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Efecto del consumo de distintas grasas de origen vegetal sobre el tejido adiposo pardo y parámetros de inflamación crónica inductores de trastornos metabólicos

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Que los trabajos llevados a cabo en la elaboración de la Tesis Doctoral titulada: “Efecto del consumo de distintas grasas de origen vegetal sobre el tejido adiposo pardo y parámetros de inflamación crónica inductores de trastornos metabólicos”, presentada por Carmen Rodríguez García, han sido realizados bajo su dirección, reuniendo las condiciones académicas necesarias para su presentación y defensa pública para optar al grado de Doctor Internacional por la Universidad de Jaén.

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ÍNDICE DE ABREVIATURAS

2-MG	2-monoacilglicerol
Acil-CoA	Acil coenzima A
AG	Ácidos grasos
AGCC	Ácidos grasos de cadena corta
AGCM	Ácidos grasos de cadena media
AGCL	Ácidos grasos de cadena larga
AGE	Ácidos grasos esenciales
AGI	Ácidos grasos insaturados
AGL	Ácidos grasos libres
AGMI	Ácidos grasos monoinsaturados
AGPI	Ácidos grasos poliinsaturados
AGS	Ácidos grasos saturados
ALA	Ácido α -linolénico
AMPc	Adenosín monofosfato cíclico
AMPK	Proteína quinasa activada por 5' adenina monofosfato
AOV	Aceite de oliva virgen
AOVE	Aceite de oliva virgen extra
Apo	Apolipoproteína



ARA	Ácido araquidónico
ATGL	Lipasa de triglicéridos adiposa
ATP	Adenosín trifosfato
DG	Diacilglicerol
DGAT	Acil-CoA diacilglicerol aciltransferasa
DHA	Ácido docosahexaenoico
ECNT	Enfermedades crónicas no transmisibles
ECV	Enfermedades cardiovasculares
EFSA	Autoridad Europea de Seguridad Alimentaria
EPA	Ácido eicosapentaenoico
EROS	Especies reactivas de oxígeno
FDA	Administración de Alimentos y Medicamentos de EE.UU
FGF15	Factor de crecimiento de fibroblastos 15
G3P	Glicerol-3-fosfato
GLP-1	Péptido similar al glucagón-1
GPDH	Glicerolfosfato deshidrogenasa
HSL	Lipasa sensible a hormonas
IDL	Lipoproteínas de media densidad
IFNγ	Interferón γ



IL	Interleucina
Keap1	Proteína 1 asociada a ECH tipo kelch
LA	Ácido linoleico
LBP	Proteína de unión a LPS
LDL	Lipoproteínas de baja densidad
LPL	Lipoproteína lipasa
LPS	Lipopolisacárido
LXR	Receptor X del hígado (Liver X receptor)
MAPK	Proteína quinasa activada por mitógenos
MD2	Proteína de diferenciación mieloide 2
MGAT	Acil-CoA monoacilglicerol aciltransferasa
MGL	Monoglicérido lipasa
myf5	Factor miogénico 5
NF-κB	Factor nuclear kappa B
Nrf2	Factor respiratorio nuclear 2
OMS	Organización Mundial de la Salud
ONU-FAO	Organización de las Naciones Unidas para la Agricultura y la Alimentación
PGC-1α	Proliferador de peroxisomas-1 α
PKA	Proteína quinasa A



PLIN1	Perilipina 1
PPAR	Receptores activados por proliferadores de proxisomas
QM	Quilomicrón
RXR	Receptor X de retinoides
SNS	Sistema nervioso simpático
TG	Triglicéridos
TGCM	Triglicéridos de cadena media
TGF-β	Factor de crecimiento transformante- β
TLR	Receptor de tipo Toll (Toll-like receptor)
TNF-α	Factor de necrosis tumoral α
UCP1	Proteína desacoplante 1
USDA	Departamento de Agricultura de los Estados Unidos
VLDL	Lipoproteínas de muy baja densidad

RESUMEN



1. RESUMEN

ANTECEDENTES

Actualmente, el 70% de las muertes a nivel mundial se deben a las enfermedades crónicas no transmisibles. Entre los principales factores de riesgo de este tipo de enfermedades se encuentra la anormal o excesiva acumulación de grasa corporal, cuya prevalencia ha incrementado drásticamente como consecuencia de la transición nutricional mundial. Esta transición se caracteriza principalmente por el abandono de las dietas tradicionales y la adquisición de patrones de dieta occidentales caracterizados por una densidad energética elevada, un alto consumo en grasas, grasas saturadas y azúcares. Se ha considerado que los aceites vegetales son los principales responsables de la transición hacia dietas altas en grasa. Los aceites vegetales más consumidos en todo el mundo son el aceite de oliva en la región mediterránea, el aceite de palma y de coco en Asia, el aceite de soja en América y el aceite de girasol, que se consume sobre todo en el norte de África y en Europa del Este. Históricamente, las grasas y los aceites han suscitado un controvertido debate sobre el tipo y las cantidades óptimas utilizadas en la dieta, su papel en la regulación del peso corporal y su importancia en la etiología de las enfermedades crónicas no transmisibles. Por su parte, las guías alimentarias mundiales recomiendan disminuir la ingesta de grasa de la dieta, sin tener cuenta ni la composición de los distintos aceites ni el impacto de su consumo en la salud. La principal diferencia entre la ingesta de aceites es su composición de ácidos grasos, siendo los ácidos grasos monoinsaturados, poliinsaturados y saturados los predominantes en aceite de oliva virgen extra, aceite de girasol y aceite de coco respectivamente. Debido a su composición, el metabolismo de los ácidos grasos que componen estos aceites difiere, siendo relevante estudiar las implicaciones de su consumo en la salud.

OBJETIVOS

Analizar en un modelo murino los efectos del consumo de una dieta alta en grasa basada en aceite de oliva virgen extra, aceite de girasol o aceite de coco, sobre la



microbiota intestinal, el tejido adiposo pardo, el tejido adiposo blanco, marcadores de inflamación y parámetros metabólicos relacionados con el tejido adiposo.

RESULTADOS Y DISCUSIÓN

Tras 16 semanas de intervención nutricional, los ratones del grupo de dieta alta en grasa de aceite de oliva virgen extra y aceite de girasol tuvieron un incremento de peso significativo, mientras que los ratones del grupo de dieta alta en grasa de aceite de coco no mostraron cambios en el peso con respecto al grupo control.

Sin embargo, el análisis de la microbiota intestinal mostró que todos los ratones intervenidos con una dieta alta en grasa presentaban una alteración en la composición de la microbiota, caracterizada principalmente por la disminución del género *Lactobacillus* y el incremento de *Lactococcus*. El resto de modificaciones en la microbiota fueron diferentes dependiendo del tipo de grasa de la dieta. Por una parte, la dieta alta en grasa de aceite de coco incrementó la abundancia de *Staphylococcus*, *Prevotella* y *Bacteroides*. La dieta alta en grasa de aceite de girasol incrementó la abundancia de *Sphingomonas* and *Neisseria*. Ambas dietas, la de aceite de coco y aceite de girasol, produjeron una disminución de *Akkermansia*, promoviendo un microambiente intestinal proinflamatorio. Por otra parte, la dieta alta en grasa de aceite de oliva virgen extra, mantuvo la abundancia de *Akkermansia*, incrementó el ratio de Firmicutes/Bacteroidetes y redujo la abundancia de *Enterococcus*, *Staphylococcus*, *Neisseria* y *Pseudomonas*, promoviendo un microambiente intestinal antiinflamatorio.

Se evaluó la capacidad de inducir termogénesis de cada una de las dietas, a través del aumento de la proteína UCP-1 en el tejido adiposo marrón interescapular. El consumo de una dieta alta en grasa con aceite de oliva virgen extra aumentó la expresión proteica de UCP-1 mientras que las otras grasas de intervención no mostraron cambios significativos.

Asimismo, se evaluó el efecto de las distintas dietas altas en grasa sobre la pardización del tejido adiposo blanco subcutáneo y visceral. Los resultados mostraron que la dieta alta en grasa de aceite de oliva virgen extra fue la única que incrementaba la expresión proteica de PGC-1 α y Nrf2 en el tejido adiposo blanco subcutáneo y de



PGC-1 α en el tejido adiposo visceral, ambos implicados en los procesos de adipogénesis y biogénesis mitocondrial.

El análisis de las citoquinas circulantes en suero, también reveló que los efectos en el perfil inflamatorio eran independientes de los efectos en el peso corporal. Por una parte, la dieta alta en grasa de aceite de coco incrementó de forma exacerbada los niveles séricos de las citoquinas proinflamatorias IL-6 y TNF- α . Mientras que la dieta alta en grasa de aceite de oliva virgen extra incrementó moderadamente los niveles séricos de IL-6 y de forma exacerbada los de TGF- β , promoviendo un ambiente antiinflamatorio. Del mismo modo la dieta alta en grasa de aceite de girasol incrementó moderadamente los niveles circulantes de TGF- β y disminuyó los de IL-6, promoviendo también un ambiente antiinflamatorio.

En lo que respecta al análisis de parámetros metabólicos relacionados con el tejido adiposo, se analizaron los niveles circulantes de dos adipoquinas: leptina y adiponectina, los triglicéridos séricos y los triglicéridos hepáticos. Los datos mostraron que la dieta alta en grasa de aceite de oliva virgen extra y aceite de girasol producían un incremento de la leptina y una disminución de la adiponectina, acorde a la ganancia de peso corporal. Sin embargo, a pesar de no producir efectos en el peso corporal, la dieta alta en grasa de aceite de coco produjo hipoleptinemia e hipoadiponectinemia, ambos relacionados con la disfunción del tejido adiposo característica de patologías como el síndrome de lipodistrofia. Del mismo modo, la dieta alta en grasa de aceite de coco produjo un incremento exacerbado de los triglicéridos séricos y junto con la dieta alta en grasa de aceite de girasol, promovieron la acumulación de triglicéridos hepáticos.

CONCLUSIONES

1. En el contexto de una dieta alta en grasa, todos los aceites comestibles de origen vegetal estudiados, independientemente de su composición en ácidos grasos, producen disbiosis intestinal.
2. A pesar de ello, las disbiosis producidas por estas dietas tienen características diferenciales, de tal forma que, la dieta alta en grasa de aceite de oliva virgen extra



produce un microambiente intestinal antiinflamatorio, mientras que, la dieta alta en aceite de coco, produciría un microambiente intestinal proinflamatorio.

3. La dieta alta en grasa en aceite de oliva virgen extra o aceite de girasol incrementa la actividad termogénica del tejido adiposo pardo interescapular. Por el contrario, la dieta alta en grasa de aceite de coco no promueve la termogénesis en el tejido adiposo pardo interescapular.

4. La dieta alta en grasa en aceite de oliva virgen extra incrementa la capacidad de pardización del tejido adiposo blanco visceral y subcutáneo. Por el contrario, la dieta alta en grasa de aceite en girasol o aceite de coco no promueve la pardización del tejido adiposo blanco.

5. La dieta alta en grasa de aceite de coco promueve un estado proinflamatorio caracterizado por un aumento de marcadores inflamatorios en suero mientras que la dieta alta en grasa de aceite de oliva virgen extra o aceite de girasol promueven un estado antiinflamatorio.

6. La dieta alta en grasa de aceite de oliva virgen extra o aceite de girasol producen hiperleptinemia e hipoadiponectinemia. A nivel metabólico, la dieta alta en grasa de aceite de girasol o aceite de coco promueven la acumulación hepática de triglicéridos. Además, la dieta alta en grasa de aceite de coco produce hipoleptinemia, hipoadiponectinemia e hipertrigliceridemia alteraciones que de forma característica aparecen en el síndrome de lipodistrofia.



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RESUMEN

SUMMARY



1. SUMMARY

BACKGROUND

Nowadays, 70% of worldwide deaths are due to chronic non-communicable diseases. Among the main risk factors for these diseases are abnormal or excessive accumulation of body fat, whose prevalence has increased dramatically as a consequence of the global nutritional transition. This transition is mainly due to the switch from traditional diets to Western dietary patterns characterised by high energy density, high intakes of fat, saturated fats and sugars. Vegetable oils have been considered to be primarily responsible for the transition to high-fat diets. The most widely consumed vegetable oils worldwide are olive oil in the Mediterranean region, palm and coconut oil in Asia, soybean oil in the Americas and sunflower oil, which is consumed mainly in North Africa and Eastern Europe. Historically, fats and oils have been a controversial topic because of the type and optimal amounts that have been used in the diet, their role in regulating body weight and their importance in the aetiology of chronic non-communicable diseases. Global dietary guidelines recommend reducing dietary fat intake, without considering either the composition of the different oils or the impact of their consumption on health. The main difference between oil intakes is their fatty acid composition, with monounsaturated, polyunsaturated and saturated fatty acids being predominant in extra virgin olive oil, sunflower oil and coconut oil respectively. Due to their composition, the metabolism of the fatty acids present in these oils differs, and it is relevant to study the implication of their consumption on health.

AIMS

To analyse in a murine model the effects of consuming a high-fat diet based on extra virgin olive oil, sunflower oil or coconut oil on gut microbiota, brown adipose tissue, white adipose tissue, markers of inflammation and metabolic parameters related to adipose tissue.



RESULTS AND DISCUSSION

After 16 weeks of nutritional intervention, mice in the group of high-fat diet based in extra virgin olive oil and sunflower oil had a significant weight gain, while mice in the high-fat diet based in coconut oil showed no change in weight with respect to the control group.

However, analysis of the gut microbiota showed that all mice treated with a high-fat diet had an altered microbiota composition, mainly characterised by a decrease in the *Lactobacillus* genus and an increase in *Lactococcus*. The other changes in the microbiota were different depending on the type of diet. On the one hand, the high-fat diet based in coconut oil increased the abundance of *Staphylococcus*, *Prevotella* and *Bacteroides*. The high-fat diet based in sunflower oil increased the abundance of *Sphingomonas* and *Neisseria*. Both the coconut oil and sunflower oil diets produced a decrease in *Akkermansia*, promoting a pro-inflammatory gut microenvironment. On the other hand, the high-fat diet based in extra virgin olive oil maintained the abundance of *Akkermansia*, increased the ratio of Firmicutes/Bacteroidetes and reduced the abundance of *Enterococcus*, *Staphylococcus*, *Neisseria* and *Pseudomonas*, promoting an anti-inflammatory gut microenvironment.

The ability of each diet to induce thermogenesis was assessed by increasing UCP-1 protein in interscapular brown adipose tissue. The consumption of a high-fat diet based in extra virgin olive oil increased UCP-1 protein expression while the other intervention oils did not show significant changes.

The effect of the different high-fat diets on the browning of subcutaneous and visceral white adipose tissue was also evaluated. The results showed that the high-fat diet based in extra- virgin olive oil was the only diet that increased the protein expression of PGC-1 α and Nrf2 in subcutaneous white adipose tissue and PGC-1 α in visceral adipose tissue, both involved in the processes of adipogenesis and mitochondrial biogenesis.

Analysis of circulating serum cytokines also revealed that the effects on the inflammatory profile were independent of the effects on body weight. On the one hand, the high-fat diet based in coconut oil dramatically enhanced serum levels of the pro-inflammatory cytokines IL-6 and TNF- α . Whereas the high-fat diet based in extra



virgin olive oil moderately increased serum levels of IL-6 and exacerbated serum levels of TGF- β , promoting an anti-inflammatory environment. Similarly, the high-fat diet based in sunflower oil moderately increased circulating levels of TGF- β and decreased those of IL-6, also promoting an anti-inflammatory environment.

Regarding the analysis of metabolic parameters related to adipose tissue, circulating levels of two adipokines (leptin and adiponectin), serum triglycerides and hepatic triglycerides were analysed. The data showed that the high-fat diet based in extra virgin olive oil and sunflower oil produced an increase in leptin and a decrease in adiponectin, proportional to body weight gain. However, despite no effects on body weight were observed, the high-fat diet based in coconut oil produced hypoleptinaemia and hypoadiponectinaemia, both associated with the adipose tissue dysfunction characteristic of pathologies such as lipodystrophy syndrome. Similarly, the high-fat diet based in coconut oil produced an exacerbated increase in serum triglycerides and, together with the high-fat diet based in sunflower oil, promoted the accumulation of hepatic triglycerides.

CONCLUSIONS

1. Within the context of a high-fat diet, all vegetable edible oils studied, independently of their fatty acid composition, promote intestinal dysbiosis.
2. However, dysbiosis produced by these diets have differential characteristics, such that the extra virgin olive oil high-fat diet produces an anti-inflammatory gut microenvironment, while the coconut oil high-fat diet produces a pro-inflammatory gut microenvironment.
3. The high-fat diet based in extra virgin olive oil or sunflower oil increases the thermogenic activity of interscapular brown adipose tissue. In contrast, the high-fat diet based in coconut oil does not promote thermogenesis in interscapular brown adipose tissue.
4. The extra virgin olive oil high-fat diet increases the browning capacity of visceral and subcutaneous white adipose tissue. In contrast, a diet high-fat diet based in sunflower oil or coconut oil do not promote the browning of white adipose tissue.



5. The coconut oil high-fat diet promotes a pro-inflammatory state characterised by an increase in serum inflammatory markers while the high-fat diet based in extra virgin olive oil or sunflower oil promotes an anti-inflammatory state.
6. The high-fat diet based in extra virgin olive oil or sunflower oil leads to hyperleptinaemia and hypoadiponectinaemia. The high-fat diet based in sunflower oil or coconut oil promotes hepatic accumulation of triglycerides at metabolic level. In addition, a coconut oil high-fat diet leads to hypoleptinaemia, hypoadiponectinaemia and hypertriglyceridaemia, which are characteristic of lipodystrophy syndrome.

ANTECEDENTES
BIBLIOGRÁFICOS



2. ANTECEDENTES BIBLIOGRÁFICOS

2.1. Nutrición en el siglo XXI y enfermedades crónicas no transmisibles

La alimentación y la nutrición son disciplinas ampliamente estudiadas a lo largo de la historia, sin embargo, la investigación sobre la implicación de ambas en el desarrollo de enfermedades crónicas no transmisibles (ECNT) como el cáncer, la diabetes y las enfermedades cardiovasculares (ECV), es relativamente reciente a pesar de que Hipócrates, el padre de la medicina moderna, recalcó hace siglos la importante función que la nutrición tiene en la promoción de la salud con su célebre frase “*Que tu medicina sea tu alimento y el alimento tu medicina*”.

Las ECNT representan actualmente más del 70% de muertes a nivel mundial (OMS, 2021a). Además, la prevalencia de las mismas en la población, se ha puesto aún más de manifiesto en la persistente pandemia por el SARS-COV-2, puesto que estas patologías se asocian a un sistema inmunitario deficiente que agrava la infección y pronóstico de COVID-19 (Dhawan & Sharma, 2020).

Por su parte, la Organización Mundial de la Salud (OMS) dentro de los Objetivos de Desarrollo Sostenible para el año 2030, engloba propuestas para reducir la mortalidad asociada a las ECNT donde prevé que la estrategia sobre alimentación saludable debería ser suficiente para prevenir un amplio rango de estas enfermedades y una de cada cinco muertes a nivel mundial (Afshin et al., 2019; OMS, 2021a). Desde la perspectiva de la salud y el bienestar humanos, una definición aceptable de una dieta saludable sería aquella que promueve el crecimiento y el desarrollo humano óptimo y previene la malnutrición en todas sus formas.

En los últimos años, los cambios en la industrialización, la urbanización, el desarrollo económico y la globalización del mercado han tenido un impacto drástico en los estilos de vida y los patrones dietéticos. Las transiciones nutricionales se han considerado un factor clave en el aumento de la prevalencia de las ECNT, en particular de las ECV en los países de ingresos bajos y medios (Shankar et al., 2017). A medida que los patrones dietéticos mundiales han ido cambiando, se ha producido un dramático empeoramiento de la alimentación. De hecho, muchas poblaciones están pasando de



las dietas tradicionales a las occidentales, caracterizadas por el elevado consumo de alimentos de alta densidad energética, ricos en grasas, grasas saturadas principalmente de origen animal, y en azúcares libres (Madrigal et al., 2020). Además, los patrones de actividad física se han modificado debido a un mayor acceso al transporte y la naturaleza cambiante de muchos trabajos, por lo que las políticas de promoción de la salud han tenido que focalizarse en tratar las dos vertientes de la malnutrición. Concepto que según la OMS engloba tanto la problemática del déficit como del exceso en la ingesta energética o de determinados nutrientes (OMS, 2021b). La desnutrición ha sido y sigue siendo un reto de salud pública principalmente en los países de ingresos bajos y medios, afectando mayoritariamente a la población infantil que se encuentra en situaciones más desfavorecidas a nivel socioeconómico y con acceso limitado a diversos recursos (Boah et al., 2019). Sin embargo, el foco de preocupación actual se encuentra en el lado opuesto a esta situación: el desequilibrio por el exceso en el consumo y déficit en el gasto energético (Stefan, 2020). Esta vertiente de malnutrición es la principal responsable de una anormal y excesiva acumulación de grasa corporal característica del sobrepeso u obesidad (Barazzoni & Gortan Cappellari, 2020). Que, a pesar de considerarse tradicionalmente un problema exclusivo de países con altos ingresos, ha incrementado drásticamente en los países de ingresos bajos y medios, donde coexisten actualmente la desnutrición y la obesidad (OMS, 2021b). Esta problemática ha alcanzado dimensiones epidémicas, ya que su prevalencia está aumentando exponencialmente y más de 2,5 millones de personas mueren anualmente como consecuencia de la anormal o excesiva acumulación de grasa, debido principalmente a que ambos son factores de riesgo de numerosas ECNT, que son las principales causas de muerte en el mundo tal y como se ha indicado previamente (OMS, 2021c; Ronto et al., 2018). A nivel global, se estima que un 40% de la población tiene sobrepeso u obesidad (Kumanyika et al., 2020; Roser, 2017). En el caso de España, según la Encuesta Europea de Salud del año 2020, en la población adulta hay un 15,5% y un 16,6% de mujeres y hombres que padecen obesidad respectivamente (INE, 2020). La mayoría de las causas del sobrepeso y la obesidad son prevenibles. Sin embargo, ningún país ha logrado aún revertir el crecimiento de esta epidemia y, actualmente las recomendaciones generales que se han establecido para disminuir el riesgo de



sobrepeso y obesidad son: reducir la cantidad de calorías consumidas procedentes de grasas y azúcares, aumentar la porción de ingesta diaria de frutos secos, cereales integrales, legumbres, verduras y frutas, y realizar actividad física regular (Gadde et al., 2018).

Históricamente, las grasas y los aceites alimentarios han suscitado un debate considerable sobre el tipo y las cantidades óptimas utilizadas en la dieta, su papel en la regulación del peso corporal y su importancia en la etiología de las ECNT. A pesar de las cuestiones controvertidas que rodean a las grasas alimentarias, se consideran nutrientes esenciales porque son necesarios para desempeñar funciones críticas en el organismo, como ser una fuente importante de nutrientes esenciales, mejorar la biodisponibilidad de los micronutrientes liposolubles y proporcionar un sustrato esencial para la síntesis de compuestos metabólicamente activos. Las grasas, también desempeñan un papel importante al mejorar el sabor y la palatabilidad de los alimentos, dado que contribuyen a la textura, sabor y aroma de los mismos (Aranceta & Pérez-Rodrigo, 2012). A pesar de estos beneficios, se ha demostrado que las dietas altas en grasas aumentan significativamente el peso corporal y se asocian a un mayor riesgo de acumulación anormal o excesiva de grasa debido principalmente a un balance energético desequilibrado (Ahmadi-Kani Golzar et al., 2019; Wang et al., 2020), así como a un mayor riesgo de desarrollar ECNT (Boateng et al., 2016). Sin embargo, se ha reconocido que la fuente y la calidad de la grasa dietética son un factor más importante que la cantidad total de la misma para la prevención de algunas ECNT (Guasch-Ferré et al., 2015; Jayawardena et al., 2020; Yusuf et al., 2004).

2.2. Grasas de origen vegetal en la dieta

2.2.1. Tipos de grasas

Las grasas alimentarias están formadas principalmente por triglicéridos (TG), formados químicamente por una molécula de glicerol que tiene esterificados sus tres grupos hidroxilo por tres ácidos grasos (AG). Los AG son moléculas orgánicas formadas por una larga cadena de hidrocarburos alifáticos que contienen un grupo



carboxilo en un extremo. Se clasifican convencionalmente por una característica química: la presencia o ausencia de dobles enlaces carbono-carbono (Wu et al., 2019). Atendiendo a esta clasificación, pueden diferenciarse en AG saturados (AGS) (cada carbono de la cadena de AG está saturado con átomos de hidrógeno), AG monoinsaturados (AGMI) (tienen un doble enlace), y AG poliinsaturados (AGPI) (tienen dos o más dobles enlaces) (Wu et al., 2019). Los AG insaturados (AGI) pueden clasificarse como *cis* o *trans*, según la configuración de sus dobles enlaces. La primera configuración se encuentra en la mayoría de los AGI de forma natural, mientras que la segunda es el resultado de un procesamiento tecnológico, como la hidrogenación (Bozza & Viola, 2010). Además, los AGPI pueden subdividirse a su vez en n-3 o n-6, según la posición del primer doble enlace desde el extremo metilo de la cadena de carbono (tercer o sexto respectivamente) (Wu et al., 2019). Debido a la ausencia de enzimas apropiadas, el cuerpo humano no puede sintetizar AGPI n-3 o n-6, por lo que se consideran AG esenciales (AGE) y deben obtenerse de modo exógeno (principalmente a través de la dieta) (Huang & Freter, 2015; Orsavova et al., 2015).

Los AG también pueden clasificarse atendiendo a la longitud de su cadena de carbono, que varía de 2 a 6 carbonos (cadena corta (AGCC)), de 8 a 12 carbonos (cadena media (AGCM)), de 14 a 18 carbonos (cadena larga (AGCL)) o de 20 a 24 carbonos (cadena muy larga) (Kihara, 2012).

2.2.2. Funciones

Las principales funciones de los lípidos de la dieta son el suministro y almacenamiento energético, el aporte de AGE, la contribución a la absorción de componentes de la dieta que son liposolubles como algunas vitaminas (A, D, E y K) y proporcionar un sustrato para la síntesis de compuestos metabólicamente activos como las hormonas esteroideas, progesterona, estrógeno y testosterona. (Aranceta & Pérez-Rodrigo, 2012). Además de estas funciones relacionadas estrechamente con la nutrición, los lípidos son moléculas complejas con una notable bioactividad y están involucrados en multitud de procesos biológicos (Wu et al., 2019).



Los lípidos desempeñan una importante función estructural, ya que forman parte de todas las membranas celulares y de los orgánulos. La membrana plasmática está formada por una bicapa de fosfolípidos permeable que contiene colesterol y proteínas. Los fosfolípidos poseen una cabeza hidrofílica situada en la cara externa de la membrana y una cola hidrofóbica en la parte interna. Los AG que componen los fosfolípidos de la membrana, que dependen de la composición de la grasa dietética, pueden ser AGS o AGI, los primeros con una conformación recta y los segundos con un doble enlace *cis*. Debido a estas conformaciones, los AGS aparecen más empaquetados en la membrana celular y, a su vez, más ordenados que los AGI, lo que afecta a algunas propiedades fisicoquímicas de la membrana como la fluidez, los AGI contribuyen a aumentarla mientras que los AGS le aportan rigidez (Fernández-Quintela et al., 2007). Además, la incorporación de AG específicos a los fosfolípidos de las membranas plasmáticas provoca alteraciones en las características de los microdominios lipídicos alrededor de los receptores transmembrana (Lemaire-Ewing et al., 2012; Lingwood & Simons, 2010). Entre ellos, los AGS pueden modular el reclutamiento de los receptores tipo Toll (TLR) así como las consiguientes respuestas inflamatorias (Cheng et al., 2012; Wong et al., 2009; Wu et al., 2019). De hecho, se ha atribuido un efecto proinflamatorio a los AGS, especialmente al ácido láurico, debido a su efecto sobre las vías de señalización TLR2 y TLR4 (B. Li et al., 2020). Al interactuar con los TLR, los AGS promueven la activación de las dos vías principales de señalización relacionadas con la inflamación en las células, la vía del factor nuclear kappa B (NF- κ B) y la vía de la proteína quinasa activada por mitógenos (MAPK). Ambas dan lugar a un aumento de la expresión y liberación de citoquinas y quimiocinas proinflamatorias, induciendo una inflamación local o sistémica, a la que también contribuye la liberación masiva de especies reactivas de oxígeno (EROS) (B. Li et al., 2020).

Según su estructura fisicoquímica, los lípidos de la dieta ejercen un impacto nutrigenómico diferente, modulando la activación transcripcional, la transducción de señales y la expresión de los genes que intervienen en el control de la inflamación, la presión arterial, el metabolismo de los lípidos y otras respuestas metabólicas (Bordoni



et al., 2021). Los AG no esterificados y sus tioésteres de acil coenzima A (acil-CoA) son ligandos naturales para numerosos receptores nucleares y factores de transcripción que son cruciales para las vías metabólicas, como los receptores activados por proliferadores de peroxisomas (PPAR), los receptores X de retinoides (RXR) y los receptores X del hígado (LXR) (Jump et al., 2013). Además de los efectos sobre los receptores nucleares, los AG también influyen en la expresión génica al actuar como ligandos de ciertos receptores de AG libres (AGL) de la superficie celular e influir en las cascadas de señalización posteriores (Nakamura et al., 2014).

Por otra parte, las grasas alimentarias se metabolizan en diversos conjuntos de metabolitos altamente bioactivos. Por ejemplo, tanto los AGPI n-6 como n-3 se convierten en eicosanoides mediante procesos enzimáticos y no enzimáticos (Mitchell & Kirkby, 2019). Los eicosanoides son derivados lipídicos oxidados que controlan el inicio, la magnitud y la duración de la inflamación (Calder et al., 2013). Los derivados de los AGPI n-6, se consideran proinflamatorios (Marion-Letellier et al., 2015), entre ellos, el ácido araquidónico (ARA) es el principal sustrato para la producción de eicosanoides y se metaboliza principalmente en leucotrienos, prostaglandinas y tromboxanos (Gleim et al., 2012; Wu et al., 2019). Sin embargo, los eicosanoides derivados de los AGPI n-3, como el ácido eicosapentaenoico (EPA) y el ácido docosahexaenoico (DHA), dan lugar a eicosanoides con funciones principalmente antiinflamatorias o pro-resolutivas, como las resolvinas (Serhan & Petasis, 2011), que aceleran la resolución de la inflamación (Serhan & Chiang, 2013), inhibiendo la migración de las células inmunitarias, la síntesis de metaloproteinasas de matriz y la liberación de citoquinas inflamatorias, como la interleucina (IL)-1 α , IL-1 β , IL-6, IL-8, IL-12, el factor de necrosis tumoral α (TNF α) y el interferón γ (IFN γ) (Calder, 2015). El principal mecanismo por el que los AGPI n-3 bloquean la tormenta de citoquinas es a través de la supresión de la señalización inflamatoria del NF- κ B (Calder, 2017). En este sentido, los AGMI como el ácido oleico o el palmitoleico, también tienen propiedades antiinflamatorias a través de la inhibición en la producción de citoquinas inflamatorias como IL-1 β , inhibiendo la formación del inflamosoma y manteniendo la

actividad de la proteína quinasa activada por 5' adenina monofosfato (AMPK), que reduce la respuesta inflamatoria (Chan et al., 2015; Murphy et al., 2015).

2.2.3. Metabolismo, biodisponibilidad, bioaccesibilidad y toxicidad

Al igual que en otros compuestos alimentarios bioactivos, el efecto nutricional de los lípidos depende de su liberación en el tracto gastrointestinal, su absorción intestinal, su biodisponibilidad, y su metabolismo.

2.2.3.1. Metabolismo

La función principal del metabolismo de los lípidos es llevarlos a los tejidos periféricos para su uso o devolverlos al hígado para su reciclaje o eliminación. Existen tres vías principales del metabolismo de los lípidos: la exógena, la endógena y el transporte inverso del colesterol.

- Vía exógena

La vía exógena se utiliza para procesar los lípidos de la dieta. En ella, las grasas dietéticas son digeridas mediante procesamientos mecánicos y químicos a lo largo del tracto digestivo. Los TG son hidrolizados en el lumen del tracto gastrointestinal por las lipasas produciendo AG y 2-monoacilglicerol (2-MG) (Mu & Høy, 2004).

La regioespecificidad de la distribución de los AG se correlaciona con la forma en que cada AG será absorbido. La hidrólisis promovida por las lipasas tiene lugar preferentemente en las posiciones sn-3 y sn-1, respectivamente (Figura 1) (Bracco, 1994).

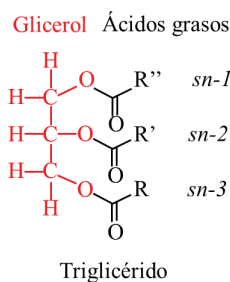


Figura 1. Estructura química de los triglicéridos



En lo que respecta a los AGCC, la lipasa pancreática presenta una alta afinidad por los situados en las posiciones sn-3 y sn-1, lo que explica una mayor actividad de la enzima y, una mejor absorción de dichos AG. Los productos generados por el proceso de hidrólisis entran en el duodeno, donde las lipasas pancreáticas se unen a la superficie de los TGs restantes junto con las co-lipasas (un componente del jugo pancreático) para seguir llevando a cabo el proceso de digestión (Mu & Høy, 2004).

Los enterocitos son células epiteliales del intestino formadas por una membrana apical y otra basolateral responsables de la captación y absorción de la mayoría de los nutrientes. Los AG atraviesan la membrana apical por difusión pasiva o por transporte mediado por proteínas (D'Aquila et al., 2016). Los AG se activan mediante su unión a coenzima A, generando acil-CoAs por la actividad de la acil-CoA sintetasa (ACS) y se utilizan para la resíntesis de TG en el retículo endoplasmático, a través de la vía acil-CoA monoacilglicerol aciltransferasa (MGAT). El primer paso implica la síntesis de diacilglicerol (DG) a partir de 2-MG y un acil-CoA graso por la actividad MGAT. El segundo paso consiste en la síntesis de TG a partir de DG y un acil-CoA graso mediante la actividad acil-CoA diacilglicerol aciltransferasa (DGAT). Los TG también se pueden sintetizar por la vía del glicerol-3-fosfato (G3P) (Yen et al., 2015).

Los TG son hidrofóbicos y requieren de ayuda para su transporte o almacenamiento en entornos acuosos como la sangre o el citoplasma. Por ello, se empaquetan en quilomicrones (QM) para su secreción y transporte en la sangre, o en gotas lipídicas citoplasmáticas comúnmente denominadas “lipid droplets” (Iqbal & Hussain, 2009; Sturley & Hussain, 2012). Los QM son la principal lipoproteína rica en TG producida por los enterocitos en respuesta a una comida que contiene grasa dietética. Los QM se sintetizan en el RE y se transportan a través del aparato de Golgi y de la vía secretoria mediante vesículas de transporte en el lado basolateral del enterocito donde se liberan al sistema linfático, adquieren la apolipoproteína (Apo) B y posteriormente pasan a la circulación. En la circulación, adquieren ApoC-II, ApoC-III y ApoE (Gaudet et al., 2017). Los QM entregan los AG de la dieta a los tejidos de todo el cuerpo a través de la actividad de la lipoproteína lipasa (LPL), que hidroliza el TG presente en el núcleo del QM liberando AGL para su absorción celular (Figura 2)

(D'Aquila et al., 2016; Hussain, 2014; Kersten, 2014). Éstos se almacenan como lípidos neutros en los adipocitos y como TG en los macrófagos, mientras que se almacenan directamente o se oxidan en las células musculares esqueléticas y cardíacas (Willnow et al., 1994).

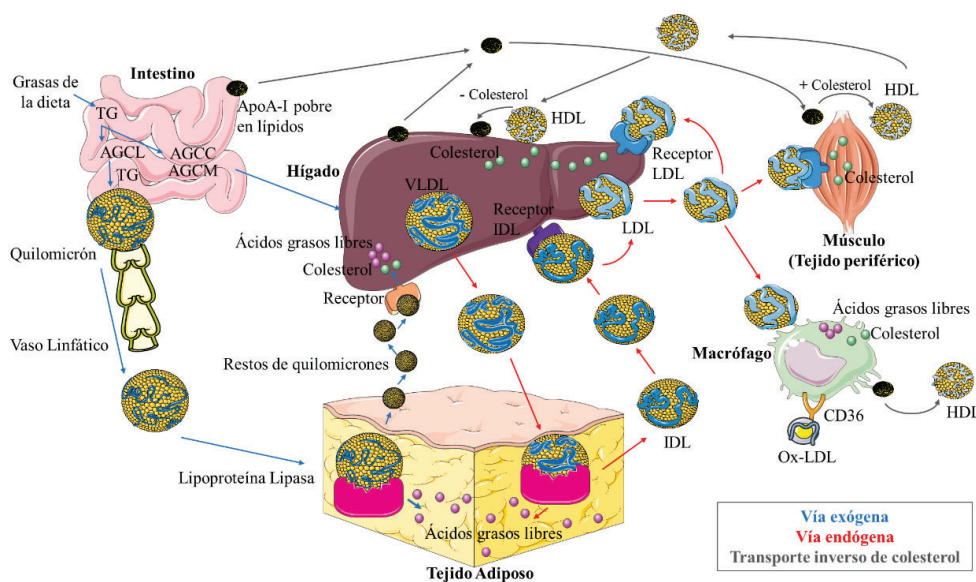


Figura 2. Metabolismo de las grasas. Figura modificada de (Remmerie & Scott, 2018)

Los TG formados por AG de cadena media (AGCM) (TGCM), se metabolizan más rápidamente que el resto. Después de que las lipasas liberen los AG de los TG, los AGCM y los AGCL tienen destinos metabólicos muy diferentes. Los AGCL se reesterifican predominantemente en TG para formar QM y favorecer la acumulación de grasa en otros tejidos. Sin embargo, los AGCM, son más solubles en agua debido a su menor longitud de cadena, y no requieren ser empaquetados en QM para ser transportados. Los AGCM son conducidos a la vena porta hepática, que une directamente el tracto gastrointestinal con el hígado (Figura 2) (Dayrit, 2014). En el hígado, los AGCM entran directamente a la mitocondria mediante difusión y sin requerir carnitina. Allí son principalmente metabolizados por la β -oxidación, produciendo acil-CoA, que entra en el ciclo de los ácidos tricarboxílicos para producir energía o se metaboliza en cuerpos cetónicos, que se transportan a tejidos como el cerebro, músculos y corazón para ser utilizados como fuente de energía (Dayrit, 2015).



- Vía endógena

La vía endógena hace referencia al procesamiento de los lípidos sintetizados en el hígado, donde se generan lipoproteínas de muy baja densidad (VLDL) que contienen TG y colesterol ensamblados con ApoB (Figura 2). Las VLDL son hidrolizadas en el tejido adiposo por la LPL en restos de VLDL (o lipoproteínas de media densidad (IDL)) y AGL para su captación celular. Las IDL son hidrolizadas por las lipasas hepáticas en lipoproteínas de baja densidad (LDL), que son las responsables de transportar la mayor parte de colesterol. La unión a sus receptores, provoca su captación y la consiguiente liberación de colesterol libre. Por su parte, las LDL restantes pueden unirse a la ApoA libre secretada por el hígado, formando la lipoproteína A, que puede unirse a la matriz extracelular y depositarse en los vasos, conduciendo al desarrollo de aterosclerosis cuando existe un exceso de LDL (Figura 3) (Nordestgaard et al., 2010).

- Transporte inverso de colesterol

El transporte inverso del colesterol es el proceso de eliminar el colesterol de los tejidos y devolverlo al hígado, es crucial para la homeostasis en el metabolismo lipídico, ya que la mayoría de las células de los órganos periféricos no pueden catabolizar el colesterol (Phillips, 2014). El hígado y los intestinos segregan ApoA-I pobre en lípidos, que se lipida en la circulación a través del eflujo de colesterol por los transportadores situados en los tejidos periféricos y los macrófagos (Figura 3). La adquisición de colesterol genera lipoproteínas de alta densidad (HDL), que llegan al hígado donde se elimina selectivamente el colesterol presente en ellas. Siendo de este modo el exceso de colesterol excretado en la bilis y las HDL hidrolizadas o devueltas a la circulación para su re-lipidación (Gaudet et al., 2017).

2.2.3.2. Biodisponibilidad

La biodisponibilidad de los lípidos se refiere a la fracción de los lípidos ingeridos que finalmente llega a la circulación sistémica, para ser distribuida posteriormente a los tejidos y órganos donde puede ser almacenada, utilizada o excretada. Sin embargo, no toda la carga lipídica de una comida es biodisponible para ejercer una función biológica. De hecho, solo la fracción lipídica bioaccesible, que es



la que se libera de la matriz alimentaria, está disponible para la absorción intestinal. Por lo que, para los lípidos, la posibilidad de ejercer un efecto biológico depende estrictamente de su bioaccesibilidad (Dima et al., 2020). Las diferentes escalas de la estructura de los lípidos, incluidas sus interacciones con la matriz alimentaria, pueden modular significativamente la bioaccesibilidad y la biodisponibilidad de los AG de la dieta, pero también su destino metabólico final. La bioaccesibilidad se define como la proporción de un nutriente o de compuestos alimentarios que se libera de la compleja matriz alimentaria, quedando disponible para la absorción intestinal y la incorporación a los QM con secreción en el sistema linfático (Vors et al., 2020).

La interacción entre las grasas alimentarias y la matriz alimentaria (sólida o líquida) es imprescindible para garantizar su accesibilidad, digestibilidad y biodisponibilidad. El contenido total de grasa de una comida puede modular la biodisponibilidad de los AG de la dieta, principalmente a través de la estimulación de secreción de jugo pancreático y la activación de las lipasas pancreáticas, lo que hace que los AG de la dieta sean más accesibles (Plourde & Cunnane, 2007). La mayoría de los lípidos de la dieta se encuentran esterificados en TG, pero solamente pueden ser absorbidos cuando se hidrolizan en AG y 2- MG después de la hidrólisis digestiva (Michalski et al., 2013). La velocidad a la que se liberan los AG de los TG durante la lipólisis, está inversamente correlacionada con la longitud de su cadena y de la posición en el esqueleto de glicerol. Las lipasas digestivas hidrolizan preferentemente los AG esterificados en las posiciones sn-1 y sn-3 del esqueleto de glicerol, pero cuando los TG contienen AGCL en estas posiciones, su actividad hidrolítica disminuye (Bordoni et al., 2021). La matriz alimentaria es especialmente crucial para los AGPI n-3 como el ácido α -linolénico (ALA), debido a que su tasa de conversión en EPA y DHA es muy limitada (Plourde & Cunnane, 2007).

2.2.3.3. Toxicidad

La lipotoxicidad surge como consecuencia de la acumulación de intermediarios lipídicos biosintéticos, como las ceramidas y los DG, en tejidos no adiposos, e induce estrés en el retículo endoplásmico, inflamación, disfunción mitocondrial y, en última



instancia apoptosis (Hirabara et al., 2010). Las células β del páncreas se encuentran entre las más afectadas, cuya secreción alterada de insulina da lugar al desarrollo de resistencia a la insulina, y otras patologías relacionadas con la obesidad, como las ECV y el síndrome metabólico (Chaurasia & Summers, 2015). Otro mecanismo por el que el desequilibrio lipídico resulta tóxico es la peroxidación lipídica. Los peróxidos lipídicos son compuestos altamente reactivos que desencadenan muchos estados patológicos, como la inflamación, el cáncer, las enfermedades neurodegenerativas y la degeneración ocular y renal. Son el resultado de reacciones de peroxidación enzimática (ciclooxigenasa, citocromo P450 y lipoxigenasa) y no enzimática ("química de Fenton") de los lípidos, preferentemente los AGPI. Los peróxidos lipídicos no sólo alteran el ensamblaje, la composición, la estructura y la dinámica de las membranas celulares, sino que pueden presentar una toxicidad adicional con sus productos de degradación que son capaces de propagar una mayor generación de EROS (Bordoni et al., 2021).

2.2.4. Fuentes alimentarias de grasas

2.2.4.1. Grasas saturadas

Los AGS son aportados tanto por alimentos de origen animal (carne, marisco, leche, queso, huevos, etc.) como vegetal (semillas oleaginosas, aceitunas, aguacates, nueces) (Bordoni et al., 2021). Las principales fuentes alimentarias de AGS son la leche, el queso, la mantequilla, la margarina, los aceites vegetales, las carnes rojas y procesadas, las aves de corral, el chocolate y los postres (Górska-Warsewicz et al., 2019; Nettleton et al., 2018). El aceite de palma y coco son las principales fuentes alimentarias de AGS en los países tropicales y cada vez están más presentes en los alimentos procesados y envasados de todo el mundo, en sustitución de las grasas parcialmente hidrogenadas (Kadandale et al., 2019). En el caso de los aceites tropicales vírgenes, además pueden contener nutrientes con beneficios para la salud como carotenoides, vitamina E, compuestos fenólicos y potasio, sin embargo, se presentan principalmente en su forma refinada en los alimentos ultraprocesados (Lima & Block, 2019).



2.2.4.2. Grasas monoinsaturadas

Los AGMI-*cis* se encuentran principalmente en alimentos de origen vegetal. Mientras que las fuentes principales de AGMI-*trans* son los alimentos de origen animal como lácteos, carne y aceites vegetales parcialmente hidrogenados (Roehm, 2009). El ácido oleico, representa más del 90% de los AGMI-*cis* de la dieta (Mehta et al., 2021). En el patrón de dieta Mediterránea, el ácido oleico se obtiene principalmente de las aceitunas y del aceite de oliva virgen extra (AOVE), mientras que en el resto de zonas se obtiene de otro tipo de aceites vegetales como aceite de colza, semillas, frutos secos, aguacate y aceite de soja alto oleico (Wu et al., 2019).

2.2.4.3. Grasas poliinsaturadas

El ácido linoleico (LA), es el principal AGPI n-6 de la dieta, está presente en los aceites de soja, girasol, maíz y otras plantas, así como en los frutos secos y semillas (Eilander et al., 2015). El ácido linoleico se convierte de forma endógena en otros AGPI n-6, como el ácido γ -linolénico, el ácido di-homo- γ -linolénico y el ácido araquidónico; estos AGPI n-6 están presentes en pequeñas cantidades en otros alimentos como los huevos y las aves de corral (Lenighan et al., 2019). El principal AGPI n-3 de origen vegetal es el ácido α -linolénico (ALA), y se encuentra en un número limitado de alimentos y sus aceites (por ejemplo, semillas, nueces, linaza, aceite de soja y aceite de colza). Por otra parte, el EPA y el DHA, se encuentran principalmente en el pescado, el marisco y las algas marinas (Schwingshackl et al., 2018).

2.2.5. Recomendaciones en las guías dietéticas mundiales

Instituciones académicas, sociedades científicas y organizaciones nacionales e internacionales, como la OMS, la Organización de las Naciones Unidas para la Agricultura y la Alimentación (ONU-FAO), la Autoridad Europea de Seguridad Alimentaria (EFSA) y la Administración de Alimentos y Medicamentos de EE.UU (FDA) han elaborado documentos de consenso con directrices y recomendaciones sobre el consumo de grasas, diferentes AG y sus efectos sobre la salud (EFSA, 2010; FAO, 2010; FDA, 2017; OMS, 2018).



La OMS recomienda que menos del 30% de la ingesta calórica diaria proceda de las grasas. Además, alega que las grasas insaturadas (presentes en pescados, aguacates, frutos secos y en los aceites de girasol, soja, canola y oliva) son preferibles a las grasas saturadas (presentes en la carne grasa, la mantequilla, el aceite de palma y de coco, la nata, el queso, la mantequilla clarificada y la manteca de cerdo) (OMS, 2018). Por su parte, la EFSA recomienda para la población adulta, una ingesta mínima de grasas de 20% y máxima de 35% de energía (EFSA, 2010). Actualmente, las directrices dietéticas estadounidenses de 2020-2025 se centran más en los alimentos y los patrones dietéticos y, aunque recomiendan limitar las grasas saturadas y *trans*, ya no recomiendan explícitamente limitar la grasa total de la dieta (USDA, 2019). Hacen referencia a que es importante tener en cuenta los aceites como parte de una dieta saludable, ya que aportan AGE. Recomiendan el consumo de aceites vegetales como maíz, oliva, soja y girasol, y excluyen los procedentes de plantas tropicales como coco y palma, debido a su mayor porcentaje en AGS (USDA, 2019).

Con respecto a las grasas saturadas, el Departamento de Agricultura de los Estados Unidos (USDA) recomienda que la ingesta diaria de las mismas no supere el 10% de las calorías totales (USDA, 2019). La OMS sugiere reducir la ingesta de grasas saturadas a menos del 10% de la ingesta total de calorías, y la de grasas *trans* a menos del 1% (OMS, 2018). Por otro lado, la EFSA, que no especifica un límite para la ingesta de grasas saturadas, recomienda que la ingesta de grasas saturadas sea lo más baja posible (EFSA, 2017).

Aunque las fuentes vegetales de AGMI se han asociado a beneficios para la salud, la mayoría de organizaciones sanitarias no ofrecen recomendaciones dietéticas específicas sobre la ingesta de AGMI para personas sanas o para personas con enfermedades crónicas. Las recomendaciones actuales de AGMI varían entre el 12% y el 25% del contenido energético total (Mehta et al., 2021). En lo que respecta a los AGPI, las recomendaciones se establecen entre el 5% y el 11% de la ingesta energética total (Schwingshackl et al., 2021), y algunas instituciones como la EFSA, establecen recomendaciones específicas para los adultos sobre la ingesta de LA y ALA en 4% y



0.5% de la ingesta energética total respectivamente, y de 250 mg al día de EPA+DHA (EFSA, 2017).

Desgraciadamente las respuestas políticas en todo el mundo son actualmente inadecuadas tanto en su fuerza como en su alcance (Institute, 2015; Roberto et al., 2015), ya que la gran mayoría de los gobiernos han implementado campañas de educación dirigidas a cambiar el estilo de vida que no terminan de llegar a la población de un modo eficaz. La gran mayoría de las guías nutricionales adoptan un enfoque centrado en los nutrientes, recomendando limitar los alimentos con alto contenido en determinados nutrientes de riesgo o los alimentos "densos en energía y pobres en nutrientes" (Baker et al., 2020). La mayoría de los países, al igual que la OMS, tienen un mensaje clave en sus guías nutricionales sobre limitar la ingesta de grasas. Solo en unas pocas guías hacen referencia a la calidad de las grasas además de a la limitación de su consumo. En algunas guías incluso agrupan dentro de la misma recomendación sobre la ingesta de grasas, a las saludables y a las que no lo son (Herforth et al., 2019).

Tratar a todas las grasas de la dieta por igual puede inducir a errores en la interpretación por parte de la población. De hecho, en un estudio reciente realizado en la población australiana, concluyeron que la mayoría de la población no es capaz de seguir las recomendaciones dietéticas porque le parecen confusas (Ramachandran et al., 2018). Los programas y las políticas alimentarias deberían fomentar patrones dietéticos que favorezcan el aumento de la ingesta de las grasas saludables y la reducción de las que no lo son, por lo que se debería hacer hincapié en la optimización de los tipos de grasa dietética y no en la reducción de la grasa total. La calidad de la grasa se especifica generalmente por la cantidad relativa de AGMI y AGPI frente a los AGS y los AG *trans* (Beulen et al., 2018).

Uno de los patrones dietéticos que cumple con estos requisitos en cuanto a la calidad de las grasas de la dieta, es la dieta Mediterránea, que está considerada como el patrón de oro de la nutrición saludable. La dieta Mediterránea se caracteriza principalmente por ser una dieta alta en grasas, lo cual podría resultar contradictorio, ya que es ampliamente conocido el papel que desempeña la adherencia a la misma en



la promoción de la salud y prevención de enfermedades (Guasch-Ferré & Willett, 2021). La dieta Mediterránea tradicional se caracteriza por la abundancia de alimentos vegetales (ej., verduras de hoja verde, tomates, cebollas, hierbas, cereales integrales, legumbres y frutos secos), cantidades moderadas de productos lácteos fermentados, marisco, vino tinto (en Europa occidental) o té (en la zona africana) y pequeñas cantidades de carne roja, y el uso de aceite de oliva virgen (AOV), como principal fuente de grasa (Trichopoulou, 2007; Willett et al., 1995). La dieta Mediterránea tradicional, que es ampliamente conocida por su efecto cardioprotector, ha demostrado mejorar los factores de riesgo de las ECV, marcadores de inflamación, presión arterial, glucosa en sangre postprandial, prevención de enfermedades neurodegenerativas, ciertos tipos de cáncer y otras enfermedades crónicas y metabólicas como el síndrome metabólico (Estruch et al., 2006; Sofi et al., 2010; Yubero-Serrano et al., 2019). La adherencia a la Dieta Mediterránea, también se ha asociado con un mantenimiento del peso corporal saludable, que se debe principalmente a los efectos de la misma en la regulación del metabolismo lipídico, la mejora de la sensibilidad a la insulina, la inducción de un efecto saciante, y al mayor efecto termogénico que tienen las grasas insaturadas frente a la oxidación de las saturadas (Camargo et al., 2014; Casas-Agustench et al., 2009; Fernández-Montero et al., 2014; Kastorini et al., 2011; Mancini et al., 2016). Por lo que podría ser un patrón de nutrición saludable a seguir a nivel mundial incorporando las peculiaridades de la disponibilidad de alimentos de cada zona.

2.2.6. Patrones poblacionales del consumo de aceites vegetales

Entender los patrones globales y el impacto de los hábitos dietéticos es esencial para implementar políticas específicas para reducir la carga de ECNT en el mundo. La transición nutricional que se está produciendo a nivel mundial, se debe en parte al incremento del acceso y consumo de alimentos de baja calidad nutricional, cuya composición se basa en lo que comúnmente se denominan “calorías vacías”. Esta se caracteriza por el abandono de las dietas ricas en hidratos de carbono y fibra, en favor de dietas con mayores proporciones de grasas (Shankar et al., 2017). Además, el



aumento de la comercialización y los bajos precios de determinados productos, han contribuido a que los países de ingresos bajos y medios hayan adoptado dietas occidentales, caracterizadas por un alto consumo de grasas (Cordain et al., 2005). En estos países, la transición nutricional suele estar marcada por una rápida expansión del consumo de aceites (Popkin et al., 2002). De hecho, los aceites han contribuido más al aumento mundial de la disponibilidad de energía desde la década de los 80 que cualquier otro grupo de alimentos (Shankar et al., 2017).

Los aceites más consumidos a nivel mundial son el aceite de girasol, que predomina principalmente en el norte de África y Oriente Medio, el aceite de soja en América Latina y el Caribe, Australasia y América del Norte y Asia central y oriental; el aceite de palma en el sur y el sureste de Asia; el aceite de coco en la región tropical y en toda Asia; y el aceite de oliva en Europa occidental (Baker et al., 2020; Unhapipatpong et al., 2021).

Los aceites vegetales también suponen una gran parte de los ingredientes suministrados por los alimentos ultraprocesados (Stuckler et al., 2012), y debido a la globalización de los sistemas alimentarios, los patrones dietéticos poblacionales se basan cada vez más en el consumo de este tipo de alimentos, lo que tiene importantes consecuencias para la nutrición mundial, la salud pública y el medio ambiente (Baker & Friel, 2016; Popkin & Reardon, 2018). A partir de la década de los 50, los alimentos ultraprocesados se han convertido en una fuente importante de energía en países como Australia, Reino Unido, Canadá y Estados Unidos (Kearney, 2010; Popkin, 2006). Aunque se ha centrado más en el aporte de azúcares libres de estos productos, la utilización de aceite vegetal para la producción de los mismos ha crecido notablemente. Siendo coherente con el pensamiento de que los aceites vegetales contribuyen más que cualquier otro grupo de alimentos al incremento del suministro de calorías en el mundo y en definitiva al engorde de la dieta global (Hawkes, 2006).

El esfuerzo de la industria para eliminar las grasas *trans* procedentes de aceites vegetales parcialmente hidrogenados, ha dado lugar al uso de aceites tropicales altamente saturados como el de palma y coco, que gracias a su estabilidad y textura,



sustituyen fácilmente a los aceites vegetales parcialmente hidrogenados (Shankar et al., 2017). Debido a su bajo coste de producción y a su versatilidad como ingrediente alimentario, el aceite de palma es el aceite vegetal más utilizado en la fabricación de alimentos. Sin embargo, ha suscitado controversia en los últimos años debido a sus efectos nocivos en la salud y a las repercusiones indirectas de su cultivo en la salud planetaria como la deforestación tropical, la emisión de gases de efecto invernadero, la degradación de la tierra y el suelo, y el agotamiento de los recursos hídricos (Ismail et al., 2018; Kadandale et al., 2019). Estos acontecimientos, han dado lugar a que debido a su bajo coste, el aceite de coco se utilice como sustituto del aceite de palma como ingrediente de los alimentos ultraprocesados, a pesar de que no reporte beneficios en la salud planetaria sobre el aceite de palma (Bajželj et al., 2021; Lima & Block, 2019).

2.2.7. Aceites vegetales e implicaciones de su consumo en la salud

La mayoría de las veces, la preocupación por el aceite dietético comienza tras la aparición de un evento cardíaco o el diagnóstico de enfermedad arterial coronaria. Se ha propuesto que uno de los factores que contribuyen son los AGS contenidos en el aceite de coco, que es el aceite más consumido (Vijayakumar et al., 2016). Por el contrario, una dieta que proporciona la principal fuente de grasa como AGMI (frecuentemente en forma de aceite de oliva) y que incluye sólo pequeñas cantidades de productos animales se ha relacionado con un menor riesgo de ECV (Martínez-González et al., 2019). Por ello, las directrices de la ONU-FAO sobre la ingesta de aceites y grasas recomiendan la ingesta de aceites con altos niveles de ácido oleico, porque además de ser estables, reducen los niveles de colesterol en sangre, ejerciendo un efecto protector contra las ECV (FAO, 2010; Salas et al., 2015).

Entre los aceites más utilizados, el aceite de coco es el que contiene más AGS (81,2-94%) seguido del aceite de palma (49,7-57,5%). El contenido en ácido oleico, disminuye desde el aceite de oliva (54,5-80,2%), hasta el aceite de coco (5-10%). La concentración de LA aumenta a partir del aceite de coco (1-2,5%) hasta el aceite de girasol (60,2-72,1%), mientras que el aceite con menor contenido de ALA es el aceite de palma (0,01-0,25%) (Bordoni et al., 2021; Y. Zhou et al., 2020).



2.2.7.1. Aceite de coco

El aceite de coco es el principal producto de la palmera cocotera (*Cocos nucifera* L.). Existen diferentes tipos dependiendo de su elaboración: el aceite de coco virgen y aceite de coco. El aceite virgen se extrae de la almendra fresca solamente mediante molienda mecánica, mientras que el aceite de coco también se somete a procesos de refinado, blanqueado y desodorizado (Deen et al., 2021). Esto hace que solamente el virgen presente compuestos bioactivos como la vitamina E, esteroides y compuestos fenólicos (Robinson et al., 2019). Independientemente de esta clasificación, ambos aceites tienen una composición similar, y muy elevada en AGS en comparación con la mayoría de aceites y grasas vegetales presentes en la dieta (Tabla 1) (Mansor et al., 2012). Los AGS representan aproximadamente el 90% de la composición del aceite de coco, entre los que se incluyen los ácidos caproico, caprílico, cáprico, láurico y mirístico (Dayrit, 2015; Eyres et al., 2016).

Tabla 1. Perfil de ácidos grasos de los aceites de coco, girasol y oliva

<i>Ácido graso (%)</i>	<i>Aceite de coco</i>	<i>Aceite de girasol</i>	<i>AOVE</i>
Caprílico C8:0	5.8	-	-
Cáprico C10:0	6.2	-	-
Láurico C12:0	46.2	-	-
Mirístico C14:0	19.3	-	0.01
Palmítico C16:0	10	5.1	13.61
Palmitoleico C16:1	-	0.1	1.21
Estéarico C18:0	2.9	2.6	2.42
Oleico C18:1	7.2	39.6	77.33
Linoleico C18:2	1.9	52.6	3.64
α -Linolenico C18:3	-	0.1	0.82

El consumo de aceite de coco es más frecuente en los países en vías de desarrollo, como varios países de África y las regiones tropicales y subtropicales del mundo (Santos et al., 2019). Sin embargo, el aceite de coco ha atraído la atención de los medios de comunicación y de la población de todo el mundo, especialmente en Europa y Norteamérica. Su popularidad ha aumentado en los últimos años y muchos profesionales de la salud y otras autoridades han respaldado el uso del aceite de coco



como medio de cocción en sustitución de otros aceites vegetales, así como ingrediente complementario (Lima & Block, 2019). De hecho, debido a su alto grado de saturación y buena estabilidad, se ha incrementado notablemente su uso en la industria alimentaria para la elaboración de productos como bebidas, suplementos y alimentos ultraprocesados (Clegg, 2017; Jakobsen et al., 2009). Sin embargo, el aceite de coco no es recomendado para cocinar debido a que su punto de humo se sitúa en torno a 177°C y las temperaturas óptimas para freír son de aproximadamente 180°C. Su uso en procesos de fritura continua, da lugar a la formación de hidrocarburos aromáticos policíclicos y aminas aromáticas, compuestos que se han considerado carcinogénicos (Srivastava et al., 2010). Comparando su uso para cocinar con el de AOV, se considera menos apropiado debido a que el segundo tiene un punto de humo de 190°C (Boateng et al., 2016; Ros et al., 2014; Vieira et al., 2015).

Tanto la literatura científica como la que no lo es, promueven el consumo de aceite de coco basándose en la suposición de que tiene propiedades saludables. Estos beneficios incluyen la disminución del colesterol, la reducción del riesgo de ECV, la pérdida de peso, la mejora de las funciones cognitivas, la mejora del síndrome del intestino irritable, las afecciones tiroideas y la diabetes, la mejora del sistema inmunológico y la promoción de la curación de heridas (DebMandal & Mandal, 2011; Deen et al., 2021; Sankararaman & Sferra, 2018; Wallace, 2019).

El aceite de coco se diferencia del resto por su elevada composición en TGCM, formados principalmente por el ácido láurico y el ácido mirístico. Tradicionalmente, se ha considerado al ácido láurico como un AGCM, debido a que tiene una longitud de cadena de 12 carbonos (Teng et al., 2019). Tal y como se ha descrito previamente, los AGCM son transportados directamente al hígado por la circulación portal, no se incorporan en los QM y entran en las mitocondrias sin el transporte dependiente de carnitina, por lo que se someten a una oxidación relativamente rápida. Este proceso, daría lugar a una disminución en la síntesis de TG y VLDL (Panth et al., 2020). Debido a su eficaz oxidación, y al mayor gasto energético a través de la activación del sistema nervioso simpático (SNS), los AGCM se depositan menos en el tejido adiposo y aumentan la termogénesis inducida por la dieta y el gasto energético que los AGCL, lo



que sugiere efectos beneficiosos para la pérdida de peso (Jayawardena et al., 2021; Neelakantan et al., 2020). Además, según esta teoría, los AGCM inducen la saciedad, impidiendo que el individuo coma en exceso (Coleman et al., 2016). Por ello, se ha sugerido, que el ácido láurico, que representa el 50% del contenido del aceite de coco, tiene efectos beneficiosos en la mejora de la composición corporal. Sin embargo, aunque el ácido láurico se considera un AGCM en base a sus propiedades bioquímicas, también se ha clasificado como AGCL en términos de digestión y metabolismo (Jayawardena et al., 2021; Nikooei et al., 2021). El 70% del ácido láurico se transporta como AGCL (en forma de QM), mientras que el otro 30% permanece como AGCM (Swift et al., 1990). Por lo tanto, hay dos formas de transportar el ácido láurico en el organismo, y el hecho de que el aceite de coco ayude a perder peso debido a su composición en el mismo, sigue siendo discutible (Maki et al., 2018).

Debido a los beneficios que presenta el consumo de TGCM en la salud, también se han atribuido al consumo de aceite de coco propiedades como reducción de los niveles de colesterol y prevención de ECV. Sin embargo, los estudios son escasos y carecen de una visión holística sobre la dieta (Eyres et al., 2016; Joshi et al., 2020; Khaw et al., 2018). De hecho, cuando el ácido láurico llega al hígado, actúa como sustrato para producir apoA1 y apoB, contribuyendo a la formación tanto de partículas de HDL como LDL (Santos et al., 2019). Está ampliamente demostrado que las dietas ricas en AGS aumentan las concentraciones plasmáticas de LDL y pueden incrementar el riesgo de ECV en comparación con los AGI. El principal problema que presenta la recomendación del consumo de aceite de coco, es que se compone principalmente de AGS y la mayoría de las directrices dietéticas recomiendan la restricción general del consumo de AGS y establecen el consumo de las mismas por debajo del 10% de las calorías totales de la dieta (Santos et al., 2019). Específicamente para la grasa de coco, la Asociación Americana del Corazón, no recomienda su consumo por encima del límite establecido por el USDA para la ingesta de grasas saturadas (Sacks et al., 2017). Del mismo modo, la EFSA también ha emitido un dictamen científico sobre las declaraciones específicas sobre los AGCM como agente de reducción de peso (EFSA, 2011a).



2.2.7.2. Aceite de oliva virgen extra

El zumo oleoso de la aceituna, procede del fruto del olivo (*Olea europaea* L.), cuyo cultivo es típico de la zona mediterránea (Trichopoulou, 2007). Según las normas del Consejo Oleícola Internacional, los AOV son aceites obtenidos únicamente por procesos mecánicos que no provocan alteraciones en el aceite. Además, los AOV no pueden someterse a ningún otro tratamiento que no sea el lavado, la decantación, la centrifugación y la filtración. Los AOV se clasifican como AOVE si su acidez libre no supera el 0,8% y no presentan defectos organolépticos. Si, en el momento de la producción, la acidez supera el 3,3%, los aceites de oliva deben ser refinados (Tomé-Carneiro et al., 2020). Los AOV contienen un 20% de AGPI, un 80% de AGMI en forma de ácido oleico (Tabla 1) y una pequeña fracción de compuestos minoritarios (Gaforio et al., 2019; Jimenez-Lopez et al., 2020). Esta fracción, se compone a su vez de esteroides, hidrocarburos, compuestos volátiles, tocoferoles, carotenos, clorofilas, alcoholes alifáticos y triterpénicos, compuestos fenólicos, alcoholes fenólicos (hidroxitirosol y tirosol), estructuras secoiridoides (oleuropeína), lignanos y flavonoides (Boskou, 2006; Rodríguez-Morató et al., 2016). En general, el hidroxitirosol, el tirosol y las formas conjugadas de los secoiridoides como la oleuropeína son los más representativos (Trichopoulou, 2007).

El AOVE es la fuente de grasa más popular de la dieta Mediterránea, sin embargo, su consumo se ha incrementado en todo el mundo durante los últimos 25 años. Existen pruebas inequívocas de que el AOVE ejerce numerosas acciones saludables. Sobre la base de estas pruebas, el consejo de utilizar preferentemente el aceite de oliva para cocinar y aliñar es científicamente sólido (Gaforio et al., 2019; Visioli et al., 2020; Visioli et al., 2018). Además, el hecho de que la molécula de AGMI sólo tenga un doble enlace, hace que el AOVE sea más resistente a la oxidación y contribuye a sus propiedades antioxidantes, a su alta estabilidad y a su larga vida útil en comparación con los aceites ricos en AGPI (Owen et al., 2000). No obstante, aunque los alimentos ricos en AGMI, son menos perjudiciales que los que contienen grasas saturadas y *trans*, su consumo sin restricciones no está exento de consecuencias, sobre



todo porque los efectos de un consumo muy elevado no están claramente establecidos (Tomé-Carneiro et al., 2020).

Durante los últimos años, han incrementado las evidencias científicas sobre los efectos beneficiosos para la salud de la ingesta del aceite de oliva, especialmente del AOVE. Sus beneficios, se atribuyen principalmente a su composición en AGMI y componentes minoritarios. Estas características, sitúan a los aceites de oliva entre los aceites más saludables del mundo (Gavahian et al., 2019).

El consumo moderado de AOVE se ha asociado con la protección frente a factores de riesgo cardiovascular, incluso en las personas con un riesgo muy elevado (Estruch et al., 2018; Schwingshackl et al., 2019). Entre sus efectos beneficiosos, se encuentran la ralentización de las primeras fases del desarrollo de la aterosclerosis, un perfil lipídico favorable, una disminución de la presión arterial, un acortamiento de la duración de la lipemia postprandial, una reducción parcial de los efectos nocivos del tabaquismo, una reducción del estrés oxidativo y la inflamación, una mejora de la función endotelial, una menor agregación plaquetaria y la creación de un entorno menos protrombótico (Buckland et al., 2012; Cruz-Teno et al., 2012; Gaforio et al., 2019; Guasch-Ferré et al., 2020; Llorente-Cortés et al., 2010; Lucas et al., 2011; Salas-Salvadó et al., 2008). La ingesta de AOVE también se ha correlacionado con una menor incidencia de ictus y menor riesgo de desarrollar enfermedad arterial periférica (Ruiz-Canela et al., 2014; Sánchez-Quesada et al., 2020). Durante el desarrollo de la aterosclerosis, se produce una infiltración de partículas de LDL en la pared del vaso. Una vez dentro de la túnica íntima del vaso, las LDL son oxidadas (oxLDL) por EROS y se vuelven más dañinas para la pared arterial que las LDL nativas, siendo los niveles plasmáticos de oxLDL un fuerte predictor de ECV. El efecto protector descrito del consumo de AOVE sobre la formación de oxLDL se debe, principalmente, a la capacidad de sus compuestos fenólicos para unirse a las partículas de LDL y protegerlas contra la oxidación (de la Torre-Carbot et al., 2010; Karantonis et al., 2002; Karantonis et al., 2006; Mehmood et al., 2020; Vicario et al., 1998; Visioli et al., 2002). También se ha demostrado que el AOVE contribuye a un mejor control glucémico con una reducción de la incidencia y el control de otras enfermedades crónicas como el



síndrome metabólico y la diabetes mellitus de tipo 2 (Yubero-Serrano et al., 2019). Además, la sustitución de una dieta rica en AGS por una dieta rica en AOVE conduce a una reducción de aproximadamente el 0,3-2,0% de la hemoglobina glicosilada, teniendo una eficacia similar a la de algunos medicamentos antidiabéticos (Yubero-Serrano et al., 2019). Estudios recientes han sugerido que el consumo moderado de aceite de oliva se asocia con un menor riesgo de todas las causas de mortalidad (Guasch-Ferré et al., 2022; Larsson, 2022).

La EFSA recomienda el consumo de aceite de oliva basándose en su composición en compuestos fenólicos y en los posibles efectos en la salud a través de su protección frente al estrés oxidativo de los lípidos sanguíneos (EFSA, 2011b). Por su parte la FDA ha sugerido que el consumo de 2 cucharadas diarias de aceite de oliva puede reducir el riesgo de enfermedad coronaria debido a su composición en AGMI, siempre que este sustituya en una cantidad similar a las grasas saturadas de la dieta y no aumente la cantidad total de calorías ingeridas en el día (FDA, 2017).

2.2.7.3. Aceite de girasol

El aceite de girasol se extrae de las semillas de girasol (*Helianthus annuus* L.) (Aly et al., 2021; Spring, 2021). El aceite de girasol se obtiene mediante extracción física o química (n-hexano), se somete a un proceso de refinado, en el que se neutraliza, desgoma, blanquea, desodoriza y winteriza (Salas et al., 2015).

El aceite de girasol se encuentra entre los cinco cultivos de aceite vegetal más importantes del mundo, su cultivo se da principalmente en las regiones de Asia y Australasia (Inturrisi, 2015). Suele ser uno de los aceites más utilizados para el consumo doméstico. En la industria, el girasol se utiliza para freír, para la producción de emulsiones y salsas, y en formulaciones de margarina. Sin embargo, los AGPI son más susceptibles a la oxidación (Sousa et al., 2021), teniendo un efecto negativo en la calidad y estabilidad del aceite de girasol (Sánchez-Muniz et al., 2016; Velasco & Ruiz-Méndez, 2015), por ello la industria alimentaria se ha focalizado en la producción de aceite de girasol alto oleico (Salas et al., 2015).



El aceite de girasol tiene un alto contenido en AGPI, principalmente en ácido linoleico (48-74%), y se caracteriza por su elevada concentración en vitamina E y fitoesteroles (Tabla 1) (Spring, 2021; Wu et al., 2020). La presencia de algunos AGS de cadena muy larga, en forma de ácidos araquídico y behénico también es notable (Salas et al., 2015).

Los mecanismos que subyacen a los efectos de las grasas alimentarias sobre las ECV siguen siendo inciertos. Las grasas alimentarias podrían contribuir a la ECV a través de mecanismos inflamatorios y de estrés oxidativo. Los AGPI pueden disminuir la producción de especies inflamatorias y EROS, mientras que los AGS y los AG *trans* pueden aumentar el estrés proinflamatorio y oxidativo, contribuyendo al desarrollo de ECV (Zhuang et al., 2019). Estudios recientes han mostrado el impacto de la ingesta de AGPI en la prevención de enfermedades coronarias y cáncer, así como enfermedades inflamatorias, trombóticas y autoinmunes e hipertensión, debido principalmente a que desempeñan funciones cruciales en muchas vías biológicas (Abedi & Sahari, 2014; De Caterina et al., 2000). Además, la ingesta de AGPI tiene un efecto bien conocido sobre la composición del colesterol de las lipoproteínas de la sangre humana, que tiene un impacto importante en la incidencia de las ECV. El aceite de girasol induce una disminución del colesterol total de las lipoproteínas del plasma, actuando sobre la LDL (Salas et al., 2015).

Los AGPI de la serie n-3 y n-6 tienen un efecto protector de la salud que va más allá del mero efecto cardiovascular. El cerebro, la retina y otros tejidos neuronales son particularmente ricos en AGPI; por lo tanto, una ingesta suficiente es crucial para el desarrollo cerebral y neurológico, la fotorrecepción y el desarrollo del sistema reproductivo en los niños. Durante la edad adulta, la ingesta de AGPI sigue siendo crucial, debido a la relación entre los AGPI n-3 y varias enfermedades crónicas, cardiovasculares e inflamatorias (Madrigal et al., 2020).

En lo que respecta a los AGPI n-3 y n-6, una proporción elevada de n-6:n-3 se asocia al aumento de peso y a la promoción de la patogénesis de muchas enfermedades, incluidas las ECV, las enfermedades inflamatorias y las autoinmunes (Patterson et al.,



2012). La relación n-6:n-3 se considera el factor clave para la síntesis equilibrada de eicosanoides y su importancia nutricional se ha debatido con frecuencia. Un consumo elevado de aceites vegetales ricos en AGPI n-6, como el aceite de girasol, y un consumo relativamente bajo de AGPI n-3 podrían hacer que esta relación fuera demasiado elevada y promover un estado proinflamatorio, lo que se observa principalmente en los países occidentales. Aparte del propio aceite, los alimentos producidos industrialmente con un alto contenido de AGPI n-6 también han adquirido popularidad en las regiones con patrones dietéticos tradicionales, promoviendo el desequilibrio en la relación n6:n3 e incrementando el riesgo de desarrollo de diversas patologías (Orsavova et al., 2015).

2.3. Tejido adiposo

En el organismo pueden diferenciarse distintos tipos de tejido adiposo, atendiendo tanto a su distribución anatómica como a su composición, ambas influenciadas por factores como edad, sexo y nutrición. A lo largo del envejecimiento, el tejido adiposo sufre cambios en cantidad, distribución y funcionalidad, desempeñando un papel importante en el estado proinflamatorio sistémico típico del propio envejecimiento (Abe et al., 2021; Zamboni et al., 2021).

