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Teresa Vezza

Doctoral Thesis

NEW THERAPEUTIC PROPERTIES FOR THE JEWEL OF ANDALUSIA

Evaluation of anti-inflammatory activity of olive leaf extract (*Olea europea* L.) in experimental models of intestinal inflammation and metabolic syndrome

Departamento de Farmacología
Universidad de Granada

Programa Oficial de Doctorado
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FACULTAD DE FARMACIA

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Evaluation of the anti-inflammatory activity of olive leaf extract (*Olea europaea* L.) in experimental models of intestinal inflammation and metabolic syndrome

Teresa Vezza

Bajo la dirección de los Doctores

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"I have finally come to the conclusion that a good reliable set of bowels is worth more to a man than any quantity of brains" *(Josh Billings)*

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RESUMEN

INTRODUCCION

La Enfermedad Inflamatoria Intestinal (EII) y el Síndrome Metabólico (SM) constituyen dos enfermedades cuya prevalencia e incidencia se encuentran en continuo incremento, especialmente en países desarrollados.

La Enfermedad Inflamatoria Intestinal comprende la enfermedad de Crohn (EC) y la colitis ulcerosa (CU). Ambas se caracterizan por una inflamación crónica del intestino derivada de una respuesta inmune intestinal exacerbada ante un determinante antigénico desconocido, en la que se alternan periodos de exacerbación de los síntomas, seguidos de intervalos más o menos prolongados de remisión de los mismos [1, 2]. Aunque hasta el momento se desconocen los mecanismos responsables de la iniciación y perpetuación en el tiempo del proceso inflamatorio intestinal, se acepta que están implicados factores genéticos, ambientales e inmunológicos. Así, numerosos estudios han propuesto que, en personas genéticamente predispuestas, una activación exagerada y descontrolada del sistema inmune intestinal frente a un determinante antigénico desconocido, puede desencadenar la aparición de la respuesta inflamatoria intestinal exacerbada [3]. Esta respuesta inmunológica genera numerosos mediadores de carácter pro-inflamatorio (citocinas, eicosanoides y metabolitos reactivos derivados del oxígeno o del nitrógeno) que actúan de forma sinérgica y simultánea promoviendo la amplificación y cronificación del proceso inflamatorio intestinal [4-6].

El sindrome metabolico (SM) es el conjunto de alteraciones metabólicas constituido por la existencia de obesidad de distribución central, conjuntamente con otras alteraciones que pueden incluir la disminución de las concentraciones del colesterol unido a las lipoproteínas de alta densidad, la elevación de las concentraciones de triglicéridos, el aumento de la presión arterial y la hiperglucemia asociada a resistencia a la insulina [7]. La obesidad, considerada como eje central en el desarrollo del síndrome metabólico, es una enfermedad crónica, de etiología compleja y multifactorial, y que se desarrolla por un desequilibrio entre la energía ingerida y la energía gastada, es decir, una acumulación anormal o excesiva de energía en forma de grasa en el tejido adiposo. El exceso de energía se almacena en los adipocitos, los cuales aumentan en tamaño y/o en número. Este desequilibrio es el resultado de la combinación de varios factores fisiológicos, psicológicos, metabólicos, genéticos, socioeconómicos y culturales. En los últimos años se ha observado que los pacientes obesos presentan un estado inflamatorio crónico subclínico como

consecuencia del incremento en la masa del tejido adiposo, que lleva a un aumento en la producción de mediadores proinflamatorios que son conjuntamente estimulados por señales de origen exógeno y/o endógeno. El tejido adiposo contiene fibroblastos, preadipocitos, adipocitos y macrófagos; estos últimos contribuyen de manera importante al proceso inflamatorio sistémico con la producción de mediadores proinflamatorios. Así, existe una asociación íntima, altamente coordinada, entre las vías inflamatorias y las metabólicas [8]. Además, recientemente se ha prestado atención al vínculo entre la composición de la microbiota intestinal, la permeabilidad intestinal y la obesidad. Así, se ha observado, en modelos animales y en humanos, que la microbiota intestinal es una fuente de endotoxinas (LPS) cuya presencia en el plasma está relacionada con la obesidad y la resistencia a la insulina, a través del aumento de la permeabilidad intestinal. El correcto funcionamiento de la barrera intestinal es esencial para evitar la excesiva translocación de estas moléculas tóxicas a la circulación y la microbiota puede influir en la integridad del epitelio intestinal y la inflamación de la mucosa, ambas involucradas en la permeabilidad intestinal.

Actualmente no existe un fármaco ideal que combine efectividad con ausencia de reacciones adversas en el tratamiento de estas dos enfermedades, por lo que se hace necesaria la investigación de nuevas estrategias terapéuticas que aúnen eficacia y seguridad. Además, distintos estudios han puesto de manifiesto la tendencia actual por parte de los pacientes con enfermedad inflamatoria intestinal y/o alteraciones metabólicas de emplear medicinas alternativas y/o complementarias en el tratamiento de sus enfermedades, probablemente como consecuencia de la falta de eficacia que el tratamiento convencional tiene en muchas ocasiones, o derivados de la elevada incidencia de reacciones adversas que les caracteriza [9, 10]. Entre la distintas terapias alternativas utilizadas destaca el empleo de plantas medicinales con propiedades antiinflamatorias: son tratamientos seguros, con empleo ancestral y escasa aparición de efectos secundarios, y que contienen una mezcla de principios activos que pueden asegurar la actuación simultanea sobre distintas dianas terapéuticas afectadas durante el proceso inflamatorio. En ocasiones, el empleo empírico de plantas medicinales antiinflamatorias o útiles en patologías intestinales, no tiene una constatación científica avalada. Este podría ser el caso del uso de extractos

vegetales procedentes de plantas medicinales de Andalucía utilizadas tradicionalmente por sus propiedades antiinflamatorias y en problemas digestivos.

El olivo (*Olea europea* L.) es una especie muy abundante en Andalucía de la cual se hace un aprovechamiento industrial, alimentario y medicinal. Entre los usos tradicionales de la hoja de olivo se encuentra el antihipertensor y el antidiabético, lo cual justifica la inclusión en este estudio.

OBJETIVO

El presente trabajo de Tesis doctoral pretende valorar el potencial efecto antiinflamatorio de un extracto bien caracterizado desde el punto de vista químico de hojas de olivo, en modelos de colitis experimental o de síndrome metabólico. La presencia de diferentes componentes activos polifenólicos en este tipo de extracto vegetal hace que pueda ser candidato para su potencial uso en pacientes con enfermedad inflamatoria intestinal y en personas obesas.

Así, se propusieron tres objetivos principales:

1. Evaluar la actividad inmunomoduladora de las hojas de olivo:
    a) *in vitro* a través de estudios en macrófagos murinos, células epiteliales murinas y humanas, células mononucleares de sangre periférica (PBMC) procedentes de individuos sanos y pacientes con Enfermedad de Crohn.
    b) *ex-vivo*, utilizando explantes humanos provenientes de pacientes con enfermedad inflamatoria intestinal.
2. Evaluar el efecto anti-inflamatorio intestinal de distintas dosis de extracto de hoja de olivo en modelos de colitis experimental inducida por ácido dinitrobencenosulfónico (DNBS) y en el modelo del sulfato de dextrano sódico (DSS) en ratones.
3. Evaluar el efecto de las hojas de olivo en un modelo experimental de obesidad en ratones con especial atención a la disfunción vascular.

En todos estos ensayos se pretendió recabar información que permitiera establecer los mecanismos responsables de los posibles efectos beneficiosos.

METODOLOGÍA

El extracto objeto de nuestro estudio fue proporcionado por CIDAF, y caracterizado químicamente [11]. Todos los protocolos que implican experimentación animal fueron aprobados por el Comité de Ética de la Universidad de Granada (Ref. No. CEEA-2010-286 ; Ref. No. 94-CEEA-OH 2015).

1. Evaluación "in vitro" de la actividad inmunomoduladora del extracto de hojas de olivo.

1.1. Ensayos "in vitro" en células.

Para ello, se valoró el efecto que distintas concentraciones del extracto de hojas de olivo tienen sobre la viabilidad y actividad de varios tipos celulares involucrados en la respuesta inmune intestinal: macrófagos murinos (RAW 264), células epiteliales murinas (CMT-93) y humanas (Caco-2), y en células mononucleares de sangre periférica (PBMC) procedentes de individuos sanos y pacientes con Enfermedad de Crohn.

Todas las células se cultivaron hasta la formación de una monocapa, se preincubaron con diferentes concentraciones de extracto de hoja de olivo que oscilaron entre 0,1 y 100 μg/ml durante 2 horas y se estimularon con el lipopolisacárido (LPS) de *Escherichia coli* (100 ng/ml) o interleucina (IL) -1β (1 ng/ml) durante 24 horas. Las células no estimuladas y no tratadas, así como las células estimuladas con LPS o IL-1β pero no incubadas con extracto, se usaron como controles negativos y positivos, respectivamente. Después del período de estimulación, se recogieron los sobrenadantes y se midió la producción de citocinas mediante ELISA usando kits comerciales.

1.2. Estudio "ex-vivo" en explantes intestinales.

Se tomaron muestras quirúrgicas de pacientes con EC colónica sometidos a cirugía por enfermedad crónica activa, que respondían poco al tratamiento médico, o de pacientes con EC ileal sometidos a cirugía debido a estenosis. Las muestras se colocaron en rejillas de hierro con la cara mucosa hacia arriba en el pocillo central de una placa de cultivo de órganos y se incubaron a 37 ° C en presencia o ausencia de extracto de hoja de olivo (0.1-100 μg/mL) y / o LPS (100 ng/mL). Después de 24 h, las muestras de la mucosa se homogenizaron y se extrajo el ARN total. Paralelamente, se recogieron los sobrenadantes de cultivo y se evaluaron las citocinas factor de necrosis tumoral (TNF)-α, IL-1β, IL-6 e IL-8 mediante ELISA.

2. Estudio de la actividad antiinflamatoria intestinal "in vivo".

Los animales de experimentación que se utilizaron en estas experiencias fueron ratones CD1 y ratones C57BL/6J de 20 g de peso, suministrados por la Universidad de Granada y/o por Janvier, y que fueron alojados en el estabulario del Servicio de Animales de Experimentación de la Universidad de Granada. Se procedió a la valoración del efecto antiinflamatorio intestinal de las hojas de olivo en dos modelos de colitis experimental: colitis inducida por DNBS [12] y colitis inducida con DSS [13]. El tratamiento oral de los animales colíticos (n=10) con las distintas dosis del extracto se inició dos días antes de la inducción del daño colónico con el DNBS o el mismo día de la inducción en el caso del DSS. En el curso de la experiencia se determinaron una serie de parámetros generales como el seguimiento del consumo de comida, la evolución del peso corporal y la aparición de heces diarreicas, lo que permitió una asignación diaria del Índice de Actividad de la Enfermedad (IAE) [14]. Tras el sacrificio de los animales se obtuvieron los segmentos de colon, y se valoró el proceso inflamatorio intestinal mediante:

a) *Parámetros macroscópicos*: existencia de adhesiones entre el colon y órganos adyacentes y la relación peso/longitud.

b) Estudio microscópico: para lo que se tomaron muestras del tejido inflamado de zonas representativas y se sometieron a técnicas estándar de microscopía óptica, que permiten valorar la alteración de la integridad intestinal a nivel microscópico (pérdida de la arquitectura de las criptas, infiltración de células inflamatorias), como consecuencia de la inducción de la colitis experimental [14] y el efecto beneficioso del tratamiento administrado.

c) Determinaciones bioquímicas: se procedió al análisis de la expresión génica y/o producción de marcadores del proceso inflamatorio. Esto incluye citocinas como TNF-α, IL-1ß, IL-6, IL-17; mediadores quimiotácticos como la proteína quimio-atrayente de monocitos MCP-1 (monocyte chemoatractant protein), la proteína inflamatoria de macrófagos MIP (macrophage inflammatory protein)-2; la molécula de adhesión intercelular ICAM (inter-cellular adhesion molecule)-1, y marcadores de la función de barrera intestinal como mucinas (MUC-2 y MUC-3), trefoil factor (TFF)-3, y proteínas de las uniones estrechas del epitelio como Zonula occludens (ZO)-1.

d) Valoración de la permeabilidad intestinal, mediante la administración oral de FITC-dextrano a los ratones sometidos a inflamación intestinal con DSS, y determinación a las cuatro horas de la concentración plasmática del mismo por espectrofluorometría.

3. Estudio del efecto de las hojas de olivo en un modelo de obesidad.

Se utilizaron ratones C57BL/6J de 5 semanas de edad que consumieron una dieta estándar o una dieta rica en grasa, en la que el 60% del aporte calórico provenía de grasa de origen animal. Los ratones se dividieron aleatoriamente en 6 grupos experimentales: grupo sano (dieta estándar), grupo control sano (administrado con la dosis mayor del extracto), grupo obeso (dieta rica en grasa) y 3 grupos obesos (dieta rica en grasa) tratados con distintas dosis del extracto de hojas de olivo. El tratamiento duró 5 semanas. Durante el periodo experimental se midió el peso corporal, el consumo de comida y bebida semanalmente [15]. Al final del tratamiento se tomaron muestras plasmáticas y tisulares (aorta, hígado, grasa epididimal e intestino). Un segmento de aorta se utilizó para estudios de la funcionalidad endotelial. Para la caracterización metabólica se midieron los niveles plasmáticos de glucosa, y colesterol (cHDL y cLDL), mientras que la insulina se calculó mediante enzimoinmunoensayo. El grado de resistencia a la insulina se evaluará con el cálculo del HOMA-IR. Muestras de tejido adiposo y de hígado se utilizaron para la extracción de ARN con el objeto de evaluar la expresión de diferentes biomarcadores que se ven alterados en condiciones de obesidad: marcadores de tipo inflamatorio (TNF-α, IL-1β, IL-6), proteínas implicadas en el mantenimiento de la homeostasis energética (leptina y adiponectina), receptores activados por proliferadores de peroxisomas (PPARα y PPARγ), la proteína trasportadora de membrana de glucosa (GLUT-4) y la proteína quinasa AMP (AMPK) involucrada en los procesos metabólicos. Las muestras de intestino grueso se utilizaron para la extracción de ARN con el objetivo de evaluar la expresión de diferentes biomarcadores relacionados con la función de barrera intestinal: MUC-2, MUC-3, ZO-1 y ocludina. Además, se recogieron los contenidos fecales para la extracción de ADN bacteriano genómico en su totalidad y se realizó la amplificación del gen 16S del ARNr. Posteriormente, se llevó a cabo la secuenciación del material genético por pirosecuenciacion de los amplicones [16].

RESULTADOS

El extracto de hojas de olivo manifestó efecto anti-inflamatorio intestinal en los dos modelos estudiados de colitis experimental en ratones: modelo experimental inducido por DNBS y el inducido por DSS. Mediante los estudios histológicos se observó una recuperación del daño intestinal y, a través de los análisis bioquímicos, se puso de manifiesto una mejora de los diferentes marcadores del proceso inflamatorio, entre los cuales se encuentran la reducción de la expresión de distintas citocinas como TNF-α, IL-6, IL-1β, IL-8 e IL-17, la quimocina MCP-1 y la molécula de adhesión ICAM-1, mejorando así la respuesta inmune alterada asociada a la inflamación del colon. Además, el extracto fue capaz de incrementar significativamente la expresión de marcadores de la integridad del epitelio intestinal como MUC-2 y TFF-3, poniendo de manifiesto una mejora de la permeabilidad del colon que se encuentra alterada en la inflamación intestinal, y que se corroboró por lo estudios funcionales llevados a cabo con el FITC-dextrano en el modelo del DSS.

El efecto inmunomodulador directo por parte del extracto se comprobó *in vitro* en varios tipos celulares involucrados en la respuesta inmune intestinal: macrófagos murinos (RAW 264), células epiteliales murinas (CMT-93) y humanas (Caco-2) y en células mononucleares de sangre periférica (PBMC) procedentes de individuos sanos y pacientes con Enfermedad de Crohn. El extracto inhibió la producción de nitritos inducida por LPS en células RAW y redujo la producción de IL-8 inducida por IL-1β en células Caco-2 y la liberación de IL-6 inducida por LPS en células CMT-93. Además, las propiedades inmunomoduladoras del extracto fueron confirmadas ya que disminuyó la producción de citoquinas proinflamatorias en PBMC estimuladas con LPS.

En forma adicional, se realizaron estudios ex-vivo. El extracto de hoja de olivo redujo significativamente la expresión de TNF-α, IL-1β, IL-6 e IL-8 en explantes de colon de pacientes con EC cuando se estimularon con LPS. Del mismo modo, la producción de estas citocinas se redujo significativamente por efecto del extracto. Sin embargo, no se pudo establecer una clara relación dosis-efecto, probablemente debido a la composición compleja del extracto. Por todo ello, podemos concluir que el extracto de hoja de olivo posee un efecto anti-inflamatorio en diferentes modelos de colitis experimental que podría ser atribuido a sus propiedades inmunomoduladoras.

Los efectos del extracto de hoja de olivo se pusieron de manifesto en un modelo de obesidad inducido por dieta rica en grasas (*high fat diet* –HFD-) en ratones. Después de

5 semanas, el peso corporal promedio de los ratones no tratados y alimentados con una dieta rica en grasa fue considerablemente mayor en comparación con los grupos de dieta standard. La administración del extracto a ratones obesos redujo significativamente este aumento de peso, aunque no se observaron diferencias significativas en el consumo de energía entre estos grupos, por lo que el efecto estaría relacionado con una disminución de la eficiencia energética. La administración del extracto redujo también la glucemia basal y la resistencia a la insulina, y mostró una mejoría en el perfil lipídico plasmático en comparación con los ratones obesos.

Además de las anomalías metabólicas, la obesidad está asociada con inflamación sistémica, que afecta tanto al hígado como al tejido adiposo [17]. Por consiguiente, en estos dos tejidos, los ratones alimentados con HFD mostraron una expresión aumentada de ARNm de las citocinas proinflamatorias TNF-α, IL-1β e IL-6. Además, en el tejido adiposo hubo una expresión incrementada del mediador quimiotáctico MCP-1 que facilitaría la infiltración y activación de macrófagos, y la posterior instauración del estado inflamatorio. Todos estos marcadores inflamatorios asociados a la obesidad se mejoraron significativamente en aquellos ratones alimentados con HFD tratados con el extracto, que mostraron principalmente una respuesta dosis dependiente. Además, las adipocinas secretadas por el tejido adiposo, la leptina y la adiponectina, muestran un papel clave en la integración del metabolismo sistémico, estando su producción y función alteradas en la obesidad [18]. En el presente estudio, la expresión tanto de leptina como de adiponectina en el tejido adiposo se vio afectada en ratones obesos cuando se comparó con los ratones alimentados con dieta standard, estando asociada a una expresión reducida del receptor de la leptina tanto en el hígado como en la grasa. La administración del extracto a ratones obesos dio como resultado una mejora significativa en la expresión de estas adipocinas en todas las dosis ensayadas. Además, la reducción de la expresión del receptor de leptina en el hígado y el tejido adiposo se mejoró significativamente con el extracto, aunque en el hígado, esto sólo se obtuvo con la dosis más alta analizada.

Por último, los estudios de expresión de marcadores en el intestino grueso indicaron que el extracto fue capaz de mejorar la función de barrera intestinal. En los ratones obesos esta función está alterada ya que se observa una reducción en la expresión colónica de ocludina y mucinas (MUC-2 y MUC-3). Sin embargo, en los ratones tratados los niveles son similares a los de los delgados.

Diferentes estudios han propuesto que la microbiota intestinal se puede considerar una diana para el tratamiento de diferentes afecciones inflamatorias, incluidas las localizadas en el sistema gastrointestinal, como la EII, y aquellas con manifestaciones sistémicas, incluyendo hipertensión y obesidad [19, 20]. Al considerar la obesidad, es bien sabido que se produce una alteración en la composición de la microbiota intestinal, que afecta principalmente a los dos principales grupos de bacterias beneficiosas dominantes en el intestino humano, los Bacteroidetes y los Firmicutes. Para evaluar la composición de la microbiota y su posible alteración en el modelo de obesidad y con los diferentes tratamientos realizamos la secuenciación del DNA extraído de las muestras fecales; y calculamos diferentes índices ecológicos como el de Shannon (parámetro que combina riqueza y uniformidad); Pielou (muestra la presencia eventual de algún individuo además de cómo se distribuye en la muestra) y por último el de Chao (índice de riqueza estimada). Además, se calculó la abundancia de los principales filos bacterianos y la ratio de los dos mayoritarios, Firmicutes y Bacteriodetes. La relación *Firmicutes y Bacteriodetes*, conocida como F/B, es un potencial marcador para evaluar una situación de disbiosis [21-23]. Los resultados revelaron que el tratamiento con el extracto en ratones obesos fue capaz de contrarrestar esta composición alterada de la microbiota intestinal, y la proporción de filo bacteriano principal se restauró a los valores normales observados en ratones alimentados con dieta estandard.

Además, las modificaciones significativas de las diferentes clases o géneros que pertenecen a los filos Actinobacteria, Bacteriodetes y Verrumicrobiota observadas en ratones obesos se restauraron parcialmente en aquellos ratones obesos tratados con el extracto de olivo. Especial atención se ha prestado al papel de *Akkermansia muciniphila* en la obesidad; se trata de una bacteria degradadora de mucina que reside en la capa de moco [24] y su abundancia se correlaciona inversamente con el peso corporal y la diabetes tipo 1 en ratones y humanos [25-28]. En el presente estudio, se observó una reducción en la proporción del género *Akkermansia* en ratones obesos control no tratados, y esto se revirtió después del tratamiento con el extracto. La restauración de la abundancia de *Akkermansia sp.* ejercida por el extracto de hojas de olivo podría estar asociada a la mejora en la función de barrera intestinal, a través del aumento de la producción de mucinas en el tejido colónico, ya que éstos son los principales nutrientes para estas bacterias. En consecuencia, la mejora en la disbiosis asociada a la obesidad en ratones obesos tratados con el extracto puede dar como resultado la modulación de la

respuesta inmune alterada, contribuyendo así a los efectos beneficiosos observados en este modelo experimental de síndrome metabólico.

Distintos estudios han demostrado que la obesidad está asociada a alteraciones cardiovasculares, incluida la disfunción endotelial [15, 29]. De hecho la aorta de ratones obesos mostró respuestas vasodilatadoras a la acetilcolina dependientes del endotelio significativamente reducidas, lo que se considera un índice de la función endotelial, en comparación con las aortas del grupo control sano. El extracto de hojas de olivo revirtió la disfunción endotelial observada en los anillos aórticos de ratones obesos, en todas las dosis ensayadas.

CONCLUSIONES

1. El extracto de hojas de olivo manifestó un efecto anti-inflamatorio intestinal en dos modelos experimentales de colitis (DSS y DNBS). Son varios los mecanismos que pueden estar involucrados, como la capacidad de restaurar la integridad de la barrera intestinal y modular la producción de mediadores inflamatorios. Esta actividad se confirmó *in vitro*, donde el extracto redujo la expresión y/o producción de citoquinas proinflamatorias inducidas por estímulos inflamatorios en diferentes tipos de células inmunes, incluyendo células mononucleares de sangre periférica humana. Además, el extracto mostró un efecto directo en los explantes de la mucosa de los pacientes con EC, inhibiendo la producción excesiva de los mediadores proinflamatorios que caracteriza la enfermedad.

2. El extracto de hoja de olivo ejerció efectos beneficiosos en un modelo de obesidad inducido por dieta rica en grasas en ratones, con una mejora en el metabolismo alterado de glucosa y lípidos. Estos efectos se asociaron a la mejora en el estado inflamatorio sistémico, junto con la restauración de la disfunción vascular que caracteriza la obesidad. Diferentes mecanismos parecen participar, e incluyen propiedades prebióticas, que contrarrestan la disbiosis asociada a la obesidad y la mejora de la función de barrera epitelial intestinal. Además, los efectos moduladores de este extracto sobre la respuesta inmune alterada también pueden colaborar en los efectos beneficiosos contra la obesidad y las complicaciones derivadas.

INTRODUCTION

INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gastrointestinal tract characterized by chronic and spontaneously relapsing inflammation of the intestine. Two main conditions are included under the heading of IDB: Crohn's disease (CD) and ulcerative colitis (UC) [1]. Both forms of IBD significantly impair quality of life, and require prolonged medical and/or surgical interventions. They share many epidemiological, pathological and therapeutic characteristics. Both have overlapping symptoms, including diarrhoea, fever, abdominal pain and cramping, blood and/or mucus in the stool, and even clinical signs of bowel obstruction [1, 30], thus justifying why in some patients is not possible to distinguish the form of IBD that is present (indeterminate colitis). However, there are important differences that discriminate these inflammatory conditions, according with the location of the inflammation and appearance (Table 1). In fact, CD is characterized by a transmural, segmental, and typically granulomatous inflammation, which may involve any part of the gastrointestinal tract, from mouth to anus. By contrast, UC is a non-transmural inflammatory disease restricted to the colon, being the inflammatory changes limited to the mucosa and submucosa, with cryptitis and crypt abscesses [31] (Figure 1).

1.1 Etiology

IBD is considered as an idiopathic and multifactorial disease. Despite of scientific efforts during the last decades, its aetiology remains rather unclear. Many theories have been proposed to explain IBD pathogenesis, ranging from infectious to psychosomatic, social, metabolic, vascular, genetic, allergic, autoimmune and immune-mediated mechanisms [2, 32, 33].

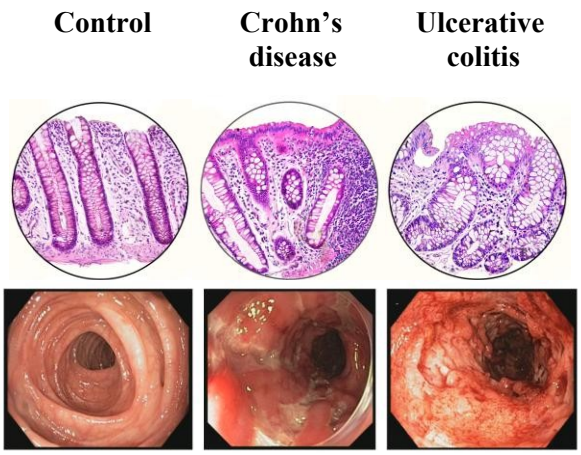


Figure 1. Histology (H&E) and endoscopic views of normal, CD and UC human intestine.

Currently, the condition seems to be the result of a combination of environmental, genetic, microbial and immunologic factors in which an uncontrolled immune response towards unknown antigens from the intestinal lumen leads to inflammation in genetically predisposed individuals. However, it is widely accepted that none of these components can by itself trigger or maintain the intestinal inflammation, being their integration and reciprocal influence responsible for its onset and the specific clinical phenotype [34].

Table 1. Differences between Crohn's disease and ulcerative colitis.

DIFFERENCES	CROHN'S DISEASE	ULCERATIVE COLITIS
LOCATION	Inflammation may occurs anywhere along the digestive tract, from mouth to anus.	Inflammation limited to the mucosal layer of the colon.
INFLAMMATION	Asymmetrical inflammation, in patches.	Continuous inflammation of the colon.
APPEARANCE	Colon wall may be thickened and may have a cobblestone appearance.	Colon wall is thinner and shows continuous inflammation.
DEPH OF INFLAMMATION	Ulcers along the digestive track are deep and may extend into all layers of the bowel wall.	Inflammation only affects the mucosa layer.
BLEEDING	Not common.	Bleeding from the rectum during bowel movements.

Genetic factors. The well-known familial occurrence of IBD has suggested that this condition could have a genetic basis [35]. In the last years, many susceptibility loci for either CD or UC have been investigated [36, 37]. Genome-wide association studies (GWAS) have identified 99 non-overlapping genetic risk loci. Although the genetic component is stronger in CD than in UC, and despite their distinct clinical features, approximately 30% of these IBD-related genetic loci are shared between both conditions, indicating that these diseases share common pathways [38, 39]. Analyses of the genes and genetic loci implicated in IBD show several mechanisms that are crucial for intestinal homeostasis, including barrier function, epithelial restitution, microbial defence, innate and adaptative responses, reactive oxygen species generation, autophagy, endoplasmic reticulum stress and metabolic pathways associated with cellular homeostasis. The intracellular nucleotide oligomerization domain 2 (NOD2)

[40, 41], caspase recruitment domain-containing protein 9 (CARD9) [42], and intelectin-1 (ITLN1) [36] in the innate immune response; interleukin (IL)-23R [36], signal transducer and activator of transcription 3 (STAT3) [36], and Tumour Necrosis Factor (TNF) Superfamily Member 15 (TNFSF15) [43] in the IL-23-Th17 pathway, autophagy-related protein 16-1 (ATG16L1) [44, 45] and immunity-related GTPase family M protein (IRGM) [46] in autophagy, X-box binding protein 1(XBP-1) [47], endoplasmic membrane protein complex (EMC1) [48] and orosomucoid like 3 (ORMDL3) [37] in endoplasmic reticulum stress, and protein–tyrosine phosphatase, non-receptor type 2 (PTPN2) [49] in T cell response, have been identified. Moreover, genetic polymorphisms can act synergically to modify the Paneth cell functions [50]. As commented above, although the vast majority of loci are shared between both diseases, some loci, like NOD2 and ATG16L1, are specifically associated with CD and others only with UC (ORMDL3 and EMC1) [48, 51]. The NOD2/caspase recruitment domain 15 gene (CARD15) variant was the first IBD gene discovered, associated with ileal CD [40]. This gene is expressed in different cellular types, including dendritic cells (DCs), Paneth cells and mucosal epithelial cells. This intracellular molecule recognizes bacterial components, like peptidoglycans, which leads to a cascade activating NF-κB [52]. Therefore, NOD2/CARD15 may represent an antibacterial factor and its mutation might result in decreased immune defence. Furthermore, this gene modulates the secretion of defensins, which are endogenous molecules produced by Paneth cells; thus, NOD2/CARD15 mutations have also a negative impact on the release of defensins [53, 54]. However, it is important to note that only ≈20% of CD patients are homozygous for NOD2 variants [3, 55], and mutations in this gene alone do not spontaneously result in IBD. Hence, genetics highlight the key part played by the interaction between the internal microenvironment in the form of gut microbial dysbiosis and associated immunological response, both processes influenced by the external environment.

Environmental influences. Epidemiological studies have pointed out a large number of risk factors for developing IBD, such us cigarette smoking, diet, oral contraceptives, vaccination history and other drugs like antibiotics, appendectomy, infections, water supply, social circumstances and perinatal and childhood factors [56]. Although there is no direct evidence except for cigarette smoking. Another feature that has been considered to explain the increasing prevalence of IBD is the "hygiene hypothesis", which proposes that the lack of proper exposure to common infections early

in life negatively affects the development of the immune system, since it becomes less "educated" and less prepared to deal with multiple new challenges later in life [57]. This is indirectly supported by evidence of improved health and the acquisition of dietary habits from western societies in countries where IBD is rising [58, 59].

Microbiota. The intestinal microbiota is the largest reservoir of bacteria in the body, and in a healthy subject consists of nearby $\sim 10^{12}$ cells, belonging to more than 1,000 different species; of note, the microbial genomes encode for a multitude of proteins as the human genome [60, 61]. Consequently, the intestinal microbiota is recognized to confer health benefits in the context of nutrition, protection from infection by pathogens, metabolic processes, and development of the immune system, among other functions. It is currently known that the healthy gut includes bacterial species that are present in the majority of individuals. Moreover, the novel technologies, including 16S-based next-generation sequencing, have facilitated the culture-independent examination of microbial composition and indicated that there is a large microbial diversity among people [62, 63]. Notably, despite this broad interindividual variability, in a given subject there is a remarkable overall temporal stability in the microbiome, thus suggesting the existence of a core microbial community that potentially depends on certain host and environmental factors [64, 65]. Colonization of the sterile foetal gut begins directly at birth with maternal vaginal and faecal microbiota and other bacteria from the environment [66]. Initially, it is characterized by the predominance of facultative anaerobes, like enterobacteria and enterococci. These bacteria consume the oxygen and are gradually associated with the colonization of anaerobic bacteria belonging to the genera *Bifidobacterium, Bacteroides, Eubacterium, Veillonella* or *Clostridium*. In consequence, the intestinal microbiota in newborns is characterized by low diversity and a relative dominance of the phyla *Proteobacteria* and *Actinobacteria*; thereafter, the microbiota becomes more and more diverse with appearance of the supremacy of *Firmicutes* and *Bacteriodetes*, which characterizes the adult microbiota [61, 62, 67]. Interestingly, several studies have reported that interactions of the host with commensal or pathogenic bacteria may be an important factor for disease development [51]. Accordingly, studies in animal models of IBD have clearly demonstrated protection from development of intestinal inflammation in a germ free environment [68]. Moreover, adoptive transfer of microbiota from mice with colitis to healthy recipients induced intestinal inflammation in these mice [69]. Notably, mucosal

T lymphocytes in IBD were shown to respond to commensals, indicating that gut microbiota is a direct trigger of intestinal inflammation [70].

In human patients, different studies have demonstrated that intestinal microbial communities are markedly shifted in IBD, a condition referred to as dysbiosis [71, 72]. Further studies have revealed that the bacteria concentrations are greater than 10^9/ml in 90-95% of IBD patients, whereas this is true in 35% of healthy controls. However, although there is an increase of bacterial concentration in IBD, the gut microbiota in patients with IBD is constituted by fewer bacterial species and is unstable over time in comparison with the healthy gut, and even in patients in remission [73]. In the healthy gut, the microbiota is dominated by the phyla *Firmicutes* and *Bacteroidetes*, followed in lesser amounts by species belonging to the *Actinobacteria* and *Proteobacteria* [61]. By contrast, both *Firmicutes* and *Bacteroidetes* are decreased in IBD, whereas *Actinobacteria* and *Proteobacteria* are considerably increased [71, 74]. Moreover, IBD patients show reduced diversity of the gut microbiota, which clearly correlated with a decline in the diversity of *Firmicutes*, particularly in the clostridial cluster IV of anaerobic bacteria [75], as well as in those genera, such as *Faecalibacterium*, *Bifidobacterium* and *Lactobacillus*, associated with mucosal protection in the intestine. In addition, species belonging to the family *Enterobacteriaceae* (*γ-Proteobacteria*) are relatively expanded in faecal samples from these patients [76], mostly including facultative aerobes and different translocating and gram negative pathogens, like adherent invasive *Escherichia coli*. Importantly, differences in *alpha* diversity were detected in IBD patients when faecal samples from inflamed and non-inflamed areas of the mucosa were directly compared [77].

Altered immune response. Under physiological conditions, a large number of innate and immune cells are located in the intestinal lamina propria, such as T and B cells, natural killer (NK), Natural Killer T (NKT) cells, macrophages, dendritic cells (DCs), mast cells, neutrophils, eosinophils, as well as stromal cells (such as fibroblasts). All of these cells coexist in a perfect equilibrium that confers tolerance and protection at the same time. However, under inflammatory conditions, a large number of activated immune cells infiltrate into the intestinal mucosa. These immune cells and some stromal cells not only express high levels of adhesion molecules and auxiliary signal molecules (such as CD54, CD62L), but also express high levels of inflammatory mediators and

chemokine receptors (such as CCR5, CCR6, and CCR9) and integrins (such as integrin α4β7). Moreover, fibroblasts and capillary endothelial cells in the intestinal mucosa also express high levels of chemokines, selectins (like selectin E) and intracellular adhesion molecule-1 (ICAM-1, or CD54), which further induce intermolecular interactions of leukocytes in the blood circulation to migrate into the intestinal mucosa, and promote a local inflammatory response [78, 79]. The adaptive immune response can clearly contribute to the pathogenesis of IBD by increasing the release of pro-inflammatory cytokines, which are important to drive the polarization of the T-helper (Th) response, but also to reduce the effectiveness of regulatory T-cells. For many years, T-helper type 1 (Th1) cells, together with their proinflammatory cytokines, interferon (IFN)-γ and tumor necrosis factor (TNF)-α, were thought to be responsible for CD pathogenesis, whereas UC was considered to be associated with Th2-like responses, characterized by enhanced production of IL-4, IL-5 and IL-13 [79, 80]. The imbalance of pro and antiinflammatory cytokines contributes to intestinal mucosal inflammation. However, studies on the IL-23/Th17 axis have demonstrated that a subtype of $CD4^+$ T cells that produce IL-17, called Th17 cells, is an essential player in the pathogenesis of IBD [81]. Recent studies have shown that RORγt (retinoic acid-related orphan nuclear receptor γt)-dependent innate lymphoid cells (ILCs) are another dominant source of IL-17 that perform both protective and pathogenic roles in the regulation of the intestinal mucosal inflammatory responses [82]. Furthermore, accumulating evidences have also suggested that the dysfunction of the regulatory immune cells, which have the capacity of suppressing excessive inflammatory responses in intestinal mucosa, contribute to the development of IBD [83]. All these events result in the failure of maintaining intestinal mucosal immune tolerance and further promoting local intestinal mucosal immune response, leading to the intestinal mucosal injury.

In the following section, it is provided a more detailed description of the role of the innate and the adaptive immune responses in IBD.

1.2 Intestinal barrier function

The intestinal mucosal barrier serves as the first defensive line against luminal environment and is crucial in the maintenance of host homeostasis [84]. It is a selectively permeable barrier since it allows the passage of water, electrolytes and nutrients, but prevents from invasion of foreign antigens, microorganisms and their

toxins [84, 85]. This epithelial intestinal barrier consists of a single layer of different cells with specific functions, including absorptive enterocytes, goblet cells, enteroendocrine cells, microfold (M) cells and Paneth cells [84]. The enterocytes are the main cell type in the intestinal epithelium and mainly perform absorptive functions. With the exception of M cells, the epithelial cells are covered with a mucus layer, which reinforces the defensive role of the epithelial barrier [86]. In fact, it serves as a diffusion barrier that prevents the direct contact of most macromolecules, particles and bacteria with the membranes of the microvilli, but is readily available for the hydrolysed food substances; also, it possesses lubricant properties that reduce the physical damage to the mucosa. Goblet cells are responsible for the secretion of bioactive molecules such as secretory mucin glycoproteins (MUC2), epithelial membrane-bound mucins (MUC1, MUC3, MUC17) and trefoil factor peptides (TFF) [87, 88]. The main structural components of the mucus layer are the gel-forming secretory mucins, predominantly MUC2, which play an essential role in keeping out the gut bacteria from the epithelial surface [87, 89]. The mucus layer is constituted of two layers – outer looser layer and an inner laye r or glycocalyx, which is tightly fitted on the epithelial surface. The colonization of the commensal flora in the colon is limited to the outer layer; on the contrary, the inner adherent layer is almost sterile due to the retention of high concentrations of antimicrobial peptides (AMPs) and secretory IgA, thus preventing the bacterial adhesion and invasion to the underlying epithelial cells [86]. The mucus layer also contains water, lipids, ions, which facilitate the clearance of the pathogenic microorganisms. M cells are specialized epithelial cells of the follicle-associated epithelium of Peyer's pathches, and mediate the transport of luminal antigens and live bacteria across the epithelial barrier to dendritic cells for its presentation to T cells [84]. In the small intestine, Paneth cells synthesize and secrete a large amount of different AMPs as well as other bactericidal molecules, thus playing a key role in the intestinal innate immunity [90].

In order to perform their barrier functions, the intestinal epithelial cells are tightly connected by intercellular junctional complexes composed of tight junctions (TJs), adherens junctions and desmosomes, whereas gap junctions provide for intercellular communication [91, 92]. These connections are necessary for the mechanical stabilization of the cells, being of great importance for the regulation of the paracellular permeability. TJs seal the intraepithelial space and form selective barriers that regulate

paracellular transport across epithelia [91]. There are four types of integral proteins as components of TJs, called occludin, claudins, tricellulin and connective adhesion molecules that are coupled to actin filaments of the cytoskeleton [92]. Intestinal TJ barrier is dynamically regulated by cytokines and pathogens [93]. Zonula occludens proteins (ZO-1, ZO-2 and ZO-3) are important intracellular TJ proteins, which link the cell cytoskeleton to the transmembrane TJ proteins.

Considering all the above, it is evident that the intestinal barrier function plays a key role in intestinal homeostasis, and an alteration in this function may result in disease. In fact, different studies have associated IBD to defects in the key components of the gut epithelial barrier. In fact, when the mucosal barrier is disrupted, the translocation of the intestinal microbiota can take place, which in turn promote the subsequent aberrant activation of the immune system [94, 95] (Figure 2). However, and similarly to that proposed for the altered microbiota composition, it is a matter of debate whether changes seen in barrier function are the result or the cause of the disease.

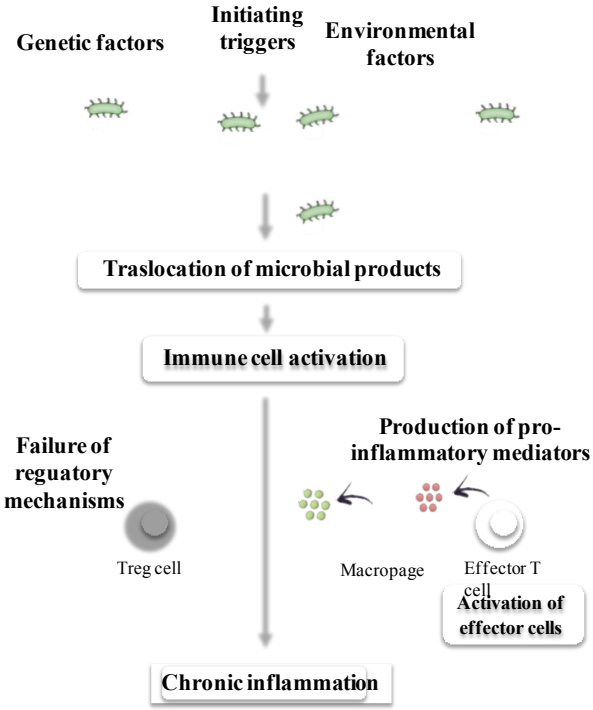


Figure 2. Genetic or environment factors may promote the onset of gut inflammation by affecting the immune balance and/or the gut microbiota. Intestinal inflammation results in increased bacterial adherence, epithelial damage and increased entry of bacteria into the intestinal lamina propria, thus sustaining a vicious inflammatory circle.

It was commented before that the inner mucus layer is devoid of bacteria in healthy controls [96]; however, it shows increased permeability in IBD patients, thus allowing interaction of the microbiota with the normally inaccessible epithelial surface [97]. The increased permeability may be due to altered composition of the mucus components secreted by goblet cells, including decreased production and/or secretion of mucin [98], glycosylation products [99] or trefoil factor [100], or due to reductions in antimicrobial factors secreted into the mucus by epithelial cells (Reg3γ), Paneth cells (defensins) and plasma cells (immunoglobulin A (IgA)) [101]. In UC, but not in CD, the mucus layers are thinner or absent, and the goblet cells responsible for mucus production are mostly depleted [97]. On the contrary, the mucus layer in CD patients is thicker in comparison to the healthy subjects [102], with an increased expression of mucins, which is accompanied by their increased sulphation [103] and altered glycosilation [104]. Interestingly, certain members of the microbiota can use the mucus as an energy source and tightly regulate its production [24, 105]; in consequence, changes in the mucus composition may result in the onset of dysbiosis , as it occurs in IBD [72].

Also, an altered network of TJs connecting epithelial cells can contribute to the increased permeability in IBD [106]. Both environmental (microbes, diet) and genetic factors can influence tight junction integrity [107]. Its disruption allows microbes to translocate beyond the mucosal surface resulting in access to the immunologically active submucosa and systemic space. Endotoxemia (lipopolysaccharide) is well documented in IBD and other microbial components (flagellin, pilli, and lipoteichoic acid) are also likely responsible for stimulating the immune system [108].

Finally, numerous experimental and clinical studies support the key role of proinflammatory cytokines, including TNF-α, IFN-γ or IL-13, in promoting the intestinal barrier disruption in IBD [109-111].

1.3 Immune response

1.3.1 Innate immune response

Innate immunity constitutes the fastest response against invading microorganisms and other harmful agents. It is nonspecific and has no immunological memory [112]. It comprises several immune cells, such as macrophages and dendritic cells (DCs), but also intestinal epithelial cells and myofibroblasts, which can sense the intestinal microbiota and respond to conserved structural motifs on microorganisms, known as

pathogen associated molecular patterns (PAMPs), in a stereotypical manner (Figure 3). This allows the initiation of rapid and effective inflammatory responses against microbial invasion.

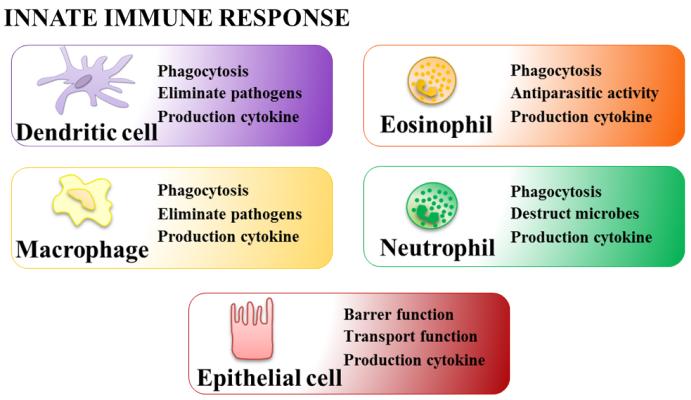


Figure 3. The innate immune response is the first line of defense against infection and includes complement proteins, granulocytes (basophils, eosinophils and neutrophils), mast cells, macrophages, dendritic cells and natural killer cells.

Macrophages

Macrophages are phagocytic cells that are present throughout the gastrointestinal mucosa. They derived from peripheral blood monocytes and are recruited to the intestine by chemoattractants, chemokines and bacterial degradation products, where they mature. In the non-inflamed/healthy mucosa, macrophages differ from peripheral monocytes in their downregulated proinflammatory function, being characterized by hyporesponsiveness to TLR ligands, diminished ability to prime adaptive immune responses, but still preserved capacity for phagocytosis and intracellular killing [113]. However, in the setting of pathogen invasion and inflammation, intestinal macrophages freshly recruited from blood monocytes rapidly change to a proinflammatory phenotype by ligation of their pattern recognition receptor (PRR). After activation, the macrophages express specific receptors for opsonized particles and pathogens, complement and common bacterial proteins, like mannose receptor, TLR, NOD; once pathogens are recognized by these receptors, they are phagocyted and subsequently killed inside the macrophage. In addition, this proinflammatory phenotype of activated macrophages results in the increased production and secretion of several cytokines, including IL-1, IL-6, IL-8, TNF-α or transforming growth factor (TGF)-β, which can affect the function of other cells. For example, TGF-β acts as a potent chemoattractant for other macrophages and neutrophils and, hence, augments the recruitment of immune cells to sites of inflammation [114]. Thus, macrophage activation not only augments its

phagocytic intracellular killing activity but also serves as a critical link between the innate and the adaptive immune systems.

Accordingly, different studies have highlighted the central role ascribed to macrophages in the pathogenesis of colitis. In fact, it has been reported that in the intestinal mucosa from IBD patients there is an increase in the macrophage populations displaying an activated phenotype, with enhanced expression of microbial receptors, which contributes to the intestinal pathology through the production and secretion of several pro-inflammatory cytokines, particularly TNF-α and IL-6 [115, 116]. Additionally, lamina propria macrophages from patients with IBD have shown un upregulated expression of T cell co-stimulating molecules (CD80 and CD86) [117], and activated NF-κB [118]. Furthermore, in CD it has been reported the presence of an increased number of CD14-expressing macrophages in the intestine that secrete TNF-α, IFN-γ and IL-23 [119-121].

Dendritic cells

In addition to macrophages, dendritic cells (DCs) are the other key cell type of the innate immune system in the gastrointestinal tract. They are phagocytic cells, which, like the macrophages, originate from the blood monocytes or a common DC progenitor in the bone marrow. DCs have the most potent ability to initiate adaptive immune responses against pathogens. Several evidences support that intestinal DCs migrate in an immature or tolerogenic state, form scavenging apoptotic cells, and acquire antigens sampled either directly from the lumen [122] or shuttled from the lumen through M cells [123]. After acquisition of the antigen, DCs process it inside the cell, load the antigenic peptide onto major histocompatibility complex (MHC) class II molecules, and display this complex on their cell surface. Then DCs migrate to the draining mesenteric lymph nodes where they present a particular MHC-peptide complex to T cells bearing the T cell receptor (TCR), specific for the antigen being presented (naive $CD4^+$helper T cells or Th0). In a normal physiologic state, these antigen-loaded DCs (intestinal DCs) express low levels of co-stimulatory molecules and cytokines and, in contact with T cells, preferentially stimulate their differentiation into T regulatory (Treg) cells that produce antiinflammatory cytokines, like IL-4, IL-10, and TGF-β. By contrast, intestinal DCs activated by their TLR and NOD2 receptors in a proinflammatory

microenvironment, migrate to T cell areas of the gut-associated lymphoid tissue (GALT), where they induce effector (Th1, Th2 and Th17) rather than tolerogenic T-cell responses. The interplay between secreted cytokines determines the balance between the different types of $CD4^+$ effector T cells [124]. In IBD, overactive DCs induce the differentiation of effector lymphocytes ($CD4^+$ and $CD8^+$) and other effector cells such as natural killer (NK) and NK T (NKT) cells while abolishing the production of Treg cells [115]. The absence of Treg lymphocytes contributes to the development of an exacerbated immune response to commensal bacteria that are normally tolerated by the mucosal immune system (peripheral tolerance). The loss of peripheral tolerance then initiates and perpetuates intestinal inflammation. The excessive activation of DCs is usually due to abnormalities in TLR and NOD receptor function in these cells and the consecutive inability to detect bacterial components. In fact, in healthy patients, TLR signalling helps to protect the epithelial barrier and assists tolerance to commensal bacteria, whereas the dysfunction in TLR signalling can induce an intestinal inflammatory response with different clinical phenotypes [125]. A major target of TLR signalling is the activation of the transcription factor NF-κB [126], which regulates the expression of a variety of pro-inflammatory cytokine genes responsible for controlling the innate response, such as IL-1, IL-2, IL-6, IL-12, and TNF-α [127].

Natural Killer cells

Natural Killer (NK) cells are innate lymphocytes that can regulate immune responses through direct killing of target cells or indirectly by secretion of a variety of cytokines, most notably IFN-γ [128]. The relevance of NK cells to IBD pathogenesis has not been clearly defined. Thus, it has been reported that NK depletion in DSS-induced colitis in mice promotes a more severe inflammation with high mortality, most probably because the depletion of these cells increases neutrophil infiltration [129]. In addition, it is speculated that NK cells maintain steady intestinal epithelium when specific cytokines are expressed and released. For example, NK cells that express IL-22 have a protective effect on initiation of IBD [130]; however, *in vitro* experiments have reported that IL-21 an strengthen cytotoxic killing activity of NK cells and increase secretion of pro-inflammatory factors such as TNF-α and IFN-γ [131].

1.3.2 Adaptative immune response

The adaptive immune system is highly specific, unlike the innate immune response, and confers long lasting immunity. It is adaptable since specificity for the antigen is the result of a complex maturation and development of the immune cells. Key players of the adaptive immune response are T and B cells, which cooperate with cells and molecules from the innate immune system to generate an effective response in order to eliminate the invading pathogens and prevent diseases (Figure 4).

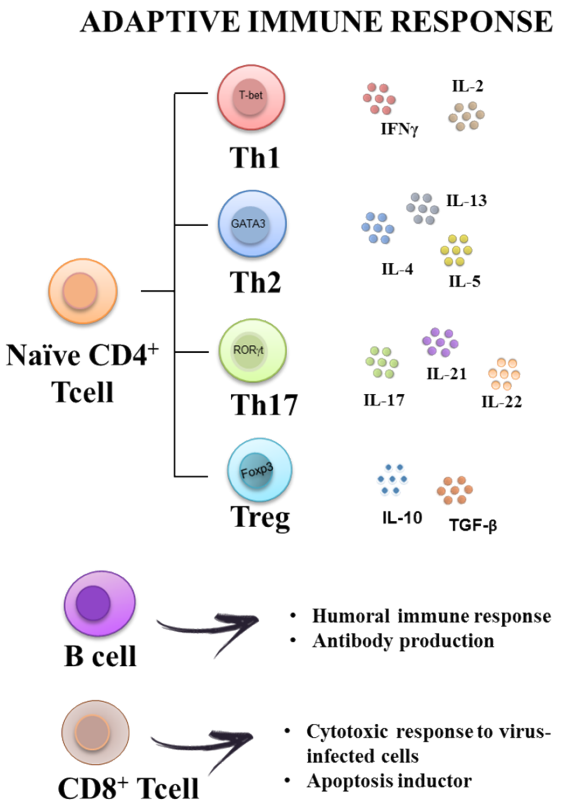


Figure 4. The adaptive immune response develops more slowly, but it is manifested as increased antigenic specificity and memory. It consists of antibodies, B cells, and $CD4^+$ and $CD8^+$ T lymphocytes.

T cells

The induction of an adaptative immune response begins when a pathogen is phagocyted by an immature DC in the infected tissue. Immature DCs express receptors on the surface that recognize common features of many pathogens, such as bacterial cell wall proteoglycans. The binding of a bacterial component to these receptors stimulates DCs to engulf the pathogen and degrade it intracellularly, like macrophages and neutrophils. However, the primary function of DCs is not destroying pathogens but carrying pathogen antigens to peripheral lymphoid organs and then presenting them to T lymphocytes. Thus, when a DC takes up a pathogen in an infected tissue, it becomes activated, and travels to a nearby lymph node. After activation, the DC matures into a highly effective antigen-presenting cell (APC) and undergoes changes that enable it to activate pathogen-specific lymphocytes located in the lymph node. In fact, upon contact with APCs, naive $CD4^+$ cells have the potential to differentiate into different Th subtypes: Th1, Th2, Treg, and Th17. Each of these subtypes has relevant immune functions [132-134].

Th1 cells secrete the cytokines IFN-γ and TNF-β. These cells are particularly effective in protecting against intracellular infections by viruses and bacteria, as well as other microorganisms that grow in macrophages, and in eliminating cancerous cells.

Th2 cells secrete IL-4, IL-5, IL-10 and IL-13, which up-regulate antibody production and target parasites. Th2 cells activate B cells, which are adapted for defence against parasites that are vulnerable to IL-4-switched immunoglobulin (Ig)E production, IL-5-induced eosinophilia, and IL-3- and IL-4-stimulated mast cell proliferation and degranulation.

Th17 cells secrete IL-17, IL-17F, IL-6, IL-22 and TNF-α and appear to play an integral role in both tissue inflammation and activation of neutrophils to combat extracellular bacteria.

Treg cells secrete IL-10 and TGF-β, which modulate Th cell activity and suppress some of their functions, inducing tolerance by suppressing abnormal immune response against commensal microbiota or food antigens.

A dysregulation in the proliferation of the different T cell subsets may result in an excessive increase of chemokines and cytokines, leading to a vicious circle that facilitates the inflammatory process. Many studies do support that activated $CD4^+$ T cells are a central player in the pathogenesis of human IBD [131]. In CD patients, Th1 response is triggered by increased mucosal levels of IL-18 and IL-12 [135-137]. Moreover, it has been observed that mucosal T cells derived from CD patients produce higher amounts of IFN-γ and IL-2 than UC patients [138]; on the contrary, UC patients produce more IL-5 than CD patients [139]. Traditionally, the hypothesis of a Th1/Th2 cytokine balance has been used to explain the pathogenesis of chronic inflammatory disorders, including IBD. However, the Th1/Th2 paradigm should be reconsidered, since it has been reported that biopsies from both UC and CD patients, when cultured *ex vivo*, are able to release high and comparable amounts of IFN-γ [140]. Moreover, IL-13 has been proposed to act as an anti-inflammatory cytokine, and its levels are reduced in both diseases [141-143].

Similarly to Th1 and Th2 cells, the cytokines released by Th17 cells have also been reported to display a pathogenic role in IBD [144]. First, high transcript levels of IL-17 are present in the intestinal mucosa from patients suffering CD or UC, but absent in

normal controls or patients with other forms of colitis [145, 146]. Although IL-17 is clearly associated with active forms of UC, this association is stronger in active CD. In addition, IL-22, similarly to IL-17, is not present in healthy intestinal mucosa, but it can be detected in inflamed mucosal tissue sections from UC and CD patients [147] and in experimental mouse colitis [148, 149]. Different studies have correlated the production of Th17 cytokines in the inflamed gut to tissue destruction [150, 151]. Furthermore, these cells are also involved in neutrophil proliferation, maturation and chemotaxis, thus contributing to the pathogenesis of these intestinal inflammatory conditions [152].

Finally, IBD pathogenesis is associated with a reduction of Tregs, which are extremely important for the maintenance of the intestinal mucosa homeostasis through the production of anti-inflammatory cytokines such as IL-10 and TGF-β [153]. Thus, it has been reported that injections of naive T cells without CD4+ and CD25+ Treg cells into T cell-depleted mice induce an exacerbated intestinal immune response to commensal bacteria, leading to autoimmune colitis, whereas injections of all T cells in the same mice inhibit inflammation [154]. A reduction in Treg cells has been also found in peripheral blood and colonic mucosa of IBD patients [155], thus suggesting that low counts of Treg cells are associated with intestinal inflammation pathogenesis [131].

Importantly, it has been reported an imbalance in the Th17/Treg ratio in IBD patients. Th17 cells and Treg cells are in dynamic equilibrium under normal circumstances, and this balance can be broken either by increasing Th17 cells or after decrease or abnormal functionality of Treg cells, thus promoting the intestinal immunogenicity that causes intestinal mucosal damages. Naive T cells differentiate into Th17 cells in the presence of high levels of IL-6 and low concentrations of TGF-β, being inhibited the proliferation of Treg cells. On the contrary, differentiation of Th17 cells is inhibited in the presence of high concentrations of TGF-β, when generation of Treg cells is promoted [134]. Of note, although Treg cells effectively repair inflammed bowel mucosa of IBD patients, they can be transformed into pathogenic Th17 cells in the presence of IL-6 and/or IL-23 [156]. There are no reports found about Th17 cells transforming into Treg cells, indicating that conversions of Treg cells into Th17 cells are irreversible [157]. Th17 cells are increased while Treg cells are decreased in peripheral blood of IBD patients. Th17/Treg ratio is significantly higher, suggesting that transformation imbalance plays an important role in IBD pathogenesis [158].

B cells

Although the main function of B cells is antibody production, they can also act as antigen presenting cells and, moreover, produce cytokines. Together with other APCs, such as macrophages and DCs, B cells are involved in maintaining mucosal immune homeostasis [159].In fact, in the gut lumen, B cells interact with food antigens, commensal bacteria and self-antigens [160], thus operating like a protective barrier for the epithelium against possible pathogens. They are specialized in IgA production [161]. IgA secretion is controlled by several compounds involved in maintaining intestinal tolerance, including IL-10 and TGF-β [161]. Similarly to T cells, B cells have been divided into two subpopulations, regulatory and pathogenic. Regulatory B cells (Breg) produce IL-10 that contributes to their suppressive function, and maintain the homeostasis that regulates the balance of the different subsets of T cells (Th1, Th17 and Treg), thus inhibiting the inflammation. They are also able to convert effector T cells in Tr1 cells, a specific subpopulation of Treg cells [162, 163]. All these properties attributed to Breg cells can justify the reported ability of these cells to suppress experimental colitis [162, 163]. Therefore, the beneficial effects of these cells are supported by the fact that the Breg cell population is reduced in CD patients [164]. Recently, it has been reported that IL-33 is an important cytokine for induction of Breg cells; in fact, in IL-10 KO mice the mucosal immune response is attenuated when IL-33-derived B cells are transferred [165]. Other evidences have shown that Breg cells can reduce intestinal inflammation via Treg since these cells can directly interact with T cells and polarize the type of response, although they are not able to induce primary responses [166]. B cells can be directly activated by DCs, which present them the processed antigens and thus influencing their differentiation and survival [167]. The survival is promoted by BAFF and APRIL, molecules released by DCs, which activate IgA production [168]. Macrophages also release BAFF, which can induce B cell differentiation [169]. Although most studies performed in the experimental models of IBD suggest that B cells suppress mucosal inflammation either by secreting cytokines or by interacting with T cells, a pathogenic role of B cells has been raised in a model of ileitis [170]. It was also shown that peripheral blood B cells constitutively secrete pro-inflammatory IL-8 in CD patients, and this cytokine production is directly correlated to disease severity [171].

