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Cardiometabolic and Renal Risk Research Group

**INSIGHTS INTO THE ROLE OF THE RAB3A-
RAB27A SYSTEM ON THE DEVELOPMENT OF
KIDNEY INJURY ASSOCIATED WITH TYPE II
DIABETES MELLITUS**

Doctoral Thesis

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ABBREVIATIONS AND ACRONYMS

AGEs	Advanced glycosylation end products
ANG-II	Angiotensin-II
BMI	Body mass index
BSA	Bovine serum albumin
CD2AP	CD2 associated protein
cDNA	Complementary DNA
CNT	Control
CP	Cell pellet
DBP	Diastolic blood pressure
DKD	Diabetic kidney disease
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle Medium
DN	Diabetic nephropathy
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
dsDNA	Double stranded DNA
DTT	Dithiothreitol
ECM	Extracellular matrix
EVs	Extracellular vesicles
FBS	Fetal bovine serum
GAP	GTPase-activating proteins
GBM	Glomerular basement membrane

GDP	Guanosin diphosphate
GEF	Guanine nucleotide exchange factor
GFB	Glomerular filtration barrier
GFR	Glomerular filtration rate
GM-130	Golgi matrix 130
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
HG	High glucose
HTN	Hypertension
IDF	International Diabetes Federation
KDa	Kilodalton
miRNA	microRNA
MPs	Microparticles
mRNA	Messenger RNA
MVB	Multivesicular body
NG	Normal glucose
NTA	Nanoparticle tracking analysis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
RAAS	Renin angiotensin aldosterone system
RNA	Ribonucleic acid
ROS	Reactive oxygen species

Rph3A	Rabphilin3A
RPM	Revolutions per minute
RPMI 1640	Roswell Park Memorial Institute
RT	Room temperature
RT-qPCR	Real Time Quantitative PCR
SBP	Systolic blood pressure
SD	Standard deviation
SDS	Sodium dodecyl sulfate
siRAB	Small interfering RNA for RAB3A or RAB27A genes
SRC	Scramble
SRNS	Steroid-resistant nephrotic syndrome
T2DM	Type II Diabetes mellitus
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline-Tween
TEM	Transmission electron microscopy
TG	Triglycerides
TGF-β	Transforming growth factor beta
TSG-101	Tumour susceptibility gene 101
UAE	Urinary albumin excretion
uEVs	Urinary extracellular vesicles
VEGF	Vascular endothelial growth factor
WT-1	Wilms's tumor 1 protein

LIST OF FIGURES

Figure 1. Estimated total number of adults (20-79 years) with diabetes in 2021	4
Figure 2. Pathophysiology of hyperglycaemia in T2DM	6
Figure 3. Glomerular appearance and structural alterations in T2DM	8
Figure 4. Effect of hyperglycaemia on the kidney	9
Figure 5. Integrative signalling networks facilitate interactions between podocyte focal adhesions, the slit diaphragm and actin dynamics.....	17
Figure 6. Mechanisms of podocyte dysfunction in DN.....	22
Figure 7. Rab GTPase cycle	26
Figure 8. Rab proteins-mediated intracellular vesicular transport	27
Figure 9. Biogenesis, secretion and uptake of EVs	34
Figure 10. EVs mediate cell-to-cell communication within the nephron	35
Figure 11. Western blot experiment workflow	56
Figure 12. RT-qPCR procedure.....	60
Figure 13. EVs differential ultracentrifugation isolation protocol.....	66
Figure 14. Urinary levels of Rab GTPases in hypertensive patients	80
Figure 15. Study of cell morphology.....	81
Figure 16. Analysis of human immortalised podocyte associated markers	83
Figure 17. Analysis of Rab GTPases in human immortalised podocytes	85
Figure 18. Podocyte apoptosis during glucose overload and Ang-II treatment.....	87
Figure 19. Morphological changes of podocytes subjected to glucose and Ang-II	88
Figure 20. Analysis of apoptosis by confocal microscopy fluorescence analysis	89
Figure 21. Cytoskeleton rearrangements in HG and Ang II-treated podocytes	90
Figure 22. Podocyte permeability assay.....	91
Figure 23. Rab GTPases gene expression under glucose treatment	92
Figure 24. Podocyte markers and Rab GTPases expression under HG treatment	94
Figure 25. Podocyte markers and Rab GTPase expression under Ang-II treatment	95
Figure 26. Podocyte markers under Ang-II treatment	96
Figure 27. Rab GTPase expression under Ang-II treatment	97

Figure 28. Immunofluorescence analysis of Rab3A-Rab27A system expression under Ang-II treatment.....	98
Figure 29. Effect of <i>RAB3A</i> and <i>RAB27A</i> gene silencing and treatment with HG on other components of the Rab3A-Rab27A system	100
Figure 30. Effect of <i>RAB3A</i> gene silencing and treatment with HG on podocyte associated markers	102
Figure 31. Effect of <i>RAB27A</i> gene silencing and treatment with HG over podocyte associated markers	103
Figure 32. Influence of <i>RAB3A</i> gene silencing and treatment with HG on podocyte apoptosis	105
Figure 33. Influence of <i>RAB27A</i> gene silencing and treatment with HG over podocyte apoptosis	106
Figure 34. Impact of Rab3A-Rab27A system gene silencing and treatment with HG on the podocyte structure	108
Figure 35. Analysis of CD63 intensity and distribution in silenced and HG overload-treated podocytes	110
Figure 36. Characterisation of EVs in the podocyte cell culture medium.	112
Figure 37. MACSPlex immunological marker analysis of podocyte-derived EVs.	113
Figure 38. Analysis of the miRNA levels in <i>RAB3A</i> -silenced and glucose overload-treated podocytes and the EVs isolated from them	115
Figure 39. Analysis of miRNA levels in <i>RAB27A</i> silenced and glucose overload-treated podocytes	116
Figure 40. Schematic illustration of siRab3A and siRab27A effect in human immortalised podocytes	133

LIST OF TABLES

Table 1. Main types and characteristics of EVs 33

Table 2. Primary antibodies used in Western blot. 55

Table 3. Secondary antibodies used in Western blot. 55

Table 4. Primer design parameters. 59

Table 5. Primers employed in mRNA RT-qPCR. 59

Table 6. RT-qPCR parameters. 61

Table 7. Primary antibodies for exosomal markers used in Western blot 68

Table 8. RT-qPCR conditions for miRNAs TaqMan assays. 72

Table 9. Clinical characteristics of patient groups. 78

RESUMEN EXTENDIDO

INTRODUCCIÓN

La diabetes mellitus (DM) es una condición crónica caracterizada por niveles elevados de glucosa en sangre debido a la incapacidad del cuerpo para producir o utilizar adecuadamente la insulina. Hay dos tipos principales: Tipo 1, causada por un proceso autoinmune que reduce la producción de insulina, y Tipo 2 (DMT2), resultante de resistencia a la insulina y producción insuficiente.

La prevalencia mundial de la DM está en aumento, siendo la DMT2 la forma más común. Según el Atlas de la Federación Internacional de Diabetes (IDF 2021), cerca de 537 millones de adultos tienen DM, y se espera que alcance los 783 millones para 2045. La DMT2 representa más del 90% de los casos de DM, caracterizándose por deficiencia de insulina y resistencia a la misma, generando alteraciones metabólicas. Diversos factores contribuyen al desarrollo de DMT2, incluyendo factores genéticos y ambientales.

Las complicaciones a largo plazo de la DMT2 incluyen lesiones macro y microvasculares, siendo estas últimas las más frecuentes. Este tipo de lesiones afectan a pequeños vasos dando lugar a complicaciones como retinopatía, neuropatía, y una de las más habituales, la nefropatía diabética (ND). La ND afecta al 40% de pacientes con DM y es la principal causa de desarrollar enfermedad renal crónica. La ND presenta cambios estructurales y funcionales en el riñón, tanto a nivel glomerular como tubular, algunos de estos cambios incluyen engrosamiento de membranas basales, pérdida de endotelio y acumulación de proteínas de matriz extracelular, produciendo hiperfiltración glomerular y la aparición de albúmina en orina (albuminuria), biomarcador utilizado en el diagnóstico de ND. La lesión renal

en la ND implica, entre otros aspectos, el daño en una de las células clave de la barrera de filtración glomerular (BFG), el podocito.

Los podocitos son células epiteliales diferenciadas que recubren los capilares glomerulares, formando la BFG, responsable de filtrar la sangre y permitir el paso de agua, iones y solutos pequeños, y evitando el paso de la mayoría de las proteínas plasmáticas, como la albúmina, hacia el espacio urinario. Su estructura se divide en tres compartimentos subcelulares: un cuerpo celular grande, los procesos primarios, que son proyecciones del citoplasma cuya forma y tamaño está regulado por microtúbulos y filamentos intermedios, y los pedicelos, prolongaciones del citoesqueleto ricos en filamentos de actina, que asumen una morfología altamente especializada y polarizada, creando interdigitaciones con podocitos vecinos.

En la biología de los podocitos, es fundamental mantener su estructura y conexión a la membrana basal glomerular (MBG) que se consigue mediante la organización precisa de los filamentos de actina en complejos de adhesión focal. Entre los pedicelos de los podocitos, se crean unas uniones que facilitan la conexión entre el citoesqueleto del podocito y la matriz extracelular, conocidas como hendiduras de filtración, del inglés *slit diaphragm* (SD). La SD incluye proteínas transmembrana como nefrina y Neph1, así como podocina y CD2AP, que proporcionan soporte estructural y participan en vías de señalización para mantener la funcionalidad de los podocitos. Para soportar la carga estructural, estas proteínas interactúan con componentes intracelulares que, a su vez, se conectan con la red citoesquelética del podocito. La proteína sinaptopodina, asociada a actina, desempeña un papel crucial en la coordinación del citoesqueleto de actina y afecta a su naturaleza contráctil. Es importante destacar que la capacidad para mantener las funciones de la BFG depende en gran medida de la integridad del citoesqueleto de los podocitos, las adhesiones focales y las SD. Es por

esto que estudiar en detalle los podocitos puede jugar un papel significativo a la hora de comprender los mecanismos involucrados en el desarrollo de la ND.

En el proceso de desarrollo de daño renal en la ND se contemplan cuatro fases: hipertrofia, transición epitelial a mesenquimal, desprendimiento de los podocitos de la membrana basal y apoptosis. Además, el tráfico a través de membranas que permite la comunicación con el entorno mediante exocitosis y endocitosis, es un proceso fundamental para la polarización celular y la comunicación intra e intercelular en los podocitos. En este contexto, las proteínas Rab, un grupo de pequeñas GTPasas monoméricas, desempeñan un papel fundamental en la regulación de la formación de vesículas y en el control de la especificidad y dirección del transporte intra e intercelular.

La familia de Rab GTPasas regula el tráfico de membranas, afectando a la señalización celular, división, supervivencia, remodelación citosquelética y migración celular. Las proteínas Rab GTPasas, actúan como interruptores moleculares que dirigen de forma específica la dirección del tráfico intercelular. Su ciclo entre formas activas (unidas a GTP) e inactivas (GDP) es esencial para su función. Estas proteínas Rab GTPasas participan en importantes procesos fisiológicos como la secreción hormonal, inflamación y neurotransmisión, así como en enfermedades neurológicas, metabólicas, cardiovasculares y cáncer.

Esta tesis doctoral se centra en el análisis de las GTPasas Rab3A y Rab27A. Las subfamilias Rab3 y Rab27 están implicadas en la regulación del tráfico vesicular y las vías de exocitosis de vesículas extracelulares (VEs) en células eucariotas. Se ha demostrado que tanto Rab3A como Rab27A son capaces de unirse a un efector común, Rabphilin3A (Rph3A), para llevar a cabo sus funciones. De hecho, hallazgos recientes indican que las proteínas Rab3A y Rab27A colaboran en la exocitosis de VEs al reclutar a su efector Rph3A.

Aunque se ha analizado el papel de las Rab GTPasas en diversos procesos, se ha profundizado menos en el estudio de su función en órganos específicos como el riñón. Nuestro grupo ha evidenciado una posible implicación de estas RabGTPasas y su efector Rph3A en el daño renal. En un estudio de asociación de genoma completo (GWAS), hallamos una conexión entre un polimorfismo de nucleótido único (SNP) en el gen *RPH3A* y la excreción urinaria de albúmina (EUA) utilizando datos genéticos y metabolómicos combinados procedentes de una población general. Más recientemente, datos del *Strong Heart Study* incluyeron variantes en los genes *RPH3A*, *RPH3AL*, *RAB3IL1* y *RAB27A* para su análisis con respecto a la EUA y el deterioro de la tasa de filtración glomerular (TFG). En el estudio se observaron asociaciones significativas entre los SNPs de los genes *RAB3IL1* y *RPH3A* con la progresión de los niveles de EUA y el deterioro de la TFG. Además, en un estudio previo en la cohorte de Framingham también se observó una asociación entre la EUA y un SNP de *RPH3A*.

Se ha descrito la presencia de las proteínas Rab3A y Rph3A en podocitos procedentes de ratones, ratas y humanos. Además, se han encontrado alteraciones en los niveles de estas proteínas en muestras de ratones y pacientes con proteinuria. Un modelo de ratón *knockout* para *Rab3a* indujo cambios importantes en el citoesqueleto de los glomérulos de animales alimentados con una dieta alta en glucosa. Del mismo modo, nuestro grupo demostró que el silenciamiento de *RPH3A* en un modelo de *Drosophila melanogaster* produjo daño estructural y funcional en los nefrocitos, con una reducción de las capacidades de filtración y en el número de nefrocitos. Los cambios en la estructura de la fibra cardíaca en el mismo modelo de interferencia de *Rph3A* abren la pregunta de si la disfunción de esta proteína podría causar daño simultáneo en las células del corazón y el riñón. Es importante destacar que las Rab GTPasas pueden influir en el transporte de VEs al controlar la maquinaria vesicular y los procesos asociados. Además, su

participación en la organización subcelular y el transporte de vesículas dentro de la célula también puede tener impacto en su liberación. Por lo tanto, comprender las interacciones entre las Rab GTPasas y las VEs es esencial para desentrañar los mecanismos subyacentes a la liberación de vesículas y su función en la comunicación celular.

El término "vesícula extracelular" comprende una población heterogénea de estructuras membranosas derivadas de células, que incluyen exosomas, microvesículas (MVs) y cuerpos apoptóticos, clasificadas según su tamaño y biogénesis. Los exosomas, vesículas redondas de 40 a 150 nm, son las VEs más pequeñas y se liberan al espacio extracelular por la fusión de compartimentos endosomales con la membrana plasmática. Las MVs, también llamadas micropartículas, son un grupo altamente heterogéneo (100-1000 nm) liberado directamente de la membrana plasmática. Los cuerpos apoptóticos son las VEs más grandes (1000-5000 nm) y son liberadas durante la apoptosis.

Las VEs son parte de un proceso evolutivo altamente conservado y son segregadas por casi la totalidad de las células del organismo. La función de cada tipo de VE depende de su contenido, que incluye proteínas, lípidos, ADN y ARN. En particular, se ha destacado la importancia de los ARN pequeños, como los microARN (miARN). La liberación del contenido de las VEs a la célula diana puede producirse mediante diversos mecanismos, como la interacción específica con receptores de membrana, la fusión directa con la membrana plasmática o la internalización por pinocitosis. Inicialmente consideradas como un mecanismo celular para la eliminación de desechos, se ha comprobado que las VEs están involucradas en importantes funciones biológicas en condiciones fisiológicas y patológicas, incluyendo el cáncer, trastornos autoinmunes, enfermedades cardiovasculares y renales.

En el riñón, las VEs juegan un papel importante en la comunicación intrarrenal, estando implicadas en funciones esenciales en la fisiología y patología renal. Las VEs urinarias (VEus) transportan moléculas a lo largo del tracto urinario cuya transmisión influye en la progresión del estado patológico o facilitan la recuperación. Por otro lado, el perfil molecular de las VEus presentes en la orina puede reflejar el origen y el estado fisiopatológico de la célula donante, lo que podría explotarse como una forma no invasiva de diagnóstico y pronóstico de enfermedades renales.

En el contexto de la DMT2 y la ND, se ha caracterizado la composición de ARN y proteínas de las VEus. Se ha observado un aumento en la expresión de ciertas proteínas, como WT-1, en VEus de pacientes diabéticos, lo que sugiere su utilidad como marcadores no invasivos tempranos para la ND. Además, las VEus también han sido implicadas en el desarrollo de la fibrosis renal en condiciones diabéticas a través de la transferencia de ARN mensajero relacionado con TGF- β 1. En cuanto a los miARN transportados por VEus, han sido identificados como posibles predictores de la ND en etapas tempranas. Es importante destacar que las VEs no ejercen exclusivamente efectos perjudiciales, ya que se ha demostrado el impacto beneficioso de las VEs derivadas de células madre en la protección contra lesiones renales en la DMT2, promoviendo la supervivencia celular, la regeneración vascular y la prevención de la apoptosis de los podocitos.

HIPÓTESIS Y OBJETIVOS

La ND surge como consecuencia del daño microvascular derivado de la DMT2. Se caracteriza principalmente por la hiperfiltración glomerular y un aumento en la EUA. La aparición de esta patología se atribuye a múltiples causas, entre las que se incluyen niveles elevados de glucosa en la sangre, la activación del sistema renina-angiotensina-aldosterona, el estrés oxidativo, la inflamación y la alteración en la regulación de diversos factores de crecimiento. Estos elementos combinados inducen modificaciones tanto estructurales como funcionales en el glomérulo, como la desaparición del endotelio fenestrado, el incremento progresivo del mesangio glomerular y la acumulación de proteínas en la matriz extracelular. Asimismo, un proceso clave en la progresión de la ND es la reducción en la cantidad de podocitos, células altamente diferenciadas, fundamentales para la integridad de la BFG. Su correcto funcionamiento está influenciado, en parte, por el tráfico intracelular de vesículas. La familia de proteínas Rab, compuesta por pequeñas GTPasas, entre las que se encuentran las proteínas Rab3A y Rab27A junto con su efector Rph3A, desempeñan un papel crucial en la regulación del tráfico de membranas. Estos procesos incluyen la formación, transporte, unión y fusión de orgánulos y vesículas delimitados por membranas. En investigaciones anteriores, nuestro grupo identificó un SNP en el gen *RPH3A* asociado con un mayor riesgo de presentar albuminuria. Además, al silenciar este gen *in vivo* en un modelo de *Drosophila melanogaster*, observamos una disminución en la capacidad de filtración por parte de los nefrocitos.

El objetivo principal de esta tesis doctoral fue evaluar los niveles de Rab3A-Rab27A en una cohorte de pacientes hipertensos con y sin diabetes y / o daño renal y el papel del sistema Rab3A-Rab27A en el desarrollo de daño renal mediante experimentos *in vitro* sometidos a un entorno diabético.

Los objetivos específicos propuestos fueron:

1. Detectar y cuantificar los niveles de ARNm de las proteínas Rab3A y Rab27A en muestras de orina de pacientes hipertensos y diabéticos, y analizar su asociación con los niveles de excreción de albúmina en la orina.
2. Investigar la presencia del sistema Rab3A-Rab27A en podocitos humanos inmortalizados y analizar los cambios en su expresión cuando se tratan con concentraciones elevadas de glucosa y Angiotensina II.
3. Silenciar la expresión génica del sistema Rab3A-Rab27A en podocitos humanos inmortalizados bajo condiciones de alta glucosa, para profundizar en su efecto sobre la función de los podocitos y determinar cómo este silenciamiento podría afectar el tráfico vesicular.
4. Aislar y caracterizar las VEs segregadas por podocitos humanos inmortalizados tratados con glucosa.
5. Cuantificar los niveles de miRNAs contenidos en las VEs derivadas de podocitos cuando se silencia el sistema Rab3A-Rab27A bajo condiciones diabéticas.

METODOLOGÍA Y RESULTADOS

Niveles del sistema Rab3A-Rab27A en muestras de orina de pacientes con hipertensión arterial con presencia o ausencia de DM2 y su relación con la excreción urinaria de albúmina

La población de estudio incluyó a 64 pacientes con hipertensión arterial esencial, 30 de los cuales tenían DM2, reclutados en el Hospital Clínico Universitario de Valencia, España. La hipertensión arterial se definió según la Sociedad Europea de Hipertensión (presión arterial sistólica ≥ 140 mmHg y/o presión arterial diastólica ≥ 90 mmHg), y la DM se definió según la Organización Mundial de la Salud. Entre los pacientes con DM, 10 fueron diagnosticados con DM sin EUA y 20 con DM y aumento de la EUA (DM EUA). Veinte pacientes no tenían DM (sin DM sin EUA), y 14 pacientes no diabéticos tenían aumento de la EUA (sin DM EUA). Todos los pacientes recibieron tratamiento antihipertensivo en el momento del estudio, y los pacientes con DM fueron tratados con agentes antidiabéticos orales. El protocolo de estudio fue aprobado por el Comité de Ética del Hospital Clínico Universitario de Valencia de acuerdo con la Declaración de Helsinki de 1975, revisada en 2013.

Inicialmente, mediante PCR cuantitativa a tiempo real (RT-qPCR) analizamos los niveles de ARNm de estas Rab GTPasas en el sedimento celular de orina de los pacientes. Los resultados mostraron niveles más altos de *RAB3A* (aumento de 2,17 veces, $p < 0,01$) y de *RAB27A* (aumento de 1,53 veces, $p < 0,05$) en el grupo de pacientes con aumento de la EUA en comparación con el grupo no albuminúrico. El ARNm de *RAB3A* estaba elevado tanto en aquellos pacientes albuminúricos sin diabetes como con diabetes respecto a los no albuminúricos (aumentos de 2,56 y 2,34 veces, $p < 0,01$ y $p < 0,05$, respectivamente). Además, los niveles de EUA

correlacionaron positivamente tanto con *RAB3A* ($r = 0,515$, $p < 0,05$) como con *RAB27A* ($r = 0,475$, $p < 0,05$).

Caracterización de podocitos humanos inmortalizados y marcadores podocitarios

En esta tesis doctoral como modelo *in vitro* se han empleado podocitos humanos inmortalizados (AB8/13) cedidos por el Prof. Moin Saleem (Unidad Renal Pediátrica y Unidad Renal Académica, Universidad de Bristol, Hospital Southmead, Bristol, Reino Unido). Esta línea celular cuenta con la particularidad de ser capaz de proliferar a la temperatura "permissiva" de 33 °C, cuando el transgen del antígeno T del virus del simio 40 (SV40) con el que ha sido modificada la línea celular está activo, manteniendo las células en un estado proliferativo no diferenciado. Al cambiar la temperatura a 37 °C, se silencia el transgen, y las células detienen su proliferación permitiendo que se diferencien. La puesta a punto de nuestro modelo *in vitro* demostró que, a nivel morfológico, los podocitos en proliferación (33 °C) presentaron cuerpos celulares pequeños y redondeados y tras la diferenciación a 37 °C durante 10-14 días, adoptaron una morfología arborizada con cuerpos celulares agrandados, procesos podocitarios y pedicelos formando uniones en forma de huso.

Mediante RT-qPCR se evaluaron los niveles de ARNm y mediante Western blot los niveles proteicos de moléculas específicas de podocitos. Los podocitos diferenciados mostraron aumentos significativos en los niveles de ARNm de sinaptopodina y nefrina, mientras que los niveles de *WT-1* disminuyeron. A nivel proteico, los podocitos diferenciados exhibieron aumentos en los niveles de sinaptopodina, *WT-1*, nefrina y CD2AP en comparación con los podocitos en proliferación. A lo largo del proceso de diferenciación, se observó un aumento general en los niveles de estos marcadores.

Expresión del sistema Rab3A-Rab27A en podocitos en proliferación y diferenciados

Mediante RT-qPCR se analizó la presencia y la expresión diferencial del sistema Rab3A-Rab27A en podocitos humanos inmortalizados. Los niveles de ARNm de *RAB3A* y *RAB27A* fueron mayores en podocitos diferenciados (aumento de 1,75 veces, $p < 0,01$, y aumento de 1,66 veces, $p < 0,05$, respectivamente). Para el gen *RPH3A*, fue necesario realizar una PCR semianidada con dos parejas de cebadores para detectar su expresión, identificándose amplicones de *RPH3A* tanto en podocitos no diferenciados como diferenciados.

Paralelamente, haciendo uso de la técnica Western blot, se analizaron los niveles de las proteínas Rab3A, Rab27A y Rabphilin3A en podocitos en estado no diferenciado y diferenciado. Las células diferenciadas mostraron una reducción en los niveles de proteínas de Rab3A (disminución de 1,22 veces, $p < 0,05$), Rab27A (disminución de 1,25 veces, $p < 0,05$) y Rabphilin3A (1,30 veces menos, $p < 0,01$).

Niveles de moléculas específicas de podocitos y del sistema Rab3A-Rab27A bajo estrés por glucosa y Angiotensina-II

Cuando analizamos los niveles de ARNm tras tratar los podocitos con elevadas concentraciones de glucosa, del inglés *high glucose* (HG) (30mM) durante 48 y 72 h, las moléculas asociadas a los podocitos, sinaptopodina, *WT-1*, nefrina y *CD2AP*, no mostraron diferencias estadísticamente significativas. En cuanto a los cambios proteicos, se observaron niveles reducidos de los marcadores WT-1 (- 1,20 veces, $p < 0,05$), CD2AP (- 1,79 veces, $p < 0,01$) y nefrina (disminución de 1,99 veces, $p < 0,05$), así como una tendencia a la baja en los niveles de la proteína sinaptopodina (-1,19 veces, $p > 0,05$). Estos resultados también fueron confirmados mediante inmunofluorescencia, en la cual, bajo condiciones de glucosa normal, del inglés *normal glucose* (NG), la sinaptopodina mostró un patrón citoplasmático, extendiendo su expresión a través de los procesos podocitarios, y la nefrina mostró

un patrón de expresión perinuclear. Bajo condiciones de HG, la sinaptopodina reveló un cambio en su patrón, no observado bajo condiciones de NG, y la nefrina mostró un aumento en la expresión citoplasmática y una reducción en la región perinuclear; en general, los niveles de marcadores podocitarios disminuyeron en comparación con las condiciones NG.

Curiosamente, las proteínas Rab GTPasas también mostraron cambios en sus niveles de proteínas bajo condiciones de HG. Rab3A y Rab27A tuvieron niveles aumentados (aumento de 1,39 veces y 1,27 veces, respectivamente, $p < 0,05$), mientras que Rabphilin3A tuvo niveles reducidos (disminución de 1,54 veces, $p < 0,01$). En experimentos de inmunofluorescencia, Rab3A, Rab27A y Rabphilin3A mostraron patrones de expresión citoplasmáticos y membranosos punteados, como se esperaba en proteínas vesiculares, alcanzando también la membrana celular. Interesantemente, bajo condiciones de HG, Rab27A reveló un cambio en su patrón de expresión en comparación con las condiciones de NG, mostrando niveles aumentados cerca de la membrana plasmática del podocito.

Tras el tratamiento con Angiotensina-II (Ang-II) durante 6, 24 y 48 h, a diversas concentraciones (0,1, 1 y 2 μM), la cuantificación de los niveles de ARNm mostró una elevación en los niveles de *WT-1* y *CD2AP*, únicamente observada en la concentración más baja de Ang-II (0,1 μM). De manera análoga, se observó un incremento en los niveles de ARNm de *RAB3A* y *RAB27A*. Por otro lado, a nivel proteico, las moléculas asociadas a los podocitos, sinaptopodina, WT-1, nefrina y CD2AP, mostraron, en general, niveles más bajos a concentraciones altas de Ang-II. Estos cambios también se reflejaron en experimentos de inmunofluorescencia, que mostraron una reducción en la expresión de sinaptopodina y un cambio en su distribución subcelular, así como niveles más bajos de nefrina, con una menor tinción perinuclear.

Las proteínas Rab3A, Rab27A y Rabphilin3A mostraron, mayoritariamente, una expresión aumentada durante los tratamientos y, también presentaron diferencias según la concentración de Ang-II y los tiempos de tratamiento. Los análisis de inmunofluorescencia mostraron un patrón similar en concordancia con los cambios a nivel proteico, con intensidad aumentada de Rab3A, Rab27A y Rabphilin3A bajo estimulación de Ang-II. Curiosamente, como se observó con el tratamiento de glucosa, Rab27A mostró un aumento en la expresión en la zona cercana a la membrana bajo tratamiento con Ang-II.

Efecto de los tratamientos de glucosa y Angiotensina-II en el proceso de apoptosis celular y la estructura del citoesqueleto

El efecto de los tratamientos sobre la apoptosis podocitaria fue analizado mediante citometría de flujo. Además, haciendo uso de la microscopía confocal, analizamos la distribución de las fibras de F-actina midiendo la anisotropía fraccional, que indica cuán organizadas están las fibras de F-actina en la célula. En cuanto a la apoptosis y los cambios en el citoesqueleto de actina inducidos por los tratamientos en podocitos, se observó un aumento de la apoptosis en el grupo de HG, en comparación con el grupo de NG, con una tendencia similar en el tratamiento más alto de Ang-II. Las observaciones morfológicas y de tinción nuclear con DAPI confirmaron los cambios apoptóticos en los podocitos tratados con HG y Ang-II. Además, se observaron alteraciones en la organización del citoesqueleto.

Efecto del silenciamiento de los genes *RAB3A* y *RAB27A* y tratamiento con glucosa elevada en el sistema Rab3A-Rab27A y marcadores de podocitos

Para entender mejor el papel de las GTPasas Rab3A y Rab27A, silenciamos mediante el uso de siRNAs ambos genes en cultivos de podocitos, incubando durante 24 horas con los siRNAs de interés, y tratando posteriormente con glucosa (30 mM) durante 24 h adicionales. Para estudiar posibles alteraciones en el sistema

Rab resultantes del silenciamiento de uno de sus componentes (siRab3A o siRab27A), y los cambios observados bajo sobrecarga de glucosa, analizamos la expresión de Rab27A cuando *RAB3A* estaba silenciada y viceversa, así como los niveles de su efector común Rph3A. Además, se estudiaron los marcadores de podocitos para observar cómo el silenciamiento de Rab junto con el tratamiento de glucosa, o una combinación de ambos, induce alteraciones en la estructura y función de los podocitos. Nuestros resultados mostraron una disminución en los niveles de ARNm de cada Rab cuando la otra estaba silenciada bajo condiciones de NG (-2,99 veces, $p < 0,05$ para siRab3A; -1,72 veces, $p < 0,05$ para siRab27A). Sin embargo, se observó el efecto contrario en el grupo con HG con una expresión aumentada de *RAB27A* cuando se silenciaba *RAB3A* y viceversa, aunque solo la expresión de *RAB3A* alcanzó significación estadística (1,71 veces, $p < 0,05$). También se analizaron los niveles proteicos de esta Rab, pero sin alcanzar significación estadística. Los niveles de proteína de Rph3A disminuyeron bajo la condición NG cuando se silenciaron *RAB3A* Y *RAB27A* (-1,16 veces, $p < 0,01$; -1,15 veces; $p < 0,05$, respectivamente). En contraste, bajo condiciones de sobrecarga de glucosa, los niveles de Rph3A aumentaron bajo el silenciamiento de *RAB27A* (1,14 veces; $p < 0,001$). Estos hallazgos sugieren que cada Rab desempeña un papel beneficioso al aumentar sus niveles de expresión durante el estrés celular para contrarrestar la disminución de la otra Rab GTPasa.

Para estudiar la influencia del silenciamiento del sistema Rab3A-Rab27A y la sobrecarga de glucosa en las proteínas estructurales y funcionales de los podocitos, nuestro siguiente paso fue analizar los niveles génicos y proteicos de los marcadores de podocitos sinaptopodina, CD2AP, WT-1 y nefrina. En el caso del silenciamiento de *RAB3A*, se observó una disminución general de ARNm en todos los marcadores bajo condiciones de NG, como se muestra para sinaptopodina (-3,93 veces, $p < 0,01$) y *CD2AP* (-2,18 veces, $p < 0,05$), mientras que bajo

sobrecarga de glucosa se observó una tendencia al aumento en todos los marcadores de podocitos, siendo significativo para sinaptopodina (3,15 veces, $p < 0,01$) y *WT-1* (4,75 veces, $p < 0,05$). Para los niveles de proteína, se observó un aumento en CD2AP y WT-1 cuando se silenció *RAB3A* a concentraciones de glucosa elevada. En resumen, estos hallazgos muestran que el silenciamiento de *RAB3A* induce una disminución general en los marcadores de podocitos, mientras que se observa el efecto contrario con el silenciamiento bajo sobrecarga de glucosa.

En el caso del silenciamiento de *RAB27A*, se encontraron niveles reducidos de ARNm de sinaptopodina (-3,12 veces, $p < 0,05$) y *CD2AP* (-2,32 veces, $p < 0,05$) en condiciones NG. Bajo condiciones de sobrecarga de glucosa, se observó una tendencia a la disminución en los niveles de sinaptopodina, mientras que, a diferencia de las condiciones NG, hubo un aumento notable en los niveles de *CD2AP* (1,70 veces, $p < 0,05$) y nefrina (4,58 veces, $p < 0,001$). En cuanto a los niveles de proteína, se observó una tendencia a la baja en los niveles de sinaptopodina y *CD2AP* bajo condiciones NG, en contraste con un aumento en nefrina (1,32 veces, $p < 0,001$). En glucosa elevada, el silenciamiento de *RAB27A* produjo una disminución en sinaptopodina (-1,19 veces, $p < 0,05$) y *CD2AP* (-1,22 veces, $p < 0,01$) y un aumento en nefrina (1,45 veces, $p < 0,05$). Similar a lo observado con el silenciamiento de *RAB3A*, el silenciamiento de *RAB27A* también parece inducir la desdiferenciación de los podocitos cuando se silencia sin tratamiento, produciendo el efecto opuesto bajo sobrecarga de glucosa. Lo que se acompaña de una reducción de los marcadores podocitarios.

Influencia del silenciamiento del sistema Rab3A-Rab27A y la sobrecarga de glucosa en la apoptosis podocitaria

Buscamos determinar el efecto del silenciamiento de Rab3A-Rab27A y el tratamiento con glucosa en la apoptosis de los podocitos. En primer lugar, los ensayos de citometría de flujo mostraron que en ambos casos (silenciamiento de *RAB3A* y *RAB27A*), se observó un aumento significativo en la apoptosis en el grupo control scramble (CNT scr) en comparación con el grupo CNT sin transfección, evidenciando un efecto de la lipofectamina en la muerte celular ($p < 0,05$). Del mismo modo, se observó un aumento en la muerte celular en los grupos tratados con concentraciones elevadas de glucosa sin silenciamiento. Sorprendentemente, el silenciamiento de *RAB3A* provocó una disminución en la apoptosis ($p < 0,05$), contrario al silenciamiento de *RAB27A*, que produjo un aumento en la apoptosis en condiciones de NG. Sin embargo, en células expuestas a concentraciones de HG, la apoptosis fue menor en ambos grupos silenciados para las proteínas Rab en comparación con los grupos HG + CNT scr. Este efecto fue más pronunciado en el grupo silenciado de *RAB3A* ($p < 0,001$) que en el grupo silenciado de *RAB27A* ($p < 0,05$).

Análisis de la citoarquitectura celular y marcadores de transporte vesicular bajo el silenciamiento del sistema Rab3A-Rab27A y estrés por glucosa

Para evaluar la influencia del silenciamiento del sistema Rab y la sobrecarga de glucosa en la estructura de los podocitos, analizamos la anisotropía de la organización de las fibras de actina mediante microscopía confocal. Se observó un aumento en la desorganización de las fibras de actina al comparar los grupos NG vs HG (valores de anisotropía cercanos a 0), este efecto también se observó en la condición NG scr como consecuencia del efecto tóxico de la lipofectamina. Se obtuvieron resultados similares tras el silenciamiento de *RAB3A* y *RAB27A*, dando

lugar a una red de fibras desorganizada en los podocitos silenciados. Curiosamente, tras el silenciamiento de las proteínas Rabs seguido del tratamiento con HG, el efecto observado previamente en la organización de las fibras se invirtió, mejorando los valores de anisotropía cuando las células se habían silenciado y tratado con HG.

Dada la participación del sistema Rab en el tráfico vesicular, a continuación, estudiamos la influencia del silenciamiento de *RAB3A* y *RAB27A* y del tratamiento con HG en la expresión de CD63 y CD9, tetraspaninas que se encuentran enriquecidas en exosomas. Para ello, realizamos análisis de inmunofluorescencia de CD63 y CD9 y cuantificamos la intensidad de fluorescencia. En condiciones de NG, el silenciamiento de *RAB3A* y *RAB27A* de manera individual resultó en un aumento de la señal de CD63, con un cambio en su distribución de un patrón punteado homogéneo y disperso en todo el citoplasma (CNT) a un patrón con mayor acumulación en la zona perinuclear. Sin embargo, tras el silenciamiento de cada una de las proteínas Rabs y el tratamiento con HG, este efecto no fue tan marcado como en NG y tendió a parecerse al patrón del CNT, especialmente en el caso de siRab27A. Por otro lado, el patrón e intensidad de CD9 no mostró cambios significativos, pero su intensidad tendió a disminuir cuando se silenciaron las Rabs tanto en condiciones de NG como de HG. Estos hallazgos podrían indicar que el silenciamiento de *RAB3A* y *RAB27A* podría causar alteraciones en la vía de transporte vesicular asociada a CD63 en los podocitos.

En conjunto, estos resultados proporcionan evidencia adicional sobre una diferente respuesta de la célula, dependiendo de si el silenciamiento génico ocurre bajo condiciones fisiológicas normales o en un entorno diabético.

Aislamiento de VEs segregadas por podocitos y su caracterización

Se utilizaron diversas estrategias para evaluar el adecuado aislamiento y purificación de fracciones ricas en exosomas. Las VEs del medio de cultivo de podocitos, se aislaron mediante ultracentrifugación diferencial. Tras su aislamiento, se emplearon técnicas complementarias que permitieron la caracterización de las muestras por tamaño, composición proteica y concentración. Las VEs se caracterizaron analizando su distribución de tamaño mediante *Nanoparticle Tracking Analysis* (NTA), su morfología y presencia de tetraspaninas CD9 y CD63 mediante microscopía electrónica de transmisión (*immunogold*) y la pureza de la suspensión de VEs mediante análisis de la expresión de marcadores de VEs mediante *Western blot*, microscopía de superresolución (ONI) y citometría de flujo.

El análisis por NTA mostró un pico marcado de las fracciones seleccionadas de VEs con tamaños que oscilaron entre 140 y 200 nm. Las imágenes de TEM revelaron muestras puras sin precipitados no deseados ni restos celulares, y mostraron vesículas teñidas con marcadores de VEs bien establecidos como CD63 y CD9. Además, los experimentos de *Western blot* confirmaron la pureza de las muestras, donde las vesículas estaban enriquecidas con marcadores específicos de superficie y citoplasmáticos, como CD63, CD9, Sintenina-1 y TSG-101. Los marcadores de orgánulos celulares implicados en la secreción como el retículo endoplásmico, mediante calnexina, y el aparato de Golgi, mediante GM-130, estaban altamente enriquecidos y expresados exclusivamente en las muestras de pellets celulares procedentes de podocitos. Además, la presencia de tetraspaninas CD63, CD9 y CD81 en VEs aisladas de podocitos no diferenciados y diferenciados se confirmó mediante microscopía de superresolución.

Asimismo, las VEs aisladas de podocitos no diferenciados, diferenciados y tratados con HG se caracterizaron mediante citometría de flujo utilizando el kit de

análisis de exosomas MACSPlex después de la inmunocaptura basada en perlas. Este kit nos permitió detectar los niveles de 37 marcadores de superficie, incluyendo tetraspaninas, marcadores mesenquimales, endoteliales/plaquetarios e inmunológicos. Las tetraspaninas CD9, CD63 y CD81 estuvieron presentes en todas las muestras medidas. Curiosamente, los niveles de los marcadores de células madre mesenquimales CD44 y CD146 fueron más altos en las células no diferenciadas, mientras que las VEs aisladas de podocitos diferenciados mostraron una expresión aumentada de marcadores renales como CD133 y CD24. Esto destaca la importancia de usar células diferenciadas cuyo perfil molecular es más similar a las condiciones fisiológicas en el riñón humano.

Efecto del silenciamiento del sistema Rab3A-Rab27A en la expresión de miARN en podocitos y sus exosomas

Los miARN son una de las moléculas más relevantes transportadas por las VEs dado su papel regulador en numerosos procesos biológicos a nivel celular. Se ha demostrado que niveles alterados de estas moléculas pueden influir en diversas patologías. Dada la relevancia de los miRNAs, analizamos los niveles de varios miARN clave en la ND en pellet celular de podocitos y en los exosomas derivados de estas células tras silenciar el sistema Rab3A-Rab27A y someter a las células a estrés por glucosa.

