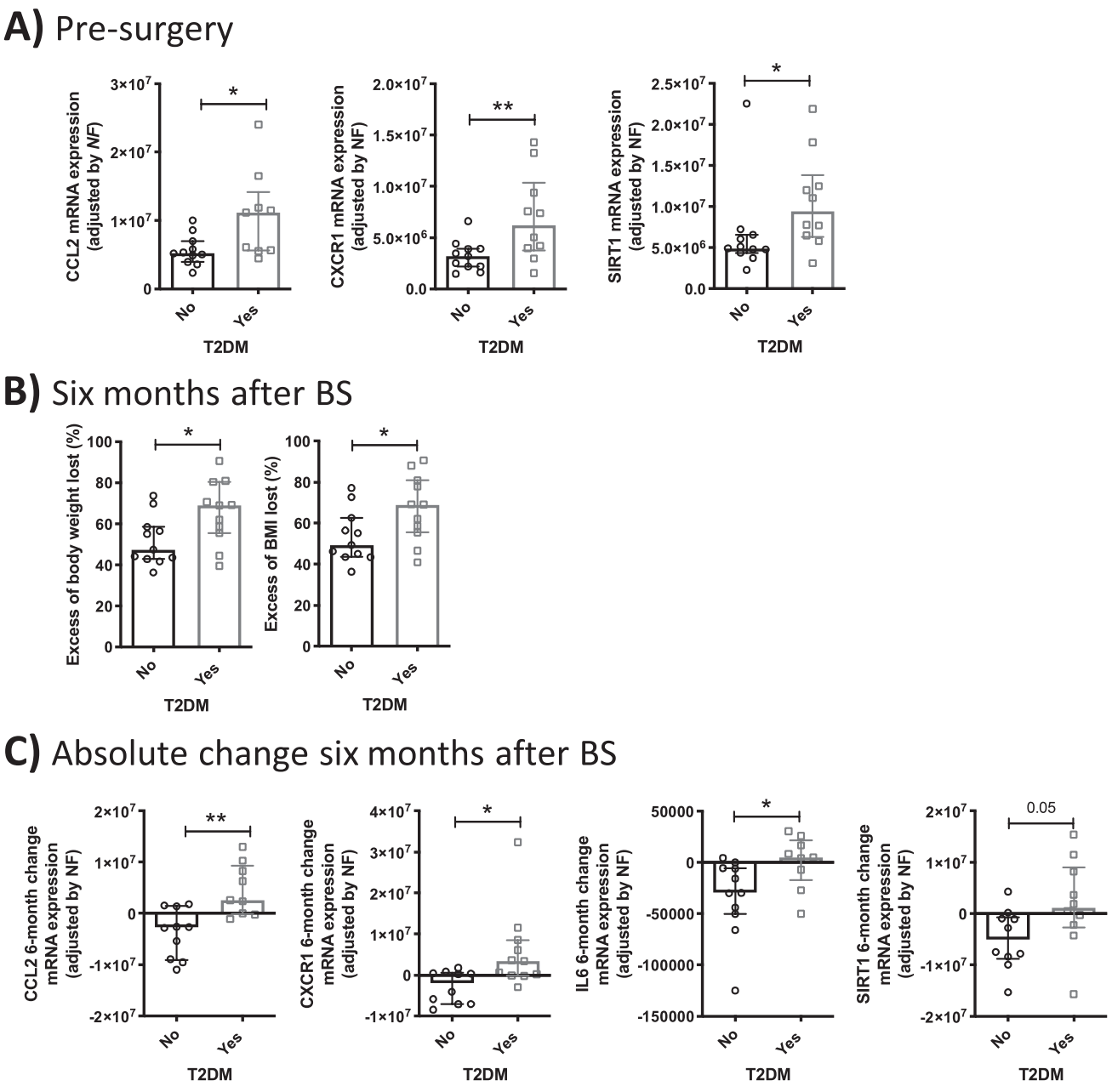

Figure 2. Significant clinical molecular associations of baseline type 2 diabetes mellitus (T2DM). (A) Messenger RNA (mRNA) expression of inflammasome components before surgery. (B) Clinical changes in patients with T2DM 6 months after surgery. (C) Absolute change in the mRNA expression of inflammasome components 6 months after surgery. Data represent the median ± interquartile range. Asterisks (*$P < 0.05$; **$P < 0.01$) indicate significant changes in the mRNA expression by paired (C) and nonpaired analysis (A and B) between the clinical variables.

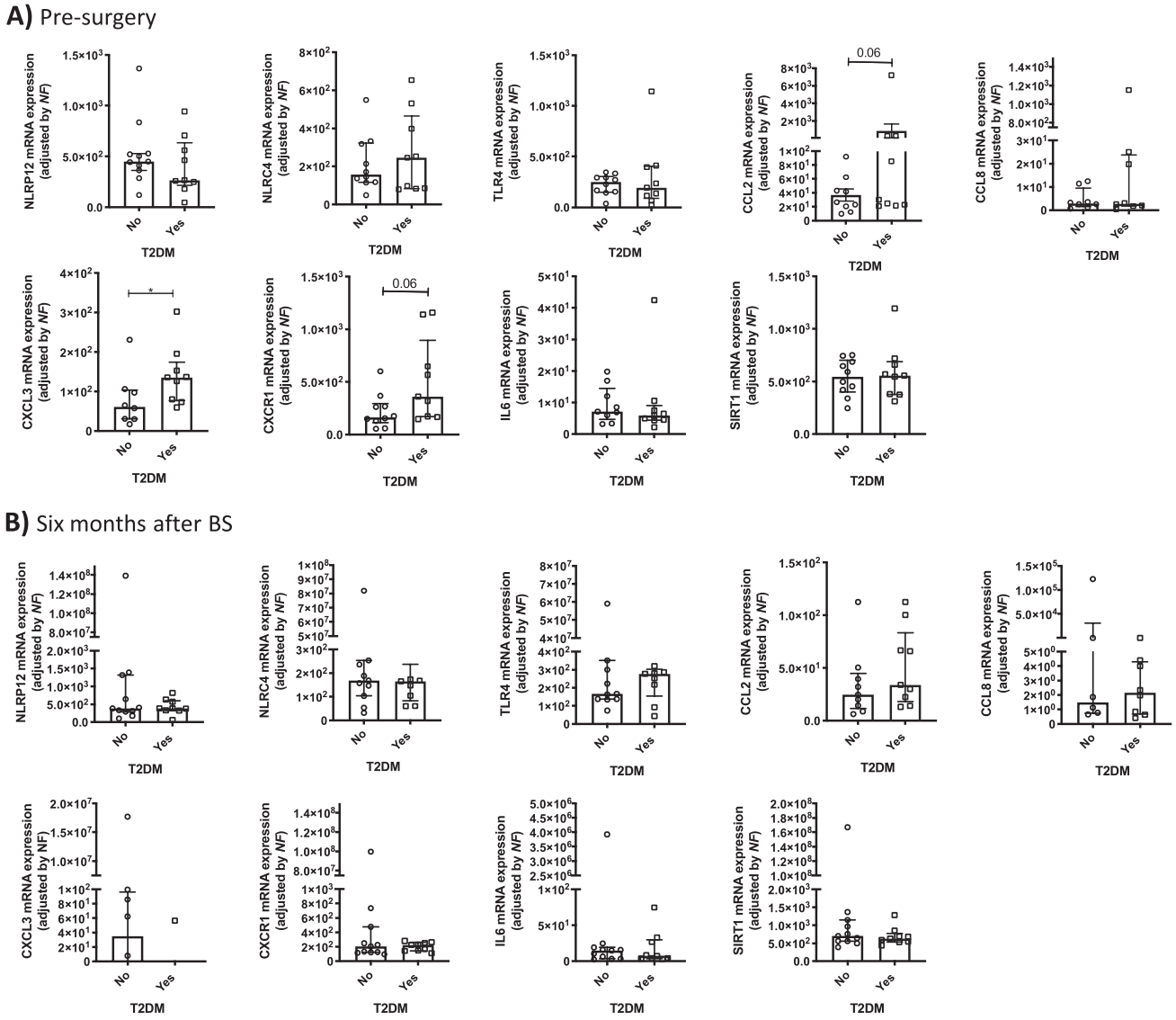

Figure 3. Clinical molecular associations of baseline type-2 diabetes mellitus (T2DM) in the validation cohort. Messenger RNA expression of inflammasome components before surgery (A) and 6 months after surgery (B). Data represent the median ± interquartile range.

and CDKN1A (Fig. 4B). Additionally, patients with HT presented higher gene expression levels of caspase 5 and lower expression levels of CXCL3 and CDKN1a at 6 months after BS (Fig. 4C).

Baseline dyslipidemia was also analyzed, which was associated to lower molecular expression levels of CXCL3 and increased mRNA levels of NLRP7 ($P < 0.05$) (Fig. 4D). After BS, only the expression levels of NLRP6 significantly increased in patients with dyslipidemia ($P < 0.05$) (Fig. 4E).

Finally, the baseline molecular profile revealed that women had higher expression levels of BIRC1 than men before BS [median 25 578 (CI 95%: 20 028-32 079) *vs* 16 194 (CI 95%: 4747-22 403); $P < 0.01$; data not shown]. Associations and correlations between all the determined components and metabolic comorbidities were evaluated, but only significant results have been presented and discussed.

Components of the Inflammasome Machinery Are Correlated to Body Composition After BS

The expression patterns of all the evaluated inflammasome components before and after BS were submitted to clustering analysis. Specifically, a nonsupervised hierarchical analysis based on the expression pattern of all components of the inflammasome machinery analyzed was not able to discriminate the expression pattern of PBMCs at baseline from 6 month after BS [Supplemental Figure 7 by a heat-map) (26)], a Variable Importance in Projection

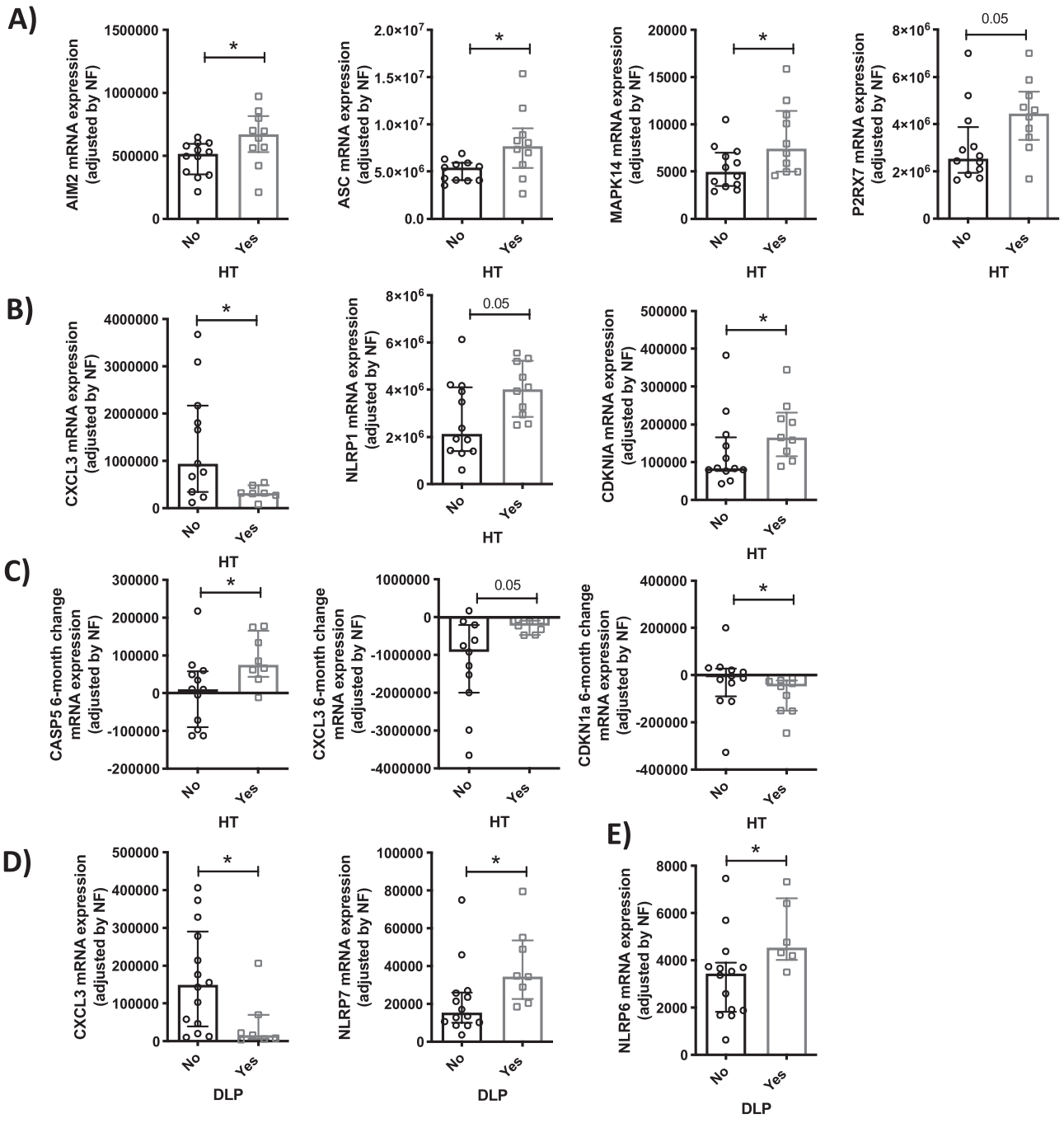

Figure 4. Significant clinical molecular associations of baseline hypertension (HT) and dyslipidemia (DLP) (A) Messenger RNA (mRNA) expression of inflammasome components before surgery in patients with baseline HT. (B) mRNA expression of inflammasome components 6 months after surgery in patients with baseline HT. (C) Absolute change in the mRNA expression of inflammasome components 6 months after surgery in patients with baseline HT. (D) mRNA expression of inflammasome components before surgery in patients with baseline DLP. (E) mRNA expression of inflammasome components 6 months after surgery in patients with baseline DLP. Data represent the median ± interquartile range. Asterisks (*$P < 0.05$) indicate significant changes in the mRNA expression by paired (C) and nonpaired (A, B, D, and E) analysis between the clinical variables.

score of the evaluated inflammasome components (Fig. 5A) indicated that CXCL3, CCL8, TLR4, NLRC4, and NLRP12 were the components that better discriminated patients before and after BS among all the evaluated inflammasome components (Fig. 5B). Therefore, based on these results, we next analyzed the correlation of the expression of these components with key clinical variables. All significant correlations are depicted in Figure 6. In particular, TLR4 levels before BS were strongly correlated to liver and lipid function expressed as alanine aminotransferase and low-density lipoprotein serum levels; in contrast, CCL8 expression was correlated

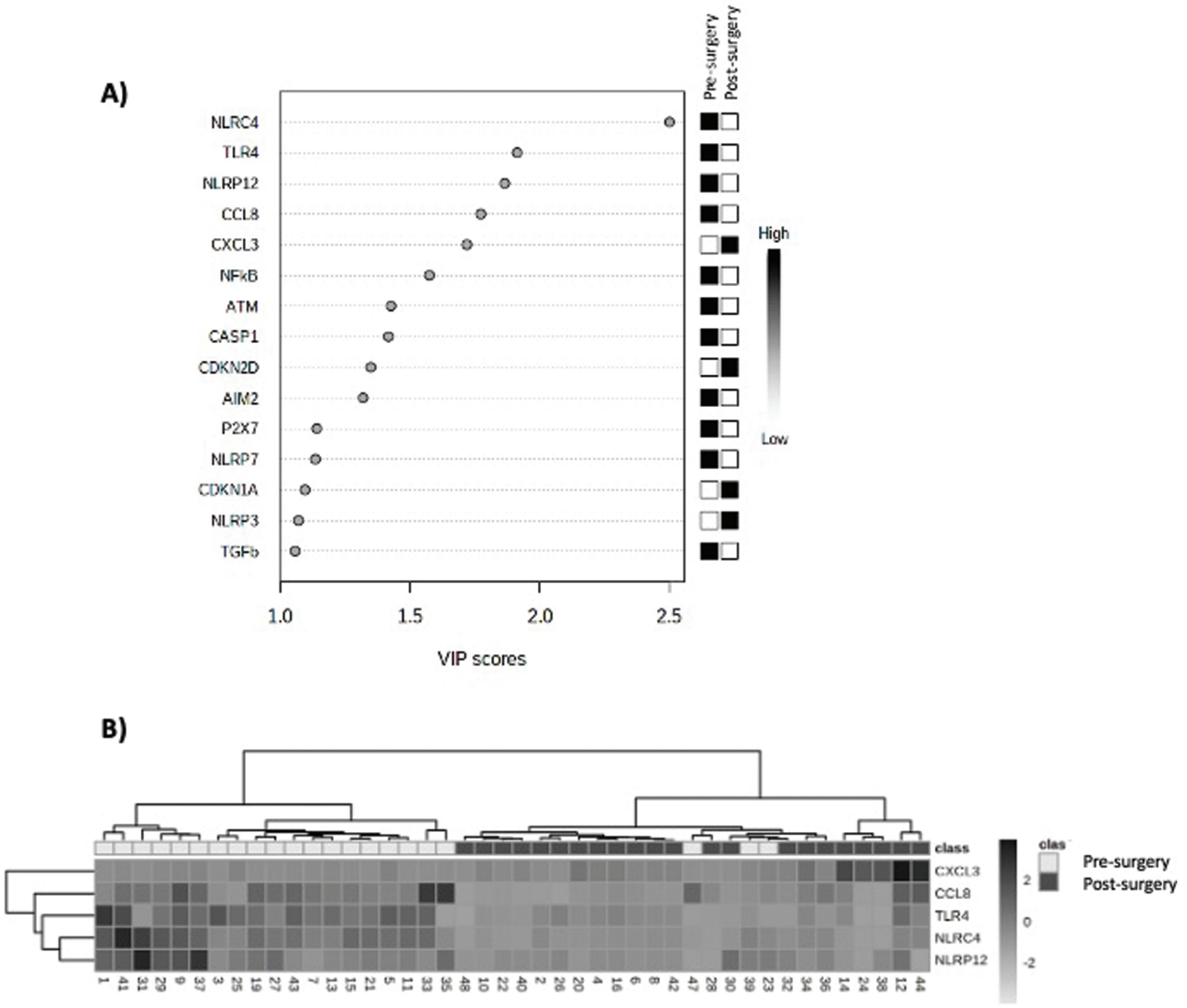

Figure 5. (A) Variable Importance in Projection scores that summarizes the contribution of the evaluated inflammasome components to discriminate pre- and postsurgery patients. (B) Heatmap obtained with the expression levels of the components that better discriminate between patients before and after surgery using bioinformatics analysis of clustering.

with age, serum, and body composition parameters ($P < 0.05$) (Fig. 6A). Interestingly, clinical correlations 6 months after BS were mostly observed between these key inflammasome components, body composition, and excess BW loss ($P < 0.05$) (Fig. 6B). Finally, NLRP12 was the only inflammasome component that was correlated not only with fat mass during follow-up but also with fat-free mass after BS ($P < 0.05$; Fig. 6B).

Alterations of NLRs Influenced Lipid Accumulation and Cell-Cycle Features in HepG2

Specific receptors of the inflammasome (NLRC4 and NLRP12) were selected for functional analysis in the HepG2 cell line based on the altered expression in the whole population of obese patients who underwent BS. Additionally, body fat mass significantly decreased in the whole cohort and in patients who underwent both surgical techniques. Effective silencing of NLRC4 and NLRP12 (Fig. 7A) resulted in a significantly increase levels in lipid accumulation after treatment with OA as compared with the percentage of OA accumulation in the control-treated cells (Fig. 7B). Additionally, the silencing of NLRC4 induced a significant increase of cell viability (Fig. 7C) and a concomitant reduction in the apoptosis rate as compared with the control cells (Fig. 7D). In contract, no changes in cell viability or apoptosis rate were observed in response to NLRP12-silenced cells (Fig. 7C and 7D).