1.4 Treatment of IBD

At present, pharmacological treatments for human IBD do not provide a cure for the disease. Thus, their main goals are to induce and maintain remission, to improve health-related quality of life and to prevent disease complications. Currently there are a variety of treatments, with different advantages and disadvantages, which mainly consist on anti-inflammatory and immunosuppressive drugs with limited specificity, displaying in some cases severe side effects and limited long term benefits.

1.4.1 Anti-inflammatory drugs

Aminosalicylates. This group comprised different drugs that are characterized by the presence of the 5-aminosalicylic acid (5-ASA) moiety, also called mesalazine, which has been shown to exert anti-inflammatory effects in human IBD, especially in UC. In fact, the aminosalicylates are indicated to treat active intestinal inflammation of slight intensity, both in CD and in UC [172, 173], and to prevent the reactivation of the UC. However, it has been reported the absence of beneficial effects after their chronic use in the maintenance of CD in remission, making difficult to support the use of this therapy for long-term. The exact mechanism of action of aminosalicylates in IBD needs to be determined, although numerous potential effectshave been proposed, like inhibition of cyclooxygenase and/or lipoxygenase activities, the reduced production of platelet-activating factor (PAF) and the proinflammatory cytokines IL-1β and TNF-α [174], the inhibition of NF-κB activation [175] and B cell activity [176], as well as a decrease in oxygen radical production, due to their ability to scavenger reactive oxygen species [177].

Corticosteroids. These drugs constitute the standard treatment for human IBD since their administration mostly results in rapid remission of disease activity [178]. Their beneficial effects are adscribed to their ability to suppress the early manifestations of inflammation, including vasodilation and the enhanced vascular permeability, thus reducing neutrophil infiltration and the subsequent inflammatory processes, including fibroblast activation, vascular proliferation and deposition of collagen. Corticosteroids also modulate immunological responses such as T-responses to antigens, by down-regulating the production of inflammatory cytokines, probably through NF-κB activation inhibition, thereby lessening the inflammatory response [179]. Unfortunately, the longstanding treatment with glucocorticosteroids is limited because

of adverse events associated with their chronic use, including hypothalamic pituitary adrenal axis suppression [173], and the possible development of steroid-dependent disease [180].

1.4.2 Immunosuppressive drugs

Immunosuppressives are a group of drugs mainly acting by inhibiting lymphocyte proliferation and activation. They include azathioprine, 6-mercaptopurine [37], tacrolimus, cyclosporine and methotrexate, among others. They are usually administered when corticosteroids fail to induce remission or to maintain it quiescent [181, 182]. The mechanisms of action involved depend on the drug considered: azathioprine and 6-mercaptopurine inhibits proliferation of activated lymphocytes in IBD and induce apoptosis in these cells [183]; the mechanism of methotrexate is not fully understood, but it seems that it inhibits pro-inflammatory cytokines synthesis and promotes lymphocyte apoptosis [184]*;* cyclosporine reduces lymphocyte activation and proliferation of lymphocytes by blocking IL-2 synthesis via calcineurin pathway [185]. The main limitations of the immunomodulators are due to their ability to provoke allergy and serious toxicity, together with the opportunistic infections that may occur [186, 187].

1.4.3 Biological therapies

Biologic therapies encompass agents with different modes of action, like blocking pro-inflammatory cytokines or interfering with inflammatory cells recruitment by antibodies against adhesion molecules.

TNF-α is a pivotal cytokine in the pathogenesis of IBD and therefore a prime target for specific immunotherapy. Several strategies to block TNF-α activity have been developed, showing a very potent clinical activity in the management of CD and UC [188-190]. Infliximab is a mouse-human chimeric monoclonal anti-TNF-α antibody, whereas CDP571 and adalimumab are monoclonal antibodies with more humanized proportion. Besides blocking TNF-α, these three drugs are able to induce apoptosis in monocytes and lymphocytes [191]. Infliximab is effective in inducing remission of both types of IBD, even in the most aggressive forms, and also in maintaining the remission in short term. However, the main problem of infliximab is the development of anti-chimeric antibodies, which can limit its use. This inconvenience has been partially solved with the use of adalimumab [192, 193].

Moreover, antibodies against other pro-inflammatory cytokines have been developed, such as MRA, a humanized monoclonal antibody against the soluble IL-6 receptor (IL-6R). It has been reported high concentrations of IL-6R in patients with CD [120], and treatment with MRA has been tested with good results in CD [179], however concerns have been raised regarding a higher rate of intestinal perforation associated with the use of this drug [194]. Additionally, according to results presented in abstract form, an IL-6 antibody (PF-04236921) yielded higher clinical response (Crohn's disease activity index-70) and remission rates than placebo therapy in CD patients [195]. Furthermore, both fontolizumab, a humanized monoclonal antibody against IFN-γ [196], and J695 (ABT-874) a human monoclonal antibody against IL-12 [197] have shown to exert beneficial effects in CD. Moreover, anti-CD4 therapy, using monoclonal antibodies as cM -T412, BF-5 and MAX.16H5, acts on CD4+ T cells and induces remission in both CD and UC patients [198]. In order to inhibit T cell proliferation, anti-IL-2 receptor therapy has been developed, basiliximab and daclizumab, which perform well in UC [199, 200]. Finally, natalizumab is a humanized mouse monoclonal antibody against α4-integrin that inhibits transendothelial migration. It results effective in remission in both CD and UC patients [201].

1.4.4 Complementary and alternative therapy

Although there are different drugs currently used in IBD that have shown efficacy, the frequency and severity of adverse effects, inconvenient dosing regimen and high price limit their long term use [202]. For this reason, the development of new therapies that combine efficacy, convenient dosing and lower side effects is an important goal in human IBD therapy. Moreover, it is interesting to note that many IBD patients look for alternative treatments; up to half of these patients have been reported to have tried or currently use complementary therapy [9]. This can be explained by the absence of complete response to conventional therapy, the more favourable safety profile of many complementary treatments, and a sense of greater control over their disease [203]. Among the many forms of complementary and/or alternative therapy, the herbal preparations or their derived compounds display an outstanding position [204]. The rationale for their use is mainly related to safety, since they have been consumed from ancient times, as well as to the presence of different active components that can act simultaneously on different targets of the inflammatory response, including the oxidative stress. Among these active components, polyphenols have been extensively

characterized because of their wide spectrum of bioactivity, particularly their potent antioxidant activity. In this context, the evaluation of plant extracts can be considered as an important approach for the development of future treatments for IBD.

The growing interest about the potential role that medicinal plant extracts may play in intestinal inflammatory conditions has promoted the development of different clinical studies, thus trying to evaluate their potential efficacy and safety. It is important to note that nowadays most botanical drugs go through a similar rigorous testing as pharmaceutical medicines, in an attempt to avoid inconsistent conclusions. Unfortunately, different factors associated with the design, execution and interpretation of these clinical trials make still difficult to easily get clear conclusions with the different strategies to be evaluated against these pathologies [205]. Among these factors, it can be highlighted the clinical heterogeneity of both intestinal conditions, UC and particularly CD, as well as the selection of appropriate therapeutic end points to evaluate the efficacy. In spite of all these concerns, there are positive examples of successful human-controlled trials within the literature of botanical drugs. Although the preclinical studies reporting the beneficial effects of plant extracts on experimental models of colitis are numerous, only a few plant extracts have been used in different clinical assays [206].

In this regard, the health benefits ascribed to the medicinal plants may be related to the high content of bioactive ingredients such as polyphenols, the most abundant secondary plant compounds, which comprise over 7000 compounds. The polyphenols can be subdivided into flavonoids (such as isoflavonoids, anthocyanins and others), the most studied group, and non-flavonoids (e.g. stilbenes, phenolic acids, coumarins, tannins). It is also important to consider that most of the polyphenols (possibly up to 90-95%) are not absorbed but reaches the colon [207], where they also become a substrate for fermentation. They may be of great utility in conditions of acute or chronic intestinal inflammation through different mechanisms including protection against oxidative stress, preservation of epithelial barrier function and immunomodulatory properties in the gut (Table 2).

Intestinal anti-inflammatory effects of polyphenols: in vivo and in vitro studies

Galsanov *et al*. (1976) reported for the first time the potential beneficial effect of polyphenols in IBD. In that study, the authors described the anti-inflammatory activity

of quercitrin, when administered at doses of 25 and 100 mg/kg, in a rat model of allergic intestinal inflammation. Since then, many studies describing the impact of polyphenols in several experimental models of colitis in rodents have been published. Among these, chemically induced models (acetic acid, trinitrobenzene sulphonic acid (TNBS) or dextrane sulphate sodium (DSS)), genetically engineered mice, (HLA-B27 rats or IL-10 knock out (KO) mice), and a T cell-transfer model have been used broadly and have been shown to share some similarities with human IBD [208]. These studies have revealed the intestinal anti-inflammatory activity of different polyphenols, including both glycosides and aglycones, and belonging to the different chemical classes, like flavonols (quercetin, quercitrin, rutin), flavanones (naringenin), flavones (baicalin, chrysin), catechins (epigallocatechin-3-gallate (EGCG)), isoflavones (genistein, daidzein, glabridin), anthocyanidins (cyanidin-3-glucoside (C3G)), chalcone (cardamonin). These beneficial effects were evidenced at acute and semi-chronic stages of intestinal inflammation, following either a preventative dosing protocol, i.e. when administered before colitis induction, or a curative administration of the test compounds once the colonic damage had been developed. At present, it is difficult to establish a structure-activity relationship, since the number of polyphenols tested until now is low. However, of all the polyphenols tested, quercitrin has been found to be the most potent, showing preventative and curative properties at doses of 1 and 5 mg/kg [209]. The range of active doses for the other flavonoids is broad, ranging from 10 to 25 mg/kg when the glycosides are considered, and between 10 and 200 mg/kg in the case of aglycones.

The polyphenols treatments have shown beneficial effects that were evidenced macroscopically by amelioration of the colonic damage. In the DSS model, characterized by intense colonic damage associated with inflammatory cell infiltration and epithelial crypt loss, which results in acute clinical symptoms, including body weight loss, bloody stools and diarrhoea, the treatment with some flavonoids such as cardamonin, chrysin, naringenin, EGCG, glabridin, rutin and quercitrin significantly reduced the Disease Activity Index (DAI), used to monitor the severity of the inflammatory process, as well as the colon shortening. Therefore, DSS-induced weight loss and histological damage were significantly ameliorated by flavonoids treatment [14, 210-216]. Associated with this finding it has been shown that quercitrin and rutin were able to decrease colonic damage reducing the length of the injury and attenuating

the diarrhoea symptoms in the TNBS model [209, 217, 218]. Moreover, rutin ameliorated histologic injury also in the acetic acid model of experimental colitis [218].

It has been proposed the involvement of different mechanisms in the intestinal anti-inflammatory activity of the polyphenols, including antioxidant properties through the interference with reactive oxygen (ROS) and nitrogen (RNS) species, inhibition of eicosanoids synthesis, immunomodulatory activity, preservation of the epithelial barrier function, and, finally, interference with the gut microbiota.

Antioxidant properties of polyphenols

Several studies have proposed that both ROS and RNS play a key role in the etiology of IBD [219]. In fact, human IBD has been associated with an intense oxidative stress, with excessive generation of ROS and RNS in the intestinal tissue that induces lipid peroxidation, protein modifications, DNA damage, apoptosis, together with impairment of the enzymatic and non-enzymatic antioxidant mechanisms, including superoxide dismutase (SOD), reduced glutathione (GSH) and catalase (CAT), and results in the colonic damage associated with intestinal inflammation [220, 221]. Different sources of free radicals have been proposed to contribute to the oxidative burst that takes place in IBD, being neutrophils one of the cell type involved in these processes [222]. The infiltration of polymorphonuclear neutrophils and mononuclear cells into the affected part of the intestine is considered as one of the main pathological features of human IBD [223]. As a consequence of the activation of the NADPH oxidase system in these cells, and the subsequent myeloperoxidase activity (MPO), massive quantities of superoxide and hypochlorous acid are generated and cause direct cytotoxicity in the intestinal tissue. This in turn facilitates the additional release of different pro-inflammatory mediators [224]. In fact, most polyphenols assayed in experimental colitis models exhibited a significant reduction of colonic myeloperoxidase activity. This enzyme is predominantly found in the azurophilic granules of the neutrophils and is considered to be a sensitive marker of leukocyte infiltration [225]. As expected, MPO activity is found increased in different experimental models of colitis induced by TNBS, DSS and T-cell transfer. The increased MPO levels were significantly reduced after the administration of genistein and quercitrin in the TNBS model [226, 227]. Similar effect was observed by quercitrin along with cardamonin, chrysin, EGCG, naringenin and rutin in the DSS model [14, 210-212, 214, 228, 229].

Finally, it is important to remark that rutin administration was also able to reduce of leukocyte infiltration in the T-cell transfer model [230]. Most of the polyphenols assayed were able to ameliorate the oxidative stress that takes place in the experimental models of colitis as evidenced by a reduced colonic lipid peroxidation, together with an improvement in different antioxidant markers, including sulfhydryl-derived compounds, or an enhancement of the different enzyme activities with antioxidant properties [230]. Specifically, different studies have suggested that both EGCG and quercitrin administration on the DSS-induced colitis model were able to increase the colonic GSH production, and naringenin and EGCG reduced the tissue malondialdehyde (MDA) levels, indicating both a reduction of lipid peroxidation and an increase of antioxidant enzymes such as SOD and GPO [14, 210, 211, 228]. Similarly, quercitrin and rutin treatment have shown to significantly increase GSH levels, thus ameliorating the colonic damage in the TNBS model [226]. Special attention can be paid to the RNS, which can be produced and released by immune cells and also play an important role in the pathophysiology of IBD. Nitric oxide (NO) is a pleiotropic free radical messenger molecule produced from L-arginine by nitric oxide synthase (NOS) enzyme. Under physiological conditions, low levels of NO are produced by the isoform of constitutive nitric oxide synthase (cNOS), which has a direct protective effect throughout the initial phases of the intestinal inflammatory process. Nevertheless, in chronic inflammation, NO synthesis is upregulated, mainly as a consequence of the increased expression of the inducible isoform of nitric oxide synthase (iNOS), which is induced, mainly in macrophages, by bacterial products and pro-inflammatory cytokines [231, 232]. The overproduction of NO contributes to the colonic damage due to its interaction with the superoxide anions, thus generating peroxynitrites, which reinforce oxidative stress and tissue damage [233](Figure 5). Numerous studies have correlated the metabolism of NO to the flavonoids, which may preserve the beneficial functions of NO through the direct capture of super oxide anions [234]. Similarly, it has been reported that polyphenols are capable of inhibiting the expression of iNOS [14], and they can act as powerful scavengers of peroxinitrite radicals [235].

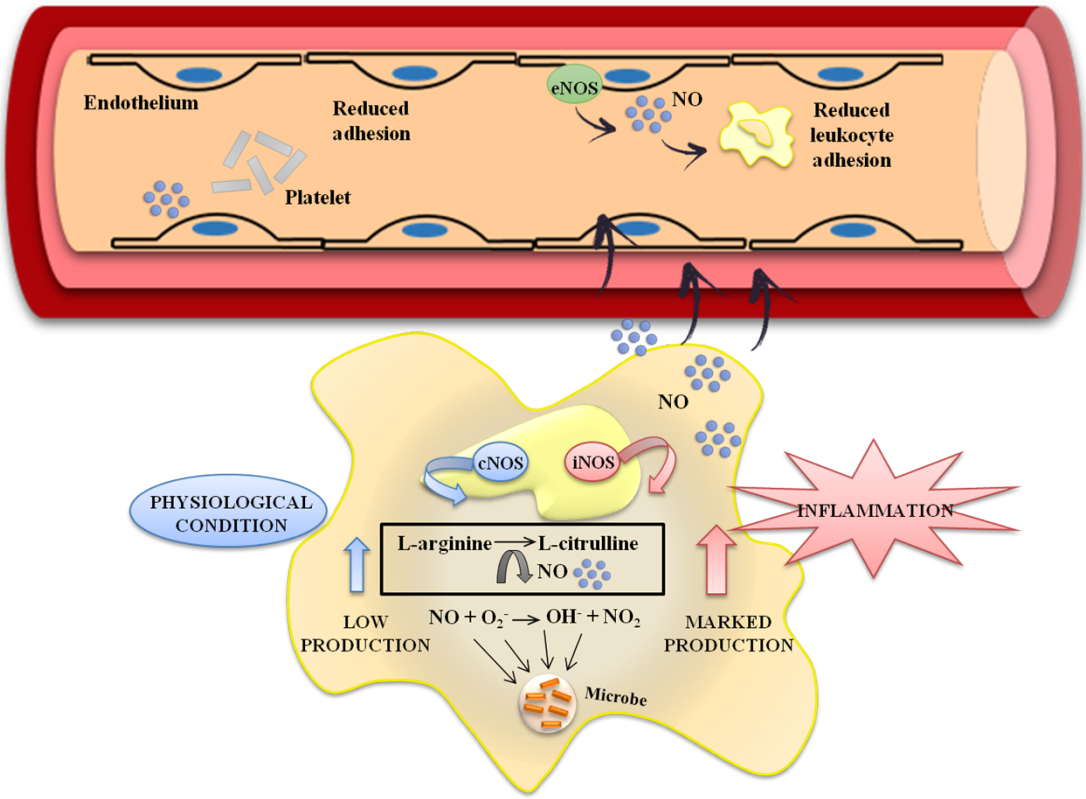


Figure 5. Nitrite oxide (NO) is a free radical molecule generated from L-arginine oxidation, and is catalyzed by the enzyme nitric oxide synthase (NOS). Different functional forms of NOS can be recognized: constitutive and inducible forms. NO synthesis by the constitutive isoform, endothelial NOS (eNOS), generates low levels of NO under normal physiological conditions which regulates the colon blood flow, bowel motility and produces reactive oxygen species (ROS) for fighting pathogens. The inducible isoform, iNOS, is expressed in cells involved in the inflammatory response and, upon different stimuli, generates high levels of NO that may be toxic to the healthy tissue, contributing to damage and upregulation of the inflammatory response. Several studies clearly demonstrated that certain flavonoids inhibit NO production in activated cells and in induced experimental colitis. Their inhibitory activity might be due to reduction of iNOS enzyme expression.

Moreover, DSS administration is associated with a significant increase of iNOS. In this regard, it has been observed that some flavonoids such as glabridin, cardamonin, naringenin and quercitrin improve the inflammatory process, reducing the expression of iNOS and, as consequence, the NO production [14, 212, 214, 216]. These results have been confirmed in *in vitro* studies using different cell lines. EGCG, naringenin, daidzenin, kaempferol, quercetin and cardamonin inhibit iNOS protein and mRNA expression and also NO production in (LPS)-activated macrophages, such as bone marrow-derived macrophages (BMDM), or murine macrophages J774 and mouse leukaemic monocyte macrophage (RAW 264.7) cell lines [212, 228, 236, 237]. Polyphenols are capable therefore, of preventing the detrimental effects generated by NO in intestinal inflammation.

Effects of polyphenols on eicosanoid metabolism and function

Eicosanoids derived from arachidonic acid metabolism, including products from cyclooxygenase (COX) (prostaglandins) and lipoxygenase (LOX) (leukotrienes) activities, seem to play a critical role in intestinal inflammation. In fact, it has been demonstrated that increased levels of eicosanoids are found in inflamed tissues areas in comparison with normal mucosa in human IBD [237]. Actually, the up-regulation of the enzymes involved in eicosanoid metabolism has also been associated with the pathophysiology of other inflammatory disorders [238].

There are two isoforms of COX: constitutive COX-1 and inducible COX-2. COX-1 has been considered crucial for mucosal integrity since it produces cytoprotective and anti-inflammatory prostaglandins like PGE_2 [239]. On the contrary, the expression of COX-2 can be induced by a variety of stimuli related to the inflammatory response. This isoform is responsible for an increased production of prostaglandins involved in IBD [240, 241] (Figure 6).

In consequence, and theoretically, the inhibition of COX-2 expression and/or activity would be also beneficial in the management of intestinal inflammation. Different studies have reported that the intestinal anti-inflammatory activity of polyphenols, such as rutin and EGCG, was associated with the inhibition of the colonic expression of COX-2 [215, 228].

Interestingly, it was reported that cyanidin 3-glucoside (C3G) has inhibitory effects on the production of several mediators during inflammation in the colonic carcinoma cell line HT29, similarly to 5-aminosalicylic acid (5-ASA), a well-established anti-inflammatory drug used in IBD. In this regard, treatment with 25 mM of C3G, 500 mM 5-ASA or both, for 1 hour before cytokine (IL-1a, TNF-α and IFN-α) stimulation, significantly reduced PGE_2 production. C3G produced the strongest inhibition (65%) while 5-ASA produced a significantly lower inhibition (50%). Additionally, C3G was able to down-regulate COX-2 expression more efficiently than 5-ASA, whereas the combination of C3G and 5-ASA afforded a much better protection than the individual compounds [242].

On the other hand, the increased generation of leukotrienes, mainly LTB_4, has also been reported to occur in IBD [243]. In this regard, it has been proposed that leukotrienes mediate intestinal inflammatory response, especially through their chemotactic effects, thus inducing the accumulation of inflammatory cells in the inflamed area of the gut. In consequence, the inhibition of lipoxygenase activity and the subsequent reduction of LTB_4 production, or the blockade of its receptor, could be proposed to exert beneficial effects in experimental colitis [244, 245]. However, although different polyphenols with beneficial effects in experimental colitis were able to reduce colonic LTB_4 production, no direct relationship between the reduced levels of this eicosanoid in the colonic tissue and the anti-inflammatory effect could be established [209, 246].

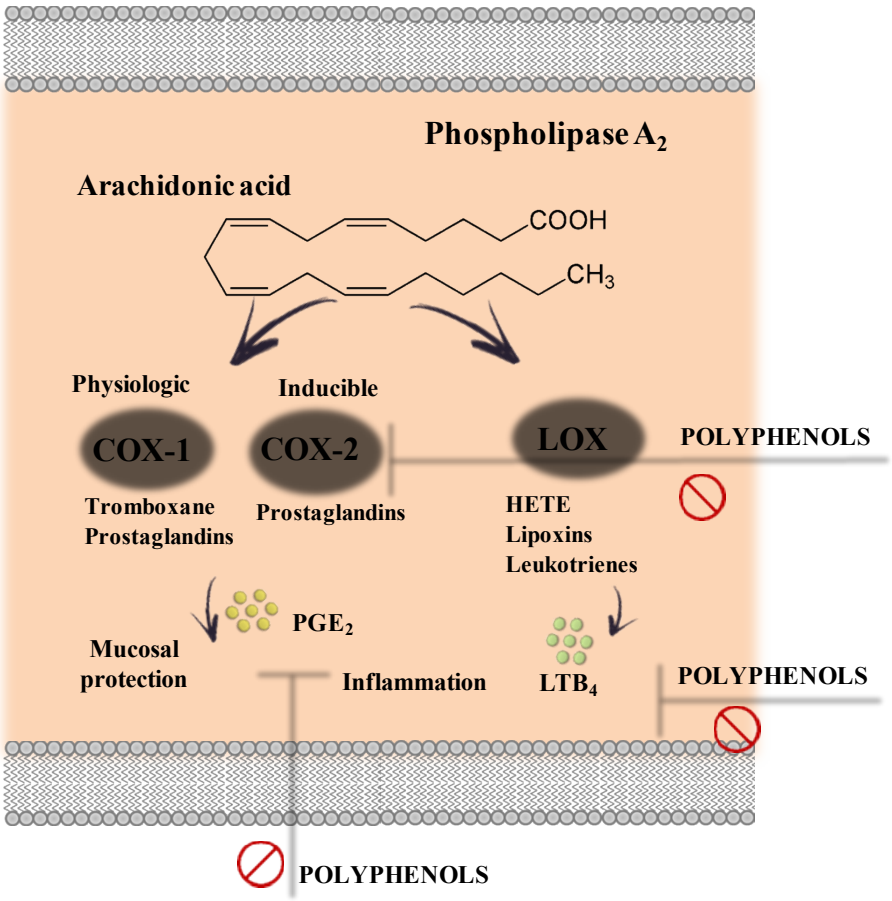


Figure 6. Eicosanoid synthesis pathways: Arachidonic acid is a polyunsaturated fatty acid that is released from the cellular membranes by cytoplasmatic phospholipase A_2 (PLA_2). Free arachidonic acid can be metabolized to eicosanoids through two major pathways: the cyclooxygenase (COX) and the lipooxygenase (LOX). The COX-1 (constitutive form) pathway results in the synthesis of prostaglandins and thromboxanes, which are important for physiological functions. The COX-2 (inducible form) pathway plays a crucial rule in the production and release of inflammatory prostaglandins. Similarly, the LOX pathway leads to the synthesis of leukotrienes and hydroxyeicosatetraenoic acid (HETE) that contribute to the inflammatory process. Different studies have associated the polyphenols anti-inflammatory effect with a suppression of these pathways.

Immunomodulatory properties of polyphenols

As mentioned previously, most of the studies performed in experimental models of colitis have proposed that an imbalance of the immune system plays a key role in IBD pathogenesis. The altered immune response is associated with an increased release of pro-inflammatory cytokines, including IFN-γ, TNF-α, IL-6, IL-1β, IL-8, GM-CSF and IL-17A, chemokines, such as MIP-2 and MCP-1, and adhesion molecules, such as ICAM-1. The ability of polyphenols to regulate the altered immune response that occurs in intestinal inflammation has been reported in different *in vivo* studies. For instance, the administration of flavonoids, such as EGCG, cardamonin, chrysin, glabridin, quercitrin, naringenin or rutin, in the DSS model remarkably decreased the increased levels of the different cytokines evaluated in the inflamed colon [210-212, 214, 247].

These immunomodulatory properties exerted by the flavonoids have been also confirmed when *in vitro* experiments were performed in different cell types involved in the immune response: epithelial cells, monocytes/macrophages, T cells, and dendritic cells. For instance, the incubation of LPS-activated macrophages, RAW 264.7 and BMDM cells with quercetin or baicalin resulted in reduced levels of IL-1β and TNF-α when compared with stimulated cells without flavonoid treatment [248, 249]. Similarly, rutin was able to significantly reduce the increased IL-1β levels produced by DSS-stimulated pMφ cells, obtained from mouse peritoneal exudate [215, 250]. In addition, quercetin exerts anti-proliferative effects by reducing IFN-γ and TNF-α production in concanavalin A-stimulated purified T lymphocytes isolated from rat splenocytes [230]. Moreover, the incubation of polyphenols, such as C3G, genistein, EGCG or chrysin in cytokine-stimulated epithelial cells, Caco-2 and HT-29 cells significantly reduced IL-8 secretion in the cell culture [242, 251]. Finally, it has been shown that treatment of THP-1 cells, a human monocytic cell line, with EGCG decreased MCP-1 and CCR2 gene expression, together with MCP-1 secretion and CCR2 expression, at the cell surface, and induced the inhibition of beta1 integrin activation [252].

Considering the role of immune cells in the development of IBD, T cells are major players [253]. Likewise, DSS-induced experimental colitis has been associated with an increased percentage of Th1 and Th17 cells in the mesenteric lymph nodes, which correlates to the overexpression of pro-inflammatory cytokines such as IFN-γ, IL-17A and IL-17F. In this regard, it has been reported that the beneficial effects observed with

the flavonoid derivative icariin in DSS-induced colitis in mice were related to a downregulation of the proportion of both Th1 and Th17 cells, and thus a reduction in the cytokine release by these cell subtypes in the colonic tissue. Different *in vitro* studies have also confirmed the ability of flavonoids to suppress T cell proliferation and activation [254-256]. Moreover, baicalin was able to reduce the expression of RORC, Foxp3 and T-bet, transcription factors associated with Th17, Treg and Th1 cells, respectively, which have been reported to be upregulated in UC patients [256]. Similarly, macrophages have been considered to be the main source of different pro-inflammatory mediators in IBD, including TNF-α, IL-1β and NO, thus actively contributing to the pathology of these intestinal conditions [257, 258]. Besides, several *in vitro* studies have shown the capacity of flavonoids to inhibit NO and cytokine production in different macrophage cell lines, including RAW 264.7 and J774.1, as well as in bone marrow–derived macrophages (BMDM) [236, 259]. *In vivo* experiments have also revealed that the beneficial intestinal anti-inflammatory effects of flavonoids, such as quercitrin, were associated with a decreased number of infiltrated macrophages in the inflamed colonic tissue induced by DSS in rats [14]. Finally, and as mentioned above, neutrophil infiltration can be considered one of the main pathological features of human IBD [223], and most active flavonoids assayed in experimental colitis models significantly reduced neutrophil infiltration into the damaged colonic tissue, as evidenced by a significant reduction of colonic myeloperoxidase [14, 210, 212-214, 226, 227, 229, 230], thus contributing to the amelioration of the intestinal inflammation.

Several studies have focused on the potential mechanisms responsible for the modulation of cytokine production; some of the mechanisms proposed are related to the inhibition of NF-κB, mitogen-activated protein kinase (MAPK) and STAT activation [260-263]. NF-κB is ubiquitously expressed, being found in its inactive form in the cytoplasm, which is bound to its high-affinity inhibitor IκB. In the presence of activating stimuli, including oxidative stress, a large signalling cascade is initiated, resulting in activation of IKK-α and IKK-β, two kinases that phosphorylate IκB. Phosphorylation of IκB results in its dissociation, and NF-κB becomes free to translocate to the nucleus, where it binds to κB regulatory elements, activating gene expression [264] (Figure 7). It has been clearly demonstrated that cardamonin and quercitrin exerted potent anti-inflammatory properties by reducing NF-κB activity in DSS-induced experimental colitis, whereas rutin reduced the IκBα phosphorylation in a

T cell transfer model [14, 230, 265]. Baicalin has also shown the ability to block this pathway in the TNBS model of rat colitis [249]. In this regard, different studies have proposed that the ability of some polyphenols to downregulate the altered immune response that occurs in intestinal inflammation may be achieved through the inhibition of the TLR4/NF-κB signalling pathway, as it has been demonstrated *in vitro*, when activated mouse macrophage J774 and RAW264.7 cells or human colonic HT-29 cells were exposed to naringenin, kaempferol, quercetin, daidzein and cardamonin [228, 237, 266, 267].

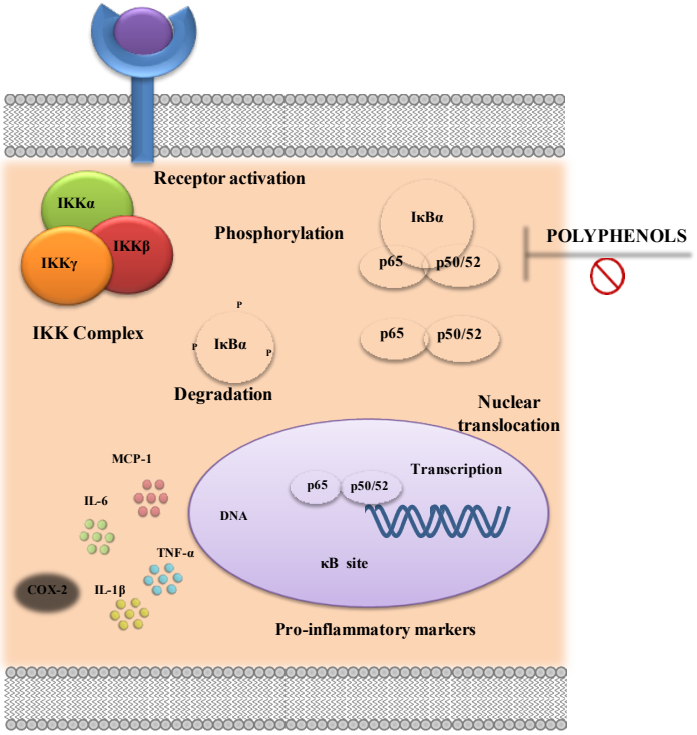


Figure 7. NF-kB signal transduction pathway. NF-κB protein complex (p65–p50) is bound and inhibited by IκB proteins. Pro-inflammatory cytokines, LPS, growth factors, and antigen receptors activate an IKK complex (IKKβ, IKKα, and IKKγ), which phosphorylates IκB proteins. Phosphorylation of IκB leads to its ubiquitination and proteasomal degradation, releasing NF-κB. Active NF-κB proteins are further activated by post-translational modifications (phosphorylation, acetylation, glycosylation) and translocate to the nucleus where they induce target gene expression, influencing a broad range of biological processes including innate and adaptive immunity, inflammation, stress responses, B cell development, and lymphoid organogenesis. Anti-inflammatory effects of several poyphenols have been related to the suppression of the NF-κB signal transduction pathway.

Similarly, it has been reported that EGCG reduced LPS-induced TNF-α production in macrophages (RAW264.7 cell line and peritoneal macrophages) by blocking NF-κB activation. In the case of baicalin and cardamonin, this effect was associated with the inhibition of NF-κB p65 subunit phosphorylation (Ren, G. *et al*., 2015; Cui, L. *et al*., 2004; Lin, Y.L. *et al*., 1997; Yang, F. *et al*., 1998), whereas quercetin was able to

reduce the IκBα phosphorylation in LPS-activated BMDM (Comalada, M. *et al*., 2005). The polyphenol's ability to interfere with NF-κB phosphorylation has been also demonstrated in IEC-6 (an intestinal epithelial cell lines), and in peripheral blood mononuclear cells (PBMC), Caco-2, as well as in PBMC and BMDM cells. Specifically, baicalin, chrysin, quercetin, and EGCG administration showed inhibition of the NF-κB pathway by up-regulation of pregname X receptor (PXP) or inhibition of IkB phosphorylation [213, 247, 251, 268].

The MAPK signaling pathway also promotes immediate early gene and transcription factor activation of cellular responses such as cytokine production, apoptosis and migration. A general feature of MAPK pathways is the participation of a three-tiered kinase canonical cascade consisting of a MAPK, a MAPK kinase (MAPKK) and a MAPK kinase kinase (MAPKKK) [269-271] (Figure 8).

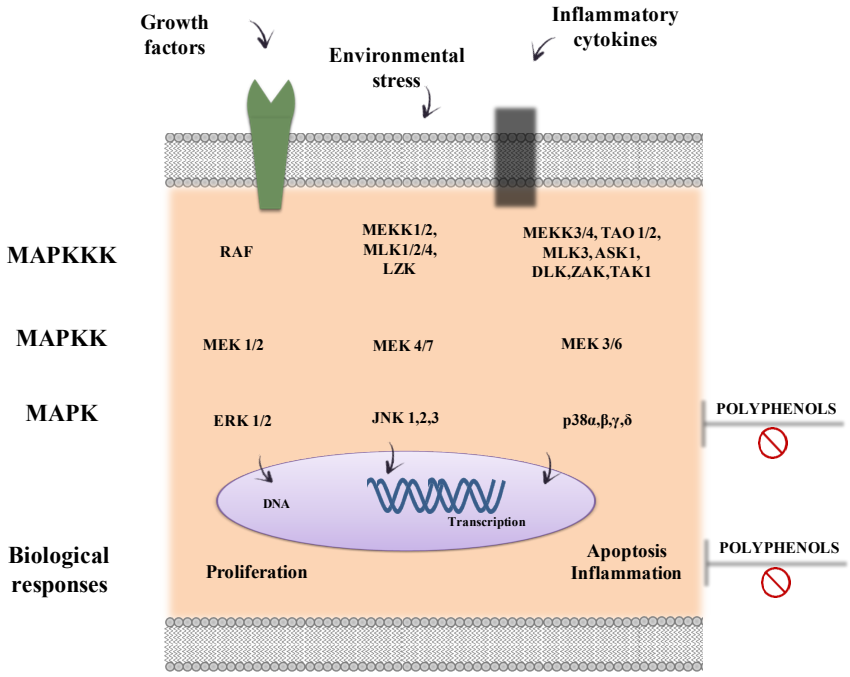


Figure 8. MAP Kinase pathway. The mitogen-activated protein kinase (MAPK) cascades are signal transduction pathways that involve a chain of three kinases activating each other in a series (MAPKKK, MAPKK, and MAPK) The result of phosphorylation of various MAP kinase isoforms is the activation of the three main MAP kinases: ERK (extracellular signal-related kinase), p38 MAPK, and JNK (c-Jun NH2-terminal kinase). Cell division, migration, and survival generally involve ERK signaling. Cellular stress activates the p38 MAPK and JNK pathways. The p38 MAPK pathway mediates transcription and cell motility. JNK signaling regulates apoptosis and inflammation. Flavonoids' immunomodulatory properties may be related to a direct inhibitory effect on the kinases themselves or by modulation of signal transduction events upstream of the relevant MAPK pathways.

Different *in vitro* studies have associated the polyphenol anti-inflammatory effect with a suppression of this pathway; for instance, EGCG was able to suppress the maturation of murine dendritic cells through the inhibition of extracellular signal-regulated kinase, p38 kinase and c-Jun NH_2-terminal kinase [271].

Finally, the JAK/STAT pathway transduces signals from a wide range of extracellular cytokine stimuli to the nucleus in order to orchestrate an appropriate cellular response through target gene expression [272, 273]. The binding of cytokines to their corresponding transmembrane receptors induces receptor dimerization of its subunits and association with JAK tyrosine kinases. Once activated, STAT proteins dissociate from the receptor, homo- or heterodimerize, and rapidly translocate from the cytoplasm into the nucleus. Thus the JAK/STAT cascade provides a direct mechanism to translate an extracellular signal into a transcriptional response [274] (Figure 9).

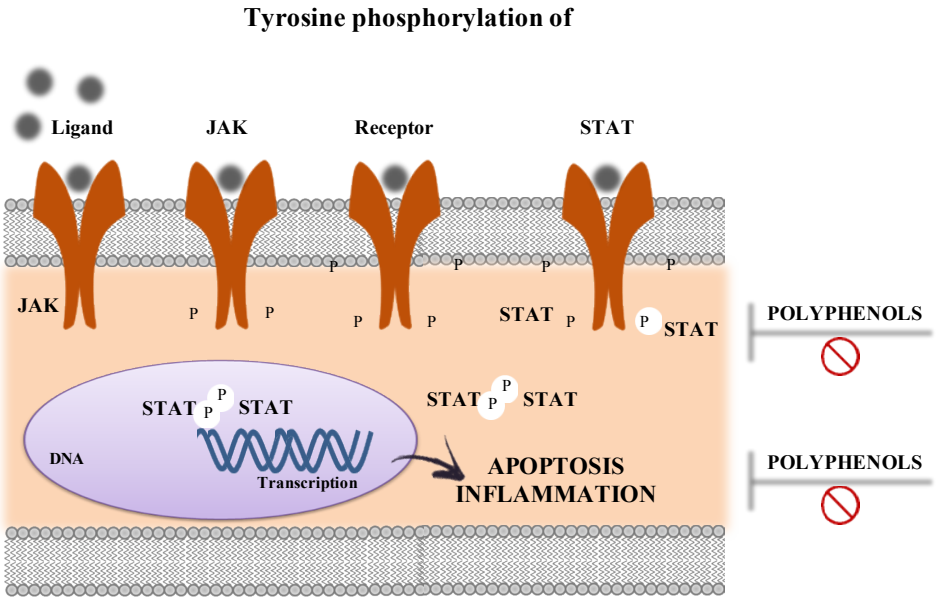


Figure 9. JAK-STAT signaling pathway. Upon the binding ligand, receptor-associated Janus Kinases (JAKs) become activated and mediate phosphorylation of specific receptor tyrosine residues. This leads to the recruitment of specific STATs (Signal Transducers and Activators of Transcription), which are then also tyrosine-phosphorylated. Activated STATs are released from the receptor, they dimerize and translocate to the nucleus to bind target genes associated with proliferation, differentiation and survival of the cells, including immune cells. The inhibition of JAK-STAT signaling pathway by flavonoids treatments leads to an immunomodulatory effect.

Many polyphenols can inhibit both JAK/STAT signalling [237, 275, 276]. Interestingly, Western Blotting analysis in HT29 cells suggested that anthocyanin cyaniding-3-glucoside remarkably reduce cytokine-induced levels of activated STAT1 [242], whose expression and activation have been shown to be upregulated in IBD patients [277].

Effects of polyphenols on intestinal barrier function

The homeostasis in the gastrointestinal tract is functionally maintained by an epithelial barrier, composed by a selectively monocellular layer between the outside lumen and host tissues, which controls the equilibrium between tolerance and immunity to microbes and non-self-antigens. Several defects related to the intestinal barrier function have been found in IBD patients. Whether mucosal barrier impairment is a consequence of the inflammatory response or a primary defect that prompts mucosal inflammation is still under debate [278]. However, transgenic animal models have clearly demonstrated that a unique defect in the intestinal epithelial barrier is enough to trigger the development of chronic gut inflammation [279]. In addition, several studies suggest that the impairment of the epithelial barrier function can be considered as one of the early events that occur in intestinal inflammation, since it facilitates the entry of antigens from the intestinal lumen to the mucosa that may start the uncontrolled and exacerbated immune response [280, 281]. For this reason, its recovery may contribute to the beneficial effects produced by polyphenols in experimental colitis models. It has been reported that different polyphenols such as quercitrin [226], rutin [218], hesperidin [282] and morin [246] improve the colonic absorptive function greatly compromised in experimental colitis, leading to a fewer diarrhoea symptoms, which are frequent in intestinal inflammation. The polyphenol anti-diarrheal effects has been also related to their capacity to inhibit muscle contractility, enhance intestinal motility, and reduce fluid intraluminal accumulation in the gut lumen, as evidenced in different experimental studies [283-285]. Moreover, Azuma *et al*. [216] also reported that naringenin treatment to colitic mice resulted in an improvement in the epithelial barrier permeability, through the preservation of the intestinal tight junction barrier function and structure, which have been described to be compromised after DSS administration [286, 287]. *In vitro* studies have confirmed the ability of polyphenols, such as naringenin, daidzenin and morin, to enhance epithelial barrier function. In particular, the incubation of these flavanones with human intestinal Caco-2 epithelial cells resulted in an increased transepithelial electrical resistance (TER) across the cell monolayers, which correlates with an improvement of tight junction integrity [288]. This was confirmed by immunoblot analysis and confocal microscopy, which demonstrated that naringenin, daidzenin and morin, increase cytoskeletal expression of the tight junction proteins as well as their assembly, thus reinforcing epithelial integrity in this cell line

[288]. In addition to a direct effect on tight protein function, indirect mechanisms can also account for the beneficial effects of flavonoids in preserving intestinal barrier function. In fact, it has been reported that pro-inflammatory cytokines, such as IFN-γ, TNF-α or IL-6, can disrupt the epithelial barrier function by apoptosis-independent mechanisms [289, 290]. In consequence, the inhibitory effect exerted by these compounds on the expression of IFN-γ and IL-6 can also contribute to the improvement of intestinal permeability observed in DSS experimental colitis [216].

Interaction of polyphenols with gut microbiota

As commented previously, there is increasing experimental evidence that support the role of luminal bacteria in the initiation and development of the intestinal inflammatory process, which would be probably related to an imbalance in the intestinal microbiota composition, known as dysbiosis [2, 291]. In fact, previous studies have tried to explain the alterations that occur in the gut microbiota or identify the bacterial populations that might be associated with the onset or recurrence of IBD, thus promoting the access of potential pathogens to the lamina propria and triggering the exacerbated immune response [32, 292-295]. Different studies have reported that diets containing bioactive compounds, such as phenolic compounds and tannins, can be considered as possible complementary treatments for IBD due to their antimicrobial and antioxidant capacity [296]. Closely related to this, it has been proposed that the impact of naringenin on microbiota composition can also contribute to the beneficial effects exerted by this compound in intestinal inflammation. In this sense, naringenin has been reported to inhibit both growth and adhesion of *Salmonella typhimurium*, a Gram-negative pathogen, to cultured human Caco-2 cells [297]. On the contrary, the same study revealed that this flavanone enhanced the proliferation and adhesion of the probiotic *Lactobacillus rhamnosus*; of note, this probiotic has been described to exert beneficial effects in human intestinal inflammation [298]. Moreover, it has been also reported that EGCG shows antimicrobial effects and capacity to disrupt bacterial growth, which may also have a positive impact on colonic inflammation [211, 299].

Tabla 2. Intestinal anti-inflammatory effects of polyphenols

Polyphenol	Plant source	Mechanism
Cyanidin-3-glucoside	*Hibiscus sabdariffa* *Hibiscus sabdariffa*	Inhibition PGE2 release by regulating COX-2 activity. Reduction pro-inflammatory mediators production. Inhibition STAT pathway.
Cardamomin	*Alpinia katsumadai* *Alpinia conchigera*	Inhibition leukocyte migration. Inhibition reactive nitrogen species generation. Reduction pro-inflammatory mediators. Inhibition NF-κB activity.
Naringenin	Grapefruit (*Citrus paradise*)	Inhibition COX-2 activity. Reduction pro-inflammatory mediators production. Inhibition NF-κB activity. Improvement epithelial barrier function. Antimicrobial effects and gut microbiota modulation.
Chrysin	*Picea crassifolia*	Inhibition leukocyte migration. Inhibition reactive nitrogen species generation. Reduction pro-inflammatory mediators. Inhibition NF-κB activity.
Baicalin	*Scutellaria baicalensis*	Modulation T cells activity. Inhibition NF-κB activity.
Quercetin	*Dysosma veitchii*	Inhibition reactive nitrogen species generation. Reduction pro-inflammatory mediator production. Inhibition NF-κB activity
Daidzein	*Pueraria mirifica* *Pueraria lobata* *Glycine max*	Inhibition reactive nitrogen species generation. Inhibition NF-κB activity.
Quercitrin	Tartary buckwheat (*Fagopyrum tataricum*) Oaks species (*Quercus* sp.)	Inhibition leukocyte migration. Inhibition reactive nitrogen species generation. Reduction pro-inflammatory mediator production. Inhibition NF-κB activity. Improvement epithelial barrier function.
Glabridin	*Glycyrrhiza glabra*	Inhibition reactive nitrogen species generation.
Epigallocatechin-3-gallate	*Camellia sinensis*	Inhibition COX-2 activity. Inhibition leukocyte migration. Inhibition reactive nitrogen species generation. Reduction pro-inflammatory mediator production. Inhibition NF-κB activity. Inhibition MAPK pathway. Antimicrobial effects and gut microbiota modulation.

2. METABOLIC SYNDROME

The metabolic syndrome (MetS) is a clinical entity of substantial heterogeneity, commonly represented by the combination of related disorders such as obesity (especially abdominal obesity), hyperglycaemia, dyslipidaemia (high levels of triglycerides (TG) and low levels of high density lipoprotein-cholesterol (HDL-c)) and hypertension [300].

It was during the period of 1910-20 when it was suggested for the first time that a cluster of associated metabolic disturbances tend to coexist together [301]. Later on, in 1947, Vague suggested the term "android obesity" to define an association of different metabolic impairments related to diabetes and cardiovascular diseases [302]. The concept of syndrome X was coined by Reaven in 1988, when he reported that several cardiovascular risk factors tend to coexist together at the same time [303]. Another name attributed to this heterogenic syndrome is the insulin resistance syndrome, as some authors consider the insulin resistance as the main clinical outcome of the MetS [304]. Nowadays, the name most used to refer to this syndrome is the MetS; however, its precise definition has not been well-established yet. During the past fifteen years, different organisms have suggested diverse definitions. Among them, the most common ones are hereafter described and summarized in Table 3. In 1999, the World Health Organization (WHO) was the first organism to suggest a definition for the MetS [305], considering the insulin resistance as the main factor for the MetS development. Soon after, the European Group of Insulin Resistance (EGIR) proposed a new definition for nondiabetic individuals [306]. Lately, in 2001 the National Cholesterol Education Program Adult Treatment Panel (NCEP ATP) III gave the same importance to all the MetS components (ATPIII, 2001) suggesting that when 3 of 5 of the listed characteristics in Table 3 are present, a diagnosis of MetS can be made.

Table 3 . Criteria to define the MEtS depending on different organisms.

WHO
One of this: - Type 2 diabetes, insulin resistance or impaired glucose tolerance. **Plus at least two:** - TG ≥ 1.7 mmol/L and/or HDL-c < 0.9 mmol/L (men) and < 1.0 mmol/L (women). - Urine albumin excretion > 20 µg/min or albumin:creatinine ratio > 30 mg/g. - SBP ≥ 140 mmHg or DBP ≥ 90 mmHg or treatment for hypertension. - Central obesity: BMI ≥ 30 kg/m^2 or waist:hip ratio > 0.90 (men), > 0.85 (women).
EGIR
- Insulin resistance defined as the top 25 % of the fasting insulin values among nondiabetic individuals. **Plus at least two:** - Central obesity: waist circumference ≥ 94 cm (men), ≥ 80 (women). - TG ≥ 2.0 mmol/L and/or HDL-c < 1.0 mmol/L or specific treatment. - SBP ≥ 140 mmHg or DBP ≥ 90 mmHg or treatment for hypertension. - Fasting glucose ≥ 6.1 mmol/L.
NCEP ATP III
At least three: - Abdominal obesity: waist circumference > 102 cm (men) > 88 cm (women). - TG ≥ 1.7 mmol/L. - HDL-c < 1.03 mmol/L in men, < 1.3 mmol/L in women. - SBP ≥ 130 mmHg or DBP ≥ 85 mmHg. - Fasting plasma glucose ≥ 6.1 mmol/L.
AHA/NHLBI
At least three: - Waist circumference ≥ 102 cm (men), ≥ 88 cm (women) or diagnosed type 2 diabetes. - TG ≥ 1.7 mmol/L or specific treatment for hypertriglyceridemia. - HDL-c < 1.03 mmol/L in men, < 1.3 mmol/L in women or specific treatment. - SBP ≥ 130 mmHg or DBP ≥ 85 mmHg or drug treatment for hypertension. - Fasting plasma glucose ≥ 5.6 mmol/L.
IDF
- Central obesity: waist circumference ≥ 94 cm (Europids men), ≥ 80 (Europids women). **Plus at least two:** - TG ≥ 1.7 mmol/L or specific treatment for hypertriglyceridemia. - HDL-c < 1.03 mmol/L in men, < 1.3 mmol/L in women or specific treatment. - SBP ≥ 130 mmHg or DBP ≥ 85 mmHg or drug treatment for hypertension. - Fasting plasma glucose ≥ 5.6 mmol/L or type 2 diabetes previously diagnosed.

AHA/NHLBI, American Heart Association/National Heart, Lung, and Blood Institute; BMI, body mass index; DBP, diastolic blood pressure; EGIR, European Group of Insulin Resistance; HDL-c, high density lipoprotein-cholesterol; IDF, International Diabetes Federation; NCEP ATP, National Cholesterol Education Program Adult Treatment Panel; SBP, systolic blood pressure; TG, triglycerides; WHO, World Health Organization.

2.1 Epidemiology

The prevalence of the MetS varies broadly around the world and among different populations from 10 % to 50 % [307]. It is increased in developed countries, sedentary people, smokers, low socioeconomic status population, as well as individuals with unhealthy dietary habits. It is also positively associated with age, although whether this association is direct, or rather correlated to the variation in body composition that occurs as people age, is not well-known. According to sex, the association with the MetS is not entirely clear, as some researchers report that the MetS is positively associated with women [308, 309], while others reported that it is more frequent in men [310]. Those who support that the prevalence is more likely in male believe that androgens may have a role in its development, since in disorders where these hormones are elevated, such as polycystic ovary syndrome, the MetS prevalence is more frequent [310]. Moreover, there are authors who reported that there is no difference between genders regarding the MetS prevalence [310]. However, due to the different definitions of the MetS, the prevalence varies depending on the source used. Nonetheless, what is clear is that the number of people suffering the MetS is increasing in epidemic proportions around the world over the last 40-50 years, mainly due to the obesogenic environment of most of the developed regions [307].

2.2 Etiology

The specific causes of the MetS triggering are still unclear. However, it is known that genetics and environmental factors, as well as their interactions, have a role in the etiology of this clinical entity [311]. Thus, the MetS is related with a general unhealthy lifestyle such as the lack of physical activity [312], smoking habits, alcoholism [313] or an unhealthy dietary pattern [314]. Moreover, genetics and epigenetics also have a role in the development of the MetS as many susceptible genes and different genetic factors are implicated in the phenotypic variation of this syndrome [311].

2.3 Physiopathology

MetS is considered as a state of chronic low grade inflammation as a consequence of complex interplay between genetic and environmental factors. Insulin resistance, visceral adiposity, atherogenic dyslipidemia, endothelial dysfunction, genetic

susceptibility, elevated blood pressure, hypercoagulable state, and chronic stress are the several factors which constitute the syndrome [300].

2.4 Central features of the metabolic syndrome

2.4.1 Insulin resistance

Insulin is a peptide hormone produced by the pancreas in response to hyperglycemia and stimulates glucose use in various tissues, such as skeletal muscle, liver and adipose tissue. In the skeletal muscle and adipose tissue, insulin stimulates glucose uptake by translocation of the GLUT4 glucose transporter to the cell surface. In the skeletal muscle and liver, insulin stimulates the synthesis of glycogen from glucose and inhibits glycogenolysis. In the liver, insulin also decreases hepatic gluconeogenesis, preventing an influx of more glucose into the bloodstream. In adipose tissue, insulin inhibits fat breakdown, or lipolysis, and stimulates glucose uptake. The net effect of all of these changes is to increase glucose uptake, reduce circulating glucose levels and increase the conversion of glucose into the storage molecules, glycogen or fat [315]. In insulin resistance, adipose, muscle and liver cells do not respond appropriately to insulin, and circulating glucose levels remain high, which leads to pathology. This is exacerbated by the deregulation of feedback mechanisms.

Physiological insulin signalling occurs following the binding of insulin to the insulin receptor, a ligand-activated tyrosine kinase. Binding of insulin results in a tyrosine phosphorylation of downstream substrates and activation of two parallel pathways: the phosphoinositide 3-kinase (PI3K)-Akt pathway and the mitogen activated protein (MAP) kinase pathway. In insulin resistance, the PI3K-Akt pathway is affected, whereas the MAP kinase pathway is not. This leads to a change in the balance between these two parallel pathways. The inhibition of the PI3K-Akt pathway leads to a reduction in endothelial NO production, resulting in an endothelial dysfunction, and a reduction in GLUT4 translocation, leading to a decreased skeletal muscle and fat glucose uptake. By contrast, the MAP kinase pathway is unaffected, so there is a continued endothelin-1 (ET-1) production, an expression of vascular cell adhesion molecules, and a mitogenic stimulus to vascular smooth muscle cells [316]. In consequence, insulin resistance leads to the vascular abnormalities that predispose to atherosclerosis.

2.4.2 Visceral obesity

A consensus has emerged that fat stored in the central segment of the body is particularly damaging since it means greater risk for diabetes, cardiovascular disease, hypertension, and certain cancers [317-319]. However, there is less information, and sometimes controversial, regarding the mechanisms that may link visceral fat with increased risk for these diseases. Different studies have suggested that one or more moieties secreted by the visceral adipocytes might mediate insulin resistance. Among these "bad actors" are probably included adipokines such as TNF-α and IL-6, which are proinflammatory that contribute to insulin resistance and vascular dysfunction [320]. In addition, visceral adiposity has been shown to be associated not only with quantitative changes in serum lipids and lipoproteins, but also with qualitative changes in lipoproteins, such as the increase of small-dense low density lipoprotein [321], thus leading to the onset of cancer and related diseases.

2.4.3 Dyslipidaemia

The key features of atherogenic dyslipidaemia are high plasma triglycerides (TG) levels, low HDL cholesterol levels and an increase in small dense LDL. Insulin resistance and visceral obesity are associated with atherogenic dyslipidaemia [322]. Insulin resistance leads to atherogenic dyslipidaemia in several ways. First, insulin normally suppresses lipolysis in adipocytes, so impaired insulin signaling increases lipolysis, resulting in increased levels of free fatty acids **(**FFAs). In the liver, FFAs serve as a substrate for synthesis of TGs. FFAs also stabilize the production of apoB, the major lipoprotein of very-low-density lipoprotein (VLDL) particles, resulting in more VLDL production. Second, insulin normally degrades apoB through PI3K-dependent pathways, so insulin resistance directly increases VLDL production. Third, insulin regulates the activity of lipoprotein lipase, the rate-limiting and major mediator of VLDL clearance (Figure 10). Thus, hypertriglyceridemia in insulin resistance is the result of both an increase in VLDL production and a decrease in VLDL clearance. VLDL is metabolized to remnant lipoproteins and small dense LDL, both of which can promote atheroma formation. The TGs in VLDL are transferred to HDL by the cholesterol ester transport protein (CETP) in exchange for cholesterol esters, resulting in TG-enriched HDL and cholesteryl ester-enriched VLDL particles. The TG-enriched HDL is a better substrate for hepatic lipase,

so it is cleared rapidly from the circulation, leaving fewer HDL particles to participate in reverse cholesterol transport from the vasculature[323].

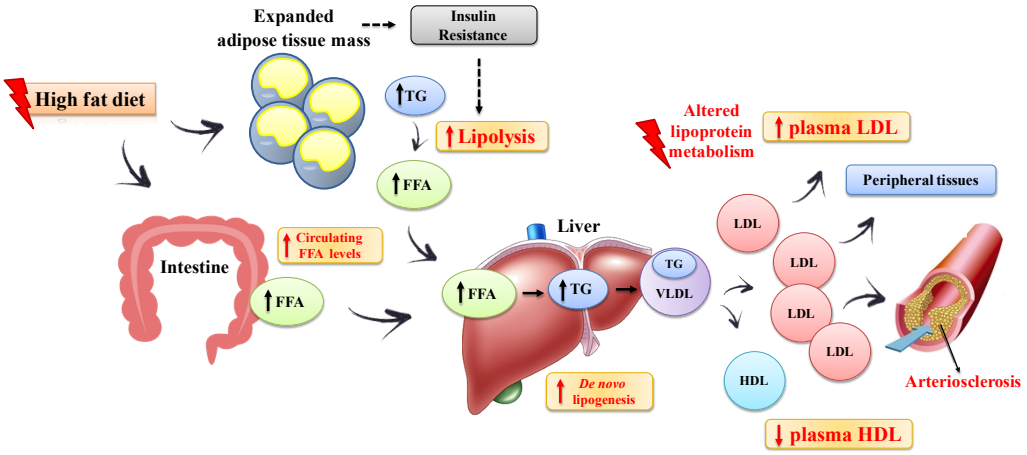


Figure 10. Insulin resistance plays a central role in the development of obese dyslipidemia. Under normal physiologic conditions, insulin suppresses lipolysis from adipose tissue and hepatic very low density lipoprotein (VLDL) production. However, in the obese state, free fatty acids (FFA) are released in abundance from an expanded adipose tissue mass. In the liver, FFA produce an increase production of glucose, triglycerides (TG) and secretion of VLDL. Associated lipid/lipoprotein abnormalities induce reduction in high density lipoprotein (HDL) cholesterol and an increased density of low density lipoproteins (LDL).

2.4.4 Hypertension

Hypertension is another core component of the MetS and an important risk factor for coronary heart disease, stroke, and renal disease [324]. Several studies have demonstrated that insulin resistance, and the resulting hyperinsulinemia, induce blood pressure elevation by activation of the sympathetic nervous system and renin-angiotensin-aldosterone system (RAAS), associated with sodium retention and volume expansion, endothelial dysfunction and alteration in renal function [325, 326].

2.4.5 Protrombotic state

The prothrombotic state present in the MetS is due to numerous changes in the coagulation and fibrinolytic pathways, as well as in platelet function [327]. Increased plasma levels of fibrinogen reflect the activation of the coagulation pathway, and increased levels of fibrinogen are associated with low-grade inflammation and insulin resistance in the MetS [328], and with impaired fibrinolysis [329]. Fibrinolytic dysfunction, as a consequence of increased levels of inhibitor of plasminogen activator type 1, also has independent value for future cardiovascular disease [330]. Insulin resistance has been associated with increased levels of coagulation factors VII-IX [331]

and increased red cell blood counts [332]. Platelet function is also disturbed in insulin resistance: Platelets from obese insulin-resistant subjects have reduced sensitivity to the known anti-aggregatory effect of insulin [333]. All these might contribute to the increased risk for the development of cardiovascular disease in people with the MetS.

