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DOCTORAL THESIS

Study of the mechanisms of oxidative stress, mitochondrial function and endoplasmic reticulum stress in obesity. Role in subclinical atherosclerosis and therapeutic approaches

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CERTIFICAN

Que **Doña Sandra López Domènech**, Licenciada en Biología por la Universitat de València, ha realizado el trabajo titulado *"Study of the mechanisms of oxidative stress, mitochondrial function and endoplasmic reticulum stress in obesity. Role in subclinical atherosclerosis and therapeutic approaches"* bajo su dirección y que a su juicio reúne los requisitos para su defensa como Tesis Doctoral para la obtención del grado de Doctor en Biomedicina y Biotecnología por la Universitat de València.

Y para que así conste firman el presente documento en Valencia a 23 de Enero de 2019.

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Dr. Antonio Hernández Mijares

"Cuando una puerta de felicidad se cierra, otra se abre"

Hellen Keller

Mamá

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REGULATIONS AND STRUCTURE

According to the regulations approved by the Doctoral School and the Academic Committee of the PhD Programme in Biomedicine and Biotechnology of the University of Valencia:

1. The present PhD thesis has been structured in the form of a **compendium of publications**:

   - The core of the thesis is composed of five original first-author (or co-first-author) papers published in Q1 journals according to the *Journal Citation Reports* database (Annex I: Articles).

   - The thesis also includes a global summary consisting of an introductory chapter explaining the consistency in the theme of the papers, a chapter containing the main results and discussion, and a chapter setting out the general conclusions.

2. Based on the present PhD thesis Ms. Sandra López Domènech would like to apply for the **International Doctorate Mention**:

   - During the training period the PhD candidate has spent six months in a prestigious research centre outside Spain (Cleveland Clinic/Pennington Biomedical Research Center, USA).

   - The thesis has been written in its entirety English and will be defended in English.

3. A summary in Spanish (Resumen) has been included, since it is one of the official languages of the University of Valencia.

ABBREVIATIONS / *ABREVIATURAS*

A1c	glycated haemoglobin / *hemoglobina glicosilada*
ATF4	activating transcription factor 4
ATF6	activating transcription factor 6
BMI	body mass index / *índice de masa corporal*
C3c	complement component 3
CAMs	cell adhesion molecules
CHOP	CCAAT/enhancer binding protein [C/EBP] homologous protein
CRP	C-reactive protein
CV	cardiovascular / *cardiovascular/es*
eIF2α	eukaryotic initiation factor 2α
eNOS	endothelial nitric oxide synthase enzyme
ER	endoplasmic reticulum / *retículo endoplasmático*
ERAD	endoplasmic reticulum-associated protein degradation
Ero1	endoplasmic reticulum oxidoreductin 1
ETC	electron transport chain
FA	fatty acids
FFA	free fatty acids
GPx	glutathione peroxidase
GPX1	glutathione peroxidase 1
GRP78	78-kDa glucose-regulated protein
GSH	glutathione
GLUT4	glucose transporter type 4
H_2O_2	hydrogen peroxide
HDL(c)	high-density lipoprotein (cholesterol) / *lipoproteínas de alta densidad*
HOCl	hypochlorite
ICAM-1	intercellular adhesion molecule-1
IKK	inhibitory κB kinase
IL6	interleukin 6
IP_3R	inositol triphosphate receptor
IR	insulin resistance / *insulín resistencia*
IRE1α	inositol requiring enzyme 1 α
IRS1/2	insulin receptor substrates 1/2
JNK	c-Jun N-terminal kinase
LCD	low-calorie diets
LDL(c)	low-density lipoprotein (cholesterol) / *lipoproteínas de baja densidad*

LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein-1
MPO	myeloperoxidase / *mieloperoxidasa*
mROS	mitochondrial reactive oxygen species
NFκB	nuclear factor κB
NLRP3	NLR family pyrin domain containing 3
NO	nitric oxide / *óxido nítrico*
NOX	nicotinamide adenine dinucleotide phosphate [NADPH] oxidase
NRF2	nuclear factor (erythroid-derived 2)-like 2
PAI-1	plasminogen activator inhibitor-1
PCOS	polycystic ovary syndrome / *síndrome de ovario poliquístico*
PERK	double-stranded RNA-activated protein kinase-like kinase
PMNs	polymorphonuclear cells
RBP4	retinol binding protein 4
RNS	reactive nitrogen species
ROS	reactive oxygen species / *especies reactivas de oxígeno*
SAT	subcutaneous adipose tissue
sdLDL	small dense low-density lipoprotein
SIRT1	sirtuin 1
SOD	superoxide dismutase
T2D	type 2 diabetes / *diabetes tipo 2*
TG	triglycerides / *triglicéridos*
TNFα	tumour necrosis factor α
UPR	unfolded protein response / *respuesta al mal plegamiento proteico*
VAT	visceral adipose tissue
VCAM-1	vascular cell adhesion molecule-1
VLDL	very low-density lipoprotein / *lipoproteínas de muy baja densidad*
VLCD	very-low-calorie diets / *dietas de muy bajo contenido calórico*
WAT	white adipose tissue
WC	waist circumference
WHO	World Health Organization
WHR	waist-to-hip ratio
(s)XBP1	(spliced) X box protein-1
ΔΨ	membrane potential / *potencial de membrana*

RESUMEN

Introducción

La obesidad es una enfermedad multifactorial caracterizada por una sobrecarga metabólica de los tejidos asociada a inflamación crónica de bajo grado, alteración del metabolismo lipídico e insulín resistencia (IR), entre otras alteraciones metabólicas. Además es un factor de riesgo para la aparición de otras patologías como la diabetes tipo 2 (T2D, del inglés *type 2 diabetes*), dislipemia, hipertensión arterial, aterosclerosis y ciertos tipos de cáncer, lo cual hace que se multiplique hasta por tres el riesgo de muerte en comparación con el de las personas no obesas. Todas estas circunstancias junto con un aumento drástico de su prevalencia en los últimos años, sobre todo en los países desarrollados, confieren a esta enfermedad una especial atención. Además de estas comorbilidades, la obesidad se ha asociado recientemente con un incremento en la prevalencia de periodontitis crónica, una enfermedad inflamatoria y destructiva de los tejidos de soporte de los dientes exacerbada por un aumento en la expresión de citoquinas proinflamatorias y hormonas derivadas del tejido adiposo. De hecho, se sabe que los pacientes con obesidad tienen hasta cinco veces más riesgo de sufrir periodontitis crónica que los sujetos sanos, aunque los mecanismos de asociación entre ambas patologías no son del todo conocidos.

Los trastornos metabólicos asociados a la obesidad están íntimamente relacionados con el desarrollo de disfunción endotelial y aterosclerosis, aunque incluso los pacientes obesos sin signos clínicos de deterioro metabólico se encuentran ante un mayor riesgo de sufrir complicaciones cardiovasculares (CV). De manera similar, la periodontitis crónica se ha convertido en un factor de riesgo potencial para el desarrollo de disfunción endotelial, y comparte con la obesidad algunos de los mecanismos implicados en la aparición de esta alteración vascular, como la inflamación y el estrés oxidativo.

La inflamación sistémica es una de las características más relevantes de la obesidad que subyace a gran parte de los procesos fisiopatológicos de la enfermedad. El excesivo almacenamiento de triglicéridos (TG) en el adipocito como consecuencia del elevado *input* de nutrientes conlleva a la hipertrofia y disfunción del tejido adiposo, caracterizada por un aumento en la secreción de citoquinas, adipoquinas y factores protrombóticos como la IL6 (del inglés *interleukin 6*), TNFα (del inglés *tumour necrosis factor α*), RBP4 (del inglés *retinol binding protein 4*), MCP-1 (del inglés *monocyte chemoattractant protein-1*) o PAI-1 (del

inglés *plasminogen activator inhibitor-1*) que contribuyen a la inflamación sistémica y la activación crónica de las células del sistema inmune, que son reclutadas hacia el tejido adiposo donde participan en el remodelado del mismo. Los macrófagos infiltrados además amplifican la señal inflamatoria y desencadenan IR en los adipocitos. El subsecuente bloqueo de la acción antilipolítica de la insulina promueve la liberación de ácidos grasos desde el adipocito al torrente sanguíneo, donde inician mecanismos de lipotoxicidad y disposición ectópica de lípidos, contribuyendo al desarrollo de IR sistémica. Este proceso está especialmente acentuado en el tejido adiposo visceral, que drena ácidos grasos y citoquinas directamente al hígado donde contribuye a la IR local y la alteración del metabolismo lipídico, desencadenando la dislipemia aterogénica. Ésta se caracteriza por el descenso de los niveles de lipoproteínas de alta densidad (HDL, del inglés *high-density lipoprotein*) y el incremento de TG y lipoproteínas de baja densidad (LDL, del inglés *low-density lipoprotein*), especialmente de LDL pequeñas y densas (sdLDL, del inglés *small and dense low-density lipoprotein*), incrementándose el riesgo de eventos CV adversos y la morbimortalidad.

Como resultado de estos procesos fisiopatológicos se produce una alteración metabólica generalizada con elevados niveles circulantes de citoquinas inflamatorias, resistencia a la insulina que favorece el incremento de los niveles circulantes de glucosa, y ácidos grasos, todos ellos factores que contribuyen a la disfunción endotelial. Este proceso se caracteriza, entre otros, por alteraciones en la actividad de la enzima eNOS (del inglés *endotelial nitric oxide synthase enzyme*) y la reducción de la biodisponibilidad de óxido nítrico (NO, del inglés *nitric oxide*), encargado del mantenimiento del tono y la homeostasis del sistema vascular. En este sentido, la alteración de la barrera endotelial favorece la migración de las sdLDL hacia la región subendotelial, donde el ambiente prooxidante favorece su oxidación que, junto con otros estímulos proinflamatorios y la hiperglicemia, conduce a la activación de las células endoteliales, que secretan factores quimioatrayentes y moléculas de adhesión como selectinas, ICAM-1 (del inglés *intercellular adhesion molecule-1*) y VCAM-1 (del inglés *vascular cell adhesion molecule-1*) favoreciendo el reclutamiento de leucocitos hacia la pared del vaso. Durante la activación de la cascada de adhesión los leucocitos disminuyen su velocidad y comienzan a rodar sobre el endotelio, tras lo cual se adhieren firmemente al mismo y comienzan el proceso de transmigración hacia la zona subendotelial en su mecanismo habitual de migración hacia el foco

inflamatorio. Sin embargo, en el contexto proaterogénico de la obesidad este proceso también constituye una etapa temprana del proceso aterosclerótico, puesto que los macrófagos infiltrados fagocitan ávidamente las moléculas oxidadas de sdLDL transformándose en células espumosas que contribuyen al remodelado de la zona subendotelial y la formación de la placa de ateroma en la región de la íntima media, cuya evolución puede llevar a complicaciones vasculares mayores como isquemia o los accidentes aterotrombóticos. Estas evidencias ponen de manifiesto la relevancia del estudio de los mecanismos subyacentes a la alteración de la dinámica entre los leucocitos y el endotelio vascular que permitan apuntar a nuevas dianas terapéuticas para disminuir el riesgo CV en esta población.

El estrés oxidativo es otro de los mecanismos fisiopatológicos asociados a la disfunción endotelial y el proceso aterogénico en las enfermedades metabólicas, donde el incremento de la producción de especies reactivas de oxígeno (ROS, del inglés *reactive oxygen species*) se suma a un sistema antioxidante defectuoso, produciéndose un desequilibrio generalizado del estado redox. Entre las mayores fuentes de ROS en la célula se encuentra la mitocondria, especialmente la cadena de transporte de electrones cuya función se encuentra sobrecargada en la obesidad debido al excesivo *input* de sustratos energéticos provenientes del metabolismo, produciendo un exceso de ROS durante la formación de ATP por fosforilación oxidativa. Este exceso de radicales libres produce daños oxidativos en las macromoléculas que pierden funcionalidad, como las que conforman la mitocondria que por proximidad a la cadena de transporte de electrones son especialmente vulnerables al daño oxidativo, produciéndose disfunción mitocondrial. La disfunción mitocondrial y el estrés oxidativo ocurren en la obesidad mediados por estímulos como la hiperglicemia o la inflamación sistémica y se han relacionado ampliamente con el desarrollo de enfermedad CV, pues interfieren notablemente en la función de la eNOS y la disponibilidad de NO en el endotelio. Sin embargo, además de la producción de ROS mitocondriales, existen otras fuentes de especies oxidantes relevantes para la disfunción endotelial, como la mieloperoxidasa (MPO) o la NOX (del inglés *nicotinamide adenine dinucleotide phosphate [NADPH] oxidase*), cuya actividad está incrementada en la obesidad.

Íntimamente ligado al estrés oxidativo y la disfunción mitocondrial se encuentra la inducción de estrés de retículo endoplasmático (ER, del inglés *endoplasmic reticulum*),

pues son mecanismos que convergen en las enfermedades metabólicas como la obesidad y la T2D. El ER es el orgánulo celular encargado de la traducción y el procesamiento de las proteínas, además de actuar como almacén de Ca^{2+}, el cual es necesario para el correcto plegamiento de estas biomoléculas. A nivel sistémico actúa como un sensor de nutrientes, lo que es especialmente relevante en la obesidad en la que, como ya hemos mencionado anteriromente, se produce un incremento de los niveles circulantes de citoquinas inflamatorias, ácidos grasos libres, glucosa, y también exceso de producción de ROS, que a su vez contribuyen al desarrollo de estrés de ER, caracterizado por la acumulación de proteínas mal plegadas en el lumen. En consecuencia se activa la respuesta al mal plegamiento proteico o UPR (del inglés *unfolded protein response*) conformada por tres cascadas de señalización iniciadas por ATF6 (del inglés *activating transcription factor 6*), IRE1α (del inglés *inositol requiring enzyme 1 α*) y PERK (del inglés *double-stranded RNA-activated protein kinase-like kinase*), cuyos factores de transcripción finales promueven en primer lugar respuestas adaptativas con el objetivo de recuperar la homeostasis celular, como la expresión de chaperonas, antioxidantes, rutas de degradación de proteínas o autofagia. Sin embargo, ante situaciones de estrés persistente como en la obesidad se desencadenan respuestas crónicas tales como la expresión de factores proapoptóticos como CHOP (del inglés *CCAAT/enhancer binding protein [C/EBP] homologous protein*), la activación de señales inflamatorias o la liberación excesiva de Ca^{2+}. Se sabe que el estrés de ER tiene un papel relevante en la fisiopatología de la obesidad, puesto que su presencia en tejidos altamente metabólicos como el hígado, páncreas y tejido adiposo se ha relacionado con la inflamación sistémica, el desarrollo de IR e incluso el fallo pancreático, siendo un mecanismo importante en la evolución hacia la T2D. Sin embargo, el papel del ER en otros tipos celulares como las células inmunes ha sido menos estudiado, aunque recientes estudios apuntan a que la activación de esta respuesta en los leucocitos podría estar influenciando su capacidad de interaccionar con el endotelio vascular. En base a las evidencias del papel del estrés oxidativo y el estrés de ER en la fisiopatología de la obesidad y la T2D ambos mecanismos se postulan como potenciales dianas terapéuticas para abordar las alteraciones metabólicas asociadas a estas patologías.

Los diferentes factores biológicos, psicológicos y sociales implicados en la obesidad hacen de ésta una enfermedad de difícil abordaje. Entre las intervenciones terapéuticas se incluyen los cambios en el estilo de vida y la alimentación, la cirugía bariátrica y el

tratamiento farmacológico. Los cambios nutricionales constituyen el tratamiento de primera elección para hacer frente a la obesidad, por su menor coste económico y sus potenciales beneficios sobre la salud. De hecho, una pérdida de peso moderada de entre un 5-10 % ha demostrado ser efectiva para la mejora de la sensibilidad a la insulina y el perfil de riesgo CV. Entre las diferentes aproximaciones, las dietas de muy bajo contenido calórico o VLCD *(del inglés very-low-calorie diets*) junto con los cambios en el estilo de vida constituyen una de las mejores herramientas para tratar la obesidad, sin embargo, no se conoce con exactitud los mecanismos mediante los cuales la pérdida de peso ejercería su papel protector. Por otro lado, el uso de inositoles vegetales como tratamiento adyuvante en las enfermedades metabólicas ha cobrado especial interés en los últimos años. En este sentido, el pinitol es un inositol vegetal al que se le atribuye propiedades sensibilizadoras de la acción de la insulina, antiinflamatorias y antioxidantes, como se ha demostrado en varios modelos de enfermedad metabólica, aunque sus dianas principales y mecanismos de acción son en gran parte desconocidos.

Hipótesis y objetivos

La obesidad es una enfermedad compleja donde una gran variedad de alteraciones metabólicas incluyendo inflamación crónica de bajo grado, IR, estrés oxidativo y alteraciones en el perfil lipídico convergen en el desarrollo de disfunción endotelial y enfermedad CV. La persistencia de un riesgo CV residual incluso en sujetos obesos metabólicamente sanos sugiere que otros factores de riesgo además de clásicos como la hipertensión o la hiperlipemia pueden estar actuando de una manera subclínica. En este contexto, las células del sistema inmune se encuentran en un estado de hiperactivación como consecuencia de la inflamación crónica y desempeñan un papel relevante en el desarrollo de complicaciones vasculares, ya que su atracción hacia a la pared vascular es un proceso clave en el inicio de la formación de la placa de ateroma. Sin embargo, poco se sabe sobre los mecanismos intracelulares que podrían estar mediando esta interacción. Hasta el momento se ha descrito que los leucocitos activados presentan una mayor producción de ROS, sin embargo, el estado de hiperactivación en el que se encuentran podría desencadenar una excesiva producción de especies oxidantes, dando lugar a una situación de estrés oxidativo, un mecanismo implicado en la disfunción endotelial. Por lo

tanto, es probable que el estrés oxidativo en los leucocitos esté alterando la dinámica entre éstos y el endotelio vascular en el contexto de la obesidad, donde el aumento de la adiposidad podría estar agravando el proceso. De manera similar, la periodontitis comparte varios mecanismos fisopatológicos con la obesidad, incluido la inflamación crónica, la hiperactivación del sistema inmune y el estrés oxidativo. De esta manera, la presencia concomitante de obesidad y periodontitis podría estar acrecentando la inflamación sistémica y el desequilibrio redox en los leucocitos, aumentando así sus interacciones con el endotelio.

En este desequilibrio redox de los leucocitos podría estar implicada la mitocondria, puesto que es el mayor productor de radicales libres en la célula. A su vez, dada la conexión entre la mitocondria y el ER, la alteración en la funcionalidad de estos orgánulos podría generar un estado de estrés que comprometería la homeostasis celular, y podría ser, en parte, responsable de la alteración de la dinámica entre leucocitos y endotelio, como se ha demostrado previamente en enfermedades metabólicas como la T2D. Por el contrario, las estrategias dietéticas para la pérdida de peso podrían estar mejorando estas situaciones patológicas al disminuir la carga metabólica del sistema. De hecho, la pérdida de peso moderada ha demostrado ser efectiva para reducir las complicaciones metabólicas y el riesgo CV, aunque los mecanismos que median estos efectos son en gran parte desconocidos. Por tanto, los estudios que profundicen en la modulación de los mecanismos de estrés intracelular y homeostasis metabólica tras la pérdida de peso serían relevantes para ampliar el conocimiento sobre la fisiopatología de la obesidad y el descubrimiento de nuevas dianas terapéuticas que pudieran mimetizar los beneficios de la pérdida de peso. Finalmente, el uso de inositoles como el pinitol ha demostrado mejorar la sensibilidad a la insulina y mejorar el perfil inflamatorio en el contexto de la enfermedad metabólica, aunque las dianas moleculares del pinitol son en gran parte desconocidas.

En base a lo expuesto anteriormente, se propusieron los siguientes objetivos para la presente tesis doctoral:

1. Evaluar la relación entre la función mitocondrial y la producción de ROS en los leucocitos y sus interacciones con el endotelio según el grado de obesidad.

2. Determinar si la presencia y el grado de severidad de la periodontitis crónica en una población con obesidad podría estar alterando la dinámica entre los leucocitos y las células endoteliales vasculares a través de mecanismos que involucren una inducción de estrés oxidativo.

3. Evaluar si una intervención dietética para la pérdida moderada de peso mejora el equilibrio redox y los marcadores de aterosclerosis subclínica en una población con obesidad mórbida.

4. Investigar cómo el estrés de RE, la disfunción mitocondrial y las vías inflamatorias pueden estar moduladas por la pérdida de peso en los leucocitos de pacientes con obesidad.

5. Explorar la posible función protectora del pinitol como un chaperona molecular capaz de mejorar el estrés de RE crónico y la señalización inflamatoria en el tejido adiposo y los leucocitos de pacientes con obesidad.

Material y Métodos

Sujetos de estudio

Se reclutaron varias cohortes de pacientes de mediana edad con normopeso, sobrepeso u obesidad que acudieron al Servicio de Endocrinología y Nutrición y/o al Servicio de Estomatología del Hospital Universitario Doctor Peset (València). De forma general se incluyeron sujetos con edad ≥ 18 años que se diagnosticaron en función de su índice de masa corporal (BMI, del inglés *body mass index*). De forma específica para los estudios de intervención se seleccionaron pacientes con un BMI ≥ 35 kg/m^2 que hubieran mantenido un peso estable en los 2 meses previos al programa dietético. Los criterios generales de exclusión fueron embarazo o lactancia, enfermedades severas incluida la oncológica, renal, hepática, inflamatoria crónica o historia de enfermedad CV, tratamiento con antiinflamatorios, abuso de alcohol o drogas y obesidad secundaria (hipotiroidismo, síndrome de Cushing). De forma específica en los estudios transversales se excluyeron además los pacientes con diabetes mellitus diagnosticada según los criterios de la *American Diabetes Association* (ADA). Para el estudio de periodontitis crónica se

excluyeron además pacientes con menos de 14 dientes, con otras enfermedades infecciosas o inflamatorias orales y aquellos bajo tratamiento con antibióticos.

Tras firmar el consentimiento informado, los pacientes se sometieron a un examen físico consistente en la evaluación del peso, talla, BMI, presión arterial y perímetro de cintura y cadera. En paralelo, se les realizó una extracción sanguínea en ayunas para la determinación de parámetros bioquímicos clínicos como el perfil lipídico – colesterol total, LDL, HDL, TG, apolipoproteinas AI y B –, parámetros de metabolismo hidrocarbonado – glucosa, insulina, hemoglobina glicosilada (A1c), HOMA-IR –, marcadores inflamatorios y de riesgo CV emergentes – proteína C reactiva (CRP, del inglés *C-reactive protein*), C3c (del inglés *component complement 3*) y RBP4 – y hemograma. El suero remanente se conservó a -80 ºC para posteriores determinaciones.

Para el estudio transversal de los diferentes grados de obesidad los pacientes se clasificaron según el BMI en no obesos (< 30 kg/m^2), obesos de grado I-II (30 - 40 kg/m^2) y obesos mórbidos (> 40 kg/m^2). Para el estudio transversal de la periodontitis crónica los pacientes pasaron una evaluación periodontal completa con determinación de la profundidad de sondaje (PD), pérdida de inserción clínica (CAL) así como el índice de sangrado y el índice de placa. Con estos parámetros los pacientes fueron diagnosticados y clasificados según el grado de enfermedad periodontal en sujetos sin periodontitis crónica o con periodontitis crónica leve, moderada o severa, según los criterios del *Center of Disease Control and Prevention/American Academy of Periodontology* (CDC/AAP).

Intervención dietética

Pacientes con BMI ≥ 35 kg/m^2 fueron sometidos a un tratamiento dietético con una duración total de 6 meses consistente en 6 semanas de una dieta VLCD de aproximadamente 654 kcal/día seguida de 18 semanas de dieta de bajo contenido calórico de entre 1200-1800 kcal/día ajustada a los requerimientos nutricionales individuales. Los exámenes físicos y las extracciones sanguíneas se llevaron a cabo a nivel basal y 6 meses tras el tratamiento dietético, sin que se pautaran cambios en la medicación ni en patrones de actividad física durante el periodo de estudio.

Evaluaciones séricas de moléculas de inflamación, adhesión, estrés oxidativo y subfracciones de LDL

Diversas metodologías fueron utilizadas para el análisis sérico de parámetros no incluidos en la analítica de referencia. Por un lado, se utilizaron kits Milliplex para el análisis simultáneo de paneles de moléculas mediante tecnología X-MAP de Luminex, con el que evaluamos los niveles de IL6, TNFα, P-selectina, ICAM-1, VCAM-1 y MPO. En paralelo se analizaron los niveles de PSGL-1 (del inglés *P-selectin glycoprotein ligand-1*), el receptor de P-selectina en los leucocitos, mediante la técnica ELISA. La actividad catalasa se midió mediante kits enzimáticos comerciales específicos. El contenido de glutatión se analizó en lisado de eritrocitos mediante un kit comercial. Además, la carbonilación de proteínas séricas se evaluó con un test colorimétrico basado en la reacción de derivatización. Por último, las subfracciones de LDL se evaluaron mediante el análisis del perfil electroforético específico con el sistema Lipoprint®.

Aislamiento de leucocitos de sangre periférica

Las muestras de sangre se incubaron con dextrano al 3 % durante 45 minutos y se sometieron a centrifugación (650 g durante 25 minutos a temperatura ambiente) en un gradiente de densidad de Ficoll-Hypaque para aislar la fracción de leucocitos. Después de la centrifugación, los eritrocitos remanentes se lisaron y el precipitado celular se lavó con HBSS.

Ensayos de interacción leucocito-endotelio

Los ensayos de adhesión se llevaron a cabo mediante el uso de un sistema de cámara de flujo paralelo acoplado a un microscopio invertido de contraste de fases, a través del cual se perfundió una suspensión de leucocitos *ex vivo* sobre una monocapa de células endoteliales humanas (aisladas de cordón umbilical mediante colagenasa) en condiciones que simulan las del flujo sanguíneo. Se registraron videos de 5 minutos en tiempo real donde se evaluaron tres parámetros de interacción leucocito-endotelio: la velocidad de rodamiento (tiempo que tardan 20 leucocitos consecutivos en recorrer una distancia de 100 µm dentro del campo de enfoque), el flujo de rodamiento (número de leucocitos que ruedan en una superficie de 100 µm^2 de células endoteliales durante un

minuto) y la adhesión (número de leucocitos que mantienen un contacto estable con las células endoteliales durante al menos 30 segundos).

Ensayos de estrés oxidativo y función mitocondrial

Los leucocitos se sembraron por duplicado en placas de 48 pocillos y se incubaron *ex vivo* durante 30 minutos a 37ºC en HBSS con diferentes sondas fluorescentes: DCFH-DA (del inglés *2',7'-dichlorodihydrofluorescein diacetate*), indicativo de la producción total de ROS; Fluo-4, que mide Ca^{2+} intracelular; CMFDA (del inglés *5-chloromethylfluorescein diacetate*), que mide contenido de glutatión; Mitosox-Red, indicativo de la producción de superóxido mitocondrial; DHE (del inglés *dihydroethidium*), que evalúa los niveles de superóxido total; TMRM (del inglés *tetramethylrhodamine, methyl ester*), indicativo del potencial de membrana (ΔΨ) mitocondrial; y Hoescht, indicativo de la morfología nuclear. Las imágenes de fluorescencia emitida fueron captadas y analizadas con un microscopio de fluorescencia IX81 de Olympus acoplado al software de citometría estática "ScanR".

Ensayos de suplementación con pinitol y tratamiento ex vivo *de adipocitos*

Para el ensayo de suplementación se reclutaron sujetos con obesidad (BMI ≥ 30 kg/m^2) cuyas pautas dietéticas fueron normalizadas previo al inicio del estudio. A continuación, se les pautó el consumo de una bebida enriquecida en pinitol (4 g/día) durante 12 semanas. Por otro lado, en otra cohorte de pacientes obesos que fueron sometidos a cirugía de bypass gástrico se obtuvieron biopsias de tejido adiposo visceral y subcutáneo, que se trataron con pinitol (30 µM) durante 48h.

Análisis de expresión de marcadores intracelulares

Se procedió a la extracción, purificación y cuantificación de proteína y mRNA de leucocitos y tejido adiposo según procedimientos estándar y/o mediante kits comerciales específicos. El análisis de expresión de proteínas se llevó a cabo tras la separación de las mismas por electroforesis SDS-PAGE, transferencia y posterior inmunoblot de las membranas de nitrocelulosa con anticuerpos específicos. La señal se detectó mediante quimioluminiscencia con reveladores específicos y se analizó por densitometría óptica. Por otro lado, la evaluación de los niveles de mRNA se realizó mediante RT-PCR cuantitativa con *primers* específicos. Con estas técnicas evaluamos marcadores de estrés de ER – GRP78 (del inglés *78-kDa glucose-regulated protein*), sXBP1 (del inglés *spliced X box*

protein-1), eIF2α (del inglés *eukaryotic initiation factor 2α*), ATF6 y CHOP – mediadores inflamatorios – NFκB (del inglés *nuclear factor κB*), SIRT1 (del inglés *sirtuin 1*), JNK (del inglés *c-Jun N-terminal kinase*) –, mediadores de la ruta de la insulina – GLUT4 (del inglés *glucose transporter type 4*), IR (del inglés *insulin receptor*), PPARγ (del inglés *peroxisome proliferator-activated receptor γ*) – y la enzima antioxidante GPX1 (del inglés *glutathione peroxidase 1*).

Análisis estadístico

El programa SPSS 19.0 se utilizó para el análisis estadístico de los resultados. Las variables continuas se expresaron como media y desviación estándar (SD, del inglés *standard deviation*), o como mediana y percentiles 25 y 75 para datos paramétricos y no paramétricos, respectivamente. Los datos cualitativos se expresaron como porcentajes. Los datos se compararon utilizando la prueba *t* de Student para muestras paramétricas para dos grupos, o el análisis de varianza de una vía (ANOVA) y una prueba *post-hoc* de Student-Newmann-Keuls para tres o más grupos. Para la comparación de proporciones se utilizó la prueba del Chi-cuadrado. Para evaluar la fuerza de asociación entre variables se llevó a cabo el cálculo del coeficiente de correlación de Pearson. En el modelo de regresión multivariante, la relación entre dos o más variables explicativas (variables independientes) y una variable de respuesta (variable dependiente) se evaluó ajustando una ecuación lineal a los datos obtenidos. Todas las pruebas tuvieron un intervalo de confianza del 95 % y las diferencias se consideraron significativas cuando el $p < 0.05$.

Resultados y discusión

En cuanto a las características generales de la población de estudio, el incremento de BMI se asoció con mayor perímetro de cintura y un aumento de la presión arterial y del índice HOMA-IR de resistencia a la insulina, si bien los niveles de A1c y glucosa en ayunas no reflejan alteraciones relevantes del control glicémico en general. Con respecto al perfil lipídico los niveles de colesterol LDL se mantuvieron dentro de los valores de referencia en todas las cohortes sin diferencias significativas entre los grupos, probablemente debido al uso de tratamientos hipolipemiantes. Por el contrario, el colesterol HDL se redujo de forma característica y los niveles de TG aumentaron en paralelo con el grado de obesidad,

mostrando características típicas de dislipemia aterogénica. De hecho, algunos de los pacientes presentaron comorbilidades metabólicas asociadas de forma común a la obesidad, como la hipertensión (19-27 %), la hiperlipemia (10-30 %) y la T2D (18 %), excepto en los estudios transversales en los que se excluyó a los pacientes diabéticos.

En línea con hallazgos anteriores, nuestros resultados describen un estado de inflamación crónica en nuestros pacientes obesos, en los que los niveles circulantes de CRP, IL6 y TNFα aumentaron en paralelo con el grado creciente de adiposidad, lo que podría estar alterando la función endotelial. Concretamente la CRP, cuya síntesis está promovida en el hígado por IL6, es un reactante de fase aguda que se ha asociado a la obesidad y la T2D y al que se le ha conferido un papel predictor de eventos CV. Esta proteína promueve la activación del endotelio y la expresión de moléculas de adhesión y factores quimioatrayentes. De forma similar, TNFα se sobreexpresa en tejido adiposo y células inmunes de pacientes obesos y es un potente promotor no sólo de resistencia a la insulina, sino también de disfunción endotelial, especialmente a través de la reducción de la biodisponibilidad de NO, lo que conlleva un mayor riesgo de eventos coronarios. Al explorar la respuesta de las células endoteliales a esta inflamación sistémica encontramos un aumento paralelo de los niveles circulantes de ICAM-1 y P-selectina con el grado de obesidad. Estas moléculas de adhesión son consideradas marcadores de activación endotelial y participan en el reclutamiento de leucocitos hacia la pared vascular durante los procesos inflamatorios; sin embargo, la elevación de sus niveles en pacientes con obesidad o T2D se considera un marcador de riesgo CV. De hecho, cuando analizamos las interacciones leucocito-endotelio observamos un descenso progresivo de la velocidad de rodamiento de los leucocitos y un flujo de rodamiento mayor, lo que indica que los leucocitos están frenando sobre el endotelio vascular. Además, se observó un mayor número de leucocitos que se adherían firmemente al endotelio, el paso previo a la transmigración hacia el espacio subendotelial. El análisis de correlación bivariada reveló la asociación entre los parámetros de interacción leucocito-endotelio con BMI, citoquinas inflamatorias y moléculas de adhesión. Estos resultados sugieren que el grado creciente de obesidad produce disfunción endotelial, inflamación y promueve las interacciones entre los leucocitos y la vasculatura.

Por otro lado, la inflamación sistémica también causa alteraciones en la respuesta inmune del huésped, lo que aumenta la susceptibilidad a la infección bacteriana y se

presenta como un posible mecanismo de conexión entre la obesidad y la periodontitis crónica. De hecho, en una cohorte de pacientes obesos ajustados por BMI encontramos un mayor recuento leucocitario en aquellos con periodontitis crónica, lo que sugiere la hiperactivación del sistema inmunológico en presencia de esta enfermedad periodontal. Además, la periodontitis crónica parece exacerbar aún más la reacción inflamatoria en pacientes con obesidad, puesto que observamos un incremento progresivo de los niveles de TNFα, CRP y RBP4 a medida que aumentaba el grado de severidad de la periodontitis. A la luz de estos hallazgos y de los muchos vínculos establecidos entre la periodontitis crónica, la obesidad y la enfermedad CV, evaluamos el efecto de esta alteración periodontal en las interacciones entre leucocitos y células endoteliales. Así, observamos que la presencia de periodontitis crónica promuevió el flujo de rodamiento y la adhesión, y que estos parámetros se correlacionaron no sólo con los marcadores periodontales clínicos sino también con TNFα y RBP4, lo que sugiere una asociación dinámica entre periodontitis crónica, inflamación y aterogénesis.

Además de la inflamación sistémica, la resistencia a la insulina y la hiperglicemia se han asociado previamente con la disfunción endotelial, en parte a través de la inducción de estrés oxidativo. Previamente en nuestro laboratorio hemos descrito que la IR podría ser un desencadenante del incremento de la producción de ROS también en los leucocitos circulantes, en base a estudios realizados en pacientes con T2D y con síndrome de ovario poliquístico (PCOS, del inglés *polycystic ovary syndrome*), donde un peor control glicémico o un mayor grado de IR respectivamente se correspondieron con mayor estrés oxidativo en estas células inmunes, lo que promovió un incremento de sus interacciones con el endotelio vascular. De forma similar, nuestros resultados describen un aumento de la producción de ROS totales y anión superóxido en los leucocitos de pacientes obesos no diabéticos, especialmente en el grupo con mayor grado de obesidad e IR. Además, tanto los marcadores de IR como el anión superóxido se correlacionaron con los parámetros de adhesión de los leucocitos, emergiendo como predictores independientes en el modelo de regresión multivariante. Estos resultados sugirieron que, de forma similar a otras enfermedades metabólicas, en la obesidad la IR podría estar alterando el estado redox intracelular de los leucocitos promoviendo su interacción con el endotelio en las primeras etapas del proceso aterosclerótico.

Dada la gran contribución de la disfunción mitocondrial al desequilibrio redox, es probable que el aumento de superóxido detectado pueda estar relacionado con una actividad mitocondrial dañada. La disfunción mitocondrial en la obesidad es una respuesta fisiológica maladaptativa al exceso de suministro de nutrientes, que aumenta el flujo de electrones hacia la cadena de transporte de electrones, incrementándose la producción de ROS y el $\Delta\Psi$ mitocondrial. De hecho, de forma similar a lo descrito por algunos autores en leucocitos de pacientes con T2D, en este estudio observamos una elevación del $\Delta\Psi$ mitocondrial en paralelo con el grado de adiposidad, lo que asociado a una presencia elevada de superóxido, indica una afectación de la función mitocondrial asociada a la obesidad. La sobreproducción de ROS también es una característica relevante de los leucocitos hiperreactivos en la periodontitis crónica. De hecho, en el estudio periodontal encontramos que, en los leucocitos de pacientes con el mismo grado de obesidad, la producción de superóxido se incrementó progresivamente con el grado de severidad de la periodontitis crónica y se correlacionó con el aumento de las interacciones de los leucocitos sobre el endotelio, similar a lo que encontramos con el grado creciente de adiposidad. Sin embargo, no hubo cambios en el $\Delta\Psi$ mitocondrial, lo que podría indicar otras fuentes mayoritarias de producción de ROS no mitocondriales. Estas evidencias sugieren de nuevo que la producción de especies oxidantes en los leucocitos es un mecanismo potencial de alteración de su dinámica con el endotelio. En conjunto, los resultados de este estudio apuntan a que la presencia concomitante de obesidad y periodontitis crónica podría estar aumentando el riesgo de desarrollar aterosclerosis en estos pacientes, entre otros, a través de la exacerbación de la respuesta inflamatoria y oxidativa.

Los datos transversales discutidos anteriormente contribuyen al conocimiento de los mecanismos y factores involucrados en las primeras etapas del proceso aterosclerótico en la obesidad. En el presente proyecto fuimos un paso más allá, investigando el efecto de la pérdida de peso en estos procesos. Previamente, la pérdida de peso ha demostrado ser una estrategia efectiva para mejorar la función cardiometabólica ejerciendo también un papel protector sobre el avance de la enfermedad aterosclerótica; sin embargo, los mecanismos que median este efecto beneficioso son en gran parte desconocidos. En nuestra población, una pérdida de peso moderada de alrededor de un 9 % mejoró la sensibilidad a la insulina, como indican los niveles de glucosa en ayunas, insulina, HOMA-IR

y A1c, y redujo los niveles circulantes de TNFα, PCR, RBP4 y P-selectina, junto con un aumento de PSGL-1 (el receptor de P-selectina en leucocitos), que podría indicar una mayor escisión del mismo de la superficie de los leucocitos. Esta disminución notable de los marcadores de resistencia a la insulina, inflamación crónica y activación endotelial se tradujo en una menor adherencia de los leucocitos al endotelio vascular. En base a los resultados del estudio transversal quisimos saber si este proceso podría estar relacionado con cambios en el estado redox de los leucocitos y en la función mitocondrial. Observamos así una reducción del ΔΨ mitocondrial tras la pérdida de peso, que se acompañó con una reducción en la producción de ROS totales y mitocondriales en los leucocitos. De forma paralela, se incrementó la expresión de la enzima GPX1 en los leucocitos, una enzima antioxidante que puede encontrarse tanto en el citosol como en la mitocondria y que se considera uno de los mayores neutralizadores de ROS celulares, lo que podría estar contribuyendo a reducir el estrés oxidativo en los leucocitos. La producción de ROS también está relacionada de forma bidireccional con la activación del NFκB, un regulador clave de la respuesta inflamatoria cuya expresión en leucocitos de pacientes obesos se ha visto aumentada junto con la producción de TNFα, contribuyendo a la hiperactivación permanente que presentan las células inmunes de estos pacientes. En el presente estudio, la expresión de NFκB en los leucocitos se redujo tras la pérdida de peso junto con el estrés oxidativo, lo que podría indicar una disminución de la activación leucocitaria. También observamos una regulación positiva de la expresión de SIRT1, cuya expresión se induce tras la restricción calórica y está implicada en mecanismos de supervivencia celular y regulación antiinflamatoria, ya que promueve la degradación de NFκB. En un estudio previo en nuestro laboratorio, el uso del antioxidante SS-31 redujo la producción de ROS en leucocitos de pacientes con T2D, a la vez que indujo la expresión de SIRT1 y redujo la activación de NFκB. Todo esto se tradujo en una disminución de las interacciones leucocito-endotelio, lo que refuerza la relación entre estas rutas intracelulares de estrés y la activación de un fenotipo adherente en los leucocitos. En su conjunto estos resultados revelan que la pérdida moderada de peso mediada por dieta es capaz de reducir la inflamación sistémica y el grado de IR en pacientes con obesidad, reduciendo la disfunción endotelial. Además, esto se asocia con una disminución de la producción de ROS en los leucocitos y de la activación intracelular, lo que podría estar disminuyendo su interacción con el endotelio vascular.

Además de las mitocondrias, en los leucocitos existen otras fuentes de especies oxidantes relevantes como la MPO, una enzima crucial para la defensa frente a patógenos; sin embargo, la liberación sistémica excesiva de MPO desde los leucocitos en un contexto inflamatorio como la obesidad puede contribuir al estrés oxidativo y la lesión vascular. De hecho, los niveles séricos elevados de MPO, característicos de la obesidad y la T2D, son considerados como un biomarcador temprano del riesgo CV. Estudios previos han descrito la capacidad de la MPO de adherirse a las células endoteliales favoreciendo el daño oxidativo y el reclutamiento de leucocitos. En el presente estudio observamos un descenso de los niveles séricos de MPO tras la pérdida de peso, que se correlacionaron con los de P-selectina. De forma paralela, observamos una mejora del equilibrio redox sistémico, puesto que disminuyeron los niveles de carbonilación de proteínas séricas (un biomarcador de estrés oxidativo sistémico), mientras que aumentó la actividad catalasa sérica y el contenido de glutatión en los eritrocitos tras la pérdida de peso, dos potentes sistemas antioxidantes. En conjunto, los resultados sugieren una recuperación parcial del equilibrio redox después de la pérdida de peso, que podría contribuir a la mejora de la función endotelial.

Otro de los factores que contribuyen a la disfunción endotelial y el proceso aterogénico es la alteración del metabolismo hepático de lípidos, promovido en la obesidad por la inflamación sistémica y la IR. El exceso de síntesis de lipoproteínas de muy baja densidad (VLDL, del inglés *very low-density lipoprotein)* en el hígado se asocia con el incremento de LDL y un cambio en el tamaño del *pool* de LDL hacia las sdLDL, que tienen mayor capacidad para atravesar el endotelio y son más susceptibles a la oxidación. Por su parte, las sdLDL oxidadas contribuyen a la activación endotelial y son más fácilmente captadas por macrófagos en el espacio subendotelial, lo que confirma su gran potencial aterogénico. En nuestro análisis de subfracciones de LDL observamos que a pesar de que los niveles de colesterol LDL total se mantuvieron estables tras la pérdida de peso, sí se produjo un descenso de las partículas sdLDL y esto se correlacionó con los niveles de MPO, que contribuye a su oxidación, sugiriendo que a falta de evidencias clínicas la pérdida de peso mejora el perfil de las LDL hacia uno menos aterogénico. En paralelo los niveles de TG se redujeron de forma significativa, mientras que el colesterol HDL aumentó, lo que indica que de forma general la pérdida moderada de peso inducida por dieta es capaz de mejorar el perfil lipídico en los pacientes con obesidad mórbida, reduciendo así el riesgo CV.

Además, otros hallazgos del presente estudio refuerzan este papel protector. Tras la intervención se redujo la presión arterial de los pacientes, un predictor clásico de riesgo CV. El ambiente prooxidante e inflamatorio característico de la obesidad contribuye al endurecimiento de las arterias por interferencia con la función endotelial y la modulación del tono vascular, lo que podría estar contribuyendo a reducir los valores de tensión arterial en nuestra población. Además, observamos una disminución de los niveles circulantes de C3c, un predictor de fallo cardiaco considerado un biomarcador de riesgo CV emergente.

A la luz de la relación observada entre la producción de ROS, la función mitocondrial de los leucocitos y su capacidad de interacción con el endotelio vascular quisimos analizar qué otros mecanismos intracelulares de estrés podrían estar siendo modulados tras la pérdida de peso. Recientemente, hemos descrito que la presencia de alteraciones metabólicas como el síndrome metabólico o la T2D, comúnmente asociadas a la obesidad, incrementa la activación del estrés de ER en los leucocitos de pacientes obesos; además hemos descrito la relación entre la activación de las vías de estrés de ER crónico, la disfunción mitocondrial y las interacciones leucocito-endotelio en pacientes con T2D. En el presente estudio cuando analizamos los cambios en la activación de la UPR tras la pérdida de peso encontramos una disminución de la expresión de ATF6, que se correlacionó con un descenso marcado de la proteína proapoptótica CHOP. También detectamos un descenso significativo de la activación de JNK, que puede estar modulada a través de la actividad quinasa de IRE1α. Sin embargo, no se encontraron cambios en mediadores de otras rutas de la UPR (eIF2α o sXBP1). Tanto CHOP como JNK son considerados marcadores de estrés de ER crónico, y de hecho la activación de JNK es el mecanismo más conocido que relaciona el estrés de ER con la inflamación y el desarrollo de IR. Por el contrario, se incrementó la expresión de GRP78, una chaperona que promueve el plegamiento de proteínas aliviando el estrés de ER y que es considerada un factor clave de la respuesta adaptativa de la UPR. Curiosamente, los niveles de GRP78 correlacionaron con la activación de SIRT1 en los leucocitos, de la que previamente habíamos descrito su potencial antiinflamatorio y antienvejecimiento. En conjunto, el aumento de GRP78 y SIRT1 y la disminución de ATF6-CHOP y JNK indican una recesión de las vías apoptóticas de la UPR a favor de respuestas de supervivencia celular. La disminución del estrés de ER tras la pérdida de peso se había descrito previamente en

otros tejidos humanos tras cirugía bariátrica, y en modelos murinos tras dieta, lo que refuerza nuestros resultados que describen este efecto por primera vez en leucocitos de pacientes obesos tras una intervención dietética.

Estudios previos han encontrado una asociación entre los marcadores de estrés de ER crónico en los leucocitos y parámetros de IR sistémica. De forma similar, en nuestro estudio el descenso de HOMA-IR después de la pérdida de peso se correlacionó con los marcadores de estrés de ER crónicos ATF6 y JNK, lo que apoya la conexión entre la función del ER y la homeostasis de la glucosa. Por otro lado, el estrés del ER en los leucocitos también podría estar implicado en su capacidad de adherencia y transmigración en el endotelio vascular. Previamente en nuestro laboratorio hemos descrito la asociación entre los marcadores de estrés de ER en los leucocitos de pacientes con T2D y mayores interacciones leucocito-endotelio. Otros autores encontraron marcadores elevados de activación de UPR en macrófagos aislados de placas ateroscleróticas. En nuestro estudio los marcadores de estrés de ER crónico y las interacciones de los leucocitos en el endotelio disminuyeron en paralelo, lo que reforzaría la asociación entre la disfunción en el ER y la activación de los leucocitos.

Por otro lado, las uniones íntimas entre ER y mitocondria favorecen el intercambio de señales de estrés como el Ca^{2+}, ROS y citoquinas inflamatorias entre otros, que actúan como mecanismos reguladores comunes en los programas celulares de muerte/supervivencia. En situaciones de estrés crónico como la obesidad se produce un bombeo excesivo de Ca^{2+} desde el ER hacia la mitocondria, donde puede incrementar el $\Delta\Psi$, la producción de ROS mitocondriales y culminar en disfunción mitocondrial. A su vez estos ROS mitocondriales, al ser transferidos al lumen del ER, pueden interferir con el plegamiento oxidativo de proteínas cronificando la situación de estrés. Si este mecanismo de retroalimentación se prolonga, ambos orgánulos inician rutas de apoptosis conjuntas que pueden llevar a la muerte celular. Nuestros resultados sugieren que la pérdida de peso podría estar mejorando la homeostasis celular y la funcionalidad del ER y la mitocondria, puesto que un descenso de los marcadores de estrés de ER crónico se acompañó de un descenso de los niveles de Ca^{2+}, $\Delta\Psi$ mitocondrial y producción de ROS mitocondriales.

