Tesis Doctoral Internacional / International PhD Thesis

Programa de Doctorado en Medicina Clínica y Salud Pública

Departamento de Enfermería, Facultad de Ciencias de la Salud

Universidad de Granada

TRANSPORTE POSTPRANDIAL DE ÁCIDO OLEANÓLICO EN ADOLESCENTES Y SU ACCIÓN TERAPÉUTICA SOBRE MONOCITOS-MACRÓFAGOS THP-1

POSTPRANDIAL TRANSPORT OF OLEANOLIC ACID IN ADOLESCENTS AND ITS THERAPEUTIC ACTION ON THP-1 MONOCYTE-MACROPHAGES

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Granada, 2023

Editor: Universidad de Granada. Tesis Doctorales
Autor: Angel Fernández Aparicio
ISBN: 978-84-1117-787-0
URI: https://hdl.handle.net/10481/81241

A mi madre y a mi padre

"*Keep your face always toward the sunshine – and shadows will fall behind you*"

(Mantén tu rostro siempre hacia la luz del sol, y las sombras caerán detrás de ti)

Walt Whitman

La memoria de la presente Tesis Doctoral Internacional es presentada por D. Ángel Fernández Aparicio con el fin de conseguir el grado de Doctor por la Universidad de Granada.

Esta Tesis Doctoral Internacional ha sido realizada en el contexto del proyecto de investigación *PREOLIA, Prevención con Ácido Oleanólico de insulinorresistencia y síndrome metabólico en adolescentes*, referencia B-AGR-287-UGR18. Dicho estudio fue financiado por los Fondos Estructurales de la Unión Europea de Desarrollo Regional (FEDER), tras su concesión por la Universidad de Granada en la convocatoria de 2018 en el marco del programa operativo FEDER Andalucía 2014-2020".

El doctorando D. Ángel Fernández Aparicio durante la realización de su Tesis Doctoral ha disfrutado de un contrato predoctoral financiado por el programa Formación de Profesorado Universitario (FPU) del Ministerio de Ciencias, Innovación y Universidades (código: FPU18/01666) del Gobierno de España, concedido en la Resolución de 4 de octubre de 2018 de la Secretaría de Estado de Universidades, Investigación, Desarrollo e Innovación.

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ABREVIATURAS

AGL	Ácidos grasos libres
AMP	Adenosín monofosfato
AMPK	Proteína quinasa activada por AMP
AO	Ácido Oleanólico
ATMs	Macrófagos del tejido adiposo
Cci	Circunferencia de la cintura
DMT2	Diabetes Mellitus tipo 2
ECV	Enfermedades cardiovasculares
EGIR	Grupo Europeo para el estudio de RI
ER	Retículo endoplásmico
ERK	Quinasa regulada por señales extracelulares
FoxO1	Forkhead box O1
GLUT4	Transportador de glucosa 4
HDL	Lipoproteínas de alta densidad
HOMA-IR	Modelo homeostático para evaluar la RI
HTA	Hipertensión arterial
IDF	Federación Internacional de Diabetes
IKK	Quinasa IκB
IL-1β	Interleuquina-1β
IL-6	Interleuquina-6
IMC	Índice de masa corporal

IR	Receptor de insulina
IRS	Sustrato del receptor de insulina
JNK	Quinasa c-Jun N-terminal
LDL	Lipoproteínas de baja densidad
LPS	Lipopolisacárido
MAPK	Proteínas quinasas activadas por mitógenos
NADPH	Nicotinamida adenina dinucleótido fosfato oxidasa
NCEP - ATPIII	Programa Nacional de Educación sobre el colesterol - Tercer panel de tratamiento para adultos
NF-κB	Factor nuclear potenciador de las cadenas ligeras kappa de las células B
NO	Óxido nítrico
Nrf2	Factor nuclear derivado de eritroide 2
OMS	Organización Mundial de la Salud
PAD	Presión arterial diastólica
PAS	Presión arterial sistólica
PI3K	Fosfatidilinositol-3-OH-quinasa
PKB/Akt	Proteína cinasa B
RI	Resistencia a la insulina
ROS	Especies reactivas de oxígeno
SM	Síndrome metabólico
SNS	Sistema nervioso simpático
SRAA	Sistema renina angiotensina aldosterona
TG	Triglicéridos

TGR5	Receptor 5 acoplado a proteína G de Takeda
TNF-α	Factor de necrosis tumoral alfa
TRLs	Lipoproteínas ricas en triglicéridos
VLDL	Lipoproteínas de muy baja densidad

RESUMEN/SUMMARY

I. RESUMEN

El concepto de síndrome metabólico (SM) se ha consolidado a lo largo del siglo XX, el cual consiste en un conjunto de anomalías cardiometabólicas que aumentan el riesgo de desarrollar diabetes mellitus tipo 2 y enfermedades cardiovasculares (ECV). Existe consenso en que la obesidad, la resistencia a la insulina (RI), la dislipidemia e hipertensión arterial constituyen los componentes esenciales de este síndrome. La prevalencia mundial de obesidad en la población adolescente se ha multiplicado por cuatro en los últimos cuarenta años. Además, los adolescentes que presentan obesidad abdominal tienen mayor probabilidad de continuar siendo obesos en la etapa adulta y, por tanto, de desarrollar eventos cardiovasculares. Aproximadamente 18 millones de personas fallecen anualmente como consecuencia de las enfermedades cardiovasculares. Se ha propuesto que la amplia morbimortalidad como consecuencia de las ECV podría reducirse mediante la identificación precoz en niños y adolescentes del SM o de sus componentes de forma individual. En esta línea, los criterios diagnósticos de SM permiten su categorización, la valoración de sus componentes relacionados y la evaluación del riesgo cardiovascular a largo plazo. A pesar del desarrollo en los últimos años por diferentes autores de criterios específicos para población infantil y adolescente, los cambios relacionados con el crecimiento y desarrollo puberal en la adolescencia dan lugar a puntos de corte sin valores fijos establecidos que dificultan la elaboración y aplicación de unos criterios homogéneos y unificados de SM.

Además de un correcto cribado del SM en adolescentes para disponer de un control óptimo de las repercusiones sanitarias y epidemiológicas de este síndrome, también resulta fundamental la búsqueda de nuevas estrategias terapéuticas y preventivas que complementen a las ya existentes. En los últimos años se ha visto incrementado el interés por las propiedades y efectos terapéuticos de ciertos compuestos bioactivos. Entre ellos se encuentra el Ácido Oleanólico (AO), un triterpeno pentacíclico disponible en grandes cantidades en la hoja del olivo, y en menor medida en el aceite de oliva. Dicho triterpeno presenta propiedades antiinflamatorias, antihipertensivas, antioxidantes, insulinosensibilizantes, hipolipemiantes e hipoglucemiantes que le confieren un gran

potencial para el control de cada uno de los componentes del SM. No obstante, aún no existe un amplio conocimiento de los mecanismos moleculares que justifican dichas propiedades.

El potencial terapéutico del AO depende de su biodisponibilidad y mecanismos de transporte. Se trata de un compuesto prácticamente insoluble en medios acuosos y parcialmente soluble en matrices lipofílicas. De hecho, se ha demostrado en humanos la presencia postprandial de AO en plasma sanguíneo tras la ingesta de una formulación de aceite enriquecido en este triterpeno. También se sabe que se transporta unido a la albúmina, pero se desconoce si se transporta formando parte de lipoproteínas ricas en triglicéridos (TRLs), tras su absorción intestinal.

Teniendo en cuenta todas estas evidencias, los objetivos generales de la presente Tesis Doctoral Internacional son caracterizar y definir el SM en adolescentes (**estudio I**); analizar los efectos terapéuticos del AO sobre monocitos-macrófagos THP-1, así como sobre la formación de células espumosas (**estudios II, III y IV**); y profundizar en los mecanismos de transporte postprandial de AO en adolescentes sanos y con normopeso (**estudio V**).

Se realizó un estudio con un diseño transversal en 981 adolescentes españoles para contrastar el grado de concordancia entre ocho criterios diferentes para definir SM en población adolescente (**estudio I**). Asimismo, se llevaron a cabo dos revisiones sistemáticas, la primera (**estudio II**) para analizar los efectos del AO sobre los componentes del SM y sobre marcadores inflamatorios y biomarcadores de estrés oxidativo; y la segunda (**estudio III**) con el fin de determinar los efectos del AO sobre los mecanismos moleculares y vías de señalización implicadas en el desarrollo de RI y el estrés oxidativo subyacente. Por último, se desarrolló un ensayo postprandial, controlado, aleatorizado y doble ciego en 22 adolescentes sanos y con normopeso con el fin de determinar la presencia de AO en TRLs postprandiales humanas (**estudio V**), así como valorar los efectos del AO tras la formación de células espumosas con TRLs (**estudio IV**). También se analizaron los efectos de diferentes concentraciones de AO en macrófagos THP-1 estimulados con LPS (**estudio IV**).

Los resultados de la presente Tesis Doctoral mostraron discrepancias en el grado de concordancia entre los ocho criterios empleados en la población adolescente. Estos datos sugieren la necesidad de avanzar en la validación de unos criterios diagnósticos uniformes y homogéneos que faciliten su uso en la práctica clínica. En cuanto a los efectos terapéuticos del AO, los resultados muestran su eficacia en el control de ciertos componentes del SM, siendo destacable su capacidad para atenuar la RI y el estrés oxidativo subyacente a través de su actividad moduladora sobre la vía de señalización de insulina IRS/PI3K/Akt/FoxO1 y las vías MAPK. Asimismo, en los estudios experimentales se ha observado la capacidad del AO de atenuar el estado inflamatorio y aumentar los niveles de AMPK-α en macrófagos THP-1 inducidos con LPS, y de disminuir los niveles de IL-6 y Akt en células espumosas. Por último, a pesar de observar indicios de presencia de AO en TRLs estos resultados deben considerarse preliminares y, por lo tanto, se necesitan futuros ensayos postprandiales que profundicen en el transporte postprandial de AO. Los hallazgos de esta Tesis Doctoral refuerzan el potencial del AO para prevenir y tratar la RI, las alteraciones aterogénicas y los diferentes factores metabólicos subyacentes al SM en adolescentes a través del uso de aceites de oliva funcionales.

II. SUMMARY

The concept of metabolic syndrome (MetS) has been consolidated throughout the 20th century and consists of a set of cardiometabolic abnormalities that increase the risk of developing type 2 diabetes mellitus and cardiovascular disease (CVD). There is consensus that obesity, insulin resistance (IR), dyslipidemia and arterial hypertension constitute the essential components of this syndrome. The worldwide prevalence of obesity in the adolescent population has quadrupled in the last forty years. Furthermore, adolescents with abdominal obesity are more likely to remain obese in adulthood and, therefore, to develop cardiovascular events. Approximately 18 million people die annually as a result of CVD. It has been proposed that the widespread morbidity and mortality as a consequence of CVD could be reduced by early identification in children and adolescents of MetS or its components on an individual basis. In this line, the diagnostic criteria for MetS allow its categorization, the assessment of its related components, and the evaluation of long-term cardiovascular risk. Despite the development in recent years by different authors of specific criteria for the child and adolescent population, the changes related to pubertal growth and development in adolescence give rise to cut-off points without established fixed values, which makes it difficult to create and apply homogeneous and unified criteria for MetS.

In addition to correct screening for MetS in adolescents in order to have optimal control of the health and epidemiological repercussions of this syndrome, it is also essential to search for new therapeutic and preventive strategies to complement the existing ones. In recent years, there has been increasing interest in the properties and therapeutic effects of certain bioactive compounds. Among them is the Oleanolic Acid (OA), a pentacyclic triterpene available in large quantities in the olive leaf, and to a lesser extent in olive oil. This triterpene has anti-inflammatory, antihypertensive, antioxidant, insulin-sensitizing, lipid-lowering and hypoglycemic properties that give it great potential for the control of each of the components of MetS. However, the molecular mechanisms underlying these properties are not yet widely understood.

The therapeutic potential of OA depends on its bioavailability and transport mechanisms. It is a compound that is practically insoluble in aqueous media and partially soluble in lipophilic matrices. In fact, the postprandial presence of OA in blood plasma after ingestion of an oil formulation enriched in this triterpene has been demonstrated in humans. It is also known to be transported bound to albumin, but it is not known whether it is transported as part of triglyceride-rich lipoproteins (TRLs) after intestinal absorption.

Taking all this evidence into account, the general objectives of this International Doctoral Thesis are to characterize and define MetS in adolescents (**study I**); to analyze the therapeutic effects of OA on THP-1 monocyte-macrophages, as well as on foam cell formation (**studies II, III and IV**); and to study the mechanisms of postprandial transport of OA in healthy and normal-weight adolescents (**study V**).

A study with a cross-sectional design was carried out in 981 Spanish adolescents to contrast the degree of agreement between eight different criteria for defining MetS in the adolescent population (**study I**). Two systematic reviews were also carried out, the first (**study II**) to analyze the effects of OA on the components of MetS and on inflammatory markers and biomarkers of oxidative stress; and the second (**study III**) to determine the effects of OA on the molecular mechanisms and signaling pathways involved in the development of IR and the underlying oxidative stress. Finally, a postprandial, controlled, randomized, double-blind, controlled trial was developed in 22 healthy, normal-weight adolescents to determine the presence of OA in human postprandial TRLs (**study V**), as well as to assess the effects of OA after foam cell formation with TRLs (**study IV**). The effects of different concentrations of OA on THP-1 macrophages stimulated with LPS were also analyzed (**study IV**).

The results of the present Doctoral Thesis showed discrepancies in the degree of concordance between the eight criteria used in the adolescent population. These data suggest the need to advance in the validation of uniform and homogeneous diagnostic criteria that facilitate their use in the clinical practice. Regarding the therapeutic effects of OA, the results show its efficacy in the control of certain components of MetS, being remarkable its ability to attenuate IR and the underlying oxidative stress through its

modulatory activity on the IRS/PI3K/Akt/FoxO1 insulin signaling pathway and MAPK pathways. Likewise, experimental studies have observed the capability of OA to attenuate the inflammatory state and increase AMPK-α levels in LPS-induced THP-1 macrophages, and to decrease IL-6 and Akt levels in foam cells. Finally, despite observing evidence of the presence of OA in TRLs, these results should be considered preliminary and, therefore, future postprandial trials that delve deeper into the postprandial transport of AO are needed. The findings of this PhD Thesis reinforce the potential of AO to prevent and treat IR, atherogenic alterations and different metabolic factors underlying MetS in adolescents through the use of functional olive oils.

INTRODUCCIÓN

I. HISTORIA DEL CONCEPTO DE SÍNDROME METABÓLICO

La obesidad central, la hipertensión arterial (HTA), la dislipidemia y la resistencia a la insulina (RI) son un conjunto de anomalías cardiometabólicas que en la actualidad conforman el denominado síndrome metabólico (SM). A largo plazo dicho síndrome favorece el desarrollo de enfermedades cardiovasculares (ECV) y diabetes mellitus tipo 2 (DMT2) (Subías-Perié et al., 2022; Succurro et al., 2022), así como otras complicaciones entre las que destacan la esteatosis hepática no alcohólica, el síndrome del ovario poliquístico, el estrés oxidativo, la apnea obstructiva del sueño (Weiss et al., 2013), la gota, el cáncer o el asma, entre otras (Subías-Perié et al., 2022). Con la finalidad de alcanzar una mejor comprensión de por qué se denomina SM a este conjunto de alteraciones cardiometabólicas, se va a realizar un breve recorrido histórico de las primeras asociaciones observadas entre determinados factores de riesgo implicados en el desarrollo de eventos cardiovasculares.

En el siglo XVIII, concretamente en el año 1761, se establecieron las bases de la anatomía patológica moderna con la publicación de "*De Sedibus et Causis Morborum per Anatomen Indagatis*". A pesar de la ausencia en la época de avances como la tomografía computarizada o la realización de analíticas sanguíneas, Joannes Baptista Morgagni describió en esta colección de registros anatomo-clínicos, a través de la observación en disecciones anatómicas, la asociación entre obesidad visceral, aterosclerosis, HTA, hiperuricemia y síndrome de apnea obstructiva del sueño en sujetos con obesidad androide (Enzi et al., 2003). Casi dos siglos después, en 1921, el Dr. Gregorio Marañón constató la asociación entre obesidad, HTA y trastornos glucémicos (Sarafidis & Nilsson, 2006). Hallazgos similares fueron descritos en 1923 por el médico Kylin, al observar en algunos pacientes la presencia simultánea de HTA, hiperglucemia e hiperuricemia, describiendo como síndrome a este grupo de anomalías metabólicas (Parikh & Mohan, 2012). En la década de los 50, el Dr. Jean Vague confirmó una mayor predisposición a desarrollar ateroesclerosis, diabetes, gota y cálculos en sujetos con obesidad de tipo androide (Vague, 1956).

En 1977, Haller englobó como SM los términos de obesidad, diabetes mellitus, hiperlipoproteinemia, hiperuricemia y esteatosis hepática al describir los efectos de los factores de riesgo de la aterosclerosis. Un año después, Gerald Phillips postuló que los factores de riesgo de infarto de miocardio constituyen una agrupación de anomalías entre las que se encuentra la hiperinsulinemia, la intolerancia a la glucosa, la hipertrigliceridemia y la HTA (Ahmed et al., 2022; Gupta & Gupta, 2010). En la década de los 80, Reaven (1988) incluyó dentro del concepto síndrome X un conjunto de alteraciones metabólicas que suponían un incremento del riesgo de padecer enfermedad arterial coronaria. Entre estas alteraciones se encontraban la resistencia a la captación de glucosa estimulada por la insulina, la intolerancia a la glucosa, la hiperinsulinemia, el incremento de lipoproteínas de muy baja densidad (VLDL), la disminución de los niveles de lipoproteínas de alta densidad (HDL) e HTA, siendo la RI el problema fisiopatológico de base. No obstante, este autor no incluyó a la obesidad como factor subyacente al desarrollo de SM.

A partir del concepto de síndrome X propuesto por Reaven, diferentes organizaciones de salud comenzaron a establecer criterios diagnósticos y de definición de SM. La Organización Mundial de la Salud (OMS), en 1998, fue la primera organización en establecer unos criterios de definición de SM, que contemplaban la presencia de una anormalidad relacionada con la diabetes mellitus (intolerancia a glucosa y/o resistencia a la insulina) junto a dos de los siguientes factores: HTA, hipertrigliceridemia o niveles bajos de HDL, obesidad central, y/o microalbuminuria (Alberti & Zimmet, 1998). En 1999, el Grupo Europeo para el estudio de RI (EGIR) estableció unos nuevos criterios que, aunque eran similares a los propuestos por la OMS, no contemplaban la presencia de microalbuminuria y la hiperglucemia debía ser determinada en ayuno en sujetos no diabéticos (Balkau & Charles, 1999). Posteriormente, el Programa Nacional de Educación sobre el colesterol - Tercer panel de tratamiento para adultos (NCEP - ATPIII) (Expert Panel on Detection, Evaluation, 2001) definió como SM la presencia de 3 o más de los siguientes criterios: obesidad abdominal, hipertrigliceridemia, niveles bajos de HDL, HTA o hiperglucemia en ayunas. No obstante, el NCEP difería de la OMS de la obligatoriedad de la presencia de RI. En resumen, las tres organizaciones destacaron

como componentes principales del SM la obesidad abdominal, la RI y la dislipidemia, aunque con matices diferentes entre ellos.

Finalmente, en 2006, la Federación Internacional de Diabetes (IDF) definió el SM como la presencia de obesidad abdominal (basados en puntos de corte de circunferencia de la cintura, CCi) junto con dos de los cuatro siguientes factores: hipertrigliceridemia (≥ 150 mg/dL o antecedentes), niveles bajos de HDL (< 40 mg/dL en hombres, y < 50 mg/dL en mujeres, o antecedentes previos), HTA (presión arterial sistólica ≥ 130 mmHg o presión arterial diastólica ≥ 85 mmHg, o HTA previa diagnosticada), o hiperglucemia en ayunas (≥ 100 mg/dL o diabetes mellitus tipo 2) (International Diabetes Federation, 2006).

II. SÍNDROME METABÓLICO Y RIESGO CARDIOVASCULAR EN ADOLESCENTES

La prevalencia de sobrepeso u obesidad en población comprendida entre 5 a 19 años era aproximadamente del 18 % en 2016 según datos de la OMS (World Health Organization, 2021), cifras que se han visto cuadriplicadas desde 1975. En esta misma línea, el informe de la quinta ronda (2018-2020) de la Iniciativa Europea de Vigilancia de la Obesidad Infantil publicado por la Oficina Regional de la OMS en Europa (World Health Organization Regional Office for Europe, 2022) ha informado que el 29 % de niños europeos de 7-9 años sufren de sobrepeso u obesidad. De acuerdo a este mismo informe, España es el tercer país de Europa con mayor tasa de sobrepeso u obesidad infantil, con una prevalencia cercana al 39 %.

La tendencia al alza, tanto a nivel internacional como nacional, de la prevalencia de obesidad en niños y adolescentes es especialmente preocupante; ya que, junto con la HTA, hiperlipidemia e hiperglucemia constituyen los principales factores de riesgo cardiometabólicos conducentes al desarrollo de enfermedades crónicas. Estas enfermedades son responsables del 74% de los fallecimientos que se producen cada año en todo el mundo, siendo de este total de defunciones 17.9 millones debidas a las ECV (World Health Organization, 2022).

No sólo la obesidad infantil, sino factores como la HTA, dislipemia e hiperglucemia contribuyen a largo plazo a la aparición prematura de aterosclerosis y ECV en la edad adulta, aumentando el riesgo cardiovascular (Drozdz et al., 2021; Serbis et al., 2020). De hecho, el estudio de cohortes Framingham iniciado en 1948 con el fin de explicar la historia natural de las ECV, especialmente de la arteriosclerosis, el ictus, las enfermedades coronarias, y la HTA (Dawber et al., 1951; Morís de la Tassa & Gómez-Morán, 2014), ha demostrado que la obesidad, la tensión arterial elevada, la diabetes mellitus y la hiperlipidemia son alteraciones metabólicas asociadas a un mayor riesgo de ECV (Eckel et al., 2005; Wilson et al., 1998). Teniendo en cuenta que todos estos factores son componentes claves del SM, la amplia morbimortalidad debida a las ECV en la población adulta podría reducirse realizando un control adecuado y una detección precoz en la infancia y adolescencia de cada uno de los componentes que conforman dicho síndrome (Drozdz et al., 2021; Serbis et al., 2020).

La capacidad del SM para predecir ECV ha sido analizada en estudios previos. En el estudio de cohortes *Princeton Lipid Research Cohort Study* se informó que las puntuaciones z para medir la gravedad del SM son útiles para la predicción de DMT2 y ECV en individuos adultos que han tenido SM durante su infancia (DeBoer et al., 2015a, 2015b). Asimismo, Koskinen et al. (2017) analizaron a un total de 5803 participantes de cuatro estudios de cohorte (Cardiovascular Risk in Young Finns, Bogalusa Heart Study, Princeton Lipid Research Study, Insulin Study) en los cuáles observaron una asociación entre la presencia de SM durante la infancia, y un mayor riesgo de presentar SM, DMT2 y un mayor grosor de la íntima-media carotídea en la edad adulta.

III. CRITERIOS DIAGNÓSTICOS DE SÍNDROME METABÓLICO EN ADOLESCENTES

Actualmente existen varios criterios consolidados para diagnosticar SM en población adulta, entre los que se encuentran los criterios de la OMS (Alberti & Zimmet, 1998), del EGIR (Balkau & Charles, 1999), del NCEP ATP III (Expert Panel on Detection, Evaluation, 2001), y de la IDF (International Diabetes Federation, 2006). A través de adaptaciones de estos criterios, durante el siglo XXI se han elaborado diferentes

criterios diagnósticos para la población infantil y adolescente (Albert Pérez et al., 2018; DeBoer, 2019). Sin embargo, diversos factores contribuyen a la heterogeneidad observada entre dichos criterios de definición de SM en población adolescente. En primer lugar destacan las propias diferencias existentes en los criterios de definición de SM para la población adulta (Jones, 2006). Otro de los factores es que las variables antropométricas, metabólicas y cardiovasculares que conforman los criterios son dependientes de la edad y el sexo (Bussler et al., 2017). Esto implica que en la población adolescente se deben utilizar valores de referencia específicos para cada edad y sexo que, sin embargo, no tienen valores fijos establecidos debido a los procesos de crecimiento y desarrollo puberal que acontecen durante la infancia y adolescencia (Albert Pérez et al., 2018; Sewaybricker et al., 2013; Tavares Giannini et al., 2014). Todos estos factores dificultan el consenso en la elaboración de unos criterios homogéneos y universales para diagnosticar SM en población adolescente (Serbis et al., 2020).

Entre los criterios diagnósticos de SM existentes para la población adolescente destacan los propuestos por de Cook et al. (2003), Weiss et al. (2004), Duncan et al. (2004), de Ferranti et al. (2004), y Cruz & Goran (2004), que fueron elaborados a partir de los criterios propuestos por el NCEP - ATP III (Expert Panel on Detection, Evaluation, 2001). También se encuentran los propuestos por Viner et al. (2005), siendo este una modificación de los establecidos por la OMS (Alberti & Zimmet, 1998); por la IDF (Zimmet et al., 2007); y por Rodríguez-Moran et al. (2004), que son una modificación de los criterios para adultos propuestos por la OMS (Alberti & Zimmet, 1998), el NCEP ATP III (Expert Panel on Detection, Evaluation, 2001) y el EGIR (Balkau & Charles, 1999).

En la Tabla 1 se muestran los principales criterios existentes en la actualidad para definir el SM en población adolescente. Destaca que todos los criterios recogidos en la tabla contemplan como componentes del SM la obesidad abdominal, la hiperglucemia, hipertrigliceridemia, bajos niveles séricos de HDL e HTA. De todos estos criterios, solo el definido por Viner et al. (2005) requiere de la presencia de 4 o más componentes para el diagnóstico de SM; mientras que el resto de criterios solo precisa de 3 o más componentes (Cook et al., 2003; Cruz & Goran, 2004; de Ferranti et al., 2004; Duncan

et al., 2004; Rodríguez-Morán et al., 2004; Weiss et al., 2004; Zimmet et al., 2007). Sin embargo, a diferencia del resto de criterios, el propuesto por la IDF es el único que contempla la presencia obligatoria de obesidad abdominal para el diagnóstico de SM (Zimmet et al., 2007).

TABLA 1. CRITERIOS DIAGNÓSTICOS PARA EL SÍNDROME METABÓLICO EN ADOLESCENTES.

Criterios	**Edad**	**Número de componentes**	**Obesidad**	**Glucosa** (mg/dL)	**TG** (mg/dL)	**HDL** (mg/dL)	**PAS** (mmHg)	**PAD** (mmHg)
IDF	10-16	Obesidad + 2	CCi > percentil 90	≥ 100	≥ 150	≤ 40	≥ 130	≥ 85
Cook	12-19	≥ 3	CCi > percentil 90	≥ 110	≥ 110	≤ 40	> percentil 90	-
de Ferranti	≥ 12	≥ 3	CCi > percentil 75	≥ 110	≥ 100	< 50 ♀; <45 ♂	> percentil 90	-
Weiss	4-20	≥ 3	IMC z-score ≥ 2	≥ 140	> percentil 95	< percentil 5	> percentil 95	-
Viner	2-18	≥ 4	IMC ≥ percentil 95	≥ 110	≥ 150	≤ 35	> percentil 95	-
Duncan	12-19	≥ 3	CCi ≥ percentil 90	≥ 110	≥ 110	< 40	≥ percentil 90	-
Rodríguez -Moran	10-18	≥ 3	CCi ≥ percentil 90	≥ 110	≥ percentil 90	-	≥ percentil 90	-
Cruz & Goran	8-13	≥ 3	CCi ≥ percentil 90	> 100	≥ percentil 90	< percentil 10	≥ percentil 90	-

IDF, Federación Internacional de Diabetes; TG, triglicéridos; HDL, lipoproteína de alta densidad; PAS, presión arterial sistólica; PAD, presión arterial diastólica; CCi, circunferencia de la cintura; IMC, índice de masa corporal.

IV. CARACTERÍSTICAS FISIOPATOLÓGICAS DEL SÍNDROME METABÓLICO

La obesidad y la RI conforman los principales factores fisiopatológicos del SM (International Diabetes Federation, 2006). A continuación, se pone en contexto los principales mecanismos moleculares y fisiopatológicos que contribuyen al desarrollo de SM, cuya comprensión es fundamental para la elaboración e implementación de estrategias preventivas y terapéuticas.

1. OBESIDAD

La obesidad se caracteriza por la acumulación excesiva de grasa en adipocitos que conduce a una hiperplasia e hipertrofia del tejido adiposo (Ros Pérez & Medina-Gómez, 2011; Zatterale et al., 2020). Las adipoquinas son un conjunto de péptidos señalizadores, secretadas por el tejido adiposo visceral, que desempeñan un rol fundamental en la regulación del metabolismo lipídico, glucídico y de la respuesta inflamatoria (Castellano et al., 2013). La inflamación crónica del tejido adiposo como consecuencia de su hiperplasia e hipertrofia asociada a la obesidad favorece la polarización de los macrófagos del tejido adiposo (ATMs) al fenotipo proinflamatorio M1 (J. Kim & Lee, 2021; J. Lee, 2013; Olefsky & Glass, 2010), promoviendo la secreción de determinadas adipoquinas, entre ellas la leptina (K. C. Wu et al., 2011), y las citoquinas proinflamatorias interleuquina-6 (IL-6), interleuquina-1β (IL-1β) y factor de necrosis tumoral-α (TNF-α) (Herrada et al., 2021; Ros Pérez & Medina-Gómez, 2011; Scarano et al., 2021; K. C. Wu et al., 2011). Dichas adipoquinas, principalmente la leptina, mantienen el estado inflamatorio al favorecer en el tejido adiposo la infiltración de macrófagos (Jang et al., 2010; Wellen & Hotamisligil, 2003). Sin embargo, los efectos de la leptina son neutralizados por la adiponectina, una adipoquina antidiabética, antiinflamatoria y antiaterogénica cuya secreción se encuentra disminuida en individuos con obesidad (Fahed et al., 2022; Frühbeck et al., 2018; Yadav et al., 2013). Se cree que la adiponectina induce la activación de los macrófagos M2 (fenotipo antiinflamatorio), inactiva la vía inflamatoria factor nuclear potenciador de las cadenas ligeras kappa de las células B (NF-

κB) (Fahed et al., 2022; Herrada et al., 2021), y aumenta la sensibilidad a la insulina (Hurrle & Hsu, 2017).

La variabilidad de los niveles séricos de las adipoquinas observados en la obesidad se consideran determinantes independientes de la RI (Mente et al., 2010). De hecho, varios autores han demostrado que tanto la leptina como la adiponectina correlacionan con el índice HOMA2-IR, un marcador de RI y SM (Esteghamati et al., 2009; Goropashnaya et al., 2009). En este sentido el estudio realizado por Aguilar et al. (2013) en 24 adolescentes españoles de 12 a 16 años observó una correlación negativa significativa entre los niveles de adiponectina y el índice HOMA2-IR, así como una correlación positiva no significativa entre los contenidos plasmáticos de leptina y el índice HOMA2-IR. En esta línea, el estudio de Al-Daghri et al. (2014) realizado en 619 sujetos con una edad comprendida entre 4-17 años observó una asociación estadísticamente significativa entre el índice HOMA-IR y los niveles séricos de leptina, así como una asociación negativa entre el índice HOMA-IR y los niveles séricos de adiponectina.

En la obesidad no solo se produce acumulación de grasa y ácidos grasos libres (AGL) en el tejido adiposo, sino también en otros tejidos dianas de la insulina como son el hígado y el músculo estriado esquelético, circunstancia que promueve la activación de las quinasas c-Jun N-terminal (JNK) e IκB (IKK) (Feng et al., 2020; Nandipati et al., 2017). De esta forma se agrava el estado inflamatorio puesto que la fosforilación de la quinasa IKK activa el factor de transcripción NF-κB. Dicho factor regula positivamente la liberación de las citoquinas proinflamatorias IL-6, IL-1β y TNF-α (Nandipati et al., 2017), las cuales tienen un efecto negativo en la señalización de la insulina (Castellano et al., 2013). No obstante, la proteína quinasa activada por AMP (AMPK) desempeña un papel clave en el control de este estado inflamatorio, dado que su activación inhibe la señalización de NF-κB. Las quinasas AMPK e IKK no solo influyen en la señalización de la insulina modulando el proceso inflamatorio de la obesidad, sino también a través de su acción sobre el sustrato del receptor de insulina (IRS) (Nandipati et al., 2017). Cabe destacar que la vía JNK junto con la vía cinasa 1/2 regulada por señales extracelulares (ERK1/2), y p38 comprenden la conocida vía proteínas quinasas activadas por mitógenos (MAPKs) (Ou et al., 2020), cuya alteración se encuentra implicada en la

inducción de RI (Nandipati et al., 2017). En definitiva, la inflamación crónica de los tejidos diana de la insulina como consecuencia de la obesidad provoca perturbaciones en el metabolismo glucídico y lipídico, bloquea la señalización de la insulina, y reduce la insulino-sensibilidad, alteraciones que contribuyen al desarrollo de la RI (Feng et al., 2020; J. Lee, 2013).

2. RESISTENCIA A LA INSULINA

En condiciones normales la insulina se une a las subunidades α del receptor de insulina (IR), induciendo la autofosforilación de dicho receptor y la activación de los IRS (Tong et al., 2022). Posteriormente, el sustrato-1 del IR se une a la fosfatidilinositol-3-OH quinasa (PI3K) y produce la fosforilación del residuo serina/treonina de la proteína cinasa B (Akt). La activación de Akt regula la translocación del transportador de glucosa 4 (GLUT4) a la membrana plasmática celular con el fin de permitir la absorción de glucosa en la célula (Khalid et al., 2021; S. Lee et al., 2022; Tong et al., 2022). En la RI, la sensibilidad en los tejidos diana a la actividad celular de la insulina en niveles normales de esta hormona se encuentra disminuida (S. Lee et al., 2022; Weiss et al., 2013), lo que resulta en una alteración de la activación de la vía IRS/PI3K/Akt (Huang et al., 2018; Tong et al., 2022). Como consecuencia, aparece una reducción de la absorción de la glucosa en el tejido adiposo y musculo estriado esquelético, un incremento de la actividad gluconeogénica y glucolítica hepática, y alteraciones del metabolismo lipídico en el hígado y tejido adiposo (Batista et al., 2021; Czech, 2017; Dilworth et al., 2021).

El exceso de grasa visceral es responsable de un desequilibrio de la secreción de adipoquinas y de un aumento de AGL, que activan la nicotinamida adenina dinucleótido fosfato oxidasa (NADPH), aumentando la secreción de especies reactivas del oxígeno (ROS) (Hurrle & Hsu, 2017). El estrés del retículo endoplásmico (ER) y el exceso de síntesis de ROS en los tejidos dianas de la insulina conduce a la activación de vías que tienen un efecto negativo sobre la señalización de la insulina, como son las vías JNK, IKK, p38 y el factor NF-κB (Batista et al., 2021; Yaribeygi et al., 2020). Asimismo, se ha informado que la hiperglucemia también induce la sobreproducción de ROS (Mahdavi et al., 2021). El mantenimiento de niveles elevados de citoquinas proinflamatorias, del estrés oxidativo, y del estrés del ER agravan el estado de RI dado que disminuyen la sensibilidad

a la insulina (Lima et al., 2022). De esta forma se establece un círculo vicioso a través del cual la RI y obesidad promueven el estrés oxidativo, y éste a su vez empeora el estado insulino-resistente (Andreadi et al., 2022).

La aparición de hiperinsulinemia compensatoria a la RI es una hipótesis ampliamente aceptada; no obstante, en los últimos años se ha planteado también la hipótesis de que la hiperinsulinemia podría preceder a la RI (Czech, 2017; James et al., 2021; Shanik et al., 2008). Independientemente del debate existente sobre si la hiperinsulinemia precede a la RI o aparece como mecanismo compensatorio de la RI, la presencia de ambos factores repercuten negativamente sobre la salud, ya que favorecen el desarrollo de DTM2 y de otras anomalías metabólicas relacionadas con el SM (Abdul-Ghani & DeFronzo, 2021).

3. DISLIPIDEMIA Y ATEROGÉNESIS

La dislipidemia aterogénica del SM se caracteriza por la presencia elevada en sangre de triglicéridos (TG), bajos niveles de HDL, y presencia de LDL con mayor capacidad aterogénica (Halpern et al., 2010). La homeostasis del metabolismo lipídico y glucídico en la obesidad se ve comprometida por la acumulación excesiva de TG, el incremento de la secreción de citoquinas proinflamatorias (A. Guilherme et al., 2008) y el aumento de la lipólisis por la acción disminuida de la insulina, hormona que tiene un papel importante en estimular la lipogénesis e inhibir la lipólisis (Batista et al., 2021). En situación de RI la lipólisis conduce a la síntesis de AGL, que son utilizados junto a la apolipoproteína (apo) B para la síntesis de VLDL en el hígado (Clavey et al., 1995; Davatgaran Taghipour et al., 2019; Kohen-Avramoglu et al., 2003). También se reprime la expresión del receptor LDL, que interviene en la captación hepática de lipoproteínas ricas en TG (TRL) (Clavey et al., 1995). Como consecuencia, los sujetos con RI presentan hipertrigliceridemia (Vergès, 2010), y una menor tasa de aclaramiento de TRL (Botham & Wheeler-Jones, 2013). La acumulación plasmática de estas TRL aumenta el riesgo de desarrollo de aterosclerosis (Clavey et al., 1995), puesto que su absorción por los macrófagos del espacio subendotelial favorece la formación de células espumosas. Estas células espumosas secretan citoquinas, moléculas de adhesión, y eicosanoides que agravan el estado inflamatorio (Botham & Wheeler-Jones, 2013).

4. HIPERTENSIÓN ARTERIAL

En la literatura científica se ha propuesto que la obesidad visceral activa diferentes mecanismos y vías, entre los que se encuentran el sistema renina angiotensina aldosterona (SRAA), el aumento de la actividad del sistema nervioso simpático (SNS) y la resistencia a la insulina/hiperinsulinemia, que inducen el desarrollo de HTA en el SM (Whaley Connell et al., 2007).

El estado inflamatorio de bajo grado que caracteriza a la obesidad promueve la secreción en el tejido adiposo visceral de adipoquinas tales como la leptina, TNF-α, IL-6, y los ácidos grasos no esterificados (NEFA). Diversos autores han propuesto que estas adipoquinas contribuyen a la activación del SRAA mediante la estimulación de la síntesis de angiotensinógeno en los adipocitos (Cabandugama et al., 2017; Yanai et al., 2008). Un aumento de la expresión del gen del angiotensinógeno, de la enzima convertidora de angiotensina y del receptor de angiotensina de tipo 1, así como una mayor síntesis de angiotensinógeno y angiotensina II se ha observado en el tejido adiposo de sujetos obesos (Mendizábal et al., 2013; Yanai et al., 2008). Además de la obesidad, también se ha asociado este aumento de angiotensina II a la resistencia a la insulina (Rochlani et al., 2017).

Otros de los mecanismos implicados en la inducción de HTA en el SM es la activación del SNS, principalmente por la exposición crónica a altos niveles de leptina que disminuye la natriuresis y el óxido nítrico (NO) vascular biodisponible (Koleva et al., 2016). Igualmente la activación del SRAA en el tejido adiposo reduce la biodisponibilidad del NO en el tejido cardiovascular (Cabandugama et al., 2017). Los niveles de este factor vasodilatador también se ven disminuidos por los efectos de los AGL, que, además, aumentan la producción de ROS, y por consiguiente la secreción de endotelina vasoconstrictora (Andreadi et al., 2022; Fahed et al., 2022). Asimismo la angiotensina II también incrementa los niveles de ROS a través de la activación de NADPH (Rochlani et al., 2017). La RI contribuye al desarrollo de HTA en el SM por la pérdida del efecto vasodilatador de la insulina (Fahed et al., 2022). Además, otros autores han informado que la angiotensina II y las ROS inhiben la vía PI3K/Akt, lo que implica una pérdida de la síntesis de NO (Mendizábal et al., 2013; Yanai et al., 2008). La disminución de la

biodisponibilidad del NO vascular, la mayor reabsorción de sodio y el aumento de la producción de ROS producen el aumento de vasoconstricción que promueve la HTA.

V. ESTRATEGIAS PREVENTIVAS Y TERAPÉUTICAS FRENTE AL SÍNDROME METABÓLICO EN EDADES TEMPRANAS

La detección precoz en niños y adolescentes de SM o de alguno de sus componentes de forma individual es fundamental para la aplicación tanto de estrategias preventivas como terapéuticas (Bussler et al., 2017) que permitan ralentizar las graves consecuencias y el riesgo cardiovascular que el SM ocasiona a largo plazo (DeBoer, 2019). Los programas de identificación temprana de SM deben incluir mediciones del IMC y de la CCi, de la presión arterial, del perfil lipídico, y de los niveles glucémicos (Bussler et al., 2017); variables consideradas en los criterios para definir SM descritos previamente. Estos criterios posibilitan la categorización del SM y la valoración de los componentes relacionados, así como la evaluación del riesgo cardiovascular a largo plazo (DeBoer, 2019).