2.4.6 Inflammatory state

Recently, the chronic inflammatory condition that often accompanies the metabolic syndrome has been implicated as a major factor both in the generation of the metabolic syndrome and in its associated pathophysiological consequences [334]. However, this inflammatory state does not completely fit into the classical definition of acute or chronic inflammation; there is no massive tissue injury and the dimension of the inflammatory activation is also not large. So it is often called 'low grade' chronic inflammation or 'meta-inflammation', meaning metabolically-triggered inflammation [335] or even 'para-inflammation' an intermediate state between basal and inflammatory states [336]. Whatever the term used, the inflammatory process that characterizes the metabolic syndrome has its own unique features but its origin is far from being fully understood. This inflammation in obesity is associated with the infiltration of macrophages in the excessive adipose tissue, resulting in the release of cytokines and promoting systemic inflammation [337-339]. Cytokines released from adipose tissue may induce insulin resistance in skeletal muscle [340], alter the pituitary-adrenal axis, and may accelerate loss of pancreatic β-cells [341]. This resistance to insulin action promotes inflammation further through an increase in free fatty acids (FFA) concentration and interference with the anti-inflammatory effect of insulin [342, 343]. Finally, low-grade inflammation within atherosclerotic lesions may increase the probability of plaque rupture, causing acute CVD [344].

2.5 Obesity, inflammation and metabolic syndrome

As commented above, one of the main components of the metabolic syndrome include obesity, especially visceral adipocyte. The worldwide epidemic of obesity has led to a dramatic increase in the metabolic diseases associated with this condition, which has focused a great deal of attention on the underlying mechanisms of obesity and its comorbidities. Several evidences suggest that many of the comorbidities of obesity, including type 2 diabetes mellitus, non-alcoholic fatty liver disease, steatohepatitis, asthma, cancer and cardiovascular diseases, are related to the generation

of the low-grade, chronic inflammation that characterizes obesity [335, 338, 345]. Although the trigger for this inflammation is uncertain, most probably there is a causal relationship between inflammation and the complications associated to obesity; in fact, little doubt remains about the fact that obesity is closely related to inflammation and that the degree of inflammation correlates well with the severity of insulin resistance and type 2 diabetes mellitus [335, 338, 345, 346], All these evidences suggest that the understanding of the inflammatory response might lead to the development of new approaches for treating these devastating diseases. Chronic inflammation typically presents three stages [346]: an initial trigger, usually a stressor of some kind, is followed by an acute, adaptive inflammatory response and then a long-term maladaptive phase that leads to complications. In the case of obesity, the initial trigger might result from homeostatic stress produced by a positive energy balance and an overall hyper-anabolic state, particularly in adipocytes. These cells respond by releasing chemokines that initiate an adaptative inflammatory response, enabling healthy expansion of adipocytes while simultaneously reducing energy storage, all of which occurs at the expense of homeostasis. However, with time, the system strives to restore homeostasis, which can be only accomplished by achieving a new set point for weight, blood levels of glucose, sympathetic tone, circulating levels of lipids and hormone levels. These changes are accompanied by reduced metabolic flexibility, long-term insulin and catecholamine resistance, abnormal tissue remodelling and fibrosis [347]

2.6 Adipose Tissue

Adipose tissue is a remarkably flexible site in terms of energy storage and release. Responding to hormonal and energetic signals, it serves as source of energy-rich fatty acids during times of negative energy balance, reducing its lipid accumulation and releasing fatty acids to target tissues when energy is needed [348]. On the contrary, adipocyte lipid uptake, esterification, and storage in the form of triglycerides within the lipid droplet allows for expansion of adipose tissue (hypertrophy), a beneficial, adaptative response to overnutrition that can prevent ectopic lipid deposition and lipotoxicity in other cell types. The lipid droplet is an active player in maintaining systemic energy homeostasis; thus, it occupies the majority of the adipocyte, placing the droplet's borders within close proximity of the endoplasmic reticulum and mitochondria, where triglycerides are esterified and hydrolysed, respectively [348]. Nowadays there is substantial evidence indicating that adipose tissue depots become

hypoxic as tissue mass expands during the development of obesity. The sheer size of large adipocytes limits the availability of oxygen, especially for those cells that are distant from the capillaries [349] Consequently, chronic hypoxia leads to derangements in lipid metabolism: fatty acids are redirected to the liver promoting dyslipidemia, characterized by elevated plasma FFA, triglycerides (TGs) and small dense low-density lipoprotein (LDL), as well as the reduction of high-density lipoproteins (HDLi) [349].

On the other hand, excess adiposity and adipocyte dysfunction may result in dysregulation of a wide range of adipose tissue-derived secretory factors, which may contribute to the development of the systemic inflammatory responses. Many of them, including MCP-1, TNF-α, IL-1β, IL-6 and IL-8, have been reported to promote macrophage infiltration in the inflamed adipose tissue [350]. Thus, adipose tissue macrophages (ATMs) accumulate in both the subcutaneous and the visceral expanding fat depots. It is well known that macrophages show an important heterogeneity in their function. In fact, different stimuli activate macrophages to express distinct patterns of chemokines, surface markers and metabolic enzymes that ultimately generate the diversity of macrophage functions. Macrophage activation has been typically defined across two separate activation status: classically activated macrophages, termed M1, and alternatively activated macrophages, termed M2. M1 cells, whose differentiation is promoted by agents such as LPS and IFN−γ, display the marker CD11c in addition to F4/8O and CD11b and produce proinflammatory mediators like TNF−α, IL−6, IL1-β, NO, IL−12, with relevant roles in inducing insulin resistance [351]. M1 macrophages also promote Th1 response and displays strong microbicidal and tumoricidal activity. On the contrary, M2 macrophages are induced by IL−4 and IL−13, and express the cell−surface markers CD11b, F4/8O, CD3O1 and CD2O6. In contrast to M1 macrophages, M2 macrophages secrete anti−inflammatory cytokines such as IL−10 [352]. Along with the increased number of ATMs in adipose tissue, obesity induces a phenotypic switch of these cells from an anti-inflammatory M2 to pro-inflammatory M1 polarization state [338] (Figure 11).

Accordingly, the accumulation of M1 macrophages in adipose tissue results in secretion of a variety of pro-inflammatory cytokines and chemokines that potentially contribute to establish a vicious circle between the fatty acids and TNF-α, produced by adipocytes and macrophages, respectively: TNF-α binds its receptor on hypertrophic adipocytes,

stimulating the production of proinflammatory cytokines and lipolysis via NF-κB dependent or independent pathways, possibly via a MAPK-dependent route.

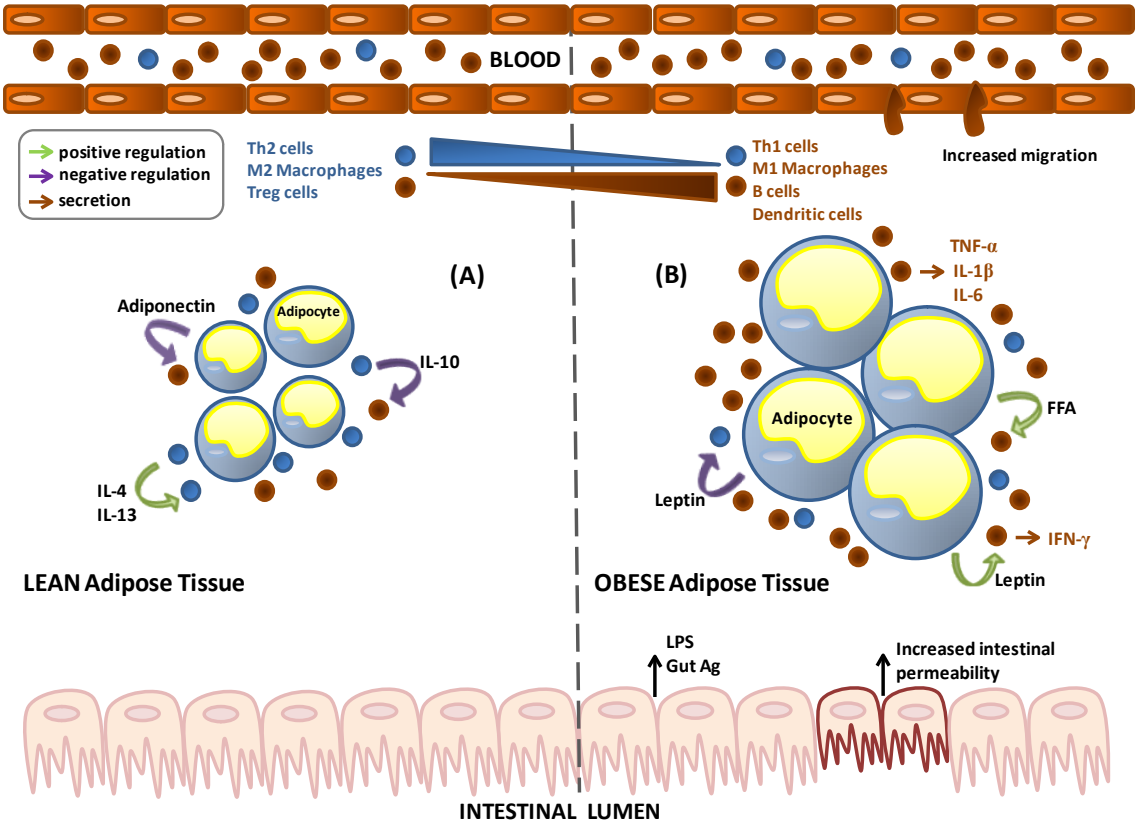


Figure 11. (**A**) Lean AT contains regulatory immune cells (blue) that suppress proinflammatory immune cells (red) and sustain alternative activation of macrophages via Th2-associated cytokines. Adipocytes in lean AT are of normal size and produce adiponectin, which has anti-inflammatory properties. (**B**) In contrast, obese AT is infiltrated with proinflammatory immune cells that produce high amounts of inflammatory cytokines and chemokines. M1 macrophages accumulate in crown-like structures around hypertrophic adipocytes that have increased rate of lipolysis, and secrete free fatty acids (FFA) that can serve as endogenous danger signals to stimulate production of inflammatory cytokines, such as TNF-α. Adipocytes in obese AT also have increased leptin production, which promotes Th1 cells and inhibits Treg expansion. The gut barrier is disrupted in obesity, causing gut Ags and PAMPs such as LPS to enter the AT and stimulate inflammation. Furthermore, immune cells in the blood migrate into the AT in response to heightened chemokine. production.

On the other hand, the fatty acids released by adipocytes bind Toll like receptor-4 (TLR-4) on the macrophages and they promote the production of proinflammatory cytokines, particularly TNF-α, which have an important role in insulin resistance. In fact, TNF−α signaling activates intracellular kinases, such as c−Jun N−terminal kinase (JNK) and IkB kinase (IKK), which inhibit insulin receptor signaling by serine phosphorylation of insulin receptor substrate ƒ (IRS−f). Furthermore, activation of the transcription factors, activator Protein−f (AP−f) and NF-κB results in a feed−forward

mechanism whereby proinflammatory cytokines production is exacerbated. If the magnitude of cytokines secretion is great enough, they can leak out of the tissue, raising circulating levels, to produce endocrine effects on distant organ systems, such as muscle and liver, exacerbating systemic insulin resistance [353].

However, the adipose tissue concentrations of cytokines are much higher than in systemic circulation, and it is likely that, in most circumstances, the major effects of these secretory products are local rather systemic [335]. Although increased adiposity causes insulin resistance, it has long been known that visceral adipose tissue (VAT) has a much greater negative metabolic effect than subcutaneous adipose tissue (SAT). In obesity, increased macrophage accumulation and other signs of inflammation occur in the VAT, but not in the SAT depots, consistent with the negative impact of VAT expansion on insuline sensitivity [354].

2.6.1 Adipose tissue as an endocrine organ

Of note, when considering obesity and metabolic syndrome, it is interesting to note that the adipose tissue acts as an endocrine organ [355]. The hormones produced by the adipose tissue are termed 'adipokines' and the expression of some of them is altered when an excess of body weight occurs [356]. Adipocytes have a key role in regulating inflammation, insulin sensitivity, glucose metabolism and a variety of other physiological processes. They can act locally in autocrine and paracrine manner but also in an endocrine way, thus eliciting effects on the rest of body [356]. There are many adipokines that have been linked to insulin resistance and metabolic syndrome:

Leptin is a protein secreted by adipocytes proportionally to the adipose tissue mass. Leptin is secreted in greater amount by SAT than VAT [357], then it circulates in plasma and enters by diffusion into the central nervous system (CNS), where acts as a satiety signal on hypothalamus, thus reducing food intake and increasing energy expenditure. The main determinant of leptin secretion is glucose metabolism, because the concentration of circulating leptin diminishes under fasting or caloric restriction conditions, and increases in response to food intake [358]. Leptin inhibits lipogenesis and stimulates lipolysis, reducing intracellular lipid levels in skeletal muscle, liver and pancreatic β−cells, thereby improving insulin sensitivity. Obesity is associated with increased leptin levels and hyperleptinemia, which is a reflection of the leptin resistance associated with obesity [359]. In fact, leptin resistance develops in obesity because the

ARH neurons expressing leptin receptors do not become further activated from baseline in response to exogenous leptin; consequently, increased leptin levels do not increase energy expenditure or decrease food intake. In addition, it is generally accepted that leptin acts as a pro−inflammatory adipokine. Indeed, leptin increases the production of TNF−α and IL−6 by monocytes and stimulates the production of CC−chemokine ligands (namely, CCL3, CCL4 and CCL5) by macrophages by activating the JAK2 (Janus kinase 2)−STAT3 (signal transducer and activator of transcription 3) pathway [360, 361]. In monocytes, leptin also stimulates the production of ROS and promotes cell proliferation and migratory responses [360, 362]. Serum leptin levels are increased in response to pro−inflammatory stimuli, including TNF-α and LPS [363]. Furthermore, leptin increases the production of the Th1−type cytokines IL−2 and IFN−γ and suppresses the production of the Th2−type cytokine IL−4 by T cells or mononuclear cells, thus polarizing T cells towards a Th1 cell phenotype [364].

Adiponectin is an anti-inflammatory, insulin-sensitizing and antiatherogenic protein, which is exclusively secreted by adipocytes. It is inversely related to obesity, diabetes and other states that cause metabolic dysfunction; thus, adiponectin deficiency may also contribute to coronary heart disease, steatohepatitis, non-alcoholic fatty liver disease, and a wide array of cancers. In the liver, it inhibits the expression of several gluconeogenic enzymes and decreases the rate of endogenous glucose production, resulting in lower fasting plasma glucose levels. In the muscle, adiponectin increases muscle fat oxidation and glucose transport via the AMP kinase pathway [365]. Adiponectin also shows vasculoprotective properties, since it inhibits TNF-α-induced expression of endothelial adhesion molecules like intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial-leukocyte adhesion molecule-1 (selectin E). Adiponectin blocks smooth muscle cell proliferation by preventing the formation of radical oxygen species (ROS) and the activity of MAPK, as well as the macrophage-to-foam cell transformation [366]. Consequently, a decrease in adiponectin plasma level, which occurs in obesity, is correlated with endothelial dysfunction, characterized by a deficiency of NO production, being a critical component of atherosclerosis development [367, 368]. Other important adipocytokines and their function are summarized in the Table 4.

Table 4: Adipocytokines and their effects on metabolism

Adipocytokine	Production site	Effect on glucose metabolism	Other effects
Vistain	Visceral adipose tissue, bone marrow, liver, muscle	Insulin sensitivity	↓ Glycemic levels Induction of adipocytic defferentation and accumulation of triglycerides in pre-adipocytes ↑ Insulin-stimulted glucose uptake
Omentin	Visceral adipose tissue, stromal vascular fraction	Insulin sensitivity	↑ Insulin-stimulted glucose uptake
TNF-α IL-6	Adipose tissue, stromal vascular fraction, macrophages	Insulin resistance	↑ Inflammation
Resistin	Immune cells, adipose tissue, pre-adipocytes	Hepatic insulin resistance	↑ Inflammation
PAI-1	Visceral adipose tissue, stromal vascular fraction	Insulin resistance	↑ Microthrombiformation and atherosclerolisis

2.7 Obesity-Associated Intestinal Inflammation

Although the main cause of obesity is excessive calorie intake compared with energy expenditure, it seems that gut microbiome-host interactions are of special significance. In fact, gut microbiota has been considered as a separate endocrine organ, which is involved, through a molecular crosstalk with the host, in the maintenance of host energy homeostasis and in the modulation of host immunity [369]. Changes in gut microbial composition, mainly caused by external factors, can result in a dramatic alteration of the symbiotic relationship between gut bacteria and the host, which promotes the development of metabolic diseases. In particular, the gut microbiota is believed to contribute to metabolic diseases through facilitating the low-grade inflammation status that characterizes these conditions [370]. This has been supported by the observation that germ-free mice do not develop obesity or insulin resistance when fed a high-fat diet (HFD) [67, 371]. In addition, it was demonstrated in mice that obesity was associated with an altered gut microbiota composition [372]. Thus, metagenomic studies in human and mice have shown that the two most abundant bacterial phyla in the gut are *Bacteroidetes* and *Firmicutes* [373]; lean individuals maintain a relative balance between these two phyla, whereas obese mice and humans have an increased ratio of *Firmicutes* to *Bacteroidetes* [63, 67, 372, 374, 375]. It has been proposed that the decreased Bacteroidetes:Firmicutes ratio found in these people leads to more efficient hydrolysis of non-digestible polysaccharides in the intestinal lumen, thus extracting more calories and fat from food than in lean individuals [67]. A more

recent study has suggested that the microbiota of obese and lean people responds differently to the calorie content in the diet [376]. It has been also reported that nutrient absorption induced a shift in the gut microbial composition in lean individuals but not in obese, increasing the relative abundance of *Firmicutes* while decreasing *Bacteroidetes* [377]. Since carrying out a controlled dietary intervention study in humans is difficult, the complex interaction between diet, age, host environment and host genetic background in the modulation of gut microbial ecosystems is not fully understood. Nevertheless, different studies have suggested that modification of the gut microbiota by behavioural interventions, including new dietary habits, and the use of antibiotics could be the main drivers of the obesity pandemic [378]. Interestingly, bacterial components from gut microbiota, such as lipopolysaccharides (LPS), peptidoglycan, lipoteichoic acid, flagellin and bacterial DNA, can cause the activation of the immune system, and among them, LPS is thought to be a major inducer of the inflammatory response [379]. LPS derives from the outer cell membrane of Gram-negative bacteria ad it is able to cross the gastrointestinal mucosa via leaky intestinal tight junctions or by infiltrating chylomicrons, the lipoproteins responsible for the absorption of dietary triglycerides and cholesterol from the intestine to the plasma [379-381]. Once it reaches the systemic circulation, LPS infiltrates tissues such as liver or adipose tissues, eliciting an innate immune response [379]. In particular, LPS bind plasma LPS-binding protein (LBP), which activates the receptor protein CD14 that is located in macrophages plasma membrane; thus, the generated complex binds Toll-like receptor 4 (TLR4) at the surface of macrophages, which triggers transduction signals that activate the expression of genes encoding several inflammatory effectors, such as NF-κB [381]. In addition, LPS participates in the recruitment of other effector molecules, such as nucleotide-binding domain leucine-rich repeat containing (NLR) protein, adaptor protein ASC, and caspase-1, which are components of the inflammasome, a multiprotein oligomer that activates the innate immune system [382].

2.7.1 Metabolic endotoxemia and altered barrier integrity in obesity

Under normal conditions, the intestinal epithelium acts as a barrier that prevents translocation of LPS. However, *in vivo* studies have demonstrated that mice fed a high-fat diet showed chronically increased plasma LPS levels; of note, a continuous infusion of LPS over 4 weeks in mice led to a metabolic state comparable to high-fat feeding, including increased macrophage infiltration into the adipose tissue, hepatic steatosis, and an increase in inflammation markers, as well as the onset of liver insulin resistance [379]. The existence of high LPS plasma levels has been defined as metabolic endotoxemia. Several mechanisms linking obesity and metabolic endotoxemia have been proposed (figure 11). During consumption of a high-fat diet, the gut microbiota is

modified, which leads to increases in gut permeability and in the systemic levels of bacterial products such as LPS [379]. Additionally, excess fat intake triggers an increase in chylomicrons in the intestine during the postprandial period (following a meal), which favours LPS infiltration into the circulation [383].

Impaired lipoprotein metabolism in patients with type 2 diabetes has also been found to reduce LPS catabolism and might increase endotoxemia-related inflammation [384]. Furthermore, changes in the gut barrier function seem to have an important role. In fact, obese mice exhibit an altered gut barrier, characterized by disruption of tight-junction (TJ) proteins (zonulin/zonula occludens (ZO)-1, occludin, claudins and actin–myosin cytoskeletal proteins) between epithelial cells [385]. Immune cells, including neutrophils, dendritic cells and monocytes, have also been directly implicated in inducing disturbances in TJ barrier function [386]. Finally, recent evidences have suggested that emulsifiers commonly used in processed food have a negative effect on gut lining by altering the intestinal microbiota composition, which results in increased microbiota invasion of the intestinal mucus layer [387]. These findings and future studies will hopefully provide the basis for new therapeutic possibilities for obesity based on diets and compounds that can improve intestinal barrier function (Figure 12).

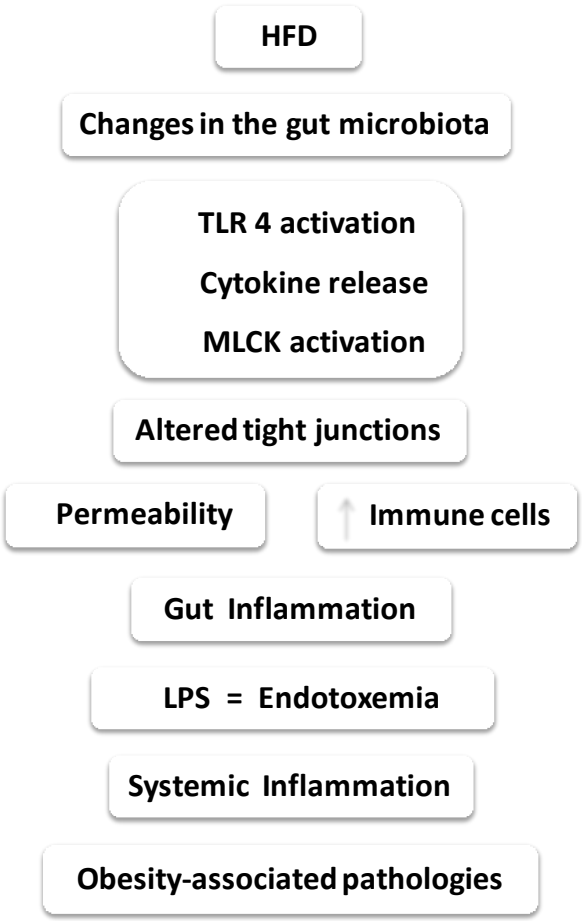


Figure 12. Changes in gut microbiota after a high-fat diet (HFD) induce an increase in intestinal permeability and activation of immune cells. Consequently, endotoxaemia increases and triggers systemic inflammation and metabolic disorders. TLR4, Toll-like receptor 4; MLCK, myosin light chain kinase; LPS, lipopolysaccharide.

2.8 Treatment of obesity

Management of obesity include both weight control, or reducing the excess in body weight, and maintaining the weight loss with time; moreover, it is quite frequent the incorporation of different measures to control the obesity-associated risk factors. To check that the objectives are achieved, periodic evaluation for obesity should be done by the measurement of BMI, measurement of waist circumference, etc., thus assessing the impact on risk factors. Based on the evaluation, appropriate treatment can be suggested. Treatment of obesity implies lifestyle changes, which can be associated to pharmacological therapies and, in certain circumstances, surgery.

2.8.1 Lifestyle changes in obesity treatment

Undoubtedly, comprehensive, or multicomponent lifestyle intervention is the cornerstone for treatment of a patient with obesity [388]. The term comprehensive refers to simultaneous implementation of three strategies: lifestyle or behavioural training, dietary change to reduce energy intake and an increase in physical activity. Therefore, increased physical activity, coupled with a calorie-restricted diet, remains the mainstay of treatment in obese individuals. Of note, although lifestyle changes are often recommended, they have modest success in limiting disease due to poor adherence of patients.

2.8.2 Pharmacological approaches in obesity treatment

While lifestyle intervention is the first line of treatment for obesity management, patients may require adjunctive therapies to meet their weight loss and health goals due to the recalcitrant nature of obesity. Most available weight loss medications are "appetite–suppressant" medications. The initial drugs used for appetite suppression were amphetamine, metamphetamine and phenmetrazine, which are no longer used in the treatment of obesity because of their high potential for abuse.

Inhibitors of 5-hyroxytryptamine (5-HT) reuptake, fenfluramine and dexfenfluramine were licensed for obesity, but proved to cause pulmonary hypertension and increased valvular heart disease [389], being withdrawn from the market. Drugs like phendimetrazine, diethylpropion, phentermine etc., are being marketed but have been classified as controlled substances that are recommended for short-term use only.

The newest agents available for weight loss are sibutramine and orlistat. They are the only weight loss medications approved by the US Food and Drug Administration (FDA) for long-term use [390] in significantly obese patients.

Sibutramine is the serotonin and norepinephrine re-uptake inhibitor, which induces decreased food intake and increased thermogensis [391]. In clinical trials, sibutramine showed a statistical improvement in amount of weight lost versus placebo (Astrup A and Toubro S, 2001). It limits decline of metabolic rate that typically accompanies weight loss [392]. However, this agent is contraindicated in patients with known seizure disorders, high blood pressure, congestive heart failure (CHF), with and a history of myocardial infraction and arrhythmias. Sibutramine was finally withdrawn from the U.S. market at the request of the U.S. FDA and also from European market in 2010, mainly due to negative cardiovascular risk shown in a post-market trial.

Orlistat is a potent and irreversible inhibitor of gastric and pancreatic lipases. It blocks the digestion of approximately 30% of the ingested dietary triglycerides. Different studies have proved that it produces 5% more weight loss than in control groups [393]. The most commonly reported side effects include dyspepsia, flatulence, abdominal pain, diarrhoea, steatorrhea and decreased absorption of fat-soluble vitamins (A, D, E and K). [394, 395].

2.8.3 Complementary and/or alternative therapy

As commented above, global strategies to weight control are focused on dietary and lifestyle modifications, i.e., restricting caloric intake and increasing physical activity to slow obesity development. However, if these strategies fail, pharmacological approaches are recommended (WHO 2007). including blocking nutrient absorption, modulating fat metabolism, regulating adipose signals, and modulating the satiety centre [396]. Although many other medications have been used to manage obesity over the years, most of them are now withdrawn due to their serious adverse effects [397]. Disappointing results of pharmacotherapy emphasize the need of other treatment modalities to prevent obesity. Indeed, much interest has been given to plant-based medications that may contribute to satiety, increased metabolism and accelerated weight loss. The anti-obesity effects of medicinal plants appear to be due to the bioactive metabolites, including the phenolic compounds, flavonoids, alkaloids, glycosteroids, and fatty acids present in a given plant part [398, 399]. Overall, the consumption of

potent anti-obesity plants is usually interconnected with improving the inflammatory, glycaemic and oxidative status in the human body, accompanied with regulation of insulin sensitivity, glucose homeostasis, lipid metabolism and hypolipidemic effects, together with the activation, at least partially, of other systems like PPAR, cAMP, β-adrenoreceptors and sympathetic nervous system [400, 401]. Therefore, management through plants extracts can be achieved by identifying active components, such polyphenols, which could modulate molecular pathways and gene/protein expressions in a beneficial way along with calorie restriction and exercise [402]

Anti-obesity effects of polyphenols: in vivo and in vitro studies

Numerous *in vitro* and *in vivo* studies support the beneficial effects of dietary polyphenols on glucose homeostasis for the prevention and treatment of obesity [403]. Thus, it has been reported that polyphenols regulate carbohydrate digestion, adipose deposition, insulin release, and glucose uptake in insulin-responsive tissues through interference with numerous cell-signalling pathways [404, 405]. Furthermore, investigation on anti-obesity action of polyphenolic compounds using animal cell cultures (3T3-L1) of adipogenesis and high fat diet-induced obese animals have suggested that polyphenols may inhibit pre-adipocyte to adipocyte differentiation, cause adipocyte apoptosis, decrease fat absorption from gut, uptake of glucose by skeletal muscles, suppress lipid biosynthesis and promote catabolism in adipose, liver and other tissues (Table 5). Moreover, they may promote anti-inflammatory molecules in adipose tissue [406]. Adipocytes generate reactive oxygen species due to fatty acid oxidation in mitochondria and peroxisomes that may lead to oxidative stress under obesity. This results in deregulated expression of pro-inflammatory adipokines. Lipid-enriched diets also cause production of reactive oxygen species as they alter oxygen metabolism leading to a significant reduction in antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase [358]. This may in turn lead to obesity-associated problems. Polyphenols bind to electrophile responsive elements on the promoters' sites and enhance gene expression of endogenous antioxidant enzymes.

Effects of polyphenols on adipose tissue metabolism

As commented previously, obesity is defined as the increase in size (hypertrophy) and number (hyperplasia) of adipocytes. Studies have shown that adipocyte number is determined during childhood and maintained through adulthood, emphasizing the

importance of preventing childhood obesity. In adulthood, hypertrophy precedes hyperplasia to accommodate initial excess of energy intake. However, when excess intake persists, as it occurs in the development of obesity, hyperplasia is accelerated [407]. Interestingly, weight loss results in a reduction in adipocyte volume but not, necessarily, adipocyte number [408]. Adipocytes are extremely resistant to apoptosis; therefore, adipose tissue that has been expanded by hyperplasia will be maintained, making difficult for an obese subject to sustain weight loss, and thus worsening the prognosis for the treatment [409, 410]. In consequence, targeting hyperplasia is essential for preventing the progression of childhood and adult obesity. Several bioactive compounds, like polyphenols, have been shown to inhibit adipogenesis, the process of developing new adipocytes. Although relatively little research exists on the effects of polyphenols on adipose cells, Kuppusamy and Das [411, 412] found that several flavonoids potentiate epinephrine-induced lipolysis in primary rat adipocytes. Also, Shisheva and Shechter [413] found that quercetin blocks insulin-mediated lipogenesis by preventing the insulin receptor tyrosine kinase from phosphorylating substrate. Quercetin was also reported to stimulate apoptosis in 3T3-L1 preadipocytes by decreasing the mitochondria membrane potential, downregulating expression of B-cell lymphoma 2 (Bcl-2) and poly(ADP-ribose) polymerase (PARP), and activating Bcl-2 homologous antagonist/killer (Bak), Bcl-2-associated X protein (Bax), and cysteine-depend0ent aspartate-directed proteases 3 (caspase 3) [414]. In growing preadipocytes, quercetin extensively decreased the expression of LPL, sterol regulatory element-binding protein 1c (SREBP1c), and PPAR, a key adipogenic transcription factor [415, 416]. Another polyphenol, curcumin, was shown to inhibit adipogenic differentiation *in vitro* as well as adipose tissue angiogenesis and obesity in C57/BL mice [417]. Similarly, naringenin has been extensively studied and found to possess anti-obesity properties, mostly dependent on the reduction in adipose tissue mass and inhibition of preadipocyte proliferation [418, 419]. Interestingly, naringenin suppressed the proliferation of preadipocytes without showing detrimental effects on subsequent adipogenesis [420]. Different potential mechanisms were also proposed to explain anti-adipogenic effects of genistein, including activation of Wnt signalling via ERs-dependent pathway [421], and inhibition of adipocyte differentiation leading to apoptosis of mature adipocytes via AMPK activation [422]. The anti-lipogenic effects of genistein supplementation include its ability to limit adipocyte hypertrophy via up-regulation of genes involved in fatty acid β-oxidation, such as PPARα, AMPK and very

long-chain acyl CoA dehydrogenase (VLCAD), as well as through the down-regulation of genes associated with adipogenesis or lipogenesis, including liver X receptor-α, SREBP1c, PPARγ, retinoid X receptor-α and acetyl CoA carboxylase (ACC) [421].

Effects of polyphenols on adipose tissue inflammation

As previously commented, the adipose tissue is an endocrine organ that secretes a variety of proinflammatory adipocytokines, including TNF-α, IL-6, MCP-1, resistin, leptin, and adiponectin. Increased visceral adiposity is associated with a higher production of these adipocytokines leading to local and generalized inflammation [423]. Among the adipocytokines, TNF-α is of particular relevance. It activates proinflammatory signaling cascades, such as the mitogen-activated protein kinases (MAPKs) and AP-1 (activator protein-1). The activation of these pathways downregulates PPAR and induces the transcription of inflammatory genes that both maintain a sustained inflammatory state and impair insulin signaling, leading to obesity-triggered insulin resistance [355]. Several studies have confirmed the ability of flavonoids to interfere with inflammatory signaling through different pathways (Figure 13). For example, in obese Zucker rats, a 10-week administration of quercetin (10 mg/kg of body weight/day) increased plasma concentration of adiponectin, reduced TNF-α secretion and the expression of the proinflammatory iNOS in visceral adipose tissue [424]. Quercetin was also able to attenuate the activation of NF-κB and MAPKs in human macrophages and adipocytes treated with macrophage-conditioned media, thus decreasing not only parameters of inflammation, but also of insulin resistance [425].

Similarly, in high fat–fed rodents, the administration of EGCG [426, 427] reduced adipose tissue inflammation and increased plasma concentration of adiponectin. In particularly, the catechin-enhanced expression and secretion of adiponectin is mediated in part via suppression of Krüppel-like factor 7 (KLF7) protein, which inhibits the expression of adiponectin and other adipogenesis-related genes, including leptin, PPARγ, CCAAT/enhancer-binding protein α (C/EBPα), and adipocyte fatty acid-binding protein (aP2) in adipocytes [428]. Moreover, several studies reported that curcumin exhibits anti-inflammatory effects, through activation of Wnt/β-catenin signalling and suppression of MAPK and NF-κB activation pathways [429].

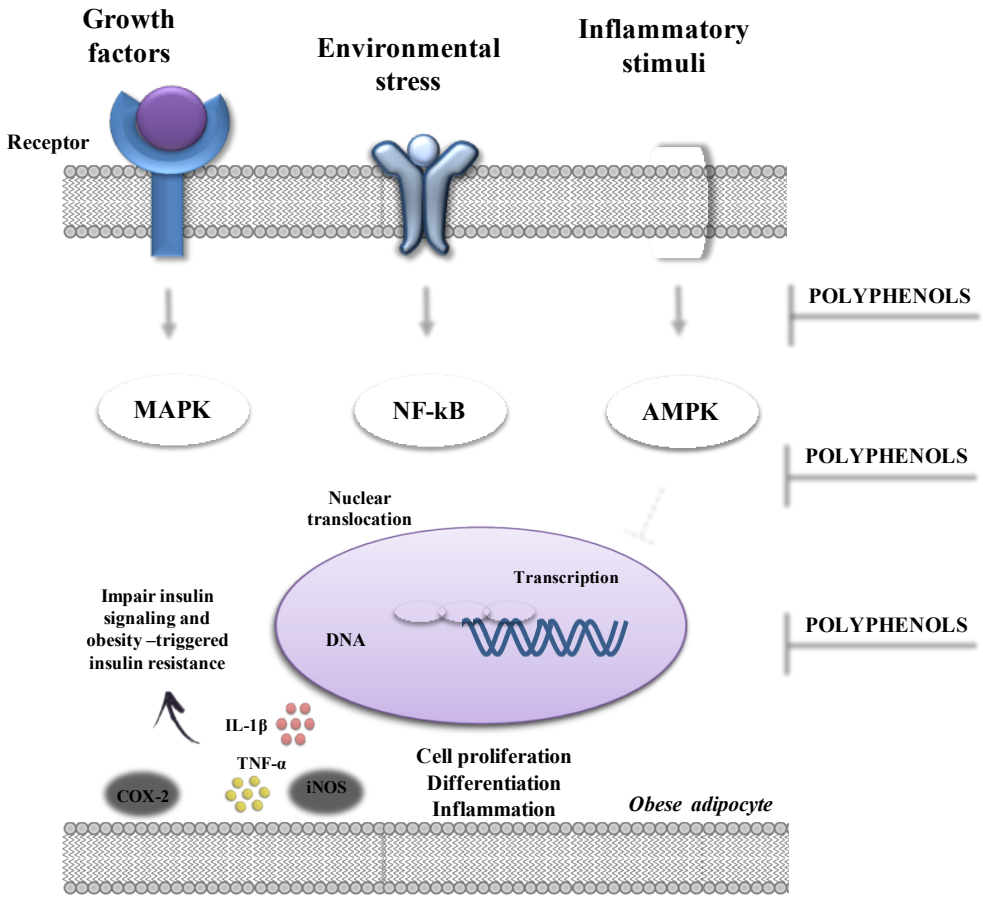


Figure 13. Anti-inflammatory effects of polyphenols on the obese adipose tissue. Obesity leads to activation of the NF-κB and MAPK signaling pathways and suppression of AMPK signaling pathway in the adipose tissue. Polyphenols are known to suppress the pro-inflammatory mediators associated with NF-κB, MAPK and AMPK pathways, thus decreasing obesity-triggered insulin resistance.

These anti-inflammatory effects exerted by curcumin were also reported in genetic obesity (ob/ob mice) and diet-induced obesity models, in which the mechanisms of action involved the reduced macrophage infiltration into adipose tissue, increased adiponectin production and decreased hepatic NF-κB activation [429]. The latter was related to the ability reported for curcumin to suppress IκB degradation and the subsequent suppression of NF-κB activation, resulting in reduced TNF-α, IL-1β, IL-6 and COX-2 gene expression in differentiated adipocytes [430]. Similarly, a recent study reported that resveratrol reduces adipose tissue inflammation [430] particularly via suppression of NF-κB and extracellular signal-regulated kinase (ERK) activation as well as activation of sirtuin-1 (silent mating type information regulation 2 homolog, Sirt1) [430, 431]. In agreement with these anti-inflammatory effects *in vitro*, resveratrol also significantly attenuated high-fat (HF) diet-induced production of TNF-α, IFN-α, IFN-β, and IL-6 and their upstream signalling molecules including TLR 2/4, myeloid differentiation primary response gene 88 (MyD88), toll interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP), TIR-domain-containing adapter-inducing interferon (TRIF), TNF receptor associated factor 6 (TRAF6),

interferon regulatory factor 5 (IRF5), p-IRF3, and NF-κB in mouse adipose tissue [432]. Finally, analysis from microarray assay reported that human adipocytes response to anthocyanins induces up-regulation of adiponectin levels and down-regulation those of plasminogen activator inhibitor-1 (PAI-1) and IL-6 [433]. Since the high expression of PAI-1 and IL-6 in adipose tissue and the low expression of adiponectin are connected with obesity and type-2 diabetes, regulation of their expression is an important therapeutic target for treating obesity and its relevant disorders [434].

Effects of polyphenols on blood glucose and insulin resistance

The term "insulin resistance" usually implies resistance to the effects of insulin on glucose uptake, metabolism or storage in the corresponding target tissues. Although many details of the mechanisms by which the enlarged adipose tissue mass that defines obesity causes systemic insulin resistance remain unknown, the past several years have witnessed an explosive increase in its understanding of what may now be referred to as the adipo-insulin axis. There are also evidences for considering the related possibility that insulin resistance and hyperinsulinemia, in addition to being caused by obesity, can contribute to the development of obesity. Insulin resistance in obesity is manifested by decreased insulin-stimulated glucose transport and metabolism in adipocytes and skeletal muscle, and by impaired suppression of hepatic glucose output [435]. These functional defects may result, in part, from impaired insulin signalling in all the three target tissues. In addition, there is also a downregulation of the major insulin-responsive glucose transporter, GLUT4. This transporter seems to be the target of many polyphenols, being able to stimulate GLUT4 translocation in adipocytes or skeletal muscle cells by activating either the insulin-mediated phosphatidylinositide 3-kinase (PI3-K)/Akt (Figure 14) or the AMP-activated protein kinase (AMPK) pathways [436, 437].

Regulation of AMPK activity can also leads to the activation of a class of protein deacetylases known as sirtuins; in fact, the activation of sirtuin 1 (Sirt1) is involved in the antiaging and anti-inflammatory effects of polyphenols such as resveratrol, querectin, catechins, and piceatannol [438]. Similarly, different anthocyanins have been reported to attenuate insulin resistance in high-fat-fed C575L/6 mice [439]. When these mice were administered a mixture of pure anthocyanins (cyaniding 3-O-galactoside,

pelargonidin 3-O-galactoside and delphinidin 3-O-galactoside), an improvement on glucose tolerance was observed after 6 weeks of treatment.

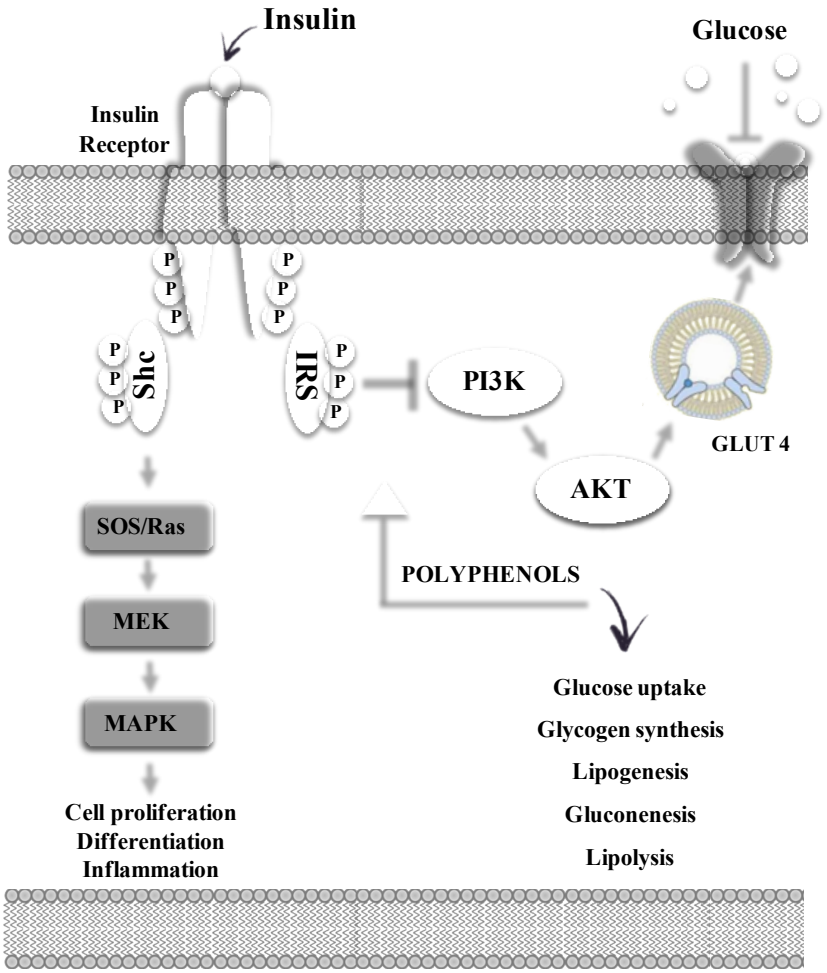


Figure 14. Physiological insulin's actions are mediated by the activation of either the phosphoinositide 3-kinase (PI3K)-Akt pathway or the mitogen activated protein (MAP) kinase pathway. In insulin resistance, the PI3K-Akt pathway is affected, whereas the MAP kinase pathway is not. This leads to a change in the balance between these two parallel pathways. The inhibition of the PI3K-Akt pathway leads to a reduction in endothelial NO production, resulting in an endothelial dysfunction, and a reduction in GLUT4 translocation, leading to a decreased skeletal muscle and fat glucose uptake. By contrast, the MAP kinase pathway is unaffected, so there is a continued cells proliferation, differentation, inflammation and an expression of vascular cell adhesion molecules. In consequence, insulin resistance leads to the vascular abnormalities that predispose to atherosclerosis and obesity-associated phatologies. Polyphenols restored insulin sensitivity and glucose uptake in skeletal muscle and adipocytes by activation of both AMPK and AKT pathways.

This beneficial effect could be due to the ability of anthocyanins to increase insulin sensitivity and/or secretion [439]. Furthermore, it has been reported that myricetin, when injected intravenously into genetically obese rats, was able to reduce the glucose-insulin index; this flavonoid augment GLUT4 expression and increased the phosphorylation of AKT and insulin receptor substrate 1 (IRS1) [440].

Diffferent *in vitro* studies have confirmed the ability of polyphenols to enhance glucose homeostasis. In particular, Scazzhocchio *et al*. [441] demonstrated that protocatechuic acid and different antocyanidins were able to increase adipocyte glucose uptake and GLUT4 expression and translocation, which was accompanied by increased nuclear PPARγ activity [441]. Moreover, it has been reported that tangeretin, curcumin and quercetin strongly induce glucose uptake in a dose dependent manner, through the AMPK/ACC pathway, in differentiated C2C12 mouse myoblast cell line [442, 443] and adipocytes [444], respectively. Also, narigenin has been reported to stimulate the glucose uptake in L6 myotubes [445] (an immortalized rat skeletal (L6) myoblast cell line that was selected for high fusion potential and endogenous expression of GLUT4) as well as in rat adipocyte primary culture [446], thus suggestion its key role in glucose disposal. Phenolic acids also exhibit potential regulation of glucose uptake in skeletal muscle and adipocytes by activation of both AMPK and AKT pathways (Lee ES *et al*., 2007).

Effects of polyphenols on obesity related dyslipidemia

Dyslipidemia has been considered as one of the major components of obesity-related disorders. It is characterized by increased Low-density lipoprotein (LDL)/very-low-density lipoproteins (VLDL)/ triglycerides (TG) and reduced level of high-density lipoprotein (HDL) [447, 448]. Dyslipidemia is also frequently associated with increased levels of certain inflammatory mediators such as IL-6, TNF-α, among many others, which play a critical role in producing insulin resistance. Under normal circumstances, insulin activates the enzyme lipoprotein lipase (LPL), which hydrolyses triglycerides. However, in obese states, insulin resistance and elevated plasma insulin levels cause increased hepatic fatty acid esterification and triglyceride formation [448, 449]. High levels of total cholesterol (TC) and LDL are considered as important coronary risk factors, although other studies also suggested that TG could independently be related to coronary heart disease. Of note, the prescription of popular lipid-lowering drugs, like statins, does not effectively address the parallel pathologies such as insulin sensitivity and inflammation. Hence, research needs to shift its attention from synthetic to natural products with a more holistic mechanism of action, without disturbing the physiological equilibrium. Polyphenols, due to their natural origin are likely to produce a more balanced therapeutic effect across the axis linking inflammation/insulin resistance with obesity/ dyslipidemia. Several dietary flavonoids have been found to lower LDL levels

and inhibit oxidative modifications [450, 451] *in vitro* and thus they have been proposed to be potential inhibitors of LDL oxidation and the consequent induction of atherogenesis. Flavonoids inhibit LDL oxidation by several mechanisms that act in concert, including the well-known scavenging properties on some radical species [452].Although there is little information available about the impact of polyphenols in obesity related dyslipidemia, several studies have reported their ability to ameliorate the altered lipid levels. In an *in vitro* study on whole blood from hypercholesterolemic patients, incubation with quercetin (10 μmol/L) reduced cholesterol concentration to 75%. [453]. Polyphenols prevent metabolic dysregulation by restricting ectopic lipid accumulation and by stimulating utilization of both fatty acids and glucose. The flavonoid naringenin was found to normalize lipids in diabetes and hypercholesterolemia by regulating the activity of nuclear receptor PPARs, such as PPARα, PPARγ and liver X receptor alpha (LXRα) [418]. Moreover, naringenin and hesperitin, included as a 0.1% and 0.2% dietary supplement in rats fed a high-cholesterol diet, reduced the cholesterol levels of plasma, as well as triacylglycerol and the cholesterol levels in hepatocytes by decreasing the activity of enzymes involved in these metabolic pathways [454, 455]. Meanwhile, studies performed on hypercholesterolemic human subjects showed increase in HDL levels after consumption of naringenin rich orange juices [456]. In addition, hesperetin has been reported to interfere with cholesterol biosynthesis, resulting in a lower intracellular supply of cholesterol and over-expression of hepatic LDL receptors, as well as increased clearance of circulating LDL particles [457]. These growing evidences indicated that polyphenols ameliorate lipid profile and metabolism.

Effects of polyphenols on gut microbiota and barrier function

It was also commented above that during the last decade new studies have emerged suggesting a role for gut microbiota in the development of obesity [385, 458]. Early studies showed that obese mice and humans had different gut microbiota composition compared to lean subjects. Indeed, an increase in bacteria from the *Firmicutes* phyla and a decrease in the *Bacteroidetes* phyla are believed to be associated with increased energy absorption from food and increased low-grade inflammation in obesity [372, 374].

Currently, a growing body of scientific evidence shows that polyphenols could exert a protective role against obesity and its related pathologies by modulating gut microbiota composition and functionality, together with their beneficial impact on intestinal inflammation and barrier integrity. Interesting results were obtained in human studies when evaluating the effect of cocoa flavanols in a randomised, double-blind, cross-over intervention study that included twenty-two human volunteers [459]. The administration of 494 mg of cocoa flavanols for 4 weeks significantly increased the number of *Lactobacillus* and *Bifidobacterium* populations, but significantly decreased the Clostridia counts. These microbial changes were correlated with reductions in plasma C-reactive protein concentrations, being considered as a blood marker of inflammation and a hallmark of the acute-phase response [459].

Of note, administration of both quercetin and resveratrol [460], or resveratrol alone [461], to obese rat fed high fat/high sucrose (HFHS) or high fat diets was reported to reduce *Firmicutes*:*Bacteroidetes* ratio [460, 461], which was increased in obese animals. Moreover, resveratrol decreased the abundance of bacteria associated with diet-induced obesity, such as *Bacillus spp*., *Eubacterium cylindroide*, and *Erysipelotrichaceae* [460] and increased the growth of *Bifidobacterium* and *Lactobacillus* [460, 461]. Interestingly, an increased level of faecal bifidobacteria has been associated with improved glucose tolerance and diminished inflammatory markers such as the interleukins IL-6, IL-1α and IL-1β, TNF-α and MCP-1 [379, 385].

On the other hand, several evidences have indicated that obese mice exhibit an altered gut barrier [385]. Interestingly, polyphenols seem to participate in the regulation of the intestinal barrier too. Thus, flavonoids like quercetin, myricetin and kaempferol, enhance barrier integrity in intestinal Caco-2 cells [386]. Quercetin increases TER and reduces paracellular flux across Caco-2 monolayers in a dose-dependent manner [462, 463]. This mechanism is accompanied by an increase in claudin-4 expression and the assembly of ZO-2, occludin and claudin-1 at the TJ level. Also, kaempferol increases TER due to promotion of the cytoskeletal association of ZO-1, ZO-2, occludin, claudin-1, claudin-3, and claudin-4 and an increase in the expression of some TJ proteins [289]. Finally, the isoflavonoid genistein inhibits the redistribution and the dissociation of occludin/ZO-1 complex protecting barrier integrity against acetaldehyde and oxidative stress [464, 465].

Table 5. Anti-obesity effects of polyphenols

Polyphenol	Plant source	Mechanism
Resveratrol	*Fallopia japonica*, grape, nuts	Reduction adipose tissue inflammation. Inhibition NF-κB activity. Gut microbiota modulation.
Quercetin	*Dysosma veitchii*	Increase apoptosis in preadipocytes Reduction pro-inflammatory mediators production in adipose tissue . Inhibition NF-κB activity. Inhibition MAPK pathway. Reduction hepatic gluconeogenesis Increase glucose uptake. Gut microbiota modulation. Improvement epithelial barrier function.
Genistein	*Glycine max*	Inhibition adipocyte differentiation.
Epigallocatechin-3-gallate	*Camellia sinensis*	Reduction adipose tissue inflammation. Increase adiponectin plasma concentration.
Kaempferol	Found in various fruits and vegetables e.g., tea, tomato, cruciferous vegetables, apple, etc.	Improvement epithelial barrier function.
Tangeretin	*Citrus* fruit peels	Increase glucose uptake.
Myricetin		Improvement epithelial barrier function.
Curcumin	*Curcuma longa*	Increase glucose uptake.
Hesperetin	*Cordia obliqua*	Improvement plasma metabolic profile.
Naringenin	Grapefruit (*Citrus paradise*)	Reduction adipose tissue mass. Inhibition preadipocyte proliferation. Improvement plasma metabolic profile.

3. Olive leaf extract

Olive tree (*Olea europaea* L.) is one of the most relevant botanical drugs in traditional Mediterranean Medicine. Remarkably, the traditional Mediterranean diet has gained robust scientific support for providing protection against different human chronic diseases, mainly those related to the cardiovascular system and the gastrointestinal tract, and including also the metabolic syndrome [466, 467]. Particularly, it has shown its ability to modulate the inflammatory response, which plays a pivotal role in these diseases. Unfortunately, the mechanisms of action behind its beneficial effects on inflammation are not entirely clear [467, 468]. They could partially be attributed to the intake of olive oil, which contains biologically active components, including oleic acid [469] and phenolic compounds [470] with antioxidant and anti-inflammatory properties. In fact, diet supplementation with olive oil, or with its polyphenol content, has previously shown intestinal anti-inflammatory activity in experimental models of colitis [471-473].

Moreover, olive leaf extracts have also been used since ancient times in traditional Mediterranean Medicine with different purposes, including antihypertensive, antiatherogenic, anti-inflammatory, hypoglycemic and hypocholesterolemic activities [474]. These extracts have been also reported to contain many potentially bioactive compounds, especially phenolic derivatives, like phenolic acids, phenolic alcohols (hydroxytyrosol), flavonoids (diosmetin, quercetin, luteolin, apigenin and their derivatives) and secoiridoids (oleuropein and its derivatives) [475], which may account for their health promoting properties. In fact, and when considering intestinal inflammation, previous studies have shown the intestinal anti-inflammatory properties of many of these compounds [472, 476-478]. For example, the effect of oleuropein administration in patients with chronic colitis was tested by Giner and collaborators, in a study that evaluated the effects of a diet supplemented with oleuropein, equivalent to 500 mg/kg of body weight for 56 days in mice with chronic colitis induced by DSS. The study showed a reduction in cellular infiltration and consequently in the recruitment of inflammatory cells (macrophage, neutrophil and eosinophil) to the location of the injury [477].

Moreover, animals and humans studies have also reported others beneficial health effects of olive leaves compounds including hypoglycmic [479] and hypocholesterolemic [480] activities, which could make it to be considered as a

coadjuvant therapy in the treatment of obesity [481]. Closely related to the latter, different studies have demonstrated the efficacy of olive leaf extracts in an experimental model of diet-induced obesity, being this activity ascribed to the ability of its phenolic compounds to regulate the number of differentiated adipocyte trough the induction of apoptosis [414]. An olive leaf extract also reduced lipid accumulation by inducing thermogenic pathways during adipogenesis in human adipose derived mesenchymal stem cells [482]. In addition, mice administrated with high-fat diet supplemented with polyphenols displayed enhanced insulin sensitivity, AMPK function and improvement of mitochondria activity as well as reduced adiposity, thus suggesting a preventive function of polyphenols against diet-induced obesity and its related disorders [483]. Interestingly, the effect of several polyphenols on intestinal epithelial integrity and on gut microbiota composition can also play an important role in their beneficial properties in IBD and obesity-related conditions [484].

AIMS

Inflammatory Bowel Disease (IBD) and Metabolic Syndrome are diseases whose prevalence and incidence are continuously increasing, especially in developed countries.

IBD mainly includes Crohn's disease (CD) and Ulcerative colitis (UC). Both conditions are characterized by chronic inflammation of the intestine, most probably due to an exacerbated immune response in the intestine against antigens that have not been determined yet, in which the alternation of periods of exacerbation and remission of symptoms takes place [1, 2]. Although the mechanisms responsible for the initiation and perpetuation over time of the intestinal inflammatory process are unknown, it is accepted that genetic, environmental and immunological factors are involved in their pathophysiology. Thus, numerous studies have proposed that, in genetically predisposed people, an exaggerated and uncontrolled activation of the intestinal immune system against an unknown antigenic determinant can trigger the exacerbated intestinal inflammatory response [135]. This immune response generates numerous pro-inflammatory mediators, including cytokines, eicosanoids and reactive metabolites derived from oxygen or nitrogen, which act in a synergistic and simultaneous manner in the intestinal inflammatory process [4-6].

The metabolic syndrome comprises a set of metabolic alterations including insulin resistance, visceral adiposity, atherogenic dyslipidemia, endothelial dysfunction, elevated blood pressure and obesity, displaying the latter a very prominent role [485]. Obesity is a multifactorial disease that appears when energy intake exceeds energy expenditure, leading to the accumulation of an excess of adipose tissue. This imbalance is the result of the combination of various physiological, psychological, metabolic, genetic, socioeconomic and cultural factors. In recent years it has been observed that obese patients show a subclinical chronic inflammatory state, as a consequence of the increase in adipose tissue mass, which leads to an increase production of proinflammatory mediators that are jointly stimulated by signals of exogenous and/or endogenous origin. Adipose tissue contains fibroblasts, preadipocytes, adipocytes and macrophages; the latter contributes significantly to the systemic inflammatory process with the production of proinflammatory mediators. Thus, there is an intimate, highly coordinated association between inflammatory and metabolic pathways [8].

In addition, recent attention has been paid to the link between the composition of the intestinal microbiota, intestinal permeability and obesity [484]. Thus, several experimental models have revealed that obesity and insulin resistance are associated with increased levels of endotoxins from intestinal microbiota in situations where an increase of intestinal permeability takes place. The correct functioning of the intestinal

barrier is essential to avoid the excessive translocation of these toxic molecules, like LPS, to the circulation. It has been reported that an altered gut microbiota composition, termed as dysbiosis, can influence the integrity of the intestinal epithelium and promoting the inflammation of the mucosa, being both involved in the obesity-associated increased intestinal permeability [484].

Currently, there is no ideal drug treatment that combines effectiveness with absence of adverse reactions in any of the two diseases, so it is necessary to research new therapeutic strategies that combine efficacy and safety. The potential of natural products for the treatment of IBD and obesity is still largely unexplored and can be an excellent alternative for the safe and effective development of anti-inflammatory and antiobesity remedies [486, 487].

Considering the botanical drugs in traditional Mediterranean Medicine, the olive (*Olea europaea L.*) leaf extract is one of the most relevant. It has been reported that olive leaves contain several different phenolic compounds that display beneficial properties, including immunomodulatory [475], antioxidant and anti-inflammatory [488, 489], anti-carcinogenic [490, 491], antimicrobial [492], antiviral [493], antiplatelet [494], as well as hypoglycemic and hypocholesterolemic activities [474]. Moreover, different studies have proven the efficacy of olive leaf extract in experimental model of diet-induced obesity, being this activity ascribed to the ability of its phenolic compounds to modulate the adipocyte physiology through the inhibition of lipid accumulation and differentiation or induction of apoptosis [414, 495, 496]. Of note, the impact of several polyphenols on intestinal epithelial integrity and on gut microbiota composition can also play a key role in their beneficial effects in IBD and obesity-related conditions [484].

With this purpose, the present study aims to assess the potential anti-inflammatory effect of a well chemically characterized olive leaf extract (OLE), in experimental models of intestinal inflammation and metabolic syndrome in mice, and to evaluate the possible mechanisms involved in the beneficial effects.

The specific objectives are:

1. To evaluate the immunomodulatory activity of OLE:

a) by *in vitro* studies in murine macrophages, murine and human epithelial cells and peripheral blood mononuclear cells (PBMC) from healthy individuals and patients with Crohn's disease (CD).

b) by *ex vivo* studies, using human explants from patients with inflammatory bowel disease.

2. To evaluate the intestinal anti-inflammatory effect of OLE in experimental colitis induced by dinitrobenzenesulfonic acid (DNBS) and sodium dextran sulfate (DSS) in mice, and analyzing its impact on the immune response and intestinal barrier function.

3. To evaluate the effects of OLE in diet-induced obesity in mice, and to investigate its impact on metabolic alterations, inflammatory status, vascular endothelial dysfunction and gut microbiota composition in obese mice.

This research would provide valuable information that establishes the basis for the future development of OLE in the treatment of these conditions in humans.