En base a éstas y otras evidencias previas, el estrés de ER se postula como una diana terapéutica relevante dentro de la fisiopatología de la obesidad, puesto que se asocia con la mejora de otras respuestas al estrés celular y alteraciones metabólicas como

la IR y la inflamación sistémica. El uso de chaperonas químicas como TUDCA y 4-PBA para facilitar el plegamiento de proteínas ha sido ampliamente estudiado en tejidos metabólicos como el adiposo o el hepático, donde reducen el estrés de ER y la señalización inflamatoria. En este sentido, los efectos antiinflamatorios del pinitol, un inositol vegetal, se han descrito previamente en la población con obesidad, aunque los mecanismos subyacentes son en gran parte desconocidos. Para evaluar si el pinitol actuaría como una chaperona aliviando el estrés de ER, y por lo tanto la inflamación, enyasamos los efectos del tratamiento con pinitol en dos de las principales fuentes de citoquinas inflamatorias, como son las células inmunes y el tejido adiposo. El consumo de una bebida enriquecida en pinitol durante 12 semanas redujo la inflamación sistémica en pacientes con obesidad, concretamente los niveles de TNFα e IL6. Sin embargo, no detectamos cambios en marcadores de la UPR (GRP78, CHOP) en leucocitos aislados. Por otro lado, tras realizar un tratamiento *ex vivo* de adipocitos de tejido adiposo visceral y subcutáneo con pinitol encontramos un descenso de la expresión de ATF6-CHOP acompañado de una disminución de la expresión de TNFα e IL6 de forma específica en el tejido subcutáneo, lo que podría contribuir a la reducción de la inflamación sistémica que habíamos observado previamente. Sin embargo, el tejido adiposo visceral no pareció responder al tratamiento con pinitol. Esta regulación diferencial podría explicarse atendiendo a las diferencias metabólicas entre ambos depósitos. Por ejemplo, los adipocitos del depósito visceral son más insulín resistentes que los del subcutáneo, y de hecho al analizar la expresión de mediadores de la ruta de la insulina (GLUT4, IR, PPARγ) en ambos tejidos observamos una menor expresión en visceral respecto al subcutáneo. Sin embargo, a pesar de la expresión diferencial entre ambos tejidos, el tratamiento con pinitol no modificó la expresión de los marcadores en ninguno de los tejidos, ni tampoco mejoró la sensibilidad a la insulina tras su consumo, por lo que parece que los efectos insulinomiméticos del pinitol observados en otros grupos de pacientes no se reproducen en pacientes con obesidad. Por otro lado, dado el potencial antiinflamatorio de SIRT1 y su relación con el estrés de ER evaluamos si se produjeron cambios en los niveles de expresión de este mediador, y observamos una regulación positiva de SIRT1 en los leucocitos de pacientes obesos después de consumir la bebida enriquecida con pinitol, lo que podría explicar en parte el efecto antiinflamatorio que se le atribuye a este inositol vegetal.

En resumen, la presente tesis doctoral comenzó con un estudio transversal donde demostramos que el patrón de interacción entre los leucocitos y el endotelio vascular está alterado en la obesidad y se incrementa en paralelo con el grado de adiposidad, los niveles crecientes de inflamación sistémica y de IR. Estas observaciones se asociaron con un mayor $\Delta\Psi$ mitocondrial y mayor producción de ROS en los leucocitos de estos pacientes, que podrían estar relacionados con el incremento de su adherencia al endotelio. Paralelamente, demostramos que el empeoramiento de la condición periodontal en una cohorte de pacientes obesos ajustados por BMI se asoció con el aumento de la inflamación sistémica y la producción de ROS en los leucocitos, promoviendo así su interacción con el endotelio. Estos resultados amplían nuestro conocimiento sobre los mecanismos subyacentes a la relación entre la obesidad, la periodontitis y la enfermedad CV. Cuando los pacientes fueron sometidos a una intervención dietética para la pérdida de peso observamos una disminución de factores proaterogénicos como la inflamación sistémica, el estrés oxidativo, la resistencia a la insulina y el perfil de la dislipemia aterogénica, reduciéndose la disfunción endotelial y la adherencia de los leucocitos al endotelio. Más tarde profundizamos en la modulación de respuestas de estrés intracelular en los leucocitos tras de la pérdida de peso y encontramos una reducción del estrés del ER, la producción de ROS y el $\Delta\Psi$ mitocondrial, asociados con un aumento en la expresión de chaperonas, mediadores antiinflamatorios y antioxidantes. En conjunto, nuestros resultados arrojan luz sobre los posibles mecanismos que subyacen en el papel protector de la pérdida de peso en el control metabólico y la homeostasis celular. Finalmente, demostramos que el pinitol alivia el estrés del ER y modula la respuesta inflamatoria en el tejido adiposo subcutáneo y los leucocitos de pacientes obesos reduciendo la inflamación sistémica, lo que demuestra el potencial efecto protector de este inositol vegetal sobre algunos de los mecanismos fisiopatológicos que subyacen a la obesidad.

Conclusiones

1. El incremento en el grado de obesidad y el nivel de resistencia a la insulina se acompañan de un aumento progresivo de la producción de especies reactivas de oxígeno (ROS) y el potencial de membrana ($\Delta\Psi$) mitocondrial en los leucocitos circulantes. Además, se asocia con un aumento de marcadores de inflamación sistémica, disfunción endotelial y mayor adherencia de los leucocitos al endotelio, lo que podría aumentar el riesgo de aterogénesis.

2. La presencia y el grado de severidad de periodontitis crónica en una población con obesidad se asocia con una mayor inflamación sistémica, un incremento en la producción de anión superóxido por los leucocitos y un aumento de las propiedades adherentes de estos a las células endoteliales, respecto a los pacientes sin enfermedad periodontal. Estas observaciones sugieren que la periodontitis crónica podría ejercer como un factor de riesgo cardiovascular en la obesidad.

3. La pérdida de peso inducida por dieta mejora el estado cardiometabólico y reduce mecanismos proaterogénicos como la señalización inflamatoria, el estrés oxidativo y la disfunción endotelial. En este contexto, se reduce la adherencia de los leucocitos a las células del endotelio vascular, lo que sugiere un papel protector de la pérdida de peso en las etapas tempranas del proceso aterosclerótico.

4. La pérdida de peso moderada reduce el estrés de retículo endoplasmático (ER) crónico favoreciendo las respuestas adaptativas en leucocitos de pacientes obesos, que se asocian con una reducción en el $\Delta\Psi$ mitocondrial. En consecuencia, las señales de estrés entre la mitocondria y el ER – Ca^{2+} y ROS – se reducen, mejorando de esta manera la homeostasis celular.

5. El pinitol modula el estrés de ER crónico en el tejido adiposo subcutáneo de pacientes obesos, lo que conlleva una disminución en la expresión de citoquinas inflamatorias y un aumento de expresión del mediador antiinflamatorio SIRT1 en los leucocitos, reduciéndose así la inflamación sistémica.

GLOBAL SUMMARY

1. BACKGROUND

1.1 Obesity overview

1.1.1 Definition, diagnosis and classification

Obesity is a chronic metabolic disease characterized by an excessive accumulation of fat. The increase in lipid storage produces adipose tissue dysfunction, which is usually accompanied by a cascade of systemic deleterious effects, such as chronic low-grade inflammation, dyslipidemia and impaired response to insulin, among other metabolic abnormalities, and is associated with an impairment of health-related quality of life and/or reduced life expectancy.

Several methods have been designed to determine the presence and extent of obesity, including specific determinations of the fat mass of an individual, such as DEXA (dual energy X-ray absorptiometry), bioelectrical impedance or body scanners, and evaluations based on anthropometry, such as body mass index (BMI), waist circumference (WC), waist-to-hip ratio (WHR) or skin-fold assessment. Of all these parameters, BMI is the most commonly used to diagnose and classify obesity in daily practice worldwide (Javed *et al*. 2015) for its simplicity and reproducibility. BMI is an indirect measurement of adiposity, obtained by dividing weight in kilograms by height in square meters (kg/m^2). The classification of obesity based on BMI was established by the World Health Organization (WHO) and adopted by other organizations including the Spanish Society for the Study of Obesity (SEEDO), and defines undernutrition as BMI < 18.5 kg/m^2, normal weight as BMI 18.5-24.9 kg/m^2, overweight as BMI 25-29.9 kg/m^2, obesity grade I as BMI 30-34.9 kg/m^2, obesity grade II as BMI 35-39.9 kg/m^2, and obesity grade III or morbid obesity as BMI ≥ 40 kg/m^2 in adulthood (Lecube *et al*. 2017).

Nevertheless, BMI is considered a gross measure of the degree of adiposity and in some circumstances is not precise enough to reflect the amount and distribution of the body fat in an individual (Blundell *et al*. 2014). In this sense, the pattern of accumulation of body fat has special relevance in the development of comorbidities, and determines obesity as abdominal, where the accumulation of fat is predominantly mesenteric and visceral, or peripheral, in which case the fat accumulation is predominantly subcutaneous. The presence of abdominal obesity, more frequent in men, has been related to the development of metabolic complications including metabolic syndrome, which particularly affects cardiovascular (CV) risk (Despres 2012). Hence, anthropometric measurements

such as WC and WHR have been proposed to define more accurately the obesity-associated risk. Indeed, WC is the most used of the two parameters due to its strong correlation with visceral fat deposits (Despres 2012). Consensus reference values are WC ≥ 102 cm for men and ≥ 88 cm for women, both indicating abdominal obesity and increased risk of morbidity and mortality (Grundy *et al*. 2005).

1.1.2 Epidemiology and risk factors

Obesity is the most prevalent metabolic disease in developed countries. According to the WHO database, in 2016 more than 1,900 million adults worldwide were overweight (39 %), of which more than 600 million were obese (13 %) (World Health Organization 2017). Moreover, the prevalence of obesity has increased drastically in the last three decades, and estimations suggest it could reach 20 % by the year 2025, which is why obesity is currently considered the 21st century epidemic (NCD Risk Factor Collaboration (NCD-RisC) 2016). The cost of the Health Services derived from obesity in developed countries ranges from 2-8 % of the total healthcare budget (Pereira J.L. 2005), and the average annual cost is estimated to be 44 % higher for an individual with BMI > 35 kg/m^2 with respect to a subject with normal weight.

From a simplistic point of view, increased fat deposition in obesity is the result of an imbalance between energy intake and caloric expenditure; i.e., overconsumption of high-caloric foods accompanied by a sedentary lifestyle. However, the aetiology of obesity has proven to be more complex, integrating a range of factors, from physiological, genetic and behavioural to environmental, social and economic aspects (Martinez 2000, Williams *et al*. 2015). Indeed, several epidemiological studies and meta-analyses have shown that obesity is associated with the shift towards increasing obesogenic environments in terms of eating choices, leisure and physical activity patterns, especially in developed countries (Bhupathiraju *et al*. 2016, Newton *et al*. 2017).

1.1.3 Physiopathology

Adipose tissue dysfunction and detrimental systemic effects

Obesity is characterized by a metabolic overload resulting from an excess nutrient supply. In this sense, adipose tissue plays a crucial role in the regulation of the whole-body

energy balance. Of the two main types of adipose tissue, white adipose tissue (WAT) is the most abundant in humans and has traditionally been considered the organ responsible for storage and mobilization of energy substrates in the form of triglycerides (TG) and fatty acids (FA), respectively. In addition, advances in the field have demonstrated that WAT is not only a fat store, but is capable of releasing a great variety of cytokines and adipokines that exert endocrine, paracrine and autocrine functions, thus ensuring a systemic regulation of the metabolism. On the other hand, brown adipose tissue adipocytes are rich in mitochondria, contributing to energy expenditure through activation of uncoupling protein 1 (UCP1)-mediated thermogenesis (Vázquez-Vela *et al*. 2008).

The excess of energy sources during obesity is channelled towards increasing lipid storage in the subcutaneous adipose tissue (SAT), a specific WAT depot located underneath the skin. SAT expansion capacity is challenged during obesity, triggering mechanisms of hyperplasia and hypertrophy to accommodate the excess energy supply over time (Gonzalez-Muniesa *et al*. 2017). In this context, adipocytes are exposed to a hypoxic and metabolically stressful environment that leads to failure of cellular function, including endoplasmic reticulum (ER) stress and mitochondrial dysfunction. As a result, several intracellular stress responses are induced, including the unfolded protein response (UPR), an inflammatory cascade mediated by c-Jun N-terminal kinase (JNK) and inhibitory κB kinase (IKK)/nuclear factor κB (NFκB) pathways, and production of reactive oxygen species (ROS) (Hotamisligil *et al*. 2008). The eventual consequence of all of this is <u>adipose tissue dysfunction</u>, characterized by a shift towards a more pro-inflammatory profile of secreted adipokine and cytokine and oxidative stress (Sun *et al*. 2013, Meijer *et al*. 2011).

For instance, increased adipocyte MCP-1 (monocyte chemoattractant protein-1) expression enhances immune cell recruitment towards adipose tissue, where they contribute to tissue remodelling and amplification of the inflammatory response (Sun *et al*. 2013, Lolmede *et al*. 2011). In addition, dysfunctional adipocytes secrete higher amounts of tumour necrosis factor alpha (TNFα), interleukin 6 (IL6), leptin, plasminogen activator inhibitor-1 (PAI-1) and retinol binding protein 4 (RBP4) and lower levels of adiponectin into the systemic circulation, leading to a <u>chronic, low-grade inflammatory response</u>, amplified and perpetuated by the activation of other tissues, including liver and circulating leukocytes (Vázquez-Vela *et al*. 2008, Schmidt *et al*. 2015).

This systemic inflammation is considered to play a central role in the development of the metabolic complications of obesity, including insulin resistance (IR), endothelial dysfunction and altered lipid management (Hotamisligil 2006). In this sense, TNFα is considered a major contributor to the development of IR in obesity, mediated by activation of JNK/IKK-NFκB pathways, which in turn leads to the phosphorylation of insulin receptor substrates 1 and 2 (IRS1/IRS2) (Yazdani-Biuki *et al*. 2004, Hotamisligil *et al*. 1995). In fact, TNFα resulting from the crosstalk between adipocytes and activated macrophages triggers IR in fat cells, which become more lipolytic and begin to release excess FA into the systemic circulation when saturated. Subsequently, free FA (FFA) initiate mechanisms of lipotoxicity and ectopic lipid depostion in other organs, including liver, muscle and pancreas, and in the visceral adipose tissue (VAT) depot, including mesenteric, perirenal and perivascular spaces (Taira *et al*. 2013). These mechanisms, together with the aberrant adipocytokine profile, contribute to an atherogenic and inflammatory systemic milieu, leading to the development of IR and impaired vascular function (Hotamisligil 2006). For instance, IR induces gluconeogenesis in the liver and reduces glucose uptake in skeletal muscle, thus resulting in systemic hyperglycaemia. Overproduction of insulin by β-cells is a compensatory mechanism that precedes hyperinsulinemia, and is characteristic of IR-obese subjects. Finally, if declining glycaemic control is not restored, the situation may progress to non-reversible β-cell failure, impaired glucose tolerance and eventually the development of type 2 diabetes (T2D) (Cusi 2010) (Figure 1).

Differences between VAT and SAT

Accumulating evidence shows that an abdominal obesity phenotype resulting from predominance of fat storage in the VAT depot correlates with the appearance of a constellation of metabolic disturbances including dyslipidemia, IR and CV alterations (Despres 2012), whereas, in peripheral obesity, SAT seems to act as a "metabolic sink" that protects against the development of cardiometabolic comorbidities (Karpe *et al*. 2015). This could be partly explained by the several morphological and metabolic differences between VAT and SAT (Misra *et al*. 2003). For instance, VAT expands predominantly by hypertrophying adipocytes and has a lower hyperplasia capacity. Higher numbers of infiltrated immune cells release increased amounts of proinflammatory cytokines in VAT than in SAT (Fontana *et al*. 2007, Ibrahim 2010). In addition, larger VAT adipocytes are

more IR and metabolically active, resulting in higher lipolytic activity, driving FFA and inflammatory cytokines directly to the liver via portal circulation (Ibrahim 2010). These molecules exert a detrimental effect on liver function, leading to local IR, expression of hepatic acute phase response mediators such as C-reactive protein (CRP) and fibrinogen, and <u>altered lipid metabolism</u>, including excess VLDL (very low-density lipoprotein) production and a rise in circulating sdLDL (small dense low-density lipoprotein) particles, all hallmarks of atherothrombotic disease (Misra *et al.* 2003).
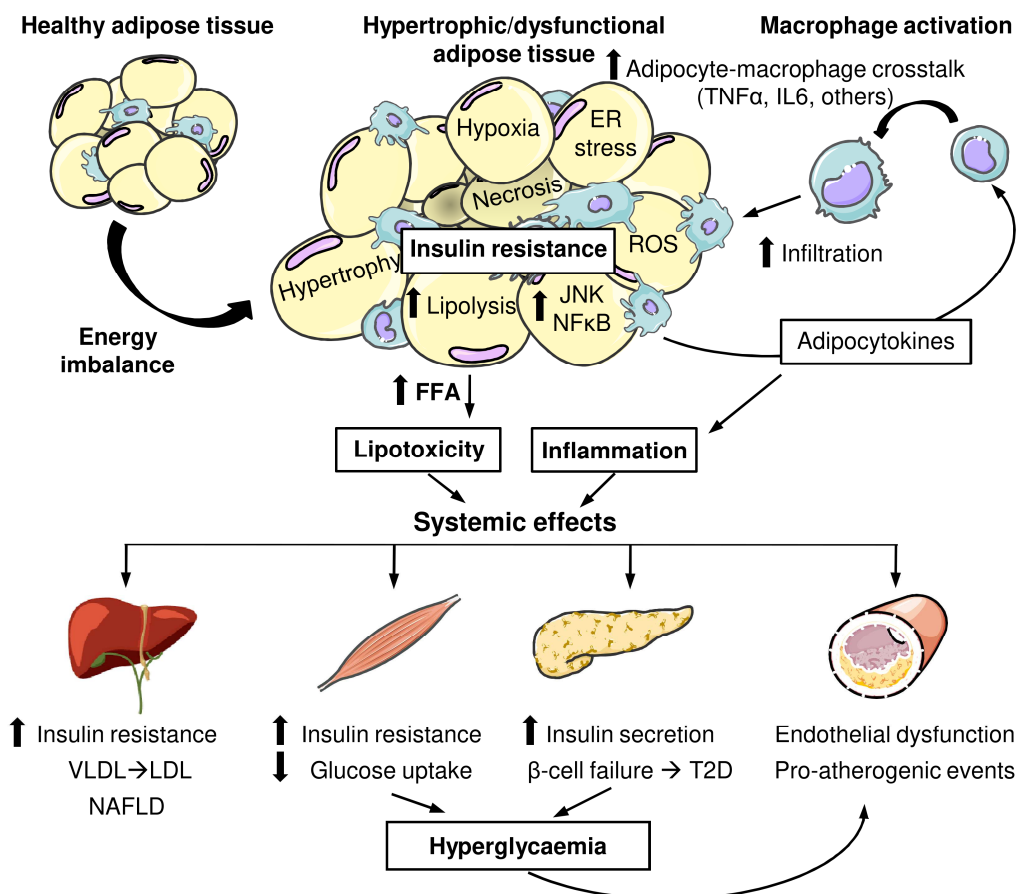

Figure 1. Adipose tissue dysfunction and pathological consequences. A persistent positive energy balance leads to excessive accumulation of fat in the adipocyte, which in turn leads to hypertrophia, hypoxia and subsequent alterations of the cell metabolism. As part of this process, stress pathways are initiated, such as inflammatory responses, oxidative stress, endoplasmic reticulum (ER) stress, and, eventually, necrosis and cell death. The crosstalk between adipocytes and macrophages is enhanced in the form of increased cytokine release from both cell types, which further undermines the adipocyte metabolism, leading to insulin resistance (IR). Finally, dysfunctional adipose tissue releases excess free fatty acids (FFA) and adipokines into the circulation, leading to systemic effects such us lipotoxicity and chronic inflammation in distal tissues such as liver, muscle, pancreas and vascular beds, resulting in metabolic complications that are characteristic of obesity, including non-alcoholic fatty liver disease (NAFLD), type 2 diabetes (T2D) and atherosclerosis. (Adapted from Cusi 2010).

1.1.4 Comorbidities, cardiovascular risk and mortality

Numerous studies have clearly demonstrated that obese subjects are at greater risk of developing several metabolic complications that significantly contribute to deterioration in health-related quality of life and an increased mortality rate (Cornier *et al.* 2011). These complications include alterations in lipid management and metabolism, IR and an aberrant inflammatory profile, key contributors to the development of obesity-related metabolic comorbidities, including metabolic syndrome, T2D, dyslipidemia, non-alcoholic fatty liver disease (NAFLD) and CV complications, such as hypertension and atherosclerosis. As mentioned above, the appearance of this cluster of damaging coexisting illnesses occurs predominantly in abdominal obesity and has been related to the increased CV risk in obese populations (Misra *et al.* 2003). Furthermore, obesity increases the likelihood and worsens the prognosis of several non-metabolic pathologies, including sleep apnoea, osteoarthritis and cancer (Lecube *et al.* 2017, Gonzalez-Muniesa *et al.* 2017).

Accumulating evidence is consistently demonstrating a positive correlation between BMI and mortality risk, and confirms that obesity is a major risk factor for all-cause mortality (Berrington de Gonzalez *et al.* 2010, Global BMI Mortality *et al.* 2016), contributing to 2.8 million deaths *per* year (World Health Organization 2017). Moreover, a rising BMI has been related to increased risk of CV disease-associated morbimortality, as shown by large prospective studies of both European (SCORE) and American cohorts (Dudina *et al.* 2011, Khan *et al.* 2018). In fact, although there is no clear consensus on the BMI cut-off points alert of the development of comorbidities such as hypertension or T2D, BMI itself (as a continuous variable) is independently associated with an increased risk of coronary heart disease, stroke and CV disease-related deaths (Lecube *et al.* 2017).

Obesity-related CV disease manifests itself through several mechanisms, including the formation of atherosclerotic plaques in the vascular beds, and an increase in arterial stiffening resulting in a rise in blood pressure. Dysfunctional adipose tissue release several factors leading to peripheral vasculature defects such as endothelial dysfunction and arterial stiffening, thus promoting the development of hypertension and atherosclerosis in obesity, two associated conditions that evolve during the development of CV disease. Highlights from the epidemiological Framingham Heart Study and other studies clearly establish a link between increased adiposity and a rise in blood pressure (Tanamas *et al.*

2015, Field *et al*. 2001) and other risk factors directly related with the development of coronary atherosclerotic disease, including diabetes and atherogenic dyslipidemia (Altman 2003).

The association between obesity and CV risk appears to be especially evident in those who accumulate fat predominantly in the abdominal area (Despres 2012), a feature considered a core component of <u>metabolic syndrome</u>; defined as the presence of ≥ 3 of the following metabolic disturbances: central obesity, high blood pressure, impaired fasting glucose and altered lipid profile (that is, elevated TG and/or low high-density lipoprotein cholesterol (HDLc)) (Alberti *et al*. 2009). In addition, when the disturbed lipid profile also includes the presence of sdLDL particles – which are proven to display enhanced atherogenic properties – the condition is known as <u>atherogenic dyslipidemia</u> (Ascaso *et al*. 2017). This lipid triad worsens as BMI and the degree of IR rise, and is the best-characterized driver of obesity-associated CV risk (Franssen *et al*. 2008). Closely linked to metabolic syndrome and dyslipidemia, the presence of <u>T2D</u> combined with obesity is a major risk factor for the development of CV complications in the overweight population. Excess adiposity is a key contributor to the systemic impairment of insulin signalling, leading to hyperinsulinemia, glucose intolerance and eventually β-cell failure and T2D development (Kahn *et al*. 2006), which explains why trends in the prevalence and incidence of diabetes closely mirror those in obesity (Menke *et al*. 2015). Several pathophysiological mechanisms in T2D, such as glucotoxicity and oxidative stress, exert deleterious effects on vascular function, leading to the development of macro and microvascular complications, including nephropathy, retinopathy, stroke and heart attack (Aronson *et al*. 2002).

On the contrary, some obese individuals seem to be cardiometabolically protected, a feature known as the metabolically healthy obese phenotype, characterized by the lack of clinical metabolic syndrome traits and typically associated with peripheral obesity (Teixeira *et al*. 2015). However, the existence and definition of a subpopulation of putative metabolically healthy obese subjects has been a major point of controversy in the last few years among experts in the field, with recent studies suggesting that these subjects are actually in a transitional state associated with a higher subclinical CV risk compared to lean subjects (Eckel *et al*. 2018).

Other emerging risk factors commonly accompanying obesity are worthy of mention. Excess adiposity drives the release of pro-inflammatory and pro-thrombotic molecules, leading to a <u>chronic low-grade inflammation</u> and <u>pro-thrombotic state</u> that may predispose obese subjects to suffer acute coronary syndromes, especially those with central obesity (Ellulu *et al*. 2017). In this sense, it is noteworthy to mention that serum levels of CRP are elevated in obesity and have consistently been demonstrated as an independent predictor of coronary heart disease, stroke, and mortality risk (Emerging Risk Factors Collaboration *et al*. 2010, Danesh *et al*. 2004).

1.1.5 Obesity, chronic periodontitis and cardiovascular risk

Besides the aforementioned metabolic comorbidities, accumulating evidence from the last decade has pointed to an association between obesity and the risk of developing chronic periodontitis (Chaffee *et al*. 2010, Jimenez *et al*. 2012, Martinez-Herrera *et al*. 2017). Periodontal disease results from the interaction between pathogenic periodontal bacteria and the host's immune response, and is characterized by an exacerbated inflammatory response and ROS production, which affects the supporting structures of the teeth and whose progression results in alveolar bone degeneration and, eventually, loss of the tooth (Gurav 2014). Similarly to obesity, periodontitis is considered an inflammatory-based disease, thus underlining its relevance not only as an oral health alteration, but as a systemic health problem. Several epidemiological studies have reported a higher prevalence of chronic periodontitis in obese populations, where it affects over 75 % of subjects, who are at a 5-6-times greater risk compared to lean subjects (Martinez-Herrera *et al*. 2017, Nishida *et al*. 2005), as well as highlighting the interfering role of obesity in non-surgical periodontal therapy (Martinez-Herrera *et al*. 2018, Suvan *et al*. 2014).

Although the causality and directionality of this relationship is not clear, systemic metabolic disturbances in obesity presumably exert an underlying role in the onset and progression of periodontal disease (Chaffee *et al*. 2010). In fact, systemic inflammation and oxidative stress share similar pathological features in both diseases, and it is likely that the concomitant presence of obesity and chronic periodontitis exacerbates the extent of these responses (Boesing *et al*. 2009). Furthermore, previous results obtained by our group and other researchers have illustrated the obesity-associated IR as a potential underlying mechanism of chronic periodontitis development (Martinez-Herrera *et al*. 2017, Song *et al*.

2016). In this sense, excess of adiposity seems to be responsible for the increased presence of chronic periodontitis, whereas IR may be involved in the extent of periodontal disease (Martinez-Herrera *et al*. 2017, Saito *et al*. 2005). However, despite there exist numerous data supporting the implication of inflammation, oxidative stress and IR in the relationship between obesity and chronic periodontitis, further studies are required to determine the molecular mechanisms underlying this association.

On the other hand, chronic periodontitis is thought to be implicated in the onset of subclinical atherosclerosis and CV disease. This association was first described by Mattila *et al*. (Mattila *et al*. 1989) and later confirmed by several epidemiological reports (Southerland *et al*. 2012, Tonetti 2009), all of which suggested that periodontitis precedes the atherosclerotic process (Haynes *et al*. 2003). The mechanisms linking periodontitis and atherosclerosis have not been fully elucidated, although exacerbated inflammatory response and ROS production in chronic periodontitis seem to trigger vascular injury and endothelial dysfunction, leading to atherosclerosis and CV complications (Gurav 2014). In this sense, chronic periodontitis shares many of the traits linking obesity and atherosclerotic disease; not surprising, since chronic periodontitis and obesity have been demonstrated to be closely related pathophysiologically (Figure 2). In the light of this evidence and the high prevalence of chronic periodontitis among obese individuals, periodontal disease would appear to be an emerging additional risk factor for CV disease in the obese population, although further prospective studies are needed to confirm this notion.

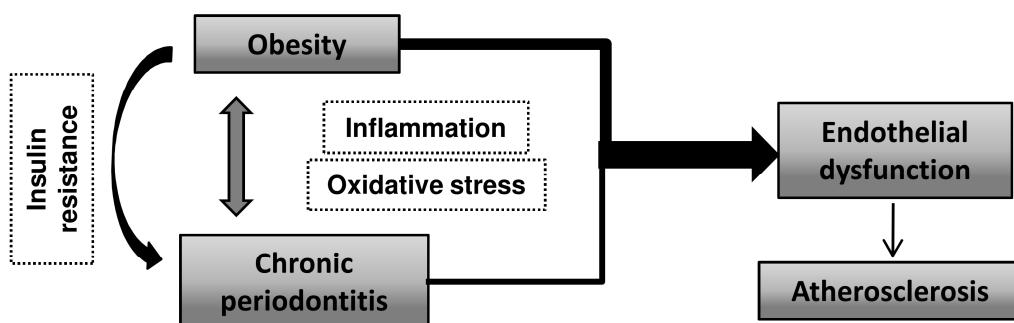Figure 2. Model of association between obesity, chronic periodontitis and endothelial dysfunction. Obesity increases the risk of developing chronic periodontitis, which tends to be more pronounced in the presence of associated insulin resistance. In addition, systemic inflammation and oxidative stress converge in both pathologies, leading to endothelial dysfunction and, eventually, the development of atherosclerosis.

1.1.6 Obesity management

The growth in rates of obesity and its morbimortality around the world fuel the need to develop strategies for the prevention and management of excess adiposity, which could reduce the global burden of the disease.

From this point of view, current trends in obesity management are aimed at weight loss, and so lifestyle interventions including nutritional changes, promotion of physical activity and behavioural modification strategies are first-line approaches. Beyond this point, a limited number of drugs have been approved for us as adjuncts to diet and exercise for weight loss purposes (Apovian *et al.* 2015). Indeed, pharmacological approaches to obesity are mostly focused on alleviating associated metabolic complications, rather than reducing weight. Finally, bariatric surgery has become the most effective treatment for excess adiposity to achieve short- and long-term weight loss and improve cardiometabolic function. In particular, gastric bypass is considered the gold standard surgical procedure, since its effectiveness in T2D remission and reducing metabolic complications compared to lifestyle interventions thus increasing survival (Sjostrom *et al.* 2014, Ikramuddin *et al.* 2013). However, gastric bypass is commonly reserved for patients with severe obesity (BMI > 40 kg/m^2) or metabolic complications, which accounts for only a tiny percentage of the obese population, and is a relatively invasive and expensive procedure. In this sense, given the limitations of pharmacotherapy and bariatric surgery use and the absence of contra-indications, lifestyle interventions still prevail as the most common strategies of obesity management and weight loss.

Lifestyle modifications and weight loss benefits

Weight loss can improve CV risk factors and the metabolic complications associated with obesity, prevent the progression of severe disease and increase health-related quality of life, among other benefits that can be achieved as long as the weight loss is maintained. For this reason, endocrine practical guidelines strongly recommend weight loss strategies based on lifestyle changes as the treatment of choice to reduce obesity burden (Lecube *et al.* 2017, Apovian *et al.* 2015). These guidelines report that sustained weight loss of only 3-5 % enhance metabolic outcomes including TG, fasting glycaemia, and reduce the risk of developing T2D. Furthermore, weight loss of 5-10 % has been shown to confer additional

benefits in terms of blood pressure levels, dyslipidemia and by reducing the need for medication for metabolic syndrome traits and T2D (Jensen *et al*. 2014). In line with this, the Finnish Diabetes Prevention Study reported long-term benefits on CV risk mediated by lifestyle interventions (Ilanne-Parikka *et al*. 2008). Among these strategies, hypocaloric diets and physical exercise are the most commonly used weight loss approaches, since their effectiveness in improving several health outcomes has been proved, especially when both strategies are prescribed as combination therapy (Clark 2015, Wu *et al*. 2009b). However, moderate levels of physical activity induce health benefits independently of weight loss, including lower risk of developing T2D and CV disease (Sadarangani *et al*. 2014), presumably by reduction of VAT depot size. Interestingly, improving overall nutrition patterns (such as adherence to a Mediterranean diet) can also induce weight loss and health improvements without explicit energy restriction (Gonzalez-Muniesa *et al*. 2017). However, the specific mechanisms through which diet-induced weight loss improves metabolic parameters are not completely understood. In this sense, further research in this field would be of great value to determine potential therapeutic targets and expanding our knowledge about the physiopathology of obesity and its metabolic disorders.

Hypocaloric diets

Caloric restriction provokes a negative energy balance that induces weight loss. In this sense, hypocaloric diets have proven to be with very effective in body weight reduction and in producing general health benefits (Lecube *et al*. 2017, Jensen *et al*. 2014). Among the wide range of diets used for the treatment of obesity, very-low-calorie diets (VLCD) decrease the weight of obese patients on average 2 kg *per* week during the first 4-6 weeks (Hernandez-Mijares *et al*. 2012, Sola *et al*. 2009). A VLCD consists of a caloric intake of under 800 kcal *per* day, usually prescribed in the form of commercially available substitutive meals to ensure subjects receive the minimal nutritional requirements. An adequate medical follow-up is required, although their safety has been largely proven when the treatment period does not exceed the maximum recommendation of 16 weeks (Gargallo Fernandez *et al*. 2012, National Task Force on the Prevention and Treatment of Obesity, NIH 1993). Nevertheless, replacement of VLCD with low-calorie diets (LCD) is recommended when there are potentially compromising effects on the subject's

nutritional status (Hernandez Mijares *et al*. 2004). General recommendations suggest that LCD should be individualized to -30 % energy restriction, or at least be prescribed according to sex differences; typically 1200 kcal *per* day for women and 1500 kcal for men (Gonzalez-Muniesa *et al*. 2017). However, correct adherence to the dietary program and weight loss maintenance are essential for maintaining the benefits achieved with the reduction of adiposity degree.

Pharmacotherapy and complementary therapies

In order to reinforce the effects of LCDs, some drugs have been developed and prescribed as adjuvant medication for patients who are struggling to lose and/or maintain body weight. For instance, liraglutide and naltrexone/bupropion have been shown to produce an average drop in body weight of 5-8 % with additional cardiometabolic benefits (Pi-Sunyer *et al*. 2015, Greenway *et al*. 2010). Standardized protocols largely depend on the respective country; in this sense, elevated costs, some low-level side effects and difficulties in dosage and administration have limited the extension of the treatment in Spain (Lecube *et al*. 2017). A more widespread use is that of treatments against the comorbidity burden of obesity; that is, drugs that combat the wide range of obesity-associated metabolic complications; i.e. hyperlipidemia, hypertension and T2D. For instance, the statins are remarkably effective in reducing LDL levels, which lowers the risk of adverse CV events in patients with hyperlipidemia. On the other hand, metformin is a widely prescribed oral antidiabetic drug that is considered the first-line medication for pre-diabetes or early stages of T2D. Both families of drugs have displayed additional synergic anti-inflammatory and atheroprotective activities that may play a relevant role in the prevention and treatment of CV disease in obese population (Krysiak *et al*. 2012). However, some studies have shown that even patients within normal LDL ranges – achieved with cholesterol-lowering medications – still have a residual CV risk (Bayturan *et al*. 2010, Lim *et al*. 2013), although the causal relationship is not understood.

On the other hand, the growing need for new therapies to diminish the metabolic burden of diseases such as obesity and T2D has fuelled research efforts to identify and develop bioactive dietary molecules, including carbohydrates such as polyols, fibre and related carbohydrates. In this sense, inositols are glucose derivatives obtained mainly from legumes, citrus fruits, whole grains and nuts that have shown several health-promoting

properties in different conditions such as T2D, obesity, polycystic ovary syndrome (PCOS) and some types of cancer (Owczarczyk-Saczonek et al. 2018).

For instance, D-chiro-inositol and its methylated form 3-O-methyl-D-chiro-inositol (D-pinitol or pinitol) is a botanical compound present in carob-pod fruit, soybeans and other legumes, and has been well documented as a sensitizer of insulin's actions in both animals and humans (Owczarczyk-Saczonek *et al*. 2018). It is thought to act downstream in the insulin-signalling pathway to mimic the effects of insulin in adipocytes and, hepatocytes (Shen *et al*. 2012). The precise mechanism is poorly understood, but seems to be mediated through activation of the PI3K/Akt axis (Gao *et al*. 2015). In this sense, we have previously demonstrated that both acute doses and chronic intake of a pinitol-enriched beverage improve glucose tolerance and insulin sensitivity in healthy subjects (Hernandez-Mijares *et al*. 2013, Bañuls *et al*. 2016). Furthermore, several studies have largely demonstrated the ability of pinitol to reduce systemic IR and improve overall glucose tolerance, not only in susceptible populations, such as T2D patients (Hernández-Mijares *et al*. 2015, Pintaudi *et al*. 2016), but also in prediabetic subjects (Davis *et al*. 2000, Bañuls *et al*. 2016) and women with PCOS or gestacional diabetes (Owczarczyk-Saczonek et al. 2018). In addition to its insulinomimmetic activity, pinitol seems to stimulate β-cell-production of insulin, thus contributing to the antidiabetic effect (Lazarenko *et al*. 2014, Lambert *et al*. 2018). Pinitol consumption has also been described to diminish oxidative stress, systemic inflammation and endothelium dysfunction in both human and murine models of diabetes (Hernandez-Mijares *et al*. 2015, Sivakumar *et al*. 2010), thus displaying a potential atheroprotective role (Choi *et al*. 2007). Our group has also reported anti-inflammatory activity in obese subjects consuming pinitol (Bañuls *et al*. 2016), although the underlying mechanism was not determined. Altogether, collected data confirm the potential therapeutic use of inositols as prophylaxis for diseases associated with IR and systemic inflammation, including obesity and T2D, although further studies are required to identify the potential targets of pinitol and describe the mechanisms implicated in the beneficial effects observed.

1.2 Pathophysiological mechanisms underlying atherosclerosis in obesity

Atherosclerosis is a CV disorder characterized by progressive accumulation of lipids, immune cells and fibrotic components in the arterial wall that causes a progressive occlusion of blood vessels. In the early stages, symptoms of ischaemia appear, with the situation worsening as the atheromatous plaque develops, ending in plaque rupture and increasing the risk of atherothrombotic events, including myocardial infarction and stroke (Bentzon *et al*. 2014).

Classic risk factors for the appearance of atherosclerosis are age, sex, hypertension, diabetes, dyslipidemia and lack of physical exercise, among others. Many of these factors converge in obesity, along with systemic inflammation, which is considered a crucial mechanism of the atherogenic process in obesity (Altman 2003, Ross 1999, Jacobs *et al*. 2009). However, its aetiology is complex, and the interaction between the different pathophysiological processes at play is not completely understood. In addition, plaque formation is a slow process, often constituting a subclinical CV risk condition in obesity, even in patients without associated comorbidities (Kim *et al*. 2017). The study of the mechanisms involved in the early asymptomatic stages of atherosclerosis, namely subclinical atherosclerosis, may open up new perspectives in the prevention and treatment of CV complications and associated morbimortality.

1.2.1 Endothelial activation and dysfunction

The endothelium is the innermost layer of the vascular wall, and is responsible for vascular tone regulation, inflammatory response, coagulation and overall vascular homeostasis. Under pathological situations such as obesity, there is an alteration of endothelial function characterized by reduced bioavailability of nitric oxide (NO), a biomolecule produced by the endothelial NO synthase enzyme (eNOS), which is involved in the relaxation of vascular tone and has additional anti-thrombotic and anti-inflammatory effects (Tousoulis *et al*. 2012). In the context of obesity, vasculature is challenged by an exacerbated pro-inflammatory state, lipotoxicity, hyperglicaemia and excess production of ROS and vasoconstrictor factors that alter endothelial homeostasis (Guzik *et al*. 2006, Reho *et al*. 2017). These mechanisms promote endothelial cell activation and stimulate the expression of chemoattractant factors, such as MCP-1, and cell adhesion molecules

(CAMs), including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E/P-selectins, which are mainly mediated through the activation of the NFκB pathway in the endothelial cells (Pierce *et al.* 2009, Zhong *et al.* 2012). These molecules further enhance leukocyte recruitment to the vessel wall and increase their infiltration in the intima space, where they contribute to the growth of the atherosclerotic plaque in a pro-oxidant and inflammatory environment (Krinninger *et al.* 2014).

The aberrant profile of cytokines and adipokines in obesity plays a key role in this process (Guzik *et al.* 2006, Chen *et al.* 2012). Studies of large cohorts of patients have demonstrated a clear association between the imbalance in pro-inflammatory (e.g., TNFα, IL6 and CRP) and anti-inflammatory (e.g., adiponectin) mediators in obesity and several CV complications, including hypertension, coronary disease, atherosclerosis and associated morbidity (Emerging Risk Factors Collaboration *et al.* 2010, Danesh *et al.* 2004, Jacobs *et al.* 2009, Bermudez *et al.* 2002, Sesso *et al.* 2003, Luc *et al.* 2003). Cytokines seem to promote a drop in the NO/ROS ratio in the vasculature, which induces oxidative stress, endothelial dysfunction and subsequent enhanced CAMs expression (Reho *et al.* 2017, Zhong *et al.* 2012, Venugopal *et al.* 2002).

On the other hand, the presence of IR and/or hyperglycaemia contributes in a large way to endothelium dysfunction (Dudina *et al.* 2011). In fact, impaired insulin sensitivity interrupts insulin vasodilatory function and stimulates the proliferation of smooth muscle cells (Wu *et al.* 2009a, Wang *et al.* 2003). Furthermore, hyperglycaemia induces the production of ROS, thus contributing to oxidative stress, apoptosis and vascular permeability (Brownlee 2005). A damaged vasculature is highly susceptible to LDL (low-density lipoprotein) infiltration in the intima zone, especially by atherogenic sdLDL particles. In the pro-oxidant subendothelial space sdLDL become oxidized, thereby acting as stimuli of an amplified inflammatory response and eNOS uncoupling and contributing to oxidative stress and endothelium dysfunction (Liao *et al.* 1995, Fleming *et al.* 2005, Gebuhrer *et al.* 1995).

1.2.2 Leukocyte activation and adhesion cascade

On the other side of the coin of the atherosclerotic process, immune cells and platelets became activated under the pro-inflammatory and pro-thrombotic status, further potentiating the inflammatory response and contributing to their adherence and

aggregation during the atherothrombotic process (Devaraj *et al.* 2003). In fact, several studies have reported that circulating leukocytes in obese patients are in a pro-inflammatory and pro-oxidant state, which in turn promotes their interaction and migration across the endothelium layer (Krinninger *et al.* 2014, Ghanim *et al.* 2004). The activation of leukocytes is usually integrated via classic inflammatory pathways such as NFκB/JNK and by enhanced production of ROS (Nguyen *et al.* 2007, Aljada *et al.* 2004). In this continuously pro-activated state, interactions between CAMs in endothelial and immune cells and chemoattractant molecules promote the recruitment of leukocytes towards the vascular endothelium and initiation of the adhesion cascade, a sequential mechanism ending in transmigration of leukocytes to the subendothelial space (Čejková *et al.* 2016, Muller 2002). Initially, leukocytes are attracted towards the endothelium, which reduces their flow velocity and allows them to establish initial contacts mediated mainly by the interaction between selectins and their respective ligands. These low-intensity transient interactions favour the rolling of leukocytes along the endothelium, although many leukocytes dissociate due to the reversible nature of the contact (Muller 2002). Later on, selectins-clustering triggers activation of high affinity unions mediated mainly by integrins, VCAM-1 and ICAM-1 (Ma *et al.* 2004). Subsequently, these high affinity unions lead leukocytes to stop rolling and firmly adhere to the endothelial surface (Tan *et al.* 2000). Finally, other molecules, including integrins, ICAM-1 and PECAM (platelet-endothelial cell adhesion molecule) promote extravasation and migration of the leukocytes, leading them to conformational changes that facilitate diapedesis and the transmigration process through endothelial cell junctions (Muller 2002). Under normal conditions leukocyte extravasation can occur as part of the physiological response against infection. However, in the context of metabolic diseases, it leads to the development of atherosclerosis and CV disease (Čejková *et al.* 2016).

1.2.3 Atheromatous plaque formation

Once in the intima, monocytes transform into macrophages, which can avidly internalize oxidized sdLDL particles by binding to specialized receptors, with the contribution of CRP cytokine (Obradovic *et al.* 2015). In this way, the macrophages transform into foam cells with a high lipid load which they cannot manage, triggering inflammatory mediators that perpetuate local vascular damage and inducing the

proliferation and migration of smooth muscle cells. If this situation persists over the time the foam cells initiate apoptotic pathways to release their lipid-filled contents and antigenic and thrombogenic residues into the necrotic core of the lesion, thereby contributing to the evolution of the atheromatous plaque (Rocha *et al*. 2009). Growing plaques tend to expand outwards and encroach on the lumen. Finally, destabilization of the plaque can lead to the rupture and release of fibrous lipidogenic deposits into the circulation, thus increasing the risk of an atherothrombotic event (Figure 3).
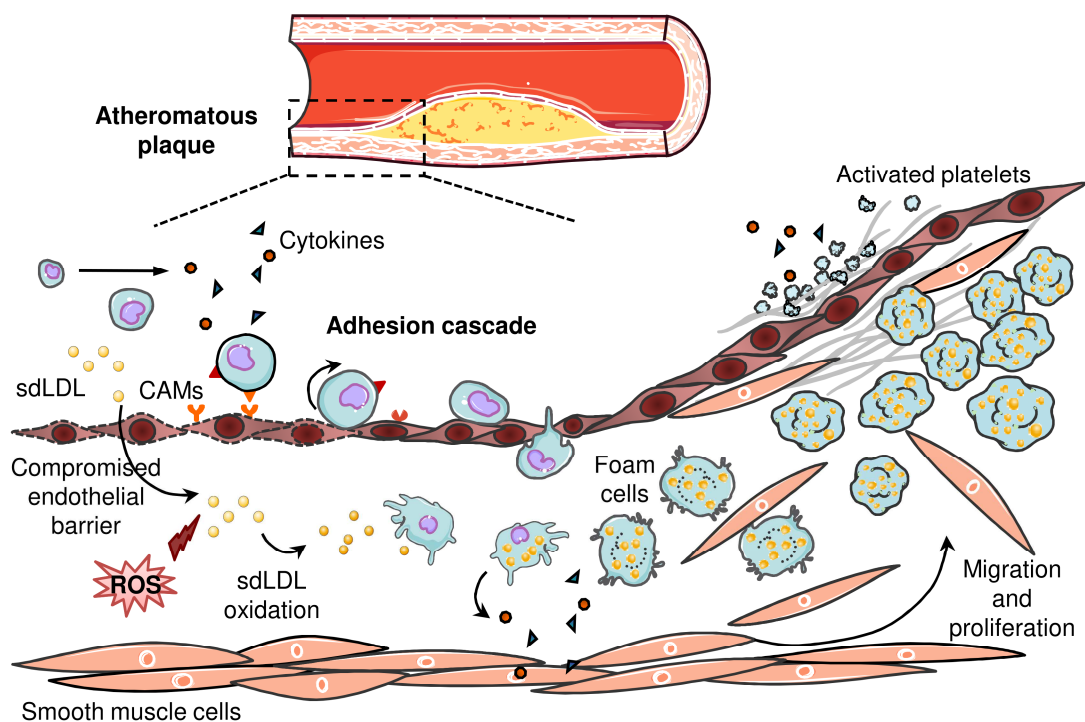

Figure 3. Leukocyte adhesion cascade and formation of the atherosclerotic plaque. A compromised endothelial barrier allows small and dense low-density lipoproteins (sdLDL) to accumulate in the subendothelial region, where they become oxidized in the pro-oxidant environtment, rendering it particularly inmunogenic. Oxidized sdLDL and inflammatory cytokines activate endothelial cells, leading to the expression of cellular adhesion molecules (CAMs), which attracts leukocytes to the endothelium and promotes initiation of the adhesion cascade. After the initial contact and rolling over the endothelium, the leukocytes firmly adhere and begin transmigration to the subendothelial space, where they differentiate into macrophages and avidly phagocyte oxidized sdLDL, thus transforming into foam cells. Lipid-filled foam cells enhance the inflammatory response, leading to proliferation and migration of smooth muscle cells and fibrotic components, thus producing a thickening of the vessel wall. Eventually, activated platelets and fibrotic components also accumulate on the luminal side of the atheroscletoric plaque, thus encroaching even more on the lumen of the vessel wall and increasing the risk of atherothrombotic events.

The abovementioned body of evidence reinforces the inflammatory basis of the atherosclerotic process, exacerbated by the status of IR, oxidative stress, lipid alteration in obesity, and highlights the relevant role of immune cells in the formation of atheromatous plaque. However, further mechanisms and factors involved are not completely determined, and thus represent potential targets to reduce CV risk in obese population.

1.3 Oxidative stress

Oxidative stress is characterized by an imbalance of the redox state, when the production of free radicals – including both ROS and reactive nitrogen species (RNS) – exceed the antioxidant capacity of the system and alter redox homeostasis. In this context, oxidation of macromolecules such as lipids, proteins and DNA is promoted, which alters their functionality, manifesting as oxidative damage to cells and tissues. These deleterious reactions contribute significantly to the aging process and are known to play a relevant role in the pathophysiology of metabolic disorders.