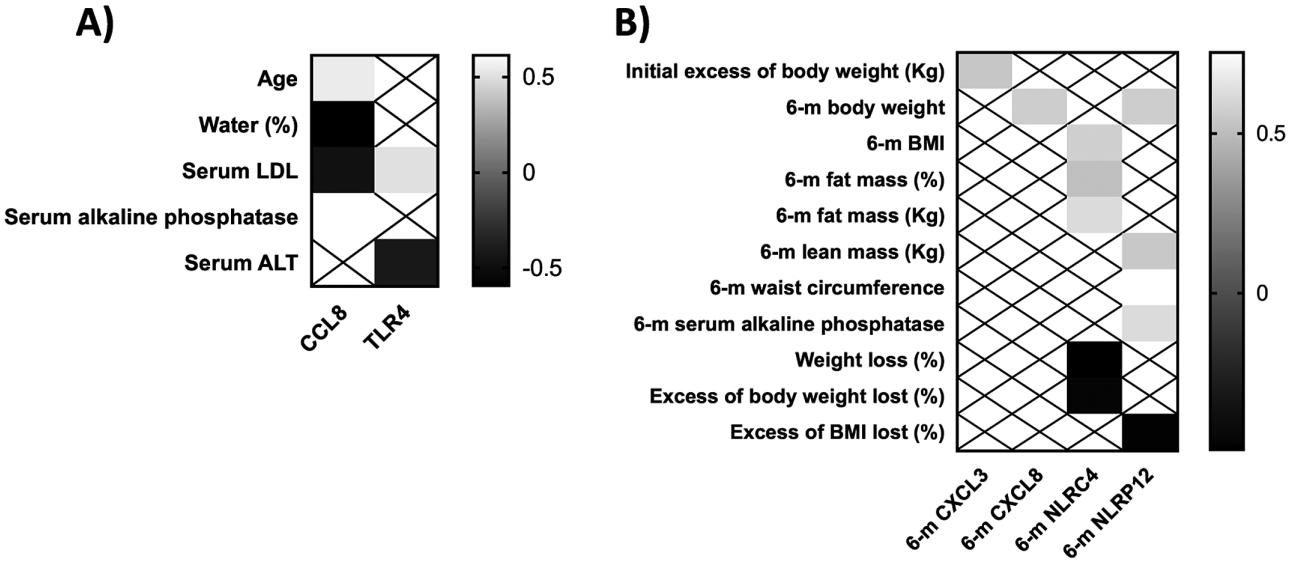

Figure 6. Clinical correlations of some inflammasome components in patients before (A) and 6 months after surgery (B). Only significant correlations ($P < 0.05$) are presented.

Discussion

In the present study, we have comprehensively evaluated the presence and changes in relevant components of the inflammasome machinery and associated-elements after BS in a well-characterized cohort of obese patients, who underwent 1 of the 2 most common BS procedures: RYGB and SG. Additionally, their relation with relevant baseline and follow-up anthropometric and biochemical characteristics was studied. To the best of our knowledge, this is the first report demonstrating that the expression of important inflammasome components is drastically dysregulated after BS (especially NLRP12, NLRC4, TLR4, CCL8, and CXCL3).

Our results demonstrate that the expression of most NLRs is decreased 6 months after BS, especially NLRP12. According to previous publications, NLRP12 seems to play an important role in reducing high-fat diet–induced OB by maintaining beneficial microbiota (43), and it also maintains colon microbiome diversity, which decreases colon inflammation and promotes specific microbes that reverse gut inflammation in chronic bowel inflammation diseases (44). Recently, an antitumorigenic effect of NLRP12 has been also described by downregulating JNK activation in mouse hepatocytes (45). JNK is activated by high-fat diet and OB, and its inhibition maintains insulin sensitivity (46). NLRP12 is also associated with metabolic-induced cancer and chronic inflammatory diseases (47), suggesting that its role as target in the treatment of OB-induced comorbidities may be relevant and requires further attention. Importantly, our data demonstrate that NLRP12 was strongly decreased in PBMCs of patients after BS, especially in women, and was significantly correlated with fat mass and abdominal perimeter after BS, but not with the presence of T2DM before BS. Thus, these data may be clinically relevant based on the critical metabolic role of NLRP12 as previously discussed.

Additionally, NLRC4 was strongly decreased after BS in the PBMCs of our cohort, and its expression was positively associated with fat mass and negatively associated with excess of BW loss. Regarding this, previous studies have reported direct actions of NLRC4 in adipocytes, where it can induce the activation of IL-1 signaling, promoting angiogenesis, and even breast cancer progression (48). It is well known that BS is associated with decreased risk of hormone-related cancer, but the specific mechanisms are not well understood (49). Therefore, based on the results of this study, we may speculate that 1 of these putative mechanisms may be related to the mediation of this inflammasome component. Another explanation for the observed changes in NLCR4 is gut microbiota. It is well-known that significant changes in the microbiota occur after BS due to changes in the metabolism of bile acids, hormones, and gastric pH (50,51). Changes in the microbiota are related to the BS procedure (52), in concordance with our results, since we observed significant differences in the inhibition of NLCR4 induced by RYGB and SG. Further studies that include gut microbiota analysis should be performed.

The effect of BS in liver disease is controversial. Some studies describe significant improvement in steatosis in nonalcoholic steatohepatitis, but changes in fibrosis are controversial, since both significant improvement and worsened condition have been described (53-56). Even

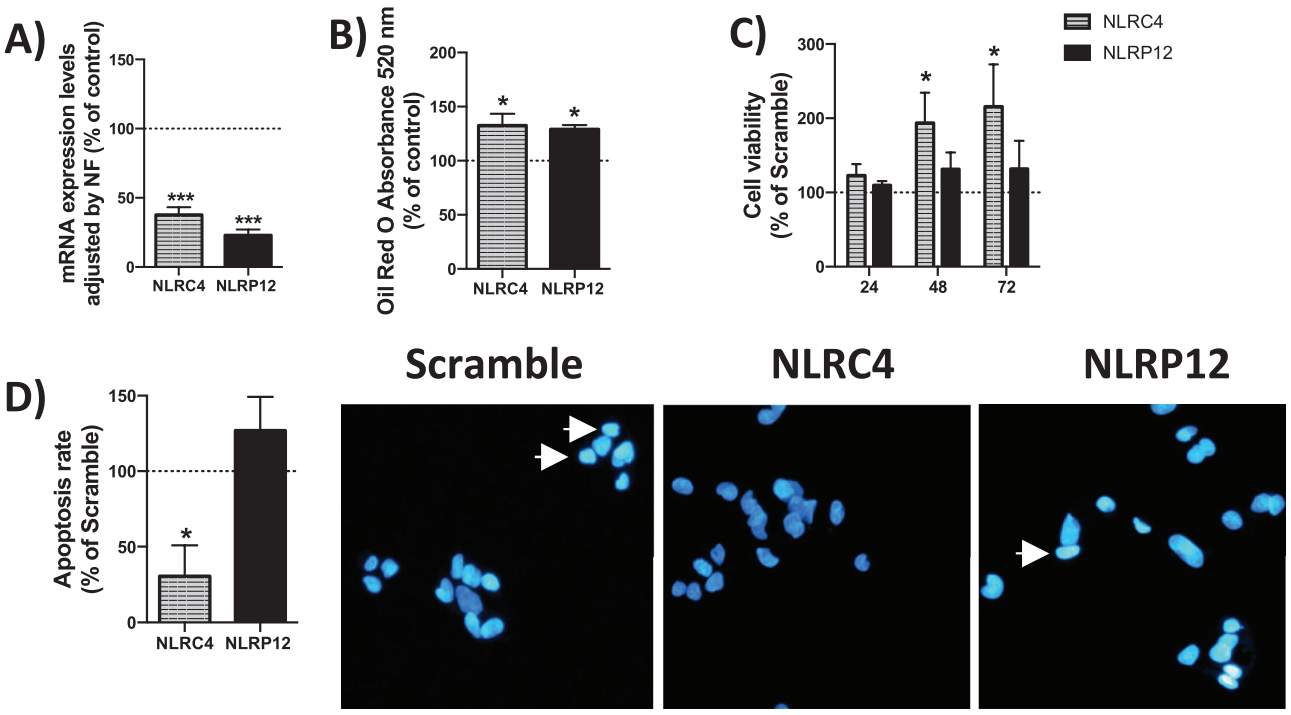

Figure 7. Alterations of Nucleotide-binding oligomerization domain-like receptors influenced lipid accumulation and cell cycle features in HepG2. (A) Quantitative polymerase chain reaction validation of the silencing with specific small inferring RNAs (siRNA). Messenger RNA expression levels adjusted by the expression level of beta actin. Data are expressed as a percentage of control siRNA (Scramble; set at 100%). (B) Validation of lipid accumulation in HepG2 cell lines by Oil Red O absorbance at 520 nm. (C) Cell viability of NLRC4 and NLRP12 silenced cells compared to scramble cells. (D) Apoptosis rate of transfected cells compared to control siRNA and representative images of stained nuclei after 24 h. Data are expressed as a percentage of the control (Scramble; normalized to 100%).

more, liver disease is not a routine indication for BS in patients with OB (54,56). In this context, we evaluated the in vitro silencing of the expression of NLRC4 (and NLRP12) in liver cells, which resulted in increased lipid accumulation despite their association with decreased fat mass and excess BW loss. These findings suggest that the modulation of inflammasome components may be cell dependent, as previously described (57), and the specific effects in liver cells should be evaluated separately. Related to this, NLRP3, NLRP6, and IL-18 can negatively modulate NAFLD progression in humans (58,59) and represent important targets for this relevant OB-related comorbidity. Results about NLCR4 and NLRP12 are still lacking.

TLR4 signaling pathway is recognized as a main trigger of OB-induced inflammatory response (60). TLR4 is expressed not only in innate immune cells but also in other cell types, including adipocytes (60). Additionally, it has been suggested that saturated fatty acids can also bind to TLR4 and activate TLR4-mediated signaling pathways (61), representing an additional link between inflammasome and OB (60). Remarkably, this relation was observed in our study when postsurgery TLR4 expression levels were correlated with postsurgery BW. Moreover, correlation between TLR4, dyslipidemia, or lipid profile before or after BS was observed. Importantly, TLR4 signaling is increased specially in nonalcoholic steatohepatitis compared to NAFLD (62). In this sense, since we did not include patients with nonalcoholic steatohepatitis, this may explain the negative correlation between baseline TLR4 levels and liver enzymes serum values.

Previous studies have reported that CCL8 is increased in obese patients compared to lean subjects and in obese patients with insulin resistance compared to noninsulin resistant patients, and it is associated with other proinflammatory cytokines in adipose tissue (63,64). In concordance with this, CCL8 levels decreased 6 months after surgery and were correlated with BW 6 months after surgery; remarkably, it was not associated with the presence of dyslipidemia at diagnosis or its reversal. In this sense, further studies should be performed to confirm our results and to determine the clinical relevance of this factor in patients with metabolic syndrome.

Besides CCL8, it has been reported that CXCL3 is widely expressed in human adipocyte tissue, showing a higher expression level in preadipocytes compared with mature adipocytes, and it seems to regulate adipocyte differentiation possibly through the JNK pathway activation (65). This fact may explain its correlation with baseline excess of BW, but in our study, CXCL3 expression was significantly increased after BS and negatively

associated with baseline dyslipidemia and postsurgical HT. Additionally, a positive correlation between its postoperative expression levels and the initial excess of BW was also observed. Therefore, although further studies would be required to determine the precise role of CXCL3, all these findings may suggest the idea that this chemokine may have a protective role for metabolic comorbidities.

Furthermore, in our cohort, T2DM was associated with the expression of some inflammasome components including CCL2, CXCR1, and SIRT1, which may have important clinical implications. These findings, specifically related to CCL2 and CXCR1, were confirmed in a second cohort of patients. Regarding this, it has been described that CCL2 is expressed by insulin-producing cells, and it can produce insulitis and, consequently, islet destruction (66). Additionally, CCL2 has been associated to diabetes-induced kidney injury (67) and delayed wound healing (68), suggesting a role as putative target for preventing diabetes-related complications. Interestingly, the inhibition of CXCR1 has been suggested as a putative mechanism for reversing type 1 diabetes according to animal models (69,70). In this context, some CXCR1/2 inhibitors are currently being evaluated for treating inflammatory diseases including diabetes and several types of cancer (71,72).

Remarkably, in our study, SIRT1 levels were also elevated in patients with T2DM, and its response to BS seemed to be dependent on the presence of T2DM. In T2DM patients, the increase in the expression levels of SIRT1 induced after BS may be associated, at least in part, to a downregulation in the expression of specific regulators, such as microRNA 448, which has been demonstrated by other groups (73). Recently, another study revealed that kallistatin, whose circulating levels were increased in obese patients after RYGB, is implicated in the upregulation of SIRT1, triggering a downregulation of many inflammatory key genes (74). These findings demonstrate that SIRT1 may have a relevant role in the regulation of many inflammatory components, especially under OB conditions. Importantly, previous publications suggest that some inflammasome components are differentially expressed in patients with T2DM despite weight loss after a bypass surgery, suggesting a persistent inflammatory state in this population (75), which explains that some evaluated components remained unchanged after BS in our cohort.

Previous publications suggest that AIM2 is associated with atherosclerosis and vascular disease (76,77). Additionally, mitogen-activated protein kinase 14 has been suggested as a cardiovascular prognostic marker (78). Accordingly, in our cohort we observed increased levels of these components in patients with high blood pressure, suggesting a putative role of these inflammasome components in the reversion of cardiovascular events after weight loss induced by BS.

Importantly, previous reports have described the link between NLRP3 and OB-associated comorbidities (23,24); even a significant reduction in NLRP3 expression levels in adipose tissue after BS has been reported (79). In our cohort, this finding was not confirmed, and, in contrast, a tendency to an increased expression of NLRP3was observed; however, we could not evaluate this component in adipose tissue, which may explain the differences observed in both studies.

Additionally, circulating IL levels also reflected the mRNA expression of some inflammasome components, suggesting an appropriate correlation between molecular and circulating markers as has been previously described in humans (80,81). Importantly, the persistent levels suggest that despite weight loss 6 months after BS, the OB-related inflammation state persists in these patients and that serum reductions would require increased BW loss as has been previously observed in other studies (82).

Taken together, our results unveiled new conceptual and functional avenues in the OB/BS field, with potential clinical implications, by demonstrating for the first time a clear dysregulation of key components of the inflammasome machinery (especially NLRP12, NLRC4, TLR4, CCL8, and CXCL3) in obese patients after BS, which may be closely related to improvement and remission of OB-related comorbidities. Specifically, our data suggest that these specific components of the inflammasome machinery may play an important role as markers for predicting OB-related comorbidities and represent putative targets for reversing OB-related complications. Moreover, we unveil novel evidence for a critical role of some NLRs (especially NLRC4) in pathophysiological processes, such as lipid accumulation, cell viability, and apoptosis rate. Although these results should be confirmed in larger cohorts, including patients of different races and may also be reinforced, for instance, by the inclusion of gut microbiota analysis and by the evaluation of different metabolic tissues and further in vitro studies, our study provides solid, convincing evidence demonstrating that some components of the inflammasome machinery are associated and may play a critical physiopathological role in the improvement and remission of OB-related comorbidities observed in the present study, offering a clinically relevant opportunity for novel targets that should be tested in humans.

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Disclosures: The authors have no conflicts of interest to declare.

Data Availability: Some or all data generated or analyzed during this study are included in this published article or in the data repositories listed in references.

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ARTICLE II

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Clinical Research Article

Clinical Research Article

Influence of Obesity in the miRNome: miR-4454, a Key Regulator of Insulin Response Via Splicing Modulation in Prostate

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Abbreviations: ACTB, Actin beta; BMI, body mass index; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GO, Gene Ontology; HOMA, Homeostatic Model Assessment; HOMA-IR, Homeostatic Model Assessment-Insulin Resistance; HPRT, hypoxanthine-guanine phosphoribosyltransferase; miRNA, microRNA; qPCR, quantitative real-time PCR; ROC, receiver operating characteristic; T2DM, type 2 diabetes mellitus

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Abstract

Context: Obesity is a major health problem associated with severe comorbidities, including type 2 diabetes and cancer, wherein microRNAs (miRNAs) might be useful as diagnostic/prognostic tools or therapeutic targets.

Objective: To explore the differential expression pattern of miRNAs in obesity and their putative role in obesity-related comorbidities such as insulin resistance.

Methods: An Affymetrix-miRNA array was performed in plasma samples from normoweight (n = 4/body mass index < 25) and obese subjects (n = 4/body mass index > 30). The main changes were validated in 2 independent cohorts (n = 221/n = 18). Additionally, in silico approaches were performed and in vitro assays applied in tissue samples and prostate (RWPE-1) and liver (HepG2) cell-lines.

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Results: A total of 26 microRNAs were altered ($P < 0.01$) in plasma of obese subjects compared to controls using the Affymetrix-miRNA array. Validation in ampler cohorts revealed that miR-4454 levels were consistently higher in obesity, associated with insulin-resistance (Homeostatic Model Assessment of Insulin Resistance/insulin) and modulated by medical (metformin/statins) and surgical (bariatric surgery) strategies. miR-4454 was highly expressed in prostate and liver tissues and its expression was increased in prostate and liver cells by insulin. In vitro, overexpression of miR-4454 in prostate cells resulted in decreased expression levels of *INSR*, *GLUT4*, and phosphorylation of AMPK/AKT/ERK, as well as in altered expression of key spliceosome components (ESRP1/ESRP2/RBM45/RNU2) and insulin-receptor splicing variants.

Conclusions: Obesity was associated to an alteration of the plasmatic miRNA landscape, wherein miR-4454 levels were higher, associated with insulin-resistance and modulated by obesity-controlling interventions. Insulin regulated miR-4454, which, in turn may impair the cellular response to insulin, in a cell type-dependent manner (i.e., prostate gland), by modulating the splicing process.

Key Words: miRNA, obesity, insulin, insulin receptor, splicing

Obesity is a major health problem that arises as a chronic long-term imbalance between calorie intake and energy expenditure. Despite all the strategies implemented to manage this pathology, the prevalence of obesity is still increasing and approximately 600 million people worldwide are predicted to be obese by 2025 according to World Health Organization estimations (1, 2). Obesity is the most common cause of insulin resistance, but overt diabetes does not develop in the majority of obese individuals because they can preserve normoglycemia through an enhanced insulin secretion, resulting in compensatory hyperinsulinemia (3). However, in susceptible individuals, compensatory hyperinsulinemia cannot be maintained, in consequence prediabetes and type 2 diabetes mellitus (T2DM) develop (4). In this sense, it is critical to understand the molecular mechanisms involved in the onset of obesity and its associated comorbidities. In fact, it has been demonstrated that the development and progression of obesity and other associated comorbidities, including insulin resistance (5), is closely linked to dysregulation of key metabolic factors, especially those implicated in the cellular control of the energy balance (e.g., insulin, leptin, glucose transporters), which are, in turn, controlled by specific cellular regulatory mechanisms, such as microRNAs.

MicroRNAs (or miRNAs) are short noncoding RNAs (20-22 nucleotides) that act at posttranscriptional level to regulate gene expression (6). Evidence is accumulating that circulating miRNAs can act as endocrine factors in that they are released to the circulation by many tissues (7). Indeed, miRNAs seem to serve as endocrine and paracrine messengers that facilitate communication between donor and target cells, thereby potentially exerting important roles in metabolic organs crosstalk (7). For these reasons, it has been postulated that circulating miRNAs could exert crucial roles in the development and progression of obesity and its related comorbidities. Previous, somewhat scattered studies have reported that some miRNAs are dysregulated in plasma samples of obesity subjects, including miR-142-3p, miR-221, or miR-222 (8-13). For example, using TaqMan technology, Heneghan et al. found in a cross-sectional study with 30 obese patients and 20 nonobese control participants that miR-17-5p and miR-132 were reduced in whole blood from obese patients compared with controls; whereas Pescador et al., using miRNA panels in a cross-sectional study with 20 obese patients and 20 control participants, found that miR-138, miR-376a, and miR-503 were reduced and miR-15b increased in obese compared with controls; and Cui et al. found that miR-222, miR-486, miR-146b, miR-146a, miR-20a, miR-15b, and miR-26b were increased in obese compared with controls, using miRNA sequencing in a cross-sectional study with 100 obese patients and 146 normal control participants. However, results generated hitherto are inconsistent among studies and cannot be compared because of several factors, including different cohort characteristics (obese, morbid obese, diabetic), study designs (cross-sectional, transversal), or detection methods (TaqMan, SYBR Green, micro- or macroarrays, small RNA, or miRNA sequencing). Hence, the whole pattern of dysregulations of miRNAs in obese condition is still to be reliably defined. In this sense, it has been shown by previous studies that high-throughput technologies may represent one of the most precise and reliable tools for the quantification of miRNAs (14). Unfortunately, although several of the previously mentioned studies

have reported the dysregulation of different miRNAs in obesity condition, to the best of our knowledge, there are no studies describing the whole miRNome using miRNAs-specific arrays.