Se observó que, al silenciar *RAB3A*, los niveles del miR-21-5p en el sedimento celular aumentaron bajo HG scr (1,94 veces, $p < 0,05$) o HG siRab3A (2,22 veces, $p < 0,05$). Por el contrario, en los exosomas secretados, estos niveles aumentaron bajo tratamiento de HG y cuando se silenció *RAB3A* (3,12 y 3,42 veces respectivamente, $p < 0,05$ para ambas condiciones), pero fueron más bajos en el grupo HG siRab3A que en el grupo NG siRab3A (-3,12 veces y -3,42 veces, $p < 0,05$), sugiriendo una influencia del silenciamiento de *RAB3A* bajo condiciones de estrés

por HG. Para el miR-26a-5p, se observó el mismo efecto que para miR-21-5p a nivel celular, un aumento en el grupo HG scr (2,01 veces, $p < 0,05$), pero una disminución bajo el silenciamiento de siRab3A sin tratamiento de glucosa (-1,56 veces, $p < 0,05$), que aumentó cuando los podocitos con la Rab silenciada fueron tratados con HG (1,62 veces, $p < 0,05$ en comparación con NG siRab3A). Respecto a la fracción exosomal, los niveles de expresión de miR-26a-5p disminuyeron significativamente bajo siRab3A (-22,2 veces, $p < 0,05$), mientras que los podocitos con *RAB3A* silenciada y sometidos a HG mostraron niveles elevados en comparación con NG siRab3A (-3,05 veces, $p < 0,05$). Se encontró una disminución en los niveles de miR-31-5p en el sedimento celular cuando se silenció *RAB3A* con o sin tratamiento de glucosa (-2,23 veces, $p < 0,01$ y -1,56 veces, $p < 0,05$ en comparación con los grupos control NG scr y HG scr, respectivamente), mientras que los niveles exosomales fueron indetectables en la mayoría de las muestras.

En el caso del silenciamiento de *RAB27A*, los niveles de miARN exosomal fueron indetectables en la mayoría de las muestras. En el sedimento celular, los niveles de miR-21-5p aumentaron en los grupos HG scr y HG siRab27A (2,06 veces, $p < 0,01$ y 2,13 veces, $p < 0,05$). Los niveles de miR-26a-5p disminuyeron en el grupo HG scr en comparación con NG scr (-3,33 veces, $p < 0,05$), mientras que, bajo el silenciamiento de *RAB27A*, los niveles de miR-26a-5p aumentaron cuando las células fueron sometidas a tratamiento de HG (1,60 veces, $p < 0,05$). Finalmente, los niveles de miR-31-5p aumentaron bajo el tratamiento de HG (1,99 veces, $p < 0,05$), pero en los grupos siRab27A, con o sin glucosa, cayeron a niveles similares a los del grupo NG scr.

En resumen, los resultados de esta tesis doctoral proporcionan nuevas perspectivas sobre el papel de las GTPasas Rab3A y Rab27A en el desarrollo de la ND. Hemos identificado alteraciones en el sistema Rab3A-Rab27A en el sedimento celular de la orina de pacientes hipertensos con y sin diabetes y / o daño renal. De

hecho, hemos descrito las GTPasas Rab3A y Rab27A como participantes clave en la lesión de podocitos y la regulación del tráfico vesicular en condiciones diabéticas. El silenciamiento génico transitorio de las moléculas *RAB3A* o *RAB27A* bajo condiciones de hiperglucemia resultó en un aumento compensatorio en el ARN mensajero de la otra Rab GTPasa, sugiriendo su cooperación e identidad como un sistema. La alteración en el patrón de expresión de CD63, acompañada de cambios en los marcadores de diferenciación de podocitos cuando se silencian *RAB3A* o *RAB27A*, subraya la importancia del sistema en el mantenimiento de la citoarquitectura de podocitos a través de su papel en el tráfico vesicular. Sin embargo, bajo condiciones de estrés glucémico, el silenciamiento de Rab mejora la expresión de moléculas de diferenciación, reduce la apoptosis e implica un patrón de distribución de vesículas menos alterado en los podocitos. Estos hallazgos, sugieren un aumento en la actividad del sistema Rab3A-Rab27A en los podocitos que, en la DM, estaría implicado en el desarrollo de daño renal a través de la alteración del tráfico vesicular, y por tanto de procesos celulares regulados por éste. Además, miARNs relevantes para la ND mostraron una expresión alterada bajo el silenciamiento y el tratamiento con glucosa. Finalmente, nuestros resultados sugieren que el sistema Rab3A-Rab27A podría orquestar parte de las alteraciones observadas en los podocitos en el desarrollo y la progresión del daño renal en la ND.

CONCLUSIONES

1. Este estudio demuestra por primera vez la presencia del sistema Rab3A-Rab27A en muestras de sedimento celular en orina de pacientes. Además, describe cambios en la expresión de ARNm del sistema Rab3A-Rab27A en muestras de pacientes con hipertensión arterial y DM2 con daño renal. Ambas proteínas Rab GTPasas muestran una asociación positiva con los niveles de albuminuria.
2. Se ha descrito la presencia del sistema Rab3A-Rab27A en podocitos humanos inmortalizados. Los patrones de aumento y distribución alterados del sistema Rab3A-Rab27A observados en podocitos tratados con glucosa y Ang-II sugieren que el tráfico vesicular guiado por el sistema Rab3A-Rab27A durante la lesión de podocitos en la ND podría tener un papel relevante en esta patología.
3. En condiciones de estrés por glucosa, el silenciamiento de cualquiera de los dos componentes del sistema Rab3A-Rab27A lleva a un aumento compensatorio en el ARNm del otro. Además, el silenciamiento de *RAB3A* o *RAB27A* induce cambios en la expresión de ARNm y niveles de proteínas de marcadores de podocitos, tanto en condiciones normales como de glucosa alta, lo que sugiere su participación en el mantenimiento de la citoarquitectura de los podocitos y su implicación en procesos de dediferenciación bajo estrés por glucosa. Además, la inhibición del sistema Rab3A-Rab27A en podocitos reduce la apoptosis causada por condiciones hiperglucémicas y restaura la distribución del marcador vesicular CD63.
4. Se ha verificado el correcto aislamiento y caracterización de VEs de podocitos humanos inmortalizados mediante varias técnicas. Además, la caracterización de las proteínas presentes en su superficie mostró diferencias entre las vesículas aisladas de podocitos en proliferación y podocitos diferenciados.

5. Los niveles de miARNs relevantes en la ND, como miR-21-5p, miR-26a-5p y miR-31-5p, se han visto alterados en el pellet celular de podocitos tras el silenciamiento de *RAB3A* o *RAB27A* y el subsiguiente tratamiento con glucosa. Además, también se han descrito cambios en los niveles de miARNs contenidos en VEs aisladas de células silenciadas para *RAB3A* y tratadas con glucosa.

ABSTRACT

Type 2 diabetes mellitus is an increasingly prevalent global health issue. It is well-recognised that type 2 diabetes mellitus is a leading factor contributing to renal damage, with up to 40% of patients progressing to diabetic nephropathy. Diabetic nephropathy is a common complication in diabetic patients with severe clinical implications, leading to end-stage renal disease. Kidney injury in diabetes entails, among others, damage in a key cell of the glomerular filtration barrier, the podocyte, which undergo loss and detachment from the basal membrane increasing the permeability of the glomerular filtration barrier. Communication between podocytes and other renal cells, mediated by extracellular vesicles, is vital for maintaining the intricate balance of renal function. This communication orchestrates the complex interplay of signalling cascades that contribute to the overall health and homeostasis of the kidneys. Within this framework, Rab GTPases play an indispensable role in regulating vesicle transport, which can impact an individual's health or contribute to disease onset. In a genetic association study, our team pinpointed a single nucleotide polymorphism in the *RPH3A* gene, an effector of Rab3A and Rab27A GTPases, to be associated with an increased risk for developing albuminuria.

The central aim of this doctoral thesis was to investigate the role of the Rab3A-Rab27A GTPase system on the development of kidney injury associated with type II diabetes mellitus. Therefore, the components of this system were quantified in urine samples from patients with hypertension and diabetes and / or renal damage and the effect of elevated glucose and angiotensin II levels on human immortalised podocytes was evaluated. Moreover, this research delved into the silencing the Rab3A-Rab27A system by transient transfection through siRNAs in podocytes exposed to high glucose concentrations to assess its impact on podocytes physiology. To achieve these objectives, mRNA levels of podocyte

markers and Rab GTPases were analysed by RT-qPCR, while protein levels were quantified through Western blot and confocal microscopy. Additionally, apoptosis, cytoskeletal arrangement, and vesicle distribution were assessed by immunofluorescence. Extracellular vesicles were characterised using several techniques, including nanoparticle tracking analysis, transmission electron microscopy, Western blot, flow cytometry, and super-resolution microscopy. Furthermore, microRNA expression levels in podocytes and podocyte-derived extracellular vesicles were quantified by quantitative RT-qPCR.

The results showed that in urine pellets from hypertensive-diabetic patients, *RAB3A* and *RAB27A* mRNA levels were increased and showed a direct correlation with albuminuria. Rab3A and Rab27A protein levels were increased under glucose overload, and Rabphilin3A decreased. Furthermore, this system exhibited higher levels under stress conditions in a manner of angiotensin II dose and time treatment. Immunofluorescence imaging indicated different expression patterns of Rab27A under treatments. Silencing *RAB3A* and *RAB27A* leads to a decrease in podocyte differentiation markers and cytoskeleton organization, an increase in apoptosis and a CD63-positive vesicle cellular pattern distribution change. Interestingly, under high glucose, Rab3A-Rab27A silencing ameliorates some of these detrimental processes. Moreover, in both podocytes and extracellular vesicles derived from podocytes, we observed significant expression changes in miRNAs that regulate key pathways involved in diabetic nephropathy following silencing and glucose treatment.

In summary, these findings suggest that the Rab3A-Rab27A system could be involved in the development of podocyte injury associated to glucose overload. Alterations of vesicular transport directed in part by this system, could be involved in a reduction in podocyte damage in diabetic nephropathy.

INDEX

I. INTRODUCTION.....	1
1. DIABETES MELLITUS.....	3
1.1. Definition and epidemiology	3
1.2. Type 2 diabetes mellitus	4
2. DIABETIC NEPHROPATHY	7
2.1. Mechanisms of kidney injury in diabetes.....	8
2.1.1. <i>Glomerular structural and haemodynamic changes</i>	10
2.1.2. <i>Renin–Angiotensin–Aldosterone System</i>	10
2.1.3. <i>Oxidative stress</i>	11
2.1.4. <i>Inflammation and fibrosis</i>	11
2.2. Treatment of diabetic nephropathy.....	12
3. PODOCYTES.....	14
3.1. Podocyte injury in diabetic nephropathy.....	18
3.1.1. <i>Cytoskeletal podocyte changes</i>	18
3.1.2. <i>Alterations in podocyte metabolism</i>	20
3.1.3. <i>Oxidative stress and mitochondrial dysfunction</i>	21
4. RAB GTPASES.....	24
4.1. Rab GTPases structure	24
4.2. Rab GTPase cycle.....	25
4.3. Rab proteins in podocytes.....	27

5. EXTRACELLULAR VESICLES	32
5.1. Biogenesis and characteristics	32
5.2. Intrarenal communication mediated by EVs.....	34
5.3. EVs in associated renal disease	37
5.4. EVs in diabetes derived renal damage	38
II. HYPOTHESIS AND OBJECTIVES	41
III. MATERIAL AND METHODS	45
1. PATIENTS.....	47
2. URINE SAMPLES.....	48
3. IN VITRO CELL CULTURE	48
3.1. Reagents.....	48
3.2. Human immortalised podocytes	48
3.3. Treatments	49
3.4. Podocyte permeability assay.....	49
3.5. <i>RAB3A</i> and <i>RAB27A</i> transient silencing	50
4. PROTEIN EXPRESSION ANALYSIS	51
4.1. Cell Sample collection and preparation	51
4.2. Whole cell protein extraction.....	51
4.3. Protein quantification: Lowry assay	51
4.4. Western blot analysis	52
4.4.1. <i>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</i> ...	52
4.4.2. <i>Electrophoresis under non-reducing conditions</i>	52

4.4.3. Electrophoresis under reducing conditions.....	53
4.4.4. Protein transfer to PVDF membrane and blocking.....	54
4.4.5. Coomassie blue staining	54
4.4.6. Protein immunodetection	54
5. QUANTIFICATION OF mRNA LEVELS	57
5.1. RNA isolation	57
5.2. Complementary DNA synthesis by reverse transcription	57
5.3. Quantitative RT-PCR.....	58
5.4. Seminested PCR for Rabphilin3A detection	61
6. CELL APOPTOSIS ASSAY.....	62
6.1. Cell apoptosis assay by flow cytometry	62
6.2. Microscopic assessment of cell apoptosis	62
7. CELL MORPHOLOGY ANALYSIS	63
7.1. Light microscopy	63
7.2. Assessment of F-actin cytoskeleton organization.....	63
8. CONFOCAL FLUORESCENCE MICROSCOPY.....	64
9. EVS ISOLATION AND CHARACTERISATION.....	65
9.1. Isolation by differential ultracentrifugation.....	65
9.2. EVs size and concentration determination	66
9.3. Transmission electron microscopy analysis	67
9.4. Identification of EVs markers	68
9.5. EVs visualization by super resolution microscopy	68

9.6. Characterisation of EVs surface markers	69
10. ANALYSIS OF EVS miRNAs	70
10.1. RNA isolation from EVs.....	70
10.2. miRNA cDNA synthesis	70
10.3. miRNAs quantification by RT-qPCR	71
10.4. spike-in cel-miR-39 and ath-miR-159a normalisation analysis	72
11. PRESENTATION OF DATA AND STATISTICAL ANALYSIS.....	73
IV. RESULTS	75
1. CLINICAL CHARACTERISTICS OF THE STUDY COHORT.....	77
2. LEVELS OF THE RAB3A-RAB27A SYSTEM IN URINE SAMPLES FROM PATIENTS WITH HTN AND CORRELATIONS WITH UAE.....	79
3. CHARACTERISATION OF DIFFERENTIATED HUMAN IMMORTALISED PODOCYTES AND PODOCYTE ASSOCIATED MOLECULES	81
4. EXPRESSION OF RAB3A-RAB27A SYSTEM IN UNDIFFERENTIATED AND DIFFERENTIATED PODOCYTES	84
5. IMPACT OF HG AND ANG-II IN HUMAN PODOCYTES	86
5.1. Podocyte apoptosis	86
5.2. Podocyte actin cytoskeleton rearrangements	89
5.1. Podocytes permeability assesment under HG conditions	91
5.2. Podocyte-specific markers and Rab3A-Rab27A system levels under glucose overload	91
5.3. Podocyte markers and Rab3A-Rab27A system levels under Ang-II treatment.....	95

6. EFFECT OF THE <i>RAB3A</i> AND <i>RAB27A</i> GENE SILENCING AND GLUCOSE TREATMENT	99
6.1. Impact of <i>RAB3A</i> and <i>RAB27A</i> silencing and glucose on Rab3A-27A system.....	99
6.2. Effect of <i>RAB3A</i> and <i>RAB27A</i> silencing and glucose on podocyte markers	101
6.3. Influence of <i>RAB3A</i> and <i>RAB27A</i> silencing and glucose overload on podocyte apoptosis	104
6.4. Influence of <i>RAB3A</i> and <i>RAB27A</i> system silencing and glucose overload on podocyte structure	107
6.5. Analysis of vesicular transport markers under Rab3A-Rab27A system silencing and glucose treatment.....	109
7. ISOLATION AND CHARACTERISATION OF PODOCYTE EVS	111
8. EFFECT OF RAB3A-RAB27A SYSTEM SILENCING ON miRNAs EXPRESSION IN CELL PELLETS AND SECRETED EXOSOMES	114
V. DISCUSSION	119
1. THE RAB3A-RAB27A SYSTEM IN URINE CELL PELLET FROM PATIENTS	122
2. THE RAB3A-RAB27A SYSTEM IN INJURED HUMAN PODOCYTES STRESSED BY GLUCOSE AND ANGIOTENSIN-II	123
3. RAB3A-RAB27A SYSTEM SILENCING AMELIORATES HIGH GLUCOSE-INDUCED INJURY IN PODOCYTES	126
4. ISOLATION AND CHARACTERISATION OF PODOCYTE EVS	129
5. miRNA ANALYSIS IN RAB3A-RAB27A-SILENCED PODOCYTES AND THE EVS DERIVED FROM THEM	131
6. STUDY LIMITATIONS	134
7. FUTURE PERSPECTIVES.....	135
VI. CONCLUSIONS.....	137

VII. BIBLIOGRAPHY	141
VIII. ANNEXES	167

I. INTRODUCTION

“Diabetes is a wonderful affection, not very frequent among men, being a melting down of the flesh and limbs into urine. Its cause is of a cold and humid nature, as in dropsy. The course is the common one, namely, the kidneys and bladder; for the patients never stop making water, but the flow is incessant, as if from the opening of aqueducts. The nature of the disease, then, is chronic, and it takes a long period to form; but the patient is short-lived, if the constitution of the disease be completely established; for the melting is rapid, the death speedy”.

Aretaeus the Cappadocian (81-138 AD)

1. DIABETES MELLITUS

1.1. Definition and epidemiology

Diabetes mellitus (DM), commonly known as diabetes, is a chronic condition characterised by high levels of blood glucose. It arises when the body either fails to produce sufficient insulin or is unable to use the produced insulin effectively (Magliano and Boyko 2021). Insulin, a vital hormone synthesised in the pancreas, plays a crucial role in facilitating the entry of glucose from the bloodstream into the cells where it is either converted into energy or stored for later use. When there is an insufficient amount of insulin or when cells do not respond effectively to it, the result is elevated levels of blood glucose, known as hyperglycaemia, which serves as a clinical indication of diabetes (Zheng *et al.* 2018, American Diabetes 2019).

There are several types of diabetes with the most common being Type 1 and Type 2 (T2DM). Type 1 diabetes is caused by an autoimmune process in which the body's immune system attacks the insulin-producing β -cells of the pancreas and as a result, the body produces little or no insulin. The causes of this destructive process are not fully understood but a likely explanation is that the combination of genetic susceptibility (conferred by a large number of genes) (Miller *et al.* 2021) and an environmental trigger such as a viral infection, initiate the autoimmune reaction. On the other hand, T2DM results from insulin resistance and insufficient insulin production, often linked to other metabolic diseases and lifestyle factors like obesity. While Type 1 usually manifests in youth, T2DM commonly develops in adulthood and is associated with factors such as poor diet and lack of exercise.

According to International Diabetes Federation (IDF) Diabetes Atlas 10th edition, approximately 537 million adults aged 20–79 years worldwide are currently living with DM, including both type 1 and T2DM, which accounts for 10.5% of all

adults in this age group, with the majority residing in low- and middle-income nations (Fig.1). Looking ahead, projections indicate that by 2030, the number is expected to rise to 643 million adults, and by 2045, it is expected to reach 783 million adults aged between 20-79 years old with DM. Furthermore, it is noteworthy that while the global population is estimated to grow 20% over during this period, the prevalence of diabetes is expected to reach 46% (Magliano and Boyko 2021).

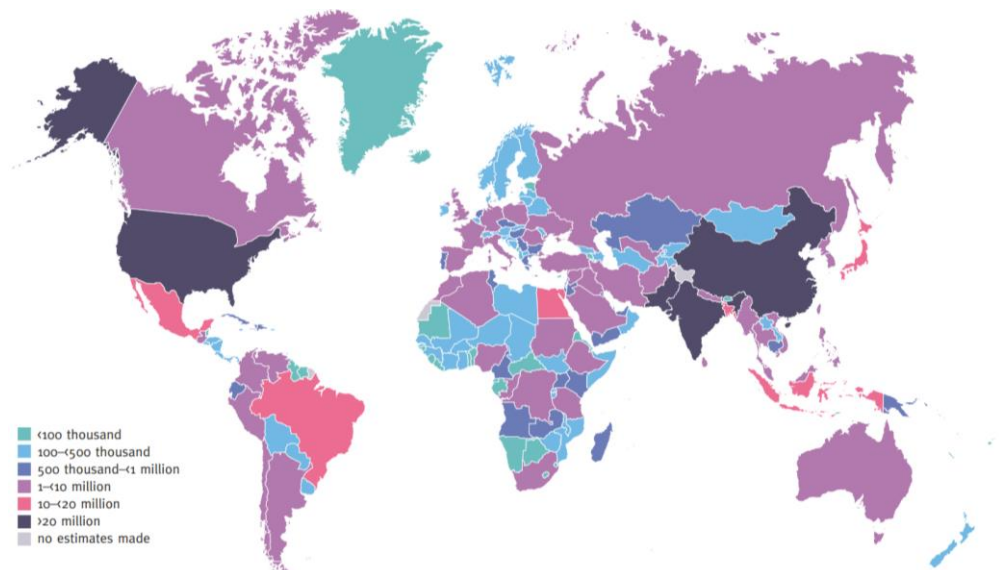


Figure 1. Estimated total number of adults (20-79 years) with diabetes in 2021. (IDF Atlas 10th edition, Magliano and Boyko 2021).

1.2. Type 2 diabetes mellitus

The predominant form of diabetes is T2DM, representing more than 90% of all diabetes cases globally (Magliano and Boyko 2021). This disease is characterised by impaired insulin secretion from β -cells coupled with insulin resistance, resulting in various metabolic disturbances such as hyperglycaemia, dyslipidemia, and obesity. Alterations in the maintenance of glucose homeostasis leads to a state of chronic high glucose blood levels and low-grade inflammation (Giri *et al.* 2018).

Proper regulation of glucose homeostasis in the body is accomplished by a complex system which includes several hormones and organs. The pancreas is responsible for releasing two crucial hormones that regulate blood glucose levels: insulin and glucagon. When blood glucose levels are low, the pancreas is stimulated to secrete glucagon, which raises endogenous blood glucose levels by breaking down glycogen in the liver (glycogenolysis). Moreover, glucagon encourages the synthesis of glucose from non-carbohydrate sources in the kidney and liver (gluconeogenesis), further elevating blood glucose levels. Conversely, when blood glucose levels are high due to the consumption of carbohydrate-rich foods, the pancreas releases insulin which facilitates the uptake of glucose in insulin-dependent muscles and adipose tissues while promoting glycogenesis, the storage of excess glucose. Any disruptions in maintaining glucose homeostasis can contribute to the development of T2DM.

While genetic predisposition sets the foundation for developing T2DM, environmental factors such as obesity, sedentary behaviour, smoking, alcohol consumption, and poor dietary choices act as catalysts, accelerating the β -cell dysfunction and insulin resistance, leading to the development of a negative risk profile, enhancing the onset of diabetes (Fig. 2). Furthermore, the influence of the intrauterine environment and gestational DM can introduce epigenetic changes that predispose the individual to a higher risk not only of T2DM but also of other diseases (Davegardh *et al.* 2018). The rising prevalence of T2DM in younger populations is intricately influenced by the interplay between genetic factors and lifestyle choices. Crucially, lifestyle modifications and early interventions are vital for mitigating the incidence of risk and for the effective management of T2DM in younger age groups.

Factors that affect insulin secretion and action

- Body weight
- Level of physical activity
- Smoking
- Heavy alcohol consumption
- Genetic predisposition
- Gene–environment interaction
- Epigenetics
- Gestational diabetes mellitus

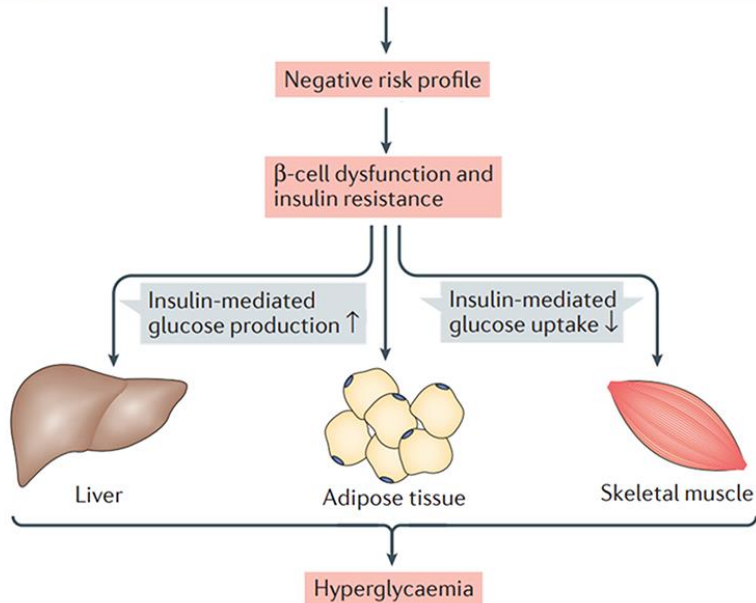


Figure 2. Pathophysiology of hyperglycaemia in T2DM. Insulin secretion from the β -cells in the pancreas normally reduces glucose output by the liver and increases glucose uptake by skeletal muscle and adipose tissue. Once β -cell dysfunction in the pancreas and/or insulin resistance in the liver, skeletal muscle or adipose tissue occur, hyperglycaemia develops, leading to an excessive amount of glucose circulating in the blood. (Zheng *et al.* 2018).

In summary, abnormally high blood glucose levels result from disrupted feedback loops between insulin secretion and its utilization. Insulin resistance leads to increased glucose production in the liver and reduced glucose uptake in muscles, liver, and adipose tissue. To compensate, β -cells release more insulin to maintain normal glucose levels. However, if the demand for insulin continually rises and β -cells cannot meet this demand, the body's ability to regulate glucose levels effectively is impaired. Although both processes contribute to the development of T2DM early on, β -cell dysfunction has a more significant impact than insulin resistance. As β -cell dysfunction worsens, hyperglycaemia intensifies, driving the progression of T2DM (Cerf 2013).

2. DIABETIC NEPHROPATHY

The primary long-term complications of T2DM are vascular complications that result from elevated glucose levels, which depending on the size of affected vessels, can be divided into macrovascular, which occur due to damage to larger arteries (such as the aorta, femoral artery, and coronary arteries) and microvascular which arise from damage to small blood vessels (such as those in the eyes, kidneys, and nervous system). The microvascular complications are more frequent than macrovascular complications and include retinopathy and neuropathy, being diabetic nephropathy (DN) especially prevalent (Faselis *et al.* 2020).

The kidney is arguably the most important target of microvascular damage in T2DM, which evolves to an end-stage renal disease. Together with the increasing incidence of T2DM worldwide, DN has become a serious health concern (IDF Atlas). Currently, DN accounts for more than 40% of patients with diabetes and is the most common cause of end stage renal disease, which poses a significant impact on the global health care (Doshi and Friedman 2017, Mohandes *et al.* 2023). Renal and cardiovascular risk events are the greatest threat to DN patients.

DN is characterised by structural and functional changes that occur in multiple compartments and cells that make up the kidney. Their pathogenesis can be characterised by the glomerular and capillary basement membranes thickening, the loss of fenestrated endothelium, progressive hypertrophy and expansion of the glomerular mesangium, combined with accumulation of extracellular matrix (ECM) proteins and reduction in the number of podocytes, key cells in glomerular filtration, and a loss of their filtration slit (Gui *et al.* 2023). These structural changes have functional repercussions and promote the appearance of glomerular hyperfiltration and increased urinary albumin excretion (UAE) also known as albuminuria. Within the context of diabetes, where albuminuria is a major risk

factor for cardiovascular and renal disease (Ruilopec *et al.* 2023), it stands out as a significant biomarker utilised in the diagnosis of DN (Hu *et al.* 2023). Clinically, all these renal insults result into glomerular basement membrane (GBM) thickening, the appearance of proteinuria and a decrease in glomerular filtration rate (GFR) (Mohandes *et al.* 2023) (Fig. 3).

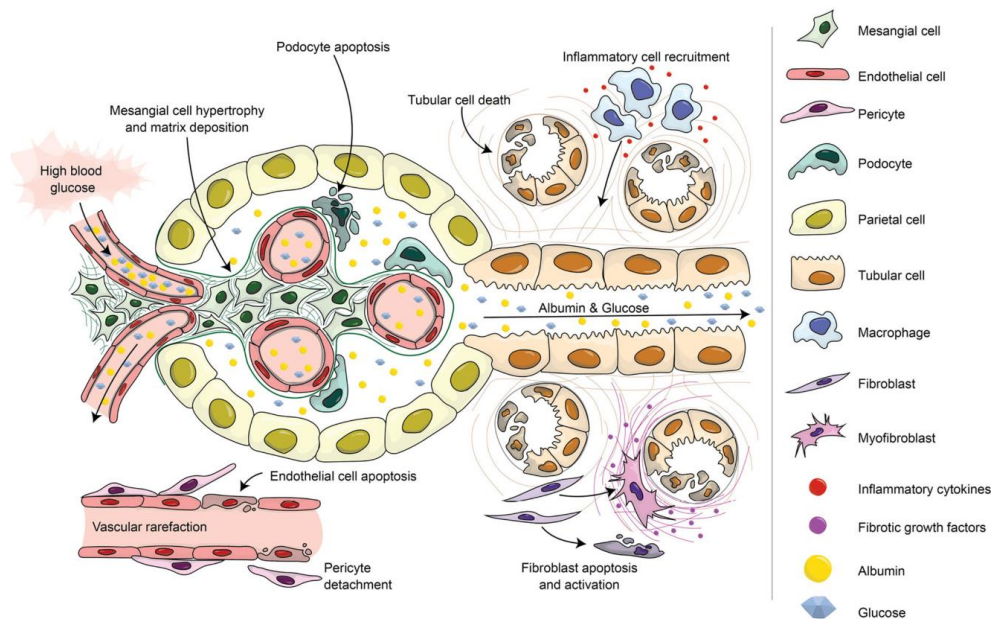


Figure 3. Glomerular appearance and structural alterations in T2DM. The kidney microenvironment in DN comprises injured tubular cells, endothelium, immune cells, activated fibroblasts, podocytes, mesangial cells, other cellular components, ECM, and a variety of secreted factors. DN leads to structural modifications, such as the thickening of the GBM, pericyte detachment, endothelial cell apoptosis, vascular rarefaction, loss of foot processes (FP) and podocyte apoptosis resulting in the exposure of the GBM, and expansion of the mesangial matrix. (Gui *et al.* 2023).

2.1. Mechanisms of kidney injury in diabetes

There is not a single pathophysiological pathway in DN but multiple mechanisms that interact with each other in a complex way, such as hyperglycaemia and related metabolic disruptions, alterations in glomerular haemodynamics, the presence of proinflammatory and profibrotic factors, cellular

stress, apoptosis, pyroptosis, and autophagy are linked to various processes occurring in renal cells, such as podocytes, mesangial cells, endothelial cells, and epithelial cells, collectively contribute to the development of kidney damage in DN (Alicic *et al.* 2017, Perez-Morales *et al.* 2019, Forst *et al.* 2022). These processes include the activation of the Renin-Angiotensin-Aldosterone System (RAAS), the buildup of advanced glycation end products (AGEs), and the appearance of oxidative stress and an increase in inflammatory processes (Hu *et al.* 2023). The pathways involved in DN kidney injury lead to glomerulosclerosis, mesangial matrix expansion, podocyte damage and loss, and tubulointerstitial damage (Fig. 4). Two of the most prominent established risk factors are hyperglycaemia and hypertension (HTN) (Tsioufis *et al.* 2014, Burnier and Damianaki 2023).

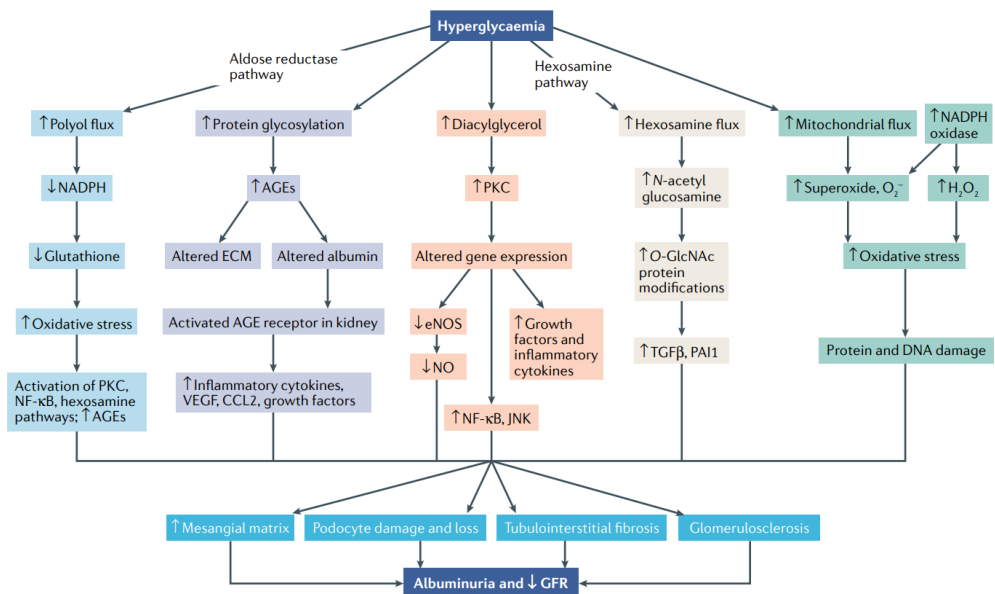


Figure 4. Effect of hyperglycaemia on the kidney. Hyperglycaemia triggers various pathways in the kidney, leading to glomerulosclerosis, mesangial expansion, podocyte loss, and tubulointerstitial damage. These pathway results in a progressive decline in kidney function and albuminuria. Image modified from DeFronzo *et al.* 2021.

2.1.1. Glomerular structural and haemodynamic changes

The earliest histopathological finding is thickening of GBM, resulting from overproduction of ECM, reduced turnover, and diminished matrix metalloproteinase expression/activity (Mason and Wahab 2003). Other glomerular changes include loss of endothelial fenestrations, mesangial matrix expansion, and as will be detailed later, loss of podocytes with effacement of foot processes (FP). Additionally to structural alterations, in DN there are significant disruptions in glomerular haemodynamics which include an initial increase in GFR due to high blood sugar levels, along with dilation of the efferent arteriole (Tuttle *et al.* 2022). This combination raises pressure within the glomerular capillaries, potentially damaging crucial structures. Moreover, the kidneys adapt with hypertrophy and hyperfiltration, but this can lead to long-term structural changes and nephron loss.

2.1.2. Renin–Angiotensin–Aldosterone System

The RAAS system is directly involved in a number of cardiovascular processes including vasoconstriction, aldosterone secretion, increased myocardial contractility, and the excitation of the sympathetic nervous system; when uncontrolled, the system can lead to HTN, fluid retention, thrombosis, and atherosclerosis (Chawla *et al.* 2010, Ruggenenti *et al.* 2010). In the kidney, evidence suggests elevated levels of prorenin, renin, angiotensin-converting enzyme (ACE), and angiotensin-II (Ang-II) in patients with DN (Rahimi 2016). Intrarenal Ang-II has several effects that can contribute to endothelial dysfunction by increasing endothelin expression, inhibiting NO production, and activating cyclooxygenase (COX) and protein kinase C (PKC). Additionally, the concomitant secretion of renin promotes vasoconstriction of the efferent arteriole (Forrester *et al.* 2018).

In summary, the overactivation of the RAAS has an important role in the pathophysiology of DN exacerbating kidney injury through a combination of

vasoconstriction, glomerular hyperfiltration, arterial HTN (causing proteinuria), stimulation of renal cell proliferation and hypertrophy, synthesis of cytokines and ECM, as well as promoting macrophage infiltration and triggering inflammation (Alicic *et al.* 2017).

2.1.3. Oxidative stress

Oxidative stress is part of many pathways that are involved in the pathogenesis of DN. An excess of reactive oxygen species (ROS) production and/or insufficiency of antioxidant systems can lead to ROS accumulation and cause oxidative stress (Lennicke and Cocheme 2021). In diabetes, the main sources of ROS include the polyol chain, AGE, and NADPH oxidase (Nox) (Yamagishi and Matsui 2010, Chen *et al.* 2018, Samsu 2021).

Kidney injury due to oxidative stress can occur by direct and indirect mechanisms. Direct damage affects specific cells like podocytes, mesangial cells, and endothelial cells, resulting in issues like protein leakage and fibrosis. The glomerulus, is particularly sensitive to this type of injury where hyperglycaemia causes damage to DNA (Yoshimoto *et al.* 2023), lipid peroxidation (Rungby *et al.* 1992), protein modifications (Degrell *et al.* 2009) and activation of both pro-inflammatory and pro-fibrotic pathways (Goldberg *et al.* 2011). Indirectly, oxidative stress can activate other harmful pathways, connected with metabolic and haemodynamic changes in the kidney. Chronic hyperglycaemia-induced oxidative stress can elevate levels of Ang-II, PKC, and increase expression of transforming growth factor- β 1 (TGF- β 1), all of which are known to stimulate oxidative stress.

2.1.4. Inflammation and fibrosis

Recent evidence shows that inflammation of the kidney is very important in initiating the development and progression of DN (Jin *et al.* 2023). The infiltration

of inflammatory cells, such as lymphocytes, neutrophils, and macrophages, contributes to kidney injury in DN (Wang *et al.* 2021).

In the context of fibrosis, TGF- β 1 is a key player in the pathogenesis of this condition. Moreover, in a diabetic environment, many factors stimulate TGF- β 1, which activates downstream pathways that ultimately trigger the secretion of the vascular endothelial growth factor (VEGF), increasing the glomerular permeability (Miaomiao *et al.* 2016). Chronic hyperglycaemia and TGF- β 1 also prompt excessive ECM deposition, contributing to glomerular fibrosis and impaired filtration. Indeed, the persistent elevation and activation of TGF- β 1, driven in part by increased ROS production, results in excessive remodelling of the ECM in the mesangium and promotes fibrotic processes in the tubular interstitium. Moreover, EMT is induced by TGF- β 1, which is closely related to tubulointerstitial fibrosis (Huang *et al.* 2018, Hong *et al.* 2022). This cascade of events ultimately contributes to kidney injury.

2.2. Treatment of diabetic nephropathy

The prevention of kidney injury in diabetes should be considered a primary aim in the overall management of the disease. Evidence suggests that the use of RAAS inhibitors, strict glycaemic and blood pressure control, cholesterol-lowering therapies, correction of metabolic acidosis and self-care, may delay the deterioration of renal function and are essential tools in the management of patients with DN. In addition, early detection of albuminuria in diabetic patients has been recognised as being a predictor of DN progression and prognosis (Papadopoulou-Marketou *et al.* 2017).

The blockade of RAAS via ACE inhibitors (ACEIs) and Ang-II receptor blockers (ARBs) has been shown to exhibit renoprotective effects in patients with DN, attenuating proteinuria. This approach continues to serve as the cornerstone of current therapeutic methods. Several clinical trials have been undertaken to affirm

the therapeutic effects of losartan, a frequently prescribed ARB. These trials have substantiated that losartan effectively addresses DN in individuals with T2DM by suppressing oxidative stress and mitigating insulin resistance (Pan *et al.* 2015). However, these drugs have nephrotoxic effects directly or indirectly, by affecting renal haemodynamic function. Despite treatment, there is substantial residual risk of DN progression with existing therapies. Therefore, during last decades new drugs targeting DN have been developed. These pharmacological agents primarily encompass second-generation sulfonylureas (such as glipizide and gliclazide), insulin therapy, DPP-4 inhibitors, GLP-1 agonists and SGLT2 inhibitors (Zac-Varghese and Winocour 2018, Hu *et al.* 2023). These antihyperglycaemic agents, in addition to metformin and thiazolidinediones, also have nephroprotective properties in addition to glucose-lowering effects in DN (Hu *et al.* 2023).

Besides pharmacotherapy, lifestyle adjustments and social support play a crucial role in managing T2DM as it directly influences the execution of self-care behaviors related to diabetes and indirectly impacts glycaemic control.

3. PODOCYTES

The renal glomerulus is a structure with entangled capillaries where the first step of urine formation occurs by filtering the plasma into the urinary space. The glomerulus has a permselective barrier function that prevents macromolecules including blood cells and proteins from leaking into the urine. The glomerular filtration barrier (GFB) consists of three layers: fenestrated endothelial cells, GBM, and the slit diaphragms (SD), which are formed by podocytes (Ebefors *et al.* 2021).

Podocytes are differentiated epithelial cells that line the glomerular capillaries, thus forming the GFB. Their structure is divided into three subcellular compartments: a large cell body, the primary processes, which are membrane extensions driven by microtubules, and the FP or pedicels, membrane extensions driven by actin filaments that assume a highly specialised and polarised morphology, creating interdigitations with neighbouring podocytes. They surround the entire endothelial vascular wall, forming extended filtration slits that are bridged by a junction called the SD (Greka and Mundel 2012, Perico *et al.* 2016). Together with the fenestrated endothelium, the GBM, and the glycocalyx, they form a physical barrier so-called GFB. This structure is responsible for filtering the blood, allowing the passage of water, ions, and small solutes, according to its molecular size, structure and charge and preventing the passage of most plasma proteins, such as albumin, into the urinary space (Gagliardini *et al.* 2010, Ebefors *et al.* 2021). The SD is considered the major restriction site to protein filtration; however, the negatively charged sialoglycoproteins that cover the podocyte abluminal surface facing the GBM also contribute to the GFB by repulsing plasmatic anionic proteins (Fissell 2020).

A pivotal aspect in the podocyte biology is the maintenance of its cytoarchitecture and its anchoring to the GBM, which is achieved thanks to a

precise organization of actin filaments that preserve their morphology and functionality through the proper assembly of numerous molecules, in the so-called focal adhesion complexes (Garg 2018). Between the pedicels, particular cell-cell junctions are developed, which exhibit elements of tight junctions and adherens junctions, also called SD. These junctions serve as connection points between the podocyte's cytoskeleton and the ECM. They encompass numerous proteins (integrins, syndecans, talin, ILK, FAK), enabling the podocyte to detect extracellular stimuli, transmit signals, and internally adapt its cellular structure to uphold the operational GFB (Lennon *et al.* 2014).