MATERIALS AND METHODS

1. Reagents

All chemicals were purchased from Sigma Chemical (Madrid, Spain) unless otherwise stated.

2. Olive leaf extract

Olive leaves samples (*Olea europaea* L.) were used in this study. The leaves were collected and dried at room temperature. The extraction of phenolic compounds was performed as follows: dry leaves (0.5 g) were crushed and extracted via Ultra-Turrax IKA T18 basic (IKA®-Werke GmbH & Co. KG, Staufen, Germany) using 30 mL of methanol/water (80/20). After solvent evaporation, the extract was redissolved in 2 mL of methanol/water (50/50). Three replicates of the sample were processed. The extracts were evaporated and concentrated in a SpeedVac concentrator Savan SC250EXP (ThermoFisher Scientific, Waltham, MA, USA). Total phenolic content was determined by high-performance liquid chromatography coupled to diode array detector and electrospray time-of-flight mass spectrometry (HPLC-DAD-ESI-TOF-MS) using the method previously described by Talhaoui *et al*. [497] and expressed as grams of phenolic compounds per 100 g of extract (%). The olive leaf extract used in the present study contained 10.64 % (w/w) phenolic compounds and its phenolic profile had been previously reported [475, 497] (Table 6). Oleuropein was the major compound, constituting 82.5% of total phenols. The sum of the other secoiridoids (oleoside, secologanoside, oleuropein aglycone, demethyloleuropein, oleuropein glucoside, 2″-methoxyoleuropein and ligstroside) was found at a percentage of 5.4 % of total phenols, whereas simple phenols such as hydroxytyrosol hexose and tyrosol glucoside represented 6.8% of total phenols. The content of other phenolic compounds, like elenolic acids, was very low, being approximately 0.3 % of total phenols.

Table 6. Quantification of the identified phenolic compounds in olive leaves extract (OLE) expressed as mg/g dry matter.

	PHENOLIC COMPOUNDS	Olive Leaf Extract
1	**Oleoside**	0.366
2	**Hydroxytyrosol-hexose isomer a**	0.438
3	**Hydroxytyrosol-hexose isomer b**	0.793
4	**Secologanoside isomer a**	2.035
5	**Tyrosol glucoside**	0.863
6	**Elenolic acid glucoside isomer a**	0.267
7	**Secologanoside isomer b**	3.677
8	**Elenolic acid glucoside isomer b**	0.904
9	**Elenolic acid glucoside isomer c**	0.186
10	**Oleuropein aglycon**	0.134
11	**luteolin-diglucoside**	0.201
12	**Elenolic acid glucoside isomer d**	1.212
13	**Demethyloleuropein**	6.382
14	**Rutin**	0.319
15	**Luteolin rutinoside**	0.199
16	**Luteolin glucoside isomer a**	3.534
17	**Verbascoside**	1.162
18	**Apigenin rutinoside**	0.230
19	**Oleuropein diglucoside isomer a**	0.430
20	**Oleuropein diglucoside isomer b**	0.680
21	**Oleuropein diglucoside isomer c**	0.900
22	**Luteolin glucoside isomer b**	1.072
23	**Chrysoeriol-7-O-glucoside/Isorhamnetin-7-O-rhamnoside**	0.581
24	**2′′-Methoxyoleuropein isomer a**	1.036
25	**2′′-Methoxyoleuropein isomer b**	0.870
26	**Oleuropein isomer a**	17.460
27	**Oleuropein isomer b**	1.279
28	**Oleuropein/Oleuroside**	2.110
29	**Ligstroside**	3.476
30	**Luteolin**	0.367
	Total	**52.129**

3. *In vitro* immunomodulatory properties of olive leaf extract

RAW 264 (murine macrophages), CMT-93 (murine epithelial cells) and Caco-2 (human epithelial cells) cells were obtained from the Cell Culture Unit of the University of Granada (Granada, Spain) while peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-stabilized blood samples of healthy and IBD patients at the Policlinico Tor Vergata, Rome (Italy) using Ficoll gradients (figure 15).

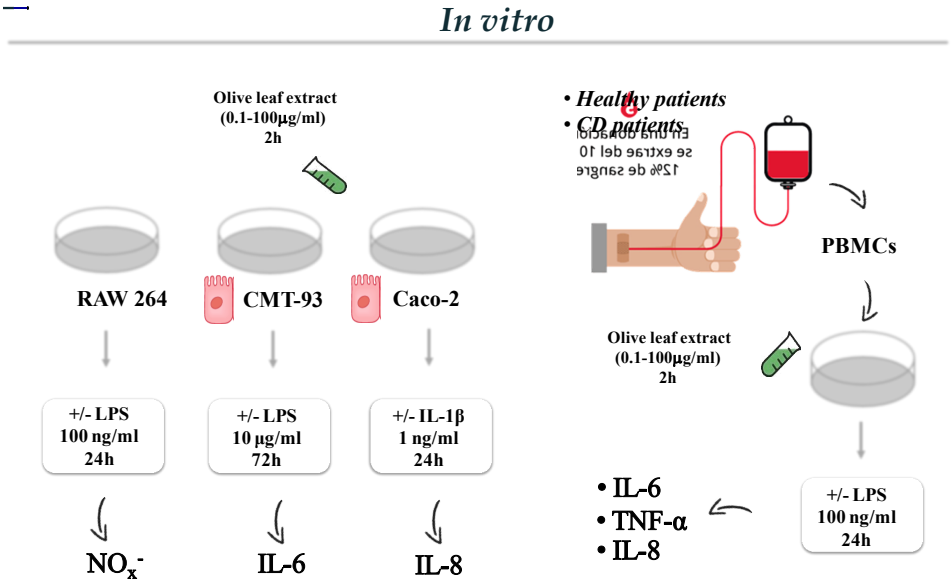


Figure 15. *In vitro* experimental designs

All cells were cultured in RPMI Medium supplemented with 10% heat-inactivated foetal bovine serum, L-glutamine (2 mmol/L), penicillin (100 units/mL) and streptomycin (1 mg/mL) in a humidified 5% CO_2 atmosphere at 37%. Cells were seeded into 96 well plates at a density of $5x10^5$ cells/well, (or 10^6 cells/mL for PBMCs), grown until the formation of a monolayer, pre-incubated with different concentrations of olive leaf extract ranging from 0.1 to 100 µg/mL for 2 h and stimulated with the lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 (100 ng/mL) for 24 h or interleukin (IL)-1β (MiltenyiBiotec, BergischGladbach, Germany) (1ng/ml) for 72 h. Untreated unstimulated cells and LPS or IL-1β stimulated cells were used as negative and positive controls, respectively. After the stimulation period, the supernatants were collected and the production of cytokines measured using sensitive commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

The effect of the olive leaf extract on cell viability was checked with the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA)

following the manufacturer's protocol [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium solution was added to each well and incubated for 1-4 h. The absorbance of the media was measured at 490 nm on a MRX Dynex microplate reader (Dynex Technologies, Chantilly, VA, USA). The cellular viability was calculated from the absorbance value and compared with that of the untreated control cells.

4. *Ex vivo* organ cultures

Freshly obtained intestinal mucosal samples were cultured as described elsewhere [498]. Briefly, surgical specimens were taken from patients with colonic CD undergoing surgery for a chronic active disease poorly responsive to medical treatment and from patients with ileal CD undergoing surgery due to stricturing disease at the Gastrointestinal Unit of Tor Vergata University Hospital (Rome, Italy). Each patient who took part in the study gave written informed consent and the study protocol was approved by the local Ethics Committees (Tor Vergata University Hospital, Rome; protocol number: 154/12). Samples were placed on iron grids with the mucosal face upward in the central well of an organ culture dish containing AQIX medium (Aqix Ltd., London, UK) supplemented with 1% L-glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml) and gentamycin (50 µg/ml) (all from Lonza, Basel, Switzerland) and incubated in the presence or absence of olive leaf extract and/or LPS (100 ng/mL). Dishes were then placed in a tight container with 95% O_2 and 5% CO_2 at 37°C. After 24 h, mucosal samples were homogenized with a Tissue Lyser II (Qiagen N.V., Hilden, Germany) in TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), and total RNA was extracted according to the manufacturer's instructions. The culture supernatants were also collected and stored at -80°C until assessed for production of cytokines (TNF-α, IL-1β, IL-6, IL-8) by ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol (Figure 16).

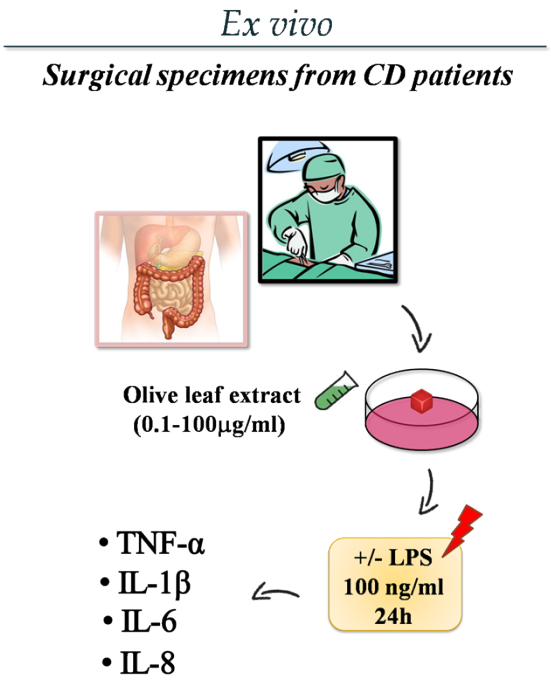


Figure 16. Experimental design of the ex vivo organ cultures

5. *In vivo* studies

The studies were carried out in accordance with the 'Guide for the Care and Use of Laboratory Animals' as promulgated by the National Institute of Health (reference number CEFA-2010-286), and the protocols approved by the Ethic Committee of Laboratory Animals of the University of Granada (Spain) (Ref. No. CEEA-2010-286).

5.1 Dinitrobenzene sulfonic acid (DNBS)-induced colitis

Male CD1 mice (8–10 weeks of age) obtained from the Laboratory Animal Service of the University of Granada (Granada, Spain) were kept in a 12 h light/dark cycle, with controlled humidity (60–80%) and temperature (22 ± 1°C). Food and water were freely available. The mice were randomly assigned to six groups (n=10). An untreated DNBS control group and a non colitic group were included for reference, which received the vehicle used to administer the extract. Four of them were treated with olive leaf extract at different doses (1, 10 and 25 mg/kg). The extract was dissolved in water and administered daily by oral gavage (Figure 17).

Colitis was induced as described previously [499]. Briefly, mice deprived of food for 1 day were lightly anaesthetized by administration of xylazine (10 mg/kg, i.p.) and ketamine (100 mg/kg, i.p). To induce colitis, DNBS (3 mg in 100 µL of 50% ethanol solution) was slowly administered by means of a Teflon catheter inserted 4 cm through

the anus. To assure the distribution of DNBS within the entire colon, mice were carefully maintained in head down position for 15 min and then returned to their cages. Mice from the non-colitic group were administered intracolonically 100 µL of phosphate buffered saline instead of DNBS.

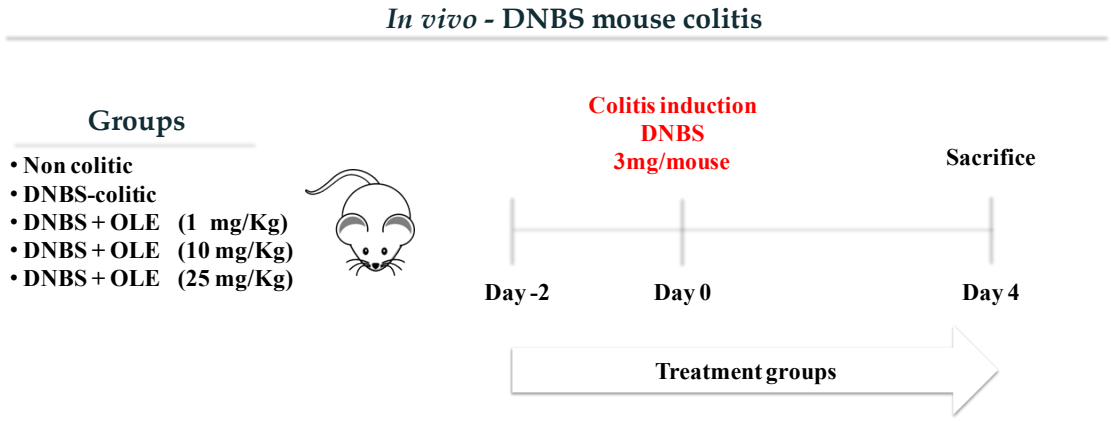


Figure 17. Experimental design in the DSS model of mouse colitis. DSS (Dextran sodium sulfate); OLE (Olive leaf extract).

The administration of the olive leaf extract started two days before colitis induction and continued until the day before of the sacrifice, which took place five days after DNBS instillation. Animal body weights, occurrence of diarrhoea, and water and food intake were recorded daily throughout all the experiment. Once the animals were sacrificed, the colon was removed aseptically and placed on an ice cold plate, longitudinally opened and cleaned from their luminal contents with cold saline. Each colon was scored macroscopically and subsequently minced, aliquoted and kept frozen at -80°C until biochemical determinations and RNA extraction were performed.

5.2 Dextran sodium sulfate (DSS)-induced colitis

Male C57BL/6J mice (7–9 weeks old) obtained from Janvier Labs (St Berthevin Cedex, France) were randomly assigned to five different groups (n=10): one non-colitic control and four DSS colitic groups. Mice were fed ad libitum standard rodent chow throughout the whole experimental period. The colitis was induced by adding 3% (w/v) DSS (36-50 KDa, MP Biomedicals, Santa Ana, CA, USA) in the drinking water for 5 days (Melgar, S. *Et al*., 2005). Three of these groups were daily treated by oral gavage with the olive leaf extract at different doses (0.5, 1, 10 mg/kg day) from the day of colitis induction. The remaining group received the vehicle (water solution) used to administer the

products. Mice were sacrificed 11 days after the beginning of the assay. Animal body weight, the presence of gross blood in the faeces, and stool consistency were individually evaluated daily by an observer unaware of the treatment.

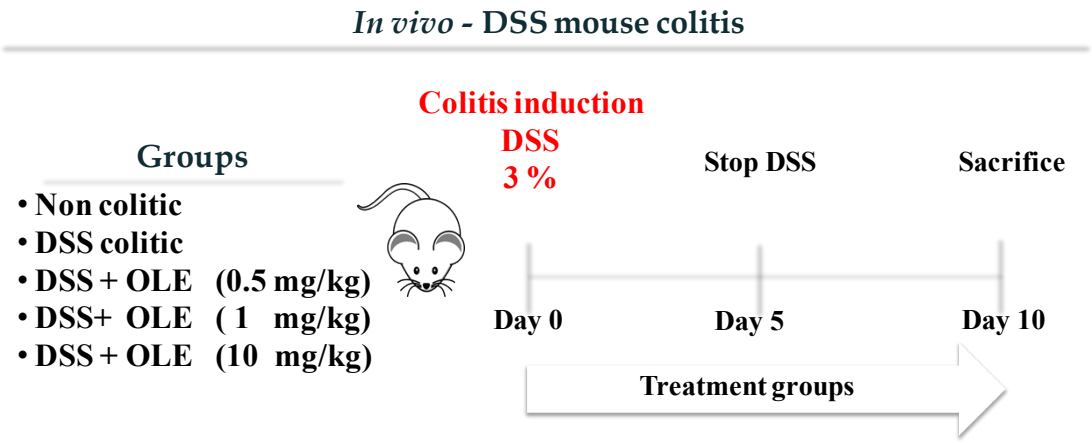


Figure 18. Experimental design in the DSS model of mouse colitis. DSS (Dextran sodium sulfate); OLE (Olive leaf extract).

Each parameter was assigned a score and used to calculate an average daily disease activity index (DAI) (Table 7).

Table 7. Disease activity index (DAI) score used to evaluate the DSS-induced colitis.

Score	Weight loss	Stool consistency	Rectal bleeding
0	None	Normal	Normal
1	1 - 5 %		
2	5- 10 %	Loose stools	
3	10 – 20 %		
4	> 20 %	Diarrhoea	Gross bleeding

Scoring of disease activity index (DAI).DAI value is the combined scores of weight loss, stool consistency, and rectal bleeding divided by 3. Adapted from Cooper *et al.*[500].

Once the animals were sacrificed, the colon was weighed and its length measured. Representative whole samples were taken from the distal inflamed area and were fixed in 4% buffered formaldehyde for histological studies. Comparable samples were

obtained from the non-colitic group. 3 intestinal explants (3 mm of diameter) were collected to measure cytokine production and the remaining colon was then minced, aliquoted and kept at -80°C.

6. *In vivo* intestinal permeability

Mice from the different experimental groups (n=7), which were fasted for 12 h and given DX-4000–FITC by oral gavage (350 mg/kg body weight). After 4 h, blood was collected by cardiac puncture, and centrifuged at 4°C, 3000 rpm for 10 min. Plasma was diluted (1:50) in PBS (pH 7.4) and analysed for DX-4000–FITC concentration with a fluorescence spectrophotometer (Fluorostart, BMG Labtechnologies, Offenburg, Germany) at an excitation wavelength of 485 nm and emission wavelength of 535 nm. Standard curves were obtained by diluting FITC–dextran in PBS [501].

7. Measurement of cytokine production in the intestine

The intestinal explants were incubated overnight at 37°C in 1 ml of RPMI culture medium containing 4.5 g/l glucose supplemented with 10% (v/v) foetal bovine serum, 1% penicillin/streptomycin, 1% amphotericin and 2% glutamine in a 5% CO2 atmosphere. Then, the medium was transferred to Eppendorf tubes and centrifuged at 4000 g for 10 min at 4°C and the supernatants were collected and kept at -80°C until levels of the cytokines IL-6 and TNF-α were determined by ELISA using kits from Peprotech (PeproTech EC Ltd., London, UK) following the manufacturer's instructions. The results are expressed as the concentration of cytokine (pg/ml).

8. Histological studies

Full-thickness intestinal cross-sections were embedded in paraffin and 5µm-thick sections were taken at different levels. They were stained with alcian blue and haematoxylin and eosin. An independent pathologist unaware of the experiment evaluated the mucin content and the histological damage according to the criteria previously described [502].

9. Diet-Induced Obesity

5-week-old C57BL/6J male mice (Janvier Labs, St Berthevin, Cedex, France) were housed in a temperature and humidity controlled facility (22 ± 1°C, 55 ± 10% relative humidity) with a 12-hour light/dark cycle. The mice were divided into several groups (*n*=9): control, control-treated, obese and obese-treated. Control mice received a normal chow diet (13% calories from fat, 20% calories from protein and 67% calories from carbohydrate) (Global diet 2014; Harlan Laboratories, Barcelona, Spain) whereas obese mice were fed a high-fat diet (HFD) in which 60% of its caloric content was derived from fat. The obese mice were daily administered the olive leaf extract at different doses (1, 10 and 25 mg/kg), dissolved in water, by oral gavage for 5 weeks (Figure 19). Animal body weight, food and water intake were controlled regularly during the treatment.

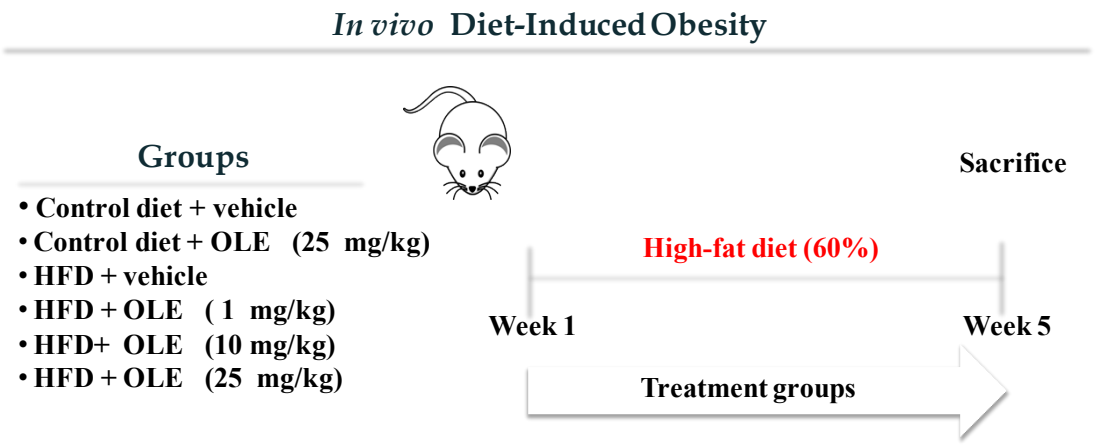


Figure 19. Experimental design in the mouse model of Diet-Induced Obesity . HFD (High-fat diet); OLE (Olive leaf extract).

10. Glucose tolerance test

One week before the sacrifice of the mice, a glucose tolerance test was performed on mice fasted for 18 h. They received a 50% glucose solution in water at a dose of 2 g/kg of body weight by intraperitoneal injection, and blood was collected from the tail vein at different time points: 0, 15, 30, 60 and 120 min after treatment.

11. Plasma determinations

At the end of the treatment, mice were sacrificed. Blood samples were collected in ice-cold tubes containing heparin and immediately centrifuged for 20 min at 5000 ***g*** at 4◦C, and the plasma frozen at −80 ◦C. Glucose, LDL (low-density lipoprotein)-cholesterol,

HDL (high-density lipoprotein)-cholesterol and total cholesterol concentrations were measured in the plasma by colorimetric methods using Spinreact kits (Spinreact, S.A., Girona, Spain). Plasma insulin concentrations were quantified using a mouse insulin ELISA kit (Alpco Diagnosis, Salem, NH, USA). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the formula: fasting glucose (mM)×fasting insulin (μ-units/ml)/22.5.

12. Morphological variables

Liver and adipose tissues were excised, cleaned, and weighed. Liver and fat weight indices were calculated by dividing the liver and fat weight by the tibia length. All tissue samples were frozen in liquid nitrogen and then stored at -80◦C.

13. Vascular reactivity studies

Descending thoracic aortic rings were dissected from animals and were suspended in a wire myograph (model 610M, Danish Myo Technology, Aarhus, Denmark) for isometric tension measurement as previously described [15]. The organ chamber was filled with Krebs solution (composition in mM: NaCl 118, KCl 4.75, NaHCO3 25, MgSO4 1.2, CaCl 2 2, KH 2PO4 1.2 and glucose 11) at 37 ºC and gassed with 95% O2 and 5% CO2 (pH ∼7.4). Length-tension characteristics were obtained via the myograph software (Myodaq 2.01) and the aortae were loaded to a tension of 5mN. After 90 min of stabilization period, cumulative concentration-response curves to acetylcholine (10-9 M-10-5 M) were performed in intact rings pre-contracted by U46619 (10-8 M). Relaxant responses to acetylcholine were expressed as a percentage of pre-contraction.

14. NADPH oxidase activity

The lucigenin-enhanced chemiluminescence assay was used to determine NADPH oxidase activity in intact aortic rings, as previously described (Zarzuelo *et al*., 2011). Aortic rings from all experimental groups were incubated for 30 minutes at 37 ºC in HEPES-containing physiological salt solution (pH 7.4) of the following composition (in mmol/L): NaCl 119, HEPES 20, KCl 4.6, MgSO4 1, Na2HPO4 0.15, KH2PO4 0.4, NaHCO3 1, CaCl2 1.2 and glucose 5.5. Aortic production of O2- was stimulated by addition of NADPH (100 μmol/L). Rings were then placed in tubes containing physiological salt solution, with or without NADPH and lucigenin was injected automatically at a final concentration of 5 μmol/L to avoid known artifacts when used a higher concentrations. NADPH oxidase activity were determined by measuring

luminescence over 200 s in a scintillation counter (Lumat LB 9507, Berthold, Germany) in 5-s intervals and was calculated by subtracting the basal values from those in the presence of NADPH. Vessels were then dried, and dry weight was determined. NADPH oxidase activity is expressed as relative luminescence units (RLU)/min/mg dry aortic ring.

15. Analysis of RNA transcripts by RT-qPCR

Total RNA from colonic samples, liver o fat was extracted using TRIzol® Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), following the manufacturer's recommendations, and was reverse transcribed using oligo(dT) primers (Promega, Southampton, UK). Real-time PCR amplification and detection were performed on optical-grade 48 well plates in an Eco™ Real time PCR System (Illumina, San Diego, CA, USA) with 20 ng of cDNA, the KAPA SYBR® FAST qPCR Master Mix (KapaBiosystems, Wilmington, MA, USA) and specific primers at their annealing temperature (Table 8). mRNA expression was normalized using the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal control. The mRNA relative quantitation was calculated using the ΔΔCt method.

Table 8. Primer sequences used in real-time qPCR assays

Gene	Organism	Sequence 5'-3'	Annealing T °C
GAPDH	Mouse	FW:CCATCACCATCTTCCAGGAG RV:CCTGCTTCACCACCTTCTTG	60
IL-1β	Mouse	FW: TGATGAGAATGACCTCTTCT RV: CTTCTTCAAAGATGAAGGAAA	60
IL-6	Mouse	FW: TAGTCCTTCCTACCCCAATTTCC RV: TTGGTCCTTAGCCACTCCTTCC	60
TNF-α	Mouse	FW: AACTAGTGGTGCCAGCCGAT RV: CTTCACAGAGCAATGACTCC	60
IL-17	Mouse	FW -CCTGGGTGAGCCGACAGAAGC RV -CCACTCCTGGAACCTAAGCAC	60
MIP-2	Mouse	FW: AGTTAGCCTTGCCTTTGTTCAG RV: CAGTGAGCTGCGCTGTCCAATG	57
ICAM-1	Mouse	FW:GAGGAGGTGAATGTATAAGTTATG RV:GGATGTGGAGGAGCAGAG	60
MCP-1	Mouse	FW: AGCCAACTCTCACTGAAG RV: TCTCCAGCCTACTCATTG	55
MUC-2	Mouse	FW: GCAGTCCTCAGTGGCACCTC RV: CACCGTGGGGCTACTGGAGAG	60
MUC-3	Mouse	FW: CGTGGTCAACTGCGAGAATGG RV: CGGCTCTATCTCTACGCTCTCC	60
iNOS	Mouse	FW:GTTGAAGACTGAGACTCTGG RV: ACTAGGCTACTCCGTGGA	67

COX-2	Mouse	FW: GGGTTGCTGGGGGAAGAAATG RV: GGTGGCTGTTTTGGTAGGCTG	60
TFF-3	Mouse	FW: CCTGGTTGCTGGGTCCTCTG RV:GCCACGGTTGTTACACTGCTC	60
ZO-1	Mouse	FW: GGGGCCTACACTGATCAAGA RV: TGGAGATGAGGCTTCTGCTT	56
JNK-1	Mouse	FW: GATTTTGGACTGGCGAGGACT RV: TAGCCCATGCCGAGAATGA	60
PPAR-a	Mouse	FW: AGGCTGTAAGGGCTTCTTTCG RV: GGCATTTGTTCCGGTTCTTC	62
PPAR-β	Mouse	FW: TAGGACTGGTGATCTGTGAG RV: TACAAGTGAGTGGGAGAGAG	60
Leptin	Mouse	FW: TTCACACACGCAGTCGGTAT RV: GCTGGTGAGGACCTGTTGAT	60
Leptin R	Mouse	FW: GCAGTCCTCAGTGGCACCTC RV: CACCGTGGGGCTACTGGAGAG	60
Adiponectin	Mouse	FW: GATGGCAGAGATGGCACTCC RV: CTTGCCAGTGCTGCCGTCAT	56
GLUT 4	Mouse	FW:GAGAATACAGCTAGGACCAGTG RV:TCTTATTGCAGCAGCGCCTGAG	62
AMPK	Mouse	FW:GACTTCCTTCACAGCCTCATC Rv:CGCGCGACTATCAAAGACATACG	60
LPL	Mouse	FW: TTCCAGCCAGGATGCAACA RV: GGTCCACGTCTCCGAGTCC	60
TLR-4	Mouse	FW: GCCTTTCAGGGAATTAAGCTCC RV: AGATCAACCGATGGACGTGTAA	60
NOX-1	Mouse	FW: TCTTGCTGGTTGACACTTGC RV: TATGGGAGTGGGAATCTTGG	50
NOX-4	Mouse	FW: ACAGTCCTGGCTTACCTTCG RV: TTCTGGGATCCTCATTCTGG	50
P47^{phox}	Mouse	FW: ATGACAGCCAGGTGAAGAAGC RV: CGATAGGTCTGAAGGCTGATGG	52
p22^{phox}	Mouse	FW: GCGGTGTGGACAGAAGTACC RV: CTTGGGTTTAGGCTCAATGG	50
TNF-a	Human	FW: AGGCGGTGCTTGTTCCTCAG RV: GGCTACAGGCTTGTCACTCG	62
IL-1β	Human	FW: AGAATGACCTGAGCACCTTC RV: GCACATAAGCCTCGTTATCC	58
IL-6	Human	FW: CCACTCACCTCTTCAGAACG RV: GCCTCTTTGCTGCTTTCACAC	61
IL-8	Human	FW:AGGAACCATCTCACTGTGTG RV:CCACTCTCAATCACTCTCAG	58

16. Analysis and isolation of adipocytes by Flow Cytometry

The cells from Visceral Adipose Tissue (VAT) were obtained following the procedure described by Anderson *et al*. with some little modifications (Anderson P. *et al*., 2015). The VAT was isolated from each mouse, washed and cut into small pieces (≤ 3 mm^3) using sterile scissors and scalpel blades. The minced fat tissue was resuspended in a HBSS solution containing 1 mg/ml collagenase Type I and the mixture was transfered

to a 50 ml tube and placed in a water bath at 37 °C for 30 min. The digested fat solution was then filtered using 100 μm- and 70 μm-cell strainers. The cells were then harvested and stained for different markers. 2×10^6 cells were counted and non-specific Fc-mediated interactions were blocked using FcγR blocking (Miltenyi Biotec, Bergisch Gladbach, Germany) before cell surface specific staining. Different specific antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) were combined for surface staining: Viability dye, anti-CD4 (PE, Clone REA 623), CD11b (APC-Vio 770,Clone REA592), CD11c (FITC, Clone N418), F4/80 (PercP-Vio700, Clone REA126), LyC6 (PE-Vio770, Clone 1G7.G10) in incubated for 15 min at 4°C in the dark. The cells were then fixed, permeabilized with the Fix/Perm Fixation/Permeabilization kit (eBioscience, San Diego, USA) and intracellular staining was made with mAbs anti- Foxp3 (APC, Clone 3G3, Miltenyi, Biotec, Bergisch Gladbach, Germany) for 30 min at 4°C in the dark. Samples were evaluated by flow cytometry using a FACS CANTO II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and the data analyzed using Flowjo™ software v.10 (FlowJo LLC, Ashland, OR, USA).

16. DNA extraction and 454/Roche pyrosequence analysis

DNA from faecal content was isolated using phenol: chloroform following a protocol modified from Sambrook & Russell (2001)[503]. To compare how 16S rRNA gene sequence recovery was affected by storage and purification methods, total DNA from stool samples was PCR amplified using primers targeting regions flanking the variable regions 1 through 3 of the bacterial 16S rRNA gene (V1-3), gel purified, and analysed using the Roche/454 GS Junior technology platform (Roche Diagnostics, Branford, CT, USA). The amplification of a 600-bp sequence in the variable region V1-V3 of the 16S rRNA gene was performed using barcoded primers. PCR was performed in a total volume of 15 μL for each sample containing the universal 27F and Bif16S-F forward primers (10 μmol/L) at a 9:1 ratio, respectively, and the barcoded universal reverse primer 534R (10 μmol/L) in addition to dNTP mix (10 mmol/L), FastStart 10× buffer with 18 mmol/L of MgCl2, FastStart HiFi polymerase (5 U in 1 mL), and 2 μL of genomic DNA. The dNTP mix, FastStart 10× buffer with MgCl2, and FastStart HiFi polymerase were included in a FastStart High Fidelity PCR System, dNTP Pack (Roche Applied Science, Penzberg, Germany). The PCR conditions were as follows: 95 °C for 2 min, 30 cycles of 95 °C for 20 s, 56 °C for 30 s, and 72 °C for 5 min, and final step at 4 °C. After PCR, amplicons were further purified using AMPure XP beads (Beckman-

Coulter, Brea, CA, USA) to remove smaller fragments. DNA concentration and quality were measured using a Quant-iTTM PicoGreen® dsDNA Assay Kit (Thermofisher, Waltham, MA, USA). Finally, the PCR amplicons were combined in equimolar ratios to create a DNA pool (109 DNA molecules) that was used for clonal amplification (emPCR) and pyrosequencing according to the manufacturer's instructions. After the sequencing was completed, all reads were scored for quality, and any poor quality and short reads were removed.

17. Taxonomic Analysis

Sequences were selected to estimate the total bacterial diversity of the DNA samples in a comparable manner and were trimmed to remove barcodes, primers, chimeras, plasmids, mitochondrial DNA and any non-16S bacterial reads and sequences <150 bp. MG-RAST (metagenomics analysis server) [504] with the Ribosomal Database Project (RDP) were used for analyses of all sequences [505]. The output file was further analysed using SPSS Statistics 17.0 Software Package (SPSS Inc.) and Statistical Analysis of Metagenomic Profiles (STAMP) software package version 2.1.3 [506, 507]. ANOVA test, Tukkey-Kramer (0.95) post-hoc and effect size Eto-squared was performed

18. Statistics

All results are expressed as the mean ± SEM. Differences between means were assessed for statistical significance using a one-way analysis of variance (ANOVA) and post hoc least significance tests. Non-parametric data (score) are expressed as the median (range) and were analysed using the Kruskal-Wallis test. All statistical analyses were performed with the GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA, USA), with statistical significance set at $P<0.05$.

RESULTS

1. Immunomodulatory properties of OLE in intestinal epithelial cells (Caco-2 and CMT-93), macrophages (RAW 264) and human PBMCs

Incubation of intestinal epithelial cells (Caco-2 and CMT-93) with increasing concentrations of OLE (0.1-100 μg/mL) did not increase production of IL-8 or IL-6 (Figures 20A and 20B). After incubation of both cell lines with IL-1β (24h) and LPS (72h), respectively, the concentration of these cytokines increased remarkably in comparison with untreated cells. Pre-treatment of such cells with OLE dose-dependently inhibited IL-1β/LPS-induced cytokine production (Figures 21A and 21B). No increase in the release of nitric oxide (NO) was seen in cultures of RAW 264 following 24 h treatment with OLE (Figure 20C).

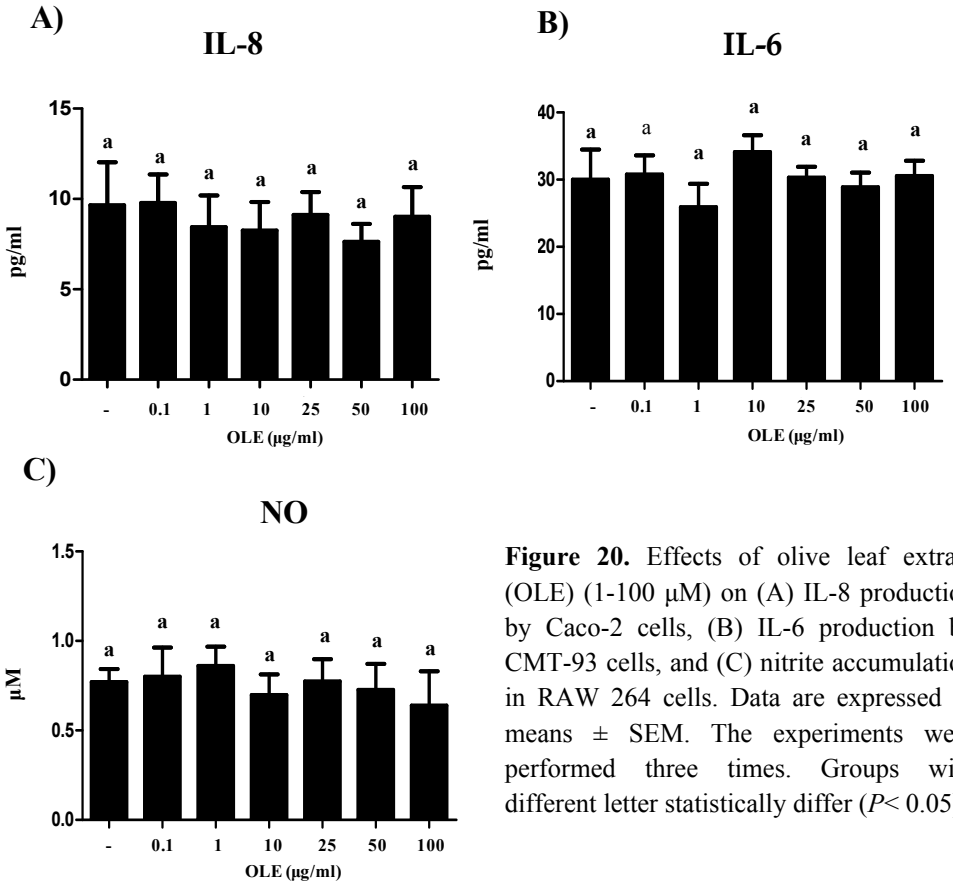


Figure 20. Effects of olive leaf extract (OLE) (1-100 μM) on (A) IL-8 production by Caco-2 cells, (B) IL-6 production by CMT-93 cells, and (C) nitrite accumulation in RAW 264 cells. Data are expressed as means ± SEM. The experiments were performed three times. Groups with different letter statistically differ ($P< 0.05$).

LPS incorporation into the culture media of these cells increased NO production and this was significantly and dose-dependently reduced by OLE (Figure 21C).

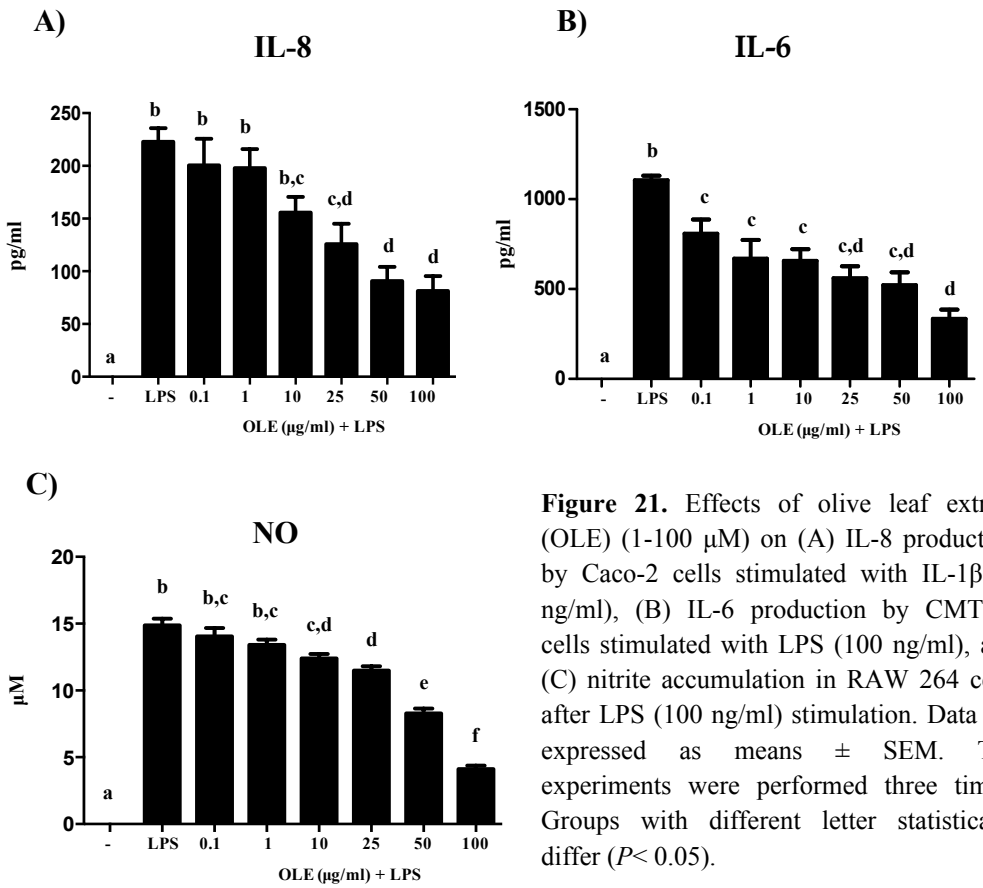


Figure 21. Effects of olive leaf extract (OLE) (1-100 µM) on (A) IL-8 production by Caco-2 cells stimulated with IL-1β (1 ng/ml), (B) IL-6 production by CMT-93 cells stimulated with LPS (100 ng/ml), and (C) nitrite accumulation in RAW 264 cells after LPS (100 ng/ml) stimulation. Data are expressed as means ± SEM. The experiments were performed three times. Groups with different letter statistically differ ($P< 0.05$).

Similarly, incubation of PBMCs from healthy donors and CD patients with different concentrations of OLE for 24 h affected neither cell viability nor production of TNF-α, IL-6 and IL-8. As expected, 24 hour-stimulation of PBMCs with LPS markedly increased production of the above cytokines (Figures 22 and 23).

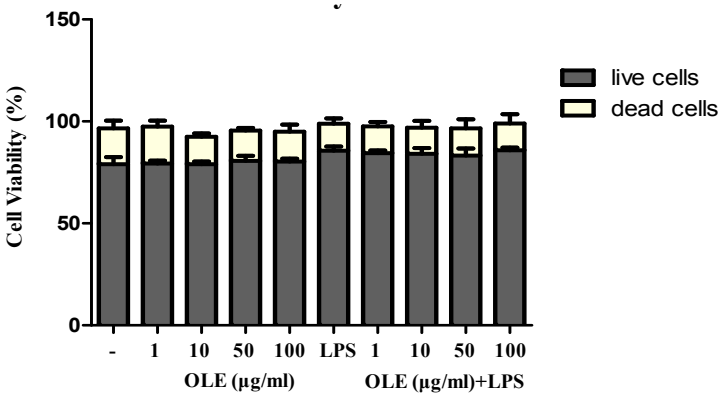


Figure 22: Effects of olive leaf extract (OLE) (1-100 µM) on PBMCs viability.

Pre-treatment of PBMCs with OLE for 2 h reduced LPS-driven TNF-α and IL-6 synthesis; such a reduction was statistically significant at the highest concentration (100

µg/mL) for IL-6 in PBMCs of both healthy donors and CD patients (Figures 23B and 23E)and for TNF-α in CD PBMCs (Figures 23A and 23D). Interestingly, the lowest concentrations tested (1 µg/mL) significantly reduced IL-8 production in PBMCs from both groups of donors (Figures 23C and 23F).

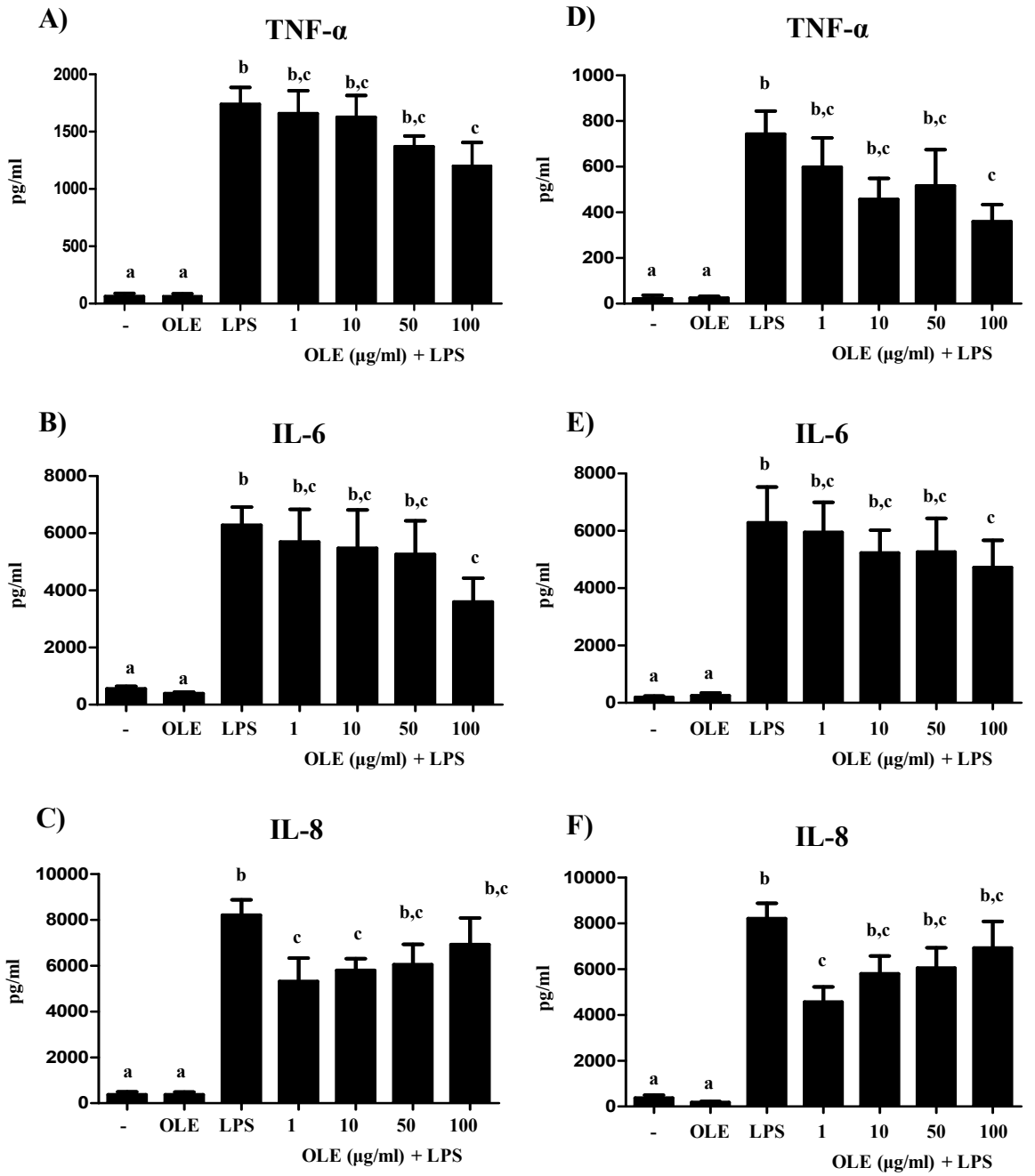


Figure 23. Effects of olive leaf extract (OLE) (1-100 µM) on (A;D) TNF-α, (B;E) IL-6 and (C;F) IL-8 production by peripheral blood mononuclear cells (PBMCs) stimulated with LPS (100 ng/ml) and isolated from (A;B;C) healthy donors and (D;E;F) Crohn's disease (CD) patients. Data are expressed as means ± SEM. The experiments were performed three times.Groups with different letter statistically differ ($P< 0.05$).

2. Effects of OLE in CD mucosal explants

To examine whether the olive leaf extract directly affects the production of cytokines by the intestinal mucosa, mucosal explants from CD patients were incubated with different concentrations of olive leaf extract, either in basal conditions or after LPS-stimulation. In basal conditions, the olive leaf extract did not affect the expression or production of the different cytokines studied: TNF-α, IL-1β, IL-6 and IL-8. However, when these explants were stimulated with LPS, which increased the expression and production of these cytokines, OLE was able to reduce IL-8 mRNA expression (Figure 24), as well as to significantly inhibit the production and/or release of IL-1β, IL-6 and IL-8, although no clear dose-effect relationship could be established (Figure 25).

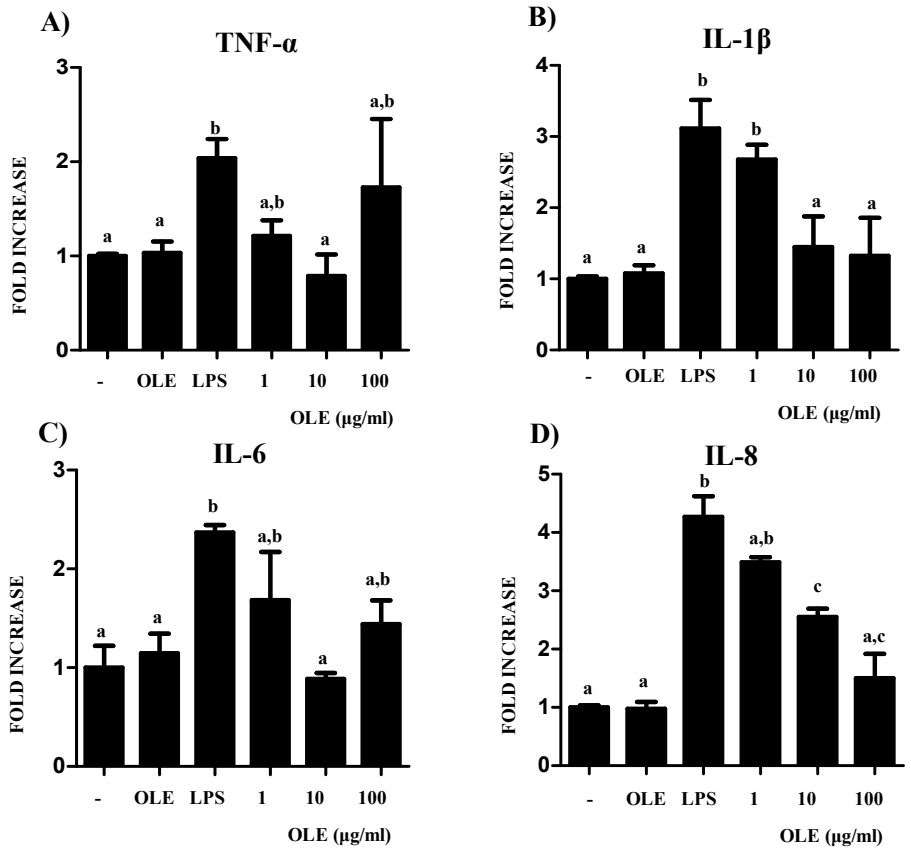


Figure 24. Effects of olive leaf extract (OLE) (1-100 µM) on (A) TNF-α, (B) IL-1β, (C) IL-6 and (D) IL-8 gene expression levels in mucosal explants from Crohn's disease (CD) patients after LPS-stimulation (100 ng/ml). Data are presented as fold-change compared to untreated colon fragments. Statistical differences were assessed by one-way ANOVA. Groups with different letter statistically differ ($P< 0.05$).

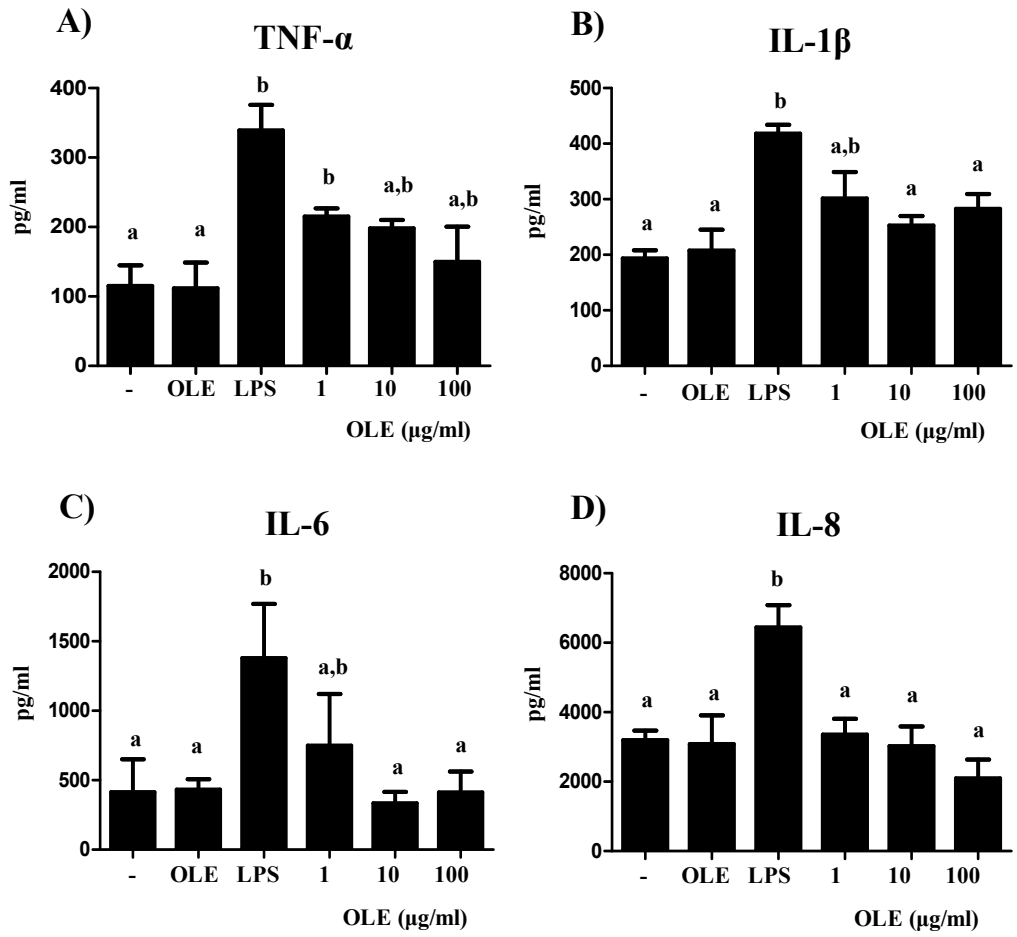


Figure 25. Effects of olive leaf extract (OLE) (1-100 µM) on (A) TNF-α, (B) IL-1β, (C) IL-6 and (D) IL-8 production by explants cultures of colonic mucosa from Crohn's disease (CD) patients, after LPS-stimulation (100 ng/ml). Data are presented as mean ± SEM. Groups with different letter statistically differ ($P< 0.05$).

3. Intestinal anti-inflammatory effect of olive leaf extract in DNBS mouse colitis

The intrarectal administration of DNBS to mice induced a colonic inflammatory status evidenced by a reduction in body weight, in contrast with the increase observed in non-colitic mice (Figure 26). The macroscopic evaluation of the colonic tissue from control colitic mice revealed the presence of inflamed and necrosed tissue, typically extending 1–2 cm along the large intestine. In addition, hyperaemia and focal adhesions to adjacent organs were also observed in most of the mice from this group.Although the administration of OLE (1–25 mg/kg) did not significantly affected body weight evolution in colitic mice, it was able to partially ameliorate the severity of the DNBS-induced colonic damage. As expected, the intestinal inflammatory process induced by DNBS was characterized by an altered expression of the different colonic markers evaluated. Thus, there was an up-regulated expression of the pro-inflammatory

cytokines IL-1β, TNF-α, IL-17 and IL-6, the chemokine macrophage inflammatory protein-2 (MIP-2) and the intercellular adhesion molecule 1 (ICAM-1) in control colitic mice in comparison with non-colitic mice. Similarly, the colonic inflammatory status was characterized by increased colonic expression of the inducible enzymes inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2. On the contrary, the expression of those proteins involved in colonic epithelial integrity (MUC-2, ZO-1 and TFF-3) was reduced in colitic mice (Figures 27-29).

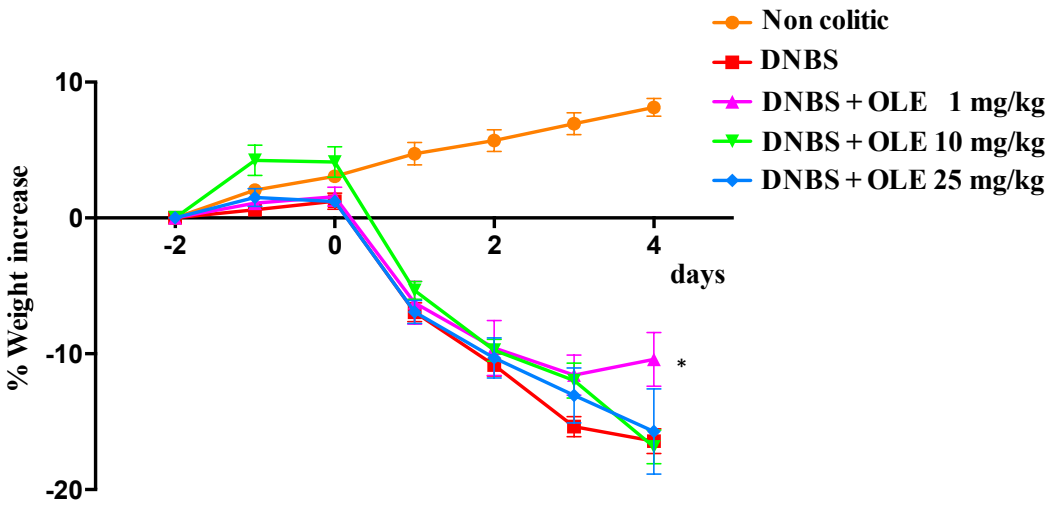


Figure 26. Effects of olive leaf extract (OLE) (1–25 mg/kg) on weight evolution in DNBS mouse colitis. Data are expressed as means ± SEM. All colitic groups significantly differ from the healthy group ($P< 0.05$). *$P<0.05$ vs DNBS colitic control group.

The treatment with OLE improved the expression of most of the proteins assayed. In fact, all the doses assayed were able to significantly reduce the expression of IL-1β, IL-17, MIP-2 and iNOS (Figures 27 and 28).

When the other markers were considered, some doses showed beneficial effects, being the lower doses more efficient. Both 1 and 10 mg/kg were able to significantly reduce the expression of ICAM-1, the dose of 10 mg/kg significantly diminished the expression of TNF-α, whereas only the dose of 1 mg/kg had a positive impact on the expression of COX-2, as well as on those proteins involved in epithelial integrity, like MUC-2, TFF-3 and ZO-1 (Figures 27-29).

