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CONEXIÓN ENTRE METABOLISMO ÓSEO Y SISTEMA VASCULAR EN DIABETES MELLITUS TIPO 2. IDENTIFICACIÓN DE NUEVAS DIANAS TERAPÉUTICAS.

Memoria presentada por **Sheila González Salvatierra** para optar a la mención de Doctor Internacional por la Universidad de Granada.

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Prof. Manuel Muñoz Torres
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Granada, 2024

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"Nada en la vida debe ser temido, solamente comprendido.
Ahora es el momento de comprender más para temer menos".
Marie Curie

A mi madre y hermana,
A Fran,
Y a mi gato Príncipe

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LISTA DE ABREVIATURAS

ACV	Accidente cerebrovascular
ADA	Asociación Americana de Diabetes
AG	Ácidos grasos
AGEs	Productos de glicación avanzada
agLDL	Lipoproteínas agregadas de baja densidad
AGLs	Ácidos grasos libres
ALP	Fosfatasa alcalina
AOPPs	Productos proteicos de oxidación avanzada
APC	Poliposis coli adenomatosa
BMP	Proteínas morfogenéticas óseas
CamKII	Proteína quinasa II dependiente de calmodulina
CK1	Caseína quinasa 1
CMLV	Células del músculo liso vascular
Cβ	Células β
DAG	Diacilglicerol
Dkk1	Dickkopf 1
DMO	Densidad mineral ósea
DM2	Diabetes mellitus tipo 2
DPP4i	Inhibidores de la dipeptidil peptidasa 4
Dsh	Dishevelled
DXA	Absorciometría radiológica de doble energía
EAC	Enfermedad arterial coronaria
EAP	Enfermedad arterial periférica
EC	Enfermedad cerebrovascular
ECV	Enfermedades cardiovasculares
ERC	Enfermedad renal crónica
ERD	Enfermedad renal diabética
ERT	Enfermedad renal terminal
FRPs	Proteínas asociadas al receptor Fzd
Fzd	Frizzled
GIP	Péptido inhibidor gástrico
GLP-1	Péptido similar al glucagón tipo 1

GLP-1a	Agonistas del péptido 1 similar al glucagón
GLUT1	Transportador de glucosa 1
GLUT2	Transportador de glucosa 2
GLUT4	Transportador de glucosa 4
GSK3	Glucógeno sintasa quinasa-3
HDL	Lipoproteínas de alta densidad
HSA	Análisis estructural de la cadera
IAPP	Polipéptido amiloide de los islotes
IDF	*International Diabetes Federation*
IGF-1	Factor de crecimiento similar a la insulina-1
IL-1	Interleucina 1
IL-6	Interleucina 6
IMC	Índice de masa corporal
IP3	Inositol trifosfato
IRS	Receptores de insulina
JNK	Quinasa Jun
LDL	Lipoproteínas de baja densidad
LEF	Factor de unión al potenciador linfoide
LPA	Ácido lisofosfatídico
LRP5/6	Proteína relacionada con el receptor de lipoproteínas de baja densidad 5 y 6
MAPK	Proteína quinasa activada por mitógenos
MGP	Proteína GLA de matriz
NADPH	Nicotinamida adenina dinucleótido fosfato
NF-κβ	Factor nuclear-kappa beta
NFAT	Factor nuclear asociado a las células T
NO	Óxido nítrico
NOS	Óxido nítrico sintasa
OC	Osteocalcina
OPG	Osteoprotegerina
OPN	Osteopontina
oxLDL	Lipoproteínas de baja densidad oxidadas
PA	Ácido fosfatídico
PCP	Polaridad celular plana
PI3K	Fosfatidilinositol 3-quinasa

PKA	Proteína quinasa A
PKB	Proteína quinasa B
PKC	Proteína quinasa C
PLC	Fosfolipasa C
PPARγ	Receptores gamma activados por el factor proliferador de peroxisomas
pQCT	Tomografía computarizada cuantitativa periférica
PTH	Parathormona
RAGE	Receptor de AGEs
RANK	Receptor activador del factor nuclear ϰβ
RANKL	Ligando del receptor activador para el factor nuclear ϰβ
ROCK	Quinasa asociada a Rho
RUNX2	Factor de transcripción 2 relacionado con Runt
SGLT2i	Inhibidores del cotransportador 2 de sodio y glucosa
SLRP	Proteoglicanos pequeños ricos en leucina
SM	Músculo liso
SMαA	SM α-actina
TBS	*Trabecular Bone Score*
TCF	Factor derivado de células-T
TFGe	Tasa de filtrado glomerular estimada
TGs	Triglicéridos
TNFα	Factor de necrosis tumoral alfa
TRAIL	Ligando citotóxico inductor de apoptosis relacionado con TNF
TZD	Tiazolidinedionas
VEGF	Factor de crecimiento endotelial vascular
VLDL	Lipoproteína de muy baja densidad

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RESUMEN/ABSTRACT

RESUMEN

La diabetes mellitus tipo 2 (DM2) se ha catalogado como la epidemia del siglo XXI. Esta enfermedad metabólica crónica representa una de las más importantes emergencias sanitarias a nivel mundial, debido a su acelerado crecimiento de prevalencia y a su elevada tasa de mortalidad causada por complicaciones asociadas, entre las que destacan las enfermedades cardiovasculares (ECV).

Cada vez hay una mayor evidencia científica que respalda la existencia de un eje de conexión entre el metabolismo óseo y el sistema vascular. En concordancia, existe una elevada prevalencia de pacientes con DM2 que padecen fragilidad ósea y ECV de forma simultánea, poniendo de manifiesto la presencia de mecanismos fisiopatológicos comunes entre ambas patologías. Este hecho ha generado un interés significativo en la identificación de biomarcadores relacionados con el metabolismo óseo en el contexto de las enfermedades vasculares. Sin embargo, en la actualidad, los conocimientos sobre los mecanismos de conexión específicos que vinculan el metabolismo óseo y el sistema vascular siguen siendo limitados.

En este contexto, este trabajo de investigación profundiza en el estudio de proteínas implicadas en el metabolismo óseo que desempeñan además un papel fundamental en la fisiopatología de la DM2 y en el desarrollo de ECV asociadas a la misma. En particular, esta Tesis Doctoral se centra en el estudio de la función de osteoglicina y esclerostina, ya que se hipotetiza que estas proteínas óseas juegan un importante papel a nivel vascular y que su modulación podría ser crucial tanto para el diagnóstico de ECV asociadas con la DM2 como para el desarrollo de nuevas estrategias terapéuticas y preventivas para abordar estas complicaciones.

Por un lado, en base a la controversia descrita en la literatura científica con respecto a la función de osteoglicina en las enfermedades vasculares, junto con la falta de comprensión sobre su papel en la homeostasis glucémica en humanos, se estudió la implicación de esta proteína en diversas ECV. En primer lugar, se evaluó su utilidad como biomarcador de la función renal alterada en DM2, para lo que se determinaron los niveles séricos de osteoglicina en pacientes con DM2 y tasa de filtrado glomerular estimada (TFGe) normal, en pacientes con DM2 y TFGe ligeramente reducida, y en sujetos control. Nuestro estudio transversal mostró un incremento de osteoglicina sérica en pacientes con DM2 y una TFGe ligeramente reducida, en comparación con los otros grupos de estudio, independientemente del sexo y la

edad. Además, los niveles circulantes de osteoglicina se asociaron de forma independiente con una función renal levemente alterada en los pacientes con DM2. Estos resultados sugieren que el nivel sérico de osteoglicina podría considerarse como un biomarcador indicativo de una incipiente disfunción renal en pacientes con DM2, independientemente de la presencia de albuminuria. Este hallazgo respalda la potencial utilidad clínica de la osteoglicina como una herramienta diagnóstica y preventiva para mitigar la progresión del daño renal en pacientes con DM2.

En segundo lugar, se analizó la implicación de la osteoglicina en el desarrollo de la aterosclerosis asociada a la DM2. En este caso, se determinaron los niveles de osteoglicina tanto en suero como en tejido vascular procedente de pacientes con DM2, con presencia/ausencia de ECV, así como de sujetos control, para analizar la asociación entre esta proteína y la aterosclerosis en DM2. Además, se evaluó el efecto de la sobreexpresión de osteoglicina en células del músculo liso vascular (CMLV) expuestas a condiciones calcificantes, simulando así el entorno fisiopatológico de la DM2. Nuestros resultados mostraron un aumento de los niveles séricos de osteoglicina en pacientes con DM2 en comparación con los controles no diabéticos; sin embargo, esta elevación no se asoció al desarrollo de aterosclerosis en esta población. Aunque, se mostró una posible relación entre la osteoglicina y marcadores de resistencia a la insulina como el nivel de triglicéridos y el índice de triglicéridos/colesterol de lipoproteínas de alta densidad; a nivel vascular, no se encontraron diferencias en la expresión de osteoglicina entre los diferentes grupos de estudio. Adicionalmente, los análisis *in vitro* revelaron que la sobreexpresión de osteoglicina en CMLV en condiciones calcificantes aumenta la proliferación celular, aunque no afecta a la apoptosis, y regula al alza la expresión de autotaxina, la cual está implicada en procesos inflamatorios. En base a estos resultados, sugerimos que la osteoglicina puede jugar un papel en la homeostasis glucémica, siendo un potencial biomarcador de resistencia a la insulina en pacientes con DM2. Además, aunque la osteoglicina no parece ser un determinante clave en la patogénesis de la aterosclerosis en pacientes con DM2 de forma directa, podría participar indirectamente en el desarrollo del proceso aterosclerótico a través de la activación de la vía autotaxina/ácido lisofosfatídico y la proliferación de las CMLV. Esto abre la puerta al estudio de la osteoglicina como potencial diana terapéutica en la DM2.

Por otra parte, se profundizó en la función de la esclerostina, una proteína antagonista de la vía Wnt/β-catenina inhibidora de la formación ósea, a nivel vascular. Recientemente se ha demostrado que su influencia no se limita exclusivamente a la regulación del metabolismo

óseo, sino que también desempeña un papel en la integridad vascular, actuando como un modulador crucial de la señalización de la vía Wnt/β-catenina en el contexto de las ECV. A pesar de estos avances, el papel específico de la esclerostina en las ECV aún no ha sido completamente esclarecido. Por tanto, en este trabajo científico analizamos por primera vez el papel perjudicial o protector de la esclerostina en el desarrollo de la aterosclerosis asociada a la DM2. Para ello, se cuantificaron los niveles de esclerostina en muestras séricas y de tejido vascular procedentes de pacientes con DM2, con presencia/ausencia de ECV, así como de sujetos control. Además, se evaluó el efecto de la sobreexpresión de esclerostina en CMLV expuestas a condiciones calcificantes, simulando en parte el entorno fisiopatológico de la DM2. Nuestros resultados demostraron un aumento de los niveles séricos de esclerostina en pacientes con DM2 y ECV en comparación con los controles sanos (especialmente en los varones). Además, los niveles séricos más elevados de esclerostina se asociaron de forma independiente con la ECV en pacientes con DM2 y se hallaron correlaciones significativas entre los niveles circulantes de esclerostina y factores de riesgo cardiovascular (edad, duración de la diabetes, TFGe, colesterol de lipoproteínas de baja densidad, calcio sérico y presión arterial diastólica) y la periostina. Además, se mostró un aumento de la expresión de esclerostina en los vasos calcificados procedentes de pacientes con DM2 y ECV en comparación con los vasos no calcificados de los sujetos de control. Por último, se observó una implicación de la esclerostina en la disminución de los depósitos de calcio, así como en la supervivencia celular y en la regulación de la expresión de diferentes marcadores óseos y de genes inflamatorios en las CMLV bajo condiciones calcificantes. Estos hallazgos sugieren que la esclerostina podría desempeñar un papel protector en el desarrollo de la aterosclerosis en pacientes con DM2. En base a estos resultados, el tratamiento con anti-esclerostina para la enfermedad ósea debe utilizarse con precaución.

En resumen, esta investigación traslacional aborda la comprensión de la función fisiopatológica de osteoglicina y esclerostina, destacando su impacto en la fisiología vascular. Este análisis resulta crucial para avanzar en el diagnóstico temprano de la ECV y en el desarrollo de estrategias preventivas y terapéuticas, abordando tanto las alteraciones en el metabolismo óseo como en el sistema vascular, mejorando así la calidad de vida de los pacientes con DM2.

ABSTRACT

Type 2 diabetes mellitus (T2D) has been catalogued as the epidemic of the 21st century. This chronic metabolic disease represents one of the most significant global health emergencies due to its accelerated prevalence growth and high mortality rate caused by associated complications, prominently cardiovascular diseases (CVD).

There is increasing scientific evidence supporting the existence of an axis of connection between bone metabolism and the vascular system. Accordingly, there is a high prevalence of patients with T2D who suffer from bone fragility and CVD, highlighting the presence of common pathophysiological mechanisms between both pathologies. This fact has generated significant interest in the identification of biomarkers related to bone metabolism in the context of vascular diseases. However, current knowledge of the specific connecting mechanisms linking bone metabolism and the vascular system remains limited.

In this context, this research delves into the study of proteins involved in bone metabolism that also play a fundamental role in the pathophysiology of T2D and in the development of CVD associated with it. In particular, this Doctoral Thesis focuses on the study of the function of osteoglycin and sclerostin, since it is hypothesized that these bone proteins play an important role at the vascular level and that their modulation could be crucial for the diagnosis of CVD associated with T2D and for the development of new therapeutic and preventive strategies to address these complications.

On the one hand, based on the controversy described in the scientific literature regarding the function of osteoglycin in vascular diseases, together with the lack of understanding of its role in glycemic homeostasis in humans, the involvement of this protein in several CVD was studied. First, we evaluated its usefulness as a biomarker of impaired kidney function in T2D by determining serum osteoglycin levels in T2D patients and normal estimated glomerular filtration rate (eGFR), in T2D patients and mildly decreased eGFR, and in control subjects. Our cross-sectional study showed an increase in serum osteoglycin level in T2D patients and mildly decreased eGFR compared to the other groups, independent of sex and age. In addition, circulating osteoglycin levels were independently associated with mildly impaired kidney function in patients with T2D. These results suggest that serum osteoglycin level could be considered as a biomarker indicative of incipient kidney dysfunction in patients with T2D, independent of the presence of albuminuria. This finding supports the potential

clinical utility of osteoglycin as a preventive tool to mitigate the progression of kidney damage in patients with T2D.

Second, the involvement of osteoglycin in the development of atherosclerosis associated with T2D was analyzed. In this case, osteoglycin levels were determined in both serum and vascular tissue from patients with T2D, with presence/absence of CVD, as well as in control subjects, to analyze the association between this protein and atherosclerosis in T2D. In addition, we evaluated the effect of osteoglycin overexpression in vascular smooth muscle cells (VSMCs) under calcified environments, simulating the pathophysiological environment of T2D. Our results showed a higher in serum osteoglycin levels in T2D patients compared to non-diabetic controls; however, this increase was not associated with the development of atherosclerosis in this population. Although, a possible relationship was shown between osteoglycin and markers of insulin resistance such as triglyceride level and triglyceride/high-density lipoprotein cholesterol ratio. At the vascular level, no differences in osteoglycin expression were found between the different study groups. Additionally, *in vitro* analyses revealed that osteoglycin overexpression in VSMCs under calcified environments increases cell proliferation, although it does not affect apoptosis, and upregulates the expression of autotaxin, which is involved in inflammatory processes. Based on these results, we suggest that osteoglycin may play a role in glycemic homeostasis, being a potential biomarker of insulin resistance in patients with T2D. Furthermore, although osteoglycin does not appear to be a key determinant in the pathogenesis of atherosclerosis in patients with T2D directly, it could participate indirectly in the development of the atherosclerotic through the activation of the autotaxin/lysophosphatidic acid pathway and the proliferation of VSMCs. This opens the door to the study of osteoglycin as a potential therapeutic target in T2D.

On the other hand, the function of sclerostin, an antagonist protein of the Wnt/β-catenin pathway that inhibits bone formation, has been further studied at the vascular level. Recently, it has been shown that its influence is not exclusively limited to the regulation of bone metabolism, but also plays a role in vascular integrity, acting as a crucial modulator of Wnt/β-catenin pathway signaling in the context of CVD. Despite these advances, the specific role of sclerostin in CVD has not yet been fully elucidated. Therefore, in this scientific study we analyzed for the first time the detrimental or protective role of sclerostin in the development of atherosclerosis associated with T2D. To this end, sclerostin levels were quantified in serum and vascular tissue samples from patients with T2D, with presence/absence of CVD, as well as in control subjects. In addition, we evaluated the effect of sclerostin overexpression in

VSMCs under calcified environments, simulating *in vitro* the pathophysiological environment of T2D. Our results demonstrated increased serum sclerostin levels in patients with T2D and CVD compared to healthy controls (especially in males). Furthermore, higher serum sclerostin levels were independently associated with CVD in patients with T2D and significant correlations were found between serum sclerostin levels and cardiovascular risk factors (age, duration of diabetes, eGFR, low-density lipoprotein cholesterol, serum calcium, and diastolic blood pressure) and periostin. Furthermore, increased sclerostin expression was shown in calcified vessels from patients with T2D and CVD compared to non-calcified vessels from control subjects. Finally, we observed an involvement of sclerostin in the decrease of calcium deposition, as well as in cell survival and in the regulation of the expression of different bone markers, and of inflammatory genes in VSMCs under calcifying conditions. These findings suggest that sclerostin could play a protective role in the development of atherosclerosis in patients with T2D. Based on these results, anti-sclerostin treatment for bone disease should be used with caution.

In summary, this translational research focuses on understanding the pathophysiological function of osteoglycin and sclerostin, highlighting their impact on vascular physiology. This analysis is crucial to advance in the early diagnosis of CVD and in the development of preventive and therapeutic strategies, addressing both alterations in bone metabolism and in the vascular system, thus improving the quality of life of patients with T2D.

INTRODUCCIÓN

1. Diabetes mellitus tipo 2

La diabetes mellitus tipo 2 (DM2) es una enfermedad metabólica crónica caracterizada por una deficiencia en la producción de insulina originada por una disfunción de las células β (Cβ)-pancreáticas y/o por una resistencia a la insulina en los órganos diana. Estas causas provocan una hiperglucemia secundaria que a lo largo del tiempo origina daño en los tejidos, dando lugar a complicaciones de salud graves e incluso potencialmente mortales (1).

1.1. Epidemiología

Actualmente, la DM2 representa una de las más importantes emergencias sanitarias a nivel mundial, debido a su rápido crecimiento y a su elevada tasa de mortalidad causada por complicaciones asociadas (2). De hecho, la DM2 se ha llegado a catalogar como la epidemia del siglo XXI.

La *International Diabetes Federation* (IDF) estimó que, a nivel mundial en 2021, 537 millones de personas padecían diabetes, 541 millones tenían intolerancia a la glucosa y, que más de 6,7 millones de personas de entre 20 y 79 años fallecieron por causas relacionadas con la diabetes. Además, se prevé un incremento de personas que padecerán diabetes a nivel mundial, estimándose aproximadamente 643 millones en 2030 y 783 millones en 2045 (Figura 1)(3). Este aumento de la prevalencia de la diabetes es multifactorial, atribuyéndose en parte al envejecimiento de la población, a la mayor supervivencia de estos pacientes gracias a la mejora de la atención médica, y a los cambios en el estilo de vida relacionados con el aumento de la urbanización, con comportamientos sedentarios y perfiles de alimentación poco saludables que conducen a un aumento de la obesidad (4).

Por otro lado, la prevalencia estimada de la diabetes en mujeres de 20 a 79 años es ligeramente inferior a la de los hombres. En 2021, había 17,7 millones más de hombres que de mujeres que padecían esta enfermedad a nivel mundial (Figura 2).

Cabe resaltar, que una proporción sustancial de individuos con diabetes no está diagnosticada. En Europa, este grupo representa el 37,9% del total de diabéticos, que, aunque es uno de los más bajos del mundo, significa que 22 millones de estas personas no diagnosticadas tienen un mayor riesgo de desarrollar enfermedades cardiovasculares (ECV) (4), las cuales son la principal causa de morbilidad y mortalidad asociada a la DM2 (5,6)

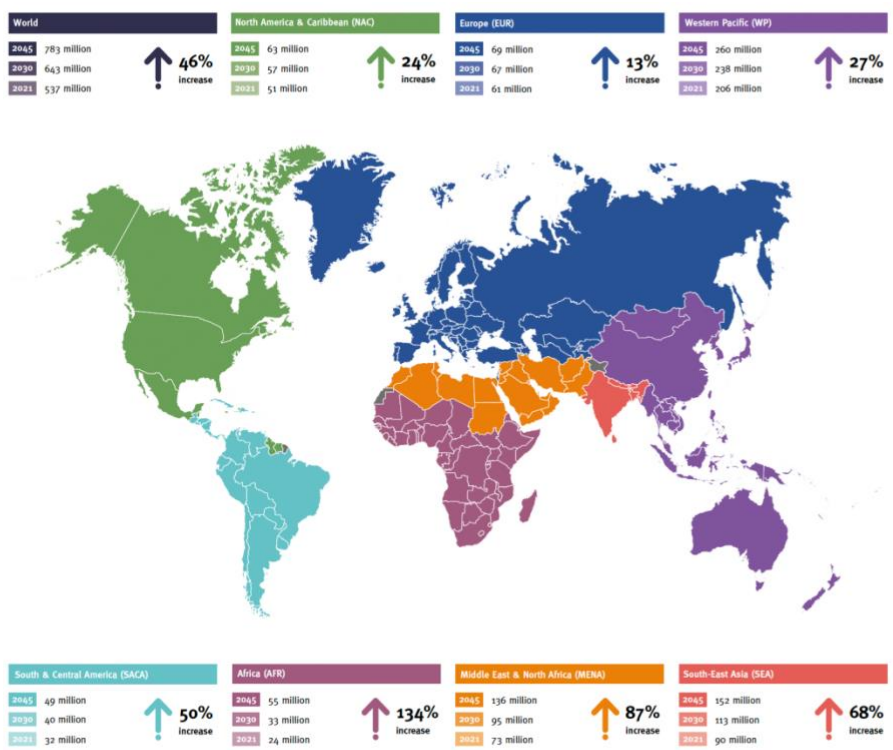

Figura 1. Atlas del número de personas con diabetes a nivel mundial, indicadas por región, en 2021-2045 (20-79 años) de la *International Diabetes Federation* (IDF) (10ª edición) (3).

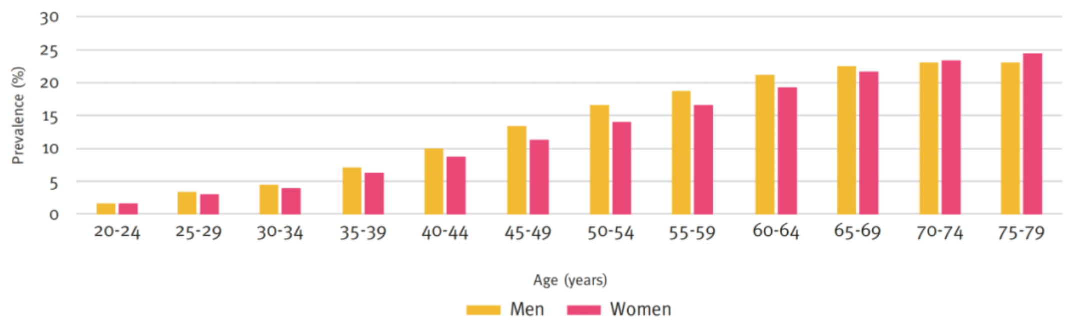

Figura 2. Prevalencia de la diabetes por sexos (20-79 años) en 2021, de la *International Diabetes Federation* (IDF) (10ª edición) (3).

En España, se observa un incremento de la prevalencia de DM2 en los últimos años y, actualmente, se estima que alcanza aproximadamente el 14,8% de la población total, posicionándose así, como el segundo país con la tasa más alta de Europa. Además, el estudio dia@bet.es llevado a cabo por CIBERDEM, halló que más de la mitad de los casos detectados correspondían a diabetes no diagnosticada (7), mostrando que la incidencia estimada de diabetes en España es de 11,6 casos/1000 personas-año, de los cuales el 7,9 casos/1000 personas-año desconoce que sufre esta patología. Además, muestran que un 8,5% adicional de españoles estarían en riesgo de desarrollar DM2. Por otro lado, también

indican que la incidencia de la diabetes aumenta con la edad y se encuentra incrementada en hombres (8).

1.2. Fisiopatología de la diabetes mellitus tipo 2

La DM2 se clasifica como un trastorno metabólico generalizado que implica la interacción entre factores ambientales y genéticos, siendo la hiperglucemia el principal desequilibrio bioquímico. Dos mecanismos principales se deben tener en cuenta en el desarrollo de la DM2: por un lado, una disminución en la secreción de insulina por parte de las Cβ-pancreáticas, y, por otro lado, un estado de resistencia a la insulina en los tejidos diana, como el músculo, el hígado y el tejido adiposo. Estos mecanismos se pueden dar de forma individual o simultánea en el organismo.

1.2.1. Disfunción de las células β-pancreáticas

Las Cβ son células especializadas del páncreas, localizadas específicamente en los islotes de Langerhans y responsables de la producción y secreción de insulina, hormona crucial en la regulación del metabolismo de la glucosa circulante.

En la DM2, la histología de los islotes de Langerhans comprende una masa de Cβ-pancreáticas reducida en un ~40% (rango 25-60%), una mayor deposición de amiloide y un contenido reducido de insulina pancreática en comparación con la de los islotes pancreáticos no diabéticos (9).

Como se ha descrito anteriormente en la DM2, la disfunción en la secreción de insulina se ha asociado a una reducción cuantitativa de las Cβ-pancreáticas. Sin embargo, el origen de esta disminución no está completamente esclarecido, pudiendo deberse a un desbalance neto negativo en la renovación de los islotes pancreáticos (10), lo que resulta en una pérdida de Cβ-pancreáticas. Por otra parte, el aumento de la apoptosis de Cβ-pancreáticas se debe a la hiperglucemia crónica (glucotoxicidad) y a los altos niveles de ácidos grasos libres (AGLs) (lipotoxicidad), que dan lugar a estrés oxidativo y a la activación de respuestas inflamatorias (11). Tanto las células T como los macrófagos activados segregan citocinas y quimiocinas proinflamatorias, lo que contribuye a la persistencia de las reacciones inflamatorias en el tejido y, posteriormente, al potencial subyacente de destrucción de las Cβ-pancreáticas mediada por la autoinmunidad (12). Además, el aumento de la apoptosis también se debe a un incremento de los depósitos amiloides que están formados por el polipéptido amiloide de los islotes (IAPP), que se agregan y desencadenan estrés (13). En consecuencia, estos factores

tienen un impacto negativo en la funcionalidad de las Cβ-pancreáticas, lo que contribuye a la reducción de la concentración de insulina y, por tanto, a un empeoramiento de la hiperglucemia.

Otro factor relacionado con la alteración de la secreción de insulina en las Cβ-pancreáticas involucra a las hormonas del tracto intestinal (incretinas), específicamente al péptido similar al glucagón tipo 1 (GLP-1) y al péptido inhibidor gástrico (GIP). Estas hormonas desempeñan un papel fundamental en la estimulación de la secreción de insulina en las Cβ-pancreáticas después de la ingesta de alimentos en sujetos sanos, contribuyendo aproximadamente al 70% de la insulina liberada. Sin embargo, en pacientes con DM2, se ha observado una reducción de la secreción insulínica entre un 30-70% en el estado postprandial, que puede deberse a una disminución en la secreción de GIP y GLP-1, o a una menor acción de estas hormonas sobre las Cβ-pancreáticas (14).

1.2.2. Resistencia a la insulina

La regulación de la homeostasis glucídica se lleva a cabo predominantemente, aunque no de forma exclusiva, mediante la acción de la insulina secretada por las Cβ-pancreáticas. Esta hormona se libera al torrente sanguíneo y se une a los receptores de insulina (IRS) de las células efectoras de los tejidos diana, principalmente en el hígado, el músculo y el tejido adiposo. Esta unión, desencadena una cascada de señales que tienen diversos efectos metabólicos, incluida la facilitación del transporte y el uso de la glucosa por parte de los tejidos diana (15,16). La insulina aumenta el transporte de glucosa a los miocitos a través del reclutamiento del transportador de glucosa 4 (GLUT4) a la membrana plasmática desde orgánulos intracelulares. GLUT4 es un transportador dependiente de insulina y está presente en todos los tejidos sensibles a esta hormona (Figura 3a). En el músculo esquelético y los adipocitos también se encuentra el transportador de glucosa 1 (GLUT1), un transportador basal de glucosa independiente a insulina. Además, en el hígado, las Cβ-pancreáticas y el epitelio gastrointestinal, se halla el transportador de glucosa 2 (GLUT2) que facilita el rápido equilibrio de la glucosa entre el espacio extracelular y el citosol celular. Por tanto, la resistencia a la insulina se manifiesta por una disminución del transporte y del metabolismo de la glucosa estimulados por la insulina en los adipocitos y el músculo esquelético, además de por una supresión deficiente de la producción hepática de glucosa (17).

Los mecanismos implicados en la resistencia a la insulina son multifactoriales y aún se están investigando las numerosas vías en las que se encuentra implicada esta hormona. Uno de los

mecanismos que conlleva a esta patología, tanto en el tejido adiposo como en el músculo, es que se reducen varios procesos como la unión de la insulina a su receptor, la fosforilación del receptor y la actividad tirosina quinasa, así como la fosforilación de los IRS (18). En los pacientes con DM2, en concreto en el músculo esquelético, los niveles de proteínas IRS-1 e IRS-2 son normales aunque la actividad de fosfatidilinositol 3-quinasa (PI3K), clave en la vía de señalización de la insulina y asociada a ambas IRS, se encuentra alterada (19), lo que limita la captación periférica de glucosa. Además, en el tejido adiposo de sujetos obesos resistentes a la insulina y específicamente en pacientes con DM2, se ha observado una reducción en la expresión de IRS-1 (20) y de GLUT4 (21), contribuyendo negativamente en las vías señalización de la insulina (Figura 3b).

Por otra parte, la insulina, además de su función en la regulación de la glucosa, juega un papel clave en el metabolismo del tejido adiposo, el cual es el principal sitio de almacenamiento de ácidos grasos (AG) que se liberan en la circulación durante la lipólisis como ácidos grasos no esterificados para ser utilizados como fuente de energía durante condiciones de ayuno (22). La principal acción de la insulina en los adipocitos es la estimulación de la adipogénesis, inhibiendo la lipólisis, estimulando la captación de glucosa y promoviendo la síntesis de triglicéridos (TGs) (15). Además, la insulina aumenta la oxidación y absorción de AGLs circulantes al estimular la actividad de la lipoproteína lipasa en el tejido adiposo (18). La obesidad provoca un aumento en el flujo de AGLs circulantes y su absorción por los miocitos o hepatocitos. Estos AG activados, conocidos como coenzima A acilada, se metabolizan principalmente por procesos de oxidación o almacenamiento. Cuando la cantidad de AG excede la capacidad de estos procesos para eliminar el coenzima A acilada, se acumulan intermediarios metabólicos como diacilglicerol (DAG), ácido fosfatídico (PA), ácido lisofosfatídico (LPA) y ceramidas. Estos productos intermedios pueden activar diversas serina quinasas que regulan negativamente la acción de la insulina. Adicionalmente, las ceramidas pueden afectar la función de la insulina a través de su interacción con Akt/proteína quinasa B (PKB). Además, se ha sugerido que la incapacidad para oxidar completamente los AG mediante la β-oxidación, lo que resulta en la acumulación de acilcarnitinas, puede contribuir a la resistencia a la insulina (23). Finalmente, los adipocitos también son capaces de secretar citocinas proinflamatorias, incluyendo el factor de necrosis tumoral alfa (TNFα) y la interleucina 6 (IL-6) (24), que tienen un papel importante en la disminución de la sensibilidad a la insulina por parte de los tejidos periféricos (25).

Además, la insulina también ejerce un potente efecto sobre el músculo liso arterial para promover la vasodilatación (26), lo que se traduce en un mayor aporte de insulina con glucosa a los miocitos poco perfundidos, y, por tanto, en una mejor eliminación de la glucosa ingerida. Cuando se activa el sistema de señalización de la insulina en las células del músculo liso vascular (CMLV) se activa la óxido nítrico sintasa (NOS), lo que produce una mayor cantidad de óxido nítrico (NO), que es un potente vasodilatador y molécula antiaterogénica (27). Así, la insulina tiene un efecto cardioprotector, mientras que la resistencia a la insulina afecta a la síntesis de NO, lo que acelera la enfermedad arterial coronaria y la hipertensión. Además, la hiperinsulinemia en casos de resistencia a la insulina estimula la vía de la proteína quinasa activada por mitógenos (MAPK), lo que aumenta la proliferación de las CMLV, la inflamación y la aterogénesis (28) (Figura 3b).

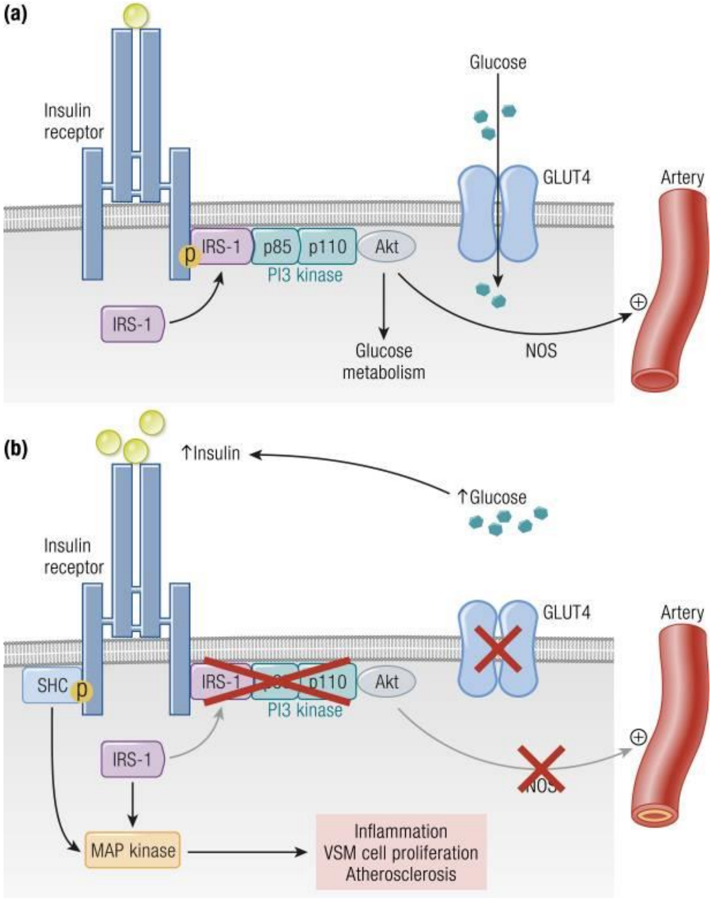

Figura 3. Sistema de transducción de señales de insulina en (a) individuos con tolerancia normal a la glucosa y en (b) individuos con resistencia a insulina (Di Pino y De Fronzo, 2019) (29). Akt, indica proteína quinasa B; GLUT4, transportador de glucosa 4; IRS-1, receptores de insulina-1; NOS, óxido nítrico sintasa; PI3 kinase, fosfatidilinositol 3-quinasa; SHC, Src colágeno homólogo; MAP kinase, proteína quinasa activada por mitógenos; VSM, músculo liso vascular.

2. Complicaciones asociadas a la diabetes mellitus tipo 2

La DM2 se caracteriza como un estado proinflamatorio y trombótico que se asocia al daño endotelial. La hiperglucemia, el exceso de AGLs y la resistencia a la insulina aumentan el estrés oxidativo, alteran la proteína quinasa C (PKC) y la transducción intracelular de señales, e incrementan los productos de glicación avanzada (AGEs), los productos proteicos de oxidación avanzada (AOPPs) y las lipoproteínas de baja densidad susceptibles de oxidación (oxLDL) (2). Como consecuencia de la alteración la señalización de la PKC se produce una disminución de la vasodilatación, un deterioro de la angiogénesis y un aumento de la adhesión de leucocitos a las células vasculares mediante la inhibición de la señalización PI3K (30). La activación de los receptores de la producción final de AGEs aumentan la producción endotelial de superóxido, lo que disminuye la activación de la NOS y suprime la producción de NO, provocando anomalías en la función de las células endoteliales vasculares (31). Además, el exceso de AGLs y glucosa contribuye a la disfunción mitocondrial y al aumento de radicales libres de oxígeno, resultando en una activación del factor nuclear-kappa beta (NF-ϰβ) y al aumento de la producción de endotelina-1 vasoconstrictora, del factor protrombótico tisular y de la producción del inhibidor-1 del activador del plasminógeno, que contribuyen a la inflamación vascular, la vasoconstricción, la trombosis y la aterogénesis (32,33). Por tanto, estos mecanismos subyacentes derivados de la hiperglucemia comprometen progresivamente la función de diversos órganos y sistemas desarrollando complicaciones secundarias asociadas a la DM2, que dan lugar a un significativo aumento de la mortalidad en estos pacientes.

2.1. Enfermedad cardiovascular

La DM2 es un factor de riesgo independiente para el desarrollo de ECV (34). Se estima que aproximadamente el 32,2% de los pacientes con DM2 sufren de ECV (35), y es responsable de una proporción sustancial de la morbilidad y la mortalidad (70-80%) en esta población (36). Los pacientes con DM2 tienen un riesgo dos veces mayor de desarrollar ECV en comparación con sujetos sin DM2, independientemente de los factores de riesgo convencionales (34).

La relación entre la DM2 y las ECV se debe en parte a una serie de factores de riesgo comunes en ambas patologías como la hiperglucemia, la resistencia a la insulina, la hipertensión arterial, la dislipidemia, la obesidad y el estado proinflamatorio (37). Como ya se ha descrito anteriormente, la hiperglucemia en la DM2 ocasiona un perfil lipoproteico

aterogénico, sintetizando AGEs, AOPPs y oxLDL y aumentando el estrés oxidativo, lo que crea un estado proinflamatorio y protrombótico que daña el endotelio y promueve la formación de lesiones ateroscleróticas (2). En cuanto a la resistencia a la insulina, ésta potencia la vía MAPK, lo que resulta en una disminución de la síntesis de NO y efectos aterogénicos (28). Con respecto a la hipertensión arterial, se encuentra una asociación con resistencia a la vasodilatación mediada por insulina, mayor sensibilidad a sustancias vasoconstrictoras, retención de sodio y proliferación de CMLV (38). Por lo que se refiere a la dislipemia, implica un aumento de las concentraciones de TGs y un incremento de los niveles de colesterol plasmático, que suele asociarse con a un aumento de colesterol de lipoproteínas de baja densidad (LDL), a un incremento de colesterol de lipoproteína de muy baja densidad (VLDL) y a una disminución en los niveles de colesterol de lipoproteínas de alta densidad (HDL). Estas alteraciones lipídicas están relacionadas con la resistencia a la insulina y promueven la formación de partículas oxLDL que dañan el endotelio y aceleran la aterogénesis (39). Por último, la obesidad, está estrechamente relacionada con la resistencia a la insulina. El tejido adiposo produce citocinas y mediadores inflamatorios que contribuyen al estado proinflamatorio y, por tanto, al desarrollo de ECV (24).

Las principales complicaciones cardiovasculares asociadas a la DM2, se clasifican dependiendo de si se encuentran afectadas las arterias de menor o mayor calibre, produciéndose complicaciones microvasculares como neuropatía, retinopatía, y nefropatía, o complicaciones macrovasculares como enfermedad arterial coronaria (EAC), enfermedad arterial periférica (EAP) y enfermedad cerebrovascular (EC) (Figura 4) (2,40).

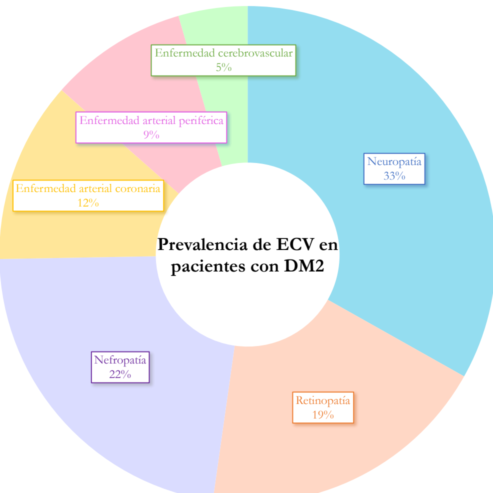

Figura 4. Principales complicaciones cardiovasculares asociadas a la DM2, indicando la prevalencia de estas en esta población. DM2, diabetes mellitus tipo 2; ECV, enfermedades cardiovasculares.

2.1.1. Complicaciones microvasculares

Las complicaciones microvasculares asociadas a la DM2 suponen un aumento en la morbilidad y un deterioro considerable de la calidad de vida de estos pacientes. Estas complicaciones microvasculares engloban principalmente la neuropatía, la retinopatía y la nefropatía. El riesgo de desarrollar estas patologías aumenta con la edad y es proporcional tanto a la magnitud como a la duración de la hiperglucemia (41).

2.1.1.1. Neuropatía

La neuropatía diabética es una complicación crónica de la DM2 que afecta a los nervios periféricos tanto del sistema nervioso autónomo como somático (42), por tanto, las neuropatías se agrupan en neuropatías difusas (incluye la polineuropatía sensitivomotora simétrica distal y la neuropatía autonómica diabética), y neuropatías focales, estas últimas son menos frecuentes y a menudo autolimitadas (43). La prevalencia de pacientes con DM2 que tienden a desarrollar diferentes síndromes de neuropatía es de aproximadamente el 59% (44). Además, el 80% de las amputaciones que se producen tras una ulceración o lesión en el pie diabético, son consecuencia de la neuropatía diabética (41). La Asociación Americana de Diabetes (ADA) reconoce la neuropatía diabética como "la presencia de síntomas y/o signos de disfunción nerviosa periférica en personas con diabetes tras la exclusión de otras causas" (37).

La neuropatía diabética es un trastorno complejo con múltiples vías de señalización involucradas en su patogénesis. La hiperglucemia, además de inducir estrés oxidativo en las neuronas, activa múltiples vías bioquímicas que constituyen la principal fuente de daño. Esta amplia gama de vías incluye la vía de los polioles, la vía de las hexosaminas, la señalización PKC, la vía de los AGEs, la vía poli ADP-ribosa polimerasa, la vía MAPK, la señalización NF-κB, la señalización TNFα, la vía de la ciclooxigenasa, las interleucinas, la vía de la lipoxigenasa, el factor de crecimiento nervioso, la señalización de quinasas dependientes de glucógeno, la autofagia y la vía de señalización Wnt/β-catenina (44). Aunque se dispone de tratamiento sintomático para la neuropatía diabética, no se encuentran opciones terapéuticas para eliminar la causa principal, por lo que causan una morbilidad grave dependiendo del órgano afectado y están relacionadas con un aumento de la mortalidad cardiovascular (45).