If we delve deeper into the composition of the SD, this is composed of very similar transmembrane proteins, including nephrin and Neph1, which form a heterophilic protein-protein interaction (Donoviel *et al.* 2001). Nephrin assembles numerous interacting partners at the SD, establishing a multi-protein complex that serves as a central hub for actin signalling and has the potential to modify the podocyte cytoskeleton. Additionally to nephrin and Neph1, several other components of the SD have been identified, such as podocin, TRPC6 and CD2-associated protein (CD2AP), which not only provide structural support for the filtration barrier, but also participate in signalling pathways that maintain the functional integrity of podocytes (Perico *et al.* 2016). Under physiological conditions, podocin forms microdomains with lipids raft to recruit nephrin and links the plasma membrane to the actin cytoskeleton. Moreover, the member of the transient receptor potential superfamily of non-selective cation channels, TRPC6, empowers podocytes to detect shifts in their immediate surroundings. These changes could encompass variations in pressure, fluid flow, or filtration rate (Moller *et al.* 2009). In response, podocytes might engage in cytoskeletal remodelling and FP contraction. CD2AP functions as a scaffold protein, bridging F-actin assembly to the establishment of the podocyte SD by interacting with nephrin and podocin

(Schwarz *et al.* 2001). Additionally, CD2AP interacts with the C-terminal domain of Rac1, a factor that regulates cytoskeletal dynamics by promoting actin nucleation (van Duijn *et al.* 2010). On the other hand, membrane proteins located in the apical zone of the podocyte, such as podocalyxin, form complexes with negatively charged monosaccharides, helping to prevent the passage of proteins into the urinary space (Kerjaschki *et al.* 1984).

To bear the structural load of podocytes, it is essential for these transmembrane proteins to interact with various intracellular proteins that in turn interact with the podocyte cytoskeletal network. Regarding that aspect, synaptopodin, a primary member of the proline-rich actin-associated proteins, is particularly prevalent in highly dynamic cells like podocytes. Through the modulation of α -actinin-4's function, synaptopodin plays a pivotal role in coordinating the arrangement of the actin cytoskeleton (Huber *et al.* 2006). Moreover, this protein significantly influences the contractile nature of the podocyte's actin cytoskeleton. It accomplishes this by activating RhoA while concurrently suppressing the activity of the small GTPase known as cell division control protein 42 homologue (Cdc42) (Yanagida-Asanuma *et al.* 2007). In addition to those located in the SD, there are other key proteins involved in maintaining podocyte function and architecture, such as Wilms' tumor 1 (WT-1). This podocyte-specific transcription factor controls the expression of major genes associated with the differentiated podocyte phenotype and has been used as a molecular indicator of podocyte loss (Guo *et al.* 2002, Macconi *et al.* 2006, Dong *et al.* 2015) (Fig.5).

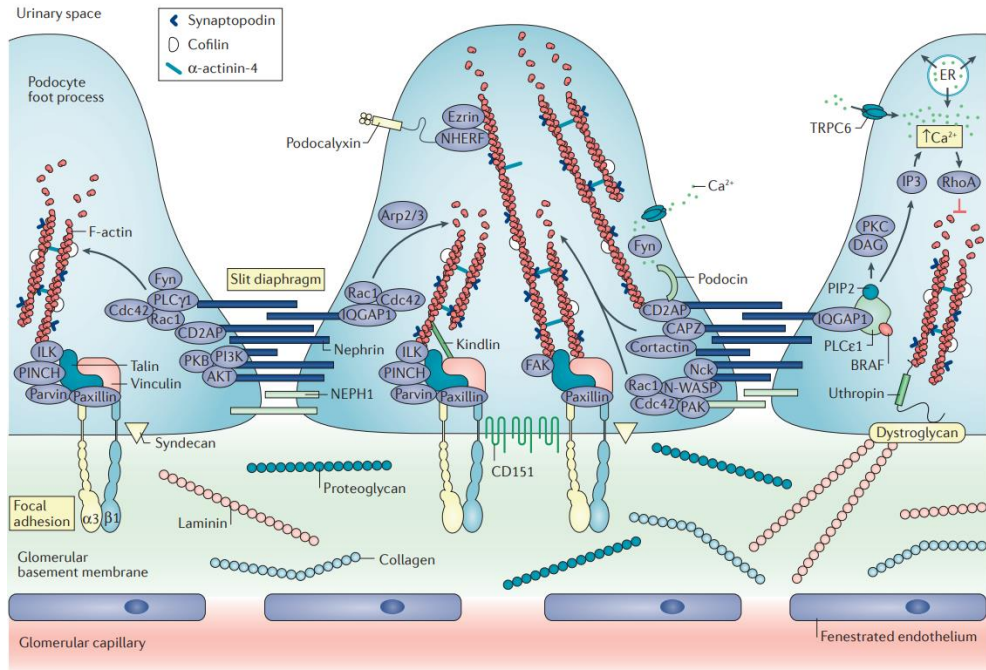


Figure 5. Integrative signalling networks facilitate interactions between podocyte focal adhesions, the slit diaphragm and actin dynamics. Podocyte FP, key components of the GFB, exhibit robust adhesions to the GBM through integrins, dystroglycans, and syndecans. Focal adhesions within the cytoplasm, comprising ILK, FAK, and α -actinin-4, support the structural integrity of podocytes and mediate intracellular signalling. SD, formed by nephrin and NEPH1, regulate cytoskeletal dynamics through interactions with podocin, TRPC6, and CD2AP. Small GTPases like RhoA, Rac1, and Cdc42 modulate cytoskeletal dynamics. (Perico *et al.* 2016).

Due to the significant role of podocyte injury in DN development, the utilization of podocyte-associated biomarkers could be crucial for promptly detecting kidney impairment associated with T2DM. These podocyte-associated molecules, due to their well-defined functions, are ideal candidates for investigating podocyte differentiation and dysfunction in renal pathological conditions like DN (Kravets and Mallipattu 2020).

3.1. Podocyte injury in diabetic nephropathy

The described pathological mechanisms in DN in section 2.1 affect glomerular cells, including podocytes. Various factors such as chronic inflammation, HTN, oxidative stress and T2DM can trigger damage to podocytes, compromising the GFB. This is why a comprehensive understanding of the biological and cellular processes occurring in podocytes in the context of DN is imperative for and maintaining optimal kidney function or efficiently managing renal pathologies.

In the last decades, a large number of studies on podocytes exposed to a high glucose milieu have proven that hyperglycaemia can induce functional and morphological alterations, including cytoskeletal and metabolic changes, hypertrophy, EMT, detachment and podocyte apoptosis, as well as oxidative stress and inflammation (Barutta *et al.* 2022). The classical albuminuric form of DN is predominantly due to glomerular injury and is characterised by both increased glomerular permeability to proteins and relentless renal function decline.

3.1.1. Cytoskeletal podocyte changes

Glomerular HTN in DN is driven by both an overactive RAAS, causing efferent arteriole vasoconstriction, and enhanced proximal tubular glucose/sodium reabsorption, leading to afferent arteriolar vasodilation. Increased filtration flow, induced by hyperfiltration is the responsible of create both, shear and mechanical stress (Barutta *et al.* 2022). This shear stress induces alterations in both the SD and the FP cytoskeleton of podocytes. Specifically, during the initial phase of FP effacement, SD are displaced from their physiologic distribution and replaced by occluding junctions (Kriz and Lemley 2015, Kriz and Lemley 2017). This simplified architecture is named FP effacement and is associated with proteinuria even in the absence of podocyte loss. Early FP effacement is reversible, but at later stage, FP retracts into the primary processes, whereas podocyte cell bodies approach and

adhere to the GBM, causing a reduction in the sub-podocyte space. In general, mature podocytes undergo hypertrophy to compensate for glomerular dilation, which contribute to cover the denuded region of the GBM. This can result in reduced podocyte density without changes in podocyte number (Godel *et al.* 2011). All of these changes lead to the deformation or loss of the GFB. Reorganization of the cytoarchitecture of podocytes — for example, changes in SD proteins (nephrin) — serves as an important signalling platform. Podocyte hypertrophy and changes in actin organization are not only critical adaptive mechanisms in glomerulomegaly but also a major contributor to chronic damage.

Elevated glucose levels alter the composition of the intracellular scaffold complex of the podocyte. Consequently, podocyte FP effacement and hypertrophy occur, ultimately leading to podocyte detachment (Anders *et al.* 2018). The mechanisms underlying podocyte loss encompass detachment resulting from the downregulation of $\alpha 3 \beta 1$ integrin expression (Regoli and Bendayan 1997) or from direct glucose- or TGF- $\beta 1$ -induced apoptosis (Schiffer *et al.* 2001). Nephrin is an important podocyte protein considered an important intracellular signalling hub and also directly involved in podocyte survival pathways and actin dynamics (Li *et al.* 2015). Its expression is reduced in murine models of diabetic kidney disease (DKD) (Doublier *et al.* 2003), in part owing to glucose-induced endocytosis of nephrin in a process orchestrated by PKC α -type and β -arrestin (Quack *et al.* 2011). Available evidence suggests that in diabetic mice model, high glucose levels alter the post-translational modifications of nephrin and other SD components by downregulating the adaptor protein CD2AP and upregulating the adaptor protein CIN85, triggering an ubiquitylation response that results in increased nephrin endocytosis (Teng *et al.* 2016).

In the adult kidney, podocytes have a limited capacity for proliferation, and lost podocytes cannot be adequately replaced (Shankland *et al.* 2017). The

reduction in podocyte number in patients with T2DM correlates with albuminuria and GFR decline and appears to be one of the best disease predictors (Sharma 2009). Following podocyte loss, the initial response of the remaining podocytes is to continue the hypertrophy process in order to cover the glomerular tuft. In the long term, this process contributes to cell detachment (Perico *et al.* 2016).

3.1.2. Alterations in podocyte metabolism

The metabolic insult of hyperglycaemia in podocyte is of paramount importance in the pathogenesis of DN (Barutta *et al.* 2022). Podocytes appear to be sensitive to insulin and express the insulin sensitive glucose transporter (GLUT4). Excessive glucose entry into podocytes through glucose transporters GLUT1 and GLUT4 leads to an increased flux through the polyol pathway, accumulation of precursors for AGEs, activation of PKC, and heightened activity in the hexosamine pathway. These processes have long been regarded as the primary mechanisms of glucotoxicity (Brownlee 2005).

Reduced nephrin expression in T2DM not only impacts the podocyte cytoskeleton but also disrupts insulin signalling, as nephrin's cytoplasmic domain plays a role in insulin recognition and intracellular signalling in podocytes. This is evidenced by the development of DKD features in mice with podocyte-specific insulin receptor deletion (Anders *et al.* 2018). Moreover, shifts in podocyte cytoarchitecture, including modifications in structural proteins like nephrin, establish a crucial signalling platform. Key regulators of intracellular calcium levels in podocytes, such as TRPC channels (TRPC5, TRPC6, and ORAI), play a significant role. Cytosolic calcium levels influence Rho and Rac proteins, along with mechanical stretch-associated pathways like YAP/TAZ, ultimately leading to reorganization of the actin cytoskeleton in podocytes (Mohandes *et al.* 2023).

Moreover, mTOR, a crucial regulator of cell processes including growth, motility, survival, protein synthesis, autophagy, and transcription, plays a central role in DN development through mTORC1 signalling in podocytes (Inoki *et al.* 2011). It has been found that mTOR-mediated podocyte hypertrophy is essential for maintaining glomerular integrity after podocyte loss, highlighting the potential for the compensatory response to become maladaptive over time, resulting in sustained damage. Additionally, through the activation of mTOR, both the serine-threonine kinase LKB1 and AMPK contribute to podocyte hypertrophy (Yao *et al.* 2016, Banu *et al.* 2021)

3.1.3. Oxidative stress and mitochondrial dysfunction

Hyperglycaemic toxicity is mediated by many abnormal glucose metabolic pathways and signalling pathways that can induce ROS overproduction and subsequent activation of nuclear factor (erythroid-related 2)–like 2 (Nrf2), leading to mitochondrial damage, apoptosis and podocyte depletion (Susztak *et al.* 2006). Evidence suggests that ROS contributes to the activation of the nucleotide leikin-rich polypeptide 3 (Nlrp3) inflammasome in glucose or AGE product stressed podocytes (Shahzad *et al.* 2015). Recent studies have reported that knocking down of NLRP3 prevented hyperglycaemia-induced mitochondrial ROS in podocytes, indicating that inhibition of NLRP3 inflammasome activation prevents podocyte injury in DN by reducing mitochondrial ROS generation and avoiding lipid accumulation (Wu *et al.* 2021). This process in podocytes has also been described in DN and leads to lipotoxicity characterised by mitochondrial oxidative stress, inflammatory responses, actin cytoskeleton remodelling, insulin resistance, and endoplasmic reticulum stress (Kim *et al.* 2021). Furthermore, *in vitro* it has been demonstrated that various harmful factors (such as adriamycin, puromycin, Ang-II, TGF- β 1, high glucose, and AGEs) lead to damage in podocytes by triggering an increase in oxidative stress (Marshall *et al.* 2006, Zhou *et al.* 2009, Zhou *et al.* 2012).

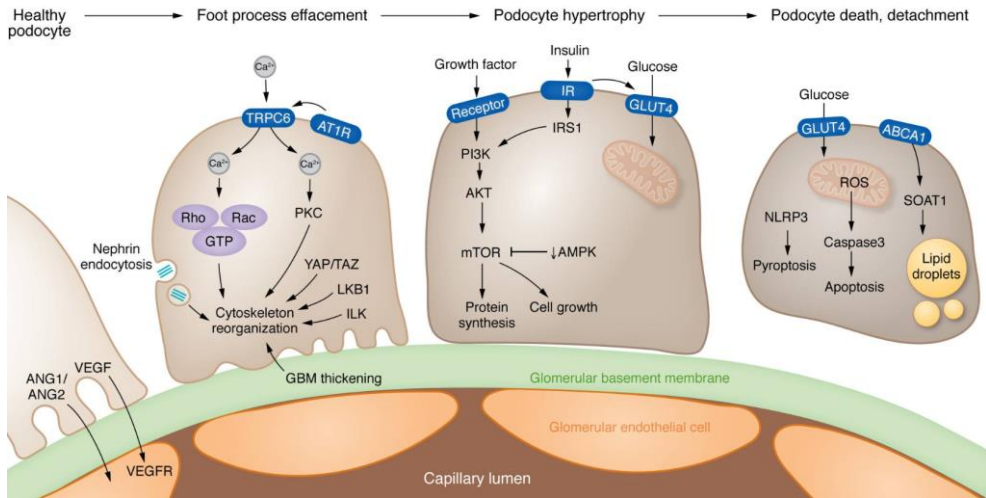


Figure 6. Mechanisms of podocyte dysfunction in DN. (Left) Early stages of DN lead to effacement of podocyte FP; multiple pathways contribute to the reorganization of the actin cytoskeleton. Mechanical stress pathways (YAP/TAZ), as well as thickening of the GBM, can induce integrin and downstream integrin linked kinase (ILK) activation. (Middle) Podocyte hypertrophy also characterizes the early stages of DKD. Growth factor and insulin signalling activates PI3K and mTOR pathways that regulate protein synthesis and cell growth. (Right) Later disease stages are characterised by podocyte death and dedifferentiation. Dysregulated insulin signalling and high glucose levels trigger oxidative stress and apoptosis. NLRP3-mediated pyroptosis also contributes to podocyte loss. ANG1/ANG2, angiotensin-I/angiotensin-II; AT1R, angiotensin II receptor type 1; GBM, glomerular basement membrane; ROS, reactive oxygen species; TRPC6, transient receptor potential channel 6. (Mohandes *et al.* 2023).

To summarize, the capacity of maintaining the functions of the GFB heavily relies on the integrity of the podocyte actin cytoskeleton, focal adhesions, and intricate signalling pathways within the SD described above. Several studies suggest that the process of albuminuria development in DN is intricate, involving four phases in sequence: FP effacement, podocyte hypertrophy, podocyte detachment, and podocyte apoptosis (Fig. 6). These phases act as warning signs for the decline in GFR in DN. In addition to the processes described, membrane trafficking is a pivotal process for cell polarization and intercellular communication in podocyte cells. Disruptions in membrane trafficking can be both a cause and a consequence of podocyte injury. In this context, Rab proteins, a group of monomeric small

GTPases, play a pivotal role in regulating vesicle formation and controlling the specificity and directionality of intracellular transport (Fukuda 2008, Zhen and Stenmark 2015, Pylypenko *et al.* 2018). Furthermore, while multiple pathogenic mechanisms of podocyte injury in DN are better understood, the complex clinical manifestations indicate that further investigation is necessary to gain a deeper understanding of the mechanisms underlying podocyte injury to identify new therapeutic targets for the treatment of DN.

4. RAB GTPASES

Eukaryotic cells have a well-structured endomembrane system that compartmentalizes different biochemical reactions and facilitates the exchange of molecules with the external environment through exocytosis and endocytosis. The Rab family of small GTPases is believed to regulate various processes of membrane trafficking, including the formation, transport, attachment, and fusion of membrane-bound organelles and vesicles (Homma *et al.* 2021). Recently, they have been shown to also regulate cell signalling, division, survival, cytoskeletal remodelling, cell polarity and migration. (Erol *et al.* 2023).

The Ras GTPase superfamily formed by small monomeric GTPases can be divided into five families: Ras, Rho, Rab, Arf and Ran (Wennerberg *et al.* 2005). Rab GTPases (Ras-like in the brain) are the largest family of the Ras GTPase superfamily, consisting of almost 70 known members in humans. Various Rab GTPases exhibit specific localizations to distinct membrane compartments, playing a pivotal role in regulating the specificity and direction of membrane trafficking routes, primarily associated with vesicular transport (Pfeffer 2013).

4.1. Rab GTPases structure

Small GTPases are single-chain polypeptides consisting of around 200–250 amino acids. Rab family proteins share a conserved globular G domain and structural elements. The G motif consists of approximately 180 residues, comprising a six-stranded beta sheet flanked by five alpha helices and a C-terminal prenylation motif (Stenmark and Olkkonen 2001). Normally, the C-terminal interacting motif contains two Cys residues available for hydrophobic geranylgeranylation addition; this crucial modification allows membrane association of Rab GTPases. On the other hand, there are structural elements consisting of switch regions, complementary determining regions, and a

hypervariable C-terminal domain. That is between 35 and 40 amino acids long and exhibits the greatest divergence among the Rab GTPases.

The structural features described above allow Rab GTPases to act as molecular switches that control the specificity and directionality of membrane trafficking within cells. The precise details of Rab protein structures can vary among individual Rab family members, contributing to their unique functions in different cellular context.

4.2. Rab GTPase cycle

Similar to other small GTPases, Rab activity is tightly regulated by the cycling between an active GTP-bound and an inactive GDP-bound form. These Rab GTPases proteins act as molecular switches, transitioning between two different conformational states: the 'on' form when bound to GTP, and the 'off' form when bound to GDP (Fig. 7). In the GDP-bound state, the switch regions seem to be in an unfolded configuration. In contrast, when GTP is bound, these regions assume specific and defined shapes, facilitating the binding of effectors (Lee *et al.* 2009, Homma *et al.* 2021). Notable examples of thoroughly characterised interactions between Rabs and effectors include the interaction of Rab3a with Rabphilin-3A (Ostermeier and Brunger 1999), as well as the interaction of Rab27a with Slp2-a (also known as Syt12-a) (Chavas *et al.* 2008). Although Rab GTPases display intrinsic GTPase activity, the Rab GDP/GTP cycle is controlled and accelerated by two sets of regulatory proteins, guanine nucleotide exchange factors (GEFs) that are responsible for the exchange of GDP for GTP and GTPase-activating proteins (GAPs) that stimulate the Rab intrinsic GTPase activity. The recruitment of Rab proteins to membranes requires Rab activation by a GEF (Muller and Goody 2018) (Fig.7). Knowing when and where Rabs are activated and which effectors are recruited by Rabs is crucial in order to understand their functions in the cells.

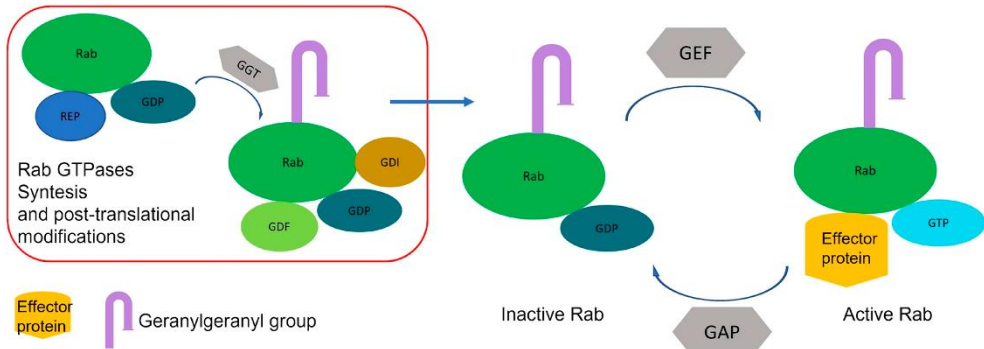


Figure 7. Rab GTPase cycle. Rab GTPases function as membrane-associated molecular switches that are ON in the GTP-bound (active) form and OFF in the GDP-bound (inactive) form. REP Rab escort protein; GDI GDP dissociation inhibitor; GEF guanine nucleotide exchange factors; GAP GTPase-activating proteins. (Erol *et al.* 2023).

Among the more than 70 Rab GTPases identified in humans, this doctoral thesis primarily focuses on the small GTPases Rab3A and Rab27A. Members of the Rab3 and Rab27 subfamilies have been implicated in regulating membrane trafficking and vesicle exocytosis pathways in eukaryotic cells, whose functions are well described in cells of the nervous system (Fig. 8) (Ghijsen and Leenders 2005, Fukuda 2013). The Rab3 family is divided into multiple isoforms, including Rab3A, Rab3B, Rab3C, and Rab3D. Each isoform is encoded by a different gene and exhibits some degree of functional specificity (Fukuda 2003). The Rab27 subfamily, which includes the Rab27a/b isoforms, modulates the exocytosis of secretory granules in a cell-type-specific manner through the utilization of multiple organelle-specific effector proteins (Izumi *et al.* 2003, Ostrowski *et al.* 2010, Fukuda 2013). Furthermore, it has been demonstrated that both of them are capable of binding to a common effector, Rabphilin3A (Rph3A), (Fukuda 2003). In fact, recent literature reports that Rab3A and Rab27A collaborate to achieve vesicle exocytosis by recruiting Rph3A, which binds to Rab27A, recruiting the Rab3IL1 activating enzyme that catalyzes the exchange of GDP for GTP in Rab3A (Quevedo *et al.* 2019).

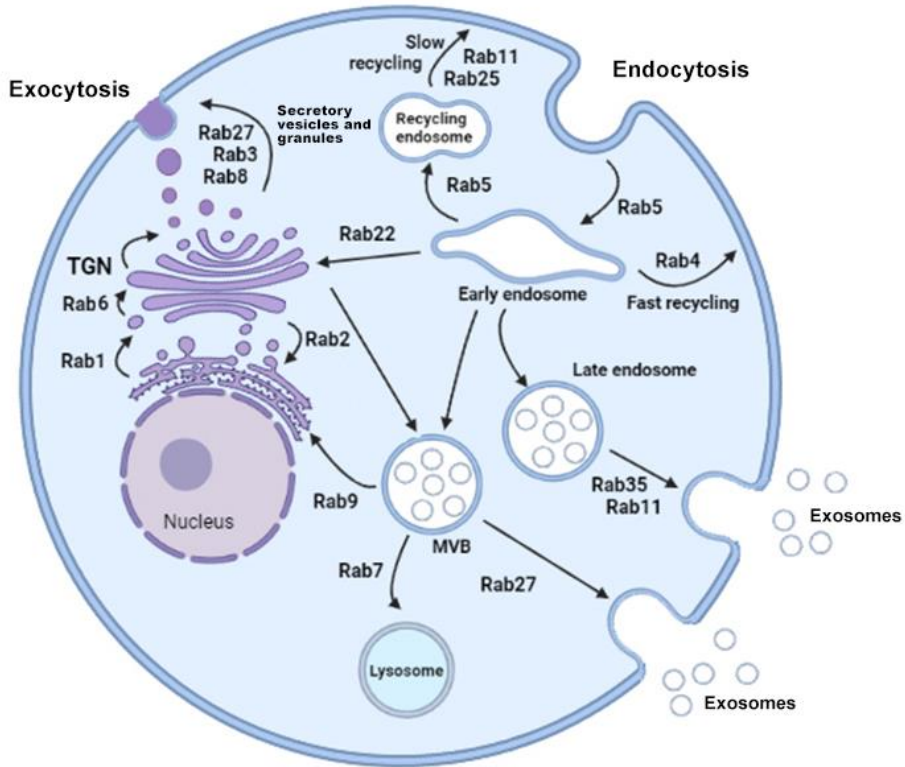


Figure 8. Rab proteins-mediated intracellular vesicular transport. Rab proteins control various aspects of cellular membrane trafficking, including endocytosis, exocytosis, exosome secretion, and the movement of vesicles between organelles. Rab proteins include Rab5, which mediates endocytosis and early endosome fusion, Rab11 and Rab25 for slow endocytic recycling, Rab4 for fast endocytic recycling, Rab7 for late endosome-lysosome maturation, Rab9 for trafficking from late endosomes to the trans-Golgi network, Rab1 for ER–Golgi trafficking, Rab2 possibly involved in Golgi–ER trafficking, Rab6 for intra-Golgi vesicle trafficking, Rab8 for constitutive trafficking from the trans-Golgi network to the plasma membrane, and Rab3, Rab27, and Rab22 for various types of regulated exocytic events. Additionally, Rab11, Rab35 and Rab27 are implicated in promoting exosome secretion in MVBs-dependent pathways. (Martinez-Arroyo *et al.* 2021) image created with BioRender.com

4.3. Rab proteins in podocytes

Rab GTPases are essential for many physiological processes such as immunity, hormone secretion and neurotransmission, and have been associated with specific syndromes and diseases such as neurologic disease, cancer, immunity,

and infections (Prashar *et al.* 2017, Guadagno and Progida 2019, Pfeffer 2023, Zeng *et al.* 2023). Nevertheless, the impact of altered Rabs expression is less understood in specific organs such as in the kidney, where vesicular trafficking has a high relevance on its structure and function.

In genetic association studies, our group found a link between certain single nucleotide polymorphism (SNP) and the risk of developing albuminuria. Specifically, we identified an association between a SNP in the *RPH3A* and UAE using combined genetic and metabolomic data from a general population (Marrachelli *et al.* 2014). More recently, data from the Strong Heart Study included variants in *RPH3A*, *RPH3AL*, *RAB3IL1* and *RAB27A* for analysis regarding UAE and GFR progression. Utilising mixed effects models to consider family relationships and adjusting for potential confounding factors, polymorphisms in *RAB3IL1* and *RPH3A* showed significant associations with the progression of UAE and GFR levels (unpublished data). Additionally, a prior study in the Framingham cohort also observed an association between UAE and a SNP of *RPH3A* (Hwang *et al.* 2007).

In 2003 was conducted the first investigation into the involvement of Rph3A and Rab3A in podocyte biology (Rastaldi *et al.* 2003). The presence of Rab3A and Rph3A has been previously described in neurons, where the Rab3A-Rph3A complex is required for the correct docking of synaptic vesicles to their target membrane (Burns *et al.* 1998). Rastaldi *et al.* described the presence of the small GTPase Rab3A and its effector Rph3A in mice, rat and human healthy kidneys, specifically surrounding FP vesicles of podocytes. Furthermore, an increase in Rab3A protein expression was observed in kidney biopsies from patients with proteinuric disease. Additionally, Rab3A expression was inversely associated with the amount of urinary proteins (Rastaldi *et al.* 2003). To further explore the potential role of these molecules in renal damage, the same research group studied the absence of Rab3A together with blockade of glutamate ionotropic N-methyl-d-aspartate receptor in

mice, resulting in an increased UAE (Giardino *et al.* 2009). Moreover, a model using Rab3A knockout mice subjected to a high glucose diet altered podocyte actin plasticity, resulting in podocyte damage and proteinuria. Additionally, the model with a HG diet displayed development of renal lesions similar to those observed in human DN (Armelloni *et al.* 2012).

In a recent research, our group has discovered that the protein Rph3A is present and co-localised with molecular markers associated with endocytic and exocytic pathways in pericardial nephrocytes of *Drosophila melanogaster* (Selma-Soriano *et al.* 2020). Nephrocytes are specialised cells that perform both podocyte and renal proximal tubule functions, playing a crucial role in eliminating waste products from the haemolymph and Malpighian tubules. This structure is considered analogous to the renal tubular system (Denholm and Skaer 2009, Helmstadter and Simons 2017). Furthermore, interference of RphA in *Drosophila melanogaster* led to both structural and functional impairments in nephrocytes, resulting in reduced filtration capacities and a decrease in the number of nephrocytes. The alterations observed in the cardiac fiber structure in the same RphA-interference model raise the intriguing possibility of concurrent damage to both heart and kidney cells due to Rab3A dysfunction (Selma-Soriano *et al.* 2021).

More recently, Fu *et al.* identified 27 distinct Rabs in *Drosophila melanogaster* nephrocytes, eleven of which were found to be functional, and among them, five played pivotal roles in maintaining functionality, with three being deemed essential. When Rab5 was silenced, its involvement in regulating endocytosis became evident. In a similar vein, experiments involving Rab7 and Rab11 revealed notable changes in protein degradation and membrane recycling processes. Consequently, when these genes were silenced in a *Drosophila melanogaster* model, disruptions in vesicle organization, the integrity of the GFB,

and subcellular structures responsible for protein reabsorption were observed (Kampf *et al.* 2019).

Steroid-resistant nephrotic syndrome (SRNS) is a rare disease that affects particularly children, with 30% of cases attributed to gene mutations. One of the gene involved is *TBC1D8B*, linked to X-linked early onset SRNS. Missense mutations in this gene lead to a decrease in Rab11b GTPase-activating protein, causing podocyte structural changes and migration due to disrupted internalization and recycling processes (Kampf *et al.* 2019). *GAPVD1* and *ANKFY1* genes, which regulates endosomes, may also contribute to SRNS. Mutations in these genes increase their binding to GTP-bound Rab5, a key protein in cellular trafficking. The interaction of *GAPVD1* with nephrin, suggests that the endocytic trafficking of nephrin may play a role in Rab5-mediated pathogenesis of SRNS (Hermle *et al.* 2018).

The impact of Rab proteins in exosome segregation has also been assessed by studying the inhibition or activation of Rab27 in podocytes and renal epithelial tubular cells. Two recent studies identify that inhibiting or activating Rab27 expression results in a reduction or increment in exosome release. Song *et al.* described in their studies, that overexpression of KIBRA stabilized Rab27, avoiding its degradation via the ubiquitin-proteasome pathway and with it the recovery of the exosomes release aborted in KIBRA knockout cell lines (Song *et al.* 2019). Following that, in a model of inflammatory response in proximal tubular epithelial cells, the knockout of Rab27a inhibited the excessive inflammatory response through the CHAC1/NF- κ B pathway. This was attributed to the intracellular accumulation of miR-26a, which consequently delayed the progression of DKD (Li *et al.* 2020).

In summary, Rab GTPases play a relevant role in kidney and podocyte injury. They are responsible for controlling the movement of vesicles within cells, facilitating processes like protein degradation, membrane recycling, and vesicle organization. This is why a better understanding of the interactions between the Rab GTPases and their effectors and deepen into the role in the development of Rab-related diseases is crucial to determine new treatment targets and to develop novel therapeutic strategies to deal with kidney diseases.

5. EXTRACELLULAR VESICLES

5.1. Biogenesis and characteristics

The term extracellular vesicle (EV) comprises a heterogeneous population of cell-derived membranous structures including exosomes, microvesicles (MVs), and apoptotic bodies that can be established based on their size and biogenesis (Gyorgy *et al.* 2011, Colombo *et al.* 2014). Exosomes, round-shaped vesicles of 40 to 150 nm, are the smallest type of EVs. Exosomes are released into the extracellular space by the fusion of endosomal compartments with the plasma membrane. This process consists of two main steps: 1) the formation of intraluminal vesicles in the multivesicular bodies (MVBs) or late endosome, and 2) the secretion of exosomes to the outside of the cell through the fusion of the MVBs with the plasma membrane, a process guided through small GTPases (Rab27A, Rab11, Rab31). MVs, also known as microparticles, are a highly heterogeneous group (100–1000 nm) stemming from and directly released by budding of the plasma membrane. Finally, apoptotic bodies are the largest EVs (1000–5000 nm) released from blebbing cells during apoptosis (Table 1). EV content can be released by different mechanisms, such as specific membrane-receptor interaction, direct fusion with plasma membrane or internalization by pinocytosis. While EVs were initially considered a cellular mechanism for waste removal (Johnstone *et al.* 1991), they have been found to be involved in numerous biological functions in both physiological and pathological conditions, including cancer, autoimmune disorders, cardiovascular, and renal diseases. (Martinez-Arroyo *et al.* 2021).

The release of EVs is a highly conserved evolutionary process, and they can be found in various biofluids like urine, saliva, plasma, breast milk, synovial, and cerebrospinal fluids. The role of each EV type depends on their content, such as functional proteins, lipids, DNAs, and RNAs. Regarding the nucleic acid content, it

has been described that EVs contain different types of RNA, of which small RNAs are especially relevant with numerous studies emphasising the effect of EV enclosed microRNAs (miRNAs) in multiple biological functions (Valadi *et al.* 2007, Mittelbrunn *et al.* 2011, Martinez-Arroyo *et al.* 2021).

Table 1. Main types and characteristics of EVs.

Type and size	Exosomes (40–150 nm)	Microvesicles (100–1000 nm)	Apoptotic bodies (1000–5000 nm)
Formation	Endolysosomal pathway. Release by exocytosis of MVs	Plasma membrane	Cell fragmentation during apoptosis
Uptake	Binding specific membrane receptor, pinocytosis, membrane fusion	Binding specific membrane receptor, pinocytosis, membrane fusion	
Markers	Tetraspanins (CD63, CD9, and CD81), Alix, PDCD6IP, phosphatidyl-serine, TSG-101, flotilin 1 and 2, chaperones (Hsp60, Hsp70, Hsp90)	Integrins, selectins, metalloproteinases, phosphatidyl-serine, CD40 ligand	Phosphatidyl-serine, calreticulin, histones, annexin V
Content	Proteins (cytokine receptors, MHC molecules, histones), lipids, mRNA, miRNA, noncoding RNAs, DNA	Membrane receptors, cytoplasmic proteins histones, mitochondrial fragments, lipids, mRNA, miRNAs, noncoding RNAs, DNA	Proteins, lipids, DNA, rRNA, mRNA, organelles, cytosol fractions

Hsp, heat shock protein; MHC, major histocompatibility complex; miRNA, microRNA; mRNA, messenger RNA; rRNA, ribosomal RNA; and TSG-101, tumor susceptibility gene 101. (Martinez-Arroyo *et al.* 2021).

The process of EVs uptake by recipient cells is facilitated by molecules present on the surface of the vesicular membrane, allowing for their identification and interaction. In this regard, EVs can interact with the target cell in several ways: a) by surface binding activating signalling pathways in the target cell through interaction with cell adhesion molecules and specific receptors without merging

with the membrane; b) through the direct fusion of both membranes and the horizontal transfer of genetic information (mRNA, miRNA, DNA), proteins, and signalling molecules; c) by initiating receptor-mediated endocytosis by the target cell and processing through the endolysosomal pathway (Fig. 9)(Ortega *et al.* 2020). However, it is still unclear whether different interaction mechanisms coexist in the same cell or vary depending on the type of recipient cell and the EV origin.

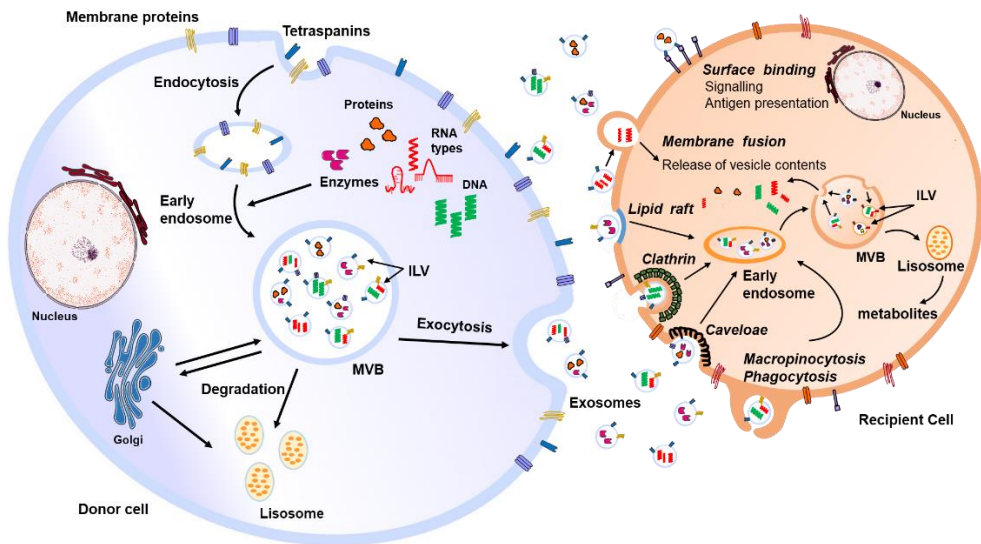


Figure 9. Biogenesis, secretion and uptake of EVs. Exosomes from intraluminal vesicles (ILVs) in multivesicular bodies (MVBs) are secreted extracellularly by fusion with the cellular membrane. Next, EVs interacting with recipient cells directly by surface binding, membrane fusion or internalization will target exogenous exosomes in the canonical endosomal pathway by micropinocytosis/phagocytosis processes, caveolae, clathrin or lipid raft. Image modified from Ortega *et al.* 2020.

5.2. Intrarenal communication mediated by EVs

Cell-to-cell communication in the kidney is essential for development and maintenance of renal physiology (Martinez-Arroyo *et al.* 2021, Grange and Bussolati 2022). Over the past few years, mounting evidence has established the central role of EVs in influencing both normal physiological processes and pathological conditions in kidney physiology. Urinary EVs (uEVs) represent a diverse

group primarily derived from cells within the urogenital tract, with the most abundant contributors being cells from the glomerulus, tubules, prostate, and bladder (Erdbrugger *et al.* 2021). They transport information within the nephron, potentially influencing disease progression or facilitating beneficial pathways for recovery, allowing intra-glomerular, glomerular-tubular, and intra-tubular communication (Fig. 10).

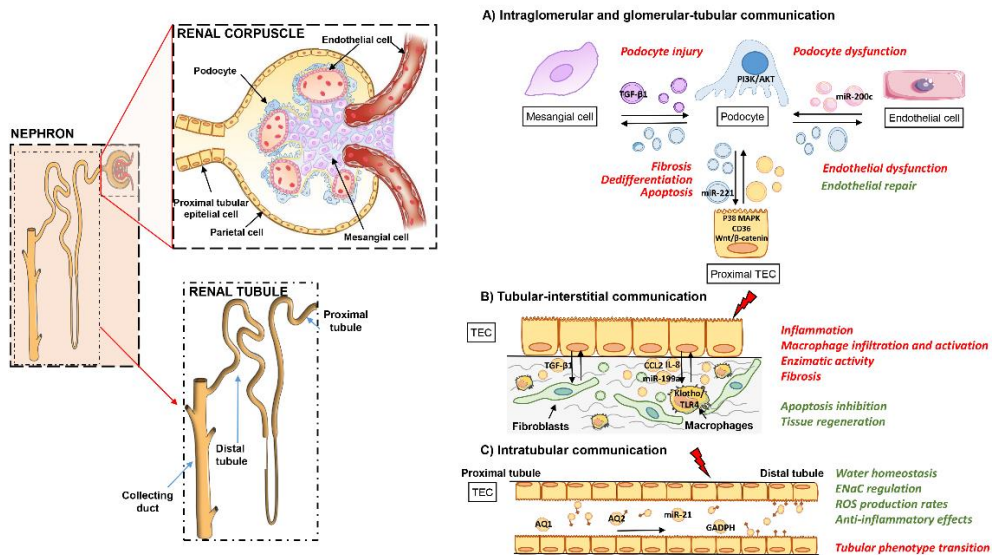


Figure 10. EVs mediate cell-to-cell communication within the nephron. Cells that comprise the nephron (podocytes, endothelial, mesangial, parietal and tubular cells), secrete uEVs along the urinary tract which mediate close cell-to-cell communication. EVs participate in the mesangial, endothelial-podocyte cell, and proximal tubular epithelial cell (proximal TEC)-podocyte communication (A). In the tubulointerstitium, TECs communicate with interstitial fibroblast (B), and proximal-to-distal signalling is also mediated by EVs (C). Representative cargos and processes mediated by EVs between urinary tract cells are indicated, beneficial in green and detrimental in red. AQP, aquaporin; CCL2, C-C motif chemokine ligand 2; ENaC, epithelial sodium channel; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-8, interleukin 8; MAPK, mitogen-activated protein kinases; PI3K/AKT, phosphatidylinositol 3-kinase/protein kinase B; ROS, reactive oxygen species; TGF- β 1, transforming growth factor beta 1; and TLR4, Toll-like receptors 4. Image obtained from Martinez-Arroyo *et al.* 2021.

