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TESIS DOCTORAL

BIOMARCADORES DE ENFERMEDAD CARDIOVASCULAR EN LA DIABETES MELLITUS TIPO 2. CONEXIÓN CON EL METABOLISMO ÓSEO Y MUSCULAR

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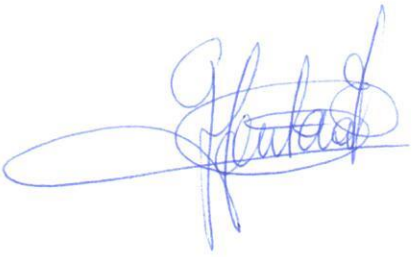
Memoria presentada para aspirar al Grado de Doctor por la
Licenciada en Bioquímica Dña. Beatriz García Fontana



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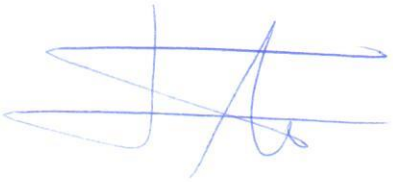

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El trabajo de investigación que se expone en esta Memoria Doctoral ha sido realizado en el Servicio de Endocrinología y Nutrición y en la Unidad de Apoyo a la Investigación del Hospital Universitario San Cecilio de Granada, bajo la dirección del Prof. D. Manuel E. Muñoz Torres y de la Dra. Dña. Sonia Morales Santana.

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El doctorando **Beatriz García Fontana** y los directores de la tesis **Manuel Muñoz Torres y Sonia Morales Santana**garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de los directores de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

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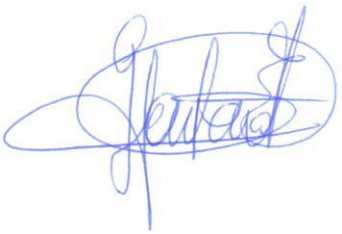

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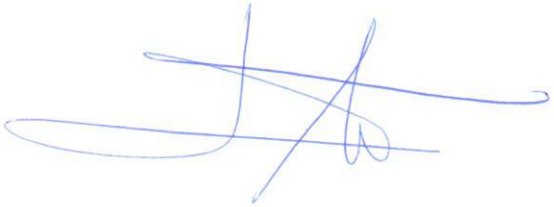

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"El mundo está en las manos de aquellos que tienen el coraje de soñar y correr el riesgo de vivir sus sueños"

Paulo Coelho.

A mi abuelo

A mis padres y hermanas

A Iván, Sergio y al pequeño que viene en camino

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CONTENIDOS

LISTA DE ABREVIATURAS

AGEs	Productos de glicación avanzada
AGLs	Ácidos grasos libres
AOPPs	Productos proteicos de oxidación avanzada
APC	Adenomatous polyposis coli
BMP	Proteínas morfogenéticas óseas
c-HDL	Colesterol HDL
CKI	Caseína quinasa I
c-LDL	Colesterol LDL
CMLV	Células del músculo liso vascular
c-VLDL	Colesterol VLDL
CPA	Células presentadoras de antígenos
Cβ	Células beta
DAG	Diacilglicerol
Dkk	Dickkopf
DM2	Diabetes mellitus tipo 2
DMO	Densidad mineral ósea
Dsh	Dishevelled
DXA	Absorciometría radiológica de doble energía
ECV	Enfermedad cardiovascular
FNDC5	Fibronectina tipo III que contiene 5 dominios
FRCV	Factores de riesgo cardiovascular
FRPs	Proteínas relacionadas con el receptor Fz
Fz	Frizzled
GIM	Grosor de íntima medio
GLP-1	Péptido similar al glucagón tipo 1
GSK3β	Glucógeno-sintetasa-cinasa 3 β
HDL	Lipoproteína de alta densidad
HTA	Hipertensión arterial
IL	Interleuquina
IMC	Índice de masa corporal
IRS-1	Sustrato del receptor de insulina tipo 1
Krm	Kremen
LDL	Lipoproteína de baja densidad
Lef1	Factor de unión al potenciador linfoide
LPA	Ácido lisofosfatídico
LRP5/6	Lipoproteína de baja densidad 5 o 6
MRO	Marcadores de remodelado óseo
NO	Óxido nítrico
oxLDL	Lipoproteínas de baja densidad susceptibles de ser oxidadas
PA	Ácido fosfatídico
PAI-1	Inhibidor del plasminógeno 1
PCR	Proteína C reactiva
PIG	Polipéptido inhibidor gástrico
PPARϒ	Receptor gamma activado por el factor proliferador de peroxisomas
PTH	Parathormona

RANKL	Ligando de receptor activador para el factor nuclear kappa B
ROS	Especies reactivas de oxígeno
TCF	Factor derivado de células T
TGs	Triglicéridos
TNFα	Factor de necrosis tumoral alfa
TZs	Glitazonas
UCP1	Proteína desacoplante 1
VLDL	Lipoproteína de muy baja densidad
Wnt	Wintless

ÍNDICE DE FIGURAS

ÍNDICE DE TABLAS

INTRODUCCIÓN

La Diabetes mellitus tipo 2 (DM2) es un problema de salud pública a nivel mundial que afecta a una gran proporción de la población. La DM2 tiene gran impacto socio-sanitario debido a su relación con el desarrollo de diversas complicaciones, siendo la enfermedad cardiovascular (ECV) su principal causa de mortalidad. Por otro lado, la fragilidad ósea y el aumento del número de fracturas en los pacientes con DM2, implica consecuencias importantes sobre la morbi-mortalidad de estos pacientes. Tradicionalmente, las complicaciones asociadas a la DM2 se han considerado como procesos independientes. Sin embargo, la elevada prevalencia de ambas patologías en la DM2 sugiere la existencia de mecanismos moleculares comunes. No obstante, los conocimientos sobre la regulación de la mineralización ósea y la calcificación vascular no se conocen en profundidad. La existencia de factores de riesgo comunes en ambas patologías podría explicar en parte el desarrollo de las mismas, sin embargo, los mecanismos y vías implicadas en el desarrollo de estos procesos no se conocen de forma completa, existiendo factores subyacentes que aún no han sido elucidados. El descubrimiento de la implicación de la vía Wnt, reguladora del metabolismo mineral, en el sistema vascular, aporta nueva información sobre los mecanismos de conexión entre el metabolismo mineral óseo y el sistema vascular que podrían explicar la prevalencia de ambas enfermedades en la DM2 debido a un desequilibrio de formación/resorción ósea tanto a nivel óseo como vascular.

1. Diabetes melliltus tipo 2

La DM2 es una afección crónica caracterizada por defectos en la secreción y/o en la acción de la insulina, dando lugar a una hiperglucemia secundaria que origina un daño de los tejidos a lo largo del tiempo causando complicaciones para la salud potencialmente letales.

1.1. Epidemiología

La DM2 ha sido catalogada como la epidemia del siglo XXI debido tanto a su creciente magnitud a nivel mundial como a su elevada tasa de mortalidad a causa de las complicaciones asociadas.

La *International Diabetes Federation* (IDF) estimó el número de personas adultas con diabetes a nivel mundial en el año 2015 en 415 millones esperándose un incremento de hasta 642 millones de personas afectadas en 2040 (Figura 1).

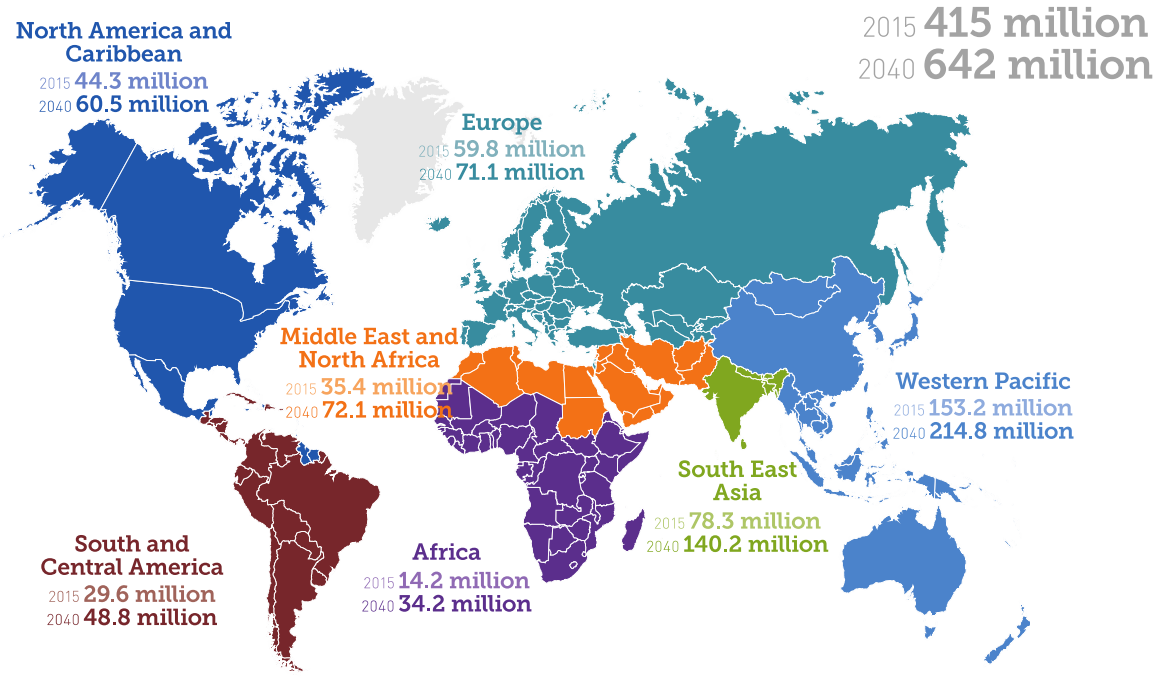

Fig. 1. Prevalencia estimada de DM2 a nivel mundial para el año 2040. (Atlas de diabetes de la IDF. 7ª edición).

En España, ha habido un aumento progresivo de la prevalencia de DM2 situánsose actualmente en un 13,8%, según los resultados del estudio di@bet.es elaborado por CIBERDEM. De este porcentaje, un 7,8% corresponde a casos de DM2 conocida mientras que un 6% de la población española desconoce que sufre esta patología (más de 2,3 millones de personas). Además, según nuevos datos aportados por este estudio, un 8% de la población española presenta tolerancia anormal de la glucemia y un 3,6% adicional presenta una glucemia basal alterada. En total, cerca de un 11,6 % adicional de españoles estarían en riesgo de desarrollar DM2 existiendo en total, un 24% de la población española con problemas de glucosa.

La edad avanzada, la obesidad, el sedentarismo, la hipertensión arterial (HTA) y los antecedentes familiares de diabetes, son entre otros, los factores de riesgo que aumentan la predisposición de desarrollar DM2. El incremento de la globalización e industrialización que da lugar cambios de dieta y a la disminución de la actividad física podría estar muy relacionado con este incremento en la prevalencia de DM2.

1.2. Fisiopatología de la DM2

La DM2 se caracteriza por un trastorno metabólico generalizado que implica la interacción de factores ambientales y genéticos, donde la hiperglucemia es el factor bioquímico dominante. En el desarrollo de la DM2 hay que considerar un mecanismo dual: por un lado, una insuficiencia secretora de las células beta pancreáticas (Cβ) y por otro, un estado de resistencia a la insulina a nivel de los tejidos diana (músculo, hígado, tejido adiposo).

1.2.1. Disfunción de las células β

Las Cβ del páncreas controlan el nivel de glucosa actuando como un sensor de los cambios del nivel de glucosa en sangre y segregando la insulina necesaria para regular la captación de carbohidratos.

Aunque el proceso de estimulación de las Cβ y la secreción de insulina no se conoce completamente, se sabe que la mediación del polipéptido inhibidor gástrico (PIG) y del péptido similar al glucagón tipo 1 (GLP-1) son de gran importancia en la regulación de la homeostasis de la glucosa, siendo los responsables del 70% de la insulina postprandial secretada en sujetos sanos. En pacientes con DM2 se ha observado una disminución de la secreción insulínica entre un 30-70% que puede deberse bien a una reducción en la secreción de PIG y GLP-1, o bien a una menor acción de estos sobre las Cβ (Herzberg-Schäfer *et al.*, 2012).

Por otro lado, la alteración de la secreción insulínica se ha relacionado también con un defecto cuantitativo de las Cβ. Así, la mayoría de pacientes con DM2, desde fases iniciales, presentan una disminución entre el 20 y el 40% del volumen de Cβ (Butler *et al.*, 2003). Aunque el origen de esta disminución no está del todo claro, parece que existe un balance neto negativo en la renovación de los islotes (Janson *et al.*, 2002) relacionado con un aumento de la apoptosis debida a la hiperglucemia crónica, los elevados niveles de ácidos grasos libres (AGLs), el estrés oxidativo y la presencia de citoquinas proinflamatorias (Rhodes, 2005), lo que afecta también negativamente a la funcionalidad de las Cβ pancreáticas contribuyendo al deterioro de la secreción de insulina en la DM2 (Poitout, 2008).

1.2.2. Resistencia a la insulina

La homeostásis glucídica está mediada en gran medida aunque no de forma exclusiva, por la insulina, hormona secretada por las Cβ del páncreas en respuesta a los niveles circulantes de glucosa. Una vez liberada al torrente sanguíneo, ésta se une a los receptores insulinicos de las células efectoras de hígado, músculo y tejido adiposo, desencadenando una cascada de señalización que da lugar a numerosos efectos metabólicos, entre ellos, la estimulación del transporte y consumo de glucosa por las células diana (White, 1998).

La insulina además, tiene un importante papel en la regulación del tejido adiposo ya que participa en la regulación de la expresión y transcripción de algunos de sus

genes (Foretz *et al.*, 1999; Kim *et al.*, 1998). Ello da lugar la estimulación de la síntesis de triglicéridos (TGs) y su almacenamiento en los adipocitos maduros e inhibición de la lipólisis. La insulina también aumenta la oxidación y absorción de AGLs circulantes mediante la estimulación de la actividad de la lipoproteín lipasa en el tejido adiposo (Kahn and Flier, 2000).

Debido a las numerosas vías en las que está implicada la insulina, la patogenia de la resistencia a la insulina es compleja y no del todo conocida.

Por un lado, se produce una ineficaz unión de la insulina con su receptor posiblemente por un defecto en la fosforilación de la tirosina del sustrato del receptor de insulina tipo 1 (IRS-1) en músculo, lo que evita la captación periférica de glucosa. Además, en pacientes con DM2, se ha observado una disminución de los receptores 1 y 2 de insulina, lo que disminuye las cascadas de señalización en las que está implicada esta hormona, y por tanto su función (Saltiel and Kahn, 2001; Virkamäki *et al.*, 1999). Asimismo, en los pacientes con DM2, existe una expresión disminuída de GLUT4, uno los principales transportadores de glucosa.

Por otro lado, debido a la estrecha relación entre la insulina y el tejido adiposo, la obesidad es uno de los principales factores implicados en el desarrollo de la resistencia a la insulina (Berman *et al.*, 2001; Kahn and Flier, 2000). De forma particular, la adiposidad central es la que se relaciona de forma más estrecha con el desarrollo de la resistencia a la insulina ya que la grasa abdominal es la que presenta mayor resistencia a los efectos antilipolíticos de la insulina (Miettinen *et al.*, 1998), además de estar asociada a una secreción alterada de diversas adipoquinas como la resistina, el factor de necrosis tumoral alfa (TNFα) o la adiponectina que agravan la resistencia a la insulina (Yadav *et al.*, 2013).

Otro de los mecanismos que se sugieren en el desarrollo de la resistencia a la insulina, es el incremento de los niveles plasmáticos de AGLs y su acúmulo a nivel muscular y hepático en forma de TGs (Kruszynska *et al.*, 2002). Aunque el mecanismo no es del todo bien conocido, se ha observado que cuando el flujo de AGLs es superior a la capacidad de las vías de almacenaje u oxidación de los mismos, se generan una serie de intermediarios del metabolismo de ácidos grasos

como diacilglicerol (DAG), ácido fosfatídico (PA), ácido lisofosfatídico (LPA) y ceramidas, que se acumulan activando una serie de diferentes serín-quinasas que pueden regular negativamente la acción de la insulina. Las ceramidas también pueden afectar a la acción de la insulina a través de interacciones con PKB/Akt. Además, la incapacidad para oxidar completamente los ácidos grasos a través de β-oxidación, conduce a una acumulación de acilcarnitinas, lo cual, también se ha planteado como hipótesis de causa de resistencia a la insulina (Schenk *et al.*, 2008) (Figura 2).

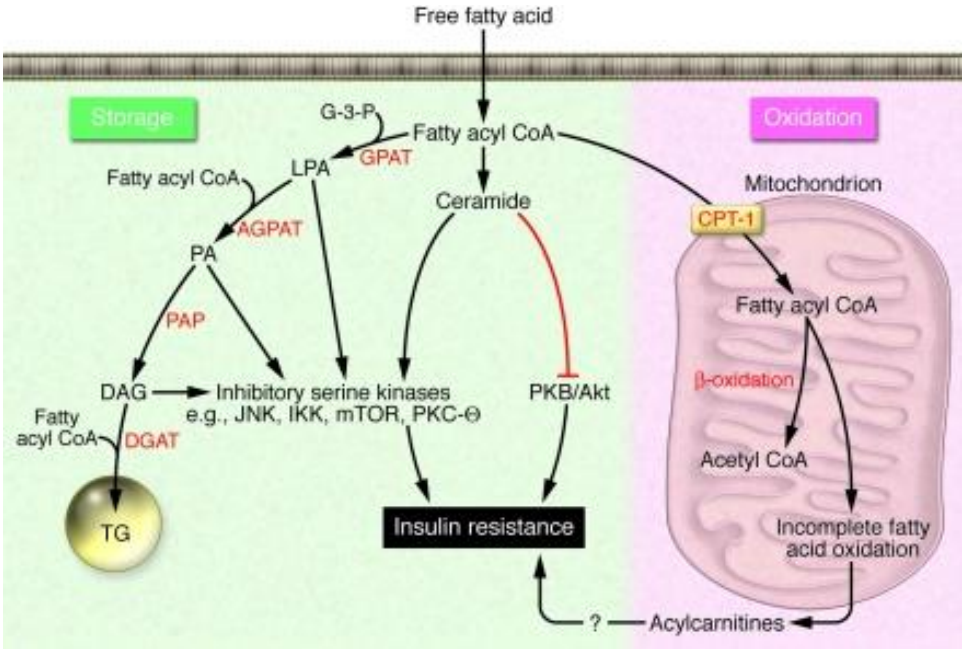

Fig. 2. Metabolismo de ácidos grasos y acción de la insulina (Schenk *et al.*, 2008); AGPAT: acilglicerol-3-fosfato aciltransferasa; PA: ácido fosfatídico; PAP: ácido fosfatídico fosfohidrolasa; DAG: diacilglicerol; DGAT: DAG aciltransferasa; GPAT: Glicerol-3-fosfato aciltransferasa.

Por otro lado, el músculo esquelético se considera un órgano endocrino activo a través de la liberación de una gran cantidad de mioquinas (Febbraio and Pedersen, 2005). Estas mioquinas, forman parte de una red compleja que media la comunicación entre el músculo y otros órganos como son el hígado, el tejido adiposo y el cerebro entre otros. Aunque la función exacta de algunas mioquinas no

se conoce por completo, datos recientes sugieren que determinadas mioquinas desempeñan un importante papel en la prevención de la resistencia a insulina, la inflamación y la disfunción metabólica asociada considerándose como potenciales dianas terapéuticas. Este es el caso de la miostatina y de la irisina que intervienen en la regulación del metabolismo de la glucosa y en la sensibilidad a la insulina (Eckardt *et al.*, 2014) aunque su papel exacto en la DM2 no se conoce con exactitud, existiendo resultados contradictorios.

1.2.1.1. Miostatina

La miostatina, también conocida como factor de crecimiento de la diferenciación 8, es secretada principalmente por el músculo esquelético actuando como un regulador negativo de la masa muscular (McPherron *et al.*, 1997a). Además, estudios recientes han mostrado que esta mioquina también está implicada en la regulación del tejido adiposo, observándose en modelos murinos, una relación entre niveles disminuídos de miostatina con una reducción de la masa y un aumento de la termogénesis. Esta reducción también se ha asociado a una mejora de los niveles plasmáticos de glucosa y de la sensibilidad insulínica. Por ello, la inhibición o bloqueo de la miostatina se han se ha planteado como estrategia para para el tratamiento de alteraciones del metabolismo energético por su efecto en la reducción de la obesidad y de la resistencia a la insulina (Cleasby *et al.*, 2014a). Sin embargo, la mayoría de estudios con miostatina han sido realizados en modelos animales por lo que el papel específico de esta mioquina y su mecanismo de actuación en humanos no está del todo claro existiendo resultados contradictorios en pacientes con DM2.

1.2.1.2. Irisina

La irisina, descubierta recientemente en el año 2012, es una hormona polipeptídica glicosilada secretada principalmente en corazón y músculo esquelético. La irisina está implicada en la homeostasis lipídica y glucídica, entre otras funciones observándose una relación entre niveles incrementados de irisina con una reducción

de peso, mejora de la tolerancia a glucosa y de la resistencia a la insulina. Aunque el mecanismo de actuación en humanos no es del todo conocido, la mejora de la resistencia a la insulina relacionada con el incremento de esta hormona podría estar relacionada con su capacidad de estimulación de la betatropina, hormona con capacidad regenerativa de las Cβ pancreáticas (Sanchis-Gomar and Perez-Quilis, 2014a).

En modelos murinos se ha observado que esta hormona actúa además sobre el tejido adiposo, estimulando la termogénesis así como la biogénesis mitocondrial, incrementando por todo ello, el gasto energético. Debido a los efectos beneficiosos de esta hormona sobre el metabolismo energético, se ha propuesto como potencial diana terapéutica para el tratamiento de enfermedades metabólicas, sin embargo, los conocimientos acerca del papel de la irisina en humanos así como su mecanismo de actuación en la regulación del tejido adiposo son muy escasos existiendo controversia sobre el efecto beneficioso de esta hormona en humanos.

En este contexto, una parte del trabajo realizado en esta Tesis Doctoral va dirigido a la profundización en el estudio de los factores no bien conocidos implicados en el desarrollo de las alteraciones del metabolismo energético en la DM2 así como a intentar elucidar los mecanismos de actuación y las vías de señalización en las cuales estos factores podrían participar con el objetivo de tener un mejor conocimiento de la patogenia de la DM2 lo que permitiría plantear nuevos abordajes terapéuticos.

2. Complicaciones asociadas a la diabetes tipo 2

La toxicidad crónica de la hiperglucemia da lugar a la síntesis de determinadas moléculas como productos de glicación avanzada (AGEs), productos proteicos de oxidación avanzada (AOPPs), así como lipoproteínas de baja densidad susceptibles de ser oxidadas (oxLDL). Los efectos de la hiperglucemia junto con otros factores como la resistencia a la insulina, dislipemia o la acción directa de la hiperinsulinemia, en los estadios iniciales, inducen modificaciones celulares, estructurales y funcionales en diversos órganos y sistemas, siendo los más relevantes

el corazón, los pequeños y grandes vasos arteriales, el riñón, el sistema nervioso central y periférico y la retina, originando así numerosas complicaciones asociadas a la DM2 que aumentan en gran medida la mortalidad asociada a esta enfermedad.

2.1. Enfermedad cardiovascular

La ECV asociada a la DM2 representa una de las principales causas de la mortalidad de esta enfermedad siendo las complicaciones cardiovasculares las responsables del 70–80% de todas las causas de muerte en los sujetos diabéticos (Goday *et al.*, 2002). La DM2 es considerada como un factor de riesgo independiente de ECV tanto en hombres como en mujeres (Wilson *et al.*, 1998a).

Las personas con DM2 presentan diversos tipos de manifestaciones clínicas tales como enfermedad macrovascular o ateroesclerótica (enfermedad arterial coronaria, enfermedad cerebrovascular y enfermedad arterial periférica), enfermedad microvascular (nefropatía y retinopatía) y afectación neuropática. La primera, es con gran diferencia la que supone mayor morbilidad y mortalidad en pacientes diabéticos. Se estima que 3 de cada 4 pacientes diabéticos fallecerán a causa de alguna manifestación de la enfermedad macrovascular.

En la Figura 3 se puede observar la tasa específica de mortalidad por enfermedades cardiovasculares en hombres y mujeres en España en 2002.

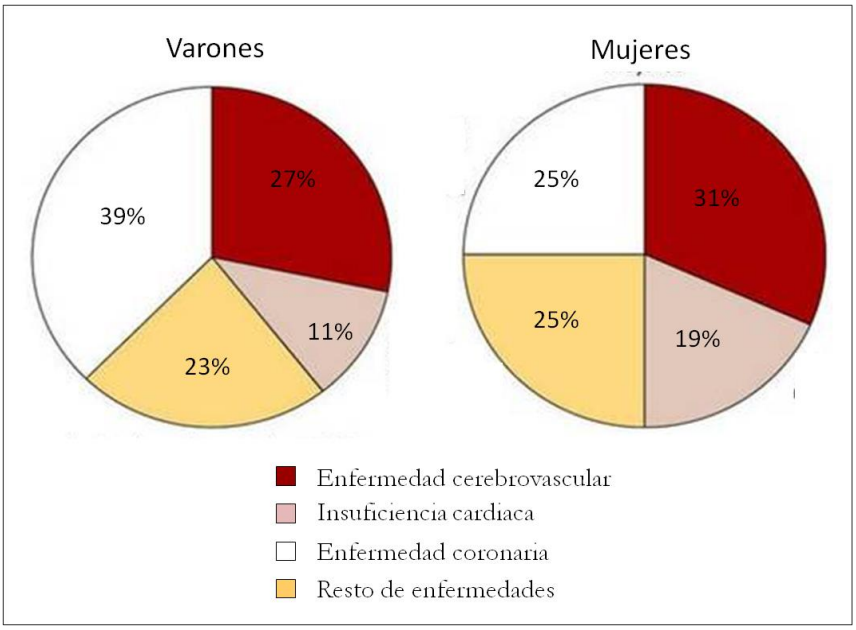

Fig. 3. Tasa específica de mortalidad por diversas causas cardiovasculares en la población española en 2002 según el sexo (Banegas *et al.*, 2006).

2.1.1. Fisiopatología de la ECV

La aterosclerosis es el mecanismo principal de la enfermedad macrovascular. Se caracteriza por el depósito focal de grasa (ateroma) y material fibroso (esclerosis) en la capa más interna de la pared de las arterias, afectando a las arterias de gran y mediano calibre, como la aorta y las arterias coronarias. Las agresiones endoteliales generadas por diversos factores de riesgo dan lugar al reclutamiento continuado de células inflamatorias, la proliferación de células del músculo liso vascular (CMLV) y el acúmulo de colesterol que constituyen los factores biológicos más importantes que determinan el crecimiento de las lesiones ateroscleróticas. Las células inflamatorias, una vez localizadas en el interior de la pared arterial, se transforman en macrófagos capaces de fagocitar las partículas LDL oxidadas. La muerte y la rotura de los macrófagos cargados de lípidos, provoca un acúmulo extracelular de colesterol formando un depósito localizado de grasa y de restos celulares en el interior de la lesión aterosclerótica. Las CMLV que migran desde la capa media de la pared arterial hasta la capa íntima donde proliferan, contribuyen a la síntesis del

colágeno y otros componentes de la matriz fibrosa extracelular dando lugar a un estrechamiento progresivo de la luz vascular (Fernández-Ortiz, 2009) (Figura 4).

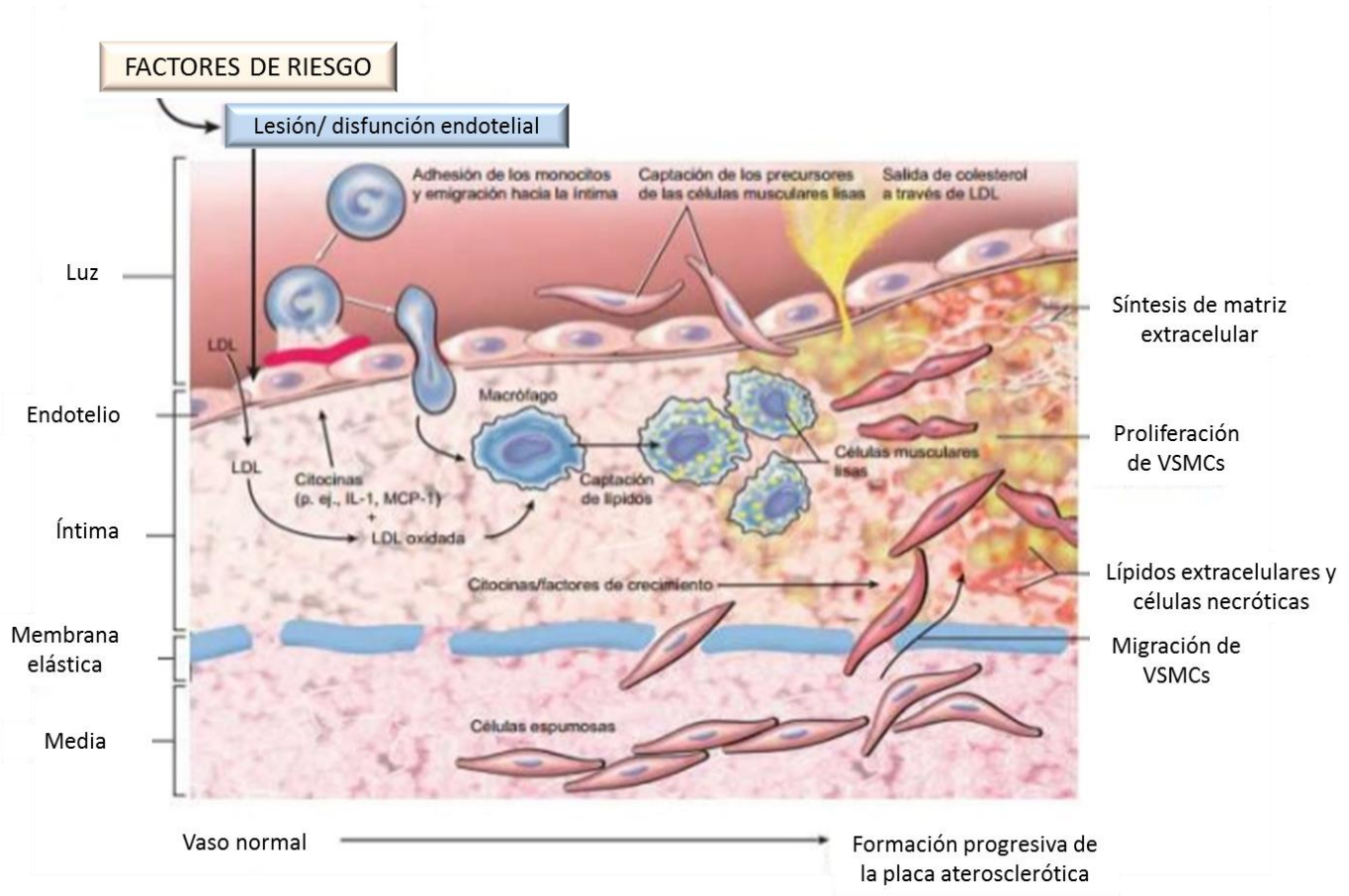

Fig. 4. Proceso de formación de la placa aterosclerótica. Adaptada de Elsevier, Kummar et al: Robbins Basic Pathology 8e.

El desarrollo de ECV en la DM2 tiene un origen multifactorial y complejo, integrado por numerosos factores de riesgo cardiovascular (FRCV) clásicos y otros propios de la diabetes (tabla 1), aunque también existen factores subyacentes implicados en el desarrollo y progresión de las alteraciones cardiovasculares asociadas a la diabetes que están aún por determinar. La interacción entre FRCV tradicionales con otros propios de la DM2 justifica en parte el exceso de riesgo cardiovascular presente en la DM2 (Fonseca, 2004).

Tabla 1. Factores de riesgo cardiovascular en la diabetes mellitus tipo 2. Adaptado de (Fonseca, 2004).

FRCV clásicos	FRCV propios de la DM2
HTA	Resistencia a la insulina
Dislipemia	Disfunción endotelial
Obesidad	Disminución de la fibrinólisis
Sedentarismo	Estado proinflamatorio
Tabaquismo	Hiperglucemia/hiperlipemia postpandrial
Antecedentes familiares de ECV	Aumento del GIM
Edad avanzada	Calcificación vascular

Los FRCV en los pacientes con DM2 actúan como verdaderos estímulos proinflamatorios capaces de alterar el normal funcionamiento de la pared vascular.

A) Hiperglucemia: la hiperglucemia ocasiona un perfil lipoproteico muy aterogénico dando lugar a la síntesis de AGEs, AOPP y oxLDL y aumentando el estrés oxidativo. Todo ello genera un estado proinflamatorio y protrombótico que desencadena un daño endotelial favoreciendo el inicio y desarrollo de lesiones ateroscleróticas en estos pacientes.

B) Resistencia a la insulina: juega un papel primordial en el inicio y progresión de la aterosclerosis debido a una potenciación de vía MAPK por el hiperinsulinismo compensador. Ello resulta en una disminución de la síntesis de óxido nítrico (NO) y en un incremento de los efectos proaterogénicos mediados por la vía MAPK (Nigro *et al.*, 2006).

C) Hipertensión arterial: La prevalencia de HTA es el doble en los pacientes con DM2 con respecto a la población general (Howard, 1996). La resistencia a la insulina juega un papel muy importante en la patogenia de la HTA por su participación en diversos mecanismos entre los que se incluyen mayor resistencia a la vasodilatación mediada por insulina, mayor sensibilidad a sustancias vasoconstrictoras, retención incrementada de sodio y una mayor proliferación de las CMLV (DeFronzo, 1988).

D) Obesidad: La obesidad es actualmente el FRCV más prevalente en personas con ECV establecida (Lopez-Jimenez *et al.*, 2004). La obesidad está directamente implicada con la aterosclerosis por su participación en numerosos procesos fisiopatológicos que interaccionan entre sí tales como HTA, dislipemia, inflamación subclínica, activación neurohormonal con aumento del tono simpático (Sierra-Johnson *et al.*, 2008), altas concentraciones de leptina (Romero-Corral *et al.*, 2008), intercambio aumentado de AGLs y aumento de factores trombogénicos, entre otros.

La obesidad y la resistencia a la insulina son las responsables del incremento de los niveles del inhibidor del plasminógeno 1 (PAI-1), principal inhibidor de la fibrinólisis y marcador de la disfunción endotelial, en pacientes con DM2. La elevación de PAI-1 en estos pacientes se ha asociado a un incremento de ECV (Chudy *et al.*, 2011).

Además, el depósito de grasa en áreas específicas, como la grasa visceral, se ha observado que ejerce una función directa en la patogenia de la aterosclerosis coronaria. El mecanismo mediante el cual la grasa visceral interviene en la patogenia de la ECV no es del todo bien conocido ya que intervienen diferentes vías fisiopatológicas, citoquinas y mediadores inflamatorios. Parece estar estrechamente relacionado con la aparición de la resistencia a la insulina y con la alteración en la secreción de determinadas adipoquinas. Así, se ha observado un incremento en los niveles de citoquinas inflamatorias como interleuquina 6 (IL-6), TNFα y proteína C reactiva (PCR) en sujetos con obesidad visceral (Berg and Scherer, 2005). En cuanto a la alteración en la secreción de adipoquinas, se ha observado una reducción en los niveles de adiponectina, con acción antiinflamatoria y sensibilizante de la insulina, en pacientes con obesidad visceral (Côté *et al.*, 2005). Por otro lado, la resistencia a la leptina en sujetos obesos da lugar a un aumento de la concentración sérica de la misma, implicada en el aumento de la actividad simpática, que potencia la trombosis y aumenta la presión arterial y la frecuencia cardiaca (López-Jiménez and Cortés-Bergoderi, 2011).

Por último, un índice de masa corporal (IMC) aumentado y el contenido de grasa corporal, particularmente obesidad central, se han asociado a disfunción endotelial la cual induce la quimiotaxis de las moléculas de adhesión e induce la diferenciación de monocitos en macrófagos, proceso crítico en la aterogénesis. Además, promueve la agregación plaquetaria y disminuye la disponibilidad de NO, aumentando así el riesgo de trombosis (Vane *et al.*, 1990).

E) Dislipemia: Se caracteriza por un aumento de los niveles de colesterol plasmático, un incremento de las concentraciones de TGs o ambos a la vez que suele corresponder a un aumento de colesterol LDL, a un incremento de colesterol VLDL o a una disminución de colesterol HDL. Estas alteraciones en el metabolismo lipídico están estrechamente relacionadas entre sí siendo la resistencia a la insulina la base de los mecanismos fisiopatológicos de las mismas (Taskinen, 2003).

El incremento de TG da lugar a una disminución de colesterol HDL (c-HDL) que se hidroliza fácilmete por el hígado dando lugar a partículas más pequeñas fáciles de eliminar dando lugar a una reducción de los niveles de c-HDL plasmáticos (Lamarche *et al.*, 1999). El hecho de que el c-HDL constituya una vía de eliminación del exceso del colesterol en el organismo a través del transporte inverso del colesterol desde los tejidos y las paredes arteriales hasta el hígado, donde se excreta por la bilis al intestino, hace que la disminución de los niveles de c-HDL sea un factor de riesgo independiente de ECV (Rosenson, 2005). Cuando hay un retraso del aclaramiento de las VLDL, la permanencia prolongada de estas partículas en el plasma favorece en enriquecimiento de colesterol LDL (c-LDL) en TG, que al hidrolizarse por la lipasa hepática, hace que se formen partículas LDL densas y pequeñas que son capaces de penetrar fácilmente en la pared arterial y son muy susceptibles a la oxidación (Chait and Wight, 2000). Existen numerosas evidencias de que los niveles elevados de c-LDL, en particular en sus formas oxidadas, tienen un efecto dañino sobre el endotelio favoreciendo la disfunción endotelial.

F) Estado proinflamatorio: Se ha observado una inflamación sistémica de bajo grado en pacientes con obesidad y DM2 debido a la alteración en producción

de adipoquinas y citoquinas pro-inflamatorias por el exceso de tejido adiposo y la hipertrofia de los adipocitos característica de la DM2. El incremento de estas moléculas desencadena efectos locales a nivel del endotelio que conducen a un incremento en la expresión de moléculas de adhesión y en la permeabilidad vascular desencadenando un aumento en la infiltración de monocitos y en la acumulación de macrófagos en el endotelio lo que contribuye al proceso aterogénico y por tanto al desarrollo de ECV (Weisberg *et al.*, 2003). Por otro lado, la elevación de las citoquinas pro-inflamatorias origina un aumento de la lipólisis y una disminución de la capacidad del tejido adiposo para acumular TGs dando lugar a un aumento de los ácidos AGL circulantes que se depositan en forma de TGs en el músculo agravando el estado pro-inflamatorio y contribuyendo significativamente al aumento de la resistencia a la insulina (Guilherme *et al.*, 2008).

Los FRCV mencionados anteriormente son factores comunes en sujetos con DM2, sin embargo, la presencia de los mismos, no representa un método del todo fiable para predecir el riesgo de desarrollar complicaciones cardiovasculares ya que sólo algunos de los pacientes diabéticos finalmente desarrollan estas complicaciones. Esto sugiere que la vía que une disglicemia y ECV no se conoce de forma completa existiendo factores subyacentes implicados en el desarrollo y la progresión de la ECV asociada a la DM2. Por ello, actualmente no existe ningún método diagnóstico claro para predecir el desarrollo de estas complicaciones en los pacientes con DM2 lo que complica la detección de aquellos que presentan un mayor riesgo. En este contexto, con el presente trabajo se pretende identificar tanto a nivel proteómico como metabolómico, nuevas moléculas implicadas en el desarrollo de estas complicaciones cardiovasculares que nos permitan conocer las diferentes vías metabólicas alteradas en estas patologías. De esta manera, se podría facilitar la identificación temprana de los sujetos diabéticos con mayor riesgo cardiovascular así como diseñar nuevas dianas terapéuticas para el tratamiento de estos pacientes.