El desarrollo de obesidad abdominal durante la infancia y adolescencia aumenta la probabilidad de padecer obesidad durante la edad adulta. De hecho, en un metaanálisis de estudios prospectivos de cohortes realizado por Simmonds et al. (2016) se observó que el 79 % de los adolescentes que fueron diagnosticados de obesidad continuaron padeciendo esta condición en la edad adulta. Estos datos muestran la dificultad de perder el exceso de peso tras el desarrollo de sobrepeso u obesidad. Teniendo en cuenta que la obesidad es uno de los principales factores conducentes al establecimiento de SM (International Diabetes Federation, 2006), una de las principales líneas de actuación para el control epidemiológico de este síndrome debe sustentarse en la prevención de obesidad en población infantil y adolescente (DeBoer, 2019) a través de estrategias basadas en el entorno o la comunidad (Weihe & Weihrauch-Blüher, 2019).

En 2016, la OMS (World Health Organization, 2017) presentó un informe que reunía una serie de recomendaciones para orientar a los países en la lucha contra la

obesidad infantil encuadradas en seis principales áreas de actuación: i) promoción de la ingesta de alimentos saludables y reducción del consumo de bebidas azucaradas, ii) fomentar la actividad física y reducir los comportamientos sedentarios, iii) atención en la preconcepción y embarazo, iv) proporcionar apoyo y orientación para la adopción de una alimentación saludable y la realización de actividad física desde la infancia, v) salud, nutrición y actividad física en edad escolar, y vi) control del peso en niños y adolescentes obesos. En este sentido las estrategias de prevención de SM deben centrarse en la promoción de un estilo de vida activo en la infancia y mantener los niveles de actividad física; impulsar en el seno familiar la ingesta de alimentos frescos y evitar alimentos hipercalóricos; y desarrollar políticas públicas que aumenten los espacios seguros disponibles para la realización de actividad física y promuevan en los espacios escolares una alimentación saludable (DeBoer, 2019).

Las intervenciones sobre los estilos de vida constituyen el eje vertebral del manejo terapéutico de SM en niños y adolescentes (Tagi et al., 2020), sobre todo en una sociedad que en la actualidad presenta una dinámica cada vez más tendente a la práctica de estilos de vida sedentarios y no saludables (Federation, 2019; World Health Organization, 2021). Se ha demostrado en dos metaanálisis de estudios observacionales realizados por Wu et al. (2022) y por Jahangiry et al. (2022), que la práctica de un sedentarismo prolongado y un tiempo excesivo frente a una pantalla incrementa el riesgo de desarrollar SM en población infantil y adolescente. Según recomendaciones generales de la OMS, la realización tanto de actividades físicas aeróbicas de intensidad vigorosa como de ejercicios de resistencia que refuercen el sistema locomotor deben implementarse en población infantil y adolescente al menos 60 minutos al día durante un mínimo de 3 días a la semana. Asimismo, el tiempo dedicado a actividades sedentarias, especialmente el dedicado a las pantallas debe ser restringido (World Health Organization, 2020). En cuanto al tipo de actividad física, varios estudios han demostrado en adolescentes obesos o con alteraciones metabólicas características del SM una mayor eficacia del entrenamiento combinado (ejercicios aeróbicos y ejercicios de resistencia) en mejorar la obesidad abdominal, el perfil metabólico y lipídico, y los marcadores inflamatorios (Campos et al., 2014; Dâmaso et al., 2014; Liang et al., 2021).

Además de la actividad física, las intervenciones dietéticas también ejercen un rol fundamental en el tratamiento de la obesidad y las alteraciones metabólicas que conforman el SM. El objetivo del tratamiento de la obesidad se orienta a la búsqueda de un balance energético negativo (E. Y. Lee & Yoon, 2018), puesto que ésta es consecuencia de un desequilibrio crónico entre el gasto energético y la ingesta calórica (Zatterale et al., 2020). Un programa de pérdida de peso consistente en realización de actividad física y una intervención dietética basada en el control de la ingesta calórica fue aplicado a 282 niños y adolescentes obesos chinos durante 3-4 semanas. Tras su conclusión observaron en adolescentes obesos con alteraciones metabólicas disminuciones significativas en los niveles de presión arterial sistólica (PAS), presión arterial diastólica (PAD), glucemia en ayunas y trigliceridemia (Yang et al., 2022). En este mismo sentido, Hoare et al. (2021) en un estudio de revisión concluyó que tanto las dietas de restricción energética como las dietas bajas en carbohidratos son efectivas para el tratamiento a corto plazo de obesidad y sus comorbilidades. Sin embargo, la disminución del cumplimiento de estas dietas durante el tiempo acorta sus beneficios a largo plazo. En población adolescente se ha observado una relación inversamente proporcional entre una mayor adherencia a la dieta mediterránea y los valores de IMC, CCi y porcentaje de grasa corporal (Seral-Cortes et al., 2022). En esta misma línea, la aplicación de una dieta mediterránea durante 16 semanas a adolescentes obesos produjo una disminución significativa del IMC, masa grasa, glucemia, colesterolemia, trigliceridemia, y niveles séricos de LDL, y un aumento significativo de los niveles plasmáticos de HDL (Velázquez-López et al., 2014).

A pesar de la eficacia demostrada de intervenciones basadas en mejorar los patrones dietéticos y de actividad física en población infantil y adolescente, su implementación en la práctica clínica requiere de un proceso intensivo y a largo plazo debido al proceso de maduración psicológica e intelectual en el que se encuentran inmersos este grupo de población (Bussler et al., 2017; E. Y. Lee & Yoon, 2018). En ocasiones, el fracaso de las intervenciones no farmacológicas en la consecución de unos estilos de vida más saludables y de resultados positivos sobre cada uno de los componentes del SM conduce al establecimiento de la terapia farmacológica (Bussler et al., 2017). Sin embargo, dada la

inexistencia de una terapia farmacológica específica y única para el SM hace que el enfoque de la misma se oriente al control de cada uno de sus componentes, recurriendo a las estatinas para tratar la dislipidemia, antiagregantes plaquetarios para disminuir el riesgo protrombótico, sensibilizadores a la insulina para disminuir el estado de RI y antihipertensivos (Ahmed et al., 2022; Bussler et al., 2017; Rochlani et al., 2017; Tagi et al., 2020). En caso de fracaso de todas las medidas no farmacológicas o farmacológicas, o en situaciones de obesidad extrema, como última opción se encuentra la cirugía bariátrica, una técnica que produce una pérdida de peso rápida (Nicolucci & Maffeis, 2022; Weihe & Weihrauch-Blüher, 2019), y que implica un mejor control de las complicaciones de la obesidad y sus comorbilidades como DTM2 y HTA (Bussler et al., 2017; Weihe & Weihrauch-Blüher, 2019). No obstante, aún son limitados los conocimientos acerca de sus efectos y complicaciones a largo plazo en adolescentes (Tagi et al., 2020; Torbahn et al., 2022).

VI. COMPUESTOS BIOACTIVOS EN EL MANEJO DEL SÍNDROME METABÓLICO

La adherencia terapéutica a la terapia farmacológica existente para cada uno de los componentes del SM se ve comprometida por la diversidad de fármacos existentes, así como por la aparición de efectos adversos no deseados derivados de su administración a largo plazo. Tanto los fármacos como las comorbilidades del SM suponen un gran costo económico para los sistemas sanitarios (Ahmed et al., 2022; Rask Larsen et al., 2018; Rochlani et al., 2017). Además, la combinación de fármacos y compuestos bioactivos podría ser más eficiente para el tratamiento, en fases avanzadas, de DMT2, una de las complicaciones del SM (Blahova et al., 2021).

En los últimos años numerosos estudios han analizado las propiedades, efectos terapéuticos y compuestos bioactivos presentes en numerosas plantas, tales como la cúrcuma, la canela, el jengibre, el olivo, goma arábica, entre otras. Dichas plantas contienen compuestos bioactivos, como polifenoles, terpenos, xantonas, carotenoides, esteroles, que presentan propiedades insulino-sensibilizantes, hipoglucemiantes, hipolipemiantes, hipotensores, antioxidantes, y de pérdida de peso y grasa corporal

(Ahmed et al., 2022; Alipour et al., 2022; Gumbarewicz et al., 2022; Mahdavi et al., 2021; Nyakudya et al., 2020). Estas propiedades presentan un gran interés y potencial para el tratamiento y prevención de las alteraciones metabólicas del SM.

Cada vez hay una tendencia más frecuente de estudios que examinan los compuestos bioactivos presentes en el aceite de oliva y sus efectos terapéuticos (Fernández-Castillejo et al., 2021; Marrone et al., 2022; Moral & Escrich, 2022; Noce et al., 2021; Rodríguez-Morató et al., 2015). El aceite de oliva está compuesto por numerosos compuestos menores como son los fitoesteroles, compuestos fenólicos, carotenoides, terpenoides y tocoferoles; que junto a los altos niveles de ácido oleico justifican sus propiedades antiinflamatorias y antioxidantes (Bagetta et al., 2020; Storniolo et al., 2019). Diversos estudios experimentales y ensayos clínicos se han llevado a cabo para analizar los efectos preventivos y terapéuticos de los compuestos menores del aceite de oliva sobre el SM. En un ensayo controlado aleatorizado llevado a cabo en 51 individuos sanos, el consumo aceite de oliva enriquecido en compuestos fenólicos y triterpénicos durante tres semanas demostró ser eficaz en la disminución de la oxidación urinaria del ADN y de biomarcadores inflamatorios plasmáticos en comparación con individuos que consumieron un aceite de oliva virgen estándar (Sanchez-Rodriguez et al., 2019). Otro estudio realizado por Claro-Cala et al. (2020), en esta ocasión en ratones obesos inducidos por la dieta, demostró la atenuación de la RI y la inflamación del tejido adiposo tras la administración de un aceite de orujo de oliva enriquecido en diferentes ácidos triterpénicos, entre ellos el ácido maslínico y el ácido oleanólico (AO). En esta misma línea, el ensayo controlado aleatorizado PREDIABOLE informó, tras la administración en 176 adultos prediabéticos durante 30 meses de un aceite de oliva enriquecido con AO, de una disminución del 55 % del riesgo de desarrollar DMT2 y una mejora del estado de RI (Santos-Lozano et al., 2019). No obstante, destaca la ausencia de ensayos clínicos que empleen AO en población adolescente con SM, RI o DMT2.

1. ÁCIDO OLEANÓLICO: PROPIEDADES EN SALUD Y MECANISMO DE ACCIÓN

En el reino vegetal se encuentra ampliamente presente el triterpeno pentacíclico AO ($C_{30}H_{48}O_3$; ácido (3β)-3-hidroixoleano-12-en-28-oico), tanto como ácido libre o

formando parte de saponinas triterpenoides (Guinda et al., 2010). La epidermis de la hoja del olivo (*Olea Europaea* L.) contiene diferentes tipos de triterpenos, y en menor medida en su fruto, siendo los triterpenos de tipo oleanano los que se encuentran en mayores concentraciones (Giménez et al., 2015; Guinda et al., 2010). De los triterpenos de tipo oleanano presentes en la hoja del olivo el principal es el AO, al representar el 3,00-3,50% del peso seco de la hoja de olivo. La variabilidad de estos porcentajes depende según la variedad del olivo (Guinda et al., 2010).

Diferentes autores han estudiado las efectos profilácticos y terapéuticos del AO y sus derivados en diversas enfermedades crónicas, como colitis ulcerosa, osteoporosis, esclerosis múltiple, hepatitis, diferentes tipos de cáncer, trastornos metabólicos, y diabetes mellitus (Ayeleso et al., 2017; Sen, 2020; Y. Wu et al., 2021). El interés cada vez mayor de estudios que analizan los efectos del AO en enfermedades crónicas se justifica por sus propiedades antivirales, antibacterianas, antifúngicas, antialérgicas (Guinda et al., 2010), y anticancerígenas (Guinda et al., 2010; Žiberna et al., 2017). También se ha informado que el AO presenta actividades antihipertensivas, hepatoprotectoras, hipolipidémicas, antiateroscleróticas, antiinflamatorias, antioxidantes, y antidiabéticas (Ayeleso et al., 2017; Castellano et al., 2013; Guinda et al., 2010; Rodriguez-Rodriguez, 2015), de gran interés para el tratamiento y prevención de SM, RI y de sus comorbilidades.

Los mecanismos moleculares que dan explicación a las diferentes propiedades del AO anteriormente mencionadas han sido ampliamente estudiados por diferentes autores (Ayeleso et al., 2017; Castellano et al., 2013; Carmen M. Claro-Cala et al., 2022; Rodriguez-Rodriguez, 2015; Sharma et al., 2018). Entre las diferentes vías se encuentra su papel activador sobre el receptor 5 acoplado a proteína G de Takeda (TGR5), que supone una mejora de la homeostasis de la glucosa, reducción de las citoquinas proinflamatorias y disminución del peso corporal TGR5 (Pols et al., 2011). Además, la activación de este receptor en células β pancreáticas estimula la secreción de insulina (Maczewsky et al., 2019). Se ha sugerido que el AO podría tener un papel represor sobre el factor de transcripción NF-κB, y activador sobre el factor nuclear derivado de eritroide 2 (Nrf2). Estos factores desempeñan un papel importante en el estado inflamatorio que se establece en la RI (Castellano et al., 2013, 2022; M. Li et al., 2015). Considerando que

la RI podría preceder en años a la DMT2, y la influencia de otras alteraciones metabólicas como la HTA, la obesidad y la dislipemia en el desarrollo de RI, es necesario una mayor compresión de los mecanismos moleculares subyacentes a través de los cuales el AO podría ejercer un efecto positivo en el SM.

2. ÁCIDO OLEANÓLICO: TRANSPORTE Y BIODISPONIBILIDAD

El interés en compuestos triterpénicos como el AO no solo radica en sus actividades biológicas, sino también en su potencial formando parte de alimentos funcionales (Guinda et al., 2010). El AO se caracteriza por ser una biomolécula prácticamente insoluble en medios acuosos (Eloy et al., 2015). No obstante, diferentes autores han informado recientemente un aumento considerable de la biodisponibilidad del AO tras su administración en matrices lipofílicas (de la Torre et al., 2020; Rada et al., 2015). Más concretamente, en su ensayo postprandial en humanos Rada et al. (2015), observaron a las tres horas de la ingesta de un aceite de orujo de oliva enriquecido con 30 mg de AO una concentración plasmática máxima de AO de 598.2 ±176.7 ng/ml. En otro ensayo postprandial realizado en 12 participantes, de la Torre et al. (2020) observaron una concentración plasmática máxima de 5.1 ± 2.1 ng/mL de AO a las 4 horas de la ingesta de 30 mL de un aceite de oliva enriquecido con 4.7 mg de este triterpeno. Además, se ha informado que aproximadamente el 99 % del AO circula unido a proteínas séricas humanas, en especial a la albúmina (Rada et al., 2011). Sin embargo, la naturaleza lipofílica del AO sugiere que podría ser transportado por TRL tras su absorción intestinal. Aunque no hay amplia evidencia científica al respecto, un estudio ha demostrado la eficacia de TRL artificiales como transportadores de AO (Espinosa et al., 2022).

JUSTIFICACIÓN, OBJETIVOS Y DISEÑO EXPERIMENTAL

I. JUSTIFICACIÓN

La práctica cada vez mayor y a edades más tempranas de hábitos no saludables en la sociedad actual está provocando un aumento de la incidencia y prevalencia de obesidad en población adolescente. La obesidad junto con la RI son los dos principales factores fisiopatológicos conducentes al desarrollo de SM, un conjunto de anomalías metabólicas que aumentan el riesgo de padecer ECV y de producir numerosas complicaciones, entre las que destaca la DMT2. Por ello, la detección precoz del SM debe constituir una prioridad en salud en edades tempranas. En este sentido, en los últimos años se han elaborado numerosos criterios para el diagnóstico de SM en adolescentes, aunque con una manifiesta heterogeneidad entre ellos, determinada por que los cambios intrínsecos al crecimiento y desarrollo puberal dan lugar a puntos de corte sin valores establecidos. De esta forma, valorar el grado de concordancia entre los diferentes criterios diagnósticos para adolescentes posibilitaría un adecuado cribado de SM, así como implementar en esta población intervenciones tempranas enfocadas a modificar estilos de vida; también permitiría avanzar en la aplicación de diversas estrategias preventivas y terapéuticas.

En ocasiones la administración de tratamientos farmacológicos a largo plazo puede producir efectos adversos; además, se ha sugerido que éstos podrían aumentar su eficacia siendo complementados con productos naturales. Entre los diferentes productos naturales existentes, se encuentran los compuestos triterpénicos, siendo uno de los más destacados el AO, cuyas propiedades antioxidantes, antiinflamatorias, insulino-sensibilizantes, hipolipidémicas, antidiabéticas, anti-ateroscleróticas y antihipertensivas han sido ampliamente probadas y son de gran interés para el abordaje terapéutico y preventivo de cada uno de los componentes del SM. Sin embargo, la evidencia disponible relativa a los mecanismos moleculares que justifican las propiedades del AO en el contexto del SM en población adolescente es muy limitada. Además, su mecanismo de transporte y la biodisponibilidad son factores esenciales a conocer para el diseño de intervenciones, dietéticas o farmacológicas, con AO. Hasta la fecha se ha demostrado la presencia de este triterpeno en plasma sanguíneo tras su administración en disolución en una matriz lipídica, y también que es transportado unido a albúmina. Sin embargo, se

desconoce si el AO se transporta unido a TRLs postprandiales. Por tanto, no solo se debe seguir profundizando en los efectos terapéuticos del AO y los mecanismos moleculares explicativos, sino también en los mecanismos de transporte con el fin de obtener una mejor eficacia de este compuesto bioactivo.

II. OBJETIVOS

1. OBJETIVOS GENERALES

- Caracterizar y definir el SM en adolescentes.
- Analizar los efectos terapéuticos del AO sobre monocitos-macrófagos THP-1, así como sobre la formación de células espumosas.
- Profundizar en los mecanismos de transporte postprandial de AO en adolescentes sanos y con normopeso.

2. OBJETIVOS ESPECÍFICOS

Para el desarrollo de los objetivos generales se definieron los siguientes objetivos específicos:

- Contrastar el grado de acuerdo entre ocho criterios diagnósticos para definir SM en adolescentes (**Estudio I**).
- Describir los efectos de formulaciones de AO sobre los componentes del SM, y sobre citoquinas proinflamatorias y enzimas antioxidantes como biomarcadores del estrés oxidativo en humanos y animales (**Estudio II**).
- Profundizar en el conocimiento existente acerca de los efectos del AO sobre los mecanismos moleculares y vías de señalización implicados en el desarrollo de resistencia a la insulina y el estrés oxidativo subyacente en modelos animales y líneas celulares inducidos de RI (**Estudio III**).
- Evaluar los efectos del AO sobre la producción de citoquinas proinflamatorias y proteínas de señalización en macrófagos THP-1 estimulados con LPS (**Estudio IV**).
- Valorar los efectos de TRLs postprandiales, obtenidas a partir de sangre de adolescentes sanos tras la ingesta de aceite de oliva enriquecido en AO, sobre la

producción de citoquinas proinflamatorias y proteínas de señalización en macrófagos THP-1; así como analizar la acumulación lipídica intracelular (**Estudio IV**).

- Determinar la presencia de AO en TRLs postprandiales tras la ingesta de aceite de oliva funcional enriquecido con AO en adolescentes sanos y con normopeso (**Estudio V**).

III. DISEÑO EXPERIMENTAL

En la Tabla 1 se muestran los títulos de las publicaciones que responden a los objetivos específicos previamente planteados, así como un resumen de la metodología de dichos estudios y las variables estudiadas.

TABLA 2. RESUMEN DE CARACTERÍSTICAS Y METODOLOGÍA DE TODOS LOS ESTUDIOS QUE CONFORMAN LA TESIS DOCTORAL.

TÍTULO	METODOLOGÍA	VARIABLES
I. Concordance among diagnostic criteria for metabolic syndrome is inconsistent in Spanish adolescents.	**Diseño:** estudio transversal. **Participantes:** 981 adolescentes. **Procedimiento:** Evaluación antropométrica, medición de presión arterial, examen sanguíneo, y diagnóstico de SM utilizando ocho criterios diferentes. **Análisis estadístico:** t-student y ANOVA para analizar diferencia entre medias, y valores Kappa para analizar el nivel de acuerdo entre los ocho criterios empleados.	Datos antropométricos y bioquímicos. Prevalencia de SM. Valores Kappa (κ) para estudiar grado de concordancia entre criterios.
II. Potential protective effect of oleanolic acid on the components of metabolic syndrome: a systematic review.	**Diseño:** revisión sistemática. **Estudios seleccionados:** 23 estudios de experimentación animal, y 1 ensayo clínico. **Criterios de inclusión:** estudios publicados entre 2013 y 2019, que analizaran los efectos del AO sobre los componentes del SM. **Estrategia de búsqueda:** Oleanolic acid AND ("metabolic syndrome"	Aquellas relacionadas con: - HTA. - Perfil lipídico. - Hiperglucemia. - RI. - Citoquinas proinflamatorias, y enzimas antioxidantes.

	OR "insulin resistance" OR obesity OR hypertension OR inflammation). **Valoración del riesgo de sesgo:** SYRCLE para estudios en animales, escala JADAD para ensayos clínicos en humanos.	
III. Potential molecular targets of oleanolic acid in insulin resistance and underlying oxidative stress: a systematic review.	**Diseño:** revisión sistemática. **Estudios seleccionados:** 13 estudios de experimentación animal, y 3 estudios sobre líneas celulares. **Criterios de inclusión:** Estudios publicados entre 2001 y 2022, que versaran sobre los efectos del OA sobre los mecanismos moleculares y vías de señalización implicados en el desarrollo de la resistencia la insulina y el estrés oxidativo subyacente, en modelos de experimentación animal y en líneas celulares inducidos de resistencia a la insulina. **Estrategia de búsqueda:** Oleanolic acid AND ("insulin resistance" OR "oxidative stress" OR "reactive oxygen species" OR cytokines OR antioxidants OR kinases). **Valoración del riesgo de sesgo:** SYRCLE para estudios en animales.	Vías de señalización de la insulina, inflamatorias y de estrés oxidativo. Biomarcadores inflamatorios y de estrés oxidativo.
IV. Oleanolic acid-enriched olive oil alleviates the interleukin-6 overproduction induced by postprandial triglyceride-rich lipoproteins in THP-1 macrophages.	**Diseño:** ensayo postprandial, controlado, aleatorizado y doble ciego; y ensayo experimental en macrófagos THP-1. **Participantes:** 22 adolescentes sanos y en normopeso; monocitos-macrófagos THP-1. **Procedimiento:** obtención de TRLs postprandiales de adolescentes, tras consumo de aceite de oliva (normal o funcional); formación de células espumosas con las TRLs; y aplicación de OA a macrófagos THP-1 previamente estimulados con LPS.	Citoquinas proinflamatorias (IL-6, IL-1β, TNF-α). Proteínas de señalización (AMPK-α, Akt). Ácidos grasos acumulados intracelularmente.

V. Analysis of triglyceride-rich lipoproteins as a possible mechanism for postprandial transport of oleanolic acid in adolescents.	**Diseño:** ensayo postprandial, controlado, aleatorizado y doble ciego. **Participantes:** 22 adolescentes sanos y en normopeso. **Procedimiento:** obtención de TRLs postprandiales de adolescentes, tras consumo de aceite de oliva (normal o funcional); extracción lipídica y análisis por cromatografía de gases acoplado a espectrometría de masas (GC-MS) de la presencia de AO en TRLs.	Datos antropométricos y bioquímicos. Cromatogramas y concentraciones de AO en TRLs.

SM, síndrome metabólico; HTA, hipertensión arterial; AO, ácido oleanólico; SYRCLE, Centro de Revisión Sistemática para Experimentación con Animales de Laboratorio; LPS, lipopolisacárido; TRLs, lipoproteínas ricas en triglicéridos; IL-6, interleucina 6; IL-1β, interleucina 1β; RI, resistencia a la insulina; TNF-α, factor de necrosis tumoral alfa.

PUBLICACIONES

ESTUDIO I

Referencia: Fernández-Aparicio, Á., Perona, J. S., Schmidt-RioValle, J., & González-Jiménez, E. (2021). Concordance among diagnostic criteria for metabolic syndrome is inconsistent in Spanish adolescents. *European Journal of Clinical Investigation*, *51*(2), e13384. https://doi.org/10.1111/eci.13384

Indicios de calidad: Impact Factor (2021) = 5,722. Edition: Science Citation Index Expanded. Category and rank: Medicine, General & Internal (39/172; Q1/T1).

I. Concordance among diagnostic criteria for metabolic syndrome is inconsistent in Spanish adolescents

Abstract

Background: The metabolic syndrome (MetS), although more frequent in adults, is a growing health problem in adolescent population. There are different criteria for the diagnosis, however without a consensus of which is the best to be used in this population. The heterogeneity of the different diagnostic criteria makes it necessary to carry out more studies that analyze the degree of agreement among these criteria. The present study was aimed to evaluate the agreement between different criteria for diagnosis of MetS in adolescents.

Materials and methods: A cross-sectional study was performed on 981 adolescents (13.2 ± 1.2 years) randomly recruited from eighteen schools in south-east Spain. MetS was diagnosed by eight different criteria.

Results: The criteria proposed by the IDF showed the highest mean values for WC and systolic blood pressure in boys and girls with MetS, and the lowest for glucose and triglycerides in boys. Depending on the diagnostic criteria used, the prevalence of MetS cases in boys ranged from 5.5% to 14.9%, while in girls varied from 3.4% to 32.6%. Both in boys and girls, the criteria proposed by the IDF was the less concordant with the other suggested criteria, while those proposed by Duncan et al, Rodriguez-Moran et al. and Cruz and Goran, were very concordant among each other. However, in girls, concordance values were not as high as those found for boys.

Conclusion: The variability observed in the agreement among the existing criteria suggests the need to validate uniform criteria for the diagnosis of MetS in adolescents.

Keywords: Anthropometric indexes; Diagnosis criteria; Metabolic syndrome; Adolescents.

1. Introduction

Metabolic syndrome (MetS) is characterized by a set of three or more metabolic disorders, including abdominal obesity, systemic arterial hypertension, elevated serum triglycerides (TG) and glycemia and low levels of high-density lipoprotein cholesterol (HDL-c).[1] Adolescents that suffer early changes in the components of MetS are associated with a high risk of developing this condition in adulthood,[2] with an increased risk of developing type 2 diabetes mellitus and cardiovascular diseases (CVD).[3]

On the other hand, the prevalence of MetS in adolescence in many studies is rather divergent, mainly due to the absence of specifically established criteria for its use in non-adult populations. This situation often involves adapting criteria to define MetS in adults to be used in adolescents.[4] This is the case of the criteria established by the National Cholesterol Education Program - Adult Treatment Panel III,[5] modified by Cook et al,[6] Weiss et al,[7] Duncan et al,[8] and de Ferranti et al.[9] The criteria defined by the International Diabetes Federation (IDF)[10] necessarily include the presence of abdominal obesity for the diagnosis of MetS. On the other hand, Cook et al,[6] Cruz & Goran,[11] de Ferranti et al[9] and Rodríguez-Moran et al[12] consider the existence of three or more impaired components of the Mets as compulsory for the diagnosis, regardless the presence of abdominal obesity. Viner et al,[13] based on the World Health Organization (WHO) criteria adapted for children by Alberti & Zimmet,[14] consider that the existence of four or more impaired components is essential for the diagnosis of MetS.

The main reasons for the heterogeneity in these criteria when adapted for the adolescent population is related to changes in growth and development during childhood and adolescence, which give rise to cut-off points without established values.[15,16] The divergence is such that some studies have shown a prevalence of MetS ranging from 20 to 300% in the same population depending on the criteria used.[17,18] The degree of agreement among these criteria is unknown at the moment. Therefore, it is necessary to define a universal criterion for the diagnosis of this condition in adolescents and that facilitates its early screening.[19] In this sense, the present study aims to contrast the degree of agreement among eight diagnostic criteria to define MetS in boys and girls.

2. Methods and materials

2.1. Study design and sample

A cross-sectional study was carried out on 981 adolescents (456 boys and 525 girls), 13.2 ± 1.2 years of age (11-16 years old), all of Spanish origin and similar socioeconomic status. The subjects attended 18 high-schools in the provinces of Granada and Almeria (South-East of Spain; 10 public and 8 private). A letter of invitation was sent to the school principals and all centers agreed to participate in the study. Of the 18 schools, two classes per grade of a total of three were randomly selected and invited to participate in the study. To be included in the study, the subjects had to be healthy and not have any type of endocrine dysfunction or physical disorder. All students who did not meet these criteria were not candidates to participate in the study. The flow diagram (Figure 1) summarizes the recruitment process. Reporting of the study conforms to broad EQUATOR guidelines.[20]

The study was previously approved by the Ethics Committee of the University of Granada and also authorized by the school principals. Written informed consent was obtained from all of the parents or legal guardians of the adolescents in accordance with the Declaration of Helsinki. Furthermore, the confidentiality of the personal information was guaranteed by coding the data.

2.2. Anthropometric measurements

Each participant underwent an anthropometric evaluation performed by a level 2 anthropometrist certified by the International Society for the Advancement of Kinanthro-pometry (ISAK), in accordance with the ISAK guidelines.[21] We performed the anthropometric measurements in the morning after a 12-hour fast and a 24-hour abstention from exercise. The anthropometric assessment was carried out individually in a classroom provided by each school, in order guarantee the privacy of the participants. We measured body weight (kg) twice (with participants wearing no shoes and in light clothes) using a self-calibrating Seca 861 class (III) digital floor scale (Saint Paul, USA) with a precision of up to 100 g. We measured height with a Seca 214* portable

stadiometer, asking participants to remove their shoes and stand erect with their backs, buttocks, and heels in continuous contact with the vertical height rod of the stadiometer and head oriented in the Frankfurt plane. We then placed the horizontal headpiece on top of the participants' heads to measure their height. We took height measurements twice to the nearest 0.5 cm. We used the average of the two values for weight and height in the analysis. We calculated body mass index (BMI) as weight divided by height squared (kg/m^2). We measured waist circumference (WC) with a Seca automatic roll-up measuring tape (precision of 1 mm) using the horizontal plane midway between the lowest rib and the upper border of the iliac crest at the end of a normal inspiration/expiration. Also measured were the triceps, biceps, subscapular, and suprailiac skinfolds. The instrument used for this purpose was a Holtain skinfold caliper (Holtain Ltd., Crymych, UK), with an accuracy of 0.1–0.2 mm. The percentage of body fat was based on these skinfold measurements. Previously, the Brook equation was used to calculate body density.[22] Once the body density value had been obtained, the body fat percentage was determined with the Siri equation.[23] A trained member of the research team collected anthropometric measures in a classroom enabled for this purpose by the principal of each school.

2.3. Serum biochemical examination

Blood collection was performed after a previous 12-h fast. At 8:00 AM, 10 mL of blood was extracted by venipuncture in the antecubital fossa of the right arm with a disposable vacuum blood collection tube. In the 4 hours after the extraction, all samples were centrifuged at 1300 g for 15 minutes (Z400 K, Hermle, Wehingen, Germany). The red blood cells were thus separated and the serum was finally frozen at −80°C for its subsequent analysis. Immediately after collection and before centrifugation, however, we measured glucose concentration using an enzymatic colorimetric method (glucose oxidase-phenol aminophenazone [GOD-PAP] method; Human Diagnostics, Germany) as well as the concentrations of HDL-C, total cholesterol, and triglycerides by means of enzymatic colorimetric methods using an Olympus analyzer. Low-density lipoprotein cholesterol (LDL-C) was estimated using the Friedewald equation ([LDLC] = [Total

Cholesterol] − [HDL-C] − ([TG]/5)), where TG = concentration of triglycerides. Serum insulin was determined by radioimmunoanalysis (Insulin Kit; DPC, Los Angeles, EEUU). Insulin resistance was quantified with HOMA (Homeostasis Model Assessment)[24] by applying the following formula: fasting glucose (mmol/L) × fasting insulin (mU/L)/22.5.

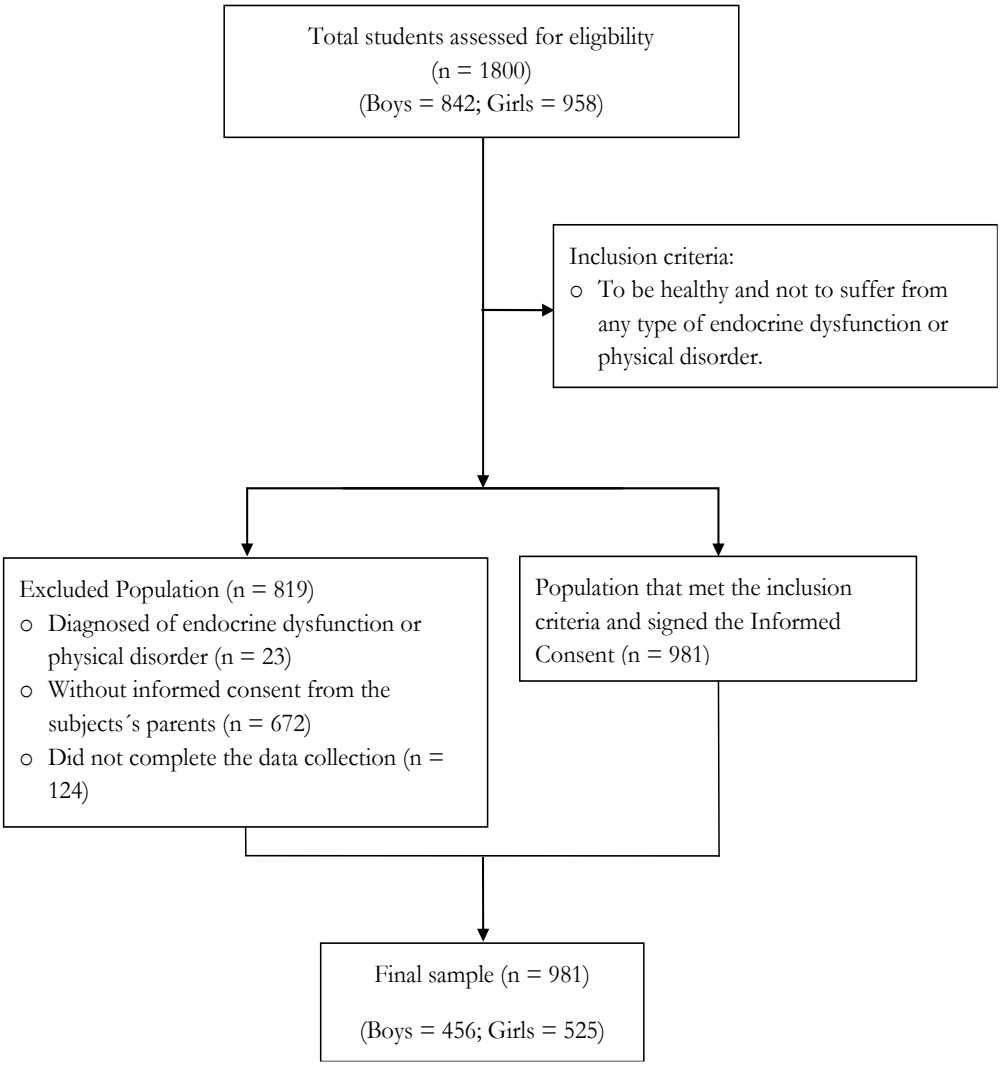

Figure 1. Flow diagram of the recruitment progress.

2.4. Blood pressure determination

We measured blood pressure (BP) levels using a previously calibrated aneroid sphygmomanometer and a Littmann® stethoscope (Saint Paul, USA), following the recommendations for blood pressure measurement of the Subcommittee of Professional and Public Education of the American Heart Association Council on High Blood Pressure Research.[25] Systolic BP ≥ 130 and/or diastolic BP ≥ 85 mm Hg were regarded as a risk factor of MetS.

2.5. Diagnostic criteria of metabolic syndrome in adolescents

Eight different criteria were used to diagnose MetS in the adolescent sample studied: Cook et al,[6] Weiss et al,[7] Duncan et al,[8] de Ferranti et al,[9] Cruz & Goran,[11] Rodríguez-Moran et al[12] and Viner et al,[13] as well as the IDF criteria as published by Zimmet et al.[10] The details of the criteria employed may be consulted in Table Supplementary.

2.6. Statistical analysis

The Kolmogorov-Smirnov test was used to assess the normality of the distribution. Results were reported as mean ± SD, except for the number of girls and boys with or without MetS, which was expressed as number. Mean differences between boys and girls were assessed using Student's t-test. Kappa for agreement was calculated for the childhood MetS definitions. Comparisons of means were assessed by ANOVA, followed by Tukey's test. There were no missing data. Statistical analyses were performed using SPSS v24.0 (IBM, Armonk, USA). Statistical significance was defined as $p<0.05$.

3. Results

3.1. Baseline characteristics of the participants

Baseline biochemical and anthropometric measurements of participants are shown in Table 1. All measured variables were within normal limits. In general terms, differences between boys and girls were not observed. However, a significant lower WC, body weight

and systolic blood pressure (SBP) and a significant higher fat content was found in girls compared to boys.

Table 1. Characteristics of participants.

	Boys (n=456)		**Girls** (n=525)	
	Mean	SD	Mean	SD
Age (y)	13.2	1.2	13.3	1.2
Weight (kg)	57.1	14.1	53.1***	11.0
Fat (%)	27.3	8.3	29.6***	7.8
BMI (kg/m^2)	21.5	4.0	21.1	3.6
WC (cm)	73.7	11.8	71.3***	9.6
Glucose (mg/dL)	86.2	31.2	85.2	28.7
Triglycerides (mg/dL)	129.2	59.3	125.0	46.2
Cholesterol (mg/dL)	81.8	17.3	81.4	15.7
LDL-c (mg/dL)	93.4	23.6	92.9	22.5
HDL-c (mg/dL)	40.1	2.8	40.0	3.1
SBP (mmHg)	119.6	15.7	116.9**	15.1
DBP (mmHg)	64.5	9.2	63.9	8.8
Insulina (mU/mL)	21.0	10.2	20.2	9.0
HOMA-IR	4.5	2.9	4.3	3.1

Fat (%), body fat percentage; BMI, body mass index; WC, waist circumference; TG, triglycerides; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol; SBP, systolic blood pressure, DBP, diastolic blood pressure, HOMA-IR, homeostatic model assessment of insulin resistance. Differences between means were assessed by an unpaired Student's t-test. **, $p < 0.05$; ***, $p < 0.001$.

3.2. Values for components of MetS in subjects with MetS according to different diagnostic criteria.

Tables 2 shows the values for components of MetS in boys and girls with MetS according to the different diagnostic criteria assessed. In boys, depending on the

diagnostic criteria employed, the number of MetS diagnoses ranged from 25 to 68. The highest mean values for WC, HDL-c, SBP and diastolic blood pressure (DBP) were found when the IDF criteria was employed. In contrast, the lowest mean values for WC and SBP were found with the Cook et al[6] criteria, and the lowest mean value for HDL-c were found with the Viner et al[13] criteria. The criteria proposed by Viner et al[13] showed the highest mean levels of glucose and triglycerides. The lowest mean levels for glucose and triglycerides were found with the IDF criteria.

A greater variability in the number of diagnosed girls was observed. In these subjects, the highest mean values for WC, SBP and DBP were found when the IDF criteria was employed. The lowest mean values of WC, SBP and DBP were found when the Cook et al[6] criteria were employed. In regard to the biochemical parameters, the highest and the lowest mean values for HDL-c were found when the Cruz and Goran[11] and Weiss et al[7] criteria were employed, respectively. The highest and the lowest mean values for glucose and triglycerides were found when the Weiss et al[7] and the Cruz and Goran[11] criteria were employed. No significant differences were observed in DBP among girls with MetS regardless the criteria used.

3.3. Prevalence of components of MetS according to the different diagnostic criteria in adolescents with Mets

Table 3 shows the prevalence of components of MetS in participants with MetS according to the different diagnostic criteria. Depending on the diagnostic criteria used, the prevalence of MetS cases in boys ranged from 5.5% to 14.9%, while in girls varied from 3.4% to 32.6%. The highest MetS prevalence in boys was observed with the Duncan et al,[8] Rodriguez-Moran et al[12] and Cruz and Goran[11] criteria. In girls, the highest MetS prevalence was observed with the Cruz and Goran[11] criteria. Girls had higher MetS prevalence than boys by all definitions except when the IDF, Weiss[7] and Viner[13] criteria were employed.

Table 2. Values for components of MetS in subjects with MetS according to diagnostic criteria.