In this context, and considering the potential relationship between metabolic disorders, such as obesity, and the alteration of cellular mechanisms that control gene expression such as miRNAs, we aimed to explore for the first time the dysregulation of the miRNoma in obesity condition. Our results unveiled a marked dysregulation of miR-4454, and thus, we sought to explore the pathophysiological role of this miRNA and the molecular events underlying its actions.

Material and Methods

Human plasma samples

Plasma samples from 3 cohorts of male patients: (1) cohort A or discovery cohort (n = 8; Table 1); (2) cohort B or validation cohort (n = 221; Table 2); and (3) cohort C or bariatric surgery cohort (n = 18; Table 3), were collected from the Reina Sofia University Hospital (cohorts A and B) and Virgen de la Victoria University Hospital (cohort C). All samples were obtained through the Andalusian Biobank (Servicio Andaluz de Salud, Spain). Inclusion and exclusion criteria have been reported previously (15-17). In summary, subjects from cohorts A and C were selected based on their normoweight (body mass index [BMI] < 25; n = 4 and 10, respectively) and obesity (BMI ≥ 30; n = 4 and 8, respectively) condition, and without any medical treatment associated to their metabolic condition (i.e., metformin, statin). Subjects from cohort B were divided in 3 groups: normoweight (BMI < 25; n = 80), overweight (BMI ≥ 25 and <30; n = 79), and obese (BMI ≥ 30; n = 62), and medical treatment associated to their metabolic condition was recorded (treatment or not with metformin and/or statins). In the bariatric surgery group (cohort C), samples from obese subjects (n = 8) were analyzed before and 12 months after bariatric surgery. This study was approved by the Ethics Committees of the Reina Sofia University Hospital (Cordoba, Spain) and Complejo Hospitalario de Málaga (Virgen de la Victoria), and was conducted in accordance with the Declaration of Helsinki and according to national and international guidelines. All patients with obesity or metabolic complications were treated according to the current clinical guidelines. Each patient or family member signed a written informed consent before inclusion into the study.

Human tissue samples

A Human Total RNA Panel (Clontech, Takara Bio, France) was used to determine the expression profile of miRNAs from different tissues (thyroid, liver, lung, testis, heart, prostate, and kidney).

Cell cultures, reagents, and transfection

The human normal-like prostate-derived RWPE-1 and the human hepatocyte-derived HepG2 cell lines were obtained from ATCC, cultured, and maintained under manufacturer recommendations. These cell lines were validated by analysis of short tandem repeats (GenePrint 10 System; Promega, Barcelona, Spain) and checked for mycoplasma contamination (18, 19). To analyze the impact of metabolic insults, RWPE-1 and HepG2 cells were plated onto 12-well tissue culture plates (150 000 cells/well), incubated for 24 hours (37°C), starved 24 hours with

Table 1. General Characteristics of the Discovery Samples

General Characteristic	Normoweight Patients	Obese Patients	*P* Value
Sex			
Male	4	4	
Age (y)	57.25 ± 6.23	59.25 ± 5.18	0.65
Waist circumference (cm)	90 ± 3.16	106.3 ± 5.31	0.03
Weight (kg)	74.38 ± 5.25	92.45 ± 2.21	0.03
Body mass index	24.48 ± 0.48	31.82 ± 1.12	0.03
Glucose (mg/dL)	96.50 ± 8.26	102.5 ± 18.81	0.99
Hemoglobin A1c (%)	5.47 ± 0.09	5.85 ± 0.78	0.28
Insulin (mU/L)	4.72 ± 2.07	5.35 ± 3.86	0.62
HOMA-IR	1.10 ± 0.44	1.29 ± 0.83	0.48
c-LDL	166.8 ± 21.55	150.5 ± 18.3	0.48
c-HDL	47.25 ± 5.43	46.5 ± 6.45	0.82
Total cholesterol (mg/dL)	238.3 ± 23.04	225 ± 22.21	0.48

Abbreviations: c-HDL, cholesterol high-density lipoprotein; c-LDL, cholesterol low-density lipoprotein; HOMA-IR, Homeostatic Model Assessment-Insulin Resistance.

Table 2. General Characteristics of the Validation Samples

General Characteristic	Normoweight Patients	Overweight Patients	Obese Patients	*P* Value
Sex				
Male	80	78	62	
Age (y)	63.38 ± 6.48	65.67 ± 5.94	63.44 ± 7.29	0.05
Waist circumference (cm)	89.53 ± 5.83	101.38 ± 6.4	111.62 ± 7.51	<0.0001
Weight (kg)	66.93 ± 6.84	78.13 ± 7.23	93.85 ± 10.95	0.0001
Body mass index	23.5 ± 1.35	27.69 ± 1.38	32.91 ± 2.45	<0.0001
Glucose (mg/dL)	102.24 ± 24.63	115.91 ± 37.06	126.63 ± 40.45	<0.0001
Hemoglobin A1c (%)	5.55 ± 0.73	5.96 ± 0.99	6.27 ± 1.22	<0.0001
Insulin (mU/L)	5.43 ± 2.47	9.6 ± 8.33	10.50 ± 5.86	<0.0001
HOMA-IR	1.41 ± 0.87	2.87 ± 2.84	3.38 ± 2.36	<0.0001
c-LDL	133.04 ± 29.24	188.62 ± 30.78	124.45 ± 33.94	0.021
c-HDL	49.03 ± 11.34	44.44 ± 9.72	42.23 ± 10.88	0.03
Total cholesterol (mg/dL)	203.44 ± 32.99	188.62 ± 30.78	193.92 ± 37.06	<0.0001

Abbreviations: c-HDL, cholesterol high-density lipoprotein; c-LDL, cholesterol low-density lipoprotein; HOMA-IR, Homeostatic Model Assessment-Insulin Resistance.

Table 3. General Characteristics of Bariatric Surgery Samples

General Characteristic	Normoweight Patients	Obese Patients (Presurgery)	*P* Value	Obese Patients (Postsurgery)	*P* Value
Sex					
Male	10	8		8	
Age (y)	44.90 ± 13.13	40.5 ± 7.81	0.012		
Waist circumference (cm)	90.30 ± 4.19	149.12 ± 16.21	0.007	115.37 ± 12.52	0.04
Weight (kg)	70.30 ± 5.98	161.01 ± 27	<0.0001	107.96 ± 16.56	<0.0001
Body mass index	24.03 ± 1.26	52.96 ± 8.23	<0.0001	35.71 ± 5.81	<0.0001
Glucose (mg/dL)	100.1 ± 10.13	95.87 ± 15.17	0.29	82.75 ± 6.18	0.04
Hemoglobin A1c (%)	5.43 ± 0.45	5.55 ± 0.49	0.77	5.12 ± 0.26	0.19
Insulin (mU/L)	6.93 ± 4.63	16.90 ± 10.14	0.14	10.87 ± 4.59	0.24
HOMA-IR	1.63 ± 0.59	4.02 ± 2.31	0.023	1.92 ± 0.87	0.09
c-LDL	134.88 ± 33.86	103.65 ± 34.33	0.65	104.02 ± 22.98	0.54
c-HDL	57.60 ± 15.63	53.75 ± 41.47	0.21	52.8 ± 6.54	0.88
Total cholesterol (mg/dL)	211.3 ± 39.82	156.12 ± 28.74	0.001	171.25 ± 25.25	0.81

Abbreviations: c-HDL, cholesterol high-density lipoprotein; c-LDL, cholesterol low-density lipoprotein; HOMA-IR, Homeostatic Model Assessment-Insulin Resistance.

serum free medium, and treated with glucose (25 mM) or insulin (100 nM) for 24 hours. In addition, RWPE-1 cells were seeded onto 12-well tissue culture plates and serum-starved for 24 hours previous to the transfection with miR-4454 miRNA Mimic (1 nM) (Qiagen, Hilden, Germany) using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA) and following the guidelines provided by the manufacturer. Transfected cells were collected for RNA and protein isolation 48 hours after the transfection.

miRNA extraction and retrotranscription

All plasma samples were processed using the Maxwell 16 miRNA Tissue Kit (Promega, WI) and extracted using the Maxwell 16 MDx Instrument (Promega) to isolate and purify microRNAs. Specifically, 300 µL of plasma was used for extraction and miRNAs were eluted in 30 µL nuclease-free water. Specific spike-in, UniSp2, UniSp4, and UniSp5 (miRCURY LNA Universal RT microRNA PCR, Exiqon, Vedbaek, Denmark) were mixed with the plasma samples to verify the extraction methodology. The quality and concentration of RNA extracted from these samples were evaluated using Nanodrop One Spectrophotometer (Thermo Fisher Scientific). Subsequently, retrotranscription of these samples was performed using the miRCURY LNA RT Kit (Qiagen), following the manufacturer instruction.

Total RNA from RWPE-1 and HepG2 cells was isolated using TRI Reagent (Sigma-Aldrich, Madrid, Spain), followed by DNAse treatment using the RNase-Free Dnase Kit (Qiagen), as previously reported (20, 21). The amount and purity of RNA recovered was determined

using the Nanodrop One Spectrophotometer (Thermo Fisher Scientific). One microgram of RNA was reverse transcribed to cDNA, using random hexamer primers and the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). To analyze the expression level of miR-4454, retrotranscription of these samples was performed using the miRCURY LNA RT Kit (Qiagen), following the manufacturer instructions.

miRNome analysis

The circulating levels of the whole miRNome was determined and compared between normoweight and obese subjects (cohort A; n = 4/group) using the Affymetrix miRNA 4.1 array (Affymetrix, Santa Clara, CA) and 50 ng of the extracted plasma miRNAs. Biotin-labeled RNA was synthesized using the FlashTag Biotin HSR RNA Labelling Kit (Thermo Fisher Scientific). Each sample was hybridized onto a GeneChip miRNA 4.1 Array Plate (Thermo Fisher Scientific) following the manufacturer's protocol.

To determine which was the most stably expressed miRNA and therefore, the one that could be used as the best housekeeping gene for our study, several standard criteria were established: (1) sufficiently high levels to allow its quantification by quantitative real-time PCR (qPCR); (2) lower difference in the levels between groups, attending to higher (closer to 1) *P* value; and (3) lower standard error of the mean. According to these parameters, our results revealed that miR-16-5p was the most stable miRNA and the most appropriate housekeeping for the determination of the levels of miRNAs in our samples. Subsequently, the most altered miRNAs in plasma from obese compared with normoweight subjects were selected according to fold-change *P* value (<0.01) and receiver operating characteristic (ROC) curve (>0.9).

qPCR

qPCR was used to determine the levels of selected miRNAs in validation cohorts of samples, in the panel of human tissues (Takara Bio) and in different cells lines; and to determine the expression of other genes of interest in cell lines. In particular, to validate the previous results from the array, the levels of 7 selected miRNAs (miR-4499, 671-3p, 21-3p, 548at-5p, 4454, 802, and 4473; based on a *P* value < 0.01 and ROC curve > 0.9) was measured in the validation cohorts of samples (A and B). Specific primers for these miRNAs (miRCURY miRNA assay, Qiagen) were used in combination with GoTaq qPCR Master Mix (Promega) using the Stratagen Mx3000P (Agilent Technologies, Madrid, Spain), following manufacturer instruction. mRNA levels were normalized according to the levels of miR-16-5p whose levels did not significantly vary among the different experimental groups in both validation cohorts B and C (data not shown). Similar approaches were used to determine the expression of miRNAs of interest in the panel of human tissues samples (Takara Bio) and in RWPE and HepG2 cell lines; however, in these cases, data were normalized using U6 expression, the most accepted housekeeping for tissues and cells.

The expression of selected transcripts of interest was quantified using specific primers (22) designed and validated by our group as previously reported (21, 23) and the Brilliant III SYBR Green Master Mix (Stratagene, La Jolla, CA) in the Stratagene Mx3000P system. The expression level of each mRNA transcript was adjusted by a normalization factor obtained from the expression levels of 3 different housekeeping genes (Actin beta [ACTB], hypoxanthine-guanine phosphoribosyltransferase [HPRT], and glyceraldehyde-3-phosphate dehydrogenase [GAPDH], which were found to be stable in the experimental conditions) using GeNorm 3.5 (24).

Measurement of splicing machinery in response to overexpression of miR-4454 using a qPCR dynamic array based on microfluidic technology

A qPCR dynamic array based on microfluidic technology (Fluidigm, San Francisco, CA) was used to simultaneously determine the expression of 48 components of the major spliceosome (n = 13), minor spliceosome (n = 4), associated splicing factors (n = 28), and 3 housekeeping genes (ACTB, GAPDH, and HPRT) in RWPE-1 cells overexpressing miR-4454. Specific primers for these transcripts were specifically designed with the Primer3 software and StepOne Real-Time PCR System software v2.3 (Applied Biosystems, Foster City, CA) (22). Preamplification, exonuclease treatment, and qPCR dynamic array based on microfluidic technology were implemented as recently reported (18, 23, 25), following manufacturer's instructions using the Biomark System and the Real-Time PCR Analysis Software (Fluidigm).

Western blotting

RWPE-1 cells were also processed to analyze protein levels by western blot after transfection with miR-4454 for 48 hours using methods previously reported (18). Specifically, 200 000 cells were seeded in 12-well plates and 48 hours after the transfection, proteins were collected using prewarmed (65°C) SDS-dithiothreitol buffer (62.5 mM Tris-HCl, 2% SDS, 20% glycerol, 100 mM dithiothreitol, and 0.005% bromophenol blue). Then, proteins were sonicated for 10 seconds and boiled for 5 minutes at 95°C.

Proteins were separated by SDS-PAGE and transferred to nitrocellulose-membranes (Millipore, Billerica, MA). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline/0.05% Tween 20 and incubated overnight with the specific antibodies for phospho-AKT (Cell Signaling Technology [CST], #9272S), AKT (CST, #4060S), phospho-AMPK (CST, #50081S), AMPK (CST, #5831S), anti-ERK1/2 (CST, #9102S) and anti-pERK (CST, #4370S), and secondary horseradish peroxidase-conjugated goat anti-rabbit IgG (CST, #7074S) were used. Proteins were detected using and enhanced chemiluminescence detection system (GEHealthcare, Madrid, Spain) with dyed molecular weight markers (Bio-Rad, Madrid, Spain). Densitometric analysis of the bands obtained was carried out with ImageJ software.

Statistical analysis

The Kolmogorov-Smirnov test was used to analyze the normality of the data sets. Parametric data were compared by 2-tailed *t* test, whereas nonparametric data were compared by Mann-Whitney test. Multiple comparison tests were performed to analyze the data from the Affymetrix array. Correlations were studied using Spearman's correlation test. Representation of volcano plot and clustering analysis by heatmaps were created using MetaboAnalyst (26). The Gene Ontology (GO) enrichment analysis for biological process and Kyoto Encyclopedia of Genes and Genomes pathway was generated by "DIANA-miRPath v3.0." All data were obtained from at least 3 independent experiments from different cellular passages and expressed as mean ± SEM. *P* values < 0.05 were considered statistically significant. All statistical analyses were performed using the GraphPad Prism 6 (La Jolla, CA) and SPSS version 17.0 (SPSS Inc., Chicago, IL).

Results

Dysregulation of plasma miRNAs landscape in obese subjects

Results using plasma samples from normoweight (n = 4) and obese subjects (n = 4) and the Affymetrix miRNA 4.1 array (which consistently detected the complete list of mature human miRNAs from the miRNome included in the array in all plasma samples analyzed with the exception of 18 transcripts) demonstrated a significant dysregulation ($P < 0.01$) in the circulating pattern of several miRNAs (n = 26), wherein 13 of them presented lower levels and 13 higher levels in obese compared with normoweight patients (Fig. 1A). Of note, 7 of these significantly dysregulated miRNAs (i.e., lower levels of miR-21-3p/miR-548at-5p/miR-802, and higher levels of miR-671-3p/miR-4454/miR-4473/miR-4499 in obese vs. normoweight subjects; $P < 0.01$) also presented a statistically significant ROC curve to distinguish between obese and normoweight subjects with an area under the curve > 0.9 (Fig. 1B), and were therefore selected for further validation. Interestingly, unsupervised clustering of normoweight and obese patients revealed that either the plasma levels of the 26 miRNAs differentially expressed (22) or the levels of these top 7 differentially expressed miRNAs (Fig. 1C) can discriminate between normoweight and obese subjects.

As mentioned previously and to corroborate these results, the top 7 selected miRNAs were analyzed by qPCR in an ampler and well-characterized cohort of subjects (cohort B). This analysis showed that the levels of 6 of these miRNAs were very low or under the detection levels in most of the samples analyzed (i.e., miR-21-3p/miR-548at-5p/miR-802/miR-671-3p/miR-4473/miR-4499), whereas only miR-4454 was robustly detected in all the samples. Importantly, results with miR-4454 observed in the initial cohort of patients were corroborated in this ampler cohort of individuals (Fig. 2A). Specifically, plasma levels of miR-4454 were significantly higher in the obese group compared with the normoweight and also to the overweight groups (Fig. 2A). Interestingly, miR-4454 was also associated with insulin resistance, inasmuch as the elevation of plasma levels of miR-4454 was also found when all subjects were classified according to the Homeostatic Model Assessment (HOMA) index (stratified in 3 groups, HOMA 1-3, that represent tertiles), being significant in the comparison between the first and the third tertiles (Fig. 2B). In close parallelism, plasma levels of miR-4454 were directly correlated with insulin and HOMA index, but not with glucose level or other available clinical parameters (such as hemoglobin A1c, C-reactive protein, or plasma lipids [cholesterol, triglycerides]) in the subjects included in cohort B (Fig. 2C). Remarkably, when obese subjects of cohort B were classified according to their treatment with metformin or statins, a significant decrease in the circulating levels of miR-4454 was observed in the treated groups compared with the nontreated groups (Fig. 2D).

To further explore the importance of miR-4454 in obesity, an independent cohort of samples was analyzed (cohort C), which confirmed the elevation in the circulating levels of miR-4454 in subjects with obesity compared with normoweight subjects (Fig. 2E), and also revealed an interesting reduction of the plasma levels of miR-4454 in obese patients after bariatric surgery (Fig. 2E). Interestingly, correlations between miR-4454 levels and insulin and HOMA-insulin resistance (HOMA-IR) were also observed

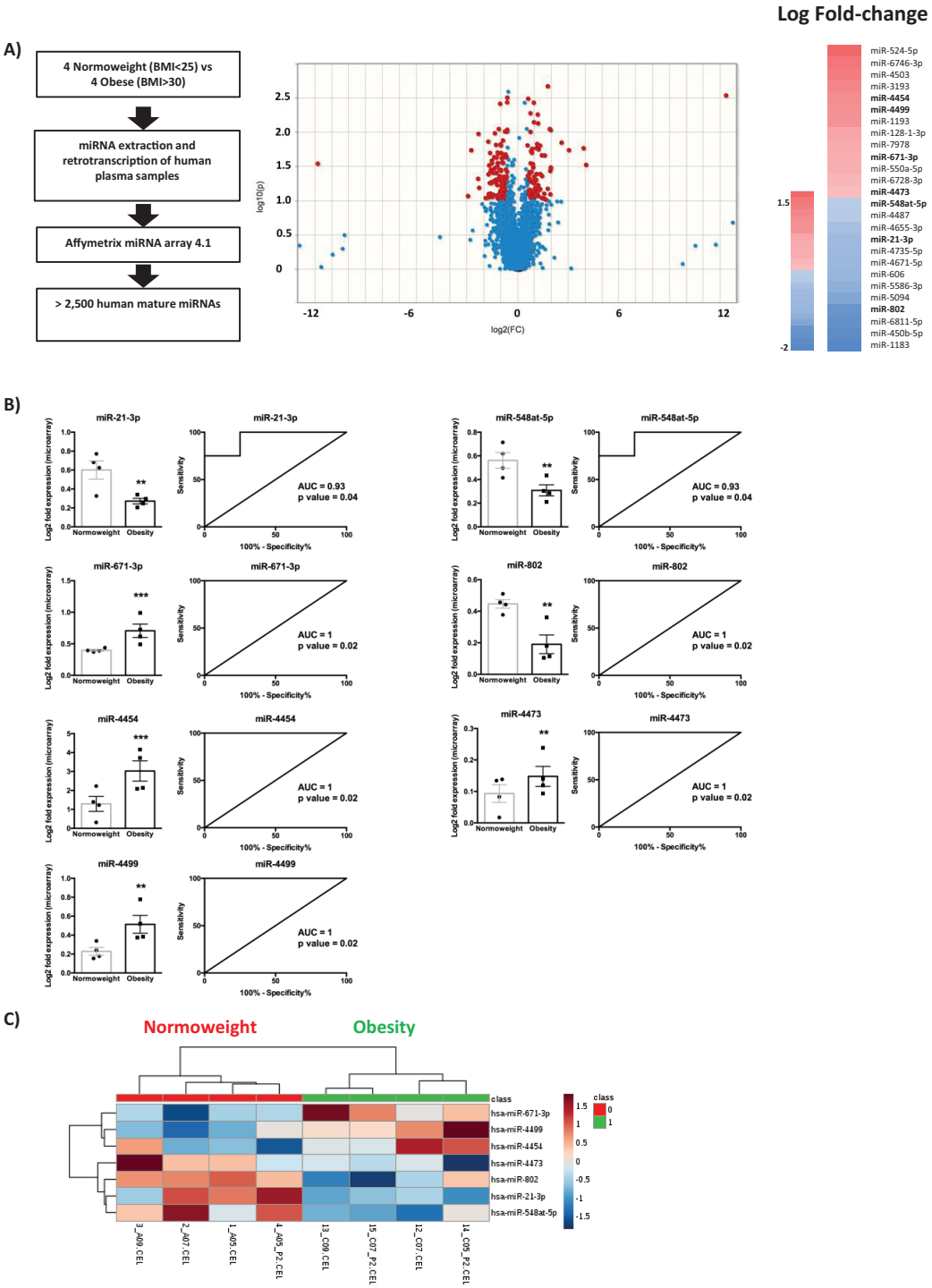

Figure 1. Landscape of circulating miRNAs in obesity. (A) (Left) Graphical summary of the study design. (Middle) Volcano plot representing the alteration in the circulating levels of the whole human miRNome using red (increase) and blue spots (decrease) for each miRNA. (Right) Schematic representation of fold-change levels of significant dysregulated miRNAs comparing plasma samples of normoweight and obese subjects, represented in red (increase) or blue (decrease). (B) Plasma level and ROC curve analysis of the selected miRNAs (21-3p, 548at-5p, 671-3p, 802, 4454, 4473, 4499) comparing plasma samples from obese and normoweight (control) individuals (cohort A). AUC, area under curve. (C) heatmap of the selected miRNAs (n = 7) in obesity compared with control subjects. All of these data derived from array analysis (n = 4 patients/group; total n = 8 patients). Asterisks indicate significant differences between compared groups (**$P < 0.01$; ***$P < 0.001$). miRNA, micro RNA; ROC, receiver operating characteristic.