2.2. Fragilidad ósea en la diabetes mellitus tipo 2

La diabetes mellitus se asocia a un aumento del riesgo de fracturas por fragilidad y un sustancial impacto sobre la morbilidad y mortalidad de la población. La mayoría de los estudios publicados han puesto de manifiesto que los pacientes con DM2 presentan un incremento del 1.5 del riesgo de fractura de cadera, húmero proximal a pesar de tener una densidad mineral ósea (DMO) normal o incluso aumentada (Ma *et al.*, 2012). Es por ello que la DMO determinada por absorciometría radiológica de doble energía (DXA), considerada hasta ahora como método de referencia para la medición de masa ósea, constituye un marcador subrrogado relativamente pobre para definir la estructura ósea en diabetes.

La evidencia científica ha puesto de manifiesto la existencia de otros factores inherentes a la propia estructura ósea que también contribuyen, en distinta medida, en el comportamiento biomecánico del hueso y que son reconocidos como factores de calidad ósea (Armas and Recker, 2012).

2.2.1. Fisiopatología de la fragilidad ósea

Aunque la patogenia de la fragilidad ósea en los pacientes con DM2 es compleja y no del todo bien conocida, las alteraciones relacionadas con la calidad del hueso como los cambios en las proteínas de la matriz ósea y en la microarquitectura, así como un bajo nivel de remodelado óseo parecen jugar un importante papel (Leslie *et al.*, 2012).

En este sentido, datos histomorfométricos sugieren que en pacientes con DM2 existe una alteración predominante de la formación ósea. Por un lado, el deterioro progresivo de las Cβ, la glicación de colágeno y la aparición de complicaciones asociadas a la diabetes, determinan una alteración de las propiedades biomecánicas del hueso. Por otro lado, se ha observado un bajo nivel de reclutamiento y un aumento de la apoptosis de osteoblastos en estos pacientes (Jackuliak and Payer, 2014). Aunque los datos no son del todo uniformes, los pacientes con DM2 muestran niveles séricos más bajos de marcadores de remodelado óseo (MRO). La

existencia de un remodelado óseo disminuido podría explicar en parte, el aumento de la DMO descrito en la DM2 así como el incremento de la fragilidad del hueso independientemente de la masa ósea a través de la acumulación de daños por fatiga y alteración de la microarquitectura (Hofbauer *et al.*, 2007a; Reyes-García *et al.*, 2013). La asociación positiva entre elevados niveles de TG, característicos en DM2, con DMO y presencia de fracturas vertebrales tras ajustar por IMC ponen de manisfiesto el aumento de fragilidad ósea independiente de la DMO en los pacientes diabéticos (Adami *et al.*, 2004; Yamaguchi *et al.*, 2002), sin embargo, el mecanismo exacto por el cual ocurre este fenómeno, no se conoce con exactitud.

Los siguientes factores patogénicos contribuyen en el incremento de la fragilidad ósea en estos pacientes (Figura 5).

A) Hiperglucemia: Los elevados niveles de glucosa derivados de una secreción y/o acción alterada de la insulina tienen efectos adversos tanto directos como indirectos sobre las células óseas. La hiperglucemia da lugar a una disminución de la expresión de los receptores de parathormona (PTH) y vitamina D e incrementa la expresión de IL-6 estimulando a los osteoclastos y por tanto la resorción ósea. 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 afectando a la calidad del hueso y acelera la pérdida de masa ósea (Gregorio *et al.*, 1994). Además, la presencia y acumulación de AGEs interfiere en la interacción entre células óseas y matriz extracelular (Blakytny *et al.*, 2011a) disminuyendo la calidad y fuerza óseas e inhibiendo la expresión de osteoblastos por parte del colágeno glicosilado (Yamagishi *et al.*, 2005). La unión de AGEs a sus receptores específicos (RAGE) a nivel óseo, reduce además la actividad de los osteoblastos y altera la osteoclastogenesis por el aumento de la liberación del ligando de receptor activador para el factor nuclear kappa B (RANKL) por parte de los osteoblastos disminuyendo por tanto la mineralización ósea. (Wijenayaka *et al.*, 2011). La relación observada entre niveles incrementados tanto en suero como en orina de pentosidina, uno de los AGEs mejor estudiado, y el aumento de la incidencia de fracturas clínicas en pacientes con DM2 sugiere, que las fibras de colágeno

deformadas por el efecto de acumulación de los AGEs pueden aumentar la fragilidad ósea en la DM2 independientemente de DMO.

La hiperglucemia, además, da lugar a un incremento de las especies reactivas de oxígeno (ROS) que producen daños en las proteínas, lípidos y DNA pudiendo afectar a la estructura ósea. Se ha observado un relación directamente proporcional entre el aumento en los niveles de algunos marcadores de oxidación como la deoxiguanosina o la 8-hidroxideoguanosina y el descenso de la formación y mineralización óseas (Hamada *et al.*, 2007).

B) Hipoglucemia: Los tratamientos hipoglucemiantes usados en la DM2 pueden modular el riesgo de fracturas en estos pacientes de diversas maneras. Se ha observado que el tratamiento con glitazonas (TZs), moléculas estimuladoras del receptor gamma activado por el factor proliferador de peroxisomas (PPARγ), reduce la densidad ósea a través de la inhibición de la diferenciación y actividad de los osteoblastos aumentando así la tasa de fracturas en los pacientes diabéticos. Además, la activación de PPARγ inducida por TZs aumenta la apoptosis de osteoblastos y estimula la diferenciación de células madre mesenquimatosas multipotentes hacia adipocitos en lugar de hacia osteoblastos favoreciendo así la adipogénesis e inhibiendo la osteoblastogénesis en los pacientes con DM2 tratados con TZs (Sorocéanu *et al.*, 2004). Por otro lado, el efecto sensibilizante de TZs a la insulina reduce los niveles circulantes de insulina y por lo tanto el efecto anabólico de la insulina en el hueso (Nyman *et al.*, 2011a).

C) Factores hormonales: Las hormonas secretadas por las Cβ pancreáticas tales como insulina, amilina y peptina, ejercen efectos anabólicos sobre el hueso. La insulina, actúa sobre el hueso a través de los receptores de insulina IRS-1 e IRS-2 expresados por los osteoblastos. La insulina interviene en la regulación de la formación ósea de forma directa a través de su efecto estimulador de la proliferación de osteoblastos y de forma indirecta a través de la inactivación de p27 (activadora de la osteoclastogénesis), la estimulación de la síntesis de colágeno, el mantenimiento de los niveles de PTH, IGF-1 y 1,25 hidroxivitamina D y el aumento de la captación de glucosa (Blakytny *et al.*, 2011a; Nyman *et al.*, 2011b).

La amilina es una hormona polipeptídica secretada por las Cβ pancreáticas perteneciente a la familia de péptidos relacionados con el gen de la calcitonina. Interviene en la regulación de la glucemia y de la sensibilidad a insulina del tejido músculo-esquelético. Estudios *in vitro* han mostrado que la amilina puede inhibir la resorción ósea a través de su unión a receptores localizados en osteoclastos (posiblemente, receptores de calcitonina) así como estimular la proliferación de osteoblastos. Aunque en humanos el papel de la amilina sobre el hueso no ha sido del todo dilucidado, se han observado niveles disminuídos de esta hormona en pacientes con DM2 postulándose que la deficiencia de amilina en estos pacientes podría contribuir a la génesis de la pérdida ósea (Blakytny *et al.*, 2011b).

El efecto de los péptidos incretínicos sobre el metabolismo óseo ha cobrado un creciente interés en los últimos años. El PIG, inhibe la actividad resortiva de los osteoclastos así como la expresión de algunos de los marcadores de diferenciación de estas células. Este péptido, actúa modulando la absorción de los carbohidratos a través de la secreción de insulina. Además, produce un aumento en la síntesis de colágeno tipo I y de la actividad fosfatasa alcalina, lo que pone de manifiesto su claro efecto antiresortivo. Bagger y colaboradores han mostrado una regulación a la baja de los receptores óseos de PIG en la DM2, postulándose que la pérdida del efecto de este péptido podría estar implicada en el aumento de la fragilidad ósea en estos pacientes. Recientemente, además de PIG, también se han propuesto GLP-1 y 2 como responsables de la inhibición de la resorción ósea tras la ingesta. (Bagger *et al.*, 2011).

D) Adiposidad: El sobrepeso y la obesidad asociados a la DM2 se han propuesto como factores protectores de la DMO a través del aumento de la carga mecánica y de la síntesis y liberación por parte del tejido adiposo de factores hormonales como la leptina, resistina y adiponectina (de Paula *et al.*, 2010), aunque la relación de estas adipoquinas con la masa ósea no está del todo clara existiendo resultados discordantes.

El papel protector de la obesidad en la regulación del metabolismo óseo sigue siendo controvertido ya que también se han descrito efectos perjudiciales derivados

de la obesidad en el tejido óseo. Así, la resistencia a la insulina y el aumento de tejido adiposo visceral observado en la DM2 se asocian a un estado de inflamación crónica que constituye un mecanismo de pérdida ósea favoreciendo el riesgo de fracturas (Schäffler *et al.*, 2006). Por otro lado, el tejido adiposo está implicado en la estimulación de la síntesis de PCR, IL6 y su receptor, estimulando así la osteoclastogenesis y por tanto la resorción ósea.

E) Complicaciones de la DM2: Un importante factor de riesgo que contribuye a un mayor riesgo de fracturas en la DM2 es la relación con las complicaciones micro y macrovasculares que incrementan la predisposición a las caídas accidentales en estos pacientes. Las alteraciones visuales, la afectación de la propiocepción, las hipoglucemias desapercibidas, las alteraciones vasculares y la nicturia, conllevan un mayor riesgo de fracturas en la población diabética de edad avanzada. Aunque la mayor propensión a las caídas debido al desarrollo de complicaciones asociadas a la DM2 no parecen ser las únicas responsables del aumento del riesgo de fracturas en esta población, el control y tratamiento de dichas complicaciones disminuye en parte el riesgo de fracturas.

Aunque los factores de riesgo mencionados anteriormente podrían explicar en parte el origen de la fragilidad ósea, se ha demostrado que no suponen un método del todo fiable para la estimación del riesgo de fracturas en los pacientes con DM2 (Giangregorio *et al.*, 2012; Vestergaard, 2007). Esto refleja un incompleto conocimiento del mecanismo fisiopatológico de la fragilidad ósea en estos pacientes que presentan un mayor riesgo de fractura a pesar de tener una DMO normal o incluso aumentada.

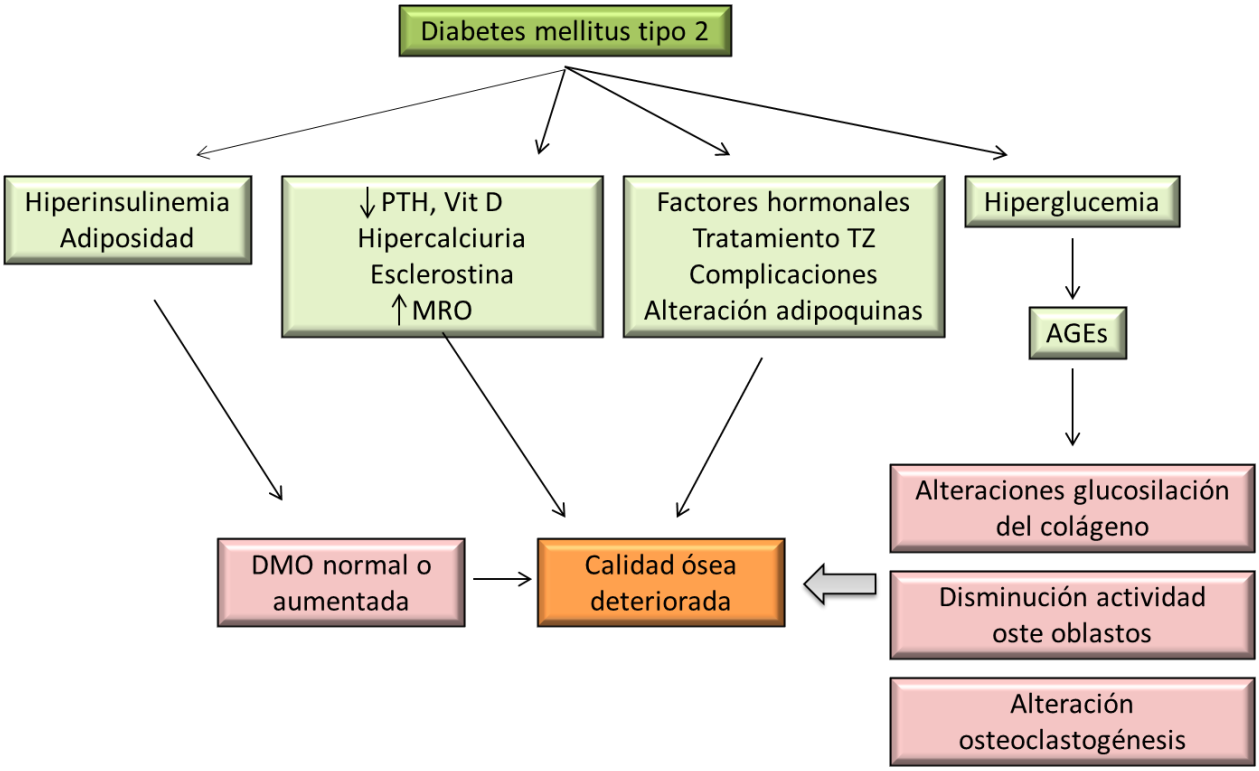

Fig. 5. Esquema de los factores implicados en la fragilidad ósea en la DM2.

La vía de señalización Wnt y sus antagonistas parece desempeñar un papel crucial en la patogenia de las alteraciones de la calidad ósea observadas en la DM2. Así, estudios recientes señalan a la esclerostina, proteína inhibidora de la vía Wnt, como un potencial marcador de fragilidad ósea independiente de la DMO (Arasu *et al.*, 2012; Starup-Linde *et al.*, 2016).

2.3. Vía de señalización Wnt

La vía de señalización Wnt/β-catenina juega un papel decisivo en los procesos de regulación, diferenciación, proliferación y muerte celular. Debido a su participación en una amplia variedad de procesos biológicos, su disfunción se relaciona con la

carcinogénesis y se ha implicado en la patogenia de diversas enfermedades degenerativas así como en numerosas anormalidades del desarrollo embrionario, del crecimiento y la homeostasis. Por ello, hoy en día constituye un objetivo potencial para la intervención terapéutica (van Amerongen and Nusse, 2009).

La señalización de la vía Wnt es inherentemente compleja debido por un lado a que tanto los ligandos como los receptores implicados en esta vía, pertenecen a grandes familias de proteínas derivadas de múltiples genes, lo que permite una amplia posibilidad en las interacciones ligando-receptor. Por otro lado, estas interacciones producen diversas respuestas intracelulares interrelacionadas entre sí (Gordon and Nusse, 2006a).

Las proteínas implicadas en la vía Wnt activan tres vías intracelulares: β-catenina (vía canónica), calcio (Ca^{2+}) y la vía de polaridad celular planar. La primera es la dominante en la regulación de la diferenciación del osteoblasto, mediante la interacción de β-catenina con los factores de transcripción del núcleo como el factor derivado de células-T (TCF) y el factor de unión al potenciador linfoide (lymphoid enhancer-binding factor o Lef) que acivan la transcripción de genes relacionados con la formación ósea.

2.3.1. Vía intracelular canónica

La vía canónica Wnt tiene principalmente efectos anabólicos óseos. Su finalidad es permitir que en el citoplasma se mantengan niveles adecuados de la proteína β-catenina. Esta vía está integrada por varios componentes que incluyen ligandos, receptores de membrana, efectores intracelulares y antagonistas. La activación de esta vía se produce tras la unión de los ligandos Wnt a un complejo receptor que engloba al receptor Frizzled (Fz), compuesto por 7 dominios transmembrana, y a las proteínas relacionadas con el receptor de la lipoproteína de baja densidad 5 o 6 (LRP5/6). En la parte intracelular del complejo se reclutan proteínas como Dishevelled (Dsh), y factores como Axin, Frat1 y adenomatous polyposis coli (APC) para el control intracelular de la vía de señalización. Esta unión desencadena una

cascada de señales que da lugar a la estabilización y acumulación citoplasmática de β-catenina que se trasloca al núcleo y activa la transcripción de genes diana a través de complejos formados por los factores de transcripción TCF / Lef1.

En ausencia de ligandos Wnt, la β-catenina se une a la Axin, APC y caseína quinasa I (CKI), constituyendo el denominado complejo de destrucción. En el mismo es fosforilada, principalmente por la enzima glucógeno-sintetasa-quinasa 3 β (GSK3β), para su degradación posterior en el proteosoma. En presencia de ligandos Wnt, este complejo se disocia impidiendo la fosforilación de la β-catenina por la GSK3β lo que permite que se alcancen niveles estables en el citoplasma para su paso al núcleo celular.

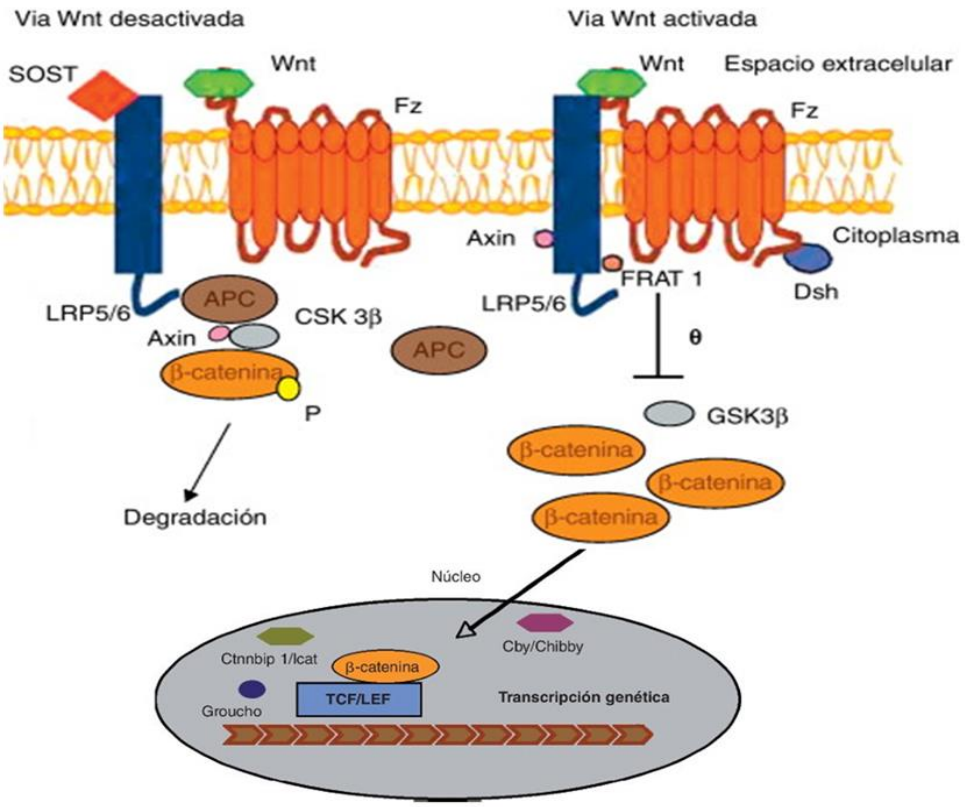

Fig. 6. Vía canónica Wnt. Adaptada de (Escobar-Gómez *et al.*, 2009). APC: adenomatosus polyposis coli; Dkk: proteína Dickkopf; Dsh: proteína dishevelled; Fz: receptor Frizzled; GSK3β: enzima glucógeno-sintetasa-quinasa 3 beta; LEF: factor de unión al potenciador linfoide; LRP: proteína relacionada con el receptor de la lipoproteína de baja densidad; SOST: esclerostina; Wnt: Wintless. TCF: factor derivado de células T.

A nivel óseo, se sabe que las proteínas Wnt ejercen diversas funciones en la génesis del esqueleto, que van desde la definición de patrones del esqueleto embrionario hasta el remodelado del esqueleto adulto. En el esqueleto adulto, las células madre mesenquimales se diferencian a células osteoprogenitoras bajo condiciones adecuadas. La vía canónica de señalización Wnt favorece la línea celular osteoblastogénica mediante el estímulo de la proliferación, diferenciación y maduración del osteoblasto a través de la inducción de los factores de transcripción osteogénicos. Además, inhibe la diferenciación a condrocito y adipocito desde las células progenitoras (Figura 7).

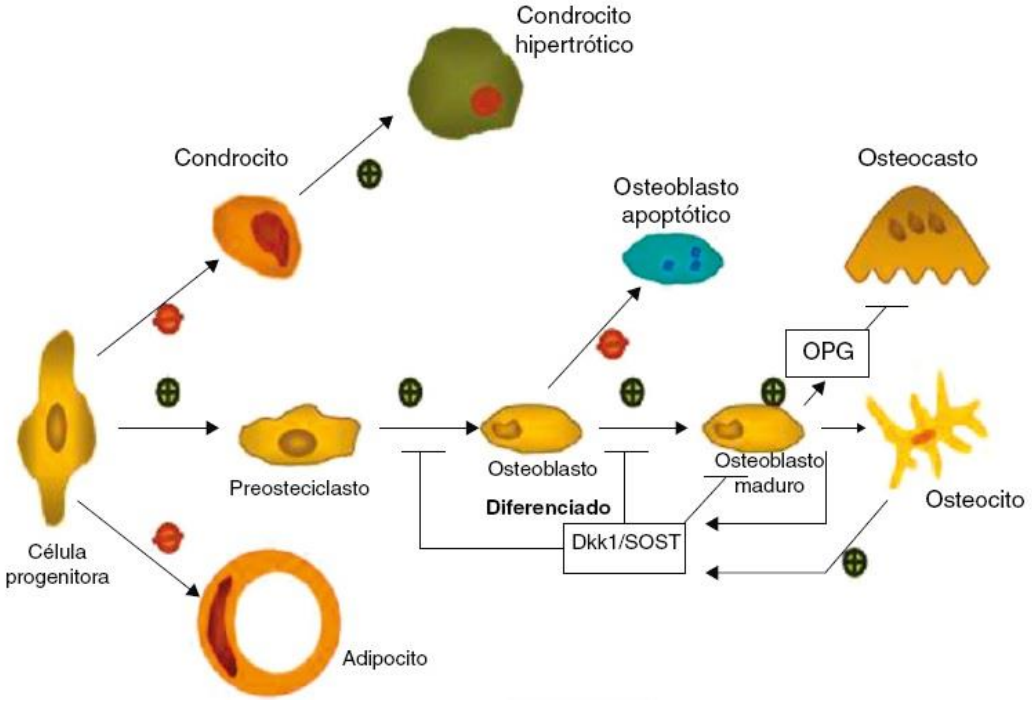

Fig. 7. Papel de la vía canónica de señalización Wnt como regulador de la formación y resorción ósea. Adaptada de (Baron and Rawadi, 2007a). Dkk1: proteína Dickkopf 1; OPG: osteoprotegerina.

Aunque la vía Wnt se había considerado hasta hace muy poco silente en el metabolismo vascular de adultos, estudios recientes ponen de manifiesto que también juega un papel importante en el mismo. Existen evidencias que implican esta vía en la regulación de la proliferación, migración y supervivencia de las CMLV

(Couffinhal *et al.*, 2006a). Además, mutaciones de genes implicados en esta vía se han asociado con hiperlipidemia, hipertensión y enfermedad arterial coronaria en pacientes con síndrome metabólico (Mani *et al.*, 2007a). En estos pacientes, la alteración de la vía Wnt se ha relacionado también con trastornos de los lípidos, glucosa y homeostasis del hueso (Jin, 2008a; Manolagas and Almeida, 2007a). Todo ello, sugiere que la vía de señalización Wnt podría estar también implicada en las patologías vasculares existiendo mecanismos moleculares comunes entre patologías óseas y vasculares. Sin embargo, aún hacen falta estudios que conduzcan a una mejor comprensión de la homeostasis entre mineralización ósea y vascular.

Existen diversos factores inhibidores de la vía Wnt que intervienen en la regulación de la formación ósea. Las proteínas relacionadas con el receptor Fz (FRPs) se unen a las proteínas Wnt, evitando la interacción entre estas Wnt y el receptor Fz. Por otro lado, existen proteínas inhibidoras de la vía Wnt como las proteínas Dickkopf (Dkk) y la esclerostina que se unen a LRP5/6 evitando que se forme el complejo Fz-LRP5/6 y por tanto la unión de Wnt (Baron and Rawadi, 2007b). El receptor transmembrana Kremen (Krm) potencia la actividad de las proteínas Dkk cuando se une con el receptor LRP5/6.

2.3.2. Esclerostina

La esclerostina es una proteína que se describió en 2001 a partir del estudio genético y molecular de 2 displasias esclerosantes poco frecuentes: la esclerosteosis, enfermedad autosómica recesiva que se caracteriza por un progresivo engrosamiento óseo con sindactilia, talla alta y agrandamiento del cráneo y la enfermedad de Van Buchem, defecto autosómico recesivo similar, de gravedad menor y en la que no se presentan los hallazgos de sindactilia ni talla alta (Staehling-Hampton *et al.*, 2002). El defecto genético que produce la esclerosteosis ha sido identificado como mutaciones con pérdida de función del gen denominado *SOST*, localizado en el cromosoma 17q12-21 y codificante de la proteína esclerostina (Brunkow *et al.*, 2001) lo que produce alteraciones óseas en las que se producen displasias esclerosantes por sobreactivación de la vía Wnt (Fgura 8).

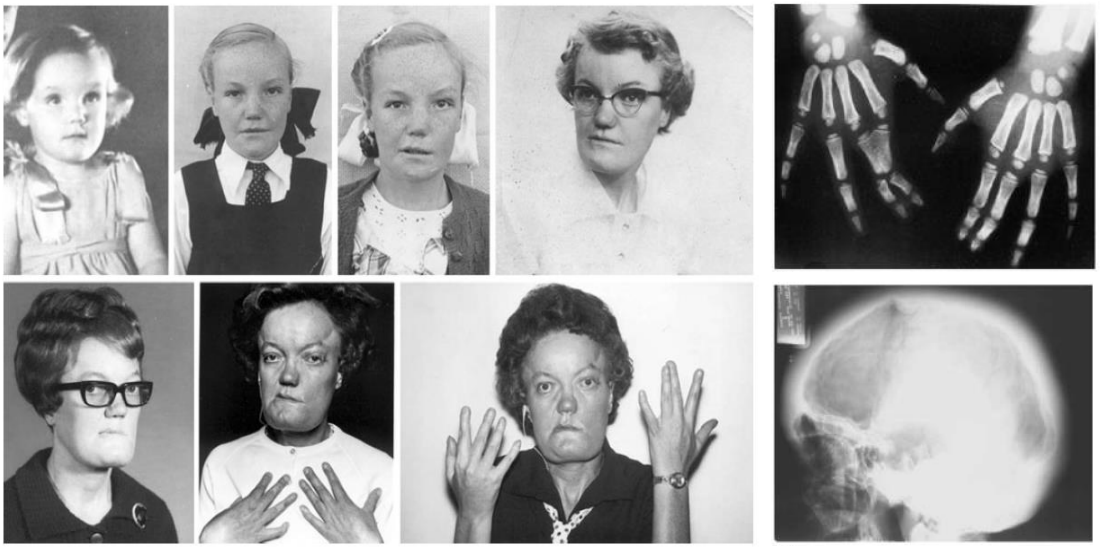

Fig. 8. Imágenes del progreso de una mujer con esclerosteosis.

La esclerostina es una glucoproteína de 190 aminoácidos miembro de la familia proteica DAN a la que también pertenecen las proteínas inhibidoras de las proteínas morfogenéticas óseas (BMP). Su estructura proteica contiene 4 enlaces disulfuro responsables de su estructura tridimensional (Figura 9).

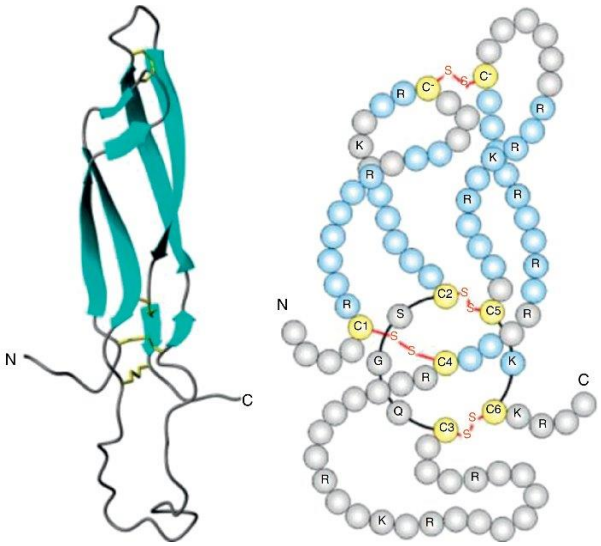

Fig. 9. Estructura proteica tridimensional de la esclerostina. Adaptada de (Veverka *et al.*, 2009).

La esclerostina es una proteína inhibidora de la formación ósea. Por un lado, interactúa con LRP5 y LRP6 induciendo un bloqueo de la vía canónica de señalización Wnt que determina la inhibición de la diferenciación, proliferación y actividad de línea osteoblástica (Li *et al.*, 2005). Por otro lado, actúa como cofactor que antagoniza la vía de las BMP que inducen la formación ósea (van Bezooijen *et al.*, 2007). Estudios recientes han mostrado, que la esclerostina, además de inhibir la osteoblastogénesis, también potencia la osteoclastogénesis ya que incrementa el ratio de RNAm entre RANKL y la osteoprotegerina (OPG), proteína inhibidora de la osteoclastogénesis gracias a su unión y bloqueo de RANKL y a la inducción de la apoptosis de los osteoclastos (Simonet *et al.*, 1997).

La mayoría de estudios describen que la esclerostina se expresa exclusivamente en osteocitos aunque también se ha detectado su expresión génica en riñón, hígado, placenta y cartílago, aunque no se ha aislado la proteína como tal. Sin embargo, recientemente, la esclerostina se ha encontrado expresada en altos niveles en tejidos calcificados de aorta en modelos murinos de diabetes (Shao *et al.*, 2005a), así como en tejido vascular de humanos (Didangelos *et al.*, 2010a) lo que sugiere un papel de la esclerostina en patologías vasculares.

En cuanto a sus funciones, la expresión de esclerostina por los osteocitos actuaría como señal de retroalimentación negativa sobre los osteoblastos para prevenir la sobrecarga de las unidades de remodelado (Poole *et al.*, 2005a). El sistema *SOST*/esclerostina está implicado en la regulación mecánica del tejido óseo observándose que la carga mecánica disminuye la expresión de *SOST* y los niveles de esclerostina *in vivo* (Robling *et al.*, 2008). Además de la carga mecánica, existen varios factores reguladores de la expresión de esclerostina. Así, la PTH, ejerce una acción inhibitoria sobre la expresión génica de *SOST* atribuyéndose parte de sus efectos anabólicos óseos a la disminución de esclerostina (O'Brien *et al.*, 2008). Los glucocorticoides inducen una incremento de esclerostina inhibiendo la vía Wnt (Sutherland *et al.*, 2004; Yao *et al.*, 2008). Asimismo, las BMP2, 4 y 6 han demostrado estimular la expresión de *SOST* en células osteoblásticas *in vitro* (Ohyama *et al.*, 2004).

El papel de la esclerostina en la inhibición de la osteoblastogénesis ha hecho que sea extensamente estudiada a nivel del metabolismo óseo abriendo una nueva área para el desarrollo de estrategias en la terapéutica de las enfermedades metabólicas óseas. Uno de los enfoques consiste en su bloqueo mediante el uso de anticuerpos monoclonales antiesclerostina para el tratamiento de la osteoporosis. Los anticuerpos monoclonales frente a esclerostina han demostrado producir un incremento de DMO, el volumen óseo y la resistencia ósea en modelos de ratas ovariectomizadas (Li *et al.*, 2009) y primates (Ominsky *et al.*, 2010). Con estos resultados, varias compañías farmacéuticas están desarrollando anticuerpos monoclonales frente a esclerostina que están siendo evaluados en ensayos clínicos que actualmente se encuentran en fase III.

Datos recientes sugieren un incremento en los niveles de esclerostina en pacientes con DM2, aunque no se corresponde con una disminución de la DMO, sugiriendo que podría existir una alteración en la vía de señalización Wnt en estos pacientes (García-Martín *et al.*, 2012a). Los niveles disminuídos de PTH en los pacientes diabéticos, al igual que la menor actividad física, podrían contribuir en parte, al incremento de los niveles séricos de esclerostina en la DM2. Asimismo, también se ha descrito una asociación positiva entre esclerostina y hemoglobina glicosilada sugiriendo que la diabetes en sí misma podría influir sobre las concentraciones de esclerostina (García-Martín *et al.*, 2012a) que podrían actuar como predictores de fragilidad ósea en pacientes con DM2 (Arasu *et al.*, 2012; Starup-Linde *et al.*, 2016).

Por otra parte, algunos estudios han descrito un incremento de los niveles de esclerostina en pacientes con calcificaciones en el tejido vascular (Didangelos *et al.*, 2010a; Shao *et al.*, 2005a) lo que sugiere que la vía de señalización Wnt y sus componentes podría estar implicada tanto en las complicaciones del metabolismo óseo como en las complicaciones cardiovasculares asociadas a la DM2.

2.3.3. Dkk1

Dkk1 es una proteína secretada por osteocitos y osteoblastos, presente en vertebrados y rica en cisteína (Figura 10). Dkk1 juega un papel importante en el desarrollo de vertebrados, donde inhibe localmente procesos regulados por Wnt. En la etapa adulta, Dkk1 está implicado en la regulación de la formación ósea.

Fig. 10. Estructura proteica tridimensional de Dkk1. Adaptada de (Veverka *et al.*, 2009).

Dkk1 antagoniza la señalización de la vía canónica Wnt mediante la unión a la región C-terminal del receptor de LRP5/6 a través de su región Cys-2. Esta interacción promueve la internalización de LRP5/6 evitando la unión del receptor con Wnt (Baron and Rawadi, 2007a). Dkk1 es capaz de unirse con elevada afinidad a la familia de receptores transmembrana Krm a través de la región Cys-2 potenciando la capacidad inhibitoria de Dkk1 sobre la señal de Wnt. De este modo, la unión de Dkk1 a LRP5/6 inhibe la vía canónica de señalización Wnt mediante la reducción del número de receptores LRP en la membrana celular.

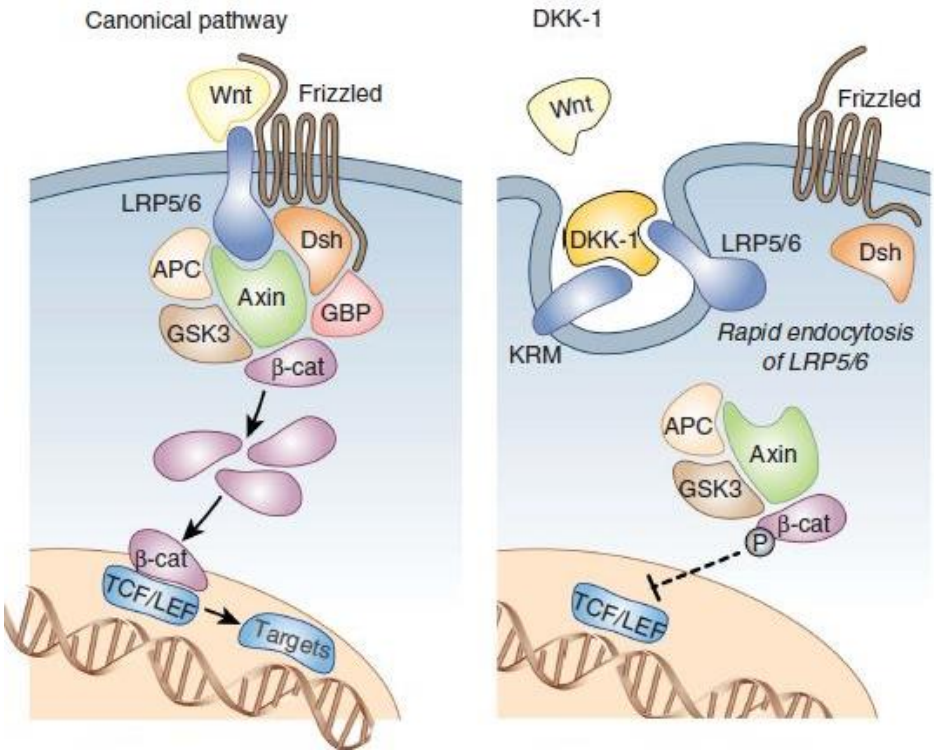

Fig. 11. Internalización del receptor LRP5/6 tras la unión de Dkk1 y Krm. (Yang *et al.*, 2015).

Diversas líneas de investigación han demostrado la importancia de Dkk1 en la regulación de formación ósea. Mientras que la expresión disminuida del gen Dkk1 da lugar a un fenotipo con elevada DMO, la expresión incrementada del mismo da lugar a osteopenia. Así, el tratamiento con anticuerpos neutralizantes frente a Dkk1 en modelos animales, ha mostrado efectos positivos sobre la DMO. Sin embargo, parece que Dkk1 no solo actúa a nivel óseo, sino que está implicado en otros procesos asociándose los niveles séricos de Dkk1 con otras patologías. Así, hay estudios que relacionan niveles elevados de Dkk1 en mieloma múltiple (Feng *et al.*, 2015) y en enfermedades neurodegenerativas como Alzheimer (Purro *et al.*, 2014). Datos recientes muestran además, una relación entre niveles séricos de Dkk1 y aterosclerosis en seres humanos (Ueland *et al.*, 2009a).

3. Relación entre metabolismo óseo y sistema vascular en la diabetes mellitus tipo 2.

En los últimos años, numerosas evidencias han puesto de manifiesto una asociación entre fragilidad ósea y ECV independiente de la edad observándose un incremento en la mortalidad cardiovascular en pacientes con DMO disminuída y/o fractura osteoporótica (Trivedi and Khaw, 2001; Van Der Klift *et al.*, 2002).

El hecho de que la fragilidad ósea y la ECV compartan factores de riesgo comunes presentes en la DM2 puede justificar en parte esta asociación. Como ya se ha mencionado anteriormente, la hiperglucemia, la obesidad, la alteración en la secreción de adipoquinas, los elevados niveles de TG y el aumento del estado inflamatorio producido durante la DM2 están implicados tanto en el desarrollo de ECV como de fragilidad ósea. Sin embargo, existen otros muchos factores implicados de los que aún no se conoce con exactitud el mecanismo de actuación en ambas patologías existiendo resultados contradictorios.