	IDF		Cook		De Ferranti		Weiss		Viner		Duncan		Rodríguez-Moran		Cruz & Goran	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Boys																
MS (number)	41		39		63		32		25		68		68		68	
WC (cm)	86.0^{a}	9.9	74.6^{b}	11.7	80.2^{ab}	12.7	76.9^{b}	14.0	77.8^{ab}	13.5	79.5^{ab}	12.4	79.5^{ab}	12.4	79.5^{ab}	12.4
Glucose (mg/dL)	128.4^{a}	53.5	172.9^{b}	47.4	139.5^{a}	56.9	191.4^{b}	27.7	193.7^{b}	24.4	136.0^{a}	56.2	136.0^{a}	56.2	136.0^{a}	56.2
Triglycerides (mg/dL)	193.0^{a}	120.1	257.9^{a}	146.4	210.1^{ac}	133.9	295.0^{bc}	143.9	338.9^{b}	132.5	204.0^{a}	130.6	204.0^{a}	130.6	204.0^{a}	130.6
HDL-c (mg/dL)	35.7^{a}	3.4	33.1^{bc}	2.2	35.0^{a}	3.4	32.3^{c}	1.6	32.4^{c}	1.8	34.9^{ab}	3.2	34.9^{ab}	3.2	34.9^{ab}	3.2
SBP (mmHg)	137.2^{a}	14.7	121.6^{b}	17.0	123.5^{b}	17.3	122.9^{b}	17.9	123.4^{b}	18.8	124.0^{b}	16.6	124.0^{b}	16.6	124.0^{b}	16.6
DBP mmHg)	74.1^{a}	11.4	64.6^{b}	8.6	65.5^{b}	9.0	65.1^{b}	9.0	63.9^{b}	9.2	66.3^{b}	8.7	66.3^{b}	8.7	66.3^{b}	8.7
Girls																
MS (number)	32		58		97		18		21		134		86		171	
WC (cm)	83.2^{a}	7.5	70.5^{b}	10.4	75.2^{bc}	10.3	73.7^{bc}	14.8	75.5^{ab}	13.3	73.6^{b}	9.1	79.5^{ac}	12.4	73.7^{b}	9.5
Glucose (mg/dL)	131.9^{abc}	52.2	148.3^{b}	51.8	121.1^{a}	52.3	194.4^{d}	7.1	188.2^{d}	22.5	110.0^{c}	48.2	139.2^{ab}	50.7	104.5^{c}	43.5
Triglycerides (mg/dL)	196.2^{ab}	120.9	191.6^{ab}	114.9	164.5^{ab}	98.1	329.8^{c}	121.9	312.9^{c}	128.1	151.9^{a}	85.9	204.0^{b}	130.6	145.5^{a}	77.0
HDL-c (mg/dL)	35.3^{ab}	2.6	33.7^{a}	4.5	36.1^{b}	4.8	32.8^{a}	1.3	33.1^{a}	1.3	37.2^{c}	4.6	34.9^{a}	3.2	38.0^{c}	4.3
SBP (mmHg)	132.3^{a}	10.2	113.4^{b}	17.1	117.7^{bc}	17.0	116.4^{bc}	20.8^{a}	118.8^{ab}	19.5	117.2^{b}	15.5	124.0^{ac}	16.6	117.5^{b}	15.3
DBP mmHg)	70.7^{a}	8.8	62.1^{a}	8.8	62.6^{a}	9.5	64.4^{a}	10.2	64.4^{a}	10.1	63.0^{a}	9.0	66.3^{a}	8.7	63.7^{a}	8.9

WC, waist circumference; HDL-c, high-density lipoprotein cholesterol; SBP, systolic blood pressure, DBP, diastolic blood pressure. Differences between means that share a letter are not statistically significant ($p < 0.05$).

The most frequently MetS component observed in boys with MetS was hypertriglyceridemia by all criteria, except for IDF, Weiss et al[7] and Viner et al[13] criteria. A greater variability on the most frequent MetS component was observed in girls. Interestingly, in girls, the same prevalence for hyperglycemia and low HDL-c concentration, 6.3% and 18.1 % respectively, was observed in all diagnostic criteria. In boys, the prevalence for hyperglycemia was 6.8% by all criteria, while the prevalence for low HDL-c was 18.0% by all criteria except for those proposed by Weiss et al.[7]

Table 3. Prevalence (%) of components of MetS according to the different diagnostic criteria in adolescents with MetS.

	Abdominal Obesity	**Hiper-glycemia**	**Hyper-triglyceridemia**	**Low HDL-c**	**Hyper-tension**	**MetS**
Boys						
IDF	48.0	6.8	3.9	18.0	39.5	9.0
Cook	48.0	6.8	99.3	18.0	17.1	8.6
De Ferranti	24.3	6.8	100.0	18.0	17.1	13.8
Weiss	5.5	6.8	11.0	13.6	1.3	7.0
Viner	48.0	6.8	3.9	18.0	1.3	5.5
Duncan	33.8	6.8	99.3	18.0	17.1	14.9
Rodriguez-Moran	33.8	6.8	99.8	18.0	17.1	14.9
Cruz & Goran	33.8	6.8	99.8	18.0	17.1	14.9
Girls						
IDF	37.0	6.3	2.7	18.1	34.7	6.1
Cook	37.0	6.3	99.0	18.1	40.8	11.0
De Ferranti	28.0	6.3	100.0	18.1	40.8	18.5
Weiss	4.6	6.3	3.2	18.1	3.0	3.4
Viner	37.0	6.3	2.7	18.1	3.0	4.0
Duncan	55.2	6.3	99.0	18.1	40.8	25.5
Rodriguez-Moran	55.2	6.3	28.6	18.1	40.8	16.4
Cruz & Goran	55.2	6.3	28.6	18.1	40.8	32.6

3.4. Agreement cases of adolescents with MetS according to Kappa after crossing diagnostic criteria

Tables 4 and 5 show the number of concordant cases of boys and girls with MetS according to Kappa values after crossing the different diagnostic criteria. Both in boys and girls, the criteria proposed by the IDF was the less concordant with the other suggested criteria, while those proposed by Viner et al,[13] Duncan et al,[8] Rodriguez-Moran et al[12] and Cruz and Goran,[11] were very concordant among each other. In fact, the highest number of MetS diagnosed boys were observed with the criteria by Duncan et al,[8] Rodriguez-Moran[12] et al and Cruz and Goran,[11] showing kappa values above 0.999 (Figure 2). All boys diagnosed of MetS with the criteria published by Weiss et al[7] (κ= 0.602), Viner et al[13] (κ=0.497) and Cook et al[6] (κ= 0.696), also presented MetS according to the criteria proposed by Duncan et al,[8] Rodriguez-Moran et al[12] and Cruz and Goran.[11] 95.2% of boys with MetS, as diagnosed with criteria by De Ferranti et al,[9] were also diagnosed of Mets with the criteria by Duncan et al,[8] Rodriguez-Moran et al[12] and Cruz and Goran[11] criteria (κ= 0.902). Good percentages of concordance were not observed between the cases diagnosed with the IDF criteria and each of the rest of the criteria.

In girls, those published by Duncan et al[8] and Cruz and Goran[11] were the criteria that diagnosed a higher number of MetS cases, 134 and 171, respectively. However, concordance values were not as high as those found for boys. Indeed, the highest kappa value observed in girls corresponded to the concordance of the criteria proposed by Weiss et al[7] and Viner et al,[13] and was 0.814. All girls diagnosed of Mets with the criteria of Cook et al[6] (κ= 0.532), Weiss et al[7] (κ= 0.188), Viner et al[13] (κ= 0.217) and Rodriguez-Moran et al[12] (κ= 0.727), also presented MetS according to the criteria proposed by Duncan et al.[8] In this line, all girls diagnosed of MetS with the criteria proposed by Weiss et al[7] (κ= 0.137), Viner et al[13] (κ= 0.159) and Rodriguez-Moran et al[12] (κ= 0.577) criteria were also diagnosed of Mets with the criteria by Cruz and Goran.[11]

Table 4. Number of concordant cases of boys with MetS according to Kappa after crossing diagnostic criteria.

	IDF	**Cook**	**De Ferranti**	**Weiss**	**Viner**	**Duncan**	**Rodriguez-Moran**	**Cruz & Goran**
IDF	**41**							
Cook	19	**39**						
De Ferranti	29	39	**63**					
Weiss	18	30	32	**32**				
Viner	17	24	25	25	**25**			
Duncan	31	39	60	32	25	**68**		
Rodriguez-Moran	31	39	60	32	25	68	**68**	
Cruz & Goran	31	39	60	32	25	68	68	**68**

Table 5. Number of concordant cases of girls with MetS according to Kappa after crossing diagnostic criteria.

	IDF	**Cook**	**De Ferranti**	**Weiss**	**Viner**	**Duncan**	**Rodriguez-Moran**	**Cruz & Goran**
IDF	**32**							
Cook	21	**58**						
De Ferranti	28	58	**97**					
Weiss	9	18	18	**18**				
Viner	14	20	21	16	**21**			
Duncan	29	58	92	18	21	**134**		
Rodriguez-Moran	29	56	70	18	21	86	**86**	
Cruz & Goran	30	56	90	18	21	130	86	**171**

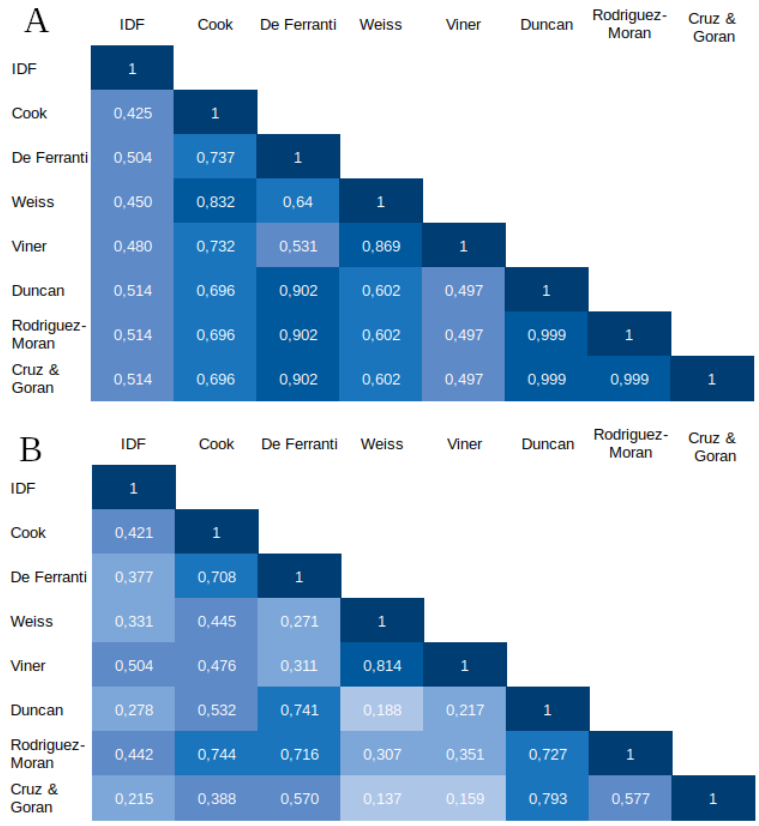

Figure 2. Kappa values for agreement for the diagnosis of metabolic syndrome in adolescents according to the eight criteria employed

4. Discussion

In the present study, we had the possibility for the first time to contrast the degree of agreement among eight diagnostic criteria to define MetS in boys and girls. The main findings of this study were the low degree of concordance observed between the IDF with the remaining diagnostic criteria, and a great agreement among the diagnostic criteria of Duncan et al.[8], Rodriguez-Moran et al.[12] and Cruz and Goran[11]. To our knowledge, this is the first study of analyzing the concordance between eight different criteria for the diagnostic of the MetS in adolescents.

Our results show significantly lower values of WC, body weight and SBP in girls, along with significantly higher fat content in girls compared to boys. These differences

among boys and girls could be explained, in part, by the variations in the body composition of the human species at that age, in particular due to a higher fat content in girls.[26]

With regard to the MetS components, in boys the highest mean values for WC, HDL-c, SBP and DBP were found after applying the criteria proposed by the IDF; in girls, applying these same criteria, higher mean values were also obtained for WC, SBP and DBP. These results partially differ from those obtained in other studies, such as the one developed by Peña-Espinoza et al,[27] with a Mexican adolescent population, who found higher WC and HDL-c values, when the criteria proposed by Ferranti et al[9] were used. In our study, the lowest mean values for WC, HDL-c and SBP were found in boys after applying the criteria of Cook et al[6] and Viner et al,[13] while in girls the lowest mean values for WC, SBP and DBP were found after applying the criteria of Cook et al,[6] followed by the lowest HDL-c values after applying the criteria of Weiss et al.[7] These results partially differ from those reported by Peña-Espinoza et al,[27] who regardless of sex obtained lower WC and HDL-c values when using the criteria proposed by Weiss et al.[7] On the other hand, in our study, after applying the criteria of Viner et al,[13] the highest mean levels of glucose and triglycerides were obtained in boys, while the lowest were found applying the criteria proposed by the IDF. These results differ from those observed by Reuter et al,[19] who in their study with an adolescent population from 10 to 17 years of age in southern Brazil found lower mean glucose levels when applying the criteria of Cook et al[6] and Ferranti et al,[9] regardless of sex. These differences observed in the values for the components of MetS with other authors might be explained by the variability in the ethnicity of the participants or the distinct number of participants diagnosed by the different diagnostic criteria.

On the other hand, our results show a high variability in the prevalence of MetS between both sexes according to the different criteria used. In boys, the prevalence ranges between 5.5% using the criteria of Viner et al[13] and 14.9% applying the criteria of Duncan et al,[8] Rodriguez-Moran et al[12] and Cruz and Goran.[11] In girls, a maximum prevalence of 32.6% was observed after applying the criteria of Cruz and Goran[11] and 3.4% when the criteria established by Weiss et al[7] are applied. According to Valdés-Villalpando et al,[28]

these differences can be explained at the different cut-off points established in each criterion to assess body composition by means of WC or BMI using both percentiles and Z-scores, together with different criteria for dyslipidemia values for TG and HDL-c, using cut-off points in percentiles or mg/dL, as well as different cut-off points to define blood pressure levels. According to Weihe et al,[29] this great variability justifies the urgent need to validate criteria to allow uniformity for the diagnosis of MetS in the adolescent population. Our results differ from those described by Reuter et al[19] with Brazilian adolescents, in which they found a MetS prevalence of 1.9, 5.0 and 2.1% using the criteria of Cook et al,[6] Ferranti et al[9] and the IDF. Other international studies have shown dissimilar prevalences according to the diagnostic criteria used. For instance, Kim et al,[30] in a representative study of the Korean adolescent population, obtained a MetS prevalence of 2.1% using the criteria defined by the National Cholesterol Education Program, Adult Treatment Panel III and IDF. Using the same criteria, in the study by Galera-Martínez et al,[31] which was carried out with adolescents from Southern Spain, a prevalence of MetS of 3.8% was reported. Another study developed in Turkey found a prevalence of MetS of 6.3% among adolescents using the criteria proposed by the IDF.[32] Therefore, a great variability in the prevalence of MetS in adolescents is observed, depending on the diagnostic criteria used and their cut-off points. Taking into account that the best prevention and treatment of MetS depends on an early diagnosis, the choice of criteria to diagnose MetS in adolescents can compromise the clinical care of these subjects.

Regarding the degree of agreement between the different criteria applied for the diagnosis of MetS, the IDF criteria showed the lowest agreement in both boys and girls with the other criteria studied (κ <0.550). These results are similar to the findings reported by Reuter et al[19] and Agudelo et al[33] who showed kappa values of 0.532 and 0.390 between the IDF vs Cook et al[6] criteria, and 0.382 and 0.14 between the IDF vs de Ferranti et al[9] criteria, respectively. Tavares Giannini et al[34] also reported confirmed a low kappa value (κ = 0.48) for the agreement between the IDF and Ferranti et al[9] criteria. However, the study carried out by Guilherme et al[4] in Brazilian adolescents reported good agreement between the IDF criteria with the criteria proposed by Cook et al[6] and

Ferranti et al,[9] κ = 0.950 and κ = 0.670, respectively. This variability on the degree of agreement in the aforementioned studies was also observed in the study by Peña-Espinoza et al,[27] where kappa values were 0.700 and 0.353 between the IDF criteria and the criteria of Cook et al[6] and De Ferranti et al,[9] respectively.

In boys, the criteria proposed by Duncan et al,[8] Rodriguez-Moran et al[12] and Cruz and Goran[11] were very concordant among each other, with kappa values above 0.999. However, in girls concordance values between these criteria were good but not as high as those found for boys. In this regard, the study carried out by Mirmiran et al[35] showed a kappa value of 0.359 between the Duncan et al[8] and Cruz and Goran[11] criteria, which differs from our results. Our findings also showed a great concordance between the criteria proposed by Duncan et al,[8] Rodriguez-Moran et al.[12] and Cruz and Goran[11] and the criteria of De Ferranti et al[9] (κ = 0.902) in boys. These results partially differ from those obtained by Mirmiran et al,[35] who in adolescent population and regardless of sex, reported kappa values of 0.616 and 0.178 between the criteria proposed by de Ferranti et al[9] and Duncan et al[8] and Cruz and Goran[11] respectively. Furthermore, Seo et al[36] reported a kappa value of 0.407 in the Korean adolescent population between the criteria of Cruz and Goran[11] and Ferranti et al,[9] regardless of sex.

The present study has some strengths and limitations. This study is the first, to our knowledge, that was employed eight different criteria for the diagnosis of MetS in Spanish adolescents and that includes an analysis of concordance between the different diagnostic criteria for MetS. In addition, we would like to emphasize the usefulness of the large sample size, which contributes to obtaining solid results that will improve comparability in future studies. In addition, all participants were of the same geographical region, with similar culture, lifestyle and eating habits, making the sample more homogeneous. The most important limitation of the present study is its cross-sectional design.

5. Conclusions

Our results showed a great concordance between the criteria proposed by Duncan et al,[8] Rodríguez-Moran et al[12] and Cruz and Goran,[11] and also between these criteria and the criteria proposed by de Ferranti et al.[9] We also report a lower degree of agreement

between the IDF criteria and the remaining 7 criteria used to diagnose MetS. However, our results differ from that reported by previous studies with similar characteristics, highlighting the differences between the different criteria for diagnosing MetS. The discrepancies observed in the agreement among the eight criteria employed in our study, and also in comparison with other authors, suggest the need to validate criteria that allow a uniformity in the diagnosis of MetS in adolescents, which is crucial for their usefulness in the clinical practice and for public health authorities in the management of this important public health problem.

Acknowledgements

The authors are grateful to schools, parents, and guardians as well as to participant students for their collaboration in the development of this study.

Disclosures

No conflict of interest, financial or otherwise is declared by the authors.

Author contributions

EGJ and JSP conceived and designed the study. AFA collected and analyzed the data. JSR interpreted the data. EGJ, JSP and AFA drafted the manuscript. All authors have revised and approved the submitted manuscript.

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ESTUDIO II

Referencia: Fernández-Aparicio, Á., Schmidt-RioValle, J., Perona, J. S., Correa-Rodríguez, M., Castellano, J. M., & González-Jiménez, E. (2019). Potential protective effect of oleanolic acid on the components of metabolic syndrome: a systematic review. *Journal of Clinical Medicine*, *8*(9), 1294. https://doi.org/10.3390/jcm8091294

Indicios de calidad: Impact Factor (2019) = 3,303. Edition: Science Citation Index Expanded. Category and rank: Medicine, General & Internal (36/165; Q1/T1).

II. Potential protective effect of oleanolic acid on the components of metabolic syndrome: a systematic review

Abstract

The high prevalence of obesity is a serious public health problem in today's world. Both obesity and insulin resistance favor the development of metabolic syndrome (MetS), which is associated with a number of pathologies, especially type 2 diabetes mellitus, and cardiovascular diseases. This serious problem highlights the need to search for new natural compounds to be employed in therapeutic and preventive strategies, such as oleanolic acid (OA). This research aimed to systematically review the effects of OA on the main components of MetS as well as oxidative stress in clinical trials and experimental animal studies. Databases searched included PubMed, Medline, Web of Science, Scopus, EMBASE, Cochrane, and CINAHL from 2013 to 2019. Thus, both animal studies (n = 23) and human clinical trials (n = 1) were included in our review to assess the effects of OA formulations on parameters concerning insulin resistance and the MetS components. The methodological quality assessment was performed through using the SYRCLE's Risk of Bias for animal studies and the Jadad scale. According to the studies in our review, OA improves blood pressure levels, hypertriglyceridemia, hyperglycemia, oxidative stress, and insulin resistance. Although there is scientific evidence that OA has beneficial effects in the prevention and treatment of MetS and insulin resistance, more experimental studies and randomized clinical trials are needed to guarantee its effectiveness.

Keywords: triterpenes; metabolic syndrome; insulin resistance; hypertension; inflammation; obesity.

1. Introduction

The increasing prevalence of overweight and obesity entails a serious global public health problem. It has been estimated that 39% of the world population over the age of 18 years is overweight, and 13% is obese, according to World Health Organization (WHO) data (2016) [1]. Furthermore, 18% of children and adolescents (5–19 years old) are either overweight or obese [1,2]. This situation is alarming because obesity and insulin resistance are two of the most important factors leading to metabolic syndrome (MetS) [3]. MetS consists of a set of cardiometabolic anomalies that lead to the development of type 2 diabetes mellitus (T2DM) and of cardiovascular disease (CVD) [4,5]. Nevertheless, MetS is also associated with other clinical conditions such as oxidative stress, hypertension, dyslipidemia, hepatic steatosis, non-alcoholic fatty liver disease, and impaired glucose tolerance, among others [6].

According to the WHO [5], insulin resistance is the main pathophysiological factor underlying MetS. It is characterized by a diminished tissue response to the cell activity of insulin [6], which implies a reduction in glucose uptake in adipocytes and muscle cells, an increase in hepatic glucose production, and altered lipid metabolism in the liver and adipose tissue [7]. Various studies highlight the relationship of insulin resistance to obesity and inflammation. Obesity-associated hyperplasia and hypertrophy of adipose tissue cause an increase in proinflammatory cytokines [8], such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) [9]. These proinflammatory cytokines, which have a negative effect on insulin signaling, are regulated by nuclear transcription factor kappa B (NF-κB), one of the pathways that activates oxidative stress [10].

The various definitions of MetS proposed by organizations such as the WHO, the European Group for the Study of Insulin Resistance (EGIR), and the National Cholesterol Education Program—Adult Treatment Panel III all underline that central MetS components are abdominal obesity, insulin resistance, hypertension, and dyslipidemia [3,5]. In this same line, in 2006, the International Diabetes Federation (IDF) defined MetS as "central obesity plus any two of the following four factors: raised triglycerides, reduced HDL cholesterol, raised blood pressure, and raised fasting plasma

glucose." Accordingly, the treatment and prevention of MetS should not only be envisaged as a whole, but each MetS component should also be considered individually.

The high prevalence of obesity and MetS because of sedentary and generally unhealthy life styles [1,2] makes the application of pharmacological interventions necessary, which have the tendency to be expensive for the health care systems. In addition, some of these drugs have several side effects that adversely affect the quality of life. In this sense, therapeutic properties of bioactive compounds are increasingly being studied [11,12]. It has been reported that oleanolic acid (OA), a naturally occurring pentacyclic triterpenoid, found at a high content in the leaves and fruit of the olive tree, among other plants [13], has various interesting pharmacological properties for the prevention and treatment of MetS and insulin resistance, such as anti-inflammatory, anti-oxidant, hypolipidemic, antidiabetic, anti-atherosclerotic [10,13,14] and antihypertensive [14,15] effects. The mechanisms of action that support these properties have been studied by different authors. OA is a selective Takeda G-protein-coupled receptor 5 (TGR5) agonist, whose activation have beneficial effects on glucose homeostasis, proinflammatory cytokines and body weight [16]. Furthermore, other authors refer that OA could suppress the NF-κB and activate the nuclear factor erythroid 2–related factor 2 (Nrf2) signaling pathways, both having important roles on the inflammatory status in insulin resistance [10,17].

Consequently, as a bioactive compound, OA has potential to be considered for the development of new and alternative therapeutic strategies for insulin resistance and MetS. Therefore, this study was aimed to systematically review the effects of OA formulations on the components of MetS and on proinflammatory cytokines and antioxidant enzymes as oxidative stress biomarkers in human subjects and animal models.

2. Materials and Methods

2.1. Databases and Search Strategy

In June 2019, a systematic review was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2009 guidelines

[18]. The objective was to systematically identify the clinical trials and experimental studies in animals that have evaluated the effects of OA on insulin resistance and the various MetS components to date. For this purpose, we conducted a bibliographic search with a time filter from January 2013 until June 2019 in the following five electronic databases: Medline, Web of Science, Scopus, EMBASE, Cochrane and CINAHL. PubMed search engine was also consulted.

The search terms were based on the following descriptors in the Medical Subject Headings (MeSH): oleanolic acid, metabolic syndrome, insulin resistance, obesity, hypertension, and inflammation. The search strategy used in all of the databases was Oleanolic acid AND ("metabolic syndrome" OR "insulin resistance" OR obesity OR hypertension OR inflammation).

2.2. Selection of Papers. Eligibility Criteria

The studies were selected in two phases. In the first phase, all titles and abstracts were read and analyzed in order to select the most potentially relevant studies, based on the following inclusion criteria: (1) oleanolic acid administration, (2) focus on insulin resistance and/or MetS components, (3) clinical trials in humans and experimental studies on animals, (4) articles published in English, and (5) access to the full text. In doubtful cases, the complete text of the article was analyzed.

In the second phase, we analyzed the full text of the articles selected in the previous phase in order to determine their eligibility. The articles excluded in this phase had one of the following criteria: (1) secondary studies, (2) no use of OA or use of an OA derivative, (3) combined administration of OA with another bioactive compounds, or (4) research based on the molecular study of OA and its biological activity. This selection process was performed by two independent reviewers (A.F-A. and J.S-R.), though a third reviewer (J.S.P.) was also consulted in doubtful cases.

2.3. Data Extraction

After selecting the studies for the qualitative synthesis of this systematic review, the next step was to extract the data. Data extracted both from the clinical trial and the animal

studies were the following: authors and publication year, subjects, sample size, type of intervention, duration of the intervention, dosage used, and outcomes obtained (hypertension, lipid profile, hyperglycemia, insulin resistance, and inflammatory and oxidative stress biomarkers). Data extraction was performed by two independent reviewers (E.G-J. and M.C-R.), though a third reviewer (J.M.C.) was consulted in cases of doubt. Each of the results measured was described in a narrative form. A meta-analysis of the animal studies included was ruled out because of the heterogeneous nature of the studies, especially in reference to their design and the animal species used in these studies.

2.4. Risk of Bias and Methodological Quality Assessment

To reduce inter-examiner bias, two independent reviewers (A.F-A. and J.S-R.) performed the methodological quality assessment and analyzed the risk of bias of all the studies included in this review. When there was any doubt, a third reviewer (J.S.P.) was consulted. The methodological quality assessment of the animal intervention studies was performed by using the SYRCLE's Risk of Bias (RoB) tool [19], whereas the Jadad scale was used to evaluate the methodological quality of the clinical trial [20].

SYRCLE's RoB tool was elaborated by the Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE) [19] for assessing the methodological quality of experimental studies of animals and is based on the Cochrane Collaboration RoB tool. SYRCLE's RoB tool contains 10 items, five of which (i.e., items 1, 3, 8, 9, and 10) coincide with those of the Cochrane RoB tool because they are applicable to animal experiments. The remaining items were adapted to the characteristics of animal experimentation studies. These items assess six types of bias: selection bias, performance bias, detection bias, attrition bias, reporting bias and another bias. In SYRCLE's RoB tool each of the 10 items is rated to a "yes" (low risk of bias), a "no" (high risk of bias), or "unclear" (insufficient information to evaluate risk of bias).

The Jadad scale [20] is a five-item tool used for reporting risk of bias of clinical trials and each of its items assess randomization, method of randomization, double-blinding, method of blinding and reporting of withdrawals, respectively. The Jadad score for ranges from 0 to 5, 0 being the lowest level of quality and 5 the highest.

3. Results

3.1. Study Selection

The search strategy, implemented in the various databases and the PubMed search engine, with a time filter from January 2013 until June 2019, produced the following 1661 results: 523 from the Web of Science, 382 from Scopus, 271 from Medline, 286 from EMBASE, 91 from PubMed, 101 from CINAHL, and 7 from Cochrane. After the duplicated publications were eliminated, the titles and abstracts of 733 articles were analyzed to ascertain whether they fulfilled the inclusion criteria. Full texts of 30 articles were then read in order to assess their suitability for the study. Finally, 24 of these articles were included in the systematic review without carrying out an inverse search of the literature. Figure 1 shows the flow diagram of the selection and exclusion process of the research studies according to the PRISMA system [18].

In the first phase of selection, four studies were excluded because of having not full-text access [21–24]. It is also noteworthy that two randomized double-blind controlled trials that studied the effects of virgin olive oils enriched with bioactive compounds, such as phenolic compounds and different triterpenes on MetS and oxidative stress were found in the first phase of selection [25,26]. However, they were excluded because the aim of this systematic review is to evaluate the effects of OA alone, without interactions with another bioactive compounds.

3.2. Characteristics of the Animal Studies Selected

Table 1 summarizes the characteristics of the animal studies selected. In addition, this table includes the results of these studies on the effect of OA on insulin resistance and MetS components. Of the animal studies selected, the majority (n = 20) used rodents, particularly rats and mice. The experimental animals in the remaining studies were quails (n = 1), rabbits (n = 1) and a mixed study of rabbits and mice (n = 1). In the studies that used rats and mice, the largest sample size was n = 122 and the smallest sample size was n = 18. The sample size in the study of quails was n = 120; in the study of rabbits, n = 24; and in the mixed study, there were 32 rabbits and 56 mice. The minimum OA dosage

administered was 5 mg/kg and the maximum OA dosage was 250 mg/kg. The maximum administration time period was 20 weeks, and the minimum time period was one week.

3.3. Characteristics of the Clinical Trial Selected

Table 2 summarizes the characteristics of the clinical trial selected.

3.4. Risk of Bias and Methodological Quality Assessment

According to the authors of the SYRCLE's RoB tool, the risk of bias assessment should be presented as a table or a figure that gives either the summary results of the assessment or the results of all individual studies. They do not recommend calculating a summary score for each individual study when using this tool since this inevitably involves assigning "weights" to specific domains in the tool, and it is difficult to justify the weights assigned [19]. Accordingly, Table 3 presents the results obtained with SYRCLE's RoB tool for each study. In addition, the Jadad score is presented in Table 4.

3.5. OA Effects on Insulin Resistance and MetS Components in Animal Studies

3.5.1. Hypertension

Three studies were performed in hypertensive animal models [27–29]. Ahn et al. [27] reported that the application of OA over a period of three months produced a significant decrease in systolic blood pressure (SBP) in hypertensive rats in comparison to non-OA-treated hypertensive rats. In this same line, the administration of OA by Bachhav et al. [28] to Nω-nitro-L-arginine methyl ester (L-NAME)-induced hypertensive rats for four weeks significantly reduced SBP and the mean arterial blood pressure (MAP). It also significantly increased urine volume and urine sodium excreted, as well as non-significantly increased serum nitrate/nitrite (NOx) levels in comparison to the group of rats that was only given L-NAME. Similarly, in a study conducted by Madlala et al. [29], the administration of various doses of OA (30, 60, and 120 mg/kg) significantly reduced MAP from the third week until the end of the intervention in OA-treated rats in comparison to the control group. Moreover, the results obtained in this study indicated

a significant increase in urine sodium excretion, but not in the volume of urine excreted. Furthermore, in the study conducted by Gamede et al. [30], a reduction of the MAP was observed in prediabetic rats. In summary, OA reduced SBP and MAP and increased urinary excretion of sodium.

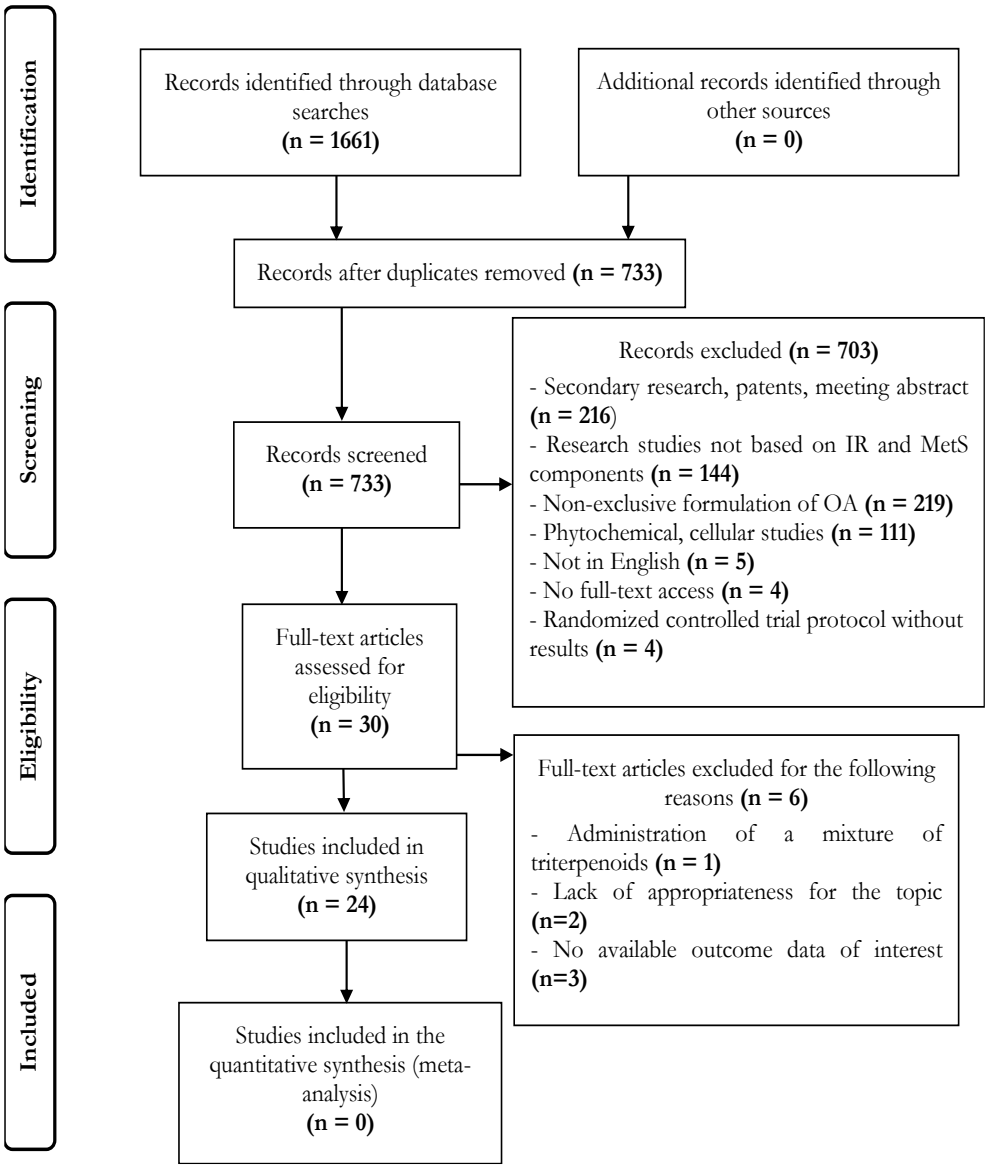

Figure 1. Flow diagram according to the PRISMA Checklist. Selection of studies. OA: oleanolic acid; IR: Insulin resistance; MetS: Metabolic syndrome.

Table 1. Characteristics and results of animal experimentation studies on the effect of oleanolic acid (OA) on insulin resistance and metabolic syndrome (MetS) components.

Author/Year	Subjects	Sample size (n)	Intervention	Dosage	Duration	Results
Ahn YM et al. [27] (2017)	Hypertensive (HTA) and normotensive rats	31	Oleanolic acid (OA) by oral gavage	30 mg/kg/day	7 weeks (OA last 3 weeks)	↓ SBP ($p < 0.001$) in OA-treated HTA rats vs HTA control.
Bachhav SS et al. [28] (2015)	L-NAME, during the intervention, induced hypertensive rats	34	Oral administration of OA	60 mg/kg/day	4 weeks	↓ SBP ($p < 0.001$) and MAP ($p < 0.05$), ↑ urine excretion and urine sodium vs L-NAME control group; ↓ non-significant (ns) of body weight and ↑ ns of serum NOx vs L-NAME control group.
Madlala HP et al. [29] (2015)	Normotense, DSS and SHR rats	18	Oral administration of OA	30, 60 and 120 mg/kg twice every three days	9 weeks	↓ MAP ($p < 0.05$), ↑ urine sodium excretion in DSS and SHR rats; ↓MDA in all tissues, and ↑ SOD and GSH-Px activities in liver and kidney in DSS and SHR rats, only in OA60.
Chen S et al. [31] (2017)	HFD-fed mice and diabetic db/db mice	20	Intraperitoneal injection of OA	20 mg/kg b.w/day	4 weeks	↓ TG, TC, LDL, HDL ($p < 0.05$) in OA-treated diabetic mice vs non-OA-treated diabetic mice.
Jiang Q et al. [32] (2015)	HFD-fed quails	120	OA via gavage	25, 50 and 100 mg/kg/day	10 weeks	↓ serum TG, TC, LDL and MDA, and ↑ HDL (4.05 ± 0.31 vs 2.63 ± 0.52 mM, $p < 0.05$), NO (37.60 ± 9.15 vs 29.49 ± 7.47 μM, $p < 0.05$), SOD, CAT, GSH and GSH-Px vs HFD control group, especially with 100 mg/kg of OA.
Luo H et al. [33] (2017)	32 rabbits 32 C57BL/6J mice 24 LDLR−/− mice	88	OA administration to animals fed with atherogenic diet	10 (rabbits) and 25 (mice) mg/kg/day	12 weeks (last 5 weeks OA)	↓ TG, TC, LDL vs non-OA-treated rabbits. ↓ TG, LDL, ↑ HDL vs non-OA-treated LDLR−/− mice. ↓ TC, LDL vs non-OA-treated C57BL/6J mice.
Pan Y et al. [34] (2018)	HFD-fed rabbits	24	OA via gavage	50 mg/kg/day	12 weeks (last 4 weeks OA)	↓ TG ($p < 0.001$), TC ($p<0.001$), LDL ($p < 0.05$) and HDL ($p < 0.01$); ↓ serum levels of IL-1β, IL-6 ($p < 0.001$), and TNFα ($p < 0.001$) vs HFD control group.
Molepo M et al. [12] (2018)	Pups rats	96	OA via oral gavage	60 mg/kg/day	16 weeks (2nd week OA)	↓ saturated FFA, and ↑ mono/polyunsaturated FFA vs control group.
Wang X et al. [35] (2013)	Non-diabetic rats and diabetic mice	34	Intraperitoneal injection of OA	20 mg/kg/day	2 weeks	↓ FBG, and FSI; ↓ body weight (36.4 ± 2.3 vs 41.7±4.1 g); ↓ TG, TC, LDL, FFA, IL-1β, IL-6, and TNFα, and ↑ HDL both in serum and ↓ liver;↓ AUC of IPGTT and IPITT. All changes ($p < 0.05$) vs non-OA-treated diabetic mice.
Li Y et al. [36] (2014)	Fructose induced insulin resistant rats	24	Oral administration of OA	5 and 25 mg/kg/day	10 weeks	↓ FSI, HOMA-IR and Adipo-IR vs non-OA-treated insulin resistant rats; ↓ AUC of FFA and ↓ non-significant of glucose in the OGTT vs non-insulin resistant rats. These changes ($p < 0.05$) only with OA 25 mg.
Lee ES et al. [37] (2016)	Non-diabetic and T2DM rats	-	OA via oral gavage	100 mg/kg/day	20 weeks	↓ Body weight vs non-diabetic rats control group. ↑ Insulinemia, HOMA-β and serum SOD, and ↓ TG vs non-OA-treated diabetic rats.
Wang X et al. [38] (2015)	Diabetic mice	24	Intragastric administration of OA	250 mg/kg/day	4 weeks	↓ FBG ($p<0.001$), HOMA-IR ($p < 0.05$) and HDL (7.54 ± 0.82 vs 9.02±0.97 mM/l, $p < 0.01$), improved glucose AUC of OGTT and ↓ non-significant of FSI vs control group.

Table 1. *Cont.*

Author/Year	Subjects	Sample size (n)	Intervention	Dosage	Duration	Results
Gamede M et al. [39] (2018)	HFHC diet induced prediabetic rats	36	Oral administration of OA	80mg/kg/3days	12 weeks	↓ Body weight ($p < 0.05$), glycemia in the OGTT ($p < 0.05$), HOMA2-IR (60.35 ± 2.05 vs 128.26 ± 2.98, $p < 0.05$), HbA1c, ghrelin, hepatic and muscular glycogen concentration vs non-OA-treated prediabetic rats.
Gamede M et al. [30] (2019)	HFHC diet induced prediabetic rats	36	Oral administration of OA	Not mentioned	12 weeks	↓ Body weight (516.75 ± 8.28 vs 679.75 ± 78.52 g), FBG, MAP, and plasma levels of TG, LDL, IL-6 and TNF-α, ↑ plasma level of HDL (1.88 ± 0.02 vs 0.85 ± 0.04 mM/l), SOD and GSH-Px, and ↓ heart MDA concentration vs prediabetic control group. All changes $p < 0.05$
Djeziri FZ et al. [40] (2018)	HFD induced obese mice	18	Oral administration of OA	Not mentioned	16 weeks	↓ Glycemia in the IPGTT; and ↓ gene expression of IL-1β, IL-6, and TNFα vs HFD control group.
Nakajima K et al. [41] (2019)	STD, HFD or HGD-fed mice	18	OA by oral gavage	20 and 40 mg/kg/day	1 week	↓ plasma octanoylated ghrelin levels and body weight gain in STD-fed rats vs non-OA-treated STD-fed rats.
Su S et al. [42] (2018)	PCBs-induced metabolic disfunction in mice	40	Oral administration of OA	50mg/kg/3days	10 weeks	↓ FBG (132 ± 14 vs 191 ± 16 mg/dl), HOMA-IR (1.02 ± 0.17 vs 1.79 ± 0.35) and serum levels of TG, FFA, cholesterol and FSI (1.35 ± 0.41 vs 2.8 ± 0.56 ng/dl); ↓ Glucose level in IPGTT and IPITT. All changes ($p < 0.05$) vs non-OA-treated PCBs-induced mice.
Wang S et al. [43] (2018)	HFF diet-fed rats	36	OA and Nano-OA by gavage	25mg/kg/day	12 weeks (last 6 weeks OA)	↓ BW, FBG and serum NO level, ↑ serum CAT activity in OA and nano-OA groups. ↓ serum levels of FSI, TG and MDA, ↑ ISI and serum SOD activity in nano-OA group. All changes ($p < 0.05$) vs non-treated insulin resistant rats.
An Q et al. [44] (2017)	Streptozotocin-induced diabetic rats.	18	Oleanolic acid	100 mg/kg/day	12 weeks (last 6 weeks OA)	↓ FBG, serum levels of IL-1β ($p < 0,001$), IL-6 ($p < 0.05$), and TNFα ($p < 0.01$); ↑ serum NO level ($p < 0.01$) vs non-OA-treated diabetic rats.
Matumba MG et al. [45] (2019)	Pups rats	40	Neonatal OA administration by orogastric gavage	60 mg/kg/day	16 weeks (2nd week OA)	↑ Adiponectin (1.5-fold, $p < 0.01$); ↓ IL-6 ($p < 0.01$) and TNFα plasma concentration; and ↓ gene expression of IL-6 ($p < 0.0001$) and TNFα $p < 0.0001$) vs non-OA-treated HF-fed rats.
Nyakudya TT et al. [46] (2018)	Pups rats. High fructose to half of the rats	112	Neonatal OA administration	60 mg/kg/day b.w.	16 weeks (2nd week OA)	↓ AUC in the OGTT, and of the HOMA-IR index in the rats treated with OA.
Nyakudya et al. [47] (2018)	Pups rats	112	Neonatal OA administration	60 mg/kg/day b.w.	16 weeks (2nd week OA)	↑ hepatic lipid content in male rats, and in terminal body mass in female rats fed with HF as neonates and as adults vs OA-treated rats.
Nyakudya et al. [48] (2019)	Pups rats in their second postnatal week	30	Neonatal OA administration by orogastric gavage	60 mg/kg/day b.w.	1 week	↑ level of GSH and CAT activity, ↓ MDA concentration in skeletal muscle tissue vs HF-fed rats.