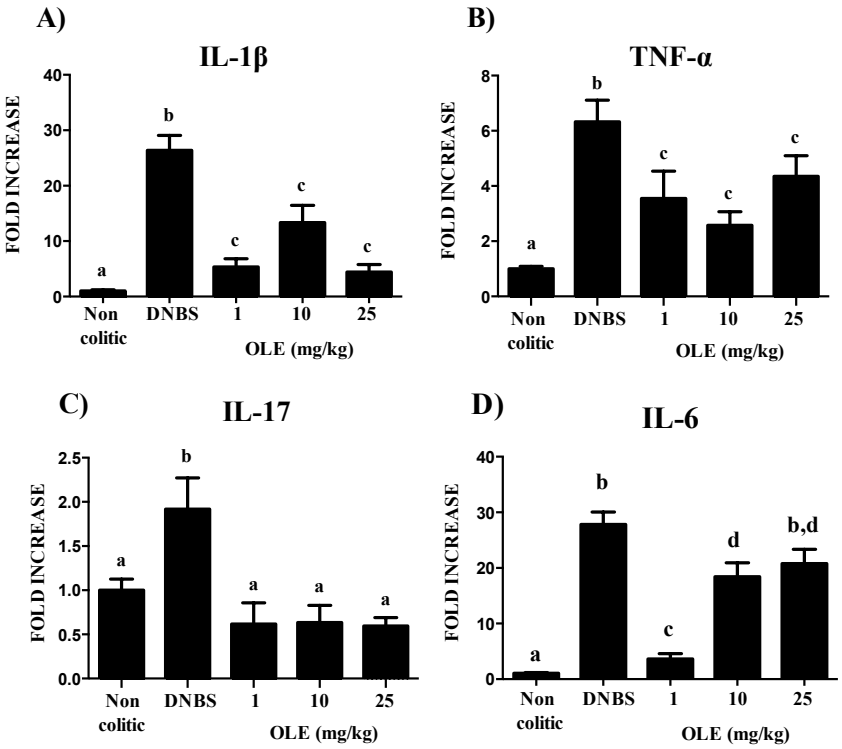


Figure 27. Effects of olive leaf extract (OLE) (1–25 mg/kg) on colonic gene expression of (A) IL-1β, (B) TNF-α, (C) IL-17 and (D) IL-6 in DNBS mouse colitis, analyzed by real-time qPCR. Data are expressed as means ± SEM (n=10). Groups with different letter statistically differ ($P< 0.05$)

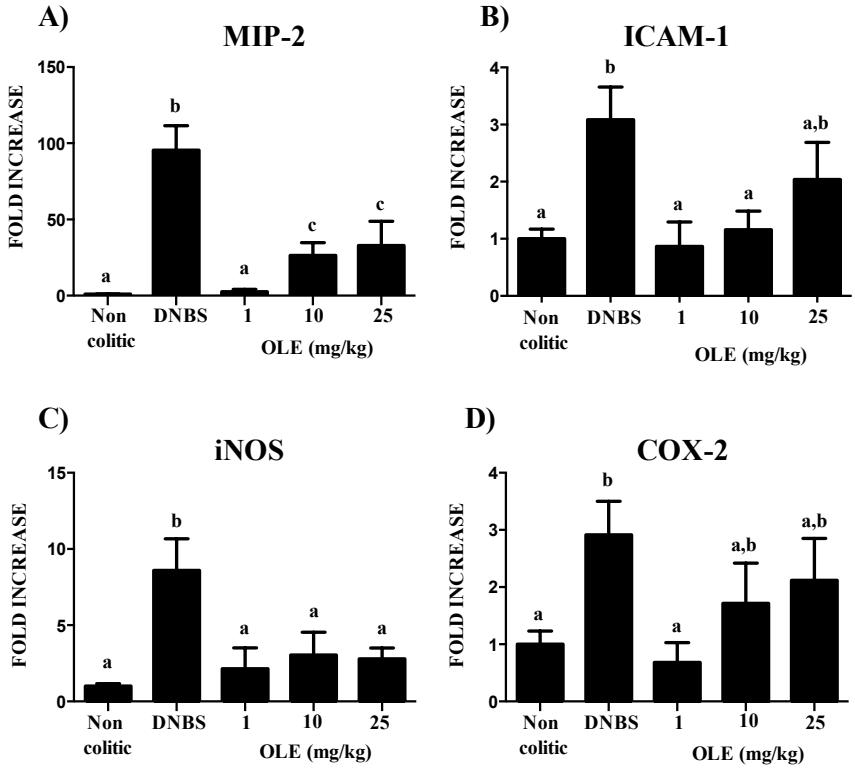


Figure 28. Effects of olive leaf extract (OLE) (1–25 mg/kg) on colonic gene expression of (A) MIP-2, (B) ICAM-1, (C) iNOS and (D) COX-2 in DNBS mouse colitis, analyzed by real-time qPCR. Data are expressed as means ± SEM (n=10). Groups with different letter statistically differ ($P< 0.05$)

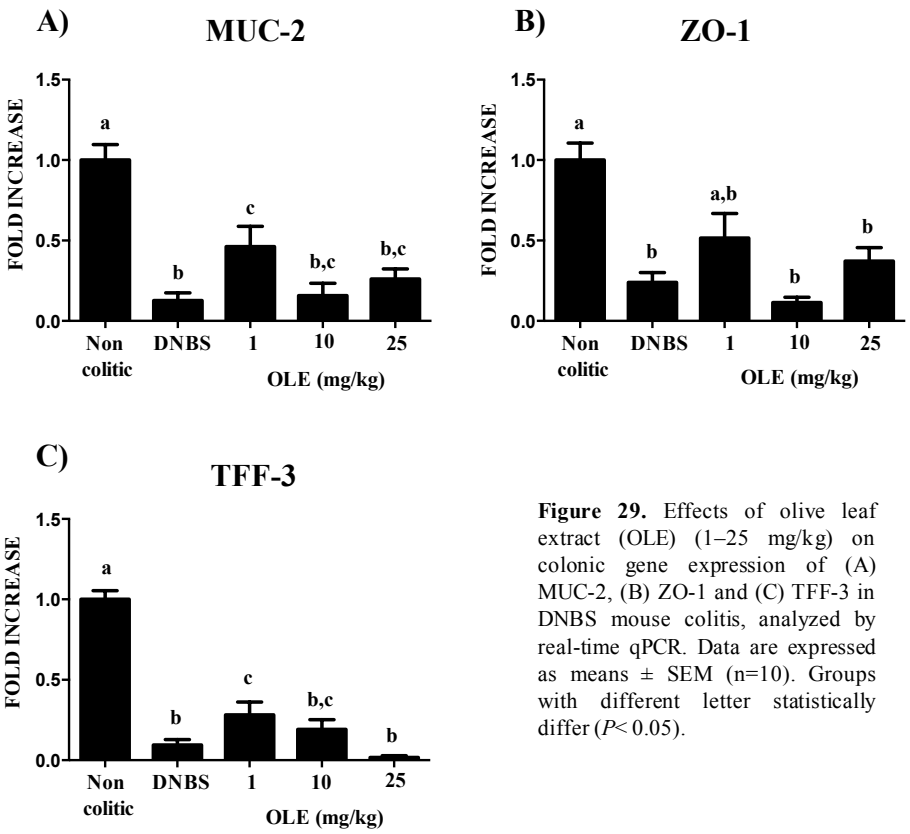


Figure 29. Effects of olive leaf extract (OLE) (1–25 mg/kg) on colonic gene expression of (A) MUC-2, (B) ZO-1 and (C) TFF-3 in DNBS mouse colitis, analyzed by real-time qPCR. Data are expressed as means ± SEM (n=10). Groups with different letter statistically differ ($P< 0.05$).

3. Intestinal anti-inflammatory effect of OLE in DSS mouse colitis

To confirm the anti-inflammatory effects observed with OLE in the DNBS model of colitis, we evaluated it in DSS induced colitis in mice. In these experiments a curative treatment protocol was followed, since the administration of the extract started once the colitis had been induced and was maintained for 5 days. Based on the results obtained in the DNBS model, the lower doses of olive leaf extract were selected for this assay (1 and 10 mg/kg), anda lower one,0.5 mg/kg, was added.

DSS intake induced a colonic inflammatory condition in the mice, which was evidenced by body weight loss and production of diarrheic faeces, frequently associated with the presence of blood. In consequence, DAI values in colitic mice increased daily, achieving the maximum value six days after the beginning of the experiment in the untreated control group (Figure 30).

The administration of the extract ameliorated DAI evolution, which was evident during the two first days of treatment as well as the last day, when all treated groups showed a reduced DAI in comparison with control group, thus revealing the improvement of the colonic inflammatory status induced by DSS (Figure 30).

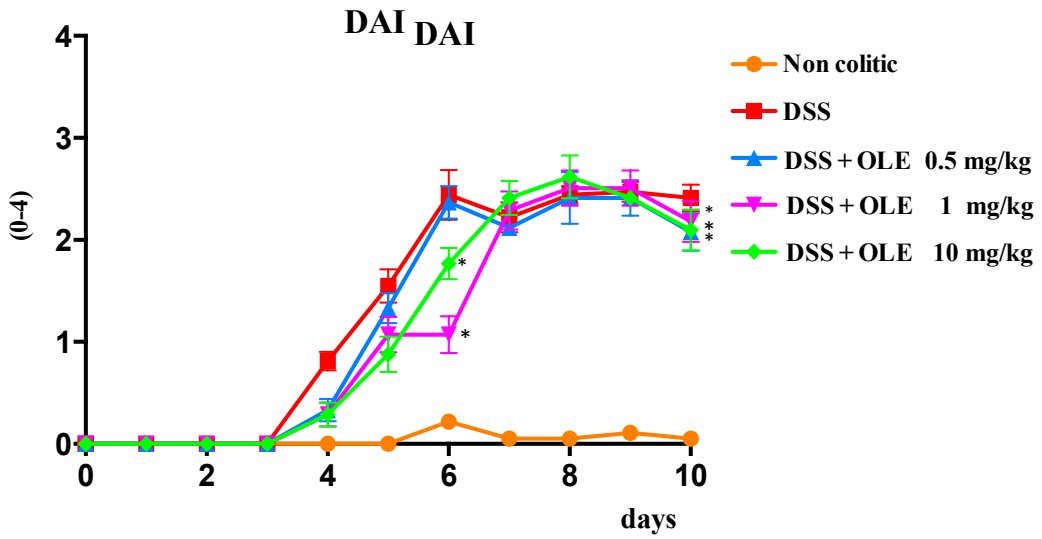


Figure 30. Effects of olive leaf extract (OLE) (0.5–10 mg/kg) on disease activity index (DAI) values in DSS colitic mice over the experimental period. Data are expressed as means s ± SEM (n=10). All colitic groups significantly differ from the non-colitic group ($P< 0.05$).

OLE enhanced gut functionality by ameliorating intestinal permeability, which was assayed *in vivo* using FITC-dextran. FITC-dextran plasma levels in the control colitic mice were significantly higher than those in non-colitic mice, which is in accordance with the alteration of the epithelial barrier function induced by DSS. However, OLE treated mice reduced significantly FITC-dextran levels ($P< 0.05$) (Figure 31A).The histological evaluation of the colonic samples confirmed the epithelial regeneration and the intestinal anti-inflammatory effects of the different doses of DXC in this model of mouse colitis (Figure 31B). Microscopically, DSS-induced colitis was characterized by epithelial ulceration (typically affecting more than 75% of the surface), intense goblet cell depletion, severe inflammatory cell infiltration in all the colonic layers and oedema between the mucosa and muscularis layers of the intestine.

An average microscopic score of 28.5 ± 3.9 was assigned to control colitic mice (Figure 31B). In contrast, the samples from the mice treated with the different doses of olive leaf extract showed a significant recovery of the inflammatory process in a doses dependent manner. The evaluation of the colonic damage in the OLE-treated groups resulted in a reduced microscopic score in comparison with the untreated control group (Figure 31B).

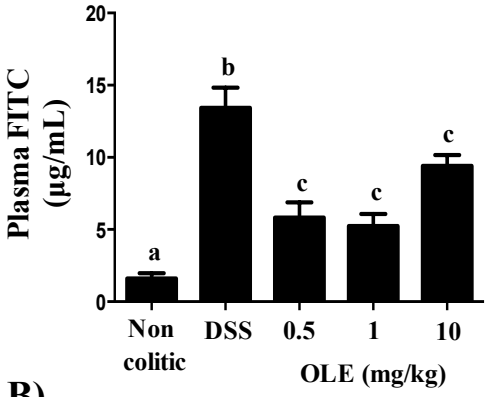
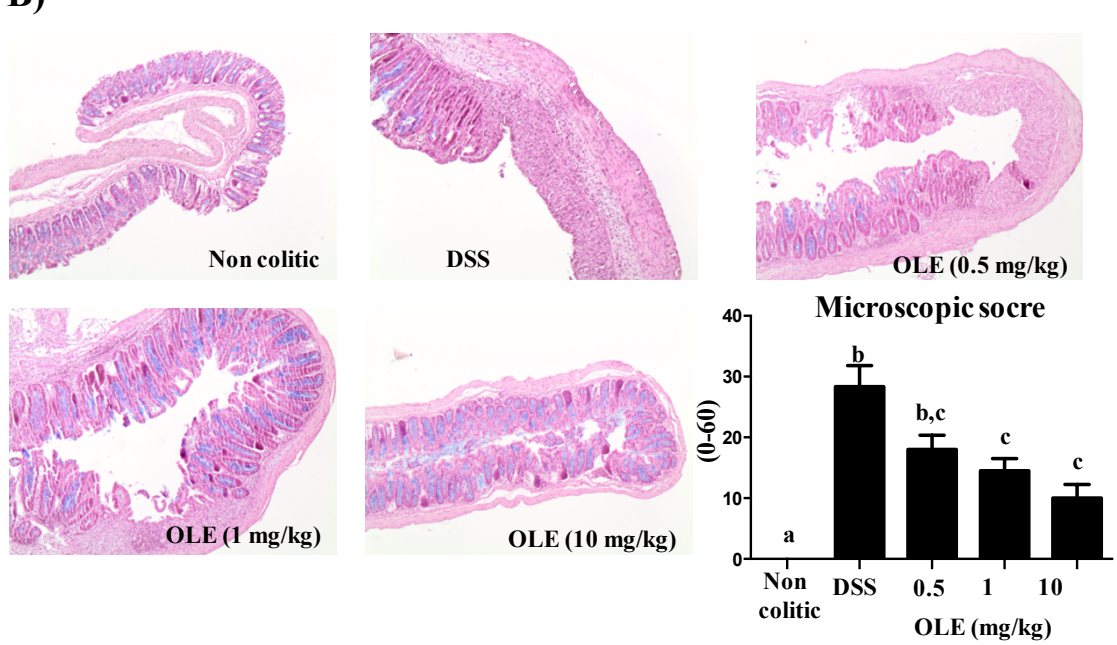


Figure 31. Effects of olive leaf extract (OLE) (0.5–10 mg/kg) in DSS mice colitis on: (A) Intestinal permeability measurement by the FITC-dextran assay *in vivo*; (B) histological sections of colonic tissue stained with hematoxylin and eosin and alcian blue. Data are expressed as means ± SEM (n = 7–10). Groups with different letter statistically differ ($p < 0.05$).



The biochemical analysis of the colonic segments confirmed the beneficial effects exerted by the different doses of the OLE assayed. As expected, DSS-induced colonic inflammation was characterized by an altered immune response, which was evidenced by an increased mRNA expression of the pro-inflammatory cytokines IL-1β, TNF-α, IL-6 and IL-17 in comparison with non-colitic mice (Figure 32).

The administration of OLE reduced the colonic expression of these cytokines, being significant at all doses assayed for IL-1β, and only for 10 mg/kg when IL-6 was considered. In addition, the extract was able to reduce the production of the same cytokines in comparison with non-colitic mice (Figure 32). Both 1 and 10 mg/kg were able to significantly reduce the expression of ICAM-1.

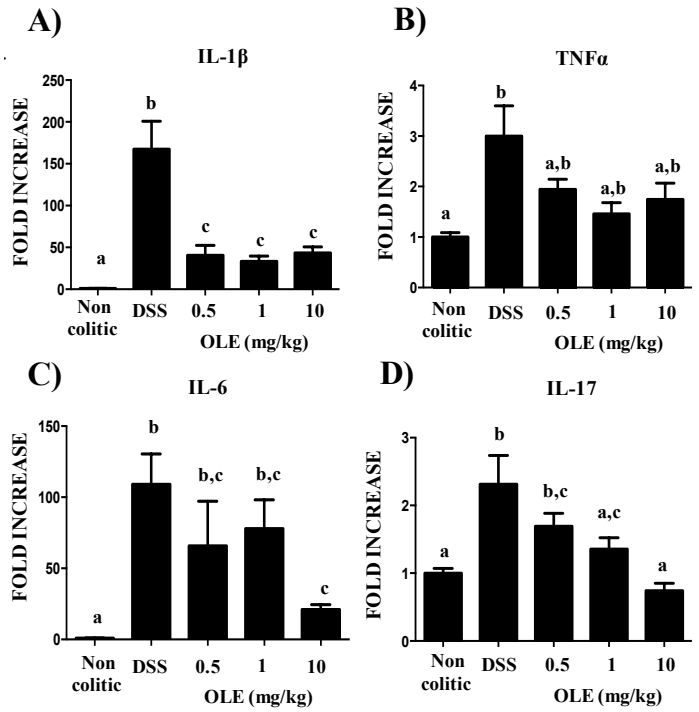


Figure 32. Effects of olive leaf extract (OLE) (0.5–10 mg/kg) on colonic gene expression of (A) IL-1β, (B) TNF-α, (C) IL-6, (D) IL-17 in DSS mouse colitis, analyzed by real-time qPCR. Data are expressed as means ± SEM (n=10). Groups with different letter statistically differ ($P< 0.05$).

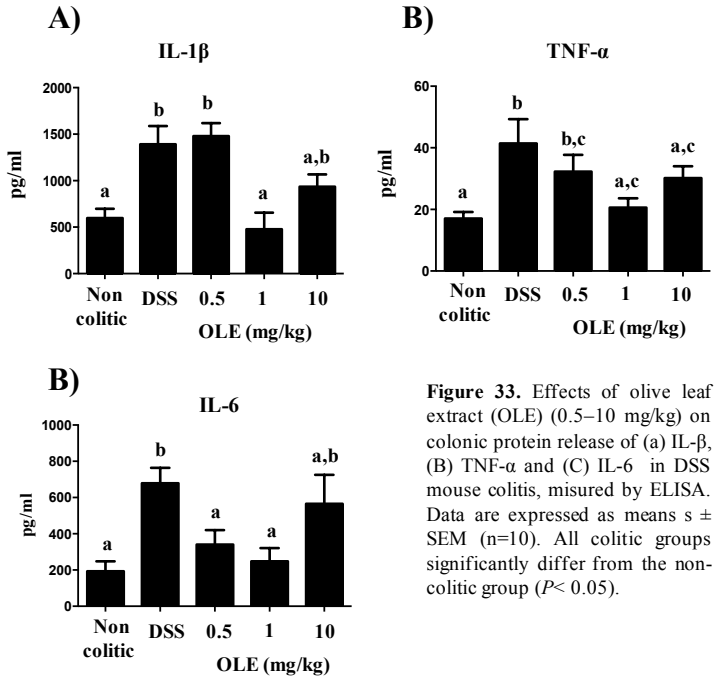


Figure 33. Effects of olive leaf extract (OLE) (0.5–10 mg/kg) on colonic protein release of (a) IL-β, (B) TNF-α and (C) IL-6 in DSS mouse colitis, misured by ELISA. Data are expressed as means s ± SEM (n=10). All colitic groups significantly differ from the non-colitic group ($P< 0.05$).

This anti-inflammatory effect was corroborated at protein level when IL-1β, TNF-α and IL-6 cytokine release was measured in colonic culture explants (Figure 33).

Similarly, and in comparison with non-colitic mice, colitic mice showed increased expression of the chemokine MIP-2, the adhesion molecule ICAM-1, as well as the inducible enzymes iNOS and COX-2. The administration of the extract to colitic mice

resulted in a significant amelioration in all these proteins, in accordance with its intestinal anti-inflammatory effect (Figure 34).

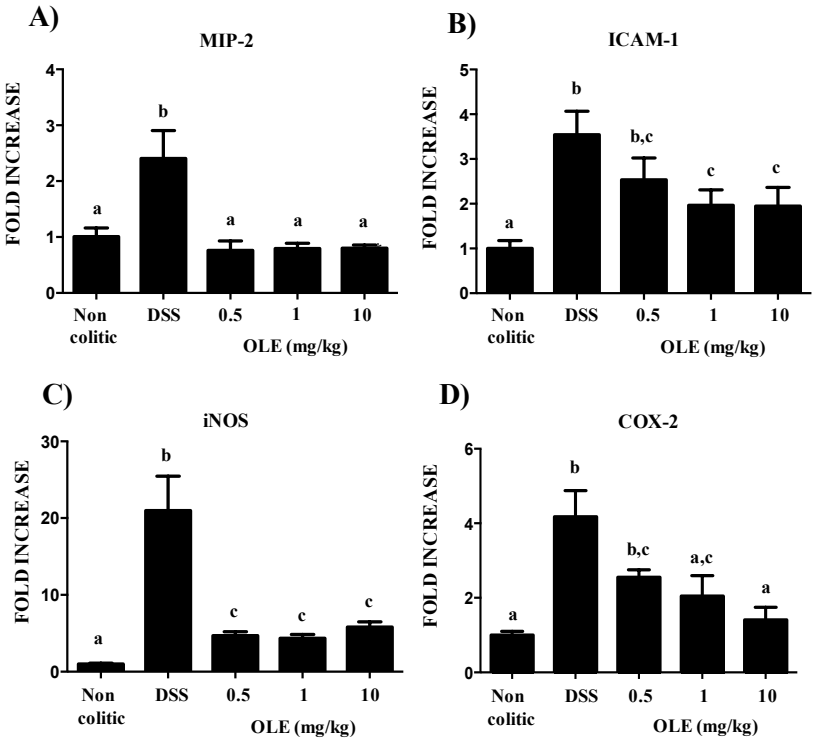


Figure 34. Effects of olive leaf extract (OLE) (0.5-10 mg/kg) on colonic gene expression of (A) MIP-2, (B) ICAM-1, (C) iNOS and (D) COX-2 in DSS mouse colitis, analyzed by real-time qPCR. Data are expressed as means ± SEM (n=10). Groups with different letter statistically differ ($P< 0.05$).

Finally, the colonic inflammation was also associated with impairment of the epithelial integrity, as revealed by the reduced expression of the mucin MUC-2 and the protein ZO-1, being the latter significantly up-regulated in those colitic mice treated with olive leaf extract at doses of 1 and 10 mg/kg (Figure 35).

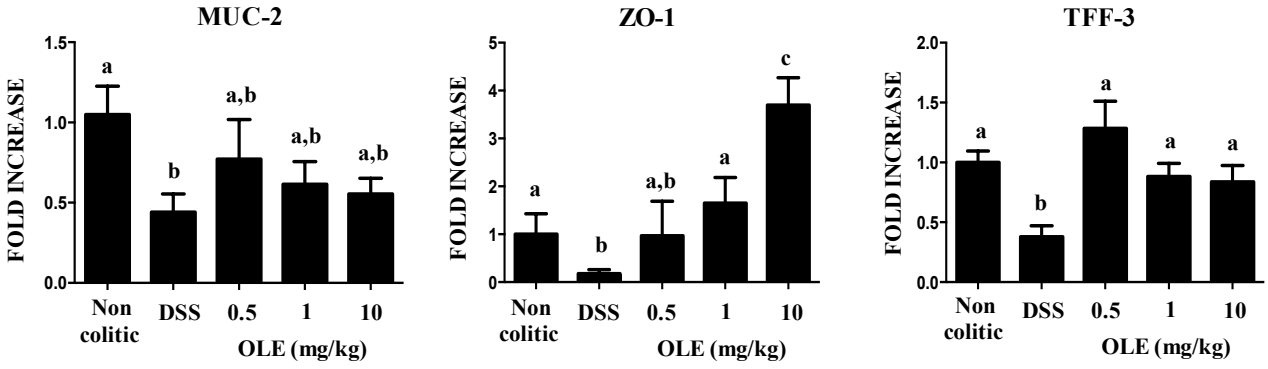


Figure 35. Effects of olive leaf extract (OLE) (1–25 mg/kg) on colonic gene expression of (A) MUC-2, (B) ZO-1 and (C) TFF-3 in DSS mouse colitis, analyzed by real-time qPCR. Data are expressed as means ± SEM (n=10). Groups with different letter statistically differ ($P< 0.05$).

4. Effects of OLE on body weight, glucose tolerance test and plasma biochemical profile.

Body weight changes of mice fed a control diet or high-fat diet (HFD) were estimated twice a week during the treatment period. As expected, after 5 weeks, the average body weight of untreated HFD-fed mice was found to be considerably higher when compared with the control diet groups (Figure 36A).

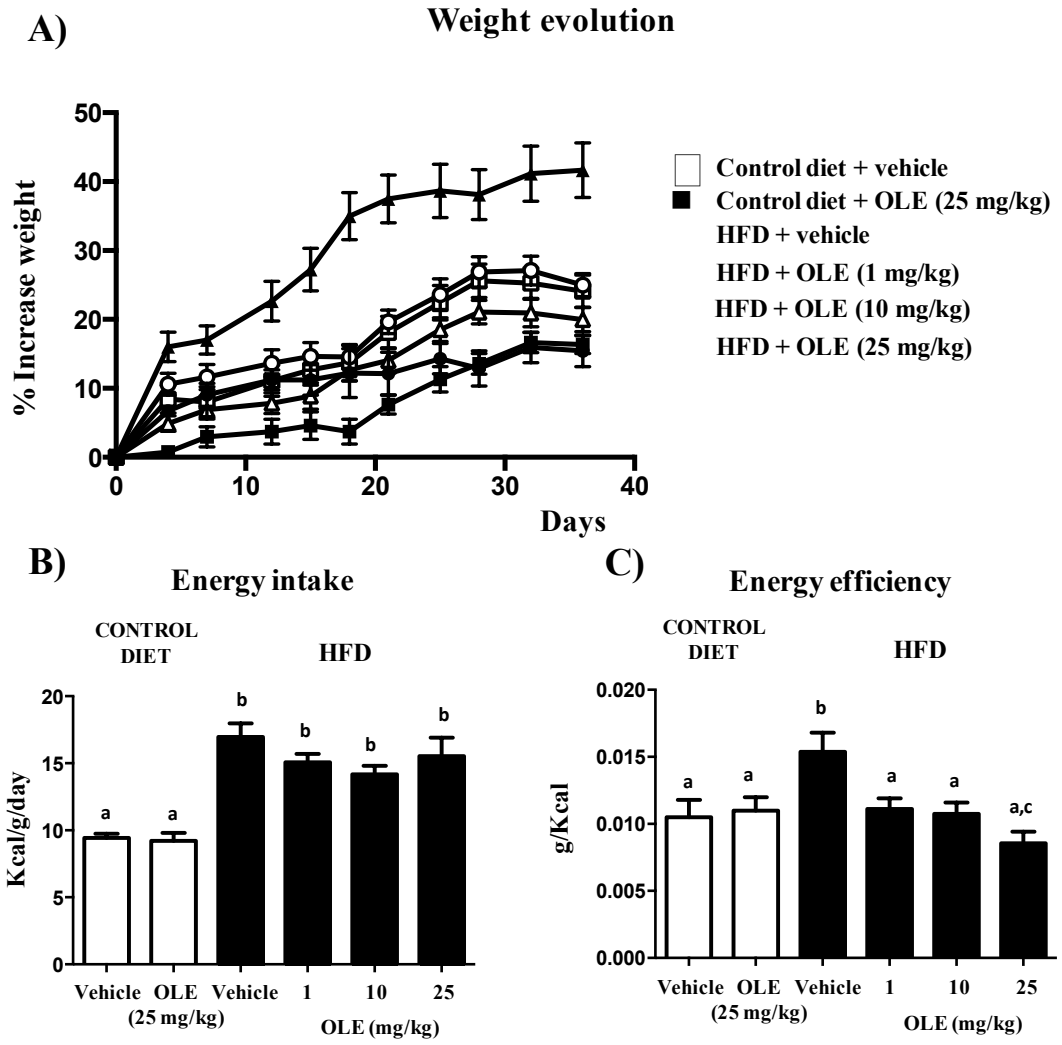


Figure 36. Effects of olive leaf extract (OLE) administration on the morphological changes.(**A**) Body weight evolution, (**B**) energy intake,(**C**) energy efficiency in control andhigh fat diet (HFD)-fed mice. Data are expressed as means ± SEM (n=10). Groups with different letter statistically differ ($P< 0.05$).

The administration of OLE to HFD-fed mice significantly reduced this weight gain, although no significant differences in energy intake were observed among these groups (Figure 36B). Moreover, the treatments were able to significantly decrease the ratio weight gain/energy intake in comparison with untreated HFD-fed mice, thus suggesting

that the effects of OLE on weight gain were probably related to a reduction in energy efficiency (Figure 36C).

The glucose tolerance test was performed one week before mice sacrifice. The plasma glucose levels of all groups reached a peak at 15 minutes after the administration of glucose (2 g/Kg, i.p.) and gradually decreased to the pre-prandial levels (Figure 16A).

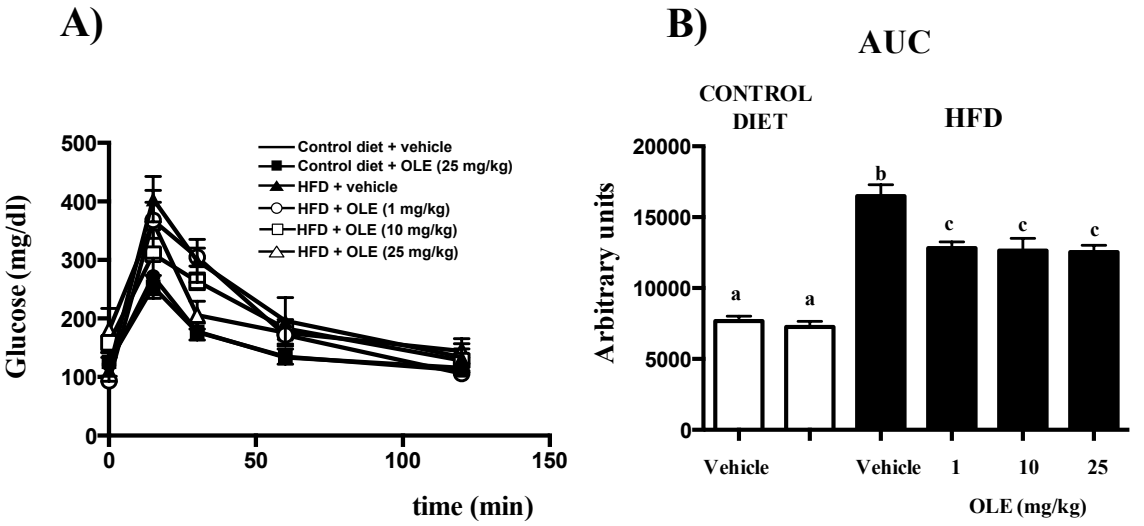


Figure 37. (A) Glucose tolerance test and (B) area under the curve (AUC) in control andhigh fat diet (HFD)-fed mice. Data are expressed as means ± SEM (n=10). Groups with different letter statistically differ ($P< 0.05$).

As expected, the glucose level peaks in HFD-fed mice were significantly higher than those obtained for control diet groups. Interestingly, the treatment with OLE reduced the plasma glucose levels compared to the HFD control group from 30 min onwards, which resulted in significant decreases in the area under the curve (AUC), with similar values in all treated groups, although these were still higher than in those groups that received the control diet (Figure 37B).

When glucose homeostasis was evaluated, the results revealed that fasting glycaemia was significantly increased in HFD-fed untreated mice, being this increase prevented by the highest dose of OLE. Similarly, fasting plasma insulin was also increased in obese mice from the untreated group in comparison with non-obese mice, and this was significantly reduced by OLE, at all doses assayed, although there were significant differences with control diet fed mice (Figures 38A and 38B).

Moreover, OLE improved insulin sensitivity in HFD-fed mice animals as evidenced by the lower HOMA-IR index values compared with their vehicle-treated controls,

obtaining at the highest dose similar values to those obtained with lean mice (Figure 38C).

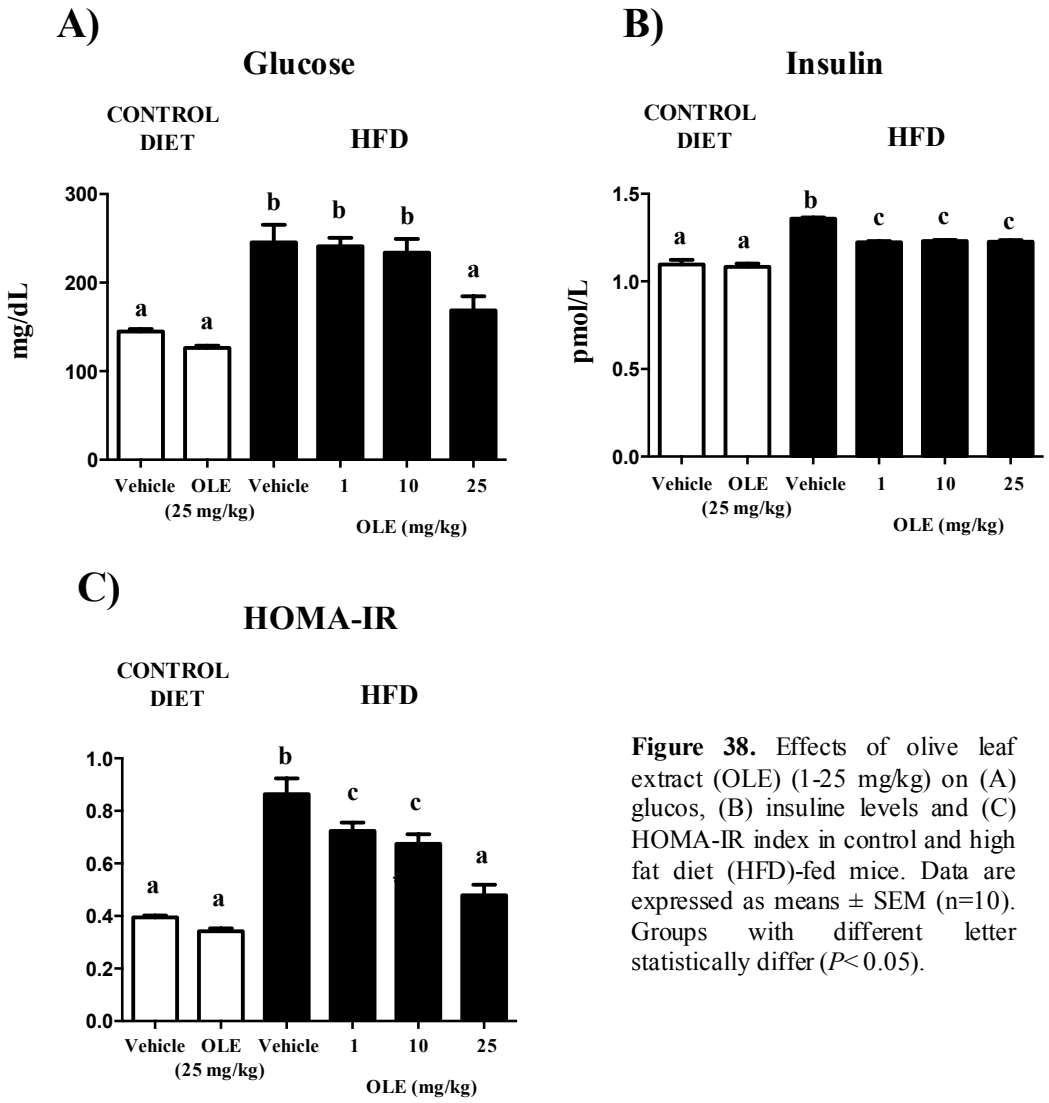


Figure 38. Effects of olive leaf extract (OLE) (1-25 mg/kg) on (A) glucos, (B) insuline levels and (C) HOMA-IR index in control and high fat diet (HFD)-fed mice. Data are expressed as means ± SEM (n=10). Groups with different letter statistically differ ($P< 0.05$).

Consistent with body weight data, the adipose tissue mass was significantly increased in the untreated HFD-fed mice in comparison with mice fed with the control diet. All the doses of OLE significantly reduced this fat content, although there were still significant differences when compared with control diet fed mice (Figure 39).

Obesity was also associated with altered plasma cholesterol profile, since the HFD control mice had hypercholesterolemia, associated with higher levels of both low-density lipoprotein (LDL)-cholesterol and high-density lipoprotein (HDL)-cholesterol.

The administration of OLE to obese mice significantly ameliorated LDL-cholesterol, but only exhibited a trend to decrease HDL-cholesterol, since no statistical differences were obtained with lean and obese control groups (Figure 40).

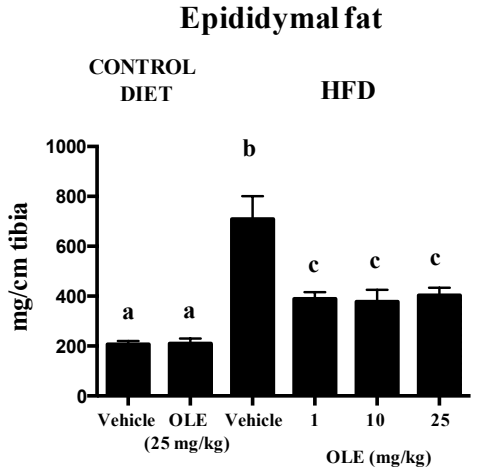


Figure 39. Effects of olive leaf extarct on adipose tissue mass in control and high fat diet (HFD)-fed mice. Data are expressed as means ± SEM (n=10). Groups with different letter statistically differ ($P< 0.05$).

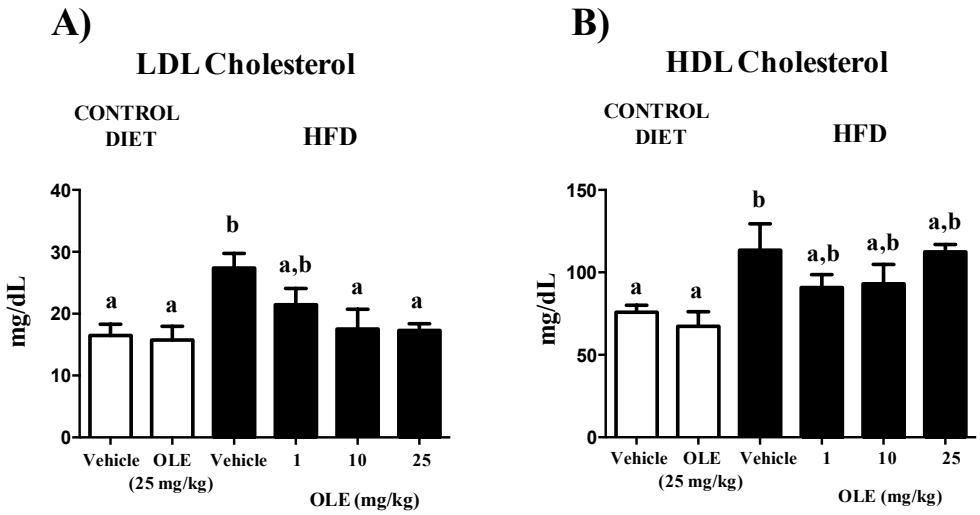


Figure 40. Effects of olive leaf extract (OLE) (1–25 mg/kg) on (A) LDL Cholesterol and (B) HDL Cholesterola plasma levelsin control andhigh fat diet (HFD)-fed mice. Data are expressed as means ± SEM (n=10). Groups with different letter statistically differ ($P< 0.05$).

5. Effects of OLEon hepatic and fat inflammation

In addition to the metabolic abnormalities, obesity has been reported to be associated with systemic inflammation, involving both liver and fat tissue [17]. Accordingly, HFD fed mice showed increased mRNA expression of the pro-inflammatory cytokines TNFα, IL-1β and IL-6 in fat and liver (Figures 41 and 42).

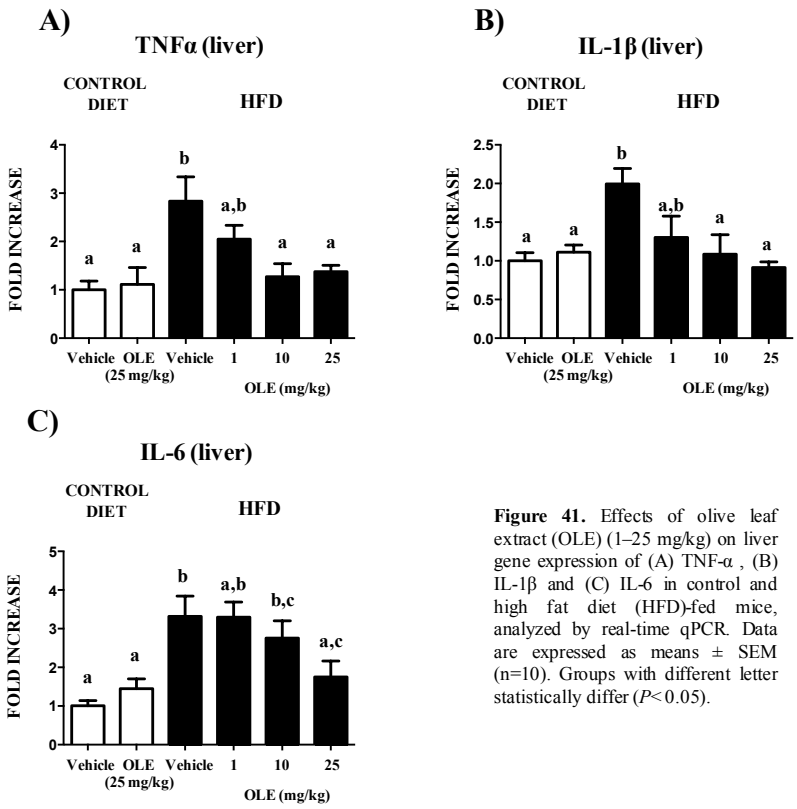


Figure 41. Effects of olive leaf extract (OLE) (1–25 mg/kg) on liver gene expression of (A) TNF-α , (B) IL-1β and (C) IL-6 in control and high fat diet (HFD)-fed mice, analyzed by real-time qPCR. Data are expressed as means ± SEM (n=10). Groups with different letter statistically differ ($P< 0.05$).

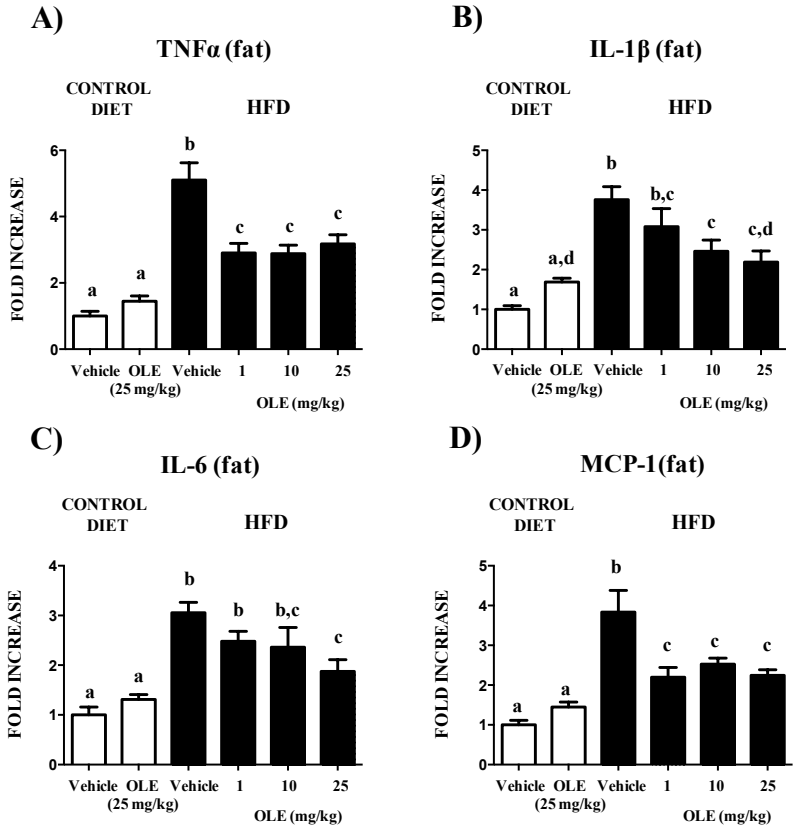


Figure 42. Effects of olive leaf extract (OLE) (1–25 mg/kg) on fat gene expression of (A) TNF-α , (B)IL-1β, (C) IL-6 and (D) MCP-1 in control and high fat diet (HFD)-fed mice, analyzed by real-time qPCR. Data are expressed as means ± SEM (n=10). Groups with different letter statistically differ ($P< 0.05$).

Moreover, in the adipose tissue there was an increased expression of the chemokine monocyte chemotactic protein-1 (Figure 42D), which would facilitate macrophage infiltration and activation, and the subsequent instauration of the inflammatory status. All these obesity-associated inflammatory markers were significantly ameliorated in those HFD-fed mice treated with OLE, mostly displaying a dose-dependent response (Figures 41 and 42).

On the other hand, investigations upstream of inflammatory cytokine expression identified the kinases c-jun N-terminal kinase (JNK) as major intracellular contributors to the induction of inflammation in metabolic tissues [508]. In fact, compared with lean controls, livers from untreated obese mice exhibited a significantincreased expression of JNK-1 that was reduced by OLE administration (Figure 43).

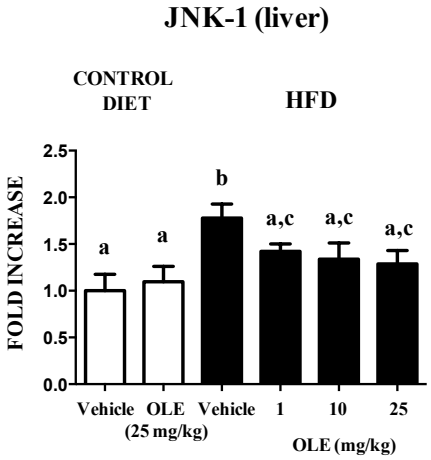


Figure 43. Effects of olive leaf extract (OLE) (1–25 mg/kg) on liver gene expression of JNK-1 in control and high fat diet (HFD)-fed mice, analyzed by real-time qPCR. Data are expressed as means ± SEM (n=10). Groups with different letter statistically differ ($P < 0.05$).

In addition, gene expressions of PPARs (PPARα and PPARγ), transcription factors that have been related to the glucose and lipid metabolism of both liver and adipose depots [509], were analysed to explore the mechanisms by which OLE improves the metabolic disorders in HFD-fed mice. As shown in Figure 44, the mRNA levels of PPARγ were significantly reduced in both tissues in HFD-mice control group compared with the control lean group; however, PPARα expression was increased in the liver, whereas it was reduced in adipose tissue. The administration of OLE to obese mice was able to restore the expression of both PPARs in the liver at the highest dose. When the adipose tissue was considered, OLE significantly increased the expression of PPARα and PPARγ at doses of 10 and 25 mg/kg, but there were still statistical differences with the untreated mice that received the control diet (Figure 44).

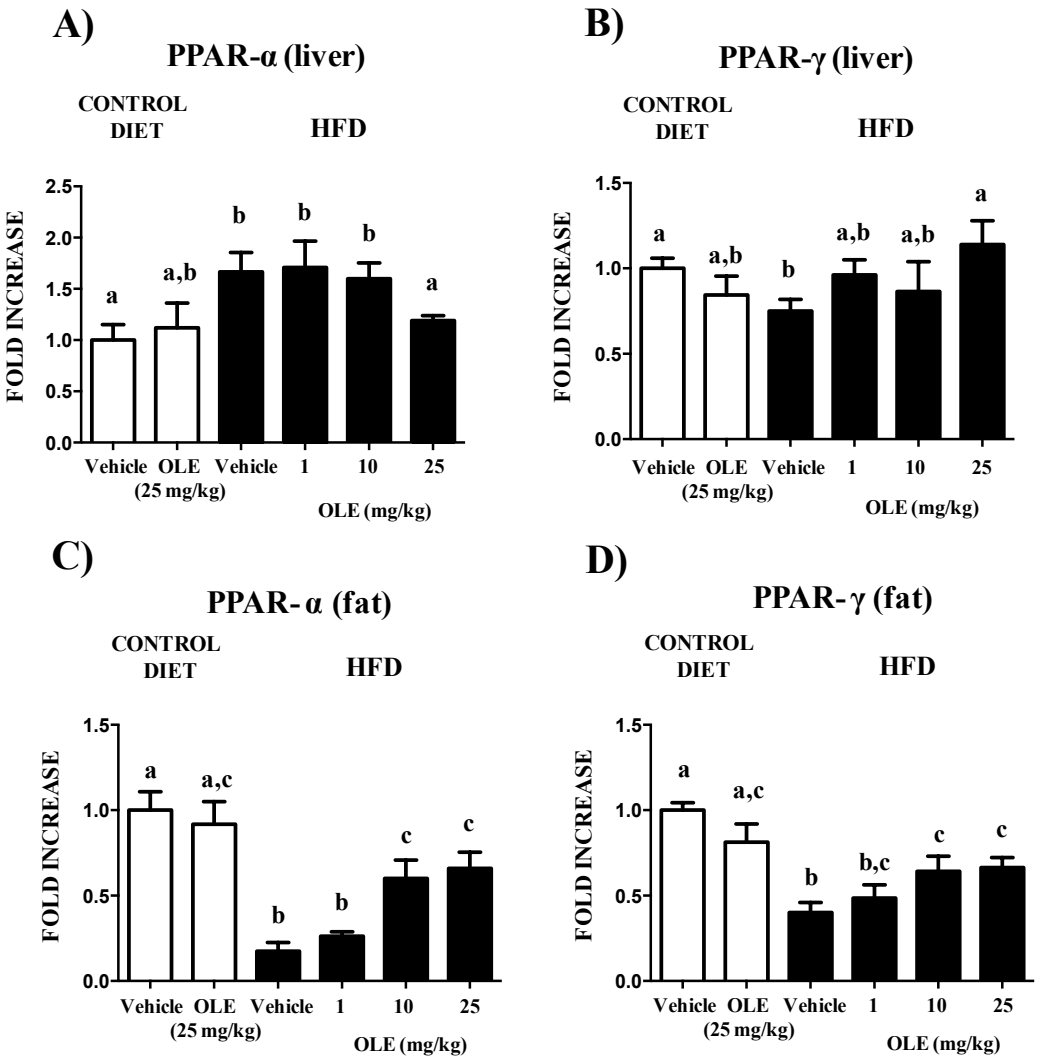


Figure 44. Effects of olive leaf extract (OLE) (1–25 mg/kg) on (A;B) liver and (C; D) fat gene expression of PPAR-α and PPAR-γ in control and high fat diet (HFD)-fed mice, analyzed by real-time qPCR. Data are expressed as means ± SEM (n=10). Groups with different letter statistically differ ($P< 0.05$).

Furthermore, the adipokines secreted by the adipose tissue, leptin and adiponectin, have been reported to display a key role in the integration of the systemic metabolism, being their production and function altered in obesity [18]. In the present study, the expression of both leptin and adiponectin in the adipose tissue was impaired in obese mice when compared with those mice fed control diet, being associated with a reduced expression of the leptin receptor in both liver and fat (Figure 45).

The administration of OLE to obese mice resulted in a significant improvement in the expression of these adipokines at all doses assayed; also, the reduced expression of leptin receptor in liver and adipose tissue was significantly ameliorated by OLE, although in liver, this was only obtained with the highest dose assayed (Figure 45A).

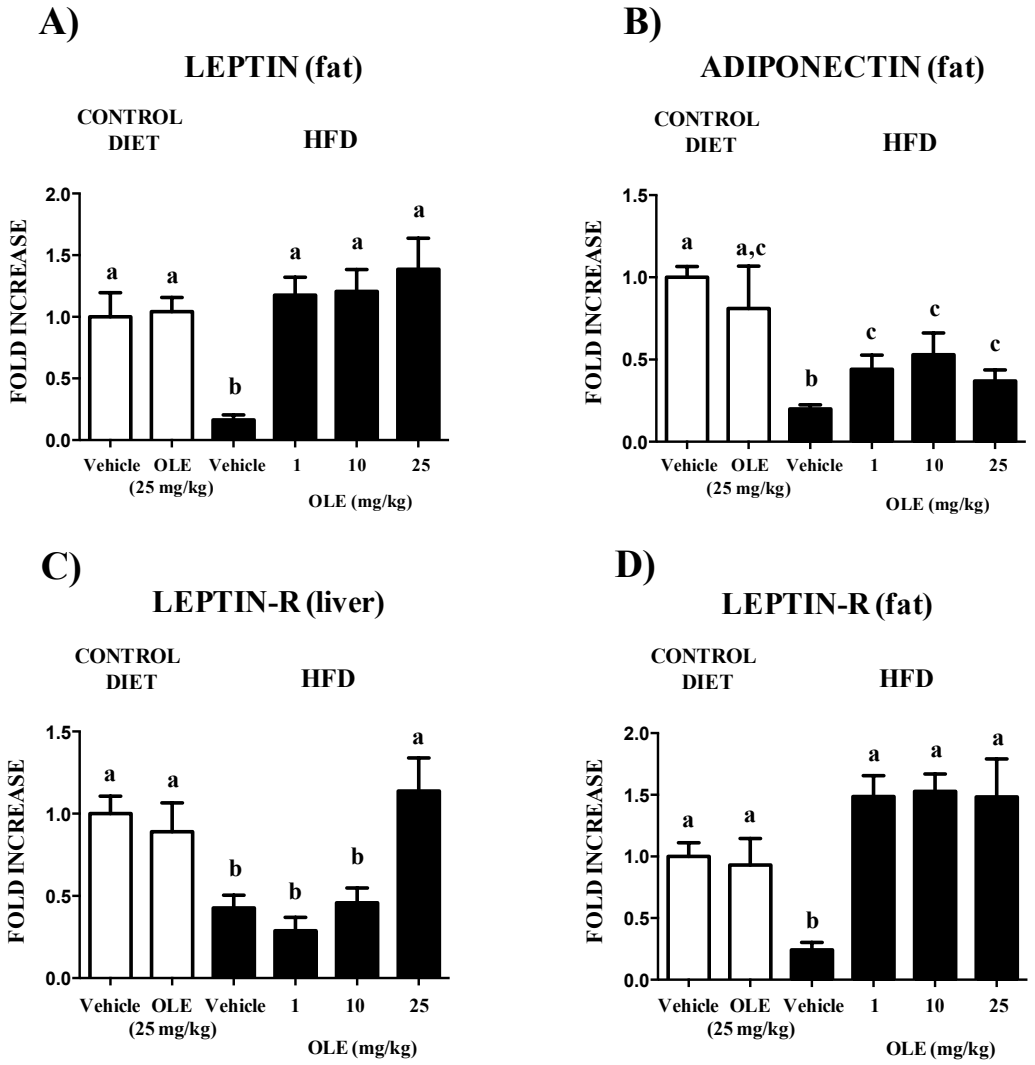


Figure 45. Effects of olive leaf extract (OLE) (1–25 mg/kg) on (A;B; D) fat gene expression of leptin, adiponectin, leptin R and (C) hepatic leptin R in control and high fat diet (HFD)-fed mice, analyzed by real-time qPCR. Data are expressed as means ± SEM (n=10). Groups with different letter statistically differ ($P< 0.05$).

The impairment in glucose metabolism and insulin resistance evidenced in obese mice were related to a decreased expression of the glucose transporter GLUT-4 in fat as well as of AMPK in both tissues studied, liver and fat (Figure 46).

Moreover, the expression of LPL in the adipose tissue was also reduced in HFD-fed mice in comparison with the corresponding lean groups (Figure 46). When obese mice were treated with the different doses of OLE, there was a significant recovery in the expression of GLUT-4 in the liver and AMPK and LPL in both liver and adipose tissues, being this recovery complete in the hepatic tissue when the dose of 25 mg/kg was used (Figure 46).

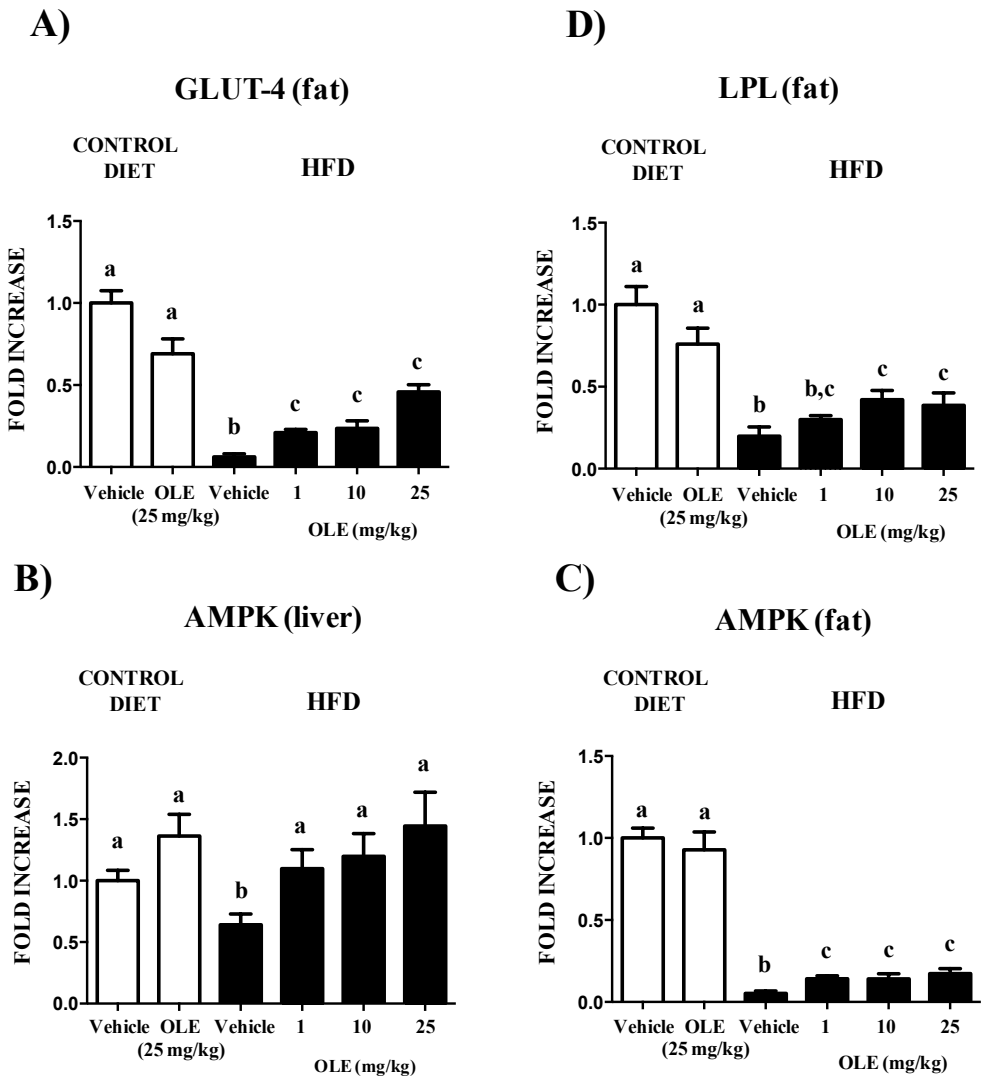


Figure 46. Effects of olive leaf extract (OLE) (1–25 mg/kg) on (A; C; D) fat and (B) liver gene expression of GLUT-4, AMPK and LPL in control and high fat diet (HFD)-fed mice, analyzed by real-time qPCR. Data are expressed as means ± SEM (n=10). Groups with different letter statistically differ ($P< 0.05$).

Additionally, the systemic inflammatory process observed in obese mice was associated with an altered immune response in the adipose tissue. Thus, HFD fed mice showed asignificant increase in the percentage of $CD11c^+$ cells, mostly dendritic cells, which was significantly reduced in those obese mice treated with the different doses of OLE (Figure 47). This correlated with an accumulation of triple$^+$ ($CD11b^+$ $CD11c^+$ $F4/80^+$) macrophages that increased twofold in obese compared with OLE (Figure 47), whereas the administration of OLE showed a dose-dependent reduction of this population (Figure 47).

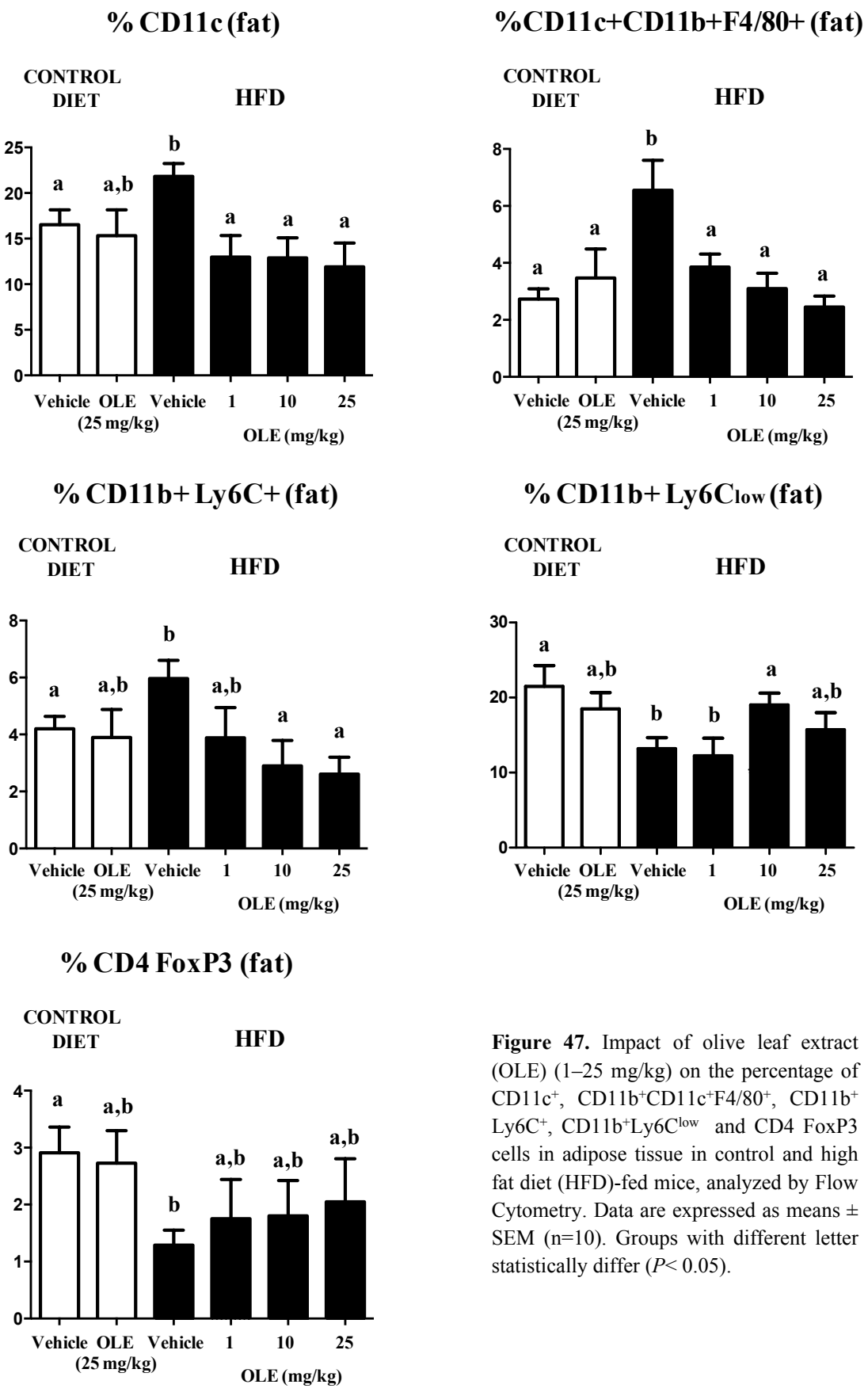


Figure 47. Impact of olive leaf extract (OLE) (1–25 mg/kg) on the percentage of $CD11c^+$, $CD11b^+CD11c^+F4/80^+$, $CD11b^+Ly6C^+$, $CD11b^+Ly6C^{low}$ and CD4 FoxP3 cells in adipose tissue in control and high fat diet (HFD)-fed mice, analyzed by Flow Cytometry. Data are expressed as means ± SEM (n=10). Groups with different letter statistically differ ($P< 0.05$).