La relación entre ambas patologías podría ser explicada por el hecho de que el desequilibrio de formación/resorción ósea está implicado también en las patologías cardiovasculares (Reyes-García *et al.*, 2011). Varios estudios ponen de manifiesto la relación entre la calcificación arterial y la fisiología ósea. Así, estudios recientes han mostrado una relación entre niveles disminuídos de DMO trabecular y un aumento de la prevalencia de calcificación de arteria coronaria y de aorta abdominal independientemente del sexo (Hyder *et al.*, 2009). Asimismo, se ha observado una mayor prevalencia de fracturas vertebrales en pacientes que han sufrido recientemente un evento coronario, independientemente de la DMO (Silva *et al.*, 2013) así como un incremento en el riesgo de sufrir enfermedad coronaria en pacientes con fractura de cadera previa (Tsai *et al.*, 2015). Teniendo en cuenta que diabetes mellitus es un factor de riesgo común para fragilidad ósea y enfermedad vascular se ha observado también una relación entre fracturas y cardiopatía isquémica en pacientes con DMT2 (Muñoz-Torres *et al.*, 2013). Sin embargo, los conocimientos sobre la homeostasis entre la mineralización ósea y la vascular son en la actualidad muy escasos.

El hecho de que la vía Wnt se haya implicado también a nivel vascular la ha puesto en el punto de mira para el estudio de la interconexión entre metabolismo óseo y sistema vascular. Así, diversos estudios en patologías vasculares implican a esta vía en la regulación de la proliferación, migración y supervivencia de las CMLV (Couffinhal *et al.*, 2006a; Goliasch *et al.*, 2012a). Asimismo, mutaciones en genes codificantes de proteínas implicadas en la vía Wnt se han asociado simultáneamente con osteoporosis y complicaciones vasculares en pacientes con síndrome metabólico (Mani *et al.*, 2007a). De especial relevancia es la esclerostina, cuya expresión, hasta ahora, estaba descrita únicamente en osteocitos. Sin embargo, estudios *in vitro* han demostrado que en un entorno calcificante, en las CMLV se puede inducir una transición fenotípica a células similares a osteocitos capaces de expresar marcadores típicos osteocíticos, entre ellos, la esclerostina. En concordancia, algunos estudios en modelos de DM2 murinos y humanos, han mostrado un incremento de esclerostina en tejidos vasculares calcificados (Didangelos *et al.*, 2010a; Shao *et al.*, 2005a).

De igual forma, se han observado moléculas del sistema OPG/RANK/RANKL implicadas tanto en osteoporosis como en enfermedad cardiovascular. Así, se ha observado expresión de OPG y RANKL en células musculares lisas así como en endoteliales de la pared arterial (Hofbauer *et al.*, 2001). Además, estudios en modelos animales han puesto de manifiesto que ratones deficientes en la producción de OPG presentan calcificación de las arterias aorta y renal y que el tratamiento con OPG previene, en modelos animales, las calcificaciones arteriales provocadas por la warfarina y /o altas dosis de vitamina D (Price *et al.*, 2001) actuando ésta como factor autocrino de supervivencia de la célula endotelial (Malyankar *et al.*, 2000). Estos resultados sugieren que la regulación de producción de OPG, su cascada de señalización y sus ligandos podrían estar implicados en la conexión existente entre la osteoporosis y la calcificación vascular (Bucay *et al.*, 1998). Contrariamente a los hallazgos en modelos animales, estudios en humanos muestran un valor predictivo de los niveles séricos de OPG en la incidencia y mortalidad de la ECV. Así, se ha mostrado una asociación significativa entre niveles séricos elevados de OPG y la presencia y severidad de arteriopatía coronaria y aterosclerosis carotídea aumentando de 3 a 4 veces el riesgo relativo de mortalidad cardiovascular (Kiechl *et*

al., 2004). Además, algunos polimorfismos del gen de OPG se han considerado como factores predictores de un grosor de íntima medio (GIM) elevado en sujetos hipertensos incrementando el riesgo de complicaciones vasculares (Brändström *et al.*, 2004).

Por otra parte, varios estudios han puesto de manifiesto la asociación entre marcadores subrogados de ECV con alteraciones óseas. Tal es el caso de la calcificación vascular que se ha asociado con la presencia de osteoporosis y fracturas vertebrales (Bagger *et al.*, 2006; Hyder *et al.*, 2010). Asimismo, se han observado depósitos de calcio en lesiones ateroscleróticas aparentemente idénticos a los que forman las trabéculas y láminas del hueso (Haust and Geer, 1970) y se ha detectado la presencia de proteínas típicas de la matriz del hueso en arterias calcificadas tales como colágeno de tipo I, proteína de la matriz GLA, osteocalcina, osteopontina y BMP2 (Boström *et al.*, 1993; O'Brien *et al.*, 1995). En pacientes diabéticos, los niveles de osteocalcina se han asociado con parámetros de arterioesclerosis sugiriendo que la osteocalcina está implicada, no solo en el metabolismo óseo, sino también en la enfermedad aterosclerótica (Reyes-Garcia *et al.*, 2012). A su vez, la fractura vertebral se asocia a un mayor riesgo de presentar placa carotídea (Kim *et al.*, 2008).

En cuanto a los niveles de PTH, existen suficientes datos que demuestran un efecto directo sobre el tejido vascular. Así, se han identificado receptores de PTH a nivel de células cardiacas y CMLV atribuyéndose un efecto hipertrófico de la PTH sobre estas células (Schlüter and Piper, 1998), así como un efecto estimulador de la migración de células progenitoras angiogénicas hacia las zonas dañadas por infarto de miocardio (Zaruba *et al.*, 2008).

Finalmente, ambas enfermedades presentan algunos tratamientos comunes. Se están empleando inhibidores del metabolismo del colesterol en el tratamiento de osteoporosis porque inhiben el proceso de diferenciación del los precursores celulares a osteoclastos. Tal es el caso de las estatinas, inhibidores de la HMG-CoA reductasa (Alegret and Silvestre, 2007). De igual forma se ha visto que los bisfosfonatos, inhibidores de la farnesil difosfato sintasa empleados para el

tratamiento de la osteoporosis, podrían ser beneficiosos también para la enfermedad cardiovascular gracias a su acción antiaterogénica y a su implicación en la inhibición de la calcificación extraósea y la reducción del acúmulo de lípidos y la fibrosis en las lesiones ateroscleróticas (Price *et al.*, 2001b). Asimismo, los bisfosfonatos endovenosos, en terapia prolongada, parecen descender los niveles de c-LDL y aumentar los de c-HDL en mujeres postmenopáusicas (Adami *et al.*, 2000). En animales de experimentación, la administración de ibandronato o alendronato a dosis similares a las necesarias para inhibir la resorción ósea, inhibe además la calcificación arterial inducida por warfarina y dosis altas de vitamina D.

El conjunto de estas observaciones sugiere que la calcificación arterial es un proceso organizado y regulado, con mecanismos celulares y moleculares similares a la formación del hueso. De igual forma sugiere la existencia de mecanismos patogénicos comunes entre la osteoporosis y la aterosclerosis que favorecerían el desarrollo de ambas enfermedades, sin embargo, los mecanismos de conexión específicos que relacionan el metabolismo vascular y óseo aún no son bien conocidos.

HIPÓTESIS Y OBJETIVOS

El conocimiento de los factores implicados tanto en la DM2 así como en el desarrollo de las complicaciones vasculares asociadas a la misma, es clave para poder llevar a cabo un mejor abordaje en el tratamiento de estos pacientes, así como un diagnóstico temprano que evite el desarrollo de complicaciones antes de que se produzcan daños irreversibles.

Hipótesis de trabajo

Existen factores subyacentes implicados en el desarrollo de las complicaciones cardiovasculares asociadas a la DM2 que no se conocen en la actualidad. La implicación de la vía de señalización Wnt, reguladora del metabolismo óseo, en el sistema vascular, podría constituir un mecanismo de conexión entre la fisiología ósea y vascular que explique la prevalencia de ambas enfermedades en la DM2.

Objetivo principal

El objetivo principal de esta tesis es la identificación de los factores subyacentes implicados en la patogenia de la DM2 y en el desarrollo de las complicaciones vasculares asociadas, con el fin de conocer las vías alteradas en estos procesos así como los mecanismos de actuación de estos factores.

Objetivos específicos

1. Determinación de los niveles séricos de las mioquinas, miostatina e irisina, para evaluar su implicación en la DM2.

2. Identificación de biomarcadores metabólicos y proteicos asociados a la ECV en pacientes con DM2.

2.1. Comparación analítica e identificación diferencial del proteoma sérico de pacientes con DM2 con presencia/ausencia de ECV y sujetos no diabéticos.

2.2. Comparación e identificación diferencal del metaboloma sérico de pacientes con DM2 con presencia/ausencia de ECV y de sujetos no diabéticos.

3. Análisis de moduladores de la vía Wnt (esclerostina y Dkk1) en sueros de pacientes con DM2 con presencia/ausencia de ECV y sujetos no diabéticos, para la evaluación de su implicación en el sistema vascular.

RESULTADOS

CAPÍTULO I

Relationship between myostatin and irisin in type 2 diabetes mellitus: a compensatory mechanism to an unfavourable metabolic state?

Adaptado de: Relationship between myostatin and irisin in type 2 diabetes mellitus: a compensatory mechanism to an unfavourable metabolic state?

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1. Abstract

Myostatin and irisin are two myokines related to energy metabolism, acting on skeletal muscle and recently suggested on adipose tissue in mice. However, the exact role of these myokines in humans has not been fully established. Our aim was to evaluate the relationship between serum levels of myostatin and irisin in type 2 diabetes mellitus patients and non-diabetic controls and to explore its links with metabolic parameters. Case–control study including 73 type 2 diabetes mellitus patients and 55 non-diabetic subjects as control group. Circulating myostatin and irisin levels were measured by enzyme-linked immunosorbent assays. Type 2 diabetes mellitus patients showed significantly lower myostatin levels ($p = 0.001$) and higher irisin levels ($p = 0.036$) than controls. An inverse relationship was observed between myostatin and irisin levels ($p = 0.002$). Moreover, in type 2 diabetes mellitus patients, after adjusting by confounder factors, myostatin was negatively related to fasting plasma glucose ($p = 0.005$) and to triglyceride levels ($p = 0.028$) while irisin showed a positive association with these variables ($p = 0.017$ and $p = 0.006$ respectively). A linear regression analysis showed that irisin and fasting plasma glucose levels were independently associated to myostatin levels and that myostatin and triglyceride levels were independently associated to irisin concentrations in type 2 diabetes mellitus patients. Our results suggest that serum levels of myostatin and irisin are related in patients with type 2 diabetes. Triglyceride and glucose levels could modulate myostatin and irisin concentrations as a compensatory mechanism to improve the metabolic state in these patients although further studies are needed to elucidate whether the action of these myokines represents an adaptative response.

2. Introduction

Myostatin, also known as growth-differentiation factor 8 (GDF-8) is a member of the transforming-growth factor beta (TGF-b) superfamily, and it is secreted mainly by skeletal muscle. It acts as a negative regulator of muscle mass, inhibiting its growth (McPherron *et al.*, 1997b). In animal models, the deletion or sequestration of myostatin results in muscle hypertrophy and reduced obesity and insulin resistance (IR) (Cleasby *et al.*, 2014b). The benefits of myostatin inhibition on glucose metabolism have been described in mice by several studies (Akpan *et al.*, 2009; Guo *et al.*, 2009, 2012; Zhang *et al.*, 2011; Zhao *et al.*, 2005). Recently, it has been shown that myostatin is also produced in adipocytes suggesting an additional role as regulator of adipocyte function or differentiation (Guo *et al.*, 2009; Lee, 2004).

Myostatin deficient mice show decreased fat mass through the induction of the expression of enzymes involved in lipolysis and fatty acid oxidation and the stimulation of brown adipocytes in white adipose tissue (WAT), thus increasing thermogenesis (Lee, 2004; McPherron and Lee, 2002; Zhang *et al.*, 2012). Accordingly, decreased levels of myostatin increase muscle mass and reduce fat mass in animal models. However, the specific role of myostatin in humans is not well elucidated and the mechanism how myostatin regulates adipose tissue remains unclear.

Irisin is a novel glycosylated polypeptide hormone derived from its precursor fibronectin type III domaincontaining protein 5 (FNDC5) located in the plasma membrane, after the cleavage of its extracellular portion (Boström *et al.*, 2012). The synthesis of irisin is mediated by the transcriptional co-activator PPAR-c co-activator-1 a (PGC1-a) that increases FNDC5 expression. There is controversial evidence on the physiology of irisin, but it has been suggested that under some conditions, such as exercise and elevated oxidative stress an increased expression of FNDC5 occurs and therefore of the circulating irisin levels (Kurdiova *et al.*, 2014; Sanchis-Gomar and Perez-Quilis, 2014b). Irisin is secreted by numerous tissues, but predominantly by the heart and skeletal muscle. Some studies have shown that irisin could be involved in diverse functions related to glucose and lipid homeostasis, such

as weight loss, improved glucose tolerance and insulin resistance associated with obesity (Boström *et al.*, 2012; Choi *et al.*, 2013; Liu *et al.*, 2014; Sanchis-Gomar and Perez-Quilis, 2014b). Once secreted, in animal and "in vitro studies" irisin acts on the adipose tissue, promoting the formation of brown like-adipocytes in WAT and stimulating genes involved in thermogenesis, such as uncoupling protein 1 (UCP1) (Sanchis-Gomar and Perez-Quilis, 2014b; Vaughan *et al.*, 2014)[13, 16]. However, studies are needed to assess whether these effects can be translated to humans.

Interestingly, recent experiments in animal models have revealed a relationship between these two myokines suggesting a close connection between muscle and adipose tissue. However, at present, the role of myostatin and irisin in normal and pathological conditions and their relationship with metabolic parameters are not well defined in humans showing discordant results (Brandt *et al.*, 2012; Han *et al.*, 2014; Sesti *et al.*, 2014; Vaughan *et al.*, 2014; Wang *et al.*, 2012).

Our objective was to evaluate the relationship between both myokines in T2DM patients and non-diabetic subjects and to study their association with metabolic parameters, in order to establish the underlying processes in which these molecules are involved.

3. Subjects and methods

3.1. Study population

Our retrospective case–control study included 128 subjects aged 35–65 years and with similar percentages of males and females. The participants were classified into two groups: 73 patients with diagnosis of diabetes according to American Diabetes Association criteria (ADA, 2005) and 55 age and gender-matched non-diabetic subjects as the control group. From January 2006 to December 2007 we consecutively recruited patients who had been referred to our outpatient clinic from

primary care centres for the treatment of diabetes. The control subjects were consecutively recruited from the general community in the same period of time.

All participants were Caucasians and ambulatory and did not have renal, hepatic, gastrointestinal or thyroid diseases apart from diabetes. The T2DM patients were on medication for diabetes, including metformin, sulfonylureas, insulin and/or a combination of these drugs. Patients treated with thiazolidinediones were excluded.

The study was conducted with the approval of the ethics committee of the San Cecilio University Hospital and conformed to the relevant ethics guidelines for human and animal research. Written informed consent was obtained from all subjects.

3.2. Clinical evaluation

Height, weight, and waist circumference were measured at baseline according to standard procedures. Weight was measured to the nearest 100 g using digital electronic scales. Height and waist circumference were measured to the nearest 1 mm using a stadiometer and a metal anthropometric tape, respectively. Body mass index (BMI) in kg/m^2 was calculated as weight divided by the square of height in metres, and obesity was defined as BMI $\geq$ 30. Blood pressure was measured in a standardised manner. After the subjects had remained at rest for at least 5 min, blood pressure was measured twice, using a standard mercury sphygmomanometer (12 cm long, 35 cm wide). The mean of the two values was used for analysis. Hypertension was defined as C140/90 mmHg and/or antihypertensive treatment.

Participants reported their alcohol use, smoking status and the level of physical activity, in response to a specific health questionnaire. Patients were classified as having a significant alcohol intake if it was higher than 40 g/day in males and 24 g/day in women. Smoking status was categorised as no tobacco use or current tobacco use. Physical activity was determined by a specific questionnaire to which the study subjects responded on a scale from 0 (none) to 10 (sport more than one

hour four times per week). Based on the results, the study sample was divided into two groups: sedentary (< 5 on the scale) and non sedentary (≥ 5 on the scale).

3.3. Serum measurements

Samples of venous blood were taken in the morning after fasting overnight. Sera were stored at -80 ºC until examination. Fasting plasma glucose (FPG), HDL cholesterol, LDL cholesterol, triglyceride (TG) levels and creatinine were measured using standard automated laboratory techniques. Dyslipidemia was defined according to the Third Report of the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) criteria or current treatment with statins. Glycated haemoglobin (HbA1c) was determined by high-performance liquid chromatography (ADAMS A1c, HA-8160; Menarini).

Serum myostatin was measured using quantitative sandwich enzyme-linked immunosorbent assay (ELISA) developed by R&D systems (Minneapolis, MN, USA) according to the manufacturer's instructions. The assay sensitivity was 5.32 pg/mL and the assay range was 31.3–2.000 pg/mL. Intra-assay and inter-assay variabilities were 5.6 and 6%, respectively.

Serum irisin levels were measured by enzyme immunoassay (Phoenix Pharmaceuticals) following the user's manual. The sensitivity of the method was 1.57 ng/ mL and the intra and inter-assay variability were 10 and 15% respectively. The assay range was 0.1–1,000 ng/mL with a linear range of 1.57–47 ng/mL.

3.4. Statistical analysis

Data for continuous variables are presented as mean ± standard deviation (SD). Data for categorical variables are presented as numbers and/or percentages. The Kolmogorov-Smirnov test was used to test the normality of distribution of continuous variables. Associations between continuous variables were described by Pearson's or Spearman's correlation coefficients for variables that were not normally

distributed. In order to correct the possible influence of some variables in this relationship, we used partial correlations, adjusting for confounding factors. Comparisons of categorical variables among groups were performed using the Chi square test. Comparisons of continuous variables among groups were performed using the unpaired Student's t test or the Mann–Whitney test. To determine the independent variables correlated with myostatin and irisin (dependent variable), the parameters that correlated significantly in univariate analysis and with others biologically linked to them were tested in multiple backward linear regression analysis. A p value of less than 0.05 was considered significant (two-tailed). Data were recorded and analysed using SPSS version 18.0 software (SPSS Inc, Chicago, IL, USA). The study variables were measured in all study subjects.

The sample size was calculated considering a statistical power of 90% and a significance level of 5% using the Student's t test for not equality, to compare means of two independent samples.

4. Results

4.1. Characteristics of the study population

The demographic, anthropometric and biochemical characteristics of the study groups are summarised in Table 2. The groups were comparable in age and percentage of males and females. As expected, the groups differed in diabetes-related parameters, associated metabolic risk factors and current medication.

Table 2. Anthropometric and biochemical parameters of the study population.

	Control Group (n = 55)	**T2DM Group (n = 73)**	**P Value**
Age (years)	57 ± 7	58 ± 6	0.160
Male/female (n)	25/30	40/33	0.472
BMI (kg/m^2)	29 ± 6	31 ± 6	0.003
Waist circumference (cm)	97 ± 12	106 ± 11	<0.001
SBP (mm Hg)	124 ± 17	135 ± 22	0.005
DBP (mm Hg)	78 ± 15	79 ± 12	0.399
Duration of diabetes (years)	-	13.5 ± 7.7	<0.001
Smoker (%)	14	18	0.603
Alcohol (%)	12	10	0.210
Sedentarism (%)	38	54	0.075
Serum parameters			
FPG (mg/dL)	89.2 ± 10.5	179 ± 60	<0.001
HbA1c (%)	4.9 ± 0.4	8.0 ± 1.9	<0.001
Creatinine (mg/dl)	0.85 ± 0.17	0.89 ± 0.19	0.209
Triglyceride (mg/dl)	104 ± 47	169 ± 149	0.002
HDL-c (mg/dl)	59 ± 12	49 ± 16	<0.001
LDL-c (mg/dl)	133 ± 28	97 ± 34	<0.001
Myostatin (pg/ml)	3267 ± 1164	2614 ± 1051	0.001
Irisin (ng/mL)	429 ± 73	462 ± 91	0.036
Current medication			
Statins (%)	14.5	70	<0.001
Oral antidiabetic drugs (%)	-	77.9	<0.001
Insulin	-	64.9	<0.001

BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; FPG: fasting plasma glucose; HbA1c: glycated haemoglobin; HDL-c: High-density lipoprotein; LDL-c: Low-density lipoprotein; Data for continuous variables are presented as mean ± SD. Data for categorical variables are presented as percentages. Unpaired *t* test or Mann-Whitney test were used for comparisons between cuantitative variables and X^2 for categorical variables.

4.2. Serum myostatin in T2DM patients and controls

Serum myostatin levels were significantly lower in the T2DM patients than in the controls; (2,614 ± 1,051 pg/mL vs 3,267 ± 1,164 pg/mL respectively; p<0.001) (Figure 12).

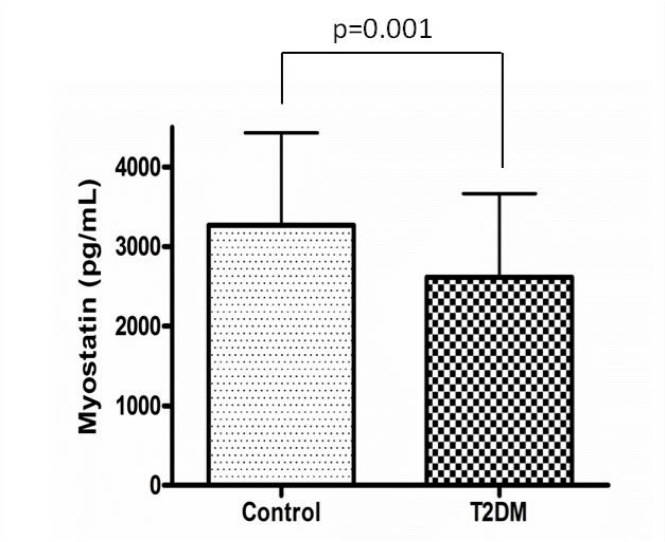


Fig. 12. Serum myostatin levels in T2DM patients and control subjects. Differences between groups were determined by the Mann-Whitney *T* test. The statistical significance was set at $p < 0.05$ (two-tailed).

When the subjects were analyzed according to sex, serum myostatin differences remained between T2DM and control group, with myostatin concentrations significantly lower in females than in males (2,583 ± 896 pg/mL vs 3,161 ± 1,109 pg/mL; p<0.05) (Figure 13).

We have found no differences on myostatin levels according to physical activity or BMI. In the T2DM patients, myostatin levels were negatively associated with irisin ($r = -0.269$, $p = 0.038$), FPG ($r = -0.241$, $p = 0.045$) and positively related to creatinine ($r = 0.363$, $p = 0.002$). After adjustment by creatinine levels, myostatin was negatively related to TG levels ($r = -0.338$, $p = 0.028$) and the relation between myostatin with irisin and FPG persisted, with a significantly increased association between myostatin and these variables ($r = -0.427$, $p = 0.002$; $r = -0.395$, $p = 0.005$ respectively). However, the association between myostatin and irisin levels was not observed in the control group ($r = -0.162$, $p = 0.262$).

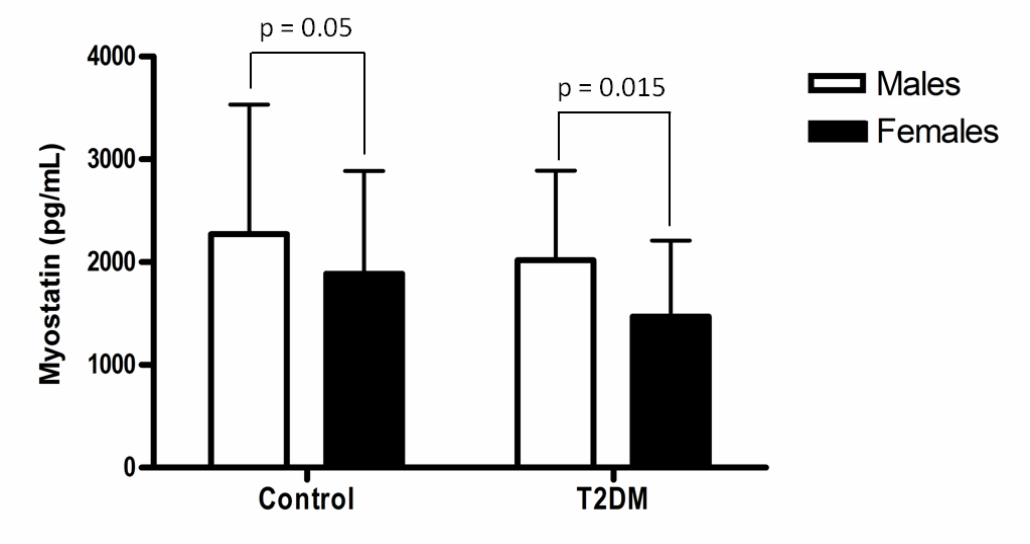
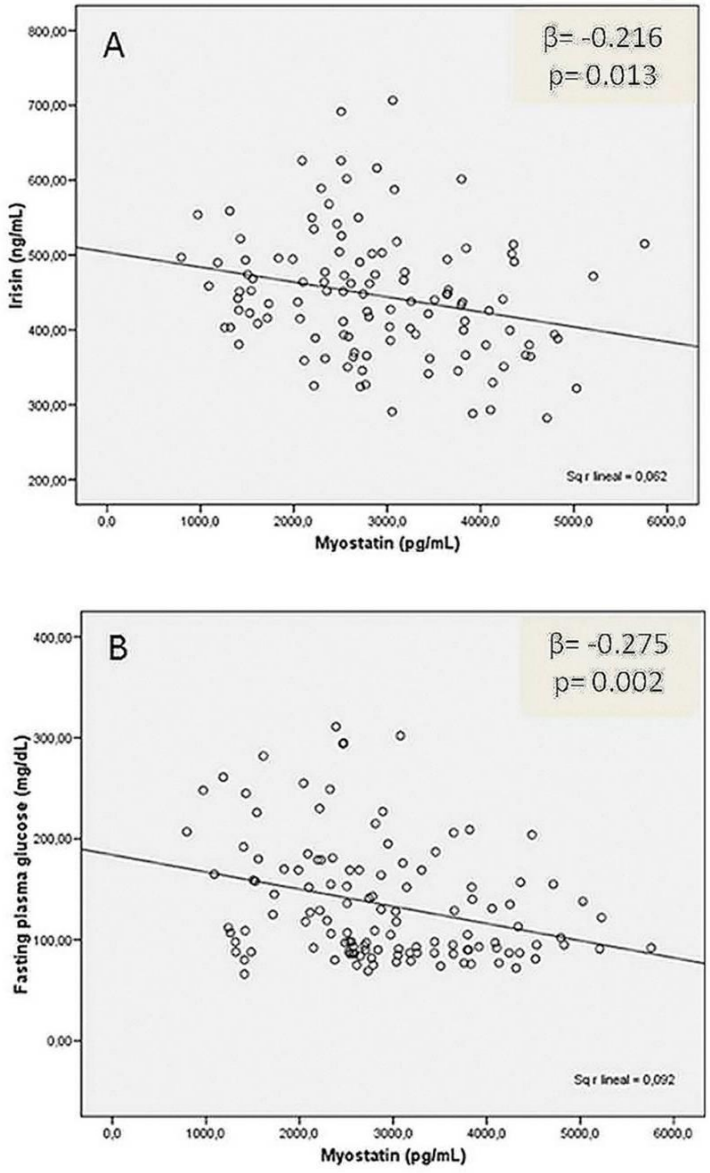


Fig. 13. Myostatin levels in T2DM and control group according to gender. Differences between groups were determined by the Mann-Whitney *T* test. The statistical significance was set at $p < 0.05$ (two-tailed).

A model of multiple linear regression was performed to evaluate the influence of sex, age, BMI, physical activity or antidiabetic drugs (insulin and antidiabetic oral drugs) on myostatin levels. The associated variables identified in the univariate correlation analysis (irisin, FPG and TG levels) were also included in the model as independent variables.

The results showed that gender (b = 0.344 [95% CI 364.340/1,072.204] p = 0.000), FPG (b = -0.275 [95% CI -8.338/-1.930] p = 0.002), irisin levels (b = -0.216 [95% CI -4.830/-0.583] p = 0.013) and age (b = -0.179 [95% CI -56.775/-1.394] p = 0.040) were associated with serum myostatin levels, independently of BMI (b = 0.029, p = 0.744), TG levels (b = -0.062, p = 0.489), physical activity (b = 0.046, p = 0.590) and T2DM drugs (insulin b = 0.143, p = 0.153; antidiabetic oral drugs b = -0.107, p = 0.260). This suggests that FPG and irisin levels might influence the serum levels of this myokine (Figure 14).



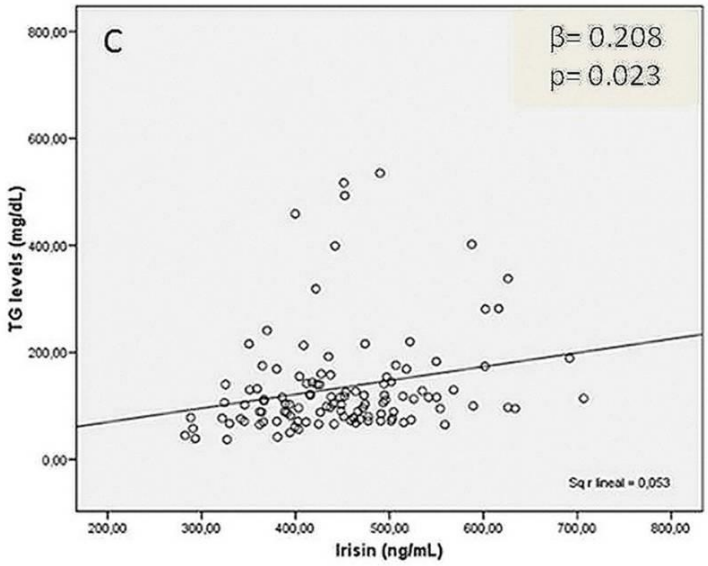


Fig. 14. Scatter plot showing the relationship between serum myostatin, irisin and other biochemical parameters by a backward multiple linear regression analysis. (A) Scatter plot between myostatin (dependent variable) and irisin. Independent variables: sex, age, BMI, physical activity, antidiabetic drugs (insulin and antidiabetic oral drugs), irisin, FPG and TG levels; **(B)** Scatter plot between myostatin (dependent variable) and FPG. Independent variables: sex, age, BMI, physical activity, T2DM drugs (insulin and antidiabetic oral drugs) irisin, FPG and TG levels; **(C)** Scatter plot between irisin (dependent variable) and TG levels. Independent variables: sex, age, BMI, physical activity, antidiabetic drugs (insulin and antidiabetic oral drugs) myostatin, FPG and TG levels.

4.3. Serum irisin in T2DM patients and controls

The T2DM patients had significantly higher serum irisin levels than the controls (462 ± 91 ng/mL vs 429 ± 73 ng/ mL respectively; $p<0.05$) (Figure 15).

We have found no differences on irisin levels according to physical activity, however we found increased levels of irisin in the group with BMI ≥ 30 in the whole sample (431.64 ± 79.87/ 467.47 ± 87.08 ng/mL; $p = 0.022$). There were no differences in irisin concentrations related to gender.

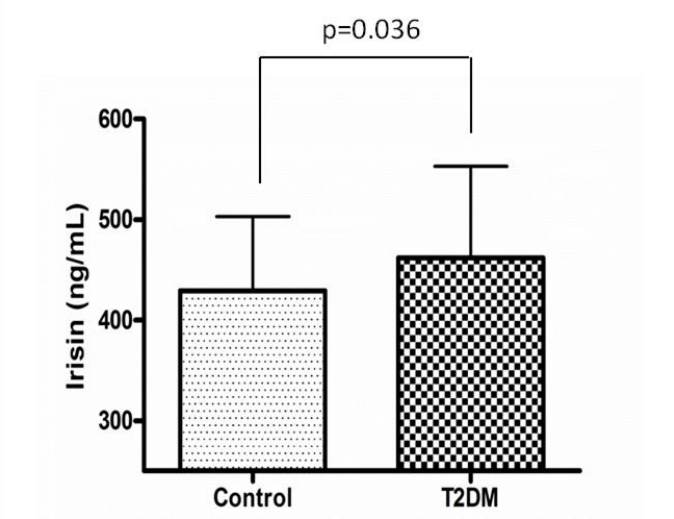


Fig. 15. Serum irisin levels in T2DM patients and control subjects.
Differences between groups were determined by the Mann-Whitney *T* test. The statistical significance was set at $p < 0.05$ (two-tailed).

Irisin levels correlated positively with BMI both in the total sample and in the T2DM group. In the T2D group, after BMI adjustment, irisin was negatively associated with myostatin (r = -0.405, p = 0.008) and positively associated with FPG and TG levels (r = 0. 368, p = 0.017; r = 0. 420, p = 0.006). Similarly as myostatin, no association was observed between the two myokines in the control group (r = -0.155, p = 0.287).

Likewise to myostatin, a multiple linear regression analysis was performed to evaluate the influence of sex, age, BMI, physical activity or antidiabetic drugs (insulin and antidiabetic oral drugs) on irisin levels. The associated variables identified in the univariate correlation analysis (myostatin, FPG and TG levels) were also included in the model as independent variables. The analysis demostrated that only myostatin (b = -0.221 [95% CI -0.032/-0.003] p = 0.017) and TG levels (b = 0.208 [95% CI 0.020/0.267] p = 0.023), were included in the model as variables independently associated to circulating irisin levels (Fig. 3). All other variables were excluded from the model (age (b = -0.048, p = 0.603), gender (b = 0.136, p = 0.156), BMI (b = 0.098, p = 0.281), FPG (b = 0.136, p = 0.171), physical activity (b

= 0.057, p = 0.529) and T2DM drugs (insulin b = -0.027, p = 0.768; antidiabetic oral drugs b = 0.080, p = 0.417)).

4.4. Association between serum myostatin and irisin levels with T2DM and obesity

Logistic regression analysis was conducted to determine whether irisin or myostatin levels were associated with T2DM or obesity. Firstly, the presence of T2DM was used as a dependent variable. Independent variables were those related to insulin resistance and cardiovascular risk factors (age, BMI, waist circumference, dyslipidaemia, HbA1c) to study the relationship of these myokines to the development of T2DM. Likewise, to evaluate the association between myostatin and irisin with the presence of obesity, a BMI C 30 was also included as a dependent variable and irisin, myostatin and the variables associated with obesity were included in the model as independent variables. Our results showed that irisin and myostatin levels were not related to the study group.

5. Discussion

Our cross-sectional study shows the presence of higher irisin levels and lower concentrations of myostatin, as well as an inverse relationship between both myokines in T2DM patients compared to the control subjects. Myostatin and irisin levels were found to be closely associated with glucose and lipid profile independently of the presence of diabetes or obesity.

Myostatin acts mainly as negative regulator of muscle mass, however, in vitro and animal studies suggest a role of myostatin in the regulation of glucose metabolism. Some evidence has reported that FPG levels and insulin sensitivity may improve after the inhibition or lowering of myostatin levels (Cleasby *et al.*, 2014b; Guo *et al.*, 2009; Zhang *et al.*, 2012). (Cleasby *et al.*, 2014b), show that the inhibition of myostatin stimulates the expression of glucose transporters GLUT1 and especially

GLUT4 increasing the capacity of basal and insulin-stimulated glucose uptake in rats. However, the results about myostatin effect in humans are inconsistent showing contradictory data (Antony *et al.*, 2007; Mitchell *et al.*, 2006; Zhang *et al.*, 2011) and little is known about the metabolic role of myostatin in T2DM patients as there is scarce and inconclusive data (Brandt *et al.*, 2012; Wang *et al.*, 2012).

Our results show decreased myostatin levels in T2DM patients compared to the control subjects. We found that myostatin was inversely associated with FPG and TG levels. Moreover, glucose levels seem to be independently associated with myostatin levels suggesting a possible inversely regulation of myostatin concentrations through blood glucose. The increased glucose and TG levels in T2DM patients could explain the lower levels of myostatin in the diabetic group compared to the controls. In agreement, a recent study has reported decreased myostatin serum levels in a large cohort of patients with metabolic syndrome compared to the control subjects and an association between myostatin levels and unfavorable metabolic parameters (Han *et al.*, 2014).

Recent studies have demonstrated that myostatin is not only linked to skeletal muscle and glucose metabolism but also plays an important role on lipid and energy metabolism. A deficiency of myostatin in mice activates lipolytic enzymes increasing fatty acid oxidation in mitochondria and peripheral tissues and induces brown adipose formation in WAT (Zhang *et al.*, 2012). However, the exact mechanism by which myostatin regulates the adipose tissue remains unknown. Recent studies suggest an involvement of irisin in the mechanism of action of myostatin during adipose tissue regulation.

The discovery of irisin has becoming important in the recent years due to its attributed beneficial effects. It was described that irisin is mainly involved in the regulation of thermogenesis through the browning of subcutaneous WAT and the stimulation of UCP1 expression. WAT is known to contain cells that can express high levels of UCP1, thus acquiring the typical appearance of brown adipocytes (Chen *et al.*, 2010). These are known as "beige adipocytes" and the process by which they are formed is known as "browning of WAT". Contrary to WAT, which stores

energy predominantly as triglycerides, brown adipose tissue dissipates energy by uncoupled mitochondrial respiration and therefore plays an important role in the regulation of body weight. Recent experimental evidence in mice demonstrated that deficiency of myostatin leads to PGC1 alpha activation and irisin secretion in muscle resulting in browning of white adipocytes (Shan *et al.*, 2013).

Our results also show an inverse association between irisin and myostatin and an independent association of the irisin levels to myostatin concentrations. These results suggest a connection between myostatin and irisin that could exert a combined action in the same direction. Supporting these findings, a recent study in human adypocites demonstrates that irisin treatment increases IGF-1 and decreases myostatin mRNA levels, the two main factors for muscle growth (Huh *et al.*, 2014). All these findings suggest a muscle-fat crosstalk through the connection between myostatin and irisin.

Some studies reveal an additional role of the irisin in glucose homeostasis (Choi *et al.*, 2013; Højlund and Boström, 2013), increasing the expression of glucose transporter 4 (GLUT4) and mitochondrial biogenesis (Vaughan *et al.*, 2014). Others have reported beneficial effects of irisin in obesity-related metabolic diseases, suggesting that it plays a protective role against insulin resistance (Hofmann *et al.*, 2014; Kurdiova *et al.*, 2014; Sesti *et al.*, 2014) making irisin a potential new target for the treatment of metabolic disorders. However, there is controversy about the health properties of irisin in humans. Some investigations question this beneficial role showing a positive correlation between irisin and related-obesity parameters (Crujeiras *et al.*, 2014; Pardo *et al.*, 2014). Little is known about the levels of irisin and the involvement of this protein in T2DM and there are inconsistencies regarding this. A few studies have found lower levels of irisin in T2DM patients compared to controls (Choi *et al.*, 2013; Liu *et al.*, 2013; Moreno-Navarrete *et al.*, 2013; Sanchis-Gomar and Perez-Quilis, 2014b). However, others have reported higher levels of irisin in prediabetic patients, women with gestational diabetes and patients with metabolic syndrome (Ebert *et al.*, 2014; Kurdiova *et al.*, 2014; Nair *et al.*, 1986; Piya *et al.*, 2014).