2.1.1.2. Retinopatía

La retinopatía diabética es una complicación microvascular frecuente asociada a la DM2, que se desarrolla como resultado del daño en los vasos sanguíneos pequeños de la retina, debido a los efectos perjudiciales de la hiperglucemia mantenida a lo largo del tiempo. La prevalencia de pacientes con DM2 que tienden a desarrollar retinopatía diabética es del 34% (42). Esta enfermedad se clasifica en retinopatía de fondo, que incluye pequeñas hemorragias, exudados duros y microaneurismas, y retinopatía proliferativa, caracterizada por la formación de nuevos vasos sanguíneos en la retina, lo que puede llevar a hemorragias y desprendimiento de la retina (46), siendo la principal causa de ceguera prematura, llegando a alcanzar una prevalencia de aproximadamente del 20% en estos pacientes (42). La ADA ha redefinido la retinopatía diabética como una "complicación neurovascular altamente específica de los tejidos que implica una alteración progresiva de la interdependencia entre múltiples tipos celulares de la retina" (47).

El riesgo de desarrollar retinopatía diabética está relacionado tanto con la gravedad de la hiperglucemia como con la presencia de hipertensión e hiperlipidemia (48). La patogénesis de la retinopatía diabética involucra varios mecanismos. La aldosa reductasa, enzima en la vía de los polioles, convierte la glucosa en sorbitol y su acumulación celular debido a niveles elevados de glucosa, se ha relacionado con la formación de microaneurismas, el engrosamiento de las membranas basales y la pérdida de pericitos (49). Además, la hiperglucemia promueve la formación de AGEs, la activación de PKC y, el estrés oxidativo que genera radicales libres y especies reactivas de oxígeno que afectan a los vasos sanguíneos, dando lugar nuevamente a la formación de microaneurismas y pérdida de pericitos (50). También se ha postulado que los factores de crecimiento, incluido el factor de crecimiento endotelial vascular (VEGF), la hormona del crecimiento y el factor de crecimiento transformante β, desempeñan funciones importantes en el desarrollo de la retinopatía diabética. La producción de VEGF aumenta en la retinopatía diabética, posiblemente en respuesta a la hipoxia (41). Por lo tanto, el control tanto glucémico, como de la tensión arterial son cruciales para prevenir la progresión de la retinopatía diabética y preservar la visión.

2.1.1.3. Nefropatía

La nefropatía diabética, también conocida como enfermedad renal diabética (ERD), es una de las complicaciones microvasculares crónicas más importantes relacionadas con la DM2

(41), con una prevalencia que alcanza hasta el 40% en esta población (51). Esta patología se caracteriza por daño renal progresivo debido a la hiperglucemia crónica, que puede llevar a la disfunción renal y, en caso graves, a la enfermedad renal terminal (ERT), la cual es la principal causa de morbilidad y mortalidad cardiovascular en estos pacientes (52). La evolución típica de la nefropatía diabética incluye hiperfiltración glomerular, albuminuria progresiva (aumenta en gravedad desde la microalbuminuria hasta macroalbuminuria), disminución de la tasa de filtrado glomerular estimada (TFGe) y, en última instancia, ERT (45). La ADA define la ERD por "la presencia de albuminuria (con excreción de albúmina en orina de al menos 30 mg/24 horas) y/o una disminución en la TFGe" (53).

Los factores de riesgo asociados al desarrollo y la progresión de la ERD abarca la prolongación de la hiperglucemia, la presencia de hipertensión, dislipidemia, tabaquismo, sedentarismo, obesidad y predisposición genética (42,45). La patogénesis de la ERD involucra varias vías metabólicas que implican cambios microvasculares y estructurales en el riñón, incluyendo en primer lugar la pérdida de podocitos, seguida de engrosamiento de la membrana basal glomerular, expansión mesangial, reducción de la densidad de la superficie de filtración glomerular y esclerosis nodular (45). La glomeruloesclerosis nodular, también conocida como la lesión de Kimmelstiel-Wilson, en la ERD representa un marcado aumento del daño de la matriz mesangial como resultado de la glicosilación no enzimática de las proteínas. Además, a medida que la enfermedad progresa se produce hialinosis arterial y fibrosis tubulointersticial (45,51). Todos estos cambios estructurales contribuyen a la disfunción renal en la ERD.

Como se ha mencionado anteriormente, la patogénesis de ERD involucra varias vías metabólicas. La hiperglucemia a largo plazo conduce a la acumulación de productos AGEs, que estimulan la transducción de señales NF-κB, aumentando la producción de anticuerpos y linfocitos B. También se ha observado un aumento en la liberación de radicales libres de oxígeno por los neutrófilos, provocando disfunción endotelial (40) y la expresión de factores inflamatorios como el TNFα y la interleucina 1 (IL-1), que desempeñan un papel perjudicial en la progresión de la ERD, contribuyendo a la inflamación y al daño en el riñón (54). Además, la mayoría de los mecanismos moleculares involucrados en la patogénesis de la ERD son comunes a los de la retinopatía diabética (55). En ambas patologías se ha demostrado que el control estricto de la glucosa en sangre, pueden retardar la progresión de estas complicaciones microvasculares.

2.1.2. Complicaciones macrovasculares

Las complicaciones macrovasculares son la principal causa de morbilidad y mortalidad en pacientes con DM2. Se estima que la frecuencia de que estos pacientes sufran una enfermedad macrovascular es de 2 a 4 veces mayor que en sujetos sin DM2 (52), observando que la mortalidad en pacientes con DM2 a causa de alguna manifestación de enfermedad macrovascular es de, al menos, el 75% (56).

El mecanismo patológico central de la enfermedad macrovascular es el proceso de aterosclerosis (41). La dislipidemia, la hipertensión, la hiperglucemia y la inflamación son los principales factores de riesgo de aterosclerosis (57), la cual se caracteriza por el estrechamiento de las paredes arteriales en todo el cuerpo (2,40).

La ateroesclerosis se caracteriza por la acumulación focal de grasa (ateroma) y material fibroso (esclerosis) en la capa más interna de las arterias de mediano y gran calibre, como las arterias coronarias y la aorta. Diversas anormalidades en el sistema vascular, el metabolismo y la coagulación, contribuyen a la elevada incidencia de la ateroesclerosis en la DM2. La ateroesclerosis comienza con lesiones en el revestimiento interno de las arterias y una respuesta inflamatoria (58). Ante la lesión, se incrementa la liberación de agentes vasoconstrictores como la angiotensina-II y la endotelina-1. La angiotensina II promueve la oxidación de las LDL que se acumulan en la pared endotelial de las arterias. Además, se observa una reducción en la actividad de la enzima NOS endotelial, lo que conlleva a una disminución en la disponibilidad de NO y en la capacidad de vasodilatación mediada por el endotelio (58). Ante la respuesta inflamatoria, los monocitos se infiltran en la pared arterial y se diferencian en macrófagos, que acumulan partículas oxLDL por fagocitosis formando células espumosas que estimulan la atracción de linfocitos T. La persistencia, la necrosis y la ruptura de las células espumosas resultan en la acumulación de colesterol y, en la formación de depósitos locales de grasa y restos celulares dentro de la lesión aterosclerótica, influyendo en la progresión y estabilidad de la placa aterosclerótica (59). A su vez, los linfocitos T liberan citocinas proinflamatorias que estimulan la migración de CMLV desde la capa media de la pared arterial hacia la capa interna, donde proliferan y contribuyen a la síntesis de colágeno y otros componentes de la matriz fibrosa extracelular, lo que conduce a un estrechamiento gradual de la luz vascular (60). El resultado neto del proceso es la formación de una lesión aterosclerótica rica en lípidos con una capa fibrosa.

Además de la formación de ateromas, en pacientes con DM2 se presentan alteraciones en la función plaquetaria y en la coagulación, lo que incrementa el riesgo de formación de trombos en las placas ateroscleróticas. Estas alteraciones incluyen la generación anormal de NO y un aumento en la producción de radicales libres en las plaquetas, así como una regulación alterada del calcio y un aumento en las glicoproteínas adhesivas IIb/IIIa y de tromboxano A-2, lo que promueve la agregación plaquetaria (41). Al mismo tiempo, se observa un aumento en los niveles de fibrinógeno, del factor inhibidor del activador de plasminógeno-1 y de los factores de coagulación VII y VIII. Esta combinación de una coagulabilidad elevada y una fibrinólisis alterada, incrementa aún más el riesgo de obstrucción vascular y eventos cardiovasculares en pacientes con DM2 (58) (Figura 5).

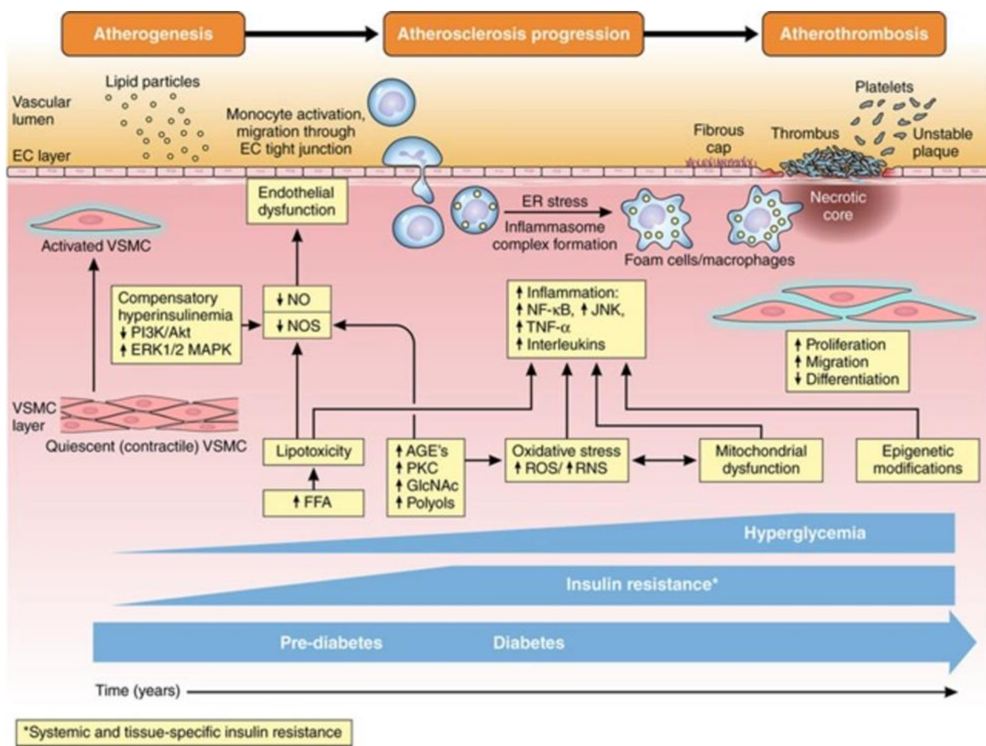

Figura 5. Desarrollo y progresión de la ateroesclerosis en la DM2, representando tanto las vías celulares como moleculares (Wang *et al.*, 2016) (39). AGEs, productos de glicación avanzada; Akt, indica proteína quinasa B; EC, células endoteliales; ER, retículo endoplasmático; ERK, quinasa regulada por señal extracelular; FFA, ácidos grasos libres; GlcNAc, N-Acetilglucosamina; JNK, quinasa Jun; MAPK, proteína quinasa activada por mitógenos; NF-κβ, factor nuclear-kappa beta; NO, óxido nítrico; NOS, óxido nítrico sintasa; PI3K, fosfatidilinositol 3-quinasa; PKC, proteína quinasa C; RNS, especies reactivas de nitrógeno; ROS, especies reactivas de oxígeno; TNFα, factor de necrosis tumoral alfa; VSMC, células de músculo liso vascular.

Estas acciones favorecen la formación de aterosclerosis y trombosis, explicando la alta prevalencia de las ECV arterioscleróticas asociadas a la DM2, como la EAC, la EAP y la EC. La prevalencia de estas enfermedades aumenta con el empeoramiento de la hiperglucemia, debido a un mayor riesgo de aterosclerosis acelerada, y otros efectos lipotóxicos y glucotóxicos resultantes. Además, otros factores de riesgo altamente relacionados con estas complicaciones son la hipertensión, la obesidad, la dislipidemia, el sedentarismo y el tabaquismo (42).

2.1.2.1. Enfermedad arterial coronaria

La EAC es la complicación macrovascular más frecuente asociada a la DM2. La prevalencia de la EAC en pacientes con DM2 llega a alcanzar el 21%, representado la principal causa de mortalidad en esta población (42). Esta afección cardiaca implica la formación de placa aterosclerótica en las arterias coronarias, lo que provoca un estrechamiento y obstrucción del flujo sanguíneo hacia el músculo cardíaco, resultando en una disminución del flujo sanguíneo y, en última instancia, a problemas cardíacos graves como la angina de pecho o el infarto de miocardio (61). Se ha descrito una fuerte asociación entre la EAC y la DM2, reportándose que la DM2 duplica tanto el riesgo de desarrollar esta patología como el riesgo de mortalidad en comparación con la población general, particularmente en mujeres (62). Estos sucesos se deben a que la ateroesclerosis en pacientes con DM2 se manifiesta de forma más temprana y acelerada (63).

La ADA y la *American Heart Association* recomiendan que la DM2 se considere un equivalente de riesgo de EAC, en lugar de un factor de riesgo, debido a que DM2 por sí sola, sin ninguna evidencia previa de EAC, indica un riesgo similar o mayor de EAC que en los pacientes sin DM2 con un evidencia previa de problemas en las arterias coronarias (64). En los pacientes con DM2, la EAC se suele detectar en etapas avanzadas debido a la potencial “isquemia silenciosa” que suelen experimentar estos pacientes. En este contexto, se ha observado que alrededor del 75% de los pacientes con DM2 que no muestran evidencia de EAC, tienen un grado significativo de aterosclerosis coronaria (65).

Un factor destacado de riesgo para el desarrollo de EAC en la DM2 es la obesidad. Los datos indican que la obesidad en pacientes con DM2 duplica el riesgo de padecer EAC. Esto se debe a que la obesidad agrava la resistencia a la insulina, lo que a su vez reduce la actividad de la lipoproteína lipasa, aumenta los niveles de AGLs y contribuye a mantener un elevado perfil lipídico que promueve el desarrollo de aterosclerosis (40).

2.1.2.2. Enfermedad arterial periférica

La EAP es una de las primeras manifestaciones más comunes de ECV en los pacientes con DM2, con una prevalencia del 16% en esta población (66). La EAP es una afección aterosclerótica caracterizada por el estrechamiento o bloqueo de las arterias que suministran sangre a las extremidades, como las piernas y los pies. Esta restricción en el flujo sanguíneo puede dar lugar a diversos problemas, como la formación de úlceras y dificultades en la cicatrización de heridas de las extremidades inferiores y, en los casos más graves, puede llevar a la necesidad de amputación (42). La estadística indica que más del 20% de los pacientes con DM2 desarrollarán el llamado pie diabético, y que al menos el 25% de las heridas y úlceras en los pies no cicatrizarán, aumentando el riesgo de que los pacientes requieran amputaciones (40). El pie diabético es una complicación que se desarrolla como consecuencia de diversos factores de riesgo, que incluyen la parestesia originada por la neuropatía diabética periférica y la isquemia provocada por la EAP. Se estima que alrededor del 50% de los pacientes con pie diabético presentan EAP (67).

Los factores de riesgo principales en la EAP, además de los mencionados anteriormente relacionados con la ECV, incluye la edad avanzada, el tiempo de evolución de la DM2 y el género. Esta patología tiende a manifestarse con mayor frecuencia en hombres (68).

2.1.2.3. Enfermedad cerebrovascular

La EC representa la segunda causa más común de mortalidad por complicación macrovascular en pacientes con DM2, después de la EAC. Se estima que la prevalencia de la EC en individuos con DM2 es de aproximadamente el 8% (42). La DM2 es un fuerte predictor independiente del riesgo de accidente cerebrovascular (ACV) y EC (41), aumentado el riesgo de ACV y duplicando el de EC (69). El ACV relacionado con la DM2 puede atribuirse a una afección en la arteria carótida extracraneal y a trastornos en los vasos intracraneales. Las manifestaciones clínicas abarcan desde oclusiones asintomáticas de la arteria carótida hasta episodios como el ataque isquémico transitorio y, en casos más severos, se incluyen ACV hemorrágico e isquémico (42).

Los factores de riesgo clave que contribuyen a padecer EC en pacientes con DM2, son la hiperglucemia, la formación de placas ateroscleróticas y la resistencia a la insulina. La hiperglucemia, provoca acidosis en células cerebrales, lo que daña las neuronas y las células gliales. Este daño conduce a la inflamación de las células endoteliales y al estrechamiento de los vasos sanguíneos, lo que resulta en isquemia cerebral (70). Además, la hiperglucemia

puede activar la enzima nicotinamida adenina dinucleótido fosfato (NADPH) oxidasa en las neuronas cerebrales, lo que provoca la producción de superóxido y, finalmente, la muerte neuronal (71). Por otro lado, la resistencia a la insulina acelera la formación de placa aterosclerótica, que a su vez, está relacionada con el estado de hipercoagulabilidad de la sangre, aumentando ambos el riesgo de recurrencia del ACV isquémico y la mortalidad (72).

2.2. Fragilidad ósea

La fragilidad ósea se considera una complicación que se relaciona a la DM2. La DM2 se asocia con un aumento de la densidad mineral ósea (DMO), pero paradójicamente, con un mayor riesgo de fracturas esqueléticas, resultando significativamente en una morbilidad grave y en un aumento de la mortalidad en esta población (73). Estos pacientes tienen un riesgo triplicado de fractura en comparación con la población en general, especialmente de cadera, muñeca, pie, e incluso fracturas vertebrales morfométricas (74,75). El método de referencia para la medición de la DMO es la absorciometría de rayos X de doble energía (DXA), resultando ser deficiente para caracterizar la estructura ósea en personas con DM2. Por tanto, se han explorado diversas técnicas de imagen, como el análisis estructural de la cadera (HSA), *Trabecular Bone Score* (TBS), y la tomografía computarizada cuantitativa periférica (pQCT) de alta resolución. Estas técnicas proporcionan información detallada sobre la arquitectura ósea y la resistencia a las fuerzas de compresión axial. También se han investigado técnicas de resonancia magnética para evaluar parámetros óseos en pacientes con DM2, demostrando una eficaz predicción del riesgo de fractura en esta población (74).

Como se ha mencionado anteriormente, los pacientes con DM2 se caracterizan por una DMO normal o alta, pero con un mayor riesgo de fracturas conociéndose este fenómeno como "la paradoja diabética de la fragilidad ósea", lo que sugiere que otros factores independientes, además de la DMO, pueden influir en el riesgo de fractura. En consecuencia, la *National Bone Health Alliance* propuso que la osteoporosis en la DM2 debería diagnosticarse sobre la base de parámetros de resistencia ósea (76).

Los mecanismos fisiopatológicos subyacentes a la fragilidad ósea inducida por la DM2 son complejos y multifactoriales. Se pueden atribuir generalmente a la acumulación de AGEs en la matriz ósea, bajas tasas de formación y remodelación ósea y, una microestructura ósea anormal (77). En este sentido, los factores principales de los mecanismos de la fragilidad ósea en la DM2 incluyen hiperglucemia crónica, resistencia a la insulina, estrés oxidativo, adiposidad alterada y, factores inflamatorios y adipocinas liberadas por la grasa visceral.

Desde un punto de vista clínico, los factores predictores de fracturas por fragilidad son la duración de la DM2, el control glucémico y la presencia de complicaciones relacionadas con la DM2 como las enfermedades microvasculares y macrovasculares. Además, varias terapias para la DM2 pueden tener un papel negativo directo sobre el metabolismo óseo (74,77).

A continuación, se exponen más detalladamente algunos de los factores patogénicos que contribuyen al aumento de la fragilidad ósea en DM2 (Figura 6):

- Hiperglucemia: Los elevados niveles de glucosa resultantes de una deficiencia de insulina o de su acción alterada, tiene un efecto perjudicial, directa o indirectamente, sobre el metabolismo óseo. En estados hiperglucémicos, la glicación no enzimática de proteínas, fosfolípidos y ácidos nucleicos conduce a la formación de AGEs, que se encuentran involucrados tanto en la matriz ósea como en diversos procesos de las células óseas (78). En condiciones fisiológicas, el colágeno tipo I en la matriz ósea mantiene la estructura ósea mediante la formación de enlaces cruzados. Sin embargo, en pacientes con DM2, la formación de AGEs causa enlaces cruzados anormales en el colágeno. Estos enlaces cruzados anormales hacen que el colágeno sea menos efectivo para proporcionar flexibilidad y resistencia al hueso, lo que contribuye a la fragilidad ósea (79). Entre los AGEs, uno de los compuestos más estudiados es la pentosidina, la cual se correlaciona con una reducción en la flexibilidad de las fibras de colágeno y una disminución en las propiedades mecánicas del hueso, a pesar de que la DMO se mantiene en niveles normales. Gracias a que los niveles de pentosidina circulante se correlacionan con la pentosidina en el hueso cortical, se ha observado una asociación significativa entre los niveles de pentosidina y el riesgo de fracturas en pacientes con DM2, ya sea determinada en suero o en orina (80). La acción de los AGEs no solo se limita a la acumulación de enlaces cruzados anómalos, sino que también ejerce un impacto directo sobre las células encargadas de la formación ósea a través del receptor de AGEs (RAGE). Se ha confirmado la presencia de RAGE tanto en osteoblastos como en osteocitos, y se ha observado que la hiperglucemia aumenta su expresión. La activación de RAGE da lugar a una reducción en los mecanismos antioxidantes y, a un aumento en la producción de citocinas inflamatorias y de especies reactivas del oxígeno, lo que coincide con el aumento de la expresión del ligando del receptor activador para el factor nuclear κβ (RANKL), que favorece la diferenciación y activación de los osteoclastos y la resorción ósea, inhibe la mineralización de células osteoblásticas (81) e induce la

apoptosis de los osteoblastos y osteocitos (82,83). Estos hallazgos indican que tanto la hiperglucemia como los AGEs interfieren en la diferenciación de las células óseas y la formación ósea, contribuyendo a una menor remodelación ósea y, por tanto, a una mayor fragilidad ósea en la DM2.

Sin embargo, los efectos perjudiciales de la hiperglucemia se extienden más allá de la acumulación de AGEs. La hiperglucemia suprime la expresión de la osteocalcina (OC), disminuye la expresión de los receptores de parathormona (PTH) y vitamina D, e incrementa la expresión de IL-6, estimulando la actividad osteoclástica y por tanto la resorción ósea (77,78). Además, la hiperglucemia aumenta la expresión de la esclerostina y de la Dickkopf 1 (Dkk1), dos importantes contribuyentes en la inhibición de la formación ósea a través de la vía Wnt/β-catenina (84). Por otra parte, la hiperglucemia conlleva una glicosuria para eliminar el exceso de glucosa en sangre, lo que da lugar a una hipercalciuria que disminuye los niveles de calcio (85), y por tanto, disminuye la captación de calcio por los osteoblastos contribuyendo de este modo a la fragilidad ósea (82).

- <u>Factores hormonales:</u> Las Cβ-pancreáticas secretan hormonas como la insulina, la amilina y la peptina, las cuales ejercen efectos anabólicos sobre el hueso. La insulina y su vía de señalización son importantes en la diferenciación osteoblástica, la síntesis de colágeno y la formación ósea. En primer lugar, la insulina estimula directamente los IRS de los osteoblastos promoviendo su proliferación y diferenciación (77) y, tiene un efecto indirecto al inactivar la proteína p27, que promueve la formación de osteoclastos. La insulina también contribuye a la síntesis de colágeno, mejora la captación de glucosa y mantiene los niveles de hormonas importantes para la salud ósea, como la PTH, el factor de crecimiento similar a la insulina-1 (IGF-1) y la vitamina D (86). Además, se ha demostrado que la insulina reduce la expresión de osteoprotegerina (OPG) en los osteoblastos mediante la fosforilación de FoxO1, lo que conduce a la osteoblastogénesis y a la resorción ósea (77). Estos múltiples mecanismos resaltan la influencia de la insulina en el metabolismo óseo demostrando que la insuficiencia/deficiencia de insulina genera pérdida ósea y deterioro de la calidad ósea, debido a una menor formación y recambio óseo, resultando en un riesgo extremadamente alto de fracturas en los pacientes con DM2. La amilina, también conocida como IAPP, es una hormona polipeptídica correspondiente a la familia de péptidos relacionados con el gen de la calcitonina. Esta hormona desempeña un

papel en la regulación de los niveles de glucosa en sangre y en la sensibilidad a la insulina en el tejido musculo-esquelético. Estudios *in vitro* han revelado que la amilina inhibe la resorción ósea al interactuar con receptores presentes en los osteoclastos, posiblemente vinculados a los receptores de calcitonina. Asimismo, se ha observado que estimula la proliferación de osteoblastos. En pacientes con DM2, se han registrado niveles reducidos de amilina, contribuyendo a la pérdida ósea en esta población (86).

Las incretinas, como GIP y GLP-1, son hormonas secretadas por el intestino que estimulan la secreción insulina, suprimen la secreción de glucagón, inhiben el vaciado gástrico y reducen el apetito. Esto se conoce como "el efecto incretina", el cual puede afectar al metabolismo óseo (74). GIP regula la absorción de carbohidratos a través de la secreción de insulina, y aumenta la síntesis de colágeno tipo I y la actividad de fosfatasa alcalina (ALP), demostrando un efecto antirresortivo. Además, esta hormona inhibe la actividad resortiva de los osteoclastos, así como, la expresión de algunos de los marcadores de diferenciación de estas células. En pacientes con DM2, se muestra una regulación a la baja de los receptores óseos de GIP. Esto plantea la posibilidad de que la disminución de la acción de esta incretina esté relacionada con el aumento de la fragilidad ósea en DM2 (87). Por otro lado, los receptores de GLP-1 se expresan en las células estromales de la médula ósea y en los osteoblastos inmaduros (88). Se ha demostrado que el GLP-1 estimula la proliferación de las células madre mesenquimales e inhibe su diferenciación en adipocitos. Las pruebas indirectas del efecto osteogénico del GLP-1 proceden del uso de análogos del GLP-1 en modelos animales. Este efecto parece estar mediado por una interacción positiva con la vía Wnt/β-catenina y la supresión de la expresión de esclerostina (89). Los pacientes con DM2 presentan una producción de GLP-1 disminuida en un estado postprandial (90), por lo que se considera responsable de la inhibición de la resorción ósea tras la ingesta.

- <u>Adiposidad:</u> Tradicionalmente el sobrepeso y la obesidad, a menudo vinculados a la DM2, han sido considerados como posibles factores de protección contra las fracturas, dado que un alto índice de masa corporal (IMC) se asocia a un aumento de la DMO (91). Sin embargo, se ha demostrado que la obesidad puede ser un factor de riesgo de fractura cuando se ajusta a la DMO (92), observándose que la obesidad se correlaciona positivamente con fracturas vertebrales y de extremidades, y

negativamente con fracturas de cadera, pelvis y muñeca (93). Por lo tanto, la obesidad en la DM2 tiene un impacto negativo en el riesgo de fracturas, pero aún es necesario dilucidar las posibles interacciones entre el IMC, la DMO y las fracturas.

La obesidad se caracteriza como una afección inflamatoria sistémica, debido a la liberación de diversas citocinas proinflamatorias por parte del tejido adiposo, principalmente IL-6 y TNFα. El incremento de estas citocinas podría estimular la actividad de los osteoclastos y la resorción ósea a través de alteraciones en la vía RANKL/ receptor activador del factor nuclear ϰβ (RANK)/OPG. Asimismo, el aumento de estas citocinas se asocia con la resistencia a la insulina, la DM2 y la aterosclerosis (94). Por tanto, la resistencia a la insulina y la acumulación de tejido adiposo visceral observado en la DM2, se relaciona con un estado de inflamación crónica que actúa como un mecanismo de deterioro óseo, aumentando el riesgo de fracturas (95).

Por otro lado, alteraciones de las hormonas liberadas por el tejido adiposo, como las adipocinas, pueden ejercer efectos nocivos sobre las células óseas favoreciendo el riesgo de fracturas. En particular la adiponectina, producida exclusivamente por el tejido adiposo, puede ejercer un efecto anabólico sobre los osteoblastos y un efecto inhibidor sobre los osteoclastos (96). Esto sugiere que las alteraciones del recambio óseo que se han observado en pacientes con DM2 podrían deberse en parte, a la desregulación de los niveles de esta hormona, cuyos niveles son bajos en estos pacientes (82). Además, se ha demostrado que los niveles de leptina, otra adipocina producida por el tejido adiposo, así como por los adipocitos de la médula ósea y las células osteoblásticas, son más bajos en pacientes con DM2, indicándose una correlación negativa entre los niveles de leptina en suero y el marcador de resorción ósea N-telopeptido de colágeno tipo I en orina (97). Las adipocinas se encuentran actualmente en vías de estudio para determinar claramente su relevancia y contribución en el desarrollo y progresión de la fragilidad ósea.

- Tratamientos para la DM2: Entre los factores que contribuyen al aumento del riesgo de fractura en la DM2, debe tenerse en cuenta el uso de los agentes antidiabéticos orales y la insulina, que pueden tener efectos directos sobre las células óseas o efectos indirectos sobre el metabolismo óseo. En este contexto, se ha observado que el tratamiento con tiazolidinedionas (TZD) aumenta el riesgo de fracturas en mujeres con DM2, independientemente de la edad y la duración de exposición (98). Las TZD

se unen y activan los receptores gamma activados por el factor proliferador de peroxisomas (PPARγ), que promueven la adipogénesis. Las células madre mesenquimales son las precursoras comunes de los adipocitos y los osteoblastos, y la activación de PPARγ induce la diferenciación preferencial de células precursoras mesenquimales hacia adipocitos en lugar de a osteoblastos (99). Contrariamente, la metformina estimula la formación de hueso al promover la diferenciación de osteoblastos y la expresión de marcadores de osteogénesis. En estudios clínicos a largo plazo en pacientes con DM2, este tratamiento muestra efectos neutrales o levemente beneficiosos en la DMO y el riesgo de fracturas (73). Por su parte, la sulfonilurea se ha asociado a un mayor riesgo de fractura, un hallazgo que podría explicarse en parte por aumentar el riesgo de hipoglucemia y caídas posteriores, que se asocian con fracturas (100). Los inhibidores del cotransportador 2 de sodio y glucosa (SGLT2i) como dapagliflozina y empagliflozina no influían sobre el riesgo de fractura, aunque la canagliflozina se ha asociado a la pérdida de DMO y al aumento del riesgo de fractura de cadera (101). Los inhibidores de la dipeptidil peptidasa 4 (DPP4i) y los agonistas del péptido 1 similar al glucagón (GLP-1a), parecen tener un riesgo de fractura neutral o incluso favorable, aunque estos hallazgos deberían confirmarse en estudios más amplios (78). Ante un fracaso del tratamiento con los medicamentos antidiabéticos orales, la terapia con insulina representa la terapia electiva para los pacientes con DM2. Sin embargo, en el contexto de la fragilidad ósea, el uso de insulina se ha asociado con un mayor riesgo de fracturas, especialmente las no vertebrales en pacientes con DM2, aunque, las insulinas de acción prolongada que tienen menos riesgo de causar hipoglucemia, se asocian a un menor riesgo de fractura en comparación con otras insulinas, lo que sugiere que la hipoglucemia podría ser un factor de riesgo (74).

- Complicaciones de la DM2: Un factor de riesgo que incrementa significativamente la probabilidad de fracturas en pacientes con DM2 es la asociación con complicaciones tanto microvasculares como macrovasculares, las cuales incrementan la predisposición a sufrir caídas accidentales en estos pacientes (102). Es probable entonces que este mayor riesgo de fracturas no sea el resultado de un efecto intrínseco de cada complicación, sino como parte del proceso de deterioro sistémico como por ejemplo las alteraciones visuales, los trastornos crónicos de la marcha o el equilibrio, las hipoglucemias inadvertidas, las alteraciones vasculares y los episodios de nicturia. En particular, se ha descrito una correlación lineal entre la función renal

y las caídas. El deterioro de la función renal podría interferir en el metabolismo de la vitamina D, lo que provoca una reducción de la fuerza muscular y neuropatía (103). La deficiencia de vitamina D produce hiperparatiroidismo secundario, que aumenta la actividad osteoclástica reduciendo la DMO, y se ha demostrado que aumenta el riesgo de caídas y de fracturas de cadera, vertebrales, y osteoporóticas (78). Además, el riesgo de caídas aumenta con la sarcopenia, afección prevalente en los pacientes con DM2, que se define como una disminución de la masa y la función muscular. En consecuencia, ésta afección puede considerarse como un factor extra-esquelético que aumenta el riesgo de fracturas en esta población (104).

Aunque los factores de riesgo mencionados anteriormente pueden ofrecer una explicación parcial del origen de la fragilidad ósea, se ha evidenciado que no son un método completamente confiable para evaluar el riesgo de fracturas en pacientes con DM2 (105).

Cabe resaltar que la vía de señalización Wnt/β-catenina y sus antagonistas desempeñan un papel fundamental en la patogénesis de las alteraciones en la calidad ósea asociadas a la DM2. De hecho, se ha destacado la esclerostina, una glicoproteína inhibidora de la vía Wnt/β-catenina, como un marcador de fragilidad ósea independiente de la DMO (106).

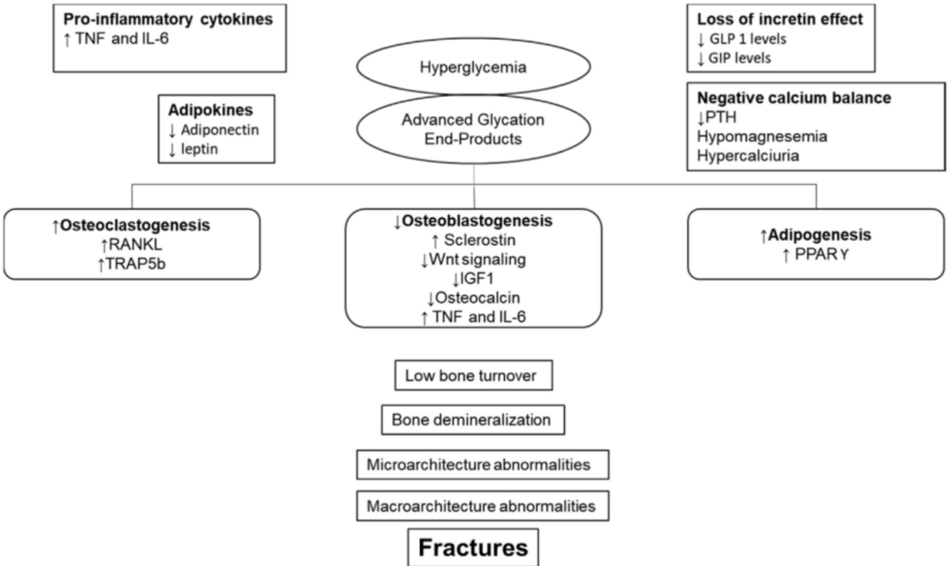

Figura 6. Mecanismos subyacentes al aumento del riesgo de fractura en la DM2 (Farooqui K.J. *et al.*, 2021) (91). DM2, diabetes mellitus tipo 2; GIP, péptido inhibidor gástrico; GLP1, péptido similar al glucagón tipo 1; IGF1, factor de crecimiento similar a la insulina-1; IL-6, interleucina 6; PTH, parathormona; PPARγ, receptores gamma activados por el factor proliferador de peroxisomas; RANKL, receptor del ligando del factor nuclear κβ; TNF, factor de necrosis tumoral; TRAP5b, fosfatasa ácida tartrato resistente específica de hueso 5b.

3. Eje óseo-vascular

Cada vez hay una mayor evidencia que respalda la existencia de un eje de conexión entre el metabolismo óseo y el sistema vascular (107), lo que ha generado un interés significativo en la identificación de biomarcadores relacionados con el metabolismo óseo en el contexto de enfermedades vasculares (108). De hecho, el metabolismo óseo y el sistema vascular están regulados por algunos mecanismos fisiopatológicos comunes, y la calcificación de las paredes vasculares se asemeja al proceso de formación ósea en diversos aspectos (109,110). La interrelación entre el sistema vascular y la remodelación ósea desempeña un papel fundamental para la correcta formación, funcionamiento fisiológico y reparación tras una lesión del tejido óseo (111). Esta interacción involucra a las células endoteliales vasculares, así como a células adyacentes que contribuyen al control del metabolismo óseo. Por ejemplo, las células madre mesenquimales tienen capacidad osteogénica intrínseca y, además, promueven la vascularización del tejido óseo mediante la comunicación con las células endoteliales vasculares. Esta comunicación se lleva a cabo a través de la acción de factores proangiogénicos, como los VEGF, los IGF-1, los factores de crecimiento derivados de las plaquetas y los factores de crecimiento de fibroblastos (112). Por tanto, las alteraciones en la conexión entre metabolismo óseo y el sistema vascular, pueden desencadenar un desequilibrio en la homeostasis ósea, lo cual es una manifestación común en patologías como la DM2 (113).

La DM2 es un factor de riesgo común para la fragilidad ósea y las ECV, por lo que podría explicar en parte, la coexistencia de estas complicaciones en la DM2. Como se ha descrito previamente, la hiperglucemia, la disfunción en la secreción de adipocinas, la obesidad y el estado crónico de inflamación que se manifiesta durante la DM2, están implicados tanto en la fragilidad ósea como en el desarrollo de ECV. En este sentido se ha demostrado que la cardiopatía isquémica se asocia a fracturas vertebrales en pacientes con DM2 (114). No obstante, la conexión entre fragilidad ósea y ECV podría deberse a que el desequilibrio en la formación/resorción ósea se encuentra también implicado en las patologías cardiovasculares (115). En esta línea, se encuentran diversos estudios que indican una interacción entre la fragilidad ósea y las ECV (114,116,117). Así, se ha observado un aumento en el riesgo de sufrir eventos cardiovasculares en pacientes que presentan una disminución en la DMO y/o fracturas osteoporóticas (116). Además, el aumento de la mortalidad por ECV se ha asociado a una baja DMO (109). Asimismo, se ha observado también una mayor prevalencia de fracturas vertebrales en pacientes que han sufrido un evento coronario, independientemente de la DMO (117,118).

La patogenia que afecta a la homeostasis de la mineralización ósea y la vascular comparten tanto factores de riesgo que incluyen la edad, la depleción estrogénica, el sedentarismo, el consumo de alcohol y tabaco, como mecanismos fisiopatológicos, que podrían fundamentar la asociación existente entre ambas patologías (115).

Los mecanismos fisiopatológicos comunes implicados tanto en la fragilidad ósea como en la calcificación vascular incluyen diversas vías, proteínas, hormonas y vitaminas (Figura 7).

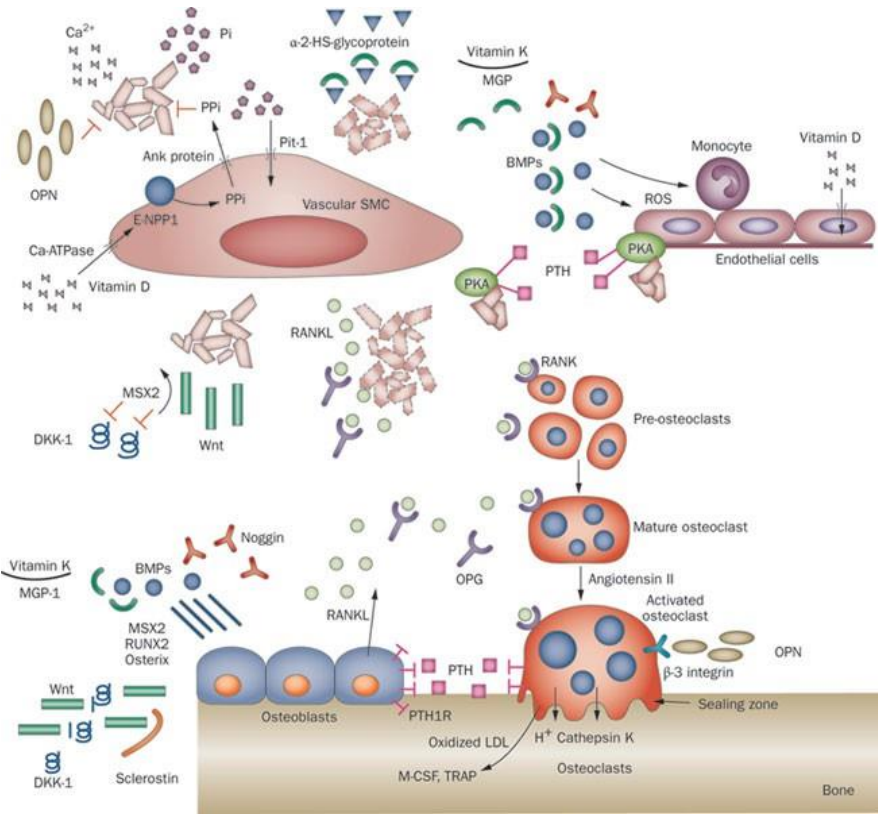

Figura 7. Mecanismos patogénicos comunes en la calcificación vascular y la fragilidad ósea (Lampropoulos C.E. *et al.*, 2012) (107). BMP, proteínas morfogenéticas óseas; Ca^{2+}, calcio; DKK-1, dickkopf-1; E-NPP1, miembro 1 de la familia de la ectonucleótido pirofosfatasa/fosfodiesterasa; M-CSF, factor estimulante de colonias de macrófagos 1; LDL, lipoproteínas de baja densidad; MGP, proteína GLA de matriz; OPG, osteoprotegerina; OPN, osteopontina; Pi, fosfato inorgánico; PPi, pirofosfato; PKA, proteína quinasa A; PTH, parathormona; PTH1R, receptor de la parathormona 1; RANKL, receptor del ligando del factor nuclear κB; ROS, especies reactivas de oxígeno; RUNX2, factor de transcripción 2 relacionado con Runt; SMC, células del músculo liso; TRAP, fosfatasa ácida resistente al tartrato de tipo 5.

La vía de señalización Wnt/β-catenina, una vía que desempeña un papel crucial en la remodelación ósea, se halla también implicada en el desarrollo y mantenimiento de la

vasculatura (119). Por ende, las complicaciones relacionadas con alteraciones de la vía Wnt/β-catenina pueden provocar una disfunción vascular. Como consecuencia, la desregulación de los componentes de esta vía genera daño inflamatorio cardiovascular, altera la plasticidad celular y provoca la acumulación de colesterol intracelular. De hecho, cada vez existen más evidencias de una contribución de las vías de señalización Wnt en la aterosclerosis y el envejecimiento vascular (52). Además, se ha demostrado que la vía Wnt/β-catenina juega un papel importante en la progresión de la enfermedad cardíaca, tanto en alteraciones metabólicas (sensibilidad a la insulina), como en remodelación cardiovascular y cambios estructurales, tales como la fibrosis, esclerosis, formación de ateromas y proliferación e hipertrofia de CMLV (120). Así, múltiples investigaciones en enfermedades vasculares señalan la participación de esta vía en la regulación de la proliferación, migración y supervivencia de las CMLV (60). Estudios han demostrado que en entornos calcificantes, las CMLV experimentan una transición fenotípica hacia células que se asemejan a los osteocitos, caracterizadas por una pérdida de marcadores de músculo liso y una regulación positiva de los marcadores osteogénicos, incluyendo esclerostina (60,121). En concordancia con estos datos, estudios en humanos, han mostrado un incremento de esclerostina en tejidos vasculares calcificados (122,123).