2.3.1. Tipos de tejido adiposo y funciones

2.3.1.1. Tejido adiposo blanco

El tejido adiposo blanco puede clasificarse en dos subtipos principales dependiendo de su localización: el tejido adiposo blanco visceral y el subcutáneo (Brandão et al., 2021). El tejido adiposo blanco visceral, comprende los tejidos adiposos blancos retroperitoneal, mesentérico, perigonadal y perirrenal. Hasta un 10-20% de la grasa es visceral en los hombres y un 5-8% en las mujeres (Wajchenberg, 2000). En el caso del tejido adiposo blanco subcutáneo, en humanos se localiza en las zonas glútea, femoral, gastrointestinal, nasal y craneal (Brandão et al., 2021). El desarrollo del tejido adiposo blanco se produce en la etapa embrionaria y la formación de los tejidos subcutáneo y visceral se completan al final de la etapa gestacional. El tejido adiposo blanco se compone de adipocitos maduros (aproximadamente el 50%

del número total de células) y de una fracción estromavascular, que incluye preadipocitos, células endoteliales, células madre mesenquimales y células inmunitarias. Morfológicamente, los adipocitos blancos se caracterizan porque contienen una única gota lipídica citoplasmática de gran tamaño (“lipid droplet”), un núcleo aplanado y pocas mitocondrias (Figura 3) (Bourgeois et al., 2021).

Adipocitos	Expresión de UCP1	Densidad Mitocondrial	Lipid Droplet	Funciones
Blanco 	-	Baja	Unilocular	Almacenamiento de energía Endocrina
Beige 	+	Media	Multilocular	Termogénesis Endocrina
Pardo 	++++	Alta	Multilocular	Termogénesis Endocrina

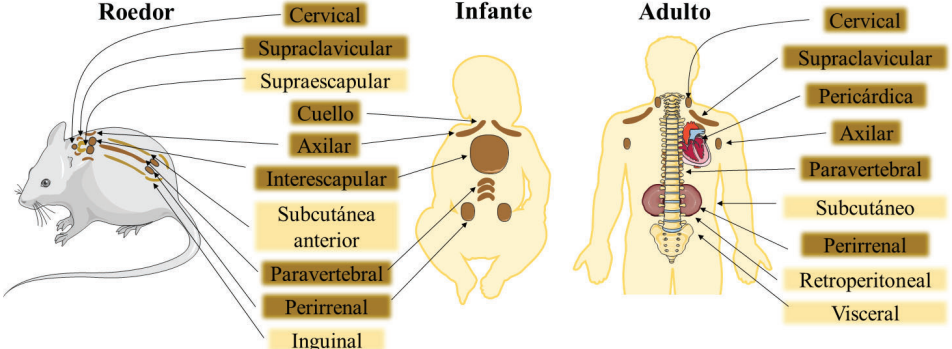


Figura 3. Adipocitos, tipos de tejido adiposo y localización en el organismo. Modificada de (Suchacki & Stimson, 2021)

El tejido adiposo blanco se expande tanto por un aumento del volumen (hipertrofia) como del número (hiperplasia) de los adipocitos. La hiperplasia se produce a través de la diferenciación de las células progenitoras (adipogénesis) bajo la influencia de varios factores de transcripción, entre los que se encuentran el receptor activado por el proliferador de peroxisomas- γ (PPAR γ) (Stenkula & Erlanson-Albertsson, 2018).



El tejido adiposo blanco es el órgano responsable del almacenamiento energético, pero también desempeña una importante función como órgano endocrino en la regulación de procesos biológicos como homeostasis metabólica e inmunidad (Coelho et al., 2013). Almacena energía en forma de TG (lipogénesis) durante los excedentes energéticos, para movilizarla en forma de AGL (lipólisis) cuando se necesita (en respuesta a estímulos hormonales), siendo el órgano responsable de la movilización y secreción lipídica en el organismo. Estos procesos están estrechamente regulados por la insulina y la epinefrina, que controlan la actividad de las enzimas lipogénicas y lipolíticas (Dhawan & Sharma, 2020).

En la lipogénesis (Figura 4), los AGCL y la glucosa acceden a los adipocitos desde la circulación. A partir de la vía de las pentosas fosfato, la glucosa impulsa el mantenimiento de una elevada relación NADPH/NADP⁺, esencial para la síntesis de TG en la célula. La glucosa oxidada también genera glicerol 3 fosfato (G3P) (dependiente de la glicerolfosfato deshidrogenasa, GPDH) que es necesario para iniciar la lipogénesis *de novo*. Además, la glucosa se oxida para generar acil-CoA mitocondrial para alimentar la síntesis de citrato mitocondrial. El citrato generado por el metabolismo de la glucosa y los AG, es exportado por las mitocondrias para formar acil-CoA citosólico, imprescindible para formar los TG tras la lipogénesis (Foster, 2012).

Los AG pueden ser oxidados por la β -oxidación mitocondrial, elevando el estado de reducción de la ubiquinona mitocondrial (QH₂/Q) y la reserva de nicotin adenin dinucleótido (NADH/NAD⁺). Los AG suelen dirigirse a la síntesis de TG para su almacenamiento, proceso que requiere una serie de aciltransferasas que facilitan la esterificación gradual de la molécula de glicerol con tres moléculas de AGL (Reshef et al., 2003).

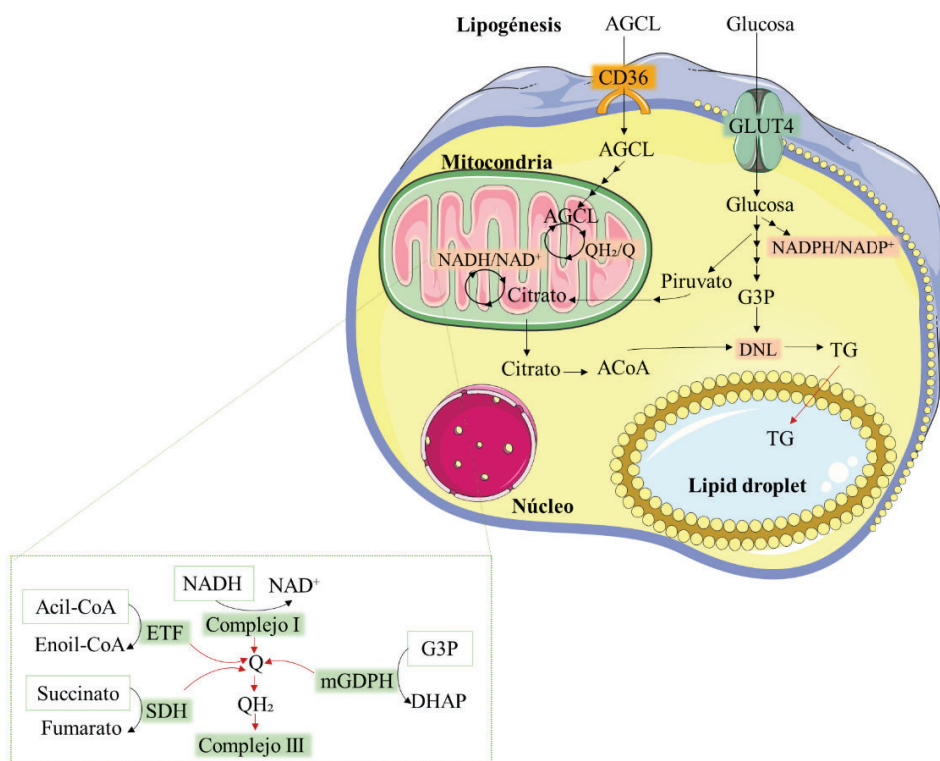


Figura 4. Lipogénesis en los adipocitos

Los TG del tejido adiposo se componen de una compleja mezcla de AG que refleja en gran medida la ingesta dietética de AG. Los TG son dinámicos ya que se forman continuamente por la afluencia de AG desde las lipoproteínas, pero también se hidrolizan para efluir AG al plasma unidos a albúmina (Fernández-Quintela et al., 2007). En condiciones de lipólisis estimulada, estos AG se movilizan selectivamente. De hecho, la movilización de AG individuales no es proporcional a su contenido en el tejido adiposo, sino que está influenciada por su estructura molecular. Depende de la longitud de la cadena, la insaturación y del isomerismo posicional *cis*. Siendo los AGS los que menos se movilizan, y los AGPI los que más (Fernández-Quintela et al., 2007).

La lipólisis (Figura 5), es catalizada por lipasas, y los AGL producidos son secretados por los adipocitos. Este proceso es muy sensible a la acción de la insulina, que constituye la principal vía antilipolítica de los adipocitos. El TG es hidrolizado a

DG y AGL por la enzima lipasa de TG adiposa (ATGL) (Zimmermann et al., 2004), mientras que la lipasa sensible a las hormonas (HSL), hidroliza el DG a MG y AGL, en el último paso, la monoglicerido lipasa (MGL) escinde el MG en glicerol y AGL. La activación de la lipólisis es el resultado de la fosforilación de la HSL en Ser563, Ser659 y Ser 660 por la proteína quinasa A (PKA) o a través de la vía ERK, la HSL se transloca a la superficie de los “lipid droplets”. Los “lipid droplets” del tejido adiposo están cubiertas por la perilipina-1 (PLIN1), uno de los miembros de la familia de las perilipinas. La actividad de la ATGL es estimulada por la CGI-58, que se une a las gotas de lípidos intracelulares mediante la interacción con la PLIN1 (Annie-Mathew et al., 2021).

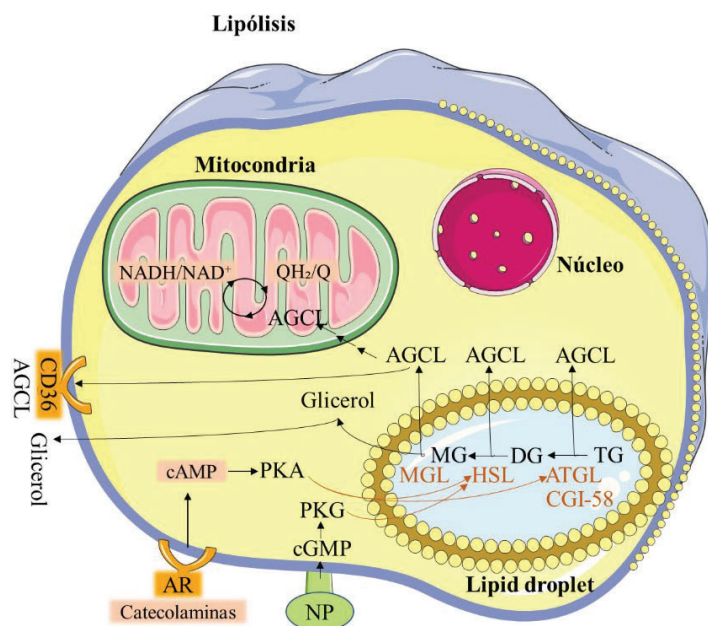


Figura 5. Lipólisis en los adipocitos



2.3.1.2. Tejido adiposo pardo

En la década de los 50 fue descrito por primera vez el tejido adiposo pardo (Wegener, 1951). Durante años se ha considerado un tejido sin relevancia fisiológica en adultos, y se creía que solamente estaba presente durante la etapa embrionaria y postnatal (Aherne & Hull, 1966; Cannon & Nedergaard, 2004). Sin embargo, en 2009 tres estudios independientes (Cypess et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009) observaron a través de técnicas de imagen la presencia de actividad termogénica en el tejido adiposo de adultos. Desde entonces, el interés en su estudio se ha incrementado exponencialmente, debido principalmente a que desempeña una importante función en la homeostasis energética del organismo, siendo una diana terapéutica excelente para patologías como diabetes y obesidad (Poher et al., 2015).

Desde el punto de vista anatómico, los depósitos de tejido adiposo pardo están presentes en las regiones del cuello y los hombros de los niños recién nacidos y en las zonas cervical, supraclavicular, paravertebral, periaórtica y perirrenal en adultos (Figura 3) (Suchacki & Stimson, 2021). Al igual que el tejido adiposo blanco, este se desarrolla durante la embriogénesis (Brandão et al., 2021).

Morfológicamente, los adipocitos marrones, se caracterizan principalmente por tener un mayor número de mitocondrias, varios “lipid droplets” pequeños y lisosomas, así como una elevada vascularización (Bourgeois et al., 2021).

Al contrario que el resto de tejidos adiposos presentes en el organismo, el tejido adiposo pardo tiene como función principal la disipación de energía en forma de calor para mantener el balance energético corporal. A nivel fisiológico, lo que diferencia a los adipocitos marrones del resto de células es que, en la mayoría se producen reacciones catapleróticas que producen un gradiente de protones a través de la membrana interna de las mitocondrias, que gracias a la ATP sintasa, genera adenosín trifosfato (ATP). Mientras que, en los adipocitos marrones no es necesaria la presencia de ATP para la formación del gradiente de protones y su consecuente producción de energía a través de la generación de calor. La mayor actividad del mecanismo desacoplador de energía es a través de la proteína desacoplante (UCP1), comúnmente



conocida como termogenina, que tiene actividad protonofórica a través de la membrana interna de la mitocondria (Wang et al., 2021).

La termogénesis adaptativa es la función metabólica más importante del tejido adiposo pardo. Existen dos tipos de termogénesis adaptativa: la termogénesis inducida por el frío y la termogénesis inducida por la dieta. Al contrario que la termogénesis en el músculo, la termogénesis del tejido adiposo pardo es sin escalofríos (Betz & Enerbäck, 2018). La termogénesis inducida por el frío se desencadena principalmente por la activación del SNS y de la tiroides (Heaton et al., 1978; Silva & Bianco, 2008).

Por otra parte, la termogénesis inducida por la dieta hace referencia al aumento del gasto energético que acompaña a la ingesta de alimentos. Esta se expresa como un porcentaje del contenido energético de los alimentos ingeridos, siendo aproximadamente el 10% para las comidas estándar. La termogénesis inducida por la dieta tiene dos componentes: un componente obligatorio asociado al coste de la digestión y el procesamiento de los macronutrientes (el 3% del contenido calórico de las grasas ingeridas, el 5-10% del de los hidratos de carbono y el 20-30% del de las proteínas) y un componente facultativo o adaptativo, que corresponde al aumento postprandial energético tras el procesamiento de los alimentos. El componente facultativo suele representar en los seres humanos el 30-40% del total de la termogénesis inducida por la dieta, se ha relacionado con la estimulación orofaríngea y parece estar bajo el control del SNS (Bonet et al., 2017; Levine & Kotz, 2005).

Además de mantener la temperatura corporal a través de la termogénesis, los adipocitos marrones también sirven como sumideros metabólicos de glucosa, AG y aminoácidos de cadena ramificada para mejorar la salud metabólica (Harms & Seale, 2013).

2.3.1.3. Tejido adiposo beige

Además de la existencia del tejido adiposo pardo, también existe tejido pardo en ciertos loci dentro del tejido adiposo blanco, denominado “beige” (Ikeda et al., 2018). A pesar de contener UCP1, los adipocitos beige proceden de un linaje de desarrollo diferente al de los adipocitos marrones. Los adipocitos beige se desarrollan a partir de redes



capilares en respuesta a factores pro-angiogénicos y, en consecuencia, deberían considerarse probablemente un tipo celular diferente (Min et al., 2016). El linaje myf5+ (factor miogénico 5), que también es común para el músculo esquelético, es determinante en los adipocitos marrones, mientras que un linaje myf5- es el precursor de los adipocitos beige. En el estado basal, los adipocitos beige muestran una baja expresión de UCP1, que aumenta significativamente ante estímulos hormonales relacionados con el adenosín monofosfato cíclico (AMPC) (Ježek et al., 2019). La exposición al frío y otras señales que reclutan y activan el tejido adiposo pardo inducen también la aparición de células beige termogénicamente competentes que expresan UCP1 en los depósitos de tejido adiposo blanco, lo que se conoce como pardeamiento del tejido adiposo blanco (Nedergaard & Cannon, 2014). Algunas señales que se originan en el intestino tras la alimentación, como el factor de crecimiento de fibroblastos 15 (FGF15) y la incretina del péptido similar al glucagón-1 (GLP-1) también pueden inducir el pardeamiento del tejido adiposo blanco (Fisher et al., 2012; Guan et al., 2016; Holst, 2007). Aunque la termogénesis clásica mediada por el tejido adiposo pardo es dominante, la UCP1 mitocondrial en los adipocitos beige es tan funcional y cualitativamente termogénica como la UCP1 de los adipocitos pardos (Shabalina et al., 2013).

2.3.2. Termogénesis

2.3.2.1. Moléculas implicadas en la actividad termogénica de los adipocitos

2.3.2.1.1. UCP1

La UCP1 fue identificada hace 40 años como una proteína de la membrana mitocondrial altamente expresada en el tejido adiposo pardo de ratas expuestas al frío y, por tanto, probablemente implicada en la termogénesis del tejido (Ricquier & Kader, 1976). La UCP1 es un desacoplador natural y regulador de la respiración mitocondrial, específico de los adipocitos pardos y beige. Su actividad disipa el gradiente de protones generado por la cadena respiratoria facilitando la reentrada de protones en la matriz mitocondrial, eludiendo la ATP sintasa (Figura 6). La actividad de la UCP1 traduce las

elevadas tasas de oxidación de los adipocitos pardos en la producción de calor en lugar de la síntesis de ATP (Cannon & Nedergaard, 2004; Divakaruni & Brand, 2011).

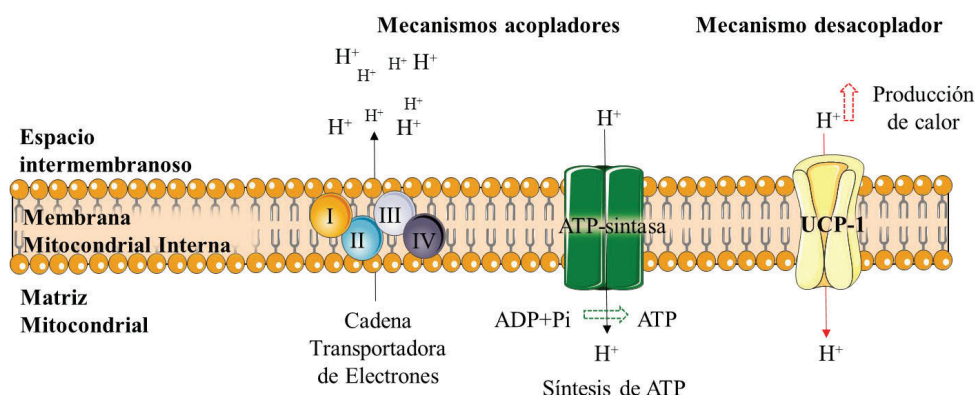


Figura 6. Actividad de UCP-1

Desde su descubrimiento, la UCP1 se ha utilizado ampliamente como marcador funcional de los adipocitos pardos y beige. De hecho, la UCP1 media la mayor parte, si no toda, de la termogénesis sin escalofríos inducida por la norepinefrina (Ruan, 2020).

Existen numerosos factores dietéticos que modulan la actividad y contenido de la UCP1. Tras el trabajo pionero de Rothwell y Stock (Rothwell & Stock, 1979), muchos estudios en animales han examinado el impacto de diferentes tipos de dietas altas en grasa en los *parámetros* del tejido adiposo pardo. Se sugirió que más bien el exceso de energía y no la dieta alta en grasa *per se* es el responsable del aumento de la termogénesis del tejido adiposo pardo (Fromme & Klingenspor, 2011). Sin embargo, no todas las grasas alimentarias son igual de eficaces para inducir la termogénesis. Diversos estudios han demostrado en comparación con la ingesta de aceites vegetales ricos en AGPI como el aceite de soja o ricos en AGMI, que las grasas animales ricas en AGS disminuyen la actividad simpática del tejido adiposo pardo, la termogénesis inducida por la dieta y la expresión de UCP1 en modelos murinos, promoviendo por lo tanto la acumulación de grasa corporal (Bonet et al., 2017). Otros estudios en animales han corroborado el efecto antiobesogénico del aceite de pescado, observando que el



EPA promueve la adipogénesis de adipocitos pardos y beige en el tejido adiposo blanco de ratón y humano, mostrando además una mayor oxidación mitocondrial y un aumento de la expresión de UCP1 (Bargut et al., 2016; Bonet et al., 2017; Flachs et al., 2005; Kim et al., 2015).

Otro AG que ha recibido una atención considerable debido a su capacidad para disminuir significativamente la masa grasa en variedad de especies, es el ácido linoleico conjugado (CLA). Los mecanismos por los que disminuye la masa adiposa pueden incluir una combinación del aumento de la apoptosis, disminución de la diferenciación preadipocitaria y de la lipogénesis, así como un aumento de la oxidación de AG en los adipocitos (Shen & McIntosh, 2016). Sin embargo, a pesar de que el tratamiento con CLA induce el pardeamiento del tejido adiposo blanco, también incrementa marcadores de inflamación de bajo grado del propio tejido (Shen et al., 2013).

Los efectos estimulantes de la alimentación con aceite de oliva sobre la expresión de las UCP pueden contribuir en parte a los beneficios para la salud de los patrones dietéticos mediterráneos, que se han demostrado repetidamente (Estruch et al., 2018). La oleuropeína, ha mostrado incrementar el contenido de UCP1 en el tejido adiposo pardo y potenciar las secreciones de noradrenalina y adrenalina en ratas (Bonet et al., 2017; Oi-Kano et al., 2008). En línea con esto, un estudio reciente ha mostrado que el AOVE es capaz de aumentar la actividad termogénica en humanos (Melguizo Rodríguez et al., 2021).

2.3.2.1.2. PGC-1 α

Las proteínas de la familia de coactivadores transcripcionales del receptor activado por el proliferador de peroxisomas-1 α (PGC-1 α) se expresan principalmente en tejidos con una elevada actividad metabólica como el tejido adiposo, el corazón, el músculo esquelético, el hígado y el cerebro (Correia et al., 2015; Knutti et al., 2000). En lo que respecta a sus funciones, está implicada en la inducción de la biogénesis mitocondrial, ya que promueve el aumento del ADN mitocondrial, así como el gran conjunto de genes mitocondriales codificados en el núcleo y en las mitocondrias (Puigserver et al., 1998). PGC-1 α induce los genes mitocondriales mediante el aumento



de los niveles del factor respiratorio nuclear 1 y 2 (Nrf1 y Nrf2) y el receptor α relacionado con el estrógeno (ERR α) (Schreiber et al., 2004). PGC-1 α también está implicada en la termogénesis adaptativa en los adipocitos pardos, donde tiene su mayor expresión, ya que coordina la expresión de genes termogénicos, entre los que UCP1 tiene una importancia central (Tiraby et al., 2003). Este mecanismo también es inducible en el tejido adiposo blanco, a través de los adipocitos beige (Kajimura et al., 2015; Waldén et al., 2012). Además, PGC-1 α es un potente regulador de la gluconeogénesis e integra múltiples aspectos de la respuesta al ayuno en el hígado (Yoon et al., 2001), aumenta el flujo del ciclo de los ácidos tricarboxílicos y promueve el metabolismo de las lipoproteínas hepáticas (Handschin & Spiegelman, 2006; Lin et al., 2005).

2.3.2.1.3. Nrf2

Nrf2 es uno de los factores de transcripción Cap'n'Collar con una región básica de cremallera de leucina, que está codificado por el gen NFE2L2 en humanos (Moi et al., 1994; Silva-Islas & Maldonado, 2018). En condiciones normales, Nrf2 es secuestrado en el citoplasma mediante su asociación con la proteína 1 asociada a ECH tipo kelch (Keap1) (Itoh et al., 1999). Durante el estrés oxidativo, Keap1 detecta el estrés oxidativo celular y libera Nrf2, momento en el que Nrf2 viaja al núcleo. En el núcleo, Nrf2 forma un complejo con las proteínas Maf y Jun y se une al elemento de respuesta antioxidante en la región promotora, iniciando la transcripción de genes antioxidantes (He et al., 2020). De este modo, Nrf2 funciona como un defensor celular primario contra los efectos citotóxicos del estrés oxidativo (Seo & Lee, 2013).

Nrf2 también desempeña un papel crucial en la restauración de la homeostasis metabólica, ya que puede mantener la forma de los “lipid droplets”, aumentar el almacenamiento de TGs y reducir la liberación de AGL, y potenciar la fosforilación de las enzimas lipolíticas (Annie-Mathew et al., 2021; Silva-Islas & Maldonado, 2018). Además, la señalización de Nrf2 regula directamente los factores pro-adipogénicos clave, como el proliferador de peroxisomas γ (PPAR γ) (Pi et al., 2010; Tonelli et al.,



2017), ambos factores dan lugar a la formación de adipocitos maduros (Brown et al., 2018).

2.3.2.2. Mecanismo

La termogénesis inducida por el frío implica el desacoplamiento de la cadena respiratoria mitocondrial, que está mediado por la UCP1. Este desacoplamiento da lugar a un aumento de la oxidación del sustrato y a la disipación de energía en forma de calor. La gran vascularización del tejido adiposo pardo ayuda a redistribuir el calor generado por todo el cuerpo para la homeostasis de la temperatura. Durante la exposición al frío, la activación de los receptores β 3-adrenérgicos por la norepinefrina conduce al catabolismo de los lípidos liberando AGL y también induce la expresión de UCP1 así como de otros genes pro-termogénicos (Rosen & Spiegelman, 2014; Warner et al., 2016). Además de las catecolaminas, se ha demostrado que otras moléculas no simpáticas, como la triyodotironina (T3), los ácidos biliares hepáticos y varios retinoides, elevan el nivel intracelular de AMPc o GMPc, desencadenan la señalización de la PKA o PKG y estimulan la lipólisis, generando AGL para el combustible mitocondrial y la activación de UCP1, además de promover la expresión de genes termogénicos en los adipocitos marrones (Ucp1, Cidea y Dio2) (Rosen & Spiegelman, 2014). La maquinaria de oxidación termogénica en los adipocitos pardos y beige también está bajo la regulación de factores transcripcionales y cofactores, como el PGC-1 α , que coopera con los factores de transcripción para inducir de forma coordinada la biogénesis mitocondrial y la oxidación de AG que requiere la termogénesis.

A pesar de tener una elevada tasa de captación de glucosa (a través del receptor GLUT4), las principales fuentes de combustible para la respiración mitocondrial por desacoplamiento en el tejido adiposo pardo son los AG (Cypess et al., 2009; Saito et al., 2009). El tejido adiposo pardo utiliza los AGL circulantes tras la hidrólisis de las lipoproteínas ricas en TG como sustrato para la termogénesis, que son captados por los adipocitos por CD36 (Khedoe et al., 2015). Tanto los AGCC como los AGCL están asociados a la UCP1 (Fedorenko et al., 2012). La glucosa puede almacenarse en forma



de glucógeno, someterse a la glucólisis o entrar en la vía de la hexosamina para la O-glicosilación de proteínas, utilizarse para la lipogénesis de *novo* o ser sustrato para la termogénesis (Ruan, 2020). Por otra parte, tras la exposición al frío, el succinato se acumula en las mitocondrias del tejido adiposo pardo activado para impulsar la termogénesis dependiente de la UCP1 a través de EROS y succinilación de proteínas mitocondriales (Wang et al., 2019). Además, el ciclo de la creatina mediado por la creatina quinasa, desencadena el recambio de ATP mitocondrial y la termogénesis independiente de UCP1 (Kazak et al., 2015; Ruan, 2020). Junto a este mecanismo de termogénesis independiente de UCP1, se incluyen la termogénesis cíclica del Ca^{2+} , que utiliza activamente la glucosa como sustrato principal (Denton, 2009), el ciclo entre la descomposición de los TG y la reesterificación de los mismos (Guan et al., 2002), y el ciclo entre la oxidación de los lípidos y la lipogénesis de *novo* en los adipocitos (Mottillo et al., 2014). También se ha demostrado que la exposición al frío aumenta la actividad de varias enzimas antioxidantes como la superóxido dismutasa, la catalasa, las glutatión peroxidasas y la glutatión reductasa en el tejido adiposo pardo, lo que sugiere un elevado estrés oxidativo en el mismo durante la termogénesis (Barja de Quiroga et al., 1991).

Un circuito regulador bien caracterizado para la respiración dependiente de la UCP1 es la interacción competitiva entre los AG y los nucleótidos de purina. La UCP1 se une a los nucleótidos de purina, siendo la especie dominante en los adipocitos el ATP. En las concentraciones existentes en la célula, esta interacción parece ser suficiente para inhibir sustancialmente la corriente de protones de la UCP1. Sin embargo, las concentraciones locales elevadas de AGCL superan la inhibición de los nucleótidos de purina e impulsan la fuga de H^+ a través de un mecanismo sinérgico entre AG y H^+ (Fedorenko et al., 2012). Esta interacción competitiva entre los AGCL y los nucleótidos de purina se produce tras la estimulación adrenérgica y la elevación de los niveles de AGL en los adipocitos termogénicos.



2.3.3. Tejido adiposo como órgano endocrino

2.3.3.1. Inmunometabolismo

El inmunometabolismo tisular se refiere a la conexión entre la inmunidad y el metabolismo en los tejidos metabólicos, como el tejido adiposo. La regulación del inmunometabolismo se basa en las interacciones entre las células del parénquima tisular, las células del estroma y las células inmunitarias.

Las células madre derivadas del tejido adiposo son las células estromales dominantes en el tejido y sirven como progenitores responsables de la regeneración y reposición de los adipocitos. Tienen un gran potencial de autorrenovación, así como una gran capacidad de diferenciación múltiple hacia adipocitos, condrocitos y células musculares (Zhou et al., 2021).

Las células inmunitarias que han sido detectadas en el tejido adiposo incluyen: eosinófilos, neutrófilos, mastocitos, macrófagos, linfocitos T, células linfoides innatas de tipo I y II, y células NK (Bourgeois et al., 2019). Los macrófagos son la población celular inmunitaria predominante en el tejido adiposo. Su función no solo reside en mediar la inflamación del tejido adiposo, sino que también participan en la eliminación de los adipocitos apoptóticos, el almacenamiento de los lípidos y la angiogénesis. A pesar de que las poblaciones de macrófagos muestran una gran plasticidad, tradicionalmente se han clasificado en subconjuntos M1 (clásicamente activados) y M2 (activados alternativamente) (Na et al., 2016; Zhou et al., 2021). En el tejido adiposo, los macrófagos M2, son el subtipo predominante y secretan en gran medida citoquinas antiinflamatorias, como la IL-10 y el TGF- β (Hong et al., 2009). Por otro lado, los macrófagos M1, que derivan de la médula ósea, secretan citoquinas proinflamatorias como IL-6 y TNF- α (Lumeng et al., 2007). A nivel inmunometabólico, al potenciar la glucólisis aeróbica mediada por la señalización mTOR/HIF-1, los macrófagos M1 se polarizan y se activan por estímulos proinflamatorios como el lipopolisacárido (LPS) y el IFN- γ , y muestran un aumento de la glucólisis aeróbica a través de la señalización mTOR/HIF-1 (Mills & O'Neill, 2016). Por el contrario, los macrófagos M2 suelen estar polarizados y potenciados por las citoquinas T-helper 2 (Th2), como la IL-4 y la IL-13,



y presentan un mayor metabolismo mitocondrial a través de la fosforilación oxidativa y la oxidación de AG mediada por STAT6/PPAR γ (Kelly & O'Neill, 2015). Así pues, el metabolismo mitocondrial regula la función de las células inmunitarias. En particular, la transición entre la glucólisis aeróbica y la fosforilación oxidativa desempeña un papel fundamental en la polarización de los macrófagos (Saitoh et al., 2021).

2.3.3.2. Adipoquinas

El tejido adiposo blanco es metabólicamente menos activo que el pardo, pero tiene un perfil endocrino único. Su principal actividad endocrina reside en la secreción de adipoquinas, siendo capaz de secretar hasta 600 adipoquinas diferentes (Bourgeois et al., 2021; Lehr et al., 2012). Las adipoquinas son proteínas de bajo peso molecular, farmacológicamente activas, que poseen una actividad pleiotrópica. Las acciones locales de las adipoquinas (autocrinas y paracrinas) incluyen la regulación de la adipogénesis, el metabolismo de los adipocitos, la migración de las células inmunitarias y la sensibilidad a la insulina. Por otra parte, los efectos sistémicos de las adipoquinas, implican la modulación y regulación de diferentes procesos biológicos, como el metabolismo de la glucosa, la secreción de insulina, la inflamación, la presión arterial, el metabolismo lipídico, el apetito y la saciedad (Fasshauer & Blüher, 2015). Algunas de estas adipoquinas son producidas por los propios adipocitos, como la leptina y adiponectina, mientras que otras son producidas principalmente por las células inmunes infiltradas en el tejido adiposo, como la IL-6 y el TNF- α (Bourgeois et al., 2021; Stenkula & Erlanson-Albertsson, 2018).

La idea de que el tejido adiposo segregaba factores hormonales fue sugerida en la década de los 50 por Kennedy (Kennedy, 1953), quien observó que existía un factor "lipostático" que actuaba en el cerebro para controlar la ingesta de alimentos en las ratas. Veinte años después, este factor se identificó como leptina (Zhang et al., 1994).



2.3.3.2.1. Leptina

La leptina es una proteína de 16 kDa no glucosilada que es producida principalmente por el tejido adiposo blanco subcutáneo (Wang et al., 1998). Fue identificada en 1994 por el grupo de Jeffrey Friedman como el producto del gen de la obesidad (ob) en ratones y del gen lep en humanos (Zhang et al., 1994). La estructura de la leptina consiste en un haz de cuatro α -hélices que están estabilizadas por enlaces disulfuro de cisteína. La leptina señala a través del receptor de leptina (LEPR en humanos, Ob-R en ratones), que es un único receptor de membrana que pertenece a la familia de receptores de citoquinas de clase I (Tartaglia et al., 1995).

En modelos animales y en humanos, los niveles circulantes de leptina sérica están positivamente correlacionados con la masa del tejido adiposo blanco (Conde et al., 2014). Esta hormona tiene un papel central en la homeostasis del peso corporal mediante la inducción de factores anorexígenos y la supresión de neuropéptidos orexígenos en los núcleos hipotalámicos (Kwon et al., 2016). El aumento de la señalización de la leptina en el hipotálamo regula el apetito y conduce a la disminución de la ingesta de nutrientes y al aumento del gasto energético, desempeñando un papel fundamental en el control de la homeostasis energética, el metabolismo y la función neuroendocrina (Park & Ahima, 2015).

La leptina también afecta a otras funciones fisiológicas, como el metabolismo óseo, la inflamación, la infección y las respuestas inmunitarias (Abella et al., 2017). El receptor de leptina se expresa en la mayoría de las células del sistema inmunitario. En general, la expresión del receptor de leptina es importante para el desarrollo de las células hematopoyéticas, la proliferación y supervivencia de las células inmunitarias (Gainsford et al., 1996). La leptina puede estimular la producción de citoquinas proinflamatorias (Santos-Alvarez et al., 1999), polarizar las células Th hacia un fenotipo proinflamatorio (Th1, que secreta $\text{IFN}\gamma$) en lugar de antiinflamatorio (Th2, que secreta IL-4) (Martín-Romero et al., 2000), aumentar la citotoxicidad de las células NK (Abella et al., 2017) y promover la quimiotaxis de los neutrófilos (Souza-Almeida et al., 2018).



2.3.3.2.2. Adiponectina

La adiponectina (también conocida como GBP28, apM1, Acrp30 o AdipoQ) es una proteína de 244 residuos con homología estructural al colágeno tipo VIII y X, y al factor del complemento C1q que fue identificada a mediados de los años 90 (Hu et al., 1996). La adiponectina humana está codificada por el gen ADIPOQ, situado en el cromosoma 3q27, un locus relacionado con la susceptibilidad a la diabetes y las ECV (Liu & Liu, 2014). Es la adipocitina más abundante en la circulación y se sintetiza principalmente en el tejido adiposo (Liu & Liu, 2014), sin embargo, sus niveles circulantes están inversamente correlacionados con el porcentaje de grasa corporal y los niveles de TG (Yamauchi et al., 2003). La adiponectina es un sensibilizador endógeno a la insulina de los órganos diana, incluidos el músculo esquelético y el hígado. La unión a su receptor, produce la reducción de la lipogénesis y la gluconeogénesis y el aumento de la absorción de glucosa por el músculo esquelético y el tejido adiposo blanco (de Oliveira Dos Santos et al., 2021). De hecho, su reducción plasmática está relacionada con la resistencia a la insulina y la intolerancia a la glucosa, lo que predispone a una lesión hepática avanzada (Buechler et al., 2017).

Los receptores de adiponectina también están presentes en la superficie de las células inmunitarias, siendo un regulador clave del sistema inmunitario y desempeñando un papel importante en la progresión de los trastornos metabólicos e inflamatorios (Luo & Liu, 2016). En los macrófagos, la adiponectina suprime la diferenciación y activación clásica de los macrófagos M1 mediante la regulación a la baja de citoquinas proinflamatorias (TNF- α e IL-6) y promueve la activación de macrófagos M2 e induce la producción de los mediadores antiinflamatorios IL-10 (Wulster-Radcliffe et al., 2004).

2.3.3.2.3. IL-6

La IL-6 es producida por adipocitos, macrófagos, músculo esquelético, células endoteliales y fibroblastos (Tanaka et al., 2014). Estructuralmente, la IL-6 es una glucoproteína que consta de cuatro α -hélices (Kaur et al., 2020). Para mediar sus efectos, la IL-6 se une a la cadena α del receptor de la IL-6 que luego se asocia con la



glicoproteína de membrana gp130 para la transducción de señales. La IL-6 derivada del tejido adiposo es una fuente importante de IL-6 circulante. Se ha estimado que aproximadamente un tercio de la IL-6 circulante deriva del tejido adiposo (Makki et al., 2013). El aumento de los niveles de IL-6 se correlaciona con el porcentaje de grasa corporal y el perímetro de la cintura en los datos clínicos (Pou et al., 2007). La IL-6, tiene efectos tanto pro como antiinflamatorios dependiendo del microambiente celular. En el caso de la IL-6 procedente del tejido adiposo, tiene un papel proinflamatorio, favoreciendo la acumulación de macrófagos en el tejido (Han et al., 2020). La IL-6 desempeña un papel central en la defensa del huésped, la inflamación, el metabolismo de los lípidos y la resistencia a la insulina (Narazaki & Kishimoto, 2018).

2.3.3.2.4. TNF- α

El TNF- α es una proteína de 26 kDa que se sintetiza como un monómero transmembrana, que luego puede ser escindido por la enzima convertidora del TNF- α para producir la forma soluble de 17 kDa. Ambas formas existen como trímeros que tienen actividad biológica (Black et al., 1997). Es una de las primeras adipoquinas descubiertas que eran producidas por el tejido adiposo. El grupo de Spiegelman determinó que las principales fuentes endógenas de TNF- α eran el bazo y el tejido adiposo visceral (Hotamisligil et al., 1995), siendo producido en este último tanto por los adipocitos como por los macrófagos infiltrados en el tejido adiposo. El desencadenante inicial que conduce a la producción de TNF- α en el tejido adiposo no se conoce del todo. Un mecanismo probable es que el aumento de la muerte de adipocitos que se produce en la expansión del tejido adiposo sirva como señal quimioatrayente para los monocitos circulantes. Además, el exceso de AG en la dieta, así como el aumento de los AGL circulantes por el incremento de la lipólisis, pueden afectar a la producción de adipoquinas, lo que provoca un aumento del TNF- α (Cawthorn & Sethi, 2008). Las acciones del TNF- α en el tejido adiposo también provocan cambios en la producción de otras adipoquinas, como la adiponectina y la leptina, y promueven la liberación de otras citoquinas proinflamatorias (Hector et al.,



2007; Kirchgessner et al., 1997). El TNF- α circulante se correlaciona de forma directa con el porcentaje de grasa corporal (Olszanecka-Glinianowicz et al., 2004).

2.3.3.2.5. TGF- β

La familia del factor de crecimiento transformante- β (TGF- β) es un grupo de citoquinas que exhibe un efecto pleiotrópico en una variedad de funciones biológicas. En 1986, Kehrl y colaboradores, informaron por primera vez de que el TGF- β es producido por las células T activadas e inhibe su proliferación, indicando que el TGF- β puede servir como un sistema de retroalimentación negativa para las funciones de las células T (Kehrl et al., 1986). Años más tarde, en 1992, el desarrollo de ratones deficientes en TGF- β 1 estableció un papel fundamental e indispensable de esta citoquina en el control de la inflamación y las enfermedades autoinmunes, debido a que morían en el útero o perinatalmente debido a la inflamación generalizada (Lutgens et al., 2002; Shull et al., 1992). Interviniendo de este modo en procesos como la embriogénesis, la carcinogénesis y la respuesta inmune. Además, se ha descrito como un importante regulador de la fisiología de los adipocitos por su papel en la inhibición de la adipogénesis así como en la remodelación del tejido adiposo (Fujio et al., 2016).

En lo que respecta a su implicación en la respuesta inflamatoria, el TGF- β muestra efectos pleiotrópicos en una variedad de células inmunitarias (Fujio et al., 2016). El TGF β 1 puede actuar tanto de forma autocrina como paracrina, ya que los macrófagos, las células T, las células dendríticas y otros tipos de células inmunitarias pueden servir tanto de fuente como de dianas fisiológicas. Principalmente se ha asociado una función inmunomoduladora debido a su regulación positiva y negativa a la vez de la diferenciación y mantenimiento de células T efectoras y reguladoras (Komai et al., 2018; Rubtsov & Rudensky, 2007).



2.4. Inflamación y reprogramación metabólica del tejido adiposo

2.4.1. Modulación inmunometabólica a través de la microbiota intestinal

El término microbiota intestinal describe las especies microbianas comensales del tracto gastrointestinal (Guarner & Malagelada, 2003). Más del 90% de los taxones de la microbiota intestinal en adultos está constituido por dos grandes filos bacterianos, Bacteroidetes o Firmicutes, con una menor abundancia de Actinobacterias, Cianobacterias, Fusobacterias, Proteobacterias y Verrumicrobia (Gilbert et al., 2018). El microbioma intestinal humano es muy dinámico y puede verse alterado por la edad, el uso de antibióticos, la genética del huésped, los patrones dietéticos y otras exposiciones ambientales (Turnbaugh et al., 2009). Actualmente, está bien establecido que la microbiota intestinal puede influir en el estado de salud de un individuo ya que desempeña un papel predominante en la formación y maduración del sistema inmunitario del huésped, en la síntesis de vitaminas, en la resistencia a la colonización o al crecimiento excesivo de microorganismos patógenos, en la desconjugación de los ácidos biliares y en la obtención de energía mediante la fermentación de carbohidratos no digeribles (Yamashiro, 2017).

La microbiota produce una amplia gama de metabolitos, como vitaminas, AGCC, ácidos biliares, trimetilamina, aminoácidos de cadena ramificada y fenoles. Estos metabolitos son absorbidos por las células epiteliales del intestino y finalmente entran en la circulación. Además, los componentes estructurales del microbioma, como el LPS, también podrían entrar en el sistema circulatorio del huésped y convertirse en compuestos bioactivos (Schroeder et al., 2008).

Los AGCC, son el producto final de la fermentación por parte del microbioma intestinal de los hidratos de carbono no digeribles. Los AGCC incluyen principalmente el ácido acético, el ácido propiónico, el ácido butírico, el ácido isobutírico, el ácido valérico, el ácido isovalérico, el ácido isocaproico y el ácido hexanoico. Siendo el ácido butírico el principal AGCC en el tracto intestinal (Serino, 2019). Los AGCC son importantes para el metabolismo del huésped y se utilizan como sustratos para la producción de energía, la lipogénesis, la gluconeogénesis y la síntesis de colesterol (G.



den Besten et al., 2013). Además, actúan como moléculas de señalización mediando el estrés oxidativo y la respuesta inmunitaria (Sanna et al., 2019).

Los ácidos biliares primarios se sintetizan a partir del colesterol en los hepatocitos. Tras la ingestión de alimentos, la vesícula biliar libera estos ácidos biliares en el duodeno, donde actúan en el intestino delgado y el colon. Después de que los ácidos biliares ayuden a la digestión de los alimentos, la mayoría se reabsorben de nuevo en el hígado en un proceso conocido como circulación enterohepática de los ácidos biliares (Boyer & Soroka, 2021). El resto de ácidos biliares no reabsorbidos, se desconjugan y siguen siendo metabolizados por las bacterias colónicas, dando lugar a un conjunto de ácidos biliares más hidrofóbicos que se excretan en heces y actúan como un importante sumidero de colesterol (Wahlström et al., 2016).

El LPS, conocido como endotoxina bacteriana, es un componente de la membrana externa de las bacterias Gram negativas y es importante para mantener la integridad estructural de la bacteria. Los cambios en la permeabilidad intestinal, permiten que el LPS entre en la circulación y active varias vías patogénicas (Neves et al., 2013). El LPS se une a la proteína de unión al LPS (LBP), que transporta el LPS a la superficie de las células inmunitarias y se une al receptor de membrana CD14. A continuación, CD14 transfiere el LPS al complejo proteico del TLR4, que activa las vías de transducción de señales intracelulares y acaba activando el factor de transcripción NF- κ B, generando citoquinas proinflamatorias como el TNF- α y la IL-6 (Medzhitov, 2007).

2.4.1.1. Disbiosis intestinal e inflamación del tejido adiposo

La disbiosis intestinal se refiere a un cambio mórbido en la composición de la microbiota intestinal y puede estar causada por varios factores como la dieta, el aumento del estrés, la inflamación y el uso de antibióticos (Carding et al., 2015). Aunque no existe una relación clara de causa y efecto entre este cambio y la enfermedad, la disbiosis puede observarse en un amplio rango de enfermedades. De hecho, se considera un proceso básico de la microbiota intestinal que influye en la disfunción del tejido adiposo (Bleau et al., 2015).



El epitelio intestinal funciona como barrera para evitar la translocación de los factores derivados de las bacterias. Sin embargo, el aumento de peso, una dieta rica en grasas y el aumento de la exposición a los AGS pueden alterar la función de barrera intestinal permitiendo la translocación de LPS (Erridge et al., 2007; Levels et al., 2001). Esto da lugar a un aumento moderado de los niveles de LPS en la sangre, lo que se define como endotoxemia metabólica, una condición vinculada a perturbaciones metabólicas como la dislipidemia, la resistencia a la insulina, la esteatosis hepática y las ECV (Cani et al., 2007; Manco et al., 2010)

La disbiosis intestinal inducida por una dieta alta en grasas es también responsable de la inflamación adiposa (Tran et al., 2020). Tras la activación de los TLR, los macrófagos del tejido adiposo son inducidos al fenotipo proinflamatorio M1 y posteriormente secretan adipocinas proinflamatorias como TNF- α , IL-6, IL-8 y MCP-1 de forma dependiente de los TLR en respuesta al LPS (Talukdar et al., 2012). Al iniciarse, este entorno inflamatorio se pueden reclutar nuevas células inflamatorias (neutrófilos, monocitos), magnificando así la respuesta inflamatoria en el tejido adiposo (Federico et al., 2016; Mencin et al., 2009).

Tras la estimulación del receptor TLR4 por el LPS, los macrófagos aumentan la lipogénesis de *ново*. Así, la estimulación microbiana aumenta la utilización de la glucosa y redirige el metabolismo de la glucosa dentro de la célula para apoyar la síntesis de AG y TG (Yan & Horng, 2020). Además de regular al alza la síntesis de lípidos, los macrófagos aumentan la captación de AGL y lipoproteínas tras la exposición a estímulos inflamatorios, lo que está relacionado con la amplificación de la respuesta inflamatoria (Feingold et al., 2012). De este modo, el LPS también aumenta la concentración de TG en sangre principalmente por la regulación de las lipoproteínas. Los niveles de LPS elevados disminuyen el catabolismo de las mismas, mientras que niveles bajos aumentan la síntesis hepática de VLDL. Por otra parte, las HDL tienen capacidad neutralizante frente a los efectos tóxicos del LPS, protegiendo contra la inflamación (Feingold et al., 1995; Pajkrt et al., 1996).



2.4.2. Disfunción del tejido adiposo

El tejido adiposo es el principal protagonista de los procesos adaptativos que se producen en el organismo, es un órgano muy dinámico que puede remodelarse rápidamente en respuesta a los cambios internos y externos, como el estado nutricional, la temperatura ambiental, las infecciones, el ritmo circadiano y el envejecimiento, entre otros. Situaciones de estrés metabólico crónico e inflamación, pueden dar lugar a una mala adaptación del tejido adiposo, que está estrechamente relacionada con el desarrollo de trastornos metabólicos, como la resistencia a la insulina, la dislipidemia, la esteatosis hepática y la diabetes de tipo 2 (Anderson & Weindruch, 2010).

La disfunción del tejido adiposo consta de dos aspectos: las anomalías en la distribución de la grasa y las anomalías en las características del tejido adiposo (Yang et al., 2021). Las anomalías en la distribución de la grasa se refieren a la acumulación excesiva de tejido adiposo visceral, o el almacenamiento de grasa en los espacios intraperitoneales y retroperitoneales, así como a la deposición de grasa ectópica, que hace referencia a depósitos de grasa en lugares del organismo donde la grasa no se almacena fisiológicamente, como el hígado, el páncreas, el corazón y el músculo esquelético (Yang et al., 2021). El tejido adiposo subcutáneo se considera un amortiguador fisiológico normal para el exceso de ingesta de energía y grasa. La energía no gastada se utiliza para la síntesis de *novo* de TG que se almacenan en el mismo (Stefan, 2020). Entre las características del tejido adiposo disfuncional se encuentran el deterioro de la capacidad de expansión del tejido adiposo y la adipogénesis, así como la hipertrofia y la alteración del metabolismo lipídico de las células grasas (Suriano et al., 2020; Yang et al., 2021). La adipogénesis es un proceso de dos pasos que implica el compromiso de las células precursoras con el linaje adipocítico y la posterior diferenciación en adipocitos maduros. El deterioro de la adipogénesis, es decir, la incapacidad del tejido adiposo de sufrir hiperplasia, predispone a los individuos a una obesidad metabólicamente insalubre, caracterizada por la resistencia a la insulina en el tejido adiposo, músculo e hígado, y da lugar a anomalías metabólicas como la hiperglucemia, la hipertensión y dislipemia (Longo et al., 2019). Esta alteración suele ir acompañada de una inflamación del tejido adiposo,



caracterizada por la infiltración y la activación de células inmunitarias proinflamatorias, como los macrófagos y las células T efectoras CD4⁺ Th1 y Th17, y linfocitos T citotóxicos CD8⁺, así como por niveles elevados de citoquinas proinflamatorias (Crewe et al., 2017; Schenk et al., 2008). Además de un mayor número de macrófagos, el entorno proinflamatorio dentro del tejido adiposo blanco promueve cambios en los fenotipos de los mismos. A pesar de clasificarse tradicionalmente en M1 y M2, recientemente se ha descubierto que en el tejido adiposo blanco inflamado hay unos macrófagos metabólicamente activos con un perfil proinflamatorio distinto (Kratz et al., 2014). Esta infiltración contribuye a su vez a la inflamación y disfunción del tejido (Chouchani & Kajimura, 2019; Hotamisligil, 2017; Man et al., 2017).

Los adipocitos hipertróficos sufren apoptosis o necrosis secundaria mediada por los macrófagos infiltrados en el tejido adiposo obeso. En este caso, se ha considerado como factor desencadenante, la metainflamación, ya que el TNF- α , que se expresa abundantemente en el tejido obeso, induce la apoptosis de los adipocitos blancos y pardos (Röszer, 2021). Además, el aumento del flujo de AGL, el incremento de los niveles de DG y ceramidas, y el exceso de lípidos en los adipocitos hipertróficos, puede dar lugar al “derrame” de lípidos en el citosol, lo que induce lipotoxicidad y apoptosis (Prieur et al., 2010; Shulman, 2014). El deterioro de la lipólisis y la hipertrofia son, por tanto, potentes desencadenantes de la apoptosis de los adipocitos (Röszer, 2021). Curiosamente, el aumento de la expresión de CD36 en los adipocitos y los macrófagos se ha asociado a una mayor inflamación del tejido adiposo y a la muerte celular en la obesidad inducida por la dieta (Cai et al., 2012; Murano et al., 2013).

La muerte de los adipocitos es un proceso que genera inflamación y es el preludio de una secuencia de acontecimientos que conducen a las enfermedades metabólicas (West, 2009). Los adipocitos blancos son más resistentes a la apoptosis que los adipocitos pardos. La apoptosis de los adipocitos es frecuente en condiciones patológicas; por ejemplo, en la lipodistrofia congénita generalizada, tanto la masa tejido adiposo blanco como la parda se pierden debido a la apoptosis y necrosis de los adipocitos (Röszer, 2021). En patologías donde se produce una disfunción del tejido adiposo como la obesidad y la lipodistrofia, la apoptosis de los adipocitos provoca el



reclutamiento y acumulación de macrófagos en el tejido adiposo, y la formación de las denominadas estructuras en forma de corona alrededor de los adipocitos apoptóticos (Figura 7) (Cinti et al., 2005).

Los macrófagos del tejido adiposo engullen los restos celulares, los "lipid droplets", las moléculas asociadas al daño, los cuerpos apoptóticos y fragmentos de adipocitos que se generan por la necrosis secundaria (Strissel et al., 2007). La muerte de los adipocitos es un acontecimiento que provoca la activación proinflamatoria de los macrófagos infiltrados en el tejido y la inflamación del propio tejido, lo que desencadena a su vez en una mayor apoptosis de los adipocitos en un círculo vicioso (Nagareddy et al., 2014). El modo en que los macrófagos responden a los adipocitos apoptóticos, depende del contexto inmunológico en el que se produce la muerte de los adipocitos, de las señales proporcionadas por las células apoptóticas y las señales inmunitarias adicionales como el factor del complemento y las citoquinas (Benoit et al., 2012; Ravichandran, 2010). En condiciones homeostáticas, cuando los adipocitos apoptóticos son escasos, los macrófagos del tejido adiposo despejan estos adipocitos y se promueve una polarización de macrófagos M2, modulando el estado inflamatorio (Fischer-Posovszky et al., 2011). Sin embargo, la prevalencia de adipocitos apoptóticos, la presencia de especies lipídicas proinflamatorias y de moléculas asociadas al daño, puede favorecer el fenotipo proinflamatorio de los macrófagos del tejido adiposo hacia la secreción de citoquinas inflamatorias y el aumento de las EROS. Si la capacidad de los macrófagos para eliminar las células apoptóticas se agota (debido a una elevada y constante presencia de las mismas), los restos celulares apoptóticos y necróticos pueden agravar la inflamación (Luo et al., 2019; Röszer, 2021).

Cada vez hay más pruebas de que los macrófagos residentes del tejido adiposo están reprogramados por su entorno, especialmente por la presencia de estresores metabólicos, como la glucosa elevada y el palmitato (Ziętak et al., 2016). Los AGS procedentes del consumo de grasas altamente saturadas o de la desregulación de la lipólisis pueden inducir y/o propagar la inflamación de bajo grado durante la obesidad (Curley et al., 2021). La liberación de un exceso de AGL y de adipocinas provoca a su vez un estrés oxidativo y una función endotelial anormal (Papaetis et al., 2015).

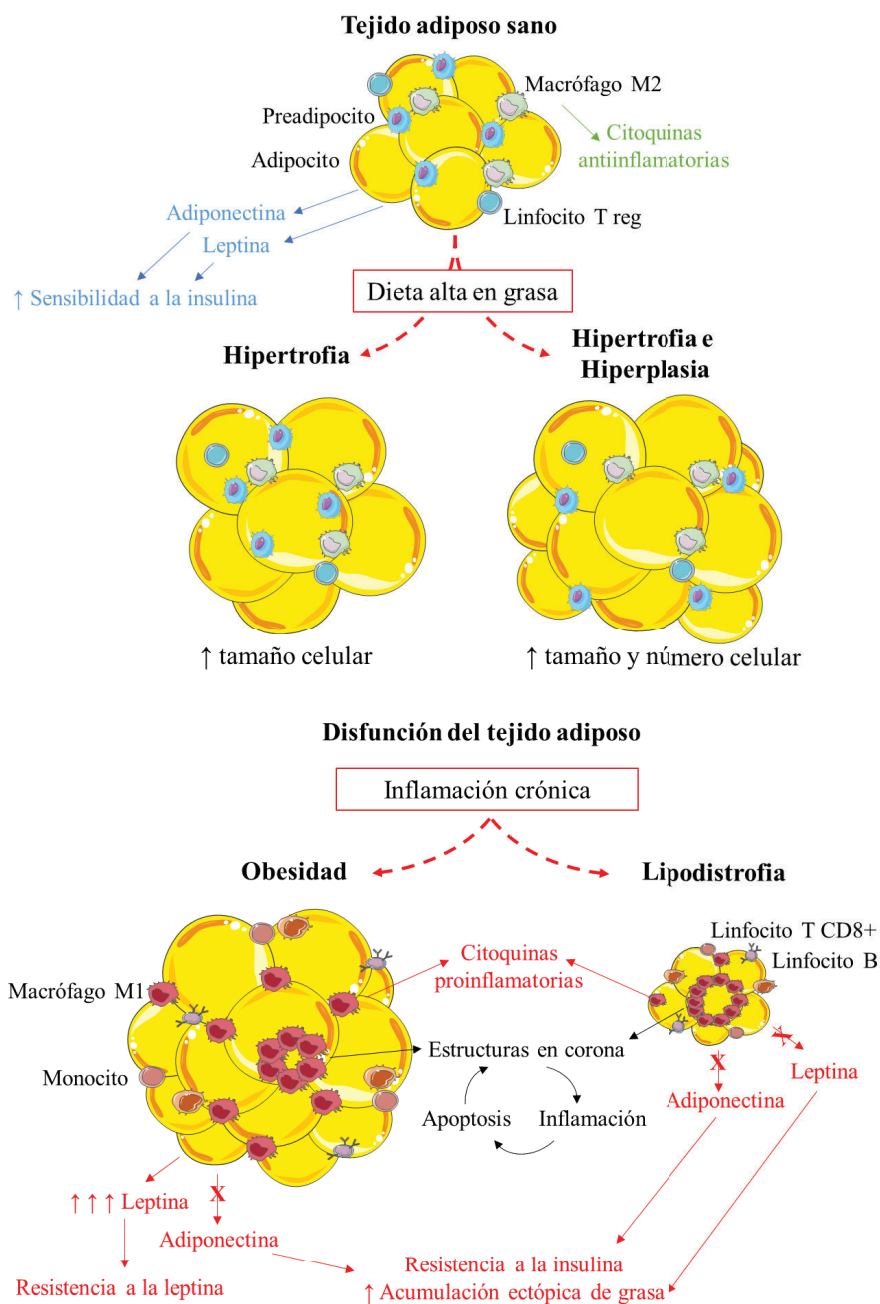


Figura 7. Tejido adiposo sano, hipertrofia, hiperplasia y tejido disfuncional



2.4.3. Enfermedades del tejido adiposo: obesidad y lipodistrofia

El exceso de tejido adiposo en pacientes con obesidad generalizada o regional, como la obesidad visceral y, la marcada reducción del mismo en pacientes con lipodistrofia congénita o adquirida, tienen bases comunes en la resistencia a la insulina y ambas patologías se caracterizan por complicaciones metabólicas similares como hipertrigliceridemia, bajos niveles de HDL y esteatosis hepática (Garg, 2006).

La disfunción del tejido adiposo en ambas patologías, provoca una limitación en el almacenamiento de TG, promoviendo una acumulación ectópica de los mismos en tejidos como el hígado. Esta disfunción está relacionada además con una limitación en la producción y secreción de adipocinas. Las adipocinas pueden afectar a su vez a la homeostasis metabólica y agravar las complicaciones metabólicas causadas por la disfunción del tejido adiposo (Garg & Misra, 2002, 2004).

Los síndromes de lipodistrofia son un grupo de enfermedades raras, muy heterogéneas, caracterizados por una pérdida de tejido adiposo blanco. Aunque la base molecular de la lipodistrofia ha revelado notablemente una amplia heterogeneidad con respecto a los genes subyacentes y sus vías biológicas, una característica común en la mayoría de las lipodistrofias es la de la resistencia a la insulina. Dado que la ausencia o la gran reducción de la masa adiposa en las lipodistrofias también significa que los factores secretados por los adipocitos están ausentes o son bajos, el responsable de la resistencia a la insulina puede encontrarse en las vías gobernadas por los factores secretados exclusivamente por los adipocitos, como la leptina y la adiponectina (Berger et al., 2017), caracterizándose la mayoría de estos síndromes por hipoleptinemia e hipoadiponectinemia.

Del mismo modo, en la obesidad, la desregulación de la leptina y la adiponectina que conducen respectivamente, a la hiperleptinemia y a la hipoadiponectinemia, ponen de relieve su importancia en la resistencia a la insulina (Frithioff-Bøjsøe et al., 2020). Así pues, la leptina y la adiponectina son denominadores comunes de la resistencia a la insulina en la lipodistrofia y la obesidad.



Aunque los niveles de leptina circulantes sean muy elevados, los pacientes obesos se caracterizan por una resistencia a la leptina, es decir, una respuesta tisular reducida a la leptina. La resistencia a la leptina agrava la condición de obesidad y contribuye a un aumento adicional de los niveles de leptina, creando un círculo vicioso denominado "resistencia a la leptina inducida por la leptina" (Izquierdo et al., 2019; Senesi et al., 2020), por lo que a pesar de que los niveles de leptina sean elevados, esta no consigue aumentar el gasto energético ni reducir la ingesta de alimentos, con el consiguiente aumento de peso corporal (Conde et al., 2014). En la lipodistrofia, la incapacidad del tejido adiposo de secretar leptina, puede modularse mediante la terapia con leptina recombinante mejorando las complicaciones metabólicas (Haque et al., 2002; Oral et al., 2002). Sin embargo, esta terapia no consigue restaurar la homeostasis metabólica, y en algunas situaciones incrementa la inflamación concomitante (Ziegler et al., 2019).

Con respecto a los niveles circulantes de adiponectina, están estrechamente ligados a la integridad funcional del tejido adiposo y disminuyen con la obesidad y la lipodistrofia. La adiponectina funciona como un potente sensibilizador a la insulina y supresor de la muerte celular y la inflamación, promoviendo directamente resultados antidiabéticos y antiateroscleróticos (Chehab, 2008; Joseph et al., 2014). Actúa en el hígado para disminuir la gluconeogénesis, en el músculo esquelético para aumentar la oxidación de los ácidos grasos, y en las células β pancreáticas y en las células musculares cardíacas como agente antilipotóxico clave (Funcke & Scherer, 2019). Además, cada vez hay más pruebas que revelan la importancia de la adiponectina en las patologías relacionadas con la inflamación debido a su modulación de la respuesta inmunitaria innata, así como de las células B y T (Luo & Liu, 2016).

Aunque la lipodistrofia y la expansión del tejido adiposo, como la obesidad, son algo opuesto en cuanto a la masa de tejido adiposo, ambos estados patológicos suelen presentar una desregulación metabólica similar (Huang-Doran et al., 2010), donde la inflamación crónica y de bajo grado desempeñan un papel principal (Donath & Shoelson, 2011). Ambas patologías se asocian con cambios en los parámetros inmunológicos, incluyendo: disminución del número de células T con función



reguladora, elevada expresión de genes proinflamatorios, mayor infiltración de monocitos en el tejido adiposo, elevada presencia de macrófagos M1 en el tejido, y sobrerrepresentación de macrófagos derivados de monocitos cargados de lípidos asociados con una mayor capacidad paracrina para inducir la acumulación de lípidos. Estas características se correlacionan estrechamente con el perfil lipídico del paciente (Berger et al., 2017; Herrero et al., 2010; Martin et al., 2012; Shimomura et al., 1998). Además, ambas patologías, se caracterizan también por unos elevados niveles circulantes de LPS y, una elevada expresión y secreción de adipoquinas proinflamatorias como TNF- α e IL-6, contribuyendo a su vez a un estado inflamatorio crónico en el tejido adiposo (Ouchi et al., 2011; Park et al., 2020).

2.4.4. Inmunonutrición en el manejo y prevención de comorbilidades asociadas a la disfunción del tejido adiposo

Tanto la obesidad como la lipodistrofia se caracterizan por la inflamación crónica debida a la actividad proinflamatoria de las células inmunitarias residentes en el tejido adiposo. El tejido adiposo, como tejido inmune desempeña un papel muy importante en la regulación inmunometabólica del organismo, pudiendo regularse su actividad y mejorar el microambiente en patologías asociadas a la disfunción del mismo, a través de la inmunonutrición.

La inmunonutrición se define como la modulación de la actividad del sistema inmunitario, o la modulación de las consecuencias de su activación, mediante intervenciones con alimentos o nutrientes específicos suministrados en cantidades superiores a las habituales en la dieta. Este proceso permite modificar la respuesta inflamatoria o inmunitaria y prevenir las comorbilidades asociadas a diversas patologías e intervenciones como la cirugía (Calder, 2003; Grimble, 2001). La inmunonutrición actúa a través de tres objetivos principales: la inflamación local o sistémica, la defensa celular y la función de barrera de la mucosa intestinal.

De hecho, algunos nutrientes y sus metabolitos desempeñan un papel clave en la maduración, diferenciación y capacidad de respuesta de las células inmunitarias. Por



lo que un suministro adecuado y equilibrado de estos nutrientes es esencial para establecer una respuesta inmunitaria adecuada (EFSA, 2016). Se han identificado diversos inmunonutrientes, entre ellos destacan algunos nucleótidos, aminoácidos que contienen azufre, glutamina, arginina, selenio, zinc, vitaminas (D, E y C), carotenoides, fitoesteroles, estilbenos, flavonoides y los AGPI n-3 (Ruiz-León et al., 2019).

El mecanismo de acción por el que los lípidos modulan el sistema inmunitario se debe a que la ingesta dietética de AGMI y AGPI dicta la composición de AG de los fosfolípidos de las membranas de las células inmunitarias y de las células de los tejidos diana sobre los que actúan las citoquinas (Herrera Vielma et al., 2021). Por su parte, los AGPI n-3 ejercen propiedades antiinflamatorias y antioxidantes, que apoyan al sistema inmunitario ayudando a resolver la respuesta inflamatoria (Calder, 2013). Se ha demostrado que la ingesta de AGPI n-3 desencadena reacciones antiinflamatorias a través de metabolitos oxigenados (oxilipinas), como las protectinas y resolvinas. Los AGPI n-3 incluyen el ALA consumido de diversas fuentes vegetales y el EPA y DHA consumidos especialmente de fuentes de pescado y marisco (Serhan & Levy, 2018). En particular, el EPA y DHA que intervienen en el proceso de inflamación se convierten enzimáticamente en mediadores pro-resolución especializados (Calder, 2010). Además, el EPA y el DHA aumentan la respuesta inmunitaria al mejorar la función de los linfocitos (Bharadwaj et al., 2016). Estas moléculas, junto con otras, funcionan conjuntamente para orquestar la resolución de la inflamación. Siendo las deficiencias de EPA y DHA las responsables de la resolución retardada o subóptima de la inflamación (Basil & Levy, 2016; Di Renzo et al., 2020). Además, se ha demostrado que las grasas ricas en AGPI n-3, o AGMI, o pobres en AGPI n-6 reducen la inflamación (Grimble, 2009; Miles & Calder, 1998).

En definitiva, debido la importancia de la nutrición como estrategia de mitigación para apoyar la función inmune, la inmunonutrición personalizada para los pacientes obesos y lipodistróficos debería ser la primera opción terapéutica para mejorar el microambiente del tejido adiposo y el reducir el riesgo de otras comorbilidades, a través de una dieta equilibrada, rica en algunos nutrientes con actividad antiinflamatoria e inmunomoduladora como los AGPI n-3.

HIPÓTESIS



3. HIPÓTESIS

Considerando que:

1. Las guías alimentarias recomiendan disminuir la ingesta de grasas de la dieta sin tener en cuenta ni su composición ni el impacto de su consumo en la salud.
2. Existe una creencia generalizada que considera que todas las grasas de origen vegetal son saludables.
3. La denominada dieta occidental se caracteriza por ser alta en grasas,

nuestra hipótesis de partida es que: tres grasas de origen vegetal en las que predominan ácidos grasos diferentes: saturados (aceite de coco), monoinsaturados (aceite de oliva virgen extra) y poliinsaturados omega-6 (aceite de girasol), actuarían de forma diferente sobre la microbiota intestinal, el tejido adiposo pardo, el tejido adiposo blanco y marcadores de inflamación.

OBJETIVOS



4. OBJETIVOS

Objetivo general

Analizar en un modelo murino los efectos del consumo de una dieta alta en grasa basada en aceite de oliva virgen extra, aceite de girasol o aceite de coco, sobre la microbiota intestinal, el tejido adiposo pardo, el tejido adiposo blanco, marcadores de inflamación y parámetros metabólicos relacionados con el tejido adiposo.

Objetivos específicos

Para desarrollar este objetivo general se han planteado los siguientes objetivos específicos:

1. Evaluar el efecto del consumo de una dieta alta en grasa en aceite de oliva virgen extra, aceite de girasol o aceite de coco en la modulación de las poblaciones bacterianas que conforman la microbiota intestinal.
2. Identificar el efecto del consumo de una dieta alta en grasa en aceite de oliva virgen extra, aceite de girasol o aceite de coco sobre la actividad termogénica del tejido adiposo pardo interescapular.
3. Analizar el efecto del consumo de una dieta alta en grasa en aceite de oliva virgen extra, aceite de girasol o aceite de coco sobre la capacidad de pardización del tejido adiposo blanco visceral y subcutáneo.
4. Estudiar el efecto del consumo de una dieta alta en grasa en aceite de oliva virgen extra, aceite de girasol o aceite de coco sobre la promoción o resolución de procesos inflamatorios a través de la modulación en la secreción de citoquinas.
5. Evaluar el efecto del consumo de una dieta alta en grasa en aceite de oliva virgen extra, aceite de girasol o aceite de coco sobre el metabolismo en base a la secreción de adipocinas y a los niveles de triglicéridos circulantes y hepáticos.

TRABAJO

EXPERIMENTAL



5. TRABAJO EXPERIMENTAL

5.1. Artículo científico I

The High-Fat Diet Based on Extra-Virgin Olive Oil Causes Dysbiosis Linked to Colorectal Cancer Prevention

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The high-fat diet based on extra-virgin olive oil causes dysbiosis linked to colorectal cancer prevention.

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Abstract

The present study aims to examine the effects of three different high-fat diet (HFD) on mice gut microbiota in order to analyse whether they create the microenvironmental conditions that either promote or prevent colorectal cancer (CRC). We evaluated colonic mucosa-associated microbiota in CD1 mice fed with HFD, based on 60% kcal from fat-containing coconut, sunflower or extra-virgin olive oil as the only source of fat. The main findings were as follows: (a) All HFD produced a decrease in the richness and diversity of the intestinal microbiota that was independent of mouse weight, (b) HFD switched *Lactobacillus* to *Lactococcus*. In general, the results showed that both sunflower- and coconut-HFD generated a pro-inflammatory intestinal microenvironment. In brief, coconut-HFD decreased *Akkermansia* and increased *Staphylococcus*, *Prevotella* and *Bacteroides* spp. abundance. Sunflower-HFD reduced *Akkermansia* and *Bifidobacterium*, while enhancing *Sphingomonas* and *Neisseria* spp. abundance. In contrast, EVOO-HFD produced an anti-inflammatory microenvironment characterised by a decreased *Enterococcus*, *Staphylococcus*, *Neisseria* and *Pseudomonas* spp. abundance. At the same time, it increased the Firmicutes/Bacteroidetes ratio and maintained the *Akkermansia* population. To conclude, EVOO-HFD produced changes in the gut microbiota that are associated with the prevention of CRC, while coconut and sunflower-HFD caused changes associated with an increased risk of CRC.

Keywords: inflammation; sunflower oil; coconut oil; gut microbiome; *Akkermansia*; *Neisseria*; *Staphylococcus*; firmicutes; bacteroidetes; *Prevotella*



1. Introduction

Nowadays, colorectal cancer (CRC) is among the most common and deadly neoplasms [1]. It is assumed that both, a Western-style diet with high-fat content and a sedentary lifestyle are responsible for the increase in their global incidence [1]. In fact, CRC is 3–4 times more common in developed than in developing nations. High fat intake is one of the characteristics of the Western pattern diet, and this correlates with an increased risk of CRC [2]. The trigger mechanism by which the risk increases is not yet well known, but it has been proposed that it is due to a change in the intestinal microbiota that favour a low-intensity inflammatory process [3].

Gut microbiota is a highly complex ecosystem, with great individual variations and thousands of microbial species. The most predominant phylum in the healthy gut are Firmicutes and Bacteroidetes, followed by Actinobacteria, Proteobacteria and Verrucomicrobia [4]. Under normal physiological conditions, gut microbiota regulate fat metabolism (by bile acids biotransformation), synthesise essential amino acids and vitamins, and facilitate the digestion of complex plant carbohydrates into short-chain fatty acids (mainly butyrate, propionate and acetate). The gut microbiome contributes significantly to host immunity and metabolic homeostasis [5,6]. However, alterations in the mucosa, together with an unfavourable genetic predisposition, favour the growth of opportunistic microbes that promote functional and morphological changes (dysbiosis) and lead to chronic inflammation. Chronic inflammation, in turn, contributes to dysbiosis [7]. Pro-inflammatory cytokines and opportunistic pathogens affect the epithelial integrity, and chronic inflammation together with oxidative stress leads to the loss of the epithelial barrier, and may result in a vicious cycle of immune hyperactivation and aggravation of barrier dysfunction [8]. In addition, defects in the host barrier enhance permeability and promote the invasion of harmful bacteria, which may lead to bacterial translocation across the epithelial layers [5], leading to the development of CRC [2].

Although high-fat diets (HFD) have been identified as promoting the development of CRC [2], not all types of fats have the same health effect. While animal fats have harmful effects on inflammation and CRC development, the effects of edible



vegetable oils on gut microbiota dysbiosis have not been studied sufficiently yet [9]. All edible vegetable oils are assumed to be healthy, but given their different fatty acid profile and minor compounds composition, their impact on gut microbiota is expected to be different.

Since HFD is typically present in Western-pattern diets and is a risk factor of CRC, the present study aims to examine, in an experimental murine model, the influence of three HFD, each one prepared with different edible vegetable oils (coconut oil, sunflower oil and extra virgin olive oil) on gut microbiota.

2. Materials and Methods

2.1. Experimental Animals

Female CD1 mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Four-week-old mice ($n = 44$) were housed (5–6 per cage) in ventilated racks and cages with environmental control (temperature: 20 ± 2 °C; humidity: 55–65%; 12 h light/12 h dark cycle).

Animal care and experiments were conducted following the guidelines of the Spanish Society for Laboratory Animal Science. The procedures applied to these animals were approved by the Ethical Committee of the University of Jaen (Record number: CEEA-100217-1) and the Ethical Committee of Animal Experiments of Regional Ministry of Agriculture, Fishing and Environment of Regional Government of Andalusia, Spain (Approval number: 16/03/2017/044).

2.2. Diets

The mice were fed with maintenance chow diet (defined as chow diet), with 13% kcal from fat (Ref. 2014S), an intermediate fat diet with 22% kcal from fat (Ref. 2019S) and custom basis (Ref. TD.170709) ready to use (fat free) for HFD with 60% kcal from fat were purchased from ENVIGO® (Madison, WI, USA). The custom basis fat free was exclusively made by ENVIGO® for the present study. In order to prepare each one of the HFD, we added one of the following oils to the custom basis: extra



virgin olive oil (EVOO), coconut oil and sunflower oil. The HFD were made and administered daily in sterile conditions (Tables S1 and S2).