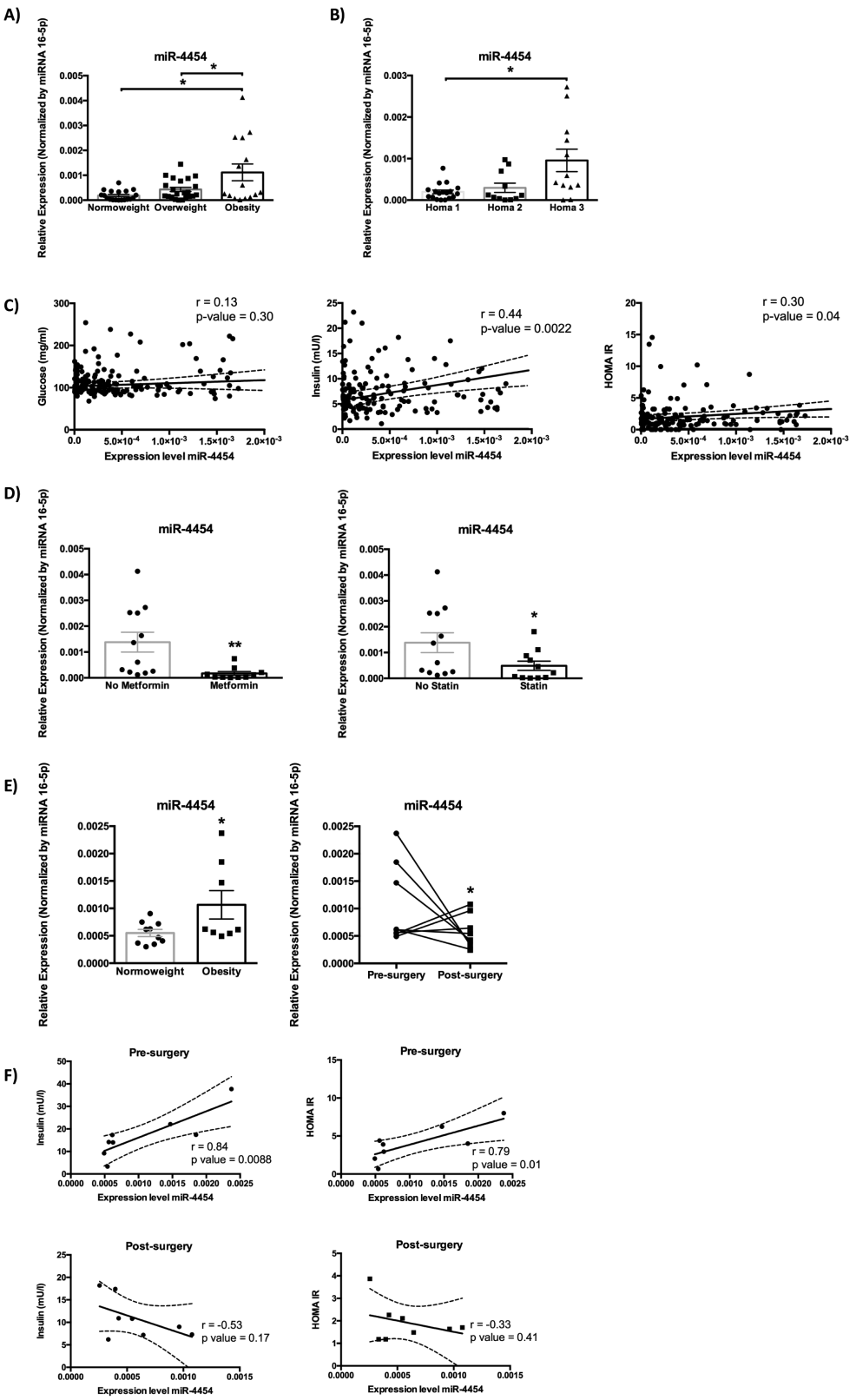

Figure 2. Circulating levels of miR-4454 are altered in obesity and associated to insulin resistance. (A) Plasma levels of miR-4454 in normoweight (BMI < 25, n = 80), overweight (BMI ≥ 25 and < 30; n = 79) and obese (BMI ≥ 30; n = 62) subjects from cohort B (n = 220). (B) Plasma levels of miR-4454 subdividing the subjects from cohort B in tertiles (HOMA 1, HOMA 2, and HOMA 3) considering their HOMA index (HOMA 1 0.34-1.64; HOMA 2 1.64-3.42; HOMA 3 3.42-9.95). (C) Correlations of miR-4454 with glucose levels, insulin, and HOMA-IR in subjects from cohort B. (D) Plasma levels of miR-4454 in treated obese subjects compared with nontreated obese subjects (cohort B). (E) Comparison between miR-4454 levels in obese subjects and normoweight subjects from cohort C and in obese subjects pre- and postbariatric surgery in the external validation cohort C. Data represent mean ± SEM. Asterisks indicate significant differences between compared groups (*$P < 0.05$; **$P < 0.01$). BMI, body mass index; HOMA, Homeostatic Model Assessment; HOMA-IR, Homeostatic Model Assessment-Insulin Resistance; miRNA, microRNA.

in plasma samples from these obese patients before surgery, whereas these correlations were not found in normoweight individuals or in obese patients after bariatric surgery.

In silico analysis of pathways regulated by miR-4454

The predicted target genes of miR-4454 were used to gain novel insights in the putative role of this miRNA by bioinformatically implementing different enrichment analyses. Firstly, GO analysis showed that miR-4454 may be involved in the insulin receptor signaling pathway (Fig. 3). Interestingly, this analysis also indicated that miR-4454 is associated with multiple categories related with RNA metabolism and splicing process, such as gene expression, mRNA metabolic process, RNA splicing, or RNA binding (Fig. 3). Complementarily, a Kyoto Encyclopedia of Genes and Genomes enrichment analysis also confirmed that miR-4454 might be implicated, among others, in the "spliceosome pathway" (Fig. 3), further supporting the results from the GO analysis.

miR-4454 is associated to the cellular response to insulin

Based on the previous observed results, an analysis of the expression of miR-4454 in a battery of commercially available human tissues (thyroid, liver, lung, testis, heart, prostate, and kidney) showed that the expression of this miRNA was higher in liver and prostate tissues compared with other tissues analyzed (Fig. 4A). For that reason, 2 different cell lines, HepG2 (liver-derived) and RWPE-1 (prostate-derived), were used to explore the association between the expression of miR-4454 and insulin pathway. Interestingly, expression of this miRNA was increased after stimulation with insulin, but not with glucose, in both cell lines (Fig. 4B). In addition, an analysis of the expression of different key genes involved in insulin metabolism (based on previous studies (8, 27)) in both cell lines in response to overexpression of miR-4454 revealed a significant downregulation of the glucose transporter *GLUT4,* of the total *INSR (IRA + IRB)* and also of *IRB*, wherein the ratio between *IRB/INSR* was also reduced in prostate, but not hepatic cells (Fig. 4C). In addition, miR-4454 overexpression reduced p-AMPK^{Thr172}, p-AKT^{Ser473}, and p-ERK$^{Thr202/Tyr204}$ levels, presumably leading to a decreased activation of AMPK, PI3K/AKT, and ERK insulin-related pathways (Fig. 4D).

miR-4454 is associated to an alteration in the splicing machinery

Based also on the results observed previously, a custom-designed qPCR array based on microfluidics of 45 spliceosome components and splicing factors revealed that overexpression of miR-4454 induced a marked dysregulation of the splicing machinery. Specifically, nonsupervised hierarchical clustering analyses showed that the expression pattern of spliceosome components and splicing factors precisely discriminated between RWPE-1 overexpressing miR-4454 vs. control mock-transfected cells (Fig. 5A). Actually, overexpression of miR-4454 altered the expression of several spliceosome components (*SNRNP200, RNU2,* and *U2AF1*) and splicing factors (*CELF1, ESRP1, ESRP2, RBM8A, RBM45, SND1, SRRM4, SRSF9,* and *SRSF10*) (Fig. 5B) and (22). Remarkably, the expression of most of these spliceosome components and splicing factors was directly correlated with the expression of INSR (IRA + IRB) and/or with that of the splicing variant IRB in prostate cells overexpressing miR-4454 (i.e., RNU2, U2AF1, CELF1, ESRP1, ESRP2, RBM8A, RBM45, SRRM4, and SRSF9; Fig. 5C, D), but not in mock-transfected control cells (22).

Discussion

Understanding the molecular mechanisms involved in the onset of obesity is a critical objective for the scientific community, in that obesity is a chronic metabolic disease of pandemic level associated with important comorbidities such as insulin resistance, T2DM, dyslipidemia, and cardiovascular disease, and with an increased risk of death (28). Herein, we explored the dysregulation of the whole miRNA landscape in human plasma samples, discovering that miR-4454 levels are higher in obesity, associated with relevant metabolic parameters such as insulin or HOMA index, and modulated by pharmacological (metformin, statins) or medical (bariatric surgery) interventions. In addition, our in silico and in vitro data also suggest that expression of miR-4454 may be related to the cellular response to insulin in endocrine-related organs such as the prostate (8, 29, 30), inasmuch as miR-4454 is associated with a dysregulation in key elements of the insulin receptor signaling pathway and with the inhibition of key cellular signaling pathways such as AKT and AMPK. Interestingly, we also show novel evidence demonstrating that the cellular and molecular alteration in response to insulin triggered by miR-4454 might be associated to a dysregulation in the splicing process and with the alteration of metabolically-relevant splicing isoforms, such as those generated from the insulin receptor gene, isoforms that have associated with the development of different pathologies, such as insulin resistance, diabetes, obesity, atherosclerosis, and cancer (31).

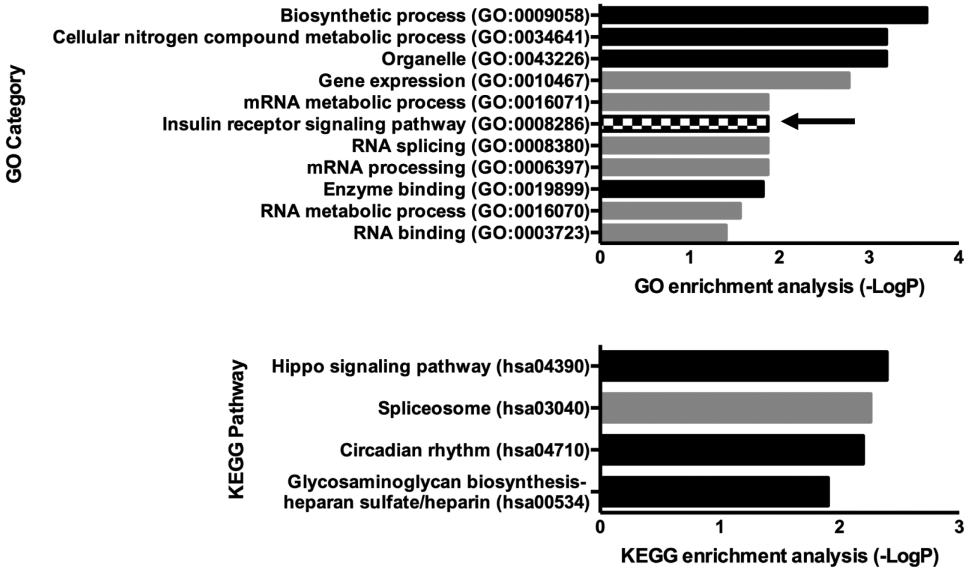

Figure 3. GO terms and KEGG pathways associated to miR-4454 target genes. Data represent -log of the *P* value of each category/pathway. Colors represent relevant categories or pathway related to mRNA processing and splicing (gray) and insulin receptor signaling pathway (black and white). GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

In particular, dysregulation of the expression pattern of miRNAs in obesity was first explored herein by using an array based on Affymetrix technology. Results from the analysis of the whole miRNome demonstrated a significant dysregulation of the plasma levels of 26 miRNAs, wherein 7 of them exhibited capacity to discriminate between normoweight and control subjects. Among them, only the reduced levels of miR-21 have been also previously found in obesity conditions in a cross-sectional study with 45 patients with T2DM and 42 control participants (32). Importantly, it should be mentioned that only the dysregulation of miR-4454 could be corroborated by qPCR in 2 ampler sets of subjects (n = 221 and n = 20, respectively). Therefore, this is the first study demonstrating that the levels of miR-4454 are increased in plasma from obese patients compared with control subjects in three independent cohorts of subjects. Actually, the functional implications of this miRNA have only been explored to date in certain tumor pathologies such as bladder cancer (33) and metastatic melanoma (34, 35). However, consistent with our study, alterations in the expression of miR-4454 have also been described in obesity-related comorbidities including cardiovascular disease (36) and loss of β-cell function (37), but its dysregulation in obesity and its putative pathophysiological role had not been reported until now. Interestingly, plasma levels of miR-4454 were found to be associated with HOMA-IR, a crucial element in obesity, used in many studies to define metabolically healthy obesity (4, 38). Intriguingly, circulating levels of miR-4454 were associated with insulin level but not with glucose level, suggesting a pathophysiological relationship between this miRNA and obesity-associated comorbidities, such as hyperinsulinemia. This is further supported by the fact that the plasma levels of this miRNA were drastically lower in subjects treated with drugs commonly used to control certain obesity-related complications, such as metformin or statins, in comparison with their control (nontreated) groups. In this sense, it should be mentioned that many studies have associated metabolic treatments with pathological alterations triggered by miRNAs (39-41). Moreover, plasma levels of miR-4454 were also downregulated in patients that underwent bariatric surgery, the most effective treatment for maintaining long-term weight loss in severe obesity (42). Altogether, these data demonstrate for the first time that miR-4454 is tightly modulated under obese conditions and that it may be implicated in the pathophysiology of obesity or its associated comorbidities, such as hyperinsulinemia and insulin resistance. Indeed, although further studies would be necessary before a precise and unequivocal conclusion can be reached in this regard, the data reported herein with miR-4454 together with previous observations indicating that specific miRNAs could be used as noninvasive diagnostic/prognostic biomarkers as well as therapeutic options in different endocrine-related pathologies (10, 43, 44), invite the suggestion that miR-4454 could represent a promising and valuable tool to develop a novel and predictive diagnostic/prognostic biomarker and/or therapeutic tool for insulin resistance.

Interestingly, a screening of the expression levels in human tissues demonstrated that miR-4454 is highly expressed in liver and prostate, 2 endocrine organs strongly influenced by metabolic factors, including insulin, one of

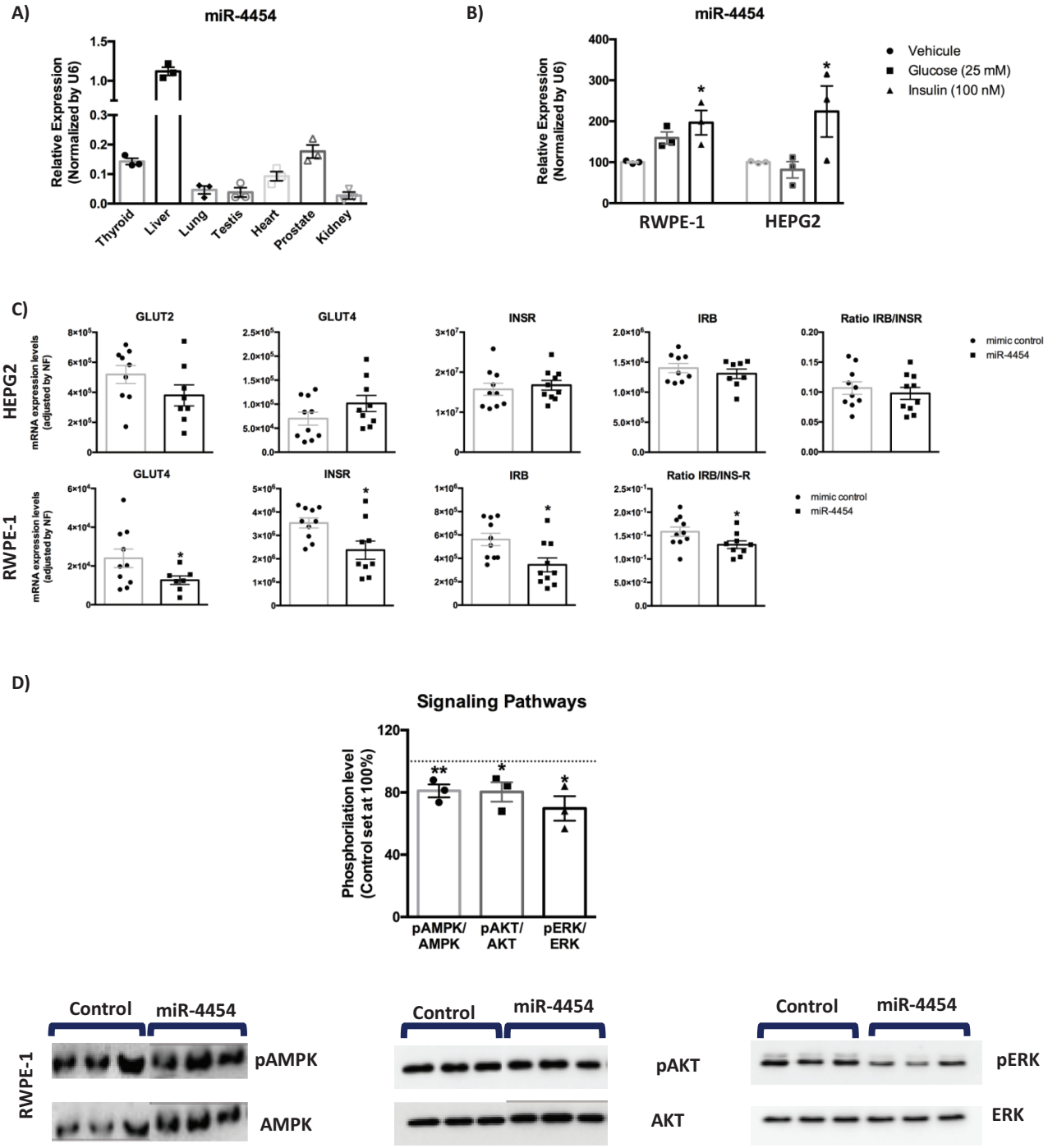

Figure 4. miR-4454 is differentially expressed in human tissues and modulates insulin signaling-related elements. (A) Expression levels of miR-4454 in a battery of human tissues (thyroid, liver, lung, testis, heart, prostate, and kidney) determined by qPCR (relative expression adjusted to U6 levels). (B) Expression levels of miR-4454 in RWPE-1 and HepG2 cell lines in basal condition and after stimulation with glucose (25 mM) or insulin (100 nM) determined by qPCR (relative expression adjusted to U6 levels). (C) Effect of miR-4454 overexpression in the modulation of the expression level of genes related to the insulin signaling in RWPE-1 and HEPG2 cell lines. mRNA levels were determined by qPCR and normalized by normalization factor calculated from ACTB, GAPDH, and HPRT expression levels. (D) Representative Western blots and quantification of p-AMPK/total AMPK, p-AKT/total AKT, and p-ERK/total ERK in RWPE-1 cell line after overexpression of miR-4454. Data are expressed as percentage of control cells (set at 100%). In B-D, values represent the mean ± SEM of at least n = 3 independent experiments. Asterisks indicate significant differences vs. controls (**P* < 0.05; ***P* < 0.01). ACTB, Actin beta; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT, hypoxanthine-guanine phosphoribosyltransferase; qPCR, quantitative real-time PCR.