La vía RANKL/RANK/OPG, implicada en la formación y resorción ósea, también tiene una implicación a nivel vascular, demostrándose que las disfunciones en esta vía se encuentran involucradas tanto en la osteoporosis como en la calcificación vascular. El sistema RANKL/OPG desempeña un papel crucial en el metabolismo óseo al definir el nivel de activación de los osteoclastos, y, en consecuencia, influye en el grado de deterioro del tejido óseo. La expresión de RANKL y OPG se ha observado tanto en células musculares lisas como en endoteliales de la pared arterial (60,124), además de en placas ateroscleróticas en humanos (125,126). Se ha reportado que RANKL participa en la aterogénesis y la desestabilización de la placa aterosclerótica (125). Además, se ha observado que RANKL soluble correlaciona positivamente con la gravedad de la enfermedad coronaria (127), y se ha propuesto como predictor del riesgo de ECV (128). Por otro lado, la OPG regula el proceso de osteoclastogénesis a través de la inhibición de RANKL. Adicionalmente, la OPG es un receptor para el ligando citotóxico inductor de apoptosis relacionado con TNF (TRAIL) y podría inhibir la apoptosis de células vasculares inducida por TRAIL (129). En estudios en humanos, esta proteína se asocia de manera positiva con la calcificación vascular, así como, con la rigidez arterial (107). Además, se encontró que niveles aumentados de OPG se relacionan con la presencia y la severidad de la EAC (130,131), de arteriopatía coronaria y de

aterosclerosis carotídea (132). En este contexto, la asociación de los niveles séricos de OPG con la DM2 y la mortalidad cardiovascular plantea la posibilidad de que esta proteína pueda ser una causa o un marcador de calcificación vascular (133). Adicionalmente, se ha observado que algunos polimorfismos en la región promotora del gen *OPG* se relacionan con marcadores de aterosclerosis subclínica como el grosor íntima-media carotídea (134). Contrariamente a los hallazgos en estudios humanos, investigaciones en modelos murinos, han revelado que la deficiencia de OPG causa osteoporosis y calcificación en las arterias aorta y renal, y que el tratamiento con OPG inhibe la calcificación arterial, actuando esta proteína como un factor autocrino de supervivencia para las células endoteliales (135). Con respecto a la DM2, se ha revelado que hay un aumento de OPG en estos pacientes desde fases iniciales de la enfermedad y que el valor predictor de la OPG sobre la mortalidad parece ser aún más significativo en esta población (133). En lo que respecta a las ECV en pacientes con DM2, se ha asociado un incremento de OPG circulante con un mayor riesgo de isquemia miocárdica silente (136) y de complicaciones microvasculares (137). Recientemente, un estudio ha mostrado que en pacientes con DM2 una elevada concentración de OPG y osteopontina (OPN) se asocia a complicaciones microvasculares lo que sugiere que estas osteocinas podrían estar implicadas en vías directamente relacionadas con la enfermedad vascular (108). Aunque la OPN es un inhibidor de la calcificación vascular, se expresa altamente en lesiones ateroscleróticas calcificadas de pacientes con DM2 e insuficiencia renal crónica, lo que se plantea como un mecanismo compensatorio de reducción de la mineralización (138).

Otra osteocina es la OC, una proteína sintetizada por los osteoblastos, considerada un marcador de formación ósea, que se ha relacionado con afección vascular. En DM2, concentraciones elevadas de OC sérica se han asociado con complicaciones macrovasculares (108) y parámetros de aterosclerosis (139). Además, se ha propuesto que la medición del OC circulante podría ser una herramienta útil para identificar un mayor riesgo cardiovascular y de DM2 en pacientes con síndrome metabólico (140).

Asimismo, se ha detectado la expresión de otras proteínas típicamente óseas en arterias calcificadas como son las proteínas morfogenéticas óseas (BMP) y la proteína GLA de matriz (MGP) (107). En cuanto a las BMP, éstas inducen la diferenciación de células mesenquimales hacia el linaje osteoblástico, aunque se ha reportado que ejercen efectos proinflamatorios y prooxidantes en las arterias sistémicas y, además, están positivamente reguladas en lesiones ateroscleróticas. Investigaciones han evidenciado que BMP-2 y BMP-4 inducen disfunción endotelial y contribuyen a aterosclerosis sistémica (141,142). Con respecto a MGP, se ha

evidenciado que esta proteína inhibe la mineralización directa e indirectamente, participando en un complejo con la glicoproteína α-2-HS e interfiriendo en la unión de BMP-2 a su receptor, respectivamente (107). Aparte, la MGP se expresa en células vasculares normales y actúa como un fuerte inhibidor de la calcificación en la matriz extracelular. Sin embargo, durante la progresión de la calcificación vascular, la expresión de MGP disminuye, y esto se asocia con un aumento en la expresión del factor de transcripción 2 relacionado con Runt (RUNX2), un factor vinculado a la inducción de osteoblastos y a la participación en el proceso de mineralización (107). Existen evidencias científicas que confirman la presencia de RUNX2 en lesiones ateroscleróticas en humanos, y su expresión en CMLV, sugiriendo la posibilidad de que juegue un papel relevante en el proceso de calcificación vascular (60,115).

En lo que respecta a la PTH, hormona que estimula la resorción ósea mediante la activación de osteoblastos, existen evidencias que respaldan un impacto directo en el tejido vascular. La PTH activa la proteína quinasa A (PKA), induciendo la calcificación independientemente del calcio o el fósforo en la vasculatura (143), y se ha atribuido a la PTH un efecto estimulante en la migración de células progenitoras angiogénicas hacia las áreas dañadas por infarto de miocardio (144). Además, la interacción entre la PTH y la vitamina D es crucial para mantener la homeostasis mineral, reportándose evidencias de que la PTH regula la vitamina D en las CMLV (145). La vitamina D participa en el metabolismo del calcio favoreciendo su absorción intestinal, mientras que la deficiencia de vitamina D es una causa secundaria de osteoporosis (107). Además, varios hallazgos han mostrado la influencia de la vitamina D en la patogénesis de las enfermedades cardiovasculares. En este contexto, niveles bajos de vitamina D se han descrito como un factor de riesgo independiente para el desarrollo de hipertensión arterial, DM2, insuficiencia cardíaca, EAP, ACV y cardiopatía isquémica (146). Por otro lado, deficiencias en vitamina K, cuya relevancia para la salud ósea y su impacto protector en la masa ósea están bien establecidos, se han relacionado con un incremento en la calcificación vascular y un aumento del riesgo de enfermedad coronaria. Por lo tanto, un aumento en la ingesta dietética de vitamina K o el uso de suplementos de esta vitamina reducen la progresión de la calcificación vascular, ofrecen protección frente a la enfermedad coronaria y mejoran la elasticidad arterial (107).

Finalmente, existen tratamientos para la patogenia de la homeostasis en la mineralización ósea y vascular que presentan efectos superpuestos, sugiriendo la conexión entre ambas enfermedades. En este contexto, se ha observado que tanto las estatinas como los bifosfonatos tienen efectos beneficiosos tanto a nivel óseo como en la pared vascular (109).

Las estatinas, conocidas como inhibidores de la 3-hidroxi-3-metilglutaril coenzima-A reductasa, son comúnmente utilizadas para tratar dislipidemia. Éstas reducen el colesterol y el riesgo de ECV, disminuyendo también el riesgo de fracturas osteoporóticas (117), ya que inhiben el proceso de diferenciación de precursores celulares hacia osteoclastos (147). En estudios epidemiológicos se ha mostrado que pacientes tratados con estatinas presentan valores más altos de DMO, tanto en mujeres menopáusicas (148) como en DM2 (149). Asimismo, los bifosfonatos que son inhibidores de la farnesil difosfato sintasa utilizados para tratar la osteoporosis, exhiben una acción antiaterogénica. Esto se debe a un mecanismo directo sobre la pared vascular y a una acción indirecta sobre otros factores de riesgo cardiovascular, como la inhibición de la calcificación extraósea, y la reducción en la acumulación de lípidos en la fibrosis de lesiones arterioescleróticas y en los depósitos de calcio (150). Se ha demostrado que el etidronato oral disminuye el grosor de la íntima-media arterial carotídea en pacientes con DM2 y osteopenia, lo que sugiere un efecto directo de los bifosfonatos sobre la pared vascular (151). Además, en mujeres posmenopáusicas con una terapia prolongada de bifosfonatos endovenosos, se ha observado una disminución de los niveles de colesterol LDL y un aumento de los niveles de colesterol HDL, mejorando así la elasticidad de la pared vascular y reduciendo la resistencia arterial de manera independiente a los cambios en las concentraciones de lípidos (152). Adicionalmente, en modelos animales, se ha demostrado que los bifosfonatos reducen la extensión de las lesiones ateroscleróticas establecidas y que ejercen múltiples efectos antiateroscleróticos (109).

El conjunto estas observaciones indican que la calcificación arterial es un proceso organizado y regulado, con mecanismos celulares y moleculares que comparten similitudes con la formación ósea. Asimismo, sugiere la presencia de mecanismos patogénicos comunes entre la fragilidad ósea y la calcificación vascular. Sin embargo, los mecanismos de conexión específicos que vinculan el metabolismo óseo y el sistema vascular aún no se han caracterizado completamente. En este contexto, a lo largo de esta Tesis Doctoral, nos hemos dedicado a profundizar en el estudio de proteínas que tienen un papel dual, implicadas tanto en el metabolismo óseo como en el sistema vascular.

3.1. Vía de señalización Wnt

El término Wnt es una combinación del gen *wingless* de Drosophila y el protooncogén de ratón homólogo *Integrase-1*. Las proteínas de la familia Wnt participan en diversas vías de señalización que desempeñan un papel clave en procesos vinculados con el desarrollo y la

fisiología como la embriogénesis, la polaridad, la migración y la diferenciación celular (153). Esta familia de proteínas incluye 19 glicoproteínas, tanto en el genoma humano como en el de ratón, que se unen a los receptores de la familia Frizzled (Fzd). Se han encontrado diez variantes de Fzd en humanos, y las proteínas correspondientes tienen siete dominios transmembrana reconocidos como una familia separada de receptores acoplados a proteína G (154). Las proteínas Wnt actúan uniéndose directamente a receptores Fzd, a través de un complejo compuesto por Fzd y la proteína relacionada con el receptor de lipoproteínas de baja densidad 5 y 6 (LRP5/6), o bien a través del complejo Fzd y receptores transmembrana tirosina quinasas ROR y RYK (119). Cuando Wnt se une a uno de estos receptores Fzd, se envía una señal a la proteína Dishevelled (Dsh) en el citoplasma, dando lugar a la activación de tres vías de señalización separadas: la vía no canónica Wnt/Ca^{2+}, la vía no canónica de polaridad celular plana (PCP) y la vía canónica Wnt/β-catenina, que involucra a proteínas Wnt y a la proteína β-catenina (154) (Figura 8).

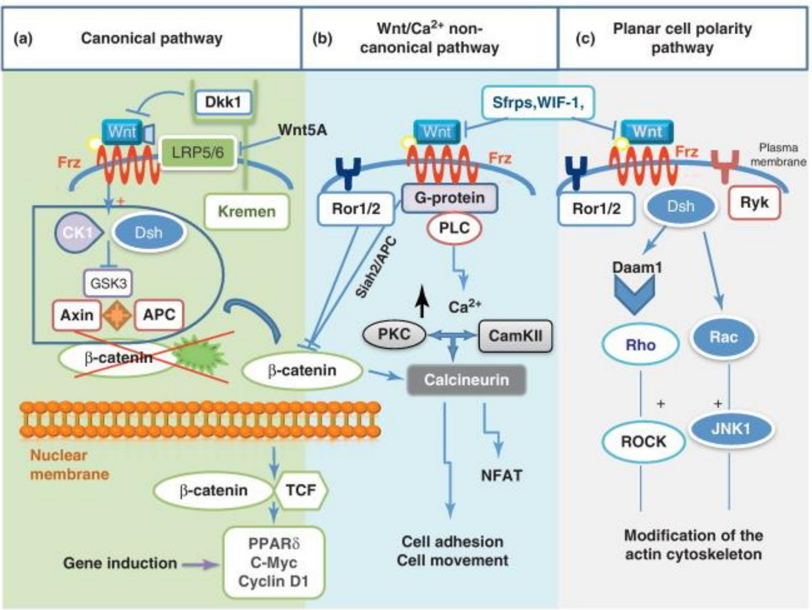

Figura 8. Descripción general de las 3 vías de señalización de Wnt: (a) vía canónica Wnt/β-catenina, (b) vía no canónica Wnt/Ca^{2+}, y (c) vía no canónica de polaridad celular plana (PCP) (Marinou K. *et al.*, 2012) (119). APC, poliposis coli adenomatosa; CK1, caseína quinasa 1; CamK2, proteína quinasa II dependiente de calmodulina; DKK, dickkopf; Dsh, Dishevelled; Frz, Frizzled; GSK3, glucógeno sintasa quinasa-3; JNK, quinasa Jun; LRP5/6, proteína relacionada con el receptor de lipoproteínas de baja densidad 5 y 6; TCF, factor derivado de células-T; PLC, fosfolipasa C; PKC, proteína quinasa C; PPARγ, receptores gamma activados por el factor proliferador de peroxisomas; ROCK, quinasa asociada a Rho; Sfrps proteínas secretadas relacionadas con el frizzled; WIF1, factor inhibidor Wnt 1.

La disfunción en estas vías tiene importantes consecuencias en el desarrollo embrionario y en el desarrollo de enfermedades degenerativas, así como, en la inflamación, en la disfunción endotelial y en la homeostasis del colesterol (153,155).

3.1.1. Vías Wnt no canónicas

Las vías de señalización Wnt no canónicas/independientes de β-catenina se caracterizan por no requerir la estabilización de la β-catenina en el citoplasma para culminar su cascada de señalización. La activación de las diferentes vías no canónicas se encuentra determinada por los distintos receptores a los que pueden unirse los ligandos Wnt, y las vías más conocidas hasta la fecha son Wnt/Ca^{2+} y Wnt/PCP (119,154).

La vía Wnt/Ca^{2+} está involucrada principalmente en la adhesión y en el movimiento celular. En esta vía el ligando Wnt se une al complejo receptor Fzd, ROR y RYK que interactúa con proteínas G heterodiméricas (156), lo que conduce a la activación de la fosfolipasa C (PLC) resultando en la producción de DAG e inositol trifosfato (IP3). IP3 activa los canales de calcio en el retículo endoplásmico, estimulando la liberación intracelular de calcio, lo que a su vez conduce a la activación de la proteína quinasa II dependiente de calmodulina (CamKII) y de la PKC, siendo la activación de esta última también dependiente de DAG. Las quinasas activadas estimulan varios factores de transcripción nuclear, y la acumulación intracelular de iones calcio activan la calcineurina, la cual se encuentra involucrada en la vía de señalización de las células T y es esencial para la activación del factor nuclear asociado a las células T (NFAT) (154).

Con respecto a la vía Wnt/PCP, la señalización Wnt a través de receptores Fzd media la organización asimétrica del citoesqueleto y la polarización de las células al inducir modificaciones en el citoesqueleto de actina (157). En esta vía se activan las GTPasas Rho y Rac. La activación de Rho requiere Daam-1 y conduce a la activación de la quinasa asociada a Rho (ROCK), en cambio, la activación de Rac es independiente de Daam-1 y estimula la actividad de la quinasa Jun (JNK) (119).

3.1.2. Vía Wnt canónica

La vía Wnt canónica es la mejor caracterizada, siendo el acontecimiento clave en esta cascada canónica la acumulación de la β-catenina en el citoplasma y su posterior translocación al núcleo celular, donde modula la transcripción de diferentes genes implicados en numerosos

procesos como el destino celular, la organogénesis y, principalmente, el metabolismo óseo (154). Esta vía presenta varios componentes involucrados incluyendo ligandos, receptores de membrana, efectores intracelulares, antagonistas y el coactivador transcripcional β-catenina, por el que se denomina esta vía como Wnt/β-catenina.

Cuando la señalización está inactiva en la vía Wnt/β-catenina, por la ausencia de Wnt o por la presencia de inhibidores de esta vía, la β-catenina intracelular es fosforilada por la glucógeno sintasa quinasa-3 (GSK3) que forma parte de un complejo multiproteico de destrucción junto a la axina, la proteína de poliposis coli adenomatosa (APC), y la caseína quinasa 1 (CK1). Posteriormente, la β-catenina fosforilada es marcada por múltiples moléculas de ubiquitina y degradada por el proteosoma. De esta forma, los niveles intracelulares de β-catenina se mantienen bajos, suprimiendo los genes diana mediante la acción de histonas desacetilasas desencadenadas por el factor derivado de células-T (TCF) y el factor de unión al potenciador linfoide (LEF) (154) (Figura 9).

La activación de la vía Wnt/β-catenina se produce tras la unión de los ligandos Wnt al complejo receptor transmembrana compuesto por Fzd y LRP5/6, que conduce a la inactivación del complejo multiproteico de destrucción, impidiendo la fosforilación de la β-catenina por la GSK3β. De esta manera, los niveles intracelulares de β-catenina se mantienen estables produciéndose la translocación de la β-catenina citoplasmática al núcleo, donde se asocia con los factores de transcripción TCF/LEF, reclutando coactivadores modificadores de histonas para activar la transcripción de genes diana (119,154) (Figura 9).

Se han identificado diversos tipos de moléculas con capacidad inhibidora de la vía Wnt/β-catenina. Por un lado, se encuentran las proteínas asociadas al receptor Fzd (FRPs) que se unen con las proteínas Wnt, impidiendo así la interacción entre estas proteínas y el receptor Fzd. Por otro lado, se encuentran proteínas inhibidoras de la vía canónica Wnt, como las proteínas Dkk1 y la esclerostina, las cuales se unen a LRP5/6, bloqueando la formación del complejo Fzd-LRP5/6 y, en consecuencia, la unión de las proteínas Wnt (158).

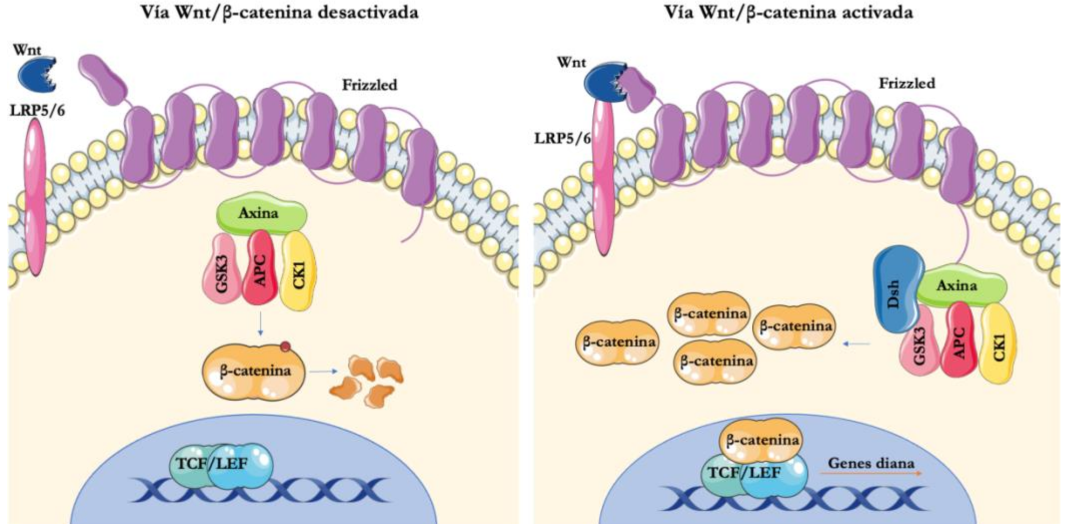

Figura 9. Vía canónica Wnt/β-catenina, desactivada y activada por ligandos Wnt. Creada con Smart Servier Medical Art. APC, poliposis coli adenomatosa; CK1, caseína quinasa 1; Dsh, Dishevelled; GSK3, glucógeno sintasa quinasa-3; LEF, factor de unión al potenciador linfoide; LRP5/6, proteína relacionada con el receptor de lipoproteínas de baja densidad 5 y 6; TCF, factor derivado de células-T.

3.1.2.1. Vía Wnt canónica a nivel óseo

La vía Wnt/β-catenina desempeña un papel crucial en la homeostasis ósea, y cada vez hay una mayor evidencia que respalda la participación de moléculas de esta vía en el metabolismo óseo y en la formación del esqueleto, abarcando desde la definición de patrones en el esqueleto embrionario hasta la remodelación en el esqueleto adulto (155).

El esqueleto adulto contiene tres tipos celulares principales, dos de origen mesenquimatoso (osteoblastos y osteocitos, estos últimos derivados de osteoblastos) y uno de origen hematopoyético (osteoclastos). En términos generales, los osteoblastos forman hueso, mientras que los osteoclastos lo reabsorben, y los osteocitos lo mantienen y contribuyen a la regulación de los osteoblastos y osteoclastos durante el modelado y remodelado óseo. La señalización canónica de Wnt afecta a todo el linaje osteoblástico. Como se ha mencionado, los osteoblastos derivan de células mesenquimales indiferenciadas, y la señalización de la vía Wnt/β-catenina es necesaria para el compromiso de estas células con el linaje de osteoblastos, inhibiendo el destino hacia células adipogénicas y condrogénicas. Una vez que se garantiza el compromiso, la señalización Wnt canónica es esencial para la proliferación, diferenciación y maduración del osteoblasto mediante la inducción de factores de transcripción osteogénicos. Asimismo, la señalización de esta vía también se ha implicado en la regulación negativa de la

apoptosis de las células osteoblásticas. Además, la señalización de Wnt-β-catenina en el linaje de osteoblastos, incluidas las células terminalmente diferenciadas de este linaje y los osteocitos, inhibe la resorción ósea osteoclástica (155). De hecho, la señalización de la vía Wnt canónica es necesaria para la expresión del factor antiosteoclástico OPG en osteoblastos y osteocitos. OPG se une a RANKL, interfiriendo de esta manera con su capacidad de unirse a su receptor, RANK, ejerciendo así una supresión significativa sobre la diferenciación de osteoclastos, y, por tanto, evitando la resorción ósea (159) (Figura 10).

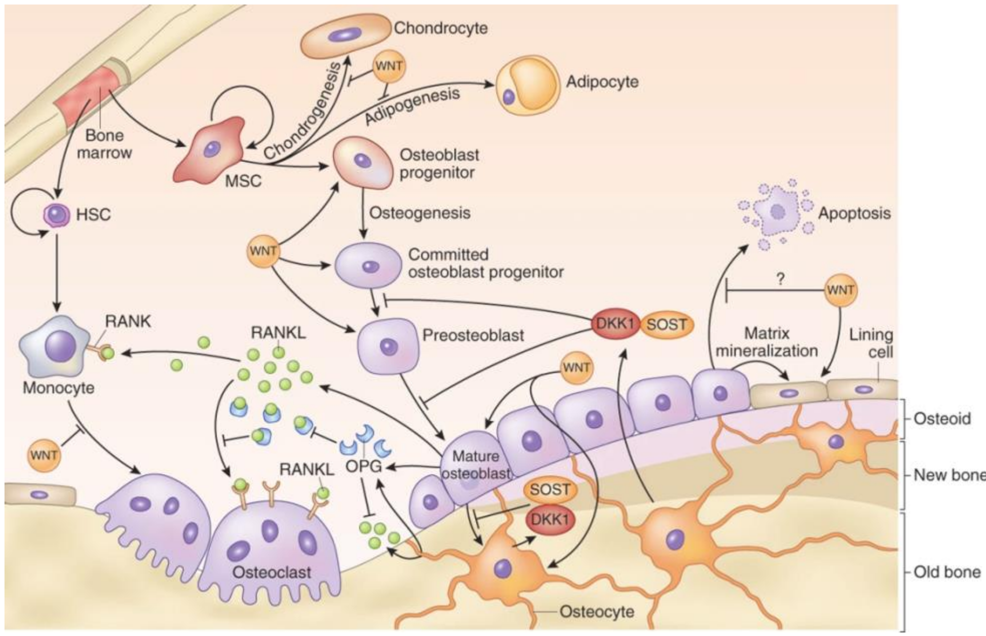

Figura 10. Impacto de la señalización de Wnt/β-catenina en las células óseas (Barón R. y Kneissel M., 2013) (155). DKK, dickkopf; HSC, células madre hematopoyéticas; MSC, células mesenquimales; OPG, osteoprotegerina; RANK, receptor activador del factor nuclear κβ; RANKL, ligando del receptor activador para el factor nuclear κβ; SOST, esclerostina.

Cabe resaltar que varios antagonistas de la vía Wnt canónica desempeñan un importante papel modulando la regulación ósea, debido a que actúan inhibiendo la formación ósea, como son Dkk1y esclerostina (160,161), esta última será explicada más adelante detalladamente. La proteína Dkk1 es secretada principalmente por osteocitos, y su sobreexpresión reduce la señalización de la vía Wnt canónica, cambiando efectivamente la vía de diferenciación de las células madre mesenquimales al inhibir la osteoblastogénesis e inducir la adipogénesis (162). Varias investigaciones han manifestado la relevancia de Dkk1 en la homeostasis ósea. La disminución de la expresión del gen que codifica Dkk1 resulta en un fenotipo caracterizado por una mayor DMO (160), mientras que un aumento en su expresión conduce a una disminución de la DMO (163). En este contexto, se ha observado en estudios con modelos

animales, que el tratamiento con anticuerpos dirigidos contra Dkk1 produce efectos beneficiosos en la DMO (162).

3.1.2.2. Vía Wnt canónica a nivel vascular

La función atribuida a la vía Wnt canónica se ha asociado convencionalmente con la regulación del metabolismo óseo y la patogénesis de las alteraciones óseas. No obstante, la evidencia científica de las últimas décadas ha revelado que la señalización de Wnt/β-catenina desempeña un papel clave en el desarrollo y mantenimiento de la vasculatura (164), estando estrechamente vinculada a los procesos ateroscleróticos (52).

La señalización Wnt/β-catenina está implicada en el desarrollo vascular. La formación de vasos sanguíneos está intrínsecamente ligada a la diferenciación de las células endoteliales derivadas de precursores hematopoyéticos. Diversos estudios con células madre embrionarias cultivadas *in vitro* respaldan la noción de que la activación de la vía Wnt canónica estimula efectivamente la diferenciación de las células endoteliales (165). Además de su importante contribución en los procesos angiogénicos del sistema nervioso central durante el desarrollo embrionario, la vía Wnt/β-catenina influye en el brote vascular, la remodelación y la especificación arteriovenosa mediante la modulación de la vía de Notch. Asimismo, la señalización de la vía Wnt canónica se ha implicado en la formación de válvulas cardíacas al iniciar la transición endotelial-mesenquimatosa (164). Por otro lado, se ha demostrado que la vía Wnt/β-catenina se encuentra involucrada en la proliferación, migración y supervivencia de las CMLV (154,166).

En consecuencia, las alteraciones en los componentes de la vía Wnt/β-catenina pueden provocar inflamación cardiovascular, alteración de la plasticidad celular, acumulación de colesterol intracelular y respuestas osteofibróticas (120). De este modo, la vía Wnt/β-catenina juega un papel importante en la disfunción endotelial (que es un cambio patológico temprano y fundamental para la DM2) y en la progresión de la enfermedad cardíaca, tanto en alteraciones metabólicas (sensibilidad a la insulina) como en remodelación cardiovascular y cambios estructurales (fibrosis, esclerosis, formación de ateromas y proliferación e hipertrofia de CMLV) (52). En este contexto, se ha revelado la implicación de la vía Wnt-β-catenina en las células vasculares activadas por una lesión vascular o por un evento de isquemia, reafirmando que esta vía parece regular la proliferación y la apoptosis de las CMLV (166). Se han descrito diversas alteraciones en diferentes componentes de la Wnt/β-catenina implicados en las complicaciones microvasculares y macrovasculares asociadas a la DM2 (52).

Por ejemplo, se ha demostrado que los niveles circulantes de Dkk1 y esclerostina tienen una asociación positiva con la aterosclerosis en pacientes con DM2 (167,168).

3.2. Células del músculo liso vascular

Las CMLV son células musculares no estriadas y contráctiles, que se encuentran en una variedad de tipos de tejidos, incluidos los vasos sanguíneos, la tráquea, el iris, la vejiga urinaria y el tracto digestivo (169).

Con respecto a los vasos sanguíneos, las arterias se componen de 3 capas (íntima, media y adventicia), las cuales tienen una distinta composición y función para mantener la integridad y funcionalidad en el sistema circulatorio. Las CMLV constituyen, en condiciones fisiológicas, el principal componente celular de la capa media arterial, siendo su principal función la vasoconstricción y vasodilatación, manteniendo así el tono vascular, la presión arterial y la distribución del flujo sanguíneo (170). Además, las CMLV también desempeñan un papel vital en el mantenimiento y la remodelación de la matriz extracelular de los vasos sanguíneos. La matriz extracelular está compuesta por microfibrillas elásticas (elastina, fibrilina), colágeno (principalmente tipos I, III, IV, V y VI), proteínas matricelulares (fibronectina, tenascina y trombospondina), factores de crecimiento y proteasas secuestradas en matriz, y proteoglicanos (171).

En el tejido adulto normal, las CMLV exhiben un fenotipo contráctil caracterizado por una proliferación lenta, funcionalidad contráctil, respuesta a señales como acetilcolina y noradrenalina, y expresión de diversas proteínas contráctiles del músculo liso (SM), entre las que se incluyen SM α-actina (SMαA), SM-22α, cadenas pesadas de miosina SM-1 y SM-2, calponina y smoothelina. Sin embargo, las CMLV no están diferenciadas terminalmente y muestran plasticidad fenotípica. Estas células pueden alterar su fenotipo en respuesta a estímulos de su entorno, como una lesión, siendo capaces de disminuir la expresión de las proteínas contráctiles, aumentar la proliferación y remodelar la matriz extracelular para facilitar la migración. Tradicionalmente esta transición fenotípica de un estado contráctil a lo que se denomina estado sintético, se consideraba un proceso binario en el que las células retornaban al estado contráctil una vez completada la reparación. Sin embargo, investigaciones recientes han revelado que las CMVL tienen la capacidad de mantener un espectro de fenotipos y pueden manifestar características de osteoblastos, condrocitos, adipocitos y células espumosas de macrófagos (60,172) (Figura 11).

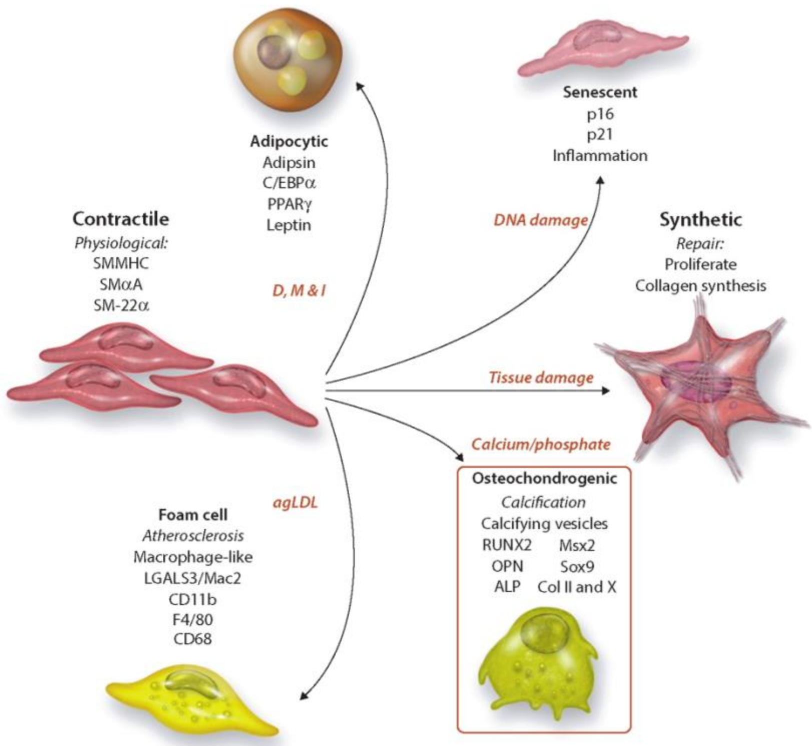

Figura 11. Representación esquemática del espectro de fenotipos de las CMLV, sus marcadores celulares (en negro), y las condiciones del entorno (en rojo) (Durham A. *et al.*, 2018) (60). ALP, fosfatasa alcalina; Col II and Col X, colágeno tipo I y X; D, Dexametasona; I, insulina; M, Metilisobutilxantina; OC, osteocalcina; OPN, osteopontina; PPARγ, receptores gamma activados por el factor proliferador de peroxisomas; RUNX2, factor de transcripción 2 relacionado con Runt; SM22α, músculo liso 22 α; SM α-actin, músculo liso α-actina.

Existen estudios *in vitro* en los que se han clasificado los distintos fenotipos de las CMLV, a través de la estimulación de las células para impulsar la diferenciación a los diferentes linajes. Por ejemplo, las CMLV cultivadas con lipoproteínas agregadas de baja densidad (agLDL) presentan una capacidad elastogénica regulada negativamente y un aumento de los marcadores de células espumosas de macrófagos (173). Asimismo, las CMLV cultivadas en medios de diferenciación apidogénicos experimentan una transición a un fenotipo adipocítico, expresando marcadores de adipocitos, como adipsina, proteína de unión a AGLs de adipocitos, PPARγ y leptina, (174). Por otro lado, las CMLV en entornos calcificantes experimentan una transición fenotípica desde un estado contráctil hacia un fenotipo osteo/condrogénico (60,175). Esta transición se caracteriza por el desarrollo de vesículas calcificantes, regulación negativa de las moléculas inhibidoras de la mineralización y elaboración de una matriz propensa a la calcificación (176). Este fenotipo se acompaña de pérdida de marcadores SM (SM22α y SM α-actina) y ganancia de marcadores

osteocondrogénicos (RUNX2, OPN, OC y ALP, Sox9, colágeno tipo II y X) (60). Además, se ha demostrado que este fenotipo de las CMLV, similar a osteocitos, expresa marcadores típicamente óseos como es la esclerostina, antagonista en la vía Wnt canónica (175).

Debido a estas transiciones fenotípicas que sufren las CMLV dependiendo del entorno en el que se encuentran, estas células desempeñan un papel fundamental en diversas enfermedades siendo la principal la aterosclerosis (170,177). Un estudio reciente ha descrito 9 fenotipos de las CMLV que participan en las lesiones ateroscleróticas (Figura 12), desempeñando diversas funciones en este proceso patológico (172); sin embargo, se requieren más investigaciones para una comprensión más completa sobre la implicación de los diferentes fenotipos de CMLV en este proceso.

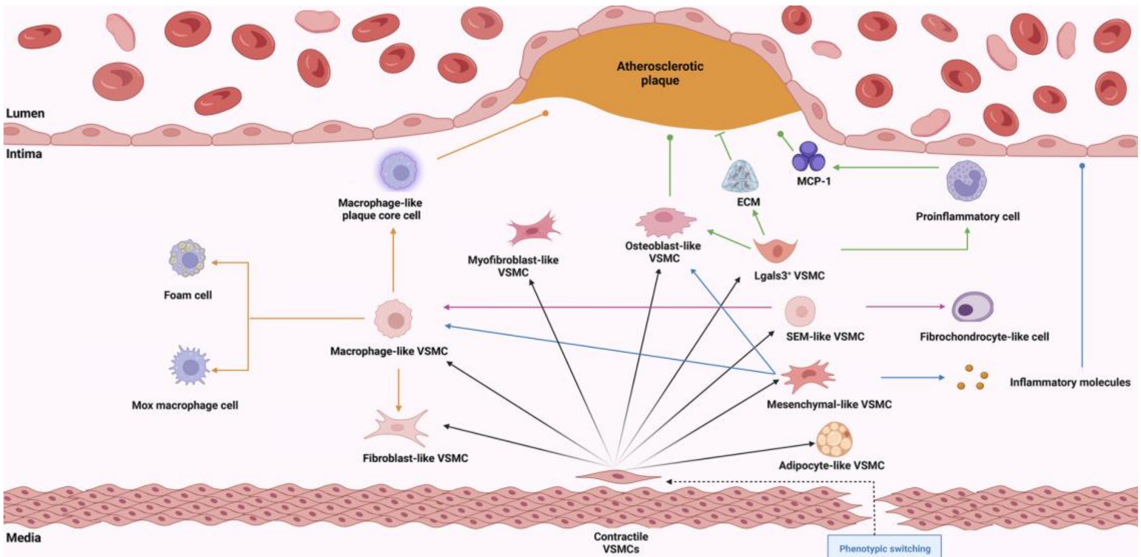

Figura 12. Cambio fenotípico de las CMLV en la aterosclerosis (Modificada de Chen R. *et al.*, 2023) (172). ECM, matriz extracelular; Lgals3, galectina 3; MCP-1, proteína 1 quimioatrayente de monocitos; SEM, célula madre/célula endotelial/monocito; VSMC, células del músculo liso vascular.

La visión histórica del papel de las CMLV en la aterosclerosis postula que su migración y proliferación en las capas íntima y media de las arterias contribuyen a la formación inicial de placas ateroscleróticas. Sin embargo en etapas avanzadas, estas células participan en la formación de capas fibrosas que, a su vez, estabilizan las placas inestables, lo cual se considera beneficioso (60). Se ha demostrado que la proliferación de CMLV puede ser beneficiosa durante toda la aterogénesis, y no solo en lesiones avanzadas, mientras que la apoptosis, la senescencia celular y las células similares a macrófagos derivadas de CMLV pueden promover la inflamación (178). Estudios de rastreo de los cambios en el linaje genético de las CMLV

durante el desarrollo de la aterosclerosis han demostrado que el cambio fenotípico de las CMLV resulta en células menos especializadas que carecen de marcadores contráctiles de CMLV, destacando principalmente la presencia de células que se asemejan a los macrófagos (178), y de manera significativa, se observa un fenotipo osteocondrogénico (60). Éstas últimas, se localizan comúnmente con depósitos de fosfato de calcio dentro de las lesiones ateroscleróticas (179).

Por otro lado, se ha revelado que la vía Wnt/b-catenina desempeña un papel crucial en la regulación de la proliferación, migración y supervivencia de las CMLV a través de la modulación de la expresión de proteínas de la matriz extracelular (170). Así, las proteínas implicadas en la vía Wnt/b-catenina como Wnt4, estimulan la proliferación de las CMLV y provocan el engrosamiento de la capa íntima vascular en la aterosclerosis (180). Por tanto, los inhibidores de la vía Wnt/b-catenina podrían facilitar el desarrollo de dianas terapéuticas para el tratamiento de la ECV.

3.3. Esclerostina

La esclerostina es una glicoproteína soluble codificada por el gen *SOST* en el cromosoma 17q12-q21. Esta proteína está compuesta por 213 aminoácidos, conservando 190 aminoácidos tras su proceso de maduración (181). Pertenece a la familia proteica DAN, la cual incluye también proteínas inhibidoras de las BMP. La esclerostina presenta una elevada variedad de características y propiedades estructurales, entre las que se incluye la presencia de brazos N y C-terminales altamente flexibles y una región rica en cisteína que contribuye a la formación de 4 enlaces disulfuro responsables de generar 3 bucles en la estructura tridimensional de la proteína (Figura 13). La identificación de la región del bucle 2 de la esclerostina como el sitio de unión para un anticuerpo que module su función, pone de manifiesto su importancia como diana terapéutica (182). Además, se ha demostrado que existen dos isoformas de la proteína, probablemente debido a diferentes estados de glicosilación (183).

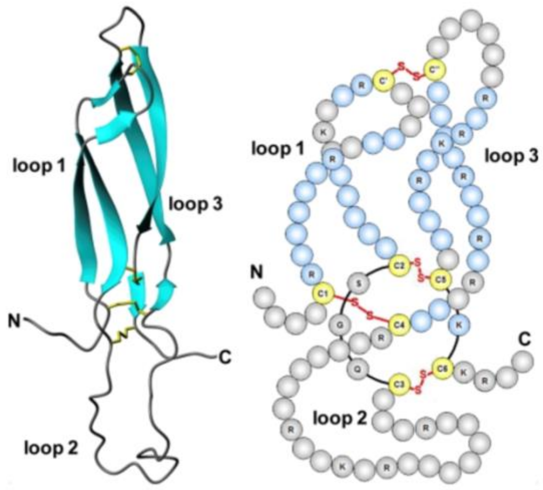

Figura 13. Estructura proteica tridimensional de la esclerostina (Adaptada de Veverka V., *et al.*, 2009) (182).

La esclerostina es segregada principalmente por osteocitos y desempeña un papel como inhibidor en la formación ósea al actuar como un antagonista de la vía Wnt/b-catenina (184). Esta proteína ejerce su función al interactuar con LRP5/6, bloqueando así la señalización de la vía Wnt canónica, y suprimiendo por tanto la diferenciación, proliferación y actividad del linaje osteoblástico (185), al mismo tiempo que induce la apoptosis (186). Además de su papel inhibidor de la osteoblastogénesis, también potencia la osteoclastogénesis a través de la vía dependiente de RANKL (187). Por otro lado, la esclerostina actúa como un cofactor que antagoniza la vía de las BMP, las cuales son inductoras de la formación ósea (188).

En el ámbito del metabolismo óseo, se han descrito tres displasias distintas debido a mutaciones genéticas en el gen *SOST:* la esclerosteosis, la enfermedad de Van Buchen y la displasia craneodiafisaria. La esclerosteosis es una displasia ósea esclerosante autosómica recesiva causada por la pérdida de la función de esclerostina. Esta patología se caracteriza por un crecimiento excesivo y progresivo generalizado del hueso que conduce a una estatura alta, distorsión facial, atrapamiento de los nervios craneales y aumento de la presión intracraneal que predispone a la muerte súbita. Las manifestaciones variables son sindactilia, desviación radial de las falanges terminales y displasia de las uñas (Figura 14). En la esclerosteosis, la formación ósea aumenta por una sobreactivación de la vía Wnt canónica, mientras que la resorción ósea no se ve afectada o disminuye levemente (184). En cuanto a la enfermedad de Van Buchen, se trata de una displasia ósea esclerosante autosómica recesiva causada por una deleción homocigótica de 52 kb de un elemento potenciador no codificante situado a unas 35 kb aguas abajo del gen *SOST* (189). Es una afección que se asemeja a la

esclerosteosis pero se diferencia por su carácter menos grave y la ausencia de sindactilia (184). En relación con la displasia craneodiafisaria, ésta surge debido a mutaciones heterocigotas en la señal de secreción del gen *SOST*, lo cual impide la secreción de esclerostina. Esta patología se caracteriza por hiperostosis generalizada masiva y esclerosis, que afecta especialmente al cráneo y a los huesos faciales (181).