2.3. Experimental Design

On arrival, the mice were randomly divided into four groups (n = 11 each), and they were assigned to a different diet:

Group 1: Chow diet (chow)

Group 2: HFD of coconut oil (coconut-HFD)

Group 3: HFD of sunflower oil (sunflower-HFD)

Group 4: HFD of extra virgin olive oil (EVOO-HFD)

Before the dietary intervention, the mice were kept in an acclimation phase for three weeks. In the first week, all the mice were fed with chow diet, and the following two weeks, the chow group continued on the same diet, but HFD groups were provided with an intermediate fat diet. Once the intervention phase started, the mice were fed either a chow diet, EVOO, coconut or sunflower HFD with ad libitum access to water and food for sixteen weeks (Figure 1).

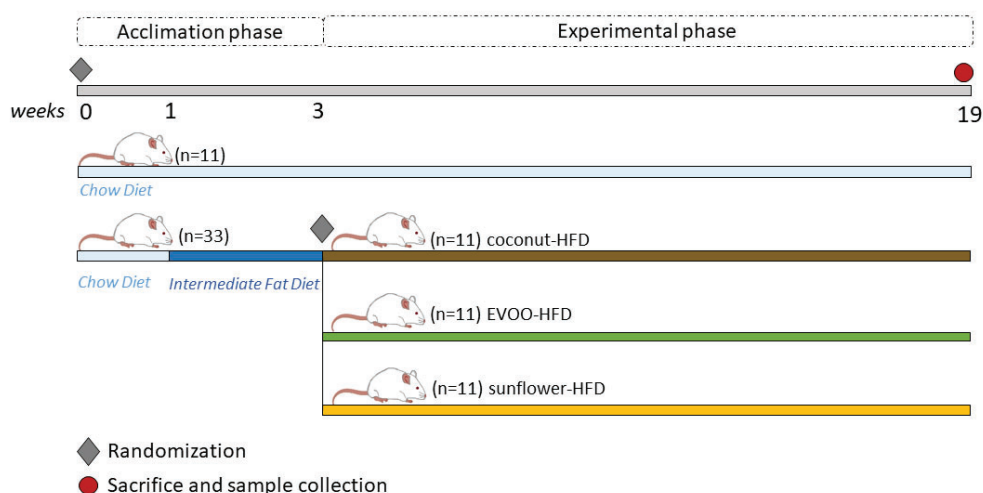


Figure 1. Experimental design



Body weight and indirect food intake were measured weekly. The mice were then sacrificed using a euthanasic mixture of ketamine (160 mg/kg) and xylazine (10 mg/kg). The intestine was completely dissected, freshly cut and stored at -80°C until analysis.

2.4. Sample Collection and Mucosa-Associated Microbiota Analysis by PCR Amplification and Sequencing of the 16S rRNA Gene

Thirty-two distal colon samples were analysed, corresponding to eight mice randomly selected from each experimental group. Samples were carefully dissected, and the colonic mucosa-associated microbiota was extracted and lysed. The nucleases were inactivated through mechanic disruption and enzymatic treatment. Genomic DNA was isolated from the bacterial colonies and purified with the QIAamp PowerFecal DNA kit (Qiagen, Hilden, Germany) following the manufacture's protocol.

The quality and quantity of the bacterial colonies were analysed by spectrophotometry using Nanodrop (Thermo Scientific, Waltham, MA, USA). DNA amplification of the 16S rRNA V3-V4 region of the bacteria's rRNA genes was carried out with a two-step PCR protocol, using the PCR primers recommended by Klindworth et al. [10]. The obtained products were verified with the PicoGreen-based DNA quantification assay (Table S3). Sequencing of the gut microbiota was performed with Illumina Miseq (Novogene, Beijing, China). Sequencing was performed using the manufacturers' protocol with a 300 pb pair-end design and 50,000 to 100,000 readings per sample were obtained. A positive control was included and another negative control was included during sequencing [11]. In both cases the result was optimal, the positive control had the expected species and the negative control had less than 50 sequences.

2.5. Bioinformatics Analysis

PEAR V.0.9.1 software (The Exelisis Lab, Heidelberg, Germany) was used to join each pair of sequences (R1 and R2) coming from the sequencing platform, taking into account a minimum overlap of 70 nts at each end, in this way a unique and complete sequence was obtained. Then, using the Cutadapt v1.8.1 program, the sequencing adapters of both ends present in each sample were eliminated.



Once the sequences without adapters were obtained, those readings that were below Q20 and less than 100 pb in length were eliminated. The reformat module of BBMap v38 was used to perform this analysis; this programme also cut out those nucleotide bases at both ends that presented a value of quality lower than that indicated (Q20). The last step in quality processing was the elimination of possible chimera sequences resulting from incomplete extension during amplification by the PCR method. This step was carried out with the Uchime programme, which allows the detection and elimination of these amplicons from a reference database (ChimeraSlayer).

The CDHit v4.8.1 program (Weizhong Li's Group, La Jolla, CA, USA) was used to determine the microbial diversity present in the sample, grouping sequences with a similarity threshold of 97% or higher in operational taxonomic units (OTUs). Each OTU was compared against the RefSeq 16S rRNA gene database (NCBI) using the BLAST tool.

For each of the samples, a rarefaction curve was performed, reaching a saturation situation of detection (plate), to corroborate that all the organisms had been detected (Figure S1). Shannon–Wiener and Chao1 biostatistical values were calculated to estimate the specific biodiversity present in the sample and the total number of species. The local contribution to beta diversity analysis (LCBD) was done representing the degree of uniqueness of the sampling units in terms of community composition and based on the number of the standardized number of counts for rarefaction.

2.6. Statistical Analysis

The analysis consisted of a differential study of the population using the DESeq2 R tool focused on microbiomes. To compare data, bacteria with minor than 0.01% of presence were eliminated. In addition, principal coordinate analysis, canonical correspondence, beta dispersion analysis of the samples, and a differential study of diversity were performed. To show the effect of different diets on the relative abundance of taxa, a PERMANOVA test was performed. p-values adjusted by FDR

has been used in DESeq2 test. Data are represented as mean $\pm$ SD and p values less or equal to 0.05 were considered statistically significant.

3. Results

3.1. High-Fat Diets Promoted Dysbiosis Independently of Body Weight and the Type of Vegetable Fat Present in the Diet

Regardless of the type of vegetable fat used, HFD led to a decrease in the richness and diversity (Simpson and Shannon test, evaluated by Chao-1) of gut microbiota. Diversity reduction for EVOO- and sunflower-HFD were statistically significant ($p < 0.05$ for both EVOO- and sunflower-HFD) (Figure 2).

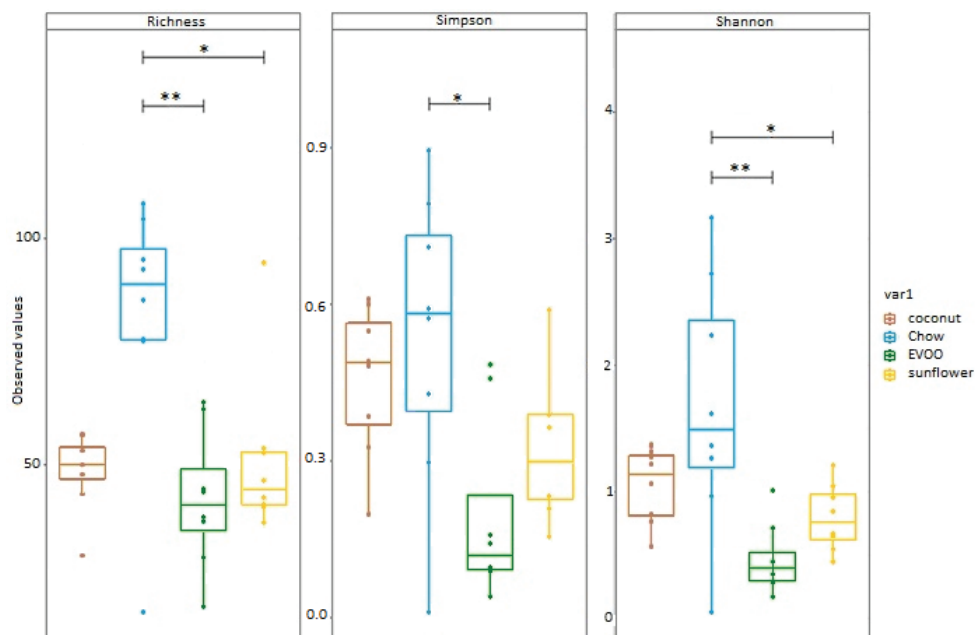


Figure 2. Gut microbiota richness and diversity (Shannon and Simpson) (evaluated by Chao-1). Boxplot figure representing the diversity of the samples according to the variables studied * $p < 0.05$ and ** $p < 0.01$ (ANOVA test).

The local contribution to the beta diversity test verified that each group's sample contribution to diversity was relatively homogeneous within the group (Figure 3).

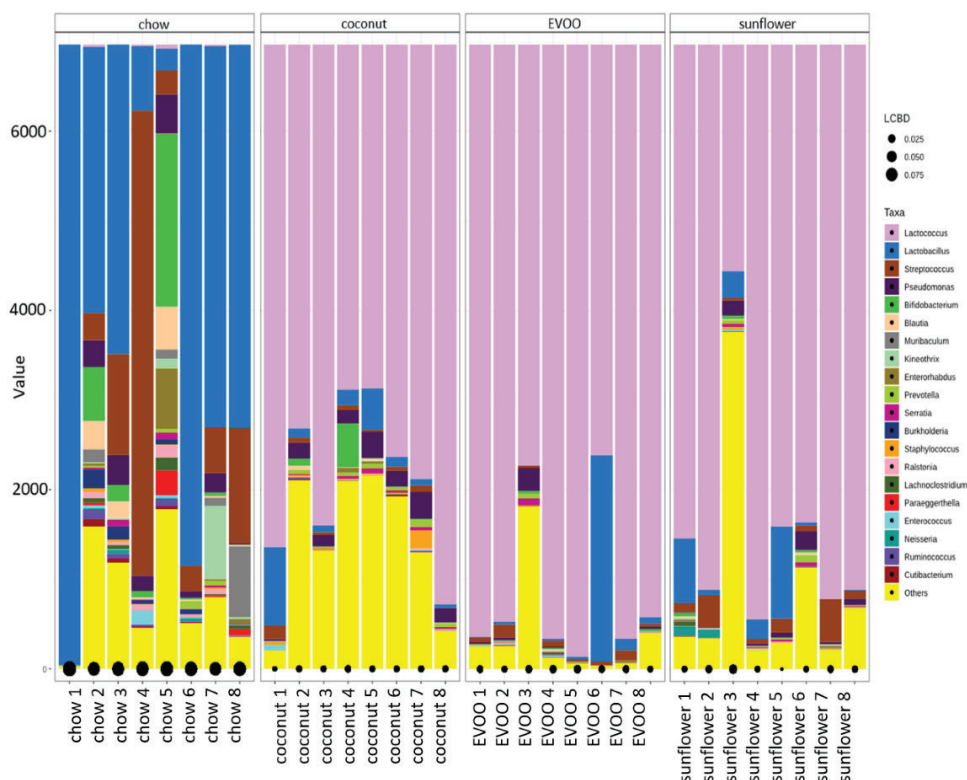


Figure 3. The local contribution to beta diversity analysis. LCBD values represent the degree of uniqueness of the sampling units in terms of community composition. Accumulative bar graph comparing the genera detected in the samples with the 16S rRNA-based bacteria profiles.

Principal coordinate analysis (PCoA) and PERMANOVA analysis corroborated that HFD promoted a change in the bacterial community in a significant way ($p = 0.002$ for coconut and EVOO-HFD, and $p = 0.01$ for sunflower-HFD) (Figure 4).

To compare if these changes in bacterial communities may be related to body weight, the initial and final mean weights of each group were analysed. Data showed that coconut-HFD increased body weight, likewise for the chow diet, while EVOO- and sunflower-HFD increased it notably (Table 1).

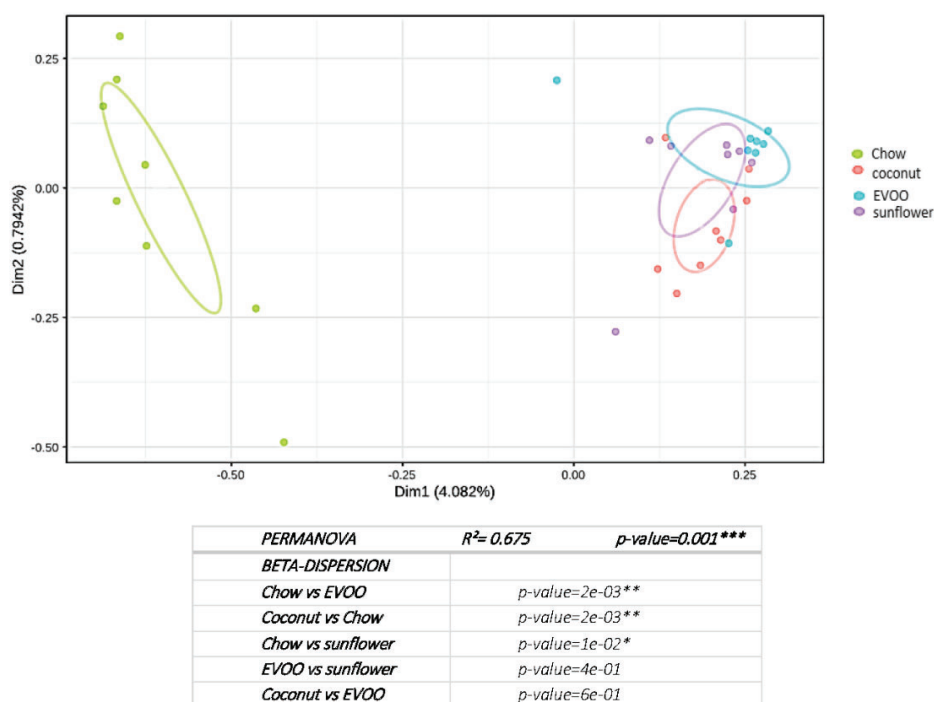


Figure 4. The principal coordinate analysis (PCoA) plot made with unweighted unifrac distances between samples of microbiome, grouped by diets. The main coordinate graph showing the differences between samples and its group, with 30% CI ellipses. A PERMANOVA analysis has also been carried out to detect whether the separation between groups is significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 1. Initial and final body weights of mice fed with chow and HFD.

Weight	Chow	Coconut	EVOO	Sunflower
Initial (g)	29.49 ± 0.37	28.65 ± 0.47	29.42 ± 0.76	31.04 ± 0.77
Final (g)	45.48 ± 1.85	44.88 ± 1.47	55.89 ± 2.65 **	55.3 ± 3.33 **

Data are shown as Mean ± SD of bodyweight (n = 11). ANOVA test, ** $p < 0.01$ for HFD (EVOO, sunflower) vs. chow diet.

3.2. Main Findings Found at the Phylum Level: EVOO-HFD Specifically Reduced Proteobacteria While Coconut-HFD Decreased Verrucomicrobia



At the phylum level, the abundance of mice gut microbiota was different between chow and all three HFD groups (Figure 5 and Table 2). However, Firmicutes remained in all diets as the majority phylum. Although there were no statistical differences between groups, EVOO- and sunflower-HFD showed an increase in this phylum. With regard to Bacteroidetes, all HFD reduced this phylum in a significant way in comparison with the chow group ($p < 0.05$). Figure 6A shows that the Firmicutes/Bacteroidetes ratio increased in EVOO-HFD while it remained almost unchanged in both coconut- and sunflower-HFD. As in Bacteroidetes, all the HFD decreased the Actinobacteria phylum in a significant way. Despite the fact that no statistical differences between HFD groups were observed, EVOO- and coconut-HFD acted differently; microbiota from EVOO-HFD mice suffered a reduction in Proteobacteria ($p < 0.001$), while coconut-HFD decreased Verrucomicrobia ($p < 0.01$). Figure 5 illustrates the taxonomic summary bar plots identification of the microbiota at the phylum level, concluding that all HFD promoted dysbiosis on gut microbiota.

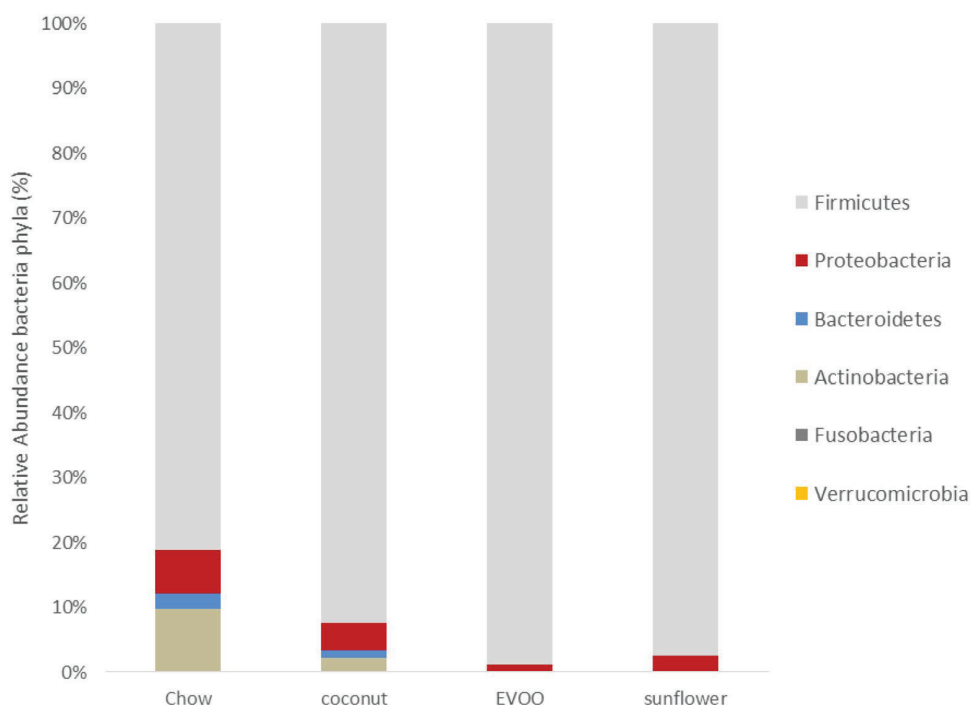


Figure 5. The relative abundance of bacterial phyla (%). The taxonomic summary bar plots identification of the microbiota at the phyla level.

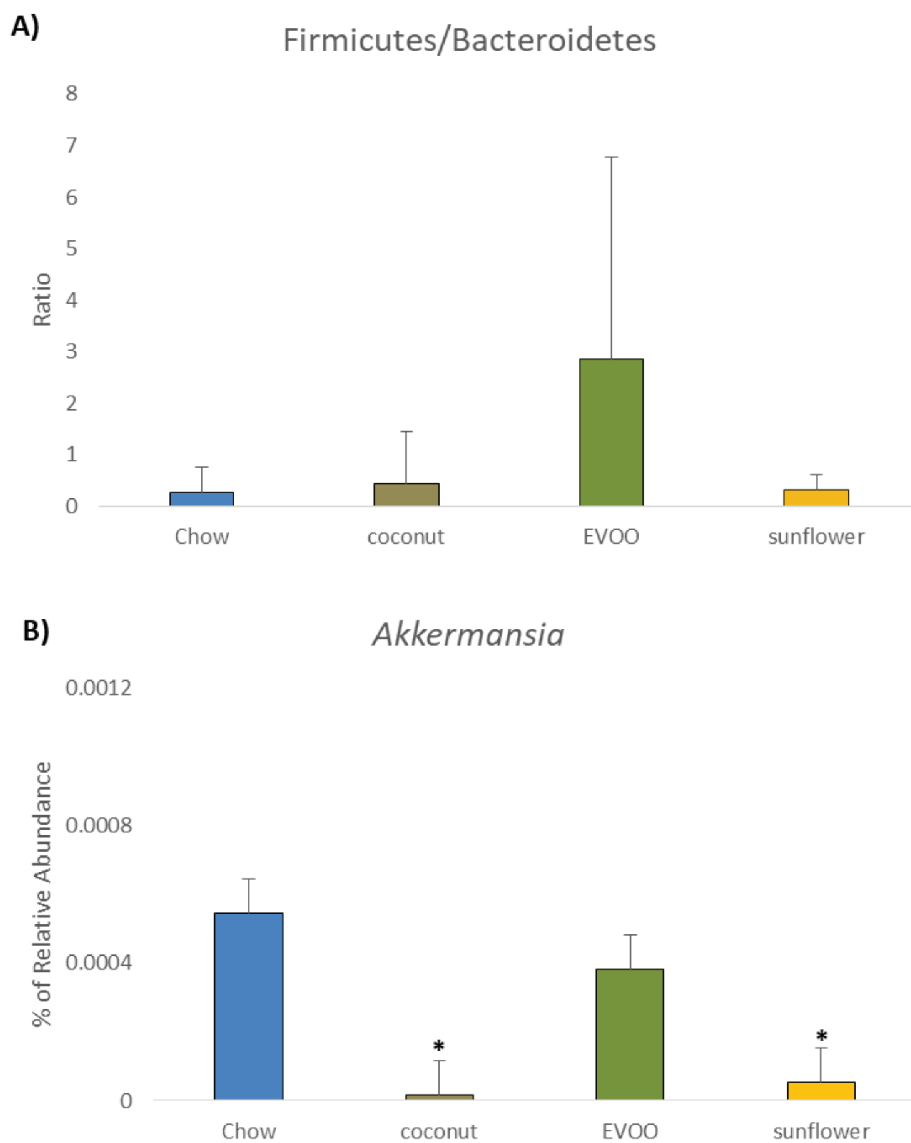


Figure 6. The relative abundance of bacterial phyla and genera (%). (A) Firmicutes/Bacteroidetes Ratio. (B) *Akkermansia*. * $p < 0.05$ for HFD (coconut and sunflower) vs. chow diet.



Table 2. Percentage of relative abundance of Phylum.

Phylum	Chow	Coconut	EVOO	Sunflower
Firmicutes	78.695 ± 20.27	75.014 ± 13.08	93.739 ± 10.56	86.081 ± 18.37
Proteobacteria	6.578 ± 5.34	3.447 ± 1.86	0.958 ± 1.61 ***	2.17 ± 1.35
Bacteroidetes	2.331 ± 3.04	0.948 ± 0.49 *	0.283 ± 0.48 ***	0.532 ± 0.46 ***
Actinobacteria	9.186 ± 15.32	1.712 ± 2.78 **	0.437 ± 0.22 ***	0.654 ± 0.45 ***
Fusobacteria	0.083 ± 0.14	0.011 ± 0.02	0.007 ± 0.01	0.046 ± 0.09
Verrucomicrobia	0.055 ± 0.12	0.002 ± 0.003 **	0.038 ± 0.06	0.011 ± 0.02

Data are shown as Mean ± SD and were analysed by the PERMANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for HFD (coconut, EVOO, and sunflower) vs. chow diet. (n = 8).

3.3. Main Findings Found at Both Genera and Species Levels: HFD Produced Both Switching *Lactobacillus* for *Lactococcus* and Only Coconut-HFD Promoting *Staphylococcus* Colonisation

Despite decreasing diversity in all HFD groups in comparison with chow, there were some relevant changes regarding some bacterial genera and species (Table 3 and Table 4). The most relevant change induced by HFD was *Lactobacillus* reduction (mainly *Lactobacillus reuteri*) and *Lactococcus* increase (mostly *Lactococcus lactis*) (Figure 7). HFD also decreased *Streptococcus*, *Turicibacter*, *Blautia*, *Clostridium*, *Ruminococcus* and *Anaerostipes* genera in a significant way in comparison with chow ($p < 0.05$). Differences between chow vs. EVOO- and sunflower-HFD were observed, reducing *Enterococcus* in a significant way (mainly *Enterococcus gallinarum*). Regarding differences between HFD groups, EVOO-HFD ($0.031 \pm 0.04\%$ of relative abundance) and coconut-HFD ($0.522 \pm 0.82\%$ of relative abundance) showed statistical differences with respect to *Staphylococcus* genera ($p < 0.05$). Coconut enhanced the relative abundance of *Staphylococcus epidermidis*.



Figure 7. The relative abundance of bacteria genera (%). (A) The taxonomic summary bar plots at the genera level, (B) chow, (C) coconut, (D) EVOO, (E) sunflower.



Table 3. Percentage of relative abundance of Genera.

Genera	Chow	Coconut	EVOO	Sunflower
FIRMICUTES				
<i>Lactobacillus</i>	51.471 ± 32.92	3.412 ± 3.99 ¹	4.717 ± 11.76 ¹	4.351 ± 5.58 ¹
<i>Streptococcus</i>	15.982 ± 24.53	0.76 ± 0.57 ¹	0.928 ± 0.66 ¹	2.428 ± 2.35 ¹
<i>Turicibacter</i>	0.152 ± 0.23	0.001 ± 0.003 ¹	0.006 ± 0.006 ¹	0.004 ± 0.004 ¹
<i>Lactococcus</i>	0.156 ± 0.19	68.83 ± 12.28 ¹	87.055 ± 13.74 ¹	77.819 ± 17.58 ¹
<i>Enterococcus</i>	0.444 ± 0.84	0.173 ± 0.4	0.125 ± 0.24 ¹	0.06 ± 0.06 ¹
<i>Blautia</i>	1.942 ± 2.71	0.129 ± 0.18 ¹	0.058 ± 0.06 ¹	0.129 ± 0.12 ¹
<i>Clostridium</i>	0.505 ± 0.64	0.01 ± 0.02 ¹	0.024 ± 0.02 ¹	0.01 ± 0.02 ¹
<i>Kineothrix</i>	1.796 ± 3.8	0.036 ± 0.07 ¹	0.014 ± 0.02 ¹	0.037 ± 0.05 ¹
<i>Ruminococcus</i>	0.411 ± 0.48	0.094 ± 0.11 ¹	0.023 ± 0.03 ¹	0.034 ± 0.03 ¹
<i>Paenibacillus</i>	0.061 ± 0.11	0.022 ± 0.05	0.003 ± 0.004 ¹	0.0003 ± 0.0007 ¹
<i>Romboutsia</i>	0.178 ± 0.24	0.046 ± 0.06 ¹	0.048 ± 0.06	0.014 ± 0.02 ¹
<i>Eubacterium</i>	0.495 ± 0.59	0.034 ± 0.05 ¹	0.03 ± 0.06 ¹	0.013 ± 0.01 ¹
<i>Lachnoclostridium</i>	0.426 ± 0.47	0.053 ± 0.07 ¹	0.046 ± 0.05 ¹	0.079 ± 0.2 ¹
<i>Gemmiger</i>	0.157 ± 0.23	0.081 ± 0.19	0.004 ± 0.004 ¹	0.033 ± 0.05
<i>Anaerostipes</i>	0.296 ± 0.45	0.012 ± 0.02 ¹	0.009 ± 0.01 ¹	0.014 ± 0.02 ¹
<i>Flintibacter</i>	0.162 ± 0.23	0.047 ± 0.06	0.009 ± 0.01 ¹	0.017 ± 0.02 ¹
<i>Staphylococcus</i>	0.194 ± 0.16	0.522 ± 0.82	0.031 ± 0.04 ^{1,2}	0.101 ± 0.1
PROTEOBACTERIA				
<i>Pseudomonas</i>	2.702 ± 2.23	2.489 ± 1.33	0.618 ± 1.19 ¹	0.999 ± 1.09
<i>Ralstonia</i>	0.696 ± 0.57	0.071 ± 0.74 ¹	0.038 ± 0.06 ¹	0.014 ± 0.01 ¹
<i>Escherichia</i>	0.095 ± 0.12	0.102 ± 0.04	0.038 ± 0.06	0.023 ± 0.02
<i>Paracoccus</i>	0.375 ± 0.98	0.024 ± 0.02	0.003 ± 0.004	0.006 ± 0.01
<i>Burkholderia</i>	0.882 ± 1.07	0.01 ± 0.02 ¹	0.018 ± 0.05 ¹	0.018 ± 0.05 ¹
<i>Moraxella</i>	0.159 ± 0.35	0.063 ± 0.14	0.02 ± 0.04	0.049 ± 0.11
<i>Neisseria</i>	0.214 ± 0.24	0.004 ± 0.008 ¹	0.002 ± 0.003 ¹	0.335 ± 0.61 ^{2,3}
<i>Acinetobacter</i>	0.073 ± 0.14	0.013 ± 0.01	0.005 ± 0.009 ¹	0.007 ± 0.01
<i>Massilia</i>	0.049 ± 0.08	0.022 ± 0.06	0.0009 ± 0.002 ¹	0.007 ± 0.01
<i>Sphingomonas</i>	0.036 ± 0.06	0.002 ± 0.005	0.001 ± 0.001	0.097 ± 0.17 ^{2,3}
BACTEROIDETES				
<i>Bacteroides</i>	0.125 ± 0.13	0.264 ± 0.21	0.103 ± 0.2	0.099 ± 0.12
<i>Prevotella</i>	0.389 ± 0.48	0.57 ± 0.29	0.134 ± 0.24	0.273 ± 0.33
<i>Parabacteroides</i>	0.004 ± 0.003	0.017 ± 0.02	0.008 ± 0.01	0.008 ± 0.01
<i>Muribaculum</i>	1.509 ± 3.26	0.003 ± 0.007 ¹	0.0003 ± 0.0007 ¹	ND ¹
ACTINOBACTERIA				
<i>Bifidobacterium</i>	5.075 ± 9.59	1.175 ± 2.52	0.138 ± 0.13 ¹	0.224 ± 0.24 ¹
<i>Paraeggerthella</i>	0.701 ± 1.38	0.005 ± 0.01 ¹	0.003 ± 0.005 ¹	0.002 ± 0.003 ¹
<i>Actinomyces</i>	0.192 ± 0.47	0.0001 ± 0.0003 ¹	0.0006 ± 0.001 ¹	0.016 ± 0.03 ¹
VERRUCOMICROBIA				
<i>Akkermansia</i>	0.055 ± 0.12	0.001 ± 0.003 ¹	0.038 ± 0.06	0.005 ± 0.008 ¹

Data are shown as Mean ± SD and were analysed by PERMANOVA test; ¹ $p < 0.05$ for groups vs. chow diet; ² $p < 0.05$ for groups vs. coconut; ³ $p < 0.05$ for groups vs. EVOO; Not detectable is shown as ND = 0.



Table 4. Percentage of Relative Abundance of Species.

Species	Chow	Coconut	EVOO	Sunflower
<i>Lactobacillus reuteri</i>	42.45 ± 29.95	0.335 ± 0.57 ¹	1.106 ± 2.84 ¹	0.658 ± 0.77 ¹
<i>Lactobacillus taiwanensis</i>	5.979 ± 7.31	0.002 ± 0.003 ¹	0.006 ± 0.01	0.002 ± 0.004 ¹
<i>Lactobacillus animalis</i>	1.71 ± 2.29	1.449 ± 2.1	0.415 ± 0.45	1.533 ± 2.76
<i>Lactobacillus caviae</i>	0.501 ± 0.34	0.004 ± 0.01 ¹	0.019 ± 0.05 ¹	0.009 ± 0.01 ¹
<i>Lactobacillus gasseri</i>	0.129 ± 0.16	0.017 ± 0.02	2.85 ± 8.05	0.019 ± 0.05
<i>Lactobacillus secaliphilus</i>	0.078 ± 0.14	0.0002 ± 0.0007 ¹	0.023 ± 0.06	0.001 ± 0.002 ¹
<i>Lactobacillus faecis</i>	0.026 ± 0.04	0.011 ± 0.02	0.01 ± 0.02	0.019 ± 0.03
<i>Lactobacillus johnsonii</i>	0.004 ± 0.004	1.351 ± 2.06	0.195 ± 0.38	2.012 ± 4.70
<i>Lactobacillus satsumensis</i>	0.002 ± 0.003	0.008 ± 0.01	0.016 ± 0.03	0.062 ± 0.12
<i>Streptococcus danieliae</i>	15.229 ± 24.65	0.711 ± 0.56 ¹	0.76 ± 0.6 ¹	1.774 ± 2.05 ¹
<i>Streptococcus oralis</i>	0.255 ± 0.30	0.022 ± 0.03 ¹	0.021 ± 0.04 ¹	0.474 ± 1.11 ^{2,3}
<i>Streptococcus sanguinis</i>	0.192 ± 0.43	0.0001 ± 0.0003 ¹	0.006 ± 0.01 ¹	0.055 ± 0.14
<i>Lactococcus lactis</i>	0.153 ± 0.19	68.407 ± 12.20 ¹	86.28 ± 13.62 ¹	77.241 ± 17.41 ¹
<i>Lactococcus taiwanensis</i>	0.003 ± 0.002	0.419 ± 0.11 ¹	0.761 ± 0.25 ¹	0.557 ± 0.21 ¹
<i>Enterococcus gallinarum</i>	0.361 ± 0.85	0.022 ± 0.06	0.003 ± 0.004 ¹	0.028 ± 0.05 ¹
<i>Blautia wexlerae</i>	0.613 ± 0.88	0.039 ± 0.06 ¹	0.012 ± 0.01 ¹	0.047 ± 0.07 ¹
<i>Blautia luti</i>	0.559 ± 0.69	0.042 ± 0.09 ¹	0.014 ± 0.02 ¹	0.015 ± 0.02 ¹
<i>Blautia obeum</i>	0.416 ± 0.90	0.012 ± 0.02 ¹	0.002 ± 0.003 ¹	0.004 ± 0.003 ¹
<i>Blautia faecis</i>	0.045 ± 0.06	0.009 ± 0.01	0.001 ± 0.001 ¹	0.005 ± 0.01 ¹
<i>Clostridium scindens</i>	0.088 ± 0.09	0.014 ± 0.02	0.027 ± 0.04	0.029 ± 0.07
<i>Clostridium spiroforme</i>	0.101 ± 0.17	0.012 ± 0.03 ¹	0.004 ± 0.01 ¹	0.0004 ± 0.0005 ¹
<i>Clostridioides difficile</i>	ND	0.001 ± 0.003	0.016 ± 0.03	0.003 ± 0.006
<i>Ruminococcus gnavus</i>	0.306 ± 0.8	0.021 ± 0.04 ¹	0.024 ± 0.04 ¹	0.051 ± 0.09 ¹
<i>Eubacterium hallii</i>	0.38 ± 0.47	0.012 ± 0.02 ¹	0.005 ± 0.01 ¹	0.008 ± 0.01 ¹
<i>Anaerostipes hadrus</i>	0.291 ± 0.45	0.012 ± 0.02 ¹	0.009 ± 0.01 ¹	0.014 ± 0.02 ¹
<i>Staphylococcus epidermidis</i>	0.065 ± 0.09	0.328 ± 0.87	0.003 ^{1,2}	0.031 ± 0.04 ²
<i>Pseudomonas migulae</i>	1.538 ± 1.56	1.199 ± 0.93	0.428 ± 0.89 ¹	0.493 ± 0.58
<i>Pseudomonas trivialis</i>	0.277 ± 0.36	0.238 ± 0.20	0.075 ± 0.16	0.169 ± 0.25
<i>Pseudomonas helmanticensis</i>	0.521 ± 1.17	0.888 ± 1.64 ¹	0.04 ± 0.1 ¹	0.277 ± 0.76 ¹
<i>Pseudomonas aeruginosa</i>	0.04 ± 0.07	0.001 ± 0.001 ¹	0.001 ± 0.001 ¹	ND ¹
<i>Neisseria mucosa</i>	0.055 ± 0.15	0.003 ± 0.007 ¹	0.0003 ± 0.0007 ¹	0.176 ± 0.32 ^{2,3}
<i>Ralstonia insidiosa</i>	0.685 ± 0.57	0.071 ± 0.07 ¹	0.038 ± 0.06 ¹	0.013 ± 0.01 ¹
<i>Prevotella oralis</i>	0.003 ± 0.01	0.004 ± 0.01	0.005 ± 0.01	0.0002 ± 0.0007
<i>Prevotella copri</i>	0.217 ± 0.32	0.543 ± 0.27	0.113 ± 0.22	0.244 ± 0.32
<i>Muribaculum intestinale</i>	1.509 ± 3.26	0.003 ± 0.01 ¹	0.0003 ± 0.0007 ¹	ND ¹
<i>Bifidobacterium longum</i>	0.683 ± 0.88	0.131 ± 0.22 ¹	0.033 ± 0.05 ¹	0.057 ± 0.08 ¹
<i>Bifidobacterium animalis</i>	4.022 ± 8.49	0.124 ± 0.14 ¹	0.094 ± 0.13 ¹	0.12 ± 0.12 ¹
<i>Bifidobacterium thermophilum</i>	0.053 ± 0.11	0.006 ± 0.02 ¹	0.001 ± 0.001 ¹	0.0001 ± 0.0003 ¹
<i>Bifidobacterium adolescentis</i>	0.189 ± 0.21	0.096 ± 0.23	0.005 ± 0.005 ¹	0.022 ± 0.04 ¹
<i>Bifidobacterium bifidum</i>	0.052 ± 0.09	0.014 ± 0.03 ¹	0.002 ± 0.005 ¹	0.004 ± 0.01 ¹
<i>Akkermansia muciniphila</i>	0.055 ± 0.12	0.001 ± 0.02 ¹	0.038 ± 0.06	0.005 ± 0.01 ¹

Data are shown as Mean ± SD and were analysed by PERMANOVA test; ¹ $p < 0.05$ for groups versus chow diet; ² $p < 0.05$ for groups versus coconut.; ³ $p < 0.05$ for groups versus EVOO.; Not detectable is shown as ND = 0.



3.4. Sunflower-HFD Enhanced *Sphingomonas* Genus

Regarding Proteobacteria, mice of the EVOO-HFD group presented a significant decrease in *Pseudomonas* abundance; in this genus, EVOO-HFD mainly reduced *Pseudomonas migulae* (Table 3 and Table 4). In comparison to EVOO- and coconut-HFD, sunflower-HFD enhanced *Neisseria*, especially *Neisseria mucosa* sp. ($p < 0.05$), while sunflower-HFD significantly increased *Sphingomonas* in comparison to chow, coconut-HFD and EVOO-HFD (Figure 7).

3.5. Coconut and Sunflower-HFD Reduced *Akkermansia Muciniphila* Abundance

With regard to the Bacteroidetes phylum, all the HFD decreased *Muribaculum* abundance (Table 3). For Actinobacteria, all the HFD decreased *Bifidobacterium*, but only EVOO- and sunflower-HFD in a significant way.

Figure 6B shows that for the Verrucomicrobia phylum, coconut and sunflower-HFD reduced *Akkermansia* (mainly *Akkermansia muciniphila*) abundance in comparison with chow ($p < 0.05$) (Table 4).

4. Discussion

Western diets characterised by high fat intake have been strongly linked to CRC in several epidemiological studies [12,13]. Indeed, diet can modulate the gut microbiota characteristics, which can eventually lead to an increased or decreased risk of CRC [14].

For the first time, our results showed that the presence of EVOO as the only source of fat in HFD, caused a dysbiosis that could be associated with the prevention of CRC, while coconut and sunflower-HFD produced a dysbiosis associated with an increased CRC risk. Although all mice were fed ad libitum during the 16 weeks of dietary intervention, surprisingly mice fed with the coconut-HFD showed a similar weight to mice of the control group (Table 1). By contrast, mice fed with the EVOO or sunflower-HFD gained considerable weight at the end of the dietary intervention. The results clearly show that HFD of coconut oil, EVOO or sunflower oil produce intestinal dysbiosis. Although similar results have been described by other researchers [15], the



present study is the only one that has used HFD containing a single type of oil as the sole source of fat; this is important because not all fats have the same impact on health. Indeed, as we can see in Figure 2, both the richness and diversity of the gut microbiota are diminished by each of the three diets administered. Similarly, beta diversity analyses corroborate these results (Figure 3). In line with these results, both the principal coordinated analysis (PCoA) and PERMANOVA analysis show a significant change in the gut microbiota of mice fed any of the three diets studied (Figure 4).

Firmicutes were the most abundant phylum found in mice gut microbiota fed with chow diet; this is consistent with other research findings [16]. Both the sunflower and EVOO-HFD diets increased the percentage of Firmicutes, although not in a statistically significant way (Table 2 and Figure 5). Interestingly, we found that EVOO-HFD increased the Firmicutes/Bacteroidetes ratio (Figure 6A). Although the increase in this ratio has been correlated with obesity, it is also associated with CRC prevention through the modulation of the inflammatory process [17,18].

Gut microbiota of all HFD studied, decreased *Lactobacillus reuteri* and promoted an increase in *Lactococcus lactis* (Table 4 and Figure 7A). Both species are probiotics that have a key role in modulating inflammatory processes. *L. reuteri* is able to improve immune activity to combat autoimmune diseases, inhibiting inflammation by reducing Th1/Th2 cell ratio and their associated cytokines [19]. On the other hand, *L. lactis* plays an important role in the antioxidant defence of gut microbiota and in the maintenance of the pro- and anti-inflammatory balance [20,21].

Bifidobacterium are a group of probiotic bacteria involved in normal colonocyte maintenance [22]. Our results showed that all three HFD produced a decrease in *Bifidobacterium*, although this only has statistical significance for EVOO- and sunflower-HFD (Table 3 and Figure 7A).

Akkermansia muciniphila has been associated with numerous human health benefits, for example, with lifespan [23]. A decrease in this bacterium has been found in individuals with inflammatory bowel disease, ulcerative colitis and Crohn's disease. Its administration can also decrease inflammatory cytokines levels in intestinal



inflammation [24]. Other authors have reported that the food additive carrageenan decreases *A. muciniphila* and promotes both inflammation and colitis in experimental animals [25].

On the contrary, some phenolic compounds such as resveratrol and caffeic acid were able to restore *A. muciniphila* after colitis induction in mice, ameliorating gut inflammation [26,27]. Interestingly, our experimental study showed that coconut- and sunflower-HFD decreased the relative abundance of *A. muciniphila* significantly, while the EVOO-HFD maintained the population of this bacterium (Table 4 and Figure 6B). A possible hypothesis is that minority compounds found in EVOO may be involved in facilitating *A. muciniphila*'s survival. It is important to note that these minority compounds are not present in either coconut or sunflower oil.

Furthermore, we have shown that the presence of opportunistic gut pathogens linked to an increase of CRC risk are also altered according to the type of diet consumed. At the genus level, we identified that *Streptococcus* and *Clostridium* decreased their abundance in mice fed any of the high-fat diets. Some species of *Clostridium*, such as *C. difficile*, produce toxic bacterial enzymes involved in bacteraemia and CRC development [28,29]. Interestingly, the coconut-HFD increased *Staphylococcus* spp., although not in a statistically significant way (Table 3 and Figure 7C). On the contrary, the EVOO-HFD significantly decreased the abundance of *Staphylococcus* spp. and especially *Staphylococcus epidermidis* (Table 3 and Figure 7D). It is well known that *S. epidermidis* is capable of forming biofilms on the colon, enhancing CRC risk [30]. All the bacteria mentioned above are considered to be opportunistic pathogens and have been found on colon specimens from patients with colitis and autoimmune diseases [31].

Another interesting finding was that EVOO- and sunflower-HFD reduced *Enterococcus gallinarum* abundance in a significant way. *E. gallinarum* is considered a pathobiont able to translocate to the liver and other tissues, triggering autoimmune responses [32]. In addition, it has been suggested that antibiotic treatment prevents mortality in mice by suppressing the growth of *E. gallinarum* in tissues [33], suggesting



that EVOO- and sunflower-HFD could act on the autoimmune response by controlling the abundance of *E. gallinarum* in the colon.

Several studies have demonstrated an increased abundance of Proteobacteria phylum in pathologies, including inflammatory bowel disease; in fact, inflammation represents a core aspect of pathologies associated with Proteobacteria [34]. In addition, its high abundance has been correlated with oxygen reactive species generation and inflammation in humans with inflammatory bowel disease, colitis and CRC [35,36,37]. Sunflower-HFD enhanced *Neisseria* and *Sphingomonas* significantly (Table 3 and Figure 7E), both of which are considered pathobionts [38]. In fact, its increase has been correlated with a pro-inflammatory response in the mucosa of the colon, promoting natural killer T-cell activity of colitis-associated cancers [39,40]. On the contrary, coconut-HFD reduced *Neisseria* abundance, and EVOO-HFD reduced not only *Neisseria* and *Pseudomonas*, but also Proteobacteria abundance (Table 3, Figure 7C,D). To our knowledge, there are no studies that have described the beneficial or harmful effects related to Proteobacteria induced by EVOO, coconut or sunflower-HFD, however, some authors have described the protective effects of EVOO on immunomodulation in murine experimental ulcerative colitis and patients with ulcerative colitis [41,42], that could be associated not only with inflammatory processes involved on the MAPK and NFκB signalling pathways but also with microbiota modulation, as our results showed [43].

In another pathogenic microbiome, *Prevotella* and *Bacteroides* abundance appeared to be higher in coconut-HFD than EVOO- and sunflower-HFD (Table 3 and Figure 7C). Both genera have negative effects on colitis through the enhancement of inflammation; in fact, its reduction ameliorates colonic inflammation [44,45]. *Prevotella* abundance is also related to the increase in epithelial inflammation and triggers autoimmune diseases [46,47]. The results suggest that coconut-HFD could produce an increased risk of CRC by stimulating the growth of *Prevotella* and *Bacteroides*, while diets rich in EVOO or sunflower oil could have a protective effect. Different research with honey polyphenols, essential oi and different vegetable oils, have shown that they were able to improve oxidative stress resistance and intestinal



inflammation by *Bacteroidetes* reduction [48,49]. We could hypothesise that the effect of EVOO is probably due to the presence of polyphenols that characterise this fat of vegetable origin.

The gut microbiota can modulate the inflammatory processes that can eventually result in the presence of a pro- or anti-tumour environment in the colon. Thus, the main objective of this study was to determine whether an HFD containing only EVOO, sunflower oil or coconut oil modifies the gut microbiota in a way that favours or prevents CRC. In brief, the main findings of this study could be summarised as follows: (a) the three HFD studied (EVOO-, sunflower- and coconut-HFD) produce a decrease in both the richness and diversity of the gut microbiota, (b) these alterations do not correlate with body weight at the end of the dietary intervention, (c) each of these HFDs has a different impact on the gut microbiota by promoting or inhibiting the growth of different bacteria that are associated with a pro- or anti-inflammatory environment (summarised in Figure 8), and that are associated with the dysbiosis linked to colorectal cancer risk (Figure 9).

This study has several limitations, including the sample size, the exploratory nature of the study, and type-II errors which could fail to detect effects present in small-scale studies. For instance, further studies are needed to assert these hypotheses. The strength of this study is that we use a single source of fat in the diet; a large number of studies use high-fat diets supplemented with different types of fat. This can be misleading, since the basis of nutritional preparations for animals has a significant amount of both vegetable (soy oil) and animal (lard) fat. Giving therefore results that could be wrong or a mixed of effect from animal and vegetable oil. Using a single type of fat allows us to observe its specific effect on health.

Finally, it is difficult to extrapolate the results obtained in this study to humans due to interspecies variability. However, the usefulness of studies using murine experimental models as a preclinical model is widely accepted [50]. In addition, it is not possible to conduct a dietary intervention study in humans in which the diet contains only one type of fat, highlighting the importance of the data obtained in this study.

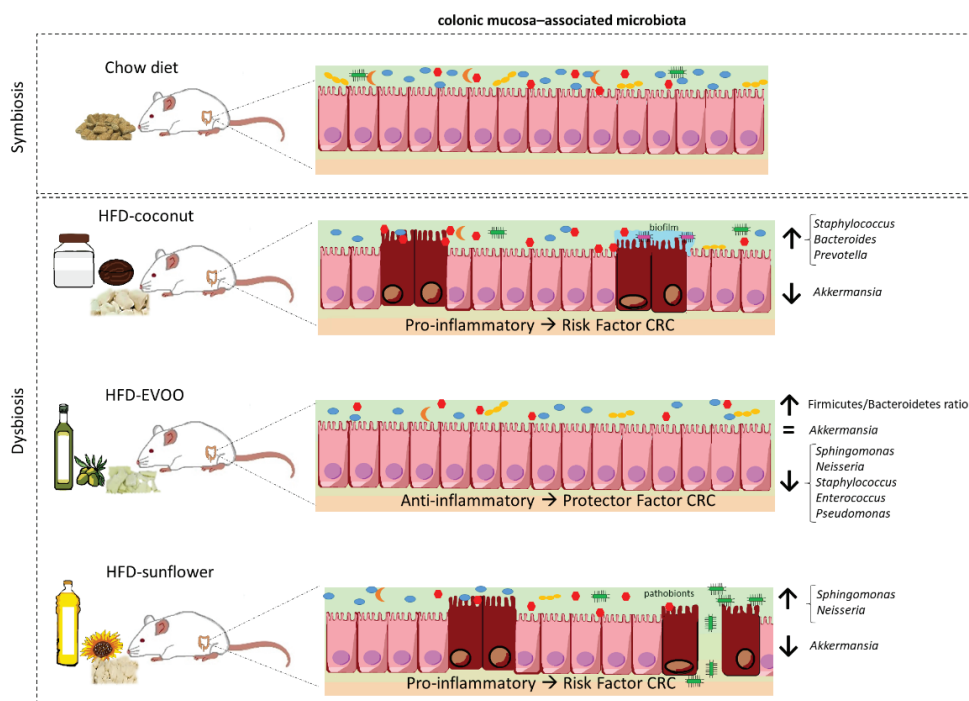


Figure 8. Summary of the main effects on gut microbiota caused by chow diet and HFD bases on coconut, EVOO or sunflower oil.

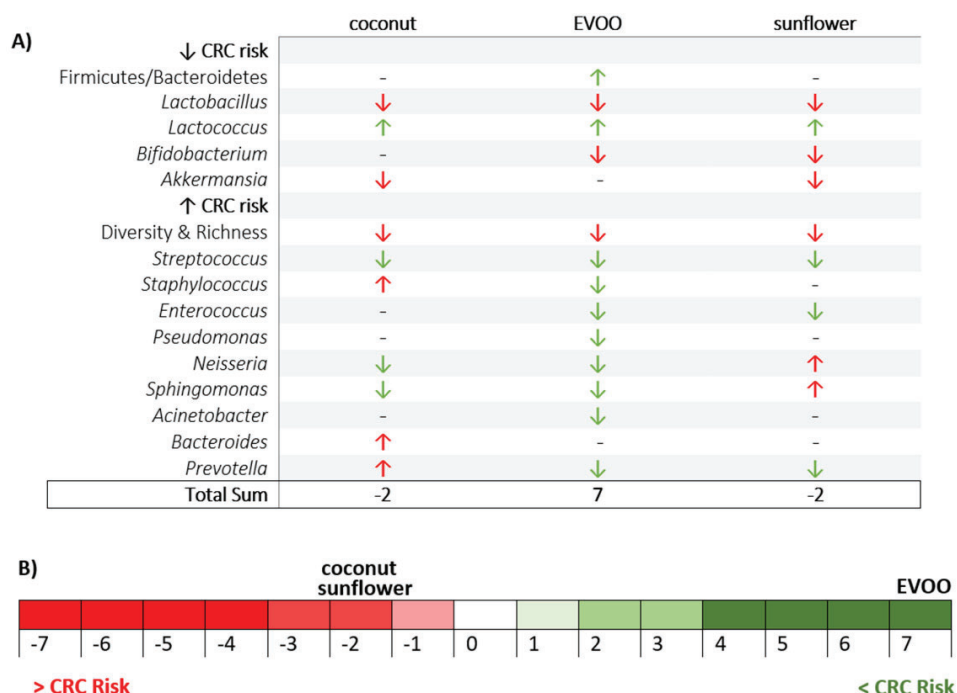


Figure 9. The effect of high-fat diets of sunflower, coconut and EVOO oils on dysbiosis linked to CRC risk. (A) Summary of HFD effects on bacteria related with CRC risk (Red arrow, increases CRC risk (minus 1); Green arrow, decreases CRC risk (plus 1); Dash, no changes (equal)). (B) Risk scale of developing CRC based on vegetable oil-HFD intake. Data are based on the total sum of Figure 9A.

Supplementary Materials

The following are available online at <https://www.mdpi.com/2072-6643/12/6/1705/s1>, Figure S1: Rarefaction curves of the amplified samples for the detection of bacterial genera, Table S1: Composition of experimental high fat diets, Table S2: Minority compounds found in EVOO, Table S3: PicoGreen-Based DNA Quantification and Quality control analysis.



Table S1. Composition of experimental high fat diets

	<i>coconut</i>	<i>sunflower</i>	<i>EVOO</i>
Diet component	<i>g/kg</i>		
Custom diet mix ¹	666.6	666.6	666.6
Coconut oil	333.4	-	-
Sunflower oil	-	333.4	-
Extra virgin olive oil	-	-	333.4
	% kcal		
Protein	18	18	18
Carbohydrate	22	22	22
Fat	60	60	60
Fatty acid composition	<i>g/100g total fatty acids</i>		
Σ SFA ²	90.4	7.7	16.04
Σ MUFA ³	7.2	39.7	78.54
Σ PUFA ⁴	1.9	52.7	4.46
Key fatty acids			
C8:0	5.8	-	-
C10:0	6.2	-	-
C12:0	46.2	-	-
C14:0	19.3	-	0.01
C16:0	10	5.1	13.61
C16:1	-	0.1	1.21
C18:0	2.9	2.6	2.42
C18:1	7.2	39.6	77.33
C18:2 ω6	1.9	52.6	3.64
C18:3 ω3	-	0.1	0.82

¹Custom diet mix supplied and formulated by ENVIGO®

²SFA: saturated fatty acids

³MUFA: monounsaturated fatty acids

⁴PUFA: polyunsaturated fatty acids

Table S2. Minority compounds found in EVOO

<i>Minority compounds</i>	<i>mg/kg</i>
Squalene	9701
Total tocopherols	378.2
α-Tocopherol	369.1
β-Tocopherol	3.6
γ-Tocopherol	5.6
δ-Tocopherol	<1.0
Tyrosol	2.5
	<i>mg/kg tyrosol</i>
Total Biophenols	265.1
Hydroxytyrosol	0.6
Oleocanthal	34
	<i>mg caffeic acid/kg</i>
Total Polyphenols	372



Table S3. PicoGreen-Based DNA Quantification and Quality control analysis

<i>Sample</i>	<i>PicoGreen-Based DNA Quantification assay</i>	<i>Quality control</i>			
	Concentration (ng/μl)	Secuencias number	Length	Total Mb	Mean quality
1	8,57	160.391	408,66	65,55	36,63
2	2,62	35.336	402,03	14,21	36,66
3	0,69	52.841	403,59	21,33	36,6
4	6,62	62.736	407,23	25,55	36,57
5	1,43	50.976	395,67	20,17	36,57
6	6,46	124.853	407,03	50,82	36,56
7	6,78	28.568	406,31	11,61	37,13
8	48,96	62.205	405,98	25,25	36,69
9	32,76	262.236	415,23	108,89	37,72
10	24	35.561	400,73	14,25	37,56
11	17,33	39.532	407,51	16,11	37,56
12	19,46	40.347	396,82	16,01	37,53
13	11,07	14.200	396,63	5,63	37,52
14	15,89	11.165	403,01	4,5	37,49
15	3,4	14.475	402,22	5,82	37,62
16	4,5	22.264	412,94	9,19	37,59
17	17,79	128.719	413,63	53,24	37,64
18	5,87	53.570	405,58	21,73	36,59
19	6,51	7.235	394,54	2,85	37,56
20	14,41	55.965	408,66	22,87	36,46
21	67,27	72.339	411,68	29,78	37,03
22	56,12	61.013	407,87	24,89	36,49
23	39,88	62.298	402,28	25,06	36,15
24	24,04	211.347	410,15	86,68	37,85
25	41,39	287.816	408,71	117,63	36,53
26	14,61	61.276	408,11	25,01	36,5
27	28,73	32.903	385,25	12,68	37,46
28	20,44	182.564	415,39	75,84	37,6
29	10,61	74.745	412,45	30,83	37,64
30	11,76	56.803	405,31	23,02	37,54
31	20,13	215.602	414,9	89,45	37,63
32	10,68	72.644	408,24	29,66	37,42

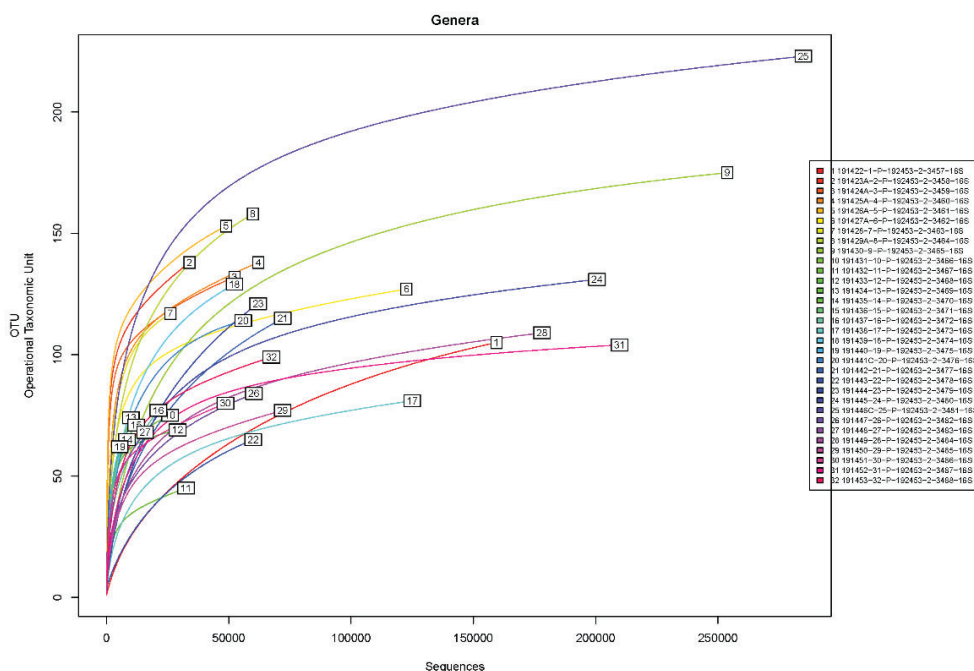


Figure S1. Rarefaction curves of the amplified samples for the detection of bacterial genera

Author Contributions

J.J.G. and C.S.-Q. conceived and designed research; C.R.-G. performed the experiments and analysed data; J.J.G. and C.R.-G. drafted the manuscript; J.J.G., I.A. and C.S.-Q. edited and revised the manuscript; All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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5.2. Capítulo de libro

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Antiinflammatory activity exerted by minor compounds found in virgin olive oils

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Chapter 46

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Abstract

Nowadays chronic inflammation is remarked as one of the main causes of several diseases such as cancer, cardiovascular diseases, or neurological diseases. Lifestyle and diet are key factors of the development of chronic inflammation. Because of that, in the last years Mediterranean diet has been granted as an antiinflammatory diet, where virgin olive oil from vegetable origin is one of the main actors because it is use as the main fat of Mediterranean diet. Virgin olive oil is mainly composed by oleic acid; but there are many minor compounds present in it, such as squalene, pinosresinol, uvaol, erythrodiol, oleanolic acid, or maslinic acid, which possess per se many bioactive activities in inflammation, as long as in other diseases as cancer or cardiovascular diseases. Because of their presence, it is believed that virgin olive oil possesses the beneficial actions described in the last decades. In this chapter, inflammation properties of several minor compounds present in virgin olive oil have been described and explained.

Keywords: Chronic inflammation; Macrophages; Immune system; Squalene; Pinosresinol; Oleanolic acid; Maslinic acid; Uvaol; Erythrodiol



Mediterranean diet

Both chronic inflammatory processes and oxidative stress play important roles in chronic diseases. Different strategies to reduce both processes will aid in the prevention of these diseases. In this context, diet could be a preventive and aggravating factor. Thus it is important to emphasize the importance of following a healthy diet. The effects of diet on human health have been widely reported in many scientific studies, providing evidence that a dietary pattern rich in certain beneficial food groups can reduce the incidence of various chronic degenerative diseases.¹ Specifically the Mediterranean diet is considered as an example of a healthy diet. Several public health-based studies have revealed that adherence to the Mediterranean diet is associated with longevity and lower incidence of major chronic degenerative diseases, such as cardiovascular diseases and several cancers.² Unfortunately, diet and lifestyle changes have accelerated over the past three decades in developing and in transition countries. Over the past 20 years, there has been a generalized decrease in adherence to the Mediterranean diet in Southern European countries. This decrease is having a significant effect on the health and nutritional status of these populations. Therefore it is important to promote a healthy diet, especially among young people.

Virgin olive oil

Although there are variations in the components of the traditional Mediterranean diet between countries, virgin olive oil (VOO) represents a distinctive element, because it is the main source of dietary lipids in the traditional Mediterranean diet. It has been reported that VOO can effectively prevent and/or reduce hypercholesterolemia, serum lipoprotein concentrations, atherosclerosis, hypertension, cardiovascular diseases and thrombotic risk, oxidation and oxidative stress, obesity, type-2 diabetes, inflammatory processes, and cancer.³ Estruch et al.⁴ conducted a primary prevention trial and observed that consuming an energy-unrestricted Mediterranean diet supplemented with extra VOO resulted in a substantial reduction in the risk of major cardiovascular events among high-risk participants.



VOO is obtained from the fruit of the olive tree (*Olea europaea sativa*) by mechanical or other physical means under conditions, particularly thermal, that do not alter the oil. Both virgin and extra VOOs are produced by simply crushing olives and extracting the juice in a press or centrifuge. Both virgin and extra VOO are not subjected to any treatment except washing, decantation, centrifugation, and filtration. This process retains most of the compounds originally present in the olive fruit, which are responsible for the characteristic taste, flavor, and color of the oil and contribute to the beneficial health effects of VOO. Consequently, VOO is a natural olive juice; it is the only vegetal fat that is obtained from the fruit, whereas others are obtained from seeds. Indeed, other edible oils such as sunflower, soybean, and rapeseed canola oils must be refined before consumption; and their original composition changes during this process.

The chemical composition of olive oils may vary depending on different factors, including olive variety, the ripening stage of the fruit at the time of collection, and the method used to process the olive fruit. Overall the chemical composition of virgin olive oil consists of major and minor components. The major components represent about 98%–99% of the total oil weight and are mainly composed of triacylglycerols. Oleic acid (a monounsaturated fatty acid) is present in a much higher concentration (55%–83%) than those of the other acids (linoleic, palmitic, or stearic acids). The minor components, present in small amounts (about 2% of oil weight), include more than 230 chemical compounds such as aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile compounds, and antioxidants. VOO is particularly appreciated by consumers for its high content of healthy compounds such as oleic acid and numerous functional bioactive components. In addition, the latter also contribute to the unique flavor and taste of virgin olive oil.

Moreover, both the US Food and Drug Administration and the European Food Safety Authority have confirmed the health benefits of some of the components present in virgin olive oil. In 2004 the US Food and Drug Administration allowed a claim to be included on olive oil labels concerning “the benefits on the risk of coronary heart disease of eating about two tablespoons (23 g) of olive oil daily due to the



monounsaturated fat in olive oil.” Moreover, in 2012 the Official Journal of the European Union published a list of health claims that may be made on food labels and packaging [Commission Regulation (EU) No. 432/2012].

Among these are two health claims directly related to olive oil. The first claim refers to oleic acid: “Replacing saturated fats in the diet with unsaturated fats contributes to the maintenance of normal blood cholesterol levels. Oleic acid is an unsaturated fat.” The second claim refers to the polyphenols in olive oil and their related health benefits: “Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress.” However, there is growing interest in studying the biological effects of the many minor components present in virgin olive oils. In recent years a large amount of scientific evidence has been published by researchers all over the world. Based on this evidence, olive oil can be categorized as a functional food that contains, in addition to a high level of oleic acid, other medicinally important minor components with multiple biological activities.

Minor compounds in virgin olive oil

There are about 230 different minor compounds included in the final composition of virgin olive oil. These compounds are divided in classes according to their chemical structure (Table 1).

Table 1. Groups of minor compounds in virgin olive oil and representative members of each group.

Group	Main compounds
<i>Sterols</i>	Stigmasterol, campesterol
<i>Tocopherols</i>	α -tocopherol, β -tocopherol, γ -tocopherol
<i>Triterpenes</i>	Oleanolic acid, maslinic acid, uvaol, erythrodiol
<i>Carotenoids</i>	β -carotene, lutein, violaxanthin, neoxanthin
<i>Hydrocarbons</i>	Squalene
<i>Phospholipids</i>	Lecithin, cephalin
<i>Phenols</i>	Hydroxytyrosol, tyrosol
<i>Lignans</i>	Pinoresinol, acetoxypinoresinol



Squalene

One interesting compound is squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,20-tetracosahexane), which is a terpenoid hydrocarbon (Fig. 1) present in high concentrations in virgin olive oils. Squalene is a polyunsaturated hydrocarbon with the formula $C_{30}H_{50}$. It is the major hydrocarbon present in virgin olive oil (> 90%), with a content ranging from 0.8 to 13 g/kg.⁵ Olive oil contains up to 300-fold more squalene than other vegetable oils and up to 5000-fold more than some vegetable foods.⁶ The squalene content in olive oil depends on the olive cultivar and oil extraction technology, and it is considerably reduced during the process of refining. It is important to note that extra-virgin olive oil contains significantly higher concentrations of squalene than refined olive oil and seed oils.

In addition to its health benefits, virgin olive oil exhibits high resistance against oxidation compared with that of other vegetable oils. This is an important feature because the physicochemical changes associated with autoxidation result in the loss of sensory and nutritional values. Indeed, it has been suggested that squalene makes a limited contribution to the oxidative stability of virgin olive oil at ambient or slightly elevated temperatures, but it seems to play a more significant role in protecting the quality of olive oil during exposure to frying temperatures.⁷

All plants and animals, including humans, are capable of producing squalene. Plant sterols, also called phytosterols, are biosynthetically derived from squalene. In olive oil, sterols are present in the range of 1800–4939 mg/kg. These compounds are known to reduce serum concentrations of low-density lipoprotein (LDL) cholesterol in both humans and animals.⁸



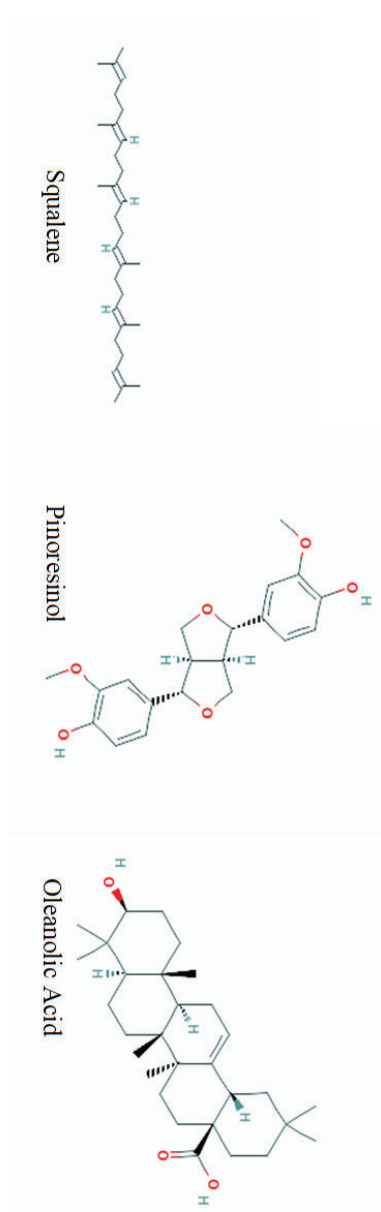
 Maslinic Acid



 Uvaol



 Erythrodil





Pinoresinol

This compound is a lignan found in olives and olive oil; and, specifically, 1-acetoxypinoresinol, which is a metabolite of plant metabolism, is found only in olive oils. These compounds act as phytoestrogens, which are natural molecules similar to human oestrogens.⁹ Pinoresinol (C₂₀H₂₂O₆) is a phenolic compound that forms a dimer, which possesses two phenol groups in its chemical structure (Fig. 1). Each phenol group consists of an aromatic ring (phenyl or benzene group) bound to a hydroxyl group (OH). The phenol group and the benzene ring are both associated with several health benefits in humans, including antioxidant and/or antiinflammatory effects.⁹

The biosynthesis of pinoresinol has been investigated in *Forsythia* spp.^{10,11} This compound is synthesized by the stereospecific coupling of two units of coniferyl alcohol in the presence of a dirigent protein (DIR). Then, it is metabolized to lariciresinol and secoisolariciresinol via pinoresinol-lariciresinol reductase (PLR) or to piperitol and sesamin via piperitol/ sesamin synthase (PSS).¹⁰

Lignans are distributed widely in the plant kingdom, with flaxseed being the major source. The consumption of VOO is associated with beneficial effects that could result from the presence of these lignans as the predominant compounds in its phenolic fraction. In fact, Owen et al.^{12,13} noted that these compounds are not present (or virtually absent) in seed oils and refined oils but are the major components of the phenolic fraction of virgin olive oils, with concentrations up to 100 mg/kg.

These researchers also found considerable variations in the concentrations of these compounds within different oils, but the average total lignan concentration was 41.53 ± 3.93 mg/kg.^{12,13}

Oleanolic acid

This triterpene is a hydroxyl pentacyclic acid (Fig. 1) present in the leaves of *Olea europaea* and olive skin.¹⁴ The leaf contains high levels of oleanolic acid (3.0%–3.5% DW); however, this amount changes during leaf ontogeny. The content of



oleanolic acid in VOO is between 8.90 and 112.36 mg/kg, depending on genetic factors of the different types of olive trees.¹⁴

In humans the plasmatic concentrations measured in healthy male volunteers after a single oral administration of 40 mg oleanolic acid were 26.5 ± 15 nM at 5.2 ± 2.9 h. These plasmatic concentrations appeared to be affected by molecular interactions between serum proteins and oleanolic acid.¹⁴

Maslinic acid

Maslinic acid is a hydroxyl pentacyclic triterpene (Fig. 1), and with oleanolic acid represent the total triterpenic fraction found in VOO. This molecule only differs in two vicinal hydroxyl groups at the C-2 and C-3 positions, besides the carboxyl radical, respect to oleanolic acid. Both are present in leaves and maslinic acid, the second most common found in leaves and VOO. The bioavailability of this compound was studied after a single oral administration (50mg/kg) to rats, and it was absorbed by the intestine and reached the blood, where it was found 10 min after the oral administration and could still be detected in plasma after 60 min. However, no study has examined its levels in humans after administration.¹⁴

Uvaol

Uvaol is a triterpene dialcohol (Fig. 1). This compound has been considered to be closely related to erythrodiol because of their similar chemical structures and because both possess two hydroxyl groups in remote positions, but they differ with regard to their methyl group location.¹⁴ This compound is present in the skin of the olives and is found at higher levels presence in pomace oil, a category of olive oil that differs from VOO mainly by the use of chemicals for the extraction of the oil. There are no available data about its bioavailability neither in humans nor in vivo.

Erythrodiol

This compound, together with uvaol, confirms the main representants of the dialcohols found in the triterpene group. The difference between compounds is the functional group present at the C-17 position (Fig. 1). The amount of uvaol-erythrodiol



determines the quality and class of an oil, and both are present in higher concentrations in pomace oil and in low concentrations in VOO.¹⁴ Erythrodiol is found mainly in the skin of the fruit, and its level can increase in the oil depending on the methods used for the extraction of olive oil. To date, there have been no bioavailability studies on its levels in humans after oral administration.

Biological activities of minor compounds in inflammation

The antioxidant, antiatherogenic, antitumoral, and detoxifying activities, among others, that these minor compounds exert have been described over the past ten years. However, the initiation of several diseases, including cancer, cardiovascular disease, or neuronal diseases such as Parkinson's or Alzheimer's disease, is directly related to inflammation processes in the early stages of the illness, as well as in later stages. Here, we describe how these compounds present in VOO can modulate and affect inflammation processes.

Inflammation is a complex process that involves immune cells as well as several signals to control homeostasis and resolve attacks from pathogens or wounds in the body (Fig. 2). In this process, we can differentiate between the innate and adaptive immune systems. The innate immune system is the first to develop an inflammatory response, and the cells that play the key role in this process are macrophages. There are different types of macrophages, with different activities. M1 macrophages are responsible for the cytotoxic response to protect the body from pathogens and/or disrupted cell growth processes in the body. M2 macrophages are responsible for the wound healing process and recovery of the injured tissue. The adaptive immune system is designed to combat infections by pathogens in a process that requires immunological memory, for which T- and B-lymphocytes are mainly responsible. This system also contributes to the inflammation process and has a close relationship with innate immune cells.¹⁵ However, when the inflammatory response is interrupted and homeostasis is difficult to restore, inflammation could result in chronic inflammation, an inflammation that lasts for prolonged periods.¹⁶ This chronic inflammation is directly related to the development of certain diseases such as cancer,

cardiovascular disease, inflammatory bowel disease, rheumatoid arthritis, systemic lupus erythematosus, and sclerosis.¹⁶

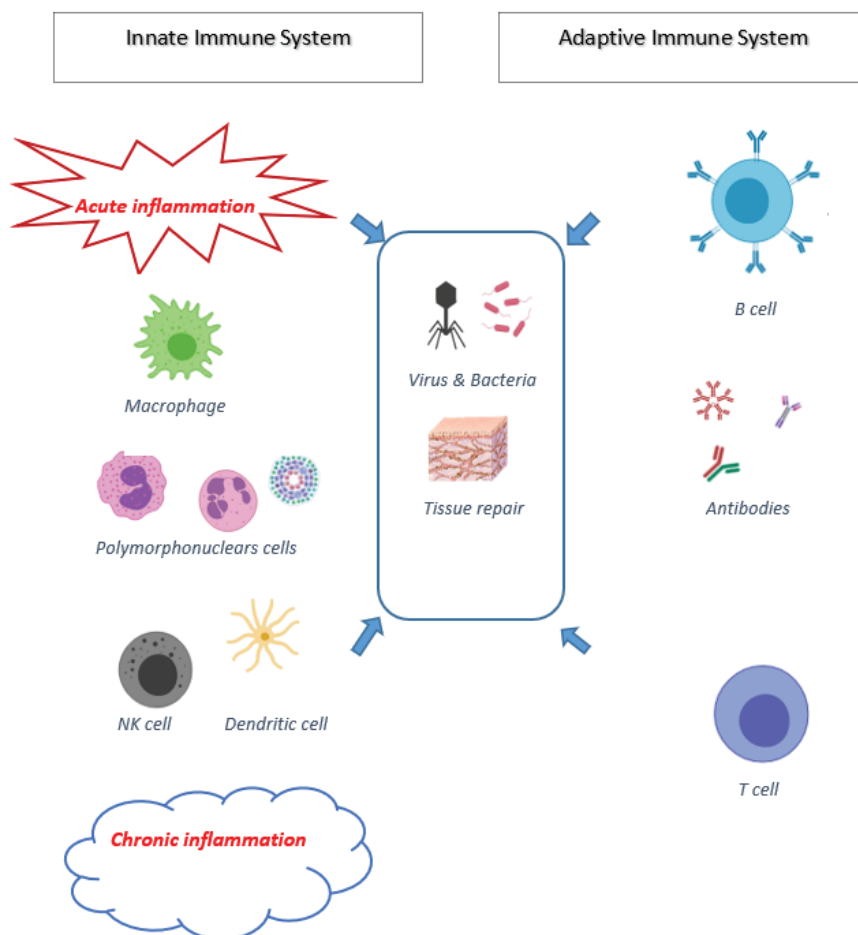


Figure 2. Main cells involved in the inflammation response to pathogens and tissue damage. PMN, polymorphonuclears; NK cells, natural killer cells.