EVs released by cells in the upper segments of tubules can be internalised by downstream cells, transferring active molecules and modulating the behaviour of the recipient cells (Street *et al.* 2011, Gildea *et al.* 2014). While the role of uEVs is not yet fully understood to date, recent findings have demonstrated their importance in several mechanisms: elimination of cellular waste, proximal-to-distal signalling, regulation of inflammation, control of ion transport (Jella *et al.* 2016), regulation of immune response (Lee *et al.* 2016) and developmental roles (Chiabotto *et al.* 2016, Krause *et al.* 2018). Indeed, uEVs can be identified by specific markers of their cells of origin. Regarding this, a proteomic analysis demonstrated that the majority of the proteins present in uEVs are characteristic of cells of the urinary system, such as is the case of podocytes or tubular cells (Gilani *et al.* 2017). For example, EVs from glomerular podocytes express podocin and podocalyxin (Hogan *et al.* 2014); proximal tubular cells derived EVs contain megalin, cubilin, aminopeptidase, and aquaporin-1 (AQP-1) (Moon *et al.* 2011) and EVs from ascending limb of the Henle's loop carry CD9, type 2 Na-K2Cl cotransporter (NKCC2), and Tamm Horsfall protein (THP) (Ranghino *et al.* 2015). Only a small fraction of uEV proteins are not derived from kidney cells, suggesting the potential origin of some of these vesicles from infiltrating kidney cells or from EVs circulating in the serum (Pisitkun *et al.* 2004). Nonetheless, this latter scenario appears improbable in the context of an intact GFB, where the SD selectively permits transit of 4-nm entities (Ndisang 2018). As in other biological barriers, it is suggested that serum EVs could potentially cross the podocyte layer via a process of transcytosis (Ramos-Zaldivar *et al.* 2022). In specific pathological conditions, such as those where basal membrane rupture allows the passage of larger structures like erythrocytes, the GFB may permit the passage of EVs. Studies in rodent models have shown an increased renal distribution of labeled EVs after acute kidney injury (AKI), with these vesicles being rapidly taken up by proximal tubule cells (Bruno *et al.* 2009, Cantaluppi *et al.* 2012, Grange *et al.* 2014).

5.3. EVs in associated renal disease

The capability of EVs to transport different cargoes from donor to acceptor cells along the nephron, leading to the regulation of gene expression and influencing the fate of target cells, has garnered substantial attention in the scientific community. Moreover, the exploration of the surface and cargo composition of these EVs has provided valuable insights into the processes taking place within the originating cells. This dual role of EVs in intercellular communication and as indicators of cellular processes has become a focal point of interest in recent years among researchers. Consequently, a substantial body of high-level studies has emerged, describing the involvement of EVs in regulating the normal function of the kidney and their role in promoting and counteracting pathophysiological responses (Li *et al.* 2019, Yates *et al.* 2022). As an example, Lv *et al.* illustrated that in the event of tubular epithelial cell damage, exosomes enriched with miR-19b-3p are secreted and taken up by macrophages. This leads to an increase in NF- κ B expression through a direct targeting of SOCS-1 by miR-19b-3p, ultimately promoting M1 macrophage activation. This mechanism, consequently contributes to tubulointerstitial inflammation (Lv *et al.* 2020).

The molecular profile of EVs reflects the type and pathophysiological status of the donor cell, so this molecular profile could potentially be exploited for diagnostic and prognostic purposes. In regard to exosomes, several studies have linked changes in exosomal levels of mRNA and proteins associated with podocytes (Zhou *et al.* 2013). A study conducted by Lv L *et al.* found that CD2AP mRNA levels in uEVs correlated negatively with 24-hour urine protein, the severity of tubulointerstitial fibrosis, and glomerulosclerosis, and could distinguish kidney disease from controls (Lv *et al.* 2014).

Moreover, previous works by other authors and our group have demonstrated that changes in the levels of one of the most relevant transported cargos in EVs, miRNAs, can be used as biomarkers for the progression of microalbuminuria and proteinuria in diseases such as HTN (Perez-Hernandez *et al.* 2021), DN, (Jia *et al.* 2016), lupus nephropathy (Garcia-Vives *et al.* 2020, Perez-Hernandez *et al.* 2021), and renal fibrosis (Sole *et al.* 2015).

5.4. EVs in diabetes derived renal damage

As exposed in the previous sections, the composition of EVs, both their surface markers and cargo represent a novel source of information that can be used to detect the presence of disease, as well as to assess its progression and evaluate potential treatments. In the case of T2DM and DN, several studies have characterised the RNA and protein composition and number of derived uEVs (Zubiri *et al.* 2015, Noren Hooten and Evans 2020, Gu *et al.* 2023). Kalani *et al.* described the predominant presence of WT-1 protein in uEVs of diabetic patients. The increase in its expression level together with the decline in renal function suggests that it could be useful as an early non-invasive marker for DN (Kalani *et al.* 2013). Recently, it has been demonstrated that high glucose-treated macrophages release more EVs and carry more TGF- β 1 mRNA. These exosomes are transferred to mesangial cells, resulting in increased TGF- β 1 expression, suggesting that exosomes carrying TGF- β 1 mRNA could promote renal fibrosis (Zhu *et al.* 2019).

As mentioned earlier, numerous studies have affirmed that podocyte injury plays a pivotal role in the onset and progression of DN. Notably, research on EVs in the context of podocyte injury related to DN has seen significant advancements (Li *et al.* 2023). Burger *et al.* observed in various diabetic rat models that levels of podocyte-derived EVs increased under hyperglycaemic conditions, predicting the onset of microalbuminuria and glomerular damage (Burger *et al.* 2014).

Furthermore, it has been demonstrated that MVs segregated from injured podocytes induced tubular fibrosis via p38 MAPK and CD36 (Munkonda *et al.* 2018).

Concerning to the miRNA cargo of uEVs, it represents an intriguing source of information for gaining a better understanding of DN pathophysiology. These uEV miRNAs have been identified as potential predictors of early-stage DN (Jia *et al.* 2016, Peng *et al.* 2022), playing a pivotal role in processes such as podocyte-derived EVs mediating dedifferentiation of renal proximal tubule cells via miRNA-221 in DN (Su *et al.* 2020). Moreover, the upregulation of miR-145 and miR-126 in EVs derived from renal cells undergoing EMT and in the urine of DN patients further highlight their diagnostic relevance (Dimuccio *et al.* 2022). These studies reinforce the idea that the quantification of miRNA levels carried by uEVs can provide new information about the changes occurring in the kidneys of T2DM patients.

It is essential to underline that EVs do not exclusively exert harmful effects. Numerous studies have elucidated the beneficial impacts of EVs, not only within the kidney but also in various organs and tissues, particularly those derived from mesenchymal stem cells. Recent findings indicate that EVs obtained from stem cell-conditioned media may confer protection against renal injury in T2DM. Derived EVs achieve this by fostering cell survival, promoting vascular regeneration, and preventing podocyte apoptosis. Jin J *et al.* described how exosomes secreted by adipose-derived stem cells alleviate DN by enhancing autophagy flux and inhibiting podocyte apoptosis (Jin *et al.* 2019). DN is also characterised by mesangial cell hypertrophy (Forbes and Cooper, 2013). Both mesenchymal stem cell-derived and human liver stem cells-derived EVs have been shown to protect mesangial cells from hyperglycaemia-induced collagen production and hyperglycaemic damage. This protection is achieved through the horizontal transfer of functional miR-222, resulting in the down-regulation of STAT5 and a decrease in miR-21 content, TGF- β 1 expression, and matrix protein synthesis (Gallo *et al.* 2016).

In summary, uEVs play a role in DN regulating various processes, including fibrosis, tubular and glomerular injury, and angiogenesis, making them potential therapeutic candidates. The analysis of the molecular content of uEVs, whose secretion is partly controlled by small GTPases center of this thesis, holds promise for better understanding the impact of T2DM on kidney health. These approaches offer valuable insights into the mechanisms underlying DN and may lead to innovative strategies for diagnosing, monitoring, and treating kidney issues in patients with T2DM.

II. HYPOTHESIS AND OBJECTIVES

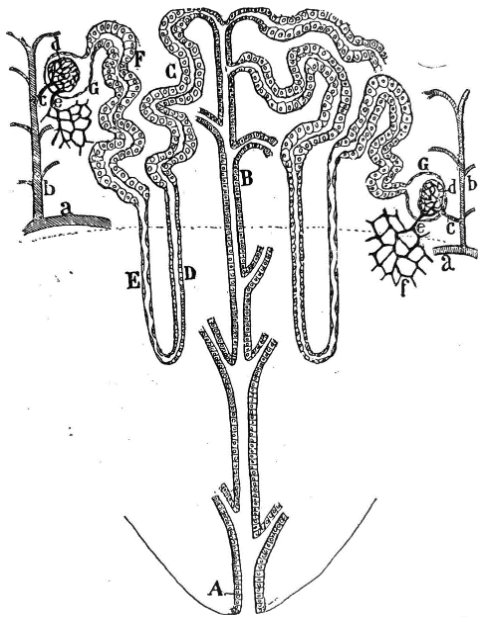


Fig. 176. — Esquema de la forma y dirección de un tubo urinífero. A, tubo terminado en la papila; B, tubo recto o de Bellini; C, tubo flexuoso que marcha por la región cortical; E, D, asa de Henle; F tubo flexuoso; G, cápsula de Müller, a, arterias arciformes yacentes entre las capas cortical y medular; b, arteriolas de donde salen las ramas aferentes del glomérulo de Malpigio; d, glomérulo; e, rama eferente; f, red capilar intersticial.

Elementos de histología normal y de técnica micrográfica-1895

Santiago Ramón y Cajal

DN emerges as a critical outcome of microvascular damage in T2DM, primarily characterised by glomerular hyperfiltration and heightened albumin excretion. The development of this pathology results from various intricate mechanisms. Notably, key contributors include chronic hyperglycaemia, activation of the RAAS, oxidative stress, inflammation, and dysregulation of various growth factors. These mechanisms collectively orchestrate structural and functional changes in the glomerulus, such as the loss of fenestrated endothelium, progressive hypertrophy and expansion of the glomerular mesangium, and the accumulation of ECM proteins. Additionally, a reduction in the number of podocytes, along with other intricacies, plays a crucial role in the progression of diabetic nephropathy. Podocytes are highly specialised cells important for preserving the structure of the glomerulus, specifically the glomerular filtration barrier. The capacity of maintaining the functions of the glomerular filtration barrier heavily relies on the integrity of the podocytes, regulated in part by intracellular vesicle trafficking. The Rab family of small GTPases, with proteins such as Rab3A, Rab27A, and its effector Rabphilin3A, plays key roles in regulating various processes of membrane trafficking. These processes include the formation, transport, attachment, and fusion of membrane-bound organelles and vesicles. Furthermore, previous studies of our group identified SNPs in *RPH3A* gene associated with the risk of albuminuria development. Indeed, the silencing of *RPH3A* in an *in vivo* model of *Drosophila melanogaster* resulted in reduced filtration capacity. Therefore, alteration of these proteins or disruption of intracellular vesicle trafficking could lead to podocyte damage and consequently to renal dysfunction.

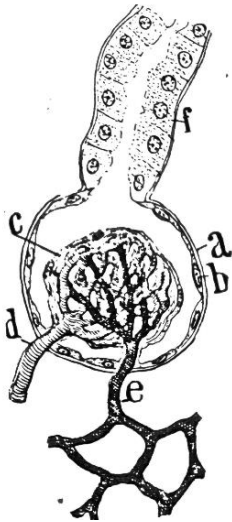
For all these reasons, the hypothesis of this thesis is that alterations in the Rab3A-Rab27A system could be involved in the presence of podocyte damage through disruptions in vesicular transport, contributing to the development of renal damage linked to T2DM.

To confirm our hypothesis, the general objective of this doctoral thesis is to assess the levels of Rab3A-Rab27A system in a cohort of diabetic patients and the role of Rab3A-Rab27A system in the development of kidney damage through *in vitro* experiments under diabetic conditions.

The specific aims proposed are:

1. To detect and quantify *RAB3A* and *RAB27A* GTPases mRNA levels in urinary samples from hypertensive and diabetic patients and their association with urinary albumin excretion levels.
2. To investigate the presence of the Rab3A-Rab27A system in human immortalised podocytes and analyze the changes in their expression when treated with elevated glucose concentrations and Angiotensin II.
3. To silence gene expression of the Rab3A-Rab27A system in human immortalised podocytes stressed by high glucose, to gain more in-depth insights into their effect on podocyte function and ascertain how this silencing could affect vesicular traffic.
4. To isolate and characterize extracellular vesicles segregated by human immortalised podocytes treated with glucose.
5. To quantify the miRNAs levels enclosed in podocytes and in the podocyte-derived extracellular vesicles when the Rab3A-Rab27A system is silenced under diabetic conditions.

III. MATERIAL AND METHODS



El carácter del epitelio es distinto en los varios segmentos mencionados. Así, en la cápsula de Müller, las células epiteliales se ensanchan y aplastan a manera de endotelio; en las porciones anchas y flexuosas, las células se engruesan, adquieren aspecto estriado, turbio, contornos indistintos, sobre todo en sus límites internos, donde se ven a menudo ciertas expansiones irregulares (fig.177).

Fig. 177.- Cápsula de Müller y glomérulo de Malpigio; a, membrana glandular; b, endotelio que la reviste interiormente; c, endotelio que rodea el glomérulo; d, arteria aferente; e, venita eferente; f, epitelio turbio del tubo flexuoso

Elementos de histología normal y de técnica micrográfica- 1895

Santiago Ramón y Cajal

1. PATIENTS

A total of 64 patients with HTN, 30 of them diagnosed with DM were recruited from the Internal Medicine Unit of Hospital Clínico Universitario of Valencia, Spain. According to the European Society of Hypertension, HTN was defined such as systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg (Gorostidi *et al.* 2022) and DM was defined according to the World Health Organization (ElSayed *et al.* 2023).

Patients were stratified in four groups based on the UAE levels and the presence or not of DM. Ten patients were diagnosed with DM (DM no UAE) and 20 patients with DM and increased UAE (DM UAE). Twenty patients were non-diabetic patients (no DM no UAE), and 14 non-diabetic patients with increased UAE (no DM UAE). All groups received antihypertensive treatment at the time of study, and diabetic patients were treated with oral antidiabetic agents. Clinical characteristics of the patient groups are described in Table 9.

UAE was measured using a nephelometric immunoassay (Behring Institute, Germany) and increased UAE was defined as an albumin (mg)-to-creatinine (g) ratio (ACR) of ≥ 30 mg/g. The level of albuminuria for each patient was considered as the mean value obtained from two measurements of UAE in the first morning urine with an interval less than one week. Data were normalised and expressed as the ACR.

The study protocol was approved by the Ethics Committee of the Hospital Clínico Universitario of Valencia (2012/072, 13 February 2013) in accordance with the Declaration of Helsinki of 1975 as revised in 2013 (World Medical 2013). All patients gave written informed consent.

2. URINE SAMPLES

Fresh first morning urine samples (200 mL) were collected from each subject of study in sterile tubes. Urinary sediment or urine cellular pellet was precipitated by centrifugation at 2,250 g for 30 min at 4 °C in a benchtop centrifuge (5804 R, Eppendorf, Hamburg, Germany). This first step allowed to obtain the urinary sediment or cell pellet, by separating the cells, subcellular fragments and cell debris, from the rest of the urine. Urinary sediment was washed with PBS (Phosphate-buffered saline) and centrifugated. Finally, dry urinary cell pellet was stored at – 80 °C until use.

3. IN VITRO CELL CULTURE

3.1. Reagents

Cell culture reagents, media and supplements for cell culture were obtained from Biowest (Nuaille, France), Invitrogen and Gibco™ (Thermo Fisher Scientific, Waltham, MA, USA), Sigma-Aldrich Chemicals (St. Louis, MO, USA), Life Technologies (Darmstadt, Germany) and Lonza (Basel, Switzerland). Reagents are summarised in Annex I.

3.2. Human immortalised podocytes

Conditionally human immortalised podocytes (cell line: AB8/13) were kindly provided by Prof. Moin Saleem (Children's Renal Unit and Academic Renal Unit, University of Bristol, Southmead Hospital, Bristol, UK). Particularly, this cell line when it is grown at the “permissive” temperature of 33 °C, the SV40 T antigen is active, maintaining cells in an undifferentiated proliferative state. Thermoswitching them to the “nonpermissive” 37 °C temperature silences the transgene, and the cells become growth arrested and differentiated (Saleem *et al.* 2002). Briefly,

podocytes were cultured on collagen I-coated (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) flasks or plates in RPMI-1640 with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (P/S) both from Biowest, (Nuaille, France), and 1% insulin-transferrin-selenium (ITS, Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Cells were propagated at 33 °C until 80% confluence was reached and were trypsinised (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and replated at proper density. Next, they were allowed to differentiate at 37 °C with 2% FBS for 10 – 14 days. Cell cultures were maintained in an incubator (BINDER CB-160 and BINDER CB-150, VWR International Eurolab, Barcelona, Spain) in a humidified atmosphere with 5% CO₂/95% air (AirLiquide Medicinal, Valencia, Spain).

3.3. Treatments

All experimental treatments were performed on differentiated podocytes starting at day 10 of differentiation and with a confluence of 80%. Cells were serum starved and then treated with normal glucose (NG; 5.5 mM D-glucose) and high glucose (HG; 30 mM D-glucose) (Sigma-Aldrich) concentrations during 72 h for Western blot, quantitative real time polymerase chain reaction (RT-qPCR) and apoptosis analyses. Differentiated podocytes were also treated with increasing concentrations (0.1, 1, and 2 µM) of Ang-II (Sigma-Aldrich) for 6, 24, and 48 h for Western blot analyses, 24 h for quantitative RT-PCR, and 48 h for flow cytometry and immunofluorescence analyses.

3.4. Podocyte permeability assay

To assess the permeability capacity of podocytes, 80,000 cells were seeded in the upper chamber of 3 µm cell culture inserts Falcon® (Corning, Glendale, AZ, USA) previously coated with human placenta collagen type IV (Sigma-Aldrich) and differentiated during 7 days at 37 °C. Then, cells were treated with HG for 48h and 72 h. After treatment, medium was removed and replaced for 1 mL of White RPMI

(Euroclone, Pero, Italy) in the upper chamber and 1 mL of 1mg/mL BSA-FITC (Sigma-Aldrich) in the lower chamber. Cells were incubated for 3 h in the incubator and later, 100 μ L from each upper chamber were placed in triplicate in a 96 well plate to be read in the Promega™ GloMax® Plate Reader (Promega, Madison, WI, USA).

3.5. *RAB3A* and *RAB27A* transient silencing

Podocytes were transiently transfected using Lipofectamine™ 3000 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Cells were differentiated for 10 days before transfection in T-75 flasks and maintained at 37 °C in a CO₂ incubator. For the transfection, 10 nmol of *RAB27A* siRNA (siRab27A) (ID: s11693) and 20 nM *RAB3A* pool siRNA (siRab3A) (ID: s11667 y s11668) and 37.5 μ L Lipofectamine™ 3000 were diluted gently in 900 μ L of serum-free Opti-MEM® in two separate tubes. As experimental control, Cy3™ Dye-Labeled Pre-miR Negative Control was employed to visualize the internalization of liposomes into cells by *in vivo* fluorescence microscopy (Leica DMI 3000-B, Wetzlar). Additionally, Silencer® Select Negative Control siRNA and siRNA targeting the constitutively expressed *GAPDH* were used at the same concentration as the siRNAs used such as negative and positive controls (Annex II). All reagents employed for siRNA experiments were from Invitrogen, Thermo Fisher Scientific.

Diluted siRNA and diluted Lipofectamine™ 3000 were combined (total volume of 1,800 μ L), mixed gently and incubated for 20 min at room temperature (RT). The transfection mixture was added to podocytes (80-90% confluence) in T-75 flask containing 13.2 mL of supplemented RPMI without antibiotics and mixed gently before incubation for 24 h at 37 °C in a cell culture incubator. After 24 h, the medium was replaced by fresh supplemented RPMI. Finally, after 24 h of transfection, cells were treated with NG and HG concentrations during 24 h.

4. PROTEIN EXPRESSION ANALYSIS

4.1. Cell Sample collection and preparation

To collect the cell samples, culture medium was removed, cells were washed once with a suitable volume of warm PBS and detached by adding trypsin-EDTA at 37 °C for 1 min. The resulting cellular suspension was centrifuged at 800 g for 3 min, at RT (5804 R, Eppendorf, Hamburg, Germany), the supernatant was then discarded and cell pellet was resuspended in 1 mL ice-cold PBS. The resulting suspension was centrifuged at 500 g for 5 min, at 4 °C (5415R, Eppendorf). Finally, the supernatant was discarded and cell pellets stored at - 80 °C.

4.2. Whole cell protein extraction

Culture cell pellets and urine cell pellets were resuspended in 40 - 60 µL of radio-immunoprecipitation assay (RIPA) lysis buffer (depending on the quantity of cell pellet), whose composition was 25 mM Tris-HCl (pH 7,6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS (Thermo Fisher Scientific) and supplemented with protease inhibitor (2 µg/mL leupeptin, 20 ng/mL aprotinin; Sigma-Aldrich). Samples were then vortexed at maximum speed for 10 seconds, incubated on ice for 15 min, vortexed again at maximum speed for 30 seconds and subsequently centrifuged at 13,500 rpm for 15 min at 4 °C (5415R, Eppendorf). Pellets were discarded, and supernatants (whole-cell protein extracts) were collected in new ice-chilled tubes and stored at – 80 °C until use.

4.3. Protein quantification: Lowry assay

The determination of the protein quantity was assessed employing the Total Protein Kit., Micro Lowry, (Sigma-Aldrich). This method is based on Peterson's (1977) modification of the spectrophotometric method of Lowry *et al.* (1951) which

is based on the reduction of Folin-Ciocalteu reagent by proteins under alkaline conditions, leading to the formation of a blue-colored complex (Winters and Minchin 2005). The intensity of the color can be measured spectrophotometrically based on the proportionality between the intensity of color in the solution and the protein concentration, according to the Lambert-Beer Law.

The absorbance was measured at a wavelength of 620 nm, using the 450 nm wavelength as a reference in a GloMax® Multidetector System spectrofluorometer (Promega). Protein concentration was calculated by absorbance interpolation using a calibration curve with bovine serum albumin (BSA) employed as standard (400 µg/mL – 0 µg/mL). After determining the protein concentration, samples were frozen at - 80 °C until their usage for protein analysis techniques.

4.4. Western blot analysis

4.4.1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Same amount of protein was loaded in the electrophoresis gel NuPAGE® Novex Bis-Tris polyacrilamide 4-12%. NuPAGE® Novex Tris-Acetate polyacrilamide 3-8% was used to separate high molecular weight proteins. Reducing and non-reducing conditions were also used depending on the conditions required for the identification of the protein of interest.

4.4.2. Electrophoresis under non-reducing conditions

Subsequently, after preparing the samples for loading onto the gel, they were heated at 70 °C for 10 min in a thermoblock. The gels were placed in an electrophoresis tank (XCell SureLock™ mini cell, Invitrogen) containing 1X MES electrophoresis buffer (20X NuPAGE® MES SDS Running Buffer, Invitrogen) for NuPAGE® Novex Bis-Tris polyacrilamide 4-12% gels and 1X Tris-Acetate SDS

Running Buffer (NuPAGE 20X, Invitrogen) for Tris-Acetate polyacrylamide gels. Next, the known molecular weight marker was loaded into the first well (Novex® Sharp Pre-Stained Protein Standard, Invitrogen), followed by the corresponding loading volume. The electrophoretic separation of the different proteins was performed according to the procedure developed by Laemmli in 1970 (Laemmli 1970), and the voltage and amperage conditions were 200 V and 200 mA, respectively, for 90 min in the case of NuPAGE® Novex Bis-Tris gels, and 150 V and 90 mA, respectively, for 120 min for NuPAGE® Novex Tris-Acetate gels. In this way, proteins with weights ranging from 3.5 to 260 kilodaltons (kDa) were separated using vertical gradient polyacrylamide gels (13 x 8.3 cm) (4-12% and 3-8% gradient for NuPAGE® Novex Bis-Tris and Tris-Acetate gels, respectively), with 12 and 15 wells. Through this type of SDS-PAGE electrophoresis, proteins were separated based on their size and charge, due to the use of the anionic detergent SDS, which denatures the proteins by eliminating their secondary and tertiary structure, and also negatively charges them in proportion to their mass.

4.4.3. Electrophoresis under reducing conditions

In SDS-PAGE under reducing conditions, in addition to protein denaturation, disulfide bonds are reduced by adding a reducing agent such as dithiothreitol (DTT) or 2-mercaptoethanol to the samples. The gels were run under reducing conditions using both Bis-Tris 4-12% polyacrylamide and Tris-acetate 3-8% polyacrylamide. After preparing the samples with the reducing agent (NuPAGE® Sample Reducing Agent (10X), Invitrogen), they were heated in a thermoblock at 70 °C for 10 min. Next, the gels were placed in an electrophoresis chamber (XCell 4 SureLock™ Midi-Cell Runner, Invitrogen) with 1X MES buffer or Tris-acetate (20X NuPAGE® SDS Running Buffer, Invitrogen) and antioxidant agent (1/400, NuPAGE® Antioxidant, Invitrogen) in the chamber area in contact with the gels (internal chambers). After electrophoresis, the gels were transferred to PVDF membrane and stained.

4.4.4. Protein transfer to PVDF membrane and blocking

Resolved proteins were transferred from the polyacrylamide gel to a PVDF membrane using the *XCell II Blot Module* (Invitrogen, Thermo Fisher Scientific). Before the transfer, sponges, gels and all the elements needed to set up the sandwich were embedded in transfer buffer (Life Technologies, Darmstadt, Germany) (25 mM Tris pH 8.3, 192 mM glycine and 10% metanol for one gel and 20% metanol for two gels). For one gel, the transfer was performed at RT adding ice to the transfer module at 30 V and 170 mA during 1 h, followed by 10 min at 110 mA and at 30V and 340 mA for two gels for 1 h. After the transfer time had elapsed, the PVDF membranes were immediately incubated with TBS-T with 0.05% Tween 20 and 1% BSA (Type V, Sigma-Aldrich) overnight (O/N) at 4 °C to block nonspecific binding sites.

4.4.5. Coomassie blue staining

The polyacrylamide gels were stained with the Coomassie staining kit (Colloidal Blue Staining Kit, Invitrogen). The gel was immersed and shaken in the staining solution (20% Stainer A, 5% Stainer B and 20% methanol) for a minimum of 3 h and a maximum of 24 h. Then, the gel was washed with distilled water to remove the excess staining, gently agitated for at least 7 h or until there was no changes in the intensity of the bands.

4.4.6. Protein immunodetection

After blocking the membrane, the blocking solution was replaced with TBS-T containing the primary antibody at an appropriate dilution (Table 2). Antibodies were incubated for 2 h at RT with gentle agitation. Then, primary antibodies were recovered and the membrane was washed 3 times for 5 min in TBS-T buffer, and incubated with a secondary monoclonal antibody against rabbit, guinea pig or

mouse, depending on the primary antibody used in each case, conjugated with alkaline phosphatase enzyme (Sigma-Aldrich) and diluted 1: 10,000 in TBS-Tween 20 + Nonidet P40, for 1 h at RT with agitation, except for nephrin, which was diluted to 1: 3,000 (Table 3).

Table 2. Primary antibodies used in Western blot.

PROTEIN	ANTIBODY	DILUTION	MW(kDa)	COMPANY
CD2AP	Rabbit policlonal	1/500	65 kDa	Thermofisher
Nephrin	Guinea Pig	1/500	200 kDa	Origene
Rab27a	Mouse monoclonal	1/500	25 kDa	Abcam
Rab3a	Mouse monoclonal	5 µg/mL	25 kDa	Sigma-Aldrich
Rabphilin-3A	Rabbit	1/500	76 kDa	Sigma-Aldrich
Synaptopodin	Rabbit monoclonal	1 µg/mL	110 kDa	Sigma-Aldrich
WT-1	Rabbit monoclonal	1/500	50 kDa	Abcam
β-actin	Mouse monoclonal	1/10,000	42 kDa	Sigma-Aldrich

kDa: Kilodaltons

Table 3. Secondary antibodies used in Western blot.

SECONDARY ANTIBODIES	DILUTION	COMPANY
Alkaline phosphatase – labeled anti-mouse-igG	1/10,000	Thermofisher
Alkaline phosphatase – labeled anti-rabbit-igG	1/10,000	Thermofisher
Alkaline phosphatase – labeled anti-guinea pig-igG	1 / 2,000	Thermofisher

Subsequently, the membrane was washed again 3 times for 5 min in TBS-T buffer, the washing process was repeated using only TBS, and band visualization was performed by incubating the membrane in darkness with a dilution of the NBT/BCIP buffered substrate tablets (Sigma-Aldrich) in distilled water (1 tablet / 10 mL) for approximately 20 min. Nitroblue tetrazolium chloride (NBT) is a compound formed by the union of two tetrazole groups with high oxidising power. It is

commonly used in immunology for the detection of alkaline phosphatase activity, which is used as a marker conjugated to an antibody. 5-bromo-4-chloro-3'-indolyl phosphate (BCIP) is an artificial chromogenic substrate used for sensitive colorimetric detection of alkaline phosphatase activity. This enzyme hydrolyzes BCIP to 5-bromo-4-chloro-3-indolyl and inorganic phosphate (Horwitz *et al.* 1966). The bromo-chloro-indol, separated from the phosphate group, undergoes tautomerization and is then oxidised by NBT, resulting in an insoluble compound of intense violet color (5,5'-dibromo-4,4'-dichloro indigo white). Once the bands appeared, the colorimetric reaction was stopped with distilled water, and then the membranes were washed with distilled water and after the membrane was dried on filter paper for at least 24 h. Finally, once the membranes were dried, were digitalised and quantified using an image analysis station (ImageQuant™ 7.0 TL software). The obtained values were normalised to β -actin protein levels and expressed as arbitrary units, and the results were compared to NG, NG scramble (scr) or untreated cells (CNT or CNT scr) as the reference, relativising levels to 100.

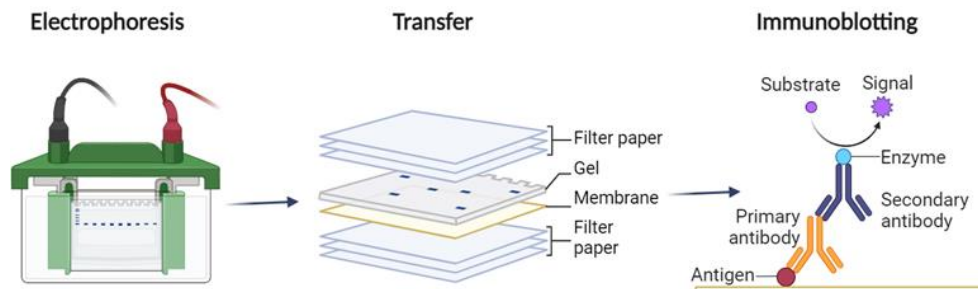


Figure 11. Western blot experiment workflow. Image created with BioRender.com.

5. QUANTIFICATION OF mRNA LEVELS

5.1. RNA isolation

RNA was extracted from podocytes and human urine cell pellets using the miRNeasy mini kit (Qiagen, Hilden, Germany), based on spin column isolation following the manufacturer's instructions as previously reported (Martinez-Arroyo *et al.* 2020). Briefly, cell pellet was resuspended in 700 μ L of Qiazol lysis buffer and was homogenised by vortex. After homogenising the sample, 140 μ L of chloroform were added and mixed again by vortex. Then, the samples were centrifuged at 12,000 g for 15 min at 4 °C (5804 R, Eppendorf). This resulted in three phases: aqueous phase rich in RNA, an interface containing DNA, and an organic phase with protein residues. The aqueous phase was isolated in a new tube and 1.5 volumes of 100% ethanol were added. Once the components were mixed, everything was loaded onto the column and centrifuged at 10,000 g for 30 seconds, discarding the eluted volume. This process was repeated using two different wash buffers. Finally, RNA was eluted from the column with 50 μ L of nuclease-free water. After extraction, RNA concentration and purity were confirmed by the 260/280-nm absorbance ratio using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific). RNA samples were stored at -80 °C until use.

5.2. Complementary DNA synthesis by reverse transcription

Total RNA was reverse transcribed to cDNA using Ready-To-Go You Prime First-Strand Beads (GE Healthcare, Buckinghamshire, UK) following the protocol indications. Briefly, 300 ng of RNA if obtained from urine cell pellet or 900 ng of RNA if obtained from human immortalised podocytes were reverse transcribed. RNA was diluted with RNase free water to a final volume of 34 μ L. Once diluted, 2 μ L of random primers were added and samples were incubated at 70 °C during 10 min. Subsequently, the dilution was incubated on ice for 2 min and the entire

volume was transferred to a 0.5 mL tube provided by the kit, which contains two beads with the enzyme (MMLV reverse transcriptase) as well as the rest of the components necessary for the reaction (dNTPs, buffers, RNA stabilizer). Finally, the entire volume was homogenised and incubated in the thermocycler for 10 min at 25 °C followed by 90 min at 37 °C. Finally, an aliquot of the cDNA obtained was diluted 1:10 in nuclease-free water and stored at -80 °C.

5.3. Quantitative RT-PCR

Real-time quantitative polymerase chain reaction (RT-qPCR) is a variant of conventional PCR used for the amplification and quantification of the amplification product. The RT-qPCR reaction monitors the real-time progression of amplification of the fragment corresponding to the gene of interest, measuring the fluorescence generated at the end of each amplification cycle. Changes in fluorescence intensity are determined by the copies of the fragment of interest (amplicon) synthesised by the polymerase enzyme in each cycle. Three phases are identified in the PCR reaction: an initial phase of linear growth, an exponential growth phase where fluorescence is proportional to the amount of product, and a final phase called the plateau phase, in which the substrates have been consumed. Thus, the data are obtained in the exponential phase by comparing the number of cycles required in each sample for the fluorescent signal to reach a specified threshold level. This value is known as Ct (cycle threshold) or Cq (quantification cycle) and is inversely proportional to the initial amount of cDNA in the sample (Bustin *et al.* 2009).

The primers used were designed *de novo* in our laboratory based on the sequences stored in the Gene Bank database of NCBI and were synthesised by Sigma-Aldrich. The parameters for their design are summarised in Table 4.

Table 4. Primer design parameters.

Parameters	Primers Characteristics
Primer length	18-24 bp
Melting temperature (T _m)	60-63 °C
Maximum difference of T _m (primers)	2 °C
Amplicon size for RT-qPCR	95-190 bp

The software Primer3 (<http://bioinfo.ut.ee/primer3-0.4.0/>) was used for this purpose, and the Basic Local Alignment Search Tool (BLAST) was used to verify the specific hybridization of the primers obtained with the corresponding cDNA region of each gene. To increase specificity, at least one of the primers in each pair was designed in the junction zone of two different exons. Finally, it was also verified in the Ensembl database that the hybridization regions did not contain SNPs, or insertions/deletions. The details of each primer pair can be found in Table 5.

Table 5. Primers employed in mRNA RT-qPCR.

GENE	FORWARD SEQUENCE 5'→ 3'	REVERSE SEQUENCE 5'→ 3'	LENGHT
ACTB	TGGAGAAAATCTGGCACCAC	CATGATCTGGGTCATCTTCTCG	125 bp
B2MG	TCCAGCGTACTCCAAAGATTC	GTCAACTTCAATGTCGGATGG	113 bp
CD2AP	AGGCATGGGAATGTAGCAAG	TGACGCTTCTGGTCTTCTTC	116 bp
NPSH1	AGCACCTGACATCACCATTCTC	GCTAGACGCCTCACAGACCAG	105 bp
RAB27A	AAAGAGTGGTGTACAGAGCCAGTG	GTCGTTAAGCTACGAAACCTCTCC	112 bp
RAB3A	CCTCATGTATGACATCACCAACG	CCTCAAAGAACTCGAACCCAAG	194 bp
SYNPO	GCCGCAAATCCATGTTTACT	CTCATCCGCTGTCTGTACCA	86 bp
WT-1	TTCGCAATCAGGGTTACAGC	AATGAGTGGTTGGGGAAC TG	97 bp

RT-qPCR was performed in the LightCycler 480 II real-time PCR system (Roche, Mannheim, Germany) using 384 well plates (Sorenson BioScience, Utah, USA), detailed below in the scheme (Fig.12). For each RT-qPCR reaction 6.8 μ L of mastermix were prepared with 5 μ L of Multiplex enzyme (Qiagen) 0.7 μ L of LCGreen such as fluorophore and nuclease free H₂O joint to 1.2 μ L of specific pair of primers added at final concentration of 0.5 μ M. Finally, 2 μ L of 1:10 diluted cDNA were added to reach a final reaction volume of 10 μ L. The reaction conditions were maintained for all genes, except for the extension temperature (Table 6). Each experiment was performed in triplicate, and the mean Ct value of the replicates was calculated using the instrument software (LightCycler® 480 SW 1.5.1, Roche, Mannheim, Germany). Furthermore, melting curves were assessed to confirm the targeted amplification of the specific gene fragment. Negative controls (lacking cDNA) were included for each primer pair, alongside a positive control derived from human kidney cDNA.

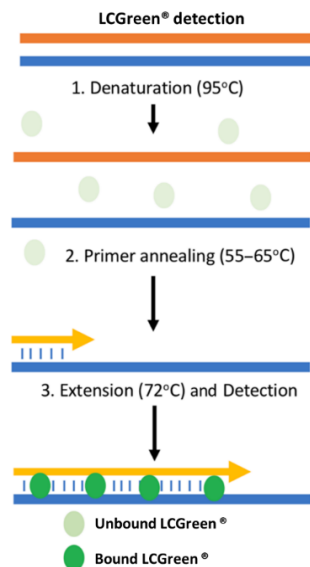


Figure 12. RT-qPCR procedure. LCGreen® detection: Following denaturation of cDNA, primers anneal and are extended. During extension, LCGreen binds to the double-stranded DNA (dsDNA), emitting a fluorescent signal detected by the qPCR instrument Image modified from DOI: 10.1042/BIO20200034. LicenseCC BY-NC-ND 4.0

The relative gene expression of the studied genes was obtained by using the comparative ΔC_t method (Pfaffl 2001). Thus, the initial C_t value of each sample for each gene, was normalised to the mean C_t value of the two constitutively expressed genes, *ACTB* (β -actin) and *B2MG* (β -2-microglobulin) used as a housekeeping gene. Once ΔC_t was obtained, $\Delta\Delta C_t$ [ΔC_t treated group – ΔC_t control group] was calculated to compare the relative expression level for each gene between the different groups of the study. Finally, $2^{-\Delta\Delta C_t}$ was obtained, and the difference between groups was expressed as fold-change. Fold change = $2^{-\Delta(\Delta C_t)}$, where $\Delta C_t = C_t$ (target gene) - C_t (housekeeping gene), and $\Delta(\Delta C_t) = \Delta C_t$ (treated) - ΔC_t (control).

Table 6. RT-qPCR parameters.

PCR step		Time	Temperature
Enzyme activation		15 min	95 °C
Amplification 40 cycles	Denaturalization	20 seg	95 °C
	Annealing	30 seg	60-62 °C
	Extension	10 seg	72 °C

5.4. Seminested PCR for Rabphilin3A detection

A seminested PCR was performed for the Rabphilin3A gene (*RPH3A*) to improve the sensitivity and specificity of cDNA amplification. Briefly, this process involved the use of two primer sets and two successive PCRs. The first set of primers was designed to anneal two sequences upstream from the second set of primers and was used in the initial PCR. Amplicons resulting from the first PCR were used as a template for a second set of primers and a second PCR. For *RPH3A* amplicon identification, we performed high-resolution capillary electrophoresis using QIAxcel (Qiagen), with fragment length assessment through QIAxcel software. A neuronal cell line (SH-SY5Y, CRL2266, ATTC) was used as a positive control of *RPH3A* expression. Appropriate controls and the non-template controls were included.

6. CELL APOPTOSIS ASSAY

6.1. Cell apoptosis assay by flow cytometry

To assess the cellular apoptosis under the different treatments and siRab3A and siRab27A podocytes, cells were trypsinised, resuspended, centrifuged for 3 min at 1,300 rpm, and washed with sterile PBS. They were then resuspended in 200 μ L of annexin V binding buffer (Immunostep, Salamanca, Spain) and incubated in darkness for 15 min at RT with 5 μ L of annexin V and 5 min with 7 μ L of 7-amino-actinomycin D. Finally, 400 μ L of annexin binding buffer were added. The number of apoptotic or necrotic cells was quantified by flow cytometry (BD LSR Fortessa X-20) with FACSDiva 8.0.1 software (BD Bioscience, Franklin Lakes, NJ). A total of 10,000 events from each replicate were analysed.

6.2. Microscopic assessment of cell apoptosis

Additionally, cell apoptosis of treated podocytes was assessed by confocal microscopy. Nuclei was stained with DAPI (4',6-diamidino-2-phenylindole) and chromatin condensation, nuclei fragmentation, and nuclear condensation of podocytes was determined following the protocol described by Cummings *et al.* analysing 250 cells/sample (Cummings and Schnellmann 2004).

7. CELL MORPHOLOGY ANALYSIS

7.1. Light microscopy

A Leica DMI1 optical microscope (Leica Microsystems, Wetzlar, Germany) and Leica LAS (version 4.9) camera software were used to follow the podocyte differentiation process. Images were taken at different stages of differentiation and also during podocyte treatments. Measures and morphological parameters were analysed in treated podocytes (membrane blebbing and area and circularity of cells) using ImageJ software (version 1.46 r, National Institutes of Health). For this analysis, measurements were taken from 150 cells of each experimental group in random and non-overlapping fields of three independent replicates.