Figura 14. Progresión de la esclerosteosis a lo largo del tiempo en una paciente (Epstein S., *et al.*, 1979) (190).

Por otro lado, se ha demostrado que una elevada expresión de esclerostina se asocia con la presencia de osteoporosis. En mujeres postmenopáusicas se describe que los niveles altos de esclerostina son un factor de riesgo fuerte e independiente de fracturas relacionadas con la osteoporosis (191). Asimismo, la esclerostina desempeña un papel clave en la mediación de la respuesta ósea a la descarga mecánica, una causa común de osteoporosis (161).

En consecuencia, el papel de la esclerostina como un agente inhibitorio en la formación ósea resulta de gran interés para el avance de estrategias terapéuticas en el tratamiento de enfermedades metabólicas óseas. Uno de los enfoques consiste en el bloqueo de esclerostina para el tratamiento de la osteoporosis. En la actualidad, en la práctica clínica se emplea un tratamiento conocido como Romozusumab para abordar la osteoporosis y reducir el riesgo elevado de fracturas. Este fármaco consiste en un anticuerpo monoclonal humanizado antiesclerostina (192), el cual bloquea la acción de la esclerostina inhibiendo su participación en la vía Wnt canónica. Una exhaustiva evaluación de este tratamiento ha demostrado beneficios óseos, aumentando la DMO de los pacientes, y por tanto, disminuyendo así el riesgo de fracturas; sin embargo, se ha identificado un potencial aumento en el riesgo de

sufrir eventos cardiovasculares adversos (193). En esta línea, el estudio *Active-Controlled Fracture Study in Postmenopausal Women With Osteoporosis at High Risk* (ARCH) reveló un aumento del riesgo de acontecimientos cardiovasculares adversos graves en mujeres postmenopáusicas durante el primer año de tratamiento con anticuerpos antiesclerostina (194). De hecho, los datos existentes hasta la fecha respaldan la idea de limitar las directrices de prescripción de este fármaco en pacientes con riesgo elevado de eventos cardiovasculares e ictus.

Estos hallazgos sugieren que la inhibición de la esclerostina podría estar vinculada al riesgo cardiovascular, aunque aún no se ha esclarecido por completo el papel de esta proteína en las ECV. En este contexto, se ha demostrado que la esclerostina puede ser expresada en CMLV en condiciones calcificantes (175). Consistentemente, se ha detectado la presencia de esclerostina en las CMLV de la capa media arterial de pacientes sometidos a endarterectomía carotídea, sugiriendo un papel de esta proteína en el desarrollo de la aterosclerosis (195). Además, se ha observado una elevada expresión de esclerostina en el tejido aórtico calcificado procedente de muestras de aorta pertenecientes a pacientes con aterosclerosis (196). A nivel sérico, varios estudios han demostrado una asociación positiva entre los niveles de esclerostina en suero con la incidencia de ECV, así como con la mortalidad cardiovascular (197–199). Asimismo, algunos estudios han confirmado una correlación positiva entre el nivel de esclerostina sérica y la aterosclerosis subclínica (200), las lesiones ateroscleróticas (168) y la mortalidad cardiovascular (199) en la población con DM2, independientemente del sexo y la edad. Adicionalmente, se ha reportado que los niveles circulantes de esclerostina suelen ser más elevados entre los hombres, tanto en pacientes con DM2 (168,201) como en sujetos de control (202).

Estos resultados apoyan firmemente que la esclerostina no se limita únicamente a la regulación del metabolismo óseo, sino que también desempeña un papel en la integridad vascular, constituyendo un importante modulador de la señalización de la vía Wnt/β-catenina en la ECV y actuando como posible marcador sérico del riesgo cardiovascular. A pesar de los significativos avances realizados hasta la fecha, aún no se ha determinado en estudios con humanos si la esclerostina juega un papel perjudicial o protector en la enfermedad aterosclerótica. Por consiguiente, uno de los objetivos principales de esta Tesis Doctoral se centra en profundizar en la comprensión de la función fisiopatológica de esta proteína, especialmente en su impacto en la fisiología vascular. Este análisis resulta crucial para avanzar en el diagnóstico temprano de la ECV y en el desarrollo de estrategias preventivas y

terapéuticas, abordando tanto las alteraciones en el metabolismo óseo como en el sistema vascular.

3.4. Osteoglicina

La osteoglicina, también conocida como factor osteoinductor o mimecan, es una proteína codificada por el gen *OGN* en el cromosoma 9q22.31. Se trata de una proteína secretora perteneciente a la clase III de los proteoglicanos pequeños ricos en leucina (SLRP), los cuales se encuentran involucrados en la regulación del ensamblaje de la matriz extracelular (203). La osteoglicina está compuesta por 298 aminoácidos (204) y, hasta la fecha, la resolución de la estructura tridimensional de esta proteína no ha sido lograda; sin embargo, se dispone de un modelo de predicción preciso de dicha estructura mediante programas de inteligencia artificial bioinformáticos (Figura 15).

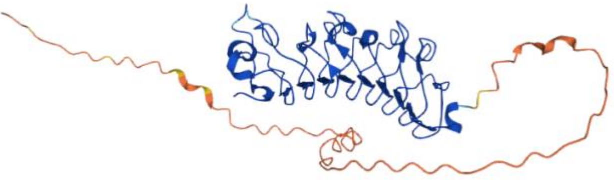

Figura 15. Predicción de la estructura proteica tridimensional de la osteoglicina mediante AlphaFoldDB.

En 1990, se identificó la osteoglicina por primera vez como un nuevo SLRP en una fracción del hueso bovino (205). Sin embargo, investigaciones actuales han confirmado la presencia de esta proteína en diversos tejidos de mamíferos. Estos incluyen, además del tejido óseo, el tejido muscular, vascular y adiposo (206). Se ha descrito que la osteoglicina tiene un sitio de glicosilación específico para cada tejido presentando diferentes modificaciones postraduccionales (207) relacionadas con su papel funcional en las diferentes localizaciones (208).

La osteoglicina está involucrada en varios procesos fisiológicos como la fibrinogénesis de colágeno, la proliferación y el desarrollo celular (208). En consecuencia, dada la amplia diversidad estructural y funcional de la osteoglicina, así como su expresión generalizada, no es sorprendente encontrar su participación en diversas patologías, como la fragilidad ósea, las enfermedades neurológicas, las enfermedades oculares, la enfermedad renal crónica (ERC), y las ECV, entre otras (208).

Aunque la principal función de la osteoglicina radica en la regulación del metabolismo óseo (209), los efectos de la osteoglicina sobre el recambio óseo son contradictorios según la evidencia actual. Se ha demostrado que la osteoglicina extracelular derivada del músculo aumenta la expresión de ALP, colágeno tipo I y β-catenina en los osteoblastos, aumentando así la diferenciación de estos y el desarrollo óseo. Asimismo, la BMP-2 aumenta la expresión de osteoglicina en los osteoblastos, potenciando su diferenciación y favoreciendo el desarrollo óseo (208). Por el contrario, se ha observado en células osteoblásticas murinas, que una sobreexpresión estable de osteoglicina disminuye significativamente los niveles de RUNX2 y Osterix, importantes promotores de la diferenciación osteoblástica mesenquimatosa y la maduración del hueso (206). Hay pocos estudios en humanos que investiguen la osteoglicina relacionada con el metabolismo óseo. No obstante, se ha demostrado que en mujeres postmenopáusicas con DM2, los niveles altos de osteoglicina se asocian con una disminución de la DMO y la presencia de fracturas vertebrales (210).

Por otro lado, destaca la importancia de la osteoglicina como un componente básico de la matriz extracelular vascular, expresada por cardiomiocitos, fibroblastos cardíacos y CMLV (211). Se ha observado expresión de osteoglicina por parte de las CMLV en arterias coronarias humanas en condiciones fisiológicas y en muestras humanas de lesiones ateroscleróticas avanzadas, mientras que las células endoteliales y los macrófagos no muestran esta expresión (211). Se ha observado que la disminución de la expresión de osteoglicina en CMLV por diversos factores de crecimiento promueve una mayor proliferación celular (208). Según estos estudios, la osteoglicina parece ser parte del sistema vascular normal y, de hecho, podría ser importante en la prevención de enfermedades cardiovasculares. Sin embargo, la función de la osteoglicina en el sistema vascular sigue siendo relativamente desconocida, lo que la convierte actualmente en un área de investigación en auge debido a la controversia reportada en la literatura científica. Algunos estudios han descrito una estrecha relación entre los niveles de osteoglicina y el riesgo de padecer ECV. En este sentido, se han descrito niveles incrementados de osteoglicina sérica en pacientes con EAC (212), así como una asociación positiva entre niveles de osteoglicina y rigidez arterial en pacientes hipertensos (213). En esta línea, se ha sugerido que la osteoglicina podría utilizarse como biomarcador del pronóstico en pacientes con EAC (214), así como de la mortalidad en pacientes con enfermedad arterial carotídea (215). Sin embargo, otros estudios apuntan en la dirección opuesta, describiendo un papel beneficioso de la osteoglicina contra el deterioro cardíaco en humanos (216). Adicionalmente, existe otra vertiente de investigaciones que apoyan la ausencia de correlación entre los niveles circulantes de

osteoglicina con el desarrollo de aterosclerosis tanto en pacientes con placa en la arteria carótida (217) como en modelos animales (218). Además, tampoco se encuentra asociación de los niveles de esta proteína con los principales acontecimientos adversos cardiovasculares, cerebrovasculares y la mortalidad en pacientes con DM2 y ERC (219). También existen resultados contradictorios en cuanto a los niveles de osteoglicina circulante en relación con alteraciones renales. Así, por un lado se ha reportado un aumento significativo de las concentraciones de osteoglicina en pacientes con DM2 y nefropatía diabética en comparación con aquellos sin nefropatía diabética y controles sanos (220), y por otro lado, se sugiere que la disminución de los niveles séricos de osteoglicina se relacionan estrechamente con el desarrollo y la patogénesis de la nefropatía diabética (221).

En el contexto de la DM2, se ha planteado que la osteoglicina podría desempeñar un papel en la homeostasis de la glucosa (206). Sin embargo, la evidencia actual es limitada y se basa principalmente en un estudio reciente que reveló que, durante una prueba de tolerancia a la glucosa en ratones, el tratamiento con osteoglicina se relacionó con una reducción de los niveles de glucosa en sangre dependiente de la dosis. Además, durante las pruebas de tolerancia a la insulina, el tratamiento con osteoglicina intensificaba la disminución de los niveles de glucosa en respuesta a la insulina, lo que sugiere que la acción de la insulina se ve reforzada por esta proteína (209). En consecuencia, estos resultados sugieren que la osteoglicina puede ser una molécula metabólicamente activa; no obstante, se requieren más investigaciones para examinar la dinámica de esta proteína, así como para explorar su relación con la DM2, especialmente en estudios con humanos.

Las discrepancias evidenciadas en la función de la osteoglicina en las enfermedades vasculares, junto con la falta de comprensión sobre su papel en la homeostasis glucémica en humanos, resaltan la necesidad de realizar investigaciones más exhaustivas. Por tanto, aclarar la relación entre los niveles de osteoglicina y estos procesos emerge como otro objetivo fundamental en la temática de la Tesis Doctoral.

HIPÓTESIS Y OBJETIVOS

Hipótesis de trabajo

El conocimiento de los factores implicados en la DM2 y sus complicaciones asociadas es esencial para el diagnóstico temprano y la prevención de enfermedades vasculares. Cada vez hay una mayor evidencia que respalda la existencia de un eje de conexión entre el metabolismo óseo y el sistema vascular. Se postula que algunas de las proteínas implicadas en el metabolismo óseo desempeñan además un papel fundamental en la fisiopatología de la DM2 y en el desarrollo de ECV asociadas a la misma. En este contexto se hipotetiza que la modulación de estas proteínas (osteoglicina y esclerostina) podría ser crucial tanto en el diagnóstico de ECV asociadas con la DM2 como en el desarrollo de nuevas estrategias terapéuticas y preventivas para abordar estas complicaciones, mejorando así la calidad de vida de los pacientes con DM2.

Objetivo principal

El propósito principal de esta Tesis Doctoral consiste en identificar proteínas que desempeñen un papel dual, con implicación tanto en el metabolismo óseo como en el sistema vascular, con el fin de identificar potenciales biomarcadores y/o dianas terapéuticas implicadas en el desarrollo de las enfermedades vasculares asociadas a la DM2, así como conocer la función que desempañan estas proteínas durante los procesos de calcificación vascular.

Objetivos específicos

1. Determinar los niveles séricos de osteoglicina en pacientes con DM2 y TFGe normal o ligeramente reducida, y en una población control, para evaluar su utilidad como biomarcador de una función renal alterada en DM2.

2. Investigar la implicación de la osteoglicina en el desarrollo de la aterosclerosis asociada a la DM2.

    2.1. Cuantificar los niveles de osteoglicina en muestras séricas y de tejido vascular procedentes de pacientes con DM2, con presencia/ausencia de ECV, así como de sujetos control, para analizar la asociación entre esta proteína y la aterosclerosis en DM2.

    2.2. Evaluar el efecto de la sobreexpresión de osteoglicina en CMLV expuestas a entornos calcificantes en el desarrollo de la aterosclerosis.

3. Analizar la función de la esclerostina en el desarrollo de la aterosclerosis asociada a la DM2.

    3.1. Cuantificar los niveles de esclerostina en muestras séricas y de tejido vascular procedentes de pacientes con DM2, con presencia/ausencia de ECV, así como de sujetos control, para analizar la asociación entre esta proteína y la aterosclerosis en DM2.

    3.2. Evaluar el efecto de la sobreexpresión de esclerostina en CMLV expuestas a entornos calcificantes en el desarrollo de la aterosclerosis.

TRABAJO CIENTÍFICO Y RESULTADOS

CAPÍTULO I

Osteoglycin as a Potential Biomarker of Mild Kidney Function Impairment in Type 2 Diabetes Patients

Sheila González-Salvatierra, Cristina García-Fontana, Francisco Andújar-Vera, Alejandro Borja Grau-Perales, Luis Martínez-Heredia, María Dolores Avilés-Pérez, María Hayón-Ponce 1, Iván Iglesias-Baena, Blanca Riquelme-Gallego, Manuel Muñoz-Torres and Beatriz García-Fontana.

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Article

Osteoglycin as a Potential Biomarker of Mild Kidney Function Impairment in Type 2 Diabetes Patients

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Abstract: Osteoglycin (OGN) could be a biomarker of mild kidney function impairment in type 2 diabetes (T2D). Our study aimed to determine the association between serum OGN and impaired kidney function risk in T2D patients and to analyze its potential role as an estimator of kidney disturbances in this population. This cross-sectional study included 147 T2D patients (65 ± 8 years, 58.5% males), and 75 healthy controls (63 ± 10 years, 36% males). Circulating OGN levels were determined by ELISA. Linear regression modeling was performed to determine the variables influencing circulating OGN, and an ROC curve was plotted to assess the usefulness of OGN as an estimator of diabetic kidney disease risk. Circulating OGN was significantly increased in T2D patients compared to controls (18.41 (14.45–23.27) ng/mL vs. 8.74 (7.03–12.35) ng/mL; $p < 0.001$). We found a progressive increase in serum OGN according to the severity of kidney impairment in T2D patients (normal kidney function: 16.14 (12.13–20.48) ng/mL; mildly impaired kidney function: 19.15 (15.78–25.90) ng/mL; moderate impaired kidney function: 21.80 (15.06–29.22) ng/mL; $p = 0.006$). Circulating OGN was an independent estimator of mildly impaired kidney function risk in T2D patients. We suggest that serum OGN could act as an albuminuria-independent biomarker of incipient kidney dysfunction in T2D patients.

Keywords: biomarker; diabetic kidney disease; kidney function impairment; osteoglycin; type 2 diabetes

1. Introduction

Osteoglycin (OGN), also known as osteoinductive factor or mimecan, is a secretory protein belonging to class III of the small leucine-rich proteoglycans [1]. OGN is involved in several biological processes [2–4] and is related to various pathologies, such as bone fragility, cardiovascular disease (CVD), neurologic disease, ocular diseases, and chronic kidney disease (CKD), among others [2,3]. OGN has a tissue-specific glycosylation site and different post-translational modifications [5] related to its functional role in different locations [2]. OGN participates mainly as a regulator of bone metabolism [6,7], as it is a bone-associated glycoprotein, expressed by osteoblasts [8,9]. Moreover, OGN is a basic

component of the vascular extracellular matrix, which is expressed by cardiomyocytes, cardiac fibroblasts, and vascular smooth muscle cells [10,11].

Diabetic kidney disease (DKD) is one of the most frequent complications of type 2 diabetes (T2D). The classic description of DKD involves progressive stages of glomerular hyperfiltration, microalbuminuria, overt proteinuria, and a decline in the estimated glomerular filtration rate (eGFR) [12]. It was widely accepted that patients with DKD develop albuminuria before a decrease in the eGFR. However, these concepts have been increasingly challenged as evidence suggests that DKD is presented in a more heterogeneous manner. Large cross-sectional studies have revealed that a significant proportion of T2D patients with impaired kidney function determined by decreased eGFR values present normal levels of albuminuria [13–18]. Therefore, determining biomarkers associated with impaired eGFR could be a useful measurement to analyze the progression to DKD in T2D patients.

Few and contradictory results are known regarding OGN levels in T2D patients with DKD [19,20]. In these studies, OGN was suggested to be a sensitive marker for early microalbuminuria; however, there is no consensus on the level of this protein in T2D patients with DKD compared to healthy subjects. Therefore, the role of OGN in kidney function has not yet been clarified in humans.

Hence, we determine the serum OGN levels in T2D patients with mildly decreased eGFR, in order to assess their significance as a biomarker of impaired kidney function.

2. Materials and Methods

2.1. Study Population

This cross-sectional study included 222 participants, 147 T2D patients (65 ± 8 years, 58.5% males), and 75 healthy controls (63 ± 10 years, 36% males). T2D was diagnosed according to the American Diabetes Association criteria from 2017 [21]. The recruitment of T2D patients was from 2017 to 2018 in the Endocrinology and Nutrition Unit of the University Hospital Clínico San Cecilio of Granada according to the following criteria: Caucasians having normal values for their blood count, hepatic function, calcium, and phosphorus. The T2D group was classified into two subgroups according to their eGFR: normal eGFR (eGFR $\geq$ 90 mL/min/1.73 m^2; mean eGFR 100 ± 7) (n = 62) and mildly decreased eGFR (eGFR < 90 mL/min/1.73 m^2; mean eGFR 69 ± 15) (n = 85). Patients with liver, gastrointestinal, and thyroid disease; cancer; dialysis; or renal transplantation were excluded.

Serum samples from healthy controls were supplied by the SSPA Biobank from blood donors of the Andalusian Regional Government Health Service. Healthy donors did not have metabolic diseases as diabetes or infectious, neoplastic, hepatic, cardiovascular, gastrointestinal, central nervous system, or renal diseases (according to section B of Annex II of Royal Decree 1088/2005, of 16 September 2005). All samples used for the study were managed by the SSPA Biobank of the University Hospital Clínico San Cecilio of Granada. Informed consent was obtained from each patient.

This study was conducted with the approval of the Ethics Committee of the University Hospital Clínico San Cecilio of Granada and conformed to the principles of the World Medical Association Declaration of Helsinki (Project ID: 0858-N-17, Research Ethics Committee of Granada Center (CEI-Granada) on 26 April 2017).

2.2. Clinical Evaluation

The height, weight, and waist circumference were measured according to standard procedures. The body mass index (BMI) was calculated by the Quetelet formula, weight (kg)/stature (m^2). The systolic and diastolic blood pressure was measured using a standard electronic sphygmomanometer. Hypertension was defined as values ≥140/90 mmHg and/or antihypertensive treatment. Dyslipidemia was characterized by serum levels of low-density lipoprotein cholesterol (LDL-c) >100 mg/dL, high-density lipoprotein cholesterol (HDL-c) <50 mg/dL, triglycerides (TG) >150 mg/dL, and/or current treatment

with lipid-lowering drugs. Patients reported their alcohol use, smoking status, and level of physical activity in response to specific health questionnaires [22].

2.3. Biochemical Measurements

Samples of venous blood were taken in the morning after fasting overnight. Serum samples were stored at −80 °C until they were analyzed at the Clinical Analysis Unit of the University Hospital Clínico San Cecilio of Granada. The fasting plasma glucose (FPG), glycated hemoglobin (HbA1c), TG, HDL-c, LDL-c, calcium, and phosphorus were measured using standard automated laboratory techniques. The eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration equation [23]. The albumin and creatinine in urine were obtained using a standardized protocol. The abnormal albuminuria was estimated from the urine albumin-to-creatinine ratio (UACR) and was defined as UACR $\geq$ 30 mg/g.

The calciotropic hormone profile included serum intact parathormone (iPTH) and 25-hydroxyvitamin D (25(OH)D) as determined with the two-site immunoassay (Roche Diagnostics SL, Barcelona, Spain) and the chemiluminescence immune assay (Beckman Coulter UniCel DxI 800, Brea, CA, USA), respectively.

The OGN was determined in duplicate by the enzyme-linked immunosorbent assay (ELISA) method developed by Cloud-Clone Corp. (Houston, TX, USA), following the manufacturer's protocol. Precision testing was performed by the determination of the intra-assay and inter-assay coefficients of variation of OGN (<10% and <12%, respectively).

Fibroblast growth factor 23 (FGF-23) was measured in duplicate using ELISA (Biomedica). The intra- and inter-assay coefficients of variation of FGF-23 were <6% and <8%, respectively.

2.4. Statistical Analysis

Analyses were performed using SPSS version 22.0 software (SPSS, Inc., Chicago, IL, USA). The data were expressed as the means ± standard deviation (SD) for the normally distributed variables and as the median with the interquartile range (IQR) for variables that were not normally distributed. The data for categorical variables are presented as percentages. A Shapiro–Wilk test was used to test the normality of the distribution of the continuous variables. The mean values between groups were compared using the unpaired Student's *t*-test for continuous and normally distributed variables. The Mann–Whitney U test and Kruskal–Wallis test were used to compare the variables that were not normally distributed. When the comparison between groups required an adjustment by covariates, an analysis of covariance (ANCOVA) was performed. The χ^2 test was used to compare categorical variables between groups.

Associations between continuous variables were described by Spearman's correlation coefficients. Multiple linear regression modeling was performed to determine the variables independently associated with the OGN (dependent variable), including the quantitative and qualitative variables linked in the bivariate analysis and other variables biologically associated with OGN as independent variables.

To identify OGN as an independent predictor of impaired kidney function, multiple logistic regression modeling was performed, including mildly decreased eGFR as a dependent variable. The independent variables included in the model were the established factors related to impaired kidney function risk in addition to the OGN level. The usefulness of serum OGN as an estimator of impaired kidney function risk was assessed using a receiver operating characteristic (ROC) curve. The area under the curve (AUC) indicates the probability of predicting an event. AUC values greater than 0.75 indicate good predictive performance.

The statistical significance was set at $p < 0.05$ (two-tailed) and $p < 0.10$ for multiple linear regression analysis.

3. Results

3.1. Characteristics of the Study Population

The clinical, anthropometric, and biochemical parameters of T2D participants according to normal or mildly impaired kidney function are summarized in Table 1.

Table 1. Comparison between eGFR ≥ 90 mL/min/1.73 m^2 and eGFR < 90 mL/min/1.73 m^2 in T2D patients.

	eGFR (mL/min/1.73 m^2)		
	eGFR ≥ 90	**eGFR < 90**	***p***
Patients *(n)*	62	85	
Men/women (%)	56/44	60/40	0.666
Age (years)	62 ± 7	68 ± 8	<0.001 *
eGFR (mL/min/1.73 m^2)	100 ± 7	69 ± 15	<0.001 *
CLINICAL EVALUATION			
Body weight (kg)	86 ± 15	87 ± 13	0.838
Height (cm)	165 ± 0.09	165 ± 0.08	0.717
BMI (kg/m^2)	32 ± 5	32 ± 4	0.944
Waist circumference (cm)	106 ± 11	106 ± 10	0.900
Diabetes duration (years)	14 ± 10	15 ± 9	0.468
Systolic blood pressure (mmHg)	133 ± 17	137 ± 18	0.133
Diastolic blood pressure (mmHg)	79 ± 9	79 ± 12	0.833
UACR ≥ 30 mg/g (%)	21	24	0.657
Hypertension (%)	76	92	0.007 *
Dyslipidemia (%)	82	93	0.045 *
CVD (%)	25.8	43.5	0.027 *
Osteoporosis (%)	9.7	5.9	0.399
Smoker or ex-smoker (%)	48	46	0.843
Alcohol consumption excessive (%)	20	13	0.271
Sedentarism (%)	15	17	0.735
CURRENT MEDICATION USE			
Insulin (%)	10	14	0.756
Oral antidiabetic drugs (%)	31	28	0.551
Insulin + Oral antidiabetic drugs (%)	59	58	0.423
BIOCHEMICAL MEASUREMENTS			
FPG (mg/dL)	150 ± 52	150 ± 55	0.989
HbA1c (mmol/mol)	62 ± 16	63 ± 15	0.792
HbA1c (%)	7.8 ± 1.4	7.9 ± 1.3	0.792
TG (mg/dL)	158 ± 71	166 ± 85	0.568
HDL-c (mg/dL)	47 ± 13	44 ± 10	0.128
LDL-c (mg/dL)	98 ± 44	88 ± 37	0.127
Calcium (mg/dL)	9.8 ± 0.4	9.7 ± 0.4	0.368
Phosphorous (mg/dL)	3.4 ± 0.5	3.3 ± 0.4	0.098
25(OH)D (ng/mL)	20 ± 8	22 ± 9	0.167
iPTH (pg/mL)	44 ± 25	56 ± 34	0.029 *
FGF-23 (pmol/L)	0.86 (0.47–1.70)	1.25 (0.77–2.44)	0.028 *
OGN (ng/mL)	16.14 (12.13–20.48)	19.59 (15.70–26.90)	0.002 *

T2D: type 2 diabetes; eGFR: estimated glomerular filtration rate; BMI: body mass index; UACR: urine albumin-to-creatinine ratio; CVD: cardiovascular disease; FPG: fasting plasma glucose; HbA1c: glycated hemoglobin; TG: triglycerides; HDL-c: high-density lipoprotein cholesterol; LDL-c: low-density lipoprotein cholesterol; 25(OH)D: 25-hydroxyvitamin D; iPTH: intact parathormone; FGF-23: fibroblast growth factor 23; OGN: osteoglycin. The data for continuous and normally distributed variables are presented as the mean ± SD. The data for continuous variables that are not normally distributed are presented as the median followed by the interquartile range in brackets. The data for categorical variables are presented as percentages. Student's *t*-test and the Mann–Whitney *U* test were used for comparisons of continuous and normally or not normally distributed variables, respectively, between groups. The χ^2 test was used for the comparison of categorical variables between groups. The * symbol represents statistically significant differences ($p < 0.05$) between groups.

The recruited groups were homogenous, and there were no differences between them in sex, weight, height, BMI, waist circumference, or diabetes duration. Regarding age, there was a significant difference between groups. Most of the clinical parameters were also comparable between groups except for hypertension, dyslipidemia, and CVD. The biochemical parameters differed between the groups in terms of the serum levels of iPTH, FGF-23, and OGN.

3.2. Influence of Diabetes Status, Sex, and eGFR on the Serum OGN Levels

The serum OGN levels were significantly higher in T2D patients ($n = 136$, 57% males) than in control subjects ($n = 75$, 36% males) (18.41 (14.45–23.27) ng/mL vs. 8.74 (7.03–12.35) ng/mL; $p < 0.001$). When T2D patients and control subjects were further divided according to sex, the significant differences in OGN levels remained for both males (16.68 (13.44–20.77) ng/mL vs. 8.79 (7.64–11.17) ng/mL; $p < 0.001$), and females (19.88 (15.70–26.30) ng/mL vs. 8.70 (6.65–12.41) ng/mL; $p < 0.001$). We found that the OGN levels were significantly higher in females than in males in the T2D group (19.88 (15.70–26.30) ng/mL vs. 16.68 (13.44–20.77) ng/mL; $p = 0.009$), but no differences were found for the healthy controls according to sex (Figure 1A).

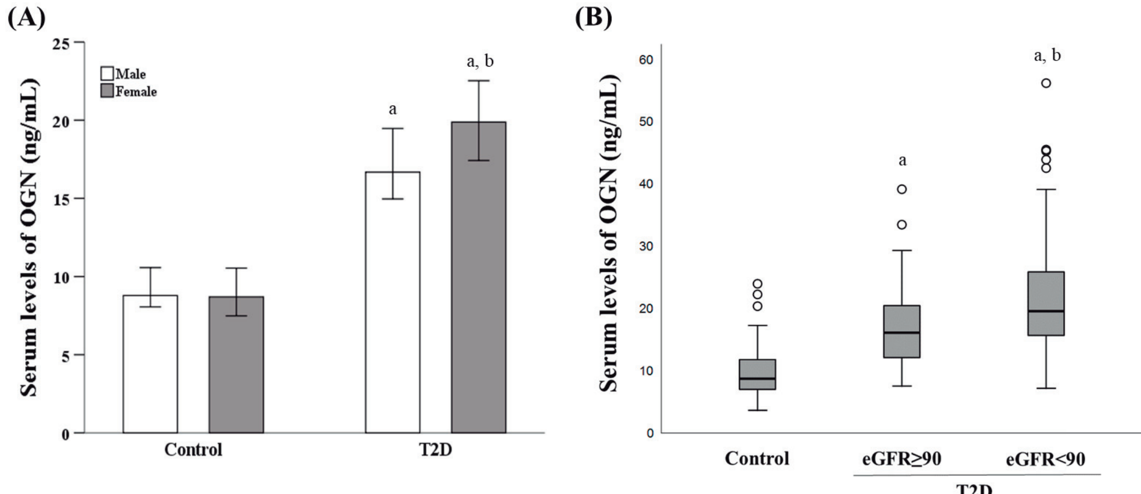

Figure 1. Serum OGN levels in control subjects and T2D patients according to sex and eGFR. (**A**) Serum levels of OGN (median ± 95% confidence interval (CI)) in the control and T2D groups for both sexes. The Kruskal–Wallis test was used for comparisons between groups. a = $p < 0.05$ vs. control of the same sex; b = $p < 0.05$ vs. male in T2D patients. (**B**) Box plot of serum OGN levels in controls, T2D patients with normal eGFR (eGFR ≥ 90 mL/min/1.73 m^2), and T2D patients with mildly decreased eGFR (eGFR < 90 mL/min/1.73 m^2). Box plot represents the minimum value, 25th percentile, median, 75th percentile, maximum value, and outliers for each group. The Kruskal–Wallis test was used for comparisons between groups. a = $p < 0.05$ vs. control; b = $p < 0.05$ vs. T2D with eGFR ≥ 90 mL/min/1.73 m^2. OGN: osteoglycin; T2D: type 2 diabetes; eGFR: estimated glomerular filtration rate.

The comparison of serum OGN levels between control subjects and T2D patients with normal ($n = 58$, 55% males) and mildly decreased ($n = 78$, 59% males) eGFR revealed significant differences between all groups ($p < 0.001$). The control group showed lower levels of serum OGN (8.74 (7.03–12.35) ng/mL). T2D patients with normal eGFR showed lower circulating OGN levels compared to T2D patients with mildly decreased eGFR (16.14 (12.13–20.48) ng/mL vs. 19.59 (15.70–25.90) ng/mL; $p = 0.013$) (Figure 1B). After adjusting by age and sex, this trend in OGN levels remained unchanged (18.02 ± 1.09 vs. 21.59 ± 0.93; $p = 0.017$).

In the group of T2D patients with mildly decreased eGFR, there is a 20.5% of T2D patients with a moderate decrease in eGFR (<60 mL/min/1.73 m^2). In this subgroup ($n = 16$,

65 ± 8 years, 60% males), the highest levels of OGN were observed (21.80 (15.06–29.22) ng/mL) with significative differences compared to normal eGFR group (p = 0.022).

3.3. Determinants of Serum OGN Levels in the T2D Group

We found a positive correlation between the serum level of OGN with age (r = 0.226; p < 0.001), iPTH (r = 0.179; p = 0.042), and FGF-23 (r = 0.324; p < 0.001) and a negative correlation with eGFR (r = −0.189; p = 0.027) in T2D patients (Figure 2).

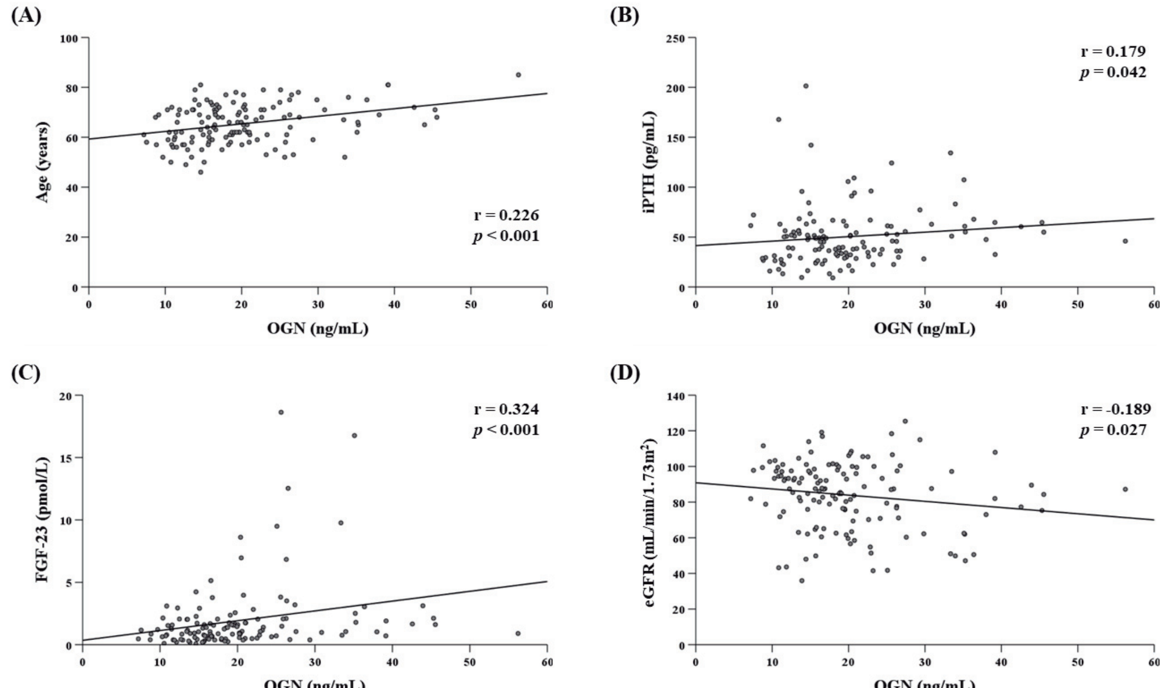

Figure 2. Scatter plots showing the correlation (Spearman's test) between OGN (ng/mL) and: age (years): (**A**), iPTH (pg/mL), (**B**), FGF-23 (pmol/L), (**C**), and eGFR (mL/min/1.73 m^2), (**D**), in T2D patients. OGN: osteoglycin; iPTH: intact parathormone; FGF-23: fibroblast growth factor 23; eGFR: estimated glomerular filtration rate; T2D: type 2 diabetes.

To analyze the variables that influence the level of OGN, a model of multiple linear regression analysis was performed including the variables associated with OGN in the previously performed bivariate analysis (age, sex, iPTH, FGF-23, and eGFR) in addition to the diabetes duration, insulin treatment, UACR, presence of osteoporosis, and presence of CVD as independent variables. The results showed that the variables independently associated with the OGN serum level were age and FGF-23, as shown in Table 2.

The presence of CVD was not independently associated with serum OGN level, although it bordered on significance (p < 0.061). The comparison of serum OGN levels in T2D patients according to the prevalence of CVD after age and sex adjustment revealed significant differences between both groups (p = 0.041). T2D patients with CVD (n = 45, 67 ± 7 years, 79% males) showed lower circulating OGN levels compared to T2D patients without CVD (n = 91, 65 ± 8 years, 47% males): (17.88 (15.39–20.38) ng/mL vs. 21.15 (19.44–22.86) ng/mL).

3.4. Association between FGF-23 Levels with Kidney Function and CVD

The serum FGF-23 levels were higher in T2D patients (n = 132, 57% males) compared to that in controls subjects (n = 75, 36% males) although not significantly (1.16 (0.63–2.05) pmol/L vs. 1.02 (0.47–1.85) pmol/L; p = 0.186). When T2D patients were divided according to

eGFR values, we observed higher circulating FGF-23 levels in the group with mildly decreased eGFR (n = 76, 59% males) compared to those with normal values (n = 56, 54% males) (p = 0.028) (Table 1). When patients with prevalent CVD were excluded from the analysis, we similarly observed higher FGF-23 levels in patients with decreased eGFR (n = 46) compared to those with normal eGFR values (n = 43): (1.55 (0.93–2.36) pmol/L vs. 0.84 (0.44–1.90) pmol/L; p = 0.005). No significative differences were found between T2D patients according to the prevalence of CVD independently of the kidney function.

Table 2. Multiple linear regression analysis of variables independently associated with serum OGN levels in T2D patients.

Variables	B	95% CI (Lower Limit/Upper Limit)	p
Age	0.319	0.091/0.547	0.007 *
Sex		−6.550/0.371	0.080
iPTH			0.554
FGF-23			0.004 *
eGFR	−0.036	−0.127/0.056	0.440
Insulin treatment	−0.089	−0.274/0.097	0.347
Current medication		−6.796/0.535	0.093
UACR ≥ 30 mg/g		−0.003/0.051	0.076
Presence of osteoporosis	−0.559	−6.128/5.010	0.842
Presence of CVD	−3.243	−6.641/0.156	0.061

OGN: osteoglycin; T2D: type 2 diabetes; CI: confidence interval; iPTH: intact parathormone; FGF-23: fibroblast growth factor 23; eGFR: estimated glomerular filtration rate; UACR: urine albumin-to-creatinine ratio; CVD: cardiovascular disease. The * symbol represents statistically significant differences ($p < 0.05$).

3.5. Usefulness of the OGN Serum Level to Estimate Impaired Kidney Function Risk in T2D Patients

Logistic regression modeling was performed to assess the variables related to impaired kidney function risk in T2D patients. The independent variables included in the model were age, hypertension, dyslipidemia, HbA1c levels (categorized according to the cutoff point of 7%), tobacco use, years of diabetes duration, presence of CVD, presence of osteoporosis, and UACR in addition to the OGN serum level. We found that, in addition to age (OR = 1.08; 95% CI (1.01/1.15); p = 0.021), the serum OGN level was an independent estimator of impaired kidney function risk (OR = 1.07; 95% CI (1.01/1.14); p = 0.029) in T2D patients.

ROC curve analysis was performed to assess the usefulness of the serum level of OGN as an estimator of impaired kidney function risk. Two different models were assessed, including the main impaired kidney function risk factors (age, hypertension, dyslipidemia, HbA1c level, tobacco use, and years of diabetes duration) with and without the serum OGN level (Figure 3).

The AUC of the model without OGN was 0.748, whereas the AUC of the model including OGN was 0.782 ($p < 0.001$ for both).

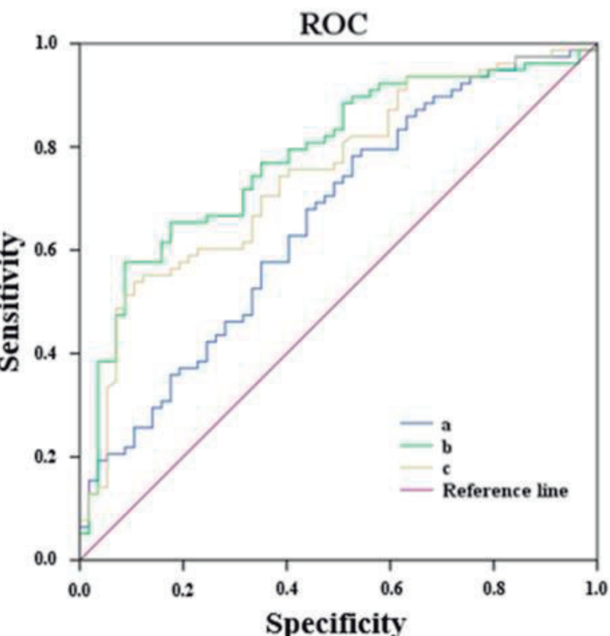

Figure 3. ROC curve for the usefulness of serum OGN level as an estimator of impaired kidney function in T2D patients. (a) OGN serum levels; AUC = 0.658; $p = 0.002$. (b) Age, hypertension, dyslipidemia, HbA1c levels (categorized according to the cutoff point of 7%), tobacco use, years of diabetes duration, and OGN serum levels; AUC = 0.782; $p < 0.001$. (c) Age, hypertension, dyslipidemia, HbA1c levels (categorized according to the cutoff point of 7%), tobacco use, and years of diabetes duration; AUC = 0.748; $p < 0.001$. ROC: receiver operating curve; OGN: osteoglycin; T2D: type 2 diabetes; AUC: area under the curve; HbA1c: glycated hemoglobin.

4. Discussion

Our cross-sectional study shows higher OGN levels in T2D patients compared to healthy controls for the first time. T2D patients with normal eGFR show lower circulating OGN levels than T2D patients with mild or moderate impaired eGFR, independent of sex and age. The serum OGN levels are independently associated with mildly impaired kidney function in T2D patients. This suggests that circulating OGN may be a biomarker of incipient impairment of kidney function, independently of the presence of albuminuria in T2D patients.

Little is known regarding the serum levels of OGN in T2D and control subjects with contradictory results. The increased levels of serum OGN observed in T2D patients compared to healthy controls, especially in those with decreased eGFR values agree with the findings reported by Wang et al., who found increased concentrations of OGN in T2D patients with diabetic nephropathy compared to T2D patients without diabetic nephropathy and healthy controls [19]. In contrast, Wei et al. reported that the decline of serum OGN levels was closely related to the development and pathogenesis of diabetic nephropathy suggesting that low serum OGN levels could act as an independent diagnostic marker of diabetic nephropathy associated with microalbuminuria in T2D patients [20]. Similarly, a study in mice with CKD showed that decreased serum OGN level was positively associated with impaired kidney function [24]. The aforementioned studies associate the OGN serum levels with kidney status independently of the presence of T2D. However, our study is the first to show higher circulating OGN levels in T2D patients regardless of kidney function.