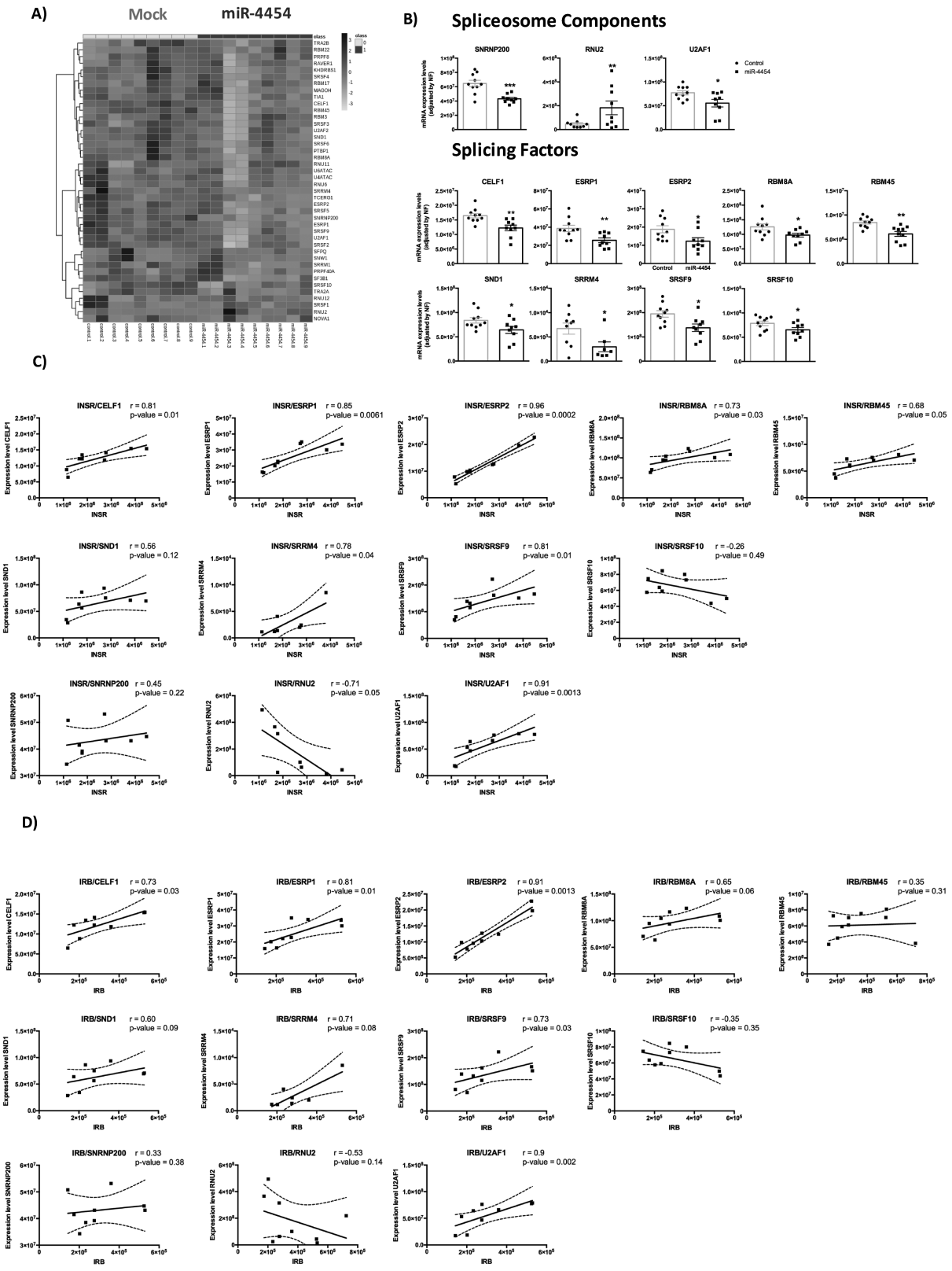

Figure 5. miR-4454 alters the expression of certain spliceosome components and splicing factors in the prostate-derived cell line RWPE-1. (A) Heatmap representing the expression levels of n = 45 spliceosome components and splicing factors in miR-4454 overexpressing RWPE-1 cells compared with mock (control) cells determined by microfluidic-based qPCR array and (B) summary of the spliceosome components and splicing factors whose expression was altered by the overexpression of miR-4454. mRNA levels were normalized by a normalization factor calculated from ACTB, GAPDH, and HPRT expression levels. (C-D) Correlations between the expression levels of splicing element (spliceosome components and splicing factors) and the expression of INSR (C) and IRB (D) in miR-4454 overexpressing miR-4454 RWPE-1 cells. Data represent mean ± SEM of n = 3

the hormones that exerts some of the most prominent physiological effects in both tissues (8, 45). In addition, the expression levels of miR-4454 in prostate and liver cells (RWPE-1 and HepG2, respectively) were altered when stimulated with a high dose of insulin, mimicking the hyperinsulinemia conditions observed in obesity subjects. In striking contrast, expression levels of this miRNA were not altered in response to high dose of glucose, reinforcing the clinical association found herein, indicating a positive correlation between miR-4454 and insulin, but not glucose, in human plasma samples, which further suggests that the expression of this miRNA may be regulated by insulin and linked to its levels.

In the same line, our in silico and in vitro studies demonstrated that miR-4454 is implicated in the cellular response to insulin in an organ or cell-type dependent manner. Indeed, in silico GO analysis demonstrated that its target genes are involved, among others, in the insulin receptor signaling pathway. This result reinforces the hypothesis that miR-4454 is tightly associated with metabolic disorders such as obesity and especially with the insulin metabolism. This may add some new clues to other studies demonstrating that the control of the metabolic homeostasis and, specially, the cellular response to insulin, is one of the pathways most regulated by miRNAs, including miR-7, miR-26a, or miR-107 (46-48). In particular, the in vitro overexpression of miR-4454 induced a significant dysregulation in key genes (insulin receptors and GLUT4) and pathways (Akt, AMPK, and ERK) involved in insulin signaling in prostate, but not in liver, cells. These changes found in prostate but not in liver cells could be explained by the sophisticated molecular mechanism of regulation of miRNAs. Transcription of miRNA genes is regulated similarly to that of protein-coding genes, and is a major level of control responsible for tissue- or development-specific expression. In keeping with this, some miRNAs can exhibit different functions depending on the tissue in which they act because of the transcriptional regulatory network present in each cell type (9, 49). As mentioned, miR-4454 overexpression in prostate cells was associated to a decrease in the expression of *INSR* and *GLUT4*. GLUT4 is a glucose carrier entirely dependent upon insulin (50) and, although the role in the insulin-induced glucose uptake by adipose and muscle tissues and its regulation by several miRNAs have been characterized (44, 47), no previous studies have demonstrated this molecular association in the prostate gland. Furthermore, a decrease in the phosphorylation levels of key insulin-related signaling pathway components was observed after the overexpression of miR-4454. In particular, AMPK regulates insulin homeostasis by reducing the phosphorylation of mTOR in the cytoplasm and also controls the glucose uptake by increasing expression and translocation of GLUT4 (43). This transcriptional regulation of GLUT4 by the activation of AMPK could also explain the reduction of the expression of this solute carrier in the cells overexpressing miR-4454. Moreover, the phosphorylation of AKT, the master downstream regulator in the insulin response, was also reduced. AKT activation also increases glycogen synthesis (through GSK-3 inhibition), protein synthesis (via mTOR signaling), and cell survival by the inhibition of pro-apoptotic factors, including Bad and FOXO transcription factors (51, 52). Concerning ERK pathway, ERK1-deficient mice are protected against diet-induced obesity and insulin resistance owing to decreased adipogenesis and elevated energy expenditure (53, 54). Therefore, the overall dysregulation in these genes and signaling pathways might be associated with the pathophysiological alterations of some of the obesity related comorbidities, such as hyperinsulinemia and hyperglycemia. Altogether, these results suggest that miR-4454 is modulated in response to insulin in prostate cells and that it can also regulate key genes and pathways involved in the cellular response to insulin, generating a regulatory feedback between insulin and miR-4454.

A particularly relevant finding of this study is that, although other miRNAs have been associated with the reduction of insulin receptor expression (55, 56), the reduction of the expression of *INSR* triggered by miR-4454 is also associated herein with a dysregulation in the ratio between both *INSR* splicing isoforms (IRA and IRB). The differential physiological and pathological role of both isoforms is not completely known, but it has been demonstrated that their role is probably determined by the different binding affinity for insulin-like growth factors (57). IRB is more abundant in adult tissues and exerts mainly the metabolic actions of insulin, whereas IRA is mainly expressed in the fetal and prenatal periods and exerts mitogenic actions (57). An increase in the IRA/IRB ratio has been shown in conditions of insulin resistance in different insulin target tissues (58). This capacity of miR-4454 to modulate the alternative splicing of the INSR gene is in complete agreement with the in silico prediction of the involvement of this miRNA with the modulation of RNA metabolism and splicing, including the regulation of the spliceosome, and could be of pathophysiological relevance, inasmuch as the control of the splicing

independent experiments. Asterisks indicate significant differences between cells overexpressing miR-4454 and control cells (*$P < 0.05$; **$P < 0.01$). ACTB, Actin beta; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT, hypoxanthine-guanine phosphoribosyltransferase; qPCR, quantitative real-time PCR.

process has been demonstrated to be tightly related to the insulin signaling pathway (31, 59, 60). Of particular importance is the regulation of the ratio between both isoforms of the insulin receptor (IRA and IRB), which has been associated to different metabolic disorders such as T2DM (57) and also with several cancer types (61, 62). Consistent with this idea, our in vitro studies revealed that overexpression of miR-4454 severely dysregulated several spliceosome components (*SNRNP200, RNU2*, and *U2AF1*) and splicing factors (*CELF1, ESRP1, ESRP2, RBM8A, RBM45, SND1, SRRM4, SRSF9*, and *SRSF10*) in prostate cells, where their expression levels correlated well with those of the total levels of IRS and with the expression of the splicing variant *IRB*, suggesting a causal association. Indeed, some of the spliceosome components and splicing factors found here to be regulated by miR-4454 have been implicated in different processes associated to the development or progression of obesity and its related comorbidities, including cancer (23). Namely, alterations in *ESRP1, SNRNP200*, and *RNU2* could augment the risk of developing T2DM (25), dysregulation of the expression levels of *ESRP2, RBM45, SND1, SRSF10*, and *U2AF1* have been observed in the liver of obese patients with steatosis (21), and SRSF10 has been described as a regulator of the production of lipin1a promoting adipocyte differentiation (63). And more importantly, a previous study has proposed that the association of specific splicing factors such as CELF1 and SRp20 acts antagonistically regulating INSR alternative splicing (causing exon skipping and increasing exon inclusion, respectively) (64), which further supports our results. Although more studies are necessary to better understand the cellular and molecular implication of miR-4454 as a regulator of the splicing process, it is clear that the dysregulation of the expression pattern of these splicing factors and spliceosome components trigger defects in the processing of splicing variants, including IR isoforms, and could, consequently, be involved in important metabolic disorders such as obesity and T2DM (21, 25). Therefore, these observations unveiled new conceptual and functional avenues, with potential therapeutic implications, which are worth exploring in future studies.

In conclusion, this study represents the first demonstration that the circulating levels of miR-4454 are increased in obesity, associated with key clinical parameters (e.g., insulin levels, HOMA-IR), and modulated by obesity-controlling interventions (metformin/statin treatment and bariatric surgery). Moreover, in vitro data revealed that miR-4454 is modulated by insulin and can impair the cellular response to insulin, in a cell type-dependent manner, through the modulation of the splicing process and important signaling pathways (Akt, AMPK, and ERK). Altogether, our results provide new, compelling evidence supporting the contention that miR-4454 represents a promising diagnostic, prognostic and/or therapeutic tool, worth to be further explored, in obesity and associated comorbidities, such as hyperinsulinemia and T2DM.

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ARTICLE III

Molecular Therapy
Nucleic Acids
Original Article

Dysregulation of the miRNome unveils a crosstalk between obesity and prostate cancer: miR-107 asa personalized diagnostic and therapeutic tool

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Prostate-specific antigen (PSA) is the gold-standard marker to screen prostate cancer (PCa) nowadays. Unfortunately, its lack of specificity and sensitivity makes the identification of novel tools to diagnose PCa an urgent medical need. In this context, microRNAs (miRNAs) have emerged as potential sources of non-invasive diagnostic biomarkers in several pathologies. Therefore, this study was aimed at assessing for the first time the dysregulation of the whole plasma miRNome in PCa patients and its putative implication in PCa from a personalized perspective (i.e., obesity condition). Plasma miRNome from a discovery cohort (18 controls and 19 PCa patients) was determined using an Affymetrix-miRNA array, showing that the expression of 104 miRNAs was significantly altered, wherein six exhibited a significant receiver operating characteristic (ROC) curve to distinguish between control and PCa patients (area under the curve [AUC] = 1). Then, a systematic validation using an independent cohort (135 controls and 160 PCa patients) demonstrated that miR-107 was the most profoundly altered miRNA in PCa (AUC = 0.75). Moreover, miR-107 levels significantly outperformed the ability of PSA to distinguish between control and PCa patients and correlated with relevant clinical parameters (i.e., PSA). These differences were more pronounced when considering only obese patients (BMI > 30). Interestingly, miR-107 levels were reduced in PCa tissues versus non-tumor tissues (n = 84) and in PCa cell lines versus non-tumor cells. *In vitro* miR-107 overexpression altered key aggressiveness features in PCa cells (i.e., proliferation, migration, and tumorospheres formation) and modulated the expression of important genes involved in PCa pathophysiology (i.e., lipid metabolism [i.e., FASN] and splicing process). Altogether, miR-107 might represent a novel and useful personalized diagnostic and prognostic biomarker and a potential therapeutic tool in PCa, especially in obese patients.

INTRODUCTION

Prostate cancer (PCa) has emerged as the most frequent tumor type among men and represents a severe health problem worldwide.[1] A key limitation in PCa management is that the gold-standard screen test is based on the plasma levels of prostate-specific antigen (PSA), a biomarker that exhibits profound drawbacks, especially in the so-called "gray zone" (defined as a PSA range of 3–10 ng/mL).[2] In fact, PSA test displays low specificity in that multiple factors can increase PSA levels without necessarily indicating the presence of a tumor, such as benign prostatic hyperplasia or inflammatory conditions. In addition, PSA test is not able to accurately distinguish clinically relevant tumors from indolent cases.[3] For these reasons, the anatomo-pathological analysis of prostate biopsies, which represent a highly invasive technique, is still necessary to appropriately diagnose PCa nowadays. Therefore, there is an important unmet clinical need for the identification and validation of new, reliable, and specific non-invasive diagnostic biomarkers, ideally showing prognostic and/or therapeutic potential.

In this context, microRNAs (miRNAs) have emerged as promising clinical tools, especially due to their potential as diagnostic and therapeutic targets.[4] miRNAs are RNA transcripts that lack protein-coding capacity. Specifically, miRNAs are short non-coding RNAs (20–22 nt)

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that act at post-transcriptional level, negatively regulating translation of target mRNAs by altering their stability, resulting either in the degradation of the target or in the translational repression through different mechanisms.[5] In fact, miRNAs are attractive biomarker candidates as they can be reproducibly extracted from a wide range of biologic samples and are generally stable and resistant to various storage conditions.[6,7] Indeed, recent studies have suggested a relationship between circulating miRNAs and PCa presence and outcome.[8–10] Specifically, recent studies have identified some miRNAs differentially present in plasma samples from PCa patients; however, only some of them seem to be specifically derived from PCa tissues.[11–13] Unfortunately, although various studies have identified some putative PCa-specific miRNAs (e.g., miR-141,[11,14] miR-375,[8,15] and miR-21[16]), there is not a consensus in the utility of plasma miRNAs as circulating non-invasive biomarkers for PCa. In fact, to the best of our knowledge, there are no studies describing the dysregulation of the whole miRNome in PCa versus healthy patients. For these reasons, this field requires further investigation in order to ascertain whether a specific miRNA or a plasma or serum miRNA signature could be associated with PCa risk and provide diagnostic and prognostic value through a fast, easy, and non-invasive test.

Likewise, it should be noted that, during the last years, many studies have demonstrated the implication of the metabolic status, especially the obese condition, in the development, progression, or aggressiveness of several pathologies, including PCa.[17–19] However, the metabolic status of the patients has not been taking into account in the previous studies implemented to define a diagnostic and prognostic model based on miRNAs expression profile, suggesting the necessity of considering the metabolic status of the patients, especially the obesity condition, when developing and validating new biomarkers to improve the diagnostic and/or prognostic models in PCa.

Therefore, this study was aimed to explore for the first time the dysregulation of the human miRNome in PCa patients, considering the obesity condition of the patients (normoweight, overweight, or obesity) in order to discover and develop new and more personalized diagnostic, prognostic, and therapeutic tools for the management of PCa. In addition, we explored the potential pathophysiological role and the molecular mechanisms underlying the role of miR-107 in PCa, the most consistently altered miRNA in PCa patients found in our study.

RESULTS

Plasma miRNAs landscape is dysregulated in PCa subjects

Results obtained from the Affymetrix miRNA array 4.1 (which analyzes the complete list of mature human miRNAs from the miRNome; n = 2,578) using plasma samples from PCa and healthy volunteers revealed that all miRNAs, with the exception of 18 transcripts, were detected in all the plasma samples analyzed. Specifically, this analysis revealed a significant dysregulation ($p < 0.01$) in the circulating pattern of 104 miRNAs, wherein 74 miRNAs showed higher levels and 30 miRNAs exhibited lower levels in PCa compared with control patients (Figure 1A). An unsupervised clustering of healthy volunteers and PCa patients revealed that the plasma levels of these 104 differentially expressed miRNAs effectively discriminated between both groups (Figure 1B). Of note, six of these significantly altered miRNAs (i.e., let-7d-5p, miR-24-5p, miR-26a-5p, miR-103a-3p, miR-107, and miR-191-5p) accurately distinguished between healthy volunteers and PCa patients, showing an area under the curve (AUC) = 1 (Figure 1C).

The expression profile of these six miRNAs was analyzed using quantitative real-time PCR (PCR) in an ampler cohort of subjects (validation cohort B). This analysis confirmed that the levels of miR-107 and miR-191-5p were higher in plasma from PCa patients compared with control patients (Figure 1D). In addition, both miR-107 and miR-191-5p significantly distinguished between PCa and control patients, exhibiting AUCs of 0.75 and 0.67, respectively (Figure 1D). Based on these data, miR-107 was selected for further analyses in order to more profoundly characterize its potential value as biomarker.

Potential value of miR-107 as a non-invasive biomarker in PCa

Further analyses of miR-107 plasma levels in the validation cohort B revealed a positive association with plasma PSA levels in PCa patients (Figure S1), but not with glucose level or other available clinical parameters (such as insulin level or plasma lipids [cholesterol and triglycerides (TGs)]; data not shown). Interestingly, miR-107 outperformed the capacity of PSA to distinguish between control and PCa patients (miR-107 [AUC = 0.75; $p < 0.0001$] versus PSA [AUC = 0.5884; $p = 0.0094$]; Figure 2A). Remarkably, when the same analysis was performed considering only patients with PSA levels in the gray zone (3–10 ng/mL; n = 238), we found that plasma levels of miR-107 were significantly higher in PCa patients compared with control patients (Figure 2B, middle panel), while no changes were observed when comparing PSA levels (Figure 2B, left panel). Consequently, miR-107 (but not PSA) levels are able to significantly distinguish between control and PCa patients (AUC = 0.66; $p < 0.001$; Figure 2B, right panel).