7.2. Assessment of F-actin cytoskeleton organization

Analysis of the F-actin cytoskeleton was performed by phalloidin staining on differentiated podocytes treated with HG and Ang-II concentrations and also in siRab27A and siRab3A podocytes. Cells were fixed in 4% paraformaldehyde for 15 min at RT, washed three times with PBS, permeabilised with 0.1% Triton X-100, washed, and incubated with phalloidin-iFluor 594 Reagent 1/1,000 (Abcam, Cambridge, UK) for 1 h at RT in darkness. Within the last half an hour of the incubation period, cells were stained with DAPI and after incubation, washed three times in PBS. Finally, fluorescence was observed with Leica TCS-SP8 confocal laser scanning unit (Leica Microsystems, Wetzlar, Germany), and images were processed using ImageJ software (version 1.46 r, National Institutes of Health). F-actin fiber orientation was measured with the FibrTool plugin (ImageJ), which quantifies the orientation and anisotropy of fibrillary structures in raw images. Values close to 0 correlate to isotropic or no ordered fibers, and values close to 1 represent perfectly orientated filaments (Boudaoud *et al.* 2014).

8. CONFOCAL FLUORESCENCE MICROSCOPY

We used a Leica TCS-SP8 confocal laser scanning unit (Leica Microsystems) which is part of “Unidad Central de Investigación de Medicina” (UCIM, Universidad de Valencia, Valencia, Spain). Images were captured at 63x magnification with HCX PL APO 40.0 x 1.32 oil UV objective or at 40X magnification with a HC PL APO 40x/1.30 Oil PH3 CS2 objective.

For immunofluorescence analysis, samples were fixed in 4% paraformaldehyde for 15 min at RT, washed in cold PBS, and blocked in PBS -BSA (1%) for 30 min at RT. After being blocked, samples were incubated O/N at 4°C with the same primary antibodies as for the Western blot analyses: anti-synaptopodin (1/40), anti-nephrin (1/50), anti-Rab3A (1/80), anti-Rab27A (1/50), and anti-Rabphilin3A (1/50) in PBS-T BSA (1%) buffer. Next, they were incubated in darkness with Alexa-conjugated secondary antibody (Invitrogen, Thermo Fisher Scientific) for 1 h at RT in the same buffer. Within the last half an hour of the secondary antibody incubation, PBS-T BSA (1%) with DAPI (1/1,000) was added to identify nuclei. After incubation, samples were washed three times for 5 min with 1% PBS BSA. Finally, samples were mounted in a slide using Prolong Gold antifade mountant agent (Invitrogen, Thermo Fisher Scientific).

9. EVS ISOLATION AND CHARACTERISATION

9.1. Isolation by differential ultracentrifugation

For the isolation of EVs present in the cell culture supernatant, we employed a technique based on differential centrifugations. With this methodology, we can separate the components of a solution according to their sedimentation coefficient, which is determined by the size and mass of each particle, as well as the viscosity of the medium in which it is suspended.

Exosomes were extracted from the podocyte cell culture medium following a protocol based on sequential ultracentrifugation (Fig. 13) (Perez-Hernandez *et al.* 2021). In summary, the medium was collected, and centrifugation was performed for 30 minutes at 2,250× g to remove cells and debris. Subsequently, the cell-free supernatant underwent a 45-minute centrifugation at 20,000× g (Ultracentrifuge Optima L 100K, 70 Ti rotor, Beckman Coulter, Indianapolis, IN, USA) to precipitate larger EVs. The supernatant was centrifuged for 70 minutes at 110,000× g to obtain the exosome-enriched pellet. This pellet was then resuspended in 0.2 µm filtered PBS and prepared for downstream analyses. Throughout the process, centrifugation was conducted at 4 °C with maximum acceleration, and no braking system was employed. The speeds are expressed in g-force (gm), considering the rotor angle at its central part. Polycarbonate bottles of 26 mL for the 70 Ti rotor were employed, and their weight was balanced before each ultracentrifugation.

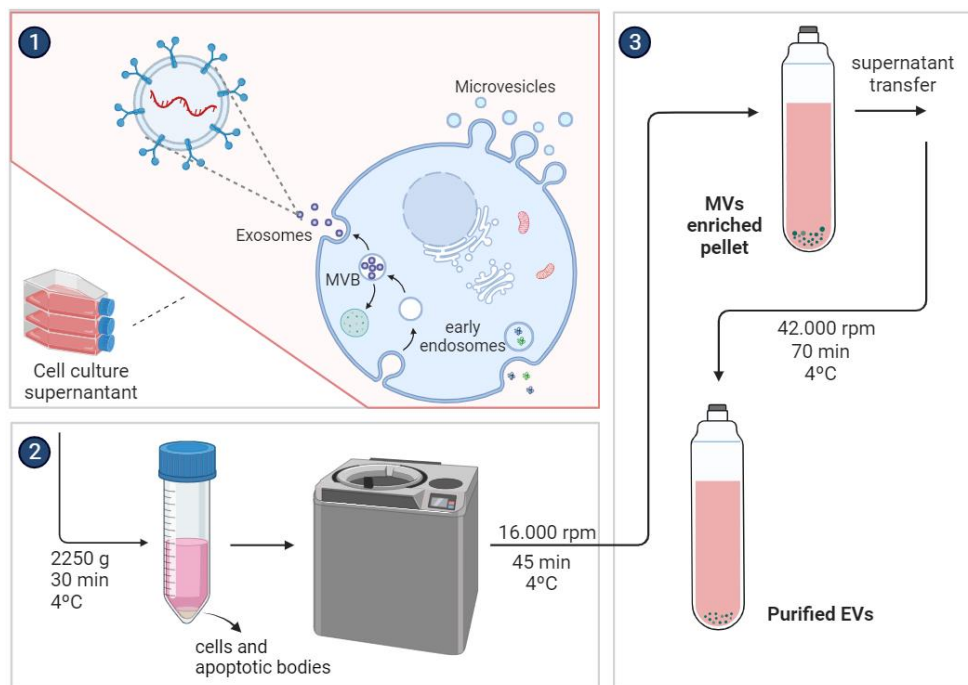


Figure 13. EVs differential ultracentrifugation isolation protocol. Image created with BioRender.com

For the experiments carried out in the laboratory of Professor Benedetta Bussolati in the Molecular Biotechnology Center of Turin, differential ultracentrifuge technique was also employed to isolate EVs from cell culture media. Briefly, cells were serum starved O/N, then the culture medium was recovered and filtered through 0.2 μm filter to eliminate cell debris and contaminants, and centrifugated for 2 h at 4 °C at 100,000 x g with no brake deceleration in a Beckman ultracentrifuge (Ultracentrifuge Optima L 100K, 70 Ti rotor, Indianapolis, IN, USA) to obtain EVs enriched pellet. Finally, EVs samples were resuspended in 0.2 μm filtered PBS and stored at -80 °C until processed for downstream analyses.

9.2. EVs size and concentration determination

To determine the size and the concentration of EVs, a nanoparticle tracking analysis (NTA) was performed in a NanoSight LM10 (Malvern Panalytical Ltd,

Malvern, UK). Samples were diluted 1/50 with 0.2 μm filtered PBS to achieve the concentration range of 20-120 particles/frame, as recommended by the manufacturer. The samples were then injected into the NanoSight with a 405 nm laser and an sCMOS camera. NTA software version 3.3 (Dev Build 3.3.104) was used to analyze the data, with the Min Track Length, Max Jump Distance, and Blur parameters set to auto and a detection threshold of 5. The camera level was set to 15, and manual temperature monitoring was conducted while five readings of 30 s at 30 frames per second were taken.

9.3. Transmission electron microscopy analysis

As described previously, EVs can be classified and differentiated based on their shape and size. One commonly used method to visualize EVs is transmission electron microscopy (TEM), where staining methods such as "Negative Staining" are employed due to their speed and simplicity. This technique allows the visualization of rounded vesicles, where the lipid bilayer characteristic of EVs is visible. Immunogold staining allowed us to detect specific tetraspanins CD9 and CD63 present in the EVs surface. Making use of colloidal gold antibodies we detected CD9 and CD63 proteins to assure the presence of EVs in our samples. For immunogold labeling, 8 μL of EV samples were first fixed in 2% paraformaldehyde - 0.1 M PBS for 30 min on carbon-coated nickel grids. After, the grids with adherent exosomes were washed in 0.1 M PBS and blocked in 0.1 M glycine and 0.3% BSA for 10 min. The grids were incubated with the primary anti-bodies CD9 and CD63 (rabbit and mouse, respectively) for 1 h. After blocking for 20 min, grids were incubated for 1 h in secondary antibodies with 6 nm and 15 nm gold particles conjugated. Finally, after washing, a standard negative staining procedure was performed and observed under a transmission electron microscope (FEI Tecnai G2 Spirit, ThermoFisher Scientific company, Oregon, USA). Images were acquired using Radius software (Version 2.1) with a Xarosa digital camera (EMSIS GmbH, Münster, Germany).

9.4. Identification of EVs markers

Western blotting immunodetection of specific proteins enriched in both the luminal space and lipid membranes can be used to characterize EVs. These markers include tetraspanins CD9 and CD63, the protein TSG-101, which is part of the ESCRT-1 complex involved in the biogenesis of MVBs containing exosomes, and syntenin-1, one of the most abundant proteins in exosomes. To identify potential contamination of EVs preparations with other subcellular fragments, non-exosomal markers such as calnexin (endoplasmic reticulum), GM-130 (Golgi apparatus), and nucleoporin-62 (nucleus) were also analysed. The protein extraction, quantification, electrophoresis, and immunodetection were conducted as previously described. Description of the antibodies used are in Table 7.

Table 7. Primary antibodies for exosomal markers used in Western blot.

PROTEIN	ANTIBODY	DILUTION	MW(KDa)	COMPANY
Calnexin	Rabbit monoclonal	1µg/ml	90KDa	Abcam
CD63	Mouse monoclonal	1µg/ml	26KDa	Abcam
CD9	Rabbit monoclonal	1/500	18 kDa	Abcam
GM-130	Rabbit monoclonal	1/500	140KDa	Abcam
Syntenin-1	Mouse monoclonal	1/500	28-38 kDa	Origene
TSG-101	Mouse monoclonal	1/500	47KDa	Abcam

9.5. EVs visualization by super resolution microscopy

Super-resolution microscopy of single EV characterisation was employed. Acquisition of EVs was obtained using Nanoimager S Mark II microscope from ONI (Oxford Nano-imaging, Oxford, UK) equipped with a 100x, 1.4NA oil immersion objective, an XYZ closed-loop piezo 736 stage, and triple emission channels split at 640, 488 and 555 nm. EV profiler Kit (ONI) was used to perform the experiments

following manufacturer's protocol. Briefly, 8 μ L of EVs suspension were added to a cocktail of conjugated antibodies provided by the kit anti CD9-488, CD63-568 and CD81-647 and the imaging buffer and samples were incubated O/N at 4 °C. The following day, samples were charged in the ONI provided chip and samples were fixed and washed before acquisition. Images were taken in dSTORM mode acquired sequentially in total reflection fluorescence (TIRF) mode. Single-molecule data was filtered using NimOS software (v.1.18.3, ONI). Data has been processed with the Collaborative Discovery (CODI) online analysis platform www.alto.codi.bio from ONI and the drift correction pipeline version 0.2.3 (Skovronova *et al.* 2021).

9.6. Characterisation of EVs surface markers

EV samples obtained from podocyte cell cultures were tested with a bead-based multiplexed analysis using the MACSPlex exosome kit (Miltenyi Biotec, Bergisch Gladbach, Germany). For this, 150 μ L of EVs samples were processed following the manufacturer's protocol. Briefly, 15 μ L of capture beads were added and samples were incubated O/N at 4 °C protected from light on an orbital shaker. Then 500 μ L of MACSPlex buffer were added to each tube and were centrifugated at 3,000x g for 5 min. After the centrifugation the supernatant was aspirated, leaving about 135 μ L of sample in the tube. 15 μ L from the kit-provided allophycocyanin-conjugated antibody mix targeting tetraspanins (CD63, CD81, and CD9) was employed and incubated for 1 h at RT in agitation. Once the incubation time finished, another 500 μ L of buffer were added and samples were centrifugated again. This last washing step was repeated twice and finally; samples were measured using BD FACSCelesta flow cytometer. Median fluorescence intensity was calculated for all 37 bead populations. The analysis of the samples was performed by subtraction of the control and the fluorescence controls. This method ensured that EVs attached to the antibody-conjugated beads were only detected if positive for CD9, CD63 or CD81.

10. ANALYSIS OF EVs miRNAs

10.1. RNA isolation from EVs

The total RNA extraction from the EVs was carried out using the Total Exosome RNA and Protein Isolation kit (Invitrogen, ThermoFisher Scientific). This kit is based on an organic extraction with a phenol-acid: chloroform solution and the use of silicon fiber columns that retain nucleic acids present in the aqueous phase. A volume of 2X Denaturing Solution, a high salt solution that denatures ribonucleases and other proteins that could degrade RNA, was added to 200 μ L of EVs suspension isolated from cell culture medium. The samples were then incubated for 5 min on ice, and 400 μ L of phenol-acid: chloroform were added to separate the total RNA from the rest of the cellular components in an aqueous phase and centrifugated at 10,000 g for 5 min at RT. The resulting aqueous phase was mixed with 1.25 volumes of ethanol 100%, vortexed and loaded onto a column provided by the kit. Then, several washing steps were performed with washing solutions buffers followed by centrifugation. Finally, with the aim of concentrate the small amount of RNA in EVs from cell culture media, RNA was eluted with 30 μ L of endonuclease-free water previously heated to 95 °C in two batches. Subsequently, the amount of RNA and its purity were quantified using the absorbance ratio of 260/280 with the NanoDrop 2000 spectrophotometer (ThermoFisher Scientific).

10.2. miRNA cDNA synthesis

TaqMan™ Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, Foster City, CA, USA) was employed to convert miRNAs into cDNA from 2 μ L of total RNA extracted from each sample. First, a poly-A tail was added to the 3' end of mature miRNAs for 45 min at 37 °C and 10 min at 65 °C employing Gene Amp 9700 thermocycler (Applied Biosystems). Then, an adapter sequence was ligated to the

5' end of mature miRNAs for 60 min at 16 °C. The reverse transcription of mature miRNAs to cDNA was performed using universal RT primers that recognize the expanded ends at 15 min at 42 °C and 5 min at 85 °C. Subsequently, the cDNA was amplified using the miR-Amp master mix (20X) and universal miR-Amp primers (20X) that recognize universal sequences to improve the detection of low-expression miRNAs. This process allows increasing the amount of cDNA while maintaining the relative levels of differential expression. The conditions in the thermocycler were 5 min at 95 °C, 14 cycles of 3 min at 95 °C and 30 s at 60 °C, and finally 10 min at 99 °C. Retrotranscription without RNA sample was carried out such as retrotranscription negative control. The cDNA obtained was diluted 1/10 and stored at -20 °C until use.

10.3. miRNAs quantification by RT-qPCR

To quantify the levels of miRNAs present in both pellet and exosomes from human immortalised podocytes, RT-qPCRs were carried out on 384-well plates. In this case, the Taqman Fast Advanced Master Mix (PCR universal master mix II, Applied Biosystems) was used, already prepared to be used with Taqman™ MicroRNA Assays probes (ThermoFisher Scientific). During the PCR reaction, the TaqMan probe hybridizes to the target sequence between the forward and reverse primers. As the DNA polymerase extends the primers, it also cleaves the TaqMan probe, which releases the reporter dye from the quencher dye. This separation of the dyes results in the fluorescence of the reporter dye, which can be detected in real-time by the PCR instrument. The reaction volumes were as follows: 7 µL of master mix, 0.5 µL of Taqman probe specific for each miRNA, and finally, 2.5 µL of cDNA diluted 1/10. Each experiment was performed in duplicate, and negative controls were included for both retrotranscription (without initial RNA) and RT-qPCR (without cDNA). The LightCycler 480 II system (Roche, Mannheim, Germany) was used following the TaqMan assays protocol (Table 8).

Table 8. RT-qPCR conditions for miRNAs TaqMan assays.

PCR step		Time	Temperature
Enzyme activation		10 min	95 °C
Amplification 40 cycles	Denaturalization	15 seg	95 °C
	Extension	1 min	60 °C

10.4. spike-in cel-miR-39 and ath-miR-159a normalisation analysis

The relative quantification of each miRNA under study was obtained by determining its normalised Ct value. The Ct values corresponding to each sample were obtained using the instrument software (LC 480 II SW v.1.5), with the threshold value being automatically set. Consensus regarding the use of a constitutive internal reference for normalising miRNA values quantified by RT-qPCR are still missing (Meyer 2010; Roberts 2014). Therefore, a normalisation procedure using the median of the spike-in was employed to normalize the data between samples (Mitchell *et al.* 2008). This procedure first quantifies the Ct value for cel-miR-39 and miR-159a in each individual sample, and then calculates the median Ct value for cel-miR-39 and miR-159a for all samples. Thus, the normalised Ct value for the miRNA of interest was calculated using the following formula:

Normalised Ct value for miRNA in the sample = initial mean Ct value - [(mean Ct value for cel-miR-39 and miR-159a) - (median Ct value for cel-miR-39 for all samples)]. Once this normalised Ct value was obtained, it was used in the calculation of $2^{(-\Delta\Delta Ct)}$, and the relative expression level of each miRNA between samples could be compared, expressed as fold-change.

11. PRESENTATION OF DATA AND STATISTICAL ANALYSIS

For clinical data, the values were expressed as the mean $\pm$ standard deviation for continuous variables, while for categorical variables, they were expressed as a percentage. Experimental data are expressed as fold change and mean $\pm$ standard deviation for n independent observations, as indicated in the figures. The Kolmogorov-Smirnov test was used to analyse the normal distribution of the variables. Comparisons between continuous variables from clinical characteristics, gene and protein expression level between two groups were analysed using Student's t test (data with normal distribution) or Mann-Whitney U test (data with non-normal distribution). In the case of categorical variables, contingency tables and Fisher's exact test were used. Pearson's and Spearman's correlation coefficients, depending on normality of data, were calculated to analyze the association between variables. The level of significance was set at $p < 0.05$. Statistical analyses were performed with SPSS software (version 20, Statistical Package for the Social Sciences), and graphical plots were created with SigmaPlot (Systat Software).

IV. RESULTS

“... and also the other claim that all this portion of the kidneys is fibrous and contains nothing but blood vessels is difficult to be accepted. In fact, in all the kidneys of whatever animal and of man himself I have happened to have in my hands so far, I have always noticed the presence of tiny corpuscles. To see these corpuscles the artery of the kidney has to be injected with a black liquid mixed with the spirit of wine until the whole kidney swells and the most external part of it turns black. Once the capsule of the kidney is removed, even by the naked eye one can see ... the small corpuscles which have turned black, hanging from the black vessels; by sectioning the kidney longitudinally, one can see among the tubules and the interstitium a large number of these corpuscles, hanging like apples from the blood vessels which, swollen with the black liquid look like a beautiful tree”.

De Renibus-1666

Marcello Malpighi

1. CLINICAL CHARACTERISTICS OF THE STUDY COHORT

The cohort employed in the study included a total of 64 hypertensive patients, 30 of them diagnosed with T2DM. Patients were stratified in four groups based on the UAE levels and the presence or not of T2DM as described in the methodology section. Patient's clinical characteristics are shown in Table 9.

When considering gender distribution, higher percentage of males were observed in the DM groups (75.0% and 77.8%) compared to the non-DM groups (64.3% and 60.0%). Blood Pressure, indicated by Systolic Blood Pressure (SBP) and Diastolic Blood Pressure (DBP), did not show differences displayed based on DM or UAE status. As expected, glucose levels (mg/dL) and glycated hemoglobin percentages revealed that individuals with T2DM generally exhibited higher values. Specifically, DM individuals with increased UAE demonstrated mean glucose levels of 154.150 mg/dL, while those without UAE had levels at 149.11 mg/dL. The lipid profile displayed significant differences between DM and non-DM groups. Total cholesterol and LDL levels were notably higher in individuals with DM, particularly in the group with increased UAE. HDL levels were lower in the DM groups compared to non-DM groups, and TG levels were significantly elevated in the DM group with increased UAE. BMI (Body Mass Index) values, reflecting weight in relation to height (kg/m^2), were highest in the DM group with increased UAE, indicating a higher prevalence of obesity ($\text{BMI} \geq 30$) in this subgroup. Finally, the ratio of UAE to creatinine (mg/g) was significantly higher in individuals with T2DM and increased UAE (307.3 mg/g) compared to all other groups. This indicates a greater level of UAE in this subgroup, which signify potential kidney-related complications.

Table 9. Clinical characteristics of patient groups.

Variables	No DM		DM	
	Increased UAE (n = 14)	No UAE (n = 20)	Increased UAE (n = 20)	No UAE (n = 10)
Age (years)	50.79 ± 9.27	54.45 ± 6.04	60.30 ± 9.33	54.78 ± 4.15
Gender (male)	64.3 %	60.0 %	75.0 %	77.8 %
SBP (mmHg)	130.79 ± 10.59	134.05 ± 18.55	143.05 ± 23.61	140.78 ± 34.06
DBP (mmHg)	82.50 ± 8.86	88.00 ± 13.53	82.95 ± 17.10	89.00 ± 18.04
Glucose (mg/dL)	97.43 ± 17.46	103.65 ± 9.51	154.150 ± 58.49†‡	149.11 ± 59.53§
HbA1c (%)	5.75 ± 0.07	5.62 ± 0.21	7.15 ± 1.17†	6.47 ± 1.05§
Total cholesterol (mg/dL)	204.86±35.98†	184.00±22.66	184.60 ± 31.14**	150.67±25.18§
LDL (mg/dL)	133.43 ± 30.13	116.85 ± 19.74	114.05 ± 27.92*	86.00 ± 20.58§
HDL (mg/dL)	56.00 ± 13.80	51.30 ± 10.85	42.20 ± 10.68	43.67 ± 8.60
TG (mg/dL)	113.86 ± 44.12	121.40 ± 57.47	244.2 ± 194.3‡	159.0 ± 65.02
Plasma creatinine (mg/dL)	0.91 ± 0.34	0.85 ± 0.17	1.00 ± 0.41	0.99 ± 0.27
GFR (mL/min/1.7m ²)	92.04 ± 29.28	89.58 ± 16.97	86.74 ± 30.23	82.81 ± 23.51
BMI (kg/m ²)	29.46 ± 5.00	30.60 ± 6.49	35.10 ± 7.57	29.51 ± 4.66
Obesity (%)	42.9	40.0	65.0	44.4
Obesity grade (%)				
Grade I	28.6	20.0	15.0	22.2
Grade II	14.3	5.0	25.0	22.2
Grade III	-	15.0	25.0	-
Dyslipidemia (%)	85.7	75.0	95.0	100
Smoking (%)	57.1	40.0	25.0	55.6
Ex-Smoking (%)	35.7	15.0	15.0	22.2
Ratio UAE/ Creatinine (mg/g)	221.7 ± 229.7+++	4.84 ± 6.45	307.3 ± 228.8†† ***	3.03 ± 1.48

Data are expressed as mean ± SD, or percentage of patients (%). BMI: body mass index; DBP: diastolic blood pressure; DM: diabetes mellitus; GFR: glomerular filtration rate; HbA1c: Glycated hemoglobin SBP: systolic blood pressure; TG: triglycerides; UAE: urinary albumin excretion. Comparisons between DM groups: * p < 0.05, ** p < 0.01, *** p < 0.001. Comparisons between DM and non-DM: † p < 0.05; †† p < 0.01; ††† p < 0.001. Comparisons between increased UAE groups: ‡ p < 0.05, ‡‡ p < 0.01. Comparisons between No UAE groups: § p < 0.05.

2. LEVELS OF THE RAB3A-RAB27A SYSTEM IN URINE SAMPLES FROM PATIENTS WITH HTN AND CORRELATIONS WITH UAE

We analysed the levels of mRNA of Rab GTPases *RAB3A* and *RAB27A* in urine pellets of hypertensive patients with and without DM and/or increased UAE. Higher *RAB3A* (2.17-fold increase, $p < 0.01$) and *RAB27A* mRNA levels (1.53-fold increase, $p < 0.05$; Fig. 14A and C) were observed in the group of patients with elevated UAE than in the non-albuminuric group. In addition, when we compared our four groups of study, *RAB3A* levels were significantly elevated in both albuminuric groups, with and without DM (2.56- and 2.34-fold increases, $p < 0.01$ and $p < 0.05$, respectively; Fig. 14B), while for *RAB27A* this difference was only significant in the group of patients with DM, being higher in the group of patients with DM and elevated UAE (2.25-fold increase, $p < 0.05$; Fig. 14D).

Moreover, we found significant associations between the levels of both mRNA with UAE. UAE levels in patients were positively correlated with *RAB3A* ($r = 0.515$, $p < 0.05$) and *RAB27A* mRNA levels ($r = 0.475$, $p < 0.05$) (Fig. 14E and F).

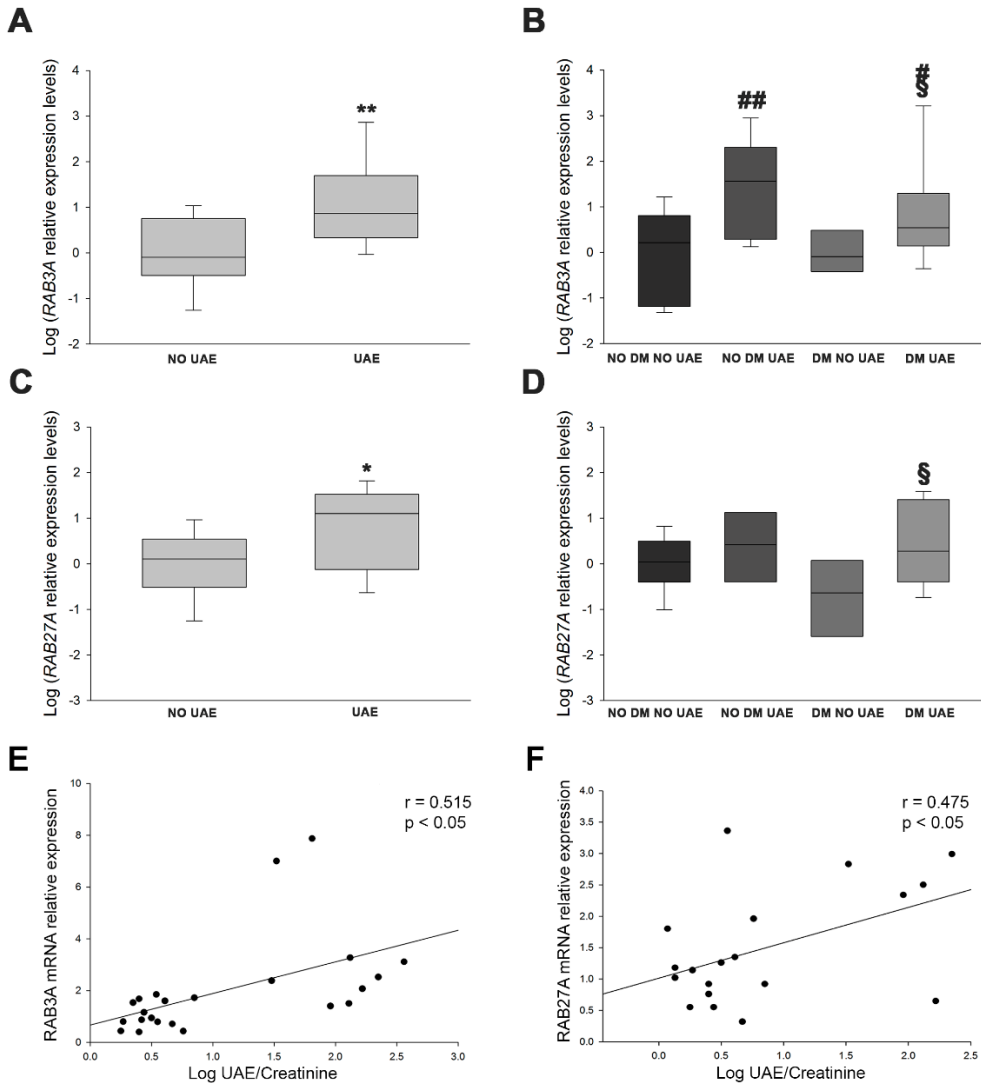


Figure 14. Urinary levels of Rab GTPases in hypertensive patients. **A** and **B**: Box plots of mRNA levels of *RAB3A* in patients with ($n = 34$) and without UAE ($n = 30$) or divided into four groups. **C** and **D**: Box plots of mRNA expression levels of *RAB27A* in patients with with ($n = 34$) and without ($n = 30$) increased UAE or divided into four groups. **E**: Correlation between mRNA levels of *RAB3A* in patients with UAE. **F**: Correlation between mRNA levels of *RAB27A* in patients with UAE. Horizontal lines represent medians and box limits interquartile range. DM, diabetes mellitus. * $p < 0.05$ and ** $p < 0.01$ vs. the no UAE group; ## $p < 0.05$ and ### $p < 0.01$ vs. the no DM, no UAE group; \$ $p < 0.05$ vs. the DM, no UAE group. UAE, urinary albumin excretion.

3. CHARACTERISATION OF DIFFERENTIATED HUMAN IMMORTALISED PODOCYTES AND PODOCYTE ASSOCIATED MOLECULES

To start with the *in vitro* cell culture model, we first set up the conditions to proliferate and differentiate human immortalised podocytes. Optical images from cultured podocytes were acquired in proliferative and differentiated state (Fig.15).

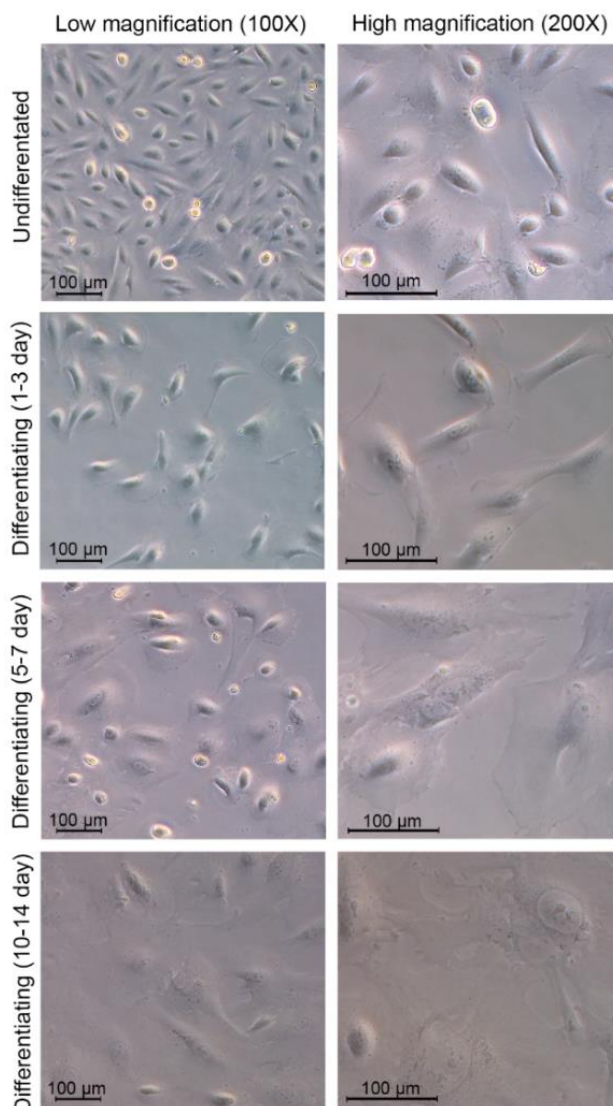


Figure 15. Study of cell morphology. Optical images at low ($\times 10$) and high ($\times 20$) magnifications of undifferentiated podocytes and at different stages of differentiation.

As described previously in literature and as shown in Figure 15, at the morphological level, proliferating podocytes showed small cell bodies and an epithelial-like cobblestone shape when cultured at 33 °C (undifferentiated state). After thermoswitching to 37 °C for 10–14 days, cells changed their phenotype into arborised cells, characterised by enlarged cell bodies with FP and spindle-like projections and junctions.

It has been described that mature podocytes express several specific proteins for which mRNA and protein levels of podocyte-specific markers were measured: synaptopodin, nephrin, WT-1 and CD2AP. Synaptopodin and nephrin mRNA levels were higher in differentiated podocytes (4.96-fold increase, $p < 0.001$, and 2.12-fold increase, $p < 0.05$, respectively), while *WT-1* levels showed a downstrain trend (2.58-fold decrease, $p < 0.001$), and *CD2AP* had no significant changes compared with undifferentiated podocytes (Fig. 16A).

At protein level, it was found that differentiated podocytes exhibited higher protein levels compared to proliferating podocytes in the podocyte-associated molecules synaptopodin (with a 2.49-fold increase, $p < 0.01$), WT-1 (with a 1.47-fold increase, $p < 0.001$), nephrin (with a 1.49-fold increase, $p < 0.05$), and CD2AP (with a 1.20-fold increase, $p < 0.05$) (Fig. 16B). Furthermore, when analysing the levels of these markers at different days of differentiation, it was observed that all of them generally increased over time (Fig. 16C).

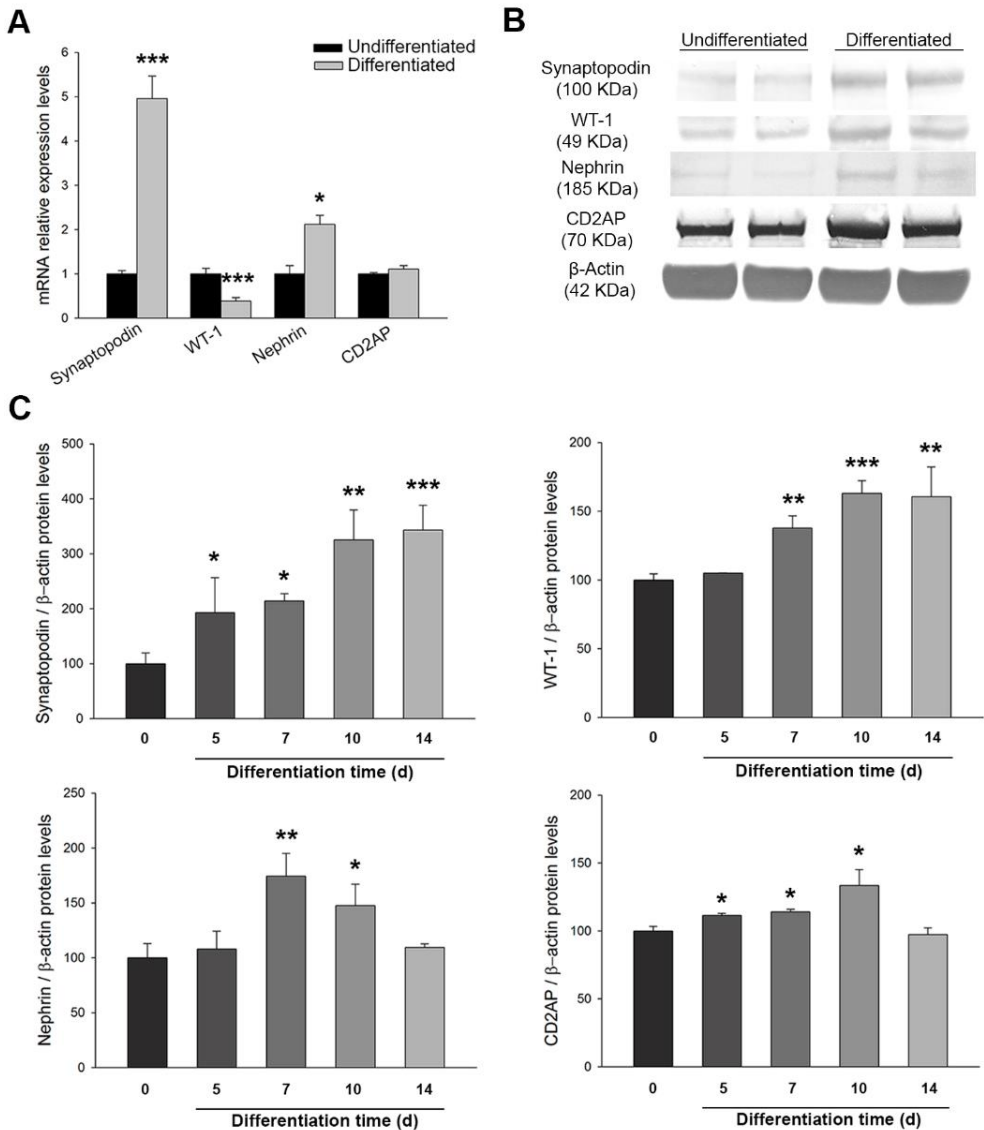


Figure 16. Analysis of human immortalised podocyte associated markers. **A:** Relative mRNA expression levels of podocyte markers at differentiation stages **B:** Western blots of undifferentiated and differentiated podocyte markers. **C:** Bar graphs representing protein levels of podocyte markers comparing undifferentiated and differentiated podocytes at different differentiation days. Data are expressed as means $\pm$ SEM; $n = 8$ for each group. Differentiation time is expressed in days (d). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. the undifferentiated group. WT-1, Wilms' tumor-1; CD2AP, CD2-associated protein.

4. EXPRESSION OF RAB3A-RAB27A SYSTEM IN UNDIFFERENTIATED AND DIFFERENTIATED PODOCYTES

The presence and differential expression of the Rab3A, Rab27A and Rabphilin3A was analysed in human immortalised podocytes cell line. Firstly, we demonstrated that Rab3A, Rab27A and Rabphilin proteins were present in our cell line, indeed, differentiated cells showed a reduction in protein levels of Rab3A (1.22-fold decrease, $p < 0.05$), Rab27A (1.25-fold decrease, $p < 0.05$), and Rabphilin3A (1.30-fold decrease, $p < 0.01$; Fig. 17A).

Additionally, when *RAB3A* and *RAB27A* mRNA levels were analysed in undifferentiated and differentiated cells, we found that mRNA levels of these GTPases were higher in differentiated podocytes (1.75-fold increase, $p < 0.01$, and 1.66-fold increase, $p < 0.05$, respectively; Fig. 17B).

Regarding the *RPH3A* gene, we conducted a seminested PCR procedure utilising two sets of primers to ascertain its presence in human immortalised podocytes, as previously documented by Rastaldi's group (Rastaldi *et al.* 2003) (Fig. 17C). Using capillary electrophoresis, we effectively identified *RPH3A* amplicons in both undifferentiated and differentiated podocytes, allowing the determination and quantification of fragment length. The images show clear bands and peaks without any other fragment contamination (Fig. 17D).

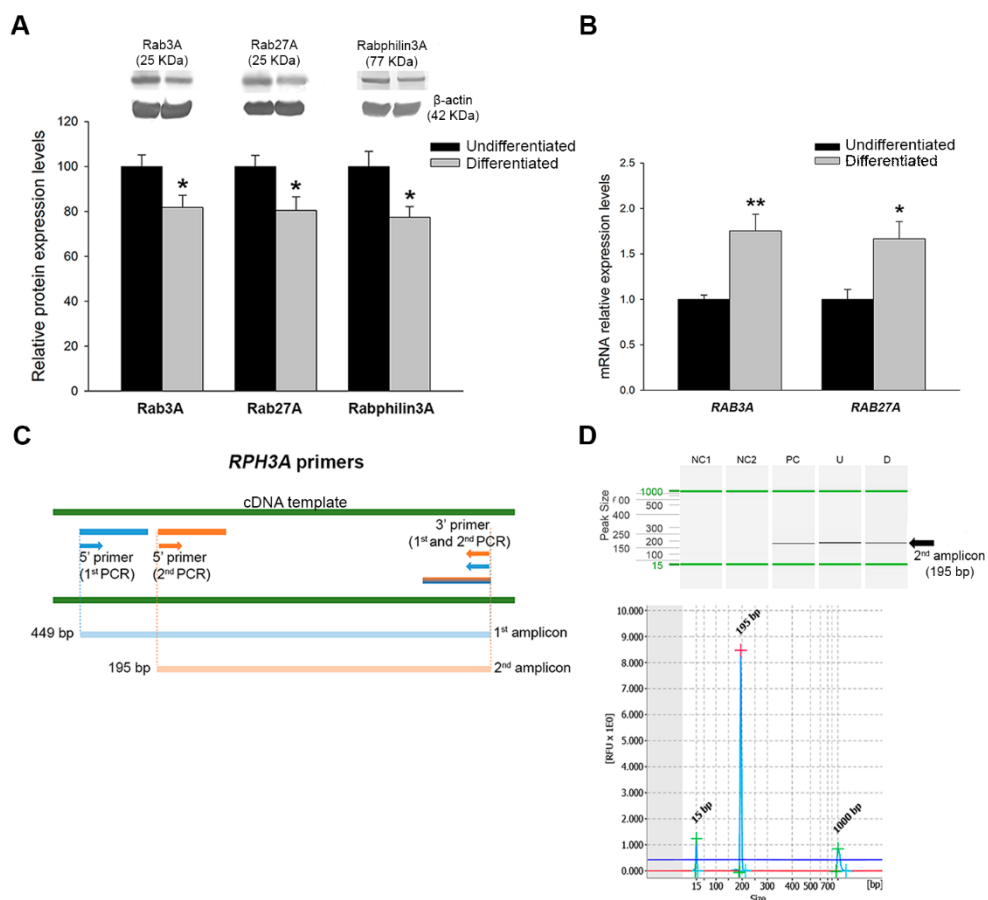


Figure 17. Analysis of Rab GTPases in human immortalised podocytes. **A:** Protein levels of Rab3A, Rab27A, and Rabphilin3A in differentiated podocytes compared with undifferentiated podocytes. Data are expressed as means $\pm$ SEM; $n = 8$ each group. **B:** Relative mRNA expression levels of *RAB3A* and *RAB27A* genes at differentiation stage compared with undifferentiated cells. **C:** *RABPHILIN3A* gene (*RPH3A*) primer set for seminested PCR experiments. **D:** Capillary electrophoresis software capture of fragments belonging to the second PCR amplicon of *RPH3A*. NC1, negative control of the first PCR; NC2, negative control of the second PCR; PC, positive control neuronal cell line; U, undifferentiated podocyte; D, differentiated podocyte; RFU, relative fluorescence units. * $p < 0.05$ and ** $p < 0.01$ vs. the undifferentiated group.