1.3.1 ROS production

Mitochondria

Mitochondria are the organelles responsible for the production of cellular energy from energy substrates in the form of ATP in a process known as oxidative phosphorylation, but they are also considered the main source of ROS in the cell. Initially, metabolic intermediaries originate during oxidative metabolism of carbohydrates, and lipids shed their electrons to the electron transport chain (ETC) embedded in the inner membrane of the mitochondria. Sequential redox reactions follow the pumping of protons towards the intermembrane space by several electron carriers of the ETC (complexes I-IV, coenzyme Q, cytochrome *c*). As a result, the proton gradient increases and the mitochondrial membrane potential ($\Delta\Psi$) is reduced by ATP synthase producing ATP from ADP during oxidative phosphorylation (Dimroth *et al*. 2000). In parallel, the ETC reduces O_2 to H_2O; but some leaking electrons do not traverse the ETC, which leads to a reduction of up to 2 % of the total O_2 to superoxide anion. This highly reactive molecule is rapidly detoxified by superoxide dismutase (SOD) into hydrogen peroxide (H_2O_2), which in turn can be reduced to the hydroxyl radical. Complexes I and III of the ETC are the main producers of superoxide, although there are other mitochondrial proteins involved whose level of contribution is not completely determined (Quinlan *et al*. 2013, Murphy 2009). Finally, mitochondrial ROS (mROS) could diffuse from mitochondria to cytosol, especially H_2O_2, where it reacts with macromolecules and affects redox balance (Turrens 2003).

Other ROS sources

In addition to the mitochondria, oxidants can also be produced in the cytosol, though to a lesser extent. One of the most relevant producers is the enzyme NADPH oxidase (nicotinamide adenine dinucleotide phosphate oxidase) or NOX, a transmembrane multiprotein complex that produces different ROS in response to changes in Ca^{2+} concentration. NOX is expressed in various cell types, such as immune cells, where ROS contribute to the elimination of pathogens after phagocytosis, or in endothelial cells, where it acts as an O_2 sensor and modulator of vascular tone.

Similarly, <u>myeloperoxidase</u> (MPO) is an enzyme that also contributes to the formation of oxidizing agents, especially in polymorphonuclear cells (PMNs) such as neutrophils, where it is stored in granules and released from the cell during degranulation as part of the host immune defence system. It catalyzes the peroxidation of chloride into hypochlorite (HOCl), which acts as a powerful destroyer of phagocytosed pathogens (van der Veen *et al*. 2009). Despite the beneficial role of MPO in immunity, an excessive activity of the enzyme contributes to local and systemic oxidative stress, inflammation and endothelial dysfunction, and has been related with the onset and progression of CV disease with a marked pro-atherogenic effect (van der Veen *et al*. 2009, Brennan *et al*. 2003).

Other sources of cytoplasmic ROS are enzymes such as xanthine oxidase, lipoxygenase and NO synthase, which in turn promotes formation of RNS. Furthermore, special conditions lead to the generation of ROS by some other organelles, including peroxisomes during the long-chain FA oxidation, or ER during oxidative protein folding and/or as downstream effectors of chronic UPR pathways in the context of inflammatory diseases (Holmstrom *et al*. 2014, Cao *et al*. 2014).

1.3.2 Antioxidant systems

Production of free radicals occurs naturally during cell metabolisms, allowing several antioxidant defence systems to cooperate to maintain redox balance and prevent oxidizing damage in healthy tissues. These defences can be distinguished in non-enzymatic molecules, including dietary antioxidants (vitamins, β-carotene, glutathione (GSH), uric acid, transferrin, albumin) and antioxidant enzymes. With regard to antioxidant enzymes,

SOD, catalase and glutathione peroxidase (GPx) are considered the most powerful ROS scavengers (Holmstrom *et al*. 2014). In this sense, GPx – expressed both in mitochondria and cytosol – and catalase – found in peroxisomes and mitochondria – are major H_2O_2 detoxifying enzymes, whereas SOD avidly targets superoxide radicals present in mitochondrial matrix and intermembrane space. Particularly in this organelle, GSH, a thiol-containing molecule with high oxidant buffering capacity, glutaredoxin and thioredoxin systems are the main ROS buffering mechanisms (McMurray *et al*. 2016). These antioxidant systems can be found in several tissues, including liver, brain, muscle, and even in blood (Vincent *et al*. 2006). Some pathologies, including obesity, are associated with a lower presence of antioxidants or inadequate antioxidant response to the rise in free radicals, ending in oxidative stress and detrimental systemic effects.

1.3.3 Redox imbalance in obesity

Low amounts of ROS are essential in some physiological processes, including immune defences, adaptive responses, cell proliferation and differentiation, and even insulin secretion by β-cells (Holmstrom *et al*. 2014, Leloup *et al*. 2009). However, excessive ROS production has deleterious effects on cell function and overall homeostasis. In fact, oxidative stress is considered a unifying mechanism underlying metabolic disturbances in obesity and other metabolic disorders. In this context, several potential contributors to redox imbalance have been described; namely, hyperglycaemia, hyperlipidemia and chronic inflammation which enhances ROS production, but also an impaired antioxidant response (Vincent *et al*. 2006); however, the degree of contribution of each of these factors depends on the metabolic status of the individual.

Hyperglycaemia and hyperlipidemia

The excess input of energetic substrates in obesity –especially from glucose and lipid metabolism – oversupplies the ETC with electrons, leading to an increase of mitochondrial ΔΨ (hyperpolarization) and an enhanced probability of electrons spinning off the ETC carriers to form disproportionate amounts of mROS (Murphy 2009). In parallel, the lipotoxic effect of elevated FFA and derivates directly interrupts correct ETC function and leads to excessive mROS and/or enzymatic ROS generation (Inoguchi *et al*. 2000,

Lambertucci *et al*. 2008). Furthermore, hyperglycaemia-mediated oxidative stress is initiated in the mitochondria and subsequently amplified by several other mechanisms. Mitochondrial superoxide inhibits enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a glycolytic enzyme whose lack of function propagates intracellular hyperglycaemia and activates additional non-mitochondrial ROS-producing pathways. Most publications describe four major mechanisms: (1) glucose leak into the polyol pathway; (2) overactivity of the hexosamine pathway; and activation of ROS-producing enzymes such as NOX or uncoupled eNOS mediated by (3) accumulation of advanced glycation end products (AGE) and (4) protein kinase C (PKC) signalling. These four mechanisms exponentially increase ROS production under high glucose conditions, although lipid excess also seems to be involved in PKC activation. Thus, it is not surprising that hyperglycaemia is considered the main driver of oxidative stress among glucose intolerant obese subjects and T2D individuals, exerting an affect on CV function (Giacco *et al*. 2010).

Impaired antioxidant capacity

Obese patients are even more prone to oxidative damage due to undermined antioxidant capacity. For instance, lower consumption of fruits and vegetables among obese subjects leads to a lack of protective antioxidants such as vitamins, minerals, β-carotene and some phytochemicals, aggravated by higher detoxification demand in obesity (Vincent *et al*. 2006). Furthermore, a defective activity of SOD, catalase and GPx has been associated with obesity (Furukawa *et al*. 2004, Ozata *et al*. 2002). Globally, total antioxidant capacity has been inversely associated with the degree of adiposity, and is specially pronounced with the presence of metabolic syndrome traits (Chrysohoou *et al*. 2007, Tabur *et al*. 2010). On the contrary, strategies to strengthen antioxidant activity, including antioxidant supplementation, physical activity and dietary interventions, seem to partially restore redox balance and protect obese patients from oxidative damage (Vincent *et al*. 2007).

1.3.4 Pathophysiological consequences of oxidative stress in obesity

Excessive ROS levels contribute to the oxidation of biomolecules such as DNA, proteins and lipids, whose accumulation can compromise cell function in a similar way to an accelerated ageing process. Accumulating data over the past years have demonstrated that obesity-induced oxidative stress in humans is associated with the development of related comorbidities (Furukawa *et al*. 2004). Progressive elevation of circulating biomarkers of oxidative stress, including lipid peroxides and carbonyl proteins, occurs in parallel to increased BMI (Keaney *et al*. 2003), and is associated with the onset of CV disturbances, especially atherosclerosis. Moreover, oxidative stress status is especially aggravated in IR-obese subjects and when obesity and diabetes exist concomitantly, which increases the likelihood of CV complications (Giacco *et al*. 2010).

Mitochondrial dysfunction

One of the most relevant targets of pro-oxidants is, precisely, mitochondria. Proximity to the ROS-overproducing ETC makes mitochondrial structures more prone to oxidation and damaging. Mitochondrial DNA and membranes are particularly affected by mROS, which produces impaired mitochondrial function, a key process during ageing but one that also accounts for the development of IR, T2D and associated CV complications (Dos Santos *et al*. 2018, Madsen-Bouterse *et al*. 2010). An excessive supply of nutrients in obesity may excessively hyperpolarize the mitochondrial membrane and overwhelm mitochondrial activity, increasing mROS production in a vicious cycle that can lead to mitochondrial dysfunction and even apoptotic pathways (Liesa *et al*. 2013). In this context, research shows that mitochondrial dysfunction is related to obesity and aggravated by several processes: an inherent redox imbalance status; alterations in mitochondrial dynamics, required for adequate mitochondrial network elongation and function; and disrupted autophagic flux, a crucial mechanism for the recycling of damaged structures, molecules and organelles, which leads to the accumulation of damaged mitochondria and exacerbates the vicious cycle of ROS production (Liesa *et al*. 2013, Sarparanta *et al*. 2017).

Mitochondrial dysfunction is defined as impaired ATP production capacity and lower O_2 consumption rate, but is also characterized by perturbations in Ca^{2+} homeostasis, catabolism and mROS production and has profound effects on global energy metabolism

(de Mello *et al*. 2018). For instance, mitochondrial dysfunction in adipocytes of obese patients has been shown to produce alterations in adipogenesis and lipid metabolism, and was associated with IR and low-grade inflammation (Heinonen *et al*. 2015). In parallel, decreased β-oxidant capacity, uncoupling of ETC and lower ATP content in muscle resulting from mitochondrial dysfunction in obesity has a profound impact on aerobic capacity and global energy expenditure (Liesa *et al*. 2013) that can be reversed by physical exercise. Apart from bioenergetic control, mitochondria play a key role in apoptosis, and extended mitochondrial dysfunction may lead to activation of programmed cell death, which usually involves cytochrome *c* release and caspase pathways (de Mello *et al*. 2018).

Accumulating data suggest that mitochondrial dysfunction impairs endothelial function/viability and induces vascular smooth muscle cell proliferation and/or apoptosis, which precedes the development of atherosclerosis and other CV alterations such as hypertension (Dos Santos *et al*. 2018). Impaired mitochondrial oxidative capacity has also been involved in cardiac dysfunction associated with obesity (Boudina *et al*. 2005). Although the underlying mechanisms of mitochondrial-mediated diseases are uncertain, ROS overproduction seems to be primordial in certain cases. Indeed, targeting mROS with specialized scavengers such as MitoQ or SS31 has proved to have antioxidant and cardiometabolic protective effects on obese and T2D populations (Apostolova *et al*. 2014).

Role of oxidative stress in IR and adipose tissue dysfunction

Mitochondrial damage plays a key role in IR and the further development of T2D and related CV complications though increased generation of pro-oxidants (Dos Santos *et al*. 2018, Holmstrom *et al*. 2012). Paradoxically, whereas physiological ROS levels may play a relevant role in adequate insulin release and sensitivity (Loh *et al*. 2009), excessive oxidative stress in metabolic diseases disturbs insulin transduction signalling, especially in liver, muscle and adipose tissue. Inhibition of insulin receptors and IRS1 mediated by activation of NFκB/JNK pathways (notably regulated by ROS) or decreased GLUT4 (glucose transporter type 4) expression in muscle is the best characterized mediators of ROS-induced IR.

Furthermore, oxidative stress also plays a key role in adipose tissue dysfunction. During adipose tissue hypertrophy NOX4 hyperactivation greatly contributes to excess ROS production, which dysregulates the profile of expression of adipocytes and enhances the

recruitment and activation of immune cells (Han *et al.* 2012). These activated macrophages amplify the pro-inflammatory and pro-oxidant response in part through activation of NOX2 (Coats *et al.* 2017), and they crosstalk back to adipocytes in a vicious cycle (Jankovic *et al.* 2015). In addition, the NLRP3 (NLR family pyrin domain containing 3) inflammasome complex is assembled in both cell types, integrating ROS and inflammatory signalling and exerting a profound impact on adipose tissue function and insulin sensitivity (Jankovic *et al.* 2015). In contrast, inhibition of NOX activity in adipocytes improves insulin sensitivity and the inflammatory profile (Furukawa *et al.* 2004, Den Hartigh *et al.* 2017). As a whole, accumulating data suggest that a vicious cycle of cytokine/ROS production by macrophages and adipocytes is involved in adipose tissue dysfunction in obesity leading to systemic deleterious effects such as IR, low-grade inflammation and oxidative damage, which may be affecting vascular function (Jankovic *et al.* 2015)

1.3.5 Oxidative stress and atherosclerosis in obesity

As stated above, oxidative stress, hyperglicaemia and systemic inflammation are considered major drivers of endothelial dysfunction in metabolic disorders such as obesity and diabetes. In this context, endothelial cells respond by overproduction of ROS, derived in part from NOX hyperactivation (Inoguchi *et al.* 2000). In addition, uncoupling of eNOS activity promotes a shift from NO formation towards superoxide production in the endothelium, contributing to an increased ROS pool while reducing NO bioavailability. Excessive superoxide radicals rapidly react with NO to form RNS such as peroxynitrite, further reducing NO availability, which is especially relevant in the regulation of vascular function. Locally, ROS and inflammatory molecules from hypertrophied perivascular adipose tissue also results in impaired vasodilatation of small arteries in obese subjects and endothelium activation. At an intracellular level, cytokines and ROS signalling induce NFκB-mediated CAM expression and further inflammatory response in endothelial cells, thus promoting the accumulation of leukocytes in the vessel wall, a crucial step in the onset of atherosclerotic processes.

In line with this, chronically activated immune cells in obesity also display a pro-oxidant phenotype, with elevated NOX and MPO activity, which can contribute to a rise in oxidized serum macromolecules and leukocyte-induced vascular injury (Nijhuis *et al.* 2009, Olza *et al.* 2012). In fact, MPO serum levels are reported to be a powerful predictor of CV

events, later mortality and progression of carotid atheromatous plaque, with several mechanisms seeming to mediate this association. For instance, MPO-derived HOCl directly oxidizes LDL particles and enhances their affinity for macrophages and the endothelium, thereby leading to the development of vascular inflammation (Lau *et al*. 2006) and formation of foam cells in the subendothelial space. In addition, MPO modifies apolipoprotein A1 in HDLc, impairing HDLc-mediated cholesterol efflux and reducing NO availability, further contributing to endothelial dysfunction (Brennan *et al*. 2003). Hence, data from human studies confirm the potential role of leukocyte-mediated oxidative stress, and particularly MPO activity in endothelial dysfunction and CV risk among the obese population. However, whether this leukocyte-induced oxidative stress could be interfering in the interaction between immune cells and the endothelium is a topic that has been explored little, though it is promising insofar as the extent to which it may prevent major CV complications in obesity.

1.3.6 Oxidative stress, inflammation and atherosclerosis in chronic periodontitis

As stated at the outset, obesity encompasses several risk factors for the development of atherosclerosis and may be a systemic condition whose underlying inflammatory state promotes periodontitis onset and progression. Together with systemic inflammation, oxidative stress is another relevant hallmark of periodontal disease produced as a result of the interaction of the host immune response and periodontal microbes.

In the pathophysiology of chronic periodontitis bacteria present in the growing subgingival plaque release immunogenic products such as lipopolysaccharide (LPS), leading to the release of cytokines from surrounding host cells. The inflammatory response promotes formation of the periodontal pocket between the gingiva and the teeth, with accumulation of crevicular fluid (Marsh *et al*. 2017). Subsequently, inflammatory and bacterial immunogenic factors are spilled into the circulation from the periodontal pocket, priming a systemic inflammatory immune response characteristic of periodontal disease (Williams *et al*. 2008). In fact, increased levels of inflammatory cytokines including IL1β (interleukin 1 β), TNFα and CRP have been associated with periodontal disease (Loos 2005). In response to periodontal inflammation, PMNs – especially neutrophils – are

activated and recruited to the crevicular pocket, where they produce high levels of ROS during the oxidative burst to facilitate the killing and destruction of microbes (Chapple *et al.* 2007). In this sense, raised levels of MPO have been found in gingival crevicular fluid in several periodontal diseases, including chronic periodontitis, and have been associated with clinical measures of the pathology (Buchmann *et al.* 2002, Wei *et al.* 2004). To a lesser extent, other cell types have been reported to locally produce ROS, including fibroblast and epithelial cells which are in front-line contact with bacteria and react to them by overproducing cytokines and oxidizing molecules (Chamulitrat *et al.* 2004).

In addition, hyperreactiveness of neutrophils in chronic periodontitis is accompanied by undermined antioxidant capacity leading to imbalanced redox status (Chapple *et al.* 2007, Buchmann *et al.* 2002, Wei *et al.* 2004). In line with this, elevated oxidative stress markers have been found in the gingiva, crevicular fluid and saliva, but also systemically, as increased lipid peroxidation and protein carbonyls levels were detected in serum from patients with chronic periodontitis (Wang *et al.* 2017). Despite activation of inflammatory and ROS-producing responses by immune cells being key mechanisms in the first barrier against an infectious challenge, in chronic periodontitis overproduction of ROS and cytokines, presence of LPS and even bacterial invasion (bacteremia) from the periodontal pocket into the circulation, resulting in systemic response (Williams *et al.* 2008). This state has several detrimental effects: locally, oxidative stress and inflammation in the pocket promote tissue damage and progressive periodontum destruction, whereas at a systemic level chronic periodontal disease leads to endothelial dysfunction promoting the development of atherosclerosis.

Several studies have reported compromised endothelial function in subjects with periodontitis (Gurav 2014). Immunogenic and inflammatory mediators such as TNFα and LPS can promote endothelial cell activation and vascular permeabilization, leading to expression of adherent molecules and decreased NO production (Gurav 2014). In addition, other studies have highlighted a more atherogenic lipid profile (Rufail *et al.* 2005, Ramirez-Tortosa *et al.* 2010) associated with markers of endothelial dysfunction and CV risk (Ramirez-Tortosa *et al.* 2010) in patients with chronic periodontitis, with one reporting a greater peroxidation of lipids in the descending aorta of a murine model of periodontitis (Ekuni *et al.* 2009a). In contrast, periodontal treatment has been proven to have beneficial

effects, not only on periodontal parameters, but also by alleviating systemic inflammatory and oxidative markers and exerting a protective role against endothelial dysfunction (Chapple *et al.* 2007, Higashi *et al*. 2009). In addition, antioxidant therapy attenuates the progression of atherosclerosis in induced periodontitis in rats (Ekuni *et al*. 2009b).

This accumulated evidence points to a potential role of chronic periodontitis in the early stages of the atherosclerotic process through a mechanism involving oxidative stress and inflammation, similarly to obesity which in turn increases the susceptibility to develop periodontal disease. However, the relationship between chronic periodontitis, obesity and atherosclerosis is still an emerging area of study that requires joint efforts from multidisciplinary teams working together to refine the disease's pathobiology.

1.4 Endoplasmic reticulum stress

1.4.1 Endoplasmic reticulum

The ER is a vast membranous organelle responsible for the synthesis, folding and trafficking of proteins, especially of those of the secretory pathway. The ER lumen provides optimum conditions for protein folding and post-translational maturation. For instance, formation of disulfide bonds is enhanced in the luminal oxidizing environment, the presence of chaperones in the lumen contributes to protein folding, and quality control of newly synthesized peptides is provided before secretion by several ER regulatory proteins. The ER also participates in lipid synthesis and trafficking, and is the primary storage site for Ca^{2+}, which is essential for the activity of Ca^{2+}-dependent chaperones in the ER lumen and for intracellular signaling (Xu *et al*. 2005).

1.4.2 Unfolded protein response

Several pathological mechanisms can challenge ER function resulting in the accumulation of misfolded proteins in the lumen, an adverse condition known as ER stress, which presents a threat to overall cell homeostasis. In response to this, the UPR is triggered, acting as an adaptive pathway designed to recover normal ER function. The UPR comprises three canonical branches initiated by three ER-transmembrane proteins: double-stranded RNA-activated protein kinase-like kinase (PERK), activating transcription factor 6 (ATF6) and inositol requiring enzyme 1 α (IRE1α). In addition, the chaperone GRP78 (78-kDa glucose-regulated protein) binds the three UPR sensors by their luminal domain and keeps them inactive. Accumulation of misfolded proteins and aggregates increases chaperone-activity demand, thus leading to the recruitment of GRP78 away from UPR leaders and stimulating their activation in different ways: PERK and IRE1α kinase activation results from autophosphorylation and dimerization of their subunits, whereas ATF6 migrates to the Golgi, where the small subunit ATF6 (p50) is cleaved by specific proteases (Hotamisligil 2010)

Subsequently, PERK phosphorylates and inactivates eIF2α (eukaryotic initiation factor 2α), thus attenuating global protein translation for a few hours in order to reduce misfolded-protein cargo in the ER lumen. Activation of PERK also promotes the expression of ATF4 (activating transcription factor 4) and NRF2 (nuclear factor (erythroid-derived 2)-

like 2). Furthermore, two different enzymatic activities have been described for IRE1α: on the one hand, IRE1α acts as an endoribonuclease, splicing the X box protein-1 (XBP1) mRNA and rendering it competent for translation, while also phosphorylating several targets (e.g., IKK and JNK) by means of kinase activity. Finally, the three resulting transcriptional factors – ATF4, XBP1 and ATF6 (p50) – migrate into the nucleus, where they regulate the transcription of a constellation of genes downstream of the UPR pathway with the primary aim of alleviating ER stress and reestablishing organelle homeostasis (Xu *et al.* 2005).

Among the most relevant UPR-adaptive responses, ATF4 and NRF2 induce the expression of an array of antioxidant response elements to counterbalance ER and cellular ROS excess. Moreover, the ER-associated protein degradation (ERAD) pathway and autophagic machinery are promoted by XBP1 and ATF6, and the IRE1α-JNK axis, respectively, to facilitate protein clearance (Ogata *et al.* 2006). In addition, the three UPR branches trigger the expression of chaperones such as GRP78 and quality-control proteins to enhance protein folding and ensure proper trafficking. However, failure of these coordinately adaptive actions or chronicity of the stress can lead UPR mediators to express pro-apoptotic factors such as CHOP (CCAAT/enhancer binding protein [C/EBP] homologous protein), caspases or by modulating B-cell lymphoma 2 (Bcl-2) family proteins, triggering programmed cell death (Xu *et al.* 2005, Hotamisligil 2010) (Figure 4).

1.4.3 Activation of the unfolded protein response in obesity

The ER has been described as a systemic nutrient sensor in several tissues; therefore, metabolic overload in obesity can induce ER stress and accumulation of misfolded proteins. The organelle faces different metabolic challenges in the context of obesity according to cell type, with secretory cells such as adipocytes, hepatocytes and β-cells being particularly affected due to the increased demand in protein synthesis (Hotamisligil 2010). Within adipocytes, hypertrophy and increased synthesis of adipokines can represent a stressful situation for the ER. In the liver, altered lipid metabolism, enhanced gluconeogenesis and protein synthesis can trigger ER stress (Fu *et al.* 2011). A high demand of insulin in response to developing IR can cause the ER to be overwhelmed in β-cells. However, higher protein synthesis cannot totally explain obesity-induced ER

stress. Although upstream regulation of the UPR in obesity is not completely understood, a combination of *in vitro* findings, animal experimental data and human studies have painted a picture of a role of FA, inflammatory cytokines, ROS and glucose in the development of ER stress (Cnop *et al.* 2012).
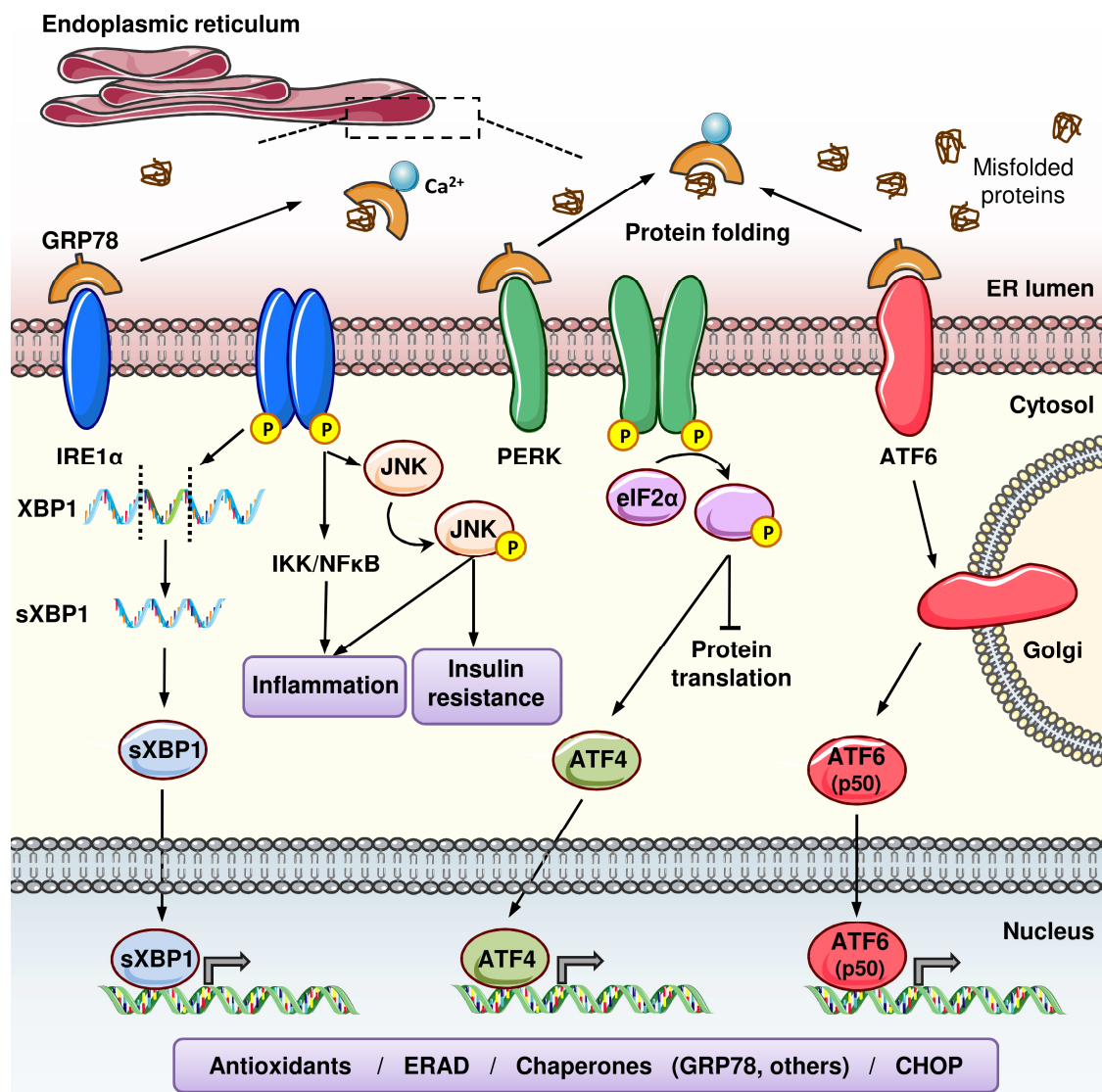

Figure 4. Activation of the unfolded protein response (UPR). Accumulation of misfolded proteins in the endoplasmic reticulum (ER) lumen leads to disassociation of GRP78 (78-kDa glucose-regulated protein) from the three leaders of the UPR: IRE1α (inositol requiring enzyme 1 α), PERK (double-stranded RNA-activated protein kinase-like kinase) and ATF6 (activating transcription factor 6) in order to address Ca^{2+}-mediated protein folding. This leads to activation of three parallel pathways ending in the translocation of the three resulting transcriptional factors into the nucleus, where they promote the expression of gens related to pro-survival responses (antioxidants, ERAD (endoplasmic reticulum-associated protein degradation), chaperones). However, chronic activation of UPR triggers the activation of inflammatory mediators such as JNK (c-Jun N-terminal kinase) or NFκB (nuclear factor κB) and the expression of proapoptotic factors such as CHOP (CCAAT/enhancer binding protein [C/EBP] homologous protein).

Insulin also seems to activate the UPR, since induced hyperinsulinemia increases GRP78, ATF4, ATF6 and XBP1 expression in adipose tissue from normoglycaemic humans (Boden *et al*. 2008). That said, insulin is unlikely to be the main stimulus of ER stress in IR individuals.

The mediators described above seem to provoke significant alterations in ER-luminal conditions, including Ca^{2+} depletion and redox imbalance, and severely interfere with ER-folding capacity and functionality (Mekahli *et al*. 2011). Deficiencies in proteasome function and autophagy in obesity may also accentuate ER stress due to an inability to process undesirable proteins and organelles, and may contribute to IR (Otoda *et al*. 2013, Yang *et al*. 2010). However, there are still important gaps in the knowledge of how specific UPR leaders are regulated by different ER stressors, with evidence suggesting this may occur differently depending on the tissue and the cluster of factors competing in a precise moment, due to the highly dynamic nature of the UPR.

1.4.4 Role in inflammation and insulin resistance

At an intracellular level, the ER integrates several intrinsic and extrinsic signals to align cell function with metabolic demand. UPR pathways intersect with a constellation of molecular processes, such as inflammation, insulin signalling, Ca^{2+} homeostasis, oxidative stress and mitochondrial function, all of which are themselves mechanisms closely related with metabolic disturbances. For this reason, ER stress is considered to play a central role in metabolic diseases, including T2D and obesity.

The interplay between UPR and inflammatory signals is thought to be the major contribution of ER stress to the physiopathology of obesity, along with the impairment of insulin signalling. It is known that UPR and inflammatory pathways converge in several stages in a bidirectional way. For instance, ATF6 and kinase activities of IRE1α and PERK are able to activate the IKK-NFκB pathway, implicated in the transcription of several inflammatory mediators and with the development of IR. Conversely, resulting inflammatory cytokines may affect ER function in some cell types, thus promoting ER stress even further. On the other hand, the activation of JNK via IRE1α kinase implies an additional ER-induced inflammatory pathway, and is also involved in the interruption of insulin signalling by means of IRS1 phosphorylation (Hotamisligil 2010) (Figure 4).

Moreover, IRE1α also triggers NLRP3 inflammasome assembly via thioredoxin-interacting protein (TXNIP) activation and ER-mitochondria crosstalk (Bronner *et al.* 2015).

The role of ER stress in inflammatory and insulin signalling in obesity and T2D has been largely described in adipose, pancreatic and hepatic tissues. Activation of UPR has been associated with increased expression of inflammatory cytokines and disturbed insulin signalling in adipocytes and hepatocytes (Boden *et al.* 2008, Kawasaki *et al.* 2012, Ozcan *et al.* 2004, Nakatani *et al.* 2005), and has also been implicated in β-cell apoptosis and necrosis of pancreatic islets (Laybutt *et al.* 2007), which further contributes to systemic IR and progression towards T2D. Treatments with chemical chaperones such as 4-phenylbutyric acid (PBA) and tauroursodeoxycholic acid (TUDCA) have been tested extensively in these tissues, demonstrating the ability to reduce UPR activation and to thus protect against IR development and inflammation (Engin *et al.* 2010). These findings largely explain why ER stress is considered a major contributor to IR in obesity and to the further development of T2D.

Sirtuin 1

An emerging body of evidence supports a connection between ER stress and Sirtuin 1 (SIRT1), a NAD$^+$-dependent protein deacetylase highly sensible to variations in nutrient availability that has been implicated in the regulation of energy homeostasis and systemic inflammatory responses. The ability of nuclear SIRT1 to target NFκB transcription factor to induce its proteasome-mediated degradation exerts a protective anti-inflammatory effect by reducing the expression of NFκB inflammatory effectors (Yang *et al.* 2012). However, in obesity, SIRT1 levels are diminished in several tissues, including adipocytes and immune cells, and this has been related with the presence of metabolic disturbances, including inflammation and IR, thus highlighting the role of SIRT1 as a relevant mediator linking metabolic homeostasis and inflammation (Vachharajani *et al.* 2016). Once again, the relationship between SIRT1 and ER stress seems to be bidirectional, since some reports have shown that SIRT1 negatively regulates UPR activation and ER-stress-dependent inflammatory responses, while others described a potential role of UPR in modulating SIRT1 expression (Koga *et al.* 2015). As the largest amount of data has been collected in cellular or animal models, further studies in humans are required to throw

light on the complex mechanisms of regulation of SIRT1 and ER stress in the setting of metabolic disorders.

Evidence of ER stress in atherosclerosis

Besides classic secretory cells, accumulating evidence suggests ER stress in the context of metabolic diseases is present in many other cell types, including neurons, leukocytes, endothelial and even skeletal muscle cells, which results in additional metabolic disturbances. For instance, ER stress also seems to contribute to the atherosclerotic process. In an *in vitro* model of advanced atherosclerosis UPR-CHOP was required for cholesterol-load-mediated expression of inflammatory cytokines such as IL6, TNFα and IL8 via IKK/NFκB in macrophages (Li *et al*. 2005). Increased markers of ER stress were also found in macrophages and smooth muscle cells isolated from atherosclerotic plaques of patients with acute coronary syndrome (Myoishi *et al*. 2007). In addition, our group has previously described a correlation between up-regulated UPR markers in leukocytes from T2D patients and enhanced leukocyte-endothelium cell interactions (Rovira-Llopis *et al*. 2014), highlighting the potential role of ER in the early stages of the atherosclerotic process.

1.4.5 Contribution to oxidative stress and mitochondrial dysfunction

The role of ER stress in metabolic diseases goes beyond inflammation and IR. Oxidative stress also results from an incorrect ER function, either because of inner ROS production or by interfering with mitochondrial function.

Oxidative protein-folding in the ER as a potential source of ROS

The ER lumen is a unique oxidizing environment where formation of disulfide bonds is enhanced during the oxidative protein-folding process. To prevent and correct illegitimate disulfide bonds, resident protein disulfide isomerases (PDI), endoplasmic reticulum oxidoreductin 1 (Ero1) and GSH cooperate. This oxidative folding machinery generates large amounts of ROS and depletes the GSH pool, thus contributing to oxidative stress. Indeed, a high percentage of the total ROS generated in the cell is estimated to arise from this process, which may be further exacerbated in obesity due to an elevated ER

folding activity. Furthermore, CHOP factor derived from UPR activation promotes greater ROS production via Ero1. Subsequently, a hyperoxidizing environment interrupts PDI function, leading to accumulation of misfolded proteins and ER stress (Malhotra *et al.* 2007).

Calcium depletion during ER

Besides optimum redox balance, the maintenance of high levels of Ca^{2+} in the ER lumen is critical for protein-folding mediated by chaperones. These levels are highly regulated by two complexes located in the ER membrane: the sarco/endoplasmic reticulum $Ca2^+$-ATPase (SERCA) pumps, which promote Ca^{2+} entry from the cytosol, and the IP_3R (inositol triphosphate receptors), which channels Ca^{2+} into the cytosol or the mitochondria. In obesity, several stimuli (e.g., FFA intermediates) can disrupt SERCA activity, thus impairing Ca^{2+} refilling (Fu *et al.* 2011). In addition, a rise in luminal ROS can activate IP_3R, which increases Ca^{2+} depletion. The consequent drop in ER-Ca^{2+} content further deteriorates protein-folding capacity and enhances ROS production in a positive feedback via oxidative folding machinery. Furthermore it also disturbs mitochondrial function and Ca^{2+} distribution within the cell. The blocking of RE-Ca^{2+} leakage alleviates ER stress and restores cell homeostasis in numerous experimental models, which highlights the crucial role of Ca^{2+} distribution.

ER and mitochondrial dysfunction

Mitochondrial and ER are closely related functionally, and an emerging body of evidence demonstrates the converging role of ER stress and mitochondrial dysfunction in the course of metabolic diseases (Arruda *et al.* 2015). In this context, ER stress has been demonstrated to promote mROS production by further increasing oxidative stress and mitochondrial dysfunction, and this mROS also feedbacks to ER stress dysfunction.

In regard to this relationship, it is worthy mentioning the existence of mitochondria-associated ER membrane (MAM), physical contacts enriched in IP_3R channels, specialized metabolite exchangers and other regulatory proteins that maintain an optimal contact between the two organelles and control Ca^{2+} transporters. Under physiological conditions, mitochondrial-ER Ca^{2+} exchange is crucial for proper organelle

function, including ATP synthesis in the mitochondria (Mekahli *et al*. 2011). However, during ER stress, excessive amounts of Ca^{2+} are pumped into the mitochondrial matrix, where Ca^{2+} overload accelerates mROS production in the ETC by several mechanisms: tricarboxylic acid cycle activity is promoted, thus increasing the flow of energetic mediators into the ETC; initially the rise in Ca^{2+} levels hyperpolarizes the mitochondrial inner membrane, which enhances ETC activity; and finally prolonged Ca^{2+} entry ends in membrane depolarization, which induces a leak of cytochrome *c* via the permeability transition pore, thus increasing ROS production in the ETC and even leading to cell death (Malhotra *et al*. 2007). In what is a vicious cycle, mROS promotes a larger Ca^{2+} load by sensitizing Ca^{2+}-release channels at the MAM and hyperoxidizing the ER lumen, thus altering protein-folding and perpetuating ER stress (Malhotra *et al*. 2007, Arruda *et al*. 2015). Finally, unresolved ER stress and prolonged Ca^{2+} overload into the mitochondria or the cytosol present a threat to cell viability. In this sense, mitochondria-dependent and - independent apoptotic programs involving caspase cascades are triggered (Mekahli *et al*. 2011).

In summary, mounting evidence suggests ER dysfunction is integrated in several vicious cycles of inflammation, oxidative stress and mitochondrial dysfunction, and plays a key role in the pathophysiology of obesity and its metabolic alterations. However, further detailed studies, especially in humans, are vital to increase our understanding of the way and the extent to which ER stress and related pathways are involved in these metabolic disturbances, which may provide the basis for the development of new therapeutic strategies.

2. HYPOTHESIS AND OBJECTIVES

Obesity is a complex disease involving a huge range of metabolic alterations, including chronic low-grade inflammation, IR, oxidative stress and altered lipid profile, which converge to promote the development of endothelial dysfunction and CV disease. The persistence of a residual risk, even in metabolically healthy or medically controlled obese subjects, suggests that factors other than those traditionally associated with CV risk exert an influence in a subclinical way. In this context, immune cells, which become activated in obesity, play a relevant role, since their attraction to the vascular wall is a key process in the formation of the atheromatous plaque. However, little is known about the intracellular mechanisms in leukocytes that may underlie this effect. Leukocytes are more capable of producing ROS for immune defence when activated; hence, it is likely that oxidative stress within leukocytes alters the dynamics between immune cells and the vascular endothelium in the context of obesity, where increased adiposity can aggravate the process. Chronic periodontitis shares several pathological mechanisms with obesity, including a hyperactivated immune system and oxidative stress. Therefore, it is feasible that the concomitant presence of obesity and periodontitis accelerates redox imbalance in leukocytes, thus leading to increased CV risk.

The main factor responsible for oxidative stress and ROS production in the cell are the mitochondria, whose functionality is altered under metabolic overload in obesity, in parallel with the development of ER stress, all of which leads to metabolic disturbances. Targeting excess fat accumulation exerts benefits by protecting against cardiometabolic alterations, although the precise mechanisms implicated are poorly understood. In this sense, weight loss interventional studies in humans that further explore the modulation of intracellular stress responses are crucial for the discovery of new therapeutic targets that mimic the benefits of weight loss. Finally, the use of inositols such us pinitol has been demonstrated to enhance insulin sensitivity and improve inflammatory profile in the context of metabolic disease, although the molecular targets of pinitol are largely unknown. In this context, we believe there is a potential role of pinitol in the modulation of ER stress and SIRT1, two pathways whose altered profile of expression is proven to contribute to increased inflammation in obesity.

On the basis of the above knowledge, the following <u>objectives</u> were proposed for the present PhD research project:

1. To evaluate the relationship between mitochondrial function and ROS production in leukocytes and the interaction of leukocytes with the endothelium according to the degree of obesity.

2. To determine whether the presence and degree of severity of chronic periodontitis alters the dynamics between leukocytes and vascular endothelial cells by a mechanism involving oxidative stress in human obesity.

3. To assess whether dietary weight-loss intervention improves redox balance and subclinical atherosclerotic markers in an obese population.

4. To investigate how ER stress, mitochondrial dysfunction and inflammatory pathways in leukocytes of obese patients can be modulated by weight loss.

5. To explore the potential protective role of pinitol as a molecular chaperone capable of ameliorating chronic ER stress and inflammatory signalling in adipose tissue and leukocytes of patients with obesity.

3. RESULTS AND DISCUSSION

The alarming rise in the prevalence of obesity worldwide and the associated heavy burden of the disease has generated a growing demand for strategies for slowing down the epidemic and/or mitigating the damaging effects of obesity on health. A massive commitment by social forces, including the international scientific community, is required not only to develop preventive strategies but to further increase understanding of the pathological events and mechanisms involved, from both basic and clinical points of view.

Indeed, the main aim of this thesis is to delve into the underlying mechanisms of obesity, especially those related to the increased risk of developing atherosclerosis, with a special focus on leukocyte activation and margination in the vessel wall and the role of certain mechanisms, including inflammation, oxidative stress, mitochondrial dysfunction and ER stress. We also address whether several therapeutic approaches can modulate these pathways and protect against metabolic disturbances.

We recruited several cohorts of middle-aged overweight and obese patients, and normoweight volunteers – all classified according to their BMI – that were attending the Department of Endocrinology and Nutrition and/or the Department of Stomatology of the University Hospital Doctor Peset (Valencia, Spain). In the first cross-sectional study non-diabetic subjects were categorized as non-obese (< 30 kg/m^2), obese grade I-II (30-40 kg/m^2) and morbid obese (> 40 kg/m^2), and measurements of WC revealed increasing visceral adiposity and blood pressure as the degree of obesity incremented. As is common among obese populations, some of the subjects in our studies presented associated metabolic comorbidities, including hypertension, hyperlipidemia and T2D (except for the cross-sectional studies, in which patients with T2D diagnosed according to ADA criteria were excluded (American Diabetes Association 2016). On average, 10 % and 19 % of the non-diabetic obese subjects were estimated to be on lipid-lowering or antihypertensive medication, respectively. However, these percentages rose to 27 % and 30 % in the cohort of the weight-loss interventional study, probably due to the presence of patients with T2D. In fact, the incidence of metabolic syndrome traits tends to be higher among T2D-obese patients when compared to non-diabetic obese subjects (Anari *et al.* 2017). In parallel, obese patients clearly presented impaired insulin sensitivity, as revealed by HOMA-IR levels above 2.5 among the different cohorts, along with an increase in fasting glucose, insulin and glycated haemoglobin (A1c) as rates of BMI rose. Nevertheless, fasting glucose and A1c remained within the normal range, thus indicating a general preservation of

glycaemic control. Regarding lipid profile, levels of LDLc were within reference values in all the cohorts, with no significant between-group differences, probably due to the use of lipid-lowering medication. In contrast, HDLc was characteristically reduced and TG levels rose as the degree of obesity increased, with typical features of atherogenic dyslipidaemia being observed.

These metabolic comorbidities – hypertension, dyslipidemia and T2D – are closely related with the development of endothelial dysfunction and atherosclerosis, although even obese patients with no clinical signs of metabolic impairment are at a higher risk of subclinical atherosclerosis (Kim *et al*. 2017). Endothelial dysfunction is one of the early events in the development of atherosclerosis and CV disease, and it has been documented that obesity *per se* causes endothelial dysfunction in several vascular beds (Grassi *et al*. 2010), a situation that worsens with higher BMI (van der Heijden *et al*. 2017). Similarly, chronic periodontitis, a highly prevalent pathology among obese patients, has emerged as a putative risk factor for the development of endothelial dysfunction and atherosclerosis, and shares with obesity many traits that lead to CV disease.

Systemic low-grade inflammation is a relevant hallmark of obesity that also underlies the atherosclerotic process resulting mainly from adipose tissue dysfunction, which overstimulate immune cells and impair vascular function. For instance, IL6 is a cytokine with pleiotropic effects produced by several tissues, especially expanding VAT, which drives large amounts of this cytokine directly to the liver via portal circulation, thereby increasing the production of CRP, an acute phase reactant widely interpreted as a predictor of CV disease risk (Ridker 2007) and promoter of a pro-atherosclerotic phenotype in the vasculature (Devaraj *et al*. 2003, Ikeda *et al*. 2003). Likewise, TNFα is overproduced by perivascular adipose tissue and immune cells and has been associated not only with IR, but also with impaired endothelial function, especially through reduced NO availability, leading to an increased risk of coronary events (Virdis *et al*. 2019, Ridker *et al*. 2000). In line with previous findings (Park *et al*. 2005), we confirmed a pro-inflammatory state in our obese patients, as revealed by increasing circulating levels of CRP, IL6 and TNFα in parallel with growing adiposity, which may alter vascular function. In this sense, it has been proposed that this inflammatory state also causes alterations in the host immune response, which increases susceptibility to bacterial infection, thus emerging

as a potential mechanism linking obesity and chronic periodontitis. Conversely, once periodontitis has developed, it also promotes systemic inflammation, thereby contributing to CV risk (D'Aiuto *et al*. 2004). We actually observed higher leukocyte count in the presence of chronic periodontitis, which suggests hyperactivation of the immune system. Subsequently, we confirmed that chronic periodontitis further exacerbates the inflammatory response in patients with obesity, as revealed by progressive increases in circulating levels of TNFα, CRP and RBP4 as periodontal disease became more severe. RBP4 is an adipokine involved in systemic IR and also in vascular oxidative damage (Yang *et al*. 2005, Wang *et al*. 2015) which has been recently associated with periodontal disease (Martinez-Herrera *et al*. 2018), reinforcing the putative link between chronic periodontitis and endothelial dysfunction.

In the presence of systemic inflammation, endothelial cells, leukocytes and platelets become activated and the expression of CAMs, including selectins, ICAM-1 and VCAM-1, is promoted. This enhances the attraction of leukocytes to the endothelium, the first step of the transmigration process in the early stages of atherogenesis. Our findings show that obesity progressively increases circulating levels of ICAM-1 and P-selectin, in accordance with previous studies showing them to be markers of endothelial activation and predictors of CV disease in obesity and T2D (Leinonen *et al*. 2003, Bielinski *et al*. 2015). To explore whether interactions between leukocytes and the endothelium are affected by escalating rates of obesity, we used a flow-chamber *in vitro* model in which a suspension of patient's leukocytes is drawn across a monolayer of human endothelial cells under conditions similar to *in vivo* blood flow. We observed a progressive reduction of leukocyte rolling velocity and enhanced rolling flux, which led to a slowing down of leukocyte flux and enhanced tethering and rolling along the vascular endothelium, in which P-selectin is a key mediator. Firm adhesion was also promoted as the grade of obesity increased, in which ICAM-1 plays a relevant role. Further correlations confirmed the association between parameters of adiposity, inflammation and CAMs with leukocyte-endothelial cell markers, with BMI proving to be a major predictor of rolling flux, which strengthens the notion that obesity impairs endothelial function and promotes interactions between the leukocytes and the vasculature. In the light of these findings and the many established links between chronic periodontitis, obesity and CV disease, we evaluated the effect of chronic periodontitis on leukocyte-endothelial cell interactions. We have observed that the

presence of this periodontal alteration promotes leukocytes rolling flux and adhesion, and that these parameters correlate, not only with clinical periodontal markers, but also with TNFα and RBP4, thus suggesting a mechanistic association between chronic periodontitis, inflammation and atherogenesis, in line with that proposed in the context of obesity.

Besides inflammatory cytokines, obesity-associated IR is a major contributor to endothelial dysfunction – as described first by Steinberg *et al.*(Steinberg *et al.* 1996) – in part through a mechanism involving oxidative stress. In this sense, hyperglycaemia seems to stimulate ROS production by endothelial cells, thus contributing to impaired vasodilatation and permeability (Brownlee 2005). On the other hand, recent reports by our group suggest that IR also triggers ROS production in leukocytes, thus promoting contacts with the vascular wall. In relation to this, the leukocytes of T2D patients showed elevated ROS production and a more pronounced adherence phenotype, especially among those with poorly glycaemic control (Rovira-Llopis *et al.* 2014). Similarly, further IR in woman with PCOS was associated with increased ROS production in leukocytes and elevated markers of endothelial dysfunction, which enhanced leukocyte-endothelium cell interactions (Bañuls *et al.* 2017). In accordance with the results of these previous studies, our present findings show rising production of ROS in leukocytes from non-diabetic obese patients that peaked in those with higher degrees of obesity and impaired insulin sensitivity. Besides BMI, markers of IR and total superoxide correlated with leukocyte adhesion parameters and showed themselves to be independent predictors in the multivariable regression model.