Remarkably, plasma miR-107 levels were higher in patients with significant PCa (Sig PCa; defined as Gleason > 6) as compared with Non-Sig PCa patients (defined as Gleason = 6; Figure 2C). Interestingly, although the levels of miR-107 could not significantly discriminate between NonSigPCa and SigPCa patients (Figure 2C), circulating levels of miR-107 were clearly associated with several key clinical parameters, such as PSA levels, tumor volume, testosterone, and C-reactive protein (CRP) levels in SigPCa patients, but not in NonSigPCa subjects (Figure 2D).

miR-107 exerts a dual suppressive/oncogenic role in PCa

We then compared the expression levels of miR-107 between tumor regions and the adjacent non-tumor regions from PCa patients included in cohort C and found that miR-107 was downregulated in PCa tissues (Figure 3A). Similarly, all PCa cell lines analyzed herein exhibited lower expression levels of miR-107 compared with the normal-like, prostate-derived cell line PNT2 (Figure 3B). Specifically, LNCaP, an androgen-sensitive PCa cell, and DU145, a more aggressive PCa cell line, showed the lowest expression level of miR-107

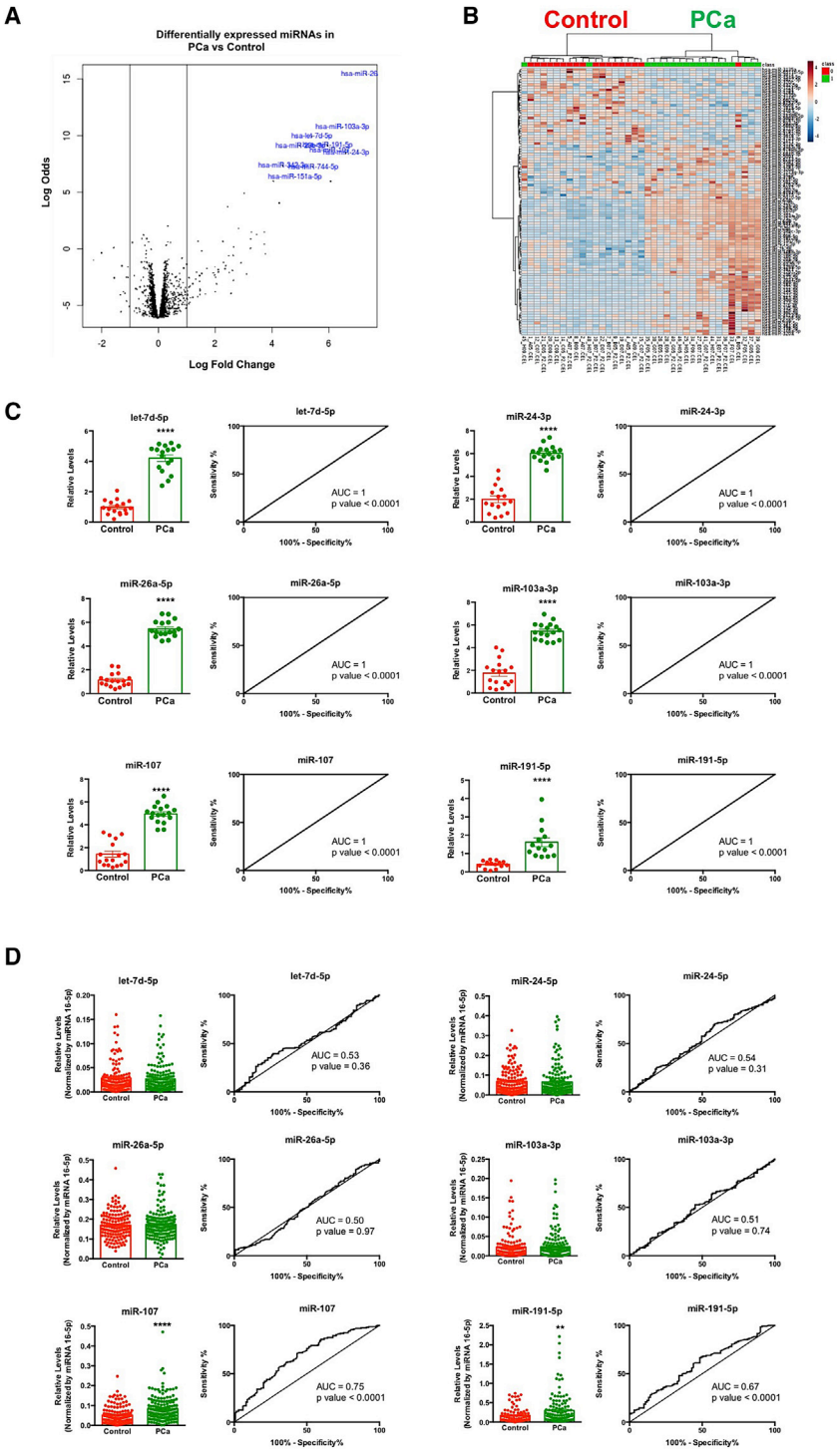


Figure 1. Landscape of circulating miRNAs in prostate cancer (PCa)

(A) Volcano plot representing the alteration in the circulating levels of the whole human miRNome. (B) Heatmap of the dysregulated miRNAs (n = 104) in PCa patients compared with healthy volunteers is shown. (C) Plasma level and ROC curve analysis of the selected miRNAs (let-7d-5p, 24-3p, 26a-5p, 103a-3p, 107, and 191-5p) comparing plasma samples from PCa patients and healthy volunteers (cohort A) are shown. All these data are derived from the array analysis. (D) Plasma level and ROC curve analysis of the selected miRNAs (let-7d-5p, 24-3p, 26a-5p, 103a-3p, 107, and 191-5p) comparing plasma samples from PCa and control individuals (cohort B) are shown. These data derive from quantitative PCR analysis. *AUC*, area under the curve. Asterisks indicate significant differences between compared groups (**p < 0.01; ****p < 0.0001).

(Figure 3B). In order to further explore this apparent contradiction between plasma and tissue levels of miR-107, we analyzed miR-107 levels in the conditioned and secreted media from PNT2, LNCaP, and DU145 cells. Interestingly, miR-107 secretion in DU145 was significantly higher compared with PNT2 and LNCaP cell lines (PNT2 = LNCaP < DU145; Figure 3C). Moreover, the ratio between the secretion and the expression of miR-107 was significantly higher in PCa DU145 and LNCaP compared with normal-like PNT2 cells (PNT2 < LNCaP < DU145; Figure 3D), suggesting that the low levels observed in PCa tissue and the high levels observed in plasma from PCa patients could be due to the prominent secretion of miR-107 from PCa cells, especially in the most aggressive models (SigPCa patients [observed in Figure 2C] and DU145 cells [Figure 3C]).

Next, and in order to study the potential role of miR-107 in functional parameters of aggressiveness, overexpression of miR-107 in DU145 and LNCaP cells were performed by transfection. The intracellular overexpression of miR-107 in DU145 cells (validation results are shown in Figure S2) significantly reduced proliferation (at 72 h; Figure 3E, right panel), migration (Figure 3F), and the number and area of tumorospheres (Figures 3G, right graphic, and 3H, respectively). However, miR-107 overexpression increased proliferation and the number and area of tumorospheres in LNCaP cells (Figures 3E, left panel; 3G, left panel; and 3H, respectively).

In silico and *in vitro* analyses of targets and pathways regulated by miR-107

The predicted target genes of miR-107 were obtained by implementing different bioinformatics enrichment analyses to gain novel insights on the putative role of miR-107. Firstly, a Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis showed that miR-107 might be implicated in 26 pathways (Figure 4A,

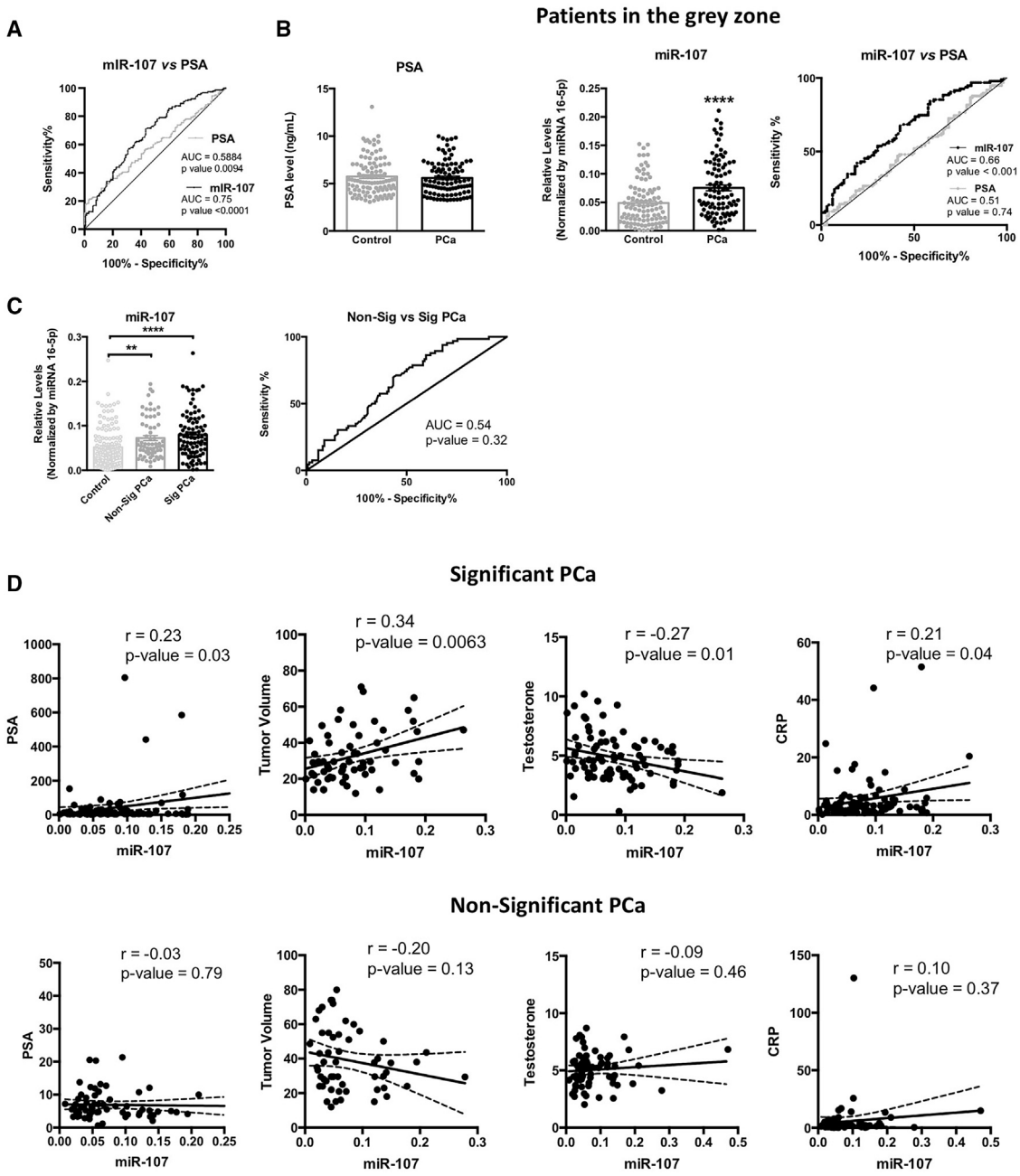


Figure 2. Circulating levels of miR-107 are altered in PCa and associated to oncogenic parameters

(A) ROC curve analysis of miR-107 and PSA comparing plasma samples from PCa and control patients (cohort B). (B) Plasma level and ROC curve analysis of miR-107 and PSA comparing plasma sample from PCa and control patients included in the gray zone of PSA are shown. (C) Plasma level and ROC curve analysis of miR-107 comparing control, non-significant, and significant PCa patients are shown. (D) Correlation between plasma level of miR-107 and PSA, tumor volume, testosterone, and CRP in non-significant and significant PCa patients is shown. Data represent mean ± SEM. Asterisks indicate significant differences between compared groups (**$p < 0.01$; ****$p < 0.0001$).

left panel). In fact, most of these pathways could be grouped in the main pathway observed, the fatty acid metabolism, which is represented by the putative targeting of 14 genes (Figure 4A, right panel). Complementary Gene Ontology (GO) analysis also confirmed that miR-107 might be involved, among others processes, in cellular lipid metabolic process (Figure 5A, black arrow). In order to further analyze the regulatory role of miR-107 in this complex metabolic pathway, the expression of these 14 genes was analyzed in LNCaP and DU145 cell

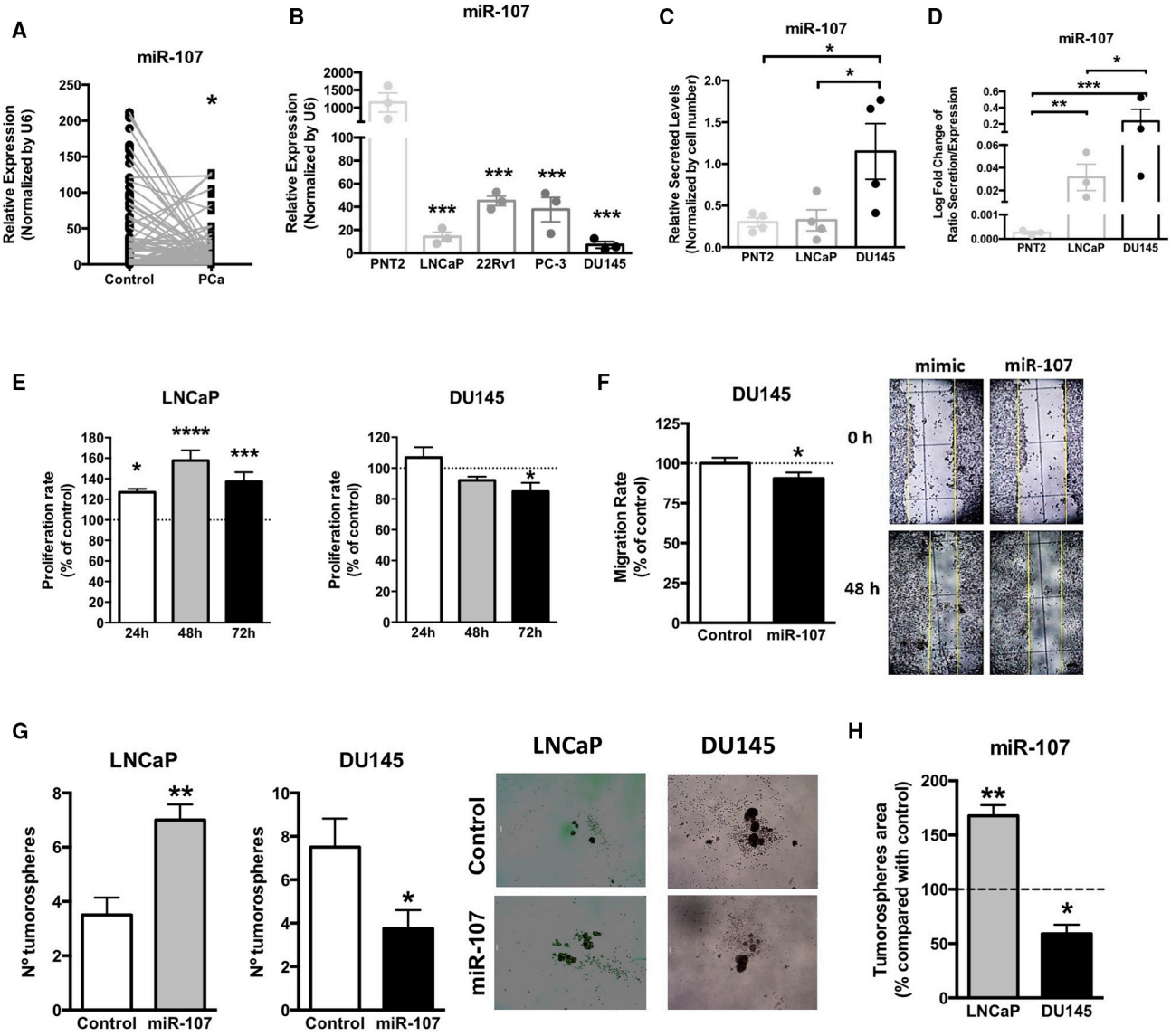
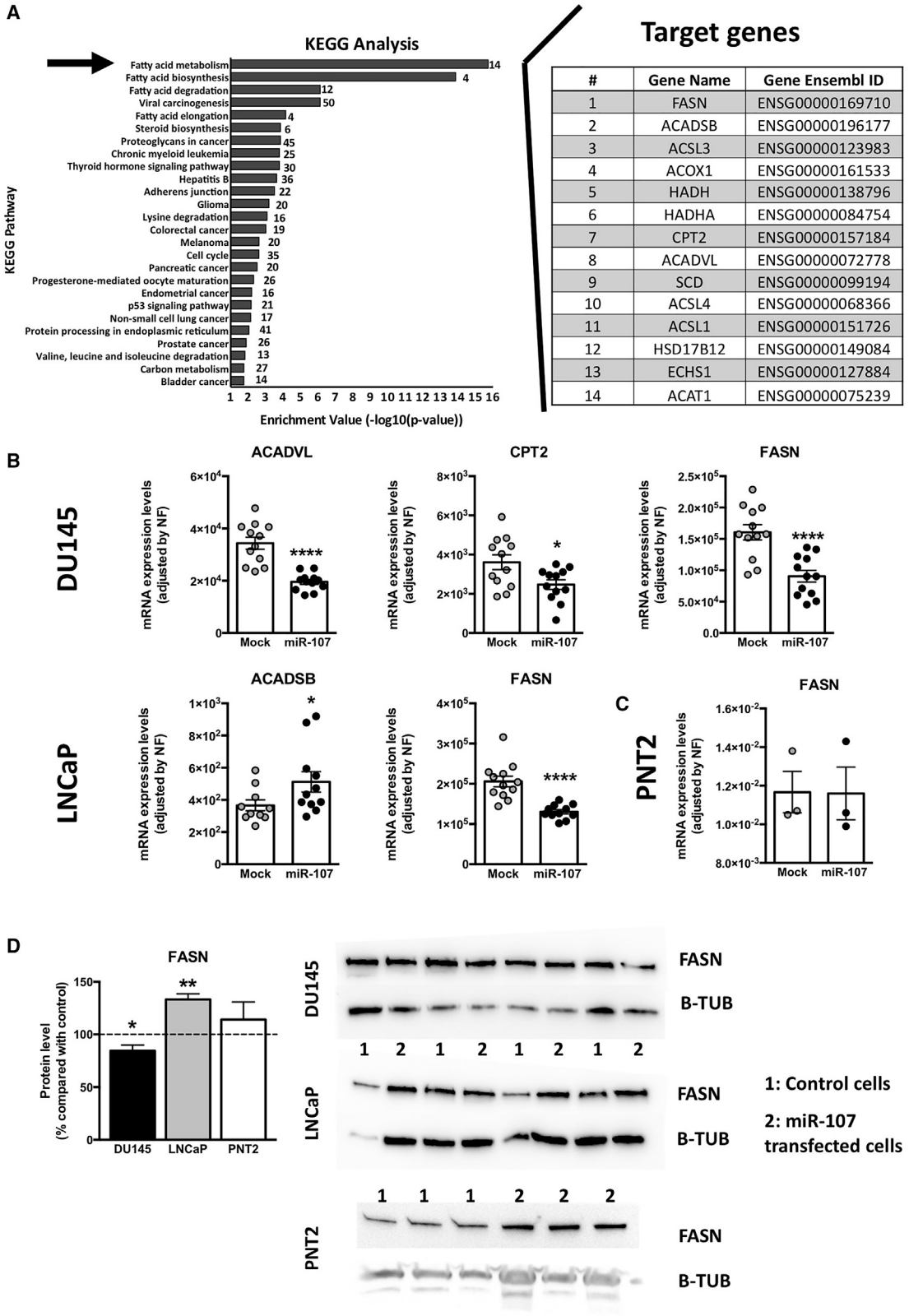


Figure 3. Dual suppressive and oncogenic role of miR-107 in PCa

(A) miR-107 expression level in PCa tissues compared with adjacent non-tumor regions from PCa patients included in cohort C. (B) miR-107 expression level in PCa cells (LNCaP, 22Rv1, PC-3, and DU145) compared with the normal-like prostate cell line (PNT2) is shown. (C) miR-107 secretion levels in media obtained from PNT2, LNCaP, and DU145 cell lines are shown. (D) Ratio between secretion and expression of miR-107 in PNT2, LNCaP, and DU145 cell lines is shown. (E) Proliferation rate analysis of LNCaP and DU145 cell lines overexpressing miR-107 is shown. (F) Migration rate and representative images of DU145 cells overexpressing miR-107 are shown. Data are expressed as percentage of control (set at 100%). (G) Analysis of tumorosphere formation assay and representative images of LNCaP and DU145 cells overexpressing miR-107 is shown. Data represent number of tumorospheres. (H) Comparison of tumorospheres area in LNCaP and DU145 cells overexpressing miR-107 compared with control cells is shown. Data are expressed as percentage of control (set at 100%). Asterisks indicate significant differences between compared groups (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

lines overexpressing miR-107, being represented in Figure 4B the genes significantly altered (results of the genes that were not altered are shown in Figure S3). Remarkably, a dysregulation in acyl-coenzyme A (CoA) dehydrogenase (ACAD) chains was observed in these cells, resulting in an upregulation of the short chain (*ACADSB*) in LNCaP and a downregulation of the long chain (*ACADVL*) in DU145 (Figure 4B). A downregulation of carnitine palmitoyltransferase 2 (*CPT2*) was also observed in DU145 (Figure 4B). Remarkably, the main gene involved in this cellular pathway, fatty acid synthase (*FASN*), was significantly downregulated in both prostate cancer cell lines (Figure 4B), but not in the normal-like prostate cell model (PNT2; Figure 4C), in response to miR-107 overexpression. However,



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at protein level, a distinct regulation was observed in both PCa cell lines since FASN protein levels were increased in LNCaP and decreased in DU145 (Figure 4D). FASN protein levels were not altered in the normal-like prostate cell model (Figure 4D).