5. IMPACT OF HG AND ANG-II IN HUMAN PODOCYTES

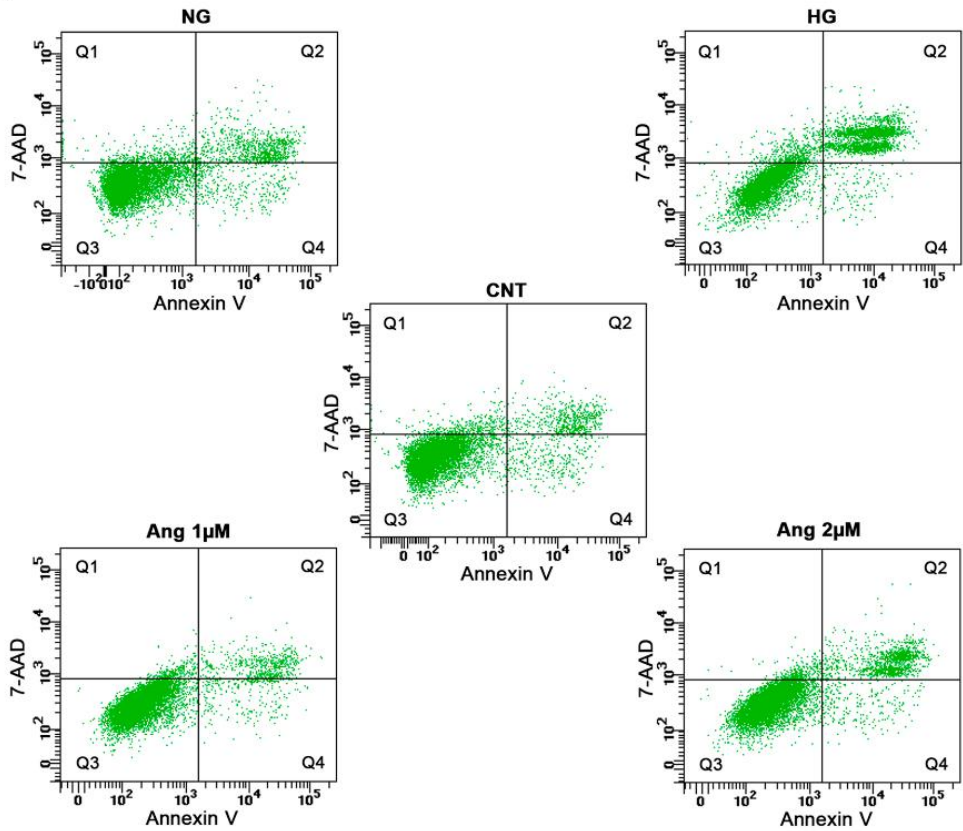
5.1. Podocyte apoptosis

To analyze the effect of treatments in our *in vitro* model, apoptosis was studied using different approaches. Firstly, flow cytometry analyses showed an apoptosis increase in HG compared with NG (22.4% vs. 13.9%, $P < 0.05$). A similar trend, reflecting increased apoptosis, was observed under a treatment with 2 μ M Ang-II (12.3% vs. 9.6%, Fig. 18).

Moreover, microscopic techniques were also employed to study podocyte apoptosis. On one hand, podocyte apoptosis studied by bright field optical microscopy revealed that both treatments, HG and Ang-II, induced typical apoptosis morphological alterations in cells, such as membrane blebbing, cell volume reduction, and rounding (Fig. 19A and B).

On the other hand, DAPI serves as a dye that can be employed as a tool for visualising alterations in the nucleus and evaluating apoptosis. When it binds to DNA, DAPI exhibits a fluorescence intensity roughly 20 times greater than that of unbound DAPI. This fluorescence is directly correlated with the quantity of DNA present. As the membrane of an apoptotic cell becomes compromised, more DAPI penetrates the cell, resulting in a more compact and pronounced blue staining. The distinct nuclear characteristics of apoptotic cells, such as chromosome condensation and fragmentation, also aid in the visual recognition of cells undergoing apoptosis. Furthermore, apoptotic cells stained with DAPI may display discernible nuclear blebbing, which can be instrumental in distinguishing them from necrotic cells, which do not exhibit this feature. Confocal microscopy analysis revealed that podocytes treated with HG and Ang-II exhibited different parameters indicative of apoptosis as described (Fig. 20A and B).

A



B

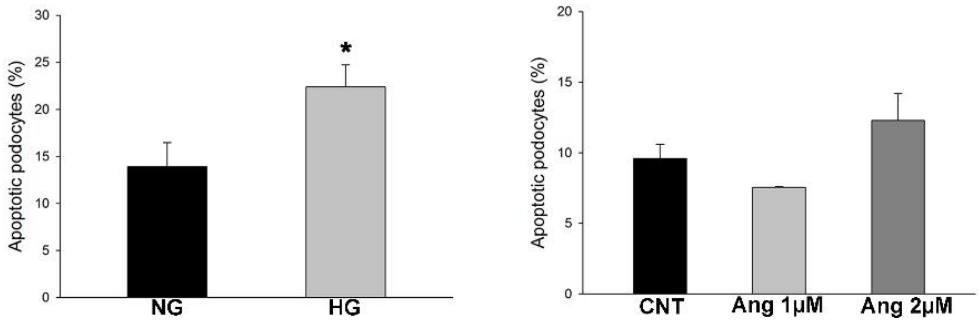
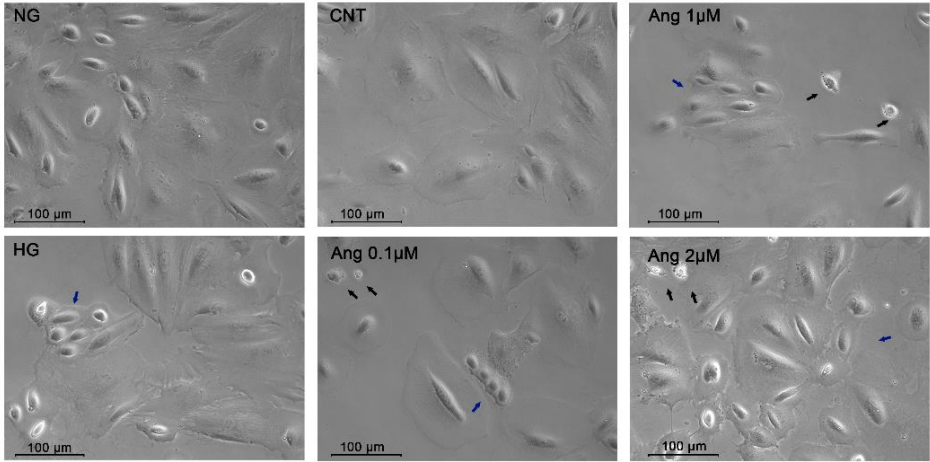


Figure 18. Podocyte apoptosis during glucose overload and Ang-II treatment. **A:** Flow cytometry images of annexin V/7-amino-actinomycin D (7-AAD) double staining-labeled cells (n = 3). **B:** Graphs of the analysis performed. The cytograms of early apoptotic cells, positive for annexin V but with still intact cell membranes (7-AAD negative), are shown in quadrant 4 (Q4). Cells with advanced stages of apoptosis or necrosis were both annexin V and 7-AAD positive and are shown in the quadrant 2 (Q2). Viable cells that did not bind annexin V or 7-AAD are depicted in quadrant 3 (Q3). Cells with lost intact cell membranes that bound 7-AAD and excluded annexin V are shown in quadrant 1 (Q1). * p < 0.05.

A



B

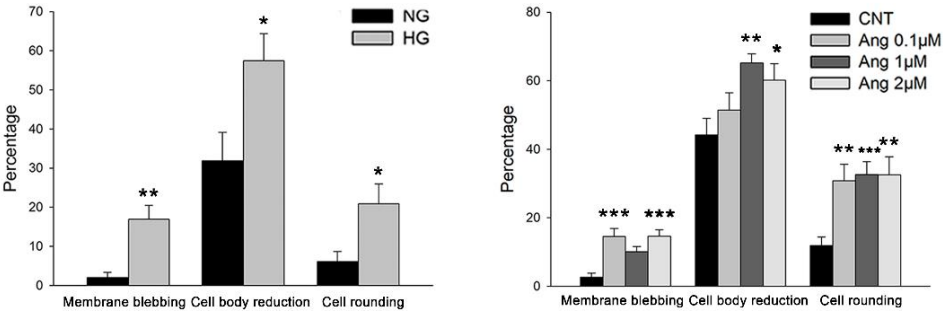


Figure 19. Morphological changes of podocytes subjected to glucose and Ang-II. **A:** Representative optical images showing morphological changes of podocytes subjected to HG and Ang-II treatments. Black arrows show membrane blebbing; blue arrows show cell body reduction and rounding. **B:** Bar graphs representing the quantification of podocyte morphological parameters under treatments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Ang-II, angiotensin-II; NG, normal glucose; HG, high glucose; CNT, control.

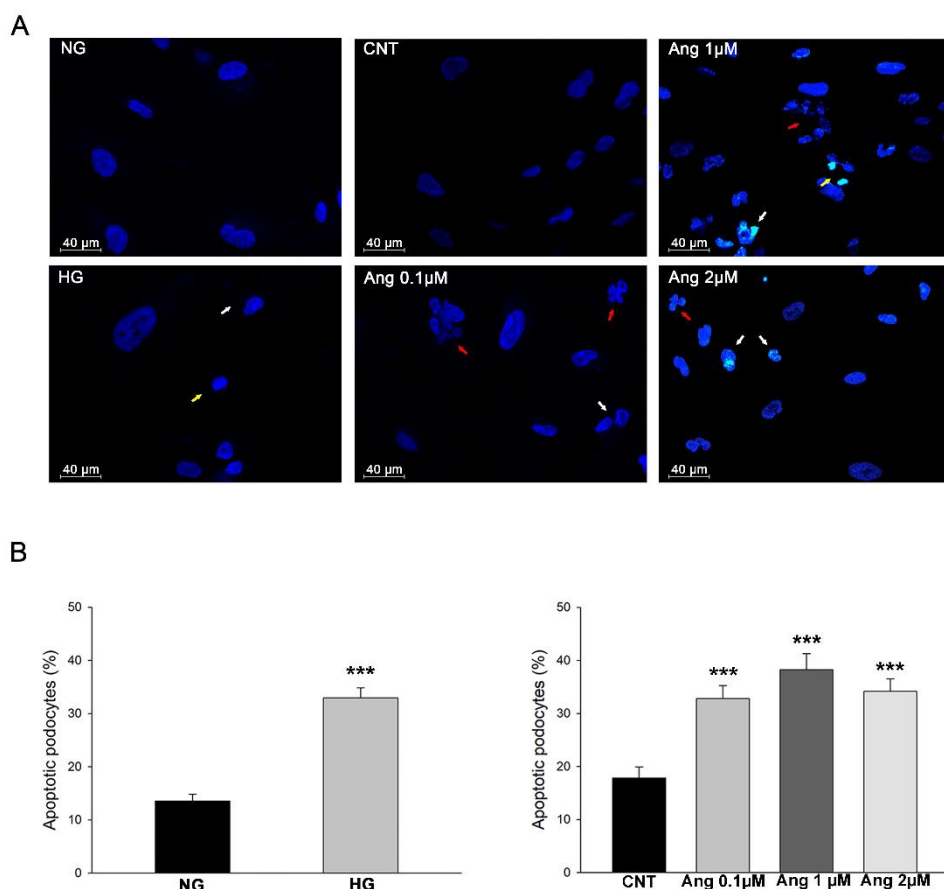


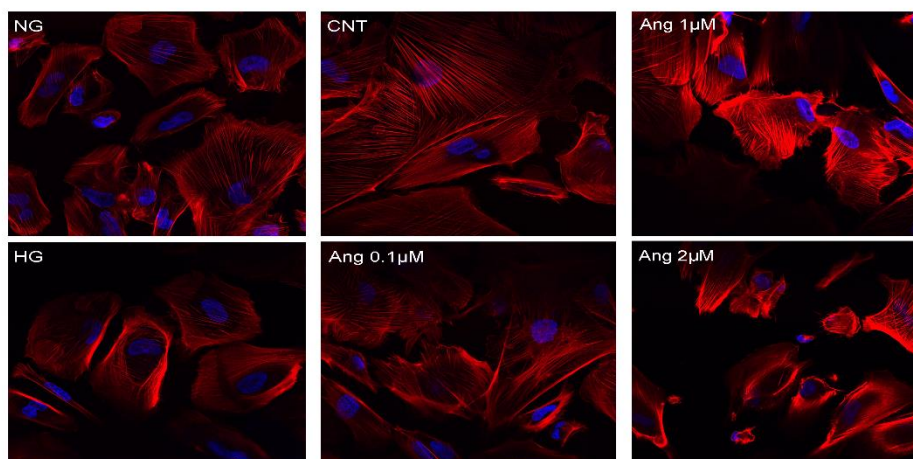
Figure 20. Analysis of apoptosis by confocal microscopy fluorescence analysis. A: Confocal images of DAPI staining showing nuclei alterations in apoptotic cells subjected to HG and Ang-II treatments. White arrows show chromatin condensation, yellow arrows show nuclear condensation, and red arrows show nuclear fragmentation. **B:** Bar graphs of percentage of apoptotic cells under NG and HG (left) and Ang-II treatments (right). Data are expressed as means $\pm$ SE. Scale bars = 40 μ m. * p < 0.05, ** p < 0.01, and *** p < 0.001 vs. the NG or CNT group. Ang-II, angiotensin-II; NG, normal glucose; HG, high glucose; CNT, control.

5.2. Podocyte actin cytoskeleton rearrangements

As emphasised throughout this doctoral thesis, podocyte cytoarchitecture plays an important function in the podocyte biology, which is why we decided to study the proper distribution of F-actin fibers by confocal microscopy. F-actin fiber identification by rhodamine phalloidin staining was also performed in podocytes treated with HG and Ang-II. In untreated cells, F-actin was distributed

homogenously in bundles along the podocyte. The HG and Ang-II treatments led to cytoskeleton rearrangements, manifesting as disorganised fibers, as indicated by the anisotropy measurements conducted, whose values decreased up to 60% in podocytes subjected to both treatments. Additionally, we noted an increased clustering of F-actin fibers in the peripheral regions of the treated podocytes after both treatments (Fig.21).

A



B

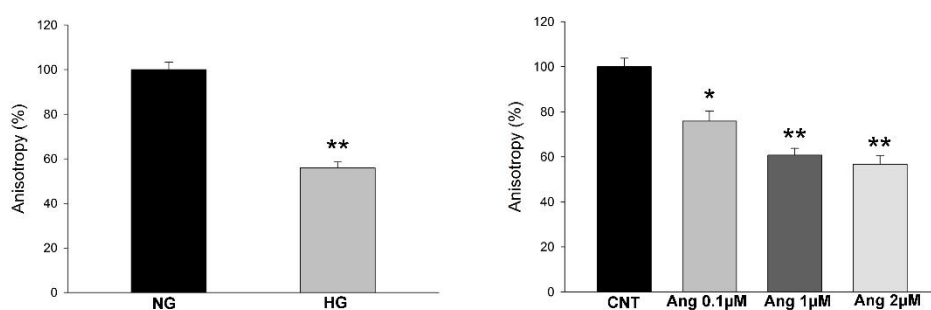


Figure 21. Cytoskeleton rearrangements in HG and Ang II-treated podocytes. A: Confocal images showing F-actin fibers organization in glucose- and Ang II-treated podocytes compared to CNTs. B: Bar graphs representing the anisotropy values displayed by F-actin fibers (n=3). CNT values were set to 100. * $p < 0.05$, ** $p < 0.01$, vs. the CNT group. Ang-II, angiotensin-II; NG, normal glucose; HG, high glucose; CNT, control.

5.1. Podocytes permeability assesment under HG conditions

Given the role that podocytes play in the filtration process within the glomerulus, attributed in part to the formation of the GFB through interdigitations between their pedicels, we aimed to investigate the filtration capacity of podocytes exposed to HG in an *in vitro* static model. We observed that HG treatment increased the permeability to BSA-FITC. Both the 48 h and 72 h treatments resulted in a reduction of the cells' filtration capacity, allowing the passage of BSA-FITC to the upper chamber of the transwell employed. Notably, the reduction in filtration was more pronounced after 72 h of treatment compared to 48 h (Fig. 22).

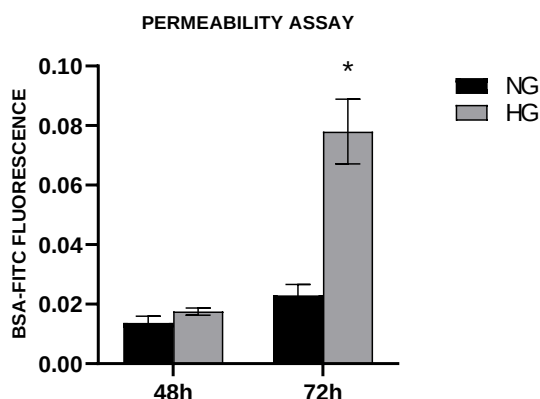


Figure 22. Podocyte permeability assay. The passage of FITC-BSA through the GFB in the static model was evaluated in HG treated podocytes at 48 and 72 h. Data were normalised to FITC-BSA (FITC-BSA solution = 1 mg/mL as 100% absorbance) (n=4). *p < 0.05 vs. the NG group at 72h. GFB, glomerular filtration barrier; NG, normal glucose; HG, high glucose.

5.2. Podocyte-specific markers and Rab3A-Rab27A system levels under glucose overload

To inquire into the possible role that the Rab system may play in glucose and Ang-II-induced damage, both, protein and mRNA levels of podocyte-associated molecules and Rab3A-Rab27A system components were measured. When we

examined mRNA levels under HG, there were no statistically significant differences in the expression of podocyte-associated molecules such as synaptopodin, *WT-1*, nephrin, and *CD2AP*. Interestingly, the Rab3A-Rab27A system exhibited alterations. Specifically, *RAB3A* demonstrated lower levels (1.51-fold decrease, $p < 0.05$), whereas *RAB27A* displayed higher levels (1.27-fold increase, $p < 0.05$) in the HG group (Fig. 23A). Nested PCR of the *RPH3A* gene in podocytes subjected to glucose overload confirmed its presence in treated podocytes. However, while the correct amplicon was detected via capillary electrophoresis, quantification of mRNA levels by RT-qPCR was not feasible (Fig. 23B).

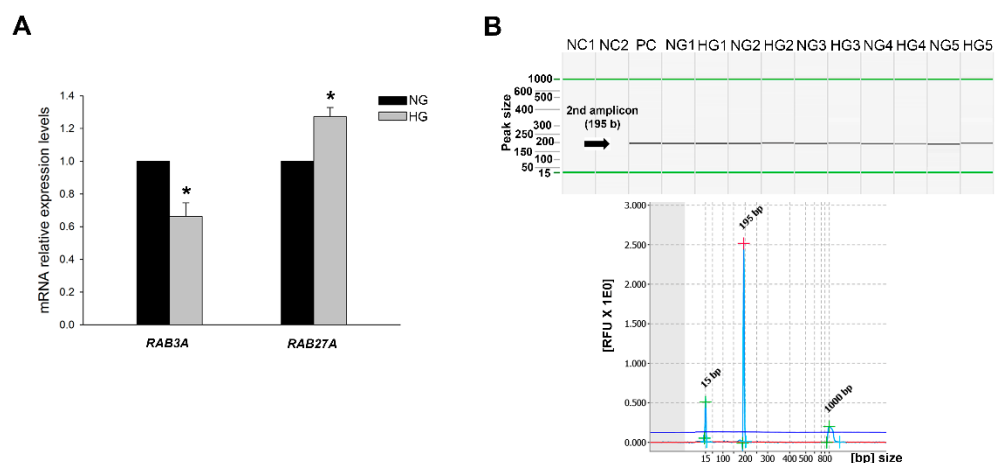


Figure 23. Rab GTPases gene expression under glucose treatment. **A:** mRNA levels of Rab GTPases under glucose treatments. **B:** Capillary electrophoresis capture of Rabphilin3A gene (*RPH3A*) amplicon under glucose treatments. Data are expressed as means $\pm$ SEM; $n = 5$ each group. * $p < 0.05$ and vs. the NG group. HG, high glucose; NC1, negative control of the first PCR; NC2, negative control of the second PCR; NG, normal glucose; PC, positive control neuronal cell line.

In relation to protein alterations, we observed diminished levels of the podocyte markers WT-1 (decreased by 1.20-fold, $p < 0.05$), CD2AP (1.79-fold decrease, $p < 0.01$), and nephrin (1.99-fold decrease, $p < 0.05$), with synaptopodin showing a slight decrease that was not statistically significant (1.19-fold decrease, $p > 0.05$) (Fig. 24A). These findings were further supported by immunofluorescence

analyses. Specifically, under NG conditions, synaptopodin exhibited a cytoplasmic distribution and was present throughout the podocyte FP, while nephrin showed a perinuclear localization. In contrast, under HG conditions, synaptopodin displayed a distinct reorganised pattern absent in NG conditions, and nephrin exhibited enhanced cytoplasmic localization with diminished perinuclear expression; overall, its protein levels were lower compared to the NG conditions (Fig. 24B).

Remarkably, the protein expression levels of Rab GTPase proteins showed significant alterations in response to HG conditions. Specifically, Rab3A and Rab27A exhibited enhanced protein levels (1.39-fold and 1.27-fold increase, respectively, $p < 0.05$), while Rabphilin3A demonstrated a decrease (1.54-fold decrease, $p < 0.01$; Fig. 24C). In immunofluorescence experiments, Rab3A, Rab27A, and Rabphilin3A displayed the expected cytoplasmic and membrane-associated dot-like expression patterns characteristic of vesicular proteins. These patterns extended to the FP, and their levels correlated with those observed in Western blot analysis. Interestingly, under HG conditions, Rab27A exhibited a shift in its expression pattern compared to NG conditions, displaying heightened levels in proximity to the podocyte plasma membrane (Fig. 24D and E).

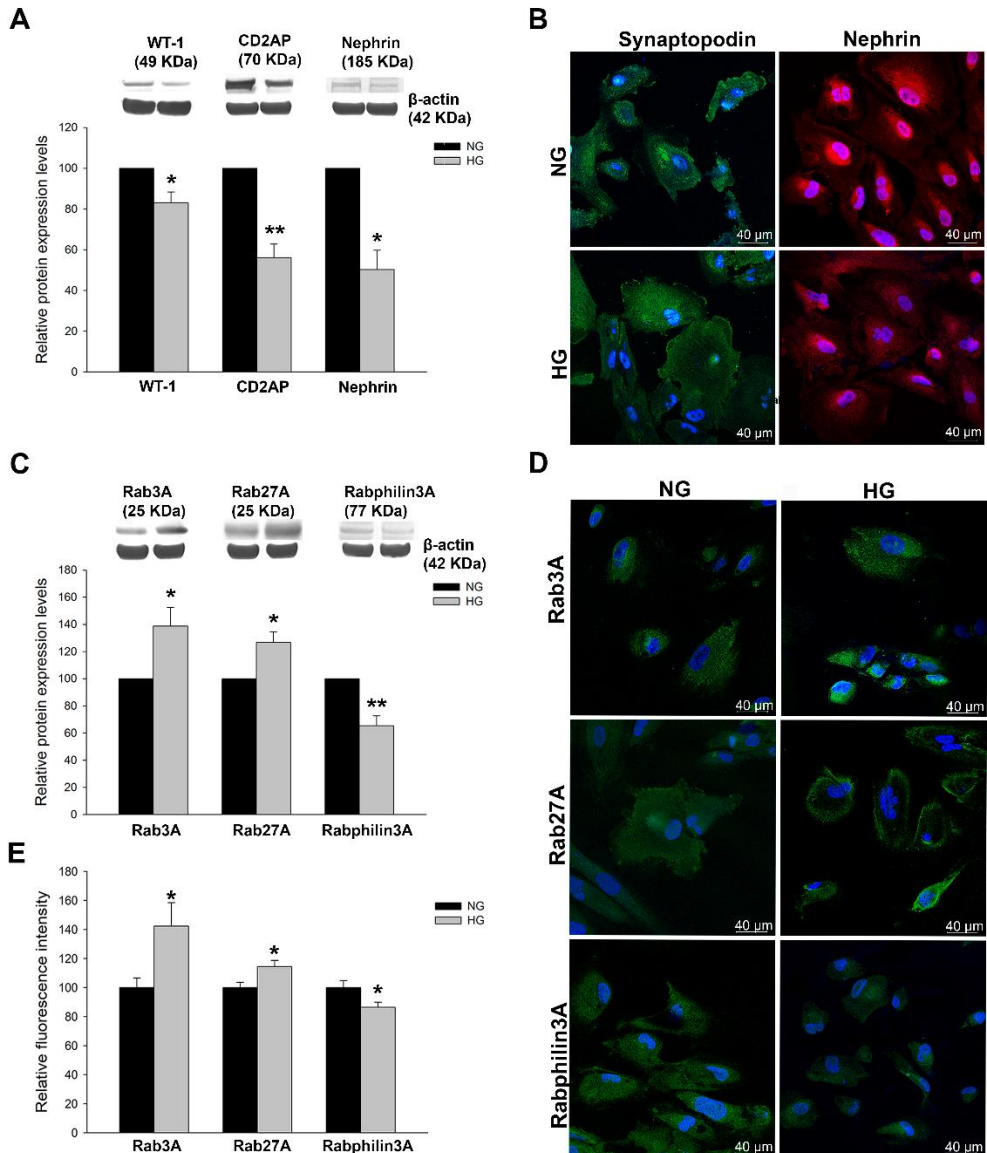


Figure 24. Podocyte markers and Rab GTPases expression under HG treatment. **A:** Protein levels of podocyte markers under NG and HG conditions. **B:** Confocal fluorescence microscopy images of podocyte markers staining in treated podocytes. **C:** Protein levels of Rab GTPases under NG and HG treatments. **D:** Confocal images of Rab GTPases in NG- and HG-treated podocytes. **E:** Fluorescence quantification in NG and HG treatments of Rab GTPases. Data are expressed as means $\pm$ SEM; $n = 5$ each group. Blue DAPI staining shows nuclei. Scale bars = 40 μ m. * $p < 0.05$ and ** $p < 0.01$ vs. the NG group. NG, normal glucose, HG, high glucose, WT-1, Wilms' tumor-1; CD2AP, CD2-associated protein.

5.3. Podocyte markers and Rab3A-Rab27A system levels under Ang-II treatment

With the addition of Ang-II, mRNA quantification revealed higher levels of *WT-1* (5.40-fold increase, $p < 0.05$) and *CD2AP* (1.41-fold increase, $p < 0.05$) only at the lowest Ang-II concentration (Fig. 25A). Likewise, *RAB3A* and *RAB27A* mRNA levels were increased (Fig. 25B).

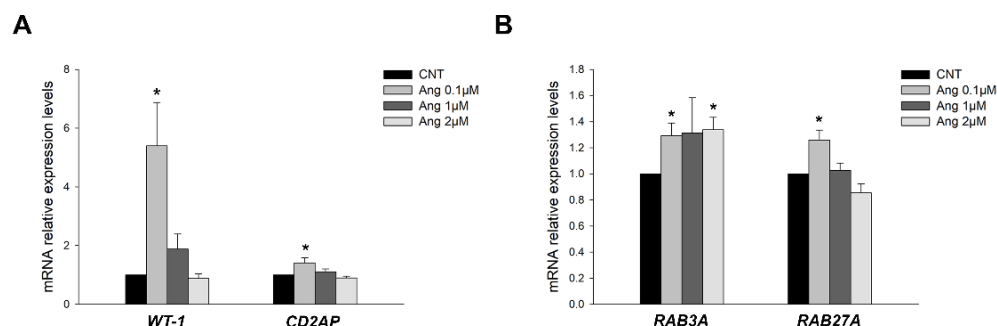
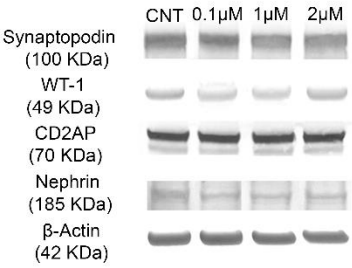


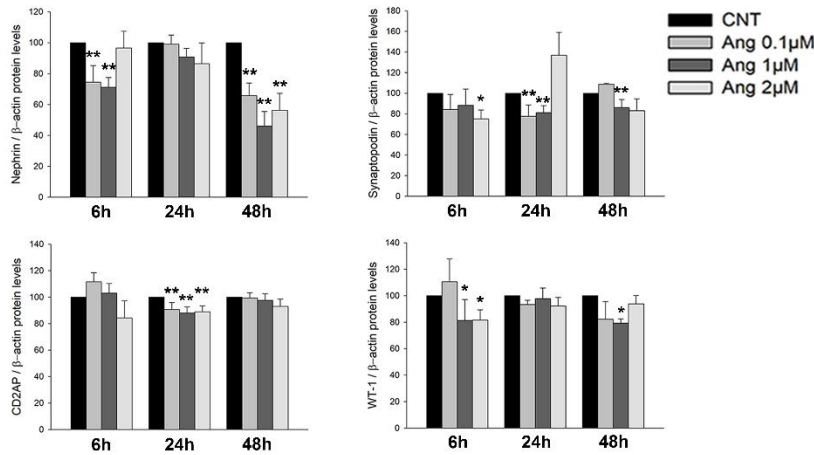
Figure 25. Podocyte markers and Rab GTPase expression under Ang-II treatment. **A:** mRNA levels of podocyte markers under Ang-II treatments. **B:** mRNA levels of Rab GTPases under Ang-II treatment. Data are expressed as means $\pm$ SEM; $n = 5$ each group. * $p < 0.05$ and ** $p < 0.01$ vs. the CNT group. CNT, control; WT-1, Wilms' tumor-1; CD2AP, CD2-associated protein.

At the protein level, the podocyte markers synaptopodin, WT-1, nephrin, and CD2AP showed generally lower levels with Ang-II increased concentrations (Fig. 26A and B). These observations were also reflected in immunofluorescence experiments, showing reduced synaptopodin expression and a rearranged pattern, and lower levels of nephrin, with less perinuclear staining (Fig. 26C).

A



B



C

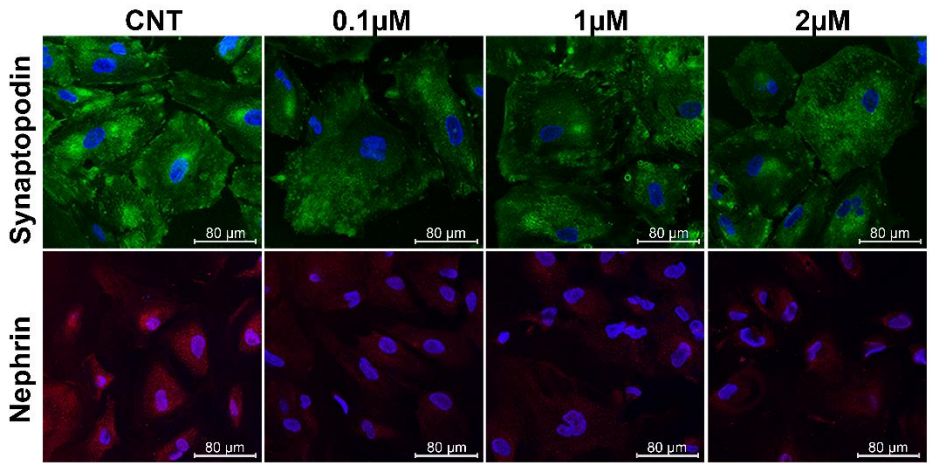


Figure 26. Podocyte markers under Ang-II treatment. A: Representative membranes of podocyte markers. B: Protein levels of podocyte markers in Ang-II treatments. C: Confocal images of podocyte markers synaptopodin and nephlin staining. Data are expressed as means ± SEM; n = 5 each group. CNT group values were set to 100. Blue DAPI staining shows nuclei. Scale bars = 80 μm. *p < 0.05 and **p < 0.01 vs. the CNT group. CNT, control; WT-1, Wilms' tumor-1; CD2AP, CD2-associated protein.

The Rab proteins, specifically Rab3A, Rab27A, and its effector Rabphilin3A, exhibited primarily elevated levels of expression during the treatments. Moreover, these expressions demonstrated variations based on both the dosage of Ang-II administered and the duration of the treatments (Fig. 27A and B).

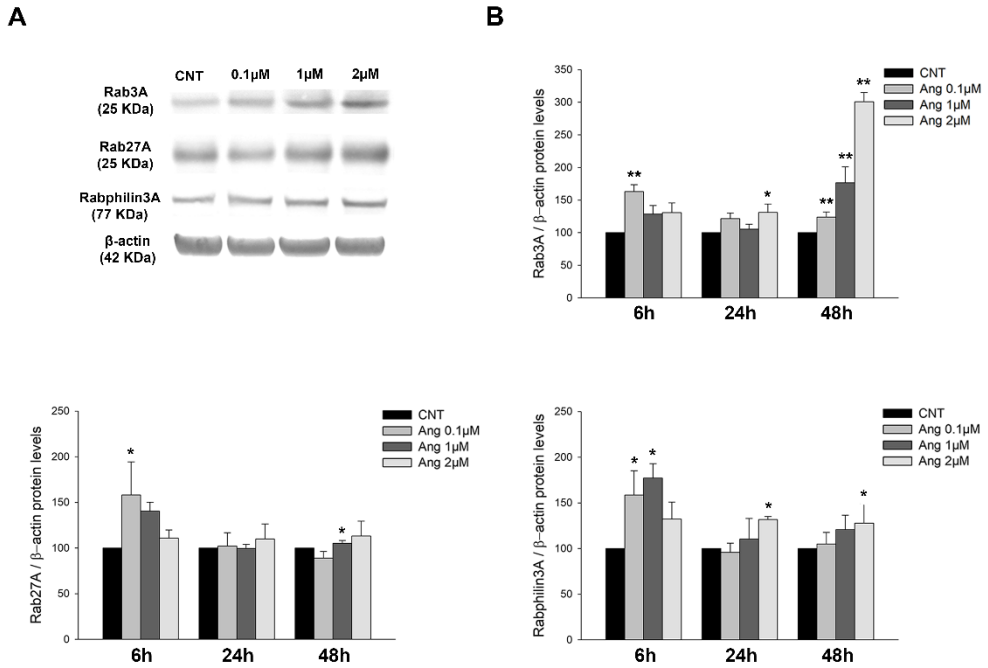


Figure 27. Rab GTPase expression under Ang-II treatment. **A:** Representative Western blots of Rab GTPase levels. **B:** Protein levels of Rab GTPases in CNT and increasing concentrations of Ang-II at 6, 24, and 48 h of treatment. Data are expressed as means $\pm$ SEM; $n = 5$ each group. * $p < 0.05$ and ** $p < 0.01$ vs. the CNT group.

After the Ang-II treatment, podocytes underwent immunostaining, and subsequent fluorescence analysis revealed similar changes consistent with alterations at the protein level. This was evidenced by heightened signals for Rab3A, Rab27A, and Rabphilin3A in response to Ang-II stimulation. Notably, Rab27A exhibited an increase in its expression on the cellular membrane in response to Ang-II treatment (Fig. 28A).

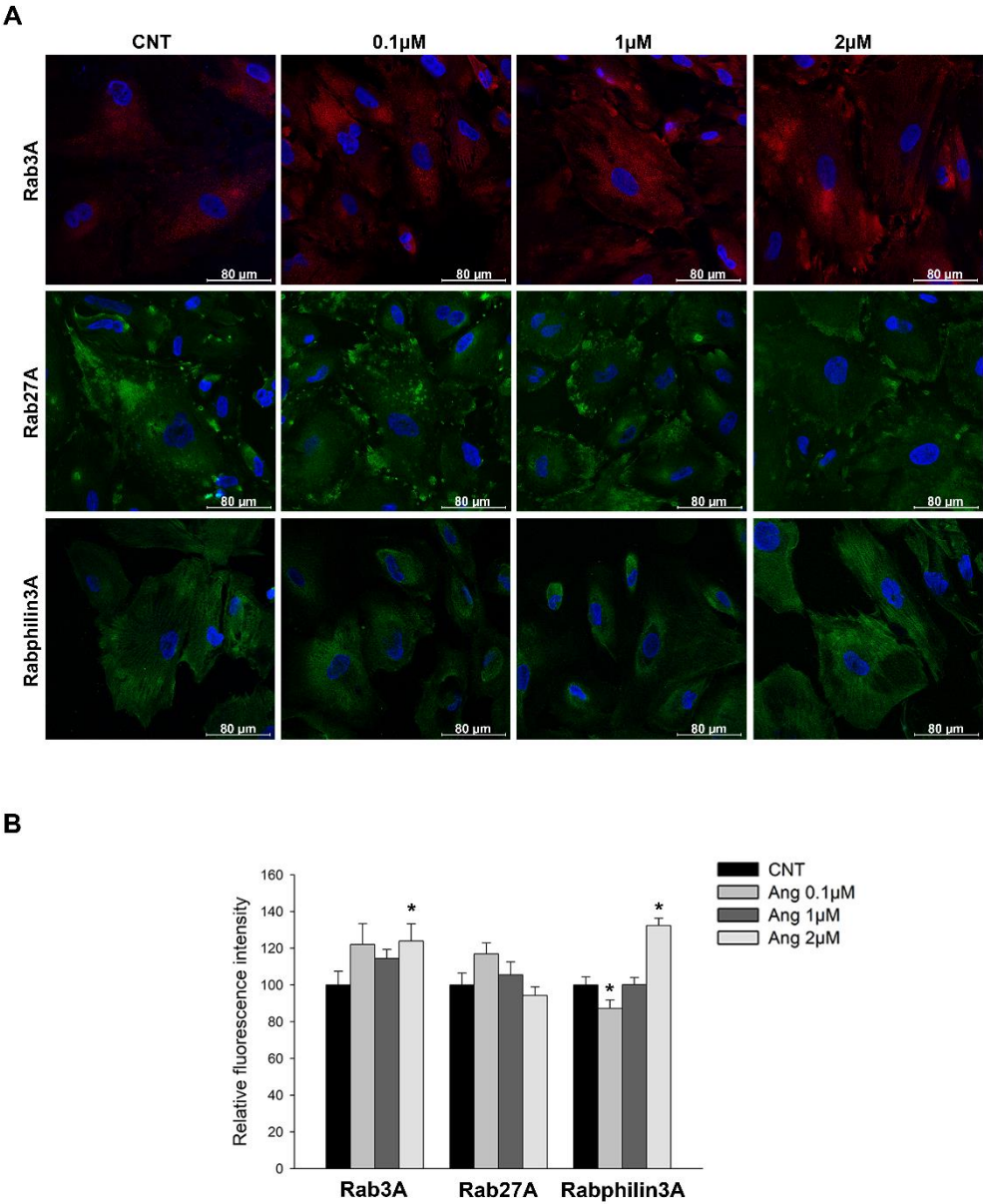


Figure 28. Immunofluorescence analysis of Rab3A-Rab27A system expression under Ang-II treatment. **A:** Confocal images of Rab GTPases. **B:** Bar graph showing the fluorescence quantification of Rab3A, Rab27A, and Rabphilin3A. Data are expressed as means $\pm$ SEM; n = 5 each group. Blue DAPI staining shows nuclei. Scale bars = 80 μ m. *p < 0.05 and **p < 0.01 vs. the CNT group.

6. EFFECT OF THE *RAB3A* AND *RAB27A* GENE SILENCING AND GLUCOSE TREATMENT

The use of siRNA is a valuable tool for investigating the functions of genes that are not yet fully understood. This is why we chose to employ siRNAs to silence *RAB3A* and *RAB27A* genes in immortalised human podocytes. In order to examine any potential changes in the Rab system when one of its components is silenced, and to assess the alterations observed during glucose overload, we analysed the expression of Rab27A when *RAB3A* was silenced, and vice versa (siRab3A and siRab27A), along with the levels of their shared effector, Rabphilin3A. Additionally, to observe how Rab silencing, glucose treatment, or a combination of both, may induce alterations in podocyte structure and function, we also investigated podocyte-associated markers.