Squalene

As mentioned earlier, squalene is a polyunsaturated triterpene composed of six isoprene units possessing antioxidant properties. Squalene is structurally similar to the carotenoids (b-carotene and lycopene) and is an intermediate metabolite in the synthesis of cholesterol and other steroids. In plants, squalene cyclizes to form phytosterols; whereas in animals, it cyclizes to form cholesterol.¹⁷



To date, anticancer, antioxidant, drug carrier, hypocholesterolemic, detoxifying, skin hydrating, and emollient activities of squalene have been reported. Because of these properties, squalene is especially useful in nutrition, pharmaceutical, cosmetic, and medicine applications.¹⁸

Its skin health maintenance activity is particularly interesting. Squalene seems to function in the skin as a quencher of singlet oxygen, protecting the human skin surface from lipid peroxidation caused by exposure to ultraviolet (UV) light and other sources of oxidative damage.¹⁹ Kohno et al.¹⁹ reported that squalene is both a highly effective oxygen-scavenging agent and resistant to peroxidation, remaining stable against attacks by peroxide radicals. These characteristics could be related to the antiinflammatory action that it exerts in the wound healing process by modulating inflammation.²⁰ This compound can modulate macrophage behavior at wound healing sites, helping to close and clean the wound by inducing the activity of M2 macrophages. This compound enhances the antiinflammatory signals responsible for ending the inflammation process, thus stopping the inflammation process and preventing chronic inflammation.²⁰ Moreover, in humans, dietary squalene is absorbed, transported in serum, and distributed ubiquitously in human tissues; the highest level of accumulation occurs in the skin because of the concentration in sebocytes.²¹ There is clear evidence that dietary bioactive compounds have beneficial effects on skin health.^{22,23} Cho et al.²⁴ reported that in healthy volunteers, a high oral dosage (> 13.5 g/day) of squalene significantly decreased wrinkles in aged human skin, increased type I procollagen, and decreased UV-induced deoxyribonucleic acid (DNA) damage. Therefore we can speculate that the regular consumption of virgin olive oil rich in squalene may help maintain healthy skin because of the wound healing effect that squalene exerts through inflammation modulation. Thus it is able to modulate neutrophils and monocytes using pro- and antiinflammatory signals and prevent their overactivation to efficiently terminate the inflammatory response²⁵ On the other hand, squalene has been described as an excellent emollient, and it is quickly and efficiently absorbed deep into the skin, restoring its suppleness and flexibility. Interestingly, it has been reported that oral



supplementation of squalene in mice resulted in a marked dose-dependent upregulation of cellular and nonspecific immune functions.²¹

In general, we can conclude that a diet with an adequate intake of oils containing squalene, such as virgin olive oil, might be sufficient to achieve the protective benefits described earlier.

Pinoresinol

This lignan present in VOO has been shown to modulate the inflammatory response in the small intestine *in vitro*. During et al. reported the reduction of proinflammatory response signals, such as IL-6 and cyclooxygenase (COX)-2-derived prostaglandin E(2). Both of these signals are elevated in patients with inflammatory bowel disease (IBD). Moreover, pinoresinol was found to attenuate chronic inflammation by blocking the NF- κ B signaling pathway, which in return is mediated by decreasing expression levels of the IL-6, MCP-1, and COX-2 genes.^{26,27} Although there have not been many scientific reports on the role pinoresinol plays in inflammation, this is a promising compound for this field because of its action in different diseases.⁹ However, more information about the molecular mechanism involved in its effects is needed to better understand the role that pinoresinol may play in inflammation.

Oleanolic acid

This triterpene is known to have antiinflammatory and anticancer properties.¹⁴ Nevertheless, very little has been reported about the molecular mechanism of this compound in inflammation. It has been described that oleanolic acid modulates the M1 response in macrophages and induces the activation of an antiinflammatory response.²⁸ These effects appear to be achieved by the inhibition of inflammatory signals as IFN- γ , IL-4, and IL-6 and the activation of IL-10 (a hallmark of the antiinflammatory response).²⁸ Phull et al. described the dedifferentiation of rabbit articular chondrocytes by inhibiting type II collagen and proinflammatory activity by increasing cyclooxygenase-2 (COX-2) expression, which could occur through the p38 and ERK pathways.²⁹



Other studies have indicated that it protects against cardiac injury in an experimental autoimmune myocarditis mouse model. Oleanolic acid is able to increase Treg cells (adaptive immune cells) and the production of IL-10 and IL-35 while reducing proinflammatory cytokines, so this compound promotes an antiinflammatory state in cardiomyopathies, including myocarditis, which will aid in protecting against the disease.³⁰ Thus this compound found in VOOs appears to function as a natural antiinflammatory molecule.

Maslinic acid

Over the last 5 years, maslinic acid has been extensively studied as a natural molecule capable of driving chronic inflammation and, as a result, preventing several diseases. The arachidonic acid pathway represents one of the major mechanisms for inflammation. Cyclooxygenase (COX) enzymes form prostaglandins from arachidonic acid, and their activity is blocked by nonsteroidal antiinflammatory drugs. Maslinic acid appears to modulate this pathway by affecting arachidonic acid metabolism. Specifically, it modulates NF- κ B signaling in the cell matrix and, therefore, chronic inflammation.^{14,31} Maslinic acid only differs from oleanolic acid by one chemical group, but its activity and molecular mechanism of its actions differ from those of oleanolic acid; it is apparently a more effective driver of inflammation than oleanolic acid, which is why this compound has been more extensively studied than oleanolic acid.

As mentioned earlier, maslinic acid is able to reduce COX, but it also reduces nitric oxide (NO), both of which are involved in chronic inflammation and tumorigenesis. Aside from the regulation of inflammation by the main molecules involved in developing chronic inflammation, maslinic acid promotes downstream targets of inflammation such as the mitogen-activated protein kinase (MAPK) pathways, which in turn are related to tumor formation.³²

Other applications of this natural triterpene are related to risk factors for chronic inflammation such as weight and diet imbalance. In this context, with VOO consumption, it is possible to achieve weight loss, which will help to prevent diseases



such as arthritis because maslinic acid improves inflammatory markers and mild knee joint pain in humans according to recently published data.³³

Uvaol

Uvaol has been reported to possess antitumor activities¹⁴ and has beneficial effects on cardiovascular disease, but very little is known about its role in inflammation. Recently an important and effective activity of this natural triterpene in allergic processes was studied. Uvaol was found to inhibit IL-5 in pleural effluent, decreasing neutrophil infiltration, mucus production, and the number of alveoli that collapsed in the lung.³⁴ Uvaol has been reported to modulate interleukin production in macrophages, decreasing proinflammatory signals such as TNF- α and I-309.³⁵

Thus this natural molecule could be considered to be an antiinflammatory biocompound. VOO could, in part, also be considered to be an antiinflammatory dietary substance because it contains compounds like this.

Erythrodiol

Erythrodiol has not been thoroughly studied, and there is little evidence of its effects on inflammatory processes. However, recent scientific reports indicate that erythrodiol acts as an ABCA1 stabilizer and exerts a positive effect on cholesterol efflux in macrophages in vitro.³⁶ These effects are key factors in atherosclerosis, and erythrodiol appears to be a promising compound for the prevention of inflammation and the development of these types of illness and even treating them. Erythrodiol has been reported to be an excellent antioxidant, decreasing reactive oxygen and nitrogen species,¹⁴ which are both cytotoxic and important tools for macrophages. This antioxidant action could be used by immune cells to drive the inflammatory response.

Although the minor compounds in VOO appear to be promising for treating and controlling inflammation processes, the effects that they could exert in humans must be carefully considered. More evidence is needed to corroborate the data published to date, but the minor compounds found in VOOs could be responsible for the antiinflammatory effects that VOO exerts in several diseases.



Summary points

- The Mediterranean diet is a well-known diet model for the prevention of major chronic diseases. These findings are relevant in terms of public health and are in agreement with current guidelines and recommendations from all the major scientific associations that strongly encourage a Mediterranean-like dietary pattern for the primary and secondary prevention of major chronic diseases related directly to inflammation.
- Virgin olive oil is considered a key component of the Mediterranean diet and is the main source of dietary lipids. Based on the scientific evidence, virgin olive oil can be categorized as a functional food not only because of its balanced lipid composition but also because of the presence of the so-called minor components.
- There are a large number of minor compounds present in virgin olive oils, and many of them have anti-inflammatory properties.
- The minor compounds in virgin olive oil can control chronic inflammation to prevent the development and appearance of chronic diseases. Thus virgin olive oil, based on its composition, can prevent the appearance of chronic diseases by managing inflammation.
- In the future, some of the minor compounds found in virgin olive oils could be used for the management of diseases, especially those in which the immune system is involved, such as allergies.

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5.3. Artículo científico II

Coconut oil-based high-fat diet develops hypoleptinaemia in an experimental murine model

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Coconut oil-based high-fat diet develops hypoleptinaemia in an experimental murine model

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Abstract

Scope: High-fat diets (HFD) are detrimental to health. But since there is a diversity of fats, in this study we compared the effect of three HFD with different compositions (coconut oil, sunflower oil and extra virgin olive oil) on adipose tissue, metabolism and inflammation.

Methods and Results: Mice were fed for 16 weeks with HFD based on coconut oil, sunflower oil or extra virgin olive oil (EVOO). HFD based on coconut oil produced significantly lower body weight than EVOO- or sunflower-HFDs. Furthermore, coconut oil HFD led to metabolic disturbances as hypoleptinaemia, hypoadiponectinaemia, hypertriglyceridaemia, hepatomegaly and liver triglyceride accumulation. Likewise, this diet produced an increase of pro-inflammatory cytokines in serum (IL-6 and TNF- α). In white (WAT) and brown (BAT) adipose tissue, HFD based on coconut oil did not cause significant changes in the expression of studied proteins that were related to thermogenesis (UCP-1), mitochondrial biogenesis and browning (PGC-1 α and Nrf2). However, HFD based on EVOO induced up-regulation of UCP-1, PGC-1 α and Nrf2 expression in BAT, increased expression of UCP-1 and PGC-1 α in inguinal WAT and enhanced the expression of PGC-1 α in epididymal WAT.

Conclusions: HFD based on coconut oil could promote hypoleptinaemia and inflammation, while EVOO based HFD increased thermogenic activity.

Keywords: browning; EVOO; inflammation; thermogenesis; UCP-1



1. Introduction

Nowadays, non-communicable diseases (NCD) as cancer, diabetes and cardiovascular disease, represent over 70% of deaths worldwide [1]. Because of its endocrine and immunomodulatory activity, the adipose tissue is a central element in the development of several NCDs [2-4]. In fact, a major risk factor for NCDs is the abnormal or excessive accumulation of body fat characteristic of overweight or obesity [5], whose prevalence has increased markedly due to the global nutritional transition [6]. This is occurring in part due to increased availability, commercialisation, low prices and higher consumption of certain products that have contributed to the adoption of Western diets, which are characterised by high intakes of calories, fats, saturated fats and sugars [7,8]. Transition to diets high in fats is usually marked by a rapid expansion in the consumption of edible oils [9,10]. The most widely consumed vegetable oils worldwide are olive oil in the Mediterranean region, palm and coconut oil in Asia, soybean oil in America and sunflower oil, which is predominantly consumed in North Africa and Eastern Europe [8,11,12]. Historically, dietary fats and edible oils have given rise to controversial debate about the optimal type and amounts used in the diet, their role in regulating body weight and their importance in the aetiology of NCDs. However, the source and quality of dietary fat has been recognised as a more important factor in the prevention of some NCDs than the total amount of fat [13-15]. The main difference between edible oils intake around the world is its fatty acids composition, being MUFAs, PUFAs and saturated fatty acids (SFAs) in the case of olive, sunflower and coconut oil respectively [16,17]. Currently, new nutritional trends promote coconut oil intake as the healthiest vegetal fat source. Among other properties, they assume that it has a positive impact on cardiovascular health, helps to maintain an adequate weight or even improves the lipid profile in the blood. One of its properties that has become popular is that of its supposed thermogenic effect, which would help to lose weight [13,18]. Non-scientific articles have had a decisive influence in creating this state of opinion.

Despite, it has been widely analysed the impact of some oils on health, the mechanisms underlying the effects of vegetable edible oils on abnormal or excessive



body fat accumulation as well as their metabolic and immunological impact remain unclear. In order to clarify this situation, it is important to obtain scientific evidence on the real impact of coconut oil on health. At the same time, it is important to compare these results with those obtained by other edible fats of regular consumption such as extra virgin olive oil (EVOO) and sunflower oil.

2. Experimental Section

2.1. Materials

The following were purchased from ENVIGO® (Wisconsin, WI, USA): Teklad Global 14% protein (chow diet with 13% kcal from fat, Ref. 2014S), Teklad Global 19% protein extruded rodent diet (intermediate fat diet with 22% kcal from fat, Ref. 2019S) and Teklad Custom diet (TD.170709). EDTA-coated tubes and spray-coated silica tubes were obtained from BD (New Jersey, NJ, USA). Mouse Leptin ELISA kit (Ref. RAB1111), Mouse Adiponectin ELISA kit (Ref. RAB1115), phosphate buffered saline (PBS; Ref. P4417), CellLytic MT (Ref. C3228), protease inhibitor cocktail (Ref. P8340), antifoam Y-30 (Ref. A5758), SDS (Ref. L3771), TEMED (Ref. T9281) and APS (Ref. A3678) were from Sigma-Aldrich (Saint Louis, MO, USA). Mouse IL-6 kit (Ref. ADI-900-045), mouse TNF- α (Ref. ADI-900-047) and mouse TGF- β 1 (Ref. ADI-900-155) were obtained from ENZO Life Sciences (Barcelona, Spain). Triglyceride assay kit was purchased from Abcam (Ref. ab65336, ON, Canada). The following were purchased from Applichem Panreac (Barcelona, Spain): Bradford protein assay (Ref. A6932,0500), Tris base (Ref. A2264,1000) and NaCl (Ref. 241659.1608). M-Tubes were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). Nonfat dry milk powder was purchased from Central Lechera Asturiana (Asturias, Spain). PVDF membranes (Ref. 88518) were obtained from Invitrogen (Carlsbad, CA, USA). Microcentrifuge tubes were purchased from VWR (Barcelona, Spain). Acrylamide (Ref. #1610156) was obtained from Bio-Rad (Madrid, Spain). Anti-PGC-1 α (D-5; Ref. sc-518025), anti-UCP-1 (4E5; Ref. sc-293418), anti-GADPH (FL-335; Ref. sc-25778), mouse m-IgGk BP-HRP (Ref. sc-516102) were from Santa Cruz Biotechnology (Dallas, TX, USA). The following were obtained from



Merck Millipore (Burlington, MA, USA): anti-Nrf2 antibody (clone 103; Ref. MABE1799) and goat anti-Rat IgG antibody, HRP conjugate (Ref. AP136P).

2.2. Murine experimental model

Female CD1 mice were obtained from Charles River Laboratories (Wilmington, MA, USA). Four-week-old mice ($n = 44$) were housed in ventilated racks and cages (5–6 per cage) with environmental control (humidity: 55–65%; temperature: 20 ± 2 °C; 12-hour day-light cycle). The trial was carried out at the Animal Production and Experimentation Centre of the University of Jaén (code ES230500000020).

Animal care and experiments were conducted following the guidelines of the Spanish Society for Laboratory Animal Science. The experimental procedures applied to these animals were approved by the Ethical Committee of the University of Jaén (Record number: CEEA-100217-1) and the Ethical Committee of Animal Experiments of Regional Ministry of Agriculture, Fishing and Environment of Regional Government of Andalusia, Spain (Approval number: 16/03/2017/044).

2.3. Diets

Mice were fed with a maintenance chow diet (defined as chow diet), with 13% kcal from fat, an intermediate fat diet with 22% kcal from fat and a custom ready-to-use (fat-free) base for high fat diets (HFDs) with 60% kcal from fat. The custom fat-free base was created exclusively by ENVIGO® for this study (TD.170709). To prepare each of the HFDs, one of the following edible oils were added to the custom basis: sunflower oil, coconut oil or EVOO from the picual olive variety. The HFDs were made (as pellets) and administered daily under sterile conditions. The composition of the HFDs is provided in Tables 1 and 2.



Table 1. Composition of experimental high fat diets.

	coconut	sunflower	EVOO
Diet component	g/kg		
Custom diet mix ¹	666.6	666.6	666.6
Coconut oil	333.4	-	-
Sunflower oil	-	333.4	-
Extra virgin olive oil	-	-	333.4
	% kcal		
Protein	18	18	18
Carbohydrate	22	22	22
Fat	60	60	60
Fatty acid composition	g/100g total fatty acids		
Σ SFA ²	90.4	7.7	16.04
Σ MUFA	7.2	39.7	78.54
Σ PUFA	1.9	52.7	4.46
Fatty acids			
Caprylic acid (C8:0)	5.8	-	-
Capric acid (C10:0)	6.2	-	-
Lauric acid (C12:0)	46.2	-	-
Myristic acid (C14:0)	19.3	-	0.01
Palmitic acid (C16:0)	10	5.1	13.61
Palmitoleic acid (C16:1)	-	0.1	1.21
Stearic acid (C18:0)	2.9	2.6	2.42
Oleic acid (C18:1)	7.2	39.6	77.33
Linoleic acid (C18:2ω6)	1.9	52.6	3.64
α- linolenic acid (C18:3ω3)	-	0.1	0.82

¹Custom diet mix supplied and formulated by ENVIGO®

²SFA: saturated fatty acids

³MUFA: monounsaturated fatty acids

⁴PUFA: polyunsaturated fatty acids



Table 2. Minority compounds found in EVOO

Minority compounds	
	mg/kg
Squalene	9701
Total tocopherols	378.2
α-Tocopherol	369.1
β-Tocopherol	3.6
γ-Tocopherol	5.6
δ-Tocopherol	<1.0
Tyrosol	2.5
	mg/kg tyrosol
Total Biophenols	265.1
Hydroxytyrosol	0.6
Oleocanthol	34
	mg caffeic acid/kg
Total Polyphenols	372

2.4. Experimental design

Upon arrival, the mice were divided randomly into four groups (n = 11) and assigned to the different diets:

Group 1: Chow diet (chow)

Group 2: HFD with coconut oil (coconut-HFD)

Group 3: HFD with sunflower oil (sunflower-HFD)

Group 4: HFD with EVOO (EVOO-HFD)

Before dietary intervention started, mice were maintained in an acclimation phase for three weeks. In the first week, all mice were fed with chow diet, followed by two weeks with an intermediate diet for acclimation to HFD transition, except for the chow group, which continued with chow diet throughout all the assays/interventions. Once the intervention phase started, mice were fed either a chow diet, EVOO-, coconut-

or sunflower-HFDs with ad libitum access to water and food for 16 weeks (Figure 1). Food was monitored indirectly through daily monitoring of intakes (Figure 2).

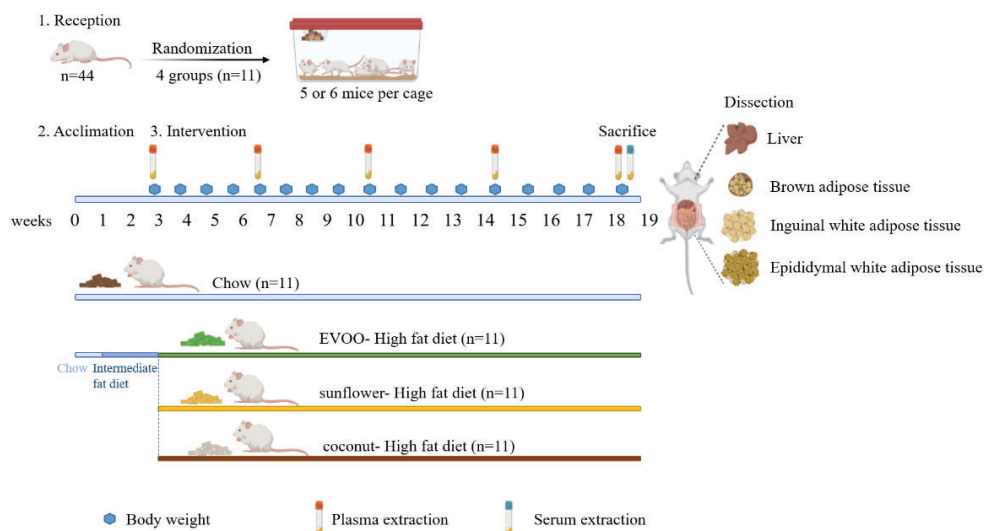


Figure 1. Experimental Design.

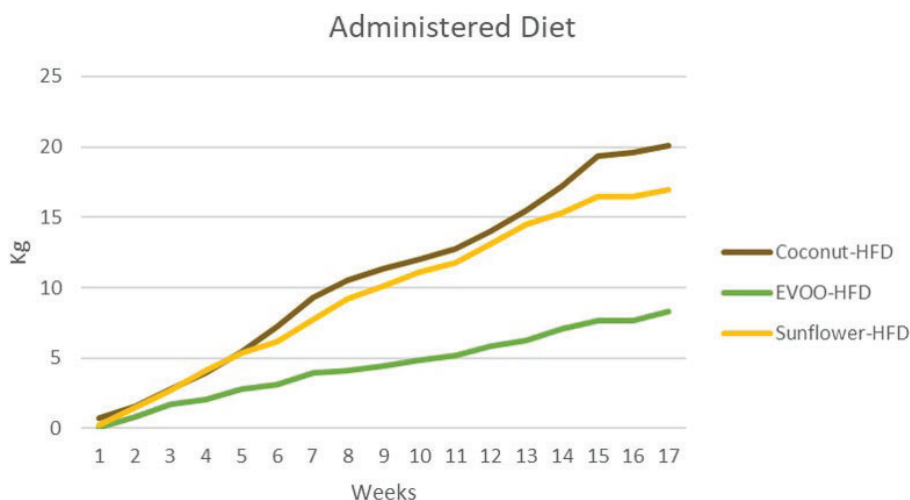


Figure 2. Administered diet per group and per week.



2.5. Measurement of body weight

Mice were weighed individually at the start of the intervention phase and weekly until the end of the study, using a 0.01g precision analytical electronic balance.

2.6. Sample collection

For plasma determination, a pool of blood from the submandibular veins of all mice in the same group was collected monthly in EDTA-coated tubes. Blood was quickly centrifuged at 1600g at 4 °C for 15 min and supernatants stored at –80 °C until further analysis.

For serum determination, blood was collected in spray-coated silica tubes as a pool of all mice in the same group from post-mortem individuals by aorta dissection at the pleural cavity level. Blood was quickly centrifuged at 1600g at 4 °C for 15 min and supernatants stored at –80 °C until further analysis.

At 16 weeks of intervention, mice were sacrificed using a euthanasia mixture of ketamine (160 mg/kg) and xylazine (10 mg/kg). Liver, epididymal WAT (gonadal region; eWAT), inguinal WAT (subcutaneous region; iWAT) and interscapular BAT were surgically removed individually from each mouse. All samples were weighed with a 0.001g precision analytical electronic balance, individually dissected, immediately frozen in liquid nitrogen, and stored at –80 °C until further analysis.

2.7. Metabolic and inflammatory markers

2.7.1. Plasma

Leptin and adiponectin were determined in each intervention group using mouse leptin ELISA kit and mouse adiponectin ELISA kit, respectively. Data were measured at 450 nm with a Tecan GENios Plus microplate reader (Tecan Group Ltd, Zürich, Switzerland). All assays were performed according to the manufacturers' protocols and each sample was assayed in triplicate.

2.7.2. Serum

IL-6, tumour necrosis factor-alpha (TNF- α) and transforming growth factor- β (TGF- β) were determined in each intervention group serum. IL-6 levels were estimated using the mouse IL-6 ELISA kit, TNF- α levels by the mouse TNF- α ELISA kit, and



TGF- β levels by using the mouse TGF- β ELISA kit. Triglyceride levels were quantified using the triglyceride assay kit. Data were measured at 450 nm with a microplate reader (Tecan GENios Plus) except for the triglyceride assay, where fluorescence was measured at $\lambda_{Ex}/\lambda_{Em}$ = 535/587 nm. Each sample was assayed in quadruplicate.

2.8. Hepatic triglycerides

A hepatic pool was made from each group after defrosting tissues (100 mg of each mouse liver). Livers were washed with cold PBS, re-suspended in 1% Tween20 and dissociated in a gentleMACS™ dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). Samples were immersed in a water bath at 80–100 °C for 5 min and previous steps repeated until all tissues were homogenated. Samples were collected and centrifuged for 2 min at 13,200g using a microcentrifuge (Eppendorf, Hamburg, Germany). Supernatants were quantified using triglyceride assay kit. Fluorescence was measured at $\lambda_{Ex}/\lambda_{Em}$ = 535/587 nm with Tecan GENios Plus. All samples were assayed in quadruplicate.

2.9. Western blotting

Prior to processing samples, the eWAT, iWAT and BAT of each mouse were removed from storage at –80 °C and kept immediately on ice. A lysis buffer (CellLytic MT, protease inhibitor cocktail and antifoam Y-30) was used with all adipose tissues to homogenise in a gentleMACS™ dissociator. Then, all tissues were centrifuged at 13,200g for 10 min at 4 °C. Finally, supernatants were collected and the proteins stored at –80 °C until further analysis.

Protein (25 ng) was run in 12% acrylamide gels. Proteins were transferred to PVDF membranes and incubated for 1 h with blocking buffer (1xTBS-T with 5% nonfat dry milk powder (w/v)). After blocking, membranes were washed three times with TBS-T, and incubated with primary antibodies O/N at 4 °C: anti-PGC-1 α (D-5; Ref. sc-518025), anti-UCP-1 (4E5; Ref. sc-293418), anti-GADPH (FL-335; Ref. sc-25778) or anti-Nrf2 (clone 103; Ref. MABE1799) diluted in 1xTBS-T containing 1% nonfat dry milk powder (w/v). Mem-branes were washed three times with TBS-T and incubated with appropriate secondary HRP-conjugated antibodies against mouse (m-IgG κ BP-HRP, sc-516102) or rabbit (Gt X Rat IgG HRP, AP136P) at room temperature



for 1 h. Finally, membranes were washed in 1xTBS-T three times for 5 min. Immunoblots were analysed with ChemiDoc XRS+ Imagin System (Bio-Rad, Madrid, Spain), and protein levels were quantified by Image Lab Software (Bio-Rad Imaging Systems, Madrid, Spain).

2.10. Statistical analysis

All results, except for those that reported the contrary, are reported as means of at least three independent experiments ($\pm$ SEM). Some results are expressed as a percentage relative to the chow group, which was set as 100%. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Fisher's LSD test. Differences between groups were considered to be significant at *p*-values of less than or equal to 0.05, 0.01 or 0.001. Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). ANOVA analysis of eWAT, iWAT or BAT weights vs body weight was carried out with Stata version 12.0 (Stata Corporation, College Station, TX, USA).

3. Results

3.1. Coconut-HFD did not promote weight increase

Mice fed sunflower-HFD or EVOO-HFD showed significant weight gain compared to those fed chow diet (Figure 3). In the case of mice fed sunflower-HFD, the differences become significant from the second week of the nutritional intervention and were maintained until the end of the experiment (Table 3). In mice fed EVOO-HFD, the differences were significant from the fourth week onwards and remained so until the end of the trial. However, mice fed coconut-HFD maintained an even lower weight than those fed chow diet, although were not statistically significant differences.

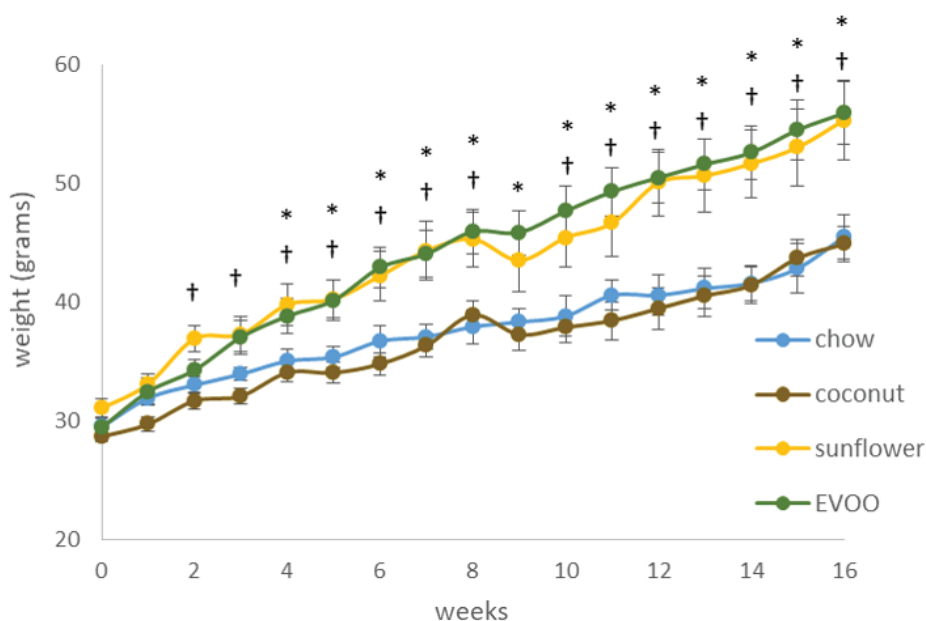


Figure 3. Bodyweight of chow and HFD groups (n=44). Data are represented as mean $\pm$ SEM of weekly mice weight (n=11). For statistical analysis, two-way analysis of variance (ANOVA) followed by Fisher's LSD test were performed; * $p < 0.05$ for EVOO-HFD, and † $p < 0.05$ for sunflower-HFD vs. chow.



Table 3. Body weight (grams).

Weeks	chow	coconut-HFD	sunflower-HFD	EVOO-HFD
0	29.49 ± 0.37	28.65 ± 0.48	31.05 ± 0.77	29.43 ± 0.76
1	31.9 ± 0.49	29.71 ± 0.59	33.09 ± 0.81	32.45 ± 1.19
2	33.01 ± 0.76	31.67 ± 0.69	36.9 ± 1.05**	34.26 ± 0.87
3	33.92 ± 0.53	32.07 ± 0.65	37.26 ± 1.48*	37.03 ± 1.41
4	35.06 ± 1.00	34.05 ± 0.77	39.77 ± 1.76*	38.81 ± 1.48*
5	35.36 ± 0.83	34.04 ± 0.83	40.24 ± 1.61*	40.14 ± 1.69*
6	36.71 ± 1.33	34.78 ± 0.94	42.21 ± 2.01*	42.89 ± 1.70**
7	37.06 ± 1.08	36.32 ± 0.98	44.31 ± 2.52**	44.05 ± 2.02**
8	37.91 ± 1.40	38.9 ± 1.20	45.3 ± 2.32**	45.93 ± 1.90**
9	38.32 ± 1.08	37.27 ± 1.31	43.51 ± 2.62	45.85 ± 1.86**
10	38.8 ± 1.68	37.87 ± 1.28	45.42 ± 2.44*	47.65 ± 2.16**
11	40.55 ± 1.25	38.43 ± 1.58	46.65 ± 2.78*	49.28 ± 2.07**
12	40.56 ± 1.72	39.45 ± 1.72	50.05 ± 2.76**	50.47 ± 2.13**
13	41.14 ± 1.73	40.52 ± 1.69	50.63 ± 3.05**	51.59 ± 2.17**
14	41.57 ± 1.45	41.41 ± 1.53	51.64 ± 2.88**	52.57 ± 2.23**
15	42.83 ± 2.06	43.7 ± 1.55	53.02 ± 3.26**	54.49 ± 2.48**
16	45.48 ± 1.85	44.88 ± 1.48	53.31 ± 3.34**	55.9 ± 2.65**

Data are represented as mean ± SEM of n=11 mice for each group. For statistical analysis, one-way analysis of variance (ANOVA) followed by Fisher's LSD test were performed; * $p<0.05$ and ** $p<0.01$ for groups vs. chow.

3.2. Coconut-HFD reduced leptin and adiponectin plasma levels

In chow-fed mice, there was an increase in leptin and adiponectin levels as body weight increased (Figure 4A). However, at the end of the intervention, mice fed sunflower-HFD or EVOO-HFD showed hypoadipopectinaemia and hyperleptinaemia (Figure 4C and D) while mice fed coconut-HFD developed hypoleptinaemia and hypoadiponectinaemia (Figure 4B).

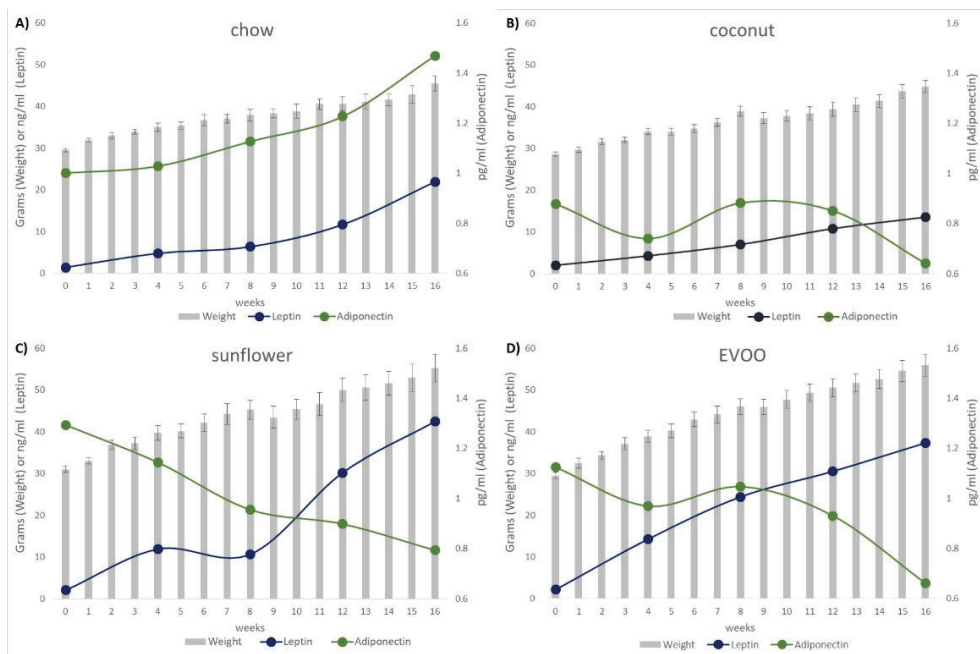


Figure 4. Evolution of weight, leptin and adiponectin. Data are represented as mean $\pm$ SEM of body weight (grams) and triplicate replies of pool serum (n=11) for each group obtained on ELISA assay. Leptin and adiponectin data of HFD groups are expressed relative to chow, which was established as 1. A) Chow, B) coconut-HFD, C) sunflower-HFD, and D) EVOO-HFD.



Table 4. Weight (grams), leptin (ng/ml) and adiponectin (pg/ml) plasma levels throughout intervention.

Weeks		chow	coconut-HFD	sunflower-HFD	EVOO-HFD
0	Weight	29.49 ± 0.37	28.65 ± 0.48	31.05 ± 0.77	29.43 ± 0.76
	Leptin	1.43 ± 0.02	2.07 ± 0.07***	2.09 ± 0.05***	2.09 ± 0.08***
	Adiponectin	1 ± 0.06	0.88 ± 0.05*	1.29 ± 0.04***	1.12 ± 0.02*
4	Weight	35.06 ± 1.00	34.05 ± 0.77	39.77 ± 1.76*	38.81 ± 1.48*
	Leptin	4.79 ± 0.13	4.33 ± 0.03***	11.95 ± 0.72***	14.19 ± 0.46***
	Adiponectin	1.03 ± 0.03	0.74 ± 0.02***	1.14 ± 0.02*	0.97 ± 0.03
8	Weight	37.91 ± 1.40	38.9 ± 1.20	45.3 ± 2.32**	45.93 ± 1.90**
	Leptin	6.39 ± 0.4	7.04 ± 0.09	10.71 ± 0.78***	24.32 ± 0.58***
	Adiponectin	1.13 ± 0.06	0.88 ± 0.03	0.96 ± 0.004	1.04 ± 0.007
12	Weight	40.56 ± 1.72	39.45 ± 1.72	50.05 ± 2.76**	50.47 ± 2.13**
	Leptin	11.68 ± 0.06	10.84 ± 0.16	30.24 ± 2.48***	30.44 ± 1.51***
	Adiponectin	1.27 ± 0.04	0.85 ± 0.01***	0.9 ± 0.02***	0.92 ± 0.004***
16	Weight	45.48 ± 1.85	44.88 ± 1.48	53.31 ± 3.34**	55.9 ± 2.65**
	Leptin	21.92 ± 1.12	13.58 ± 0.64***	42.52 ± 1.07***	37.25 ± 1.44***
	Adiponectin	1.46 ± 0.11	0.64 ± 0.02***	0.79 ± 0.008***	0.66 ± 0.005***

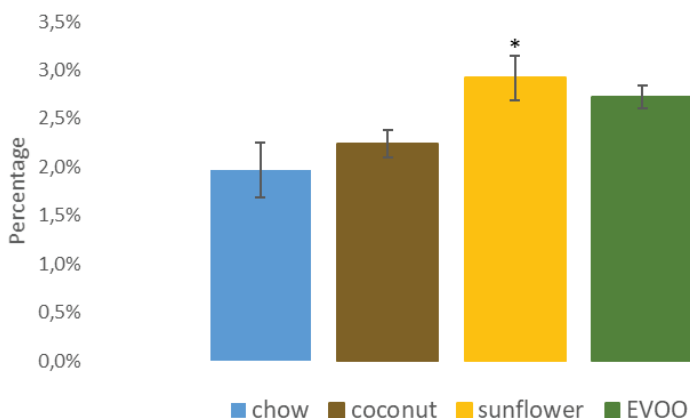
Data are represented as mean ± SEM of n=11 mice for each group. For statistical analysis, one-way analysis of variance (ANOVA) followed by Fisher's LSD test were performed; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ for groups vs. chow.

3.3. Consumption of EVOO, sunflower and coconut-HFD led to different alterations in adiposity

In order to study the effect of each of the diets administered on adipose tissue, three different types of indices were calculated as follows: (fat weight/body weight) × 100, where 'fat' was replaced by iWAT, eWAT or BAT (Figure 5). Mice fed sunflower oil-HFD showed a significant increase in the relative weight of iWAT ($p = 0.018$). Furthermore, sunflower-HFD and EVOO-HFD produced a relative increase in eWAT ($p < 0.01$). None of the diets administered altered BAT weight. On the other hand, coconut-HFD had no impact on any of the adipose tissues studied.

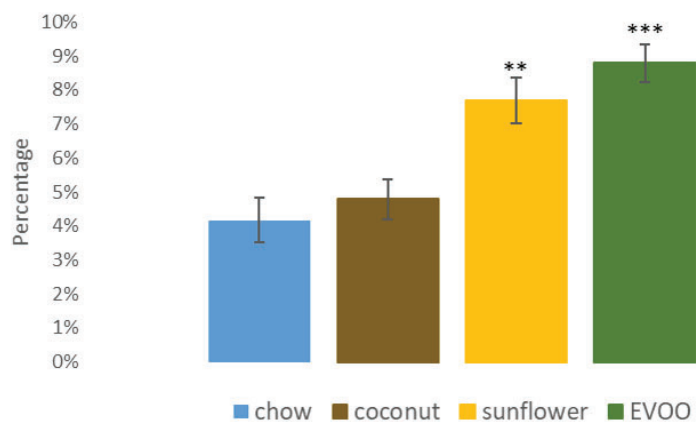


A) iWAT weight/ body weight index (%)

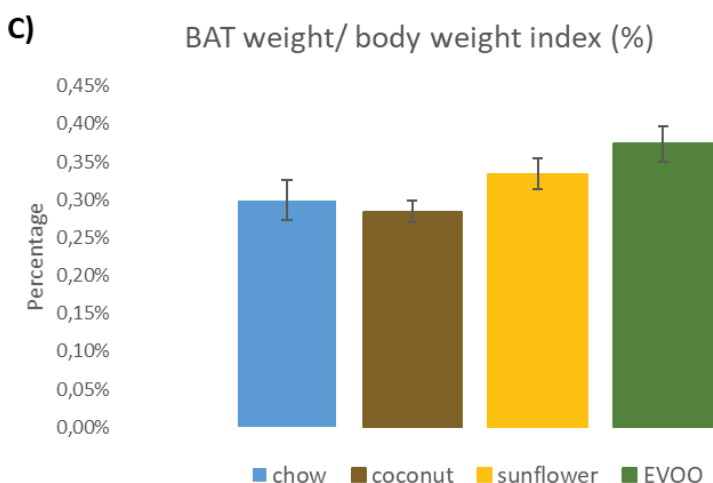


iWAT weight /body weight index (%)			
Group	Mean	SD	P value
Chow	1.98	0.94	
Coconut	2.24	0.45	1.000
Sunflower	2.92	0.75	0.018
EVOO	2.73	0.40	0.093

B) eWAT weight/ body weight index (%)



eWAT weight/body weight index (%)			
Group	Mean	SD	P value
Chow	4.17	2.14	
Coconut	4.79	1.99	1.000
Sunflower	7.69	2.28	0.002
EVOO	8.79	1.83	0.000



BAT weight/ body weight index (%)			
Group	Mean	SD	P value
Chow	0.30	0.08	
Coconut	0.28	0.05	1.000
Sunflower	0.33	0.06	1.000
EVOO	0.37	0.08	0.107

Figure 5. Ratio of adipose tissue/ body weight. A) iWAT, B) eWAT and C) BAT. Data are represented as mean $\pm$ SD of $n=11$ mice for each group and are expressed in percentages. ANOVA followed by Fisher's LSD test comparing all groups vs chow. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

3.4. Coconut-HFD appeared to promote low-grade systemic inflammation while sunflower-HFD and EVOO-HFD may modulate inflammatory process

To assess inflammatory status, serum levels of two pro-inflammatory cytokines (IL-6 and TNF- α) and one anti-inflammatory cytokine (TGF- β) were determined at the end of the dietary intervention (Figure 6). Coconut-HFD produced a statistically significant increase in serum levels of IL-6 and TNF- α and a minor increase in TGF- β . EVOO-HFD produced a moderate increase in IL-6, no change in serum TNF- α levels and a very significant increase in TGF- β levels. Sunflower-HFD induced a moderate decrease in IL-6 levels, no change in plasma TNF- α levels and produced a moderate increase in TGF- β .

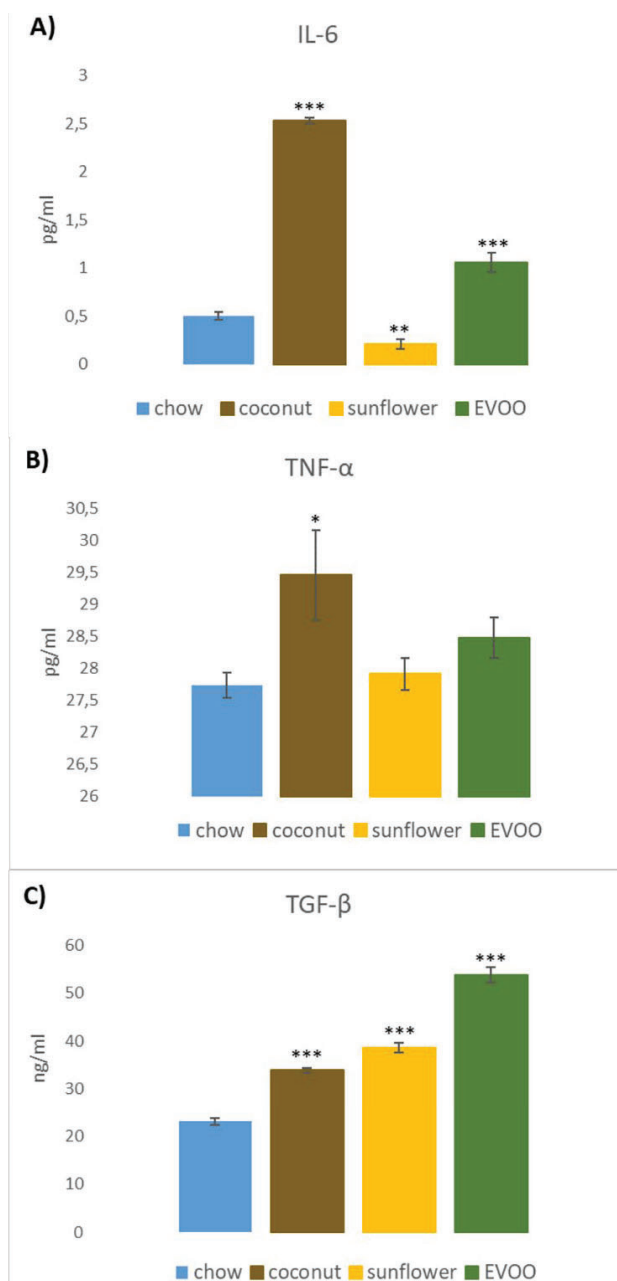


Figure 6. Serum inflammatory cytokine levels. Data are represented as mean $\pm$ SEM of quadruplicate replies of pool serum (n=11) for each group obtained on ELISA assay for A) IL-6, B) TNF- α , and C) TGF- β . For statistical analysis, one-way analysis of variance (ANOVA) followed by Fisher's LSD test were performed; * p <0.05, ** p <0.01 and *** p <0.001 for groups vs. chow.

3.5. Coconut-HFD promoted hypertriglyceridaemia

Determination of serum triglyceride levels showed that coconut-HFD significantly increased triglyceride values ($p < 0.001$). On the other hand, EVOO-HFD increased it very slightly, while sunflower-HFD had no effect (Figure 7).

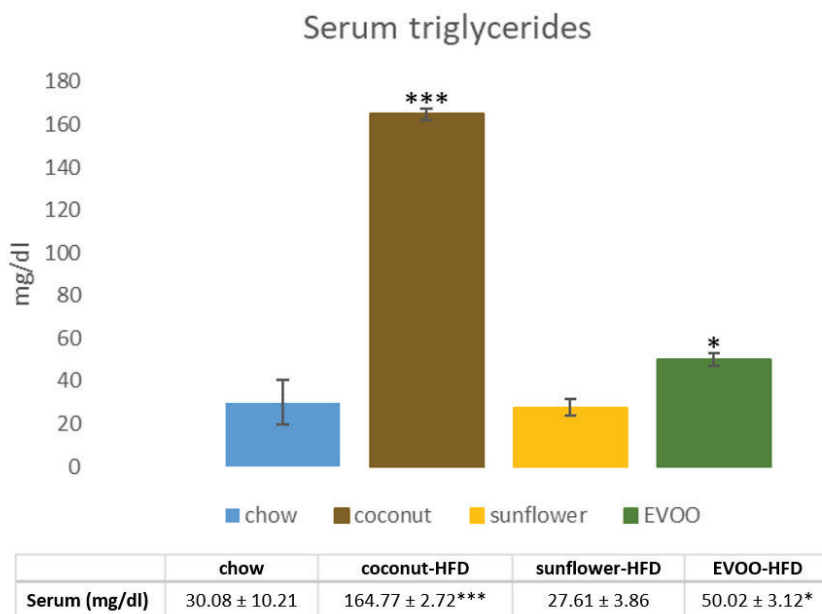


Figure 7. Serum Triglycerides. Data are represented as mean ± SD of quadruplicate replies of pool serum (n=11) for each group obtained on ELISA assay. For statistical analysis, one-way analysis of variance (ANOVA) followed by Fisher's LSD test were performed; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ for groups vs. chow.

3.6. Coconut-HFD promoted hepatomegaly and liver triglyceride accumulation

As Figure 8 and Figure 9 show, coconut-HFD produced a very significant increase in liver weight relative to body weight ($p < 0.001$) as well as an increase in the quantity of triglycerides stored in the liver ($p < 0.05$). Therefore, this diet could lead to hepatomegaly and hepatic steatosis. Sunflower-HFD did not change the relative liver weight compared to the chow diet but did induce an increase in liver triglycerides ($p < 0.05$). Finally, EVOO-HFD produced a moderate increase in relative liver weight ($p < 0.05$) although there was no increase in hepatic triglycerides.

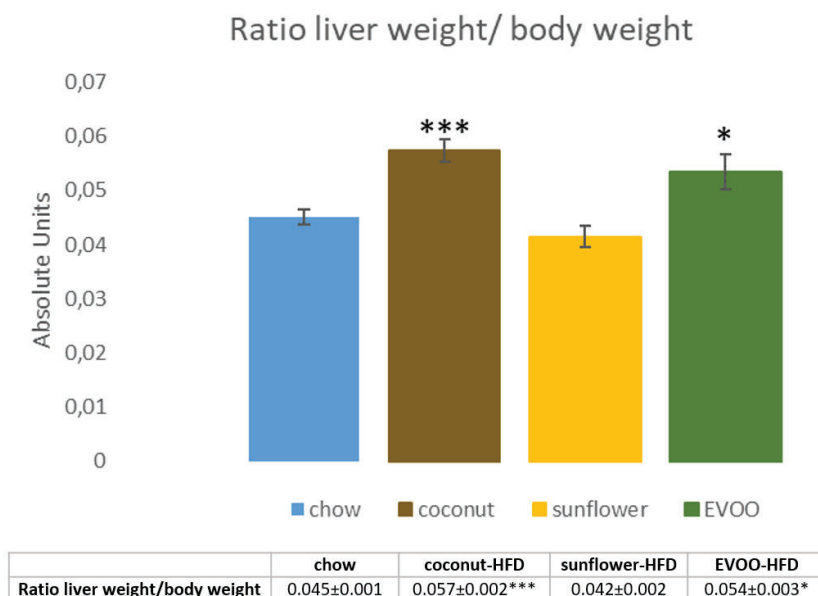


Figure 8. Ratio liver weight/ body weight. Data are represented as mean $\pm$ SEM of $n=11$ mice for each group. For statistical analysis, one-way analysis of variance (ANOVA) followed by Fisher's LSD test were performed; * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ for groups vs. chow.

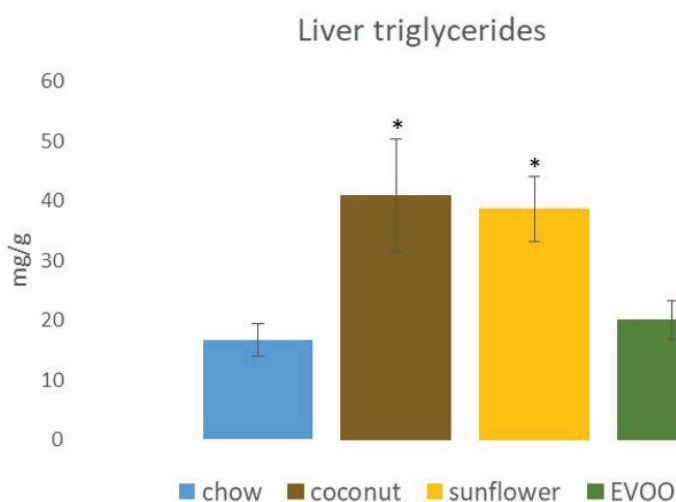


Figure 9. Liver Triglycerides. Data are represented as mean $\pm$ SEM of quadruplicate replies of pool livers ($n=11$) for each group obtained on ELISA assay. For statistical analysis, one-way analysis of variance (ANOVA) followed by Fisher's LSD test were performed. * $p<0.05$ for groups vs. chow.



3.7. EVOO-HFD increased UCP-1 expression in BAT and iWAT

Uncoupling protein 1 (UCP-1) is the protein responsible of thermogenesis in adipocytes. Determination of UCP-1 expression by western blotting showed that EVOO-HFD produced a significant increase in its expression in both iWAT and BAT but not in eWAT (Figures 10.1a, 10.2a, 10.3a). Sunflower-HFD produced an increase in UCP-1 expression only in BAT, although to a lesser extent than EVOO-HFD. In contrast, coconut-HFD did not modify UCP-1 expression in any of the adipose tissues studied.

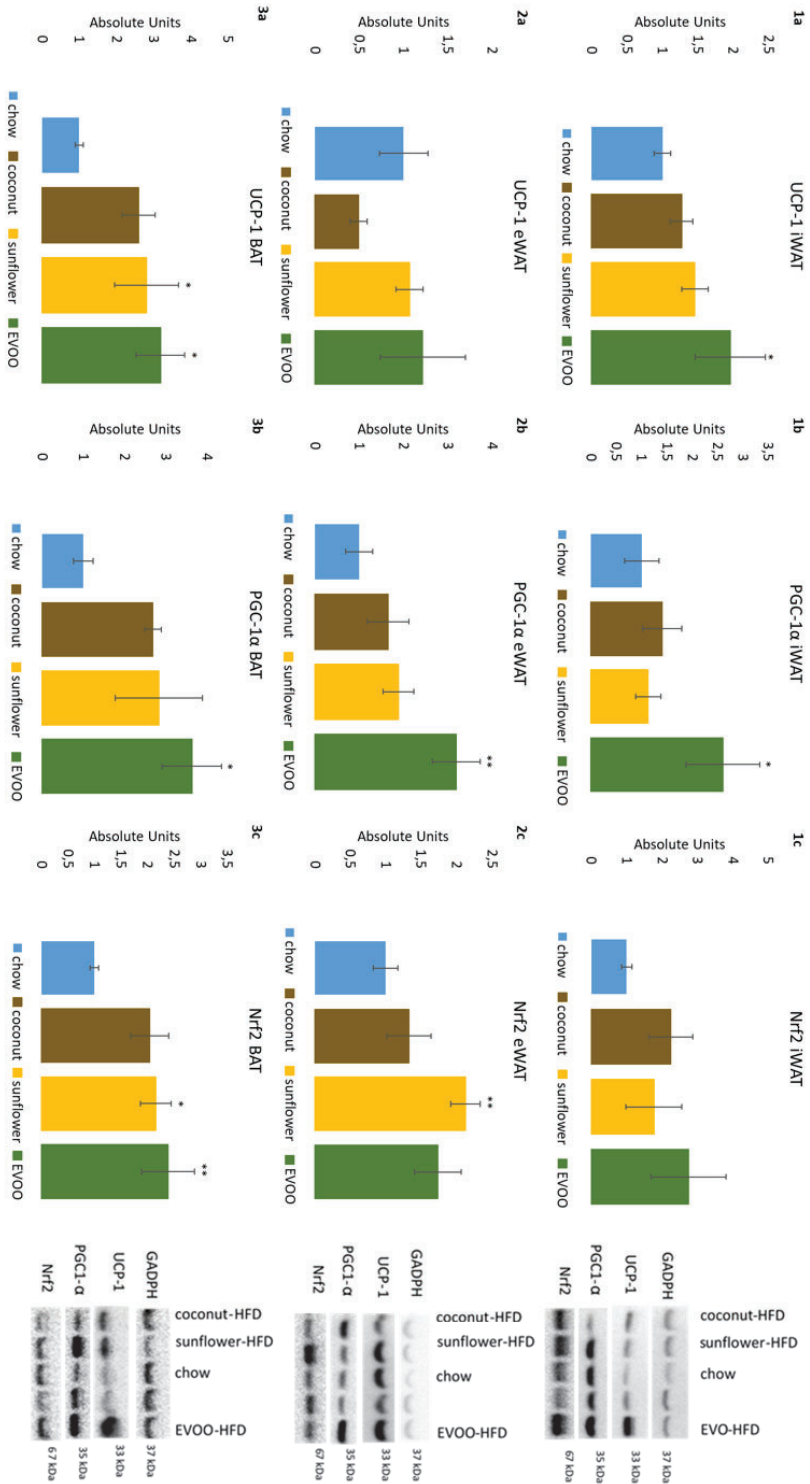
3.8. EVOO-HFD appeared to promote mitochondrial biogenesis in WAT and BAT

Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 α) is a protein involved in mitochondrial biogenesis and thermogenesis in WAT and BAT. As shown in Figures 10.1b, 10.2b and 10.3b. EVOO-HFD was the only diet administered that significantly increased PGC-1 α expression in all adipose tissues. Neither coconut-HFD nor sunflower-HFD modified PGC-1 α expression in any of the adipose tissues studied.

3.9. Sunflower-HFD and EVOO-HFDs enhanced Nrf2 expression in adipose tissue

Nuclear factor E2-related factor 2 (Nrf2) is involved indirectly adipocyte differentiation and has antioxidant activity in response to oxidative stress. As shown in Figures 10.1c, 10.2c and 10.3c. none of the diets administered modified Nrf2 expression in iWAT. However, sunflower-HFD was the only diet that increased Nrf2 expression in eWAT. On the other hand, both EVOO-HFD and sunflower-HFD significantly increased Nrf2 expression in BAT ($p < 0.01$ and $p < 0.05$ respectively).

Figure 10. Quantitative analysis (left) and representative western blot (right) of a UCP-1, b PGC1 α and c Nrf2 protein expression in 1 Inguinal White Adipose Tissue (iWAT). 2 Epididymal White Adipose Tissue (eWAT). 3 Brown Adipose Tissue (BAT). GADPH, loading control. Data are represented as mean of $n=11$ per group with triplicate independent experiment. For statistical analysis, we performed on-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference. * $p < 0.05$ and ** $p < 0.01$ for groups vs chow.





4. Discussion

Given the controversy in the literature regarding the impact of vegetable oils on health, and the growing epidemic among the population caused by excess and abnormal accumulation of body fat related to a high fat intake. The aim of this study was to analyse the effect of different high-fat diets based on coconut oil, EVOO or sunflower oil on adipose tissue, alterations in metabolism and modulation of inflammation in mice.

Adipose tissue is responsible for energy storage but also plays an important role as an endocrine organ in the regulation of biological processes as immunity and metabolic homeostasis [19]. It secretes adipokines that have a significant role in the pathogenesis of fat accumulation disturbances and its metabolic complications [20,21].

Regarding fat accumulation and metabolism, our results showed that mice fed coconut-HFD had significantly lower body weight than those fed EVOO- or sunflower-HFDs. Coconut oil has been recommended for its weight-loss properties although these effects remain controversial [18,22]. It has been suggested that this effect could be related to its composition in medium-chain fatty acids (MCFAs), which are quickly used as an energy substrate and are less susceptible to accumulation in adipose tissues [23,24]. Traditionally, the main coconut oil fatty acid, lauric acid, has been considered as a MCFA. However recent evidence has shown that it should be classified as a long-chain fatty acid due to its metabolic destiny after gut absorption [25,26]. In this sense, the effects of coconut-HFD on body weight regulation may be due to its satiating activity through leptin, which is directly related to appetite regulation because of its activity on hypothalamic centres [27]. However, our results showed that coconut-HFD was the only diet that produced hypoleptinaemia.

In relation to adiponectin, whose plasma levels are inversely correlated with fat mass [28], all mice fed HFDs developed hypoadiponectinaemia. This disturbance is related to insulin resistance and metabolic complications [29]. Regardless of their effects on body weight, all HFD resulted in altered adipokine levels.

The intake of a high-calorie diet should promote an excessive accumulation of fat. However, depending on the source of fat, the HFD resulted in different types of fat



accumulation. Mice fed coconut-HFD showed no differences compared to mice fed chow diet. Sunflower-HFD increased subcutaneous and visceral fat while EVOO-HFD only increased significantly subcutaneous fat.

With respect to immune modulation, different pro-inflammatory (IL-6 and TNF- α) and anti-inflammatory (TGF- β) cytokines serum levels were analysed. Our data showed that mice fed coconut-HFD had high levels of pro-inflammatory cytokines. While TGF- β serum levels were highest in mice fed EVOO-HFD, followed by sunflower-HFD and coconut-HFD. This cytokine has an essential role in the suppression of inflammation [30]. These results are in concordance with previous data published by our group where gut microbiota analysis showed that sunflower-HFD and coconut-HFD promoted a pro-inflammatory intestinal microenvironment in these mice while EVOO-HFD produced an anti-inflammatory microenvironment [31]. Collectively, these data suggest that coconut-HFD could induce a low-grade systemic inflammation. These alterations may be associated with TLR4 modification induced by a diet supplemented with coconut oil in health mice that observed Veras et al. [24]. TLR4 plays a key role in the activation of inflammatory pathways [24].

Furthermore, concerning the effects on lipid metabolism, mice fed coconut-HFD developed serum hypertriglyceridaemia. Likewise, liver triglycerides were significantly elevated in coconut-HFD and sunflower-HFD mice. Furthermore, the mice with highest ratio of liver weight to body weight were those fed coconut-HFD. Suggesting that this diet, despite not increasing body weight, could produce hepatomegaly and metabolic alterations.

Adipocytes can be classified as white, brown and beige. White adipocytes are distributed in subcutaneous and visceral adipose tissue, and their function is to store excess energy in form of triglycerides. Brown adipocytes are located in limited areas of the body, and their primary physiological function is energy dissipation. Finally, beige adipocytes are inducible brown-like thermogenic adipocytes found sporadically in WAT deposits [32,33]. Brown and beige adipocytes trigger non-shivering thermogenesis in response to cold through increased activity of UCP-1 [34].

In our study, we observed that the EVOO-HFD significantly increased UCP-1 expression in iWAT and BAT, similar to results obtained by Oi-Kano et al. [35].



Furthermore, they found that EVOO increased triglyceride catabolism and thermogenesis in BAT by increasing UCP-1 level, suggesting that oleuropein (a minor compound in EVOO) was responsible for this action. Similarly, Rodriguez et al. [36] found that olive oil induces an increase in UCP-1 mRNA expression in BAT of rats. Reinforcing our results, a recently published clinical trial has observed that a dietary intervention with EVOO was able to increase the thermogenic activity of BAT as well as increase leptin levels [37].

On the other hand, the results obtained in the present study showed that a coconut-HFD did not produce any significant change in UCP-1 expression. Therefore, the mechanism to ameliorate weight gain after a coconut-HFD is not associated with an increase in thermogenic activity.

PGC-1 α is a cold-inducible transcription coactivator of adaptive thermogenesis in adipose tissue. Furthermore, its expression is necessary to promote differentiation to the brown-adipocyte lineage and is the major regulator of mitochondrial biogenesis and oxidative metabolism in BAT [38,39].

Our results showed that only the EVOO-HFD was able to increase significantly PGC-1 α expression in all adipose tissues studied. Interestingly, Zhang et al. [40] reported that berberine, a plant-derived compound, induced development of murine beige adipocytes in iWAT through PGC-1 α signaling. Similarly, have been described other dietary factors that promote the development of brown and beige adipocytes and induce thermogenesis [41]. In line with our results, some authors have suggested that phenolic compounds, which are in high amounts in EVOO, may be responsible for this browning effect via PGC-1 α activation [42,43].

Nrf2 is a transcription factor that plays a critical role in mitochondrial biogenesis and controls the capacity of adipose tissue expansion [44]. Nrf2 also protects cells against oxidative stress and has a potent anti-inflammatory effect [45]. Expression of Nrf2 protein in BAT was upregulated in EVOO-HFD mice. Taking into account UCP-1 and PGC-1 α data, EVOO-HFD could have a positive function in the promotion of thermogenesis by increasing the mitochondrial synthesis in adipocytes.



Literature shows that a HFD leads to a metabolic and inflammatory disorders as well as alterations in adipose tissue function that could be mediated by TLR4 modulation [24]. On the other hand, coconut oil is rich in myristic and palmitic acids that could induce lipoapoptosis [46], it could explain obtained results of coconut-HFD. However, more studies are needed to understand the different vegetable edible oils' mechanism of action.

In summary, as shown in Table 5 coconut-HFD caused numerous metabolic disorders including hypoleptinaemia and hypoadiponectinaemia as well as a pro-inflammatory status. In addition, a HFD based on coconut oil ameliorated body weight gain relative to diets that included EVOO or sunflower oil. Our results exclude that this effect was due to the thermogenic effect of coconut oil. On the other hand, results showed that EVOO-HFD increases thermogenic activity and could promote browning of WAT.



Table 5. Summary of the effects of HFD

Parameters		Groups		
		Coconut-HFD	EVOO-HFD	Sunflower-HFD
Body weight		-	↑	↑
Leptin		↓	↑	↑
Adiponectin		↓	↓	↓
IL-6		↑↑↑	↑↑	↓↓
TNF- α		↑	-	-
TFG- β		↑	↑↑↑	↑↑
Triglyceridaemia		↑↑↑	↑	-
Liver weight		↑↑↑	↑	-
Liver triglycerides		↑	-	↑
iWAT	weight	-	-	↑
	UCP1	-	↑	-
	PGC1 α	-	↑	-
	Nrf2	-	-	-
eWAT	weight	-	↑↑↑	↑↑
	UCP1	-	-	-
	PGC1 α	-	↑↑	-
	Nrf2	-	↑↑	↑↑
BAT	weight	-	-	-
	UCP1	-	↑	↑
	PGC1 α	-	↑	-
	Nrf2	-	↑↑	↑

↑ or ↓ indicate difference compared to control. Dash, no significant changes.

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Data Availability Statement: All data are available in the manuscript or upon request to the authors.

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5.4. Artículo científico III

PPAR γ as a Link Between Acquired and Congenital Lipodystrophy and its Modulation by Dietary Fatty Acids

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PPAR γ as a Link Between Acquired and Congenital Lipodystrophy and its Modulation by Dietary Fatty Acids

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Abstract

Lipodystrophy syndromes are rare diseases that could be of genetic or acquired origin. The main complication of lipodystrophy is the dysfunction of adipose tissue, which leads to an ectopic accumulation of triglycerides in tissues such as the liver, pancreas and skeletal muscle. This abnormal fat distribution is associated with hypertriglyceridemia, insulin resistance, liver steatosis, cardiomyopathies and chronic inflammation. Although the origin of acquired lipodystrophies remains unclear, these patients show alterations in genes related to genetic lipodystrophy, suggesting that this disease could be improved or aggravated epigenetically, for example by diet. Nowadays the main responsible for adipose tissue dysfunction is the imbalance in metabolism, caused in other pathologies associated with adipose tissue dysfunction, such as obesity, by high-fat diets. However, not all dietary fats have the same health implications. Therefore, the aim of this review is to summarize the main genes involved in the pathophysiology of lipodystrophy, identify connections between them, and make a systematic review, from January 2017 to January 2022, of the dietary fats that can epigenetically modulate the development of lipodystrophy.

Keywords: lipids, lipotoxicity, inflammation, metabolism, prognosis, STRING



1. Introduction

Lipodystrophy syndromes are rare disorders characterized by adipose tissue (AT) dysfunction (Hussain & Garg, 2016). The atrophy of adipocytes leads to immunometabolic complications such as chronic inflammation, ectopic fat accumulation, insulin resistance, hypoleptinemia, hypoadiponectinemia and hypertriglyceridemia (Brown et al., 2016). These comorbidities should be managed to avoid the development of non-alcoholic fatty liver disease (NAFLD), diabetes mellitus type 2 (DMT2) and cardiomyopathies associated with lipodystrophy syndromes (Akinci et al., 2019). Lipodystrophy syndromes may be congenital or acquired origin. Some genes have been selected as the main responsible of different types of lipodystrophy (Brown et al., 2016). However, in the case of acquired lipodystrophy, the origin remains unclear although the expression of some genes as peroxisome proliferator-activated receptor gamma (PPAR γ) is implicated in lipodystrophy associated with antiretroviral therapy (ART) (Caron et al., 2009).

Some exogenous factors such as pharmacological or nutritional factors can modulate the AT epigenetically. Obesogenic diets, characterized by high-fat composition, can downregulate some gene expression in adipocytes that are related to adipogenesis and lipid metabolism alteration (Pinel et al., 2021). However, dietary fats have different metabolic targets based on their composition in fatty acids, and not all have the same effects on health (Amin et al., 2019). Therefore, the aim of this review was to summarize the main genes involved in the pathophysiology of lipodystrophy, to identify the connection between them, and to analyze in a systematic review the dietary fats that can epigenetically modulate the development of lipodystrophy.

2. Methods

The methodology has been divided into:

- 1) Initial research on the most common types of lipodystrophy, physiopathology and main genes involved in the development of congenital lipodystrophy and genes implicated in acquired lipodystrophy.



- 2) Analysis of the proteins involved in lipodystrophy by Search Tool for the Retrieval of Interacting Proteins 11 (STRING 11) (a database under a Creative Commons by 4.0' license) (Szkłarczyk et al., 2020) with a minimum required interaction score (high confidence (0.700)). This analysis was performed to determine the interaction between the different proteins implicated in lipodystrophy, to identify which ones played a more relevant role and the main biological processes in which they were involved. In addition, it allowed us to know the relationship between the implicated proteins in congenital and acquired lipodystrophy.
- 3) Systematic review of the scientific evidence on the modulation of the expression and activity of the selected genes (PPAR γ and PLIN1) by dietary lipids. PubMed database was searched from January 2017 to January 2022. Due to the lack of studies analyzing the impact of diet on lipodystrophy, the following search strategy was used: ((PPARG) OR (peroxisome proliferator-activated receptor-gamma) OR (PLIN1) OR (PERILIPIN1)) AND ((oil) OR (fatty acid) OR (high fat diet) OR (dietary lipid) OR (capric acid) OR (lauric acid) OR (myristic acid) OR (palmitic acid) OR (stearic acid) OR (arachidic acid) OR (behenic acid) OR (caprylic acid) OR (oleic acid) OR (linoleic acid) OR (eicosapentaenoic acid) OR (linolenic acid) OR (arachidonic acid) OR (docosatetraenoic acid) OR (palmitoleic acid)) NOT (review[Publication Type]).

3. Results and Discussion

3.1. Lipodystrophy syndromes classification

Lipodystrophy syndromes are disorders characterized by a redistribution of AT. It affects either localized areas (partial) or the whole body (generalized) (Sharawat et al., 2019). Lipodystrophy may appear as an undesirable effect of certain drugs (i.e. insulin, antiretroviral therapies, etc.), due to autoimmune mechanisms, or has a genetic origin (autosomal dominant or recessive subtypes) (Hussain & Garg, 2016). Acquired lipodystrophy occurs with metabolic syndromes, human immunodeficiency virus (HIV), connective tissue disorders and some inflammatory conditions.

The different types of lipodystrophy syndromes can be classified as follows (Figure 1):

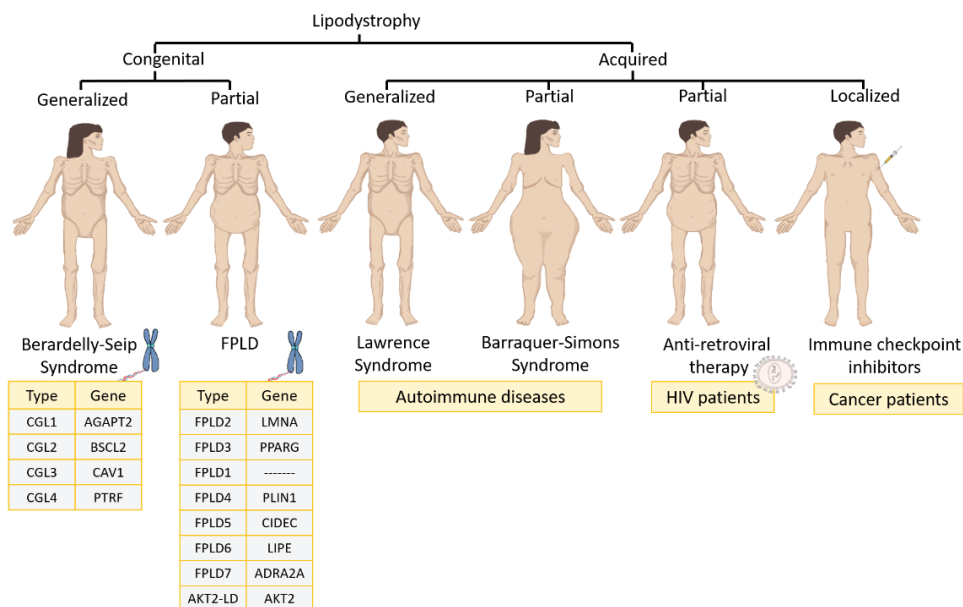


Figure 1. Lipodystrophy syndromes classification. FPLD, Familial partial lipodystrophy.

Congenital lipodystrophies

- Familial partial lipodystrophy (FPLD) is usually an autosomal dominant syndrome characterized by a selective loss of fat from the lower and upper extremities, as well as the trunk (Hussain & Garg, 2016). During childhood, patients have a normal fat distribution but at puberty start to lose fat from the chest, anterior abdomen and extremities. There are eight varieties of FPLD: 1) FPLD2, the most common subtype (also called the Dunnigan type), characterized by mutations in the LMNA gene; 2) FPLD3, the second most common subtype, based on mutations in the PPARG gene; 3) FPLD1 (or the Kobberling type) whose genetic mutation is unknown; 4) FPLD4, characterized by heterozygous mutations in the PLIN1 gene; 5) and 6) FPLD5 and FPLD6, both autosomal recessive disorders in the CIDEA and LIPE genes, respectively; 7) FPLD7, with a genetic mutation in the ADRA2A gene; and 8) AKT2-linked lipodystrophy (AKT2-LD), based on a mutation in the AKT2 gene (Hussain & Garg, 2016).



- Congenital generalized lipodystrophy (CGL), or Berardinelli-Seip syndrome, is an autosomal recessive disease that is distinguished by the absence of AT both at birth and in early childhood (Hussain & Garg, 2016). Four distinct subtypes are depending on the gene that is altered: 1) CGL1 is the most common subtype and the altered gene is AGPAT2, which is involved in triglyceride biosynthesis; 2) CGL2 is the second most common subtype and the altered gene is BSCL2, which plays a relevant role in adipocyte differentiation and small lipid droplet fusion in the adipocytes; 3) CGL3 has only been reported in one patient and the altered gene is CAV1, which translocate fatty acids to lipid droplets; 4) CGL4 has been reported in 20 patients and is very close to CGL3 because its gene (PTRF) is regulated by CAV1 expression (Hussain & Garg, 2016).

Acquired lipodystrophies

- Acquired generalized lipodystrophy, or Lawrence syndrome, is characterized by generalized loss of subcutaneous fat. The loss of fat usually begins in childhood or adolescence (Hussain & Garg, 2016). Most of the patients have related autoimmune diseases (Childs et al., 2019; Corvillo et al., 2018; Manzel et al., 2014; Sahinoz et al., 2018; Sharawat et al., 2019; Vieira et al., 2014). In some autoimmune diseases, the role of PPAR γ is essential to modulate inflammation. In fact, therapy is based on agonists of PPAR γ (Liu et al., 2020).
- Acquired partial lipodystrophy, or Barraquer-Simons syndrome, is characterized by a gradual loss of subcutaneous fat from the upper trunk, upper extremities, neck and face. In the case of females, after puberty excess fat could accumulate in the lower extremities, hips and lower abdomen. It is often related to autoimmune diseases (Corvillo, Ceccarini, et al., 2020; Corvillo, Nozal, et al., 2020; Heidemann et al., 2016; Hussain & Garg, 2016).
- Antiretroviral therapy-induced lipodystrophy occurs in patients infected with human immunodeficiency virus (Hussain & Garg, 2016) who, after 2-4 years of treatment with ART, start to have an increased accumulation of both intra-abdominal and upper trunk fat, while they lost subcutaneous fat in the lower and upper extremities (Akita et al., 2021; Hussain & Garg, 2016). This is related to



PPAR γ downregulation. However, stopping ART, PPAR γ expression is restored in macrophages and adipocytes (Caron et al., 2009).

- Recent cases of acquired lipodystrophy have been associated with the use of immune checkpoint inhibitors to treat cancer (Falcao et al., 2019; Gnanendran et al., 2020; Hosokawa et al., 2019). Among them, childhood cancer survivors transplanted with hematopoietic stem cells and treated with chemotherapy developed acquired lipodystrophy over time (Nagayama et al., 2019). Furthermore, other types of cancer, such as craniopharyngioma, may lead to chronic inflammatory demyelinating polyneuropathy together with acquired lipodystrophy (Lockemer et al., 2018). This pathology is associated with some cancer therapies against programmed cell death protein 1 (PD-1), which promotes apoptosis in antigen-specific T cells in lymph nodes and leads to adverse events of an immune nature (Eigentler et al., 2020).

3.2. Physiopathology of lipodystrophy

The physiopathology of acquired lipodystrophy is characterized mainly by the disfunction of AT, which leads to several metabolic complications such as inability to stored triglycerides in adipocytes, ectopic fat accumulation in tissues such as the liver, insulin resistance and hypertriglyceridemia. These alterations are closely related to NAFLD and DMT2. Furthermore, the dysfunction of AT promotes chronic inflammation that exacerbates the comorbidities associated with the disease (Bloomgarden, 2007; Brown et al., 2016; Fiorenza et al., 2011).