Notes: All results referenced are statistically significant unless otherwise noted; SBP, systolic blood pressure; MAP, mean arterial blood pressure; L-NAME, Nω-nitro-L-arginine methyl ester; NOx, serum nitrate/nitrite level; DSS, Dahl salt-sensitive; SHR, spontaneously hypertensive rats; MDA, malonaldehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; HFD, high-fat diet; TG, triglycerides; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; NO, nitric oxide; CAT, catalase; GSH, total glutathione; FFA, free fatty acids; FBG, fasting blood glucose; FSI, fasting serum insulin; AUC, area under the curve; IPGTT, intraperitoneal glucose tolerance test; IPITT, intraperitoneal insulin tolerance test; T2DM: 2 type diabetes mellitus; OGTT, oral glucose tolerance test; HFHC, high-fat high-carbohydrate; HOMA-IR and HOMA2-IR: insulin resistance index; STD: standard diet; HGD, high-glucose diet; PCBs, polychlorinated biphenyls; HFF, High fat and fructose; BW, body weight; ISI, insulin sensitivity index; HF, high fructose. ↓means reduction/decrease, etc of the results; and ↑means an increase.

Table 2. Characteristics and results of the clinical trial on the effect of OA.

Author/Year	Subjects	Sample Size (n)	Intervention	Dosage	Duration	Results
Luo HQ et al. [49] (2018)	Hyperlipidemic patients	15	Oleanolic acid	Not mentioned	4 weeks	↓ TC, TG, LDL, glucose and FSI; ↑ HDL and Leptin; slight ↓ of HbA1c

Notes: TC, total cholesterol; TG, triglycerides; LDL, Low-density lipoprotein; HDL, High-density lipoprotein; FSI, fasting serum insulin; HbA1c, glycosylated hemoglobin A1c. ↓ means reduction/decrease, etc of the results; and ↑ means an increase.

Table 3. SYRCLE's RoB tool results for each study.

Items of the tool	Ahn YM et al. (2017) [27]	Bachhav SS et al. (2015) [28]	Madlala HP et al. (2015) [29]	Chen S et al. (2017) [31]	Jiang Q et al. (2015) [32]	Luo H et al. (2017) [33]	Pan Y et al. (2018) [34]	Molepo M et al. (2018) [12]	Wang X et al. (2013) [35]	Li Y et al. (2014) [36]	Lee ES et al. (2016) [37]	Wang X et al. (2015) [38]	Gamede M et al. (2018) [39]	Gamede M et al. (2019) [30]	Djeziri FZ et al. (2018) [40]	Nakajima K et al. (2019) [41]	Su S et al. (2018) [42]	Wang S et al. (2018) [43]	An Q et al. (2017) [44]	Matumba MG et al. (2019) [45]	Nyakudya TT et al. (2018) [46]	Nyakudya TT et al. (2018) [47]	Nyakudya TT et al. (2019) [48]
1. Was the allocation sequence adequately generated and applied?	?	?	?	?	+	?	?	?	?	?	?	?	?	—	?	?	?	?	?	?	?	+	+
2. Were the groups similar at baseline or were they adjusted for confounders in the analysis?	+	+	+	+	+	?	+	?	?	+	?	+	+	+	+	?	?	+	?	?	+	+	+
3. Was the allocation to the different groups adequately concealed during?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
4. Were the animals randomly housed during the experiment?	?	+	?	?	?	+	?	?	+	+	+	+	?	?	?	?	?	?	?	?	?	+	+
5. Were the caregivers and/or investigators blinded from knowledge which intervention each animal received during the experiment?	?	?	+	+	?	?	?	?	?	+	?	?	?	?	?	?	?	?	?	?	?	?	?
6. Were animals selected at random for outcome assessment?	?	?	?	?	?	+	?	+	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
7. Was the outcome assessor blinded?	?	+	?	?	+	+	?	+	?	+	?	+	+	+	+	+	+	+	+	+	?	+	+
8. Were incomplete outcome data adequately addressed?	+	?	?	?	?	+	+	+	?	+	?	+	+	+	+	?	+	+	+	+	?	+	+
9. Are reports of the study free of selective outcome reporting?	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10. Was the study apparently free of other problems that could result in high risk of bias?	?	+	?	?	?	+	+	?	?	+	+	+	+	?	?	?	?	?	?	?	?	?	?

Notes: + (*low risk of bias*); — (*high risk of bias*); *?* (*item not reported, unknown risk of bias*). 1–3 considers selection bias; 4–5 performance bias; 6–7 detection bias; 8 attrition bias; 9 reporting bias; and 10 other biases

Table 4. Jadad score results for the clinical trial selected.

Authors (year)	Randomization	Method of Randomization	Double Blinding	Method of Blinding	Dropouts/Withdrawals	Jadad Score
Luo HQ et al. (2018) [49]	No	No	No	No	Yes	1

3.5.2. Lipid Profile and Obesity

In a study performed by Chen et al. [31], the administration of OA over a four-week period produced a significant decrease in serum levels of triglycerides (TG), total cholesterol (TC), LDL, and HDL in diabetic mice in comparison to non-OA-treated diabetic mice.

One mixed-animal study and two trials that used non-rats/mice animal models assessed the effects of OA on high-fat diet (HFD)-induced atherosclerosis [32–34]. In the study conducted by Jiang et al. [32], it was reported that all OA-treated quails fed along with a HFD for 10 weeks experienced a significant reduction in TG, TC, and LDL, as well as a significant increase in HDL and nitric oxide (NO) serum levels. The experimental study conducted by Luo et al. [33] for 12 weeks was performed on rabbits, LDL receptor knockout (LDLR−/−) mice, and C57BL/6J mice. Only the experimental groups of these animal models received OA in the last five weeks of the study. The OA-treated rabbits showed a significant reduction in TG, TC, and LDL levels, with a slight increase in HDL levels. OA-treated (LDLR−/−) mice experienced a significant decrease in TG and LDL levels, as well as a significant increase in HDL compared to the HFD control group. Finally, the OA-treated C57BL/6J mice not only showed a significant reduction in TC and LDL levels but also no changes in their HDL and TG levels in comparison to non-OA-treated HFD mice. At the end of their study, Pan et al. [34] reported that there was a significant reduction in TG, TC, LDL, and HDL levels in the OA-treated rabbits.

The administration of OA to diabetic mice by Wang et al. [35] showed a significant decrease of TG, TC, LDL, and free fatty acids (FFA) serum levels and a significant increase of HDL levels in comparison to non-OA-treated diabetic mice. Similarly, in the study conducted by Gamede et al. [30], OA significantly reduced body weight, TG, and LDL plasma levels, and also led to a significant increase of HDL levels in prediabetic rats in comparison to non-OA-treated prediabetic rats.

Molepo et al. [12] administered OA to neonatal rats fed with a HF diet and observed a decrease of saturated FFA, and an increase of mono/polyunsaturated FFA. OA

administration by Nakajima et al. [41] significantly reduced the plasma levels of octanoylated ghrelin levels and the body weight gain in comparison to non-OA-fed rats.

Therefore, according to the results reported, OA improved lipid profile, as evidenced by the decrease in serum levels of TG, TC, and LDL. However, regarding serum HDL levels, some studies reported an increase, while others reported a decrease.

3.5.3. Hyperglycemia and Insulin Resistance

Wang et al. [35] showed that, after administrating OA to diabetic mice, body weight, fasting blood glucose (FBG), and fasting serum insulin (FSI) decreased significantly. The results of the glucose area under the curve (AUC), obtained from the intraperitoneal glucose tolerance test (IPGTT) and intraperitoneal insulin tolerance test (IPITT) (both performed after the intervention), were significantly lower in diabetic OA-treated mice than in those that had not received OA. Similarly, in the study performed by Li et al. [36] in fructose-induced insulin-resistant rats treated with OA, an oral glucose tolerance test (OGTT) was performed on the eighth day of the intervention, and it was observed that the AUC for FFA was significantly lower, whereas AUC for glucose experienced a slight decrease. Moreover, they also found a significant reduction of the homeostatic model assessment of insulin resistance (HOMA-IR) and the adipose tissue insulin resistance (Adipo-IR) scores in comparison to non OA–treated insulin-resistant rats.

Lee et al. [37] administered OA for 20 weeks to type 2 diabetic rats and observed a significant increase in the homeostasis model assessment of β-cell function (HOMA-β) and insulinemia in comparison to non-OA-treated type 2 diabetic rats. Furthermore, the OA-treated rats were found to have a slightly lower glucose AUC after the IPGTT and the intravenous insulin tolerance test (IVITT). Similarly, a study performed on diabetic mice by Wang et al. [38] showed that OA administration significantly reduced FBG, HOMA-IR, and serum HDL levels as well as non-significantly reduced FSI in comparison to the control group. Moreover, there was a significant improvement in glucose AUC.

Two similar studies were conducted by Gamede et al. [30,39] in high-fat high-carbohydrate (HFHC) diet-induced prediabetic rats. In both studies, rats were

administered OA after prediabetes induction. During OA administration, the rats continued to receive either an HFHC diet or a normal diet. In the first study [39], both OA-treated groups experienced a significant reduction in body weight, in glycemia in the OGTT test and in the HOMA2-IR and HbA1c indexes as well as a reduction in the hepatic and muscle glycogen concentration in comparison to the non-OA-treated prediabetic rats. In the other study [30] a reduction of FBG was observed in comparison to non-OA-treated prediabetic rats.

Djeziri et al. [40] showed that after administrating OA for 16 weeks to HFD-induced obese mice, a signification reduction in glycemia levels at 15, 30, 60, and 120 min after the administration of glucose was observed in the IPGTT test in comparison to non-OA-treated HFD-induced obese mice. In addition, from six week until the end of the intervention, the increase in body weight of the OA-treated mice was significantly lower than the HFD control group.

Su et al. [42] administered OA to polychlorinated biphenyls (PCBs)-induced metabolic disfunction mice and observed a significant decrease of FBG, FSI, HOMA-IR, adipocyte size, and serum levels of TG, FFA, and TC. It was also observed a significant decrease of glycemia in the IPGTT and IPITT tests. In the study performed by Wang et al. [43] in high fat and fructose (HFF) diet-fed rats, OA administration reduced significantly body weight, FBG, FSI, and serum levels of NO and TG, as well as increased the insulin sensitivity index (ISI).

OA administration by An et al. [44] to diabetic rats with a carotid artery injury showed a significant reduction of levels of FBG and a significant increase of serum NO level in comparison to non-OA-treated diabetic rats with the same injury.

Two studies performed by Nyakudya et al. [46,47] assessed the protective long-term effects of neonatal intake of OA in rats. These animals were administered OA, a high-fructose (HF) solution, or OA combined with the HF solution during their second week of life. From postnatal day 56 until the end of the experiment, half of each group received either distilled water or a fructose-rich solution. At the end of the first experiment [46], a significant increase in glucose in the OGTT test was observed in both administration

periods in comparison to all of the OA-treated female rats. Moreover, both HF solution-fed male and female rats had a significant higher HOMA-IR index than their respective OA-fed male and female rats in both periods. In the another study [47], a significant increase of hepatic lipid content in male rats, and a significant raise in terminal body mass in female rats fed with a HF solution, was observed in both periods in comparison to their respective OA-treated rats.

In summary, the reported results showed that OA improved the AUC of glucose and insulin in the glucose tolerance tests, decreased the serum levels of FBG and FSI and reduced the HOMA-IR index.

3.5.4. Inflammatory and oxidative stress biomarkers. Antioxidant enzymes

In the studies conducted by Pan et al. [34], Wang et al. [35] and An et al. [44], the administration of OA led to significant decreases in serum levels of IL-1β, IL-6, and TNFα. Similarly, a reduction of plasma levels of IL-6 and TNFα was observed by Gamede et al. [30]. In agreement with these results, a significant reduction in the gene expression of IL-1β and IL-6 in liver and adipose tissue, as well as of TNFα in adipose tissue of mice was observed by Djeziri et al. [40]. In addition, Matumba et al. [45] administered OA to neonatal rats fed with a high fructose (HF) diet, and they observed a decrease of IL-6 and TNFα plasma levels and a reduction of their gene expression at the end of the experiment. An increase of plasma level of adiponectin was also observed by these authors [45]. In summary, OA was able to decrease both the serum levels and gene expression of the proinflammatory cytokines IL-1β, IL-6, and TNFα.

A number of authors showed an increase of serum superoxide dismutase (SOD) activity [30,32,37,42,43]. In addition to SOD, Jiang et al. [32] and Gamede et al. [30] also reported an increase in glutathione peroxidase (GSH-Px) activity. Moreover, the administration of OA in the study conducted by Madlala et al. [29] decreased malonaldehyde (MDA) concentration in the heart, liver, and kidney and increased the activities of SOD and GSH-Px in the liver and kidney. In this same line, a reduction of heart MDA concentration was found in the study of Gamede et al. [30], as well as a decrease of serum MDA in the studies conducted by Jiang et al. [32] and Wang et al. [43].

Furthermore, Jiang et al. [32], Su et al. [42], and Wang et al. [43] also showed an increase of serum catalase (CAT) activity. Moreover, Nyakudya et al. [48] administered OA to neonatal rats fed with a HF diet and observed a decrease of MDA concentration, as well as an increase of CAT activity by the end of the study. Accordingly, OA increased the activity of antioxidant enzymes and decreased MDA levels.

3.6. Hypolipidemic Effects of OA in Human Patients

In a study performed by Luo et al. [49], OA administration for four weeks to hyperlipidemic patients elicited a decrease of TC, TG, LDL, glucose, and FSI serum levels, as well as an increase of leptin serum levels. A slight decrease of HbA1c (%) and a slight increase of HDL was also observed.

4. Discussion

The aim of this systematic review was to investigate the effects of OA on parameters concerning on components of Mets, including central obesity, lipid profile, blood pressure, hyperglycemia, as wells as insulin resistance and/or oxidative stress biomarkers. The findings provided in this study derive mostly from experimental studies in animals, while only one non-randomized clinical trial in humans was included. The main findings of this study are (i) OA administration improves the hypertensive status, (ii) the disturbance of the lipid profile in hyperlipidemic and metabolic dysfunction situations is attenuated by OA, (iii) OA reduces the oxidative stress status, and (iv) the insulin resistance condition is improved by the action of OA. Taken together, these findings suggest that OA has potential to be a new or alternative therapeutic strategy to the insulin resistance and metabolic syndrome treatments.

In this review, some studies showed that OA improves hypertension, one of the Mets components. More specifically, in the studies of Ahn et al. [27] and Bachhav et al. [28], there was a significant reduction of SBP. Additionally, a significant decrease of MAP was reported by Bachhav et al. [28], Madlala et al. [29], and Gamede et al. [30]. This improvement in hypertension could be due to the reported hypotensive effect of OA, probably by the modulation of the renin-angiotensin-aldosterone system and the

synthesis of atrial natriuretic peptide [27,50], since Bachhav et al. [28] and Madlala et al. [29] showed that OA increased the quantity of urine sodium excreted. Another possible hypotensive mechanism of action of OA could be the increase of the production of nitric oxide (NO), a vasodilator factor that is diminished in endothelial disfunction induced by cardiovascular risk factors such as hypertension, obesity, diabetes, and dyslipidemia [15]. This is in agreement with the increase of serum NO levels produced by the action of OA reported by Bachhav et al. [28], Jiang et al. [32], and An et al. [44]. However, Wang et al. [43] reported the opposite, which might be explained by the fact that they studied rats fed with a high fat and fructose diet. High fructose diets are closely linked to a higher oxidative stress status [51], which increases NO through enhancing inducible nitric oxide synthase (iNOS) expression [52,53].

With regard to the lipid profile, the significant reduction in TG and LDL serum levels [30–35], as well as the significant increase in HDL serum levels observed in animal studies [30,32,33,35] suggest that OA could prevent oxidative stress-induced CVD, since low HDL levels and high LDL levels in obesity caused an overproduction of reactive oxygen species (ROS), especially in obesity-induced oxidative stress [54]. However, in the studies carried out by Chen et al. [31], Pan et al. [34], and Wang et al. [38], OA significantly decreased serum HDL levels. These differences found in HDL serum levels might be explained by the different animal models or clinical contexts studied, as well as the different OA dosage applied. Interestingly, the decrease in serum levels of TG and LDL in hyperlipidemic patients treated with OA reported by Luo et al. [49] is consistent with all the results obtained in animals. However, the slight increase of HDL serum levels in hyperlipidemic patients is only in agreement with the results from animal studies by Gamede et al. [30], Jiang et al. [32], Luo et al. [33], and Wang et al. [35].

Dysregulation of adipokines plays a main role in the association between insulin resistance and oxidative stress [55]. Matumba et al. [45] showed that OA enhanced adiponectin plasma concentrations, which could result on greater insulin sensitivity [56] due to its insulin-sensitizing properties through enhancing hepatic IRS-2 expression [57]. In the present review, we found that OA increased serum leptin levels in hyperlipidemic patients [49], an adipokine that favors the synthesis of proinflammatory cytokines [54].

This result of OA in leptin serum levels might be due to an impairing leptin signaling in hyperlipidemic conditions [58]. Although the mechanism by which OA reduces body weight is not yet clear, two of the studies that reported a decrease in body weight also observed a reduction of ghrelin levels (Gamede et al. [39] and Nakajima et al. [41]). Ghrelin has been shown to induce body weight gain through increasing food intake [59].

On the other hand, the reduction of plasma levels of FFA [12,35,42] as well as the lower Adipo-IR index [36] caused by the action of OA, might imply an increase of insulin secretion because chronic exposure to elevated levels of FFA leads to ROS overproduction [60] and thus to impaired insulin signaling and beta-cell failure [54,55]. In this line, the literature supports that OA increases insulin biosynthesis and secretion [10], which is in accordance with the increased insulin levels and HOMA-β index [37], as well as the reduced FSI levels [35,36,38,43]. In addition, Li et al. [36], Wang et al. [38], Su et al. [42], and Nyakudya et al. [46] reported an improvement in the HOMA-IR index, whereas Gamede et al. [39] reported an improvement of the HOMA-2 IR index. These findings might be explained by the availability of OA to modulate insulin signaling pathways such as glycogen synthase (GS)/glycogen phosphorylase (GP) signaling pathway [61,62] or the insulin receptor substrate 1 (IRS1)-glucose transporter 4 (GLUT4) pathway via NF-κB [17]. Interestingly, a synthetic-biology-inspired therapeutic strategy based on OA-triggered short human glucagon-like-peptide 1 (GLP-1) expression through TGR5 pathways has been successfully developed and applied in hepatogenous diabetic mice [63]. Nonetheless, according to the literature, OA improves beta-cell function through increasing insulin biosynthesis and secretion and also improves glucose tolerance [10]. This idea coincides with different studies included in our review that reported improvements of glucose tolerance both in glucose [35,36,38–40,42,46] and in insulin [35,42] tolerance tests, as well as reductions of FBG [30,35,38,42–44].

It is noteworthy that the decrease in both serum levels [30,34,35,44,45] and gene expressions [40,45] of proinflammatory cytokines reported in our review, probably due to the modulatory effect of OA on NF-κB [17], could result in an improvement of insulin secretion and beta-cell function because proinflammatory cytokines, whose levels are higher in obese individuals, have an important role in the development of insulin

resistance [8–10]. Moreover, the ability of OA to alleviate oxidative stress and to improve pancreatic beta-cell function could also be related to the increase of SOD [29,30,32,37,42,43], GSH-Px [29,30,32], and CAT [32,42,43,48] activities, as well as the reduction of MDA [29,30,32,43,48]. Since OA might activate the transcription factor Nrf2, which increases the transcription of antioxidant enzymes (SOD, CAT, and GSH-Px), the aforementioned variations observed in the antioxidant enzymes might be explained by a possible activation of Nrf2 by OA [9,10].

Therefore, OA has potential effects on the components of MetS and insulin resistance. However, the results reported in our review have shown that OA has inconsistent effects on serum levels of HDL and NO, since they are increased in some studies and decreased in others. In this sense, these parameters should be further investigated, and the animal models and clinical situations studied should be standardized to better understand the effects of OA. Thus, a better understanding of the effects of OA may allow more randomized controlled trials to be carried out.

This study has some strengths and limitations. One of its strengths is the use of the PRISMA 2009 checklist, one of the most prestigious sets of guidelines for the reporting of systematic reviews [18]. Another strength is the use of the SYRCLE's Risk of Bias (RoB) tool and the Jadad scale for assessing the methodological quality of the animal studies and the clinical trial included, respectively. Moreover, the initial selection of studies, data extraction, and the evaluation of the studies finally included in our review were performed by two independent reviewers, who consulted a third reviewer when there was any doubt in order to reduce the risk of subjectivity [64,65]. Furthermore, a wide number of databases were consulted. This review has some limitations, such as that unpublished material sources were not consulted, which might have resulted in a selection bias [66]. Another limitation is the presence of only one clinical trial and its non-randomized design.

5. Conclusions

In summary, from the data from studies in experimental animals assessed in the present systematic review, we conclude that OA administration may improve

hypertension, attenuate the disturbance of the lipid profile in metabolic dysfunction situations, reduce oxidative stress status, and improve insulin resistance. In reference to the non-randomized clinical trial assessed in the present work, we conclude that OA may improve the hyperlipidemic status, as well as glycemia in hyperlipidemic patients. These findings confirm the potential of the OA to be effectively used in the treatment of the MetS and insulin resistance. However, there is need for performing further animal studies that include all parameters involved in the development of insulin resistance and MetS in order to provide a more in-depth understanding of the OA effects on these metabolic disorders. More importantly, it is peremptory to conduct randomized clinical trials whose results will open the door to the possible use of OA in humans as a therapeutic alternative or as a complement to conventional therapy.

Author Contributions: A.F.-A., E.G.-J. and J.S.-R. conceived and designed the review; A.F.-A., J.S.-R., and J.S.P. contributed to the selection process and analysis of the risk of bias; E.G.-J., M.C.-R., and J.M.C. contributed to the data extraction; A.F.-A. and E.G.-J. drafted the paper. All authors read and approved the final manuscript.

Acknowledgements: The results reported in the study are from the doctoral thesis of the main author and belong to the Clinical Medicine and Health Public Programme (B 12.56.1) of the University of Granada, Spain. F.-A. Á is granted with a pre-doctoral FPU grant (University of Granada, Spain). This work was also supported by a research initiation fellowship of the University of Granada, Spain.

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

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ESTUDIO III

Referencia: Fernández-Aparicio, Á., Correa-Rodríguez, M., Castellano, J. M., Schmidt-RioValle, J., Perona, J. S., & González-Jiménez, E. (2022). Potential molecular targets of oleanolic acid in insulin resistance and underlying oxidative stress: a systematic review. *Antioxidants*, *11*(8), 1517. https://doi.org/10.3390/antiox11081517

Indicios de calidad: Impact Factor (2021) = 7,675. Edition: Science Citation Index Expanded. Category and rank: Food Science & Technology (12/143; D1)

III. Potential molecular targets of oleanolic acid in insulin resistance and underlying oxidative stress: a systematic review

Abstract

Oleanolic acid (OA) is a natural triterpene widely found in olive leaves that possesses antioxidant, anti-inflammatory, and insulin-sensitizing properties, among others. These OA characteristics could be of special interest in the treatment and prevention of insulin resistance (IR), but greater in-depth knowledge on the pathways involved in these properties is still needed. We aimed to systematically review the effects of OA on the molecular mechanisms and signaling pathways involved in the development of IR and underlying oxidative stress in insulin-resistant animal models or cell lines. The bibliographic search was carried out on PubMed, Web of Science, Scopus, Cochrane, and CINHAL databases between January 2001 and May 2022. The electronic search produced 5034 articles but, after applying the inclusion criteria, 13 animal studies and 3 cell experiments were identified, using SYRCLE's Risk of Bias for assessing the risk of bias of the animal studies. OA was found to enhance insulin sensitivity and glucose uptake, and was found to suppress the hepatic glucose production, probably by modulating the IRS/PI3K/Akt/FoxO1 signaling pathway and by mitigating oxidative stress through regulating MAPK pathways. Future randomized controlled clinical trials to assess the potential benefit of OA as new therapeutic and preventive strategies for IR are warranted.

Keywords: *Olea europaea*; bioactive compounds; triterpenes; oleanolic acid; insulin resistance; type 2 diabetes mellitus; oxidative stress; inflammation; insulin signaling; pathways

1. Introduction

Diabetes mellitus (DM) is a major public health problem since it currently affects about 420 million people worldwide, and its prevalence is expected to increase by approximately 38% by 2030 [1]. Around 90% of DM world cases are accounted for by type 2 diabetes mellitus (T2DM) [2]. Insulin resistance (IR) affects individuals for many years before the development of T2DM [3], and consists of a diminished sensitivity of insulin target tissues to healthy insulin levels [4]. IR disrupts the insulin receptor substrate (IRS)/phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) pathway activation [5,6], which provokes a reduction of the glucose uptake in the adipose tissue and skeletal muscle, an increment of the gluconeogenic and glycolytic activity in the liver, and lipid metabolism disturbances in the liver and adipose tissue [7,8].

Authors have proposed possible hypotheses with regard to the development of IR, such as fat accumulation in the liver and skeletal muscle [4]. Obesity-associated chronic inflammation of insulin target tissues implies an impairment of glucose and lipid metabolism, a blockage of insulin signaling, and lower insulin sensitivity [9], since accumulation of fatty acids favors the activation of c-Jun N-terminal kinase (JNK) and of inhibitor of κB kinase (IKK) [8]. Phosphorylation of IKK activates the nuclear transcription factor kappa B (NF-κB) pathway, which upregulates the induction of the typical inflammatory status in IR through releasing proinflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) [10]. Moreover, JNK, the extracellular signal-regulated kinase 1/2 (ERK1/2), and p38 pathways comprise the pathway known as mitogen-activated protein kinases (MAPKs) [11], whose impairment has been reported to be involved in inducing IR [10].

The insulin-resistant state is closely linked to the overproduction of reactive oxygen species (ROS) and endoplasmic reticulum (ER) stress in insulin target tissues, which also leads to the activation of JNK and IKK [8]. In this line, elevated levels of proinflammatory cytokines, oxidative stress (OS), and ER stress have been suggested to trigger IR by negatively regulating insulin signaling [12]. In this way, a vicious circle is established by which IR increases the OS, which in turn aggravates the insulin-resistant

state [13]. Moreover, research highlighting the relevance of OS in the induction of IR has gained importance in the last few years [13–16]; in adults, it has been reported that IR is significantly associated with OS parameters [17,18].

Early and appropriate management of IR is essential not only to avoid the development of T2DM, but also to avoid the associated macrovascular and microvascular complications. Dietary and lifestyle interventions have been shown to be effective in the prevention and treatment of T2DM [3,19]. When these interventions fail, pharmacotherapy is considered, whose administering over a prolonged period of time unavoidably leads to the occurrence of undesirable adverse effects. Moreover, it has been suggested that the combination of drugs with bioactive compounds could be more efficient [20]. In the last few years, there has been increasing clinical interest in the potential properties of pentacyclic triterpenes to fight metabolic syndrome [21]. The study conducted by Claro-Cala et al. [22] demonstrated that the application of an olive pomace oil containing triterpenic acids, among them maslinic acid and oleanolic acid (OA), attenuated IR and adipose tissue inflammation in diet-induced obese mice. In this line, a randomized controlled trial known as the PREDIABOLE study reported a 55% lower risk of developing T2DM and an improvement of IR state in 176 prediabetic participants after consuming OA-enriched olive oil for 30 months [23].

Several studies have focused on analyzing the antidiabetic [24], anti-obesity [25], anti-inflammatory [24], and antioxidant properties [21,25] of OA, which may exert a positive effect in the treatment and prevention of IR. Interestingly, authors have proposed prophylactic and therapeutic roles of OA and its derivatives in several chronic diseases such as ulcerative colitis, multiple sclerosis, metabolic disorders, diabetes, hepatitis, different cancers, and osteoporosis [26–28]. Note that our group has also previously reviewed the potential protective effect of OA on the components of MetS. In this systematic review, we found that OA may improve blood pressure levels, hypertriglyceridemia, hyperglycemia, oxidative stress, and IR [29]. Furthermore, in a recent review, we summarized the biological activities of OA and its underlying mechanisms of action [30]. However, to our knowledge, no previous study has systematically examined the molecular targets of OA in insulin resistance and underlying

oxidative stress. Taking into account that IR precedes the development of T2DM, a better understanding of potential molecular targets by which OA exerts its ameliorating actions on insulin resistance and underlying OS is needed. On the other hand, the great variability of the studies that continue to be carried out must be considered; thus, it is essential to study the latest original studies published in depth. Therefore, the aim of this study is to systematically review the effects of OA on the molecular mechanisms and signaling pathways involved in the development of IR and underlying OS in insulin-resistant animal models or cell lines.

2. Materials and Methods

A systematic review was conducted following the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) 2020 protocol [31]. The protocol of this systematic review is published in PROSPERO (reference CRD42022344225).

2.1. Search Strategy

The bibliographic search was carried out in five electronic databases: PubMed, Web of Science, Scopus, Cochrane Plus, and CINAHL. All publications in English between January 2001 and May 2022 were included. The following categories were used in the database search strategy: oleanolic acid AND ("insulin resistance" OR "oxidative stress" OR "reactive oxygen species" OR cytokines OR antioxidants OR kinases). Table 1 shows information on the additional filters used and on the search field in which the search strategy was applied in each database.

Table 1. Specific filters applied on the search in each database.

Database	Search Field	Additional Filters
PubMed	All fields	Not applicable
Web of Science [1]	Title, Abstract, and Keywords	Document types: articles, others, and clinical trial
Scopus	Title, Abstract, and Keywords	Document types: article
Cochrane	Title, Abstract, and Keywords	Document types: article
CINAHL [2]	Full text	Not applicable

[1] Search performed in "All databases"; [2] via EBSCOhost.

2.2. Selection Process. Inclusion and Exclusion Criteria

The selection of potentially relevant studies was conducted in two phases. In the first one, the titles and abstracts were reviewed to assess their eligibility. In case of doubt, the full text was also read. In the second phase, the full text of the records previously selected was analyzed. Á.F.-A. and M.C.-R. independently performed the screening process. A third reviewer (J.M.C.) resolved differences and disagreements by consensus.

The inclusion criteria were established according to the PICOS framework:

- Patients: insulin-resistant animal models or cell lines.
- Intervention: administration of OA.
- Comparison: control substances and/or another bioactive compound.
- Outcomes: signaling pathways that are altered in states of IR and inflammatory and OS biomarkers (proinflammatory cytokines, antioxidant enzymes, transcription factors, and ROS).
- Study type: experimental studies.

The following exclusion criteria were established:

- Narrative and systematic reviews, letter to editor, book chapter, and other kinds of secondary studies.
- Studies that are focused on the extraction of bioactive compounds from natural plants and on the analysis of their biological activities without a more in-depth explanation of the molecular mechanisms implied.
- Studies that are focused on treating comorbidities or short-term or long-term complications of T2DM, without the approach of analyzing the effects of OA on signaling pathways involved in the development of IR.
- Administration of OA derivatives or OA combined with another bioactive compound.
- Non-English studies.

2.3. Data Extraction

Data were extracted once the selection process was finished. Data extracted both from the animal studies and the cell experiments were the following: authors, year of publication, subjects and sample size, type of intervention, period of administration and dosage used, and outcomes obtained (insulin signaling, inflammatory and OS pathways; and inflammatory and OS biomarkers). Two independent reviewers (Á.F.-A. and M.C.-R.) performed the data extraction. A third reviewer (E.G.-J.) resolved differences and disagreements by consensus. A meta-analysis was not possible because of the heterogeneous design of the studies included in the review. Therefore, we carried out a narrative description of each of the results measured.

2.4. Study Risk of Bias Assessment

Á.F.-A. and J.S.-R. independently analyzed the risk of bias of all the studies included in this review. A third reviewer (J.S.P.) resolved disagreements by consensus. For this purpose, the SYRCLE's Risk of Bias (RoB) tool [32] was used for the animal studies. The risk of bias for "in vitro" studies was not assessed because of the absence of a standard risk of bias tool for these kinds of studies.

SYRCLE's RoB tool was published by the Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE) [32] for assessing the methodological quality of experimental studies of animals. SYRCLE's RoB tool contains 10 items, and each of them is rated as a "yes" (low risk of bias), a "no" (high risk of bias), or "unclear" (insufficient information to evaluate risk of bias). These items assess six types of bias: selection bias, performance bias, detection bias, attrition bias, reporting bias, and another bias.

3. Results

3.1. Study Selection

Figure 1 shows the flow chart for the process of selection and exclusion of studies according to the PRISMA system [31]. A total of 5034 records were identified: 1446 from

PubMed, 1832 from Web of Science, 1402 from Scopus, 347 from CINAHL, and 7 from Cochrane. Once the duplicates were removed, 2702 records were screened, from which 2645 were excluded. Then, 57 reports were sought for retrieval, and 5 of them were not retrieved [33–37]. Later, 52 full-text articles were read to assess their eligibility, and finally 16 studies were included. An inverse search from the included studies was not conducted.

3.2. Characteristics of the Cell Experiments Selected

The characteristics and the main findings of the cell experiments included in this review are shown in Table 2. Of the three studies selected, two used the HepG2 cell line, and the other one used a human normal hepatocyte line (QZG). To induce IR, different components were used in each study (tert-butyl hydroperoxide (tBHP), sodium oleate, and high concentrations of insulin).

3.3. Characteristics of the Animal Studies Selected

The characteristics of the animal studies selected and their main findings are summarized in Table 3. All of them (n = 13) employed rodents, especially rats and mice. Of the 13 studies included, 2 of them employed T2DM mice models. Of the remaining 11, 5 used high-fat diet, 3 used high-fructose diet, 1 used high-fat and fructose diet, 1 used high-fat, high-carbohydrate diet, and 1 used Aroclor 1254 (PCB) to induce IR in rodents. The largest sample size was n = 40 and the smallest sample size was n = 21. The maximum and the minimum OA dosage administered in the studies were 250 mg/kg/day and 5 mg/kg/day, respectively. The maximum exposition time period to OA was 12 weeks, and it was 1week for the minimum time period.

3.4. Reporting Risk of Bias Assessment

The results of the risk of bias assessment of the thirteen animal studies included are reported in Figure 2. The SYRCLE's RoB tool assesses selection bias (items 1–3), performance bias (items 4–5), detection bias (items 6–7), attrition bias (item 8), reporting bias (item 9), and other biases (item 10).

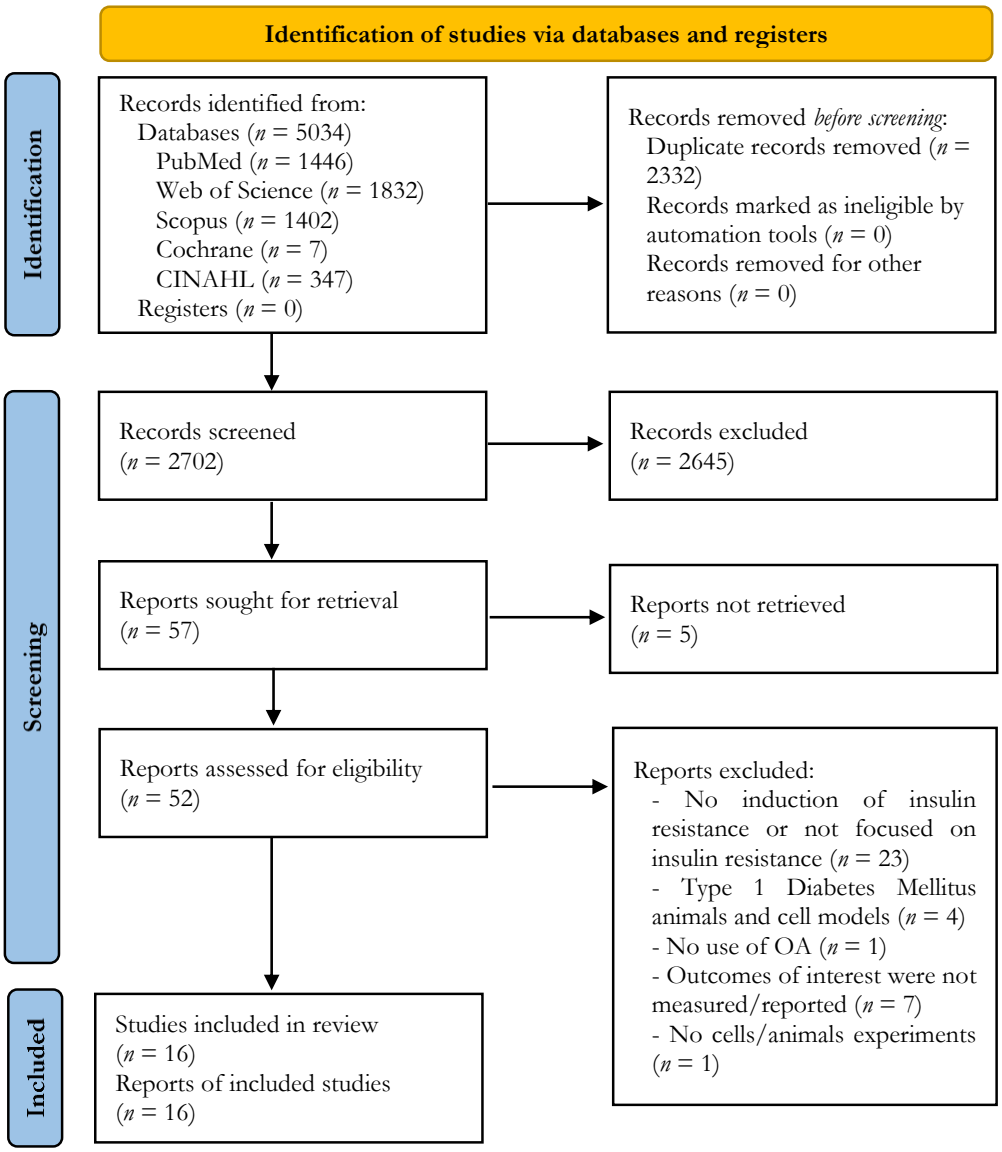

Figure 1. Flow diagram of the study selection process.

Table 2. Characteristics and results of the "in vitro" studies on the OA effects on insulin-resistant cell lines.

Author/Year	Subjects	Intervention and Groups	Main Findings
Wang X et al. [38] (2011)	QZG cells induced for IR with tBHP.	Intervention: Treatment with or without OA at 10 μM for 4 h, later exposition to tBHP, and finally stimulation with insulin. Groups: - CG: Non-insulin-resistant cells, non-treated with OA - Non-insulin-resistant cells only stimulated with insulin - Non-OA-treated insulin-resistant cells - OA-treated insulin-resistant cells	The exposition to tBHP decreased the insulin-stimulated phosphorylation of Akt and ERK, but it was inhibited by OA ($p < 0.05$). Non-significant (ns) actions on PGC-1α gene expression and on phosphorylation of p38 were observed.
Li M et al. [39] (2015)	HepG2 cells induced for IR with sodium oleate, except the CG.	Intervention: Insulin-resistant HepG2 cells treated with different doses of OA. Groups: - CG (non-insulin-resistant cells, non-treated with OA) - Non-treated insulin-resistant cells - Positive control ➔ Rosiglitazone (RSG) - Experimental groups ➔ OA-5 μM; OA-10 μM; OA-25 μM	↓ Content of glucose, IL-6, and TNF-α ($p < 0.05$) and ↓ protein expression of NF-κB ($p < 0.01$); ↑ IRS and GLUT4 protein expression ($p < 0.01$) in all OA groups vs. non-treated insulin-resistant cells. ↓ ns of TNF-α levels at OA-5 μM vs. non-treated insulin-resistant cells.
Zhang Y et al. [40] (2020)	Insulin-resistant HepG2 cells induced with high concentrations of insulin.	Intervention and Groups: - CG: non-OA-treated insulin-resistant cells - OA group: OA-treated insulin-resistant cells - PGA-OA group: OA-loaded PGA-OA-treated insulin-resistant cells	↓ PTP1B protein expression and ↑ protein expression of Akt and IRS-1 in both insulin-resistant cell models treated with OA or PGA-OA. All changes $p < 0.05$.