Given the extent of the contribution of mitochondrial dysfunction to redox imbalance, it is likely that the increased superoxide detected is linked with impaired mitochondrial activity. Mitochondrial dysfunction in obesity is a maladaptive physiological response to excess nutrient supply, which increases electron input into the ETC, thus leading to increased ROS production and imbalance of proton flux. In a previous study, hyperpolarisation of the mitochondrial membrane and enhanced superoxide production has been reported in the leukocytes of T2D (Widlansky *et al.* 2010). In the same way, we found that mitochondrial ΔΨ in leukocytes gradually increased with the degree of obesity, in parallel with superoxide production. Moreover, accumulating evidence points to the contribution of mitochondrial dysfunction and oxidative stress to vascular damage, altered

leukocyte dynamics and progression of CV complications in metabolic disorders, all of which are particularly pronounced in patients with T2D (Madsen-Bouterse *et al*. 2010, Bañuls *et al*. 2017, Hernandez-Mijares *et al*. 2013), which is in accordance with our observations in obese patients. Altogether, these findings point to a role of IR, mitochondrial dysfunction and oxidative stress within leukocytes in the activation of the adhesion cascade.

Overproduction of oxidizing species is also a relevant characteristic of hyperreactive leukocytes in chronic periodontitis, however, how this state can be modulating their interactions with the endothelium is a mechanism largely unknow. In this study, we found that, in leukocytes from patients with the same degree of obesity, superoxide production increased progressively with the degree of severity of periodontitis and correlated with increased rolling of leukocytes over the endothelium, similar to what we found in the study of obesity degrees. Furthermore, it is likely that concomitant presence of obesity and chronic periodontitis exacerbates the oxidative response. In this regard, obesity has been shown to be a predictive factor of enhanced oxidative response in humans with chronic periodontitis compared to lean subjects (Atabay *et al*. 2017), conversely, among obese subjects those with chronic periodontitis displayed higher systemic markers of oxidative stress (Suresh *et al*. 2016). In addition, obese rats showed higher basal levels of oxidative stress than lean rats; when periodontal disease was induced both groups displayed an oxidative stress response, which was more severe in the obese group, with enhanced infiltration of PMNs in the periodontal lesion (Tomofuji *et al*. 2009). Although none of these evidences confirm causality, they highlight a novel and significant connection between chronic periodontitis, obesity and CV disease, and also suggests that the presence and deterioration of periodontal condition in obese subjects may be an additional risk factor for CV disease.

The benefits for cardiometabolic function on targeting excess weight have been largely demonstrated, and a potential protective role of weight loss on the progression of carotid atherosclerosis has been proposed (Shai *et al*. 2010), although the mechanisms by which these benefits are achieved remain largely unknown. The above discussed cross-sectional data and prior studies have contributed to our understanding of some mechanisms involved in the early stages of the atherosclerotic process in obesity, such as

changes in leukocytes activation. In the present project, we went a step further by investigating the effect of weight reduction on these and other pro-atherogenic processes. For this purpose, a cohort of morbid obese patients was enrolled in a 6-month dietary weight-loss program. A weight loss of ~9 % improved insulin sensitivity in our obese population, as revealed by decreased levels of fasting glucose, insulin, A1c and HOMA-IR. These data were accompanied by favourable changes in serum TNFα – a potent activator of endothelial cells–, P-selectin and its receptor on the surface of leukocytes PSGL-1 (P-selectin glycoprotein ligand-1). As expected, adherence of leukocytes to the endothelium was reduced, thus suggesting an amelioration of endothelial dysfunction and recruitment of leukocytes to the vessel wall, which our previous findings suggest are associated with improved insulin sensitivity.

When we explored the potential intracellular changes underlying these observations, we confirmed a fall in mitochondrial $\Delta\Psi$ associated with reductions in total superoxide and mROS production, thus suggesting that weight loss is an effective strategy to diminish mitochondrial dysfunction and subsequent excess ROS production in leukocytes. The wide range of pathophysiological implications of mitochondrial dysfunction and oxidative stress has given rise to an intense field of research into therapeutic strategies against the spectrum of metabolic disturbances in obesity, a body of work to which our findings about the role of lifestyle interventions may contribute significantly. In this sense, targeting mROS in leukocytes with specific mitochondrial antioxidant molecules, such as SS-31 or MitoQ, has been proven to exert benefits by diminishing oxidative stress, inflammation and leukocyte-endothelium cell interactions (Escribano-Lopez *et al*. 2018, Escribano-Lopez *et al*. 2016), reinforcing the role of mitochondrial function and ROS production in leukocytes in the initiation of the adhesion cascade. Interestingly, the GPX1 (glutathione peroxidase 1) antioxidant enzyme was up-regulated after weight loss, and was likely to be contributing to the drop in ROS signalling within the leukocytes. In addition, the expression of NFκB in leukocytes was reduced in parallel with intracellular ROS content. The transcriptional NFκB cascade integrates several cell stress pathways (including ROS signalling) in a bidirectional way, and is considered a master regulator of cell activation and inflammatory response. Circulating mononuclear cells in obesity are known to be in a pro-activated state, displaying higher levels of NFκB and TNFα (Ghanim *et al*. 2004), which may enhance their adherence to the endothelium; conversely, weight loss diminished NFκB

regulatory pathways (de Mello *et al*. 2008), in line with our findings. As a whole, our results show that diet-induced weight loss exerts beneficial effects on leukocyte homeostasis by improving redox balance and preventing the activation of intracellular stress pathways.

Besides mitochondria, other sources of oxidizing species play relevant roles in immune cells. Of note, the pro-oxidant activity of MPO in leukocytes is vital for cells to defend themselves against pathogens; however, excess release of MPO from leukocytes into the circulation in an inflammatory context such us obesity, can contribute to oxidative stress and vascular injury. Previously, our group has shown an association between elevated serum MPO levels and the presence of nephropathy in T2D patients, as well as significant correlations with leukocyte-endothelial cell interaction parameters (Rovira-Llopis *et al*. 2013), while Kinkle and *cols*. have also described a potential role of MPO in the interaction of leukocytes with the vessel wall by means of electrostatic forces (Klinke *et al*. 2011), athought mechanistic data are inconclusive. Serum analysis after 6 months of dietary treatment revealed a decline in MPO levels, which may have protected against leukocyte adhesion and endothelial dysfunction. In fact, changes in MPO positively correlated with soluble P-selectin, a marker of endothelial dysfunction. MPO is known to impair eNOS function, leading to activation of immune and endothelial cells, impaired vascular function and the subsequent expression of inflammatory cytokines and CAMs (Vita *et al*. 2004). Therefore, decreased levels of MPO, TNFα and P-selectin after weight loss may be indicative of enhanced endothelial function in our obese population.

Systemic oxidative stress result of an imbalance of pro-oxidant and antioxidant systems, and is reflected by the presence of oxidised circulating macromolecules. Thus, lipid peroxidation and protein carbonylation in serum are considered relevant biomarkers of systemic oxidative damage, whose levels are elevated in obesity as a result of excessive ROS production and undermined activity of the serum detoxifying systems, which can be found in plasma, circulating immune cells and erythrocytes (Vincent *et al*. 2006). In our study, moderate weight loss displayed a systemic antioxidant effect, as revealed by enhanced serum antioxidant capacity and diminished systemic markers of oxidative stress. In this sense, catalase activity was stimulated and erythrocyte glutathione content increased after weight loss. In addition, we observed a decline of carbonyl groups in serum proteins, as well as reduced ROS production and enhanced GPX1 expression in leukocytes, which is in accordance with previously published data (Dandona *et al*. 2001). In relation to

this issue, other authors have demonstrated that the use of antioxidants to target leukocytes ameliorates ROS production and systemic markers of oxidative stress, resulting in improved vascular function in obese individuals (Garg *et al*. 2000). As a whole, given the known role of oxidative stress in eNOS uncoupling and reduced NO availability in the endothelium (Matsuda *et al*. 2013), a partial recovery of redox balance after weight loss may contribute to the enhancement of endothelial function described in the present PhD project.

Altered hepatic lipid metabolism is another mechanism contributing to CV risk in metabolic diseases. In obesity, elevated FFA and IR are involved in excess synthesis and failed processing of VLDL, which in turn leads to increases in LDLc levels and a shift of the LDL pool towards sdLDL particles. Elevated serum LDLc concentration is considered a classic CV risk factor; however, the qualitative characteristics of these particles with respect to the atherosclerotic process are also relevant. In this sense, the smallest and densest LDL – known as phenotype B – have a higher capacity of penetration through the vascular endothelium. In addition, sdLDL particles are more prone to oxidation in which MPO plays a relevant role, which further promotes endothelial dysfunction and increases the immunogenic power of sdLDL (Liao *et al*. 1995, Fleming *et al*. 2005, Gebuhrer *et al*. 1995). This phenomenon could partly explain the presence of residual CV risk in patients with adequate LDL control (Bayturan *et al*. 2010). In this context, as we stated at the outset, the average LDLc in our obese population did not reveal a notable CV risk derived from clinical LDLc levels, which were similar among the different BMI groups and remained surprisingly constant after weight loss. However, qualitative assays of LDL particles revealed a significant reduction of the percentage of sdLDL particles and a beneficial change in LDL size pattern, thus endorsing the protective role of moderate weight loss on subclinical LDL profile. Interestingly, these changes correlated with the decrease of MPO, which may be indicating not only a decrease in the number of sdLDL particles, but also lower oxidation. Atherogenic process is aggravated by the decrease in HDLc associated with obesity, since this lipoprotein exerts an atheroprotective role through the uptake of lipids from macrophages, the reverse transport of cholesterol and its anti-oxidant, anti-inflammatory and anti-thrombotic function (Tall 2008, Badrnya *et al*. 2013). Typically, HDLc levels in our study population decreased with as the degree of obesity escalated, and were partially restored by weight loss. Conversely, TG levels rose with BMI and fell after dietary

intervention, thus leading to an overall improvement in lipid profile that may have protected against CV risk.

Other remarkable findings from the present interventional study give credence to the cardiometabolic protective role of moderate weight loss in middle-aged morbid obese populations. The elevated blood pressure observed initially with growing rates of obesity – a big contributor to endothelial dysfunction – was reduced after weight loss, along with circulating inflammatory CRP, C3c (complement component 3), RBP4 and TNFα. On the other hand, there is some controversy about the role of weight loss in systemic inflammation in obesity, and the extent of this effect. While some authors have failed to observe significant changes in inflammatory markers (Sola *et al*. 2009), other studies have reported marked reductions in plasma CRP, C3c, RBP4 and TNFα following moderate weight loss (Hermsdorff *et al*. 2009, Hernandez-Mijares *et al*. 2012), which is in accordance with our findings. This discrepancy could be explained by the amount of weight loss achieved or different therapeutic approaches, both of which would impede the comparison between studies.

On the whole, there is a broad consensus on the considerable benefits of caloric-restriction-mediated weight loss and subsequent maintenance of weight-loss on cardiometabolic function and CV risk in obese and T2D populations, even with moderate weight reduction of 5-10 % (Cornier *et al*. 2011), although less is known about the mechanisms involved in the observed benefits. Our findings confirm this notion and provide new insights on the processes underlying protective effects of weight loss, namely, modulation of pro-atherogenic factors and intracellular mechanisms involved in activation of leukocytes and their subsequent arrest on the endothelium.

Among the molecular mechanisms involved in the pathophysiology of obesity, ER stress is activated in several tissues such us the pancreas, liver and adipose tissue, where protein trafficking and secretory pathways play a significant part in cell function, contributing to the impairment of insulin sensitivity, β-cell apoptosis and inflammation (Cnop *et al*. 2012). In addition, several *in vitro* studies have investigated the activation of UPR pathways under individual stimuli such as FFA and glucose (Hotamisligil 2010). However, there are still many gaps in our knowledge of how UPR and ER stress are modulated in obesity *in vivo,* where a cluster of activating mechanisms occur simultaneously, and beyond metabolic tissues. Herein, we have explored the modulation

of ER stress responses in leukocytes and WAT of obese patients by means of two interventional approaches: a dietary weight-loss intervention and the use of pinitol, a bioactive plant compound with insulin-like and anti-inflammatory properties.

The role and modulation of ER stress in immune cells is much less clear than that in metabolic tissues and has been the focus of a growing research field over the last decade owing to the discovery that immune cells also contribute to the pathophysiology of obesity, T2D and associated CV complications. In this context, previous studies have reported elevated ER stress in leukocytes of T2D patients, and have associated it with the activation of cell-death pathways and impaired immune function (Rovira-Llopis *et al*. 2014, Komura *et al*. 2010). In addition, markers of ER stress have been detected in peripheral mononuclear cells of patients with obesity and metabolic syndrome (Sage *et al*. 2012, Degasperi *et al*. 2009, Bañuls *et al*. 2017), and, recently, in woman with PCOS (Bañuls *et al*. 2017). This accumulating evidence confirms alterations of ER function in circulating immune cells of patients with IR-related metabolic diseases. Part of the present research aimed to explore whether weight loss can reverse this intracellular stress response in obesity.

When ER function is challenged, early adaptive UPR pathways are promoted in order to restore cell homeostasis. However, under severe or persistent imbalances, pro-survival efforts are abandoned in favor of pro-death responses. To determine how weight loss could be modulating this dichotomy, we evaluated several mediators of the three branches of the UPR and several downstream effectors. We observed a marked down-regulation of the activated ATF6 (p50) transcriptional factor, whereas no changes were detected in either phosphorylated eIF2α or spliced XBP1, both considered to be mediators of the PERK and IRE1α-endoribonuclease pathways, respectively. When we analyzed downstream targets of the UPR after weight loss, we observed decreased JNK activation and CHOP expression, both of which are markers of chronic ER stress (Schonthal 2012). In fact, pro-apoptotic factor CHOP –regulated by ATF6 and/or other UPR leaders– is a crucial executor of cell-death decisions under chronic ER stress (Nishitoh 2012). The strong correlation between changes in ATF6 and CHOP may indicate, predominantly, an ATF6-mediated CHOP regulation. Conversely, GRP78 expression was up-regulated after weight loss in our patients. This chaperone is a master regulator of ER stress through its role in protein-folding, by which the ER lumen is relieved of misfolded proteins; therefore, it is

considered a major contributor to the pro-survival response (Schonthal 2012). Together, increasing GRP78 and a drop in ATF6-CHOP and JNK indicate an amelioration of apoptotic pathways of UPR in favor of adaptive responses, as suggested previously (Rutkowski *et al*. 2006). The only two previous studies that have explored the effect of weight loss on ER stress also reported a decrease in UPR activation in WAT and liver from humans after bariatric surgery and from rats after diet-induced weight reduction, which in accordance with our findings and underline the important role of body weight in ER function (Gregor *et al*. 2009, Tsutsumi *et al*. 2011).

As outlined above, ER stress has been widely described as a mechanism underlying IR and inflammation in obesity and T2D. For instance, IRE1α-kinase activity triggers NFκB and JNK signaling pathways, which are key mediators of ER-induced inflammation (Hotamisligil 2010); both markers decrease after weight loss, thus indicating reduced inflammatory activation within leukocytes. Stimulation of JNK is also considered a major contributor to ER-mediated impairment of insulin signaling. Previous studies have found associations between chronic ER stress markers in leukocytes and indicators of systemic IR and metabolic disturbances (Bañuls *et al*. 2017). In line with this, we found that a drop in HOMA-IR after weight loss correlated with a decrease in chronic ER stress markers ATF6 and JNK, thus supporting the connection between ER function and glucose homeostasis. In addition, ER stress may also be involved in leukocyte activation and transmigration thought the endothelium. In this sense, the presence of ER stress in leukocytes has previously been associated with enhanced properties of leukocyte adherence to the vessel wall (Bañuls *et al*. 2017). In addition, elevated markers of UPR activation have been found in macrophages isolated from atherosclerotic plaques (Myoishi *et al*. 2007), while targeting ER stress with chaperones seems to protect against the development of atherosclerosis (Erbay *et al*. 2009). In the present PhD project changes in ER stress activation after weight loss occurred in parallel with decreased interactions of leukocytes with the endothelium, which may have reinforce this association.

Stress signals, including Ca^{2+}, ROS and inflammatory cytokines traveling from ER to mitochondria and *viceversa,* play a key role in determining cellular viability (Cao *et al*. 2014). In this context, previous studies have shown synergistic activation of ER stress, mitochondrial dysfunction and ROS production in leukocytes from patients with T2D and obesity (Rovira-Llopis *et al*. 2014, Degasperi *et al*. 2009, Bañuls *et al*. 2017). In contrast,

targeting ER with chemical chaperones diminished ROS production within leukocytes, which highlights the close interplay between ER and oxidative stress (Degasperi *et al.* 2009). Similarly, we now demonstrate concomitant improvements in ER and mitochondrial function and reduced ROS production in immune cells of obese patients after weight loss. By way of explaining these findings, a drop in cytosolic Ca^{2+} suggests a partial restoration of ER-intraluminal Ca^{2+} depots, which may in turn be associated with a decrease in mitochondrial ΔΨ and mROS production since excess of Ca^{2+} pumping during ER stress is a key mediator of disturbances in the ETC of mitochondria (Mekahli *et al.* 2011). Excess Ca^{2+} depletion from the ER lumen also has far-reaching effects, since Ca^{2+} is required for adequate protein-folding by several chaperones (Fu *et al.* 2011). Up-regulation of chaperone GRP78 and partial restoration of ER Ca^{2+} depots after weight loss indicate an enhanced protein-folding capacity, which would ameliorate misfolded protein aggregates and ER stress.

Based on the accumulating findings of the present interventional study, in which ER stress relief was associated with amelioration of several other cell stress responses and metabolic disturbances, targeting ER stress emerges as a potential therapeutic strategy to diminish and/or slow down the progression of maladaptive responses underlying obesity and T2D. In this sense, the use of chemical chaperones such as TUDCA and 4-PBA for ER stress amelioration in the context of metabolic disorders is extensively documented in the literature (Engin *et al.* 2010). For instance, administration of these chemical chaperones to a mouse model of obesity decreased several markers of ER stress in WAT, together with inflammatory mediators such as TNFα and IL6 (Chen *et al.* 2016). In this regard, the anti-inflammatory effects of pinitol have been previously described in obese populations (Bañuls *et al.* 2016, Sivakumar *et al.* 2010), although the underlying mechanisms are largely unknown. To assess whether pinitol acts as a chemical chaperone by alleviating ER stress and hence inflammation, we tested the effects of pinitol treatment on two of the major sources of inflammatory cytokines: immune cells and WAT. Our findings showed that pinitol exerts an anti-inflammatory systemic effect, as revealed by decreased levels of TNFα and IL6 in serum of patients after consuming a pinitol-enriched beverage for 12 weeks. However, we did not detect changes in the ER stress markers GRP78 or CHOP in isolated leukocytes. Intriguingly, further *ex vivo* culture of human VAT and SAT with pinitol revealed differential responses; whereas VAT did not show changes in either inflammatory

or ER stress markers, pinitol exerted a beneficial effect on SAT by down-regulating the ATF6-CHOP chronic pathway, a response that was associated with reduced expression of TNFα and IL6 in the tissue, similarly to that reported by Chen *et al.* after treatment with chemical chaperones (Chen *et al.* 2016). Thus, targeting ER stress and production of cytokines in SAT may be a potential mechanism behind the anti-inflammatory properties displayed by pinitol.

Differential responses between VAT and SAT could be explained by the alternative metabolic-activity patterns displayed by both tissues (Misra *et al.* 2003). For instance, adipocytes from VAT are more IR than those from SAT. In fact, VAT exhibited undermined expression of mediators of the insulin-signalling pathway in our comparative analysis, further confirming this belief. Despite this, incubation of VAT and SAT with pinitol did not modify the insulin-signalling pathway – GLUT4, IR (insulin receptor) and PPARγ (peroxisome proliferator-activated receptor γ). In fact we did not find either changes in systemic insulin sensitivity after oral consumption of pinitol for 12 weeks in our obese population, indicating that insulinomimetic activity of pinitol described in other populations (Owczarczyk-Saczonek *et al.* 2018) is not occurring within obese subjects.

On the other hand, SIRT1 is a powerful nutrient-sensing regulator of a wide range of cellular processes, including cell survival, whose expression is undermined in obesity, with harmful effects on overall energy balance and metabolic control. For instance, down-regulation of SIRT1 expression was found in macrophages of patients with metabolic syndrome and was related with impaired insulin sensitivity and atherosclerotic plaque formation (de Kreutzenberg *et al.* 2010). Moreover, SIRT1 physically interacts with NFκB, and mediates its degradation, thus exerting anti-inflammatory properties. In fact, stimulation of SIRT1 in immune cells led to inhibition of pro-inflammatory pathways and improved insulin sensitivity (Yoshizaki *et al.* 2010). Our results revealed SIRT1 up-regulation in leukocytes from obese patients after consuming the pinitol-enriched beverage, which may have contributed to the anti-inflammatory effect of the inositol. Caloric restriction is another powerful inducer of SIRT1 expression (Bordone *et al.* 2005). Interestingly, we observed up-regulation of SIRT1 in the leukocytes of obese patients after weight-loss in the dietary interventional study, in association with a drop in NFκB signaling, ROS production and leukocyte-endothelial cell interactions. Additionally, our research group has previously reported similar findings after stimulation with SS-31 – an antioxidant targeting to

mitochondria – in leukocytes of T2D patients (Escribano-Lopez *et al*. 2018). Finally, SIRT1 has been described as a sensor of ER function, as it participates in a UPR-SIRT1-UPR regulatory loop. In fact, increased expression of SIRT1 after weight loss correlated with enhanced adaptive GRP78. In the light of this accumulating evidence of the converging role of SIRT1, cellular stress responses and metabolic homeostasis in obesity, SIRT1 emerges as a potential therapeutic target. In this sense, here we described two promising strategies to achieve SIRT1 stimulation, namely weight loss and pinitol supplementation.

In summary, by means of a cross-sectional study, we show that adherence between leukocytes and the vascular endothelium is enhanced in obesity in parallel with the rising degree of adiposity, with morbidly obese patients being particularly affected. This response is associated with systemic conditions such as inflammation, IR and endothelial dysfunction, but also with increased ROS production and mitochondrial dysfunction in leukocytes, suggesting a role of altered redox balance within leukocytes in the onset of the atherosclerotic process. At the same time, we demonstrate that a worsening of the periodontal condition in a cohort of obese patients adjusted by BMI was associated with increasing systemic inflammation and ROS production in leukocytes, thus promoting their interaction with the endothelium. These results are an important contribution to our knowledge of the potential mechanisms underlying the relationship between obesity, chronic periodontitis and CV disease. Interestingly, when morbid obese patients underwent dietary weight-loss intervention we found that moderate weight loss partially reversed this situation by improving lipid profile, insulin sensitivity and reducing inflammatory and oxidative response both in leukocytes and at systemic level, resulting in a better profile of endothelial function and lesser interactions between leukocytes and the endothelium. Further analysis of the modulation of intracellular stress responses in leukocytes after weight loss revealed ameliorated ER stress and mitochondrial dysfunction, which were associated with increased expression of chaperones and anti-inflammatory and antioxidant mediators. Altogether, these results shed light on the potential mechanisms underlying the protective role of weight loss on metabolic control and cellular homeostasis. Finally, we demonstrate that pinitol targets ER stress and inflammatory pathways in adipose tissue and leukocytes of obese patients and may represent a novel adjunctive treatment to reduce metabolic complications in this pathology.

4. CONCLUSIONS

1.   Oxidative stress and mitochondrial dysfunction are progressively promoted in leukocytes in parallel with a growing degree of obesity and state of insulin resistance. Furthermore, rising obesity is associated with markers of systemic inflammation, endothelial dysfunction and enhanced adherence of leukocytes to the endothelium, which can increase the risk of atherogenesis.

2.   The presence and degree of severity of chronic periodontitis in an obese population is associated with further systemic inflammation, superoxide production in leukocytes and enhanced properties of leukocyte adherence to the vessel wall with respect to those without periodontal disease. These observations suggest that chronic periodontitis may be an added risk factor for CV disease in obesity.

3.   Diet-induced weight loss improves several cardiometabolic outcomes and reduces pro-atherogenic mechanisms including inflammation, oxidative stress and endothelial dysfunction. In this context, the adherence between less activated leukocytes and endothelial cells is reduced, thus suggesting a protective role of weight loss in the early stages of the atherosclerotic process.

4.   Moderate weight loss provokes a switch from endoplasmic reticulum (ER) chronic/apoptotic pathways to more adaptive responses in the leukocytes of obese patients, which are in turn associated with a reduction in mitochondrial membrane potential. Therefore, ER-mitochondria crosstalk signals – namely, Ca^{2+} and reactive oxygen species (ROS) – are undermined, which improves cellular homeostasis and reduces leukocyte activation.

5.   Pinitol modulates chronic ER stress specifically in subcutaneous adipose tissue of obese patients, leading to a drop in inflammatory cytokine expression and up-regulation of anti-inflammatory SIRT1 in leukocytes, thus reducing systemic inflammation.

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ANNEX I: Articles

The core of the thesis is composed by the following original articles:

1. **López-Domènech, S**; Bañuls, C (co-first author); Díaz-Morales, N; Escribano-López, I; Morillas, C; Veses, S; Orden, S; Álvarez, A; Víctor, VM; Hernández-Mijares, A; Rocha, M. Obesity impairs leukocyte-endothelium cell interactions and oxidative stress in humans. *European Journal of Clinical Investigation*. 2018;48(8):e12985. https://doi.org/10.1111/eci.12985

   Impact factor: **3.086**
   Category: General & Internal Medicine (**Q1**)

2. Martínez-Herrera, M; **López-Domènech, S (co-first author)**; Silvestre, FJ; Silvestre-Rangil, J; Bañuls, C; Víctor, VM; Rocha, M. Chronic periodontitis impairs polymorphonuclear leukocyte-endothelium cell interactions and oxidative stress in humans. *Journal of Clinical Periodontology*. 2018;45(12):1429-1439. https://doi.org/10.1111/jcpe.13027

   Impact factor: **4.046**
   Category: Dentistry, Oral surgery & Medicine (**D1**)

3. **López-Domènech, S**; Martínez-Herrera, M (co-first author); Abad-Jiménez, Z; Morillas, C; Escribano-López, I; Díaz-Morales, N; Bañuls, C; Víctor, VM; Rocha, M Dietary weight loss intervention improves subclinical atherosclerosis and oxidative stress markers in leukocytes of obese humans. *International Journal of Obesity*. 2019 (*epub*). https://doi.org/10.1038/s41366-018-0309-5

   Impact factor: **5.159**
   Category: Nutrition & Dietetics (**Q1**)

4. **López-Domènech, S**; Abad-Jiménez, Z; Iannantuoni, F; de Marañón, AM; Rovira-Llopis, S; Morillas, C; Bañuls, C; Víctor, VM; Rocha, M. Moderate weight loss attenuates chronic endoplasmic reticulum stress and mitochondrial dysfunction in human obesity. *Molecular Metabolism*. 2019;19:24-33. https://doi.org/10.1016/j.molmet.2018.10.005

   Impact factor: **6.291**
   Category: Endocrinology & Metabolism (**D1**)

5. **López-Domènech, S**; Bañuls, C; de Marañón, AM; Abad-Jiménez, Z; Morillas, C; Gómez-Abril, SA; Rovira-Llopis, S; Victor, VM; Hernández-Mijares, A; Rocha, M. Pinitol alleviates systemic inflammatory cytokines in human obesity by a mechanism involving unfolded protein response and sirtuin 1. *Clinical Nutrition*. 2017;37(6 pt A):2036-2044. https://doi.org/10.1016/j.clnu.2017.09.015

   Impact factor: **5.496**
   Category: Nutrition & Dietetics (**D1**)

WILEY

ORIGINAL ARTICLE

Obesity impairs leukocyte-endothelium cell interactions and oxidative stress in humans

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Abstract

Background: To evaluate the relationship between leukocyte-endothelial cell interactions and oxidative stress parameters in non-diabetic patients with different grades of obesity.

Material and methods: For this cross-sectional study, 225 subjects were recruited from January 1, 2014 to December 31, 2016 and divided into groups according to BMI (<30 kg/m^2, 30-40 kg/m^2 and >40 kg/m²). We determined clinical parameters, systemic inflammatory markers, soluble cellular adhesion molecules, leukocyte-endothelium cell interactions—rolling flux, velocity and adhesion —, oxidative stress parameters—total ROS, total superoxide, glutathione—and mitochondrial membrane potential in leukocytes.

Results: We verified that HOMA-IR and hsCRP increased progressively as obesity developed, whereas A1c, IL6 and TNFα were augmented in the BMI > 40 kg/m² group. The cellular adhesion molecule sP-selectin was increased in patients with obesity, while sICAM, total ROS, total superoxide and mitochondrial membrane potential were selectively higher in the BMI > 40 kg/m² group. Obesity induced a progressive decrease in rolling velocity and an enhancement of rolling flux and leukocyte adhesion.

Conclusion: Our findings reveal that endothelial dysfunction markers are altered in human obesity and are associated with proinflammatory cytokines and increased oxidative stress parameters.

KEYWORDS
atherogenesis, humans, mitochondrial membrane potential, obesity, reactive oxygen species

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López-Domènech and Bañuls have contributed equally.

1 | INTRODUCTION

Obesity is a low-degree chronic inflammatory disease associated with a variety of metabolic disorders, including the development of insulin resistance (IR), dyslipidemia, arterial hypertension, atherosclerosis and diabetes mellitus.[1]

Mitochondrial dysfunction and high reactive oxygen species (ROS) production are considered adverse cellular responses to nutrient excess in obesity, which are generated during glucose or free fatty acid (FFAs) oxidation, mainly by mitochondria. When ROS production increases, the balance between oxidant and antioxidant factors is disturbed and oxidative stress occurs. These conditions can damage cellular structures and trigger an inflammatory response.[2] Several studies have shown that an increase in ROS production in adipose tissue of experimental animal models of diabetes and obesity or in cultured adipocytes can cause altered synthesis and secretion of adipokines and promote cell senescence, IR and an inflammatory response.[3-6] More recently, human adipose tissue has not only been associated with mitochondrial activity and enhanced ROS production in the context of obesity,[7,8] but has also been related with downregulation of the mitochondria-related transcriptional signature.[9]

Proinflammatory adipokines and elevated levels of FFAs, in particular those released by visceral adipose tissue, cause IR and are pathogenic factors that can induce endothelial dysfunction in the earlier stages of obesity, which further deteriorates insulin signalling pathways in endothelial cells, thus leading to initiation of the atherosclerotic process.[10] This process is initiated by an interaction between the adhesion molecules expressed on white blood and/or endothelial cells. Different cellular adhesion molecules (CAMs) have been implicated in atherogenesis, including selectins, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1).[11]

Peripheral polymorphonuclear leukocytes (PMNs) are one of the main inflammatory cell types. Once activated, PMNs release ROS, which contributes to oxidative stress, the inflammation and endothelial damage that follow.[12] We have previously described that oxidative stress occurs in the PMNs of insulin resistant patients—type 2 diabetes and polycystic ovary syndrome—and is related to an impairment of mitochondrial function and endothelial dysfunction.[12-15] Although several studies have shown that the presence of IR is determinant for the endothelial dysfunction associated with obesity,[16,17] little is known about how the redox status and mitochondrial function of PMNs influence this process.

Therefore, the current study was performed to throw light on the relationship between leukocyte activation, mitochondrial dysfunction and enhanced leukocyte-endothelium cell interactions according to BMI, in addition to exploring a possible correlation between these factors.

The primary endpoint was leukocyte-endothelium cell interactions in a population of non-diabetic subjects with different grades of obesity. Secondary endpoints were the redox status and mitochondrial function of leukocytes, and a possible relationship between these parameters.

2 | MATERIAL AND METHODS

2.1 | Subjects

The participants in this cross-sectional study were recruited at the Outpatient's Department of the Endocrinology and Nutrition Service of the University Hospital Dr. Peset (Valencia, Spain) between January 2014 and December 2016. Subjects between the ages of 18-68 years (inclusive) were eligible for inclusion in the study and were clustered in three groups depending on their body mass index (BMI): Non-obese condition group (BMI < 30 kg/m²), subjects with grade I and II of obesity (BMI = 30-40 kg/m²) and group with morbid and extreme obesity (BMI > 40 kg/m²), defined according to the criterion of the Spanish Society for the Study of Obesity.[18] Exclusion criteria were pregnancy or lactation, severe disease including malignancies, severe renal or hepatic disease, alcohol or drug abuse, psychiatric disorders, history of cardiovascular or chronic inflammatory disease, diabetes mellitus with fasting glycaemia ≥126 mg/dL on at least two occasions or glycated haemoglobin (A1c) ≥6.5% and pharmacological treatment for diabetes, and secondary obesity (hypothyroidism, Cushing's syndrome).

The study—a human observational study structured according to STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) and the broader EQUATOR guidelines[19]—was conducted according to the ethical principles stated in the Declaration of Helsinki, and all procedures were approved by the Ethics Committee of the Hospital. Written informed consent was obtained from all subjects.

Anthropometrical parameters including weight (kg), height (m), body mass index (BMI; kg/m²), waist circumference (cm), hip circumference (cm), waist-hip ratio (WHR) and systolic and diastolic blood pressure (SBP/DBP mm Hg) were obtained from all the participants.

2.2 | Blood sampling

Venous blood samples were collected from subjects after 12 hours overnight fasting. Serum and plasma were obtained after centrifugation (1500 g, 10 minutes) at 4°C. Fresh samples were used to measure biochemical parameters and the remaining aliquots were stored at −80°C for subsequent measurement of inflammatory parameters and soluble CAMs.

2.3 | Biochemical determinations

All biochemical determinations were carried out in our hospital's Clinical Analysis Service. Levels of glucose, total cholesterol (TC) and triglycerides (TG) in serum were determined by means of an enzymatic method. HDL cholesterol (HDLc) levels were obtained with a Beckman LX20 analyzer (Beckman Coulter, Inc., Brea, CA, USA) using a direct method. The intraserial variation coefficient was <3.5% for all determinations. LDL cholesterol (LDLc) concentration was calculated using the method of Friedewald when TG were lower than 300 mg/dL. Insulin was determined by an immunochemiluminescence assay and IR was estimated using the homeostasis model of assessment (HOMA-IR = (fasting insulin (μU/mL) × fasting glucose (mg/dL)/405)). Obese patients were classified as IR obese when the HOMA index was >2.5 and non-IR obese when the HOMA index was <2.5, as in a previously published study.[20] Percentage of A1c was measured with an automatic glycohemoglobin analyzer (Arkray Inc., Kyoto, Japan), and high-sensitive C-reactive protein (hsCRP) levels were quantified by an immunonephelometric assay.

2.4 | Measurement of proinflammatory cytokines and soluble CAMs

Serum levels of interleukin 6 (IL6) and tumor necrosis factor alpha (TNFα) and soluble CAMs—sP-selectin, sICAM-1 and sVCAM-1—were analysed with a Luminex 200 analyzer system (Austin, TX, USA). Both kits were purchased from Millipore Corporation (Billerica, MA, USA). The intraserial and interserial variation coefficients were <5.0% and <15.0%, respectively, for all determinations.

2.5 | Leukocyte isolation

Human PMNs were isolated from citrated blood samples and incubated with dextran (3%) for 45 minutes. The supernatant was collected, released over Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden) and centrifuged (650 g, 25 minutes) to isolate leukocytes. The pellet was treated with lysis buffer and centrifuged at room temperature (240 g, 5 minutes) to remove the remaining erythrocytes. After being washed and resuspended in Hanks' balanced salt solution (HBSS) (Sigma-Aldrich, Inc., St. Louis, MO, USA), cells were counted with a Scepter 2.0 cell counter (Millipore Corporation, Billerica, MA, USA).

2.6 | Leukocyte-endothelial interaction assay

A flow-condition adhesion assay based on an in vitro model of leukocyte-endothelial cell interactions was carried out. In short, human umbilical vein endothelial cells (HUVEC) were seeded on coverslips until confluent and inserted in the bottom plate of a flow chamber. One million leukocytes were resuspended in 1 mL of RPMI medium (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) and drawn across the HUVEC monolayer at a flow rate of 0.36 mL/min. A 5 × 25 mm portion of the endothelial cells was recorded during a 5-minute period of flow with a video camera (Sony Exware HAD; Koeln, Germany) connected to an inverted microscope (Nikon Eclipse TE 2000-S, Nikon Corporation, Tokio, Japan) to evaluate different leukocyte parameters: rolling velocity was calculated by measuring the time it took 20 consecutive leukocytes to travel a distance of 100 μm within the field of focus; rolling flux was calculated by counting the number of leukocytes rolling over 100 μm^2 of the HUVEC monolayer during a 1-minute period; and adhesion was evaluated by counting the number of leukocytes that maintained stable contact with endothelial cells for 30 seconds. Platelet-activating factor (1 μmol/L, 1 h) was used as a positive control for leukocytes, and tumoral necrosis factor (10 ng/mL, 4 h) for HUVEC. Both reagents were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA).

2.7 | Evaluation of oxidative stress parameters

Leukocytes were seeded in a 48-well plate and incubated with different fluorescence probes diluted in HBSS for 30 minutes at 37°C. The plate was read in a fluorescence microscope (IX81; Olympus Corporation, Tokio, Japan) coupled with the static cytometry software "ScanR" (Olympus Corporation, Tokio, Japan) to evaluate oxidative stress parameters: total ROS production was assessed with the 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorochrome (5×10^{-6} mol/L), glutathione (GSH) content was measured with the 5-chloromethylfluorescein diacetate (CMFDA,) probe (1×10^{-6} mol/L), tetramethylrhodamine methylester (TMRM) at 5×10^{-6} mol/L was used to assess mitochondrial membrane potential ($\Delta\Psi$m), and total superoxide was detected with 5×10^{-6} mol/L of dihydroethidium (DHE) dye. All fluorescent probes were purchased from Life Technologies (Thermo Fisher Scientific, Waltham, MA, USA).

2.8 | Statistical analysis

The study was designed based on preliminary data[12,15] to detect a 20% and 80% difference in the variation of leukocyte-endothelium interactions (measured by rolling velocity, rolling flux and adhesion of PMNs) between and within groups, respectively, with a power of 90% and an α risk of 0.05. Under these premises, at least 26 subjects per group were considered.

The statistics programme SPSS 19.0 (SPSS Statistics Inc., Chicago, IL, USA) was employed for statistical analysis. All experiments were performed in duplicated with the exception of anthropometric and biochemical determinations. Continuous variables were expressed as mean and standard deviation (SD), or as median and 25th and 75th percentiles for parametric and non-parametric data, respectively. Qualitative data was expressed as percentages. Data were compared with a one-way analysis of variance (ANOVA) by a Student-Newmann-Keuls post-hoc test. Pearson's correlation coefficient was employed to measure the strength of the association between variables. A Chi-square test was employed to compare proportions. In the multivariable regression model, the relationship between two or more explanatory variables (independent variables) and a response variable (dependent variable) was evaluated by fitting a linear equation to the data obtained. All the tests had a confidence interval of 95% and differences were considered significant when $P < 0.05$.

3 | RESULTS

This study analysed a total of 225 subjects (62 men and 163 women) with a BMI < 30 kg/m² (106 subjects),

BMI = 30-40 kg/m² (45 subjects) or BMI > 40 kg/m² (74 subjects).

Anthropometric and biochemical parameters are shown in Table 1. As the rate of obesity augmented, waist circumference, DBP, insulin, HOMA-IR and TG increased significantly, whereas HDLc decreased. WHR, SBP and glucose increased to the same extent in subjects with obesity independently of grade of obesity, while A1c, a determination of average blood glucose level for previous 2-3 months, was significantly increased in the higher BMI group.

3.1 | Measurement of proinflammatory molecules and soluble CAMs

Acute phase reactants, such as hsCRP, was associated with BMI, increasing with grade of obesity (Figure 1a) ($P < 0.001$). Other systemic inflammatory markers were also altered, though only in the group with the highest grade of obesity, in which there was an increase in IL6 (Figure 1b) ($P = 0.008$) and TNFα (Figure 1c) ($P < 0.001$). In line with this, soluble CAMs levels showed a rise as BMI increased, which was evident in sP-selectin (Figure 2a) and sICAM-1 levels, the latter of the two occurring only among subjects with the highest grade of obesity (BMI > 40 kg/m²) (Figure 2b) ($P = 0.023$ and $P = 0.008$, respectively).

TABLE 1 Anthropometric and biochemical parameters in subjects according to BMI

	BMI < 30	BMI 30-40	BMI > 40	*P*-value
n (females)	106 (73)	45 (34)	74 (56)	0.35
Age (years)	37.9 ± 14.5	42.2 ± 11.9	41.5 ± 9.6	0.08
BMI (kg/m²)	23.4 ± 2.9[a]	35.7 ± 3.2 [b]	45.2 ± 4.7[c]	<0.001
Waist circumference (cm)	78.9 ± 12.2[a]	109.5 ± 13.5[b]	127.3 ± 13.1[c]	<0.001
WHR	0.798 ± 0.097[a]	0.895 ± 0.090[b]	0.892 ± 0.093[b]	<0.001
SBP (mm Hg)	120 ± 18[a]	130 ± 17[b]	135 ± 16[b]	<0.001
DBP (mm Hg)	72 ± 11[a]	80 ± 11[b]	86 ± 12[c]	<0.001
Glucose (mg/dL)	86 ± 11[a]	93 ± 11[b]	96 ± 14[b]	<0.001
A1c (%)	5.20 ± 0.30[a]	5.33 ± 0.44[a]	5.51 ± 0.44[b]	<0.001
Insulin (μU/mL))	7.1 ± 2.7[a]	12.8 ± 5.0[b]	21.3 ± 15.7[c]	<0.001
HOMA-IR	1.52 ± 0.73[a]	2.98 ± 1.27[b]	5.19 ± 4.12[c]	<0.001
TC (mg/dL)	189 ± 35	187 ± 40	185 ± 31	0.80
HDLc (mg/dL)	58 ± 13[a]	47 ± 10[b]	40 ± 9[c]	<0.001
LDLc (mg/dL)	112 ± 29	118 ± 35	118 ± 28	0.36
TG (mg/dL)	70 (57,106)[a]	101 (76,141)[b]	125 (90,168)[c]	<0.001
Treatment				
Lipid-lowering drugs (%)	—	9.8	6.8	0.72
Hypotensive drugs (%)	—	7.7	24.3	0.04

A1c, glycated haemoglobin; BMI, body mass index; DBP, diastolic blood pressure; HDLc, HDL cholesterol; LDLc, LDL cholesterol; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides; WHR, waist-to-hip ratio.

Data are expressed as mean ± SD. Values of serum triglycerides were normalized using a log transformation. Different superscript letters indicate significant differences among groups ($P < 0.05$) when compared by means of one-way ANOVA followed by a Student-Newman-Keuls as post-hoc test. Hence, means with the same superscript are not significantly different from each other ($P > 0.05$), while means that have no superscript in common are significantly different from each other ($P < 0.05$).

3.2 | Leukocyte-endothelial cell interaction assay

These variations were associated with an impairment in adhesion under flow conditions; ie, a progressive reduction in rolling velocity as obesity developed (Figure 2d) and an increase in PMN rolling flux in all the obese groups (Figure 2e) ($P < 0.001$ for both) and cellular adhesion only in the highest grade of obesity (BMI > 40 kg/m²) (Figure 2f) ($P = 0.046$).

3.3 | Evaluation of oxidative stress parameters

To investigate whether obesity impairs leukocyte-endothelial interactions by altering oxidative stress and mitochondrial function, we employed static cytometry to determine total ROS production, total superoxide, GSH levels and mitochondrial membrane potential in PMNs. As shown in Figure 3, total ROS production (Figure 3a; $P < 0.001$ and Figure 3e (representative images) and total superoxide (Figure 3b; $P = 0.027$) were increased in patients with obesity with higher BMI, whereas GSH levels were unchanged (Figure 3c; $P = 0.82$). These variations were associated with an increase in mitochondrial membrane potential, as shown in Figure 3d ($P < 0.001$) and Figure 3f (representative images).

3.4 | Correlation analysis

Pearson's correlation coefficients between leukocyte-endothelium cell interactions and different clinical are shown in Table 2. Summing up, BMI and hsCRP were correlated with rolling velocity, rolling flux and adhesion. Waist circumference, WHR, A1c, insulin, HOMA-IR and TNFα were associated to rolling velocity and rolling flux whereas total superoxide, sICAM, HDLc and mitochondrial membrane potential were specifically associated with velocity, rolling and adhesion, respectively. The multivariable regression model showed that total superoxide ($\beta = -0.373$) and HOMA-IR ($\beta = -0.370$) were independent predictors of rolling velocity, explaining 31% of the dependent variable. BMI ($\beta = 0.288$) and A1c ($\beta = 0.216$) were independently associated with rolling flux and mitochondrial membrane potential ($\beta = -0.383$) was independently associated with adhesion, explaining 15% and 14% of the dependent variable, respectively.

Finally, since BMI and HOMA-IR are closely related, we investigated the effect of IR on leukocyte-endothelial cell interactions and mitochondrial function, and categorized the results according to HOMA. For this purpose, we divided the population into normoweight subjects (Control group) and obese subjects without (HOMA-IR < 2.5) or
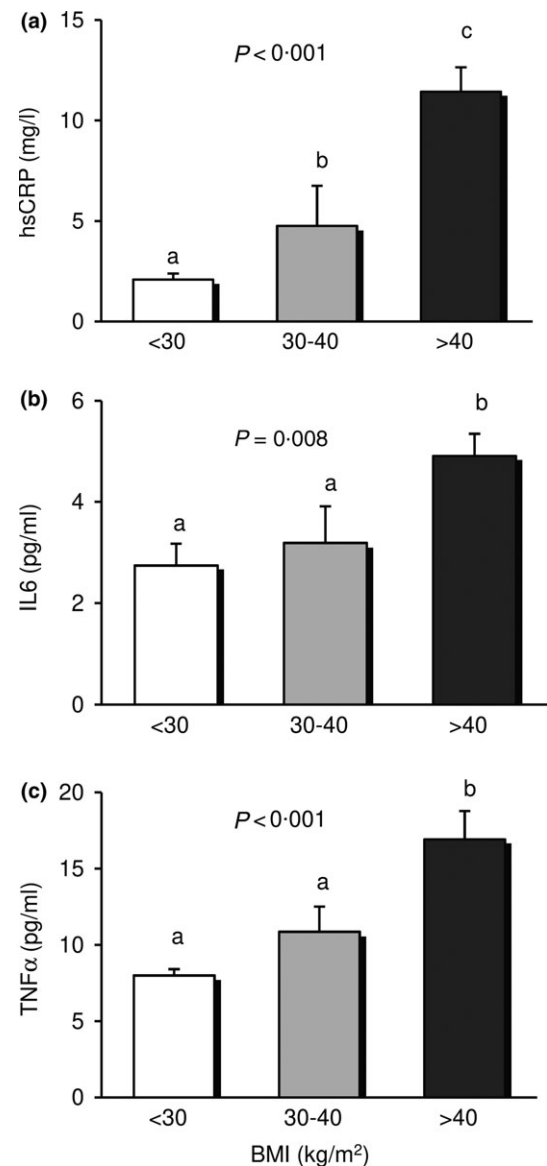

FIGURE 1 Inflammatory Parameters in Subjects According to BMI. Levels of hsCRP (a) in subjects with BMI < 30 kg/m² (n = 89), BMI = 30-40 kg/m² (n = 33) and BMI > 40 kg/m² (n = 65), IL6 (b) in subjects with BMI < 30 kg/m² (n = 38), BMI = 30-40 kg/m² (n = 22) and BMI > 40 kg/m² (n = 22) and TNFα (C) in subjects with BMI < 30 kg/m² (n = 38), BMI = 30-40 kg/m² (n = 22) and BMI > 40 kg/m² (n = 22). Data are represented as mean + standard error. Different superscript letters indicate significant differences among groups ($P < 0.05$) when compared by means of one-way ANOVA followed by a Student-Newman-Keuls as post-hoc test. Hence, means with the same superscript are not significantly different from each other ($P > 0.05$), while means that have no superscript in common are significantly different from each other ($P < 0.05$). BMI, body mass index; hsCRP, high-sensitive C-reactive protein; IL6, Interleukine 6; TNFα, Tumor necrosis factor alpha

with (HOMA-IR > 2.5) IR. As shown in Figure S1, sP-selectin and sICAM levels rose in the obese population with IR. In addition, these changes were associated with
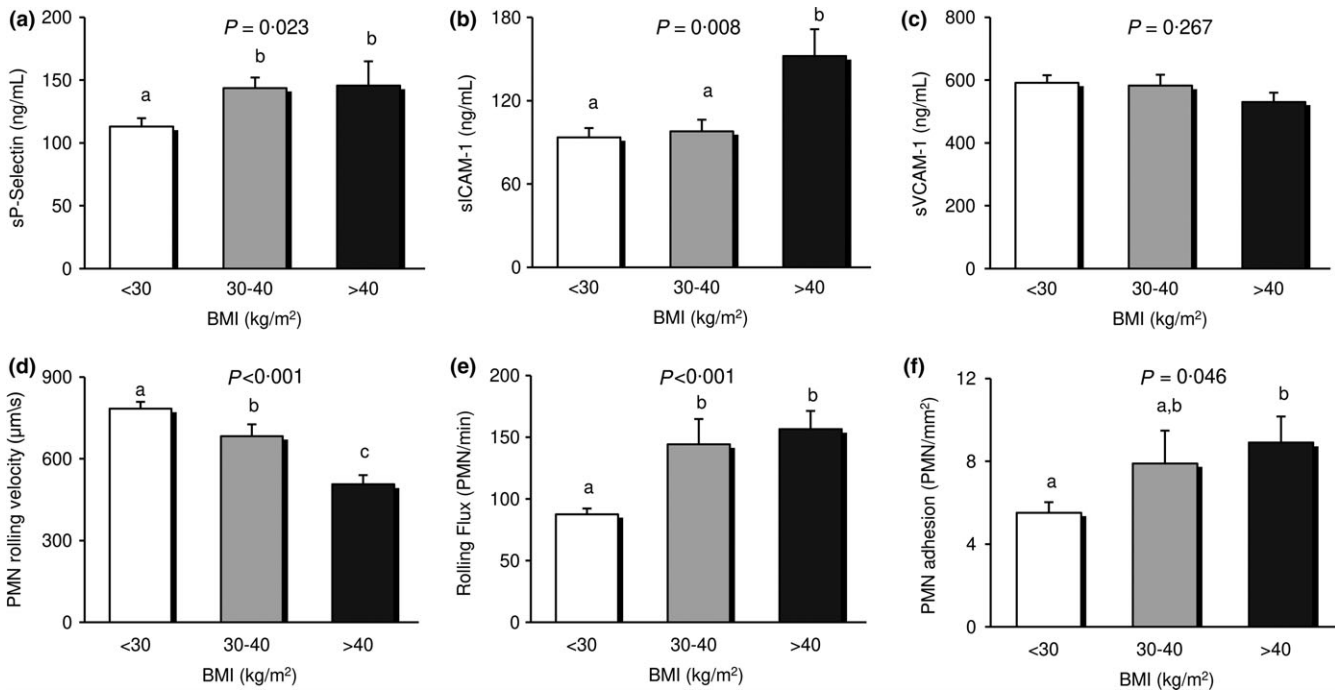
FIGURE 2 Endothelial Function According to BMI, Determined by Cellular Adhesion Molecules and Leukocyte-Endothelial Interactions. Levels of cellular adhesion molecules represented by sP-selectin (a) in subjects with BMI < 30 kg/m² (n = 38), BMI = 30-40 kg/m² (n = 22) and BMI > 40 kg/m² (n = 22), sICAM-1 (b) in subjects with BMI < 30 kg/m² (n = 38), BMI = 30-40 kg/m² (n = 22) and BMI > 40 kg/m² (n = 22) and sVCAM-1 (c) in subjects with BMI < 30 kg/m² (n = 38), BMI = 30-40 kg/m² (n = 22) and BMI > 40 kg/m² (n = 22). Leukocyte-endothelial interactions were evaluated by leukocyte rolling velocity (d) in subjects with BMI < 30 kg/m² (n = 45), BMI = 30-40 kg/m² (n = 26) and BMI > 40 kg/m² (n = 36), leukocyte rolling flux (e) in subjects with BMI < 30 kg/m² (n = 45), BMI = 30-40 kg/m² (n = 26) and BMI > 40 kg/m² (n = 36) and leukocyte adhesion (f) in subjects with BMI < 30 kg/m² (n = 45), BMI = 30-40 kg/m² (n = 26) and BMI > 40 kg/m² (n = 36). Data are represented as mean + standard error. Different superscript letters indicate significant differences among groups ($P < 0.05$) when compared by means of one-way ANOVA followed by a Student-Newman-Keuls as post-hoc test. Hence, means with the same superscript are not significantly different from each other ($P > 0.05$), while means that have no superscript in common are significantly different from each other ($P < 0.05$). BMI, body mass index; sICAM, soluble intercellular adhesion molecule; sVCAM, soluble vascular cell adhesion molecule; PMNs, polymorphonuclear leukocytes

impairment of leukocyte-endothelial cell interactions; we detected a slowing of rolling flux velocity and an increase in rolling flux in the obese population, and enhanced leukocyte adhesion to the endothelium in the insulin-resistant obese group. Leukocyte function was also altered; total ROS production and mitochondrial membrane potential were increased in both obese groups and total superoxide was selectively augmented in obese subjects with IR (Figure S2), which is in line with the results obtained following stratification of the population by different grades of BMI.