Interestingly, GO analysis also showed that miR-107 might be also involved in the RNA splicing process (Figure 5A), a molecular event that we have recently demonstrated to be critically altered in PCa and associated with PCa aggressiveness.[20] For this reason, we analyzed the expression of key components of the splicing machinery and associated splicing factors in LNCaP and DU145 cell lines overexpressing miR-107. Intriguingly, a profound dysregulation of several genes was observed in both cell lines (11 altered genes; Figure 5B).

miR-107 as a personalized non-invasive biomarker in the pathophysiological crosstalk between PCa and obesity

Based on the results obtained from the *in silico* and *in vitro* analyses linking the dysregulation of miR-107 with the metabolic milieu of PCa cells, we further analyzed the plasma levels of miR-107 in the patients included in cohort B, considering the metabolic status of the patients (i.e., obesity state). To that end, control and PCa patients from cohort B were subdivided according to their body mass index (BMI) in three different subgroups (normoweight [18.5 < BMI < 25], overweight [25 $\leq$ BMI < 30], and obese [BMI $\geq$ 30]), as shown in Table 3. This analysis showed a clear influence of the obese status in miR-107 plasma levels, especially in PCa patients (Figure 6A). Specifically, miR-107 levels were not affected by obesity in control patients, while in PCa patients, miR-107 levels were significantly higher in overweight and obese patients (Figure 6A, left panel). This was confirmed by the receiver operating characteristic (ROC) curve analysis, wherein the maximal difference, represented by an AUC = 0.73, was found in the comparison between obese PCa and obese control patients (Figure 6A, right panel).

Moreover, when considering those patients included in the gray zone of the PSA (3–10 ng/mL), plasma miR-107 levels exhibited significantly improved ROC curve analysis compared with PSA, when these comparative analyses were carried out in overweight (n = 100; Figure 6B, middle right panel) and obese (n = 69; Figure 6B, bottom right panel) conditions, but not in the normoweight condition (n = 69; Figure 6B, top right panel). Notably, the highest statistical difference with the ROC curve analyses was observed when comparing PSA and miR107 in obese PCa patients (Figure 6B, right panels). Specifically, we observed an increasing AUC of the miR-107 ROC curve analysis dependent on the obesity state (AUC normoweight [NW] = 0.53, overweight [OW] = 0.65, and obese [OB] = 0.74), a result that was not observed on the PSA ROC curve analyses (AUC NW = 0.68, OW = 0.51, and OB = 0.52; Figure 6B, right panels).

Remarkably, when comparing PCa patients stratified by Gleason score and obese status, we found that the levels of miR-107 were significantly higher in obese-SigPCa patients compared with obese-NonSigPCa patients (Figure 6C, bottom panel). In fact, obese patients with SigPCa exhibited the highest AUC in the ROC analysis performed herein (AUC = 0.8025; $p < 0.0001$; Figure 6C). In this sense, the plasma levels of miR-107 were able to discriminate between SigPCa and NonSigPCa in obese patients, exhibiting a potential prognostic value (Figure 6C).

DISCUSSION

Plasma PSA levels remain the current gold-standard biomarker to diagnose PCa, which represents one of the tumor types with the highest incidence worldwide.[21] Unfortunately, PSA continues to show important limitations (especially in the range of 3–10 ng/mL, also named the "gray zone"), including compromised specificity, inasmuch as non-tumor conditions (e.g., infections and inflammation) can also increase PSA levels. Therefore, considerable research efforts have been focused on the identification of novel biomarkers that could complement or even replace plasma PSA in order to improve the diagnosis of PCa. In this line, we explored herein for the first time the dysregulation of the whole known miRNome in human plasma samples from PCa patients compared with those from healthy volunteers in order to identify novel and useful personalized diagnostic and prognostic biomarkers and potential therapeutic tools in PCa.

In particular, results from the analysis of the whole miRNome demonstrated a significant dysregulation of the plasmatic levels of 104 miRNAs, wherein six of them exhibited a great capacity to discriminate between PCa patients and healthy volunteers. The dysregulation of these miRNAs in PCa patients further supports previous data reporting the alteration of some of these miRNAs, including miR-107,[9,10,22–25] in certain studies. However, any of these studies have explored its putative role as a diagnostic biomarker as well as prognostic or therapeutic tool. Interestingly, the dysregulation of miR-107 and miR-191-5p was corroborated by qPCR in a second, ampler, and independent cohort of subjects (n = 295). The fact that the dysregulation of other miRNAs was not corroborated may be explained by the fact that control patients from this ampler cohort are patients with suspect of PCa but negative results in the biopsy instead of healthy volunteers, as used in cohort A. Nonetheless, we demonstrated that diagnostic capacity of plasma

Figure 4. Fatty acid metabolism pathway alteration by miR-107

(A) (Left) Data represent −log of the p value of each category and pathway. The arrow indicates fatty acid metabolism, the main pathway associated to miR-107. (Right) Representation of target genes of this pathway and gene ensemble ID are shown. (B) Effect of miR-107 overexpression in the modulation of the expression level of genes related to the fatty acid metabolism pathway in LNCaP and DU145 cell lines is shown. (C) Effect of miR-107 overexpression in the modulation of the expression level of FASN in the normal-like prostate cell model (PNT2) is shown. mRNA levels of (B) and (C) were determined by quantitative PCR and normalized by a normalization factor calculated from *ACTB*, *GAPDH*, and *HPRT* expression levels. (D) Representative western blots and quantification of FASN and B-TUB in LNCaP, DU145, and PNT2 cell lines after overexpression of miR-107 are shown. Data are expressed as percentage of control cells (set at 100%). In (B)–(D), values represent the mean ± SEM of at least n = 3 independent experiments. Asterisks indicate significant differences versus controls (*$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$).

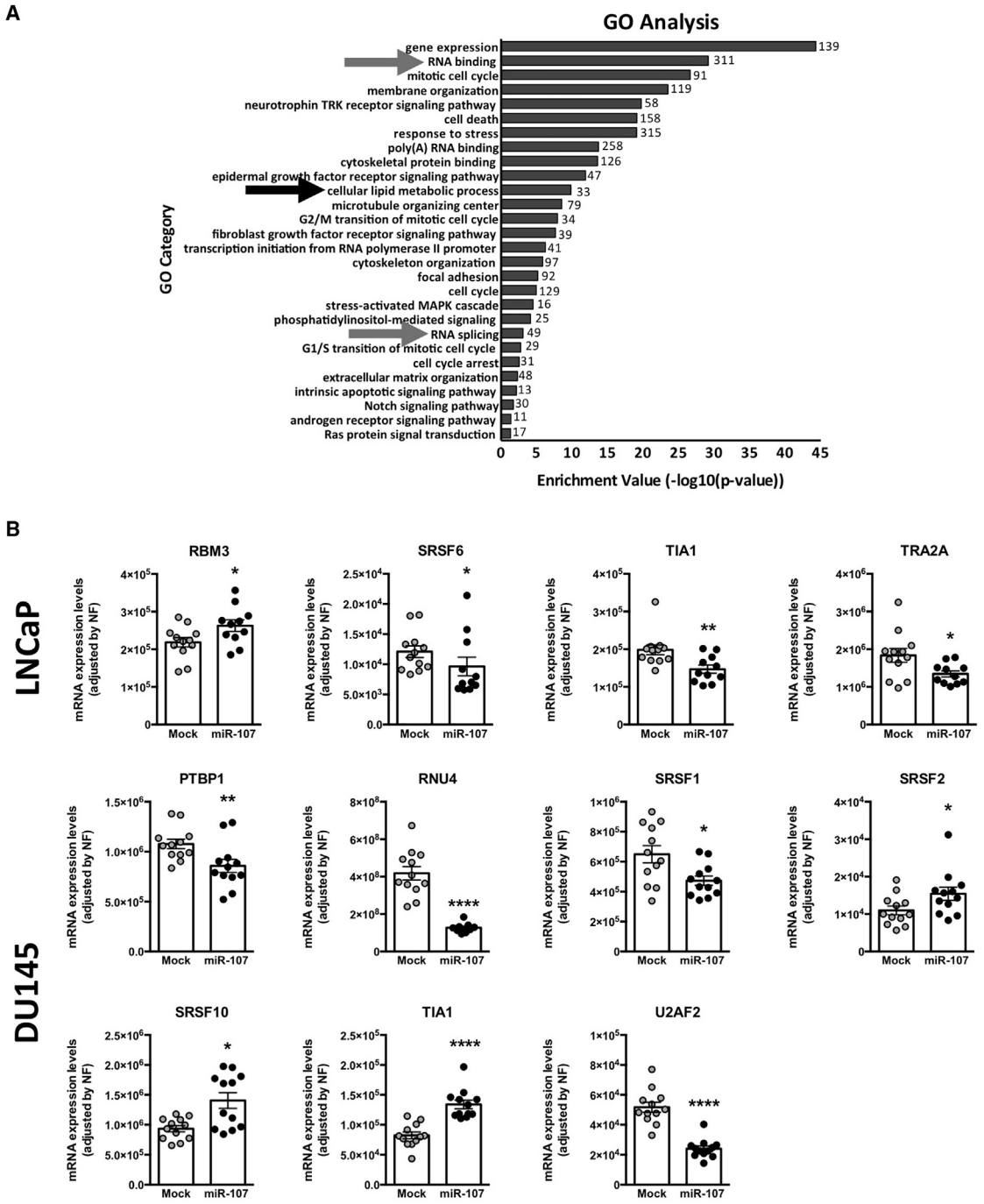

Figure 5. GO terms associated with miR-107 target genes

(A) Data represent −log of the p value of each category and pathway. The black arrow indicates the association of miR-107 with the category of the cellular lipid metabolic process, while gray arrows indicate the association of miR-107 with the category of the splicing process. (B) Effect of miR-107 overexpression in the modulation of the expression level of key genes related with the category of splicing process in LNCaP and DU145 cell lines is shown. mRNA levels were determined by quantitative PCR and normalized by a normalization factor calculated from the expression levels of three housekeeping genes (ACTB, GAPDH, and HPRT). Values represent the mean ± SEM. Asterisks indicate significant differences versus controls (*$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$).

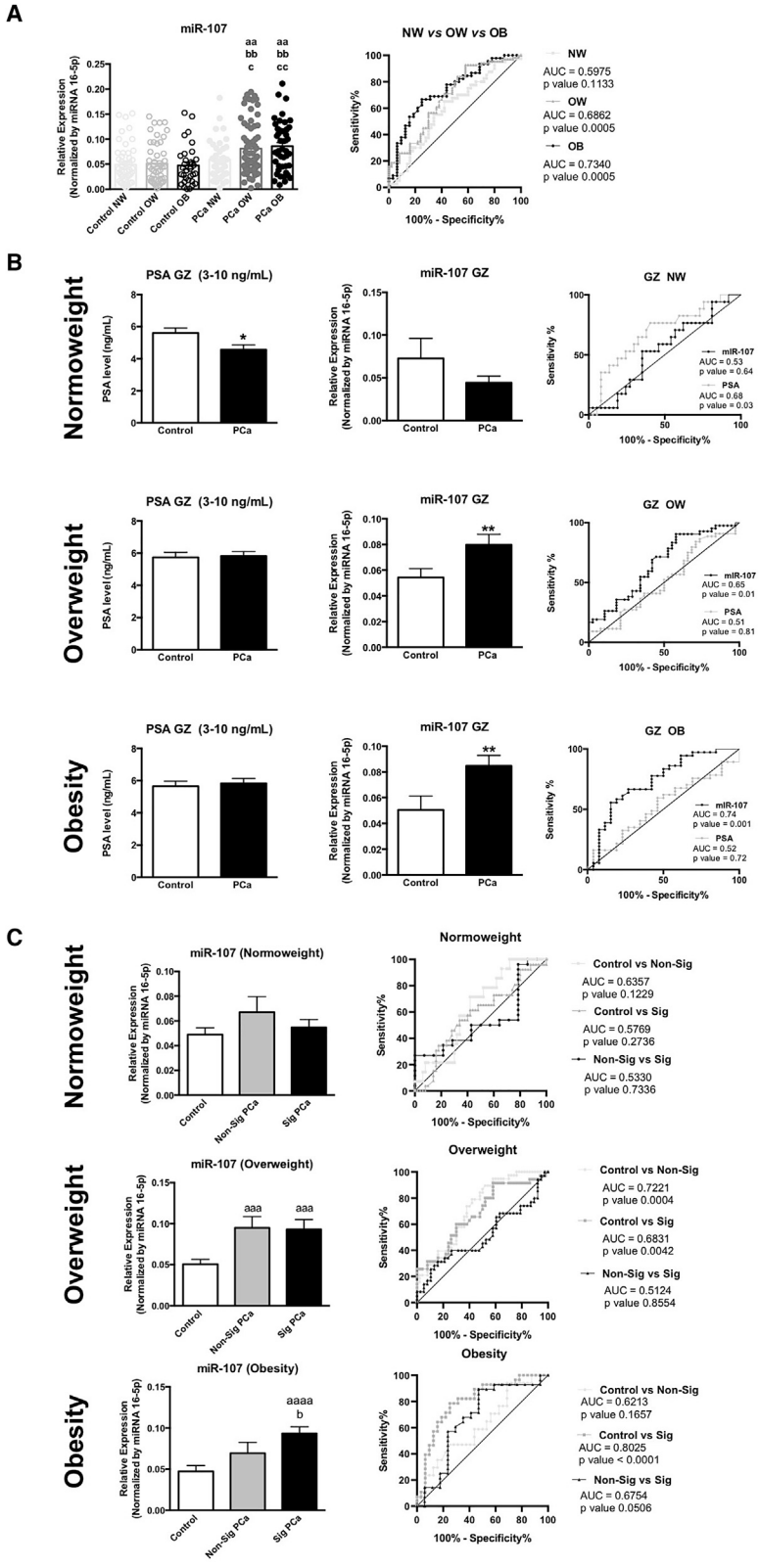

Figure 6. Circulating levels of miR-107 represent a personalized biomarker in the pathological association between PCa and obesity

(A) Plasma level and ROC curve analysis of miR-107 comparing plasma samples from PCa and control patients categorized in normoweight, overweight, and obese groups (cohort B). *a*, *b*, and *c* indicate significant differences compared with normoweight (NW), overweight (OW), and obese OB) control groups, respectively (*a, b, c*, $p < 0.05$; *aa, bb, cc*, $p < 0.01$). (B) Plasma level and ROC curve analysis of PSA and miR-107 comparing plasma sample from PCa and control patients included in the gray zone of PSA subdividing in NW, OW, and OB groups (cohort B) is shown. Asterisks indicate significant differences between compared groups (*$p < 0.05$; **$p < 0.01$). (C) Plasma level and ROC curve analysis of miR-107 comparing between control, non-significant, and significant PCa patients and subdividing in NW, OW, and OB groups (cohort B) are shown. *a* and *b* indicate significant differences compared with control and non-significant PCa groups, respectively (*a, b,* $p < 0.05$; *aaa*, $p < 0.001$; *aaaa,* $p < 0.0001$). GZ means gray zone. Data represent mean ± SEM.

miR-107 levels to discriminate between tumor and control patients significantly outperformed that of PSA levels. Most importantly, this improvement in the diagnostic ability of miR-107 persists when comparing only patients in the gray zone (wherein the capacity of PSA is extremely low). Remarkably, although miR-107 levels were higher in SigPCa patients compared with controls, these levels did not allow to discriminate between SigPCa and NonSigPCa patients; however, miR-107 levels correlated with relevant oncogenic parameters, such as PSA levels,[26] tumor volume,[27] testosterone levels,[28] and CRP[29] in SigPCa patients, but not in NonSigPCa patients, reinforcing also a putative prognostic capacity of plasma miR-107 levels.

Despite miR-107 plasma levels being increased in PCa patients, this miRNA exhibits an apparently controversial reduced expression in PCa tissue samples compared with their non-tumoral adjacent tissues as well as in all PCa cell lines analyzed compared with a non-tumor prostate cell line. These results are consistent with a previous study, which also suggests a tumor suppressor role of miR-107 in PCa cells.[25] However, it has not been demonstrated the reason why the levels of this miRNA increase in plasma samples from PCa patients while their levels are reduced in PCa cells. Here, we found that this discrepancy could be possibly explained by the higher capacity of PCa cells to secrete miR-107 to the extracellular medium compared with control prostate cells (possibly through a direct extracellular vesicle release and/or by a PCa-derived exosomal miRNA release), resulting in a lower level of miR-107 in PCa tissues compared with the plasma levels. These results are in line with several studies demonstrating the same cellular phenomenon in the case of other miRNAs.[30,31] Moreover, this is also consistent with the previously suggested tumor suppressor role of miR-107 in PCa,[25] which was corroborated in the present study. Indeed, when miR-107 is overexpressed in androgen-independent PCa cells (i.e., DU145), several oncogenic features, including cell proliferation, migration, and tumorosphere formation, were significantly reduced. However, it should be noted that the overexpression of miR-107 in an androgen-dependent PCa cell line (i.e., LNCaP) triggered an increase in cell proliferation and tumorosphere formation. These results obtained from androgen-dependent versus androgen-independent PCa cells have not been reported before and suggest the idea that miR-107 could play a different endogenous role in PCa cells, depending on the degree of tumor aggressiveness, maybe due to the sophisticated molecular mechanism of regulation of miRNAs in different cell types.[5] Therefore, although differential responses in androgen-dependent versus androgen-independent PCa cells have been previously reported,[32–34] this particular phenomenon observed in our study warrants further investigation in order to unveil its clinical implication.