QZG: human normal hepatocyte line; IR: insulin resistance; tBHP: tert-butyl hydroperoxide; CG: control group; Akt: protein kinase B; ERK: extracellular signal-regulated kinase; PGC-1α: PPARγ coactivator 1α; PPARγ: peroxisome proliferator-activated receptor γ; IL-6: interleukin-6; TNF-α: tumor necrosis factor-α; NF-κB: nuclear transcription factor kappa B; IRS: insulin receptor substrate; GLUT4: glucose transporter type 4; PGA: polygalacturonic acid; PTP1B: protein tyrosine phosphatase 1B.

Table 3. Characteristics and results of animal experimentation studies on the effect of oleanolic acid (OA) on signaling pathways impaired in insulin resistance and inflammatory/oxidative stress biomarkers in insulin-resistant animal models.

Author/Year	Subjects (n)	Intervention	Groups	Main Findings
Wang X et al. [41] (2015)	24 male C57BL/KsJ-Lepdb (db/db) mice	Intragastric administration of vehicle (0.5% CMC-Na), OA (250 mg/kg/day), or metformin (100 mg/kg/day) for 28 days.	Groups (n = 6 per group): - CG - OA - Metformin - OA + metformin	↓ HOMA-IR; ↓ mRNA expression levels in liver ($p < 0.001$) of GP, PEPCK1, G-6-Pase, and GLUT2; ↓ ns of mRNA expression levels in liver of PGC-1α and ↑ ns of GS. ↑ AMPK, ACC, Akt, and PI3K and ↓ mTOR and CREB phosphorylation in livers; ↓ liver protein levels of PEPCK and of G-6-Pase. All changes reported are of OA group compared with CG.
Wang X et al. [42] (2013)	24 male C57BLKS/Jlar-Lep$^{db/db}$ mice, and 10 wild-type mice as control	Intraperitoneal injection of OA (20 mg/kg/day) for 14 days.	- CG - Untreated diabetic mice - OA-treated diabetic mice	↓ Liver protein expression of G-6-Pase and PEPCK; and ↑ PGC-1α gene expression and AMPK phosphorylation in livers. ↑ Insulin-stimulated phosphorylation of Akt in livers. ↓ Mitochondrial ROS production and GSSG, and ↑ mitochondrial GSH in liver. ↑ Protein expression of Nrf2, GCLC, SOD, and CAT in liver. ↓ IL-6, IL-1β, and TNF-α both in serum and in liver. All changes ($p < 0.05$) in OA-treated diabetic mice compared with non-OA-treated diabetic mice.
Zeng X et al. [43] (2012)	Male C57BL/6J mice	Mice fed with a normal diet or an HFD during 10 weeks, and injection of STZ into HFD-fed rats to establish a T2DM model. Later, administration of 100 mg/kg/day of OA to T2DM mice for 2 weeks.	- CG (non-diabetic mice) - Untreated T2DM mice - OA-treated T2DM mice	Phosphorylated-/total-Akt ratio similar in both diabetic mice groups; ↓ levels of p-Akt in non-treated T2DM mice vs. non-diabetic mice, while treatment with OA ↑ p-Akt levels in T2DM mice to levels of non-diabetic mice. In OA-treated T2DM mice, ↑ of phosphorylated-/total-FoxO1 ratio and ↓ in total FoxO1 protein ($p < 0.05$) compared to untreated T2DM mice and non-diabetic mice. All results measured in livers of mice.
Zhou X et al. [44] (2014)	Male C57BL/6J mice	Idem to Zeng et al. [43], but measurements of variables were undertaken after 4 weeks of OA administration (during OA treatment experiment), or after 2 weeks of OA administration followed by an OA-free HFD diet during 4 weeks (post-OA treatment).	- CG (non-diabetic mice) - Untreated T2DM mice - OA-treated T2DM mice	In liver of diabetic mice, the following was observed: ↓ total content of FoxO1, ↑ phosphorylation of FoxO1 and acetylation of FoxO1, and ↓ gene expression of G-6-Pase during and post OA treatment. ↑ Phosphorylation of AMPK-α and ACC; ↓ of the mature form of SERBP-1c in the livers of diabetic mice during OA treatment. All changes $p < 0.05$ vs. untreated diabetic mice.

Table 3. *Cont.*

Author/Year	Subjects (n)	Intervention	Groups	Main Findings
Yunoki K et al. [45] (2008)	24 male Sprague Dawley rats	Administration of OA (50 mg/kg/day) or PEE (450 mg/kg/day) for 4 weeks	- CG: Normal fat diet - HFD group - HFD + OA - HFD + PEE	Downregulation of ACC, G-6-Pase, FoxO1, TNF-α, and IL-1β genes; and upregulation of genes of insulin receptor substrates and AMPK β-2 regulatory subunit in rat livers. All changes $p < 0.01$ vs. HFD-fed rats.
Xue C et al. [46] (2021)	30 Sprague Dawley Rats	Administration of OA (25, 50, and 100 mg/kg/day) for 8 weeks. Rats were simultaneously fed with an HFD or normal diet for 12 weeks.	Groups (n = 6 per group): - Normal-diet-fed rats - HFD-fed rats - HFD + OA 25 fed rats - HFD + OA 50 fed rats - HFD + OA 100 fed rats	↓ MDA and ↑ SOD, GPx, and CAT content; ↓ IL-1β, IL-6, and TNF-α overexpression; inhibition of the phosphorylation of IκB-α and p65 in liver tissues. All changes ($p < 0.05$), especially in rats treated with OA 50 or 100 vs. HFD-fed rats.
Li Y et al. [47] (2014)	24 male Sprague-Dawley rats	Fructose induced insulin- resistant rats and oral administration of 5 or 25 mg/kg/day of OA for 10 weeks.	Groups (n = 6 per group): - Control group: non- insulin-resistant rats. - Insulin-resistant rats: non-treated insulin-resistant rats; OA-5 mg; and OA 25 mg	↓ Adipo-IR; ↑ adipose mRNA expression of insulin receptor, IRS-1, PI3K, and Akt. ↑ IRS-1 protein expression, ↓ fructose-stimulated pIRS-1 protein expression, and of the ratio of pIRS-1 to total IRS-1 protein expression. ↑ Ratio of pAkt protein to Akt protein. All changes ($p < 0.05$) in the adipose tissue of insulin-resistant rats treated with OA 25 mg vs. non-treated insulin-resistant rats.
Li W et al. [48] (2021)	21 C57BL/6J male mice	All mice fed with HFD during 12 weeks. Later, administration with distilled water, or OA 25 mg/kg or OA 50 mg/kg per day by intragastric gavage for 4 weeks.	Groups (n = 7 per group): - CG: distilled water - OA-25: 25 mg/kg/day - OA-50: 50 mg/kg/day	↓ HOMA-IR and Adipo-IR index; ↑ phosphorylation of Akt and ↓ gene expression of iNOS, IL-6, TNF-α, IL-1β, and Caspase 1; ↓ macrophages M1 and ↑ macrophages M2 in eWAT of mice treated with OA-25 and -50. ↓ phosphorylation of ERK and JNK in eWAT of mice treated with OA-50. All changes ($p < 0.05$) vs. non-OA-treated HFD-fed mice.
Su S et al. [49] (2018)	40 male C57B6/J mice	Exposure to vehicle or Aroclor 1254 (100 μg/kg; PCBs) every 3 days for 10 weeks. Pretreatment with 50 mg/kg of OA for 1 h every 3 days for 10 weeks.	Groups (n = 10 per group): - CG - PCBs - PCBs + OA - PCBs + Vitamin C	↓ HOMA-IR, ↓ serum MDA, and ↑ serum SOD and CAT activity; ↓ mRNA expression of NOX4, GCLC, and GCLM in adipose tissue. All changes ($p < 0.05$) in favor of OA-pretreated mice vs. PCBs-treated mice.
Matumba MG et al. [50] (2019)	40 male Sprague Dawley rat pups	OA (60 mg/kg/day) administration by orogastric gavage during the second neonatal week. Duration of the experiment was 16 weeks.	- CG - OA - HF diet - HF diet + OA - HF diet + Met	↑ AMPK, GLUT4 and ↓ IL-6 and TNF-α gene expression ($p < 0.001$) in the skeletal muscle; and ↓ plasma concentration of IL-6 ($p < 0.0001$) and ns of TNF-α. All changes in OA-treated HF-fed rats vs. HF-fed rats.

Table 3. *Cont.*

Author/Year	Subjects (n)	Intervention	Groups	Main Findings
Nyakudya T et al. [51] (2019)	30 Sprague Dawley rat pups	Administration of OA (60 mg/kg/day b.w.) by orogastric gavage in the second postnatal week. Duration of the experiments 14 days since their birth.	- CG (distilled water + DMSO) - OA - HF - OA + HF	The ↓ of GSH and CAT activity in the skeletal muscles of HF-fed rats was attenuated in OA-treated HF-fed rats ($p < 0.05$). Non-significant changes on GPx, SOD, and MDA.
Gamede M et al. [52] (2019)	36 male Sprague-Dawley rats	Treatment with OA or OA + dietary intervention for 12 weeks after previous administration of HFHC diet during 20 weeks to induce prediabetes.	Groups (n = 6 per group): Non-prediabetic control (NC); prediabetic control (PC); metformin (MET); MET + dietary intervention; OA; OA + diet	↓ Plasma levels of IL-6 and heart MDA concentration, and ↑ plasma level of SOD and GPx in OA-treated rats vs. prediabetic control group. All changes $p < 0.05$.
Wang S et al. [53] (2018)	36 male Sprague-Dawley rats	6 normal-diet-fed rats and 30 HFF-fed rats during the first four weeks. HFF-fed rats were intraperitoneally injected with tBHP during the last eight weeks of the experiments.	6 Groups (n = 6 per group): 6 HFF-fed rats were treated with OA and another 6 with nano-OA during the last 6 weeks of the experiments. 25 mg/kg/day	↓ Serum NO levels and ↑ serum CAT activity in OA and nano-OA groups; ↓ serum levels of MDA and ↑ serum SOD activity and ISI in nano-OA group. All changes ($p < 0.05$) vs. non-treated insulin-resistant rats.

CG: control group; OA: oleanolic acid; HOMA-IR: homeostatic model assessment for insulin resistance; GP: glycogen phosphorylase; PEPCK1: phosphoenolpyruvate carboxykinase 1; G-6-Pase: glucose-6-phosphatase; GLUT2: glucose transporter type 2; PGC-1α: PPARγ coactivator 1α; PPARγ: peroxisome proliferator-activated receptor γ; GS: glycogen synthase; AMPK: AMP-activated protein kinase; ACC: acetyl-CoA carboxylase; Akt: protein kinase B; PI3K: phosphatidylinositol-3-kinase; mTOR: mammalian target of rapamycin; CREB: cAMP-response element-binding protein; ROS: reactive oxygen species; GSSG: glutathione, oxidized form; GSH: glutathione; Nrf2: nuclear factor erythroid 2-related factor 2; GCLC: glutathione cysteine ligase catalytic subunit; SOD: superoxide dismutase; CAT: catalase; IL-6: interleukin-6; IL-1β: interleukin-1β; TNF-α: tumor necrosis factor-α; HFD: high-fat diet; STZ: streptozotocin; T2DM: type 2 diabetes mellitus; pAkt: phosphorylated-Akt protein at serine-473; FoxO1: forkhead box O1; SREBP-1c: sterol regulatory element-binding protein-1c; MDA: malondialdehyde; IRS: insulin receptor substrate; pIRS: phosphorylated-IRS at serine; eWAT: epididymal white adipose tissue; JNK: c-Jun N-terminal kinase; ERK: extracellular signal-regulated kinase; NOX4: NADPH oxidase; GCLm, glutamate-cysteine ligase modifier subunit; RSG: rosiglitazone; HF: high-fructose; GPx: glutathione peroxidase; PEE: pomace ethanol extract; GLUT4: glucose transporter type 4; HFHC: high-fat high-carbohydrate; HFF: high-fat and fructose; tBHP: tert-butyl hydroperoxide; NO: nitric oxide; ISI: insulin sensitivity index.

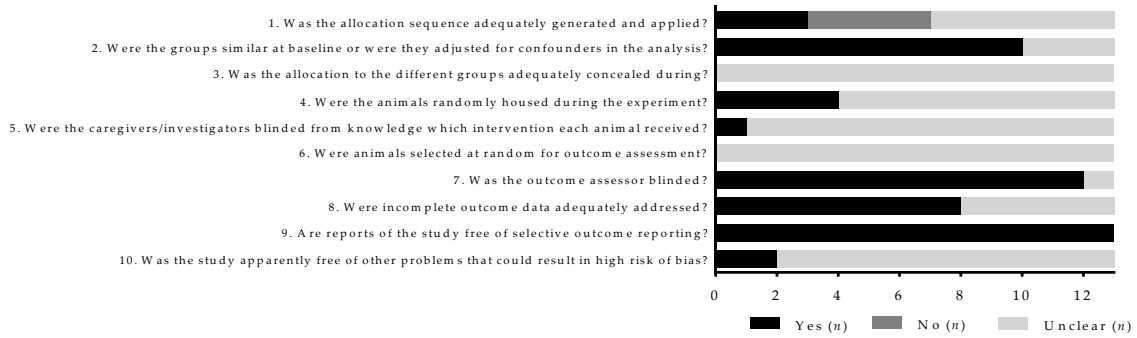

Figure 2. SYRCLE's RoB tool results for each study. Yes (low risk of bias); No (high risk of bias); Unclear (item not reported, unknown risk of bias); *n* (number of studies).

3.5. OA Effects in Insulin-Resistant Cell Lines

The IR state was improved by OA actions in the three "in vitro" studies included [38–40]. Pretreatment with OA at 10 μM in QZG cells by Wang et al. [38] inhibited the reduction of insulin-stimulated phosphorylation of Akt and ERK induced by tBHP. Li et al. [39], in a study performed on insulin-resistant HepG2 cells, showed an increment in the protein expression of IRS and glucose transporter type 4 (GLUT4) and a decrease of NF-κB protein expression after the administration of OA at 10 μM and 25 μM. Moreover, protein levels of IL-6 and of TNF-α were diminished by OA actions [39]. In another study conducted on insulin-resistant HepG2 cells, OA increased Akt and IRS-1 protein expression and reduced the protein expression of protein tyrosine phosphatase 1B (PTP1B) [40].

3.6. OA Effects on Impaired Signaling Pathways and on OS in Insulin-Resistant Animal Models

3.6.1. Hepatic IR

Five studies analyzed the actions of OA on livers of insulin-resistant rodents [41–46]. The experiments of Wang et al. [41] and Wang et al. [42] reported that OA decreased the protein expression of glucose-6-phosphatase (G-6-Pase) and phosphoenolpyruvate carboxykinase (PEPCK) and increased the AMP-activated protein kinase (AMPK) and Akt phosphorylation in livers of type 2 diabetic mice. In the first one, the following was

also reported: a downregulation of the liver gene expression of glycogen phosphorylase (GP), PEPCK1, G-6-Pase, and glucose transporter type 2 (GLUT2), and a non-significant downregulation of peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α). Moreover, in this study, OA augmented the phosphorylation of acetyl-CoA carboxylase (ACC) and of PI3K, and diminished the phosphorylation of mammalian target of rapamycin (mTOR) and of cAMP-response element-binding protein (CREB) [41]. In the second one, OA also increased the gene expression on mice livers of PGC-1α [42].

The administration of OA by Zeng et al. [43] restored the levels of phosphorylated-Akt in livers of type 2 diabetic mice to the same levels as those of non-diabetic mice. These authors also showed an increment of the ratio phosphorylated/total forkhead box O1 (FoxO1), and the diminution of the total FoxO1 protein by OA actions. In the same line, Zhou et al. [44] observed a significant decrease of the hepatic total content of FoxO1, an increase of FoxO1 phosphorylation and acetylation, and a reduction in the gene expression of G-6-Pase during the OA administration and 2 weeks after the OA treatment. They also reported that during the OA administration, the phosphorylation of AMPK-α and of ACC was increased in the livers of type 2 diabetic mice. Similarly, Yunoki et al. [45] evidenced that OA was responsible for downregulating ACC, G-6-Pase, and FoxO1 genes, and for upregulating IRS and AMPK-β-2 genes in the livers of HFD-fed rats.

3.6.2. IR in Adipose Tissue and Skeletal Muscle

Two studies undertook to investigate the IR in adipose tissues [47,48]. Li et al. [47] reported an overexpression of IRS-1, PI3K, and Akt genes after the application of OA for 10 weeks to fructose-induced adipose tissue insulin-resistant rats. In this same study, OA inhibited the phosphorylation of IRS-1 induced by fructose, and increased the ratio of phosphorylated-Akt/Akt. Previous research has shown that OA enhanced the phosphorylation of Akt and reduced the phosphorylation of ERK and JNK in the adipose tissue of HFD-fed mice. Moreover, the HOMA-IR and Adipo-IR index of these mice achieved lower levels because of OA actions [48]. In regards to OA effects on IR in skeletal muscle, Matumba et al. [50] administered OA to high-fructose (HF)-diet-fed

neonatal rats, and they observed an increase of the gene expression of AMPK and GLUT4 in the skeletal muscle at the end of the experiment.

3.6.3. OA Effects on Proinflammatory Cytokines and OS Biomarkers

OS and inflammation in insulin target tissues are closely linked in the establishment of IR. In the study conducted by Wang et al. [42], OA led to a reduction of ROS and L-glutathione oxidized (GSSG), and an increase of glutathione (GSH) in liver mitochondria of treated diabetic mice in comparison to non-OA-treated diabetic mice. OA also increased the protein levels of nuclear factor erythroid 2-related factor 2 (Nrf2), superoxide dismutase (SOD), and catalase (CAT), stabilized those of glutathione cysteine ligase catalytic subunit (GCLC) in the liver of these treated diabetic mice, and decreased serum and liver gene expression of IL-6, IL-1β, and TNF-α [42].

Xue et al. [46] demonstrated that the administration of OA for eight weeks mitigated the overexpression of IL-6, TNF-α, and IL-1β in liver tissues of HFD-fed rats. Together with these OA effects, the following were also observed: lower content of malondialdehyde (MDA) and higher levels of SOD, glutathione peroxidase (GPx), and CAT, as well as the inhibition of IκB-α and p65 phosphorylation. Yunoki et al. [45] reported a gene downregulation of TNF-α and IL-1β in livers of HFD-induced rats treated with OA. In the study carried out by Li et al. [48], OA produced a reduction of the gene expression of iNOS, IL-6, TNF-α, IL-1β, and caspase 1 in HFD-fed mice. Moreover, in these mice, OA changed the polarization of adipose tissue macrophages, since macrophages M1 diminished and macrophages M2 increased in these OA-treated HFD-fed mice [48].

In the study conducted by Su et al. [49], the exposition to PCB produced in the adipose tissue of mice showed high levels of serum MDA, and also increased the gene expression of NADPH oxidase 4 (NOX4), GCLC, and glutamate-cysteine ligase modifier subunit (GCLM), but it was inhibited by OA. Moreover, it was reported that OA increased the serum SOD and CAT activity in these PCBs-exposed mice. The administration of OA to HF-fed neonatal rats by Nyakudya et al. [51] attenuated the fructose-induced decrease of GSH and CAT activity in the skeletal muscle by the end of

the study. In another study performed on HF-fed neonatal rats by Matumba et al. [50], it was observed that OA reduced the plasma levels and gene expression of IL-6 and TNF-α in the skeletal muscle at the end of the experiment.

Gamede et al. [52] induced prediabetic rats through administering high-fat high-carbohydrate (HFHC) diet before the administration of OA. Rats continued to receive either an HFHC diet or a normal diet during OA administration. These authors also observed a mitigation of plasma levels of IL-6, an increase of serum SOD activity, and an increase in GPx activity because of OA actions. In the study performed by Wang et al. [53], OA produced an increase of serum SOD and CAT activity and a diminution of serum MDA and serum NO in high-fat and fructose (HFF)-diet-fed rats. Moreover, the insulin sensitivity index (ISI) was improved.

Figures 3 and 4 highlight the potential molecular action of OA on impaired insulin signaling pathways and underlying oxidative stress as consequence of the induction of IR in animal experimentation or cell experiments.

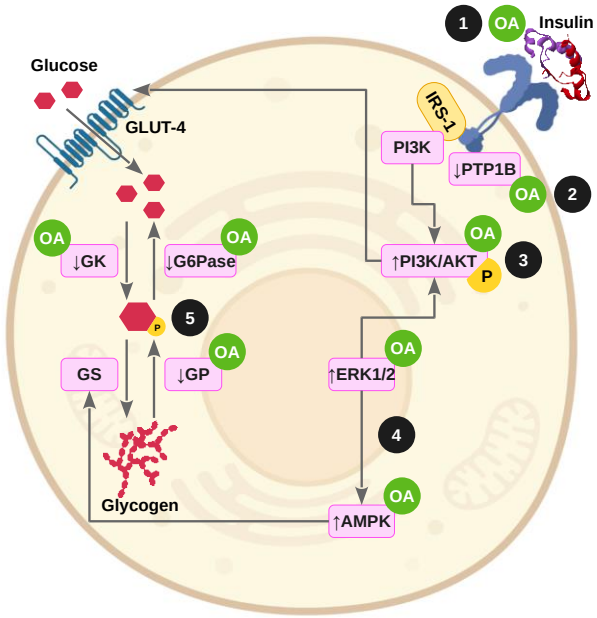

Figure 3. Oleanolic acid improves insulin signaling in peripheral tissues through a multimolecular mechanism. (**1**) OA is an activator of the insulin receptor, exerting an insulin mimetic role; (**2**) OA upregulates insulin sensitivity by inhibition of the tyrosine phosphatase PTP1B and TCPTP; (**3**) OA increases glucose uptake by activation of the PI3K/Akt pathway and GLUT-4 translocation; (**4**) OA also enhances glucose uptake and fatty acid oxidation in muscle and liver by activating the ERK1/2-AMPK axis; (**5**) OA preserves the glycogen pool in muscle and liver by stimulating glucokinase and repressing the glucose-6-phosphatase and glycogen phosphorylase activities.

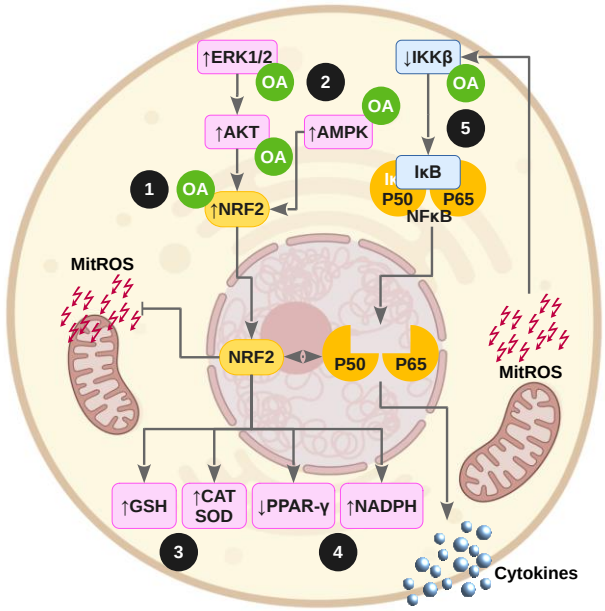

Figure 4. Oleanolic acid exerts antioxidant and anti-inflammatory actions against the supraphysiological production of mitochondrial ROS (mitROS) via transcription factors NRF2 and NFκB. (**1**) OA activates NRF2 by direct interaction with Keap1, the primary sensor that retains NRF2 for ubiquitin-dependent degradation in cytoplasm; (**2**) OA also activates NRF2 through the stimulation of stress kinase pathways such as ERK1/Akt and AMPK; (**3**) OA preserves the glutathione pool and increases the expression of antioxidant enzymes; (**4**) OA conserves the NADPH levels by upregulating genes of the pentose phosphate pathway and downregulating lipogenic genes; (**5**) OA reduces the production of inflammatory mediators, avoiding NFκB activation through competitive inhibition of IKKβ.

4. Discussion

This systematic review aimed to provide a better understanding of how OA acts on signaling pathways involved in the development of IR and underlying OS. To our knowledge, no previous studies have been carried out to review the potential molecular targets of OA in IR, as presented in this study. All results provided in this review derive from experimental studies where OA was administered to animal or cell lines induced for IR. The present systematic review shows that OA is capable of attenuating IR through enhancing insulin sensitivity and glucose uptake and suppressing the hepatic glucose production, probably by modulating the IRS/PI3K/Akt/FoxO1 insulin signaling pathway. The modulation of MAPK pathways by OA mitigates underlying OS and inflammation, and consequently improves insulin sensitivity. Therefore, these results

suggest the potential molecular targets of the therapeutic and preventive actions of OA on signaling pathways leading to IR.

In the three "in vitro" studies included, OA attenuated the IR status on hepatic cell lines [38–40]. More specifically, the induction of IR to QZG cells by Wang et al. [38] reduced the insulin-stimulated phosphorylation of Akt and ERK, but it was reversed by OA. Another of the studies included was the one conducted by Li et al. [39] on HepG2 cells, where OA attenuated IR through increasing the protein levels of IRS and GLUT4, and repressing the protein expression of NF-κB, IL-6, and TNF-α. It is well known that the activation of the NF-κB pathway negatively affects insulin signaling and promotes the release of proinflammatory cytokines [24,54]. In addition, ROS-induced activation of the NF-κB pathway in skeletal muscle of HFD-fed mice has been associated with increased expression of PTP1B [55], a negative regulator of insulin signaling [56]. In this line, the transfection with PTP1B to HepG2 cells by Niu et al. [57] decreased the transcription of IRS1 and GLUT4. Considering that several authors have previously reported inhibitory activities of OA and its derivatives on PTP1B [56,58–60], one of the possible mechanisms by which OA improves insulin sensitivity could be through inhibiting PTP1B. In fact, Zhang et al. [40] showed that OA enhanced the protein levels of IRS and of Akt, and reduced those of PTP1B on IR-induced HepG2 cells.

In regard to the studies performed on rodents induced for IR, five of them undertook the molecular mechanisms of OA involved in its ability to ameliorate hepatic IR [41–45]. In the studies conducted by Wang et al. [41] and Wang et al. [42], OA led to a decrease of the protein levels of G-6-Pase and PEPCK in type 2 diabetic mice. Moreover, Wang et al. [41] reported that OA had downregulating effects in the liver gene expression of GP, G-6-Pase, GLUT2, and PEPCK1, and reduced mTOR and CREB phosphorylation. The repressive action of OA in the gene expression of G-6-Pase was also observed by Zhou et al. [44] and Yunoki et al. [45]. All these OA-inhibitory effects on gluconeogenic enzymes might be explained by the OA actions on IRS, PI3K, and Akt, since the activation of the insulin IRS-1/PI3K/Akt pathway has been reported to be suppressed in IR [61]. In fact, increased phosphorylation of Akt and of PI3K in livers of type 2 diabetic mice due to OA was reported by Wang et al. [41] and increased

phosphorylation of Akt was reported by Wang et al. [42]. The existing literature supports the hypothesis that insulin-stimulated phosphorylation of Akt2 activates glycogen synthase and reduces the transcription of gluconeogenic enzymes through inactivating FoxO1 [62]. Moreover, deacetylation of FoxO1 has been proposed to intensify its activity, favoring gluconeogenesis [63]. Another mechanism by which OA enhances glucose uptake and regulates gluconeogenesis is through increasing phosphorylation of Akt and FoxO1 [43,44], and also acetylation of FoxO1 [44]. Similarly, Yunoki et al. [45] observed gene downregulation of FoxO1 in livers of OA-treated HFD-fed rats.

Two studies were focused on OA actions in adipose tissue insulin-resistant rodents and they reported an enhancement of the insulin-stimulated phosphorylation of Akt [47,48]. These results are in consonance with those previously discussed for hepatic insulin-resistant rodents [41–44]; moreover, they are in consonance with Wang et al. [38] and Zhang et al. [40], two of the studies carried out on hepatic cell lines. In the first one, Li et al. [47] observed that OA attenuated adipose tissue IR through increasing gene expression of IRS-1, PI3K, and Akt, and by inhibiting the fructose-induced phosphorylation of IRS-1. These results suggest that OA could improve insulin signaling and glucose uptake through modulating the IRS-1/PI3K/Akt insulin pathway. In fact, previous studies have studied the role of PI3K/Akt in enhancing insulin signaling [5,64]. In the second one, OA improved the HOMA-IR and ADIPO-IR in adipose tissue insulin-resistant mice, probably by increasing effects on Akt phosphorylation and decreasing effects on JNK phosphorylation [48], since JNK activation has been shown to reduce insulin action through increasing phosphorylation of IRS-1 at serine [8,65]. Moreover, Li et al. [48] also showed that OA modified the polarization of macrophages in favor of M2 macrophages, which could also explain the improvement effects of OA in adipose tissue IR, since JNK phosphorylation has also been reported to promote polarization of ATMs to M1 macrophages [66], which are responsible for releasing proinflammatory cytokines in adipose tissue IR [67]. This idea coincides with the alleviating effects of OA on gene expression of iNOS, IL-6, TNF-α, IL-1β, and caspase 1, also reported by Li et al. [48]. A decrease in the phosphorylation of ERK in the adipose tissue of insulin-resistant mice was also reported by Li et al. [48], while in the study of

Wang et al. [38], OA increased phosphorylation of ERK. This difference might be explained by the fact that OA was used in different kinds of experimental individuals.

The eukaryotic enzyme AMPK's main function is to regulate glucose uptake [68] through stimulating GLUT4 translocation [21,65,69], which is in consonance with the study conducted by Matumba et al. [50]. These authors reported a higher gene expression of both AMPK and GLUT4 in high-fructose-diet-fed neonatal rats treated with OA. AMPK has also been demonstrated to stimulate PI3K/Akt pathway [69], which could support the suppression of hepatic gluconeogenesis due to OA by increasing phosphorylation of AMPK, Akt, and PI3K in livers of type 2 diabetic mice [41]. This OA-induced phosphorylation of AMPK was also observed in rodent livers by Yunoki et al. [45] and by Wang et al. [42], demonstrating that OA lowers gene expression of TNF-α, IL-6, and IL-1β. The relief of inflammation might be explained by the OA-induced inactivation of NF-κB via dephosphorylation of IκB-α and of p65 [10,70], as was observed in livers of HFD-fed rats [46]. These results could indicate an improvement of insulin sensitivity and of glucose uptake, because NF-κB promotes the release of proinflammatory cytokines by suppressing the phosphorylation of AMPK [10].

Long-term inflammation leads to the generation of ROS in insulin target tissues [71], which supposes a greater activation of JNK and IKK [8], and consequently, higher levels of proinflammatory cytokines, further impairing insulin signaling [12]. Thus, combating OS is essential for alleviating IR, and in the present review, OA has exhibited antioxidant activities. Specifically, OA mitigated the production of ROS and GSSG, and also increased GSH in liver mitochondria of diabetic mice [42]. Moreover, OA augmented SOD [42,46], CAT [42,46], and GPx [46] activities, and decreased serum MDA [46] in livers of rodents with IR [42,46]. Since Wang et al. [42] reported an increment in the protein levels of Nrf2, these increased activities of antioxidant enzymes could be explained by the activation of OA of the transcription factor Nrf2, as was shown by previous authors [72,73]. Moreover, OA attenuated OS induced by Aroclor 1254 in the adipose tissue of insulin-resistant mice through downregulating the gene expression of NOX4, GCLC, and GCLM, decreasing serum MDA, and increasing serum SOD and CAT activities. OA also seems to mitigate the fructose-induced IR and OS, as was

observed in the increasing effects of OA on GSH and CAT activities in the skeletal muscle of high-fructose-diet-fed neonatal rats [51].

All the aforementioned modulatory activity of OA on proinflammatory cytokines and on antioxidant enzymes was also shown by Gamede et al. [52] and Wang et al. [53]. In the first one, the application of OA mitigated in prediabetic rats the reduction of serum levels of SOD and GPx activities [52], suggesting that OA favors antioxidant activities. In the second one, OA also led to higher SOD and CAT activities, as well as a reduction in the serum levels of MDA and nitric oxide [53]. Moreover, Wang et al. [53] observed an enhancement in the insulin sensitivity in HFF-diet-fed rats treated with OA. Thus, OA could promote better sensitivity to insulin through reducing OS.

This work has some strengths and limitations. The main strength of this research is that it provides a broad and overall picture of the effects of OA on the molecular mechanisms and signaling pathways involved in the development of IR and underlying OS in insulin-resistant animal models or cell lines. The large number of databases that have been used for the review and the utilization of the PRISMA 2020 protocol for the reporting of systematic reviews are also noteworthy [31]. One should note that the SYRCLE's Risk of Bias (RoB) tool was also used for assessing the risk of bias of the animal studies. In addition, it should be noted that in order to reduce inter-examiner bias, the quality of the studies was evaluated by two independent reviewers. Moreover, a third reviewer resolved potential differences [74,75]. However, this review has several limitations. First, the selected articles are written in only English, possibly ignoring articles written in other languages; this is found to be a limitation. Second, the fact that we did not include grey literature in this systematic review would be another limitation of this study.

5. Conclusions

OA presents potential antioxidant, anti-inflammatory, and insulin-sensitizing properties of special interest for the treatment and prevention of IR. OA attenuates IR through improving insulin sensitivity and insulin signaling, which results in a better glucose homeostasis and inhibition of gluconeogenesis, as well as the mitigation of the OS that aggravates IR status by impairing insulin signaling pathways. Thus, the potential

molecular targets of OA for alleviating IR are the modulation of the IRS-1/PI3K/Akt insulin signaling pathway, inactivation of FoxO1, and regulation of different MAPK pathways, which are implicated in the development of OS and of inflammation. However, more in vitro and in vivo studies should be addressed to obtain greater in-depth knowledge on the exact mechanism of interaction between OA and its target proteins, as well as the main source of ROS (activated NADPH oxidases, mitochondrial electron transport chain, and so on) targeted by OA. Moreover, the performance of randomized clinical trials is needed in order to elucidate its future use potential as a new and alternative therapeutic and preventive strategy for IR.

Author Contributions: Conceptualization, Á.F.-A., E.G.-J., and M.C.-R.; methodology, Á.F.-A., J.M.C., and M.C.-R.; validation, E.G.-J., J.S.P., and J.S.-R.; formal analysis, Á.F.-A., J.S.P., and J.S.-R.; investigation, Á.F.-A., E.G.-J., and J.S.-R.; data curation, Á.F.-A. and M.C.-R.; writing—original draft preparation, Á.F.-A. and M.C.-R.; writing—review and editing, Á.F.-A., M.C.-R., and E.G.-J.; visualization, Á.F.-A. and J.M.C.; supervision, E.G.-J.; project administration, E.G.-J.; funding acquisition, E.G.-J. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Andalusia 2014–2020 European Regional Development Fund (ERDF) Operative Program, grant number B-AGR-287-UGR18.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data is contained within the article.

Acknowledgments: The results of this review belong to Ángel Fernández-Aparicio's doctoral thesis; he is enrolled in the Official PhD Program of Clinical Medicine and Public Health (University of Granada, Spain).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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ESTUDIO IV

Referencia: Fernández-Aparicio, Á., Perona, J. S., Castellano, J. M., Correa-Rodríguez, M., Schmidt-RioValle, J., & González-Jiménez, E. (2021). Oleanolic acid-enriched olive oil alleviates the interleukin-6 overproduction induced by postprandial triglyceride-rich lipoproteins in THP-1 macrophages. *Nutrients*, *13*(10), 3471. https://doi.org/10.3390/nu13103471

Indicios de calidad: Impact Factor (2021) = 6,706. Edition: Science Citation Index Expanded. Category and rank: Nutrition & Dietetics (15/90; Q1/T1)

IV. Oleanolic acid-enriched olive oil alleviates the interleukin-6 overproduction induced by postprandial triglyceride-rich lipoproteins in THP-1 macrophages

Abstract

Oleanolic acid (OA), a triterpene that is highly present in olive leaves, has been proposed as a component of functional foods for the prevention of metabolic syndrome, due to its anti-inflammatory activity. We analyzed the effects of OA on inflammatory parameters and signaling proteins in LPS-stimulated THP-1 macrophages. Thus, THP-1 macrophages were incubated with LPS for 48 h after pretreatment with OA at different concentrations. Pretreatment with OA was significantly effective in attenuating IL-6 and TNF-α overproduction induced by LPS in macrophages, and also improved the levels of AMPK-α. We also evaluated the effects of human triglyceride-rich lipoproteins (TRLs) derived from individuals consuming an OA-enriched functional olive oil. For this purpose, TRLs were isolated from healthy adolescents before, 2 and 5 h postprandially after the intake of a meal containing the functional olive oil or common olive oil, and were incubated with THP-1 macrophages. THP-1 macrophages incubated with TRLs isolated at 2 h after the consumption of the OA-enriched olive oil showed significant lower levels of IL-6 compared to the TRLs derived from olive oil. Our results suggest that OA might have potential to be used as a lipid-based formulation in functional olive oils to prevent inflammatory processes underlying metabolic syndrome in adolescents.

Keywords: oleanolic acid; olive oil; functional foods; postprandial trial; human triglyceride-rich lipoproteins; metabolic syndrome; obesity; insulin resistance; adolescents; THP-1 macrophages

1. Introduction

Metabolic syndrome (MetS) is a worrying health public problem that affects approximately 31% of the world population, and it is expected that its prevalence will increase by about 53% by 2035 [1,2]. The International Diabetes Federation defines central obesity as the unique fixed component of MetS [3]. In fact, inadequate control of abdominal obesity and insulin resistance (IR), among others, not only lead to the development of Type 2 Diabetes Mellitus (T2DM) and cardiovascular diseases, but also to other clinical disorders, such as oxidative stress, non-alcoholic fatty liver disease and hepatic steatosis [4–6].

Obesity is characterized by an excess of fat accumulation in the adipose tissue [7], in which the inflammatory response is closely linked to the development of IR [8]. Chronic inflammation of adipose tissue in obesity causes an impairment in the polarization of adipose tissue macrophages (ATMs), resulting in a higher presence of the M1 proinflammatory phenotype [8–10]. As a consequence, ATMs are involved in the release of proinflammatory cytokines, such as interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) [11,12]. These proinflammatory cytokines are positively regulated by the nuclear transcription factor kappa B (NF-κB), which is translocated to the cell nucleus after activation of IκB kinase (IKK). Another kinase with an important role in NF-κB function is AMP-activated protein kinase (AMPK), in which its activation inhibits NF-κB signaling. Both AMPK and IKK also participate in insulin signaling through acting on the insulin receptor substrate [13]. These pathways that are associated with obesity-induced inflammation have been considered as potential targets in the research of new bioactive compounds or drugs for treating obesity and IR, two of the main factors underlying MetS.

Sedentary and unhealthy lifestyles, both in adults and in adolescents, makes the search of new preventive and therapeutic interventions to fight MetS necessary [14]. Moreover, the worrying comorbidities derived from MetS together with the expensive tendency of the pharmacological interventions used for preventing and controlling this syndrome jeopardize the stability of the healthcare systems [15]. The Seven Country

Study accomplished in the 1950s first proved the cardioprotective abilities and health benefits of the Mediterranean diet [16]. Since then, intervention studies based on the Mediterranean diet have gained importance for their beneficial effects on the components of MetS, such as obesity, hyperlipidemia, hyperglycemia and hypertension [17]. The key constituent of the Mediterranean diet is virgin olive oil, which possesses anti-inflammatory and antioxidant properties that are not only due to the high levels of oleic acids, but also to the presence of minor compounds, such as phenolic derivatives, phytosterols, carotenoids, tocopherols and terpenoids [18,19].

There is increasing interest in performing clinical trials to study the preventive and therapeutic effects of virgin olive oil minor compounds on MetS. Sánchez-Rodríguez et al. [20] have recently presented the results of a randomized controlled trial with 51 healthy individuals who consumed a functional olive oil enriched in their phenolics and triterpenes for three weeks, showing that this dietary intervention decreased urinary DNA oxidation and plasma inflammatory biomarkers, in comparison with the intake of a standard virgin olive oil. In addition, the PREDIABOLE study, a randomized controlled trial with 176 prediabetic patients demonstrated that the consumption of an oleanolic acid-enriched olive oil for 30 months improved insulin resistance and diminished the risk of developing T2DM by 55% [21]. Oleanolic acid (OA; (3β)-3-hydroxyolean-12-en-28-oic acid) is a pentacyclic triterpene widely distributed in the plant kingdom, which is significantly abundant in the fruit and leaf from the olive tree [22], and is therefore a natural component of virgin olive oil [23]. This triterpene possesses anti-inflammatory, anti-oxidant, anti-atherosclerotic and anti-hypertensive activities [22,24], but the pathways involved in these properties are not fully understood. However, it is suggested that OA could be entangled in the repression of the NF-κB and in the activation of the nuclear factor erythroid 2–related factor 2 (Nrf2), two important transcription factors that modulate the inflammatory status in insulin resistance [25,26].

Therefore, a better understanding of the action of OA in a metabolic syndrome context is still necessary. For this reason, our study aimed to evaluate the effects of OA on the production of the pro-inflammatory cytokines and on signaling proteins in LPS-stimulated THP-1 macrophages. We also studied for the first time the potential capacity

of OA to be used in functional foods in order to prevent IR and MetS, by assessing the effects of postprandial TRLs obtained from healthy adolescents after the intake of a meal containing an OA-enriched olive oil.