4 | DISCUSSION

In the present study, we demonstrate an alteration in leukocyte-endothelium cell interactions in subjects with obesity, with rising impairment as adiposity increases. This response is also associated with altered mitochondrial function and increased oxidative stress in PMNs and with

systemic release of proinflammatory cytokines, and is more evident in patients with higher grades of obesity.

Obesity, IR and cardiovascular disease are closely related. Recently, it has been published that even in a cohort of metabolically healthy subjects, increasing BMI reported a positive association with the incidence of subclinical carotid atherosclerosis, suggesting that visceral obesity favours the development of this pathology.[21,22] In regards to the involvement of IR on the development of endothelial dysfunction, several studies revealed its determinant role on impaired endothelium-dependent vasodilatation in patients suffering morbid obesity.[17,23] Even small changes in insulin can have a significant effect on endothelial function in populations with obesity that are not yet classified as insulin resistant.[24] In accordance with such reports, we have shown that IR and BMI are main predictors of leukocyte-endothelial cell interactions; namely rolling velocity and rolling flux, respectively. Insulin could be involved in endothelial dysfunction through several mechanisms. In in vitro experiments, insulin stimulates the
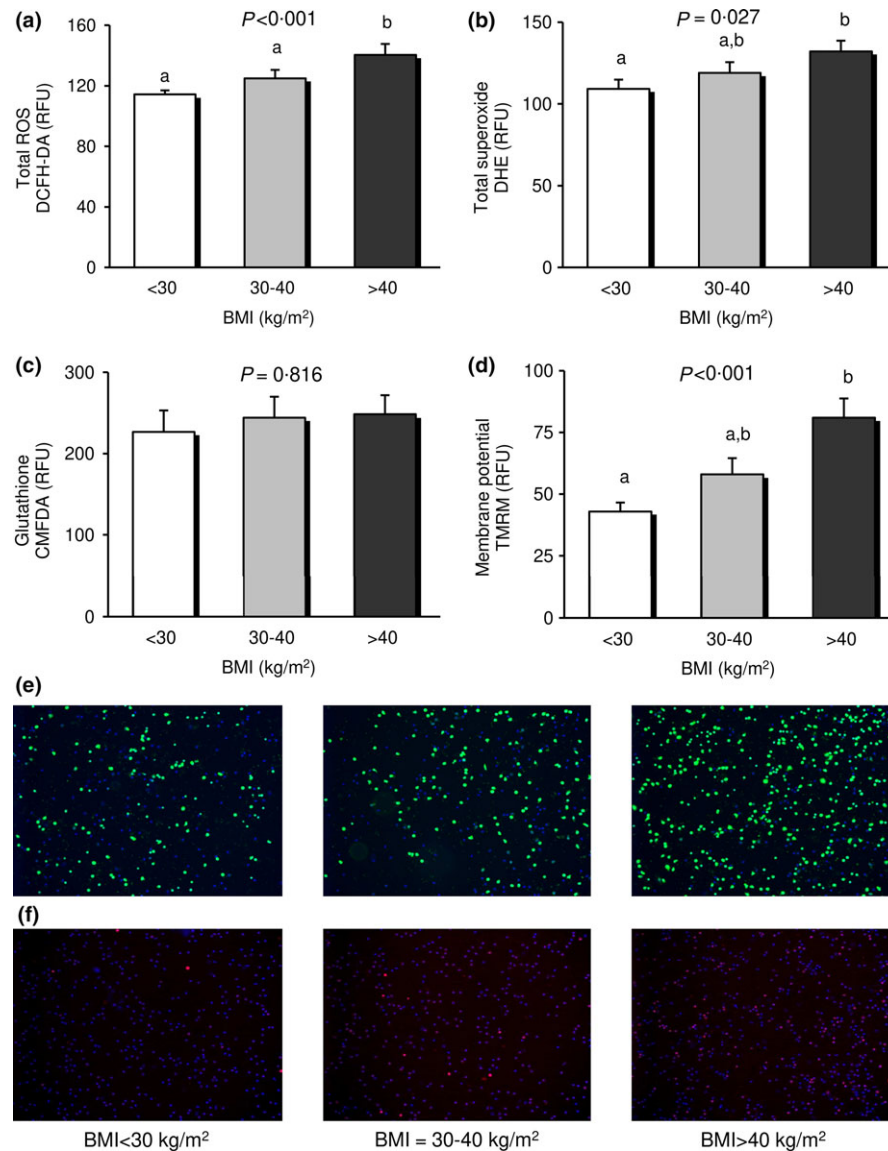
FIGURE 3 Mitochondrial Function Parameters in Subjects According to BMI. Mean of fluorescence intensity of DCFH-DA (a) in subjects with BMI < 30 kg/m^2 (n = 56), BMI = 30-40 kg/m^2 (n = 26) and BMI > 40 kg/m^2 (n = 38), DHE (b) in subjects with BMI < 30 kg/m^2 (n = 38), BMI = 30-40 kg/m^2 (n = 27) and BMI > 40 kg/m^2 (n = 35), CMFDA (c) in subjects with BMI < 30 kg/m^2 (n = 32), BMI = 30-40 kg/m^2 (n = 26) and BMI > 40 kg/m^2 (n = 38) and TMRM (d) in subjects with BMI < 30 kg/m^2 (n = 55), BMI = 30-40 kg/m^2 (n = 25) and BMI>40 kg/m^2 (n = 27). Representative fluorescent images depicting DCFH-DA and TMRM intensities (green and red signals) are shown in panels (e) and (f), respectively. The nuclei were visualized using the specific nuclear stain Hoechst 33342 (blue). Data are represented as mean ± standard error. Different superscript letters indicate significant differences among groups ($P < 0.05$) when compared by means of one-way ANOVA followed by a Student-Newman-Keuls as post-hoc test. Hence, means with the same superscript are not significantly different from each other ($P > 0.05$), while means that have no superscript in common are significantly different from each other ($P < 0.05$). BMI, body mass index; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; CMFDA, 5-chloromethyl fluorescein diacetate; TMRM, Tetramethylrhodamine methyl ester; RFU, relative fluorescent units

expression of VCAM-1 and E-selectin in the endothelium and increases both rolling interaction and adhesion of monocytes,[25] suggesting a central role of insulin in endothelial dysfunction. Indeed, we have previously established a relationship between endothelial dysfunction and IR-related pathologies, such as type 2 diabetes and polycystic ovary syndrome.[12,15] In line with this, leukocyte-endothelial cell interactions were activated in the PMNs of our subjects with obesity, and an increase in CAMs—sP-selectin and sICAM—was observed among those with the highest grade of obesity, insulin and HOMA-IR index. Although the adhesive strength of P-selectin was low, became stronger with the induction of ICAM-1. Noticeably, sICAM-1 was markedly upregulated

TABLE 2 Pearson's correlation coefficients between leukocyte-endothelium cell interactions and biochemical, inflammation, cellular adhesion molecules and oxidative stress parameters

	Rolling velocity		Rolling flux		Adhesion	
Rolling velocity	–	–	$r = -0.639$	$P < 0.001$	$r = -0.527$	$P < 0.001$
Rolling flux	–	–	–	–	$r = 0.362$	$P < 0.001$
BMI	$r = -0.505$	$P < 0.001$	$r = 0.400$	$P < 0.001$	$r = 0.301$	$P = 0.004$
Waist circumference	$r = -0.433$	$P < 0.001$	$r = 0.417$	$P < 0.001$	–	–
WHR	$r = -0.219$	$P = 0.041$	$r = 0.284$	$P = 0.007$	–	–
HDLc	–	–	$r = -0.230$	$P = 0.022$	–	–
TG	–	–	–	–	–	–
A1c	$r = -0.307$	$P = 0.004$	$r = 0.336$	$P = 0.001$	–	–
Insulin	$r = -0.425$	$P < 0.001$	$r = 0.359$	$P = 0.001$	–	–
HOMA-IR	$r = -0.406$	$P < 0.001$	$r = 0.375$	$P < 0.001$	–	–
hsCRP	$r = -0.275$	$P = 0.010$	$r = 0.469$	$P < 0.001$	$r = 0.360$	$P < 0.001$
TNFα	$r = -0.340$	$P = 0.021$	$r = 0.484$	$P < 0.001$	–	–
DHE	$r = -0.405$	$P = 0.002$	–	–	–	–
sICAM	–	–	$r = 0.341$	$P = 0.039$	–	–
TMRM	–	–	–	–	$r = -0.339$	$P = 0.009$

A1c, glycated haemoglobin; BMI, Body mass index; DHE, dihydroethidium; HDLc, HDL cholesterol; hsCRP, high-sensitive C-reactive protein; sICAM, soluble intercellular adhesion molecule; TG, triglycerides; TMRM, Tetramethylrhodamine methyl ester.; TNFα, Tumor necrosis factor alpha; WHR, waist-hip ratio.

in the group with the highest grade of obesity, suggesting stronger adhesion and slower rolling velocity, which would, in turn, promote diapedesis, plaque formation and atherosclerosis.

In addition to insulin, glucotoxicity also contributes to endothelial dysfunction through a mechanism that impairs blood-flow, vascular permeability, angiogenesis, vascular and capillary occlusion and induces an increase in proinflammatory gene expression and in ROS production, resulting in endothelial dysfunction.[26] In this sense, we have previously shown that endothelial function is worsened in patients with type 2 diabetes whose glycaemia is poorly controlled.[27] Although type 2 diabetic patients were excluded from our study, we have shown that A1c is involved in leukocyte-endothelium cell interactions, which suggests that small changes in long-term glycaemia have a significant effect on endothelial function and consequently in cardiovascular diseases. In keeping with this, it has been shown that glucose metabolism is the main predictor of carotid intima media thickness in morbid obesity.[28]

The increased levels of CRP and proinflammatory cytokines—IL6 and TNFα—indicate the presence of a chronic low-grade inflammation associated with obesity and which can be involved in atherosclerosis.[1] In fact, TNFα has been reported to be an independent predictor of coronary endothelial function.[29] More recently, it has been shown that pharmacological blockade of TNFα improves endothelial function in mesenteric and omental vessels of subjects with obesity.[17,30] Our results reveal a correlation

between inflammatory markers—TNFα and hsCRP—and leukocyte-endothelium cell interactions, although the multivariable regression model showed they were not significant predictors. These results are in accordance with a previous study in which, despite the presence of inflammation in morbid obesity, endothelial dysfunction was observed only in insulin resistant subjects.[17]

It has been suggested that oxidative stress is a major pathophysiological mechanism involved in endothelial dysfunction associated with obesity. The underlying mechanism seems to involve an increased secretion of ROS and adipokines by adipose tissue, which impairs bioavailability of nitric oxide.[31] Mitochondrial membrane potential is critical for maintaining the physiological function of the respiratory chain. In fact, a significant loss of mitochondrial membrane potential results in the death of cells with depleted energy levels, whereas a surplus nutrient supply can hyperpolarize mitochondria, leading to the accumulation of incompletely oxidized substrates or intermediates (eg, FFAs and diacylglycerol) and overproduction of ROS,[32-34] especially superoxide under hyperglycaemic conditions.[26] This is in line with the association between glucose and superoxide and ROS demonstrated by the present study.

The present study has some limitations, including the size of the study population, which, although relatively small, was supported by sample size calculation. In addition, although we did not determine the presence of the atherosclerotic plaque in our patients, we did evaluate the

onset of the atherosclerotic process; in other words, the first stages of endothelial dysfunction, which is heralded by the movement and accumulation of leukocytes in the vessel wall and enhanced levels of CAMs in a proinflammatory environment. Our results point to an association between higher BMI and enhanced endothelial dysfunction, manifested in leukocyte-endothelium cell interactions, adhesion molecules and inflammation. Whether changes in intracellular signalling in PMNs are related to the interaction of these cells with the endothelium and the subsequent risk of developing atherosclerosis and cardiovascular disease is a question that needs to be explored.

In conclusion, our data reveal that obesity is characterised by an increase in endothelial dysfunction markers and proinflammatory cytokines, which is associated with altered mitochondrial function and increased oxidative stress in PMNs. Moreover, these characteristics are more evident in patients with higher grades of obesity. These findings help to explain the link between obesity, IR, oxidative stress and atherosclerosis and point to new targets for specific interventions to prevent the development of cardiovascular disease although further studies are needed to throw light on the mechanisms involved in these processes.

CONFLICT OF INTEREST

All authors have read the journal's authorship agreement. All authors have read the journal's policy on conflicts of interest, and all authors declared no conflicts of interest.

AUTHOR CONTRIBUTION

The authors' responsibilities were as follows: V.M.V., A.H-M. and M.R. conducted the study. C.M. and S.V. provided overall supervision and the follow-up of the patients in the study. S.L-D., C.B., N.D-M. and I.E.-L. performed the laboratory analyses and collected data. S.O. and A.A. performed adhesion assays. VM.V. assisted in the design of the experiment and provided support throughout the course of the trial and analysis. S.L-D, C.B. and M.R. analysed the data, performed the statistical analysis and redacted the manuscript. S.L-D, C.B., V.M.V., A.H-M and M.R were responsible for its final content. All authors read and approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.
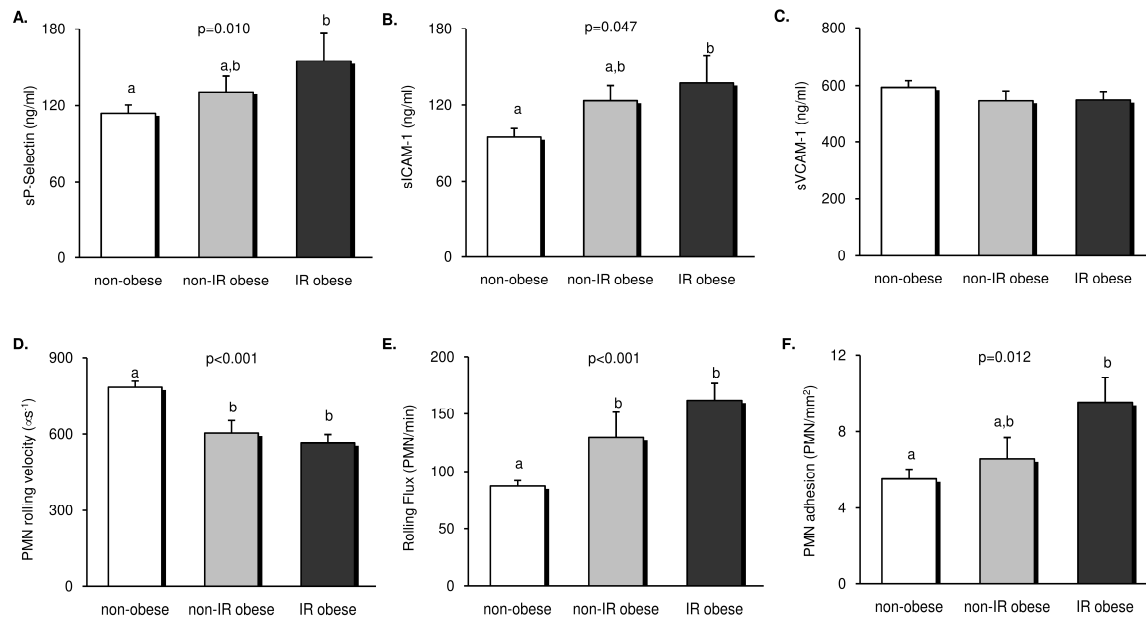
FIGURE S1 Endothelial function according to BMI and HOMA-IR, determined by cellular adhesion molecules and leukocyte-endothelial interactions. Levels of cellular adhesion molecules represented by sP-selectin (A), sICAM-1 (B) and sVCAM-1 (C); leukocyte-endothelial interactions were evaluated by leukocyte rolling velocity (D), leukocyte rolling flux (E) and leukocyte adhesion (F) in non-obese (normoweight subjects), non-IR obese (obese subjects without IR (HOMA-IR < 2.5)) and IR obese (obese subjects with IR (HOMA-IR > 2.5)). BMI, body mass index; IR, insulin resistance: PMN, polymorphonuclear leukocytes; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular adhesion molecule-1;
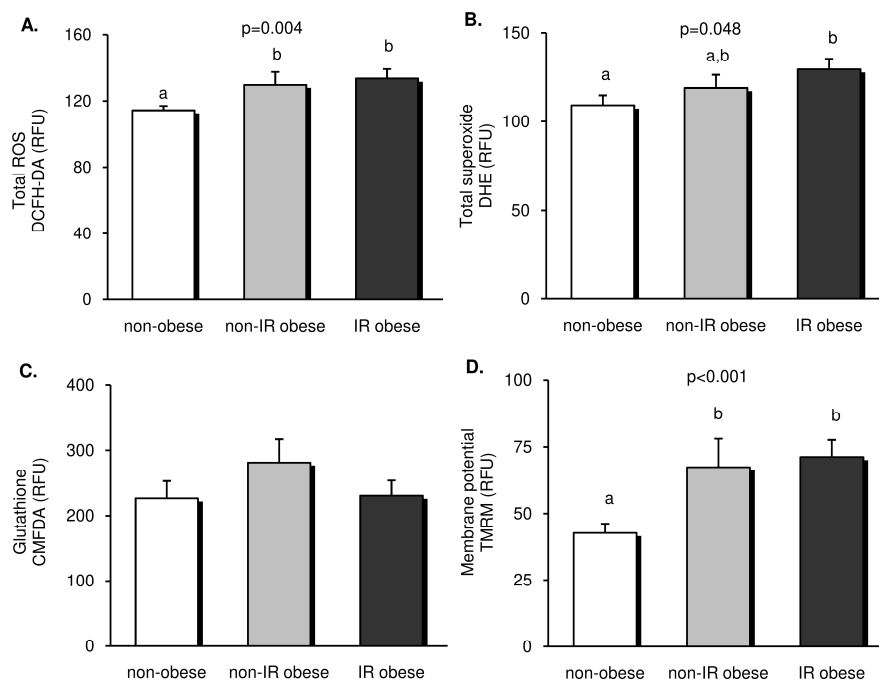
FIGURE S2 Mitochondrial function parameters in subjects according to BMI and HOMA-IR. Mean of fluorescence intensity of DCFH-DA (A), DHE (B), CMFDA (C) and TMRM in non-obese (normoweight subjects), non-IR obese (obese subjects without IR (HOMA-IR<2.5)) and IR obese (obese subjects with IR (HOMA-IR > 2.5)). BMI, body mass index; DCFH-DA, 2´7´-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; CMFDA, 5-cholomethyl fluorescein diacetate; IR, insulin resistance; ROS, reactive oxygen species; RFU, relative fluorescence units; TMRM, tetramethylrhodamine methyl ester.

EPIDEMIOLOGY (COHORT STUDY OR CASE-CONTROL STUDY)

WILEY *Journal of* Clinical Periodontology

Chronic periodontitis impairs polymorphonuclear leucocyte–endothelium cell interactions and oxidative stress in humans

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Abstract

Aim: To evaluate the relationship between oxidative stress parameters in polymorphonuclear leucocytes (PMNs) and PMN–endothelial cell interactions in patients with chronic periodontitis (CP) according to different degrees of severity of the disease.

Materials and methods: For this cross-sectional study, 182 subjects were divided into four groups according to degree of CP: without CP ($n = 37$), mild CP ($n = 59$), moderate CP ($n = 51$), and severe CP ($n = 35$). We determined anthropometric and biochemical variables, periodontal parameters, inflammatory markers, oxidative stress parameters (superoxide and mitochondrial membrane potential), and PMN–endothelium cell interactions (rolling flux, velocity, and adhesion).

Results: Systemic inflammatory markers—C-reactive protein, leucocyte count, TNFα, and retinol-binding protein 4—were altered in the group with CP. Total superoxide was augmented in patients with moderate and severe periodontitis, whereas mitochondrial membrane potential did not change. Furthermore, PMNs adhesion and rolling flux were increased in subjects with CP.

Conclusion: In a systemic proinflammatory environment, PMNs from patients with CP exhibit hyperactivity and produce higher amounts of superoxide. In parallel with this, an increase in PMNs rolling flux and cell adhesion to the endothelium suggests the presence of alterations of PMN–endothelium interactions in patients with CP that can lead to atherosclerosis and cardiovascular complications.

KEYWORDS
endothelial dysfunction, humans, oxidative stress, periodontitis, reactive oxygen species

1 | INTRODUCTION

Periodontitis is a multifactorial chronic inflammatory disease characterized by breakdown of the tooth-supporting tissues. It results from a complex interaction between periodontopathogens

and the host immune system caused by dysregulation of the host inflammatory response to bacterial infection (Page & Kornman, 1997).

In periodontitis, host cells release proinflammatory cytokines against pathogens in the gingival sulcus which stimulate infiltration

of polymorphonuclear leucocytes (PMNs), the first line of cellular host defences. An exacerbated inflammatory response to bacterial plaque leads to the release of reactive oxygen species (ROS) such as hydrogen peroxide and superoxide from leucocytes that, together with an imbalance of antioxidant defences, results in oxidative stress and apoptosis of connective periodontal tissue (Chapple & Matthews, 2007; Kanzaki et al., 2017). Previous studies have reported oxidative stress in periodontitis, both locally (periodontal tissues, gingival crevicular fluid [GCF], and saliva) and peripherally (serum and plasma) (for review see Wang, Andrukhov, & Rausch-Fan, 2017), pointing to potential mechanistic links between periodontitis and systemic inflammatory diseases. However, only few studies have focused on the production of ROS by PMNs and most have been carried out in stimulated conditions (Fredriksson, Gustafsson, Bergström, & Asman, 2003; Gustafsson, Ito, Asman, & Bergström, 2006; Matthews, Wright, Roberts, Cooper, & Chapple, 2007 and Matthews, Wright, Roberts, Ling-Mountford, et al., 2007; Ling, Chapple, & Matthews, 2016).

On the other hand, epidemiological studies have shown that periodontitis may play a role in subclinical atherosclerotic cardiovascular diseases in humans (Tonetti, 2009; Southerland et al., 2012). In fact, systemic inflammation caused by periodontitis contributes to the development and maintenance of atherosclerosis, which is preceded by endothelial dysfunction (Gurav, 2014). In brief, the first stage of endothelial dysfunction is heralded by the movement and accumulation of leucocytes in the vessel wall, which are mediated by an interaction between the adhesion molecules expressed on white blood and/or endothelial cells. We have previously demonstrated an association between oxidative stress in PMNs and endothelial dysfunction in type 2 diabetes and obesity (Hernández-Mijares et al., 2013; López- Domènech et al., 2018). Although there have been recent reports of a significant connection between chronic periodontitis (CP) and endothelial dysfunction (Orlandi et al., 2014; Moura et al., 2017), to the best of our knowledge, no previous study has evaluated the association between oxidative stress and endothelial dysfunction in PMNs of patients with CP.

Therefore, since leucocytes play a critical role in mediating oxidative stress and the destruction of connective periodontal tissues, and given the strong evidence of an association between periodontitis and subclinical atherosclerosis markers, the primary outcome of the present study was to evaluate the relationship between oxidative stress parameters in PMNs and PMN–endothelial cell interactions in patients with CP, according to degree of severity of the disease. A second aim was to explore a possible correlation between these factors and different clinical periodontal parameters.

2 | MATERIALS AND METHODS

2.1 | Subjects

Patients between the ages of 20 and 65 years attending the Outpatient's Department of the Stomatology Service of the University Hospital Dr. Peset (Valencia, Spain) from June 2015 to

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Clinical Relevance

Scientific rationale for the study: Recent studies have reported a significant association among chronic periodontitis (CP), oxidative stress, and endothelial dysfunction, but no previous study has evaluated the redox status and PMN–endothelial cell interactions in PMNs of patients with CP.

Principal findings: PMNs from patients with CP showed higher adhesion to the endothelium and rolling flux associated with the presence of impaired redox status and a pro-inflammatory profile augmented, being more evident in patients with severe periodontitis.

Practical implications: Special importance should be given to the diagnosis and treatment of periodontitis in patients with cardiovascular risk to avoid cardiovascular complications.

March 2017 were recruited for the present cross-sectional study. Exclusion criteria were fewer than 14 teeth, infectious or other oral inflammatory diseases, to have received periodontal or antibiotic treatment in the previous 6 or 3 months, respectively, to be under systemic anti-inflammatory treatment, pregnancy or lactation, severe disease including malignancies, alcohol or drug abuse, psychiatric disorders, and a history of cardiovascular or chronic inflammatory disease or diabetes mellitus according to the American Diabetes Association criteria. Data concerning current medication and smoking habit (yes or no) were recorded.

Study subjects were clustered in four groups depending on the degree of CP: without CP, mild CP, moderate CP, and severe CP, defined according to the Centers for Disease Control and Prevention/American Academy of Periodontology (CDC/AAP) (Eke, Page, Wei, Thornton-Evans, & Genco, 2012). Subjects without CP were matched by sex, age, and BMI.

Mild periodontitis was defined as the existence of at least two interproximal sites with clinical attachment loss (CAL) ≥3 mm and at least two interproximal sites with probing depth (PD) ≥ 4 mm (not in the same tooth) or one site with PD ≥ 5 mm. Moderate periodontitis was defined as at least two interproximal sites with CAL ≥ 4 mm (not in the same tooth) or at least two interproximal sites with PD ≥ 5 mm (in different teeth). Severe periodontitis was defined as at least two interproximal sites with CAL ≥ 6 mm (not in the same tooth) and at least one interproximal site with PD ≥ 5 mm.

This human observational study—reported according to Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines—followed the ethical principles stated in the Declaration of Helsinki. All procedures involving humans were approved by the hospital's Ethics Committee, and all the participants, as well as umbilical cord donors, gave their written informed consent.

2.2 | Clinical periodontal determinations

A full-mouth periodontal examination was performed to measure PD, CAL, and gingival bleeding on probing (BOP) at six sites per tooth for all teeth, excluding third molars, and the Silness and Löe simplified Plaque Index was employed to score six representative Ramfjörd teeth as described previously (Martinez-Herrera et al., 2017). All periodontal assessments were recorded using a conventional manual periodontal probe PCP UNC-15 (Hu-Friedy, Chicago, IL, USA).

2.3 | Anthropometric and biochemical determinations

The following data were collected for all the participants: weight (kg), height (m), body mass index (BMI; kg/m^2), and systolic and diastolic blood pressure (SBP/DBP mmHg). Weight was determined by an electronic scale (with an approximation of 0.1 kg), height was measured with a stadiometer with an approximation of 0.5 cm, BMI was calculated by dividing the weight in kilograms by the square of the height in metres (kg/m^2), and blood pressure was measured using an automatic sphygmomanometer following a 5-min rest period (Omron M3, Kyoto, Japan).

Blood samples were drawn from the antecubital vein after 12 hr overnight fasting. Glucose, total cholesterol (TC), and triglycerides (TG) levels were evaluated in serum using an enzymatic method. HDL cholesterol (HDLc) was determined by a direct method in a Beckman LX20 analyser (Beckman Corp., CA, USA). The Friedewald formula was used to calculate LDL cholesterol (LDLc) when TG were below 300 mg/dl. Insulin levels were measured by immunoassay, and insulin resistance was calculated (HOMA-IR = (fasting insulin (μU/ml) × fasting glucose (mg/dl)/405)).

Immunonephelometric assay (Dade Behring BNII, Marburg, Germany) was used to quantify circulating high-sensitive C-reactive protein (hsCRP), serum levels of tumour necrosis factor alpha (TNFα) were determined with a Luminex® 200 analyser system (Austin, TX, USA), retinol-binding protein four (RBP4) systemic levels were assessed by means of nephelometry assay (Dade Behring, Marburg, Germany), and leucocytes count was determined using a Sysmex ME-8000 autoanalyser.

2.4 | Cell isolation

Citrated blood samples were incubated with dextran 3% for 45 min. The resulting supernatant was centrifuged by density gradient in Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden) for 25 min at 650 *g* in order to isolate PMNs. Erythrocyte lysis was performed, and the pellet was then washed and resuspended in HBSS (Sigma Aldrich, MO, USA). Finally, cells were counted using a Scepter 2.0 cell counter (Millipore Corporation, Billerica, MA, USA).

2.5 | Evaluation of oxidative stress parameters

In order to evaluate oxidative stress parameters, we employed a life cell imaging method to detect fluorescent markers in which cells remain adherent and vital during the whole procedure. PMNs were seeded in a 48-well plate at $1.5 × 10^5$ cells/well for fluorescence determinations. Dihydroethidium dye (DHE) and tetramethylrhodamine methyl ester (TMRM) working solutions were prepared at 5 μM in HBSS immediately before use, and cells were incubated for 30 min at 37°C to detect cytoplasmic superoxide (DHE) and mitochondrial membrane potential (TMRM). The nuclei were visualized using the specific nuclear stain Hoechst 33342. Fluorescence was detected with an IX81 Olympus fluorescence microscope, and CellR software (Olympus, Shinjuku, Tokyo, Japan) was employed to capture individual images. The fluorescent signal was quantified individually (20 live cell images/well) by static cytometry software "ScanR" version 2.03.2 (Olympus). Fluorescence arbitrary units of DHE and TMRM from each subject were normalized with the values of an external cell line, Hep3B because of their fast growing ratio and metabolic stability and competence (Zhu, Wang, & Tong, 2007). FCCP 10 mM (uncoupler of oxidative phosphorylation) and rotenone 25 μM (complex I inhibitor) were used as positive controls (Labbe, Pessayre, & Fromenty, 2008).

2.6 | Adhesion assay

Polymorphonuclear leucocytes interaction with the human endothelium was assessed in vitro using a parallel plate flow chamber. Previously, human umbilical vein endothelial cells (HUVECs) had been harvested from fresh umbilical cords of healthy donors and seeded on coverslips at $1 × 10^3$ cells/mm^2. Cells were grown in complete EMB-2 culture medium (Lonza, Basel, Switzerland) until confluent and the coverslips were then inserted in the bottom plate of a flow chamber. A PMN suspension ($1 × 10^6$ cell/ml) in RPMI medium (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) was drawn across a monolayer of HUVECs (flow rate 0.36 ml/min) and visualized by an inverted microscope (Nikon Eclipse TE 2000-S; Minato, Tokyo, Japan) coupled to a video camera (Sony Exware HAD; Koeln, Germany). A five-minute period of flow across a 5 × 25 mm portion of the coverslip was recorded and then used to evaluate rolling velocity, rolling flux, and adhesion, as described previously (López-Domènech et al., 2018).

No agonists were added in the course of the experiments to promote expression of adhesion molecules. However, platelet-activating factor (1 μM, 1 hr) and TNFα (10 ng/ml, 4 hr; Sigma Aldrich) were used in parallel to the main experiments as positive controls for activation of PMNs and HUVECs, respectively.

2.7 | Statistical analysis

The study was designed based on preliminary data (Hernández-Mijares et al., 2013) to detect a 20% and 80% difference in the variation of PMN–endothelium interactions (measured by rolling velocity, rolling flux, and adhesion of PMNs) between and within groups, respectively, with a power of 90% and an α risk of 0.05. Under these premises, at least 26 subjects per group were considered.

The statistics software SPSS 19.0 (SPSS Statistics Inc., Chicago, IL, USA) was employed for data analysis. Continuous variables were expressed as mean and standard deviation (*SD*) for parametric data, whereas non-parametric data were expressed as median and 25th and 75th percentiles. When data did not show normal distribution, values were normalized using a log transformation. Qualitative data were expressed as percentages, and proportions were compared by means of a chi-square test. Data were compared using a Student's *t* test for parametric samples or one-way ANOVA followed by a Student–Newman–Keuls post hoc test. Pearson's correlation coefficient was used to evaluate the strength of linear association between two variables. A confidence interval of 95% was determined for all the tests, and differences were considered statistically significant when $p < 0.05$.

3 | RESULTS

This study analysed a total of 182 subjects (66 men and 116 women) classified according to CP diagnosis: 37 subjects without CP and 145 subjects with CP, of which 59 had mild periodontitis, 51 had moderate periodontitis, and 35 had severe periodontitis. Most of the patients in the present cohort were obese with BMI ≥ 30 kg/m^2 (63.7%), which is in line with the fact that a high prevalence of periodontitis has been described in obese population (Martinez-Herrera et al., 2017).

Anthropometric, biochemical, and periodontal parameters of the study population are outlined in Table 1. Severe periodontitis was associated with ageing, and moderate and severe periodontitis were associated with alterations in the lipid profile showing higher

TABLE 1 Anthropometric, biochemical, and representative periodontal parameters of the study population according to the presence or absence of chronic periodontitis

		With CP			
	Without CP	**Mild**	**Moderate**	**Severe**	**All**
Anthropometric variables					
n (% females)	37 (56.8)	59 (71.2)	51 (70.6)	35 (48.6)	145 (65.5)
Age	40.0 ± 11.4[a]	42.3 ± 11.3[a]	43.7 ± 10.2[a]	48.6 ± 9.2[b]	44.3 ± 10.7
BMI (kg/m^2)	32.5 ± 9.8	35.3 ± 11.7	35.7 ± 8.7	37 ± 9.4	36.2 ± 10.2
SBP (mmHg)	125 ± 14	127 ± 17	131 ± 20	135 ± 16	130 ± 17
DBP (mmHg)	77 ± 10	79 ± 11	82 ± 13	83 ± 12	81 ± 12
Biochemical parameters					
Glucose(mg/dl)	90 ± 11	93 ± 12	93 ± 13	96 ± 13	93 ± 12
Insulin (μU/ml)	12.0 ± 10.3	14.9 ± 14.9	14.7 ± 8.6	19.3 ± 15.4	15.9 ± 13
HOMA-IR	2.78 ± 2.69	3.51 ± 3.78	3.42 ± 2.12	4.75 ± 4.02	3.77 ± 3.37
TC (mg/dl)	188 ± 34	189 ± 31	181 ± 33	190 ± 34	187 ± 32
HDLc (mg/dl)	50 ± 14[a]	50 ± 13[a]	44 ± 13[b]	43 ± 9[b]	46.4 ± 12
LDLc (mg/dl)	120 ± 28	119 ± 25	112 ± 26	120 ± 32	116 ± 27
TG (mg/dl)	73 (55,125)[a]	101 (62,133)[a,b]	116 (82,163)[b]	139 (86,172)[b]	111 (71,159) *
Medication and life style habits					
Antihypertensive (%) (n)	16.0 (4)	11.9 (7)	25.5 (13)	28.6 (10)	20.7 (30)
Statin medication (%) (n)	4.0 (1)	11.9 (7)	11.8 (6)	20.0 (7)	13.8 (20)
Current smokers (%) (n)	24.0 (6)	27.1 (16)	25.5 813)	22.9 (8)	25.5 (37)
Periodontal parameters					
PD (mm)	2.46 ± 0.21[a]	2.76 ± 0.20[b]	2.97 ± 0.30[c]	3.55 ± 0.51[d]	3.02 ± 0.45***
CAL (mm)	2.46 ± 0.22[a]	2.76 ± 0.21[b]	2.99 ± 0.31[c]	3.66 ± 0.62[d]	3.06 ± 0.52***
BOP (%)	11.4 ± 6.5[a]	20.7 ± 11.3[b]	26.6 ± 11.7[c]	38.1 ± 15.0[d]	27.0 ± 14.1***
Plaque index (A.U)	0.587 ± 0.473[a]	0.916 ± 0.542[b]	1.02 ± 0.64[b]	1.31 ± 0.66[c]	1.05 ± 0.62**

Notes. A.U: arbitrary units; BMI: body mass index; BOP: bleeding of probing; CAL: clinical attachment loss; CP: chronic periodontitis; DBP: diastolic blood pressure; HDLc: HDL cholesterol; HOMA-IR: homoeostasis model assessment of insulin resistance; LDLc: LDL cholesterol; PD: probing depth; SBP: systolic blood pressure; TC: total cholesterol; TG: triglycerides.

Data are presented as mean ± *SD* or percentage (n). For TG are represented as median and IQ range. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ when patients with CP and individuals without CP were compared with an unpaired Student's *t* test. Values with different superscript letters (a,b,c,d) were significantly different when the four groups were compared by one-way ANOVA followed by a Student–Newman–Keuls post hoc test. Hence, means with the same superscript are not significantly different from each other ($p > 0.05$), while means that have no superscript in common are significantly different from each other ($p < 0.05$).

TG levels and lower levels of HDLc. However, no differences were found in sex, BMI, SBP, DBP, glucose, insulin, HOMA-IR, TC, LDLc, medical treatment, or smoking habit among the groups. Periodontal clinical parameters that indicate disease—PD, CAL, and BOP— worsened progressively as the severity of periodontitis increased, whereas plaque index was higher in subjects with CP and peaked in the severe CP group.

Inflammatory parameters, such as TNFα and RBP4, were associated with CP and increased with the severity of periodontitis ($p < 0.001$ and $p = 0.025$, respectively), even after adjustment for age (Figure 1a,d). Other systemic inflammatory markers were also altered in the group with CP, in which there was an increase in hsCRP levels ($p = 0.033$) and leucocytes count ($p = 0.020$), although no differences were observed according to the degree of periodontitis (Figure 1b,c).

3.1 | Evaluation of oxidative stress parameters

To investigate whether periodontitis promotes oxidative stress and alters mitochondrial function, we employed static cytometry to determine total superoxide and mitochondrial membrane potential in PMNs. As shown in Figure 2, total cytoplasmic superoxide (Figure 2a and representative images in Figure 2c) was increased in patients with CP ($p = 0.038$), particularly so in subjects with moderate and severe periodontitis ($p = 0.040$), whereas mitochondrial membrane potential (Figure 2b) was unaltered by the presence/absence of CP or grade of the disease.
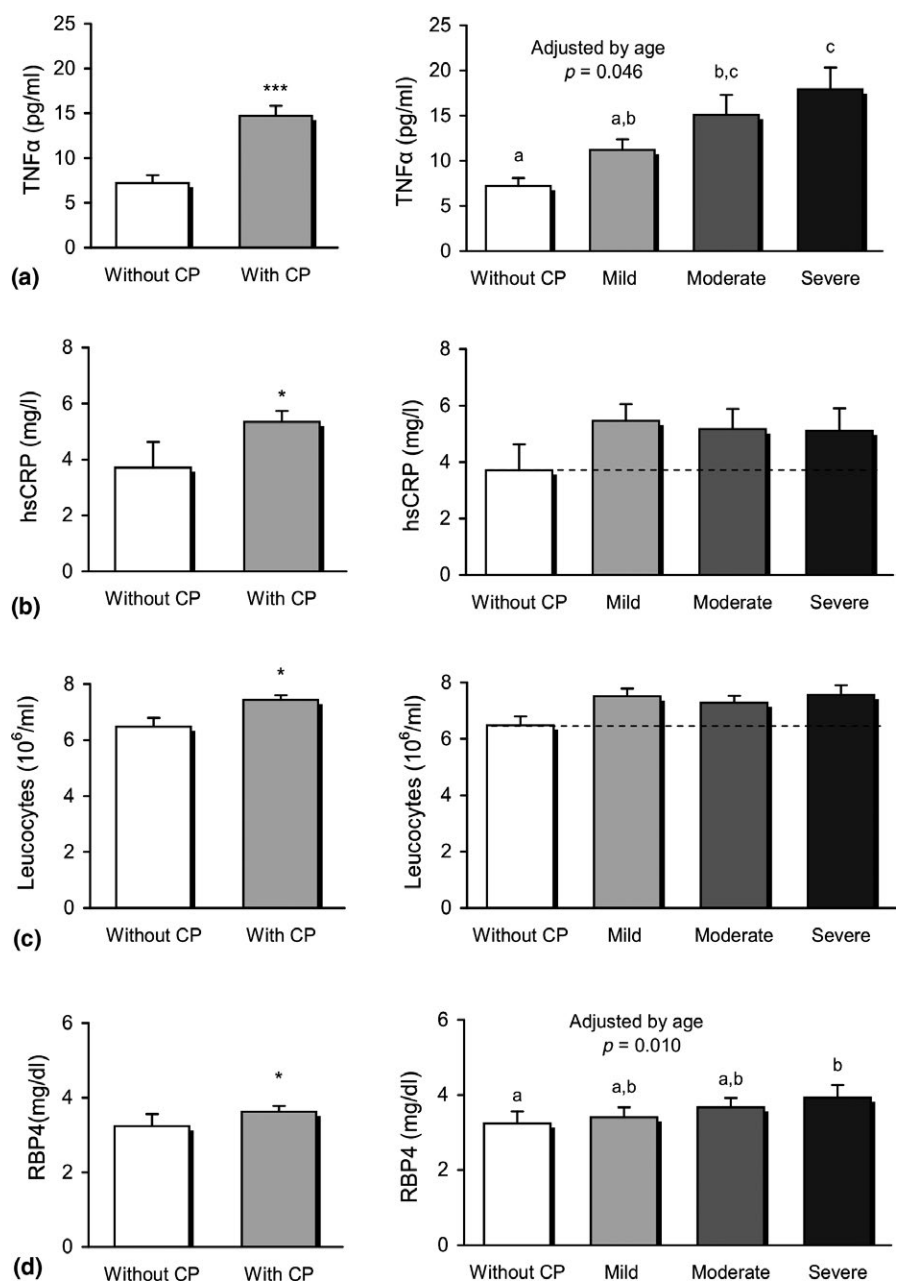
FIGURE 1 Inflammatory parameters of the study population according to the presence or absence of chronic periodontitis. Levels of TNFα (a) and hsCRP (b), leucocytes cell count (c) and RBP4 levels (d). Data are presented as mean + standard error. * $p < 0.05$; *** $p < 0.001$ when data of patients with CP and individuals without CP were compared with an unpaired Student's *t* test. Values with different superscript letters [a,b,c] were significantly different ($p < 0.05$) when the four groups were compared by one-way ANOVA followed by a Student–Newman–Keuls post hoc test. Hence, means with the same superscript are not significantly different from each other ($p > 0.05$), while means that have no superscript in common are significantly different from each other ($p < 0.05$). CP: chronic periodontitis; hsCRP: high-sensitive C-reactive protein; RBP4: retinol-binding protein 4; TNFα: tumour necrosis factor alpha
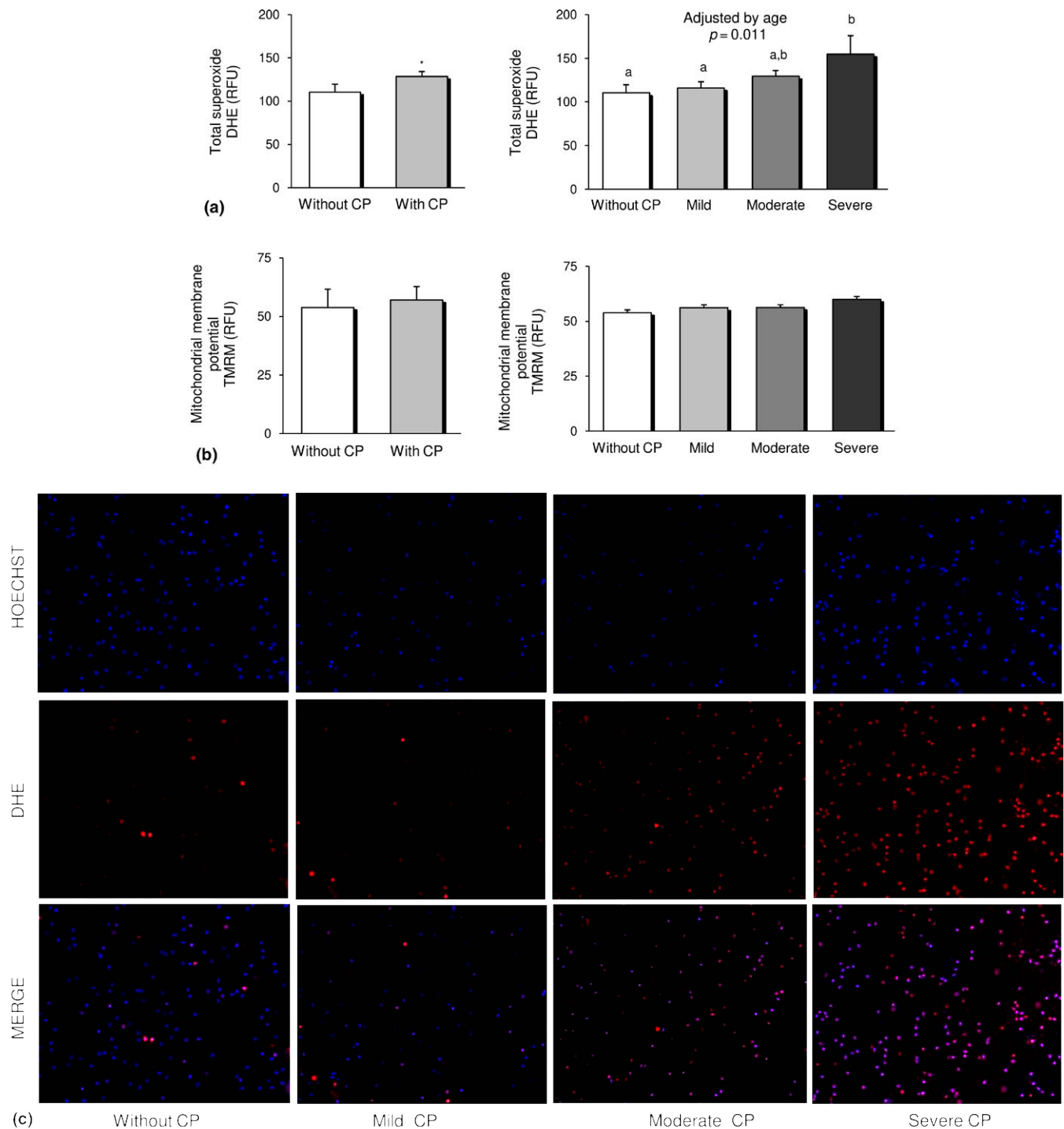
FIGURE 2 Oxidative stress parameters of the study population according to the presence or absence of chronic periodontitis. Mean of fluorescence intensity of DHE (a) and TMRM (b) dyes measuring superoxide and mitochondrial membrane potential, respectively. Representative fluorescence images showing DHE (c) intensity (red signal). The nuclei were visualized using the specific nuclear stain Hoechst 33342 (blue). Data are presented as mean + standard error. * $p < 0.05$ when data of patients with CP and individuals without CP were compared with an unpaired Student's *t* test. Values with different superscript letters [a,b] were significantly different when the four groups were compared by one-way ANOVA followed by a Student–Newman–Keuls post hoc test. Hence, means with the same superscript are not significantly different from each other ($p > 0.05$), while means that have no superscript in common are significantly different from each other ($p < 0.05$). CP: chronic periodontitis, DHE: dihydroethidium, TMRM: tetramethylrhodamine methyl ester, RFU: relative fluorescent units

3.2 | PMN–endothelial cell interaction assay

We also evaluated PMN–endothelium interactions under flow conditions, observing a slight and progressive reduction in PMN rolling velocity as severity of periodontitis increased, though this was not significant (Figure 3a). As a whole, PMN rolling flux (Figure 3b; $p = 0.026$) and cellular adhesion (Figure 3c; $p = 0.038$) increased in subjects with CP. Moreover, we observed that PMN rolling flux increased with the degree of severity of periodontitis ($p = 0.037$) and that these differences were maintained even after adjusting for age (Figure 3b).