Our results show higher irisin levels in T2DM patients compared to non-diabetic subjects and a positive correlation of irisin with TG and glucose levels which are significantly higher in these patients, independently of the gender. Moreover, TG levels were independently associated to irisin serum levels suggesting a possible influence of lipid profile on irisin concentrations. A few studies have reported a positive association between irisin, TG levels and atherogenic factors in patients with metabolic syndrome (de la Iglesia *et al.*, 2014). Accordingly, a recent study showed that under certain conditions of elevated oxidative stress, inflammation and high levels of free fatty acids, such as in obesity, metabolic syndrome or diabetes, the expression of PGC1a in muscle could be stimulated increasing FNDC5 and subsequently irisin levels which stimulate the production of brown adipocytes, increasing UCP1 expression and thermogenesis and decreasing insulin resistance (Choi *et al.*, 2013; Sanchis-Gomar and Perez-Quilis, 2014b). In agreement with these findings, recent evidences in human adipocytes show that increased irisin levels also decrease lipid accumulation through an increase of adipose triglyceride lipase (ATGL) and a decrease of fatty acid synthase (FAS) expression. This implies that irisin promotes the decrease of stored fat increasing UCP1 expression and thermogenesis and inhibiting the synthesis of lipids. As a result, T2DM patients have greater energy expenditure which has been reported in classical studies in this field (Bogardus *et al.*, 1986; Fontvieille *et al.*, 1992; Nair *et al.*, 1986).

According to our results we propose the following mechanism of action of these two myokines in T2DM. The elevated glucose levels would act as an alarm signal that downregulates the myostatin expression. The lack of myostatin concentrations would act on glucose metabolism inducing the expression of glucose transporters and on lipid metabolism inducing the activation of lipolytic enzymes that increase fatty acid oxidation and stimulating the formation of brown adipocytes. Consequently, decreased myostatin levels along with the high TG content, increased oxidative stress and inflammatory state typical of T2DM patients would lead to upregulated irisin levels. Increased irisin levels would act in parallel on the same targets that myostatin through the stimulation of glucose transporter expression, mitochondrial biogenesis as well as the formation of brown adipocytes activating

thermogenesis and lipid consumption. Under these conditions, glycolysis and oxidative metabolism increase to consume energy and to improve glucose and lipid metabolism in these population. This mechanism of action could explain the positive association between irisin and related-obesity parameters reported in previous studies (Crujeiras *et al.*, 2014; Pardo *et al.*, 2014).

Although there are several studies that support the offsetting effect of these hormones, there are also other hypotheses that attempt to explain the levels of these myokines in patients with metabolic disorders. Thus, some studies have proposed that increased irisin levels could represent an irisin resistance process similar to insulin or leptin resistance in patients in risk for or with metabolic disturbances (Boström and Fernández-Real, 2014; Panagiotou *et al.*, 2014).

According to our results, we speculate that the effects of irisin and myostatin represent an adaptive response to counteract the metabolic disturbances in T2DM reflecting the need for action of these myokines in muscle and adipose tissue although direct evidence is needed to elucidate the exact role of these myokines in humans.

Our study has certain limitations. Firstly, a case–control study does not allow us to establish a cause-effect relationship and the directionality of the results. Therefore, further studies are needed to elucidate whether decreased myostatin levels and increased irisin levels represent a compensatory mechanism to restore the metabolic disturbances in T2DM or whether their levels represent a reflection of these metabolic disturbances. Secondly, specific studies are needed to properly evaluate the posible influence of the antidiabetic drugs in the levels of these myokines. Thirdly, we did not measure other markers of interest such as UCP1, glucose transporters, plasma insulin levels or free fatty acids that would provide additional information contributing to a better understanding of the mechanism of action of these myokines. Finally, our study was conducted in a specific population, and it cannot be assured that the same results would be obtained in other ethnic or study groups.

The strengths of this study lie in the novel evaluation of circulating serum myostatin and irisin levels in humans supporting that these myokines could act as the same way as reported in animal models. Moreover, we provide the explanation of a possible mechanism of action which involves the connection between muscle and adipose tissue through the action of these myokines in patients with T2DM opening new lines of research in this field.

In conclusion, we suggest that irisin and myostatin levels could be interconnected, acting on the same targets in response to an unfavourable metabolic state, increasing glucose uptake and beige adipocyte formation and activating energy metabolism and thermogenesis in diabetic patients. This comprehensive process could occur as a compensatory mechanism to regain the metabolic balance. However, future studies are needed to confirm these preliminary results.

CAPÍTULO II

Relationship between Proinflammatory and Antioxidant Proteins with the Severity of Cardiovascular Disease in Type 2 Diabetes Mellitus

Adaptado de: Relationship between Proinflammatory and Antioxidant Proteins with the Severity of Cardiovascular Disease in Type 2 Diabetes Mellitus.

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1. Abstract

Type 2 diabetes mellitus patients are at significant risk of cardiovascular disease, however, the pathophysiology of these complications is complex and incompletely known in this population. The aim of this study was to compare the serum proteome of patients with type 2 diabetes mellitus presenting or not presenting cardiovascular disease with non-diabetic subjects to find essential proteins related to these cardiovascular complications.

This cross-sectional study compares the serum proteome by a combination of protein depletion with 2D-DIGE (2-dimension Difference Gel Electrophoresis) methodology. The proteins differentially expressed were identified by MALDI TOF/TOF (Matrix-assisted laser desorption/ionization and Time-Of-Flight ion detector) or LC-MS/MS (Liquid Chromatography coupled to Mass-Mass Spectrometry). Type 2 diabetes mellitus patients with cardiovascular disease showed higher expression of plasma retinol binding protein and glutathione peroxidase-3 compared to those without cardiovascular disease and non-diabetic controls. These results show that proteins related to the inflammatory and redox state appear to play an important role in the pathogenesis of the cardiovascular disease in the type 2 diabetes mellitus patients.

2. Introduction

In developed countries, type 2 diabetes mellitus (T2DM) represents a major public health problem mainly by its relationship with different cardiovascular diseases (CVD) (Jensen *et al.*, 2014). Long-term hyperglycemia results in the synthesis of a number of molecules like advanced glycation end products (AGEs), advanced oxidation protein products (AOPPs), and low-density lipoprotein susceptibility to oxidation (oxLDL).

Besides chronic hyperglycemia, other factors such as insulin resistance, dyslipidemia and states of inflammation and oxidation are related to vascular injury in diabetes through several underlying processes (Tousoulis *et al.*, 2013). However, only some diabetic patients develop cardiovascular disease while others do not develop these complications despite having the same common risk factors. The etiologic pathway linking impaired glucose tolerance and cardiovascular disease remains to be clarified. Many factors, including genetic components may be involved and not all are well established. Currently, multiple areas of research are open to explain this complex phenomenon (Paneni *et al.*, 2015) and the precise role of the different disturbed metabolic pathways is not well established. In this context, the identification of new molecules that take part in the development of these vascular complications in T2DM may be of great importance for improving the outcomes of this population or to design new therapeutic targets. Thus, the underlying factors that may lead to the development of CVD require further in-depth research. The proteomic analysis is a hypothesis-free approach integrating genetic and epigenetic influences by examining the protein expression profiles and is not limited by the above knowledge.

Human serum is rich in potential biomarkers reflecting the pathophysiological state of the human body and related to the impaired metabolic pathways present in different disorders. In this way, it is able to facilitate the early detection of many diseases. However, finding biomarkers in serum is difficult due to the interference of the main proteins that provide a significant background covering low abundance proteins (Zhang *et al.*, 2004). To overcome this problem, serum depletion is essential

for removing the major proteins and to improve the sensitivity of detection methods for these minor proteins.

The main objective of this cross-sectional study was to compare the serum proteome of the T2DM patients with and without CVD and non-diabetic controls to find protein differences in the protein profile expression of the study groups. Candidate proteins were identified by MALDI TOF/TOF or LC-MS/MS in order to identify essential proteins related to severe cardiovascular disease in patients with T2DM.

Here we report that proteins involved in inflammatory and redox state, such as retinol binding protein (RBP4) and glutathione peroxidase 3 (GPx-3) have increased levels in the serum of T2DM patients with severe CVD compared to those without CVD and non-diabetic controls. These differentially expressed proteins could be potential markers used for diagnosis or prognosis of CVD.

3. Subjects and methods

3.1. Study Population

Our cross-sectional study included 18 males divided according to three study groups: (i) T2DM patients with CVD; (ii) T2DM patients without CVD; (iii) Non-diabetic subjects as controls. To achieve 85% statistical power to detect differences among groups it is necessary to include six experimental units per group, resulting in a total sample size of 18 subjects in the experimental study.

Diagnosis of diabetes was according to the American Diabetes Association criteria (2005). From January 2006 to December 2007 we consecutively recruited patients who had been referred to our outpatient clinic from primary care centers for treatment of diabetes. The inclusion criteria for patients with CVD were coronary

heart disease (previous myocardial infarction or coronary revascularization surgery), cerebrovascular disease (ischemic stroke), or ischemic peripheral arterial disease.

The control group consisted of non-diabetic subjects consecutively recruited from the general community in the same period of time and matched with diabetic patients for demographic characteristics.

All were Caucasians, ambulatory and had neither renal, hepatic, gastrointestinal nor thyroid diseases. All T2DM patients were receiving medications for diabetes, including metformin, sulfonylureas, insulin or a combination of these drugs.

Samples of venous blood were obtained from patients in the Endocrinology Unit of University Hospital San Cecilio in Granada. Samples of venous blood in vacutainer tubes containing no anticoagulant were taken in the morning after fasting overnight. Samples were incubated at room temperature for 30–45 min and centrifuged for 15 min at 7500 rpm. Then, the supernatant was carefully aspirated by a serologic pipette, aliquoted into 50 μL cryovials and stored at −80 °C until the examination.

The study was conducted with the approval of the ethical committee of the San Cecilio University Hospital and conformed to the relevant ethical guidelines for human and animal research (Project ID: PI 0514-2012. Research Ethics Committee of Granada Center (CEI-Granada) at 26 November 2012). Written informed consent was obtained from all subjects.

3.2. Depletion, Concentration and Cleaning of Serum Samples

A chromatographic removal of the 14 most abundant serum proteins (albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin, fibrinogen, alpha 2-macroglobulin, alpha 1-acid glycoprotein, IgM, apolipoprotein AI, apolipoprotein AII, complement C3 and transthyretin) was performed in 50 μL of whole serum using Multiple Affinity Removal Spin Cartridges (Agilent, Santa Clara, CA, USA) according to the user manual. The depleted fractions were concentrated by a 5 kDa Mw cut off spin concentrators (Agilent) at 4000 rpm, 60 min at 10 °C. The ReadyPrep 2D cleanup

kit (BioRad, Hercules, CA, USA) was used to reduce streaking, background staining, and other gel artifacts that may contaminate the 2D/IEF samples. The resultant pellet was dispersed into the adequate volume of 2D gel electrophoresis buffer (8 M Urea, 4% CHAPS) to solubilize protein samples. The samples were separated in aliquots of 11.5 μL for DIGE assay and 0.5 μL for protein determination, pH adjustment and 1D electrophoresis.

3.3. 2D-DIGE

3.3.1. CyDye Fluors Preparation and Sample Labeling

The CyDye DIGE fluors (Kit labeling minimal CyDye DIGE fluor 1 × 5 nmol (GE Healthcare, Freiburg, Germany)) were dissolved in dimethylformamide up to 400 pmol/mL of working solution. Sodium hydroxide at 0.1 M diluted 1:10 was added to each sample to adjust pH at 8.5–9 for the labeling reaction. Randomization of samples across gels removes any bias from the experiments.

Fifty μg of protein from each sample was minimally labeled on ice for 30 min with 1 μL of corresponding fluorescent dye working solution according to the Ettan DIGE System user manual (GE Healthcare). The internal standard (IS) was generated by combining equal amounts of each sample and labeled with Cy2 fluorescent dye. The reaction was stopped by addition and incubation of 10 mM lysine for 10 min on ice in the dark (1 μL Lys per 50 μg protein). To prepare the samples for each gel, two samples were mixed with 50 μg of IS according to the user manual of 2D-DIGE GE Healthcare (Table 3). Twenty mM DTT and 0.5 mM PMSF were added to the mix. Finally, a total of nine 2D gels were run.

Table 3. Sample arrangement on each gel and staining with each corresponding fluorophore. A: T2DM + CVD group; B: T2DM − CVD group; IS: Internal standard.

Gel Number	Cy2	Cy3	Cy5
1	IS	Control 1	Sample B4
2	IS	Sample A1	Control 4
3	IS	Sample B1	Sample A4
4	IS	Control 2	Sample B5
5	IS	Sample A2	Control 5
6	IS	Sample B2	Sample A5
7	IS	Control 3	Sample B6
8	IS	Sample A3	Control 6
9	IS	Sample B3	Sample A6

3.3.2. 2D Gel Electrophoresis

IPG (0.5%) buffer was added to destreak solution (GE Healthcare) after 30 min to temper the solution at RT. The adequate volume was added to the sample and placed into the strip holders. The IPG strips (pH 3–10, 24 cm, GE Healthcare) were located over the sample, covered with mineral oil and focused on an IPGphor III (GE Healthcare) following these steps: 1 h at 500 V, 1 h at 1000 V, 3 h at 8000 V, 8000 V until 20,000 V/h and 500 V as maintenance step. The run was monitorized by IPGphor III software (GE Healthcare) ensuring that isoelectric focusing (IEF) process was successful. The strips were twice equilibrated for 15 min in the adequate buffer containing 75 mM Tris buffer (pH 8.8), 6 M Urea, 30% glycerol, 2% SDS and Bromophenol Blue. The first equilibration was with 1% DTT, and the second one with 2.5% Iodoacetamide in the equilibration buffer. The second dimension was carried out using 12% polyacrylamide gels at 13 mA per gel for 1 h in the first step and 17 mA per gel for 5.50 h in the second step. The proteins were visualized by Typhoon 9400 fluorescence scanner (GE Healthcare).

The image analysis was performed by the specific software DeCyder 7.0 (GE Healthcare), that performs an automatic analysis and a co-detection of the fluorescent gels images by background subtraction, normalization, inter-gel matching and quantization. By using the statistical analysis by Decyder 7.0 (GE Healthcare) the sum of the pixel values within a spot minus background for each experimental sample is compared directly to the internal standard. Thereby, the protein abundance for each spot is expressed as a normalized ratio relative to the internal standard. This analysis compares the average ratio and variation within each group to the average ratio and variation in the other groups to see if any change between the groups is significant. Thus, it is possible to compare the protein abundance for a protein of interest in different samples. The total proteins were detected by Sypro staining (BioRad).

3.3.3. Identification of the Candidate Biomarker by MALDI-TOF/TOF

The analysis by Decyder 7.0 performs the matching of the gels and creates a pick list after the scan. Selected protein spots are automatically picked from 2D representative gel by Ettan Spot Picker (GE Healthcare) using the pick list generated. The gel plugs are transferred into microplates for further digestion prior to identification. The gel pieces were reduced with DTT for 60 min at room temperature and cysteines were carbamidomethylated with iodoacetamide for 30 min. The proteins were digested with trypsin (Gold, MS Grade, Promega, Madison, WI, USA) for 8 h at 37 °C. The resulting peptide mixture was spotted on a MALDI plate with CHCA and analyzed using the 4800 MALDI TOF/TOF mass spectrometer (AB-Sciex, Madrid, Spain). The MALDI-TOF/TOF spectra were interpreted by database search MASCOT (Matrix Science, Boston, MA, USA) with a significance threshold of the MOWSE score of $p < 0.05$. The database used for identification was NCBI restricted to mammalian proteins with the following parameters: peptide mass tolerance 100 ppm, fragment tolerance 0.2 Da, enzyme set as trypsin and allowance up to one missed cleavage, variable modification of methionine oxidation (+16 Da), fixed modification of cysteine

carbamidomethylation (+57 Da). Only the proteins with the better identification parameters and the best score during the search in databases have been included in the list of results. Other identified proteins with lower score were identified (Data not shown).

3.3.4. LC-MS/MS Analysis

The tryptic extracts were reanalyzed by high resolution LC-MS/MS in data-dependent mode. The MS system used was an Orbitrap XL (ThermoFisher, Waltham, MA, USA) equipped with a microESI ion source (Proxeon). The tryptic extracts were diluted to 20 μL with 5% methanol and 1% formic acid. Then, extracts were loaded into a chromatographic system consisting of a C18 preconcentration cartridge (Agilent, Santa Clara, CA, USA) connected to a 15 cm long, 100 μm i.d. C18 column (Nikkyo Technos Co., Tokyo, Japan). The separation was done at 0.4 μL/min in a 30 min acetonitrile gradient from 3% to 40% (solvent A: 0.1% formic acid, solvent B: acetonitrile 0.1% formic acid). The HPLC system was composed of a 1200 capillary nano pump (Agilent), a binary pump, a thermostated micro injector and a micro switch valve. The Orbitrap XL was operated in the positive ion mode with a spray voltage of 2 kV. The scan range for full scans was m/z 400–2000. The spectrometric analysis was performed in a data-dependent mode, acquiring a full scan followed by 10 MS/MS scans of the 10 most intense signals detected in the MS scan. An exclusion time of 30 s was included to avoid repetitive MS/MS analysis of the dominant MS signals. LC-MS/MS spectra were searched using SEQUEST (Proteome Discoverer v1.3, ThermoFisher) with the following parameters: peptide mass tolerance 10 ppm, fragment tolerance 0.8 Da, enzyme set as trypsin and allowance up to two missed cleavages, dynamic modification of methionine oxidation (+16 Da), fixed modification of cysteine carbamidomethylation (+57 Da).

The database used for searching included human proteins (Uniprot taxonomy 9606). The peptide identifications were filtered for 0.1% FDR and only proteins identified

with two or more peptides were considered. Only the proteins with the better identification parameters and the best score during the search in databases have been included in the list of results. Other proteins with lower score were identified (Data not shown).

3.4. Western Blot Analysis

A biochemical validation of protein candidates was performed by western blot analysis using standard techniques. In order to get a better approximation to the physiologic state of the individuals we used whole serum samples. We performed the division of 15 samples from each study group into 3 groups of 5 individuals each one, giving a total of 9 groups (3 groups of Control, 3 groups of T2DM + CVD and 3 groups of T2DM − CVD). The 9 groups were compared in three independent Western blots. Equal volumes of the pooled samples were separated on a 1D SDS-PAGE using 15% polyacrylamide gels during 60 min at 150 V. The separated proteins were transferred to PVDF membrane on Trans-Blot Turb Transfer System (BioRad), following the transfer protocol for 1.5 mm gels. The membranes were blocked in phosphate-buffered saline (PBS) supplemented with 5% (*w/v*) non-fat dry milk (BioRad) for 2 hours at RT. Afterwards the membranes were washed with PBS 0.05% Tween 20 (PBST) during 10 min and subsequently they were incubated overnight with polyclonal human anti-goat primary antibodies: Anti-serum retinol binding protein (RBP4) ((SAB2500875), Anti-Transthyretin (TTR) (SAB25001931), Anti-glutathione peroxidase 3 (GPx-3) (SAB2500485) Sigma-Aldrich, Madrid, Spain); Anti-C3 complement ((A304) Quidel, San Diego, CA, USA), at 4 °C in PBS containing 0.1% albumin (PBSA). After three washes with PBST, the membranes were incubated with corresponding secondary horseradish peroxidase-conjugated antibody (A5420, Sigma-Aldrich) diluted 1:20,000 in PBSA for 1 h at RT. The proteins were detected by chemiluminiscence using Clarity Western ECL Substrate (BioRad) and the ChemiDoc gel imaging system (BioRad). The bands were quantified in three independent experiments by Quantity One software (BioRad), normalized to value 1 corresponding to the

control and expressed as intensity difference relative to the control group (ratio disease group/control group).

3.5. Statistical Analysis

The data for continuous variables are presented as mean ± standard deviation (SD). The data for categorical variables are presented as numbers and/or percentages. Kolmogorov-Smirnov test was used to test the normal distribution of continuous variables. Comparisons of categorical variables among groups were performed using the Chi-square test. Comparison of continuous variables between case-control was performed using the unpaired Student's *t* test, or the Mann-Whitney test. The statistical significance was set at $p < 0.05$ (two-tailed) and performed with the specific software, SPSS 18.0 (Chicago, IL, USA).

For statistical analysis by Decyder 7.0, the individual gels were processed in Differential In-gel Analysis module (DIA) to spot detection and first in-gel analysis for the images from each single gel removing background and artefacts and normalizing the gel images. The Biological Variation Analysis module (BVA) was used to analyze DIA spot maps of all gels in one DIGE experiment, to merge gel spot maps, to normalize intensities and to obtain statistical differences between groups. The analysis of variance (ANOVA) was applied to matched spots. The data was filtered to retain spots with ANOVA *p*-values of 0.05 or less.

To get a power of 85% to find differences in testing the null hypothesis "The proportions of the three groups are equal using a Chi-square test for three independent samples, considering that the significance level is 5% and assuming that the expected proportions of the three groups are specified by the researcher (85%, 10%, 10%)", it is necessary to include 18 experimental units distributed equally among the 3 groups (6 units per group).

4. Results

4.1. Baseline Characteristics of the Study Population

The clinical and demographic characteristics of the study groups (Control, T2DM + CVD, T2DM − CVD) are summarized in Table 4.

Table 4. Anthropometric and biochemical parameters of the study population.

Group	**Control** (***n*** **= 6)**	**T2DM + CVD** (***n*** **= 6)**	**T2DM – CVD** (***n*** **= 6)**
Age (years)	56 ± 4	58 ± 4	49 ± 11
Measurements			
BMI (kg/cm^2)	28.9 ± 3.8	29.2 ± 3.2	25.6 ± 4.2
Fasting glucose (mg/dL)	88.1 ± 7.8	202.6 ± 64.4 *	156.6 ± 64.9 *
HbA_{1C} (%)	4.56 ± 0.20	9.60 ± 2.89 *	7.76 ± 2.42 *
sBlood pressure (mm Hg)	133.3 ± 15.1	123.3 ± 34.4	121.6 ± 27.1
dBlood pressure (mm Hg)	83.3 ± 13.6	66.6 ± 13.6	77.5 ± 7.5
LDL (mg/dL)	129 ± 21	83.5 ± 59.5	127.1 ± 20.5
HDL (mg/dL)	52.3 ± 10.2	54.3 ± 24.1	47.0 ± 9.9
TG's (mg/dL)	150.1 ± 70.5	169.1 ± 151	177.5 ± 155.5
IMT (mm)	0.62 ± 0.10	0.88 ± 0.13 *	0.77 ± 0.18
Creatinine (mg/dL)	0.92 ± 0.20	0.97 ± 0.17	0.90 ± 0.08
GFR (mL/min)	90.53 ± 16.66	84.85 ± 13.71	97.31 ± 11.94
Medical history			
Duration of diabetes (years)	-	15 ± 8 **	10 ± 5 **
Cerebrovascular disease	-	(1/6) 16.7%	-
Peripheral artery disease	-	(2/6) 33.3% *	-
Coronary heart disease	-	(5/6) 83.3% **	-
Carotid plaques (%)	-	33.3 *	16.7

Aortic calcifications (%)	-	40 *	16.7
Active smokers (%)	66.7	83.3	66.7
Sedentarism (%)	50	50	33.3
Alcohol (%)	16.7	50 *	33.3
Current medication			
Statins (%)	16.7	83.3 *	33.3
Oral antidiabetic drugs (%)	-	100 **	83.3 **
Insulin (%)	-	50 *	33.3

BMI: body mass index; HbA_{1C}: Glycated haemoglobin; s: Systolic; d: Dyastolic; LDL: Low density lipoproteins; HDL: High density lipoproteins; TG's: Triglycerides; IMT: Intima media thickness; GFR: Glomerular filtration rate. The data for continuous variables are presented as mean ± SD. The data for categorical variables are presented as percentages. ANOVA model or Kruskal Wallis test; X^2 for comparisons of categorical variables: * $p < 0.05$ for the control group *vs.* T2DM + CVD/T2DM−CVDgroups; ** $p < 0.001$ for the control group *vs.* T2DM + CVD/T2DM − CVD groups.

All groups were comparable in anthropometric and biochemical parameters except for those associated with T2DM, CVD and the related medication. As expected, fasting glucose and glycated haemoglobin (HbA1c) were significantly higher in the T2DM patients compared to the controls. The T2DM group presenting CVD showed a longer duration of diabetes compared to the diabetic group without CVD. Moreover, there was significantly higher percentage of patients with abnormal intima-media thickness (IMT) and aortic calcifications in T2DM patients with CVD compared to the control group.

4.2. Identification of Candidate Biomarkers

Using 2D-DIGE and MALDI TOF/TOF or LC-MS/MS spectrometry methodology, we have identified five spots differentially expressed between groups. The MASCOT database search allowed the protein identification of two spots from MALDI TOF/TOF analysis, and the other three spots were identified by using LC-MS/MS methodology and SEQUEST data analysis program.

The identified spots corresponded to serum retinol binding protein (RBP4), glutathione peroxidase 3 (GPx-3), which were increased, and to transthyretin (TTR), that was decreased in serum of the T2DM patients with CVD compared to the other groups. The chain A of C3b Complement was decreased in the serum of the T2DM patients independently of the presence of CVD compared to the control subjects.

The proteins identified by gel trypsin digestion and MALDI-TOF/TOF or LC-MS/MS with detailed information and concentrations compared to the control subjects are summarized in Table 5.

Table 5. List of the candidate biomarkers identified by MALDI-TOF/TOF or LC-MS/MS analysis.

				DeCyder analysis		MALDI TOF/TOF			LC-MS/MS				
Pos[a]	Protein name	ID[b]	E MW/pI[c]	*p*[d]	App[e]	Score[f]	Sec Cov[g]	Nºpep[h]	Score[i]	Sec Cov[j]	Nºpep		Change[k]
1	Plasma retinol binding protein	Q5VY30	22.5/5.2	0.004	27/27				17.38	12.06	3	↑	CVD
2	Transthirretyn	P02766	13.8/5.4	0.037	27/27				47.21	68.71	8	↓	CVD
3	Gluthathione Peroxidase	P22352	22.7/5.3	0.047	24/27	57	96	2				↑	CVD
4	Chain A of C3b Complement	P01024	70/7.0	0.012	27/27	495	50	34				↓	T2DM
5	Chain A of C3b Complement	P01024	70/6.8	0.023	27/27	310	41	28				↓	T2DM

[a] Position of the the spot in 2D-DIGE representative map; [b] Protein accession number in Uniprot Database; [c] Experimental molecular weight (kDa) / isoelectric point; [d] p value of the DeCyder analysis according to the ANOVA model; [e] number of the maps in which the spot appears from a total of 27 maps; [f] MALDI TOF/TOF protein score; [g] amino acid sequence coverage for the identified protein in percentage; [h] number of peptides matched by mass-mass spectrometry; [i] LC-MS/MS protein score; [j] amino acid sequence coverage for the identified protein in percentage; [k] expression change in the T2DM+CVD and T2DM-CVD serum compared to control serum.

Figure 16 shows a Sypro stained representative map with the pick location of the spots differentially expressed in the serum of the T2DM patients with or without CVD compared to the control subjects.

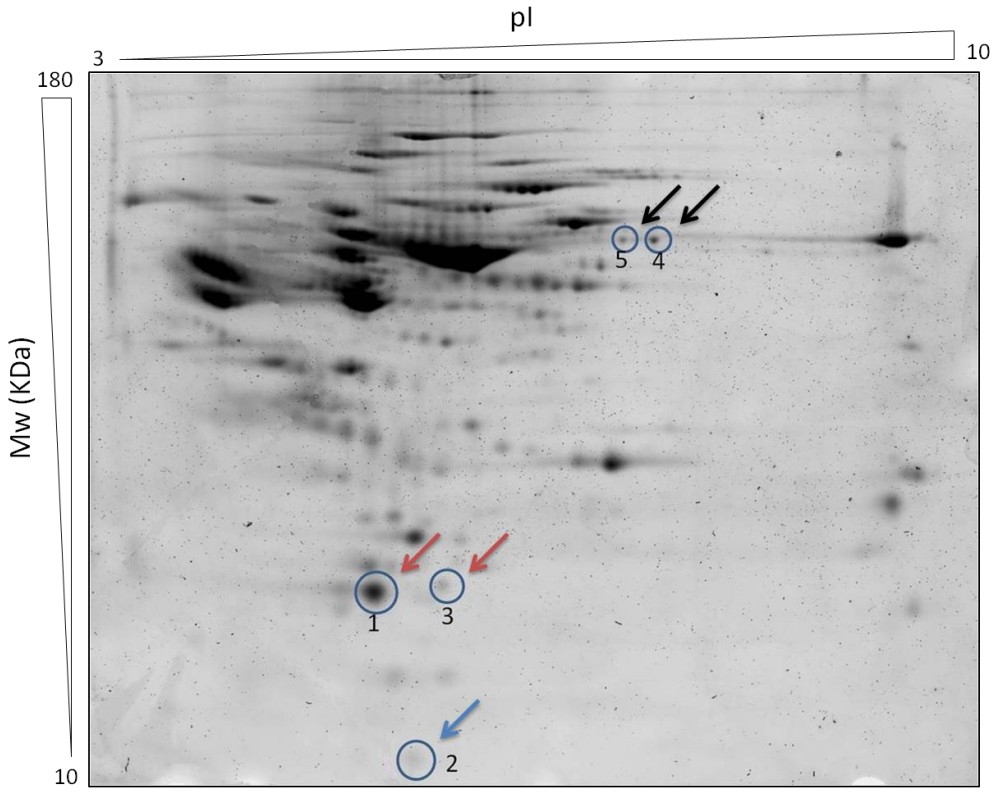

Fig. 16. Sypro stained representative 2D-DIGE map of the depleted serum showing the pick location of the proteins differentially expressed. The protein spots found significantly increased ($p < 0.05$) in T2DM + CVD patients compared to the others groups are marked with red arrows; The protein spot found significantly decreased ($p < 0.05$) in T2DM + CVD patients compared to the others groups are marked with blue arrow; The protein spots found significantly decreased in T2DM patients ($p < 0.05$) in regard to the control group are marked with black arrows.

In order to confirm the results obtained by 2D-DIGE, we performed a Western blot analysis from 15 whole and pooled serum samples of each group. In agreement with our results, the spot 1 corresponding to serum RBP4, and the spot 3 correponding to GPx-3, showed significative increased concentrations in the T2DM patients presenting CVD compared to the controls (Figure 17).

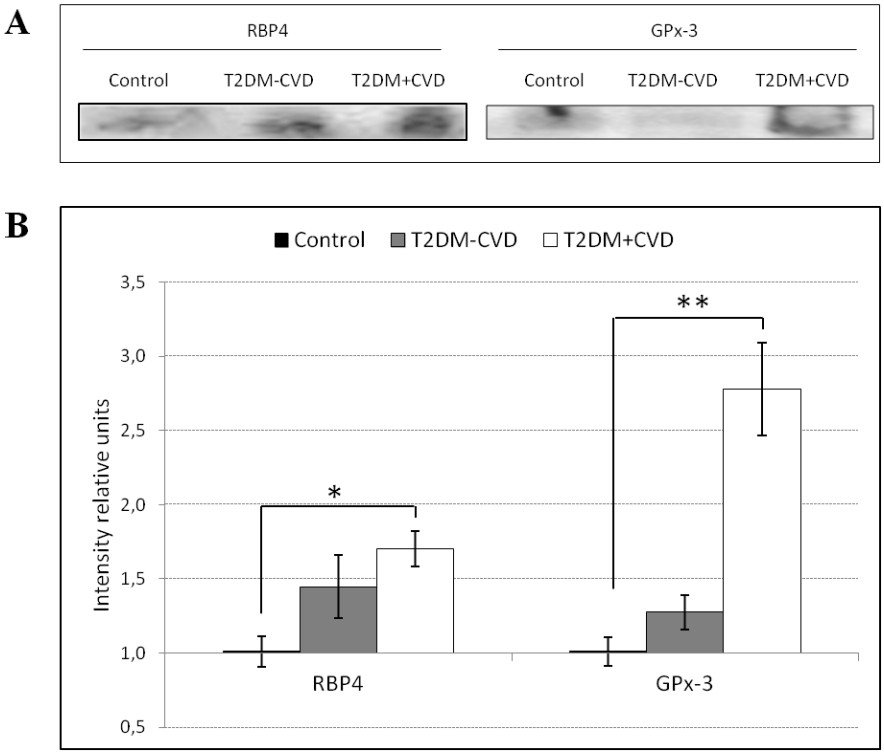

Fig. 17. Analysis of RBP4 and GPx-3 expression levels in whole pooled serums from the T2DM patients with and without CVD and the control subjects in three independent experiments of five subjects per group (45 study subjects in total). (**A**) Representative western blot analysis of correspondent RBP4 and GPx-3 pattern showing an increase of RBP4 and GPx-3 in T2DM patients compared to control group; (**B**) Quantification of the protein levels by densitometry analysis of the three western blots showing a significant increase of RBP4 and GPx-3 between T2DM patients with CVD and control subjects. The gel bands were normalized to value 1 corresponding to the control and the protein expression from the T2DM patients with and without CVD is represented relative to the control group. Differences between groups were determined by the Mann-Whitney U test. * $p < 0.05$; ** $p < 0.001$.

However, we were unable to detect TTR and complement C3b in the analysis conducted by western blot.

5. Discussion

Diabetes mellitus currently represents a serious health problem since affects a large proportion of the population (Chen *et al.*, 2012b). The patients with type 2 diabetes mellitus have increased risk for developing many complications mainly cardiovascular events depicting the major cause of mortality of this disease (Fruchart *et al.*, 2014; Iglesias *et al.*, 2015). The probability of developing vascular complications in this population depends on a number of traditional factors and others that are not fully known. The knowledge of new factors involved in these disorders may facilitate the identification of the subjects in whom an intensive approach on cardiovascular risk factors should be established before irreversible damage occurs.

The present study was conducted to analyze the differences in the serum proteome profile of the T2DM patients with and without severe CVD in order to identify the proteins which could be related to higher cardiovascular risk in this population. Since the majority of patients with prolonged duration of T2DM usually show some sign of subclinical vascular disease, we select patients with established cardiovascular disease to identify the proteomic profile associated with more severe and advanced cardiovascular disease.

Our proteomic analysis by using 2D-DIGE and LC-MS/MS revealed changes in proteins related to the inflammatory and redox state. Over recent years, the inflammatory state is becoming more important as a factor involved in the cardiovascular disorders. We found increased RBP4 concentrations in the T2DM patients, being significantly higher in those with CVD compared to those without CVD and the control subjects. RBP4 is a protein, member of the lipocalin family, involved in diverse functions like sensory transduction and carrier of retinol. In plasma, the RBP4-retinol complex interacts with transthyretin, preventing its loss by filtration through the kidney glomeruli. RBP4 is generated by mature adipocytes (Friebe *et al.*, 2011) and activated macrophages (Broch *et al.*, 2010). The involvement of RBP4 in insulin resistance has been described in some studies (Friebe *et al.*, 2011; Park *et al.*, 2014; Shaker *et al.*, 2011). Clinical studies have shown that exercise, or

other weight loss interventions, could improve the insulin sensitivity by reducing RBP4 serum levels (Balagopal *et al.*, 2007; Haider *et al.*, 2007). Furthermore, a very recent study determines the mechanism by which RBP4 induces insulin resistance through activation of the antigen presenting cells (APC) of the adipose tissue. This activation induces higher levels of proinflammatory cytokines resulting in an inflammation of the adipose tissue that leads to insulin resistance (Moraes-Vieira *et al.*, 2014).

However, the association of RBP4 with cardiovascular events and their potential utility as a biomarker of CVD is not well known. In our study we found an increased expression of RBP4 in diabetic patients with cardiovascular disease in regard to the others groups. These findings are consistent with two preliminary studies which show a relationship between RBP4 levels and common cardiovascular risk factors in the elderly population (Ingelsson *et al.*, 2009; Sasaki *et al.*, 2010). Furthermore, (Farjo *et al.*, 2012) have recently shown that RBP4 promotes the expression of proinflammatory molecules such as vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), *E*-selectin and interleukin 6 (IL-6) through activation of NF-$\varkappa$B and NADPH oxidase in an *in vitro* study. This effect is RBP4-dose dependent and induces the adhesion of leukocytes to the endothelium leading to endothelial inflammation. Our results are in agreement with these recent data suggesting that the elevation of RBP4 could be one of the main causes involved in the development and the progression of CVD associated to T2DM.

Another factor involved in the inflammatory state and vascular lesions is the oxidative stress due to the increase of reactive oxygen species (ROS) (Abe *et al.*, 2013). To offset the toxic effects of free radicals produced during vascular lesions over cells, a cascade of redox reactions between nitric oxide (NO), a neurotransmitter with vasodilatory function, and ROS takes place. This process increases lipid peroxidation (Beckman *et al.*, 1990) causing cell damage of the vascular system and aggravating CVD in these patients. Since oxidized lipoproteins constitute one of the most important factors involved in the development of

atherosclerosis (Ehara *et al.*, 2001), the endogenous defense systems are essential in the prevention of these complications. According to this, our results revealed an increase of GPx-3 in the serum of diabetes patients with CVD compared to those without CVD and controls. GPx-3 belongs to the family of glutathione peroxidases (GPx's), a group of enzymes that reduce the oxidative stress decreasing vascular injury. GPx-3 is a homotetrameric protein present in high density lipoproteins (HDL) particles and secreted to the plasma that protects cells and enzymes catalyzing the reduction of hydrogen peroxide, lipid peroxides and organic hydroperoxides. It contributes to maintaining the vascular bioavailability of NO. We postulate that in patients with CVD an increase of GPx-3 levels takes place as a protective mechanism to reduce lipid peroxidation and to maintain the NO levels. There is some evidence supporting this potential protective effect. Regarding this, some studies link increased levels of GPx-3 with vascular complications (Russo *et al.*, 1998), and a deficiency or a reduction in GPx-3 are related to an increase in the predisposition to developing a thrombotic disorder (Jin *et al.*, 2011). Thus, levels of GPx-3 could act as a predictor of cardiovascular risk.

Our results also show a significant decrease in TTR levels in the serum of the T2DM patients presenting CVD as well as a decrease in the chain A of C3 complement levels in the T2DM group compared to the other groups. Although several studies support these findings (Ingenbleek and Young, 1994; Lappas, 2011), our results about C3 and TTR are not conclusive since these proteins are depleted by the spin cartridges. Thus, results obtained by 2D-DIGE referents to C3 complement and TTR may not reflect the pathophysiological serum levels of patients and controls. So, future studies are needed to clarify the role of these proteins in the development of T2DM and CVD.

The strengths of this study lie in the novel evaluation of the differences in the serum proteome between T2DM patients with and without severe CVD and the identification of the proteins probably involved in the pathogenesis of CVD associated to T2DM and in the strict selection of study subjects. This allows the minimization of confounding factors resulting in a high reproducibility of the results

in all replicates. However, our study has certain limitations. A case-control study does not allow us to establish a cause-effect relationship. Moreover, a biochemical validation of the protein candidates by ELISA in a larger T2DM study population will be helpful in order to confirm these preliminary results.

In conclusion, our study shows that proteins involved in the inflammatory state and oxidative stress are associated with cardiovascular complications in patients with type 2 diabetes. The usefulness of these proteins as biomarkers of cardiovascular risk or potential therapeutic targets of vascular disease will require further study.

CAPÍTULO III

Metabolomic profile related to cardiovascular disease in patients with type 2 diabetes mellitus: A pilot study.

Adaptado de: Metabolomic profile related to cardiovascular disease in patients with type 2 diabetes mellitus: A pilot study.

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1. Abstract

Type 2 diabetes mellitus (T2DM) patients have an increased risk of cardiovascular disease (CVD) that represents one of the main causes of mortality in this population. The knowledge of the underlie factors involved in the development of CVD and the discovery of new biomarkers of the disease could help to early identification of high-risk patients.