2. Materials and Methods

2.1. Materials

A scanning multi-well spectrophotometer (Multiskan spectrum, Thermo Fisher Scientific, Waltham, MA, USA) was utilized to measure absorbances. RPMI 1640 medium, fetal bovine serum (FBS), penicillin, streptomycin and phosphate buffered saline (PBS) were purchased from Biowest (Nuaillé, France). Lipopolysaccharide (LPS) (from E. coli 0111:B4) and phorbol 12-myristate-13-acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. OA Obtaining and Elaboration of the Functional Olive Oil

2.2.1. OA Obtaining

OA was obtained from olive tree leaves following the method developed by Albi et al. [27]. In brief, OA was extracted from leaves using 96% ethanol (20 mL/g) at room temperature. To facilitate the formation of OA crystals, the extract was filtered and vacuum concentrated. OA crystals were washed with cold 96% ethanol (5–7 ºC) and filtered to remove pigment traces and other possible contaminants. Finally, the crystals were heated at 165 ºC, and homogenized to a powder. OA purity was determined by gas chromatography.

2.2.2. Analysis of OA by Gas Chromatography (GC)

Prior to derivatization, 100 µL of a methanolic solution of betulinic acid (0.5 mg/mL) was added as an internal standard to 100 µL of the OA-containing sample. The mixture was evaporated to dryness under a N2 stream, and the residue dissolved in 200 µL of the silylating reagent (BSTFA+1%TMCS in pyridine).

OA was identified and quantified using a coupled GC–mass spectrometry detector (GC-MS) QP2010 Ultra (Shimadzu Europa GmbH, Duisburg, Germany) with an AOC-20i autosampler, an ion source of electron impact and a quadrupole detector. The analysis was carried out in splitless mode, with an injector temperature of 290°C. Helium was used as a carrier gas at 53.1 kPa and of 1mL/min. The oven temperature program was as follows: initial temperature, 50°C/1min; 50–200°C at 40°C/min; 200–280°C at 10°C/min; and finally held for 2 min. Total run time was 14.75 min. The MS conditions were: interface temperature, 280°C; ion source temperature, 220°C; electron impact, 70 eV; acquisition mode, scan (m/z 50–600). OA was identified by comparing the retention times and abundance ratios of two fragments ions (203 and 189m/z).

2.3. Elaboration and Characteristics of the Functional Olive Oil

The functional olive oil used in the postprandial trial was elaborated by adding OA at a concentration of 610 mg/kg to a commercial olive oil. No adjuvants were added. The control olive oil was the same as that of the functional olive oil, but without adding OA. Green-colored flasks were used for bottling of the functional and non-functional olive oils, and were labeled with a bicolor code system for blinding. Each dose of the functional oil consisted of 55 ml, containing 30 mg of OA.

Representative chemical compositions of the oils are shown in Table S1 (see the supplementary materials). OA concentrations in the control and in the functional olive oil were 3.8 ± 0.1 mg/kg oil and 610.4 ± 16.2 mg/kg oil, respectively.

2.4. Preparation of OA Used in the LPS-Stimulated THP-1 Macrophages

When OA was directly administered to LPS-stimulated THP-1 macrophages, it was dissolved in DMSO. To achieve the concentrations of 0.5, 1, 5, 10 and 25 μM, increased volumes of this solution were employed. Thus, for higher OA concentrations, higher volumes of DMSO were added to cells.

2.5. Postprandial Trial

2.5.1. Design and Implementation of the Study

We performed a randomized, controlled and double-blind postprandial trial with adolescents to obtain postprandial TRLs as circulating OA vehicle when the triterpene was administered as functional olive oil. The study was carried out in accordance with the Helsinki declaration and authorized by the research ethics committee of the University Hospitals Virgen de la Macarena and Virgen del Rocío in Seville, Spain. This trial was registered at ClinicalTrials.gov (Identifier: NCT05049304).

2.5.2. Setting and Participants

Twenty-two healthy and normal weight adolescents of both sexes (aged 16–17 years-old) participated in the postprandial trial. The participants were recruited from a high school in the province of Granada (Spain), and their health status was checked through a complete biochemical analysis. Participants did not suffer from digestive, metabolic or oncologic disorders, or any other pathology. For their inclusion in the trial, it was also required that their parents granted the written consent to the approved protocols, after being conveniently informed both orally and by written means. The fieldwork was performed in the high school. Eleven adolescents were randomly assigned to the intervention group (OA-enriched functional olive oil), and the other eleven participants to the control one (the same commercial olive oil not enriched in OA).

The determination of the sample size for the postprandial trial was calculated considering plasma OA concentration as the quantitative variable. As there are no population data, a cohort of 60 participants from the control group of the PREDIABOLE study was used. A confidence of 95% (α risk= 0.05; 1.645) and a power of 90% ($z\beta$=1.282), were used, yielding a sample size of 15 participants. Taking into account possible losses, sample size was finally augmented to 22 participants.

2.5.3. Anthropometric Assessment and Body Composition Analysis

The intervention was carried out after 12 hours of overnight fasting. Anthropometric measurements were performed individually according to the

recommendations of the International Society for the Advancement of Kinanthropometry (ISAK) [28] in a classroom provided by the high school to guarantee the privacy of each adolescent. We used a body composition analyzer (TANITA® model BC-418MA, West Drayton, United Kingdom) to measure the body weight and the percentage of body fat. Height was measured twice with a ± 5 mm precision with a Seca 214* portable stadiometer, and the average of the two values was used in the analysis. The body mass index (BMI) was obtained by dividing the body weight in kilograms by the height in meters squared. We measured waist circumference (WC) in the middle distance from the lowest rib to the upper border of the iliac crest at the end of a normal exhalation using a Seca automatic roll-up measuring tape (precision of 1 mm). Blood pressure (BP) levels were determined using a calibrated aneroid sphygmomanometer and a Littmann® stethoscope (Saint Paul, MN, USA), according to international recommendations [29]. Adolescents self-reported their pubertal development based on the Tanner stages [30].

2.5.4. Intervention

On the trial day, and after 12 hours of overnight fasting, blood samples were drawn from the cubital vein. Subsequently, participants took an experimental breakfast that consisted of 150 mL skimmed milk, a tablespoon of crushed tomato, three slices of whole meal bread, and 55 mL of the assigned oil (equivalent dose of OA 30 mg, in the case of the functional olive oil). After eating the respective breakfasts, aliquots of cubital blood were drawn at 2 and 5 hours in the postprandial period. During that time, free access to water intake was allowed.

2.5.5. Biochemical Determinations and Isolation of TRLs

Aliquots of cubital blood samples were drawn before, and at 2 and 5 hours after the breakfast intake. All blood samples were centrifuged at 3000 rpm for 5 minutes at 20 ºC, and aliquots of serum were frozen until their use for determining biochemical parameters at baseline or isolating TRLs.

Serum levels of fasting glucose, high-density lipoprotein cholesterol (HDL-c), total cholesterol (TC) and triglycerides (TG) were measured by means of enzymatic

colorimetric methods. The Friedewald equation ((LDLC) = (Total Cholesterol) – (HDL-c) − ([TG]/5)) was used for estimating low-density lipoprotein cholesterol (LDL-c) [31]. Fasting insulin serum levels were determined using an ELISA kit (Diaclone, Besançon, France) according to the manufacturer's instructions. HOMA-IR was calculated using the following equation: fasting glycemia (mmol/L) x fasting insulin (mU/L)/22.5.

Once thawed, 4-4.5 mL of serum was layered under 6 mL of 0.9% NaCl solution (d= 1.006 Kg/L), and ultracentrifuged at 39.000 rpm for 16 hours at 12ºC in a preparative ultracentrifuge Beckman Coulter L90K (Beckman Instruments, Inc., Palo Alto, CA, USA). Finally, the upper off-white phase of each tube, containing TRLs, was collected.

2.6. THP-1 Monocytes Culture

THP-1 monocytes (3-8 x 105 cells/mL) were cultured in RPMI-1640 medium supplemented with antibiotics (100 U/mL streptomycin and 100 U/mL penicillin) and 10% heat-inactivated fetal bovine serum (FBS), under a 100% humidified atmosphere of air +5% CO2 at 37 ºC. Cells were passaged every 2 to 3 days to maintain growth.

2.7. Differentiation of THP-1 Monocytes into Macrophages

THP-1 monocytes were seeded at 1x106 cells/mL in cell culture dishes, and incubated with PMA (0.2 μg/mL) at 37ºC in 5% CO2–95% air to induce their differentiation into macrophages. After incubation for 72 h, cells were washed with preheated RPMI-1640 medium supplemented with antibiotics to eliminate the excess of PMA and undifferentiated monocytes. The confluency of adherent cells was 70–80% in all cell-culture experiments, and cell viability was > 95%. Finally, THP-1 macrophages were maintained with RPMI-1640 medium supplemented with antibiotics for 24 h at 37ºC in 5% CO2–95% air, until the addition of the experimental (OA or TRL) or vehicle.

2.8. Cell Viability Assay in THP-1 Macrophages Pretreated with OA

THP-1 macrophages were pretreated with OA at different concentrations (0.5 to 25 μM) for 1 hour. LPS was added to OA-pretreated THP-1 macrophages for 48 hours at 37ºC in 5% CO2–95% air. DMSO was added to control cells. The XTT assay was

performed following the instructions of the manufacturer's kit (Canvax Biotech, Córdoba, Spain) to determine the cell viability. Absorbance was measured at 450 nm. Cell viability was calculated using the equation: (mean OD treated cells/mean OD control cells) x 100.

2.9. Determination of Proinflammatory Cytokines and Kinases in LPS-Stimulated THP-1 Macrophages Pretreated with OA

THP-1 macrophages were pretreated for 1 hour with OA (0.5, 1, 5, 10 and 25 μM), followed by LPS (100 ng/mL) for 48 hours at 37º C in 5% CO2–95% air. DMSO was added to control cells. All experiments were performed in triplicate. Once the incubation period was finished, culture media were collected and the IL-6, IL-1β and TNF-α levels were measured using ELISA kits (Diaclone, Besançon, France; RayBiotech, Norcross, GA, USA; and Cloud-Clone, Katy, TX, USA, respectively). Furthermore, THP-1 macrophages were washed with PBS, scraped off the plates and lysed by sonication twice for 50 seconds at 50 W. The protein content of the lysate was measured using the Bradford protein assay [32]. Cell lysates were used for measuring the presence of Akt and AMPK-α with ELISA kits (ThermoFisher, Carlsbad, CA, USA). ELISA assays were performed according to the manufacturer's instructions and absorbance was read at 450 nm.

2.10. TRL Stimulation of THP-1-Derived Macrophages and Determination of Proinflammatory Cytokines and Kinases

Appropriate volumes of TRL to achieve a concentration of 25 μg apoB/total protein were added to THP-1 macrophages. PBS (50 μL per mL of cell culture) was added to macrophages as a vehicle in triplicate. After 48 hours of incubation at 37 ºC in 5% CO2–95% air, culture media were collected and THP-1 macrophages were washed with PBS, scraped off the plates and lysed twice for 50 seconds at 50 W by sonication. The Bradford protein assay [32] was performed to measure the protein content of the lysates.

The levels of IL-6, IL-1β and TNF-α produced in the media were measured by ELISA kits (Diaclone, Besançon, France; RayBiotech, Norcross, GA, USA; and Cloud-Clone, Katy, TX, USA). In addition, ELISA kits were used to analyze the content of Akt, AMPK-α and NF-κB (ThermoFisher, Carlsbad, CA, USA for Akt and AMPK-α; and Cloud-Clone, Katy, TX, USA for NF-κB) using the cell lysates. Absorbance was read at 450 nm.

2.11. Oil Red O Staining

THP-1 macrophages were incubated with TRLs at the same conditions as described in Section 2.10. After incubation, macrophages were washed with PBS and treated with 1 mL of isopropanol 60% for 2 minutes. Once isopropanol was removed, 1 mL of Oil Red O (0.2% (m/v) in 40% isopropanol/water (v/v)) was added and cells were incubated at room temperature for 10 minutes. Oil Red O was removed, and cells were washed with PBS. Finally, 2 mL of glycerol 30% in water (v/v) was added for preservation for 3–4 days. An inverted microscope Motic AE21 Series (Barcelona, Spain) coupled to a Moticam 2500 5.0M Pixel Live Resolution camera (Motic, Barcelona, Spain) was used for microphotography.

2.12. Intracellular Fatty Acid Composition

For the analysis of fatty acids accumulated intracellularly, THP-1 macrophages were incubated at the same conditions as described in Section 2.10. After incubation, cells were washed with PBS, scraped off the plates and lysed twice for 50 seconds at 50 W by sonication. A direct method of fatty acid methylation was carried out [33]. In brief, 10 μL of 15:0 fatty acid at 1mg/mL was added as an internal standard to 50 μL of cell lysates in a glass tube. In total, 1.5 mL of methanolic HCl 3N was added and shaken for 30 seconds. Tubes were heated at 85ºC for 45 minutes, and after cooling down to room temperature, 0.5 mL of hexane was added to each glass tube, shaken for 30 seconds and centrifuged at 1000 rpm for 5 minutes twice. Finally, the upper phase of each tube was collected, evaporated under a stream of nitrogen and concentrated to 75 μL of hexane. An aliquot of 1 μL was injected in a model 5890 series II GC (Hewlett-Packard Co, Avondale, AZ, USA) equipped with a flame ionization detector and a capillary silica column Supelcowax

10 (Supelco Co., Bellefonte, PA, USA) of 60 m length and 0.25 mm internal diameter. Fatty acids were identified by comparison of their retention times against those of standards and quantified by internal standard using peak area integration.

2.13. Statistical analysis

The data were expressed as mean ± standard error of mean (SEM), except olive oil composition, which was expressed as mean ± standard deviation (SD). Values of Akt, AMPK-α, NF-κB and intracellular fatty acids were normalized by the protein content of the lysates. ANOVA followed by Tukey's test was used for assessing the mean differences among control and experimental groups in the cell experiments. Mean differences of the baseline characteristics of adolescents were assessed by Student's t-test. Statistical analyses were carried out using IBM SPSS Statistics 24.0 (IBM Corp., Armonk, NY, USA) software. GraphPad Prism 6 (San Diego, CA, USA) was used for figures.

3. Results

3.1. OA Effects on LPS-Stimulated THP-1 Macrophages

3.1.1. Effects of OA Pretreatment on THP-1 Cell Viability

The XTT assay was performed to determine cell viability when treated with OA. Cell viability was not threatened by the presence of OA at all concentrations assayed with a proportion of active cells that remained always above 87%. It was found that both DMSO and LPS, at the applied concentrations, did not affect the viability of THP-1 cells (data are shown in Figure S1).

3.1.2. IL-1β, IL-6 and TNF-a Release in LPS-Stimulated THP-1 Macrophages Pretreated with OA

Our results show significant higher levels of IL-1β in LPS-stimulated macrophages pretreated with OA at 10 μM and 25 μM, in comparison to lower OA concentrations (Figure 1a). IL-1β levels at the highest OA concentrations were similar to those of non-stimulated cells treated with DMSO only. Concerning IL-6 (Figure 1b) and TNF-α (Figure 1c), a significant increase in the release of these cytokines in THP-1 macrophages

treated with LPS was observed in comparison to THP-1 macrophages only exposed to DMSO. However, pretreatment with OA attenuated the release of IL-6 and TNF-α, especially at 25 μM.

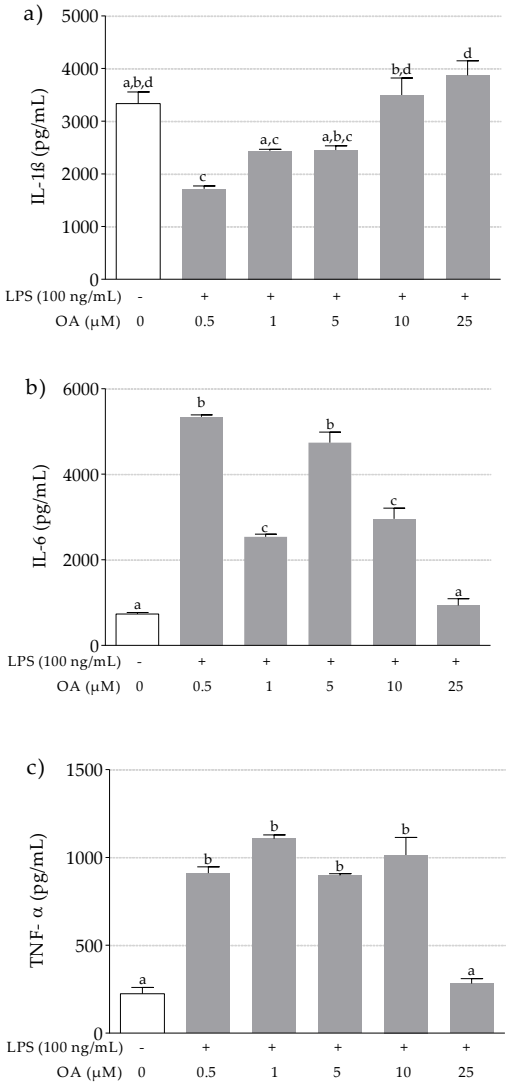

Figure 1. Effects of OA on proinflammatory cytokines production by THP-1 macrophages induced by LPS. Control cells were incubated with DMSO. Experiments performed in triplicate. (a) IL-1β; (b) IL-6; (c) TNF-α. Different letters indicate significant difference ($p < 0.05$) by one-way ANOVA analysis and Tukey's post hoc test.

3.1.3. Levels of AMPK-a and Akt in LPS-Stimulated and OA-Pretreated THP-1 Macrophages

AMPK-α levels in LPS-stimulated THP-1 cells increased dose-dependently up to 10 μM. In contrast, at 25 μM, the concentration was lower than at 10 μM (Figure 2a). The LPS-stimulated THP-1 macrophages pretreated with 0.5 and 1 μM of OA had significantly lower levels of AMPK-α in comparison to non-LPS-stimulated THP-1 macrophages treated with DMSO. Therefore, 10 μM was the concentration at which OA was more effective in attenuating the dephosphorylation of AMPK-α induced by LPS. No significant differences were observed in the levels of Akt (Figure 2b).

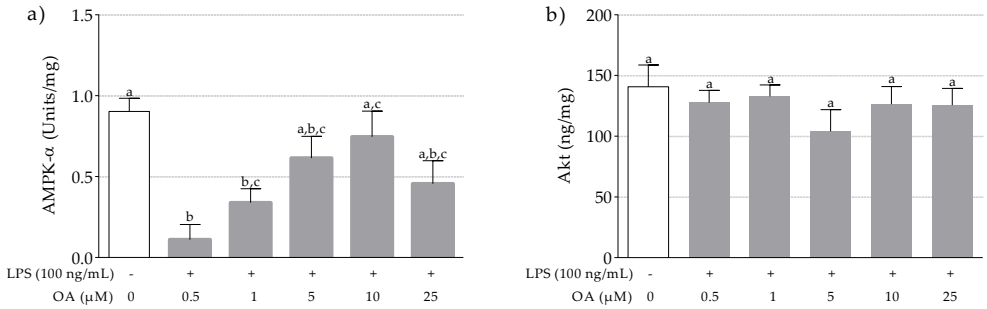

Figure 2. Effects of OA on AMPK-α (a) and Akt (b) in THP-1 macrophages induced by LPS. Control cells were incubated with DMSO. Experiments performed in triplicate. Different letters indicate significant difference ($p < 0.05$) by one-way ANOVA analysis and Tukey's post hoc test.

3.2. Postprandial Clinical Trial. Activation of THP-1 Macrophages with Human TRL

3.2.1. Anthropometric and Biochemical Data of Adolescents at Baseline

Table S2 shows the anthropometric and biochemical characteristics of adolescents, whose data were assessed prior to the postprandial trial. All data were within normal limits.

3.2.2. Effects of Human TRLs on the Production of Proinflammatory Cytokines in THP-1 Macrophages

TRL-induced production of the proinflammatory cytokines IL-1β, IL-6 and TNF-α on THP-1 macrophages is shown in Figure 3. At the same time point, cells stimulated with 2 hour postprandial TRL in adolescents that ingested the OA-enriched olive oil produced lower levels of IL-6 than those treated with 2 hour postprandial TRL after the intake of the non-functional olive oil. Non-statistically significant differences were observed for IL-1β and TNF-α.

We did not find statistically significant differences for any variable among the different time points in the macrophages incubated with either olive oil derived TRLs or OA-enriched olive oil TRLs.

3.2.3. Effects of Human TRLs on the Levels of Kinases and NF-κB in THP-1 Macrophages

The levels of AMPK-α and Akt proteins after incubation of THP-1 macrophages with postprandial TRLs are shown in Figure 4. At the same time point, levels of Akt were higher in macrophages stimulated with 2 hour postprandial TRL of adolescents that ingested the non-functional olive oil compared to PBS. In contrast, THP-1 macrophages stimulated with 2 hour postprandial TRL after the intake of the functional olive oil presented levels of Akt similar to PBS. No significant differences in levels of AMKP-α and NF-κB were observed.

No significant differences for kinases and NF-κB were observed at the different time points in either the macrophages incubated with olive oil-derived TRLs or with OA-enriched olive oil-derived TRLs.

3.2.4. Intracellular Lipid Accumulation

Figure 5 shows microphotographs of intracellular lipids stained with Oil Red O. The lipid content was higher in TRL-treated cells, independent of the presence of OA. The fatty acid content of total lipids accumulated intracellularly by THP-1 macrophages after being incubated for 48 h in the absence (control) or presence of TRLs isolated at baseline, 2 and 5 hours after the consumption of the OA-enriched or non-functional

olive oils are shown in Table 1. A higher oleic acid (18:1) content was found in cells incubated with TRLs obtained 2 h after the intake of the experimental meals compared to control but not compared to the other time points studied. For the rest of the fatty acids, no differences were found compared to either the control or other time points.

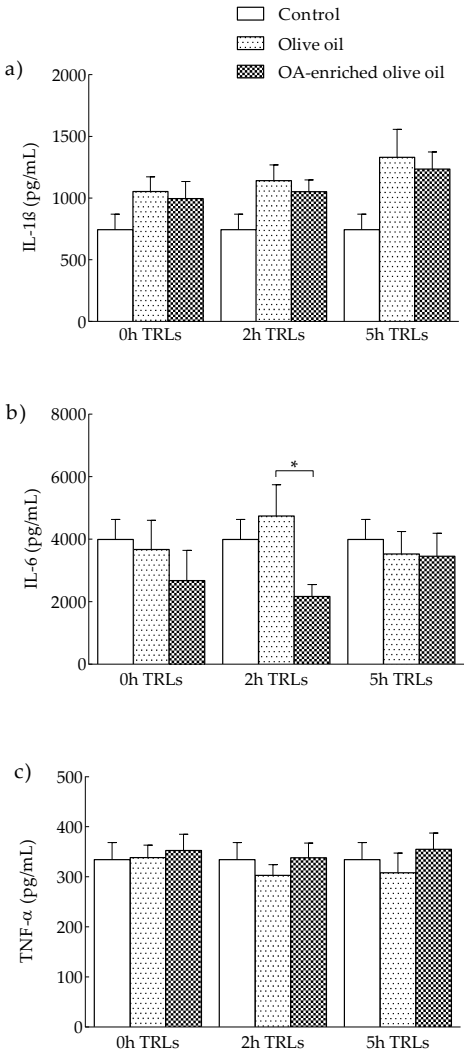

Figure 3. TRL production of proinflammatory cytokines by THP-1 macrophages incubated with TRLs obtained from healthy adolescents before, and at 2 and 5 h after the intake an OA-enriched olive oil or non-functional olive oil. Control cells incubated with PBS. (a) IL-1β; (b) IL-6; (c) TNF-α. * $p < 0.05$.

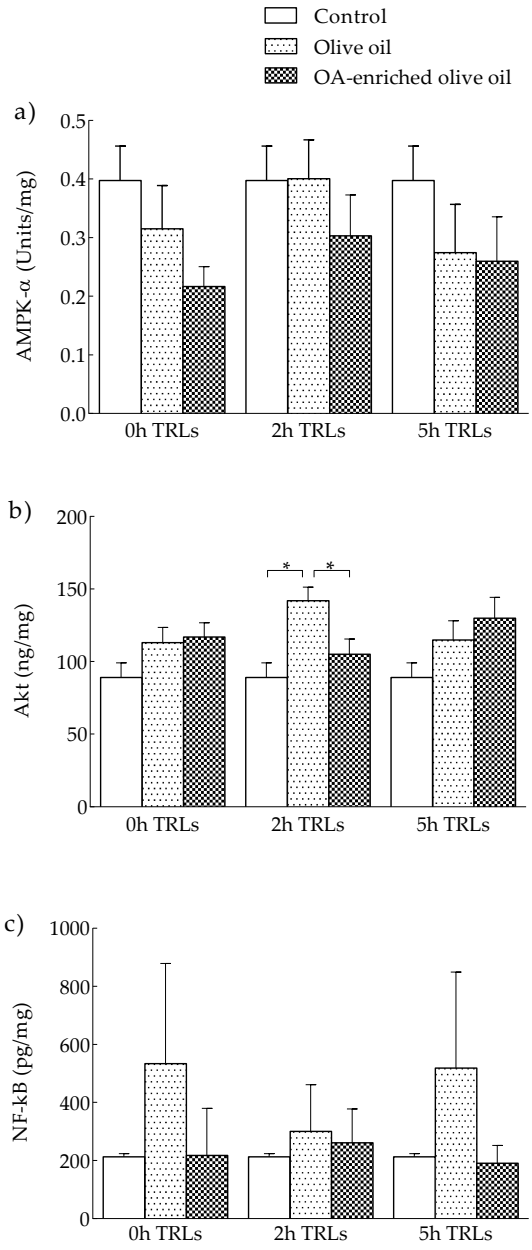

Figure 4. Levels of AMPK-α (**a**), Akt (**b**) and NF-κB (**c**) in THP-1 macrophages incubated with TRL obtained from healthy adolescents before, and at 2 and 5 h after the intake an OA-enriched olive oil or non-functional olive oil. Control cells incubated with PBS. * $p < 0.05$.

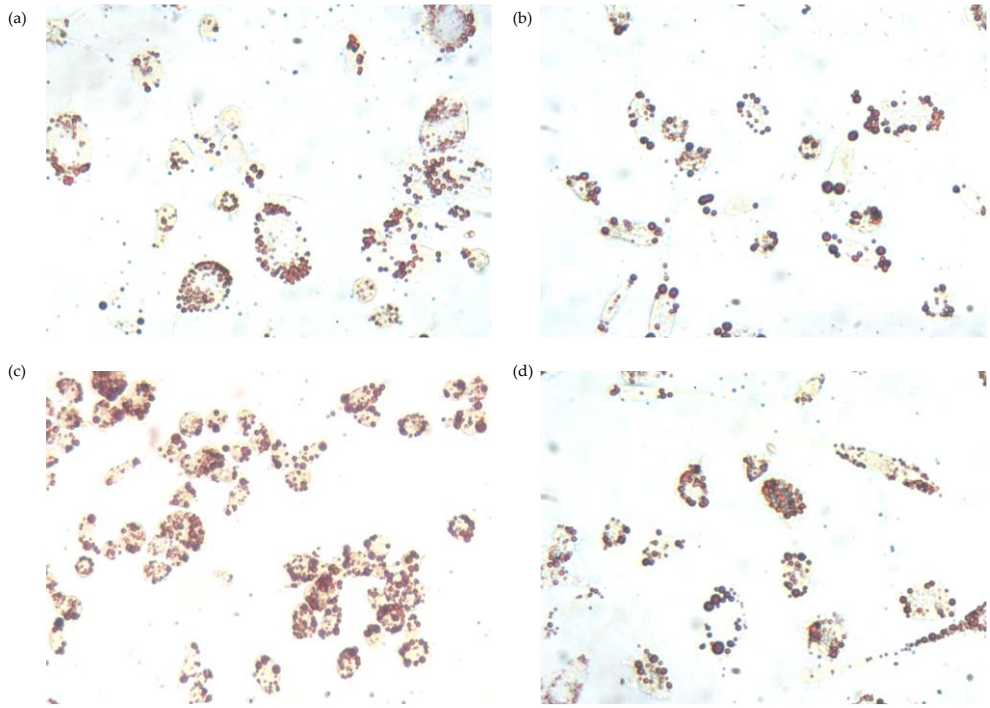

Figure 5. Oil red O microphotographs of THP-1 macrophages incubated for 48 h in the presence or absence (control) of TRLs obtained before, and at 2 and 5 h after the consumption of the olive oils. **(a)** Control; **(b)** 0h TRL; **(c)** 2h TRL; **(d)** 5h TRL.

Table 1. Intracellular fatty acid composition (μg/mg cell protein) of total lipids in THP-1 macrophages after incubation with TRLs derived from two different olive oils.

Fatty Acids	Control	0 Hour		2 Hours		5 Hours	
		Olive Oil	OA-Enriched Olive Oil	Olive oil	OA-Enriched Olive Oil	Olive Oil	OA-Enriched Olive Oil
12:0	41.46 ± 3.41	41.68 ± 4.26	38.93 ± 3.23	42.10 ± 5.51	41.02 ± 2.21	35.63 ± 4.93	40.06 ± 6.80
14:0	45.00 ± 3.33	48.13 ± 4.26	42.41 ± 2.38	48.18 ± 4.30	40.71 ± 3.10	40.94 ± 2.95	41.41 ± 4.56
16:0	95.68 ± 16.74	158.11 ± 26.64	151.96 ± 26.97	171.74 ± 19.10	148.57 ± 24.76	130.98 ± 20.06	180.53 ± 48.50
18:1	60.16 ± 6.51	119.58 ± 24.85	104.55 ± 22.00	126.60 ± 13.02 *	119.88 ± 19.83	102.02 ± 14.42	145.63 ± 44.69
18:2	46.68 ± 6.07	45.22 ± 4.12	40.83 ± 3.43	56.26 ± 11.56	41.65 ± 3.45	37.49 ± 2.99	40.59 ± 4.35
20:4	41.74 ± 1.30	51.22 ± 16.64	63.37 ± 17.74	46.04 ± 4.73	43.44 ± 6.23	42.60 ± 11.64	35.02 ± 1.65
Others	79.37 ± 9.00	ND	59.97 ± 26.24	ND	ND	ND	155.12 ± 0.57

Data are presented as mean ± SEM. Controls performed in triplicate. Experimental groups (n = 11). * $p < 0.05$ vs. control. Statistical differences were analyzed among control, olive oil and the OA-enriched olive oil at the same time point and at different time points. ND, not detected.

4. Discussion

In the present work, we evaluated the ability of OA to attenuate the overproduction of proinflammatory cytokines and to modulate signaling pathways in THP-1 macrophages stimulated by LPS, as well as its activity when forming part of human TRLs obtained after the intake of an olive oil enriched in the triterpene. The main findings presented here are (i) pretreatment with OA attenuated the LPS-induced overproduction of pro-inflammatory cytokines, especially of IL-6, but also TNF-α, in THP-1 macrophages; (ii) levels of AMPK-α were increased by OA in a dose-dependent manner in LPS-stimulated THP-1 macrophages; and (iii) OA-enriched olive oil alleviated the levels of IL-6 and Akt in THP-1 macrophages stimulated with 2 h postprandial TRLs.

LPS is widely used in experiments to induce an inflammatory response, and numerous studies have shown an overproduction of IL-1β, IL-6 and TNF-α in THP-1 cells stimulated by LPS [34–36]. In our study, the activation of THP-1 macrophages with LPS produced an abundant release of IL-6, IL-1β and TNF-α in the cell culture supernatants. However, pretreatment with OA attenuated IL-6 levels, especially at 25 μM. At that concentration, OA was also able to reduce TNF-α release by the cells. Similar results were reported by Castellano et al. [37] in LPS-activated BV-2 microglia, where the overproduction of IL-6 was attenuated in a dose-dependent manner by OA. In contrast, unexpected results were reported in our study for LPS-induced production of IL-1β, since a dose-dependent increase of this cytokine by OA was observed. Interestingly, LPS-stimulated THP-1 macrophages pretreated with the highest concentrations of OA released similar values of IL-1β than the control. It has been reported that DMSO induces IL-1β secretion in THP-1 macrophages by activating the enzyme caspase-1 [38,39]. This could partly explain our findings, since OA was dissolved in DMSO, and the cells that received the higher doses of OA also received higher DMSO volumes. Nevertheless, DMSO is a common solvent for water-insoluble substances, such as OA, in this kind of experiment [39,40].

The effects of OA on proinflammatory cytokines produced by THP-1 macrophages, especially IL-6, might be explained by the modulatory role of AMPK on

inflammation processes. In order to become active, AMPK needs to be phosphorylated at its α-subunit (Thr172 residue) [41,42]. It has been reported that LPS induces the dephosphorylation of AMPK [42]. Therefore, to achieve a better understanding of the anti-inflammatory ability of OA in THP-1 macrophages, the levels of phosphorylated AMPK-α were measured. A significant reduction of LPS-induced dephosphorylation of AMPK-α was observed at 10 μM of OA. In line with our study, Liu et al. [43] reported that δ-oleanolic acid could stimulate the phosphorylation of AMPK in THP-1 macrophages. In the same way, Matumba et al. [44] reported an increase in the AMPK gene expression and a reduction of IL-6 and TNF-α gene expression in high-fructose diet-fed rats treated with OA. Furthermore, the application of an OA derivative, 3-acetyl-oleanolic acid, to rats fed with a high-fructose diet has been shown to significantly increase the phosphorylation of AMPK in the liver tissues [45]. Therefore, OA seems to have a key role in the alleviation of inflammatory status through regulating AMPK phosphorylation.

Indeed, AMPK is believed to be one of the main mechanisms of the biological activity of OA, as well as other triterpenic compounds. OA modulates glucose uptake, fatty acid oxidation in the muscle and fatty acid synthesis and gluconeogenesis in the liver by stimulating AMPK phosphorylation [46]. Another reported signaling pathway underlying the metabolic effects of OA is PI3K/Akt. For instance, OA and its derivatives have been shown to stimulate Akt in vascular smooth muscle cells [47], and to relieve inflammation in different experimental models [48–50]. However, not all OA derivatives have the same inflammatory effect through Akt. Jin et al. [51] prepared eleven oxooleanolic acid derivatives using OA as starting compounds for structural modification, and found that only two of them exerted significant anti-inflammatory effects on BV-2 cells by the activation of NF-κB, MAPKs and PI3K/Akt. In addition, in bone marrow macrophages, OA showed no significant inhibitory effects on receptor activator of nuclear factor kappa-B ligand-activated expression of NF-κB, p38, JNK and Akt [52]. However, in our study, OA was unable to significantly reduce Akt levels. There are three homologous Akt isoforms that are activated by similar mechanisms, Akt1, Akt2 and Akt3 [53]. In THP-1 macrophages, Shiratsuchi and Basson [54] found that Akt2 but

not Akt1 and Akt3 mediated phagocytosis stimulated by pressure. Since we measured total Akt, the absence of a clear effect might be related to opposing roles of the different isoforms. In the same way, a Mg-based alloy inhibited the inflammatory response of THP-1 cell-derived macrophages through the inhibition of Akt1 but not total Akt [55]. Therefore, further studies are needed to clarify the participation of Akt isoforms in the regulation of proinflammatory cytokines in THP-1 macrophages by OA.

Postprandial TRL participates in the atherosclerotic process through promoting foam cell formation from macrophages, in which the secretion of pro-inflammatory substances plays an important role [56,57]. The induction of THP-1 macrophages in our study with 2 h postprandial TRLs derived from the OA-enriched olive oil showed significant lower levels of IL-6 but not IL-1β and TNF-α, compared to the TRLs derived from olive oil. Our results have some similarities with the study performed by Graham et al. [58] with postprandial TRLs from healthy adults. They reported lower levels of IL-6 in THP-1 cells incubated with 4 h TRLs derived from pomace olive oil, which was attributed to the presence of OA in the oil. In addition, and in agreement with our results, chylomicron remnants, a form of TRL, but not native chylomicrons or oxLDL, did not increase IL-1β and TNF-α in vascular smooth muscle cells, which are also involved in atherogenesis, despite increased monocyte chemoattractant protein (MCP)-1 mRNA expression [59,60].

The potential role of OA might be due to its repressive action on NF-κB, which has been shown to be activated by TRLs [61]. Although we did not find significant influences of TRL on NF-κB, levels of this factor were slightly lower in macrophages treated with postprandial TRL derived from OA-enriched olive oil. The effect of TRLs on NF-κB is highly dependent upon their fatty acid composition. De Pascale et al. [62] used lab-made TRLs to induce foam cell formation in PMA-activated THP-1 macrophages, and found a lowering effect on the NF-κB binding of polyunsaturated fatty acids, but not by monounsaturated fatty acids. Since the TRLs employed in the present study were obtained after the intake of olive oil, which is rich in monounsaturated fatty acids, the lack of a statistically significant effect of these particles on NF-κB might be related to their fatty acid composition, with no intervention of OA. In contrast, at 2 h,

TRLs obtained from the control olive oil significantly increased Akt levels, which were ameliorated when these lipoproteins were isolated after the intake of the OA-enriched oil. The application of postprandial very low-density lipoproteins (VLDL) to peripheral blood mononuclear cells and THP-1 monocytes by den Hartigh et al. [63] produced an overexpression of Akt phosphorylation, which, in contrast to our study, was accompanied by increased gene expression levels of TNF-α and IL-1β. Unfortunately, these authors did not measure IL-6 in the culture medium.

The intracellular lipid accumulation after administering postprandial TRLs into THP-1 macrophages varies depending on several factors, including the postprandial time at which TRLs are isolated [56]. Cabello-Moruno et al. [56], Graham et al. [58], Perona et al. [64] and Cabello-Moruno et al. [65] have repeatedly showed that the intracellular lipid composition is very dependent on the composition of TRLs, which in turn, reflects that of the ingested oils. Nevertheless, we previously demonstrated that minor components of pomace olive oil can also modify postprandial TRL composition and the clearance of TG molecular species of postprandial TRL [66]. Therefore, it was reasonable to suggest that the presence of OA in TRL would affect the intracellular lipid composition of THP-1 macrophages. However, we only found slight changes in the oleic acid content of intracellular lipids. This is, in fact, in agreement with previous reports. In the study by Graham et al. [58], no differences were found in the intracellular lipid content when THP-1 macrophages were incubated with TRLs obtained 2 h or 4 h after the intake of virgin, pomace and OA-enriched pomace olive oil.

The present study shows that OA is capable of alleviating the proinflammatory cytokines overproduction in THP-1 macrophages, probably due to the enhancement of AMPK-α levels. The effect was also observed when cells were treated with OA as part of postprandial TRLs, as obtained from the blood of adolescents after the intake of a functional olive oil enriched with the triterpene. Therefore, OA has potential anti-atherogenic activity and might be considered to be used in functional foods, at least in adolescents.

This work has some strengths and limitations. For the first time a formulation of olive oil enriched with the triterpene OA has been used in a postprandial trial performed

on a population of adolescents. TRLs isolated from adolescent blood and different concentrations of OA were supplied to THP-1 macrophages, obtaining interesting results that must be interpreted with caution, due to its difficult generalization to other cells or whole organisms. Nevertheless, our results offer new data on OA effects on signaling pathways, allowing a better understanding of the mechanisms of OA action.

5. Conclusions

In summary, pretreatment of THP-1 macrophages with OA attenuated the overproduction of proinflammatory cytokines, especially of IL-6, and enhanced the AMPK-α levels in a dose-dependent manner. Therefore, we conclude that OA has potential to treat insulin resistance due to its capability to alleviate the inflammatory response through modulating the activity of AMPK-α. In regard to the results reported on the postprandial trial, TRLs obtained from adolescents 2 hours after the ingestion of the functional olive oil led to lower levels of IL-6 and Akt in macrophages. These results suggest that OA could have the potential to be used as a lipid-based formulation in functional olive oils to prevent and treat insulin resistance, atherogenic impairments and the different metabolic factors underlying MetS in adolescents. However, further cell experiments using OA and clinical trials with OA-enriched olive oil must be performed in order to obtain more solid data to allow the future use of OA as a nutraceutical in the preventive strategies of MetS and insulin resistance.

Supplementary Materials: The following are available online at www.mdpi.com/article/10.3390/nu13103471/s1, Table S1: Composition of the olive oil used in the study, Figure S1: Cell viability in OA-pretreated THP-1 macrophages at different concentrations as measured by XTT, Table S2: Baseline anthropometric and biochemical characteristics of adolescents according to the olive oil ingested at breakfast.

Author Contributions: Conceptualization, E.G.-J. and J.S.P.; methodology, J.S.P. and J.M.C.; validation, J.S.P. and J.M.C.; formal analysis, Á.F.-A.; investigation, Á.F.-A., J.S.-R. and M.C.-R.; resources, J.S.P. and J.M.C.; data curation, Á.F.-A.; writing—original draft preparation, Á.F.-A.; writing—review and editing, Á.F.-A., E.G.-J., and J.S.P.; visualization, Á.F.-A; supervision, E.G.-J.; project administration, E.G.-J.; funding acquisition, E.G.-J. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Andalusia 2014–2020 European Regional Development Fund (ERDF) Operative Program, grant number B-AGR-287-UGR18.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the ethics committees of the University Hospitals Virgen de la Macarena and Virgen del Rocío, Seville, Spain (protocol code 0952-N-17 and date of approval: August 6th, 2017).

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

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: This manuscript is part of Ángel Fernández-Aparicio's doctoral thesis, included in the Official PhD Program of Clinical Medicine and Public Health at the University of Granada, Spain. Ángel Fernández-Aparicio is awarded with a pre-doctoral FPU grant (University of Granada, Spain). We would like to thank the school, parents and the students for participating in this study.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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ESTUDIO V

Referencia: Fernández-Aparicio, Á., et al. Analysis of triglyceride-rich lipoproteins as a possible mechanism for postprandial transport of oleanolic acid in adolescents. [Análisis de lipoproteínas ricas en triglicéridos como posible mecanismo de transporte postprandial de ácido oleanólico en adolescentes].