3.3 | Correlation analysis

Correlation coefficients between periodontal, inflammatory, oxidative stress, and PMN–endothelium cell interactions parameters are shown in Table 2. All periodontal parameters were positively correlated with leucocytes count, suggesting a main inflammatory component of CP. In addition, PD and CAL correlated with PMN rolling

flux ($r = 0.273$, $p = 0.040$ and $r = 0.285$, $p = 0.032$, respectively) and plaque positively correlated with cellular adhesion of PMNs ($r = 0.271$, $p = 0.042$). In reference to oxidative stress parameters, superoxide positively correlated with inflammatory parameters— TNFα ($r = 0.448$, $p = 0.025$), hsCRP ($r = 0.344$, $p = 0.007$ and RBP4 ($r = 0.284$, $p = 0.024$), mitochondrial membrane potential ($r = 0.360$, $p = 0.011$), and cellular adhesion of PMNs ($r = 0.313$, $p = 0.045$), and negatively with rolling velocity of PMNs ($r = -0.290$, $p = 0.047$). In addition, TNFα and RBP4 correlated positively with PMN rolling flux ($r = 0.464$, $p = 0.022$; $r = 0.301$, $p = 0.032$, respectively).

4 | DISCUSSION

In the present study, we demonstrate an alteration of PMN–endothelium cell interactions in subjects with CP, in whom PMN adhesion and rolling flux increase with the presence of periodontitis. Moreover, this response is associated with the presence of impaired
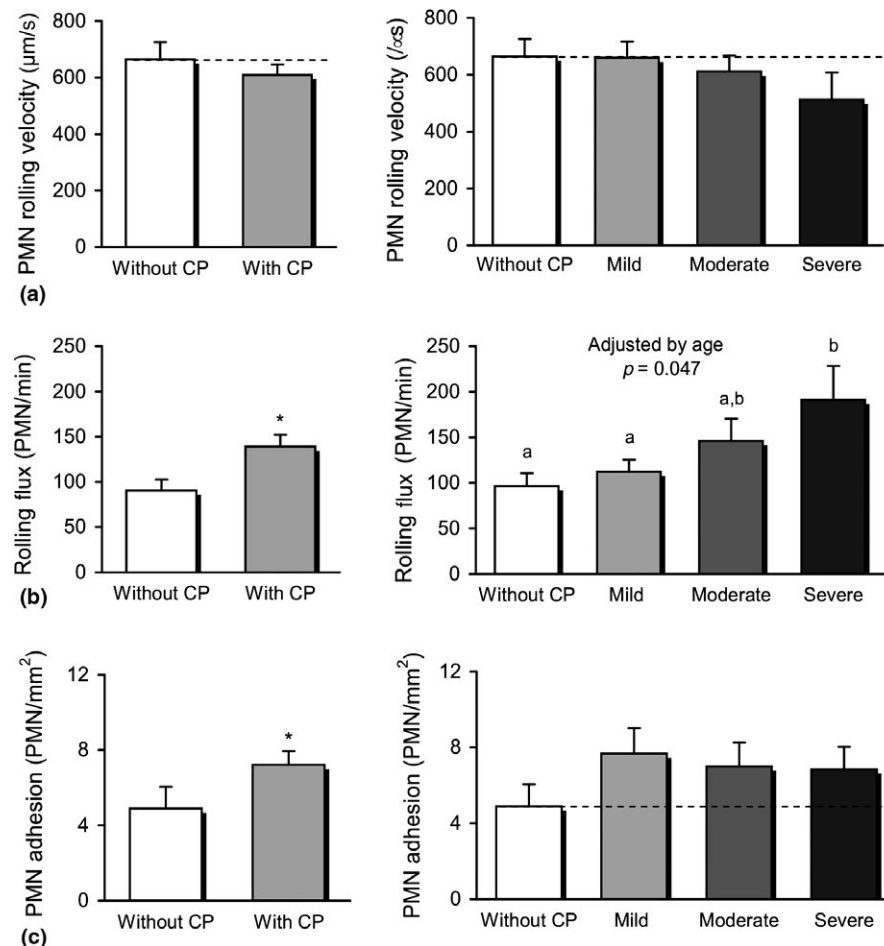
FIGURE 3 Endothelial function of the study population according to the presence or absence of chronic periodontitis determined by PMN–endothelial interactions evaluated by PMN rolling velocity (a), PMN rolling flux (b), and PMN adhesion (c). Data are presented as mean + standard error. * $p < 0.05$ when data of patients with CP and individuals without CP were compared with an unpaired Student's *t* test. Values with different superscript letters [a,b] were significantly different when the four groups were compared by one-way ANOVA followed by a Student–Newman–Keuls post hoc test. Hence, means with the same superscript are not significantly different from each other ($p > 0.05$), while means that have no superscript in common are significantly different from each other ($p < 0.05$). CP: chronic periodontitis, PMN: polymorphonuclear leucocytes

TABLE 2 Correlation coefficients between periodontal, inflammatory, oxidative stress, and PMN–endothelium interaction parameters in the study population

	PD	CAL	BOP	Plaque	TNFα	hsCRP	Leucocytes	RBP4	DHE	TMRM	Rolling velocity	Rolling flux	Adhesion
PD	–	0.984***	0.604***	0.406***	n.s	n.s	0.172*	n.s	n.s	n.s	n.s	0.273*	n.s
CAL	–	–	0.567***	0.394***	n.s	n.s	0.166*	n.s	n.s	n.s	n.s	0.285*	n.s
BOP	–	–	–	0.575***	n.s	0.235**	0.159*	n.s	n.s	n.s	n.s	n.s	n.s
Plaque	–	–	–	–	n.s	0.198*	0.204**	n.s	n.s	n.s	n.s	n.s	0.271*
TNFα	–	–	–	–	–	0.279*	n.s	0.315**	0.448*	0.545**	n.s	0.464*	n.s
hsCRP	–	–	–	–	–	–	0.315***	n.s	0.344**	0.463***	n.s	n.s	n.s
Leucocytes	–	–	–	–	–	–	–	0.178*	n.s	0.355**	n.s	n.s	n.s
RBP4	–	–	–	–	–	–	–	–	0.284*	0.326*	n.s	0.301*	n.s
DHE	–	–	–	–	–	–	–	–	–	0.360*	−0.290*	n.s	0.313**
TMRM	–	–	–	–	–	–	–	–	–	–	n.s	n.s	n.s
Rolling velocity	–	–	–	–	–	–	–	–	–	–	–	0.704***	−0.451***
Rolling flux	–	–	–	–	–	–	–	–	–	–	–	–	0.339**
Adhesion	–	–	–	–	–	–	–	–	–	–	–	–	–

Notes. BOP: bleeding of probing; CAL: clinical attachment loss; DHE: dihydroethidium (to detect total superoxide); hsCRP: high-sensitive C-reactive protein; PD: probing depth; PMNs: polymorphonuclear leucocytes; RBP4: retinol-binding protein-4; TMRM: tetramethylrhodamine methyl ester (to assess mitochondrial membrane potential); TNFα: tumour necrosis factor alpha.

Data are expressed as Pearson's correlation and statistical significance *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ for each pair of variables. When correlation is not significant, we represent it as n.s.

redox status in PMNs and a pronounced proinflammatory profile, being more evident in patients with severe periodontitis.

Oxidative stress is associated with the pathogenesis of many systemic diseases, including CP. Increased ROS levels are a hallmark of the inflammation induced by neutrophils when they combat invading bacteria, and are involved both directly and indirectly in periodontal tissue destruction (Chapple & Matthews, 2007; Kanzaki et al., 2017). However, oxidative damage is not exclusive to gingival tissues, saliva, and GCF; several studies demonstrate an increase in oxidant status and oxidative damage in the systemic circulation of individuals with periodontitis compared with periodontally healthy controls, as well as a generalized imbalance of antioxidant capacity (Akalin, Baltacıoğlu, Alver, & Karabulut, 2007; D'Aiuto et al., 2010; Baltacıoğlu et al., 2014; Ahmadi-Motamayel, Goodarzi, Jamshidi, & Kebriaei, 2017). Nonetheless, studies of ROS generation by peripheral blood neutrophils in periodontal disease are scarce. While the most consistent evidence for neutrophil hyper-reactivity in CP has been provided by a series of reports by a Swedish research group that have observed higher levels of ROS generation by Fcγ-receptor-stimulated peripheral neutrophils (Fredriksson et al., 2003; Gustafsson et al., 2006), only two studies have shown that neutrophils of periodontitis patients release more extracellular ROS and superoxide than neutrophils of healthy controls, even in the absence of any stimulation (Matthews, Wright, Roberts, Cooper, et al., 2007; Ling et al., 2016). In accordance with this, we now report an imbalance in redox status in subjects with CP due to higher cytoplasmic levels of total superoxide that rise as the severity of periodontal disease increases. However, superoxide assessment with DHE involves some limitations; namely, instability of the probe and its products, complex chemistry, and potential interference with heme enzymes (Zielonka et al., 2008; Dikalov & Harrison, 2014), which could have interfered with our findings. Therefore, superoxide determinations must be interpreted carefully. As far as we know, this is the first research to be performed in which superoxide and mitochondrial membrane potential have been determined in PMNs of patients with different grades of CP. In this context, mitochondrial membrane potential is critical for maintaining the physiological function, as it mediates the cell's capacity to generate ATP by oxidative phosphorylation. In fact, a decrease in the mitochondrial membrane potential may be linked to apoptosis (Lemasters et al., 2002), as has been shown in type 2 diabetes patients (Hernández-Mijares et al., 2013), whereas an increase has been associated to a surplus nutrient supply, as we have recently demonstrated in obese patients (López-Domènech et al., 2018). However, in the present study we have not observed changes in mitochondrial membrane potential, suggesting that different underlying molecular mechanisms are involved in the PMNs dysfunction that characterizes different inflammatory-related pathologies.

Despite mitochondrial membrane potential levels remaining unchanged and limitations of DHE to determine cytoplasmic superoxide, our results suggest an imbalance in oxidant status and PMNs dysfunction that could promote the hyperactive PMN phenotype seen in CP. Recently, it has been shown that superoxide release by neutrophils significantly correlates with hsCRP in plasma (Ling et al.,

2016), which is in line with our findings. This association could be partially explained by the fact that hsCRP enhances TLR-mediated superoxide release from neutrophils, potentially increasing oxidative stress (Ling, Chapple, Creese, & Matthews, 2014). Nevertheless, in our study population no significant association between severity of periodontitis and hsCRP levels was found, which is in accordance with the findings of a recent study (Delange et al., 2018).

In this line, other inflammatory molecules, including TNFα (Nishimura et al., 2003; Gonçalves et al., 2015) and RBP4 (Martinez-Herrera et al., 2018), have generally been found to be higher in periodontitis patients than in healthy controls and have been related with atherosclerosis. It has been shown that TNFα activates endothelial cells at the site of inflammation, leading to oxidative stress via escalating ROS production by endothelial cells (Yan et al., 2015). This, in turn, mediates local leucocyte accumulation, adherence, and subsequent transmigration into the subendothelial space, which is an early phase of the atherosclerotic process. In addition, a previous study showed that RBP4 induced vascular oxidative damage and endothelial dysfunction, thus accelerating the development of atherosclerosis in periodontal disease (Wang et al., 2015), which is in accordance with the positive correlation between PMN rolling flux and inflammatory markers—TNFα and RBP4—we report herein.

Previous studies have revealed compromised endothelial function in subjects with periodontitis (Amar et al., 2003; Higashi et al., 2008; Moura et al., 2017) and an improvement after periodontal treatment (Mercanoglu et al., 2004; Tonetti et al., 2007; Higashi et al., 2008; Piconi et al., 2009). In the current study, evaluation of PMN–endothelium cell interactions revealed impaired endothelial function—enhanced PMN rolling flux and cell adhesion to the endothelium—in subjects with CP. Moreover, when we divided the population into groups according to severity of periodontitis we observed that the rolling flux of PMNs was greater in subjects with moderate and severe periodontitis. In accordance with our results, a previous study found that, when compared to controls, endothelial function was significantly worsened in patients with severe periodontitis, but not in those with mild periodontitis (Amar et al., 2003). Chronic systemic inflammation caused by periodontitis is likely to culminate in endothelial dysfunction through a decrease in nitric oxide (NO) bioavailability, a decrease in NO production, and/or an increase in NO inactivation, which in turn promotes inflammation of the vascular wall, contributing to a vicious circle of endothelial dysfunction and low-grade inflammation (Higashi et al., 2008; Gurav, 2014). The underlying mechanism of this process seems to involve increased oxidative stress in the cardiovascular system (Cai & Harrison, 2000). In fact, in a rodent model, it has recently been shown that local periodontal inflammation induces systemic endothelial dysfunction caused by overproduction of ROS in the systemic artery (Yamamoto et al., 2016), which is in line with our findings. In addition, our data reveal an association among inflammatory markers (TNFα and RBP4), superoxide production, and PMN–endothelium cell interaction, suggesting a role of oxidative stress and inflammation as underlying mechanisms associated to the atherosclerotic process in CP.

To the best of our knowledge, previous studies reporting impaired endothelial function in patients with periodontitis have

employed flow-mediated dilatation of the brachial artery and vascular ultrasound to obtain measurements. In this sense, our study is the first to evaluate endothelial dysfunction by means a flow-condition adhesion assay based on an in vitro model of PMN–endothelial cell interactions and PMNs function and assessment of oxidative stress parameters. However, the present study has some limitations, including the relatively small size of the study population, although it has been supported by sample size calculation. In addition, we did not determine cellular adhesion molecules or the presence of the atherosclerotic plaque in our patients, parameters which we will evaluate in the future. Furthermore, despite being widely used for superoxide detection, the DHE fluorescence probe has some limitations, since other nonspecific redox reactions could act as confounders of DHE-superoxide determinations. More specific techniques, such as HPLC, should be carried out to corroborate our findings. On the other hand, whether changes in intracellular signalling in PMNs are related to the interaction of these cells with the endothelium and the subsequent risk of atherosclerosis and cardiovascular disease in patients with CP is a question that needs exploring. Finally, the cross-sectional nature of this study limits its interpretability.

To sum up, the data in the current study demonstrate that, in a systemic proinflammatory environment, PMNs from CP patients exhibit hyperactivity by increasing cytoplasmic production of ROS (such as superoxide), which alters PMN–endothelium interactions by promoting an increase in PMN rolling flux and cell adhesion to the endothelium. This can lead to atherosclerosis, resulting in cardiovascular complications. Therefore, special importance should be given to the presence and treatment of periodontitis in patients with risk of cardiovascular disease.

CONFLICT OF INTEREST

The authors declare no potential conflict of interests with respect to the authorship and/or publication of this article.

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ARTICLE

Molecular Biology

Dietary weight loss intervention improves subclinical atherosclerosis and oxidative stress markers in leukocytes of obese humans

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Abstract

Background The relationship between caloric restriction-mediated weight loss and the generation of ROS and its effects on atherosclerotic markers in obesity is not fully understood. Therefore, we set out to investigate whether dietary weight loss intervention improves markers of oxidative stress in leukocytes and subclinical parameters of atherosclerosis.

Subjects and Methods This was an interventional study of 59 obese subjects (BMI > 35 kg/m^2) who underwent 6 months of dietary therapy, including a 6-week very-low-calorie diet (VLCD) followed by an 18-week low-calorie diet (LCD). We determined clinical parameters, inflammatory markers—hsCRP, TNFα and NFκB –, oxidative stress parameters—total superoxide, glutathione, catalase activity and protein carbonyl groups–, soluble cellular adhesion molecules—sICAM, sP-selectin, sPSGL-1 –, myeloperoxidase (MPO), leukocyte-endothelium cell interactions—rolling flux, velocity and adhesion—and LDL subfractions, before and after the dietary intervention.

Results After losing weight, an improvement was observed in the patients' anthropometric, blood pressure and metabolic parameters, and was associated with reduced inflammatory response (hsCRP, TNFα and NFκB). Oxidative stress parameters improved, since superoxide production and protein carbonyl content were reduced and antioxidant systems were enhanced. In addition, a significant reduction of subclinical markers of atherosclerosis—small and dense LDL particles, MPO, sP-selectin and leukocyte adhesion—and an increase in soluble PSGL-1 were reported.

Conclusions Our findings reveal that the improvement of subclinical atherosclerotic markers after dietary weight loss intervention is associated with a reduction of oxidative stress in leukocytes and inflammatory pathways, suggesting that these are the underlying mechanisms responsible for the reduced risk of cardiovascular disease in obese subjects after losing weight.

Introduction

Obesity is a low-degree chronic inflammatory disease associated with an increased risk of developing a variety of

metabolic disorders, including insulin resistance, dyslipidemia, arterial hypertension, diabetes mellitus, coronary heart disease and stroke, as well as some types of cancers [1].

Mitochondrial dysfunction and high reactive oxygen species (ROS) production are considered adverse cellular responses to nutrient excess. In fact, mitochondrial dysfunction and enhanced ROS production have been observed in leukocytes and adipocytes of omental and subcutaneous tissues from obese subjects [2–4]. Therefore, an increase in ROS production favors an imbalance between oxidant and antioxidant factors, which can lead to oxidative stress.

Oxidative stress and inflammation are closely interrelated and play a key role in the pathogenesis of atherosclerosis [5, 6]. Atherosclerosis is triggered by endothelial dysfunction and induction of inflammation, which is accompanied by an increased expression of cell adhesion molecules (CAMs) such as intercellular adhesion molecule-1 (ICAM-1),

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vascular cell adhesion molecule-1 (VCAM-1) and P-selectin, which stimulates the adhesion of leukocytes and their transmigration into the vascular subendothelial space [7, 8].

Myeloperoxidase (MPO) is a heme enzyme derived mainly from neutrophils and monocytes, and plays a key role in leukocyte-mediated vascular injury responses. Such responses include oxidation of LDL, rendering it atherogenic and HDL, impairing its capacity to promote cholesterol efflux [9].They also lead to a reduction in nitric oxide (NO) bioavailability, which leads to endothelial dysfunction [10]. This series of detrimental effects has promoted the idea that MPO is an active mediator of atherogenesis [11].

We have previously reported that a proinflammatory state can stimulate the release of ROS from leukocytes, which contributes to the oxidative stress, mitochondrial impairment and endothelial dysfunction that follow insulin resistance in pathologies such as type 2 diabetes, polycystic ovary syndrome and obesity [4, 12–15].

However, the effect of dietary weight loss intervention on the generation of ROS and its consequences on atherosclerotic markers in obesity has been poorly studied. It is possible that dietary modifications help to ameliorate the inflammatory response, to reduce leukocyte ROS generation and/or to enhance the antioxidant system, consequently improving leukocyte function and cardiometabolic risk factors.

Therefore, the current study was performed to throw light on the effect of dietary therapy on leukocyte activation, oxidative stress and endothelial dysfunction. The primary endpoint was the effect of dietary weight loss intervention on leukocyte-endothelium cell interactions. Clarifying whether weight loss improves markers of oxidative stress in leukocytes and exploring its association with subclinical markers of atherosclerosis were secondary endpoints.

Subjects And Methods

Subjects

The study was an interventional study carried out in fifty-nine patients with a BMI > 35 kg/m² who were referred to the outpatient department of the Endocrinology and Nutrition Service at the Dr. Peset University Hospital in Valencia (Spain) to be treated for their obesity. The study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the hospital's Ethics Committee. Written informed consent was obtained from all patients.

Exclusion criteria were pregnancy or lactation, severe disease, history of cardiovascular disease or chronic inflammatory disease and secondary obesity (hypothyroidism, Cushing's syndrome).

After an initial evaluation, patients underwent treatment consisting of a 6-week VLCD (very-low-calorie diet) in a liquid formula (Optisource Plus®, Nestlé S.A., Vevey, Switzerland) containing 52.8 g protein, 75 g carbohydrates, 13.5 g fat and 11.4 g of fibre. The energy provided by this formula was 2738 kJ per day (654 kcal per day). The formula provided the vitamins, minerals and trace elements that are essential according to Recommended Dietary Allowances. After this period, patients were submitted to a low-calorie diet (LCD) for 18 weeks with an average daily energy intake of 5023–7535 kJ (1200–1800 kcal) (recommended according to caloric requirements), of which 15–20% was proteins, 50–55% was carbohydrates and 28–33% was fats. A daily ingestion of more than two litres of calorie-free liquids was recommended.

Anthropometric parameters were evaluated as follows: weight was determined using electronic scales with an approximation of 0.1 kg and a capacity of up to 200 kg; height was measured with a stadiometer with an approximation of 0.5 cm; BMI was calculated by dividing the weight in kilograms by the square of the height in meters; blood pressure was measured twice consecutively using a sphygmomanometer; waist circumference was measured at the natural indentation between the 10th rib and the iliac crest using a metric tape with approximations of 0.5 cm.

Venous blood samples were collected from patients after 12 h overnight fasting at baseline and after 6 months of the dietary treatment.

Biochemical determinations

Levels of glucose, total cholesterol and triglycerides were determined in serum by an enzymatic method. HDL levels were obtained with a Beckman LX20 analyzer (Beckman Corp., Brea, CA, US) using a direct method. The intraserial variation coefficient was < 3.5% for all determinations. LDLc concentration was calculated using the Friedewald method. Insulin was determined by an immunochemiluminescence assay and insulin resistance was estimated using the Homeostasis Model of Assessment (HOMA-IR = (fasting insulin (µU/ml) x fasting glucose (mg/dl)/405)). Percentage of glycated hemoglobin (A1c) was measured with an automatic glycohemoglobin analyzer (Arkray Inc., Kyoto, Japan) and high-sensitive C-reactive protein (hsCRP) levels were quantified by an immunonephelometric assay (intra-assay CV < 4%). Leukocytes and neutrophils were determined in a COULTER® LH 500 Hematology Blood Analyzer from Beckman Coulter (Brea, CA, US).

Serum levels of TNFα and MPO, and the adhesion molecules soluble ICAM (sICAM-1) and soluble P-selectin (sP-selectin) were measured with a Luminex 200 analyzer system (Austin, TX, USA). Soluble P-selectin glycoprotein

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ligand-1 (sPSGL-1) was also determined in serum samples according to the manufacturer's instructions (Human PSGL-1 Platinum ELISA, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). All samples were tested in duplicate.

Oxidative stress parameters

Cell isolation

Leukocytes were isolated by incubating peripheral blood samples with 3% dextran for 45 min followed by a Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) density gradient separation. After an erythrocyte-removing step, the pellet was washed and resuspended in Hank's Balanced Salt Solution (Capricorn, Ebsdorfergrund, Germany).

Superoxide production

Aliquots of 10^5 cells were seeded in duplicate in 48-well plates and incubated with 5 μM dyhidroethidium (DHE, Thermo Scientific, Rockford, USA) and Hoechst 33342 nucleic acid stain (4 μM, Sigma-Aldrich, MO, USA) fluorescence dyes for 30 min at 37 °C. Images were obtained with an IX81 Olympus microscope coupled with the static cytometry software "ScanR" (Olympus, Hamburg, Germany) and analyzed to assess leukocyte superoxide production.

Total glutathione, catalase activity, and protein carbonyl content

Antioxidant status was determined based on total glutathione content in erythrocyte lysates, since they contain the highest concentrations of glutathione. This was done using a commercially available test kit (Glutathione Assay Kit, Cayman Chemical, MI, USA), according to the manufacturer's instructions. In addition, we determined serum catalase (CAT) activity and protein carbonyl content, again according to the manufacturer's instructions (Catalase Assay Kit, Cayman Chemical, MI, USA and Protein Carbonyl Content Assay Kit, Sigma-Aldrich, MO, USA, respectively). Experiments were performed in duplicate.

Western blotting

Total protein extraction from leukocytes was performed on ice. Cells were lysed for 15 min with an extraction buffer (20 mM HEPES pH 7.5, 400 mM sodium chloride, 20% Glycerol, 0.1 mM EDTA, 10 μM Na_2MoO_4, 0.5% NP-40) containing protease inhibitors (10 mM NaF, 1 mM $NaVO_3$, 10 mM PNP, 10 mM β-glycerolphosphate). The supernatant was collected after centrifugation for 15 min at $16,000 \times g$.

The total concentration of proteins was quantified in both cases using a bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, Waltham, MA, USA). Twenty-five μg of protein were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Target proteins were detected by incubating the membranes with mouse monoclonal anti-NFκB p65 antibody (#33–9900, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), and rabbit anti-actin antibody (Sigma-Aldrich, MO, USA) was used to assess loading protein control. HRP goat anti-mouse (Thermo Fisher Scientific, Waltham, MA, USA) and HRP goat anti-rabbit (Merck Millipore, MA, US) were employed as secondary antibodies. ECL plus reagent (GE Healthcare, Uppsala, Sweden) was used to detect the protein signal by chemiluminiscence, visualized by means of the Fusion FX5 acquisition system (Vilbert Lourmat, Marne La Vallée, France). Data were analyzed by densitometry with the Bio1D software (Vilbert Lourmat, Marne La Vallée, France).

Flow-chamber assay

An in vitro model of leukocyte-endothelial cell interactions was designed using a flow chamber coupled to an inverted microscope (Nikon Eclipse TE 2000-S). In short, coverslips with confluent monolayers of human umbilical vein endothelial cells (HUVEC) were inserted in the bottom plate of the flow chamber. One million leukocytes in 1 ml of RPMI medium (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) were drawn across the HUVEC at a flow rate of 0.36 ml/min. A video camera (Sony Exware HAD; Koeln, Germany) connected to the microscope was used to record a 5×25 mm portion of the endothelial cells during a 5-min period to evaluate different leukocyte parameters: rolling velocity was calculated by measuring the time it took 20 consecutive leukocytes to travel a distance of 100 μm within the field of focus; rolling flux was calculated by counting the number of leukocytes rolling over $100 \, \mu m^2$ of the HUVEC monolayer during a 1-min period; and adhesion was evaluated by counting the number of leukocytes that maintained stable contact with endothelial cells for 30 s. Platelet-activating factor (1 μM, 1 h) and tumoral necrosis factor (10 ng/ml, 4 h) were used as a positive control for leukocytes and HUVEC, respectively.

LDL subfractions

LDL subfractions were separated using the Quantimetrix Lipoprint® system (Redondo Beach, CA,USA) and were then identified and quantified using a computerized method developed for the Quantimetrix Lipoprint® system and NIH image program version 1.62 (Bethesda, MD, USA) for research purposes. The Liposure® (Quantimetrix

Table 1 Anthropometric and biochemical parameters of the study population before and after weight loss

	Before	After
n (females %)	59 (72.9)	
Age (years)	45.1 ± 9.3	
BMI (Kg/m^2)	44.3 ± 5.6	40.4 ± 4.7***
Weight (Kg)	120.3 ± 18.0	109.4 ± 15.5***
Waist (cm)	123 ± 15	115 ±13***
SBP (mmHg)	133 ± 17	127 ± 15**
DBP (mmHg)	85 ± 11	78 ± 9***
Glucose (mg/dl)	101 ± 21	97 ± 22*
Insulin (µU/ml)	18.2 ± 10.3	15.8 ± 9.2*
HOMA-IR	4.63 ± 3.06	3.94 ± 3.03*
A1c (%)	5.75 ± 0.72	5.61 ± 0.78*
TC (mg/dl)	184 ± 34	182 ± 36
HDLc (mg/dl)	40.8 ± 8.2	43.1 ± 9.5**
LDLc (mg/dl)	115 ± 31	115 ± 33
TG (mg/dl)	125 (97,177)	109 (83,146)**
hsCRP (mg/l)	6.99 (4.63,13.00)	6.20 (3.07,11.48)*
TNFα (pg/ml)	18.0 ± 8.89	14.6 ± 5.9*
Leukocytes (cellsx 10^3/µl)	7.53 ± 2.18	7.60 ± 2.02
Neutrophils (cellsx 10^3/µl)	4.51 ± 1.61	4.66 ± 1.59
Neutrophils (percentage)	59.3 ± 7.95	60.3 ± 7.64
Type 2 diabetes (%) (n)	22 (13)	
Treatment		
Hypertension (%) (n)	30.0 (18)	
Hyperlipidemia (%)	26.7 (16)	

Data are presented as mean ± SD or percentage (n). TG data are represented as median and IQ range. *p<0.05; **p<0.01; ***p<0.001 when compared with a paired Student's t-test or Wilcoxon test.

BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, A1c: glycated haemoglobin, TC: total cholesterol; LDLc: LDL cholesterol, HDLc: HDL cholesterol, TG: triglycerides, Apo: Apolipoprotein

Corporation, Redondo Beach, CA, USA) was used for quality control. VLDL, 3 intermediate-density lipoprotein (IDL) and 7 LDL were quantified. The LDL electrophoretic profile allows 3 patterns to be defined: pattern A / large and buoyant LDL (cut-off size of over 268 Å); an intermediate pattern (cutoff size over 265 and equal to or less than 268 Å); and pattern B / small and dense LDL (sdLDL) (cut-off size less than or equal to 265 Å). All samples were tested in duplicate.

Statistical analysis

The study was designed based on preliminary data [13, 15] in order to have a power of 80% and to detect differences between two paired means in relation to the primary efficacy criterion (minimum expected difference in leukocyte adhesion) ≥ 5 cells/mm^2, assuming a common standard deviation of 10 units. Under these premises, at least 32 subjects were considered.

For the statistical analysis of the data we employed the statistics program SPSS 19.0 software (SPSS Statistics Inc., Chicago, IL, USA). Continuous variables in the tables are expressed as mean ± SD, or as median and 25th and 75th percentiles for parametric and non-parametric data, respectively, whereas qualitative data are expressed as percentages. Data in the figures are represented as mean + SE. The data were analyzed using a paired Student's t test or a Wilcoxon test for parametric and non-parametric data, respectively. A X^2 test was employed to compare proportions. The correlation between variables was determined using Pearson's correlation coefficients. All the tests used a confidence interval of 95% and differences were considered significant when $p < 0.05$.

Results

This study analyzed a total of 59 patients (16 men and 43 women) with an average BMI of $44.3 ± 5.6$ kg/m^2. After 6 month adherence to a VLCD + LCD, anthropometric parameters—body weight, BMI, and waist circumference, systolic and diastolic blood pressure –, tryglicerides and hydrocarbonated metabolism parameters—glucose, insulin, HOMA-IR and A1%—all decreased significantly ($p < 0.05$), whereas HDLc increased ($p < 0.01$) (Table 1). Total cholesterol and LDLc remained unchanged, probably due to the antihyperlipidemic treatment in 26.7% of patients. In addition, the leukocyte defence system did not seem to be altered, since the number of total leukocytes or neutrophils remained within their normal range after weight loss (Table 1). However, systemic inflammatory markers were altered by weight loss. Specifically, acute phase reactants, such as hsCRP—which is known to be associated with BMI —and TNFα decreased after dietary therapy (Table 1). Furthermore, the nuclear factor NFκB p65, which has long been considered a prototypical proinflammatory signaling pathway, was also markedly reduced after weight loss (Fig. 1a) ($p < 0.01$).

To investigate whether weight loss improved oxidative stress, we employed static cytometry to determine ROS production. Total superoxide (Fig. 1b and Supplementary Figure 1) was significantly lower in leukocytes after dietary weight loss intervention ($p < 0.001$). Furthermore, an increase in antioxidant defences was confirmed, as glutathione levels were significantly higher in erythrocyte lysates (Fig. 1c) ($p < 0.001$), while a significant increase in serum catalase activity (Fig, 1d) ($p < 0.05$) was

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Fig. 1 Inflammatory and oxidative stress parameters in obese patients before and after dietary weight loss intervention. **a** Levels of NFκB p65 protein expression and representative western blot images ($n = 15$). **b** Total superoxide production, measured as arbitrary units of DHE fluorescence ($n = 33$). **c** Erythrocytes glutathione content ($n = 36$). **d** Catalase activity ($n = 38$) and (**e**) protein carbonyl content ($n = 29$) in serum. Data are represented as mean + SE. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ when compared using a paired Student's t test. DHE dihydroethidium, NFκB p65 nuclear factor κB p65, RFU relative fluorescence units
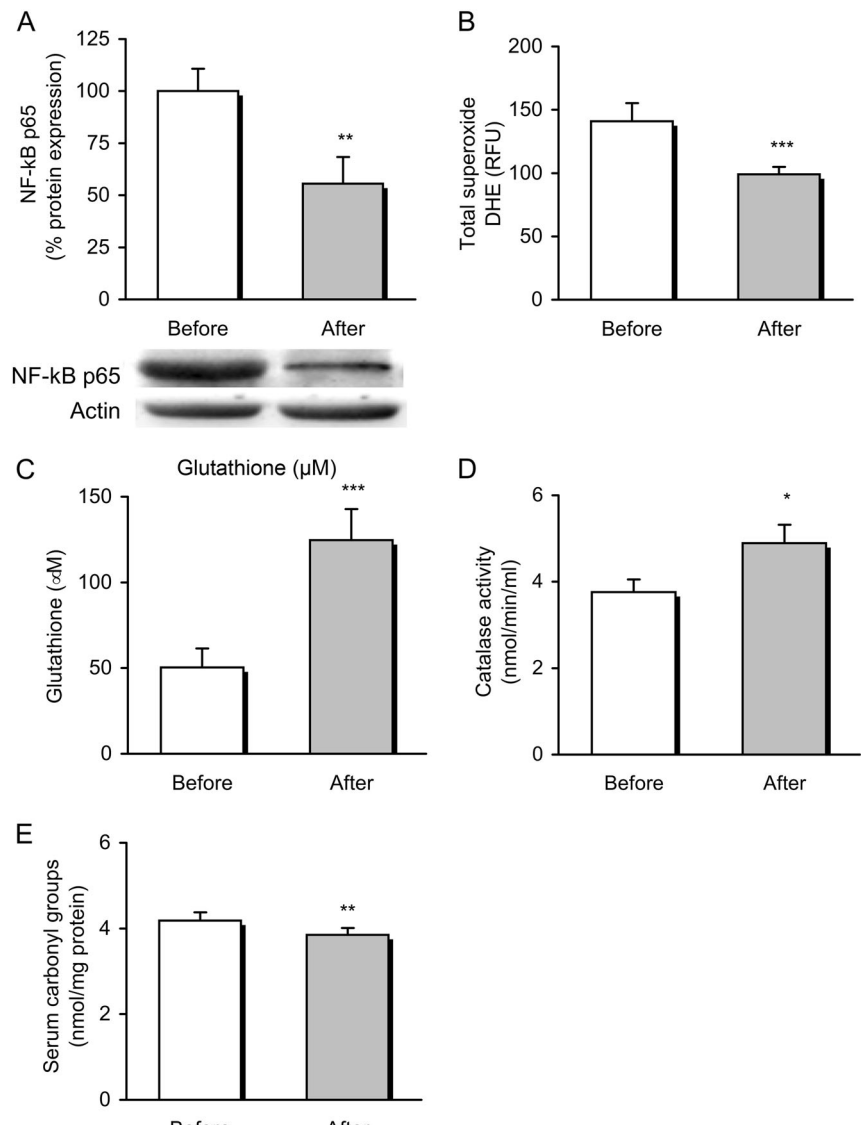
also observed. However, superoxide generation, total glutathione levels and catalase activity are not strictly markers of oxidative stress, as they only reflect either pro-oxidant agents or antioxidant agents. Thus, we determined protein carbonyl content in serum samples, detecting a reduction after dietary weight loss intervention (Fig. 1e) ($p < 0.01$). These findings suggest an undermining of oxidative stress parameters in blood cells. We next investigated whether or not these changes in oxidative stress parameters were associated with an improvement in adhesion under flow conditions. Dietary weight loss intervention induced a significant reduction in serum levels of soluble P-selectin (Fig. 2a) ($p < 0.05$), which binds to PSGL-1 on leukocytes. Strikingly, we observed an increase in soluble serum PSGL-1 ($p < 0.05$) after dietary weight loss intervention (Fig. 2b), suggesting that cleavage of the protein from the cell surface is one of the mechanisms involved in the deactivation process. These changes were associated with a reduced

cellular adhesion of leukocytes to the endothelium (Fig. 2g) ($p < 0.001$). Since MPO is a potent pro-oxidant derived mainly from neutrophils that mediate vascular damage, and is involved in the formation of proatherogenic LDL particles, we evaluated circulating MPO and LDL subfractions. Our results showed that MPO was markedly reduced after weight loss (Fig. 2d) ($p < 0.001$), despite the total leukocyte or neutrophil count remaining unchanged (Table 1), suggesting a reduced MPO expression by the leukocyte defence system. In addition, the percentage of small LDL particles decreased (Fig. 3a), while LDL particle size increased (Fig. 3b). As a consequence, the LDL electrophoretic pattern became less atherogenic, changing from a profile of 64% pattern A, 21% intermediate pattern and 15% pattern B to one of 85% pattern A, 6% intermediate pattern and 9% pattern B ($p < 0.05$). Finally, a negative correlation was observed between MPO levels and LDL particle size, both at baseline and after 6 months ($r = -0.523$ and $r = -0.542$,
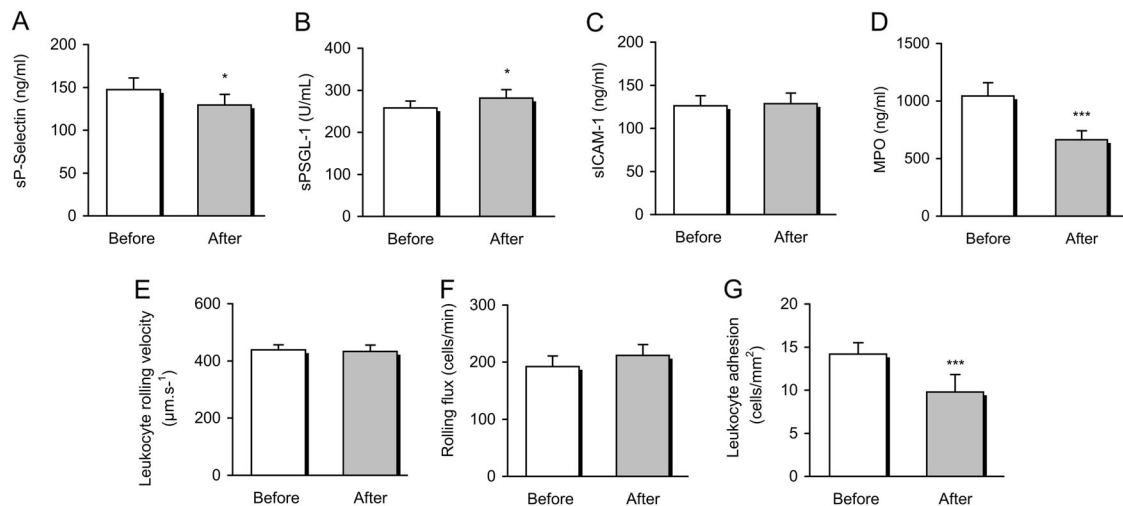
Fig. 2 Serum levels of atherosclerotic markers determined by commercial kits and leukocyte-endothelium cell interactions in obese patients before and after dietary weight loss intervention. Serum levels of (**a**) sP-selectin ($n = 20$), (**b**) sPSGL-1 ($n = 20$), (**c**) sICAM-1 ($n = 20$) and (**d**) MPO ($n = 20$). **e** Leukocyte rolling velocity expressed as μm/s ($n = 34$). **f** Leukocyte rolling flux measured as cells/min ($n = 34$). **g** Leukocyte adhesion expressed as cells/mm^2 ($n = 34$). Data are represented as mean + SE. *$p < 0.05$ and ***$p < 0.001$ when compared using a paired Student's t test. sP-Selectin soluble P-selectin and sPSGL-1 soluble P-selectin glycoprotein ligand-1, MPO Myeloperoxidase, sICAM-1 soluble intercellular adhesion molecule 1

respectively) (Fig. 4a, b), and a positive correlation existed between MPO and sP-selectin ($r = 0.545$ and $r = 0.415$, respectively) (Fig. 4c, d), suggesting an involvement of MPO in the atherosclerotic process.

Discussion

In our population of middle-aged morbid obese subjects, the moderate weight loss achieved by adherence to a 6-week VLCD followed by LCD for 18 weeks improved the main anthropometric and biochemical parameters and ameliorated the inflammatory response. In addition, pro-oxidant agents such as total superoxide and MPO were reduced and antioxidant capacity increased by higher production of glutathione and strengthened enzymatic antioxidant systems such as catalase activity: This contributed to a reduction of oxidative stress, as determined by protein carbonylation. These responses were associated with an increase of LDL particle size, a reduction of cellular adhesion molecules and less adherence of leukocytes to the endothelium, suggesting that these molecular mechanisms are involved in the diminished cardiovascular risk factor associated with dietary therapy in obese populations.

Cardiovascular disease and obesity are closely linked and take a substantial toll on the health of individuals when both are present. High-calorie diets and the resulting obesity are major risk factors for hypertension and coronary artery diseases. Modest weight loss of 5–10% ameliorates cardiometabolic risk factors, including hypertension and dyslipidemia, and improves health outcomes [16–18]. The role of lipids in the formation and evolution of the atheromatous plaque has been well documented. Numerous studies have demonstrated that the predominance of sdLDL particles correlated with the development and progression of atherosclerosis and earlier and more severe cardiovascular disease, even when LDL cholesterol is low [19–21]. As expected, our results showed a clear improvement in blood pressure and atherogenic dyslipidemia, including HDL cholesterol, triglycerides and LDL particle size, although total and LDL cholesterol remain unchanged, probably due to antihyperlipidemic treatment.

Similarly, we have observed that dietary weight loss intervention also produces a drop in blood glucose levels and insulin, resulting in a reduction of insulin resistance, which is in accordance with the results of previous research [22].

Growing evidence has highlighted an important role for oxidative stress in obesity, mainly in organs involved in energy metabolism, such as the pancreas, liver, skeletal muscle, white adipose tissue and heart. However, only a few studies have focused on leukocytes as the main mediators of the inflammatory response and atherogenesis. Previous studies have shown that circulating mononuclear cells in obese patients are in a proinflammatory state characterized by an increase in intranuclear NFκB and transcription of proinflammatory cytokines [23, 24]. Dietary weight loss intervention in overweight and obese individuals was shown to result in a decreased expression of genes involved in the activation of NFκB [25], which is in line with the results of the present study.

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Fig. 3 LDL subfractions in obese patients before and after dietary weight loss intervention determined by the Quantimetrix Lipoprint® system. **a** Percentage of small LDL particles from total lipoproteins containing cholesterol. **b** Mean LDL particle size, and (**c**) Changes in LDL electrophoretic profile are expressed as a percentage of sample size. Data are represented as mean + SE or as a percentage of LDL patterns of 35 patients. $*p < 0.05$ when compared using a paired Student's t test or a X^2 test. The LDL patterns A, intermediate and B refer to the size of LDL cholesterol particles in the blood. Pattern A (cut-off size of over 268 Å); intermediate pattern (cut-off size over 265 and equal to or less than 268 Å); and pattern B (cut-off size less than or equal to 265 Å). LDL low-density lipoproteins
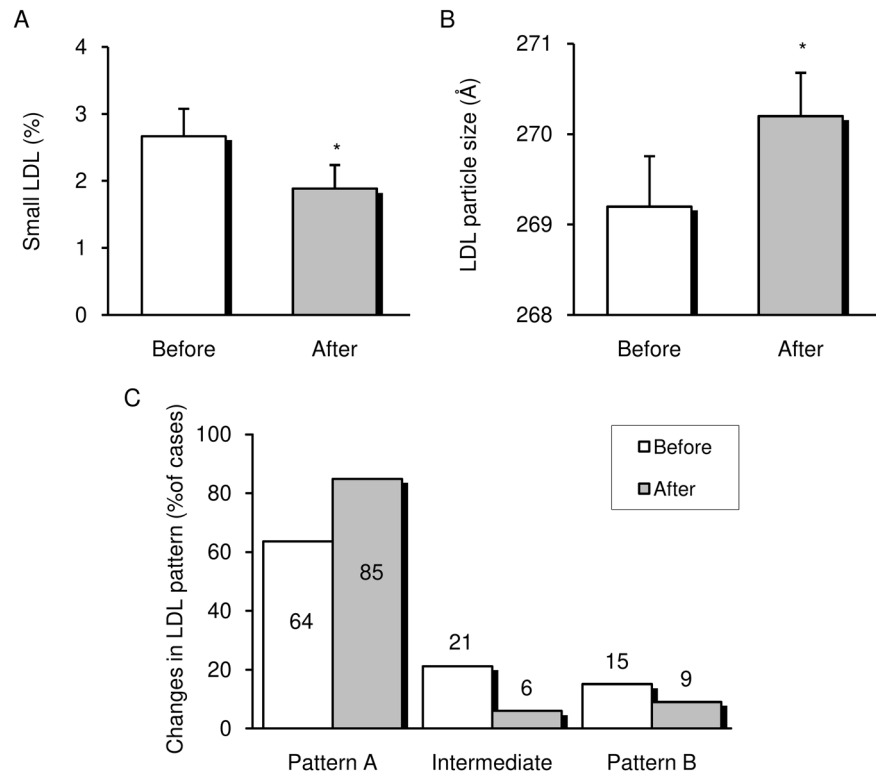
Regarding endothelial dysfunction, recent studies have demonstrated impaired brachial artery endothelial function and microvascular endothelial dysfunction in obese subjects, which was associated with an increase in the activity of NADPH oxidase [26, 27], one of the main enzymes producing superoxide. However, since vascular dysfunction may occur differentially in vascular beds, and endothelial cells differ in phenotype and structure depending on vessel type [28], we have focused on how the activation of leukocytes is involved in the atherosclerotic process. In this context, obesity has recently been associated with chronic oxidative and inflammatory stress, which leads to mitochondrial dysfunction, increased vascular damage and enhanced endothelial dysfunction markers [4, 27].

The findings of the present study represent a step further, since we have assessed the influence of dietary weight loss intervention. Previously, Dandona et al. showed that superoxide generation by polymorphonuclear leukocytes and peripheral blood mononuclear cells falls markedly after 4-week dietary restriction and the resulting weight loss [29]. Similarly, exercise has been shown to reduce ROS levels and to restore microvascular endothelial function to levels similar to those found in lean subjects [27], suggesting that the negative energy balance in obesity reduces oxidative stress and improves endothelial function. In the present study, we report that dietary weight loss intervention reduces total superoxide and strengthens antioxidant systems such as glutathione and catalase activity, leading to an improvement in oxidative damage, manifested by a reduction in protein carbonylation. It is widely known that superoxide is a highly reactive molecule with well-documented detrimental effects on vascular function, such as increased endothelial cell permeability, limited NO bioavailability and apoptosis [30, 31].