6.1. Impact of *RAB3A* and *RAB27A* silencing and glucose on Rab3A-27A system

Our results showed a decrease in the mRNA levels of each Rab when the other was silenced under NG conditions (–2.99-fold decrease, $p < 0.05$ for siRab3A; –1.72-fold decrease, $p < 0.05$ for siRab27A). However, the opposite effect was observed in the HG group, with increased expression of *RAB27A* when *RAB3A* is silenced and vice versa, although only the expression of siRab27A reached statistical significance (1.71-fold increase, $p < 0.05$) (Fig. 29A and B). Protein levels were also analysed, but without reaching statistical significance. Rabphilin3A protein levels were decreased under NG conditions when *RAB3A* and *RAB27A* were silenced (–1.16, $p < 0.01$; –1.15 $p < 0.05$, fold decrease respectively). In contrast, under HG conditions, Rabphilin3A levels were augmented under *RAB27A* silencing (1.14-fold increase; $p < 0.001$) (Fig. 29C and D). These findings suggest that each

Rab plays a collaborative role by increasing its expression levels during cellular stress to counteract the decrease of the other Rab.

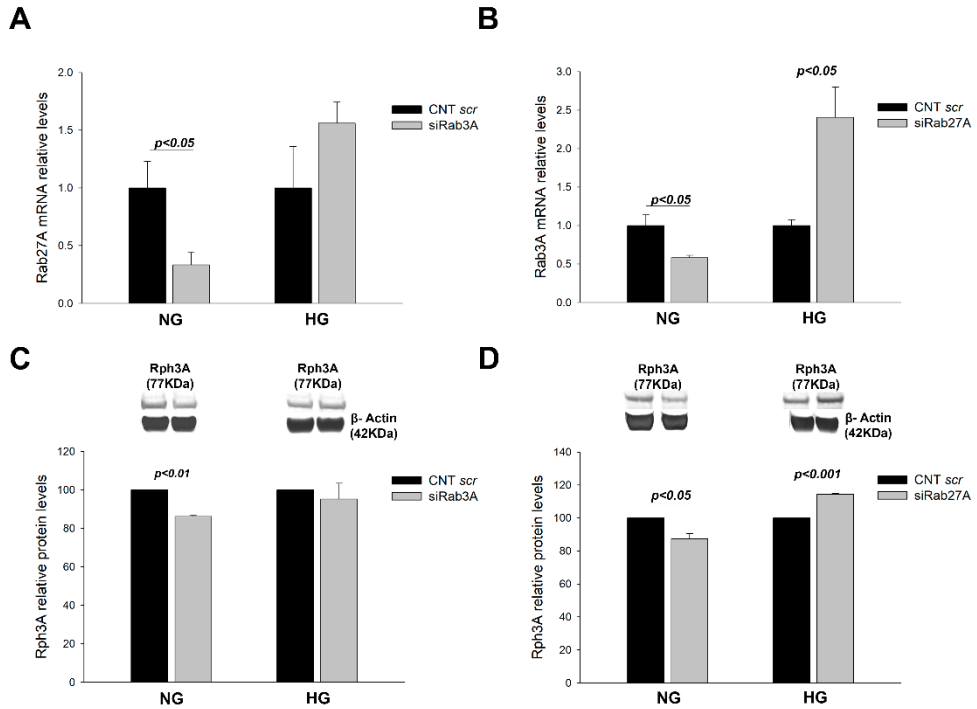


Figure 29. Effect of *RAB3A* and *RAB27A* gene silencing and treatment with HG on other components of the Rab3A-Rab27A system. A: mRNA levels of *RAB27A* when *RAB3A* is silenced under NG and HG conditions. B: mRNA levels of *RAB3A* when *RAB27A* is silenced under NG and HG conditions. C, D: Protein levels of Rph3A when *RAB3A* and *RAB27A* are silenced, respectively. Data are shown as mean $\pm$ SEM. N = 5 for each group. CNT scr: control scramble; NG: normal glucose; HG: high glucose; siRab3A: Rab3A siRNA; siRab27A: Rab27A siRNA; Rph3A: Rabphilin3A.

6.2. Effect of *RAB3A* and *RAB27A* silencing and glucose on podocyte markers

To investigate the impact of Rab3A-Rab27A system silencing and glucose overload on the structural and functional proteins of podocytes, our subsequent analysis focused on examining the gene and protein expressions of these markers. When *RAB3A* was silenced, a comprehensive reduction in mRNA levels across all markers was evident in NG conditions, notably observed for synaptopodin (-3.93-fold decrease, $p < 0.01$) and *CD2AP* (-2.18-fold decrease, $p < 0.05$). Conversely, under glucose overload, there was a tendency for an increase in all podocyte markers, reaching statistical significance for synaptopodin (3.15-fold increase, $p < 0.01$) and *WT-1* (4.75-fold increase, $p < 0.05$) (Fig. 30A). Regarding protein levels, *CD2AP* and *WT-1* exhibited an increase when *RAB3A* was silenced under HG concentrations (Fig. 30B). To summarize, these findings indicate that *RAB3A* silencing leads to a general reduction in podocyte markers, while the opposite effect is observed when silencing is conducted under HG treatments.

In the scenario of *RAB27A* silencing, a reduction in synaptopodin (-3.12-fold decrease, $p < 0.05$) and *CD2AP* (-2.32-fold decrease, $p < 0.05$) mRNA levels was identified in NG conditions. Under HG conditions, there was a downstream trend for synaptopodin levels, while, in contrast to NG, a significant increase in *CD2AP* (1.70-fold increase, $p < 0.05$) and nephrin levels (4.58-fold increase, $p < 0.001$) was observed (Fig. 31A). Examining protein levels, there was a declining trend in synaptopodin and *CD2AP* levels under NG conditions, contrasting with an elevation in nephrin (1.32-fold increase, $p < 0.001$) in line with the mRNA level changes. In HG, *RAB27A* silencing resulted in a decrease in synaptopodin (-1.19-fold decrease, $p < 0.05$) and *CD2AP* (-1.22-fold decrease, $p < 0.01$) and an increase in nephrin (1.45-fold increase, $p < 0.05$) (Fig. 31B). Similar to the observations with *RAB3A* silencing, *RAB27A* silencing also appears to induce podocyte dedifferentiation when conducted without treatment, leading to an opposing effect under HG conditions.

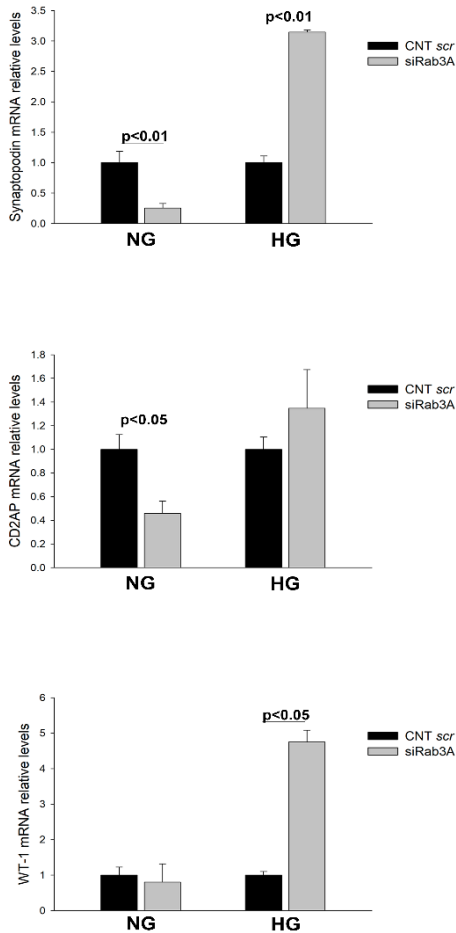
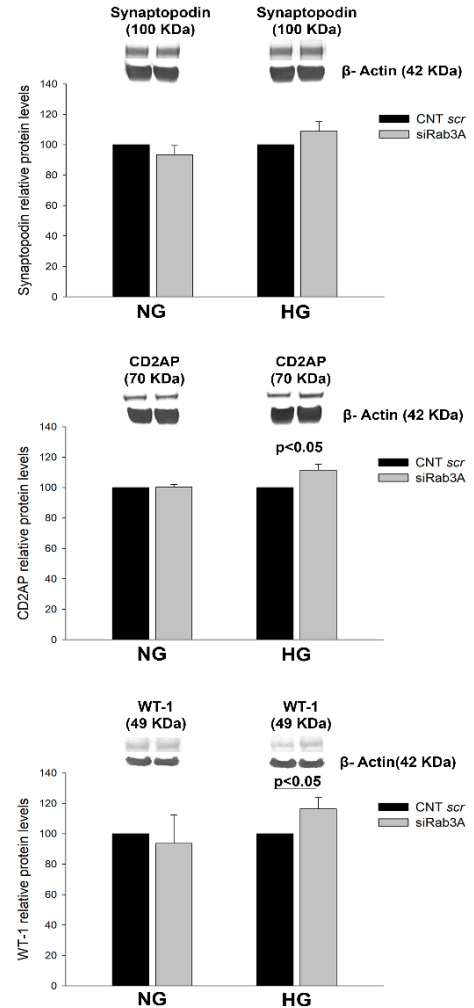
A**B**

Figure 30. Effect of *RAB3A* gene silencing and treatment with HG on podocyte associated markers. **A:** mRNA levels of podocyte markers synaptopodin, CD2AP and WT-1 when *RAB3A* is silenced under NG and HG conditions. **B:** Protein levels of podocyte markers synaptopodin, CD2AP and WT-1 when *RAB3A* is silenced under NG and HG conditions. Data are shown as mean $\pm$ SEM. N = 5 for each group. CNT scr: control scramble; NG: normal glucose; HG: high glucose; siRab3A: Rab3A siRNA

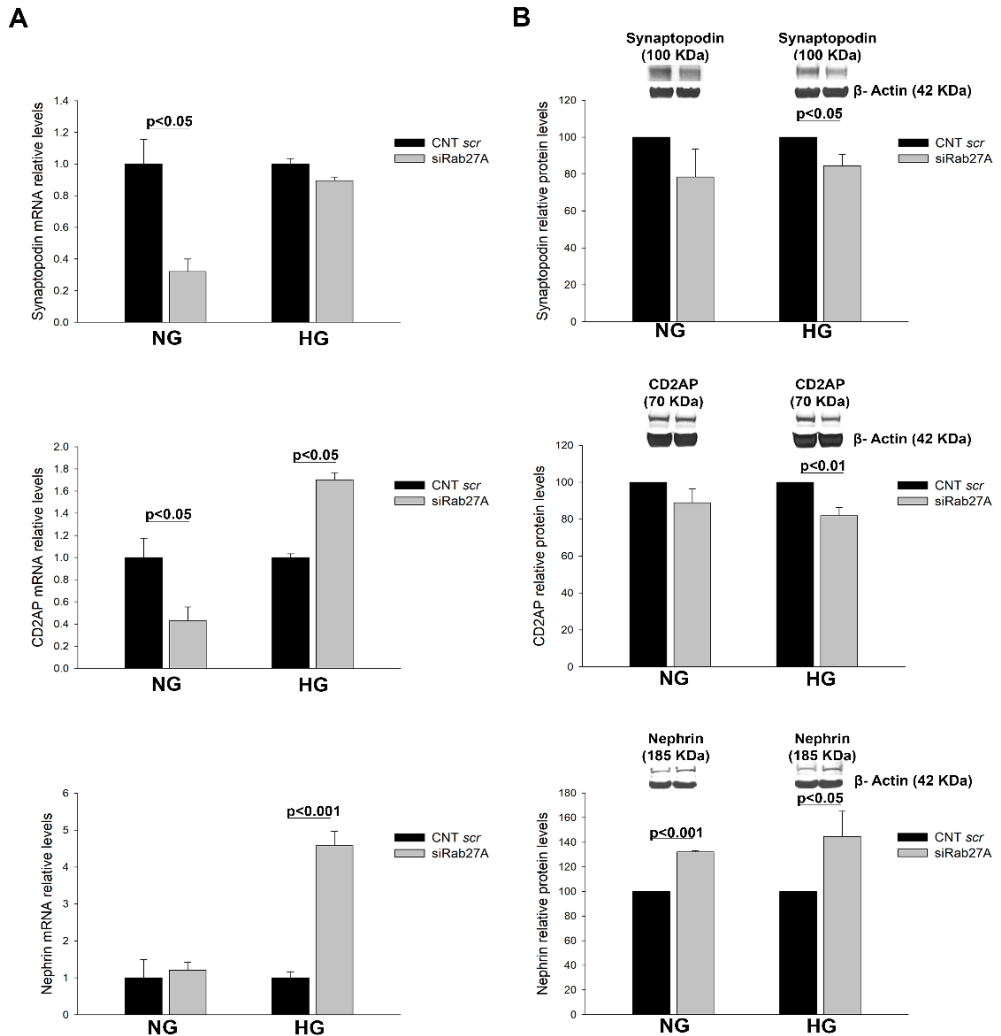


Figure 31. Effect of *RAB27A* gene silencing and treatment with HG over podocyte associated markers. A: mRNA levels of podocyte markers synaptopodin, CD2AP and nephrin when *RAB27A* is silenced under NG and HG conditions. B: Protein levels of podocyte markers synaptopodin, CD2AP, and nephrin when *RAB27A* is silenced under NG and HG conditions. Data are shown as mean $\pm$ SEM. N = 5 for each group. CNT scr. control scramble; NG: normal glucose; HG: high glucose; siRab27A: Rab27A siRNA.

6.3. Influence of *RAB3A* and *RAB27A* silencing and glucose overload on podocyte apoptosis

Our subsequent objective was to assess the impact of both *RAB3A* and *RAB27A* silencing and glucose treatment on podocyte apoptosis and cellular structure. Initially, flow cytometry assays revealed a significant increase in apoptosis in the CNT scr group for both *RAB3A* and *RAB27A* silencing compared to the CNT without transfection group, indicating an influence of lipofectamine on cell death ($p < 0.05$) (Fig. 32 and 33). Similarly, elevated cell death was observed in groups treated with HG without silencing. Notably, *RAB3A* silencing resulted in a reduction in apoptosis at NG levels ($p < 0.05$, Fig. 32), a phenomenon not observed in *RAB27A* silencing (Fig. 33). However, under conditions of HG exposure, apoptosis was lower in both silenced Rabs compared to the HG + CNT scr groups, with a more pronounced effect in *RAB3A* silencing ($p < 0.001$, Fig. 32) than *RAB27A* silencing ($p < 0.05$, Fig. 33). This suggests that silencing Rab genes appears to contribute to a decrease in podocyte apoptosis when exposed to HG concentrations.

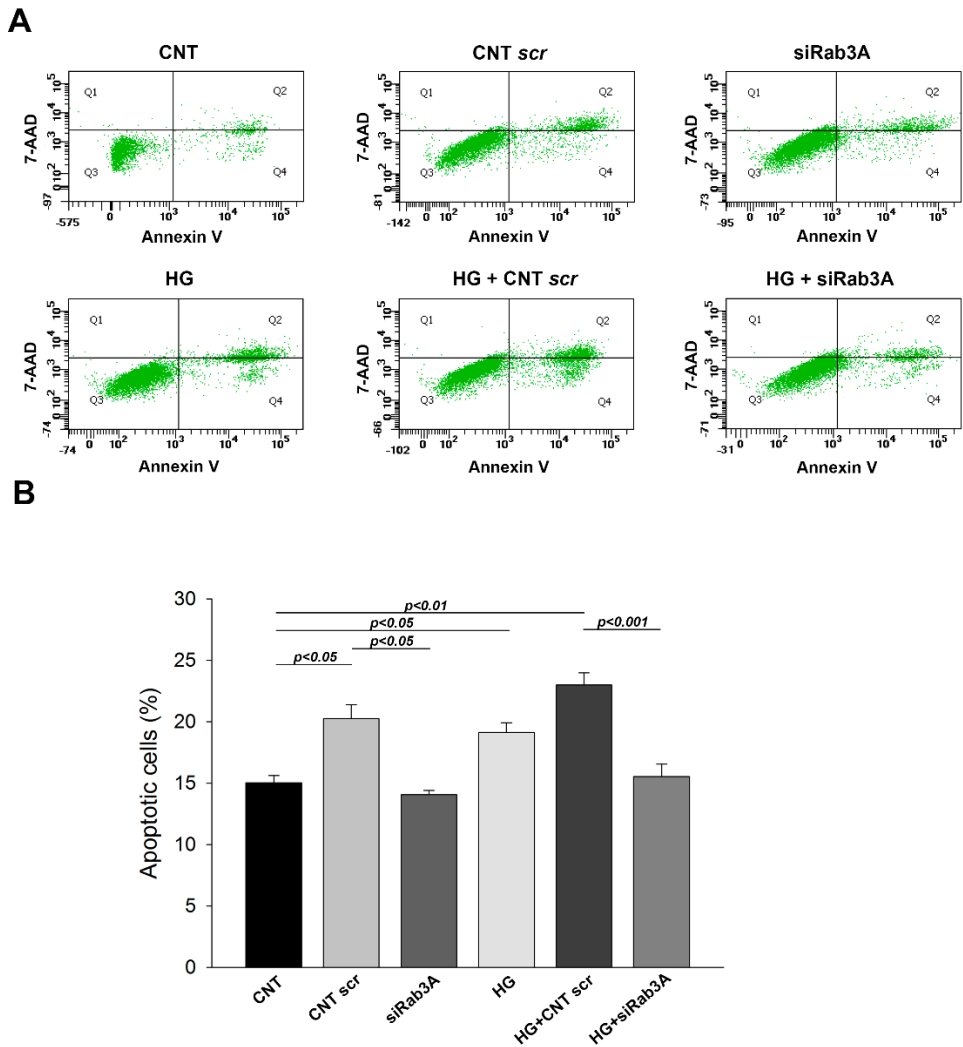


Figure 32. Influence of *RAB3A* gene silencing and treatment with HG on podocyte apoptosis. **A:** Flow cytometry images showing cells double-stained with annexinV/aminocyanine (7- AAD). The cytograms of cells with lost intact cell membranes that bound 7-AAD and excluded annexin V are shown in quadrant 1 (Q1). Cells with advanced stages of apoptosis or necrosis were both annexin V- and 7-AAD-positive and are shown in quadrant 2 (Q2). Viable cells that did not bind annexin V or 7-AAD are depicted in quadrant 3 (Q3). Early apoptotic cells, positive for annexin V but with still intact cell membranes (7-AAD negative), are shown in quadrant 4 (Q4). **B:** Graphs showing apoptosis percentages. Data are shown as mean $\pm$ SEM. N = 5 for each group. CNT: control; CNT scr: control scramble; NG: normal glucose; HG: high glucose; siRab3A: Rab3A siRNA.

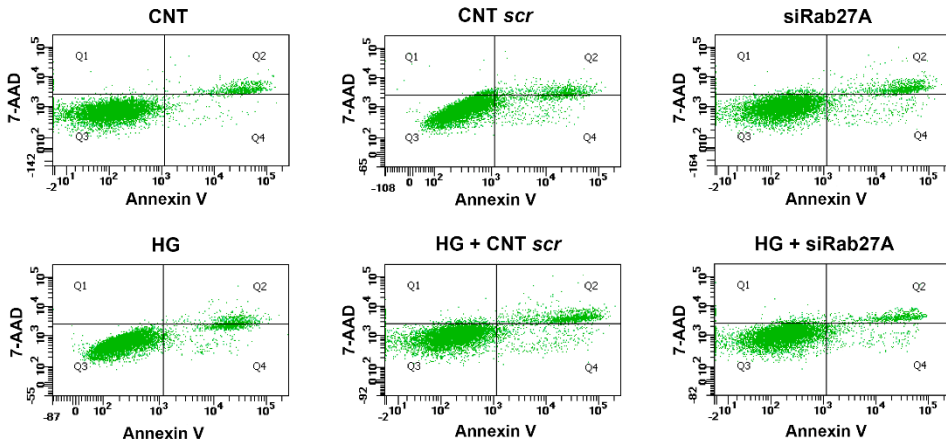
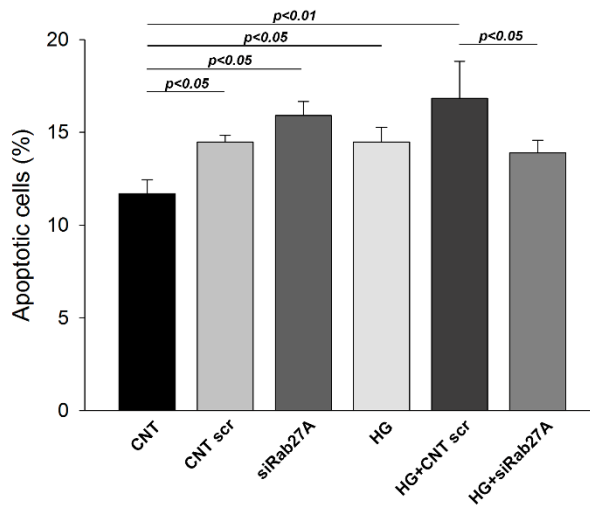
A**B**

Figure 33. Influence of *RAB27A* gene silencing and treatment with HG over podocyte apoptosis. **A:** Flow cytometry images showing cells double-stained with annexinV/-amino-actinomycin (7-AAD). The cytograms of cells with lost intact cell membranes that bound 7-AAD and excluded annexin V are shown in quadrant 1 (Q1). Cells at advanced apoptosis or necrotic stages were both annexin V- and 7-AAD-positive and are shown in quadrant 2 (Q2). Viable cells that did not bind annexin V or 7-AAD are depicted in quadrant 3 (Q3). Early apoptotic cells, positive for annexin V but with still intact cell membranes (7-AAD negative), are shown in quadrant 4 (Q4). **B:** Graphs showing apoptosis percentages. Data are shown as mean $\pm$ SEM. N = 5 for each group. CNT: control; CNT scr: control scramble; NG: normal glucose; HG: high glucose; siRab27A: Rab27A siRNA

6.4. Influence of *RAB3A* and *RAB27A* system silencing and glucose overload on podocyte structure

To evaluate the impact of *RAB3A* and *RAB27A* silencing and glucose overload on podocyte structure, we examined the distribution of F-actin fibers by measuring fractional anisotropy, an indicator of the organization of F-actin fibers in the cell. Comparable findings were observed for both silenced Rabs, revealing a disrupted fiber network (anisotropy values close to 0) in HG-treated podocytes compared to the NG group. Consequence of lipofectamine employment for siRNA transfection, disorganization of F-actin fibers was also noted in the CNT scr. Interestingly, under NG conditions, the silencing of both Rabs was linked to a decrease in anisotropy, a trend that reversed when silencing both Rabs under HG (Fig. 34).

Taken together, these results provide additional evidence of distinct cellular responses to detrimental processes depending on whether gene silencing occurs under normal physiological conditions or in a diabetic environment.

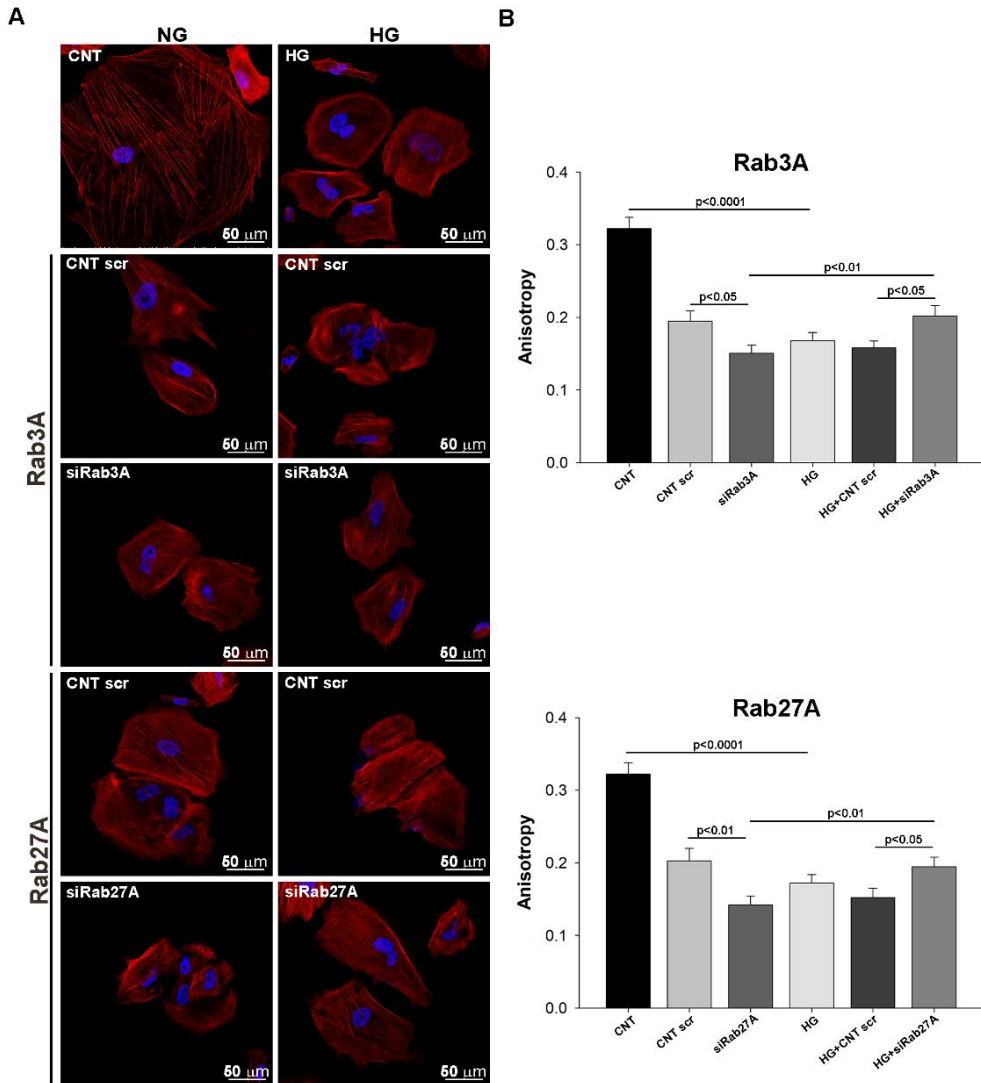


Figure 34. Impact of Rab3A-Rab27A system gene silencing and treatment with HG on the podocyte structure. **A:** Confocal images of podocytes subjected to the gene silencing of *RAB3A* and *RAB27A* and under HG, showing F-actin fibre staining with phalloidin. **B:** Graphs showing the anisotropy index in each group for *RAB3A* and *RAB27A* silencing. Data are shown as mean $\pm$ SEM. N = 5 for each group. CNT: control; CNT scr: control scramble; NG: normal glucose; HG: high glucose; siRab3A: Rab3A siRNA; siRab27A: Rab27A siRNA

6.5. Analysis of vesicular transport markers under Rab3A-Rab27A system silencing and glucose treatment

Considering the involvement of the Rab system in vesicular trafficking, our subsequent investigation focused on examining the impact of *RAB3A* and *RAB27A* silencing, as well as HG conditions, on the expression of CD63 and CD9, common tetraspanins enriched in EVs. We conducted fluorescence analyses of CD63 and CD9, quantifying image intensity. In NG conditions, silencing of both Rabs led to an increased CD63 signal, accompanied by a shift in distribution from a homogenous and scattered dotted pattern throughout the cytoplasm to a pattern exhibiting more accumulation in the perinuclear zone. However, in HG and siRab condition, this effect was less pronounced compared to NG and tended to resemble the pattern of the CNT, particularly in the case of siRab27A (Fig. 35). Conversely, the CD9 pattern and intensity did not exhibit significant changes, but there was a tendency for its intensity to decrease when Rabs were silenced, under both NG and HG conditions (Annex III). These observations suggest that *RAB3A* and *RAB27A* gene silencing may induce alterations in the vesicular transport pathway in podocytes.

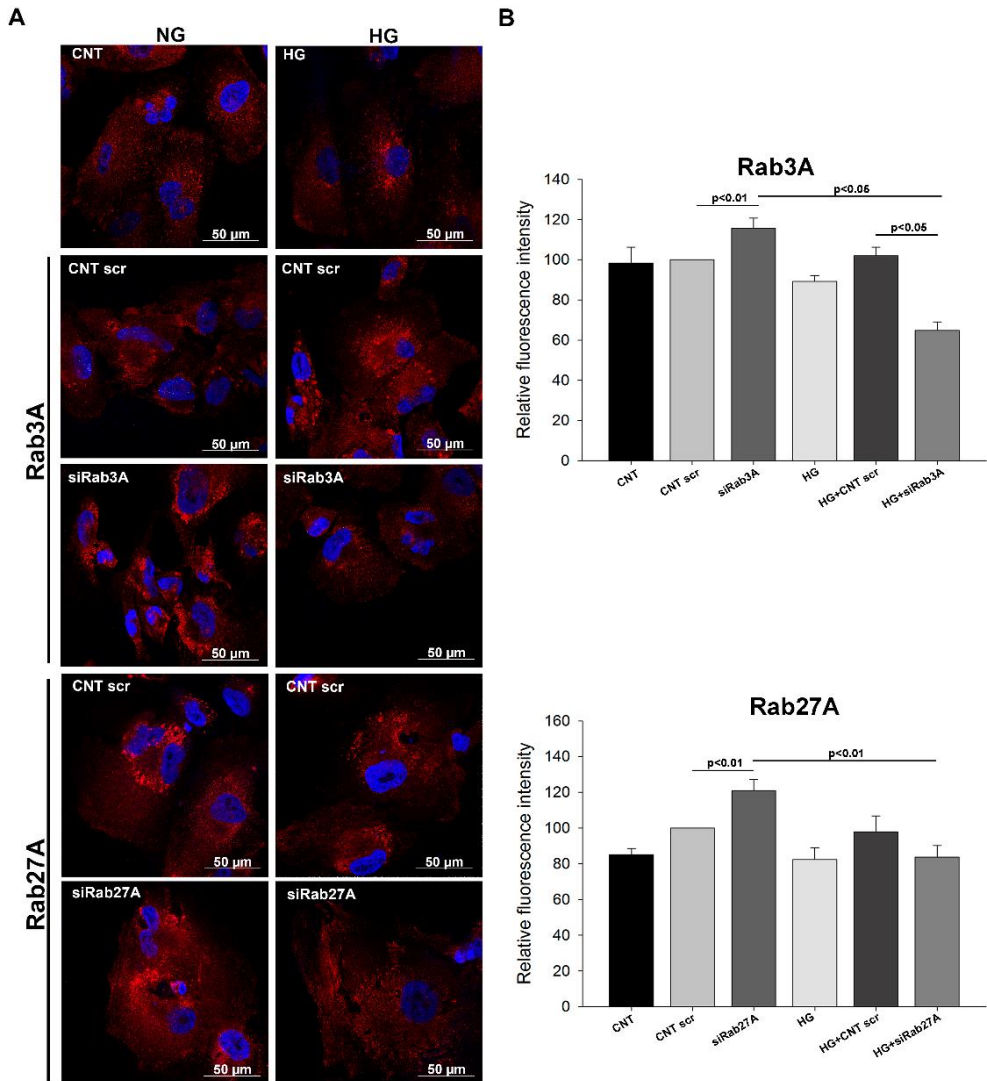


Figure 35. Analysis of CD63 intensity and distribution in silenced and HG overload-treated podocytes. A: Confocal images of podocytes treated with siRab3A and siRab27A and under glucose overload, showing CD63 staining and cell distribution. **B:** Graphs showing the fluorescence intensity of CD63 in each group for *RAB3A* and *RAB27A* silencing. Data are shown as mean $\pm$ SEM. N = 5 for each group. CNT: control; CNT scr: control scramble; NG: normal glucose; HG: high glucose; siRab3A: Rab3A siRNA siRab27A: Rab27A siRNA.

7. ISOLATION AND CHARACTERISATION OF PODOCYTE EVS

Different strategies were used to assess the proper characterisation of EVs enriched fractions. To do this, several complementary techniques were employed, which allowed for the characterisation of the samples by size, protein composition, and concentration. EVs were characterised by analysing their size distribution by NTA, morphology by TEM and purity of EV suspension through analysis of EVs marker expression by WB, superresolution microscopy (ONI) and flow cytometry.

NTA analysis showed a marked peak of the selected EV fractions ranging from 140 to 200nm (Fig. 36A). TEM images revealed pure samples without undesired precipitates or cell debris, and showed vesicles stained with well-established EV markers CD63 and CD9 (Fig. 36B). In addition, Western blot experiments confirmed the purity of samples, where vesicles were enriched in surface and cytoplasmic EV markers CD63, CD9, syntenin-1, and TSG-101. The intracellular compartment markers calnexin and GM-130 were highly enriched and exclusively expressed in the cellular pellet samples (Fig. 36C). Furthermore, the presence of tetraspanins CD63, CD9 and CD81 in EVs isolated from undifferentiated and differentiated podocytes were confirmed by superresolution microscopy (Fig. 36D).

The different techniques employed evidence that the isolation and purification of EVs has been performed correctly and that we have obtained an enriched EVs pellet.

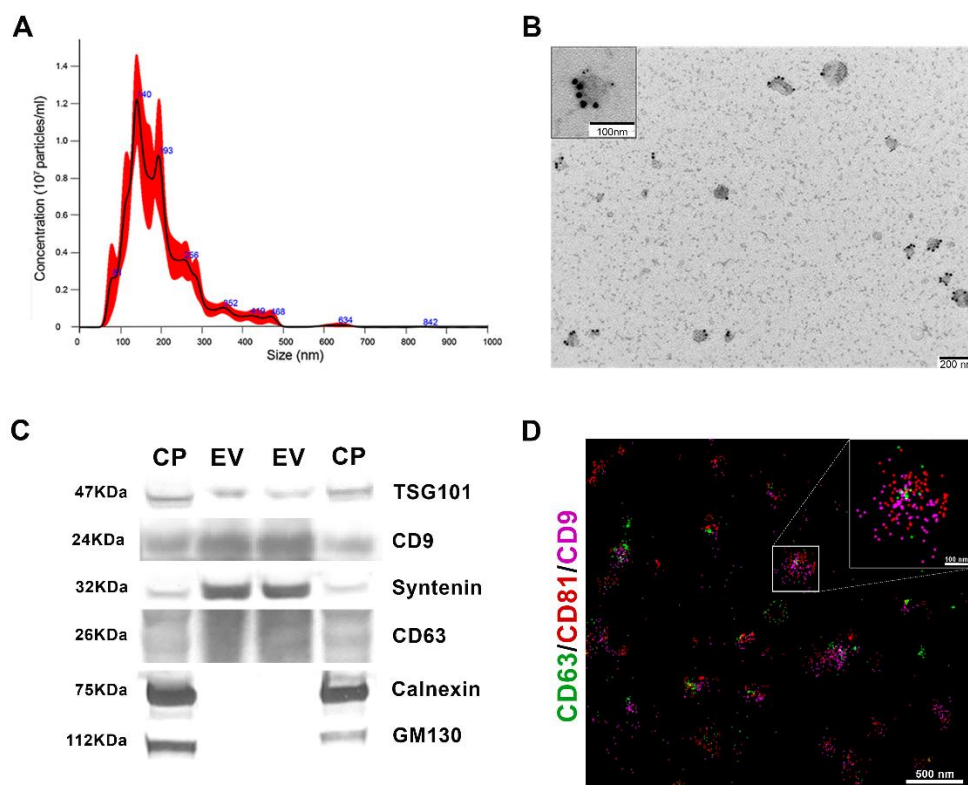


Figure 36. Characterisation of EVs in the podocyte cell culture medium. **A:** Graph showing the NTA results with the concentration and size of EV particles. **B:** TEM micrographs of EVs with CD9 (6 nm) and CD63 (15 nm) after immunogold labelling. **C:** Western blot analysis of EV markers (TSG-101; CD9, syntenin-1, CD63) and cell organelle markers (calnexin and GM130) in EV and CP. **D:** Representative super-resolution microscopy images of podocyte-EVs showing double, single and triple expression of CD63 (green), CD81 (red), CD9 (pink). CNT: control; CNT scr: control scramble; CP; cell pellet; EV: extracellular vesicles; GM130: Golgi matrix protein 130; NTA: nanoparticle tracking analysis; TEM: transmission electron microscopy; TSG-101: tumour susceptibility gene 101 protein.

In addition, EVs isolated from undifferentiated, differentiated and HG-treated podocytes were characterised by their surface marker expressions, including tetraspanins, mesenchymal, endothelial/platelet, and immunological markers, using a MACSPlex Exosome analysis kit after bead-based immunocapture (Fig. 37). The levels of tetraspanins CD9, CD63, and CD81 were high in all the samples measured. Interestingly, levels of the mesenchymal stem cell markers CD44 and CD146 were higher in undifferentiated cells, while EVs isolated from

differentiated podocytes showed an increased expression of renal markers such as CD133 and CD24. This highlights the importance of working with differentiated cells to attempt to replicate the physiological conditions in the human kidney.

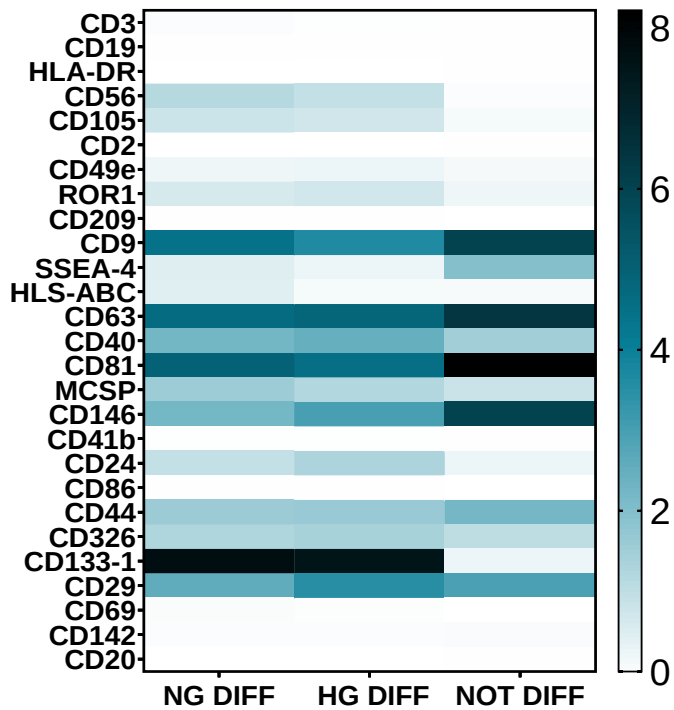


Figure 37. MACSplex immunological marker analysis of podocyte-derived EVs. MACSplex representative heatmap showing the podocyte-EV distribution of allophycocyanin (APC)-stained bead populations; captured EVs are counterstained with APC-labeled detection antibodies using a mixture of anti-CD9, anti-CD63 and anti-CD81 antibodies.

8. EFFECT OF RAB3A-RAB27A SYSTEM SILENCING ON miRNAs EXPRESSION IN CELL PELLETS AND SECRETED EXOSOMES

miRNAs represent crucial cargoes within EVs, playing pivotal roles in cellular functions and displaying altered levels in various pathologies. Given their significance, we investigated the expression levels of key miRNAs associated with podocyte biological functions and DN in both podocyte cell pellets and exosomes derived from these cells, examining alterations under conditions of Rab silencing and glucose-induced stress. In the case of *RAB3A* silencing, the levels of miR-21-5p in the cell pellet increased under HG scr (1.94-fold increase, $p < 0.05$) and HG siRab3A (2.22-fold increase, $p < 0.05$) compared to CNT scr. Additionally, miR-21-5p levels were also increased when compared siRab3A cells and HG siRab3A (1.62-fold increase, $p < 0.05$). Conversely, in secreted exosomes, these levels were elevated under HG treatment and *RAB3A* silencing (3.12 and 3.42-fold increase, respectively, $p < 0.05$ for both compared to CNT scr) but lower in the HG siRab3A group compared to HG+CNT scr and NG siRab3A (-3.12 and -3.42-fold decrease, $p < 0.05$ for both), indicating an impact of *RAB3A* silencing under HG stress conditions (Fig. 38A and B). For miR-26a-5p, a similar effect was observed in the cell pellet, there was an increase in the HG scr group (2.01-fold increase, $p < 0.05$), but a significant decrease under *RAB3A* silencing with HG treatment (-1.92-fold decrease, $p < 0.05$, compared to HG+CNT scr group). This decrease was reversed when *RAB3A*-silenced cells were treated with HG (1.62-fold increase, $p < 0.05$ compared to NG siRab3A). Regarding exosomes fraction, miR-26a-5p expression levels were markedly decreased under siRab3A (-22.2-fold decrease, $p < 0.05$), while podocytes with *RAB3A* silenced and subjected to HG showed increased levels compared to siRab3A (8-fold increase, $p < 0.05$) (Fig. 38C and D). A decrease in miR-31-5p levels was observed in the cell pellet when *RAB3A* was silenced without or with HG treatment (-2.23 and -2.32-fold decrease, $p < 0.01$ compared to CNT scr

and HG CNT scr groups, respectively), while exosomal levels were undetectable in most samples (Fig. 38E).