Interestingly, more profound *in silico* (KEGG analysis) and *in vitro* studies demonstrated for the first time that miR-107 is implicated in the modulation of fatty acid metabolism, one of the main energy pathways used by tumor cells and whose dysregulation represents a hallmark of PCa.[35] In this sense, we observed several transcriptional dysregulations in genes implicated in this pathway in response to the overexpression of miR-107 in LNCaP and DU145, such as *ACADSB*, *ACADVL*, and *CPT2*. Remarkably, only the expression level of *FASN*, the main driver of fatty acid metabolism,[36] was decreased at mRNA level in both cancer cell lines. However, it should be noted that the protein levels of FASN were decreased in the androgen-independent PCa cell line DU145, while its levels were increased in LNCaP. Notably, these alterations could explain, at least in part, the differential functional *in vitro* results previously discussed in DU145 versus LNCaP cells, as FASN is a well-known oncogenic driver.[37,38] Interestingly, the alteration in the expression of *FASN* (at mRNA and protein levels) observed in both prostate cancer cell models in response to the overexpression of miR-107 was not found in the normal-like prostate cell model (PNT2). These results might suggest that the association of miR-107 with the cellular lipid metabolic process could be specific of prostate cancer cells that reinforce the idea that this metabolic cellular process could be one of the main energy pathways used by prostate tumor cells to enhance their aggressiveness features. Likewise, the *in silico* GO analysis revealed that miR-107 could be also associated with another relevant cellular process, the RNA splicing, which has been recently reported by our laboratory to be tightly implicated in PCa aggressiveness.[39] In support of this idea, we found that several relevant splicing-related genes (such as *PTBP1*, *SRRM1*, and *SRSF6*) were significantly altered in response to miR-107 overexpression in prostate cancer cells. Although more studies are necessary to better understand the cellular and molecular implication of miR-107 in PCa, our results clearly suggest that the dysregulation of the expression of this miRNA triggers defects in the splicing process but especially in the fatty acid metabolism (possible due to the dysregulation of FASN), which are two relevant cellular and molecular processes that represent hallmarks of cancer.[40,41] Therefore, these observations unveiled new conceptual and functional avenues, with potential therapeutic implications, which are worth to be explored in future studies.

Finally, our study also revealed that miR-107 is tightly associated with obese condition in patients, which suggests its putative role as a personalized diagnostic biomarker based on the obese status of the patients. In this sense, it should be emphasized that PCa is strongly influenced by metabolic dysregulations, including obesity.[17,42] Particularly, we observed that the diagnostic capacity of miR-107 significantly increased together with the increase of BMI, reaching its higher AUC value when comparing obese PCa patients versus obese control patients. Furthermore, miR-107 significantly outperformed the diagnostic capacity of the gold-standard PSA, especially when considering patients in the gray zone of PSA. Indeed, in the case of patients with obesity, miR-107 did not only discriminate between tumor and non-tumor patients but also between NonSigPCa and SigPCa, which further reinforces its potential value as prognostic biomarker for this pathology.

In conclusion, this study represents the first demonstration that the plasma levels of miR-107 might represent a useful personalized diagnostic biomarker of PCa since its levels are increased in plasma from PCa patients compared with control subjects using two independent cohorts. In addition, we found that plasma miR-107 levels are associated with key oncogenic parameters, such as PSA levels, tumor volume, testosterone, or CRP levels, suggesting that high plasma miR-107 levels could also be related to PCa aggressiveness and

Table 1. General characteristics of the samples included in the discovery cohort (cohort A)

	Control patients	Prostate cancer patients	p value
n	18	19	
Age	59.61 ± 7.29	67 ± 8.01	0.90
Body mass index (BMI)	27.73 ± 3.72	28.76 ± 3.85	0.41
PSA levels (ng/mL)	0.7 ± 0.41	5.51 ± 2.11	<0.0001

Control patients represent healthy volunteers who donated blood samples. Data are represented as mean ± SD.

progression. Moreover, *in vitro* and *in silico* data revealed that miR-107 is implicated in the regulation of the splicing process and the fatty acid metabolism, altering the expression of the main driver FASN, resulting in a reduction of aggressiveness features in androgen-independent cells. Interestingly, this study also shows novel evidence demonstrating that miR-107 could represent a promising personalized biomarker for PCa, in that miR-107 levels were higher in plasma from obese patients with PCa compared with PCa patients with normoweight. Indeed, miR-107 also allowed a strong discriminatory capacity between SigPCa and NonSigPCa in obese PCa patients, thus representing not only a diagnostic but also a potential prognostic biomarker in that an obesity condition has been reported to represent a risk factor for PCa development, aggressiveness, and mortality.[43,44] Altogether, our results provide new, compelling evidence supporting the contention that miR-107 represents a promising diagnostic, prognostic, and/or therapeutic tool, worth to be further explored, in the pathological association between PCa and obesity.

MATERIALS AND METHODS

Patients and samples

The study protocol was approved by the Reina Sofia University Hospital Ethics Committee, according to institutional and Good Clinical Practice guidelines (protocol number 20052020) and in compliment with the Declaration of Helsinki. Informed consent was obtained from all patients or their relatives. Plasma samples from two cohorts of male patients were collected: (1) cohort A or discovery cohort (n = 37; Table 1) divided in controls (healthy volunteers, n = 18) and PCa patients (n = 19) and (2) cohort B or validation cohort (n = 295; Table 2) divided in controls (patients with suspect of PCa but with a negative result in the biopsy; n = 135) and PCa patients (n = 160; who also were divided in patients with non-significant PCa [NonSigPCa; defined as Gleason score of 6 in the biopsy; n = 70] or with significant PCa [SigPCa; defined as Gleason score $\geq$7 on the biopsy; n = 90]). In addition, patients included in cohort B were also classified according to their BMI in normoweight (BMI < 25), overweight (BMI $\geq$ 25 and <30), and obese (BMI $\geq$ 30) for additional analyses as shown in Table 3. All samples were obtained through the Andalusian Biobank (Nodo Cordoba, Servicio Andaluz de Salud, Spain). Inclusion and exclusion criteria have been reported previously.[45] This represents a retrospective analysis wherein patients were enrolled between 2013 and 2015 by consecutive recruitment of individuals with suspicion of PCa that underwent a transrectal ultrasound (TRUS)-guided prostate biopsy according to clinical practice in the Urology Service of Reina Sofia Hospital (Córdoba, Spain). Specifically, blood and plasma samples were collected early in the morning after an overnight fast and just before the prostate biopsy. Recommendations for biopsy indication were 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 in patients with previous biopsies, a persistent suspicion of PCa (i.e., persistently elevated PSA, suspicious DRE, etc.). For transrectal prostate biopsy, 12 biopsy cores were obtained from patients undergoing the first biopsy procedure and a minimum of 16 biopsy cores for those who had a previous biopsy. All biopsy specimens were analyzed by experienced urologic pathologists according to the International Society of Urological Pathology 2005 modified criteria.[46]

In addition, a set of available formalin-fixed paraffin-embedded (FFPE) tissue samples were used from a cohort of 84 patients with clinically localized PCa subjected to radical prostatectomies (PCa tumor regions [n = 84] and their adjacent non-tumor region [used as control tissues; n = 84] isolated by expert urologic pathologists; cohort C; Table 4) to isolate RNA and perform gene expression analyses, as previously reported.[47,48]

Determination of plasma PSA, testosterone, and C-reactive protein (CRP) levels

As previously reported,[29,39] measurement of PSA, testosterone, and CRP levels were performed in the laboratory service of the Reina Sofia University Hospital of Cordoba using technology of Chemiluminescent Microparticle Immunoassays (References 7k70, 7k73, and 6k26-30/41, respectively; Abbott, Madrid, Spain), following the manufacturer's instructions.

miRNA extraction and retrotranscription

All plasma and FFPE samples were processed using the Maxwell 16 miRNA Tissue Kit and Maxwell 16 miRNA FFPE kit (Promega, Wisconsin, USA) respectively, and extracted using the Maxwell 16 MDx Instrument (Promega) in order to isolate and purify miRNAs as previously described.[49] The quality and concentration of RNA extracted from these samples were evaluated using Nanodrop One Spectrophotometer (Thermo Fisher Scientific). Retrotranscription of these samples was performed using the miRCURY LNA RT Kit (Qiagen, Hilden, Germany), following manufacturer instructions.

Total RNA from the human normal-like, prostate-derived PNT2 and the PCa-derived LNCaP, 22Rv1, PC-3, and DU145 cell lines was isolated using TRI Reagent (Sigma-Aldrich, Madrid, Spain), followed by DNAse treatment using the RNase-Free Dnase Kit (Qiagen), as previously reported.[50] The amount and purity of RNA recovered was determined using the Nanodrop One Spectrophotometer (Thermo Scientific). One microgram of RNA was retrotranscribed to cDNA, using random hexamer primers and the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). To analyze the expression level of miR-107, retrotranscription of the samples was performed using the miRCURY LNA RT Kit (Qiagen), following the manufacturer instructions.

Table 2. General characteristics of the samples included in the validation cohort (cohort B)

	Control patients	Prostate cancer patients	p value
n	135	160	
Age	66 ± 5.67	64 ± 7.21	0.90
BMI	27.43 ± 0.36	28.16 ± 0.3	0.11
PSA levels (ng/mL)	7.13 ± 0.42	27.87 ± 7.14	0.008
Sig PCa (n [%])	–	90 [56.25%]	–
Tumor volume (cm^3)	–	35.64 ± 1.14	–
Testosterone (ng/mL)	5.30 ± 0.17	4.95 ± 0.13	0.11
CRP (mg/L)	4.05 ± 0.59	5.37 ± 0.95	0.26

Control patients represent subjects with suspect of prostate cancer but with a negative biopsy result. Data are represented as mean ± SD or no. total (% [no./total]). BMI, body mass index; CRP, C-reactive protein; PSA, prostate-specific antigen; Sig PCa, significant prostate cancer.

Moreover, miRNAs secreted by PNT2, LNCaP, and DU145 cells were evaluated by isolating miRNAs from the cell culture media (conditioned media) using the miRNeasy Serum/Plasma kit (Qiagen) following the manufacturer's protocol. Retrotranscription of these samples was performed using the miRCURY LNA RT Kit (Qiagen) as described below.

miRNome analysis

Circulating levels of the whole miRNome were determined and compared between control (healthy volunteers; n = 18) and PCa patients (n = 19) from cohort A using 50 ng of the extracted plasma miRNAs and the Affymetrix miRNA 4.1 array (Affymetrix, Santa Clara, USA), as previously described.[49] This array is able to detect all mature human miRNAs identified to date (total of 2,578 miRNAs). Biotin-labeled RNA was synthesized using the FlashTag Biotin HSR RNA Labeling Kit (Thermo Fisher Scientific). Each sample was hybridized onto a GeneChip miRNA 4.1 Array Plate (Thermo Fisher Scientific) following the manufacturer's protocol.

In order to determine the most stably expressed miRNA and therefore the best housekeeping gene for our study, several standard criteria were established: (1) sufficiently high levels in order to allow its quantification by qPCR; (2) lower difference in the levels between different experimental groups, attending to higher (closer to 1) p value; and (3) lower standard error of the mean. According to these parameters, the results revealed that miR-16-5p was the most stable miRNA and the most appropriate housekeeping for the normalization of miRNA levels in these samples. Subsequently, the most altered miRNAs in plasma from PCa patients compared with healthy volunteers were selected according to lower fold-change p value (<0.0001) and higher AUC of the ROC curve analysis.

Analysis of miRNA levels by quantitative real-time PCR

Quantitative real-time PCR was used to determine the levels of selected miRNAs in the validation cohort of human patients (cohort B), in the different human cell lines, and in the conditioned media of the specific cell lines mentioned above. Particularly, to validate the previous results obtained from the Affymetrix miRNA 4.1 array, the levels of six miRNAs (let-7d-5p, miR-24-3p, 26a-5p, 103a-3p, 107, and 191-5p; based on a $p < 0.0001$ and ROC curve with AUC = 1) were measured. Specific primers for these miRNAs (miRCURY miRNA assay, Qiagen) were used in combination with GoTaq qPCR Master Mix (Promega) using the Stratagene Mx3000P (Agilent Technologies, Madrid, Spain), following manufacturer's instructions. mRNA levels were normalized according to the levels of miR-16-5p, whose levels did not significantly vary among the different experimental groups in both human cohorts A and B (data not shown). Similar approaches were used to determine the expression of miR-107 in the different cell lines (PNT2, LNCaP, 22Rv1, PC-3, and DU145) as well as in the conditioned media collected from PNT2, LNCaP, and DU145 cells. In these cases, data were normalized using *RNU6* expression, the most accepted housekeeping for tissues and cells.

Cell cultures, reagents, and transfection with miR-107

PNT2 was a kindly gift from Prof. De Bono lab (London), and LNCaP, 22Rv1, PC-3, and DU145 cell lines were obtained from ATCC, cultured, and maintained under manufacturer's recommendations, as previously reported.[18,20] These cell lines were validated by analysis of short tandem repeats (GenePrint 10 System; Promega, Barcelona, Spain) and systematically checked for mycoplasma contamination.[49,51]

Moreover, LNCaP and DU145 cells were seeded onto 12-well tissue culture plates and serum starved for 24 h previous to the transfection with miR-107 Mimic (1 nM; Qiagen, Hilden, Germany) using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, USA) and following the guidelines provided by the manufacturer. Proliferation, migration, and tumorospheres assays were performed in transfected (mock versus miR-107 overexpression) cells. Transfected cells were collected for RNA and protein isolation. Conditioned media (incubated during 24 h) from transfected cells were also collected for miRNA isolation.

Cell proliferation, migration, and tumorospheres formation assays in response to miR-107 overexpression

Cell proliferation in LNCaP and DU145 cells was evaluated using Alamar-Blue assay (Bio-Source International, Camarillo, CA, USA), as previously reported.[20,52] Briefly, cells were seeded in 96-well culture plates at a density of 3,000–5,000 cells/well and serum starved for 24 h. Then, fluorescence (560 nm) was evaluated using the FlexStation III system (Molecular Devices, Sunnyvale, CA, USA) after 3 h of incubation with Alamar-Blue compound at 10%. Cell proliferation was measured at 24, 48, and 72 h after miR-107 overexpression. Cell culture media was replaced by Alamar-Blue free fresh media after each measurement.

Cell migration was evaluated by wound-healing assay in DU145 cells, as previously reported.[20] Briefly, cells were serum starved for 24 h to achieve cell synchronization, and then the "wound" was made using a

Table 3. General distribution of patients from validation cohort (cohort B) based on body mass index (BMI) (normoweight [BMI < 25], overweight [BMI ≥ 25 and <30], and obese [BMI ≥ 30])

	Control patients (n = 135)			Prostate cancer patients (n = 160)			
	Normoweight[a]	Overweight[b]	Obesity[c]	Normoweight[a]	Overweight[b]	Obesity[c]	p value
n	52	50	33	40	74	46	
Age	65.24 ± 6.31	67.3 ± 5.58	63.26 ± 8.12	65.65 ± 7.5	62.8 ± 5.12	62.43 ± 8.31	0.99[a]; 0.9[b]; 0.83[c]
BMI	23.44 ± 1.46	27.8 ± 1.47	33.17 ± 2.73	23.83 ± 1.1	27.65 ± 1.13	32.76 ± 2.84	0.91[a]; 0.99[b]; 0.93[c]
PSA levels (ng/mL)	7.66 ± 6.09	6.89 ± 3.98	6.64 ± 4.04	37.75 ± 127.5	32.35 ± 91.66	12.37 ± 23.98	0.26[a]; 0.29[b]; 0.99[c]
Sig PCa (n [%])	–	–	–	26 (65%)	35 (47.29%)	29 (63.04)	–

Control patients represent subjects with suspect of prostate cancer but with a negative biopsy result. Data are represented as no. total, mean ± SD. or no. total (% [no./total]).
[a]Refers to the comparison between normoweight control patients and normoweight prostate cancer patients.
[b]Refers to the comparison between overweight control patients and overweight prostate cancer patients.
[c]Refers to the comparison between obese control patients and obese prostate cancer patients.

200-μL sterile pipette tip. Wells were rinsed with sterile PBS, and cells were then incubated for 48 h with supplemented medium without FBS. Wound healing was compared with the area just after the wound was performed. Images were acquired along the "wound" to calculate the area by ImageJ software. Results were expressed as percentage referred to control.

Tumorospheres formation assay was carried out in LNCaP and DU145 cells cultured in a Corning Costar ultra-low attachment plate (no. CLS3473) with DMEM F-12 (Gibco, no. 11320033) with epidermal growth factor (EGF) (20 ng/μL; no. SRP3027) for 10 days (refreshed every 48 h), as previously reported.[47] After 10 days of incubation, an inverted microscope coupled to a digital camera was used to take photographs to visualize and measure tumorospheres morphology and area in order to calculate the number of generated tumorospheres.

qPCR dynamic array based on microfluidic technology

A qPCR dynamic array based on microfluidic technology (Fluidigm, San Francisco, CA, USA) was used to simultaneously determine the gene expression of 14 components of the fatty acid metabolism, as well as of different components of the major spliceosome (n = 13), minor spliceosome (n = 4), and associated splicing factors (n = 14) in LNCaP and DU145 cell lines overexpressing miR-107 (Table S1). The expression of FASN was also determined in the prostate normal-like cell model (PNT2) in response to miR-107 overexpression. Moreover, the expression of three housekeeping genes (*ACTB*, *GAPDH*, and *HPRT*) was analyzed in the same samples. Specific primers for these transcripts were specifically designed with the Primer3 software and StepOne Real-Time PCR System software v.2.3 (Applied Biosystems, Foster City, CA, USA). Preamplification, exonuclease treatment, and qPCR dynamic array based on microfluidic technology were implemented as previously reported,[49,52] following manufacturer's instructions, using the Biomark System and the Real-Time PCR Analysis Software (Fluidigm).

Table 4. Demographic, biochemical, and clinical parameters of the patients with low aggressive PCa (cohort C)

General characteristic	
n	84
Age, years (median [interquartile range (IQR)])	61 (57–66)
PSA levels, ng/mL (median [IQR])	5.2 (4.2–8.0)
Sig PCa (n [%])	76 (90.5%)
pT ≥ 3a (n [%])	59 (70.2%)
PI (n [%])	72 (85.7%)
VI (n [%])	8 (9.52%)
Recurrence (n [%])	35 (41.7%)
Metastasis (n [%])	0 (0%)

PI, perineural invasion; pT, pathological primary tumor staging; VI, vascular invasion.

Western blotting

Prostate cancer cells (LNCaP and DU145) and the prostate normal-like cell model (PNT2) were processed to analyze protein levels by western blot after transfection with miR-107 using methods previously reported.[20,53] Specifically, 200,000 cells were seeded in 12-well plates, and 48 h after transfection, proteins were collected using pre-warmed (65°C) SDS-dithiothreitol (DTT) buffer (62.5 mM Tris-HCl, 2% SDS, 20% glycerol, 100 mM DTT, and 0.005% bromophenol blue). Then, proteins were sonicated for 10 s and boiled for 5 min at 95°C. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Billerica, MA). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline/0.05% Tween 20 and incubated overnight with the specific antibodies for FASN (sc-55580 mouse monoclonal immunoglobulin G [IgG], Santa Cruz Biotechnology) and β-tubulin (rabbit monoclonal antibody [mAb] 2128S, Cell Signaling Technology) and secondary horseradish peroxidase (HRP)-conjugated antibodies (anti-mouse IgG no. 7076S and anti-rabbit IgG antibody no. 7074S, Cell Signal). Proteins were detected using an enhanced chemiluminescence detection system (GE Healthcare, Madrid, Spain). Densitometric analysis of the bands obtained was carried out with ImageJ software.

In silico analyses

The GO enrichment analysis for biological process and KEGG pathway were generated by DIANA-miRPath v.3.0. Predicted miR-107 target

genes were obtained from top KEGG pathway and evaluated using the different *in vitro* approaches described above.

Statistical analysis

Kolmogorov-Smirnov test was used to analyze the normality of the datasets. Parametric data were compared by two-tailed t test, while nonparametric data were compared by Mann-Whitney test. AUC from ROC curves were compared by DeLong test.[54] Correlations were studied using Spearman's correlation test. Representation of volcano plot and clustering analysis by heatmaps were created using MetaboAnalyst.[55] All data were obtained from at least three independent experiments from different cellular passages and expressed as mean ± SEM. p values lower than 0.05 were considered statistically significant. All statistical analyses were performed using the GraphPad Prism 6 (La Jolla, CA, USA) and SPSS v.17.0 (SPSS, Chicago, IL, USA).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2022.02.010.

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AUTHOR CONTRIBUTIONS

V.H.-A., P.S.-M., J.M.J.V., A.D.H.-M., A.S.-C., M.D.G., and R.M.L. designed the project. V.H.-A., P.S.-M., J.M.J.V., M.T.M.-M., A.J.M.-H., J.M.P.-G., J.L.L.-C., F.P.-P., and A.I.-C. carried out the experiments and analyzed data. J.C.-V., F.J.A., E.G.-G., and E.M.Y.-S. organized, collected, and analyzed human samples. A.D.H.-M. and A.S.-C. performed formal analysis. M.D.G. and R.M.L. supervised and acquired funding resources. V.H.-A., P.S.-M., J.M.J.V., M.D.G., and R.M.L. wrote the original draft. All authors revised and edited the submitted manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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