In line with this, our findings show that the improvement in redox status was associated with a reduction in systemic P-selectin levels and TNFα, which may have mediated the reduction in leukocyte recruitment. Since P-Selectin binds to PSGL-1 on leukocytes, it is likely that PSGL-1 expression in leukocytes decreases after dietary intervention, and therefore less leukocytes adhere to the vessel wall. Strikingly, we report an increase in soluble serum PSGL-1 after dietary weight loss intervention. Although there is little information available about the regulation of PSGL-1, previous studies have shown that stimulation of human neutrophils decreases the surface expression of PSGL-1 and increases its release from the surface, suggesting that cleavage of the protein from the cell surface is one of the mechanisms involved in the deactivation process. In addition, a decrease in surface expression of PSGL-1 on neutrophils has been shown to correlate with a decrease in neutrophil adhesion to P-selectin under both static and dynamic conditions [32], which is in line with the present findings. Regarding TNFα, there is a large body of evidence of TNFα-induced adhesion of leukocytes to endothelial cells [33, 34], and it

Fig. 4 Correlation coefficients between circulating MPO levels and atherosclerotic markers in obese patients before and after dietary weight loss intervention. Graphs represent correlations between MPO and LDL particle size (**a**) before and (**b**) after treatment and between MPO and serum levels of soluble P-selectin (**c**) before and (**d**) after weight loss. The data are shown as r correlation coefficient, p value and sample size. Correlation coefficients were estimated by Pearson's correlation for all parameters. MPO mieloperoxidase, LDL low-density lipoprotein
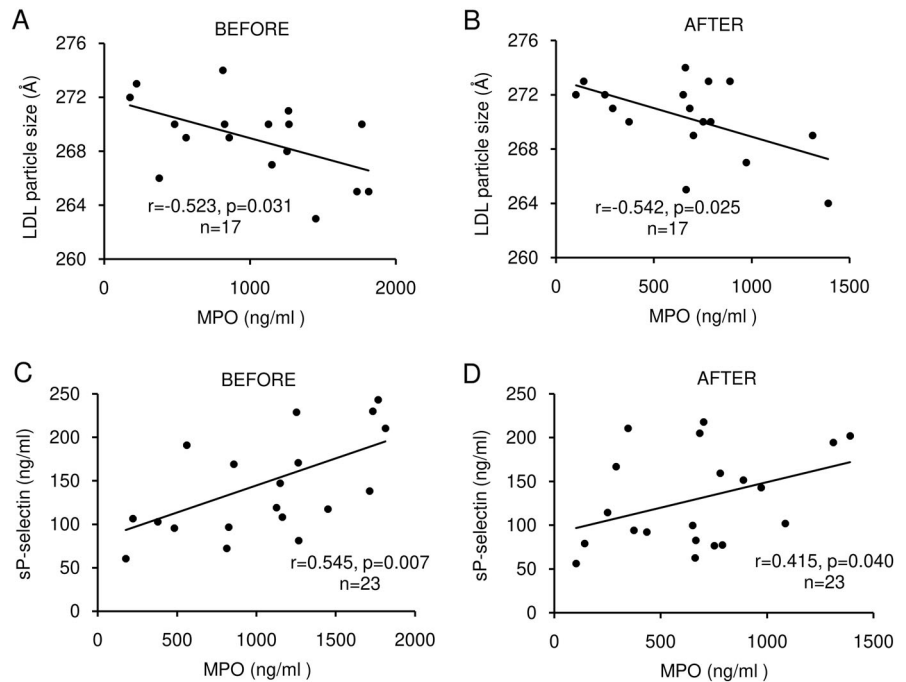
has also been shown that raised plasma levels of P-selectin can influence the early progression of vascular disease by promoting leukocyte adhesion to the endothelium [35].

Vascular damage may also be mediated by MPO, which binds to the surface of LDL [36], promoting the formation of oxidized lipoproteins that are not recognized by the LDL receptor, which in turn leads to the activation of endothelial cells and monocyte/macrophages and induces the release of proinflammatory cytokines such as TNFα [37]. In fact, clinical studies have highlighted elevated serum levels of MPO as a prognosis factor in patients with acute coronary syndromes [38] or chest pain [39], or in apparently healthy individuals with an increased risk of coronary artery disease [40]. This would suggest that leukocyte activation is stimulated many years before the onset of overt coronary artery disease, thus supporting the use of leukocytes to evaluate cardiovascular risk. The present study's demonstration of a parallel drop in reactive oxygen species generation and MPO activity in leukocytes, together with an enhancement of antioxidant defences, has important implications with respect to atherosclerosis; namely, leukocyte-mediated oxidative stress could be the mechanism underlying oxidative damage to LDL. In fact, we have observed a significant association between MPO and sdLDL and levels of soluble P-selectin.

Our data show clearly, to our knowledgr, for the first time, that generation of reactive oxygen species by leukocytes is undermined markedly and antioxidant systems are improved by dietary restriction, suggesting an amelioration of oxidative stress parameters. In addition, the reduction of

subclinical markers of atherosclerosis that we report—sdLDL, MPO, sP-selectin and leukocyte adhesion –may improve endothelial function. However, the present study has some limitations, including the size of the study population, which, although relatively small, was supported by sample size calculation. In addition, although we did not determine the presence of the atherosclerotic plaque in our patients, we did evaluate the onset of the atherosclerotic process; in other words, the first stages of endothelial dysfunction, which is heralded by the movement and accumulation of leukocytes in the vessel wall and enhanced levels of cellular adhesion molecules in a proinflammatory environment. Whether changes in intracellular signaling in leukocytes are related to the interaction of these cells with the endothelium and the subsequent risk of developing atherosclerosis and cardiovascular disease is a question that needs to be explored further.

To sum up, dietary weight loss intervention in obese patients is effective in diminishing cardiometabolic risk factors. Leukocytes could be largely responsible for this response, since they are one of the main mediators of inflammatory response and atherogenesis. The underlying mechanism appears to involve an improvement in oxidative stress status and leukocyte function that causes LDL particles to increase in size and undermines adhesion of leukocytes to the endothelium, thereby reducing the risk of cardiovascular events. Future exploration of this oxidative stress may help to clarify the nature of the molecular mechanisms involved and the physiological significance of weight loss as an effective therapy to reduce cardiovascular risk. Such knowledge would no doubt help to develop

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strategies to reduce the risk of the development of cardiovascular disease in obese populations.

Author contribution The authors' responsibilities were as follows. MR and VM-V contributed to the conception and design of the study. CB assisted in the design of the experiments and provided support throughout the course of the study. CM, SL-D and MM-H carried out the recruitment, diagnosis and follow-up of the patients in the study. SL-D, MM-H, ZA-J, IE-L and ND-M performed the laboratory analyses and collected data. SL-D, MM-H and MR analyzed the data, performed the statistical analysis and drafted the manuscript. MR, and VM-V critically revised the manuscript and were responsible for its final content. All authors read and approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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MOLECULAR
METABOLISM

Moderate weight loss attenuates chronic endoplasmic reticulum stress and mitochondrial dysfunction in human obesity

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ABSTRACT

Objective: In obese patients undergoing caloric restriction, there are several potential mechanisms involved in the improvement of metabolic outcomes. The present study further explores whether caloric restriction can modulate endoplasmic reticulum (ER) stress and mitochondrial function, as both are known to be mechanisms underlying inflammation and insulin resistance (IR) during obesity.

Methods: A total of 64 obese patients with BMI $\geq$35 kg/m^2 underwent a dietary program consisting of 6 weeks of a very-low-calorie diet followed by 18 weeks of low-calorie diet. We evaluated changes in the metabolic and inflammatory markers -TNFα, hsCRP, complement component 3 (C3c), and retinol binding protein 4 (RBP4)-, in the ER stress markers and modulators -eIF2α-P, sXBP1, ATF6, JNK-P, CHOP, GRP78, and SIRT1-, and in mitochondrial function parameters -mitochondrial reactive oxygen species (mROS), glutathione peroxidase 1 (GPX1), cytosolic Ca^{2+}, and mitochondrial membrane potential.

Results: The dietary intervention produced an 8.85% weight loss associated with enhanced insulin sensitivity, a less marked atherogenic lipid profile, and a decrease in systemic inflammation (TNFα, hsCRP) and adipokine levels (RBP4 and C3c). Chronic ER stress was significantly reduced (ATF6-CHOP, JNK-P) and expression levels of SIRT1 and GRP78 — a Ca^{2+}-dependent chaperone — were increased and accompanied by the restoration of Ca^{2+} depots. Furthermore, mROS production and mitochondrial membrane potential improvement were associated with the up-regulation of the antioxidant enzyme GPX1.

Conclusions: Our data provide evidence that moderate weight loss attenuates systemic inflammation and IR and promotes the amelioration of ER stress and mitochondrial dysfunction, increasing the expression of chaperones, SIRT1 and antioxidant GPX1.

1. INTRODUCTION

Obesity is a multifactorial disease associated with the appearance of several comorbidities, such as dyslipidemia, hypertension, and type 2 diabetes (T2D), the prevalence of which has risen significantly in the past decades in parallel with the rise in the obesity rate worldwide [1]. Metabolic overload and increase in fat accumulation during obesity favors the release of several adipokines and cytokines, contributing to systemic chronic low-grade inflammation, which is closely related to

the development of insulin resistance (IR) and other metabolic abnormalities [2]. Despite the emerging body of evidence supporting the role of inflammatory and stress responses in the context of obesity, the molecular pathways and mechanisms underlying these processes remain unclear.

It is known that the endoplasmic reticulum (ER) acts as a systemic nutrient sensor in peripheral tissues during obesity, in which elevated circulating levels of free fatty acids, glucose, or inflammatory cytokines may act as stress signals for the organelle [3]. The accumulation of

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Abbreviations: ATF6, activating transcription factor 6; BMI, body mass index; CHOP, CCAAT/enhancer binding protein [C/EBP] homologous protein; C3c, complement component 3; eIF2α-P, phosphorylated eukaryotic translation initiation factor 2 subunit 1; ER, endoplasmic reticulum; GPX1, glutathione peroxidase 1; GRP78, 78-kDa glucose regulated protein; IR, insulin resistance; IRE1α, inositol requiring enzyme 1 α; JNK, cJun NH$_2$-terminal kinase; LCD, low-calorie diet; PERK, protein kinase RNA-like endoplasmic reticulum kinase; ROS, reactive oxygen species; sXBP1, spliced X-box binding protein 1; SIRT1, Sirtuin 1; T2D, type 2 diabetes; UPR, unfolded protein response; VLCD, very-low-calorie diet

misfolded proteins during ER stress triggers the activation of the unfolded protein response (UPR) through three different leaders: the inositol requiring enzyme 1 α (IRE1α), activating transcription factor 6 (ATF6), and protein kinase RNA-like endoplasmic reticulum kinase (PERK). However, the failure of the adaptive response and the chronicity of the stress lead the UPR to generate the expression of pro-apoptotic factors such as CCAAT/enhancer binding protein [C/EBP] homologous protein (CHOP). Previous findings have described the role of CHOP in the cytokine-ER-stress-mediated apoptosis of pancreatic β-cells [4]. On the other hand, IRE1α kinase activity has been associated with IR through the cJun NH$_2$-terminal kinase (JNK) inflammatory pathway, partly as a result of serine phosphorylation of insulin receptor substrates (IRS1) [5]. In addition, JNK activation in macrophages has been related to increased tissue infiltration [6] and is known to play a key role in chronic inflammation in obesity [7]. In contrast, it has been reported that chemical chaperones that reduce ER stress improve insulin sensitivity in *ob/ob* mice [8] and β-cell function in humans [9], and we have recently described an improvement in ER stress and inflammatory markers in subcutaneous adipose tissue that was mediated by an insulin sensitizer [10]. This accumulated evidence of the adaptive capacity of ER supports a role for ER stress in human metabolic disease and points to potential novel therapeutic targets for the treatment of obesity and related disorders. However, how ER stress is modulated *in vivo* is a question yet to be answered.

Sirtuin 1 (SIRT1), a NAD$^+$-dependent protein deacetylase, is an important regulator of energy homeostasis in response to nutrient availability; its expression is down-regulated in adipose tissue [11] and peripheral blood mononuclear cells in obese populations and has been related with IR and metabolic syndrome [12]. Accumulating evidence shows that SIRT1 helps to regulate inflammatory [13] and ER stress responses in obesity, since both endogenous induction and over-expression of SIRT1 exert a protective role by alleviating ER stress and inflammatory markers in the liver [14,15] and adipose tissue [10,16]. Furthermore, an excess of energy substrates in obesity is believed to lead to increased mitochondrial dysfunction and reactive oxygen species (ROS) signaling, which may underlie IR [17,18], metabolic syndrome [19] and impaired endothelium function [20]. In fact, enhanced oxidative stress is reported to be increased in leukocytes and adipose tissue from obese patients and has been correlated with body mass index (BMI) [20,21].

Caloric restriction displays several metabolic benefits in the obese population, improving insulin signaling and reducing cardiovascular risk [22]. The molecular mechanisms implicated in these effects could be targeted to decelerate the progressive deterioration in the health of obese subjects, but, unfortunately, they are poorly understood. Since nutrient overload has been related to ER stress and mitochondrial dysfunction [23], the aim of the present study was to explore whether caloric restriction modulates UPR pathways during ER stress and improves redox status and mitochondrial function in human obesity, and to determine the role of inflammatory mediators such as SIRT1 and JNK.

2. MATERIALS AND METHODS

2.1. Subjects

Patients attending the Endocrinology and Nutrition Department at the University Hospital Dr. Peset (Valencia, Spain) were consecutively recruited as they were referred for treatment for their obesity.

Eligible participants were obese patients between 18 and 60 years of age that had maintained a stable weight (± 2 kg) over the 3 months prior to the study and whose disease duration was at least five years.

The inclusion criteria were BMI ≥ 35 kg/m^2, with or without associated comorbidities, including T2D diagnosed according to the American Diabetes Association Guidelines [24]. Exclusion criteria were pregnancy or lactation, severe disease, history of cardiovascular disease or chronic inflammatory disease and secondary obesity (hypothyroidism, Cushing's syndrome).

The study protocol was approved by the Ethics Committee of the Hospital (Code: 96/16) and was conducted according to the guidelines laid down in the Declaration of Helsinki. The dietary weight loss intervention was designed in accordance with the guidelines of the Spanish Society for the Study of Obesity (SEEDO) [25]. Written informed consent was signed by all the participants.

After an initial evaluation, patients underwent treatment consisting of a 6-week very-low-calorie diet (VLCD) in liquid formula (Optisource Plus$^®$, Nestlé S.A., Vevey, Switzerland), containing 52.8 g protein, 75.0 g carbohydrates, 13.5 g fat and 11.4 g of fiber and the vitamins, minerals and trace elements that are essential according to Recommended Dietary Allowances (RDA). The energy provided by this formula was 2738 kJ/day (654 kcal/day). Participants replaced their usual 3 meals a day with the commercially available meal replacement provided by the National Healthcare System, under prescription from the endocrinologist. After this period, patients met the dietician for dietary counseling. During the appointment, the patient was interviewed, weighed, and prescribed a further 18 weeks of low-calorie diet (LCD) following an estimate of the caloric requirements of each individual according to sex, height, weight, and physical activity level. This diet consisted of an average daily energy intake of 5023−7535 kJ (1200−1800 kcal) in accordance with the recommended caloric requirement: 15−20% proteins, 50−55% carbohydrates and 28−33% fats. After the experimental period, patients were re-evaluated by the dietician.

Throughout the study, subjects were given detailed written and oral instructions about their diet, including precise amounts to be eaten, and cooking methods. A daily ingestion of more than two litres of calorie-free liquids was recommended. Patients were encouraged to maintain their normal pattern of activity and to ask for dietary counseling if necessary. No modifications were made to drug prescriptions during the course of the study.

Anthropometrical parameters, including weight (kg), height (m), BMI (kg/m^2), waist circumference, and systolic and diastolic blood pressure (SBP and DBP) (mmHg) were measured in all the participants both at baseline and 6 months after dietary weight loss intervention. Blood samples of the patients were drawn from the antecubital vein during both appointments, after a 12 h fasting period.

2.2. Biochemical parameters

Biochemical determinations were performed at the Hospital's Clinical Analysis Service. An enzymatic method was used to determine serum levels of glucose, total cholesterol (TC) and triglycerides (TG). HDL cholesterol (HDLc) levels were obtained with a Beckman LX20 analyzer (Beckman Corp., Brea, CA, US) using a direct method. The intra-serial variation coefficient was <3.5% for all determinations. The Friedewald method was used to calculate levels of LDL cholesterol (LDLc) when triglycerides were under 300 mg/dl. Insulin was measured by a chemiluminescence immunoassay, and IR was estimated with the Homeostasis Model of Assessment (HOMA-IR = (fasting insulin (μU/ml) $\times$ fasting glucose (mg/dl)/405)). Percentage of glycated hemoglobin (A1c) was measured using an automatic glycohemoglobin analyzer. Levels of apolipoprotein (Apo) AI and B, high-sensitive C-reactive protein (hsCRP) and complement component 3 (C3c) were determined with an immunonephelometric assay whose intra-assay

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variation coefficient was <5.5%. Serum retinol binding protein 4 (RBP4) concentrations were measured by nephelometry with intra- and inter-assay coefficients of variation of 3.1% and 2.2%, respectively.

2.3. TNFα levels

Levels of TNFα in the serum were measured with a Luminex 200 analyzer system (Austin, TX, USA) by means of a Milliplex® MAP Kit (Merck Millipore, Burlington, MA, USA). The intra-serial and inter-serial variation coefficients were <5.0% and <15.0% respectively.

2.4. Cell isolation

Blood samples were incubated with dextran 3% for 45 min and subjected to centrifugation (650 g for 25 min at room temperature) in a Ficoll-Hypaque density gradient to isolate leukocyte fraction. After centrifugation, remnant erythrocytes were lysed and the pellet was washed with HBSS (Capricorn Scientific, Ebsdorfergrund, Germany).

2.5. Protein expression

Proteins were extracted by incubating leukocytes on ice for 15 min with lysis buffer (20 mM HEPES pH 7.5, 400 mM NaCl, 20% Glycerol, 0.1 mM EDTA, 10 μM Na$_2$MoO$_4$, 0.5% NP-40, 10 mM NaF, 1 mM NaVO$_3$, 10 mM PNP, 10 mM β-glycerolphosphate, 1 mM dithiothreitol). BCA protein Assay Kit (Thermo Scientific, Waltham, MA, USA) was used to quantify total protein content of the samples. 25 μg of protein were resolved in a SDS-PAGE, transferred onto nitrocellulose membranes and blotted with the following primary antibodies: monoclonal anti-SAPK/JNK-P (Thr183/Tyr185) from Cell Signaling (Danvers, MA, USA), polyclonal anti-SIRT1 from Merck Millipore (Burlington, MA, USA), monoclonal anti-GPX1, polyclonal anti-eIF2α-P (Ser52) and monoclonal anti-CHOP from Thermo Scientific (Waltham, MA, USA), monoclonal anti-ATF6 and polyclonal anti-GRP78 (BiP) from Abcam (Cambridge, UK). HRP-goat anti-mouse (Thermo Scientific, Waltham, MA, USA) and HRP-goat anti-rabbit (Sigma Aldrich, Kawasaki, Kanagawa, Japan) were used as secondary antibodies. A chemiluminescence signal was detected after incubation of the membrane with ECL plus reagent (GE Healthcare, Little Chalfont, UK) or Supersignal West Femto (Thermo Scientific, Waltham, MA, USA). Images were acquired and bands densitometrically analyzed with the Fusion FX5 system coupled to the Bio1D software (VilbertLourmat, Marne LaVallée, France).

2.6. Fluorescence microscopy

Leukocytes were seeded in duplicate in 48-well plates and incubated for 30 min at 37 °C with the following fluorogenic dyes: MitoSOX Red (5 μM) was used to assess mitochondrial ROS (mROS) production, Fluo-4 (1 μM) indicated levels of cytosolic Ca^{2+}, and tetramethylrhodamine methyl ester (TMRM, 5 μM) was used to evaluate changes in mitochondrial membrane potential. All the fluorescent probes were purchased from Invitrogen (Life Technologies, Carlsbad, CA, USA). Imaging was performed with an IX81 Olympus inverted fluorescence microscope and 20 images/well were analyzed with the static cytometry ScanR software 2.03.2 (Olympus, Hamburg, Germany).

2.7. Gene expression

Total RNA was extracted from leukocytes using the GeneAllR Ribospin™ total RNA extraction kit (Geneall Biotechnology, Seoul, Korea) according to the manufacturer's instructions. A total of 1 μg of RNA samples were reverse-transcribed using the RevertAid first-strand cDNA synthesis kit (Thermo Scientific, Waltham, MA, USA). Quantitative RT-PCR analysis was then performed using the FastStart Universal

SYBR Green Master (Roche Applied Science, Penzberg, Germany) and a 7500 Fast RT-qPCR system (Life technologies, Carlsbad, CA, USA), as described previously [26]. Spliced X-box binding protein 1 gene (s-xbp1; 103 pb) was amplified using the following primers: Forward 5′-CTGAGTCCGCAGCAGGTG-3′ and Reverse 5′-AACAGGATATCA-GACTCTGAATCTGAA-3′. The internal control gene gapdh (168 pb) was amplified using the following primers: Forward 5′-CGCATCTTCTTTTGCGTCG-3′ and Reverse 5′-TTGAGGTCAAT-GAAGGGGTCA-3′.

2.8. Statistical analysis

The study was designed based on preliminary data [22] in order to have a power of 80% and to detect differences between two paired means in relation to the primary efficacy criterion (minimum expected difference in mROS) ≥50 relative fluorescence units (RFU), assuming a common standard deviation of 100 units. Under these premises, at least 32 subjects were considered.

Statistical differences between variables before and after the dietary treatment were analyzed using the paired Student's t-test or the Mann Whitney U-test for non-parametric variables with SPSS 19.0 statistics software (SPSS Statistics Inc., Chicago, IL, USA). The strength of the association between variables was measured by means of Pearson's correlation coefficient. Continuous variables in tables are expressed as mean ± standard deviation (SD) for parametric data or as median and 25th and 75th percentiles for non-parametric data. Qualitative data are expressed as percentages. In the figures, data are represented as mean +standard error (SE). All the tests used a confidence interval of 95% and the threshold of significance for all the analyses was set at $p < 0.05$.

3. RESULTS

In the present study, we analyzed a total of 64 obese patients of middle age (43.5 ± 9.9 years) — mainly females of reproductive-age (n = 14), pre-menopausal (n = 16) and menopausal status (n = 16) — with an average BMI of 44.0 ± 5.7 kg/m^2. The 6-month VLCD + LCD treatment resulted in a significant reduction of body weight and BMI ($p < 0.001$), with an average weight loss of 8.85 ± 4.16%. Waist circumference, SBP and DBP ($p < 0.01$), as well as hydrocarbonated metabolic parameters such as insulin, HOMA-IR and A1c, decreased significantly ($p < 0.05$), whereas fasting glucose levels did not change. Regarding blood lipid profile, triglycerides were significantly decreased and HDLc increased after weight loss ($p < 0.01$), although we did not observe changes in either total cholesterol or LDLc (Table 1).

The dietary weight loss intervention induced significant changes in systemic inflammatory markers and adipokines. Serum levels of TNFα (Figure 1A) and hsCRP (Figure 1B) were lower after weight loss ($p < 0.05$). In addition, reductions in the adipokine RBP4 (Figure 1C) ($p < 0.05$) and in the cardiovascular risk marker C3c (Figure 1D) ($p < 0.01$) were detected at the end of the experimental period ($p < 0.05$).

The effect of dietary intervention on ER stress was evaluated by assessing differential expression of markers among the three branches of the UPR, as represented in Figure 2. No changes were observed in the activity of the PERK-eIF2α-P branch (Figure 2A) or in the endoribonuclease activity of IRE1α, determined as mRNA levels of spliced XBP1 (Figure 2B). On the contrary, the dietary weight loss intervention seemed to have a profound effect on the ATF6-UPR branch, since we observed a marked decrease of p50/cleaved ATF6 levels (Figure 2C) that was associated with a down-regulation of the proapoptotic

Table 1 — Anthropometric and biochemical parameters of the study population before and after dietary weight loss intervention.

	Before	After
n (females %)	64 (71.9)	64 (71.9)
Age (years)	43.5 ± 9.9	—
Weight (kg)	120 ± 18	109 ± 15
BMI (Kg/m^2)	44.0 ± 5.7	40.0 ± 4.8***
Waist (cm)	122 ± 14	114 ± 13***
SBP (mmHg)	134 ± 17	127 ± 15**
DBP (mmHg)	85 ± 11	78 ± 10***
Glucose (mg/dl)	100 ± 21	97 ± 22
Insulin (μU/ml)	17.5 ± 10.2	15.3 ± 9.1*
HOMA-IR	4.43 ± 3.02	3.79 ± 2.95*
A1c (%)	5.73 ± 0.70	5.60 ± 0.75*
TC (mg/dl)	184 ± 34	183 ± 41
HDLc (mg/dl)	41.4 ± 9.4	43.7 ± 10.8**
LDLc (mg/dl)	115 ± 30	116 ± 36
TG (mg/dl)	120 (89,174)	103 (83,143)**
Apo AI (mg/dl)	139 ± 25	143 ± 28
Apo B (mg/dl)	99 ± 25	96 ± 26
Treatment		
Oral antidiabetic drugs (%)	14.1	
Antihypertensive drugs (%)	29.7	
Lipid-lowering drugs (%)	26.6	

Data are presented as mean ± SD or percentage (n). For TG are represented as median and IQ range. *p < 0.05; **p < 0.01; ***p < 0.001 when compared with a paired Student's t-test or Wilcoxon test.

Apo, Apolipoprotein; A1c, glycated hemoglobin; DBP, diastolic blood pressure; HDLc, HDL cholesterol; LDLc, LDL cholesterol; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides.

molecule CHOP (Figure 2D). In addition, we detected a drop in levels of phosphorylated JNK (Figure 2E), a major regulator of the inflammatory process in leukocytes, which can be activated through IRE1α kinase activity during ER stress.

Based on the enhanced UPR expression profile, we decided to assess changes in ER stress modulators. Chaperones are major regulators of protein trafficking and processing in the ER. In line with this, protein expression of the chaperone 78-kDa glucose regulated protein (GRP78) was significantly up-regulated after dietary weight loss intervention (Figure 2F). In addition, increased expression of the anti-inflammatory mediator SIRT1 was observed in parallel with ER stress alleviation (Figure 2G).

The known link between ER and mitochondrial function led us to explore whether the dietary weight loss intervention modulated mitochondrial function in our obese population. We found that ER stress relief was associated with an improvement in oxidative stress and mitochondrial function parameters. In fact, glutathione peroxidase 1 (GPX1) expression was induced after dietary treatment (Figure 3A) (p < 0.05), and was accompanied by a significant decrease in mROS production of leukocytes after dietary treatment (Figure 3B). Simultaneously, leukocytes showed a significant drop off in cytosolic Ca^{2+} content (p < 0.05) (Figure 3C), which was indicative of reduced ER Ca^{2+} depletion and a marked decrease of mitochondrial membrane potential (p < 0.001) (Figure 3D), pointing to a restoration of mitochondrial function and cellular homeostasis following the dietary weight loss intervention.

Finally, when we explored possible associations among variations in molecular markers and clinical metabolic outcomes after dietary weight loss intervention, we found that percentage of change of HOMA-IR was correlated significantly with that of ATF6 (r = 0.478, p = 0.018, n = 24), JNK-P (r = 0.442, p = 0.016, n = 24) and CHOP — although in this latter case it did not reach statistical significance (r = 0.371, p = 0.075, n = 24) — pointing out to a relationship
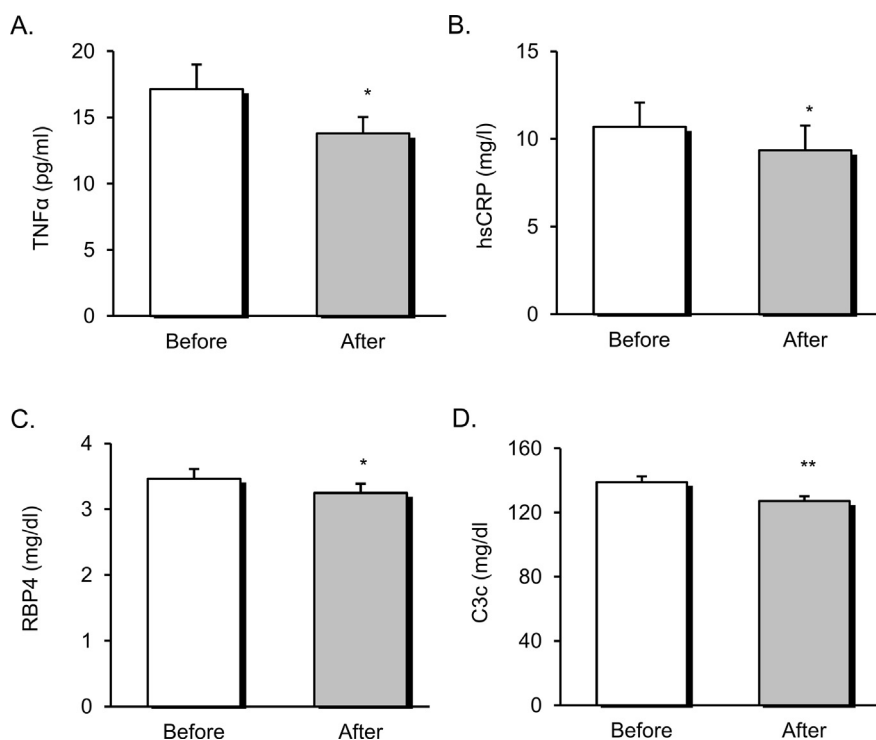

Figure 1: Systemic inflammatory markers and adipokines of obese patients before and after dietary weight loss intervention. Serum levels of TNFα (A), hsCRP (B), RBP4 (C), and C3c (D). Data are represented as mean +standard error. *p < 0.05; **p < 0.01, when compared using a paired Student's t-test. TNFα, tumor necrosis factor α; hsCRP, high sensitivity C-reactive protein; RBP4, retinol binding protein 4; C3c, complement component 3.
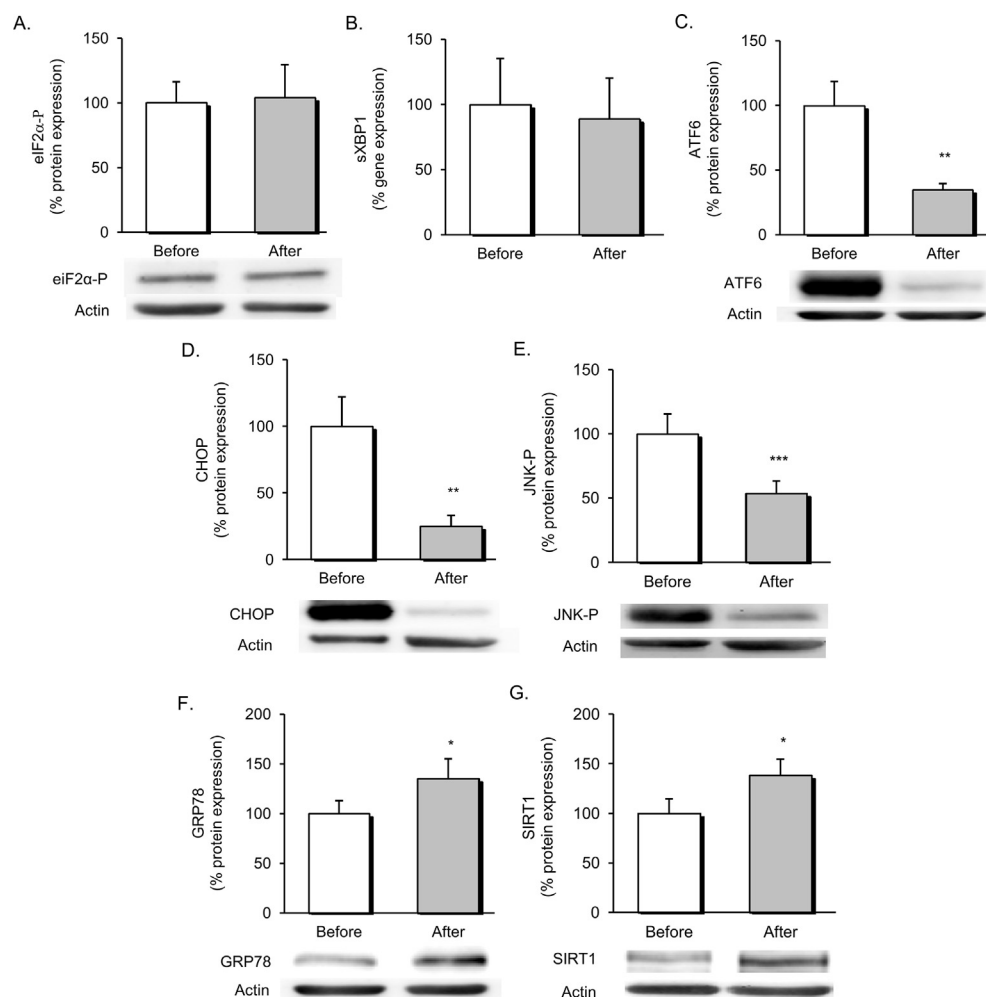
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Figure 2: Evaluation of UPR markers and ER stress modulators in obese patients before and after dietary weight loss intervention. Relative protein expression of eIF2α-P (n = 14) (A) in the PERK-UPR pathway and representative western blot images. Endoribonuclease activity of IRE1α expressed in mRNA levels of sXBP1 (n = 11) (B). Protein levels of p50/ activated ATF6 (n = 21) (C) and representative western blot images. Regulation of chronic downstream targets of the UPR, proapoptotic molecule CHOP (n = 21) (D) and phosphorylated JNK (n = 28) (E). Protein levels of major UPR chaperone GRP78 (n = 21) (F) and SIRT1 (n = 23) (G) and representative western blot images. Data are represented as mean +standard error. **p < 0.01; ***p < 0.001 when compared using a paired Student's t-test. UPR, unfolded protein response; ER, endoplasmic reticulum; eIF2α-P, phosphorylated eukaryotic translation initiation factor 2 subunit 1; PERK, protein kinase RNA-like endoplasmic reticulum kinase; IRE1α, inositol requiring enzyme 1 α; sXBP1, spliced X-box binding protein 1; ATF6, activating transcription factor 6; JNK, cJun NH2-terminal kinase; CHOP, CCAAT/enhancer binding protein [C/EBP] homologous protein; GRP78, 78-kDa glucose regulated protein; SIRT1, Sirtuin 1.

between changes in IR, ER stress and proinflammatory signals. There were also correlations among ER stress markers: percentage of change of GRP78 correlated positively with that of spliced XBP1 (r = 0.883, p = 0.001, n = 10); percentage of change of eIF2α-P correlated with that of ATF6 (r = 0.656, p = 0.003, n = 18) and JNK-P (r = 0.666, p = 0.003, n = 18); and percentage of change of CHOP correlated with that of ATF6 (r = 0.963, p < 0.001, n = 26), eIF2α-P (r = 0.691, p = 0.001, n = 18) and JNK-P (r = 0.850, p < 0.001, n = 26). In addition, a positive correlation between the percentages of change of GRP78 and SIRT1 was observed (r = 0.548, p = 0.018, n = 18), suggesting a relationship between the UPR adaptive response and SIRT1 expression (Table 2).

4. DISCUSSION

In this interventional study a population of middle-aged obese subjects underwent a dietary weight loss intervention consisting of 6 weeks of VLCD diet followed by 18 weeks of LCD. After this dietary program, we confirmed a moderate weight loss, which was associated with the improvement of anthropometric and cardiometabolic parameters and was accompanied by a reduction in the inflammatory response. When we examined the effect of the intervention on ER homeostasis we found that apoptotic pathways of the UPR were ameliorated and chaperone expression up-regulated. In parallel to this, we observed an improvement in oxidative stress and mitochondrial function in leuko-cytes. Altogether, these results suggest that the dietary weight loss intervention induced a partial recovery of cellular homeostasis medi-ated by better ER function and mitochondrial redox status, which were associated with an enhanced metabolic profile.

Several studies have described the benefits of caloric restriction and moderate weight loss for the metabolic profile of patients with obesity and related disorders. In fact, both obese and T2D patients have been shown to display improved insulin sensitivity and cardiovascular risk factor profiles when weight loss of 5—10% is achieved [22,27]. In line
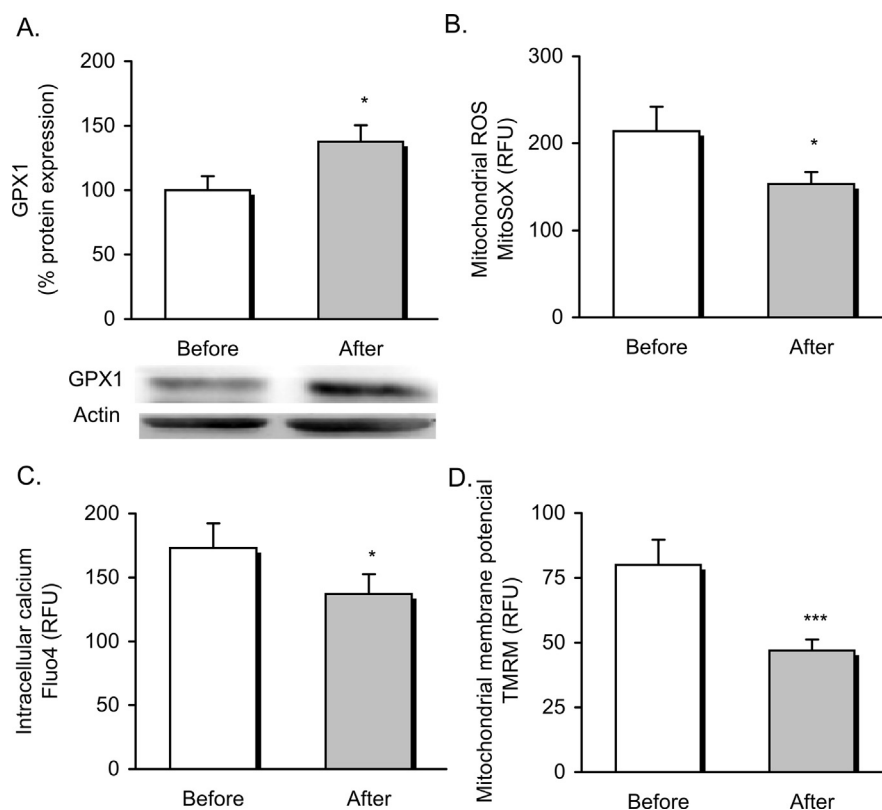
Figure 3: Oxidative stress and mitochondrial function parameters in obese patients before and after dietary weight loss intervention. Expression of the antioxidant enzyme GPX1 ($n = 16$) and protein representative images (A) and levels of mROS production measured as arbitrary units of MitoSOX fluorescence dye ($n = 31$) (B), cytosolic Ca^{2+} measured as arbitrary units of Fluo-4 fluorescence dye ($n = 18$) (C) and mitochondrial membrane potential measured as arbitrary units of TMRM fluorescence dye ($n = 30$) (D). Data are represented as mean +standard error. *$p < 0.05$; ***$p < 0.001$ when compared using a paired Student's t-test. RFU, relative fluorescence units; GPX1, glutathione peroxidase 1; mROS, mitochondrial reactive oxygen species; TMRM, tetramethylrhodamine methyl ester.

with this, VLCDs have been shown to be an effective strategy for weight loss in obese patients, although the reported long-term effect of malnutrition has led to them being replaced by LCDs [28]. In our study population, the dietary weight loss intervention reduced BMI and abdominal fat accumulation, which was associated with the reduction of classic cardiovascular risk factors and circulating C3c levels. An association between C3c and metabolic syndrome [29] and IR in obesity has previously been described, and weight loss is reported to reduce levels of this adipokine, which is in accordance with the results published by our group and other researchers [30,31]. In parallel,

caloric restriction improved the lipid profile of our patients, including increased HDLc and lower circulating triglycerides.

Increased levels of adiposity in obesity are known to be responsible for the aberrant profile of circulating inflammatory markers and adipokines that may underlie IR in these patients. TNFα is overproduced by adipocytes and macrophages during obesity [32], triggering an impairment in insulin signaling at a systemic level. In the present study, reduced circulating levels of TNFα and hsCRP were detected after dietary weight loss intervention. In addition, lower levels of RBP4, an adipokine contributing to systemic IR and recently associated with

Table 2 — Pearson correlation coefficients of percentage of changes between insulin resistance and UPR markers and ER stress modulators in obese patients.							
	HOMA-IR	GRP78	eIF2α-P	ATF6	sXBP1	CHOP	JNK-P	SIRT1
HOMA-IR	—	n.s	n.s.	0.478*	n.s.	n.s.	0.442*	n.s.
GRP78	—	—	n.s.	n.s	0.883***	n.s	n.s	0.548*
eIF2α-P	—	—	—	0.656**	n.s.	0.691***	0.666**	n.s.
ATF6	—	—	—	—	n.s.	0.963***	0.842***	n.s.
sXBP1	—	—	—	—	—	n.s.	n.s.	n.s.
CHOP	—	—	—	—	—	—	0.850***	n.s.
JNK-P	—	—	—	—	—	—	—	n.s.
SIRT1	—	—	—	—	—	—	—	—

Data are expressed as Pearson's correlation and statistical significance *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ for each pair of variables. When correlation is not significant, it is represented as n.s.

Percentage of change was calculated following the formula: ((after-before)/before)*100.

GRP78, 78-kDa glucose regulated protein; eIF2α-P, phosphorylated eukaryotic translation initiation factor 2 subunit 1; ATF6, activating transcription factor 6; sXBP1, spliced X-box binding protein 1; CHOP, CCAAT/enhancer binding protein [C/EBP] homologous protein; JNK, cJun NH$_2$-terminal kinase; SIRT1, Sirtuin 1.

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hsCRP in the progression of metabolic syndrome [33], were detected. As a whole, inflammatory parameters were reduced after weight loss, suggesting a reduction in systemic inflammation mediated by caloric restriction.

It is interesting to speculate about the molecular mechanisms associated with metabolic improvements in obese populations following caloric restriction and moderate weight loss. In this context, ER stress has been reported to be activated in several tissues under conditions related to obesity and T2D, contributing to the development of IR and inflammation. In response to this, the UPR, a highly dynamic pathway, is activated to align ER functional capacity with demand according to external and intrinsic stress signals, such as alterations in metabolism and body weight. In the present study, our findings highlight a decrease in CHOP expression and in JNK activation in leukocytes from obese patients after weight loss, pointing to an alleviation of chronic ER stress, in accordance with previous findings [34].

It is known that all three UPR-branches, IRE1α, PERK, and ATF6, trigger adaptive and apoptotic responses and are involved in CHOP regulation. Our results suggest that dietary weight loss intervention modulates this apoptotic pathway, mainly by a decrease in ATF6 activation, since we found lower levels of p50-activated ATF6 to be correlated with a drop in CHOP expression. However, despite no significant changes in PERK-eIF2α-P being detected after the intervention, a positive correlation was observed between alterations in CHOP and eIF2α-P, suggesting a role of this branch in the regulation of CHOP. Finally, bearing in mind the role of the IRE1α-JNK axis in obesity-induced ER stress [7,35,36], it is likely that changes in JNK-P after dietary weight loss are mediated by a reduction in IRE1α kinase activity, although further analyses are required to confirm this hypothesis. Previous studies have shown reduced ER stress upon weight loss in murine models [34] and patients undergoing bariatric surgery [36], thus highlighting the relevance of body weight in ER homeostasis. However, to our knowledge, this is the first report of UPR modulation by dietary weight loss intervention in humans with obesity.

When exploring the mechanisms involved in ER restoration we have observed increased levels of GRP78, a chaperone that is a major regulator of the UPR. In previous studies, GRP78 upregulation was associated with a decrease in hepatic UPR markers and the IRE1α-JNK activation axis, and an improvement of insulin action and lipid profile in a murine model of obesity [37] and in hepatic cells [38], which is in line with the results of the present study. On the other hand, a growing body of evidence suggests an important role of SIRT1 in ER stress regulation and IR in metabolic disorders. Moreover, several authors have shown that caloric restriction and weight loss are powerful inducers of SIRT1 [39,40] and have demonstrated a role for SIRT1 as an anti-inflammatory molecule in obesity [10,41], which once again is compatible with our results. Interestingly, we found a positive correlation between changes in SIRT1 and GRP78 after the dietary weight loss intervention. These findings provide new insights into the association between ER stress adaptive response and SIRT1. However, since causality cannot be inferred from our data, further analyses are required to elucidate how these two intracellular signaling pathways are interrelated in the context of dietary weight loss in obesity.

Recent studies have provided new insight into the contribution of leukocyte-ER homeostasis to metabolic disease. In this sense, we have previously reported higher ER stress levels in leukocytes from obese subjects with metabolic disturbances when compared with healthy counterparts [19] and also in immune cells from T2D patients, especially in those with poor glycemic control [26]. In line with this, Sage

et al., 2012 [42] demonstrated that induced UPR markers in mononuclear cells correlated with indicators of impaired glucose tolerance in metabolic syndrome. In accordance, we show here that changes in ATF6 and JNK-P in leukocytes from obese patients after dietary intervention correlate with changes in HOMA-IR, supporting a connection between ER homeostasis, glucose management and development of IR.

UPR pathways in immune cells have also been implicated in the progression of cardiovascular disease. Increased ER stress markers have been found in peripheral blood mononuclear cells, as well as in smooth muscle cells and infiltrated macrophages isolated from atherosclerotic plaques of patients with coronary disease [43,44]. In another study, treatment with chaperones that reduce ER stress in macrophages was associated with a delay in the progression of atherosclerosis [45]. In line with this, when we previously explored the association between UPR activation and leukocyte-endothelium cell interactions, an enhancement of the GRP78 adaptive response in leukocytes was found to correlate with a lower level of interaction with the endothelium, whereas increased expression of CHOP seemed to promote adherence [26], which is the first step of the atherosclerotic process. In the present study, increasing levels of GRP78 and a drop in CHOP expression were observed in leukocytes of obese patients after dietary intervention, in parallel with the improvement of some cardiovascular risk factors. Nevertheless, how these changes are related to the interaction of these cells with the endothelium and the subsequent risk of developing cardiovascular disease remains to be confirmed.

Mitochondria are closely linked to the ER by physical contact and Ca^{2+} interchange, and accumulating evidence suggests a converging role of the two organelles in the progression of metabolic disorders [46]. During ER stress, mitochondrial Ca^{2+} overload, among other stress signals, disturbs mitochondrial membrane potential and causes excess ROS production. The imbalance between pro- and anti-oxidants leads to oxidative stress and mitochondrial dysfunction, a mechanism that has been related to the appearance of obesity-related comorbidities and IR [17,47]. In fact, decreased levels of GPX1, an antioxidant enzyme located both in the cytosol and the mitochondria, have been reported in adipose tissue from a genetic murine model and related to impaired insulin signaling [48] and endothelial dysfunction [49]. Our present data demonstrate that caloric restriction reduces abnormal Ca^{2+} distribution and mitochondrial membrane potential, pointing to a restoration of cell homeostasis and mitochondrial function. Furthermore, redox balance was improved in our patients, since lower levels of mROS molecules and higher GPX1 expression were found in their leukocytes after dietary treatment. These results demonstrate that dietary weight loss intervention can modulate mitochondrial function and oxidative stress. However, further analyses are required to assess the degree of implication of these changes in the enhancement of metabolic status in obese patients under caloric restriction.

Finally, most of the literature supports an orchestrated response among leukocytes, adipocytes, and muscle cells in obesity. In fact, we have recently published results showing that total ROS, total superoxide, and mitochondrial membrane potential are selectively higher in obese patients [20], which is in line with impaired mitochondrial activity and enhanced ROS production in subcutaneous adipocytes and white adipose tissue [21,50,51]. In reference to oxidative stress and mitochondrial function in human skeletal muscle, considerable debate exists about whether alterations in mitochondrial respiratory capacity and/or content play a causal role in the development of IR during obesity. Previous reports have shown that mitochondrial content is significantly lower in muscle samples of obese individuals [52—54],

whereas others have not reported such results [55], despite that elevations in H_2O_2 emission rates and reductions in cellular glutathione [52,55,56] are correlate with those measured in leukocytes and adipocytes.

This study presents some limitations. Firstly, it does not clarify whether the main findings were mediated by weight loss or caloric restriction *per se*, since no period of eucaloric stability was programmed after weight loss intervention. In addition, further analyses are required to elucidate the directionality of changes in ER and mitochondrial function and metabolic improvements in obese patients after dietary weight loss intervention. Nevertheless, the present results provide vital new insight into the modulation of ER stress and mitochondrial function *in vivo* that could have important implications for the treatment or prevention of obesity and T2D.

5. CONCLUSIONS

In summary, the results of the present study extend our understanding of the molecular changes and metabolic improvements that obese patients display when moderate weight loss is achieved by caloric restriction. Interestingly, the improvement in systemic inflammation and glucose tolerance was mirrored by an attenuation of chronic ER stress and mitochondrial dysfunction after dietary weight loss intervention, and was accompanied by enhanced expression of chaperones, SIRT1 and antioxidants. These findings highlight the relevance of restoration of ER homeostasis and mitochondrial function as potential targets for treating metabolic complications in obesity.