The higher levels of FGF-23 in T2D patients compared to control subjects could partly explain the elevation of serum OGN in this group. Consistently, studies reported increased FGF-23 serum levels in the T2D population [25,26]. The presence of T2D implies

a progressive deterioration of kidney function, which is associated with increased serum levels of FGF-23 and iPTH as we observed in the T2D subjects with an incipient decrease in glomerular filtration. The elevation of iPTH in these patients could be explained by its positive correlation with FGF-23 as has been reported in CKD patients [27,28]. However, a review addressing the role of FGF-23 in clinical outcomes in T2D patients has shown that the mechanism by which an increase in FGF-23 occurs in T2D patients is unclear [29]. Most studies suggest that the increase in FGF-23 may be associated with the presence of CVD and may act as a predictor of cardiovascular mortality in the T2D population [30,31]. Considering this, the increased FGF-23 levels observed in the T2D group of patients with decreased eGFR levels could be due to the higher prevalence of CVD in this group. However, our results suggest that the increased FGF-23 is associated more with kidney function disturbances rather than with CVD in our study population.

Most of the studies have shown a strong and independent association between FGF-23 concentrations and greater risk of end-stage renal disease in advanced-stage CKD patients [32,33], thereby making it an independent risk factor for mortality in this population [32,34]. However, a long-term prospective study including patients with nondiabetic CKD has identified FGF-23 as a novel risk marker for the progression of CKD in mild to moderate CKD patients [27].

Regarding the T2D population, there is some controversy. Several studies have pointed to serum levels of FGF-23 as predictors for renal outcomes and progression to end-stage renal disease in T2D patients [35,36]. It has been revealing that FGF-23 is a novel independent predictor of the progression of renal disease in patients with macroalbuminuric diabetic nephropathy [27]. However, a recent multicenter study, reported a lack of association between FGF-23 and early kidney decline in T2D patients [37]. Based on our findings, we suggest that the ability of FGF-23 to predict early stages of renal impairment could be limited [30,31]. We consider that it could be due to its relationship with albuminuria since most studies associate elevated FGF-23 levels with nephropathy including albuminuria. In our study population, most of the T2D patients with mildly decreased eGFR had normoalbuminuric (75.9%). Our results showed that serum OGN levels are related to eGFR values, independently of the albuminuria. In this context, we suggest that, as OGN is an albuminuria-independent biomarker, it could be a better predictor than FGF-23 in T2D patients with early kidney impairment (data not shown).

Although there are markers associated with kidney impairment, the predictions for the progression of DKD in T2D patients based on clinical parameters are currently poor. The majority of the criteria for early kidney function deterioration considers albuminuria in combination with eGFR as the main clinical factors despite its modest predictive ability [25]. However, a decrease in eGFR may occur as an initial sign of kidney impairment without the presence of albuminuria as reported by Penno et al., who showed an association between decreased eGFR and the risk of death irrespective of albuminuria in T2D patients [15]. Accordingly, research reported that the majority of T2D patients with reduced eGFR had normoalbuminuric [16] and a significant risk for coronary artery disease in T2D patients with reduced eGFR independently of albuminuria [26].

Considering this, there is a need to search for effective intervention strategies and public health policies focused on the detection of incipient impaired kidney function in T2D patients, due to the rising mortality rate associated with this non-albuminuria DKD phenotype [15]. In this context, much of the research conducted in the last decade has endeavored to identify biomarkers for DKD progression. Some proposed useful biomarkers to measure the progression of advanced kidney damage [35,36]. However, few studies have determined biomarkers that can predict the eGFR decline in T2D patients when combined with commonly available clinical risk factors. Heinzel et al. identified some candidate biomarkers associated with eGFR in a longitudinal study conducted in T2D patients with the eGFR maintained at baseline. However, their predictive power was low [37]. Our results showed that the OGN serum level was an independent estimator of impaired kidney function risk in T2D patients by increasing the risk by 8% for a 1 ng/mL

increase in the serum OGN regardless of other comorbidities such as CVD or osteoporosis. Our ROC curve analysis revealed that the inclusion of serum OGN levels, in addition to age and impaired kidney function–related variables improved the prediction model for mildly impaired kidney function in T2D patients.

The positive correlation observed between serum OGN levels and age may be due to their close relationship with age-related renal function loss in T2D patients [38,39]. Although the T2D patients with eGFR values below 90 mL/min/1.73 m^2 were significantly older than those with normal eGFR values, the differences between groups remained significant after adjustment for age and sex. Our results showed that in addition to age, the serum FGF-23 levels could influence serum OGN. Based on these findings, the age and the higher serum levels of iPTH, and in particular, of FGF-23, could explain the increased OGN in T2D patients with eGFR values below 90 mL/min/1.73 m^2.

On the other hand, the fact that prevalent CVD is so close to significance as a predictor variable of OGN as shown in our results suggests that there may be a relationship between OGN levels and CVD. The role of OGN in CVD is controversial to date. Some studies' results have reported a close relationship between OGN levels and the risk of suffering CVD although the mechanism of action of OGN is unclear. In this line, a couple of studies found an association between high serum OGN levels and poor coronary collateralization in patients with coronary artery disease [40,41], as well as increased arterial stiffness in hypertensive patients [42]. Cheng et al. pointed out that OGN could be used as a prognostic biomarker in patients with coronary artery disease, and it proved to be a predictor for the incidence of cardiovascular events and mortality within this population [11]. Although most of the studies postulate an association between higher OGN levels and vascular damage, Van Aelst et al. reported that the increased OGN expression is essential in the infarct scar promoting proper collagen maturation and protecting against cardiac disruption in humans [4]. In contrast, another study found no association between the circulating levels of OGN and the progression of atherosclerosis [43]. We found lower serum circulating OGN levels in T2D patients with CVD than in T2D patients without CVD. Therefore, our results suggest that the increased serum OGN levels observed in T2D patients with mildly impaired kidney function (eGFR < 90 mL/min/1.73 m^2) are mainly related with renal rather than vascular damage. Endorsing our findings, a recent study observed increased immunostaining of OGN in human atherosclerotic carotid plaques from patients with lower eGFR values [44]. However, further studies are needed to clarify the relationship between OGN and CVD.

Although future longitudinal studies are needed, our preliminary results place OGN as a promising biomarker that deserves future research to confirm its potential role as an early predictor of kidney damage in daily clinical practice.

Our cross-sectional study has some limitations. First, the cross-sectional design precludes any determination of causality in our findings. Thus, we cannot assure whether FGF-23 influences the increase in OGN levels or the other way around. Second, we do not have many biochemical determinations in the control group, which could provide valuable information for the comparative study of OGN between T2D and control subjects. Finally, our study was conducted in a specific population of Caucasian T2D and healthy subjects, which prevents guaranteeing the same results in other ethnic or study groups. The strengths of this study lie in the evaluation of circulating OGN in T2D patients with an exhaustive evaluation of the biochemical and clinical parameters. In addition, we considered potential confounders, such as age, sex, diabetes duration, and current medications.

5. Conclusions

The main findings of this study suggest that the elevation of OGN related to mildly impaired kidney function could involve a specific role of OGN in this process acting as an albuminuria-independent biomarker of incipient impaired kidney function in T2D patients. Future longitudinal studies are required to understand the mechanisms through which the upregulation of OGN influences the risk of impaired kidney function in T2D patients and to

confirm the potential usefulness of serum OGN as a potential biomarker of kidney status in clinical settings to establish preventive and therapeutic approaches in the T2D population.

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Institutional Review Board Statement: This study was conducted with the approval of the Ethics Committee of the University Hospital Clínico San Cecilio of Granada and conformed to the principles of the World Medical Association Declaration of Helsinki (Project ID:0858-N-17, Research Ethics Committee of Granada Center (CEI-Granada) on 26 April 2017).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data sets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflicts of Interest: The authors declare no conflict of interest.

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CAPÍTULO II

Exploring the Role of Osteoglycin in Type 2 Diabetes: Implications for Insulin Resistance and Vascular Pathophysiology

Sheila González-Salvatierra, Beatriz García-Fontana, Luis Martínez-Heredia, Jesus Lacal, Francisco Andújar-Vera, Raquel Sanabria-de la Torre, Enrique Moratalla-Aranda, Silvia Lozano-Alonso, Cristina García-Fontana, and Manuel Muñoz-Torres.

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RESEARCH ARTICLE

Clinical Metabolism

Exploring the role of osteoglycin in type 2 diabetes: implications for insulin resistance and vascular pathophysiology

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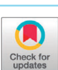
Abstract

Osteoglycin, a fundamental proteoglycan within the vascular extracellular matrix, is expressed in vascular smooth muscle cells (VSMCs). Type 2 diabetes (T2D) is associated with cardiovascular disease (CVD) but the role of osteoglycin in the development of CVD is controversial to date. Therefore, our aims are to determine and compare the level of osteoglycin in T2D patients with/without CVD versus control subjects both at serum and vascular tissue and to analyze in vitro role of osteoglycin in VSMCs under calcified conditions. For this, serum osteoglycin levels were determined by enzyme-linked immunosorbent assay (ELISA) in 117 controls and 129 patients with T2D (46 with CVD and 83 without CVD), revealing a significant increase in patients with T2D compared with controls. Osteoglycin level was not an estimator of CVD but correlated with markers of insulin resistance (triglycerides and triglycerides/high-density lipoprotein cholesterol index) in patients with T2D. At the vascular level, osteoglycin expression was assessed by RT-qPCR and immunohistochemistry, and no significant differences were observed between calcified arteries from patients with T2D and noncalcified arteries from controls. In vitro experiments using VSMCs (mock and overexpressing osteoglycin) under calcifying conditions were performed to analyze the osteoglycin function. The overexpression of osteoglycin in VMSCs under calcifying conditions revealed an increase of cell proliferation without effect on apoptosis and an upregulation of the expression of autotaxin (*ATX*) involved in inflammatory processes. In conclusion, osteoglycin could play a role in glycemic homeostasis, being a potential biomarker of insulin resistance in patients with T2D. Furthermore, osteoglycin could indirectly participate in the development of atherosclerosis through its regulatory effect on *ATX* and by proliferating VSMCs.

NEW & NOTEWORTHY This study uncovers an increase of serum osteoglycin levels in patients with type 2 diabetes, which does not appear to be associated with the development of atherosclerosis, but rather with insulin resistance in this population. Overexpression of osteoglycin increased proliferation and upregulated the expression of autotaxin in vascular smooth muscle cells within calcified environments. Osteoglycin could be a biomarker of insulin resistance for type 2 diabetes and could be indirectly involved in the development of atherosclerosis.

cardiovascular disease; insulin resistance; osteoglycin; type 2 diabetes; vascular smooth muscle cells



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INTRODUCTION

Type 2 diabetes (T2D) is associated with an increased risk of cardiovascular disease (CVD; 1), affecting ~35% of this population (2). Atherosclerosis is the main pathological mechanism underlying CVD in T2D, leading mainly to coronary heart disease, cerebrovascular disease, and peripheral arterial disease (3). The development of atherosclerosis is a complex process involving numerous cells, including immune cells, endothelial cells, and vascular smooth muscle cells (VSMCs; 4). There is a strong evidence that phenotypic switching of the VSMCs plays a key role in the development of atherosclerosis (5). Indeed, during disease development, VSMCs proliferate and migrate from the media layer of the arterial wall into the intima layer, contributing to atherosclerotic plaque formation through extracellular matrix (ECM) production and lipid accumulation (5, 6). The ECM contains different proteins, including collagens, elastin, glycoproteins, glycosaminoglycans, and proteoglycans, which provide the structural integrity of the plaques and are involved in several key events, such as cell migration and proliferation, lipoprotein retention, and thrombosis (7).

Osteoglycin, also known as osteoinductive factor or mimecan and encoded by the osteoglycin (*OGN*) gene, is a class III member of the small leucine-rich proteoglycan (SLRP; 8), a distinct group of extracellular proteoglycans, being a basic component of the vascular ECM, expressed mainly by cardiomyocytes, cardiac fibroblasts, and VSMCs (9). Furthermore, this protein is involved both in bone remodeling and in vascular metabolism (10). This indicates that osteoglycin could have a relevant role in vascular pathophysiology; however, few studies have been carried out to elucidate the mechanism of action of this protein at vascular level, which remains unclear to date. Some studies have reported an association between elevated osteoglycin levels in serum with increased cardiovascular risk (10–13) and mortality (14, 15). In this line, it has been reported that reduced osteoglycin levels in patients with complex cardiovascular lesions suggesting a role of osteoglycin in the stabilization of coronary plaque (16). Although most of the studies postulate a detrimental role for osteoglycin. Van Aelst et al. (10) reported that increased osteoglycin expression is essential in the infarct scar, as it promotes proper collagen maturation and protects against cardiac impairment in humans. On the other hand, no association was found between circulating levels of osteoglycin and progression of atherosclerosis in patients with carotid artery plaque (17), and it was shown that osteoglycin deficiency did not affect the progression of atherosclerosis in mice (18). Furthermore, osteoglycin levels were not associated with major adverse cardiovascular, cerebrovascular events, and mortality in T2D patients with chronic kidney disease (CKD; 14).

This study aims to investigate the involvement of osteoglycin in the development of atherosclerosis in humans, which is highly controversial. To research our main objective, we first proposed to quantify osteoglycin levels in serum and vascular tissue samples obtained from patients with T2D, both with and without CVD, as well as from healthy controls. This analysis allowed us to evaluate the association between osteoglycin and atherosclerosis in a clinical context. Second, we conducted experiments using osteoglycin in VSMCs exposed to calcified environments in vitro, to examine the direct impact of this protein on the development of atherosclerosis. By combining clinical and experimental investigations, our study aims to provide comprehensive insights into the role of osteoglycin in the processes of atherosclerosis in humans with T2D.

MATERIALS AND METHODS

Study Population

In this cross-sectional study, a total of 246 participants were included: 117 healthy subjects (65 ± 9 yr, 55.6% males) and 129 patients with T2D (65 ± 8 yr, 58.1% males). The healthy subjects were recruited between 2020 and 2022 from the reference population of the Hospital Universitario Clínico San Cecilio of Granada (Spain). The patients with T2D were diagnosed based on the criteria established by the American Diabetes Association (19) and were recruited between 2017 and 2018 from the Endocrinology and Nutrition Unit of the same hospital. Rigorous selection criteria, including Caucasian ethnicity and normal values for blood count and hepatic function, were applied to both groups to ensure the inclusion of individuals suitable for the study. The T2D group was further divided into two subgroups depending on the presence of CVD ($n = 46$) or not ($n = 83$). A statistical power analysis considering the proportion of patients with T2D suffering from CVD in our study population (35.6%) was performed. The scientific literature corroborates this proportion describing a mean prevalence of 32.2% of CVD in patients with T2D worldwide (20, 21). Considering our sample comprises 46 T2D patients with CVD and 83 T2D patients without prevalent CVD our statistical power is >85% (e.g., 87.7%). Calculations were obtained using G*Power (v.3.1.9.7.), using proportions for two independent groups (Fisher's exact test) and an α significance of 0.05. Both groups of patients with T2D received statin treatment, and specifically the T2D patients with CVD were treated with high-intensity statins. The inclusions criteria for CVD were coronary heart disease (previous myocardial infarction, a diagnosis of stable or unstable angina, or previous coronary revascularization surgery), ischemic cerebrovascular disease (transient ischemic attack or ischemic stroke), or ischemic peripheral arterial disease. Exclusion criteria involved patients with hepatic, gastrointestinal, thyroid, or bone diseases, as well as those with an estimated glomerular filtration rate (eGFR) below 45 mL/min/1.73 m^2 or receiving treatment with thiazolidinediones.

Vascular tissue samples were obtained from the artery of lower limbs at the Angiology and Vascular Surgery Unit of the Hospital Universitario Clínico San Cecilio of Granada. Calcified vessels were obtained from T2D patients with ischemic diabetic foot with criteria of critical ischemia, who were not suitable candidates for revascularization (primary amputation) or had experienced failed revascularization (secondary amputation; $n = 6$). Noncalcified vessel samples were obtained from healthy subjects who provided informed consent at the Hospital Universitario Clínico San Cecilio of Granada ($n = 3$).

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The study adheres to the general ethical principles of the Declaration of Helsinki. Before starting the study, the project was reviewed and approved by the Research Ethics Committee of Granada on April 26, 2017 (Project ID: 0858-N-17). Before participation, informed written consent was provided for each participant. The Biobank of the Andalusian Public Health System at the Hospital Universitario Clínico San Cecilio of Granada managed all the samples used in this study.

Clinical Evaluation and Biochemical Measurements of Study Population

Baseline height, weight, and waist circumference measurements were obtained from the entire study population using standard procedures. The body mass index (BMI) was calculated using the Quetelet formula: weight (kg)/stature (m^2). Moreover, for patients with T2D, dyslipidemia was characterized by serum levels of low-density lipoprotein cholesterol (LDL-c) >100 mg/dL, high-density lipoprotein cholesterol (HDL-c) <50 mg/dL, triglycerides (TG) >150 mg/dL, and/or current treatment with lipid-lowering drugs. Systolic and diastolic blood pressures were measured using a standard electronic sphygmomanometer. Hypertension was defined as values equal to or exceeding 140/90 mmHg and/or the use of antihypertensive medication. Alcohol consumption, smoking, and physical activity levels were assessed using the Spanish version of the Rapid Assessment of Physical Activity questionnaire (22). Regarding biochemical measurements, venous blood samples were collected in the morning after an overnight fast, and serum samples were stored at −80°C until analysis, which was carried out at the Clinical Analysis Unit of the Hospital Universitario Clínico San Cecilio of Granada (Spain) of the entire study population. Fasting plasma glucose (FPG), glycated hemoglobin (HbA1c), TG, HDL-c, LDL-c, eGFR, calcium, and phosphorus were measured using standard automated laboratory techniques. The TG/HDL-c index was calculated as an indicator of insulin resistance. eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration equation (CKD-EPI; 23). Osteoglycin level was determined by the enzyme-linked immunosorbent assay (ELISA) method, following the manufacturer's protocols (Cloud Clone Corp.), and precision testing was performed by the determination of intraassay and interassay variances (10% and 12%, respectively).

Immunohistochemistry and Data Acquisition of Osteoglycin in Vascular Tissue

Formalin-fixed paraffin-embedded biopsy tissues were obtained from the calcified lower limb artery of patients with T2D and noncalcified from control subjects. To enable antigen retrieval, the tissue sections (3 μm) were subjected to high temperature (200°C) incubation in 1× citrate buffer using a steamer machine for 20 min. Then, the tissue sections were deparaffined using a standard protocol involving xylenes and ethanol. Subsequently, the sections were rinsed in phosphate-buffered saline (PBS; 0.01 M, pH 7.4), treated with 3% hydrogen peroxide for 15 min, and rinsed again. A solution containing 3% normal goat serum and 0.1% PBS-tween20 was applied to the sections for 1 h. The slices were exposed overnight at 4°C to an anti-osteoglycin primary antibody (1:200, SC-374463, Santa Cruz). Following a rinse with PBS, the tissue sections were incubated with Goat Anti-Mouse IgG secondary antibody [1:5,000, Goat Anti-Mouse IgG H&L (HRP), No. ab6789, Abcam] at room temperature for 2 h. Both antibodies were diluted in 3% normal goat serum and 0.1% PBS-tween20. The sections were rinsed, then processed using the ABC-kit (Vector Laboratories), and the reaction was visualized using the peroxidase substrate kit DAB (Vector Laboratories). The tissue sections were rinsed, rehydrated with ethanol and xylenes, and cover slipped. For data acquisition of tissue immunohistochemistry, microphotographs were captured using a light microscope, Olympus BX41. Slices containing the regions of interest were identified by Stereo Investigator Software (Bioscience) from coronal sections of the samples (Fig. 4*A*). In each sample, microphotographs of the intima-media and adventitia layers were captured at ×20 magnification (Fig. 4*B*). Image J Software was used to quantify osteoglycin expression. For each microphotograph, the software automatically identified proteins as threshold objects. These objects appeared as black circular dots against a white background and met specific size criteria (ranging from 35 to 150 μm^2) and circularity values (from 0.35 to 1.00). To ensure consistency across all microphotographs and eliminate potential background noise, we first converted them into 8-bit type images. Then, we adjusted the background by increasing its brightness to 150.0 pixels. The threshold settings for all images were standardized to a range of 0–150.

RNA Isolation and RT-qPCR

In this study, we performed RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to measure the expression of osteoglycin in vascular tissue and to assess the efficiency of lentiviral transduction in primary human aortic smooth muscle cells (HAoSMCs). Furthermore, we quantified the expression of various genes under different conditions in HAoSMCs in vitro. To obtain total RNA from vascular tissue, we isolated 23 transversal sections from the calcified lower limb artery of patients with T2D and peripheral artery disease, as well as from the noncalcified lower limb artery of healthy donors. We used Trizol reagent (Thermo Fisher Scientific) and a manual homogenizer for RNA extraction. For cell samples, we used the RNeasy mini kit (QIAGEN) following the manufacturer's instructions. In both cases, Turbo DNase (Ambion) was used to treat the RNA, and we assessed the RNA concentration and quality using the Qubit Flex Fluorometer (Thermo Fisher Scientific). Only RNA samples with an A260/280 ratio between 1.8 and 2.0 were included for cDNA synthesis, performing reverse transcription with the iScript cDNA synthesis kit (BioRad), according to the manufacturer's protocol. For qPCR analysis, we used the PowerUp SYBR Green Master Mix in a QuantStudio 7 Flex Real-Time PCR system (Thermo Fisher Scientific). The qPCR protocol was as follows: 95°C 2 min; 40× (95°C 20 s, 65°C 20 s); 65°C to 95°C with an increment of 0.5°C every 4 s. We designed the primers using Clone Manager Suite program (Table 1). To normalize the mRNA data, we used the expression of a constitutive gene. Each real-time PCR reaction was performed in triplicate for each sample. Relative expression of each gene of interest was assessed using the $2^{-\Delta\Delta Ct}$ method (25).

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Table 1. *Primers used in this study*

Gene	Sequence (5′–3′)	Amplicon (pb)	Application	Reference
OGN		151	Check the efficiency of HAoSMCs transduction. Expression in vascular tissue. Quantify the expression under different conditions in HAoSMCs in vitro.	This study
Forward	CCTCACCTTCCTCTACTTGGACCATA			
Reverse	CCGGATGTAACTGGTGTCATTAGC			
ATX		158	Quantify the expression under different conditions in HAoSMCs in vitro.	(24)
Forward	ATGCCTGAATGACTCCACTGTT			
Reverse	AGGACTAAATGTGGCAACTGTG			
RPL13		228	Constitutive gene	This study
Forward	CGTAAGATCCGCAGACGTAAGGC			
Reverse	GGACTTGTTCCGCCTCCTCGGAT			

ATX, autotaxin; HAoSMCs, primary human aortic smooth muscle cells; *OGN*, osteoglycin; *RPL13*, ribosomal protein L13.

Cell Cultures

Human embryonic kidney 293 T cells (HEK293T) (ATCC) were cultured in DMEM/F-12 GlutaMAX (Gibco) supplemented with 10% fetal bovine serum (FBS) [NeoBiotech RNase A (Powder)].

HAoSMCs (ATCC) were cultured using vascular cell basal medium (ATCC) supplemented with the VSMC growth kit (ATCC), which consists of various components including recombinant human (rh) fibroblast growth factor, rh insulin, rh epidermal growth factor, L-glutamine, ascorbic acid, and FBS.

Both cell cultures were maintained under standard conditions, which consists of an incubation temperature of 37°C, 5% CO_2, and a humid atmosphere. Cells were grown to confluence and used for experiments from passages 4–5.

Second-Generation Lentiviruses and Transduction for Generation of Stable Lines of HAoSMCs

To generate stable lines of HAoSMCs with overexpression of osteoglycin, a second-generation lentiviral packaging system protocol was performed using the following vectors: pVSV-G, which expresses the envelope gene of the VSV-G virus; psPAX2, which expresses the reverse transcriptase gene, protease gene, and gene for assembly of the HIV-1 virus; and the pLVX:*OGN* construct or empty pLVX. All plasmids were obtained from Addgene. HEK293T cells were transfected with a mixture of the above plasmids using polyethyleneimine (Quimigen). The transfected cells were then cultured in DMEM/F-12 GlutaMAX supplemented with 10% FBS under standard conditions for 24 h. Lentivirus particles were harvested, filtered, ultracentrifuged, and resuspended in PBS.

The HAoSMCs were transduced using the lentivirus particles in the presence of polybrene infection reagent (8 mg/mL, Merck) and subsequently selected with hygromycin B (50 mg/mL, Thermo Fisher Scientific). Control cells (mock) were transduced with lentiviruses generated from the empty pLVX vector. The transductions were performed in triplicate, and the cells were cultured under standard conditions. RT-qPCR was performed to test for osteoglycin overexpression in this stable cell line.

Induction of Calcifying Conditions In Vitro

HAoSMCs that had been transduced with osteoglycin overexpression and mock were seeded on six-well plates at a confluency of 1,000 cells/well. The cells were cultured in a growth medium containing 1.5 mM $CaCl_2$ and 10 mM β-glycerophosphate for a maximum of 20 days to stimulate matrix calcification. Throughout the incubation period, the cells were maintained under standard conditions, and the growth medium was changed every 2–3 days.

Cell Proliferation Assay

Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HAoSMCs transduced with osteoglycin overexpression and mock were seeded in a 96-well plate at a density of 250 cells/100 μL/well under calcified conditions. The cells were maintained in standard conditions for 10 days, and the cell proliferation assay was performed every 2 days. At different time points, 10 μL of MTT solution (5 mg/mL) was added to each well, and the plate was incubated for 6 h under standard conditions. Subsequently, 100 μL of lysis buffer (20% SDS in 50% formamide, pH 4.7) was added, and the plate was kept under standard conditions overnight to allow for cell lysis. Cell proliferation was quantified by measuring the optical density (OD) at 570 nm using a spectrophotometer. Four replicates were performed for each condition, and the values were corrected by subtracting the background signal from cell-free media controls.

Cell Apoptosis Assay

The percentages of cell apoptotic under calcified conditions were analyzed using a FITC annexin V apoptosis detection kit (BD Biosciences). HAoSMCs with osteoglycin overexpression and mock were washed twice with PBS, and a density of 10^5 cells/100 μL was incubated with annexin V-FITC and propidium iodide for 15 min at room temperature and in darkness for each condition. The samples were analyzed using a BD FACSAria IIIu flow cytometer (Becton Dickinson, BD Biosciences). To calculate the percentage of apoptosis, the sum of early apoptotic cells (Annexin-FITC+/PI−) and late apoptotic cells (Annexin-FITC+/PI+) was determined. Each condition was performed in duplicate.

Statistical Analysis

For statistical analyses, SPSS 28.0 (IBM Corp.) and GraphPad Prism v7.03 (GraphPad Software) were used.

Serum data were presented as means ± standard deviation (SD) or median with interquartile range (IQR) depending on

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normality. Categorical variables were expressed as percentages. Normality of continuous variables was assessed using the Kolmogorov–Smirnov test. Group comparisons for normally distributed variables were performed using unpaired Student's *t* test, whereas the Mann–Whitney *U* test was used for nonnormally distributed variables. One factor analysis of variance single factor (One-Factor ANOVA) was used to compare several groups. Covariate-adjusted group comparisons were conducted using univariate analysis of covariance (ANCOVA). Categorical variables were compared using the χ^2 test. Correlations between continuous variables were assessed using Spearman's correlation coefficients. Multiple linear regression was utilized to identify independent variables associated with osteoglycin levels in serum, and the data were expressed as B; 95% confidence interval (CI; lower limit/upper limit). Multiple logistic regression was performed to determine if osteoglycin was an independent predictor of CVD. Statistical significance was set at $P < 0.05$ (two tailed) and $P < 0.10$ for multiple linear and logistic regression analysis.

Immunohistochemistry and RT-qPCR were performed on vascular tissue to assess osteoglycin expression. Data were presented as means ± SD, and the differences between different groups and tissue layers were compared using unpaired Student's *t* test, providing a statistical assessment of the significance of the observed variations. In vitro analysis of HAoSMCs presented data as mean ± SD. Group differences were assessed using unpaired Student's *t* test to determine significance of observed variations between groups.

RESULTS

Characteristics of the Study Population

Table 2 summarizes the baseline characteristics of the entire population consisting of healthy subjects and patients with T2D. Both groups were comparable in age and sex. As expected, patients with T2D showed a significantly worse metabolic profile in terms of BMI, waist circumference, FPG, HbA1c, and lipid profile. In addition, T2D group showed a significant increase in serum osteoglycin levels compared with control group ($P < 0.001$).

When the patients with T2D were divided according to the presence of CVD, the groups were comparable in most of the variables. The groups differed in sex, age, and various factors defining CVD risk, such as blood pressure, lipid profile, and eGFR. No significant differences were found between the groups in serum osteoglycin levels (Table 3).

Influence of Diabetes Status, Sex, and CVD on Serum Osteoglycin Levels

Osteoglycin levels in serum were significantly increased in patients with T2D (n = 129, 58.1% males) compared with healthy subjects (n = 117, 55.6% males) [1,802 (1,403–2,444) pg/mL vs. 905 (776–1,155) pg/mL, $P < 0.001$]. When patients with T2D and healthy subjects were further divided according to sex, the significative differences in osteoglycin levels remained for both males [patients with T2D: 1,079 (1,366–2,444) pg/mL vs. healthy subjects: 864 (756–1,047) pg/mL, $P < 0.001$], and females [patients with T2D: 1,953 (1,606–2,449) pg/mL vs. healthy subjects: 950 (798–1,247) pg/mL, $P < 0.001$]. Moreover, no significant differences in serum osteoglycin levels were found between males and females in the T2D group [1,079 (1,366–2,444) pg/mL vs. 1,953 (1,606–2,449) pg/mL, $P = 0.082$] and the control group [864 (756–1,047) pg/mL vs. 950 (798–1,247) pg/mL, $P = 0.060$; Fig. 1*A*]. Furthermore, when patients with T2D were divided according to the presence of CVD no significant differences were observed between the groups [1,866 (1,377–2,861) pg/mL vs. 1,788 (1,474–2,331) pg/mL, $P = 0.363$]. In addition, serum osteoglycin differences after adjusting by age and sex were only observable between the control and T2D groups ($P < 0.001$) regardless of the presence of CVD ($P = 0.374$; Fig. 1*B*). There were also no differences in serum osteoglycin levels according to the presence of major CVDs in the T2D group [coronary artery disease ($P = 0.837$); ischemic cerebrovascular disease ($P = 0.616$); and peripheral artery disease ($P = 0.241$)]. These findings suggest that serum osteoglycin levels are not tightly involved in CVD in the population with T2D.

Table 2. *Comparison of baseline characteristics between the control and T2D groups*

Baseline Characteristics	Control	T2D	P
Men/women, *n*	65/52	75/54	0.683
Age, yr	65 ± 9	65 ± 8	0.352
Body weight, kg	74.6 ± 14.2	86.3 ± 14.3	<0.001*
Height, cm	163 ± 0.1	165 ± 0.09	0.111
BMI, kg/m^2	27.9 ± 4.5	31.7 ± 4.5	<0.001*
Waist circumference, cm	96.9 ± 10.6	105.9 ± 10.4	<0.001*
FPG, mg/dL	91 (84–99)	143 (110–176)	<0.001*
HbA1c, %	5.6 (5.4–5.8)	7.6 (7–8.6)	<0.001*
HDL-c, mg/dL	54 ± 12	46 ± 11	<0.001*
LDL-c, mg/dL	116 ± 32	93 ± 41	<0.001*
TG, mg/dL	102 (77–144)	139 (99–196)	<0.001*
TG/HDL-c index	2 (1.3–3)	3.3 (2.3–4.5)	<0.001*
eGFR, mL/min/1.73 m^2	87.2 (74.5–94.5)	87.2 (74.7–97.5)	0.585
Calcium, mg/dL	9.7 (9.5–10.1)	9.7 (9.5–9.9)	0.401
Phosphorous, mg/dL	3.2 (2.9–3.5)	3.3 (2.9–3.6)	0.293
Osteoglycin, pg/mL	905 (776–1,155)	1802 (1,402–2,444)	<0.001*

Data for continuous and normally distributed variables are presented as means ± standard deviation. Data for continuous variables not normally distributed are presented as median followed by interquartile range in brackets. Data for categorical variables are presented as percentages. Student's *t* test and Mann–Whitney *U* test were used for comparisons of continuous and normally or not normally distributed variables, respectively, between groups. χ^2 test was used for comparison of categorical variables between groups. BMI, body mass index; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; HDL-c, high-density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; T2D, type 2 diabetes; TG, triglycerides; TG/HDL-c index, triglycerides/high-density lipoprotein cholesterol index. *$P < 0.05$ between groups.

Determinants of Serum Osteoglycin Levels in the Patients with T2D

A positive correlation between the serum osteoglycin levels and variables such as age ($r = 0.247$; $P = 0.005$), TG ($r = 0.206$; $P = 0.019$), and TG/HDL-c index ($r = 0.174$; $P = 0.048$) was observed in patients with T2D. In addition, a negative correlation was found between serum osteoglycin levels and eGFR ($r = -0.338$; $P < 0.001$; Fig. 2).

In addition, our study showed that circulating osteoglycin levels in patients with T2D increased stepwise from the lowest quartile to the highest quartile of TG/HDL-c index, with

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Table 3. *Intergroup comparison for patients with T2D according to the presence of CVD*

	T2D without CVD	T2D with CVD	*P*
Men/women, *n*	39/44	36/10	<0.001*
Age, yr	64 ± 7.6	67 ± 7.4	0.017*
Clinical Evaluation			
Body weight, kg	87.1 ± 14	84.9 ± 15	0.204
Height, cm	164 ± 0.09	166 ± 0.09	0.185
BMI, kg/m^2	32.2 ± 4.6	30.8 ± 4.29	0.043*
Waist circumference, cm	106.6 ± 10.6	104.3 ± 10	0.133
Diabetes duration, yr	13 ± 8.6	17 ± 10.2	0.011*
Dyslipidemia, %	83.1	97.8	0.013*
Systolic blood pressure, mmHg	136.2 ± 18	135.3 ± 16.5	0.392
Diastolic blood pressure, mmHg	81.3 ± 9.4	75.5 ± 12.3	0.002*
Hypertension, %	79.5	95.7	0.013*
Coronary heart disease, %		56.5	<0.001*
Cerebrovascular disease, %		23.9	<0.001*
Peripheral artery disease, %		37.0	<0.001*
Smoker or ex-smoker, %	41.5	54.3	0.161
Alcohol consumption, %	15.9	17.4	0.822
Sedentarism, %	11.6	22.2	0.150
Current Medication Use			
Insulin, %	68.7	78.3	0.245
Oral antidiabetic drugs, %	31.3	21.7	0.245
Biochemical Measurements			
FPG, mg/dL	147.9 ± 48.8	153.9 ± 57.6	0.266
HbA1c, %	7.9 ± 1.3	7.9 ± 1.6	0.463
HDL-c, mg/dL	47 ± 11	43 ± 11	0.029*
LDL-c, mg/dL	101 ± 42	79 ± 36	0.002*
TG, mg/dL	139 (110–205)	142.5 (91–186)	0.192
TG/HDL-c index	3.1 (2.3–4.5)	3.4 (2.2–4.8)	0.906
eGFR, mL/min/1.73 m^2	86.7 ± 18.3	79.1 ± 19.4	0.014*
Calcium, mg/dL	9.8 (9.5–9.9)	9.6 (9.3–9.8)	0.012*
Phosphorous, mg/dL	3.3 (3–3.7)	3.3 (2.9–3.6)	0.492
Osteoglycin, pg/mL	1,788 (1,474–2,331)	1,866 (1,377–2,861)	0.363

Data for continuous and normally distributed variables are presented as means ± standard deviation. Data for continuous variables not normally distributed, are presented as median followed by interquartile range in brackets. Data for categorical variables are presented as percentages. Student's *t* test and Mann–Whitney *U* test were used for comparisons of continuous and normally or not normally distributed variables, respectively, between groups. χ^2 test was used for comparison of categorical variables between groups. BMI, body mass index; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; HDL-c, high-density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; TG, triglycerides; TG/HDL-c index, triglycerides/high-density lipoprotein cholesterol index; T2D, type 2 diabetes. *$P < 0.05$ between groups.

significant differences between *quartile 1* and *quartile 4* ($P = 0.039$; Fig. 3), suggesting that osteoglycin is highly related to this index.

To examine the factors influencing osteoglycin levels, a multiple linear regression analysis model was performed. The model included variables identified as being associated with osteoglycin in previous bivariate analysis, such as age, TG, TG/HDL-c index, and eGFR. In addition, sex, current medication, and the presence of CVD were included as independent variables in the analysis. The results showed that the variables independently associated with the serum osteoglycin level were age ($B = 0.232$; 95% CI [6.3/44.0]; $P = 0.009$), TG ($B = 0.217$; 95% CI [0.6/4.3]; $P = 0.009$), and eGFR ($B = -0.251$; 95% CI [−18.4/−3.4]; $P = 0.005$).

Analysis of the Relation between Serum Osteoglycin Level and CVD Risk in Patients with T2D

Logistic regression modeling was performed to assess the variables related to CVD risk in population with T2D. The independent variables included in the model were age, sex, dyslipidemia, hypertension, years of diabetes duration, TG/HDL-c index, eGFR, tobacco use, and sedentarism, in addition to serum osteoglycin level. We showed that the independent variables associated with CVD risk in patients with T2D were sex (OR = 0.228; 95% CI [0.088/0.591]; $P = 0.002$), dyslipidemia (OR = 0.118; 95% CI [0.013/1.099]; $P = 0.061$), hypertension (OR = 0.217; 95% CI [0.043/1.092]; $P = 0.064$), and years of diabetes duration (OR = 1.048; 95% CI [0.993/1.105]; $P = 0.086$). These results revealed that circulating osteoglycin level does not appear to be a predictor of CVD risk in population with T2D.

Osteoglycin Expression Level in Vascular Tissue

Immunohistochemistry and RT-qPCR were performed on calcified lower limb arteries of patients with T2D ($n = 6$) and noncalcified arteries of control subjects ($n = 3$). Analysis of the total average of osteoglycin-positive cells using immunohistochemistry revealed no significant differences in osteoglycin expression in calcified vessels from patients with T2D compared with noncalcified vessels from control subjects (136.5 ± 46.3 vs. 100.8 ± 44.9, $P = 0.321$). When examining the location of osteoglycin-positive cells, no significant differences were observed between the intima-media layer and the adventitial layer in both patients with T2D (95 ± 29.7 vs. 178 ± 63.6; $P = 0.132$) and control groups (77 ± 42.7 vs. 124 ± 48.1; $P = 0.251$; Fig. 4*C*). In addition, no change in osteoglycin mRNA regulation was found between calcified lower limb arteries of patients with T2D and noncalcified lower

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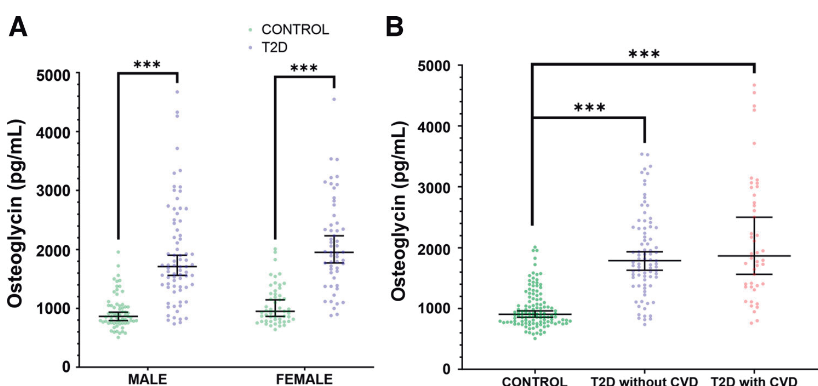

Figure 1. Serum osteoglycin level of the study population. *A*: serum osteoglycin level in male and females for both control (65/52) and T2D groups (75/54). The dot-plot represent median (95% confidence interval) and *P* values between sexes were performed using the Mann–Whitney *U* test. ***$P < 0.001$. *B*: dot-plot of serum osteoglycin levels in controls ($n = 117$), T2D patients without CVD ($n = 46$), and T2D patients with CVD ($n = 83$), adjusting by age and sex. Dot-plot represent median (95% confidence interval) and *P* values between the different were performed by ANCOVA. ***$P < 0.001$. CVD, cardiovascular disease; T2D, type 2 diabetes.

limb arteries of control subjects (1.37-fold change; $P = 0.288$; Fig. 4*D*). These results indicate that there is no variation in the expression of osteoglycin in vascular tissue associated with the pathogenesis of vascular calcification in patients with T2D.

Effect of Osteoglycin Overexpression on Mechanisms Involved in Calcification in HAoSMCs

To examine the influence of osteoglycin on HAoSMCs in a calcified condition, we used a second-generation lentiviral packaging system to establish stable overexpression of osteoglycin in vitro. The effectiveness of osteoglycin overexpression in HAoSMCs was confirmed through RT-qPCR. Remarkably, HAoSMCs transduced with *OGN* gene exhibited a significant 1,364-fold increase in osteoglycin mRNA levels compared with the mock group, indicating successful and robust osteoglycin overexpression ($P < 0.001$) under the same experimental conditions. Furthermore, RT-qPCR was also used to check the impact of osteoglycin overexpression on the regulation of the autotaxin (*ATX*) gene, which encodes the ATX protein, also

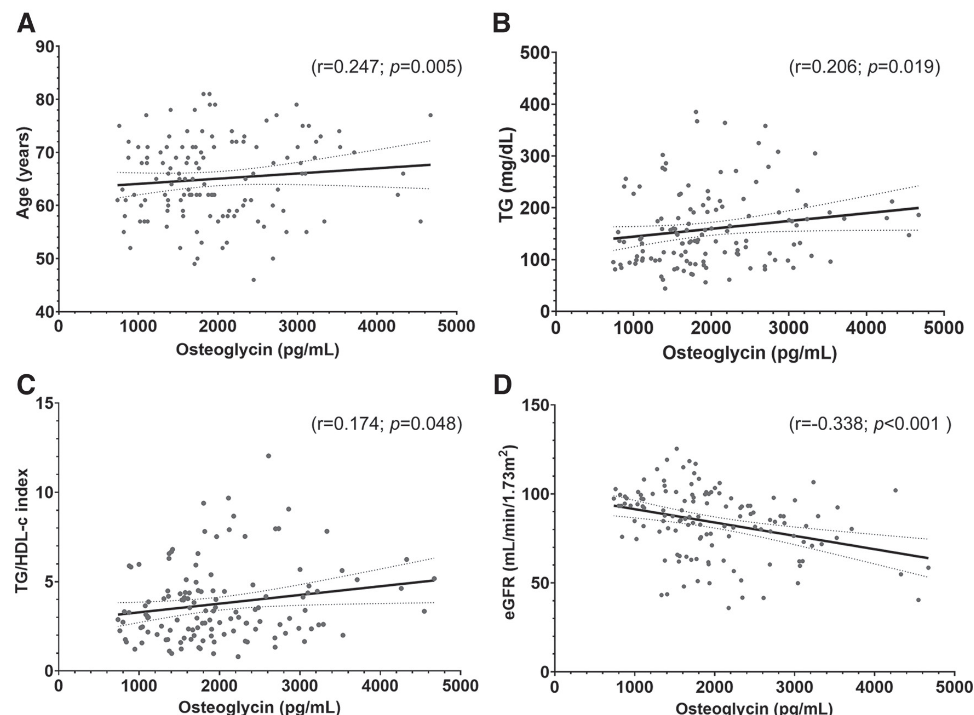

Figure 2. Scatter plots showing the correlation between serum osteoglycin (pg/mL) and: age (years; *A*), TG (mg/dL; *B*), TG/HDL-c index (*C*), and eGFR (mL/min/1.73 m^2; *D*) in patients with T2D ($n = 129$). The *P* values between the different associations were performed by Spearman's correlation coefficients (showing $P < 0.05$ in each scatter plot). eGFR, estimated glomerular filtration rate; TG, triglycerides; TG/HDL-c index, triglycerides/high-density lipoprotein cholesterol index; T2D, type 2 diabetes.