3.2.1. Adipose tissue

In humans, there are two types of AT: white (WAT) and brown (BAT). They differ mainly in the type of adipocytes that constitute them (Berger et al., 2017). White adipocytes are involved in lipid storage through a single large lipid droplet, being involved in energy storage mainly. WAT is extremely important for nutrient storage, which confers an important role in the maintenance of energetic homeostasis (Berger et al., 2017). Traditionally, it has been suggested that the function of WAT was only



lipid storage (Fernández-Galilea et al., 2020). However, recent research has shown that it plays an important role as an active endocrine organ because it is involved in synthesizing a wide range of bioactive peptides (Clemente-Postigo et al., 2020). On the other hand, brown adipocytes are multilocular lipid storage cells that contain more mitochondria than white adipocytes. BAT is involved in body temperature maintenance, specifically burning calories to create heat (thermogenesis) (Xiao & Kang, 2020). Furthermore, there are beige adipocytes that are an inducible form of brown adipocytes. They emerge from white adipocytes in response to certain environmental causes (such as cold temperatures), a process called “browning” of WAT (Sidossis & Kajimura, 2015; Xiao & Kang, 2020).

Both the type and amount of AT and the distribution of body fat contribute to maintaining a normal metabolic state (Fiorenza et al., 2011). In acquired lipodystrophy, there is a dysfunction of AT that alters the normal distribution of body fat with an increase in ectopic fat accumulation and a reduction in the production of adipokines (Figure 2) (Qiang et al., 2016). These adipokines (omentin, apelin, vaspin, visfatin, chemerin, resistin, adiponectin and leptin) can act systematically as endocrine factors, regulating and modulating different biological processes, such as satiety, appetite, lipid and glucose metabolism, cardiomyocyte contraction, blood pressure and insulin secretion (Suriano et al., 2020). Furthermore, they can act locally as paracrine and autocrine factors, modulating insulin sensitivity, immune cell migration, adipocyte metabolism and adipogenesis (Suriano et al., 2020).

Usually, patients with lipodystrophy have extremely low serum levels of adipokines, such as adiponectin and leptin (Hussain & Garg, 2016). Both of them are important in AT atrophy and its associated metabolic complications. Regarding leptin, once secreted by adipocytes, circulates in the bloodstream and exerts its effects through binding to the leptin receptor, a single transmembrane-spanning protein in the central nervous system (Moon et al., 2013; Triantafyllou et al., 2016). Leptin activates hypothalamic centers involved in the synthesis of anorexigenic peptides, promoting satiety and counteracting the effect of orexigenic hormones like ghrelin (Figure 2) (Triantafyllou et al., 2016). In addition to its function in the central nervous system, leptin has an important role in other metabolically important tissues, such as the liver,

muscle and AT (Triantafyllou et al., 2016). In the liver, leptin reduces hepatic gluconeogenesis. In muscle, it increases lactate formation, glycogen synthesis, glucose uptake and oxidation. Regarding AT, leptin promotes the “browning” process, reduces lipolysis, improves insulin sensitivity and limits lipid storage in non-ATs (Münzberg et al., 2020; Triantafyllou et al., 2016).

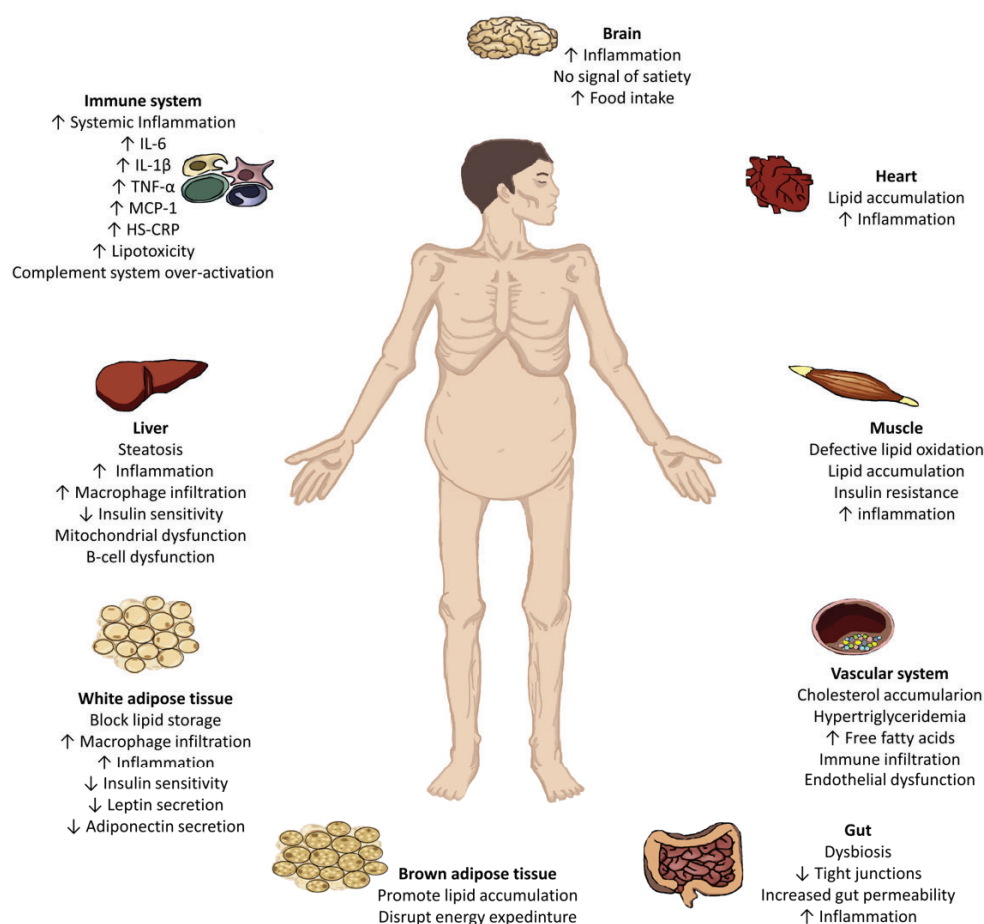


Figure 2. Physiopathology of lipodystrophy

Interestingly, leptin deficiency or absence leads to clinical phenotypes of dyslipidemia, liver steatosis, hyperinsulinemia, recurrent bacterial infections, hyperphagia and impaired satiety (Foss-Freitas et al., 2020). Therefore, Metreleptin replacement is routinely used in lipodystrophy therapy to treat the complications of



leptin deficiency in patients with acquired or congenital generalized lipodystrophy (Vahid et al., 2020).

Apart from its metabolic and endocrine functions, leptin has an important role in systemic autoimmunity and inflammation (Navarini et al., 2018). Leptin has a relevant peripheral role in immune system-metabolism interplay, acting as an immunomodulatory factor (Francisco et al., 2018). Indeed, several inflammatory cytokines (such as IL-1 β and TNF- α) provide a positive feedback mechanism, modulating the synthesis and secretion of leptin (Angelucci et al., 2020), while it stimulates immune cells, activating lymphocytes, polymorphonuclear cells and monocytes. Its deficiency is related to the downregulation of the inflammatory response, impaired phagocytosis, enhanced susceptibility to infections and immune suppression (Angelucci et al., 2020). Finally, leptin can regulate the differentiation, function and metabolism of a variety of intestinal epithelial cells and immune cell subpopulations (Ziegler et al., 2019).

3.2.2. Immune system

It is widely recognized the bidirectional interaction between the immune system and metabolism. Immune system dysfunction occurs in situations of depleted or excessive fat mass (Berger et al., 2017). This dysfunction has been extensively studied in obese individuals, making obese people more susceptible to infections because of impaired immune defense. However, the role of the immune system in lipodystrophy has been studied mainly in the context of leptin replacement (Berger et al., 2017).

Despite the lack of available data on the role of the immune system in the genesis and development of lipodystrophy, the following are the main abnormalities of the immune system found in patients with lipodystrophy: high pro-inflammatory cytokine levels, increased pro-inflammatory M1 macrophages, formation of crown-like structures, increased B cell counts and decreased the number of T cells with regulatory function (Figure 2) (Berger et al., 2017). Some cases have reported disorders such as low platelet, low lymphocyte and low white blood cell counts (Ishiguro et al., 2002); autoimmune thrombocytopenia (Cunningham et al., 2017); predominance of CD4+



over CD8⁺ lymphocytes (Gdynia et al., 2009); greater accumulation of lipid droplets in CD8⁺ T cells; and intracellular accumulation of lipid droplets in NK cells (Ziegler et al., 2019). This lipid accumulation has been related to a disruption of the capacity of lymphocytes to flux calcium, which is essential to control the function and metabolism of immune cells, as well as impair the killing capacity of cytotoxic NK cells by metabolic reprogramming (Ziegler et al., 2019).

Interestingly, an increase in proinflammatory M1 macrophages is found in lipodystrophy syndromes (Berger et al., 2017). Monocyte-derived macrophages are plastic cells that can display different phenotypes, such as pro-inflammatory M1 or anti-inflammatory M2, with the former being predominant in the WAT of obese individuals and the latter in lean individuals (Berger et al., 2017). Despite these widely recognized phenotypes, another phenotype has been described that is more widely represented in patients with lipodystrophy, the lipophages. Lipophages are macrophages that contain small lipid vacuoles (or fat lobules) from ingested cells or extracellular fat material (Goeritzer et al., 2015). These cells are developed by the internalization of the oxidized form of low-density lipoprotein (Berger et al., 2017). In lipodystrophy, the presence of lipophages is related to a higher expression of M1 markers (Berger et al., 2017). Some cases of acquired lipodystrophy have shown lipophage infiltration in AT (Gnanendran et al., 2020; Haddad et al., 2020; Jehl et al., 2019), suggesting that macrophages become lipophages within the inter-adipocyte stroma in these patients (Ahmed, 2006; Sharma et al., 2019; Zalla et al., 1995). Lipophages could be a useful target for treatment, but they are not discerned in routine tissue analysis.

Another alteration of the immune system in patients with acquired lipodystrophy is the overactivation of the complement system (Corvillo & Akinci, 2019; Gdynia et al., 2009). The disruption of the activation-inactivation of the complement system has been related to several diseases, such as infections or autoimmune conditions. Some studies have associated adipocyte destruction and complement activation with cases of acquired lipodystrophy (Eren et al., 2010; Savage et al., 2009). In fact, in some cases of Barraquer-Simons Syndrome were detected



autoantibodies targeting complement system proteins (Corvillo, Ceccarini, et al., 2020; Corvillo & López-Trascasa, 2018).

Finally, lipodystrophy is related to pro-inflammatory alterations in the immune system, including the overrepresentation and infiltration of lipophages in AT, which is related to the enhanced paracrine capacity to induce lipid droplet accumulation, depletion of the number of NK and regulatory T cells and disruption of complement system homeostasis (Berger et al., 2017).

3.2.3. Gut microbiota

There is no clear evidence about the link between acquired lipodystrophy and microbiota alteration. However, some evidence correlates AT inflammation with gut microbiota changes (Cheru et al., 2019). Gut microbiota is involved in the metabolism of amino acids, lipids and carbohydrates (Wang et al., 2020). Besides, the gut's mucosa is an important barrier between the bloodstream and the luminal contents of the gut, developing a relevant role in the maintenance of intestinal barrier homeostasis (Cheru et al., 2019). Thus, the host's immune system plays an important role in health and suffers alterations in different diseases, such as metabolic diseases, HIV infection and inflammatory bowel disease (Cheru et al., 2019).

The appropriate balance in intestinal microbiota is crucial to maintain the barrier function of the gut's epithelium (Torres et al., 2019). Changes in intestinal microbiota (dysbiosis) compromise the integrity of the intestinal barrier (Torres et al., 2019). Concerning dysbiosis and body weight, a study in a murine experimental model suggested that diet-induced dysbiosis may be related to failure to gain weight (Rodríguez-García et al., 2020). Furthermore, dysbiosis may lead to an increase in lipopolysaccharide (LPS)-related bacterial translocation, endotoxemia and a rise in intestinal permeability. LPS is a component of the cell wall of gram-negative bacteria that triggers a proinflammatory cytokine cascade (Hersoug et al., 2016; Torres et al., 2019). The activation of this cascade promotes a vicious cycle of AT inflammation and increases gut permeability by Toll-Like Receptor 4 (TLR4) activation (Cheru et al., 2019).



The gut microbiota of healthy individuals has beneficial effects by producing short-chain fatty acids (SCFAs), antioxidants and vitamins. Bacteria help the gut to utilize energy by degrading undigested carbohydrates to SCFAs, such as butyrate, propionate and acetate (Hersoug et al., 2016; Wang et al., 2020). These SCFAs can prevent bacterial translocation across the gut barrier. Furthermore, SCFAs may suppress the pro-inflammatory effects of LPS on macrophages (Hersoug et al., 2016). Under healthy conditions, the intestinal epithelium acts as an effective barrier for LPS. It has been suggested that the human gut contains around 1 g of LPS and its concentrations in blood are low (1-200 pg/ml) (Hersoug et al., 2016). However, elevated levels of LPS in the blood are associated with metabolic endotoxemia, which promotes metabolic complications. Patients with HIV-related lipodystrophy have correlated markers of metabolic alterations (such as insulin resistance) with high plasma LPS levels (Hersoug et al., 2016; Pedro et al., 2018; Viladés et al., 2014), being a marker that could be analyzed in the diagnosis of patients with acquired lipodystrophy.

Although more evidence is needed to show this, gut dysbiosis could be related to the development of acquired lipodystrophy through the induction of AT inflammation by TLR4.

3.2.4. Liver

Since the AT of patients with lipodystrophy is unable to effectively store triglycerides, fat is accumulated ectopically in non-ATs, such as the liver, promoting metabolic abnormalities characterized by lipotoxicity (such as hyperlipidemia and hepatic disease) (Polyzos et al., 2019; Zammouri et al., 2021). This happens as a result of increased uptake of fatty acids arising from insulin resistance (Tajima et al., 2017). Regarding patients with lipodystrophy, they show a high predisposition to the development of hepatic steatosis (Polyzos et al., 2019). It has been reported that patients with lipodystrophy have a higher risk of NAFLD and non-alcoholic steatohepatitis (Hadizadeh et al., 2017). This liver alteration may lead to hepatomegaly, inflammation, fibrosis, cirrhosis and hepatic cancer (Polyzos et al., 2019; Safar Zadeh et al., 2013). NAFLD is characterized by the upregulation of several soluble



inflammatory mediators and the overactivation of inflammatory cells (Braunersreuther et al., 2012). Among the different immune markers related to NAFLD, it is common to find low platelet counts and high levels of pro-inflammatory cytokines. The latter can lead to activation of harmful pathogenic pathways by decreasing HDL cholesterol and stimulating the production of C-Reactive Protein by the liver (Hadizadeh et al., 2017). The rate of patients with acquired lipodystrophy that develop hepatomegaly is around 80% (Polyzos et al., 2019). Furthermore, other research observed that 90% of patients with lipodystrophy had fatty liver disease (Safar Zadeh et al., 2013), being some of the cases related to autoimmune hepatitis (Safar Zadeh et al., 2013).

Finally, it can summarize that, in the physiopathology of acquired lipodystrophy, there is important crosstalk between the metabolism and immune system, and its management must be focused on targeting both of them (Figure 2).

3.3. Relationship of genes involved in the development of lipodystrophy

The main genes involved in the development of lipodystrophy of congenital origin and those implicated in some cases of acquired lipodystrophy are summarized below:

- BSCL2: a protein expressed mainly in AT, which is involved in lipid droplet biogenesis, in the regulation of energy homeostasis and adipocyte differentiation (Zhou et al., 2020).
- LIPE or hormone-sensitive lipase: promotes the hydrolysis of triglycerides stored in lipid droplets during adipocyte differentiation (Sollier et al., 2021).
- CAV1: a protein located in lipid droplets of adipocytes and developed a key role in cholesterol homeostasis, endothelial transcytosis and cellular metabolism (Schrauwen et al., 2015)
- LMNA: a protein involved in telomere dynamics, nuclear membrane, chromatin organization and nuclear assembly (Vigouroux et al., 2018)
- AKT2: a kinase involved in processes such as angiogenesis, cell growth, proliferation and metabolism (Shearin et al., 2016).



- ADRA2A: a receptor involved in inhibition of adenylate cyclase induced by catecholamine (Garg et al., 2016).
- PPARG: a nuclear receptor that controls insulin sensitivity, glucose metabolism and adipocyte differentiation (Guo et al., 2020). PPAR γ is a major adipogenic factor (Zammouri et al., 2021).
- AGPAT2: an acyltransferase involved in the transformation of lysophosphatidic acid into phosphatidic acid, which belongs to the triglyceride biosynthetic pathway (Ceccarini et al., 2020).
- CIDEA: a protein that modulates triglyceride storage by restricting lipolysis, and is involved in the enlargement of lipid droplets (Zammouri et al., 2021).
- PLIN1: a modulator of lipid metabolism in adipocytes. Protect lipid droplets from breakdown by HSL, and its interaction with CIDEA promotes the enlargement of lipid droplets (Jéru et al., 2019).

STRING11 software was used to analyze if there was any relationship between the main genes implicated in the different types of lipodystrophy. The results of the analysis showed that there was an interaction between some of the proteins: PPARG, CIDEA, CAV1, LIPE, PLIN1, BSCL2 and AGPAT2 (Figure 3). We observed that there were strong interactions between PPARG and PLIN1 with most proteins. In fact, the network had significantly more interactions than expected, this means that proteins have more interactions among themselves than what would be expected from a random set of proteins, which shows that proteins are partially biologically connected as a group. This group is mainly involved in biological processes that are closely related to lipodystrophy such as lipid metabolism and lipid droplet formation. Thus, data suggested that PPARG and PLIN1 could develop an important role in the dysfunction of adipose tissue modulating the activity of other proteins. Surprisingly PPARG expression is also altered in some types of acquired lipodystrophy, which could be the link between congenital and acquired lipodystrophy and could be a key target for the management of the disease. Therefore, the next step was to identify how the expression of both proteins can be modulated by nutritional factors such as dietary fatty acids.

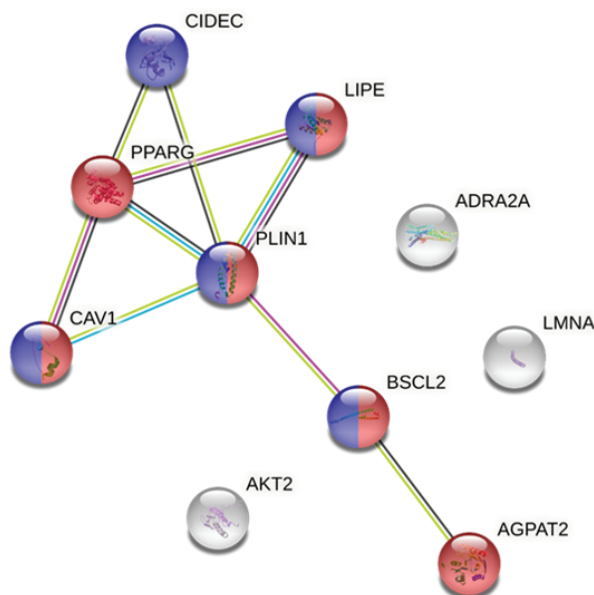


Figure 3. Protein-protein interaction network. Colored nodes in red: proteins involved in lipid metabolism. Colored nodes in blue: proteins involved in lipid droplet formation. Colored nodes in white: the second shell of interactions. Interaction lines in blue: from curated databases. Interaction lines in pink: experimentally determined. Interaction lines in green: gene neighborhood. Interaction lines in black: co-expression.

3.4. Nutrigenomic effects on PPAR γ and PLIN1 induced by dietary lipids

The epigenetic changes in AT can be produced by diet. The dietary fat composition can affect ectopic lipid accumulation and dietary fatty acids are involved in the composition of cellular membranes, organelles membranes and can regulate the TLR activity, contributing to an inflammatory response (Cheng et al., 2012; Fernández-Quintela et al., 2007). However, studies that analyzed the effect of dietary lipids in lipodystrophy are limited. After a review of the scientific literature, 37 articles have been selected for discussion in this section (Supplementary Figure 1).

Interventions with oils have been developed mainly in murine models, although there are some clinical trials on diabetic and healthy adults (Table 1). Patients with metabolic dysregulation as DMT2, have common complications such as cardiomyopathy, a typical outcome in some lipodystrophic patients (Sanon et al., 2016). A study analyzed the effect of Krill oil (an oil rich in n-3 polyunsaturated acid



(PUFA) from marine origin) in the prevention of cardiomyopathy in diabetic mice (Sun et al., 2022). This oil was able to upregulate the peroxisome proliferator-activated receptor- γ coactivator 1 α (PGC-1 α), which is involved in the inhibition of the inflammasome (NLRP3). In the same way, the supplementation with flaxseed oil (rich in n-3 PUFAs) in diabetic patients with coronary heart disease, downregulated in a significant way the expression of TNF- α and upregulated PPAR γ in the peripheral blood mononuclear cells (PBMCs) of patients (Hashemzadeh et al., 2017). Supplementation with other oil rich in n-3 PUFA was closely related to the fatty acid composition of different tissues, and regulated positively the expression of PPAR γ in the AT of turkeys, regulating the fat metabolism (Szalai et al., 2021). The oils' composition is directly associated with their impact on health. In the case of palm oil or butter, which is composed of saturated fatty acids, are related to the development of NAFLD, while the substitution of these oils with other rich in unsaturated fatty acids as rapeseed oil, can attenuate the progression of NAFLD by reducing the levels of LPS, downregulating the activation of TLR4 and increasing the PPAR γ activity in the small intestine (Brandt et al., 2020). While peanut oil and lard induced inflammation, hepatic steatosis, and high blood pressure, a blended oil rich in oleic acid and ALA were able to reduce low-density lipoprotein cholesterol (LDL), serum triglycerides, TLR4 expression, TNF- α , and C-Reactive Protein, but increased PPAR γ expression. These results demonstrated that an appropriate proportion of monounsaturated and n-6:n-3 PUFAs ratio could prevent immunometabolic disturbances (Uriho et al., 2019).

Virgin olive oil and fish oil have been demonstrated to be one of the healthiest fat sources in maintaining body weight, regulating metabolism and promoting an anti-inflammatory status. However, there is no evidence about PPAR γ modulation by virgin olive oil with the search strategy used. Regarding fish oil, its supplementation can enhance significantly the lipoprotein lipase, PPAR γ and PGC-1 α expression even during a high-fat diet intervention in rats (Liu et al., 2019). Supplementation with fish oil enriched in docosahexaenoic acid (DHA) in diabetic patients increased PPAR γ activity significantly in PBMCs (Naeini et al., 2020), improving their metabolism.

On the other hand, palm oil and palmitic acid-induced lipotoxicity in the liver enhance hepatic fatty acid and triglycerides uptake by the upregulation of CD36, a very-



low-density lipoprotein receptor (VLDLR) and PPAR γ (Hajri et al., 2021). Despite the different effects on health, overfeeding promotes the methylation of PGC-1 α and TNF- α in AT (Perfilyev et al., 2017).

Regarding studies that analyze the effect of n-3 PUFAs in different research models (Table 2), all conclude that n-3 PUFAs can suppress metabolic disturbances associated with insulin resistance and the development of liver fibrosis. In the WAT of rats, these fatty acids induced browning by enhancing the activity and expression of PPAR γ and enhancing the expression of Nrg4, which is involved in the prevention of lipid accumulation in hepatic cells (Yang et al., 2021). Furthermore, in murine models of metabolic syndrome, the n-3 PUFAs supplementation increased the expression of PPAR γ and GLUT4, improving insulin sensitivity and lipid profile (Zayed et al., 2018). Similarly, the supplementation with n-3 PUFAs in athletes produced an upregulation in the protein levels of UCP2 and PPAR γ in PBMCs, improving the energy expenditure and controlling body weight (Moradi et al., 2021).

Drug therapy for patients with DMT2 includes the use of drugs such as metformin, which improves insulin sensitivity in tissues such as the liver and skeletal muscle. N-3 PUFAs supplementation alone or in combination with metformin restore metabolism by increasing the PGC-1 α expression, preventing mitochondrial degeneration, having a hypoglycemic effect and improving the myocardial structure to prevent cardiomyopathies in rats (Eraky & Ramadan, 2021). Depending on the genes altered in DMT2, the response to n-3 PUFAs supplementation may vary. However, n-3 PUFAs can modulate the expression of genes involved in metabolism maintenance as PPAR γ , NOS3 and CD36, improving the lipid profile of patients (Zheng et al., 2018). During insulin resistance, the liver is one of the most damaged organs due to the excessive accumulation of triglycerides. N-3 PUFAs can prevent liver fibrosis in hepatic cells, due mainly to their agonist effect on PPAR γ , being most potent the treatment with DHA and EPA than ALA (Hu et al., 2020). Despite the health benefits of other PUFAs as n-6, the amount of n-3 to n-6 in the diet should be in a 3:1 ratio to control the macrophage recruitment to adipocyte during AT dysfunction, to improve fatty acid oxidation and to reduce inflammation via TLR4 and TNF- α (Pandurangan et al., 2020).

Table 1. Effect of oils on PPAR γ modulation

Intervention	Duration	Study Design	Outcome	Ref
Krill oil (KO) AING-93G diet for control and 1.5% KO-containing high-fat diet	24 weeks	<i>In vivo</i> Diabetic C57BL/6 mice	KO increases cardiac PGC1- α protein expression in cardiac tissue, reducing apoptosis of cardiomyocytes and regulated negatively the NRLP3 inflammasome.	(Sun et al., 2022)
Flaxseed Oil Placebo or 1000mg flaxseed oil supplements (400mg α -linolenic acid) (Twice a day)	12 weeks	Randomized double-blind placebo-controlled trial Diabetic patients with coronary heart disease (40-85 years) n=60	Flaxseed oil supplementation up-regulates PPAR γ gene expression and reduces TNF- α levels.	(Hashemzadeh et al., 2017)
Linseed oil (LO) A basic diet or a basic diet supplemented with 0.5% or 1% of LO	22 weeks	<i>In vivo</i> <i>Meleagris gallopavo</i> n=140	LO supplementation lead to higher levels of PPAR γ in adipose tissue	(Szalai et al., 2021)
Rapeseed oil Control diets with 12% of energy from soybean oil or 25% of energy from the butterfat. Diet with 12% of energy from rapeseed oil or diet with rapeseed oil, fructose and cholesterol	13 weeks	<i>In vivo</i> C57BL/6J mice with NAFLD n=28	Rapeseed oil exerts protective effects against NAFLD by increasing PPAR γ activity, lowering portal endotoxin levels and attenuating TLR4 signal	(Brandt et al., 2020)





Blended oil	Low-fat, middle. A fat and high-fat diet based on peanut oil, lard oil diet or blended oil (canola oil, corn oil, olive oil, peanut oil, sunflower oil)	12 weeks	<i>In vivo</i> Wistar rats n=90	A blended oil diet enhances genes related to lipid catabolism as PPARG in the liver, due mainly to their n-6:n-3 PUFAs ratio. Blended oil reduces TNF- α and C-Reactive Protein in serum	(Uribe et al., 2019)
Fish oil	High-fat diet, or a high-fat diet with 5% of fish oil (30,6% of EPA and 19,2% of DHA)	8 weeks	<i>In vivo</i> Sprague-Dawley rats n=18	High-fat diet in fish oil may reverse the protein expression in soleus muscle of PPARG and PGC-1 α protein, improving lipid metabolism	(Liu et al., 2019)
DHA-enriched fish oil	Soft gels of 2400mg of fish oil with 600mg of n-3 PUFAs or paraffin oil. 4 gels per day	8 weeks	A randomized trial Patients with DMT2 n=50	DHA-rich fish oil increases PPARG activity in PBMCs	(Naeini et al., 2020)
Palm oil	Low-fat diet or A high-fat diet based on palm oil (60%)	12 weeks	<i>In vivo</i> C57BL/6 mice n=32	A high-fat diet based on palm oil increases the hepatic expression of VLDLR, CD36, and PPARG, leading to excess hepatic lipid accumulation	(Hajri et al., 2021)
Palm oil and Sunflower oil	High-caloric muffins that contain refined sunflower oil or refined palm oil.	7 weeks	A randomized trial Healthy adults (20-38years) n=31	After overfeeding the methylation of PGC-1 α and TNF- α in adipose tissue was increased.	(Perfilyev et al., 2017)

DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; KO: Krill oil; LO: Linseed oil; NAFLD: non-alcoholic fatty liver disease; PUFA: polyunsaturated acid



Table 2. Effect of n-3 PUFAs on PPAR γ modulation

Intervention	Duration	Study Design	Outcome	Ref
n-3 PUFAs A high-fat diet rich in saturated fats, or n-3 PUFAs, or a normal diet.	13 weeks	<i>In vivo</i> Male Sprague-Dawley n=30	n-3 PUFAs induce browning in white adipose tissue by the PPAR γ pathway, and EPA increases PPAR γ in HPAs.	Yang et al., 2021)
0, 5, 10 and 20 μ mol/L of EPA	Data not shown	<i>In vitro</i> Human preadipocytes		
n-3 PUFAs Chow diet, High-fat diet +260mg/kg n-3 PUFA, High-fat diet +100mg/kg metformin, or High-fat diet + 200mg/kg L-carnitine	12 weeks	<i>In vivo</i> Sprague Dawley rats n=30	n-3 PUFAs decrease body weight, glucose, insulin, and increase adiponectin by GLUT4 and PPAR γ regulation.	(Zayed et al., 2018)
n-3 PUFA Supplement of two Omega 3 gel capsules per day (240mg of DHA and 360mg EPA) or placebo (paraffin oil capsules)	3 weeks	A randomized double-blind controlled trial Peripheral mononuclear cells of athletes (PBMCs) n=36	Omega 3 supplementation lead to the up-regulation of PPAR γ protein levels in the blood	(Moradi et al., 2021)
n-3 PUFAs Normocaloric diet or a high-fat diet supplemented with metformin, 300mg/kg/d of n-3 PUFAs, or a combination of both.	8 weeks	<i>In vivo</i> Diabetic Sprague-Dawley rats n=38	n-3 PUFAs alone or in combination with metformin can suppress metabolic changes related to diabetes by increasing gene expression of PGC-1 α	(Eraky & Ramadan, 2021)



n-3 PUFAs	4 capsules/day of 0.5 g of n-3 PUFAs from fish oil, 0.62 g of α -linolenic acid from flaxseed oil and corn oil as a control	180 days	A randomized, double-blind, controlled trial	T2D patients with different genotypes at PPAR γ , NOS3, and CD36 respond differentially to the intervention of omega-3 supplements in blood lipid profiles.	(Zheng et al., 2018)
			Patients with DMT2 (35-80 years)		
			n=185		
n-3 PUFAs	Treatment with Palmitic acid, Oleic acid, Linoleic acid, ALA, EPA, and DHA at 50 μ M	24 hours	<i>In vitro</i> LX-2 cells	α -linolenic acid, EPA and DHA prevent liver fibrosis development by PPAR γ regulation.	(Hu et al., 2020)
			Primary human and mouse hepatic stellate cells		
n-6:n-3 PUFAs	0.1, 0.2 and 0.4 μ g/ml of chia seed extract fatty acid with a ratio of 3:1 of omega 3 and omega 6	14 days	<i>In vitro</i> Human bone marrow-derived mesenchymal stem cells	Chia seed extracts fatty acid decrease lipid accumulation and enhances mitochondrial fatty acid oxidation in mature adipocytes by increasing the expression of PGC-1 α . Furthermore, this treatment suppresses macrophage recruitment in adipocytes and reduce protein levels of TNF- α	(Pandurangan et al., 2020)
			PBMCs from healthy volunteer		

ALA: α -linolenic acid; DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; PBMCs: Peripheral blood mononuclear cells; PUFA: polyunsaturated fatty acids



Based on the protective effects of n-3 PUFAs supplementation, several studies have analyzed individually the effects of EPA and DHA to elucidate their mechanism of action (Table 3). Both EPA and DHA fatty acids have shown an anti-lipotoxic effect in different types of cells. In a high-fat diet or during induction of lipotoxicity with palmitic acid *in vitro*, the supplementation with EPA was able to promote fatty acid oxidation and lipid droplet formation (Yamamoto et al., 2021). Similarly, the supplementation in obese mice attenuated the dysfunction of AT by enhancing the expression of PPAR- γ and reducing the inflammation associated with high levels of IL-6 and TNF α , attenuating the inflammatory-metabolic state (Illesca et al., 2020). Furthermore, in UCP1 knockout mice fed a high-fat diet, who are unable to regulate diet-induced thermogenesis and are at increased risk for obesity, EPA supplementation exerted protective effects increasing PGC1 α expression in BAT and improving the glucose tolerance (Pahlavani et al., 2019). The treatment with EPA and DHA, alone or combined, upregulated different genes involved in the mitochondrial function. In skeletal muscle cells, EPA improved the response to insulin via PGC1- α and countered the inflammation induced by palmitic acid, inhibiting the NF κ B signaling (Sergi et al., 2021). In the same way, EPA promotes adipogenesis in mesenchymal stem cells through activation of PPAR γ , while the treatment with EPA, DHA or furan fatty acid 9-(3-methyl-5-pentylfuran-2-yl)-nonanoic acid (9M5) induced adipogenesis in preadipocytes (Lauvai et al., 2019; Martins et al., 2020; Riera-Heredia et al., 2019). Comparing the effects of EPA and DHA, the last one has demonstrated to be more effective in immunometabolic regulation, enhancing fat oxidation in muscle cells (via PGC1- α), reducing the expression of TNF α R (Katsnelson & Ceddia, 2020), improving the adipocyte functionality through PPAR γ and increasing adiponectin secretion (Song et al., 2017). In combination with arachidonic acid (ARA), DHA attenuated the AT dysfunction induced by an obesogenic diet and can reduce inflammatory cytokines as TNF- α IL-6 in the AT (Mao et al., 2018).

The effects of conjugated linoleic acid (CLA) on metabolism remain controversial (Table 4). Its supplementation has been shown to decrease the abundance of genes related to fatty acid oxidation and lipolysis, and to increase genes involved in lipogenesis as PPAR γ (Dirandeh et al., 2021). In murine models, high doses of CLA



are associated with lipid accumulation in the liver by regulating negatively PCG-1 α and leading to steatogenic effects (Cristofano et al., 2021), as well as reduction of body fat through modulation of PPAR γ (Abdelatty et al., 2019). Contrary to the effects of n-3 PUFAS, CLA does not stimulate mitochondrial biogenesis or PCG-1 α expression in murine models (Barone et al., 2017). On the other hand, in vitro effects of CLA in combination with alpha-lipoic acid exerted anti-inflammatory activity in murine macrophages through modulation of the ERK1/PPAR γ pathway (Lee et al., 2020).

Other fatty acids studied individually are listed in Table 5. Among them, sterol ester of α -linolenic acid stimulated mitochondrial biogenesis by PCG-1 α regulation and reduced oxidative stress in a murine model with NAFLD (Han et al., 2021). However, in vitro studies showed the different effects of unsaturated fatty acids as stearidonic and palmitoleic acid. While stearidonic acid inhibits adipocyte regulation by downregulating PPAR γ (Li et al., 2017), palmitoleic acid not only increased the transdifferentiation of bovine satellite cells into adipocytes by PPAR γ but also upregulated PLIN1 (Zhang et al., 2021). This is the only study to analyze the effect on PLIN1 expression. Regarding saturated fatty acids (SFA), the two studies analyzing the effect of palmitic acid showed that induced lipid accumulation and suppressed lipolysis by stimulating PPAR γ in hepatic cells, promoting hepatotoxicity, and favored the M1 macrophage polarization, enhancing TNF- α and IL-6 secretion and stimulating TLR4/ NF- κ B signaling (Wu et al., 2020; Zhao et al., 2018).



Table 3. Effect of eicosapentaenoic acid, docosahexaenoic acid and arachidonic acid on PPAR γ and PLIN1 modulation

Intervention	Duration	Study Design	Outcome	Ref
EPA Normocaloric diet, High-fat diet, or High-fat diet mixed with 3.6% of EPA (weight/weight)	8 weeks	<i>In vivo</i> GFP-MAP1LC3 transgenic, atg5-cKO (Atg5 ^{FF} ; Kap-Cre), and atg5-iKO (Atg5 ^{FF} ; Ndtgl-Cre) mice n=21	EPA promotes lipid droplet formation and a fatty acid transfer from them to the mitochondria for beta-oxidation, attenuating the lipotoxicity induced by a high-fat diet	(Yamanoto et al., 2021)
EPA Control diet or a high-fat diet supplemented with saline, EPA (50mg/kg) or Hidroxytyrosol (5mg/kg), or a combination of both.	12 weeks	<i>In vivo</i> C57BL/6J mice n=80	Supplementation with EPA improved the activity and mRNA levels of PPAR- γ , and reduce the levels of TNF- α and IL-6 in white adipose tissue	(Illesca et al., 2020)
EPA High-fat diet or a high-fat diet supplemented with 36g/kg of EPA-enriched fish oil	14 weeks	<i>In vivo</i> Wild type and UCP1 knockout C57BL/6J mice n=40	EPA can rescue the glucose tolerance in UCP1 knockout mice via PGC1- α .	(Pahlavani et al., 2019)
EPA 500 μ M of palmitic acid, 24 hours 500 μ M of EPA, or 500 μ M of palmitic acid combined with 100 μ M of EPA		<i>In vitro</i> Human primary myotubes derived from the abdominal rectus muscles of male individuals 31.00 $\pm$ 5.67 years	EPA increases the expression of PGC1- α and is coupled with the inhibition of the inflammatory response induced by palmitic acid.	(Sergi et al., 2021)



EPA	EPA, DHA, Linoleic acid or α -linolenic acid at 1,10 or 100 μ M	6, 24, 48 and 72 hours	<i>In vitro</i> Primary cultures of gillhead sea bream bone-derived MSCs	EPA treatment up-regulates the genes involved in adipogenesis as PPAR- γ . (Riera-Heredia et al., 2019)
EPA/DHA	10 μ M of EPA, 50 μ M of DHA, or 100 μ M of EPA + 50 μ M of DHA	24 hours	<i>In vitro</i> Murine 3T3-L1 cell line	EPA and DHA, alone or combined, modulate the adipogenesis of adipocytes via PPAR γ -CIDEc suppression (Martins et al., 2020)
EPA/DHA	10mM of DHA, or 10mM of EPA	24, 72 or 120 hours	<i>In vitro</i> Rat L6 skeletal muscle cells	DHA increases the expression of PGC1- α , regulating metabolism and fat oxidation more effectively than EPA, and reduces the expression of TNF α R (Katsnelson & Coddia, 2020)
EPA / DHA	EPA and DHA at 25,50,100 or 200 μ mol/L	24 hours	<i>In vitro</i> 3T3-L1 preadipocytes	DHA led to an increase of PPAR γ expression and secretes adiponectin at relatively low concentrations (Song et al., 2017)
DHA + ARA	A low-fat diet, High-fat diet, High-fat diet supplemented with ARA+DHA, High-fat diet supplemented with eHC, or High-fat diet supplemented with ARA+DHA+eHC	12 weeks	<i>In vivo</i> Ucp1-2A-Luciferase Knock-In C57BL/6J n=60	ARA + DHA supplementation improves metabolic flexibility and attenuates adipose tissue dysfunction during a high-fat diet, as well as systemic inflammation-reducing IL-6 and TNF- α in adipose tissue. (Mao et al., 2018)
EPA/ DHA/ 9M5	Treatment with 10 μ M of 9M5, 50 μ M of EPA, or 50 μ M of DHA alone or in different combinations.	48 hours	<i>In vitro</i> 3T3-L1 preadipocytes	9M5 increases protein expression of PPAR γ and lipid accumulation during the differentiation process of 3T3-L1 preadipocytes into adipocytes (Lauvai et al., 2019)

ARA: arachidonic acid; DHA: docosahexaenoic acid; eHC: extensively hydrolyzed casein; EPA: eicosapentaenoic acid; MSCs: mesenchymal stem cells; 9M5: Furan fatty acid 9-(3-methyl-5-pentylfuran-2-yl)-nonanoic acid

Table 4. Effect of Conjugated linoleic acid on PPAR γ and PLIN1 modulation

	Intervention	Duration	Study Design	Outcome	Ref
CLA	7.5g/d of CLA or 8.78 g/d of palm oil as a control	3 weeks	<i>In vivo</i> Holstein cows n=16	CLA decreases mRNA abundance of genes related to fatty acid oxidation and lipolysis, and increases mRNA abundance of genes related to lipogenesis, such as PPAR γ , in the adipose tissue of dairy cows	(Dirandeh et al., 2021)
CLA	Supplementation 5 days/week with 600, 2000 or 3000mg/kg of CLA, or fish oil doses of 600 or 3000mg/kg	4 weeks	<i>In vivo</i> BALB/c mice n=30	Fish oil increases mitochondrial respiration in the liver. High doses of CLA produce steatogenic effects and promote lipid accumulation by downregulating PGC-1 α	(Cristofano et al., 2021)
CLA	A basal diet with 1% of oleic acid, a diet supplemented with 0.5% of CLA and 1% of oleic acid, or a diet supplemented with 1% isomer-mix of CLA	8 weeks	<i>In vivo</i> V-line rabbits n=75	Dietary supplementation of CLA produces a lower fat percentage via PPAR γ regulation	(Abdelatty et al., 2019)
CLA	Placebo sedentary/trained, or CLA sedentary/trained (84% CLA, 12% oleic acid, 3% stearic acid, 0.5% palmitic acid, 0.5% linoleic acid)	6 weeks	<i>In vivo</i> BALB/cAnNHsd mice n=32	CLA does not stimulate mitochondrial biogenesis or PGC-1 α expression	(Barone et al., 2017)
CLA + α -lipipoic acid	CLA at 0, 25, or 50 μ M, α -lipipoic acid at 0, 25, or 50 μ M, or a combination of both.	24 hours	<i>In vitro</i> Murine macrophage RAW264.7 cells	CLA α -lipipoic acid increases the expression of PPAR γ and shows anti-inflammatory activity through ERK1	(Lee et al., 2020)

CLA: Conjugated linoleic acid

Table 5. Effect of different fatty acids on PPAR γ and PLIN1 modulation

Intervention	Duration	Study Design	Outcome	Ref
Sterol ester of α-linolenic acid	Control diet, a high-fat diet rich in cholesterol, or based in plant sterol, or ALA acid or sterol ester of ALA	16 weeks <i>In vivo</i> C57BL/6J mice n=50	Sterol ester of α -linolenic acid stimulates mitochondrial biogenesis by PGC-1 α regulation and reduced oxidative stress in the NAFLD.	(Han et al., 2021)
	0.9mM of oleic acid, 0.1mM of plant sterol, 0.1mM of α -linolenic acid, or 0.1mM sterol ester of ALA	24 hours <i>In vitro</i> HepG2 cells		
Stearidonic acid	50 or 200 μ M of Stearidonic acid, EPA, DHA or ALA.	24, 75, and 144 hours <i>In vitro</i> 3T3-L1 preadipocytes	Stearidonic acid can inhibit adipocyte differentiation and reduce lipid accumulation by downregulating PPAR γ	(Li et al., 2017)
Palmitoleic acid (POA)	50 μ M, 100 μ M and 200 μ M of POA	96 hours <i>In vitro</i> Bovine satellite cells (BSC)	All treatment of POA increased the protein expression of PPAR γ and induced transdifferentiation of BSC into adipocytes. After treatment PLIN1 is up-regulated.	(Zhang et al., 2021)
Palmitic acid	Palmitate at 50, 100 or 200 mM	24 hours <i>In vitro</i> HepG2 cells	Palmitate induced lipid accumulation and suppressed lipolysis in HepG2 cells via PPAR γ stimulation	(Zhao et al., 2018)
Palmitic acid/DHA	Normocaloric diet or High-fat diet with rosiglitazone	12 weeks <i>In vivo</i> C57BL/6 mice	Palmitic acid induces M1 polarized macrophage that promotes lipid accumulation in hepatocytes via PPAR γ , and enhances the expression of IL-6 and TNF- α , DHA promotes M2 phenotype.	(Wu et al., 2020)

ALA: α -linolenic acid; BSC: Bovine satellite cells; HEPG2: Human hepatocellular carcinoma cell line



Overall, these results suggest that while SFA and CLA induce dysfunction in AT and regulate negatively adipogenesis and lipid metabolism, n-3PUFAs and some monounsaturated fatty acids can reverse the effects caused by saturated fatty acids and a high-fat diet (Figure 4).

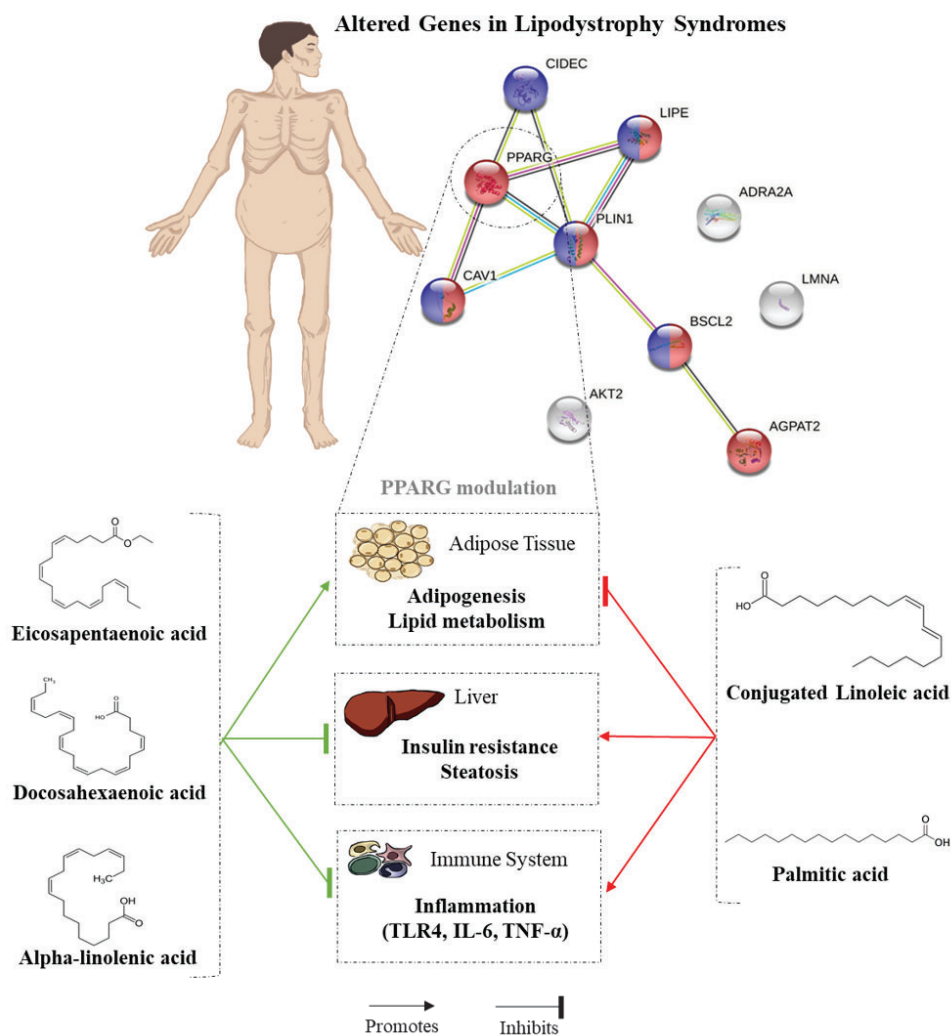


Figure 4. Genetic modulation of PPAR γ through dietary fatty acids



4. Conclusions

Congenital and acquired lipodystrophy share common pathophysiology: adipose tissue dysfunction. An alteration that results in most cases in hypertriglyceridemia, ectopic fat accumulation, insulin resistance, chronic inflammation, and low levels of leptin and adiponectin. However, in this review, we described that the main genes involved in lipodystrophy are partially biologically connected as a group, and some types of congenital and acquired lipodystrophies appears to share a common link in the alteration of certain proteins such as PPAR γ . Which could be considered as a key target in the management of the disease, because it could be modulated through exogenous factors such as diet. In the case of dietary fatty acids, those responsible for positive modulation of PPAR γ , are mainly omega-3 polyunsaturated fatty acids such as EPA, DHA and ALA, while other saturated fatty acids such as palmitic acid, produce a down-regulation leading to the development of typical comorbidities of lipodystrophy such as type 2 diabetes mellitus, NAFLD and cardiomyopathies, worsening the symptomatology of lipodystrophy. However, more clinical studies are required to determine the role of dietary fatty in PPAR- γ modulation to control adipose tissue dysfunction in lipodystrophic patients.

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RESULTADOS



6. RESUMEN GLOBAL DE LOS RESULTADOS

6.1. Artículo científico I. The High-Fat Diet Based on Extra-Virgin Olive Oil Causes Dysbiosis Linked to Colorectal Cancer Prevention

Las dietas altas en grasa inducen disbiosis independientemente de su efecto en el peso corporal

En comparación con el grupo control, todas las dietas produjeron una disminución en la riqueza y la diversidad de la microbiota intestinal, a pesar de que la dieta alta en grasa de aceite de coco no indujo cambios en el peso con respecto al grupo control.

Alteraciones a nivel de filo

Todas las dietas altas en grasa indujeron una disminución significativa del filo Bacteroidetes y Actinobacteria. Solamente el grupo AOVE mostró un incremento en la ratio Firmicutes/Bacteroidetes. También la dieta alta en grasa de AOVE indujo una reducción del filo Proteobacteria ($p < 0.001$) mientras que la dieta alta en grasa de aceite de coco produjo una disminución del filo Verrucomicrobia ($p < 0.01$).

Alteraciones a nivel de género y especie

Una de las alteraciones más relevantes de las dietas altas en grasa con respecto al grupo control fue la reducción de *Lactobacillus* (principalmente *Lactobacillus reuteri*) y el incremento de *Lactococcus* (principalmente *Lactococcus lactis*). Las dietas altas en grasa también incrementaron los géneros *Streptococcus*, *Turicibacter*, *Blautia*, *Clostridium*, *Ruminococcus* y *Anaerostipes* ($p < 0.05$).

Las dietas AOVE y girasol redujeron el género *Enterococcus* (principalmente *Enterococcus gallinarum*) significativamente con respecto al grupo control. Comparando las dietas altas en grasa, la de coco produjo un incremento del género *Staphylococcus* con respecto al grupo AOVE ($p < 0.05$). El grupo AOVE también presentó una disminución significativa de *Pseudomonas* (principalmente *Pseudomonas migulae*). En comparación con las dietas de coco y AOVE, el girasol incrementó el



género *Neisseria* (principalmente *Neisseria mucosa*) ($p < 0.05$), y el género *Sphingomonas*, en comparación con el resto de grupos. Todas las dietas altas en grasa redujeron la abundancia de *Bifidobacterium*, pero solo el AOVE y girasol de forma significativa. Los ratones de los grupos coco y girasol mostraron una reducción significativa de *Akkermansia* (principalmente *Akkermansia muciniphila*) en comparación con el grupo control ($p < 0.05$).

6.2. Capítulo de libro. Antiinflammatory activity exerted by minor compounds found in virgin olive oils

Componentes minoritarios de los aceites de oliva vírgenes

En base a su estructura química los compuestos minoritarios pueden dividirse en distintas clases, entre ellos se encuentran:

- Escualeno (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,20-tetracosahexane), es un hidrocarburo terpenoide presente en altas concentraciones en los aceites de oliva vírgenes. Todas las plantas y animales, incluido el ser humano, son capaces de producir escualeno. Los esteroides vegetales, también llamados fitoesteroides, son derivados biosintéticos del escualeno. En el aceite de oliva, los esteroides están presentes en un rango de 1800-4939 mg/kg.
- Pinoresinol o 1-acetoxipinoresinol, es un lignano presente en las aceitunas y aceite de oliva, y es un metabolito procedente del metabolismo de las plantas. Es un compuesto fenólico que forma un dímero, que posee dos grupos fenólicos en su estructura química. Cada grupo fenólico está formado por un anillo aromático (grupo fenilo o benceno) unido a un grupo hidroxilo (OH).
- Ácido oleanólico, es un triterpeno presente mayoritariamente en las hojas del olivo u *Olea europaea* y en la piel de la aceituna. El contenido de ácido oleanólico en el aceite de oliva virgen oscila entre 8,90 y 112,36 mg/kg, dependiendo del tipo de olivo.
- Ácido maslínico, es un triterpeno pentacíclico hidroxilado, que junto con el ácido oleanólico, representa la fracción triterpénica total encontrada en el aceite de oliva virgen.



- Uvaol, es un dialcohol triterpénico que se ha relacionado con el eritrodiol debido a su similitud en las estructuras químicas. Este compuesto está presente en la piel de las aceitunas y se encuentra en mayores niveles de presencia en el aceite de orujo, una categoría de aceite de oliva que se diferencia del VOO principalmente por el uso de productos químicos para la extracción del aceite.
- Eritrodiol, es compuesto que junto con el uvaol, confirma los principales representantes de los dialcoholes encontrados en el grupo de los triterpenos. La diferencia entre los compuestos es el grupo funcional presente en la posición C-17. La cantidad de uvaol-eritrodiol determina la calidad y la clase de un aceite, y ambos están presentes en mayores concentraciones en el aceite de orujo y en bajas concentraciones en el aceite de oliva virgen.

Propiedades biológicas de los compuestos minoritarios en inflamación

En los últimos diez años se han descrito las actividades antioxidantes, antiaterogénicas y antitumorales que ejercen los compuestos minoritarios de los aceites de oliva vírgenes. Sin embargo, el inicio de varias enfermedades, incluyendo el cáncer, las enfermedades cardiovasculares o las enfermedades neuronales como el Parkinson o el Alzheimer, está directamente relacionado con los procesos de inflamación en las primeras etapas de la enfermedad, así como en las etapas posteriores. En estas etapas, los compuestos minoritarios presentes en los aceites de oliva vírgenes pueden modular los procesos de inflamación.

La inflamación es un proceso complejo en el que intervienen las células inmunitarias. Entre las diferentes células implicadas, los macrófagos desempeñan un papel importante tanto en el inicio como la resolución del proceso inflamatorio. Los macrófagos M1 son responsables de la respuesta citotóxica para proteger al organismo de los patógenos y/o de los procesos de crecimiento celular interrumpidos en el cuerpo. Los macrófagos M2 son responsables del proceso de curación de las heridas y de la recuperación del tejido lesionado.

En relación a la modulación de la polarización de los macrófagos, el escualeno es capaz de potenciar la respuesta antiinflamatoria, inducir la actividad de macrófagos M2 y



promover la cicatrización de heridas. Por otra parte, el pinoresinol y el ácido maslínico han mostrado tener actividad antiinflamatoria mediante la inhibición de la vía de señalización NF- κ B, la reducción de los niveles de IL-6 y la prostaglandina derivada de la ciclooxigenasa 2. La actividad antiinflamatoria de otros triterpenos como el ácido oleanólico, se debe a la capacidad de modular la respuesta de los macrófagos de tipo M1, inhibiendo las señales inflamatorias como el IFN- γ , la IL-4 y la IL-6 y activando la IL-10. Por su parte, la actividad antiinflamatoria del uvaol se ha relacionado con su capacidad para disminuir el TNF- α , mientras que el eritrodilol ha mostrado tener actividad antiinflamatoria y antioxidante mediante la reducción de las especies reactivas de oxígeno y nitrógeno.

Aunque los compuestos menores de los aceites de oliva vírgenes parecen ser prometedores para tratar y controlar los procesos de inflamación en estudios *in vitro* e *in vivo*, es necesario realizar más estudios para determinar los efectos que podrían ejercer en los humanos. Sin embargo, es posible afirmar que los compuestos minoritarios presentes en los aceites de oliva vírgenes son los responsables del efecto beneficioso para la salud de su consumo.

6.3. Artículo científico II. Coconut oil-based high-fat diet develops hypoleptinaemia in an experimental murine model

Efectos de las dietas altas en grasa en la composición corporal

Tras una intervención nutricional de 16 semanas, los ratones alimentados con una dieta alta en grasa de aceite de oliva virgen extra (grupo AOVE) o de aceite de girasol (grupo girasol) tuvieron una ganancia de peso corporal significativa en comparación con los ratones alimentados con la dieta control ($p < 0.05$). Sin embargo, los ratones del grupo de dieta alta en grasa de aceite de coco (grupo coco) no sufrieron una ganancia de peso corporal excesiva, sino que por el contrario mantuvieron un peso similar al del grupo control.

En lo que respecta a la composición corporal, los ratones del grupo girasol tuvieron un incremento relativo de la cantidad de grasa subcutánea y epididimal, mientras que los del grupo AOVE solo sufrieron un incremento en la grasa epididimal.



Alteraciones metabólicas inducidas por las dietas altas en grasa

Al final de la intervención, los efectos sobre las adipoquinas difirieron entre las distintas dietas, mientras que los ratones del grupo AOVE y girasol desarrollaron hiperleptinemia e hipoadiponectinemia, el grupo coco desarrolló hipoleptinemia e hipoadiponectinemia. Ambos marcadores del estado de salud del tejido adiposo, que indicaron que todas las dietas altas en grasa indujeron una disfunción del mismo.

En lo que respecta a la alteración metabólica directa, la dieta alta en grasa de aceite de coco indujo hipertrigliceridemia, mientras que el grupo de AOVE sufrió un ligero incremento de los triglicéridos circulantes.

A pesar de no producir alteraciones en el peso corporal, la dieta de coco, produjo un aumento del tamaño y peso del hígado, que se relacionó a su vez con un incremento de la acumulación de triglicéridos en el mismo.

Desarrollo de inflamación crónica de bajo grado

A pesar de que los efectos de las citoquinas son pleiotrópicos y dependiendo del microambiente y del tejido en el que se encuentren pueden inducir un estado pro o antiinflamatorio, de las citoquinas analizadas en suero, se utilizaron como proinflamatorias la IL-6 y TNF- α , y como antiinflamatoria TGF- β .

La dieta que más incrementó la producción de IL-6 fue la dieta de coco, seguida de la dieta de AOVE. Sin embargo, la única dieta que incrementó de forma significativa la producción de TNF- α fue la de coco ($(p < 0.05)$).

Por el contrario, la dieta que más incrementó la secreción de TGF- β fue la de AOVE mientras que la de coco fue la que indujo un menor incremento.

Influencia de las dietas altas en grasa sobre la actividad termogénica del tejido adiposo

Para determinar la actividad termogénica, se analizó la expresión de UCP1 en el tejido adiposo blanco inguinal y epididimal, y en el tejido adiposo pardo.



La única grasa que indujo un incremento de la expresión de UCP1 tanto en el tejido inguinal como en el pardo fue la de AOVE, mientras que la de aceite de girasol la incrementó solamente en el tejido adiposo pardo.

Al contrario de lo esperado debido a su efecto en el peso corporal, la dieta alta en grasa de coco no produjo un incremento de la actividad termogénica en ninguno de los tejidos analizados.

Implicación de las dietas altas en grasa sobre el proceso de pardización

Se analizó la expresión de las proteínas PGC-1 α y Nrf2 en los tejidos adiposos blanco y pardo, para determinar la capacidad de las distintas dietas para promover la biogénesis mitocondrial e inducir adipogénesis. De todas las dietas, solo la de AOVE incrementó estadísticamente la expresión de PGC-1 α en todos los tejidos. Por otra parte, solo la dieta de girasol incrementó la expresión de Nrf2 en el tejido adiposo inguinal y el pardo, mientras que la de AOVE lo incrementó solo en el tejido adiposo pardo.

6.4. Artículo científico III. PPAR γ as a Link Between Acquired and Congenital Lipodystrophy and its Modulation by Dietary Fatty Acids

Base inmunometabólica de la lipodistrofia

Los síndromes de lipodistrofia son un grupo heterogéneo de enfermedades raras que afectan al tejido adiposo de forma localizada o generalizada. Este tipo de patologías están acompañadas normalmente de alteraciones metabólicas como hipoleptinemia, resistencia a la insulina, esteatosis hepática e hipertrigliceridemia. La lipodistrofia puede ser de carácter congénito o adquirida, esta última, relacionada normalmente con el tratamiento antirretroviral en pacientes con el virus de la inmunodeficiencia humana, y con el tratamiento de inhibidores del control inmunitario típico en pacientes oncológicos.

En los pacientes con lipodistrofia, la patología cursa con alteraciones del sistema inmune como niveles elevados de citoquinas proinflamatorias, un incremento



de los macrófagos M1, un elevado conteo de linfocitos B y una marcada reducción de linfocitos T reguladores. En algunos casos, los pacientes presentan además una gran acumulación de TG en los “lipid droplets” de las células inmunitarias infiltradas en el tejido adiposo como las células Natural Killer. Algunos pacientes muestran en sus biopsias de tejido adiposo lipófagos, que son macrófagos de tipo M1 con una elevada actividad metabólica.

Esta patología se asocia además con cambios en la microbiota intestinal y una elevada permeabilización del intestino, que promueve unos niveles elevados de LPS circulante. Situación que está estrechamente relacionada con la inflamación y disfunción del tejido adiposo, característica de esta patología.

Debido a la disfunción del tejido adiposo, este es incapaz de secretar adipocinas como la leptina y la adiponectina, alterando por lo tanto procesos biológicos como la saciedad, el apetito, el metabolismo glucídico y lipídico. Pero la alteración más relevante es la inducción de resistencia a insulina en distintos tejidos, dando lugar en su conjunto (bajos niveles de leptina y adiponectina, y resistencia a la insulina) a la acumulación ectópica de lípidos, afectando principalmente a tejidos como el hígado y promoviendo la aparición de esteatosis hepática y esteatohepatitis. Alteraciones que a su vez están implicadas en la desregulación del metabolismo y que pueden dar lugar en última instancia al desarrollo de diabetes mellitus tipo 2 y cardiopatías.

Nexo de unión entre la lipodistrofia adquirida y congénita

Los distintos tipos de lipodistrofia congénita se han asociado con determinadas alteraciones en algunos genes como: BSCL2, LIPE, CAV1, LMNA, AKT2, ADRA2A, PPAR γ , AGPAT2, CIDEC y PLIN1. Por otra parte, se ha observado que algunas lipodistrofias adquiridas, debido a la terapia antirretroviral en pacientes con síndrome de inmunodeficiencia adquirida, o a algunos inmunoterápicos basados en la inhibición de PD-L1 dan lugar a la alteración de PPAR γ . Para identificar, si existía relación entre las distintas proteínas implicadas, se utilizó STRING11. El análisis mostró que algunas



proteínas como PPAR γ , CIDEA, CAV1, LIPE, PLIN1, BSCL2 y AGPAT2 tenían una fuerte interacción, mostrando que estaban conectadas biológicamente como un mismo grupo. Este grupo de proteínas, está involucrado principalmente en procesos biológicos como la regulación del metabolismo lipídico y la formación de “lipid droplets”. Los datos mostraron que PPAR γ y PLIN1 eran los que más interacciones tenían con el resto.

Debido a los efectos observados de la dieta alta en grasa con aceite de coco en nuestro estudio, el trabajo de revisión se focalizó en la búsqueda de evidencia sobre el efecto de ácidos grasos de la dieta sobre la modulación de PPAR γ y PLIN1.

Efectos nutrigenómicos en PPAR γ y PLIN inducidos por los lípidos de la dieta

Tras realizar una revisión sistemática exhaustiva que incluía modelos *in vitro*, *in vivo* y ensayos en humanos, se recopilaron aquellos estudios que analizaban el efecto de los ácidos grasos de la dieta de forma individualizada y se excluyeron aquellos que combinaban el tratamiento o suplementación con otros compuestos bioactivos que pudiesen interferir en la propia actividad de los ácidos grasos.

Tras revisar todos los artículos, se concluyó que dependiendo del tipo de ácido graso la modulación en la expresión y actividad de PPAR γ difería. Mientras los ácidos grasos poliinsaturados omega 3 en su conjunto, o de forma aislada como el ácido eicosapentaenoico, docosahexaenoico y α -linolénico eran capaz de promover la adipogénesis, regular el metabolismo lipídico, e incluso prevenir la resistencia a la insulina y esteatosis hepática inducida por una dieta alta en grasa o fármacos a través de la regulación al alza de PPAR γ , los ácidos grasos saturados como el ácido palmítico, y el ácido linoleico conjugado, comúnmente denominado CLA, promovían la acumulación ectópica de triglicéridos, bloqueaban la adipogénesis y causaban resistencia a la insulina.

A pesar de la necesidad de realizar más ensayos en pacientes con lipodistrofia, estos resultados podrían ayudar a focalizar la terapia y manejo de la lipodistrofia tanto congénita como adquirida a través de la modulación de PPAR γ con los ácidos grasos de la dieta.

DISCUSIÓN



7. DISCUSIÓN

Artículo científico I. The High-Fat Diet Based on Extra-Virgin Olive Oil Causes Dysbiosis Linked to Colorectal Cancer Prevention

Las dietas occidentales, caracterizadas por una elevada ingesta de grasas, están estrechamente relacionadas con un mayor riesgo de desarrollar cáncer colorrectal, este proceso se produce principalmente por la alteración de la composición de la microbiota intestinal (Mehta et al., 2017; Moss & Nalankilli, 2017). Todas las dietas altas en grasa producen una disbiosis caracterizada por una reducción significativa de la diversidad y abundancia de la microbiota (Schulz et al., 2014). Entre ellas, reducen las bacterias del género *Bifidobacterium*, implicadas en el mantenimiento de la homeostasis colónica (Gijs den Besten et al., 2013).

La microbiota intestinal de todos los ratones intervenidos con cualquier dieta alta en grasa produjo una disminución de *Lactobacillus reuteri* y un incremento de *Lactococcus lactis*. Ambos son probióticos que tienen un papel importante en la modulación de los procesos inflamatorios. Por su parte, *L. reuteri* es capaz de mejorar la actividad del sistema inmune a través de la reducción del ratio de células Th1/Th2 y sus citoquinas asociadas, inhibiendo de ese modo la inflamación (He et al., 2016). Del mismo modo, *L. lactis* está implicado en la defensa antioxidante e interviene en la modulación de la inflamación (Ballal et al., 2015; Souza et al., 2016).

Con respecto a bacterias que han mostrado desempeñar un papel beneficioso en la salud, la abundancia de *Akkermansia muciniphila*, se ha asociado a la longevidad. La reducción de esta especie se ha encontrado en patologías como colitis ulcerosa, enfermedad de intestino irritable y enfermedad de Crohn (Bian et al., 2019). Algunos aditivos alimenticios como carragenanos están relacionados con la disminución de esta especie (Shang et al., 2017). Por el contrario, algunos compuestos fenólicos como el resveratrol y el ácido cafeico son capaces de restaurar los niveles de la misma y disminuir la inflamación intestinal (Alrafas et al., 2019; Zhang et al., 2016). Nuestros resultados muestran que las dietas altas en grasa de aceite de coco y girasol disminuyen



de forma significativa la abundancia de esta especie bacteriana. Una posible hipótesis para el efecto que ejerce la dieta alta en grasa de AOVE es que sus componentes minoritarios podrían estar involucrados en el restablecimiento de *A. muciniphila*.

La presencia de patógenos oportunistas está estrechamente relacionada con el riesgo de desarrollar cáncer colorrectal. La dieta alta en grasa de AOVE produjo una reducción significativa de *Staphylococcus* spp. y especialmente de *Staphylococcus epidermidis*, que está involucrado en la formación de biofilms colónicos. Esta bacteria está presente en pacientes con colitis y enfermedades autoinmunes (Dinakaran et al., 2019; Sánchez et al., 2013). Otra especie considerada oportunista es *Enterococcus gallinarum*, que es capaz de translocarse al hígado y producir endotoxemia, desencadenando respuestas autoinmunes (Vieira et al., 2018). La abundancia de esta especie suele tratarse con antibiótico para prevenir la mortalidad asociada a su sobrecrecimiento y translocación, las dietas de AOVE y girasol disminuyeron su abundancia de forma significativa, pudiendo actuar como inmunomoduladores (de la Visitación et al., 2019).

Otro filo relacionado con un estado proinflamatorio, mayor generación de especies reactivas de oxígeno y con el desarrollo de patologías del colon como colitis y cáncer colorrectal es el filo Proteobacteria (Bäumler & Sperandio, 2016; Carvalho et al., 2012; Mukhopadhyaya et al., 2012). La dieta alta en grasa de girasol incrementó de forma significativa los géneros *Neisseria* y *Sphingomonas*, ambos considerados oportunistas (Awdisho & Zulfikar, 2016). De hecho, el incremento de su abundancia está relacionado con un ambiente colónico proinflamatorio, una elevada actividad de células Natural Killer y la colitis asociada a cáncer (El Aidy et al., 2014; Richard et al., 2018). Por el contrario, la dieta alta en grasa de AOVE produjo una disminución de ambos géneros, así como de la totalidad del filo Proteobacteria. Algunos autores han descrito que el AOVE ejerce efectos protectores a través de la inmunomodulación en ratones y humanos con colitis ulcerosa, que podría deberse no solo a la modulación de la inflamación a través de las vías MAPK y NFkB sino también a la modulación de la microbiota como muestran nuestros resultados (Morvaridi et al., 2020; Sánchez-Fidalgo et al., 2013; Sánchez-Fidalgo et al., 2010).



Otras bacterias patógenicas son las del género *Prevotella* y *Bacteroides*, cuya abundancia fue más alta en los ratones del grupo coco. Ambas tienen efectos negativos en los procesos patológicos del colon ya que incrementan el estado inflamatorio, mientras que la reducción de su abundancia se ha asociado a una disminución del mismo (Chen et al., 2019; Praengam et al., 2017). De este modo, aunque todas las dietas altas en grasa dan lugar a disbiosis, una dieta alta en grasa de aceite de coco podría incrementar el riesgo de cáncer colorrectal mediante la estimulación de géneros patógenicos, mientras que las dietas altas en grasa de AOVE y girasol podrían tener efectos protectores. Algunas investigaciones con compuestos fenólicos y diferentes aceites vegetales han mostrado que la reducción del filo Bacteroidetes podría mejorar la inflamación intestinal, lo que podría justificar los efectos que tiene la ingesta de una dieta alta en grasa de AOVE (A.-I. Li et al., 2020; Zhao et al., 2019).

Artículo científico II. Coconut oil-based high-fat diet develops hypoleptinaemia in an experimental murine model

El tejido adiposo desempeña un rol fundamental en la regulación inmunometabólica del organismo debido a su relación directa con la dieta. El exceso de calorías, así como el exceso de grasa en la dieta, da lugar a un exceso en la acumulación de grasa en el tejido adiposo. Esta situación conduce a un proceso cíclico en el que aumenta la infiltración de monocitos en el tejido, predominan los macrófagos M1, se incrementa la secreción de citoquinas proinflamatorias y conduce a un ambiente proinflamatorio, que, si se mantiene en el tiempo, culmina en la disfunción del tejido adiposo. Esta disfunción conduce a su vez a una alteración en el metabolismo y al mantenimiento de la inflamación sistémica crónica.

Dependiendo del tipo de fuente de grasa de la dieta, estos efectos sobre el tejido adiposo difieren tal y como se expone a continuación.

En lo que respecta al peso corporal, las dietas altas en grasa de AOVE y girasol, produjeron una ganancia de peso significativa. Sin embargo, a pesar de haber sido intervenidos todos los ratones del grupo intervención con dietas altas en grasa durante



16 semanas, los ratones del grupo coco, no sufrieron un incremento de peso exacerbado, sino que incrementaron su peso igual que si hubiesen sido alimentados con la dieta control. Este hallazgo, podría estar en consonancia con los efectos beneficiosos que se han promocionado del consumo de aceite de coco en fuentes no científicas (Clegg, 2017). Estos efectos se han atribuido a su elevada composición en triglicéridos de cadena media (Coleman et al., 2016; Tsuji et al., 2001). Sin embargo, estudios recientes han mostrado que el ácido láurico presente en el aceite de coco sólo se metaboliza un 30% como ácido graso de cadena media, el 70% restante se metaboliza como un ácido graso de cadena larga, mediante el transporte en quilomicrones, por lo que los efectos del aceite de coco en el peso corporal no se justifican por el destino metabólico de sus ácidos grasos tras la ingesta (Dayrit, 2015; Kinsella et al., 2017).

En lo que respecta a la composición corporal, tanto los ratones del grupo AOVE como girasol, sufrieron hipertrofia en el tejido adiposo blanco epididimal, mientras que solo la dieta alta en grasa de girasol produjo esto en el tejido blanco subcutáneo.

Estas modificaciones en el tejido adiposo, dieron lugar a una alteración en la secreción de adipoquinas como la leptina y adiponectina. La leptina está implicada en la regulación de la saciedad mediante su actividad en los centros hipotalámicos y en la sensibilidad a la insulina mediante su acción en los tejidos periféricos (Francisco et al., 2018). Cuando se produce un incremento exacerbado de grasa corporal como ocurre en patologías como la obesidad, los niveles de leptina se incrementan de modo exacerbado dando lugar a hiperleptinemia, que suele estar acompañada de resistencia a la leptina en los tejidos periféricos. Con respecto a la adiponectina, está íntimamente relacionada con la sensibilidad a la insulina, siendo la hipoadiponectinemia uno de los factores implicados en la resistencia a la insulina en los tejidos periféricos (Funcke & Scherer, 2019; Straub & Scherer, 2019). Ambas situaciones, la hiperleptinemia e hipoadiponectinemia, producen una alteración en el metabolismo glucídico y lipídico y dan lugar a la acumulación ectópica de grasa, en tejidos como el hígado, el páncreas y el músculo esquelético. Los ratones del grupo de AOVE y girasol, desarrollaron hiperleptinemia e hipoadiponectinemia al final de la intervención, acorde a la ganancia de peso corporal que mostraron.



A pesar de no inducir ningún efecto en la ganancia de peso corporal, la dieta alta en grasa de aceite de coco, produjo hipoleptinemia e hipoadiponectinemia, reflejando que, aunque estaban aparentemente “delgados”, los ratones del grupo de coco tenían una disfunción en el tejido adiposo diferente a la observada en los del grupo AOVE y girasol. La hipoleptinemia es un marcador común de algunos tipos de lipodistrofia, patología que se caracteriza por una pérdida de masa grasa localizada o generalizada, y que conlleva un estado de inflamación crónica de bajo grado. Al igual que en la obesidad, en el tejido adiposo lipodistrófico predominan los macrófagos M1, hay una elevada secreción de citoquinas proinflamatorias, se produce resistencia a la insulina y se promueve la acumulación ectópica de grasa, pero, ambas patologías difieren en los niveles circulantes de leptina (Berger et al., 2017; Vegiopoulos et al., 2017).

Otros marcadores analizados fueron los triglicéridos en suero y en hígado, indicadores del estado de metabolismo lipídico. Al contrario de lo esperado tras los efectos observados en el peso corporal, los ratones del grupo coco desarrollaron hipertrigliceridemia, y fueron los que mayor acumulación de triglicéridos hepáticos mostraron, junto con un mayor ratio de peso de hígado/peso corporal, sugiriendo que el aceite de coco en una dieta alta en grasa podría producir esteatosis hepática.