Myeloid derived suppress cells (MDSCs) are a heterogeneous population comprised of myeloid cell progenitors and precursors of granulocytes, macrophages and dendritic cells. In the present study, the percentage of total MDSCs, identified by CD11b and Ly6C co-staining, was increased in obese mice in comparison with mice fed with control diet, thus suggesting a blockade in the normal differentiation process of these cells with consequent accumulation of this population in the adipose tissue.

The treatment with OLE, mainly at the highest doses, was able to significantly decrease the percentage of this population, and restore the normal values from non-obese mice (Figure 47). Furthermore, when the CD11b+Ly6C^{low} macrophages population was analysed, an important cell type involved in the resolution of inflammation, it was observed that it was reduced in obese mice, while it was significantly increased after OLE treatment, particularly at doses of 10 and 25 mg/kg (Figure 47). Moreover, MDSCs cells have been reported to exert an important role also in the expansion of T regulatory cells; in fact, in obese mice there was a reduced Treg population in comparison with lean mice, and the treatment with OLE showed a trend to increase this population at all doses tested. All these data suggest the possible role of OLE in the modulation of adaptive immune response in obese mice.

6. Effects of OLE on gut microbiota composition and barrier function

When the faecal microbiota was evaluated, the 16S ribosomal DNA sequencing and the bioinformatics alignment comparison against RDP database revealed significant faecal microbial alterations in the untreated HFD-fed group. The composition of bacterial communities was evaluated by calculating two major ecological parameters, including Chao richness (an estimate of a total community) and Shannon diversity (the combined parameter of richness and evenness). Microbial richness, evenness, and diversity did not show any significant modification (Figure 48).

Furthermore, 2-dimensional scatterplot was generated by principal coordinated analysis (PCA) to visualize whether the experimental groups in the input phylogenetic tree had significantly different microbial communities. This method allows to present the dissimilarities of the data in terms of distance [510], and each PC axis percentage describes how much one dimension can account for. The composition of faecal microbial communities clearly differed between control diet- and HFD-fed groups, thus

indicating two extremely different gut environments (Figure 49). Of note, when different doses of OLE were administered to obese mice, the PCA plot showed marked differences in comparison with the untreated group, showing clear similarities with lean control groups when the highest dose of OLE was assayed (Figure 49).

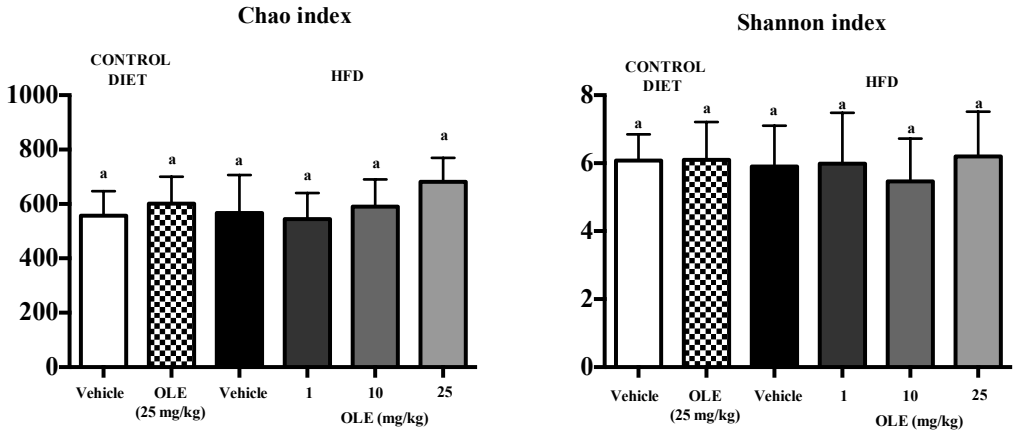


Figure 48. Gut microbial communities in in control and high fat diet (HFD)-fed mice. Microecological parameters of the gut. Fecal samples were collected from each group (n=4), and bacterial 16S ribosomal DNA were amplified and sequenced to analyze the compositions of microbial communities. Results were compared by Student t test; bars with a different letter differ statistically (P<0.05).

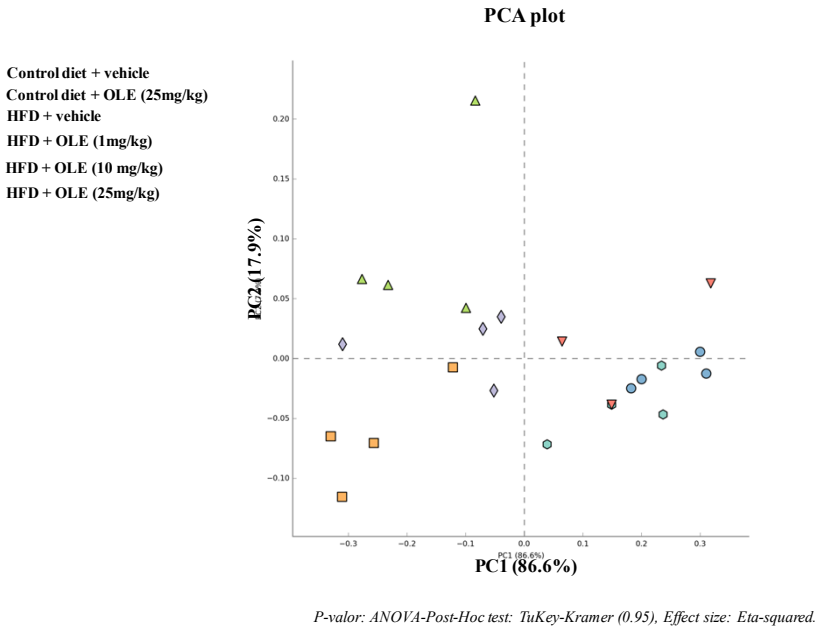


Figure 49. Effects of olive leaf extract (OLE) (1–25 mg/kg) on microbiota composition in control and high fat diet (HFD)-fed mice. Phylum breakdown of the most abundant bacterial communities in the different groups.

In order to further investigate this remarkable shift in the gut microbial environment after OLE treatment, the bacteria composition of principal phyla was evaluated (Figure

50), and the results revealed that there significant differences between lean and obese control gropus in all bacterial phyla except for Proteobacteria phylum (Figure 50).

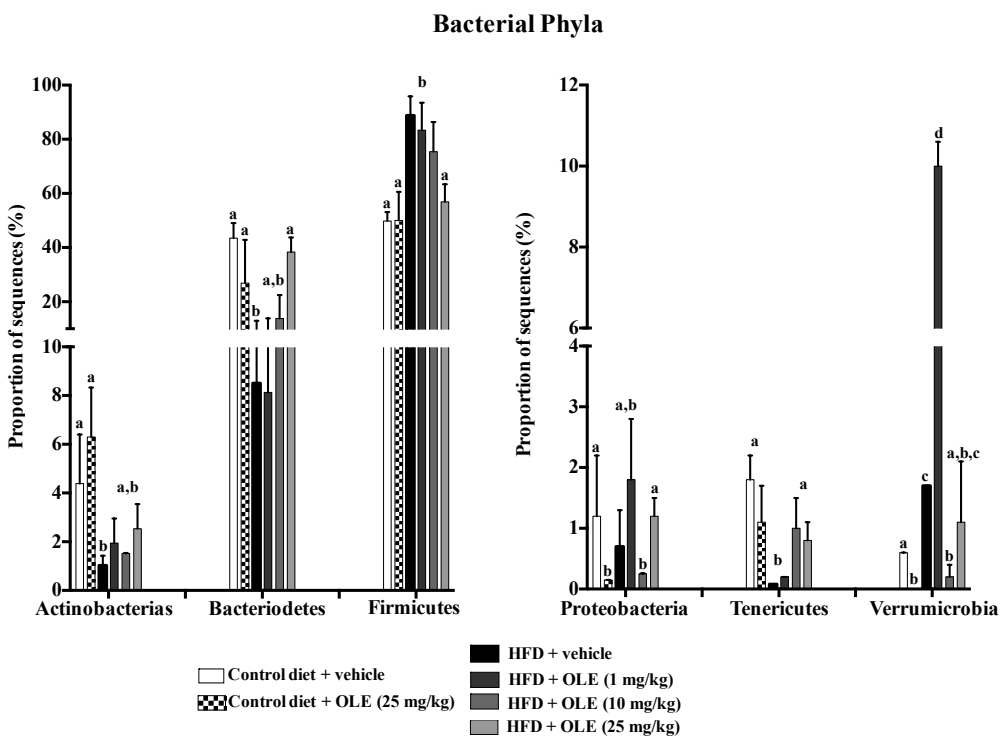


Figure 50. Effects of olive leaf extract (OLE) (1–25 mg/kg) on microbiota composition in control and high fat diet (HFD)-fed mice. Phylum breakdown of the most abundant bacterial communities in the different groups.

Furthermore, OLE treatment was able to restore most of the changes produced in these phyla, specifically when the highes dose of OLE was considered (Figure 50).

These changes were also evidenced when the ratio of the microbiome communities *Firmicutes* (F) and *Bacteriodetes* (B), known as the F/B ratio, was determined. This ratio can be used as biomarker for pathological conditions as obesity, inflammation and diarrhea [21-23] (Figure 51). The F/B ratio in HFD-fed mice was significantly increased (≈ 10-fold) when compared with control diet-fed group (Figure 51). Of note, when this parameter was evaluated in OLE-treated groups, only the lowest dose was not able to decrease this ratio significantly (Figure 51), thus showing the ability of this extract to restore the Firmicutes and Bacteriodetes populations and reshape the altered microbiota composition in obesity (Figure 51).

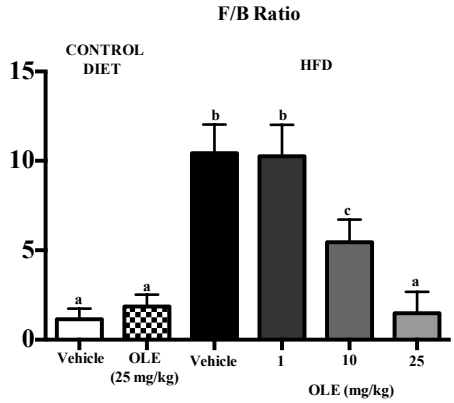


Figure 51. Effects of olive leaf extract (OLE) (1–25 mg/kg) on microbiota composition in control and high fat diet (HFD)-fed mice. The *Firmicutes/Bacteriodetes* (F/B) ratio was calculated as a biomarker of gut dysbiosis. Results were expressed as the mean ± SEM. Bars with a different letter differstatistically ($P<0.05$).

Finally, we also investigated the specific bacteria that had a notable alteration of abundance in the two different classes belong to the phyla, *Actinobacteria* and Bacteroidetes. In this sense, HFD-fed mice showed a significant reduction of *Actinobacteria* (Class) and *Bacteroidia*, being once again counteracted, at least partially, after OLE treatment, mainly with the highest dose (Figure 32). At genera level, untreated HFD-fed mice showed reduced proportion in the sequences in two genera, *Cytophaga* and *Akkermansia*, belong to *Bacteriodetes* and *Verrumicrobia*, respectively, in comparison with lean mice, being significantly ameliorated after OLE treatment to obese mice (Figure 52).

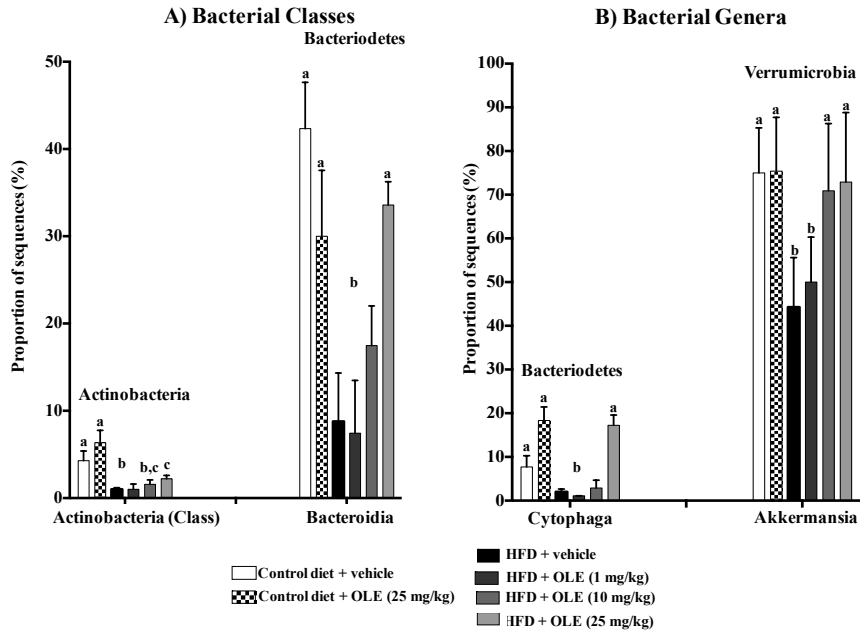


Figure 52. Effects of olive leaf extract (OLE) (1–25 mg/kg) on microbiota composition in control and high fat diet (HFD)-fed mice.Clases and Genera of the most abundant bacterial phyla in the different groups.

When the parameters of intestinal barrier function were evaluated, obesity was associated with a reduced colonic expression of occludin, as well as the mucins MUC-2 and MUC-3, in comparison with the control diet fed mice (Figure 53); however, no significant modification was observed with the colonic expression of ZO-1 between obese and lean mice.

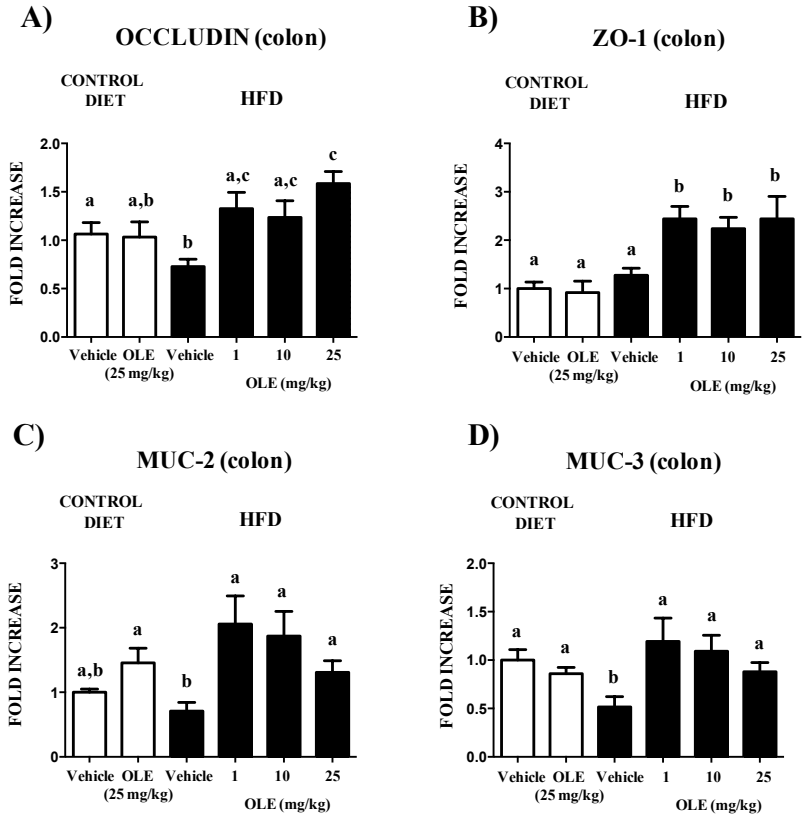


Figure 53. Effects of olive leaf extract (OLE) (1–25 mg/kg) on colonic gene expression of (A) Occludin, (B) ZO-1, (C) MUC-2 and (D) MUC-3 in control and high fat diet (HFD)-fed mice, analyzed by real-time qPCR. Data are expressed as means ± SEM (n=10). Groups with different letter statistically differ ($P<$ 0.05).

The altered permeability would result in an increased translocation of bacterial components, including LPS, thus reaching portal circulation and liver that in turn would be responsible for the increased expression of TLR4 in the liver from obese control mice in comparison with non-obese groups (Figure 54).

The administration of OLE resulted in a significant amelioration in the colonic expression of occludin and mucins (MUC-2 and MUC-3), obtaining similar levels to those in lean mice; of note, the colonic expression of ZO-1 was further increased after OLE treatment to obese mice in comparison with both lean and obese control groups

(Figure 53). All these data support the improvement of gut barrier function exerted by OLE in obese mice, thus preventing the systemic access of bacterial products, as evidenced by reduced expression of TLR4 in the liver from obese mice (Figure 54).

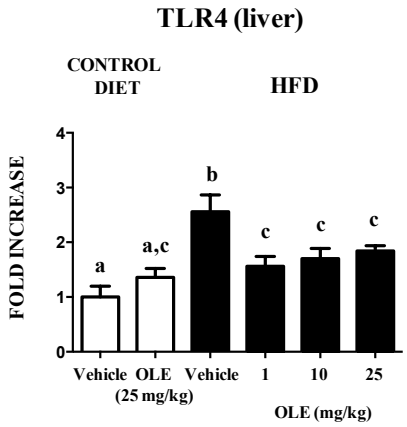


Figure 54. Effects of olive leaf extract (OLE) (1–25 mg/kg) on liver gene expression of TLR-4 in control and high fat diet (HFD)-fed mice, analyzed by real-time qPCR. Data are expressed as means ± SEM (n=10). Groups with different letter statistically differ ($P< 0.05$).

7. Effect of OLE on endothelial function

Obesity has been reported to be associated to cardiovascular alterations, including endothelial dysfunction [15, 29]. Aortae from mice fed a HFD showed significant reduced endothelium-dependent vasodilator responses to acetylcholine, which is considered as an index of endothelial function, as compared with aortae from the control group (Figure 55 A). In fact, the concentration-response curves revealed that there was a reduction in the maximal relaxant response in untreated HFD in comparison with non-obese control mice (Emax values were 59±7%and 79±4% in the HFD and control groups, respectively; P<0.05), although no significant changes were observed in the concentration of acetylcholine required for half-maximal relaxation (−logIC50 values were 7.25±0.10 and 7.34±0.09 in the HFD and control groups respectively; P>0.05).

OLE administration to obese mice almost restored the altered endothelium-dependent relaxation induced by acetylcholine, obtaining similar E_{max} values to those in non-obese control mice. Since it has been previously reported that the obesity-associated endothelial dysfunction is mainly associated with reactive oxygen species production within the vascular wall, and that NADPH oxidase is the major source of ROS in this location[15], it was evaluated both the NADPH oxidase activity (Figure 55 B) and the gene expression of the main NADPH oxidase subunits in the aorta from all experimental groups (Figure 56). Thus, the NADPH oxidase activity and the expression

of the NADPH oxidase subunits NOX-1, NOX-4 and p47^{phox}, but not p22^{phox}, was significant increased in the rings from HFD-fed mice compared with control diet fed mice (Figure 55B). OLE administration to obese mice reduced the increased activity and the upregulated expression of all these subunits, including p22^{phox}, in comparison with the untreated HFD-fed mice, typically obtaining a dose-response relationship.

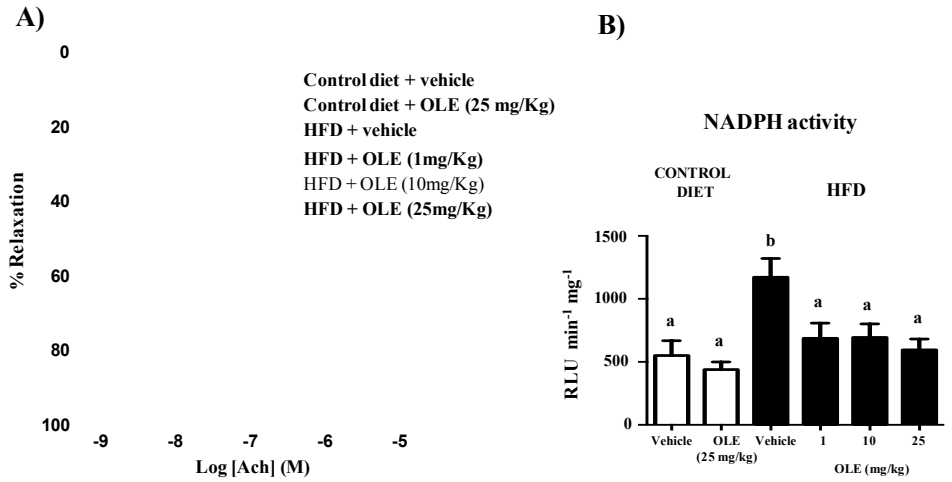


Figure 55. (A) Effects of of olive leaf extract (OLE) (1–25 mg/kg) administration on endothelial function. (B) Effects of olive leaf extract (OLE) (1–25 mg/kg) on aortic NADPH activity in control and high fat diet (HFD)-fed mice, analyzed by real-time qPCR. Data are expressed as means ± SEM (n=10). Groups with different letter statistically differ ($P< 0.05$).

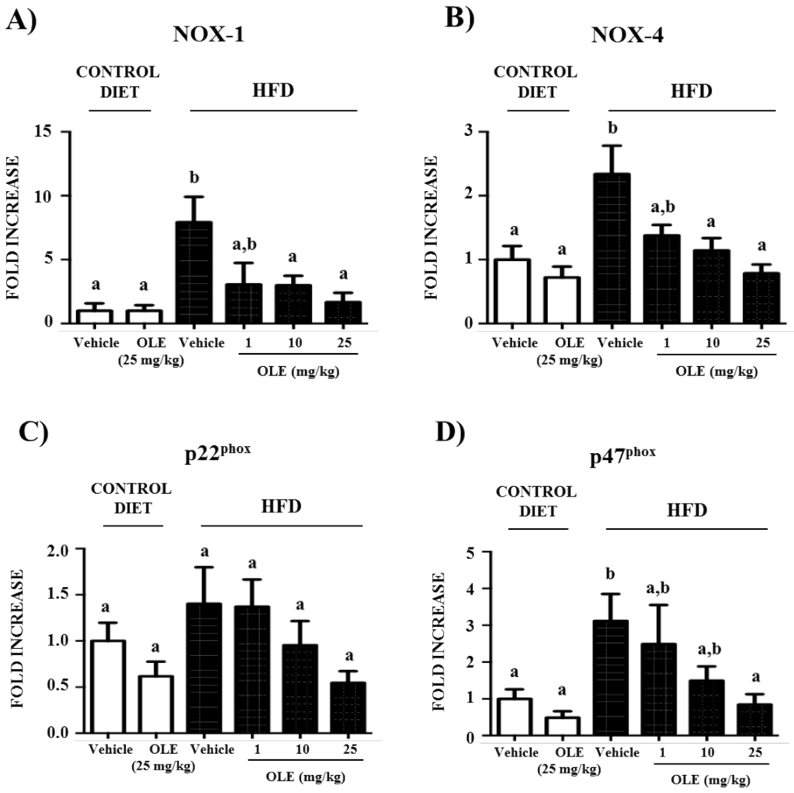


Figure 56. Effects of olive leaf extract (OLE) (1–25 mg/kg) on aortic gene expression of NADPH oxidase subunits (A) NOX-1, (B)NOX-4, (C) p22^{phox}, (D) p47^{phox}in control and high fat diet (HFD)-fed mice, analyzed by real-time qPCR. Data are expressed as means ± SEM (n=10). Groups with different letter statistically differ ($P< 0.05$).

Finally, and similarly to that observed in the liver, the expression of TLR-4 in aortic homogenates was significantly increased in HFD-fed mice in comparison with non-obese groups, whereas OLE administration significantly ameliorated these TLR-4 levels (Figure 57).

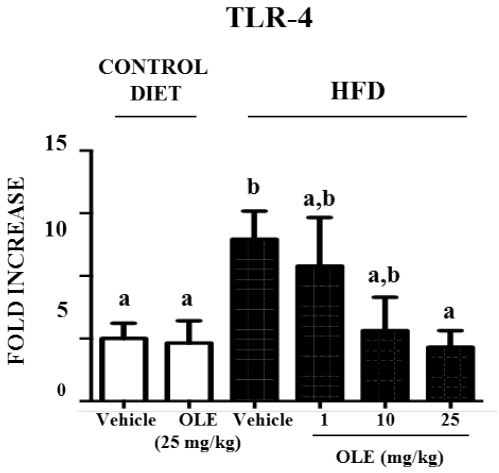


Figure 57. Effects of olive leaf extract (OLE) (1–25 mg/kg) on aortic gene expression of TLR-4 in control and high fat diet (HFD)-fed mice, analyzed by real-time qPCR. Data are expressed as means ± SEM (n=10). Groups with different letter statistically differ ($P< 0.05$).

DISCUSSION

PART I

The term IBD mainly refers to two gastrointestinal conditions, Crohn's disease (CD) and ulcerative colitis (UC). They are characterized by a chronic gastrointestinal inflammation disorder with alternating periods of relapses and remissions. The most common symptoms include abdominal pain, fever, body weight loss, bowel obstruction or diarrhoea with passage of blood and/or mucus diarrhoea, nausea, etc., which clearly lead to poor quality of life of the patients, and require prolonged medical and/or surgical interventions [511]. The incidence and prevalence of IBD is continuously increasing over the past decades in different regions around the world, especially in the most developing countries, causing an important socioeconomic burden [512]. At present, the etiology of IBD has not been completely elucidated unravelled, but it has been suggested that both genetic and environmental factors could initiate alterations in the epithelial barrier function. These impairments could permit luminal antigens translocation into the bowel wall and the generation of an exacerbated and uncontrolled immune response response [513]. As a result, there is an up-regulation of the synthesis and release of different proinflammatory mediators, including reactive oxygen and nitrogen metabolites, eicosanoids, chemokines and cytokines, which actively contribute to the harmful cascade that initiates and perpetuates the inflammatory response in the gut [514]. The existing treatments, including aminosalicylates, corticosteroids, immunosuppressants, antibiotics or, more recently, biologic agents (like infliximab or adalimumab) [515] address the inflammation and the related symptoms. However, although they show efficacy, their chronic use are frequently associated with severe side effects [173, 202].

The latter has prompted the development and validation of new treatments combining efficacy and safety, and the frequent use of alternative therapies in gastrointestinal diseases [516]. In this regard, the use of complementary and alternative medicine is widely prevalent among IBD patients, especially in those that experience adverse effects to conventional dugs [9, 517]. Considering the different alternative therapies, botanical drugs seems to be of special relevance for the treatment of intestinal inflammation. They are considered safe, since they have been consumed from old times, as well as effective, given the presence of several active compounds that can modulate the inflammatory response at different levels [206, 518]. In this context, the preclinical studies reporting the beneficial effects of plant extracts on experimental models of colitis are numerous,

only a few plant extracts have been shown efficacy in different clinical assays. This would justify the search of new plant extracts with potential application in intestinal inflammation based on their consideration as suitable remedies for digestive disorders in Traditional Medicine. With this aim we have focused our study in the olive leaf extract (OLE).

Olive tree (Olea europaea L.) is one of the most relevant botanical drugs in traditional Mediterranean Medicine. Remarkably, Mediterranean diet has been proposed to confer protection against different human chronic diseases, mainly those related to the cardiovascular system and the gastrointestinal tract, like IBD [466, 467]. These beneficial effects could partially be attributed to the intake of olive oil, which contains biologically active components, including oleic acid [469] and phenolic compounds [470] with antioxidant and anti-inflammatory properties. In fact, diet supplementation with olive oil, or with its polyphenol content, has previously shown intestinal anti-inflammatory activity in experimental models of colitis [471-473].

Moreover, OLE have also been used since ancient times in traditional Mediterranean Medicine with different purposes, including antihypertensive, antiatherogenic, anti-inflammatory, hypoglycemic and hypocholesterolemic activities [474]. These extracts have also been reported to contain many potentially bioactive compounds, especially phenolic derivatives, like phenolic acids, phenolic alcohols (hydroxytyrosol), flavonoids (diosmetin, quercetin, luteolin, apigenin and their derivatives) and secoiridoids (oleuropein and its derivatives) [475], which may account for their beneficial effects. In fact, and when considering intestinal inflammation, previous studies have shown the intestinal anti-inflammatory properties of many of these compounds [472, 476-478]. However, so far, only one study has investigated the intestinal anti-inflammatory properties of the OLE [519]. In this study, different doses of the extract (250, 500 and 750 mg/kg) showed intestinal anti-inflammatory effects in the acetic acid model of rat colitis, which were associated with a modulation of the altered immune response, particularly of the nitrergic and opioidergic system, due to the well-described antioxidant properties of the extract.

The aim of the present study is to evaluate the intestinal anti-inflammatory effects of a standardized extract from olive leaves, both *in vitro* and *in vivo*. Thus, several doses of OLE were assayed in two different experimental models of intestinal inflammation:

DNBS- and DSS-induced colitis in mice, being probably the most commonly used models to initially evaluate the potential application of a given treatment in intestinal inflammation. They show some resemblance to the main manifestations of IBD. Whereas the DNBS model of mouse colitis induces a transmural lesion with pathological characteristics similar to Crohn's disease, the DSS model in mice produces inflammation limited to the colonic mucosa that is more closely related to human ulcerative colitis [520].

The beneficial effects of the extract were associated with an amelioration of the colonic damage induced by the insulting agents, as evidenced both histologically, by a recovery of the inflammatory process and a reduction in the inflammatory infiltrate, and biochemically, by an amelioration in most of the inflammatory markers evaluated, that have been involved in the pathogenesis of IBD [79, 521]. Different mechanisms might contribute to these beneficial effects, as evidenced both *in vivo* and *in vitro*.

The positive impact of extract treatment was demonstrated biochemically in both models, when the colonic expression of cytokines, chemokines, adhesion molecules or proteins involved in the intestinal epithelial integrity were evaluated by qPCR. Particularly, the extract significantly reduced the expression of the different proinflammatory cytokines studied, like TNF-α, IL-1β, IL-6 or IL-17 in DNBS model and the expression and production of the same cytokines in the DSS one. These results confirm the immunomodulatory properties ascribed to the extract that account for its beneficial effects in intestinal inflammation [477]. In fact, among the numerous cytokines, IL-1β, IL-6 and TNF-α have been considered as important inflammation mediators of innate and/or adaptive immunity, and to have a key role driving intestinal inflammation [522]. Typically, activation of innate and adapatative immune responses during the progression of IBD also implies the increased expression of other mediators involved in the inflammatory response, like chemokines or adhesion molecules. Interestingly, the olive leaf treatment significantly modify the expression of MIP-2 and ICAM-1 in the DSS model of mouse colitis as well as in the DNBS one, thus reducing the activation and migration of leukocytes to the inflamed area, and subsequently ameliorating their deleterious impact on the intestinal tissue [523, 524].

During the last two decades, it has become increasingly clear that NO overproduction by iNOS is deleterious to intestinal function, thus contributing significantly to

gastrointestinal immunopathology during the chronic inflammatory events that take place in IBD [258]. In the present study, iNOS expression was upregulated in both models of experimental intestinal inflammation used; interestly, OLE was able to significantly reduce the increased iNOS expression in colitic mice. It has been proposed that this probably results from the intense activation of macrophages, which took place as a consequence of the inflammatory insult [14]. In fact, macrophages are considered an important source of pro-inflammatory mediators, such as NO and TNF-α, playing a key role in the pathophysiology of IBD [146]. Moreover, this beneficial effect was also proved in the *in vitro* studies, since the extracts reduced NO production in LPS-stimulated RAW 264 cells.

Besides, an inhibitory effect on the activity of immune cells could participate in the intestinal antiinflammatory effect. This was confirmed by others *in vitro* experiments performed with different immune cell types involved in the inflammatory response, ntestinal epithelial cells (Caco-2 and CMT-93) and human PBMC. These assays revealed that the incubation of these cells with the extract before LPS or IL-1β stimulation, which were used as inductors of an inflammatory milieu, resulted in decreased production of the proinflammatory cytokines studied, both in epithelial cell lines and in human PBMCs. It is remarkable that the extract showed a similar efficacy when these PMBCs were obtained from healthy subjects or from CD patients. In the later, PBMCs functions are altered, since the disease is active, and they can contribute to the production of intestinal pro-inflammatory mediators [525]. Thus, these results show that OLE may exert beneficial effects even in pathological conditions.

Finally, it has been reported that the increased expression and/or production of different inflammatory cytokines can facilitate the breaking of the intestinal subepithelial basement membrane, and thus promote the presence of activated mononuclear cells in close proximity to the intestinal epithelial cells [526]. Such situation may lead to the disruption of junctional complexes in the mucosal epithelium, as evidenced in the present study, by the decrease in the expression of ZO-1 (zonula occludens) and/or TFF-3 (trefoil factor) in the experimental models of colitis This may result in a dysfunctional intestinal epithelium barrier with increased tight junction permeability, which could also be worsen by the reduced expression of mucins that cover the epithelial cell line in the intestine and also contribute to the maintenance of the epithelial integrity [88]. However, OLE improved the epithelial barrier function in both

models of experimental colitis as demonstrated by the increasing in the expression of the mucin MUC-2, the tight junction protein ZO-1 and the TFF-3, which were shown at the mRNA level and when intestinal permeability was assayed *in vivo* using FITC-dextran in the DSS model. Besides, the mucin content in the mucosa was confirmed by the histological evaluation with alcian blue staining in the DSS model. This effect can also contribute to the downregulation of the immune response, especially when considering that the weakening of the epithelial barrier function allows access of luminal antigens that could trigger the exacerbated immune response. In fact, this has been proposed to be one of the main events that occur in IBD [280].

Of note, OLE was also able to exert beneficial effects when assayed in colonic explants from CD patients. It significantly reduced LPS stimulated IL-1β, IL-6, and IL-8 production, although no clear dose-effect relationship could be established. So the extract confirmed its capacity to downregulate the altered immune response that characterizes this intestinal condition, thus supporting its intestinal anti-inflammatory properties evidenced *in vitro* as well as *in vivo*.

The beneficial effects exerted by OLE can be ascribed to the presence of different phenolic compounds, as previously reported [11]. Among these, oleuropein constitutes the major component (approximately 80% of the phenolic content). It is a secoiridoid that has been described to possess intestinal anti-inflammatory effects in experimental models of colitis, most probably due to its antioxidant properties as well as to its ability to modulate the altered immune response and facilitate the recovery of the intestinal epithelial cell damage that characterize these intestinal conditions [477, 478]. Furthermore, the presence of other phenolic compounds in the extract could also contribute to its effect, especially when considering the active doses of the extract used in the present study, 1 and 10 mg/kg, much lower than those used with oleuropein in experimental colitis (500 mg/kg) [477].

PART II

The absence of a pharmacological clear treatment against obesity that combines efficacy and safety has prompted the frequent use of complementary and alternative therapies in these patients. Among these, botanicals have an obvious role because, in addition to their consideration as safe remedies, many of them have proven their efficacy [527], most probably due to the presence of several active compounds that act synergically and simultaneously at different levels in this condition. In the present study, a well chemically-characterized OLE [528] was assayed in mice fed HFD, by exploring its effects on different constituents of the metabolic syndrome. Thus, OLE administration, at doses of 1, 10 and 25 mg/kg, to these mice reduced the HFD-induced weight gain and, consequently, the amount of epididymal fat tissue. Previous studies have also reported the ability of OLE to attenuate obesity in HFD fed mice, although these were made by using a least 5-fold higher doses than those used in the present study [496]; this can be considered specially relevant, since the risk of side effects following chronic treatment with this extract is considerably further reduced if lower doses are used. It is interesting to note that the reduction in body weight obtained with OLE was not due to a lower energy intake in these mice, but it was related to a significant decrease in energy efficiency, reaching similar levels to those observed in mice receiving control diet.

The preventative beneficial effects exerted by OLE on obesity were associated with an improvement in the systemic glucose intolerance and insulin resistance found in obese mice, as evidenced by lower HOMA-IR index in OLE-treated HFD-fed mice. Similarly, the obesity-associated alterations in lipid metabolism evaluated were also ameliorated after OLE administration to obese mice, since both LDL- and HDL-cholesterol levels in plasma were reduced in comparison with the corresponding control group. It has been proposed that all these metabolic changes induced by HFD, which are associated with the impaired insulin signalling in the corresponding target tissues, such as skeletal muscle, adipose tissue and liver, are probably due to the development of a tissue inflammatory process [379]. In fact, obesity is nowadays considered as a state of chronic inflammation characterized by increased production of pro-inflammatory cytokines and chemokines, such as TNF-α, IL-6, and MCP-1, in association with the activation of different inflammatory signalling networks, including inhibitor of NF-κB (Iκβ) kinaseβ (IKK-β)/NF-κB and the c-JunNH2-terminal kinase (JNK) pathways in key metabolic tissues as well as in macrophages [335, 529, 530]. Moreover, the

development of obesity-induced insulin resistance has been clearly correlated with these inflammatory pathways [529]. Consequently, we have shown that hepatic and adipose tissue mRNA levels of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, were higher in HFD-fed mice than in control diet-fed mice. OLE administration to obese mice reduced their expression in both tissues, effects that can account for its ability to improve glucose metabolism, given the role attributed to these pro-inflammatory cytokines in the impairment of insulin action [531]. Moreover, *in vitro* studies have reported the specific actions attributed to these cytokines that can account for the pathogenesis of obesity; IL-6 reduces the expression of adiponectin, glucose transporter-4 (GLUT-4) and insulin receptor substrate-1 (IRS-1), whereas TNF-α causes an elevated secretion of MCP-1 and IL-6 from pre-adipocytes [532]. Similar observations have been obtained *in vivo* in the present study.

When insulin resistance occurs, the cells in insulin dependent tissues, including adipose tissues and muscles, fail to effectively respond to this hormone, thus inducing high levels of serum glucose. In both adipocytes and skeletal muscle, intracellular glucose uptake is insulin-dependent via GLUT-4 [533]. In normal conditions, the excess blood glucose is diffused into adipocytes through GLUT-4, thus stimulating the synthesis of fatty acid and glycerol, while suppressing lipolysis. However, in obesity-associated insulin resistance there is a reduced GLUT-4 gene expression in adipose tissue [534], similarly to that observed in the present study. OLE administration to obese mice significantly upregulated GLUT-4 expression in fat tissue, which can be considered as a manifestation of the improvement in insulin sensitivity, thus increasing blood glucose uptake into adipocytes, and contributing to the reduced serum glucose levels observed in OLE treated obese mice groups.

Moreover, it has been reported that the deleterious effects of inflammatory cytokines on adipocytes, i.e. increase in lipolysis and decrease in triglyceride synthesis, are associated to the negative regulation of the nuclear hormone receptor PPAR-gamma, which is considered as an essential transcriptional regulator of adipogenesis and is required for maintenance of an adequate mature adipocyte function [535]. Indeed, TNF-α affects PPAR-gamma levels by interference in all the steps involved in its production, including the transcription, translation and turnover of PPAR-gamma mRNA and protein; which require the participation of JNK-related signalling pathways [536]. Different studies have identified JNK-1 as the major isoform contributing to the

development of obesity-associated insulin resistance, since mouse mutants deficient for JNK-1, but not those for JNK-2, are largely protected from the development of obesity and insulin resistance in both diet- and genetically-induced obesity models [529, 537]. The results obtained in the present study confirm these observations, since the increased mRNA levels of pro-inflammatory cytokines evidenced in untreated HFD-fed mice were associated to a reduced expression of PPAR-gamma in the adipose tissue and liver, together with and increase in the hepatic expression of JNK-1. All these modifications were effectively counteracted by OLE administration to obese mice, thus confirming its beneficial effect in the prevention the obesity-associated inflammatory response and the subsequent tissue altered function that can occur in this conditions.

AMP-activated protein kinase (AMPK) is a signalling protein that, together with SIRT1, is considered as a cell nutrient sensor, being proposed as a candidate linking nutrient metabolism and inflammation in target tissues. AMPK regulates metabolic pathways in lipid and glucose metabolism by integrating nutritional and hormonal signals in the periphery and hypothalamus [538]. Thus, AMPK activation stimulates ATP production by increasing FA oxidation, muscle glucose transport, mitochondrial biogenesis and caloric intake, whereas provokes the inhibition of ATP-consuming anabolic pathways, including FA (fatty acid) synthesis, cholesterol and isoprenoid synthesis, as well as hepatic gluconeogenesis [539, 540]. Furthermore, AMPK regulates inflammatory signaling in different cell types, including macrophages, which can contribute to insulin resistance in obesity [541]. In fact, it has been proposed that inactivation of AMPK signaling promotes inflammatory response in macrophages, whereas its activation prevents it [542]. Furthermore, the activation of AMPK prevents LPS-induced expression of pro-inflammatory cytokines and NF-κB signaling in several cell types [541, 543]. In consequence, AMPK displays a key physiological function in preventing inflammation-induced insulin resistance. It is well known that the antidiabetic drugs, metformin and rosiglitazone, which act as insulin sensitizers, can activate AMPK [544, 545], thus supporting the role of AMPK in regulating insulin sensitivity. Indeed, different studies have reported that the activation of AMPK increases basal and insulin-stimulated glucose uptake in skeletal muscle and inhibits gluconeogenesis in the liver, leading to improved systemic insulin sensitivity and glucose homeostasis [546, 547]. In the present study, as expected, AMPK expression was significantly downregulated in untreated HFD-fed mice both in liver and adipose tissue. The administration of OLE to

obese mice resulted in a significant increase in the expression of AMPK in both tissues; however, although a complete restoration was observed in the liver, this was not the case in adipose tissue, since this was still significantly reduced in comparison with lean mice. The altered expression of AMPK in obese mice was associated to a decrease in the expression of LPL in adipose tissue, which is involved in the uptake of free fatty acids from triglycerides circulating in lipoproteins [548]. In HFD-fed mice OLE treatment was able to significantly increase the LPL expression in this tissue, similarly to the effects observed when AMPK expression was evaluated. The effect on LPL expression exerted by OLE can collaborate in the improvement of systemic metabolism observed in obesity, since previous studies have proposed that increasing adipocyte lipoprotein lipase ameliorated glucose metabolism in high fat diet-induced obesity. [549]. The impact of OLE on AMPK activity was previously demonstrated *in vitro* in 3T3-L1 adipocytes, a hypertrophic and insulin resistant adipocyte model, since this extract was able to decrease intracellular lipid accumulation through AMPK-dependent mechanisms [528]. This effect was ascribed to the presence in OLE of compounds that belonged to the secoiridoids, cinnamic acids, phenylethanoids and phenylpropanoids, flavonoids and lignans subclasses, as proposed by the *in silico* results reported in the same study [528].

Closely related to the altered insulin sensitivity, obesity is associated with a dysregulation of several adipose-derived factors, including adipokines like leptin and adiponectin, which can act locally and systemically to regulate immune response, cardiovascular function, and many other physiological processes [320]. In fact, this adipokine dysregulation was also observed in the present study since the mRNA expression of adiponectin was impaired in the adipose tissue, in comparison with those mice fed control diet, and the expression of the leptin receptor in both liver and fat was reduced, which is considered as a marker of the leptin intolerance previously reported in obesity. Leptin is produced and secreted by white adipocytes in proportion to fat mass, and physiologically, it decreases food intake and body weight by activating leptin receptors in the arcuate [504]. It is well known that leptin production increases proportionally with adiposity, and thus, plasma leptin levels are increased in obese human and in rodent models of diet induced obesity (DIO), which leads to decreased central leptin sensitivity and increased leptin receptor resistance [550]. Leptin is considered a proinflammatory adipokine, being described to exert many of its actions by

affecting several cells of the immune system, including monocytes/macrophages, dendritic cells, neutrophils, eosinophils, basophils, natural killer cells, and lymphocytes [551]. In this context, leptin promotes activation of adipose tissue macrophages, increasing the levels of proinflammatory M1 macrophages, and facilitating phagocytosis, inducing the expression of inducible enzymes, like COX-2 and iNOS, and increasing the production and release of proinflammatory cytokines, including TNF-α and IL-1β, which subsequently promote insulin resistance [552, 553]. Adiponectin is another adipocyte-derived protein that decreases body weight and plasma lipid levels, and enhances the ability of insulin to suppress hepatic glucose production [554]. In contrast to leptin, an inverse correlation between plasma adiponectin and insulin resistance occurs in obese humans [555]. In fact, it has been reported that adiponectin shows insulin-sensitizing and anti-inflammatory effects on several immune cell types, and, for instance, it is able to inhibit the phagocytic activity and the production of proinflammatory cytokines, including IL- 6 and TNF-α by macrophages [551, 556]. In consequence, the restoration of both leptin receptor sensitivity and adiponectin expression in adipose tissue observed after the administration of OLE to obese mice can account in ameliorating the insulin resistance status that characterizes obesity.

Considering all the above, it is evident that the impact of OLE treatment on the immune response can contribute to the beneficial effects, being this achieved by modulating the infiltration and composition of immune cells into adipose tissue, both from innate and adaptive immunity. Among these cells, dendritic cells play a prominent role; they are a heterogeneous population of professional antigen-presenting cells that modulate the activity of conventional and regulatory T cells, being also involved in the recruitment and activation of macrophages at sites of immune responses [531]. As previously commented, adipose tissues in obese individuals and in animal models of obesity are infiltrated by a large number of macrophages, and this recruitment is linked to systemic inflammation and insulin resistance [557]. In obesity, adipocytes produce low levels of TNF-α that induce pre-adipocytes and endothelial cells to secrete MCP-1, being this chemokine one of the critical factors responsible for macrophage recruitment [558]. In fact, in the present study, the characterization of the inflammatory status in the adipose tissue from HFD-fed mice revealed an increased expression of MCP-1 in comparison with lean mice, which was significantly reduced after OLE administration, thus indicating a lower ability of the adipose tissue to promote macrophage infiltration and

activation, and, consequently, attenuating the impact of these cells in the development of the obesity-associated inflammatory response.

The flow cytometry analysis in adipose tissue corroborated these observations. In this tissue it has been described the presence of a significant population of proinflammatory macrophages that express the marker CD11c but also CD11b and F4/80 and for that reason, these cells are defined as "triple-positive" (triple+) [338, 559]. However, CD11c is commonly known as a marker of dendritic cells (DC), which can also express CD11b and F4/80 [560]. Consequently, it is possible that the increased percentage of CD11c+ cells the obesity-induced model is also correlated with an increased DC population in adipose tissue. In any case, OLE treatment showed a significant reduction of both populations, CD11c+ and triple+, thus confirming the inhibitory effect exerted by this extract against macrophage/DC cell infiltration. Moreover, myeloid derived suppress cells (MDSCs) are a heterogeneous population comprised of myeloid cell progenitors and precursors of granulocytes, macrophages and dendritic cells. In normal conditions, immature myeloid cells (IMCs) generated in the bone marrow differentiate into mature granulocytes, macrophages or dendritic cells. In pathological condition, like inflammatory disorders, cancer and infections, different cytokines and other soluble factors are released. These mediators induce the proliferation of IMCs and a partial block of their differentiation that results in the accumulation of MDSCs, which then migrate to secondary lymphoid organs and tissues where they exert their effects on other cell populations. Furthermore, MDSC-mediated immune suppression can also be associated with the expansion of Treg cell populations [561]. In the present study, the percentage of total MDSCs (CD11b+Ly6C+) was increased in obese mice in comparison with control mice, thus suggesting a block of normal differentiation process of these cells with consequent accumulation of this population in the adipose tissue. The treatment with OLE was able to restore the normal values of this population. Furthermore, the percentage of CD11b+Ly6Clow macrophages, which have been reported to be essential in the resolution of inflammation [562], were reduced in obese mice, whereas OLE-treated obese mice showed a significant increase, at doses of 10 and 25 mg/kg. Of note, MDSCs have an important role in the expansion of T regulatory cells, and accordingly, in obese mice there was a reduced Treg population in comparison with lean mice, as previously reported in mice and humans [563]. The treatment with OLE extract showed a trend to increase this population at all doses

tested, suggesting the beneficial role of OLE in modulating the adaptive immune response that is compromised in obesity.

Remarkably, in addition to the metabolic effects shown by OLE, the present study has also demonstrated that oral administration of this extract improved endothelial dysfunction in HFD fed mice, thus corroborating previous studies performed with other OLE in high carbohydrate–, HFD–fed rats [495]. The beneficial effects of OLE on cardiovascular function has been also previously reported in experimental models of hypertesion [564-566] as well as in humans [567], being these hypotensive actions attributed to the presence of specific components in OLE, like oleuropein and its principle colonic metabolite, hydroxytyrosol, due to their antiinflammatory and/or antioxidant properties in the vascular wall [566]. Aortas from untreated HFD-fed mice significantly displayed a reduction in the endothelium-dependent vasodilator responses to acetylcholine in comparison with control group fed the control diet. This altered reponse to acetylcholine is considered as a marker of endothelial dysfunction, which is a hallmark underlying vascular disease that has been previously reported to occur in different conditions, including obesity and diabetes [568, 569]. In the aorta, NO is the major factor accounting for endothelium-dependent relaxation as shown by the almost full inhibitory action of L-NAME in previous studies, and the diminished acetylcholine-induced relaxation indicates an impaired agonist-induced NO bioactivity [15, 29]. In obesity, a key mechanism of endothelial dysfunction involves the vascular production of reactive oxygen species (ROS), particularly superoxide anion, which reacts rapidly with NO and inactivates it [570]. NADPH oxidase, a multi-enzymatic complex formed by gp91phox or its vascular homologues NOX-1 and NOX-4, rac, p22phox, p47phox and p67phox, is considered the major source of superoxide anion in the vascular wall in HFD-fed rodents [571] and in obese humans [572]. Confirming these observations, in the present study, vascular dysfunction was associated with increased NADFPH activity in aortas from untreated obese mice, together with increased aortic expression of the catalytic NADPH oxidase subunits NOX-1 and NOX-4 and the regulatory subunit $p47^{phox}$, being without effect on $p22^{phox}$. The administration of OLE in obese mice inhibited the increased NADPH activity in the aortic tissue and reduced the expression of those subunits that were upregulated in HFD-fed mice. This effect could be involved in the reduction of vascular ROS levels that in generated in aorta in obesity-related

conditions, thus promoting the restoration in the endothelial vascular function observed in OLE-treated obese mice.

It is evident that obesity-associated vascular dysfunction in HFD-fed mice can be considered as another manifestation of the subclinical systemic inflammatory process that characterizes this metabolic condition, which, as commented before, is involved in the onset of insulin resistance [379]. In fact, it has been proposed that a moderate increase in the plasma concentration of the inflammatory mediator LPS occurs during a fat-enriched diet [379], and, consequently, those strategies able to reduce endotoxemia or impair LPS/TLR4 signalling would improve glucose homeostasis [385]. Accordingly, in the present study, increased expression of TLR4 expression was obtained in both liver and aorta tissues in untreated HFD-fed mice when compared with lean groups, thus suggesting the existence of an endotoxemia that promotes a systemic inflammatory status. In accordance with the beneficial effects obtained when metabolic and vascular functions were evaluated, OLE treatment to obese mice resulted in a significant decrease in the expression of TLR4 in both tissues, which confirms the beneficial effect exerted by this extract on the obesity-associated inflammatory status in the experimental model used in the present study.

Furthermore, different studies have proposed that an increase in intestinal permeability is a crucial mechanism for the development of metabolic endotoxemia in obesity [379, 385]. The present results support this suggestion, since altered gut barrier function can occur in the large intestine in untreated obese mice, in which there was a reduced expression of the tight-junction protein occludin, together with a decreased expression of the mucins MUC-2 and MUC-3. OLE administration to obese mice restored the colonic expression of all these proteins. Of note, although there were no significant differences between lean and obese control group, OLE treatment to obese mice significantly increased the expression of ZO-1, an important linker protein in tight junctions that, in association with the transmembrane protein occludin, contributes to maintain the intestinal epithelial integrity [573]. Moreover, the defensive mechanisms of the intestinal epithelial barrier were reinforced in OLE-treated obese mice by increasing the expression of the mucins MUC-2 and MUC-3, primary constituents of the mucus layer in the colon [88], and therefore preventing the access of bacterial components from the intestinal lumen, including LPS, one of the main factors that promote the obesity-associated endotoxemia and the subsequent systemic inflammatory

response and the metabolic alterations in obese mice. Similarly, the beneficial effects exerted by the probiotic *Lactobacillus coryniformis* CECT5711 in HFD-fed mice have been associated with the preservation of the intestinal barrier function [15]. Moreover, the ability showed by OLE to improve intestinal barrier has been previously reported to participate in the intestinal antiinflammatory effects of this extract in experimental models of colitis in mice, since, in addition to increase the colonic expression of the mucin MUC-2 and tight-junction proteins ZO-1 and TFF-3, it ameliorated intestinal permeability when evaluated *in vivo* using the FITC-dextran assay.

The beneficial effects exerted by OLE in obese mice can be ascribed to the presence of different phenolic compounds, as previously reported [528]. Among these, oleuropein constitutes the major component (approximately 80%), and can be considered as one of the active components of OLE. In fact, and supporting this, a previous study demonstrated that n extract from olive leaves with oleuropein as the major component, decreases body weight gain and improves the lipid profiles in the plasma of HFD mice by regulating the expression of genes involved in adipogenesis and thermogenesis in the visceral adipose tissue [496]. However, the additive/synergic effects of the different products that are present in this extract can also play a key role, being difficult to discriminate and correlate each activity with specific compounds.

Finally, and among these effects, there is an increasing interest in considering plant extracts that contain polyphenols, like OLE, as prebiotic products able to modulate gut microbiota composition. Prebiotics are defined as compounds able to promote the selective stimulation of growth and/or activity(ies) of one or a limited number of microbial genus(era)/species in the gut microbiota that confer(s) health benefits to the host [574].

Interestingly, different studies have proposed that the gut microbiota can be considered as a target for the management of different inflammatory conditions, including those located in the gastrointestinal system, like inflammatory bowel disease, and those with systemic manifestations, including hypertension and obesity [19, 20]. When considering obesity, it is well known that an altered composition of gut microbiota occurs, mainly involving the two main groups of dominant beneficial bacteria in the human gut, the Bacteroidetes and the Firmicutes. Two decades ago, Ley et al described for the first time that the relative proportion of Bacteroidetes is decreased in obese people by comparison with lean people, and that this proportion increases with weight loss [63]. This

observation has been also reported in subsequent studies, both in human and in experimental models in rodents [575-577], and confirmed in the present study, since an increase F/B ratio was observed in HFD-mice in comparison with lean mice. OLE treatment to obese mice was able to counteract this altered composition in the gut microbiota, and the proportion of the main bacterial phyla was restored to the normal values observed in control-diet fed mice. This was confirmed in the PCA analysis, since we observed a clear separation between the clusters of OLE-treated obese mice and lean mice in comparison with untreated HFD-fed group, indicating a shift in the gut bacterial composition by the OLE administration, associated with an amelioration in the obesity-associated dysbiosis. Additionally, the significant modifications observed in control HFD-fed mice in the proportions of the different classes or genera belonging to the phyla Actinobacteria, Bacteriodetes and Verrumicrobiota were partially restored in those obese mice treated with OLE. Special attention has been paid to the role of *Akkermansia muciniphila* in obesity, which has been identified as mucin-degrading bacteria that resides in the mucus layer, being considered as the dominant human bacterium that abundantly colonizes this nutrient-rich environment [24]. In fact, it has been reported that *A. muciniphila* may represent 3–5% of the microbial community in healthy subjects [24], and its abundance inversely correlates with body weight and type 1 diabetes in mice and humans [25-28]. In fact, it has been reported that *A. muciniphila* treatment reversed high-fat diet-induced metabolic disorders, including fat-mass gain, metabolic endotoxemia, adipose tissue inflammation, and insulin resistance. Moreover, those treatments that promote a higher abundance of this bacterium have shown to exert beneficial metabolic effects through improving HFD-induced features of the metabolic syndrome in obese mice [578]. In the present study, a reduction in the proportion of the genus *Akkermansia* was observed in untreated control obese mice, and this was reversed after treatment to these mice with OLE. The restoration of the abundance of *Akkermansia sp.* exerted by OLE could be associated to the improvement in the gut barrier function, through the increased production of mucins in the colonic tissue, since these are the main nutrients for these bacteria. In consequence, the improvement in obesity-associated dysbiosis in OLE-treated obese mice may result in modulation of the altered immune response, thus contributing to the beneficial effects observed in this experimental model of metabolic syndrome.

CONCLUSIONS

CONCLUSIONS

1. Olive leaf extract has shown intestinal anti-inflammatory effects in two experimental models of mouse colitis (DSS and DNBS). Several mechanisms appear to be involved, like capacity to restore the intestinal barrier integrity and to modulate the production of inflammatory mediators. This activity was confirmed *in vitro*, where the extract reduced the expression and/or production of proinflammatory cytokines induced by inflammatory stimuli in different immune cell types, including human peripheral blood mononuclear cells. Besides, the extract displayed a direct effect on mucosal explants from CD patients inhibiting the excessive production of the proinflammatory mediators that characterize the disease.

2. Olive leaf extract has exerted beneficial effects in high-fat diet-induced obesity in mice, revealing an improvement in the altered glucose and the lipid metabolism. These effects were associated withamelioration of the systemic inflammatory status, together with restoration of the vascular dysfunction that characterizes obesity. Different mechanisms seem to participate, and include prebiotic properties, which counteract the obesity-associated dysbiosis, and the improvement of the intestinal epithelial barrier function. Moreover, the immunomodulatory effects of this extract can also contribute to the beneficial effects against obesity and its derived complications.