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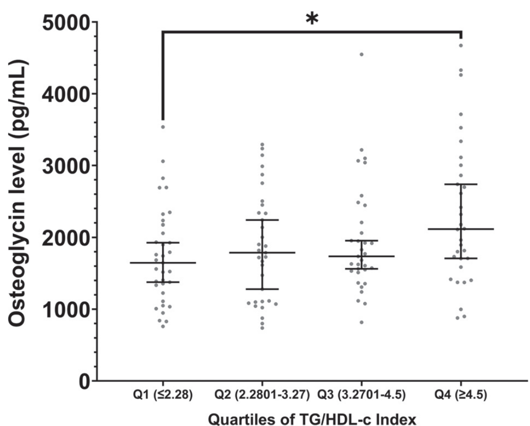

Figure 3. Comparison of circulating osteoglycin levels in the different quartiles (Q) of the TG/HDL-c index in patients with T2D. The dot-plot represents the median of the osteoglycin serum levels and the 95% confidence interval within each Q of TG/HDL-c index (Q1, $n = 33$; Q2, $n = 32$; Q3, $n = 32$; Q4, $n = 32$). The P values between quartiles were performed by one-factor ANOVA. $*P < 0.005$. Q, quartile; TG/HDL-c index, triglycerides/high-density lipoprotein cholesterol index.

called ectonucleotide pyrophosphatase/phosphodiesterase family member 2 (*ENPP2*), a protein involved in inflammatory processes. The results revealed that HAoSMCs with osteoglycin overexpression exhibited a significant upregulation of *ATX* compared with mock (16.9-fold; $P < 0.001$).

The effect of osteoglycin overexpression on the proliferation and survival of HAoSMCs was evaluated in calcified conditions. The MTT assay was performed to determine how osteoglycin overexpression affected the proliferation of HAoSMCs. The results revealed a slight significant increase in the proliferation rate of HAoSMCs with osteoglycin overexpression compared with the mock group. Specifically, at 4 days, there was a 16% increased ($P = 0.042$), at 6 days a 3% increased ($P = 0.122$), at 8 days a 2% increased ($P = 0.297$), and at 10 days an 8% increased ($P = 0.003$; Fig. 5*A*). Furthermore, flow cytometry was used to investigate apoptosis-induced cell death after annexin V and propidium iodide staining (Fig. 5*B*). No discernible differences in the percentage of apoptosis between mock compared with HAoSMCs with osteoglycin overexpression were observed (6.8 ± 0.3%, vs. 6.8 ± 0.6%, $P = 0.476$; Fig. 5*C*).

DISCUSSION

Our study reported for the first time an increase in serum osteoglycin levels in patients with T2D compared with nondiabetic controls. This elevation does not appear to be associated with the development of atherosclerosis in this population. However, there is a potential relationship of osteoglycin with markers of insulin resistance.

The function of osteoglycin at the vascular level is controversial and not well understood. Some studies have reported a close relationship between osteoglycin levels and the risk of suffering CVD showing an association between an increase of osteoglycin in serum and patients with coronary artery disease (12, 16), as well as, increased arterial stiffness in hypertensive patients (13). Within this line, it has been suggested that osteoglycin could be used as a prognostic biomarker in patients with coronary artery disease (15). In addition, this protein may act as a predictor for the incidence of cardiovascular events in patients with acute coronary syndrome or CKD (14, 15), as well as for mortality in patients with carotid artery disease (11). However, some studies point in the opposite direction, describing the beneficial role for osteoglycin against cardiac impairment in humans (10). Despite this evidence, our results found no association between serum osteoglycin levels with the presence of CVD overall nor with the presence of specific manifestations of CVD in patients with T2D. In addition, our statistical results did not position the presence of CVD as a variable influencing osteoglycin levels. Likewise, osteoglycin was not an independent estimator of CVD risk in patients with T2D. On the other hand, our results also revealed no significant differences in osteoglycin expression in calcified vessels from patients with T2D, both in the intima-media and adventitia layers, compared with noncalcified vessels from control subjects. This indicates that the elevation of serum osteoglycin in patients with T2D relative to controls is not derived from vascular expression. By contrast, a previous in situ hybridization study in human coronary arteries showed that osteoglycin mRNA was expressed by normal medial VSMCs but was downregulated in a subset of intimal VSMCs (9). Furthermore, an upregulation of osteoglycin in the thick neointima and in the front edge of migrating VSMCs has been described in rabbits with atherosclerotic lesions (26). Consistently with our results, some studies support the noncorrelation between circulating osteoglycin levels with major adverse cardiovascular, cerebrovascular events, and mortality in T2D patients with CKD (14) nor with atherosclerosis development in patients with carotid artery plaque (17) or animal models (18).

Based on this, osteoglycin does not appear to be a key determinant in the pathogenesis of atherosclerosis in patients with T2D directly, although it may play an indirect role through its involvement in insulin resistance. The elevation of circulating osteoglycin levels in patients with T2D compared with controls could be explained partially by influencing variables such as age, circulating TG level, TG/HDL-c index, and eGFR. Osteoglycin is a hormone highly expressed in adipose tissue and secreted into the circulation, in both mice (27, 28) and humans with obesity (29). Our results showed a positive correlation between serum osteoglycin levels and the TG level and TG/HDL-c index which are associated with glucose disturbances (30). Specifically, the TG/HDL-c index is used as a predictor of insulin resistance and CVD risk (31). Our results showed a positive correlation between serum osteoglycin levels and the TG/HDL-c index in patients with T2D observing an increase in circulating osteoglycin levels according to the TG/HDL-c index quartiles. Thus, the T2D patients with higher insulin resistance (fourth quartile of TG/HDL-C index) are those who showed higher levels circulating osteoglycin levels. This finding suggests that osteoglycin could be a mediator of insulin resistance. We propose that the osteoglycin-mediated insulin resistance may be related to the ATX pathway. Our in vitro experimental analysis showed for first time that an osteoglycin overexpression leads to an upregulation of *ATX* encoding for ATX, which is a protein with lysophospholipase D activity

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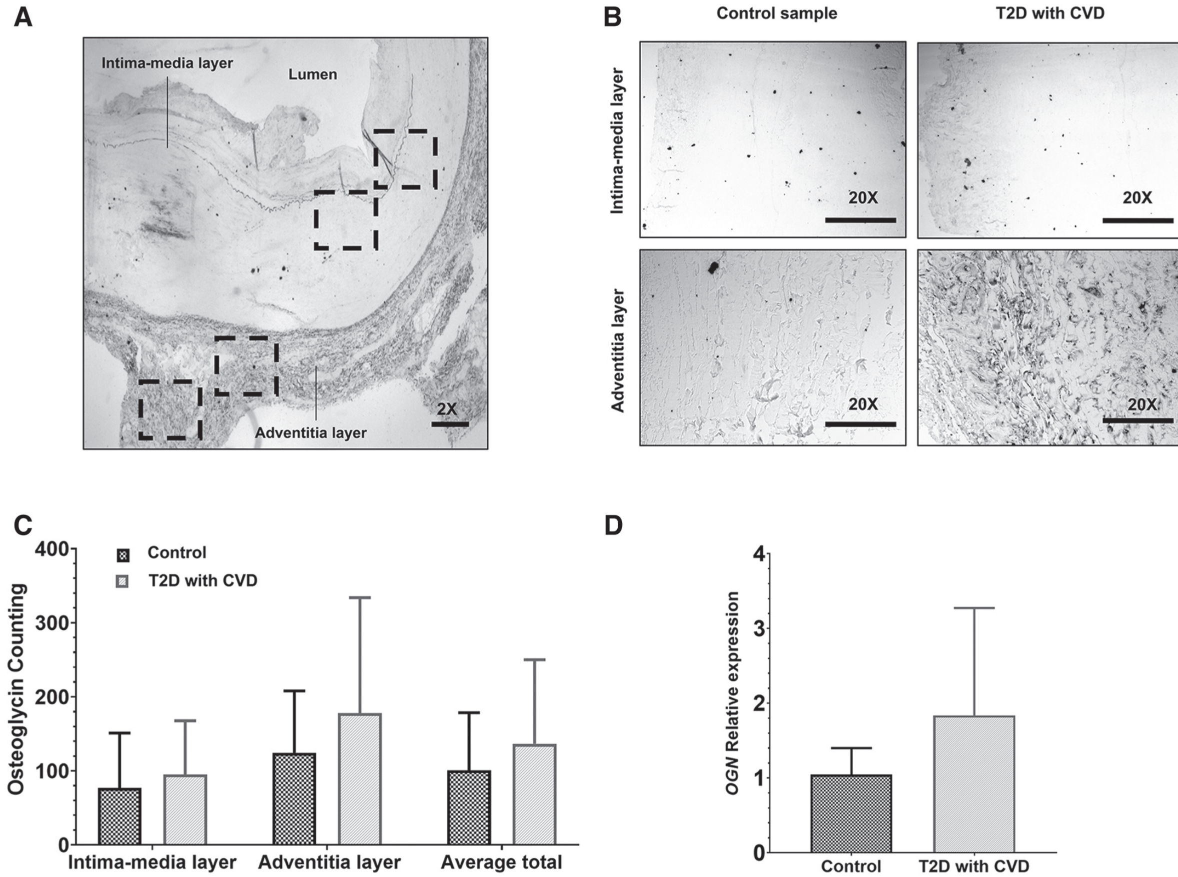

Figure 4. Osteoglycin expression at vascular tissue, specifically in calcified lower limb arteries of patients with T2D ($n = 6$) and noncalcified arteries of control subjects ($n = 3$). *A*: schematics of the microphotographs (×2) of the artery sections used for osteoglycin immunohistochemistry. The microphotographs were systematically captured following a medio-lateral axis to cover the intima-media and the adventitia layer of the artery. The dashed boxes represent the schematic location of the microphotographs captured for both layers. *B*: representative microphotographs obtained at ×20 magnification of the artery for the intima-media layer (*top*) and the adventitia layer of the artery (*bottom*) for control and T2D patients with CVD groups. *C*: counting of osteoglycin labeled proteins in the intima-media and the adventitia layer of the artery and the total average for control and T2D with CVD groups, was performed by immunohistochemistry. *D*: evaluation of osteoglycin relative expression in both groups using the $2^{-\Delta\Delta Ct}$ method (fold-change), was performed by RT-qPCR. All data are presented as the means ± standard error and Student's *t* test was used for the comparison. CVD, cardiovascular disease; *OGN*, osteoglycin; T2D, type 2 diabetes.

catalyzing the hydrolysis of lysophospholipids into lysophosphatidic acid (LPA). Studies have demonstrated that the ATX/LPA axis is involved in T2D, insulin resistance, and obesity (32, 33). Accordingly, it has been reported an increase in ATX expression among patients with T2D, suggesting a potential involvement of this protein in the development of the disease (34), whose expression could be influenced by alterations in glucose homeostasis affecting insulin and glucose levels in these patients with T2D (32). In addition, the ATX/LPA axis has been involved in CVD, particularly atherosclerosis (33), by enhancing the penetration of blood monocytes into the subendothelial space of vascular tissue, endothelial dysfunction, and proliferation of VSMCs (35). An increase in the proliferation of VSMCs implies an increase in collagen synthesis promoting artery stiffening and the formation of atherosclerotic plaque (36, 37). Regarding cell apoptosis, there is evidence supporting that a high rate of VSMCs apoptosis can promote vascular calcification (38) and contribute to plaque instability (39) favoring the atherosclerotic process. Few studies have attributed to osteoglycin a reducing effect on the proliferation of VMSCs (40, 41). However, our experimental results showed an increase in the proliferation rate of HAoSMCs associated with osteoglycin overexpression without influence on HAoSMCs apoptosis under calcifying conditions. These findings suggest that osteoglycin plays a key role mainly in insulin resistance and could indirectly participate in the development of atherosclerotic process through activation of the ATX/LPA pathway and proliferation of VSMCs within the population with T2D. However, future studies are needed to fully determine the molecular mechanism of osteoglycin in this regard.

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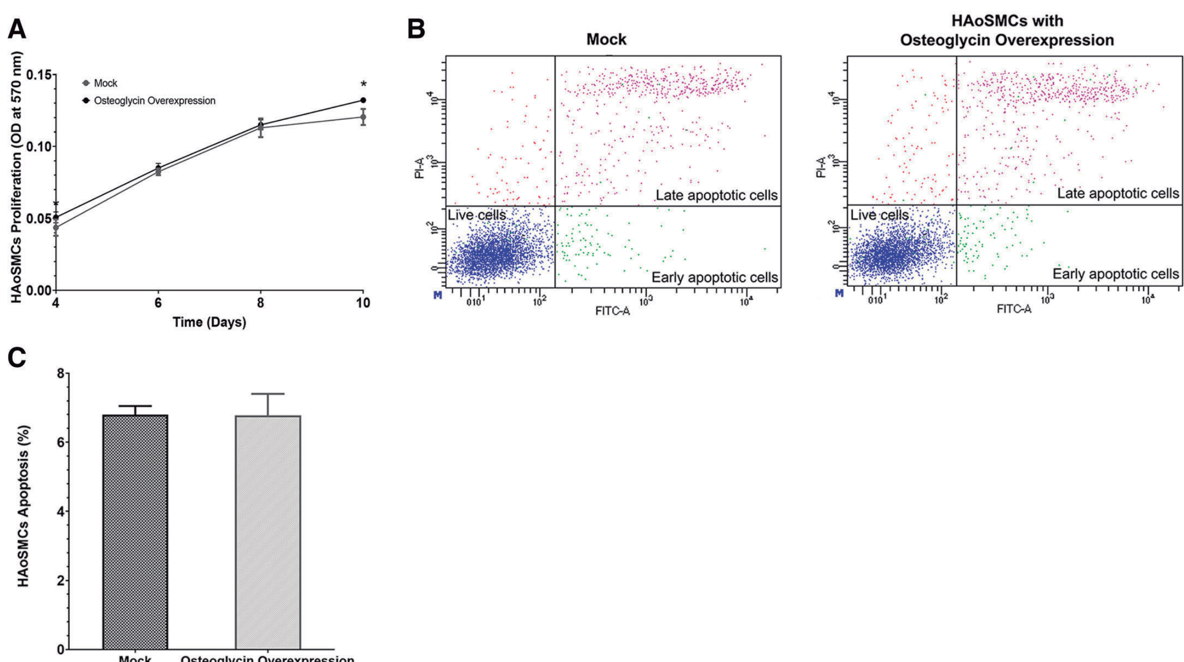

Figure 5. Effect of osteoglycin overexpression on proliferation and apoptosis in HAoSMCs under calcified conditions. *A*: analysis of proliferation in mock compared with HAoSMCs with osteoglycin overexpression ($n = 4$ biological replicates in each time/group). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed for proliferation, and each result was expressed as OD at 570 nm. Raw data of apoptosis analysis by flow cytometry in mock (*left*) and HAoSMCs with osteoglycin overexpression (*right*) (*B*) and determination of the percentage of apoptosis between both cultures (*C*). To calculate the percentage of apoptosis, the sum of early apoptotic cells (Annexin-FITC+/PI−) and late apoptotic cells (Annexin-FITC+/PI+) was determined ($n = 3$ biological replicates/group and $n = 2$ technical replicates/biological replicates). All data represent as the means ± standard deviation of experiments performed and the *P* values between groups were determined by the unpaired Student's *t* test. $*P < 0.05$ vs. mock. HAoSMCs, primary human aortic smooth muscle cells; OD, optical density.

On the other hand, it is well known that cardiovascular and renal alterations are closely related in patients with T2D (42). Some studies have reported higher osteoglycin levels in serum in T2D patients with nephropathy (43). Consistently, our previous study reported increased serum osteoglycin levels associated with mild kidney function impairment in patients with T2D compared with healthy subjects proposing osteoglycin as an early biomarker of kidney impairment (44). In agreement, our results have shown a negative correlation between circulating osteoglycin levels and eGFR in patients with T2D. In contrast, Wei et al. (45) showed that low serum osteoglycin levels were an independent diagnostic marker of diabetic nephropathy with microalbuminuria in patients with T2D (45). This discrepancy may be due to differences in patient populations regarding to age, comorbidities, and variability in the duration of the observational period.

Our study provides valuable data, although it also has some limitations. The cross-sectional design prevents us from establishing a cause-effect relationship, offering only associations between variables. In addition, our study population included only Caucasian individuals from a specific area, and the use of antihyperlipidemic, antihypertensive, and antidiabetic drugs may influence the clinical results. Another limitation is the small number of vascular tissue samples obtained from both controls and T2D patients with CVD. This scarcity is mainly due to the difficulties in acquiring such samples, especially for healthy controls. As a consequence, it is imperative to interpret these results with caution, underscoring the need for future investigations to further validate and expand upon our findings. Despite these limitations, our research presents several strengths such as comprehensive evaluation of osteoglycin at both the clinical level (serum and vascular tissue) and the basic level (in vitro assays) within the same study. Moreover, we meticulously assessed various clinical, anthropometric, and biochemical parameters, covering all the variables that could influence cardiovascular risk, integrating these findings with experimental results. Furthermore, we used rigorous statistical analyses to ensure the reliability of our conclusions. Overall, while acknowledging the limitations, our study contributes significantly to the understanding the role of osteoglycin in the context of atherosclerosis and T2D.

In conclusion, our study describes for the first time that patients with T2D present increased serum osteoglycin levels compared with nondiabetic controls, although we found no evidence to support a relevant role for this protein in the development of atherosclerosis in this population. We demonstrated that osteoglycin plays a key role in glycemic homeostasis and could be a potential biomarker

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of insulin resistance in patients with T2D. Furthermore, this protein could indirectly participate in the development of atherosclerosis through its regulatory effect on other genes involved in inflammation, such as *ATX* and by VSMCs proliferation. Although further research is necessary to elucidate the intricate pathways in which osteoglycin is involved, these results open the door for the study of osteoglycin as a potential therapeutic target in T2D.

DATA AVAILABILITY

The data sets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.G.-S., B.G.-F., L.M.-H., E.M.-A., S.L.-A., C.G.-F., and M.M.-T. conceived and designed research; S.G.-S., B.G.-F., J.L., R.S.-d.l.T., and C.G.-F. performed experiments; S.G.-S., B.G.-F., L.M.-H., F.A.-V., C.G.-F., and M.M.-T. analyzed data; S.G.-S., B.G.-F., F.A.-V., C.G.-F., and M.M.-T. interpreted results of experiments; S.G.-S. prepared figures; S.G.-S., B.G.-F., L.M.-H., C.G.-F., and M.M.-T. drafted manuscript; S.G.-S., B.G.-F., C.G.-F., and M.M.-T. edited and revised manuscript; S.G.-S., B.G.-F., L.M.-H., J.L., F.A.-V., R.S.-d.l.T., E.M.-A., S.L.-A., C.G.-F., and M.M.-T. approved final version of manuscript.

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CAPÍTULO III

Cardioprotective function of sclerostin by reducing calcium deposition, proliferation, and apoptosis in human vascular smooth muscle cells

Sheila González-Salvatierra, Cristina García-Fontana, Jesus Lacal, Francisco Andújar Vera, Luis Martínez-Heredia, Raquel Sanabria-de la Torre, María Ferrer-Millán, Enrique Moratalla-Aranda, Manuel Muñoz-Torres, Beatriz García-Fontana.

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Cardiovascular Diabetology

RESEARCH **Open Access**

Cardioprotective function of sclerostin by reducing calcium deposition, proliferation, and apoptosis in human vascular smooth muscle cells

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Abstract

Background Sclerostin is an inhibitor of the Wnt/b-catenin pathway, which regulates bone formation, and can be expressed in vascular smooth muscle cells (VSMCs). Type 2 diabetes (T2D) is associated with an increased risk of cardiovascular disease (CVD) and increased serum and tissue expression of sclerostin. However, whether the role of sclerostin is detrimental or protective in the development of CVD is unknown. Therefore, our aims are to determine the level of sclerostin in T2D patients with/without CVD and in controls, both at serum and vascular tissue, and to analyze the role of sclerostin in VSMCs under calcified environments.

Methods Cross-sectional study including 121 controls and 139 T2D patients with/without CVD (48/91). Sclerostin levels in serum were determined by ELISA, and sclerostin expression was analyzed by RT-qPCR and immunohistochemistry in calcified and non-calcified artery of lower limb from T2D patients (n = 7) and controls (n = 3). *In vitro* experiments were performed in VSMCs (mock and sclerostin overexpression) under calcifying conditions analyzing the sclerostin function by determination of calcium and phosphate concentrations, and quantification of calcium deposits by Alizarin Red. Proliferation and apoptosis were analyzed by MTT assay and flow cytometry, respectively. The regulation of the expression of genes involved in bone metabolism was determined by RT-qPCR.

Results A significant increase in serum sclerostin levels in T2D patients with CVD compared to T2D patients without CVD and controls ($p < 0.001$) was observed. Moreover, higher circulating sclerostin levels were independently associated with CVD in T2D patients. Increased sclerostin expression was observed in calcified arteries of T2D patients

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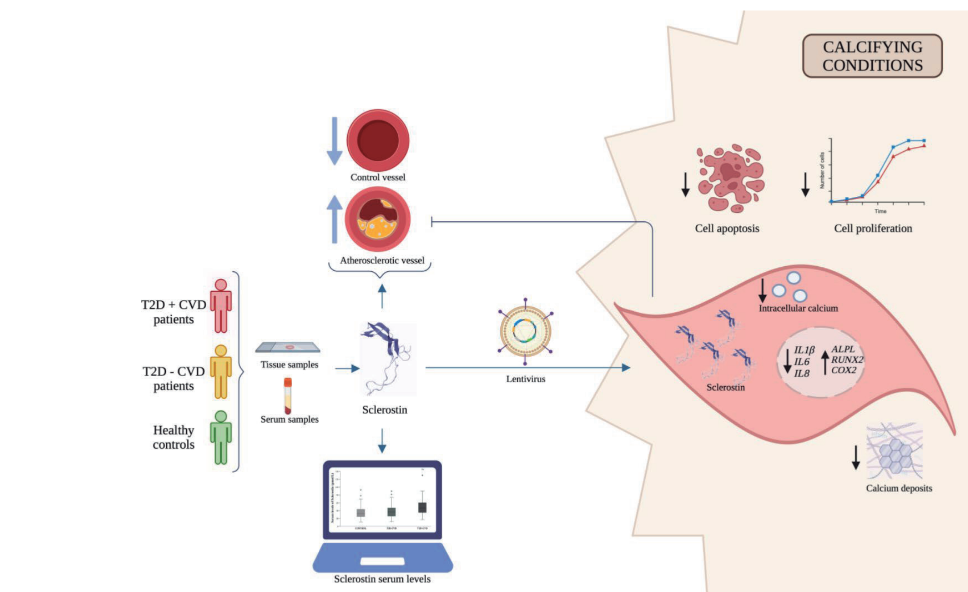
compared to non-calcified arteries of controls ($p = 0.003$). *In vitro* experiments using VSMCs under calcified conditions, revealed that sclerostin overexpression reduced intracellular calcium ($p = 0.001$), calcium deposits ($p < 0.001$), cell proliferation ($p < 0.001$) and promoted cell survival ($p = 0.015$). Furthermore, sclerostin overexpression exhibited up-regulation of *ALPL* ($p = 0.009$), *RUNX2* ($p = 0.001$) and *COX2* ($p = 0.003$) and down-regulation of inflammatory genes, such as, *IL1β* ($p = 0.005$), *IL6* ($p = 0.001$) and *IL8* ($p = 0.003$).

Conclusions Sclerostin could play a protective role in the development of atherosclerosis in T2D patients by reducing calcium deposits, decreasing proliferation and inflammation, and promoting cell survival in VSMCs under calcifying conditions. Therefore, considering the bone-vascular axis, treatment with anti-sclerostin for bone disease should be used with caution.

Keywords Type 2 Diabetes, Cardiovascular Diseases, Atherosclerosis, Sclerostin, Vascular smooth muscle cells, Protective role

Graphical Abstract



Background

Type 2 diabetes (T2D) is associated with an increased risk of cardiovascular disease (CVD) [1] affecting approximately 35% of T2D patients [2]. The major CVDs associated with T2D include ischemic heart disease, coronary artery disease, and peripheral artery disease [2]. Atherosclerosis is the main pathological mechanism underlying CVD in T2D, due to hypertension, hyperglycemia and dyslipidemia [3]. The development of atherosclerosis is strongly associated with the proliferation and migration of vascular smooth muscle cells (VSMCs) and endothelial activation [4]. To repair vascular damage in atherosclerosis, VSMCs switch from the contractile phenotype (characterized by high levels of contractile protein production) to synthetic phenotype (characterized by an increase in the production of cytokines and extracellular matrix). This facilitates proliferation and mobility as a physiological response induced by proinflammatory stimuli and oxidative stress [5, 6]. Moreover, VSMCs are capable of undergoing a phenotypic transition to osteocyte-like cells in a calcifying microenvironment capable of expressing typical osteocyte markers [7], as occurs in patients with T2D [8]. At this point, the Wnt/b-catenin pathway plays a crucial role in regulating VSMCs proliferation, migration and survival via modulation of the expression of matrix proteins [5, 9]. Proteins involved in the Wnt/b-catenin pathway, such as WNT4 and dickkopf-related protein, have been shown to stimulate the proliferation of VSMCs causing intimal thickening in atherosclerosis [10, 11]. Therefore, inhibitors of the Wnt/b-catenin pathway may facilitate the development of therapeutic targets for the treatment of CVD.

Sclerostin, a protein synthesized by the *SOST* gene, is an inhibitor of the Wnt/b-catenin pathway that

regulates bone formation [12]. Under physiological condition, sclerostin is mainly expressed by osteocytes [13], although it is also expressed by VSMCs in calcifying environment [7]. In the serum level, sclerostin had been found highly expressed in T2D patients with atherosclerotic lesions [3, 14, 15]. Also, several studies have shown an association between sclerostin levels in serum and the occurrence of CVD and cardiovascular mortality [12, 16–18]. These findings strongly support that sclerostin, in addition to regulating bone metabolism, is involved in vascular homeostasis, constituting an important modulator of the Wnt/b-catenin pathway in CVD. Sclerostin has also been found highly expressed in calcified aortic tissue derived from human aortic samples from patients with atherosclerosis [19]. Sclerostin expression in the tunica media of VSMCs in plaques from patients undergoing carotid endarterectomy, suggests a role in the development of atherosclerosis [20]. A recent study in mice showed that calcifications in the aortic medial layer and renal vessels were significantly more pronounced when warfarin treatment was combined with anti-sclerostin antibody treatment, suggesting a protective effect of sclerostin during vascular calcification [21]. Despite these important advances, no human studies have demonstrated whether sclerostin plays a detrimental or protective role in atherosclerotic disease. Currently, a humanized monoclonal anti-sclerostin antibody, is being used in the clinical practice for the treatment of osteoporosis and high fracture risk [22]. Likewise a recent evaluation of this treatment has shown benefits on bone but an potential increased rate of adverse cardiovascular events [23–25]. These findings suggest that sclerostin inhibition could be associated with cardiovascular risk, although the role of this protein in CVD has not yet been clarified in humans. It is therefore crucial to clarify the role of sclerostin in pathophysiological vascular mechanisms in order to prevent and reduce the high morbidity and mortality rate associated with CVD.

This study aims to investigate the potential protective role of sclerostin in the development of atherosclerosis in humans, which has not been previously explored. To accomplish this, we pursued two primary objectives. Firstly, we quantified sclerostin levels in serum and vascular tissue samples obtained from individuals with T2D, both with and without CVD, as well as from control subjects. This analysis allowed us to assess the association between sclerostin and atherosclerosis in a clinical context. Secondly, we conducted experiments using sclerostin in VSMCs exposed to calcified environments in vitro. This approach enabled us to examine the direct impact of sclerostin on VSMCs under conditions relevant to atherosclerosis development. By combining clinical and experimental investigations, our study aims to provide comprehensive insights into the potential protective role of sclerostin in atherosclerotic processes, thereby contributing to a deeper understanding of the pathophysiology of this condition in humans.

Methods

Study population

This cross-sectional study included 260 participants, 121 healthy controls (65±9 years, 56.2% males) and 139 T2D patients (65±8 years, 56.1% males). T2D was diagnosed according to American Diabetes association criteria [26]. Healthy controls were recruited from Nuclear Medicine Unit at the University Hospital Clínico San Cecilio of Granada (Spain) between 2020 and 2022. T2D patients, on the other hand, were recruited from the Endocrinology and Nutrition Unit of the same hospital between 2017 and 2018. In both groups, recruitment was based on specific criteria, including Caucasian ethnicity and normal values for blood count, and hepatic function. These rigorous criteria ensured the selection of suitable individuals for the study, minimizing potential confounding factors and enhancing the reliability of the research findings. T2D group was classified in two subgroups according to the presence of CVD: CVD group (n=48) and non-CVD group (n=91). Inclusion criteria for CVD were cerebrovascular disease (transient ischemic attack or ischemic stroke), coronary heart disease (previous myocardial infarction, angina diagnosed with stable or unstable coronary artery bypass graft surgery), or ischemic peripheral arterial disease. Patients with hepatic, gastrointestinal, thyroid or bone diseases and those with an estimated glomerular filtration rate (eGFR) below 45 mL/min/1.73m^2 or treated with thiazolidinediones, warfarin or other drugs that affect to bone metabolism were excluded.

Vascular tissue samples were obtained from artery of lower limbs at the Angiology and Vascular Surgery Unit of the University Hospital Clínico San Cecilio of Granada. Calcified vessels were obtained from T2D patients with ischemic diabetic foot with criteria of critical ischemia, in whom major lower limb amputation was indicated because they were not candidates for revascularization (primary amputation) or because it had failed (secondary amputation) (n=7). The sample obtained was a fragment (10 mm) of the distal third of the superficial femoral artery. The stiffer or even stenotic or occluded arterial segment with visible atherosclerotic plaque was the one extracted for the research study. Samples of non-calcified vessels without any visible atherosclerotic lesions proceed from healthy subjects with no history of vascular risk factors or any ischemic event at any level with informed consent at the University Hospital Clínico San Cecilio of Granada (n=3).

The Biobank of the Andalusian Public Health System at the University Hospital Clínico San Cecilio of Granada was responsible for the management of all samples used

in this study. Prior to participation, informed consent was obtained from each subject, ensuring their voluntary involvement. The study was conducted in accordance with the guidelines and regulations set forth by the Ethics Committee of the University Hospital Clínico San Cecilio of Granada, and it adhered to the principles outlined in the World Medical Association Declaration of Helsinki (Project ID:0858-N-17. Research Ethics Committee of Granada Center at 26th April 2017).

Clinical evaluation of study population

The height, weight, and waist circumference were measured at baseline according to standard procedures. The body mass index (BMI) was calculated by the Queletet formula, weight (kg)/stature (m^2). The systolic and diastolic blood pressures were measured using a standard electronic sphygmomanometer. Hypertension was defined as values 140/90 mmHg and/or antihypertensive treatment. Dyslipidemia was characterized by serum levels of low-density lipoprotein cholesterol (LDL-c) > 100 mg/dL, high-density lipoprotein cholesterol (HDL-c) < 50 mg/dL, triglycerides (TG) > 150 mg/dL, and/or current treatment with lipid-lowering drugs. Patients reported their alcohol use, smoking status, and level of physical activity was recorded using the Spanish version of the questionnaire for Rapid Assessment of Physical Activity [27].

Biochemical measurements of study population

Samples of venous blood were taken in the morning after fasting overnight. Serum samples were stored at -80ºC until analysis at the Clinical Analysis Unit of the University Hospital Clínico San Cecilio of Granada. The parameters as fasting plasma glucose (FPG), glycated haemoglobin (HbA1c), TG, HDL-c, LDL-c, phosphorus, and calcium were measured using standard automated laboratory techniques. eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration equation (CKD-EPI) [28].

The calciotropic hormone profile included serum intact parathormone (iPTH) was determined by the two-site immunoassay for iPTH (Roche Diagnostics) and 25-hydroxyvitamin D (25(OH)D) was determined using a chemiluminescence immune assay (CLIA) (Beckman Coulter UniCel DxI 800). Total osteocalcin (OC) was determined by CLIA (N-Mid Osteocalcin; Immunodiagnostic Systems iSYS automated analyzer). The procollagen type 1 N-terminal propeptide (P1NP) and serum carboxy-terminal crosslinked telopeptide of type I collagen (CTX) were determined by electrochemiluminescence immune assay (ECLIA) (Roche Diagnostics). The alkaline phosphatase (ALP) levels were measured by a colorimetric method in an AU5800 analyzer (Beckman Coulter). Sclerostin and periostin levels were determined by the enzyme-linked immunosorbent assay (ELISA) method, following the manufacturer's protocols (Biomedica). Precision testing was performed by the determination of intra-assay and inter-assay variations for each ELISA assay (5% and 1% for sclerostin; 6% and 3% for periostin).

RNA isolation and RT-qPCR

RNA isolation and RT-qPCR were performed to quantify the expression of sclerostin in vascular tissue, to check the efficiency of transduction in primary human aortic smooth muscle cells (HAoSMCs) with lentiviral particles, and to quantify the expression of different genes under different conditions. For vascular tissue, total RNA was obtained by isolation of 23 transversal sections (3 μm) of calcified lower limb artery of T2D patients with peripheral artery disease and of non-calcified lower limb artery from healthy donors. RNA extractions were carried out with Trizol Reagent (ThermoFisher Scientific) by a manual homogenizer. For cells, RNA was isolated using a RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. In both cases, RNA was treated with Turbo DNase (Ambion), and the RNA concentration and quality were assessed using the Qubit Flex Fluorometer (ThermoFisher Scientific). Only RNA samples with a A260/280 ratio between 1.8 and 2.0 were used for cDNA synthesis.

The RNA was reverse-transcribed to synthesize cDNA using the iScript cDNA synthesis kit (BioRad), following the manufacturer's protocol.

qPCR was carried out using PowerUp SYBR Green Master Mix (ThermoFisher Scientific) in a QuantStudio™ 7 Flex Real-Time PCR System (ThermoFisher Scientific) as follows: 95 °C for 2 min, 40 cycles of 95 °C for 20 s and 65 °C for 20 s. The analysis of the melting curve was performed from 65 to 95ºC with increment of 0.5ºC/4 sec. Primers were designed using the Primer Blast software (NCBI) and Clone Manager Suite program (Table 1). The expression of a constitutive gene was used to normalize the mRNA data. All real-time PCR reactions for each sample were performed in triplicate. Relative expression of each gene of interest was assessed using the $2^{-\Delta\Delta Ct}$ method [29].

Immunohistochemistry and immunofluorescence of vascular tissue

Formalin-fixed paraffin-embedded biopsy tissues from calcified lower limb artery of T2D patients and non-calcified of healthy controls were obtained from archival paraffined in slides. The samples were deparaffined using a standard protocol by a combination of xylenes and ethanol. In order to perform the antigen retrieval, the tissue sections were incubated in 1X citrate buffer

Table 1 Primers used in the different experiments of this study

Gene	Sequence (5´-3´)		Amplicon (pb)	Application
SOST	Forward	ATGCCACGGAAATCATCCCC	185	Expression in vascular tissue.
	Reverse	GTCACGTAGCGGGTGAAGTG		Check the efficiency of cells transduction. Quantify the expression under different conditions in VSMCs in vitro.
RPL13	Forward	CGTAAGATCCGCAGACGTAAGGC	228	Constitutive gene
	Reverse	GGACTTGTTCCGCCTCCTCGGAT		
ALPL	Forward	GGCTGGAGATGGACAAGTTC	152	Quantify the expression under different conditions in VSMCs in vitro.
	Reverse	ACGCTCAGTGGCTGCGCTTA		
RUNX2	Forward	CGCCGTGGTCCTATGACCAGTCTTA	169	Quantify the expression under different conditions in VSMCs in vitro.
	Reverse	AGGCAGAAGTCAGAGGTGGCAGTGT		
IL1β	Forward	CTTCAGGCAGGCCGCGTCAGTTGTT	202	Quantify the expression under different conditions in VSMCs in vitro.
	Reverse	CCGGAGCGTGCAGTTCAGTGATCGT		
IL6	Forward	AGACAGCCACTCACCTCTTCAGAAC	208	Quantify the expression under different conditions in VSMCs in vitro.
	Reverse	CCAGGCAAGTCTCCTCATTGAATCC		
IL8	Forward	GAGAGTGATTGAGAGTGGACCAC	112	Quantify the expression under different conditions in VSMCs in vitro.
	Reverse	CACAACCCTCTGCACCCAGTTT		
COX2	Forward	CCGCCATCATCCTAGTCCTCATCGC	143	Quantify the expression under different conditions in VSMCs in vitro.
	Reverse	TAGTCCGCCGTAGTCGGTGTACTCG		
ACTA2	Forward	CATCGTGCTGGACTCTGGAGATGGT	151	Quantify the expression under different conditions in VSMCs in vitro.
	Reverse	GAAGGAATAGCCACGCTCAGTCAGG		

SOST, sclerostin; *RPL13*, ribosomal protein L13; *ALPL*, alkaline phosphatase, biomineralization associated; *RUNX2*, runt-related transcription factor 2; *IL1β*, interleukin 1 beta; *IL6*, interleukin 6; *IL8*, interleukin 8; *COX2*, cyclooxygenase 2; *ACTA2*, actin aortic smooth muscle; VSMCs, vascular smooth muscle cells.

inside a steamer machine at high temperature (200 °C) for 20 min.

For immunohistochemistry, tissue sections were then rinsed in phosphate-buffered saline (PBS) (0.01 M, pH 7.4), incubated for 15 min with 3% hydrogen peroxide, rinsed again, and incubated in a solution of 3% normal goat serum and 0.1% PBS-tween20 for 60 min. Slices were transferred to an anti-sclerostin primary antibody (1:500, #ab85799, Abcam) solution overnight at 4ºC. After being rinsed with PBS, the sections were incubated with a secondary antibody (1:5000 Goat Anti-Rabbit IgG (H&L) HRP, #ab205718, Abcam) solution for 120 min at room temperature. Primary and secondary antibody solutions were mixed in a solution of 3% normal goat serum and 0.1% PBS-tween20. The sections were rinsed for further processing using the ABC-kit (Vector Laboratories). The reaction was visualized using the peroxidase substrate kit DAB (Vector Laboratories). Finally, the sections were rinsed, rehydrated with ethanol and xylenes and cover slipped. Images of lower limb artery of T2D patients and controls were captured using a light microscope (Olympus BX41). Slices containing the regions of interest were identified using the *Stereo Investigator* Software (mbf Bioscience) from coronal sections of the samples (Fig. 4A). In each sample, 4 microphotographs were captured at 20X magnification (Fig. 4B). The quantification of sclerostin was obtained using the *Image J* Software. For each microphotograph threshold objects (black circular dots over the white background) having specific size (35–150 μm^2) and circularity (0.35-1.00) values matching those positive nuclei were automatically identified by the software as proteins. In order to equalize all the microphotographs and to cancel out possible background noise, they were previously converted into 8-bit type image and the background was lightened (150.0 pixels). The threshold was set up to 0-150 for all images.

For immunofluorescence, tissue sections were then rinsed in PBS (0.01 M, pH 7.4) and incubated in a solution of 3% normal goat serum and 0.1% PBS-tween20 for 60 min. Slices were transferred to an anti-sclerostin primary antibody (1:200; #ab85799, Abcam) solution overnight at 4ºC. After being rinsed with PBS, they were incubated in a secondary antibody (1:500, Goat Anti-Rabbit IgG (H+L) cross-adsorbed Secondary Antibody, Alexa fluor 488, #A-11008, Thermo Fisher) solution for 120 min at room temperature. Primary and secondary antibody solutions were mixed in a solution of 3% normal goat serum and 0.1% PBS-tween20. The sections were washed four times for 10 min in PBS and were mounted on glass slides with Fluoroshield Mounting Medium with 4',6-diamidino-2-306 phenylindole (DAPI) (#ab104139, Abcam) and cover-slipped. The slices were then kept in the dark at 4°C and later observed using an upright Olympus BX41 confocal microscope. In each sample microphotographs at 20X magnification were captured using the blue (DAPI) and green (sclerostin) filters. The quantification of sclerostin (green circular dots) and cell nuclei (blue circular dots) were performed using the

Image J Software. Representative microphotographs of the different groups are shown in Fig. 4D.

Cell culture and reagents

HAoSMCs (ATCC) were grown using Vascular Cell Basal Medium (ATCC) supplemented with VSMC Growth Kit (ATCC) that contains the following components: recombinant human (rh) fibroblast growth factor (FGF-b), rh insulin, ascorbic acid, L-glutamine, rh epidermal growth factor (EGF), and fetal bovine serum (FBS). Cells were grown to confluence and used from passages 4 to 5.

Human embryonic kidney 293T cells (HEK293T) (ATCC) were cultured in DMEM/F-12 GlutaMAX (Gibco) supplemented with 10% FBS (NeoBiotech RNase A (Powder)).

Both cell cultures were grown under standard conditions (37 °C and 5% CO_2 in a humid atmosphere).

Second-generation lentiviruses and transduction for generation of stable lines of HAoSMCs

Overexpression of sclerostin was carried out by producing a second-generation lentiviral packaging system protocol using the vectors pVSV-G (Addgene), that expresses the envelope gene of the VSV-G virus, psPAX2 (Addgene) that expresses the reverse transcriptase gene, the protease gene and the gene for assembly of the HIV-1 virus and, the pLVX:*SOST* construct or empty pLVX (Addgene). HEK293T cells were transfected with the mix of the above plasmids using polyethylenimine (Quimigen) and were cultured in DMEM/F-12 GlutaMAX (Gibco) supplemented with 10% FBS and grown under standard conditions for 24 h. Lentivirus particles were harvested, filtered, ultracentrifuged, resuspended in PBS and stored at -80ºC.

HAoSMCs were transduced using lentivirus particles with polybrene infection reagent 8 mg/mL (Merck) and selected with Hygromycin B 50 mg/mL (ThermoFisher Scientific). Control cells (mock) were transduced with lentiviruses generated from the empty pLVX vector. Transductions were performed in triplicate and cells were cultured under standard conditions using Vascular Cell Basal Medium supplemented with VSMC Growth Kit. Finally, sclerostin overexpression in this stable cell line was tested by RT-qPCR.