Estos hallazgos son característicos de la lipodistrofia, en la que debido a la incapacidad de almacenar lípidos en el tejido adiposo, se produce la acumulación ectópica en tejidos como el hígado. Una posible explicación para entender el mecanismo por el que la dieta alta en grasa de aceite de coco produce este efecto, es a través de la proteína Perilipin 1 (PLIN1). PLIN1 pertenece a un grupo de proteínas implicadas en el almacenamiento de triglicéridos en los “lipid droplets” y en la regulación de la lipólisis a través de su función en la regulación de la lipasa sensible a hormonas (HSL) y la lipasa de triglicéridos adiposa (ATGL). La pérdida de la función de este gen, da lugar a lipodistrofia parcial, caracterizada por hipoadiponectinemia, hipertrigliceridemia y esteatosis hepática (Knebel et al., 2020). Algunos estudios, han analizado el efecto de ácidos grasos como el ácido linoleico conjugado (CLA) en el metabolismo de los adipocitos, observando que su ingesta produce una disminución de



la grasa corporal relacionada con una disminución en la expresión de PLIN1 y un incremento en la expresión de TNF- α en el tejido adiposo (Green et al., 2004; House et al., 2005). De hecho, la ingesta de CLA se ha asociado con la promoción de apoptosis en el tejido adiposo y el desarrollo de lipodistrofia en ratones (Tsuboyama-Kasaoka et al., 2000). En lo que respecta a la composición del aceite de coco utilizado en la intervención, se compone de un 10% de ácido palmítico y un 19.3% de ácido mirístico. A pesar de que el ácido mirístico *per se* no tiene efectos lipotóxicos, cuando se combina con ácido palmítico incrementa su capacidad natural de producir lipoapoptosis y está relacionado con el desarrollo de lipodistrofia (Martínez et al., 2015).

En lo que respecta a las citoquinas proinflamatorias analizadas IL-6 y TNF- α , los ratones del grupo coco fueron los que mayores niveles séricos presentaron de ambas tras la intervención nutricional, mientras que los del grupo AOVE mostraron los niveles séricos más altos de la citoquina antiinflamatoria TGF- β . Los resultados sugieren que una dieta alta en grasa con aceite de coco puede promover inflamación crónica de bajo grado. En línea con nuestros resultados, se ha observado que los niveles elevados de IL-6 promueven la lipólisis en el tejido adiposo blanco, promoviendo la pérdida de peso corporal en pacientes de cáncer (Han et al., 2018). Del mismo modo, el TNF- α induce la producción de citoquinas inflamatorias y promueve la lipólisis en los adipocitos (Langin & Arner, 2006). Como resultado, los ácidos grasos libres, especialmente los ácidos grasos saturados, se liberan localmente en el tejido adiposo y activan el receptor TLR4 en los macrófagos, lo que conduce a la activación de la vía NF- κ B y a una mayor expresión de citoquinas proinflamatorias (Korbecki & Bajdak-Rusinek, 2019; H. Zhou et al., 2020). Nuestros resultados, están en consonancia con los obtenidos por Veras y colaboradores (Veras et al., 2021), que observaron que la suplementación con aceite de coco daba lugar a alteraciones metabólicas e inflamatorias a través de la activación de TLR4.

En lo que respecta a las alteraciones intrínsecas del tejido adiposo, se analizaron distintas proteínas implicadas en la actividad termogénica, la adipogénesis y la biogénesis mitocondrial en el tejido adiposo blanco inguinal, epididimal y pardo. Los resultados mostraron que los ratones del grupo AOVE tenían una mayor expresión



de UCP1 en el tejido adiposo inguinal y pardo, similar a los resultados obtenidos por Oi-Kano y colaboradores (Oi-Kano et al., 2007). En su estudio, encontraron que tras la ingesta de AOVE, se incrementaba el catabolismo lipídico y la termogénesis en el tejido adiposo pardo (a través de UCP1), sugiriendo que la oleuropeína era la principal responsable de esta acción. Del mismo modo, otro estudio ha observado que el aceite de oliva incrementa la expresión del mRNA de UCP1 en el tejido adiposo pardo de ratas (Rodríguez et al., 2002). Apoyando nuestros resultados, un ensayo clínico con una intervención dietética de AOVE ha mostrado incrementar la actividad termogénica del tejido adiposo pardo, así como un incremento en los niveles de leptina en humanos (Monfort-Pires et al., 2020). En conjunto estos resultados sugieren que el AOVE es capaz de incrementar la actividad termogénica del tejido adiposo pardo y beige, mientras que el mecanismo por el que el coco controla la ganancia de peso corporal es independiente de la inducción de termogénesis.

Con respecto al proceso de pardización (aparición de adipocitos beige en el tejido adiposo blanco), la expresión de las proteínas analizadas PGC-1 α y Nrf2 fue diferente dependiendo de la dieta y el tipo de tejido analizado. La dieta de AOVE, incremento de forma significativa la expresión de PGC-1 α en todos los tejidos. Este efecto, se ha observado en otros estudios en los que han analizado el papel de compuestos de origen vegetal, así como compuestos fenólicos (presentes de forma notable en el AOVE), observando que promueven la adipogénesis de adipocitos marrones y beige mediante la activación de PGC-1 α (Okla et al., 2017; Silvester et al., 2019; Suchacki & Stimson, 2021; Zhang et al., 2014). Nrf2, que ha mostrado tener un papel crítico en la biogénesis mitocondrial y el control en la capacidad de expansión del tejido adiposo, también estaba incrementada en el tejido adiposo pardo de los ratones del grupo AOVE.

El conjunto global de los resultados sugiere que la dieta alta en grasa de AOVE induce un incremento de peso, pero no produce alteraciones inmunometabólicas, de hecho, es probable que debido a los compuestos minoritarios presentes en el AOVE, se contrarresten los efectos producidos como consecuencia de la dieta alta en grasa. Así



pues, aunque el AOVE es saludable y debe utilizarse preferentemente en el contexto de una dieta equilibrada, debe evitarse el uso desmesurado de cualquier grasa.

Por el contrario, la dieta alta en grasa de aceite de coco, a pesar de ser capaz de controlar la ganancia de peso inducida por una dieta alta en grasa, produce alteraciones en el metabolismo que se asemejan al síndrome de lipodistrofia e induce un estado de inflamación crónica que puede aumentar el riesgo de padecer otro tipo de patologías como cáncer.

CONCLUSIONES



8. CONCLUSIONES

Tras analizar los resultados obtenidos, se han alcanzado las siguientes conclusiones:

Primera conclusión

En el contexto de una dieta alta en grasa, todos los aceites comestibles de origen vegetal estudiados, independientemente de su composición en ácidos grasos, producen disbiosis intestinal.

Segunda conclusión

A pesar de ello, las disbiosis producidas por estas dietas tienen características diferenciales, de tal forma que, la dieta alta en grasa de aceite de oliva virgen extra produce un microambiente intestinal antiinflamatorio, mientras que, la dieta alta en aceite de coco, produciría un microambiente intestinal proinflamatorio.

Tercera conclusión

La dieta alta en grasa en aceite de oliva virgen extra o aceite de girasol incrementa la actividad termogénica del tejido adiposo pardo interescapular. Por el contrario, la dieta alta en grasa de aceite de coco no promueve la termogénesis en el tejido adiposo pardo interescapular.

Cuarta conclusión

La dieta alta en grasa en aceite de oliva virgen extra incrementa la capacidad de pardización del tejido adiposo blanco visceral y subcutáneo. Por el contrario, la dieta alta en grasa de aceite en girasol o aceite de coco no promueve la pardización del tejido adiposo blanco.

Quinta conclusión

La dieta alta en grasa de aceite de coco promueve un estado proinflamatorio caracterizado por un aumento de marcadores inflamatorios en suero mientras que la dieta alta en grasa de aceite de oliva virgen extra o aceite de girasol promueven un estado antiinflamatorio.



Sexta conclusión

La dieta alta en grasa de aceite de oliva virgen extra o aceite de girasol producen hiperleptinemia e hipoadiponectinemia. A nivel metabólico, la dieta alta en grasa de aceite de girasol o aceite de coco promueven la acumulación hepática de triglicéridos. Además, la dieta alta en grasa de aceite de coco produce hipoleptinemia, hipoadiponectinemia e hipertrigliceridemia alteraciones que de forma característica aparecen en el síndrome de lipodistrofia.

CONCLUSIONS



8. CONCLUSIONS

Based on the analysis of the results obtained, the following conclusions have been reached:

First conclusion

Within the context of a high-fat diet, all vegetable edible oils studied, independently of their fatty acid composition, promote intestinal dysbiosis.

Second conclusion

However, dysbiosis produced by these diets have differential characteristics, such that the extra virgin olive oil high-fat diet produces an anti-inflammatory gut microenvironment, while the coconut oil high-fat diet produces a pro-inflammatory gut microenvironment.

Third conclusion

The high-fat diet based in extra virgin olive oil or sunflower oil increases the thermogenic activity of interscapular brown adipose tissue. In contrast, the high-fat diet based in coconut oil does not promote thermogenesis in interscapular brown adipose tissue.

Fourth conclusion

The extra virgin olive oil high-fat diet increases the browning capacity of visceral and subcutaneous white adipose tissue. In contrast, a diet high-fat diet based in sunflower oil or coconut oil do not promote the browning of white adipose tissue.

Fifth conclusion

The coconut oil high-fat diet promotes a pro-inflammatory state characterised by an increase in serum inflammatory markers while the high-fat diet based in extra virgin olive oil or sunflower oil promotes an anti-inflammatory state.



Sixth conclusion

The high-fat diet based in extra virgin olive oil or sunflower oil leads to hyperleptinaemia and hypoadiponectinaemia. The high-fat diet based in sunflower oil or coconut oil promotes hepatic accumulation of triglycerides at metabolic level. In addition, a coconut oil high-fat diet leads to hypoleptinaemia, hypoadiponectinaemia and hypertriglyceridaemia, which are characteristic of lipodystrophy syndrome

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MÉRITOS

INVESTIGADORES



10. MÉRITOS INVESTIGADORES

10.1. Estancias de investigación internacionales

Entidad de realización: Liverpool John Moores University

Tipo de entidad: Universidad

Facultad, Instituto, Centro: Faculty of Science, Sport and Exercise Sciences

Ciudad entidad realización: Liverpool

Fecha de inicio-fin: 25/03/2019 – 27/06/2019

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10.2. Otras aportaciones científicas

Naturally Lignan-Rich Foods: A Dietary Tool for Health Promotion?

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Review

Naturally Lignan-Rich Foods: A Dietary Tool for Health Promotion?

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Abstract: Dietary guidelines universally advise adherence to plant-based diets. Plant-based foods confer considerable health benefits, partly attributable to their abundant micronutrient (e.g., polyphenol) content. Interest in polyphenols is largely focused on the contribution of their antioxidant activity to the prevention of various disorders, including cardiovascular disease and cancer. Polyphenols are classified into groups, such as stilbenes, flavonoids, phenolic acids, lignans and others. Lignans, which possess a steroid-like chemical structure and are defined as phytoestrogens, are of particular interest to researchers. Traditionally, health benefits attributed to lignans have included a lowered risk of heart disease, menopausal symptoms, osteoporosis and breast cancer. However, the intake of naturally lignan-rich foods varies with the type of diet. Consequently, based on the latest humans' findings and gathered information on lignan-rich foods collected from Phenol Explorer database this review focuses on the potential health benefits attributable to the consumption of different diets containing naturally lignan-rich foods. Current evidence highlight the bioactive properties of lignans as human health-promoting molecules. Thus, dietary intake of lignan-rich foods could be a useful way to bolster the prevention of chronic illness, such as certain types of cancers and cardiovascular disease.

Keywords: lignans; diet; antioxidants; health promotion; chronic diseases

1. Introduction

Polyphenol-rich diets are suggested to possess health benefits. Polyphenols are micronutrients found in plants, and include flavonoids, stilbenes, phenolic acids, lignans and others [1]. They are secondary plant metabolites implicated in protection against pathogens and ultraviolet radiation [2]. Given their diverse chemical structures, different polyphenol classes likely possess differing health benefits [3]. It is therefore important to elucidate the specific potential benefits of each polyphenolic compound. Significant interest has been elicited by lignans, due to their steroid-analogous chemical structure. Accordingly, they are considered to be phytoestrogens. Lignans are bioactive compounds exhibiting various biological properties, including anti-inflammatory, antioxidant and antitumor

activities [4]. Additionally, some epidemiological studies have proposed that lignans decrease the risk of cardiovascular disease, but their effects on other chronic diseases (e.g., breast cancer) remain controversial [5].

Lignans are found in relatively low concentrations in various seeds, grains, fruits and vegetables, and in higher concentrations in sesame and flax seeds [6]. Therefore, the level of lignan ingestion—and, thus, lignan bioavailability, depends on the type of diet consumed [7,8] and can be highly variable. The present review attempts to describe the potential beneficial effects of lignan intake on human chronic disease, depending on the dietary source.

2. Biosynthesis, Classification and Presence of Lignans in Foods

Lignans are a type of secondary plant metabolite exhibiting diverse structures [9]. Plants derive a complex array of secondary metabolites from only a handful of relatively simple propenyl phenols [10]. Biosynthesis of lignans is characterized by a remarkable increase in molecular complexity [10].

Lignans share common biosynthetic pathways, consist of two propyl-benzene units coupled by a β,β' -bond [11], and thus belong to the group of diphenolic compounds [12].

Lignans may be organized into eight structural subgroups (according to the manner in which oxygen is incorporated and the pattern of cyclization): Dibenzylbutyrolactol, dibenzocyclooctadiene, dibenzylbutyrolactone, dibenzylbutane, aryl-naphthalene, aryltetralin, furan and furofuran (Figure 1). Each subgroup can be further subdivided according to lignan molecule oxidation level and identities of non-propyl aromatic rings present on side chains [13,14].

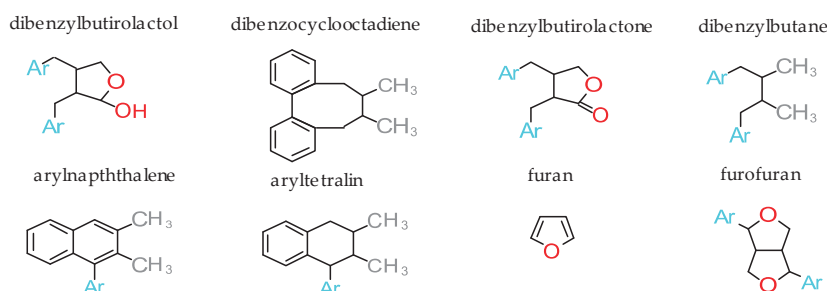


Figure 1. Structural subgroups of lignans (Ar=Aryl).

Of the eight lignan subclasses, synthesis of furofurans—which exhibit a 2,6-diaryl-3,7-dioxabicyclooctane skeleton—is initiated by the enantioselective dimerization of two coniferyl alcohol units derived from the shikimate biosynthetic pathway (Figure 2) [14]. To date, 53 species of furofuran lignans have been reported in 41 genera of 27 plant families, including Thymelaeaceae, Styracaceae, Scrophulariaceae, Saururaceae, Rutaceae, Rhizophoraceae, Piperaceae, Pedaliaceae, Orobanchaceae, Myristicaceae, Magnoliaceae, Lauraceae, Lamiaceae, Geraniaceae, Dioscoreaceae, Cyperaceae, Cupressaceae, Compositae, Combretaceae, Cactaceae, Aristolochiaceae, Arecaceae, Araliaceae, Aquifoliaceae, Apocynaceae, Acoraceae and Acanthaceae. Furofuran lignans are present in the bark, bulbs, leaves, seeds, stems and roots of these plants [14].

However, depending on the enzyme that catalyzes modification of the precursor metabolite, a variety of lignans can be synthesized (Figure 2). The major lignans—which possess numerous pharmacological properties—are artigenin, enterodiol, enterolactone, sesamin, syringaresinol, medioresinol, (−)-matairesinol, (−)-secoisolariciresinol, (+)-lariciresinol and (+)-pinoresinol, among others [15].

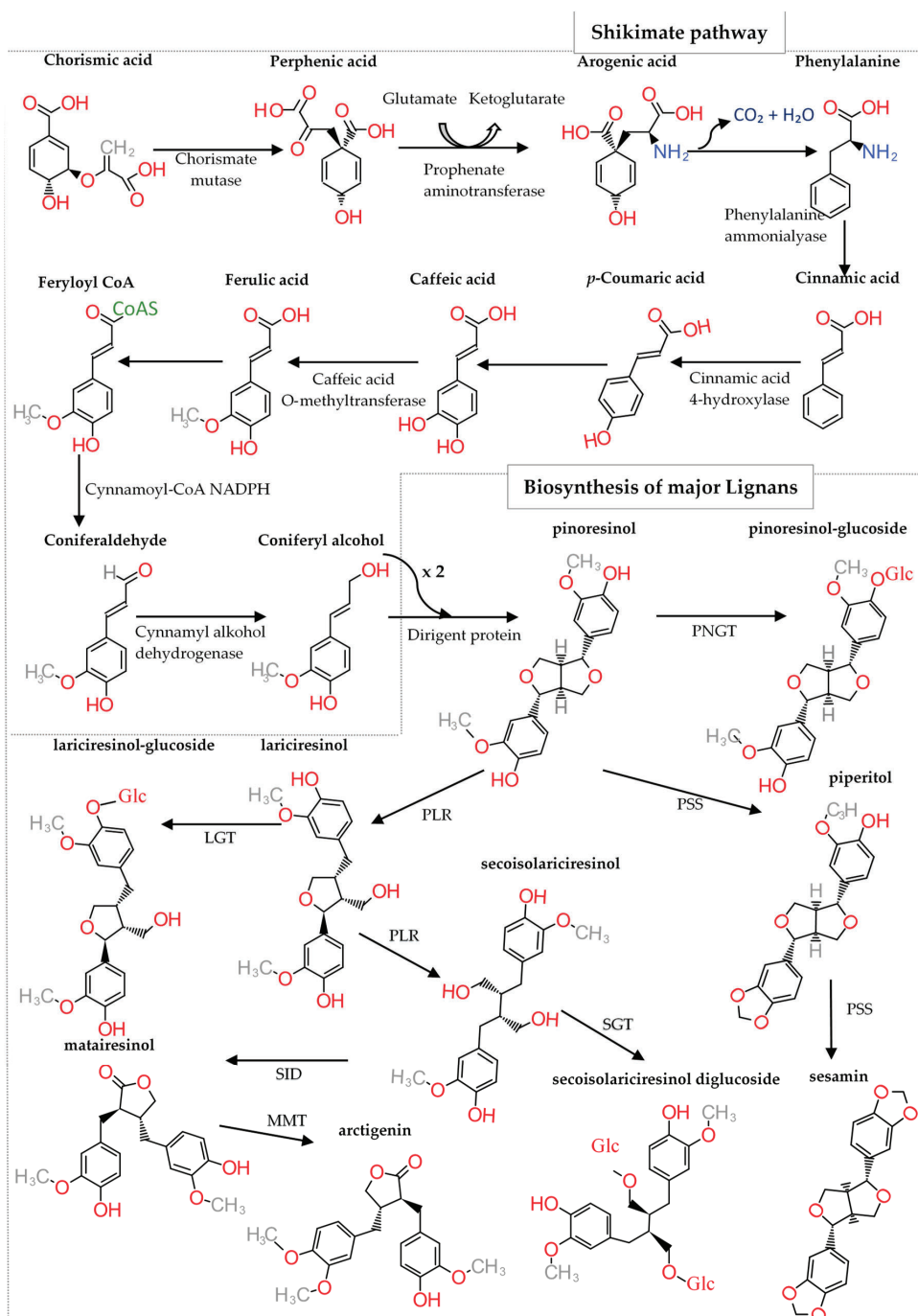


Figure 2. Biosynthetic pathway of lignans. NGT (pinoresinol glucosyltransferase), PSS (piperitol/sesamin synthase), PLR (pinoresinol/lariciresinol reductase), LGT (lariciresinol glycosyltransferase), SGT (secoisolariciresinol glycosyltransferase), SID (matairesinol O-methyltransferase), MMT (matairesinol O-methyltransferase), Glc (Glucoside).

Currently, there is a growing interest in the presence of lignans in foodstuffs, given the potentially beneficial bioactive properties of the former (anti-estrogenic, antioxidant and anti-carcinogenic activities) [16]. The chief sources of dietary lignans are various vegetables and fruits, legumes, whole grain cereals and oilseeds [16,17]. Among edible plant components, the most concentrated lignan sources are sesame and flax seeds (Tables 1 and 2) [6]. Specifically, flax seeds contain approximately 294.21 mg/100 g lignan, at present the maximal known content of any foodstuff. Sesame seeds exhibit the second-highest lignan concentration, with sesaminol as the major constituent, at 538.08 mg/100 g [6]. Flaxseed and cashew nuts are also relatively rich in lignans (containing 257.6 and 56.33 mg/100 g, respectively) [6].

Table 1. Lignan content of sesame seed (mg/100g food). Data collected from phenol explorer [18].

Seeds	HMA	HSE	OXO	ARC	CYC	CON	DIM
Sesame seed	7.2	0.01	0.7	0.01	1.77	0.75	0.39
	ISO	LAR	LAS	MAT	MED	NOR	SEC
	1.61	10.37	0.08	29.79	4.15	0.08	0.1
	SECS	SES	SEI	SEN	SYR	TOD	Total
	0.01	538.08	102.86	133.94	0.2	2.47	834.57

Lignans: 7-Hydroxymatairesinol (HMA), 7-Hydroxysecoisolaricresinol (HSE), 7-Oxomatairesinol (OXO), Arctigenin (ARC), Conidendrin (CON), Cyclolaricresinol (CYC), Dimethylmatairesinol (DIM), Isohydroxymatairesinol (IHM), Isolaricresinol (ISO), Laricresinol (LAR), Laricresinol-sesquigignan (LAS), Matairesinol (MAT), Medioresinol (MED), Nortrachelogenin (NOR), Secoisolaricresinol (SEC), Secoisolaricresinol-sesquigignan (SECS), Sesamin (SES), Sesaminol (SEI), Sesamol (SEN), Syringaresinol (SYR), Todolactol A (TOD).

Table 2. Lignan content of seeds (mg/100g food) [18].

	LAR	MAT	MED	SEC	SYR	Total
Other Seeds						
Flaxseed	11.46	6.68	-	257.6	-	257.6
Sunflower seed	0.67	0.67	-	0.18	-	1.52
Nuts						
Almond	0.03	3×10^{-4}	-	0.07	-	0.10
Brazil nut	-	0.01	-	0.77	-	0.78
Cashew nut	49.6	2.5×10^{-3}	-	6.73	-	56.33
Chesnut	7.8×10^{-3}	8.42×10^{-3}	-	0.2	-	0.21
Hazelnut	0.01	3.3×10^{-3}	-	0.05	-	0.06
Peanut	4.1	2.5×10^{-3}	-	2.7	-	6.8
Pecan nut	8.4×10^{-3}	3.15×10^{-3}	-	0.01	-	0.02
Pistachio	0.12	1×10^{-4}	-	0.04	-	0.16
Walnut	7.2×10^{-3}	3.8×10^{-3}	-	0.12	-	0.13
Pulses-Beans						
Common bean white	0.12	1×10^{-3}	-	0.08	8×10^{-3}	0.2
Broad bean seed whole	-	8.9×10^{-4}	-	0.09	-	0.09
Mung bean	-	-	-	0.18	-	0.18
Soy and soy products						
Soy paste, miso	0.02	3.6×10^{-3}	-	0.01	-	0.03
Soy flour	-	7.5×10^{-3}	-	0.3	-	0.3
Soy tempe	0.01	5×10^{-4}	-	0.01	-	0.02
Soy tofu	0.04	7.27×10^{-5}	8.5×10^{-3}	9.91×10^{-3}	0.04	0.09
Soy yogurt	0.01	3×10^{-3}	-	0.02	-	0.03
Soybean edamame	0.07	-	0.02	0.07	0.2	0.3
Soybean sprout	0.03	5×10^{-4}	0.01	0.03	0.05	0.12

Regarding cereal grains (Table 3), lignans are largely concentrated in their outer layers [19,20]. In cereal grains, the highest lignan concentration is found in the fiber-rich outer layers (seed coat

and pericarp), as well as the aleurone layer, whereas the lowest concentration is found in the inner endosperm [21,22].

Table 3. Lignan content of cereals (mg/100g food) [18].

	LAR	MAT	MED	SEC	SYR	Total
Cereal products						
Bread (whole grain flour)	0.05	3.1×10^{-4}	-	8.68×10^{-3}	-	0.05
Bread (refined flour)	0.01	1.23×10^{-3}	-	7.19×10^{-3}	0.04	0.05
Bread, rye, whole grain flour	0.01	0.02	-	0.14	-	0.17
Breakfast cereals, bran	0.01	4.87×10^{-3}	-	0.03	-	0.04
Breakfast cereals, corn	-	1.67×10^{-3}	-	5.5×10^{-3}	-	0.007
Breakfast cereals, muesli	0.14	5.6×10^{-3}	-	0.08	-	0.22
Breakfast cereal, oat	-	0.06	-	0.02	-	0.08
Pasta	-	1.85×10^{-3}	-	2.3×10^{-3}	-	0.004
Pasta Whole Grain	-	1.5×10^{-3}	-	5×10^{-3}	-	0.006
Cereals						
Barley, whole grain flour	0.08	3×10^{-3}	0.01	0.03	0.16	0.28
Buckwheat, whole grain flour	0.36	1×10^{-3}	0.03	0.13	0.24	0.76
Common wheat, germ	-	9×10^{-3}	-	0.02	-	0.02
Common wheat, refined flour	0.18	2.14×10^{-4}	-	0.02	-	0.2
Common wheat, whole grain flour	0.1	9×10^{-4}	0.03	0.02	0.37	0.52
Hard wheat, semolin	-	-	-	2×10^{-3}	-	0.002
Maize, whole grain	0.12	6.55×10^{-5}	-	0.14	0.07	0.33
Oat, whole grain flour	0.18	0.07	0.04	0.01	0.35	0.65
Rye, whole grain flour	0.32	0.01	0.14	0.02	0.97	1.46

Ordering species by lignan content produces the following list: Dhurra < brown rice < red rice < quinoa < millet < corn < amaranth < barley < buckwheat < wild rice < Japanese rice < spelt < oat < triticale < wheat < rye [6]. Regarding vegetables (Table 4), the brassica family may contain between 185 and 2.321 mg /100 g of lignan, mainly pinoresinol. Peppers, French beans, carrots and courgettes also exhibit a relatively high lignan content, ranging from 0.113 to 0.273 mg/100 g. Other foods, such as spinach, white potatoes and mushrooms—contain below 0.1 mg/100 g of lignan. Fruits exhibit a lower lignan content than seeds or vegetables (Tables 5 and 6), ranging from 11.57 mg/100 g for apricots to 0 mg/100 g for banana, with green grapes and kiwi fruit falling somewhere between these extremes [6].

Table 4. Lignan contents of vegetables (mg/100g food) [18].

	LAR	MAT	MED	SEC	SYR	Total
Cabbages						
Broccoli	97.2	2.44×10^{-5}	-	1.31	-	98.51
Brussel sprouts	49.3	4×10^{-5}	-	1.06	-	50.36
Cauliflower	9.31	2.4×10^{-5}	0.02	0.13	0.02	9.48
Collards	0.06	4×10^{-4}	-	5.9×10^{-3}	-	0.06
Green cabbage	0.03	3.5×10^{-5}	-	9.2×10^{-3}	-	0.03
Red cabbage	17.8	4.44×10^{-5}	-	0.3	-	18.1
White cabbage	21.2	-	-	0.31	-	21.51
Kale	59.9	1.2	-	1.9	-	63
Sauerkraut	11.6	-	-	6.7	-	18.3

Table 4. Cont.

	LAR	MAT	MED	SEC	SYR	Total
Fruit vegetables						
Avocado	0.03	7.67×10^{-3}	0.24	0.02	0.44	0.73
Eggplant purple	0.05	-	7×10^{-3}	7.79×10^{-3}	6×10^{-3}	0.07
Black olive	0.03	5.62×10^{-3}	-	5.75×10^{-3}	-	0.04
Green olive	3.9×10^{-3}	3.34×10^{-3}	-	0.02	-	0.02
Green sweet pepper	12.32	-	1×10^{-3}	0.22	4×10^{-3}	12.54
Red sweet pepper	7.97	-	-	0.24	-	8.21
Yellow sweet pepper	0.07	-	-	5.5×10^{-3}	-	0.07
Tomato (Cherry)	0.03	-	3×10^{-3}	0.01	4.5×10^{-3}	0.04
Tomato (Whole)	2.1	8.33×10^{-6}	3.5×10^{-3}	0.05	4.5×10^{-3}	2.15
Gourds						
Cucumber	3.55	-	-	0.25	-	3.8
Pumpkin	0.01	2.5×10^{-5}	-	0.1	-	0.11
Squash	-	-	-	9×10^{-3}	-	0.009
Zucchini	6.4	-	-	0.62	-	7.02
Leaf vegetables						
Arugula	-	2×10^{-4}	-	0.1	-	0.1
Chicory (green)	0.6	1.24×10^{-4}	-	0.57	-	1.17
Lettuce (green)	0.3	2.24×10^{-4}	-	0.18	-	0.48
Spinach	0.06	2.37×10^{-5}	-	4.85×10^{-3}	-	0.06
Broad bean pod	-	-	-	0.02	-	0.02
Pod vegetables						
Green bean	22	-	-	0.67	-	22.67
Pulse vegetables						
Fresh pea	0.05	-	3.5×10^{-3}	7.56×10^{-4}	-	0.0542
Root vegetables						
Carrot	4.5	3.89×10^{-3}	-	3.16	-	7.66
Celeriac	-	3×10^{-5}	-	0.02	-	0.02
Parsnip	-	0.02	-	0.03	-	0.05
Radish	0.01	1.25×10^{-4}	5.5×10^{-3}	6.57×10^{-3}	0.02	0.04
Swede	-	7.43×10^{-5}	-	4.93×10^{-3}	-	0.005
Turnip root	0.1	-	4×10^{-3}	9.83×10^{-3}	0.03	0.14
Shoot vegetables						
Asparagus	0.07	3.97×10^{-3}	4×10^{-3}	0.25	0.05	0.37
Fennel	-	0.01	-	0.05	-	0.06
Stalks vegetables						
Celery stalks	-	-	-	5.99×10^{-3}	-	0.005
Tubers						
Potato	2.8	7.69×10^{-4}	-	0.09	-	2.89
Sweet potato	0.07	0.1	-	0.12	-	0.29

Table 5. Lignan contents of fruits berries (mg/100g food) [18].

	HMA	OXO	CON	CYC	LAR	LAS
Fruit Berries						
Bilberry	-	-	-	6.24×10^{-3}	0.04	0.09
Blackberry	-	-	-	7.96×10^{-3}	0.15	0.15
Blackcurrant	-	-	-	0.01	7.3×10^{-3}	0.01
Cloudberry	-	-	-	-	0.65	0.25
Black grape	-	-	-	-	5.2	-
Green grape	-	-	-	-	1.88	-
Lingonberry	-	-	1.04×10^{-3}	0.03	0.03	0.01
Strawberry	8.55×10^{-4}	4.59×10^{-4}	9.45×10^{-3}	0.01	5.87	0.1
	MAT	MED	SEC	SECS	SYR	Total
Bilberry	-	0.08	0.06	0.01	0.12	0.4
Blackberry	9.07×10^{-4}	0.05	0.1	0.13	0.19	0.77
Blackcurrant	1.47×10^{-3}	0.01	0.09	0.03	-	0.15
Cloudberry	-	0.48	0.05	0.01	0.41	1.85
Black grape	0.11	-	0.09	-	-	5.4
Green grape	0.09	-	0.28	-	-	2.25
Lingonberry	-	0.23	0.37	0.02	0.14	0.83
Strawberry	1.58×10^{-5}	0.03	0.14	0.01	0.03	6.2

Table 6. Lignan contents of fruits (mg/100g food) [18].

	LAR	MAT	MED	SEC	SYR	Total
Fruits Citrus						
Grapefruit	7.13	0.05	-	0.26	-	7.44
Lemon	-	-	-	0.02	-	0.02
Orange	2.4	0.05	9.5×10^{-3}	0.14	0.12	2.71
Tangerine	5.7	0.02	-	0.08	-	5.8
Fruits Drupes						
Apricot	10.5	3.11×10^{-5}	-	1.07	-	11.57
Nectarine	4.1	-	-	0.61	-	4.71
Peach	6	1.71×10^{-4}	-	0.83	-	6.83
Plum	0.31	2.22×10^{-4}	1×10^{-3}	0.09	-	0.4
Fruits-Gourds						
Cantaloupe	1.8×10^{-3}	-	-	4.7×10^{-3}	-	0.006
Melon	4.4	1.05×10^{-5}	-	0.09	-	4.49
Watermelon	0.04	-	1×10^{-3}	0.02	0.02	0.08
Fruits-Pomes						
Apple	0.1	2.71×10^{-5}	-	1.79×10^{-3}	-	0.1
Pear	15.5	4.3×10^{-5}	-	0.06	-	15.56
Fruits-Tropical						
Banana	2.2×10^{-3}	5.45×10^{-5}	-	7.73×10^{-5}	0.01	0.01
Kiwi	1.03	1.93×10^{-3}	4.5×10^{-3}	3.13	4×10^{-3}	4.17
Mango	-	1.06×10^{-3}	-	0.01	-	0.01
Passion fruit	-	-	-	0.02	-	0.02
Papaya	-	2×10^{-3}	-	-	-	0.002
Persimmon	-	-	-	4×10^{-3}	-	0.004
Pineapple	0.2	0.16	2×10^{-3}	0.21	0.09	0.66
Pomegranate	-	9×10^{-3}	-	0.29	-	0.29

The highest lignan content is observed in non-alcoholic beverages, such as tea (0.0392–0.0771 mg/100 g), which also contains other polyphenols (Table 7). Coffee is another

important source of lignans, although concentration varies by type of coffee, ranging from 0.0187 to 0.0313 mg/100 g. Regarding alcoholic beverages, red wine contains an average of 0.080 mg/100 mL, whereas white wine contains only approximately 0.022 mg/100 g [23].

Table 7. Lignan content of beverages (mg/100g drink and mg/100 mL wine) [18].

	ISO	LAR	MAT	SEC	SYR	Total
Alcoholic Beverages						
Red Wine	0.07	7.56×10^{-3}	5.51×10^{-3}	0.04	3.43×10^{-3}	0.12
White Wine	0.03	6.65×10^{-3}	2.68×10^{-3}	7.45×10^{-3}	1.45×10^{-3}	0.04
Dark Beer	-	-	-	0.04	-	0.04
Beer	-	-	-	0.03	-	0.03
Cider	-	-	-	0.04	-	0.04
Scotch whisky	-	-	-	4×10^{-3}	-	0.004
Sherry	-	-	-	0.02	-	0.02
Non-alcoholic Beverages						
Cocoa	-	-	-	0.03	-	0.03
Coffee	-	9×10^{-4}	4×10^{-4}	8.67×10^{-3}	-	0.009
Decaffeinated Coffe	-	1.1×10^{-3}	4.25×10^{-4}	8.35×10^{-3}	-	0.009
Roman camomile	-	-	5×10^{-4}	1×10^{-3}	-	0.001
Lemon juice	-	-	-	2×10^{-3}	-	0.002
Orange juice	-	2×10^{-4}	-	8×10^{-3}	-	0.008
Soy milk	-	6.17×10^{-3}	5×10^{-5}	2.25×10^{-3}	-	0.008
Black Tea	-	2×10^{-4}	2.65×10^{-3}	0.03	-	0.03
Green Tea	-	1×10^{-4}	3.38×10^{-3}	0.03	-	0.03
Oolong Tea	-	-	1.8×10^{-3}	0.02	-	0.02

Furthermore, the chief source of dietary fat in Mediterranean countries—extra virgin olive oil (EVOO)—has garnered much interest regarding its beneficial properties, largely attributable to its polyphenol profile (Table 8). Lignans are the second most abundant polyphenolic class present in EVOO; of these, the most abundant across different EVOO types are pinoresinol (1.17–4.12 mg/ 100 g) and 1-acetoxypinoresinol (0.27–6.69 mg/ 100 g) [7,24,25].

Table 8. Lignan content of oils (mg/100 g food) [18].

Fruit oils	ACE	LAR		MAT		PIN	SEC	Total
Extra virgin Olive Oil	0.66	3.43×10^{-3}		7.5×10^{-5}		0.42	2.5×10^{-4}	1.08
Nut oils								
Peanut, butter	-	8.8×10^{-3}		7.52×10^{-3}		-	0.05	0.06
Other seed oils	EPI	EPL	SES	SEI	SEO	SEN	SEL	Total
Sesame seed oil	192.6	51.97	420.99	305.43	24.92	243.13	55.71	1294.75
Sesame seed black oil	-	-	644.5	226.92	21.55	287.33	43	1223.3

1-Acetoxypinoresinol (ACE), Episesamin (EPI), Episesaminol (EPL), Pinoresinol (PIN), Sesamol (SEO), Sesamolol (SEL).

Thus, given the presence of lignan in many common foodstuffs and beverages, its intake occurs frequently, on a near-daily basis. For example, in a Dutch population, the major dietary sources of lignan were fruits (7%), bread (9%), seeds and nuts (14%), vegetables (24%), and beverages (37%) [6]. Similarly, in a cohort of French women, the major dietary sources of lignan were vegetables and fruits (0.2% from legumes, 0.6% from potatoes, 30% from vegetables, and 35% from fruits), followed by alcoholic beverages (5%), coffee (5%), cereals (7%) and tea (11%) [6,26,27].

3. Bioavailability

Only a handful of studies exist regarding post-consumption lignan bioavailability, including only very limited human pharmacokinetic studies. After ingestion, plant lignans are metabolized by intestinal bacteria, undergoing transformation to mammalian lignans (enterolactones and enterodiols (Figure 3)) prior to absorption [16,28]. This apparently considerably decreases the risk of diverse types of cancer, particularly of the colon, prostate and breast [16,29].

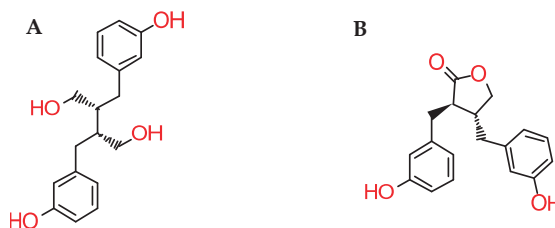


Figure 3. Chemical structure of enterodiol (A) and enterolactone (B).

Many studies demonstrate a positive correlation between plant lignan intake and plasma enterolignan levels [30]. After lignan ingestion, enterolactone and enterodiol are the first lignans to become detectable in human biological fluids [28]. The half-lives of these compounds in plasma are approximately 13 and 5 h, respectively [31], and they remain detectable even up to 8–10 h after plant lignan consumption [32]. Furthermore, their intestinal metabolism into mammalian forms appears indispensable for colonic absorption, and the colonic barrier is capable of conjugating enterolignans [28,33].

The concentration of enterodiol and enterolactone in biological fluids varies significantly by geographic region [28]. A study examining mammalian lignan pharmacokinetics in both men and women after lignan solution intake found that enterodiol and enterolactone, respectively, exhibit absorption half-lives of 3.4 and 8.4 h, reach maximum plasma concentrations of 65 and 42 mmol/L [28], exhibit elimination half-lives of 4.6 and 15.1 h, and exhibit maximum retention times of 23.9 and 43.2 h [28,34]. Thus, while enterolactone is more rapidly absorbed than enterodiol, the former attains a lower maximum plasma concentration [28].

During lignan metabolism, the initial (cytochrome P450-mediated) step involves conjugation to glucuronic acid and sulfate, followed by enterohepatic recirculation [35]. Chaojie et al. (2013) that glucuronidation of flax seed lignans significantly involves liver and intestinal microsomes [36]. Some studies demonstrate that flax seed-derived lignan metabolites distribute mainly to the intestine (largely to the caecum), kidneys, uterus, prostate and liver [37]. Of these locations, the highest concentration of lignan metabolites is observed in the liver [37].

Human breast cyst, prostatic, and seminal fluid (as well as prostate tissue) lignan concentration has been determined [38,39]. As in circulation, the common mammary form of lignan is enterolignan, while urinary forms are essentially monoglucuronides [28]. Furthermore, inter-individual variations in gut microbiota and hepatic enzymes may modulate mammalian lignan metabolism and bioactivity [33].

Moreover, lignan bioavailability also depends on diet. For example, diets rich in flax seed increase production of gut microbiota-derived enterolignans in a murine model, and lead to high tissue and plasma concentrations of sulfate and glucuronide conjugates (the major flax-derived lignan metabolites) [8,40].

Other studies have demonstrated that plant lignans, such as sesamin are quickly absorbed, apparently from the small intestine and become detectable in systemic circulation within a few hours after ingestion [22,41]. For example, lignans have been observed in porcine plasma 3 h after cereal intake [42]. On the one hand, it has been empirically demonstrated that plant lignans are rapidly absorbed from the small intestine after intake of a diet rich in cereals [22]. On the other hand,

various factors—e.g., the use of oral antibiotics and inter-individual variations in gut microflora, as well as diet—impact lignan pharmacokinetics [43]. For example, seed maturation state can alter oral lignan bioavailability [44].

4. Lignan Content of Various Regional Diets

Dietary lignan consumption varies mainly with geographic location, but diet patterns are also subject to cultural and ethnic group influences.

4.1. Mediterranean Diet

The traditional Mediterranean diet is predominantly plant-based, characterized by a low intake of sweets; low meat products and red meat; a moderate intake of fish, poultry and fermented dairy products; a high intake of unprocessed cereals, legumes, nuts, fruits and vegetables [45]; the use of EVOO as the principal source of added fat; and moderate consumption of red wine [45]. Health benefits of this diet are essentially attributable to increased consumption of fiber and bioactive compounds (including antioxidants and functional fatty acids and lipids), as well as to a low intake of saturated fats [45,46].

Lignan sources in the diet of a Mediterranean population included garlic, onions, vegetables, including leafy greens, grains and seasonal fruits, including citrus, with each accounting for diverse proportions (11–70%) and subtypes of total polyphenols consumed [47].

Indeed, many typical Mediterranean diet foods (e.g., cereals) exhibit a high concentration of both lignans and other phenolic compounds [48].

Recently, the role of whole grain cereal intake in chronic disease prevention has been evaluated. Numerous studies propose a connection between lignan intake—as part of a wholegrain-based diet—and decreased incidence of chronic diseases, including cardiovascular disease, cancer and diabetes [5].

Thus, the major dietary lignan sources in the Mediterranean diet are vegetables and fruits, legumes, wholegrain cereals and oilseeds [3]. Additionally, another component of the Mediterranean diet, the chestnut, represents an excellent source of calcium, antioxidants and phenolic compounds [16,49]. Furthermore, EVOO consumption is an essential part of the Mediterranean diet. In fact, regular EVOO consumption is associated with a lower incidence of atherosclerosis, cardiovascular disease and some types of cancer [50–52]. This effect may be attributable to the high concentrations of (+)-1-acetoxypinoresinol and (+)-pinoresinol present in EVOO [53,54].

4.2. Northern Hemisphere Diet

This diet is observed in Northern and Nordic European regions, and is characterized by a high level of consumption of seaweed, shellfish, fatty fish (such as mackerel, herring and salmon), lean meats, rapeseed oil, legumes, nuts (such as almonds), vegetables, fruits (such as berries), whole grains (such as oats), low-fat dairy, and restricted salt and sugar intake [55,56]. In Nordic countries, the major dietary sources of plant lignans are vegetables, fruits and wholegrain cereals [57].

Among the many frequently-consumed plant species exhibiting a high lignan content, some species occur mainly in the Northern Hemisphere (e.g., *Cirsium spp.* of the family Asteraceae) [58]. The vegetative structures of these plants contain triterpenes, polyacetylenes, phenolic acids, flavonoids and alkaloids [58]. The most recent phytochemical studies of European *Cirsium spp.* demonstrate that their seeds are rich sources of neolignans and lignans [58,59].

4.3. Indian Diet

Various categories of food products make up a significant portion of the typical Indian diet, including fish, grapes, chocolate, oils, coffee, tea, biscuits and bread [60].

The fruit of *Morinda citrifolia* (Indian mulberry) has been extensively traditionally utilized in the treatment of cancer, diabetes, high blood pressure, diarrhea, headache and inflammation, largely due to its high lignan content [61,62].

Sesame is a typical component of the Indian diet, and both sesame seeds and oil are rich in lignans [63]. Sesame oil is recognized for both its notable resistance to oxidation and its nutritional value [64–66]. Despite lignans comprising only a small proportion (0.5 to 1.0%) of total sesame seed mass, the main sesame lignans—such as (+)-sesaminol, (+)-sesamolin and (+)-sesamin glucosides—have garnered attention for their notable health-promoting properties (demonstrated both in vitro and in vivo), including anti-inflammatory, antioxidant and anti-hypertensive activities [63].

Long-term intake of (+)-sesaminol has been proposed to inhibit the pathogenic extracellular β -amyloid aggregation observed in Alzheimer's Disease [67]. Similarly, (+)-sesamin exhibits protective activity against prostate and breast cancers [68], and is a precursor to enterodiol and enterolactone (which have been shown to possess anti-cancer, antidiabetic and anti-ageing properties [64]).

4.4. Asian Diet

The Asian diet is characterized by an elevated consumption of rice, noodles, spices and vegetables, sesame seeds and oil [69]. Additionally, seafood, tofu and other soy products are commonly consumed [70]. Many major plant sources of lignans occur in Asia; these are habitually included in the diet, and in China are also used as medicinal plants. Such plants include *Articum lappa*, whose fruit extracts and seeds are a rich source of bioactive lignans [70], including arctiin and arctigenin. These two lignans exhibit anti-inflammatory activities (e.g., inhibition of lipopolysaccharide-induced nitric oxide production and release of pro-inflammatory cytokines in murine macrophages in vivo) [70,71]. In addition, when tested on diverse cancer cell lines, arctigenin possesses potent apoptotic and anti-proliferative activities [70,72].

Certain medicinal herbs are usually used as an aqueous infusion. Among them, *Isodon spp.* and *Tripterygium spp.*

The genus *Isodon* comprises nearly 150 species found in the subtropical and tropical regions of Asia and represents an excellent lignan source [73]. Some species, such as *Isodon japonica*, have been used in traditional Chinese medicine to treat (for example) arthralgia, stomach-ache, mastitis, gastritis and hepatitis [73,74]. *Isodon rubescens* has also been used in traditional medicine for its hypotensive, antioxidant, immunological, antimicrobial, antitumor and anti-inflammatory properties [73].

Tripterygium wilfordii Hook f., a traditional medicinal herb, may ameliorate symptoms of rheumatoid arthritis and other autoimmune diseases [75]. Several phytochemical research studies have isolated hundreds of bioactive compounds—including lignans—from the root of this plant [75,76].

Chinese traditional medicine has long made use of *Schisandra chinensis* Baill. fruit as a sedative and antitussive tonic [77]. This fruit is additionally used in other countries in the production of functional foods, jam and beverages. Dibenzocyclooctadiene lignans isolated from *S. chinensis* exhibit anti-inflammatory and antioxidant properties, as well as improving cognitive functions (e.g., memory) [77]. In addition, prior studies have reported that *S. chinensis* fruit extracts—in which the major bioactive constituents are lignans—exert a neuroprotective effect and possess bioactivity which may help prevent Alzheimer's Disease [78]. Furthermore, *S. chinensis* fruit may have positive effects on the liver, as well as on the gastrointestinal, immune, sympathetic and central nervous systems [79,80]. Lignan extracts have been shown to successfully suppress hepatocellular carcinoma cell proliferation and to prevent chemical toxin-induced hepatic injury [79]. However, only 2% of the total *S. chinensis* fruit is made up of lignans, and most of these are present in the seeds, which are usually removed during manufacture of fruit-derived products [79].

The *Schisandra glaucescens* Diels vine is extensively distributed across the Southeastern Sichuan and Western Hubei regions of China [81]. The stem of this vine has been used as an analgesic in diverse conditions, including arthritis, rheumatism, and contusions. As yet, one sesquiterpenoid, 25 lignans

and 43 triterpenoids have been isolated from *S. glaucescens* [81]. In addition, *S. glaucescens* berries are thought to exert beneficial effects on the kidneys and lungs, relieving the symptoms of asthma for example [82].

Crataegus pinnatifida has been employed by the functional foods industry. Some studies have reported that it has the ability to protect against low-density lipoprotein (LDL) oxidation, to scavenge free radicals, and to exert an anti-inflammatory effect [83,84]. *C. pinnatifida* is mostly consumed as fresh fruit, processed juice or jam. Juice and jam manufacture results in a significant quantity of by-products, including seeds and leaves [84].

Schisandra sphenanthera is mainly located in Southwest China. A diversity of triterpenoids and lignans has been isolated from its leaves, stems, and fruit [85].

The roots, stems, fruit, and leaves of *Kadsura coccinea* are used medicinally, and its fruit, particularly, exhibits significant medicinal and nutritional properties [86]. Its bioactive triterpenoids and lignans have garnered interest for their reported bioactivities, including anti-inflammatory and anti-tumor effects [86–88].

Zanthoxylum schinifolium has been employed to stimulate blood circulation, as well as in the treatment of various diseases [89,90]. Due to its exceptional taste and characteristic aroma (usually described as green, spicy, floral, and fresh), *Z. schinifolium* fruit is used as a spice in many traditional Asiatic cuisines [89]. Prior pharmacological studies have demonstrated that the leaves and fruit of this plant possess medicinal properties, including antitumor, anti-inflammatory, and antioxidant activities, as well as inhibition of both platelet aggregation and monoamine oxidase production [89,91].

4.5. Latin-American Diet

The basis of the Latin-American diet consists of maize (corn), potatoes, peanuts and beans. This diet also includes flax seed. As mentioned above, *Linum usitatissimum* L. (flax seed) represents one of the best dietary sources of lignans, exhibiting a higher lignan content than legumes or grains [8]. Diets rich in flax seed are associated with a reduced risk of various diseases, including cardiovascular disease, osteoporosis, diabetes, and prostate and breast cancers [8,92]. Likely mechanisms include the ability to decrease circulating glucose, LDL and total cholesterol levels [93,94]. Furthermore, *L. usitatissimum* has significant commercial applications, in the manufacture of linen fiber for example [94]. In terms of lignans, flax seed contains mainly secoisolariciresinol and secoisolariciresinol diglucoside, but matairesinol is also present in small quantities [95]. Indeed, >95% of total flax seed mass consists of secoisolariciresinol diglucoside, which is predominantly localized in the seed's fibrous hull [96] rather than its interior [97].

Asian diet appears to facilitate the highest intake of lignans, in forms which also result in higher bioavailability. This is due largely to a high level of vegetable consumption, as well as the use of lignan-rich plant infusions in traditional medicine.

5. Human Studies Concerning Lignan Bioactivity

Recently, interest in identifying new sources of health-promoting natural compounds has increased. However, there are few human epidemiological studies that evaluate lignans bioactivity. Laboratory research, carried out on cell and animal models, concluded that lignans possess antimicrobial, anti-inflammatory and anti-oxidant activities, among others.

About antimicrobial activity, various lignans have exhibited antiviral and antibacterial activity, e.g., against Gram-positive bacteria through alteration of biofilm formation, bacteria metabolites, membrane receptors and ion channels [98]. For instance, pinoresinol has demonstrated activity against some virus [99].

Concerning anti-inflammatory activity, some lignans have the capacity to inhibit NF- κ B activity (transcription factor involves on the expression of inflammatory cytokines) on human mast cells (HMC-1). Thus, reduced pro-inflammatory cytokines production. Furthermore, lignans are able to suppress nitric oxide (NO) generation and decrease inflammatory cell infiltration [100–102].

Regarding anti-oxidant activity, various bioactive natural compounds—including phenols from grains, vegetables and fruits—are rich dietary sources of phytochemicals and vitamins, both of which guard against oxidative stress [84,103]. A free radical formation is an inevitable byproduct of cellular metabolism, and cells also *require* a certain level of reactive oxygen species (ROS) to carry out a normal cellular process [70]. Nevertheless, accumulation and/or overproduction of ROS can damage cellular constituents, including DNA [70], and play an important role in the pathogenesis of various severe disorders, including chronic inflammation, cancer, neurodegeneration and atherogenesis [84].

Many studies have demonstrated the strong antioxidant activity of plant extracts, attributable to several highly-effective antioxidants, including lignans (e.g., lariciresinol, matairesinol, secoisolariciresinol, pinoresinol, and nortrachelogenin) [104]. Among the natural antioxidants, lignans exhibit particularly high antioxidant efficiency and thus have potential as preventive and/or therapeutic clinical tools [105].

In recent years, a significant effort has been devoted to analyzing the lignan consumption of various populations (Table 9). Most studies have focused on post-menopausal women, due to lignans being phytoestrogens that ameliorate menopausal symptoms and consequences (e.g., climacteric symptoms, osteoporosis and estrogen-dependent cancers) [106].

5.1. Cancer

Various cohort studies have investigated dietary lignan anticancer bioactivity. As McCann et al. (2010) describe in the “Western New York Exposures and Breast Cancer” study, lignan intake among post-menopausal women with breast cancer significantly reduced the risk of mortality from breast cancer (Hazard Ratio (HR) 0.29, 95% Confidence interval (CI) 0.11–0.76), as well as significantly reducing the risk of all-cause mortality (HR 0.49, 95% CI 0.26–0.91) [107]. Other research based on the Swedish Mammography Cohort (SMC) also detected a statistically significant inverse association between breast cancer risk and lignan consumption among post-menopausal breast cancer patients [108]. Interestingly, the “Ontario Women’s Diet and Health Study” reported that neither lignan nor isoflavone consumption by a Canadian cohort correlated with a significant reduction in breast cancer risk [109]. Nonetheless, some studies do propose that isoflavone consumption correlates with a minor reduction in breast cancer risk in both pre- and post-menopausal women [109,110]. In addition, a cohort study examining the association between flax seed and flax bread intake and breast cancer risk demonstrated that flax seed intake was associated with a significant reduction in breast cancer risk (Odds Ratio (OR) 0.82, 95% CI 0.69–0.97) [111]. Furthermore, Buck et al. (2011) demonstrated that high serum enterolactone levels in post-menopausal breast cancer patients are associated with improved overall survival rates [109,112].

Another study, based on data from the United States Cancer Center Support Grant, investigated the association between individual breast cancer estrogen receptor (ER) status and lignan intake [113]. Higher lignan consumption was inversely correlated with the risk of ER[−] breast cancer among premenopausal women (OR 0.16, 95% CI 0.03–0.44) and with the risk of ER⁺ breast cancer among post-menopausal women (OR 0.64, 95% CI 0.42–1.00) [113]. Although this effect was largely independent of specific lignan class, it predominantly correlated with matairesinol and lariciresinol intake levels [113]. In addition, this study examined associations between breast tumor subtype and dietary lignan intake, demonstrating that a reduction in premenopausal triple-negative (HER2[−] PR[−] ER[−]) breast cancer risk (OR 0.16, 95% CI 0.04–0.62) was associated with higher lariciresinol and pinoresinol intake [113]. This finding agrees with that of a German case-control study that demonstrated a correlation between high intake of pumpkin and sunflower seeds (rich sources of lariciresinol and pinoresinol) and a statistically significant reduction in post-menopausal ER⁺ breast cancer risk (OR = 0.88, 95% CI = 0.77–0.99, *p* for trend = 0.02) [109,114].

Two recent meta-analyses have corroborated that high levels of plant lignan consumption correlate with a modest reduction in post-menopausal breast cancer risk (13 studies; Risk Estimated (RE) 0.86, 95% CI 0.78–0.94) [115,116].

Dietary lignan intake is also associated with a reduced risk for other cancer types (e.g., esophageal and gastric adenocarcinoma, as well as colon cancer), but very few human studies have been conducted.

A Swedish study indicates that dietary lignan intake correlates with decreased risk of gastroesophageal junction adenocarcinoma [117]. However, another Swedish study examining the Swedish Cancer Registry database did not find a clear association between dietary lignan consumption and development of gastric or esophageal adenocarcinoma [118]. Yet another (case-control) study indicated that a diet rich in resveratrol, quercetin and lignans (characterized by low intake of milk, but high intake of wholegrain bread, vegetables, wine and tea) may decrease the risk of developing such cancers [103].

Regarding colorectal cancer, Zamora-Ros et al. (2015) evaluated the association of lignan and flavonoid consumption with overall survival time and risk of recurrence in Barcelona (Spain) [119]. After a mean of 8.6 years' follow-up, 77 of the 319 (24.1%) patients in the cohort had experienced recurrence (excluding cases with metastasis that could not be resected), 133 of 409 (32.5%) patients had died, and no association was noted between consumption of any flavonoid subclass or total lignans and colorectal cancer risk [119].

Concerning prostate cancer risk, it has been studied its association with plasma enterolactone concentrations. Wallström et al. (2018) evaluated a population of Swedish men with 1010 cases and 1817 controls. After a mean follow-up of 14.6 years; there were no significant associations between the incidence of prostate cancer and plasma enterolactone (OR 0.99, 95% CI 0.77–1.280) [120]. Other study carried out at Danish men, neither found an association between prostate cancer mortality and plasma enterolactone [121]. However, two other pieces of research on humans, from 2003 and 2006, obtained positive results based on dietary phytoestrogen intake [122,123]. A Swedish case-control study indicated that lower prostate cancer risk is related to certain phytoestrogen-rich foods [123].

Given such mixed results, additional studies examining the effect of human lignan intake on cancer risk are necessary. Specifically, most existing studies have not examined the relevance of the specific dietary lignan source.

5.2. Cardiovascular Disease

Neolignans and flax lignans are reportedly relevant in diabetes, hypercholesterolemia and cardiovascular disorders [124]. In addition, the anti-aging role of lignans has recently been described [125]. Such lignan characteristics may be relevant to the reduction of cardiovascular disease risk in post-menopausal women. Indeed, an inverse association exists between high lignan consumption and the development of hypertension and cardiovascular disease [126]. Furthermore, prospective and cross-sectional epidemiological evidence suggests that dietary lignan intake reduces cardiovascular disease risk in post-menopausal women and elderly men by modifying traditional risk factors [127].

Jacobs et al. (2000) demonstrated that the risk of mortality is inversely associated with whole grain consumption in post-menopausal women [128]. Another study described how four weeks' consumption of a whole grain cereal-rich diet exerted a reasonable cholesterol-lowering effect in healthy post-menopausal women [17].

Table 9. Association between naturally lignan-rich foods and health promotion.

Author, Year	Methods	Results
Breast Cancer		
Lowcock, E.C. et al. (2013) [111]	Case-control study (2999 cases and 3370 controls) FFQ	Consumption of flaxseed and flax bread was associated with a significant reduction in breast cancer risk (OR 0.82, 95% CI 0.69–0.97; and OR 0.77, 95% CI 0.67–0.89), respectively.
McCann et al. (2012) [113]	Case-control study (638 cases and 611 controls) BioRepository at Roswell Park Cancer Institute FFQ	Lignan intakes were inversely associated with risk of ER (–) breast cancer among premenopausal women (OR 0.16, 95% CI 0.03–0.44) and particularly triple negative tumors (OR 0.16, 95% CI 0.04–0.62). High and low consumption of soybeans, as well as of sunflower and pumpkin seeds were associated with significantly reduced breast cancer risk compared to no consumption (OR 0.83, 95% CI 0.70–0.97; and OR 0.66, 95% CI 0.77–0.97, respectively).
Zaineddin AK et al. (2012) [114]	Case-control study (2884 cases and 5509 controls) FFQ	Serum enterolactone was associated with a significantly reduced risk of death only for estrogen receptor-negative tumors (HR 0.27; 95% CI 0.08 to 0.87)
Buck K et al. (2011) [112]	1140 postmenopausal patients (age 50 to 74 years) FFQ Serum Enterolactone	Lignan exposure was not associated with overall breast cancer risk (RE 0.92; 95% CI 0.81, 1.02).
Buck K et al. (2010) [116]	Meta-analyses Medline search to identify epidemiologic studies published between 1997 and August 2009	Lignan intake among post-menopausal women with breast cancer significantly reduced risk of mortality from breast cancer (HR 0.29, 95% CI, 0.11–0.76), as well as significantly reducing risk of all-cause mortality (HR 0.49, 95% CI 0.26–0.91).
McCann, S.E et al. (2010) [107]	Breast cancer patients; National Death Index Food frequency questionnaire (FFQ), DietSys (3.7)	Overall, there was little association between high plant lignan intake and breast cancer risk (11 studies, OR 0.93, 95% CI 0.83–1.03).
Velentzis LS et al. (2009) [115]	Meta-analyses Medline, BIOSIS and EMBASE databases publications up to 30 September 2008	Total phytoestrogen intake in pre-menopausal women was associated with a significant reduction in breast cancer risk among overweight women (OR 0.51, 95% CI 0.30, 0.87).
Cotterchio, M et al. (2008) [109]	Ontario Cancer Registry; Controls: Age-stratified random sample of women FFQ Swedish Mammography Cohort FFQ and Swedish National Food database	A significant 17% risk reduction for breast cancer overall in high lignan intake was observed, but no heterogeneity across Estrogen Receptor/Progesterone Receptor subtypes.
Suzuki, R. et al. (2008) [108]	Serum Enterolactone: Fluoroimmunoassay Receptor status of tumors: Immunohistochemical	High soy intake was discretely associated with reduction of breast cancer risk (OR 0.86, 95% CI: 0.75 to 0.99); association was not statistically significant among women in Asian countries (OR 0.89, 95% CI 0.71 to 1.12).
Trock BJ et al. (2006) [110]	Meta-analysis of 18 epidemiologic studies published from 1978 through 2004	

Table 9. Cont.

Author, Year	Methods	Results
Gastroesophageal Cancer		
Lin Y et al. (2012) [117]	Case-control study (1995–1997); 806 controls, 181 cases of esophageal adenocarcinoma, 255 cases of gastroesophageal junctional adenocarcinoma, and 158 cases of esophageal squamous cell carcinoma. Interviews and questionnaires; FFQ	No clear associations were found between risk of esophageal carcinoma and lignan intake.
Lin Y et al. (2012) [118]	Cohort study in Sweden, 81,670 (followed up 1998 to 2009). Cancer cases: Swedish Cancer Register FFQ	There was no statistically significant association between dietary intake of lignans and any of the studied adenocarcinomas.
Colon Cancer		
Zamora-Ros, R. et al. (2015) [119]	409 CRC cases in Barcelona (Spain). FFQ; Phenol-Explorer database.	No associations were also observed with either total lignans or any flavonoid subclass intake.
Prostate Cancer		
Wallström P et al. (2018) [120]	Case-control study (1010 cases and 1817 controls) National registers and hospital records FFQ Plasma Enterolactone: Fluoroimmunoassay	There were no significant associations between plasma enterolactone and incidence of prostate cancer (OR 0.99, 95% CI 0.77–1.280)
Eriksen AK et al. (2017) [121]	1390 men diagnosed with prostate cancer from the Danish Diet, Cancer and Health cohort Plasma Enterolactone: Fluoroimmunoassay	No associations between plasma enterolactone concentrations and prostate cancer aggressiveness.
Hedelin M et al. (2006) [123]	Swedish case-control study (1499 prostate cancer cases and 1130 controls) FFQ	No association was found between dietary intake of total or individual lignans or isoflavonoids and risk of prostate cancer.
Bylund A. et al. (2003) [122]	10 men with prostate cancer were randomized to a daily supplement of rye bran bread and 8 men of wheat bread Blood and urine samples. Ultrasound-guided core biopsies of the prostate.	In the rye group, there was a significant increase in plasma enterolactone. However, only small changes were observed in plasma concentrations of prostate specific antigen (PSA).
Cardiovascular disease		
Witkowska AM et al. (2018) [126]	2599 postmenopausal women, participants of the Multi-center National Population Health Examination Surveys. 24-h Dietary recall and food databases. Cross-sectional study in 151 men and 91 post-menopausal women.	In postmenopausal women, total and individual lignan intakes (secoisolaricresinol, pinoresinol, matairesinol) were not associated with the prevalence of CVD and its risk factors.
Pellegrini N et al. (2010) [127]	Anthropometric characteristics. Soluble intercellular adhesion molecule-1 (sICAM-1), CRP, insulin, glucose, total cholesterol, HDL-cholesterol and triacylglycerols. Three-day weighed food record	No relationship between intake of pinoresinol, laricresinol or total lignans and sICAM-1 values was observed.

Table 9. Cont.

Author, Year	Methods	Results
Jacobs DR. et al. (2000) [128]	11,040 postmenopausal women enrolled in the Iowa Women's Health Study Followed from baseline 1986–1997.	Women who consumed on average 1.9 g refined grain fiber/2000 kcal and 4.7 g whole grain fiber/2000 kcal had a 17% lower mortality rate (RR = 0.83, 95% CI = 0.73–0.94) than women who consumed predominantly refined grain fiber.
Vanharanta M. et al. (2003) [129]	A prospective study of Finnish men. 1889 men aged 42 to 60 years. Followed up 12.2 years.	Multivariate analyses showed significant associations between elevated serum enterolactone concentration and reduced risk of CVD-related mortality.
Other diseases		
Franco OH. et al. (2005) [130]	Community-based survey among 394 postmenopausal women. FFQ; Cognitive function: Mini-Mental Examination	Increasing dietary lignans intake was associated with better performance on the MMSE (OR 1.49, 95% CI 0.94–2.38). Results were most pronounced in women who were 20–30 years.
Eichholzer M. et al. (2014) [131]	2028 participants of NHANES 2005–2008 and 2628 participants of NHANES 1999–2004 (aged ≥ 18 years) Inflammatory marker: CRP	Statistically significant inverse associations of urinary lignan, enterodiol, and enterolactone concentrations with circulating CRP counts were observed in the multivariate-adjusted models.

FFQ: Food Frequency Questionnaire; CI: Confidence Interval; HR: Hazard Ratio; OR: Odds Ratio; CVD: Cardiovascular Disease; MMSE: Cognitive function Mini-Mental Examination; CRP: C-Reactive Protein.

However, a Warsaw population-based cross-sectional study conducted by the National Institute of Cardiology demonstrated that total dietary lignan consumption does not correlate with the occurrence of cardiovascular diseases, nor with cardiovascular risk factors (including central obesity, hypercholesterolemia and hypertension) in post-menopausal women [126]. Nevertheless, this study attributed a potentially-beneficial effect of lignan intake on hypercholesterolemia specifically to lariciresinol [126].

In a Finnish population, the highest serum enterolactone concentrations correlated with a lower risk of all-cause mortality, including from cardiovascular disease [129]. Enterolactone is a metabolite of lariciresinol, pinoresinol, secoisolariciresinol and matairesinol, and very low matairesinol intake does demonstrate an inverse relationship with endothelial dysfunction and vascular inflammation [127].

5.3. Other Diseases

Most studies have focused on the effects of lignan-rich food consumption in the prevention of cancer and cardiovascular disease. However, some observational studies have investigated the relationship between regular consumption of plant lignans and the risk of developing other lifestyle-related diseases. A study based on the European Prospective Investigation into Cancer and Nutrition cohort proposed that improved cognitive performance in post-menopausal women is associated with higher dietary phytoestrogen consumption (predominantly lignans in Western diets) [130]. Thus, it has been suggested that low-grade chronic inflammation contributes to the prevalence of chronic lifestyle-related diseases. The relationship between lignan consumption and inflammatory markers (e.g., C-reactive protein (CRP)) was studied in a United States cohort, demonstrating that a beneficial inflammatory marker profile is associated with adult lignan consumption [131].

6. Conclusions

Taken together, reviewed data support the recently increased interest in lignan health-promoting properties. Due to their various bioactive properties, dietary intake of lignan-rich foods may prevent certain types of cancers (e.g., breast cancer in post-menopausal women and colon cancer). Regarding chronic lifestyle-related diseases, some pieces of evidence indicate that lignan intake is associated with a lower risk of developing cardiovascular disease. Nonetheless, further human studies are warranted to evaluate lignan bioavailability resulting from different traditional dietary patterns, in order to influence the rational promotion of healthy lignan-rich diets.

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Dietary Flavonoids as Cancer Chemopreventive Agents: An Updated Review of Human Studies

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Review

Dietary Flavonoids as Cancer Chemopreventive Agents: An Updated Review of Human Studies

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Abstract: Over the past few years, interest in health research has increased, making improved health a global goal for 2030. The purpose of such research is to ensure healthy lives and promote wellbeing across individuals of all ages. It has been shown that nutrition plays a key role in the prevention of some chronic diseases such as obesity, cardiovascular disease, diabetes, and cancer. One of the aspects that characterises a healthy diet is a high intake of vegetables and fruits, as both are flavonoid-rich foods. Flavonoids are one of the main subclasses of dietary polyphenols and possess strong antioxidant activity and anti-carcinogenic properties. Moreover, some population-based studies have described a relationship between cancer risk and dietary flavonoid intake. In this context, the goal of this review was to provide an updated evaluation of the association between the risk of different types of cancers and dietary flavonoid intake. We analysed all relevant epidemiological studies from January 2008 to March 2019 using the PUBMED and Web of Science databases. In summary, this review concludes that dietary flavonoid intake is associated with a reduced risk of different types of cancer, such as gastric, breast, prostate, and colorectal cancers.

Keywords: flavonoids; diet; antioxidants; cancer

1. Introduction

Cancer is among the diseases that have the greatest impact on society [1]. Even though its incidence has increased over the years, its mortality has decreased because of advances in treatment [2]. However, efforts to improve cancer prevention are needed. The aetiology of cancer is multifactorial, involving both environmental and genetic factors [3]. Diet is one of the lifestyle factors that affect cancer incidence and mortality [4]. Recently, several studies have reported that diets based on high levels of vegetables and fruits are strongly associated with a significant reduction in cancer risk [5,6].

Furthermore, there are some bioactive compounds in foods that have potential health benefits, such as flavonoids, carotenoids, stilbenes, lignans, and phenolic acids [7,8]. Flavonoids are a large group of phenolic compounds and are usually involved in protection against harsh environmental conditions, UV radiation, and microorganism attacks in plants [9,10]. Because of their potent antioxidant activity against oxidative stress, the interest in flavonoids has recently increased [11]. In vitro and in vivo studies have demonstrated that they have anti-carcinogenic properties against different types of cancers [5,12]. Moreover, many population-based studies have described an association between dietary flavonoids and cancer risk [13,14]. Hence, the goal of this review is to

perform an updated evaluation of the association between the risk of different types of cancers and dietary flavonoids, as well as the intake of each flavonoid subclass.

2. Methodology

Recently, interest in flavonoids has increased because their strong antioxidant and anti-carcinogenic activities may have possible beneficial effects on cancer. Thus, in this review, we analysed all relevant cancer epidemiological studies from January 2008 to March 2019 using the PUBMED and Web of Science databases [15,16]. Since different reviews have already been published on flavonoids and cancer before 2008. Search entries included [flavonoids and cancer], [flavonoids and “breast cancer”], [flavonoids and “lung cancer”], [flavonoids and “prostate cancer”], [flavonoids and “gastric cancer”], [flavonoids and “pancreatic cancer”] and [flavonoids and “colorectal cancer”]. Selection criteria applied were the following: human studies, randomized controlled trials, cross-sectional, cohort and case-control studies and information about dietary intake. Reviews studies and Meta-Analyses were excluded. Besides, population studies were catalogued based on type of study: case-control or cohort study and the type of cancer.

3. Biosynthesis and Subclasses of Flavonoids

Flavonoids are secondary metabolites synthesised mainly by plants [9]. To date, more than 6000 different flavonoids have been identified, and they are distributed in a wide range of plants [17]. The general structure of flavonoids is composed of a 15-carbon skeleton, containing 2 benzene rings connected by a 3-carbon linking chain (Figure 1) [9]. Therefore, they are depicted as C6-C3-C6 compounds. Their biosynthesis involves two different biosynthetic pathways: the shikimic acid pathway and the acetate pathway (Figure 1) [9].

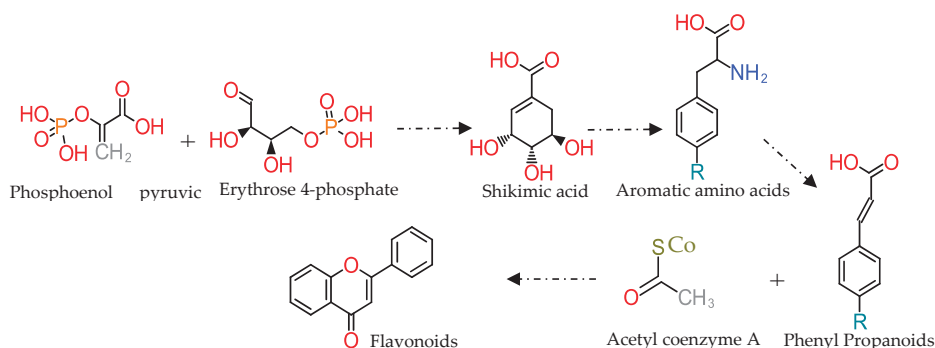


Figure 1. Flavonoid biosynthesis pathways (general structure of flavonoids).

Depending on the chemical structure, degree of oxidation, and unsaturation of the linking chain (C3), flavonoids can be classified into different groups, such as anthocyanidins, chalcones, flavonols, flavanones, flavan-3-ols, flavanonols, flavones, and isoflavonoids (Figure 2). Furthermore, flavonoids can be found in plants in glycoside-bound and free aglycone forms [9]. The glycoside-bound form is the most common flavone and flavonol form consumed in the diet [9].

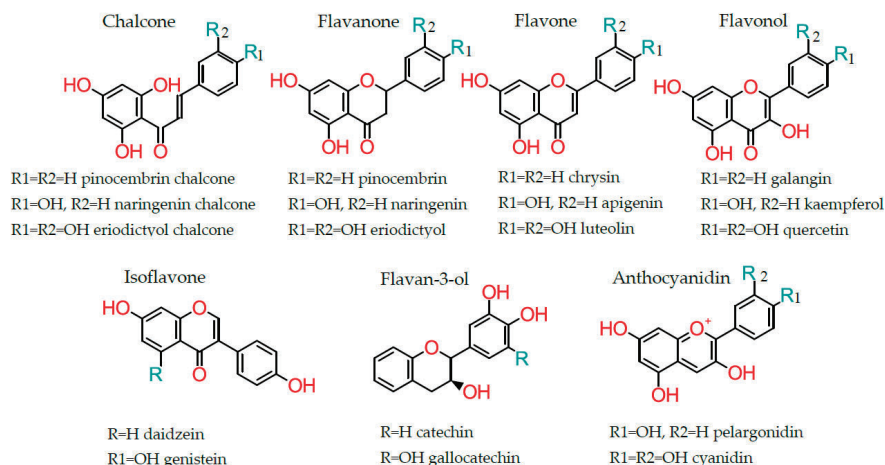


Figure 2. Flavonoid subclasses.

4. Dietary Flavonoids

Flavonoids are widely spread in different foods and beverages (such wine and tea), but the sources with the highest levels are fruits and vegetables [10]. Among the fruits (Table 1), the highest levels of flavonoids are found in berries, such as black elderberry (1358.66 mg/100 g) and black chokeberry (1012.98 mg/100 g) [18,19]. In the drupes group, some fruits such as plum and sweet cherry have higher levels of flavonoids than the rest of the group, 101.67 mg/100 g and 185.05 mg/100 g, respectively [20,21]. In the pomes group, apple has the level (56.35 mg/100 g) [21,22]. Furthermore, tropical fruits have a very low flavonoid content [23]. Depending on the type of fruit, the main flavonoid subclass groups vary: anthocyanins predominate in berries, and flavanols predominate in pomes, tropical fruits, and drupes (except in sweet cherry).

Table 1. Flavonoid contents of fruits (mg/100 g food). Data collected from Phenol Explorer [24].