Estado: Pendiente de traducción y envío a revista.

V. Analysis of triglyceride-rich lipoproteins as a possible mechanism for postprandial transport of oleanolic acid in adolescents. [Análisis de lipoproteínas ricas en triglicéridos como posible mecanismo de transporte postprandial de ácido oleanólico en adolescentes]

Resumen

Introducción: La biodisponibilidad de compuestos bioactivos como el Ácido Oleanólico (AO) condiciona el éxito de su uso en cualquier intervención, dietética o farmacológica. Algunos autores han demostrado la presencia de AO en plasma sanguíneo, así como su transporte unido a albúmina. No obstante, se desconoce si se transporta formando parte de lipoproteínas ricas en triglicéridos (TRLs). Por ello, el objetivo fue determinar la presencia de AO en TRLs postprandiales extraídas de adolescentes sanos y en normopeso tras el consumo de un aceite de oliva enriquecido en AO.

Métodos: Ensayo postprandial, aleatorizado, controlado y doble ciego. Se administró a adolescentes sanos y en normopeso un aceite de oliva enriquecido en AO (n=11) o un aceite de oliva control (n=11). Se extrajeron TRLs basales, a las 2 y a las 5 horas del consumo del aceite de oliva. La presencia de AO en TRLs se analizó por espectrometría de masas (GC-MS).

Resultados: En las TRLs basales no se determinó presencia de AO. De las TRLs postprandiales extraídas a las 2 horas, se objetivó AO en 4 de los participantes que consumieron aceite de oliva enriquecido en AO, y en 2 que ingirieron el aceite control. De las TRLs postprandiales extraídas a las 5 horas, se identificó AO en 1 de los participantes que consumieron el aceite de oliva funcional, y en 2 adolescentes que ingirieron el aceite control.

Conclusiones: Se ha observado en adolescentes una tendencia de que, tras la previa ingesta de aceite de oliva funcional, el AO se transporta postprandialmente unido a TRLs humanas. Sin embargo, se necesitan realizar más estudios que confirmen estos hallazgos preliminares.

Palabras clave: Oleanolic acid; triterpenes; adolescents; postprandial; transport

1. Introducción

El olivo, también denominado científicamente como Olea Europaea L., es una planta rica en compuestos triterpénicos, principalmente de Ácido Oleanólico (AO) (Guinda et al., 2010). El AO presenta un amplia y variada actividad biológica, entre las que destacan sus efectos antidiabéticos, hipolipidémicos, antiateroscleróticos, antioxidantes, antiinflamatorios (Ayeleso et al., 2017; Castellano et al., 2013; Guinda et al., 2010), y antihipertensivos (Ayeleso et al., 2017; Rodriguez-Rodriguez, 2015). Estas propiedades podrían ser de gran interés y utilidad para el tratamiento y prevención de hipertensión arterial, hiperglucemia, trastornos del perfil lipídico y obesidad; todos ellos factores de riesgo metabólico que contribuyen al desarrollo de enfermedades crónicas (World Health Organization, 2021, 2022).

Diferentes estudios han demostrado la eficacia de los triterpenos, entre ellos el AO, tras su administración en aceite de oliva funcionales para la prevención y tratamiento de trastornos metabólicos (Fernández-Castillejo et al., 2021; Sanchez-Rodriguez et al., 2019; Santos-Lozano et al., 2019), enfermedades neurodegenerativas (Rodríguez-Morató et al., 2015), cáncer (Moral & Escrich, 2022) y enfermedades renales (Marrone et al., 2022; Noce et al., 2021). Además del estudio de la actividad biológica del AO, su biodisponibilidad y transporte postprandial son factores esenciales a conocer para garantizar su eficacia al ser usado en intervenciones dietéticas o farmacológicas (Feng et al., 2020). En esta línea, dos estudios realizados por Rada et al. (2015) y de la Torre et al. (2020), han demostrado la presencia plasmática de AO en adultos tras el consumo de aceite de oliva enriquecido en AO. Se ha informado además de la presencia de AO en proteínas plasmáticas como la albúmina (Rada et al., 2011).

Considerando que el AO es una biomolécula de carácter lipófilo y prácticamente insoluble en medios acuosos (Castellano et al., 2022), se plantea la hipótesis de que el AO podría unirse a lipoproteínas ricas en triglicéridos (TRLs) tras su absorción intestinal como mecanismo de transporte postprandial. De hecho, Espinosa et al. (2022) ha empleado previamente de manera eficaz TRLs artificiales como transportador de AO en experimentos realizados en células de la glía. Por tanto, el objetivo de este estudio fue

determinar la presencia de AO en TRLs extraídas de adolescentes sanos y con normopeso tras la ingesta de un aceite de oliva funcional enriquecido en AO.

2. Métodos

2.1. Obtención del AO y elaboración del aceite de oliva funcional

2.1.1. Obtención del AO

La extracción de AO se realizó de acuerdo al método desarrollado por Albi et al. (2001), utilizando para ello hojas de olivo y etanol al 96% (20 mL/g) a temperatura ambiente. Se filtró el extracto y se concentró al vacío para la obtención de cristales. Se utilizó etanol frío al 96 % (5–7 ° C) para lavar los cristales, y se filtraron para eliminar restos de pigmentos y otros posibles contaminantes. Posteriormente, los cristales de AO fueron calentados a 165 ºC, y homogeneizados hasta su conversión en polvo.

2.1.2. Análisis de AO por cromatografía de gases (GC)

La determinación del grado de pureza del AO obtenido se midió mediante cromatografía de gases. Se añadieron 100 µL de una solución metanólica de ácido betulínico (0.5 mg/mL) como patrón interno a 100 µL de la muestra que contenía AO. La mezcla se evaporó hasta sequedad bajo una corriente de N_2, y el residuo se disolvió en 200 µL del reactivo de sililación (N,O-Bistrifluoroacetamida, BSTFA, + 1% de Cloruro de trimetilsililo, TMCS, en piridina).

El AO se identificó y cuantificó utilizando la técnica cromatografía de gases acoplado a espectrometría de masas (GC-MS) QP2010 Ultra (Shimadzu Europa GmbH, Duisburg, Alemania) con un automuestreador AOC-20i, una fuente de iones de impacto de electrones y un detector cuadrupolar. El análisis se realizó en modo splitless, con una temperatura del inyector de 290°C. Se utilizó helio como gas portador a 53.1 kPa y de 1mL/min. El programa de temperatura del horno fue el siguiente: temperatura inicial, 50°C/1min; 50-200°C a 40°C/min; 200-280°C a 10°C/min; y finalmente se mantuvo durante 2 min. El tiempo total de ejecución fue de 14.75 min. Las condiciones de la espectrometría de masas fueron: temperatura de la interfaz, 280°C; temperatura de la

fuente de iones, 220°C; impacto de electrones, 70 eV; modo de adquisición, barrido (m/z 50-600). El OA se identificó comparando los tiempos de retención y las relaciones de abundancia de tres iones fragmentos (203, 202 y 189 *m/z*).

2.1.3. Elaboración del aceite de oliva funcional

El aceite de oliva funcional usado en el ensayo postprandial se obtuvo a través de la adición de AO a una concentración de 610 mg/kg a un aceite de oliva comercial. No se añadieron adyuvantes. El aceite de oliva control fue el mismo que el aceite de oliva funcional, pero sin añadir OA. Ambos aceites fueron embotellados en frascos de color verde y etiquetados mediante un sistema bicolor para asegurar el cegamiento. Cada dosis del aceite funcional consistía en 55 mL, que contenía 30 mg de OA.

2.2. Ensayo postprandial

2.2.1. Diseño de estudio y muestra

Ensayo postprandial, aleatorizado, controlado y doble ciego llevado a cabo en estricto cumplimiento con la declaración de Helsinki, y autorizado por los Comités de Ética de los Hospitales Universitarios Virgen de la Macarena y Virgen del Rocío de Sevilla, España. Se reclutaron 22 adolescentes sanos y con normopeso de ambos sexos, y con una edad comprendida entre 16-17 años de un instituto de la provincia de Granada (España). Para su inclusión en el estudio, los adolescentes no debían padecer trastornos digestivos, metabólicos u oncológicos, ni ninguna otra patología, lo que fue chequeado a través de un análisis bioquímico completo. También fue requisito para participar en el ensayo el consentimiento por escrito de los padres de los participantes, después de ser convenientemente informados tanto oral como por escrito de los protocolos del estudio. El trabajo de campo se realizó en el propio centro educativo. Once adolescentes fueron aleatoriamente asignados al grupo intervención (desayuno con aceite de oliva funcional enriquecido en AO), y los otros once participantes en el grupo control (desayuno con aceite de oliva control, sin enriquecer con AO). El desayuno consistió en tres rebanadas de pan tostado, una cucharada de tomate triturado, 150 mL de leche desnatada y el aceite de oliva.

El cálculo del tamaño muestral del ensayo postprandial se determinó considerando la concentración plasmática de AO como variable cuantitativa, y usando una cohorte de 60 participantes del grupo control del estudio PREDIABOLE. Se utilizó una confianza del 95% (riesgo $\alpha = 0.05$; 1.645), y una potencia del 90% ($z\beta=1.282$), obteniendo un tamaño muestral de 15 participantes. Teniendo en cuenta las posibles pérdidas, el tamaño muestral fue finalmente aumentado a 22 participantes.

2.2.2. Valoración antropométrica, análisis de composición corporal y determinaciones de presión sanguínea

Entre las 8:30 y las 10:30 horas, después de 12 horas de ayuno nocturno, se realizaron las mediciones antropométricas según las recomendaciones de la International Society for the Advancement of Kinanthropometry (ISAK) (Stewart et al., 2011). La privacidad de cada adolescente se garantizó al realizar la valoración de forma individual en un aula facilitada por el centro educativo. El peso corporal y el porcentaje de grasa corporal se midió a través de un analizador de composición corporal (TANITA® modelo BC-418MA). La medición de la estatura se realizó usando un estadiómetro portátil Seca 214*. Las medidas de altura se tomaron por duplicado con una precisión de 0.5 cm y se utilizó la media de ambos valores en el análisis. A través de una cinta métrica automática Seca (precisión de 1 mm) se determinó la circunferencia de la cintura (Cci) y la circunferencia de la cadera (Cca). La Cci se midió en el punto equidistante entre la última costilla y el borde superior de la cresta ilíaca al final de una espiración normal. La Cca se midió en el plano horizontal en la zona de mayor relieve de los glúteos y coincidiendo con la sínfisis isquiopúbica. El índice cintura-cadera (ICC) se calculó como la relación entre la Cci en centímetros dividido por la Cca en centímetros. El índice de masa corporal (IMC) se calculó dividiendo el peso del individuo en kilogramos por la altura en metros al cuadrado ($IMC = kg/m^2$). Los niveles de presión sanguínea fueron determinados usando un esfigmomanómetro aneroide calibrado y un estetoscopio Littmann® (Saint Paul, USA) de acuerdo a las recomendaciones internacionales (Pickering et al., 2005). Según los estadios de Tanner (1962), los adolescentes autoinformaron de su desarrollo puberal. También proporcionaron un registro alimentario de 72 horas, realizado los días previos al ensayo postprandial.

2.2.3. Parámetros bioquímicos y hematológicos

Después de la valoración antropométrica se extrajeron alícuotas de sangre cubital basales, así como a las 2 y a las 5 horas de la ingesta de los aceites. Se centrifugaron todas las muestras sanguíneas a 3000 rpm durante 5 minutos a 20 º C, y las alícuotas de suero se congelaron hasta su uso para la determinación de los parámetros bioquímicos basales o el aislamiento de TRLs postprandiales humanas.

En cuanto a los parámetros bioquímicos basales determinados, los niveles séricos de glucosa en ayunas, lipoproteína de alta densidad (HDL), colesterol total (CT) y triglicéridos (TG) se midieron mediante métodos enzimáticos colorimétricos. El nivel sérico de lipoproteína de baja densidad (LDL) se estimó usando la ecuación de Friedewald (1972) ((LDL) = (Colesterol Total) – (HDL) – ([TG]/5)). La insulinemia en ayunas se determinó usando un kit ELISA (Diaclone, Besançon, France) de acuerdo a las instrucciones del fabricante. El índice HOMA-IR se calculó con la siguiente ecuación: glucemia en ayunas (mmol/L) x insulinemia en ayunas (mU/L)/22.5.

2.3. Determinación de la presencia de AO en TRLs

2.3.1. Extracción de TRLs postprandiales humanas

Un volumen máximo de 4-4.5 mL de suero humano (siempre que fue posible) fue depositado dentro de tubos de polialómero TM (Beckman Instruments, Inc., Palo Alto, EEUU) debajo de 6 mL de una solución de 0.9% NaCl (densidad 1.006 Kg/L). Estos tubos de polialómero se introdujeron dentro de un rotor SW 41 Ti, y éste en una ultracentrífuga preparativa Beckman Coulter L90K (Beckman Instruments, Inc., Palo Alto, EEUU) programada a 39.000 rpm durante 16 horas a 12º C con el fin de aislar TRLs (densidad <0.93 Kg/L). Finalmente, se recogió la fase superior blanquecina de cada tubo.

2.3.2. Extracción de lípidos y preparación de TRLs

A cada alícuota de 1 mL de TRL se le añadió como patrón interno 15 μL de una solución metanólica de ácido betulínico (0.1 mg/mL). La mezcla fue expuesta a una corriente de N_2 durante 5-10 min para eliminar las trazas de metanol. A continuación, se

llevó a cabo el proceso de extracción de lípidos siguiendo el método Butanol-Metanol (BUME) propuesto por Cruz et al. (2016). Una cantidad de 300 µL de butanol/metanol (3:1, v/v) fue añadida a los tubos de ensayo, y se agitó enérgicamente la solución en vórtex durante un minuto. Se añadió a la solución 150 µL Heptano/acetato de etilo (EtAc) (3:1, v/v), y se volvió a agitar la solución en vórtex durante otro minuto. Se volvió a añadir Heptano/EtAc (150 µL, 3:1, v/v) y a agitar en vórtex durante otro minuto. Con el fin de inducir la separación de fases, a esta solución se le añadió 300 µL de LiCl 50 mM, y se centrifugó a 2.700 g durante 10 minutos. La fase orgánica superior obtenida fuera recogida y guardada en un nuevo tubo de ensayo. A la capa acuosa restante se le volvió a añadir heptano/EtAc (3:1) por dos veces, 320 µL y 250 µL respectivamente. Tras cada adición heptano/EtAc (3:1) se volvió a agitar en vórtex durante un minuto y a centrifugar la mezcla a 2.700 g durante 10 minutos. Se recogieron las capas orgánicas superiores resultantes y se combinaron con la fase orgánica previamente guardada. Los 870 µL de fase orgánica obtenidos se evaporaron a sequedad con una corriente de N_2, y la mezcla se disolvió en 200 µL del reactivo de sililación (BSTFA+1%TMCS en piridina) agitando de nuevo en vórtex durante 30 segundos. La mezcla finalmente obtenida se sometió a un baño de agua a 40 º C durante 25 minutos.

2.3.3. Determinación de OA en TRLs mediante espectrometría de masas

Los extractos lipídicos obtenidos tal y como se ha explicado en la sección 2.4.2. fueron analizados por espectrometría de masas GC (GC-MS) con el fin de determinar la presencia de AO. El análisis por GC-MS se realizó siguiendo el mismo procedimiento explicado previamente en la subsección 2.2.2. El OA se identificó comparando los tiempos de retención y las relaciones de abundancia de tres iones fragmentos (203, 202 y 189 *m/z*). Teniendo en cuenta que a los extractos lipídicos se le añadió como patrón interno ácido betulínico, un isómero del ácido oleanólico cuyo factor de respuesta es 1; a partir de las áreas obtenidas en los cromatogramas se cuantificó el AO.

2.4. Análisis estadísticos

Las diferencias entre medias de las características basales de los adolescentes fueron analizadas con el test-t de Student's. Los análisis estadísticos se llevaron a cabo usando el software IBM SPSS Statistics 24.0 (IBM Corp., Armonk, NY, USA).

3. Resultados y Discusión

3.1. Características basales de los participantes y de los aceites ingeridos.

En la Tabla 1 se muestran tanto las características antropométricas como bioquímicas basales de los adolescentes según el aceite de oliva ingerido. Tal y como muestran los datos no hubo ninguna diferencia estadísticamente significativa entre los adolescentes que consumieron el aceite de oliva funcional y el aceite de oliva control, lo que refleja la homogeneidad de los participantes.

La Tabla 2 refleja la composición química de los aceites usados en el ensayo postprandial. Los datos de la tabla muestran diferentes concentraciones de AO en ambos aceites, siendo de 3.8 ± 0.1 mg/kg de aceite en el aceite de oliva, y de 610.4 ± 16.2 mg/kg de aceite en el aceite de oliva funcional.

3.2. Presencia de AO en TRLs postprandiales.

La Figura 1 y la Figura 2 representan los cromatogramas obtenidos tras el análisis por GC-MS de dos muestras de TRLs. En el cromatograma de la Figura 1 que corresponde a una muestra de TRL basal, no se observa ningún pico cromatográfico indicativo de presencia de AO. En el cromatograma de la Figura 2 se observa un pico cromatográfico correspondiente al AO.

En cuanto a la interpretación de un cromatograma obtenido por GC-MS, cualquier compuesto se asocia a un pico cromatográfico concreto siempre que se cumplan los siguientes dos requisitos: i) que el compuesto eluya de la columna cromatográfica con un tiempo de retención que coincida con el exhibido por el compuesto que actúa como

patrón interno; ii) que aparezcan los fragmentos m/z característicos del compuesto en el sistema cromatográfico (Viñas & Campillo, 2019).

Tabla 1. Características antropométricas y bioquímicas basales de los adolescentes según el aceite de oliva ingerido en el desayuno.

Variables	Aceite de oliva n = 11	Aceite de oliva enriquecido en AO n = 11
Sexo (%)		
Chicos	2 (18.2)	3 (27.3)
Chicas	9 (81.8)	8 (72.7)
Edad (años)	16.00 ± 0.00	16.18 ± 0.40
Peso corporal (kg)	57.26 ± 9.08	59.57 ± 8.03
Estatura (cm)	166.31 ± 5.80	167.91 ± 7.40
IMC (kg/m^2)	20.67 ± 2.87	21.10 ± 2.24
Grasa corporal (%)	21.43 ± 7.81	21.33 ± 7.22
Cci (cm)	68.55 ± 6.08	70.64 ± 6.89
Cca (cm)	90.68 ± 6.37	90.32 ± 4.92
ICC	0.76 ± 0.04	0.78 ± 0.06
TAS (mmHg)	124.36 ± 10.48	122.91 ± 10.62
TAD (mmHg)	76.73 ± 9.66	76.91 ± 6.79
TG (mg/dL)	56.45 ± 15.96	58.82 ± 28.50
CT (mg/dL)	172.10 ± 15.16	168.09 ± 19.26
LDL (mg/dL)	115.36 ± 20.85	96.64 ± 28.50
HDL (mg/dL)	45.18 ± 12.54	59.10 ± 21.76
Glucosa (mg/dL)	70.73 ± 5.27	72.64 ± 6.25
Insulina (μU/ml)	7.27 ± 2.95	7.50 ± 5.20
HOMA-IR	1.27 ± 0.52	1.32 ± 0.92
Estadio puberal (%)[1]		
Estadio Tanner 1/2	0 (0)	0 (0)
Estadio Tanner 3	3 (27.3)	1 (9.1)
Estadio Tanner 4	6 (54.5)	4 (36.4)
Estadio Tanner 5	2 (18.2)	6 (54.5)

Los datos se presentan como media ± desviación estándar (SD), o %. No se encontraron diferencias significativas. IMC, índice de masa corporal; Cci, circunferencia de la cintura; Cca, circunferencia de cadera; ICC, índice cintura cadera; TAS, tensión arterial sistólica; TAD, tensión arterial diastólica; TG, triglicéridos; CT, colesterol total; LDL, lipoproteína de baja densidad; HDL, lipoproteína de alta densidad; HOMA-IR, modelo homeostática para evaluar la resistencia a la insulina. [1]Basado en estadios Tanner auto reportados.

Tabla 2. Composición de los aceites de oliva usados en el ensayo postprandial

Componente	**Media ± SD**
Ácidos Grasos (%)	
Palmítico (16:0)	11.0 ± 0.4
Palmitoleico (16:1 n-7)	1.0 ± 0.1
Esteárico (18:0)	3.2 ± 0.1
Oléico (18:1 n-9)	75.7 ± 0.2
Linoléico (18:2, n-6)	7.0 ± 0.0
α-linolénico (18:3 n-3)	0.3 ± 0.1
Araquídico (20:0)	0.4 ± 0.0
Gadoleico (20:1 n-11)	0.6 ± 0.1
Fenoles totales (μg/g de aceite)	60.7 ± 0.8
Hidroxitirosol y derivados	21.9 ± 0.2
Tirosol y derivados	38.6 ± 0.3
Lignanos	0.4 ± 0.0
Flavonoides	0.3 ± 0.1
Fenoles simples	0.1 ± 0.0
Esteroles totales (μg/g de aceite)	1295.3 ± 3.9
β-sitosterol	1124.0 ± 1.9
δ5-avenasterol	46.5 ± 0.7
Campesterol	44.0 ± 0.1
Estigmasterol	11.7 ± 0.1
Clerosterol	14.2 ± 0.0
δ5,24-estigmastadienol	14.9 ± 0.7
δ5,23-estigmastadienol	9.1 ± 0.0
Tocoferoles totales (μg/g de aceite)	263.8 ± 4.1
α-tocoferol	230.6 ± 0.8
Triterpenoides (μg/g de aceite)	
Eritrodiol	37.6 ± 0.1
Uvaol	3.6 ± 0.8
Ácido Oleanólico (aceite control/enriquecido)	3.8 ± 0.1 / 610.4 ± 16.2
Ácido Maslínico	4.8 ± 0.1
Clorofilas totales (μg/g de aceite)	1.1 ± 0.1
Carotenoides totales (μg/g oil)	1.5 ± 0.1

SD, Desviación estándar

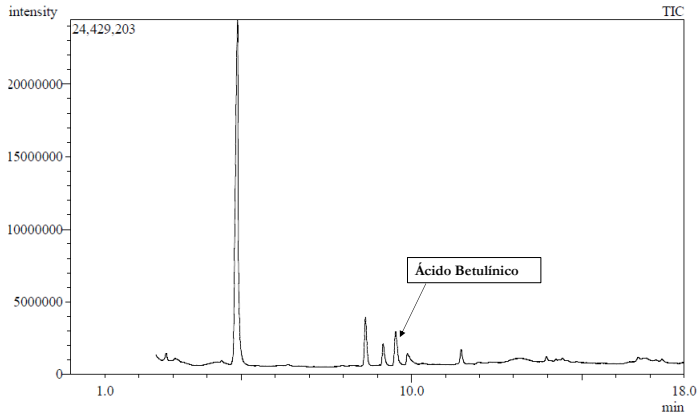

Figura 1. Cromatograma de una muestra de TRL basal.

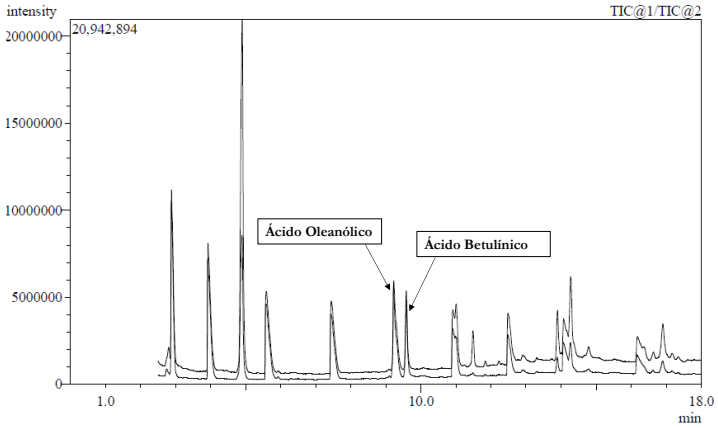

Figura 2. Cromatograma de una muestra de TRL postprandial extraída a las 2 horas del consumo de aceite de oliva funcional.

Cada compuesto tiene una serie de fragmentos moleculares característicos. En el caso del AO, la molécula se fracciona mediante ionización por electrospray (ESI) a 1-5 KV, dando tres picos mayoritarios característicos con m/z 203, 202 y 189 (Castellano et al., 2022; Guinda et al., 2004). En mezclas complejas, como es el caso del plasma o TRLs, puede haber otros compuestos orgánicos silanizables a través de sus grupos OH que eluyan en tiempos próximos o iguales al AO, tal y como se muestra en la Figura 1. Sin embargo, la observación de sus espectros de masas permite afirmar o descartar si se trata de AO o de otro compuesto.

No se detectó presencia de AO en las TRLs basales, lo que indica que ninguno de los adolescentes participantes presentaba AO procedente de su dieta. En cuatro muestras de TRLs extraídas a las 2 horas de la ingesta del aceite de oliva funcional se cuantificó 0.24, 0.13, 0.14, y 0.08 μg AO totales en fracción de TRL, respectivamente. En el adolescente que se cuantificó 0.14 μg AO total/fracción TRL; a las 5 horas se detectó también AO, al cuantificarse 0.13 μg AO total/fracción TRL.

En cuanto a los adolescentes que consumieron el aceite de oliva control, se detectó 0,38 y 0,06 μg AO totales en fracción de TRL postprandial aisladas a las dos horas del consumo del aceite. En el adolescente que se cuantificó 0.38 μg AO total/fracción TRL; se cuantificó 0.19 μg AO total/fracción TRL a las 5 horas de la ingesta del aceite de oliva control. Asimismo, en otro participante se cuantificó 0.42 μg AO total/fracción TRL a las 5 horas.

En base a estos resultados preliminares, se puede interpretar con cautela que el AO se transporta unido a TRLs tras la ingesta de un aceite de oliva enriquecido en AO. No obstante, la presencia también de AO en TRLs postprandiales de adolescentes que ingirieron el aceite de oliva control puede deberse a factores como el propio metabolismo de los participantes, dado que el AO se encuentra de forma natural minoritaria en el aceite de oliva o tomate, ambos ingeridos en el ensayo postprandial. Puesto que estos hallazgos deben considerarse como preliminares, se necesita la realización de futuros ensayos postprandiales para su validación.

El presente estudio tiene fortalezas y limitaciones que deben de ser consideradas. Entre las fortalezas destacar la naturaleza pionera del estudio en el que se analiza en adolescentes el posible transporte de AO unido a TRLs postprandiales tras la ingesta de un aceite de oliva funcional. La principal limitación fue la dificultad encontrada en el proceso de extracción sanguínea en adolescentes, factor que dificultó la posterior extracción de TRLs necesarias para el estudio del transporte de AO.

4. Conclusiones

En conclusión, nuestros resultados muestran la presencia de AO en TRLs postprandiales aisladas de adolescentes que previamente consumieron un aceite de oliva enriquecido en AO. A pesar de este hallazgo preliminar en cuanto al transporte postprandial de AO, se debe continuar realizando en adolescentes nuevos ensayos postprandiales con un mayor tamaño muestral que permitan avanzar y consolidar los resultados sobre el transporte postprandial de AO formando parte de las TRLs.

Agradecimientos: Los resultados informados en este estudio forman parte de la tesis doctoral de D. Ángel Fernández Aparicio, perteneciente al programa de doctorado de Medicina Clínica y Salud Pública (B 12.56.1) de la Universidad de Granada.

Financiación: Esta investigación ha sido financiada por el Programa Operativo del Fondo Europeo de Desarrollo Regional (FEDER) de Andalucía 2014-2020, número de subvención B-AGR-287-UGR18.

Conflictos de interés: Los autores no declaran ningún tipo de conflicto de interés.

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RESUMEN DE RESULTADOS Y DISCUSIÓN

La presente Tesis Doctoral Internacional ha abordado la caracterización y definición del SM en adolescentes (**estudio I**), los efectos terapéuticos del AO sobre monocitos-macrófagos THP-1 (**estudios II, III y IV**), y el mecanismo de transporte postprandial de AO en adolescentes sanos y con normopeso (**estudio V**). De acuerdo a los objetivos generales planteados, en esta sección se resumen y se discuten los principales resultados de los cinco estudios que conforman esta Tesis Doctoral Internacional.

I. Caracterización y definición del síndrome metabólico en adolescentes

El cribado del SM o de sus componentes en población infantil y adolescente es un paso previo para identificar a la población en riesgo de desarrollar ECV durante la edad adulta, y reducir este riesgo mediante la aplicación de intervenciones preventivas, terapéuticas y de educación sanitaria (DeBoer, 2019). Actualmente existen numerosos criterios para definir SM en población infantil y adolescente, entre los que destacan los propuestos por de Cook et al. (2003), Weiss et al. (2004), Duncan et al. (2004), de Ferranti et al. (2004), Cruz & Goran (2004), Rodríguez-Moran et al. (2004), Viner et al. (2005), y por la IDF (Zimmet et al., 2007). Dado que cada uno de estos criterios diagnósticos presenta diferencias en cuanto a los puntos de corte para evaluar cada uno de los componentes de SM, en la presente Tesis Doctoral se analizó en un estudio con diseño transversal (estudio I) el grado de concordancia entre ellos, y se determinó la prevalencia de SM en 981 adolescentes.

En nuestro estudio la prevalencia de SM en los adolescentes participantes difirió según los criterios empleados. En varones la menor prevalencia de SM observada fue de un 5.5% según el criterio de Viner et al. (2005), mientras que la mayor fue de un 14.9 % con los criterios de Duncan et al. (2004), Rodríguez-Morán et al. (2004), y Cruz & Goran (2004). En el caso de las chicas el menor promedio de prevalencia observada fue de un 3.4% con el criterio de Weiss et al (2004), y el mayor de un 32.6 % con el criterio de Cruz & Goran (2004). Las diferentes prevalencias encontradas en nuestro estudio según el

criterio utilizado también fueron informadas por otros estudios (Galera-Martínez et al., 2015; F. R. Guilherme et al., 2019; S. Kim & So, 2016; Reuter et al., 2018).

En cuanto a los análisis de grado de concordancia, por un lado, los criterios diagnósticos propuestos por Duncan et al. (2004), Rodríguez-Morán et al. (2004), y Cruz y Goran (2004) mostraron una gran concordancia entre sí, así como con el criterio diagnóstico propuesto por de Ferranti et al. (2004). Sin embargo, estos resultados no coinciden con los resultados informados por otros autores (Mirmiran et al., 2010; Seo et al., 2008). Por otro lado, el criterio diagnóstico que mostró un peor grado de concordancia, tanto en chicas como en chicos, con el resto de criterios usados en nuestro estudio fue el propuesto por la IDF (Zimmet et al., 2007). Resultados similares fueron observados por Reuter et al (2018), y Agudelo et al. (2014) entre los criterios de la IDF (Zimmet et al., 2007), y los propuestos por Cook et al (2003) y de Ferranti et al (2004); mientras que Guilherme et al. (2019) informó en adolescentes brasileños un buen grado de concordancia entre IDF (Zimmet et al., 2007), y los criterios de Cook et al. (2003) y de Ferranti et al. (2004). Igualmente, Peña-Espinoza et al. (2017) evidenció un buen grado de concordancia entre los criterios de la IDF (Zimmet et al., 2007) y de Cook et al. (2003), aunque bajo entre los criterios de la IDF (Zimmet et al., 2007) y de Ferranti et al. (2004). Estos resultados podrían explicarse porque los criterios propuestos por la IDF son los únicos en incluir como componente obligatorio para su diagnóstico a la obesidad abdominal (Zimmet et al., 2007).

En definitiva, nuestros resultados muestran disparidad tanto en la prevalencia de SM como en el grado de concordancia entre los ocho criterios diagnósticos empleados en los adolescentes participantes, disparidad que también ha sido informada por otros autores.

II. Análisis de los efectos terapéuticos del Ácido Oleanólico sobre macrófagos THP-1 y células espumosas

En el presente trabajo de Tesis Doctoral se han llevado a cabo dos revisiones sistemáticas con la finalidad de exponer los efectos del AO sobre los componentes del

SM (**estudio II**), y sobre los mecanismos moleculares y vías de señalización implicados en el desarrollo de RI y el estrés oxidativo subyacente (**estudio III**). Además, se ha llevado a cabo un ensayo postprandial y estudios experimentales sobre macrófagos THP-1 estimulados con LPS y células espumosas para analizar los efectos del AO sobre variables inflamatorias y las vías de señalización asociadas (**estudio IV**).

En la primera revisión sistemática (estudio II) se evidenció la capacidad del AO de reducir significativamente el estado hipertensivo en diferentes modelos de experimentación animal (Ahn et al., 2017; Bachhav et al., 2015; Gamede et al., 2019; Madlala et al., 2015), así como de aumentar la cantidad de sodio excretado por vías urinarias (Bachhav et al., 2015; Madlala et al., 2015). Los niveles séricos del factor vasodilatador NO también se vieron incrementados por acción del AO (An et al., 2017; Bachhav et al., 2011; Jiang et al., 2015). Estos datos sugieren que el efecto hipotensor del AO podría deberse a su capacidad moduladora sobre el sistema renina-angiotensina aldosterona, la síntesis del péptido natriurético auricular, y el aumento de niveles NO, mecanismos cuya alteración promueve el desarrollo de HTA (Cabandugama et al., 2017; Mendizábal et al., 2013; Whaley Connell et al., 2007; Yanai et al., 2008).

En diferentes modelos de experimentación animal (S. Chen et al., 2017; Gamede et al., 2019; Jiang et al., 2015; H. Luo et al., 2017; Pan et al., 2018; Xin Wang et al., 2013), y en un ensayo realizado en pacientes hiperlipidémicos (H. Q. Luo et al., 2018) el AO redujo significativamente los niveles séricos de TG y LDL (estudio II). Además, todos estos autores informaron de un aumento de los niveles de HDL, a excepción de S. Chen et al. (2017) y Pan et al. (2018), probablemente por la variabilidad en las dosis de AO o por los distintos modelos animales clínicos estudiados. Estos resultados constatan la actividad hipolipidémica del AO; sin embargo, con la finalidad de valorar el rol del AO sobre el proceso aterosclerótico, en el estudio IV se indujo la formación de células espumosas, las cuales según la literatura científica liberan sustancias proinflamatorias al espacio subendotelial (Botham & Wheeler-Jones, 2013; Cabello-Moruno et al., 2014; Napolitano et al., 2013). En nuestros resultados se observó que la inducción de macrófagos THP-1 con TRLs postprandiales de 2 h derivados del aceite de oliva enriquecido con AO, mostró niveles significativamente más bajos de IL-6 en

comparación con las TRLs derivadas del aceite de oliva. En esta línea Graham et al. (2012) obtuvo resultados similares en macrófagos THP-1 incubados con TRLs postprandiales de 4 h de adultos sanos derivados de aceite de orujo de oliva. Los niveles del factor NF-κB también fueron medidos en nuestro estudio dado que se ha demostrado que es activado por las TRLs (Botham & Wheeler-Jones, 2013). Los niveles de este factor fueron ligeramente inferiores en macrófagos tratados con TRL postprandial derivado de aceite de oliva enriquecido con AO.

En cuanto a los efectos del AO sobre la RI (estudio II), diferentes autores informaron de una disminución de los valores del índice HOMA-IR (Y. Li et al., 2014; Nyakudya et al., 2018; Su et al., 2018; Xue Wang et al., 2015). Asimismo, en términos generales la administración de AO a diferentes modelos de animales con RI mejoró la tolerancia a la glucosa y redujo los niveles de glucemia en ayunas (An et al., 2017; Djeziri et al., 2018; Gamede et al., 2018, 2019; Y. Li et al., 2014; Nyakudya et al., 2018; Su et al., 2018; S. Wang et al., 2018; Xin Wang et al., 2013; Xue Wang et al., 2015). Estos resultados podrían explicarse por el papel regulador del AO sobre la vía de señalización de la insulina IRS-1/PI3K/Akt, vía que se encuentra suprimida en estados de RI (Z. Zhang et al., 2021). De hecho, en los resultados expuestos en el estudio III, el AO aumentó la fosforilación de Akt (Xin Wang et al., 2013; Xue Wang et al., 2015; Zeng et al., 2012; Zhou et al., 2014), e inactivó el factor FoxO1 (Zeng et al., 2012; Zhou et al., 2014) en hígados de ratones diabéticos tipo 2. Esto podría implicar un aumento de la absorción de glucosa y disminución de la gluconeogénesis, puesto que la fosforilación estimulada por la insulina de Akt2 activa la glucógeno sintasa y reduce la transcripción de enzimas gluconeogénicas mediante la inactivación de FoxO1 (Samuel & Shulman, 2016). En este sentido en esta misma revisión se ha informado de la actividad antigluconeogénica del AO en roedores con RI hepática (Xin Wang et al., 2013; Xue Wang et al., 2015; Yunoki et al., 2008; Zeng et al., 2012; Zhou et al., 2014).

En el estudio IV no se observaron cambios significativos en los niveles de Akt en macrófagos THP-1 pretratados con diferentes dosis de AO y estimulados con LPS. Mencionar que en nuestro estudio se midieron los niveles de Akt totales, independientemente de su estado de fosforilación. Además, existen tres isoformas

homólogas de Akt (Akt1, Akt2, y Akt3) que se activan por mecanismos similares y que presentan funciones diferentes (Shiratsuchi & Basson, 2007; Song et al., 2005). No obstante, en dos estudios incluidos en la revisión del estudio III, el AO revirtió la desfosforilación de Akt en diferentes líneas celulares hepáticas con RI (Xin Wang et al., 2011; Y. Zhang et al., 2020). Además, Y. Zhang et al. (2020) junto a otro de los estudios incluidos en la revisión (M. Li et al., 2015), demostraron un aumento de los niveles proteicos de IRS por acción del AO en células HepG2 insulinorresistentes. Asimismo, M. Li et al. (2015) observaron una reducción de la expresión proteica de NF-κB, IL-6 y TNF-α. Por tanto, la reducción del estado inflamatorio y del estrés oxidativo subyacente a la RI podría ser uno de los mecanismos a través de los cuales el AO mejora la sensibilidad a la insulina.

En consonancia con los resultados expuestos en roedores con RI hepática y en líneas celulares hepáticas insulinorresistentes, en el estudio III también se informó de un aumento de la fosforilación de Akt en roedores con RI en el tejido adiposo tratados con AO (W. Li et al., 2021; Y. Li et al., 2014). Además, W. Li et al. (2021) advirtieron de una disminución de la fosforilación de JNK, vía cuya activación está implicada en reducir la acción de la insulina a través del aumento de la fosforilación de IRS-1 en serina (Batista et al., 2021; James et al., 2021). La fosforilación de JNK está implicada en el aumento de la secreción de citoquinas proinflamatorias en la RI del tejido adiposo (Rohm et al., 2022) al fomentar la polarización de ATMs a macrófagos M1 (Han et al., 2013). En esta línea W. Li et al. (2021), demostraron también un modificación de la polarización de ATMs en favor de macrófagos M2 (fenotipo antiinflamatorio), y una atenuación de la expresión génica de IL-6, TNF-α e IL-1β.

Combatir el estrés oxidativo es esencial para aliviar la RI. La perduración de la inflamación en los tejidos dianas de la insulina promueve la síntesis de ROS (Hurrle & Hsu, 2017), lo que implica una mayor activación de JNK e IKK (Batista et al., 2021) y, en consecuencia, la activación del factor NF-κB que induce la liberación de citoquinas proinflamatorias, que impiden la señalización de la insulina (Lima et al., 2022). En diferentes modelos de experimentación animal (estudio II y estudio III) se ha demostrado una disminución de los niveles séricos de diferentes citoquinas proinflamatorias (An et

al., 2017; Gamede et al., 2019; Matumba et al., 2019; Pan et al., 2018; Xin Wang et al., 2013). Igualmente el AO favoreció las actividades de superóxido dismutasa (Gamede et al., 2019; Jiang et al., 2015; E. S. Lee et al., 2016; Madlala et al., 2015; Su et al., 2018; S. Wang et al., 2018; Xin Wang et al., 2013; Xue et al., 2021), glutatión peroxidasa (Gamede et al., 2019; Jiang et al., 2015; Madlala et al., 2015; Xue et al., 2021) y catalasa (Jiang et al., 2015; Nyakudya et al., 2019; Su et al., 2018; S. Wang et al., 2018; Xin Wang et al., 2013; Xue et al., 2021), así como redujo la actividad del malondialdehído (Gamede et al., 2019; Jiang et al., 2015; Madlala et al., 2015; Nyakudya et al., 2019; S. Wang et al., 2018; Xue et al., 2021). El aumento de la actividad antioxidante inducido por el AO podría deberse por su acción sobre el factor de transcripción Nrf2, el cual aumenta la transcripción de las enzimas antioxidantes (superóxido dismutasa, catalasa y glutatión peroxidasa) (Castellano et al., 2013; S. Li et al., 2020; Tan & de Haan, 2014; K. C. Wu et al., 2011). En esta línea, Xin Wang et al. (2013) informaron de un aumento de los niveles proteicos de Nrf2 por acción del AO en hígado de ratones diabéticos. Asimismo, en ratones con RI hepática el AO indujo la inactivación del factor NF-κB a través de la desfosforilación de IκB-α y de p65 (Xue et al., 2021), confirmando lo propuesto por otros autores (L. Chen et al., 2015; Nandipati et al., 2017).