Induction of calcification

Transduced HAoSMCs with sclerostin overexpression and mock were seeded on 6-well plates at a confluency of 1000 cells/well. The cells were maintained in growth medium supplemented with 1.5 mM $CaCl_2$ and 10 mM β-glycerophosphate for up to 20 days to induce matrix calcification. Incubation cells were grown under standard conditions and the medium was changed every second/third day.

Calcium measurement

Extra- and intracellular calcium concentration was determined by the Calcium Colorimetric Assay Kit (BioVision). Extracellular calcium was measured from the culture medium. Cells were treated with 0.1 M NaOH and 0.1% Sodium Dodecyl Sulfate (SDS) to measure intracellular calcium. Quantification of calcium was measured with an optical density (OD) at 570 nm by spectrophotometry. The results were normalized to the total protein concentration, which was measured in the cultures using a protein assay reagent (Bio-Rad), based on the Bradford dye binding procedure, and albumin was used as a standard. Each condition was performed in triplicate and expressed as µg calcium/µg protein.

Additionally, calcium mineral deposition was assessed by Alizarin Red S staining. Cells were washed twice with PBS, fixed in 4% paraformaldehyde for 30 min at room temperature, rinsed with distilled water 3 times, stained with 2% alizarin red (pH 4.2) for 30 min with gentle shaking at room temperature and the dye was removed and cells were washed 5 times with distilled water. Alizarin red stained cultures were extracted with 10% acetic acid and incubated at room temperature for 30 min with shaking. Cells were collected in a microcentrifuge tube and vortexed. The tubes were heated at 85°C for 10 min, incubated on ice for 5 min and centrifuged. 10% ammonium hydroxide was added, then the OD of the dissolved dye was measured at 405 nm spectrophotometrically. The results were normalized to the total protein concentration, which was measured in the cultures using a protein assay reagent (Bio-Rad), based on the Bradford dye binding procedure, and albumin was used as a standard. Each condition was performed in triplicate and expressed as OD 405 nm/µg protein.

Phosphate measurement

Extra- and intracellular phosphate concentration was determined by Phosphate Colorimetric Assay Kit (BioVision). Extracellular phosphate was measured from the culture medium. Cells were treated with 0.1 M NaOH and 0.1% SDS to measure intracellular phosphate. Quantification of phosphate was measured with an OD at 650 nm by spectrophotometry. The results were normalized to the total protein concentration, which was measured in the cultures using a protein assay reagent (Bio-Rad), based on the Bradford dye binding procedure, and albumin was used as a standard. Each condition was performed in triplicate and expressed as nmol phosphate/µg protein.

Cell proliferation assay

Cell proliferation of HAoSMCs with overexpression of sclerostin and mock was analyzed with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

(MTT) assay. Cells were seeded in a 96-well plate (250 cells/100µL per well) in calcified condition for 10 days in standard condition and performed cell proliferation assay every 2 days. After the different times have elapsed, 10 µL of MTT (5 mg/mL) was added and the plate was incubated for 6 h at standard condition. Subsequently, 100 µL of lysis buffer (20% SDS in 50% formamide, pH 4.7) was added, and the plate was kept under standard condition overnight. Cell proliferation was measured with an OD at 570 nm by spectrophotometry. Four replicates per condition were carried out and were corrected by cell free media.

Cell apoptosis assay

The percentages of apoptotic HAoSMCs with overexpression of sclerostin and mock in calcified condition were analyzed using a FITC Annexin V Apoptosis Detection Kit (BD Biosciences). Cells were washed with PBS twice and 10^5 cells/100µL were incubated with Annexin V-FITC and propidium iodide at room temperature and in darkness for 15 min per condition. The samples were analyzed using a BD FACSAria IIIu flow cytometer (Becton Dickinson, BD Biosciences). The percentage of apoptosis was calculated by considering the sum of percentages of apoptotic cells (Annexin-FITC+/PI–) and late apoptotic cells (Annexin-FITC+/PI+). Each condition was performed in duplicate.

Statistical analysis

Analyses were performed using SPSS version 28.0 software (SPSS, Inc., Chicago, IL) and GraphPad Prism v7.03 (GraphPad Software). Data were expressed as means ± standard deviation (SD) for variables normally distributed and as median with the interquartile range (IQR) for variables not normally distributed. The data for categorical variables were presented as percentages. A Kolmogorov-Smirnov test was used to test the normality of distribution of the continuous variables. The mean values between groups were compared using the unpaired Student´s t-test for continuous and normally distributed variables. The Mann-Whitney U test was used to compare variables not normally distributed. When the comparison between groups required an adjustment by covariates, a univariate analysis of covariance (ANCOVA) was performed. The χ^2 test was used to compare categorical variables between groups. Associations between continuous variables were described by Spearman's correlation coefficients. A multiple lineal regression model was performed to determine the variables independently associated with sclerostin (dependent variable), including the quantitative and qualitative variables linked in the bivariate analysis, and other variables biologically associated to sclerostin as independent variables. Data were expressed as B; 95% confidence interval (CI) (lower limit/upper limit). To identify sclerostin as an independent predictor of CVD, a multiple logistic regression model was performed, including prevalent CVD as a dependent variable. Statistical significance was set at $p<0.05$ (two tailed) and $p<0.10$ for multiple linear and logistic regression analysis. The independent variables included in the model were the established cardiovascular risk in addition to sclerostin levels. The usefulness of serum sclerostin as an estimator of CVD risk was assessed using a receiver operating characteristic curve (ROC). The area under the curve (AUC) indicates the probability to predict an event. AUC values greater than 0.75 indicate a good predictive performance.

Immunohistochemistry was performed on vascular tissue to assess the expression of sclerostin. The mean number of sclerostin-positive cells, along with the standard error of the mean (SEM), was determined for the different groups as well as for the intima-media and adventitia layers. This analysis allowed us to evaluate potential variations in sclerostin expression. To compare the differences in sclerostin-positive cells between the two vascular tissue layers, Student´s t-test were employed, providing a statistical assessment of the significance of the observed variations.

Furthermore, in the in vitro analysis of HAoSMCs, data were presented as means ± SD. To determine differences between the groups, the unpaired Student´s t-test was used for mean value comparisons. This statistical approach enabled us to evaluate the significance of observed variations in the experimental data between different groups of HAoSMCs.

Results

Characteristics of the study population

Table 2 summarizes the baseline characteristics of the entire population consisting of healthy subjects and T2D patients. Both groups were comparable in age and sex. As expected, patients with T2D showed a significantly worse metabolic profile in terms of BMI, waist circumference, FPG, HbA1c, and lipid profile. In addition, T2D group showed a significant increase in serum sclerostin level compared to control group ($p=0.003$).

Furthermore, clinical, anthropometric, biochemical and bone parameters of the T2D participants according to the absence or presence of CVD are summarized in Table 3. In terms of clinical evaluation, notable variations were observed in CVD-defining factors, including hypertension, dyslipidaemia, and duration of diabetes. Additionally, significant differences were observed between the groups with respect to sex and age, indicating potential demographic influences on CVD development (Table 3). On the other hand, there were no significant differences between the groups in weight, height, BMI, or waist circumference (Table 3). Most biochemical

Table 2 Comparison of baseline characteristics between the control and T2D groups

Baseline Characteristics	Control	T2D	*p*
Men/women (n)	61/53	78/61	0.989
Age (years)	65 ± 9	65 ± 8	0.267
Body weight (kg)	74.9 ± 14.6	86.2 ± 14.4	< 0.001*
Height (cm)	163 ± 0.1	164 ± 0.09	0.298
BMI (kg/m^2)	28 ± 4.8	31.8 ± 4.6	< 0.001*
Waist circumference (cm)	97 ± 10.7	105.9 ± 10.8	< 0.001*
FPG (mg/dL)	91 (84–99)	143 (107–173)	< 0.001*
HbA1c (%)	5.6 (5.4–5.8)	7.6 (6.9–8.6)	< 0.001*
TG (mg/dL)	103 (78–144)	139 (99–197)	< 0.001*
HDL-c (mg/dL)	54 ± 12	46 ± 11	< 0.001*
LDL-c (mg/dL)	116 ± 32	93 ± 40	< 0.001*
eGFR (mL/min/1.73 m^2)	86.3 (73.5–94)	87 (71.1–97.5)	0.568
Calcium (mg/dL)	9.7 (9.5–10.1)	9.7 (9.5–9.9)	0.553
Phosphorous (mg/dL)	3.2 (2.9–3.5)	3.3 (2.9–3.6)	0.169
25(OH)D (ng/mL)	25.6 ± 8.5	20.7 ± 8.5	< 0.001*
P1NP (ng/mL)	43.8 ± 21	37.2 ± 15	0.002*
ALP (µg/L)	12.6 (10.3–16.9)	16.6 (13.3–21.4)	< 0.001*
iPTH (pg/mL)	52 (40.1–69.3)	46.8 (31.25-61)	0.007*
OC (ng/mL)	21.7 ± 17.4	10.8 ±	< 0.001*
Periostin (pmol/L)	1208.8 (972–1452)	1147 (936–1546)	0.975
Sclerostin (pmol/L)	32.6 (24.82–43.14)	39.02 (28.20-49.47)	0.003*

T2D, type 2 diabetes; BMI, body mass index; FPG, fasting plasma glucose; HbA1c, glycated haemoglobin; TG, triglycerides; HDL-c, high-density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; 25(OH)D, 25-hydroxyvitamin D; P1NP, procollagen type 1 N-terminal propeptide; ALP, alkaline phosphatase; iPTH, intact parathormone; OC, osteocalcin. Data for continuous and normally distributed variables are presented as mean±standard deviation. Data for continuous variables not normally distributed, are presented as median followed by interquartile range in brackets. Data for categorical variables are presented as percentages. Student´s t-test and Mann-Whitney U test were used for comparisons of continuous and normally or not normally distributed variables, respectively, between groups. χ^2 test was used for comparison of categorical variables between groups. * = $p < 0.05$ between groups

parameters exhibited similar values between the two groups, except for HDL-c, LDL-c, eGFR, and calcium levels (Table 3). These variations suggest potential associations between these parameters and the presence of CVD in T2D patients. Furthermore, notable discrepancies were observed between the groups in terms of serum levels of proteins involved in bone metabolism, specifically periostin and sclerostin (Table 3). These findings indicate potential links between altered bone metabolism and the development of CVD in T2D patients.

Influence of diabetes status, sex and CVD on serum sclerostin levels

Serum sclerostin levels were significantly higher in T2D patients (n = 139, 56.1% males) than in control subjects (n = 121, 56.2% males) (39.02 (28.20-49.47) pmol/L *vs.* 32.60 (24.82–43.14) pmol/L, $p = 0.003$). When T2D patients and control subjects were further divided according to sex, the significative differences in serum sclerotin levels remained for both males (T2D patients: 45.99 (32.05–55.36) pmol/L *vs.* controls subjects: 35.65 (27.62–47.35) pmol/L, $p = 0.031$), and females (T2D patients: 36.64 (26.88–43.02) pmol/L *vs.* controls subjects: 29.08 (21.51–34.82) pmol/L, $p = 0.012$). We found serum sclerostin levels significantly higher in males than females in the T2D group (45.99 (32.05–55.36) pmol/L *vs.* 36.64 (26.88–43.02) pmol/L, $p = 0.002$) and healthy controls (35.65 (27.62–47.35) pmol/L *vs.* 29.08 (21.51–34.82) pmol/L, $p < 0.001$).

The comparison of circulating sclerostin levels between healthy controls and T2D patients without CVD (n = 91, 45% males) and with CVD (n = 48, 77% males) revealed significant differences between groups ($p < 0.001$). The control group showed the lowest levels of circulating sclerostin levels , whereas T2D patients with CVD has the highest levels ($p < 0.001$). No significative differences were found between control group and T2D without CVD. After adjusting by age and sex, this trend in serum sclerostin levels remained unchanged among all groups ($p < 0.001$), except for control group *versus* T2D without CVD ($p = 0.073$) (Fig. 1). Specifically, our study revealed that T2D patients with peripheral arterial disease exhibited higher levels of sclerostin in serum compared to T2D patients without peripheral arterial disease.

Determinants of serum sclerostin levels in the T2D patients

We found a positive correlation between the circulating sclerostin level and CVD-defining factors such as age (r = 0.193; $p = 0.024$) and duration of diabetes (r = 0.275; $p < 0.001$). Whereas we found a negative correlation with eGFR (r=-0.295; $p < 0.001$), diastolic blood pressure (r=-0.185; $p = 0.031$), LDL-c (r=-0.198; $p = 0.020$), and calcium (r= -0.183; $p = 0.031$). In addition, we found a positive correlation between serum sclerostin levels and the bone marker periostin (r = 0.238; $p = 0.005$), in T2D patients (Fig. 2).

To investigate the factors influencing the level of sclerostin, a multiple linear regression analysis model was performed. The model included variables that were found to be associated with sclerostin based on prior bivariate analysis, including age, diabetes duration, eGFR, diastolic blood pressure, LDL-c, calcium, and periostin. Additionally, sex, current medication, and the presence of CVD were included as independent variables in the analysis. The results of this analysis will provide valuable

Table 3 Intergroup comparison for T2D patients according to the presence of CVD.

	T2D without CVD	T2D with CVD	*p*
Men/women (n)	41/50	37/11	< 0.001*
Age (years)	65 ± 8	67 ± 7	0.040 *
Clinical Evaluation			
Body weight (kg)	86.51 ± 14.05	85.70 ± 15.18	0.377
Height (cm)	164 ± 0.09	166 ± 0.09	0.132
BMI (kg/m^2)	32.14 ± 4.67	31.11 ± 4.49	0.107
Waist circumference (cm)	106.32 ± 11.08	105.03 ± 10.24	0.268
Diabetes duration (years)	13.43 ± 8.57	17.17 ± 9.99	0.011*
Systolic blood pressure (mmHg)	135.16 ± 18.14	134.79 ± 16.39	0.454
Diastolic blood pressure (mmHg)	81.25 ± 9.00	75.60 ± 12.04	0.003*
Hypertension (%)	80.2	95.8	0.013*
Dyslipidemia (%)	83.5	97.9	0.011*
Coronary heart disease (%)		56.3	< 0.001*
Cerebrovascular disease (%)		27.1	< 0.001*
Peripheral artery disease (%)		35.4	< 0.001*
Nephropathy (%)	13.2	25	0.080
Smoker or ex-smoker (%)	40	54.2	0.111
Alcohol consumption (%)	14.4	18.4	0.510
Sedentarism (%)	13	21.1	0.263
Fractures (%)	12.2	16.7	0.470
Osteopenia (%)	42.1	40.9	0.898
Osteoporosis (%)	10.5	6.8	0.498
Current Medication Use			
Insulin (%)	67	79.2	0.133
Oral antidiabetic drugs (%)	33	20.8	0.133
Biochemical Measurements			
FPG (mg/dL)	145.99 ± 48.68	152.88 ± 56.88	0.228
HbA1c (%)	7.79 ± 1.26	7.89 ± 1.60	0.340
TG (mg/dL)	139 (107–205)	142.5 (91.5–183)	0.232
HDL-c (mg/dL)	45 (39–52)	41 (35.50–48)	0.012*
LDL-c (mg/dL)	91 (67–126)	75 (51–103)	0.004*
eGFR (mL/min/1.73 m^2)	89.70 (75.90–99.50)	80.90(62.30-93.35)	0.016*
Calcium (mg/dL)	9.8 (9.5–9.9)	9.6 (9.35–9.8)	0.010*
Phosphorous (mg/dL)	3.3 (3-3.7)	3.3 (2.9–3.6)	0.398
25(OH)D (ng/mL)	20.82 ± 8.22	20.55 ± 9.19	0.431
P1NP (ng/mL)	38.19 ± 15.57	35.15 ± 13.58	0.138
ALP (µg/L)	16.60 (13.50–22.50)	16.65 (12.10–19.30)	0.424
CTX (ng/mL)	1.69 (1.10–2.50)	1.35 (0.83–2.21)	0.218
iPTH (pg/mL)	45.95 (32.5–56.5)	47.95 (29.4–65.6)	0.970
OC (ng/mL)	11.24 ± 5.97	10.04 ± 4.72	0.255
Periostin (pmol/L)	1101.79 (853.41-1407.14)	1368.30 (1078.22-1734.54)	0.002*
Sclerostin (pmol/L)	36.64 (26.02–47.05)	45.99 (35.24–62.13)	< 0.001*

T2D, type 2 diabetes; CVD, cardiovascular disease; BMI, body mass index; FPG, fasting plasma glucose; HbA1c, glycated haemoglobin; TG, triglycerides; HDL-c, high-density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; 25(OH)D, 25-hydroxyvitamin D; P1NP, procollagen type 1 N-terminal propeptide; ALP, alkaline phosphatase; CTX, carboxy-terminal crosslinked telopeptide of type I collagen; iPTH, intact parathormone; OC, osteocalcin. Data for continuous and normally distributed variables are presented as mean ± standard deviation. Data for continuous variables not normally distributed, are presented as median followed by interquartile range in brackets. Data for categorical variables are presented as percentages. Student´s t-test and Mann-Whitney U test were used for comparisons of continuous and normally or not normally distributed variables, respectively, between groups. χ^2 test was used for comparison of categorical variables between groups. * = $p < 0.05$ between groups

insights into the multifactorial nature of sclerostin regulation and its associations with various clinical parameters in the context of our study population. The results showed that the variables independently associated with the serum sclerostin level were sex (B = 0.182; 95% CI [0.857/13.128]; $p = 0.026$), diabetes duration (B = 0.198; 95% CI [0.085/0.730]; $p = 0.014$), eGFR (B= -0.237; 95% CI [-0.388/-0.081]; $p = 0.003$), and presence of CVD (B = 0.176; 95% CI [0.483/13.689]; $p = 0.036$).

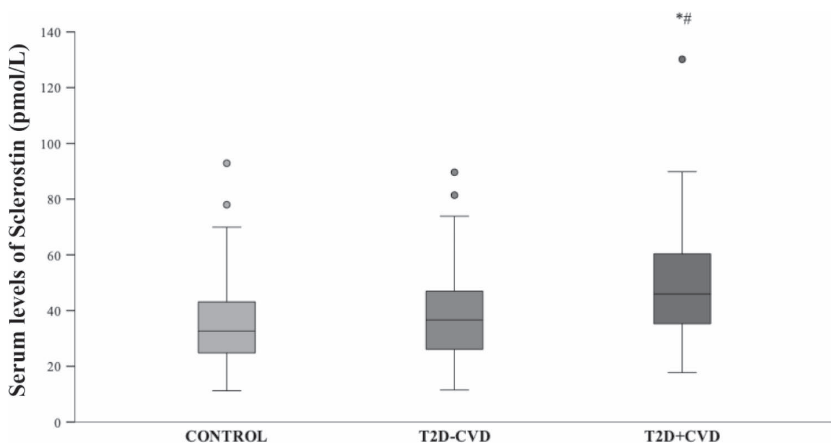


Fig. 1 Box plot of serum sclerostin levels in controls (n = 121), T2D patients without CVD (n = 48), and T2D patients with CVD (n = 91). Box plot represents the minimum value, 25th percentile, median, 75th percentile, maximum value, and outliers for each group. The *p*-values between the different were performed by ANCOVA. * = $p < 0.05$ *vs.* Control; # = $p < 0.05$ *vs.* T2D without CVD. T2D, type 2 diabetes; CVD, cardiovascular disease.

Evaluating the sclerostin serum level as a potential indicator of CVD risk in T2D patients

Logistic regression modelling was performed to assess the variables related to CVD risk in T2D patients. The independent variables included in the model were age, sex, hypertension, dyslipidaemia, eGFR, sedentarism, tobacco use and years of diabetes duration, in addition to serum sclerostin level. We found that, in addition to sex (OR=0.305; [0.120/0.771]; $p=0.012$), hypertension (OR=0.213; [0.043/1.069]; $p=0.060$) and dyslipidaemia (OR=0.142; [0.160/1.236]; $p=0.077$), the serum sclerostin level was an independent estimator of CDV risk (OR=1.026; [0.999/1.054]; $p=0.064$) in T2D patients.

To evaluate the predictive value of serum sclerostin level for CVD risk estimation, a ROC analysis was conducted. Two distinct models were assessed. The first model consisted of the main CVD risk factors, namely age, sex, hypertension, dyslipidaemia, eGFR, sedentarism, tobacco use, and years of diabetes duration. The second model included the same CVD risk factors along with serum sclerostin level as an additional variable. By comparing the performance of these two models, we aimed to determine the contribution of serum sclerostin level in improving the accuracy of CVD risk estimation. AUC of the model without sclerostin was 0.757; $p<0.001$, whereas the AUC of the model including sclerostin was 0.795; $p<0.001$ (Fig. 3).

Sclerostin expression level in vascular tissue

Immunohistochemistry and immunofluorescence were performed on calcified lower limb arteries of T2D patients (n=7) and non-calcified arteries of healthy controls (n=3). For immunohistochemistry, the analysis of the total average of sclerostin-positive cells revealed a significantly higher expression of sclerostin in T2D patients' vessels compared to control subjects' vessels (96.18±13.61 *vs.* 17.25±2.18, $p=0.003$). When examining the location of sclerostin-positive cells, a significant increase was observed in both the intima-media layer (51.71±14.19 *vs.* 16.50±4.01, $p=0.025$) and the adventitia layer (140.64±26.59 *vs.* 18.00±0.76, $p=0.010$) in calcified vessels compared to healthy vessels. Furthermore, a higher expression of sclerostin was detected in the adventitia layer compared to the intima-media layer in calcified lower limb arteries of T2D patients (140.64±26.59 vs. 51.71±14.19, $p=0.006$). However, no significant differences were observed between the layers in non-calcified vessels of healthy controls (18±0.76 vs. 16.5±4.01, $p=0.373$) (Fig. 4C).

Immunofluorescence analysis revealed significantly increased sclerostin expression in T2D patients' vessels compared to control subject' vessels (24.4±6.4 *vs.* 7.5±2.8, $p=0.022$) without significant differences in the total cell nuclei count between groups ($p=0.496$) (Fig. 4E).

Additionally, the calcified lower limb arteries of T2D patients (n=7) exhibited a significant upregulation of sclerostin mRNA compared to the non-calcified lower limb arteries of healthy controls (n=3), with a 4.71-fold increase ($p=0.010$) (Fig. 4F). These findings highlight the elevated expression of sclerostin in calcified lower limb arteries of T2D patients, both at the protein level and mRNA level, indicating its potential involvement in the pathogenesis of arterial calcification in T2D.

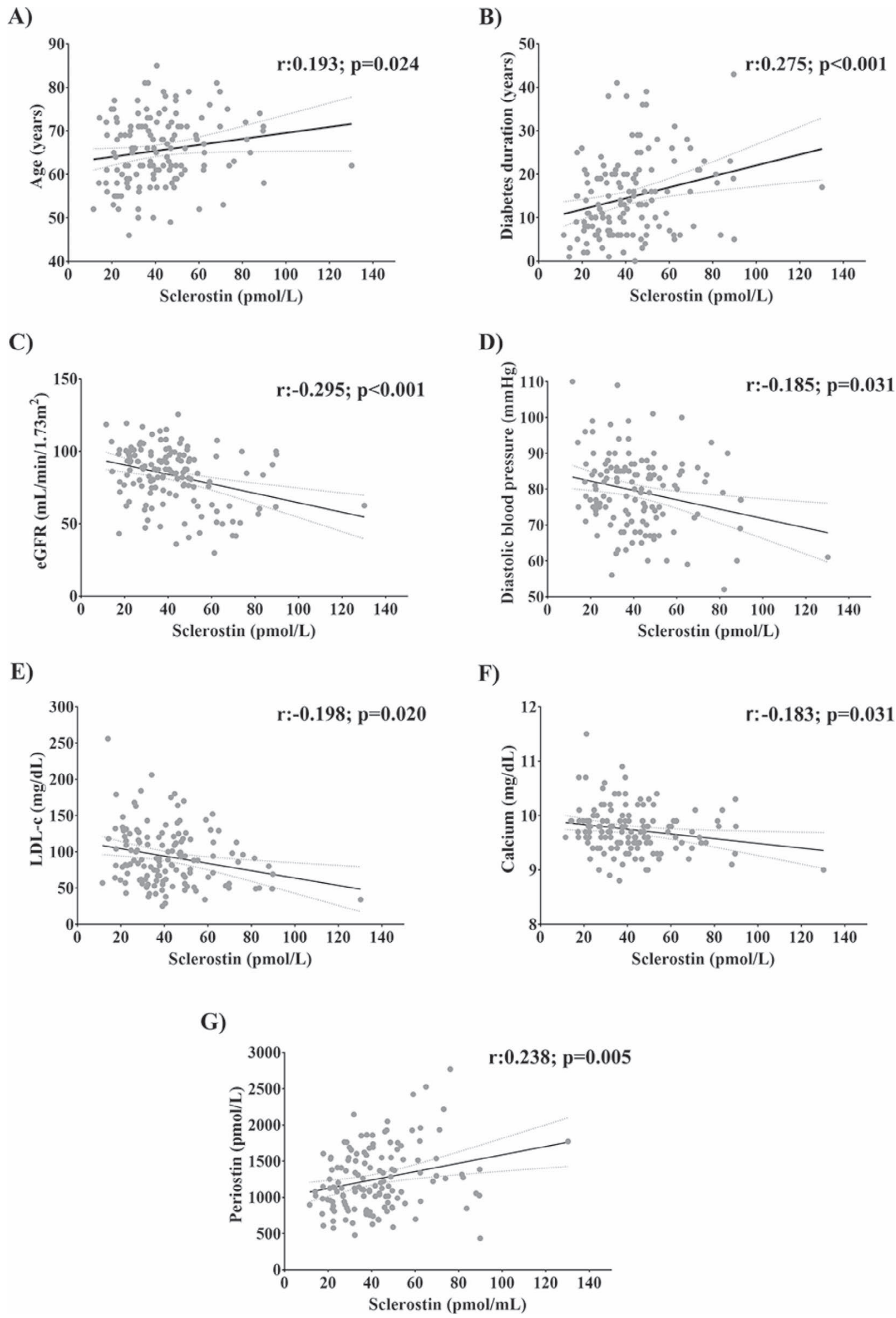


Fig. 2 Scatter plots showing the correlation between sclerostin (pmol/L) and: **(A)** age (years), **(B)** diabetes duration (years), **(C)** eGFR (mL/min/1.73m^2), **(D)** diastolic blood pressure (mmHg), **(E)** LDL-c (mg/dL), **(F)** calcium (mg/dL), and **(G)** periostin (pmol/L), in T2D patients (n = 139). The *p*-values between the different associations were performed by Spearman's correlation coefficients (showing $p < 0.05$ in each scatter plot). eGFR, estimated glomerular filtration rate; LDL-c, low density lipoprotein cholesterol; T2D, type 2 diabetes.

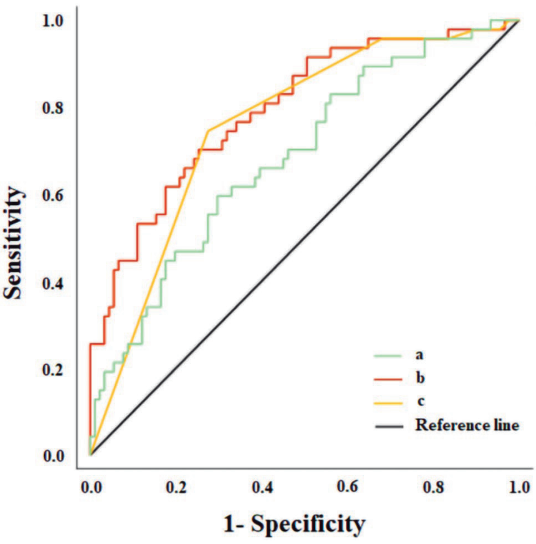


Fig. 3 ROC curve for the usefulness of serum sclerostin level as an estimator of CVD in T2D patients (n = 139). **(a)** Serum sclerostin levels; AUC = 0.685; $p = 0.003$. **(b)** Age, sex, hypertension, dyslipidaemia, eGFR, sedentarism, tobacco use and years of diabetes duration and serum sclerostin level; AUC = 0.795; $p < 0.001$. **(c)** Age, sex, hypertension, dyslipidaemia, eGFR, sedentarism, tobacco use and years of diabetes duration; AUC = 0.757; $p < 0.001$. The independent variables included in the models were the established cardiovascular risk in addition to sclerostin levels. The usefulness of serum sclerostin as an estimator of CVD risk was assessed using a ROC curve. The AUC indicates the probability to predict an event and the values greater than 0.75 indicate a good predictive performance. ROC, receiver operating curve; eGFR, estimated glomerular filtration rate; T2D, type 2 diabetes; AUC, area under the curve.

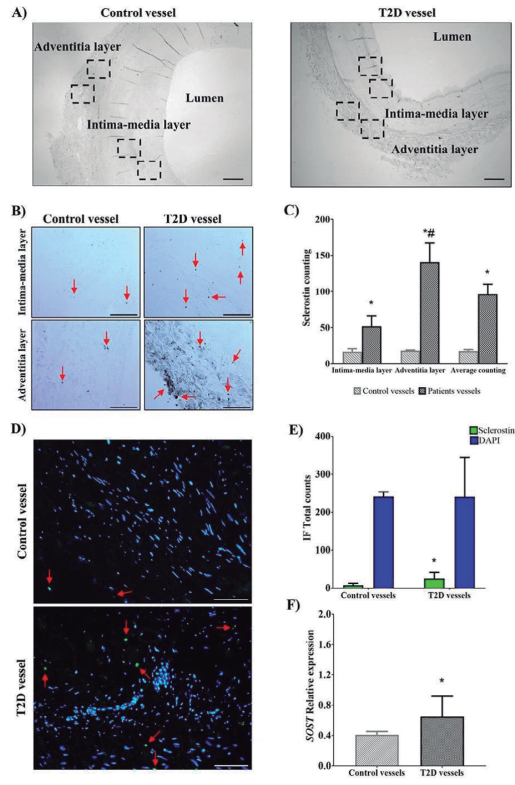


Fig. 4 Sclerostin expression at vascular tissue. **(A)** Schematics of the immunohistochemistry microphotographs (2X) of the control subject vessel (left) and T2D patient vessel (right). All the microphotographs were captured following a medio-lateral axis in order to cover the two layers of the artery. The dashed boxes represent the schematic location of the microphotographs captured for the intima-media and the adventitia layer. (**B**) Representative microphotographs obtained at 20X magnification of the artery for the intima-media layer (top panel) and the adventitia layer of the artery (bottom panel) for T2D and control groups. Scalebars: 100µm. The red arrows show examples of sclerostin location. (**C**) Sclerostin count in calcified lower limb arteries of T2D patients (n = 7) and non-calcified arteries of control subjects (n = 3) by immunohistochemistry. Data are presented as the mean ± standard error of the mean of sclerostin labeled proteins in the intima-media and the adventitia layer of the artery and the total average for both groups. The *p*-values were determined by the unpaired Student´s t-test. * = $p < 0.05$ *vs.* control subjects' vessels. # = $p < 0.05$ *vs.* intima-media layer of T2D patients' vessels. **(D)** Representative microphotographs of immunofluorescence of sclerostin (green color), counterstained with DAPI (blue color) of control subject vessel (left) and T2D patient vessel (right). The red arrows show examples of sclerostin location. **(E)** Quantification of the immunofluorescence images of calcified lower limb arteries of T2D patients (n = 7) and non-calcified arteries of control subjects (n = 3). Data are presented as the mean ± standard error of the mean of sclerostin labeled proteins and DAPI in the total count of both groups. The *p*-values were determined by the unpaired Student´s t-test. * = $p < 0.05$ *vs.* control subjects' vessels. **(F)** Sclerostin relative expression in both groups by RT-qPCR using the $2^{-\Delta\Delta Ct}$ method (fold-change). All data are presented as the mean ± standard error and Student´s t-test was used for the comparison. * = $p < 0.05$. T2D, type 2 diabetes; DAPI, 4′,6-diamidino-2-306 phenylindole; IF, immunofluorescence; *SOST*, sclerostin.

Effect of sclerostin overexpression on mechanisms involved in calcification in HAoSMCs

To investigate the impact of sclerostin on HAoSMCs in a calcified medium, we employed a second-generation lentiviral packaging system to establish stable sclerostin overexpression in vitro. The effectiveness of sclerostin overexpression was confirmed through RT-qPCR. Remarkably, HAoSMCs transduced with *SOST* gene exhibited a significant 12,370-fold increase in sclerostin mRNA levels compared to the mock group, indicating successful and robust sclerostin overexpression ($p < 0.001$) under the same experimental conditions.

Extra- and intracellular calcium and phosphate concentration were measured in HAoSMCs with sclerostin overexpression and mock under calcification conditions. The normalized results showed that the extracellular calcium and phosphate concentrations in both mock and HAoSMCs with sclerostin overexpression under non-calcifying conditions is significantly lower than those of the cultures under calcified conditions ($p < 0.05$). The calcium and phosphate concentrations were similar in both groups ensuring proper calcification of the medium

(Fig. 5AC). The results showed that HAoSMCs overexpressing sclerostin notably decrease the intracellular calcium concentration compared to mock under calcifying conditions (0.007±0.001 μg calcium/μg protein *vs.* 0.017±0.003 μg calcium/μg protein; $p<0.001$) revealing the calcification-inhibitory effect of sclerostin (Fig. 5B). However, no significant differences were observed in intracellular phosphate between mock and HAoSMCs with sclerostin overexpression under non-calcifying and calcifying conditions ($p>0.05$) (Fig. 5D).

On the other hand, calcium mineral deposition was assessed by Alizarin Red staining followed the protocol of Alizarin Red S Staining Quantification Assay (ScienCell) and normalized by total protein content. The results showed decreased mineral deposits in HAoSMCs overexpressing sclerostin compared to mock under calcification conditions (0.0005±0.000008 OD 405 nm/μg protein *vs.* 0.0012±0.00004 OD 405 nm/μg protein; $p<0.001$) (Fig. 5E), indicating the role of sclerostin in decreasing calcification.

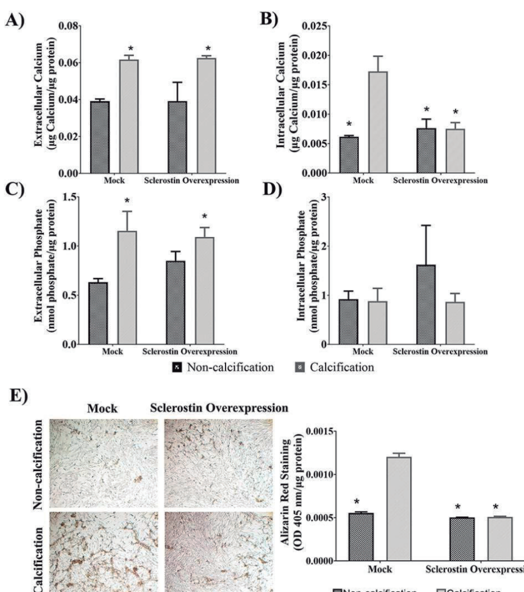


Fig. 5 Calcium and phosphate determinations under different calcifying conditions in HAoSMCs. **(A) (B)** Extracellular and intracellular calcium concentrations (μg calcium/μg protein), **(C) (D)** extracellular and intracellular phosphate (nmol phosphate/μg protein). All determinations were measured in mock and HAoSMCs overexpressing sclerostin, under non-calcifying and calcifying conditions. **(E)** Representative microphotographs obtained at 5X magnification of Alizarin Red staining of mock and HAoSMCs overexpressing sclerostin, under both non-calcifying (top) and calcifying (bottom) conditions and normalized by total protein content. Scalebars: 200 μm. Calcium mineral depositions were analyzed in the different conditions and results were expressed as the OD 405 nm/μg protein; n = 3 biological replicates/group and n = 3 technical replicates/ biological replicates were performed. Data are represented as the mean ± standard deviation. The *p*-values between groups were determined by the unpaired Student´s t-test. In the figure A and C * = $p<0.05$ *vs.* non-calcifying conditions. In figure B, * = $p<0.05$ *vs.* calcified mock. HAoSMCs, Primary Human Aortic Smooth Muscle Cells; OD, optical density.

The impact of sclerostin overexpression on the proliferation and apoptosis of HAoSMCs was assessed. The MTT assay was used to assess the effect of sclerostin overexpression on HAoSMCs' proliferation. The results demonstrated a significant decrease in the proliferation rate of HAoSMCs with sclerostin overexpression compared to the mock group. Specifically, at 6 days, there was a 12.5% reduction, at 8 days a 22.24% reduction, and at 10 days a 22.74% reduction ($p<0.001$ for all conditions) (Fig. 6A). Furthermore, apoptosis-induced cell death was analyzed using annexin V and propidium iodide staining, followed by flow cytometry. Notably, HAoSMCs with sclerostin overexpression exhibited a significantly lower percentage of apoptosis compared to the mock group (6.02 ± 0.32% *vs.* 6.8 ± 0.25%, $p=0.015$) (Fig. 6B).

Additionally, the impact of sclerostin overexpression on the regulation of genes involved in bone metabolism, inflammation and contractility was evaluated by qPCR. The analysis revealed that HAoSMCs with sclerostin overexpression exhibited up-regulation of genes such as alkaline phosphatase, biomineralization associated *(ALPL)* encoding for phosphatase alkaline (ALP) (2.5-fold; $p=0.009$), runt-related transcription factor 2 (*RUNX2*) (1.97-fold; $p=0.001$) and cyclooxygenase 2 *(COX2)* (1.78-fold; $p=0.003$), and down-regulation the genes such as interleukin 1 beta (*IL1β*) (0.43-fold; $p=0.005$), interleukin 6 (*IL6*) (0.15-fold; $p=0.001$) and interleukin 8 (*IL8*) (0.07-fold; $p=0.003$). No significant differences were observed in the expression of actin aortic smooth muscle (*ACTA2*) encoding for α-smooth muscle actin (αSMA) (0.92-fold; $p=0.218$) (Fig. 7). These findings provide valuable insights into the effects of sclerostin overexpression on HAoSMCs, indicating its role in inhibiting proliferation and promoting cell survival, as well as its potential influence on the regulation of genes associated with bone metabolism.

Discussion

Our study examines for the first time whether sclerostin, a bone formation inhibitor protein, plays a detrimental or protective role in the development of atherosclerotic process in T2D population. Firstly, this study showed increased serum sclerostin levels in T2D patients with CVD compared to healthy controls (especially in males). Furthermore, higher serum sclerostin levels were independently associated with CVD in patients with T2D and significant correlations were found between serum sclerostin levels and cardiovascular risk factors such as age, diabetes duration, eGFR, LDL-c, calcium, diastolic blood pressure and periostin. Secondly, increased

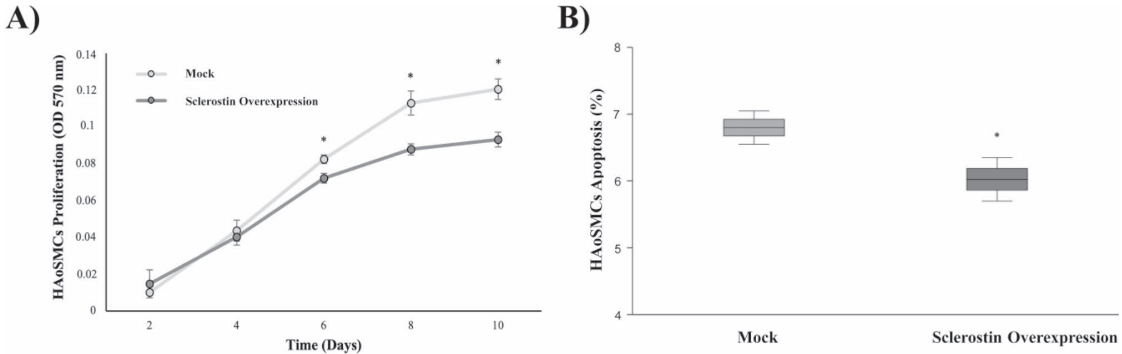


Fig. 6 Effect of sclerostin overexpression on proliferation and apoptosis in HAoSMCs. **(A)** Effect of sclerostin overexpression on proliferation in HAoSMCs compared to mock (n = 4 biological replicates in each time/group). Each result was expressed as the OD at 570 nm. **(B)** Percentage of apoptosis in HA-oSMCs with sclerostin overexpression compared to mock (n = 3 biological replicates/group and n = 2 technical replicates/ biological replicates). Data represent as the mean ± standard deviation of experiments performed. The *p*-values between groups were determined by the unpaired Student´s t-test. * = $p < 0.05$. HAoSMCs, Primary Human Aortic Smooth Muscle Cells; OD, optical density.

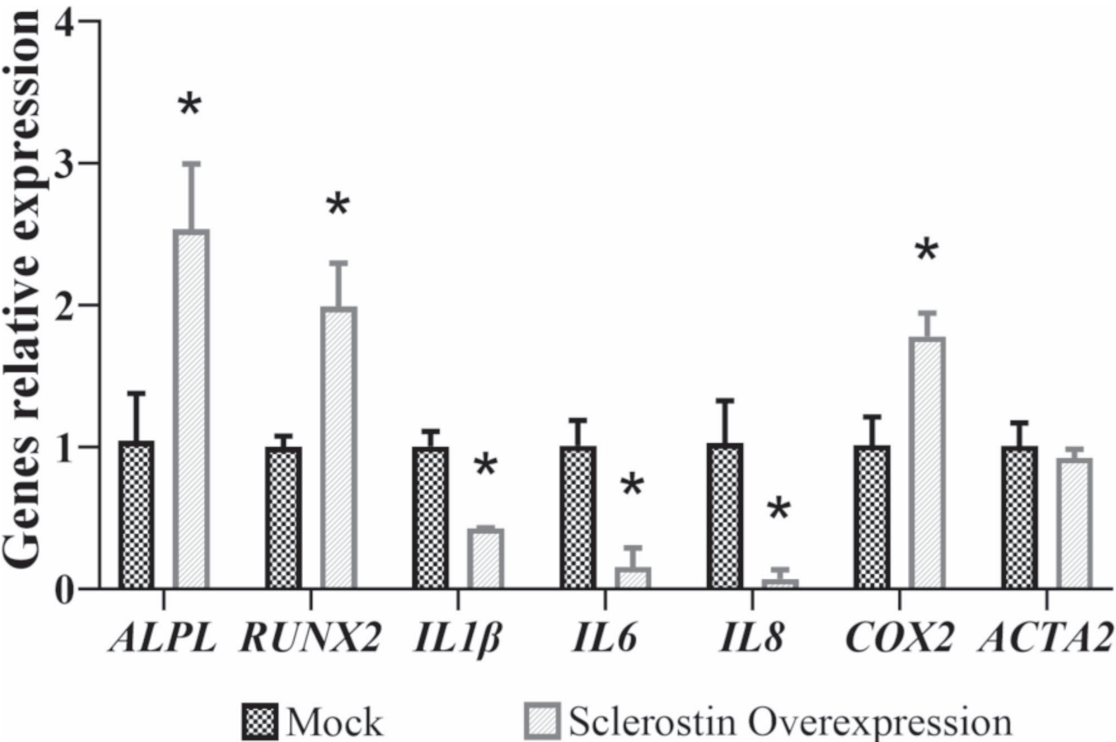


Fig. 7 Relative expression of genes involved in bone metabolism, inflammation and contractility evaluated by qPCR in calcified HAoSMCs overexpressing sclerostin *vs.* calcified mock. Relative expression of each gene of interest was assessed using the $2^{-\Delta\Delta Ct}$ method. Data are represented as the mean ± standard deviation. The *p*-values between groups were determined by the unpaired Student´s t-test. * = $p < 0.05$. *ALPL*, alkaline phosphatase, biomineralization associated; *RUNX2*, runt-related transcription factor 2; *IL1β*, interleukin 1 beta; *IL6*, interleukin 6; *IL8*, interleukin 8; *COX2*, cyclooxygenase 2; *ACTA2*, actin aortic smooth muscle.

sclerostin expression was observed in calcified lower limb arteries of T2D patients compared to non-calcified vessels of control subjects. Thirdly, we found that sclerostin overexpression in VSMCs, in vitro, is involved in the decrease of calcium deposits in a calcified environment, as well as, in cell survival and in the regulation of the expression of different bone markers such as *ALPL* and *RUNX2* and inflammatory genes such as *IL1β*, *IL6* and *IL8*. Therefore, these findings suggest that the sclerostin increase may have a protective role on atherosclerosis development in T2D population.