Fruits	ANT	DYC	FVA	FVO	Total
Berries					
Aestivalis grape	79.74	-	-	1.7	81.44
American cranberry	49.89	-	-	43.84	93.73
Black chokeberry	878.11	-	-	134.87	1012.98
Black elderberry	1316.66	-	-	42	1358.66
Black raspberry	-	-	-	19	19
Blackberry	172.59	-	13.87	16.87	203.33
Blackcurrant	593.58	-	1.17	13.68	608.43
Black grape	72.15	-	14.03	4.01	90.19
Green grape	-	-	3.78	2.49	6.27
Green currant	-	-	-	11.07	11.07
Highbush blueberry	156.6	-	1.11	54.77	212.48
Lingonberry	60.21	-	-	48.98	109.19
Lowbush blueberry	204.56	-	-	-	204.56
Red raspberry	72.47	-	5.73	16.26	94.46
Redcurrant	33.13	-	4.68	0.77	38.58
Strawberry	26.87	-	9.1075	2.32	38.29
Drupes					
Nectarine	0.86	-	17.65	1.35	19.86
Peach	0.28	-	45.18	1.42	46.88
Plum	47.79	-	46.9	6.98	101.67

Sour cherry	54.43	-	0.2	-	54.63
Sweet cherry	170.18	-	14.87	-	185.05
Pomes					
Apple	0.93	5.38	39.42	10.62	56.35
Pear	-	-	4.98	0.84	5.82
Quince	-	-	7.49	0.67	8.16
Tropical Fruits					
Banana	-	-	1.55	-	1.55
Kiwi	-	-	0.7	-	0.7
Mango	-	-	1.72	-	1.72
Persimmon	-	-	1.28	-	1.28
Pomegranate	-	-	1.1	-	1.1

ANT: Anthocyanins, DYC: Dihydrochalcones, FVA: Flavan-3-ols, FVO: Flavonols.

Regarding vegetables (Table 2), the foods with the highest levels of flavonoids are broad bean pod (189.54 mg/100 g) [25], black olive (159.83 mg/100 g) [26], red onion (131.51 mg/100 g) [27], spinach (119.27 mg/100 g), and shallot (112.22 mg/100 g) [28,29]. Except for broad bean pod, the predominate flavonoid subclass in vegetables is flavanols.

Table 2. Flavonoid contents of vegetables (mg/100 g food) [24].

Vegetables	ANT	CHA	FVA	FNE	FVE	FVO	Total
Cabbages							
Broccoli	-	-	-	-	-	27.8	27.8
Fruit Vegetables							
Avocado	-	-	0.55	-	-	-	0.55
Black olive	82.97	-	-	-	27.43	49.43	159.83
Green olive	-	-	-	-	0.56	-	0.56
Green sweet pepper	-	-	-	-	2.11	5.49	7.6
Red sweet pepper	-	-	-	-	0.05	0.24	0.29
Tomato	-	-	-	0.14	-	0.014	0.15
Leaf Vegetables							
Curly	-	-	-	-	-	24.06	24.06
Escarole	-	-	-	-	-	18.23	18.23
Green lettuce	-	-	-	-	0.4	3.99	4.39
Red lettuce	3.53	-	-	-	2.51	16.74	22.78
Spinach	-	-	-	-	-	119.27	119.27
Onion-Family Vegetables							
Red onion	9	-	-	-	-	122.51	131.51
White onion	-	-	-	-	-	5.4	5.4
Yellow onion	-	-	-	-	-	59.1	59.1
Shallot	-	-	-	-	-	112.22	112.22
Pod Vegetables							
Broad bean pod	-	0.08	154.45	-	0.37	34.64	189.54
Green bean	-	-	2.42	-	-	5.55	7.97
Shoot Vegetables							
Asparagus	-	-	-	-	-	23.19	23.19
Globe artichoke, heads	-	-	-	-	57.8	-	57.8

ANT: Anthocyanins, CHA: Chalcones, FVA: Flavan-3-ols, FNE: Flavanones, FVE: Flavones, FVO: Flavonols.

Regarding seeds (Table 3), although common bean has high levels of flavonoids (from anthocyanins and flavonols), the foods with the highest levels are those derived from soy, and soy products have been suggested to play a key role in the prevention of different diseases [30].

Table 3. Flavonoid contents of seeds (mg/100 g food) [24].

Seeds	ANT	FVA	FNE	FVE	FVO	IFA	Total
Nuts							
Almond	-	4.93	0.5	-	3.81	0.06	9.3
Cashew nut	-	1.1	-	-	-	-	1.1
Chestnut	-	0.05	-	-	-	-	0.05
Hazelnut	-	5.7	-	-	-	-	5.7
Peanut	-	-	-	-	-	0.51	0.51
Pecan nut	-	16.7	-	-	-	-	16.7
Pistachio	-	6.9	0.12	0.103	0.07	-	7.193
Common Bean							
Black common bean	41.05	-	-	-	10	1.4	52.45
Others common bean	7.42	-	-	-	69.58	0.2	77.2
White common bean	0.13	-	-	-	49.96	0.5	50.59
Other Beans							
Broad bean seed whole	-	49.37	-	-	-	-	49.37
Sunflower seed meal	-	-	-	-	-	0.02	0.02
LENTILS							
Lentils	-	5.17	-	0.95	1.09	-	7.21
Soy Products							
Soy paste miso	-	-	-	-	-	63.09	63.09
Soy tempeh	-	-	-	-	-	147.74	147.74
Soy tofu	-	-	-	-	-	39.24	39.24
Soybean roasted	-	-	-	-	-	253.11	253.11

ANT: Anthocyanins, FVA: Flavan-3-ols, FNE: Flavanones, FVE: Flavones, FVO: Flavonols, IFA: Isoflavonoids.

Regarding cereals (Table 4), some such as barley, buckwheat, and common wheat contain average levels of flavonoids (35.2 mg/100 g, 37.04 mg/100 g, and 77.4 mg/100 g, respectively). However, it is important to note that the highest levels are found in whole grains, and levels are greatly reduced when grains are heat treated or refined [30,31].

Table 4. Flavonoid contents of cereals (mg/100 g food) [24].

Cereals	FVA	FVE	FVO	Total
Cereals				
Barley, whole grain flour	35.2	-	-	35.2
Buckwheat groats, thermally treated	-	-	8.96	8.96
Buckwheat, refined flour	-	-	5.86	5.86
Buckwheat, whole grain flour	-	0.9	36.14	37.04
Common wheat, refined flour	-	18.4	0.08	18.48
Common wheat, whole grain flour	-	77.29	0.11	77.4

FVA: Flavan-3-ols, FVE: Flavones, FVO: Flavonols.

Cocoa and its products, such as dark and milk chocolate, are flavonoid-rich foods (Table 5). In these foods, the main flavonoids are flavanols, with cocoa containing 511.63 mg/100 g [32,33].

Table 5. Flavonoid contents of cocoa (mg/100 g food) [24].

Cocoa	FVA	FVO	Total
Cocoa			
Chocolate dark	212.36	25	237.36
Chocolate milk	19.22	-	19.22
Cocoa powder	511.62	-	511.62

FVA: Flavan-3-ols, FVO: Flavonols.

Regarding oils, the data collected from the Phenol Explorer database refer only to oils made from olives (Table 6). In ascending order, refined, virgin, and extra virgin olive oil contain 0.15 mg, 0.23 mg, and 1.53 mg of flavones in 100 g, respectively [34,35].

Table 6. Flavonoid contents of oils (mg/100 g oil) [24].

Oils	FVE	Total
Oils		
Extra virgin olive oil	1.53	1.53
Virgin olive oil	0.23	0.23
Refined olive oil	0.15	0.15

FVE: Flavones.

For beverages, a distinction can be made between non-alcoholic (Table 7) and alcoholic drinks (Table 8). The non-alcoholic drinks with the highest levels of flavonoids are tea infusions, particularly black (83.35 mg/100 g) and green tea (77.44 mg/100 g), and these are mainly flavanols [36,37]. The second most flavonoid-rich beverages are fruit juices, notably pure apple juice (54.99 mg/100 g), pure orange juice (48.02 mg/100 g), pure grapefruit juice (47.12 mg/100 g), and pure lemon juice (37.43 mg/100 g) [38]. The main flavonoids in citrus juices and grapefruit juice are flavanones [39]. However, the main flavonoids in pome juices are flavanols. Regarding alcoholic beverages, wine red contains the highest flavonoid level (83.96 mg/100 mL) [40,41].

Table 7. Flavonoid contents of non-alcoholic beverages (mg/100 g drink) [24].

non-alcoholic beverages	ANT	DYC	FVA	FNE	FVE	FVO	IFA	Total
Cocoa Beverage								
Chocolate, milk	-	-	20.33	-	-	-	-	20.33
Fruit Juices								
Berry Juices								
Fox grape juice	-	-	5.9	-	-	-	-	5.9
Green grape juice	-	-	3.88	-	-	-	-	3.88
Grapefruit juice	-	-	-	46.44	-	0.68	-	47.12
Citrus Juices								
Lemon juice	-	-	-	32.66	4.77	-	-	37.43
Lime juice	-	-	-	19.61	-	-	-	19.61
Orange juice	3.17	-	-	37.63	6.14	1.08	-	48.02
Pummelo juice	-	-	-	8.48	-	-	-	8.48
Red raspberry juice	-	-	-	-	-	9.58	-	9.58
Rowanberry	-	-	-	-	-	7.04	-	7.04
Drupe Juices								
Plum juice	-	5.85	24.7	-	-	-	-	30.55
Pome Juices								
Apple juice	-	4.39	48.45	-	-	2.15	-	54.99
Apple (cider) juice	-	4.78	22.66	-	-	-	-	27.44
Pear juice	-	-	3.24	-	-	-	-	3.24
Tropical Juices								
Kiwi juice	-	-	0.38	-	-	0.09	-	0.47
Pomegranate juice	10.13	0.1	-	-	-	0.25	-	10.48
Herb Infusions								
German chamomile, tea	-	-	2.07	-	-	-	-	2.07
Lemon verbena	-	-	10.6	-	-	-	-	10.6
Peppermint, tea	-	-	10.23	-	-	-	-	10.23
Tea Infusion								
Fennel tea	-	-	-	-	-	3.26	-	3.26
Black tea	-	-	73.29	-	-	10.06	-	83.35
Green tea	-	-	71.18	-	-	6.26	-	77.44
Oolong tea	-	-	35.72	-	-	-	-	35.72
Soy Products								
Soy milk	-	-	-	-	-	-	18	18

ANT: Anthocyanins, DYC: Dihydrochalcones, FVA: Flavanols, FNE: Flavanones, FVE: Flavones, FVO: Flavonols, IFA: Isoflavonoids.

Table 8. Flavonoid contents of alcoholic beverages (mg/100 g drink and mg/100 mL wine) [24].

Alcoholic beverages	ANT	DYC	DYF	FVA	FNE	FVE	FVO	IFA	Total
Beer									
Beer (alcohol free)	-	0.0003	-	0.11	0.01	-	-	-	0.12
Beer (ale)	-	0.01	-	0.38	0.24	-	-	0.02	0.65
Beer (dark)	-	0.03	-	0.03	0.15	-	-	-	0.21
Beer (regular)	-	0.001	-	0.61	0.04	0.004	0.09	0.02	0.77
Wines									
Red wine	23.3	-	5.44	47.02	0.85	-	7.35	-	83.96
Rosé wine	-	-	0.38	2	-	-	-	-	2.38
White wine	0.04	-	0.57	2.07	0.23	-	0.695	-	3.61

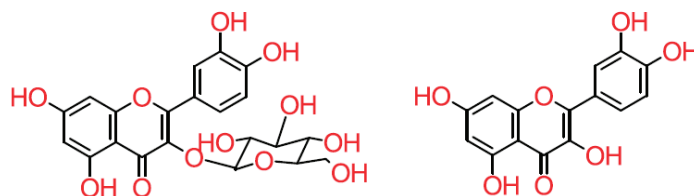
ANT: Anthocyanins, DYC: Dihydrochalcones, DYF: Dihydroflavonols, FVA: Flavan-3-ols, FNE: Flavanones, FVE: Flavones, FVO: Flavonols, IFA: Isoflavonoids.

Therefore, a diet rich in fruits, vegetables, seeds, and cereals will provide large amounts of flavonoids. However, it is important to know that there are some foods which contain high quantities of flavonoids, including berries, black olives, spinach, onions, soy products, cocoa, whole grain cereals, tea infusions, and red wine.

5. Pharmacokinetics

In order to determine the biological activity and physiological functions of flavonoids *in vivo*, their bioavailability must be known. Hence, it is necessary to understand the processes of absorption, digestion, metabolism, and excretion in the digestive tract.

Although dietary flavonoids are mostly found in their glucoside form (Figure 3), they are not found in plasma [42,43] because, once flavonoids enter the oral cavity, they begin to be hydrolysed [42]. In addition, their absorption throughout the digestive tract is associated with the hydrolysing activity of different enzymes [44]. In the small intestine, deglycosylation occurs in which two enzymes that act as β -glucosidases are involved: lactase-phlorizin hydrolase (LPH) and cytosolic β -glucosidase (CBG), which are located in the brush border of epithelial cells and enterocytes, respectively [42,45]. Flavonoid-O- β -D-glucosides, for which LPH has high specificity, can enter into cells by passive diffusion. However, glucosides enter enterocytes via sodium-glucose co-transporter type 1 (SGLT1) [42,44,46]. Although β -glucosidases cannot hydrolyse non-monoglucosidic glycosides, gut microbiota compensate for this through the production of absorbable aglycon in the large intestine and cecum (Figure 3) [42].

**Figure 3.** Structure of glycoside and aglycone flavonoids.

Once flavonoids and aglycons are absorbed via the small and the large intestine, respectively, the second phase of enzymatic metabolism begins [42,44]. In this stage, three types of enzymes are involved (uridine-5'-diphosphate-glucuronosyltransferases, sulfotransferases, and catechol-O-methyltransferases) that can conjugate flavonoids with glucuronic acid, sulphate, and methyl groups, making them more water-soluble [13,47]. This phase begins in the wall of the small intestine where metabolites pass to the portal vein and are transported to the liver. In the liver, metabolites are conjugated by sulphation and methylation processes [42]. In the systemic circulation and urine, there are different chemical forms of flavonoids. However, in human plasma, aglycons are rarely detected [42,48–50]. Certain plasmatic metabolites are usually excreted into the intestine

through bile, and here, they are deconjugated by microbiota and reabsorbed [42,51]. Thus, enterohepatic circulation increases the half-life of flavonoids in human plasma [40].

The gut microbiome plays a main role in the metabolism and absorption of flavonoids. However, these processes could be modified due to flavonoids interaction with other nutrients [52,53]. Among them, flavonoids could alter glucose absorption after high carbohydrate food intake, because inhibit carbohydrate-hydrolyzing enzymes (α -amylase and α -glucosidase) [54]. Besides, flavonoids inhibit glucose transporter in the brush border [54]. However, flavonoid bioavailability is modified with fats intake that improves flavonoid intestinal absorption due to the increment of bile salts secretion which enhances micellar incorporation of flavonoids [54]. However, regarding proteins intake, flavonoid bioavailability became worse [55]. It has been demonstrated that the interaction of phenolic acids with proteins affects antioxidant efficacy and protein digestibility [56].

Depending on the type of flavonoid and its source, bioavailability may differ. Quercetin is one of the most frequently consumed flavonoids (the main sources of quercetin are onions, apples, tea, and wine), being mainly found in its glycosylated form [13]. For example, quercetin glycosides from apples have lower bioavailability than those from onions [13,57]. The plasma levels of quercetin metabolites range from 0.7 to 7.6 μ M [13].

Other studies have analysed the levels of flavonoids in human plasma after the intake of flavonoid-rich foods [13]. They could be grouped according to the flavonoid subclass. Flavonols present in apples, onions, and buckwheat tea are found after intake at plasma levels of 0.30 μ M, 0.74–7.60 μ M, and 2.10 μ M, respectively [13,57]. For flavanols in red wine, black tea, green tea, and cocoa, the plasmatic concentration after intake is around 0.08 μ M, 0.09–0.34 μ M, 1.00–1.80 μ M, and 4.92–5.92 μ M, respectively [13,58–60]. The base plasma levels of flavanones in orange juice and grapefruit juice are around 0.06–0.64 μ M and 5.99 μ M, respectively, after intake [61]. Finally, the plasma levels of anthocyanidins after consuming red wine, elderberry extract, and blackcurrant juice are around 0.01 μ M, 0.10 μ M, and 0.11 μ M, respectively [13,61,62].

The highest concentration of plasma flavonoids in humans usually occurs 1 to 2 h after the consumption of flavonoid-rich foods [36]. However, the level depends on the type of flavonoid, as anthocyanins and catechins have a half-life elimination that is 5 to 10 times less than that of flavonols [55]. Although data on the concentration of flavonoids in human tissue are scarce, flavonoids have been shown to play an important role in antioxidant defence in both cells and tissues [13].

6. Worldwide Flavonoid Intake

The intake of flavonoids depends not only on the food itself and its bioavailability but also on geography, agricultural practices, climate stress, and cultural factors. Diets may differ in different locations [63]. Therefore, based on food frequency questionnaires (FFQs) administered in different studies, we extracted the following distribution of flavonoid consumption around the world (Figure 4).

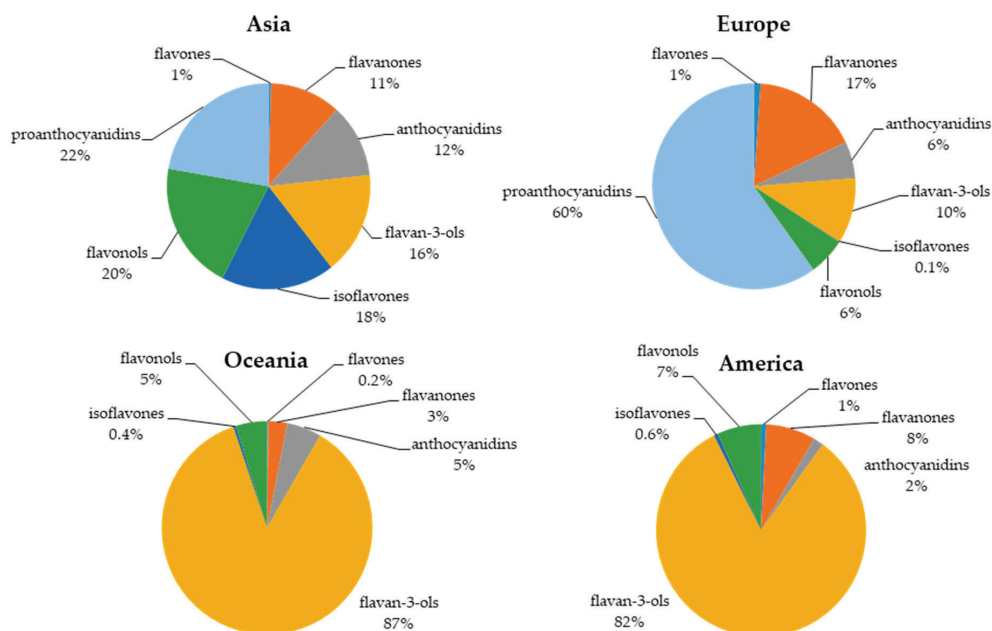


Figure 4. Worldwide intake of flavonoid subclasses.

6.1. Asia

A study performed by Ying Zhang et al. (2010) in China investigated the main sources of flavonoids in adults [64]. The mean intakes of total flavonoids, flavones, and flavonols were 19.13, 4.19, and 13.38 mg/day, respectively. The total intake of flavones and flavonols was attributable to fruits and vegetables. The main sources of flavonoids were *Actinidia* (5%), eggplant (7%), celery (7%), potato (8%), and apple (12%) [64]. Similarly, another study on female adolescents of northern China observed that the mean total flavonoid intake was 20.60 mg/day, with flavone and flavonol intakes of 4.31 and 16.29 mg/day, respectively. From lowest to highest, the food sources of flavonoids were aubergine (3.9%), leeks (3.9%), soybean sprouts (4.2%), celery (4.2%), tomatoes (4.2%), Chinese cabbage (4.7%), oranges (7%), lettuce (7.3%), potatoes (9.9%), and apple (11.7%) [65].

The major sources of dietary flavonoids in Korean adults were identified in a study performed by You Kin Kim et al. (2015) [66]. In this study, they observed that the total daily flavonoid intake was 107 mg/day and that the anthocyanidin, flavan-3-ol, flavanone, flavone, flavonol, and isoflavone intakes were 24.3, 21.8, 8.81, 0.97, 27.8, and 24.3 mg/day, respectively. The main food sources of flavonoids were, in ascending order, tangerine, radish, tofu, onion, soybeans, persimmons, green tea, and Kimchi (traditional fermented vegetable product) [66]. Another study performed by Shinyoung Jun et al. (2015) evaluated the dietary flavonoid intake in Korean adults (33,581 subjects, aged 19 years and older) [67]. The mean total flavonoid intake was 318 mg/day. The intakes of flavonoid subclasses were, in ascending order, flavones (0.4%), flavanones (11.3%), anthocyanidins (11.6%), flavan-3-ols (16.2%), isoflavones (18.1%), flavonols (20.3%), and proanthocyanidins (22.3%) [67].

6.2. Europe

Diets can differ according to region. Hence, Europe can be divided into southern and northern diets, known as the Mediterranean and Non-Mediterranean (UK and Nordic) diets, respectively.

The Mediterranean diet has been closely studied. Therefore, there is a large amount of data on Mediterranean nutrition. Among these studies, the European Prospective Investigation into Cancer and Nutrition Study (EPIC) is one of the most important. This study included 477,312 subjects from

different countries in Europe, aged 35 to 70 years. For the Spanish cohort (40,683 subjects) in 2010, the mean total flavonoid intake was 313.26 mg/day [68]. From lowest to highest, the flavonoid subclass intakes were isoflavones (<0.01%), flavones (1.1%), anthocyanidins (5.8%), flavonols (5.9%), flavan-3-ols (10.3%), flavanones (16.9%), and proanthocyanidins (60.1%). The main dietary food sources of flavonoids were tea (2.2%), chocolate (2.6%), peaches (3.3%), pears (4%), beans (4.9%), oranges (9.3%), red wine (21%), and apples (23%) [68]. The main sources of proanthocyanidins were apples, red wines, and beans. Similarly, the most abundant sources of flavan-3-ols were, in ascending order, some fruits (such as plums, grapes, apricots, pears, and peaches), chocolate, apples, tea, and red wine. However, the most abundant sources of flavanones were citrus fruits and their derived products (such as juices) [69]. In the European region, the main food sources of anthocyanidin were fruits (such as pears, apples, and grapes), seeds, and nuts. These were followed by wine, isotonic drinks (in the Northern region), juices (Central region), and vegetables [70].

Another study performed by EPIC (36037 subjects aged 35–74 years) demonstrated that there are differences in the in flavonoid intake between European countries (Norway, Sweden, Denmark, the UK, the Netherlands, Germany, France, Italy, Spain, and Greece) [71]. The daily proanthocyanidin intake was the lowest in Greece and the highest in Spain. In contrast, the lowest intake of flavan-3-ols was observed in Greek women and men (124.8 and 160.5 mg/day, respectively), and the highest total intake was observed in women of the UK General population cohort (377.6 mg/day) and health-conscious men (453.6 mg/day). Likewise, flavan-3-ol monomer intake was the lowest in Greece (20.7 and 26.6 mg/day in women and men, respectively) and the highest in the UK general population (178.6 and 213.5 mg/day in women and men, respectively) [71]. The most important sources of flavan-3-ols in Mediterranean countries, non-Mediterranean countries, and the UK are non-citrus fruit, mainly apples, followed by wine and tea. Tea is responsible for the high flavan-3-ol intake in the UK [71]. For proanthocyanidins, the most important sources in Mediterranean countries are non-citrus fruits, and those in the UK are tea, wine, puddings, and pulses. However, in non-Mediterranean countries, the most important sources are non-citrus fruits, wine, and chocolate [71].

A study performed by Anna Vogiatzoglou et al. (2015) identified the main sources of flavonoids in the European Union [64]. The mean intake of total flavonoids was 428 mg/day, with the lowest intake in the Southern Region (301 mg/day), followed by the Northern Region (348 mg/day), and the highest intake was in the Central Region (506 mg/day), with flavan-3-ols the main flavonoid subclass consumed. Except for flavones and anthocyanidins (which had the highest intakes in the Northern Region), the highest intakes of all other flavonoid subclasses were in the Central Region. Regarding flavonoid sources, in the Southern region, the main sources of flavonoids were fruits and fruit products (mainly pome fruits and berries), but in the Northern and Central regions, tea was the main source of total flavonoids [72]. There were many regional differences, and in the Northern region, the intakes of flavanones and anthocyanidins were the highest, mainly in Finland where the primary sources are citrus fruits and berries, respectively. Nevertheless, in the Southern region, France had the highest intake of anthocyanidins and flavan-3-ols. This study also reported that Germany and Belgium had very low intakes of flavonoid-rich foods [72].

6.3. Oceania

A study that estimated the flavonoid intake in the Australian population (13858 participants) obtained an average total flavonoid intake of 351 mg/day (of which 75% was flavan-3-ols and 15% was flavanones). In ascending order, the most important flavonoids sources were stalk vegetables, leaf, apples, wine, grapes, oranges, and black tea (which provided 76% of the flavonoid intake) [73]. In the Australian diet, the predominant sources of flavonols and flavan-3-ols were green and black tea as well as pears, apples, and wine for the latter [74]. Other significant sources of flavonols were beans, grapes, apple, broccoli, and onion. Wine was the main source of anthocyanidin. The main sources of flavone and flavanone were spinach and oranges, respectively [74]. However, the most recent Australian population study performed by Murphy KJ et al. (2019) reported an average total flavonoid intake of 660 and 566 mg/day for women and men, respectively [75]. In ascending order,

the contributions to total flavonoids intake by subclass were flavones (0.2%), isoflavones (0.4%), flavanones (2.9%), flavonols (4.8%), anthocyanidins (5.3%), and flavan-3-ols (86.5%) [75]. Regarding the dietary sources of flavonoids, tea was responsible for 85% of the total flavonoid intake, followed by fruit juice (2.4%), apple (2.2%), wine (1.7%), berries (1.6%), banana (1.1%), cocoa (0.6%), citrus fruit (0.6%), plum (0.4%), grapes (0.4%), and nuts (0.4%) [75].

6.4. North America

In America, diets differ depending on the region. In North America, processed foods predominate in diets, whereas in South America, fruits and vegetables are the main components of the diet [76]. A study performed by Monica L Bertoia et al. (2016) analysed the dietary flavonoid intake of three prospective cohorts in United States, finding estimated averages of 236 mg/day and 224 mg/day for women and men, respectively [77]. Another prospective study in the United States estimated the intake of flavonoid subclasses, in increasing order, as isoflavones (0.6%), flavones (0.8%), anthocyanidins (1.6%), flavonols (6.8%), flavanones (7.6%), and flavan-3-ols (82.5%) [78]. Thus, the major dietary flavonoid sources were citrus fruits, wine, citrus fruit juices, and tea. In fact, tea was the main source for flavan-3-ols and flavonols [78]. Another study performed by Kim K et al. (2016) estimated the intake and major food sources of flavonoids in adults in the United States [79]. The major dietary sources were apples, wine, citrus fruit, berries, citrus fruit juices, and tea, with tea as the major contributor of flavan-3-ols and flavonols, at 155.9 and 164.4 mg/day, respectively, of the total flavonoids [79].

For Europe and the United States, numerous descriptive studies on flavonoid intake have been published, but for Latin-American countries, there are insufficient data available. However, Raul Zamora-Ros et al. (2018) analysed polyphenol dietary intake in the Mexican Teachers' Cohort, reporting an average total flavonoid intake of 235 mg/day. In this population, the main food sources of total polyphenols were orange juice (4.8%), mandarins (5.1%), apples (7.2%), and coffee (47.4%) [80].

There are some differences between the intake of flavonoid subclasses around the world (Table 9). However, it is unclear if these differences are related to differences in cancer incidence. To clarify this issue, for this review, the latest epidemiological studies and GLOBOCAN data (2018) [2] were collected.

Table 9. Flavonoids intake and main food sources worldwide.

Country	Intake (mg/d)	Subclass	Food sources
Asia	107	Protanthocyanidins>flavonols>isoflavones>flavan-3-ols>anthocyanidins>flavanones>flavones	Kimchi, green tea, persimmons, soybeans, onions
Southern europe	313	Proanthocyanidins>flavanones>flavan-3-ols>flavonols>anthocyanidins>flavones>isoflavones	Apples, red wine, oranges, beans, pears, peaches
Northern europe	348	Flavan-3-ols>flavones>anthocyanidins>flavonols>flavanones> isoflavones	Tea, citrus fruits, berries
Central europe	506	Flavan-3-ols>anthocyanidins>proanthocyanidins>flavanones> flavonols >flavones>isoflavones	Tea, non-citrus fruits, wine
Oceania	351	Flavan-3-ols> anthocyanidins> flavonols> flavanones> isoflavones> flavones	Black tea, oranges, grapes, wine, apples
North America	230	Flavan-3-ols> flavanones > flavonols> anthocyanidins > flavones > isoflavones	Apples, wine, citrus fruit juices and tea.

7. Antioxidant Activity of Dietary Flavonoids and Cancer Incidence

All biological processes in an organism must remain in homeostasis. When the pro-oxidant load and antioxidant defence are unbalanced, reactive oxygen species (ROS) are produced, and free

radicals are generated [81]. Oxidative stress is characterised by the amount of ROS produced and is closely related to development of some diseases such as cancer caused by oxidative lesions in DNA. However, there are other mechanisms that protect organisms against oxidation, including good nutrition [81]. Thus, the interest in finding compounds with antioxidant activity such as flavonoids has increased. Among them, apigenin (a plant-derived food polyphenol, with sources such as chamomile tea and celery) seems to have strong antioxidant activity in neurological disorders [82]. Myricitrin has been isolated from Daebong persimmon peel, and this flavonoid has strong antioxidant activity through its ferric ion reducing antioxidant ability [83]. Another flavonoid, hesperetin, was shown to ameliorate oxidative stress in disease conditions such as dyslipidaemia and hyperglycaemia in a murine model [84]. In addition, in diabetic rats, galangin reduced hyperglycaemia-mediated oxidative stress and improved the antioxidant status [85]. Under abnormal conditions such as hyperammonemia in rats, quercetin was found to protect against oxidative stress and exert anti-inflammatory activity [86]. During induced oxidative stress in rats, rutin was found to act as a strong antioxidant protecting against oxidative effects [87]. Moreover, in another in vitro study, it was demonstrated that kaempferol has moderate oxygen radical absorption capacity and strong radical-scavenging activity [80]. In murine tissues, quercetin protects against induced oxidative damage [88]. Several studies have investigated the antioxidant activity of flavonoids in humans [89]. Among them, a study performed by Alipour B. et al. (2016) suggested an association between serum total antioxidant capacity and total flavonoid consumption [90]. However, they attributed antioxidant activity to anthocyanins [90]. Thus, there is evidence indicating the strong antioxidant activity of flavonoids in vitro and in vivo, and many epidemiological studies have shown that dietary flavonoids are associated with a lower incidence of cancer. Therefore, because cancer is a major health problem worldwide, it would be of value to determine if its incidence is associated with dietary flavonoid intake and what intake amount would reduce cancer risk.

The latest data collected from GLOBOCAN [2] indicate differences in total cancer incidence around the world. Asia is responsible for 48% of the total cancer incidence (Figure 5).

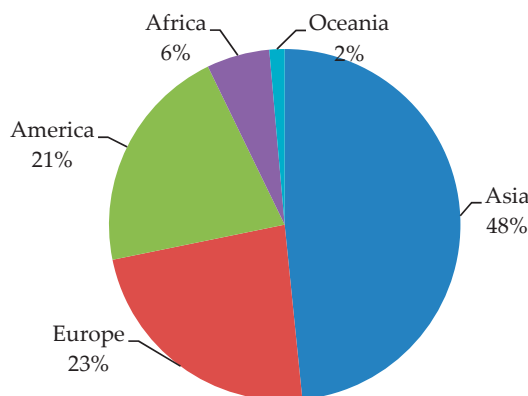


Figure 5. Worldwide cancer incidence. Data collected from GLOBOCAN (2018) [2].

However, incidence can vary according to gender for different types of cancer around the world. According to GLOBOCAN [2] data, the cancer types with the highest incidence in males are, in decreasing order, lung, prostate, stomach, liver, and colorectal cancers (Figure 6). For females, breast cancer accounts for the highest number of cancer cases, followed by lung cancer (Figure 7).

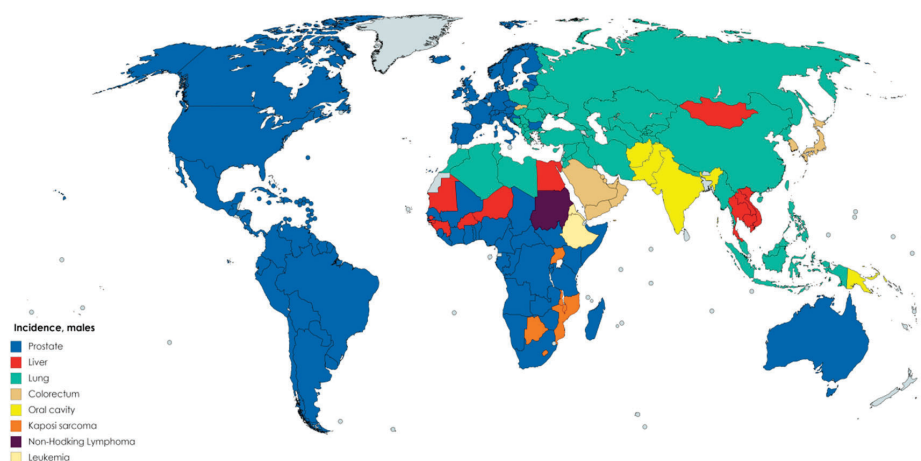


Figure 6. Worldwide cancer incidence by type in males [2]. Created with mapchart.net.

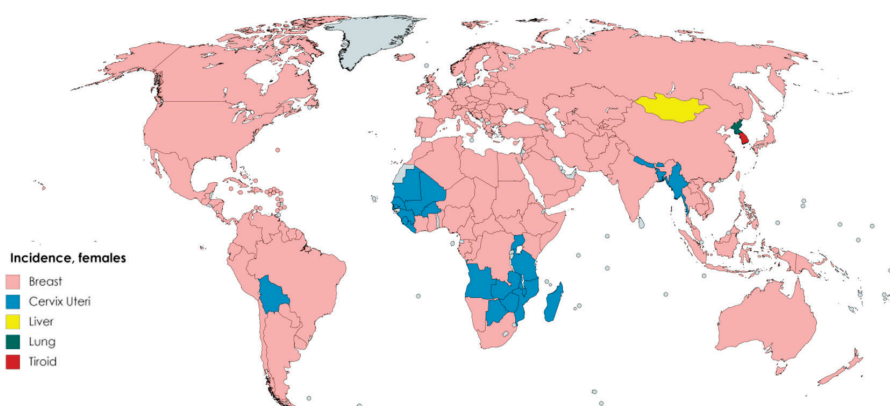


Figure 7. Worldwide cancer incidence by type in females [2]. Created with mapchart.net.

Regarding breast cancer (Table 10), in the European Prospective Investigation into Cancer and Nutrition (EPIC) study, flavonoid dietary intake and breast cancer risk were analysed in a cohort of 334,850 women with an 11.5 year follow up [91]. Within this cohort were 11,576 breast cancer cases. However, there was no statistically significant association between total flavonoid (Hazard Ratio (HR) 0.97, 95% Confidence Interval (CI): 0.90–1.07) and isoflavone (HR 1, 95%CI: 0.91–1.10) intakes and breast cancer risk [91]. Another prospective study evaluated coffee and tea intake and its relationship with breast cancer risk in black women [92]. The results showed that among the 52,062 participants, there were 1,268 incident cases of breast cancer during 12 years of follow up. The data showed that the intake of coffee (Internal Rate of Return (IRR): 1.03, 95% CI: 0.77–1.39) or tea (IRR: 1.13, 95% CI: 0.78–1.63) was not associated with the risk of breast cancer [92]. Regarding tea and coffee intake, a study performed in Sweden suggested that tea intake is positively associated with oestrogen and progesterone receptor-positive breast cancer, but that coffee consumption is negatively associated with the risk of oestrogen receptor-positive, progesterone receptor-negative breast cancer [93]. Another study performed in Shanghai attempted to associate urinary polyphenols with breast cancer risk [94]. They measured tea flavonols (kaempferol and quercetin) and polyphenols as epicatechin in a cohort with 353 cases and 701 controls. They observed an inverse

association between breast cancer risk and urinary excretion of epicatechin (Odds Ratio (OR) 0.59, 95% CI: 0.39–0.88) [94]. Thus, it was concluded that epicatechin-rich foods could reduce breast cancer risk.

There is controversy regarding the association between breast cancer and isoflavone intake because of its possible role in oestrogen metabolism. Thus, a case-control study in south-western China investigated the relationship between oestrogen metabolism, soy isoflavones, and breast cancer risk [95]. The findings suggested a protective effect of a high soy isoflavone intake on breast cancer risk based on the relation of oestrogen metabolites and breast cancer [95]. Furthermore, regarding other flavonoid subclasses, a meta-analysis of epidemiologic studies performed in 2013 demonstrated that breast cancer risk had a direct association with flavone (Relative Risk (RR): 0.83, 95% CI: 0.76–0.91) and flavonol intake in women (RR: 0.88, 95% IC: 0.80–0.98) [96]. Likewise, in another study performed by Cutler et al. (2008) that analysed cancer risk in postmenopausal women in relation with dietary flavonoid intake, an inverse association was obtained between isoflavone intake and cancer incidence (HR: 0.93, 95% CI: 0.86–1.00), and an inverse association was found between proanthocyanidin (HR: 0.75, 95% CI: 0.57–0.97) and flavanone (HR: 0.68, 95% CI: 0.53–0.86) intake and lung cancer incidence [14].

Regarding total polyphenol intake and breast cancer risk, a study performed by Gardezabal et al. (2018) that included more than 22,000 Spanish university graduates showed that menopausal status is an important factor in breast cancer risk [97]. Thus, they found no significant association between breast cancer risk and total polyphenol intake. However, in postmenopausal women, they observed an inverse association between breast cancer risk and total polyphenol intake (HR: 0.31, 95% CI: 0.13–0.77) [97]. Because they have antioxidant activity and similar chemical compositions as oestrogens, flavonoids are able to reduce menopause symptoms [98]. However, further research is needed to demonstrate the effect of flavonoid intake on pre- and post-menopause breast cancer risk.

A case-control study performed by Christensen et al. (2012) (Table 10), which analysed the association of lung cancer risk with flavonoid intake, did not find an association between flavonoid intake and risk reduction. However, a low intake of total and different subclasses of flavonoids was related to an increased risk of lung cancer. The ORs (95%CI) were 0.63 (0.47–0.85) for total flavonoids, 0.70 (0.53–0.94) for flavanones, 0.62 (0.45–0.84) for flavonols, 0.68 (0.50–0.93) for flavones, 0.67 (0.50–0.90) for flavan-3-ols, and 0.82 (0.61–1.11) for anthocyanidins [99].

A population-based case-control study carried out on a population of Sicilian men analysed the association between dietary factors, such as flavonoids, and prostate cancer risk (Table 10). The results suggested that prostate cancer risk could be reduced by a high intake of catechins (OR: 0.12, 95% CI: 0.04–0.36) and flavonol (OR: 0.19, 95% CI: 0.06–0.56). However, the risk seemed to increase with a high intake of flavanones [100].

Table 10. Association between flavonoid intake and risk of breast, lung, and prostate cancers.

Authors	Methods	Results
		Breast
Itziar Gardezabal et al. (2018) [97]	Prospective Cohort Study 10,713 Spanish Women	There was not a statistically significant association between total flavonoids and breast cancer risk. However, in postmenopausal women, the data indicate an inverse association between breast risk cancer and total polyphenol intake (HR: 0.31, 95% CI: 0.13–0.77)
	Food Frequency Questionnaire (FFQ) Phenol-Explorer database HPLC	
Oh, J.K et al. (2015) [93]	Prospective Cohort Study 42,099 Swedish Women 30–49 years FFQ	Data showed that compared with no consumption, women who consumed >1 cup tea/day had an increased breast cancer risk (Relative Risk (RR): 1.19, 95% Confidence Interval (CI): 1.00–1.42), but women with a high intake of coffee (3–4 cups/day) had a decreased breast cancer risk (RR: 0.87, 95% CI: 0.76–1.00).

Raul Zamora-Ros et al. (2013) [91]	Prospective Cohort Study 334,850 women, 35–70 years 11.5 years follow up FFQ Phenol-Explorer database	There was no statistically significant association between total flavonoid (Hazard Ratio (HR) 0.97, 95% CI: 0.90–1.07) and isoflavone (HR 1, 95%CI: 0.91–1.10) intake and breast cancer risk.
Wang et al. (2011) [95]	Case-Control Study. 400 cases and 400 controls. Daily intake of soy isoflavones data Gene sequencing	They suggested a protective role of high soy isoflavone intake against breast cancer risk based on the relation of oestrogen metabolites, breast cancer, and isoflavone metabolism.
Boggs et al. (2010) [92]	Prospective Cohort Study 52,062 women, 21–69 years 12 years follow up FFQ	Data showed that the intake of coffee (Internal Rate of Return (IRR): 1.03, 95% CI: 0.77–1.39) or tea (IRR: 1.13, 95% CI: 0.78–1.63) was not associated with risk of breast cancer in participants.
Luo JF et al. (2010) [94]	Case-Control Study 353 cases, 701 controls, 40–70 years FFQ Liquid chromatography	There was an inverse association between breast cancer risk and urinary excretion of epicatechin (Odds Ratio (OR) 0.59, 95% CI: 0.39–0.88).
Lung		
Christensen KY et al. (2012) [99]	Case-Control Study 1,061 cases and 1,425 controls FFQ	A low intake of flavonoids was related to an increased risk of lung cancer. OR: 0.63, 95% CI: 0.47–0.85.
Cutler et al. (2008) [14]	Prospective Cohort Study USDA database FFQ	There was an inverse association between isoflavone intake and cancer incidence (HR: 0.93, 95% CI: 0.86–1.00) and an inverse association between proanthocyanidin (HR: 0.75, 95% CI: 0.57–0.97) and flavanone (HR: 0.68, 95% CI: 0.53–0.86) intake with lung cancer incidence.
Prostate		
Giulio Reale et al. (2018) [100]	Case-Control study 118 cases and 222 controls FFQ Prostate Specific Antigen	High intake of some subclasses of flavonoids (catechin (OR: 0.12, 95% CI: 0.04–0.36) and flavonol (OR: 0.19, 95% CI: 0.06–0.56) significantly reduces the risk of prostate cancer.

Gastric cancer is the second main cause of cancer deaths and the fourth most common cancer worldwide (Table 11) [68]. In a case-control study performed in Korea, a significant association was found between total flavonoid intake and gastric cancer risk reduction in women (OR 0.33, 95% CI 0.15–0.73) but not in men [101]. Furthermore, the EPIC study investigated the association between gastric adenocarcinoma risk and flavonoid intake [68]. They observed an inverse association between gastric adenocarcinoma risk and total flavonoid intake in women (HR 0.81, 95% CI 0.70, 0.94). This association was observed for some flavonoid subclasses such as flavanols, flavones, flavonols, and anthocyanidins [68]. However, in a prospective study carried out in the United States that analysed all cancers, researchers observed that flavonoid intake was associated with protection against neck and head cancer risk but not gastric cancer risk [102].

Pancreatic cancer has the worst prognosis of all cancers (Table 11), and its mortality/incidence ratio is 0.98 [96]. However, a study performed on the EPIC cohort examined the association between pancreatic cancer risk and flavonoid intake and found an inverse association between them, although it was not statistically significant [103].

Regarding colorectal cancer (Table 11), it has been demonstrated that flavonoids are able to inhibit the growth of colon cancer cells in vitro [104]. However, in human-based studies, the results are different. In a prospective study that examined daily flavonoid intake and its relationship with

colorectal cancer, the data showed there was no association between the risk of colorectal cancer and flavonoid intake [104]. Important results were obtained in a study performed by Xu M. et al. (2016). Their data showed that there was an inverse association between flavonoid intake as anthocyanidins, flavanones, and flavones and colorectal cancer risk. However, this only occurred when the sources of flavonoids were fruits and vegetables [105]. A case-control study performed on a Spanish population found an inverse association between the risk of colorectal cancer and intake of total flavonoids (OR: 0.59, 95% CI, 0.35–0.99) and some flavonoid subclasses (such as proanthocyanidins and flavones) [106]. The same researchers performed a case-control study to analyse the relationship between flavonoid intake and colorectal cancer recurrence and survival. However, their results did not support the beneficial effects of flavonoids on colorectal cancer prognosis [107].

Table 11. Association between flavonoid intake and risk of gastric, pancreatic, and colorectal cancers.

Authors, Year	Methods	Results
Gastric Adenocarcinoma		
Sun L et al. (2017) [102]	Prospective cohort study 469,008 participants 12 years follow up FFQ	Data suggested that total flavonoid intake was associated with a reduced risk of neck and head cancer (HR: 0.76, 95% CI: 0.66–0.86).
Hae Donw Woo et al. (2014) [101]	Case-Control study. 334 cases and 334 controls, aged 35–75 years, from Korea. FFQ	Total flavonoids and their subclasses were significantly associated with a reduced risk of gastric cancer in women (OR 0.33, 95% CI 0.15–0.73) but not in men.
Zamora-Ros et al. (2012) [68]	USDA database Observational study 477,312 subjects, aged 35–70 years, from 10 European countries. Average Follow-up of 11 years FFQ USDA and Phenol Explorer Databases.	Total dietary intake was associated with a significant reduction in the risk of gastric adenocarcinoma in women (HR 0.81, 95% CI 0.70, 0.94).
Pancreatic Cancer		
Molina-Montes E. et al. (2016) [103]	Prospective cohort 477,309 participants Average Follow-up of 11 years FFQ USDA and Phenol Explorer Databases.	There was no association with pancreatic cancer risk and dietary flavonoid intake (HR: 1.09, 95% CI: 0.95–1.11); however, there was an inverse association, but not statistically significant, between prostate cancer risk and flavanone intake.
Colorectal Cancer		
Nimptsch K et al. (2016) [104]	Prospective cohort 42,478 male 76,364 female 26 years follow up FFQ	Data did not show an association between colorectal cancer risk and flavonoid subclass intake. RR (95% IC) values were 0.98 (0.81, 1.19) for anthocyanins, 1.07 (0.95, 1.21) for flavan-3-ols, 0.96 (0.84, 1.10) for flavanones, 1.01 (0.89, 1.15) for flavones, and 1.04 (0.91, 1.18) for flavonols.
Xu M. et al. (2016) [105]	Case-Control study 1,632 cases and 1,632 controls FFQ	There was an inverse association between colorectal cancer risk and flavone (OR: 0.54, 95% CI 0.43, 0.67), flavanone (OR: 0.28, 95% CI: 0.22–0.36), and anthocyanidin (OR: 0.80, 95% CI: 0.64–1.00) intake.

Zamora Ros et al. (2015) [107]	Case-control study 523 participants FFQ	Flavonoid intake was not associated with colorectal cancer survival or recurrence.
Zamora Ros et al. (2013) [108]	Case-Control study 424 cases and 401 hospital-based controls FFQ Phenol Explorer Database	Data showed an inverse correlation between risk of colorectal cancer and intake of total flavonoids (OR: 0.59, 95% CI, 0.35–0.99) and some flavonoid subclasses (such as proanthocyanidins and flavones)

8. Conclusions

In summary, there remains controversy regarding the possible protective effect of flavonoids on cancer in epidemiological studies. However, this association could vary depending on many factors such as geographical location and diet. It appears that some flavonoid subclasses suggest a decrease of the risk of different types of cancer, such as catechin and flavonols for prostate cancer, epicatechin for breast cancer, proanthocyanidins for lung cancer, flavones for colorectal cancer, and total flavonoids for gastric cancer. Thus, because the main sources of these flavonoids are different, the risk of cancer could be reduced by including them in a healthy diet, which would be mainly based on vegetables and fruits, whole grain cereals, legumes, seeds, and nuts, as well as cocoa, coffee, fruit juices, and tea. However, further studies are needed to investigate and confirm this hypothesis that a healthy diet can help decrease the incidence of different types of cancer.

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Protein interventions augment the effect of resistance exercise on appendicular lean mass and handgrip strength in older adults: a systematic review and meta-analysis of randomized controlled trials

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Protein interventions augment the effect of resistance exercise on appendicular lean mass and handgrip strength in older adults: a systematic review and meta-analysis of randomized controlled trials

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ABSTRACT

Background: Increased protein intake is suggested as a strategy to slow or reverse the loss of muscle mass and strength observed in sarcopenia, but results from studies that directly tested this possibility have been inconsistent.

Objectives: We assessed the evidence on the effects of whole protein supplementation or higher-protein diets, without the use of amino acids or supplements known to stimulate hypertrophy, alone or in combination with resistance exercise (RE) interventions, on lean body mass (LBM) and strength in older adults.

Methods: A systematic search was conducted using PubMed, Medline, Web of Science, and Cochrane CENTRAL databases from January 1990 to July 2021. Randomized controlled trials that assessed the effects of protein supplementation and/or higher-protein dietary interventions in older adults (mean age ≥ 50 y) on total LBM, appendicular lean mass (ALM), and handgrip (HG) and knee extension (KE) strength were included.

Results: Twenty-eight studies were identified. In pooled analysis, compared with lower protein controls, protein supplementation did not have a significant positive effect on total LBM [weighted mean difference in change (WMD): 0.34; 95% CI: -0.21, 0.89; $I^2 = 90.01\%$], ALM (WMD: 0.4; 95% CI: -0.01, 0.81; $I^2 = 90.38\%$), HG strength (WMD: 0.69; 95% CI: -0.69, 2.06; $I^2 = 94.52\%$), or KE strength (WMD: 1.88; 95% CI: -0.6, 4.35; $I^2 = 95.35\%$). However, in interventions that used also RE, statistically significant positive effects of protein were observed for ALM (WMD: 0.54; 95% CI: 0.03, 1.05; $I^2 = 89.76\%$) and HG (WMD: 1.71; 95% CI: 0.12, 3.30; $I^2 = 88.71\%$). Meta-regression revealed no significant association between age, per-meal protein dose, duration, and baseline protein intake with change in any outcome. Subgroup analysis revealed the statistically significant effects on ALM occurred only in sarcopenic/frail populations (WMD: 0.88; 95% CI: 0.51, 1.25; $I^2 = 79.0\%$). Most studies ($n = 22$) had some risk of bias.

Conclusions: In older adults performing RE, increased protein intake leads to greater ALM and HG strength compared with lower protein controls. Without RE, protein has no additional benefit on changes in total LBM, ALM, or HG strength. *Am J Clin Nutr* 2021;00:1–17.

Keywords: protein, resistance exercise, lean body mass, sarcopenia, aging, frailty, strength, elderly

Introduction

By 2050, more than 1 in 5 people worldwide will be >60 y old (1), but although life expectancy is increasing, health span (years free of disease and disability) is not keeping pace (2). A major contributor to poor health and disability in later life is sarcopenia, the age-associated decline in muscle size, strength, and quality (3), which accelerates considerably in one's 50s (4). Sarcopenia is positively associated with a great variety of

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Data availability: Data described in the manuscript, code book, and analytic code will be made available upon request pending application and approval.

Supplemental Table 1 and Supplemental Figure 1 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at <https://academic.oup.com/ajcn/>.

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Abbreviations used: ALM, appendicular lean mass; BIA, bioimpedance analysis; HG, handgrip; IPD, individual participant data; ITT, intention to treat; KE, knee extension; LBM, lean body mass; MPS, muscle protein synthesis; PP, per protocol; RCT, randomized controlled trial; RE, resistance exercise; RM, repetition maximum; RNI, reference nutrient intake; WMD, weighted mean difference in change.

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Synergistic Effect of Squalene and Hydroxytyrosol on Highly Invasive MDA-MB-231 Breast Cancer Cells

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Article

Synergistic Effect of Squalene and Hydroxytyrosol on Highly Invasive MDA-MB-231 Breast Cancer Cells

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Abstract: Several studies relate Mediterranean diet and virgin olive oil (VOO) intake with lower risk of several chronic diseases, including breast cancer. Many of them described antitumor properties of isolated minor compounds present in VOO, but beneficial properties of VOO arise from the effects of all its compounds acting together. The aim of the present study was to test the antitumor effects of two minor compounds from VOO (hydroxytyrosol (HT) and squalene (SQ)) on highly metastatic human breast tumor cells (MDA-MB-231) when acting in combination. Both isolated compounds were previously analyzed without showing any antitumoral effect on highly invasive MDA-MB-231 breast cancer cells, but the present results show that HT at 100 μ M, combined with different concentrations of SQ, could exert antitumor effects. When they are combined, HT and SQ are able to inhibit cell proliferation, promoting apoptosis and DNA damage in metastatic breast cancer cells. Therefore, our results suggest that the health-promoting properties of VOO may be due, at least in part, to the combined action of these two minor compounds.

Keywords: virgin olive oils; breast cancer; antitumor; proliferation; apoptosis; DNA damage; comet assay



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1. Introduction

Among all types of cancer, breast cancer is one of the most common causes of mortality among women in developed countries [1]. Many environmental factors, such as diet, can affect breast cancer development [2]. In this context, the Mediterranean diet pattern is associated with low incidence of breast cancer [3]. Several authors associate these health effects with virgin olive oil (VOO), the main source of fat in the Mediterranean diet [4,5]. Growing scientific evidence suggests that healthy properties of VOO reside in their minority compounds such as polyphenols, triterpenes, tocopherols and lignans [6,7]. Among them, polyphenols were found to be related to the prevention of diabetes, neurological and cardiovascular diseases, and cancer [8].

Hydroxytyrosol (HT) is one of the main phenolic compounds present in VOO, although it appears in a smaller amount than tyrosol [9]. Several studies have demonstrated biological activities of HT, both in vitro and in vivo. HT is a strong antioxidant that exerts a scavenger function of oxygen and nitrogen free radicals [10]. Furthermore, HT protects DNA against oxidative damage in neuronal hybridoma cells [11]. It also has anti-inflammatory and analgesic properties [12], and exerts antitumor activities in colon and breast cancer cells [13,14], with pro-apoptotic effects through the modulation of gene expression [15]. The mechanism by which HT exerts its effects on cancer cells is not clear, but it could produce a reduction in Pin1 levels that causes the translocation of cyclin D1 to

the cytoplasm, where it is degraded. Cyclin D1 is a necessary protein to the G1/S cell cycle transition and, consequently, for tumor cell growth [16]. Furthermore, in a previous study conducted by our group, HT was able to protect DNA in breast normal cells in vitro [17], pointing it out as a protector against the development of breast cancer.

Squalene (SQ) is the main triterpene hydrocarbon present in VOO. VOO is the highest source of SQ compared to other vegetable fats [18]. SQ has a lot of interesting activities such as antioxidant and antitumor effects [19]. Among other bioactivities, SQ exerts a potent inhibition of aberrant hyperproliferation in mammary epithelial cells [20].

SQ is a metabolite involved in the biosynthesis of cholesterol and it is oxidized by monooxygenase in the early stages of metabolism. However, its monooxygenase activity is highly suppressed by the accumulation of cholesterol [21]. Therefore, when squalene is ingested by diet, squalene-derived sterols accumulate in the cells and β -hydroxy- β -methylglutaryl-CoA reductase (HMG-CoA reductase) is inhibited [21]. Furthermore, recent evidence has shown that squalene excess leads to an accumulation of farnesyl pyrophosphate (FFP), which could be related to the suppression of carcinogenesis [22,23]. In fact, dietary ingestion of SQ can inhibit colon cancer development in vivo [24]. On the other hand, it was suggested that SQ enhances the action of the immune system against tumors [25] and protects breast cells against the accumulation of mutagenic lesions in their DNA, according to a previous work published by our group [26].

The bioprotective and antitumoral activities of VOO have been linked to its contents of minor compounds. Several studies remarked on different bioactivity properties of isolated VOO compounds [6,9–26], but VOO's health-promoting properties could be attributed to synergistic effects of the nutrients present in it, such as minor compounds and fatty acids. HT and SQ are two of the minor compounds of VOO and both isolated compounds have been previously described to possess a preventive role in breast epithelial cells but without significant antitumoral activity in highly invasive breast cancer cells [17,26]. We hypothesized that the well-known chemopreventive effects of VOO against breast cancer may be due not to an isolated compound, but the synergistic activity of several minor compounds acting together. The present study attempts to demonstrate the possible synergistic antitumoral effect of these two minority compounds in breast cancer cells.

2. Materials and Methods

2.1. Materials

TrypLE Express and Minimum Essential Medium with Eagle's salts (MEM) were obtained from Gibco® Life Technologies Ltd. (Paisley, UK). Fetal bovine serum (FBS) was purchased from de PAA Laboratories GmbH (Pasching, Austria). The following products were obtained from Sigma-Aldrich Co. (St Louis, MO, USA): 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt tablets (CAS 30931-67-0 (ABTS)); (S)-(+)-camptothecin (CAS 7689-03-4 (CPT)) purity $\geq 90\%$; 2,2-Diphenyl-1-picrylhydrazyl (DPPH) purity $\sim 90\%$; 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox™ CAS 53188-07-1 (TR)) purity $\geq 97\%$; Phosphate Buffer Saline (PBS); DL-all-rac- α -tocopherol (Vitamin E-CAS 10191-41-0 (TOC)) purity $\geq 96\%$; Dimethyl sulfoxide (DMSO); 2',7'-dichlorofluorescein diacetate (DCFH-DA); Non-Essential Amino Acids mixture 100 $\times$ (NEAA); Sodium Pyruvate; Hepes Buffer; 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,20-tetracosahexane (Squalene CAS 111-02-4). 2-(3,4-dihydroxyphenyl)-ethanol (Hydroxytyrosol CAS 10597-60-1) was acquired from Cayman Chemical (Ann Arbor, MI, USA). Binding Buffer and FITC-conjugated Annexin V were purchased from Miltenyi Biotec (Cologne, Germany). The PI/RNase Staining Buffer kit was purchased from BD Biosciences Pharmingen (San Diego, CA, USA). Phosphate Buffer Saline (PBS 1x) (Ca^{2+} / Mg^{2+} free) was obtained from (Gatersleben, Germany). CellTiter-Blue® Cell Viability Assay was obtained from Promega Corporation (Madison, WI, USA).

2.2. Scavenging Radical Activity Estimation by DPPH Assay

Antioxidant activity of the HT-SQ combination (HT-SQ) against DPPH radical was performed as previously described [27], incorporating certain changes. An ethanolic solu-

tion 100 μM of DPPH was mixed in 96-well plates with various solutions of antioxidant standard (α -tocopherol) or HT-SQ in ratios of 0.03, 0.13, 0.5 and 2 moles of antioxidant/ moles of DPPH. Absorbance at 520 nm was read every 5 min during 2 h using a microplate reader (TECAN GENios Plus, Tecan Group Ltd., Männedorf, Switzerland). Triplicate measurements were conducted in 3 independent experiments. Furthermore, DPPH samples were measured as blank controls. The radical scavenging activity percentage (% RSA) was estimated using the formula below:

$$\% \text{ Radical Scavenging Activity} = 100 (A_{C(0)} - A_{A(t)}) / A_{C(0)}$$

$$A_{C(0)} = \text{Control absorbance at } t_0$$

$$A_{A(t)} = \text{HT-SQ/standard absorbance at } t_{60}$$

2.3. ABTS Radical Scavenging Assay

ABTS cation radical scavenging activity was analyzed according to a previously described protocol [28], adding certain modifications. ABTS radical cations ($\text{ABTS}^{\bullet+}$) were generated through the reaction of 2.45 mM $\text{K}_2\text{S}_2\text{O}_8$ with 7 mM ABTS over 16 h at room temperature (RT) in darkness. $\text{ABTS}^{\bullet+}$ obtained was diluted in ultrapure water (UPW) until absorbance ranged between 0.7 and 1 at 734 nm. Antioxidant standard (Ethanol (EtOH) solutions of TroloxTM) were diluted in UPW to achieve concentrations of 50 to 800 μM . HT-SQ were diluted in UPW to reach concentrations between 0.01 and 100 μM . TroloxTM, UPW (blank) or EtOH control (8%) were added in a flat-bottom 96-well plate. Different HT-SQ concentrations were added in the 96-well plate. Reactions were initiated by adding $\text{ABTS}^{\bullet+}$. Immediately, absorbance was measured at 734 nm every 5 min for 2 h at 30 °C with a microplate reader. Triplicate measurements were conducted in 3 independent experiments. % RSA was calculated as previously described (at t_{60}).

2.4. Cell Culture

MDA-MB-231 cells (highly invasive and triple-negative human breast cancer cells) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in monolayer cultures in MEM supplemented with 1% NEAA, 1% Sodium Pyruvate, 1% HEPES Buffer and 10% FBS. Cells were subcultured using TrypLE Express solution and were maintained at 37 °C in a humidified atmosphere with 5% CO_2 . For all experiments, cells in the exponential growth phase were used. Different combinations of HT-SQ were tested for DPPH, ABTS, cytotoxicity and proliferation assays: HT ranged from 0.01 to 100 μM combined with SQ from 0.01 to 100 μM .

2.5. Cytotoxicity Assay

Cell viability was tested after treatment of MDA-MB-231 with different concentrations of HT-SQ using the CellTiter-Blue[®] Cell Viability Assay based on the manufacturer's specifications with certain amendments. Briefly, a total of 5×10^3 /well cells in 100 μL were cultured in 96-well plates. After overnight (O/N) incubation to ensure cell adhesion, cells were treated with increasing HT-SQ combinations (0.01 to 100 μM) for a further 24 h. Thereafter, cells were incubated for 3 h in darkness at 37 °C in 5% CO_2 with CellTiter-Blue Cell Viability. Cells were treated with EtOH and DMSO alone and a combination of both as vehicle control. Relative fluorescence was measured in a plate reader (Ex. λ_{485} /Em. λ_{595} , Gain 60). All treatment combinations were conducted in triplicate in 3 independent experiments. Cell viability was estimated using the formula:

$$\text{Cell viability percentage} = [A (\text{treated cells}) / A(\text{control})] \times 100$$

$$A = \text{relative fluorescence units of each sample}$$

2.6. Cell Proliferation Assay

Cell proliferation was conducted using a CellTiter-Blue Cell Viability Assay based on the manufacturer's specifications, with some adaptations. It was measured as cell growth of untreated controls versus treated cells. Briefly, a total of 1×10^3 /well cells in 100 μ L were cultured into 96-well plates. After O/N incubation to ensure cell fixation, cells were treated with increasing combinations of HT-SQ, from 0.01 to 100 μ M. After 24 h of treatment, fresh medium was added and cells were incubated for another 24 h. Then, cells were incubated for 3 h in darkness at 37 °C in 5% CO₂ with the CellTiter-Blue Cell Viability Assay kit and relative fluorescence units were measured in a plate reader (Ex. λ_{485} /Em. λ_{595} , Gain 60). Measurements were repeated after 72 and 96 h of treatments. As vehicle control, cells were treated with EtOH and DMSO alone and a combination of both. All treatment combinations were conducted in triplicate in 3 independent experiments. Cell viability was estimated with the following equation:

$$\text{Cell viability percentage} = [A(\text{treated cells})/A(\text{control})] \times 100$$

A = relative fluorescence units of each sample

2.7. Cell Cycle Assay

A total of 5×10^4 /well cells in 1 mL were cultured into 12-well plates and incubated O/N to ensure cell attachment. Afterward, cells were treated with 100 μ M of HT combined with a range of SQ, from 0.01 to 100 μ M, for 48 h. Thereafter, cells were harvested with TrypLE Express and washed with cold $1 \times$ PBS (300 $\times$ g, 10 min at 4 °C). Eventually, cells were fixed in cold 70% EtOH and kept at −20 °C for at least 24 h. After propidium iodide labeling (PI/RNase Staining Buffer), cells were analyzed by flow cytometry (MACSQuant[®] Analyzer 10, Miltenyi Biotec, Bergisch Gladbach, Germany). The MACSQuantify Software program was used for calculating the % of cells in different phases (G0/G1, S and G2/M). Each experiment was run independently 3 times.

2.8. Analysis of Apoptosis

Apoptotic cell percentage was assessed by a double staining assay with PI and FITC-conjugated Annexin V. A total of 5×10^4 /well cells in 1 mL were cultured in 12-well plates and incubated O/N to ensure attachment of cells. Afterward, cells were treated with 100 μ M of HT combined with a range of SQ, from 0.01 to 100 μ M, for 48 h. Thereafter, cells were harvested with TrypLE Express, centrifuged (300 $\times$ g; 10 min at 4 °C) and resuspended in 100 μ L of Annexin Binding Buffer 1x. Cells were stained with PI and Annexin V-FITC solution; then, they were gently vortexed and incubated for 15 min at RT in darkness prior to flow cytometry analysis. Cells were treated with camptothecin (CPT) as positive control. Every experiment was repeated independently 3 times.

2.9. Detection of Intracellular Reactive Oxygen Species

Intracellular reactive oxygen species (ROS) levels were evaluated using a cell-permeable fluorescent probe, 2',7'-dichlorofluorescein diacetate (DCFH-DA), as previously described by Wang and Joseph [29], adding some modifications. Briefly, 3.5×10^3 /well cells in 100 μ L were cultured in 96-well plates. After O/N incubation to allow cell attachment, cells were treated with 100 μ M of HT combined with a range of SQ, from 0.01 to 100 μ M, for 48 h. Thereafter, DCFH-DA (100 μ M) was added during 30 min at 37 °C with 5% CO₂. Fluorescence was measured in a plate reader during 30 min (Ex. λ_{485} /Em. λ_{535} , Gain 60). Intracellular ROS levels were calculated using the following formula:

$$F = [(F(t = 30 \text{ min}) - F(t = 0 \text{ min})) / F(t = 0 \text{ min}) \times 100]$$

In culture cells, the addition of H₂O₂ was shown to increase oxidative stress and to damage DNA directly [30]. Therefore, 400 μ M H₂O₂ was added 30 min prior to fluorescence

quantification to assess the protective capacity of HT-SQ treatments against oxidative stress induction.

All tests were performed six-fold for each experimental situation, and each experiment was repeated 3 times. Experiments were carried out using iron-free media (MEM).

2.10. Alkaline Single-Cell Gel Electrophoresis (Comet Assay)

Cells were cultured into a 12-well plate (1×10^5 cells/well) and incubated O/N to reach cell attachment. Cells were treated with different HT-SQ combinations. Then, cells were scraped and washed twice with cold 1X PBS ($300 \times g$ 10 min, $4^\circ C$). They were resuspended in 1 mL of cold 1x PBS. Cells were treated with $50 \mu M$ H_2O_2 for 10 min at $4^\circ C$ to assess the ability of HT ($100 \mu M$)-SQ (100, 10 and $1 \mu M$) in protecting against oxidative DNA damage. Then, the comet assay was conducted as described in Warleta et al. [17]. Each experiment was repeated independently at least 3 times.

DNA strand breaks were screened using the Komet 5.5 software package (Kinetic Imaging Ltd., Liverpool, UK) in a fluorescence microscope (Zeiss Axiovert 200) equipped with a Luca EMCCD camera (Andor Technology, Belfast, UK) (Ex. 494 nm/Em; 521 nm wavelength). We randomly characterized 25 cell images per sample at a magnification of $20\times$. Relative fluorescence between head and tail, determined through the olive tail moment (Olive_TM), was used to determine DNA damage. Olive_TM is defined as the product of the Tail Moment Length and the fraction of DNA in the tail.

$$\text{Olive\_TM} = [(\text{tail (mean)} - \text{head (mean)}) \times (\% \text{ DNA})]/100$$

2.11. Statistical Analysis

The results are shown as the mean of 3 independent experiments ($\pm$ SEM), and are expressed as a relative percentage of untreated control (set as 100%). Statistical analysis was conducted using a one-way analysis of variance (ANOVA) followed by Fisher's LSD test with the STATGRAPHICS Centurion XVI software (version 16.0.10) (Statpoint Technologies, Inc., Warrenton, VA, USA). p values < 0.05 were considered statistically significant.

3. Results

3.1. Estimation of Radical Scavenging Activity by the DPPH Test

The antiradical activity of the HT and SQ combinations, measured by DPPH assay, showed that HT at 2 mol ratio combined with all ratios of SQ (2, 0.5, 0.13 and 0.03 mol ratio) exhibited antioxidant activity (RSA $> 40\%$ in the four combinations) (Table 1). This antioxidant capacity was not observed when HT was below 2 mol ratio (data not shown). α -tocopherol was used as an antioxidant standard control.

Table 1. Percentage of Free Radical Scavenging Activity of HT-SQ and α -tocopherol measured by the reduction in the DPPH Radical (% RSA at $t = 60$).

mol HT/mol DPPH-mol SQ/mol DPPH	HT-SQ (%)	mol α -tocopherol/mol DPPH	α -tocopherol (%)
2-2	43.66 ± 5.4	2	67.47 ± 3.26
2-0.5	41.12 ± 4.93	0.5	64.34 ± 3.71
2-0.13	52.16 ± 3.22	0.13	30.85 ± 10.06
2-0.03	44.62 ± 3.3	0.03	4.26 ± 8.8

Data are represented as mean $\pm$ SEM of 3 independent experiments at $t = 60$ min.

3.2. Radical Scavenging Activity by the ABTS Assay

The ABTS antiradical assay showed that HT at $100 \mu M$, combined with all concentrations of SQ (from 0.01 to $100 \mu M$), exhibited a high scavenging activity (RSA $> 60\%$ at five combinations) (Table 2). HT at $10 \mu M$ combined with all the concentrations of SQ showed weak antiradical activity. HT below $10 \mu M$ combined with SQ did not present scavenging activity (data not shown).

Table 2. Percentage of Free Radical Scavenging Activity of the combination of both HT and SQ and TroloxTM measured by ABTS assay (% RSA at t = 60). Values in the first and third columns represent HT and SQ concentrations, respectively. Values in the fifth column represent TroloxTM concentrations.

HT-SQ (μM)	% RSA	Trolox TM (μM)	% RSA
100–100	83.07 ± 1.27	800	85.92 ± 0.47
100–10	64.05 ± 8.57	400	76.79 ± 2.12
100–1	84.96 ± 0.68	200	43.41 ± 1.54
100–0.1	82.02 ± 1.76	100	24.82 ± 1.71
100–0.01	79.61 ± 2.07	50	14.75 ± 2
10–100	11.45 ± 2.43		
10–10	11.16 ± 2.25		
10–1	10.92 ± 2.73		
10–0.1	10.54 ± 2.43		
10–0.01	8.07 ± 2.39		

Data are represented as mean ± SEM of 3 independent experiments at t = 60 min.

3.3. Cytotoxicity Assay

To analyze the potential cytotoxic effects of HT-SQ, cells were treated with combinations of both HT and SQ, from 0.01 μM to 100 μM, for 24 h. The results are expressed as % of cell survival with respect to the untreated control, which was set as 100%. None of the tested combinations promoted cell death with statistical differences compared to the untreated control (Figure 1).

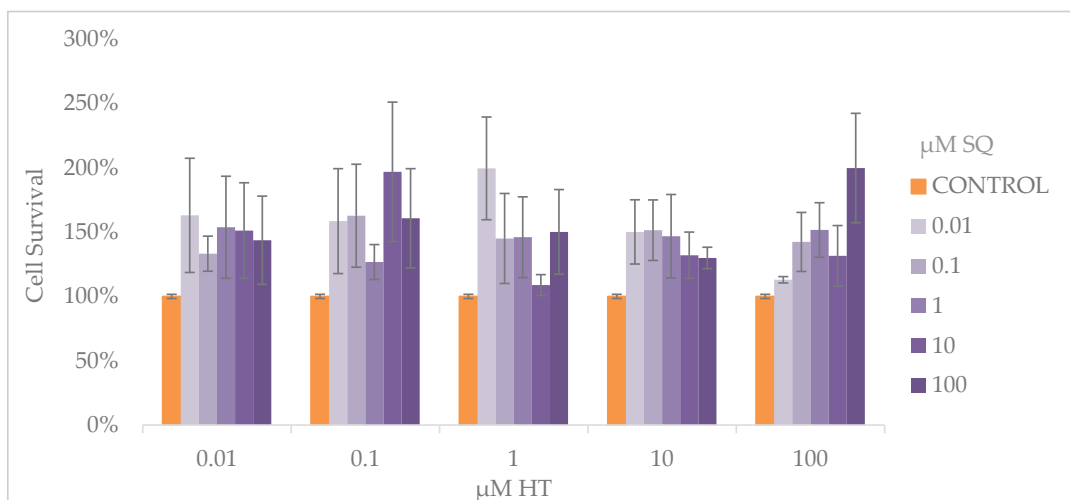


Figure 1. Cytotoxicity of HT-SQ from 0.01 μM to 100 μM in MDA-MB-231 cells. Data are represented as mean ± SEM of 3 independent experiments.

3.4. Cell Proliferation Assay

The proliferation of MDA-MB-231 cells was determined after treatment of HT-SQ from 0.01 to 100 μM for 24 h, and was measured again after another 24 h with fresh medium. After each 24 h, proliferation was measured in a microplate reader. The results are expressed as % of cell survival concerning the untreated control that was set as 100% (Figure 2). After 48 h, inhibition of the cell survival was observed with HT at 100 μM and all SQ concentrations assayed (Figure 2a). The same antiproliferative effect was maintained by these combinations at 72 h (Figure 2b) and after 96 h (Figure 2c) with statistical differences.



Figure 2. Cell proliferation after 48 h (a), 72 h (b) and 96 h (c) of a combination of HT-SQ from 0.01 μM to 100 μM in MDA-MB-231 cells. Data are represented as mean $\pm$ SEM of 3 independent experiments (* $p < 0.05$ compared to untreated control).

3.5. Cell Cycle Assay

Due to the results observed during the proliferation assay, MDA-MB-231 cells were treated with HT at 100 μM combined with SQ (from 0.01 to 100 μM) for 24 h and fresh medium was added for another 24 h. The results are expressed as cell percentages in the different phases of the cell cycle. None of the selected combinations produced cell cycle alterations in a statistically significant way (Figure 3). However, all the tested combinations produced an increment of the percentage of cells in the S phase. They also diminished (without statistical significance) the percentage of cells in the G0/G1 phase.

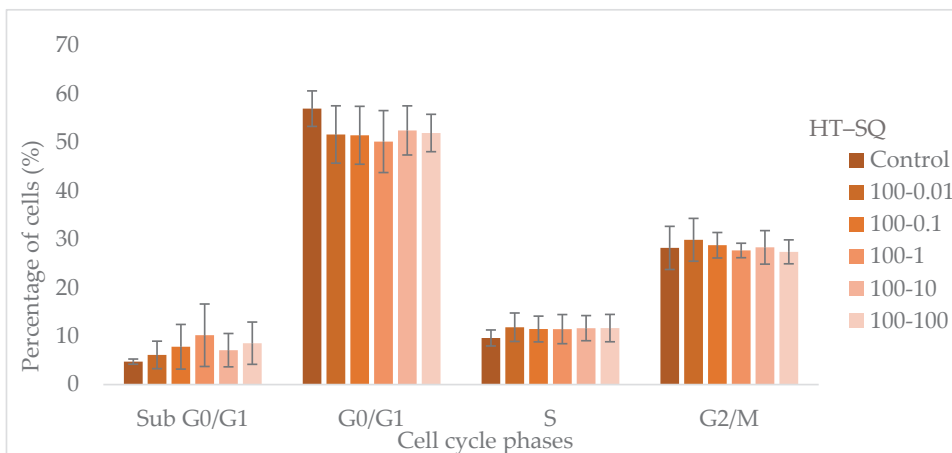


Figure 3. Distribution of cells in phases of the cell cycle for MDA-MB-231 cells after 24 h of treatment with HT at 100 μM combined with SQ at different concentrations (from 0.01 to 100 μM) and another 24 h of fresh medium. Data are represented as mean $\pm$ SEM of 3 independent experiments.