Using liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) we analyzed the serum metabolomic profile of 30 subject distributed according three groups: i) T2DM patients with CVD; ii) T2DM patients without CVD; iii) non-diabetic subjects as controls (C) in order to identify potential biomarkers of the CVD related to T2DM.

A partial least squares discriminant analysis (PLS-DA) and one-way analysis of variance (ANOVA) were applied to identify differential metabolites between different groups.

Four glycerophospholipids were further identified as potential biomarkers of CVD in T2DM patients. Specifically, a reduction in phosphatidylcholine, lysophosphatidylcholine and lysophosphatidylethanolamine (LPE) serum levels were found in T2DM patients compared to controls, presenting the patients with CVD the lowest serum levels of these metabolites.

These results show a generalized reduction of circulating phospholipids species in T2DM patients which is more pronounced in those with CVD providing information of the pathways involved in the pathogenesis and progression of CVD associated to T2DM.

2. Introduction

T2DM is a major public health problem worldwide disease affecting a large proportion of the population. It is predicted that T2DM could increase worldwide to more than 300 million individuals by 2025 (Lowell and Shulman, 2005). T2DM is related to several complications being cardiovascular events one of the main causes of mortality of this disease. Moreover, diabetic patients that develop clinical CVD have a worse prognosis for survival than CVD patients without diabetes (Grundy *et al.*, 1999). Several studies reveal that T2DM is an independent risk factor of CVD in both men and women (Wilson, 1998; Wilson *et al.*, 1998b). The development of vascular complications is determined by the presence of common risk factors in T2DM such as obesity, chronic hyperglycemia, insulin resistance, dyslipidemia and states of inflammation and oxidation. However, only some of T2DM patients will develop these complications. This suggests that the pathway linking disglycemia and CVD is not well elucidated and the underlying factor involved in the development and progression of CVD associated with diabetes, should be studied in an intensive way. Therefore, effective strategies for earlier detection of CVD could reduce morbidity and mortality in this population. Little is known about the serum metabolites in T2DM and CVD in humans. Applying a novel technology to study serum metabolome may provide useful information to elucidate the whole mechanism involved in the development of CVD associated to T2DM.

Metabolomics is an emerging and powerful discipline that provides an accurate and dynamic image of the phenotype of biological systems through the study of endogenous and exogenous metabolites in cells, tissues and biofluids. It is a technology that allows the biomarker identification and improves clinical diagnosis and treatment of diverse disorders since altered metabolite profiles can be the indicators of changes in disease-relevant metabolic processes. Metabolic fingerprinting, the non-targeted global analysis of tissues and biofluids, strives to fingerprint and semiquantify metabolites and their changes revealing information about the general metabolic state of the individuals. Metabolomic analysis of human serum is very useful since serum is a rich source of potential biomarkers which are

able to reflect the impaired pathways during different disorders (Nicholson *et al.*, 2002).

The purpose of this study is to identify metabolites related to CVD in T2DM patients which could be used as predictors of the progression of the disease as well as to act as therapeutic targets for the prevention and treatment of CVD in this population.

For this goal, serum samples from non-diabetic controls and T2DM patients with and without CVD were collected and analyzed by LC-HRMS using a non-targeted approach. The use of HR mass spectrometry and Q-TOF mass analyzer in metabolomic experiments is essential for tentative identification of unknown interest compounds (Theodoridis *et al.*, 2008). A significant difference in metabolic profile was observed between all different groups by using the partial least squares discriminant analysis (PLS-DA). PLS-DA and one-way analysis of variance (ANOVA) were successfully used to screen out potential biomarkers from complex mass spectrometry data. Several metabolite ions were selected as potential biomarkers according to the variable importance in the project (VIP) value. The VIP parameter indirectly reflects the correlation of the metabolites with the disease and it is a widely used method for biomarker selection. According to "greater than one rule," variables with VIP values higher than one were selected in this study (Chong and Jun, 2005). The identification of molecular components of selected potential biomarkers was achieved through some molecular properties (such as experimentally determined accurate mass) and mass spectral patterns that were used to define molecular and empirical formulae from which metabolites were derived or identified by comparative searches of available spectral, compound and metabolic pathway databases (Brown *et al.*, 2009).

As a result, 4 compounds in 3 Phospholipid (PL) classes were found significant down regulated in T2DM patients compared to control subjects and were selected as potential biomarkers.

3. Subjects and methods

3.1. Study Population

Our cross-sectional study included 30 males matched for age and equally distributed according three study groups: i) T2DM patients with CVD (T2DM+CVD); ii) T2DM patients without CVD (T2DM-CVD); iii) non-diabetic subjects as controls (C).

Diagnosis of diabetes was according to American Diabetes Association criteria (2005). From January 2006 to December 2007 we consecutively recruited patients who had been referred to our outpatient clinic from primary care centers for treatment of diabetes. The inclusion criteria for patients with CVD were coronary heart disease (previous myocardial infarction or coronary revascularization surgery), cerebrovascular disease (ischemic stroke), or ischemic peripheral arterial disease.

The control group consisted in non-diabetic subjects consecutively recruited from the general community in the same period of time and matched with diabetic patients for demographic characteristics.

All were Caucasians, ambulatory and had neither renal, hepatic, gastrointestinal nor thyroid diseases. All T2DM patients were receiving medications for diabetes, including metformin, sulfonylureas, insulin or combination of these drugs.

Samples of venous blood were obtained from patients in Endocrinology Unit of University Hospital San Cecilio in Granada. Samples were taken in the morning after fasting overnight, incubated at room temperature for 30–45 min and centrifuged for 15 min at 6500g. Then, the supernatant was carefully aspirated by a serologic pipette, aliquoted into 50 μL cryovials and stored at −80 °C until the examination.

The study was conducted with the approval of the ethical committee of the San Cecilio University Hospital and conform the relevant ethical guidelines for human and animal research. The written informed consent was obtained from all subjects.

3.2. Metabolomic Analysis

All serum samples were kept at 4°C throughout the analytical process. The proteins were removed from serum samples using methanol (1:3 serum: methanol), shaken for 60 s and incubated at -25°C for 20 min. The samples were then centrifuged at 15.000g for 30 min at 4°C. The supernatants were lyophilized (Savant, Holbrook, NY) and stored at -20°C prior to analysis. The samples were reconstituted in 50% H_2O/ acetonitrile. The supernatants were transferred to the analytical vials, stored in the autosampler at 4°C and analyzed in triplicate within 48 h of reconstitution using AB SCIEX TripleTOF 5600 quadrupole-time-of-flight mass spectrometer (Q-TOF-MS) in positive ESI mode (AB SCIEX, Concord, ON, Canada). Reversed stationary phases (especially C18 column) are widely used for chromatographic separations in the field of metabolomics, providing acceptable retention of compounds with medium and low polarity. However, in for serum samples, these separation systems do not give good results for retain polar and ionic compounds (Theodoridis *et al.*, 2008). To overcome, this drawback, an analytical column with stationary phase containing embedded polar groups has been used in this study to increase the retention of polar metabolome components of the serum, such as amino acids and sugars. Thus, chromatographic separation was performed by Agilent Series 1290 LC system (Agilent Technologies, Santa Clara, CA, USA) using a Waters Atlantis T3 HPLC column (C18: 2.1 mm x 150 mm, 3 μm) (Waters Corporation, Milford, MA, USA) kept at 35 ºC. The injected sample volume was 5 μL. The mobile phase consisted of 0.1% formic acid-[water:AcN][90:10] (eluent A) and 0.1% formic acid-[AcN:water][90:10] (eluent B). The gradient elution was performed as follows: 0–0.5 min 0% eluent B; 0.5–11 min 100% eluent B; 11-15.50 min 100% eluent B; 15.50-15.60 min 0% eluent B and 15.60-20 min 0% eluent B. The elution flow rate was 0.3 ml/ min. Triple TOF 5600 used a Duo Spray source with separated electrospray ionization ESI and atmospheric pressure chemical ionization (APCI) probes. The ESI was used for the measurement of sample metabolomic profiling, while APCI probe worked as the second gas heater and for exact mass calibration of the QTOF-MS. Regarding to similarity of respective ion

sources, identical parameter settings were used. Positive ESI mode parameters were as follows:

Capillary voltage: 5000 V; nebulizing gas pressure: 50 psi; drying gas pressure: 50 psi: temperature: 500 ºC and declustering potential: 100V. TripleTOF 5600 was operated using an information dependent acquisition (IDA) method to collect full scan MS and MS/MS information simultaneously. The method consisted of high resolution survey spectra (mass resolving power 30,000 fwhm) from m/z 100 to m/z 1000 collected for 250 ms. Subsequently, the 8 most intense ions were selected for acquiring MS/MS fragmentation spectra (each collected for 100 ms) after each scan. The collision voltage was linearly ramped from15 to 55 V. The exact mass calibration was automatically performed before each analysis by the Automated Calibration Delivery System. It should be noted that the in-batch sequence of the samples was random (established based on random number generation) to avoid any possible time dependent changes in HPLC–MS chromatographic profiling. Within the sequence run, blank solvent (BS) samples and quality control (QC) samples were analyzed. QC samples were prepared by pooling equal volume of all serum samples included in this study. QC samples were analyzed throughout the run every five injections to provide a measurement of the stability and performance of the system. BS samples were run alongside with QC samples to identify either impurities of the solvents or extraction procedure and for checking carry over contamination from intense analytes (Dunn *et al.*, 2011).

3.3. Data Treatment Steps

3.3.1. Data Set Creation

MarkerView software (version 1.2.1, AB SCIEX, Concord,ON, Canada) was used for processing the LC-HRMS records. This is an adaptive processing software package designed LC-HRMS data that performs peak detection, alignment and data filtering generating a feature table where the measured m/z, retention time (RT) and

integrated ion intensity are defined. Data mining was performed by an automated algorithm in the RT range 0.6–19 min and m/z range 100–1000. Extraction intensity threshold was set to 10.00 cps. Then, RT and m/z tolerances of 0.2 min and 15 ppm, were used to alignment of the respective peaks. The background noise was removed by the tool of MarkerView software. To create features, the algorithm uses the accuracy of mass measurement to group ions related to charge-state envelope and isotopic distribution. The filter by "presence" was performed in order to retain only masses that appear at least in 20 samples within the study case groups.

To evaluate the high resolution MS and MS/MS spectra obtained within LC–QTOF-MS analysis and to estimate elemental formulae of pre-selected marker compounds, PeakView software (version 1.0 with Formula Finder plug-in version 1.0, AB SCIEX, Concord, ON, Canada) was used. Next steps of the procedure were carried out using Metaboanalyst 3.0 Web Server (Xia *et al.*, 2009, 2012).

3.3.2. Data Pre-treatment

The normalization process removes systematic bias in ion intensities between measurements, making samples and metabolite concentrations more comparable. For data normalization prior to analysis, different approaches were assessed including maximum peak height, mean and median normalization. Finally, normalization by a QC reference sample (probabilistic quotient normalization) (Dieterle *et al.*, 2006) was performed. The data transformation process is crucial for relatively small sets of replicates within groups. Different types of data transformation and scaling were tested to reduce the influence of potential outliers(Steuer R, Morgenthal K, Weckwerth W, Selbig J, 2007) to transform the data matrix into a more Gaussian-type distribution (Fiehn O and Kind T, 2007) and to adjust the importance assigned to the elements of the data in fitting the model. Moreover, transformation increases the weight of low-intensity metabolites and compresses the upper end of the measurement scale (Morgenthal *et al.*, 2007). In this study, cubic root transformation of normalized data was performed.

3.3.3. Data treatment

Filtration according to significative differences was carried out based on statistical analysis including both univariate (UVA) and multivariate analysis (MVA). For UVA, a double filtering procedure with fold change (> 2) and t test (p-value < 0.05) were used in order to identify differentially expressed metabolites between both blank and case samples removing the background and contaminants and preserving the peaks from true metabolites from LC-QTOF/MS data. Additionally, An ANOVA-based filter (p=0.01) was used to eliminate redundant signals from the dataset and to detect differences between the study groups related to each variable providing a quality criterion to assess variable relevance for further data analysis(Boccard *et al.*, 2007).. For MVA, principal component analysis (PCA) and PLS-DA were applied. PCA was applied to validate quality of the analytical system performance and to observe possible outliers. To discriminate the variables which are responsible for variation between the groups, PLS-DA was used(Wold H, 1966). Afterwards, VIP tool was used to select metabolites according to the different groups. Briefly, VIP is a weighted sum of squares of the PLS loadings taking into account the amount of Y-variation in each dimension. VIP scores are calculated for each component. When more than 2 components are used to calculate the feature importance, the average of the VIP scores is used(Wold *et al.*, 2001).

3.4. Analytical validation

The QCs presentation on PCA plot was used for analytical validation: Variables with unacceptable reproducibility (RSD > 30%) or detected in less than 50% of QCs were rejected from the data matrix. Model quality description by goodness of fit (R^2) and goodness of prediction (Q^2) was the first step in statistical validation. Obtained models were also validated using cross validation (Franklin, 2008).

3.5. Identification of marker compounds

Elemental formula estimation was achieved from both single MS and MS/MS accurate mass spectra, which was followed by a spectral database search for structural identification. The automatic elemental formula estimation was carried out with the use of: (i) accurate single MS mass of parent ion, (ii) isotopic profile of parent ion, and (iii) accurate MS/ MS fragment ions. For calculations, the follow atoms were considered: C (n > 50), H (n > 100), N (n > 10), O (n > 20), P (n > 15), and S (n > 5). The FormulaFinder software (AB SCIEX, Framingham, MA, USA) used in this study enabled to sort the proposed formulas according to "MS rank" and "MS/MS rank", reflecting the differences between calculated and measured m/z values for both parent and fragment ion(s), and the match of experimental and theoretical isotope pattern in terms of isotope spacing and relative intensities. In the next step, stepwise search of the molecular formula of the candidates in several online databases (MassBank, Metlin, Human metabolome data base, Lipid mass, PubChem) and MS/MS libraries, was performed. From all of the compounds obtained, only candidates, presence of which was probable in humans, were further examined by comparing experimental fragmentation mass spectra with those provided either in databases (MassBank, Human metabolome data base, Metlin, NIST 2012 MS/MS library) and/or in scientific literature (Vaclavik *et al.*, 2011).

4. Results

4.1. Baseline Characteristics of the Study Population

The clinical and demographic characteristics of the study groups are summarized in Table 6.

Table 6. Anthropometric and biochemical parameters of the study population.

	Control	T2DM+CVD	T2DM-CVD
Age (years)	57 ± 5	58 ± 4	55 ± 8
Measurements			
BMI (Kg/cm^2)	27.2 ± 3.7	30.4 ± 3.4	28.3 ± 3.4
Fasting glucose (mg/dL)	88.1 ± 7.8	205.2 ± 63.2**	172.3 ± 67.7**
HbA_{1C}	4.75 ± 0.35	9.18 ± 2.52**	8.63 ± 2.17**
sBlood pressure (mm Hg)	128.3 ± 16.8	125.5 ± 21.6	129.0 ± 17.9
dBlood pressure (mm Hg)	80.0 ± 13.1	73.5 ± 15.2	78.5 ± 7.4
LDL (mg/dL)	136.9 ± 22.9	92.2 ± 51.7*	109.8 ± 29.2
HDL (mg/dL)	57.8 ± 11.1	46.4 ± 22.7*	44.0 ± 8.4*
TG's (mg/dL)	136.5 ± 59.0	223.7 ± 148.4	229.5 ± 158.3
Medical history			
Cerebrovascular disease (%)	-	20*	-
Peripheral artery disease (%)	-	30*	-
Coronary heart disease (%)	-	90**	-
Active smokers (%)	20	20	30
Sedentarism (%)	40	40	40
Alcohol (%)	20	20	30
Current medication			
Oral antidiabetic drugs (%)	-	90**	80**
Insulin (%)	-	50**	60**

BMI: body mass index; HbA_{1C}: Glycated hemoglobin; s: Systolic; d: Dyastolic; LDL: Low density lipoproteins; HDL: High density lipoproteins; TG's: Triglycerides.

Data for continuous variables are presented as mean ± SD. Data for categorical variables are presented as percentages.

Student *t*-test or Mann-Whitney test were used for comparisons of continuous variables; X^2 for comparisons of categorical variables: *: $p < 0.05$ for the control group *vs.* T2DM + CVD/T2DM − CVD groups; **: $p < 0.001$ for the control group *vs.* T2DM + CVD/T2DM − CVD groups.

All groups were comparable in anthropometric and biochemical parameters except for those associated with T2DM, CVD and related medication. As expected, fasting glucose and glycated haemoglobin (HbA1c) were significantly higher in the T2DM patients compared to the controls. Moreover, only patients with CVD present cardiovascular events such as cerebrovascular disease, peripheral artery disease or coronary heart disease in regard to the other groups.

4.2. HPLC–MS metabolomic analysis

Generic parameter settings for chromatographic separation and MS detection were performed to obtain specific metabolomic fingerprints of the study groups. Figure 18 shows representative LC–QTOF-MS total ion current (TIC) chromatograms of the different study groups, recorded in positive ionization mode. It shows that good chromatographic separation of samples has been obtained.

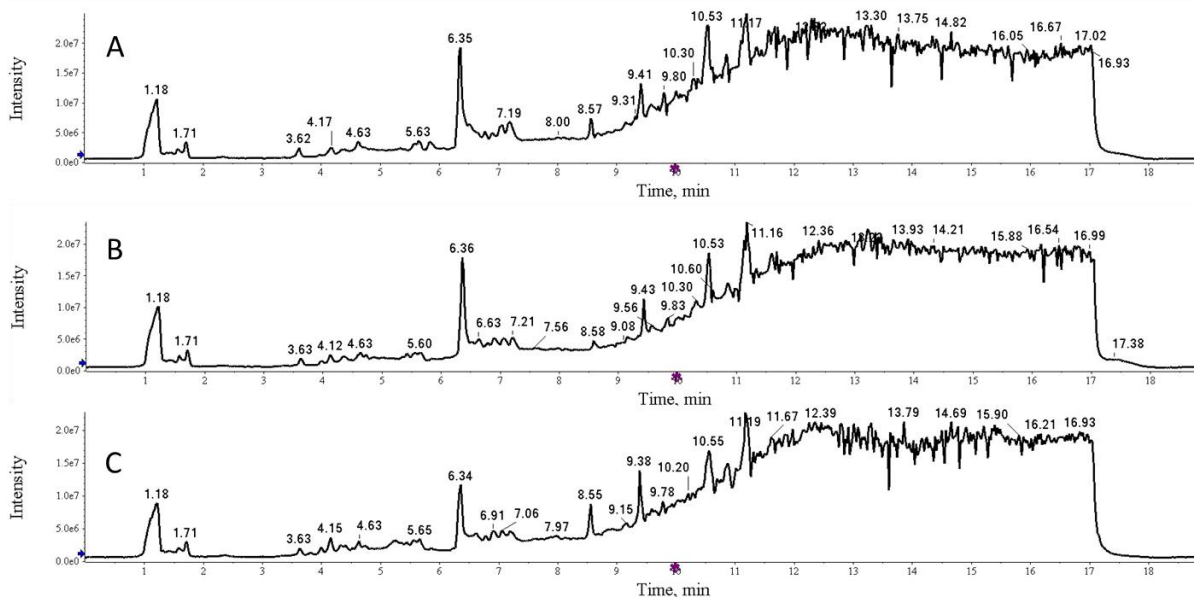

Fig.18. Representative LC/MS total ion chromatograms of the serum samples scanned by positive ion mode. (A) Control subject; **(B)** T2DM patients without CVD; **(C)** T2DM patients with CVD.

Under experimental conditions, the typical base widths of chromatographic peaks were below 10 s. The reproducibility of both m/z and RT plays an important role in successful processing of metabolomic data, especially in the peak aligning step. The minimization of m/z values fluctuation was ensured by regular mass spectrometer calibration, however, the in-batch stability of RT, should be examined. For this purpose, repeated measurements (n = 6) of randomly selected samples were performed in a batch with all other samples (random sequence) and the RT variability of three peaks eluting in RTs 1.8, 6.36 and 10.53 min was evaluated. Based on these data, typical RT peak variability was found to be below 3 s (SD) or a relative SD less than 3%, which can be considered as acceptable values. Besides of full scan mass spectra recording, simultaneous automatic acquisition of MS/MS fragmentation mass spectra was performed for ions exceeding the intensity threshold setting, allowing obtaining additional information on respective markers.

4.3. Chemometric analysis

A positive ionization data matrix of 1764 mass signals was obtained as an outcome of the data mining and alignement procedures. To filter the results of the data matrix and to minimize the signal redundancy, only peaks representing monoisotopic ions (signals with the lowest m/z value within an isotope pattern), were selected (409 peaks) and subjected to the chemometric analysis. Data normalization was performed by a QC reference sample through which the best clustering was obtained and followed by a cubic root transformation of normalized data to reduce uninformative features avoiding model overfitting and deterioration of its performance. Pareto scaling of the data was performed to reduce, but not eliminate completely the abundance differences of the input variables (van den Berg *et al.*, 2006). It provides good results when it is applied to LC–MS data, as it considers the larger peaks more reliable, but all variables are equivalent (Vaclavik *et al.*, 2012). Then, data was filtered using "by presence" approach to retain variables which appear at least in 70% of all study samples in order to select mainly endogenous metabolites. 17% of metabolites (301 candidates) with two fold change

and t test ($p < 0.05$) were differentially expressed in study case samples *versus* SB samples. ANOVA filtering demonstrates a relevant effect on group discrimination. A p-value of 0.01 was selected as the best compromise for relevant metabolite detection. With this value, about 91% of the original mass information was discarded. The 9% (154) remaining m/z were analysed by PCA. Within PCA of study case sample data, 5 PCs explaining 66 % of total variance were calculated. In order to assess the quality of the analytical system performance PCA was also applied to track the clustering or spreading of QC samples. The PCA scores plot (data not shown) revealed a close clustering of the QC samples indicating that the separation observed between study case groups was mainly due to biological reasons. Control samples were clearly separated from patient samples (T2DM-CVD/ T2D+CVD) along PC1 and PC2 which describe about 33.9 and 16.6% respectively of the total data variability remaining after ANOVA filtering (Figure 19A).

Most control samples shared a negative coordinate on PC1and PC2, while patient samples mainly presented a positive score. As expected, loadings were useful to understand differences among samples in each metabolite level. The PLS-DA score plots are shown in Figure 19B.

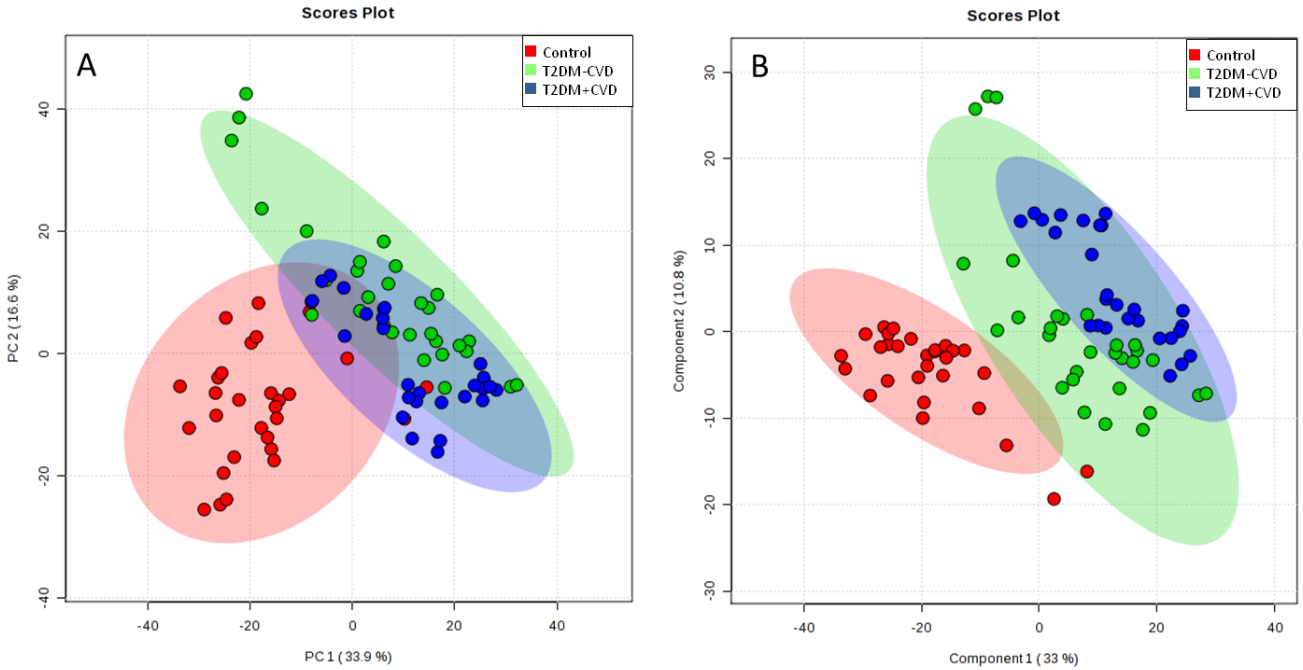

Fig. 19. PCA (A) and PLS-DA (B) scores plots based on LC/MS data of serum samples. Control group (red), T2DM-CVD group (green), and T2DM + CVD group (blue).

Based on the PLS-DA models, patient samples and control samples were discriminated with an R^2 of 0.85, and a Q^2 of 0.83. It is assumed that for metabolomics data acceptable values are $R^2 \geq 0.8$ and $Q^2 \geq 0.5$ and not vary more than 0.2–0.3 to ensure the model is powerful for diagnostics (Godzien *et al.*, 2013). To assess the impact of outliers on statistical outcome, a new data set without outliers was generated. Random Forest methodology allowed the identification of 4 potential outliers in the control group despite attempts to modify the method of standardization (data not shown). Another 3 samples from T2DM-CVD group, lying outside Hotelling T^2 ellipse in PLS-DA score plot (Figure 19B) were detected as potential outliers. No differences in terms of PLS-DA modelling were observed for model quality: Either R2 (0.86) or Q2 (0.84) were comparable, no loss of the model predictability by the presence of outliers.

4.4. Selection of the potential biomarkers

The VIP parameter and ANOVA test ($p < 0.005$) were used to select significantly different variables as potential biomarkers. The ions at m/z 536.3348, 478.2928, 482.3598, 468.3056 have significant differences among all of the groups. Additionally, the ions at m/z 478.2928 and 482.3598 were also significantly different between T2DM patients with and without CVD. Totally, 4 phospholipids (PLs) species were found as potential biomarkers by PLS-DA and ANOVA. Among them, two candidates (478.2928 and 482.3598) could be used to differentiate the study groups. The detailed information related to the identified candidate biomarkers is shown in Table 7.

Table 7. Potential biomarkers found by LC/MS analysis of Control, T2DM+CVD and T2DM-CVD groups ($p < 0.005$).

Peak nº	Ret. Time	m/z	Tentative Molecular Formula	Mass Error (ppm)	Tentative ID	VIP	*p* value	Fold change[a]	Fold change[b]	Fold change[c]
1	8.12	536.3348	C26H50NO8P	0.2	PC (16:1(9Z)/2:0)	1.5022	8.08E-06	1.833	2.398	N.A.
2	10.46	478.2928	C23H44NO7P	0	LPE (18:2(9Z,12Z)/0:0)	1.5980	5.93E-09	1.505	1.982	1.317
3	11.56	482.3598	C24H52NO6P	-0.7	LPC (O-16:0/0:0)	1.6011	1.26E-06	1.250	1.805	1.443
4	9.59	468.3056	C22H46NO7P	-2.8	PC (O-12:0/2:0)	1.9260	4.77E-09	1.703	2.150	N.A.

a Fold change expressed as the ratio of the two averages (C/T2DM-CVD)

b Fold change expressed as the ratio of the two averages (C/T2DM+CVD)

c Fold change expressed as the ratio of the two averages (T2DM-CVD/T2DM+CVD)

A decreasing trend of these biomarkers from control group through T2DM-CVD to T2DM+CVD groups is observed in Figure 20.

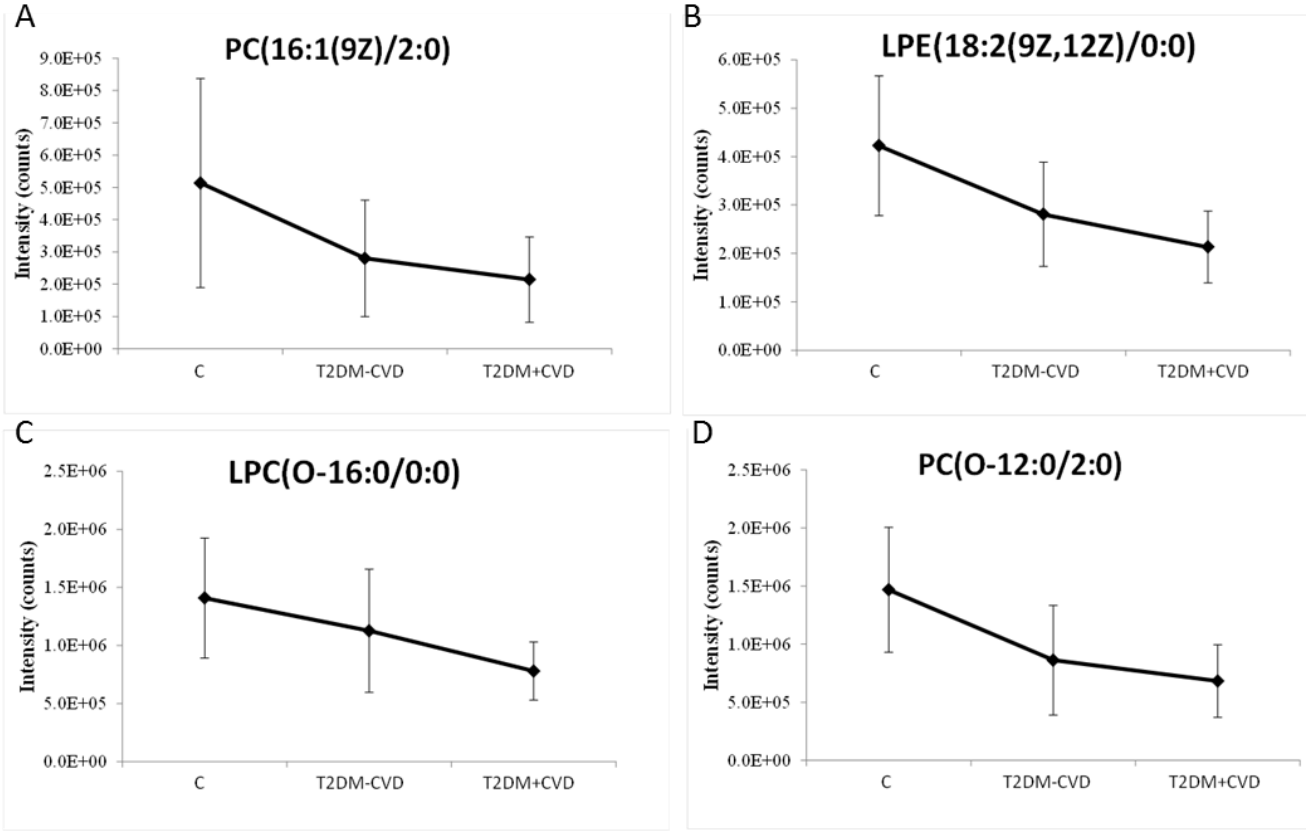

Fig. 20. Serum levels of the four selected biomarkers represented as mean ± SD in the control, T2DM-CVD and T2DM +CVD groups. (A) PC(16:1(9Z)2:0) levels; **(B)** PE(18:2(9Z,12Z)/0:0) levels; **(C)** PC(O-16:0/0:0) levels; **(D)** PC(O-12:0/2:0) levels.

4.5. Structural identification of the potential biomarkers

Characterization of individual potential biomarkers was achieved using electrospray ionization mass spectrometry (ESI-MS) through a production analysis after collision-induced dissociation (CID). Using the previously mentioned procedure, the 4 potential biomarkers were tentatively identified as PLs: m/z 536.3348 corresponded to phosphatidylcholine (PC) (16:1(9Z)/2:0); m/z 478.2928 corresponded to lysophosphatidylcholine (LPC)(O-16:0/0:0); m/z 482.3598

corresponded to lysophosphatidylethanolamine (LPE) (18:2(9Z,12Z)/0:0) and m/z 468.3056 corresponded to PC (O-12:0/2:0)](Table 7).

5. Discussion

The development of cardiovascular complications in patients with T2DM is a serious problem that compromises the quality of life and survival of these patients. The precise mechanism linking CVD to T2DM is not completely known and there still unknown factors. To get a more insight into the changes that occur in diabetic patients who develop vascular disorders, we have conducted a metabolomic study to get an overview of what is going on at the current moment in these patients. Metabolomic approach could provide information about the changes in the metabolic pathways involved in CVD associated to diabetes mellitus and could lay the basis for the development of new prevention strategies.

The present study revealed that phospholipids (PLs) are important metabolites which are involved in T2DM and CVD.

We found that 4 compounds in 3 PLs classes were significant down regulated in T2DM patients compared to the control subjects and they were selected as potential biomarkers. PLs are molecules with amphiphilic character constituted of a glycerol, a phosphate group and two fatty acid chains and categorized according to their different polar heads which bind the phosphate group. They have principally a structural role being the main constituents of biological membranes but also they have many other cellular functions such as activating enzymes and cells, messengers in signal transmission within the cell, synthesis precursors of prostaglandins, thromboxanes and leukotrienes among others (Zhu *et al.*, 2011a). Over recent years, the PLs and their metabolism is becoming more important due to its association with many diseases, like Alzheimer's, obesity and cancer (Su *et al.*, 2005; Tomiki *et al.*, 2004) increasing the attention in many fields. Several studies have shown a disturbed lipid metabolism directly associated with T2DM. Dyslipemia and the alteration of a number of lipid species contribute in a very important way to

development of insulin resistance and to the pathogenesis of T2DM (Boden and Shulman, 2002). In this way, several studies have identified some PLs as biomarkers of T2DM (Hsu *et al.*, 2000; Tan *et al.*, 2003).

The PLS-DA score plot and the concentration variation of potential biomarkers revealed that an abnormal phospholipid metabolism takes place in T2DM and CVD. This analysis showed a metabolomic discrimination among groups related to the metabolites identified as PC(16:1(9Z)/2:0), PC(O-12:0/2:0)], LPC(O-16:0/0:0) and LPE (18:2(9Z,12Z)/0:0). These metabolites showed decreased concentrations in the T2DM patients compared to the control subjects. The mechanism responsible for the reduction in these PLs may be due to an increase of protein kinase C (PKC) and the related activation of the phospholipase A2 (PLA2). PLA2 catalyzes the decomposition of phospholipids resulting in free fatty acids. An environment of high glucose concentrations as in diabetes leads the activation of the PKC pathway and the subsequent activation of PLA2 decreasing the concentrations of PCs (Zhu *et al.*, 2011a). Decreased levels of LPE and LPC which derived from the degradation of PE and PC respectively, could be partially explained by increased levels of phospholipase A1 (PLA1) which increases its levels in diabetic patients (Abu El-Asrar *et al.*, 2013) and catalizes lysophophospholipids degradation by a complex process of metabolic pathways (Aoki *et al.*, 2008; Bolen *et al.*, 2011). In agreement with our results, some studies have reported decreased levels of LPC in glucose intolerant, insulin resistant and obese T2DM patients (Barber *et al.*, 2012; Graessler *et al.*, 2009; Rhee *et al.*, 2011; Zhao *et al.*, 2010).

A very recent study has reported a beneficial effect of LPC in glucose homeostasis in mice showing an activation of glucose uptake by an insulin independent mechanism that increases GLUT4 levels in 3T3-L1 adipocytes (Yea *et al.*, 2009). Based on these findings, LPC may be a novel insulin independent signal for the regulation of blood glucose levels and the reduction in circulating LPC levels in T2DM patients could contribute to the worse glycemic control observed in these patients.

It is important to highlight that not all T2DM patients develop CVD despite having the same typical risk factors. However, few studies have focused on the pursuit of serum phospholipids related to development or progression of CVD in T2DM patients.

In this regard, our results also showed significantly decreased expression of LPC(O-16:0/0:0) and LPE (18:2(9Z,12Z)/0:0) in diabetic patients with CVD indicating their strong influence on PC scores that separated T2DM patients with CVD from T2DM patients without CVD (Figure 3B).

T2DM patients have increased CVD risk factors such as hypertension, hypercholesterolemia, and obesity and it is well known the fact that T2DM patients who develop vascular complications have increased levels of proinflammatory cytokines and reactive oxygen species (ROS) that unleash these complications. A very recent study of our group showed increased expression levels of protein binding 4 (RBP4) in T2DM patients with CVD, a protein that promotes the expression of proinflammatory molecules and induces the adhesion of leukocytes to the endothelium leading to endothelial inflammation typical of vascular disorders (García-Fontana *et al.*, 2015).

Several studies have reported an increased expression of Autotaxin (ATX), also known as ectonucleotide pyrophosphatase/ phosphodiesterase 2 (ENPP2 or NPP2), in many chronic inflammatory disorders (Hammack *et al.*, 2004; Nakasaki *et al.*, 2008; Oikonomou *et al.*, 2012; Umemura *et al.*, 2006). Accordingly, tumor necrosis factor (TNF), the major pro-inflammatory factor, was shown to induce ATX expression (Oikonomou *et al.*, 2012; Wu *et al.*, 2010). The origin of plasma ATX remains to be determined, but likely sources are the lymphatic high endothelial venules (HEVs) (Kanda *et al.*, 2008) and adipose tissue, which express and secrete ATX at high levels. ATX possesses lysophospholipase D activity, catalyzing the hydrolysis of lysophospholipids into lysophosphatidic acid (LPA) which exerts its action through binding to specific G protein-coupled receptors (GPCRs). Possible pathophysiological effects of increased local concentrations of LPA at inflamed sites as a result of increased expression of ATX have been described. The relationship

between serum LPA and vascular complications has been reported in some studies and it was suggested to induce atherosclerosis by enhancing the penetration of blood monocytes into the subendothelial space of vascular tissue, proliferation of SMCs and endothelial dysfunction via decreased eNOS expression and increased oxidative stress (Chen *et al.*, 2012a).

The ATX/LPA axis is also involved in obesity, diabetes and insulin resistance. Some studies in humans have reported an upregulation of ATX mRNA expression of adipose tissue in obese patients with glucose intolerance compared to obese patients with no diabetic symptoms (Boucher *et al.*, 2005) as well as an visceral fat ATX upregulation was observed in obese than in non-obese patients (Rancoule *et al.*, 2012). These evidences suggest that the regulation of ATX expression from adipocytes is closely associated with the development of hyperglycemia. Moreover, it has been observed that LPA plays a role in decreasing plasma insulin levels.

The above findings and to our results suggest that ATX expression could be influenced by alterations in glucose homeostasis affecting insulin and glucose levels in diabetic patients and decreasing the levels of LPLs in these patients. Moreover, an increased inflammation state of some T2DM patients could induce higher levels of ATX and thus also of LPA further decreasing the content of LPLs in diabetic patients who develop CVD. According to these findings, we propose that identified PLs are involved in the T2DM and that the variation of these biomarker species could reflects the development and the progression of CVD in T2DM patients. However, the usefulness of these metabolites as biomarkers of cardiovascular risk or potential therapeutic targets of vascular disease will require further study.