The increased serum sclerostin levels observed in T2D patients with CVD are consistent with previous studies suggesting the potential role of sclerostin in vascular pathology. Some studies reported a positive correlation between serum sclerostin level and subclinical atherosclerosis [3], atherosclerotic lesions [14, 30] and cardiovascular mortality [17] in T2D population regardless of sex and age. Circulating sclerostin levels are generally higher among men, a trend that has been demonstrated in our study, both in T2D patients and control subjects. These results are supported by several studies in T2D patients [14, 15, 17] and healthy subjects [15, 31]. Mödder *et al.* reported than the larger skeletal size in men may explain the gender differences in circulating sclerostin production and release [31]. Regarding to age and in accordance with previously reported [31, 32], we found a positive association between serum sclerostin level and age that may be due to the skeletal remodeling or to imbalances in vascular remodeling associated with aging [14]. Furthermore, consistent with previous studies [3, 14], we found a significant positive correlation between serum sclerostin level and the duration of diabetes. The up-regulation of sclerostin in T2D patients could be produced by the hyperglycemia that has both a direct effect on bone cells and indirect effects through the formation of advanced glycation end-products affecting bone [15].

Based in previous studies, cardiovascular and renal alterations are closely related in T2D patients [33]. Several studies have reported higher serum sclerostin level in chronic kidney disease (CKD) patients with cardiovascular events [34], such as coronary [35] and aortic calcification [36] and the effect of sclerostin levels on CVD and all-cause cardiovascular mortality in patients with CKD [16, 37]. Accordingly, our results have shown a negative correlation between serum sclerostin level and eGFR observing more impaired renal function in patients with CVD and higher sclerostin levels. By contrast, some studies have described a positive association between higher levels of circulating sclerostin and better cardiovascular survival in dialysis patients [38, 39]. This discrepancy may be due to differences in patient populations with regard to age, comorbidities, duration of time on dialysis, and variability in the duration of the observational period.

Although the elevation of serum sclerostin level associated with cardiovascular alterations has been widely described, the function of this protein at the vascular level has not been studied in depth to date. Our biochemical results suggest a potential beneficial role of sclerostin on CVD in T2D patients due to its inverse association with some cardiovascular risk factors such as LDL-c, calcium, and diastolic blood pressure, which are considered the main factors contributing to susceptibility to atherosclerosis. It has been shown that sclerostin is stimulated in endothelial cells by pro-atherosclerotic factors including hyperglycemia, which increases the susceptibility of LDL-c to oxidation [33]. In T2D patients, oxidized LDL-c is efficiently recognized by scavenger receptors on macrophages that have accumulated within arterial walls, leading to lipid accumulation in arterial wall macrophages promotes atherogenesis and vascular stiffening [30]. This suggests that sclerostin could reduce lipid accumulation through LDL-c, thus decreasing vascular damage. Regarding calcium, our results suggest a protective role of sclerostin by inhibiting vascular calcification, since the activation of the canonical Wnt/b-catenin pathway releases calcium, activating the Wnt/Ca^{2+} route necessary for inflammatory process [18, 40]. These findings imply that elevated sclerostin levels in individuals T2D and CVD could contribute to the decrease in atherosclerotic plaque formation through the reduction of LDL-c and calcium levels. Consequently, this mechanism could potentially lead to a decline in blood pressure as we have observed. To our knowledge, this study is the first report revealing an association between serum sclerostin level with calcium and diastolic blood pressure in patients with T2D. However, future studies are required to corroborate these results. In addition, we found a significant positive correlation between serum sclerostin level and periostin, an extracellular matrix protein that is considered a biomarker for CVD [41] and it is involved in cardiac remodeling [42]. Some studies have shown an increase in serum periostin levels in diabetic vascular complications [42, 43]. The positive association observed between serum levels of periostin and sclerostin suggests that both proteins could play a protective role against the development of cardiovascular damage.

Our results showed that the serum sclerostin level was an independent estimator of CVD risk in T2D patients. In this context, the literature has reported a positive association between circulating sclerostin levels and CVD [44], and indicates that sclerostin is a predictive marker of these pathologies [45]. Based on this, our ROC curve analysis reveals that the inclusion of serum sclerostin level , in addition to variables related to CVD development, improved the CVD risk prediction model.

Considering these finding and the fact that sclerostin acts as an inhibitor of bone formation, it is suggested that the increase in serum levels of this protein in patients who develop atherosclerotic processes could be a compensatory mechanism to block or attenuate the canonical Wnt/b-catenin pathway, with the aim of slowing vascular calcification. Although, studies have been carried out to elucidate the protective or pathological role of sclerostin at the vascular level in humans, all of them are merely observational. In this context, vascular effect of sclerostin has recently been of concern following the development of a new anti-osteoporotic treatment based on the monoclonal anti-sclerostin antibody, which simultaneously increases bone formation and, to a lesser extent, decreases bone resorption [18, 24]. This drug has shown a remarkable increase in bone formation and a reduction of fractures, although there are concerns regarding the degree of cardiovascular safety. A systematic review and meta-analysis report that treatment with anti-sclerostin antibody does not significantly increase the risk of composite cardiovascular outcomes [46]. However, significant cardiovascular adverse effects were reported during one study with anti-sclerostin antibody [23]. Moreover, the Active-Controlled Fracture Study in Postmenopausal Women With Osteoporosis at High Risk (ARCH) study, revealed an increased risk of serious adverse cardiovascular events in postmenopausal women during the first year treatment with anti-sclerostin antibody [25]. Currently, the existing data so far backs the notion of limiting the prescription guidelines outlined in the data sheet, which suggests that patients with a high risk of cardiovascular disease and stroke should not be eligible for treatment with anti-sclerostin antibody. Consistently, a study in animal models had described the potential protective role of sclerostin in vascular calcification. De Maré *et al.* showed evidence for a protective role of sclerostin during the development of vascular calcification by examining sclerostin expression in a mouse model of warfarin-induced vascular calcification. Serum levels and aortic expression of sclerostin were up-regulated in response to warfarin administration and increased vascular calcification was observed when warfarin was combined with anti-sclerostin antibody treatment [21].

Despite the clinical importance of sclerostin in vascular calcification, the precise biochemical processes that regulate this protein in this pathological process are not yet fully understood. Thus, it is necessary to develop experimental studies focused on discerning the potential protective role of sclerostin at vascular level. An essential step during the development of vascular calcification is the trans-differentiation of VSMCs to osteocyte-like cells capable of expressing typical osteocyte markers, including sclerostin [7]. The present study first established a cell model stably overexpressing sclerostin in HAoSMCs using a lentivirus system. This novel cell model offers a valuable tool for studying the role of sclerostin in VSMCs under calcification conditions.

Our study demonstrates that sclerostin overexpression leads to a reduction in intracellular calcium levels in HAoSMCs under a calcified environment. Intracellular calcium is required for the Wnt/Ca^{2+} pathway activated by Wnt5 and involved in endothelial inflammatory regulation [18, 40]. Notably, Wnt5 is expressed in human inflammatory diseases, including atherosclerotic plaques, and is expressed in VSMCs, supporting a pathophysiological role of Wnt5 in inflammatory regulation [40]. Moreover, in pulmonary arterial smooth muscle cells, an increase in the cytosolic calcium concentration is involved in physiological processes such as cell proliferation [47]. VSMCs switch from the contractile to the synthetic phenotype, facilitating proliferation as a physiological response induced by proinflammatory stimuli and oxidative stress for repair vascular damage in atherosclerosis [5, 6]. Increased calcium uptake has been shown to the phenotype switch to synthetic VSMCs and the development of vascular calcification [48]. Therefore, it is plausible to propose that the overexpression of sclerostin could play a role in decreasing the inflammatory response and inhibiting the proliferation of HAoSMCs, consequently leading to a reduction in atherosclerotic plaque formation. In this line, our results revealed a significant down-regulation in proinflammatory cytokines, such as *IL1β*, *IL6* and *IL8* in HAoSMCs overexpressing sclerostin compared to mock, suggesting a potential inhibitory role of sclerostin in inflammatory process in VSMCs. In addition, IL8 participates in the recruitment of neutrophils that adhere to and infiltrate the endothelial wall, favoring arterial stiffness [49]. Therefore, overexpression of sclerostin, in addition to reducing inflammation, would favor arterial elasticity via inhibition of these cytokines. In addition, quantitative alizarin red staining confirms that sclerostin overexpression reduces calcium deposition. We found less calcium deposition when sclerostin is overexpressed in HAoSMCs suggesting that this protein is acting as an inhibitor of calcification development. In this context, the scientific evidence has reported a role of COX2 in vascular calcification; however, there is controversy on its function with studies in opposite directions [50, 51]. The observed increased expression of *COX2* related to sclerostin overexpression showed in our results could have a protective role on vascular calcification supporting the beneficial function of sclerostin at vascular level. Agreeing, Cheng Gao *et al.*, reported that COX2 may decrease the abnormal vascular calcification in humans [51]. These results support our hypothesis about the protective role of sclerostin in the atherosclerotic process.

Furthermore, our study reveals that sclerostin overexpression plays a role in regulating proliferation and apoptosis in HAoSMCs in calcified environment. The switch from contractile to synthetic phenotype promoting VMSCs proliferation has been identified as a crucial factor in the development of atherosclerotic plaque [52]. In response to vascular injury, VSMCs have been observed to significantly increase its rate of cell proliferation [5, 48], which implies an increase in collagen synthesis, further contributing to artery stiffening [30] and narrowing. While the proliferation rate of VSMCs may be elevated during the initial stages of lesion formation, it is not high in advanced mature lesion [48]. Specifically, we observed a decreased proliferation rate in HAoSMCs overexpressing sclerostin in a calcified medium. This reduction in proliferation rate could be attributed to the inhibitory effect of sclerostin on calcification, decreasing the intracellular calcium concentration, resulting in decreased proliferation, and consequently slowing the development of atherosclerotic plaque. Additionally, it is possible that the reduced proliferation in sclerostin overexpressing in HAoSMCs is influenced by the energy expenditure associated with the process of protein overexpression. On the other hand, there is extensive evidence that apoptosis of VSMCs can promote vascular calcification [53]. *In vitro*, apoptosis takes place before calcification occurs, and it is believed that apoptotic bodies contain elevated levels of calcium. These calcium-rich apoptotic bodies are subsequently deposited on the extracellular matrix, leading to the process of calcification. Furthermore, it has been shown that inhibition of apoptosis, for example by caspase inhibitors, significantly decrease both calcifying vesicle release and calcification [52]. Our results showed lower percentage of apoptosis in HAoSMCs with sclerostin overexpression. This finding suggests that the up-regulation of sclerostin could have a protective effect on HAoSMCs by reducing the formation of apoptotic calcium bodies, cell apoptosis and ultimately the calcification process. This observation aligns with the potential role of sclerostin in inhibiting calcification and promoting cell survival. These results provide evidence of the protective role of sclerostin in the development of CVD in the T2D population and confirm what was observed at both serum and tissue levels in these patients in our study and in previously described studies [3, 14].

This study has demonstrated the up-regulation of genes involved in bone formation, specifically *ALPL* and *RUNX2*, associated to sclerostin overexpression. ALP plays a crucial role in the production of inorganic phosphate, a significant molecule involved in calcification [6]. The induction of ALP in VSMCs implies an irreversible transformation towards calcified vascular cells [6]. As for *RUNX2*, a central transcriptional factor, is expressed by VSMCs to drive calcification [52]. It has been observed that cells expressing sclerostin also coexpress RUNX2 in calcified aortic valves [54]. Furthermore, it is noteworthy that the mineralization of VSMCs was associated by the up-regulation of key calcification genes, including *ALPL* and *RUNX2* [6]. Hence, we suggest that the up-regulation of *ALPL* and *RUNX2* could act as a compensatory mechanism in response to sclerostin overexpression that promotes inhibition of vascular calcification. Further research is necessary to elucidate the role of sclerostin in the up-regulation of genes involved in bone metabolism under calcified conditions.

At vascular level, our results revealed a significant increased expression of sclerostin in calcified artery of T2D patients both in the intima-media and adventitia layers compared to non-calcified vessels. *In vitro* results indicating that overexpression of sclerostin in calcified HAoSMCs leads to decreased intracellular calcium levels, calcium deposition, proliferation, and apoptosis, suggest that sclerostin plays a role in the intima-media layer of calcified artery of T2D patients reducing atherosclerotic plaque formation. Moreover, the increased sclerostin expression showed in the adventitia layer could be partly explained due to the expression of sclerostin in VSMCs from the intima-media layer could be transitioning to the adventitia layer to suppress the Wnt/b-catenin pathway limiting wall inflammation by decreasing inflammatory cytokines along with macrophage reduction [55] in the adventitia layer [56], thereby reducing arterial stiffness. However, future studies are required to elucidate the effect of increased sclerostin in the adventitial layer, as this is the first time that the increase of sclerostin has been described specifically in the adventitia layer of human arteries. These finding suggest that the elevation of serum sclerostin levels in T2D patients with CVD could be due to the increase of sclerostin at vascular level. According to our results sclerostin has been detected in the aorta of patients undergoing aortic valve replacement and is up-regulated in calcifying VSMCs and calcified valvular plaques compared non-calcified control valves [54]. Recently, sclerostin was identified in the media layer of VSMCs in plaques isolated from carotid arteries in subjects affected by severe vascular disease, irrespective of history of T2D [20].

These results at vascular levels, in conjunction with the outcomes derived from this research, indicate a potential protective function of sclerostin in the context of vascular calcification. However, it is important to note that further experimental studies are needed to validate this hypothesis in humans.

Our study presents some limitations. First, the cross-sectional design does not allow establishment of a cause-effect relationship. Moreover, our study population included only Caucasian individuals, from a specific area, and the use of common antihypertensive,

antihyperlipidemic and antidiabetic drugs in patients may have influenced the results. Second, the number of vascular tissue samples from both controls and T2D patients with CVD is very small due to the difficulty of obtaining such samples, mainly for healthy controls, so these results should be interpreted with caution, therefore future investigations are necessary. However, our work has several strengths. Sclerostin has been evaluated both at the clinical level (serum and vascular tissue) and at the basic level (in vitro in VSMCs) in the same study establishing for the first time a cellular model that stably overexpressing sclerostin in HAoSMCs. Our cross-sectional study presents an exhaustive evaluation of clinical, anthropometric, and biochemical parameters, integrating all variables that could influence cardiovascular risk with experimental results. In addition, we performed rigorous statistical analyses, in order to obtain reliable results.

Conclusions

We provide evidence supporting the protective role of sclerostin in the development of vascular calcification by reducing calcium deposition, decreasing proliferation and inflammation, and promoting cell survival associated with sclerostin overexpression. This suggest that sclerostin may mitigate the susceptibility to atherosclerosis by decreasing atherosclerotic plaque related to improved cardiovascular risk factors such as LDL-c, calcium, and diastolic blood pressure. These findings, both basic and clinical, contribute to the current understanding of the shared mechanisms between systemic bone and vascular physiology and pathology. Thus, our results emphasize the importance of considering the bone-vascular axis when designing therapeutic approaches for the treatment of impaired bone metabolism or vascular diseases.

Abbreviations

ACTA2	Actin, aortic smooth muscle
ALP	Alkaline phosphatase
ALPL	Alkaline phosphatase, biomineralization associated
BMI	Body mass index
CKD	Chronic kidney disease
COX2	Cyclooxygenase 2
CVD	Cardiovascular disease
eGFR	Estimated glomerular filtration rate
FPG	Fasting plasma glucose
HAoSMCs	Human primary aortic smooth muscle cells
HbA1c	Glycated haemoglobin
HDL-c	High-density lipoprotein cholesterol
HEK293T	Human embryonic kidney 293T cells
IL1β	Interleukin 1 beta
IL6	Interleukin 6
IL8	Interleukin 8
LDL-c	Lipoprotein cholesterol
RPL13	Ribosomal protein L13
RUNX2	Runt-related transcription factor 2
SOST	Sclerostin
T2D	Type 2 diabetes
TG	Triglycerides
VSMCs	Vascular smooth muscle cells

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Author contributions
Conceptualization, M.M.-T. and B.G.-F.; methodology, S.G.-S., L.M.-H., C.G.-F., and B.G-F.; formal analysis, B.G.-F., S.G.-S., F.A.-V., E.M.-A., M.F.-M., and L.M.-H.; investigation, J.L., R.S.-dlT., C.G.-F., B.G.-F., and S.G.-S.; writing—original draft preparation, S.G.-S., B.G.-F., and M.M.-T.; writing—review and editing, S.G.-S., B.G.-F., M.M.-T., C.G.-F., J.L., and F.A.-V.; funding acquisition, B.G.-F., and M.M.-T. All authors revised the manuscript for intellectual content and approved the final version.

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Data Availability
The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
This study was conducted with the approval of the Ethics Committee of the University Hospital Clinico San Cecilio of Granada and conformed to the principles of the World Medical Association Declaration of Helsinki (Project ID:0858-N-17, Research Ethics Committee of Granada Center (CEI-Granada) on 26 April 2017). Informed consent was obtained from all subjects involved in the study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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CONCLUSIONS

1. T2D patients with normal eGFR show lower circulating osteoglycin levels than T2D patients with mild or moderate impaired eGFR, independent of sex and age. The serum osteoglycin levels are independently associated with mildly impaired kidney function in T2D patients. This suggests that circulating osteoglycin may be a biomarker of incipient impairment of kidney function, independently of the presence of albuminuria in T2D patients.

2. T2D patients show increased serum osteoglycin levels compared with nondiabetic controls. This finding suggests that osteoglycin may play a key role in glycemic homeostasis and could be a potential biomarker of insulin resistance in T2D patients.

3. Osteoglycin does not appear to be a key determinant in the pathogenesis of atherosclerosis in T2D patients directly, although it could participate indirectly in the development of atherosclerotic process through activation of the ATX/LPA pathway and the proliferation of VSMCs in the T2D population. This opens the door to the study of osteoglycin as a potential therapeutic target in T2D.

4. T2D patients with CVD show an increase in serum sclerostin levels compared to healthy controls, especially in males. Furthermore, higher serum sclerostin levels are independently associated with CVD in patients with T2D and significant correlations are found between serum sclerostin levels and cardiovascular risk factors (age, duration of diabetes, eGFR, LDL-c, serum calcium, diastolic blood pressure) and periostin, suggesting that elevated serum sclerostin levels appear to be associated with a better cardiometabolic profile.

5. Calcified artery of T2D patients shows higher sclerostin expression both in the intima-media and adventitia layers than non-calcified vessels. This finding suggests that sclerostin could play a role in the pathogenesis of atherosclerotic process in T2D patients with CVD.

6. Sclerostin could play a protective role in the development of vascular calcification by reducing calcium deposition, decreasing inflammation, and reducing proliferation and apoptosis of VSMCs. These findings contribute to the understanding of the common mechanisms between systemic bone and vascular physiology and pathology. Therefore, it is necessary to emphasize the importance of considering the

bone-vascular axis when designing therapeutic approaches for the treatment of impaired bone metabolism or vascular diseases.

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PRODUCCIÓN CIENTÍFICA

La producción científica generada durante esta etapa de investigación ha sido difundida mediante su publicación en distintas revistas científicas indexadas, y/o ha sido presentada en diversas comunicaciones a congresos, como se detalla a continuación:

Artículos derivados de esta Tesis Doctoral:

1. *Osteoglycin as a Potential Biomarker of Mild Kidney Function Impairment in Type 2 Diabetes Patients.* Sheila González-Salvatierra; Cristina García-Fontana; Francisco Andújar-Vera; Alejandro Borja Grau-Perales; Luis Martínez-Heredia; María Dolores Avilés-Pérez; María Hayón-Ponce; Iván Iglesias-Baena; Blanca Riquelme-Gallego; Manuel Muñoz-Torres; Beatriz García-Fontana. *J Clin. Med. 2021 May* 20; 10(10):2209. doi: 10.3390/jcm10102209.

2. *Exploring the role of osteoglycin in type 2 diabetes: implications for insulin resistance and vascular pathophysiology.* Sheila González-Salvatierra; Beatriz García-Fontana; Luis Martínez-Heredia; Jesus Lacal; Francisco Andújar-Vera; Raquel Sanabria-de la Torre; Enrique Moratalla-Aranda; Silvia Lozano-Alonso; Cristina García-Fontana; Manuel Muñoz-Torres. *Am J Physiol Endocrinol Metab. 2023 Nov 1;* 325(5):E649-E660. doi: 10.1152/ajpendo.00320.2023.

3. *Cardioprotective function of sclerostin by reducing calcium deposition, proliferation, and apoptosis in human vascular smooth muscle cells.* Sheila González-Salvatierra; Cristina García-Fontana; Jesus Lacal; Francisco Andújar-Vera; Luis Martínez-Heredia; Raquel Sanabria-de la Torre; María Ferrer-Millán; Enrique Moratalla-Aranda; Manuel Muñoz-Torres; Beatriz García-Fontana. *Cardiovasc Diabetol. 2023 Nov 2;* 22(1):301. doi: 10.1186/s12933-023-02043-8.

Otros artículos

4. *Systemic effects of hypophosphatasia. characterization of two novel variants in the ALPL gene.* Luis Martínez-Heredia; Manuel Muñoz-Torres; Raquel Sanabria-de La Torre; Ángela Jiménez-Orta; Francisco Andújar-Vera; Trinidad González-Cejudo; Victoria Contreras-Bolívar; Sheila González-Salvatierra; José María Gómez-Vida; Cristina García-Fontana; Beatriz García-Fontana. *Frontiers in Endocrinology. Accepted: 2023 Dec 01.*

5. *Mild hypophosphatasia may be twice as prevalent as previously estimated: an effective clinical algorithm to detect undiagnosed cases.* Trinidad González-Cejudo; Juan M Villa-Suárez; María Ferrer-Millán; Francisco Andújar-Vera; Victoria Contreras-Bolívar; María C Andreo-López; José M Gómez-Vida; Luis Martínez-Heredia; Sheila González-Salvatierra; Tomás de Haro Muñoz; Cristina García-Fontana; Manuel Muñoz-Torres; Beatriz García-Fontana. *Clin Chem Lab Med.* doi: 10.1515/cclm-2023-0427.

6. *Analysis of the genetic relationship between atherosclerosis and non-alcoholic fatty liver disease through biological interaction networks.* Francisco Andújar-Vera; María Ferrer-Millán; Cristina García-Fontana; Beatriz García-Fontana; Sheila González-Salvatierra;

Raquel Sanabria-de la Torre; Luis Martínez-Heredia; Blanca Riquelme-Gallego; Manuel Muñoz-Torres. *Int J Mol Sci. 2023 Feb 18;*24(4):4124. doi: 10.3390/ijms24044124.

7. *Do patients with type 2 diabetes have impaired hip bone microstructure? A study using 3D modeling of hip dual-energy X-ray absorptiometry.* Esther Ubago-Guisado; Enrique Moratalla-Aranda; Sheila González-Salvatierra; José J Gil-Cosano; Beatriz García-Fontana; Cristina García-Fontana; Luis Gracia-Marco; Manuel Muñoz-Torres M. *Front Endocrinol (Lausanne). 2023 Jan 9;* 13:1069224. doi: 10.3389/fendo.2022.1069224.

8. *Exploring the Role of Sclerostin as a Biomarker of Cardiovascular Disease and Mortality: A Scoping Review* Raquel Sanabria-de la Torre; Sheila González-Salvatierra; Cristina García-Fontana; Francisco Andújar-Vera; Beatriz García-Fontana; Manuel Muñoz-Torres; Blanca Riquelme-Gallego. *INT J Environ Res Public Health. 2022 Nov 30.* doi: 10.3390/ijerph192315981.

9. *Undercarboxylated osteocalcin: a promising target for early diagnosis of cardiovascular and glycemic disorders in patients with metabolic syndrome: a pilot study.* Blanca Riquelme-Gallego; Laura García-Molina; Naomi Cano-Ibáñez, Francisco Andújar-Vera, Sheila González-Salvatierra; Cristina García-Fontana; Aurora Bueno-Cavanillas; Manuel Muñoz-Torres; Beatriz García-Fontana. *Nutrients 2022 Jul 21*;14(14):2991. doi: 10.3390/nu14142991.

10. *The contribution of Wnt signaling to vascular complications in type 2 diabetes mellitus.* Raquel Sanabria-de la Torre; Cristina García-Fontana; Sheila González-Salvatierra; Francisco Andújar-Vera; Luis Martínez-Heredia; Beatriz García Fontana; Manuel Muñoz-Torres. *Review Int J Mol Sci. 2022 Jun 23;*23(13):6995. doi: 10.3390/ijms23136995.

11. *Characterization of genetic variants of uncertain significance for the ALPL gene in patients with adult hypophosphatasia.* Raquel Sanabria-de la Torre; Luis Martínez-Heredia; Sheila González-Salvatierra; Francisco Andújar-Vera; Iván Iglesias-Baena, Juan Miguel Villa-Suárez; Victoria Contreras-Bolívar; Mario Corbacho-Soto; Gonzalo Martínez-Navajas; Pedro Real-Luna; Cristina García-Fontana; Manuel Muñoz-Torres; Beatriz García-Fontana. *Front Endocrinol (Lausanne). 2022 Apr 14:* 13:863940. doi: 10.3389/fendo.2022.863940.

12. *Identification of potential targets linked to the cardiovascular/alzheimer's axis through bioinformatics approaches.* Francisco Andújar-Vera; Cristina García-Fontana; Raquel Sanabria-de la Torre; Sheila González-Salvatierra; Luis Martínez-Heredia; Iván Iglesias-Baena; Manuel Muñoz-Torres; Beatriz García-Fontana. *Biomedicines. 2022 Feb 6*;10(2):389. doi: 10.3390/biomedicines10020389.

13. *Lower trabecular bone score in type 2 diabetes mellitus: A role for fat mass and insulin resistance beyond hyperglycaemia.* María Hayón-Ponce; Beatriz García-Fontana; María Dolores Avilés-Pérez; Sheila González-Salvatierra; Francisco Andújar-Vera; Enrique Moratalla-Aranda; Manuel Muñoz-Torres. *Diabetes Metab. 2021 Nov;* 47(6):101276. doi: 10.1016/j.diabet.2021.101276.

14. *Hypophosphatasia: a unique disorder of bone mineralization.* Juan M Villa-Suárez; Cristina García-Fontana; Francisco Andújar-Vera; Sheila González-Salvatierra; Tomás de Haro-Muñoz; Victoria Contreras-Bolívar; Beatriz García-Fontana; Manuel Muñoz-Torres. *Int J Mol Sci. 2021 Apr 21*;22(9):4303. doi: 10.3390/ijms22094303.

15. *3D DXA hip differences in patients with acromegaly or adult growth hormone deficiency.* Luis Gracia-Marco; Sheila González-Salvatierra; Antonia García-Martin; Esther Ubago-Guisado; Beatriz García-Fontana; José Juan Gil-Cosano; Manuel Muñoz-Torres. *J Clin Med. 2021 Feb 9;* 10(4):657. doi: 10.3390/jcm10040657.

16. *Association between oxidative-stress-related markers and calcified femoral artery in type 2 diabetes patients.* Francisco Andújar-Vera, Cristina García-Fontana, Silvia Lozano-Alonso, Sheila González-Salvatierra, Iván Iglesias-Baena, Manuel Muñoz-Torres, Beatriz García-Fontana. *J Pharm Biomed Anal. 2020 Aug 13*; 190:113535. doi: 10.1016/j.jpba.2020.113535.

17. *Circulating undercarboxylated osteocalcin as estimator of cardiovascular and type 2 diabetes risk in metabolic syndrome patients.* Blanca Riquelme-Gallego, Laura García-Molina, Naomi Cano-Ibáñez, Guillermo Sánchez-Delgado, Francisco Andújar-Vera, Cristina García-Fontana, Sheila González-Salvatierra, Enrique García-Recio, Virginia Martínez-Ruiz, Aurora Bueno-Cavanillas, Manuel Muñoz-Torres, Beatriz García-Fontana. *Sci Rep. 2020 Feb 4*; 10(1):1840. doi: 10.1038/s41598-020-58760-7.

18. *Epidemiological, clinical and genetic study of hypophosphatasia in a spanish population: identification of two novel mutations in the ALPL gene.* Cristina García-Fontana, Juan M Villa-Suárez; Francisco Andújar-Vera; Sheila González-Salvatierra; Gonzalo Martínez-Navajas; Pedro Real; José M Gómez-Vida; Tomás de Haro; Beatriz García-Fontana; Manuel Muñoz-Torres. *Sci Rep. 2019 Jul 2*; 9(1):9569. doi: 10.1038/s41598-019-46004-2.

Comunicaciones a congresos nacionales e internacionales

1. Cristina García Fontana; Sheila González Salvatierra; María Ferrer Millán; Francisco Andújar Vera; Luis Martínez Heredia; Raquel Sanabria de la Torre; Enrique Moratalla Aranda; Manuel Muñoz Torres; Beatriz García Fontana. Comunicación Póster: Función cardioprotectora de esclerostina mediante la reducción de los depósitos de calcio, la proliferación y la apoptosis en células de músculo liso vascular humanas. XXVII Congreso de la Sociedad Española de Investigación Ósea y Metabolismo Mineral (SEIOMM). Sevilla, España; 22-24 noviembre 2023.

2. Beatriz García Fontana; Esther Ubago Guisado; Enrique Moratalla Aranda; Sheila González Salvatierra; José Juan Gil Cosano; Cristina García Fontana; Mirella López Picazo; Ludovic Humbert; Luis Gracia Marco;Manuel Muñoz Torres. Comunicación Póster: *Impaired Cortical thickness at the femoral neck by 3D-DXA in type 2 diabetes patients.* XXVII Congreso de la Sociedad Española de Investigación Ósea y Metabolismo Mineral (SEIOMM). Sevilla, España; 22-24 noviembre 2023.

3. Sheila González Salvatierra; Beatriz García Fontana; Cristina García Fontana; Jesus Lacal; María FerrerMillán; Francisco Andújar Vera; Luis Martínez Heredia; Raquel Sanabria de la Torre; Enrique Moratalla Aranda; Manuel Muñoz Torres. Comunicación Oral Premium: Papel protector de la esclerostina en aterosclerosis en pacientes con diabetes tipo 2. 64 Congreso de la Sociedad Española de Endocrinología y Nutrición (SEEN). Barcelona, España; 18-20 octubre 2023.

4. Cristina García Fontana; Francisco Andújar Vera; Sheila González Salvatierra; Luis Martínez Heredia;Beatriz García Fontana; Manuel Muñoz Torres. Comunicación Póster: Estudio de redes de interacción biológica para la identificación de las vías de conexión entre enfermedad de hígado graso no alcohólico y aterosclerosis. 64 Congreso de la Sociedad Española de Endocrinología y Nutrición (SEEN). Barcelona, España; 18-20 octubre 2023.

5. Sheila González Salvatierra; María Ferrer Millán; Raquel Sanabria de la Torre; Luis Martínez Heredia; Francisco Andújar Vera; Jesús Lacal Romero; Beatriz García Fontana; Manuel Muñoz Torres; Cristina García Fontana. Comunicación Póster: Evaluación de la función de osteoglicina en células renales. 63 Congreso de la Sociedad Española de Endocrinología y Nutrición (SEEN). Las palmas de Gran Canaria, España; 26-28 octubre 2022.

6. Cristina García Fontana; Francisco Andújar Vera; Raquel Sanabria de la Torre; Sheila González Salvatierra;Luis Martínez Heredia; Iván Iglesias-Baena; Nuria Cabrera-Gómez; Manuel Muñoz Torres; Beatriz García Fontana. Comunicación Póster: Identificación de factores comunes implicados en enfermedad cardiovascular y enfermedad de alzheimer. 63 Congreso de la Sociedad Española de Endocrinología y Nutrición (SEEN). Las palmas de Gran Canaria, España; 26-28 octubre 2022.

7. Sheila González Salvatierra; María Ferrer Millán; Raquel Sanabria de la Torre; Luis Martínez Heredia; Francisco Andújar Vera; Jesús Lacal Romero; Cristina García Fontana; Manuel Muñoz Torres; Beatriz García Fontana. Comunicación Póster: Evaluación de la función de esclerostina en células renales. XXVI Congreso de la Sociedad Española de Investigación Ósea y Metabolismo Mineral (SEIOMM). Madrid, España; 05-07 octubre 2022.

8. Cristina García Fontana; Nuria Cabrera Gómez; Luis Martínez Heredia; Francisco Andújar Vera; Raquel Sanabria de la Torre; Sheila González Salvatierra; Manuel Muñoz Torres; Beatriz García Fontana. Comunicación Póster: Identificación de potenciales biomarcadores asociados a enfermedad cardiovascular subclínica en pacientes con diabetes mellitus tipo 2. XXVI Congreso de la Sociedad Española de Investigación Ósea y Metabolismo Mineral (SEIOMM). Madrid, España; 05-07 octubre 2022.

9. Cristina García Fontana; Francisco Andújar Vera; Raquel Sanabria de la Torre; Sheila González Salvatierra;Luis Martínez Heredia; Iván Iglesias Baena; Nuria Cabrera Gómez; Manuel Muñoz Torres; Beatriz García Fontana. Comunicación Póster:

Identificación de potenciales biomarcadores comunes en enfermedad cardiovascular yenfermedad de alzheimer mediante herramientas bioinformáticas. XXVI Congreso de la Sociedad Española de Investigación Ósea y Metabolismo Mineral (SEIOMM). Madrid, España; 05-07 octubre 2022.

10. Sheila González Salvatierra; Cristina García Fontana; Francisco Andújar Vera; Luis Martínez Heredia;Manuel Muñoz Torres; Beatriz García Fontana. Comunicación Póster: Osteoglicina como potencial biomarcador del deterioro leve de la función renal en pacientes con diabetes tipo 2. III Congreso Nacional-V Jornadas de Investigadores/as en Formación. Granada, España; 22- 24 junio 2022.

11. Sheila González Salvatierra; Beatriz García Fontana; Francisco Andújar Vera; Luis Martínez Heredia;Raquel Sanabria de la Torre; María Dolores Avilés Pérez; María Hayón Ponce; Manuel Muñoz Torres; Cristina García Fontana. Comunicación Póster: Osteoglicina como biomarcador de enfermedad renal diabética temprana. II Congreso de Investigación PTS. Granada, España; 09- 11 febrero 2022.

12. Luis Martínez Heredia; Francisco Andújar Vera; Raquel Sanabria de la Torre; Sheila González Salvatierra;Manuel Muñoz Torres; Beatriz García Fontana; Cristina García Fontana. Comunicación Póster: Validación de *miRNAs* como posibles biomarcadores no invasivos para patologías asociadas a la diabetes mellitus tipo 2. II Congreso de Investigación PTS. Granada, España; 09- 11 febrero 2022.

13. Sheila González Salvatierra; Cristina García Fontana; Francisco Andújar Vera; Luis Martínez Heredia; Leyre Villar Ballesteros; María Dolores Avilés Pérez; María Hayón Ponce; Manuel Muñoz Torres; Beatriz García Fontana. Comunicación Póster: Potencial biomarcador del deterioro leve de la función renal en pacientes con diabetes tipo 2: osteoglicina. XXV Congreso de la Sociedad Española de Investigación Ósea y Metabolismo Mineral (SEIOMM). Vitoria-Gasteiz, España; 27-29 octubre 2021.

14. Sheila González Salvatierra; Cristina García Fontana; Francisco Andújar Vera; Luis Martínez Heredia; Leyre Villar Ballesteros; María Dolores Avilés Pérez; María Hayón Ponce; Manuel Muñoz Torres; Beatriz García Fontana. Comunicación Oral: Osteoglicina como biomarcador de disfunción renal temprana en pacientes con diabetes mellitus tipo 2. 62 Congreso de la Sociedad Española de Endocrinología y Nutrición (SEEN). Sevilla, España; 13- 15 octubre 2021.

15. Sheila González Salvatierra; Cristina García Fontana; Francisco Andújar Vega; Alejandro Borja GrauPerales; Luis Martínez Heredia; Manuel Muñoz Torres; Beatriz García Fontana. Comunicación Póster: Relationship of osteoglycin with renal function and vascular tissue in patients with type 2 diabetes. XXXII Congreso de la Sociedad Española de Diabetes. Virtual; 16- 18 junio 2021.

16. Sheila González Salvatierra; Cristina García Fontana; Francisco Andújar Vera; Alejandro Borja Grau Perales; Silvia Lozano Alonso; Luis Martínez Heredia; María

Dolores Avilés Pérez; María Hayón Ponce; Iván Iglesias Baena; Manuel Muñoz Torres; Beatriz García Fontana. Comunicación Póster: Osteoglycin as potential marker of mild kidney function impairment in type 2 diabetes patients. IV Congreso Nacional Jóvenes Investigadores en Biomedicina. Granada, España; 04- 06 noviembre 2020.

17. Luis Martínez Heredia; Sheila González Salvatierra; Cristina García Fontana; Francisco Andújar Vera; Manuel Muñoz Torres; Beatriz García Fontana. Comunicación Póster: Optimization of the transfection protocol in Human Aortic Smooth Muscle cells. IV Congreso Nacional Jóvenes Investigadores en Biomedicina. Granada, España; 04- 06 noviembre 2020.

18. Sheila González Salvatierra; Francisco Andújar Vera; Cristina García Fontana; Manuel Muñoz Torres;Beatriz García Fontana. Comunicación Póster: Expresión de esclerostina y osteoglicina en tejido vascular y estudio de su relación con la calcificación vascular en pacientes con diabetes tipo 2. XXXI Congreso Nacional de la Sociedad Española de Diabetes. Virtual; 21- 23 junio 2020.

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22. Sheila González Salvatierra; Francisco Andújar Vera; Cristina García Fontana; Teresa Márquez Hernández;Manuel Muñoz Torres; Beatriz García Fontana. Comunicación Póster: Expresión transcripcional de esclerostina y osteoglicina en tejido vascular. Relación con la calcificación vascular de la placa aterosclerótica en pacientes con diabetes tipo 2. XXIII Congreso de la Sociedad Española de Investigación Ósea y Metabolismo Mineral (SEIOMM). Granada, España; 07-09 noviembre 2018.

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24. Sheila González Salvatierra; Cristina Novo Rodríguez; Francisco Andújar Vera; Cristina García Fontana; Teresa Márquez Hernández; Silvia Lozano Alonso; Sonia Morales Santana; Pedro Rozas Moreno; Antonia García Martín; Francisco O'valle Ravassa; Juan De Dios Luna Del Castillo; Manuel Muñoz Torres; Beatriz García Fontana. Comunicación Oral: *Levels of sclerostin in serum and femoral vascular tissue and its relationship withvascular calcification in patients with and without type 2 diabetes. 45th European Calcified Tissue Society Congress.* Valencia, España; 26-29 mayo 2018.

25. Francisco Andújar Vera; Cristina García Fontana; Sheila González Salvatierra; Antonia García Martín; Manuel Muñoz Torres; Beatriz García Fontana. Comunicación Póster: *Common gene signature for osteoporosis, diabetes mellitus and peripheral neuropathy by translational bioinformatic approach. 45th European Calcified Tissue Society Congress.* Valencia, España; 26-29 mayo 2018.

26. Beatriz García Fontana; Cristina Novo Rodríguez; Francisco Andújar Vera; Cristina García Fontana; Sheila González Salvatierra; Teresa Márquez Hernández; Silvia Lozano Alonso; Sonia Morales Santana; Pedro Rozas Moreno; Antonia García Martín; Francisco O'valle Ravassa; Juan De Dios Luna Del Castillo; Manuel Muñoz Torres. Comunicación Póster: *Levels of sclerostostin in serum and femoral vascular tissue and its relationship with atherosclerosis and cardiovascular mortality in patients with and without type 2 diabetes. 20th European Congress of Endocrinology.* Barcelona, España; 19-22 mayo 2018.

27. Francisco Andújar Vera; Cristina García Fontana; Sheila González Salvatierra; Teresa Marquez; AntoniaGarcía Martín; Manuel Muñoz Torres; Beatriz García Fontana. Comunicación Oral: Identificación de marcadores genéticos comunes para la diabetes, aterosclerosis y enfermedad renal crónica mediante un análisis integrador por herramientas bioinformáticas. XXIX Congreso Nacional de la Sociedad Española de Diabetes. Oviedo, España; 18-20 abril 2018.

28. Beatriz García Fontana; Cristina García Fontana; Sheila González Salvatierra; Francisco Andújar Vera; Silvia Lozano Alonso; Antonia García Martín; Manuel Muñoz Torres. Comunicación Póster: Niveles de esclerostina en suero y tejido vascular femoral y su relación con aterosclerosis en pacientes con diabetes tipo 2. XXIX Congreso Nacional de la Sociedad Española de Diabetes. Oviedo, España; 18-20 abril 2018.

Producción Científica

Simposios

1. IV Symposium de Medicina de Precisión. Comunicación oral: Papel protector de la esclerostina en aterosclerosis en pacientes con diabetes tipo 2. Hospital Universitario San Cecilio, Granada, España; 30 noviembre 2023.

2. II Symposium de Medicina de Precisión. Comunicación oral: Osteoglicina: un nuevo biomarcador de enfermedad renal en pacientes. Hospital Universitario San Cecilio, Granada, España; 26 julio 2021.

Premio

Premio a la comunicación oral DIABETES en el 64 Congreso de la Sociedad Española de Endocrinología y Nutrición (SEEN). Barcelona, España; 18-20 octubre 2023. Sheila González-Salvatierra; Beatriz García-fontana; Cristina García-fontana; Jesús Lacal-Romero; María Ferrer-Millán; Francisco Andújar-Vera; Luis Martínez-Heredia; Raquel Sanabria de la-Torre; Enrique Moratalla-Aranda; Manuel Muñoz-Torres. Título: Papel protector de la esclerostina en aterosclerosis en pacientes con diabetes tipo 2.