El factor NF-κB es conocido por afectar negativamente a la señalización de la insulina y promover la liberación de citoquinas proinflamatorias mediante la supresión de la fosforilación de AMPK (Camer et al., 2014; Castellano et al., 2013; Nandipati et al., 2017). El enzima AMPK presenta entre sus funciones la absorción de glucosa mediante la estimulación de la traslocación de GLUT4 (Entezari et al., 2022; James et al., 2021; Sharma et al., 2018; Szewczuk et al., 2020). En el estudio III se incluyeron diferentes estudios con resultados del AO sobre el enzima AMPK. Una mayor expresión génica de AMPK y de GLUT4, acompañada de una menor expresión génica de IL-6 y TNF-α fue observado por Matumba et al. (2019) en ratas neonatas alimentadas con una dieta rica en fructosa y tratadas con AO. En otro estudio el AO promovió la fosforilación de AMPK, Akt y PI3K en hígados de ratones diabéticos de tipo 2 (Xue Wang et al., 2015). Igualmente, Yunoki et al. (2008) y por Wang et al. (2013), demostraron una menor expresión génica TNF-α, IL-6 e IL-1β tras la inducción de la fosforilación de AMPK por

el AO en hígados de roedores insulinorresistentes. Estos resultados refuerzan el concepto de que el AO interviene en la captación de glucosa, e inhibe la gluconeogénesis en el hígado mediante la estimulación de la fosforilación de la AMPK (Ha et al., 2009).

En consonancia con los efectos expuestos del AO sobre la enzima AMPK, en el estudio IV el AO, a 10 μM, revirtió la desfosforilación de AMPK-α inducida por LPS en macrófagos THP-1. Resultados similares fueron obtenidos por Liu et al. (2021), dado que informaron que el ácido δ-oleanólico podía estimular la fosforilación de AMPK en macrófagos THP-1. Además, también se observó que el pretratamiento realizado con AO a macrófagos THP-1, especialmente a 25 μM, atenuó la liberación inducida por LPS de IL-6 y TNF-α en los sobrenadantes del cultivo celular (estudio IV). Por lo tanto, la regulación de la fosforilación de AMPK parece ser uno de los mecanismos mediante el cual el AO alivia el estado inflamatorio característico de la RI.

III. Estudio del transporte postprandial del Ácido Oleanólico en adolescentes sanos y con normopeso

Avanzar en el conocimiento sobre el transporte y biodisponibilidad del AO tras su ingesta es un factor clave que condiciona su efectividad al ser usado en cualquier intervención dietética o farmacológica. Por ello, en la presente Tesis Doctoral se analizó la presencia de AO en TRLs postprandiales aisladas de adolescentes sanos y con normopeso tras la ingesta de un aceite de oliva enriquecido en AO. En los análisis por GC-MS se detectó la presencia de AO en algunas de las TRLs analizadas. No obstante, se necesitan nuevos ensayos postprandiales que profundicen en el transporte postprandial de AO, y confirmen estos resultados preliminares.

LIMITACIONES

I. LIMITACIONES

Las publicaciones que conforman esta Tesis Doctoral Internacional presentan una serie de limitaciones que, aunque aparecen expuestas en cada uno de los artículos, van a ser resumidas en esta sección. En cuanto al estudio I la principal limitación encontrada fue su naturaleza transversal, aspecto que limita el establecimiento de una relación de causalidad.

Las principales limitaciones de las revisiones sistemáticas de los estudios II y III fue el riesgo de incurrir en un sesgo de selección al seleccionar artículo publicados únicamente en inglés, pudiendo ignorar estudios escritos en otros idiomas y que cumplieran el resto de criterios de inclusión. Además, tampoco fue consultada la literatura gris o fuentes de materiales no publicados. En el estudio II, el único ensayo clínico incluido para los resultados de la revisión fue un ensayo no aleatorizado. Además, en los estudios II y III, las características heterogéneas de los estudios incluidos en sendos resultados limitaron la realización de metaanálisis, lo que podría haber aportado una mayor consistencia a los resultados sobre el efecto del AO en los diferentes componentes del SM, y sobre los mecanismos moleculares a través de los cuales el AO modula su acción terapéutica sobre la RI y el SM.

En relación al estudio IV, al haberse realizado los experimentos celulares en una única línea celular, en este caso monocitos macrófagos THP-1, dificulta la generalización de los resultados a otras líneas celulares u organismos completos. Además, tanto en el estudio IV como en el V, se utilizaron TRLs derivadas de adolescentes que consumieron aceite de oliva enriquecido en AO o aceite de oliva control. La dificultad encontrada en la extracción sanguínea de algunos adolescentes influyó inexorablemente en el volumen de plasma sanguíneo obtenido, y por ende en el volumen final de TRLs obtenidas, necesarias para la realización de los experimentos de los estudios IV y V.

CONCLUSIONES/CONCLUSIONS

I. CONCLUSIONES

De acuerdo a las publicaciones que abordan los objetivos específicos planteados en esta Tesis Doctoral Internacional, se pueden extraer las siguientes conclusiones:

1. Los criterios diagnósticos para el SM en adolescentes propuestos por Duncan et al. (2004), Rodríguez-Morán et al. (2004), y Cruz y Goran (2004) no solo mostraron una gran concordancia entre sí, sino también con el criterio diagnóstico propuesto por de Ferranti et al. (2004). Sin embargo, los criterios de la IDF (Zimmet et al., 2007) presentaron un bajo grado de acuerdo con los otros siete criterios diagnósticos. Por lo tanto, se identificaron discrepancias en el grado de concordancia entre los ocho criterios empleados en la población adolescente. Estos datos sugieren la necesidad de avanzar en la validación de unos criterios diagnósticos uniformes y homogéneos para diagnosticar SM en adolescentes que faciliten su uso en la práctica clínica.
2. La administración de AO parece mejorar los estados hipertensivos, atenuar la alteración del perfil lipídico, reducir el estado de estrés oxidativo y mejorar la RI en modelos de animales en situaciones de disfunciones metabólicas. Asimismo, el AO puede mejorar la hiperlipidemia y la hiperglicemia en pacientes hiperlipidémicos. De esta forma estos resultados sugieren que el AO presenta un potencial efecto protector sobre los componentes del SM.
3. El AO alivia el estado de RI al mejorar la sensibilidad a la insulina y la señalización de la insulina, no solo por su modulación sobre la vía de señalización de la insulina IRS-1/PI3K/Akt e inactivación de FoxO1, sino también por su actividad reguladora en diferentes vías MAPK, las cuales están implicadas en el estrés oxidativo subyacente a la RI.
4. El pretratamiento con AO atenuó la sobreproducción de citoquinas proinflamatorias, especialmente de IL-6, y aumentó los niveles de AMPK-α de manera dependiente de la dosis en macrófagos THP-1 inducidos con LPS. Por lo tanto, el AO tiene potencial para tratar la RI debido a su capacidad para aliviar la respuesta inflamatoria a través de la modulación la actividad de la AMPK-α.

5. Con respecto a los resultados observados en el ensayo postprandial, las TRLs obtenidas de los adolescentes 2 h después de la ingestión del aceite de oliva funcional condujeron a niveles más bajos de IL-6 y Akt en comparación a aquellos macrófagos que fueron estimulados con TRLs obtenidos de adolescentes 2 h después del consumo de aceite de oliva no enriquecido. Estos resultados avalan el potencial que el AO podría tener para ser utilizado como una formulación basada en lípidos en los aceites de oliva funcionales para prevenir y tratar la RI, las alteraciones aterogénicas y los diferentes factores metabólicos que subyacen al SM en adolescentes.
6. Los resultados preliminares del ensayo postprandial muestran la presencia en adolescentes de AO en TRLs postprandiales tras la ingesta de aceite de oliva enriquecido en AO.

II. CONCLUSIONS

According to the publications that address the objectives set out in this International Doctoral Thesis, the following conclusions can be drawn:

1. The diagnostic criteria for SM in adolescents proposed by Duncan et al. (2004), Rodriguez-Moran et al. (2004), and Cruz and Goran (2004) not only showed high agreement with each other, but also with the diagnostic criteria proposed by de Ferranti et al. (2004). However, the IDF criteria (Zimmet et al., 2007) showed a low degree of agreement with the other seven diagnostic criteria. Therefore, discrepancies were identified in the degree of agreement between the eight criteria used in the adolescent population. These data suggest the need to advance in the validation of uniform and homogeneous diagnostic criteria for diagnosing MS in adolescents that will facilitate their use in clinical practice.
2. The administration of OA appears to improve hypertensive states, attenuate the alteration of the lipid profile, reduce the state of oxidative stress, and improve RI in animal models in situations of metabolic dysfunction. Likewise, OA can improve hyperlipidemia and hyperglycemia in hyperlipidemic patients. Thus, these results suggest that OA has a potential protective effect on the components of MS.
3. OA alleviates the state of IR by improving insulin sensitivity and insulin signaling, not only by its modulation on the IRS-1/PI3K/Akt insulin signaling pathway and FoxO1 inactivation, but also by its regulatory activity on different MAPK pathways, which are involved in the oxidative stress underlying IR.
4. Pretreatment with OA attenuated the overproduction of proinflammatory cytokines, especially IL-6, and increased AMPK-α levels in a dose-dependent manner in LPS-induced THP-1 macrophages. Therefore, OA has potential to treat IR due to its ability to alleviate the inflammatory response through modulation of AMPK-α activity.
5. Regarding the results observed in the postprandial assay, TRLs obtained from adolescents 2 h after ingestion of functional olive oil led to lower levels of IL-6

and Akt compared to those macrophages that were stimulated with TRLs obtained from adolescents 2 h after consumption of non-enriched olive oil. These results support the potential that OA could have to be used as a lipid-based formulation in functional olive oils to prevent and treat IR, atherogenic alterations, and different metabolic factors underlying MS in adolescents.

6. Preliminary results of the postprandial assay show the presence of AO in postprandial TRLs in adolescents after the intake of olive oil enriched in AO.

FUTURAS LÍNEAS DE INVESTIGACIÓN

I. FUTURAS LÍNEAS DE INVESTIGACIÓN

Los resultados expuestos en la presente Tesis Doctoral Internacional posibilitan el planteamiento de diferentes líneas futuras de investigación. En cuanto a la caracterización y definición de SM en adolescentes, las discrepancias observadas en el grado de concordancia entre los ocho criterios diagnósticos empleados para diagnosticar SM en los adolescentes participantes de nuestro estudio, así como con estudios similares realizados por otros autores, pone de manifiesto la necesidad de validar criterios uniformes y homogéneos para el diagnóstico de SM en población adolescente. Por ello, se deben realizar nuevos estudios con muestras de mayor tamaño, considerando las particularidades socioeconómicas, demográficas y anatomo-fisiológicas de la población adolescente mundial en el diseño y búsqueda de unos criterios universales para la definición de SM en adolescentes. La resolución de esta problemática permitirá mejorar la utilidad de los criterios diagnósticos de SM tanto en la práctica clínica como en el control por parte de las autoridades sanitarias del importante problema de salud pública que constituye el SM en adolescentes; también haría posible evitar la aparición a largo plazo de enfermedades cardiovasculares.

En este trabajo también se han analizado los efectos terapéuticos del AO sobre componentes del SM y parámetros relacionados con el desarrollo de este síndrome y de la RI. La primera de las revisiones sistemáticas que conforman los resultados de esta Tesis Doctoral incluyó estudios que no abordaban conjuntamente todos los parámetros implicados en el desarrollo de SM y RI. Similarmente, en la segunda revisión sistemática incluida en esta Tesis Doctoral también se incluyeron estudios de experimentación animal o en líneas celulares que no eran homogéneos entre sí. Por tanto, la realización de estudios de experimentación que aborden simultáneamente todos los componentes del SM y la RI, empleando modelos de experimentación animal de características similares, y con las mismas dosis de AO permitiría obtener unos hallazgos más sólidos en cuanto a los efectos del AO sobre estos trastornos metabólicos y de los mecanismos moleculares implicados. Además, en nuestros estudios experimentales en macrófagos THP-1 se observó una atenuación de los niveles de citoquinas proinflamatorias, así como un efecto

modulador sobre determinadas quinasas por parte del AO. A pesar de los resultados obtenidos tanto de las revisiones sistemáticas como de los experimentos celulares, sería necesario la realización de más estudios *in vitro* e *in vivo* para conocer en profundidad el mecanismo exacto de interacción entre el AO y sus proteínas diana.

Por último, sería conveniente realizar más ensayos postprandiales con un mayor tamaño muestral en población adolescente para continuar avanzando en el estudio del transporte postprandial de AO formando parte de las TRLs. Asimismo, la realización de ensayos clínicos aleatorios en adolescentes diagnosticados de SM y RI permitiría dilucidar el potencial uso futuro de AO como terapia complementaria, en forma de nutracéutico, a la terapia convencional del SM y la RI.

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ANEXOS

ANEXO I. COMITÉ DE ÉTICA

JUNTA DE ANDALUCIA

CONSEJERÍA DE IGUALDAD, SALUD Y POLÍTICAS SOCIALES
Dirección General de Calidad, Investigación, Desarrollo e Innovación
Comité Coordinador de Ética de la Investigación Biomédica de Andalucía

DICTAMEN ÚNICO EN LA COMUNIDAD AUTÓNOMA DE ANDALUCÍA

D/Dª: Jose Salas Turrents como secretario/a del **CEI de los hospitales universitarios Vírgen Macarena-Virgen del Rocío**

CERTIFICA

Que este Comité refrendará de oficio en la reunión celebrada en el día 20/07/2017 la propuesta de (No hay promotor/a asociado/a) para realizar el estudio de investigación titulado:

Título del estudio:	Prevención con Ácido Oleanólico de Insulinorresistencia y Síndrome Metabólico en Adolescentes ,(PREOLIA)
Protocolo, Versión:	1
HIP, Versión:	1
CI, Versión:	1

Y que considera que:

Se cumplen los requisitos necesarios de idoneidad del protocolo en relación con los objetivos del estudio y se ajusta a los principios éticos aplicables a este tipo de estudios.

La capacidad del/de la investigador/a y los medios disponibles son apropiados para llevar a cabo el estudio.

Están justificados los riesgos y molestias previsibles para los participantes.

Que los aspectos económicos involucrados en el proyecto, no interfieren con respecto a los postulados éticos.

Y que este Comité considera, que dicho estudio puede ser realizado en los Centros de la Comunidad Autónoma de Andalucía que se relacionan, para lo cual corresponde a la Dirección del Centro correspondiente determinar si la capacidad y los medios disponibles son apropiados para llevar a cabo el estudio.

Lo que firmo en a 06/08/2017

D/Dª. Jose Salas Turrents , como Secretario/a del CEI de los hospitales universitarios Vírgen Macarena-Virgen del Rocío

CERTIFICA

Que este Comité refrendará de oficio en la sesión a celebrar el 20/07/2017 la propuesta del/de la Promotor/a (No hay promotor/a asociado/a), para realizar el estudio de investigación titulado:

Título del estudio:	Prevención con Ácido Oleanólico de Insulinorresistencia y Síndrome Metabólico en Adolescentes ,(PREOLIA)
Protocolo, Versión:	1
HIP, Versión:	1
CI, Versión:	1

Lo que firmo en a 06/08/2017

ANEXO II. CONSENTIMIENTO INFORMADO

Prevención con Ácido Oleanólico de Insulinorresistencia y Síndrome Metabólico en Adolescentes (Estudio PREOLIA)

CONSENTIMIENTO INFORMADO

PREOLIA es un proyecto financiado por el Programa Operativo FEDER Andaucía 2014-2020. Convocatoria 2018.

He sido invitado a participar voluntariamente en este estudio por parte del equipo de investigación del proyecto.

He leído la hoja de información acerca de este proyecto que se me ha entregado

He comprendido las explicaciones ofrecidas y he podido hacer las preguntas que he creído convenientes.

Atendiendo a todas las consideraciones anteriores

Yo D. _____,
madre/padre/tutor legal, autorizo la participación de mi hijo/a en el mencionado estudio y comprendo que mi hijo/a puede retirarse del estudio cuando quiera sin tener que dar explicaciones.

Fecha de la aceptación: _____

Firma del representante legal

Firma del investigador

DNI del representante legal

DNI del investigador

Debido a un cambio en mis circunstancias personales, decido revocar el anterior consentimiento y dejar de participar en el estudio sin que esto tenga ningún tipo de consecuencias negativas para mí.

Firma del representante legal

Fecha de la revocación

Para cualquier aclaración o gestión relacionada con el proyecto, puede consultar con Emilio González Jiménez en el siguiente email**: emigoji@ugr.es** En caso de autorizar la participación de su hijo/a en el estudio, el comienzo de cada una de las fases mencionadas le será comunicado con suficiente antelación.

1

Table Suplemmentary. Diagnostic criteria for the MetS in adolescents.

	IDF	**Cook**	**de Ferranti**	**Weiss**	**Viner**	**Duncan**	**Rodríguez-Moran**	**Cruz & Goran**
Age (years)	10-16	12-19	≥ 12	4-20	2-18	12-19	10-18	8-13
Number of components	Obesity + 2 components	≥ 3	≥ 3	≥ 3	≥ 4	≥ 3	≥ 3	≥ 3
Obesity	WC > 90 percentile	WC > 90 percentile	WC > 75 percentile	BMI z-score ≥ 2	BMI ≥ 95 percentile	WC ≥ 90 percentile	WC ≥ 90 percentile	WC ≥ 90 percentile
Glucose (mg/dL)	≥ 100	≥ 110	≥ 110	≥ 140	≥ 110	≥ 110	≥ 110	> 100
TG (mg/dL)	≥ 150	≥ 110	≥ 100	> 95 percentile	≥ 150	≥ 110	≥ 90 percentile	≥ 90 percentile
HDL-cholesterol (mg/dL)	≤ 40	≤ 40	< 50 girls <45 boys	< 5 percentile	≤ 35	< 40	-	< 10 percentile
SBP (mmHg)	≥ 130	> 90 percentile	> 90 percentile	> 95 percentile	> 95 percentile	≥ 90 percentile	≥ 90 percentile	≥ 90 percentile
DBP (mmHg)	≥ 85	-	-	-	-	-	-	-

IDF, International Diabetes Federation; TG, triglycerides; HDL, high-density lipoprotein; SBP, systolic blood pressure, DBP, diastolic blood pressure, WC, waist circumference; BMI, body mass index.

ANEXO IV. MATERIAL SUPLEMENTARIO ESTUDIO IV

Table S1. Composition of the olive oil used in the study

Component	Mean ± SD
Fatty acids (%)	
Palmitic (16:0)	11.0 ± 0.4
Palmitoleic (16:1 n-7)	1.0 ± 0.1
Estearic (18:0)	3.2 ± 0.1
Oleic (18:1 n-9)	75.7 ± 0.2
Linoleic (18:2, n-6)	7.0 ± 0.0
α-linolenic (18:3 n-3)	0.3 ± 0.1
Arachidic (20:0)	0.4 ± 0.0
Gadoleic (20:1 n-11)	0.6 ± 0.1
Total phenolics (μg/g oil)	60.7 ± 0.8
Hydroxytyrosol and derivatives	21.9 ± 0.2
Tyrosol and derivatives	38.6 ± 0.3
Lignanes	0.4 ± 0.0
Flavonoids	0.3 ± 0.1
Simple phenols	0.1 ± 0.0
Total sterols (μg/g oil)	1295.3 ± 3.9
β-sitosterol	1124.0 ± 1.9
δ5-avenasterol	46.5 ± 0.7
Campesterol	44.0 ± 0.1
Stigmasterol	11.7 ± 0.1
Clerosterol	14.2 ± 0.0
δ5,24-stigmastadienol	14.9 ± 0.7
δ5,23-stigmastadienol	9.1 ± 0.0
Total tocopherols (μg/g oil)	263.8 ± 4.1
α-tocopherol	230.6 ± 0.8
Triterpenoids (μg/g oil)	
Erythrodiol	37.6 ± 0.1
Uvaol	3.6 ± 0.8
Oleanolic acid (control/enriched oils)	3.8 ± 0.1 / 610.4 ± 16.2
Maslinic acid	4.8 ± 0.1
Total chlorophylls (μg/g oil)	1.1 ± 0.1
Total carotenoids (μg/g oil)	1.5 ± 0.1

SD, Standard deviation

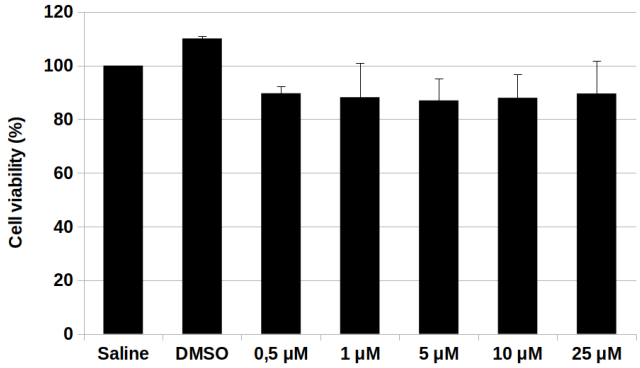

Figure S1. Cell viability in OA-pretreated THP-1 macrophages at different concentrations as measured by XTT.

Table S2. Baseline anthropometric and biochemical characteristics of adolescents according to the olive oil ingested at breakfast.

Variables	Olive oil n = 11	OA-enriched olive oil n = 11
Gender (%)		
Boys	2 (18.2)	3 (27.3)
Girls	9 (81.8)	8 (72.7)
Mean age (years)	16.00 ± 0.00	16.18 ± 0.12
Body weight (kg)	57.26 ± 2.74	59.57 ± 2.42
BMI (kg/m^2)	20.67 ± 0.87	21.10 ± 0.67
Body fat (%)	21.43 ± 2.36	21.33 ± 2.18
WC (cm)	68.55 ± 1.90	70.64 ± 2.08
SBP (mmHg)	124.36 ± 3.16	122.91 ± 3.20
DBP (mmHg)	76.73 ± 2.91	76.91 ± 2.05
TG (mg/dL)	56.45 ± 4.81	58.82 ± 4.08
TC (mg/dL)	172.10 ± 4.57	168.09 ± 5.81
LDL-c (mg/dL)	115.36 ± 6.29	96.64 ± 8.59
HDL-c (mg/dL)	45.18 ± 3.78	59.10 ± 6.88
Glucose (mg/dL)	70.73 ± 1.59	72.64 ± 1.88
Insulin (µU/ml)	7.27 ± 0.93	7.50 ± 1.65
HOMA-IR	1.27 ± 0.17	1.32 ± 0.29
Pubertal stage (%)[1]		
Tanner stage 1/2	0 (0)	0 (0)
Tanner stage 3	3 (27.3)	1 (9.1)
Tanner stage 4	6 (54.5)	4 (36.4)
Tanner stage 5	2 (18.2)	6 (54.5)

Data are presented as mean ± SEM or %. Significant differences were not found. BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglycerides; TC, total cholesterol; LDL, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance. [1]Based on self-reported Tanner stages.

ANEXO V. ARTÍCULOS DERIVADOS DE LA TESIS DOCTORAL

En este anexo se encuentran enumerados los artículos derivados de la presente Tesis Doctoral Internacional y que se han incluido previamente en el apartado de "Publicaciones", tanto los publicados, como el artículo pendiente de envío. Asimismo, se presentan a continuación la portada y última página de los artículos ya publicados.

1. Fernández-Aparicio, Á., Perona, J. S., Schmidt-RioValle, J., & González-Jiménez, E. (2021). Concordance among diagnostic criteria for metabolic syndrome is inconsistent in Spanish adolescents. *European Journal of Clinical Investigation*, *51*(2), e13384. https://doi.org/10.1111/eci.13384
2. Fernández-Aparicio, Á., Schmidt-RioValle, J., Perona, J. S., Correa-Rodríguez, M., Castellano, J. M., & González-Jiménez, E. (2019). Potential protective effect of oleanolic acid on the components of metabolic syndrome: a systematic review. *Journal of Clinical Medicine*, *8*(9), 1294. https://doi.org/10.3390/jcm8091294
3. Fernández-Aparicio, Á., Correa-Rodríguez, M., Castellano, J. M., Schmidt-RioValle, J., Perona, J. S., & González-Jiménez, E. (2022). Potential molecular targets of oleanolic acid in insulin resistance and underlying oxidative stress: a systematic review. *Antioxidants*, *11*(8), 1517. https://doi.org/10.3390/antiox11081517
4. Fernández-Aparicio, Á., Perona, J. S., Castellano, J. M., Correa-Rodríguez, M., Schmidt-RioValle, J., & González-Jiménez, E. (2021). Oleanolic acid-enriched olive oil alleviates the interleukin-6 overproduction induced by postprandial triglyceride-rich lipoproteins in THP-1 macrophages. *Nutrients*, *13*(10), 3471. https://doi.org/10.3390/nu13103471
5. Fernández-Aparicio, Á., et al. Analysis of triglyceride-rich lipoproteins as a possible mechanism for postprandial transport of oleanolic acid in adolescents. [Análisis de las lipoproteínas ricas en triglicéridos como posible mecanismo de transporte postprandial de ácido oleanólico en adolescentes]. Pendiente de traducción y envío.

Received: 30 June 2020 | Revised: 23 July 2020 | Accepted: 6 August 2020

DOI: 10.1111/eci.13384

ORIGINAL ARTICLE

WILEY

Concordance among diagnostic criteria for metabolic syndrome is inconsistent in Spanish adolescents

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Funding information
This work was supported by funds from Andalusia ERDF 2014-2020 Operative Programme (B-AGR-287-UGR18).

Abstract

Background: The metabolic syndrome (MetS), although more frequent in adults, is a growing health problem in adolescent population. There are different criteria for the diagnosis, however without a consensus of which is the best to be used in this population. The heterogeneity of the different diagnostic criteria makes it necessary to carry out more studies that analyse the degree of agreement among these criteria. The present study was aimed to evaluate the agreement between different criteria for diagnosis of MetS in adolescents.

Materials and methods: A cross-sectional study was performed on 981 adolescents (13.2 ± 1.2 years) randomly recruited from 18 schools in south-east Spain. MetS was diagnosed by eight different criteria.

Results: The criteria proposed by the IDF showed the highest mean values for WC and systolic blood pressure in boys and girls with MetS, and the lowest for glucose and triglycerides in boys. Depending on the diagnostic criteria used, the prevalence of MetS cases in boys ranged from 5.5% to 14.9%, while in girls varied from 3.4% to 32.6%. Both in boys and girls, the criteria proposed by the IDF was the less concordant with the other suggested criteria, while those proposed by Duncan et al, Rodriguez-Moran et al and Cruz and Goran, were very concordant among each other. However, in girls, concordance values were not as high as those found for boys.

Conclusion: The variability observed in the agreement among the existing criteria suggests the need to validate uniform criteria for the diagnosis of MetS in adolescents.

KEYWORDS

adolescents, anthropometric indexes, diagnosis criteria, metabolic syndrome

1 | INTRODUCTION

Metabolic syndrome (MetS) is characterized by a set of three or more metabolic disorders, including abdominal obesity, systemic arterial hypertension, elevated serum triglycerides (TG) and glycaemia and low levels of high-density lipoprotein cholesterol (HDL-c).[1] Adolescents that suffer early changes in the components of MetS are associated with a high risk of developing this condition in adulthood,[2] with an increased risk of developing type 2 diabetes mellitus and cardiovascular diseases (CVD).[3]

On the other hand, the prevalence of MetS in adolescence in many studies is rather divergent, mainly due to the absence of specifically established criteria for its use in non-adult

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Fernández-Aparicio Á, Perona JS, Schmidt-RioValle J, González-Jiménez E. Concordance among diagnostic criteria for metabolic syndrome is inconsistent in Spanish adolescents. *Eur J Clin Invest*. 2021;51:e13384. https://doi.org/10.1111/eci.13384

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Review

Potential Protective Effect of Oleanolic Acid on the Components of Metabolic Syndrome: A Systematic Review

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Received: 19 July 2019; Accepted: 21 August 2019; Published: 23 August 2019

Abstract: The high prevalence of obesity is a serious public health problem in today's world. Both obesity and insulin resistance favor the development of metabolic syndrome (MetS), which is associated with a number of pathologies, especially type 2 diabetes mellitus, and cardiovascular diseases. This serious problem highlights the need to search for new natural compounds to be employed in therapeutic and preventive strategies, such as oleanolic acid (OA). This research aimed to systematically review the effects of OA on the main components of MetS as well as oxidative stress in clinical trials and experimental animal studies. Databases searched included PubMed, Medline, Web of Science, Scopus, EMBASE, Cochrane, and CINAHL from 2013 to 2019. Thus, both animal studies ($n = 23$) and human clinical trials ($n = 1$) were included in our review to assess the effects of OA formulations on parameters concerning insulin resistance and the MetS components. The methodological quality assessment was performed through using the SYRCLE's Risk of Bias for animal studies and the Jadad scale. According to the studies in our review, OA improves blood pressure levels, hypertriglyceridemia, hyperglycemia, oxidative stress, and insulin resistance. Although there is scientific evidence that OA has beneficial effects in the prevention and treatment of MetS and insulin resistance, more experimental studies and randomized clinical trials are needed to guarantee its effectiveness.

Keywords: triterpenes; metabolic syndrome; insulin resistance; hypertension; inflammation; obesity

1. Introduction

The increasing prevalence of overweight and obesity entails a serious global public health problem. It has been estimated that 39% of the world population over the age of 18 years is overweight, and 13% is obese, according to World Health Organization (WHO) data (2016) [1]. Furthermore, 18% of children and adolescents (5–19 years old) are either overweight or obese [1,2]. This situation is alarming because obesity and insulin resistance are two of the most important factors leading to metabolic syndrome (MetS) [3]. MetS consists of a set of cardiometabolic anomalies that lead to the development of type 2 diabetes mellitus (T2DM) and of cardiovascular disease (CVD) [4,5]. Nevertheless, MetS is also associated with other clinical conditions such as oxidative stress, hypertension, dyslipidemia, hepatic steatosis, non-alcoholic fatty liver disease, and impaired glucose tolerance, among others [6].

According to the WHO [5], insulin resistance is the main pathophysiological factor underlying MetS. It is characterized by a diminished tissue response to the cell activity of insulin [6], which implies

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Systematic Review

Potential Molecular Targets of Oleanolic Acid in Insulin Resistance and Underlying Oxidative Stress: A Systematic Review

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Abstract: Oleanolic acid (OA) is a natural triterpene widely found in olive leaves that possesses antioxidant, anti-inflammatory, and insulin-sensitizing properties, among others. These OA characteristics could be of special interest in the treatment and prevention of insulin resistance (IR), but greater in-depth knowledge on the pathways involved in these properties is still needed. We aimed to systematically review the effects of OA on the molecular mechanisms and signaling pathways involved in the development of IR and underlying oxidative stress in insulin-resistant animal models or cell lines. The bibliographic search was carried out on PubMed, Web of Science, Scopus, Cochrane, and CINHAL databases between January 2001 and May 2022. The electronic search produced 5034 articles but, after applying the inclusion criteria, 13 animal studies and 3 cell experiments were identified, using SYRCLE's Risk of Bias for assessing the risk of bias of the animal studies. OA was found to enhance insulin sensitivity and glucose uptake, and was found to suppress the hepatic glucose production, probably by modulating the IRS/PI3K/Akt/FoxO1 signaling pathway and by mitigating oxidative stress through regulating MAPK pathways. Future randomized controlled clinical trials to assess the potential benefit of OA as new therapeutic and preventive strategies for IR are warranted.

Keywords: *Olea europaea*; bioactive compounds; triterpenes; oleanolic acid; insulin resistance; type 2 diabetes mellitus; oxidative stress; inflammation; insulin signaling; pathways

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Citation: Fernández-Aparicio, Á.; Correa-Rodríguez, M.; Castellano, J.M.; Schmidt-RioValle, J.; Perona, J.S.; González-Jiménez, E. Potential Molecular Targets of Oleanolic Acid in Insulin Resistance and Underlying Oxidative Stress: A Systematic Review. *Antioxidants* **2022**, *11*, 1517. https://doi.org/10.3390/antiox11081517

Academic Editors: Milagros Rocha, Victor M. Victor and Sandra López-Doménech

Received: 29 June 2022
Accepted: 1 August 2022
Published: 3 August 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

Diabetes mellitus (DM) is a major public health problem since it currently affects about 420 million people worldwide, and its prevalence is expected to increase by approximately 38% by 2030 [1]. Around 90% of DM world cases are accounted for by type 2 diabetes mellitus (T2DM) [2]. Insulin resistance (IR) affects individuals for many years before the development of T2DM [3], and consists of a diminished sensitivity of insulin target tissues to healthy insulin levels [4]. IR disrupts the insulin receptor substrate (IRS)/phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) pathway activation [5,6], which provokes a reduction of the glucose uptake in the adipose tissue and skeletal muscle, an increment of the gluconeogenic and glycolytic activity in the liver, and lipid metabolism disturbances in the liver and adipose tissue [7,8].

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Article

Oleanolic Acid-Enriched Olive Oil Alleviates the Interleukin-6 Overproduction Induced by Postprandial Triglyceride-Rich Lipoproteins in THP-1 Macrophages

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Abstract: Oleanolic acid (OA), a triterpene that is highly present in olive leaves, has been proposed as a component of functional foods for the prevention of metabolic syndrome, due to its anti-inflammatory activity. We analyzed the effects of OA on inflammatory parameters and signaling proteins in LPS-stimulated THP-1 macrophages. Thus, THP-1 macrophages were incubated with LPS for 48 h after pretreatment with OA at different concentrations. Pretreatment with OA was significantly effective in attenuating IL-6 and TNF-α overproduction induced by LPS in macrophages, and also improved the levels of AMPK-α. We also evaluated the effects of human triglyceride-rich lipoproteins (TRLs) derived from individuals consuming an OA-enriched functional olive oil. For this purpose, TRLs were isolated from healthy adolescents before, 2 and 5 h postprandially after the intake of a meal containing the functional olive oil or common olive oil, and were incubated with THP-1 macrophages. THP-1 macrophages incubated with TRLs isolated at 2 h after the consumption of the OA-enriched olive oil showed significant lower levels of IL-6 compared to the TRLs derived from olive oil. Our results suggest that OA might have potential to be used as a lipid-based formulation in functional olive oils to prevent inflammatory processes underlying metabolic syndrome in adolescents.

Keywords: oleanolic acid; olive oil; functional foods; postprandial trial; human triglyceride-rich lipoproteins; metabolic syndrome; obesity; insulin resistance; adolescents; THP-1 macrophages

Citation: Fernández-Aparicio, Á.; Perona, J.S.; Castellano, J.M.; Correa-Rodríguez, M.; Schmidt-RioValle, J.; González-Jiménez, E. Oleanolic Acid-Enriched Olive Oil Alleviates the Interleukin-6 Overproduction Induced by Postprandial Triglyceride-Rich Lipoproteins in THP-1 Macrophages. *Nutrients* **2021**, *13*, 3471. https://doi.org/10.3390/nu13103471

Academic Editor: Satoshi Nagaoka

Received: 6 September 2021
Accepted: 27 September 2021
Published: 29 September 2021

1. Introduction

Metabolic syndrome (MetS) is a worrying health public problem that affects approximately 31% of the world population, and it is expected that its prevalence will increase by about 53% by 2035 [1,2]. The International Diabetes Federation defines central obesity as the unique fixed component of MetS [3]. In fact, inadequate control of abdominal obesity and insulin resistance (IR), among others, not only lead to the development of Type 2 Diabetes Mellitus (T2DM) and cardiovascular diseases, but also to other clinical disorders, such as oxidative stress, non-alcoholic fatty liver disease and hepatic steatosis [4–6].

Obesity is characterized by an excess of fat accumulation in the adipose tissue [7], in which the inflammatory response is closely linked to the development of IR [8]. Chronic inflammation of adipose tissue in obesity causes an impairment in the polarization of adipose tissue macrophages (ATMs), resulting in a higher presence of the M1 proinflammatory phenotype [8–10]. As a consequence, ATMs are involved in the release of proinflammatory cytokines, such as interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) [11,12]. These proinflammatory cytokines are positively regulated by the nuclear

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AGRADECIMIENTOS

Y llegó el momento de escribir los agradecimientos, tan deseado por unos, tan temido por quién presenta dificultades para exteriorizar los sentimientos. Sin embargo, este momento es positivo, dado que se cierra una ardua, pero bonita y enriquecedora etapa, la finalización de la Tesis Doctoral.

Me gustaría empezar dándoles las gracias a mis padres, los dos pilares más importantes de mi vida. Mamá, papá, gracias por haber estado siempre apoyándome estos cuatro años, y por confiar en mí en los momentos difíciles, en los que parecía que luz al final del túnel nunca llegaría. También gracias por haberme inculcado desde pequeño que con los valores de la constancia, sacrificio y humildad se puede conseguir cualquier meta que uno se proponga. Nuria, querida hermanita, gracias por soportar mis "chistes malos" y "tonterías" que solo me salen contigo y en momentos de mayor estrés, y también por los ánimos recibidos. Aunque no lo suelo decir, os quiero.

Tío Basi, fuiste un ejemplo de superación en estos últimos años. Siempre estarás en nuestros pensamientos y corazones. Y sé, que, en el día de hoy, junto a mis dos abuelos tocayos me estaréis acompañando y apoyando en el día de la defensa de la tesis.

Ahora, es el turno de mis directores de tesis. Emilio. Recuerdo perfectamente el primer día que te conocí cuando fui a presentarme tras firmar mi primer contrato de trabajo, y como Personal Técnico de Apoyo. Gracias por la confianza ofrecida hasta tal punto de aventurarnos a solicitar la ayuda FPU, por darme la oportunidad de realizar la Tesis Doctoral, y por orientarme en este camino que no ha sido para nada sencillo. María, gracias por contagiarme durante este camino de ese ímpetu y constancia que te caracteriza.

Jacqueline, siempre te admiré en mi etapa como alumno en el Grado en Enfermería, y en nuestra etapa como compañeros aún más; tu sabiduría y visión de la vida me han servido para seguir adelante en muchas ocasiones tanto en el ámbito profesional como en el personal. Agradecer a todos los miembros de mi grupo de investigación "CTS-436, Aspectos Psicosociales y Transculturales de la Salud y la Enfermedad" todo el apoyo y ánimo brindado. Especial mención a Dani, además de compañero de trabajo, amigo y compañero de promoción del Grado en Enfermería. Gracias porque mi andadura como

profesional universitario en parte comenzó gracias a que me informaste de la existencia de unos desconocidos contratos de personal técnico de apoyo. Gracias también por tu visión realista de lo complicada que es la carrera universitaria.

Sevilla tiene un color especial, según Los del Río. Aunque no empezó especialmente bien mi estancia predoctoral en esta preciosa ciudad, cuando el COVID-19 obligó a todo un país, con cierta incertidumbre, a estar confinados. No fue un confinamiento sencillo, lejos de mis seres queridos y de mis compañeros de trabajo. Pero todo mejoró cuando se pudieron retomar las actividades de investigación en los laboratorios del Instituto de la Grasa del CSIC. Gracias Javi y José María por la oportunidad de poder trabajar en los laboratorios del CSIC, y poder aprender numerosas técnicas y procedimientos imprescindibles para la realización de esta Tesis Doctoral. Y no todo fue rigurosidad científica, también salmorejos y cervecitas en el almuerzo con Javi después de mañanas intensas de trabajo.

Melilla no puede quedarse sin ser nombrada. Dos etapas distintas en el transcurso de la tesis, la primera al poco de matricularme en el programa de doctorado, y la segunda en el intenso último año de tesis. Agradecer a mis primos Pauli y Emi por hacerme más fácil el comienzo de mi primera etapa en Melilla. Lucía, gracias por devolverme el oxígeno que necesitaba cuando el dichoso COVID me obligó a estar encerrado en las cuatro paredes de la habitación del Hotel Ánfora, y también por seguir estando ahí como amiga, sabiendo escuchar y dando buenos consejos. Raúl, el creador de la mítica frase, pero si solo es un día a la semana, que al final acababan siendo más de uno, pero llenos de momentos necesarios para desconectar. Karol, dichoso el momento en el que me hiciste el lío para apuntarme a las clases de bachata. Miriam, eres pura energía y alegría, gracias por los ánimos que me has dado en estos últimos meses. María Olmo, una magnífica enfermera con vocación asistencial, pero sin miedo a aventurarse en el mundo del profesorado universitario. Y, sobre todo, una excelente amiga, que, a pesar de mis cambios de humor provocados por la Tesis, no ha dudado en intentar hacerme reír y darme la energía necesaria para cerrar esta etapa.

Soy una persona que siempre cumple sus promesas. Rocío, tanto cuando estábamos juntos como cuando decidimos separar nuestros caminos te hice la promesa de que te

mencionaría en los agradecimientos. Fuiste un gran apoyo cuando comencé la tesis, creíste en mí y en mis posibilidades, por ello te doy las gracias.

Mencionar a dos importantes personas del colectivo motrileño. Meli. Nos conocimos hace ya ocho años y medio, cuando comenzamos a ser vecinos en el mismo bloque en Granada, cuando empezaste a estudiar Bellas Artes. Qué buenos momentos pasamos ese año, en mi último curso como estudiante de grado. Pasaba más tiempo en tu piso que en el mío jaja. Y a pesar del transcurrir de los años, continuamos siendo buenos amigos, y tengo el honor de lucir tu talento en la portada de mi Tesis Doctoral. Encarni, nos conocimos casualmente a través de la desconexión y diversión que nos ofrecía una app de trivial. Desde entonces me has mostrado tu calidez humana, humildad y el sentido de la amistad, escuchándome siempre que lo he necesitado, dándome sabios consejos, y apoyándome con la tesis.

Por último, agradecer al Ministerio de Ciencias, Innovación y Universidades la concesión de la ayuda FPU; y también a todas aquellas personas que han colaborado en la realización de la tesis y a los participantes voluntarios del ensayo postprandial.