REFERENCES

1. Baumgart, D.C. and W.J. Sandborn, *Inflammatory bowel disease: clinical aspects and established and evolving therapies.* Lancet, 2007. **369**(9573): p. 1641-57.
2. Fiocchi, C., *Inflammatory bowel disease: etiology and pathogenesis.* Gastroenterology, 1998. **115**(1): p. 182-205.
3. Podolsky, D.K., *The current future understanding of inflammatory bowel disease.* Best Pract Res Clin Gastroenterol, 2002. **16**(6): p. 933-43.
4. Abraham, C. and J.H. Cho, *IL-23 and autoimmunity: new insights into the pathogenesis of inflammatory bowel disease.* Annu Rev Med, 2009. **60**: p. 97-110.
5. Chin, A.C. and C.A. Parkos, *Neutrophil transepithelial migration and epithelial barrier function in IBD: potential targets for inhibiting neutrophil trafficking.* Ann N Y Acad Sci, 2006. **1072**: p. 276-87.
6. Sartor, R.B., *Pathogenesis and immune mechanisms of chronic inflammatory bowel diseases.* Am J Gastroenterol, 1997. **92**(12 Suppl): p. 5S-11S.
7. Park, Y.W., et al., *The metabolic syndrome: prevalence and associated risk factor findings in the US population from the Third National Health and Nutrition Examination Survey, 1988-1994.* Arch Intern Med, 2003. **163**(4): p. 427-36.
8. Sur, G., et al., *The relevance of inflammatory markers in metabolic syndrome.* Maedica (Buchar), 2014. **9**(1): p. 15-8.
9. Weizman, A.V., et al., *Characterisation of complementary and alternative medicine use and its impact on medication adherence in inflammatory bowel disease.* Aliment Pharmacol Ther, 2012. **35**(3): p. 342-9.
10. Moro, C.O. and G. Basile, *Obesity and medicinal plants.* Fitoterapia, 2000. **71 Suppl 1**: p. S73-82.
11. Talhaoui, N., et al., *Phenolic compounds and in vitro immunomodulatory properties of three Andalusian olive leaf extracts.* Journal of Functional Foods, 2016. **22**: p. 270-277.
12. Garrido-Mesa, N., et al., *The intestinal anti-inflammatory effect of minocycline in experimental colitis involves both its immunomodulatory and antimicrobial properties.* Pharmacol Res, 2011. **63**(4): p. 308-19.
13. Camuesco, D., et al., *The intestinal anti-inflammatory effect of dersalazine sodium is related to a down-regulation in IL-17 production in experimental models of rodent colitis.* Br J Pharmacol, 2012. **165**(3): p. 729-40.
14. Camuesco, D., et al., *The intestinal anti-inflammatory effect of quercitrin is associated with an inhibition in iNOS expression.* Br J Pharmacol, 2004. **143**(7): p. 908-18.
15. Toral, M., et al., *The probiotic Lactobacillus coryniformis CECT5711 reduces the vascular pro-oxidant and pro-inflammatory status in obese mice.* Clin Sci (Lond), 2014. **127**(1): p. 33-45.
16. Zhang, L., et al., *An improved RT-PCR assay for rapid and sensitive detection of grass carp reovirus.* J Virol Methods, 2010. **169**(1): p. 28-33.
17. Gregor, M.F. and G.S. Hotamisligil, *Inflammatory mechanisms in obesity.* Annu Rev Immunol, 2011. **29**: p. 415-45.
18. Ouchi, N., et al., *Adipokines in inflammation and metabolic disease.* Nat Rev Immunol, 2011. **11**(2): p. 85-97.
19. Bouter, K.E., et al., *Role of the Gut Microbiome in the Pathogenesis of Obesity and Obesity-Related*

Metabolic Dysfunction. Gastroenterology, 2017. **152**(7): p. 1671-1678.
20. McIlroy, J., et al., *Review article: the gut microbiome in inflammatory bowel disease-avenues for microbial management.* Aliment Pharmacol Ther, 2017.
21. Mariat, D., et al., *The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age.* BMC Microbiol, 2009. **9**: p. 123.
22. Sanz, Y. and A. Moya-Perez, *Microbiota, inflammation and obesity.* Adv Exp Med Biol, 2014. **817**: p. 291-317.
23. Youmans, B.P., et al., *Characterization of the human gut microbiome during travelers' diarrhea.* Gut Microbes, 2015. **6**(2): p. 110-9.
24. Derrien, M., et al., *Akkermansia muciniphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium.* Int J Syst Evol Microbiol, 2004. **54**(Pt 5): p. 1469-76.
25. Everard, A., et al., *Responses of gut microbiota and glucose and lipid metabolism to prebiotics in genetic obese and diet-induced leptin-resistant mice.* Diabetes, 2011. **60**(11): p. 2775-86.
26. Everard, A., et al., *Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity.* Proc Natl Acad Sci U S A, 2013. **110**(22): p. 9066-71.
27. Hansen, C.H., et al., *Early life treatment with vancomycin propagates Akkermansia muciniphila and reduces diabetes incidence in the NOD mouse.* Diabetologia, 2012. **55**(8): p. 2285-94.
28. Karlsson, C.L., et al., *The microbiota of the gut in preschool children with normal and excessive body weight.* Obesity (Silver Spring), 2012. **20**(11): p. 2257-61.
29. Diaz-de-Cerio, E., et al., *The hypoglycemic effects of guava leaf (Psidium guajava L.) extract are associated with improving endothelial dysfunction in mice with diet-induced obesity.* Food Res Int, 2017. **96**: p. 64-71.
30. Vucelic, B., *Inflammatory bowel diseases: controversies in the use of diagnostic procedures.* Dig Dis, 2009. **27**(3): p. 269-77.
31. Khor, B., A. Gardet, and R.J. Xavier, *Genetics and pathogenesis of inflammatory bowel disease.* Nature, 2011. **474**(7351): p. 307-17.
32. Xavier, R.J. and D.K. Podolsky, *Unravelling the pathogenesis of inflammatory bowel disease.* Nature, 2007. **448**(7152): p. 427-34.
33. Fiocchi, C., *Inflammatory bowel disease: evolutionary concepts in biology, epidemiology, mechanisms and therapy.* Curr Opin Gastroenterol, 2013. **29**(4): p. 347-9.
34. Triantafillidis, J.K., E. Merikas, and F. Georgopoulos, *Current and emerging drugs for the treatment of inflammatory bowel disease.* Drug Des Devel Ther, 2011. **5**: p. 185-210.
35. Orholm, M., et al., *Familial occurrence of inflammatory bowel disease.* N Engl J Med, 1991. **324**(2): p. 84-8.
36. Barrett, J.C., et al., *Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease.* Nat Genet, 2008. **40**(8): p. 955-62.
37. McGovern, D.P., et al., *Genome-wide association identifies multiple ulcerative colitis susceptibility loci.* Nat Genet, 2010. **42**(4): p. 332-7.
38. Franke, A., et al., *Genome-wide association study for ulcerative colitis identifies risk loci at 7q22 and 22q13 (IL17REL).* Nat Genet, 2010. **42**(4): p. 292-4.

39. Andersen, V., et al., *Assessment of heterogeneity between European Populations: a Baltic and Danish replication case-control study of SNPs from a recent European ulcerative colitis genome wide association study.* BMC Med Genet, 2011. **12**: p. 139.
40. Hugot, J.P., et al., *Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease.* Nature, 2001. **411**(6837): p. 599-603.
41. Ogura, Y., et al., *A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease.* Nature, 2001. **411**(6837): p. 603-6.
42. Zhernakova, A., et al., *Genetic analysis of innate immunity in Crohn's disease and ulcerative colitis identifies two susceptibility loci harboring CARD9 and IL18RAP.* Am J Hum Genet, 2008. **82**(5): p. 1202-10.
43. Yamazaki, K., et al., *Single nucleotide polymorphisms in TNFSF15 confer susceptibility to Crohn's disease.* Hum Mol Genet, 2005. **14**(22): p. 3499-506.
44. Prescott, N.J., et al., *A nonsynonymous SNP in ATG16L1 predisposes to ileal Crohn's disease and is independent of CARD15 and IBD5.* Gastroenterology, 2007. **132**(5): p. 1665-71.
45. Hampe, J., et al., *A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1.* Nat Genet, 2007. **39**(2): p. 207-11.
46. Parkes, M., et al., *Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility.* Nat Genet, 2007. **39**(7): p. 830-2.
47. Kaser, A., et al., *XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease.* Cell, 2008. **134**(5): p. 743-56.
48. Fisher, S.A., et al., *Genetic determinants of ulcerative colitis include the ECM1 locus and five loci implicated in Crohn's disease.* Nat Genet, 2008. **40**(6): p. 710-2.
49. Wiede, F., et al., *T cell protein tyrosine phosphatase attenuates T cell signaling to maintain tolerance in mice.* J Clin Invest, 2011. **121**(12): p. 4758-74.
50. VanDussen, K.L., et al., *Genetic variants synthesize to produce paneth cell phenotypes that define subtypes of Crohn's disease.* Gastroenterology, 2014. **146**(1): p. 200-9.
51. Jostins, L., et al., *Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease.* Nature, 2012. **491**(7422): p. 119-24.
52. Bonen, D.K., et al., *Crohn's disease-associated NOD2 variants share a signaling defect in response to lipopolysaccharide and peptidoglycan.* Gastroenterology, 2003. **124**(1): p. 140-6.
53. Kobayashi, K.S., et al., *Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract.* Science, 2005. **307**(5710): p. 731-4.
54. Wehkamp, J., et al., *NF-kappaB- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by Escherichia coli Nissle 1917: a novel effect of a probiotic bacterium.* Infect Immun, 2004. **72**(10): p. 5750-8.
55. Cuthbert, A.P., et al., *The contribution of NOD2 gene mutations to the risk and site of disease in inflammatory bowel disease.* Gastroenterology, 2002. **122**(4): p. 867-74.
56. Ye, Y., et al., *The epidemiology and risk factors of inflammatory bowel disease.* Int J Clin Exp Med, 2015. **8**(12): p. 22529-42.
57. Strachan, D.P., *Hay fever, hygiene,*

and household size. BMJ, 1989. **299**(6710): p. 1259-60.
58. Yazdanbakhsh, M., P.G. Kremsner, and R. van Ree, *Allergy, parasites, and the hygiene hypothesis.* Science, 2002. **296**(5567): p. 490-4.
59. Feillet, H. and J.F. Bach, *Increased incidence of inflammatory bowel disease: the price of the decline of infectious burden?* Curr Opin Gastroenterol, 2004. **20**(6): p. 560-4.
60. Dave, M., et al., *The human gut microbiome: current knowledge, challenges, and future directions.* Transl Res, 2012. **160**(4): p. 246-57.
61. Qin, J., et al., *A human gut microbial gene catalogue established by metagenomic sequencing.* Nature, 2010. **464**(7285): p. 59-65.
62. Eckburg, P.B., et al., *Diversity of the human intestinal microbial flora.* Science, 2005. **308**(5728): p. 1635-8.
63. Ley, R.E., et al., *Microbial ecology: human gut microbes associated with obesity.* Nature, 2006. **444**(7122): p. 1022-3.
64. Faith, J.J., et al., *The long-term stability of the human gut microbiota.* Science, 2013. **341**(6141): p. 1237439.
65. Martinez, I., C.E. Muller, and J. Walter, *Long-term temporal analysis of the human fecal microbiota revealed a stable core of dominant bacterial species.* PLoS One, 2013. **8**(7): p. e69621.
66. Dominguez-Bello, M.G., et al., *Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns.* Proc Natl Acad Sci U S A, 2010. **107**(26): p. 11971-5.
67. Backhed, F., et al., *The gut microbiota as an environmental factor that regulates fat storage.* Proc Natl Acad Sci U S A, 2004. **101**(44): p. 15718-23.
68. Wirtz, S. and M.F. Neurath, *Mouse models of inflammatory bowel disease.* Adv Drug Deliv Rev, 2007. **59**(11): p. 1073-83.
69. Garrett, W.S., et al., *Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system.* Cell, 2007. **131**(1): p. 33-45.
70. Belkaid, Y., N. Bouladoux, and T.W. Hand, *Effector and memory T cell responses to commensal bacteria.* Trends Immunol, 2013. **34**(6): p. 299-306.
71. Frank, D.N., et al., *Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases.* Proc Natl Acad Sci U S A, 2007. **104**(34): p. 13780-5.
72. Tamboli, C.P., et al., *Dysbiosis as a prerequisite for IBD.* Gut, 2004. **53**(7): p. 1057.
73. Andoh, A., et al., *Multicenter analysis of fecal microbiota profiles in Japanese patients with Crohn's disease.* J Gastroenterol, 2012. **47**(12): p. 1298-307.
74. Morgan, X.C., et al., *Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment.* Genome Biol, 2012. **13**(9): p. R79.
75. Kang, S., et al., *Dysbiosis of fecal microbiota in Crohn's disease patients as revealed by a custom phylogenetic microarray.* Inflamm Bowel Dis, 2010. **16**(12): p. 2034-42.
76. Nicholson, J.K., et al., *Host-gut microbiota metabolic interactions.* Science, 2012. **336**(6086): p. 1262-7.
77. Walker, A.W., et al., *High-throughput clone library analysis of the mucosa-associated microbiota reveals dysbiosis and differences between inflamed and non-inflamed regions of the intestine in inflammatory bowel disease.* BMC Microbiol, 2011. **11**: p. 7.

78. Latella, G. and C. Papi, *Crucial steps in the natural history of inflammatory bowel disease.* World J Gastroenterol, 2012. **18**(29): p. 3790-9.
79. Baumgart, D.C. and W.J. Sandborn, *Crohn's disease.* Lancet, 2012. **380**(9853): p. 1590-605.
80. Ordas, I., et al., *Ulcerative colitis.* Lancet, 2012. **380**(9853): p. 1606-19.
81. Liu, Z., et al., *The increased expression of IL-23 in inflammatory bowel disease promotes intraepithelial and lamina propria lymphocyte inflammatory responses and cytotoxicity.* J Leukoc Biol, 2011. **89**(4): p. 597-606.
82. Spits, H. and T. Cupedo, *Innate lymphoid cells: emerging insights in development, lineage relationships, and function.* Annu Rev Immunol, 2012. **30**: p. 647-75.
83. Buckner, J.H., *Mechanisms of impaired regulation by CD4(+)CD25(+)FOXP3(+) regulatory T cells in human autoimmune diseases.* Nat Rev Immunol, 2010. **10**(12): p. 849-59.
84. Peterson, L.W. and D. Artis, *Intestinal epithelial cells: regulators of barrier function and immune homeostasis.* Nat Rev Immunol, 2014. **14**(3): p. 141-53.
85. Groschwitz, K.R. and S.P. Hogan, *Intestinal barrier function: molecular regulation and disease pathogenesis.* J Allergy Clin Immunol, 2009. **124**(1): p. 3-20; quiz 21-2.
86. Johansson, M.E., J.M. Larsson, and G.C. Hansson, *The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions.* Proc Natl Acad Sci U S A, 2011. **108 Suppl 1**: p. 4659-65.
87. Dharmani, P., et al., *Role of intestinal mucins in innate host defense mechanisms against pathogens.* J Innate Immun, 2009. **1**(2): p. 123-35.
88. Kim, Y.S. and S.B. Ho, *Intestinal goblet cells and mucins in health and disease: recent insights and progress.* Curr Gastroenterol Rep, 2010. **12**(5): p. 319-30.
89. Bischoff, S.C., et al., *Intestinal permeability--a new target for disease prevention and therapy.* BMC Gastroenterol, 2014. **14**: p. 189.
90. Muniz, L.R., C. Knosp, and G. Yeretssian, *Intestinal antimicrobial peptides during homeostasis, infection, and disease.* Front Immunol, 2012. **3**: p. 310.
91. Schneeberger, E.E. and R.D. Lynch, *The tight junction: a multifunctional complex.* Am J Physiol Cell Physiol, 2004. **286**(6): p. C1213-28.
92. Schulzke, J.D., et al., *Epithelial tight junctions in intestinal inflammation.* Ann N Y Acad Sci, 2009. **1165**: p. 294-300.
93. Lee, S.H., *Intestinal permeability regulation by tight junction: implication on inflammatory bowel diseases.* Intest Res, 2015. **13**(1): p. 11-8.
94. Merga, Y., B.J. Campbell, and J.M. Rhodes, *Mucosal barrier, bacteria and inflammatory bowel disease: possibilities for therapy.* Dig Dis, 2014. **32**(4): p. 475-83.
95. Vindigni, S.M., et al., *The intestinal microbiome, barrier function, and immune system in inflammatory bowel disease: a tripartite pathophysiological circuit with implications for new therapeutic directions.* Therap Adv Gastroenterol, 2016. **9**(4): p. 606-25.
96. Johansson, M.E., et al., *The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria.* Proc Natl Acad Sci U S A, 2008. **105**(39): p. 15064-9.
97. Johansson, M.E., et al., *Bacteria penetrate the normally*

impenetrable inner colon mucus layer in both murine colitis models and patients with ulcerative colitis. Gut, 2014. **63**(2): p. 281-91.
98. Moehle, C., et al., *Aberrant intestinal expression and allelic variants of mucin genes associated with inflammatory bowel disease.* J Mol Med (Berl), 2006. **84**(12): p. 1055-66.
99. Theodoratou, E., et al., *The role of glycosylation in IBD.* Nat Rev Gastroenterol Hepatol, 2014. **11**(10): p. 588-600.
100. Aamann, L., E.M. Vestergaard, and H. Gronbaek, *Trefoil factors in inflammatory bowel disease.* World J Gastroenterol, 2014. **20**(12): p. 3223-30.
101. Hooper, L.V. and A.J. Macpherson, *Immune adaptations that maintain homeostasis with the intestinal microbiota.* Nat Rev Immunol, 2010. **10**(3): p. 159-69.
102. Rhodes, J.M., *Mucins and inflammatory bowel disease.* QJM, 1997. **90**(2): p. 79-82.
103. Dorofeyev, A.E., et al., *Mucosal barrier in ulcerative colitis and Crohn's disease.* Gastroenterol Res Pract, 2013. **2013**: p. 431231.
104. Dupaul-Chicoine, J., M. Dagenais, and M. Saleh, *Crosstalk between the intestinal microbiota and the innate immune system in intestinal homeostasis and inflammatory bowel disease.* Inflamm Bowel Dis, 2013. **19**(10): p. 2227-37.
105. Png, C.W., et al., *Mucolytic bacteria with increased prevalence in IBD mucosa augment in vitro utilization of mucin by other bacteria.* Am J Gastroenterol, 2010. **105**(11): p. 2420-8.
106. Michielan, A. and R. D'Inca, *Intestinal Permeability in Inflammatory Bowel Disease: Pathogenesis, Clinical Evaluation, and Therapy of Leaky Gut.* Mediators Inflamm, 2015. **2015**: p. 628157.
107. Ulluwishewa, D., et al., *Regulation of tight junction permeability by intestinal bacteria and dietary components.* J Nutr, 2011. **141**(5): p. 769-76.
108. Klapproth, J.M. and M. Sasaki, *Bacterial induction of proinflammatory cytokines in inflammatory bowel disease.* Inflamm Bowel Dis, 2010. **16**(12): p. 2173-9.
109. Heller, F., et al., *Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution.* Gastroenterology, 2005. **129**(2): p. 550-64.
110. Gibson, P.R., *Increased gut permeability in Crohn's disease: is TNF the link?* Gut, 2004. **53**(12): p. 1724-5.
111. Hering, N.A., M. Fromm, and J.D. Schulzke, *Determinants of colonic barrier function in inflammatory bowel disease and potential therapeutics.* J Physiol, 2012. **590**(5): p. 1035-44.
112. Medzhitov, R. and C. Janeway, Jr., *Innate immunity.* N Engl J Med, 2000. **343**(5): p. 338-44.
113. Smith, P.D., C. Ochsenbauer-Jambor, and L.E. Smythies, *Intestinal macrophages: unique effector cells of the innate immune system.* Immunol Rev, 2005. **206**: p. 149-59.
114. Wahl, S.M., et al., *Transforming growth factor type beta induces monocyte chemotaxis and growth factor production.* Proc Natl Acad Sci U S A, 1987. **84**(16): p. 5788-92.
115. Hart, A.L., et al., *Characteristics of intestinal dendritic cells in inflammatory bowel diseases.* Gastroenterology, 2005. **129**(1): p. 50-65.
116. Varol, C., E. Zigmond, and S. Jung, *Securing the immune tightrope: mononuclear phagocytes in the intestinal lamina propria.* Nat Rev Immunol, 2010. **10**(6): p. 415-26.

117. Rugtveit, J., A. Bakka, and P. Brandtzaeg, *Differential distribution of B7.1 (CD80) and B7.2 (CD86) costimulatory molecules on mucosal macrophage subsets in human inflammatory bowel disease (IBD).* Clin Exp Immunol, 1997. **110**(1): p. 104-13.
118. Neurath, M.F., et al., *Cytokine gene transcription by NF-kappa B family members in patients with inflammatory bowel disease.* Ann N Y Acad Sci, 1998. **859**: p. 149-59.
119. Kamada, N., et al., *Unique CD14 intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-gamma axis.* J Clin Invest, 2008. **118**(6): p. 2269-80.
120. Reinecker, H.C., et al., *Enhanced secretion of tumour necrosis factor-alpha, IL-6, and IL-1 beta by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease.* Clin Exp Immunol, 1993. **94**(1): p. 174-81.
121. Reimund, J.M., et al., *Increased production of tumour necrosis factor-alpha interleukin-1 beta, and interleukin-6 by morphologically normal intestinal biopsies from patients with Crohn's disease.* Gut, 1996. **39**(5): p. 684-9.
122. Niess, J.H., et al., *CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance.* Science, 2005. **307**(5707): p. 254-8.
123. Lelouard, H., et al., *Peyer's patch dendritic cells sample antigens by extending dendrites through M cell-specific transcellular pores.* Gastroenterology, 2012. **142**(3): p. 592-601 e3.
124. Baumgart, D.C. and S.R. Carding, *Inflammatory bowel disease: cause and immunobiology.* Lancet, 2007. **369**(9573): p. 1627-40.
125. Zhang, S.Z., X.H. Zhao, and D.C. Zhang, *Cellular and molecular immunopathogenesis of ulcerative colitis.* Cell Mol Immunol, 2006. **3**(1): p. 35-40.
126. Karin, M. and Y. Ben-Neriah, *Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity.* Annu Rev Immunol, 2000. **18**: p. 621-63.
127. Caamano, J. and C.A. Hunter, *NF-kappaB family of transcription factors: central regulators of innate and adaptive immune functions.* Clin Microbiol Rev, 2002. **15**(3): p. 414-29.
128. Cella, M., H. Miller, and C. Song, *Beyond NK cells: the expanding universe of innate lymphoid cells.* Front Immunol, 2014. **5**: p. 282.
129. Hall, L.J., et al., *Natural killer cells protect mice from DSS-induced colitis by regulating neutrophil function via the NKG2A receptor.* Mucosal Immunol, 2013. **6**(5): p. 1016-26.
130. Zenewicz, L.A., et al., *Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease.* Immunity, 2008. **29**(6): p. 947-57.
131. Huang, Y. and Z. Chen, *Inflammatory bowel disease related innate immunity and adaptive immunity.* Am J Transl Res, 2016. **8**(6): p. 2490-7.
132. Kidd, P., *Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease.* Altern Med Rev, 2003. **8**(3): p. 223-46.
133. Romagnani, S., *Lymphokine production by human T cells in disease states.* Annu Rev Immunol, 1994. **12**: p. 227-57.
134. Korn, T., et al., *IL-17 and Th17 Cells.* Annu Rev Immunol, 2009. **27**: p. 485-517.
135. Podolsky, *The current future understanding of inflammatory bowel disease.* Best Practice & Research Clinical Gastroenterology, 2002. **16(6)**: p. 933-43.
136. Monteleone, G., et al., *Bioactive IL-18 expression is up-regulated in*

Crohn's disease. J Immunol, 1999. **163**(1): p. 143-7.
137. Monteleone, G., et al., *Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells.* Gastroenterology, 1997. **112**(4): p. 1169-78.
138. Breese, E., et al., *Interleukin-2- and interferon-gamma-secreting T cells in normal and diseased human intestinal mucosa.* Immunology, 1993. **78**(1): p. 127-31.
139. Fuss, I.J., et al., *Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5.* J Immunol, 1996. **157**(3): p. 1261-70.
140. Rovedatti, L., et al., *Differential regulation of interleukin 17 and interferon gamma production in inflammatory bowel disease.* Gut, 2009. **58**(12): p. 1629-36.
141. Vainer, B., et al., *Colonic expression and synthesis of interleukin 13 and interleukin 15 in inflammatory bowel disease.* Cytokine, 2000. **12**(10): p. 1531-6.
142. Kadivar, K., et al., *Intestinal interleukin-13 in pediatric inflammatory bowel disease patients.* Inflamm Bowel Dis, 2004. **10**(5): p. 593-8.
143. Wilson, M.S., et al., *Colitis and intestinal inflammation in IL10-/- mice results from IL-13Ralpha2-mediated attenuation of IL-13 activity.* Gastroenterology, 2011. **140**(1): p. 254-64.
144. Fouser, L.A., et al., *Th17 cytokines and their emerging roles in inflammation and autoimmunity.* Immunol Rev, 2008. **226**: p. 87-102.
145. Fujino, S., et al., *Increased expression of interleukin 17 in inflammatory bowel disease.* Gut, 2003. **52**(1): p. 65-70.
146. Nielsen, O.H., et al., *Upregulation of interleukin-12 and -17 in active inflammatory bowel disease.* Scand J Gastroenterol, 2003. **38**(2): p. 180-5.
147. Andoh, A., et al., *Interleukin-22, a member of the IL-10 subfamily, induces inflammatory responses in colonic subepithelial myofibroblasts.* Gastroenterology, 2005. **129**(3): p. 969-84.
148. Brand, S., et al., *IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration.* Am J Physiol Gastrointest Liver Physiol, 2006. **290**(4): p. G827-38.
149. Wolk, K., et al., *IL-22 induces lipopolysaccharide-binding protein in hepatocytes: a potential systemic role of IL-22 in Crohn's disease.* J Immunol, 2007. **178**(9): p. 5973-81.
150. Stockinger, B., M. Veldhoen, and B. Martin, *Th17 T cells: linking innate and adaptive immunity.* Semin Immunol, 2007. **19**(6): p. 353-61.
151. Wynn, T.A., *T(H)-17: a giant step from T(H)1 and T(H)2.* Nat Immunol, 2005. **6**(11): p. 1069-70.
152. Matsuzaki, G. and M. Umemura, *Interleukin-17 as an effector molecule of innate and acquired immunity against infections.* Microbiol Immunol, 2007. **51**(12): p. 1139-47.
153. Valencia, X., et al., *TNF downmodulates the function of human CD4+CD25hi T-regulatory cells.* Blood, 2006. **108**(1): p. 253-61.
154. O'Garra, A. and P. Vieira, *Regulatory T cells and mechanisms of immune system control.* Nat Med, 2004. **10**(8): p. 801-5.
155. Boden, E.K. and S.B. Snapper, *Regulatory T cells in inflammatory bowel disease.* Curr Opin

Gastroenterol, 2008. **24**(6): p. 733-41.
156. Kitani, A. and L. Xu, *Regulatory T cells and the induction of IL-17.* Mucosal Immunol, 2008. **1 Suppl 1**: p. S43-6.
157. Geremia, A., et al., *Innate and adaptive immunity in inflammatory bowel disease.* Autoimmun Rev, 2014. **13**(1): p. 3-10.
158. Elson, C.O., et al., *Monoclonal anti-interleukin 23 reverses active colitis in a T cell-mediated model in mice.* Gastroenterology, 2007. **132**(7): p. 2359-70.
159. Cerutti, A., I. Puga, and M. Cols, *Innate control of B cell responses.* Trends Immunol, 2011. **32**(5): p. 202-11.
160. Pabst, O., *New concepts in the generation and functions of IgA.* Nat Rev Immunol, 2012. **12**(12): p. 821-32.
161. Cerutti, A., *The regulation of IgA class switching.* Nat Rev Immunol, 2008. **8**(6): p. 421-34.
162. Mizoguchi, A., et al., *Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation.* Immunity, 2002. **16**(2): p. 219-30.
163. Fillatreau, S., et al., *B cells regulate autoimmunity by provision of IL-10.* Nat Immunol, 2002. **3**(10): p. 944-50.
164. Oka, A., et al., *Role of regulatory B cells in chronic intestinal inflammation: association with pathogenesis of Crohn's disease.* Inflamm Bowel Dis, 2014. **20**(2): p. 315-28.
165. Sattler, S., et al., *IL-10-producing regulatory B cells induced by IL-33 (Breg(IL-33)) effectively attenuate mucosal inflammatory responses in the gut.* J Autoimmun, 2014. **50**: p. 107-22.
166. Batista, F.D. and N.E. Harwood, *The who, how and where of antigen presentation to B cells.* Nat Rev Immunol, 2009. **9**(1): p. 15-27.
167. Jego, G., et al., *Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6.* Immunity, 2003. **19**(2): p. 225-34.
168. Shang, L., et al., *Toll-like receptor signaling in small intestinal epithelium promotes B-cell recruitment and IgA production in lamina propria.* Gastroenterology, 2008. **135**(2): p. 529-38.
169. Craxton, A., et al., *Macrophage- and dendritic cell--dependent regulation of human B-cell proliferation requires the TNF family ligand BAFF.* Blood, 2003. **101**(11): p. 4464-71.
170. Olson, T.S., et al., *Expanded B cell population blocks regulatory T cells and exacerbates ileitis in a murine model of Crohn disease.* J Clin Invest, 2004. **114**(3): p. 389-98.
171. Noronha, A.M., et al., *Hyperactivated B cells in human inflammatory bowel disease.* J Leukoc Biol, 2009. **86**(4): p. 1007-16.
172. Klotz, U., *The role of aminosalicylates at the beginning of the new millennium in the treatment of chronic inflammatory bowel disease.* Eur J Clin Pharmacol, 2000. **56**(5): p. 353-62.
173. Stein, R.B. and S.B. Hanauer, *Comparative tolerability of treatments for inflammatory bowel disease.* Drug Saf, 2000. **23**(5): p. 429-48.
174. Egan, L.J., et al., *Inhibition of interleukin-1-stimulated NF-kappaB RelA/p65 phosphorylation by mesalamine is accompanied by decreased transcriptional activity.* J Biol Chem, 1999. **274**(37): p. 26448-53.
175. Weber, C.K., et al., *Suppression of NF-kappaB activity by sulfasalazine is mediated by direct inhibition of IkappaB kinases alpha and beta.*

Gastroenterology, 2000. **119**(5): p. 1209-18.
176. Nikolaus, S., U. Folscn, and S. Schreiber, *Immunopharmacology of 5-aminosalicylic acid and of glucocorticoids in the therapy of inflammatory bowel disease.* Hepatogastroenterology, 2000. **47**(31): p. 71-82.
177. Allgayer, H. and W. Kruis, *Aminosalicylates: potential antineoplastic actions in colon cancer prevention.* Scand J Gastroenterol, 2002. **37**(2): p. 125-31.
178. Travis, S.P., et al., *European evidence based consensus on the diagnosis and management of Crohn's disease: current management.* Gut, 2006. **55 Suppl 1**: p. i16-35.
179. Ito, K., K.F. Chung, and I.M. Adcock, *Update on glucocorticoid action and resistance.* J Allergy Clin Immunol, 2006. **117**(3): p. 522-43.
180. Aceituno, M., et al., *Steroid-refractory ulcerative colitis: predictive factors of response to cyclosporine and validation in an independent cohort.* Inflamm Bowel Dis, 2008. **14**(3): p. 347-52.
181. Shibolet, O., et al., *Cyclosporine A for induction of remission in severe ulcerative colitis.* Cochrane Database Syst Rev, 2005(1): p. CD004277.
182. Nayar, M. and J.M. Rhodes, *Management of inflammatory bowel disease.* Postgrad Med J, 2004. **80**(942): p. 206-13.
183. Schroll, S., et al., *Effects of azathioprine and its metabolites on repair mechanisms of the intestinal epithelium in vitro.* Regul Pept, 2005. **131**(1-3): p. 1-11.
184. Kozuch, P.L. and S.B. Hanauer, *Treatment of inflammatory bowel disease: a review of medical therapy.* World J Gastroenterol, 2008. **14**(3): p. 354-77.
185. Sandborn, W.J. and W.J. Tremaine, *Cyclosporine treatment of inflammatory bowel disease.* Mayo Clin Proc, 1992. **67**(10): p. 981-90.
186. Bermejo, F., et al., *Acute pancreatitis in inflammatory bowel disease, with special reference to azathioprine-induced pancreatitis.* Aliment Pharmacol Ther, 2008. **28**(5): p. 623-8.
187. Rayner, C.K., et al., *Long-term results of low-dose intravenous ciclosporin for acute severe ulcerative colitis.* Aliment Pharmacol Ther, 2003. **18**(3): p. 303-8.
188. Olesen, C.M., et al., *Mechanisms behind efficacy of tumor necrosis factor inhibitors in inflammatory bowel diseases.* Pharmacol Ther, 2016. **159**: p. 110-9.
189. Razanskaite, V., et al., *Biosimilar Infliximab in Inflammatory Bowel Disease: Outcomes of a Managed Switching Programme.* J Crohns Colitis, 2017. **11**(6): p. 690-696.
190. Dulai, P.S., et al., *Systematic review: Monotherapy with antitumour necrosis factor alpha agents versus combination therapy with an immunosuppressive for IBD.* Gut, 2014. **63**(12): p. 1843-53.
191. Lugering, A., et al., *Apoptosis as a therapeutic tool in IBD?* Ann N Y Acad Sci, 2006. **1072**: p. 62-77.
192. Hanauer, S.B., et al., *Human anti-tumor necrosis factor monoclonal antibody (adalimumab) in Crohn's disease: the CLASSIC-I trial.* Gastroenterology, 2006. **130**(2): p. 323-33; quiz 591.
193. Colombel, J.F., et al., *Four-year maintenance treatment with adalimumab in patients with moderately to severely active ulcerative colitis: Data from ULTRA 1, 2, and 3.* Am J Gastroenterol, 2014. **109**(11): p. 1771-80.
194. Shipman, L., *Rheumatoid arthritis: Tocilizumab and the risk of intestinal perforation.* Nat Rev Rheumatol, 2016. **12**(9): p. 499.
195. Neurath, M.F., *Current and emerging therapeutic targets for*

IBD. Nat Rev Gastroenterol Hepatol, 2017. **14**(5): p. 269-278.
196. Reinisch, W., et al., *Fontolizumab in moderate to severe Crohn's disease: a phase 2, randomized, double-blind, placebo-controlled, multiple-dose study.* Inflamm Bowel Dis, 2010. **16**(2): p. 233-42.
197. Mannon, P.J., et al., *Anti-interleukin-12 antibody for active Crohn's disease.* N Engl J Med, 2004. **351**(20): p. 2069-79.
198. Ardizzone, S. and G. Bianchi Porro, *Inflammatory bowel disease: new insights into pathogenesis and treatment.* J Intern Med, 2002. **252**(6): p. 475-96.
199. Creed, T.J., et al., *Basiliximab (anti-CD25) in combination with steroids may be an effective new treatment for steroid-resistant ulcerative colitis.* Aliment Pharmacol Ther, 2003. **18**(1): p. 65-75.
200. Van Assche, G., *A pilot study on the use of the humanized anti–interleukin-2 receptor antibody daclizumab in active ulcerative colitis.* The American Journal of Gastroenterology, 2003. **98**(2): p. 369-376.
201. Sakuraba, A., et al., *Novel Topics in Inflammatory Bowel Disease.* Biomed Res Int, 2016. **2016**: p. 8958751.
202. Siegel, C.A., *Review article: explaining risks of inflammatory bowel disease therapy to patients.* Aliment Pharmacol Ther, 2011. **33**(1): p. 23-32.
203. Burgmann, T., P. Rawsthorne, and C.N. Bernstein, *Predictors of alternative and complementary medicine use in inflammatory bowel disease: do measures of conventional health care utilization relate to use?* Am J Gastroenterol, 2004. **99**(5): p. 889-93.
204. Singh, U.P., et al., *Alternative medicines as emerging therapies for inflammatory bowel diseases.* Int Rev Immunol, 2012. **31**(1): p. 66-84.
205. Carty, E. and D.S. Rampton, *Evaluation of new therapies for inflammatory bowel disease.* Br J Clin Pharmacol, 2003. **56**(4): p. 351-61.
206. Vezza, T., et al., *Flavonoids in Inflammatory Bowel Disease: A Review.* Nutrients, 2016. **8**(4): p. 211.
207. Clifford, M.N., *Diet-derived phenols in plasma and tissues and their implications for health.* Planta Med, 2004. **70**(12): p. 1103-14.
208. Mizoguchi, A., *Animal models of inflammatory bowel disease.* Prog Mol Biol Transl Sci, 2012. **105**: p. 263-320.
209. Sanchez de Medina, F., et al., *Effect of quercitrin on acute and chronic experimental colitis in the rat.* J Pharmacol Exp Ther, 1996. **278**(2): p. 771-9.
210. Bruckner, M., et al., *Green tea polyphenol epigallocatechin-3-gallate shows therapeutic antioxidative effects in a murine model of colitis.* J Crohns Colitis, 2012. **6**(2): p. 226-35.
211. Oz, H.S., T. Chen, and W.J. de Villiers, *Green Tea Polyphenols and Sulfasalazine have Parallel Anti-Inflammatory Properties in Colitis Models.* Front Immunol, 2013. **4**: p. 132.
212. Ren, G., et al., *The anti-inflammatory effect and potential mechanism of cardamonin in DSS-induced colitis.* Am J Physiol Gastrointest Liver Physiol, 2015. **309**(7): p. G517-27.
213. Dou, W., et al., *Chrysin ameliorates chemically induced colitis in the mouse through modulation of a PXR/NF-kappaB signaling pathway.* J Pharmacol Exp Ther, 2013. **345**(3): p. 473-82.
214. Kwon, H.S., S.M. Oh, and J.K. Kim, *Glabridin, a functional compound of liquorice, attenuates colonic inflammation in mice with dextran sulphate sodium-induced colitis.*

Clin Exp Immunol, 2008. **151**(1): p. 165-73.
215. Kwon, K.H., et al., *Dietary rutin, but not its aglycone quercetin, ameliorates dextran sulfate sodium-induced experimental colitis in mice: attenuation of pro-inflammatory gene expression.* Biochem Pharmacol, 2005. **69**(3): p. 395-406.
216. Azuma, T., et al., *Supplemental naringenin prevents intestinal barrier defects and inflammation in colitic mice.* J Nutr, 2013. **143**(6): p. 827-34.
217. Cruz, T., et al., *Oral administration of rutoside can ameliorate inflammatory bowel disease in rats.* Life Sci, 1998. **62**(7): p. 687-95.
218. Galvez, J., et al., *Rutoside as mucosal protective in acetic acid-induced rat colitis.* Planta Med, 1997. **63**(5): p. 409-14.
219. Rezaie, A., R.D. Parker, and M. Abdollahi, *Oxidative stress and pathogenesis of inflammatory bowel disease: an epiphenomenon or the cause?* Dig Dis Sci, 2007. **52**(9): p. 2015-21.
220. Piechota-Polanczyk, A. and J. Fichna, *Review article: the role of oxidative stress in pathogenesis and treatment of inflammatory bowel diseases.* Naunyn Schmiedebergs Arch Pharmacol, 2014. **387**(7): p. 605-20.
221. Achitei, D., et al., *Different profile of peripheral antioxidant enzymes and lipid peroxidation in active and non-active inflammatory bowel disease patients.* Dig Dis Sci, 2013. **58**(5): p. 1244-9.
222. Mariani, F., P. Sena, and L. Roncucci, *Inflammatory pathways in the early steps of colorectal cancer development.* World J Gastroenterol, 2014. **20**(29): p. 9716-31.
223. Alzoghaibi, M.A., *Concepts of oxidative stress and antioxidant defense in Crohn's disease.* World J Gastroenterol, 2013. **19**(39): p. 6540-7.
224. Pavlick, K.P., et al., *Role of reactive metabolites of oxygen and nitrogen in inflammatory bowel disease.* Free Radic Biol Med, 2002. **33**(3): p. 311-22.
225. Veljaca, M., et al., *BPC-15 reduces trinitrobenzene sulfonic acid-induced colonic damage in rats.* J Pharmacol Exp Ther, 1995. **272**(1): p. 417-22.
226. Sanchez de Medina, F., et al., *Effect of quercitrin on the early stages of hapten induced colonic inflammation in the rat.* Life Sci, 2002. **70**(26): p. 3097-108.
227. Seibel, J., et al., *Oral treatment with genistein reduces the expression of molecular and biochemical markers of inflammation in a rat model of chronic TNBS-induced colitis.* Eur J Nutr, 2009. **48**(4): p. 213-20.
228. Dou, W., et al., *Protective effect of naringenin against experimental colitis via suppression of Toll-like receptor 4/NF-kappaB signalling.* Br J Nutr, 2013. **110**(4): p. 599-608.
229. Al-Rejaie, S.S., et al., *Protective effect of naringenin on acetic acid-induced ulcerative colitis in rats.* World J Gastroenterol, 2013. **19**(34): p. 5633-44.
230. Mascaraque, C., et al., *Rutin has intestinal antiinflammatory effects in the CD4+ CD62L+ T cell transfer model of colitis.* Pharmacol Res, 2014. **90**: p. 48-57.
231. Alderton, W.K., C.E. Cooper, and R.G. Knowles, *Nitric oxide synthases: structure, function and inhibition.* Biochem J, 2001. **357**(Pt 3): p. 593-615.
232. Korhonen, R., et al., *Nitric oxide production and signaling in inflammation.* Curr Drug Targets Inflamm Allergy, 2005. **4**(4): p. 471-9.
233. Miller, M.J. and M. Sandoval, *Nitric Oxide. III. A molecular prelude to intestinal inflammation.* Am J

Physiol, 1999. **276**(4 Pt 1): p. G795-9.
234. Sichel, G., et al., *In vitro scavenger activity of some flavonoids and melanins against O2-(.).* Free Radic Biol Med, 1991. **11**(1): p. 1-8.
235. Haenen, G.R., et al., *Peroxynitrite scavenging by flavonoids.* Biochem Biophys Res Commun, 1997. **236**(3): p. 591-3.
236. Comalada, M., et al., *Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: analysis of the structure-activity relationship.* Biochem Pharmacol, 2006. **72**(8): p. 1010-21.
237. Hamalainen, M., et al., *Anti-inflammatory effects of flavonoids: genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-kappaB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-kappaB activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages.* Mediators Inflamm, 2007. **2007**: p. 45673.
238. Surh, Y.J., et al., *Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-kappa B activation.* Mutat Res, 2001. **480-481**: p. 243-68.
239. Dubois, R.N., et al., *Cyclooxygenase in biology and disease.* FASEB J, 1998. **12**(12): p. 1063-73.
240. Morita, I., *Distinct functions of COX-1 and COX-2.* Prostaglandins Other Lipid Mediat, 2002. **68-69**: p. 165-75.
241. Willoughby, D.A., A.R. Moore, and P.R. Colville-Nash, *COX-1, COX-2, and COX-3 and the future treatment of chronic inflammatory disease.* Lancet, 2000. **355**(9204): p. 646-8.
242. Serra, D., et al., *Cyanidin-3-glucoside suppresses cytokine-induced inflammatory response in human intestinal cells: comparison with 5-aminosalicylic acid.* PLoS One, 2013. **8**(9): p. e73001.
243. Sharon, P. and W.F. Stenson, *Enhanced synthesis of leukotriene B4 by colonic mucosa in inflammatory bowel disease.* Gastroenterology, 1984. **86**(3): p. 453-60.
244. Fretland, D.J., et al., *Effect of the leukotriene B4 receptor antagonist SC-41930 on colonic inflammation in rat, guinea pig and rabbit.* J Pharmacol Exp Ther, 1990. **255**(2): p. 572-6.
245. Bertran, X., et al., *Intracolonic administration of zileuton, a selective 5-lipoxygenase inhibitor, accelerates healing in a rat model of chronic colitis.* Gut, 1996. **38**(6): p. 899-904.
246. Ocete, M.A., et al., *Effects of morin on an experimental model of acute colitis in rats.* Pharmacology, 1998. **57**(5): p. 261-70.
247. Shin, E.K., et al., *Chrysin, a natural flavone, improves murine inflammatory bowel diseases.* Biochem Biophys Res Commun, 2009. **381**(4): p. 502-7.
248. Comalada, M., et al., *In vivo quercitrin anti-inflammatory effect involves release of quercetin, which inhibits inflammation through down-regulation of the NF-kappaB pathway.* Eur J Immunol, 2005. **35**(2): p. 584-92.
249. Cui, L., et al., *The anti-inflammation effect of baicalin on experimental colitis through inhibiting TLR4/NF-kappaB pathway activation.* Int Immunopharmacol, 2014. **23**(1): p. 294-303.
250. Kwon, K.H., et al., *In vitro and in vivo effects of macrophage-stimulatory polysaccharide from leaves of Perilla frutescens var.*

crispa. Biol Pharm Bull, 2002. **25**(3): p. 367-71.
251. Romier, B., et al., *Modulation of signalling nuclear factor-kappaB activation pathway by polyphenols in human intestinal Caco-2 cells.* Br J Nutr, 2008. **100**(3): p. 542-51.
252. Melgarejo, E., et al., *Targeting of histamine producing cells by EGCG: a green dart against inflammation?* J Physiol Biochem, 2010. **66**(3): p. 265-70.
253. Yamaji, O., et al., *The development of colitogenic CD4(+) T cells is regulated by IL-7 in collaboration with NK cell function in a murine model of colitis.* J Immunol, 2012. **188**(6): p. 2524-36.
254. Tao, F., et al., *Inhibition of Th1/Th17 responses via suppression of STAT1 and STAT3 activation contributes to the amelioration of murine experimental colitis by a natural flavonoid glucoside icariin.* Biochem Pharmacol, 2013. **85**(6): p. 798-807.
255. Song, B., et al., *Suppressive effects of fisetin on mice T lymphocytes in vitro and in vivo.* J Surg Res, 2013. **185**(1): p. 399-409.
256. Xu, J., et al., *Stat4 is critical for the balance between Th17 cells and regulatory T cells in colitis.* J Immunol, 2011. **186**(11): p. 6597-606.
257. Grip, O., S. Janciauskiene, and S. Lindgren, *Macrophages in inflammatory bowel disease.* Curr Drug Targets Inflamm Allergy, 2003. **2**(2): p. 155-60.
258. Grisham, M.B., et al., *Nitric oxide and chronic gut inflammation: controversies in inflammatory bowel disease.* J Investig Med, 2002. **50**(4): p. 272-83.
259. Kim, H.P., et al., *Anti-inflammatory plant flavonoids and cellular action mechanisms.* J Pharmacol Sci, 2004. **96**(3): p. 229-45.
260. Hoesel, B. and J.A. Schmid, *The complexity of NF-kappaB signaling in inflammation and cancer.* Mol Cancer, 2013. **12**: p. 86.
261. Pedersen, J., et al., *Inflammatory pathways of importance for management of inflammatory bowel disease.* World J Gastroenterol, 2014. **20**(1): p. 64-77.
262. Coskun, M., et al., *Involvement of JAK/STAT signaling in the pathogenesis of inflammatory bowel disease.* Pharmacol Res, 2013. **76**: p. 1-8.
263. Roy, P.K., et al., *Role of the JNK signal transduction pathway in inflammatory bowel disease.* World J Gastroenterol, 2008. **14**(2): p. 200-2.
264. Niederberger, E. and G. Geisslinger, *Proteomics and NF-kappaB: an update.* Expert Rev Proteomics, 2013. **10**(2): p. 189-204.
265. Dou, W., et al., *Mangiferin attenuates the symptoms of dextran sulfate sodium-induced colitis in mice via NF-kappaB and MAPK signaling inactivation.* Int Immunopharmacol, 2014. **23**(1): p. 170-8.
266. Dou, W., et al., *Alleviation of gut inflammation by Cdx2/Pxr pathway in a mouse model of chemical colitis.* PLoS One, 2012. **7**(7): p. e36075.
267. Vecchi Brumatti, L., et al., *Curcumin and inflammatory bowel disease: potential and limits of innovative treatments.* Molecules, 2014. **19**(12): p. 21127-53.
268. Yu, F.Y., et al., *Effects of baicalin in CD4 + CD29 + T cell subsets of ulcerative colitis patients.* World J Gastroenterol, 2014. **20**(41): p. 15299-309.
269. Jeffrey, K.L., et al., *Targeting dual-specificity phosphatases: manipulating MAP kinase signalling and immune responses.* Nat Rev Drug Discov, 2007. **6**(5): p. 391-403.
270. Broom, O.J., R. Massoumi, and A. Sjolander, *Alpha2beta1 integrin*

signalling enhances cyclooxygenase-2 expression in intestinal epithelial cells. J Cell Physiol, 2006. **209**(3): p. 950-8.
271. Ahn, M.R., et al., *Antioxidant activity and constituents of propolis collected in various areas of Korea.* J Agric Food Chem, 2004. **52**(24): p. 7286-92.
272. Stark, G.R. and J.E. Darnell, Jr., *The JAK-STAT pathway at twenty.* Immunity, 2012. **36**(4): p. 503-14.
273. O'Shea, J.J. and R. Plenge, *JAK and STAT signaling molecules in immunoregulation and immune-mediated disease.* Immunity, 2012. **36**(4): p. 542-50.
274. Levy, D.E. and J.E. Darnell, Jr., *Stats: transcriptional control and biological impact.* Nat Rev Mol Cell Biol, 2002. **3**(9): p. 651-62.
275. Lee, I.T., et al., *Protective effects of (-)-epigallocatechin-3-gallate against TNF-alpha-induced lung inflammation via ROS-dependent ICAM-1 inhibition.* J Nutr Biochem, 2013. **24**(1): p. 124-36.
276. Liu, S., et al., *Inhibition of pancreatic lipase, alpha-glucosidase, alpha-amylase, and hypolipidemic effects of the total flavonoids from Nelumbo nucifera leaves.* J Ethnopharmacol, 2013. **149**(1): p. 263-9.
277. Schreiber, S., et al., *Activation of signal transducer and activator of transcription (STAT) 1 in human chronic inflammatory bowel disease.* Gut, 2002. **51**(3): p. 379-85.
278. Teshima, C.W., L.A. Dieleman, and J.B. Meddings, *Abnormal intestinal permeability in Crohn's disease pathogenesis.* Ann N Y Acad Sci, 2012. **1258**: p. 159-65.
279. Blumberg, R.S., L.J. Saubermann, and W. Strober, *Animal models of mucosal inflammation and their relation to human inflammatory bowel disease.* Curr Opin Immunol, 1999. **11**(6): p. 648-56.
280. Mankertz, J. and J.D. Schulzke, *Altered permeability in inflammatory bowel disease: pathophysiology and clinical implications.* Curr Opin Gastroenterol, 2007. **23**(4): p. 379-83.
281. Vivinus-Nebot, M., et al., *Functional bowel symptoms in quiescent inflammatory bowel diseases: role of epithelial barrier disruption and low-grade inflammation.* Gut, 2014. **63**(5): p. 744-52.
282. Crespo, M.E., et al., *Anti-inflammatory activity of diosmin and hesperidin in rat colitis induced by TNBS.* Planta Med, 1999. **65**(7): p. 651-3.
283. Di Carlo, G., et al., *Inhibition of intestinal motility and secretion by flavonoids in mice and rats: structure-activity relationships.* J Pharm Pharmacol, 1993. **45**(12): p. 1054-9.
284. Galvez, J., et al., *Inhibitory effects of quercetin on guinea-pig ileum contractions.* Phytother Res 1996. **10**: p. 66-69.
285. Meli, R., et al., *Inhibitory action of quercetin on itestinal transit time.* Phytother Res 1990. **4**: p. 201-202.
286. Kong, J., et al., *Novel role of the vitamin D receptor in maintaining the integrity of the intestinal mucosal barrier.* Am J Physiol Gastrointest Liver Physiol, 2008. **294**(1): p. G208-16.
287. Iwaya, H., et al., *Mucosal permeability is an intrinsic factor in susceptibility to dextran sulfate sodium-induced colitis in rats.* Exp Biol Med (Maywood), 2012. **237**(4): p. 451-60.
288. Noda, S., S. Tanabe, and T. Suzuki, *Differential effects of flavonoids on barrier integrity in human intestinal Caco-2 cells.* J Agric Food Chem, 2012. **60**(18): p. 4628-33.
289. Suzuki, T., N. Yoshinaga, and S. Tanabe, *Interleukin-6 (IL-6) regulates claudin-2 expression and*

tight junction permeability in intestinal epithelium. J Biol Chem, 2011. **286**(36): p. 31263-71.
290. Bruewer, M., et al., *Proinflammatory cytokines disrupt epithelial barrier function by apoptosis-independent mechanisms.* J Immunol, 2003. **171**(11): p. 6164-72.
291. Geier, M.S., R.N. Butler, and G.S. Howarth, *Inflammatory bowel disease: current insights into pathogenesis and new therapeutic options; probiotics, prebiotics and synbiotics.* Int J Food Microbiol, 2007. **115**(1): p. 1-11.
292. Bellaguarda, E. and E.B. Chang, *IBD and the gut microbiota--from bench to personalized medicine.* Curr Gastroenterol Rep, 2015. **17**(4): p. 15.
293. Elson, C.O., et al., *Experimental models of inflammatory bowel disease reveal innate, adaptive, and regulatory mechanisms of host dialogue with the microbiota.* Immunol Rev, 2005. **206**: p. 260-76.
294. Swidsinski, A., et al., *Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease.* J Clin Microbiol, 2005. **43**(7): p. 3380-9.
295. Loftus, E.V., Jr., *Management of extraintestinal manifestations and other complications of inflammatory bowel disease.* Curr Gastroenterol Rep, 2004. **6**(6): p. 506-13.
296. Larrosa, M., et al., *Polyphenol metabolites from colonic microbiota exert anti-inflammatory activity on different inflammation models.* Mol Nutr Food Res, 2009. **53**(8): p. 1044-54.
297. Parkar, S.G., D.E. Stevenson, and M.A. Skinner, *The potential influence of fruit polyphenols on colonic microflora and human gut health.* Int J Food Microbiol, 2008. **124**(3): p. 295-8.
298. Ghouri, Y.A., et al., *Systematic review of randomized controlled trials of probiotics, prebiotics, and synbiotics in inflammatory bowel disease.* Clin Exp Gastroenterol, 2014. **7**: p. 473-87.
299. Steinmann, J., et al., *Anti-infective properties of epigallocatechin-3-gallate (EGCG), a component of green tea.* Br J Pharmacol, 2013. **168**(5): p. 1059-73.
300. Eckel, R.H., et al., *The metabolic syndrome.* Lancet, 2010. **375**(9710): p. 181-3.
301. Sarafidis, P.A. and P.M. Nilsson, *The metabolic syndrome: a glance at its history.* J Hypertens, 2006. **24**(4): p. 621-6.
302. Vague, J., *[Obesity in the development of arteriosclerosis and diabetes].* Sem Hop, 1954. **30**(58): p. 3244-6.
303. Reaven, G., F. Abbasi, and T. McLaughlin, *Obesity, insulin resistance, and cardiovascular disease.* Recent Prog Horm Res, 2004. **59**: p. 207-23.
304. DeFronzo, R.A. and E. Ferrannini, *Insulin resistance. A multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease.* Diabetes Care, 1991. **14**(3): p. 173-94.
305. Alberti, K.G. and P.Z. Zimmet, *Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation.* Diabet Med, 1998. **15**(7): p. 539-53.
306. Balkau, B. and M.A. Charles, *Comment on the provisional report from the WHO consultation. European Group for the Study of Insulin Resistance (EGIR).* Diabet Med, 1999. **16**(5): p. 442-3.
307. Hanefeld, M. and F. Pistrosch, *The metabolic syndrome and cardiovascular disease - an ongoing tsunami.* Pediatr Endocrinol

Diabetes Metab, 2013. **20**(1): p. 5-11.
308. Gundogan, K., et al., *Metabolic syndrome prevalence according to ATP III and IDF criteria and related factors in Turkish adults.* Arch Med Sci, 2013. **9**(2): p. 243-53.
309. Soares, G.M., et al., *Metabolic and cardiovascular impact of oral contraceptives in polycystic ovary syndrome.* Int J Clin Pract, 2009. **63**(1): p. 160-9.
310. Grundy, S.M., *Metabolic syndrome pandemic.* Arterioscler Thromb Vasc Biol, 2008. **28**(4): p. 629-36.
311. Kosa, Z., et al., *Prevalence of metabolic syndrome among Roma: a comparative health examination survey in Hungary.* Eur J Public Health, 2015. **25**(2): p. 299-304.
312. Lee, I.M., et al., *Effect of physical inactivity on major non-communicable diseases worldwide: an analysis of burden of disease and life expectancy.* Lancet, 2012. **380**(9838): p. 219-29.
313. Sun, K., et al., *Alcohol consumption and risk of metabolic syndrome: a meta-analysis of prospective studies.* Clin Nutr, 2014. **33**(4): p. 596-602.
314. Bernabe Garcia, J., et al., *Biochemical and nutritional markers and antioxidant activity in metabolic syndrome.* Endocrinol Nutr, 2014. **61**(6): p. 302-8.
315. Govers, R., *Cellular regulation of glucose uptake by glucose transporter GLUT4.* Adv Clin Chem, 2014. **66**: p. 173-240.
316. Kim, J.A., et al., *Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms.* Circulation, 2006. **113**(15): p. 1888-904.
317. Kissebah, A.H. and M.M. Hennes, *Central obesity and free fatty acid metabolism.* Prostaglandins Leukot Essent Fatty Acids, 1995. **52**(2-3): p. 209-11.
318. Zimmet, P., K.G. Alberti, and J. Shaw, *Global and societal implications of the diabetes epidemic.* Nature, 2001. **414**(6865): p. 782-7.
319. Reaven, G.M., *Insulin resistance: the link between obesity and cardiovascular disease.* Med Clin North Am, 2011. **95**(5): p. 875-92.
320. Kershaw, E.E. and J.S. Flier, *Adipose tissue as an endocrine organ.* J Clin Endocrinol Metab, 2004. **89**(6): p. 2548-56.
321. Chan, D.C., H.P. Barrett, and G.F. Watts, *Dyslipidemia in visceral obesity: mechanisms, implications, and therapy.* Am J Cardiovasc Drugs, 2004. **4**(4): p. 227-46.
322. Semenkovich, C.F., *Insulin resistance and atherosclerosis.* J Clin Invest, 2006. **116**(7): p. 1813-22.
323. Feingold, K.R. and C. Grunfeld, *Obesity and Dyslipidemia*, in *Endotext*, L.J. De Groot, et al., Editors. 2000: South Dartmouth (MA).
324. Chobanian, A.V., et al., *Seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure.* Hypertension, 2003. **42**(6): p. 1206-52.
325. Wang, C.C., M.L. Goalstone, and B. Draznin, *Molecular mechanisms of insulin resistance that impact cardiovascular biology.* Diabetes, 2004. **53**(11): p. 2735-40.
326. Sowers, J.R. and E.D. Frohlich, *Insulin and insulin resistance: impact on blood pressure and cardiovascular disease.* Med Clin North Am, 2004. **88**(1): p. 63-82.
327. Nieuwdorp, M., et al., *Hypercoagulability in the metabolic syndrome.* Curr Opin Pharmacol, 2005. **5**(2): p. 155-9.
328. Raynaud, E., et al., *Relationships between fibrinogen and insulin resistance.* Atherosclerosis, 2000. **150**(2): p. 365-70.