Additionally, the use of this method, based on multivariate data analysis of serum metabolic profile, is a successfully approach to find out and to identify potential biomarkers. The combination of partial least squares discriminant analysis and one-way analysis of variance was successfully applied to screen out biomarker candidates from complex mass spectrometry data and it could be an integral component of HPLC/Q-TOF MS studies of metabolic profile in human serum samples.

CAPÍTULO IV

Atherosclerotic disease in type 2 diabetes is associated with an increase in sclerostin levels.

Adaptado de: Atherosclerotic disease in type 2 diabetes is associated with an increase in sclerostin levels.

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1. Abstract

Wnt/b-catenin signaling is related to the pathogenesis of several diseases. Sclerostin is an inhibitor of Wnt/b-catenin signaling. However, there are few data regarding the sclerostin levels and vascular disease. Our aim was to examine the relationship between serum sclerostin and atherosclerotic disease (AD) in type 2 diabetes mellitus (T2DM).

We performed a cross-sectional study including 78 T2DM patients (45.3% females, mean age 59 ± 5.7 years; 54.7% males, 57.4 ± 6.7 years).

Serum sclerostin concentrations of T2DM patients in the AD group were significantly higher than in the non-AD group (P = 0.006). For each increase of 1 pmol/L in sclerostin level, there was a 4% increase of the risk of AD in T2DM patients. A concentration of ≥ 42.3 pmol/L showed a sensitivity of 69% and a specificity of 54.8% to detect an increased risk of AD. In males, sclerostin levels were higher in those with AD (P = 0.04), abnormal intima-media thickness (IMT) (P = 0.004), carotid plaques (P < 0.001), and aortic calcification (P < 0.001). In females, higher levels of sclerostin were related to abnormal IMT (P = 0.03) and aortic calcifications (P = 0.004). Homocysteine (b = 0.319 [95% CI 0.561–2.586], P = 0.003) and IMT (b = 0.330 [14.237–67.693], P = 0.003) were positive correlated with sclerostin.

Circulating sclerostin is increased in T2DM patients with atherosclerotic lesions. Although the sample size of our study was small, these data suggest that sclerostin levels could be a major modulator of Wnt signaling in AD with implications in T2DM patients.

2. Introduction

Type 2 diabetes mellitus (T2DM) enhances the risk of macrovascular complications (coronary artery disease, peripheral artery disease, and cerebrovascular disease) and disorders of bone metabolism with serious consequences on morbidity and mortality. Atherosclerosis is the main pathological mechanism in macrovascular disease, inducing an inappropriate proliferation of vascular smooth muscle cells (VSMCs), which is linked to thickening of the arterial wall, atheroma plaque formation, and vascular calcification (Madonna and De Caterina, 2011a).

The canonical Wnt or Wnt/b-catenin pathway is increasingly related to the regulation of proliferation, migration, and survival of VSMCs (Couffinhal *et al.*, 2006b; Tsaousi *et al.*, 2011; Wang *et al.*, 2002). Furthermore, a gene mutation implicated in this pathway has been associated with hyperlipidemia, hypertension, and early coronary artery disease inmetabolic syndrome patients (Mani *et al.*, 2007b). In these patients, abnormal canonical Wnt signaling has been also implicated in disturbances of the lipids, glucose, and bone homeostasis (Chafey *et al.*, 2009; Jin, 2008b; Manolagas and Almeida, 2007b; Ueland *et al.*, 2009b).

The Wnt/b-catenin pathway results from Wnt proteins binding to its receptors Frizzled and its coreceptors LRP-5 and -6 on the cell surface. The formation of the complex increases the stability of b-catenin, which leads to its translocation in the nucleus and induces transcription of Wnt target genes (Rao and Kühl, 2010). The canonical Wnt pathway is modulated by several Wnt antagonists, including a family of proteins such as soluble Frizzled-related receptors (sFRPs) and dickkopfs (DKKs), which have been shown in physiological and pathological processes to be related to vascular injury in experimental mice (Barandon *et al.*, 2003; Mastroiacovo *et al.*, 2009; Román-García *et al.*, 2010; Ueland *et al.*, 2009b) and humans (Goliasch *et al.*, 2012b; Ueland *et al.*, 2009b).

On the other hand, sclerostin is an endogenous antagonist secreted almost always exclusively by osteocytes, and it has been extensively studied as a major regulator of canonical Wnt pathway in bone metabolism (van Bezooijen *et al.*, 2005; Poole *et al.*,

2005b). We have previously reported that circulating sclerostin is increased in T2DM and its relationship with bone turnover and bone mass. Moreover, in T2DM sclerostin levels are related to duration of T2DM and HbA1c (García-Martín *et al.*, 2012b). Notably, sclerostin was highly expressed in calcified aorta tissues from a diabetic murine model (Shao *et al.*, 2005b) and in human aortic samples from three patients with atherosclerosis (Didangelos *et al.*, 2010b). Recently, besides sclerostin production by osteocytes, in vitro assays under a calcifying environment, showed sclerostin expression in VSMCs (Zhu *et al.*, 2011b) that were able to undergo phenotypic transition to mineralizing osteoblast-like cells, expressing several osteogenic genes, among them, the protein product of the SOST gene (sclerostin). These findings suggest an additional role for sclerostin on vascular pathology, but at present this fact remains to be evaluated. In this context, our aim was to study the relationship between serum sclerostin and atherosclerotic disease (AD) and vascular calcification in T2DM.

3. Subjects and methods

3.1. Study Population

Our cross-sectional study included 78 T2DM patients with diagnosis of diabetes according to American Diabetes Association criteria (2005). From January 2006 to December 2007, we consecutively recruited patients who had been referred to our outpatient clinic from primary care centers for treatment of diabetes. Patients were classified into two groups according to the presence of AD: AD group (n = 44) and non-AD group (n = 31). Inclusion criteria for patients with AD were cerebrovascular disease (ischemic stroke or transient ischemic attack), coronary heart disease (previous myocardial infarction, diagnosed stable or unstable angina, or coronary revascularization surgery), or ischemic peripheral arterial disease. There are some local administrative constraints for referring patients to Endocrinology in our

area, and patients with longer diabetes duration and with comorbidities are more likely to be referred than those without.

All were Caucasians and ambulatory, had normal values of serum calcium and phosphorus, and did not have renal, hepatic, gastrointestinal, or thyroid diseases. All patients were on medications for diabetes, including metformin, sulfonylureas, insulin, and a combination of these drugs. None of them had been treated with calcium supplements, vitamin D preparations, hormone therapy, antiresorptive therapy, thiazides, steroids, or other medications that might affect bone metabolism. Patients treated with thiazolidinediones were also excluded.

The study was conducted with the approval of the ethics committee of the San Cecilio University Hospital and conformed to the relevant ethics guidelines for human and animal research. Written informed consent was obtained from all subjects.

3.2. Clinical evaluation

Height, weight, and waist circumference were measured at baseline according to standard procedures. Weight was measured to the nearest 100 g using digital electronic scales. Height and waist circumference were measured to the nearest 1 mm using a stadiometer and a metal anthropometric tape, respectively. BMI was calculated as weight divided by the square of height in meters.

Blood pressure was measured in a standardized manner. After subjects remained at rest for at least 5 min, blood pressure was measured twice using a standard mercury sphygmomanometer (12 cm long and 35 cm wide). The mean of the two values was used for analysis. We defined hypertension as values ≥ 140/ 90 mmHg and/or antihypertension treatment. Participants reported alcohol use, smoking status, and level of physical activity with a specific health questionnaire. Patients were classified as having a significant alcohol intake if it was > 40 g/day in males and 24 g/day in women. Smoking status was categorized as no tobacco use or current tobacco use.

Physical activity was collected through a specific questionnaire in which study subjects considered activity levels on a scale from 0 (none) to 10 (sport > 1 h four times per week). Based on the results, the study sample was divided into two groups: sedentary (< 5 on the scale) and not sedentary (≥ 5 on the scale).

3.3. Biochemical measurements

Biochemical parameters, including fasting plasma glucose (FPG), HDL cholesterol, LDL cholesterol, triglycerides, and creatinine, were measured by standard biochemical methods. Dyslipidemia was defined according to Third Report of the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) criteria or current treatment with statins. HbA1c was determined by high-performance liquid chromatography (ADAMS A1c, HA-8160; Menarini), and homocysteine levels were determined by immunoassay (Abbott, Wiesbaden, Germany).

Serum sclerostin was measured using a commercially available ELISA (Biomedica, Vienna, Austria) according to the manufacturer's instructions. In our laboratory, we assay duplicates for all values. Precision was tested by determination of intra-assay and interassay variation. Two samples of known concentrations were tested six times for assessment of intra-assay variability, which resulted in 4%. Moreover, two samples of known concentrations were tested in three assays from two different operators to assess interassay variability, which was 3%. Sclerostin measurements are reported in picomoles per liter, and the lower limit of detection was < 10 pmol/L.

3.4. Carotid intima-media thickness and aortic calcification measurements

Ultrasonographic examination of the carotid arteries was performed with patients in the supine position using Doppler ultrasonography (Toshiba PowerVision 6000). The maximum intima-media thickness (IMT) at the carotid bifurcation (BIF) was

determined between the near and far walls of the BIF on the right and left sides. Each part was measured from views of both longitudinal and vertical sections at the BIF. If a discrepancy was observed in the measured values between longitudinal and vertical sections, the smaller value was selected to avoid overestimation. BIF-IMT was defined as the mean of the measurements from the right and left sides. The value for each side was obtained from the mean of 10 wall measurements. The BIF-IMT, measured in millimeters, was considered pathological if it was ≥ 0.9 mm and was considered carotid atherosclerosis if the BIF-IMT was ≥ 1.2 mm or 50% greater than the BIF-IMT in the adjacent area (Junyent *et al.*, 2005a). Plaques were identified as calcified by findings of bright white echoes on sonography. A single trained sonographer performed the ultrasonographic study in all subjects.

The presence of aortic calcification was evaluated on lateral-view conventional X-rays of the thoracic and lumbar spine (T4–L5). Severity of anterior and posterior aortic calcifications was graded individually on a scale of 0–3 at each lumbar segment, and the results were summarized (Kauppila *et al.*, 1997). Patients with calcification in one or more lumbar segments were considered to have aortic calcification. The radiographs were analyzed by two independent investigators who were blinded to each other readings. In instances of disagreement, M.M.-T. analyzed the radiograph to confirm the diagnosis of aortic calcification.

3.5. Statistical analysis

Data were expressed as means ± SD. Data for categorical variables are presented as percentages. Kolmogorov-Smirnov test was used to test the normality of distribution of continuous variables. For continuous variables, mean values between two groups were compared by unpaired Student t test for normally distributed variables and Mann-Whitney U test for skewed variables. The x2 test was used to compare categorical variables between groups. Pearson (normal distribution) and Spearman correlation analyses (nonnormal distribution) were used to assess the

correlations between serum sclerostin and other continuous parameters, and we used partial correlations to correct the possible influence of age on sclerostin values.

Multiple backward model of logistic regression analysis was performed to identify sclerostin as an independent predictor of AD (dependent variable) in T2DM patients. The model included established atherosclerotic risk factors (age, sex, BMI, hypertension, dislipidemia, smoking, sedentarism, HbA1c, creatinin, homeocysteine, and IMT). AD-defining parameters (cerebrovascular disease, coronary heart disease, or ischemic peripheral arterial disease) were not included in the multiple logistic regression model. To determine the independent variables correlated with sclerostin (dependent variable), the parameters that correlate significantly in univariate analysis and others that are biologically linked to sclerostin were tested in multiple backward model linear regression analysis. The usefulness of serum sclerostin as a marker of high risk of AD in T2DM was analyzed using a receiver operating characteristic (ROC) curve. A p value of 0.05 was considered significant (two tailed). Data were recorded and analyzed using SPSS, version 18.0, software (SPSS, Chicago, IL).

4. Results

4.1. Baseline Characteristics of the Study Population

The clinical characteristics of the T2DM patients are summarized in Table 8. Both groups were comparable in clinical, anthropometric, and biochemical parameters except AD-defining parameters and AD surrogate markers.

Table 8. Anthropometric and biochemical parameters of study subjects according to AD status.

	AD (n=44)	Non-AD (n=31)	p value
Men/women (%)	61.3/38.6	45.2/54.8	0.16
Age (years)	59.4±4.5	56.3±7.9	0.06
Medical history:			
Duration of diabetes (years)	14.3±7.6	12.6±7.5	0.36
Hypertension (%)	84.1	70.9	0.17
Dislipidaemia (%)	97.7	83.9	0.032**
Abnormal intima-media thickness (%)	71.1	38.7	0.007**
Carotid plaques (%)	41.2	14.8	0.023**
Aortic calcifications (%)	51.4	20.7	0.011**
Coronary heart disease (%)	61.4	0	<0.001**
Cerebrovascular disease (%)	36.4	0	<0.001**
Peripheric arthery disease (%)	23.8	0	<0.001**
Smoker or ex-smoker (%)	52.2	67.7	0.18
Alcohol (%)	15.9	16.1	0.98
Sedentarism (%)	59.1	45.1	0.23
Current medication use:			
Antihypertensives (%)	77.3	61.3	0.13
Statins (%)	83.7	51.6	0.003**
Insulin (%)	67.4	64.5	0.79
Oral antidiabetic drugs (%)	66.6	85.7	0.88
Measurements:			
Body mass index (kg/m^2)	31.4±5.9	31.6±5.0	0.91
Waist circumference (cm)	106.9±11.1	106.5±11.2	0.89
Systolic blood pressure (mm Hg)	135.4±20.4	135.6±24.6	0.97

Diastolic blood pressure (mm Hg)	77.5±12.1	82.8±11.4	0.06
Fasting plasma glucose (mg/dL)	174.6±61.9	173.6±67.5	0.95
HbA1c (%)	8.0±2.0	8.1±1.8	0.96
Triglyceride (mg/dL)	159.2±120.4	181.6±179.2	0.79
High-density lipoprotein (mg/dL)	47.5±16.0	52.3±17.2	0.21
Low-density lipoprotein (mg/dL)	87.9±33.0	108.5±33.7	0.010*
Creatinin (mg/dL)	0.9±0.2	0.8±0.1	0.021*
Homocysteine (mmol/L)	11.3±5.16	8.98±3.84	0.05
Sclerostin (pmol/L)	59.0±26.2	44.8±16.5	0.006*

Data are expressed as n (%) or mean ± SD; unpaired *t* test or Mann-Whitney test: *P<0.05 Non-AD *vs* AD; χ^2 for comparisons of categorical variables: **P<0.05 for Non-AD *vs* AD.

4.2. Association of serum sclerostin levels in T2DM patients with AD

Serum sclerostin concentrations of T2DM patients in the AD group were significantly higher than in the non-AD group (59.0 ± 26.2 vs. 44.8 ± 16.5 pmol/L, $P = 0.006$). When subjects were further divided according to sex (Figure 21), we found that serum sclerostin differences were maintained in AD males compared with non-AD males and females. Furthermore, serum sclerostin levels were higher in males than in females in both groups but only reached significance in the AD group.

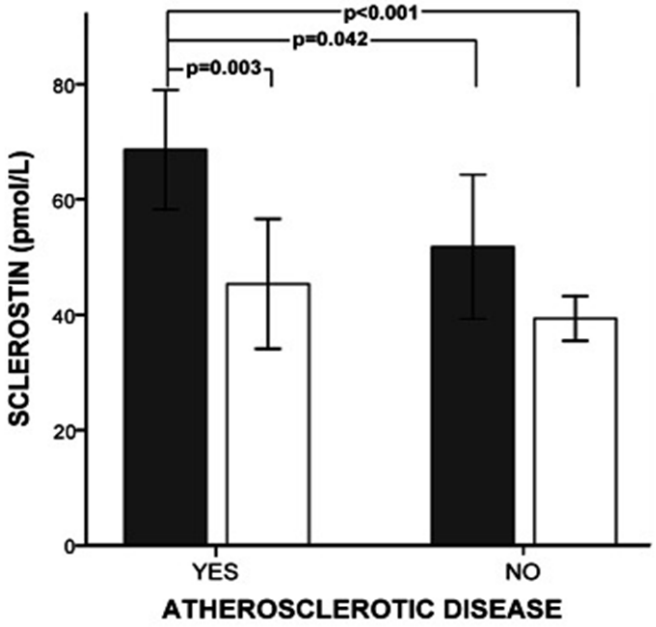

Fig. 21. Sclerostin serum levels in female (white bars) and male (gray bars) T2DM patients with and without AD. Data are means ± 95% CI. Significant differences between group regions are indicated by a bar with the *P* value given above.

A model of logistic regression analysis was performed using the presence of AD as a dependent variable. Independent variables were serum sclerostin levels and atherosclerotic risk factors (age, sex, BMI, hypertension, dislipidemia, smoking, sedentarism, HbA_{1c}, creatinin, homocysteine, and IMT). Only serum sclerostin levels were independent predictors of the presence of AD in T2DM (odds ratio 1.040 [95% CI 1.009–1.072]; $P = 0.012$). Therefore, for each picomole per liter of serum sclerostin level increase, there is a 4% increase risk in AD in T2DM patients.

In the ROC curve analysis to evaluate the usefulness of sclerostin as a marker for high risk of AD (Figure 22), the area under the curve was 0.654 ($P = 0.025$). A concentration of ≥ 42.3 pmol/L showed a sensitivity of 69% and a specificity of 54.8% for identifying an increased risk of AD.

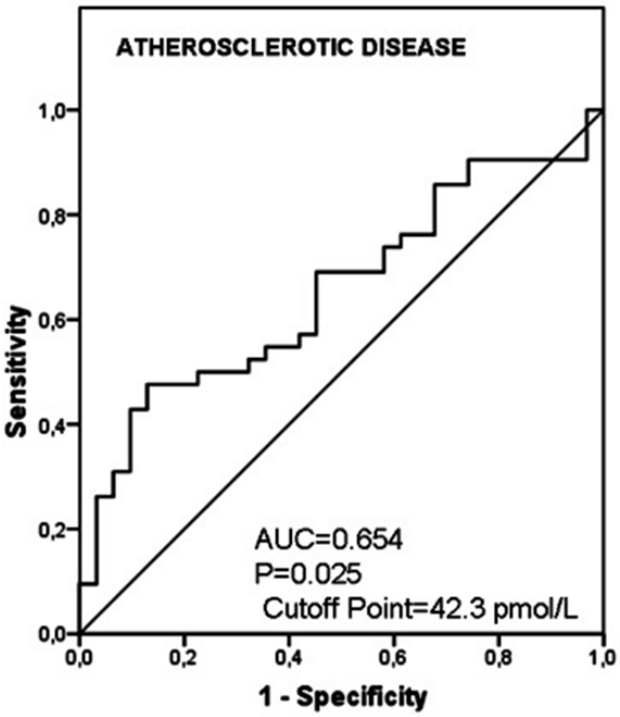

Fig. 22. Sclerostin ROC curve for AD. AUC, area under the curve.

4.3. Association of serum sclerostin levels in T2DM patients with surrogate markers of AD

In addition, in the entire cohort we analyzed the associations between serum sclerostin levels and surrogate markers of AD, such as abnormal IMT (Figure 23A), carotid plaque (Figure 23B) and aortic calcifications (Figure 23C). Male patients had higher concentrations of serum sclerostin compared with females.

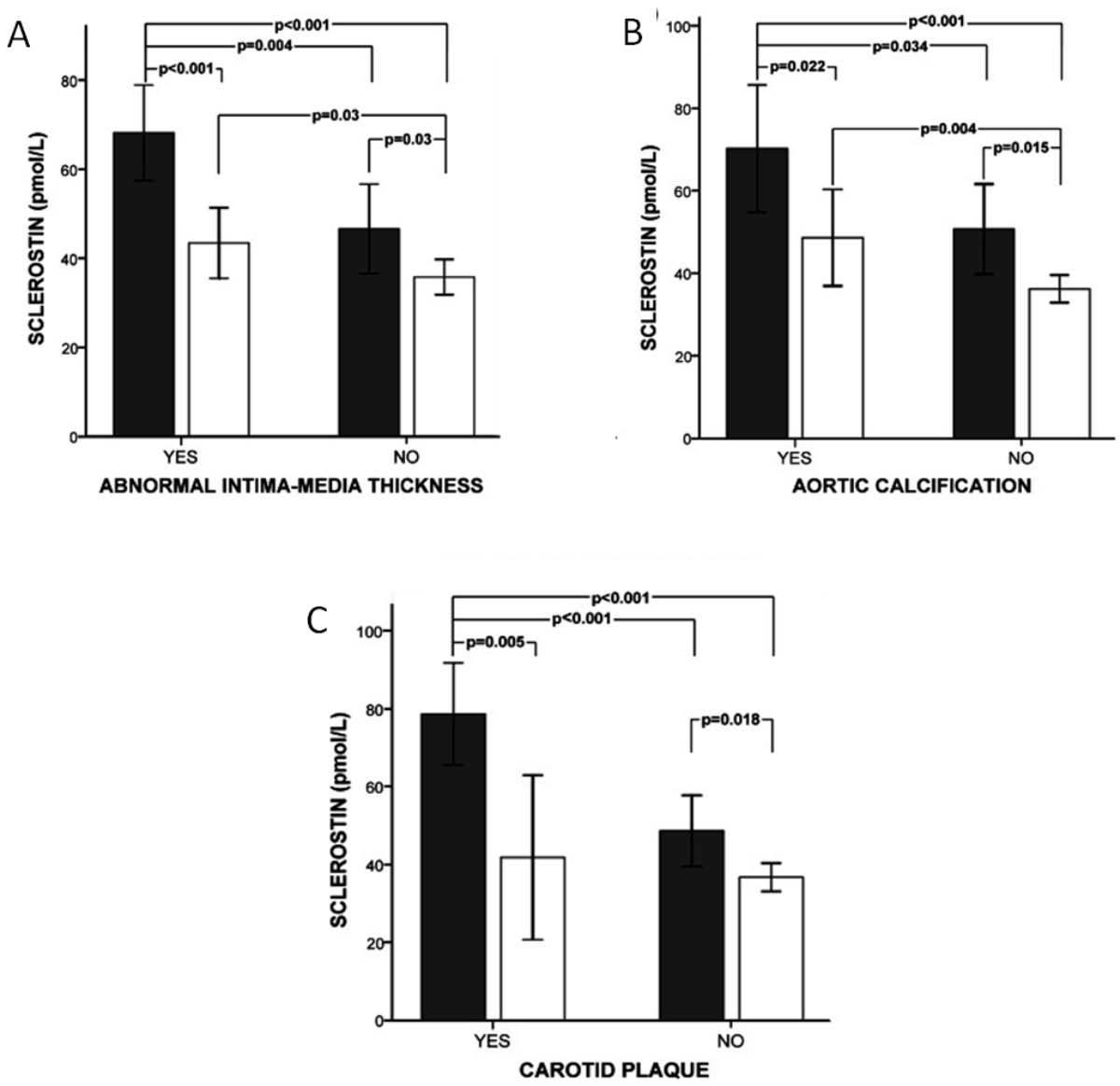

Fig. 23. Sclerostin serum levels in female (white bars) and male (gray bars) T2DM patients with and without abnormal IMT (A), carotid plaque (B), and aortic calcifications (C). Data are means ± 95% CI. Significant differences between group regions are indicated by a bar with the P value given above.

In males, serum sclerostin levels were higher patients with versus without abnormal IMT, carotid plaques, and aortic calcifications. In females, similar results were found, except for a lack of significance in serum sclerostin concentrations in patients with versus without carotid plaques.

The ROC curve analysis to evaluate the usefulness of sclerostin as a marker for high risk showed an area under the curve of 0.754 ± 0.059 ($P < 0.001$) for abnormal IMT thickness (Figure 24A), 0.800 ± 0.064 ($P < 0.001$) for carotid plaque (Figure 24B), and 0.760 ± 0.062 ($P = 0.001$) for aortic calcification (Figure 24C).

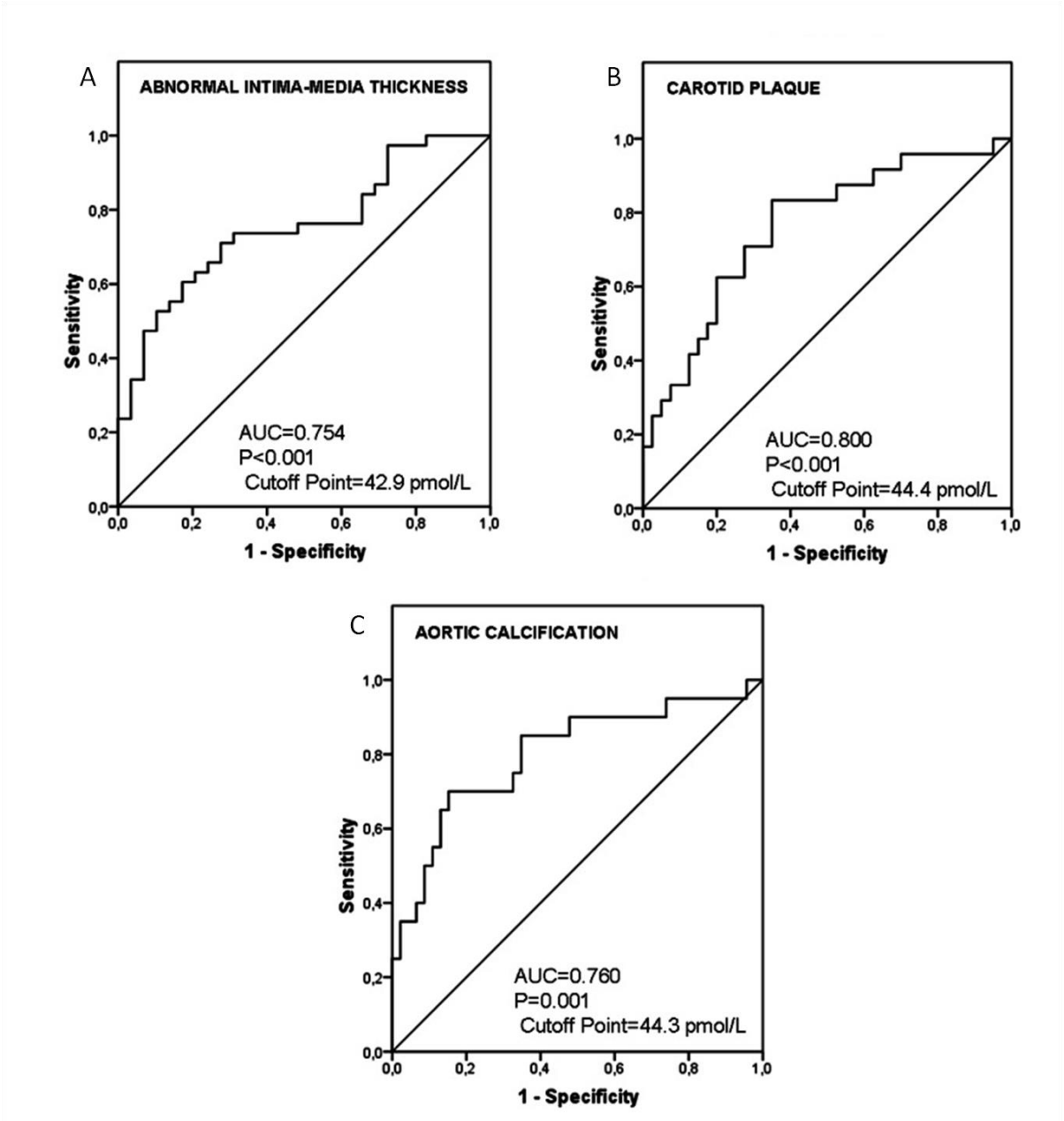

Fig. 24. Sclerostin ROC curve for abnormal IMT (A), carotid plaque (B), and aortic calcifications (C). AUC, area under the curve.

A concentration of ≥42.9 pmol/L showed a sensitivity of 73.7% and a specificity of 69% to identify an increased risk for abnormal IMT. A concentration of ≥44.4 pmol/L showed a sensitivity of 85% and a specificity of 65.2% to identify an increased risk for carotid plaque, and a concentration of ≥44.3 pmol/L showed a sensitivity of 75% and a specificity of 65% to identify an increased risk for aortic calcification.

4.4. Relationship of sclerostin serum levels in T2DM patients with anthropometric and biochemical parameters of AD risk

In male T2DM patients, significant positive correlations were observed between serum sclerostin levels and age (r = 0.34, P = 0.03), IMT (r = 0.48, P = 0.002), serum creatinin (r = 0.43, P = 0.006), and homeocysteine (r = 0.33, P = 0.047). After further adjustment for age, only creatinin (r = 0.43, P = 0.02) and homocysteine (r = 0.42, P = 0.02) remained significant. In contrast, we found no relation between sclerostin levels and age in female T2DM patients. However, in this group, sclerostin concentrations were positive related to HbA_{1c} (r = 0.46, P = 0.008) and homocysteine (r = 0.56, P = 0.0.002) and remained significant after adjustment for age. Moreover, in the entire cohort we found a positive correlation between levels of sclerostin and IMT (r = 0.52, P < 0.001) (Figure 25).

Linear regression analysis was performed to determine the influence of independent factors identified in univariate correlation analysis, including sex, age, HbA_{1c}, creatinin, homocysteine, and IMT as independent variables that explain serum sclerostin levels. The analysis demonstrated that homocysteine (β = 0.319 [95% CI 0.561–2.586], P = 0.003) and IMT (β = 0.330 [14.237–67.693], P = 0.003) were positively associated with serum sclerostin, independently of sex.

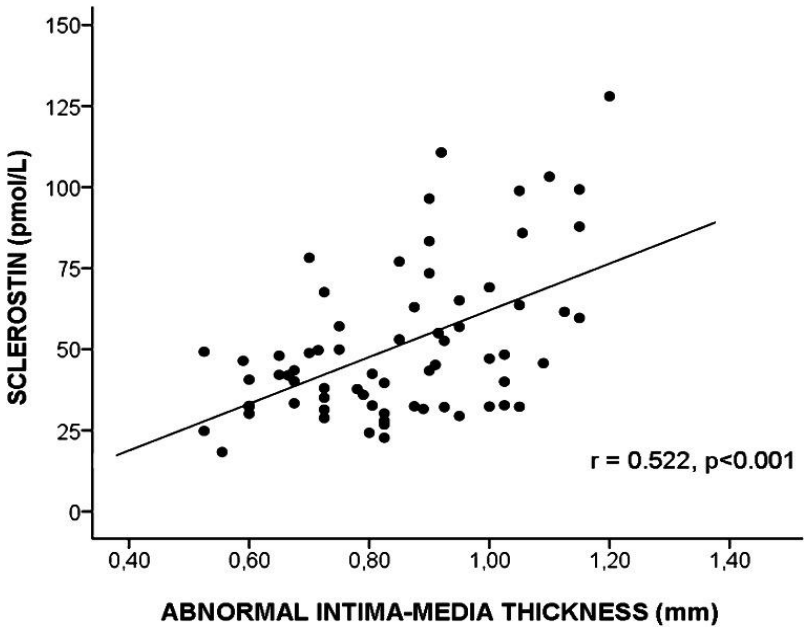

Fig. 25. Statistically significant positive correlation between sclerostin concentration and IMT in the whole T2DM population.

5. Discussion

Our cross-sectional study shows for the first time that higher sclerostin levels are associated independently with AD in T2DM patients. Secondly, high concentrations of sclerostin were associated with abnormal IMT, carotid plaques, and aortic calcifications in T2DM males. We found similar differences in T2DM females with abnormal IMT and aortic calcifications. Thirdly, we found a significant positive correlation among sclerostin levels, homocysteine, and IMT in T2DM patients, independently of sex.

The higher levels of sclerostin observed in T2DM patients with AD suggest a specific role of sclerostin in this process, confirming data from preclinical studies. Previous reports on the Wnt signaling antagonist sFRP (Barandon *et al.*, 2003; Román-García *et al.*, 2010) illustrated that its upregulation after injury was involved in healing and homeostasis of vascular tissue. In support of this hypothesis, data with the Wnt signaling agonist Dishevelled (Dvl) (Malekar *et al.*, 2010), which acts as a positive regulator of the Wnt pathway, showed that activation of Wnt signaling

was sufficient and critical for the induction of vascular injury. Therefore, sclerostin might also be indicative of a defensive mechanism activated in order to block or to attenuate the canonical Wnt pathway. The overexpression of sclerostin may influence disease progress, leading to restoration of quiescent Wnt signaling observed under health conditions.

We found an increase of serum sclerostin in T2DM patients with abnormal IMT and advanced aortic calcification. Moreover, T2DM males with carotid plaques had increased levels of sclerostin compared with those without carotid plaques. Our finding of a relationship between sclerostin and vascular status might be explained, at least in part, by sclerostin production not only by osteocyte cells from skeleton but also, mainly, by sclerostin upregulation in vascular cells previously transformed to osteocytic phenotype after osteogenic regulation, such as has been shown recently in VSMCs under calcifying conditions (Zhu *et al.*, 2011b). Several studies on Wnt inhibitors have shown an increase of its expression in advanced carotid plaques (Dkk1 antagonist) and in calcified aortas (SFRP1, -2, and -4), supporting the role of inhibitors in the establishment of a defensive response to reduce the activation of the Wnt pathway and, accordingly, to reduce ossification and avoid further atherosclerotic progression (Román-García *et al.*, 2010; Ueland *et al.*, 2009b). By contrast, the positive association between IMT and serum sclerostin seen in our study with T2DM patients was different from the findings of another study in chronic kidney disease (Thambiah *et al.*, 2012a), which showed a lack of correlation between sclerostin and a surrogate marker of arterial stiffness.

Several groups have previously reported opposed findings concerning the correlation between serum sclerostin concentrations and age (Cejka *et al.*, 2012a; García-Martín *et al.*, 2012b; Mödder *et al.*, 2011a). We found positive correlations in serum sclerostin levels with aging in male but not in female T2DM patients. This finding may reflect imbalances in vascular remodeling seen with aging in males in addition to skeletal remodeling. Also, higher sclerostin levels were observed in males compared with females as previously documented (García-Martín *et al.*, 2012b; Mödder *et al.*, 2011a). This sex difference could result from an influence of sex

hormones on sclerostin production (Mödder *et al.*, 2011b). In particular, large differences were found when sclerostin levels were compared in male versus female T2DM patients with AD or presence of abnormal IMT, carotid plaque, or aortic calcification. Differences can be explained by the scarce association between traditional risk factors for AD in females compared with males (Braunwald, 1997; Manolio *et al.*, 1992; Rimm *et al.*, 1993).

The significant positive correlation between sclerostin and creatinin values in male T2DM patients can be explained by a lack or reduction of clearance of the protein sclerostin, which, owing to its molecular weight of 22 KD, would be cleared by the kidney. Thus, we noticed that sclerostin concentrations were positively associated with HbA_{1c} levels in female T2DM patients. Hyperglycemia has effects on vascular complications (Cefalu, 2005) and also on the formation of advanced oxidation protein products that induce vascular calcification by promoting osteoblastic trans-differentiation of VSMCs (You *et al.*, 2009), which could explain, at least in part, overexpression of sclerostin in T2DM patients. To determine the factors independently associated with sclerostin, we included sex and all significant variables correlating with sclerostin (age, HbA_{1c}, creatinin, homocysteine, and IMT) in a multiple linear regression analysis. We found that homocysteine and IMT were factors independently associated with sclerostin levels. High plasma homocysteine level is an independent risk factor for the development of atherosclerosis, cardiovascular events, and stroke (Petramala *et al.*, 2009). Thus far, there is only one report showing the relationship between sclerostin metabolism and homocysteine in postmenopausal women (Urano *et al.*, 2012). On the other hand, carotid IMT is a strong predictor of vascular events (Lorenz *et al.*, 2007). To our knowledge, this is the first report that reveals a relationship between serum sclerostin levels and IMT. Future studies are required to uncover the relationship among sclerostin, homocysteine metabolism, and AD.

Our cross-sectional study has some limitations. First, the cross-sectional design does not allow establishment of a cause-effect relationship. Second, the sample size is relatively small and might affect the statistical power. However, we believe that our

findings are consistent. Moreover, pharmacologic treatment of patients with vascular disorders may have influenced the results. Strengths of our study are the evaluation of circulating serum sclerostin in patients with T2DM and AD for the first time and the exhaustive evaluation of biochemical and clinical parameters of atherosclerotic risk.

In summary, our observation that sclerostin circulates in a significant amount in T2DM patients with atherosclerotic lesions may support the hypothesis that sclerostin action is not only on the regulation of bone formation. Although the sample size of our study is small, we suggest that sclerostin circulating levels are a major modulator of Wnt signaling in AD, and they are implicated in the vascular integrity in T2DM. Nevertheless, it remains to be established whether higher levels of sclerostin have a protective role in the survival of patients with AD. The usefulness of sclerostin as a serum marker of atherosclerotic risk and vascular lesions in T2DM patients, merits further prospective studies.

CAPÍTULO V

Relationship of Dickkopf1 (Dkk1) with cardiovascular disease and bone metabolism in Caucasian type 2 diabetes mellitus.

Adaptado de: *Relationship of Dickkopf1 (Dkk1) with cardiovascular disease and bone metabolism in Caucasian type 2 diabetes mellitus.*

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1. Abstract

Dickkopf-1 (Dkk1) is a potent inhibitor of Wnt signalling, which exerts anabolic effects on bone and also takes part in the regulation of vascular cells. Our aims were to evaluate serum Dkk1 in type 2 diabetes (T2DM) patients and to analyze its relationships with cardiovascular disease (CVD). We also evaluated the relationship between Dkk1 and bone metabolism.

We conducted a cross-sectional study in which we measured serum Dkk1 (ELISA, Biomedica) in 126 subjects: 72 patients with T2DM and 54 non-diabetic subjects. We analysed its relationship with clinical CVD, preclinical CVD expressed as carotid intima media thickness (IMT), and bone metabolism.

T2DM patients with CVD ($P=0.026$) and abnormal carotid IMT ($P=0.038$) had higher Dkk1 concentrations. Dkk1 was related to the presence of CVD in T2DM, independently of the presence of risk factors for atherosclerosis. Therefore, for each increase of 28 pg/ml of serum Dkk1 there was a 6.2% increase in the risk of CVD in T2DM patients. The ROC curve analysis to evaluate the usefulness of Dkk1 as a marker for high risk of CVD showed an area under the curve of 0,667 (95% CI: 0.538–0.795; $P=0.016$). In addition, there was a positive correlation between serum Dkk1 and spine bone mineral density in the total sample ($r=0.183$; $P=0.048$).

In summary, circulating Dkk1 levels are higher in T2DM with CVD and are associated with an abnormal carotid IMT in this cross-sectional study. Dkk1 may be involved in vascular disease of T2DM patients.

2. Introduction

Patients with type 2 diabetes mellitus (T2DM) have a higher risk of cardiovascular disease (CVD). This association has serious consequences on the morbidity and the mortality in this population. Atherosclerosis is the main pathological mechanism in macrovascular disease in T2DM, which causes the development of atheroma plaques and the major life-threatening events in atherosclerosis, including myocardial infarction and stroke (Madonna and De Caterina, 2011b).

Patients with osteoporosis have a higher prevalence of cardiovascular disease, and it has been proposed that both pathologies share common pathophysiologic pathways (Magnus and Broussard, 2005). In T2DM there is an increased risk of fractures at any skeletal site even with greater bone mineral density (BMD) (Epstein and Leroith, 2008; Hofbauer *et al.*, 2007b), and there are data from epidemiological studies supporting a relationship between low bone mineral density (BMD) and atherosclerosis in T2DM (Carr *et al.*, 2008; Divers *et al.*, 2011). Thus, a better characterization of the pathways that could be involved in both processes is of interest, and may help in the development of new therapeutic tools.