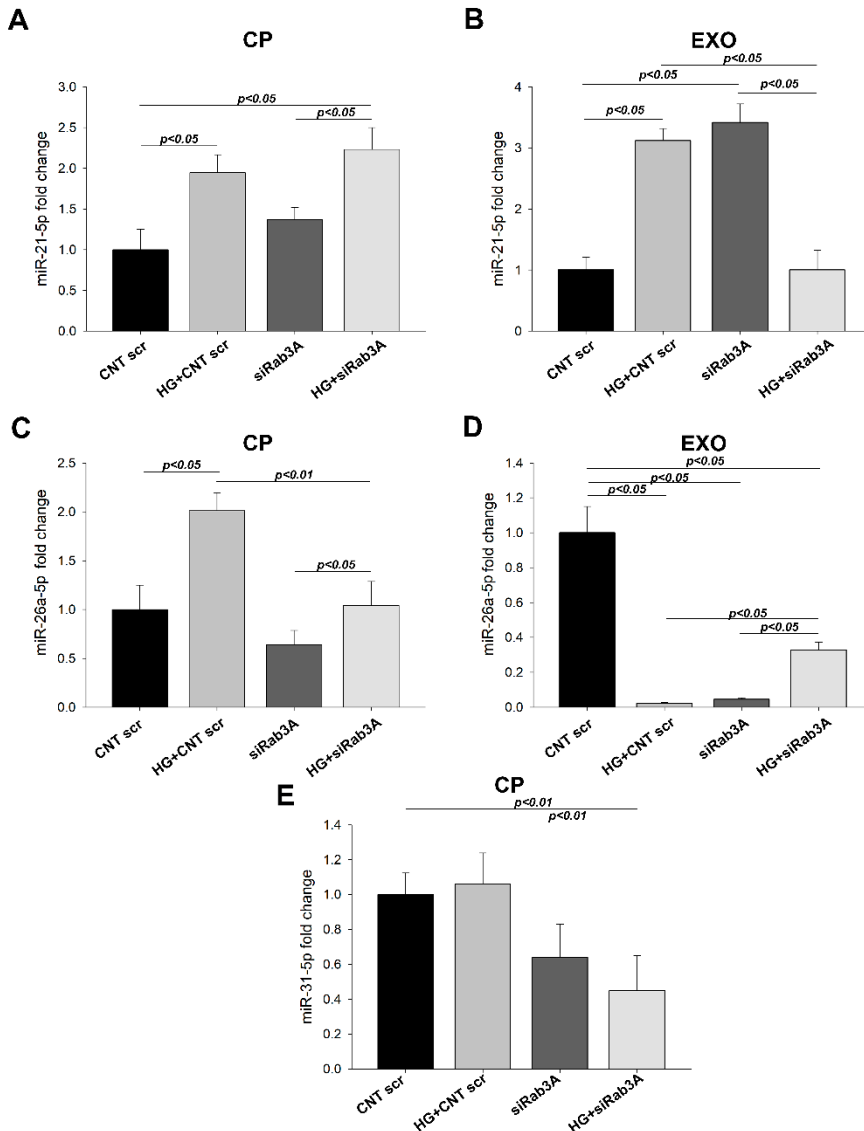


Figure 38. Analysis of the miRNA levels in *RAB3A*-silenced and glucose overload-treated podocytes and the EVs isolated from them. A: miR-21-5p CP levels. B: miR-21-5p EXO levels. C: miR-26a-5p CP levels. D: miR-26a-5p EXO levels. E: miR-31-5p CP levels. Data are shown as mean $\pm$ SEM. N = 5 for each group. miRNA levels for CNT scr group are normalised to 1. CNT: control; CNT scr: control scramble; CP: cell pellet; EXO: exosome; HG: high glucose; siRab3A: Rab3A siRNA

In the context of *RAB27A* silencing, the majority of samples from cells in which this gene has been silenced exhibited undetectable levels of exosomal miRNA. Within the cell pellet, the HG groups demonstrated an increase in miR-21-5p levels (2.06 and 2.13-fold increase, $p < 0.05$, compared to CNT scr; Fig. 39A). miR-26a-5p levels showed an increase in the HG siRab27A group compared to CNT scr and siRab27A (1.88 and 2.07-fold increase, respectively, $p < 0.05$ for both; Fig. 39B). Lastly, miR-31-5p levels exhibited an increase under HG treatment (1.99-fold increase, $p < 0.05$), but in siRab27A groups, both with and without glucose, they declined to levels similar to those of the NG scr group (Fig. 39C).

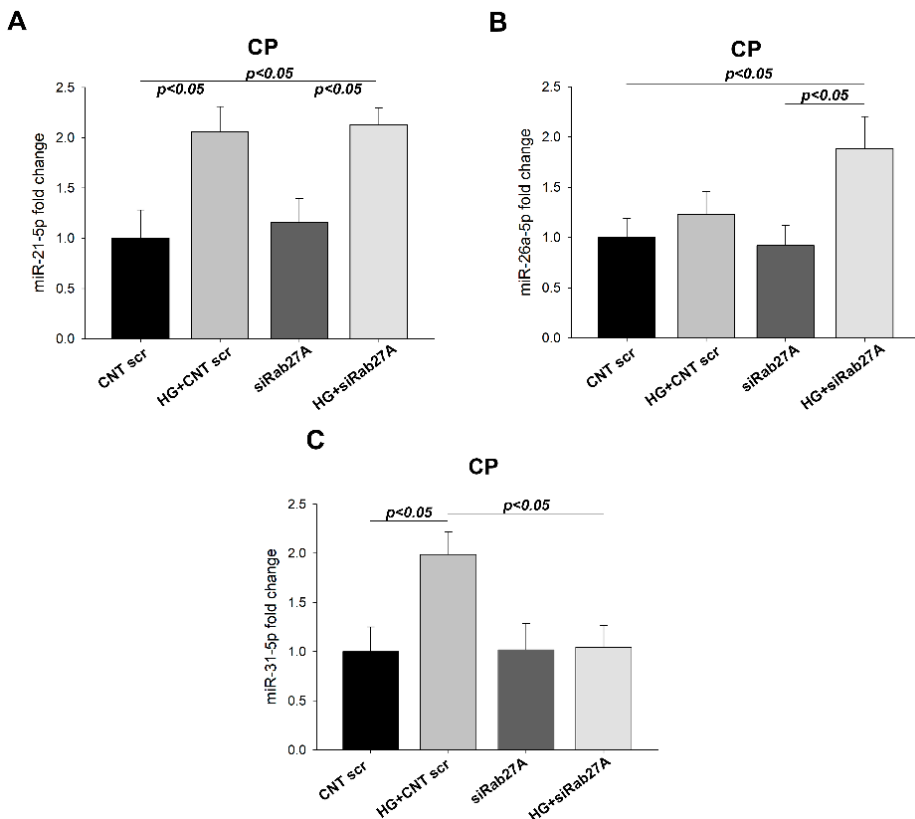


Figure 39. Analysis of miRNA levels in *RAB27A* silenced and glucose overload-treated podocytes. A: miR-21-5p CP levels. **B:** miR-26a-5p CP levels **C:** miR-31-5p CP levels. Data are shown as mean $\pm$ SEM. N = 5 for each group. CNT: control; CNT scr: control scramble; CP: cell pellet; HG: high glucose; siRab27A: Rab27A siRNA.

miRNAs are essential components of EVs and play critical roles in cellular functions, with their dysregulation being associated with various pathologies. In this study, we investigated the expression patterns of select miRNAs related to podocyte functions and DN in both podocyte cell pellets and the exosomes they release after gene silencing of *RAB3A* and *RAB27A*. Our findings revealed distinct alterations in miRNA levels under conditions of *RAB3A* and *RAB27A* silencing, as well as glucose-induced stress. These observations highlight the intricate regulatory roles of Rab proteins in miRNA expression and secretion, particularly under conditions mimicking diabetic stress.

V. DISCUSSION

“I marvel at this, that so many different substances are separated through these glands by the process of Nature, for water with salty, sulphurous and similar particles goes through...And whatever is of larger size or of different shape does not enter the little pores and small spaces of the excreting body, and is not excreted”.

De Renibus-1666

Marcello Malpighi

T2DM is a widespread and growing health concern globally. It is widely acknowledged that T2DM is a primary cause of renal damage as up to 40% of the patients develop DN (Magliano and Boyko 2021). Podocytes, key glomerular cells, which act as crucial regulators of the integrity of the GFB, are significantly injured during T2DM, leading to alterations in GFR, influencing the progression of renal disease, like DN. Consequently, reducing podocyte damage has become a priority.

Intra- and intercellular communication between podocytes and other renal cell types is essential, with EVs and the cargo that they transport playing a key role in this process. In this context, Rab GTPases are crucial in controlling vesicular trafficking, influencing an individual's health status, or contributing to disease. Regarding Rab GTPases, in genetic association studies, our group identified a SNPs in *RPH3A* gene, effector of the Rab3A and Rab27A GTPases, related with the risk to develop albuminuria by using combined genetic and metabolomic data from a general population (Marrachelli *et al.* 2014).

For all these reasons, we aimed to investigate the role of Rab3A-Rab27A system in glucose-induced podocyte injury to shed light on the system's role in glomerular damage under diabetic conditions. In this way, this work has allowed us to obtain new and more specific information about the role of Rab3A-Rab27A system in the development of podocyte injury in T2DM. The results suggest that the Rab3A-Rab27A system plays a pivotal role in podocyte injury and vesicular traffic regulation within the diabetic context.

1. THE RAB3A-RAB27A SYSTEM IN URINE CELL PELLET FROM PATIENTS

In this doctoral thesis, we have quantified and compared mRNA levels of the Rab3A-Rab27A system in urine samples from patients with HTN and with and without DM and/or increased UAE. The importance of UAE as an indicator of renal damage is based on cross-sectional studies demonstrating the development of cardiovascular risk and organ damage associated with UAE (Pascual *et al.* 2014).

Our results revealed elevated mRNA expression levels for *RAB3A* and *RAB27A* in urine cell pellets from patients with kidney disease, establishing a positive correlation between UAE levels and Rab mRNAs. Specifically, there was an increase in *RAB3A* gene expression in both albuminuric groups (with and without associated DM), which suggests that Rab3A could be involved in the presence of incipient renal damage or at least indicate this damage. This result aligns with the findings reported by Rastaldi *et al.*, which demonstrated elevated Rab3A levels in the glomerular tissue of patients with proteinuric diseases (Rastaldi *et al.* 2003). In connection to this, a *Rab3A* knockout mice model revealed enhanced UAE and increased damage to glomerular and podocyte structures under HG conditions (Giardino *et al.* 2009, Armelloni *et al.* 2012). Regarding *RAB27A* levels, the observed increase reached statistical significance when examining both albuminuric groups collectively. This implies that Rab27A may exert a less potent but still significant influence on renal injury. While Rab27A has been investigated in the context of the kidney, it has not been directly linked to UAE (Yasuda *et al.* 2012, Song *et al.* 2019).

Remarkably, this doctoral thesis represents the initial evidence of the presence of Rab GTPases from the Rab3A-Rab27A secretory system in human urinary cell pellets. Additionally, important expression changes of this system were found in patients with HTN and T2DM with renal injury, along with an association between these Rab GTPases and UAE.

2. THE RAB3A-RAB27A SYSTEM IN INJURED HUMAN PODOCYTES STRESSED BY GLUCOSE AND ANGIOTENSIN-II

Rab GTPases play a significant role in establishing polarity by regulating the trafficking of junctional proteins and defining epithelial transport circuits and recycling endosomes (Schwartz *et al.* 2007). In podocytes, the vesicular trafficking directed by Rab GTPases represents a pivotal process for maintaining their polarised cell bodies, facilitating FP formation, and contributing to recycling and cell communication routes (Simons *et al.* 1999, Yasuda *et al.* 2012, Dorval *et al.* 2019).

Regarding the results obtained, in differentiated podocytes, we observed a reduction in the protein levels of Rab3A, Rab27A, and their effector Rabphilin3A, coupled with an increase in *RAB3A* and *RAB27A* mRNA levels compared to the proliferative stage. This suggests a compensatory mechanism in response to the observed reduction of protein levels at the end of the differentiation process. Previous studies attribute these disparities to variations in half-lives and production rates between mRNA and proteins, as well as to a significant regulatory influence of processes occurring downstream of transcription. These processes can result in alterations, including changes in the degradation rates of proteins (de Sousa Abreu *et al.* 2009, Maier *et al.* 2009, Vogel and Marcotte 2012, Liu *et al.* 2016). Furthermore, under conditions of HG and Ang-II, the decreased protein levels of podocyte differentiation markers, combined with the elevated levels of Rab3A and Rab27A proteins (in contrast to levels during differentiation), indicate a potential occurrence of dedifferentiation. In accordance with our results, previous studies (Saleem *et al.* 2002, Chittiprol *et al.* 2011, Li *et al.* 2017) have described that protein levels of podocyte-associated markers (synaptopodin, WT-1, nephrin, and CD2AP) exhibited an increase in *in vitro* differentiated human podocytes, with a decrease observed under different treatment conditions such as HG and Ang-II, indicating that podocytes were losing their differentiation state. Indeed, our research group

has previously demonstrated alterations in podocyte-specific markers in urine cell pellet from patients with HTN and lupus nephritis, where viable podocytes were detected in urine, and changes in podocyte markers suggested a dedifferentiation process rather than apoptosis, identifying them as potential biomarkers for the disease (Perez-Hernandez *et al.* 2016, Perez-Hernandez *et al.* 2018). Herman-Edelstein *et al.* (Herman-Edelstein *et al.* 2011) also observed this process in stressed human podocytes subjected to TGF- β 1 and Ang-II treatments. When examining the effects of HG and Ang-II on podocytes, we detected features indicative of apoptosis, consistent with those reported previously in a diabetic mice model (Gu *et al.* 2016) and *in vitro* podocyte models subjected to HG and Ang-II, where the maintenance of podocyte number and FP architecture was assessed (Huang *et al.* 2014, Huang *et al.* 2017). Furthermore, in the morphological analysis performed we revealed characteristic features of apoptosis in treated podocytes, including nuclei and chromatin condensations observed in DAPI-stained micrographs. Surprisingly, the lack of statistically significant changes in the number of apoptotic cells under Ang-II treatment suggests that this may not be a result of marked apoptotic cell death but rather initial dedifferentiation followed by cell death, as reported by other authors in human podocyte cultures (Sanchez-Nino *et al.* 2012, Li *et al.* 2020). Prior studies have underscored the involvement of changes in cytoskeleton components in the initiation and advancement of renal diseases (Niranjan *et al.* 2008, Perico *et al.* 2016, Hurcombe *et al.* 2019). Our results from morphological assessments highlighted cytoskeletal disturbances in podocytes treated with HG and Ang-II. Specifically, F-actin staining showcased a disorganised cytoskeleton in podocytes, with fiber grouping at the cell periphery and a more diffuse pattern in the cytoplasm, emphasising the structural vulnerability of podocytes under these stress conditions (Macconi *et al.* 2006, Shankland 2006, Tian *et al.* 2014, Lv *et al.* 2016, Huang *et al.* 2019).

Additionally, immunofluorescence analyses unveiled altered levels and distinct subcellular distributions of podocyte markers and Rab proteins under stressed conditions. Synaptopodin expression rearranged under HG conditions, as previously noted (Li *et al.* 2013). Nephryn expression shifted from a perinuclear to a more cytoplasmic pattern and was reduced under HG and Ang-II exposure. These findings are consistent with prior research, which indicated that human podocytes treated with lipopolysaccharide exhibited increased cytoplasmic nephryn expression and a concomitant decrease in perinuclear expression (Succar *et al.* 2016). These changes in the protein subcellular distribution and the reduced expression support the occurrence of dedifferentiation in these cells. Notably, imaging analysis of Rab GTPases revealed a distinctive Rab27A expression pattern. Unlike the cytoplasmic distribution observed under normal conditions, higher expression near the podocyte membrane was observed under HG and Ang-II treatments. Considering Rab27A's role in vesicle exocytosis, these observations suggest intensified exosomal traffic under these stresses.

In summary, this doctoral thesis provides initial evidence of the presence of Rab GTPases Rab3A and Rab27A in human podocyte cell cultures. Consistent with this, our research highlighted the seemingly significant involvement of the Rab3-Rab27A system in podocyte damage under HG and Ang-II stress conditions. We observed an elevation in Rabs when cells were exposed to glucose overload, coupled with a general reduction in podocyte differentiation markers. This observation suggests the potential occurrence of a dedifferentiated state under such stress situations, indicating that the Rab3A-Rab27A system might play a role in this process (Martinez-Arroyo *et al.* 2020).

3. RAB3A-RAB27A SYSTEM SILENCING AMELIORATES HIGH GLUCOSE-INDUCED INJURY IN PODOCYTES

Taking into consideration the obtained results, we investigated the role of silencing the Rab3A-Rab27A system in HG-induced podocyte injury to shed some light on the system's role in glomerular damage under diabetic conditions. Limited prior investigations have explored the role of silenced Rabs within this system at the renal level. Armelloni *et al.* examined the consequences of *Rab3A* knockout in mice subjected to a HG diet, revealing podocyte damage and subsequent proteinuria (Armelloni *et al.* 2012). In contrast, Li *et al.* demonstrated that inhibiting Rab27A resulted in an amelioration of renal tubular epithelial cells, reducing inflammation and mitigating the development of DKD (Li *et al.* 2020).

In line with the results discussed above, we have observed that the silencing of *RAB3A* in podocytes, followed by exposure to glucose overload, is associated with a general increase in podocyte markers (Synaptopodin, CD2AP and WT-1). This suggests an absence of a dedifferentiation state in podocytes. Rab3A plays a role in the exocytosis of secretory vesicles and granules (Fukuda 2008, Martinez-Arroyo *et al.* 2021), but its functional studies have yielded conflicting roles in exocytosis processes (Darchen and Goud 2000). Some studies indicate that Rab3A negatively controls calcium dependent exocytosis in neuroendocrine cells (Johannes *et al.* 1994, Geppert *et al.* 1997), while more recent studies show that together with Rab27A, Rab3A work sequentially during sperm densecore granule exocytosis and promote melanin exocytosis from melanocytes (Bustos *et al.* 2012, Cabaco *et al.* 2022). Both studies support the idea that Rab27A and Rab3A represent a Rab cascade involved in the regulation of exocytosis. Interestingly, these opposing functions have been reported to depend on the secretory cell type (Darchen and Goud 2000). Our results indicate that Rab3A, through its function, could participate in certain mechanisms of podocyte damage under diabetic conditions.

The impact of silencing *RAB27A* and subsequent treatment of podocytes is less notable in the levels of the proteins and mRNA of *SYNPO*, *CD2AP* and *WT-1* compared to the effects seen with the silencing of *RAB3A*. Both Rabs share common and distinct roles in vesicle traffic. Interestingly, some publications report a cooperative role between the two Rabs to achieve vesicle exocytosis at plasma membranes (Tolmachova *et al.* 2004, Handley *et al.* 2007). Consistent with this, our observation is that when one Rab is silenced without glucose treatment, the expression levels of the other Rab and their common effector Rph3A generally decrease. However, under HG, their expression levels increased, suggesting a potential compensatory mechanism of Rabs in a cellular stress situation.

Significantly, we identified similar trends regarding apoptosis, podocyte cell structure alterations, and vesicular transport markers. Generally, under conditions of silenced Rabs and no treatment, we observed an increase in cell death and a decrease in anisotropy, indicating disorganization of F-actin fibers. A study conducted by Giardino *et al.* (Giardino *et al.* 2009) demonstrated that *Rab3A* knockout led to dysregulation of exocytosis and alterations in podocyte cytoskeleton, underscoring the significance of the Rab3A in the vesicular transport of podocytes, supporting the results showed in this thesis. Furthermore, there were alterations in the CD63 pattern, transitioning from cytoplasmic dispersal to accumulation in the perinuclear zone. However, intriguingly, when cells are exposed to glucose overload and Rabs are silenced, adverse processes like apoptosis, cellular structure organization, and CD63 accumulation appear to be reduced. As demonstrated in previous studies, processes such as apoptosis tend to increase under glucose treatment (Cao *et al.* 2021). These findings suggest that different mechanisms may be at play when Rabs are silenced, depending on whether the cell is under stress or not. Under normal glucose conditions, Rab silencing might decrease the secretion of CD63-positive vesicles, as indicated by

their accumulation pattern in the perinuclear zone. Nevertheless, when cells are damaged by treatment, the signals they would normally send could be negative and are partially disrupted by silencing the Rab system.

Ultimately, Rab3A-Rab27A system silencing ameliorates some of the detrimental processes derived from HG insults such as podocyte apoptosis, cytoskeleton disorganization and an improvement in the protein levels of podocyte associated markers, suggesting a role influence depending on the presence or absence of cellular stress. Our results suggest that Rab system GTPases may orchestrate the observed alterations in podocytes during insults, influencing the development and progression of renal damage.

4. ISOLATION AND CHARACTERISATION OF PODOCYTE EVs

Although there is no single technique for EV isolation, the method employed in this study, differential ultracentrifugation, is widely used for separating EVs from various sample types (Consortium *et al.* 2017, Witwer *et al.* 2021). As there is no standardised technique for EVs isolation, a fundamental criterion when describing EVs preparations is the characterisation through diverse approaches to ensure that isolation has been carried out properly. In this context, fundamental guidelines and databases, such as "EV-TRACK" and the Minimal Information for Studies of Extracellular Vesicles (MISEV), have been developed (Thery *et al.* 2018). The utilization of information provided by authors on the isolation and characterisation of EVs is integral to a field-consensus rigor initiative by the International Society for Extracellular Vesicles (ISEV).

The proper isolation of an enriched population of EVs from the supernatant of human immortalised podocyte cultures has been confirmed through various techniques, including immunogold TEM and NTA (Nanosight technology). TEM images show EVs with very similar morphology and size, consistent with literature descriptions (Huang *et al.* 2020, Jeon *et al.* 2020). Additionally, immunogold TEM revealed positive signal for tetraspanins CD9 and CD63 on the surface of EVs. Meanwhile, size profiles obtained by Nanosight and image size of EVs showed vesicles around 100 nm, demonstrating the correct separation of EVs.

Additionally, we conducted a characterisation of EVs protein markers by Western blot. We utilised membrane markers (CD9 and CD63 tetraspanins) and those associated with endosomal transport (TSG-101) to ensure that we were working with fractions enriched in exosomes as well as syntenin-1, which has been shown to interact with several binding partners that are involved in exosome biogenesis. Similar to other studies which employed the same podocyte cell line (Su

et al. 2020), the presence of these markers was identified in our EVs preparations. Furthermore, as previously reported by our group, we employed other markers such as calnexin (endoplasmic reticulum marker), and GM-130 (Golgi apparatus marker) to verify that no fragments or subcellular organelles were carried over during the process of the differential centrifugation (Perez-Hernandez *et al.* 2015).

In the experiments conducted in Prof. Bussolati's laboratory, the ultracentrifugation protocol entailed a single step at 38,000 rpm, precipitating all types of EVs. In contrast, our group's standard experiments involved two ultracentrifugation steps, enabling the separation of only the smallest EVs. Consequently, it is anticipated that samples isolated using a single ultracentrifugation step may exhibit a greater diversity in EVs size. Despite this, super-resolution images of podocyte EVs revealed similar EVs measuring 100 nm. Caution is warranted when comparing data from different experimental protocols; investing time and effort in standardising isolation protocols for all experiments is essential. Additionally, experiments utilising MACSPlex kits for flow cytometry ensured accurate vesicle isolation, with detection limited to vesicles bound to tetraspanin markers CD9, CD63, and CD81. Notably, EVs from differentiated podocytes exhibited renal cell-specific markers, underscoring the importance of working with vesicles isolated from differentiated podocytes to closely mimic renal physiological conditions in our experiments.

While no singular technique dominates EVs isolation, this study employed differential ultracentrifugation, a prevalent method in the field. Indeed, we have meticulously validated EVs isolation from human immortalised podocyte cultures using diverse techniques.

5. MiRNA ANALYSIS IN RAB3A-RAB27A-SILENCED PODOCYTES AND THE EVS DERIVED FROM THEM

Involved in many cellular processes, miRNAs are important EV cargoes. Taking this into consideration, we investigated the levels of several miRNAs with involvement in DN and podocyte damage. In fact, the differential expression levels of miRNAs have been associated with renal damage and they been used as biomarkers of the disease and/or proposed as potential therapeutic targets (Perez-Hernandez *et al.* 2021, Perez-Hernandez *et al.* 2021, Dimuccio *et al.* 2022, Liu *et al.* 2022). Considering this, we sought to determine their levels in cellular pellets and EVs from podocytes silenced and treated with glucose. Our results showed that for both silenced Rabs, miR-21-5p cell pellet levels increased in both HG-treated podocytes, suggesting their involvement in podocyte damage as positive or negative influencers. Interestingly, in exosomes from *RAB3A* silenced podocytes, miR-21-5p levels were decreased in HG siRAB3A, pointing to a decrease in this miRNA loading to vesicles when RAB3A is silenced and treated, whereas under silencing with no treatment, its levels were greatly increased. MiR-21-5p is one of the most characterised miRNAs and has demonstrated a pathological role in renal physiology, although with certain controversy (Krichevsky and Gabriely 2009, Larrue *et al.* 2022). Increased levels of miR-21-5p have been observed in mesangial and tubular cells from a diabetic mice model. Furthermore, this was associated with microalbuminuria, inflammation, and renal fibrosis (Zhong *et al.* 2013). However, in another study, streptozotocin-induced diabetic mice showed increased kidney levels of miR-21, and lack of miR-21 was associated with accelerated glomerular injury and increased proteinuria, with a reduction in podocyte density, which leads to the conclusion that miR-21 ameliorates TGF- β 1 and hyperglycaemia-induced glomerular injury through repression of pro-apoptotic signals (Lai *et al.* 2015).

TGF- β 1 signalling pathway is intrinsically involved in various cellular processes, particularly in the context of DN. A specific microRNA, miR-26a-5p, has emerged as a significant player within this pathway. Notably, miR-26a-5p levels were elevated in podocytes treated with HG and in groups where Rabs were silenced. This microRNA has garnered attention for its potential protective role against diabetes and DN and is being explored as a potential therapeutic target (Fu *et al.* 2015, Zheng *et al.* 2016, Jiang *et al.* 2020, Perez-Hernandez *et al.* 2021). In this way, Koga *et al.* reported that downregulation of miR-26a favours progression to DN by enhancing TGF- β /CTGF signalling (Koga *et al.* 2015). Conversely, miR-26a-5p levels in exosomes were potentially downregulated in the HG group that was partially recovered in the HG siRab3A group. These results coincide with those of Li *et al.*, who found decreased levels of this miRNA in exosomes from HG-treated fibroblasts (Li *et al.* 2021). Additionally, we found similar results in urinary exosomes from hypertensive patients with renal damage and in exosomes from TGF- β 1-treated podocytes (Perez-Hernandez *et al.* 2021).

Finally, miR-31-5p was increased in the EVs in the HG group, and its levels decreased significantly with Rab silencing, as well as in treated and silenced podocytes, which suggests that Rab silencing seems to influence miR-31-5p expression in podocytes. Although little evidence has been accumulated to support this Rab system as a regulator of miR-31-5p, one study proposed Rab27A as a target for miR-31-5p, together with target prediction tools such as Targetscan, a line which deserves further investigation (Asangani *et al.* 2012). Moreover, this miRNA has been linked with DN and was proposed as a serum disease biomarker related with inflammation (Rovira-Llopis *et al.* 2018).

Summarising, miR-21-5p showed varied expression patterns in podocytes based on Rab silencing and HG treatment. Meanwhile, miR-26a-5p, known for its protective potential against DN, exhibited altered levels in HG-treated and Rab-

silenced conditions, aligning with its significance in renal damage pathways. Additionally, miR-31-5p levels were influenced by Rab silencing, hinting at the potential regulatory role of Rabs on this miRNA, with implications for DN.

This doctoral thesis highlights the roles of GTPases Rab3A and Rab27A in DN. Alterations in these GTPases were found in urine cell pellets from human patients. Indeed, we have described GTPases Rab3A and Rab27A as a key participant in podocyte injury. Transitory gene silencing of *RAB3A* or *RAB27A* molecules under hyperglycaemic conditions resulted in a compensatory increase in the mRNA of the other Rab GTPase, suggesting their cooperation and identity as a system. While silencing impacted podocyte cytoarchitecture and vesicle distribution, it also mitigated apoptosis and improved differentiation under glucose stress (Fig. 40). Moreover, miRNAs relevant for DN showed altered expression under silencing and glucose treatment. Our results suggest that Rab3A-Rab27A system may orchestrate the observed alterations in podocytes during insults, influencing the development and progression of renal damage in DN.

Rab3A-Rab27A system silencing has beneficial effects in HG derived podocyte injury and vesicular traffic regulation

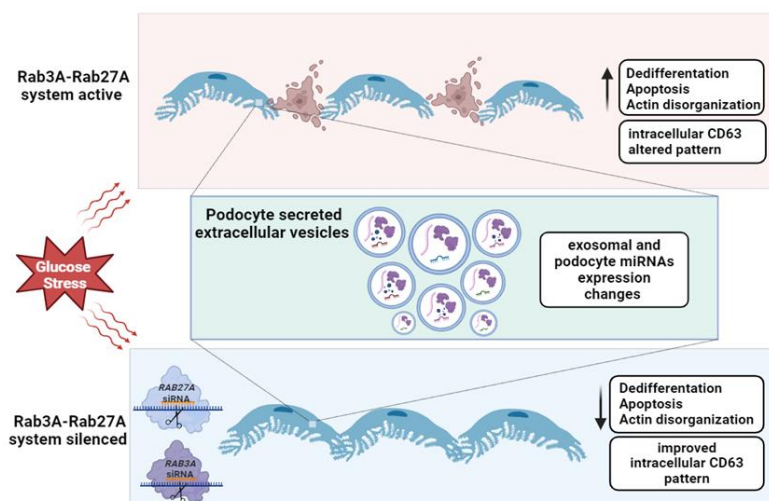


Figure 40. Schematic illustration of siRab3A and siRab27A effect in human immortalised podocytes. Image created with BioRender.com

6. STUDY LIMITATIONS

The results of this thesis should be interpreted within the framework of several limitations. Accessing biopsies from patients with DN is challenging. Due to this difficulty, working with primary cells derived directly from these biopsies becomes impractical for many researchers. Moreover, while cells *in vitro* offer a valuable model, they may not precisely replicate the physiological conditions of a human diabetic kidney. Additionally, we observed that the use of siRNA for transient transfections imposes a time constraint on the effectiveness of silencing, limiting the duration of the treatment with HG and Ang-II. However, despite these limitations, this is an experimental study, employing human immortalised podocyte cell line widely used by researchers in this field, allowing to compare the reproducibility of our data (Saleem *et al.* 2002, Liu *et al.* 2018, Ma *et al.* 2023). This is still considered a robust approach for studying cellular mechanisms under controlled conditions that has allowed significant progress in studying the effect of hyperglycaemia on podocytes. Therefore, this study serves as a reference point for future experiments focused on the impact of the Rab3A-Rab27A system in kidney podocytes and its influence on vesicular traffic and in the cargo that EVs transport.

Regarding EVs results, we should be particularly cautious with this novel field of research, since standardised procedures are still needed to make studies comparable, for example, in which cell line should be used, number of secretory cells and the selection of EVs isolation and characterisation techniques. The isolation method of EVs introduces a certain degree of imprecision in studies, as the differential ultracentrifugation fails to completely separate EVs from other protein aggregates that sediment alongside them.

7. FUTURE PERSPECTIVES

The findings from this doctoral thesis offer a deeper insight into the molecular processes underlying podocyte damage in DN. These insights hold potential for identifying novel targets that could enhance clinical interventions and pave the way for developing new therapeutic approaches and refining the management strategies for treating podocyte injury in patients with DN.

The impact of altered Rabs expression is less understood in specific organs where vesicular trafficking has a high relevance on their structure and function, such as in the kidney. Looking ahead, research on Rab GTPases like Rab3A and Rab27A in renal disease, along with their role in regulating vesicular trafficking, sets the stage for promising future studies. This could be particularly relevant for examining the roles of these GTPases in DN and, intriguingly, in patients with early-stage renal damage. Future studies could delve into the dynamic interplay between Rab GTPases and the biogenesis, secretion, and uptake of EVs. Additionally, advancements in imaging techniques and molecular tools may provide unprecedented insights into the spatiotemporal regulation of Rab GTPase-mediated vesicular trafficking. Understanding the intricate mechanisms governing these processes is essential not only for deciphering fundamental cell biology but also for exploring the therapeutic potential of manipulating Rab GTPases in the context of EV-mediated communication in several diseases. Moreover, the integration of omics approaches and systems biology is anticipated to unravel comprehensive networks and pathways involving Rab GTPases and EVs, paving the way for targeted interventions in various physiological and pathological conditions.

Importantly, exploring the small non-coding RNA landscape within EVs present in a non-invasive biofluid like urine, offers an intricate molecular signature reflective of the dynamic cellular processes underlying kidney pathophysiology. In

this context, the application of advanced high-throughput sequencing technologies and bioinformatics has enabled researchers to comprehensively decode the complete small non-coding RNA repertoire within EVs in the recent years. This is why we have proposed to sequence the small non-coding RNAs inside EVs isolated from overload HG podocytes silenced for Rab3A and Rab27A genes. This omic analysis holds tremendous promise for advancing our understanding of the role of EV-packaged small RNAs in intercellular communication within the renal microenvironment, and explore the role of Rab3A-Rab27A system in this process.

VI. CONCLUSIONS

“I have also found that these [water of consumptives] when placed on the fire, soon become milky, really smelled like milk, and had the taste of sweet milk; when a drop or two of acetic acid were added to this, and it was then placed in the cool air, shortly a white rennet, namely the caseous part, sank to the bottom, and the oily or buttery part floated up and there was a sweet whey, quite homogeneous, free now of the different parts; wherefrom we might conclude that this urine is a kind of clear and very thin watery chyle, or nourishing fluid”.

First description of albuminuria-1694

Frederick Dekkers

1. This study, for the first time, demonstrates the presence of the Rab3A-Rab27A system in human urine cell pellet samples. Furthermore, an upward trend in mRNA expression levels of the Rab3A-Rab27A system in samples from patients with HTN and T2DM with renal injury has been described. Both Rab GTPases showed a positive association with UAE.
2. It has been described the presence of Rab3A-Rab27A system in a cell line of human immortalised podocytes. The observed increase and altered distribution patterns of the Rab3A-Rab27A system in podocytes treated with glucose and Ang-II suggest that there may be an enhanced vesicular trafficking process occurring through the Rab3A-Rab27A system during podocyte injury in diabetic nephropathy.
3. Under glucose stress conditions, silencing either of the two components of the Rab3A-Rab27A system leads to a compensatory increase in mRNA of the other component. Additionally, silencing Rab3A or Rab27A induces changes in the expression of mRNA and protein levels of podocyte markers under both normal and high glucose conditions, suggesting their involvement in maintaining podocyte cytoarchitecture and implication in dedifferentiation processes under glucose stress. Furthermore, inhibiting Rab3A-Rab27A system in podocytes reduced the apoptotic cell death caused by hyperglycaemic conditions and restored the distribution of CD63 vesicle marker.
4. A proper isolation and characterisation of EVs from human immortalised podocytes through ultracentrifugation has been verified by several techniques. Additionally, characterisation of several proteins presents on the EVs surface showed that EVs isolated from undifferentiated or differentiated cells were different.

5. The levels of miRNAs relevant for DN such as miR-21-5p, miR-26a-5p and miR-31-5p, have been altered in podocytes after the silencing of *RAB3A* or *RAB27A* and the subsequent treatment with HG. Changes in the miRNAs levels enclosed in EVs isolated from high glucose and *RAB3A* silenced cells have been also described.

VII. BIBLIOGRAPHY

“These tubes consist of an external tunic of transparent homogeneous tissue (which I have termed the basement membrane) lined by epithelium. The Malpighian bodies, I saw to be a rounded mass of minute vessels invested by a capsule of precisely similar appearance to the basement membrane of the tubes. Seeing these similar tissues in such close proximity, it was not easy to resist the conviction that the capsule was the basement membrane of the tubes expanded over the vessels, but, after many trials, I could not at that time succeed in gaining an unequivocal view of their continuity. All that I could accomplish was to perceive here and there an ambiguous approach to such an arrangement sufficient to make it appear probable”.

On the structure and use of the Malpighian bodies of the kidney, with observations on the circulation through that gland- 1842

William Bowman

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VIII. ANNEXES

ANNEX I

Table I. Cell culture reagents and their suppliers.

REAGENT	COMPANY
Roswell Park memorial institute (RPMI) 1640	Biowest
Fetal bovine serum (FBS)	Biowest
Insulin-Transferrin-Selenium (ITS)	Gibco
Penicillin/streptomycin (P/S)	Biowest
Phosphate-buffered saline (PBS, pH 7.4) Gibco	Sigma-Aldrich
Trypsin-Ethylenediaminetetraacetic acid (EDTA) 0.25%	Gibco
D-glucose	Sigma-Aldrich
OptiMem	Gibco
Lipofectamine	Invitrogen
Collagen-I	Gibco
Dulbecco's Modified Eagle Medium	Life Technologies
Ham's F-12 nutrient mix	Life Technologies
GlutaMAX	Life Technologies
Epidermal growth factor	Sigma-Aldrich
Hydrocortisone	Sigma Aldrich

ANNEX II

For the study of gene silencing of *RAB3A* and *RAB27A* in podocytes, the transfection efficiency was previously tested. For this purpose, the Cy3 transfection control with red fluorescent labeling was used to assess the internalization of liposomes, and the positive control *GAPDH* small interfering RNA (siRNA) was used to evaluate the successful gene silencing through RNA interference in podocytes (Fig. I). In section A, the Cy3 control labeling is observed inside the podocytes at a lipofectamine concentration of 3nM, indicating that the liposomes with the corresponding siRNA are internalised and that the transfection protocol is efficient at that concentration. On the other hand, in section B, the expression levels of *GAPDH* mRNA are observed in the CNT scr samples and in cells transfected with *GAPDH* siRNA, showing a decrease in expression in the latter (fold change -8.93, $p < 0.01$), indicating that the silencing protocol is correct.

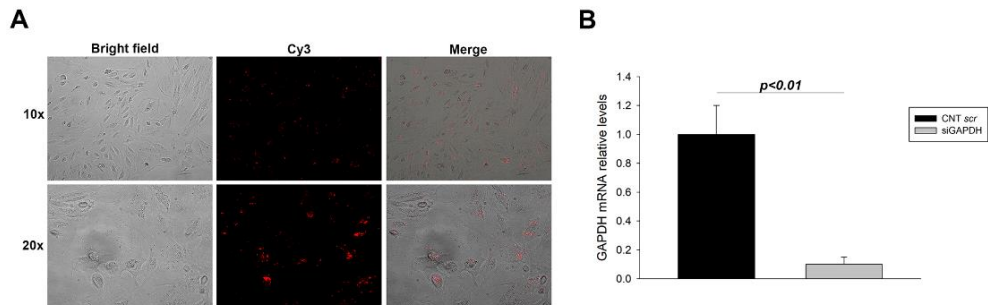


Figure I. Evaluation of transfection efficiency in podocytes. A. Bright-field and fluorescence images at different magnifications (10x and 20x) of podocytes transfected with Cy3 control. B. *GAPDH* mRNA levels. CNT scr, scramble control; siGAPDH, siRNA against the *GAPDH* gene. Data are presented as mean $\pm$ SE; $n=4$ for each group. mRNA levels are expressed as fold change, with values from the CNT scr group set to 1.

ANNEX III

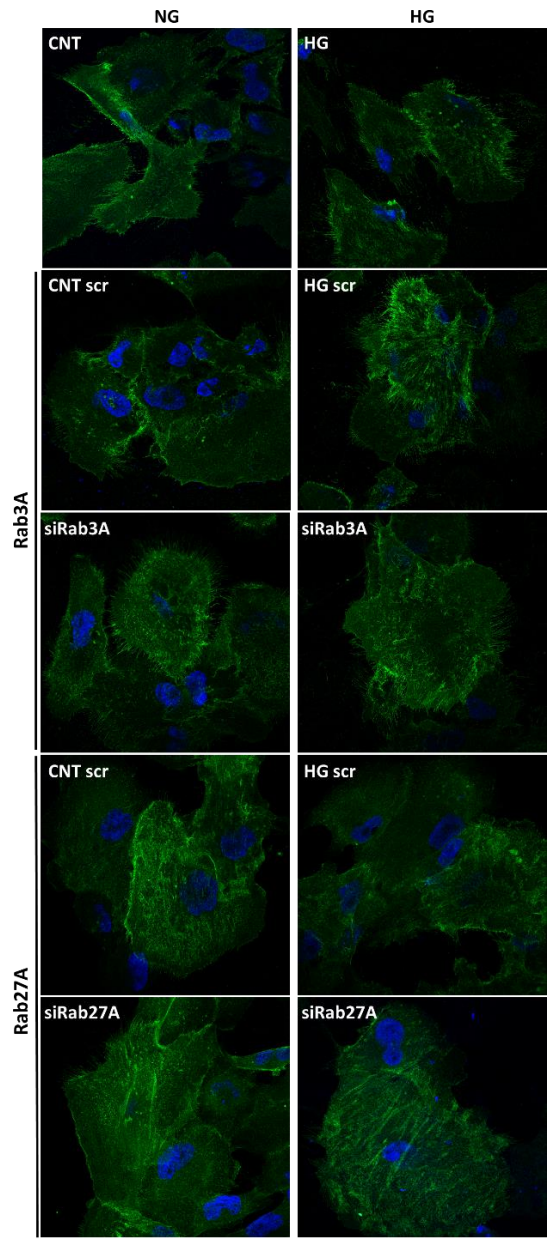


Figure III. Analysis of CD9 intensity and distribution in silenced and HG overload-treated podocytes: Confocal images of podocytes treated with siRab3A and siRab27A and under glucose overload, showing CD9 staining and cell distribution. CNT: control; CNT scr: control scramble; NG: normal glucose; HG: high glucose; siRab3A: Rab3A siRNA siRab27A: Rab27A siRNA.

ANNEX IV

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