3.6. Analysis of Apoptosis

The percentages of living, apoptotic, and necrotic cells are represented with respect to the total, which was set as 100%. HT at 100 μM combined with SQ (from 0.01 to 100 μM) for 24 h plus 24 h of fresh medium were analyzed by flow cytometry. HT at 100 μM combined with all concentrations of SQ produced apoptosis in MDA-MB-231 cells (Table 3). HT at 100 μM combined with SQ at 10 and 100 μM produced apoptotic cells with statistical significance.

Table 3. Apoptosis of MDA-MB-231 cells treated with HT at 100 μM combined with SQ (from 0.01 to 100 μM) after 24 h of treatment plus 24 h of fresh medium.

HT-SQ (μM)	Live Cells	Apoptotic Cells	Necrotic Cells
Control	85.63 $\pm$ 3.06	11.38 $\pm$ 2.02	2.98 $\pm$ 1.14
100-0.01	76.13 $\pm$ 4.66	19.29 $\pm$ 2.61	4.58 $\pm$ 2.08
100-0.1	73.64 $\pm$ 1.99	22.52 $\pm$ 2.03	3.84 $\pm$ 1.67
100-1	75.32 $\pm$ 4.31	20.82 $\pm$ 2.18	3.87 $\pm$ 1.45
100-10	69.17 $\pm$ 5.87 *	25.68 $\pm$ 2.77 *	5.15 $\pm$ 2.12
100-100	69.34 $\pm$ 5.86 *	26.6 $\pm$ 2.98 *	4.07 $\pm$ 1.48

Data are represented as mean $\pm$ SEM of 3 independent experiments (* $p < 0.05$ compared to untreated control).

3.7. Detection of Intracellular Reactive Oxygen Species

Cells treated for 24 h with HT combined with SQ (from 0.01 to 100 μM), followed by another 24 h with fresh medium, were analyzed to determine ROS levels. None of the selected combinations decreased the ROS levels in MDA-MB-231 cells (Figure 4a).

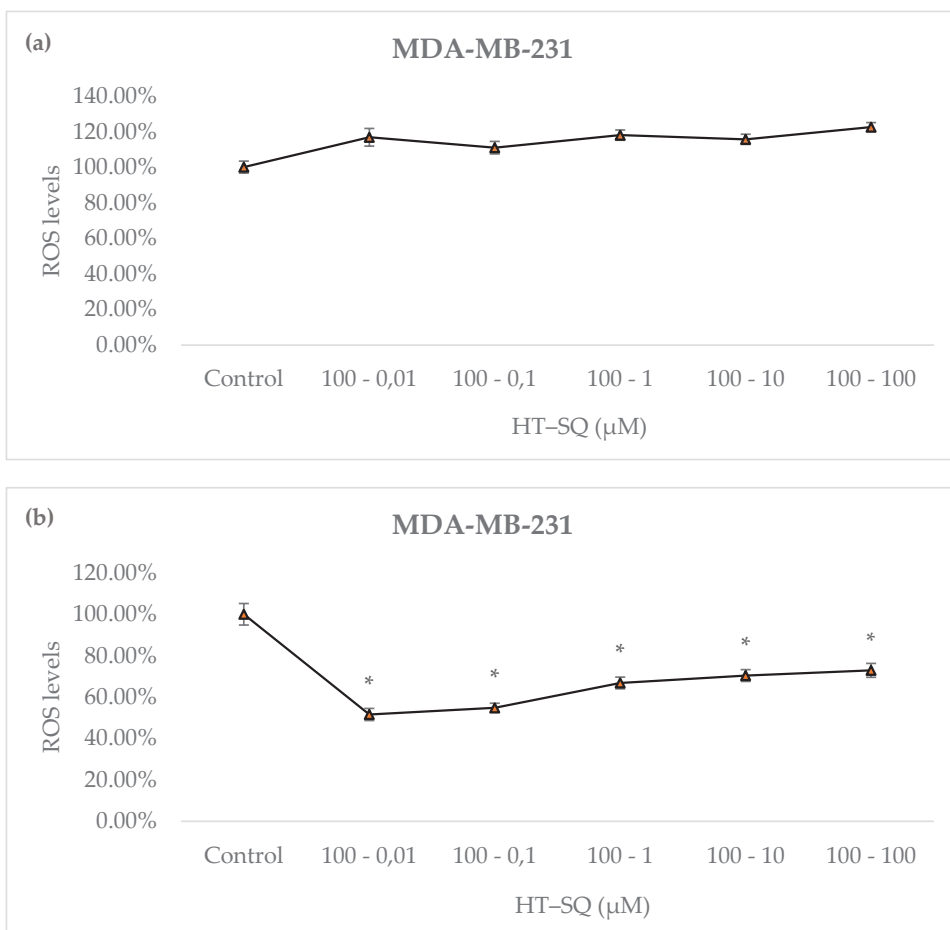


Figure 4. ROS levels represented in MDA-MB-231 cells in basal state (a) and with H_2O_2 burst (b) after treatment with HT at 100 μM , combined with SQ (from 0.01 to 100 μM) for 24 h, followed by another 24 h with fresh medium. Data are represented as mean $\pm$ SEM of 3 independent experiments (* $p < 0.05$).

H_2O_2 was added prior to fluorescence measurement to induce intracellular oxidative stress. To investigate the in vitro preventive effect of HT-SQ against H_2O_2 oxidative injury, intracellular levels of ROS were measured in cells that were previously treated with HT at 100 μM combined with all concentrations of SQ (from 0.01 to 100 μM) for 24 h followed by another 24 h with fresh medium. The results showed that at the lowest SQ concentration (0.01 μM), ROS levels were decreased (~50%). At higher SQ concentrations, levels of ROS increased in a dose-dependent manner (Figure 4b).

3.8. Analysis of DNA Damage

DNA damage in MDA-MB-231 was measured by unicellular alkaline electrophoresis after HT (100 μM)-SQ (100, 10 and 1 μM) treatments for 24 h followed by another 24 h in fresh medium. Due to apoptosis results, only three different concentrations of SQ were used to analyze the formation of possible DNA breaks. As Table 4 shows, HT-SQ treatment did not damage DNA, except for HT (100 μM)-SQ (1 μM), which promoted about 20%

more damage to DNA than observed in control. After H₂O₂ burst, all assayed treatments promoted the prevention of DNA damage (Table 4).

Table 4. DNA damage in MDA-MB-231 cells in basal state (blue) and with H₂O₂ burst (orange) after treatment with HT at 100 µM combined with SQ (100, 10 and 1 µM) for 24 h followed by another 24 h with fresh medium.

	HT-SQ (µM)			
	Control	(100–100)	(100–10)	(100–1)
Basal	100 ± 5.79	92.88 ± 5.46	98.60 ± 6.54	129.45 ± 6.31 *
H ₂ O ₂	100 ± 7.07	44.71 ± 3.64 *	34.17 ± 2.76 *	57.77 ± 6.60 *

Data are represented as mean ± SEM of 3 independent experiments expressed as percentage of Olive_{TM} (* $p < 0.05$ compared to untreated control).

4. Discussion

There is now scientific evidence linking the Mediterranean dietary pattern with the low incidence and prevalence of some chronic diseases such as certain types of cancer. VOO is the main source of fat consumed in this dietary pattern, and its regular intake reduces the incidence of some illnesses, such as cardiovascular diseases and a wide variety of cancers [5]. The minor compounds present in VOO could have a key role in its healthy properties [4–9,31–34]. However, most of these studies are focused mainly on the health benefits of isolated compounds from VOO. In the present article, we found that HT and SQ, both minor compounds present in VOO, have antitumor effects on highly invasive breast cancer cells only when they act in combination, but not when they act independently. HT is one of the main phenols present in VOO with interesting health benefits [11–14]. On the other hand, SQ, another important compound present in VOOs and olives, also has antitumor properties against several types of cancer, such as skin, breast and colon cancer [18–24]. Interestingly, both isolated compounds did not promote the inhibition of highly invasive breast cells in previous published works [17,26], but together at certain concentrations, appeared to promote the inhibition of breast cancer cells' growth and apoptosis, as well as increasing DNA damage.

The combination of HT and SQ showed high radical scavenging activity in the DPPH and ABTS assays. Warleta et al. [26] showed that SQ does not present any radical scavenging activity in the DPPH and ABTS tests. On the contrary, HT has been reported to be a potent scavenger of free radicals [10,16]; for this reason, the antioxidant activity observed in this study could be due to the antioxidant activity per se of HT (Tables 1 and 2). Further, the higher free radical scavenger activity occurred when HT was at the highest concentration used in both assays (DPPH and ABTS) regardless of the concentration of SQ, which reinforces the idea that HT is the compound responsible for this antioxidant effect.

This antioxidant effect of both compounds in combination could be related to oxidative radical accumulation that collaborates with breast neoplastic transformation. In fact, reactive oxygen species (ROS) play a crucial role in tumor progression [35]. In this context, SQ is a potent intracellular antioxidant [36]. An experimental study showed that SQ is able to reduce basal and H₂O₂-induced ROS levels in human breast MCF10A cells, but not in human breast cancer MCF-7 cells and MDA-MB-231 cells [26]. Unless SQ could have a protective activity against tumor development, this "selective antioxidant sensitivity" of SQ remains unknown. Moreover, HT is a well-known antioxidant phenol present in VOO but it is not able to reduce basal ROS levels in MDA-MB-231 breast cancer cells. Indeed, our group previously observed that in conditions of H₂O₂-induced ROS levels, it reduced ROS levels in a dose-dependent manner in these cancer cells [17]. According to that, our results showed that the combination of both HT and SQ did not reduce basal ROS levels in MDA-MB-231 cells; furthermore, when H₂O₂ was added, HT at 100 µM combined with SQ (from 0.01 to 100 µM) reduced ROS levels.

Due to the above, once a high-stress microenvironment is present in breast cancer, a combination of both compounds is not able to induce higher ROS levels, which could

promote the inhibition of breast cancer growth. It is well known that the formation of neoplasms promotes a higher stress microenvironment than that observed in normal states, and cancer cells are able to adapt their metabolism to the new stress conditions [35]. However, once they are adapted, a change in ROS levels (a small enhancement or decrease) could be derived from the inhibition of cancer growth, due to the lack of oxidative stress enzymes [35]. Interestingly, combination of HT-SQ is able to increase DNA damage in normal conditions (Table 4), which suggests that the combination of these two compounds could inhibit breast cancer growth, but with another pathway that is different from ROS management (Figure 4a). However, once a stress condition is induced in these cells (Table 4), the HT-SQ combination is able to reduce DNA damage, causing them to lose their antitumoral effect.

Indeed, an inhibition of proliferation was observed with HT-SQ at certain concentrations (Figure 2) as well as an apoptosis activity (Table 3), which reinforces the idea that the combination of both HT-SQ could be a useful tool to treat or even prevent some breast cancers. These HT-SQ effects were not observed in our previous works [17,26], where the research was focused on their antitumoral effects as isolated compounds.

The antiproliferative activity of HT was previously described in human colon adenocarcinoma cells by Corona et al. [13]. HT exerts its antiproliferative effects through the inhibition of the 1/2 phosphorylation of extracellular signal-regulated kinase (ERK) and a decrease in cyclin D1 expression [13]. This interesting activity of HT also was observed in human breast cancer cells. HT was capable of inhibiting cell proliferation in human breast cancer MCF-7 cells [16,37,38]. Extracts from dry olive mill residue with increasing contents of HT were a strong cell growth inhibitor [39]. We evaluated the antiproliferative effect of HT in MDA-MB-231 cells, but HT was found to only have a slight antiproliferative activity at 200 μ M [17]. In contrast, SQ does not have any antiproliferative effect in MDA-MB-231 cells [26]. In the present work, we demonstrated that if HT was combined with SQ, the antiproliferative activity of HT appears at a lower concentration (100 μ M) in these highly invasive breast cancer cells, but in combination with SQ.

The pro-apoptotic activity of HT has also been widely studied. Some authors described the apoptotic effect of HT in promyelocytic leukemia cells and colon adenocarcinoma cells [40,41]. HT also induces apoptosis in human breast MCF-7 cancer cells, but at higher concentrations tested than in the present study [38]. In our previous work, HT did not promote apoptosis in MDA-MB-231 [17]. Furthermore, SQ also did not exert pro-apoptotic activity in our previous studies [26]. Surprisingly, our results showed a pro-apoptotic activity of HT at 100 μ M when it was combined with SQ, especially at high concentrations of SQ (10 and 100 μ M) (Table 3). In addition, the amount of HT required to induce apoptosis in tumor cells was found to be lower when combined with SQ. As we observed in cell proliferation assay, it seems that SQ potentiates the activity of HT in some way.

It seems clear that SQ is, in some way, capable of enhancing the pro-apoptotic and antiproliferative activities of HT in human breast tumor cells. Since the existing research on the biological properties of VOO has focused on the study of isolated minority compounds, there are no references about how SQ could produce this interesting reinforcing effect. Nevertheless, Nakagawa et al. [42] observed that several anticancer agents such as Adriamycin, 5-fluorouracil, bleomycin and cis-dichlorodiamminoplatinum potentiated their antitumor activity when they were combined with SQ. The synergism of SQ with anticancer agents has also been described *in vivo*. SQ is able to potentiate the antitumor activity of 3-[(4-amino-2-methyl-5-pyrimidinyl) methyl]-1-(2-chloroethyl)-1-nitrosourea (ACNU) in a tumor murine model of leukemia, maybe by a modification of membrane permeability [43]. According to the authors, the synergism of SQ and antitumor agents may be due to the accumulation of anticancer drugs inside the cell because it interferes with drug efflux [43]. The anti-carcinogenic effects of SQ have been investigated together with Geranylgeranoic acid in murine models and it has been suggested that they could be due to the accumulation of FPP [21–23]. To our knowledge, only Bullon et al. [44] described

the effects of HT and SQ in periodontal disease, where both compounds reversed gingival vascular damage after atherosclerotic diet, but there are no scientific data in cancer disease.

Unfortunately, the apoptotic and antiproliferative effects of HT–SQ combination only occur at high and non-physiological concentrations. However, as seen before, when HT is combined with SQ, the concentration of HT needed to maintain these antitumoral effects is dramatically reduced, compared to previous reports [17]. In fact, Yamaguchi et al. [40] claimed that the combination of anticancer agents with membrane-active molecules such as SQ could decrease the needed dose of anticancer drugs. Nowadays, the physiological concentration of HT after the ingestion of VOO remains unclear, but it could be between 10 and 100 µM [45]. In addition, SQ has the advantage that it is another minority compound that is naturally present in VOO. It is also present in animal cells, as a cholesterol biosynthesis intermediate. Therefore, the consumption of HT and SQ could be achieved by the Mediterranean diet, including VOO as the main fat.

5. Conclusions

The low risk of breast cancer associated with VOO intake may be a consequence of the synergistic activity of different minor compounds rather than the action of an individual compound. HT and SQ are two minor compounds present in VOO that possess antitumor activity on highly invasive breast tumor cells when acting together. However, we cannot extrapolate in vitro results to human populations; for this reason, further studies are needed to confirm its possible preventive role.

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10.3. Otras actividades de divulgación

Título de la aportación: Disminución de la expresión de CD36 en células de cáncer de mama humanas altamente metastásicas inducida por componentes de los aceites de oliva vírgenes

Congreso: III Congreso Internacional sobre Aceite de Oliva Virgen, Olivar y Salud

Tipo de evento: Comunicación oral en congreso internacional (Jaén)

Fecha: 18/05/2018

Autores: Rodríguez-García, Carmen; Sánchez-Quesada, Cristina; Gaforio-Martínez, José Juan

Título de la aportación: Immune Response Modulation By Triterpenes Present In Virgin Olive Oil

Congreso: XIX Simposio Científico-Técnico de EXPOLIVA 2019

Tipo de evento: Comunicación en congreso internacional (Jaén)

Fecha: 15/05/2019

Autores: Rodríguez-García, Carmen; Sánchez-Quesada, Cristina; Gaforio-Martínez, José Juan

Título de la aportación: Promoting Tissue Regeneration Through Squalene

Nombre del congreso: VII International Symposium SRUK/CERU

Tipo de evento: Póster en Congreso Internacional (Liverpool)

Fecha: 28/06/2019

Autores: Rodríguez-García, Carmen; Sánchez-Quesada, Cristina; Gaforio-Martínez, José Juan

Título de la aportación: Effect of high fat diets in extra virgin olive oil or coconut oil on metabolic and inflammatory markers in an experimental murine model

Nombre del congreso: 17 th Euro Fed Lipid Congress and Expo

Tipo de evento: Comunicación en congreso Internacional (Sevilla)

Fecha: 22/10/2019

Autores: Rodríguez-García, Carmen; Sánchez-Quesada, Cristina; Algarra-López De Diego, Ignacio; Gaforio-Martínez, José Juan

ANEXO I



Article

The High-Fat Diet Based on Extra-Virgin Olive Oil Causes Dysbiosis Linked to Colorectal Cancer Prevention

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Abstract: The present study aims to examine the effects of three different high-fat diet (HFD) on mice gut microbiota in order to analyse whether they create the microenvironmental conditions that either promote or prevent colorectal cancer (CRC). We evaluated colonic mucosa-associated microbiota in CD1 mice fed with HFD, based on 60% kcal from fat-containing coconut, sunflower or extra-virgin olive oil as the only source of fat. The main findings were as follows: (a) All HFD produced a decrease in the richness and diversity of the intestinal microbiota that was independent of mouse weight, (b) HFD switched *Lactobacillus* to *Lactococcus*. In general, the results showed that both sunflower- and coconut-HFD generated a pro-inflammatory intestinal microenvironment. In brief, coconut-HFD decreased *Akkermansia* and increased *Staphylococcus*, *Prevotella* and *Bacteroides* spp. abundance. Sunflower-HFD reduced *Akkermansia* and *Bifidobacterium*, while enhancing *Sphingomonas* and *Neisseria* spp. abundance. In contrast, EVOO-HFD produced an anti-inflammatory microenvironment characterised by a decreased *Enterococcus*, *Staphylococcus*, *Neisseria* and *Pseudomonas* spp. abundance. At the same time, it increased the Firmicutes/Bacteroidetes ratio and maintained the *Akkermansia* population. To conclude, EVOO-HFD produced changes in the gut microbiota that are associated with the prevention of CRC, while coconut and sunflower-HFD caused changes associated with an increased risk of CRC.

Keywords: inflammation; sunflower oil; coconut oil; gut microbiome; *Akkermansia*; *Neisseria*; *Staphylococcus*; firmicutes; bacteroidetes; *Prevotella*

1. Introduction

Nowadays, colorectal cancer (CRC) is among the most common and deadly neoplasms [1]. It is assumed that both, a Western-style diet with high-fat content and a sedentary lifestyle are responsible for the increase in their global incidence [1]. In fact, CRC is 3–4 times more common in developed than in developing nations. High fat intake is one of the characteristics of the Western pattern diet, and this correlates with an increased risk of CRC [2]. The trigger mechanism by which the risk increases is not yet well known, but it has been proposed that it is due to a change in the intestinal microbiota that favour a low-intensity inflammatory process [3].

Gut microbiota is a highly complex ecosystem, with great individual variations and thousands of microbial species. The most predominant phylum in the healthy gut are Firmicutes and Bacteroidetes,

followed by Actinobacteria, Proteobacteria and Verrucomicrobia [4]. Under normal physiological conditions, gut microbiota regulate fat metabolism (by bile acids biotransformation), synthesise essential amino acids and vitamins, and facilitate the digestion of complex plant carbohydrates into short-chain fatty acids (mainly butyrate, propionate and acetate). The gut microbiome contributes significantly to host immunity and metabolic homeostasis [5,6]. However, alterations in the mucosa, together with an unfavourable genetic predisposition, favour the growth of opportunistic microbes that promote functional and morphological changes (dysbiosis) and lead to chronic inflammation. Chronic inflammation, in turn, contributes to dysbiosis [7]. Pro-inflammatory cytokines and opportunistic pathogens affect the epithelial integrity, and chronic inflammation together with oxidative stress leads to the loss of the epithelial barrier, and may result in a vicious cycle of immune hyperactivation and aggravation of barrier dysfunction [8]. In addition, defects in the host barrier enhance permeability and promote the invasion of harmful bacteria, which may lead to bacterial translocation across the epithelial layers [5], leading to the development of CRC [2].

Although high-fat diets (HFD) have been identified as promoting the development of CRC [2], not all types of fats have the same health effect. While animal fats have harmful effects on inflammation and CRC development, the effects of edible vegetable oils on gut microbiota dysbiosis have not been studied sufficiently yet [9]. All edible vegetable oils are assumed to be healthy, but given their different fatty acid profile and minor compounds composition, their impact on gut microbiota is expected to be different.

Since HFD is typically present in Western-pattern diets and is a risk factor of CRC, the present study aims to examine, in an experimental murine model, the influence of three HFD, each one prepared with different edible vegetable oils (coconut oil, sunflower oil and extra virgin olive oil) on gut microbiota.

2. Materials and Methods

2.1. Experimental Animals

Female CD1 mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Four-week-old mice ($n = 44$) were housed (5–6 per cage) in ventilated racks and cages with environmental control (temperature: 20 ± 2 °C; humidity: 55–65%; 12 h light/12 h dark cycle).

Animal care and experiments were conducted following the guidelines of the Spanish Society for Laboratory Animal Science. The procedures applied to these animals were approved by the Ethical Committee of the University of Jaen (Record number: CEEA-100217-1) and the Ethical Committee of Animal Experiments of Regional Ministry of Agriculture, Fishing and Environment of Regional Government of Andalusia, Spain (Approval number: 16/03/2017/044).

2.2. Diets

The mice were fed with maintenance chow diet (defined as chow diet), with 13% kcal from fat (Ref. 2014S), an intermediate fat diet with 22% kcal from fat (Ref. 2019S) and custom basis (Ref. TD.170709) ready to use (fat free) for HFD with 60% kcal from fat were purchased from ENVIGO® (Madison, WI, USA). The custom basis fat free was exclusively made by ENVIGO® for the present study. In order to prepare each one of the HFD, we added one of the following oils to the custom basis: extra virgin olive oil (EVOO), coconut oil and sunflower oil. The HFD were made and administered daily in sterile conditions (Tables S1 and S2).

2.3. Experimental Design

On arrival, the mice were randomly divided into four groups ($n = 11$ each), and they were assigned to a different diet:

Group 1: Chow diet (chow)

Group 2: HFD of coconut oil (coconut-HFD)

Group 3: HFD of sunflower oil (sunflower-HFD)

Group 4: HFD of extra virgin olive oil (EVOO-HFD)

Before the dietary intervention, the mice were kept in an acclimation phase for three weeks. In the first week, all the mice were fed with chow diet, and the following two weeks, the chow group continued on the same diet, but HFD groups were provided with an intermediate fat diet. Once the intervention phase started, the mice were fed either a chow diet, EVOO, coconut or sunflower HFD with ad libitum access to water and food for sixteen weeks (Figure 1).

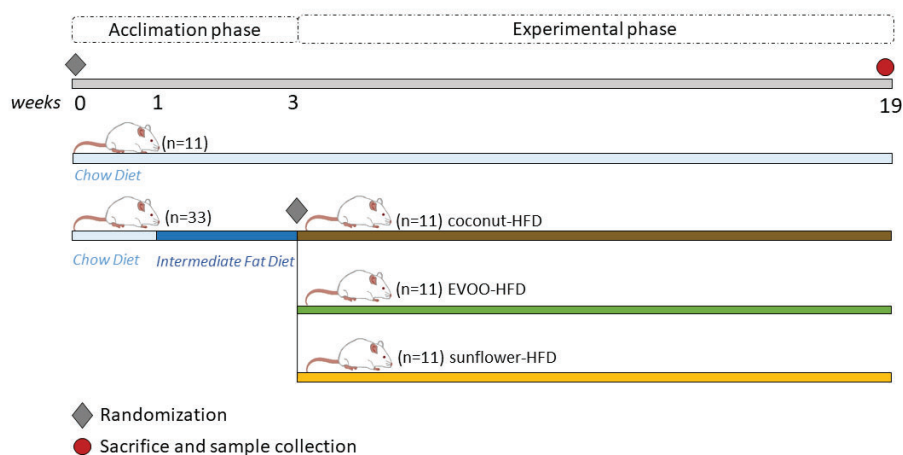


Figure 1. Experimental design.

Body weight and indirect food intake were measured weekly. The mice were then sacrificed using a euthanasic mixture of ketamine (160 mg/kg) and xylazine (10 mg/kg). The intestine was completely dissected, freshly cut and stored at -80°C until analysis.

2.4. Sample Collection and Mucosa-Associated Microbiota Analysis by PCR Amplification and Sequencing of the 16S rRNA Gene

Thirty-two distal colon samples were analysed, corresponding to eight mice randomly selected from each experimental group. Samples were carefully dissected, and the colonic mucosa-associated microbiota was extracted and lysed. The nucleases were inactivated through mechanic disruption and enzymatic treatment. Genomic DNA was isolated from the bacterial colonies and purified with the QIAamp PowerFecal DNA kit (Qiagen, Hilden, Germany) following the manufacture's protocol.

The quality and quantity of the bacterial colonies were analysed by spectrophotometry using Nanodrop (Thermo Scientific, Waltham, MA, USA). DNA amplification of the 16S rRNA V3-V4 region of the bacteria's rRNA genes was carried out with a two-step PCR protocol, using the PCR primers recommended by Klindworth et al. [10]. The obtained products were verified with the PicoGreen-based DNA quantification assay (Table S3). Sequencing of the gut microbiota was performed with Illumina Miseq (Novogene, Beijing, China). Sequencing was performed using the manufacturers' protocol with a 300 pb pair-end design and 50,000 to 100,000 readings per sample were obtained. A positive control was included and another negative control was included during sequencing [11]. In both cases the result was optimal, the positive control had the expected species and the negative control had less than 50 sequences.

2.5. Bioinformatics Analysis

PEAR V0.9.1 software (The Exelisis Lab, Heidelberg, Germany) was used to join each pair of sequences (R1 and R2) coming from the sequencing platform, taking into account a minimum overlap

of 70 nts at each end, in this way a unique and complete sequence was obtained. Then, using the Cutadapt v1.8.1 program, the sequencing adapters of both ends present in each sample were eliminated.

Once the sequences without adapters were obtained, those readings that were below Q20 and less than 100 pb in length were eliminated. The reformat module of BBMap v38 was used to perform this analysis; this programme also cut out those nucleotide bases at both ends that presented a value of quality lower than that indicated (Q20). The last step in quality processing was the elimination of possible chimera sequences resulting from incomplete extension during amplification by the PCR method. This step was carried out with the Uchime programme, which allows the detection and elimination of these amplicons from a reference database (ChimeraSlayer).

The CDHit v4.8.1 program (Weizhong Li's Group, La Jolla, CA, USA) was used to determine the microbial diversity present in the sample, grouping sequences with a similarity threshold of 97% or higher in operational taxonomic units (OTUs). Each OTU was compared against the RefSeq 16S rRNA gene database (NCBI) using the BLAST tool.

For each of the samples, a rarefaction curve was performed, reaching a saturation situation of detection (plate), to corroborate that all the organisms had been detected (Figure S1). Shannon–Wiener and Chao1 biostatistical values were calculated to estimate the specific biodiversity present in the sample and the total number of species. The local contribution to beta diversity analysis (LCBD) was done representing the degree of uniqueness of the sampling units in terms of community composition and based on the number of the standardized number of counts for rarefaction.

2.6. Statistical Analysis

The analysis consisted of a differential study of the population using the DESeq2 R tool focused on microbiomes. To compare data, bacteria with minor than 0.01% of presence were eliminated. In addition, principal coordinate analysis, canonical correspondence, beta dispersion analysis of the samples, and a differential study of diversity were performed. To show the effect of different diets on the relative abundance of taxa, a PERMANOVA test was performed. *p*-values adjusted by FDR has been used in DESeq2 test. Data are represented as mean $\pm$ SD and *p* values less or equal to 0.05 were considered statistically significant.

3. Results

3.1. High-Fat Diets Promoted Dysbiosis Independently of Body Weight and the Type of Vegetable Fat Present in the Diet

Regardless of the type of vegetable fat used, HFD led to a decrease in the richness and diversity (Simpson and Shannon test, evaluated by Chao-1) of gut microbiota. Diversity reduction for EVOO- and sunflower-HFD were statistically significant ($p < 0.05$ for both EVOO- and sunflower-HFD) (Figure 2).

The local contribution to the beta diversity test verified that each group's sample contribution to diversity was relatively homogeneous within the group (Figure 3).

Principal coordinate analysis (PCoA) and PERMANOVA analysis corroborated that HFD promoted a change in the bacterial community in a significant way ($p = 0.002$ for coconut and EVOO-HFD, and $p = 0.01$ for sunflower-HFD) (Figure 4).

To compare if these changes in bacterial communities may be related to body weight, the initial and final mean weights of each group were analysed. Data showed that coconut-HFD increased body weight, likewise for the chow diet, while EVOO- and sunflower-HFD increased it notably (Table 1).

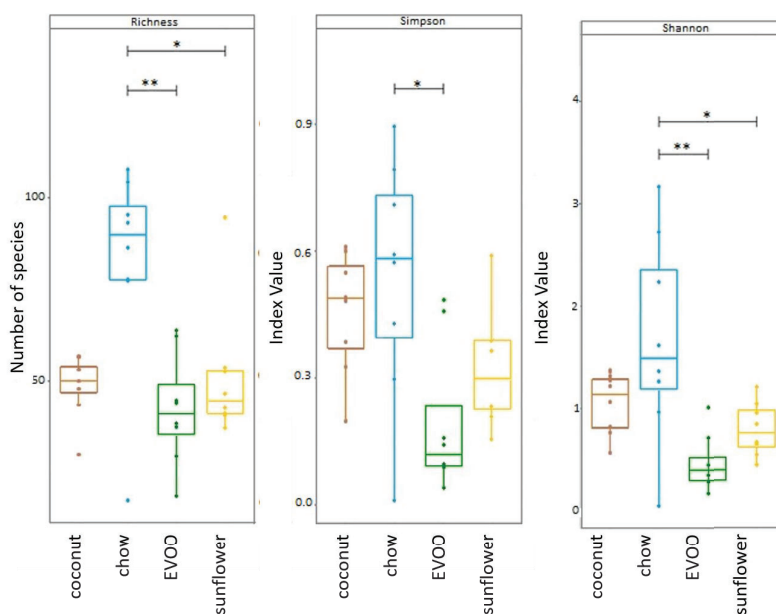


Figure 2. Gut microbiota richness and diversity (Shannon and Simpson) (evaluated by Chao-1). Boxplot figure representing the diversity of the samples according to the variables studied * $p < 0.05$ and ** $p < 0.01$ (ANOVA test).

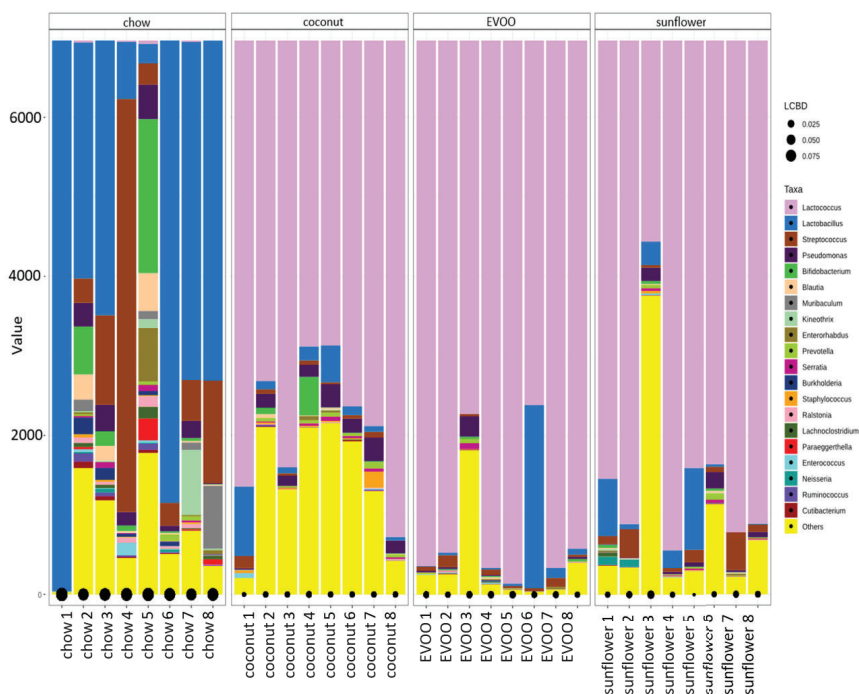


Figure 3. The local contribution to beta diversity analysis. LCBD values represent the degree of uniqueness of the sampling units in terms of community composition. Accumulative bar graph comparing the genera detected in the samples with the 16S rRNA-based bacteria profiles.

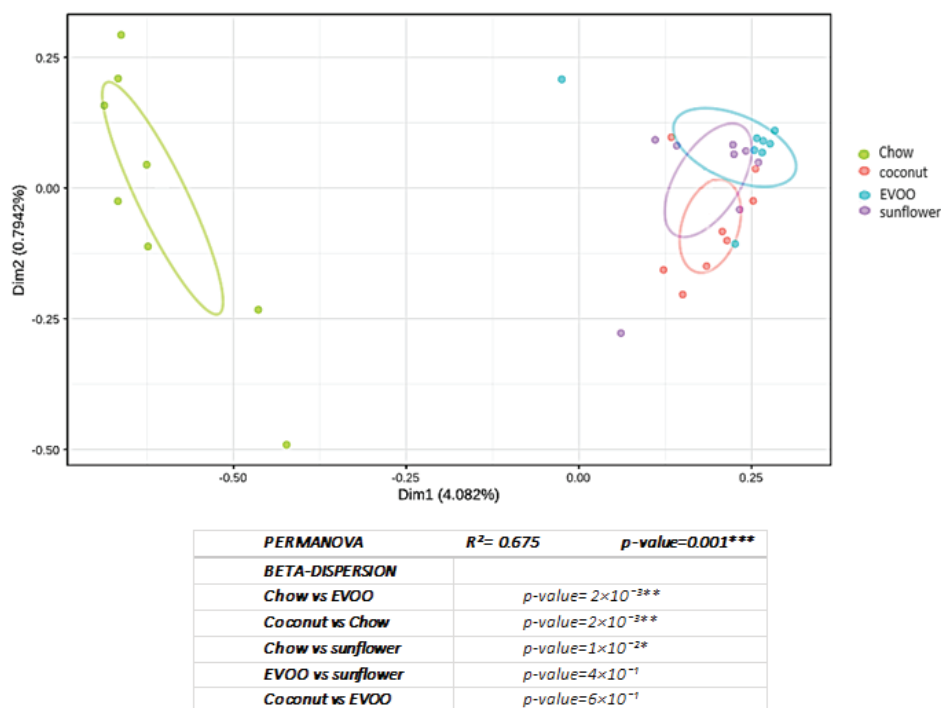


Figure 4. The principal coordinate analysis (PCoA) plot made with unweighted unifrac distances between samples of microbiome, grouped by diets. The main coordinate graph showing the differences between samples and its group, with 30% CI ellipses. A PERMANOVA analysis has also been carried out to detect whether the separation between groups is significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 1. Initial and final body weights of mice fed with chow and HFD.

Weight	Chow	Coconut	EVOO	Sunflower
Initial (g)	29.49 ± 0.37	28.65 ± 0.47	29.42 ± 0.76	31.04 ± 0.77
Final (g)	45.48 ± 1.85	44.88 ± 1.47	55.89 ± 2.65 **	55.3 ± 3.33 **

Data are shown as Mean ± SD of bodyweight ($n = 11$). ANOVA test, ** $p < 0.01$ for HFD (EVOO, sunflower) vs. chow diet.

3.2. Main Findings Found at the Phylum Level: EVOO-HFD Specifically Reduced Proteobacteria While Coconut-HFD Decreased Verrucomicrobia

At the phylum level, the abundance of mice gut microbiota was different between chow and all three HFD groups (Figure 5 and Table 2). However, Firmicutes remained in all diets as the majority phylum. Although there were no statistical differences between groups, EVOO- and sunflower-HFD showed an increase in this phylum. With regard to Bacteroidetes, all HFD reduced this phylum in a significant way in comparison with the chow group ($p < 0.05$). Figure 6A shows that the Firmicutes/Bacteroidetes ratio increased in EVOO-HFD while it remained almost unchanged in both coconut- and sunflower-HFD. As in Bacteroidetes, all the HFD decreased the Actinobacteria phylum in a significant way. Despite the fact that no statistical differences between HFD groups were observed, EVOO- and coconut-HFD acted differently; microbiota from EVOO-HFD mice suffered a reduction in Proteobacteria ($p < 0.001$), while coconut-HFD decreased Verrucomicrobia ($p < 0.01$). Figure 5 illustrates the taxonomic summary bar plots identification of the microbiota at the phylum level, concluding that all HFD promoted dysbiosis on gut microbiota.

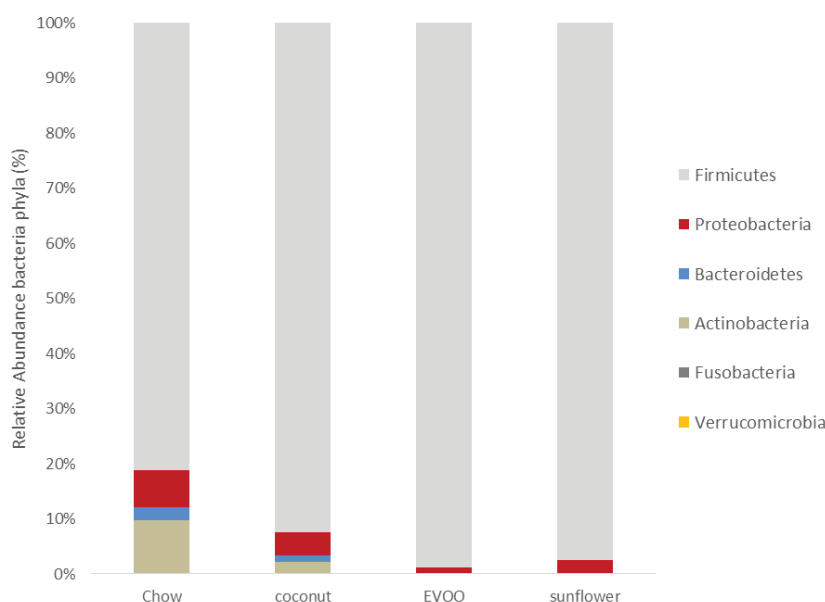


Figure 5. The relative abundance of bacterial phyla (%). The taxonomic summary bar plots identification of the microbiota at the phyla level.

Table 2. Percentage of relative abundance of Phylum.

Phylum	Chow	Coconut	EVOO	Sunflower
Firmicutes	78.695 ± 20.27	75.014 ± 13.08	93.739 ± 10.56	86.081 ± 18.37
Proteobacteria	6.578 ± 5.34	3.447 ± 1.86	0.958 ± 1.61 ***	2.17 ± 1.35
Bacteroidetes	2.331 ± 3.04	0.948 ± 0.49 *	0.283 ± 0.48 ***	0.532 ± 0.46 ***
Actinobacteria	9.186 ± 15.32	1.712 ± 2.78 **	0.437 ± 0.22 ***	0.654 ± 0.45 ***
Fusobacteria	0.083 ± 0.14	0.011 ± 0.02	0.007 ± 0.01	0.046 ± 0.09
Verrucomicrobia	0.055 ± 0.12	0.002 ± 0.003 **	0.038 ± 0.06	0.011 ± 0.02

Data are shown as Mean ± SD and were analysed by the PERMANOVA test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for HFD (coconut, EVOO, and sunflower) vs. chow diet. ($n = 8$).

3.3. Main Findings Found at Both Genera and Species Levels: HFD Produced Both Switching *Lactobacillus* for *Lactococcus* and Only Coconut-HFD Promoting *Staphylococcus* Colonisation

Despite decreasing diversity in all HFD groups in comparison with chow, there were some relevant changes regarding some bacterial genera and species (Tables 3 and 4). The most relevant change induced by HFD was *Lactobacillus* reduction (mainly *Lactobacillus reuteri*) and *Lactococcus* increase (mostly *Lactococcus lactis*) (Figure 7). HFD also decreased *Streptococcus*, *Turicibacter*, *Blautia*, *Clostridium*, *Ruminococcus* and *Anaerostipes* genera in a significant way in comparison with chow ($p < 0.05$). Differences between chow vs. EVOO- and sunflower-HFD were observed, reducing *Enterococcus* in a significant way (mainly *Enterococcus gallinarum*). Regarding differences between HFD groups, EVOO-HFD ($0.031 \pm 0.04\%$ of relative abundance) and coconut-HFD ($0.522 \pm 0.82\%$ of relative abundance) showed statistical differences with respect to *Staphylococcus* genera ($p < 0.05$). Coconut enhanced the relative abundance of *Staphylococcus epidermidis*.

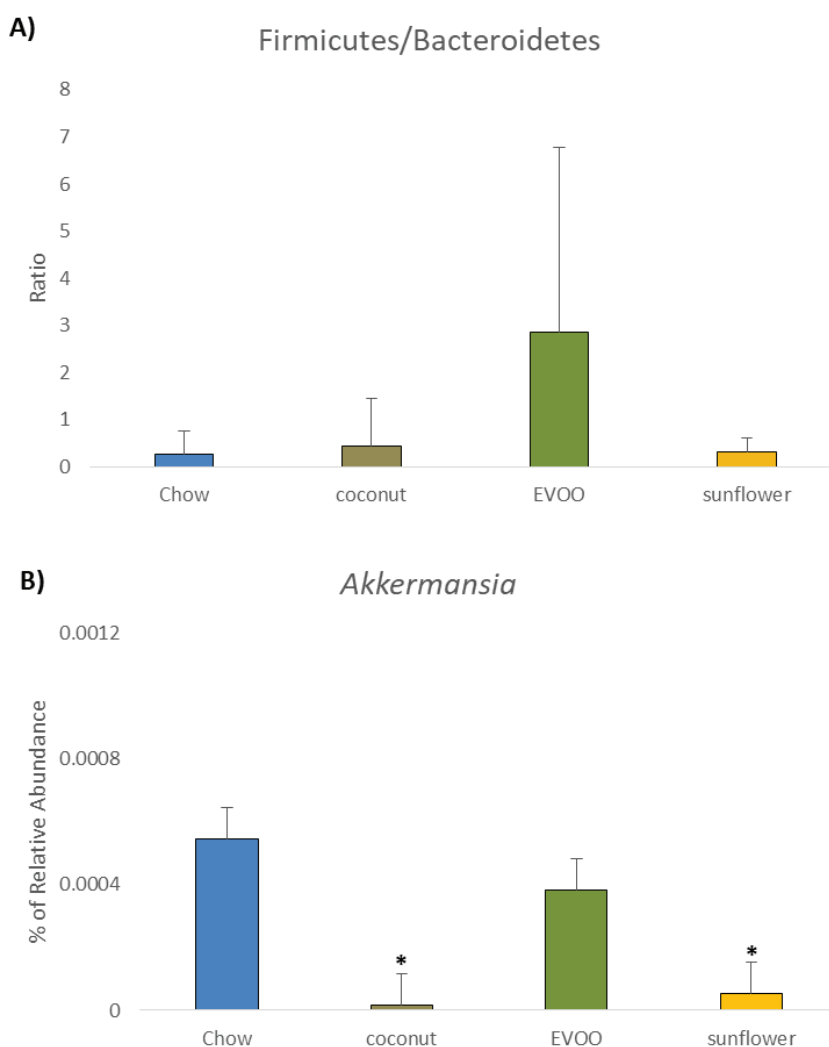


Figure 6. The relative abundance of bacterial phyla and genera (%). **(A)** Firmicutes/Bacteroidetes Ratio. **(B)** *Akkermansia*. * $p < 0.05$ for HFD (coconut and sunflower) vs. chow diet.

Table 3. Percentage of relative abundance of Genera.

Genera	Chow	Coconut	EVOO	Sunflower
FIRMICUTES				
<i>Lactobacillus</i>	51.471 ± 32.92	3.412 ± 3.99 ¹	4.717 ± 11.76 ¹	4.351 ± 5.58 ¹
<i>Streptococcus</i>	15.982 ± 24.53	0.76 ± 0.57 ¹	0.928 ± 0.66 ¹	2.428 ± 2.35 ¹
<i>Turicibacter</i>	0.152 ± 0.23	0.001 ± 0.003 ¹	0.006 ± 0.006 ¹	0.004 ± 0.004 ¹
<i>Lactococcus</i>	0.156 ± 0.19	68.83 ± 12.28 ¹	87.055 ± 13.74 ¹	77.819 ± 17.58 ¹
<i>Enterococcus</i>	0.444 ± 0.84	0.173 ± 0.4	0.125 ± 0.24 ¹	0.06 ± 0.06 ¹
<i>Blautia</i>	1.942 ± 2.71	0.129 ± 0.18 ¹	0.058 ± 0.06 ¹	0.129 ± 0.12 ¹
<i>Clostridium</i>	0.505 ± 0.64	0.01 ± 0.02 ¹	0.024 ± 0.02 ¹	0.01 ± 0.02 ¹

Table 3. Cont.

Genera	Chow	Coconut	EVOO	Sunflower
<i>Kineothrix</i>	1.796 ± 3.8	0.036 ± 0.07 ¹	0.014 ± 0.02 ¹	0.037 ± 0.05 ¹
<i>Ruminococcus</i>	0.411 ± 0.48	0.094 ± 0.11 ¹	0.023 ± 0.03 ¹	0.034 ± 0.03 ¹
<i>Paenibacillus</i>	0.061 ± 0.11	0.022 ± 0.05	0.003 ± 0.004 ¹	0.0003 ± 0.0007 ¹
<i>Romboutsia</i>	0.178 ± 0.24	0.046 ± 0.06 ¹	0.048 ± 0.06	0.014 ± 0.02 ¹
<i>Eubacterium</i>	0.495 ± 0.59	0.034 ± 0.05 ¹	0.03 ± 0.06 ¹	0.013 ± 0.01 ¹
<i>Lachnospirillum</i>	0.426 ± 0.47	0.053 ± 0.07 ¹	0.046 ± 0.05 ¹	0.079 ± 0.2 ¹
<i>Gemmiger</i>	0.157 ± 0.23	0.081 ± 0.19	0.004 ± 0.004 ¹	0.033 ± 0.05
<i>Anaerostipes</i>	0.296 ± 0.45	0.012 ± 0.02 ¹	0.009 ± 0.01 ¹	0.014 ± 0.02 ¹
<i>Flintibacter</i>	0.162 ± 0.23	0.047 ± 0.06	0.009 ± 0.01 ¹	0.017 ± 0.02 ¹
<i>Staphylococcus</i>	0.194 ± 0.16	0.522 ± 0.82	0.031 ± 0.04 ^{1,2}	0.101 ± 0.1
PROTEOBACTERIA				
<i>Pseudomonas</i>	2.702 ± 2.23	2.489 ± 1.33	0.618 ± 1.19 ¹	0.999 ± 1.09
<i>Ralstonia</i>	0.696 ± 0.57	0.071 ± 0.74 ¹	0.038 ± 0.06 ¹	0.014 ± 0.01 ¹
<i>Escherichia</i>	0.095 ± 0.12	0.102 ± 0.04	0.038 ± 0.06	0.023 ± 0.02
<i>Paracoccus</i>	0.375 ± 0.98	0.024 ± 0.02	0.003 ± 0.004	0.006 ± 0.01
<i>Burkholderia</i>	0.882 ± 1.07	0.01 ± 0.02 ¹	0.018 ± 0.05 ¹	0.018 ± 0.05 ¹
<i>Moraxella</i>	0.159 ± 0.35	0.063 ± 0.14	0.02 ± 0.04	0.049 ± 0.11
<i>Neisseria</i>	0.214 ± 0.24	0.004 ± 0.008 ¹	0.002 ± 0.003 ¹	0.335 ± 0.61 ^{2,3}
<i>Acinetobacter</i>	0.073 ± 0.14	0.013 ± 0.01	0.005 ± 0.009 ¹	0.007 ± 0.01
<i>Massilia</i>	0.049 ± 0.08	0.022 ± 0.06	0.0009 ± 0.002 ¹	0.007 ± 0.01
<i>Sphingomonas</i>	0.036 ± 0.06	0.002 ± 0.005	0.001 ± 0.001	0.097 ± 0.17 ^{2,3}
BACTEROIDETES				
<i>Bacteroides</i>	0.125 ± 0.13	0.264 ± 0.21	0.103 ± 0.2	0.099 ± 0.12
<i>Prevotella</i>	0.389 ± 0.48	0.57 ± 0.29	0.134 ± 0.24	0.273 ± 0.33
<i>Parabacteroides</i>	0.004 ± 0.003	0.017 ± 0.02	0.008 ± 0.01	0.008 ± 0.01
<i>Muribaculum</i>	1.509 ± 3.26	0.003 ± 0.007 ¹	0.0003 ± 0.0007 ¹	ND ¹
ACTINOBACTERIA				
<i>Bifidobacterium</i>	5.075 ± 9.59	1.175 ± 2.52	0.138 ± 0.13 ¹	0.224 ± 0.24 ¹
<i>Paraeggerthella</i>	0.701 ± 1.38	0.005 ± 0.01 ¹	0.003 ± 0.005 ¹	0.002 ± 0.003 ¹
<i>Actinomyces</i>	0.192 ± 0.47	0.0001 ± 0.0003 ¹	0.0006 ± 0.001 ¹	0.016 ± 0.03 ¹
VERRUCOMICROBIA				
<i>Akkermansia</i>	0.055 ± 0.12	0.001 ± 0.003 ¹	0.038 ± 0.06	0.005 ± 0.008 ¹

Data are shown as Mean ± SD and were analysed by PERMANOVA test; ¹ $p < 0.05$ for groups vs. chow diet; ² $p < 0.05$ for groups vs. coconut; ³ $p < 0.05$ for groups vs. EVOO; Not detectable is shown as ND = 0.

Table 4. Percentage of Relative Abundance of Species.

Species	Chow	Coconut	EVOO	Sunflower
<i>Lactobacillus reuteri</i>	42.45 ± 29.95	0.335 ± 0.57 ¹	1.106 ± 2.84 ¹	0.658 ± 0.77 ¹
<i>Lactobacillus taiwanensis</i>	5.979 ± 7.31	0.002 ± 0.003 ¹	0.006 ± 0.01	0.002 ± 0.004 ¹
<i>Lactobacillus animalis</i>	1.71 ± 2.29	1.449 ± 2.1	0.415 ± 0.45	1.533 ± 2.76
<i>Lactobacillus caviae</i>	0.501 ± 0.34	0.004 ± 0.01 ¹	0.019 ± 0.05 ¹	0.009 ± 0.01 ¹
<i>Lactobacillus gasseri</i>	0.129 ± 0.16	0.017 ± 0.02	2.85 ± 8.05	0.019 ± 0.05
<i>Lactobacillus secaliphilus</i>	0.078 ± 0.14	0.0002 ± 0.0007 ¹	0.023 ± 0.06	0.001 ± 0.002 ¹
<i>Lactobacillus faecis</i>	0.026 ± 0.04	0.011 ± 0.02	0.01 ± 0.02	0.019 ± 0.03
<i>Lactobacillus johnsonii</i>	0.004 ± 0.004	1.351 ± 2.06	0.195 ± 0.38	2.012 ± 4.70
<i>Lactobacillus satsumensis</i>	0.002 ± 0.003	0.008 ± 0.01	0.016 ± 0.03	0.062 ± 0.12
<i>Streptococcus danieliae</i>	15.229 ± 24.65	0.711 ± 0.56 ¹	0.76 ± 0.6 ¹	1.774 ± 2.05 ¹
<i>Streptococcus oralis</i>	0.255 ± 0.30	0.022 ± 0.03 ¹	0.021 ± 0.04 ¹	0.474 ± 1.11 ^{2,3}
<i>Streptococcus sanguinis</i>	0.192 ± 0.43	0.0001 ± 0.0003 ¹	0.006 ± 0.01 ¹	0.055 ± 0.14
<i>Lactococcus lactis</i>	0.153 ± 0.19	68.407 ± 12.20 ¹	86.28 ± 13.62 ¹	77.241 ± 17.41 ¹
<i>Lactococcus taiwanensis</i>	0.003 ± 0.002	0.419 ± 0.11 ¹	0.761 ± 0.25 ¹	0.557 ± 0.21 ¹

Table 4. Cont.

Species	Chow	Coconut	EVOO	Sunflower
<i>Enterococcus gallinarum</i>	0.361 ± 0.85	0.022 ± 0.06	0.003 ± 0.004 ¹	0.028 ± 0.05 ¹
<i>Blautia wexlerae</i>	0.613 ± 0.88	0.039 ± 0.06 ¹	0.012 ± 0.01 ¹	0.047 ± 0.07 ¹
<i>Blautia luti</i>	0.559 ± 0.69	0.042 ± 0.09 ¹	0.014 ± 0.02 ¹	0.015 ± 0.02 ¹
<i>Blautia obeum</i>	0.416 ± 0.90	0.012 ± 0.02 ¹	0.002 ± 0.003 ¹	0.004 ± 0.003 ¹
<i>Blautia faecis</i>	0.045 ± 0.06	0.009 ± 0.01	0.001 ± 0.001 ¹	0.005 ± 0.01 ¹
<i>Clostridium scindens</i>	0.088 ± 0.09	0.014 ± 0.02	0.027 ± 0.04	0.029 ± 0.07
<i>Clostridium spiroforme</i>	0.101 ± 0.17	0.012 ± 0.03 ¹	0.004 ± 0.01 ¹	0.0004 ± 0.0005 ¹
<i>Clostridioides difficile</i>	ND	0.001 ± 0.003	0.016 ± 0.03	0.003 ± 0.006
<i>Ruminococcus gnavus</i>	0.306 ± 0.8	0.021 ± 0.04 ¹	0.024 ± 0.04 ¹	0.051 ± 0.09 ¹
<i>Eubacterium hallii</i>	0.38 ± 0.47	0.012 ± 0.02 ¹	0.005 ± 0.01 ¹	0.008 ± 0.01 ¹
<i>Anaerostipes hadrus</i>	0.291 ± 0.45	0.012 ± 0.02 ¹	0.009 ± 0.01 ¹	0.014 ± 0.02 ¹
<i>Staphylococcus epidermidis</i>	0.065 ± 0.09	0.328 ± 0.87	0.003 ^{1,2}	0.031 ± 0.04 ²
<i>Pseudomonas migulae</i>	1.538 ± 1.56	1.199 ± 0.93	0.428 ± 0.89 ¹	0.493 ± 0.58
<i>Pseudomonas trivialis</i>	0.277 ± 0.36	0.238 ± 0.20	0.075 ± 0.16	0.169 ± 0.25
<i>Pseudomonas helmanticensis</i>	0.521 ± 1.17	0.888 ± 1.64 ¹	0.04 ± 0.1 ¹	0.277 ± 0.76 ¹
<i>Pseudomonas aeruginosa</i>	0.04 ± 0.07	0.001 ± 0.001 ¹	0.001 ± 0.001 ¹	ND ¹
<i>Neisseria mucosa</i>	0.055 ± 0.15	0.003 ± 0.007 ¹	0.0003 ± 0.0007 ¹	0.176 ± 0.32 ^{2,3}
<i>Ralstonia insidiosa</i>	0.685 ± 0.57	0.071 ± 0.07 ¹	0.038 ± 0.06 ¹	0.013 ± 0.01 ¹
<i>Prevotella oralis</i>	0.003 ± 0.01	0.004 ± 0.01	0.005 ± 0.01	0.0002 ± 0.0007
<i>Prevotella copri</i>	0.217 ± 0.32	0.543 ± 0.27	0.113 ± 0.22	0.244 ± 0.32
<i>Muribaculum intestinale</i>	1.509 ± 3.26	0.003 ± 0.01 ¹	0.0003 ± 0.0007 ¹	ND ¹
<i>Bifidobacterium longum</i>	0.683 ± 0.88	0.131 ± 0.22 ¹	0.033 ± 0.05 ¹	0.057 ± 0.08 ¹
<i>Bifidobacterium animalis</i>	4.022 ± 8.49	0.124 ± 0.14 ¹	0.094 ± 0.13 ¹	0.12 ± 0.12 ¹
<i>Bifidobacterium thermophilum</i>	0.053 ± 0.11	0.006 ± 0.02 ¹	0.001 ± 0.001 ¹	0.0001 ± 0.0003 ¹
<i>Bifidobacterium adolescentis</i>	0.189 ± 0.21	0.096 ± 0.23	0.005 ± 0.005 ¹	0.022 ± 0.04 ¹
<i>Bifidobacterium bifidum</i>	0.052 ± 0.09	0.014 ± 0.03 ¹	0.002 ± 0.005 ¹	0.004 ± 0.01 ¹
<i>Akkermansia muciniphila</i>	0.055 ± 0.12	0.001 ± 0.02 ¹	0.038 ± 0.06	0.005 ± 0.01 ¹

Data are shown as Mean ± SD and were analysed by PERMANOVA test; ¹ $p < 0.05$ for groups versus chow diet;

² $p < 0.05$ for groups versus coconut; ³ $p < 0.05$ for groups versus EVOO.; Not detectable is shown as ND = 0.

3.4. Sunflower-HFD Enhanced *Sphingomonas* Genus

Regarding Proteobacteria, mice of the EVOO-HFD group presented a significant decrease in *Pseudomonas* abundance; in this genus, EVOO-HFD mainly reduced *Pseudomonas migulae* (Tables 3 and 4). In comparison to EVOO- and coconut-HFD, sunflower-HFD enhanced *Neisseria*, especially *Neisseria mucosa* sp. ($p < 0.05$), while sunflower-HFD significantly increased *Sphingomonas* in comparison to chow, coconut-HFD and EVOO-HFD (Figure 7).

3.5. Coconut and Sunflower-HFD Reduced *Akkermansia Muciniphila* Abundance

With regard to the Bacteroidetes phylum, all the HFD decreased *Muribaculum* abundance (Table 3). For Actinobacteria, all the HFD decreased *Bifidobacterium*, but only EVOO- and sunflower-HFD in a significant way.

Figure 6B shows that for the Verrucomicrobia phylum, coconut and sunflower-HFD reduced *Akkermansia* (mainly *Akkermansia muciniphila*) abundance in comparison with chow ($p < 0.05$) (Table 4).

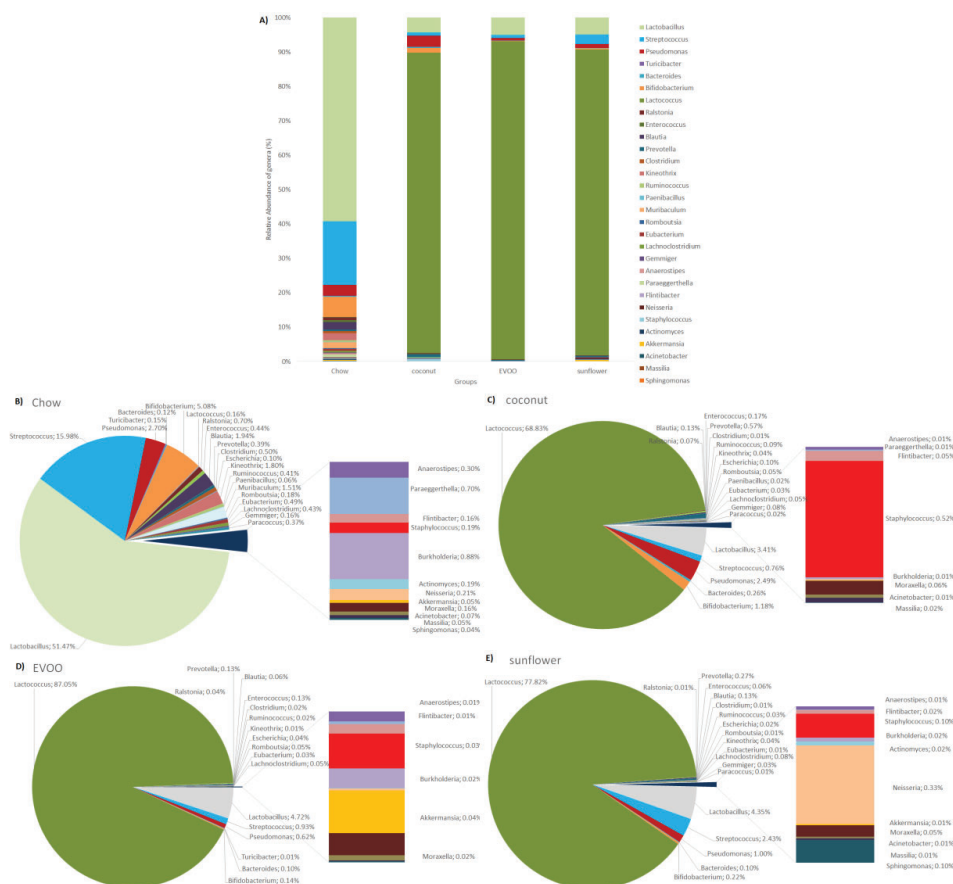


Figure 7. The relative abundance of bacteria genera (%). (A) The taxonomic summary bar plots at the genera level, (B) chow, (C) coconut, (D) EVOO, (E) sunflower.

4. Discussion

Western diets characterised by high fat intake have been strongly linked to CRC in several epidemiological studies [12,13]. Indeed, diet can modulate the gut microbiota characteristics, which can eventually lead to an increased or decreased risk of CRC [14].

For the first time, our results showed that the presence of EVOO as the only source of fat in HFD, caused a dysbiosis that could be associated with the prevention of CRC, while coconut and sunflower-HFD produced a dysbiosis associated with an increased CRC risk. Although all mice were fed ad libitum during the 16 weeks of dietary intervention, surprisingly mice fed with the coconut-HFD showed a similar weight to mice of the control group (Table 1). By contrast, mice fed with the EVOO or sunflower-HFD gained considerable weight at the end of the dietary intervention. The results clearly show that HFD of coconut oil, EVOO or sunflower oil produce intestinal dysbiosis. Although similar results have been described by other researchers [15], the present study is the only one that has used HFD containing a single type of oil as the sole source of fat; this is important because not all fats have the same impact on health. Indeed, as we can see in Figure 2, both the richness and diversity of the gut microbiota are diminished by each of the three diets administered. Similarly, beta diversity analyses corroborate these results (Figure 3). In line with these results, both the principal coordinated analysis (PCoA) and PERMANOVA analysis show a significant change in the gut microbiota of mice fed any of the three diets studied (Figure 4).

Firmicutes were the most abundant phylum found in mice gut microbiota fed with chow diet; this is consistent with other research findings [16]. Both the sunflower and EVOO-HFD diets increased the percentage of Firmicutes, although not in a statistically significant way (Table 2 and Figure 5). Interestingly, we found that EVOO-HFD increased the Firmicutes/Bacteroidetes ratio (Figure 6A). Although the increase in this ratio has been correlated with obesity, it is also associated with CRC prevention through the modulation of the inflammatory process [17,18].

Gut microbiota of all HFD studied, decreased *Lactobacillus reuteri* and promoted an increase in *Lactococcus lactis* (Table 4 and Figure 7A). Both species are probiotics that have a key role in modulating inflammatory processes. *L. reuteri* is able to improve immune activity to combat autoimmune diseases, inhibiting inflammation by reducing Th1/Th2 cell ratio and their associated cytokines [19]. On the other hand, *L. lactis* plays an important role in the antioxidant defence of gut microbiota and in the maintenance of the pro- and anti-inflammatory balance [20,21].

Bifidobacterium are a group of probiotic bacteria involved in normal colonocyte maintenance [22]. Our results showed that all three HFD produced a decrease in *Bifidobacterium*, although this only has statistical significance for EVOO- and sunflower-HFD (Table 3 and Figure 7A).

Akkermansia muciniphila has been associated with numerous human health benefits, for example, with lifespan [23]. A decrease in this bacterium has been found in individuals with inflammatory bowel disease, ulcerative colitis and Crohn's disease. Its administration can also decrease inflammatory cytokines levels in intestinal inflammation [24]. Other authors have reported that the food additive carrageenan decreases *A. muciniphila* and promotes both inflammation and colitis in experimental animals [25].

On the contrary, some phenolic compounds such as resveratrol and caffeic acid were able to restore *A. muciniphila* after colitis induction in mice, ameliorating gut inflammation [26,27]. Interestingly, our experimental study showed that coconut- and sunflower-HFD decreased the relative abundance of *A. muciniphila* significantly, while the EVOO-HFD maintained the population of this bacterium (Table 4 and Figure 6B). A possible hypothesis is that minority compounds found in EVOO may be involved in facilitating *A. muciniphila*'s survival. It is important to note that these minority compounds are not present in either coconut or sunflower oil.

Furthermore, we have shown that the presence of opportunistic gut pathogens linked to an increase of CRC risk are also altered according to the type of diet consumed. At the genus level, we identified that *Streptococcus* and *Clostridium* decreased their abundance in mice fed any of the high-fat diets. Some species of *Clostridium*, such as *C. difficile*, produce toxic bacterial enzymes involved in bacteraemia and CRC development [28,29]. Interestingly, the coconut-HFD increased *Staphylococcus* spp., although not in a statistically significant way (Table 3 and Figure 7C). On the contrary, the EVOO-HFD significantly decreased the abundance of *Staphylococcus* spp. and especially *Staphylococcus epidermidis* (Table 3 and Figure 7D). It is well known that *S. epidermidis* is capable of forming biofilms on the colon, enhancing CRC risk [30]. All the bacteria mentioned above are considered to be opportunistic pathogens and have been found on colon specimens from patients with colitis and autoimmune diseases [31].

Another interesting finding was that EVOO- and sunflower-HFD reduced *Enterococcus gallinarum* abundance in a significant way. *E. gallinarum* is considered a pathobiont able to translocate to the liver and other tissues, triggering autoimmune responses [32]. In addition, it has been suggested that antibiotic treatment prevents mortality in mice by suppressing the growth of *E. gallinarum* in tissues [33], suggesting that EVOO- and sunflower-HFD could act on the autoimmune response by controlling the abundance of *E. gallinarum* in the colon.

Several studies have demonstrated an increased abundance of Proteobacteria phylum in pathologies, including inflammatory bowel disease; in fact, inflammation represents a core aspect of pathologies associated with Proteobacteria [34]. In addition, its high abundance has been correlated with oxygen reactive species generation and inflammation in humans with inflammatory bowel disease, colitis and CRC [35–37]. Sunflower-HFD enhanced *Neisseria* and *Sphingomonas* significantly (Table 3 and Figure 7E), both of which are considered pathobionts [38]. In fact, its increase has been correlated with a pro-inflammatory response in the mucosa of the colon, promoting natural killer T-cell activity of colitis-associated cancers [39,40]. On the contrary, coconut-HFD reduced *Neisseria* abundance, and EVOO-HFD reduced not only *Neisseria* and *Pseudomonas*, but also Proteobacteria abundance (Table 3, Figure 7C,D). To our knowledge, there are no studies that have described the beneficial or harmful effects related to Proteobacteria induced by EVOO, coconut or sunflower-HFD, however, some authors have described the protective effects of EVOO on immunomodulation in murine experimental ulcerative colitis and patients with ulcerative colitis [41,42], that could be associated not only with inflammatory processes involved on the MAPK and NFκB signalling pathways but also with microbiota modulation, as our results showed [43].

In another pathogenic microbiome, *Prevotella* and *Bacteroides* abundance appeared to be higher in coconut-HFD than EVOO- and sunflower-HFD (Table 3 and Figure 7C). Both genera have negative effects on colitis through the enhancement of inflammation; in fact, its reduction ameliorates colonic inflammation [44,45]. *Prevotella* abundance is also related to the increase in epithelial inflammation and triggers autoimmune diseases [46,47]. The results suggest that coconut-HFD could produce an increased risk of CRC by stimulating the growth of *Prevotella* and *Bacteroides*, while diets rich in EVO or sunflower oil could have a protective effect. Different research with honey polyphenols, essential oil and different vegetable oils, have shown that they were able to improve oxidative stress resistance and intestinal inflammation by Bacteroidetes reduction [48,49]. We could hypothesise that the effect of EVOO is probably due to the presence of polyphenols that characterise this fat of vegetable origin.

The gut microbiota can modulate the inflammatory processes that can eventually result in the presence of a pro- or anti-tumour environment in the colon. Thus, the main objective of this study was to determine whether an HFD containing only EVOO, sunflower oil or coconut oil modifies the gut microbiota in a way that favours or prevents CRC. In brief, the main findings of this study could be summarised as follows: (a) the three HFD studied (EVOO-, sunflower- and coconut-HFD) produce a decrease in both the richness and diversity of the gut microbiota, (b) these alterations do not correlate with body weight at the end of the dietary intervention, (c) each of these HFDs has a different impact on the gut microbiota by promoting or inhibiting the growth of different bacteria that are associated with a pro- or anti-inflammatory environment (summarised in Figure 8), and that are associated with the dysbiosis linked to colorectal cancer risk (Figure 9).

This study has several limitations, including the sample size, the exploratory nature of the study, and type-II errors which could fail to detect effects present in small-scale studies. For instance, further studies are needed to assert these hypotheses. The strength of this study is that we use a single source of fat in the diet; a large number of studies use high-fat diets supplemented with different types of fat. This can be misleading, since the basis of nutritional preparations for animals has a significant amount of both vegetable (soy oil) and animal (lard) fat. Giving therefore results that could be wrong or a mixed of effect from animal and vegetable oil. Using a single type of fat allows us to observe its specific effect on health.

Finally, it is difficult to extrapolate the results obtained in this study to humans due to interspecies variability. However, the usefulness of studies using murine experimental models as a preclinical model is widely accepted [50]. In addition, it is not possible to conduct a dietary intervention study in humans in which the diet contains only one type of fat, highlighting the importance of the data obtained in this study.

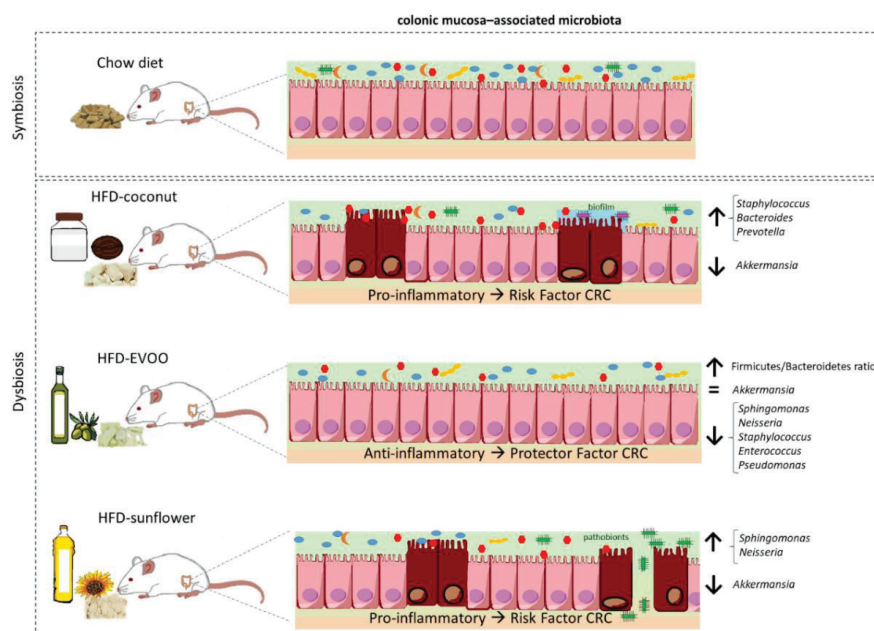


Figure 8. Summary of the main effects on gut microbiota caused by chow diet and HFD bases on coconut, EVOO or sunflower oil.

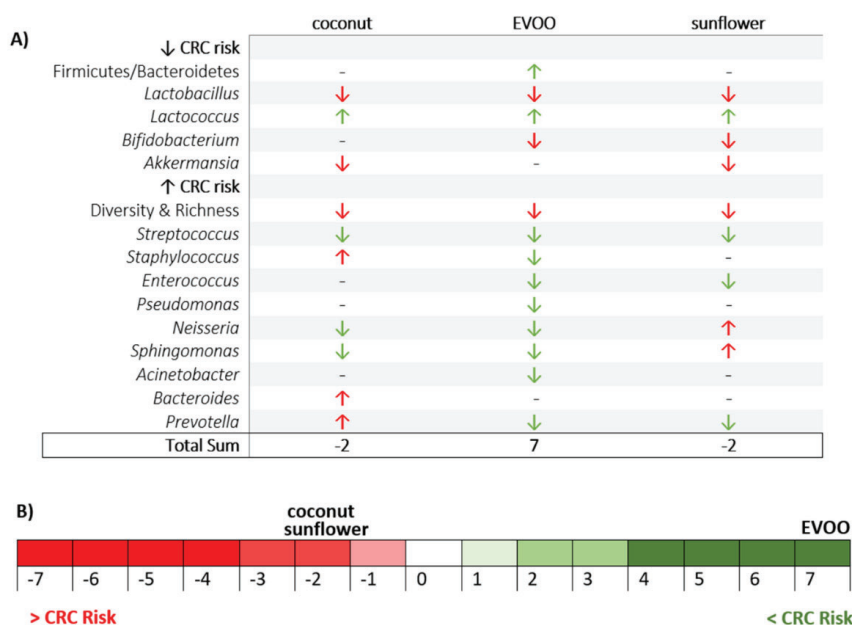


Figure 9. The effect of high-fat diets of sunflower, coconut and EVOO oils on dysbiosis linked to CRC risk. (A) Summary of HFD effects on bacteria related with CRC risk (Red arrow, increases CRC risk (minus 1); Green arrow, decreases CRC risk (plus 1); Dash, no changes (equal)). (B) Risk scale of developing CRC based on vegetable oil-HFD intake. Data are based on the total sum of Figure 9A.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2072-6643/12/6/1705/s1>, Figure S1: Rarefaction curves of the amplified samples for the detection of bacterial genera, Table S1: Composition of experimental high fat diets, Table S2: Minority compounds found in EVOO, Table S3: PicoGreen-Based DNA Quantification and Quality control analysis.

Author Contributions: J.J.G. and C.S.-Q. conceived and designed research; C.R.-G. performed the experiments and analysed data; J.J.G. and C.R.-G. drafted the manuscript; J.J.G., I.A. and C.S.-Q. edited and revised the manuscript; All authors have read and agreed to the published version of the manuscript.

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

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ANEXO II

The Mediterranean Diet

An Evidence-Based Approach

Second edition

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Antiinflammatory activity exerted by minor compounds found in virgin olive oils

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Mediterranean diet

Both chronic inflammatory processes and oxidative stress play important roles in chronic diseases. Different strategies to reduce both processes will aid in the prevention of these diseases. In this context, diet could be a preventive and aggravating factor. Thus it is important to emphasize the importance of following a healthy diet. The effects of diet on human health have been widely reported in many scientific studies, providing evidence that a dietary pattern rich in certain beneficial food groups can reduce the incidence of various chronic degenerative diseases.¹ Specifically the Mediterranean diet is considered as an example of a healthy diet. Several public health-based studies have revealed that adherence to the Mediterranean diet is associated with longevity and lower incidence of major chronic degenerative diseases, such as cardiovascular diseases and several cancers.² Unfortunately, diet and lifestyle changes have accelerated over the past three decades in developing and in transition countries. Over the past 20 years, there has been a generalized decrease in adherence to the Mediterranean diet in Southern European countries. This decrease is having a significant effect on the health and nutritional status of these populations. Therefore it is important to promote a healthy diet, especially among young people.

Virgin olive oil

Although there are variations in the components of the traditional Mediterranean diet between countries, virgin olive oil (VOO) represents a distinctive element, because it is the main source of dietary lipids in the traditional Mediterranean diet. It has been reported that VOO can effectively prevent and/or reduce hypercholesterolemia, serum lipoprotein