The Wnt signalling pathways are involved in several developmental and physiological processes, including the cell differentiation and tissue/organ morphogenesis (Gordon and Nusse, 2006b). The discovery of the Wnt signaling pathway and its relevance in bone homeostasis has contributed to a better knowledge of the cellular and molecular mechanisms of bone biology (Huang and He, 2008). The activation of this pathway results in an expansion of osteoprogenitor cells and also determines a reduction in the apoptosis of osteoblasts, which leads to anabolic effects on bone (Huang and He, 2008). The canonical Wnt pathway is regulated by several families of secreted antagonists, such as the soluble frizzled-related receptors, dickkopf-1 (Dkk1) and sclerostin. Dkk1 regulates the Wnt signaling by binding to the Wnt co-receptor, the low-density lipoprotein-related receptor (LRP) 5/6. In addition to its binding to LRP 5/6, Dkk1 binds to other trans-membrane molecules as proteins Kremen, which increases their inhibitory activity on the Wnt signaling pathway (Mao *et al.*, 2002).

The role of Wnt signaling pathways in atherosclerosis it is a matter of growing interest. In preclinical studies the Wnt signaling pathways are involved in atherosclerosis-related processes as vascular calcification (Shao *et al.*, 2005c), inflammation (Sen and Ghosh, 2008), and the monocyte adhesion and its trans-endothelial migration (Christman *et al.*, 2008). In a recent study conducted in bovine aortic endothelial cells (ECs) (Cheng *et al.*, 2013), the authors found that Msx2 and Wnt7 family members stabilize the aortic ECs phenotype, whereas Dkk1 promotes an endothelial–mesenchymal transition in cultured bovine aortic ECs, a process associated to the pathogenesis of myocardial fibrosis with ischemia (Krenning *et al.*, 2010) and valve calcification (Wylie-Sears *et al.*, 2011). In addition, in animal models of CKD-MBD (chronic kidney disease-mineral bone disease) (Fang *et al.*, 2014) there is an increased renal production of Wnt inhibitor family members and higher levels of circulating Dkk1, sclerostin, and secreted klotho. In this model, the neutralization of Dkk1 by administration of a monoclonal antibody after renal injury stimulated bone formation rates, corrected the osteodystrophy, and prevented CKD-stimulated vascular calcification. Besides, the neutralization of Dkk1 suppressed aortic expression of the osteoblastic transcription factor Runx2, increased expression of vascular smooth muscle protein 22-α, and restored aortic expression of klotho"

There is also recent data showing a relationship between serum Dkk1 and atherosclerosis in humans (Kim *et al.*, 2011; Register *et al.*, 2013). In a previous study from Register and colleagues, Dkk1 concentrations were inversely related to coronary artery disease and aortic calcification. However, as the authors point, this study has been conducted in Afro-Americans (AAs) with T2DM, which has a different prevalence of atherosclerotic disease and osteoporosis compared to European populations. These differences make it interesting to explore this relationship in Caucasian type 2 diabetes. Besides, the relationship between Dkk1 concentrations and bone mass has been explored with conflicting data (Butler *et al.*, 2011; Cejka *et al.*, 2012b; Polyzos *et al.*, 2012), so its evaluation in a population with a high risk of bone fragility such as T2DM is of interest.

In this context, the objectives of our study were to evaluate serum Dkk1 levels in a cohort of T2DM patients and to analyze its relationships with CVD and bone metabolism. In addition, we compared serum Dkk1 concentrations between T2DM and non-diabetic subjects. Thus, our hypothesis was that Dkk1 concentrations may be related to CVD and bone metabolism in T2DM patients.

3. Subjects and methods

3.1. Study Population

Our study was a cross-sectional one that included 126 subjects. The estimated size of the sample considering an error of 5% and a statistical power of 80% was 84 subjects. The T2DM group included 72 patients with a diagnosis of diabetes according to the American Diabetes Association criteria (ADA, 2005). From January 2006 to December 2007 we consecutively recruited patients who had been referred to our outpatient clinic from community clinics for treatment of diabetes. The control group included 54 non-diabetic subjects who were consecutively recruited from the general community in the same period of time.

All participants were recruited according to the following criteria: Caucasian, free-living, aged 35 to 65 years and normal values for blood count, renal creatinine, hepatic function, calcium and phosphorus. We considered as exclusion criteria the presence of chronic diseases apart from T2DM in the non-diabetic group, the presence of diseases affecting bone (Paget's disease, rheumatoid arthritis, hyperparathyroidism, hypercortisolism, malignant tumors, renal bone disease, chronic liver disease and post-transplantation bone disease) and also previous or current treatment with drugs affecting bone metabolism (calcium supplements, vitamin D preparations, selective estrogen receptor modulators, calcitonin, estrogens therapy, antiresorptive therapy, thiazides, steroids, glucocorticoids or anticonvulsants). Patients treated with thiazolidinediones were also excluded.

The presence of cardiovascular disease was recorded by the medical history and after that it was checked in medical records. T2DM patients were classified into two groups according to the presence of clinical CVD: CVD group and non-CVD group. Inclusion criteria for patients with CVD were cerebrovascular disease (ischemic stroke or transient ischemic attack); coronary heart disease (previous myocardial infarction, diagnosed stable or unstable angina, or coronary revascularization surgery) or ischemic peripheral arterial disease (diagnosed by a positive Doppler ultrasound or arteriogram, or by a previous revascularization surgery).

The study was approved by the ethical review board of the Hospital Universitario San Cecilio and it was done conformed to the ethical guidelines for research in humans. All the participants in the study provided written informed consent after a full explanation of the purpose of the study and the nature of all the procedures that will be used in the study. Written informed consent was approved for the ethical review board of our hospital, and after that it was filed in the medical record of each patient.

3.2. Clinical evaluation

Height, weight, and waist circumference were measured at baseline according to standard procedures. Weight was measured to the nearest 100 grams using digital electronic scales. Height and waist circumference were measured to the nearest 1 mm using a stadiometer and a metal anthropometric tape, respectively. Body mass index (BMI) in kg/m^2 was calculated as weight divided by the square of height in meters.

Blood pressure was measured in a standardized manner. The subjects remained at rest for at least 5 minutes, and after that blood pressure was measured twice using a standard mercury sphygmomanometer (12 centimeters long, 35 cm wide). The mean of the two values was used for analysis. We defined hypertension when blood

pressure values were higher than 140/90 mmHg and/or where subjects were on antihypertensive treatment.

The participants reported their alcohol use, smoking status and their level of physical activity with a specific health questionnaire. Patients were classified as having a significant alcohol intake if it was higher than 40 grams/day in males and 24 grams/day in women; smoking status was categorized as no tobacco use or current tobacco use. Physical activity was collected through a specific questionnaire in which study subjects scored their physical activity on a scale from 0 (none) to 10 (sport more than one hour four times per week). Based on the results, the study sample was divided into two groups: sedentary (<5 on the scale) and no sedentary (≥5 on the scale).

3.3. Serum measurements

Samples of venous blood were taken in the morning after a fasting overnight. The sera were stored at −80°C until examination. Fasting plasma glucose (FPG), glycated hemoglobin (HbA1c), calcium, phosphorus and creatinine were measured using standard automated laboratory techniques. Glomerular filtration rate (GFR) was calculated by MDRD/CKD-EPI formula (Levey *et al.*, 2009). High-density lipoprotein (HDL), low-density lipoprotein (LDL) cholesterol and triglycerides (TGs) were measured by standard biochemical methods. Dyslipidemia was defined according to third report of the expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (ATP-III) criteria or current treatment with statins.

Calciotropic hormones measurement included serum intact parathormone (Two-site immunoassay for iPTH, Roche Diagnostics SL, Barcelona, Spain; intra and interassay variability of 3%) and 25-hydroxyvitamin D (25-OH-D, radioimmunoassay, DiaSorin, Stillwater, Minnesota USA. Serum Dkk1 was measured using quantitative sandwich enzyme-linked immunosorbent assay (ELISA) developed by Biomedica (Biomedica Medizinprodukte GmbH and Co.

KG, Wien, Austria) according to the manufacturer's instructions. The Biomedica Dkk1 ELISA (BI-20412) detects free Dkk1. Intra-assay and interassay variability were of 7% and 9%, respectively. Dkk1 measurements are reported throughout in pg/mL after conversion from pmol/L. One pmol/L is equivalent to 28.68 pg/mL.

3.4. Bone density measurement and vertebral fractures assessment

Bone mineral density at lumbar spine (LS) L2–L4, femoral neck (FN) and total hip (TH) was performed in all patients by dual-energy X-ray absorptiometry (DXA) using Hologic QDR 4500 densitometer (Whatman, MA; variation coefficient <1%). All the BMD measurements were done by the same experienced operator. We used the World Health Organization criteria for osteoporosis (Kanis JA, 1994).

Standardized spinal X-rays were taken for morphometric analysis of all participants of the study and they were interpreted according to the algorithm developed by McCloskey and colleagues (McCloskey *et al.*, 1993).

Carotid intima-media thickness Ultrasonographic examination of the carotid arteries was performed with patients in supine position using Doppler ultrasonography (Toshiba PowerVision 6000). The maximum intima-media thickness (IMT) at the carotid bifurcation (BIF) was determined between the near and far walls of the carotid bifurcation on the right and left sides. Each part was measured from views of both longitudinal and vertical sections at the bifurcation. If a discrepancy was observed in the measured values between longitudinal and vertical sections, the smaller value was selected to avoid overestimation. BIF–IMT was defined as the mean of the measurements from the right and left sides. The value for each side was obtained from the mean of 10 wall measurements. The BIF–IMT, measured in millimeters (mm), was considered pathological if it was ≥0.9 mm, and was considered to be carotid atherosclerosis if the BIF–IMT was ≥1.2 mm or 50% greater than the BIF–IMT in the adjacent area (Junyent *et al.*, 2005b). Plaques were identified as calcified by findings of bright white echoes on sonography. A single trained sonographer performed the study in all subjects.

3.5. Statistical analysis

Data for continuous variables are presented as mean ± standard deviation (SD). Data for categorical variables are presented as numbers and/or percentages. Kolmogorov–Smirnov test was used to test the normality of distribution of continuous variables. The association between continuous variables was described by Pearson's or Spearman's correlation coefficients. The comparison of categorical variables among groups was performed using Chi-square test or Fisher test. The comparison of continuous variables among groups was performed using unpaired Student's *t* test or Mann-Whitney test. To determine the independent variables which correlated with Dkk1 (dependent variable), the parameters that correlate significantly in univariate analysis and other which are biologically linked to Dkk1 were tested in multiple backward model linear regression analysis. Multiple backward model of logistic regression analysis was performed to identify Dkk1 as independent predictor for CVD (dependent variable) in T2DM patients. The model included established atherosclerotic risk factors (age, gender, body mass index, hypertension, dislipidaemia, smoking, sedentarism, HbA1c, GFR and intima-media thickness). CVD-defining parameters (cerebrovascular disease, coronary heart disease or ischemic peripheral arterial disease) were not included in the multiple logistic regression model. The usefulness of serum Dkk1 as a marker of high risk of atherosclerotic disease in T2DM was analyzed using a receiver operating characteristic curve (ROC Curve). A p value of less than 0,05 was considered to be significant (two-tailed). Data were recorded and analyzed using SPSS version 18.0 software (SPSS Inc, Chicago, IL, USA).

4. Results

4.1. Baseline Characteristics of the Study Population

The clinical characteristics of the study population are summarized in Table 9.

Table 9. Characteristics of the study population.

	Total Group (n=126)	**T2DM Group (n=72)**	**Non-diabetic Group (n=54)**	**P Value**
Age (years)	57±6	58±6	55±7	0.018
Male/female (n)	62/64	39/33	25/29	0.472
Medical history				
Duration of diabetes (years)	-	13.7±7.6	-	
Hypertension (%)	53.2	80.6	46.3	<0.001
Dyslipidaemia (%)	65.9	94.4	70.4	<0.001
Albuminuria (%)	20.5	35.0	4.0	0.006
Chronic kidney disease (KDOQI stages) (%)				
Stage 1	51	52	52	0.938
Stage 2	46	44	48	0.815
Stage 3	3	4	0	0.155
Smoker or ex-smoker (%)	15.1	16.7	13	0.623
Alcohol (%)	8.7	6.9	11.1	0.104
Sedentarism (%)	47.6	55.6	37.0	0.048
Clinical evaluation:				
BMI (kg/m^2)	102.6±12.4	31.4±5.7	29.3±5.9	0.043
Waist circumference (cm)	130±20	106.4±11.4	97.4±11.9	<0.001
SBP (mm Hg)	80±13	134±97	124±17	0.002
DBP (mm Hg)	30.5±5.9	80±12	79±15	0.705
Serum parameters:				
FPG (mg/dL)	137.2±61.9	173±60.1	89.4±10.4	<0.001
$HbA1_c$ (%)	6.7±2.2	8±1.9	4.8±0.4	<0.001
GFR (MDR/CKD-EPI)(ml/min/1,73 m^2)	92±23	92±23	93±22	0.745
Calcium (mg/dL)	9.5±0.5	9.6±0.5	9.3±0.4	0.001
Phosphorus (mg/dL)	3.6±0.5	3.7±0.5	3.5±0.5	0.01

PTH (pg/mL)	43.6±19.5	38.5±18.4	50.4±19.1	<0.001
25(OH) D (ng/mL)	19.5±11.3	17.8±11.5	21.6±10.9	0.06
Triglyceride (mg/dl)	142±121	169.9±149.8	104.9±47.7	<0.001
HDL-c (mg/dl)	53.5±15.5	49±16	59.5±12.5	<0.001
LDL-c (mg/dl)	111.7±35.5	96.9±34.1	130.8±27.4	<0.001
Dkk1 (pg/ml)	629±374	669±395	575±340	0.163
DXA parameters and VF:				
BMD LS (g/cm^2)	0.977±0.148	0.954±0.146	1±0.148	0.068
BMD FN (g/cm^2)	0.820±0.124	0.817±0.132	0.823±0.117	0.792
BMD TH (g/cm^2)	0.906±0.135	0.903±0.145	0.911±0.125	0.772
T-score LS	−1.08±1.36	−1.3±1.3	0.82±1.3	0.058
T-score FN	−0.55±1.01	−0.6±1.04	−0.49±0.99	0.565
T-score TH	−0.55±0.98	−0.62±1	−0.51±0.92	0.557
Osteoporosis (%)	15.9	24.6	9.4	0.047
Morphometric VF (%)	23.0	30.3	20.0	0.274
Cardiovascular disease:	35.7	58.3	5.6	<0.001
Cerebrovascular disease (%)	11.9	19.4	1.9	0.002
Coronary heart disease (%)	23.8	38.9	3.7	<0.001
Peripheral artery disease (%)	7.9	13.9	0	0.005
Abnormal intima-media thickness (%)	35.7	54.2	11.1	<0.001
Carotid plaques (%)	15.9	29.4	0	<0.001

Data for continuous variables are presented as mean ± SD. Data for categorical variables are presented as numbers and/or percentages. The comparison between groups was done by Student's t test (continuous variables) or Chi-square test (categorical variables).

T2DM: type 2 diabetes mellitus; BMI: body mass index; SBP: sistolic blood pressure; DBP: diastolic blood pressure; FPG: fasting plasma glucose; HbA1c: glycated hemoglobin; GFR: glomerular filtration rate; MDR/CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration; PTH: parathormone; 25(OH) D: 25-hydroxyvitamin D; HDL-c: High-density lipoprotein; LDL-c: Low-density lipoprotein; BMD: bone mineral density; LS: lumbar spine; FN: femoral neck; TH: total hip; VF: vertebral fractures.

Serum Dkk1 was significantly higher (P=0.026) in T2DM patients with CVD (757±416 pg/ml) compared with those without CVD (547±333 pg/ml). T2DM

patients with abnormal IMT (756±433 pg/ml *vs* 563±322 pg/ml; *P*=0.042) and cerebrovascular disease (854±480 pg/ml *vs* 625±363; *P*=0.045) had higher Dkk1 concentrations. (Figure 26 and Table 10). However, we did not find differences in Dkk1 concentrations according to the presence of carotid plaque (*P*=0.522), coronary heart disease (*P*=0.677) or ischemic peripheral artery disease (*P*=0.762).

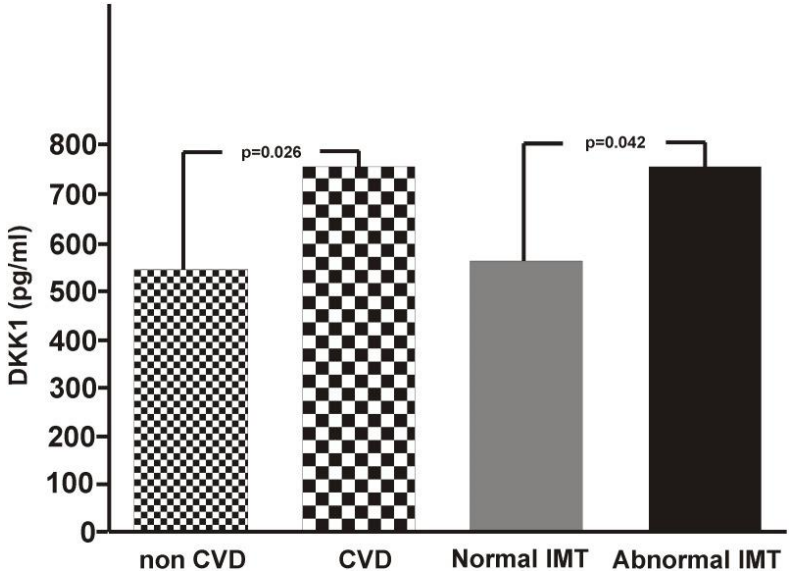

Fig. 26. Dkk1 serum levels in T2DM patients according to the presence of cardiovascular disease and abnormal intima-media thickness. Differences between groups are indicated through a bar with the P-value given above.

Table 10. Dkk1 concentrations according to the presence of cardiovascular disease in the T2DM group.

	Yes	No	P value
Cardiovascular disease	757±416	547±333	0.026
Cerebrovascular disease	854±480	625±363	0.045
Coronary heart disease	694±379	654±408	0.677
Peripheral artery disease	634±247	675±415	0.762
Abnormal intima-media thickness	756±433	567±322	0.042
Carotid plaque	730±422	661±395	0.522

The comparison between groups was done by Student's t test.

A model of logistic regression analysis was performed using the presence of CVD as a dependent variable. Independent variables were serum Dkk1 levels and risk factors for atherosclerosis (age, gender, body mass index, hypertension, dislipidaemia, smoking, sedentarism, HbA1c, GFR, and IMT). Dkk1 levels were independently associated with the presence of CVD in T2DM (odds ratio: 1.062, 95% confidence interval: 1,003–1,125; $P=0.04$). Therefore, for each increase of 28 pg/ml of serum Dkk1 there was a 6.2% increased risk of CVD in T2DM patients.

In the ROC curve analysis to evaluate the usefulness of Dkk1 as a marker for high risk of CVD, the area under the curve was 0.667 (95% confidence interval: 0.538–0.795; $P=0.016$) (Figure 27). A concentration of 494 pg/ml or higher showed a sensitivity of 71.4% and a specificity of 60% to identify an increased risk of CVD.

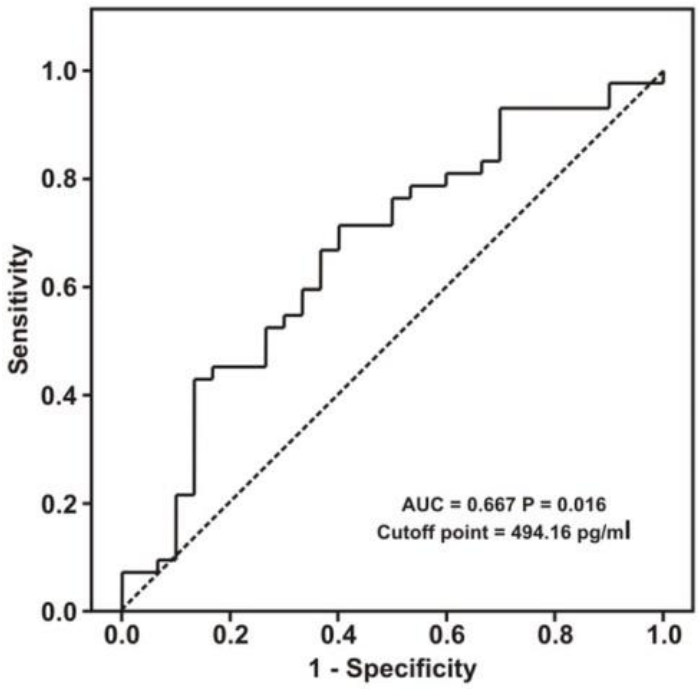

Fig. 27. Dkk1 ROC curve for cardiovascular disease. AUC, area under the curve.

We found no differences in Dkk1 between study groups: T2DM 669±395 vs non-diabetic 575±340 pg/ml, $P=0.163$. Serum Dkk1 concentrations were significantly

higher in females than in males in total sample (697±434 vs 563±293 pg/ml, $P=0.046$) and in T2DM group (788±493 vs 569±255 pg/ml, $P=0.025$) but were not significantly increased in control females (593±335 vs 553±349 pg/ml, $P=0.682$).

Linear regression analysis was performed to determine the influence of independent factors identified in univariate correlation analysis, including group, gender, age, HbA1c, CVD and IMT as independent variables that explain serum Dkk1 levels. The analysis demonstrated that gender ($\beta=-0.299$, $P=0.002$) and CVD ($\beta=0.292$, $P=0.007$) were positively associated with serum Dkk1.

Regarding bone metabolism, lumbar spine BMD was positively related to serum Dkk1 in total sample ($r=0.183$, $P=0.048$). In contrast, we did not found a significant relationship with bone remodelling markers osteoporosis diagnosis or morphometric vertebral fractures (data not shown), in the total sample or in the study groups separately.

5. Discussion

To our knowledge, there is no previous study evaluating Dkk1 concentrations and its relationship with CVD in Caucasian T2DM patients. There is also scarce data concerning the relationship between Dkk1 and bone metabolism in T2DM. Our results show that higher levels of Dkk1 are associated with clinical CVD and abnormal IMT in T2DM patients. These findings suggest that serum Dkk1 are related to the presence of CVD in this population. However, our data showed no differences in serum Dkk1 between T2DM patients and non-diabetic patients. Regarding bone metabolism, we found a significant relationship with bone mineral density.

In our study, higher Dkk1 levels were positively related to cardiovascular disease in T2DM patients independently of the presence of others risk factors for atherosclerosis, and high concentrations of Dkk1 were related to abnormal IMT in

these patients. Our results are in accordance with previous reports showing the relationship between vascular disease and Dkk1 in other populations. Patients with cerebrovascular disease have higher serum Dkk1 levels compared with control subjects (Seifert-Held *et al.*, 2011), and serum Dkk1 correlated with coronary artery calcification and atherosclerotic plaques evaluated by coronary computed tomography and coronary artery calcium scoring (Kim *et al.*, 2011). Previously, Ueland and colleagues (Ueland *et al.*, 2009c) demonstrated that Dkk1 expression is enhanced in advanced carotid plaques and that Dkk1 is a novel mediator in platelet-mediated endothelial cell activation, which could occur within the atherosclerotic lesions. Moreover, current data clearly demonstrate a novel role for Wnt proteins in the proliferation, migration, and survival of smooth muscle cells (SMCs) (Mill and George, 2012). Regarding this, higher levels of Dkk1 are present in advanced carotid plaques and Dkk1 promotes platelet-mediated activation of endothelial cells. Thus, it has been proposed that this could lead to enhanced inflammation in the atherosclerotic lesion; however, it may also lead to a reduced survival, proliferation, and migration of SMCs, thereby reinforcing the potential complexity of the participation of Wnt proteins in atherosclerosis (Mill and George, 2012).

In contrast to our findings, Dkk1 concentrations were negatively associated with atherosclerotic calcified plaque in African-Americans (AAs) patients with T2DM (Register *et al.*, 2013). There are several reasons that might contribute to this discrepancy. As the authors point, AAs have lower rates of vascular calcification. It has been reported lower levels of coronary artery calcified plaque relative to European Americans (EAs) despite similar or more detrimental cardiovascular risk factor profiles (Bild *et al.*, 2005; Lee *et al.*, 2003). This observation is consistent both in T2DM and subjects without diabetes (Freedman *et al.*, 2005; Wade *et al.*, 2011), and suggests a differential impact of CV risk factors on atherosclerosis based on ethnicity although the underlying causes of ethnic differences in vascular calcification are not well established (Divers *et al.*, 2013). Reinforcing these ethnic differences, AAs are at lower risk for myocardial infarction based on less coronary artery calcification, and they have significantly lower rates of myocardial infarction than EAs, provided equal access to healthcare (Karter *et al.*, 2002; Young *et al.*,

2003). It has been suggested that AAs may be less susceptible than whites to hyperglycaemia–induced macrovascular disease (Cannata-Andia *et al.*, 2011). Thus, these findings may explain the differences observed in the relationship between vascular disease and Dkk1 between our study and the data from Register and colleagues.

Recent data suggests that the association between vascular calcification and osteoporosis is not simply a consequence of age, stressing that the co-incidence of vascular calcification with low bone activity and osteoporosis could be biologically linked. During the development of vascular calcification, there is a transition of vascular SMCs towards an osteoblast- like phenotype, which promotes the mineralization within these structures. In this process there are several players, including those related to mineral metabolism, like phosphorus, calcium or parathyroid hormone, which influences the expression of osteogenic factors. There is emerging evidence suggesting that some inhibitors of the Wnt pathway, such as secreted frizzled Proteins 2 and 4 and Dkk1, may play a role linking vascular calcification and bone loss (Mill and George, 2012; Ueland *et al.*, 2009c). AAs manifest a skeletal resistance to the effects of parathyroid hormone (Aloia *et al.*, 1996; Bell *et al.*, 2001) and exhibit opposite relationships between arterial calcification and serum vitamin D concentrations compared with Europeans (Freedman and Register, 2012). In our opinion, these ethnic differences in the effects of calciotropic hormones on bone and vessels may indicate that differences in other pathways, as Wnt pathway, may be expected according to race. However, this hypothesis must be confirmed.

There are other differences between the study from Register and colleagues and ours, which may explain the discrepancy of results. First, the prevalence of cardiovascular disease is lower, 27%, compared to our study where a 58% of patients had previous cardiovascular disease. Second, in the study from Register and colleagues 28.3% of subjects were taking hormonal therapy while no women were on hormonal therapy in our study. Estradiol and progesterone has shown to regulate Wnt pathways in endometrial tissue (Wang *et al.*, 2009) and brain (Scott and

Brann, 2013), so an influence of hormonal therapy in Dkk1 concentrations can not be excluded. Finally, despite the low expected incidence of arterial calcification in AAs, in the study from Register and colleagues there is a high prevalence of aortic calcification. Thus, differences between study populations apart from the ethnicity may influence the discrepancy of results.

There are no previous studies evaluating differences in serum Dkk1 concentrations according to the presence of diabetes. We did not find differences in Dkk1 between T2DM patients and subjects without diabetes. These results contrast with our previous data showing higher sclerostin concentrations in the same group of type 2 diabetes patients (García-Martín *et al.*, 2012c). However, serum Dkk1 and sclerostin have shown to be barely correlated (Thambiah *et al.*, 2012b), as we found in our sample (data not shown). In addition, the effects of Dkk1 and sclerostin may differ due to differences in Kremen binding. While Dkk1 is always an inhibitor of Wnt signaling, the effect of sclerostin is variable depending on context (Kawano and Kypta, 2003). Unlike our previous results about sclerostin, women had higher Dkk1 concentrations both in the total sample and in the T2DM group. Estradiol and progesterone have shown to take part in the regulation of the Wnt pathways in endometrial tissue (Wang *et al.*, 2009) and brain (Scott and Brann, 2013), so a sex steroid-induced effect may explain gender differences in Dkk1 although this hypothesis must be confirmed.

Regarding the relationship between bone metabolism and Dkk1 we only found a weak correlation with BMD at lumbar spine in the total sample, and no relationship with bone turnover markers, osteoporosis or morphometric vertebral fractures (data not shown). In addition, lumbar spine BMD may be affected by aortic calcification. Our findings confirm previous data showing no relationship between Dkk1 and bone turnover markers in postmenopausal osteoporosis (Polyzos *et al.*, 2012). The relationship between Dkk1 and BMD is not totally established. It has been reported no relationship with volumetric BMD in Afro-American T2DM patients (Register *et al.*, 2013). However, is also been described and inverse relationship between Dkk1 and BMD, and higher Dkk1 concentrations in patients with osteoporosis (Butler *et*

al., 2011). Therefore, in our opinion the data about serum Dkk1 and bone metabolism are controversial and thus no clear conclusions can be drawn.

Our study has some limitations. First, the cross-sectional design does not allow to establishing a cause-effect relationship. Second, the sample size is relatively small and might affect the statistic power. Our study has also several strengths, as the evaluation of circulating serum Dkk1 in Caucasian patients with T2DM for the first time, the comparison to non-diabetic subjects and the evaluation both of bone metabolism and atherosclerotic disease.

In summary, serum levels of Dkk1 are elevated in Caucasian patients with T2DM presenting CVD. These findings suggest that Wnt signaling pathway is involved in vascular disease in diabetic subjects. However, the relations of Dkk1 with bone metabolism are inconsistent.

DISCUSIÓN GENERAL

La DM2 se considera uno de los principales problemas de salud a nivel mundial debido al aumento de su prevalencia en los últimos años, alcanzando dimensiones de epidemia en los países desarrollados, suponiendo un gran impacto socio-económico. Aunque la aparición de la diabetes está en gran medida determinada por factores genéticos y por factores de riesgo convencionales, no se conocen de manera exacta la totalidad de los factores implicados en el desarrollo de la enfermedad.

Durante la última década, el concepto de músculo esquelético se ha modificado considerándose actualmente como un tejido metabólicamente activo, con funciones autocrinas y endocrinas que pueden regular el metabolismo energético y el tejido graso a través de la liberación de determinadas mioquinas. Algunas de ellas tienen funciones bien conocidas. Sin embargo, existen otras con funciones casi desconocidas en humanos.

En los últimos 5 años se ha acumulado una fuerte evidencia de que el músculo esquelético juega un papel importante en la fisiopatología de determinadas alteraciones metabólicas, observándose que algunas mioquinas secretadas por el músculo están involucradas en la regulación de la sensibilidad/resistencia a la insulina y del metabolismo hepático y graso. Este es el caso de la miostatina e irisina, que intervienen en la regulación del metabolismo de la glucosa y en la sensibilidad a la insulina (Eckardt *et al.*, 2014) aunque su papel exacto en la DM2 no se conoce con exactitud,existiendo resultados contradictorios.

En este contexto, uno de los objetivos de este trabajo fué determinar los niveles de miostatina e irisina en pacientes con DM2 para tratar de elucidar la implicación de estas mioquinas en la diabetes.

La miostatina actúa principalmente como regulador negativo de la masa muscular. Sin embargo, estudios *in vitro* y en animales, sugieren que además ejerce un papel en la regulación del metabolismo de la glucosa, existiendo evidencias de una mejora en los niveles de glucosa plasmática en ayunas y sensibilidad a la insulina después de la inhibición o disminución de los niveles de miostatina (Cleasby *et al.*, 2014b; Guo *et al.*, 2009; Zhang *et al.*, 2012). Cleasby y colaboradores (2014), explican el incremento

de la capacidad de captación de glucosa basal estimulada por insulina observado tras la inhibición de la miostatina, a través de la estimulación de la expresión de transportadores de glucosa GLUT1 y especialmente GLUT4 en modelos murinos. Sin embargo, los resultados sobre el efecto de la miostatina en seres humanos no están del todo claros, observándose datos contradictorios (Antony *et al.*, 2007; Mitchell *et al.*, 2006; Zhang *et al.*, 2011).

Según estudios previos, la disminución de miostatina da lugar a efectos metabólicos beneficiosos. Sin embargo, en contra de lo esperado, nuestros resultados muestran niveles disminuídos de miostatina en pacientes con DM2 en comparación con sujetos no diabéticos, mostrando una relación inversa entre los niveles de miostatina y los de FPG y TG. Además, también observamos un efecto independiente de los niveles de glucosa sobre los niveles circulantes de miostatina. De esta forma, la hipertrigliceridemia e hiperglucemia característica de los pacientes diabéticos, podría explicar la disminución de los niveles de miostatina en los pacientes con DM2. Existen estudios que apoyan estos resultados mostrando niveles disminuídos de miostatina en pacientes con síndrome metabólico con respecto a controles sanos (Han *et al.*, 2014).

Diversos estudios en modelos animales evidencian que el papel de la miostatina no solo está restringido al músculo esquelético y la homeostasis glucídica, sino que también desempeña un papel importante en la regulación del metabolismo lipídico y del tejido adiposo. Así, se ha observado que la deficiencia de miostatina en ratones, activa enzimas lipolíticas incrementando la oxidación de ácidos grasos e induciendo la formación de tejido adiposo marrón en el tejido adiposo blanco (Zhang *et al.*, 2012). Sin embargo, el mecanismo exacto mediante el cual la miostatina regula el tejido adiposo, sigue siendo desconocido. Estudios recientes sugieren una implicación de la irisina en el proceso de regulación del tejido adiposo por parte de la miostatina.

La irisina, descubierta recientemente el el año 2012, es una mioquina a la que se le han atribuído numerosos efectos beneficiosos. Fundamentalmente participa en el pardeamiento del tejido adiposo blanco, incremetando la formación de adipocitos

marrones en el tejido adiposo blanco, capaces de expresar elevados niveles de UCP-1, proteína implicada en la respiración mitocondrial y en la disipación de energía. Es por eso, que a la irisina se la conoce de forma coloquial como la "hormona quemagrasas".

En cuanto a la implicación de la irisina en la regulación del tejido adiposo por parte de la miostatina, existen evidencias experimentales en ratones que han demostrado que la deficiencia de miostatina da lugar a la activación PGC1α que incrementa la expresión de FNDC5 dando lugar por tanto a un incremento de la secreción de irisina en el músculo. Esto resulta en el pardeamiento de los adipocitos blancos (Shan *et al.*, 2013) aumentando así el gasto energético. Nuestros resultados también apuntan en este sentido, mostrando una conexión entre ambas mioquinas. Así, hemos observado una asociación inversa entre irisina y miostatina, así como un efecto independiente de los niveles de irisina sobre las concentraciones circulantes de miostatina. Apoyando nuestros resultados, un estudio reciente en adipocitos humanos, ha demostrado que el tratamiento con irisina aumenta los niveles de IGF-1 y disminuye los niveles de ARNm de miostatina, incrementando así el crecimiento muscular (Huh *et al.*, 2014). Todos estos hallazgos avalan la conexión entre miostatina e irisina que podrían ejercer una acción combinada en la misma dirección. Así, al igual que la miostatina, algunos estudios revelan un papel adicional de la irisina en la homeostasis glucídica (Choi *et al.*, 2013; Højlund and Boström, 2013), observándose una estimulación de la expresión del transportador 4 de la glucosa (GLUT4) y de la biogénesis mitocondrial en modelos animales (Vaughan *et al.*, 2014). Sin embargo, poco se sabe sobre las funciones concretas de la irisina en humanos, existiendo controversia acerca de sus propiedades beneficiosas (Crujeiras *et al.*, 2014; Pardo *et al.*, 2014).

En cuanto a la implicación de la irisina en la DM2, existen algunos estudios cuyos resultados no son concluyentes mostrando datos contradictorios (Choi *et al.*, 2013; Liu *et al.*, 2013; Moreno-Navarrete *et al.*, 2013; Sanchis-Gomar and Perez-Quilis, 2014b) (Ebert *et al.*, 2014; Kurdiova *et al.*, 2014; Nair *et al.*, 1986; Piya *et al.*, 2014).

Nuestros datos muestran niveles más elevados de irisina en pacientes con DM2 en comparación con sujetos no diabéticos. Además, observamos una correlación positiva entre los niveles de irisina y los niveles de TG y de glucosa. Por otra parte, observamos un efecto independiente de los niveles de TG sobre los niveles circulantes de irisina, lo que sugiere una posible influencia del perfil lipídico en concentraciones séricas de irisina. De esta forma, los niveles incrementados de TG y de glucosa en los pacientes diabéticos, así como la influencia de los niveles de TG sobre los niveles de irisina, podrían explicar la elevación de los niveles de esta mioquina en pacientes diabéticos con respecto a controles no diabéticos. En la línea de nuestros resultados, algunos estudios han mostrado una asociación positiva entre irisina, niveles de TG y factores aterogénicos en pacientes con síndrome metabólico (de la Iglesia *et al.*, 2014), así como un aumento en la expresión de PGC1α en el músculo esquelético en condiciones de estrés oxidativo, inflamación y altos niveles de AGLs (Vaughan et al., 2014), como ocurre en la obesidad, el síndrome metabólico o la diabetes. Esto daría lugar a un incremento en los niveles de irisina en estas situaciones, estimulando la producción de adipocitos marrones en el tejido adiposo blanco, la expresión de UCP1, la termogénesis y la disminución de resistencia a la insulina (Choi *et al.*, 2013; Sanchis-Gomar and Perez-Quilis, 2014b). Estudios recientes en adipocitos humanos, han mostrado que el aumento de los niveles de irisina también está implicado en la disminución de lípidos acumulados a través de un aumento de la triglicérido lipasa de adipocitos (ATGL) y una disminución de la expresión de la ácido graso sintasa (FAS). Esto sugiere que una de las acciones de la irisina estaría encaminada a disminuir la grasa acumulada en el tejido adiposo blanco a través de ATGL y del aumento de la expresión UCP1 y la termogénesis así como a inhibir la síntesis de lípidos posiblemente en respuesta a los niveles incrementados de TG en la DM2 que según nuestros resultados estimularían la síntesis de irisina. Apoyando estos resultados, los pacientes con DM2 tienen un mayor gasto energético basal con respecto a sujetos sanos como ha sido descrito en estudios clásicos (Bogardus *et al.*, 1986; Fontvieille *et al.*, 1992; Nair *et al.*, 1986).

Aunque son necesarios estudios futuros que confirmen nuestros resultados, sugerimos que estas mioquinas podrían actuar en la misma línea que los resultados

mostrados en modelos animales y en estudios *in vitro* y proponemos el siguiente mecanismo de acción que implica la conexión entre el músculo y el tejido adiposo a través de la acción de la miostatina y la irisina en pacientes con DM2: La hiperglucemia actuaría como una señal de alarma que regula a la baja la expresión de miostatina. Esta disminución en las concentraciones séricas de miostatina tendría un efecto, por un lado, sobre el metabolismo de la glucosa estimulando la expresión de transportadores de glucosa, y por otro lado, sobre el metabolismo lipídico, induciendo la activación de enzimas lipolíticas que aumentan la oxidación de ácidos grasos y estimulando la formación de adipocitos marrones. Asimismo, la disminución de los niveles séricos de miostatina junto con el elevado contenido de TGs así como el aumento del estrés oxidativo y del estado inflamatorio característico de la DM2, daría lugar a un incremento de los niveles circulantes de irisina. Los efectos de la irisina actuarían, al igual que la miostatina, a nivel glucídico, estimulando la expresión del transportador de glucosa GLUT4 y la biogénesis mitocondrial así como la formación de adipocitos marrones, la termogénesis y el consumo lipídico. Por tanto, la variación en los niveles de estas mioquinas podría representar una respuesta de adaptación para contrarrestar los trastornos metabólicos de la DM2, reflejando la necesidad de las acciones de estas mioquinas en el músculo y el tejido adiposo encaminadas a estimular el metabolismo oxidativo para aumentar el consumo energético y mejorar el metabolismo glucídico y lipídico en esta población (Figura 28).