AUTHORS CONTRIBUTION

The authors' responsibilities were as follows. MR and VMV contributed to the conception and design of the study. CB and SR-L assisted in the design of the experiments and provided support throughout the course of the study. CM carried out the recruitment and diagnosis of the patients in the study. SL-D, ZA-J, FI, and AM performed the laboratory analyses and collected data. SL-D and MR analyzed the data, performed the statistical analysis and drafted the manuscript. MR and VMV critically revised the manuscript and were responsible for its final content. All authors have read and approved the final version of the manuscript.

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CONFLICT OF INTEREST

The authors have no conflict of interest to disclose.

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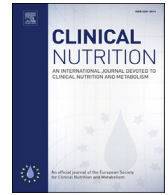
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Original article

Pinitol alleviates systemic inflammatory cytokines in human obesity by a mechanism involving unfolded protein response and sirtuin 1

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A R T I C L E   I N F O

S U M M A R Y

Background & aims: It is known that pinitol acts as a mediator of the insulin-signaling pathway, though little is known about its anti-inflammatory effect in human obesity. Therefore, this study aimed to evaluate the effect of pinitol on peripheral blood mononuclear cells (PBMCs) and visceral (VAT) and subcutaneous adipose tissues (SAT), focusing on the involvement of endoplasmic reticulum (ER) stress and sirtuin 1 (SIRT1).
Methods: In the intervention study, thirteen obese subjects consumed a pinitol-enriched beverage (PEB) for 12 weeks. In the *ex vivo* study, a biopsy of VAT and SAT was removed from thirty-four obese patients and incubated with D-pinitol for 48 h.
Results: The consumption of a PEB reduced circulating levels of IL6 and TNFα and increased SIRT1 protein expression in PBMCs. *Ex vivo* experiments showed a decline in gene expression and protein levels of IL6 and TNFα in SAT and a reduction in ER stress parameters (ATF6 and CHOP), while VAT markers remained unaltered. Differential gene expression profiles revealed an up-regulation of SIRT1 and insulin-signaling pathways in SAT with respect to VAT.
Conclusions: Our results suggests that pinitol down-regulates the inflammatory pathway which may lead to novel treatment options for obesity and its metabolic disorders.

1. Introduction

Obesity is a highly prevalent condition characterized by systemic low-grade inflammation and is related to a wide variety of metabolic disturbances, including insulin resistance, dyslipidemia, hypertension and, eventually, development of diabetes mellitus [1]. Chronic inflammation in obese subjects is manifested by increased circulating levels of proinflammatory cytokines, such as tumor necrosis factor α (TNFα) and interleukin-6 (IL6) [2,3]. Although adipose tissue inflammation is well characterized in obese patients, the molecular mechanisms that trigger the chronic inflammatory response are not completely understood.

Sirtuin 1 (SIRT1) is a nicotinamide adenine dinucleotide (NAD^+)-dependent protein deacetylase that regulates energy homeostasis in response to nutrient availability and whose levels and activity are reduced in obesity [4,5]. Mounting evidence shows that SIRT1 participates in the regulation of inflammatory responses in several tissues. Since nuclear SIRT1 deacetylates p65 subunit of NF-κB and support its proteasome degradation, decreased nuclear SIRT1 levels amplify proinflammatory gene expression during chronic inflammation [6,7]. NF-κB is also reported to be activated *in vitro* and in white adipose tissue (WAT) of obese mice under endoplasmic reticulum (ER) stress conditions [8,9]. Moreover, previous studies have reported increased ER stress in WAT of obese mice and humans [10,11], highlighting a causal relation between ER stress and chronic inflammation [12]. Chaperones are a group of multifunctional proteins responsible for the proper folding and conformation of newly synthesized proteins in the ER. They also facilitate the trafficking of mutant proteins to restore ER homeostasis. Theoretically, chaperones may alleviate WAT inflammation in obesity by reducing ER stress and improving tissular functionality. When the folding capacity of the ER cannot be restored, the unfolded protein response (UPR) promotes the expression of pro-apoptotic factors, such as CHOP [10].

The growing interest in medicinal plants used to fight metabolic disorders including obesity and diabetes has led to the development of dietary bioactive compounds, such as polyols and related carbohydrates. Among these compounds, a potential mediator of the insulin-signaling pathway, known as pinitol (3-O-methyl-D-chiro-inositol), has been developed [13]. Interestingly, we have previously reported that chronic consumption of a pinitol-enriched beverage (PEB) influences parameters of systemic glucose tolerance and insulin sensitivity, not only in healthy subjects, but also in prediabetic and diabetic patients [14—16]. In addition, pinitol and its derivatives are thought to have other properties, including anti-inflammatory activity [17]. In this sense, we have recently reported a systemic anti-inflammatory effect of an inositol-enriched beverage in obese patients [16] and a protective role in diabetic patients that prevented the increase of IL6 associated with consumption of a sweetened beverage in a diabetic population [15], although the underlying mechanism remained unclear.

Therefore, the aim of this study was to assess the mechanism by which pinitol mediates the anti-inflammatory effect on the main producers of cytokines; that is, peripheral blood mononuclear cells (PBMCs) and visceral (VAT) and subcutaneous adipose tissues (SAT) in *in vivo* and *ex vivo* models of human obesity. In particular, we have focused on the involvement of UPR and the role of SIRT1 as an inflammatory mediator.

2. Materials and methods

2.1. Subjects

All the study's subjects were recruited at the Outpatient's Clinic of the Endocrinology and Nutrition Department of the University Hospital Dr. Peset. In the intervention study, a subpopulation of thirteen obese patients was recruited from a larger population previously registered in clinicaltrials.gov under study number NCT01754792.

In the *ex vivo* study, thirty-four subjects underwent a laparoscopy according to the Roux-en-Y gastric bypass (LRYGB) technique. During surgery, a biopsy of VAT and SAT white adipose tissue was performed.

The inclusion criteria were BMI >30 kg/m2, absence of kidney, liver or heart dysfunction and normal protein and hematological clinical status. The patients were excluded from the study in the following cases: severe diseases, malignancies, chronic diseases affecting kidney or cardiovascular function, psychiatric disorders, inflammatory disease or treatment with systemic anti-inflammatory drugs, alcohol or drug abuse, subjects with diabetes mellitus (more than two episodes with fasting glucose

We evaluated anthropometrical parameters as follows: weight and height were determined using a electronic scale and a stadiometer with an approximation of 0.1 kg and 0.5 cm, respectively. BMI was calculated by dividing weight in kg by height in m^2; brachial artery blood pressure was measured twice consecutively in the upper arm of sitting patients after a 5-min resting period, using a sphygmomanometer.

2.2. Intervention study design

For stabilizing subjects' dietary patterns before intervention, they initiated a 1-month run-up period of a normocaloric diet. After this period, subjects received a PEB and followed the dietary recommendations throughout the 12-week study period. The PEB beverage was consumed as a snack between main meals (mid-morning and mid-afternoon).

The PEB consisted of a natural mixture of soluble carbohydrates (with mono-di, oligosaccharides, polyalcohols and soluble fiber) and lower concentrations of other nutrients such as organic acids, aminoacids and minerals obtained from carob fruit.

The PEB (prepared with the commercially available natural food ingredient Fruit Up®) was produced by Wild-Valencia SAU (Spain). The drink was packaged in cans of 250 ml. Each can contained 2.29 g of inositols (2.00 g of pinitol, 0.23 g of myoinositols plus D-chiro-inositol and 0.08 g of other polyols). Detailed information about the intervention drink has been previously published [15]. The recommendation was to ensure a daily energy intake of 7118—9630 KJ (1700—2300 Kcal), of which 50—55% were carbohydrates, 28—33% fats and 15—20% proteins.

2.3. Blood sampling and biochemical determinations

Extraction of the venous blood samples was performed after 12 h overnight fasting at baseline (t = 0) and after 12 weeks for the intervention study and in a preoperative state for the *ex vivo* model. We obtained freshly separated serum by centrifuging at 2000*g* for 15 min at 4 °C. An aliquot of the serum was employed to determine lipid and hydrocarbonated parameters. The remaining serum was stored at −80 °C for subsequent determinations of proinflammatory cytokines.

HDL, total cholesterol, and triglycerides were measured with a Beckman LX-20 autoanalyzer (Beckman Coulter, La Brea, CA, USA). LDL-cholesterol levels were assessed by the Friedewald formula only when triglycerides were lower than 300 mg/dl. Carbohydrate metabolism parameters (glucose and insulin) were measured by employing enzymatic assays. The homeostatic model assessment (HOMA-IR) formula was calculated as follows: fasting insulin (μU/mL) × fasting glucose (mg/dl)/405. For all the measurements, the intraserial variation coefficient was <3.5%. Serum levels of proinflammatory cytokines IL6 and TNFα were analyzed with a Luminex® 200 analyzer system (Austin, TX, USA) following the Milliplex-Kit manufacturer's procedure (Millipore Corporation, Billerica, MA, USA).

2.4. Leukocyte isolation

Blood samples were incubated for 45 min with 3% dextran at room temperature. The supernatant was placed over Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden) and centrifuged for 25 min at 650g. Erythrocytes were removed by 5-min incubation in lysis buffer followed by 5-min centrifugation at 240g. Hank's Balanced Salt Solution (HBSS; Sigma Aldrich, St. Louis, MO) was added to the isolated leukocytes (pellet) in order to wash and resuspend the cells.

2.5. Ex vivo experiments

Explants of VAT and SAT from obese patients were obtained by splicing samples into 5-mg portions and washing them with PBS. After 30-min preincubation with PBS supplemented with 5% BSA, explants were incubated in DMEM-F12 medium (Biowest, Nuaillé, France) with 10% fetal bovine serum (FBS), 3% penicillin/streptomycin (Capricorn, Portland, Maine, USA), 1% Amphotericin B (Biowest, Nuaillé, France) and 10 nM insulin with the addition, or not, of 30 μM D-pinitol (Sigma Aldrich, St. Louis, MO). After 48 h at 37 °C explants were collected, washed twice with PBS and subsequently frozen and stored at −80 °C for later use.

2.6. mRNA expression

Explants were processed with an Ultra-Turrax® homogenizer (IKA, Staufen, Germany) and a RNAse Free DNAse kit (Qiagen, Hilden, Alemania) was added later. The RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Alemania) was used to extract total RNA from VAT and SAT explants following the manufacturer's procedure. Nanodrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA) was employed to quantify the total amount of RNA, and the 260/280 ratio was calculated to assess the purity of these samples (a ratio between 1.8 and 2 was considered optimal).

The Revert Aid cDNA First-Strand Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to synthesize first-strand cDNA from 1 μg of RNA. One microliter of this cDNA was used to assess *TNFα, IL6, SIRT1, GRP78, sXBP1, CHOP, glucose transporter 4 (GLUT4), insulin receptor (IR)* and *PPARγ* expression by real-time RT-PCR using SYBR green (Roche Applied Science, Basilea, Sweden) in a 7500 Fast RT-PCR system (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA).

In Supplementary Table 1, the primer sequences and details of the procedure are specified. All samples were referred to GAPDH gene expression, and the relative quantification was calculated with the comparative $2^{-\Delta\Delta Ct}$ formula using Expression Suite Software (Thermo Fisher Scientific, Waltham, MA, USA).

2.7. Western blotting

Total protein extraction from leukocytes was performed on ice lysing cells with an extraction buffer (20 mM HEPES pH 7.5, 400 mM sodium chloride, 20% Glycerol, 0.1 mM EDTA, 10 μM Na$_2$MoO$_4$, 0.5% NP-40) containing protease inhibitors (10 mM NaF, 1 mM NaVO$_3$, 10 mM PNP, 10 mM β-glycerolphosphate) for 15 min. The supernatant was collected after centrifugation for 15 min at 13,000g. Explants were homogenized with Ultra-Turrax® in the protein lysis buffer provided by Ne-Per® Kit (Thermo Fisher Scientific, Waltham, MA, USA) and in the presence of phosphatase inhibitors (Sigma Aldrich, St. Louis, MO, USA). After following the manufacture's protocol, explants were centrifuged twice for 20 min at 4 °C to remove superficial fat. The total concentration of proteins was quantified in both cases using a bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, Waltham, MA, USA). Twenty-five μg of protein were resolved by SDS-PAGE and proteins were transferred to nitrocellulose membranes. Detection of target proteins was performed by incubating the membranes with anti-SIRT1 polyclonal rabbit antibody (Millipore, MA, USA), anti-TNFα polyclonal rabbit antibody (Abcam, Cambridge, UK), anti-GRP78 polyclonal rabbit antibody (Abcam, Cambridge, UK), anti-IL6 monoclonal rabbit antibody (Millipore Corporation, Billerica, MA, USA), anti-CHOP monoclonal mouse antibody (Thermo Fisher Scientific, Waltham, MA, USA), anti-ATF6 monoclonal mouse antibody (Abcam, Cambridge, UK), anti-EIF2α-P polyclonal rabbit antibody (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), and anti-actin rabbit polyclonal antibody (Sigma Aldrich, St. Louis, MO, USA). The secondary antibodies employed were goat anti-rabbit IgG (Abcam, Cambridge, UK) and goat anti-mouse IgG (Thermo Fisher Scientific, Waltham, MA, USA). ECL plus reagent (GE Healthcare, Uppsala, Sweden) or Supersignal West Femto (Thermo Fisher Scientific, Waltham, MA, USA) were used to detect the protein signal by chemiluminescence, visualized by means of the Fusion FX5 acquisition system (Vilbert Lourmat, Marne La Vallée, France). Data were analyzed by densitometry with the Bio1D software (Vilbert Lourmat, Marne La Vallée, France).

2.8. Statistical analysis

For the statistical analysis of the data we employed the statistics program SPSS 19.0 software (SPSS Statistics Inc., Chicago, IL, USA). In the tables continuous variables are expressed as mean ± standard deviation (SD), or as median and 25th and 75th percentiles for parametric and non-parametric data, respectively, whereas qualitative data are expressed as percentages. In figures, data are represented as mean + standard error (SE). Data were analyzed using a paired Student's t-test or a Mann Whitney U-test for non-parametric samples when comparing baseline conditions vs pinitol treatment. Differential gene expression analysis between visceral and subcutaneous adipose tissues was compared using an unpaired Student's t-test. The confidence interval was 95% for all the tests and significance was established when the difference between variables was $p < 0.05$.

3. Results

In the intervention study, we analyzed a total of 13 obese patients − mainly females in pre-menopausal (n = 3) and menopausal status (n = 7) with a mean age of 53.0 ± 14.0 years − who received a PEB for 12 weeks. The intervention did not modify any of the anthropometric or metabolic parameters assessed, with the exception of a significant reduction in systemic inflammatory cytokines such as IL6 and TNFα ($p < 0.05$ for both) (Table 1).

In order to clarify the origin of this reduction, we evaluated the protein expression of ER stress markers and SIRT1 in PBMCs − one of the main inflammatory cell types − at the beginning and at the end of the experimental period. GRP78 − the chaperone responsible for initiation of UPR − and CHOP − a proapoptotic marker whose expression is increased when ER stress is maintained for a long period of time − were not altered by consumption of the PBE, whereas SIRT1 levels increased significantly (Fig. 1).

We also wished to explore the effect of pinitol on other cell types that also play a central role in inflammatory response; thus, we evaluated the expression of VAT and SAT after treating *ex vivo* explants with pinitol for 48 h. Subjects participating in this study were submitted to LRYGB, during which biopsies were collected. This study analyzed a total of 34 middle-aged subjects of which 24 were females (8 were reproductive-aged woman, 10 were pre-menopausal, and 6 were at menopausal status) with a BMI of 38.0 ± 4.7 and a mean age of 43.6 ± 11.7 years. Analysis of gene

Table 1
Anthropometric and metabolic parameters in obese subjects at baseline and after consumption of an inositol-enriched beverage (IEB).

	Baseline	12 weeks
n (% females)	13 (92.3)	13 (92.3)
Age (years)	53.0 ± 14.0	53.0 ± 14.0
BMI (Kg/m^2)	34.9 ± 3.2	35.1 ± 3.3
Systolic BP (mmHg)	144 ± 15	139 ± 9
Diastolic BP (mmHg)	87.0 ± 6.9	85.2 ± 7.4
Total cholesterol (mg/dl)	179 ± 29	187 ± 38
LDLc (mg/dl)	109 ± 28	116 ± 33
HDLc (mg/dl)	44.2 ± 5.5	45.3 ± 6.9
Triglycerides (mg/dl)	121 (92, 144)	130 (78, 155)
Glucose (mg/dl)	107 ± 9	106 ± 8
Insulin (μU/ml)	15.3 ± 8.1	15.2 ± 8.0
HOMA-IR	4.12 ± 2.45	4.07 ± 2.36
hsCRP (mg/l)	4.43 (2.01, 7.21)	4.77 (2.89, 7.02)
IL6 (pg/ml)	7.31 ± 9.23	4.68 ± 6.39*
TNFα (pg/ml)	8.14 ± 3.38	5.78 ± 2.34*

Data are expressed as mean ± SD. *p < 0.05 when compared by a paired Student's t-test.
hsCRP: high sensitivity C-reactive protein.

expression and protein levels of inflammatory markers showed that VAT explants did not respond to pinitol treatment, since none of the mRNAs — TNFα, IL6 and SIRT1 — or proteins — TNFα and IL6 — were modified (Fig. 2A). On the contrary, SAT explants showed a significant decrease in IL6 (gene and protein expression; both p < 0.05) and TNFα, although statistical differences were found only at the protein level (p < 0.05) (Fig. 2B), which could partly explain the drop of IL6 and TNFα serum levels observed in the intervention study. However, unlike that observed with PBMCs, SIRT1 was unaltered after treatment of explants with pinitol in both VAT and SAT (Fig. 2B), suggesting that other pathways were involved in the anti-inflammatory response. An alleviation of ER stress or a potentiation of the insulin-signaling pathway could be involved in this process. To analyze the effect of pinitol on ER stress markers, we evaluated GRP78, the three pathways involved in the UPR — ATF6, IRE1 and PERK — and CHOP. Since we did not detect changes in inflammatory markers in VAT after pinitol treatment, we did not expect, a priori, to observe differences in the expression of ER stress markers or the insulin-signaling pathway. Indeed, mRNA — GRP78, sXBP1, or CHOP — or protein — ATF6, eIF2α or CHOP — expression was not altered by treatment of explants with pinitol (Fig. 3A). Furthermore,

gene expression of GLUT4, IR or PPARγ was also unaltered in VAT (Fig. 4A). However, incubation of SAT with pinitol produced a selective down-regulation of the ATF6 pathway and CHOP (both gene and protein expression (p < 0.01)) and a downward trend in GRP78 and sXBP1 (p < 0.100) (Fig. 3B), thus pointing to pinitol as a possible mediator in the mitigation of ER stress. Finally, treatment with pinitol did not modify the expression of GLUT4, IR or PPARγ (Fig. 4B), suggesting that it does not exert its beneficial effect on the inflammatory response by enhancing the insulin-signaling pathway. In fact, these results are in accordance with the interventional study (Table 1), since chronic consumption of the PEB for 12 weeks did not modify hydrocarbonated metabolism parameters.

Differential gene expression analysis revealed a generally higher expression level in SAT than in VAT. In particular, we observed a significant increase in SIRT1, GLUT4, IR and PPARγ, whereas ER stress markers and proinflammatory cytokines showed similar levels of mRNA expression in both tissues (Table 2).

4. Discussion

The present study is the first to provide experimental evidence for the anti-inflammatory effects of pinitol on the main producers of cytokine in obesity. Thus, in PBMCs of obese patients that consumed a PEB over 12 weeks we witnessed an increase in SIRT1 protein levels that was associated with a drop in circulating levels of IL6 and TNFα. On the other hand, in *ex vivo* adipose tissue explants incubated with pinitol we observed a decrease in gene expression and protein levels of these proinflammatory cytokines in SAT, which could have been mediated, at least in part, by a reduction in ER stress parameters, while markers of VAT remained unaltered throughout the experimental period. In addition, gene expression profiles varied between the two types of adipose tissues. Specifically, SIRT1 and insulin-signaling pathway genes were up-regulated in SAT. Thus, our findings suggest that the underlying mechanism mediating the anti-inflammatory effects of pinitol differs depending on the nature of the cytokine producers and their depot-specific location.

Although most previous studies have focused on evaluating the effect of pinitol supplementation on glycemic control, only a few have evaluated its anti-inflammatory effects. To date, the effects of pinitol on proinflammatory cytokines have been evaluated mainly in rodent models and have proved to be mostly beneficial and
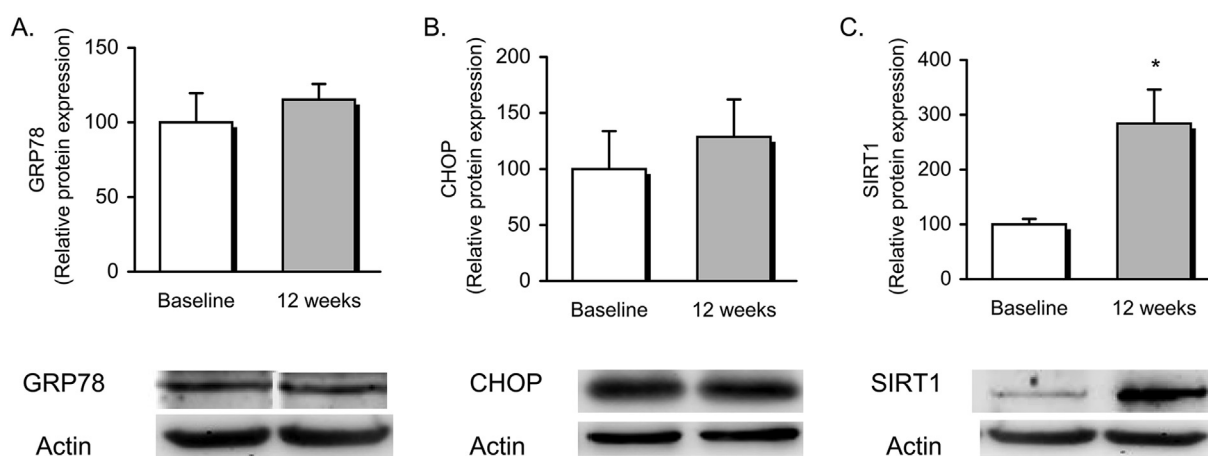

Fig. 1. Effect of chronic consumption of a pinitol-enriched beverage on PBMCs of obese subjects. (A) Protein levels of GRP78 and representative western blot images of GRP78 and actin at baseline and after 12 weeks. (B) Protein levels of CHOP and representative western blot images of CHOP and actin at baseline and after 12 weeks. (C) Protein levels of SIRT1 and representative western blot images of SIRT1 and actin at baseline and after 12 weeks. Data are represented as mean + SE.*p < 0.05 when compared with baseline using a paired Student's t-test. SIRT1: sirtuin 1.
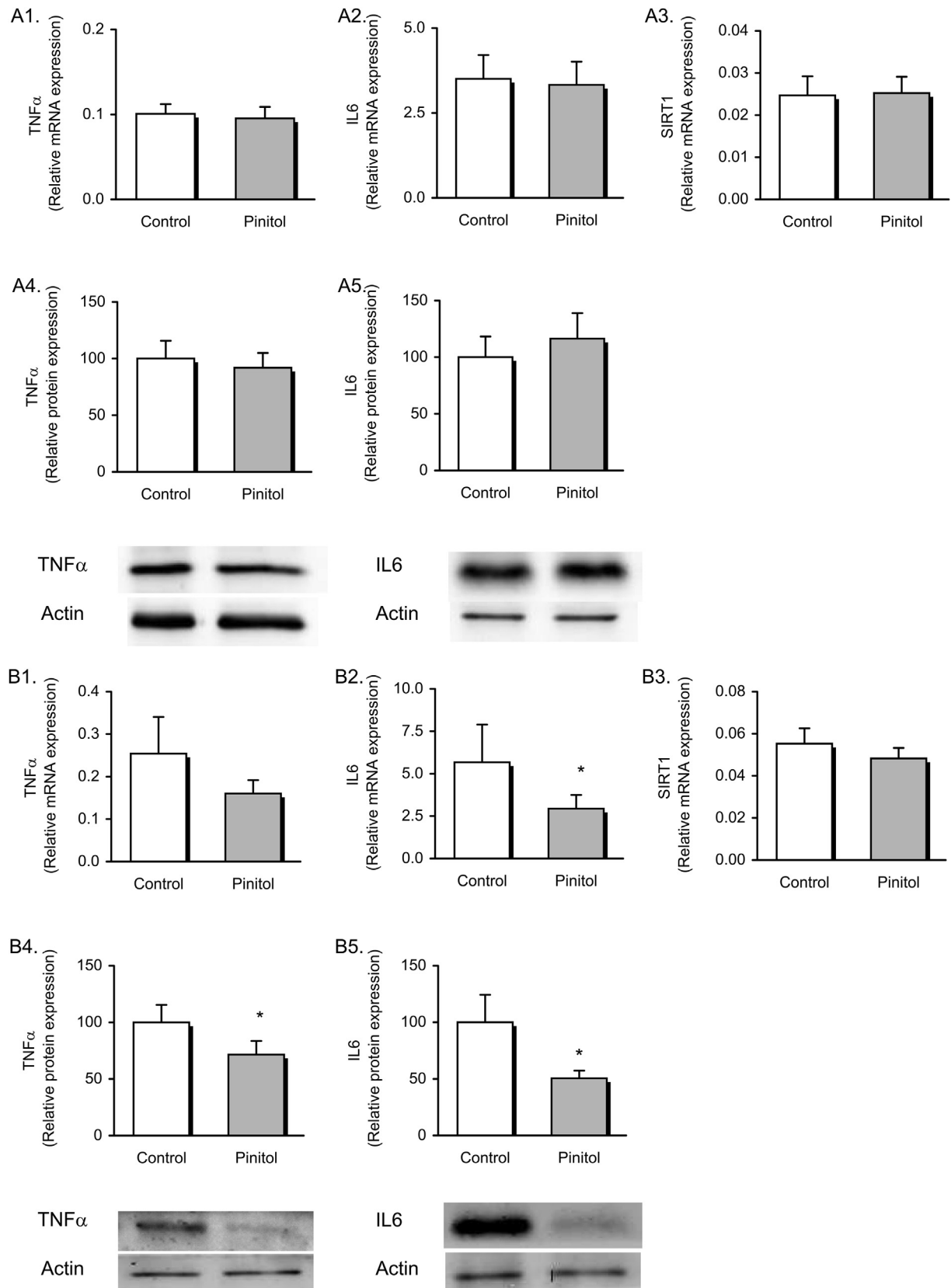
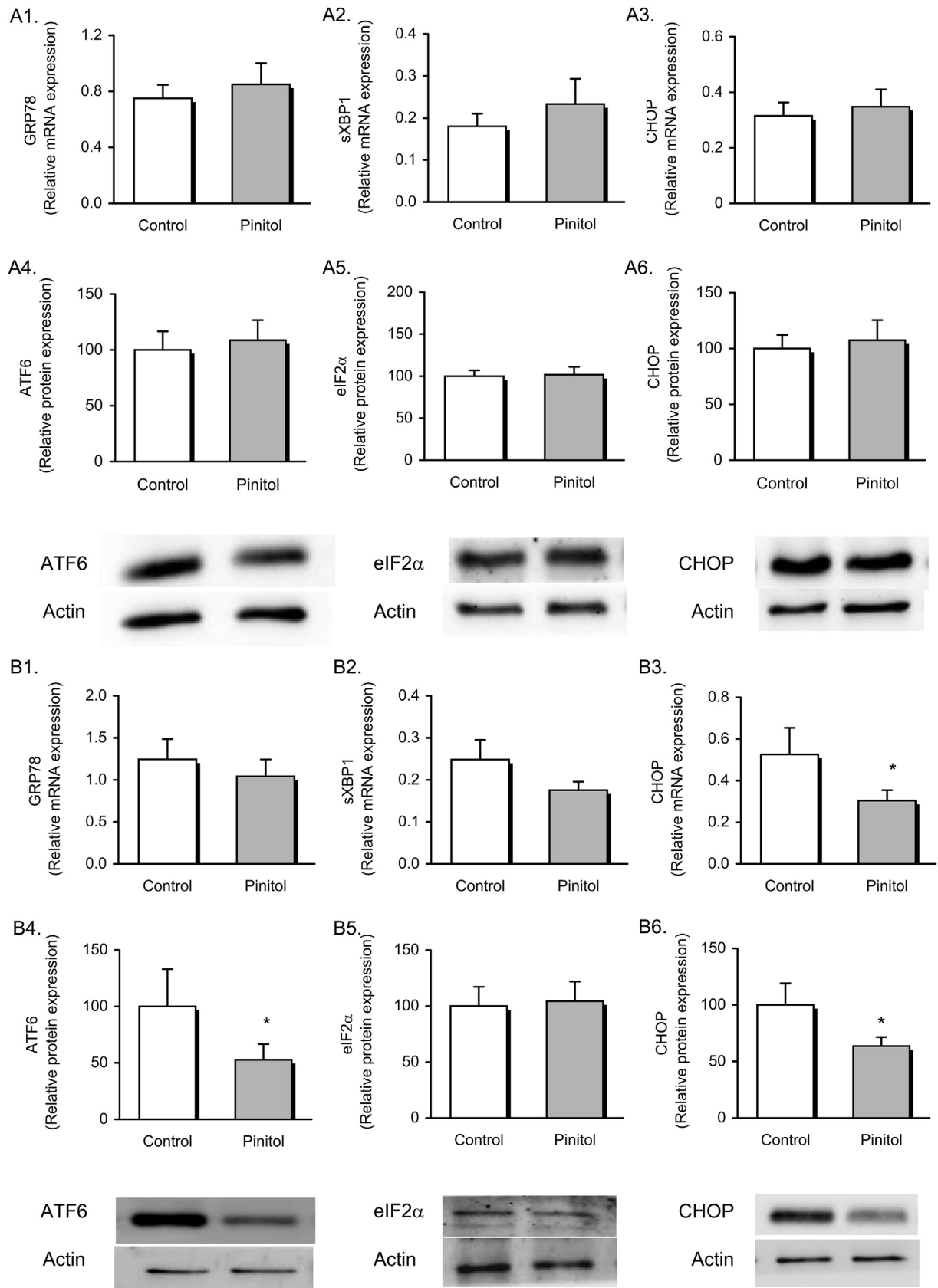**Fig. 2.** Effect of pinitol on inflammatory parameters in (A) visceral white adipose tissues and (B) subcutaneous white adipose tissue in controls and patients treated with pinitol explants. (A1 and B1): mRNA expression of TNFα, (A2 and B2): mRNA expression of IL6, (A3 and B3): mRNA expression of SIRT1, (A4 and B4): Protein levels of TNFα and representative western blot images of TNFα and actin and (A5 and B5): Protein levels of IL6 and representative western blot images of IL6 and actin. Data are represented as mean + SE.*p < 0.05 when compared with controls using a paired Student's t-test. TNFα: tumor necrosis factor α; IL6: interleukin 6; SIRT1: sirtuin 1.

Fig. 3. Effect of pinitol on endoplasmic reticulum stress parameters in (A) visceral white adipose tissues and (B) subcutaneous white adipose tissue in controls and patients treated with pinitol explants. (A1 and B1): mRNA expression of GRP78, (A2 and B2): mRNA expression of sXBP1, (A3 and B3): mRNA expression of CHOP, (A4 and B4): Protein levels of ATF6 and representative western blot images of ATF6 and actin (A5 and B5): Protein levels of eIF2α and representative western blot images of eIF2α and actin and (A6 and B6): Protein levels of CHOP and representative western blot images of CHOP and actin. Data are represented as mean + SE.*p < 0.05 when compared with controls using a paired Student's t-test.
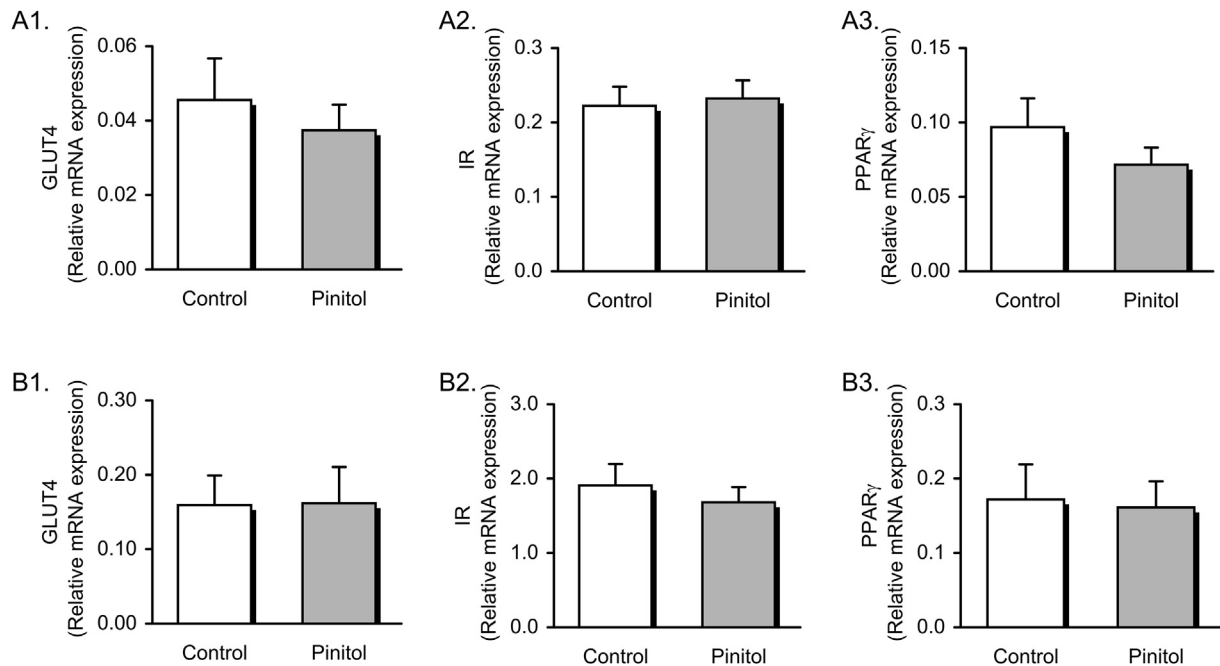
Fig. 4. Effect of pinitol on insulin-signaling pathway parameters in (A) visceral white adipose tissues and (B) subcutaneous white adipose tissue in controls and patients treated with pinitol explants. (A1 and B1): mRNA expression of GLUT4, (A2 and B2): mRNA expression of IR, (A3 and B3): mRNA expression of PPARγ. Data are represented as mean + SE. IR: insulin receptor.

protective in several inflammatory diseases, such us diabetes, asthma and paw edema, in both chronic and subchronic conditions [18–20]. Recently, pinitol has been shown to have an inhibitory effect on tumor growth by decreasing interleukins − TNFα and IL6 − and inducing apoptosis through inhibition of NF-κB, which could mediate the anti-inflammatory response [21,22].

As far as we know, only two previous studies in humans have explored the effects of pinitol as an inhibitor of TNFα activity in whole blood and neutrophils after stimulation with LPS [23,24]. In both studies, pinitol displayed an average inhibition of the activity or expression of TNFα of 30%, which is in the line with the systemic drop of TNFα we have observed after chronic consumption of the PEB in the present study. In this context, we have previously reported that consumption of a PEB prevents the increase of systemic IL6 associated with consumption of a sweetened beverage in a diabetic population [15] and exerts a marked anti-inflammatory effect in obese subjects [16], thereby reinforcing its role as an anti-inflammatory agent.

Since adipocytes are one of the main sources of synthesis of proinflammatory cytokines in obesity [2,3], we aimed to evaluate

Table 2
Differential gene expression analysis in visceral and subcutaneous white adipose tissues in baseline conditions.

	Visceral	Subcutaneous	p
TNFα	0.106 ± 0.056	0.192 ± 0.262	0.300
IL6	4.26 ± 4.07	3.69 ± 5.08	0.748
SIRT1	0.032 ± 0.027	0.057 ± 0.028	0.022
sXBP1	0.209 ± 0.194	0.227 ± 0.171	0.802
GRP78	0.844 ± 0.496	1.040 ± 0.647	0.432
CHOP	0.263 ± 0.194	0.449 ± 0.473	0.168
GLUT4	0.043 ± 0.053	0.123 ± 0.127	0.026
IR	0.248 ± 0.121	1.863 ± 1.134	<0.001
PPARγ	0.061 ± 0.058	0.176 ± 0.197	0.021

Data are expressed as mean ± SD of 12 samples and normalized with an external sample. *p < 0.05 when compared by a paired Student's t-test.

the influence of pinitol on the synthesis of IL6 and TNFα by white adipose tissues. Our results show that there was a depot-specific response in SAT. The incubation of explants with pinitol induced a reduction of TNFα and IL6 in both gene expression and protein levels, suggesting that pinitol alleviates the up-regulation of IL6 and TNFα in SAT and systemically, though the molecular mechanism involved in its anti-inflammatory effect in obesity was not elucidated. To our knowledge, this is the first study to delve deeply into this matter. ER stress has previously been reported to be enhanced in SAT of obese human subjects [11]. Under this metabolic condition, we have shown that incubation of SAT explants with pinitol down-regulates the gene expression and protein content of inflammatory cytokines and ER stress markers, suggesting that its beneficial effect on the inflammatory response is mediated by an alleviation of ER stress acting as chemical chaperone. Several studies have shown that the chemical chaperones TUDCA and 4-PBA decrease levels of TNFα and IL6 in epididymal WAT of obese mice by reducing ER stress markers such as GRP78, sXBP1, CHOP and ATF4 [9,12]. Our results are in accordance with these findings, as we show that pinitol inhibits ER stress markers (sXBP1, ATF6 and CHOP) that may mediate the down-regulation of proinflammatory cytokines, probably involving the down-regulation of the NF-κB signaling pathway [9]. However, we did not detect alterations to VAT after incubation of explants with pinitol, which is in the line with the existence of inherently different progenitor cells that mediate different patterns of gene expression [25].

While VAT has been associated with metabolic dysfunction mainly due to greater lipolytic potential and to the release of free fatty acids into the portal circulation, SAT has a protective role and responds better to the antilipolytic effects of insulin, shared by other molecules or drugs involved in signal transduction [26]. In effect, we have shown that SAT responds differentially to pinitol and has an alternative gene expression profile by which it up-regulates genes involved in insulin signal transduction and SIRT1. Previous studies have reported that SIRT1 is undermined in endothelial cells isolated from VAT and in the whole visceral VAT

of obese subjects when compared with those from SAT [27,28]. In reference to PPARγ, higher mRNA levels have been detected in SAT than in VAT in obese subjects [29,30]. Furthermore, thiazolidine-dione — an agonist of PPARγ — is more responsive to the differentiation in subcutaneous than in visceral preadipocytes, which would lead to an improvement in insulin sensitivity [31]. In addition, the capacity of adipocytes to respond to insulin stimulation may be reflected indirectly by the expression of IR and GLUT4. In this sense, it has been reported that the mRNA expression levels of GLUT4 and insulin receptor substrate 1 (IRS-1) are significantly higher in SAT than in VAT in a population of overweight women [32], which is in accordance with our findings. This divergent pattern of expression could be responsible for a reduced capacity of VAT to respond to insulin stimulation, suggesting a role in the development of obesity-related complications. Despite this, incubation of VAT and SAT with pinitol did not modify the insulin-signaling pathway, which is in the line with the results of our intervention study, in which we found no differences in hydrocarbonated metabolism parameters after chronic consumption of a PEB. This response is specifically associated with the obese state, since we have previously reported that consumption of a PEB clearly improves hydrocarbonated metabolism parameters in non-obese subjects [14,16], suggesting that insulin resistance associated with obesity impairs the insulin-signaling pathway and that the effectiveness of pinitol supplementation is likely to be higher in individuals without an underlying defect of insulin action.

PBMCs are also involved in chronic low-grade inflammation, which confers a proinflammatory phenotype and contributes to endothelial dysfunction and atherosclerosis. This inflammatory process may be repressed by SIRT1, since it has been shown to regulate acetylation of several lysine residues of the p65 subunit of NF-κB [7]. In fact, SIRT1 is reported to inhibit inflammatory pathways in macrophages [33]. In addition, SIRT1 is reduced in PBMCs from patients with insulin resistance [34] and up-regulated after weight loss in obese patients, thus decreasing the expression of IL6 [35]. Further support of a role of SIRT1 in chronic inflammation is provided by evidence that increasing the activity of SIRT1 with the polyphenol resveratrol reduces chronic inflammation and rebalances metabolism and bioenergetics towards homeostasis [36]. In accordance with this, we now report that chronic consumption of pinitol by obese subjects induces an evident increase of SIRT1 in PBMCs, suggesting its involvement in the systemic down-regulation of the inflammatory response.

One of the strengths of this study is that we have evaluated the anti-inflammatory effect of pinitol on the main producers of cytokines in *in vivo* and *ex vivo* models of human obesity. On the other hand, the main limitation of the present study was the reduced although homogeneous sample size. Further interventional studies involving larger patient samples are necessary to corroborate these findings and to better understand the underlying molecular mechanisms responsible for this effect on different tissues and pathologies.

5. Conclusions

To sum up, as far as we know this is the first study in humans in which pinitol has been demonstrated to reduce the inflammatory response. The underlying mechanism appears to involve an alleviation of ER stress — which is likely to act as a chaperone — in SAT and an increase in SIRT1 in PBMCs. Although further efforts are necessary to explore these signaling pathways in obesity, our data point to the potential of inhibition of ER stress and an increase in SIRT1 as novel therapeutic strategies in the down-

regulation of inflammation associated with obesity and its metabolic disorders.

Statement of authorship

A.H-M. and M.R. conducted the study. C.M. provided overall supervision and the follow-up of the patients in the study. SA.G-A. performed the LRYGB and collected adipose tissue samples. S.L-D., C.B., A.M-M, and Z.A-J. performed the laboratory analyses and collected data. VM.V. and S.R-L. assisted in the design of the experiment and provided support throughout the course of the trial and analysis. S.L-D and M.R. analyzed the data, performed the statistical analysis and redacted the manuscript. S.L-D, C.B., A.H-M, and M.R were responsible for its final content. All authors read and approved the final version of the manuscript.

Conflict of interest

None of the authors have any personal or financial conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.clnu.2017.09.015.

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ANNEX II: Additional scientific production during the PhD training

Original articles

1.   Martínez-Herrera, M; **López-Domènech, S (co-first author)**; Silvestre, FJ; Silvestre-Rangil, J; Bañuls, C; Hernández-Mijares, A; Rocha, M. Dietary therapy and non-surgical periodontal treatment in obese patients with chronic periodontitis. *Journal of Clinical Periodontology.* 45(12);1148-1457.

     https://doi.org/10.1111/jcpe.13030

     Impact factor: **4.046**
     Category: Dentistry, Oral surgery & Medicine (**D1**)

2.   Escribano-López, I; Díaz-Morales, N; Iannantuoni, F; **López-Domènech, S**; de Marañón, AM; Abad-Jiménez, Z; Bañuls, C; Rovira-Llopis, S; Herance, JR; Rocha, M; Víctor, VM. The mitochondrial antioxidant SS-31 increases SIRT1 levels and ameliorates inflammation, oxidative stress and leukocyte-endothelium interactions in type 2 diabetes. *Scientific Reports*. 2018;8(1):e15862.

     https://doi.org/10.1038/s41598-018-34251-8

     Impact factor: **4.122**
     Category: Multidisciplinary Sciences (**Q1**)

3.   Rovira-Llopis, S; Escribano-Lopez, I; Díaz-Morales, N; Iannantunoni, F; **López-Domènech, S**; Andújar, I; Jover, A; Pantoja, J; Pallardó, LM; Bañuls, C; Víctor, VM. Downregulation of miR-31 in diabetic nephropathy and its relationship with inflammation. *Cellular Physiology and Biochemistry*. 2018;50(3):1005-1014.

     https://doi.org/10.1159/000494485

     Impact factor: **5.5**
     Category: Physiology (**D1**)

4.   Díaz-Morales, N; **López-Domènech, S**; Iannantuoni, F; López-Gallardo, E; Solá, E; Morillas, C; Rocha, M; Ruiz-Pesini, E; Víctor, VM. Mitochondrial DNA haplogroup JT is related to impaired glycaemic control and renal function in type 2 diabetic patients. *Journal of Clinical Medicine*. 2018;7(8):pii E220.

     https://doi.org/10.3390/jcm7080220

     Impact factor: **5.583**
     Category: Medicine, General & Internal (**D1**)

5.   Martínez-Herrera, M; Silvestre, FJ; Silvestre-Rangil, J; **López-Domènech, S**; Bañuls, C; Milagros Rocha. Levels of serum retinol binding protein 4 before and after non-surgical periodontal treatment in lean and obese subjects: an interventional study. *Journal of Clinical Periodontology*. 2018;45(3):336-344.

https://doi.org/10.1111/jcpe.12840

Impact factor: **4.046**
Category: Dentistry, Oral surgery & Medicine (**D1**)

6.   Bañuls, C; Rovira-Llopis, S; **López-Domènech, S**; Díaz-Morales, N; Blas-García, A; Veses, S; Morillas, C; Víctor, VM; Rocha, M; Hernández-Mijares, A. Oxidative and endoplasmic reticulum stress is impaired in leukocytes from metabolically unhealthy vs healthy obese individuals. *International Journal of Obesity*. 2017;41(10):1556-1563.

https://doi.org/10.1038/ijo.2017.147

Impact factor: **5.159**
Category: Nutrition & Dietetics (**Q1**)

7.   Díaz-Morales, N; Rovira-Llopis, S; Bañuls, C; **López-Domènech, S**; Escrbano-López, I; Veses, S; Jover, A; Rocha, M; Hernández-Mijares, A; Víctor, VM. Does metformin protect diabetic patients from oxidative stress and leukocyte-endothelium interactions? *Antioxidants & Redox Signaling*. 2017;27(17):1439-1445.

https://doi.org/10.1089/ars.2017.7122

Impact factor: **6.53**
Category: Endocrinology & Metabolism (**D1**)

8.   Bañuls, C; Rovira-Llopis, S; **López-Domènech, S**; Veses, S; Rocha, M; Víctor, VM; Hernández-Mijares, A. Effect of consumption of a carob pod inositol-enriched beverage on insulin sensitivity and inflammation in middleaged prediabetic subjects. *Food & Function*. 2016;7(10):4379-4387.

https://doi.org/10.1039/C6FO01021K

Impact factor: **3.289**
Category: Food Science & Technology (**Q1**)

9. Díaz-Morales, N; Rovira-Llopis, S; Bañuls, C; Escribano-López, I; de Marañón, AM; **López-Domènech, S**; Orden, S; Roldán, I; Álvarez, A; Veses, S; Jover, A; Rocha, M; Hernández-Mijares, A; Víctor, VM. Are mitochondrial fusion and fission impaired in leukocytes of type 2 diabetic patients? *Antioxidants & Redox Signaling.* 2016;25(2):108-115.

   https://doi.org/10.1089/ars.2016.6707

   Impact factor: **6.53**
   Category: Endocrinology & Metabolism (**D1**)

10. Rovira Llopis, S; Díaz-Morales, N; Bañuls, C; Blas-García, A; Polo, M; **López-Domènech, S**; Jover, A; Rocha, M; Hernández-Mijares, A; Víctor, VM. Is autophagy altered in the leukocytes of type 2 diabetic patients? *Antioxidants & Redox Signaling*. 2015;23(13):1050-1056.

    https://doi.org/10.1089/ars.2015.6447

    Impact factor: **6.53**
    Category: Endocrinology & Metabolism (**D1**)

11. Víctor, VM; Rovira-Llopis, S; Bañuls, C; Díaz-Morales, N; **López-Domènech, S**; Escribano-López, I; Rios-Navarro, C.; Álvarez, A; Gomez, M; Rocha, M; Hernández-Mijares, A. Metformin modulates human leukocyte/endothelial cell interactions and proinflammatory cytokines in polycystic ovary syndrome patients. *Atherosclerosis*. 2015;242(1):167-240.

    https://doi.org/10.1016/j.atherosclerosis.2015.07.017

    Impact factor: **4.467**
    Category: Peripheral Vascular Disease (**Q1**)

Book chapters

1.   Rocha, M; Bañuls, C; Rovira-Llopis, S; Morillas, C; Veses, S; López-Domènech, S; Díaz-Morales, N; Víctor, VM; Hernández-Mijares, A. Influence of factors that modulate efficacy of phytosterols. *In series "Progress in Food Science and Technology", Book Title: "Phytosterols: Food Sources, Functions and Health Benefits"*. 2015;8:127-162. (USA): Deanna Garner, Nova Science Publishers, Inc., NY (USA). ISBN 978-1-63483-477-3.

Reviews

1.   Díaz-Morales, N; Rovira-Llopis, S; Escribano-López, I; Bañuls, C; **López-Domènech, S**; Falcón, R; de Marañón, AM; Solá, E; Jover, A; Roldán, I; Diez, JL; Rocha, M; Hernández-Mijares, A; Víctor, VM. Role of oxidative stress and mitochondrial dysfunction in skeletal muscle in type 2 diabetic patients. *Current Pharmaceutical Design*. 2016;22(13):2650-2656.

https://doi.org/10.2174/1381612822666160217142949

Impact factor: **2.757**
Category: PHARMACOLOGY & PHARMACY (**Q2**)