329. Aso, Y., et al., *Metabolic syndrome accompanied by hypercholesterolemia is strongly associated with proinflammatory state and impairment of fibrinolysis in patients with type 2 diabetes: synergistic effects of plasminogen activator inhibitor-1 and thrombin-activatable fibrinolysis inhibitor.* Diabetes Care, 2005. **28**(9): p. 2211-6.
330. Mavri, A., M.C. Alessi, and I. Juhan-Vague, *Hypofibrinolysis in the insulin resistance syndrome: implication in cardiovascular diseases.* J Intern Med, 2004. **255**(4): p. 448-56.
331. Wannamethee, S.G., et al., *The metabolic syndrome and insulin resistance: relationship to haemostatic and inflammatory markers in older non-diabetic men.* Atherosclerosis, 2005. **181**(1): p. 101-8.
332. Barbieri, M., et al., *New aspects of the insulin resistance syndrome: impact on haematological parameters.* Diabetologia, 2001. **44**(10): p. 1232-7.
333. Trovati, M., et al., *Impaired insulin-induced platelet antiaggregating effect in obesity and in obese NIDDM patients.* Diabetes, 1995. **44**(11): p. 1318-22.
334. de Ferranti, S. and D. Mozaffarian, *The perfect storm: obesity, adipocyte dysfunction, and metabolic consequences.* Clin Chem, 2008. **54**(6): p. 945-55.
335. Hotamisligil, G.S., *Inflammation and metabolic disorders.* Nature, 2006. **444**(7121): p. 860-7.
336. Medzhitov, R., *Origin and physiological roles of inflammation.* Nature, 2008. **454**(7203): p. 428-35.
337. Weisberg, S.P., et al., *CCR2 modulates inflammatory and metabolic effects of high-fat feeding.* J Clin Invest, 2006. **116**(1): p. 115-24.
338. Lumeng, C.N., J.L. Bodzin, and A.R. Saltiel, *Obesity induces a phenotypic switch in adipose tissue macrophage polarization.* J Clin Invest, 2007. **117**(1): p. 175-84.
339. Johnson, A.R., J.J. Milner, and L. Makowski, *The inflammation highway: metabolism accelerates inflammatory traffic in obesity.* Immunol Rev, 2012. **249**(1): p. 218-38.
340. Wieser, V., A.R. Moschen, and H. Tilg, *Inflammation, cytokines and insulin resistance: a clinical perspective.* Arch Immunol Ther Exp (Warsz), 2013. **61**(2): p. 119-25.
341. Oh, Y.S., et al., *Interleukin-6 treatment induces beta-cell apoptosis via STAT-3-mediated nitric oxide production.* Diabetes Metab Res Rev, 2011. **27**(8): p. 813-9.
342. Dandona, P., et al., *Metabolic syndrome: a comprehensive perspective based on interactions between obesity, diabetes, and inflammation.* Circulation, 2005. **111**(11): p. 1448-54.
343. Galisteo, M., J. Duarte, and A. Zarzuelo, *Effects of dietary fibers on disturbances clustered in the metabolic syndrome.* J Nutr Biochem, 2008. **19**(2): p. 71-84.
344. Libby, P., *Inflammation in atherosclerosis.* Nature, 2002. **420**(6917): p. 868-74.
345. Olefsky, J.M. and C.K. Glass, *Macrophages, inflammation, and insulin resistance.* Annu Rev Physiol, 2010. **72**: p. 219-46.
346. Kotas, M.E. and R. Medzhitov, *Homeostasis, inflammation, and disease susceptibility.* Cell, 2015. **160**(5): p. 816-27.
347. Saltiel, A.R. and J.M. Olefsky, *Inflammatory mechanisms linking obesity and metabolic disease.* J Clin Invest, 2017. **127**(1): p. 1-4.
348. Scherer, P.E., *Adipose tissue: from lipid storage compartment to*

endocrine organ. Diabetes, 2006. **55**(6): p. 1537-45.
349. Ye, J., *Emerging role of adipose tissue hypoxia in obesity and insulin resistance.* Int J Obes (Lond), 2009. **33**(1): p. 54-66.
350. Bruun, J.M., et al., *Monocyte chemoattractant protein-1 release is higher in visceral than subcutaneous human adipose tissue (AT): implication of macrophages resident in the AT.* J Clin Endocrinol Metab, 2005. **90**(4): p. 2282-9.
351. Lauterbach, M.A. and F.T. Wunderlich, *Macrophage function in obesity-induced inflammation and insulin resistance.* Pflugers Arch, 2017. **469**(3-4): p. 385-396.
352. Sica, A. and A. Mantovani, *Macrophage plasticity and polarization: in vivo veritas.* J Clin Invest, 2012. **122**(3): p. 787-95.
353. Osborn, O. and J.M. Olefsky, *The cellular and signaling networks linking the immune system and metabolism in disease.* Nat Med, 2012. **18**(3): p. 363-74.
354. Johnson, A.M. and J.M. Olefsky, *The origins and drivers of insulin resistance.* Cell, 2013. **152**(4): p. 673-84.
355. Hotamisligil, G.S., N.S. Shargill, and B.M. Spiegelman, *Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance.* Science, 1993. **259**(5091): p. 87-91.
356. Balistreri, C.R., C. Caruso, and G. Candore, *The role of adipose tissue and adipokines in obesity-related inflammatory diseases.* Mediators Inflamm, 2010. **2010**: p. 802078.
357. Fain, J.N., et al., *Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans.* Endocrinology, 2004. **145**(5): p. 2273-82.
358. Fernandez-Sanchez, A., et al., *Inflammation, oxidative stress, and obesity.* Int J Mol Sci, 2011. **12**(5): p. 3117-32.
359. Friedman, J.M. and J.L. Halaas, *Leptin and the regulation of body weight in mammals.* Nature, 1998. **395**(6704): p. 763-70.
360. Santos-Alvarez, J., R. Goberna, and V. Sanchez-Margalet, *Human leptin stimulates proliferation and activation of human circulating monocytes.* Cell Immunol, 1999. **194**(1): p. 6-11.
361. Kiguchi, N., et al., *Leptin enhances CC-chemokine ligand expression in cultured murine macrophage.* Biochem Biophys Res Commun, 2009. **384**(3): p. 311-5.
362. Zarkesh-Esfahani, H., et al., *Leptin indirectly activates human neutrophils via induction of TNF-alpha.* J Immunol, 2004. **172**(3): p. 1809-14.
363. Grunfeld, C., et al., *Endotoxin and cytokines induce expression of leptin, the ob gene product, in hamsters.* J Clin Invest, 1996. **97**(9): p. 2152-7.
364. Lord, G.M., et al., *Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression.* Nature, 1998. **394**(6696): p. 897-901.
365. Okamoto, Y., et al., *Adiponectin: a key adipocytokine in metabolic syndrome.* Clin Sci (Lond), 2006. **110**(3): p. 267-78.
366. Motoshima, H., et al., *Adiponectin suppresses proliferation and superoxide generation and enhances eNOS activity in endothelial cells treated with oxidized LDL.* Biochem Biophys Res Commun, 2004. **315**(2): p. 264-71.
367. Cao, Y., et al., *Endothelial dysfunction in adiponectin deficiency and its mechanisms involved.* J Mol Cell Cardiol, 2009. **46**(3): p. 413-9.
368. Ouchi, N., et al., *Association of hypoadiponectinemia with*

impaired vasoreactivity. Hypertension, 2003. **42**(3): p. 231-4.
369. Clarke, G., et al., *Minireview: Gut microbiota: the neglected endocrine organ.* Mol Endocrinol, 2014. **28**(8): p. 1221-38.
370. Marchesi, J.R., et al., *The gut microbiota and host health: a new clinical frontier.* Gut, 2016. **65**(2): p. 330-9.
371. Backhed, F., et al., *Mechanisms underlying the resistance to diet-induced obesity in germ-free mice.* Proc Natl Acad Sci U S A, 2007. **104**(3): p. 979-84.
372. Ley, R.E., et al., *Obesity alters gut microbial ecology.* Proc Natl Acad Sci U S A, 2005. **102**(31): p. 11070-5.
373. Gill, S.R., et al., *Metagenomic analysis of the human distal gut microbiome.* Science, 2006. **312**(5778): p. 1355-9.
374. Turnbaugh, P.J., et al., *An obesity-associated gut microbiome with increased capacity for energy harvest.* Nature, 2006. **444**(7122): p. 1027-31.
375. Turnbaugh, P.J. and J.I. Gordon, *The core gut microbiome, energy balance and obesity.* J Physiol, 2009. **587**(Pt 17): p. 4153-8.
376. Le Chatelier, E., et al., *Richness of human gut microbiome correlates with metabolic markers.* Nature, 2013. **500**(7464): p. 541-6.
377. Kotzampassi, K., E.J. Giamarellos-Bourboulis, and G. Stavrou, *Obesity as a consequence of gut bacteria and diet interactions.* ISRN Obes, 2014. **2014**: p. 651895.
378. David, L.A., et al., *Diet rapidly and reproducibly alters the human gut microbiome.* Nature, 2014. **505**(7484): p. 559-63.
379. Cani, P.D., et al., *Metabolic endotoxemia initiates obesity and insulin resistance.* Diabetes, 2007. **56**(7): p. 1761-72.
380. Vreugdenhil, A.C., et al., *Lipopolysaccharide (LPS)-binding protein mediates LPS detoxification by chylomicrons.* J Immunol, 2003. **170**(3): p. 1399-405.
381. Neal, M.D., et al., *Enterocyte TLR4 mediates phagocytosis and translocation of bacteria across the intestinal barrier.* J Immunol, 2006. **176**(5): p. 3070-9.
382. Tanti, J.F., et al., *Implication of inflammatory signaling pathways in obesity-induced insulin resistance.* Front Endocrinol (Lausanne), 2012. **3**: p. 181.
383. Ghoshal, S., et al., *Chylomicrons promote intestinal absorption of lipopolysaccharides.* J Lipid Res, 2009. **50**(1): p. 90-7.
384. Verges, B., et al., *Changes in lipoprotein kinetics associated with type 2 diabetes affect the distribution of lipopolysaccharides among lipoproteins.* J Clin Endocrinol Metab, 2014. **99**(7): p. E1245-53.
385. Cani, P.D., et al., *Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice.* Diabetes, 2008. **57**(6): p. 1470-81.
386. Suzuki, T., *Regulation of intestinal epithelial permeability by tight junctions.* Cell Mol Life Sci, 2013. **70**(4): p. 631-59.
387. Chassaing, B., et al., *Dietary emulsifiers impact the mouse gut microbiota promoting colitis and metabolic syndrome.* Nature, 2015. **519**(7541): p. 92-6.
388. Ryan, D. and M. Heaner, *Guidelines (2013) for managing overweight and obesity in adults. Preface to the full report.* Obesity (Silver Spring), 2014. **22 Suppl 2**: p. S1-3.
389. Connolly, H.M., et al., *Valvular heart disease associated with fenfluramine-phentermine.* N Engl J Med, 1997. **337**(9): p. 581-8.
390. James, W.P., et al., *Effect of sibutramine on weight maintenance after weight loss: a*

randomised trial. STORM Study Group. Sibutramine Trial of Obesity Reduction and Maintenance. Lancet, 2000. **356**(9248): p. 2119-25.
391. Mun, E.C., G.L. Blackburn, and J.B. Matthews, *Current status of medical and surgical therapy for obesity.* Gastroenterology, 2001. **120**(3): p. 669-81.
392. Luque, C.A. and J.A. Rey, *Sibutramine: a serotonin-norepinephrine reuptake-inhibitor for the treatment of obesity.* Ann Pharmacother, 1999. **33**(9): p. 968-78.
393. Sjostrom, L., et al., *Randomised placebo-controlled trial of orlistat for weight loss and prevention of weight regain in obese patients. European Multicentre Orlistat Study Group.* Lancet, 1998. **352**(9123): p. 167-72.
394. Hill, J.O., et al., *Orlistat, a lipase inhibitor, for weight maintenance after conventional dieting: a 1-y study.* Am J Clin Nutr, 1999. **69**(6): p. 1108-16.
395. Bray, G.A. and D.H. Ryan, *Drug treatment of the overweight patient.* Gastroenterology, 2007. **132**(6): p. 2239-52.
396. Padwal, R.S. and S.R. Majumdar, *Drug treatments for obesity: orlistat, sibutramine, and rimonabant.* Lancet, 2007. **369**(9555): p. 71-7.
397. Kang, J.G. and C.Y. Park, *Anti-Obesity Drugs: A Review about Their Effects and Safety.* Diabetes Metab J, 2012. **36**(1): p. 13-25.
398. Kimura, H., et al., *Antiobese effects of novel saponins from edible seeds of Japanese horse chestnut (Aesculus turbinata BLUME) after treatment with wood ashes.* J Agric Food Chem, 2008. **56**(12): p. 4783-8.
399. Xia, D.Z., et al., *Anti-obesity and hypolipidemic effects of ethanolic extract from Alpinia officinarum Hance (Zingiberaceae) in rats fed high-fat diet.* J Med Food, 2010. **13**(4): p. 785-91.
400. Sheng, X., et al., *Improved Insulin Resistance and Lipid Metabolism by Cinnamon Extract through Activation of Peroxisome Proliferator-Activated Receptors.* PPAR Res, 2008. **2008**: p. 581348.
401. Dallas, C., et al., *Lipolytic effect of a polyphenolic citrus dry extract of red orange, grapefruit, orange (SINETROL) in human body fat adipocytes. Mechanism of action by inhibition of cAMP-phosphodiesterase (PDE).* Phytomedicine, 2008. **15**(10): p. 783-92.
402. Meydani, M. and S.T. Hasan, *Dietary polyphenols and obesity.* Nutrients, 2010. **2**(7): p. 737-51.
403. Kim, Y., J.B. Keogh, and P.M. Clifton, *Polyphenols and Glycemic Control.* Nutrients, 2016. **8**(1).
404. Milenkovic, D., et al., *Modulation of miRNA expression by dietary polyphenols in apoE deficient mice: a new mechanism of the action of polyphenols.* PLoS One, 2012. **7**(1): p. e29837.
405. Kawser Hossain, M., et al., *Molecular Mechanisms of the Anti-Obesity and Anti-Diabetic Properties of Flavonoids.* Int J Mol Sci, 2016. **17**(4): p. 569.
406. Moghe, S.S., et al., *Effect of blueberry polyphenols on 3T3-F442A preadipocyte differentiation.* J Med Food, 2012. **15**(5): p. 448-52.
407. Suwa, A., T. Kurama, and T. Shimokawa, *Adipocyte hyperplasia and RMI1 in the treatment of obesity.* FEBS J, 2011. **278**(4): p. 565-9.
408. Spalding, K.L., et al., *Dynamics of fat cell turnover in humans.* Nature, 2008. **453**(7196): p. 783-7.
409. Daquinag, A.C., Y. Zhang, and M.G. Kolonin, *Vascular targeting of adipose tissue as an anti-obesity approach.* Trends Pharmacol Sci, 2011. **32**(5): p. 300-7.

410. Wing, R.R. and S. Phelan, *Long-term weight loss maintenance.* Am J Clin Nutr, 2005. **82**(1 Suppl): p. 222S-225S.
411. Kuppusamy, U.R. and N.P. Das, *Effects of flavonoids on cyclic AMP phosphodiesterase and lipid mobilization in rat adipocytes.* Biochem Pharmacol, 1992. **44**(7): p. 1307-15.
412. Kuppusamy, U.R. and N.P. Das, *Potentiation of beta-adrenoceptor agonist-mediated lipolysis by quercetin and fisetin in isolated rat adipocytes.* Biochem Pharmacol, 1994. **47**(3): p. 521-9.
413. Shisheva, A. and Y. Shechter, *Quercetin selectively inhibits insulin receptor function in vitro and the bioresponses of insulin and insulinomimetic agents in rat adipocytes.* Biochemistry, 1992. **31**(34): p. 8059-63.
414. Hsu, C.L. and G.C. Yen, *Induction of cell apoptosis in 3T3-L1 pre-adipocytes by flavonoids is associated with their antioxidant activity.* Mol Nutr Food Res, 2006. **50**(11): p. 1072-9.
415. Yang, J.Y., et al., *Enhanced inhibition of adipogenesis and induction of apoptosis in 3T3-L1 adipocytes with combinations of resveratrol and quercetin.* Life Sci, 2008. **82**(19-20): p. 1032-9.
416. Eseberri, I., et al., *Doses of Quercetin in the Range of Serum Concentrations Exert Delipidating Effects in 3T3-L1 Preadipocytes by Acting on Different Stages of Adipogenesis, but Not in Mature Adipocytes.* Oxid Med Cell Longev, 2015. **2015**: p. 480943.
417. Batra, P. and A.K. Sharma, *Anti-cancer potential of flavonoids: recent trends and future perspectives.* 3 Biotech, 2013. **3**(6): p. 439-459.
418. Goldwasser, J., et al., *Transcriptional regulation of human and rat hepatic lipid metabolism by the grapefruit flavonoid naringenin: role of PPARalpha, PPARgamma and LXRalpha.* PLoS One, 2010. **5**(8): p. e12399.
419. Jung, U.J., et al., *Naringin supplementation lowers plasma lipids and enhances erythrocyte antioxidant enzyme activities in hypercholesterolemic subjects.* Clin Nutr, 2003. **22**(6): p. 561-8.
420. Harmon, A.W. and Y.M. Patel, *Naringenin inhibits phosphoinositide 3-kinase activity and glucose uptake in 3T3-L1 adipocytes.* Biochem Biophys Res Commun, 2003. **305**(2): p. 229-34.
421. Kim, M.H., K.S. Kang, and Y.S. Lee, *The inhibitory effect of genistein on hepatic steatosis is linked to visceral adipocyte metabolism in mice with diet-induced non-alcoholic fatty liver disease.* Br J Nutr, 2010. **104**(9): p. 1333-42.
422. Hwang, J.T., et al., *Genistein, EGCG, and capsaicin inhibit adipocyte differentiation process via activating AMP-activated protein kinase.* Biochem Biophys Res Commun, 2005. **338**(2): p. 694-9.
423. Guzik, T.J., D. Mangalat, and R. Korbut, *Adipocytokines - novel link between inflammation and vascular function?* J Physiol Pharmacol, 2006. **57**(4): p. 505-28.
424. Rivera, L., et al., *Quercetin ameliorates metabolic syndrome and improves the inflammatory status in obese Zucker rats.* Obesity (Silver Spring), 2008. **16**(9): p. 2081-7.
425. Overman, A., C.C. Chuang, and M. McIntosh, *Quercetin attenuates inflammation in human macrophages and adipocytes exposed to macrophage-conditioned media.* Int J Obes (Lond), 2011. **35**(9): p. 1165-72.
426. Bose, M., et al., *The major green tea polyphenol, (-)-epigallocatechin-3-gallate, inhibits obesity, metabolic syndrome, and fatty liver disease in high-fat-fed*

mice. J Nutr, 2008. **138**(9): p. 1677-83.
427. Terra, X., et al., *Modulatory effect of grape-seed procyanidins on local and systemic inflammation in diet-induced obesity rats.* J Nutr Biochem, 2011. **22**(4): p. 380-7.
428. Cho, S.Y., et al., *(-)-Catechin suppresses expression of Kruppel-like factor 7 and increases expression and secretion of adiponectin protein in 3T3-L1 cells.* Am J Physiol Endocrinol Metab, 2007. **292**(4): p. E1166-72.
429. Weisberg, S.P., R. Leibel, and D.V. Tortoriello, *Dietary curcumin significantly improves obesity-associated inflammation and diabetes in mouse models of diabesity.* Endocrinology, 2008. **149**(7): p. 3549-58.
430. Gonzales, A.M. and R.A. Orlando, *Curcumin and resveratrol inhibit nuclear factor-kappaB-mediated cytokine expression in adipocytes.* Nutr Metab (Lond), 2008. **5**: p. 17.
431. Olholm, J., et al., *Anti-inflammatory effect of resveratrol on adipokine expression and secretion in human adipose tissue explants.* Int J Obes (Lond), 2010. **34**(10): p. 1546-53.
432. Kim, S., et al., *Resveratrol exerts anti-obesity effects via mechanisms involving down-regulation of adipogenic and inflammatory processes in mice.* Biochem Pharmacol, 2011. **81**(11): p. 1343-51.
433. Tsuda, T., *Regulation of adipocyte function by anthocyanins; possibility of preventing the metabolic syndrome.* J Agric Food Chem, 2008. **56**(3): p. 642-6.
434. Vanhoutte, P.M., *Obesity and vascular dysfunction: the fat-e of rich and poor.* Br J Pharmacol, 2012. **165**(3): p. 541-3.
435. Reaven, G.M., *Pathophysiology of insulin resistance in human disease.* Physiol Rev, 1995. **75**(3): p. 473-86.
436. Eid, H.M., et al., *The molecular basis of the antidiabetic action of quercetin in cultured skeletal muscle cells and hepatocytes.* Pharmacogn Mag, 2015. **11**(41): p. 74-81.
437. Hajiaghaalipour, F., M. Khalilpourfarshbafi, and A. Arya, *Modulation of glucose transporter protein by dietary flavonoids in type 2 diabetes mellitus.* Int J Biol Sci, 2015. **11**(5): p. 508-24.
438. Chung, S., et al., *Regulation of SIRT1 in cellular functions: role of polyphenols.* Arch Biochem Biophys, 2010. **501**(1): p. 79-90.
439. Jayaprakasam, B., et al., *Amelioration of obesity and glucose intolerance in high-fat-fed C57BL/6 mice by anthocyanins and ursolic acid in Cornelian cherry (Cornus mas).* J Agric Food Chem, 2006. **54**(1): p. 243-8.
440. Liu, I.M., et al., *Myricetin, a naturally occurring flavonol, ameliorates insulin resistance induced by a high-fructose diet in rats.* Life Sci, 2007. **81**(21-22): p. 1479-88.
441. Scazzocchio, B., et al., *Cyanidin-3-O-beta-glucoside and protocatechuic acid exert insulin-like effects by upregulating PPARgamma activity in human omental adipocytes.* Diabetes, 2011. **60**(9): p. 2234-44.
442. Kim, M.S., et al., *Tangeretin stimulates glucose uptake via regulation of AMPK signaling pathways in C2C12 myotubes and improves glucose tolerance in high-fat diet-induced obese mice.* Mol Cell Endocrinol, 2012. **358**(1): p. 127-34.
443. Kang, C. and E. Kim, *Synergistic effect of curcumin and insulin on muscle cell glucose metabolism.* Food Chem Toxicol, 2010. **48**(8-9): p. 2366-73.
444. Xu, M., et al., *Quercetin differently regulates insulin-mediated glucose transporter 4 translocation under*

basal and inflammatory conditions in adipocytes. Mol Nutr Food Res, 2014. **58**(5): p. 931-41.
445. Zygmunt, K., et al., *Naringenin, a citrus flavonoid, increases muscle cell glucose uptake via AMPK.* Biochem Biophys Res Commun, 2010. **398**(2): p. 178-83.
446. Szkudelska, K., et al., *In vivo metabolic effects of naringenin in the ethanol consuming rat and the effect of naringenin on adipocytes in vitro.* J Anim Physiol Anim Nutr (Berl), 2007. **91**(3-4): p. 91-9.
447. Repas, T., *Obesity and dyslipidemia.* S D Med, 2011. **64**(7): p. 241-3, 245, 247 passim.
448. Klop, B., J.W. Elte, and M.C. Cabezas, *Dyslipidemia in obesity: mechanisms and potential targets.* Nutrients, 2013. **5**(4): p. 1218-40.
449. Brunzell, J.D. and J.E. Hokanson, *Dyslipidemia of central obesity and insulin resistance.* Diabetes Care, 1999. **22 Suppl 3**: p. C10-3.
450. Monforte, M.T., et al., *Biological effects of hesperidin, a Citrus flavonoid. (note II): hypolipidemic activity on experimental hypercholesterolemia in rat.* Farmaco, 1995. **50**(9): p. 595-9.
451. Pietta, P.G., *Flavonoids as antioxidants.* J Nat Prod, 2000. **63**(7): p. 1035-42.
452. Vaya, J., et al., *Inhibition of LDL oxidation by flavonoids in relation to their structure and calculated enthalpy.* Phytochemistry, 2003. **62**(1): p. 89-99.
453. Duchnowicz, P., et al., *Hypolipidemic and antioxidant effects of hydroxycinnamic acids, quercetin, and cyanidin 3-glucoside in hypercholesterolemic erythrocytes (in vitro study).* Eur J Nutr, 2012. **51**(4): p. 435-43.
454. Lee, S.H., et al., *Cholesterol-lowering activity of naringenin via inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase and acyl coenzyme A:cholesterol acyltransferase in rats.* Ann Nutr Metab, 1999. **43**(3): p. 173-80.
455. Cha, J.Y., et al., *Effect of hesperetin, a citrus flavonoid, on the liver triacylglycerol content and phosphatidate phosphohydrolase activity in orotic acid-fed rats.* Plant Foods Hum Nutr, 2001. **56**(4): p. 349-58.
456. Kurowska, E.M., et al., *HDL-cholesterol-raising effect of orange juice in subjects with hypercholesterolemia.* Am J Clin Nutr, 2000. **72**(5): p. 1095-100.
457. Bawazeer, N.A., et al., *Role of hesperetin in LDL-receptor expression in hepatoma HepG2 cells.* BMC Complement Altern Med, 2016. **16**: p. 182.
458. Cox, A.J., N.P. West, and A.W. Cripps, *Obesity, inflammation, and the gut microbiota.* Lancet Diabetes Endocrinol, 2015. **3**(3): p. 207-15.
459. Tzounis, X., et al., *Prebiotic evaluation of cocoa-derived flavanols in healthy humans by using a randomized, controlled, double-blind, crossover intervention study.* Am J Clin Nutr, 2011. **93**(1): p. 62-72.
460. Etxeberria, U., et al., *Reshaping faecal gut microbiota composition by the intake of trans-resveratrol and quercetin in high-fat sucrose diet-fed rats.* J Nutr Biochem, 2015. **26**(6): p. 651-60.
461. Qiao, Y., et al., *Effects of resveratrol on gut microbiota and fat storage in a mouse model with high-fat-induced obesity.* Food Funct, 2014. **5**(6): p. 1241-9.
462. Suzuki, T. and H. Hara, *Quercetin enhances intestinal barrier function through the assembly of zonula [corrected] occludens-2, occludin, and claudin-1 and the expression of claudin-4 in Caco-2 cells.* J Nutr, 2009. **139**(5): p. 965-74.
463. Amasheh, M., et al., *Quercetin enhances epithelial barrier function and increases claudin-4 expression*

in Caco-2 cells. J Nutr, 2008. **138**(6): p. 1067-73.
464. Rao, R.K., et al., *Tyrosine phosphorylation and dissociation of occludin-ZO-1 and E-cadherin-beta-catenin complexes from the cytoskeleton by oxidative stress.* Biochem J, 2002. **368**(Pt 2): p. 471-81.
465. Atkinson, K.J. and R.K. Rao, *Role of protein tyrosine phosphorylation in acetaldehyde-induced disruption of epithelial tight junctions.* Am J Physiol Gastrointest Liver Physiol, 2001. **280**(6): p. G1280-8.
466. Delgado-Lista, J., et al., *Mediterranean Diet and Cardiovascular Risk: Beyond Traditional Risk Factors.* Crit Rev Food Sci Nutr, 2016. **56**(5): p. 788-801.
467. Marlow, G., et al., *Transcriptomics to study the effect of a Mediterranean-inspired diet on inflammation in Crohn's disease patients.* Hum Genomics, 2013. **7**: p. 24.
468. Panunzio, M.F., et al., *Randomized, controlled nutrition education trial promotes a Mediterranean diet and improves anthropometric, dietary, and metabolic parameters in adults.* Ann Ig, 2011. **23**(1): p. 13-25.
469. Bermudez, B., et al., *Oleic acid in olive oil: from a metabolic framework toward a clinical perspective.* Curr Pharm Des, 2011. **17**(8): p. 831-43.
470. Cardeno, A., M. Sanchez-Hidalgo, and C. Alarcon-de-la-Lastra, *An up-date of olive oil phenols in inflammation and cancer: molecular mechanisms and clinical implications.* Curr Med Chem, 2013. **20**(37): p. 4758-76.
471. Camuesco, D., et al., *Intestinal anti-inflammatory activity of combined quercitrin and dietary olive oil supplemented with fish oil, rich in EPA and DHA (n-3) polyunsaturated fatty acids, in rats with DSS-induced colitis.* Clin Nutr, 2006. **25**(3): p. 466-76.
472. Sanchez-Fidalgo, S., et al., *Influence of extra virgin olive oil diet enriched with hydroxytyrosol in a chronic DSS colitis model.* Eur J Nutr, 2012. **51**(4): p. 497-506.
473. Takashima, T., et al., *Feeding with olive oil attenuates inflammation in dextran sulfate sodium-induced colitis in rat.* J Nutr Biochem, 2014. **25**(2): p. 186-92.
474. El, S.N. and S. Karakaya, *Olive tree (Olea europaea) leaves: potential beneficial effects on human health.* Nutr Rev, 2009. **67**(11): p. 632-8.
475. Talhaoui, N., et al., *Phenolic compounds and in vitro immunomodulatory properties of three Andalusian olive leaf extracts.* Journal of Functional Foods, 2016. **22**: p. 270-277.
476. Campolo, M., et al., *Effects of a polyphenol present in olive oil, oleuropein aglycone, in a murine model of intestinal ischemia/reperfusion injury.* J Leukoc Biol, 2013. **93**(2): p. 277-87.
477. Giner, E., et al., *Oleuropein protects against dextran sodium sulfate-induced chronic colitis in mice.* J Nat Prod, 2013. **76**(6): p. 1113-20.
478. Giner, E., et al., *Chemopreventive effect of oleuropein in colitis-associated colorectal cancer in c57bl/6 mice.* Mol Nutr Food Res, 2016. **60**(2): p. 242-55.
479. Kontogianni, V.G., et al., *Olive leaf extracts are a natural source of advanced glycation end product inhibitors.* J Med Food, 2013. **16**(9): p. 817-22.
480. Jemai, H., A. El Feki, and S. Sayadi, *Antidiabetic and antioxidant effects of hydroxytyrosol and oleuropein from olive leaves in alloxan-diabetic rats.* J Agric Food Chem, 2009. **57**(19): p. 8798-804.
481. Santiago-Mora, R., et al., *Oleuropein enhances osteoblastogenesis and inhibits adipogenesis: the effect on*

differentiation in stem cells derived from bone marrow. Osteoporos Int, 2011. **22**(2): p. 675-84.
482. Palmeri, R., et al., *Olive Leaf Extract from Sicilian Cultivar Reduced Lipid Accumulation by Inducing Thermogenic Pathway during Adipogenesis.* Front Pharmacol, 2016. **7**: p. 143.
483. Baur, J.A., et al., *Resveratrol improves health and survival of mice on a high-calorie diet.* Nature, 2006. **444**(7117): p. 337-42.
484. Gil-Cardoso, K., et al., *Effects of flavonoids on intestinal inflammation, barrier integrity and changes in gut microbiota during diet-induced obesity.* Nutr Res Rev, 2016. **29**(2): p. 234-248.
485. Parker, L., et al., *A lifecourse study of risk for hyperinsulinaemia, dyslipidaemia and obesity (the central metabolic syndrome) at age 49-51 years.* Diabet Med, 2003. **20**(5): p. 406-15.
486. Joo, Y.E., *Natural product-derived drugs for the treatment of inflammatory bowel diseases.* Intest Res, 2014. **12**(2): p. 103-9.
487. Vasudeva, N., N. Yadav, and S.K. Sharma, *Natural products: a safest approach for obesity.* Chin J Integr Med, 2012. **18**(6): p. 473-80.
488. Brahmi, F., et al., *The efficacy of phenolics compounds with different polarities as antioxidants from olive leaves depending on seasonal variations.* Industrial Crops and Products, 2012. **38**: p. 146-152.
489. Briante, R., et al., *Olea europaea L. leaf extract and derivatives: antioxidant properties.* J Agric Food Chem, 2002. **50**(17): p. 4934-40.
490. Anter, J., et al., *A pilot study on the DNA-protective, cytotoxic, and apoptosis-inducing properties of olive-leaf extracts.* Mutat Res, 2011. **723**(2): p. 165-70.
491. Taamalli, A., et al., *Use of advanced techniques for the extraction of phenolic compounds from Tunisian olive leaves: phenolic composition and cytotoxicity against human breast cancer cells.* Food Chem Toxicol, 2012. **50**(6): p. 1817-25.
492. Lee, O.H. and B.Y. Lee, *Antioxidant and antimicrobial activities of individual and combined phenolics in Olea europaea leaf extract.* Bioresour Technol, 2010. **101**(10): p. 3751-4.
493. Lee-Huang, S., et al., *Anti-HIV activity of olive leaf extract (OLE) and modulation of host cell gene expression by HIV-1 infection and OLE treatment.* Biochem Biophys Res Commun, 2003. **307**(4): p. 1029-37.
494. Singh, I., et al., *The effects of polyphenols in olive leaves on platelet function.* Nutr Metab Cardiovasc Dis, 2008. **18**(2): p. 127-32.
495. Poudyal, H., F. Campbell, and L. Brown, *Olive leaf extract attenuates cardiac, hepatic, and metabolic changes in high carbohydrate-, high fat-fed rats.* J Nutr, 2010. **140**(5): p. 946-53.
496. Shen, Y., et al., *Olive leaf extract attenuates obesity in high-fat diet-fed mice by modulating the expression of molecules involved in adipogenesis and thermogenesis.* Evid Based Complement Alternat Med, 2014. **2014**: p. 971890.
497. Talhaoui, N., et al., *Determination of phenolic compounds of 'Sikitita' olive leaves by HPLC-DAD-TOF-MS. Comparison with its parents 'Arbequina' and 'Picual' olive leaves.* Lwt-Food Science and Technology, 2014. **58**(1): p. 28-34.
498. Monteleone, G., et al., *Blocking Smad7 restores TGF-beta1 signaling in chronic inflammatory bowel disease.* J Clin Invest, 2001. **108**(4): p. 601-9.
499. Cannarile, L., et al., *Glucocorticoid-Induced Leucine Zipper Is Protective in Th1-Mediated Models of Colitis.* Gastroenterology, 2009. **136**(2): p. 530-541.

500. Cooper, H.S., et al., *Clinicopathologic study of dextran sulfate sodium experimental murine colitis.* Lab Invest, 1993. **69**(2): p. 238-49.
501. Bergstrom, K.S., et al., *Muc2 protects against lethal infectious colitis by disassociating pathogenic and commensal bacteria from the colonic mucosa.* PLoS Pathog, 2010. **6**(5): p. e1000902.
502. Arribas, B., et al., *Di-D-fructose dianhydride-enriched caramels: effect on colon microbiota, inflammation, and tissue damage in trinitrobenzenesulfonic acid-induced colitic rats.* J Agric Food Chem, 2010. **58**(10): p. 6476-84.
503. *Sambrook J, Russell D (2001) Molecular Cloning A Laboratory Manual, 3rd edn. Cold Spring Harbor, NY Cold Spring Harbor Laboratory Press.pdf>.*
504. Meyer, F., et al., *The metagenomics RAST server - a public resource for the automatic phylogenetic and functional analysis of metagenomes.* BMC Bioinformatics, 2008. **9**: p. 386.
505. Wang, Q., et al., *Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy.* Appl Environ Microbiol, 2007. **73**(16): p. 5261-7.
506. Parks, D.H. and R.G. Beiko, *Identifying biologically relevant differences between metagenomic communities.* Bioinformatics, 2010. **26**(6): p. 715-21.
507. Parks, D.H., et al., *STAMP: statistical analysis of taxonomic and functional profiles.* Bioinformatics, 2014. **30**(21): p. 3123-4.
508. Solinas, G. and M. Karin, *JNK1 and IKKbeta: molecular links between obesity and metabolic dysfunction.* FASEB J, 2010. **24**(8): p. 2596-611.
509. Tailleux, A., K. Wouters, and B. Staels, *Roles of PPARs in NAFLD: potential therapeutic targets.* Biochim Biophys Acta, 2012. **1821**(5): p. 809-18.
510. Caporaso, J.G., et al., *PyNAST: a flexible tool for aligning sequences to a template alignment.* Bioinformatics, 2010. **26**(2): p. 266-7.
511. Kaser, A., S. Zeissig, and R.S. Blumberg, *Inflammatory bowel disease.* Annu Rev Immunol, 2010. **28**: p. 573-621.
512. Molodecky, N.A. and G.G. Kaplan, *Environmental risk factors for inflammatory bowel disease.* Gastroenterol Hepatol (N Y), 2010. **6**(5): p. 339-46.
513. Silva, F.A., et al., *The Immunological Basis of Inflammatory Bowel Disease.* Gastroenterol Res Pract, 2016. **2016**: p. 2097274.
514. Strober, W. and I.J. Fuss, *Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases.* Gastroenterology, 2011. **140**(6): p. 1756-1767.
515. Bernstein, C.N., *Treatment of IBD: where we are and where we are going.* Am J Gastroenterol, 2015. **110**(1): p. 114-26.
516. Hung, A., et al., *Complementary and Alternative Medicine Use Is Prevalent Among Patients with Gastrointestinal Diseases.* Dig Dis Sci, 2015. **60**(7): p. 1883-8.
517. Opheim, R., et al., *Complementary and alternative medicine in patients with inflammatory bowel disease: the results of a population-based inception cohort study (IBSEN).* J Crohns Colitis, 2012. **6**(3): p. 345-53.
518. Algieri, F., et al., *Botanical Drugs as an Emerging Strategy in Inflammatory Bowel Disease: A Review.* Mediators Inflamm, 2015. **2015**: p. 179616.
519. Fakhraei, N., et al., *Protective effect of hydroalcoholic olive leaf extract on experimental model of colitis in rat: involvement of nitrergic and opioidergic systems.*

Phytother Res, 2014. **28**(9): p. 1367-73.
520. Randhawa, P.K., et al., *A review on chemical-induced inflammatory bowel disease models in rodents.* Korean J Physiol Pharmacol, 2014. **18**(4): p. 279-88.
521. Danese, S. and C. Fiocchi, *Ulcerative colitis.* N Engl J Med, 2011. **365**(18): p. 1713-25.
522. Korzenik, J.R. and D.K. Podolsky, *Evolving knowledge and therapy of inflammatory bowel disease.* Nat Rev Drug Discov, 2006. **5**(3): p. 197-209.
523. Muzes, G., et al., *Changes of the cytokine profile in inflammatory bowel diseases.* World J Gastroenterol, 2012. **18**(41): p. 5848-61.
524. Thomas, S. and D.C. Baumgart, *Targeting leukocyte migration and adhesion in Crohn's disease and ulcerative colitis.* Inflammopharmacology, 2012. **20**(1): p. 1-18.
525. Kaliora, A.C., et al., *Alterations in the function of circulating mononuclear cells derived from patients with Crohn's disease treated with mastic.* World J Gastroenterol, 2007. **13**(45): p. 6031-6.
526. Al-Ghadban, S., et al., *Cross-talk between intestinal epithelial cells and immune cells in inflammatory bowel disease.* Sci Rep, 2016. **6**: p. 29783.
527. Mopuri, R. and M.S. Islam, *Medicinal plants and phytochemicals with anti-obesogenic potentials: A review.* Biomed Pharmacother, 2017. **89**: p. 1442-1452.
528. Jimenez-Sanchez, C., et al., *AMPK modulatory activity of olive-tree leaves phenolic compounds: Bioassay-guided isolation on adipocyte model and in silico approach.* PLoS One, 2017. **12**(3): p. e0173074.
529. Hirosumi, J., et al., *A central role for JNK in obesity and insulin resistance.* Nature, 2002. **420**(6913): p. 333-6.
530. Shoelson, S.E., J. Lee, and A.B. Goldfine, *Inflammation and insulin resistance.* J Clin Invest, 2006. **116**(7): p. 1793-801.
531. Asghar, A. and N. Sheikh, *Role of immune cells in obesity induced low grade inflammation and insulin resistance.* Cell Immunol, 2017. **315**: p. 18-26.
532. Chung, S., et al., *Preadipocytes mediate lipopolysaccharide-induced inflammation and insulin resistance in primary cultures of newly differentiated human adipocytes.* Endocrinology, 2006. **147**(11): p. 5340-51.
533. Wilcox, G., *Insulin and insulin resistance.* Clin Biochem Rev, 2005. **26**(2): p. 19-39.
534. Shepherd, P.R. and B.B. Kahn, *Glucose transporters and insulin action--implications for insulin resistance and diabetes mellitus.* N Engl J Med, 1999. **341**(4): p. 248-57.
535. Tamori, Y., et al., *Role of peroxisome proliferator-activated receptor-gamma in maintenance of the characteristics of mature 3T3-L1 adipocytes.* Diabetes, 2002. **51**(7): p. 2045-55.
536. Guilherme, A., et al., *Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes.* Nat Rev Mol Cell Biol, 2008. **9**(5): p. 367-77.
537. Tuncman, G., et al., *Functional in vivo interactions between JNK1 and JNK2 isoforms in obesity and insulin resistance.* Proc Natl Acad Sci U S A, 2006. **103**(28): p. 10741-6.
538. Xue, B. and B.B. Kahn, *AMPK integrates nutrient and hormonal signals to regulate food intake and energy balance through effects in the hypothalamus and peripheral tissues.* J Physiol, 2006. **574**(Pt 1): p. 73-83.

539. Bijland, S., S.J. Mancini, and I.P. Salt, *Role of AMP-activated protein kinase in adipose tissue metabolism and inflammation.* Clin Sci (Lond), 2013. **124**(8): p. 491-507.
540. Carling, D., et al., *AMP-activated protein kinase: new regulation, new roles?* Biochem J, 2012. **445**(1): p. 11-27.
541. Sag, D., et al., *Adenosine 5'-monophosphate-activated protein kinase promotes macrophage polarization to an anti-inflammatory functional phenotype.* J Immunol, 2008. **181**(12): p. 8633-41.
542. Yang, Z., et al., *Macrophage alpha1 AMP-activated protein kinase (alpha1AMPK) antagonizes fatty acid-induced inflammation through SIRT1.* J Biol Chem, 2010. **285**(25): p. 19051-9.
543. Xue, B., et al., *Neuronal protein tyrosine phosphatase 1B deficiency results in inhibition of hypothalamic AMPK and isoform-specific activation of AMPK in peripheral tissues.* Mol Cell Biol, 2009. **29**(16): p. 4563-73.
544. Pilon, G., P. Dallaire, and A. Marette, *Inhibition of inducible nitric-oxide synthase by activators of AMP-activated protein kinase: a new mechanism of action of insulin-sensitizing drugs.* J Biol Chem, 2004. **279**(20): p. 20767-74.
545. Zou, M.H., et al., *Activation of the AMP-activated protein kinase by the anti-diabetic drug metformin in vivo. Role of mitochondrial reactive nitrogen species.* J Biol Chem, 2004. **279**(42): p. 43940-51.
546. Fisher, J.S., et al., *Activation of AMP kinase enhances sensitivity of muscle glucose transport to insulin.* Am J Physiol Endocrinol Metab, 2002. **282**(1): p. E18-23.
547. Foretz, M., et al., *Short-term overexpression of a constitutively active form of AMP-activated protein kinase in the liver leads to mild hypoglycemia and fatty liver.* Diabetes, 2005. **54**(5): p. 1331-9.
548. Wang, H. and R.H. Eckel, *Lipoprotein lipase: from gene to obesity.* Am J Physiol Endocrinol Metab, 2009. **297**(2): p. E271-88.
549. Walton, R.G., et al., *Increasing adipocyte lipoprotein lipase improves glucose metabolism in high fat diet-induced obesity.* J Biol Chem, 2015. **290**(18): p. 11547-56.
550. Levin, B.E. and A.A. Dunn-Meynell, *Reduced central leptin sensitivity in rats with diet-induced obesity.* Am J Physiol Regul Integr Comp Physiol, 2002. **283**(4): p. R941-8.
551. Tilg, H. and A.R. Moschen, *Adipocytokines: mediators linking adipose tissue, inflammation and immunity.* Nat Rev Immunol, 2006. **6**(10): p. 772-83.
552. Acedo, S.C., et al., *Participation of leptin in the determination of the macrophage phenotype: an additional role in adipocyte and macrophage crosstalk.* In Vitro Cell Dev Biol Anim, 2013. **49**(6): p. 473-8.
553. Bai, Y. and Q. Sun, *Macrophage recruitment in obese adipose tissue.* Obes Rev, 2015. **16**(2): p. 127-36.
554. Kadowaki, T. and T. Yamauchi, *Adiponectin and adiponectin receptors.* Endocr Rev, 2005. **26**(3): p. 439-51.
555. Matsuzawa, Y., *Establishment of a concept of visceral fat syndrome and discovery of adiponectin.* Proc Jpn Acad Ser B Phys Biol Sci, 2010. **86**(2): p. 131-41.
556. Turer, A.T. and P.E. Scherer, *Adiponectin: mechanistic insights and clinical implications.* Diabetologia, 2012. **55**(9): p. 2319-26.
557. Weisberg, S.P., et al., *Obesity is associated with macrophage accumulation in adipose tissue.* J Clin Invest, 2003. **112**(12): p. 1796-808.

558. Panee, J., *Monocyte Chemoattractant Protein 1 (MCP-1) in obesity and diabetes.* Cytokine, 2012. **60**(1): p. 1-12.
559. Nguyen, M.T., et al., *A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways.* J Biol Chem, 2007. **282**(48): p. 35279-92.
560. Engin, A., *The Pathogenesis of Obesity-Associated Adipose Tissue Inflammation.* Adv Exp Med Biol, 2017. **960**: p. 221-245.
561. Xia, S., et al., *Gr-1+ CD11b+ myeloid-derived suppressor cells suppress inflammation and promote insulin sensitivity in obesity.* J Biol Chem, 2011. **286**(26): p. 23591-9.
562. Nahrendorf, M., et al., *The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions.* J Exp Med, 2007. **204**(12): p. 3037-47.
563. Deiuliis, J., et al., *Visceral adipose inflammation in obesity is associated with critical alterations in tregulatory cell numbers.* PLoS One, 2011. **6**(1): p. e16376.
564. Khayyal, M.T., et al., *Blood pressure lowering effect of an olive leaf extract (Olea europaea) in L-NAME induced hypertension in rats.* Arzneimittelforschung, 2002. **52**(11): p. 797-802.
565. Zarzuelo, A., et al., *Vasodilator effect of olive leaf.* Planta Med, 1991. **57**(5): p. 417-9.
566. Romero, M., et al., *Antihypertensive effects of oleuropein-enriched olive leaf extract in spontaneously hypertensive rats.* Food Funct, 2016. **7**(1): p. 584-93.
567. Susalit, E., et al., *Olive (Olea europaea) leaf extract effective in patients with stage-1 hypertension: comparison with Captopril.* Phytomedicine, 2011. **18**(4): p. 251-8.
568. Quintela, A.M., et al., *Activation of peroxisome proliferator-activated receptor-beta/-delta (PPARbeta/delta) prevents endothelial dysfunction in type 1 diabetic rats.* Free Radic Biol Med, 2012. **53**(4): p. 730-41.
569. Toral, M., et al., *Chronic peroxisome proliferator-activated receptorbeta/delta agonist GW0742 prevents hypertension, vascular inflammatory and oxidative status, and endothelial dysfunction in diet-induced obesity.* J Hypertens, 2015. **33**(9): p. 1831-44.
570. Kobayasi, R., et al., *Oxidative stress and inflammatory mediators contribute to endothelial dysfunction in high-fat diet-induced obesity in mice.* J Hypertens, 2010. **28**(10): p. 2111-9.
571. Bourgoin, F., et al., *Endothelial and vascular dysfunctions and insulin resistance in rats fed a high-fat, high-sucrose diet.* Am J Physiol Heart Circ Physiol, 2008. **295**(3): p. H1044-H1055.
572. Silver, A.E., et al., *Overweight and obese humans demonstrate increased vascular endothelial NAD(P)H oxidase-p47(phox) expression and evidence of endothelial oxidative stress.* Circulation, 2007. **115**(5): p. 627-37.
573. Furuse, M., et al., *Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions.* J Cell Biol, 1994. **127**(6 Pt 1): p. 1617-26.
574. Roberfroid, M., et al., *Prebiotic effects: metabolic and health benefits.* Br J Nutr, 2010. **104 Suppl 2**: p. S1-63.
575. De Filippo, C., et al., *Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa.* Proc

Natl Acad Sci U S A, 2010. **107**(33): p. 14691-6.
576. Rajilic-Stojanovic, M. and W.M. de Vos, *The first 1000 cultured species of the human gastrointestinal microbiota.* FEMS Microbiol Rev, 2014. **38**(5): p. 996-1047.
577. Bagarolli, R.A., et al., *Probiotics modulate gut microbiota and improve insulin sensitivity in DIO mice.* J Nutr Biochem, 2017. **50**: p. 16-25.
578. Anhe, F.F., et al., *A polyphenol-rich cranberry extract protects from diet-induced obesity, insulin resistance and intestinal inflammation in association with increased Akkermansia spp. population in the gut microbiota of mice.* Gut, 2015. **64**(6): p. 872-83.

ABBREVIATIONS

ABBREVIATIONS

Akt Protein kinase B

AMP Antimicrobial peptide

APC Antigen presenting cell

AUC Area under curve

CD Crohn's disease

CFU Colony forming units

DAI Disease activity index

DC Dendritic cell

DNBS Dinitrobenzene sulfonic acid

DSS Dextran sulfate sodium

GADPH glyceraldehyde-3-phosphate dehydrogenase

GALT Gut associated lymphoid tissue

HDL High-density lipoprotein

HFD High-fat diet

HOMA-IR Homeostatic model assessment of insulin resistance

IBD Inflammatory bowel disease

ICAM-1 Inter-Cellular Adhesion Molecule - 1

IL Interleukin

iNOS inducible NOS

IRS-1 Insulin receptor substrate-1

JNK c-Jun N-terminal kinase

LDL Low-density lipoprotein

L-NAME N^G-nitro-L-arginine methyl ester

LPS Lipopolysaccharide

MAPK Mitogen-activated protein kinase

MCP-1 Monocyte chemoattractant protein-1

MS Metabolic syndrome

MUC Mucin

NO Nitric oxide

OLE Olive leaf extract

PAMP Patogen-associated molecular pattern

PBMC Peripheral blood mononuclear cell

PCA Principal component analysis

PPAR Peroxisome proliferator activated receptors

PRR Pattern recognition receptor

ROS Reactive oxygen species

sIgA secretory Immunoglobulin A

SNPs Single Nucleotide Polymorphism

TFF Trefoil factor

Th T helper cell

TLR Toll-like receptor

TLR4 Toll-like receptor 4

TNF-α Tumor necrosis factor α

Treg Regulatory T cell

UC Ulcerative colitis

VLDL Very low density lipoprotein

ZO Zonula occludens protein

ANNEX

PUBLICACIONES

1. Rodríguez-Nogales A, Algieri F, Garrido-Mesa J, **Vezza T**, Utrilla MP, Chueca N, Garcia F, Olivares M, Rodríguez-Cabezas ME, Gálvez J. Differential intestinal anti-inflammatory effects of Lactobacillus fermentum and Lactobacillus salivarius in DSS mouse colitis: impact on microRNAs expression and microbiota composition. Molecular Nutrition & Food Research 2017 Jul 28

2. **Vezza T**, Algieri F, Rodríguez-Nogales A, Garrido-Mesa J, Utrilla MP, Talhaoui N, Gómez-Caravaca AM, Segura-Carretero A, Rodríguez-Cabezas ME, Monteleone G, Gálvez J. Immunomodulatory properties of Olea europaea leaf extract in intestinal inflammation. Molecular Nutrition & Food Research 2017 Oct;61(10).

3. Rodriguez-Nogales A., Algieri F., De Matteis L., Lozano-Perez AA., Garrido-Mesa J., **Vezza T**, de la Fuente JM., Cenis JL., Gálvez J., Rodriguez-Cabezas ME. Intestinal anti-inflammatory effects of RGD-functionalized silk fibroin nanoparticles in trinitrobenzenesulfonic acid-induced experimental colitis in rats. Int J Nanomedicine. 2016 Nov 10;11:5945-5958.

4. Rodriguez-Nogales A, Lozano-Pérez AA, Aznar-Cervantes SD, Algieri F, Garrido-Mesa J, Garrido-Mesa N, **Vezza T**, Utrilla MP, Cenis JL, Rodríguez-Cabezas ME, Gálvez J.Effect of aqueous and particulate silk fibroin in a rat model of experimental colitis. International Journal of Pharmaceutics 2016 Sep 10;511(1):1-9.

5. Algieri, F., Rodríguez-Nogales, A., **Vezza T**, Garrido-Mesa, J., Garrido-Mesa, N., Utrilla, MP., González-Tejero, MR., Casares-Porcel, M., Molero-Mesa, J., Contreras, MM., Segura-Carretero, A., Pérez Del Palacio, J., Diaz, C., Vergara, N., Vicente, F., Rodríguez-Cabezas, ME., Gálvez, J. Anti-inflammatory activity of hydroalcoholic extracts of Lavandula dentata L. and Lavandula stoechas. Journal of Ethnopharmacology, 2016, Aug 22;190:142-58.

6. Bribi N., Algieri F., Rodriguez-Nogales A., **Vezza T**, Garrido-Mesa J., Utrilla MP., Del Mar Contreras M., Maiza F., Segura-Carretero A., Rodriguez-Cabezas ME., Gálvez J. Intestinal anti-inflammatory effects of total alkaloid extract from Fumaria capreolata in the DNBS model of mice colitis and intestinal epithelial CMT93 cells. Phytomedicine. 2016 Aug 15;23(9):901-13.

7. Hidalgo-Cantabrana, C., Algieri, F., Rodríguez-Nogales, A., **Vezza T**, Martínez-Camblor, P., Margolles, A., Ruas-Madiedo, P., Gálvez, J. A mucoid-ropy exopolysaccharide-producing Bifidobacterium animalis subsp. Lactis strain orally administered attenuates DSS-induced colitis in mice. Frontiers in Microbiology,2016, Jun 9;7:868.

8. Talhaoui N., **Vezza T**, Gómez-Caravaca AM., Fernández-Gutiérrez A., Gálvez J., Segura-Carretero A. Phenolic compounds and *in vitro* inmunomodulatory properties of three Andalusian olive leaf eztracts. Journal of Functional Foods 22(2016) 270-277.

9. **Vezza T**, Rodríguez-Nogales, A., Algieri, F., Utrilla, P., Rodríguez-Cabezas, ME., Gálvez, J. Flavonoids in inflammatory bowel disease: a Review. Nutrients. 2016. Apr 9;8(4).

10. Rodríguez-Nogales, A., Algieri, F., Garrido-Mesa, J., Camuesco, D., **Vezza T**, Garrido-Mesa, N., Utrilla, MP., Rodriguez-Cabezas, ME., Pischel, I., Gálvez, J. Intestinal anti-inflammatory activity of calcium pyruvate in the TNBS model of rat colitis: comparison with ethyl pyruvate. Biochemical Pharmacology. 2015. Mar 8. pii: 1087057116635517.

11. Bribi N., Algieri F., Rodriguez-Nogales A., Garrido-Mesa J., **Vezza T**, Maiza F., Utrilla MP., Rodriguez-Cabezas ME., Galvez J. Antinociceptive and Anti-Inflammatory Effects of Total Alkaloid Extract from Fumaria capreolata. Evidence-Based Complementary and Alternative Medicine, 2015; 2015:736895.

12. Rodríguez-Nogales A, Algieri F, **Vezza T**, Garrido-Mesa, Olivares M, Comalada M, Riccardi C, Utrilla MP, Rodríguez-Cabezas ME, Galvez J. The viability of Lactobacillus fermentum CECT5716 is not essential to exert intestinal antiinflammatory properties. Food & Function, 2015, 6, 1176.

13. Algieri F, Rodriguez-Nogales A, Garrido-Mesa N, **Vezza T**, Garrido-Mesa J, Utrilla MP, Montilla A, Cardelle-Cobas A, Olano A, Corzo N, Guerra-Hernandez E, Zarzuelo A, Rodriguez-Cabezas ME, Galvez J. Intestinal Anti-inflammatory Effects of Oligosaccharides Derived from Lactulose in the Trinitrobenzenesulfonic Acid Model of Rat Colitis. J. Agric. Food Chem. 2014, 62, 4285−4297

CONTRIBUCIONES A CONGRESOS
(incluidas solo las relativas a esta tesis)

Vezza T, Algieri F, Rodriguez-Nogales A, Garrido-Mesa J, Utrilla MP, Talhaoui N, Gomez-Caravaca AM, Segura-Carretero A, Monteleone G, Rodriguez-Cabezas ME, Galvez J. Titulo: Olive leaf extract exhibits immunomodulatory activity in human PBMCs and antiinflammatory effect in the DSS model of mouse colitis. Tipo de participación: Poster Congreso: Falk Symposium 209 2017. Publicación: Abstract book. Lugar celebración: Berlin (Alemania) Fecha: 06-07/10/2017

Vezza T, Algieri F., Rodríguez-Nogales A., Garrido-Mesa J., Utrilla M.P., Talhaoui N., Gómez-Caravaca A.M., Segura-Carretero A., Rodríguez-Cabezas ME., Gálvez J. Olive leaf extract exhibits immunomoduladory activity in human PBMCS and anti-inflammatory effect in the DSS model of mouse colitis. Tipo de participación: Poster Congreso: 37 CONGRESO SEF 2017 Publicación: Abstract book . Lugar de celebración: Barcelona (España) Fecha: 18-21/06/2017

Diez-Echave P., **Vezza T**, Algieri F., Rodríguez-Nogales A., Garrido-Mesa J., Toral M., Romero M. , Sanchez M. , Rodriguez-Perez C., Gómez-Caravaca A.M., Segura-Carretero A., Rodríguez-Cabezas ME., Gálvez J. Titulo: Effects of olive leaf extract in high-fat diet-fed mice.Tipo de participación: Poster .Congreso: 37 CONGRESO SEF 2017. Publicación: Abstract book. Lugar celebración: Barcelona (España) Fecha: 18-21/06/2017

Vezza T, Algieri F., Rodríguez-Nogales A., Garrido-Mesa J., Utrilla M.P., Talhaoui N., Gómez-Caravaca A.M., Segura-Carretero A., Rodríguez-Cabezas ME., Gálvez J. Olive leaf extract exhibits immunomoduladory activity in human PBMCs and anti-inflammatory effect in the DSS model of mouse colitis. Tipo de participacion: Comunicacion oral.Congreso: IX Reunion de Jovenes Farmacologos. Publicacion: Abstract book. Lugar celebracion: Sevilla (España). Fecha: 07-06-2017

Diez-Echave P., **Vezza T**, Algieri F., Rodríguez-Nogales A., Garrido-Mesa J., Toral M., Romero M., Sanchez M., Rogriguez-Perez C., Gómez-Caravaca A.M., Segura-Carretero A., Rodríguez-Cabezas ME., Gálvez J. Titulo: Effects of olive leaf extract in high-fat diet-fed mice: impact on vascular dysfunction. Tipo de participacion: Comunicacion oral. Congreso: IX Reunion de Jovenes Farmacologos. Publicacion: Abstract book. Lugar celebracion: Sevilla (España). Fecha: 07-06-2017

Vezza T, Algieri F, Rodríguez-Nogales A, Garrido-Mesa J, Utrilla MP, Talhaoui N, Gomez-Caravaca AM, Segura-Carretero A, Rodríguez-Cabezas ME, Gálvez J. Intestinal anti-inflammatory effect of olive leaf extract in the DSS model of mouse colitis. Tipo de participación: poster. Congreso: 7th European Congress of Pharmacology (EPHAR 2016) Publicación: Abstract book. Lugar celebración: Estambul (Turquia) Fecha: 26-30/06/2016

Vezza T, Algieri F, Rodríguez-Nogales A ,Camuesco D, Garrido-Mesa J, Utrilla MP, Talhaoui N, Gómez-Caravaca AM, Segura-Carretero A, Rodríguez-Cabezas ME, Gálvez J. Intestinal anti-inflammatory effect of olive leaf extract in the DSS model of mouse colitis. Tipo de participacion: Comunicacion oral. Congreso: VIII Reunion de Jovenes Farmacologos. Publicacion: Abstract book. Lugar celebracion: Malaga (España). Fecha: 31-05-2016

Vezza T, Algieri F, Rodríguez-Nogales A, Garrido-Mesa J, Utrilla MP, Talhaoui N, Gomez-Caravaca AM, Segura-Carretero A, Rodríguez-Cabezas ME, Gálvez J. Intestinal anti-inflammatory effect of olive leaf extract in the DSS model of mouse colitis. Tipo de participación: poster. Congreso: Falk Symposium 200 Publicación: Abstract book. Lugar celebración: Friburgo (Alemania) Fecha:16-17/10/2015

Talhaoui N, **Vezza T**, Gómez-Caravaca AM, Algieri F, Rodríguez Nogales A, Gálvez J, Fernández- Gutiérrez A, Segura-Carretero A. *In vitro* immunomodulatory effect of olive leaf extracts. Tipo de participación: poster. Congreso: XVIII EuroFood Chem Publicación: Abstract book. Lugar celebración: Madrid (España) Fecha:13-16/10/2015

Otros méritos

- Curso formativo "Métodos alternativos en toxicologia usando el pez cebra" (19-20 de Octubre de 2016)

- Curso de manipulación de animales de experimentación Categoria B en el Centro de Investigacion Biomedica de Granada desde el 26 de enero 2015 hasta el 18 de febreo 2015

- Partecipacion a la Jornada Cientifica “LC-MS CLINICAL APPLICATIONS (25 de Febrero de 2015)

- Curso de Prevencion de riesgos laborales en laboratorios en el Centro de Investigacion Biomedica de Granada desde el 20 de enero 2015 hasta el 22 de enero 2015

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- Curso de Histologia Pratica en el Centro de Investigacion Biomedica de Granada (Octubre 2013)

- Estancia en el Departamento de Farmacología de la Universidad de Granada, dentro del programa LLP/ERASMUS PLACEMENT desde el 22 de noviembre de 2012 hasta el 15 de abril 2013.

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