Aunque hay varios estudios que apoyan el efecto compensador de estas hormonas, también existen otras hipótesis que tratan de explicar los niveles de estas mioquinas en pacientes con trastornos metabólicos. Así, algunos estudios han propuesto que el aumento de irisina podría representar un proceso de resistencia similar al de la resistencia a la insulina o la leptina característico de pacientes con trastornos metabólicos (Boström and Fernández-Real, 2014; Panagiotou *et al.*, 2014).

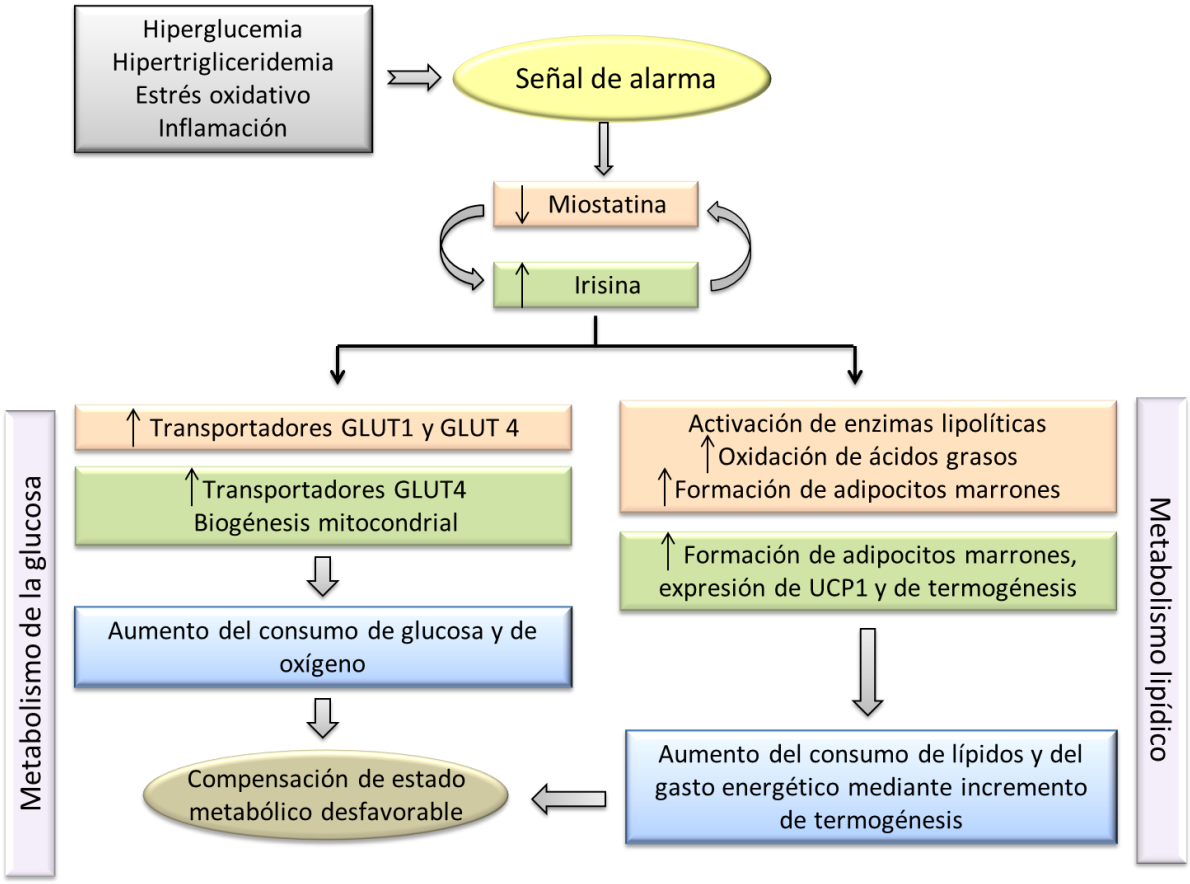

Fig. 28. Esquema del mecanismo de acción de la miostatina y la irisina en la DM2.

En conclusión, el músculo ha empezado a considerarse como un tejido metabólicamente más complejo y el mayor conocimiento de sus funciones puede contribuir al desarrollo de futuras estrategias preventivas, intervenciones eficientes y herramientas para el diagnóstico y seguimiento de pacientes con diversas enfermedades metabólicas.

Por otro lado, la DM2 aumenta el riesgo de desarrollar diversas complicaciones, entre ellas la ECV que representan la principal causa de mortalidad de esta enfermedad (Fruchart *et al.*, 2014; Iglesias *et al.*, 2015). La probabilidad de desarrollar complicaciones vasculares en los pacientes con DM2 depende de una serie de factores tradicionales y de otros que no se conocen completamente. Actualmente no

existen herramientas eficaces para predecir el riesgo de desarrollar o no complicaciones vasculares, ya que sólo algunos de los pacientes diabéticos desarrollan estas complicaciones a pesar de tener los mismos factores de riesgo tradicionales. Por ello, el conocimiento de nuevos factores implicados en estos trastornos, es fundamental y puede contribuir a la identificación precoz de los sujetos con un mayor riesgo cardiovascular para poder establecer un protocolo de actuación antes de que se produzcan daños irreversibles.

Así, algunos de los objetivos de esta Tesis Doctoral, van encaminados a la identificación de factores implicados en las complicaciones vasculares asociadas a la DM2 a través del uso de técnicas proteómicas y metabolómicas.

El análisis proteómico ofrece un enfoque libre de hipótesis no limitado por conocimientos previos, en el cual se integran tanto las influencias genéticas como epigenéticas mediante el estudio de los perfiles de expresión de proteínas. En nuestro análisis proteómico, utilizando 2D-DIGE y LC-MS/MS, hemos seleccionado sujetos no diabéticos como grupo control, pacientes con DM2 sin ECV establecida y pacientes con DM2 con manifestaciones clínicas de ECV, para identificar así el perfil proteómico asociado con la ECV más severa y avanzada.

Nuestros resultados, han revelado cambios en los niveles de expresión de proteínas relacionadas con el estado inflamatorio y redox que se sabe que desempeñan un papel importante en la patogénesis de las complicaciones vasculares, aunque no se conocen con exactitud la totalidad de los mecanismos implicados. Nuestros datos muestran niveles incrementados de RBP4 en los pacientes con DM2, siendo significativamente mayores en aquellos pacientes con presencia de ECV establecida. RBP4 es una proteína generada por adipocitos maduros (Friebe *et al.*, 2011), que participa en diversas funciones como la transducción sensorial, la activación de macrófagos (Broch *et al.*, 2010) y el transporte plasmático de retinol. El complejo RBP4-retinol interactúa con la transtiretina, evitando su pérdida por filtración a través de los glomérulos renales. Además, algunos estudios han descrito la participación de RBP4 en la resistencia a la insulina (Friebe *et al.*, 2011; Park *et al.*, 2014; Shaker *et al.*, 2011) observándose que la mejora de la sensibilidad a la insulina

producida por el ejercicio u otras intervenciones de pérdida de peso podría estar mediada por la reducción de los niveles séricos de RBP4 (Balagopal *et al.*, 2007; Haider *et al.*, 2007). Según estos estudios, RBP4 podría estar implicada en la inducción de la resistencia a la insulina. En concordancia con estos datos, un estudio reciente ha determinado el mecanismo de inducción de la resistencia a la insulina mediante la activación de las células presentadoras de antígeno (CPA) del tejido adiposo por parte de RBP4. Esta activación induce mayores niveles de citoquinas proinflamatorias que resultan en una inflamación del tejido adiposo que conduce a la resistencia a la insulina (Moraes-Vieira *et al.*, 2014). Esto explicaría los niveles elevados de RBP4 en pacientes con DM2 respecto a sujetos no diabéticos, sin embargo, la implicación de RBP4 en las complicaciones cardiovasculares y su posible utilidad como un marcador biológico de las mismas, no se conoce con exactitud existiendo escasos estudios al respecto. Los niveles incrementados de RBP4 en pacientes con DM2 y ECV observados en nuestro estudio, son consistentes con dos estudios preliminares que muestran una relación entre los niveles de RBP4 y FRCV comunes en la población anciana (Ingelsson *et al.*, 2009; Sasaki *et al.*, 2010). Un estudio reciente realizado por Farjo y colaboradores, ha demostrado que el efecto inflamatorio de RBP4 no está únicamente asociado a la resistencia a la insulina, sino que existe un efecto dosis-dependiente de RBP4 en la activación de NF-kB y NADPH oxidasa, que promueve la expresión de moléculas proinflamatorias tales como la molécula vascular de adhesión celular 1 (VCAM-1), molécula de adhesión intercelular 1 (ICAM-1), E-selectina e interleuquina 6 (IL-6). Esto da lugar a la adhesión de leucocitos al endotelio y por tanto a la inflamación endotelial característica de las complicaciones vasculares. Nuestros resultados apuntan en la misma dirección que estos últimos datos, sugiriendo que el incremento de los niveles séricos de RBP4 podría ser una de las causas implicadas en el desarrollo y progresión de la ECV asociada a la DM2 a través del aumento de la inflamación endotelial.

Nuestros resultados, además han mostrado un incremento de los niveles de GPx-3 en el suero de los pacientes diabéticos con ECV en comparación con aquellos sin ECV establecida y sujetos no diabéticos. GPx-3 es una proteína perteneciente a la

familia de las glutatión peroxidasas (GPx) que participan en la reducción de ROS, disminuyendo así el estrés oxidativo. GPx-3 cataliza la reducción de peróxido de hidrógeno, peróxidos de lípidos e hidroperóxidos orgánicos y contribuye al mantenimiento de la biodisponibilidad vascular de NO.

Es bien conocido que en las complicaciones vasculares se produce un incremento tanto de los procesos inflamatorios como del estrés oxidativo, debido a un aumento de ROS (Abe *et al.*, 2013). En condiciones normales, para tratar de compensar los efectos tóxicos de los radicales libres, se produce una cascada de reacciones redox entre el NO y ROS que aumenta la peroxidación lipídica la cual origina graves daños celulares (Beckman *et al.*, 1990). El aumento de la peroxidación de lípidos en pacientes con complicaciones cardiovasculares es bien conocido, constituyendo uno de los factores más importantes implicados en el desarrollo de la aterosclerosis (Ehara *et al.*, 2001). Por ello, el buen funcionamiento de los sistemas de defensa endógenos, es esencial en la prevención de estas complicaciones. Según nuestros resultados, se postula que los pacientes con DM2 y ECV presentan niveles incrementados de GPx-3 para reducir la peroxidación lipídica así como para mantener los niveles de NO. Algunos estudios han mostrado evidencias que apoyan este efecto protector, observándose niveles aumentados de GPx-3 en pacientes con complicaciones vasculares (Russo *et al.*, 1998). Asimismo, niveles deficientes de GPx-3, se han asociado con un aumento de la predisposición a desarrollar alteraciones trombóticas debido a un aumento de la disfunción vascular que promueve la trombosis arterial dependiente de plaquetas. (Jin *et al.*, 2011). Según estos resultados, los niveles de GPx-3 podrían actuar como un predictor de riesgo cardiovascular.

Nuestros resultados también muestran una disminución significativa en los niveles de TTR en el suero de pacientes con DM2 que presentan ECV con respecto a aquellos sin manisfestaciones clínicas de ECV. TTR, también conocida como prealbúmina, además de su función en el transporte de hormonas tiroideas y retinol, también actúa como proteína de respuesta de fase aguda. De acuerdo con nuestros resultados, algunos hallazgos muestran una disminución en los niveles séricos de

TTR en procesos inflamatorios agudos y crónicos a través de la liberación de citoquinas proinflamatorias como IL-1, IL-6 y TNF-α en el área de la lesión, implicadas en la disminución de la transcripción de TTR (Ingenbleek and Young, 1994). Asimismo, nuestros resultados muestran una disminución en los niveles de la cadena A del complemento C3 en el grupo de pacientes con DM2 independientemente de la presencia de ECV, en comparación con sujetos no diabéticos. El complemento C3 se produce principalmente en el hígado en respuesta a citoquinas inflamatorias (IL-1, IL-6) en condiciones inflamatorias tales como la diabetes y las complicaciones cardiovasculares (Engström *et al.,* 2005), actuando como biomarcador de la resistencia a la insulina (Muscari *et al.,* 2007), y probablemente como factor de riesgo de eventos cardiovasculares (Hertle *et al.*, 2012). Aunque la mayoría de los estudios llevados a cabo en DM2 y ECV asociada muestran un aumento en las concentraciones séricas de complemento C3, también hay algunos datos que muestran una disminución de los niveles de C3 en varias enfermedades como la diabetes gestacional (Lappas, 2011) o en sujetos con síndrome de ovario poliquístico, un trastorno endocrino asociado con un mayor riesgo cardiovascular e inflamación crónica (Insenser *et al.*, 2010). Sin embargo, los resultados referentes a TTR y complemento C3 no son concluyentes, ya que estas proteínas son algunas de las que quedan retenidas en las columnas usadas durante el proceso de depleción proteica, por lo que los niveles observados en suero no son representativos de los niveles séricos fisiopatológicos de estos pacientes.

En conclusión, nuestro estudio sugiere que las proteínas implicadas el estado inflamatorio y en el estrés oxidativo como RBP4 y GPx-3, se asocian con la presencia de complicaciones cardiovasculares en pacientes con DM2. Sin embargo, hacen falta estudios que permitan extrapolar los resultados a una población más extensa con el fín de confirmar estos resultados y de determinar la utilidad de estas proteínas como biomarcadores de riesgo cardiovascular o como potenciales dianas terapéuticas de las complicaciones vasculares.

En cuanto a la identificación de factores implicados en la ECV a través de estudios de metabolómica, los resultados situaron a los PLs como importantes factores

implicados en la patogénesis de la DM2 y de las complicaciones vasculares asociadas.

En los últimos año, los PLs y su metablismo están cobrando creciente importancia debido a su relación con diversas patologías como el Alzheimer, la obesidad y el cáncer (Su *et al.*, 2005; Tomiki *et al.*, 2004). Diversos estudios han puesto de manifiesto la estrecha asociación entre un metabolismo lipídico alterado y el desarrollo de la resistencia a la insulina y la patogénesis de la DM2 (Boden and Shulman, 2002) identificándose de esta manera, algunos PLs como potenciales biomarcadores de la DM2 (Hsu *et al.*, 2000; Tan *et al.*, 2003).

Nuestro estudio metabolómico ha mostrado una discriminación entre los diferentes grupos de estudio (sujetos no diabéticos y pacientes diabéticos con y sin ECV establecida) relacionada con los metabolitos identificados como fosfatidil colina (PC) (16: 1 (9Z) / 2: 0), PC (O-12: 0/2: 0), lisofosfatidil colina (LPC) (O-16: 0 / 0: 0) y lisofosfatidil-etanol-amina (LPE) (18: 2 (9Z, 12Z) / 0: 0). Estos metabolitos han mostrado niveles disminuídos en el grupo de DM2 con respecto al de sujetos no diabéticos. En un entorno de altas concentraciones de glucosa se produce un aumento de los niveles de la proteína quinasa C (PKC) que lleva a la activación de la fosfolipasa A2 (PLA2), la cual cataliza la degradación de fosfolípidos hasta AGLs (Zhu *et al.*, 2011a). En pacientes diabéticos, se ha observado además un aumento de los niveles de fosfolipasa A1 (PLA1) (Abu El-Asrar *et al.*, 2013) que cataliza la degradación de lisofosfolípidos a través de una compleja cascada de vías metabólicas (Aoki *et al.*, 2008; Bolen *et al.*, 2011). El aumento de estas enzimas en condiciones de hiperglucemia, podría explicar la disminución de los niveles tanto de PC como de LPE y LPC en el grupo de DM2 en comparación con el grupo control. En concordancia con nuestros resultados, estudios recientes han mostrado niveles disminuídos de LPC en pacientes con intolerancia a la glucosa, resistencia a la insulina así como en pacientes con DM2 obesos (Barber *et al.*, 2012; Graessler *et al.*, 2009; Rhee *et al.*, 2011; Zhao *et al.*, 2010). Por otra parte, LPC podría tener efectos beneficiosos en la homeostasis glucídica según el estudio de (Yea *et al.*, 2009), que observa la activación de la captación de glucosa en adipocitos 3T3-L1 mediante un

aumento de los niveles de GLUT4 mediado por LPC a través de un mecanismo independiente de insulina, lo que abre la puerta a nuevos estudios para la investigación de LPC como molécula reguladora de los niveles de glucosa en sangre. Según estos resultados, la reducción de los niveles circulantes de LPC observados en el grupo de pacientes con DM2, podría constituir una de las causas que contribuyen al peor control glucémico característico de la DM2.

En cuanto al desarrollo de complicaciones cardiovasculares, pocos estudios se han centrado en la búsqueda de fosfolípidos séricos relacionados con la aparición o la progresión de ECV en la DM2. Nuestros resultados han mostrado una reducción de los niveles de LPC Y LPE en pacientes diabéticos con presencia de ECV establecida en comparación con aquellos sin ECV establecida, lo que indica una posible influencia de estos PL en la ECV. Esto queda reflejado en los gráficos PC, donde se observa la separación de los grupos de pacientes diabéticos con y sin ECV en función de los niveles de LPE y LPC.

Es bien conocido que los pacientes con DM2 que desarrollan ECV, presentan un estado proinflamatorio aumentado, observándose niveles elevados de citoquinas proinflamatorias y ROS. Además, el elevado aumento de los niveles de RBP4 en el grupo pacientes diabéticos con ECV observado en nuestro estudio proteómico, favorecería el estado inflamatorio en estos pacientes a través de la expresión de moléculas proinflamatorias y la adhesión de leucocitos al endotelio. Algunos estudios han mostrado que en condiciones de inflamación crónica, se produce un incremento de la expresión de autotaxina (ATX), también conocida como ectonucleótido pirofosfatasa / fosfodiesterasa 2 (ENPP2 o NPP2) (Hammack *et al.*, 2004; Nakasaki *et al.*, 2008; Oikonomou *et al.*, 2012; Umemura *et al.*, 2006). En concordancia, TNF, un importante factor proinflamatorio, ha demostrado inducir la expresión ATX (Oikonomou *et al.*, 2012; Wu *et al.*, 2010). ATX es una enzima que posee actividad lisofosfolipasa D, catalizando la hidrólisis de lisofosfolípidos hasta ácido lisofosfatídico (LPA). Aunque el origen sérico de ATX no se conoce de forma exacta, parece que podría provenir de las vénulas endoteliales linfáticas altas (HEV) y del tejido adiposo (Kanda *et al.*, 2008). Existen evidencias que implican el eje

ATX/LPA en la obesidad, la diabetes y la resistencia a la insulina, observándose una regulación positiva de la expresión del ARNm de ATX de tejido adiposo en pacientes obesos con intolerancia a la glucosa (Boucher *et al.*, 2005), así como una sobreexpresión de ATX en grasa visceral en pacientes obesos (Rancoule *et al.*, 2012). Además, se ha observado que los niveles séricos de LPA están implicados en la disminución de los niveles de insulina en plasma. Todo ello sugiere que la regulación de la expresión de ATX en los adipocitos y los niveles de LPA podrían estar estrechamente relacionados con el desarrollo de hiperglucemia.

Por otra parte, se han descrito efectos fisiopatológicos debidos al aumento local de las concentraciones de LPA en sitios inflamados, como resultado de una mayor expresión de ATX, asociándose los niveles de este metabolito con alteraciones vasculares. Así, se ha sugerido que los niveles de LPA podrían inducir el desarrollo de aterosclerosis mediante la estimulación de la penetración de monocitos a nivel subendotelial del tejido vascular, así como de la proliferación de CMLV dando lugar a una disfunción endotelial mediada además por la disminución de la expresión de eNOS y el aumento del estrés oxidativo (Chen *et al.*, 2012a).

El conjunto de estos hallazgos, sugieren que la expresión de ATX podría estar implicada en la homeostasis glucídica, regulando los niveles de insulina y glucosa plasmática además de catalizar la degradación de lisofosfolípidos en pacientes con DM2. El incremento del estado inflamatorio de algunos pacientes diabéticos, podría inducir niveles más altos de ATX disminuyendo aún más el contenido de lisofosfolípidos en los pacientes diabéticos que desarrollan ECV e incrementando de esta forma los niveles de LPA en estos pacientes que a su vez estarían implicados en el desarrollo de complicaciones vasculares.

De acuerdo con estos resultados, proponemos que los PLs identificados están involucrados en la DM2 y que la variación de estas especies podría reflejar el desarrollo y la progresión de la ECV en estos pacientes, aunque la utilidad de estos metabolitos como biomarcadores de riesgo cardiovascular o potenciales dianas terapéuticas requiere estudios futuros.

Además de la identificación de factores metabólicos y proteicos implicados en el desarrollo de la ECV en la DM2, debido a las evidencias científicas que ponen de manifiesto una posible conexión entre metabolismo óseo y sistema vascular, hemos querido estudiar la implicación de algunas de las proteínas implicadas en la regulación del metabolismo óseo, sobre el sistema vascular en estos pacientes. Así, hemos determinado los niveles séricos de esclerostina y Dkk1, proteínas inhibidoras de la vía Wnt implicada en la formación ósea, en nuestra cohorte de pacientes diabéticos con y sin ECV.

Con respecto a la esclerostina, nuestros resultados, han mostrado niveles séricos significativamente más elevados en el grupo de pacientes con DM2 que presentaban ECV establecida con respecto a aquellos que no presentaban ECV. Además, se ha observado una asociación independiente entre los niveles de esclerostina y la presencia de ECV en la DM2 que muestra que para cada pmol/L de aumento en los niveles séricos de esclerostina, se produce un incremento de riesgo de ECV de un 4% en pacientes con DM2. De forma similar, encontramos niveles incrementados de Dkk1 en el grupo de pacientes con ECV clínica. Los niveles séricos de Dkk1 también se asociaron de forma independiente con la presencia de ECV mostrando un incremento de un 6,8% en el riesgo de sufrir ECV en pacientes con DM2 por cada 28 pg/mL de incremento en los niveles séricos de Dkk1.

Nuestros resultados, muestran además un incremento tanto de esclerostina como de Dkk1 en suero de pacientes con DM2 que presentan un GIM anormal así como niveles incrementados de esclerostina en pacientes con calcificación aórtica avanzada y en varones diabéticos con presencia de placas carotídeas. Estos resultados sugieren que los inhibidores de la vía de señalización Wnt, esclerostina y Dkk, podrían estar implicados en la ECV, aunque su papel específico en este proceso aún no es bien conocido.

La elevación de los niveles de esclerostina y Dkk1 observada en pacientes diabéticos con ECV establecida, se podría explicar, al menos en parte, por su producción en células vasculares previamente transformadas en células de fenotipo similar a osteocitos, capaces de expresar proteínas típicas osteocíticas. Estudios recientes han

demostrado que las CMLV en condiciones de calcificación son capaces de sufrir esta transición fenotípica y expresar proteínas del metabolismo óseo (Zhu *et al.*, 2011b). La hiperglucemia tiene efectos negativos sobre las complicaciones vasculares (Cefalu, 2005) dando lugar a la formación de productos oxidación avanzada que inducen la calcificación vascular a través de la estimulación de la trans-diferenciación de las CMLV (You *et al.*, 2009). En concordancia, varios estudios han mostrado un aumento en la expresión de proteínas inhibidoras de la vía Wnt tales como SFRP1, 2 y 4 en aortas calcificadas (Román-García *et al.*, 2010) y Dkk1 en pacientes con ECV en comparación con los sujetos control (Seifert-Held *et al.*, 2011) así como en placas carótideas calcificadas (Kim *et al.*, 2011; Ueland *et al.*, 2009d).

Aunque el papel específico que desempeñan las proteínas del metabolismo óseo en las complicaciones vasculares aún no es del todo bien conocido, el hecho de que la esclerostina y Dkk1 actúen como inhibidores de la formación ósea, hace pensar que el incremento de sus niveles en pacientes diabéticos que desarrollan procesos de aterosclerosis y calcificación vascular pudiera constituir un mecanismo defensivo con el fin de bloquear o atenuar la vía Wnt canónica para disminuir la osificación a nivel vascular y evitar una mayor progresión de la aterosclerosis en estos pacientes. De acuerdo a esta hipótesis, estudios previos han mostrado una regulación al alza del antagonista de la vía de señalización Wnt sFRP (Barandon *et al.*, 2003; Román-García *et al.*, 2010) después de una lesión del tejido vascular, sugiriendo su implicación en el proceso de curación del tejido dañado. En concordancia, los datos del estudio de Malekar y colaboradores (2010), mostraron que la activación de la vía Wnt a través de su agonista Dvl era crítica y suficiente para la inducción de lesiones vasculares. Según estos resultados, la sobreexpresión de esclerostina podría influir en el progreso de la ECV en la DM2. Sin embargo, también existen estudios que apuntan en dirección contraria, atribuyéndole efectos anti-inflamatorios a la vía Wnt. Así, se ha observado que la estabilización de β-catenina, como resultado de la activación de la vía Wnt canónica, juega un papel importante en la promoción de la supervivencia de las células T reguladoras con efectos potencialmente anti inflamatorios (Ding *et al.*, 2008). Asimismo, la activación de la vía Wnt parece regular a la baja los niveles de RANKL implicado en la aterogénesis y

desestabilización de la placa aterosclerótica (Spencer *et al.*, 2006). En ésta línea, Ueland y colaboradores (2009d)), han mostrado una implicación de Dkk1 en la activación de endotelial mediada por plaquetas que liberan y expresan mediadores inflamatorios induciendo una respuesta inflamatoria en los leucocitos y las células endoteliales adyacentes de gran importancia en el desarrollo del proceso aterogénico.

Por último, nuestros resultados han mostrado una asociación independiente de la homocisteína y el GIM con los niveles séricos de esclerostina independientemente del sexo. Es bien conocido que la hiperhomocisteinemia en plasma es un factor de riesgo independiente para el desarrollo de aterosclerosis, eventos cardiovasculares y de accidentes cerebrovasculares (Petramala *et al.*, 2009). Sin embargo, hasta la fecha, sólo se ha descrito la relación entre homocisteína y esclerostina en un estudio en mujeres postmenopáusicas (Urano *et al.*, 2012). En cuanto al GIM, éste se considera como un fuerte predictor de eventos vasculares (Lorenz *et al.*, 2007), sin embargo, hasta ahora, no se ha descrito su relación con los niveles de esclerostina. Nuestros resultados muestran que los niveles elevados de homocisteína en pacientes con elevado riesgo cardiovascular así como valores anormales de GIM podrían estar implicados en el aumento de los niveles de esclerostina, aunque son necesarios estudios futuros que aclaren la relación entre la esclerostina y el metabolismo de la homocisteína así como el mecanismo de actuación mediante el cual estos factores inducirían niveles incrementados de esclerostina.

Estos resultados sugieren, que el incremento de los niveles circulantes de esclerostina y Dkk1 observado en pacientes con DM2 con lesiones ateroscleróticas, apoya la hipótesis de que la acción de la esclerostina y Dkk1 no está únicamente restringida a la regulación de la formación ósea, sino que estas proteínas pueden están implicadas además, en la integridad vascular constituyendo un importante modulador de la señalización Wnt en la ECV asociada a la DM2, así como constituir potenciales marcadores séricos de riesgo cardiovascular. Sin embargo, son necesarios estudios futuros para establecer si los niveles incrementados de estas

proteínas tienen un papel protector en la supervivencia de los pacientes con ECV o por el contrario, están implicados en la patogénesis de dichas complicaciones.

Sin embargo, en cuanto a la relación entre niveles séricos de esclerostina y Dkk1 con parámetros del metabolismo óseo en estos pacientes, no se han obtenido resultados concluyentes. En uno de los estudios previos de nuestro grupo, en contra de lo esperado debido al papel fisiopatológico de la esclerostina en la inhibición de la osteoblastogénesis, se observaron niveles séricos más bajos de esclerstina en pacientes con DM2 con osteoporosis densitométrica en comparación con aquellos sin osteoporosis aunque no se observó relación con la presencia de fracturas vertebrales morfométricas. Además, también se observó una asociación positiva entre niveles circulantes de esclerostina y DMO en pacientes diabéticos (García-Martín *et al.,* 2012). De igual forma, en el caso de Dkk1, sólo observamos una débil correlación con la DMO a nivel lumbar en la muestra total, sin ninguna relación entre niveles séricos de Dkk1 con MRO, presencia de osteoporosis o fracturas vertebrales morfométricas. En base a estos y otros resultados, los datos sobre los niveles de esclerstina y Dkk1 séricos y el metabolismo óseo actualmente son controvertidos, no pudiendo extraer conclusiones claras a este respecto.

De acuerdo con los resultados obtenidos en esta Tesis Doctoral, se abren numerosas líneas de investigación para profundizar tanto en el conocimiento del mecanismo de acción de los factores identificados implicados en la DM2, como en el papel de los inhibidores de la vía Wnt en el desarrollo de las complicaciones vasculares asociadas a la DM2 y su relación con la fragilidad ósea en estos pacientes. Para ello, actualmente, nuestro grupo está desarrollando diferentes proyectos de investigación con el objetivo de aclarar todas las cuestiones planteadas. Así, en nuestro proyecto "Identificación de las vías de conexión entre calcificación vascular y mineralización ósea en diabetes mellitus tipo 2. Cristalización, abordaje estructural y evaluación de su bloqueo como aproximación terapéutica para el tratamiento de la osteoporosis y aterosclerosis" vamos a tratar de identificar las proteínas implicadas en la ECV a nivel de tejido vascular calcificado estudiando su implicación en el metabolismo óseo en pacientes con DM2 con el objetivo de ensayar diferentes

tratamientos que puedan actuar conjuntamente sobre las alteraciones óseas y del sistema vascular. De forma paralela, en nuestro proyecto "Relación entre parámetros de trabecular bone score (TBS), enfermedad cardiovascular y fragilidad ósea en pacientes con diabetes tipo 2" estamos analizando la microarquitectura ósea mediante TBS, en pacientes con DM2 con y sin ECV establecida con el objetivo de evaluar la relación de la fragilidad ósea y la ECV observada en estos pacientes a través de parámetros de microarquitectura ósea.

CONCLUSIONES

1. Los pacientes con DM2 presentan niveles séricos reducidos de miostatina e incrementados de irisina en comparación con sujetos no diabéticos, los cuales están asociados entre sí. Los niveles séricos de TGs y glucosa podrían modular las concentraciones de miostatina e irisina como un mecanismo compensador para restablecer el equilibrio metabólico en estos pacientes.

2. Los niveles séricos de RBP4 están incrementados en pacientes con DM2 en comparación con sujetos no diabéticos, participando en el mecanismo de resistencia a la insulina.

3. Los niveles de RBP4 están incrementados en pacientes con DM2 con presencia de ECV establecida, respecto a pacientes con DM2 sin ECV establecida participando en la patogenia de las complicaciones vasculares, a través un incremento de la inflamación endotelial.

4. Los niveles séricos de GPx-3 están incrementados en pacientes con DM2 con ECV establecida, disminuyendo la peroxidación lipídica producida por el incremento de ROS en estos pacientes.

5. En la DM2 se produce una reducción generalizada de los fosfolípidos circulantes PC, LPE y LPC. Esta disminución es más pronunciada en los pacientes diabéticos con ECV, proporcionando información sobre las rutas implicadas en la patogénesis de la ECV asociada a la DM2.

6. Los pacientes con DM2 y ECV establecida presentan niveles séricos incrementados de esclerostina respecto a aquellos sin ECV, mostrándose más elevados en los pacientes que presentan marcadores subrrogados de ECV, como GIM anormal, calcificaciones aórticas y placa carotídea.

7. Los pacientes con DM2 y ECV establecida presentan niveles séricos incrementados de Dkk1 respecto a aquellos sin ECV, asociándose con un GIM patológico.

8. Esclerostina y Dkk1 son importantes moduladores la vía Wnt en pacientes con DM2 y ECV, actuando como moléculas predictoras de las complicaciones vasculares asociadas a la DM2.

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RESUMEN

La diabetes mellitus tipo 2 (DM2) tiene grandes repercusiones socio-sanitarias debido a su relación con el desarrollo de diversas complicaciones, entre ellas, la enfermedad cardiovascular (ECV), que representa la principal causa de mortalidad en estos pacientes. Sin embargo, la vía que une la disglicemia y la ECV no está bien dilucidada y los factores subyacentes implicados en el desarrollo y progresión de las alteraciones cardiovasculares asociadas a la diabetes, no se conocen en profundidad.

Por otra parte, la elevada prevalencia de ECV y de fracturas por fragilidad en estos pacientes, sugiere la existencia de mecanismos moleculares comunes entre las alteraciones óseas y vasculares, aunque los conocimientos sobre la regulación de la mineralización ósea y la calcificación vascular son en la actualidad muy escasos.

En este contexto, este trabajo de investigación se centra en la profundización de los mecanismos implicados tanto en el desarrollo de la DM2 como de las alteraciones vasculares y su conexión con el metabolismo óseo y muscular, mediante el uso de diferentes abordajes metodológicos.

Por un lado, basándonos en evidencias que apoyan el papel de músculo esquelético en la regulación de la homeostasis glucídica y su implicación en determinados desórdenes metabólicos a través de la liberación de determinadas mioquinas, se determinaron los niveles séricos de miostatina e irisina para estudiar su implicación en la DM2. Nuestros resultados muestran un descenso de los niveles séricos de miostatina, así como un incremento de los niveles séricos de irisina en pacientes con DM2 respecto a sujetos no diabéticos. Además, se observa una asociación inversa entre estas mioquinas cuyas funciones confluyen en las mismas respuestas metabólicas. El cambio en los niveles circulantes de irisina y miostatina así como la influencia de la una sobre la otra, podría constituir un mecanismo compensatorio para restablecer el equilibrio metabólico en pacientes con DM2.

Además, en este trabajo, se identificaron factores implicados en la DM2 y las complicaciones vasculares asociadas, mediante el análisis sérico con técnicas proteómicas y metabolómicas en pacientes con DM2 con presencia y ausencia de ECV así como en sujetos no diabéticos, explorando así, las vías alteradas durante estos trastornos.

A nivel proteómico, nuestros resultados muestran un incremento en los niveles séricos de *retinol binding protein 4* (RBP4) en pacientes diabéticos. Esta proteína está implicada en la inducción de la resistencia a la insulina del tejido adiposo mediante un mecanismo de activación de las células presentadoras de antígenos (CPA) que liberan diversas citoquinas proinflamatorias en el tejido adiposo dando lugar a la inflamación del mismo y a la resistencia a la insulina. Además, RBP4, parece estar implicada en el desarrollo y la progresión de la ECV asociada a la DM2 a través de la activación de NF-kB y NADPH oxidasa, lo que da lugar a un aumento de la inflamación endotelial, siendo éste el primer paso para el desarrollo de complicaciones vasculares en la DM2. Así, hemos observado niveles más elevados de RBP4 en el grupo de pacientes con DM2 que presentaban ECV establecida con respecto a aquellos sin ECV. La glutatión peroxidasa 3 (GPx-3), también parece estar implicada en la ECV asociada a la DM2, encontrando niveles significativamente aumentados en el grupo de pacientes diabéticos con ECV respecto al grupo sin ECV, que podrían actuar en respuesta a los elevados niveles de especies reactivas de oxígeno (ROS) producidas en las lesiones vasculares.

A nivel metabolómico, nuestros resultados muestran una posible implicación de los fosfolípidos fosfatidil-colina (PC), lisofosfatidil-etanol-amina (LPE) y lisofosfatidil-colina (LPC) en la DM2 y en el desarrollo de las complicaciones vasculares asociadas. Así, encontramos niveles disminuídos de todas las especies de fosfolípidos identificadas en el grupo de DM2 respecto al grupo de sujetos no diabéticos. Esto se relaciona con el aumento de la expresión de las enzimas que catalizan la degradación de los mismos en condiciones de hiperglucemia. Asimismo, los niveles séricos de LPE y LPC se muestran significativamente más disminuídos en el grupo de pacientes con DM2 y ECV, lo que se corresponde con el aumento de autotaxina (ATX), fosfolipasa con función catalítica de lisofosfolípidos en condiciones de inflamación crónica, como ocurre en presencia de lesiones vasculares. La degradación de estos lisofosfolípidos, da lugar a un aumento de ácido lisofosfatídico (LPA) que agrava las complicaciones vasculares.

Por otro lado, debido a la reciente implicación de la vía osteoblastogénica Wnt sobre el sistema vascular, se determinaron los niveles séricos de los antagonistas esclerostina y Dkk1, con el objeto de evaluar su implicación en las alteraciones vasculares en pacientes con DM2. Nuestros resultados muestran niveles elevados tanto de esclerostina como de Dkk1 en pacientes con DM2 con ECV establecida en comparación con aquellos que no presentan manifestaciones clínicas de ECV. Además, se pueden observar niveles elevados de estas proteínas en aquellos pacientes con marcadores subrogados de ECV. Así, pacientes con grosor de íntima media carotídeo (GIM) anormal presentan niveles incrementados tanto de esclerostina como de Dkk1. Pacientes con presencia de placa carotídea y calcificaciones aórticas presentan niveles aumentados de esclerostina. Estos resultados sugieren que esclerostina y Dkk1 son importantes moduladores la vía Wnt en pacientes con DM2 y ECV, pudiendo actuar como moléculas predictoras de las complicaciones vasculares, aunque hacen falta estudios futuros que aclaren el papel específico de estas proteínas en el desarrollo de la ECV.

En resumen, mediante este trabajo de investigación, abordamos desde diferentes perspectivas el conocimiento de los factores y vías subyacentes implicadas en el desarrollo y/o progresión de las complicaciones vasculares asociadas a la DM2, con el objetivo de identificar moléculas predictoras del riesgo cardiovascular en estos pacientes, así como posibles nuevas dianas terapéuticas para el tratamiento de dichas complicaciones.

PRODUCCIÓN CIENTÍFICA

La producción científica desarrollada en esta etapa de investigación ha sido publicada, o está en vías de publicación en distintas revistas científicas, o ha sido presentada en distintas comunicaciones a congresos, como se detalla a continuación:

Artículos derivados de esta tesis doctoral:

1. Metabolomic profile related to cardiovascular disease in patients with type 2 diabetes mellitus: A pilot study.
García-Fontana B, Morales-Santana S, Díaz Navarro C, Rozas-Moreno P, Genilloud O, Vicente Pérez F, Pérez del Palacio J, Muñoz-Torres M.
Talanta. 2016 Feb 1;148:135-43.

2. Relationship between myostatin and irisin in type 2 diabetes mellitus: a compensatory mechanism to an unfavourable metabolic state?
García-Fontana B, Reyes-García R, Morales-Santana S, Ávila-Rubio V, Muñoz-Garach A, Rozas-Moreno P, Muñoz-Torres M.
Endocrine. 2016 Apr;52(1):54-62.

3. Relationship between Proinflammatory and Antioxidant Proteins with the Severity of Cardiovascular Disease in Type 2 Diabetes Mellitus.
García-Fontana B, Morales-Santana S, Longobardo V, Reyes-García R, Rozas-Moreno P, García-Salcedo JA, Muñoz-Torres M.
Int J Mol Sci. 2015 Apr 27;16(5):9469-83.

4. Atherosclerotic disease in type 2 diabetes is associated with an increase in sclerostin levels.
Morales-Santana S, García-Fontana B, García-Martín A, Rozas-Moreno P, García-Salcedo JA, Reyes-García R, Muñoz-Torres M.
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